ELSEVIER

Ultrasonics Sonochemistry 7 (2000) 63–68

Ultrasonics SONOCHEMISTRY

www.elsevier.nl/locate/ultsonch

Sonication of chitin–glucan, preparation of water-soluble fractions and characterization by HPLC

Danica Mislovičová [a,*], Jana Masárová [a], Katarína Bendžálová [b], Ladislav Šoltés [c], Eva Machová [a]

[a] *Institute of Chemistry, Slovak Academy of Sciences, SK-84238 Bratislava, Slovak Republic*
[b] *Faculty of Natural Sciences of Comenius University, SK-84215 Bratislava, Slovak Republic*
[c] *Institute of Experimental Pharmacology, Slovak Academy of Sciences, SK-84216 Bratislava, Slovak Republic*

Received 28 June 1999

Abstract

A water-insoluble chitin–glucan complex, isolated from the mycelium of *Aspergillus niger*, was swollen in various aqueous media and treated subsequently by high-energy sonication. The concentration of the resulting water-soluble polysaccharide fractions was dependent on the swelling medium, the amount of the chitin–glucan complex in the suspension, and on the time of sonication. The yields of water-soluble chitin–glucan were within the range 13.6 to 24.4% relative to the mass of the original chitin–glucan. The nitrogen content obtained for the samples of water-soluble chitin–glucan indicated a higher content of chitin (3.45% of nitrogen in high-molecular fraction) than in the original water-insoluble chitin–glucan sample (1.8%). The distribution of the molecular weights of the water-soluble fractions prepared was determined by high-performance liquid chromatography.

Keywords: Fractionation; HPLC; Sonication; Water-soluble chitin–glucan

1. Introduction

The alkali-insoluble cell-wall residue of the *Aspergillus niger* biomass consists mainly of chitin and (1→3, 1→6)-β-D-glucan. Chitin is thought to be present as microfibrils physically embedded in the β-glucan matrix [1,2]. Polysaccharides of the (1→3)-β-D-glucan type belong to the group of so-called biological response modifiers [3–5]. Immunopharmacological activity of β-glucan involves enhancement of the host resistance to viral, bacterial, fungal, and parasitic infections. Chitin-poly-*N*-acetyl-(1→4)-β-D-glucosamine — a cellulose-like biopolymer — belongs to the most abundant organic compounds on Earth [6]. It is present in a large number of life forms, including insects, fungi, and crustaceans. It has a combination of properties that makes it an attractive biomaterial. Medical applications of chitin are based on its immunological properties and hypocholesterolemic activity [7].

Chitin–glucan (CG) from the mycelium of *A. niger* was isolated in 1979 by Muzzarelli [8]. The method comprises treatment of mycelia with aqueous NaOH solution (2.5%) at ambient temperature overnight and subsequently with concentrated NaOH (40–45%) at 130°C for 4–6 h. On applying this procedure, the insoluble residue is left as a white powder material containing 32% of the polyaminosaccharide and 15–20% glucan. Other procedures for the isolation of CG from the mycelium of *A. niger* involve the alkaline extraction to remove the proteins and alkali-soluble polysaccharides [9–12].

The linkage region between chitin and (1→3, 1→6)-β-D-glucan in the cell wall was studied in detail in *Saccharomyces cerevisiae* yeast by Kollar and coworkers [13,14]. They established that the major component of the yeast cell-wall is a (1→3)-β-D-glucan with some (1→6)-β-D-linked side chains. To these further (1→3)-β-D-glucan chains are linked. *N*-Acetylglucosamine at the reducing terminus of the chitin chain is bound to the glucose units in the side chains either by (1→4)-β- or (1→2)-β-linkages.

Sonication can be used to degrade the (1→4)-β-

* Corresponding author. Tel.: +421-7-5941-0263; fax: +421-7-5941-0222.
E-mail address: mislovicova@savba.sk (D. Mislovičová)

1350-4177/00/$ - see front matter
PII: S1350-4177(99)00030-9

linkage (depolymerization) and effect the deacetylation of chitinous materials. The principle behind the above physical means is to provide the added energy needed to break the chemical bonds [15]. Wang and Lin [16] reported that ultrasonic treatment decreases the molecular weight without deacetylation of the treated chitosan. However, Muzzarelli and Rocchetti [17] reported that sonication leads to an immediate chain degradation and to detectable deacetylation after more prolonged periods of treatment, especially at low pH. The ultrasonic effect on the degradation of other carbohydrates was also studied [18,19].

The aim of the present work was to prepare a water-soluble CG from the mycelium of *A. niger*, the waste material obtained upon the industrial production of citric acid, by using of high-energy sonication. The influence of the material swelling conditions and of the time of sonication on the yield of a water-soluble product was investigated. The high-performance liquid chromatography (HPLC) method was applied to determine the influence of sonication on the distribution of molecular weight of water-soluble CG and also to characterize the products after fractionation.

2. Experimental

2.1. Materials

The *A. niger* mycelia were obtained as a by-product of surface production of citric acid from Lachema (Kaznejov, Czech Republic). Mycelia were digested with boiling 1 M aqueous NaOH solution for 1 h. The remaining insoluble CG was then washed exhaustively with water and dried by lyophilization [11].

A set of the pullulan standards, P-82, with the weight-average molecular weights M_w within the range 5.8–853.0 kDa, were purchased from Shodex (Macherey–Nagel GmbH + CoKG, Düren, Germany).

2.2. Swelling and sonication

The insoluble CG sample was swollen by stirring in aqueous alkaline media (0.0–1.0 M NaOH) for various times (48 h–2 weeks). CG in the amount 25–200 mg per 25 ml of the swelling media was applied. After the sample neutralization with concentrated HCl, the CG suspension was sonicated in a UZD 300 ultrasound generator (Person–Ultragen, Nitra, Slovak Republic) operating at 90–100 W and 20 kHz. The generator was equipped with a 1.5 cm diameter probe. The duration of sonication was 10, 20, 30, and 40 min. The suspension was cooled during sonication. The average temperature was 25°C.

2.3. Fractionation

The water-soluble CG for the fractionation procedure was prepared as follows: a suspension of 200 mg/50 ml of 1 M NaOH was swollen (for 2 days) and sonicated (20 min). The water-soluble polysaccharides were separated using a MPW-310 centrifuge (Mechanika Precyzyjna, Warszawa, Poland). The desalted supernatant was fractionated by ultrafiltration in a stirred cell (Amicon, Beverly, USA) with the membrane ultrafilters YM 3, 10, and 30. Concentration of the water-soluble polysaccharides before and after sonication was determined by the phenol–sulfuric acid method [20].

Another method of fractionation was based on the solubility of the water-soluble CG at different temperatures and the amount of fractions was determined as the mass of the lyophilizates. The supernatant, the preparation of which is described above, was divided into two fractions (using the YM 30 membrane). The fraction up to $M_w < 30$ kDa was desalted by ultrafiltration through YM 3 membrane. The second fraction with $M_w > 30$ kDa was subsequently fractioned according to the biopolymer's solubility in cold water (80 ml; 70 h; 20°C), and of the insoluble residue in hot water (80 ml; 4 h; 80°C). All three water-soluble fractions obtained were lyophilized. The lyophilizates were analyzed for C, H, N, O content using an EA 1108 CHN-O analyzer (Milan, Italy).

2.4. HPLC analysis

The molecular-weight parameters of the water-soluble CGs were investigated using a Schimadzu HPLC system (Wien, Austria) containing an LC-10AD high-pressure pump, a GT-104 membrane degasser, and two detectors connected in series: a RID-6A differential refractometer and a SPD-10AV spectrophotometer set at 215 nm or 280 nm. The HPLC separations were performed on a HEMA-BIO 1000 column (8×250 mm) packed with 10 μm sorbent (Tessek, Prague, Czech Republic). The mobile phase, 0.1 M aqueous NaCl in a 0.05 M phosphate buffer (pH 7.0), was run at a flow rate of 0.5 ml/min. The samples were dissolved in the eluent, and introduced into the device with a Rheodyne 7725i injector. The sample volume injected was 20 μl. The HPLC system was calibrated using pullulan standards. The datastation Class-VP-Chromatography (Shimadzu) was used to control the separation and to evaluate the HPLC results obtained.

3. Results and discussion

3.1. Swelling and sonication of CG

The water-insoluble CG complex (25 mg) was swollen in 25 ml of various swelling media. The recovery of the

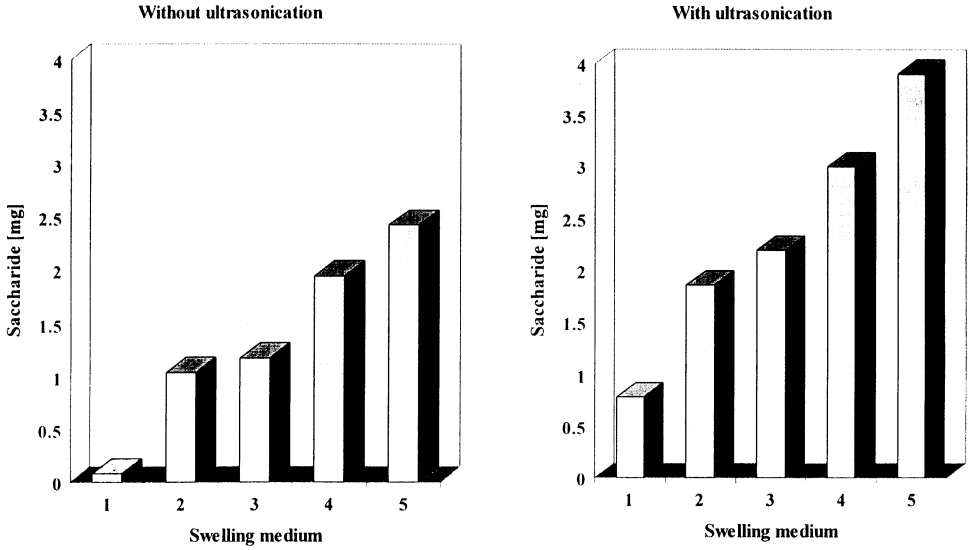

Fig. 1. Production of water-soluble CG in various swelling media. Swelling media: (1) water, (2) 0.25 M NaOH, (3) 0.5 M NaOH, (4) 0.75 M NaOH, (5) 1 M NaOH. The time of sample sonication was 20 min; the polysaccharides were determined by the phenol–sulfuric acid method.

water-soluble biopolymer, after swelling for 48 h in various media, as well as after additional 20 min sonication, is shown in Fig. 1. Prolongation of swelling beyond 48 h did not increase the yields of the water-soluble products. Production of the water-soluble polysaccharide depended mainly on the concentration of NaOH.

The influence of the duration of sonication on the biopolymer pretreated for 48 h with 1 M NaOH is presented in Table 1. The results show that for increasing the time of sonication the yield of water-soluble polysaccharide increases up to 3.8 mg, i.e. 15.2%.

The results presented in Table 2 document that by applying a higher load of CG (200 mg) to 25 ml of 1 M NaOH, the yield of the water-soluble saccharides determined by the phenol–sulfuric acid method in the supernatants increases to 17.8 mg (without sonication) and 14.6 mg (with sonication). In the experiments, the CG:1 M NaOH ratio was changed from 25 mg/25 ml to 200 mg/25 ml and the time of sonication was 20 min. The non-reactivity of *N*-acetylglucosamine in the phenol–sulfuric acid method caused the differences between the results obtained by such an assay and those obtained from the mass of lyophilizates. The real yields of the water-soluble CG as lyophilizates (Table 3) at higher CG:1 M NaOH ratios (above 50 mg/25 ml ratio) were greater than those determined by the phenol–sulfuric acid method (Table 2). Yields of up to 32.6 mg after sonication for the same set of experiments have been obtained. The lyophilizates prepared from CG fractions were less soluble — 20 to 50 wt% of lyophilizate were insoluble. The low concentration of oligosaccharide fragments of CG obtained at high sample loads (the two last lines in Table 2) was due to an increased chitin content, which cannot be determined by the phenol–sulfuric method. A possible coagulation of high-

Table 1
Dependence of the yield of the water-soluble polysaccharide on the time of sonication

Ultrasonication (min)	0	10	20	30	40
Water-soluble polysaccharide[a] (mg)	2.4	2.8	3.4	3.5	3.8
Water-soluble polysaccharide[b] (%)	9.6	11.2	13.6	14.0	15.2

[a] Determined by the phenol–sulfuric acid method.
[b] Relative to the initial mass of the sample.

Table 2
The yield of the water-soluble polysaccharide at various CG-complex loadings

Sample load (mg/25 ml 1 M NaOH)	Without sonication		With sonication	
	(mg)[a]	(%)[b]	(mg)[a]	(%)[b]
25	2.4	9.6	3.4	13.6
50	3.8	7.6	6.1	12.2
100	8.2	8.2	8.5	8.5
200	17.8	8.9	14.6	7.3

[a] Determined by the phenol–sulfuric acid method.
[b] Relative to the initial mass of the sample.

Table 3
Influence of the ratio CG:NaOH on the yield of water-soluble sonicated CG lyophilizate

Sample load (mg/25 ml NaOH)	Weight of lyophilizate (mg)	Yield of lyophilizate[a] (%)
25	3.9	15.6
50	8.8	17.6
100	24.4	24.4
200	32.6	16.3

[a] Relative to the initial mass of the sample.

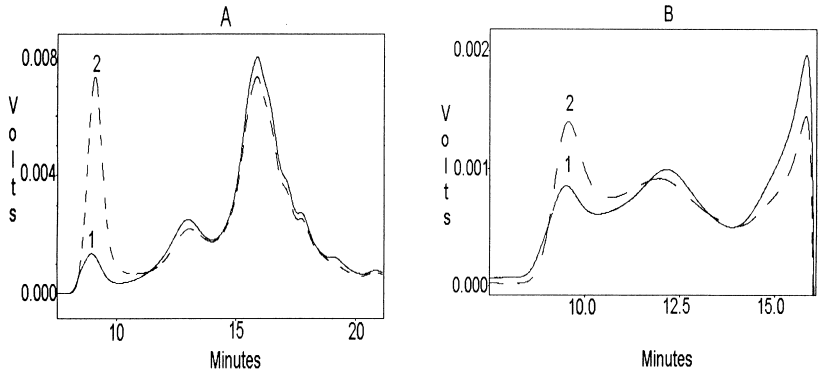

Fig. 2. Chromatograms of the water-soluble CG prepared without (1) and with (2) ultrasound treatment. (A) Record of the UV detector set at 280 nm; (B) record of the RI detector.

molecular weight aggregates of CG at the highest sample load (200 mg/25 ml) might cause a drop of the yield of water-soluble product (Tables 2 and 3). Therefore, an absolute extraction improvement by means of sonication (in percent) is found to be difficult to calculate.

3.2. HPLC chromatography of sonicated CG

Fig. 2 shows the chromatograms (at UV and RI detection) of the water-soluble biopolymers prepared on applying 100 mg CG per 25 ml of 1 M NaOH for 48 h, with and without 20 min action of ultrasound. The chromatograms revealed three peaks corresponding to the following M_w values: (I) ~600 kDa (9.57 and 9.52 min); (II) ~80 kDa (11.93 and 12.15 min); (III) ~8 kDa (15.61 and 15.62 min). As evident from Fig. 2, the action of ultrasound led to a significant increase in the content of the fraction with the highest molecular weight, i.e. fraction I. With prolonged time of sonication (up to 40 min), the content of fraction I increased further

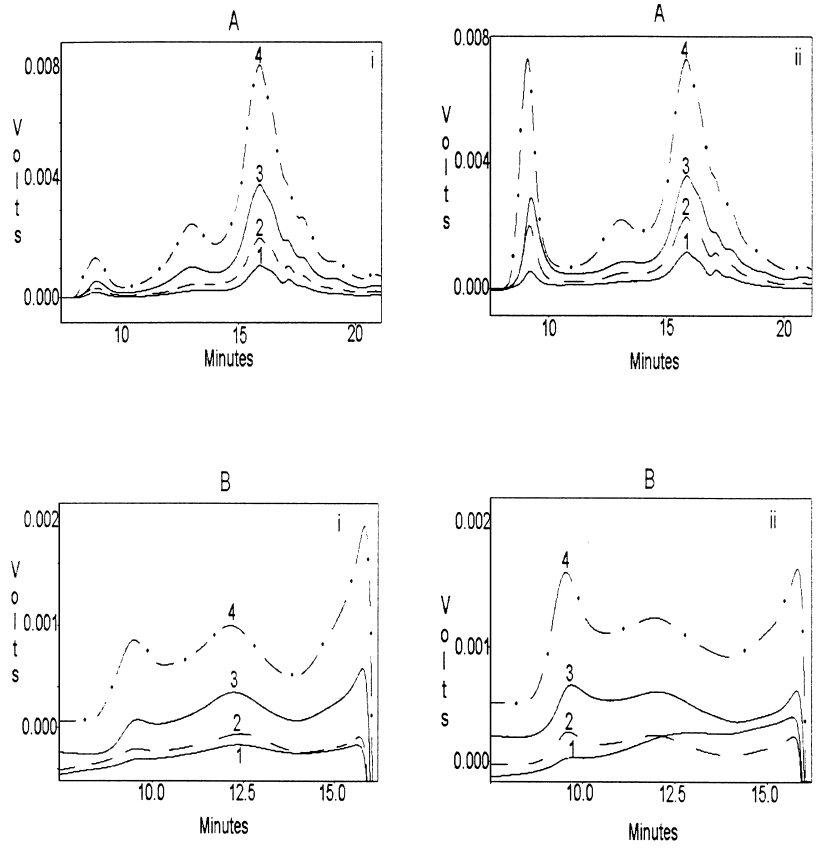

Fig. 3. Chromatograms of the water-soluble CG prepared without (i) and with (ii) ultrasound treatment at various ratios CG:1 M NaOH. (A) Record of the UV detector at 280 nm; (B) record of the RI detector; (1) 25 mg/25 ml, (2) 50 mg/25 ml, (3) 100 mg/25 ml, (4) 200 mg/25 ml.

Table 4
Fractionation of water-soluble CG by ultrafiltration

Sample M_w	GC[a] (mg)	GC[b] (%)
below 3 kDa	0.33	6.0
from 3 to 10 kDa	0.43	7.1
from 10 to 30 kDa	0.52	8.6
above 30 kDa	4.36	77.3

[a] Determined by the phenol–sulfuric acid method.
[b] Relative to the sum of determined fractions masses (1+2+3).

(data not shown). This increase was shown better by recording the UV (280 nm) adsorption of the sample.

Comparison of the chromatograms (Fig. 3) of samples prepared by using various CG:1 M NaOH ratios, without and with sonication, revealed that along with proportional increases of fractions II and III according to the content of CG in the suspension treated, a significant increase of peak I after sonication occurred. This fact can be evidenced with RI detection and by a clearer UV tracing at 280 nm. From the aforementioned results it is evident that the molecular weight distribution of the water-soluble CG samples is represented by three peaks (i.e. it is trimodal) (Figs. 2 and 3). These three peaks are detectable by both RI and UV detectors. The amounts of fractions I, II, and III depended on the conditions of the sample swelling and its sonication (swelling medium, CG:NaOH ratio, time of sonication). The fraction with the high molecular weight (peak I) is more easily detectable by UV than by RI detection. This is caused, most probably, by a higher content of chitin in fraction I, which was confirmed by its adsorption spectrum which resembled that of chitooligosaccharides. (Glucan does not adsorb light at $\lambda = 215$ or 280 nm.) The determination of the low molecular-weight sample component (fraction III) by HPLC was hindered by the closeness of the peak of salts present in the samples.

3.3. Fractionation of water-soluble CG

The solution of water-soluble CG prepared by swelling in 1 M NaOH (200 mg/50 ml; 2 days), neutralization with aqueous HCl, sonication (for 20 min), and centrifugation, was fractionated in the ultrafiltration cell. The supernatant (18 ml) was divided using three different membranes into four fractions followed (Table 4). From the amounts of the saccharides in individual fractions, determined by the phenol–sulfuric acid method, the major one was the fraction having the highest molecular weight. Its content was 77.3%, whereas those of the other fractions were below 10%.

Table 5
Yields of the water-soluble CG fractions

Fraction no.	Characterization	Lyophilizate of soluble CG (mg)	Lyophilizate of soluble CG[a] (%)	Nitrogen (%)
0	Unfractionated sample	–	100	2.40
1	Fraction $M_w < 30$ kDa, desalted	3.2	11.9	3.22
2	Fraction $M_w > 30$ kDa soluble in cold water	15.9	59.1	3.45
3	Fraction $M_w > 30$ kDa insoluble in cold water/soluble in hot water	7.8	29.0	2.51

[a] Relative to the sum of lyophilizates masses (1+2+3).

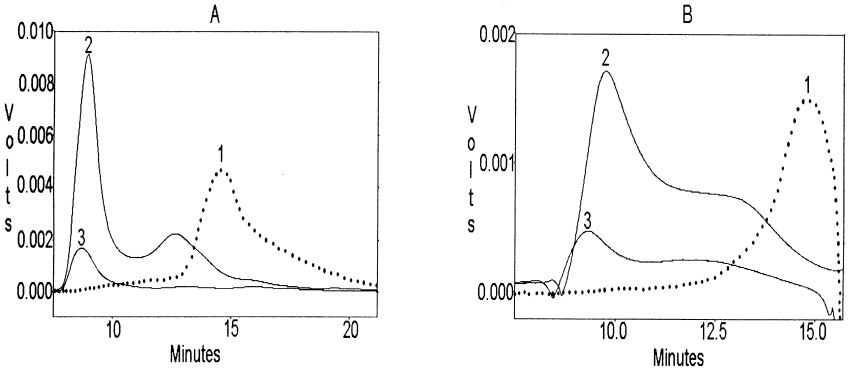

Fig. 4. Chromatograms of the water-soluble CG fractions prepared by ultrafiltration and by fractionation according to their water solubility. (A) Record of the UV detector at 280 nm; (B) record of the RI detector; (1) fraction with $M_w < 30$ kDa, (2) fraction with $M_w > 30$ kDa soluble in cold water (20°C), (3) fraction with $M_w > 30$ kDa insoluble in cold water, but soluble in hot water (80°C).

Another procedure to obtain a fraction with $M_w > 30$ kDa, which would be soluble in cold water, has been developed. 200 mg CG was swollen in 50 ml 1 M NaOH for 48 h, neutralized, sonicated 20 min, and subsequently processed. The low molecular-weight fraction was separated by ultrafiltration (using YM 30 and YM 3 membranes). Further fractionation of the high molecular-weight component ($M_w > 30$ kDa) was performed according to the solubility in cold and in hot water. The results are presented in Table 5. The chromatograms of fractions 1, 2, and 3 are show in Fig. 4. The major fraction 2 (submitted to the bioactivity tests), was composed of polysaccharides having $M_w \approx 660$ kDa and $M_w \approx 50$ kDa (detected by RI and UV). The percentage of nitrogen in the individual fractions presented in the last column of Table 5 indicated a higher content of chitin in fraction 2 (3.45% of nitrogen). This implied a higher content of chitin than in the original water-insoluble CG sample (1.8% of nitrogen) and in water-soluble CG prepared by the action of ultrasound (2.4% of nitrogen).

The experiments of concentration, heating of solution and lyophilization of water-soluble CG fractions have shown changes in its solubility and the distribution of molecular weight. This fact caused problems with separation of the pure individual fractions. The changes were estimated by means of HPLC of the treated samples.

In conclusion, it can be said that by the action of highly energetic sonication on water-insoluble CG, a chain cleavage of water-soluble fragments with high chitin content is achieved from the surface of swollen particles (cavitation near the surface). Subsequently, these fragments under sonication form aggregates of high molecular weight (ca. 600 kDa), which can at a higher concentration partially coagulate. The content of the CG fragments of high molecular weight increases with the sonication time, the temperature and the CG:1 M NaOH ratio.

Acknowledgement

This work was supported by the VEGA Grants of Slovak Agency for Science (Grant No. 2/5062/98 and 2/5059/98).

References

[1] R.F. Rosenberger, The cell wall, in: J.E. Smith, D.R. Berry (Eds.), The Filamentous Fungi, vol. 2, Edward Arnold, London, 1976, pp. 328–344.
[2] J.H. Sietsma, J.G.H. Wessels, J. Gen. Microbiol. 125 (1981) 209–212.
[3] T. Kiho, M. Katsuragawa, K. Nagai, S. Ukai, Carbohydr. Res. 224 (1992) 237–243.
[4] E. Machová, G. Kogan, J. Alföldi, L. Šoltés, J. Šandula, J. Appl. Polym. Sci. 55 (1995) 699–704.
[5] W.-M. Kulicke, A.I. Lettau, H. Thielking, Carbohydr. Res. 297 (1997) 135–143.
[6] D. Knorr, Food Technol. 38 (1) (1984) 85–97.
[7] R. Muzzarelli, Chitin, in: G.O. Aspinall (Ed.), The Polysaccharides, vol. 3, Academic Press, Orlando, 1985, p. 417.
[8] R. Muzzarelli, Patent FGR, COSB 37/08. No 293 802, 1979.
[9] I. Stojanov, C. Panajotov, A. Atev, P. Dalev, S. Ilieva, V. Balutzov, E. Kolev, Nahrung 30 (3–4) (1986) 411–412.
[10] M. Leštan, A. Pečavar, D. Leštan, A. Perdih, Amino Acids 4 (1993) 169–176.
[11] D. Chorvatovičová, J. Šandula, Mutation Res. 346 (1995) 43–48.
[12] V.M. Tereshina, A.C. Memorskaja, E.P. Feofilova, D.V. Nemcev, V.M. Kozlov, Mikrobiologia 66 (1) (1997) 84–89.
[13] R. Kollar, E. Petráková, G. Ashwell, P.W. Robbins, E. Cabib, J. Biol. Chem. 270 (1995) 1170–1178.
[14] R. Kollar, B.B. Reinhold, E. Petráková, H.J.C. Yeh, G. Ashwell, J. Drgonová, J.C. Kapteyn, F.M. Klis, E. Cabib, J. Biol. Chem. 272 (1997) 17 762–17 775.
[15] R.H. Chen, J.R. Chang, J.S. Shyur, Carbohydr. Res. 299 (1997) 287–294.
[16] W. Wang, W. Lin, Huaxue Tougbao 44 (1989) 41, Chem. Abstr. 112 (1990) 58627g.
[17] R.A.A. Muzzarelli, R. Rocchetti, Carbohydr. Polym. 5 (1985) 461–472.
[18] J.P. Lorimer, T.J. Mason, T.C. Cuthbert, E.A. Brookfield, Ultrason. Sonochem. 2 (1995) 55–57.
[19] S.C.S. Szu, G. Zou, R. Schneerson, J.B. Robbins, Carbohydr. Res. 152 (1986) 7–20.
[20] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Anal. Chem. 28 (1956) 350–356.