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7 Production of Bioactive Chitosan Oligosaccharides and Their Potential Use as Nutraceuticals

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7.1 INTRODUCTION

Chitosan and its derivatives have many interesting properties that make them attractive for a wide variety of applications in many fields such as food [1], cosmetics [2], biomedicine [3], agriculture, and wastewater management. Their antibacterial, antifungal, and antiviral properties make them particularly useful for biomedical applications, such as wound dressings, surgical sutures, and as aids in cataract surgery and periodontal disease treatment. Moreover, researches have shown that chitosan derivatives are nontoxic and nonallergenic; so the body is not likely to reject them as foreign invaders. Chitosan is commercially obtained by deacetylation of chitin, the second most abundant natural biopolymer

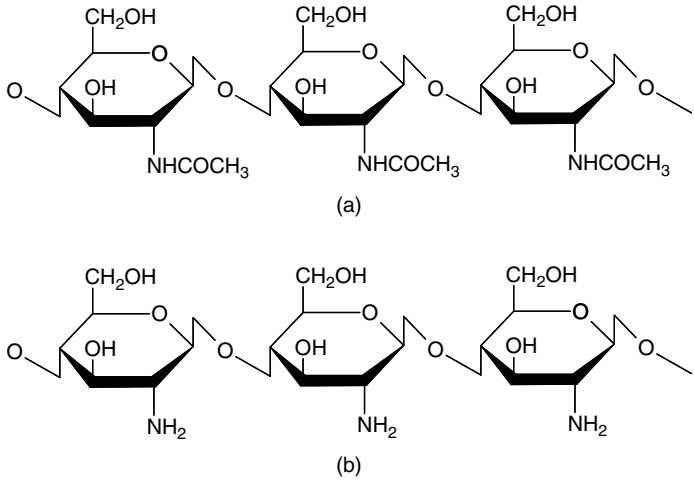

FIGURE 7.1 Chemical structures of (a) chitin and (b) chitosan.

on earth after cellulose. Chitin, a major structural component in the exoskeleton of crustaceans, insects, and cell walls of fungi is identified as a polymer of *N*-acetylglucosamine (β-1,4 linked 2-acetamido-D-glucosamine). Therefore, chitosan, the *N*-deacetylated form of chitin, can be named as a polymer of β-1,4 linked D-glucosamine units (Figure 7.1).

Normally chitin is water-insoluble, but chitosan can be solubilized in water at slightly acidic pH. Unlike chitin and chitosan, chitosan oligosaccharides (COSs), the hydrolyzed products of chitosan are readily soluble in water because of their shorter chain length and free amino groups of D-glucosamine units [4]. Therefore, over the past decade, researchers in Asia, Europe, and North America have tested COSs in biomedical applications. Chitosan-based researches have also been focused on the food and nutrition areas, including development of edible films and coatings to preserve the quality and texture of foods. In addition, numerous studies have also been undertaken to find out unraveled new properties of COSs and sequential developments were achieved in continuous production of COSs in large scale. This chapter provides an overview of the development of new continuous production methods of COSs by enzymatic means and some biological activities of COSs those may contribute to improve their value as nutraceuticals and functional food ingredients.

7.2 CHITOSAN OLIGOSACCHARIDES: A POTENTIAL SOURCE FOR NUTRACEUTICALS

Although chitin and chitosan are known to have important functional properties in many areas, their application as nutraceuticals is restricted because of poor absorption through the human intestine. This is mainly because chitinase- or

chitosanase-like enzymes that are required to break down chitin and chitosan into smaller chain molecules for absorption are absent in the human digestive track. In addition, the insoluble nature and high viscosity of chitinous material at neutral pH act as major barriers for their absorption into the body. However, results of early studies on chitin and chitosan conducted *in vitro* and *in vivo* demonstrated that they are capable of binding dietary fats and thereby prevent fat absorption from the gut. Despite their beneficial effects to act as antihypertensive [5], hypocholesterolemic [6] and weight-loss materials, chelation of some metal ions from the dietary sources becomes unfavorable for human nutrition. However, there is a renewed interest to identify biological properties of COSs because they can be readily absorbed into the bloodstream. Observations have proven that COSs exert many favorable biological properties, including increase of body resistance to diseases [7,8] that makes them attractive for a wide variety of health applications. Owing to their health benefits, COSs have the potential to be used as physiological functional foods. Several production methods of COSs have already been developed and some methods are capable of producing safe and nontoxic COSs suitable for human consumption. Especially, the higher-grade COSs are highly purified and their safety has been verified scientifically through many tests for medical and food applications. Korea, China, and Japan have a long history for using chitosan and COSs for their health benefits. Currently, COSs are available in the market as different commercial products highlighting their beneficial effects. However, the high price and limited knowledge about the beneficial health effects of COSs may limit their widespread use among consumers.

The proven bioactivities and possibility of large-scale and safe production of COSs as well as availability of raw materials have stimulated the potential use of COSs as nutraceuticals. In large-scale production of COSs, exoskeletons of crab and shrimp are utilized as starting material. Every year a large volume of crustacean exoskeletons is discarded as processing waste. According to the fisheries statistics of Food and Agriculture Organization, the global crustacean harvest is increasing annually, and more than 40% of the catch is utilized for processing. Hence, a considerable amount of crustacean shells is discarded as by-products. Therefore, the raw material for production of COSs is readily available at low cost.

7.3 PRODUCTION OF BIOACTIVE CHITOSAN OLIGOSACCHARIDES

The degree of polymerization (DP) is crucial for the bioactivities of COSs and COSs with relatively high DP (five to seven D-glucosamine units) are favored in this regard [9,10]. Therefore, bioactivities of COSs relate to their structural features. For the production of COSs, both chemical and enzymatic hydrolysis can be employed. In the chemical method, COSs are produced by partial hydrolysis of chitosan with concentrated HCl. However, experimental results have shown that chemical hydrolysis produces low yields of COSs and a larger amount of

monomeric D-glucosamine units. In addition, the COSs prepared by acid hydrolysis may not be suitable for human consumption because of possibility of production of toxic compounds during hydrolysis [11]. Therefore, enzymatic hydrolysis of chitosan for production of COSs has become the preferred method during the past few decades. Chitosanases obtained from microbes produce a relatively higher proportion of COSs from chitosan. Initially, enzymatic hydrolysis was carried out in batch reactors, where chitosanase was mixed with its substrate, and allowed to break down glycosidic bonds of chitosan under optimum conditions [12]. This batch method had some disadvantages such as low yields and higher cost associated with the use of large quantities of expensive chitosanase enzyme. Later, it was found that a number of different enzymes can also be used for the hydrolysis of chitosan. For instance, Lysozyme and chitinase can act on partially *N*-acetylated chitosan by recognizing *N*-acetylglucosamine residues in the chitosan sequence [13,14]. However, due to inefficient production of desirable chain lengths of COSs, these methods were not applicable for large-scale production of bioactive COSs, thus limiting the potential use of COSs as nutraceuticals.

In 1998, a new method for producing COSs with higher degree of polymerization by means of a column reactor packed with an immobilized enzyme was introduced [15]. However, in this method the yield of COSs was lower, because the immobilized enzyme showed a lower affinity and lower reaction rate than the free enzyme. Therefore, an enzyme reactor system together with an ultrafiltration (UF) membrane reactor was tested to produce COSs with higher degrees of polymerization and yield [16]. The optimum conditions for the production of relatively high proportions of COSs were determined by changing reaction temperature, incubation time, permeation rate, and amount of reducing sugar, which indicates the degree of hydrolysis. This reactor system could hydrolyze at least 11 batches of substrate for the same amount of enzyme used in the batch reactor and was effective for the production of relatively longer-chain oligosaccharides that have shown interesting biological activities in other studies. The most important factor in the UF reactor system was the control of permeation rate that determines the components of the resultant oligosaccharides. However, this method did not allow continuous production of COSs and resulted in increased transmembrane pressure, possibly because of the fouling of membrane by highly viscous chitosan and accumulation of substrate. Therefore, to improve this method to a more effective continuous production system, efforts were made to reduce chitosan viscosity prior to treatment in the UF membrane system.

The continuous production of COSs was found to be feasible with the combination of the above two methods in which a column reactor packed with immobilized enzyme was coupled to a UF membrane reactor and the new system was named as dual reactor system [17]. In this system, production of COSs may be performed in two steps (Figure 7.2). In the first step, chitosan is partially hydrolyzed by the immobilized enzyme packed in the column reactor and it is supplied to the enzyme reactor in UF membrane reactor system for the production of COSs. As expected, the low viscosity of partially hydrolyzed chitosan did not create fouling problems under the controlled conditions, and continuous production

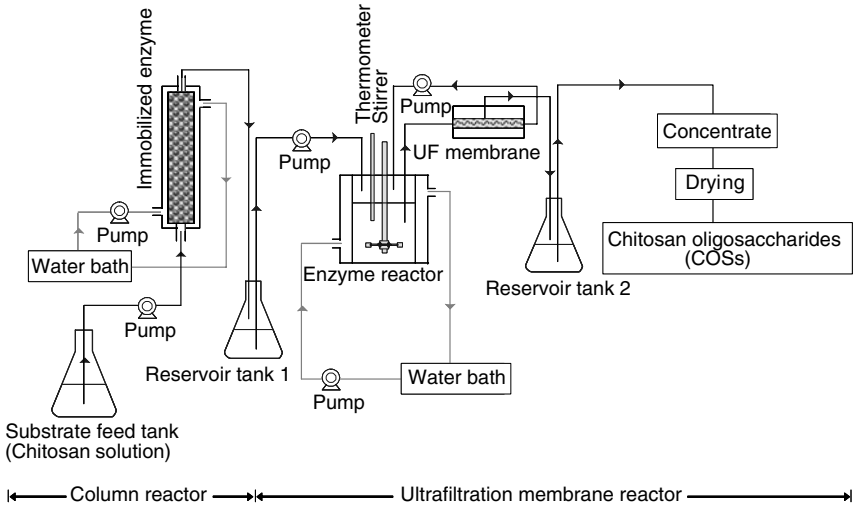

FIGURE 7.2 Schematic diagram of the dual reactor system developed for continuous production of chitosan oligosaccharides. (From Jeon, Y.J. and Kim, S.K., *Process Biochem.,* 35, 623–632, 2000. With permission.)

of COSs was achieved. A greater productivity per unit enzyme, ability to control molecular weight distribution, and more efficient continuous production process were obtained by utilizing the dual reactor compared to conventional methods. Therefore, this method is commonly used to produce different molecular size COSs to study their bioactivities.

In recent years, many researchers have attempted to utilize enzymatic methods to produce bioactive COSs. Currently, few methods are used to produce COSs with a particular molecular size distribution. In these methods complex enzyme systems have been used to produce COSs with a preferred degree of polymerization range [18]. Later, Kuroiwa et al. [19] reported optimum conditions for production of pentamers and hexamers of COSs using a packed-bed enzyme reactor. In addition to enzymatic methods, several other approaches such as chemoenzymatic synthesis of chitosan oligomers have also been identified to produce bioactive COSs [20]. Although COSs can be produced using different methods, enzymatic hydrolysis of chitosan is the most reliable and effective method to obtain bioactive COSs with higher purity, used for human consumption.

7.4 BIOLOGICAL ACTIVITIES OF CHITOSAN OLIGOSACCHARIDES

Low-molecular-weight water-soluble chitosan or COSs are known to have many desirable biological activities such as antifungal activity [21], antibacterial activity [17,22,23], antitumor activity [24,25], immuno-enhancing effects [26], and protective effects against infection [4]. The molecular weight or chain length,

which is generally referred to as the degree of polymerization (DP) and the degree of acetylation (DA) are considered as principal characteristics of COSs related to their biological activities. Therefore, by altering substrate and enzyme conditions, desired properties of COSs that are necessary to exhibit different biological activities can be obtained. As described earlier [15–17], many researchers have demonstrated that enzymatic hydrolysis is useful for preparation of COSs as it gives greater yields of oligomers with higher DP than those in acid hydrolysis [11]. In this chapter, several biological activities of COSs prepared by enzymatic hydrolysis of differentially acetylated chitosans are discussed. These beneficial biological activities of COSs may encourage their utilization as functional foods as well as in improving food quality.

7.4.1 Antibacterial Activity

Chitosans are capable of inhibiting the growth of some microorganisms including several bacterial strains. Positively charged amino groups on chitosan are presumed to be the reason for this bioactivity. The cationic amino groups may form polyelectrolyte complexes with negatively charged carboxylic anion groups present on cell walls of bacteria, thus inhibiting their growth and functions. In addition, antibacterial activity of COSs has been shown to be greatly dependent on their DP. Therefore, to identify the antibacterial activities of COSs, three fractions of COSs with different molecular weights were prepared by employing the dual reactor system and tested against Gram-negative, Gram-positive, and lactic acid bacterial strains [15,23]. COSs with molecular weights higher than 1 kDa seemed to be suitable for antibacterial activity; inhibitory effects were slightly varied depending on the type of bacteria (Table 7.1). In addition, COSs exhibited a more effective suppression against Gram-positive bacteria compared to Gram-negative bacteria. Interestingly, COSs were more effective against pathogenic bacteria associated with human diseases in comparison to nonpathogenic bacteria. For examples, *Streptococcus mutans*, which induces tooth decay, was completely inhibited by 0.1% of COSs and *Staphylococcus aureus*, which is responsible for pimples on human skin, was inhibited 93–100% at the same concentration of COSs. In the case of Gram-negative bacteria, all fractions of COSs could effectively inhibit *Salmonella typhi* that causes typhoid fever.

In addition to COSs, hetero-COSs (oligosaccharides derived from partially deacetylated chitosan) also have proven their ability to serve as antibacterial compounds. Antibacterial activity of hetero-COSs is dependent on the degree of deacetylation as well as the molecular size. To identify the effect of these two parameters on antibacterial activity of hetero-COSs, differentially deacetylated chitosans and their oligosaccharides were tested against a number of bacterial strains [27,28]. Interestingly, hetero-COSs followed the same pattern of inhibition as that exhibited by COSs in other studies. It was also found that treatment with all hetero-COSs could reduce the growth of bacteria significantly. However, high-molecular-weight hetero-COSs were more effective in inhibiting bacterial growth. In addition, 75% deacetylated hetero-COSs rendered a better antibacterial activity

TABLE 7.1
Antibacterial Activities of COS Fractions with Different Molecular Weight Ranges

Bacteria		Antibacterial Activity (%)[a]		
		HMWCOSs[b]	MMWCOSs[c]	LMWCOSs[d]
Gram-negative bacteria	*Escherichia coli*	98 ± 0	62 ± 6	51 ± 7
	Escherichia coli O-157	71 ± 3	56 ± 4	60 ± 2
	Salmonella typhi	91 ± 2	88 ± 0	89 ± 0
	Pseudomonas aeruginosa	47 ± 5	35 ± 5	22 ± 8
Gram-positive bacteria	*Streptococcus mutans*	100 ± 0	99 ± 0	99 ± 0
	Staphylococcus aureus	97 ± 3	95 ± 0	93 ± 9
	Staphylococcus epidermidis	82 ± 0	57 ± 3	23 ± 1
	Bacillus subtilis	63 ± 5	60 ± 5	63 ± 7
	Micrococcus luteus	70 ± 0	67 ± 3	63 ± 7

[a] Following the incubation of bacterial culture with 0.1% different COSs fractions, the number of colonies formed on the medium was calculated as a percentage compared to the control.
[b] High-molecular-weight COSs (molecular weight range 10–5 kDa).
[c] Medium-molecular-weight COSs (molecular weight range 5–1kDa).
[d] Low-molecular-weight COSs (molecular weight less than 1 kDa).
Source: Jeon, Y.J., Park, P.J. and Kim, S.K., *Carbohyd. Polym.*, 44, 71–76, 2001.

than that of 50 and 90% deacetylated oligomers. Especially, the growth of *Vibrio parahaemolyticus*, a food-borne Gram-negative bacterial strain, was inhibited by the 75% deacetylated hetero-COSs at a high rate [24]. Therefore, antibacterial property of COSs and deacetylated forms of them better reflects their functional properties, which could stimulate their use as potential nutraceuticals.

7.4.2 Antitumor Activity

Early studies demonstrated that chitosan and COSs could inhibit the growth of tumor cells by exerting immuno-enhancing effects. Some studies have suggested that the observed antitumor activity is not because of the direct killing of tumor cells but possibly because of the increased production of lymphokines [29]. *In vivo* studies carried with hexamer COSs ingested mice have shown significant antimetastatic effects on lung carcinoma [26]. However, the antitumor activities observed in COSs also depend on their structural characteristics such as degree of deacetylation and molecular weight. Thus, a study was carried out to identify the antitumor activities of different molecular weight COSs prepared using UF membrane reactor system. The mean molecular weight COSs 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 cell–bearing mice [25]. In addition,

the optimum dose of this COS (89% deacetylated) for inhibiting these tumors was approximately 20 mg/kg/day, which resulted in 66.6 and 73.6% tumor inhibition rates against S180 and U14-bearing mice, respectively (Table 7.2). Many reports suggest that these antitumor compounds exert effects on immune system to stimulate leucocytes, cytotoxic T cells, and natural killer cells. The observed increase in thymus weight of S180 and U14 tumor cell–bearing animals after the COSs treatment implied an improvement in the immune system particularly by activation of T lymphocytes. Furthermore, studies on antitumor activity of chitosans and their derivatives revealed that partially deacetylated chitin and carboxymethylchitin with appropriate degrees of substitution were effective toward controlling various tumor cells [30]. Unlike many other biological molecules, COSs could exert their biological activities following oral administration and effects were more or less similar to those of intraperitoneal injection. Qin et al. [31] have demonstrated that water-soluble COSs prepared with a mixture of tetramer and pentamer could inhibit the growth of S180 tumor cells in mice after oral and intraperitoneal administration. Therefore, COSs and their *N*-acetylated analogs that are soluble in basic physiologic environments may serve as good candidates for developing nutraceuticals. Further, very few reports have hypothesized that free amine groups of COSs play an important role for antitumor activity in tumor-bearing animals.

TABLE 7.2
Effect of COSs on Thymus Growth and Tumor Growth Inhibition in BALB/c Mice

		Sarcoma 180 (S180)		Uterine Cervix Carcinoma (U14)	
Sample	Dose (mg/kg/day)	Thymus (mg/10g)	Tumor Inhibition (%)	Thymus (mg/10g)	Tumor Inhibition (%)
Control		29.2 ± 9.5		25.4 ± 11.5	
HMWCOSs[a]	50	6.5 ± 4.5	–	14.5 ± 4.8	–
	20	31.4 ± 15.9	12.7	21.6 ± 11.9	11.9
	10	31.6 ± 8.5	61.7	25.9 ± 9.0	15.3
MMWCOSs[b]	50	41.4 ± 5.5	66.6	36.7 ± 14.0	73.6
	20	37.1 ± 9.7	35.5	33.8 ± 8.4	61.4
	10	34.4 ± 16.7	28.4	28.0 ± 10.0	26.6
LMWCOSs[c]	50	31.9 ± 6.7	12.4	27.8 ± 6.7	27.1
	20	34.1 ± 6.6	15.3	31.6 ± 17.5	7.8
	10	33.2 ± 10.5	5.7	30.9 ± 7.5	3.5

[a] High-molecular-weight COSs (molecular weight range 12.0–6.5 kDa).
[b] Medium-molecular-weight COSs (molecular weight range 5.5–1.5 kDa).
[c] Low-molecular-weight COSs (molecular weight range 1.4–0.5 kDa).
Source: Jeon, Y.J. and Kim, S.K., *J. Microbiol. Biotechnol*., 12, 503–507, 2002.

This was attributed to the improvement in the antitumor activity with the increase of deacetylation of COSs.

7.4.3 Radical Scavenging Activity

Aerobic organisms must deal with free radicals that are generated from sequential reduction of oxygen during the normal course of aerobic metabolism. Uncontrolled formation of these free radicals is toxic as they cause cellular damage leading to a number of pathological conditions including atherosclerosis, arthritis, diabetes, and carcinogenesis [32]. The body has developed natural antioxidant systems to fight against these free radicals; however, the capacity of such systems gradually decreases with ageing, resulting in imbalances in the redox status. Therefore, the body must be nourished with a diet that includes adequate antioxidants. Scavengers of free radicals are preventive antioxidants, and presence of radical scavenging compounds break the oxidative sequence at different levels. Therefore, there has been a growing interest to identify natural antioxidant compounds from many 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. In addition, COSs and hetero-COSs have shown radical scavenging properties depending on their DA and molecular weight [33,34]. Low-molecular-weight COSs (1–3 kDa) scavenged different radicals as evidenced by electron spin trapping technique using ESR spectroscopy. In addition, highly deacetylated (90%) COSs are more effective for scavenging DPPH, hydroxyl, superoxide, and carbon-centered radicals (Figure 7.3). Especially, the hydroxyl radical, which is one of the most reactive free radicals involved in the oxidation of biomolecules such as lipids and proteins can be effectively scavenged by these COSs. However, scavenging effects on these harmful free radical species *in vivo* should be studied to identify the precise application of COSs or their heteroderivatives as radical scavengers in the human body.

7.4.4 Other Biological Activities

In addition to the bioactivities discussed above, COSs and hetero-COSs have been shown to possess some other important properties such as anticoagulant activity [35,36], angiotensin I converting enzyme (ACE)-inhibitory activity [37], calcium absorption acceleration activity [38], and antifungal activity [21]. Similar to other observed biological activities of COSs, these properties are also dependent on the DA. For antifungal activity, chitosan was more effective than its oligomers. However, low-molecular-weight COSs had better inhibition effects on some fungal species than low- and medium-molecular-weight COSs (Figure 7.4).

In two separate studies, COSs were found to exert anticoagulant and ACE-inhibitory activities regardless of their molecular weights. ACE, a dipeptidylcarboxypeptidase present in mammals plays a major role in high blood pressure. Owing to the undesirable side effects of synthetic ACE inhibitors, increased

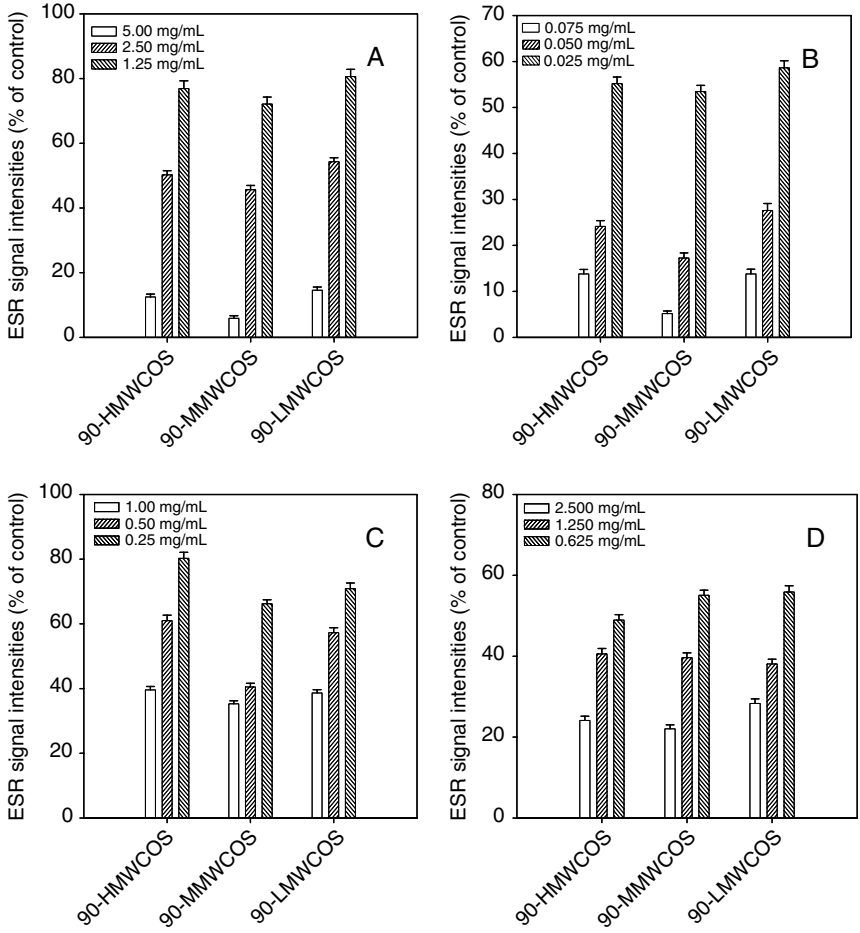

FIGURE 7.3 DPPH (A), hydroxyl (B), superoxide, and (C) carbon-centered radical (D) scavenging activities of COSs tested with their different molecular weight fractions. Values represent means ±S.E ($n = 3$); 90-HMWCOS, 90% deacetylated high-molecular-weight COS (MW 10,000–5,000 kDa); 90-MMWCOS, 90% deacetylated medium-molecular-weight COS (MW 5000–1000 kDa); 90-LMWCOS, 90% deacetylated low-molecular-weight COS (MW <1000 kDa). (From Je, J.Y., Park, P.J. and Kim, S.K., *Food Chem. Toxicol.,* 42, 381–387, 2004.)

attention has been paid toward natural and safe ACE inhibitors such as peptides, carbohydrates, and phenolic compounds. In addition to their inhibitory activity, these natural compounds may offer other beneficial effects to be used them as physiological functional foods or nutraceuticals. COSs with a relatively low degree of deacetylation (50%) are better ACE inhibitors than other deacetylated forms.

Heparin, a widely used anticoagulant polysaccharide in clinical therapy, has been shown to exert a number of complications in patients, and for a long time

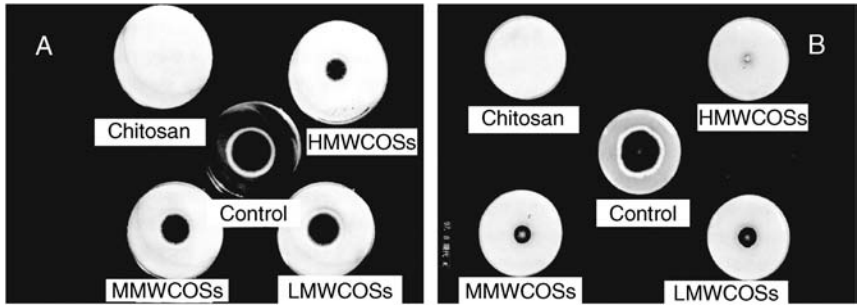

FIGURE 7.4 Antifungal activity of chitosan and COSs. Different molecular weights of COSs affect the growth of *Aspergillus niger* (A) and *Alternaria mali* (B). (From Kim, S.K., *Food Ind. Nut.,* 8, 1–8, 2003. With permission.)

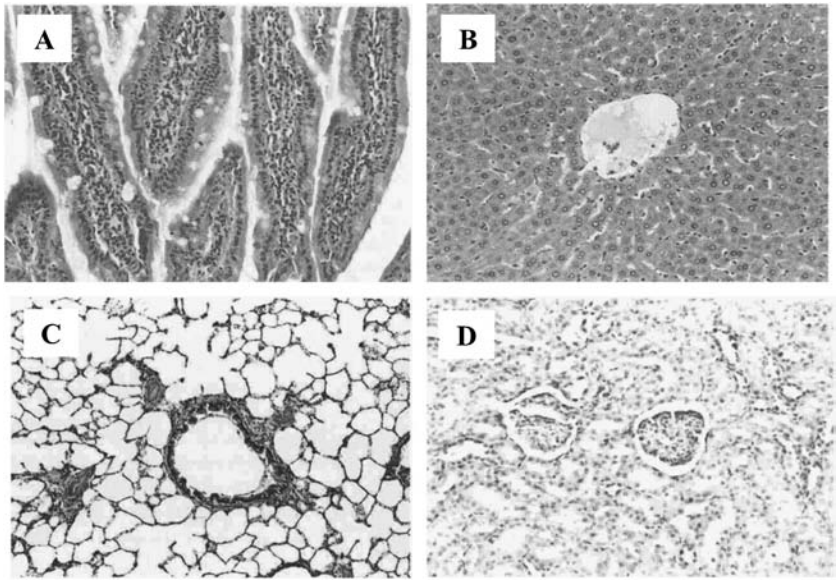

FIGURE 7.5 Microscopic views of small intestine (A), liver (B), lung (C), and kidney (D) sections obtained from Sprague–Dawley rats after 4 weeks of COSs (5000 mg/kg) ingestion. (From Jeon, Y.J. and Kim, S.K., *J. Chitin Chitosan,* 4, 115–120, 1999. With permission.)

researchers have been searching for better natural alternate sources. In this regard, sulfated forms of COSs have displayed promising results for acting as anticoagulants [35]. However, their low activity compared to heparin does not negate their use as natural anticoagulants, because they do not impart any unfavorable side effects. In addition, COSs with high degrees of deacetylation had a better performance as anticoagulants than their less deacetylated counterparts.

7.5 SAFETY OF COSs

Even though COSs have very strong biological properties *in vitro*, little information is available on their cytotoxicity and bioavailability in the human body. Interestingly, experiments on subacute toxicity of COSs in Sprague–Dawley (SD) rats revealed that COSs do not induce any mortalities, or change in blood chemistry, urinalysis, and body weights of rats [39]. Therefore, it can be presumed that COSs may not have any influence on acute toxicity and side effects in humans. Furthermore, histopathological findings revealed that COSs do not cause any lesions in tissues of rats under the tested dose range of COSs (Figure 7.5) [40]. These findings further confirm that intake of COSs will not cause any undesirable side effects, at least in animals.

COSs have become popular during the past few decades because of their biological effects related to their structural properties. In addition, availability of raw materials at low cost and recently developed methods ensure continuous production of COSs. Therefore, it is expected that these biomolecules play an important role in fulfilling the current demand for nutraceuticals.

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