Kinetics study of the antioxidant capacities of humic and humic-like substances

Olga KOROLEVA[a*], Olga KLEIN[a], Ivan FILIMONOV[a], Natalia KULIKOVA[ab], and Andrey KONSTANTINOV[b]

[a] Bach Institute of Biochemistry, Research Center of Biotechnology of the Russian Academy of Sciences, 33, bld. 2, Leninsky Ave., 119071, Moscow, Russia. [b] Lomonosov Moscow State University, Leninskie Gory, 119991, Moscow, Russia.

* Tel. & Fax. No. +7-499-952-8499; E-mail: koroleva57@gmail.com

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Abstract Humic substances (HS) play protective functions by interrupting radical reactions and preventing damage to cell membranes due to their antioxidant activity. With HS, antioxidant capacity (AOC), rather than antioxidant activity, is usually used to describe antioxidant properties. Our work aimed at quantifying the main kinetic parameters of antioxidant activity for HS and humic-like substances (HLS). The ten-hour kinetic profiles of four standard HS and two fungi-produced HLS were established using the ABTS decolorization assay. Three pH levels (3.75, 4.25 and 6.80) and a broad range of humic material concentrations (0.5–10 mg L^{-1}) were examined. Our results demonstrated that during the first 40 min, the determined AOCs did not exceed 50% of the endpoint AOCs for studies of humic materials, indicating that short-term measures of the AOCs of humic materials provide artificially low values due to the presence of slow-acting antioxidant compounds. The results showed also a clear-cut distinction in reaction rate constants and AOCs between HS and HLS. The difference thus discovered was visualized by cluster analyses, in which HS and HLS formed two groups.

Introduction

HS and HLS can exert protective functions in ecosystems by interrupting radical reactions and preventing damage to cell membranes and biological macromolecules due to their antioxidant activity. Quantitative estimation of antioxidant properties is based upon the kinetics parameters of the interaction between antioxidant molecules and free radicals, and the reaction rate constant for this interaction can be used as a measure of antioxidant activity. In the case with humic materials, which are heterogeneous organic macromolecules, the antioxidant capacity (AOC) rather than the antioxidant activity is usually used for such an evaluation. Due to difficulties in measuring the individual antioxidant components of HS, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)-equivalency is used as a measure for the antioxidant capacity of such a complex mixture. Trolox-equivalent antioxidant capacity (TEAC) is most often measured using the ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) decolorization assay (Re et al., 1999). Considering that the TEAC approach is an endpoint assay, the kinetics parameters for the reactions between the antioxidant and the $ABTS^{\bullet+}$ radical are also of great importance. As HS and HLS are complex mixtures of different molecules, a kinetics study into their antioxidant properties allows for a deeper understanding of the nature of the anti-radical activities of HS in the environment. Our work aimed to estimate the main kinetics parameters of the antioxidant activity of HS and HLS. To this end, AOCs and the reaction rate constants for the reaction with $ABTS^{\bullet+}$ were estimated for a set of standard HS and two HLS produced by fungi.

Materials and methods

Standard samples of HS including Suwannee River humic (SRHA) and fulvic acids (SRFA) and dissolved organic matter (SRDOM) were kindly provided by the coordinator of the Russian Chapter of the IHSS Prof. I.V. Perminova. Commercially available coal humic acid (AldHA) was purchased from Sigma-Aldrich (USA). Two samples of humic-like substances (HLS), HLS45 and HLS70, were produced in our laboratory by the basidiomycete *Trametes maxima 0275* cultivated on oat straw. HLS45 and HLS70 were isolated after 45 and 70 days of solid phase cultivation, as described earlier (Koroleva et al., 2007). Humic materials were characterized by elemental analysis, size-exclusion chromatography and ^{13}C NMR spectroscopy.

The AOCs of HS and HLS were measured based on a decrease in the absorbance of $ABTS^{\bullet+}$ in the presence of the studied substances. The reactions were monitored by measuring the absorbance at 734 nm (A_{734}). The radicals were generated according to a published report (Re et al., 1999). For measurements, $ABTS^{\bullet+}$ stock solution was diluted with an appropriate buffer (0.1 M sodium citrate buffer (pH 3.75 or 4.25) or 0.1 M potassium phosphate buffer (pH 6.80) to obtain final concentrations of 43 μM for the measurements.

Samples of HS and HLS were dissolved in 1 mL of 0.1 M NaOH and then diluted to 100 mL with deionized water to obtain final stock concentrations of 100 mg L^{-1}. Further dilutions of HS and HLS were made in such a manner that the final concentrations for the measurements would be 0.5, 1, 2, 4, 6, 8, and 10 mg L^{-1}. The reaction was initiated by mixing 20 μL of each dilution with 180 μL of $ABTS^{\bullet+}$, followed by measurement of A_{734} for 10 h using a kinetics program on a Synergy 2 96-well plate reader (BioTek, USA). The assay was carried out at 25°C. All solutions were prepared in 18 MΩ cm water or HPLC-grade ethanol from Pancreac-Quimica (Spain). All measurements were performed on four replicates.

Trolox was used as a benchmark for the AOC determinations. A standard Trolox (Sigma-Aldrich, USA) stock solution was prepared by dissolving a sample of Trolox of known weight in 1 mL of ethanol

and then diluting it with water to 100 mL to obtain a final concentration of 100 μM.

Interval treatment of kinetic curve and calculation of AOC was performed considering that HS is a set of individual antioxidants, and the reaction between $ABTS^{\bullet+}$ and each antioxidants can be considered to be pseudo-first order due to an excess of $ABTS^{\bullet+}$.

Results and Discussion

Results clearly demonstrated the instability of the $ABTS^{\bullet+}$ cation radical at pH 6.80. After 10 h of incubation, the A_{734} level dropped to approximately 32% of the initial value, whereas 10 h of incubation at pH 3.75 or 4.25 led to an A_{734} value equal to 96% of the initial value. An excess of the $ABTS^{\bullet+}$ cation radical in the reaction mixture at pH 6.80 (a value equal to at least 90% of the initial concentration) was observed only during the initial 40 min of the reaction. Therefore, the data obtained at pH 6.8 were excluded from further examination.

The reduction of $ABTS^{\bullet+}$ by the studied humic compounds at pH 3.75 or 4.25 at different concentrations demonstrated a linear dependence between ABTS and the concentration of humic materials only in the range of 0.5–4 mg L^{-1}, indicating that there is a substantial consumption of $ABTS^{\bullet+}$ at higher humic concentrations. Therefore, the $ABTS^{\bullet+}$: humic ratio should not be higher than 43 μmol : 0.4 mg under selected conditions. Data on the AOCs and the reaction rate constants for the scavenging of $ABTS^{\bullet+}$ by humics, calculated as averages of the values obtained with humics in the concentrations 0.5–4 mg L^{-1} are presented in Table 1.

Table 1 AOCs and reaction rate constants for the scavenging of $ABTS^{\bullet+}$ by HS and HLS, determined at pH 3.75 and 4.25.

Humic material	Constant rate, $(sec \times M)^{-1}$		Antioxidant capacity, mmol TE/g		
	k_I	k_{II}	AOC_I	AOC_{II}	AOC
pH 3.75					
SRDOM	18±3*	1.6±0.5	1.3±0.1	1.0±0.1	2.3±0.2
SRFA	16±3	1.3±0.1	1.2±0.2	1.1±0.1	2.2±0.2
SRHA	20±4	1.3±0.3	1.8±0.5	1.4±0.3	3.2±0.3
AldHA	36±5	1.8±0.6	1.3±0.5	1.1±0.4	2.3±0.2
HLS45	40±7	1.6±0.3	1.5±0.4	1.9±0.7	3.4±0.3
HLS70	47±7	1.6±0.4	1.4±0.3	1.9±0.5	3.3±0.3
pH 4.25					
SRDOM	24±4	1.3±0.2	1.2±0.1	1.2±0.2	2.5±0.2
SRFA	19±1	1.1±0.2	1.10±0.08	1.3±0.1	2.4±0.2
SRHA	25±5	1.3±0.4	1.5±0.2	1.4±0.2	3.0±0.2
AldHA	27±5	1.3±0.4	1.2±0.5	1.0±0.3	2.5±0.3
HLS45	37±3	1.2±0.1	1.2±0.6	2.0±0.6	3.3±0.1
HLS70	44±6	1.5±0.5	1.2±0.3	1.7±0.3	2.9±0.3

During the first 40 min, only approximately 50% of the total AOC was reached for both HS and HLS. The latter was indicative of undervaluation of the AOC of humic materials using short-term measuring due to the presence of slow-acting antioxidant compounds.

Among the humic materials studied, those produced by the basidiomycete were characterized by the highest AOCs and reaction rate constants, i.e., HLS45 and HLS70 possessed faster antioxidant moieties or greater amounts of fast antioxidant compounds compared to HS. Besides, the results showed a clear-cut distinction in reaction rate constants and AOCs between HS and HLS. For the first interval (0–40 min), reaction rate constants ranged from 14 to 47 $(sec \times M)^{-1}$ at pH 3.75 and from 20 to 37 $(sec \times M)^{-1}$ at pH 4.25, reaching their maximum values in both cases for HLS. HS and HLS manifested different behaviors in response to pH increases from 3.75 to 4.25. Along with these increases in pH values, the reaction rate constant k_I rose for HS, but declined for HLS. The difference thus discovered was visualized by cluster analyses (Fig. 1), in which HS and HLS formed two groups.

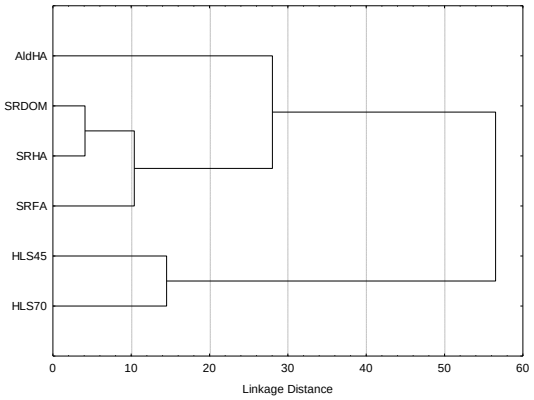

Fig. 1 A tree diagram for the HS and HLS studied. Clustering was performed based antioxidant property data (Table 1).

When the dataset of the physicochemical and antioxidant properties of humic materials was subjected to correlation analysis, values related to antioxidant capacity (endpoint AOC, AOC_I and AOC_{II}) almost always correlated with the C/N ratio. The antioxidant activity of humic materials under selected conditions therefore cannot be attributed to phenol contents alone. This is most likely due to the leading roles of N-containing structural moieties such as peptides and amino acid residues or insole derivatives in determining the antioxidant properties of HS and HLS.

In conclusion, our results demonstrated that short-term measures of the AOCs of humic materials provide artificially low values due to the presence of slow-acting antioxidant compounds. Strongly pronounced differences in the antioxidant properties of HS and HLS were discovered. Most likely, the differences observed could be attributed to their differences in chemical composition and structural features.

References

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