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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