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Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review

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Abstract

Many researchers have focused chitosan as a source of potential bioactive material during past few decades. However, chitosan has several drawbacks to be utilized in biological applications, including poor solubility under physiological conditions. Therefore, a new interest has recently been emerged on partially hydrolyzed chitosan, chitosan oligosaccharides (COS). During the resent past, several technological approaches have been taken to prepare COS and, enzymatic preparation methods captured a great interest due to safe and non-toxic concerns. With time, new improvements were introduced to enzymatic production and presently it has been developed to a continuous production process. Many of the biological activities reported for COS, such as antimicrobial, anticancer, antioxidant, and immunostimulant effects are depend on their physico-chemical properties. In this review, we have summarized different enzymatic preparation methods of COS and some of their reported biological activities.

Keywords: Chitosan oligosaccharides (COS); Low molecular weight chitosan (LMWC); Bioactivity; Enzymatic hydrolysis; Dual reactor

1. Introduction

Since the discovery of chitosan in late 1850s, many researches were performed to find out its fundamental physico-chemical properties. Even though researches on chitosan progressed slowly in early period, a renewed interest encouraged around 1970s to explore their novel applications. Chitosan and its derivatives have shown various functional properties and made them possible to be used in many fields including, food (Shahidi & Synowiecki, 1991), cosmetics (Majeti & Kumar, 2000) biomedicine (Felt, Buri, & Gurny, 1998), agriculture (Yamada, Shibbuya, Komada, & Akatsuka, 1993), environmental protection (Peniche-covas, Alwarez, & Arguelles-Monal, 1987) and wastewater management (Jeuniaux, 1986). Further, biodegradable, non-toxic and non-allergenic natures of chitosan especially encourage its potential use as a bioactive material (Kurita, 1998). Even though chitosan is known to have important functional activities, poor solubility makes them difficult to use in food and biomedicinal applications. Unlike chitosan, its hydrolyzed products and chitosan oligosaccharides (COS) are readily soluble in water due to their shorter chain lengths and free amino groups in D-glucosamine units (Jeon, Shahidi, & Kim, 2000). The low viscosity and greater solubility of COS at neutral pH have attracted the interest of many researchers to utilize chitosan in its oligosaccharide form. Especially, researches on COS in food and nutrition arenas have emphasized their ability to improve food quality and human health progression. Recent advances have insighted into the health benefits of COS including lowering of blood cholesterol (Macchi, 1996), lowering of high blood pressure (Giustina & Ventura, 1995), protective effects against infections (Tokoro, 1989), controlling arthritis (Lee, Suh, Kim, Lee, Song and Lee, 2003) and enhancing antitumor properties (Nishimura, Nishimura, Nishi, Saiki, Tokura and Azuma, 1984).

Chemical and enzymatic methods are the widely used COS production approaches and among them chemical hydrolysis is used more commonly in the industrial-scale

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production. However, chemical hydrolysis has some drawbacks to be commercialized, due to development of some toxic compounds, higher risk associated with the environmental pollution, and lower production yields. Therefore, lack of proper technology for the large-scale manufacturing of COS with desired molecular weights made it difficult for human use in the past years. The enzymatic processes are generally carried out in batch reactors and are preferable over chemical methods. This is due to minimized adverse chemical modifications of products during enzymatic hydrolysis and promotion of their biological activities. However, the higher cost associated with hydrolytic enzymes demote the application of enzymatic methods. To reduce this production cost, reuse of hydrolytic enzymes is recommended, instead of a single use in batch reactors. This makes possible to develop a continuous and sequential production of COS using enzymatic hydrolysis and it is recommended as one of the promising methods to produce safe and bioactive COS.

2. Physico-chemical properties of chitin, chitosan and COS

Chitin, the starting material of chitosan, is a white, hard and inelastic structural polysaccharide found in cell walls of fungi and in exoskeletons of crustaceans. The molecular structure of chitin is identified as a high-molecular weight linear polymer of *N*-acetyl-D-glucosamine units (GlcNAc) linked by β-1,4 bonds. The hydrophobic nature of chitin has made it insoluble in water as well as in most organic solvents. In contrast, chitosan, the *N*-deacetylated form of chitin is readily soluble in dilute organic acids at low pH (Peniston & Johnson, 1980). The most important parameter that determines the solubility of chitosan is the degree of deacetylation (DD). Conversion of chitin into chitosan increases DD, and thereby alters the charge distribution of chitosan molecules. In general, degree of acetylation (DA) of chitin is about 90% and following partial or fully deacetylation with alkaline treatment, it is converted into chitosan. In addition to the DD, degree of polymerization (DP) also contributes to the alteration of physico-chemical properties of chitosan. Moreover, COS (relatively lower DP) are better soluble than low molecular weight chitosans (LMWC) with relatively higher DP. However, there is no specific DP to distinguish COS and LMWC. Generally, molecular weight of COS can be considered up to 10 kDa or less, and during preparation of different molecular weight chitosans, viscosity is used as a parameter to determine the molecular weight.

Unlike most polysaccharides, chitosan and COS have positive charges resulted following removal of acetyl units from D-glucosamine residues. This chemical feature allows chitosan and COS to bind strongly to negatively charged surfaces and responsible for many of observed biological activities. In addition to that, non-toxicity, biodegradability and biocompatibility of chitosan and COS promote their biological applications compared to other synthetic polymers (Kurita, 1998).

3. COS preparation methods

Similar to all polysaccharides, chitosan can also be cleaved by hydrolyzing agents due to the presence of rather unstable glycosidic bonds. Degradation of *O*-glycosidic linkages of chitosan by different methods leads production of COS varying in the DP as well as number and sequence of glucosamine (GlcN) and GlcNAc units. Some of these methods include, acid hydrolysis (Il'ina & Varlamov, 2004), enzymatic hydrolysis (Kuroiwa, Ichikawa, Sato, Hiruta, Sato and Mukataka, 2002; Zhang, Du, Yu, Mitsutomi, & Aiba, 1999), oxidative degradation (Shirui, Xintao, Florian, Michael, Dianzhou and Thomas, 2004), ultra sonic degradation (Chen & Chen, 2000), chemo-enzymatic (Akiyama, Kawazu, & Kobayashi, 1995) and recombinant approaches (Samain, Drouillard, Heyraud, Driguez, & Geremia, 1997). Moreover, electromagnetic radiation, sonication and mechanical energy generated by microfiltration can also be used for the production of COS without employing chemical agents (Hai, Diep, Nagasawa, Yoshii, & Kume, 2003). Absorption of energy by chitosan molecules in any of above methods results in scission of chemical bonds, and if the broken bond belongs to the backbone of the polymer (*O*-glycosidic bond), a decreased molecular weight will be resulted.

Even though, chitin and chitosan can be isolated from different sources (Muzarelli, Ilari, Tarsi, Dubini, & Xia, 1994), crab and shrimp shell wastes are currently utilized as the major industrial source of biomass for the large-scale production of COS. These crustacean shell wastes are composed of protein, inorganic salts, chitin and lipids as main structural components. Therefore, extraction of chitin and chitosan (the starting materials of COS) is mainly employed stepwise chemical methods. In the first step, shrimp or crab shells are treated with 3–5% aqueous NaOH solution to remove proteins attached to the shells and thereby prevent the contamination of chitin products with proteins. Deproteinized shells are then neutralized and calcium is removed by treating with 3–5% aqueous HCl solution to afford a white or slightly pink precipitate of chitin. Then the chitin is *N*-deacetylated with 40–45% NaOH to form chitosan with a cationic nature. The resulting crude sample is dissolved in 2% acetic acid and the supernatant is neutralized with aqueous NaOH solution to afford purified chitosan as a white precipitate (Hirano, 1996). After alkaline deacetylation, some of the amino groups may remain acetylated and distribute randomly along the whole polymer chain.

For large-scale production of COS, acid hydrolysis is commonly used to cleave glycosidic linkages of chitosan. However, chemical hydrolysis results low yields of COS

and a larger amount of monomeric D-glucosamine units (Uchida, Izume, & Ohtakara, 1989). Therefore, COS prepared by industrial-scale acid hydrolytic methods are generally not considered to serve as bioactive materials due to the possibility of contamination of toxic chemical compounds. As a result, enzymatic hydrolysis of chitosan has been proposed as a preferred method for the production of bioactive COS during past few decades.

4. Enzymatic preparation of COS

Chitosan is generally susceptible to a number of different enzymes and that indicates its broad substrate specificity (Aiba, 1994a,b). Up-to-date a range of chitosanolytic enzymes (chitosanase) has been reported from different of microorganisms including fungi (Kim, Shon, & Lee, 1998; Muzarelli et al., 1994) and bacteria (Lee, Choi, Han, Park, Lee and Yi, 1996; Varum, Holme, Izume, Stokke, & Smidsrod, 1996). In addition, some other common carbohydrases and proteases also have proven their hydrolytic ability on chitosan to produce COS with various molecular weights (Aiba, 1994a; Zhang et al., 1999). Further, it has been revealed that the structure of the glycosidic bonds in chitosan affects enzymatic hydrolysis process. Differentially deacetylated chitosans have four different types of randomly distributed glycosidic bonds in their structures. These include linkages between two *N*-acetylated units (A–A), acetylated and deacetylated units (A–D), deacetylated and acetylated units (D–A) and two deacetylated units (D–D). The specificity of chitosanases with respect to the cleavage of four different glycosidic linkages in partially *N*-deacetylated chitosan is determined by the identity of the reducing and non-reducing ends and DD of chitosan. For examples, egg white lysozyme is found to be almost exclusive towards the cleavage of glycosidic linkage between two acetylated units, while *Bacillus* chitosanase is found to be highly specific towards the deacetylated glycosidic linkages (Varum et al., 1996). In addition, chitinase could act on partially *N*-acetylated chitosan by recognizing GlcNAc residues in the chitosan sequence (Aiba, 1994b). However, a clear distinction between chitosanase and chitinase for the hydrolysis of differentially deacetylated chitosans cannot be identified. Moreover, chitosanases from different organisms also differ in their catalytic action and that is mainly dependent on DD of chitosan (Kurita, 1998). However, it has been generally observed that chitosanases obtained from microbes produce relatively a higher yield of COS compared to chitosanases from other sources.

Even though, microbial chitosanases have shown to have excellent performances in COS production, they are too expensive to be utilized in large-scale industrial applications. Therefore, other commercial enzymes are utilized under specific conditions to produce COS with relatively a low cost (Zhang et al., 1999).

4.1. Batch reactors

During the early period of enzymatic production of COS, hydrolysis was carried out in batch reactors (Fig. 1), where chitosanase was mixed with its substrate, and allowed to breakdown glycosidic bonds (Izume & Ohtakara, 1987). Jeon and Kim (2000a) reported that optimum conditions for hydrolyzing 1% chitosan (DD, 89%) using chitosanase from *Bacillus pumilus* BN-262 are 45 °C, 5.5 pH and for 1 h. While, Varum et al. (1996) hydrolyzed 65% deacetylated citosan for 5 h under 5.5 pH and at 37 °C using chitosanase obtained from *Bacillus* sp. No. 7-M. However, the main disadvantage of the batch reactor system is the higher cost associated with large quantities of expensive enzymes that cannot be reused. Also in these systems lower yields are generally resulted due to the limited ability to control DP and hence the final product is obtained as a mixture of COS with broader molecular weight range. Therefore, many researches have been performed to develop new strategies to produce COS with desired molecular weights and make these systems economical.

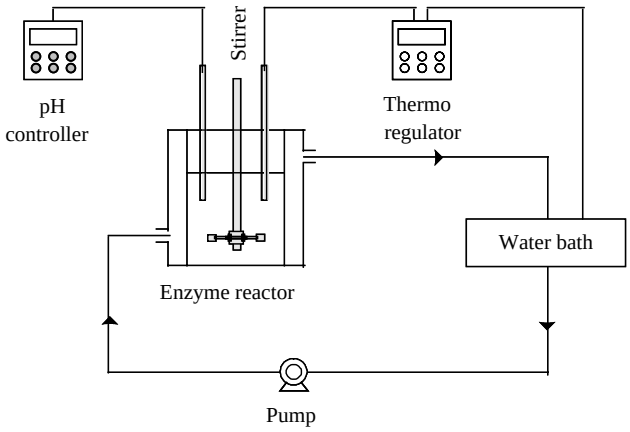

Fig. 1. Typical schematic diagram of a batch reactor for the hydrolysis of chitosan, using free enzyme.

4.2. Column reactors immobilized with enzyme

In general, immobilization of enzymes offers a number of technological advantages including reusability of enzymes, continuous operation, rapid termination of reactions and controlled product formation. A new method to produce COS with higher DP was introduced by Jeon, Park, Byun, Song, and Kim (1998) using an immobilized chitosanase. In this system, the immobilization was tested with different carriers and the chitosanase immobilized on chitin exhibited the highest enzymatic activity. In addition, few investigators have studied the effects of various conditions on immobilization of chitosanases and yields of target COS and their intermediates (Kuroiwa et al., 2002; Kuroiwa, Ichikawa, Sato, & Mukataka, 2003). Kuroiwa et al. (2002) studied the reaction conditions for effective production of pentamers

and hexamers and observed that it is greatly dependent on surface enzyme density, support particle size, temperature, aggregation speed, and initial substrate concentration. However, as observed in many related studies, immobilization of enzymes could also not provide promising results as expected. The main limitation associated with this method was poor affinity of immobilized enzyme to chitosan substrate than that of free enzyme, resulting in inefficient production of desirable chain lengths of COS. Therefore, these methods did not capture much interest in large-scale production systems.

4.3. Ultrafiltration (UF) membrane reactors

Following several attempts, an enzyme reactor system along with an ultrafiltration (UF) membrane reactor was developed to produce COS with relatively a higher DP (Jeon & Kim, 2000a). This system could hydrolyze substrate that equivalent to 11 batches used in the batch reactor with the same amount of enzyme, and enabled effective production of relatively large COS at a low cost. The most important factor in the usage of an UF reactor system was the control of permeation rate that determines molecular size of COS. In a related study, Perea and Ugalde (1996) produced whey peptides using a membrane reactor and identified that this method was suitable for the production of peptides with specific functional properties. However, UF membrane method did not allow continuous production of COS due to the increased transmembrane pressure during the reaction. This was due to high viscosity of chitosan solution and fouling of membrane by accumulated substrate. Therefore, reduction of viscosity of chitosan prior to treatment in the UF membrane system was a requisite for a more effective continuous production system.

4.4. Continuous production of COS by dual reactor system

Continuous production of COS was feasible with combination of a column reactor packed with immobilized enzyme and the UF membrane reactor. The new system was named as dual reactor system (Jeon & Kim, 2000b) and is shown in Fig. 2. In the first step, chitosan is partially hydrolyzed by the immobilized enzyme prepacked in the column reactor and the product is supplied to the UF membrane system for the production of COS. Partially hydrolyzed chitosan possess low viscosity and does not create fouling problems under controlled conditions. This method ensures a greater productivity per unit enzyme, ability to control molecular weight distribution and more efficient continuous production process compared to those of conventional methods.

In recent years, many researchers have attempted to improve enzymatic methods to produce bioactive COS. As a result, few of these methods are currently practiced to obtain continuous production of COS with desired molecular sizes. In some methods, complex enzyme systems are incorporated to produce COS within preferred molecular weight range (Zhang et al., 1999). Kuroiwa et al. (2003) have determined the optimum conditions for continuous production of pentamers and hexamers of COS using a packed-bed enzyme reactor. Under the optimum conditions, continuous production of pentamers and hexamers was achieved for a month without significant decrease in products.

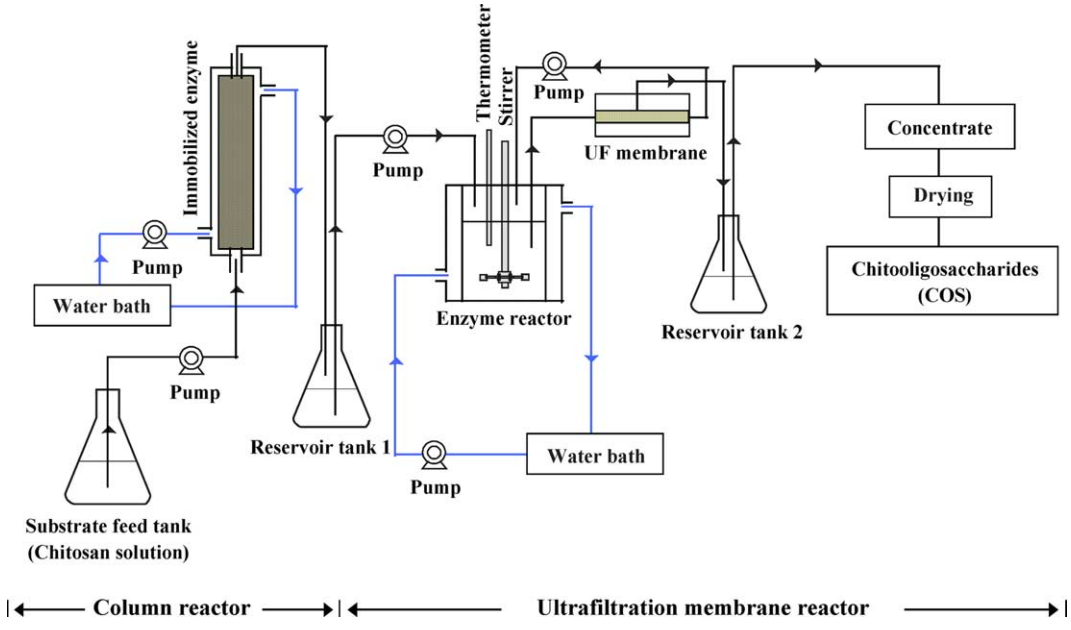

Fig. 2. Schematic diagram of the dual reactor system developed for continuous production of chitosan oligosaccharides (COS). Adapted from Jeon and Kim (2000b).

5. Biological activities of COS

COS are known to possess many biological activities such as antifungal activity (Hirano & Nagao, 1989), antibacterial activity (Jeon & Kim, 2000a; Jeon & Kim, 2001; Jeon, Park, & Kim, 2001), antitumor activity (Jeon & Kim, 2002; Nam, Shon, Kim, Kim, & Nam, 1999), immunoenhancing effects (Suzuki, Mikami, Okawa, Tokoro, Suzuki and Suzuki, 1986), and protective effects against infection (Jeon et al., 2000). Properties of COS, such as DP, DA, charge distribution and nature of chemical modification to the molecule strongly influence its observed biological activities (Muzzarelli, 1996). Unlike high molecular weight chitosan, COS are easily absorbed through the intestine, quickly get into the blood flow and have a systemic biological effects in the organism. In food industry, COS attract a greater interest as antimicrobial agents, antioxidants and enhancers of nutritional quality of food (Shahidi, Vidana Arachchi, & Jeon, 1999). Therefore, molecular weight is considered as a principal characteristic of COS that highly correlates to their biological activities. In this section, we will discuss a number of recently reported biological activities of COS and few LMWC prepared by enzymatic means.

5.1. Antimicrobial activity

Antimicrobial activity of chitosan and its derivatives against several bacterial species has been recognized and is considered as one of the most important properties linked directly to their possible biological applications. The antibacterial activity of these compounds is influenced by a number of factors such as degree of polymerization (Park, Je, Byun, Moon, & Kim, 2004; Park, Kim, & Lee, 2002; Yun, Kim, & Lee, 1999), level of deacetylation (Chung, Su, Chen, Jia, Wang and Wu, 2004; Tsai, Su, Chen, & Pan, 2002), type of microorganism (Gerasimenko, Avdienko, Bannikova, Zueva, & Varlamov, 2004; Park, Lee, & Kim, 2004; Uchida et al., 1989) and some other physico-chemical properties (Table 1).

5.1.1. Antibacterial mechanisms of COS

Unlike chitin, chitosan and COS possess primary amino groups in their structures. The number of these amino groups has proven to play a major role in antibacterial activity and several mechanisms have been proposed to describe this activity (Chen, Chung, Wang, Chen, & Li, 2002). The mostly accepted mechanism explains that COS can alter permeability characteristics of microbial cell membrane and further prevent the entry of materials or cause leakage of cell constituents that finally leads to death of bacteria (Sudharshan, Hoover, & Knorr, 1992). To study this mechanism, Choi, Kim, Yoo, Oh, Choi and Kim (2001) carried out a study assessing the antibacterial activity of a mixture of COS and LMWC (MW 2∼30 kDa and DD 91.5%) against clinically important bacterial pathogen, *Actinobacillus actinomycetemcomitans*. SEM and TEM images of those treated bacterial cells exhibited a change of their morphology from spherical shape to irregular condensed masses with bleb-like structures, indicating a separation of cytoplasmic membrane from cell wall and coagulation of cytosolic components. The authors suggest that, the site of COS action is probably the bacterial envelope and killing of the organism could be due to membrane disruption. Another suggested mechanism for antibacterial activity of COS is the blockade of RNA transcription by adsorption of penetrated chitosans to bacterial DNA (Kim, Lee, Lee, & Park, 2003). To furnish this mechanism, the molecular weight of COS must be less than a critical value, which enable penetration of molecules into bacterial cells. However, adequate evidences have not gathered up-to-date to strengthen this propose. In addition,

Table 1
Minimum inhibitory concentrations of different COS and LMWC against various bacterial strains

Bacterial strain	DD (%)	MW (kDa)	MIC (%)[a]	Ref.
Gram-negative bacteria				
Escherichia coli	85	12	0.1	Gerasimenko et al. (2004)
Escherichia coli	85	6	0.06	Gerasimenko et al. (2004)
Escherichia coli O-157	90	5–10	0.12	Jeon et al. (2001)
Vibrio parahaemolyticus	75	1–10	0.4	Park et al. (2004b)
Salmonella typhimurium	75–90	1–10	0.125	Park et al. (2004a)
Pseudomonas aeruginosa	50–90	5–10	0.25	Park et al. (2004a)
Gram-positive bacteria				
Micrococcus luteus	90	5–10	0.031	Jeon et al. (2001)
Streptococcus mutans	90	5–10	0.008	Jeon et al. (2001)
Streptococcus faecalis	90	5–10	0.03	Jeon et al. (2001)
Staphylococcus epidermis	75–90	5–10	0.063	Park et al. (2004a)
Staphylococcus aureus	50–90	1–10	0.125	Park et al. (2004a)
Bacillus subtilis	75–90	5–10	0.125	Park et al. (2004a)
Bacillius cereus	75–90	1–10	0.125	Park et al. (2004a)
Lactobacillus plantarum	85	12	0.06	Gerasimenko et al. (2004)
Bifidobacterium bifidum	85	12	0.0005	Gerasimenko et al. (2004)

[a] Minimum inhibitory concentration (MIC) is defined as the lowest concentration of COS at which the bacterial growth is completely inhibited.

deprivation of metals, trace elements or essential nutrients by chelating action of COS has also been proposed as a factor that limits the growth of bacteria.

5.1.2. Effect of positive charge and molecular weight of COS on antibacterial activity

In general, positively charged nature of chitosan and COS molecules facilitates their binding with bacterial cell wall and leads to the inhibition of bacterial cell growth. This is because positively charged amino group at C-2 position of the glucosamine monomer interacts with negatively charged carboxylic acid group of the macromolecules of bacterial cell surface and form polyelectrolyte complexes (Choi et al., 2001; Kim et al., 2003). This could act as impermeable layer around the cell and suppress the metabolic activity of the bacteria by blocking of nutrient permeation through the cell wall. With respect to formation of polyelectrolyte complexes, it is possible that the higher number of primary amino groups present in COS can make stronger interactions with bacterial cells (Tsai et al., 2002). The number of primary amino groups is dependent on DD and DP of COS and it has been observed that the death rate of bacterial cells tends to increase upon the increase in DD of COS (Tsai et al., 2002). In most cases, 85–95% deacetylation of COS has shown to be responsible for perform the highest antibacterial activity (Chung et al., 2004). Also there is a general tendency to increase the antibacterial activity of COS with the increase of molecular weight (Park, et al., 2004). Uchida et al. (1989) have reported that the chitosan hydrolysate, which was obtained by partial hydrolysis of chitosan with chitosanase, was more effective as antibacterial agent than highly hydrolyzed chitosan materials. In another study, Ueno, Yamaguchi, Sakairi, Nishi, and Tokura (1997) have reported that COS with an average molecular weight less than 2.2 kDa, was not capable of suppressing the microbial growth, but COS with molecular weight around 5.5 kDa suppressed the growth dose dependently. In addition, LMWC ranging their average molecular weight from 5 to 27 kDa are generally accepted to be effective in suppressing bacterial growth (Gerasimenko et al., 2004). However, LMWC with molecular weights greater than 30 kDa are not used as antibacterial agents due to their poor solubility in aqueous solutions at neutral pH (Sekiguchi, Miura, Kaneko, Nishimura, Nishi and Iwase, 1994).

Since positive charge favors the antibacterial activity of COS, modification of amino group at C-2 position of glucosamine by positively charged potent groups was expected to enhance the antibacterial activity. And hence Jeon and Kim (2001) attempted to improve the positive charge of COS by introducing asparagine to the C-2 position of glucosamine via *N*-conjugation. Asparagine was expected to enhance the positive charge due to the presence of two functional amino groups on its backbone and side chain. The resultant COS derivatives that *N*-conjugated with asparagines (Asn-COS) were observed to possess improved bactericidal activity. The minimum inhibitory concentration (MIC) of Asn-COS on *E. coli* was much lower compared to that of COS. Asn-COS with 0.01% or more MIC completely inhibited the growth of *E. coli* after 3 days of culture period. In addition, Muraki and Aiba (1996) reported that partially *N*-lauroyl (PNL)-chitooligosaccharides (DP 7–8) with approximately 50% degree of *N*-lauroylation could exhibit strong growth inhibition against *E. coli*. Sudharshan et al. (1992) also revealed that water-soluble chitosans, such as chitosan lactate and chitosan hydroglutamate, exhibit bactericidal activity against both Gram-positive and Gram-negative bacteria. Moreover, this activity was in the range of 1—5 log cycle reduction within 1 h. Further, Kim et al. (2003) synthesized a COS derivative with quaternary ammonium functionality by coupling COS with glycidyl trimethylammonium chloride (GTMAC) and the antibacterial activity was evaluated against *Streptococcus mutans*. The COS with quaternary ammonium functionality (COS-GTMAC) markedly inhibited the growth of *S. mutans*, compared to COS, indicating antimicrobial activity resulted from the substitution of positively charges functional group. Moreover, these observations are in line with general understanding of antimicrobial activity of COS and their derivatives. Therefore, above information further strengthen the fact that structural modification of COS by introducing positively charged groups can improve the antibacterial activity ensuring better interaction with negative charged groups on bacterial cell wall.

5.1.3. Charge properties of bacterial cell wall

In addition to positively charged characteristics of COS, charge distribution of bacterial cell wall seems to play a considerable role for observed antibacterial activities of COS and their derivatives. Chung et al. (2004) studied the cell surface characteristics of a number of Gram-positive and Gram-negative bacterial species and revealed that there is a close relationship between hydrophilicity and negative charge distribution of bacterial cell surface. Cell surface negative charge distribution of Gram-negative bacteria is higher than that of Gram-positive bacteria and it leads to a higher hydrophobicity. Moreover, negative charge distribution on the cell surface also varies among Gram-negative and Gram-positive bacteria. Therefore, adsorption of COS to the cell surface is normally in the order of higher negatively charged Gram-negative bacteria to less negatively charged Gram-positive bacteria. Interestingly, this was further confirmed by the results of inhibition degree of COS against tested bacteria (Wang, 1992). This clearly explains the reason why, most Gram-negative bacteria become sensitive to COS and charge distribution on the cell surfaces is apparently a factor determining antibacterial activity.

5.1.4. Effect of COS on the inhibition of fungal growth

Polycationic nature of chitosan and COS allows them to react with negatively charged groups of fungi, thereby

exhibiting the antifungal activity on mold and yeast. The inhibition mechanism of COS against fungi is also similar to that of bacteria explained in above section. The formation of polyelectrolyte complexes between COS and negatively charged groups on the cell surface directly interferes the growth and normal physiological functions of fungi, suggesting that the charge distribution of COS has a correlation with its antifungal activity (Hirano & Nagao, 1989). Also, antifungal activity of chitosan and COS appeared to be more pronounced at lower pH and lower temperatures.

5.2. Antiviral activity of COS

Chitosan and COS are reported to suppress viral infections in various biological systems. In most cases, the mechanism of antiviral activity is not well understood. Further, different studies carried out up-to-date involving factors that are presumed to be involved in determining the antiviral activity of chitosan and COS, have resulted conflicting observations with regard to the inhibition mechanism. However, one possible explanation is that, cationic charges of amino groups of chitosan and COS may have additional functions to activate the immune and defense systems in plants and animals.

5.2.1. Inhibitory activity on plant viruses

Researches on viral infections in plants have revealed that the treatment of chitosan on leaf surfaces can decrease the number of local necroses caused by different mosaic viruses (Pospieszny, Chirkov, & Atabekov, 1991). In addition, they have also confirmed that the treatment of chitosan could suppress infections regardless of type of virus as well as plant species. Some plants with genetic resistance to particular viruses can form local lesions and that could prevent spreading of viruses. This defense mechanism is referred to as hypersensitivity response of host plants (Pospieszny & Atabekov, 1989). Decreased local lesions after treatment of chitosan suggests that it could improve the plant resistance against viral infections. Further, antiviral activity is affected by molecular structural properties of chitosan derivatives. Generally, anionic derivatives of COS result a lower antiviral activity and increased DD can improve antiviral activity indicating that positively charged groups of COS are responsible for their antiviral activity.

5.2.2. Inhibitory activity on animal viruses

Chitosan and COS are reported to stimulate immune and defense systems in animal cells. Stimulation of functional activity of macrophages with the treatment of COS helps to increase the generation of active oxygen species in mouse models and these reactive radical species lead viral destruction. Bacon, Makin, Sizer, Jabbal-Gill, Hinchcliffe and Illum (2000) showed that co-treatments of chitosan with antigen to mice could strongly increase the local and systemic immune and defense responses to influenza A and B viruses. Another possible mechanism of antiviral activity of COS can be explained in relation to interactions between protein receptors on viral coat and blood leucocytes. Gama Sosa, Fazely, Koch, Vercellotti, and Ruprecht (1991) observed that carboxymethyl and sulfated derivatives of chitosan could inhibit the replication of HIV-1 in cultured T-cells and human MT-4 lymphocytes. Further, they have suggested that this activity was due to the prevention of interactions between viral coat glycoprotein receptors and target proteins on lymphocytes.

5.2.3. Inhibitory activity on bacteriophages

COS are also effective in preventing several phage infections. Antiviral activity on phages is known to be dependent on properties of COS such as molecular weight, molecular structure, DD and also type of bacteriophage. However, it is hard to predict the level of antiviral activity of COS only based on the above factors. Kochkina and Chirkov (2000a) have shown that COS are more effective against replication of 1-97A phage in *Bacillus thuringiensis* than that of LMWC. In contrast, LMWC with higher polymerization degree has been observed to become more beneficial in inhibiting coliphage infection than that of LMWC with lower polymerization degree (Kochkina & Chirkov, 2000b). Therefore, it can be expected that COS and LMWC can be involved in the inhibition of the replication of bacteriophages by several different mechanisms. Interestingly, electron microscopic observations have revealed that COS containing lower DP could change the structure of phage particles. Furthermore, these changes make the phage inactive directly through receptor-recognizing structures on the phage particles. However, the ability of COS to prevent phage infection is not well elucidated.

5.3. Antitumor activity

Biological activities of chitosan and COS have reported that COS could inhibit the growth of tumor cells by exerting immunoenhancing effects. Results of some related studies suggest that, the observed antitumor activities were not due to direct killing of tumor cells, but might be due to increased production of lymphokines, leading to manifestation of antitumor effect through proliferation of cytolytic T-lymphocytes (Tokoro, Tatewaki, Suzuki, Mikami, Suzuki and Suzuki, 1998).

The antitumor activities observed in COS are dependent on their structural characteristics such as DD and molecular weight (Table 2). Jeon and Kim (2002) carried out a study to identify the antitumor activity of different molecular weight COS prepared by UF membrane reactor system. It could be observed that, medium molecular weight COS ranging from 1.5 to 5.5 kDa could effectively inhibit the growth of Sarcoma 180 solid (S180) or Uterine cervix carcinoma No. 14 (U14) tumor in BALB/c mice. Further, Suzuki et al. (1986) reported that 100 and 55% inhibition of S180

Table 2
Effect of COS and LMWC on the growth of different tumors in mice

COS			Tumor type	Inhibition (%)[a]	Ref.
MW (kDa)	DD (%)	Dose (mg/(kg/day))			
~1	100	10	Meth-A solid tumor	41	Tokoro et al. (1998)
~1	100	300	Sarcoma 180 solid tumor	93	Suzuki et al. (1986)
~1	100	500	MM 46 solid tumor	55	Suzuki et al. (1986)
6.5–12	90	10	Sarcoma 180 solid tumor	61.7	Jeon and Kim (2002)
1.5–5.5	90	10	Sarcoma 180 solid tumor	66.7	Jeon and Kim (2002)
1.5–5.5	90	50	Uterine cervix tumor	73.6	Jeon and Kim (2002)
1.4	85	50	Sarcoma 180 solid tumor	50.4	Qin et al. (2002)
3–10	80	200	Sarcoma 180 solid tumor	56.9	Qin et al. (2002)

[a] Inhibition of tumor growth is calculated as a percentage, by comparing the reduction in tumor weight following COS treatment with the controlled tumor weight.

and MM46 solid tumors in mice was observed following intravenous administration of chitohexaose. Several other reports suggest that these antitumor compounds exert effects on immune system to stimulate leucocytes, cytotoxic T cells and natural killer cells (Tokoro et al., 1998). Furthermore, studies on the antitumor activity of chitosan and COS revealed that partially deacetylated chitin and carboxymethyl chitin with an adequate degree of substitution were effective toward controlling various tumor cells (Nishimura, Nishi, Tokura, Nishimura, & Azuma, 1986). Unlike many other biological molecules, COS could exert their biological activities following oral administration and effects are more or less similar to those of intraperitoneal injection. Moreover, Qin, Du, Xiao, Li, and Gao (2002) have demonstrated that water soluble COS prepared with a mixture of tetramer and pentamer could inhibit the growth of S180 tumor cells in mice after oral and intraperitoneal administration. Therefore, COS and their *N*-acetylated analogues that soluble in basic physiologic environments could be considered good candidates to develop potential nutraceuticals.

5.4. Antioxidant and radical scavenging activities

Scavengers of free radicals are preventive antioxidants and presence of radical scavenging compounds can break the oxidative sequence at different levels. Therefore, there has been a growing interest to identify natural antioxidants from many natural sources to overcome the radical mediated deleterious effects in biological systems. Many biological compounds including carbohydrates, peptides and some phenolic compounds have been identified as potent radical scavengers. Recently, the antioxidant activity of chitosan and its derivatives attracted a greater attention (Chiang, Yao, & Chen, 2000). Further, it has been observed that the radical scavenging properties of COS are dependent on their DD and molecular weights. Based on the results obtained from studies carried out using electron spin trapping techniques, COS with low molecular weight range (1 ~ 3 kDa) have been identified to have a higher potential to scavenge different radicals (Park, Je, & Kim, 2003a). In contrast, another study reported that LMWC could exhibit more than 80% of superoxide radical scavenging activity at 0.5 mg/ml concentration (Yin, Lin, Zhang, & Yang, 2002). In addition, highly deacetylated (90%) COS are more preferable to scavenge DPPH, hydroxyl, superoxide and carbon-centered radicals (Je, Park, & Kim, 2004). Even though the precise mechanism of radical scavenging activity of COS is not clear, it is attributed that amino and hydroxyl groups attached to C-2, C-3 and C-6 positions of the pyranose ring react with unstable free radicals to form stable macromolecule radicals.

However, there are some discrepancies about hydroxyl radical scavenging activities of COS and some of their derivatives (Xie, Xu, & Qing, 2001). The latest studies of Huang, Mendis and Kim (2005) revealed that, metal ion uptake ability of COS has a great influence on their hydroxyl radical scavenging ability. According to their results, hydroxyl radical scavenging potency of COS is partly due to chelating ability of transition Fe^{2+}, molecular charge properties and proton donation via hydroxyl and amino groups. The uptake of metal ions by COS can be proceeded through different mechanisms including, chelation via lone electron pairs of amino groups and ion exchange mechanisms of protonated amino groups (Guzman, Saucedo, Revilla, Navarro, & Guibal, 2003). Therefore, above information further strengthen the effect of DD on radical scavenging which is directly correlated with protonation of amine groups. Hoverer, there is no much information about the relationship between ion chelation and antioxidant properties of COS up-to-date.

5.5. Fat lowering and hypocholesteromic effects of COS

In early studies on properties of chitosan, several investigators demonstrated that chitosan is capable of binding dietary fats and it can prevent their absorption through the gut (Kanauchi, Deuchi, Imasato, Shizukuishi, & Kobayashi, 1995). Several other related studies also have shown that chitosan has a greater ability to absorb fat from the body. Those studies have further revealed that chitosan could absorb fat up to five times of its weight. Furthermore,

it was also shown to lower the level of LDL cholesterol while boosting the HDL cholesterol level. Some studies have shown that chitosan can decrease blood cholesterol levels up to more than 50% and called it as an effective hypocholesterolemic agent (Jameela, Misra, & Jayakrishnan, 1994; Maezake, Tsuji, & Nakagawa, 1993). Further, in a preliminary human study, intake of 3–6 g of chitosan in a day for 2 weeks could drop the blood cholesterol level by 6% and increase HDL level by 10% (Maezake et al., 1993). In addition to chtosan, COS applications also lead to control of blood cholesterol level. Especially, COS are capable of decreasing cholesterol level in the liver. Unlike high molecular weight chitosan, COS application does not lead to enhancement compensatory cholesterol synthesis, decrease in essential fatty acids, fat soluble vitamins and microelements form the organisms (Muzzarelli, 1997). Especially, COS prevent development of fatty liver caused by the action of hepatotrope poisons.

Despite few researches carried out to search for the ability of chitosan and COS to bind with bile salts and lipids, up-to-date their exact mechanism on lowering blood cholesterol level has not been completely elucidated. However, several hypotheses have been suggested to explain the possible actions of COS in reducing blood cholesterol levels. One hypothesis suggests that, ionic binding of COS with bile salts and bile acids may inhibit micelle formation during lipid digestion in the digestive track (Remunan-Lopez, Portero, Vila-Jato, & Alonso, 1998). Another hypothesis suggests that chitosan and its oligomers can directly trap lipids and fatty acids (Tanaka, Tanioka, Tanaka, Tanigawa, Kitamura and Minami, 1997). However, contradictory results have been observed with regard to ionic interactions of COS, and thus the fat binding and cholesterol lowering effect of chitosan cannot be explained only using ion binding hypothesis (Sugano, Watanabe, Kishi, Izume, & Ohtakara, 1988). Moreover, some other evidences from animal studies suggest that, the effective lowering of cholesterol level can be explained in relation to the ability of COS to increase the excretion of neutral sterol and indigestion of dietary fats.

Despite observations that COS involves in the reduction of blood cholesterol following ingestion in animal models, few other reports reveal that some low molecular weight COS (2000 Da) fail to absorb and excrete dietary fat in the digestive track (Ikeda, Sugano, Yoshida, Sasaki, Iwamoto and Hatano, 1993). However, Sugano, Watanabe, et al. (1988) have studied the relationship between intake of different molecular weight COS and hypocholesteromic effect in mice, and have reported that almost all chitosan preparations could prevent increase in blood cholesterol level at 5% dietary level. Further, blood cholesterol levels are decreased considerably with the administration of 2% of chitosan diet regardless of their molecular weights, suggesting that hypocholesteromic action of COS is not dependent on their molecular weights. In addition, Sugano, Yoshida, Hashimoto, Enomoto, and Hirano (1992) have reported that highly depolymerized chitosans are not effective in lowering cholesterol level.

5.6. Immunostimulant effects of COS

The non-specific immune system in animals is responsible for initiating rapid and general responses against invasion of microorganisms. Immunostimulants are generally identified as compounds that stimulate non-specific immune system by enhancing the capability of defending activity of phagocytes (macrophages and neutrophils). Most immunostimulants can bind specifically with the cell surface receptor proteins of phagocytes or lymphocytes, and it stimulates the production of immune response compounds such as interferon, interleukin and complement proteins that activate the immune system. In addition, some immunostimulants are capable of competing with specific receptor molecules in the target cells of infectious organisms. A number of carbohydrate derivatives including mannan oligosaccharides, peptidoglycan and COS have been reported to possess immunostimulting properties (Matsuo & Miyazono, 1993).

Okamoto, Inoue, Miyatake, Ogihara, Shigemasa and Minami (2003) reported that both oligomers of chitin and chitosan are effective in enhancing migratory activity of macrophages. Moreover, this activity can be explained by their chemotatic effects on macrophages. In contrast, chitin and chitosan are reported to reduce the migration of macrophages and it suggested that chitin and chitosan might absorb some substances in culture medium that are involved in migration of macrophages (Okamoto, Kawakami, Miyatake, Morimoto, Shigemasa and Minami, 2002). Further, water-soluble chitosan can activate the production of cytokines such as interleukin-1 (IL-1β), tumor necrosis factor-alpha (TNF-α), and reactive oxygen intermediates to promote the defense system against microbial infections (Feng, Zhao, & Yu, 2004; Shibata, Metzger, & Myrvik, 1997).

5.7. Other biological activities of COS

Angiotensin I converting enzyme (ACE) plays an important role in regulating blood pressure in mammals. Researches on chitosan and its oligomers have identified their potential to inhibit ACE activity. Even though enough studies have not been carried out, it is presumed that COS may have desirable properties to inhibit ACE and also this activity is dependent on DD (Park, Je, & Kim, 2003b). Further, Hong, Kim, Oh, Han, and Kim (1998) studied ACE inhibitory activity of different COS and identified that chitosan trimer is more effective in lowering blood pressure compared to other oligomers.

Also chitosan and sulfated COS reports to possess anticoagulant activity tested in vitro (Park, Je, Jung, & Kim, 2004). Binding or agglutination of red blood cells in

the presence of chitosan is dependent on its molecular size and other physical characters. The ionic attraction between negatively charged red blood cell membranes and positively charged groups in chitosan is a possible explanation for the anticoagulant activity of chitosan. Therefore, positively charged chitosan is more effective than chitin as a blood coagulant (Klokkevoid, Fukuyama, Sung, & Bertolami, 1999; Rao & Sharma, 1997). However, according to Okamoto, yano, Miyatake, Tomohiro, Shigeemase, and Mihami, (2003), chitin is more effective on aggregation of platelets than that of chitosan.

6. Concluding remarks

COS possess various biological activities and have a considerable potential to be utilized in number of useful applications. However, many of the studies carried out to search bioactivities of COS do not provide detailed molecular mechanisms. In fact, it is hard to explain how exactly these molecules exert their activities. Therefore, future research should be directed towards understanding their molecular level details which may provide an insight into the unrevealed molecular level functions of COS and help to accelerate their future applications.

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