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Isolation and purification of *Mucor circinelloides* intracellular chitosanolytic enzymes

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ABSTRACT

This study aimed at isolation, purification and characterization of a chitosanase from *Mucor circinelloides* mycelium. The latter contains also a mycelium-bound lipase and lipids. The chitosanase and lipase were extracted from defatted *M. circinelloides* mycelium with a detergent and purified through a two-step procedure comprising chromatography on bacitracin–CNBr-Sepharose 4B and gel filtration on Sephadex G-100. Purification degree of the chitosanase (endo-type enzyme) and lipase was 23 and 12, respectively. These enzymes were optimally active at pH of 5.5–6.0 (chitosanase) and 7.2 (lipase in olive oil hydrolysis) and at 37 °C. Both purified enzymes were activated by Ca^{2+} and Mg^{2+} ions. The preferred substrates of chitosanase were chitosan preparations with a high degree of deacetylation. This enzyme showed no activity for colloidal chitin, Na-CMC and starch. SDS–PAGE of both purified enzymes showed two bands with molecular masses of 42 and 43 kDa. Our results suggest that *M. circinelloides* synthesizes an oligomeric (bifunctional) lipase which also efficiently depolymerizes chitosan.

1. Introduction

Chitosan, a copolymer composed of 2-amino-2-deoxy-β-D-glucose and 2-acetamido-2-deoxy-β-D-glucose units is one of natural, nontoxic, biodegradable and bioactive polymers. Recently, attention has been paid to water-soluble chito-oligosaccharides (CHOS, with an average molecular weight below 3.9 kDa) and low molecular weight chitosan (LMWC, with an average molecular weight between 3.9 kDa and 20 kDa) because these chitosan derivatives display numerous biological activities (antibacterial, antifungal, antiviral, antitumor, antioxidant and radical scavenging), stimulate immune system and exert fat lowering and hypocholesteromic effects (Harish Prashanth & Tharanathan, 2007; Kim & Rajapakse, 2005). The oligoaminosaccharides can by prepared by chemical (acid hydrolysis, oxidative–reductive and nitrous acid depolymerization), physical (thermal and gamma irradiation, ultrasound degradation) and enzymatic methods (Choi, Ahn, Lee, Byun, & Park, 2002; Holme, Foros, Pettersen, Dornish, & Smidsrød, 2001; Kang, Dai, Zhang, & Chen, 2007; Liu, Bao, Du, Zhou, & Kennedy, 2006; Roberts, 1992). The CHOS with a relatively high degree of polymerization (DP) as well as LMWC obtained by enzymatic degradation of chitosan display more diversified and stronger biological activities as compared to that shown by the high molecular weight biopolymer and oligomers with low DP that are produced through its acid hydrolysis (Harish Prashanth & Tharanathan, 2007; Kim & Rajapakse, 2005; Shahidi, Arachchi, & Jeon, 1999).

Chitosanases (EC 3.2.1.132) are endo-hydrolytic enzymes acting on glycosidic bonds throughout chitosan chains thereby releasing low molecular weight oligomers. Numerous bacteria and fungi secrete extracellular chitosanases. Only some intracellular enzymes are found in plants and zygomycetous fungi like *Mucor rouxii* (Alfonso, Martinez, & Reyes, 1992; Kołodziejska, Malesa-Ciećwierz, Górna, & Wojtasz-Pająk, 1996) or *Absidia orchidis* (Jaworska, Konieczna, & Kusaoke, 2002). Due to high prices and scarcity of specific enzymes like chitosanase and chitinase the chitosan oligomers are produced using some non-specific enzymes such as: lysozyme, cellulase, hemicellulase, lipase, papain, pectinase, pepsin or pronase (Kittur, Vishu Kumar, & Tharanathan, 2003; Lee, Xia, & Zhang, 2008; Muzzarelli, 1997; Nordtveit, Varum, & Smidsrød, 1996; Qin, Du, Xiao, Li, & Gao, 2002; Roncal, Oviedo, Lopez de Armentia, Fernandez, & Villaran, 2007; Vishu Kumar & Tharanathan, 2004; Vishu Kumar, Varadaraj, Lalitha, & Tharanathan, 2004; Xia, Liu, & Liu, 2008).

The chitosanolytic activity of lipases (EC 3.1.1.3., triacylglycerol acylhydrolases) isolated from several microorganisms was frequently reported. Many lipase preparations (e.g. the wheat germ lipase (Muzzarelli, 1997; Muzzarelli, Xia, Tomasetti, & Ilari, 1995), recombinant lipase B from *Candida antarctica* (Muzzarelli, 1997), lipase from *Candida cylindracea* (Luckachan & Pillai, 2006), porcine pancreas lipase (Pantaleone, Yalpani, & Scollar, 1992)) were found to depolymerize chitosan and its derivatives. There are two hypotheses on this unspecific activity of lipases: (1) the occurrence of chitosanase contaminating the lipase preparations, and (2) the similarity of active sites of both these enzymes (Muzzarelli, Francescangeli, Tosi, & Muzzarelli, 2004).

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The fungal strain of *Mucor circinelloides* from Institute of Technical Biochemistry of TUL is a known producer of an intracellular membrane-bound lipase and chitosanase (Szczęsna-Antczak et al., 2006; Struszczyk, Szczęsna-Antczak, Antczak, & Gajewska, 2007; Struszczyk et al., 2006). Crude preparation of these enzymes (dried and defatted mycelium) can be applied in large-scale chitosan hydrolysis yielding functional, biologically active chitosan oligomers. In this study we have developed the method of purification of *M. circinelloides* intracellular proteins yielding the purified enzymatic preparation displaying both lipolytic and endo-chitosanolytic activities. This purified preparation was successfully used in chitosan oligomers production.

2. Materials and methods

2.1. Chemicals

CNBr-Sepharose 4B, Sephadex G-100, chitin, glucosamine, N-acetylglucosamine, sodium carboxymethyl cellulose and starch were purchased from Sigma (USA). Bacitracin was obtained from Fluka. Chitosan preparations with various viscosity average molecular weight ($\overline{Mv}$) ranging from 121 to 421 kDa and different deacetylation degree (DD) ranging from 66% to 97% were obtained from Vanson, Redmont (USA) and Chemopol Complex Pvt. Ltd. Tada (India). Molecular weight markers: bovine albumin (66 kDa), egg albumin (45 kDa), horseradish peroxidase (40 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.7 kDa) and bovine milk α-lactalbumin (14.2 kDa) were supplied by Sigma, USA. All other reagents were of analytical grade.

2.2. Microorganism and culture conditions

The strain of *M. circinelloides* from the culture collection of the Institute of Technical Biochemistry of TUL was cultivated for 72 h at 30 °C with agitation at 180 rpm. The culture medium (optimized for intracellular lipase biosynthesis) contained corn steep liquor (3.7% w/v) and olive oil (2.7% v/v) (Szczęsna-Antczak et al., 2006). Its pH was adjusted to 4.7 prior to autoclaving. Mycelium of *M. circinelloides* was harvested by filtration, carefully washed with water, defatted with acetone and air-dried at room temperature.

2.3. Extraction of proteins from defatted M. circinelloides mycelium

2.3.1. Extraction by detergents

Air-dried and defatted *M. circinelloides* mycelium (1 g) was suspended in 0.1 M phosphate buffer, pH 7.2 (12 ml) and one of the following detergents was added (0.5% w/v): Triton X-100, Brij 35, Tween 80 or sodium cholate. The mixtures were stirred for 30 min at 4 °C, centrifuged at 13,000*g* for 20 min and the supernatants were used as crude enzymatic extracts.

2.3.2. Sonication

Air-dried and defatted mycelium of *M. circinelloides* (1 g) was suspended in 0.1 M phosphate buffer, pH 7.2 (12 ml). The mixture was sonicated by using two frequencies of ultrasounds (22 and 30 kHz) for 6 min at 4 °C and centrifuged at 13,000*g* for 20 min. The supernatants were used as crude enzymatic extracts.

2.3.3. Homogenization

Air-dried and defatted *M. circinelloides* mycelium (1 g) was suspended in 0.1 M phosphate buffer, pH 7.2 (12 ml) and either supplemented with Triton X-100 (0.5% w/v) or not. The suspensions were homogenized (DI 25 basic IKA-Ultra Turrax homogenizer) for 5 min at 4 °C and centrifuged at 13,000*g* for 20 min. The supernatants were used as crude enzymatic extracts.

2.3.4. Freezing and grinding

Air-dried and defatted *M. circinelloides* mycelium (1 g) was suspended in 0.1 M phosphate buffer, pH 7.2 (12 ml) either supplemented with Triton X-100 (0.5% w/v) or not, frozen at −20 °C and ground (2 times) with glass ballotines in a mortar (at 0 °C for 10 min). The homogenates were centrifuged at 13,000*g* for 20 min and the supernatants were used as crude enzymatic extracts.

2.4. Enzyme purification

2.4.1. Chromatography on bacitracin–CNBr-Sepharose 4B

The crude protein extract obtained by mycelium extraction with 0.5% w/v Triton X-100 (one of the methods described in Section 2.3) was applied on bacitracin–CNBr-Sepharose 4B column (2 × 50 cm) previously equilibrated with 0.2 M phosphate buffer (pH 7.2). The unbound proteins were eluted with the same buffer. The adsorbed proteins were eluted with 0.2 M phosphate buffer (pH 7.2) supplemented with 0.15% w/v Brij 35 or with 25% isopropanol. The elution was carried out at a flow rate of 14 ml cm^{-2} h^{-1} and 2.5 ml fractions were collected. Fractions displaying chitosanolytic and lipolytic activities were pooled and concentrated by ultrafiltration (30 kDa membrane, Amicon).

2.4.2. Gel filtration on Sephadex G-100

The concentrated fractions derived from the chromatography on Sepharose 4B–bacitracin were applied to a Sephadex G-100 column (2 × 100 cm) equilibrated with 0.2 M phosphate buffer (pH 7.2). The elution was carried out at a flow rate of 8 ml cm^{-2} h^{-1} and 4.0 ml fractions were collected. Fractions containing chitosanolytic and lipolytic enzymes were pooled and concentrated by ultrafiltration (30 kDa membrane, Amicon). Albumin (66 kDa), peroxidase from horseradish (40 kDa) and lysozyme (14.7 kDa) were used as standards for the molecular mass determination.z

2.5. Determination of enzymatic activities

2.5.1. Chitosanolytic activity

The chitosanolytic activity of extracted and purified proteins was determined both on the basis of a decrease in an average molecular weight of chitosan (endo-chitosanolytic activity) and on the basis of a rise in reducing sugars concentration after the hydrolysis of this biopolymer (exo-chitosanolytic activity).

2.5.1.1. Reduction of an average molecular weight of chitosan. Reaction mixture contained: 1 ml of 2% chitosan in 2% acetic acid, 0.85 ml of 1 M CH_3COONa and 0.15 ml of enzyme solution (pH 5.5). Chitosan digestion was carried out at 37 °C for 60 min and was stopped by boiling in a water bath for 5 min. Controls with the same composition as the samples were incubated for 5 min in a boiling water bath to inactivate the enzyme and then incubated for 60 min at 37 °C.

The viscosity average molecular weight ($\overline{Mv}$) of chitosan and its digestion products was determined by the viscometric method using one of the following solutions: (1) 0.1 M sodium chloride, 0.2 M acetic acid and 4.0 M urea (for chitosan with ($\overline{Mv}$) between 113 and 492 kDa), (2) 0.30 M sodium chloride and 0.33 M acetic acid (for chitosan with ($\overline{Mv}$) between 13 and 135 kDa) and calculated according to the Mark–Houwink's equation $[\eta = k\overline{Mv}^{\alpha}]$ with (1) $k = 8.93 \times 10^{-4}$ and $\alpha = 0.71$ or (2) $k = 3.41 \times 10^{-3}$ and $\alpha = 1.02$, respectively (Roberts, 1992). The viscosity measurements

were conducted at 25.0 ± 0.1 °C using an Ubbelohde's viscometer (Shott GmbH, type 53110/I).

The hydrolytic activity of endo-chitosanolytic enzymes ($A_{endo\text{-}CH}$) was expressed in units equivalent to an amount of enzyme necessary to decrease the viscosity average molecular weight of chitosan by 1 kDa per 1 min under the conditions described above [1 U = 1 kDa min^{-1}].

2.5.1.2. Saccharification of chitosan. Reaction mixture contained: 1 ml of 2% chitosan in 2% acetic acid, 0.70 ml of 0.1 M phosphate buffer (pH 7.2) and 0.3 ml of enzyme solution. pH of reaction mixture was 5.5. Chitosan digestion was carried out at 37 °C for 24 h and was terminated by 5 min incubation in a boiling water bath. Controls were incubated for 5 min in a boiling water bath (to inactivate enzymes) and next for 24 h at 37 °C.

The content of reducing amino-chitooligomers was determined by the Somogyi–Nelson method (Wood & Bhat, 1988) using glucosamine or N-acetylglucosamine as standards.

One unit (U) of hydrolytic activity of exo-chitosanolytic enzymes ($A_{exo\text{-}CH}$) was equivalent to an amount of enzyme necessary to produce 1 μmol of reducing sugar per 1 min, [1 U = 1 μmol min^{-1}].

2.5.2. Lipase activity

The hydrolytic activity of lipase was determined using 20% olive oil emulsion stabilized with 2% polyvinyl alcohol (PVA). Reaction mixture contained 2.5 ml of oil emulsion, 1.5 ml of 0.1 M phosphate buffer (pH 7.2) and 0.5 ml of enzyme solution. The enzymatic reaction was carried out at 37 °C for 30 min with agitation at 120 rpm and terminated by adding 10 ml of ethanol. The amount of fatty acids released during olive oil hydrolysis was determined by titration with 0.05 M NaOH up to pH 10 (Schott-titrator TitroLine).

One unit [U] of lipolytic activity (A_L) denoted the release of 1 μmol of fatty acid in 1 min under the conditions described above, [U = 1 μmol min^{-1}].

2.6. Protein assay and electrophoresis

Protein concentration was determined by the Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951) using bovine serum albumin as the standard. Protein concentration in fractions derived by column chromatography was estimated by measurements of absorbance at 280 nm.

The purity and molecular mass of the separated proteins were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970) using 12.5% or 15% polyacrylamide gels. Bovine albumin (66 kDa), egg albumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20.1) and bovine milk α-lactalbumin (14.2 kDa) were used as standards for the molecular mass determination. Protein bands were visualized by silver staining.

2.7. Enzyme characterization

The effect of pH on activity of chitosanolytic enzymes (endo-hydrolases) was estimated on the basis of assays carried out at 37 °C and over pH range between 3.0 and 8.0.

The temperature optimum of chitosanolytic enzymes was determined by measuring relative activity (Section 2.5.1.1) at pH 5.5 over a temperature range between 5 and 60 °C.

The pH stability of the purified enzymes was determined by measuring residual activity (Section 2.5.1.1) after protein pre-incubation (for 60 min at 4 °C) in 0.1 M citrate buffer (pH 3.0–6.0) or in 0.1 M phosphate buffer (pH 6.0–8.0).

Thermostability of chitosanolytic enzymes was evaluated by their incubation for 30 min at temperature varying from 5 to 100 °C followed by residual activity assays under standard conditions (Section 2.5.1.1).

The effect of various metals ions (Ag^{2+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Sn^{2+}, Zn^{2+}) and other chemicals (Tween 20, Tween 80, Triton X-100, EDTA, SDS) on the activity of *Mucor* lipase and chitosanase was investigated by their pre-incubation with these compounds for 30 min at 37 °C followed by measuring residual activity under standard conditions (Section 2.5.1.1).

For determination of *substrate specificity* of *M. circinelloides* chitosanolytic enzymes, various substrates such as chitosan ($\overline{Mv}$) ranging from 121 to 421 kDa, DD ranging from 66% to 97%, colloidal chitin, sodium carboxymethyl cellulose and starch were used.

2.8. Characteristics of the chitosan degradation products

The gel permeation chromatography (GPC) of chitosan and its digestion products was done using Hewlett Packard 1050 system equipped with two PLaquagelOH Mixed columns and RI HP 1047 detector. The eluent was acetate buffer, pH 4.3. The temperature of column was 30.0 ± 0.1 °C and the flow rate – 8 ml min^{-1}. The standards used to calibrate the column were: polyethylene glycol and polyethylene oxide.

3. Results and discussion

3.1. Comparison of various methods of intracellular protein extraction

Protein extraction from defatted mycelium of *M. circinelloides* was carried out by various methods, e.g.: extraction with detergents, homogenization, freezing and grinding, and sonication. The efficiency of extraction of chitosanolytic and lipolytic enzymes from fungal mycelium by these methods is presented in Table 1.

Table 1
Extraction of intracellular enzymes from mycelium of *M. circinelloides*.

Method of protein extraction	Protein [mg]	Specific chitosanolytic activities		Specific lipolytic activity, A_L [U mg^{-1}]
		Endo-$A_{endo\text{-}CH}$ [U mg^{-1}]	Exo-$A_{exo\text{-}CH}$ [U g^{-1}]	
Extraction by mixing with detergent solution[a]				
Triton X-100	50.52	14.74	4.24	5.95
Brij 35	46.20	9.49	1.62	5.72
Sodium cholate	48.00	8.74	3.36	5.57
Tween 80	37.08	8.32	0.91	4.25
Homogenization				
With Triton X-100	51.18	15.31	4.87	5.76
Without Triton X-100	45.23	10.48	0.97	4.08
Freezing and grinding				
With Triton X-100	52.20	16.65	5.41	5.98
Without Triton X-100	48.84	11.11	1.10	4.22
Sonication with ultrasound				
Frequencies [kHz] 22	39.12	3.74	1.33	1.91
30	40.68	3.06	1.05	1.25

[a] Concentration of detergents of 0.5% (w/v).

The results shown in Table 1 indicate that enzymes that are present in cell-free extracts of *M. circinelloides* mycelium catalyze the hydrolysis of both glycosidic bonds in chitosan and ester bonds in triacylglycerols. It is to note that the endo-chitosanolytic and lipolytic activities in extracts from *M. circinelloides* mycelium were strictly correlated.

Relatively high yield of protein extraction (50.5 mg) was achieved by treatment of mycelium (1 g) with non-ionic detergent – Triton X-100. These extracts displayed the high endo-chitosanolytic (14.74 U mg^{-1}) and lipolytic (5.95 U mg^{-1}) activities. The other methods of protein extraction like mycelium disintegration by homogenization or freezing and grinding coupled with detergent treatment gave only a slightly higher yield of chitosanolytic enzymes. Relatively high amount of extracted proteins (39.15 mg from 1 g of mycelium) and very low activities of endo-chitosanase (3.74 U mg^{-1}) and lipase (1.91 U mg^{-1}) were obtained when ultrasounds were used for mycelium disintegration, which presumably inactivated the enzymes.

3.2. Purification of enzymes

Crude enzymatic solutions obtained by extraction of *Mucor* mycelium with Triton X-100 (0.5% w/v) were purified by chromatography on CNBr-Sepharose 4B–bacitracin. As shown in Fig. 1, only one protein peak (fractions from 123 to 136) containing endo-, exo-chitosanolytic and lipase activities was eluted from this column. It was found that only 1.5% proteins applied on the column were adsorbed (e.g. 1.3 mg from 91.2 mg).

The protein fractions from the first step of purification were concentrated and subjected to gel chromatography on Sephadex G-100 (gel filtration conditions were optimized earlier). This step of purification gave one main peak of proteins that displayed endo-, exo-chitosanolytic and lipase activities (Fig. 2). Molecular mass of these proteins (estimated by gel filtration) was close to 42–43 kDa.

Results of the two-step purification procedure are summarized in Tables 2 and 3. The chitosanase (endo-type enzyme) was puri-

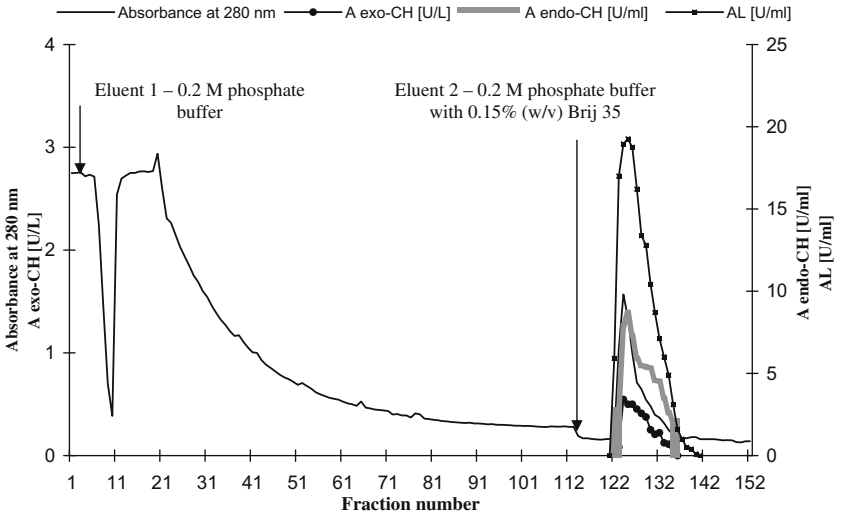


Fig. 1. Chromatography of chitosanase/lipase on CNBr-Sepharose 4B–bacitracin. Prior to protein separation, the column was equilibrated with 0.2 M phosphate buffer (pH 7.2). Unbound proteins were eluted with 280–300 ml of this buffer and the adsorbed proteins were eluted with about 100 ml of the buffer supplemented with Brij 35.

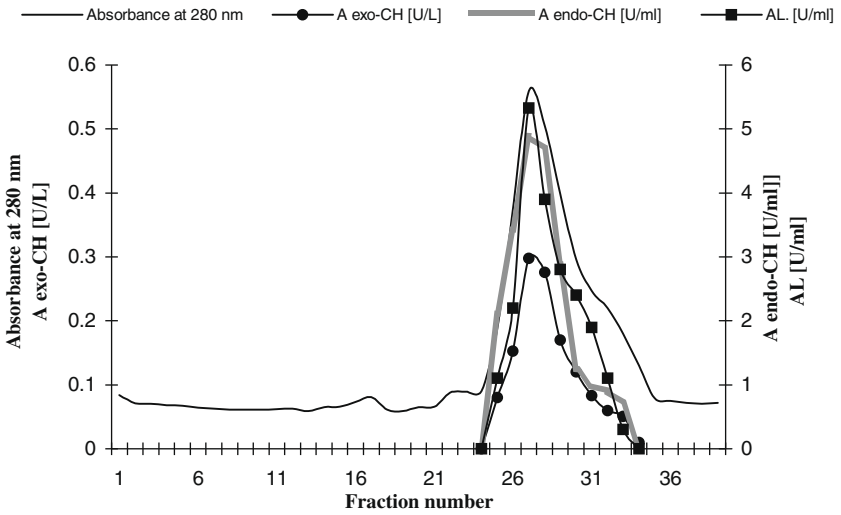


Fig. 2. Gel filtration of *M. circinelloides* intracellular proteins on Sephadex G-100. The column was equilibrated with 0.2 M phosphate buffer (pH 7.2) prior to separation of proteins. The latter were eluted with the same buffer.

Table 2
Purification of *M. circinelloides* chitosanase.

Step	Total activity [U]	Total protein [mg]	Specific activity $A_{endo\text{-}CH}$ [U mg^{-1}]	Yield [%]	Purification degree [fold]
Crude extract	908.64	91.20	9.96	100	1
CNBr-Sepharose 4B–bacitracin	161.40	1.30	124.15	17.8	12
Sephadex G-100	41.67	0.18	231.50	4.6	23

Table 3
Purification of *M. circinelloides* lipase.

Step	Total activity [U]	Total protein [mg]	Specific activity A_L [U mg^{-1}]	Yield [%]	Purification degree [fold]
Crude extract	691.20	91.20	7.58	100	1
CNBr-Sepharose 4B–bacitracin	82.00	1.30	63.08	11.9	8
Sephadex G-100	16.38	0.18	91.00	2.4	12

fied 23-fold with 4.6% recovery of its activity and the lipase was purified 12-fold with 2.4% recovery of activity. The ultimate specific activities of chitosanase and lipase preparations were 231.5 and 91.0 U mg^{-1}, respectively.

It is to note that both the purified *M. circinelloides* enzymes migrated in SDS–PAGE as two protein bands with the molecular mass of 42 and 43 kDa (Fig. 3A).

Also other column chromatography methods such as hydrophobic chromatography on octyl-sepharose and ion exchange on the strong anionite Q (unpublished data) that were used for purification of these two enzymes resulted in two protein bands in SDS–PAGE (an example is shown in Fig. 3B, line 4). Unfortunately, all trials of native-PAGE and zymogram analysis of the purified enzymatic preparations were unsuccessful (native proteins contained in these preparations escaped from penetration into polyacrylamide gels with various concentrations, which could result from strong interactions between aggregated proteins).

Application of the authors' new, two-step method of purification of *M. circinelloides* intracellular proteins, comprising chromatography on CNBr-Sepharose 4B–bacitracin and gel filtration on Sephadex G-100, yielded the preparation with the relatively high activity of both endo-chitosanase and lipase. Usually, chitosanases from various sources have been purified using fractionation by ammonium sulfate (salting out), molecular sieving and ion-exchange chromatography (Somashekar & Joseph, 1996). Also the affinity chromatography was used for this purpose and usually chitosan, chitin or products of their degradation were employed as specific ligands. For example, the chitosanase of *Bacillus circulans* was purified on immobilized chitosan carbodiimidazole-activated agarose beads (Davis & Eveleigh, 1984) while enzyme from *Nocardia orientalis* - on Sepharose CL-4B with immobilized chitotriose (Sakai, Katsumi, Isobe, & Nanjo, 1991). In our studies chromatography on CNBr-Sepharose 4B coupled with bacitracin, was used to purify *M. circinelloides* chitosanase. Bacitracin, the antibiotic-cyclopeptide produced by *Bacillus licheniformis,* is known as an efficient ligand of serine, aspartyl and metalloproteins and is used for separation of serine enzymes like lipases and proteases (Stephanov & Rudenskaya, 1983). On the other hand, bacitracin can also interact with several different types of biomolecules, including DNA, RNA, lipoproteins, receptors, and lipids from various sources (Ming, 2003). It also binds transition metal ions, including Zn(2+), Mn(2+), Co(2+), Ni(2+),

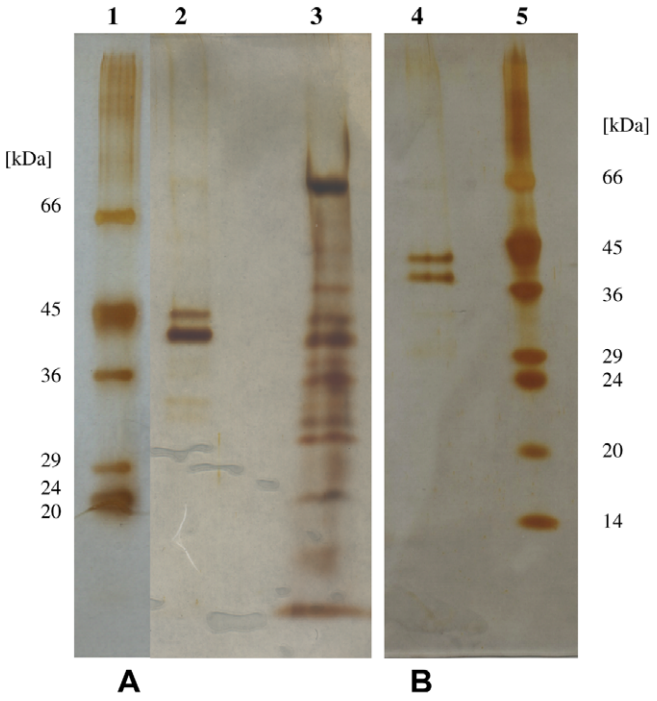


Fig. 3. SDS–PAGE of *M. circinelloides* chitosanolytic enzymes in 12.5% (A) and in 15% (B) polyacrylamide gel (16.0 × 17.5 cm). The molecular mass markers (lanes 1 and 5), proteins purified by CNBr-Sepharose 4B–bacitracin chromatography and gel filtration on Sephadex G-100 (lane 2) and crude extract of *M. circinelloides* proteins (lane 3), proteins purified by CNBr-Sepharose 4B–bacitracin chromatography and ion-exchange chromatography (lane 4).

and Cu(2+) (Ming & Epperson, 2002). The mechanism of separation of chitosanases by using CNBr-Sepharose 4B–bacitracin is still unknown for us and we suppose that some proteins extracted from *Mucor* mycelium adhere to this matrix because they form aggregates with proteins displaying high affinity for the ligand (e.g. lipases). We have already mentioned that many preparations of lipases are a source of chitosanases.

The purified *Mucor* enzymes significantly lowered the chitosan viscosity average molecular weight ($\overline{Mv}$) and their exo-chitosanolytic activity was minor. For comparison, all the preparations of lipases described in literature (Lee et al., 2008; Luckachan & Pillai, 2006; Muzzarelli, 1997; Muzzarelli et al., 1995; Pantaleone et al., 1992; Roncal et al., 2007) and applied for chitosan digestion also rapidly depolymerized this biopolymer and rapidly decreased viscosity of its solutions. Also commercial preparation of lipase, purchased from Novozymes corp. acted on chitosan in both endo- and exo-manner (Lee et al., 2008) while the purified recombinant lipase from *C. antarctica* (Muzzarelli, 1997) preferred polymers of glucosamine with the high degree of polymerization.

Most of the described in literature endo-chitosanases and lipases from bacteria and fungi are characterized by the low molecular mass, usually ranging from 20 to 50 kDa and from 20 to 80 kDa, respectively, while that of exo-chitosanases (β-GlcNase) varies between 97 and 135 kDa. For example, the molecular mass of fungal endo-hydrolase (EC 3.2.1.132) from *Penicillum islandicum* was 30 kDa (Fenton & Eveleigh, 1981) from *Aspergillus* sp. CJ22-326 – 29 kDa (Chen, Xia, & Yu, 2005) and from *Aspergillus oryzae* IAM2660 – 40 kDa (Zhang et al., 2000).

Reassuming, results of SDS–PAGE and problems with separation of both the activities suggest that either these intracellular *Mucor* enzymes form stable aggregates or the oligomeric *Mucor* lipase displays also the chitosanolytic activity.

3.3. Properties of *M. circinelloides* chitosanolytic enzymes

3.3.1. The effect of pH and temperature on activity and stability

The highest endo-chitosanolytic activity of the purified *M. circinellloides* enzymatic preparation was observed at temperature around 37 °C and in pH range between 5.5 and 6.0 (Figs. 4 and 5). The lowering or increasing in pH by only 1 U significantly decreased activity of chitosanase. The enzyme reveals approximately 70% activity at the temperature 25 °C, what can be advantageous for its industrial applications. The chitosanase was relatively stable below 40 °C (as much as 80% activity retained at 50 °C) and at pH between 4.5 and 7.5 (Figs. 4 and 5). The same preparation displayed the highest lipolytic activity in olive oil hydrolysis at temperature around 37 °C and at pH 7.2. The lipase was relatively stable below 50 °C and in pH range from 8 to 10 (Baddour, 1989).

The optimum pH and temperature for activity of *M. circinelloides* endo-chitosanase were similar to those of other fungal chitosanases. For example, endo-hydrolytic enzymes from *P. islandicum* were optimally active at pH between 4.5 and 6.0 and 45 °C (Fenton & Eveleigh, 1981), while two chitosanases from *M. rouxii* were optimally active at pH 5.0 and either 55 °C (type A) or 50 °C (type B) (Alfonso et al., 1992). The other fungal chitosanolytic enzymes were found to be stable up to 50 °C (Somashekar & Joseph, 1996). Different preparations of lipases that catalyzed chitosan hydrolysis displayed the chitosanolytic activity at temperature between 25 and 50 °C and pH between 3.6 and 7.0 (Lee et al., 2008; Muzzarelli et al., 1995). The commercial lipase preparation from Novozymes was found to be more efficient in chitosan degradation at 37 °C (the yield of 24 h hydrolysis at pH 4.8 was 93.8%) than at 55 °C (the yield of 24 h hydrolysis at the same pH was only 67.9%) (Lee et al., 2008).

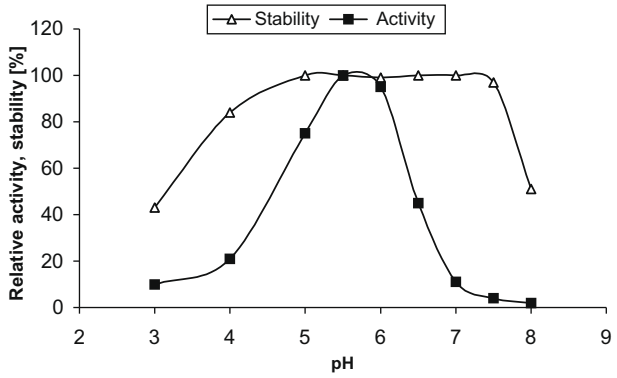


Fig. 4. Effect of pH on the activity and stability of *M. circinelloides* endo-chitosanase.

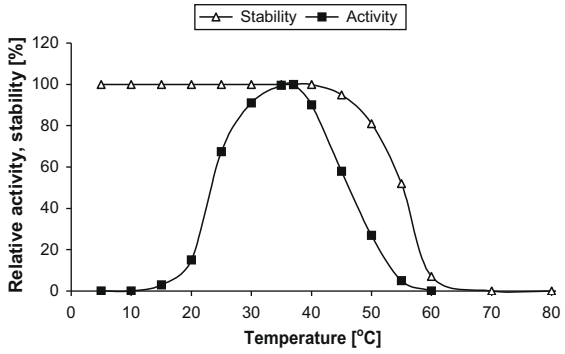


Fig. 5. Effect of temperature on the activity and stability of *M. circinelloides* endo-chitosanase.

3.3.2. Effect of various chemicals on activity of enzymes

Effects of various chemicals on *M. circinelloides* endo-chitosanase activity are presented in Table 4. This enzyme was activated by 1 mM Ca^{2+} (148%), Mn^{2+} (110%) and Mg^{2+} (106%) ions and inhibited by 1 mM Hg^{2+}, Cu^{2+} and Ag^{2+} ions (by about 100%, 92% and 89%, respectively). It was found that the activity of *M. circinelloides* enzymes was increased by 0.1% (w/v) non-ionic surfactant Triton X-100 and strongly inhibited when its concentration was higher than 1.0% w/v. The lipolytic activity of the purified enzymatic preparation was also activated by Ca^{2+} and Mg^{2+} ions and Triton X-100 (Baddour, 1989).

Also other fungal endo-type chitosanases, e.g. from *M. rouxii* (Alfonso et al., 1992) and *P. islandicum* (Fenton & Eveleigh, 1981) were activated by Ca^{2+} ions while chitosanases of *Aspergillus* CJ22-326 (Chen et al., 2005), *A. fumigatus* KH-94 (Kim, Shon, & Lee, 1998) and *A. oryzae* (Zhang et al., 2000) were activated by Mn^{2+} ions. These enzymes were inhibited by Hg^{2+}, Cu^{2+}, Ag^{2+},

Table 4
Effect of selected compounds on the activity of *M. circinelloides* chitosanolytic enzymes.

Sample	Concentration of additives	Relative chitosanolytic activity[a], %
Control	–	100
Metal ions addition (mM)		
Ca^{2+}	0.5	111
	1.0	148
Mn^{2+}	0.5	104
	1.0	110
Mg^{2+}	0.5	102
	1.0	106
Ba^{2+}	0.5	98
	1.0	89
Cd^{2+}	0.5	96
	1.0	87
Co^{2+}	0.5	93
	1.0	87
Pb^{2+}	0.5	92
	1.0	86
Sn^{2+}	0.5	91
	1.0	80
Zn^{2+}	0.5	81
	1.0	76
Fe^{2+}	0.5	81
	1.0	74
Ag^{2+}	0.5	21
	1.0	11
Cu^{2+}	0.5	16
	1.0	8
Hg^{2+}	0.5	5
	1.0	0
Other additives % (w/v)		
Tween 20	0.1	97
	1.0	28
Tween 80	0.1	94
	1.0	21
Triton X-100	0.1	115
	1.0	20
SDS	0.1	92
	1.0	9
EDTA	1.0	83
	5.0	13

[a] The relative chitosanolytic activity was determined on the basis of a decrease in an average molecular weight of chitosan (DD 97%, $\overline{M\nu}$ 235 kDa) and expressed as a percentage of the specific activity (U mg^{-1}) of the purified *M. circinelloides* chitosanase. Reaction mixture composition and hydrolysis conditions are described in Section 2.5.1.1.

Fe^{2+} and Cd^{2+} ions. For comparison, the recombinant lipase from *C. antarctica* (Muzzarelli, 1997) was activated by calcium chloride (10 mM).

3.4. Substrate specificity

Chitosanases from various sources display different hydrolytic patterns that depend on the chitosan deacetylation degree and/or its molecular weight. The purified *M. circinelloides* enzymatic preparation was incubated for 60–300 min at 37 °C and pH 5.5 with different chitosans – viscosity average molecular weight ($\overline{Mv}$) ranging from 121 to 421 kDa, degree of deacetylation (DD) ranging from 66% to 97%. Product patterns were determined as described in Section 2.5.1.

The results shown in Fig. 6 indicate that the purified *M. circinelloides* enzymatic preparation degraded chitosan with the different degree of deacetylation (DD) and preferred the substrate with the highest DD. The highest drop in $\overline{Mv}$ of the chitosan (36%) was observed within the first h of substrate (DD 97%, $\overline{Mv}$ 236 kDa) degradation while the reducing sugar concentration reached only 0.425 μmol ml^{-1}. After 5 h of the process the $\overline{Mv}$ of chitosan decreased by circa 70% while the reducing sugar concentration was still rather low (0.842 μmol ml^{-1}).

Chitosanases from different sources degrade chitosan, its derivatives (glycol chitosan, colloidal chitosan, carboxymethyl chitosan) and other carbohydrates, like glycol chitin, colloidal chitin, carboxymethyl chitin, carboxymethyl cellulose (Somashekar & Joseph, 1996). As shown in Table 5 the purified *M. circinelloides* enzyme preferred the chitosan with high DD and showed no activity for colloidal chitin, sodium carboxymethyl celulose (Na-CMC) and starch.

Table 5
Degradation of selected polysaccharides by the purified *M. circinelloides* chitosanase.

Substrate, 1% (w/v)	Relative chitosanolytic activity (%)[a]
Chitosan (DD 97%)	100
Chitosan (DD 83%)	90.2
Chitosan (DD 79%)	59.9
Chitosan (DD 66%)	27.3
Colloidal chitin	0.5
Na-CMC	0
Starch	0

[a] The relative chitosanolytic activity was determined on the basis of reducing sugars concentration after the hydrolysis of substrates and expressed as a percentage of the specific activity (U mg^{-1}) of the purified *M. circinelloides* chitosanase. Reaction mixture composition and hydrolysis conditions are described in Section 2.5.1.2.

Results of gel permeation chromatography of hydrolysis products indicate that the purified *M. circinelloides* proteins contain endo-enzymes because of the bimodal character of the curve presenting distribution of molecular mass of chitosan hydrolysis products after 60 min (Fig. 7). After 2 h of substrate hydrolysis (under optimum conditions) with the purified *M. circinelloides* endo-chitosanase this curve becomes a typical Gaussian plot. The digest contained as much as 69% of 5–50 kDa oligoaminosaccharides and

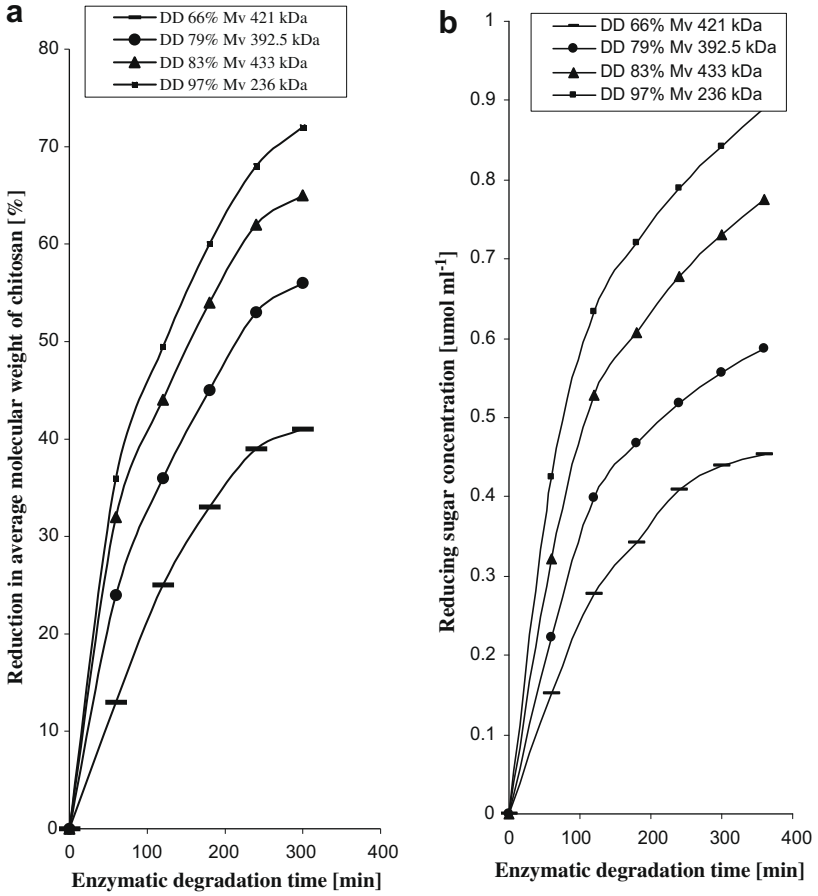
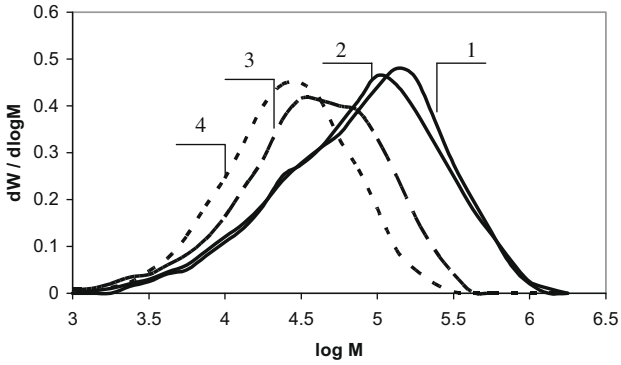


Fig. 6. Dynamics of: reduction in average molecular weight (a), and reducing sugars concentration (b) for various variants of chitosans hydrolysis catalyzed by *M. circinelloides* enzymes. Reaction mixture composition and chitosan hydrolysis conditions are described in Section 2.5.1.1. The endo-chitosanolytic activity used in reaction was 9.15 U ml^{-1}.



No	Sample	Fraction content [% (w/w)]						
		Molecular mass: M x 10^3 Da						
		<5	5-50	50-100	100-200	200-400	400-800	>800
1.	Undigested chitosan	2	31	22	24	15	6	0
2.	Chitosan contained in control (120 min)	2	34	25	22	12	4	1
3.	Chitosan degradation products (time 60 min.)	3	55	24	14	4	0	0
4.	Chitosan degradation products (time 120 min.)	5	69	19	6	1	0	0

Fig. 7. Molecular weight distribution of chitosan (samples 1 and 2) and enzymatically degraded chitosan (samples 3 and 4). The preparation of *M. circinelloides* enzymes that was used in the experiment displayed the endo-chitosanolytic activity of 18.4 U ml^{-1}. Chitosan digestion lasted for 60 min. (no 3) or 120 min. (no 4). In the control (no 2) chitosan was treated for 120 min. with inactivated enzymes. Other details on reaction mixture composition and conditions of chitosan hydrolysis are described in Section 2.5.1.1.

only 5% of products with molecular mass below 5 kDa. When duration of chitosan hydrolysis was longer the concentration of monoaminosaccharides was only slightly increased. This result demonstrates that *M. circinelloides* chitosanase can be used for production of biologically active CHOS.

4. Conclusion

Our earlier studies (Struszczyk et al., 2006, 2007) showed that the crude *M. circinelloides* enzymatic preparation (simply the defatted fungal mycelium) can be considered as a very useful tool for industrial production of biologically active chitosan oligomers. Therefore we tried to separate chitosanases from this mycelium, purify them to homogeneity and characterize.

The efficient yields of extraction of both the endo-chitosanase and lipase from the mycelium were achieved by using the non-ionic detergent Triton X-100. The purified enzymatic preparation was obtained within the scope of this study through the chromatography of proteins extracted from *M. circinelloides* mycelium on CNBr-Sepharose-4B column with covalently linked bacitracin followed by molecular sieving on Sephadex G-100. This preparation appeared as two protein bands in SDS–PAGE (molecular masses of 43 and 42 kDa) and hydrolyzed both glycosidic bonds in chitosan and ester bonds in triacylglycerols.

Our findings suggest that we have identified the new oligomeric lipase, which efficiently depolymerizes chitosan. In the nearest future we intend to determine 3D structure and molecular properties of the protein(s).

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