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Microbial (1 → 3)-β-D-glucans, their preparation, physico-chemical characterization and immunomodulatory activity

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Abstract

(1 → 3)-β-D-glucans have the ability to stimulate the immune system and are classified as biological response modifiers (BRMs). Glucans from two technologically important species, baker's yeast *Saccharomyces cerevisiae* and filamentous fungus *Aspergillus niger* were isolated and characterized. Water-insoluble yeast glucan has a low branched structure with a ratio of the glucose units in the side chains and the backbone of 1:8. Using ultrasonic treatment and subsequent chemical derivatization, water-soluble derivatives (carboxymethyl and sulfoethyl) of yeast glucan were obtained with high yield. The glucan isolated from *A. niger* forms a complex with chitin and is relatively resistant to solubilization. The yield of its carboxymethylated derivative was only 30%. A method of determination of the relative ratio of α- and β-glycosidic linkages in the glucans using FTIR spectroscopy has been developed as well as a method for ultrasonic purification of the isolated glucans. Using ultrasonic treatment, glucan derivatives with decreased molecular weight (90–100 kDa) have been prepared, which show broader application possibilities in comparison with the initial high molecular-weight derivatives (300–600 kDa). It has been found that the derivatives prepared reveal high mitogenic and comitogenic activities, as well as radioprotective and antimutagenic effects.

Keywords: Glucans; Physico-chemical characterization; Immunomodulatory activity

1. Introduction

In recent years, increased attention has been paid to β-glucans isolated from the cell walls of fungi that act as non-specific modulators of the immune system and have found application as immunoadjuvants, antitumor and radio-protective agents. By stimulation of the host immune system, they exert a beneficial effect on a variety of experimental disease states of bacterial, viral, fungal, and parasitic origin (DiLuzio, 1983; Stone and Clarke, 1992; Bohn and BeMiller, 1995). Most fungal β-glucans exhibit immunomodulatory activity when administered intravenously or intraperitoneally. The problem is that insoluble or hardly soluble β-glucans cause significant adverse effects (granuloma formation, microembolization, inflammation, pain) when administered by parenteral routes (Maeda et al., 1988). From this point of view, oral administration of the water-soluble glucan preparations would have several advantages. In order to improve the solubility of β-1,3-D-glucans, several derivatization procedures, e.g. carboxymethylation (Šoltés et al., 1993), sulfation (Williams et al., 1991a), phosphation (Williams et al., 1991b) were developed. Lowering of the molecular weight of the polysaccharides may also contribute to their improved solubility and facilitate chemical derivatization. Many different methods, e.g. acid and alkaline hydrolysis, enzymic digestion and ultrasound irradiation have been applied to depolymerize biopolymers into lower molecular-weight fragments. Among those, ultrasonication proved to be very advantageous because it did not change the chemical nature of the polymer but simply reduced its molecular weight by splitting the most susceptible chemical bonds. It is now well established that prolonged exposure of solutions of macromulecules to high-energy ultrasonic waves leads to a permanent reduction in the solution viscosity (Lorimer et al., 1995). This method has been used to depolymerize various biopolymers including DNA, dextran, and bacterial capsular polysaccharides (Szu et al., 1986). In the present paper, we present the results of the application of targeted ultrasonic depolymerization of the microbial β-glucans in the processes of their isolation, purification and derivatization in order to obtain highly biologically active compounds.

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2. Experimental

2.1. Materials

Baker's yeast glucan was isolated from the commercial yeast biomass purchased from Slovlik, Slovak Republic. The chitin–glucan complex was isolated from the cell walls of the industrial strain of filamentous fungus *Aspergillus niger* used for the commercial production of citric acid (Biopo, Slovak Republic).

Laminarin was purchased from Sigma-Aldrich. Glycogen was obtained from Charles Druce, London. Curdlan, a bacterial β-1,3-glucan from *Alcaligenes faecalis*, was a generous gift of Dr Kozo Ogawa, Osaka Prefecture University. Zymosan, the polysaccharide complex from baker's yeast cell walls was a gift from Likospol, Bratislava.

Endo-β-1,3-D-glucanase, from the extracellular production of *Trichoderma reesei*, was a generous gift from Dr Farkaš of the Slovak Academy of Sciences. The enzyme was purified by liquid chromatography on Bio-Gel P-100 and Ostsorb DEAE columns.

2.2. Methods

2.2.1. Isolation of the baker's yeast glucan

The water-insoluble (1-3)-β-D-glucan was obtained by means of the extraction of *Saccharomyces cerevisiae* cells using 6% NaOH at 60°C followed by 4% phosphoric acid extraction at room temperature as described by Machová et al. (1995). After the removal of all soluble components, β-glucan was left as the insoluble residue.

2.2.2. Isolation of the chitin–glucan complex from A. niger

The fungal mycelium was subjected to a hot alkaline (1 M NaOH) digestion for 1 h. The alkali-insoluble sample was subsequently extensively washed with distilled water, acetone, and finally with diethylether. The dry sample contained 2.24% nitrogen which corresponded to a content of ca. 30% chitin.

2.2.3. Methylation analysis

Permethylation of baker's yeast glucan was carried out according to the procedure developed by Ciucanu and Kerek (1984), using powdered NaOH. The methylated polysaccharide was hydrolyzed with 4 M tetrafluoroacetic acid and the fragments were analyzed as alditol acetates using gas chromatography–mass spectrometry. Methylation of the chitin–glucan complex was unsuccessful due to its poor solubility.

2.2.4. Carboxymethylation of the glucans

Derivatization of the glucan and the chitin–glucan complex was performed using the modified procedure described by Machová et al. (1995). Briefly, 10 g of the glucan or chitin–glucan complex was suspended in a mixture of 12.4 mL of aqueous NaOH (300 g L^{-1}) and 125 mL of isopropanol. The suspension was vigorously stirred at 10°C for 1 h. Subsequently, the sodium salt of monochloroacetic acid was added (7.9 g for achievement of the substitution degree 0.5) in 14 mL of water, and the mixture was stirred at 70°C for 2 h. Excess NaOH was neutralized with 6 N HCl and the salts were removed by dialysis. The non-dialyzable portion was dried, dissolved in water, centrifuged and freeze-dried. The degree of substitution of the carboxymethylated glucan was 0.56 or 0.91 depending on the amount of monochloroacetic acid, and that of the chitin–glucan complex was 0.43 as determined by potentiometric titration with a KOH solution according to Rinaudo and Hudry-Clergeon (1967).

2.2.5. Sulfoethylation of the glucans

Sulfoethylation was performed according to Pastýr et al. (1997) using sodium β-chloroethylsulfonate in isopropanol solution.

2.2.6. Ultrasonication of the samples

Ultrasonication of the carboxymethylated samples of the yeast glucan and chitin–glucan complex (250 mg of the lyophilized sample dissolved in 25 ml distilled water) was performed at 20 kHz and 100 W in an ice bath using a horn-type (ϕ = 1.5 cm) ultrasound generator UZD 300 (PERSON-Ultragen, Slovak Republic).

2.2.7. High-performance liquid chromatography

All HPLC experiments were performed at ambient temperature with a system that included a high-pressure pump (LCP 3001; Laboratorní přístroje, Czech Republic), an eight-port switching valve equipped with two 100 μl loops (Model PK 1; Vývojové dílny, Czechoslovak Academy of Sciences), and two in-series-connected stainless-steel HPLC columns (250 × 8 mm) packed with SEPARON HEMA-BIO sorbent (mean particle size = 10 μm; Tessek, Prague). The separation process was monitored with a differential refractometric detector (RIDK 102; Laboratorní přístroje). The mobile phase used was 0.1 M aqueous $NaNO_3$ solution. The flow rate was 0.4 ml min^{-1}. A set of pululans P-5, P-100, P-200, P-400, and P-800 (Shodex Standard P-82; Macherey-Nagel, Germany) was used for the calibration of the HPLC system.

Samples containing 0.1 mg of the pullulan standard dissolved in 100 μl of the mobile phase were loaded onto the HPLC column. the elution volumes corresponding to the the maxima of the chromatographic curves were assigned by the values of $M_{peak} = (M_w \times M_n)^{1/2}$ of the pullulan calibrants, where M_w and M_n are the weight- and the number-average molecular weights, respectively. Taking into account that the HPLC system was calibrated using pullulan standards as the reference materials, the molecular-weight characteristics of M_{peak}, M_w, and M_n of all CM-CG samples should be regarded as *relative* values.

2.2.8. Infrared spectroscopy

Infrared spectra were measured on a NICOLET Magna

750 spectrometer with DGTS detector and OMNIC 3.2 software. The samples were pressed into KBr pellets with a sample/KBr ratio of 2/200 mg. In order to obtain more exact band positions, Fourier self-deconvolution was applied using the Omnic 3.2 software (bandwidth 50 cm^{-1}, enhancement factor 2.6).

2.2.9. Elemental analysis

The solid biopolymers were analyzed for their carbon, hydrogen, and nitrogen content using the EA 1108 device (Fisons Instruments, UK). The standard procedure for the C, H, N configuration provided by the manufacturer was utilized.

2.2.10. Gel filtration

Gel filtration of the ultrasonicated carboxymethyl derivatives of yeast glucan and the chitin–glucan complex was performed on the glass column (150 × 1.5 cm) packed with Sepharose CL-6B (Pharmacia). Sodium-phosphate buffer (0.1 M, pH 7.5) was applied as the mobile phase at a flow rate of 0.2 ml min^{-1}. The 250 mg sample, dissolved in 6 ml of the mobile phase, was loaded onto the column. The separation process was monitored with a differential refractometric detector RIDK-102 (Laboratorní přístroje). Fractions (4 ml) were collected and dialyzed against distilled water for 48 h. The dialyzed samples were freeze-dried.

2.2.11. ^{13}C-NMR spectroscopy

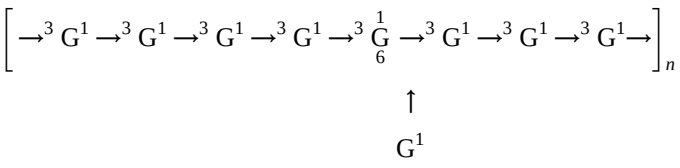
The ^{13}C-NMR spectra were recorded at 298 K in D_2O solutions (30 mg ml^{-1}) using a Bruker AM-300 instrument. Acetone was used as an internal standard (CH_3 resonance at 31.07 ppm).

3. Results and discussion

3.0.1. Isolation, structural characterization and chemical modification of the polysaccharides

β-Glucan extracted from fungal cells by alkaline treatment still contains relatively large amounts of impurities (5–10%), mainly mannan, proteins, water-soluble α-glucan and amorphous β-1,6-glucan trapped in the β-glucan fibrils. Usually, mild acid hydrolysis or enzymatic (pronase or mannanase) treatment can be used to further purify, but in this way a significant amount of β-glucan is lost. We developed an ultrasound-assisted purification—1% glucan suspension in water is treated with ultrasound during 10–15 min then centrifuged and the sediment washed several times with distilled water and dried. In this way the more soluble amorphous impurities are released from the β-glucan network and the resulting β-glucan is of high purity (96–99%). The ultrasonic treatment simplifies glucan preparation and the final yield of water-insoluble β-glucan was increased by 10–15%.

Methylation analysis of the β-glucan showed that it contained ca. 10% terminal non-reducing glucosyl units and 1,3,6-linked glucosyl units each, as well as ca. 80% 1,3-linked glucosyl units. Thus, the tentative structure of the ''repeating unit'' of the yeast glucan can be presented as follows:



3.0.2. Solubilization of the glucans

Fibrillar glucan is insoluble in water and has some undesirable side effects in intravenous application. Therefore water-soluble glucan products are prepared by its derivatization. Solubility of the glucan derivatives depends on the degree of substitution (DS). Water-soluble carboxymethyl glucans with a DS ranging from 0.4 to 1.15 have been prepared. It has been found, on the one hand, that samples with DS lower than 0.4 were insoluble or only partially soluble in water, while on the other hand, the biological activity of the samples with DS > 1.0 was gradually decreasing, probably due to the change in the molecule conformation with increasing net charge. The optimum DS for the carboxymethyl glucan was found to be 0.6–0.8 (Šandula et al., 1995).

Solubility of the glucan derivatives depends also on the nature of the anionic group introduced into the molecule. For example, sulfoethyl glucan with DS 0.3 was fully soluble in water, while for the carboxymethyl derivatives, higher DS was required. The data on solubility, molecular weight

^{13}C-NMR spectra of baker's yeast glucan and its carboxymethylated derivatives have been published by Kogan et al. (1988) and Machová et al. (1995). Chemical characterization of the chitin–glucan complex and preparation of its carboxymethyl derivatives has been described by Machová et al. (1998). The chitin content in the complex was estimated to be ca. 30% based on the data of the nitrogen content, and this result was corroborated by the ^{13}C-NMR spectra of the chitin–glucan fractions obtained by ultrasonic depolymerization. With both polysaccharides, the efficiency of ultrasonication as a tool for producing better soluble lower molecular-weight preparations has been demonstrated.

Table 1
Some physiochemical characteristics and biological activity (quoted from Šandula et al. (1995)) of the investigated fungal β-glucans

Compound	Chemical composition	Solubility in water	Molecular weight (kDa)	Comitogenic activity for 100 μg ml^{-1} dose
Zymosan	80% β-glucan + α-glucan + mannan + protein	Insoluble	Not determined	7.1
Baker's yeast glucan	98% β-glucan	Insoluble	Not determined	19.1
Carboxymethyl glucan DS 0.56	Pure compound	Soluble	220	1.2
Carboxymethyl glucan DS 0.75	Pure compound	Soluble	220	6.7
Carboxymethyl glucan DS 1.15	Pure compound	Soluble	220	1.2
Sulfoethyl glucan DS 0.3	Pure compound	Soluble	660	10.7
Chitin–glucan	70% β-glucan + 30% chitin	Insoluble	Not determined	Not determined
Carboxymethyl chitin–glucan DS 0.43	Pure compound	Soluble	650	Not determined

and comitogenic activity of some investigated glucan derivatives are summarized in Table 1.

Carboxymethylation of fibrillar glucan gives an 80–85% yield of the soluble derivatives. With sonication of glucan (10–15 min) prior to the chemical derivatization, the efficiency of the reaction can be increased up to 90–95%. Glucans containing high quantities of chitin that occur in the cell walls of various filamentous fungi e.g. *A. niger*, *Penicillium chrysogenum*, etc. are very resistant to chemical derivatization. Carboxymethylation of the chitin–glucan complex from *A. niger* renders only 15–20% of the soluble derivative. Ultrasonic pretreatment of chitin–glucan in alkaline solution allowed an increase in the yield of water-soluble derivative of up to 30–35%.

3.0.3. Application of Fourier-transform infrared spectroscopy for characterization of the glucans

Application of FT-IR spectroscopy (Wilson et al., 1988; Breierová et al., 1997) has been shown to be a useful tool in monitoring structural changes in biopolymers. In spite of the fact that on the one hand the infrared characteristics of glucose are well known (Mathlouthi and Koenig, 1986), on the other hand, information about glucans is rather insufficient. Therefore we applied this technique to glucans in the 1800–700 cm^{-1} region. The (C–O) and ring vibrations ranging at 1200–950 cm^{-1} as well as in the ''anomeric region'' at 950–750 cm^{-1} (Mathlouthi and Koenig, 1986) show differences in the α- and β-glucan spectra. In the case of laminarin and curdlan, the characteristic bands for (1,3)- and/or (1,6)-linked β-D-glucans are at ca. 1160, 1078, 1041, and 889 cm^{-1}. In the case of the α-(1,4;1,6)-linked glycogen, the characteristic bands are at ca. 1155, 1023, 930, 850, and 765 cm^{-1} (Fig. 1, Table 2). Consequently, the intensity ratios 1160/1155, 1041/1023, 1160/1023, and 889/930, 889/765 cm^{-1} could be applied for ratio of α- and β-linkage determination. Determination of this α/β ratio is important for the estimation of the purity of the glucan preparation.

Carboxymethylation introduces into the molecule the carboxylate anions which dominate the glucan's IR spectra at about 1600 and 1421 cm^{-1} (Table 2). The 1200–750 cm^{-1} region becomes less resolved and the determination of the α/β ratio can be more complicated.

FT-IR spectroscopy can also be applied to chitin–glucan complex characterization. The most characteristic chitin

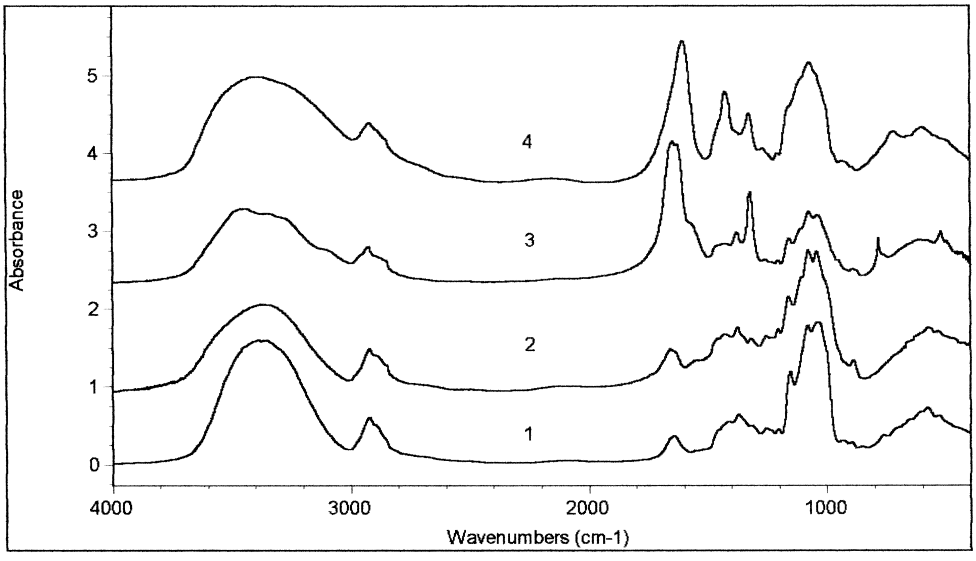


Fig. 1. FT-IR spectra of (1) β-D-glucan with α-glucan concomitant; (2) pure β-D-glucan; (3) glucan–chitin complex, and (4) carboxymethyl β-D-glucan.

Table 2
Frequencies of the FT-IR bands for glucans

Frequency (cm^{-1})	Assignment	Comment
3415	ν OH	
2922	ν CH	
1730	ν (C=O)	UA[a] protonated form
1651	Amide I: ν (C=O)	Chitin, acetamide
1620	ν (C=0)	Chitin, acetamide
~1600	ν_{as} (COO^-)	CM[b]
1556	Amide II: ν NH	Chitin
1540	Amide II: ν NH	
1421	ν_s (COO^-)	CM[b]
1375		Chitin
1328		CM[b]
1316	Amide III: δ(CN)	Chitin
1161	ν (COC), ν (CC)	β Glc
1153	ν (COC), ν (CC)	α Glc
1078	ν (CO), (CC)	β Glc
1047	ν (CO), (CC)	CM[b]
1038	ν (CO), (CC)	β Glc
1023		α Glc
930		Ring
890	δ CH	(C-1-H), β Glc
850	δ CH	(C-1-H), α Glc
783		(C-1-H), β Glc
765	δ CH	(C-1-H), α

[a]UA—uronic acid.
[b]CM—carboxymethyl.

bands (Focher et al., 1992; Wlochowicz et al., 1987) appear at 1650 and 1620 cm^{-1} and at 1561 cm^{-1}. In the yeast glucans, however, band broadening may appear up to 1530 cm^{-1}. This may be due to some aromatic components. Because of the presence of chitin, the supramolecular structure of the glucan is changed and therefore the changes occur also in the 1150–750 cm^{-1} region. The final shape of the spectrum in this region is due to the amount of chitin as well as the carboxymethyl content in the molecule. Because of the effect on IR spectra caused by carboxymethylation and chitin–glucan complex formation, FT-IR spectroscopy can be applied also for a quantitative characterization of these glucans. The application of FT-IR spectroscopy can provide fast and valuable information about the quality of the glucan preparations.

3.0.4. Application of ultrasound for targeted depolymerization of the glucans

After the extraction of the (1 → 3)-β-D-glucan from the cell walls of baker's yeast *S. cerevisiae*, enzymatic and ultrasonic degradations of this biopolymer were studied (Machová et al., 1995). The depolymerization of the carboxymethylated glucan by means of ultrasonication was effective for the two prepared glucan derivatives with DS 0.56 and 0.91. The initial M_w value of the untreated polymer was 3.46×10^5 Da. After 20 min ultrasonic treatment, the M_w of the carboxymethylated (1 → 3)-β-D-glucan with DS 0.56 decreased by 64% and reached a value of 1.24×10^5 Da. Comparison of the M_w values of the samples obtained by ultrasonication shows the degradation to be more effective in the first 5 min when the M_w value decreased by 35%. The higher substituted carboxymethylated β-1,3-D-glucan with DS 0.91 was also depolymerized and after 20 min of ultrasonication, M_w decreased by 34% and reached a value of 2.4×10^5 Da. The resulting M_w after the termination of the enzymatic degradation of the carboxymethylated (1 → 3)-β-D-glucan with DS 0.56 (after 192 h) was 1.17×10^5 Da, corresponding to a 66% decrease in the initial M_w value. The higher substituted glucan (DS 0.91) was found to be an inappropriate substrate for the β-1,3-D-glucanase used (Machová et al., 1995). Our results demonstrated that ultrasonic degradation of the carboxymethylated glucan was more effective for the production of samples with lower molecular weights than enzymatic hydrolysis. Application of the ultrasonically depolymerized fractions of carboxymethyl glucan in the antimutagenic protection of mice has been described by Chorvatovičová et al. (1996).

In the case of chitin–glucan, ultrasonication has also proved to be a potent instrument for the preparation of fragments with desired properties. In our previous study (Chorvatovičová and Šandula, 1995), oral administration of the high molecular carboxymethyl chitin–glucan complex failed to exhibit antimutagenic effects against cyclophosphamide, effects which *were* manifested in its peritoneal and intravenous administration. The complex structure of high molecular-weight chitin–glucan may have accounted for its failure to pass through the wall of the gastrointestinal tract. We therefore attempted to prepare a carboxymethylated chitin–glucan complex with ultrasonically lowered molecular weight with conserved biological activity against cyclophosphamide (Chorvatovičová et al., 1998). The ultrasonic degradation of the carboxymethylated chitin–glucan and preparation of the two fractions with decreased molecular weight are described by Machová et al. (1998). Untrasonicated carboxymethylated chitin–glucan with molecular weight 0.19×10^5 was administered either intraperitoneally or orally prior to cyclophosphamide injection, and its effect on the frequency of micronuclei in polychromatic erythrocytes of mouse bone marrow was evaluated. Both ways of carboxymethylated chitin–glucan administration significantly decreased the clastogenic effect of cyclophosphamide. The protective effect of carboxymethylated chitin–glucan was concentration dependent. Ultrasonic depolymerization of high molecular carboxymethylated chitin–glucan resulted in its anticlastogenic effect against cyclophosphamide not only at intraperitoneal but also at oral administration, achieved by decreasing its molecular weight. Ultrasonication proved to be an efficient way of obtaining molecules of carboxymethylated chitin–glucan able to pass through the cell walls of the gastointestinal tract (Chorvatovičová et al., 1998).

3.0.5. Immunomodulatory activity of the glucans

The immunomodulatory activity of the investigated glucans and their soluble derivatives have been studied in collaboration with several research institutions of medical

or pharmacological specialization. Fibrillar and partially hydrolyzed baker's yeast glucan as well as its soluble derivatives prepared by means of carboxymethylation and sulfoethylation exhibited anti-infective activity against *Klebsiella pneumoniae* after intravenous or subcutaneous prophylactic application to mice (Kogan et al., 1989). Carboxymethyl glucan was shown to exert a potent protective effect in acute massive blood loss in mice (Vereschagin et al., 1994, 1997). The radioprotective effects of carboxymethyl glucan and its enhancement of haemopoiesis was investigated by Hofer et al. (1995a), Hofer et al. (1995b), Pospíšil et al. (1991) and Pospíšil et al. (1992). Sulfoethyl glucan revealed protective effects against mutagenicity induced by potassium bichromate in mice. It has been suggested that the protective effect of sulfoethyl glucan can be associated with formation of Cr^{VI} ion complexes with sulfoethyl groups of sulfoethyl glucan and/or by scavenger activity of sulfoethyl glucan towards emerging hydroxyl radicals (Chorvatovičová et al., 1993). Mitogenic activity of the insoluble yeast glucan and its water-soluble derivatives has been examined by Šandula et al. (1995). The antimutagenic activity of chitin–glucan and its carboxymethyl derivatives with different molecular weights and degrees of substitution has been investigated by Chorvatovičová and Šandula (1995) and Chorvatovičová et al. (1998).

4. Conclusions

Glucan-based preparations obtained from industrial biotechnologically important fungal strains can serve as broad spectrum immunomodulators. Because the source of these valuable polysaccharides is quite common, abundant and inexpensive (in the case of *A. niger* the fungal mycelia is the waste of the industrial production of citric acid), more attention should be paid to utilization of these microbial sources in polysaccharide biotechnology.

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References

Bohn, J.A., & BeMiller, J.N. (1995). (1 → 3)-*β*-Glucans as biological response modifiers: a review of structure-functional activity relationships. *Carbohydr. Polym., 28,* 3–14.

Breierová, E., Kačuráková, M., & Stratilová, E. (1997). The effect of higher concentration of NaCl in the cultivation medium on changes in the composition of extracellular yeast glycoproteins. *System. Appl. Microbiol., 20,* 348–355.

Chorvatovičová, D., Kováčiková, Z., Šandula, J., & Navarová, J. (1993). Protective effect of sulfoethylglucan against hexavalent chromium. *Mutation Res., 302,* 207–211.

Chorvatovičová, D., & Šandula, J. (1995). Effect of carboxymethyl-chitin-glucan on cyclophosphamide induced mutagenicity. *Mutation Res., 346,* 43–48.

Chorvatovičová, D., Machová, E., & Šandula, J. (1996). Effect of ultrasonic carboxymethylglucan on cyclophosphamide induced mutagenicity. *Mutation Res., 371,* 115–120.

Chorvatovičová, D., Machová, E., & Šandula, J. (1998). Ultrasonication: the way to achieve antimutagenic effect of carboxymethyl-chitin-glucan by oral administration. *Mutation Res., 412,* 83–89.

Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res., 131,* 209–217.

DiLuzio, N.R. (1983). Immunopharmacology of glucan: a broad spectrum enhancer of host defense mechanisms. *Trends Pharmacol. Sci., 4,* 344–347.

Focher, B., Naggi, A., Torri, G., Cosani, A., & Terbojevich, M. (1992). Structural differences between chitin polymorphs and their precipitates from solutions—evidence from CP-MAS ^{13}C-NMR, FT-IR and FT-Raman spectroscopy. *Carbohydr. Polym., 17,* 97–102.

Hofer, M., Pospíšil, M., Pipalová, I., Holá, J., & Šandula, J. (1995a). Hemopoiesis-enhancing effects of repeatedly administered carboxymethylglucan in mice exposed to fractionated irradiation. *Folia Biologica (Prague), 41,* 249–256.

Hofer, M., Pospíšil, M., Viklická, Š., Pipalová, I., Holá, J., Netíková, J., & Šandula, J. (1995b). Effects of postirradiation carboxymethylglucan administration in mice. *Int. J. Immunopharmac., 17,* 167–174.

Kogan, G., Alföldi, J., & Masler, L. (1988). ^{13}C-NMR spectroscopic investigation of two yeast cell wall *β*-D-glucans. *Biopolymers, 27,* 1055–1063.

Kogan, G., Masler, L., Šandula, J., Navarová, J., & Trnovec, T. (1989). Recent results on the structure and immunomodulating activities of yeast glucan. In V. Crescenzi, I.C.M. Dea, S. Paoletti, S.S. Stivala & I.W. Sutherland (Eds.), *Biomedical and biotechnological advances in industrial polysaccharides* (pp. 251–258). New York: Gordon and Breach.

Lorimer, J.P., Mason, T.J., Cuthbert, T.C., & Brookfield, E.A. (1995). Effect of ultrasound on the degradation of aqeous native dextran. *Ultrasonics Sonochemistry, 2,* S55–S57.

Machová, E., Kogan, G., Alföldi, J., Šoltés, L., & Šandula, J. (1995). Enzymic and ultrasonic depolymerization of carboxymethylated *β*-1,3-D-glucans derived from *Saccharomyces cerevisiae*. *J. Appl. Polym. Sci., 55,* 699–704.

Machová, E., Kogan, G., Šoltés, L., Kvapilová, K., & Šandula, J. (1998). Ultrasonic depolymerization of the chitin-glucan isolated from *Aspergillus niger*. *React. Funct. Polym.,* in press.

Maeda, Y.Y., Watanabe, S.T., Chihara, C., & Rokutanda, M. (1988). Denaturation and renaturation of a *β*-1,6:1,3-glucan, lentinan, associated with expression of T-cell-mediated responses. *Cancer Res., 48,* 671–675.

Mathlouthi, M., & Koenig, J.L. (1986). Vibrational spectra of carbohydrates. *Adv. Carbohydr. Chem. Biochem., 44,* 7–89.

Pastýr, J., Šandula, J., Masler, L., Trnovec, T., & Beneš, L. (1997). Preparation of water-soluble sulfoderivatives of microbial *β*-1,3-D-glucans. Slovak Patent No. 278783.

Pospíšil, M., Šandula, J., Pipalová, I., Hofer, M., & Viklická, Š. (1991). Hemopoiesis stimulating and radioprotective effects of carboxymethylglucan. *Physiol. Res., 40,* 377–380.

Pospíšil, M., Hofer, M., Pipalová, I., Viklická, Š., & Šandula, J. (1992). Enhancement of hematopoietic recovery in gamma-irradiated mice by the joint use of diclofenac, an inhibitor of prostoglandin production, and glucan, a macrophage activator. *Exp. Hematol., 20,* 891–895.

Rinaudo, M., & Hudry-Clergeon, G. (1967). Etude des O-carboxymethylcellulose a degre de substitution variable. I. Preparation et caracterisation des produits. *J. Chim. Phys., 64,* 1746–1752.

Stone, B.A., & Clarke, A.E. (1992). *Chemistry and biology of (1 → 3)-β-glucans*. Victoria, Australia: La Trobe University Press.

Szu, S.C., Zon, G., Schneerson, R., & Robbins, J.B. (1986). Ultrasonic irradiation of bacterial polysaccharides. Characterization of the depolymerized products and some applications of the process. *Carbohydr. Res.*, *152*, 7–20.

Šandula, J., Machová, E., & Hříbalová, V. (1995). Mitogenic activity of particulate yeast β-(1 → 3)-D-glucan and its water-soluble derivatives. *Int. J. Biol. Macromol.*, *17*, 323–326.

Šoltés, L., Alföldi, J., & Ŝandula, J. (1993). HPLC and ^{13}C-NMR study of carboxymethyl-β-(1 → 6)-D-gluco-β-(1 → 3)-D-glucan derived from *Saccharomyces cerevisiae*. *J. Appl. Polym. Sci.*, *48*, 1313–1319.

Vereschagin, E.I., Korolenko, T.A., & Šandula, J. (1994). Protective effect of β-1,3-D-carboxymethylglucan in acute massive blood loss (in Russian). *Patolog. Fiziolog. Exp. Terap.*, *3*, 33–35.

Vereschagin, E.I., Schwartz, Y.Sh., Tzyrendojev, D.D., Korolenko, T.A., & Šandula, J. (1997). Carboxymethyl β-1,3-D-glucan has potent protective effect against acute massive hemorrhage. In E. Faist, A.E. Baue & F.W. Schildberg (Eds.), *The immune consequences of trauma, shock and sepsis—mechanisms and therapeutic approaches* (Vol. 2, pp. 1033–1037). Berlin: Pabst Science Publishers.

Williams, D.L., Pretus, H.A., McNamee, R.B., Jones, E.L., Ensley, H.E., & Browder, I.W. (1991). Development of a water-soluble, sulfated (1 → 3)-β-D-glucan biological response modifier derived from *Saccharomyces cerevisiae*. *Carbohydr. Res.*, *235*, 247–257.

Williams, D.L., McNamee, R.B., Jones, E.L., Pretus, H.A., Ensley, H.E., Browder, I.W., & DiLuzio, N.R. (1991). A method for the solubilization of a (1 → 3)-β-D-glucan isolated from *Saccharomyces cerevisiae*. *Carbohydr. Res.*, *219*, 203–213.

Wilson, R.H., Goodfellow, B.J., & Belton, P.S. (1988). Fourier transform infrared spectroscopy for the study of food biopolymers. *Food Hydrocolloids*, *2*, 169–178.

Wlochowicz, A., Przygocki, W., & Polowinski, S. (1987). Crystallinity of krill chitin and its derivatives. *Acta Polymerica*, *38*, 184–189.