Polymerization of dissolved humic substances catalyzed by peroxidase. Effects of pH and humic composition

A. Cozzolino, A. Piccolo*

Dipartimento di Scienze Chimico-Agrarie, Università di Napoli “Federico II”, Via Università 100, 80055 Portici, Italy

Abstract

High performance size exclusion chromatography (HPSEC) was used to follow the changes in molecular size distribution of three dissolved humic materials of various origins brought about by oxidative coupling of humic constituents under the combined action of hydrogen peroxide and horseradish peroxidase (HRP). Increase in weight-average molecular weight \( M_w \) occurred invariably for all humic substances with the oxidative polymerization catalyzed by HRP. Polymerization was found to occur to a further extent at pH 7 than at pH 4.7. This was attributed to the larger mobility of reacting molecules in the hydrated and smaller humic associations stabilized only by weak dispersive forces at pH 7. Conversely, hydrogen bonds confer a conformational rigidity to humic associations at pH 4.7 and depress molecular reactivity. Comparison with chromatograms and \( M_w \) values obtained by treating humic solutions with acetic acid to pH 3.5 before HPSEC injection confirmed that the increase in molecular size by HRP catalysis was stable and due to the formation of covalent bonds among reacting humic molecules. Humic polymerization was somewhat inhibited when humic substances were rich in alkyl carbons and poor in carboxyl carbons. These molecular characteristics are conducive to thermodynamically stable hydrophobic domains in aqueous solution and may limit the mobility of potentially reactive humic molecules towards the catalytic sites of HRP. Control of the conformational structure of dissolved humic substances may be of importance in regulating their reactivity in the environment. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

It is generally recognized that humic substances in both soils and waters are partially composed by phenolic compounds (Stevenson, 1994; Saiz-Jimenez, 1996). There is a large body of literature showing that a number of naturally-occurring phenolic monomers can undergo oxidative coupling reactions catalyzed by oxidative enzymes, such as laccases and peroxidases, to produce humic-like substances (Martin and Haider, 1971; Haider et al., 1975; Sjoblad and Bollag, 1981). Laccases and peroxidases are thought to couple phenolic compounds by way of a radical mechanism (Nakamoto and Machida, 1992; Zhang and Nicell, 2000).

While the oxidative enzymes have been also largely proved, by chromatographic and spectroscopic experiments, to transform organic contaminants such as chlorophenols and anilines into their oligomers by a radical mechanism (Berry and Boyd, 1985; Ruggiero et al., 1989; Simmons et al., 1989; Bollag, 1992; Kim et al., 1997; Itoh et al., 2000), less is known on the reciprocal effect between dissolved humic substances and enzymatic catalysts. It has been ascertained that humic materials, depending on origin and concentration, may form association with peroxidase (Serban and Nissenbaum, 1986), influence activity of the enzyme (Pflug, 1980), catalyze the degradation of phenoxyalkanoic acids (Piccolo et al., 2001b) and, in the presence of peroxidase, covalently incorporate xenobiotics into the humic structure (Hatcher et al., 1993; Park et al., 2000). However, the heterogeneity and chemical complexity of
humic substances has so far prevented workers from evaluating the changes in the humic structure brought about by an oxidative reaction catalyzed by peroxidase.

Recent results have shown that high performance size exclusion chromatography (HPSEC) can be used to evaluate both the differences in conformational structure of humic substances from various sources (Conte and Piccolo, 1999a) and their fractionation into smaller components induced by interactions with mineral and organic acids (Conte and Piccolo, 1999b; Piccolo et al., 1999; Cozzolino et al., 2001). As opposed to humic materials, undisputed covalently-linked polymers such as polysaccharides and polystyrenesulphonates did not change their HPSEC behaviour when treated with mineral and organic acids under the same conditions as for the humic matter (Piccolo et al., 2000a, 2000b). Organic acids were also reported to significantly affect the fluorescence and thermal behaviour of humic substances (Kenworthy and Hayes, 1997; Buurman et al., 1997). These findings suggested that humic substances, rather than being constituted by macromolecular polymers as traditionally believed, may be better described as supramolecular associations of relatively small heterogeneous molecules (possibly lower than 600–1000 Daltons). Humic molecules are then randomly associated by weak dispersive forces (van der Waals, π–π, CH/π) into apparently high molecular dimensions (Piccolo and Conte, 2000; Piccolo, in press).

By such understanding, one should expect that phenolic compounds, present in the loosely-bound humic supramolecular structures, would undergo the same radical-mediated intermolecular polymerization observed by phenolic monomers when catalyzed by the enzymes. An increase in molecular size of humic matter following an oxidative polymerization reaction catalyzed by peroxidase was effectively observed by HPSEC (Piccolo et al., 2000b). In contrast to the non polymerized material, interaction with an organic acid was not able to alter the molecular size of the polymerized humic matter, thereby indicating that its conformation should have been stabilized by covalent bonds. DRIFT spectra of polymerized humic matter showed IR bands typical of alkyl- and aryl-ethers, which suggested formation of C–O–C bonds upon oxidative reaction (Piccolo et al., 2000b).

This work has the objective to further study, by HPSEC, the effect of the oxidation reaction catalyzed by a peroxidase on the molecular size of three humic substances of various origins as a function of their different molecular characteristics and pH of their solution.

2. Materials and methods

2.1. Humic substances

Three humic acids (HAs) were isolated from different raw materials: HA–A from a volcanic soil (Typic Xero-

fluence near Rome (Italy), HA–B from an oxidized coal provided by Eniricerche SpA (Italy), and, HA–C from North Dakota Leonardite (Mammoth, Int. Chem. Co.). The HAs were extracted and purified by common procedures (Stevenson, 1994). The original materials were shaken overnight in a solution of 0.5 M NaOH and 0.1 M Na2P2O7 under N2 atmosphere. The HAs were precipitated from the alkaline extracts by lowering the pH to 1 with 6 M HCl. The HAs were extensively purified by three cycles of dissolution in 0.1 M NaOH solution and subsequent precipitation in 6 M HCl. The precipitated HAs were then treated with a 0.5% (v/v) HCl–HF solution for 36 h, dialyzed (spectrapore 3 dialysis tubes, 3500 Mw cut-off) against distilled water until chloride-free, and freeze-dried. The HAs were then dissolved in 0.5 M NaOH and passed through a strong cation-exchange resin (Dowex 50) to further eliminate divalent and trivalent metals and freeze-dried again. Purified HA samples (50 mg) were subsequently suspended in distilled water (50 ml) and titrated to pH 7 by an automatic titrator (VIT 90 Videotitrator, Radiometer, Copenhagen), with a CO2-free 0.5 M NaOH solution under N2 atmosphere and continuous stirring. After having reached the constant pH 7, the solution containing sodium-humates was left under titration for 2 h, filtered through a Millipore 0.45 μm filter, and freeze-dried.

2.2. Characterization of humic samples

Elemental content of HAs (Table 1) was determined with a Fisons EA 1108 elemental analyzer and the ash content, obtained by burning 50–100 mg of the sample in an oven at 750 °C for 8 h, resulted lower than 5% (w/w) for all humic materials. Cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CPMAS13C-NMR) experiments were carried out on a Bruker AMX400 instrument operating at 100.6 MHz on the carbon-13. A recycle time of 1 s and an acquisition time of 13 ms were used. All the experiments were conducted with a variable contact time (VCT) pulse sequence in order to find the optimum contact time (OCT) for each sample, and to minimize the error on the evaluation of the peak areas (Conte et al., 1997).

<table>
<thead>
<tr>
<th>Humic sample</th>
<th>C (%)</th>
<th>H (%)</th>
<th>N (%)</th>
<th>C/H</th>
<th>C/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA–A</td>
<td>53.7</td>
<td>4.9</td>
<td>4.3</td>
<td>11.0</td>
<td>12.5</td>
</tr>
<tr>
<td>HA–B</td>
<td>48.0</td>
<td>3.0</td>
<td>1.0</td>
<td>16.0</td>
<td>48.0</td>
</tr>
<tr>
<td>HA–C</td>
<td>45.9</td>
<td>3.7</td>
<td>1.0</td>
<td>12.4</td>
<td>45.9</td>
</tr>
</tbody>
</table>

* HA–A, humic acid from a volcanic soil; HA–B, humic acid from an oxidized coal; HA–C, humic acid from a lignite.
OCT ranged between 0.8 and 1.0 ms. A line broadening of 50 Hz was used to transform all Free Induction Decays. The area in the 110–140 ppm region was corrected for the side band area in the 190–230 ppm region from that of the 110–140 ppm region. The areas of each region of the spectra in Table 2 were attributed to non-polar carbons such as the alkyl (0–45 ppm), and aromatic (110–160 ppm) ones, and to polar carbons such as the C–O, C–N groups and anomeric carbons (45–110 ppm), and carboxyl carbons (160–190 ppm). The areas of the 0–45 and 110–160 ppm regions were used to calculate hydrophobicity (HB), whereas those of the 45–60, 60–110, and 160–190 ppm regions were used to obtain hydrophilicity (HI) of HAs. The HI/HB ratios are given in Table 2.

2.3. Horseradish peroxidase

Horseradish peroxidase (HRP) was purchased from Sigma Chemical Co. (Milano). The HRP activity was assayed by oxidizing ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate)]. The reaction mixture (1.5 ml) contained 4 nM (0.75 ml) of ABTS, 4 mM (0.75 ml) of H₂O₂, dissolved in 0.1 M citrate-phosphate buffer (pH 4.5), and 15 ml (4.4 mg/ml) of peroxidase. Oxidation of ABTS was followed by absorbance increase at 420 nm (extinction coefficient: 24 l mmol⁻¹ cm⁻¹). One unit was defined as the amount of HRP that oxidized 1 mmol of ABTS in 1 min at 25 °C and pH 4.5. The enzymatic specific activity of enzyme was calculated by dividing the unit measured in 1 ml of enzyme for the mass of enzyme contained in the volume.

2.4. Oxidation of humic substances by peroxidase catalysis

Control humic solutions for HPSEC analysis were prepared by dissolving 2.0 mg of each sodium-humate sample in 10 ml of 0.1 M phosphate buffer at either pH 4.7 or 7 to obtain a final humic concentration of 0.2 mg ml⁻¹. Catalytic oxidation of humic substances was performed by adding 0.58 ml of a 0.1 mg ml⁻¹ HRP solution (corresponding to 64 units) and 139 µl of H₂O₂ (1%) to a 0.2 sodium humate solution in both phosphate buffers up to a final 10 ml volume. Both control and peroxidase-treated solutions were left standing for 2 h at 25 °C and injected in the HPSEC system. Immediately after the completion of the first chromatogram, glacial acetic acid was used to bring the phosphate buffer solutions of reaction mixtures either from pH 4.7 to 3.5 or from pH 7 to 4.5. The modified solutions were again injected into the HPSEC system.

2.5. Size exclusion chromatography

The HPSEC system consisted of a high pressure Perkin-Elmer LC200 solvent pump, and of two detectors in series: a UV/vis variable wavelength detector (Perkin-Elmer LC295) set at 280 nm, and a refractive index (RI) detector (Fisons Instruments, Refractomonitor IV). A rheodyne rotary injector, equipped with a 100 µl sample loop, was used to load the calibration standard and humic solutions. Size exclusion separation occurred through a G3000SW (600 mm per 7.5 mm i.d.) TSK column (Toso Haas). The column was preceded by a 7.5 cm TSK Guard-Column (7.5 mm i.d.) packed with G3000SW, and by a 0.2 µm stainless-steel inlet filter. The column system was thermostated at 25 °C in a water bath. The column manufacturer only reports a calibration for globular proteins from 5 to 300 kD as nominal separation range. Details on column specifications, performance and separation capacities were previously reported (Conte and Piccolo, 1999a). The flow rate was set to 0.6 ml min⁻¹ and the HPSEC eluent was a 0.05 M NaNO₃ and 4.0×10⁻³ M NaN₃ solution (the latter as a bacteriostatic agent). The mobile phase was made with MilliQ water and HPLC-grade reagents, filtered through Millipore 0.45 µm and He degassed. The void volume (V₀=11.18 ml) and total permeation

Table 2

Distribution (%) of 33C in resonance intervals (ppm) of CPMAS–NMR spectra and HI/HB ratios of humic acids

<table>
<thead>
<tr>
<th>Humic samplea</th>
<th>0–45</th>
<th>45–60</th>
<th>60–110</th>
<th>110–160</th>
<th>160–190</th>
<th>HI/HBb</th>
<th>Aromaticityc</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA–A</td>
<td>35.3</td>
<td>12.8</td>
<td>30.8</td>
<td>16.0</td>
<td>10.2</td>
<td>1.05</td>
<td>15.2</td>
</tr>
<tr>
<td>S.D.</td>
<td>±1.41</td>
<td>±0.81</td>
<td>±1.30</td>
<td>±0.90</td>
<td>±0.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA–B</td>
<td>22.1</td>
<td>9.8</td>
<td>16.4</td>
<td>36.9</td>
<td>23.4</td>
<td>0.84</td>
<td>34.0</td>
</tr>
<tr>
<td>S.D.</td>
<td>±1.30</td>
<td>±1.70</td>
<td>±1.60</td>
<td>±2.70</td>
<td>±1.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA–C</td>
<td>25.3</td>
<td>8.4</td>
<td>18.0</td>
<td>39.4</td>
<td>16.6</td>
<td>0.66</td>
<td>36.5</td>
</tr>
<tr>
<td>S.D.</td>
<td>±2.10</td>
<td>±0.50</td>
<td>±1.10</td>
<td>±2.45</td>
<td>±1.20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a HA–A, humic acid from a volcanic soil; HA–B, humic acid from an oxidized coal; HA–C, humic acid from a lignite.
b HI/HB = [(45–60) + (60–110) + (160–190)]/[(0–45) + (110–160)].
c Aromaticity = (110–160)/(0–190).
d S.D., standard deviation.
volume \((V_i = 22.57 \text{ ml})\) of the columns were determined using Blue Dextran 2000 and water, respectively.

2.6. Molecular weight determination

Size exclusion chromatograms for both the UV and RI detectors were evaluated by using Perkin–Elmer–Nelson Turbochrom 4-SEC peak integration and molecular weight software, a SEC noise threshold of 5, and a filter size of 5 for the Savitzky-Golay smoothing. Calculation of weight-average \((M_w)\) molecular weights was done as previously described (Piccolo et al., 1999) by the following equation:

\[
M_w = \frac{\sum_{i=1}^{N} M_i h_i}{\sum_{i=1}^{N} h_i}
\]

where \(M_i\) and \(h_i\) are the molecular weight and the height of the \(i\)-th chromatographic slice in the chromatogram of each sample eluted at volume \(i\), respectively. The \(M_w\) values from chromatograms of control and treated HAs as well as from chromatograms of the same HAs added with acetic acid were obtained by using calibration curves with standard polysaccharides (Polymer Sciences Laboratories, UK) of known molecular weights (100, 48, 23.7, and 12.2 kDa). The system dispersion was found to be less than the error \((<5\%)\) and the chromatograms were evaluated without additional correction factors. The relative standard deviation of calculated values among triplicates of each chromatogram varied only to a maximum of 7%, thereby confirming the good reproducibility of the HPSEC system previously reported (Becher et al., 1985; Conte and Piccolo, 1999a,b).

3. Results and discussion

The HAs of this study had distinct elemental characteristics with the HA–B from an oxidized coal showing the highest degree of condensed or unsaturated carbon compounds (C/H in Table 1), followed by HA–C from a lignite and HA–A from a forested volcanic soil. CPMAS-\(^{13}\)C-NMR spectroscopy was used to determine the molecular composition of the different HAs. Integrated values from NMR spectra (Table 2) indicated that HA–A had a higher content of polar carbons, such as oxidized (53–56 ppm) and carbohydrate (70–105 ppm) carbons, than HA–B and HA–C. The HA–A had also the highest content of alkyl carbons (30–32 ppm) whereas both HA–B and HA–C were significantly richer in aromatic and carboxyl carbons (around 130 and 170 ppm, respectively). Areas from different resonance intervals of NMR spectra were used to quantify potential humic reactivity due to polar and apolar carbons (Table 2). The HI/HB ratio varied in the order: HA–A > HA–B > HA–C indicating that HA–A was the most potentially hydrophilic material, while HA–C was the most hydrophobic of HAs. Aromaticity (Table 2) of humic substances resulted in the following order: HA–C ≥ HA–B ≥ HA–A.

It is assumed that hydrophobic humic components in aqueous solutions should be pulled together and isolated from water in order to decrease the total free energy of solution (Tanford, 1991; Schwarzenbach et al., 1993), whereas the hydrophilic constituents should preferentially accommodate in the outer sites of humic associations. Values of \(M_w\) show that HA–A had the largest apparent molecular dimension followed by HA–C and HA–B (Table 3). However, UV-detected HPSEC chromatograms alone cannot reveal whether the UV-absorbing chromophores in the high-molecular-size peaks are in hydrophobic or in hydrophilic associations. The HPSEC separation combined to pyrolysis (PYR–GC–MS) and \(^1\)H-NMR analysis indicated that strong associations of relatively small apolar molecules were eluted at the void volume whereas progressively hydrophilic molecules including aromatic phenols were excluded at larger eluting volumes (Piccolo et al., in press).

Fig. 1 reports the size-exclusion chromatograms of HA–A before and after oxidative polymerization catalyzed by peroxidase in a phosphate buffer solution at pH 4.7. Peroxidase is supposed to have the maximum catalytic activity at this pH (Bollag et al., 1987). The control chromatogram was somewhat modified by lowering pH to 3.5 with acetic acid prior to the injection in the HPSEC system. In fact, both the first sharp peak and the diffused absorption at higher elution volumes resulted in lower absorbance intensity. A similar decrease in peak absorbance was shown by HA–A after the enzyme-catalyzed oxidative reaction both at the initial pH 4.7 and at pH 3.5 reached after acetic acid treatment. No substantial chromatographic difference was noticeable between the control and the reacted sample except for a further lowering of absorbance of the diffused peak in the reacted sample brought to pH 3.5 with acetic acid. Despite these slight variations, the elaborated \(M_w\) values (Table 3) showed an increase in molecular size going from the control to the reacted (mixture) samples before and after treatment with acetic acid.

The same control and mixture samples of HA–A produced rather different chromatograms when in solution buffered at pH 7 (Fig. 2). In comparison to pH 4.7, the chromatogram of control HA–A at pH 7 showed a first peak intensity reduced by half while the diffused peak maintained a similar absorbance. This was reflected in the \(M_w\) value which was 35% less than for pH 4.7 (Table 3). Addition of acetic acid to control solution to lower pH from 7 to 3.5 increased somewhat the absorbance of the first peak and shifted its maximum to lower elution volumes while reduced dramatically the intensity
of the diffused peak whose maximum was centered at higher elution volumes. The \( M_w \) values describe (Table 3) quantitatively this effect by showing a 65\% increase in molecular dimension when the HA–A control solution was brought from pH 7 to 3.5 with acetic acid.

This behaviour cannot be attributed to a modification of the column separation capacity because of the acetic acid added to the injected sample (Piccolo et al., 2000a) but rather to a conformational change of the humic supramolecular structure. A HA sample at pH 7 has a larger number of dissociated acidic functions than at pH 4.7 (Stevenson, 1994). By loosening the inter- and intramolecular hydrogen bonds, which stabilize the conformational structure at pH 4.7, the humic association at pH 7 has a lower molecular size and is stabilized by only dispersive forces (Piccolo and Conte, 2000).

The enzyme-catalyzed oxidative reaction undergone by the HA–A sample altered significantly the chromatographic pattern of the resulting mixture (Fig. 2C). The intensity of the first peak almost doubled as compared to control solution, while the diffused peak not only increased in absorbance but also shifted to lower retention volumes. Calculation of \( M_w \) values indicated that the reacted material increased its molecular size by 28\% in comparison to control (Table 3). These changes cannot be attributed to formation of intermolecular hydrogen bonds because no change in pH occurred in the control and mixture solution before HPSEC injection. A condensation between the catalytic quantity of peroxidase and humic components was also excluded (Piccolo et al., 2000b). The changes should be rather explained by a stabilization of the humic conformation through covalent C–O–C or C–C bonds formed during the enzyme-catalyzed oxidative reaction (Piccolo et al., 2000b). The increase in molecular size of the reacted sample was indicated by the appearance of a peak of higher intensity and of lower retention time than for the control sample at pH 7 (Fig. 2B). The association of chromophore molecules did not have the same impact on molecular size (Table 3) when the HA–A sample was brought from pH 4.7 to 3.5 with acetic acid (Fig. 1) because the intermolecular hydrogen bonds present at pH 4.7 already conferred a stabilization of humic molecules into association of larger molecular size.

Table 3

<table>
<thead>
<tr>
<th>Samplea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>HA–A pH 4.7</td>
</tr>
<tr>
<td>( M_w )</td>
</tr>
<tr>
<td>( \Delta )</td>
</tr>
<tr>
<td>HA–A pH 7.0</td>
</tr>
<tr>
<td>( M_w )</td>
</tr>
<tr>
<td>( \Delta )</td>
</tr>
<tr>
<td>HA–B pH 7.0</td>
</tr>
<tr>
<td>( M_w )</td>
</tr>
<tr>
<td>( \Delta )</td>
</tr>
<tr>
<td>HA–C pH 7.0</td>
</tr>
<tr>
<td>( M_w )</td>
</tr>
<tr>
<td>( \Delta )</td>
</tr>
<tr>
<td>HA–A pH 4.7</td>
</tr>
<tr>
<td>( M_w )</td>
</tr>
<tr>
<td>( \Delta )</td>
</tr>
<tr>
<td>HA–C pH 7.0</td>
</tr>
<tr>
<td>( M_w )</td>
</tr>
<tr>
<td>( \Delta )</td>
</tr>
</tbody>
</table>

\( ^a \) HA–A, humic acid from a volcanic soil; HA–B, humic acid from an oxidized coal; HA–C, humic acid from a lignite.
The sample is confirmed by the chromatogram obtained after addition of acetic acid to lower pH from 7 to 3.5 (Fig. 2D). The first peak of the reacted material was reduced significantly in intensity but shifted to even lower retention volumes than for the reacted sample at pH 7. Moreover, the diffused peak showed considerably less absorbance, whose values were lower than for control after addition of acetic acid (Fig. 2B). The $M_w$ value of the reacted sample brought to pH 3.5 with acetic acid was 85% larger than for control and about 20% larger than for control plus acetic acid (Table 3). This shows that the enzyme-catalyzed reaction increased the mole-
cular size of this sample more than could be only attributed to intermolecular hydrogen bonds formed during a pH change.

Nevertheless, the changes in peaks absorbance of the mixture sample before and after acetic acid treatment cannot be easily interpreted. The reported variations of molecular absorptivity of humic substances with molecular dimension (Summers et al., 1987) have been generally explained by deviations from the Lambert-Beer law (Stevenson, 1994). Changes in chromatographic peaks absorbance were recently ascribed to the effect of chromism when the supramolecular association of

Fig. 2. HPSEC chromatograms of the control HA–A solution (HA from a volcanic soil) in phosphate buffer at pH 7.0 (A), of the same solution as in A but brought to pH 4.5 with acetic acid (B), of the same solution as in A but with both H₂O₂ and peroxidase added (C), of the same solution as in C but brought to pH 4.5 with acetic acid (D).
humic substances was perturbed (Conte and Piccolo, 1999b; Piccolo et al., 1999; Piccolo and Conte, 2000; Cozzolino et al., 2001). According to the reciprocal orientation of the dipolar moments of chromophores present in a humic association the resulting molar absorptivity may vary considerably, giving rise to either a hypochronic or a hyperchromic effect (Cantor and Shimmel, 1980). Also in this study, the conformational perturbation due to formation of covalent bonds among humic molecules, otherwise randomly associated by weaker bonds, should be responsible for the changes in reciprocal orientation of the dipolar moments of chromophores, thereby altering the light absorbance of the eluting peaks.

Addition of acetic acid to the HA–B solution to lower the pH from 4.7 to 3.5 disrupted the original conformation (Fig. 3A and B) and resulted in an increase of the \( M_w \) value by 17% in respect to control (Table 3). When the HA–B solution was subjected to the oxidative reaction catalyzed by peroxidase, the elution pattern was further modified and showed a significant shift of absorption towards lower elution volumes and a concomitant intensity decrease for the diffused peak (Fig. 3C). The addition of acetic acid to bring the reacted mixture of HA–B to pH 3.5 before injection produced an even larger modification of the elution profile with peaks absorption being enhanced and shifted to lower elution volumes. The \( M_w \) values for the reaction mixture at pH 4.7 and for that at pH 3.5 were about 39 and 45%, respectively, larger than control (Table 3). Also these findings suggest an increase in molecular size of the humic material due to polymerization catalyzed by peroxidase.

Even more evident was the result of the oxidative reaction on the molecular size distribution of the HA–B sample when the pH of the reaction solution was set at 7 (Fig. 4). The control solution brought to pH 3.5 with acetic acid showed a marked peak shift towards much lower elution volumes, while the absorbance of the diffused peak significantly decreased in respect to control at pH 7 (Fig. 4B). As in the case of HA–A, the conformation of HA–B at pH 7 was less stable and smaller (see Table 3) than at pH 4.7 because the dissociation of acidic functions disrupted the stabilizing intermolecular hydrogen bonds. However, when the pH was lowered from 7 to 3.5 by acetic acid, hydrogen bonds were formed and the loosely-bound humic molecules were assembled into a larger association showing a 33% \( M_w \) increase over control (Table 3). A significant increase in molecular size is also visible in the chromatogram of the reacted HA–B sample at pH 7 (Fig. 4C) which produced a \( M_w \) value 52% larger than the control (Table 3). The addition of acetic acid to this mixture (Fig. 4D) shifted the first peak towards lower elution volumes. The persistent peak intensity and the relative \( M_w \) value, that resulted in being 36% larger than control (Table 3), suggested a degree of stable polymerization for this material.

Changes in molecular absorptivity with either acetic acid or polymerization treatments were observed also in the chromatograms of the HA–C sample (Figs. 5 and 6). The elution profiles of this humic material as well as the relative \( M_w \) values (Table 3) were similar in both pH solutions and similarly altered by addition of acetic acid to bring the pH either from 4.7 to 3.5 (Fig. 5) or from 7 to 3.5 (Fig. 6). In both solutions, there was a significant reduction of the diffused peak intensity accompanied by a marked shift of its maximum towards higher elution volumes. However, such changes were not reflected in the appearance of the first peak, which was only slightly reduced (Figs. 5 and 6). This lack of consistencies in the intensity changes between the two peaks of the HPSEC chromatogram further suggests that the molecular absorptivity of humic conformations depends on the mutual orientation and degree of association of chromophores.

Subjecting HA–C dissolved at pH 4.7 to oxidative coupling catalyzed by peroxidase resulted in dramatic changes (Fig. 5C). Not only the first peak was enhanced in intensity and shifted to significantly lower elution volumes, while the diffused peak was decreased in absorbance, but also a third peak appeared between the two absorptions. When acetic acid was added to the reacted mixture (Fig. 5D), the first two peaks eluted at the same retention volumes but increased substantially in absorbance, whereas the diffused peak was reduced even more in intensity and somewhat shifted again to larger elution volumes. These changes in peak intensities should be ascribed to re-positioning of chromophores in the new conformations created by the presence of acetic acid. However, the appearance of new peaks in the high molecular-size range and their persistence in both elution volume and intensity even after addition of acetic acid should be interpreted as evidence of an occurred polymerization of humic material. The \( M_w \) values confirmed the formation of stable intermolecular bonds by showing an increase in molecular size (56.7%) over control (Table 3).

The HA–C material subjected to the oxidative reaction behaved similarly when dissolved at pH 7 (Fig. 6). However, the intensity enhancement of both the first and second peak, after the oxidative reaction, was much more dramatic than for the solution at pH 4.7 but no new peaks were observed in this chromatogram (Fig. 6C). Decreasing the pH to 3.5 with acetic acid increased absorbance of the first peak while reducing the intensity of the diffused peak to that of control but to larger elution volumes (Fig. 6D). These changes and the relative \( M_w \) values suggest that the enzyme-catalyzed oxidative reaction caused a persistent increase in the molecular size of HA–C. The significantly larger chromic effect than at pH 4.7 may be due to a more weakly
stable conformation at pH 7 in which chromophores are allowed a larger reciprocal mobility.

The HPSEC chromatograms showed that oxidative polymerization of humic molecules through peroxidase catalysis occurs to a different extent according to pH. While some of the differences have been ascribed to the degree of dissociation of HA acidic functions at various pHs, much depended on the molecular composition of the humic superstructures. The most dramatic differences in size distribution between the polymerized products at pH 4.7 and that at pH 7 were found for HA–A that produced a more advanced polymerization at pH 7. The first peak of the HA–A material showed a much stronger absorption (Figs. 1 and 2) than for the control

Fig. 3. HPSEC chromatograms of the control HA–B solution (HA from an oxidized coal) in phosphate buffer at pH 4.7 (A), of the same solution as in A but brought to pH 3.5 with acetic acid (B), of the same solution as in A but with both H₂O₂ and peroxidase added (C), of the same solution as in C but brought to pH 3.5 with acetic acid (D).
samples of both HA–B and HA–C (Figs. 3–6). The halving of absorption intensity of this peak for the HA–A control when solution pH passed from 4.7 to 7.0 is to be attributed to a deaggregation of the humic supramolecular structure into smaller associations following deprotonation of acidic functions.

It has been shown that the apparent high molecular dimension of humic matter is due to a tight association of the most hydrophobic humic components such as long aliphatic chains (Piccolo et al., in press). This suggests that the extent of oxidative coupling among humic molecules depends on their availability to enter in con-

Fig. 4. HPSEC chromatograms of the control HA–B solution (HA from an oxidized coal) in phosphate buffer at pH 7.0 (A), of the same solution as in A but brought to pH 4.5 with acetic acid (B), of the same solution as in A but with both H₂O₂ and peroxidase added (C), of the same solution as in C but brought to pH 4.5 with acetic acid (D).
tact with the catalyst. The large content of alkyl carbons as well as the low amount of carboxyl carbons found in the HA–A sample (Table 2) may indicate that potentially reactive humic molecules are confined at pH 4.7 in strong hydrophobic associations of apparently large molecular size and are hardly available to sterically interact with the enzyme. This limitation is partly solved at pH 7.0 when the negative charges arising from the dissociation of humic carboxyl groups disrupt the hydrophobic associations and humic constituents may become available to react by a free-radical mechanism. At higher pHs, the increased hydration of humic molecules enhances their mobility in solution and the probability of a concomitant contact among each other, the

Fig. 5. HPSEC chromatograms of the control HA–C solution (HA from a lignite) in phosphate buffer at pH 4.7 (A), of the same solution as in A but brought to pH 3.5 with acetic acid (B), of the same solution as in A but with both H₂O₂ and peroxidase added (C), of the same solution as in C but brought to pH 3.5 with acetic acid (D).
oxidant and the catalyst. In accordance to this mechanism, HAs were also found to possess a greater reactivity towards the transformation of the herbicide 2,4-D into 2,4-dichlorophenol when dissolved at pH 7.0 than at pH 4.7 (Piccolo et al., 2001a).

The generally high content of carboxyl carbons and low amount of alkyl carbons in HA–B and HA–C should have prevented the formation of tight hydrophobic domains of large molecular size and kept the humic molecules relatively mobile in solution even at pH 4.7. For both samples a degree of polymerization was in fact evident even at pH 4.7. The higher reactivity of HA–B and HA–C may be also attributed to their large content of aromatic carbons (Table 2). Aromatic...
moieties are potentially highly reactive in free-radical reactions such as those catalyzed by peroxidase (Sjoblal and Bollag, 1981) especially when they are sufficiently mobile to approach the catalyst. The involvement of aromatic groups in the polymerization of humic matter was confirmed by the formation of aryl ethers during the oxidative coupling of humic molecules (Piccolo et al., 2000a). Moreover, aromatic groups hardly contribute to the hydrophobic stability of humic aggregates of large molecular dimension but are preferentially positioned in smaller and more hydrated humic associations (Piccolo et al., in press).

The large amount of alkyl carbons and low content of carboxyl carbons in HA–C (Table 2) may explain the particularly intense first peak in the HPSEC chromatograms of HA–C (Figs. 5 and 6). The specific molecular composition of HA–C may have favored tight hydrophobic domains of larger size than for HA–B. The distinct differences in extent of polymerization for HA–C at the two pHs (Figs. 5 and 6) may then be attributed to changes of molecular associations with pH and relative mobility of reactive species.

4. Conclusions

This work indicates that peroxidase catalyzed the transformation of weakly-bound humic superstructures into stable conformations of truly large molecular size through formation of covalent bonds among the reactive humic molecules. The HPSEC elution profiles showed that the extent of polymerization generally increased when the pH of reacting solutions was raised from 4.7 to 7.0. The reactive humic molecules appeared to be more mobile in the smaller and looser conformations at neutral pH thereby rendering more efficient the enzyme-catalyzed polymerization. The molecular composition of humic matter seemed to control the extent of polymerization: the larger the alkyl carbons and the lower the carboxyl carbons in HA–C (Table 2) may explain the particularly intense first peak in the HPSEC chromatogram of HA–C (Figs. 5 and 6). The specific molecular composition of HA–C may have favored tight hydrophobic domains of larger size than for HA–B. The distinct differences in extent of polymerization for HA–C at the two pHs (Figs. 5 and 6) may then be attributed to changes of molecular associations with pH and relative mobility of reactive species.

Acknowledgements

This work was partially supported by the Italian Ministry of University and Scientific and Technological Research (MURST) through the project No. 9807352092.

References


Piccolo, A., Conte, P., Cozzolino, A., Paci, M., 2001a. Combined effects of an oxidative enzyme and dissolved humic substances on 13C-labelled 2,4-D herbicide as revealed by high-resolution 13C-NMR spectroscopy. Journal of Industrial Microbiology and Biotechnology 26, 70–76.


