CHITIN, CHITOSAN, AND CO-PRODUCTS: CHEMISTRY, PRODUCTION, APPLICATIONS, AND HEALTH EFFECTS

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I. INTRODUCTION

Chitin is derived from the Greek word *chiton*, which means a coat of nail. It is the major component of the exoskeleton of invertebrates, crustaceans, insects, and the cell wall of fungi and yeast (Knorr, 1984; Lower, 1984; Tan *et al*., 1996) in which chitin acts as a supportive and protective component. Chitin is the second most plentiful natural polymer on earth after cellulose (Brzeski, 1987; Ornum, 1992). At least 10 gigatons (1×10^{13} kg) of chitin

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is produced and hydrolyzed each year in the biosphere (Muzzarelli, 1999). Chitin, poly-(164)-*N*-acetyl-D-glucosamine, is a cellulose-like biopolymer found in a wide range of products in nature (Shahidi *et al*., 1999). It was discovered in 1811 by a French scientist Henri Braconnot, who isolated it from mushroom (Winterowd and Sandford, 1995). In 1823 Odier found the same compound in the cuticles of insects (Muzzarelli, 1977; Winterowd and Sandford, 1995). The biosynthesis of chitin occurs in the membrane-bound protein complex chitin synthase. In arthropod outerskeleton and most of the fungi, uridine diphosphate-*N*-acetyl-D-glucosamine is polymerized into chitin by chitin synthase (EC 2.4.1.16) (Hirano, 1996).

Chitosan, a copolymer of D-glucosamine and *N*-acetyl-D-glucosamine with β-(164) linkage, is obtained by alkaline or enzymatic deacetylation of chitin and is an abundant polymeric product in nature. Chitosan was first discovered by Rouget in 1859 when he heated chitin to the boiling point in a concentrated KOH solution (Dunn *et al*., 1997). Chitosan is found in different morphological forms such as a primary, unorganized structure, crystalline and semicrystalline forms. For different reasons, especially problems of environmental toxicity, these two biopolymers are considered interesting substances for producing polymers (Sorlier *et al*., 2001). Because of their unique structures, they possess high biological and mechanical properties as they are biorenewable, biodegradable, and biofunctional (Hirano *et al*., 2000). Two methods, namely chemical and enzymatic, are known for preparation of chitin, chitosan and their oligomers, with different degrees of deacetylation, polymerization, and molecular weight (MW). Chitin, chitosan, and their oligomers can be produced chemically using concentrated HCl followed by column chromatographic fractionation (Jeon *et al*., 2000). Three methods are known for modification of the process of isolation of chitin and chitosan oligomers (Jeon *et al*., 2000). These are acetolysis, fluorohydrolysis, and sonolysis. Meanwhile, chitin and chitosan oligomers can be prepared through microbiological and fungal treatments (enzymatic preparation). Certain enzymes are involved in the degradation of chitin and chitosan such as chitinases and chitosanases, respectively, and the process is environmentally friendly. Chitin, chitosan, and their oligomers have different applications such as medical uses as a wound-healing agent, dietary uses as hypocholesterolemic agents, antitumor, and antiulcer agents, and as a coating of artificial parts of the body such as leg, tooth, and arm, among others. They may also be used in food preservation such as for seafoods (Shahidi *et al*., 1999) and fruits (EL-Ghaouth *et al*., 1992c), as well as for acidity adjustment (Scheruhn *et al*., 1999), and as antibacterial and antifungal agents (Shahidi *et al*., 1999).

The following section presents the chemistry and applications of chitin, chitosan and their oligomers.

II. CHEMISTRY

A. STRUCTURE, PHYSICAL, AND CHEMICAL PROPERTIES OF CHITIN AND CHITOSAN

Chitin is a high-molecular-weight polymer of *N*-acetyl-D-glucosamine (NAG) units linked by β-D (164) bonds, having 1000–3000 units (Lower, 1984). The chemical structure of chitin is the same as that of cellulose, with the hydroxyl group at position C2 replaced by an acetamido group (Figure 1). Chitin can be deacetylated to produce chitosan, which is soluble in dilute acidic solutions (Table I) and is highly viscous when dissolved; this

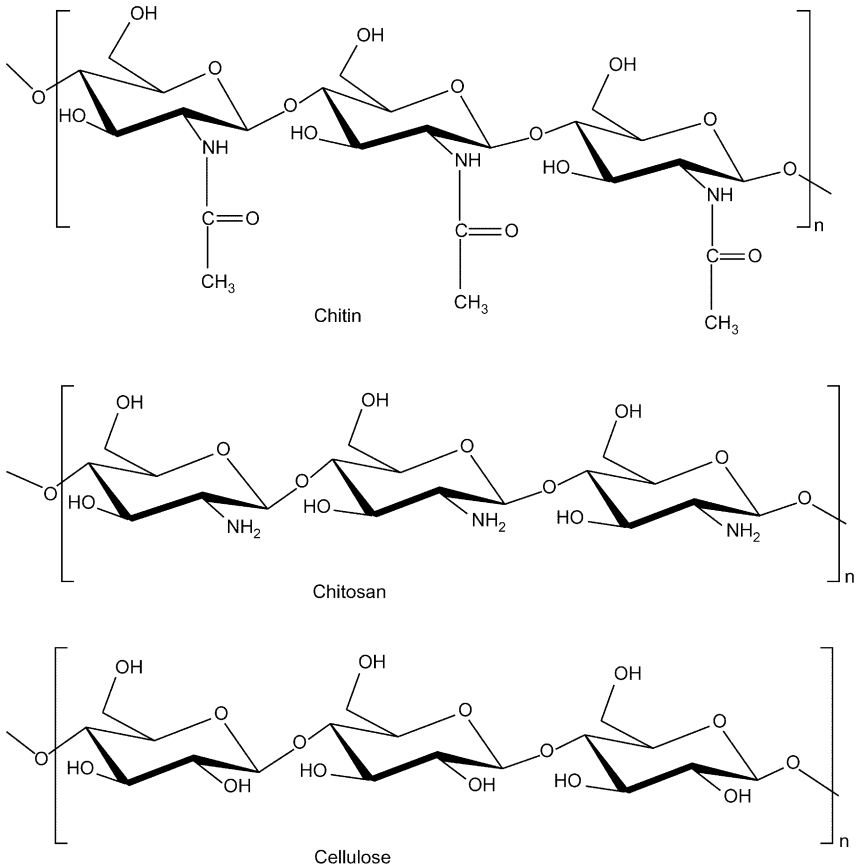


FIG. 1 The chemical structures of chitin, chitosan, and cellulose.

TABLE I
COMMON SOLVENTS FOR CHITIN AND CHITOSAN

Compound	Solvent
Chitin	Dimethylformamide/lithium chloride; diethylformamide/lithium chloride; hexafluoroisoacetone sesquihydrate; hexafluoroisopropanol (Capozza, 1975); 1,2-chloroethanol/sulfuric acid (Austin, 1975)
Chitosan	Formic acid/water; acetic acid/water; lactic acid/water; glutamic acid/water, etc.

makes it distinctly different from chitin (Jeon *et al*., 2000; Shahidi *et al*., 1999).

Chitosan has many useful applications in different fields, mainly because of the presence of amino groups at the C2 position, and because of the primary and secondary hydroxyl groups at the C3 and C6 positions, respectively (Furusaki *et al*., 1996; Kurita, 1986). Chitosan is the simplest and least expensive derivative of chitin (Ornum, 1992). Unlike with most polysaccharides, the presence of positively charged amino groups repeatedly placed along the chitosan polymer chain allows the molecule to bind to negatively charged surfaces via ionic or hydrogen bonding (Muzzarelli, 1973; Rha, 1984; Shahidi, 1995). The term *chitosan* is favored when the nitrogen content of the molecule is higher than 7% by weight (Muzzarelli, 1985) and the degree of deacetylation (DD) is more than 70% (Li *et al*., 1992).

B. CHEMICAL REACTIONS OF CHITIN AND CHITOSAN

1. Neutralization

Chitin and chitosan are weak bases. They go through the usual neutralization reactions of basic compounds. The non-bonding pair of electrons on the primary amino group of the glucosamine unit accepts a proton, and thus becomes positively charged (Winterowd and Sandford, 1995).

2. Nucleophilic reactions

Chitosan is a strong nucleophile because of the presence of a nonbonding pair of electrons on its primary amino groups. Chitosan reacts readily with most aldehydes to produce imines (Kurita *et al*., 1988). It also reacts with acyl chlorides to form the corresponding acylated derivatives (Hirano *et al*., 1976) (Figure 2).

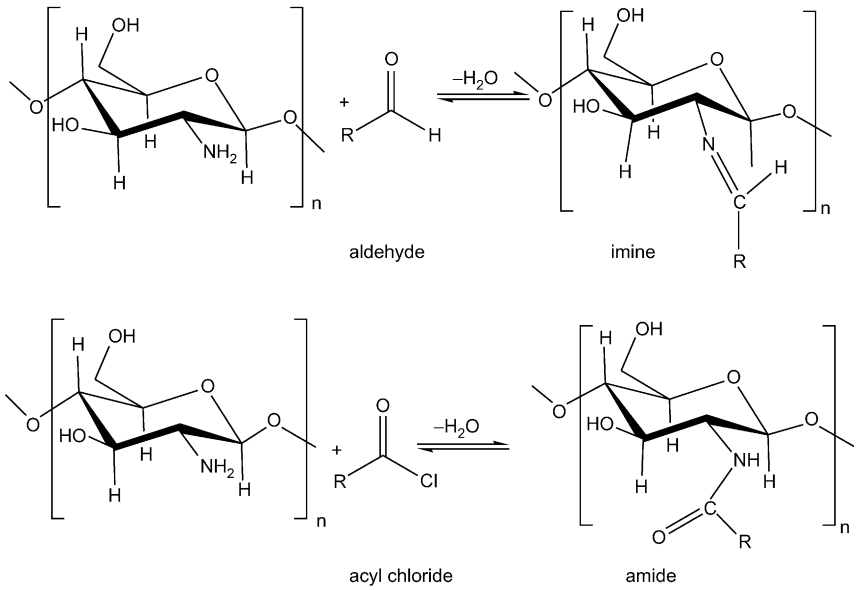


FIG. 2 Reactions of chitosan with an aldehyde or acyl chloride.

3. *Acid- or base-assisted hydrolysis*

Chitin and chitosan are labile to acid- or alkaline-assisted degradation. Under acidic or basic conditions, acetic acid can be freed as *N*-acetyl groups at the C2 positions of *N*-acetyl glucosamine units are released, leaving behind primary amine groups (Muzzarelli, 1977). In addition, presence of the primary amino groups in chitosan presents further potentials for modification of the molecule such as *N*-acylation, *N*-alkylation, and *N*-alkylidenation. Acidic conditions also cause some degree of depolymerization as degradation of the β-glycosidic bonds occurs (Madhaven and Ramachandran, 1974). Depolymerization under basic conditions occurs, but to a lesser extent, and chitosan can be hydrolyzed using nitrous acid (Allan and Peyron, 1989) (Figure 3).

4. *Sulfation reactions*

Although most reactions of chitosan involve the primary amino groups, it is possible to selectively derivatize the hydroxyl groups. This can be done by protecting the amino groups via reaction with formic or acetic acid to produce a polysaccharide formate or acetate salt. The chitosan formate or acetate salt may then be reacted with an electrophile (Muzzarelli, 1977).

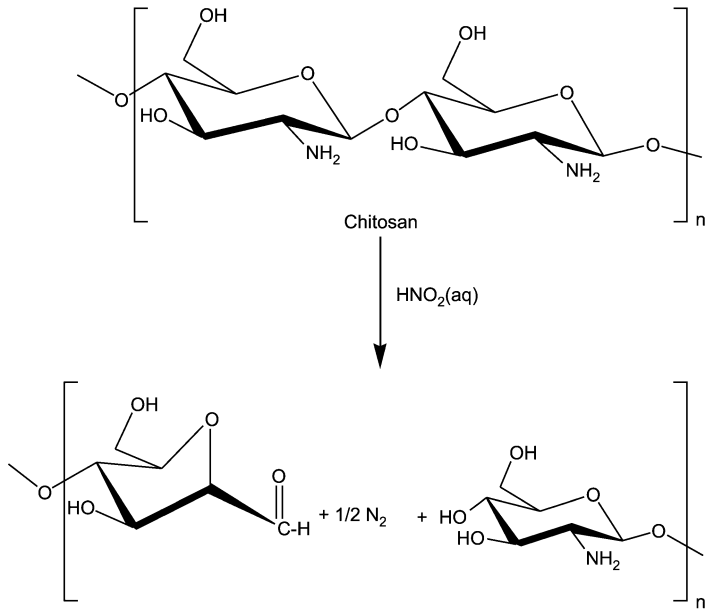


FIG. 3 Hydrolysis of chitosan with aqueous nitrous acid.

The hydroxyl group at the C6 position is more active than that at the C3 position and is, therefore, derivatized favorably. Figure 4 shows the sulfation as a common example of this type of reaction. When this type of reaction (Figure 4) is conducted on chitin, the end products behave like heparin, an anti–blood-clotting agent (Wolform and Shen-Han, 1959).

5. *Heavy metal complexes*

Chitin and chitosan are capable of forming complexes with many of the transition metals and some of those from groups three through seven of the periodic table (Muzzarelli, 1973). The heavy metal complexes are supposed to form as a result of donation of a nonbonding pair of electrons on the nitrogen and/or on the oxygen of the hydroxyl groups to a heavy metal ion. Cupric ion appears to form one of the strongest metal complexes with chitosan in the solid state (Domard, 1987; Kentaro *et al.*, 1986; McKay *et al.*, 1986). Koshijima *et al.* (1973) observed that ferrous ion has the ability of binding to chitosan. Under experimental conditions (100 mg of powdered chitosan mixed with a solution of ferrous nitrate [25 mg] in 50 ml of water, at 30 °C, and reaction time of 74 hours), about 28% of the ferrous ions were complexed with chitosan. The rate of formation and stability of these

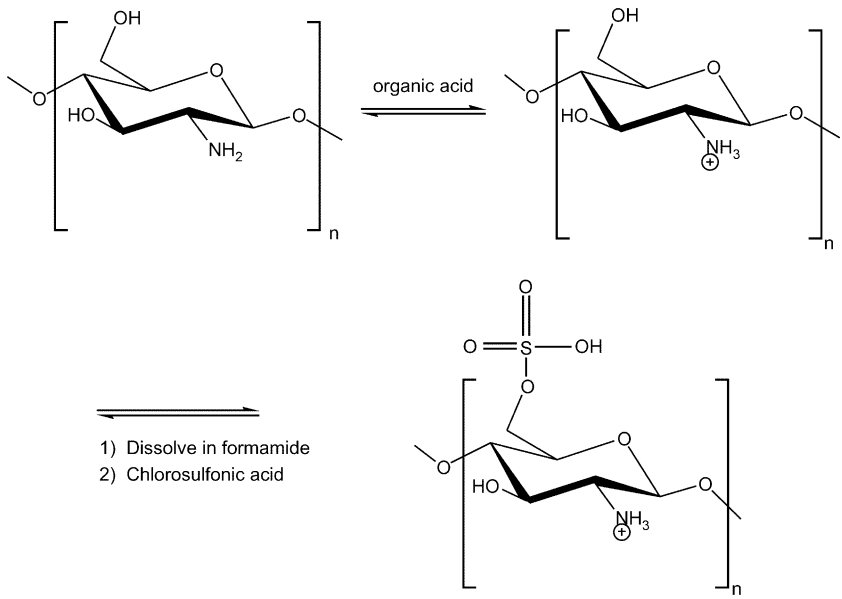


FIG. 4 Sulfation of chitosan in the presence of an organic acid such as formic or acetic acid.

complexes is affected by the presence of counterions, competing heavy metal ions, temperature, and pH of the solution, as well as particle size, crystallinity, and the degree of *N*-acetylation of chitin and chitosan (Winterowd and Sanford, 1995).

C. SOLUBILITY

The most remarkable difference between chitin and chitosan is their solubility characteristics. There are few solvents for chitin, whereas almost all aqueous acids dissolve chitosan (Table I). Most solvents used for dissolution of chitin are toxic (Table I) and hence cannot be used in food processing applications. Nonetheless, when chitin is ground to a fine mesh, it could be used to increase viscosity of liquids. Solvents for chitosan are generally safe to consume, allowing the formation of solutions that are appropriate for gel production. Thus, chitosan is better matched to the viscosity of foods (Winterowd and Sandford, 1995). The solubility characteristics of chitosan are governed mostly by the extent of *N*-acetylation, the distribution of acetyl groups, the pH, and the ionic strength (Anthonsen *et al.*, 1993). The amino group in chitosan has a pKa value of 6.2 to 7.0, which makes chitosan a

polyelectrolyte at low pH values (Claesson and Ninham, 1992). It was reported that chitin molecules with a highly deacetylated chitin with a number of acetylated chitin joining each other in a block, and then a number of units with free amino groups in a block could produce products that are soluble in water (Kristbergsson *et al.*, 2003). However, no details are available in the nonproprietary literature in this regard.

The solubility problems associated with chitin and chitosan may limit their use in physiological and functional foods. The intestines of most animals lack the ability to produce chitinase and chitosanase. These two enzymes have the ability to hydrolyze chitin and chitosan, respectively. Therefore, they will be excreted unchanged in the feces. On the other hand, chitin and chitosan oligomers are considered to have more physiological functions because they are water soluble and their solutions are less viscous, so they are readily absorbed in the human intestine (Jeon *et al.*, 2000).

1. Molecular weight of chitin and chitosan

The MW of natural chitin is normally higher than 1,000,000 Da and that of commercially available chitosan is around 100,000–1,200,000 Da (Li *et al.*, 1992; Lower, 1984). Numerous forces during commercial production may influence the MW of chitosan. Factors such as high temperature (>280 °C thermal degradation of chitosan occurs and the polymer chains quickly break down), dissolved oxygen concentration, and shear stress may cause these changes to occur (Li *et al.*, 1992; Muzzarelli, 1977).

D. PREPARATION OF CHITIN AND CHITOSAN

Two hydrolytic methods were reported to prepare chitin and chitosan. These are acid hydrolysis (chemical treatment) and enzymatic hydrolysis (Shahidi *et al.*, 1999).

1. Chemical treatment

The normal procedure for preparation of chitin from crustacean shells includes the use of NaOH, HCl, and decoloring agents to remove the remaining proteins, calcium, and color, respectively. The chitin that is produced can then be deacetylated with sodium hydroxide to produce chitosan (Tsai *et al.*, 2002). Jaworska and Konieczna (2001) reported that chitosan can be prepared via chemical means using concentrated hydroxides (40–50%) at high temperatures (100–130 °C).

Oh *et al.* (2001) reported that deacetylation proceeds rapidly during the first hour of treatment with 50% NaOH at 100 °C and the product is 68%

deacetylated. This is followed by a slower step, and by the end of 5 hour, about 78% deacetylation is achieved. Increasing time does not deacetylate chitin any further, but it lowers the MW of the product.

The resulting chitosans from both chemical and enzymatic methods are different with respect to their DD, distribution of acetyl groups, chain length, and conformational structure of chitin and chitosan molecules. These factors together will affect the characteristics of chitin and chitosan. Optimum conditions for chitosan pretreatment (deacetylation by 45% alkali solution for 1 hour) were studied by investigating the coagulation efficiencies of chitosan prepared under different conditions (Huang *et al.*, 2000). The procedure involved crushing crab shells to a powder and isolating the chitin. The next step included deacetylation of chitin using NaOH at 100 °C (Kamil *et al.*, 2002) followed by rinsing the product several times with deionized water to reach a pH level of 7, and finally drying at 80 °C for 48 hours. The resulting chitosan was dissolved in different concentrations of acetic acid and hydrochloric acid, stirring at room temperature, until it was completely dissolved. It was noticed that as the concentration of acid increased, the viscosity of dissolved chitosan coagulants decreased due to binding of positively charged chitosan to the negatively charged acid anion in the solution. The conformation of chitosan polymers changes and becomes more compact in the acidic solution and thus lowers the viscosity of the solution; the best solution was obtained at a pH level of 2.

a. Effect of degree of acetylation, degree of deacetylation, and molecular weight of chitosan and its activity. Chitin can be found with varying degrees of acetylation (DA), ranging from fully acetylated to totally deacetylated. The degree of acetylation is very important because of its effects on physical properties of chitin. For example, as the degree of acetylation increases, the degree of solubility in different solvents decreases. Oh *et al.* (2001) reported that the DD of chitosan is affected by the concentration of alkali, temperature, reaction time, previous treatment of chitin, particle size, and chitin concentration.

Heux *et al.* (2000) found that after partial deacetylation (<50%), the product of chitin becomes soluble in acidified water. Therefore, chitosan is characterized by its DA, which is the average mole fraction/percentage of *N*-acetyl-D-glucosamine units within the macromolecular chain (Desbrieres, 2002). Alternatively, Heux *et al.* (2000) calculated DA by measuring all carbonyl or methyl groups divided by the integral of all the carbon atoms in the backbones. The DD may be determined by a titration method in which chitosan is dissolved in 0.1% acetic acid to form a 0.01% solution. This is followed by titration with 0.0025 N poly (vinyl sulfate) potassium salt (PVSK) with 1% toluidine blue (TBO) as an indicator. The acetyl content of

chitosan was measured from the amount of titrant used (Huang *et al.*, 2000). Many techniques are used to evaluate the average degree of acetylation of chitosan, such as infrared, solid-state nuclear magnetic resonance (NMR), ultraviolet spectrometry and potentiometric titration, ^{1}H liquid-state NMR, and elemental analysis (Heux *et al.*, 2000), as well as ^{13}C solid-state NMR and elemental analysis; these techniques do not require solubilization of the polymer. Three techniques are used for evaluation of DA of chitin and chitosan over the whole range of DA. These are ^{13}C and ^{15}N cross-polarization/magic angle spin (CP-MAS) solid-state NMR and ^{1}H liquid-state NMR. These three methods are found to afford results in good agreement, but the limitation of solid-state NMR is that it requires a detection threshold not higher than 5%. Meanwhile, the ^{15}N CP-MAS technique was found to be a powerful technique to assess the acetyl content in the case of complex association of chitin and other polysaccharides (Heux *et al.*, 2000).

Circular dichroism and viscometric methods have been used successfully to determine the degree of acetylation and the MW of chitin and chitosan, respectively (Zhang and Neau, 2001). The DD has no effect on the acid-binding properties of chitosan (Scheruhn *et al.*, 1999). Chitosans have a relatively high DD and strongly enhance fibroblast proliferation, whereas chitosans with lower levels of deacetylation show less activity. The MW and polymer chain length were of little importance (Howling *et al.*, 2001).

Many authors have considered the MW of chitosan to have an important effect on its activity. Chitosan preparations with a MW of 5–50 kDa had the ability to reduce serum cholesterol levels in rats (Ikeda *et al.*, 1993). Meanwhile, it has been reported that chitosan with an MW of 8 kDa was more effective as a hypocholesterolemic agent in rats than chitosan with a MW of 2 or 220 kDa (Enomoto *et al.*, 1992). Oh *et al.* (2001) reported that chitosan with an MW of 12,000 Da (DDA, 87%) was most effective against *L. fructivorans,* and chitosan with an MW of 32,500 Da (DDA, 80%) was most effective against *L. plantarum*. The MW of chitosan had no effect on *S. liquifaciens*. From these results, it is clear that there is a relationship between the type of microorganism and antimicrobial activity of different MW chitosans. Recent studies have reported that chitosans with an average MW of more than 10 kDa have a positive effect on enhancing fecal excretion of neutral steroids. Moreover, as the viscosity or the DD of chitosan preparation increased, the more clear the effects on the apparent fat digestibility became (Ylitalo *et al.*, 2002). Tsai *et al.* (2002) explained that with an increase in the DD and hence the number of NH_2 groups, the antimicrobial activity of chitosan became stronger. This result agrees with the findings of Chang *et al.* (1989), Darmadji and Izumimoto (1994), Simpson *et al.* (1997), and Wang (1992).

b. Depolymerization. Thermal depolymerization of chitosan chloride in solid state has been examined (Holme *et al*., 2001). After depolymerization, the apparent and intrinsic viscosity values were measured. Intrinsic viscosity data indicated that the initial constants for chitosan were clearly increasing with the increasing degree of acetylation, which is an important parameter for thermal degradation. The presence of oxygen had no effect on the rate of chitosan degradation, whereas pH had a very important effect on the degradation of chitosan. Moreover, acid hydrolysis was the primary mechanism involved in thermal depolymerization of chitosan chloride in the solid state (Holme *et al*., 2001). Chitosan, like other polysaccharides, is influenced by several degradation mechanisms, including oxidative-reductive free radical depolymerization, and acid-, alkaline-, and enzymatic-catalyzed hydrolysis (Holme *et al*., 2001).

Degradation of polysaccharides usually occurs via cleavage of glycosidic bonds; it is very important to control the depolymerization of chitosan to maintain other properties such as viscosity, solubility, and biological activity. It has been reported that the decomposition (release of material) of chitosan starts at 200 °C. However, Holme *et al*. (2001) reported that chitosan chlorides were thermally degraded at 60, 80, 105, and 120 °C. They also found that the degradation rate of chitosan increased by acid hydrolysis with increasing temperature and degree of acetylation (Holme *et al*., 2001).

c. N-*acetylation.* Hirano *et al*. (2000) reported that the filament surface and inside chitosan fibers were *N*-acylated by treatment with a series of carboxylic anhydrides in methanol at room temperature. The *N*-acylation has little effect on mechanical properties of chitin filaments such as tenacity and elongation values. Treatment of chitin fiber and chitin–cellulose mixed fiber with 40% NaOH at 95–100 °C for 4 hours in suspension afforded a chitosan fiber and a novel cellulose–chitosan mixed fiber, respectively. Novel *N*-acylchitosan fibers produced were *N*-acetyl, *N*-propionyl, *N*-butyryl, *N*-hexanoyl, and *N*-octanoyl chitosans. These fibers were insoluble in water and aqueous basic and acidic solutions.

d. Comb-shaped chitosan. Chitosan has a considerable advantage over chitin for modification purposes because it possesses free amino groups. *N*-substituted chitosan derivatives may be obtained using reducing sugars, aldehydes, or ketones via reductive alkylation, which is a typical example of reactions of chitosan. The comb-shaped chitosan derivatives have been prepared by reductive alkylation with monoaldehydes that were synthesized from tri and tetra (ethylene glycol) monosubstituted derivatives. The introduction of such branches clearly increased the affinity of molecules for both water and organic solvents without loosening the attractive characteristics of

chitosan, such as metal ion adsorption capacity (Kurita *et al.*, 1999). To prepare comb-shaped chitosan derivatives, chitosan may be completely deacetylated via treatment with monoaldehyde derived from tri(ethylene glycol) under homogeneous conditions in an acetic acid-methanol solution. Sodium cyanoborohydride was added to the solutions, which afforded a weak gel. Then the resultant mixtures were dialyzed against deionized water to afford clear solutions, then concentrated and freeze-dried; the compounds obtained were slightly yellowish solids (Kurita *et al.*, 1999).

e. N-*alkylation.* Chitin remains in nature because of its lack of solubility, except in fluorinated solvents, *N*,*N*-dimethylacetamide/LiCl, and methanol/$CaCl_2$. Meanwhile, randomly 50% deacetylated chitin and chitin derivatives having tosyl, iodo, trimethylsilyl, and glycosyl groups are soluble in water or organic solvents (Kurita *et al.*, 2002). The *N*-alkylation of chitin is a potential process for preparation of simple chitin analogues with lowered crystallinity and for improving solvent affinity. The resulting structure around C2 is analogous to that of *N*,*N*-dimethylacetamide, which is an exceptionally good solvent, exhibiting high affinity for a wide variety of substances. Many experiments were conducted to synthesize polymers having *N*,*N*-dimethylacetamide moieties in their backbone by ring-opening polymerization of 2-oxazolines.

Kurita *et al.* (2002) succeeded in introducing alkyl groups, such as methyl, ethyl, and pentyl groups, into chitin at the nitrogen of C2 acetamido moiety via an adjusted five-step modification process (Figure 5). Chitosan was completely deacetylated and treated with three types of aldehydes, namely formaldehyde (methanol), acetaldehyde (ethanol), and valeraldehyde (pentanol). The Schiff bases of chitosan were reduced to *N*-alkylated chitosan using sodium cyanoborohydride ($NaCNBH_3$).

The *N*-alkyl chitosans were subsequently changed into corresponding *N*-alkyl chitins via acetylation using acetic anhydride followed by transesterification to eliminate partially formed *O*-acetyl groups. This synthetic pathway is direct and effective to provide well-defined novel chitin derivatives. The resulting *N*-methyl, *N*-ethyl, and *N*-pentyl chitins were amorphous and displayed a high affinity for solvents (Kurita *et al.*, 2002).

i. Trapping: Retention of heavy metals. Cardenas *et al.* (2001) described a method for preparing chitosan mercaptan derivatives with mercaptoacetic acid and 1-chloro-2,3-epoxypropane and evaluated their retention capacities using different concentrations of copper and mercury. Chitosan (5.0 g) was dissolved in 50 ml of mercaptoacetic acid followed by addition of benzene (100 ml) and kept under reflux for 46 hours. The solid product was washed with benzene and ethanol and then treated with 5% NaOH and water at a

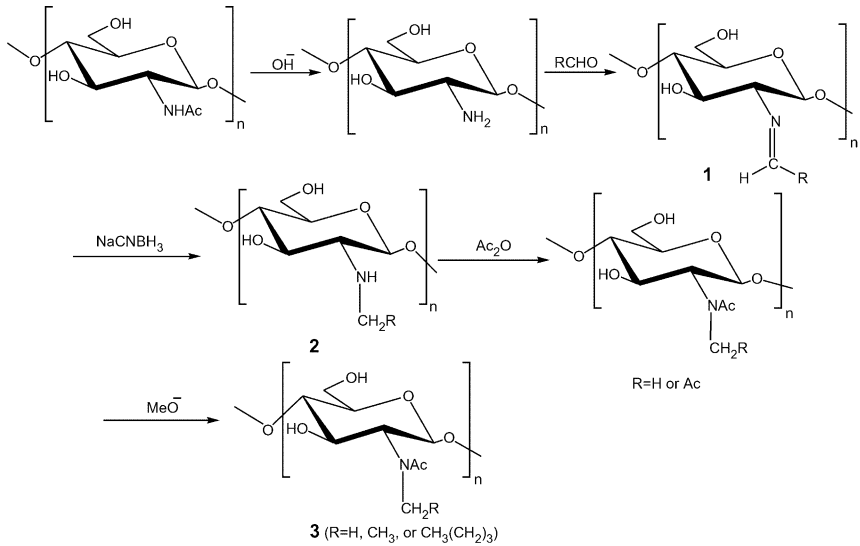
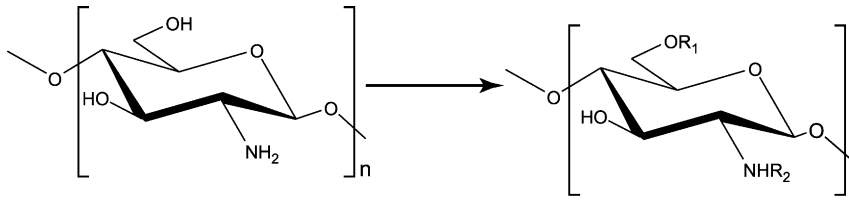


FIG. 5 Schiff base formation with aldehydes, reduction, and *N*-acetylation of chitin. Ac, acetyl; Ac_2O, acetic anhydride.

neutral pH level. The solid was dried under vacuum at 50 °C for 2 hours. These derivatives are shown in Figure 6 and include *N*-hydroxy-3-mercaptopropylchitosan (chitosan 1), and *N*-(2-hydroxy-3-methylaminopropyl-chitosan, (chitosan 2). Thermal stability studies showed that all chitosan derivatives were thermally stable; *N*-hydroxy-3-mercaptopropylchitosan showed the highest thermal stability at 314 °C compared with chitosan at 290 °C. The adsorption behavior of chitosan derivatives for heavy metal ions was investigated and the results showed that chitosan 1 was better for both Cu and Hg; there was a decline in Cu adsorption, from a pH of 4.5 to a pH of 2.5. The highest mercury adsorption was at 556 mg/g (concentration of ion adsorbed per gram of adsorbent) at a pH level of 2.5 and 588 mg/g at a pH level of 4.5. Chitosan 2 showed better adsorption for Hg at a pH level of 4.5 than that at a pH level of 2.5. The copper ion adsorption was less than that of mercury ions at a pH level of either 2.5 or 4.5, suggesting a lower selectivity for Cu (Cardenas *et al.*, 2001).

2. *Enzymatic hydrolysis*

The enzymatic hydrolysis of chitin and chitosan might occur because of the action of chitinases, chitosanases, lysozymes, and cellulases (Shahidi *et al.*, 1999). The products of chitin hydrolysis are of high degree of polymerization



R_1=H; R_2= -$CH_2CHOHCH_2SH$ chitosan (1)

R_1=H; R_2= -$CH_2CHOHCH_2NHCH_3$ chitosan (2)

R_1=R_2= -$COCH_2SH$ chitosan (3)

FIG. 6 Production of mercaptan derivatives of chitosan via conversion with 1-chloro-2,3-epoxypropane.

(DP). Tsigos *et al.* (2000) reported the necessity for pretreatment (alkali treatment) of crystalline chitin before adding the enzyme to increase the rate of deacetylation to produce new polymers with new physical and chemical characteristics. The compounds are easily soluble if produced with different distribution of *N*-deacetylated residues. A synthetic procedure for chitin with *N*-acetyl-D-glucosamine and chitosan derivatives with D-glucosamine branches has been reported (Kurita *et al.*, 2000). These resulting nonnatural branched chitin and chitosan have extra amino sugars in branches that render them much improved properties in comparison with linear ones, such as the affinity for solvents and hygroscopicity. These characteristics would be of great interest in different applications, such as moisturizers for cosmetics and antimicrobial substances for fiber and textile treatment (Kurita *et al.,* 2000).

E. PREPARATION OF CHITIN AND CHITOSAN OLIGOMERS

Preparation of oligomers of chitin and chitosan may be carried out by acid hydrolysis or biological hydrolysis (Figure 7).

1. Preparation of chitin oligomers by chemical hydrolysis

Several reports on chemical hydrolysis (including acid hydrolysis) for preparation of chitin oligomers have been cited (Bosso *et al.*, 1986; Defaye *et al.*, 1989; Hirano and Nagano, 1989; Inaba *et al.*, 1984; Kendra *et al.*, 1989; Kurita *et al.*, 1993; Rupley, 1964; Sakai *et al.*, 1990; Takahashi *et al.*, 1995). A series of chitin oligomers, up to hexamer, has been prepared by

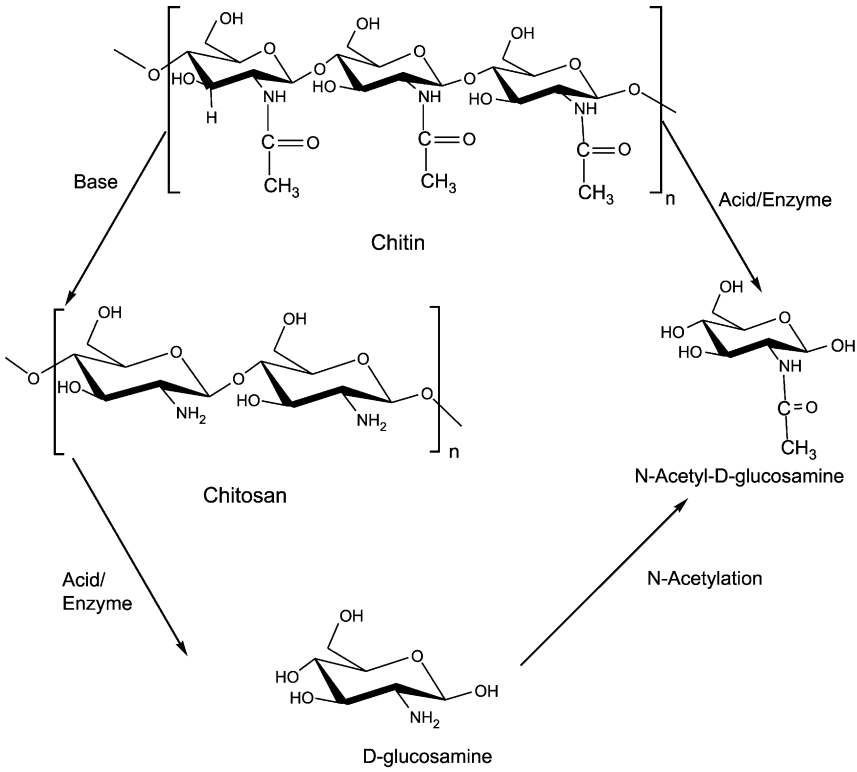


FIG. 7 Preparation of products from chitin.

partial hydrolysis of chitin with concentrated HCl, followed by fractionation using column chromatography. These oligomers are commercially available (Rupley, 1964).

There are many traditional methods for isolation of chitin oligomers. The procedures involved acid hydrolysis, neutralization, demineralization, fractionation by charcoal-celite column, fractionation by high-performance liquid chromatography (HPLC), and lyophilization.

Several disadvantages, such as being time consuming, laborious, and environmentally unfriendly, have been recorded for these methods (Tsigos *et al*., 2000). In addition, these methods may afford a low yield of oligomers with a high degree of polymerization (Takahashi *et al*., 1995). To overcome drawbacks associated with the conventional methods, procedures such as acetolysis (Bosso *et al*., 1986; Defaye *et al*., 1989; Hirano and Nagano, 1989; Inaba *et al*., 1984; 1989; Kendra *et al*., 1989; Kurita, 1993; Rupley, 1964;

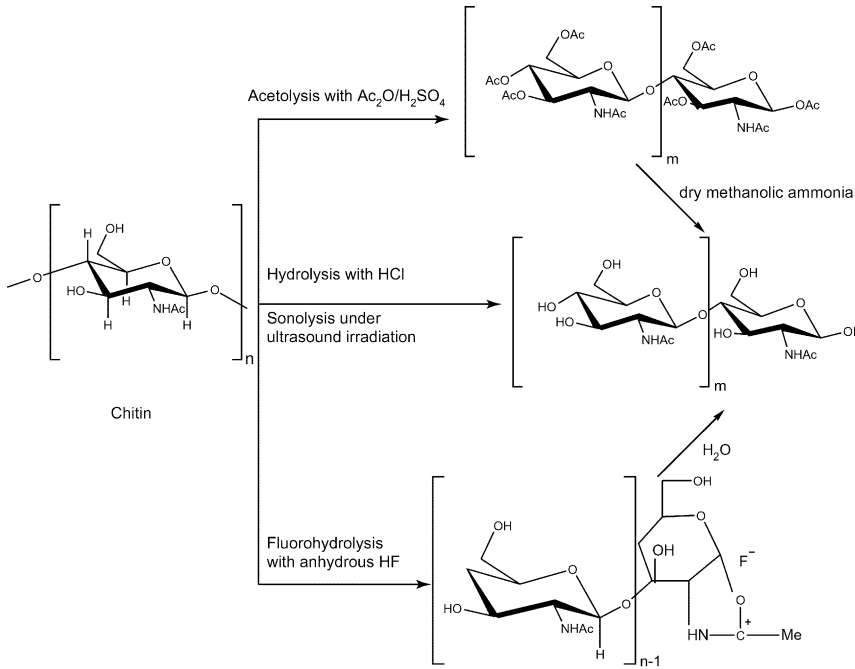


FIG. 8 Mechanism for acid hydrolysis of chitin.

Sakai *et al.*, 1990; Takahashi *et al.*, 1995), fluorosis (Bosso *et al.*, 1986), fluorohydrolysis (Defaye *et al.*, 1989), and sonolysis (Takahashi *et al.*, 1995) (Figure 8) have been considered.

a. Acetolysis. Acetolysis is a procedure for preparation of oligomers from chitin using acetic anhydride and sulfuric acid (Figure 8). β-Chitin from squid has been suggested as a starting material for simple acetolysis, leading to the formation of *N*-acetylchitooligosaccharide peracetate in high yields with reasonable reproducibility (Kurita *et al.*, 1993).

b. Fluorohydrolysis. Fluorohydrolysis is a method for preparation of chitin oligomers using anhydrous hydrogen fluoride (HF) (Figure 8). Defaye *et al.* (1989) reported that fluorohydrolysis of chitin in anhydrous HF yields chitin oligomers in a nearly quantitative manner. In addition, conditions can be easily controlled to optimize the preparation of specific oligomers ranging from two to nine residues and chitin oligomer isomer (β-[166]-linked acetamino-2deoxy-D-glycosyloligosaccharides).

c. Sonolysis. Sonolysis is a method used to prepare oligomers from chitin using hydrochloric acid hydrolysis under ultrasound irradiation (Figure 8). Takahashi *et al.* (1995) reported that the best way for production of chitin oligomers is through the combination of a mild acid hydrolysis and sonolysis. The combined method was able to hydrolyze polymers independent of temperature of the bulk solution and degradation of chitin by HCl under ultrasound irradiation (Takahashi *et al.*, 1995). This method saves time and does not require more than 2 hours. However, caution should be exercised to avoid deacetylation of the acetamido group.

2. Preparation of chitosan oligomers by chemical hydrolysis

Chitosan oligomers were first prepared by Horowitz *et al.* (1957). They demonstrated that acid hydrolysis of chitosan with concentrated HCl leads to the production of chitosan oligomers with a low degree of polymerization (DP), but in a quantitative manner.

Several studies have described the production of chitosan oligomers with a DP of less than six residues (Sakai *et al.*, 1990; Takahashi *et al.*, 1995; Tsukada and Inoue, 1981). On the other hand, Domard and Cartier (1989) reported that a wide distribution of glucosamine oligomers could be easily produced and separated up to a DP of 15 in the pure form. Defaye *et al.* (1994) prepared chitosan oligomers by fluorolysis in anhydrous hydrogen fluoride. They obtained oligomers with a DP of 2–11.

Most acidic hydrolysis methods have reported production of chitosan oligomers with a low DP, mainly from monomer to tetramer in quantitative amounts. The yields of relatively higher DP (pentamer to heptamer) oligomers were low. However, physiological function is rendered best by high DP oligomers.

3. Biological preparation

a. Preparation of chitin and chitosan oligomers by enzymatic hydrolysis. Chitin and chitosan oligomers can be prepared by enzymatic methods, as shown in Figure 9. Enzymatic methods offer many benefits over chemical hydrolysis. They produce desirable oligomers with a high DP and the reaction is performed under milder conditions (Jeon *et al.*, 2000).

b. Preparation of chitin from fungal cell wall. Jaworska and Konieczna (2001) investigated the effect of supplemental components (Fe^{2+}, Co^{2+}, Mn^{2+}, trypsin, and chitin) on the *in vivo* activity of two enzymes (chitin synthase and chitin deacetylase) to produce chitosan from fungus *Absidia*

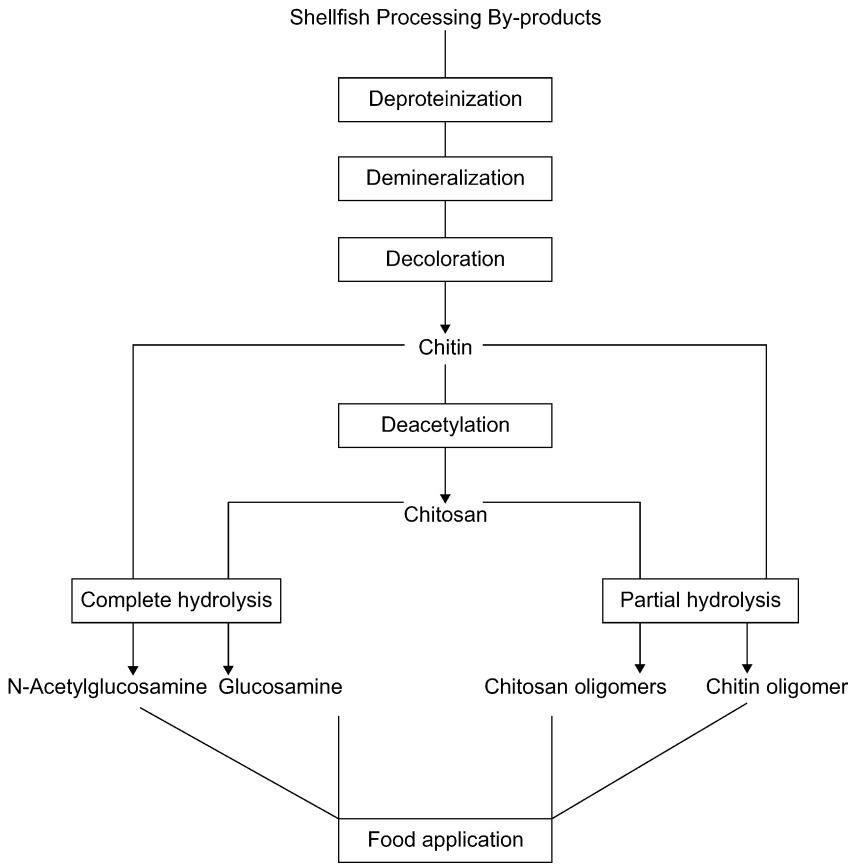


FIG. 9 Flowsheet for preparation of chitin, chitosan, and their oligomers and monomers from shellfish processing by-products.

orchodis. Manganese ions (Mn^{2+}) and ferrous ions (Fe^{2+}) gave rise to the highest increase in the amount of biomass rather than chitosan content in cell walls of the fungus. The effects of trypsin and chitin on biomass and chitosan content in cell walls were not significant, whereas Co^{2+} totally inhibited the growth of fungi. Ferrous ions decreased the activity of chitin deacetylase. Chitosan from fungi cultivated with Fe^{2+} ions had a higher DD (26–30%) than chitosan from unsupplemented medium (15%). The same trend was observed for Mn^{2+}. The amount of chitosan from fungi cultivated in the presence of Mn^{2+} was higher (about 30%) than that produced in an uncultivated medium (15%).

4. *Effects of preparation procedures and degree of deacetylation on chitosan activity*

Tsai *et al*. (2002) evaluated the effects of the DD and preparation procedures for chitosan upon its antimicrobial activity. Chitin was chemically (CH-chitin) and microbiologically (MO-chitin) prepared from shrimp shells. The resulting chitins were subsequently deacetylated chemically to produce chitosan with DDs ranging from low (47–53%) to medium (74–76%) to high (95–98%). The antimicrobial activities of both chemically and microbiologically prepared chitin/chitosan were the same, and in both cases the activity increased with increasing DD. Moreover, the size and conformational characteristics of chitin and chitosan appear to be crucial for their antimicrobial function. In general, chitosan has a stronger effect against bacteria than fungi. Chitosan with a high DD (98%) efficiently inhibited various bacteria (Tsai *et al*., 2002). Therefore, chitosan displays potential for increasing the shelf life of refrigerated fish fillets (Shahidi *et al*., 1999).

Uchida *et al*. (1989) showed that enzymatic hydrolysis produced a high amount of high DP oligomers from chitin and chitosan when compared to acid hydrolysis.

F. CHITINASES, CHITOSANASES, AND THEIR FUNCTIONS

1. *Chitinases*

Chitinases from different organisms have been studied and their genes cloned. Chitinases, if combined with cellulases, develop substrate structures that are similar in that both are crystalline and have β-1,4-glycosidic bonds. Cellulose is hydrolyzed by microorganisms and needs multiple enzymes (Bagnara-Tardif *et al*., 1992). Cellulases have been classified into 12 families (Henrissat, 1997), but chitinases have been classified into 2 families: family number 18 and family number 19 (Henrissat and Bairoch, 1993). Chitinases belonging to family 19 include classes I, II, and IV of the plant chitinases (Meins *et al*., 1992). Family 18 chitinases include most of the chitinases from bacteria, fungi, insects, plants (class III and V chitinases), and animals (Lee *et al*., 2000b). Amino acid sequence revealed that the chitinase gene contains two presumed chitin-binding domains and a single catalytic domain. Two proline-threonine replicate areas, linking catalytic and substrate-binding domains in some cellulases and xylans, were found. The chitinase gene binds to colloidal chitin and other substrates (chitosan, avicel, and xylan). However, the binding affinity of avicel, chitosan, and xylan is ten times less than that of colloidal chitin (Lee *et al*., 2000b).

Kawachi *et al.* (2001) described the production, purification, and characterization of extracellular chitinases from parasitic fungus *Isaria japonica*. Two chitinases (P-1 and P-2) were separated using chromatography on a DEAE Biogel agarose column. The enzymes were electrophoretically similar and had an MW of almost 43 kDa for P-1 and about 31 kDa for P-2. The optimum activity of P-1 chitinase was noticed at a pH of 3.5–4.0 and a pH of 4.0–4.5 for P-2. Hydrolysis products of chitin and chitosan hexamers were investigated by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). It was revealed that the products from chitin hexamer, obtained from P-1 chitinase, were all dimers with only a minor amount of trimer. However, those from P-2 were principally trimers with few dimers and tetramers. High homology (39–48%) for chitinase P-1 was shown by chitinases from *Trichoderma harzianum*, *Candida albicans*, and *Saccharomyces cerevisiae*. The highest homology (66%) for chitinase P-2 was displayed by endochitinase from *Metarchizium anisopliae* (Kawachi *et al.*, 2001).

Purification and characterizations of extracellular chitinases from the marine bacterium *Bacillus* sp. LJ-25 were described by Lee *et al.* (2000a). The purified chitinase so obtained showed a single band on SDS-PAGE and had an MW of approximately 50 kDa. The chitinase was most active and relatively stable at a pH of 7.0. The optimum temperature for this enzyme was around 35 °C when the pH of the reaction was kept at 7.0. The effect of metal ions on chitinase activity showed that Zn^{2+} strongly inhibited the enzyme activity. However, Ba^{2+}, Co^{2+}, Mn^{2+}, and Cu^{2+} showed slight inhibition of the enzyme. Substrate specificity studies indicated that colloidal chitin (a substrate of the endo type of chitinase) was efficiently degraded by the chitinase. However, chitin and chitosan were ineffectively hydrolyzed by this enzyme. This chitinase did not hydrolyze *N*,*N*-diacetylchitobiose, *p*-nitrophenol-*N*-acetyl-β-D-glucosamine, and *Micrococcus lysodeikticus* cells, which are known to be the substrates of the exo type of chitinases.

2. *Chitosanases*

Chitosanases are useful enzymes to hydrolyze chitosan, thus producing dichitooligosaccharides, trichitooligosaccharides, and tetrachitooligosaccharides (Kurakake *et al.*, 2000). A chitosanase with an MW of 45 kDa from *Bacillus cereus* S1 was purified and characterized (Kurakake *et al.*, 2000). Optimum pH for reaction incubation was about 6 at an optimum temperature of about 60 °C. This chitosanase was stable in basic solutions. Purified chitosanase has been used for many substrates, such as

soluble chitosan, colloidal chitosan, colloidal chitin, carboxymethylcellulose (CMC), and crystalline cellulose. The hydrolysis of colloidal chitosan was around 30% that of soluble chitosan. Kurakake *et al.* (2000) reported that the type of functional group on the C2 position in the glycoside residue is important for the adsorption of S1 chitosanase. They found that the degree of adsorption for enzyme was on the order of acetamide ($-NHCOCH_3$) > hydroxyl ($-OH$) > amino group ($-NH_2$). They also concluded that colloidal chitin binds with chitosanase as a competitive inhibitor because of their similar structure.

a. Chitin deacetylase. Chitin deacetylases are found in several fungi and insects (Tsigos *et al.*, 2000). They hydrolyze chitin by acting on *N*-acetamido bonds to produce chitosan. The use of these enzymes offers a controlled, non-degradable process leading to the production of novel well-defined chitosan oligomers and polymers (Tsigos *et al.*, 2000).

Win and Stevens (2001) studied shrimp chitin as a substrate for chitin deacetylase for fungus *Absidia coerulea*. The chitin was exposed to physical and chemical treatment to obtain a better accessibility of its acetyl groups for deacetylation process. Chitin was exposed to physical treatments such as heating, sonicating, and grinding. None of these treatments increased the enzymatic deacetylation efficiency.

Partially deacetylated shrimp chitin was produced before and after grinding of chitin. Chitin was treated with 50% NaOH for 20 hours at 40 °C and subsequently ground to various particle sizes (75–250 μm). Another sample of chitin was ground first and then treated with 50% NaOH. The results showed higher enzyme activity with decreasing particle size in both treatments. Chitin was exposed to different chemicals such as succinic anhydride, hot phosphoric acid, and 2-propanol. Solvent effect studies revealed that the best results were obtained when chitin was dissolved in $CaCl_2.2H_2O$ and methanol. A good substrate for chitin deacetylase from *A. coerulea* was obtained when mixing superfine chitin with formic acid, followed by neutralization. The MW of chitin was reduced from 2×10^5 to 1.2×10^4 kDa. The pretreated chitin could be deacetylated by enzyme up to a DD of 80–90% (Win and Stevens, 2001). Furthermore, this process was scaled up without the use of NaOH using a four-step procedure. Ten steps were involved and led to production of superfine chitin by (1) dissolving chitin in a solution of $CaCl_2.2H_2O$ and methanol as a solvent; (2) dissolving the superfine chitin in 18% formic acid; (3) adjusting the pH to an optimum level for the enzyme; and (4) enzymatic deacetylation with Absidia CDA.

III. APPLICATIONS OF CHITIN, CHITOSAN, AND THEIR OLIGOMERS

A. MEDICAL APPLICATIONS

1. Wound-healing agent

Chitin and chitosan have been tested to have both material and biological properties that might be beneficial to enhance wound repair. In addition, both of them have great influence on different stages of wound healing in experimental animal models (Howling *et al.*, 2001). Howling *et al.* (2001) found that chitosan polymers can interact with and modulate the migration behavior of neutrophils and macrophages modifying subsequent repair process such as fibroplastica and reepithelialization. It was reported that chitin and chitosan have both stimulatory and inhibitory effects on proliferation of human dermal fibroblasts and keratinocytes (Howling *et al.*, 2001). They also have an enhancing effect on the survival function of osteoblasts and chondrocytes (Lahiji *et al.*, 2000). The procedure for promoting wound healing by chitosan was tested as follows: Chitosan was coated onto plastic coverslips that had been filled into 24-well plates. Human osteoblasts and articular chondrocytes were seeded on either uncoated or chitosan coated coverslips. The incubation temperature of the culture was 37 °C, 5% CO_2 for 7 days. By using a fluorescent molecular probe, cell viability was judged. Reverse transcriptase polymerase chain reaction and immunocytochemistry were used for phenotyping expression of osteoblasts and chondrocytes. The results showed that the chondrocytes and osteoblasts appeared spherical and refractile of the chitosan-coated coverslips, whereas 90% of the cells on the plastic coverslips were elongated and spindle shaped after this period of incubation (Lahiji *et al.*, 2000). It was reported that the wound recovering material composed of polyelectrolytic complexes of chitosan and sulfonated chitosan that speeded up wound healing and afforded a good-looking skin surface (Lahiji *et al.*, 2000).

Chitosan has the ability to promote wound healing; this is due to the tendency to form polyelectrolyte complexes with polyanion heparin, which possesses anticoagulant and angiogenic properties (Lahiji *et al.*, 2000). By forming a complex with heparin and acting to lengthen the half-life of growth factors, chitosan supports tissue growth and helps wound healing.

Other studies have examined the effect of chitin and chitosan samples with different deacetylation levels and polymer chain length on the proliferation of human dermal fibroblasts *in vitro* (Howling *et al.*, 2001). It was found that chitosans with a high DD strongly motivated fibroblast proliferation; meanwhile, samples with lower degrees of deacetylation showed less activity.

Cho *et al.* (1999) used water-soluble chitin (WSC) prepared at room temperature through depolymerization by ultrasonication after alkaline treatment of chitin. The DD and MW were controlled. Chitin with DD of 8.60%, chitosan with DD of 83.9% and WSC were embedded to the wounded backs of rats after full thickness skin cuts. It was noticed that the WSC had the highest efficiency in recovering strength of the wounded skin due to the hydrophilicity and high biodegradability of WSC that maximized its activity as a wound-healing accelerator. In addition, the arrangement of the collagen fibers in the wound was the same as that of the normal skin. Hirano and Zhang (2000) described the preparation of a novel blend fiber. This fiber is a mixture of cellulose with each of hyaluronate (HA), heparin (Hep), chondroitin 4-sulfate, chondroitin 6-sulfate, and a chitin-chondroitin 6-sulfate blend using an aqueous 10% sulfuric acid solution containing 40–43% ammonium sulfate as a coagulating solution. These blend fibers could be used as covering materials for the wound-healing tissues of animals and plants.

Recently, bandages made of chitosan were investigated in the field of military in the new war in Iraq (Brown, 2003). Z-Medica, a small company, supplied these products to the U.S. ground troops in Iraq and Afghanistan (Becker, 2003). These bandages are used immediately after injury to control bleeding and were found to save numerous lives (Becker, 2003). Arterial bleeding was stopped in about a minute when these bandages were applied with pressure to a wound (Brown, 2003). The use of such bandages was approved by the Food and Drug Administration (FDA) in November 2002 (Mientka, 2003). They called it "shrimp" bandage that contains chitosan. This bandage can stop capillary bleeding and stanch severe arterial hemorrhaging (Mientka, 2003). Mientka (2003) reported that chitosan bandages had the ability to stop bleeding at a rate of 600 ml/min. Moreover, there was no sign of allergenicity for use of these bandages in soldiers who were allergic to shrimp (Mientka, 2003).

2. Dietary applications

Chitosan may be considered as a dietary supplement for reducing body weight in humans. Industrial production of chitosan tablets (Muzzarelli *et al.*, 2000) and chitosan dietary fibers (Hughes, 2002) has occurred. Furthermore, Schiller *et al.* (2001) reported that a rapidly soluble chitosan (LipoSan Ultra that has a higher density and solubility than chitosan itself) facilitated weight loss and reduced body fat. This effect was due to the fact that this chitosan was able to prevent dietary fat absorption in overweight and mildly obese individuals that consumed a high-fat diet.

a. Chitosan tablets. Although the reactivity of chitosan toward lipids is not clear, it is claimed that chitosan, because of its cationic nature, binds to appropriate bile and fatty acids and brings about their excretion (Muzzarelli *et al*., 2000). This claimed efficacy of chitosan in reducing the body weight, hypercholesterolemia, and hypertension stimulated production of chitosan tablets. Muzzarelli *et al*. (2000) studied the capacity of chitin, chitosan, *N*-lauryl chitosan, and *N*-dimethylaminopropyl chitosan on sequestering steroids. They reported that chitin might be more effective in holding olive oil and enriching the retained oil fraction with steroids sequestering than chitosan. In addition, chitin derivatives were able to distinguish between different lipids. These results put into question the need for high cationity for sequestering cholesterol. The use of chitosan monomer glucosamine sulfate for joint building is also commonplace.

b. Chitosan dietary fibers. Dietary fibers have many health advantages such as lowering low-density-lipoprotein (LDL) cholesterol levels and hence reduce heart disease and lower the risk of colon cancer. Moreover, dietary fibers are involved in weight loss. They increase the feeling of fullness (satiety) after meals and slow the sugar absorption from the gut (as brush buries), balancing blood sugar and insulin levels (Hughes, 2002).

Chitosan is considered a dietary supplement, fat-blocking fiber that can be used in weight management by slowing sugar absorption (Hughes, 2002). The fat-absorbing mechanism of chitosan has been explained by Hughes (2002); the chitosan with its positively charged amino groups ($-NH_3^+$) is attracted to the anionic carboxyl groups of fatty acids and bile acids forming films passing through the digestive system undigested (Hughes, 2002; Ylitalo *et al*., 2002).

Different results concerning the role of chitosans in weight loss have been recorded. One study proved the ability of chitosan in reducing weight without controlled diets (Hughes, 2002). Others suggested that the effect of chitosan could be related to a calorie-restricted diet. An Italian clinical study used chitosan supplements with a low-calorie diet to achieve weight loss (Hughes, 2002).

Chitosans have also been used to prevent body weight increase in animals (Hughes, 2002). Meanwhile, negative results were recorded regarding chitosan effectiveness in this field (Hughes, 2002). During a high-fat diet and chitosan supplementation, no increase in fecal fat content was noticed (i.e., chitosan had no effect on fat absorption) (Hughes, 2002). Some researchers do not recommend the use of chitosan in the diet of individuals who are allergic to crustaceans (Ylitalo *et al*., 2002).

3. *Antihypercholesterolemic agent*

Chitosan has been reported to cause significant hypocholesterolemic activity in different experimental animals (Hirano *et al.*, 1990; Sugano *et al.*, 1978, 1980; Ylitalo *et al.*, 2002). Sugano *et al.* (1988) noted that chitosan oligomers did not exhibit this effect. The studies were carried out on rat groups fed on a diet rich in cholesterol to find the effect of chitosan hydrolysates with different MWs and viscosity on the hypocholesterolemic activity. The lower the MW of chitosan, the better its cholesterol-lowering potential.

The mechanism of antihypercholesterolemic activity of chitosan has been described by Ylitalo *et al.* (2002). In the stomach, and because of the acidic condition, the ($-NH_2$) groups of chitosan accept protons (H^+) to form positively charged amino groups ($-NH_3^+$). Consequently chitosan becomes a soluble salt in the presence of hydrochloric acid (HCl). Fats, fatty acids (oleic, linoleic, palmitic, stearic, and linolenic acids), and other lipids, as well as bile acids, due to their negative charge ($X\text{-}COO^-$), attach themselves strongly to the positively charged amino groups ($-NH_3^+$) of chitosan. This binding might inhibit their absorption and recycling from the intestine to the liver. However, this interruption of enterohepatic circulation of cholic acid and other bile acids can lead to an increase in the biosynthesis of cholic acid from cholesterol in the liver. The cholesterol content of liver cells is thus decreased, which may lead to activation of LDL-receptor expression and could further increase LDL uptake via LDL receptors in the liver (Ylitalo, 2002).

Shahidi *et al.* (1999) reported that production of dietary cookies, potato chips, and noodles enriched with chitosan is commonplace in certain countries. The products enriched with chitosan have high hypocholesterolemic effects. In addition, vinegar products containing chitosan are produced and sold in Japan because of their cholesterol-lowering ability (Shahidi *et al.*, 1999).

4. *Antitumor activity*

Suzuki (1996) reported that chitin and chitosan oligomers can act as inhibitors of growth tumor cells via their immunoenhancing effects. Suzuki *et al.* (1985) found that chitin oligomers from $(GlcNAc)_4$ to $(GlcNAc)_7$ have strong attracting responses to peritoneal exudate cells in BALB/c mice. However, chitooligosaccharides from $(GlcN)_2$ to $(GlcN)_6$ did not exhibit such an effect.

With regard to hexamers, both $(GlcNAc)_6$ and $(GlcN)_6$ were reported to process growth inhibitory effects against allogenic and syngeneic mouse

systems (Suzuki *et al.*, 1986a). These results indicate that the effect was not by direct cytocidal action on tumor cells but was host mediated.

5. *Antiulcer agent*

Ito *et al.* (2000) reported that chitosans with different MWs had ulcer-healing actions. The effects of low MW (LMW) chitosan, high MW (HMW) chitosan, and chitin on ethanol-induced gastric mucosal injury and on the healing of acetic acid–induced gastric ulcers in rats were compared. It was found that orally administrated LMW chitosan could prevent ethanol-induced gastric mucosal injury. Repeated oral administration of LMW chitosan in a dose-dependent manner accelerated the gastric ulcer healing. The effects of HMW chitosan and chitin on gastric ulcer healing were less than those of LMW chitosan.

6. *A coating agent for prosthetic articles (artificial parts of the body)*

Muzzarelli *et al.* (2000) described a method for coating prosthetic articles with chitosan-oxychitin. Plates of titanium (Ti) and its alloys were plasma sprayed with hydroxyapatite and glass layers, and subsequently a chitosan coat was deposited on the plasma-sprayed layers using chitosan acetate. These layers were treated with 6-oxychitin to form a polyelectrolytic complex. This complex was optionally contacted with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at 4 °C for 2 hours to form amide links between the two polysaccharides, or acetylation with acetic anhydride in methanol to obtain a chitin film. In all cases, the modified coats were insoluble, uniformly flat, and smooth. Prosthetic materials coated with chitosan–oxychitin were capable of provoking colonization by cells, osteogenesis, and osteointegration.

There were two main reasons behind the selection of chitosan-oxychitin–coated orthopedic plates. First, chitosan enhances the integration of the implant, and second, chitosan stimulates bone regeneration.

B. FOOD APPLICATIONS OF CHITIN, CHITOSAN, AND THEIR OLIGOMERS

New applications of chitin and its oligomers led to more than 50 patents in the 1930s and the early 1940s. However, commercialization of these products was hindered by inadequate manufacturing services and competition from synthetic polymers (Averbach, 1981). However, after the 1970s, industrial use of chitin and its oligomers increased (Kaye, 1985). Furthermore, improvement in research and small-scale production of chitin and chitosan

has extended the number and varieties of potential applications of chitinous materials. In addition, environmental problems and cost for disposal of shellfish processing discards have increased the urgency for development of environmentally safe alternatives for numerous plastic or polymeric products (Ashford *et al*., 1976; Berkeley, 1979; Shahidi and Synowiecki, 1991). Some food applications of chitin, chitosan, and their oligomers are summarized in Table II.

Chitin, chitosan, and their derivatives offer a wide range of applications including bioconversion for the production of value-added food products, preservation of foods from microbial spoilage, formation of biodegradable films, recovery of waste material from food processing discards, purification of water, and clarification and deacidification of fruit juices (Shahidi *et al*., 1999) (Table II).

1. Antimicrobial activity

Chitin, chitosan, and their derivatives have antimicrobial activity against bacteria, yeast, and fungi (Yalpani *et al*., 1992). The exact mechanism of antimicrobial action of chitin and chitosan and their derivatives remains illusive, but different mechanisms have been proposed (Shahidi *et al*., 1999). Chitosan has the ability to produce phytoalexins, cell wall phenols and callose (Tsai *et al*., 2002). Chitosan is considered to be a soluble chelating agent and activator due to the presence of a positive charge on the C-2 of its glucosamine monomer at pH values less than 6. This characteristic gives it a higher antimicrobial activity than chitin (Chen *et al*., 1998). A leakage of proteineous and intercellular components occurs due to the interaction between the positively charged chitosan molecules and the negatively charged microbial cell membranes (Chen *et al*., 1998; Papineau *et al*., 1991; Sudharashan *et al*., 1992; Young *et al*., 1982). This is affected by the MW of chitosan (Tsai *et al*., 2002). Being a chelating agent, chitosan has the ability to selectively bind trace metals, which prevents production of toxins and microbial growth (Cuero *et al*., 1991). Chitosan is also an activator for several defense processes in the host tissue (EL-Ghaouth *et al*., 1992b), having the ability to bind water and inhibit various enzymes (Young *et al*., 1982).

Tsai *et al*. (2002) studied the effects of DD and preparation methods for chitin and chitosan on their antimicrobial activity. It was found that chemically (ch-chitin) and microbiologically prepared chitin (MO-chitin) could undergo further chemical deacetylation to produce chitosan with different DDs. However, MO-chitin that was deacetylated by various proteases had no antimicrobial activity (Tsai *et al*., 2002). However, for chitosan, as the DD increased, its antimicrobial effect on bacteria increased, even to a greater extent than that on fungi.

TABLE II
FOOD APPLICATIONS OF CHITIN, CHITOSAN, AND THEIR DERIVATIVES

Area of application	Examples
Antimicrobial agent	Bactericidal
	Fungicidal
	Measure of mold contamination in agricultural commodities
Edible film	Controlled moisture transfer between food and the surrounding environment
	Controlled release of antimicrobial substances
	Controlled release of antioxidants
	Controlled release of nutrients, flavors, and drugs
	Reduction of oxygen partial pressure
	Controlled rate of respiration
	Temperature control
	Controlled enzymatic browning in fruits
	Reverse osmosis membranes
Food additive	Clarification and deacidification of fruit juices
	Natural flavor extender
	Texture adjusting agent
	Emulsifying agent
	Food mimetic
	Thickening and stabilizing agent
	Color stabilization
Nutrition	Dietary fiber
	Hypocholesterolemic agent
	Livestock and fish feed additive
	Reduction of lipid absorption
	Production of single cell protein
	Antigastritis agent
	Infant food ingredient
Water treatment	Recovery of metal ions, pesticides, phenols, and PCBs
	Removal of dyes, radioisotopes
Agriculture	Seed and fruit covering
	Fertilizer
	Fungicide
Cosmetics	Skin and hair products
Biomedical and pharmaceutical materials	Artificial skin
	Surgical structures
	Contact lens
	Treating major burns
	Blood dialysis membranes
	Artificial blood vesicles
Others	Enzyme immobilization
	Encapsulation of nutraceuticals
	Chromatography
	Analytical reagent
	Synthetic fiber
	Chitosan-coated paper
	Manufacturing material for fiber
	Film and sponges

Genetically, chitosan can enter the nuclei of a microorganism and bind with DNA. This binding inhibits the mRNA and protein synthesis (Hadwiger *et al*., 1985; Sudharashan *et al*., 1992).

a. Antibacterial agent. Several studies have examined the effect of concentration of chitosan to complete inactivation of certain types of bacteria (Shahidi *et al*., 1999). Wang (1992) observed that a much higher concentration of chitosan (1–1.5%) was required for complete inactivation of *Staphylococcus aureus* after 2 days of incubation at pH 5.5 or 6.5 in the medium. Furthermore, Chang *et al*. (1989) found that chitosan concentrations of 0.005 or more were sufficient to elicit complete inactivation of *S. aureus*. This was in accordance with the findings of Darmadji and Izumimoto (1994) on the effect of chitosan in meat preservation. Simpson *et al*. (1997) studied the effect of different concentrations of chitosan on the growth of different cultures of bacteria on raw shrimp. They found that *Bacillus cereus* required chitosan concentrations of 0.02% for antibacterial effect, whereas *Escherichia coli* and *Proteus vulgaris* exhibited minimal growth at 0.005% and growth was inhibited at 0.0075% more. Numerous studies have shown the effect of different concentrations of chitosan on *E. coli* growth. Complete inhibition was observed by Wang (1992) after 2 days incubation with 0.5% or 1% chitosan at a pH level of 5.5. It was also reported that if chitosan concentration increased by about 1% in the broth, it could afford complete inactivation. However, Darmadji and Izumimoto (1994) reported that growth inhibition of *E. coli* required a 0.1% chitosan concentration. Simpson *et al*. (1997) found that only 0.0075% chitosan was required to inhibit the growth of the same species. The observed variations are possibly due to the existing differences in the degree of acetylation of chitosans employed (Shahidi *et al*., 1999).

Iida *et al*. (1987) and Nishimura *et al*. (1984) have reported that if chitin is partially deacetylated, especially at 70%, it has the ability to stimulate nonspecific host resistance against *E. coli* and Sendai virus infection in mice. Meanwhile, chitin and chitosan have the ability to increase the number of mouse peritoneal exudate cells that generate reactive oxygen intermediates and then display candidacidal activities (Suzuki *et al*., 1984). Suzuki *et al*. (1986) reported that chitin hexamer $(GlcNAc)_6$ had a strong candidacidal activity.

b. Antifungal agent. Chitosan decreased the *in vitro* proliferation of many fungi with the exception of Zygomycetes (Allan and Hadwiger, 1979). Chitosan acts as an antifungal agent via the formation of gas-permeable coats, interference with fungal growth, and stimulation of many defense processes, including accumulation of chitinases, production of proteinase

inhibitors, and lignifications and stimulation of callous synthesis (Bai *et al.*, 1988; EL-Ghaouth *et al.*, 1992a).

EL-Ghaouth *et al.* (1992b) studied the antifungal influence of chitosan on *in vitro* growth of common postharvest fungal pathogens in strawberry fruits. These authors found that chitosan with 7.2% NH_2 significantly decreased the eradial proliferation of *Botrytis cinerea* and *Rhizopus stolonife*, with a greater impact at higher concentrations. In an *in vivo* study, EL-Ghaouth *et al.* (1992b) observed signs of infection in chitosan-covered fruits after 5 days of storage at 13 °C compared with 1 day for the control treatment. After 14 days of storage, chitosan coating (at 15 mg/ml) decreased spoilage of strawberries induced by the same fungi by more than 60% and noticed that coated fruits were grown normally and did not show any clear sign of phytotoxicity. Fang *et al.* (1994) reported the preservative influence of chitosan on low-sugar candied Kumquat (fruit). Chitosan (at 0.1–5 mg/ml) inhibited the growth of *Aspergillus niger*, whereas chitosan at less than 2 mg/ml was ineffective in inhibiting mold proliferation and aflatoxin synthesis by *Aspergillus parasitius*. Cuero *et al.* (1991) conducted a similar study and observed that *N*-carboxymethylchitosan decreased aflatoxin formation in *A. flavus* and *A. parasitius* by more than 90% while fungal growth was decreased to less than half. Furthermore, Savage and Savage (1994) reported that apples coated with chitosan reduced the rate of molds occurring on them over a period of 12 weeks. Cheah and Page (1997) found that chitosan coating of carrot with a 2% or 4% chitosan solution considerably reduced their Sclerotinia rotting from 28% to 88%.

El-Katatny *et al.* (2001) reported the characterization of a chitinase and endo-β-1,3-glucanase from *Trichoderma harzianum* strain Rifai T_{24}. These two enzymes are the key enzymes in the lyses of cell walls during their mycoparasitic effect against plant diseases caused by fungi, including *S. rolfsii*. The chitinase from *T. harzianum* was purified in two steps using ammonium sulfate precipitation followed by hydrolytic interaction chromatography. SDS-PAGE showed that the enzyme exhibited a single band at 43 kDa. The β-1,3-glucanase was purified in three steps using ammonium sulfate precipitation, hydrophobic interaction chromatography, and gel filtration, showing a molecular mass of 74 kDa. The optimum pH level of both enzymes was 4.5. The optimum temperature of the T_{24} chitinase was 40 °C, whereas the optimum temperature of β-1,3-glucanase was 50–60 °C. Both the *T. harzianum* T_{24} chitinase and β-1,3-glucanase were strongly inhibited by Hg^{2+}, suggesting that sulfhydryl groups are involved in the catalytic reaction (El-Katatny *et al.*, 2001). The pure form of the two enzymes from *T. harziaanum* T_{24} inhibited the growth of *S. rolfsii* in an additive manner showing a promising effective dose of 50% (ED_{50}) at a 2.7-g/ml concentration.

A mixture of these enzymes, which showed thermostability and low effective dose (ED_{50}) values against *S. rolfsii*, may be considered a potential tool for controlling of plant's pathogens.

i. Chitin as a measure of mold contamination of agriculture commodities and food products. Chemically, when chitin in the cell wall of fungi is determined, it has a benefit that indicates the total content of mycelium based on chitin (Bishop *et al.*, 1982; Donald and Mirocha, 1977). Bishop *et al.* (1982) used chitin to evaluate the presence of mold in tomato products, ketchup, paste, and puree. They noticed variations in chitin content among different fungal species depending on the cultural age and growth conditions; values ranged from 5.7 to 43 μg of glucosamine per milligram of dry weight.

2. Preservation of foods

Chitosan can be used for food preservation to inhibit the growth of spoilage microorganisms in mayonnaise (Oh *et al.*, 2001). By treating crude chitin with various NaOH concentrations (45%, 50%, 55%, and 60% w/v), four kinds of chitosans were prepared (chitosan-45, -50, -55, and -60, respectively) (Ylitalo *et al.*, 2002). Four species of food spoilage microorganisms were treated by chitosans to examine their effects on microbial activity (Oh *et al.*, 2001). These were *Lactobacillus plantarum, Lactobacillus fructivorans, Serratia liquefaciens*, and *Zygosacchaomyces bailii*. Chitosan had a biocidal effect; the number of cells grown was clearly reduced. It has been found that after an extended phase, some strains recovered and started to grow. As the concentration of chitosan increased, the activities of these strains increased. It was noticed that chitosan-50 had the most efficiency against *L. fructivorans*; meanwhile, the inhibition of *L. plantarum* growth was mostly by chitosan-55 and no difference was found among the chitosans against *S. liquefaciens* and *Z. bailii*. Thus, for mayonnaise, during its storage at 25 °C, the addition of chitosan decreased the viable cell counts of *L. fructivorans* and *Z. bailii*.

a. Preservation of seafood and meats. Tsai *et al.* (2002) found that 1% chitosan solution with a high DD can be added to certain kinds of fish to increase their shelf life from 5 to 9 days. Kamil *et al.* (2002) showed that chitosans prepared from snow crab shells had different viscosities, closely correlated to the time of deacetylation. Different viscosity chitosans (14, 57, 360 cP chitosans) were prepared and used to examine the impact of chitosan covering on fish quality during refrigerated storage. This study showed the potential of chitosan as a protective coating for herring and

cod in decreasing or preventing moisture loss, lipid oxidation, and microbial growth. Cod samples coated with 57 and 360 cP chitosans demonstrated a considerably ($p < .05$) lower relative moisture loss in comparison with those of uncoated samples and those coated with 14 cP chitosan throughout the storage period. Furthermore, crab chitosan showed a medium to high viscosity-dependent protective effect in both fish model systems. In general, 360 cP chitosan exhibited a better preservative effect in comparison with 57 and 14 cP chitosans in both systems at 4 ± 1 °C. In this study, the effects of different viscosity chitosans on lipid oxidation of cooked comminuted fish were also tested. The chitosan showed antioxidant activity in cooked comminuted fish model system, as revealed in their peroxide value and content of 2-thiobarbituric acid–reactive substances (TBARS), which were reduced in a concentration-dependent manner. However, the antioxidant efficiency of relatively high viscosity chitosan in both model systems was lower than that of the low-viscosity chitosan at the same concentration. The mechanism of action appears to be a result of chelation of metal ions found in fish muscle proteins, gas exchange adjustment (particularly oxygen) between fish meat and the surrounding environment, and the bactericidal effect of chitosan itself. Thus, chitosan as an edible coating would enhance the quality of seafoods during storage (Jeon *et al.*, 2002).

Weist and Karel (1992) studied the effect of using chitosan powders in a fluorescence sensor for monitoring lipid oxidation in muscle foods. The efficiency of chitosan powders was explained to be due to the ability of the primary amino groups of chitosan to form a stable fluorosphere with volatile aldehydes such as malonaldehyde, which is derived from the breakdown of fats (Weist and Karel, 1992). On the other hand, chitosan was used to improve the preservation of vacuum-packaged processed meats stored under refrigerated conditions (Quattar *et al.*, 2000). These authors used chitosan matrix to produce antimicrobial films by adding acetic or propionic acid (with or without addition of lauric acid or cinnamaldehyde) to this matrix (Quattar *et al.*, 2000). These films were applied to bologna, regular cooked ham, or pastrami. The amounts of antimicrobial agents found in the chitosan matrix were measured several times during storage. It was found that within the first 48 hours of application, propionic acid was nearly completely released from the chitosan matrix. Whereas, 2–22% of acetic acid remained in chitosan even after 168 hours of storage. With regard to the presence of lauric acid, but not cinnamaldehyde, it was found that the release of acetic acid was reduced significantly and more limited to bologna than to ham or pastrami (Quattar *et al.*, 2000). In another study, Li *et al.* (1996) found that addition of 3000 ppm of *N*-carboxymethylchitosan to cooked pork was sufficient to prevent the oxidative rancidity of the product.

b. Fruit preservative agent. Chitin, chitosan, and their derivatives have been used as food wraps, because of their film-forming properties. The chitosan film controls moisture movement between food and the surrounding environment. The presence of the film results in a decreased rate of metabolism, respiration, and high impermeability to certain substances such as fats and oils, in addition to temperature. These would lead to a delay in ripening of fruits (Shahidi *et al*., 1999).

The coating of fruits with chitosan delays the rate of ripening and the occurrence of decay in tomato (EL-Ghaouth *et al*., 1992c), bell pepper, cucumber (EL-Ghaouth 1991b), and strawberries (EL-Ghaouth *et al*., 1991a). The control of disease in fruits by chitosan could account for the antifungal activity of chitosan and its capacity to provoke defense enzymes and phytoalexins in the plant tissue or a combination of both (EL-Ghaouth *et al*., 1992a). Chitosan (7.2% NH_2) inhibited the growth of postharvest pathogens, namely *B. cinerea, A. alternata, C. gloesporioides*, and *R. stolonifer* (EL-Ghaouth *et al*., 1992b). Among the fungi examined, *R. stolonifer* was least affected by chitosan (EL-Ghaouth *et al*., 1992b). Whereas chitin did not influence the growth of any of the fungi tested, the growth delay of fungi provoked by chitosan increased with increasing DD (EL-Ghaouth *et al*., 1992a). The inhibitory effects of chitosan correlated with its cationic nature and the size of the polymers. Moreover, the importance of cationic groups and the length of the polymer chain was demonstrated by the low fungicidal activity displayed by *N*,*O*-carboxymethyl chitosan compared to that of chitosan, and by the improved activity of chitosan with increasing levels of deacetylation. The antifungal effects may, in part, account for the capacity of chitosan to enhance membrane permeability and result in cellular leakage.

Three mechanisms may be involved in the action of chitosan as an antifungal agent in the preservation of postharvest crops. First, the treatment of potato with chitosan, challenged with *Erwinia carotovora* (the soft rot pathogen of potato), showed a declined count of bacteria and tissue maceration, thus resulting in an increase in cell viability. Second, potatoes treated with chitosan showed an inhibition in bacterial reproduction and secretion of pectic enzymes (produced by pathogenic bacteria capable to attack the plant tissue). The third mechanism of chitosan action is by controlling the pH level. The pathogenic bacteria that cause decay of crops after harvesting such as potatoes secrete macerating enzymes (negatively charged proteins), leading to an outflow of protons and cations from the cell wall of the plant and hence a pH increase in the cell and cell wall.

c. Acidity adjusting agent. Chitosan could be used for deacidification of fruit juices because chitosan salts carry a strong positive charge that could interact with proteins and hence act as dehazing agents in fruit juice (Shahidi

et al., 1999). Scheruhn *et al.* (1999) reported that treating coffee drinks with chitosan increased the pH level and decreased the acid content of the coffee drinks because of the acid-binding properties of chitosan in the coffee. This treatment depended on the concentration of chitosan and the acid content of the drinks, as well as the raw material of chitosan and its processing (Scheruhn *et al.*, 1999).

d. Antioxidant agent. Muscle food products containing a high content of unsaturated lipids are highly labile to off-flavor and rancidity development. Warmed-over flavor (WOF) is developed in cooked poultry and uncured meat upon storage, resulting in the loss of attractive meaty flavor (Shahidi *et al.*, 1999). Darmadji and Izumimoto (1994) noticed that 1% chitosan added to meat resulted in a decline of 70% in the 2-thiobarbituric acid (TBA) values after 3 days of storage at 4 °C. St. Angelo and Vercellotti (1989) reported that *N*-carboxymethylchitosan was effective in preventing the formation of WOF over a broad range of temperatures. Moreover, ground beef treated with 5000 ppm of *N*-carboxymethylchitosan exhibited 93% inhibition of TBA values and a 99% reduction in hexanal content. Furthermore, Shahidi *et al.* (1999) reported that *N*,*O*-carboxymethylchitosan (NOCC) and its lactate, acetate, and pyrrolidine carboxylate salts were effective in controlling the oxidation and off-flavor development in cooked meat stored for 9 days at refrigerated temperature. The mechanism by which this inhibition occurred was thought to be related to the chelation of free iron, which was released from hemoproteins during heat processing. These results were further confirmed by Li *et al.* (1996) who found that addition of 3000 ppm *N*-carboxymethylchitosan to cooked pork was sufficient for inhibiting the development of oxidative rancidity in the product.

C. AGRICULTURAL APPLICATIONS

1. Retention of nutrients and nutrient cycle in the soil

Smither-kopperl (2001) found that chitin exhibits several functions, including retention of nutrients, in the soil. Chitin contributes to their cycling of nutrients such as nitrogen. When chitin decomposes, it produces ammonia, which takes part in the nitrogen cycle. Furthermore, chitin is a main constituent in geochemical recycling of both carbon and nitrogen. Fungi, arthropods, and nematodes are the major contributor of chitin in the soil. Among these, the fungi provide the largest amount of chitin in the soil (6–12% of the chitin biomass, which is in the range 500–5000 kg/ha).

In another study, Kokalis-Burelle (2001) reported that chitin contributes significantly to soil enrichment. It was found that chitin could control plant

pathogens and pathogenic nematodes and provoke the development of host plant resistance against these pathogens. Chitin led to an increase in microorganism population; this sharp increase could shift and prompt their action as anti-plant pathogens in two ways. First, the microorganism may act as parasite for plant pathogens. Second, they can kill or inhibit these pathogens through production of toxins or metabolites or enzymes. Furthermore, the increase in microorganism numbers increases the number of non-parasitic nematodes, which results in a decline in the number of pathogenic nematodes.

D. INDUSTRIAL APPLICATIONS

1. Purification of water

There is an increasing demand for treatment of industrial wastewater before their use or disposal because of the environmental and health difficulties associated with heavy metals and pesticides and their deposit through the food chain (Shahidi *et al*., 1999). Traditional methods for the elimination of heavy metals from industrial wastewater may be inefficient or costly, particularly when metals are present at low concentrations (Deans and Dixon, 1992; Volesky, 1987). Recovering of metal ions from discards can be achieved using a chelation ion exchange process. Biopolymers, such as chitin and chitosan, have the ability to lower the concentration of transition metal ions to parts per billion levels. These biopolymers should be ecologically safe and commercially available and bear a number of functional groups, such as hydroxyl and amino groups in their backbones (Deans and Dixon, 1992). Chitosan can be used as a means for treatment of wastewater because it has a good sorption capacity (Jeuniaux, 1986). In Japan, chitin and chitosan have been used for water purification because of their ability to complex metal ions via their amino groups (Simpson *et al*., 1994). Chitosan powder and dried films of it have free amino groups above the pKa of their NH_2 groups. Therefore, chitosan powder and dried films have potential use in complexing metal ions (Tirmizi *et al*., 1996). The U.S. Environmental Protection Agency (USEPA) has approved the use of commercially available chitosan for wastewater treatment up to a maximum level of 10 mg/L (Knorr, 1984). Muzzarelli *et al*. (1989) demonstrated the efficiency of cross-linked *N*-carboxymethylchitosan in removing lead and cadmium from drinking water. Micera *et al*. (1986) showed that chitosan has a high binding capacity with metals such as copper and vanadium. Deans and Dixon (1992) observed that unfunctionalized chitosan was efficient in eliminating Cu^{2+}, but not Pb^{2+}. Thome and Daele (1986) examined the ability of chitosan to remove polychlorinated biphenyls (PCBs) from polluted stream water. The authors

showed that chitosan was highly effective, compared to activated charcoal, for purification of PCB-polluted water. Use of chitosan for purification of potable water is also in practice.

IV. SAFETY AND REGULATORY STATUS

Chitosan has many industrial, agricultural, pharmaceutical, and cosmetic applications. Consequently, safety and toxicological studies have been performed on chitosan to address issues related to its regulatory status. Rao and Sharma (1997) reported no toxicity for 2% chitosan solution in acetic acid, when applied on punctured bleeding capillaries in mice, rabbits, and guinea pigs. These researchers further observed that eye irritation tests in rabbits and skin irritation tests in guinea pigs did not produce any toxic effect due to chitosans. Similar results were obtained by Mou *et al.* (2003), who reported no obvious toxic reaction using a mixture of polylactic acid and chitin as a basic scaffold material in tissue engineering. Chitosan received the "Generally Recognized as Safe" (GRAS) status by the FDA in the United States in 1983 for use as animal feed component; its use in pet food was also reported by Shepherd *et al.* (1997). The use of chitosan for purification of potable water was approved by the USEPA, up to a maximum concentration of 10 mg/L (Knorr, 1986). In 1992, Japan's health department approved the use of chitin and its derivatives as functional food ingredients. Based on its definition of functional foods, chitin and chitosans possess most of the required attributes related to enhancement of immunity, prevention of illness, delaying of aging, recovery for illness, and control of biorhythm (Subasinghe, 1999). Thus, the use of chitosan in foods such as potato chips has been in commercial practice for some time. Therefore, regulatory status of chitosan varies from country to country and its use in food requires further studies to address issues of concern (Lenz and Hamilton, 2004).

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