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Fungal mycelium—the source of chitosan for chromatography

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Abstract

Mycelium of the mold Aspergillus niger was used as a raw material for the preparation of microbial chitosan. Aspergillus niger, the mold used for the production of citric acid, contains approx. 15% of chitin, which can be separated, transformed into chitosan, and used as a sorbent for chromatography. The main advantage of this material in comparison with krill chitosan is the uniformity of particle size leading to the low back-pressure in the column. The other advantage is the fact, that original fibrous structure of mycelial pellets could be stabilized before chitosan preparation by cross-linking with glutaraldehyde. The product prepared by this way – crosslinked chitosan of uniform particle size, is highly porous, with high water regain and, as a result, low sedimentation velocity. Low sedimentation velocity is not disadvantage in chromatographic application, but may form some problems in batchwise operation. Chitosan as a polymer of glucosamine is anion exchanger in nature and the chromatographic properties of this anion exchanger was demonstrated by the chromatography of bovine blood plasma, glucose oxidase, and chicken pepsinogen. In all cases, the course of chromatography on crosslinked chitosan was compared with the chromatography on MONO Q (bovine blood plasma) or DEAE-cellulose (glucose oxidase, chicken pepsinogen) under the same protocol.

Keywords: Fungal mycelium; Chitosan

1. Introduction

The best matrices for the sorbents must be highly porous, must be of regular shape, and the pressure drop in column must be as low as possible. The last condition is fulfilled best if the particle size is uniform or, in reality, as near to uniformity as possible.

The mold mycelial pellets, on the other hand, are formed from a single initial cell by growing the fibers of diameter equal to the cell thickness and the length ruled by the cultivation conditions. The fibers are coiled to form the pellets which diameter is done by the conditions of growth, such as aeration, stirring, and, to a lower extent, by geometrical parameters of the fermentor [1–3].

Cultivation of the mold is carried-out most frequently for the production of citric acid [4]. Factories producing citric acid must keep the cultivation conditions constant to get maximum production of citric acid and, therefore, the pellets diameter is constant also in repeating cultivations. The material obtained after harvesting contains citric acid in filtrate, and mycelial pellets as waste. The mycelial pellets are usually destroyed by anaerobic digestion process.

Washed mycelial pellets were successfully utilized for the purification of water contaminated with carcinogenic polyaromatic compounds [5], as well as for the separation of heavy metals from water [6].

Chitin or chitosan were also used as a matrices for preparation of affinity sorbents [7] and ion-exchanger [8]. The materials for this purpose were usually prepared from crustacean shells [9]. The preparation of chitin and chitosan were also described from the mold cells [10], but the methods used destroy the fine structure of microbial pellets.

The aim of this communication was to prepare chitosan in the form of microbial pellets suitable for its application as chromatography sorbent.

2. Material and methods

- Protein concentration was determined according to Lowry in the modification of Hartree [11].
- Pepsin activity was determined and pepsinogen was activated according the method described by Bohak [12].
- Pellet size was determined by microphotography.

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- Mycelium of Aspergillus niger was obtained from Lachema Kaznejov, CR as waste material from production of citric acid.
- Mycelial pellets were washed exhaustively before use, to eliminate contaminants stemming from the cultivation process (saccharose, mineral salts, citric acid).
- Moisture content was determined by drying material at 105 °C to constant weight.
- Glucose oxidase was produced in an indoor semi-pilot plant, bovine blood was obtained from Agriculture Cooperation Cechtice, CR and blood plasma was prepared by centrifugation (1000 × *g*, 60 min, 4 °C).
- Chicken fore-stomach was a gift form the Czech Agricultural University Prague.
- All chromatographic experiments were carried-out on the Fast Protein Liquid Chromatography (FPLC) system model LCCC-500 (Pharmacia, Uppsala, Sweden).

3. Results and discussion

Preparation of the sorbent was done in three steps, namely alkaline extraction, cross-linking, and conversion to chitosan.

3.1. Alkaline extraction

The primary alkaline washing is to open the cells and wash-out as much as possible of the soluble cells content. Alkaline bacterial protease (Alcalase-NOVO) was used to enforce this effect.

Hundred grams of dry mycelium of Aspergillus niger were suspended in 1000 ml of 1 M Na_2CO_3 and different amounts of bacterial protease (from 50 mg to 1 g) were added. Reaction mixture was stirred for 2 h and an aliquot of this suspension was used for further processing. Mycelium aliquots were separated by filtration, washed with distilled water and dried to constant weight (105 °C). The changes of mycelium concentration in suspension were taken as a criterion of effectiveness of the process. Fig. 1 shows the dependence of final solid content on the amount of alkaline protease added.

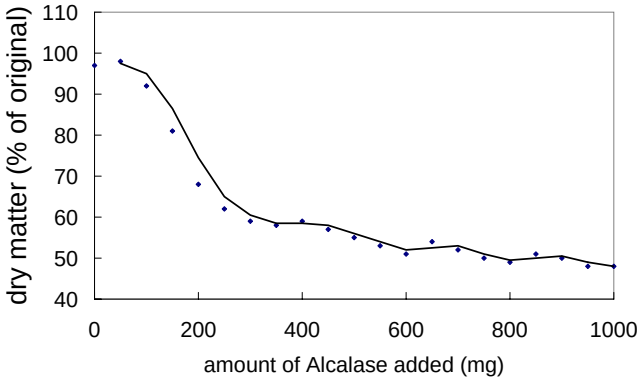

Fig. 1. Dependence of residual mycelium dry mater on alkaline protease concentration during alkaline treatment.

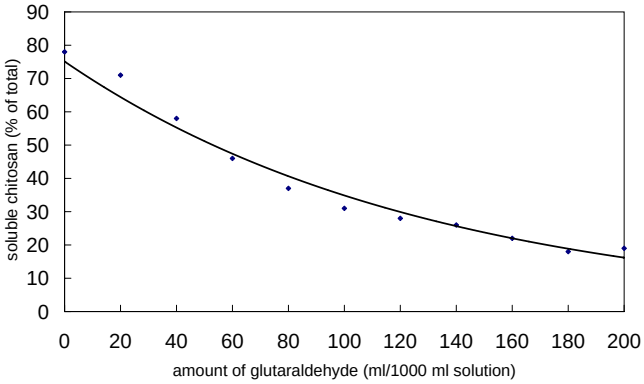

Fig. 2. Solubility of cross-linked mycelial pellets in strong acid as a result of glutaraldehyde concentration used for crosslinking.

3.2. Cross-linking

Mycelium must be crosslinked before treatment to conserve its internal structure. Cross-linking was realized by using glutaraldehyde in 0.2 M acetate buffer pH 6.5.

Hundred grams of washed and dried mycelium was suspended in 800 ml of acetate buffer, different volume of 20% glutaraldehyde was added (from 10 to 200 ml), and the final volume was adjusted to 1000 ml with acetate buffer. The result of cross-linking is lowering the chitosan solubility in strong acid. The efficiency of cross-linking can be, therefore, determined as solubility percentage of the crosslinked mycelium in 1 M HCl. This was assayed by suspension the mycelium after this operation in 1 M HCl and stirring the suspension for 1 h. The suspension was afterwards filtered though sintered glass and the solubility percentage was determined after drying. The results are summarized in Fig. 2. The figure shows that 2% of final concentration of glutaraldehyde is necessary to reach satisfactorily low solubility at this condition.

3.3. Conversion to chitosan

Crosslinked chitin was then hydrolyzed to chitosan by using strong alkali (2 M NaOH). Chitin from the previous operation (suction dried) was suspended in 2 M NaOH (100 ml for each 20 g of chitin) and stirred 4 h at 90 °C. Hydrolyzed chitin (chitosan) was left to settle and clear dark brown fluid was separated. The sediment containing chitosan and water soluble impurities was repeatedly washed with distilled water and finally separated by centrifugation (1500 × *g*, 30 min, 4 °C). Crosslinked chitosan pellets prepared by this way were dried at 50 °C in vacuum. The yield of that chitosan was 13.6 ± 1.76% of dry mater of mycelium (mean value from five experiments).

3.4. Physical properties of crosslinked mycelial chitosan

The properties of crosslinked chitosan were determined. These properties are summarized in Table 1. Particle size distribution is seen in the Fig. 3.

Table 1
Physical properties of crosslinked mycelial pellets

Property	Value
Water regain (g/g)	28
Sedimentation velocity (cm/min)	0.8
Mean particle size (μ)	120
Flow rate at 4 MPa (cm/min)	8.2

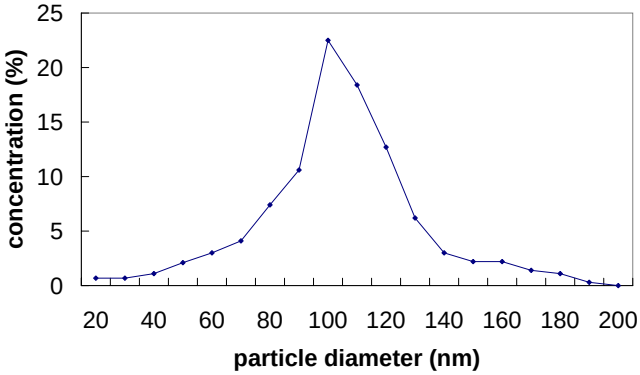

Fig. 3. Particle size distribution of cross-linked mycelial pellets.

3.5. Chromatography

Crosslinked chitosan prepared as described above was tested for the application as a chromatographic matrix. Its properties as an ion-exchanger were tested with glucose oxidase, bovine serum, and chicken pepsin as a model samples. It was assumed that the free amino groups of chitosan can serve as the active sites of the chitosan ion exchanger.

3.5.1. Chromatography of bovine serum on mycelial chitosan

Chromatography of bovine blood serum on ion exchangers is well known separation method, which has broad practical application (see e.g. [13]). The results obtained with chitosan were compared with MONO Q chromatography. The samples (200 μl) are injected into the column (V_t = 5.6 ml in the case of chitosan and 5.2 ml in the case of MONO Q) in 5 mM Tris–HCl buffer pH 7.4 (buffer A). The column is first eluted with the same buffer and then the gradient (from 0 to 1 M NaCl in buffer A) was applied. The results are shown in Fig. 4. The peaks are broader in the case of chitosan, but the chromatographic patter is very similar.

3.5.2. Chromatography of glucose oxidase on mycelial chitosan

Years ago we have described chromatography of an commercially available enzyme – glucose oxidase on DEAE-cellulose [14]. This ion exchange procedure allows separating most contaminating enzymes in food-grade glucose oxidase. This method is used in many laboratories with good reproducibility. With crosslinked chitosan the same chromatographic protocol was used. Glucose oxidase (food-grade) was desalted and transferred to buffer A (50 mM acetate pH 4.7) and this sample (200 μl) was injected into the column (V_t = 12.8 ml in the case of DEAE cellulose and 12.4 in the case of crosslinked chitosan) equilibrated with the same buffer. The column was washed with buffer A and contaminating proteins were eluted with buffer B (100 mM acetate pH 4.5). Finally, glucose oxidase was eluted with buffer C (100 mM acetate pH 3.7). The results are shown in Fig. 5.

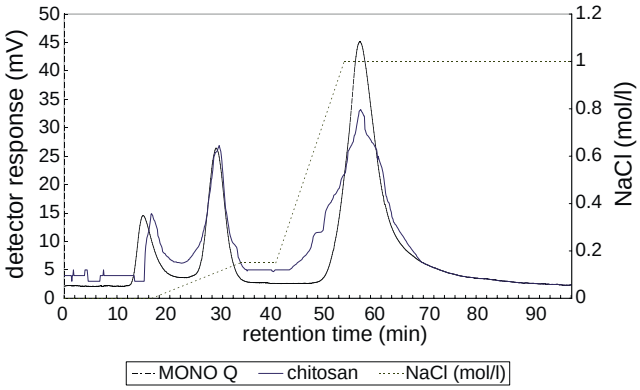

Fig. 4. Chromatography of bovine serum and cross-linked chitosan pellets compared with the chromatography on MONO Q.

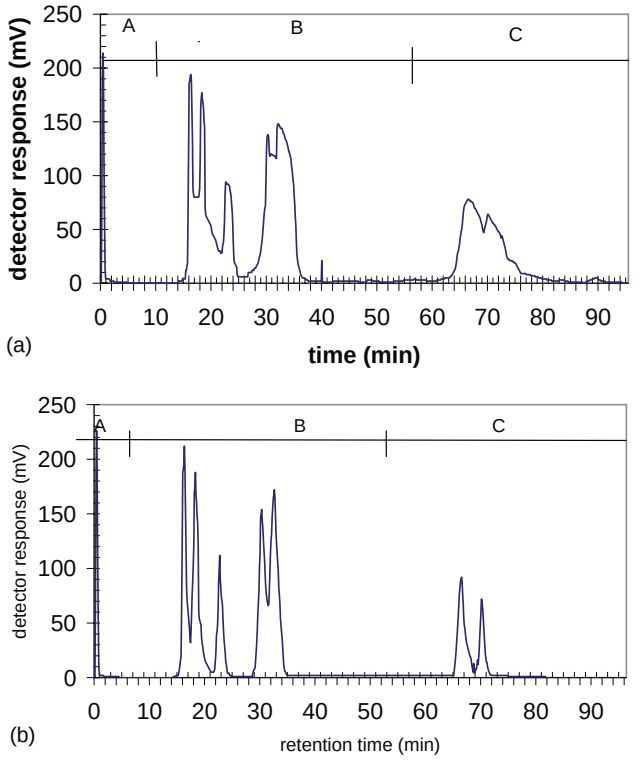

Fig. 5. (a) Chromatography of glucose oxidase on cross-linked chitosan pellets. (A) 50 mM acetate buffer pH 4.7; (B) 100 mM acetate buffer pH 4.5; (C) 100 mM acetate buffer pH 3.7. (b) Chromatography of glucose oxidase on DEAE-cellulose. Conditions are the same as in (a).

Table 2
Large scale separation of chicken pepsinogen by batchwise adsorption on crosslinked mycelial chitosan

Purification step	Volume (l)	Total activity (unit × 10^5)	Activity yield (%)	Specific activity (unit/mg protein)
Chicken fore-stomach	1 kg	Not determined	–	–
Crude extract	4.6	28.6	100	–
Dialysate of ammonium sulfate precipitate	6.9	26.3	92	0.98
Desalted eluate from crosslinked chitosan	2.8	20.6	72	3.2
Lyophilized material	0.022 kg	19.4	68	3.6

3.5.3. Chromatography and large-scale separation of chicken pepsinogen from chicken fore-stomach extract on crosslinked mycelial chitosan

Separation of chicken pepsinogen from chicken fore-stomach was described earlier [12]. Crosslinked chitosan was used by the same way as described by Bohak [12] and compared with the same chromatography on DEAE-cellulose. Chicken fore-stomach was extracted and precipitated with ammonium sulfate according to Bohak [12]. Extract was desalted and transferred to 5 mM Tris–HCl buffer pH 7.4 and injected into the column (500 ml, V_t = 16.8 ml in the case of DEAE-cellulose and 17.2 ml in the case of crosslinked mycelium). The column was washed with the starting buffer and gradient of NaCl (from 0 to 1 M) was applied. The results are shown in Fig. 6.

Afterwards the large-scale batchwise adsorption of the extract of chicken fore-stomach was carried-out. One kilogram of chicken fore-stomach was homogenized in 5 l of 50 mM phosphate buffer pH 7.5, centrifuged (10,000 × *g*, 30 min, 5 °C), and precipitated with ammonium sulfate (20–70 saturation). Precipitate was centrifuged again (10,000 × *g*, 60 min, 5 °C). Ammonium sulfate precipitate was dissolved in minimum of 5mM Tris–HCL buffer and dialyzed against the same buffer overnight. Dialysate was clarified by centrifugation under the same conditions as before and 4.6 l of solution was obtained. Fifty grams of suction dried crosslinked mycelial chitosan equilibrated with the same buffer was then added to each 1 l of clarified dialysate and stirred at room temperature for 2 h. Chicken pepsinogen adsorbate was separated by filtration. Filter cake was washed three times with 500 ml of the same buffer and dried by suction. This adsorbate (218 g) was afterwards suspended in the same buffer containing 1 M NaCl (2 l) and stirred 2 h. The solution was then desalted on Sephadex G-25 and dried by lyofilization. The separation results are summarized in Table 2.

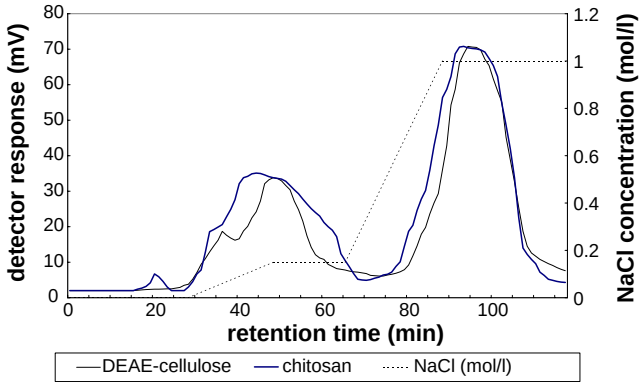

Fig. 6. Chromatography of chicken pepsinogen on cross-linked chitosan pellets compared with the chromatography on DEAE-cellulose.

4. Conclusion

Pellets of Aspergillus niger from citric acid production could be used for the preparation of microbial chitosan in pellet form and stabilized by cross-linking with glutaraldehyde. Mycelium was then extracted with strong alkali and used as chromatography sorbent.

Crosslinked chitosan pellets were used as anion-exchanger for the chromatography of bovine blood serum and compared with the same chromatography on MONO Q. The results of this chromatography shows peak broadening in the case of crosslinked mycelium, but, nevertheless, the chromatographic pattern was similar to that obtained with the same chromatography on MONO Q.

The crosslinked chitosan was used also for the chromatography of food grade glucose oxidase and chicken pepsinogen. Chicken pepsinogen was also separated by batchwise adsorption.

The results obtained demonstrate that crosslinked microbial chitosan could be used as sorbent for preparative chromatography.

Acknowledgements

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