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Ultrasonic depolymerization of the chitin–glucan isolated from *Aspergillus niger*

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Abstract

By means of ultrasonication two fractions of the carboxymethylated chitin–glucan complex isolated from the cell walls of the filamentous fungus *Aspergillus niger* were obtained. Elemental analysis as well as ^{13}C NMR investigation of the high-molecular weight (M_w 680 kDa) and low-molecular weight (M_w 75 kDa) fractions revealed their essentially different chitin content. HPLC analysis of the fractions produced by ultrasonic treatment of different duration, combined with nitrogen assay showed steadily increasing chitin content in the faster eluted fraction that allows one to suggest the different susceptibility of the two components of the chitin–glucan complex to the ultrasonication.

Keywords: Chitin; Glucan; Aspergillus; Ultrasonication; HPLC

1. Introduction

In recent years increased attention has been attracted to the β-glucans isolated from the cell walls of yeasts and molds that act as nonspecific modulators of the immune system and have found application as immunoadjuvants, antitumor agents, etc. Recently, the antimutagenic activity of a carboxymethylated derivative of the chitin–glucan complex (CM–CG) isolated from the cell walls of the filamentous fungus *Aspergillus niger* was demonstrated: a concentration-dependent protective effect was observed at both intraperitoneal and intravenous administration of the CM–CG complex to mice. On the other hand, oral administration was not effective most probably due to the failure to resorb a large CM–CG molecule from the gastro-intestinal tract into blood [1]. Therefore, the depolymerization of the above mentioned macromolecule would be desirable.

Many different methods, e.g. acid and alkaline hydrolysis, thermal denaturation, enzymic digestion and ultrasonic irradiation have been applied to depolymerize biopolymers into lower-molecular-weight fragments [2]. Among those, ultrasonication proved to be very advantageous because it did not change the chemical nature of the polymer, simply reducing its molecular weight by splitting the most suscep-

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tible chemical bonds. It is now well established that the prolonged exposure of solutions of macromolecules to high-energy ultrasonic waves produces permanent reduction in the solution viscosity [3]. Ultrasonic irradiation, which can generate velocity gradients as high as 10^{-5} cm, has been used to depolymerize polymers, such as polystyrene, polyvinyls, collagen, DNA, and bacterial Vi polysaccharide [2]. Ultrasonication proved to be the best way to prepare xanthan with a controlled molecular weight [4]. Sonication led to the fragmentation of cinerean, a β-(1→3, 1→6)-D-glucan of *Botryris cinerea,* while approximately exponential decrease of mass in the range of 250–50 kDa has been observed [5], and was effective for the degradation of carboxymethylated glucan of *Saccharomyces cerevisiae* [6]. Schizophyllan, a water-soluble β-D-glucan, was partially depolymerized by ultrasonic irradiation and both native and degraded polysaccharides exhibited pronounced antitumor activity against Sarcoma-180 ascites [7].

The cell wall of *Aspergilli* contains mainly glucan, chitin, and galactomannan [8]. The alkali-insoluble part of the cell wall of *Aspergillus fumigatus* consists of a covalently linked glucan–chitin complex which seems to be present in almost all fungal species and probably plays a critical role in the fungal morphogenesis. In this organism, (1→3)-β-glucan is apparently linked to chitin through a linkage that involves glucosamine [9].

The aim of the present work was to prepare samples of CM–chitin–glucan with reduced molecular weight using ultrasonic depolymerization and to broaden our knowledge about the chemical composition and some physico-chemical characteristics of the carboxymethylated derivative of the chitin–glucan complex isolated from the cell walls of *Aspergillus niger.*

2. Experimental

2.1. Isolation of the crude chitin–glucan complex

The crude, water-insoluble chitin–glucan complex was isolated from the cell walls of the industrial strain *Aspergillus niger* used for the commercial production of citric acid (Biopo, Leopoldov, Slovak Republic). The mycelium was subjected to a hot alkaline (1 M NaOH) digestion for 1 h. The alkali-insoluble sample was subsequently washed five times with distilled water, acetone, and finally with diethylether. The dry sample contained 2.24% nitrogen which corresponded to a content of ca. 30% chitin. Elemental analyses were calculated from multiple determinations within ±0.2% agreement.

2.2. Preparation of the carboxymethylated chitin–glucan complex (CM–CG complex)

Derivatization of the chitin–glucan complex was performed using the modified procedure described by Sasaki et al. [10]. Briefly, 10 g of the chitin–glucan complex were 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, 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 nondialyzable portion was dried, dissolved in water, centrifuged and freeze-dried. The degree of substitution of the obtained carboxymethylated chitin–glucan complex was 0.43 as determined by the potentiometric titration with a KOH solution [11]. The content of carbon in this sample was 38.3%, hydrogen 5.74%, and nitrogen 1.61%.

2.3. Ultrasonication of the CM–CG complex

The obtained CM–CG complex (250 mg of the lyophilized sample dissolved in 25 ml distilled water) was treated by ultrasonication (20 kHz, 100 W) in the ice bath using a horn

type ($\phi = 1.5$ cm) ultrasound generator UZD 300 (PERSON-Ultragen, Nitra, Slovak Republic). Aliquots (1 ml) of the biopolymer solution were withdrawn from the ultrasound vessel at the defined time intervals of 5, 10, 20, 30, and 50 min. These aliquots, as well as the final 60-min ultrasonicated sample were further analyzed by the combined methods of high-performance liquid chromatography (HPLC) and elemental analysis. The final sample of the 60-min ultrasonicated CM–CG complex was further fractionated by gel filtration, and the two fractions obtained were subsequently investigated by ^{13}C-NMR spectroscopy.

2.4. 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, Prague, Czech Republic), an eight-port switching valve equipped with two 100-μl loops (Model PK 1; Vývojové dílny, Czechoslovak Academy of Sciences, Prague), and two in series connected stainless-steel HPLC columns (250×8 mm) packed with SEPARON HEMA-BIO 1000 sorbent (mean particle size = 10 μm; Tessek Ltd., 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. A set of pullulans P-5, P-100, P-200, P-400, and P-800 (Shodex Standard P-82; Macherey-Nagel GmbH + CoKG, Düren, 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.

The original and the ultrasonicated CM–CG samples were analyzed by HPLC applying both analytical and semipreparative separation modes. The biopolymer concentrations used while operating HPLC in the analytical separation mode were 3–4 mg/ml, whereas for the scaled-up procedure they were increased up to 70 mg/ml. During the HPLC run under the semipreparative separation mode, defined fractions were collected for the subsequent elemental analysis. All analyzed samples were characterized by the corresponding values of M_{peak}. The M_w and M_n molecular-weight averages of the samples were calculated using the computer program described by Šoltés et al. [12]. 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 being relative values.

2.5. Elemental analysis

The solid biopolymers, obtained by dialysis and subsequent freeze-drying of the CM–CG samples collected upon semipreparative HPLC, were analyzed for their carbon, hydrogen, and nitrogen content using the EA 1108 device (FISONS Instruments, UK).

2.6. Gel filtration

Gel filtration of the final 60-min ultrasonicated CM–CG complex was performed on the glass column (150×1.5 cm) packed with Sepharose CL-6B (Pharmacia, Uppsala, Sweden). 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 using a sample collector (SF62; Mikrotechna, Prague). The two pooled fractions – tubes 16–22 and tubes 34–65 – were further dialyzed (Dialysis Tubing –

Art. No. 44146; Servapor, SERVA, Germany) against distilled water for 48 h. During the first hours of the dialysis, a significant amount of mono- and oligosaccharides was detected in the waste water using the phenol-sulfuric acid assay [13]. The dialyzed samples were freeze-dried. The yields of the two lyophilizates, designated as samples I and II, were 3.3 and 49.5%, respectively, relative to the initial amount of the CM–CG sample prior to ultrasonication.

2.7. ^{13}C-NMR spectroscopy

For the quantitative determination of the glucan:chitin ratio in samples I and II ^{13}C-NMR spectroscopy was applied. The ^{13}C-NMR spectra were recorded at 298 K in D_2O solutions (30 mg/ml) using the Bruker AM-300 instrument.

3. Results

The HPLC analysis of the original CM–CG complex and of the high-intensity-sonicated CM–CG samples revealed the bimodal elution profiles (Fig. 1, curves 1–3). As evident from Fig. 2, already after a short-time ultrasonication ($\approx$20 min) the M_{peak} value of the earlier eluted high-molecular-weight component, equaling originally to 650 kDa, increased up to 680 kDa and did not change significantly upon further treatment (Fig. 2, curve A). On the other hand, the prolonged ultrasonication led to a continual decrease of the M_{peak} value of the later eluted component (Fig. 2, curve B). The molecular-weight averages (M_w and M_n) as well as the carbon, hydrogen, and nitrogen contents of the ultrasonicated CM–CG samples are listed in Table 1. It should be pointed out that while the nitrogen content of the original CM–CG complex was 1.61%, in the high-molecular-weight component of the final 60-min ultrasonicated sample that was equal to 3.89%. On the other hand, in the low-molecular-weight component the content of nitrogen (1.67%) was comparable to the value found in the original CM–CG complex.

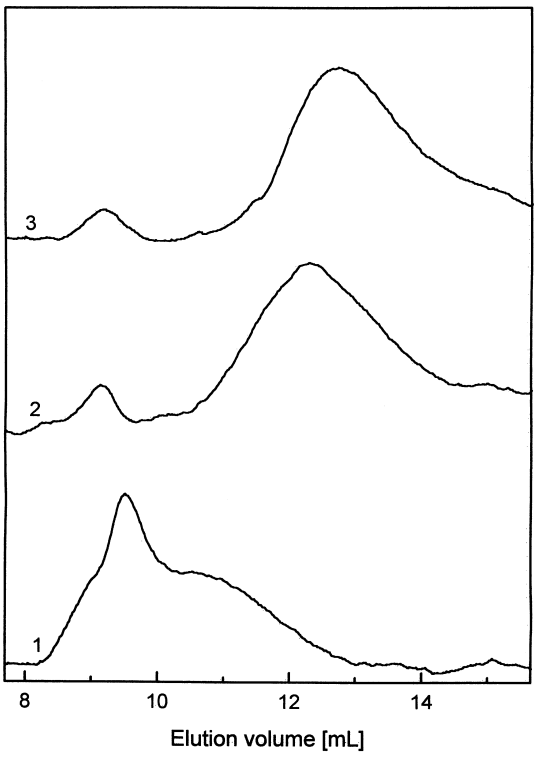

Fig. 1. HPLC elution profiles of the original CM–CG complex (curve 1) and of the ultrasonicated CM–CG samples. Time of ultrasonication: 20 min (curve 2), 60 min (curve 3).

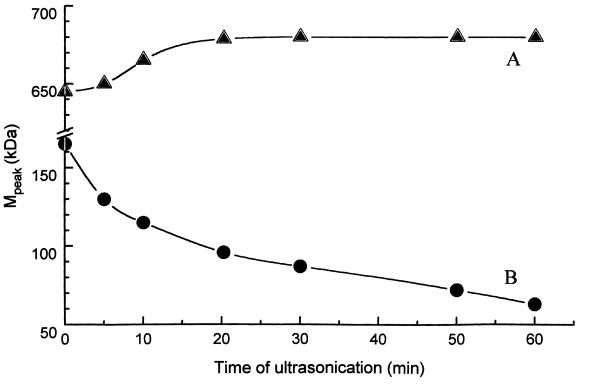

Fig. 2. Dependence of the molecular weight (M_{peak}) on the time of sample ultrasonication. Curve A relates to the earlier eluted (HPLC) sample component, curve B corresponds to the later eluted sample component.

The gel filtration record of the final 60-min ultrasonicated CM–CG sample is depicted in

Table 1
The carbon, hydrogen, and nitrogen content and the molecular-weight averages of the ultrasonicated CM–CG samples

Time of sonication (min)	High-molecular-weight component					Low-molecular-weight-component				
	C (%)	H (%)	N (%)	M_w (kDa)	M_n (kDa)	C (%)	H (%)	N (%)	M_w (kDa)	M_n (kDa)
5	42.9	6.21	2.84	650	640	37.9	6.31	1.75	138	95
10	39.8	5.78	3.02	662	654	37.1	6.24	1.69	120	85
20	39.8	5.79	3.46	680	670	36.8	6.09	1.66	101	72
30	39.9	5.78	3.56	680	671	36.1	6.10	1.50	95	69
60	39.8	5.69	3.89	680	670	36.9	6.12	1.67	75	57

Fig. 3. As can be seen from this record, sample I is represented by a narrower elution curve than the one corresponding to sample II. This finding correlates well with the polydispersity values (M_w/M_n) of ca. 1.01 and ca. 1.32 (cf. data in Table 1) calculated for the high- and low-molecular weight components of the final 60-min ultrasonicated CM–CG complex, respectively.

The quantitative estimation of the glucan-:chitin ratio in samples I and II was carried out on the basis of their ^{13}C-NMR spectra (Fig. 4).

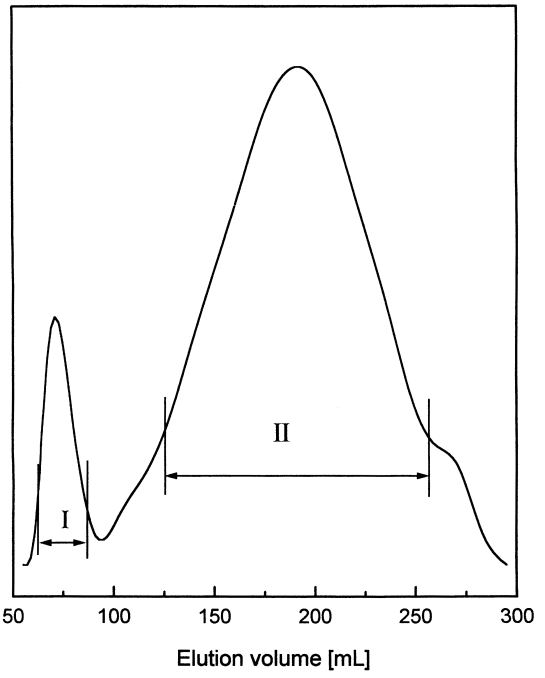

Fig. 3. Gel filtration record of the final 60-min ultrasonicated CM–CG sample. The two collected fractions containing the high-molecular-weight (I) and the low-molecular-weight (II) components are indicated by the vertical lines.

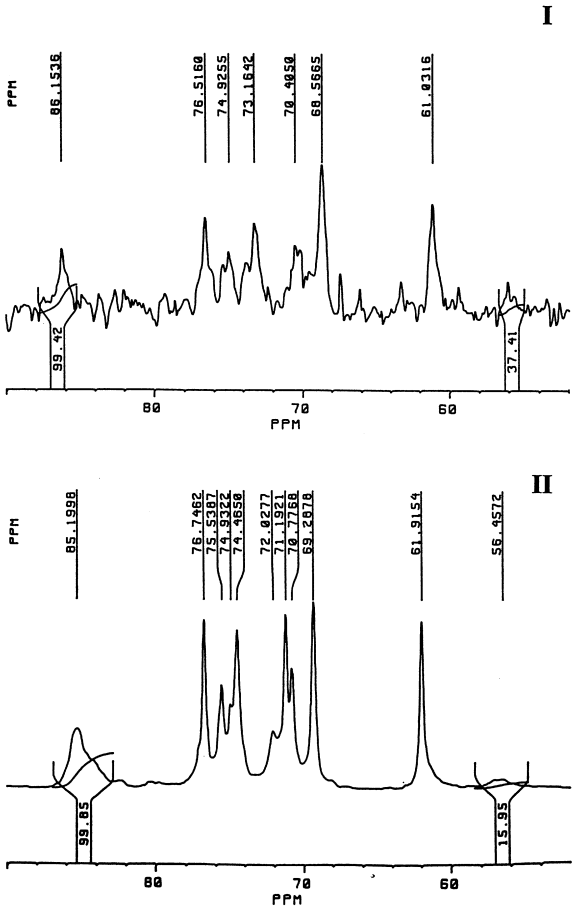

Fig. 4. ^{13}C-NMR spectra of sample I (I) and sample II (II).

The glucan component in the spectrum is represented by its characteristic signal of C-3 at ca. 86 ppm, while the chitin component is represented by the signal of N-linked C-2 at ca. 56 ppm. The C-2 signal of the chitin component has a shape of a doublet due to the partial N-acetylation of the amino group. The integra-

tion of the specified signals revealed that the content of chitin in sample I was 27.1%, while in sample II its content was only 13.8%.

4. Discussion

In our recently published paper [1], an inhibition effect of the original CM–CG complex towards the micronuclei formation in the reticular system of mice induced by a mutagen cyclophosphamide was demonstrated. However, this effect was not observed when CM–CG was applied orally. The reason for such ineffectiveness of the orally administered CM–CG probably lies in its inability to pass to the bloodstream from the gastro-intestinal tract. In order to overcome this hindrance, we have prepared a series of CM–CG fragments with lower molecular weight. Ultrasonication was selected as the method for depolymerization because it has been found to be very effective at controlled depolymerization of biopolymers [3,5,14–18].

Our results demonstrated that ultrasonication was efficient for depolymerization of the CM–CG complex prepared from the cell walls of *Aspergillus niger*. The original CM–CG complex was shown to contain two components (Fig. 1, curve 1). Although the bicomponental character of CM–CG complex was preserved through the 60 min of ultrasonication (Fig. 1, curve 3), changes in certain parameters (e.g. nitrogen content, M_w and M_{peak} values) were observed in the higher and lower molecular weight components (samples I and II, respectively). The rise of the M_{peak} value, i.e. the increase of the hydrodynamic parameter of the hydrated macromolecule within the first 20 min of sonication (Fig. 2, curve A) might have resulted from the unwinding of the biopolymer chains due to the action of ultrasonication [19]. The M_{peak} value of the low-molecular-weight component of the CM–CG complex decreased significantly (Fig. 2, curve B), while its content of nitrogen remained almost unchanged with the extended ultrasonication (Table 1).

The observed consistent increase of nitrogen content in the high-molecular-weight component with the prolongation of the ultrasonic treatment (Table 1) implies that the ultrasonication caused a preferential depolymerization of the glucan component of the CM–CG complex. Degradation of the polymeric glucan to the oligomeric molecules and their subsequent elution in sample II resulted in accumulation of the chitin component in sample I, which was observed as a relative increase of the nitrogen content. It is very plausible that in the chitin–glucan complex the linear rod-like chitin chain segments form a more crystalline structure [20], while the helical chains of the branched $(1\rightarrow6)$, $1\rightarrow3)$-β-D-glucan constitute a more amorphous moiety [21] which is more susceptible to the depolymerizing action of the ultrasonication. The glucan chains are most probably initially deprived of the most exposed $(1\rightarrow6)$-linked side chains which are degraded to mono- and oligosaccharides that were subsequently removed by dialysis, while the larger glucan fragments remained in the dialysate and were eluted at HPLC or gel-filtration together with the degraded chitin fragments in the lower-molecular-weight component (sample II).

The comparison of the ^{13}C-NMR spectra of the samples I and II (Fig. 4) indicates that sample II has lower molecular weight which is evident from the narrower shape of the signals in its spectrum. The spectra also provide information on the heterogeneity in N-acetylation of the amino group in chitin component (which results in doublet appearance of the signal of N-linked C-2 atom at ca. 56 ppm) as well as on the relative content of chitin in both samples. Since the signal at ca. 86 ppm is unambiguously ascribable to C-3 of $(1\rightarrow3)$-β-D-glucan [22] and the signal at ca. 56 ppm is known to correspond to C-2 of *N*-acetyl-D-glucosamine [23, 24], relative intensities of these two signals can be used for evaluation of the chitin content in the samples. Integration of these signals indicated that chitin content in sample I was 27.1%, while in sample II it was 13.8%. In

other words, sample I contained approximately twice as much chitin as sample II. Comparison of the data on the nitrogen content obtained by means of the elemental analysis (Table 1) shows that the ratio of the chitin contents in samples I and II is 2.3, which is in quite reasonable agreement with the spectrometric data. These results indicate that ^{13}C-NMR spectroscopy may be used for the off-line monitoring of the chromatographic separation of different polysaccharides.

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