1. Introduction

Chitosanolytic enzymes have a wide-ranging applications such as generation of glucosamine, N-acetylg glucosamine and size-specific chitooligosaccharides which have strong biological activity (antimicrobial, antiviral, antitumor, and antioxidant activities, immunological effects) [1 - 2]. Chitosanases (EC 3.2.1.132) are endo-hydrolytic enzymes acting on internal glycosidic bonds within the biopolymer chains thereby releasing low molecular weight oligomers. Numerous bacteria and fungi secrete extracellular chitosanases. Some intracellular enzymes are found in plants and zygomycetes fungi like Mucor rouxii [3] or Absidia orchidis [4]. Beside the chitosanase, the unspecific chitosanolytic activity of lipase (EC 3.1.1.3., glycerol ester hydrolases) preparations from several microorganisms was frequently reported [5-11]. All lipase preparations, which have been tested in various laboratories (wheat germ lipase [5-6, 9], recombinant lipase B from Candida antartica [6], lipase from Candida cylindracea [8], porcine pancreas lipase [9-10]) significantly depolymerized chitosan and its derivatives. The unspecific activity of lipase may result from the occurrence of chitosanase contaminating the preparation or the similarity of active sites of both these enzymes [7]. On the other hand, the activity of the recombinant lipase B from Candida antartica in the chitosan depolymerization [6] definitely rules out the action of unidentified impurities in enzyme preparations.

Mucor circinelloides strain from Institute of Technical Biochemistry TUL, is a known producer of intracellular lipase, crude preparations of which have been used in chitosan hydrolysis [12 - 14]. In the present study we have developed the method of purification of Mucor circinelloides intracellular proteins yielding the preparation displaying both lipolytic and chitosanolytic activities, which could be used for large-scale production of chitosan oligomers.
2. Materials and methods

2.1. Microorganism and culture conditions

The strain of *Mucor 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 r.p.m. The culture medium was composed of corn steep liquor (3.7% w/v) and olive oil (2.7% v/v) - the medium was optimized for lipase biosynthesis [12]. The initial pH of the medium was 4.7. Mycelium of *M. circinelloides* was harvested by filtration, washed with water, defatted with acetone and air-dried at room temperature.

2.2. Chemicals and substrates

CNBr-Sepharose 4B, Sephadex G-100, bacitracin, chitin, glucosamine, N-acetylglucosamine and sodium carboxymethyl cellulose were purchased from Sigma (USA). Chitosan preparations with various molecular weight (Mv) ranging from 121 kDa to 421 kDa and different deacetylation degree (DD) ranging from 66% to 98% were obtained from Vanson, Redmont (USA) and Chemopol Complex Pvt. Ltd. Tada (India). All other reagents were of analytical grade.

2.3. Enzymes extraction from *Mucor* 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 cm³) and supplemented with one of the following detergents (0.5% w/v): Triton X-100, Brij35, Tween 80 or sodium cholate. The mixture was stirred for 30 min at 4 °C and centrifuged at 13,000 × g for 20 min and the supernatant was used as a crude enzymatic extract.

2.3.2. Sonification

Air-dried and defatted *M. circinelloides* mycelium (1 g) was suspended in 0.1 M phosphate buffer, pH 7.2 (12 cm³). The mixture was sonicated by using two frequencies of ultrasounds (22 kHz and 30 kHz) for 6 min at 4 °C and centrifuged at 13 000 × g for 20 min. The supernatant was used as a crude enzymatic extract.

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 cm³) either supplemented with Triton X-100 (0.5% w/v) or not. The suspension was homogenized for 5 min and centrifuged at 13 000 × g for 20 min. The supernatant was used as a crude enzymatic extract.

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 cm³) 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 homogenate was centrifuged at 13 000 × g for 20 min and the supernatant was used as a crude enzymatic extract.
2.4. Enzyme purification

2.4.1 Chromatography on bacitracin-CNBr-Sepharose 4B
The enzyme preparation obtained by mycelium extraction with 0.5% w/v Triton X-100 (method described in section 2.3.1.) was applied on bacitracin-CNBr–Sepharose 4B column (2 cm × 50 cm) previously equilibrated with 0.2 M phosphate buffer (pH 7.2). The column was washed 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. The elution was carried out at a flow rate of 14 cm³cm⁻²h⁻¹ and 2.5 cm³ fractions were collected. Fractions containing chitosanolytic and lipolytic enzymes were pooled and concentrated by ultrafiltration (30 kDa membrane, Amicon).

2.4.2. Gel filtration on Sephadex G-100
The concentrated fractions from affinity chromatography were applied on a Sephadex G-100 column (2 cm × 100 cm) equilibrated with 0.2 M phosphate buffer (pH 7.2). The elution was carried out at a flow rate of 8 cm³cm⁻²h⁻¹ and 4.0 cm³ fractions were collected. Fractions containing active 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.

2.5. Determination of enzymatic activity

2.5.1. Chitosanolytic activity
The chitosanolytic activity was determined 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 content after the hydrolysis of this biopolymer (exo-chitosanolytic activity).

✓ Reduction of an average molecular weight of chitosan
Reaction mixture contained: 1 cm³ of 2 % chitosan in 2 % acetic acid, 0.85 cm³ of 1 M CH₃COONa and 0.15 cm³ of enzyme solution (pH 5.5). Chitosan digestion was carried out at 37 °C for 60 min and was terminated by 5 min incubation in a boiling water bath. To prepare the respective control, the mixture with the same composition as above was incubated for 5 min in the boiling water bath to inactivate the enzyme and for 60 min at 37 °C.

An average molecular weight of chitosan and its digestion products was determined by the viscometric method described in [15]. The viscosity measurements were conducted at 25 °C using an Ubbelohde’s viscometer (Shott, K ≈ 0.01 mm²s⁻¹).

The hydrolytic activity of endo-chitosanolytic enzymes (A_{endo-CH}) was expressed in units defined as an amount of enzyme necessary to decrease an average molecular weight of chitosan by 1 kDa per min under the conditions described above [unit = 1 kDa min⁻¹].

✓ Saccharification of chitosan
Reaction mixture contained: 1 cm³ of 2% chitosan in 2% acetic acid, 0.70 cm³ of 0.1 M phosphate buffer (pH 7.2) and 0.3 cm³ of enzyme solution (pH 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. As a control the same mixture was first incubated for 5 min in the boiling water bath and
next for 24 h at 37 °C.

The content of reducing amino-chitooligomers was determined by the Somogyi-Nelson method [16] using glucosamine or N-acetylglucosamine as the standards.

One unit (U) of hydrolytic activity of exo-chitosanolytic enzymes ($A_{exo-CH}$) was expressed as an amount of enzyme necessary to produce 1 µmol of reducing sugar per 1 min.

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 cm$^3$ of oil emulsion, 1.5 cm$^3$ of 0.1 M phosphate buffer (pH 7.2) and 0.5 cm$^3$ of enzyme solution. The enzymatic reaction was carried out at 37 °C for 30 min with agitation at 120 r.p.m. and terminated by adding 10 cm$^3$ of ethanol. The amount of 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.

2.6. Protein assay and electrophoresis

Protein was determined by the Lowry method [17] using bovine serum albumin as the standard. During enzyme purification the protein concentration 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) using a 12% acrylamide gel [18].

2.7. Enzyme characterization

The effect of pH on activity of chitosanolytic enzymes (exo- and endo-hydrolases) was estimated on the basis of assays carried out at 37 °C and over pH range between 3.5 and 8.0. The temperature optimum of chitosanolytic enzymes was determined by measuring residual activity (section 2.5.1.) over a temperature range between 5 °C and 60 °C at pH 5.5.

The pH stability of the purified chitosanolytic enzymes was determined by measuring residual activity (section 2.5.1.) after pre-incubating (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.).

For determination of substrate specificity of *M. circinelloides* chitosanolytic enzymes, various substrates e.g. chitosan (Mv ranging from 121 kDa to 421 kDa, DD ranging from 66% to 98%), colloidal chitin and sodium carboxymethyl cellulose were used.
3. Results and discussion

3.1. Methods of intracellular protein extraction

Protein extraction from defatted mycelium of *Mucor circinelloides* was carried out by various methods, e.g.: extraction with detergents, homogenization, freezing and grinding, and sonification. The efficiencies of protein extraction from fungal mycelium are presented in Table 1.

**Table 1.** Extraction of intracellular enzymes from mycelium of *M. circinelloides*; 1) Concentration of detergents equal to 0.5% (w/v), 2) Sonification with ultrasound frequencies of 22 kHz (method 1) and 30 kHz (method 2).

<table>
<thead>
<tr>
<th>Method of protein extraction</th>
<th>Protein, mg</th>
<th>Specific chitosanolytic activities</th>
<th>Specific lipolytic activity, A_L [U/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Endo-CH [unit/mg]</td>
<td>Exo-CH [U/g]</td>
</tr>
<tr>
<td><strong>Extraction by mixing with detergents</strong></td>
<td></td>
<td>Endo-CH [unit/mg]</td>
<td>Exo-CH [U/g]</td>
</tr>
<tr>
<td>Tween 80</td>
<td>37.08</td>
<td>8.32</td>
<td>0.91</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>50.52</td>
<td>14.74</td>
<td>4.24</td>
</tr>
<tr>
<td>Brij 35</td>
<td>46.20</td>
<td>9.49</td>
<td>1.62</td>
</tr>
<tr>
<td>Sodium cholate</td>
<td>48.00</td>
<td>8.74</td>
<td>3.36</td>
</tr>
<tr>
<td><strong>Homogenization</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without Triton X-100</td>
<td>45.23</td>
<td>10.48</td>
<td>0.97</td>
</tr>
<tr>
<td>With Triton X-100</td>
<td>51.18</td>
<td>15.31</td>
<td>4.87</td>
</tr>
<tr>
<td><strong>Freezing and grinding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without Triton X-100</td>
<td>48.84</td>
<td>11.11</td>
<td>1.10</td>
</tr>
<tr>
<td>With Triton X-100</td>
<td>52.20</td>
<td>16.65</td>
<td>5.41</td>
</tr>
<tr>
<td><strong>Sonification</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method 1</td>
<td>39.12</td>
<td>3.74</td>
<td>1.33</td>
</tr>
<tr>
<td>Method 2</td>
<td>40.68</td>
<td>3.06</td>
<td>1.05</td>
</tr>
</tbody>
</table>

The results shown in Table 1 indicate that cell-free extracts of *M. circinelloides* mycelium catalyzed the hydrolysis of both glycosidic bonds in chitosan and ester bonds in triacylglycerols. The highest yield of protein extraction from this mycelium was achieved by treatment with non-ionic detergent – Triton X-100. These extracts displayed high activities of both chitosanase (14.74 unit/mg) and lipase (5.95 U/mg). The laborious and time-consuming methods like homogenization, freezing and grinding of mycelium connected with detergent extraction gave only slightly higher yield of chitosanolytic enzymes extraction. The high amount of extracted proteins and very low activity of chitosanase and lipase was obtained by using ultrasounds, which probably considerably inactivated the enzymes.

3.2. Purification of enzymes

Usually, chitosanases from various sources have been purified using standard protein purification methods, like fractionation by ammonium sulfate (salting out), molecular sieving and ion-exchange chromatography [19]. Affinity chromatography has been used very rarely and usually chitosan, chitin or products of their degradation were employed as specific ligands [19]. In our studies the affinity chromatography on CNBr-Sepharose 4B coupled with bacitracin was used to purify *M. circinelloides* chitosanase. Bacitracin, the antibiotic-
cyclodeptide, is known as an efficient ligand of serine, aspartyl and metalloproteins and is used for separation of serine enzymes like e.g. lipases [20]. On the other hand, bacitracin can also interact with several different types of biomolecules, including DNA, RNA, lipoproteins, receptors, and lipids from various sources [21]. It also binds transition metal ions, including Zn(+2), Mn(2+), Co(2+), Ni(2+), and Cu(2+) [22].

Crude enzymatic solution obtained by extraction of Mucor mycelium with Triton X-100 (0.5% w/v) was purified by affinity chromatography on CNBr-Sepharose 4B-bacitracin. As is shown in Figure 1, the proteins from the same peak (fractions from 123 to 136) displayed endo-, exo-chitosanolytic and lipase activities.

The fractions from affinity chromatography were concentrated and applied to gel chromatography on Sephadex G-100. Also this step of purification gave only one peak of proteins displaying endo-, exo-chitosanolytic and lipase activities (Figure 2). Their molecular mass was close to 42-43 kDa.

The results of purification are displayed in Tables 2 and 3. The purification procedure yielded a 23–fold purified chitosanase (endo-type enzyme) with 4.6% recovery of its activity and a 12–fold purified lipase with 2.4% recovery of activity. The ultimate specific activities of chitosanase and lipase were 231.5 unit/mg and 91.0 U/mg, respectively.

**Figure 1.** Affinity chromatography of chitosanase/lipase on CNBr-Sepharose 4B-bacitracin. Before protein separation, the column was equilibrated with 0.2 M phosphate buffer (pH 7.2). Unbound proteins from the protein extract (24 cm³) were washed with 280 cm³ of the latter buffer and the adsorbed proteins were eluted with 100 cm³ of the same buffer supplemented with Brij 35 (0.15% w/v).
Isolation and purification of intracellular chitosanolytic enzymes of Mucor circinelloides

The purified Mucor enzymes significantly lowered the chitosan Mv (average molecular weight) and their exo-chitosanolytic activity was minor. For comparison, all the preparations of lipases described in literature [5 - 11] and applied for chitosan digestion also rapidly depolymerized this biopolymer and rapidly decreased viscosity of its solutions.

It is to note that the purified enzymes preparation migrated as only one protein band with the molecular mass of 42.5 kDa in the SDS-polyacrylamide gel electrophoresis (gel surface

---

**Figure 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) and proteins from CNBr-Sepharose 4B-bacitracin (4.5 cm³) were applied. The column was eluted with the same buffer.

**Table 2.** Purification of M. circinelloides chitosanase.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity, unit</th>
<th>Total protein, mg</th>
<th>Specific activity A_{endo-CH}, unit/mg</th>
<th>Yield, %</th>
<th>Purification folds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>908.64</td>
<td>91.20</td>
<td>9.96</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>CNBr-Sepharose 4B + bacitracin</td>
<td>161.40</td>
<td>1.30</td>
<td>124.15</td>
<td>17.8</td>
<td>12</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>41.67</td>
<td>0.18</td>
<td>231.50</td>
<td>4.6</td>
<td>23</td>
</tr>
</tbody>
</table>

**Table 3.** Purification of M. circinelloides lipase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity [U]</th>
<th>Total protein [mg]</th>
<th>Specific activity A_{L}, U/mg</th>
<th>Yield, %</th>
<th>Purification folds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>691.20</td>
<td>91.20</td>
<td>7.58</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>CNBr-Sepharose 4B + bacitracin</td>
<td>82.00</td>
<td>1.30</td>
<td>63.08</td>
<td>11.9</td>
<td>8</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>16.38</td>
<td>0.18</td>
<td>91.00</td>
<td>2.4</td>
<td>12</td>
</tr>
</tbody>
</table>

The purified Mucor enzymes significantly lowered the chitosan Mv (average molecular weight) and their exo-chitosanolytic activity was minor. For comparison, all the preparations of lipases described in literature [5 - 11] and applied for chitosan digestion also rapidly depolymerized this biopolymer and rapidly decreased viscosity of its solutions.

It is to note that the purified enzymes preparation migrated as only one protein band with the molecular mass of 42.5 kDa in the SDS-polyacrylamide gel electrophoresis (gel surface...
of 8.0 cm × 7.3 cm, **Figure 3**). But, when a distance of protein migration was longer (gel surface of 16.0 cm × 17.5 cm), the SDS-PAGE revealed two main protein bands with the molecular mass of 42 kDa and 43 kDa (**Figure 4**).

Most of the described in literature chitosanases and lipases from bacteria and fungi are characterized by the low molecular mass, usually ranging from 10 kDa to 50 kDa [19] and from 20 kDa to 80 kDa, respectively [23]. For example, the molecular masses of two chitosanases (A and B) from *Mucor rouxii* were 58 kDa and 76 kDa [24], from *Penicillium islandicum* - 30 kDa [19] and from *Aspergillus niger* - 29 kDa [25].

### 3.3. The effect of pH and temperature on *M. circinelloides* chitosanolytic enzymes

The optimum pH for activity of *M. circinelloides* endo- and exo-chitosanolytic enzymes was 5.5-6.0 and these enzymes were stable in the pH range between 4.5 and 7.5. The highest chitosanolytic activities (endo- and exo-) were observed at temperature around 37 °C and these enzymes were relatively stable below 50 °C.

The optimum pH and temperature for activity of *M. circinelloides* chitosanolytic enzymes were similar to those of other fungal chitosanases. For example, enzymes from *P. islandicum* were optimally active at pH between 4.5 and 6.0 and 45 °C, while chitosanase from *F. solani* f. sp. *phaseoli* was optimally active at pH 5.6 and 40 °C. The other fungal chitosanolytic enzymes were found to be stable up to 50 °C [19].

**Figure 3.** SDS-PAGE (8.0 cm × 7.3 cm) of *M. circinelloides* chitosanolytic enzymes. Molecular weight markers (lane 1), the purified *M. circinelloides* proteins (lane 2).

**Figure 4.** SDS-PAGE (16.0 cm × 17.5 cm) of *M. circinelloides* chitosanolytic enzymes. The purified *M. circinelloides* proteins (lane 1), crude extract of proteins (lane 2).
Different preparations of lipases that catalyzed chitosan hydrolysis, displayed the endo-chitosanolytic activity at temperature between 25 °C and 50 °C and pH between 3.6 and 7.0 [5 - 6, 8, 10].

3.4. Substrate specificity

The purified enzymatic preparation was incubated with different substrates (1.0% w/v) such as chitosan (Mv ranging from 121 kDa to 421 kDa, DD ranging from 66% to 98%), colloidal chitin and sodium carboxymethyl cellulose at 37 °C and pH 5.5 for 1 h (endo-chitosanolytic activity) or 24 h (exo-chitosanolytic activity). Further steps were as described in 2.5.1.

Table 4. Substrate specificity of M. circinelloides enzymes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative chitosanolytic activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_{\text{endo-CH}}$</td>
</tr>
<tr>
<td>Chitosan (DD 97.1%, Mv 236 kDa)</td>
<td>100</td>
</tr>
<tr>
<td>Chitosan (DD 83.3%, Mv 433 kDa)</td>
<td>86.7</td>
</tr>
<tr>
<td>Chitosan (DD 78.9%, Mv 392.5 kDa)</td>
<td>57.9</td>
</tr>
<tr>
<td>Chitosan (DD 66.2%, Mv 421.3 kDa)</td>
<td>29.4</td>
</tr>
<tr>
<td>Colloidal chitin</td>
<td>-</td>
</tr>
<tr>
<td>NaCMC</td>
<td>-</td>
</tr>
</tbody>
</table>

The results shown in Table 4 indicate that the purified *Mucor* chitosanase prefers chitosan with the high degree of deacetylation (DD). The colloidal chitin and Na-CMC were not hydrolyzed by *M. circinelloides* enzymes.

4. Summary

Application of the authors’ new, two-step (bacitracin affinity chromatography and gel filtration on Sephadex G-100) method of purification of *M. circinelloides* intracellular enzymes gave the preparation with the relatively high activity of both endo-chitosanases and lipases. Our investigations indicate that this purified preparation contains two proteins with almost the same molecular masses and different activities. We have been trying to separate these proteins and purify them to homogeneity. We also intend to determine their 3 D structures and properties. However, the hitherto published results and our findings suggest that we may also identify the new oligomeric lipase, which efficiently depolymerizes chitozan.

5. Acknowledgment

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6. References