Carbohydrate Polymers 90 (2012) 491–495

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

A comparison study on the interactions of two oligosaccharides with tobacco cells by time-resolved fluorometric method

Weihua Guo[a], Heng Yin[b], Zhiqiang Ye[c], Xiaoming Zhao[b], Jingli Yuan[c,*], Yuguang Du[b,*]

[a] School of Life Science and Technology, Dalian Ocean University, Dalian 116023, China
[b] Department of Biotechnology, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[c] State Key Laboratory of Fine Chemicals, Department of Chemistry, Dalian University of Technology, Dalian 116024, China

ARTICLE INFO

Article history:
Received 3 March 2012
Received in revised form 17 April 2012
Accepted 19 May 2012
Available online 2 June 2012

Keywords:
Oligosaccharide
Tobacco cell
Interaction
Europium complex
Time-resolved fluorescence

ABSTRACT

The interactions of tobacco cells with two oceanic oligosaccharides, chitosan oligosaccharide (COS) and alginate derived oligosaccharide (AOS) were investigated and compared by time-resolved fluorometric method using two Eu^{3+} complexes as luminescence probes. The binding processes of two oligosaccharides and tobacco leaf cells (epidermal and stomatal guard cells) were characterized by the luminescence imaging method based on the reaction of tobacco cells with oligosaccharide (COS or AOS) conjugated to a highly fluorescent Eu^{3+} complex. In addition, the concentration changes of indole-3-acetic acid (IAA) and IAA-related peroxidase produced in the cells during the interaction of oligosaccharides (COS or AOS) and tobacco cells were also determined. The results indicate that the bioactivity of COS, with faster binding rate to the tobacco cells and stronger effect on the IAA generation in the cells, is remarkably higher than that of AOS. The comparison study on bioactivities of the two oligosaccharides reveals the relationship between structures and bioactivities of oligosaccharide, and suggests that COS should be more favorable to be used as a promotion reagent of plant growth in agriculture field.

1. Introduction

Oligosaccharide is a kind of oligomer which consist 2–10 molecules of monosaccharide linked by glucosidic bonds (Albersheim & Darvill, 1985). During past few decades, many researchers have focused on oligosaccharides due to their biological activities, such as antimicrobial (Mengibar et al., 2011), anticancer (Huang, Mendis, Rajapase, & Kim, 2006), antioxidant (Dai-Hung et al., 2011), and immunostimulant effects (Kim & Rajapakse, 2005). Recent advances have demonstrated that bioactive oligosaccharides can act as signal molecules to regulate plants growth and development (Yin, Zhao, & Du, 2010). In plants, oligosaccharides can effectively elicit increase in net photosynthesis, transpiration rate (Guo, Zhao, & Du, 2008), nitrogen metabolism (Zhang et al., 2011) and cell division in differentiated cells, which are beneficial for plant growth and development. Recently, the generation of an important native auxin, indole-3-acetic acid (IAA), induced by oligosaccharides in plant cells was also reported (Guo et al., 2009). The most widely used oligosaccharides in the agricultural and biomedical fields include chitosan oligosaccharide (COS) and alginate-derived oligosaccharide (AOS). The activity of the two oligosaccharides is different maybe due to their different structures (Cote & Hahn, 1994) (Fig. 1). COS has a substituting NH_2 group which can form ammonium groups NH_3^+ by absorbing hydrion from the solution, and this positively charged character make it easy to bind or react with other molecules, such as reactive oxygen species (ROS), through addition reaction. At the same time, AOS has a substituting carboxylic group, a strong electron-withstanding group, which can decline the dissociation energy of O—H and effectively improve the reactive activity of OH groups in oligosaccharide molecules (Xie, Xu, & Liu, 2001). These two oligosaccharides can be easily prepared by either chemical or enzymatic hydrolysis of chitosan and sodium alginate, respectively. Although oligosaccharides have considerable potentials to be utilized in agriculture (Muzzarelli et al., 2012), it is still hard to explain exactly how these molecules exert their bioactivities. The interaction study of different oligosaccharides with plant cells is expected to be able to provide an insight about the unrevealed molecular functions of oligosaccharides.

Time-gated luminescence imaging based on lanthanide complexes is a powerful tool for investigating the interaction of biological molecules and cells, especially in the complicated biological environments (Bünzli, 2010). By the introduction of an appropriate delay time, the short-lived background fluorescence from the raw biological samples and nearby optics can be

* Corresponding authors. Fax: +86 411 84986041/84379061.
E-mail addresses: jingliyuan@yahoo.com.cn (J. Yuan), duyg@dicp.ac.cn, dyguang@gmail.com (Y. Du).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.05.070

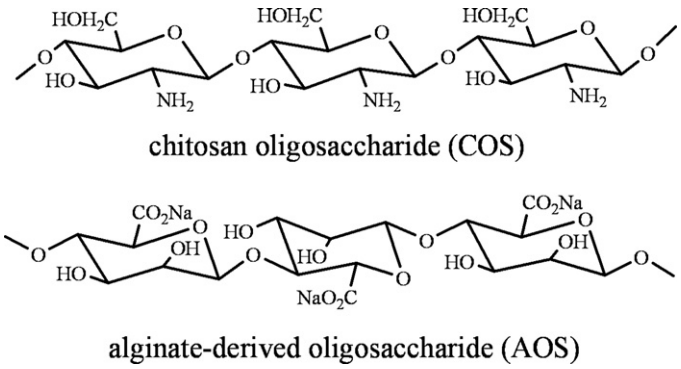

Fig. 1. Structures of chitosan oligosaccharide (COS) and alginate-derived oligosaccharide (AOS).

effectively eliminated, and therefore the long-lived signals are selectively imaged with high signal-to-noise ratios. Recently, we established a fluorescence imaging method to monitor the interaction between COS and tobacco cells based on the reaction of tobacco cells with COS conjugated with a highly fluorescent Eu^{3+} complex 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl) chlorosulfo-*o*-terphenyl–Eu^{3+} (COS–BHHCT–Eu^{3+} conjugate), and developed a highly specific and sensitive time-resolved fluorescence assay method for the determinations of indole-3-acetic acid (IAA) and IAA-related peroxidase in tobacco cells by using a Eu^{3+} complex [4′-(10-methyl-9-anthryl)-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)tetrakis(acetate)–Eu^{3+} (MTTA–Eu^{3+}) as a probe (Guo et al., 2009).

Herein we describe a comparison study on the interactions of two oligosaccharides, COS and AOS, with tobacco leaf cells. After the two oligosaccharides were covalently conjugated with BHHCT–Eu^{3+}, kinetic processes of the interactions of the two oligosaccharides with the tobacco leaf cells (epidermal and stomatal guard cells) were monitored with a time-resolved fluorescence imaging method. In addition, the IAA and IAA-related peroxidase concentration changes in the tobacco cells during this process were also measured and compared. The results revealed the relationship of structure and bioactivity of the different oligosaccharide molecules.

2. Materials and methods

2.1. Plant materials and cell culture

Tobacco plants (*Nicotiana tabacum* var. Samsun NN) were grown from seed in a greenhouse and were used at the 4–6 leaf stage after 2 months in culture. The plants were kept in a growth chamber at $23 \pm 1\,^{\circ}C$ with a photoperiod of 16 h and 70–80% relative humidity for several days before use.

The plant cell line used was derived from young Samsun NN tobacco leaves using MS medium, and cell suspension cultures were maintained in MX medium in 250 mL Erlenmeyer flasks with a liquid volume of 100 mL in each capped flask. All cultures were maintained at 25 °C with a 12 h photoperiod and a photon flux density of 36 mol m^{-2} s^{-1}, agitated at 120 rpm on an orbital shaker. The suspension culture was sub-cultured every 2 weeks and kept at least 1 year.

2.2. Observation of oligosaccharides binding to tobacco leaf epidermis

The tobacco leaf epidermis was incubated in the MES (4-morpholine ethanesulfonic acid)–KCl buffer (MES–KOH, 10 mM; KCl, 50 mM; $CaCl_2$, 100 mM, pH 6.15) for 3 h in light, and then immersed into 2.0 mL of the Tris–KCl buffer (Tris, 10 mM; KCl, 50 mM, pH 7.20). After 20 μL of the conjugate solution of oligosaccharide–BHHCT–Eu^{3+} was added to the mixture, the mixture was incubated in the dark at room temperature. The epidermal strip was carefully washed 3 times with water, and then subjected to luminescence microscopy imaging detection.

2.3. Time-resolved luminescence assay for IAA in tobacco cell solution

Fresh tobacco cells were collected by filtration and washed twice with distilled water. The cells were re-suspended in distilled water, and the density of the suspension was adjusted to 1.5×10^6 cells/mL. The cell suspension, mixed with an appropriate amount of COS (0.01 mg/L) or AOS (1.0 mg/L) was incubated at room temperature with shaking at 150 rpm. At different treatment times, the cells were centrifuged for 5 min at 10,000 rpm and 4 °C. One milliliter of supernatant solution was added to 1.0 mL of 0.1 M acetate buffer of pH 4.0 containing 0.05 μM of HRP and 0.2 μM of MTTA–Eu^{3+}. After incubated at room temperature with shaking for 20 min, the solutions were subjected to the time-resolved fluorometric measurement by using Perkin Elmer Victor 1420 multilabel counter. A control experiment in the absence of oligosaccharide was carried out with the same method.

2.4. Time-resolved luminescence assay for IAA-related peroxidase in tobacco cells

The tobacco cells (1.5×10^6 cells/mL)–oligosaccharides (0.01 mg/L for COS or 1.0 mg/L for AOS) suspension was prepared as mentioned above. At different treatment times, the cells were centrifuged for 5 min at 10,000 rpm and 4 °C. The precipitate was ground with liquid nitrogen and quartz sands for 3 min, and the mixture was stirred in 0.1 M phosphate buffer of pH 7.0 for 30 min. After centrifuged for 5 min at 10,000 rpm and 4 °C, 1.0 mL of supernatant was added to 1.0 mL of 0.1 M sodium acetate buffer of pH 4.0 containing 2.0 μM of IAA and 0.2 μM of MTTA–Eu^{3+}. The solution was incubated at room temperature with shaking for 20 min, and then subjected to the time-resolved luminescence measurement by using Perkin Elmer Victor 1420 multilabel counter. A control experiment in the absence of oligosaccharide was carried out with the same method.

3. Results and discussion

COS and AOS possess various biological functions maybe due to the difference in substituted groups and binding patterns of monosaccharide. The kinetic process of COS and AOS binding to tobacco epidermis were investigated by using COS–BHHCT–Eu^{3+} conjugate and AOS–BHHCT–Eu^{3+} conjugate with the fluorescence imaging method and the time-gated fluorescence imaging method, respectively. As shown in Fig. 2, it is very difficult to identify the specific fluorescence signal from the steady-state fluorescence images (Fig. 2A, C, E, and G) due to the presence of strong autofluorescence from the tobacco tissues. After time-resolved mode was used, the images (Fig. 2B, D, F, and H) showed only the specific Eu^{3+} fluorescence signals from the tobacco leaf cells (epidermal and stomatal guard cells) and the short-lived autofluorescence from the sample was completely eliminated. These clear time-resolved fluorescence images demonstrated that AOS is bound to epidermal cells at ~90 min reaction (Fig. 2F), and both epidermal cells and stomatal guard cells are bound by AOS at ~180 min reaction (Fig. 2H). Compared with AOS, COS shows faster binding with the cells. It was bound to epidermal cells and stomatal guard cells at ~30 and ~60 min reactions, respectively (Fig. 2B and D).

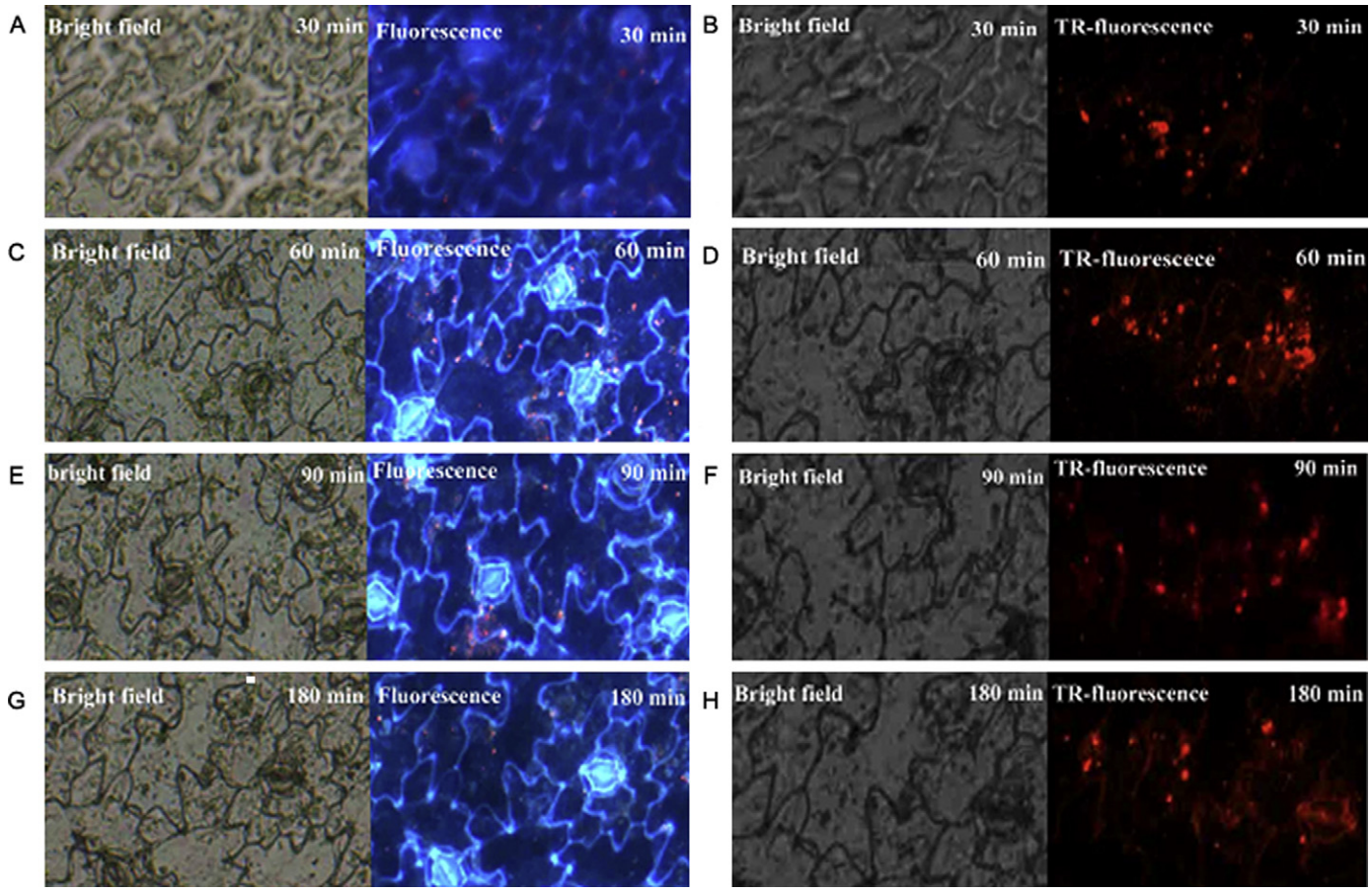

Fig. 2. Bright-field (left) and fluorescence (right, A, C, E, and F: steady-state mode; B, D, F, and H: time-resolved mode) images of the BHHCT–Eu^{3+} labeled oligosaccharides binding to tobacco epidermises at different reaction times. (A–D) The epidermises were reacted with the COS–BHHCT–Eu^{3+} conjugate for 30 and 60 min, respectively; (E–H) the epidermises were reacted with the AOS–BHHCT–Eu^{3+} conjugate for 90 and 180 min, respectively.

The different binding patterns of tobacco cells with AOS and COS could be caused by the differences of the oligosaccharides' structures, negatively charged AOS and positively charged COS. It is known that the plant cell surface is negatively charged with zeta potentials of −60 to −160 mV; thus, positively charged COS is easier to be bound to the negatively charged plant cell surface to form polyelectrolyte complexes than that of AOS (Mølhøj, Verma, & Reiter, 2004). The relatively slower binding rate of the oligosaccharides to stomatal guard cells than that to epidermal cells maybe is caused by the different structures of stomatal guard cells and epidermal cells. The stomatal guard cell has a thicker cell wall than that of the epidermal cell enabling the guard cell to be more stable in the processes of differential expansion and shape change in the stomatal movement (Appleby & Devies, 1983), which inhibits the combination of the cell with oligosaccharide molecules.

In a previous work, we have demonstrated that COS could effectively induce tobacco cells to generate IAA, an important native auxin beneficial for plant growth and development (Ding et al., 2008). By using the established method, the effects of COS and AOS on IAA concentration in tobacco cell solution were investigated. After the tobacco cells were treated by different concentrations of COS or AOS for 8 h, respectively, the IAA concentrations are shown in Fig. 3. It is apparent that the IAA generation in tobacco cell remarkably depend on the COS or AOS concentrations. The highest IAA concentration was obtained when the COS and AOS concentrations are 0.1 mg/L and 1.0 mg/L, respectively. The result perfectly matches the result in a planting experiment, in which 1.0 mg/L of AOS and 0.01 mg/L of COS have been demonstrated to be the best concentration to promote the growth of tobacco plants in comparison with the other concentrations.

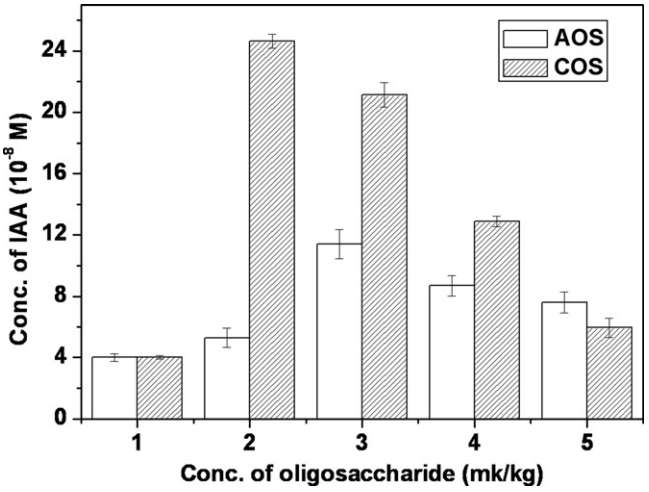

Fig. 3. Concentrations of IAA in tobacco cells treated by different concentrations of AOS (A) and COS (B) for 8 h.

Then the concentration changes of IAA in the tobacco suspension-cultured cell solutions at different treatment times in the presence of the oligosaccharides (1.0 mg/L of AOS or 0.01 mg/L for COS) were measured. As shown in Fig. 4, the IAA concentration

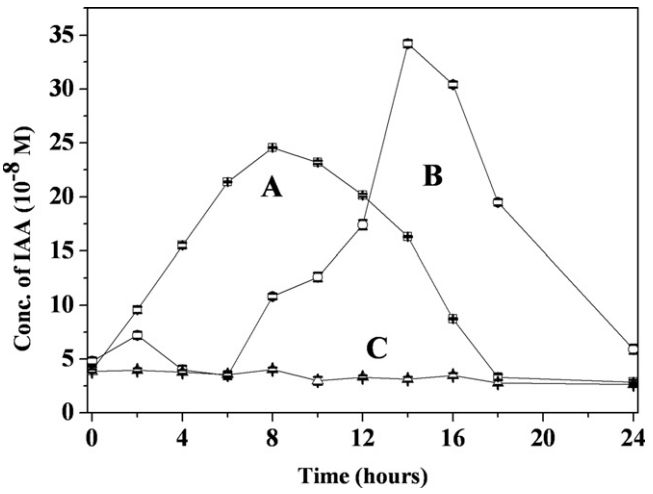

Fig. 4. The concentration changes of IAA in tobacco cells in the presence of COS (A, 0.01 mg/L) or AOS (B, 1.0 mg/L) at different treatment times. (C) Concentration in untreated tobacco cells.

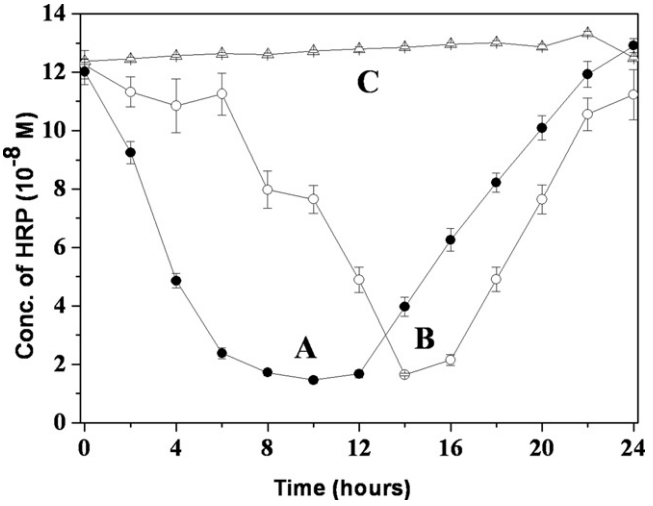

Fig. 5. The concentration changes of IAA-related peroxidase in tobacco cells in the presence of COS (A, 0.01 mg/L) or AOS (B, 1.0 mg/L) at different treatment times. (C) Concentration in untreated tobacco cells.

in the COS treated tobacco cell solution increased up to the maximum value of ~0.24 μM at 8 h and gradually returns to original level from 8 to 18 h. Unlike COS, the IAA concentration in the AOS treated tobacco cell solution increased significantly from 6 h treatment, gave the maximum value of ~0.35 μM at 14 h. After 14 h, the IAA concentration decreased gradually and returned to original concentration. These results indicate that AOS requires longer inducement time for the IAA generation, which is in agreement with the result that AOS–tobacco cell binding is remarkably slower than that of COS–tobacco cell binding. Besides the difference of the inducement time, the IAA generation abilities of the two oligosaccharides are also very different. Although the concentration of AOS was 100-fold higher than COS, the maximum concentrations of IAA in the tobacco suspension cells induced by the two oligosaccharides were at the same level. These results clearly indicate that the bioactivity of COS is remarkably higher than that of AOS, and suggest that COS could be more useful for agricultural and biomedical applications.

To investigate the effects of two oligosaccharides on the regulation of IAA generation in tobacco cells, the concentration changes of IAA-related peroxidase were also measured using the established method (Guo et al., 2009). The results are shown in Fig. 5. In the case of COS, the peroxidase concentration in the cells decreased dramatically with the increase of treatment time, gave the minimum value of ~1.7 nM at ~8 h, and then gradually returned to its original level. In the case of AOS, the peroxidase concentration in the cells decreased significantly after ~6 h treatment, gave the minimum value of ~1.7 nM at 14 h, and then gradually returned to its original level. This phenomenon is corresponding with the results of the oligosaccharide-induced IAA generation, and can be explained as follows. When the IAA concentration in tobacco cells was increased by the inducement of oligosaccharide, the tobacco cells could adjust the IAA concentration to return to the normal level by a series of physiological and biochemical processes, such as the change of the IAA-related peroxidase concentration, facilitating the degradation of the IAA in the cells.

4. Conclusion

In this work, the interactions of COS and AOS with tobacco leaf cells were investigated by time-resolved fluorometric method. The comparison study on the interactions of tobacco leaf cells with the two oligosaccharides revealed the relations of the oligosaccharide structures and their bioactivities. It is believed that our findings may contribute to a better understanding not only about the functions of oligosaccharides in biological systems, but also the potential applications of oligosaccharides in agricultural fields.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Nos. 20835001, 31100203), and Hi-tech Research and Development Program of China (No. 2011AA090704).

References

Albersheim, P., & Darvill, A. G. (1985). Oligosaccharins. *Scientific American*, *253*, 58–64.

Appleby, R. F., & Devies, W. J. (1983). The structure and orientation of guard cells in plants showing stomatal responses to changing vapour pressure difference. *Annals Botany (London)*, *52*, 459–468.

Bünzli, J. C. G. (2010). Lanthanide luminescence for biomedical analyses and imaging. *Chemical Reviews*, *110*, 2729–2755.

Cote, F., & Hahn, M. G. (1994). Oligosaccharins – Structures and signal-transduction. *Plant Molecular Biology*, *26*(5), 1379–1411.

Dai-Hung, N., Qian, Z. J., Thanh-Sang, V., Ryu, B., Dai-Nghiep, N., & Kim, S.-K. (2011). Antioxidant activity of gallate-chitooligosaccharides in mouse macrophage *RAW264.7* cells. *Carbohydrate Polymers*, *84*(4), 1282–1288.

Ding, X., Cao, Y., Huang, L., Zhao, J., Xu, C., Li, X., et al. (2008). Activation of the indole-3-acetic acid-amido synthetase GH3–8 suppresses expansin expression and promotes salicylate- and jasmonate-independent basal immunity in rice. *Plant Cell*, *20*, 228–240.

Guo, W., Ye, Z., Wang, G., Zhao, X., Yuan, J., & Du, Y. (2009). Measurement of oligochitosan–tobacco cell interaction by fluorometric method using europium complexes as fluorescence probes. *Talanta*, *78*, 977–982.

Guo, W., Zhao, X., & Du, Y. (2008). Effects of oligochitosan on the seedling growth and photosynthetic characters of tobacco. *Plant Physiology Communications*, *44*(6), 1155–1157.

Huang, R. H., Mendis, E., Rajapakse, N., & Kim, S. K. (2006). Strong electronic charge as an important factor for anticancer activity of chitooligosaccharides (COS). *Life Sciences*, *78*, 2399–2408.

Kim, S. K., & Rajapakse, N. (2005). Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate Polymers*, *62*, 357–368.

Mengibar, M., Ganan, M., Miralles, B., Carrascosa, A. V., Martinez-Rodriguez, A. J., Peter, M. G., et al. (2011). Antibacterial activity of products of depolymerization of chitosans with lysozyme and chitosanase against *Campylobacter jejuni*. *Carbohydrate Polymers*, *84*(2), 844–848.

Mølhøj, M., Verma, R., & Reiter, W. (2004). The biosynthesis of D-galacturonate in plants. Functional cloning and characterization of a membrane-anchored UDP-D-glucuronate 4-epimerase from *Arabidopsis*. *Plant Physiology*, *135*, 1221–1230.

Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate Polymers*, *87*(2), 995–1012.

Xie, W., Xu, P., & Liu, Q. (2001). Antioxidant activity of water-soluble chitosan derivatives. *Bioorganic Medicinal Chemistry Letters*, *11*(13), 1699–1701.

Yin, H., Zhao, X., & Du, Y. (2010). Oligochitosan: A plant diseases vaccine: A review. *Carbohydrate Polymers*, *82*(1), 1–8.

Zhang, Y., Zhang, G., Liu, L., Zhao, K., Wu, L., Hu, C., et al. (2011). The role of calcium in regulating alginate-derived oligosaccharides in nitrogen metabolism of *Brassica campestris* L. var. *utilis* Tsen et Lee. *Plant Growth Regulation*, *64*(2), 193–202.