THE USE OF ENZYMES FROM *Mucor circinelloides* IBT-83 IN THE SYNTHESIS OF CHITOOLIGOSACCHARIDES – PRELIMINARY RESEARCH

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Abstract

Chitooligosaccharides (COS) have numerous biological activities, including antibacterial, antifungal, antiviral, anticancer and antioxidant. Unlike high-molecular-weight chitosan, COS have a number of advantages in large-scale commercial applications due to their water solubility and low viscosity. COS are used in the food, agricultural, medical and pharmaceutical industries. The present study demonstrated that intracellular, partially purified enzymes from Mucor circinelloides IBT-83 can hydrolyse chitosan and effectively catalyse the synthesis of a heterodimer composed of D-glucosamine and N-acetyl-D-glucosamine (carried out at a 2.5-fold molar excess of N-acetyl-D-glucosamine relative to D-glucosamine in an acetate buffer [pH 5.5] containing 20% ammonium sulfate). The obtained product was analysed using high-performance liquid chromatography.

Keywords: *chitooligosaccharides, chitosanolytic enzymes, reverse hydrolysis, Mucor circinelloides*

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1. Introduction

Complete hydrolysis of partially *N*-acetylated chitosan to free D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) requires the synergistic action of a multienzyme complex composed of both chitosanolytic and chitinolytic enzymes. These include chitosanase [EC 3.2.1.132], which catalyses the hydrolysis of β-1,4-glycosidic bonds between GlcN units occurring inside the biopolymer chain, and exo-1,4-β-D-glucosaminidase [EC 3.2.1.165], which cleaves GlcN monomers from non-reducing ends. Enzymatic hydrolysis of partially acetylated chitosan involves chitinase [EC 3.2.1.14] and β-*N*-acetylhexosaminidase [EC 3.2.1.52]. These enzymes show activity similar to the abovementioned endo- and exo-chitosanases, but they are specific for β-1,4-glycosidic bonds between the GlcNAc units [1]. In addition to hydrolytic activity, chitosanolytic and chitinolytic enzymes have the ability to catalyse the transglycosylation and/or reverse hydrolysis reactions [2, 3].

The possibility of using the aforementioned enzymes to produce oligosaccharides in the transglycosylation or reverse hydrolysis reactions has been confirmed. This enables the production of chitin/chitosan oligomers with the assumed properties, as well as their derivatives. Chitosanase from *Streptomyces griseus* HUT 6037 synthesises two types of oligomers: $(GlcN)_2$-$(GlcNAc)_3$ and $(GlcN)_3$-$(GlcNAc)_3$ [4]. In turn, chitosanase from *Paenibacillus dendritiformis* produces $(GlcN)_6$ from smaller chitooligosaccharides (COS) [5]. Moreover, enzymes with exohydrolytic activity are a promising catalyst for the production of COS with different degrees of polymerisation. Purified exo-1,4-β-D-glucosominidase from *Aspergillus fumigatus* IIT-004 was used to produce a chitodimer composed of GlcN and GlcNAc [6]. β-*N*-acetylhexosaminidase obtained from *Aspergillus oryzae* was used to produce higher COS (chitohexaose-chitooctaose) from a mixture of smaller chitooligomers formed by acid hydrolysis of chitin [7].

Previous studies have confirmed that the mycelium of *Mucor circinelloides* IBT-83 is a promising source of enzymes that hydrolyse chitosan. They can be used to produce chitosan oligomers with different degrees of polymerisation: low-molecular weight chitosan, COS and GlcN [8–11]. Moreover, this strain produces intracellular chitin deacetylase [12, 13]. The present study demonstrated that the enzymes from *M. circinelloides* IBT-83 have the ability to effectively catalyse the synthesis of a heterodimer composed of GlcN and GlcNAc.

2. Materials and Methods

2.1. Materials

GlcN and GlcNAc were purchased from Sigma-Aldrich (USA). COS standards (from dimer to hexamer) were purchased from Seikagaku Co. (Japan). Chitosan with a degree of deacetylation of 95% and a viscosity of 1500 mPas was obtained from Heppe Medical Chitosan GmbH (Germany). All other reagents were of analytical grade.

2.2. Microorganisms and Culture Conditions

The filamentous fungus *M. circinelloides* IBT-83 was obtained from the culture collection of the Institute of Molecular and Industrial Biotechnology of Lodz University of Technology (GenBank database with accession numbers KR056084 and KR056083). It was cultured in a 30-l Techfors-S laboratory fermenter (Infors HT, Switzerland). The fermenter was filled with 18 l of culture medium containing corn steep liquor powder (3.7%, w/v)

and rapeseed oil (2.7%, v/v), pH 4.7. The culture medium in the fermenter was inoculated with 5×10^7 cells per 1 ml of medium. The inoculum was grown in a 1000-ml Erlenmeyer flask containing 200 ml of medium at 30°C for 12 h with shaking at 180 rpm. After inoculation, the fungus was cultured at 30°C for 72 h with a stirring speed of 100 rpm and aeration at 1 vvm. After cultivation, the mycelium was harvested by filtration, carefully washed with water, defatted with acetone and air-dried at room temperature.

2.3. Protein Extraction and Salting Out

Protein was extracted from defatted and air-dried mycelium by freezing and grinding the biological material in a 0.5% (w/v) Triton-X100 solution according to the method described by Struszczyk et al. [8]. The homogenate was centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was used as the crude enzyme extract. Protein salting out was performed using ammonium sulfate (saturation degree = 60%). The obtained partially purified enzymes were dissolved in 0.05 M acetate buffer (pH 5.5) and dialysed against the same buffer for 12 h at 4°C in a dialysis bag (regenerated cellulose with a molecular weight cut-off of 10 kDa).

2.4. Chitosanolytic Activity Determination

Chitosanolytic activity was determined based on an increase in the reducing sugar concentration after chitosan hydrolysis, according to the method described previously by Struszczyk et al. [8]. The reaction mixture contained 1 ml of 2% chitosan in 2% acetic acid, 0.85 ml of 1 M sodium acetate and 0.15 ml of enzyme solution (pH 5.5). Chitosan hydrolysis was carried out at 37°C for 60 min and 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 at 37°C for 60 min. One unit (U) of chitosanolytic activity (A_{CH}) was equivalent to the amount of enzyme necessary to produce 1 µmol of reducing sugar per 1 min, [1 U = 1 µmol/min]. The content of reduced amino chitooligomers was determined by the Somogyi–Nelson method [14].

2.5. Protein Assay

The protein concentration was determined by the Lowry method [15] using bovine serum albumin as the standard.

2.6. Synthesis Reaction Conditions

The reaction mixture contained 1 mmol GlcN and 2.5 mmol GlcNAc in 50 ml of 0.05 M acetate buffer medium (pH 5.5) containing ammonium sulfate (20%, w/v). The enzyme preparation was introduced at an enzyme-to-reaction medium ratio of 1:25 (v:v). The mixture was incubated at 37°C for 24 h. After the reaction was completed, the sample was freeze-dried. The presence of chitosan oligomers in the sample was analysed using high-performance liquid chromatography (HPLC).

2.7. Analysis of Chitosan Oligomers by High-Performance Liquid Chromatography

HPLC was performed using a Gold Beckman chromatography system equipped with a TSKgel Amide-80 column (4.6 mm × 25 cm), a precolumn of the same type (3.2 mm × 1.5 cm) and a refractometer detector. A mixture of acetonitrile, water, methanol, and tetrahydrofuran (THF) (6:2.4:1.5:1.0, v/v/v/v) was used as the mobile phase. The analysis was carried out at 24°C. GlcN, GlcNAc and COS (from dimer to hexamer) were used as standards.

3. Results and Discussion

3.1. Preparation of Chitosanolytic Enzymes From *Mucor circinelloides* IBT-83

The filamentous fungus *M. circinelloides* IBT-83 produces intracellular chitosanase, exo-1,4-β-D-glucosominidase, and chitin deacetylase [8–13]. We investigated whether the enzymes produced by this strain catalyse the synthesis of COS. First, we extracted intracellular enzymes using a previously developed method (freezing and grinding of mycelium with a 0.5% [w/v] Triton X-100 solution) [8], and then salting out proteins using ammonium sulfate. Table 1 shows the results of the enzyme purification steps. We used partially purified chitosanolytic enzymes as catalysts to synthesise GlcN and GlcNAc oligomers.

Table 1. Salting out proteins using ammonium sulfate precipitation.

Purification step	Total activity [U] × 10^{-3}	Total protein [mg]	Specific activity [U/g]	Yield [%]	Purification degree [fold]
Crude extract	618.2	98.1	6.3	100	–
Salting out by ammonium sulfate (60%, w/v)	483.9	57.5	8.4	78	1.3

3.2. Chitosanolytic Enzymes in the Oligomer Synthesis Reaction

We prepared reaction mixture variants with both monomers together or separately as substrates. We also tested various GlcN and GlcNAc concentrations. Moreover, we performed the reaction using different ammonium sulfate concentrations. We synthesised oligomers under the optimal conditions for the hydrolytic activity of chitosanase from *M. circinelloides* IBT-83: 37°C and pH 5.5 [8]. The reaction occurred exclusively using 1 mmol GlcN and 2.5 mmol GlcNAc in 50 ml of 0.05 M acetate buffer medium (pH 5.5) containing 20% (w/v) ammonium sulfate. Conducting the reaction for 24 h produced a sample containing a dimer with total consumption of GlcN. Table 2 shows the HPLC analysis of the components of the post-reaction mixture. Of note, the other tested variants of the reaction mixture did not yield oligomers as reaction products.

Figure 1 shows the HPLC chromatogram of the initial sample (containing GlcNAc and GlcN as substrates) and the chromatogram of the reaction product. The post-reaction mixture also contains an unused substrate, namely GlcNAc.

Table 2. Reaction products of chitooligosaccharide synthesis using chitosanolytic enzymes from *Mucor circinelloides* IBT-83.

Sample	Content [%]		
	GlcNAc	GlcN	GlcNAc-GlcN
Components of the initial sample (substrates)	65	32	0
Mixture after synthesis for 12 h	59	26	12
Mixture after synthesis for 24 h	42	0	56

Note. Abbreviations: GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine.

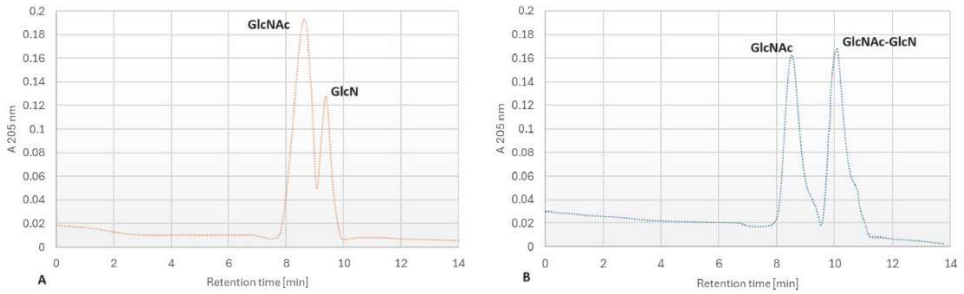

Figure 1. High-performance liquid chromatogram of (A) the components of the initial sample (substrates) and (B) the components of the post-reaction mixture. *Note:* The reaction was carried out according to the methodology described in Section 2.6, reaction time 24 hours. D-Glucosamine (GlcN), *N*-acetyl-D-glucosamine (GlcNAc) and chitooligosaccharides were used as standards.

In the future, it will be necessary to evaluate the possibility of synthesising oligomers with a higher degree of polymerisation. It is also necessary to identify the enzyme that catalyses this reaction, which is related to the need to use highly purified protein in the reaction. Next, attention should be paid to optimising the reaction conditions, including temperature, pH and additives, to increase enzymatic activity. The addition of co-solvents (acetonitrile, dimethylsulfoxide, *N,N* dimethylformamide, dioxane, 1,3-butanediol and 1,2,4-butanetriol), water-soluble solutes, or inorganic, soluble salts (most often ammonium sulfate) can alter water activity, which contributes to increase the synthetic activity of hydrolases [16]. In the present study, we only tested ammonium sulfate. The presence of a high concentration of ammonium sulfate (30%) initiated the conversion of $(GlcNAc)_4$ into $(GlcNAc)_2$ (55.7%) and $(GlcNAc)_6$ (39.6%) using chitinase from *Trichoderma reesei* KDR-11 [17]. Another example is the GlcN-GlcNAc dimer synthesis reaction carried out using exo-1,4-β-D-glucosominidase from *A. fumigatus* IIT-004 in a 15% ammonium sulfate environment [6]. β-*N*-Acetyl glucosaminidase from *Chitinolyticbacter meiyuanensis* SYBC-H1 has reverse hydrolysis activity towards GlcNAc, synthesising various linked GlcNAc dimers. In this case, the dimer synthesis reaction took place in an environment with a high substrate concentration (10 g/l GlcNAc) [3].

4. Conclusions

This paper includes the preliminary results of research on the activity of intracellular enzymes obtained from *M. circinelloides* IBT-83 towards the synthesis of chitosan oligomers. We confirmed that these enzymes effectively catalyse the synthesis of a heterodimer consisting of GlcN and GlcNAc when using a 2.5-fold molar excess of GlcNAc relative to GlcN and performing the reaction at 37°C and pH 5.5 for 24 h.

5. References

[1] Kaczmarek MB, Struszczyk-Świta K, Li X, Szczęsna-Antczak M, Daroch M; (2019) Enzymatic modifications of chitin, chitosan, and chitooligosaccharides. Front Bioeng Biotechno 7, 243. **DOI:**10.3389/fbioe.2019.00243

[2] Jung WJ, Park RD; (2014) Bioproduction of chitooligosaccharides: present and perspectives. Mar Drugs 12(11), 5328–5356. **DOI:**10.3390/md12115328

[3] Zhang A, Mo X, Zhou N, Wang Y, Wei G, Chen J, Chen K, Ouyang P; (2020) A novel bacterial β-*N*-acetyl glucosaminidase from *Chitinolyticbacter meiyuanensis* possessing transglycosylation and reverse hydrolysis activities. Biotechnol Bifuels 13, 115. **DOI:**10.1186/s13068-020-01754-4

[4] Tanabe T, Morinaga K, Fukamizo T, Mitsutomi M; (2003) Novel chitosanase from Streptomyces griseus HUT 6037 with transglycosylation activity. Biosci Biotechnol Biochem 67(2), 354–364. **DOI:**10.1271/bbb.67.354

[5] Sun H, Zhao L, Mao X, Cao R, Liu Q; (2023) Identification of a key loop for tun-ing transglycosylation activity in the substrate-binding region of a chitosanase. J Agric Food Chem 71(14), 5585–5591. **DOI:**10.1021/acs.jafc.3c00110

[6] Sinha S, Chand S, Tripathi P; (2016) Enzymatic production of glucosamine and chitooligosaccharides using newly isolated exo-β-D-glucosaminidase having transglycosylation activity. 3 Biotech 6(1), 13. **DOI:**10.1007/s13205-015-0330-5

[7] Dvořáková J, Schmidt D, Huňková Z, Thiem J, Křen V; (2001) Enzymatic rearrangement of chitine hydrolysates with β-*N*-acetylhexosaminidase from *Aspergillus oryzae*. J Mol Catal B Enzym 11(4–6), 225–232. **DOI:**10.1016/S1381-1177(00)00075-8

[8] Struszczyk K, Szczęsna-Antczak M, Walczak M, Pomianowska E, Antczak T; (2009) Isolation and purification of *Mucor circinelloides* intracellular chitosanolytic enzymes. Carbohydr Polym 78(1), 16–24. **DOI:**10.1016/j.carbpol.2009.04.010

[9] Struszczyk K, Szczęsna-Antczak M, Walczak M, Pomianowska E, Wojciechowska J, Antczak T; (2009) Enzymatic preparations from *Mucor* moulds and their application in oligoaminosaccharides production. Prog Chem Appl Chitin Deriv 14, 89–100.

[10] Struszczyk K, Szczęsna-Antczak M, Pomianowska E, Stańczyk Ł, Wojciechowska J, Antczak T; (2010) Process of continuous production of oligoaminosaccharides in a column reactor. Prog Chem Appl Chitin Deriv 15, 177–188.

[11] Struszczyk-Świta K, Stańczyk Ł, Szczęsna-Antczak M, Antczak T; (2017) Scale-up of PUF-immobilized fungal chitosanase-lipase preparation production. Prep Biochem Biotechnol 47(9), 909–917. **DOI:**10.1080/10826068.2017.1365240

[12] Kaczmarek MB, Struszczyk-Świta K, Florczak T, Szczęsna-Antczak M, Antczak T; (2016) Isolation, molecular cloning and characterisation of two genes coding chitin deacetylase from Mucor circinelloides IBT-83. Prog Chem Appl Chitin Deriv 21, 93–103. **DOI:**10.15259.PCACD.21.09

[13] Kaczmarek MB, Struszczyk-Świta K, Szczęsna-Antczak MH, Antczak T, Gierszewska M, Steinbuchel A, Daroch M; (2021) Polycistronic expression system for Pichia pastoris composed of chitin deacetylase, chitinase, and chitosanase enabling one-pot enzymatic modification of chitin substrates. Front Bioeng Biotechnol 9, 710922. **DOI:**10.3389/fbioe.2021.710922

[14] Nelson W; (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem 153, 375–380. **DOI:**10.1016/S0021-9258(18)71980-7

[15] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ; (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193, 265–275. **DOI:**10.1016/S0021-9258(19)52451-6

[16] Muschiol J, Vuillemin M, Meyer AS, Zeuner B; (2020) β-*N*-Acetylhexosaminidases for Carbohydrate Synthesis via Trans-Glycosylation. Catalysts 10(4), 365. **DOI:**10.3390/catal10040365

[17] Usui T, Matsui H, Isobe K; (1990) Enzymic synthesis of useful chito-oligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate. Carbohydr Res. 203(1), 65–77. **DOI:**10.1016/0008-6215(90)80046-6