CHAPTER II

LITERATURE REVIEW

Chitosan

"Chitosan is a natural carbohydrate biopolymer derived by deacetylation of chitin"

1.1 Chitosan origin and preparation

Chitin is the second most ubiquitous natural biopolymer on earth after cellulose. It is composted of 2-acetamido-2-deoxy-β-D-glucose (*N*-acetyl-D-glucosamine) and 2-amino-2-deoxy-β-D-glucan (D-glucosamine) attached via β-(1, 4) linkages (Figure 2.1a). Chitin is a common constituent of crustacean exoskeletons, insect cuticle, and cell walls of some fungi and microorganisms (Austin *et al.*, 1981; Tsigos *et al.*, 2000). In nature, chitin occurs in three polymorphs, namely α-, β-, and γ-chitins, which differ in the arrangement of molecular chains. α-chitin, an antiparallel chain, is the most abundant in nature and usually isolated from the exoskeleton of crustaceans, particularly from crabs, lobsters, and shrimps. β-chitin, a parallel chain, can be extracted from squid pens. While γ-chitin, the mixture of α- and β-chitins, obtained from fungi and yeast (Yen *et al.*, 2008).

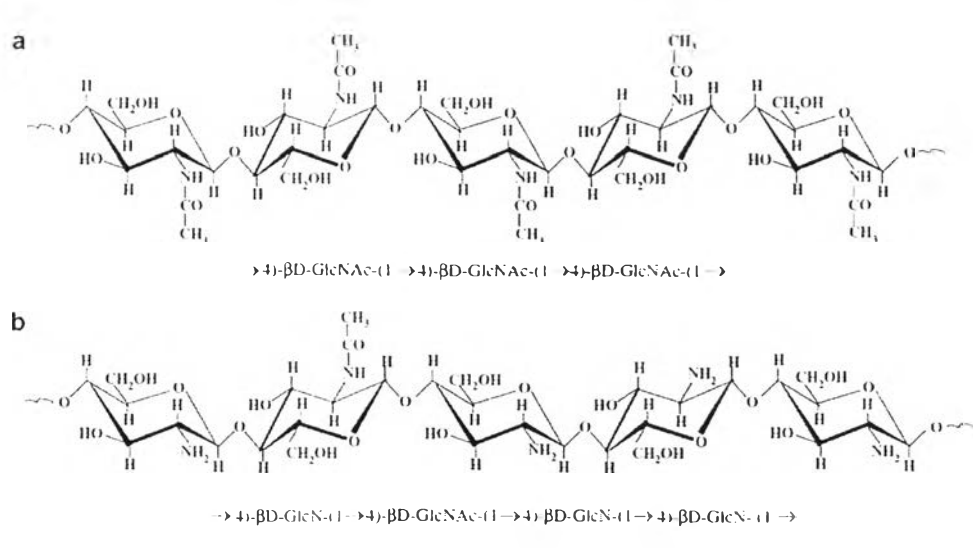

Figure 2.1 The primary structure of chitin (a) and chitosan (b) (Prashanth and Tharanathan, 2007)

Chitosan is converted from chitin by enzymatic methods or alkali deacetylation. Several sources of chitin have been used for the production of chitosan. The famous source is the processing waste of crustaceans from food industry, especially crab, lobster, shrimp, and squid. The crustacean shells contain chitin (about 15-40% dry weight), proteins, pigment, minerals (particularly calcium carbonate) and other components such as cellulose, and glucan (Rasmussen and Morrissey, 2007). Chitin must be purified by decalcification, deproteination and decolorization followed by deacetylation (by NaOH) to obtain chitosan (Figure 2.2) (Je and Kim, 2012).

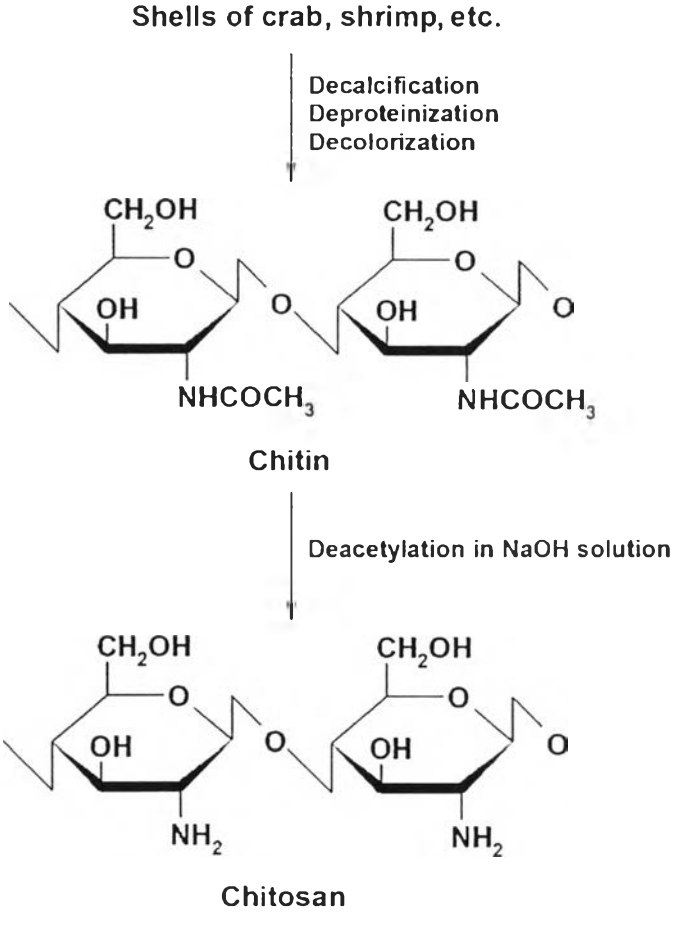

Figure 2.2 Preparation of chitosan (Je and Kim, 2012)

1.2 Chitosan structure

Chitosan is a linear polysaccharide. The primary units in the chitosan is copolymerization of 2-acetamido-2-deoxy-β-D-glucan (*N*-acetyl-D-glucosamine or GlcNAc) and 2-amino-2-deoxy-β-D-glucan (D-glucosamine or GlcN) combined by β-(1, 4) glycosidic bonds (Figure 2.1b) in varied proportions (Trombotto *et al.*, 2008).

1.3 Physical and chemical characteristics

Reactive functional group of chitosan has been identified to three types, which are an amino/acetamido group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions (Xia *et al.*, 2011). The availability of free amino groups in chitosan makes it carries a positive charge and thus in turn reacts with many negatively charged surfaces or polymers (Fukuda, 1980).

Chitosan is non-toxic, biodegradable, and biocompatible with many cells both animal and plant. It can be processed into several products, for example, bead, fiber, fine powders, flakes, gel, and membrane (Badawy and Rabea, 2011).

1.3.1 Degree of deacetylation (DD)

Chitosan is fully or partially *N*-deacetylation derivatives with a DD of more than 70%. The ratio of GlcNAc to GlcN structural units has a striking effect on chitosan solubility and solution properties. The DD is calculated from the integral ratio between the proton on C-2 and the glucose unit protons (Hirai *et al.*, 1991).

The increasing of DD means the increased number of amino groups on chitosan that correlated to the increasing number of protonated amino groups in an acidic condition and dissolves in water completely, which leads to an increased chance of interaction between chitosan and negatively charged cell walls of microorganisms (Tokura *et al.*, 1997).

1.3.2 Molecular weight (M_w)

The most direct method for molecular weight characterization is gel filtration chromatography. It provides the number-average molecular weight (M_n), and the weight-average molecular weight (M_w) and the polydispersity index M_w/M_n in a single measurement (Terbojevich *et al.*, 1993). Commercial

chitosan is available with molecular weights between 100 and 1000 kDa. It is accepted that chitosan with low M_W is less than 50 kDa, medium M_W is between 50 and 150 kDa, and high M_W is larger than 150 kDa (Goy *et al.*, 2009).

1.3.3 Solubility

The solution properties of chitosan depend on degree of deacetylation, the distribution of acetyl groups along the main chain, and molecular weight (Rinaudo and Domard, 1989). Chitosan is insoluble in organic solvents and water at neutral and alkaline pH, but soluble in most organic acidic solutions at pH less than 6.5 such as acetic acid, ascorbic acid, formic acid, glutamic acid and lactic acid. Chitosan is available in a wide range of molecular weight, viscosity, and degree of deacetylation (Ilium, 1998; Romanazzi *et al.*, 2009).

1.4 Biological properties

The information of chemical and physical properties is essential for the understanding the relationship between the structure and biological response of chitosan-based biomaterials. The first perspective of the biological properties of chitosan is an elicitor of diverse activities when contact with plant cells. The response was dose-dependent and increased with degree of polymerization (DP) and degree of deacetylation (DD) until reach a plateau. The result also depends on molecular weight of chitosan, and plant species (Domard, 2011).

1.4.1 Antioxidant activity

Reactive oxygen species (ROS) are considered as a harmful byproduct from normal respiration, metabolism, and xenobiotic stresses. The production of ROS is essential to maintain homeostasis of cell in living organism. Excessive ROS production is effectively eliminated by antioxidant enzymes and compounds (Mittler, 2002). Antioxidant is defined as a substance, which significantly delays or inhibits the oxidation of other molecules (Park *et al.*, 2001). Chitosan is capable to act as an effective antioxidant. Three different partially deacetylated hetero-chitosans, 50%, 70% and 90% deacetylated, were prepared from crab chitin to measure their antioxidative activity using electron spin resonance (ESR) spectromer. All the hetero-chitosan possess alkyl, hydroxyl, and superoxide-radical scavenging activity, and 90% chitosan, a high degree of deacetylation, showed the highest radical scavenging effects on the

hydroxyl and superoxide radicals. In addition, the effect of these hetero-chitosans depends on their degree of deacetylation and concentration (Park *et al.*, 2004).

1.4.2 Antimicrobial activity

Chitosan and its derivatives have broad-spectrum antimicrobial effects, which inhibit the growth of some microorganisms (Allan and Hadwiger, 1979). The minimum inhibitory concentration (MIC) of chitosan, for both Gram-negative and Gram-positive bacteria, ranges from 10 to 1000 ppm depending on a multitude of factors such as the molecular weight, degree of deacetylation, solubility, positive charge density, chemical modification, pH of medium, concentration, and type of microorganism (Uchida *et al.*, 1989; Seo *et al.*, 1992).

1.4.3 Fungicidal activity

Chitosan revealed antifungal activity on inhibition of the mycelial growth of *Rhizopus stolonifer* isolated from naturally infected tomatoes. The results showed that chitosan of molecular weight 17.4 kDa, 23.8 kDa, and 30.7 kDa increased protein release (cell membrane damage determination), the glucose consumption (loss of membrane permeability determination), and hexokinase activity (glycolysis determination) (Guerra-Sánchez *et al.*, 2009).

The study of relative molecular weights of chitosan on the inhibition of plant pathogenic fungi indicated that chitooligosaccharides and partially degraded low molecular weight chitosan showed higher inhibitory activities on *Fusarium oxysporum*, *Phomopsis fukushi* and *Alternaria alternata* than high-molecular-weight chitosan (Hirano and Nagao, 1989).

1.4.4 Insecticidal activity

The physiologically active pesticides without danger for human, beneficial organism, and the environment have been considered. As mention, chitosan could be served as a good alternative persistent pesticide due to non-toxic, biodegradable, and potential insecticidal and microbicidal properties (Rabea *et al.*, 2005).

The insecticidal activity has been detected when supply artificial diet containing *O*-(decanoyl) chitosan against the larvae of the cotton leafworm *Spodoptera littoralis*. The chitosan derivative showed high inhibition of the larvae growth after 5 days of feeding (Badawy *et al.*, 2005). In addition, chitosan had a very high insecticidal activity against *Hyalopterus pruni* (Goffroy), *Rhopalosiphum padi* L., *Metopolophium dirhodum* (Walker), and *Aphis gossypii* (Glover), while *Sitobion avenae* (Fabricius) and *Myzus persicae* (Sulzer) showed a lower susceptibility to chitosan (Zhang *et al.*, 2003).

Studies on the biological activities of chitosan and its oligomers have been increasing. Different chitosan derivatives and modified products have different structures and physicochemical properties leading to the discoveries of novel bioactivities or novel findings in known bioactive compounds. These implied that no single type of chitosan or its derivatives possess all of the above biological activities.

1.5 Agricultural application of chitosan

As a result of characteristic and properties, chitosan has been extensively used in agriculture, biomedical and pharmaceutical materials, chromatographic media and analytical reagents, cosmetics, and water treatment (Tsigos *et al.*, 2000). Base on our interest, the examples of chitosan utilization focus on agricultural application.

1.5.1 Seed coating

Chitosan has an excellent film-forming property lead to easily forming a semi-permeable film on seed coat surface. The film is considered as a good selective permeability that can maintain seed moisture, absorb water from soil, prevent oxygen passing through the film, maintain a high concentration of CO_2, and control seed respiration (Zeng *et al.*, 2012). Furthermore, chitosan can increase soluble sugar content, enhance the protease activity, and increasing free amino acid content which has conclusive inhibition effect for plant pathogenic fungi (Chen and Xu, 2004).

The chitosan coating on soybean seeds is an appropriate option to control pathogenic insects and pests, which can decrease soybean aphid emergence percentage, the number of soybean aphid per plant, and leaf roll emergence percentage (Zeng *et al.*, 2012).

1.5.2 Seed priming

Seed priming has been used to improve seed performance under adverse environmental condition. It can increase a suitable range of environment for seed germination and convey faster and more uniformly seedling emergence (Lin and Sung, 2001). Seed priming with chitosan can increase germination energy, germination percentage, lipase activity, and hormone level such as gibberellic acid (GA) and indole acetic acid (IAA) in germinating peanut seeds (Zhou *et al.*, 2002).

Besides, chitosan could reduce the mean germination time, enhanced germination index, and also increased shoot height, root length, and shoot and root dry weights of maize. It also increases soluble sugars and proline contents, peroxidase and catalase activities after priming with chitosan for about 60-64

hours. This denoted that chitosan might improve the speed of seed germination and benefit for seed growth enhancement (Guan *et al.*, 2009).

1.5.3 Foliar application

Plants are able to absorb a small amount of essential and additional supplied elements by directly spraying to leaves. Considerations for foliar application of chitosan include crop protection, pests and diseases control, plant growth improvement. Chitosan formulation Elexa™ was sprayed to test for control downy mildrew disease caused by *Sclerospora graminicola* in pearl millet. Disease protection was studied by maintaining spatial and temporal separation of the inducer and the pathogen inoculation and observing for the downy mildew disease reaction. The protection was detected at 2, 7, and 14 days after chitosan spraying on seedlings inoculated with *S. graminicola* (Sharathchandra *et al.*, 2004).

Chitosan pentamer foliar application could increase net photosynthetic rate of soybean correlated with increasing in stomatal conductance and transpiration rate, but not affected the intercellular CO_2 concentration and plant growth parameters such as plant height, root length, leaf area, shoot and root dry mass after 10 days of treatment (Khan *et al.*, 2002). In the other research, chitosan was applied on strawberry seedlings in field experiment. Results showed that chitosan foliar application improved plant height, number of leaves, leaf fresh and dry weight, yield components (number and weight of fruit), and fruit quality (Abdel-Mawgoud, 2010). The difference of growth induction effects might be the consequence of type of chitosan and plant species as mention above.

Addition effect of chitosan foliar application, it can reduce plant transpiration of pepper plants supported by stomatal conductance, scanning electron microscopy (SEM) and histochemical analysis that illustrated partial or full closure of plant stomata. Chitosan also reduced water use of pepper plants while maintaining biomass production and yield in field experiment. These supposed that chitosan might be an effective antitranspirant in agriculture (Bittelli *et al.*, 2001).

1.5.4 Postharvest

A large amount of research is available on the effectiveness of chitosan treatment in postharvest to maintain the properties and extend the storage of fresh-cut fruits and vegetables. Chitosan inhibits the development of fungi involving in fruit decay, and induces resistance responses in host plants. Chitosan can reduce gray and blue mold infection which may result from enhancement of pathogenesis related enzyme activities such as phenylalanine ammonia-lyase (PAL), chitinase, chitosanase, β-1,3 glucanase after chitosan treatment (Romanazzi, 2010).

Chitosan can form an edible film when applied to the surface of fruit and vegetable. It is effective in conferring to moisture loss, delaying dehydration, and fruit shriveling. Thus, it can prolong storage shelf-life, delay a decline sensory quality, and control the decay of strawberry fruit. Chitosan coating could be used as a carrier for combination of functional ingredients, i.e. antimicrobials and nutraceutical compounds, to enhance the effects or improve the nutritional value of strawberries (Perdones *et al.*, 2012; Romanazzi *et al.*, 2013).

Chitosan pre-harvest spraying on strawberry plants significantly reduced postharvest fungal rot on strawberry fruits and maintain the quality of the fruit compared to untreated group. The chitosan-treated fruits were firmer and ripened at a slower rate (Reddy *et al.*, 2000).

Due to the unique biological properties, chitosan has been considered as an ideal coating material. The effectiveness of chitosan treatment in extending strawberry fruit shelf-life was detected on reducing darkening, delaying changes in less weight loss, ripening, progress of fruit decay and firmness, and greater visual acceptability (Hernández-Muñoz *et al.*, 2008).

1.6 Chitosan recognition as PAMPs

Chitosan is a powerful elicitor that has been shown to trigger several intricate networks in plant especially in plant basal immunity. Chitosan has been recognized as pathogen-associated molecular patterns (PAMPs) (Henry *et al.*, 2012).

PAMPs are recognized as the molecule that perceived at the plant cell surface by pattern-recognition receptors (PRRs), which typically contained an extracellular ligand-binding domain with leucine-rich repeats (LRR) or lysine motifs (LysM), a single transmembrane domain and an intracellular serine/threonine kinase domain (Nicaise *et al.*, 2009). Most of PRRs are receptor-like kinases (RLK), receptor-like proteins (RLPs) (Seifert and Blaukopf, 2010). A large number of *RLKs* and *RLPs* are transcriptionally induced upon PAMP activation. This illustrates the large diversity of perception systems and suggests their role in plant basal immunity. The common PAMPs responses as shown in Table 2.1

Additionally, partially deacetylated chitosan has been reported to weakly bind to the major chitin receptor, CERK1 and rapidly induced *in vivo* phosphorylation on its kinase domain (Petutschnig *et al.*, 2010). However, the comparison of transcription profiling between chitin and chitosan treatment in *Arabidopsis* revealed the divergence of signaling cascade. The *cerk1* mutant failed to block the expression of camalexin biosynthesis genes that are responsive to chitosan. Besides, some transcription factors for defense response could be activated by chitin and chitosan. These demonstrated that the perception of chitosan is independent of CERK1, but the downstream pathway might converge (Povero *et al.*, 2011).

The war between the plant and their pathogens appears to be in balance of the signaling systems to cause disease or enhance host defense. Fast and strong activation of the plant immune responses aids the host plants to win the war against their pathogens.

Table 2.1 Common PAMPs responses

Category	Responses	Reference
Ion flux	Calcium transient	Zuppini *et al.*, 2004
	Plasma membrane H^+-ATPase inhibition	Amborabé *et al.*, 2008
Signal transduction	Mitogen-activated protein (MAP) kinases	Lizama-Uc *et al.*, 2007
	Phospholipase C	Raho *et al.*, 2011
	WRKY transcription factors	Povero *et al.*, 2011
	MYB transcription factors	Povero *et al.*, 2011
Stress responses	Callose apposition	Franco and Iriti, 2007
	Reactive oxygen species	Lin *et al.*, 2005
Plant hormones	Abscisic acid	Iriti and Faoro, 2008
	Jasmonic acid	Doares *et al.*, 1995
	Salicylic acid	Liu *et al.*, 2011
	Ethylene	Boutrot *et al.*, 2010
Secondary metabolites	Anthraquinones	Baque *et al.*, 2012
	Camalexins	Povero *et al.*, 2011
	Phytoalexins	Hadwiger *et al.*, 1994
Defense response	Oxidative burst	Arnott and Murphy, 1991
	Pathogenesis-related proteins	Agrawal *et al.*, 2002
	Systemic acquired resistance	Iriti *et al.*, 2006
	Hypersensitive response and cell death	Ning *et al.*, 2004

1.7 Chitosan effects on plant growth induction in 'LPT123' rice seedlings

In the laboratory's study conducted by Wasinee Pongprayoon, The combination of degree of deacetylation, molecular weight and concentration of chitosan has been investigated in the 'LPT123' rice seedling growth. There were oligomeric (MW approximately 20 kDa) and polymeric (MW approximately 200 to 500 kDa) chitosan with a degree of deacetylation of 80% or 90% (named as O80, O90, P80, and P90, respectively) at concentration of 20 or 40 mg/L. For the best plant growth enhancement, O80 applied at 40 mg/L significantly enhanced the vegetative growth of rice seedlings, in term of the leaf and root fresh weights and dry weights of rice seedlings comparing with the control. In addition, chlorophyll *a*, chlorophyll *b* and carotenoid content were increased by chitosan application. These physiological responses of rice seedlings to chitosan indicated the potential of chitosan to be an elicitor for plant growth induction.