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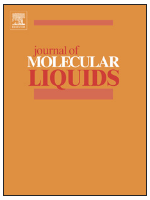

# Binding of proton and iron to lignite humic acid size-fractions in aqueous matrix

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ABSTRACT

The bioavailability of trivalent iron ($Fe^{3+}$) to plants can be enhanced using fertilizer solutions containing humic acids (HA) as manifested from the increased crop yield at an iron stress conditions. The lignite-derived HA ($HA_{lignite}$) facilitates higher diffusion of $Fe^{3+}$ between the soil layers as attributable to more number of reactive sites in the assemblage compared to those from other origins. In the current work, the proton-binding of $HA_{lignite}$ size-fractions (5–10, 10–30, 30–100, and >100 kDa), as segmented based on the molecular weight distribution, and their complexation with $Fe^{3+}$ have been studied at varying pH ranging from low to high. The protonation or formation of $Fe^{3+}$-complexes exhibited a comparable pattern despite the differences in the conformational distribution of $HA_{lignite}$ size-fractions. The protonation behavior specified that the behavior of $HA_{lignite}$ size-fractions has similarity with that of a dibasic acid. The results are interpreted using reactive structural units (RSU) concept to show that the carboxyl and phenolic-hydroxyl groups in the $HA_{lignite}$ size-fractions simultaneously available as the $Fe^{3+}$-binding sites. The stability constants for larger MW fractions of $HA_{lignite}$ (>100 kDa) was the lowest, as attributed to the increased aggregation rate in an aqueous matrix. The trend in conditional stability constants of $HA_{lignite}$-size fractions and other Fe-chelators point to a better Fe-binding capability of $HA_{lignite}$ (30–100 kDa) size-fraction than the biodegradable alternatives (GLDA, HIDS, EDDS, IDSA, or NTA), while the Fe-interaction was stronger with classical synthetic chelators (EDTA, DTPA, or EDDHA).

## 1. Introduction

Iron (Fe) was recognized as an essential nutrient for plants because of its significant role in the photosynthesis, nitrogen fixation, hormone production, or several other cellular activities in plants [1,2]. The widespread abundance of Fe in soils, fourth-most to be precise, however, cannot ensure the supply of adequate Fe to plants due to the formation of sparingly soluble $Fe^{3+}$ species, such as $Fe_2O_3 \cdot nH_2O$, $Fe(OH)_2^+$, $Fe(OH)_3$, and $Fe(OH)_4^-$ in aerobic environments [3,4]. The Fe-stress conditions has been accomplished in plants by any of the following routes: a) Fe-solubilization using proton-release, reducing agent secretion and membrane-bound $Fe^{+3}$ reductase oxidase activity followed by diffusion of Fe to roots with an iron-regulated-transporter, (b) Fe-binding to root-released phytosiderophore to form soluble complexes as recognizable to specific membrane transporters [5–7]. The lack in Fe-nutrition impedes crop growth or yields despite the plant physiology factors, and the alleviation approaches include bioavailability escalation of indigenous soil-Fe, supplement with an external iron-available solution or modification of Fe-uptake mechanisms in plants [4].

The characteristics of synthetic chelators in keeping metal ions at solutions within a wider pH range were exploited to facilitate the increased-diffusion of already available Fe in soils or to supply as Fe-chelates with the fertilizer solutions [8,9]. The application of EDTA, DTPA, EDDHA in liquid formulations to increase the bioavailability of soil-Fe to plants has been introduced since the 1950s [10]. The prolonged persistence of the classical Fe-chelators with a harmful impact on the surrounding biota evoke concerns [11,12], which were proposed to be abated using eco-friendly alternatives (e.g., GLDA, HIDS, EDDS, or IDSA) [13–17]. The rapid biodegradation of the Fe-chelators [18,19], nevertheless, can be a limiting factor considering the time-lag required for fertilizer to complete its role as a metal complexone [4,13].

The organomineral fertilizers primarily consist of humic substances (HS) [20,21], as isolated from naturally oxidized lignite derivatives [22], enhance the rates of seed germination, nutrient uptake, root growth, and crop yields [23–25]. The effect of HS in increasing bioavailability of plant-micronutrients (e.g., Fe, Zn, Mn, and so forth) was attributable to the complexation between HS and metals [26]. The HS also

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aids in the diffusion of plant-nutrients among the soil-layers and uptake in plants without any negative impact [21,26,27]. A higher bioavailability of HS-Fe complexes than those with EDTA [28], desferrioxamine B or ferrichrome [29] has also been reported. An increase in Fe-bioavailability to plants with the lignite-derived HS application compared to those from other origins has also been observed [30]. The humic (HA) and fulvic acids are the acid-base reactive fractions in the HS that are usually separated using their corresponding solubility in alkalis and acids [22].

The proton- and metal-binding behavior of HS in solution have been an issue of interest because of their role in the acid-base equilibria of natural aqueous systems, and impact of solution pH on the complexation between metal and HS [31]. The acid-base equilibria of —COOH (carboxyl), Ph-OH (phenolic-hydroxyl) groups control the proton- or ion-binding properties of HS, which can be measured using potentiometric titrations as reported earlier for natural-source-derived HS [27,31,32].

The structural chemistry of lignite-derived HA ($HA_{lignite}$) is different from those derived-from other origins because of the extensive-pretreatments with oxidizing reagents or mineral acids of the parent materials [20], which increases the contents of oxygenated functional groups, such as carboxyl, phenolic-hydroxyl and carbonyl ($>C{=}O$), as well as the reactivity or solubility of the HA [22]. Thus, the $HA_{lignite}$ could offer several reactive sites for ion-binding [32], and interactions could occur through the water-bridge formation, electrostatic bonding with $COO^-$ group, coordinate linkage with a single donor functional group, or combined-chelation in carboxyl and phenolic-hydroxyl sites [4]. The HAs are assumed to be an assorted-assemblage of several smaller molecular fragments that are bound by weak dispersive forces, such as, van dar waals, π–π or CH–π interactions and so forth, to form supramolecular aggregates [33]. The disparity in conformational distribution among the $HA_{lignite}$ size-fractions via molecular characterization [34], or the corresponding impact of $HA_{lignite}$-fractionation on iron solubility [35] has been reported.

The current work aimed to study the proton- and iron-binding behavior of $HA_{lignite}$ size-fractions in the aqueous matrix, which has not been reported before. The results supposed to extend the knowledge on the suitability of $HA_{lignite}$ size-fractions compared to the classical or biodegradable Fe-chelators as a micronutrient carrier in soil environment.

## 2. Experimental

### 2.1. Reagents

The $HA_{lignite}$, as supplied by Denka Co., Ltd. (Tokyo, Japan), was isolated from lignite samples of Berezovsky, Russia using an $HNO_3$-assisted degradation followed by a modified version of the IHSS recommended scheme [36]. The ultimate analysis of the dried (at 105 °C) $HA_{lignite}$ sample using a CHN-O analyzer (Flash 2000; Thermo Scientific, Waltham, MA, USA) confirmed the following elemental composition (wt%): C, 52.0; H, 3.8; N, 4.0; O, 34.6. The distribution of carbon in the functional groups was determined using the protocols from Tsutsuki et al. [37] to confirm the following ($mmol\ g^{-1}$ C): OH + COOH, 13.9; COOH, 12.3.

The $HA_{lignite}$ was subsequently fractionated in a Vivaflow 200 system (Sartorius Stedim, Surrey, UK) using tangential-flow ultrafiltration technique. The Fig. S1 (Appendix A: Supplementary information) shows the complete scheme for $HA_{lignite}$ isolation and $HA_{lignite}$ fractionation. The average molecular weight and total organic carbon content in the $HA_{lignite}$ size-fractions are listed in Table 1.

**Table 1**
Characteristics of the lignite-derived humic acid ($HA_{lignite}$) size-fractions.

| | $HA_{lignite}$ (5–10 kDa)† | $HA_{lignite}$ (10–30 kDa)† | $HA_{lignite}$ (30–100 kDa)† | $HA_{lignite}$ (>100 kDa)† |
|---|---|---|---|---|
| AMW (Da)[a] | 1200 | 2000 | 2700 | 4300 |
| TOC ($mg\ L^{-1}$)[b] | 2000 | 880 | 1900 | 2600 |

† The values in the parenthesis indicate the size of the filter used for size-fractionation. The filter size is decided according to the ratios of 'molecular length (size)' and 'molecular weight' of proteins. Although the shape and ratio of humic acids used in the current work were different from the filter size, it has been mentioned to describe the variation in fractionation assembly.

[a] AMW stands for 'average molecular weight' as found in reference to sodium polystyrenesulfonate markers.

[b] TOC stands for 'total organic carbon', which indicate the HA concentrations in a solution that separated directly from the ultrafiltration procedure as shown in the Fig. S1 (Appendix A: Supplementary information).

All the other chemicals or solvents are of analytical reagent grade. Titrisol® iron standard ($FeCl_3$ in 15% HCl) (Merck KGaA, Darmstadt, Germany) was used to prepare the iron stock solution. The carbonate-free potassium hydroxide (KOH) (Kanto Chemical, Tokyo, Japan) was standardized using potassium hydrogen phthalate (Wako Pure Chemical, Osaka, Japan. The pre-standardized hydrochloric acid (HCl) (Kanto Chemical, Tokyo, Japan) solution was used for neutralization of the initially added KOH in $HA_{lignite}$ samples. The potassium chloride (KCl) (Wako Pure Chemical, Osaka, Japan) was used to adjust the solution ionic strength ($I$).

### 2.2. Sample preparation and potentiometric measurements

The $HA_{lignite}$ solutions in the aqueous matrix (50 $cm^3$, total volume) were prepared at the following compositions: (a) $HA_{lignite}$, 160 $mg\ L^{-1}$ + HCl, 0.1 $mol\ dm^{-3}$ + KCl, 1.0 $mol\ dm^{-3}$; (b) solution (a) + $Fe^{3+}$, $1.0 \times 10^{-3}\ mol\ dm^{-3}$. The solution (a) was used to determine the protonation constants, and the solution (b) was used for the determination of stability constants of $HA_{lignite}$-Fe systems. The intermolecular interactions between the ionic charges occur more effectively in the solvent systems at $I \geq 0.1\ mol\ dm^{-3}$ due to the compressed ionic atmosphere [38,39], which prompted to select $I = 0.1\ mol\ dm^{-3}$ for all the experiments.

The solution systems have been titrated with 0.1 $mol\ dm^{-3}$ carbonate-free KOH using the KEM AT-710 automatic titrator (Kyoto Electronics; Kyoto, Japan). The potentiometric titration assembly includes a pH-combination electrode, a temperature probe, and a magnetic stirrer. The solutions were placed in a sealed vessel (100 $cm^3$) containing electrode, temperature probe, and titrant dosing nozzle inlets, plus in-out options for $N_2$ gas. The solution in vessel was placed in the SKG-01 jacketed heat exchanger bath (AS ONE, Tokyo, Japan), while the system temperature was maintained at 25 ± 0.1 °C using a combination of the TBK202HA constant temperature water heater (Advantec, Tokyo, Japan) and the Eyela CTP-1000 thermo-controlled water circulator (Tokyo Rikakikai, Tokyo, Japan). The flow of $N_2$ gas was continued through the solution system to eliminate the $CO_2$ ingress and to achieve inert atmosphere. The standard pH solutions (Horiba Scientific, Kyoto, Japan) were used to calibrate the electrode at pH 4.0, 7.0 and 9.0 before each titration. The electrode can measure solution pH up to the third decimal place of pH units with a precision of ±0.001, and the potential were simultaneously recorded with a precision of ±0.1 mV. The rate of drift of the electrode was recorded over a pre-fixed interval (3 min) with a constant increment of titrant volume (0.02 mL), and an adequate mixing of the components was ensured through continuous stirring. The titrant addition was continued, starting from pH 2.5, until the solution pH changes to 11, as achieved within an average time-lag of 14 ± 2 h, to obtain a real-time titration curve from the recorded data. All the titrations were carried out from the acid side due to the presumed possibility of higher pH limit of system homogeneity in such a case [40]. Each experiment was repeated at least five times, and the potentiometric data points used for analysis were not less than 150.