

BIOLOGICAL CONVERSION OF LIGNIN WASTE PRODUCTS FROM CHEMICAL WOOD TREATMENT INTO ANTICARCINOGENIC PREPARATIONS

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ABSTRACT

In this study biologically modified lignin by-products derived from industrial wastes of pulp production were examined for potential chemo-prevention in development of cancer as natural antimutagenic agents in practical fields instead of those prepared exclusively by organic synthesis. Biological transformation of isolated lignin was performed by using of yeast-like strain *Geotrichum klebahnii*. The structural and molecular changes in the lignin during a cultivation process were determined by ¹³C NMR spectroscopy and gel permeation chromatography analysis. The effect of biologically modified lignin on decrease of the level of deoxyribonucleic acid (DNA) strand breaks was measured using comet assay on the different lines of mammalian cells exposed to oxidative treatment with hydrogen peroxide as well as to alkylating damage with N-methyl-N-nitro-N-nitrosoguanidine.

KEY WORDS: lignin, biotransformation, *Geotrichum klebahnii*, ¹³C NMR spectroscopy, deoxyribonucleic acid (DNA)

INTRODUCTION

Lignin is a non-toxic polymer composed of phenylpropane units, e.g. guaiacyl and syringyl with hydroxyl and carbonyl substituents. The chemical treatment of wood for paper production yields about 50×10^6 t of lignin per year. Mostly it is used as a source of energy in the pulp mills. Novel methods of lignin use include application of chemically modified lignin preparations for production of phenol-formaldehyde resins (Hon and Shirashi 1991), polyurethane foams (Saraf and Glasser 1984), dispersants (Demianová et al. 1995, Košíková et al. 2000), lignin-polypropylene composites (Košíková et al. 1993, Gregorová et al. 2005) and rubber blends (Košíková and Gregorová 2005). It is known that living organisms are susceptible to attack by molecular oxygen. The damage of DNA by oxygen radicals and various chemicals is important factor in the development of chronic diseases such as

cancer. As to several drawbacks of synthetic compounds for human organism, the natural compounds received more attention in recent years for potential application in biomedicine (Hertog et al. 1993). In our research lignin fractions derived from industrial wastes of pulp production were investigated for this purpose (Slameňová et al. 2000). As to our knowledges about the ability of some yeast species to biotransform the lignin macromolecule (Košíková and Sláviková 2000), in this paper, *Geotrichum klebahnii* was used for modification of lignin biopolymer with the aim to prepare anticarcinogenic and antimutagenic compounds.

MATERIAL AND METHODS

Lignin preparations

Lignin fraction of molecular weight 2000 was obtained by fractionation of co-product of first stage of kraft pulp production from beech wood. Biologically modified lignin was prepared by treatment of this lignin fraction with yeast-like strain *Geotrichum klebahnii* (CCY 74-6-2) obtained from the Culture Collection of Yeasts (Institute of Chemistry, Slovak Academy of Sciences, Bratislava). This strain was cultivated in a medium containing water 6.7 g yeast nitrogen base (Difco) and lignin sample (3 g) per litre of solution in distilled in the presence or absence of glucose (20 g). The pH was adjusted to 6.5. Incubation took place on rotary shaker at 2.7 Hz and 28 °C for 16 days.

¹³C NMR spectra were recorded in deuterated dimethylsulfoxide at 303 K with a Bruker AM 300 NMR spectrometer operating at 75.47 MKz in the inverse gated decoupling mode.

Gel permeation chromatography was performed on a column (53 x 0.8 cm) of Sephadex LH 60 using a mixture of dioxane and water (7:3) containing 0,005 M aqueous NaOH and 0.001 M LiCl as the eluant (Košíková et al. 1990).

Cell lines

The diploid human cell line VH10, derived from foreskin of a healthy 10-year-old boy, obtained from Dr. A. Kolman (University of Stockholm). Quasidiploid Chinese hamster V79 cells were obtained from Dr. A. Abbondandolo (University of Genoa), and epithelial colon cancer cells Caco-2 from Dr. A. Collins (The Rowet Research Institute, Aberdeen, Scotland).

The level of DNA damage (DNA strand breaks) was measured using single-cell-gel electrophoresis, i.e. comet assay.

RESULTS AND DISCUSSION

The lignin fraction of molecular mass 2100 isolated from waste products of first stage of beech wood pulping was treated by the microorganism *Geotrichum klebahnii*. Production of cell protein during cultivation in the absence or presence of glucose is summarized in Tab. 1.

Tab. 1: Production of cell protein (g/L) during cultivation of *G. klebahnii*

Glucose in cultivation medium (g/L)	Cultivation time (days)			
	0	4	8	10
20	0.150	0.181	0.200	0.209
0	0.150	0.172	0.180	0.181

It is evident that growth of *G. klebahnii* was lower when lignin was the only source of carbon in the cultivation medium. After incubation of *G. klebahnii* in the presence of lignin in the medium without glucose, it was possible to isolate approximately 35 % of the original sample from the dialyzed and lyophilized supernatant by methanol extraction. This lignin fraction and original lignin were analysed by gel permeation chromatography to characterize molecular changes in the lignin. Fig. 1 shows the changes in the molecular mass distribution of the lignin sample after biological treatment, which are indicative of partial degradation of the lignin. The average molecular mass of the lignin sample was reduced to 1650 from 2100 in the original.

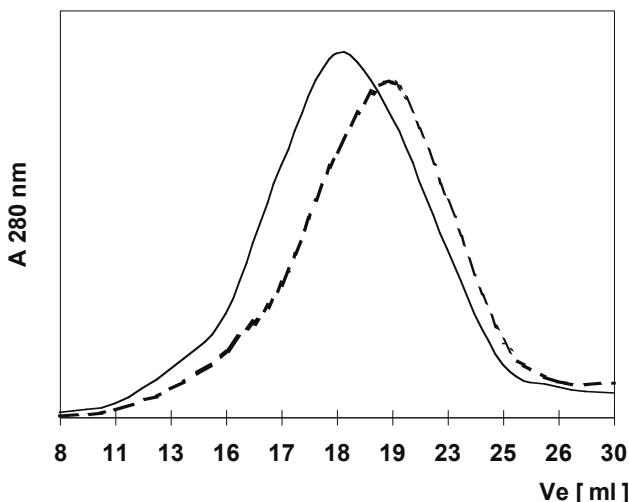


Fig. 1: The effect of *G. klebahnii* on the molecular weight distribution of lignin. Original lignin sample (solid line). Yeast-treated sample in the absence of glucose (broken line)

A comparison of the spectra of lignin samples before and after treatment with *G. klebahnii* in the absence of glucose for 16 days (Fig. 2) shows a decrease of the aromatic signals 3, 4, 5, and 8 ($\delta = 152.1, 148.3, 135.3$, and 104.2 ppm) and methoxyl signal 12 at 55.6 ppm. The structural changes of lignin tested indicate an oxidative cleavage of $C_\alpha - C_\beta$ linkages between lignin units similarly as it was described for treatment of lignin by white-

rot basidiomycetes (Chua et al. 1982) resulting in the formation of Ar-O-CH₂-COOH units (173.5 and 172.1 ppm) and COOH in Ar-COOH (165.1 ppm).

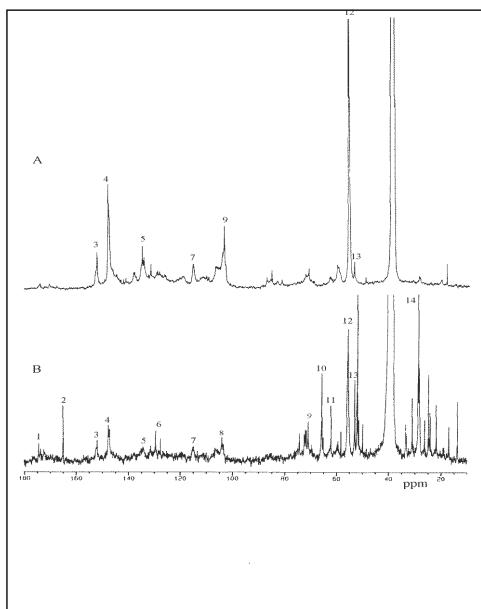


Fig. 2: ¹³C NMR spectra of lignin before modification (A) and after modification (B) with *G. klebahnii*

In further experiments, the modified lignin preparation was investigated from the view point of its ability to protect DNA of different cell lines towards oxidation and alkylation. Toxicity of this lignin sample before and after biological modification was tested on hamster cells V79 under conditions nearly identical to those of the standard assay (Fig. 3).

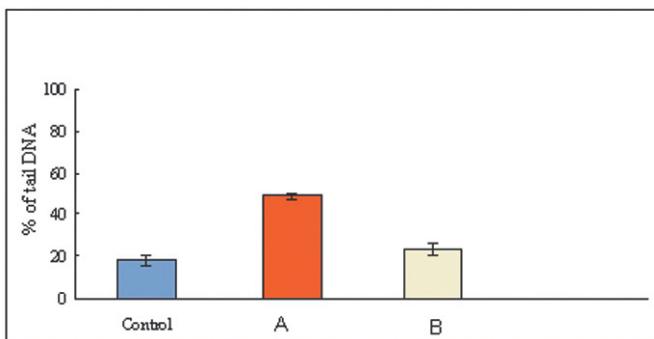


Fig. 3: The level DNA breaks in V79 cells treated with lignin before (A) and after biological modification (B)

The measure of DNA damage was expressed as „% of the tail DNA“. The obtained data illustrate that biologically modified lignin does not induce statistically significant increase of single-strand breaks. In contrast, lignin sample before modification was toxic as it is evident from an increase of single strand breaks on DNA. Antioxidative effect of biologically modified lignin sample on DNA in human cells VH10 exposed to oxidative treatment with H_2O_2 is illustrated in Fig. 4.

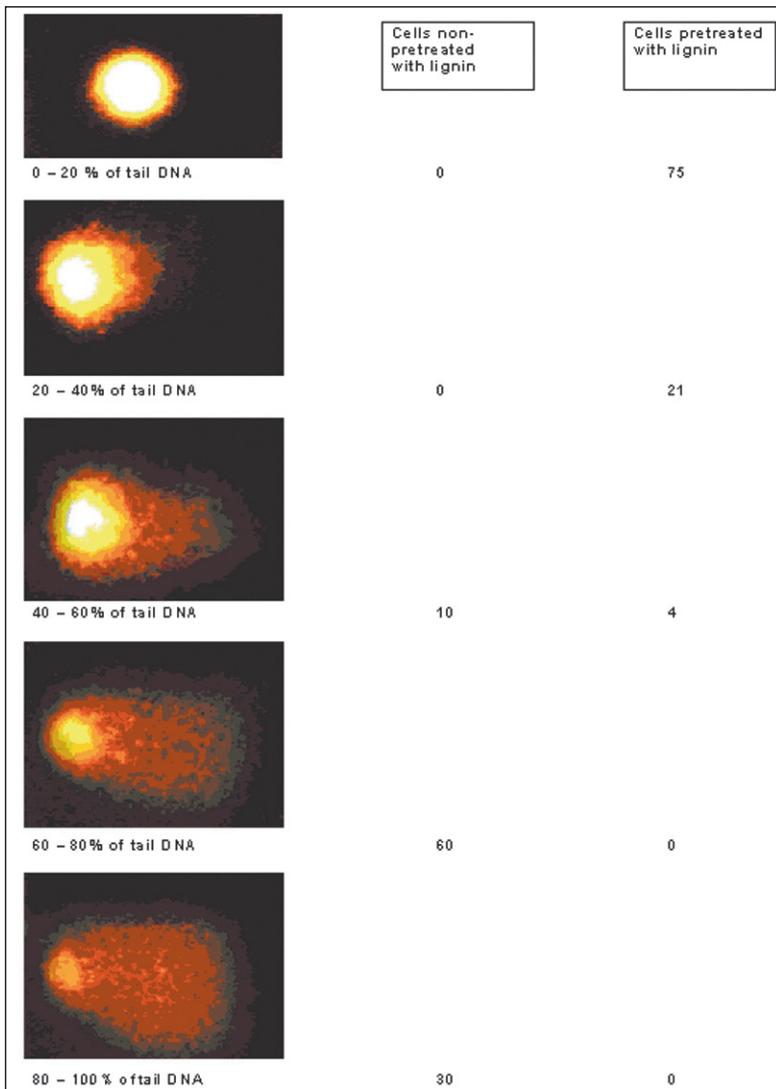


Fig. 4: Influence of biologically modified lignin on distribution of DNA strand breaks in H_2O_2 damaged human cells VH10 determined by comet assay

The obtained data confirm that cells pre-incubated with lignin before oxidative treatment contain significantly fewer heavily damage cells (percent tail DNA>40) than in those treated with H₂O₂ alone. This observation could be explained by the presence of steric hindered phenolic hydroxyl groups which act as radical scavenger. The potential protective role of the prepared lignin sample is also evident from the results performed on human Caco-2 colon carcinoma cells isolated from a primary colonic tumor exposed to oxidative damage (Fig. 5).

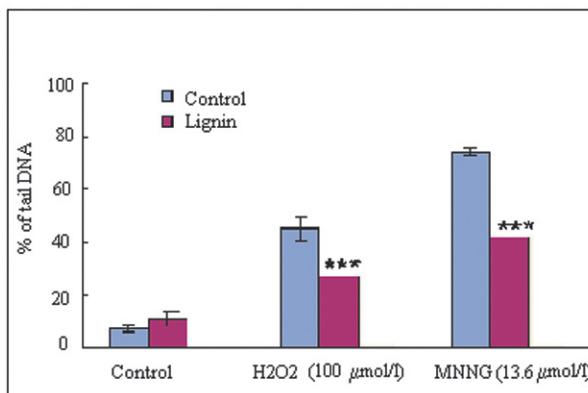


Fig. 5: Influence of biologically modified lignin on DNA strand breaks in VH10 cells induced by H₂O₂ and MNNG

The followed experiments were performed with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). It is known that it alkylates several positions on DNA. The results in Fig. 5 illustrated that the level of single-strand breaks of DNA induced by MNNG was also reduced by modified lignin in carcinoma cell line. Based on our previous study (Košíková et al. 1990) the anticarcinogenic activity of the yeast-treated lignin sample towards MNNG can be explain by ability to adsorb carcinogenic compounds as N-nitrosoamines, or to intercept the arising free radicals from MNNG decomposition.

CONCLUSION

The obtained results indicate that biologically modified non-toxic lignin preparation exhibits a high level of protection of DNA towards oxidative damage by scavenging of OH- radicals. The observed reduction of alkylating activity of MNNG to cause instability of the N-glycosyl bond on DNA may correlate with adsorption affinity of lignin towards N-nitrosoamins. This dual ability of lignin tested to decrease genotoxic activity of chemicals seems to be very promising for its prospective application in chemoprevention of cancer instead of compounds prepared by organic synthesis. Moreover, biological conversion of lignin waste products into natural antimutagenic and anticarcinogenic agents contributes to their valorization as well as to protection of environment.

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