



Review

Advances in preparation, analysis and biological activities of single chitooligosaccharides



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ABSTRACT

Chitooligosaccharides (COS), as a source of potential bioactive material, has been reported to possess diverse bioactivities. These bioactivities of COS are often tested using relatively poorly characterized oligomer mixtures during past few decades, resulting in difficult identification of COS molecules responsible for biological effects. Therefore, a new interest has recently been emerged on highly purified COS of defined size. Several technological approaches have been used to produce single COS and new improvements were introduced to their characterization in order to understand the unrevealed structure-function relationship. Here we provide an overview of techniques that were used to prepare and analyze reasonably well-defined COS fractions. Based on the latest reports, several applications of single COS for plants and animals, are also presented, including antitumor, immunostimulatory, antioxidant, antimicrobial, elicitors of plant defence and neural activity.

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Contents

1. Introduction	179
2. Preparation of single COS	179
2.1. Chemical and enzymatic synthesis	179
2.2. Separation of single COS	180
2.2.1. Size exclusion chromatogram (SEC)	180
2.2.2. Metal affinity chromatography	180
2.2.3. Absorption chromatography	180
2.2.4. Ion exchange chromatography	180
2.2.5. Electrodialysis with ultrafiltration membrane (EDUF)	181
3. Analysis of single COS	181
3.1. Thin-layer chromatography (TLC)	181
3.2. High performance liquid chromatography (HPLC)	183
3.3. Capillary electrophoresis (CE)	184
3.4. Mass spectrometry (MS)	185
4. Bioactivities of single COS	185
4.1. Antitumor	185
4.2. Immunostimulatory	186
4.3. Antioxidant	186
4.4. Antimicrobial	186
4.5. Elicitors of plant defence	186
4.6. Other bioactivities	187
Concluding remarks	187
Acknowledgement	187
References	187

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1. Introduction

Chitin is a natural linear polysaccharide consisting of $\beta(1\rightarrow4)$ -linked *N*-acetyl-D-glucosamine (GlcNAc) residues, that occurs mainly as a structural component in the cell walls of fungi and yeasts and in the exoskeletons of insects, nematodes and arthropods (e.g., crabs, crawfish, lobsters and shrimps). Chitin is often considered the second most abundant polysaccharide in nature following plant cellulose. Despite the widespread distribution of chitin, up to now the main commercial sources of chitin are crab and shrimp shells. In industrial processing, chitin is extracted from crustaceans by two isolation steps: demineralization with HCl and deproteination with aqueous NaOH (Rinaudo, 2006).

Chitosan is a heteropolymer of GlcNAc and D-glucosamine (GlcN) residues and can be produced by the deacetylation of chitin. At the elemental analysis, chitosan generally has nitrogen content higher than 7% and degree of acetylation (DA) lower than 0.40. Removal of the acetyl group is a harsh treatment usually performed with concentrated NaOH solution (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). In combination with their non-toxicity, biocompatibility and biodegradability, both chitin and chitosan exhibits a variety of interesting physicochemical and biological properties, which makes chitin and chitosan suitable for use in numerous applications in agriculture, cosmetics, water treatment and medicine (Domard, 2011; Kim & Rajapakse, 2005; Muzzarelli, 2010; Xia, Liu, Zhang, & Chen, 2011). However, chitin is insoluble in water and most organic solvents, while chitosan has high viscosity in dilute aqueous acid solutions and poor solubility at neutral pH conditions. This property has restricted their use, particularly in medicine and food industry.

Chitooligosaccharides (COS), the hydrolyzed product of chitin and chitosan, is $\beta(1\rightarrow4)$ linked homo- or heterooligomers of GlcNAc and/or GlcN with high solubility in neutral aqueous solutions, and exhibits numerous biological functions such as antifungal (Li, Xing, Liu, Li, Qin, et al., 2012; Li, Xing, Liu, Qin, Li et al., 2012; Li, Xing, Liu, Qin, Meng, & Li, 2012; Oliveira, El Gueddari, Moerschbacher, Peter, & Franco, 2008), antibacterial (Benhabiles et al., 2012; Fernandes et al., 2008), antitumor (Huang, Mendis, Rajapakse, & Kim, 2006; Park, Chung, Choi, & Park, 2011; Shen, Chen, Chan, Jeng, & Wang, 2009), anti-inflammatory (Fernandes et al., 2010) and antioxidant activities (dos Santos, El Gueddari, Trombotto, & Moerschbacher, 2008; Ngo, Kim, & Kim, 2008; Xu et al., 2009), bone strengthening (Jung, Moon, & Kim, 2006), enzyme inhibition (Henning Cedervik, Parmer, Vårum, Eijnsink, & Sørli, 2008), hypocholesterolemic (Liu, Zhang, & Xia, 2008), anti-allergic activity (Vo, Kong, & Kim, 2011), immunity-enhancing (Kim et al., 2006), wound healing (Minagawa, Okamura, Shigemasa, Minami, & Okamoto, 2007), activation of plant defense (Day, Shibuya, & Minami, 2003; de Jonge et al., 2010; Liu et al., 2012) and so on. At present, large amounts of COS can be prepared by a variety of techniques, including acid hydrolysis, oxidative degradation and enzymatic methods (Horn et al., 2006; Tishchenko et al., 2011; Trombotto, Ladaviere, Delolme, & Domard, 2008; Xia, Liu, & Liu, 2008). Moreover, some physical methods can be applied to improve degradation of chitosan, including microwave (Shao, 2003; Sun, Zhou, Xie, & Mao, 2006), ultrasonication (Popa-Nita, Lucas, Ladaviere, David, & Domard, 2009; Yue, Yao, Wei, & Mo, 2008), ultraviolet light (Wang, Huang, & Wang, 2005) and γ -irradiation (Kang, Dai, Zhang, & Chen, 2007). All these reported techniques generally produce a complicated COS mixture, which was classified according to degree of *N*-acetylation (DA), the degree of polymerization (DP) or the molecular weight (MW), and the molecular weight distribution (PD, for polydispersity). Therefore, the bioactivities of COS are often determined using heterogeneous and/or relatively poorly characterized oligomer mixtures, and it is difficult to determine which molecules are responsible for the observed biological effects. The

activity mechanisms are unknown. In addition, some controversial results regarding the biological activities of COS exist due to the use of COS mixtures with various DAs and DPs and the reproducibility can be an issue (Aam et al., 2010; Mourya, Inamdar, & Choudhari, 2011). It is clearly essential that well-characterized and highly purified oligosaccharides preparations of defined size should be used in both cell biological and structural studies so as to understand in-depth knowledge on the mode of the bioactivity of COS. Herein, we summarize both preparation approaches and analysis methods for the single COS as well as their biological activities.

2. Preparation of single COS

At present, single COS could be prepared by two ways: synthesis and separation. Some COSs of defined size could be obtained by chemical or enzymatic synthesis using GlcN/GlcNAc as donors. In addition, single COS could also be separated from COS mixture produced by hydrolysis of chitin/chitosan using various chromatographic methods.

2.1. Chemical and enzymatic synthesis

The glycosylation to construct the $\beta(1\rightarrow4)$ glycosidic linkage is the key reaction in the synthesis of COS. The leaving groups in the glycosyl donors play a prominent role in the glycosylation, which determine the activation reagents and reaction conditions. Another utmost important parameter in the synthesis of COS is the *N*-substituent in the GlcN/GlcNAc donor, which affects critically the glycosylation efficiency and also the overall protecting group manipulation. Before the 1970s, synthesis of COS was a difficult task. In 1977, *N*-phthalyl group was first introduced to COS synthesis (a chitobiose derivative) by Lemieux, Takeda, and Chung (1977). Then COS synthesis has been extensively explored with a number of the glycosylation strategies and various GlcN donors/acceptors (Blatter, Beau, & Jacquinet, 1994; Kanie, Ito, & Ogawa, 1994). It has been reported that several COSs with single DP was synthesized including tetra-*N*-acetylchitotetraose and hexa-*N*-acetylchitohexaose (Aly, Ibrahim, El Ashry, & Schmidt, 2001). Recent progress in chemical synthesis of COS has been reviewed by Yang and Yu (Yang & Yu, 2014). Herein, we will not go into details.

Chemical methods for the synthesis of oligosaccharides have been considerably improved during the past twenty years, but they still require complicated processes for multiple protection and deprotection. As the number of steps increases with the size of the oligosaccharide, preparation of large quantities of COS longer than trisaccharide is not practical. Enzymatic methods allow regioselective synthesis under mild conditions and without complicated protection. Some researchers began to focus on the enzymatic synthesis of COS. Akiyama et al. reported a novel method for the synthesis of COS utilizing a transglycosylation reaction catalyzed by hen egg-white lysozyme and a COS with DP4–12 was prepared using *N,N,N'*-tri(monochloro)acetylchitotriose and tri-*N*-acetylchitotriose as glycosyl donors followed by *N*-demonochloroacetylation (Akiyama, Kawazu, & Kobayashi, 1995). Kobayashi et al. synthesized di-*N*-acetylchitobiose via enzymatic glycosylation using a 1, 2-oxagoline derivative of GlcNAc as new glycosyl donor for chitinase (Kobayashi, Kiyosada, & Shoda, 1997). In addition, Tokuyasu et al. found that a chitin deacetylase from *Colletotrichum lindemuthianum* could acetylate free amino sugar residues into *N*-acetylated forms in the presence of 3.0M sodium acetate. They used the chitin deacetylase to synthesize GlcNAc-(1 \rightarrow 4)-D-GlcN and β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)-D-GlcN and found the enzymatic reaction allowed a regioselectivity that was hard to achieve by chemical *N*-acetylation (Tokuyasu, Ono, Mitsutomi, Hayashi, &

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