BIOLOGICAL EFFECTS AND EXTRACTION PROCESS FOR MARINE CHITOSAN

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ABSTRACT

In this chapter, the different existing methods of obtaining chitin, chitosan and chitooligosaccharides are reviewed. Chitin is the main constituent of exoskeleton of crustaceans and traditional chemical processes for its recovery creates a disposal problem due to the large amounts of toxic wastes produced. A description of alternative methods, such as the use of different proteolytic enzymes, fermentation with selected microorganisms, ultrasonication or microwave treatment, together with the use of ionic liquids have been described. Also, methods to obtain chitosan based on alkaline or enzymatic deacetylation of chitin as well as the strategies to increase its solubility in water, such as hydrolysis of the glycosidic bonds in chitosan releasing chitooligosaccharides or formation of different types of chitosan derivatives have also been depicted. Biological activities such as antioxidant, hypocholesterolemic, antimicrobial, antihypertensive, prebiotics and ion binding and the potential applications in foods, medicine, pharmacy, agriculture, cosmetics are also presented.

KEYWORDS: biological properties; chitin; chitosan; chitooligosaccharides; food.

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1. Introduction

Chitin, a linear polymer of *N*-acetyl-D-glucosamine units linked through β -(1-4) bonds, is the most abundant organic compound on earth after cellulose. Cellulose and chitin are mainly found as primary structural components and protective materials in plant or animal kingdoms, respectively. As Figure 1 shows, both biopolymers are structurally similar, cellulose being formed by β -(1-4)-linked D-glucose units while chitin possesses an acetamide group at C-2 in place of the hydroxyl group in cellulose (Kurita 2006). Chitin occurs in nature as the main structural component in the exoskeleton material of crustaceans, cuticle of insects, wall of some fungi, and in other organisms such as algae, marine diatoms and yeasts. Marine shellfish, including lobster, crab, krill, cuttlefish, shrimp, and prawn, are richer in chitin compared to terrestrial organisms; thus, marine chitin is an important by-product of the fishing industry, being its production estimated about 10^9 metric tons annually (Je & Kim 2012).

2. Extraction processes of chitin

Although chitin is the main constituent of crustacean's exoskeleton, other minor components such as proteins, mineral salts and carotenoids are present. Moreover, it has been described that content of chitin on crustacean and molluscan organisms is variable depending on its source, specie, or body part (shell or head portion), among others (Venugopal 2011). Due to the fact that chitin is intimately associated with proteins and salts, its isolation requires the removal of these constituents from the raw materials.

The traditional processes (chemical method) of chitin production from crustacean shells involve treatment with bases for protein removal, strong acids for demineralization (Kjartansson *et al.* 2006; Setoguchi *et al.* 2012) and decoloration using salts as potassium permanganate and oxalic acid (Je & Kim 2012). To ensure complete protein removal, the alkaline treatment is repeated several times and if necessary, the product is again treated with diluted hydrochloric acid and alkali (Kurita 2006). A flow scheme of chitin production is summarized in Figure 2. These traditional processes may cause deacetylation or reduction of molecular weight (MW) of the resulting chitin, which can alter its properties (Setoguchi *et al.* 2012). Moreover, the chemical method creates a disposal problem due to the large amounts of toxic waste that must be treated before it is sent to landfill (see Table 1).

An alternative to the chemical process is the use of biological methods (Table 1). A wide variety of enzymatic procedures have been developed based on the use of proteolytic enzymes for the deproteinization of crustacean wastes (Valdez-Peña *et al.* 2010).

Fermentation technology may be also used to remove the minerals and proteins. It has been described that using a selected *Lactobacillus plantarum* strain, an 88% deproteinization and 90% demineralization of shrimp waste is achieved (Rao *et al.* 2000; Khorrami *et al.* 2012). Microorganisms such as *Bacillus subtilis* produce sufficient quantities of acid to remove minerals whereas the protease present in *Bacillus* species cause deproteinization (Sini *et al.* 2007). Co-fermentation of the shells using two types of strains, a lactic-acid-producing and a protease-producing bacterium, are efficient and applicable for the one-step extraction of crude chitin from red crab shell waste (Jung *et al.* 2006). Although considerable work has been done on these processes, more research is needed before this technology becomes commercially available.

Ultrasonication has also been used to extract chitin from shrimp shells. Although this treatment did not enhance the elimination of minerals, it improved the removal of the protein; besides, it did not affect the degree of chitin acetylation (Venugopal 2011).

Ionic liquids (ILs), salts with melting points below 100 °C, have been reported to be effective solvents for cellulose and other polysaccharides extraction (Swatloski *et al.* 2002; Mora-Pale *et al.* 2011). However, few data are available about the dissolution of chitin in ILs, most of them concerning the dissolution behaviour of pure chitin (Wu *et al.* 2008). Recent studies have shown that ILs can be used for chitin isolation from crustacean shells to avoid depolymerisation. 1-Ethyl-3-methyl-imidazolium acetate can completely dissolve raw shrimp shells, allowing the recovery of a high MW chitin powder (Qin *et al.* 2010) while the production of chitin from crab shells is easily performed using 1-ethyl-3-methyl-imidazolium bromide and citric acid (Setoguchi *et al.* 2012).

The process of dissolving chitins in ILs is very complex and depends on the structure of the particular ILs employed and the source and purification process of chitins. A study on the dissolving behaviour of chitins with different physico-chemical characteristics in series of ILs indicated that the dissolution behaviour of chitin in ILs was affected by the degree of deacetylation (DD), the crystallinity, and the MW of chitin, as well as the nature of the anion of the IL (Wang *et al.* 2010b).

Chitin has been considered for many years as an abundant waste material with limited industrial uses; however, research during recent decades has shown its remarkable properties that are of particular interest in a wide range of industrial applications.

3. Obtaining of chitosan

Chitosan consists of a wide group of partially and fully deacetylated chitins with a wide range of MW distribution. Partial deacetylation of chitin gives rise to chitosan (Figure 1), a cationic polysaccharide composed of units of glucosamine (2-amino-2-deoxy-D-glucose) and N-acetyl glucosamine (2-acetamido-2-deoxy-D-glucose) linked by β -(1-4) bonds. Chitosan may be obtained by enzymatic or alkaline deacetylation of chitin, the latter being the most commonly used methodology. Alkaline deacetylation is performed at different combinations of temperature (up to 140°C) and time (up to 10 h) using concentrated (30 to 60% w/v) sodium or potassium hydroxide solutions (Figure 2). In the homogeneous deacetylation process, chitin is dissolved in an alkali solution during the deacetylation process (at low temperature and using strong stirring) while in the heterogeneous deacetylation process, chitin is kept insoluble in a hot alkali solution, meaning that this is a two-phase process (Aam *et al.* 2010). The operating conditions of the process strongly influence the resulting MW and MW distribution, as well as on the DD and the distribution of deacetylated units along the polysaccharide chain (Synowiecki & Al-Khateeb 2003). This fact implies that different chitosan products, including oligomers, can be obtained by varying the basic manufacturing process conditions. Moreover, the presence of oxygen during chitin deacetylation may cause chain degradation, decreasing the MW and viscosity of the product. Such changes can be limited through the processing of chitin under nitrogen (Bough *et al.* 1978). A minimum deacetylation of 70% is required to produce chitosan with acceptable properties.

On the other hand, chemical deacetylation may cause unwanted by-products and the alkali wash effluent has to be treated to avoid environmental pollution. Therefore, there is a growing interest in the utilization of the environmental friendly biological processes based on the use of chitin deacetylase (CDA, EC 3.2.1.41) for enzymatic conversion of chitin to chitosan under mild reaction conditions. Although CDA may be produced by a wide variety of microorganisms, most of the strains isolated so far are intracellular producers with low activity and yield. Thus, many studies are devoted to

the isolation and screening of microorganisms with CDA activity for purification and characterization of enzyme and optimization of nutritional requirements to improve their extracellular production (Tsigos & Bouriotis 1995; Cai *et al.* 2006; Kim *et al.* 2008b; Pareek *et al.* 2011).

3.1. Properties of chitosan

Chitosan is the only natural polysaccharide that presents cationic character due to free amino groups present in its structure, fact highly advantageous for providing distinctive biological functions and for conducting modifications reactions (Kurita 2001). At low pH values, these amino groups are protonated and can interact with negatively charged compounds such as proteins, anionic polysaccharides (alginates, carragenates, pectins, etc.), fatty acids, bile acids, and phospholipids (Agulló *et al.* 2003; Guo *et al.* 2007). Moreover, in contrast to chitin, the presence of free amino groups along the chitosan chain allows this macromolecule to be dissolved in diluted aqueous acidic solvents (Aranaz *et al.* 2009). This behaviour along with its biocompatibility and biodegradability, as well as its non-toxicity, has led chitosan to being used in such diverse fields such as food, cosmetics, medicine, biotechnology, agriculture and paper industry (Dutta *et al.* 2004; Kim & Rajapakse 2005; Kurita 2006), as it can be observed in Table 2.

Besides, chitosan presents biological activities of interest such as antibacterial, antihypertensive, bifidogenic, hypocholesterolemic, hypoglycaemic, antioxidant, anti-inflammatory, immune modulatory and antiproliferative activities (Xia *et al.* 2011). In this chapter some of these properties will be expanded later.

3.2. Chemical modifications of chitosan

Despite the broad spectrum of properties of chitosan, its applications in foods are partly limited by its insolubility at neutral and basic pH (Sugimoto *et al.* 1998; Yang *et al.* 2002). Consequently, several strategies have been applied in order to expand its range of solubility. In general, high MW chitosans are insoluble in water whereas depolymerised chitosans with low MW are soluble in water, thereby, increasing their potential applications.

One of the most used strategies for chitosan modification is the introduction of hydrophilic residues in the chitosan molecule through the formation of covalent bonds with the free amino groups. Moreover, chitosan possesses also primary and secondary

hydroxyl groups to be used for chemical modifications and give new functional products with improved properties. The reactive groups of chitosan may be subjected to a number of reactions such as acylation, tosylation, alkylation, *O*-carboxy methylation and *N*-carboxy alkylation (Figure 3). Chitosan may be also treated with aldehydes or ketones to prepare the corresponding Schiff's base, which can be easily converted into an *N*-alkyl derivative by reduction with sodium borohydride or sodium cyanoborohydride (Kurita 2001, 2006; Prashanth & Tharanathan 2007; Aranaz *et al.* 2010).

In the last years, a number of new chitosan derivatives with well-defined structures are being developed with the aim of improving the industrial applications of chitosans, which include dietary supplements, coagulants for water treatment, food preservatives, oligosaccharides, ingredients for cosmetics and toiletries, and bacteriostatic fabrics (Kurita 2006).

3.3. Physical forms of chitosan

Chitosan may be processed into a number of forms including solution, powder and flake, hydrogels, coating, films, fibres, nanoparticles, beads and microspheres, nanofibrils and lyophilized scaffolds. Each of these forms has its own advantages for handling and applications in different fields. However, chitosan is mechanically weak and unable to keep a predefined shape when swelling takes place, limiting its use for some applications (Martino *et al.* 2011). Chitosan may be blended with other biopolymers or polymers from renewable resources (starch, alginate, collagen, pullulan, polylactic acid or polyhydroxybutyrate, for instance) to improve its mechanical properties or moisture-resistance, which limit its applications for food packaging when used in edible coatings. Therefore, polymer blending is an alternative to produce new materials with tailored properties by adjusting the blend composition (Yu *et al.* 2006; El-Hefian *et al.* 2011). It is necessary to note that the physical forms are as important as the chemical structures (Muzzarelli & Muzzareli 2005). Thus, the selection of one particular physical form depends mainly on the particular application.

Chitosan/silk fibroin blend films have been prepared and evaluated for feasibility of using them as biomaterial for skin tissue engineering application (Luangbudnark *et al.* 2012). The blends of chitosan and gelatinized starch have been tested as thickeners in textile screen printing (Abdou *et al.* 2013). The results proved that chitosan increased the dye uptake on fabrics and the treated products were found to

be antimicrobial. Fastness properties of the printed fabrics to washing, rubbing, perspiration, and light were also improved. Xyloglucan and chitosan has also been blended to form versatile hydrogels, non-toxic, renewable, transparent, with good thermal, antimicrobial and texture properties, which are very promising in food and cosmetic applications (Simi & Abraham 2010).

New chitosan-based materials have also been produced from blending with synthetic polymers. The most widely used are poly(vinyl alcohol), γ -poly(glutamic acid), poly- ϵ -caprolactone, poly(acrylonitrile), poly(N-vinyl-2-pyrrolidone), poly-L-lactic acid, 3-aminopropyltriethoxysilane and polyethylene glycol. All these materials are biocompatible, can be cheaply produced from reliable raw material sources and have been extensively used in biomaterial applications (Julkapli *et al.* 2011). A review describing the chitosan blends with synthetic polymers has been recently published (Sionkowska 2011).

4. Obtaining of chitooligosaccharides

Chitosan glycosidic bonds can be hydrolysed releasing compounds of lower MW called chitooligosaccharides (COS), which are soluble in water and with a wide range of industrial applications. COS are homo- or heterooligomers of N-acetylglucosamine and D-glucosamine with molecular weights of 10 kDa or less (Xia *et al.* 2011). Hydrolysis of chitosan can be carried out by chemical, physical and enzymatic methods.

4.1. Chemical methods

Chemical treatments are very common and fast methods for producing COS, which may be fractionated by chromatography on a charcoal/celite column or high-performance liquid chromatography (HPLC). However, these treatments have the main disadvantage of causing environmental pollution (Liu *et al.* 2006).

Chemical hydrolysis is performed at high temperatures under highly acidic conditions, mainly using hydrochloric acid (Cabrera & Van Cutsem 2005; Osorio-Madrado *et al.* 2010) or nitrous acid (Tommeraaas *et al.* 2001; Morris *et al.* 2009) producing a large amount of glucosamine (chitosan monomer) (Cabrera & Van Cutsem 2005; Mourya *et al.* 2011). Owing to difficulties in controlling the progress of the reaction, these treatments also give rise to the formation of secondary compounds which are difficult to eliminate (Mourya *et al.* 2011).

Other chemical hydrolysing agent is hydrogen peroxide (H_2O_2), a strong oxidant that produces free radicals which are able to attack β -D-(1-4) glycosidic bond of chitosan decreasing the average MW of chitosan. However, the formation of radical groups is inefficient when H_2O_2 is used alone (Wang *et al.* 2005; Huang *et al.* 2010). Degradation of chitosan by H_2O_2 is enhanced by ultraviolet light (UV) irradiation, γ -ray irradiation and microwaves (Makuuchi 2010). The MW of chitosan decreases with increasing irradiation time when UV (Wang *et al.* 2005) and γ -ray (Kang *et al.* 2007) are used in combination with H_2O_2 . However, when these radiations are employed alone the reduction of MW of chitosan with irradiation time reaches a plateau (Wang *et al.* 2005; Kang *et al.* 2007; Makuuchi, 2010).

Degradation of chitosan using H_2O_2 under phosphotungstic acid as catalyst has proved to be an effective method for obtaining low MW chitosan (LMWC). However, it is difficult to separate phosphotungstic acid from the degradation medium due to its high solubility in water (Huang *et al.* 2010). An assembly crystal synthesized with phosphotungstic acid and 1,2-diaminobenzene with low solubility in aqueous solution makes suitable the preparation process. Consequently, this degradation technique may have a potential application for the preparation of LMWC with average MW of 4900 Da (Huang *et al.* 2010).

Hydrolysis of high MW chitosan (HMWC) can also be performed in aqueous ILs solutions using H_2O_2 as catalyst for obtaining LMWC. This is an effective method since chitosans can be easily separated from the solvent which may be reused (Li *et al.* 2012a).

4.2. Physical methods

The chitosan glycosidic bond can also be cleaved by physical methods such as irradiation with ultrasound, microwave, γ radiation and pulse electric fields (PEF) without employing chemical agents. These methods are environmentally friendly, highly efficient, and use low reaction temperature, resulting in small side-effects (Chen *et al.* 1997; Choi *et al.* 2002; Feng *et al.* 2008; Luo *et al.* 2010). With low frequency ultrasound (20 kHz) partial depolymerisation of chitosan can be obtained, reducing the average MW of chitosan from 2000 kDa up to 450 kDa (Kasaai *et al.* 2008) or from 300 kDa up to 50 kDa (Wu *et al.* 2008); therefore the reduction on MW is limited (Chen, *et al.* 2012; Mourya *et al.* 2011). When chitosans were irradiated with Co-60 γ -ray, an inverse relationship between the irradiation dose and MW was observed; acetic solution

of chitosan (MW 110 kDa) irradiated with 100 kGy dose experimented a rapid decrease of viscosity and monomers up to hexamers (1 kDa) were formed (Choi *et al.* 2002). In another study, 20 kGy was used and the MW decrease recorded was from 210 to 2.1 kDa (Feng *et al.* 2008). The effect of γ -radiation (25 kGy) over chitosan (MW 128 kDa) in a solid state was minor, obtaining a final MW of 58 kDa. Also the PEF technique has been described as a possible method to obtain LMWC (Luo *et al.* 2010); MW decreased with the increasing electric field strength, so that a treatment of 25 kV/cm could reduce the initial MW to half. However, the effectiveness of treatment with microwave over chitosan was low: irradiation during 20 min at 98°C and 650 W only produced a 55% of reduction on MW respect to the initial chitosan (Tishchenko *et al.* 2011).

The use of ILs along with microwave treatment has been described to improve the hydrolysis of chitosan (Chen *et al.* 2012). ILs interact very efficiently with microwaves through the ionic conduction mechanism and are rapidly heated at rates easily exceeding 10°C/s without any significant pressure build-up. Chen *et al.* (2012) observed that when the total reducing sugars yield reached 92%, the average MW of chitosan dropped from 560 to 0.45 kDa. This method can dramatically reduce reaction time since under optimal reaction conditions the yield of total reduced sugars reached over 90% within 2 min. Moreover, results obtained by nuclear magnetic resonance pointed out that the chemical structure of hydrolysed chitosan was not apparently modified (Chen *et al.* 2012).

4.3. Enzymatic methods

The enzymatic method, such as the application of chitosanases and non-specific enzymes (Jeon *et al.* 2001; Qin *et al.* 2004) seems to be generally preferable to chemical methods because the reaction is under more gentle conditions and the MW distribution of the product is more controllable. Non-specific enzymes are inexpensive, commercially available and have been used in the industry for years to produce COS with relatively low cost (Zhang *et al.* 1999; Vishu Kumar *et al.* 2005). However, the expensive cost of chitosanases limits its wide application on industrial scale, even using immobilized enzymes (Jeon *et al.* 2001; Zeng & Zheng 2002; Lin *et al.* 2009; Luo *et al.* 2010).

Previous studies have reported the hydrolytic susceptibility of chitosan to a wide range of enzymes, including glycanases, proteases, and lipases derived from

bacterial, fungal, mammalian and plant sources (Pantaleone *et al.* 1992; Kim & Rajapakse 2005).

The enzymatic hydrolysis of chitosan can be performed either using the enzyme in soluble form (batch reactor) (Cabrera & Van Cutsem 2005; Xie *et al.* 2009) or immobilized in batch (Ichikawa *et al.* 2002; Roy & Gupta 2003; Sardar *et al.* 2003; Li *et al.* 2006), column or membrane reactor (Jeon *et al.* 2001; Lin *et al.* 2009). Advantages of using immobilized enzymes onto solid phase have been widely reported. Among them, an increase in the biocatalyst stability, the possibility to reuse the immobilized biocatalyst with the concomitant reduction of the process costs, as well as the fact that continuous operation becomes feasible, can be highlighted (Kim & Rajapakse 2005). Also, it is important to obtain a reaction media free of enzyme at the end of the process; COS free of protein are more suitable for biomedical and food applications, avoiding the undesirable level of pyrogenicity caused by the presence of protein (Li *et al.* 2006).

5. Biological activities of chitosan and chitooligosaccharides (COS)

As commented before, the reactive amino and hydroxyl groups confer to chitosan important biological properties such as antitumor, antimicrobial, anti-inflammatory, bone regeneration, human haemostasis, antioxidant, hypocholesterolemic, antihypertensive, prebiotic and ion binding activities, allowing the use of chitosan in many practical applications. The extent of acetylation and MW have been described to be the most important factors influencing the properties of chitosan and COS; therefore reactions of hydrolysis and deacetylation need to be controlled to obtain products with well-defined structures to improve their functionality. Also, in order to better understand the mechanisms of chitosan activities, accurate methods of characterization and purification must be developed. Due to the high number of properties and applications of chitosan and COS that can be found in literature, this chapter will focus on the most important aspects from the point of view of food and nutrition applications. Moreover, only selected articles will be commented as representative examples of the current state of the art.

5.1. Antioxidant activity

Antioxidant activity is one of the well-known functionalities of chitosan. Many studies on the relationship between structure and antioxidant properties revealed that both MW and DD of chitosan are ones of the most important factors affecting this activity (Je & Kim 2012). The hydroxyl groups (OH^-) and amino groups (NH_2^+) in chitosan contribute to the overall antioxidant capacity since they can react with unstable free radicals to form stable macromolecule radicals. However, they are difficult to dissociate due to the semi-crystalline structure of chitosan with strong hydrogen bonds (Jung & Zhao 2012).

A number of studies have examined the antioxidant properties of different types of chitosan. Tomida *et al.* (2009) studied the relationship between antioxidant properties and MW of chitosans through *in vitro* assays. They measured the ability of seven chitosans from different MW (2.8-931 kDa) to protect plasma protein from oxidation by peroxy radicals derived from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). The obtained results showed that the LMWCs have impressive antioxidant properties through the ability to scavenge hydroxyl radicals and to reduce cupric acid ions; however, HMWCs were less effective. Fernandes *et al.* (2010) demonstrated the antioxidant activity of COS mixtures and LMWC upon two biological oxidizable substrates, erythrocytes and phages, subjected to accelerated oxidation conditions. However, they recommended the use of low concentrations in order to prevent enhancement of the oxidative damage (i.e. a prooxidant effect). Also, for the assessment of antioxidant properties of biological substrates, it is necessary to use different methodologies since chemical methods alone may suggest misleadingly high antioxidant activities.

Physical modifications using high-intensity ultrasounds have been induced modifying the functional properties of chitosan. Weerakkody *et al.* (2011) studied the modification of antioxidant activity using sonicated and untreated water-soluble chitosan and their Maillard conjugates. Ultrasonication led to reduction in solution viscosity indicating its influence on MW reduction and the increase availability of amino groups; however, their reactivity was not enhanced and no improvement of antioxidant activity was observed when compared with un-sonicated chitosan.

Antioxidant properties of chitosan have also been evaluated through *in vivo* assays. Anraku *et al.* (2011) investigated the effect of HMWC supplement in normal volunteers. Treatment with chitosan for 8 weeks produced a decrease on oxidized to reduced albumin ratio and an increase in total plasma antioxidant activity. Also, a

significant decrease in total cholesterol levels, and atherogenic index, as well as an increase of levels of high density lipoprotein (HDL) was observed. These findings demonstrated the antioxidative potential of HMWC in the systemic circulation in human volunteers. These authors suggested that HMWC significantly reduced the levels of pro-oxidants such as cholesterol and uremic toxins in gastrointestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation in humans. Besides, they indicated that HMWC can be co-administered with conventional antioxidants such as vitamins and *N*-acetyl-cysteine as a new strategy for antioxidative treatment in cases of several diseases.

In food industry, the antioxidant activity of chitosan makes it suitable to preserve food or to be used as functional additive. Chien *et al.* (2007) used LMWC (12 kDa), medium MW chitosans (MMWC, 95 kDa) and HMWC (318 kDa) to study the influence of MW on antioxidant activity (measured through different *in vitro* assays, namely, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS, hydrogen peroxide and superoxide anion radicals and metal ion chelating capacity) as well as the possible antioxidant effects of different MW chitosans in apple juice. LMWC exhibited stronger scavenging activity toward DPPH radicals, superoxide anion radicals and hydrogen peroxide compared with the other chitosan tested. Besides, LMWC were able to increase antioxidant activity in apple juice.

Ying *et al.* (2011) studied antioxidant activity using branched-chain chitosan derivatives formed through Maillard reaction or further reductive alkylation of Schiff's bases. Formation of these derivatives improved functional properties such as solubility and the ability of scavenging DPPH radical. These authors proposed the water-soluble chitosan derivatives produced through Maillard reaction as a promising commercial additive in cosmetic and foods.

5.2. Hypocholesterolemic and hypolipidemic effects

Chitosan has become a useful dietary ingredient due to its beneficial plasma cholesterol level lowering effect. Because of this activity, chitosan plays an important role in the alleviation and treatment of cardiovascular diseases (Prashanth & Tharanathan 2007).

Since the first reported study on the hypocholesterolemic activity of chitosan on humans (Maezaki *et al.* 1993), growing evidence indicates that chitosan can diminish plasma triacylglycerol and total cholesterol levels (Bokura & Kobayashi 2003; Liao *et*

al. 2007). Recently, Choi *et al.* (2012) have shown that oral administration of COS can also reduce the plasma level of lipids in healthy men.

Numerous studies on animal models have revealed that chitosan reduces plasma triglycerides and total cholesterol levels. These effects are dependent on the DD and MW. Those with higher DD and MW effectively reduce plasma triglyceride, total cholesterol and LDL-cholesterol levels (Liu *et al.* 2008). Also, chitosan interacts with fatty acids and bile salts through amino groups present at C-2 position of chitosan structure. Primary and secondary hydroxyl groups at the C-3 and C-6 positions are also implicated in this interaction (Aranaz *et al.* 2009).

Xia *et al.* (2011) reviewed the mechanisms of hypocholesterolemic and hypolipidemic effects of different types of chitosan using *in vitro* and *in vivo* models. They indicated that physicochemical properties such as DD, MW and the viscosity-average molecular weight of chitosan affected to its fat, cholesterol and bile-salt binding capacities and its hypocholesterolemic and hypolipidemic activities.

Also, Zhang *et al.* (2012) studied rats fed high-fat diets containing HMWC, LMWC or cellulose. A decrease on levels of total cholesterol, LDL and liver triglyceride (TG) were observed in the group fed LMWC. This could be due to an increase on activities of liver and serum lipoprotein lipase. Moreover, in rats fed a high sucrose diet surplus with chitosan (5-7%, DD 90%, MW 835 kDa) during 16 weeks, a decrease on the total cholesterol and an improvement on the lipid metabolism was also observed. Because it has been described that MW has influence on hypocholesterolemic activity, Wang *et al.* (2011a) studied the influence of COS on plasma lipid regulation. A decrease of LDL-cholesterol and triacylglycerol contents, and an increase of HDL-cholesterol content was observed in rats fed a high-fat diet and COS.

A recent study of hypolipidemic activity of chitosan using guinea pigs has been developed. These animals were fed high-fat isocaloric diets containing soluble fiber or chitosan. The latter reduced the fat absorption and increased bile acid excretion indicating that chitosan inhibited the intestinal bioconversion of cholesterol and primary bile acids to secondary metabolites (Santas *et al.* 2012).

Other factor influencing on hypocholesterolemic activity of chitosan is the form and the size of particle. Powdered chitosans with a fine particle size exhibited better cholesterol-binding capacity than the flake form. The effect was more pronounced when the particles were finer, and DD and MW were relatively high (Xia *et al.* 2011). Thus, it has been shown that chitosan in form of nanoparticles with small particle size and

exceptionally large surface area for adsorbing organic compounds is effective in lowering serum lipid levels in rats (Zhang *et al.* 2011).

5.3 Antimicrobial activity

Since the first report on the fungicidal effect of chitosan on many fungi, including plant and animal pathogens (Allan & Hadwiger 1979), there have appeared many published studies on the antimicrobial activity of chitosan. Chitosan has a wide spectrum of activity and high killing rate against gram-negative and gram-positive bacteria (Shahidi *et al.* 1999). The functional groups for growth inhibition are the cationized amino groups of chitosan. Once chitosan is dissolved in diluted acid, the protonated polyelectrolyte can interact with the negative charges of macromolecules on the microbial cell surface, interfering with bacterial metabolism. Since gram-positive and gram-negative bacteria possess different cell wall structures, different mechanisms have been proposed for each type of bacteria. In gram-positive bacteria, with a continuous cell wall composed of peptidoglycan, chitosan can form a polymer membrane on the surface of the cell inhibiting the access of nutrients into the cell. In gram-negative bacteria, which cell wall is made up of a thin membrane of peptide polyglycogen and an outer membrane mainly constituted of lipoprotein, lipopolysaccharide and phospholipids, the polycationic structure of chitosan can interact with the anionic components of the outer membrane resulting in a great alteration of its structure and the release of proteinaceous material from the cell.

Other mechanisms that have been proposed are: (i) the chelation of metals, spore elements and essential nutrients (Cuero *et al.* 1991), and (ii) the interaction of chitosan with DNA and inhibition of mRNA and protein synthesis, which would occur via chitosan penetrating the nuclei of the microorganisms (Shahidi *et al.* 1999).

Rabea *et al.* (2003) did an excellent review about the use of chitosan as antimicrobial agent where they highlighted its applications against bacteria, fungi and viruses and as a potential elicitor of plant defence responses. Chitosan inhibits the growth of a wide variety of bacteria (*Agrobacterium tumefaciens*, *Bacillus cereus*, *Corinebacterium michiganence*, *Erwinia* sp., *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Xanthomonas* sp., among others).

Different works have shown the influence of chitosan structure on its antibacterial properties; indeed, an improvement of bactericidal effect against

microorganisms such as *Listeria monocytogenes* and *Salmonella typhimurium* was observed when quaternary ammonium salts chitosan derivatives were used. Besides, their incorporation in hydroxypropylcellulose matrices allowed the elaboration of effective antibacterial biopackagings. This could lead to the development of high-performance food packaging, although further research is still needed (Belalia *et al.* 2008).

Since antimicrobial activity of chitosan is a biological molar-mass dependent property, different studies have been carried out in order to evaluate the influence of MW and the polysaccharide concentration on bacteria growth. Fernandes *et al.* (2008) studied the relationship between MW of chitosans and their antimicrobial activity upon selected common food-borne spoilage and pathogen microorganisms (*Staphylococcus aureus* and *Escherichia coli*). Also, with the objective to study the possible interactions of chitosan with food ingredients, these authors studied chitosan effects upon the activity in typical liquid food products such as milk and apple juice. The results showed that antimicrobial effect of chitosan is strongly dependent on the type of target microorganisms (gram-negative versus gram-positive) and on the MW of chitosan, being higher for lower MW in the case of gram-negative bacterium, and the contrary in the case of gram-positive. The stronger antibacterial activity observed in apple juice with respect to milk matrix, suggests that the use of chitosans (irrespective of MW) may be limited to low protein content food products.

The majority of experiments carried out to study antimicrobial activity of chitosan and/or COS are focused on the study of pathogenic or industrially important species; however, scarce information is available about the effects on bacteria colonizing the gastrointestinal tract of humans or animals. Simunek *et al.* (2006) studied the antimicrobial effect of chitosan upon pure cultures isolated of human colonic bacteria and found that it markedly inhibited the growth of all bacteria strains tested. However, inhibition effects differed to a certain extent depending on the chitosan concentration and on the bacterial strain. The most susceptible to chitosan effect were bacteria belonging to genera *Bacteroides* and *Clostridium* (91-97% growth inhibition). In a later work, the study on the effect of MW of chitosan on the microbiological growth was presented (Simunek *et al.* 2012), showing that the antimicrobial activity increased with the DP, being the antimicrobial activity of COS (MW of 2 and 3 kDa) weak.

Chitosan has also been used to control postharvest fungal contamination. This polysaccharide presents fungicidal properties against several fungi, without chitin in their cellular wall, such as *Botrytis cinerea*, *Fusarium oxysporum*, *Drechstera sorokiana*, *Micronectriella nivalis*, *Piricularia oryzae*, *Rhizoctonia solani*, and *Trichophyton aquinum*. The minimum inhibitory concentration (MICs) for specific target organisms are influenced by different factors such as pH of the grown medium, DP of chitosan, and the presence or absence of interfering substances such as lipids and proteins (Rabea *et al.* 2003). In the food industry, antifungal properties of chitosan are of interest because they make it a safe biopolymer, suitable for oral administration. Rabea *et al.* (2003) observed the inactivation of 15 species of yeasts and moulds associated with food spoilage by chitosan at various concentrations, pH values and temperatures in apple juice. Besides, it has been described a nematicidal activity of chitin and chitosan derived from shrimp shell wastes against the root-knot nematode *Meloidogyne incognita*, infecting tomato in glasshouses (Radwan *et al.* 2012).

Although chitosan has been recognised for its antimicrobial properties for years, its molecular mechanism of action remains unclear, and additional work is needed to understand it more precisely (Zhong *et al.* 2012; Meng *et al.* 2012).

5.4. Antihypertensive activity

Hypertension is one of the most common cardiovascular diseases and has become a worldwide problem of epidemic proportions affecting 15-20% of adult population. Moreover, it is the most common serious chronic health problem because it carries a high risk factor for arteriosclerosis, stroke, myocardial infarction, and end-stage renal disease (Park *et al.* 2003). Angiotensin I converting enzyme (ACE) is a dipeptidyl carboxypeptidase (E.C. 3.4.15.1) and plays a crucial role in the regulation of blood pressure as it promotes the conversion of angiotensin-I to the potent vasoconstrictor angiotensin-II as well as inactivates the vasodilator bradykinin, which has a depressor action in the rennin-angiotensin system (Wijesekara & Kim 2010). Different studies have revealed that chitosan presents antihypertensive activity. Park *et al.* (2003) showed the ACE inhibitory activity of heterochitoooligosaccharides (heteroCOS) prepared from different deacetylated chitosans. As it can be appreciated in Figure 4, ACE inhibitory activity of heteroCOS depends on DD of chitosan. Later, more studies have been carried out to improve ACE inhibitory activity of chitosans through formation of different derivatives such as carboxylated (Huang *et al.* 2005), and grafted

with 2-chloroethylamino hydrochloride (Je *et al.* 2006; Ngo *et al.* 2008). Huang *et al.* (2005) revealed, through of Lineweaver-Burk plot analysis, that inhibition was competitive via obligatory binding site of the enzyme.

Wijesekara & Kim (2010) indicated that in addition to ACE, rennin also plays a vital role in the rennin-angiotensin system. Rennin (also known as angiotensinogenase) cleaves plasma angiotensinogen to angiotensin I; thereby, inhibition of rennin activity is also a target in hypertension therapy. This activity was previously described by Park *et al.* (2008) who found that COS with medium MW (1-5 kDa) and a 90% of deacetylation exerted the strongest rennin inhibition (IC₅₀ value of 0.51 mg/mL) that was dose dependent.

Xia (2003) also demonstrated antihypertensive activity of chitosan when high-salt diets were used because chitosan binds Cl⁻ and remove it, favouring blood pressure drop. Park *et al.* (2009) observed that the combination of NaCl plus 3% of chitosan may decrease the systolic blood pressure greater than the conventional use of NaCl plus KCl. However, further research is needed to identify anti-hypertensive mechanism of action of NaCl plus chitosan.

5.5. Prebiotic effects

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth of beneficial bacteria and/or by suppressing the growth of harmful bacteria in the colon, which have the potential to improve the host health (Gibson & Roberfroid 1995). Non-digestible oligosaccharides are known to promote the growth of beneficial bacteria in the colon, mainly bifidobacteria species and are the recognized as prebiotics (Mussato & Mancilha 2007). Usually prebiotics are oligosaccharides, therefore, due to their structural similarities, chitosan and COS may behave as potential prebiotics.

First research works on this topic were focused on obtaining information regarding the digestion and absorption of chitin and chitosan in the gastrointestinal tract. Okamoto *et al.* (2001) indicated a serie of works which demonstrated that about 35-83% of chitin and chitosan were digested by rabbits, and 88-98% by hens and broilers. The absorption of chitosan in the gastrointestinal tract was demonstrated by ¹⁴C-labeled chitosan; however, currently it is unclear in which site takes place the digestion. The same authors evaluated the physical changes suffered by chitosan and chitin in the canine gastrointestinal tract. Chitin did not undergo any changes in weight and shape

whereas chitosan did. Besides, chitosan was found to be affected in the stomach and large intestine but not in the small one. This study clarifies the site where chitosan is influenced in the gastrointestinal tract of dogs. Pan *et al.* (2009) investigated the ability of COS (DP 3-6 and DD 90%) to act as prebiotics using a mouse model system. Bifidobacteria and lactobacilli growth was favoured while a decrease in the concentration of unfavourable enterococci and enterobacteria in the *caecum* of mice treated with COS was observed. Authors suggested the potential of COS to be used as prebiotics in the food industry.

Other researchers have studied the effect of dietary supplementation with copper-loaded chitosan nanoparticles (100 mg/kg day) over intestinal microflora in broilers (Wang *et al.* 2011c) and piglets (Wang *et al.* 2012). In both cases, a beneficial effect over intestinal microflora and morphology was observed when compared with chlortetracycline. Results from these studies indicate the potential of these compounds as substitutes in animal diets.

Other studies have been aimed to investigate the behaviour of bacteria in presence of chitosan and/or derivatives as carbon source. Lee *et al.* (2002) investigated the growth stimulatory effect of COS on bifidobacteria and lactic acid bacteria pure culture. COS (completely deacetylated and composed of 2-8 DP oligomers) did not inhibit the growth of both bifidobacteria and lactic acid bacteria, and they did not have any antimicrobial effect. Fully deacetylated chitosan is non-digestible, while partially acetylated is degraded by lysozymes. COS had a bifidogenic effect at concentrations between 0.1 and 0.5% and a growth stimulatory effect on *Lactobacillus casei* and *Lactobacillus brevis* at a concentration of 0.1%. This shows that COS may have prebiotic properties and that they could be used as ingredients in the food industry.

Simunek *et al.* (2010) studied the *in vitro* effect of COS, chitosan and LMWC on different members of bifidobacterial family, namely, *B. adolescentis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. infantis* and *B. longum* spp. *longum*. LMWC strongly inhibited the growth of all assayed bifidobacterial strains but not inhibitory effect of COS was detected. In contrast, rather increased growth rate of *B. bifidum*, *B. catenulatum* and *B. infantis* was observed. However, the *in vitro* prebiotic effect of COS is still in doubt; Fernandes *et al.* (2012) described that neither *B. animalis* nor *L. acidophilus* were capable of using COS as a primary source of carbon.

Vernazza *et al.*, (2005) studied the fermentation properties of chitosan derivatives by the human gut microbiota using mixed culture systems in batch culture

fermenters. Low, medium and high MW chitosan and COS caused a decrease in bacteroides, bifidobacteria, clostridia and lactobacilli. To study COS metabolism, they used a three-stage fermentation model of the human colon. Different behaviour of bacteria was observed depending on the region. Groups of bacteria considered to be negative in the gut microflora increased in the gut model, principally Clostridia. COS were more easily utilised and when they were added to *in vitro* colonic model led to increase production of butyrate; however, some population of potentially detrimental bacteria also increased.

The first attempt to monitor the response of human fecal microbiota to the dietary adjuvant chitosan was performed by Mrazek *et al.* (2010). Ten healthy human subjects consumed chitosan (3 g/day) during 4 weeks and the overall bacterial composition of fecal samples was monitored. Volunteers maintained their usual diet and documented the type of food consumed; energy was not restricted. The predominant bacterial groups in the large intestine of humans belonged mainly to the genera *Bacteroides*, *Eubacterium*, *clostridium*, *Ruminococcus*, *Bifidobacterium* and *Fusobacterium*. Practically, no inhibition was found in bifidobacteria, which are beneficial for human health; however, an increase in the number of bacteroides population was observed. Therefore, from this point of view, chitosan supplementation could be considered as potentially harmful or at least no health promoting. These results suggest that chitosan influences both, the composition and the numbers of fecal bacteria.

5.6. Ion binding properties

Chitosan has the capacity to bind metals and it has been demonstrated to have the best chelating properties among natural polymers (Badawy *et al.* 2011). The interaction of chitosan with metal ions could involve several complex actions including adsorption, ion-exchange and chelation. Metal binding by chitosan can take place under acidic or near neutral pH. Under acidic conditions, the amino groups of chitosan and hydroxyl groups are protonated and interaction with metal anions proceeds by electrostatic attraction and adsorption. At pH close to neutrality, the free electron doublets on the nitrogen atoms can react with metal cations by a chelation mechanism. The process depends not only on the pH, ion type and composition of the solution, but also on the chitosan characteristics such as DD and MW. Jung & Zhao (2012) studied the chelating ability on ferrous ions of chitosan with a wide range of MW and observed

the highest values for 4–5 kDa chitosan, while 280–300 kDa chitosan did not exhibit this property.

The binding efficiency of chitosan is very useful in order to consider its application to remediate metal contaminated soil and water. Until the mid-1980s, a major application of chitosan was the treatment of wastewater. In last years, new chitosan forms have been studied to enhance the recovery of different ions of residual waters. Different solid particles coated with chitosan have been used to enhance mechanical strength of chitosan and ion binding ability. Bravo-Osuna *et al.* (2007) used core-shell nanoparticles to bind calcium, and Albarelli *et al.* (2012) used glass beads coated with chitosan for the adsorption of Cu^{2+} from aqueous solution, enhancing chitosan use for wastewater treatment. Also, chitosan/poly(acrylic acid) magnetic composite microspheres that could be easily regenerated at lower pH and reused have been used to remove Cu^{2+} from aqueous solutions (Yan *et al.* 2012). The chelating capacity of chitosan can be improved using nanofibers, with a huge surface area. They can be obtained via electrospinning of chitosan/agarose solution to reduce the viscosity of the blend (Elsabee *et al.* 2012). Chitosan can also be used for the absorption of anions, as boron species, that may interact with the NH_2 groups of chitosan at basic pH (Bursali *et al.* 2011; Wei *et al.* 2011).

As a result of the adsorption and chelation capacity of chitosan, its intake can accelerate the absorption of some minerals such as calcium, iron, zinc and copper, although there is some controversy about this possibility (Xia *et al.* 2011). Deuchi *et al.* (1995) described a decrease in the absorption of calcium and the calcium content of bones in rats after oral administration of chitosan for two weeks. Yonekura *et al.* (2004) observed that in rats with deficit of zinc originated by diet with phytic acid, the normal level of zinc was restored with an addition of 1 or 2% of chitosan to diet. In the same way, Bravo-Osuna *et al.* (2007) indicated that core-shell nanoparticles chelated with Ca^{2+} or Fe^{3+} could be used as nutritional supplement as the cations remain soluble in alkaline conditions, increasing the absorption of these ions in the intestines. More recently, Wang *et al.* (2010a, 2011b) observed a decrease in the level of iron and zinc in mice fed a diet supplement with 0.75% (w/w) of carboxymethyl chitosans or N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan during 30 days.

6. Food Applications

In the food industry, chitosan can be used in various applications such as fruit juice clarificant and desacidificant agent (Chatterjee *et al.* 2004), as flocculant in wastewater treatment (Fernandez & Fox 1997; Dutta *et al.* 2004; Bhatnagar & Sillanpää 2009), as a support in the immobilization of enzymes (Shahidi *et al.* 1999; Agulló *et al.* 2003), in the recovery of lipids and proteins from by-products (Fernandez & Fox 1997; Dutta *et al.* 2004), and for the selective separation of proteins such as β -lactoglobulin from whey cheese (Casal *et al.* 2006; Montilla *et al.* 2007). Table 3 presents the wide range of processes in which chitin, chitosan and their derivatives are used. No *et al.* (2007) did an extensive review detailing a wide number of scientific publications which report the applications of chitosan for improving quality and shelf life of foods from agriculture, poultry, fruits and vegetables such as bread, egg, mayonnaise, meat, milk, noodle, rice cake, sausage, soybean curd (tofu), soybean sprouts, starch jelly, vinegar and seafood origin.

Based on the strong antimicrobial effects and film-forming capacity of chitosan and its derivatives, they can effectively be used as food preservatives or edible coating materials to maintain quality and extend shelf-life of different food products. Coma *et al.* (2003) evaluated the potential of chitosan as a bioactive (antibacterial) packaging material against growth of two pathogens (*Staphylococcus aureus* and *Listeria monocytogenes*) and one strain involved in food alteration (*Pseudomonas aeruginosa*). The effectiveness of chitosan coating was validated on Emmental cheese. Edible coatings of chitosan and lysozyme have been used to extend shelf-life of hard boiled eggs (Kim *et al.*, 2008a). Siripatrawan & Noipha (2012) studied the feasibility of using a chitosan film incorporating green tea extract as an active packaging to extend shelf-life of pork sausages. The results showed that the incorporation of this extract enhanced antioxidant and antimicrobial properties of the chitosan and those pork sausages maintained their physical and organoleptic qualities by retarding lipid oxidation and microbial growth. In the same way, Kanatt *et al.* (2012) obtained an active film prepared from chitosan and polyvinyl alcohol containing aqueous mint extract and pomegranate peel extract with enhanced physical, mechanical, antimicrobial and antioxidant properties. These active films have potential to meet consumer demand for foods without chemical preservatives.

Kerch *et al.* (2008) studied the effect of chitosan on the structure and properties of bread (crumb and crust). They indicated that during bread staling chitosan increases

water migration rate from crumb to crust, prevents amylose-lipid complexation, and increases dehydration rate both for starch and gluten.

Other important characteristic of chitosan in food applications is its excellent emulsifying capacity, which depends on concentration, DD and MW. Cho *et al.* (1998) reported the capacity of chitosan to enhance the emulsifying properties of egg yolk. Also, this property was improved when chitosan was conjugated via Maillard reaction with proteins such as β -lactoglobulin or lysozyme (Hattori *et al.* 2000; Song *et al.* 2002).

Moreover, chitosan has also been used to encapsulate highly labile functional ingredients to improve its stability (Alishahi *et al.* 2011; Abbas *et al.* 2012) as well as polyphenolic antioxidants from medicinal plant extracts using alginate-chitosan microbeads (Belscak-Cvitanovic *et al.* 2011). Microencapsulation with chitosan-coated alginate beads can also be used to maintain the viability of probiotic microorganisms during food product processing and storage; and to provide protection in simulated gastric conditions (Rokka & Rantamaki 2010).

An interesting review of applications of chitosan in the seafood industry and aquaculture has recently been performed by Alishahi & Aider (2012).

7. Regulatory aspects

The legislation about chitosan uses as food additives varies according to the country. In USA, the Food and Drug Administration (FDA) approved its use in 1983 only as a feed additive, although in 2005 this organization accepted chitosan as a Generally Recognised As Safe (GRAS) component (Barreteau *et al.* 2006). Chitosan is approved as a food additive in Japan and Korea since 1983 and 1995, respectively, being used a preservative in many food products (No *et al.* 2007).

Currently, chitosan is not listed in the General Standard for Food Additives and has not been authorized as a food ingredient in the EU (Aranaz *et al.* 2009). The European Food Safety Authority (EFSA) asked the panel of dietetic products, nutrition and allergies a scientific opinion on the substantiation of health claims related to chitosan and reduction in body weight, maintenance of normal blood LDL-cholesterol concentrations, reduction of intestinal transit time and reduction of inflammation (EFSA 2011). The permitted health claim for chitosan is that contributes to the maintenance of normal blood cholesterol levels and the beneficial effect is obtained with a daily intake of 3 g of chitosan.

In the food industry, edible chitosan (more than 83% DD) and COS have been used as dietary food additives due to their interesting properties. In China and Norway, functional products based on chitosan and COS capsules are available. Also, an edible chitosan biofilm has been prepared for food storage because of its antimicrobial activity (Xia *et al.* 2011).

8. Conclusions

This chapter highlights the large amount of research about properties and applications of chitosan and its derivatives. However, further studies are needed in the fields of analytical and biological research. The establishment of analytical methodology for appropriate analysis and purification, as well as characterization of chitosan and derivatives structures is necessary. The understanding of chitosan structure may provide insights into the unknown biochemical functions as well as to enlarge their range of applications. Also, research should be oriented to obtain new chitosan derivatives with improved biological and/or functional properties.

The use of chitosan as a functional ingredient in foods must still save legal barriers; therefore, further research to obtain more data on their efficacy and safety should be carried out, in order to fulfil the requirements of legal organization (in Europe and in worldwide) to include chitosan, COS and their derivatives as food additives.

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Figure legends

Figure 1.- Chemical structures of cellulose and chitin (Reproduced with permission from Kurita 2001).

Figure 2.- Chitin and chitosan production flow scheme (Reproduced with permission from Radwan *et al.* 2012).

Figure 3.- Multifaceted derivatization potential of chitin/chitosan (Reproduced with permission from Prashanth & Tharanathan 2007).

Figure 4.- ACE inhibitory activity of COSs with different molecular masses prepared from 90, 75, and 50% deacetylated chitosan. HMWCOSs: high molecular weight chitooligosaccharides; MMWCOSs: medium molecular weight chitooligosaccharides; LMWCOSs: low molecular weight chitooligosaccharides (Reproduced with permission from Park *et al.* 2003 with some modifications).

Table 1. Main types of treatments used for chitin production

Chitin production	Demineralization	Deproteinization	Advantages	Drawbacks
Chemical method	Inorganic acids	Strong alkali	<ul style="list-style-type: none"> - Efficient - Fast - Cheap 	<ul style="list-style-type: none"> - Source of pollution - Produce depolymerization - Reduce chitin quality - Produce deacetylation - Reduce molecular weight - Renders the protein content useless (cannot be used as animal feed or nutritional additive)
Biological method	Organic acids-production bacteria	Protease-producing bacteria	<ul style="list-style-type: none"> - Protein and organic acid salts are recovered for feed, fertilizer and chemical reagents purposes - Reduce environmental pollution - Reduce chitin depolymerization 	<ul style="list-style-type: none"> - Lower efficiency and quality - Time- consuming - Higher cost

Table 2.- Potential applications of chitin, chitosan and their derivatives.

FIELDS	APPLICATIONS	REFERENCES
Food /Nutrition	Preservative agent; Edible film; Fining, texture controlling, emulsifying and natural flavor agent; colour stabilizer; Recovery of solid materials. Dietary fibre. Hypocholesterolemic agent; Reduction of lipid absorption; Antimicrobial; Antioxidant; Prebiotics; Accelerating the calcium and ferrum absorption; Antigastritis agent. Use in lactose intolerance; infant fee ingredient. Livestock and fish feed additive. Production of single cell protein.	Shahidi et al. (1999); Agulló et al. (2003); Chien <i>et al.</i> (2007); Prashanth &Tharanathan (2007); Aranaz <i>et al.</i> (2010); Vidanarachchi <i>et al.</i> (2010); Xia <i>et al.</i> (2011); Ying <i>et al.</i> (2011)
Pharmaceutical and biomedical	Wound dressing; accelerator for wound healing; tissue engineering: Artificial skin; Burn treatment, Skin grafts,; Suture threads. Scaffolds for cartilage and bone replacement therapies. Drug delivering systems (oral, parenteral, nasal, ocular, pulmonary, transdermal administration), DNA delivery system, gene delivery. Oral hygiene, periodontal applications. Protective effect on bacterial infection. Immuno-enhancing agent. Antitumor agent. Antiulser agent. Blood anticoagulants. Use in anticholesterolemic drugs. Contact lens; Excipient; Orthopaedic; Radiopharmaceuticals.	Dutta et al., (2004) Prashanth &Tharanathan (2007); Aranaz <i>et al.</i> (2010); Vidanarachchi <i>et al.</i> (2010); Xia <i>et al.</i> (2011); Luangbudnark <i>et al.</i> (2012)
Cosmetics	Maintain skin moisture; Protect epidermis; Acne treatment ; Tone skin. Reduce static electricity in hair.	Dutta et al., (2004) Aranaz <i>et al.</i> (2010); Ying <i>et al.</i> (2011)

Biotechnology	Carrier for immobilized enzymes and cells; heterogeneous catalyst. Water engineering. Porous beads for bioreactors. Molecular Imprinting. Textiles. Nanomaterials. Biosensors. Resin for chromatography; Membrane material; Paper industry.	Dutta et al., (2004) Aranaz <i>et al.</i> (2010); Xia <i>et al.</i> (2011)
Agriculture	Gene elicitor. Antibacterial; Antifungal. Seed coatings. Activator of plant cells. Extension of cut flowers life.	Rabea et al., (2003) Aranaz <i>et al.</i> (2010); Xia <i>et al.</i> (2011)
Miscellaneous	Wastewater treatment; Flocculating agent; Reverse osmosis membranes. Polymeric nanoparticles; Synthetic polymer blends. Animal feed.	Dutta et al., (2004) Prashanth & Tharanathan (2007); Xia <i>et al.</i> (2011); Vidanarachchi <i>et al.</i> (2010)

Table 3.- Food application of chitin, chitosan and their derivatives in the food industry
(From Shahidi *et al.* 1999 with some modification).

AREA OF APPLICATION	EXAMPLES
Antimicrobial agent	Bactericidal Fungicidal Measure of mould contamination in agricultural commodities
Edible film industry	Controlled moisture transfer between food and surrounding environment Controlled release of antimicrobial substances Controlled release of antioxidants Controlled release of nutrients, flavours and drugs Reduction of oxygen partial pressure Controlled rate of respiration Temperature control Controlled enzymatic browning in fruits Reverse osmosis membranes
Additive	Clarification and deacidification of fruits and beverages Natural flavour extender Texture controlling agent Emulsifying agent Food mimetic Thickening and stabilizing agent Colour stabilization
Nutritional quality	(See Table 2)
Recovery of solid materials from food processing wastes	Affinity flocculation Fractionation of agar
Purification of water	Recovery of metal ions, pesticides, phenols and PCB's Removal of dyes
Other applications	Enzyme immobilization Encapsulation of nutraceuticals Chromatography Analytical reagents