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Investigation of the Dependence of Biological Activity on the Structural Parameters of Native and Modified Humic Acids from Brown Coal

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Abstract

The samples of humic acids (HA) isolated from brown coal, both native and modified with hydrogen peroxide and *n*-butanol, were obtained and studied by means of elemental and technical analysis. The changes of structural parameters and the concentration of paramagnetic centres (PMC) of modified HA were revealed by means of ^{13}C NMR, IR, EPR spectroscopy. The biological activity of native and modified HA was evaluated by means of phytotesting with the seeds of Iren wheat as an example. A trend to a decrease in the biological activity of HA with a decrease in PMC content was discovered. An increase in biological activity is connected with an increase in parameter F_1 depicting the relations between hydrophilic and hydrophobic elements in HA structure.

Keywords: humic acids, modification, biological activity, phytoactivity index

INTRODUCTION

Humic acids (HA) are natural high-molecular compounds of irregular structures that are present in peat, brown and oxidized black coal, soil, bottom sediments. HA from different sources differ from each other in elemental composition, condensation degree, the substitution of aromatic cores, the relations between hydrophilic and hydrophobic fragments.

HA macromolecules include various functional groups: carbonyl, carboxyl, hydroxyl. Due to the unique nature of HA structure, these molecules are able to enter oxidation-reduction reactions, complexing and ion exchange with metal cations, which defines a broad range of their application [1]. HA as the sorbents of various metals are used to cope with chemical pollution, to purify industrial waste waters, to detoxify contaminated soil, etc. They are used for remediation and recultivation of degraded soil improving its structure and enhancing its fertility [2]. Positive results are known for the application of the salts of HA (humates) in plant cultivation, poultry keeping, animal husbandry [3]. During recent years, the attention to the application of HA and related preparations in agriculture as the stimulators of plant growth has increased. It is known that the effect of HA on plants involves activation of the synthesis of proteins, nucleic acids, phosphorus-containing compounds acting as energy transferring agents. Humic acids affect the enzymatic activity of a plant cell, enhance photochemical processes, electron transport, phosphorylation in chloroplasts [4]. These processes involve an increase in the permeability of cell membranes, which simplifies the transport of nutrients and microelements inside cells and accelerates plant breathing [3]. As a result, ripening time decreases, crop productivity increases, resistance to diseases and

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stability to unfavourable environmental conditions improve, the income of heavy metals and radionuclides into plants decreases. By present, substantial experimental experience has been accumulated proving the positive effect of humic substances on the crop productivity and quality [5]. However, the ideas concerning the nature of the biological activity of HA are ambiguous and contradictory. The opinion exists that the biological activity of HA is determined by their ability to participate in oxidation-reduction processes in plant cells and depends on the content of phenol and quinoid groups [6, 7]. It was demonstrated that the biological activity of HA increases with an increase in the structural parameter describing the degree of aromaticity f_a [6, 8]. The interrelation between biological activity and the content of paramagnetic centres (PMC) was discovered [9], as well as the effect of the ratio of hydrophilic to hydrophobic fragments in HA structure on the biological activity [10, 11]. The higher extent of condensation, aromaticity, paramagnetism, and the higher content of functional groups, in particular quinoid ones, provide higher biological activity of HA extracted from brown coal, in comparison with HA of peat [12].

During the recent years, substantial attention of researchers is attracted to chemical modification of HA, which provides the possibility to obtain HA-based preparations possessing valuable properties exceeding the characteristics of initial HA. Directed chemical modification due to the changes in the functional group composition may be used to govern oxidation-reduction properties, to enhance sorption capacity and biological activity, etc. [13, 14]. Various reactions are used to modify HA, for example oxidation-reduction, hydrolysis, alkylation and so on. It was demonstrated [15] that HA oxidized with potassium permanganate and alkylated with methyl are much more efficient in stimulating plant growth than initial HA. At present, it is impossible to enhance the productivity and quality of agricultural products without advanced technologies, in particular those involving biologically active substances. Changing the structural group composition as a result of the application of chemical methods to HA macromolecule, one may predict its biological activity.

The goal of the present work was to study the biological activity of native humic acids extracted from brown coal and the acids modified with hydrogen peroxide and *n*-butanol.

EXPERIMENTAL

Materials

To obtain HA, we used brown coal samples from the Tyulgan deposit of the Southern Ural basin (BCT), from the Tisyl deposit of the Kansk-Achinsk basin (BCTS) and its bed-oxidized form (BCTSO). Humic acids were extracted from coal by treating it with a solution of sodium hydroxide and precipitation with hydrochloric acid [16]. The characteristics of the samples of brown coal under investigation and HA obtained from them are listed in Table 1. Oxidized coal (BCTSO) is distinguished by higher degree of aromaticity (C/H = 1.7) and higher yield of HA.

Investigation procedures

HA modification with hydrogen peroxide was carried out as follows: hydrogen peroxide (concentration 32 %, volume V = 5, 10, 15 mL) was added drop by drop from a burette at a constant rate to a definite volume of a solution containing 5 g of sodium humate (HumNa), under perma-

TABLE 1

Technical and elemental analysis of the studied samples of coal and humic acids, %

Sample	W^a	A^d	V^{daf}	Elemental composition per daf			C/H атом.	$(HA)_t^{daf}$
				C	H	(O + N +S), from difference		
BCTS	8.0	6.1	48.1	64.3	4.7	31.0	1.1	21.3
HA BCTS	4.9	9.2	–	59.1	4.9	36.1	1.0	–
BCTSO	13.5	46.6	90.8	55.1	2.7	42.3	1.7	60.9
HA BCTSO	7.0	15.2	–	61.6	5.4	33.1	1.0	–
BCT	9.1	21.5	64.4	63.7	5.9	30.4	0.9	38.5
HA BCT	0.7	13.4	–	53.2	10.5	36.3	0.4	–

Note. 1. W^a is analytical moisture; A^d – ash content per the dry sample; V^{daf} is the content of volatile substances; C, H, O, N, S – content of elements; $(HA)_t^{daf}$ is the yield of humic acids; daf is the dry ash-free state of the sample. 2. Dash means that the parameter was not determined.

nent mixing with a magnetic mixer. After the addition of H_2O_2, mixing went on for 5 min, and then HA precipitation with hydrochloric acid was carried out. The precipitates were separated by filtering, washed to achieve pH of distilled water, dried at a temperature of 70 °C to a constant mass.

For alkylation, a weighted HA portion (5 g) was dissolved in 100 mL *n*-butanol, acidified with hydrochloric acid to pH 2–3, boiled for 3 h at the boiling point of butanol, and cooled to the room temperature. Thus obtained mixture was diluted with two litres of distilled water. The precipitate was separated using a Bunsen flask with a funnel, then it was washed with a large amount of water, and dried to a constant mass.

To establish a relation between the structural parameter and biological activity, phyto-tests were carried out [17, 18] using the grains of the Iren wheat variety. The biological activity of HA in the form of sodium humates (concentration: 0.01, 0.005 and 0.0005 %) obtained both from initial HA samples and from those modified using hydrogen peroxide and butanol (HA-H_2O_2 and HA-Bu, respectively) was determined according to GOST 12038–84 and GOST 54221–2010 [19, 20]. For this purpose, the seeds were germinated at a constant temperature 20 °C in the dark in special germinating trays. The biological activity of native and modified HA was evaluated on the basis of the integral index of phytoactivity (IP) taking into account three test functions: the power of seed germination (PG), root length (RL), and seedling height (SH). The index of phytoactivity is a generalizing parameter depicting deviations from the reference and is calculated as an arithmetic mean of the sum of PG, RL and SH expressed in portions of unity:

$$IP = \frac{PG + RL + SH}{3 \cdot 100}$$

where PG, RL and SH are average values over three trays.

The experiment was made in the triple with 50 seeds in each tray for each HA kind, and the same number for reference. Only distilled water was used for a reference test. The parameters PG, SH and RL, as well as the amount of roots (AR), were measured on the fifth day. Relative error was 3–5 % for the significance level α = 0.05 in all experiments.

Methods of investigation

The EPR spectra of the samples under investigation were recorded at room temperature with the help of an EMX-m40X EPR spectrometer (Bruker, Germany) at the frequency of 9.86 GHz with the power level of 1.8–1.9 mW and modulation frequency 100 kHz. To determine the amount of organic paramagnetic centres (PMC), Mn^{2+} ions in MgO were used as a reference. The characteristics of EPR spectra were calculated with the help of BrukerWinEPR software.

High-resolution ^{13}C NMR spectra in solids were recorded with an AVANCE III 300 WB instrument (Bruker, Germany) at the frequency of 75 MHz using the standard procedure of cross polarization and magic angle spinning (CPMAS).

IR spectra were recorded with an Infralyum-FT 801 IR-Fourier transform spectrometer (Russia) in tablets with KBr.

RESULTS AND DISCUSSION

Humic acids are characterized by typical absorption bands differing from each other in intensities in the IR spectra [21]. A broad absorption band with the maximum at ~3400 cm^{-1} points to the presence of hydroxyl groups involved in hydrogen bonds. The bands at 2940–2920 and 2860–2840 cm^{-1} correspond to the stretching vibrations of CH_3 and CH_2 groups; 1630–1610 cm^{-1} – to the stretching vibrations of conjugated C=C double bonds and aromatic fragments; 1370–1450 cm^{-1} – to the bending vibrations of C–H bonds in aliphatic groups CH_3 and CH_2; 1280–1240 cm^{-1} – C–O bonds in carboxylic acids, esters, O–H bonds in phenols; 1100–1030 cm^{-1} – C–O bonds in cyclic and aliphatic ethers and alcohols; 800 cm^{-1} – C=C and C–H bonds in aromatic rings. For all samples of initial HA under investigation, the bands of different intensities appear within the region of 1720–1710 cm^{-1} – these are stretching vibrations of C=O bonds in carboxylic acids. In the IR spectra of HA from BSTS and BST series modified with hydrogen peroxide, the intensities of the bands at 1710, 1260, 1100–1030 cm^{-1} are increased. he spectra of HA from BCTSO are distinguished by a decrease in the intensity of the band related to C=O bonds of carboxylic groups. IR spectra of HA modified with *n*-butanol display an increase (to different extents) in the intensities of the bands at 2930 and 2870, 1450 and 1380, 1240 cm^{-1}.

Experimental data on the determination of the biological activity of HA by means of phyto-testing are presented in Table 2. These procedures revealed not only plant stimulating effects but also suppressions of one or another test function. One of the factors affecting the biological activity

TABLE 2

Test functions and the integral index of phytoactivity of humic preparations

Sample	Concentration, %	AR	RL	SH	PG	IP	PMC · 10^{-18}, spin/g (HA)
		% with respect to the reference					
HumNa BCTS	0.005	115	118	109	115	1.14	7.57
HumNa BCTS	0.0005	102	96	108	107	1.04	–
HumNa BCTS	0.01	102	94	100	93	0.96	–
HumNa BCTS-H_2O_2(5)	0.005	113	104	118	112	1.11	3.77
HumNa BCTS-H_2O_2(10)	0.005	107	91	88	93	0.91	3.12
HumNa BCTS-H_2O_2(15)	0.005	103	83	78	101	0.87	2.72
HumNa BCTS-Bu	0.005	106	125	137	110	1.24	4.40
HumNa BCTSO	0.005	104	123	138	106	1.22	0.87
HumNa BCTSO	0.0005	–	101	103	108	1.04	–
HumNa BCTSO-H_2O_2(5)	0.005	101	101	125	103	1.10	0.56
HumNa BCTSO-H_2O_2(10)	0.005	99	105	120	98	1.08	0.29
HumNa BCTSO-H_2O_2(10)	0.0005	–	101	103	108	1.04	–
HumNa BCTSO-H_2O_2(15)	0.005	101	85	79	107	0.90	0.28
HumNa BCTSO-Bu	0.005	102	130	145	106	1.27	1.93
HumNa BCT	0.005	–	125	149	107	1.27	0.31
HumNa BCT	0.0005	–	112	149	107	1.22	–
HumNa BCT-H_2O_2(10)	0.005	–	122	147	112	1.27	0.47
HumNa BCT-H_2O_2(10)	0.0005	–	116	134	108	1.19	–
HumNa BCT-Bu	0.005	–	151	142	124	1.39	1.42

Note. 1. Dash means that the parameter was not determined. 2. Here and in Table 3: the amount of H_2O_2 (mL) used for sample modification is shown in parentheses; Bu is the sample modified with *n*-butanol

is the concentration of the applied humates. For the concentration of 0.005 %, the highest IP is observed for all the studied samples (see Table 2). In the reference experiment with water, IP = 1. A positive effect of HA on PG, RL and SH should be stressed. Higher biological activity is inherent in humates obtained from the naturally oxidized form of brown coal (HumNa BCTSO): its application leads to the maximal increase in SH and RL (+38 and +23 % with respect to the reference, respectively).

Chemical modification with hydrogen peroxide and *n*-butanol changes the functional group composition of HA and affects the biological activity of HA. Indeed, the use of HA modified with hydrogen peroxide leads to a decrease in all test functions and IP for all samples under study, in comparison with native samples (see Table 2). An insignificant decrease in AR is observed. After seed treatment with the preparations HumNa BCTS(10), HumNa BCTS(15), HumNa BCTSO(15) (the volume (mL) of H_2O_2 used for modification is shown in parentheses) IP values are lower than in reference experiments with water. All test functions RL, SH and PG in these experiments are lower than the reference values, too. The index of phytoactivity of humates modified with *n*-butanol exceeds the corresponding parameter of native preparations and is equal to 1.24 (+24 % increase with respect to the reference) for HumNa BCTS; 1.27 (+27 %) for HumNa BCTSO and 1.39 (+39 %) for HumNa BCT. The use of preparations alkylated with *n*-butanol has a positive effect on SH (+37 % for HumNa BCTS; +45 % for HumNa BCTSO; +42 % for HumNa BCT with respect to the reference) and RL. A maximal increase in RL (by a factor of 1.5) is observed for the application of HumNa BCT. So, modification with hydrogen peroxide causes a decrease in IP, while modification with *n*-butanol leads to a decrease in IP as a sequence: HA-Bu > HA > HA-H_2O_2(5) > HA-H_2O_2(10) > HA-H_2O_2(15).

The data shown in Table 2 provide evidence of a decrease in IP with a decrease in PMC content during HA modification with hydrogen peroxide. Evaluating a correlation of the biological activity of HA with the paramagnetic properties, it should be stressed that a straight dependence of IP on PMC content is characterized by a rather low correlation coefficient: 0.77 (for HA BCTS) and 0.83 (for HA BCTSO) (Fig. 1). As mentioned above, the modification of HA with *n*-butanol causes an

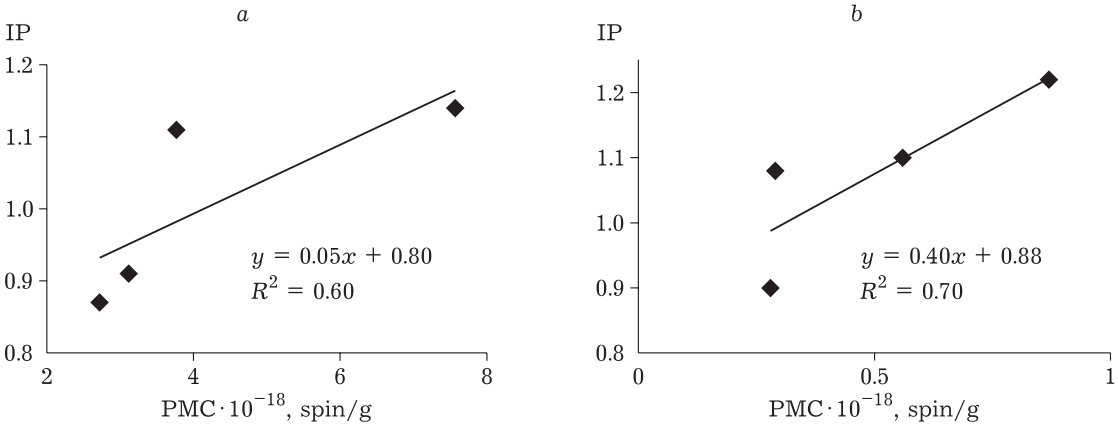

Fig. 1. Dependence of the index of phytoactivity (IP) on the content of paramagnetic centres (PMC) in native and H_2O_2-modified humic acids obtained from brown coal BCTS (*a*) and BCTSO (*b*).

increase in IP for all samples. For HA of BCTSO and BCT series, an increase in PMC content is observed, while for HA BCTS – a decrease. So, it is probable that a correlation between PMC content and biological activity is not so simple and unambiguous. An opinion exists that the paramagnetic properties of HA are due to the synergic effect of the interaction of aromatic systems of polyconjugation and hydrogen bonds formed by the functional groups, first of all, carboxylic ones [12]. Quinoid groups and phenol hydroxyls able to form free radicals also may be a reason for HA paramagnetism [7].

Though HA under investigation have a common structural type, which is confirmed by the appearance of NMR signals in the same regions, they differ from each other in the integral intensities of these signals. Analysis of the data shown in Table 3 suggests that the changes in the structural group composition caused by HA modification depend on the nature of the initial raw material. For example, HA extracted from the oxidized coal BCTSO differ from HA of BCTS by a higher content of carboxylic groups, which decreases during the oxidation with H_2O_2, perhaps because of decarboxylation. An inverse trend is observed for HA of BCTS: the content of carboxylic groups increases as a result of oxidation. Esterification of carboxylic acids during alkylation [22] leads to a decrease in their content in all HA samples. The

TABLE 3

Integral intensities of spectral regions according to the data of ^{13}C NMR spectra of samples, %

Sample	Chemical shift, ppm							F_1
	220–187	186–180	179–165	164–140	140–106	106–54	54–0	
	C=O	C_{quin}	COOH	$C_{ar}O$	$C_{ar}C,H$	$C_{alk}O$	C_{alk}	
HA BCTS	0.99	<0.2	5.70	10.30	24.37	3.57	55.07	0.80
HA BCTS-H_2O_2 (5)	1.21	<0.2	6.01	9.16	25.10	4.06	54.44	0.76
HA BCTS-H_2O_2(10)	1.29	<0.2	6.43	9.40	24.88	4.15	53.86	0.80
HA BCTS-H_2O_2(15)	1.18	<0.2	6.75	9.00	24.84	4.60	53.63	0.82
HA BCTS-Bu	0.47	0.30	5.32	15.18	37.55	13.13	28.05	0.89
HA BCTSO	0.59	1.03	14.48	3.61	43.82	4.27	32.22	0.51
HA BCTSO-H_2O_2(5)	1.17	0.67	11.76	3.18	37.52	5.80	39.90	0.55
HA BCTSO-H_2O_2(10)	0.50	0.80	13.16	3.84	44.38	5.26	32.06	0.50
HA BCTSO-H_2O_2 (15)	0.80	0.70	12.89	2.81	39.00	5.36	38.45	0.54
HA BCTSO-Bu	0.42	0.68	9.70	7.94	34.19	10.69	36.37	0.82
HA BCT	0.75	<0.2	7.30	7.67	33.19	8.33	42.81	0.70
HA BCT-H_2O_2(5)	0.70	<0.2	6.84	7.14	31.43	8.60	45.30	0.72
HA BCT-H_2O_2(10)	0.51	<0.2	7.61	7.32	33.28	7.99	43.29	0.69
HA BCT-Bu	0.66	<0.2	6.52	7.46	28.40	14.37	42.76	0.99

Note. For designations, see Table 2.

dependence of IP on the content of quinoid groups cannot be revealed because of the low content of these groups (less than 1 %).

An integrated structural parameter F_1 characterising the ratio of active functional groups and $C_{alk}O$-fragments in HA to the value of the aromatic component is shown in Table 3. It is calculated relying on the data of ^{13}C NMR spectroscopy: $F_1 = (COOH + C_{ar}O + C_{alk}O)/C_{ar}C,H$ [11]. This parameter also determines the relations between hydrophilic and hydrophobic properties of HA macromolecule. Almost for all the studied HA samples modified with *n*-butanol, the intensity of NMR signals in the region of 160–140 ppm ($C_{ar}O$) and 106–54 ppm ($C_{alk}O$) increases, which causes a substantial increase in F_1 for alkylated samples and an increase in IP.

These results allow us to conclude that modification with hydrogen peroxide and *n*-butanol causes substantial changes in the structural group composition of HA, in PMC content, in hydrophilic-hydrophobic properties and, as a consequence, in biological activity.

CONCLUSION

It was established in the experiments with wheat seeds that the index of phytoactivity of humic acids extracted from brown coal increases by 14–27 % in comparison with the reference test and depends on the concentration of the applied humates. The largest effect is for humate concentration equal to 0.005 %. As a result of chemical modification, the structural group composition and biological activity of humic acids change. Humic acids modified with hydrogen peroxide exhibit lower biological activity in comparison with native samples, while the samples modified with *n*-butanol demonstrate higher biological activity. A trend to a decrease in the index of phytoactivity of humic acids with a decrease in the content of paramagnetic centres caused by modification with hydrogen peroxide was revealed. However, this parameter increases with an increase in the hydrophilic-hydrophobic factor F_1 as a result of modification with *n*-butanol.

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