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Short communication

Sample preparation in separation of the extracellular chitinolytic enzymes of the human intestinal bacterium *Clostridium paraputrificum* J4 from the culture fluids

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ABSTRACT

Membrane ultrafiltration (UF) was used in sample preparation of the culture fluids of the human intestinal bacterium *Clostridium paraputrificum* strain J4 containing seven extracellular chitinolytic isoenzymes (38–90 kDa). The subsequent filtration of the bacteria-free supernatants was carried out through Millipore membranes with cut-off 100 and 30 kDa for separation of undigested components of the culture medium and bacterial metabolites with molecular weight higher and lower than that of the target enzymes. The chitinolytic enzymes, which were the minor components in the culture fluids, were concentrated at UF as well. The aim of the research consisted in evaluation of the effect of component composition of bacteria-free supernatants and the chemical nature of membrane active layer on partial fractionation of the chitinolytic enzymes, their recovery in retentates and purification degree. On the basis of the obtained experimental results, the sample preparation procedure of the culture fluids of *C. paraputrificum* J4 was established to be used further in chromatographic separations of the chitinolytic enzymes.

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1. Introduction

In both analytical, preparative [1–3] and industrial separations [4,5] of highly purified proteins and enzymes from microbial culture fluids, the membrane ultrafiltration (UF) is used very often as the first separation step owing to its high throughput [4–6], easy scaling and automation [7,8]. As concerns the large-scale separations, the sieving through membranes is especially effective in separation of the solutes, the difference in molecular sizes of which achieves approximately one order of magnitude [9].

The studied culture fluids of the human intestinal bacterium *Clostridium paraputrificum* strain J4 contained an extracellular complex of the chitinolytic enzymes (at least six chitinases and β, *N*-acetylglucosaminidase, NAGase) with molecular weight ranging from 38 up to 90 kDa) [10,11]. In the human body, these enzymes participate not only in digestion of the chitin/chitosan containing food (e.g. mushrooms) but also in the defense of humans from invasive fungal infections by splitting (1 → 4)-β bonds between *N*-acetyl-D-glucosamine (GlcNAc) moieties in chitin-containing cell walls of pathogenic fungi. The chitinolytic enzymes of *C. paraputrificum* J4 together with other chitinases working in humans such as the highly conserved chitotriosidase expressed by activated phagocytes in plasma [12] and acid mammalian chitinase (AMCase) found in the gastrointestinal tract and lungs take a special significance in modern medicine. In contrast to many synthetic drugs, the human chitinolytic enzymes are absolutely harmless for humans [13] and can be considered as promising antifungal medicines, especially in patients with suppressed immunity. It is known that chitotriosidase [14] is an effective biomarker in treatment of Gaucher's patients [15] and in earlier diagnostics of sarcoidosis, visceral leishmaniasis, β-thalassemia and the extended atherosclerosis (Tangier disease) [16,17]). The acid mammalian chitinase (AMCase) [14,18] has been successfully used in both diagnostics and therapeutic or prophylactic treatment of lung diseases [14]. On this basis, the design of a separation process for preparation of highly purified chitinolytic enzymes of *C. paraputrificum* J4 is extremely important practical task.

In this paper, the subsequent filtration of bacteria-free supernatants through 100- and 30-kDa Millipore membranes was carried out to decrease the content of contaminating solutes with molecular weight higher and lower than the membrane cut-off limits and to concentrate the target enzymes. The effect of the membrane active layer (its nature and pore sizes) and the culture fluid composition on the recovery, fractionation and purification degree of the extracellular chitinolytic enzymes was estimated.

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Table 1
Recovery of chitinolytic enzymes and proteins at clarification of culture fluids of *C. paraputrificum* J4 by centrifugation.

Clarified solution	Recovery (%)		
	NAGase	Chitinases	Proteins
[a]S	65.8 ± 8.5	83.0 ± 3.5	83.9 ± 5.1
[b]CS	31.4 ± 4.8	50.3 ± 3.1	60.3 ± 4.9

[a] S – supernatant obtained by centrifugation of the initial culture fluids.
[b] CS – solution obtained by centrifugation followed by ultafiltration through a 10-kDa Millipore PES membrane. CS solutions were tenfold more concentrated than S ones.

Table 2
Recovery of chitinolytic enzymes and proteins in filtration of the bacteria *C. paraputrificum* J4-free supernatants through a set of Millipore PES membranes.

Retentate	Recovery, %					
	NAGase		Endochitinases		Proteins	
	S	CS	S	CS	S	CS
R100	16.1 ± 0.7	21.1 ± 1.1	3.8 ± 0.8	8.7 ± 1.5	15.7 ± 2.3	27.9 ± 5.0
R30	27.5 ± 1.4	15.3 ± 0.4	14.3 ± 2.1	9.3 ± 0.3	5.7 ± 0.6	5.2 ± 0.5
R10	9.7 ± 1.1	7.2 ± 0.6	14.4 ± 5.8	13.8 ± 0.7	8.6 ± 0.7	8.7 ± 0.5
Sum	53.3	43.6	32.5	31.8	30.0	41.8

Conductivity of R30 retentates varied from 3.95 to 5.83 mS/cm; pH ranged from 8.05 to 8.20.

2. Experimental

2.1. Culture fluids of C. paraputrificum J4

In cultivation of *C. paraputrificum* strain J4,[1] the modified medium M10 (1 l) contained colloidal chitin (4 g), previously filtered and sterilized Rumen fluid (5 vol.%), redox indicator (resazurin), growth stimulator (hemin), salts such as $NaHCO_3$, NaCl, $(NH_4)_2SO_4$, $CaCl_2$, $KHSO_4$, $MgSO_4$, KH_2PO_4 and $MnCl_2$ at a relatively high total concentration (about 0.04 mol/l). The sixteenth batches of the crude culture fluids of *C. paraputrificum* J4 were used in UF experiments. In the crude fluids, the NAGase and chitinases specific activity varied in the range 180–440 pkat/mg and 1900–4000 pkat/mg, respectively.

At first, the bacteria of *C. paraputrificum* J4 were separated from the culture fluids by centrifugation at 35,000 × *g*, 4 °C for 15 min. A half of the bacterium-free supernatants (S solutions, conductivity about 7–8 mS/cm, pH 7.3–8.0) were tenfold concentrated by filtration through a 10-kDa polyethersulfone (PES) membrane (Millipore, USA) to prepare CS solutions (conductivity about 5–6, pH 8–8.4) with different composition of solutes.

2.2. Sample preparation procedure by membrane ultrafiltration

The S or CS solutions were subsequently filtered through a set of UF membranes (9.6 cm in diameter) having active layer from PES or regenerated cellulose, RC with cut-off 100, 30 and 10 kDa or 100 and 10 kDa, respectively (Millipore, USA). The experiments were carried out under the applied pressure 50 kPa using an Amicon 200-ml or 50-ml membrane cells (Millipore, USA). At first, S or CS solutions (800 or 80 ml, respectively) were filtered through a membrane with cut-off 100 kDa and each retentate (R100) was washed three times with 0.05 M phosphate ($KHPO_4$/Na_2HPO_4) buffer solution (PB), pH

[1] The bacterium *C. paraputrificum* J4 was grown in the modified medium M10 for 16 h. The obtained inoculate (50 ml) was added to the same medium (1 l) and cultivated at 39 °C for 120 h. Both the component composition of M10 medium and the preparation procedure of colloidal chitin have been described in [19,20], respectively.

Table 3
Changes in the specific activity of chitinolytic enzymes in UF retentates.

Retentate	NAGase		Chitinases	
	S	CS	S	CS
R100	1.0 ± 0.4	0.8 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
R30	4.8 ± 0.9	2.9 ± 1.6	2.5 ± 0.5	1.8 ± 1.3
R10	1.1 ± 0.5	0.8 ± 0.3	1.7 ± 0.2	1.6 ± 0.5

The values in Table 3 are ratio of the specific activity of NAGase or chitinases in the retentates and in S or CS solutions, used in UF experiment.

6. Permeates (P100) were pooled and UF procedure was repeated using a membrane with cut-off 30 kDa. For permeates P30, the same UF procedure was carried out using a 10-kDa membrane. As a rule, each UF step was stopped when concentration degree, i.e. the ratio of volumes of the used permeates and obtained retentate reached about 25.

2.3. Enzyme and protein assays

The total activity of chitinases, NAGase and total content of proteins were determined in both retentates and permeates to evaluate their recovery in retentates and adsorption in membranes. The chitinase activity was determined using a 0.5% aqueous solution of (carboxymethyl) chitin as a substrate prepared from chitin (Sigma) [21]. After UF, the culture filtrates or permeates/retentates were incubated at 40 °C for 60 min together with the substrate solution in 100 mM PB, pH 6. The enzymatic reaction was stopped with a mixture of 300 mM $ZnSO_4$ (A) and 300 mM $Ba(OH)_2$ (B) (at the volume ration of a sample/A/B = 1/1/1) and the reduced groups were detected with 4-hydroxy-benzohydrazide [22]. In the same way, NAGase was assayed with 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma) according to [23]). The enzymatic activity (pkat/ml) was measured in triplicate. The accuracy in analysis of chitinase/NAGase activity did not exceed ± 14.4%/ ± 11.4%, respectively. Protein concentration was determined according to Bradford [24] with the accuracy ± 8.8%. In all spectrophotometric analyses, the absorbance of solutions was measured using a Biomate 5 spectrophotometer (Thermo-Spectronics, USA).

2.4. SDS–PAGE

Denaturing gel electrophoresis (SDS–PAGE) was carried out using 10% or 8.5%-resolving gels in a vertical mini SE280 (Hoefer Inc.) under nonreducing conditions described by Laemmli [25]. A standard protein mixture (Invitrogen, Carlsbad, California, USA and Roti-Mark Standard, Roth, Germany) was used as molecular-weight protein markers. In analysis of protein composition in retentates, samples (10 or 30 μl) twice diluted with 100 mM PB, pH 6 were loaded on a gel. The proteins in gels were visualized by silver staining [26].

2.5. Zymography

Zymograms were prepared according to the modified method [27] with separation gels (10% or 8.5%) containing 0.1% (carboxymethyl)chitin. According to the optimized procedure, the samples were mixed with 10% aqueous SDS solution (1:2) and left in the fridge for a night to disrupt the tight intermolecular bindings and improve the resolution of the chitinolytic enzymes. After SDS–PAGE, the renaturation of the enzymes was achieved by washing the gels with 1% Triton X-100 for 15 min followed by their rinsing with 50 mM PB, pH 6 (3× 15 min). To visualize all chitinases the gels were stained with 0.1 wt% solution of Congo red.

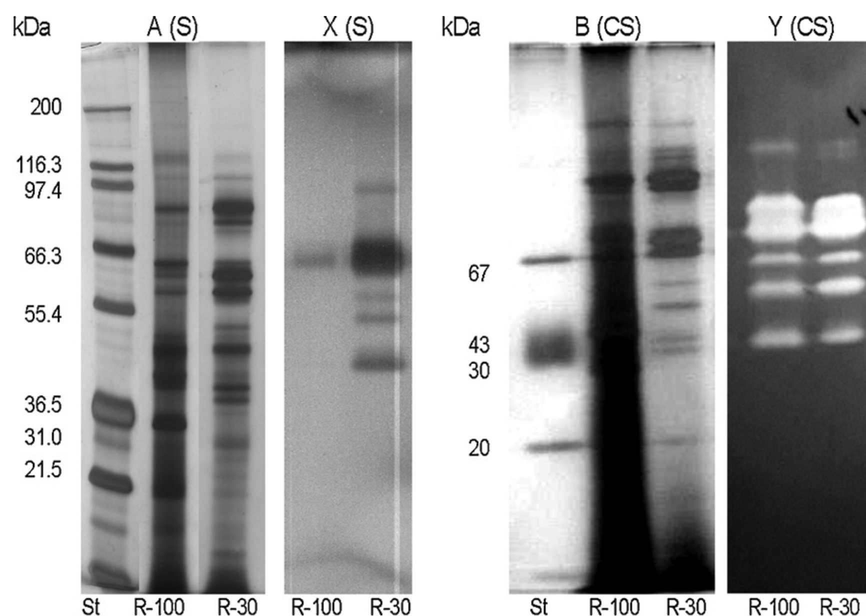

Fig. 1. Compositions of proteins and chitinases in UF retentates. A and B: SDS–PAGE gels. X and Y: zymograms. St – protein standards (20–120 kDa) (Roth, Germany) or (21.5–200 kDa) (Invitrogen, USA). R100 and R30 are UF retentates.

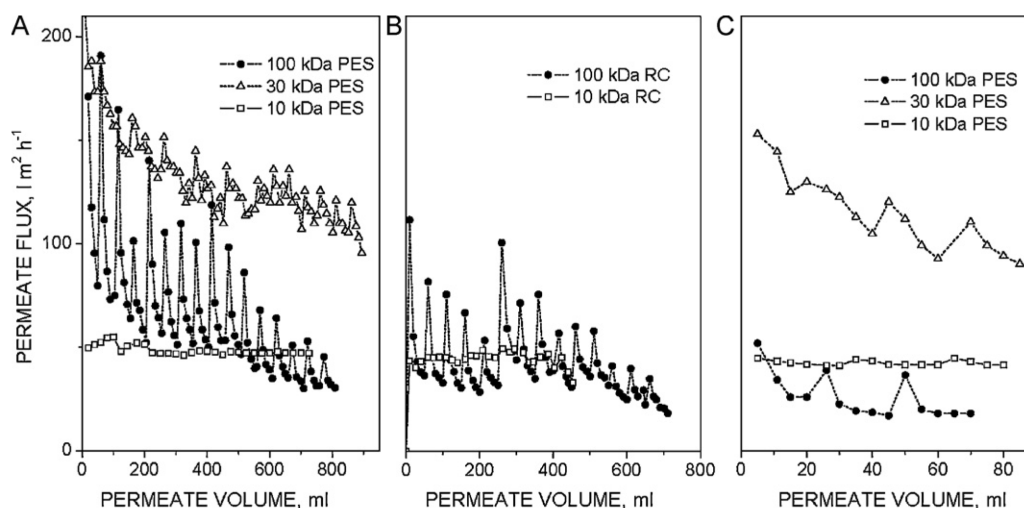

Fig. 2. Ultrafiltration of S and CS solutions of *C. paraputrificum* J4 through Millipore membranes with cut-off 100, 30 and 10 kDa. (A, B) S solutions – bacterium-free supernatants obtained by centrifugation of the culture fluids, (C) CS solutions – S solutions tenfold concentrated by ultrafiltration through a 10 kDa-membrane. Membranes: (A) 100 kDa; 30 and 10 kDa from polyethersulphone, PES; (B) 100 kDa and 10 kDa from regenerated cellulose, RC; (C) 100 kDa, 30 kDa and 10 kDa from PES.

3. Results and discussion

The total activity of chitinases and NAGase in S solutions clarified by centrifugation was 83% and 65%, respectively, of that in the crude culture fluids. The total amount of proteins was 84% (Table 1). The losses of enzymes and proteins are believed to be mainly due to their co-sedimentation with the bacterial cells. Irreversible binding within the membranes and partial inactivation of the enzymes contributed to their losses during UF as well. The yield of the total activities of the enzymes and the content of proteins in CS solutions decreased to 50%, 32% and 60%, respectively, in comparison with their amounts in the crude culture fluids. The chitinase and NAGase activity recovery in CS solutions was 90–92% of that in S solutions. In all further calculations, the total enzymatic activity or content of proteins in the pooled UF retentates were compared with those in S or CS solutions.

In UF of both S and CS solutions through a 100-kDa PES membrane, the recovery of NAGase (∼40 kDa) activity was higher than that of chitinases (48–90 kDa) (Table 2). Very likely, higher aggregation of NAGase than chitinases with accompanying proteins took place. In R100 retentates, the recovery of total enzymatic activity and proteins was higher if more concentrated CS solutions filtered through a 100-kDa membrane. As before, this can be explained by high disposition of the chitinolytic enzymes towards intermolecular bindings and formation of aggregates, which did not penetrate into the membrane pores. The chitinolytic enzymes have high tendency towards aggregation. We have realized that at thirtyfold and higher concentrating the retentates, the slight opalescence appeared but it absolutely disappeared with diluting the retentates. In subsequent UF of the pooled permeates through a 30-kDa PES membrane, higher recovery of the chitinolytic enzymes in R30 retentates was observed if more diluted S solutions were used in previous filtration through a 100-kDa

membrane. The recovery of proteins in R30 retentates did not depend on their concentration in the origin solutions. There were no practically difference in recovery of both enzymatic activity and proteins in R10 retentates when S or CS solutions were used originally in the previous filtration steps. The total recovery of NAGase was higher (S: 53% and CS: 44%) than that of chitinases (S: 33% and CS: 32%). It is obvious that more intensive adsorption of enzymes/proteins within membrane pores occurred with increase in pore diameters and concentration of solutes. Higher aggregation of the enzyme/protein molecules in CS solutions contributed to this phenomenon as well. As a result, the increase (times) in the specific activity of NAGase (S: 4.8 and CS: 2.5) and chitinases (S: 2.9 and CS: 1.8) in R30 retentates was lower when CS solutions instead of S ones were filtered (Table 3). In R100 retentates, the specific activity of NAGase and chitinases was lower than that in the loaded S and CS solutions. The decrease in the specific activity was more pronounced for chitinases than for NAGase. It means that a 100-kDa membrane was more permeable for chitinases than for NAGase aggregates and proteins with higher molecular dimensions.

Comparison of the protein/enzyme compositions of the chitinolytic enzymes in R100 and R30 retentates has shown that the main quantity of the contaminating proteins and only small amount of the chitinolytic enzymes were present in R100 retentates when S solution was filtered through a 100-kDa membrane (Fig. 1A, X). The most contaminating proteins together with remarkably higher amount of the chitinolytic enzymes were retained if CS solutions were filtered through a100-kDa membrane (Fig. 1B, Y).

The effect of concentration polarization on efficiency of UF can be seen in Fig. 2. The comparison of the dependences of the permeate flux on permeate volume has shown that in filtration of S solutions, the initial permeate flux was rather high $180\,l \times m^{-2} \times h^{-1}$ and $115\,l \times m^{-2} \times h^{-1}$ through a 100-kDa PES and RC membranes but it progressively dropped to $80\,l \times m^{-2} \times h^{-1}$ and $40\,l \times m^{-2} \times h^{-1}$, respectively. For maintaining the filtration efficiency, the experiment was stopped after collecting each 50-ml portion of permeate and solution in the membrane cell was stirred for a minute at zero pressure. After starting the filtration again, the values of the permeate flux achieved high values. Nevertheless, the exponential decrease in values of permeate flux was observed with time due to the gradual narrowing the membrane pores because of adsorption of proteins/enzymes. RC membranes exhibited lower filtration efficiency compared with PES ones (Fig. 2A vs. B). Tenfold higher enzymes/proteins concentration in CS solutions assisted the essential decrease in the permeate flux through 100-kDa membranes. Higher proteins/enzymes adsorption on the surface of pores of a cellulosic membrane (Fig. 2C) promoted considerably higher decrease in the permeate flux compared with PES membrane (Fig. 2A). This can be explained by higher affinity of the undigested chitooligosaccharides from substrate (chitin) in the bacteria-free culture fluids to cellulosic membranes than to PES ones. The permeate flux was practically the same in filtration of both S and CS solutions through membranes with cut-off to 30 kDa and 10 kDa. The blocking of pores was negligible in filtration of P30 permeates through a 10-kDa membrane.

4. Conclusions

The optimized procedure for sample preparation of culture fluids of *C. paraputrificum* J4 consisted in two steps:

- removal of bacterial cells by centrifugation (35,000 × g, 4 °C for 15 min) and
- partial separation of solutes with molecular weight higher and lower than that of the target enzymes by the subsequent filtration of the bacteria-free supernatants through a 100-kDa and 30-kDa PES Millipore membranes under the applied pressure 50 kPa.

The main fraction of the chitinolytic enzymes was retained with a 30-kDa PES membrane. Conductivity of R30 retentates was about 4–6 mS/cm, pH 8–8.2. It contained 28% of NAGase, 15% of chitinases and only 6% of proteins from their content in the crude culture fluids. The NAGase and chitinase specific activity was 4.8 and 2.5 times, respectively, higher than that in the culture supernatants.

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