CHARACTERIZATION OF CHITOSAN ISOLATED FROM ASPERGILLUS NIGER INDUSTRIAL WASTE MYCELIUM

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INTRODUCTION

Fungal mycelial wastes of the antibiotic or citric acid industry can become free and rich alternative sources of chitin - chitosan materials, beside the traditional industrial source - shellfish waste materials. Moreover, the fungal chitosans can have unique properties compared with those derived from Crustacea.
We have considered the possibility of industrial production of chitosan from Aspergillus niger mycelial wastes from citric acid production in a biotechnological plant in Czech republic.
The main part of the mycelial waste is discarded to the sewage treatment system or burnt.
The alkali-insoluble cell-wall residue of the Aspergillus niger biomass consists mainly of chitosan, chitin and ß-glucans, with a significant preponderance of (1→3)-ß-D-glucan. Chitin is thought to be present as microfibrils physically embedded in the ß-glucan matrix.

EXPERIMENTAL

Hyphal wall isolation and chitosan extraction

The A. niger mycelia were obtained as a by-product of submerse production of citric acid from Lachema (Kaznějov, Czech republic). Mycelia were washed exhaustively with water and dried by freeze-drying. Chitosan and chitin-glucan complex were extracted from Aspergillus niger mycelial wastes of citric acid production with KOH and HCl using a method developed by combination and modification of methods by Muzzarelli [1] and White et al.[2]. Mycelium was treated with 60% aqueous KOH solution (10 ml per 1 g of dry mycelium) at 130 °C for 2 - 3 h to remove proteins, lipids and alkali-soluble polysaccharides. The insoluble material was washed with DEMI water to obtain neutral pH and concentrated ethanol and freeze-dried. On applying this procedure, an insoluble fraction of cell walls consisting of chitosan and chitin - glucan complex is left as a white powder. For chitosan isolation, this freeze-dried alkali insoluble fraction was treated with diluted 2 % HCl solution (50 ml per 1 g of dry matter) for 1-10 h at 25° or 95°C. The pH value of the acidic supernatant was increased to 9,5 with 2M NaOH. The alkali-insoluble precipitate (presumable chitosan) obtained was repeatedly centrifuged and washed with distilled water, freeze dried and weighed.

Measurement of IR spectrum of the isolated chitosan fraction

The IR spectra of the KBr discs containing the isolated alkali-insoluble precipitate after the acidic extraction (presumable chitosan), and a commercial chitosan, medium molecular weight (Sigma-Aldrich) were measured from 4 000 cm^{-1} to 450 cm^{-1} with a Nicolet-Impact 410 FT-IR spectrophotometer.

Measurement of ^{1}H NMR spectrum of the isolated chitosan fraction

10 mg of the alkali-insoluble precipitate (presumable chitosan) and a commercial chitosan, medium molecular weight (Sigma-Aldrich) were dissolved in D_2O (pH=3,1). Chemical shifts in ppm units are related to DSS standard (sodium dimethylsilylsulphonate). From the ratio of acetyl CH_3 group (2,0 ppm) and H_2 group (deacetylated form, 3,13 - 3,10 ppm) signal intensities degree of acetylation of the isolated chitosan was estimated.

Acid hydrolysis of the isolated chitosan fraction

20 g of the freeze-dried alkali insoluble fraction of cell walls after the alkaline extraction was suspended in 800 ml of 2 % (v/v) HCl and treated at 60°C under stirring using a reflux condenser. Samples of 100 ml of reaction mixture were collected in selected time intervals and centrifuged at 11 600 g. Supernatants were analysed by gel permeation chromatography (GPC) and used for chitosan precipitation by increasing pH value with a concentrated KOH solution to 9,5. The precipitated chitosan samples were repeatedly washed with DEMI water, freeze - dried and used for viscosimetric determination of MW.

Viscosimetric determination of MW of the isolated chitosan fraction
To determine the rate of the acid hydrolysis of the O-glycosidic linkages (depolymerization) molecular weights of the chitosan samples were determined using a method described by M.Terbojevich and A.Cosani [3].

GPC determination of acetic acid
To determine the rate of the acid hydrolysis of the N-acetyl linkage (deacetylation) acetic acid formed was determined by gel permeation chromatography under following conditions:
TSK gel GMPW column; 7,5 x 300 mm (Supelco); UV-VIS „photodiode array" detection; eluent: 3 mM HCl in water; flow rate: 0,5 ml.min^{-1}; injection: 20 μl.

RESULTS and DISCUSSION

Hyphal wall isolation and chitosan extraction

Yields of the insoluble material after the treatment of the fungal biomass with KOH solution were 25 - 33.2 % on a dry cell weight basis. The result is in a good agreement with results published by other authors.
The total yields of the chitosan fraction after the repeated acidic extraction were 12 - 15% on a dry cell weight basis. We found that one hour is a sufficient time period to form an equilibrium chitosan concentration in the acidic solution at both the temperatures tested. Chitosan yields did not already increase with prolonged extraction time. The material is a fine-grained white powder. Miyoshi, et al. [4] obtained yields varied from 1.2 to 10.4 % (of a dry fungal cell weight) of a chitosan-like material extracted from 5 different fungal strains other than Aspergillus sp. Muzzarelli, et al., [5] found 11 - 14 % of chitosan in A. niger biomass under comparable conditions of alkali extraction.
The final yields of the acid insoluble chitin-glucan fraction were 10 - 21% on a dry cell weight basis, according to the conditions of the isolation procedure.

Measurement of IR spectrum of the isolated chitosan fraction

To prove that the acid extractable material contains chitosan, its IR spectrum was measured in comparison with IR spectrum of a commercial chitosan purchased from Sigma-Aldrich (see Fig. 1). The isolated fraction gave IR spectrum similar to that of the commercial chitosan. The result indicates a strong similarity of both the compounds.

Measurement of ^{1}H NMR spectrum of the isolated chitosan fraction

Fig. 2 shows ^{1}H NMR spectrum of the isolated chitosan fraction. 1H NMR spectrum of the commercial chitosan sample is not shown because of its low solubility at the conditions of the method. However comparisons with the published chitosan spectra proved that the isolated fraction is chitosan. Minor signals at 0.8 and 1.1 ppm were found in the spectrum which does not belong to any form of chitin - chitosan. The signals have not been identified. From the ratio of acetyl CH_3 group (2.0 ppm) and H_2 group (deacetylated form, 3.13 - 3.10 ppm) signal intensities degree of acetylation (DA) of the isolated chitosan was calculated as cca 4%. The result is in a good correspondence with our previous result obtained with a direct spectrophotometric method.
The calculation of DA: $DA = [(0.118/3)/1] \times 100 \cong 4$
Kobayashi, et al. [6] prepared a chitosan product from mycelia of absida strains with a degree of acetylation of 8 - 21 %. Degree of acetylation of different fungal chitosans ranged from 6 to 15 %, according to Rane and Hoover [7].

Acid hydrolysis of the isolated chitosan

Time course of the acid hydrolysis of the O-glycosidic linkages, as determined by the viscosimetric determination of molecular weights of the collected chitosan samples, is shown on Fig. 3.
The depolymerization time course shows a steep decrease of chitosan MW from 500 kDa to 50 kDa in the first 2,5 hours of the reaction. From 2,5 hour chitosan MW decreased slowly to a value corresponding to a small chitooligosaccharides in approximately 10 more hours. Because the results can be distorted by aggregation effects of chitosan particles they are still to be verified by other method, like GPC or light scattering.
To determine the rate of the acid hydrolysis of the N-acetyl linkage (deacetylation) acetic acid formed in the reaction mixture was determined in the selected time intervals by GPC method (Fig. 4). In contrast to the results of Varum, et al. [8], who found the rate of hydrolysis of the N-acetyl linkage to be equal to the rate of hydrolysis the glycosidic linkages of in dilute HCl, we have observed a quick production of just a low equilibrium concentration of acetic acid (about 0,1 % w/w). The equilibrium concentration was constant and independent on degree of acetylation of the tested chitosans . To allow the reaction to proceed it would be probably necessary to remove the acetic acid from the reaction mixture continuously to shift the reaction equilibrium.

CONCLUSIONS

A mixture of chitosan and the chitin - glucan complex was obtained by treating the prewashed mycelia with 60 % aqueous KOH at 130°C for 2 - 3 hours with yields in the range 25 - 33% on a dry cell weight basis. In this alkali-insoluble fraction 2,3% of total glucans were found. 35 - 50% part of the alkali-insoluble material (12 - 15% on a dry cell weight basis) was extractable by 2 % HCl at 95°C. The acid extractable fraction was identified as chitosan. Molecular weights of the isolated chitosan fraction vary significantly from 588 kDa to a few kDa according to the conditions of the acidic extraction - time and temperature. Degree of acetylation is 4 - 5%.
Results of IR spectral analysis indicate that chitin is the predominant constituent of the remaining acid-insoluble chitin - glucan fraction. 6,3% of total glucans were found in the chitin - glucan complex.
The production of chitosan from mycelia of filamentous fungi is hereby proposed as an alternative to the production from crab and shrimp shells. However the proposed manufacturing process turned out to be too expensive in the industrial scale because of the necessity of usage of large excess of caustic alkali solutions. That is the reason why we are looking for alternative production methods at present e. The alternative methods should consist from a cheap isolation of the chitin - glucan complex from the mould biomass and subsequent deacetylation of chitin to chitosan without of the usage of the caustic alkali solutions. Fermentation treatment or autolysis of the biomass are examples of the cheap methods for the chitin - glucan complex isolation.
An interesting alternative to the traditional method of chitosan production can also be a manufacture of water soluble chemical derivates of chitin - glucan complex, such as carboxymethylated derivates.

REFERENCES:

[1] R.Muzzarelli, Patent FGR, COSB 37/08. No 293802, 1979.
[2] S.A.White, P.R. Farina, and I. Fulton, *Appl. Environ. Microbiol.* **38** (2) (1979), 323-8.
[3] M.Terbojevich and A.Cosani: Molecular weight determination of chitin and chitosan. In: Chitin Handbook, R.A.A. Muzzarelli and M.G.Peter, eds., European Chitin Society. 1977.
[4] H. Miyoshi, et al., *Biosci. Biotechnol. Biochem.* **56** (12) (1992), 1901- 05.
[5] A.A.R. Muzzarelli, F. Tanfani, and G. Scarpini, *Biotech. Bioeng.* **22** (1980), 885-896.
[6] T. Kobayashi, et al., *Nippon Nogei Kogaku Kaishi* **62** (10) (1988), 1463-69.
[7] K.D. Rane, and D.G. Hoover, *Process Biochem.* **28** (1992), 115-118.
[8] K.M Varum, M.H. Ottoy, and O. Smidsron, *Carbohydrate Polymers* **46** (2001), 89-98.

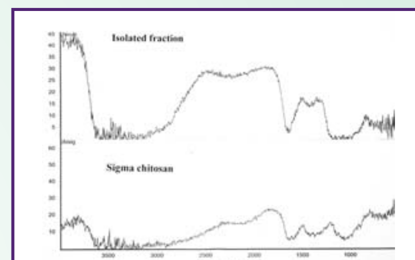

Fig.1. Comparison of IR spectra of the isolated chitosan fraction (below) and a commercial chitosan sample from Sigma-Aldrich (above)

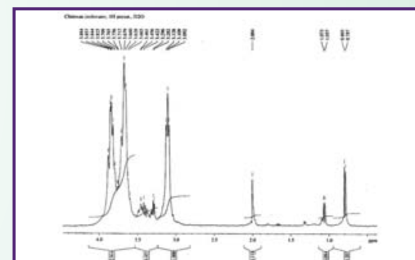

Fig. 2. ^{1}H NMR spectrum of the isolated chitosan fraction

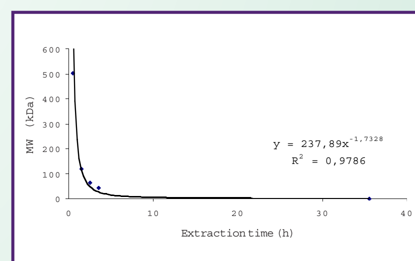

Fig. 3. Time course of chitosan depolymerization in 2% HCl at 60°C

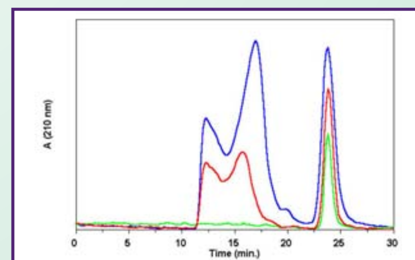

Fig. 4. Identification of acetic acid peak in chitosan solution in 2% HCl by GPC method.
Blue curve: isolated chitosan; Green curve: 0,1% acetic acid in water; Red curve: isolated chitosan with a standard addition of acetic acid