Carbohydrate Research 383 (2014) 27–33

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Production of chitooligosaccharides from *Rhizopus oligosporus* NRRL2710 cells by chitosanase digestion

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ARTICLE INFO

Article history:
Received 24 April 2013
Received in revised form 1 June 2013
Accepted 4 June 2013
Available online 14 June 2013

Keywords:
Rhizopus oligosporus
Fungal cell wall
Chitosanase
Partially N-acetylated chitooligosaccharides
CP/MAS ^{13}C NMR

ABSTRACT

The intact cells of *Rhizopus oligosporus* NRRL2710, whose cell walls are abundant source of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN), were digested with three chitinolytic enzymes, a GH-46 chitosanase from *Streptomyces* sp. N174 (CsnN174), a chitinase from *Pyrococcus furiosus*, and a chitinase from *Trichoderma viride*, respectively. Solubilization of the intact cells by CsnN174 was found to be the most efficient from solid state CP/MAS ^{13}C NMR spectroscopy. Chitosanase products from *Rhizopus* cells were purified by cation exchange chromatography on CM-Sephadex C-25 and gel-filtration on Cellulofine Gcl-25 m. NMR and MALDI-TOF-MS analyses of the purified products revealed that GlcN–GlcNAc, $(GlcN)_2$–GlcNAc, and $(GlcN)_2$ were produced by the enzymatic digestion of the intact cells. The chitosanase digestion of *Rhizopus* cells was found to be an excellent system for the conversion of fungal biomass without any environmental impact.

1. Introduction

Chitin, a water-insoluble polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc), is the most abundant biomass next to cellulose, and widely distributed in organisms, such as crustacean shells, insect cuticles, and fungal cell wall.[1] Commercial chitin is mainly extracted by demineralization and deproteinization of the shells of crustaceans such as shrimps, crabs, lobsters, and krill, which are supplied in large quantities by shellfish processing.[2] Chitosan, a β-1,4-linked polymer of glucosamine (GlcN), has been produced on an industrial scale by the N-deacetylation of chitin using sodium hydroxide. The enzymatic hydrolysis of chitin and chitosan has been conducted to produce corresponding oligosaccharides,[3] which have been drawing attention because of their presumed biological functions, such as the inhibition of tumor angiogenesis,[4] control of cell growth, differentiation, and development in vertebrates,[5,6] antioxidative effect,[7] and elicitation of defensive actions in plants.[8,9] However, the extraction process of chitin from crustacean shells is well known to produce a significant impact on the environment. In this context, it is highly desirable to establish a strategy for directly producing chitin and chitosan oligosaccharides by the enzymatic digestion of raw materials containing chitin and chitosan.

Chitin is associated with proteins and minerals in crustacean shells,[2] resulting in a sclerotized and recalcitrant structure, and further protecting the polysaccharide chain from enzymatic digestion. However, fungal cell wall consists of chitin, branched β-1,3-glucan, and β-1,6-glucan, which are cross-linked to each other. Chitosan is also found in the fungal cell wall,[10] and makes the cell wall structure more pliable. Therefore, the chitinous components of the fungal cell wall appear to be degradable by enzymatic actions. In fact, the digestion of the cell wall fraction of *Fusarium oxysporum* by chitinase and chitosanase was found to produce oligosaccharides composed of GlcNAc and GlcN.[11,12] However, most studies on the chitinous components of fungal cell wall thus far have been devoted to efficient utilization of fungi as the source of polysaccharide chitin/chitosan[13] or GlcN monomer.[14,15] Experimental data on the oligosaccharide production from the fungal cell wall have not been fully accumulated. In order to utilize the chitooligosaccharide products obtained from the fungal cell wall as

Abbreviations: GlcNAc, 2-acetamido-2-deoxy-β-D-glucopyranose; GlcN, 2-amino-2-deoxy-β-D-glucopyranose; $(GlcN)_n$, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of *n*; MALDI-TOF-MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; NMR, nuclear magnetic resonance.

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http://dx.doi.org/10.1016/j.carres.2013.06.002

food additives or pharmaceutics, fungi as the source materials should be harmless; therefore, pathogenic fungi such as the *Fusarium* species could not be used for this purpose.

Some *Rhizopus* species have been used in the fermentation food industry in Asia, and their products have been generally recognized as safe.[16] Thus we determined the chitin/chitosan content in several *Rhizopus* species, and the content in *R. oligosporus* was found to be the highest (32%, Yamashita et al., unpublished). In this study, the intact cells of *R. oligosporus* were digested by chitinolytic enzymes, and the products were analyzed by NMR spectroscopy and MALDI-TOF-MS. We found that the chitosanase digestion of *Rhizopus* cells is an excellent strategy for chitooligosaccharide production without any impact on the environment.

2. Results and discussion

2.1. Enzymatic digestibility of *Rhizopus oligosporus* cells

To evaluate the enzymatic digestibility of *Rhizopus* cells, three enzymes were tested, an endo-splitting chitosanase from *Streptomyces* sp. N174 (CsnN174), a thermophilic chitinase from *Pyrococcus furiosus* (PfChit), and a chitinase from *Trichoderma viride* (TvChit). These two chitinases belong to the GH18 family and hydrolyze the internal β-1,4-glycosidic linkages (endo-splitting mode) through substrate-assisted mechanism.[17] The production systems of these enzymes were highly efficient and available in our laboratory. Individual digestions of the cells were conducted for 24 h. We confirmed that the release of reducing sugars into the supernatant fraction completely ceased after 24 h incubation. The amounts of the insoluble materials remaining after the enzymatic digestion were determined and compared with the starting amounts of the cells. The results are listed in Table 1. The amount of insoluble materials remaining was the lowest in the digestion with the chitosanase, CsnN174, indicating that the chitosanase has the highest digestibility (67%). The digestibilities of the chitinase preparations (PfChit and TvChit) were comparable to each other (35–36%), and much lower than those of CsnN174. Since the chitinase (PfChit) used in this experiment was thermophilic, the enzymatic reaction was also conducted at a higher temperature, 70 °C. However, solubilization was not enhanced by elevating the temperature. The accessibility of the enzymes to the chitin polysaccharide chain might have been restricted in the *Rhizopus* cell wall probably due to the ordered polysaccharide structure. When the control experiments were conducted in the absence of enzymes, reducing sugars did not significantly increase in the reaction mixture. It is evident that the cell wall polysaccharides were digested by the exogenous enzymes, but not by the endogenous ones.

2.2. Solid-state CP/MAS ^{13}C NMR analysis of *Rhizopus* cells

The insoluble materials remaining after the enzymatic digestion of *Rhizopus* cells were used for solid-state CP/MAS ^{13}C NMR analysis. The NMR spectra are shown in Figure 1. We initially compared the spectrum of intact *Rhizopus* cells (Fig. 1D) with those of authentic chitin and chitosan preparations (Fig. 1E and F) to roughly estimate the overall structure of the cell wall. Based on the previous assignment data,[18] the resonances for polysaccharides were assigned as labeled in the figure. The resonances designated by asterisks and some portion of the carbonyl resonance were supposed to be derived from the protein and lipid components of *Rhizopus* cells. Since carbonyl and methyl carbon resonances were clearly observed, the *Rhizopus* cell wall was found to contain a significant portion of chitin. Line broadening of the resonances for the pyranose-ring carbons (C1–C6) indicated that a major fraction of the cell wall is a mixture of polysaccharides, such as chitin/chitosan and β-1,3- and β-1,6-glucans. However, the shape of the C6 and C2 resonances of *Rhizopus* cells appeared to be similar to that of chitosan. *Rhizopus* cells are likely to contain chitin/chitosan abundantly in their cell walls.

When *Rhizopus* cells were digested by TvChit or PfChit, the resonance intensities of the methyl and carbonyl carbons were reduced by the enzymatic digestion (Fig. 1B and C), indicating the significant digestion of chitin polysaccharides. However, the relative intensities of C1–C6 in the spectra appeared to be similar to those of the intact cells. The major polysaccharide components were not markedly disrupted by the treatment with TvChit or PfChit. On the other hand, the insoluble fraction obtained after digestion of *Rhizopus* cells by CsnN174 exhibited lower intensities of C1–C6 (Fig. 1A). Major polysaccharide components were intensively disrupted by CsnN174. These results indicated again that solubilization of *Rhizopus* cells by CsnN174 was the most efficient. In the intact fungal cells, it appears that the polysaccharide structure of chitosan region is flexible, but the chitin region is rigid. This situation might have made the chitosanase enzyme more accessible to the polysaccharide chain, resulting in the more efficient degradation by CsnN174.

2.3. Purification of the oligosaccharide products obtained by CsnN174 digestion

The soluble fraction (1.38 g) obtained after the chitosanase digestion of *Rhizopus* cells was applied onto a charcoal column, and the adsorbed fraction was further employed for cation-exchange chromatography. The profile is shown in Figure 2. After the elution of a void-volume fraction, several reducing-sugar fractions were obtained, and the three major fractions I, II, and III were further purified by gel-filtration. The three purified fractions were dialyzed against distilled water, lyophilized, and employed for solution NMR spectroscopy and MALDI-TOF-MS. The recoveries of fractions I, II, and III were 21, 8, and 25 mg, respectively.

2.4. Structure analysis of the oligosaccharide products

Figure 3 shows the ^{13}C NMR spectra of fractions I, II, and III in solution. Two major resonances were observed in the carbonyl and methyl carbon regions of fractions I and II, and assigned to the α- and β-anomers of the reducing end GlcNAc residue based on their chemical shifts. In the C1 carbon region (90–100 ppm) of fraction I, one resonance (C1′) was found at 97.8 ppm in addition to the resonances corresponding to the α- and β-anomers of the reducing end GlcNAc (C1α and C1β), and assigned to the non-

Table 1
Amounts of the insoluble and soluble products obtained from the enzymatic digestion of *Rhizopus* cells

Enzyme	Intact cells* (g)	Insoluble products after enzymatic digestion* (g)	Soluble products (g)	Hydrolysis %
CnsN174	2.06 ± 0.04	0.68 ± 0.06	1.38	67
PfChit	2.10 ± 0.05	1.34 ± 0.04	0.76	36
TvChit	2.05 ± 0.02	1.34 ± 0.06	0.71	35

* Mean values of three independent reactions with ± SD (standard deviations).