Applications of mushroom chitosans in medical biomaterials

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INTRODUCTION

Chitosans and its derivatives have been studied widely for use in biomedical applications such as wound dressings, drug delivery systems and materials for tissue engineering. Chitosan has been reported to be a promising candidate as a scaffold material for engineered human tissue such as skin, cartilage and bone due to its biocompatibility and resorbability.

Wastes after industrial processing of edible mushrooms, such as *Agaricus* or *Pleurotus* species, can become free and alternative source of chitin - chitosan materials, beside the traditional industrial source - shellfish waste materials. Moreover, the mushroom chitosans can have unique properties compared with those derived from *Crustacea.*

Chitosans have been extracted from *Pleurotus ostreatus* and *Agaricus hortensis* using successive alkali extraction and characterized by physiochemical methods.

Several medical applications of the mushroom chitosans have been tested:

1) composite chitosan - collagen superporous sponges for wound healing and scaffolds
2) nonwoven composite fabrics from nanofibers containing chitosan
3) tissue adhesive material for medical usage

EXPERIMENTAL

Chitosan isolation

Mushrooms (*Pleurotus ostreatus* or *Agaricus hortensis*) were washed exhaustively with water after adjusting pH with KOH to 8-9.

Chitosan was extracted from the washed mushrooms with KOH and HCl using a method developed by combination and modification of methods by Muzzarelli [1] and White et al. [2]. Mushroom biomass was treated with 60% (w/v) 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. For chitosan isolation, this freeze-dried alkali insoluble fraction was boiled with diluted 0,1 - 1% (v/v) HCl or acetic acid solutions (50 ml per 1 g of dry matter) for 10-40 min. 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.

Viscosimetric determination of MW of isolated chitosan

Molecular weight of the isolated alkali-insoluble precipitate (presumable chitosan) was determined using a viscosimetric method described by M.Terbojevich and A.Cosani [3].

Measurement of ^{13}C NMR spectra of isolated chitosans

Solid-state ^{13}C CP/MAS (cross polarization/magic angle spinning) NMR spectra were measured on a Bruker Avance 500 spectrometer at 125.8 MHz with spinning frequency 10 kHz and contact time 2 ms. Chemical shifts in the ^{13}C NMR spectra were referred to the carbonyl line of glycine (with a signal at 176.0 ppm from TMS) by sample replacement.

Measurement of IR spectra of the isolated fractions

The IR spectra of the KBr discs containing the isolated alkali-insoluble precipitate (presumable chitosan) were measured from 4 000 cm^{-1} to 450 cm^{-1} with a Nicolet-Impact 410 FT-IR spectrophotometer. A ratio KBr / sample in the discs was 180 : 1.

Preparation of nonwoven fabric containing fungal chitosan

The nonwoven fabric was prepared containing nanofibres composed from the mushroom chitosan (10 - 90%) and polyvinylalcohol using an unique electrospinning technology *Nanospider* which enables the production nanofiber textiles on an industrial scale in Elmarco company (Liberec, Czech republic).

Preparation of test tissue adhesives

Principle of the proposed tissue adhesive is crosslinking gelatin, collagen, or other proper proteins with a commercial microbial enzyme transglutaminase to form strong and irreversible gels. This acyl-transfer enzyme catalyzes transamidation reactions that lead to the formation of N-ε-(γ-glutamyl) lysine crosslinks in proteins [4].

Gel formation was initiated by adding 0.25 - 1% (w/v) of transglutaminase ACTIVA WM (Ajinomoto Co., Inc., Tokyo, Japan) to the solutions containing gelatin (5-10 w/v %) from porcine skin (Type A, 300 bloom, Sigma), or blends containing different ratios of gelatin and mushroom chitosan (1.6-3.2 w/v %). Transglutaminase activity: 81 - 135 U/g (Hydroxamate method). Experiments were performed at pH 6.0 and 37°C.

Viscosimetric measurements of gelation time of the tissue adhesives

Viscosimetric measurements of gel formation were performed at constant rotation speed and temperature 37°C using viscosimeter RHEOTEST 2.1 (MLW Prufgerate- Werke Medingen, GDR). Following the addition of transglutaminase into each test blend, polymerization of a gelatin hydrogel is distinguished by the occurrence of gelation at some point in the crosslinking. At this point, the adhesive loses fluidity or its viscosity increases abruptly.

Bonding strength measurement

Bonding strength of the proposed tissue adhesive (gelatin - transglutaminase (TGA) - chitosan) was compared with bonding strengths of gelatin - TGA mixture, pure gelatin, and a commercially used mixture gelatin - resorcinol crosslinked with formaldehyde (GRF glue). The adhesive characteristics of each tested sample were determined by measuring its bonding strength between two thin muscle slices (8 x 2.5 cm) from a fresh porcine heart by an texture analyser TA-XT 2 (Stable Micro System, UK).

Preparation of composite chitosan - collagen sponge materials

Solutions containing 1% (w/v) of the mushroom chitosan, commercial chitosan *Primex TM326* (Primex Ingredients ASA, Norway), or commercial bovine collagen (M6, Medicol, Czech republic) in 0.5% acetic acid were prepared. After adjustment of pH of the prepared solutions to 6.5 with ammonia, each of the chitosan solutions was mixed with the collagen solution in 1 : 1 ratio (v/v). The suspensions obtained as described above were whisked in a high speed mixer, poured into dishes, frozen immediately and freeze dried to obtain a three-dimensional porous structure.

Before biocompatibility testing the sponges were sterilized by gama-irradiation.

Preparation of composite superporous foams containing chitosan

Superporous foams containing gelatin crosslinked by transglutaminase (TGA) and mushroom chitosan were prepared by:

1) Method using cooking soda

25 ml of 15% (w/v) water solution of gelatin (300 Bloom, Sigma-Aldrich) was mixed with 10 ml of 5% (w/v) solution of the mushroom chitosan (*Pleurotus ostreatus*) in 0,5% (v/v) acetic acid at 50°C, pH was adjusted with NaHCO3 addition to 7.0. After addition 0,25 - 1% (w/v) TGA to the solution, pH was adjusted to 6.0 by 5% (v/v) HCl. The foam formed was poured into a pre-frozen Petri dish.

2) Method using foam extrusion

The experimental solution was prepared as above, except the cooking soda addition, pH 6.0. The solution was pressed with nitrogen in a special chamber to 5MPa and expelled through a narrow atomizing jet into a pre-frozen Petri dish. Porous structures fixed by crosslinking with TGA were analyzed by macro-photography.

RESULTS and DISCUSSION

Chitosan isolation

Chitosan yields were within the range of 3 - 7% of the mushroom dry weight, depending on stage of growth and mushroom parts. According to literature chitin content of the mushrooms differs significantly from 1,8 to 19 % (d.w.), with great intra- and extraspecies variabilities.

Viscosimetric determination of MW of isolated chitosan

Molecular weights of the isolated chitosan fractions varied significantly according to the conditions of the acidic extraction - time and temperature in the range from 10 to100 kDa, relative lower in comparison with crustacean chitosans, in accordance with literature [5,8].

Measurement of ^{13}C NMR spectra of isolated chitosans

Figures 1 a)-d) show ^{13}C NMR spectra of the isolated mushroom chitosans *(Pleurotus ostreatus* 1c, *Agaricus hortensis* 1d) in comparison with spectra of a commercial chitosan Fluka (1a) and chitosan of fungus *Penicillium oxalicum* (1b, our previous result).

Signals of C1-C6 carbons of chitosan structures are in the range 50-110 ppm [6].

In spectra of samples 1c and 1d signals of carbonyl and methyl carbons from residual acetyl groups at 173.2 ppm and 22.7 ppm, respectively, are also visible.

The degree of acetylation (DA) was calculated using the formula $DA = I_{CO}/(I_{\Sigma}/6)$, where I_{CO} is the integrated intensity of the signal of carbonyl carbons and I_{Σ} is the integrated intensity of signals of chitosan structures in the range 50-110 ppm. DA of the isolated mushroom chitosans were in the range form 0.18 to 0.5, in accordance with literature [5,8], between the values for commercially available chitosans.

Intensive signal at 33.1 ppm and other signals in the region 10-45 ppm are probably due to residual proteins and/or lipids [6,7]. The identical signals in the region 10-45 ppm were observed in all the spetra of analyzed chitosan samples of *Pleurotus ostreatus* or *Agaricus hortensis.* It indicates that the contaminant is very tightly bound to chitosan macromolecules and can be a residual of natural structure of mushroom cell walls. Characterization of the contaminant particle and further chitosan purification, followed by biocompatibility assessment in vivo are inevitable for potential commercial medical applications.

Measurement of IR spectra of the isolated fractions

Figures 2 a) - d) show IR specta of intact dried musroom *Pleurotus ostreatus* (2b), isolated chitosan *Pleurotus ostreatus* (2c), isolated chitosan *Agaricus hortensis* (2d), in comparison with IR spectrum of standard chitin sample (Sigma) with assignments of the bands (1a). Spectra 2c and 2b shows typical patterns for chitosans. The distinct group of bands between 900 and 1260 cm^{-1} in the spectrum 2b appertains to the presence of glucans, which were removed during the isolation process.

Preparation of nonwoven fabric containing fungal chitosan

A sample of the nonwoven fabric containing fungal chitosan is shown in the Fig. 3. Fig.4 shows structure of the nanofibers as depicted by electron microscopy. Possible areas of medical applications of the nonwoven fabric include artificial organs, tissue engineering, artificial blood vessels, targeted drug delivery, wound dressing or mouth-screens.

Measurement of properties of the tissue adhesive

The highest rate of gel formation, about 15 minutes, was observed when gelatin concentration was 10% (w/v) and TGA concentration 1% (w/v). This gelation time should be sufficient for most applications of the biological glue.

Chitosan addition slightly increased the rate of gel formation. However, chitosan was not required for transglutaminase - catalysed reaction. We have no direct evidence to support or reject suggestion that transglutaminase can transfer the protein directly onto chitosan's amino groups.

The bonding strength of the tested sample (5 w/v % of gelatine - 3 w/v % of TGA) is comparable with the bonding strength of the sample 3 (3.3 w/v % of gelatine - 6.7 w/v % of resorcinol - 1.2 w/v % of formaldehyde), a commercially used mixture. Chitosan presence didn't influence the bonding strength significantly.

Therefore, transglutaminase provide an alternative method for crosslinking collagen, gelatine or other proteins and may offer interesting opportunities for in situ applications. The enzyme can replace the cytotoxic crosslinking agents such as formaldehyde (GRF glue) or glutaraldehyde (GRG) in many cases. Chitosan presence is important because of its healing and haemostatic properties.

Preparation of composite chitosan - collagen sponge materials

Prepared chitosan - collagen sponges have uniform porous structure, good mechanical strength and stability, and water absorption capacity, in connection with haemostatic and wound healing properties. They can be used for wound healing covers and in scaffolds applications. Moreover, chitosan has also been used frequently for drug controlled release.

Fig. 5 shows a sample of the sponge containing the mushroom chitosan.

Preparation of composite superporous foams containing chitosan

Fig. 6 shows a macro-photograph of the superporous foams containing chitosan and gelatin fixed by crosslinking with transglutaminase. The superporous hydrogel foams have a lot of interesting food and medical applications. The superporous structure and mechanical properties are especially proper for scaffold preparation with the advantages described in the above paragraph.

CONCLUSIONS

Beside their traditional usage as a source of many pharmacologically active compounds, mushrooms can be used for cheap production of medical-grade chitosan, promising biocompatible and biodegradable candidate for many medical applications, including materials for tissue engineering. Moreover, chitosan has wound healing and haemostatic properties and can be used for controlled release of biologically active compounds.

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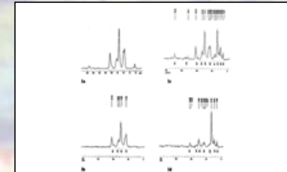

Fig.1: 13C NMR spectra of standard sample of chitosan (Fluka), 1a; chitosan isolated from fungus Penicillium oxalicum, 1b; mushroom Pleurotus ostreatus, 1c; and Agaricus hortensis, 1d.

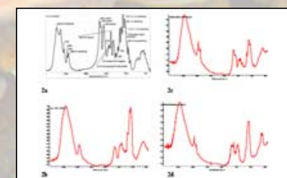

Fig. 2: IR spektra of standard chitin sample (Sigma) with assignments of the bands, 2a; dried intact mushroom biomass Pleurotus ostreatus, 2b; chitosan isolated from Pleurotus ostreatus, 2c; and Agaricus hortensis mushroom, 1d.

Fig. 3: A sample of a nonwoven fabric prepared from nanofibres composed of the mushroom chitosan (10%) and polyvinylalcohol (90%).

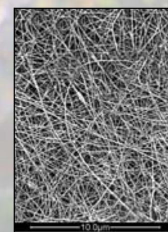

Fig. 4: Chitosan containing nanofibers as depicted by electron microscopy

Fig. 5: composite chitosan - collagen sponge.

Fig. 6: macro-photograph of the superporous foams containing chitosan and gelatin fixed by crosslinking with transglutaminase.

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