Role of paramagnetic polyconjugated clusters in lignin antioxidant activity (*in vitro*)

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Abstract. Using physico-chemical methods (EPR, SEC, Py-GC/MS and UV/VIS spectroscopy) and wet chemical analysis, the characteristics of 6 hardwood lignins in terms of functionality, molecular weight and composition of lignin substructures were determined and considered together with the results of $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and $O_2^{\bullet-}$ antioxidant assays with the aim to understand the relationships governing antioxidant properties of lignin. The strong positive linear correlation between lignin antioxidant capacity in the three assays used and the extent of conjugation of paramagnetic polyconjugated clusters in lignin macromolecules was found. The biological activity of the most active alkaline lignins was assessed by *in vitro* experiment with human blood.

1. Introduction

The application of natural antioxidants instead of the synthetic ones is a subject widely investigated with emphasis on reduction of the ecological problems. Phenylpropanoid polymer lignin *in situ* (Figure 1) serves to protect plants against chemical, biological and mechanical stresses. Antioxidant activity of technical lignins obtained from plant biomass processing in multi-tonnage scale are well documented [2-4]. Polymeric antioxidants due to their higher compatibility with polymeric systems and slower degradation rate often can be successfully applied in those fields in which the employment of a single molecule with antioxidant activity is inefficient. For example, lignins, comparing to

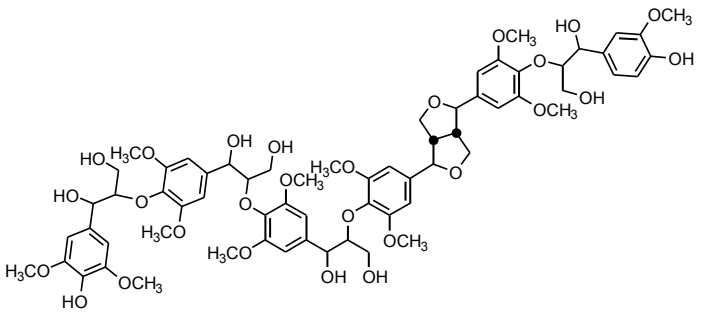

Figure 1. Potential structure of a repeated unit in macromolecule of hardwood lignin *in situ* [1]

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flavanoids, have longer circulation time in human and animal organisms and are much less toxic.

However, due to molecular complexity of lignins, it is difficult to assign their antioxidant activity to specific structural elements and further search of the relationship "structure-activity" is needed to determine directions for goal-oriented tuning of lignin structure, in order to use these naturally originated products successfully. The chemical structure of lignins (functionality, molar mass, cross linking density, etc.) depends on a number of factors including the botanical origin, the environmental conditions of growth and also the conditions of isolation from plant tissue due to all the isolation techniques significantly influence lignin intermonomeric linkages. Technical lignins, in particular those obtained from processing of lignocelluloses under conditions promoting double bonds formation (e.g. alkaline delignification, acid hydrolysis, thermal and hydrothermal treatment), are characterized with formation in their macromolecules of sub-structures with spatially extended π-bonding systems, so called polyconjugated systems (PCS). The polymers with high-developed PCS display high antioxidant, radioprotective and photoprotective properties [5]. The specific feature of PCS is the presence of paramagnetic centres of high-stability responsible for scavenging (deactivation) of labile free radicals [3,6].

The aim of the present work was to assess antioxidant and biological activity *in vitro* for a set of hardwood lignins and to investigate the relevance of lignin structural features, in particular development of polyconjugated clusters, molecular weight, functionality (phenoxy- and methoxy groups) and lignin antioxidant activity.

2. Experimental

Lignins isolated from hardwood species (alder, aspen and ash-tree) by alkaline delignification (Alk-L) and fast pyrolysis (Py-L) were the objects of the study. EPR spectroscopy and analytical pyrolysis (Py-GC/MS) were used as the main analytical tools for obtaining direct information on the extent of conjugation ("delocalization length") for lignin PCS and macromolecule condensity, respectively.

Ash tree (*Fraxinus excelsior*), alder (*Alnus incana*), aspen (*Populus tremula*) wood sawdust (fraction 0.1-1.0 mm, moisture 8.5-9%) were used as raw-materials for lignins obtaining by the alkaline delignification and fast pyrolysis treatment [7].

The polyconjugated paramagnetic clusters in lignin samples were characterized in terms of extent of polyconjugation estimated using the value of the conjugation length of π-polyconjugation systems calculated from the EPR spectra as the number of CH fragments (n) included in the region of unpaired electron delocalization [3]. The EPR spectra were recorded at 293K using an EMX-6/1 spectrometer (BRUKER) working at X-band frequency with 100 kHz modulation.

The condensity of lignin macromolecules as well as the content of carbohydrates admixtures were characterized by Py-GC/MS with Shimadzu GC/MS-QP 2010, column RTX-1701, 60 m x 0.25 mm x 0.25 μm. The key functionalities of lignin macromolecule, in particular phenolic hydroxyl and methoxy-groups were determined, respectively, by conductometric titration and the Viebock-Schwappach method [8]. All analytical data are shown on oven dry samples. The molecular weight of lignins was determined by the size exclusion chromatography (SEC) method (Agilent 1100 system, UV-DAD/RI, 60^{o}C, column AGI_PLgel 5μm MIXED-D 300x7.5 mm, eluent DMSO).

Antioxidant activity was evaluated using three radical scavenging assays: deactivation of $ABTS^{\bullet+}$ cation-radicals and $DPPH^{\bullet}$ radicals, inhibition of generation of superoxide anion-radicals $O_2^{\bullet-}$ in the biosystem hypoxanthine-xanthine oxidase. Radical scavenging activity was expressed by IC_{50}, i.e. the concentration of lignin required for 50% inhibition of the radical species. The values of IC_{50} were calculated from the dependence of the free radical inhibition on lignin concentration determined by UV/VIS spectroscopy [3,9]. Trolox (water soluble analogue of E vitamin) was used as the reference antioxidant.

Biological activity of lignins was tested in the experiment *in vitro* with blood samples taken from voluntaries. The blood markers relevant to oxidative stress, namely the indices of blood lipids peroxidation (MDA) and proteins oxidation (PC) as well as glucose level, were analyzed using standardized biochemical methods.

All the analyses were carried out five times and the data were compared by linear regression analysis using the SPSS Statistics 17,0. Differences were considered significant for $p < 0.05$. The graphs were plotted using mean values.

3. Results and Discussion

All investigated lignin samples revealed high dose-dependant radical scavenging activity in three tests applied (Figure 2). Pyrolytic lignins had the lowest IC_{50} values, i.e. the maximum antioxidant activity; their antioxidant activity was close and even higher than that for Trolox.

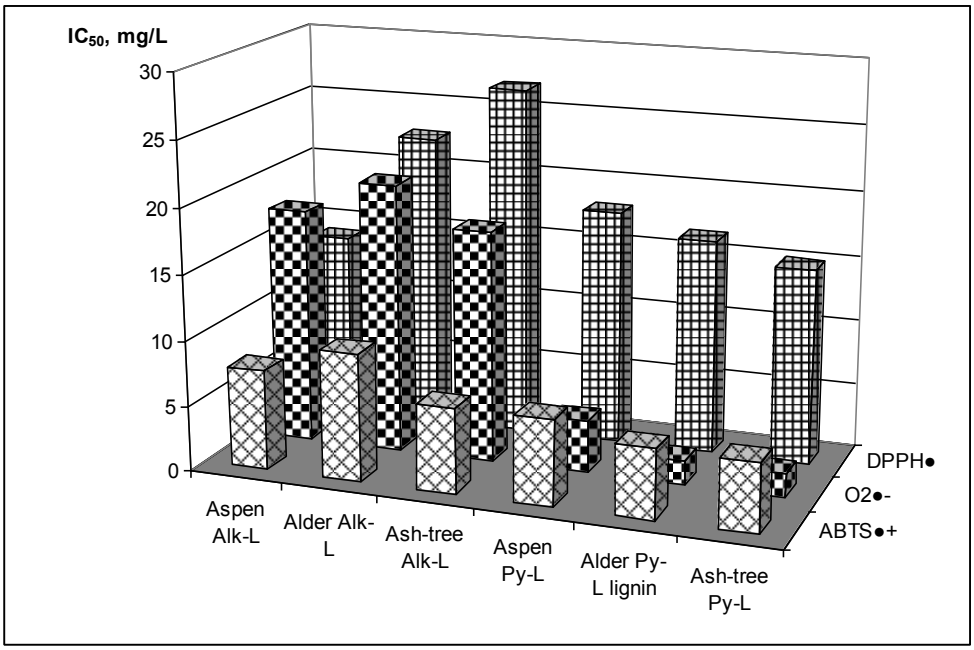

Figure 2. Half inhibition concentrations (IC_{50}) of lignins. For Trolox, IC_{50} values of 4.2, 4.8 and 17.7 mg/L in $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and $O_2^{\bullet-}$ assays, respectively, were found.

For all lignins investigated, the results of EPR spectroscopy show an intensive narrow single line spectrum with a g-factor of about 2.0030 and a line width of 0.3 – 0.5 mT that is characteristic for an unpaired electron localized in a conjugated polymer matrix. The comparison of the values of extent of conjugation calculated from the EPR spectra for alkaline and pyrolytic lignins (Table 1) gave evidence of the intensive development of polyconjugated clusters in lignin macromolecules as the result of fast pyrolysis. Formation of phenylpropanoid aroxyl radicals is an essential step in the realization of antioxidant activity of lignin. The stability of the aroxyl radicals strongly depends on unpaired electron delocalization. The development of polyconjugated clusters in lignin macromolecules leads to the extension of electron delocalization thus decreasing dangerous prooxidant potential of polyphenols.

The data obtained using Py-GC/MS and chemical analysis showed distinctions between the lignins in terms of condensity, carbohydrates admixtures content, OH_{phen} and OCH_3 groups contents (Table 1).

For searching of correlation between antioxidant activity of lignin and its structure parameters, the term of radical scavenging index (RSI), which was defined by as the inverse of IC_{50}, was used as a dependent variable. It was found (Table 2) that a growth of extent of conjugation of paramagnetic polyconjugated clusters in lignin resulted in linear increasing antioxidant capacity (the Pearson's correlation coefficient of 0.9932). OH_{phen} and OCH_3 groups contents are lower influential features. The heterogeneity of technical lignin had negative impact on the lignin radical scavenging capacity.

The results of the tests *in vitro* clearly demonstrated dose-dependent reducing action of the ash tree and alder lignins on the indices of blood lipids peroxidation (MDA) and proteins oxidation (PC). The

decreasing action of the lignin on glucose level in blood (by 32%) was also found in tests with blood taken from voluntaries – patients with metabolic syndrom (initial glucose level of 7.4 ± 0.2 mkM/L). These results indicate the multifunctional activity of the investigated lignins.

Table 1. The characteristics of hardwood lignins structure and chemical composition

Sample	Index				
	Poly-conjugation extent, **n**	-OH_{phen} content, %	-OCH_3 content, %	Condensity	Carbohydrates content, %
Aspen Alk-L	16	5.0	18.5	2.9	23.0
Alder Alk-L	14	4.7	17.1	4.0	28.5
Ash-tree Alk-L	17	5.6	19.5	3.2	10.0
Aspen Py-L	26	6.1	9.8	13.3	20.2
Alder Py-L	30	7.1	10.9	11.0	23.2
Ash-tree Py-L	30	7,3	12.2	11.7	16.9

Table 2. Correlation between structure parameters and radical scavenging indices of hardwood lignins

Independent variables	Pearson's linear correlation coefficient, r		
	Dependent variables		
	RSI in $DPPH^{\bullet}$ test	RSI in $ABTS^{\bullet+}$ test	RSI in $O_2^{\bullet-}$ test
-OH_{phen} content	+0.77 [a]	+0.92 [b]	+0,93 [b]
Molecular weight, M_w	ns	ns	ns
-OCH_3 content	ns	ns	ns
Condensity	+0.78 [a]	ns	+0.95 [b]
Carbohydrates content	-0.97 [b]	ns	ns
Extent of conjugation	+0,88 [b]	+0.88 [b]	+0.99 [b]

[a] $P < 0.05$; [b] $P < 0.01$; «ns» indicates non-significant correlation ($P > 0.05$)

4. Conclusions

Lignins isolated from hardwood species by alkaline delignification and pyrolysis treatment demonstrated high antioxidant activity close to that of widely used reference material Trolox, which implies their application foreground as polymeric antioxidants of natural origin, capable of competing with low-molecular commercialized antioxidants. The extent of conjugation of paramagnetic clusters in lignin was found to be the major parameter predetermining antioxidant activity. Phenolic and methoxyl functional groups contents influence is of lesser importance. These regularities will bring the opportunity to determine directions for targeted modification of different types of lignins for the production of polymeric antioxidants utilizable in various technical and non-technical systems.

Acknowledgments

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