CHAPTER II
LITERATURE SURVEY

2.1 Chitin-Chitosan

The name ' chitin' was derived from the Greek word 'chiton', meaning a coat of mail named by Odier in 1823 (Shahidi *et al.*, 1999). Due to the slow biodegradation of chitin in crustacean shell waste and the accumulation of large quantities of discards from processing of crustaceans, making use of chitin-chitosan becomes a major concern demanded by the seafood processing industry. Its annual biological production is about 150 000 tons worldwide. Therefore, much attention has been paid to converse the shell waste or by-products value added products. Chitin-chitosan has unique properties as biopolymers in terms of bioactivity, biocompatibility and biodegradability. Structure of chitin-chitosan is concerned to be reactive more than cellulose owing to its amino, and the primary and secondary hydroxyl groups. Therefore, much attention has been paid to chitin-chitosan to modify either physical appearance to obtain gel, powder, film, liquid or chemical modification to obtain unique derivatives and extend the applications.

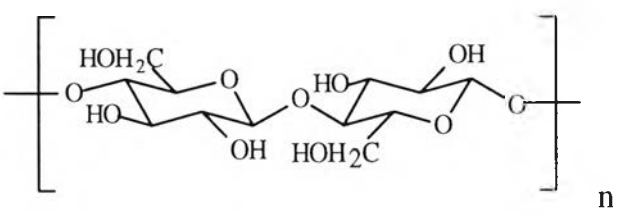
2.1.1 The Source and the Structure

Chitin is the second most abundant polysaccharides found mainly in the shells of crustaceans such as lobster and crab where the content of chitin is as much as 20 to 50% (Rathke *et al.*, 1994). Other sources of chitin-chitosan can be raised as fungi, bacterial cell walls and insect cuticles. Chitin has the same backbone as cellulose but an acetamide group is replaced the 2-hydroxy, β-(1→4)-2-amino-2-deoxy-D-glucopyranose structural unit (GlcNAc).

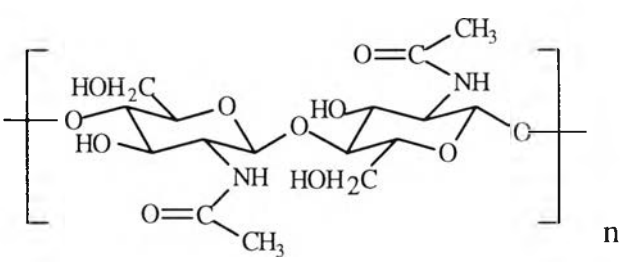
Chitosan is the N-deacetylated derivatives of chitin, β-(1→4)-2-amino-2-deoxy-D-glucopyranose structural unit (GlcNA), though this N-deacetylation is almost never complete (Scheme 2.1).

Scheme 2.1 Chemical structure of (a) cellulose, (b) chitin, and (c) chitosan

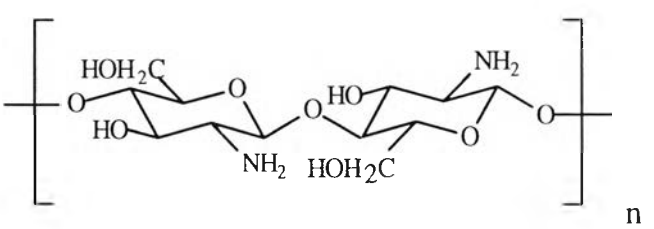
a) cellulose



b) chitin



c) chitosan



2.1.2 Physical and Chemical Properties

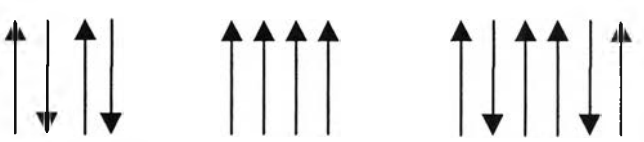
Since the source of chitin-chitosan is a main factor to make the variation of the properties. Moreover, the process to purify chitin-chitosan from the source effects the viscosity, degree of deacetylation, molecular weight, and polymorphous structure. The productions of chitin-chitosan in lab scale and industrial scale still are the issues for discussion.

Kurita *et al.* (1977) found that the homogeneous hydrolysis process could give a chitosan product with a higher adsorption ability for metal ions than one prepared by a heterogeneous process with the same degree of deacetylation.

Bough *et al.* (1978) found that the chitosan product with high viscosity and high molecular weight could be prepared by grinding the dry shrimp hulls to 1 mm prior to treatment, using alkali deproteination, purging nitrogen into the reaction vessel, and increasing the deacetylation time.

2.1.2.1 Polymorphs

Chitin has three known polymorphs, i.e., α, β, and γ, in a 2_1 helical conformation similar to that of cellulose. The α-form is arranged in an antiparallel fashion, which found in crustaceans, insects, and fungi. While β-chitin is in parallel form found in squid and marine diatoms. The γ-chitin form has not been totally classified but an arrangement of two parallel chains and one antiparallel chain has been suggested (Figure 2.1). These polymorphs have the same helical conformation but different packing of adjacent chains (Figures 2.2-2.3).



(a) **(b)** **(c)**

Figure 2.1 Arrangement of 3 polymorphs (a) α-chitin, (b) β-chitin, and (c) γ-chitin.

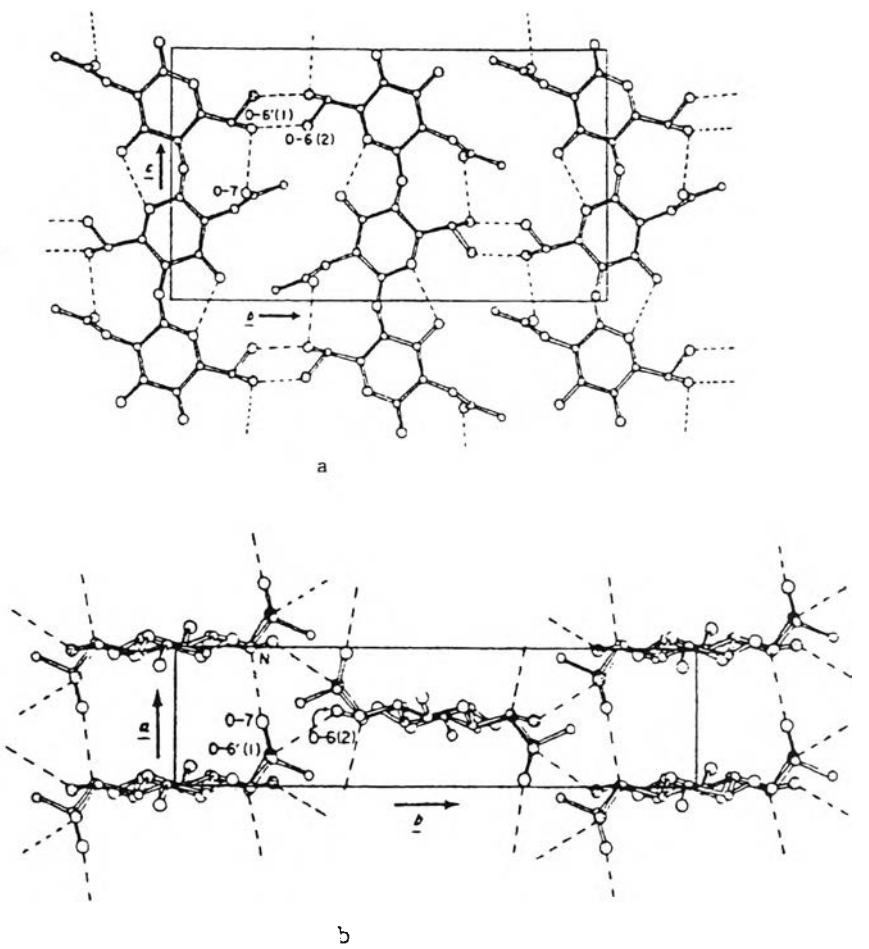


Figure 2.2 Structure of α-chitin; (a) *bc* projection, (b) *ab* projection (Minke and Balckwell, 1978).

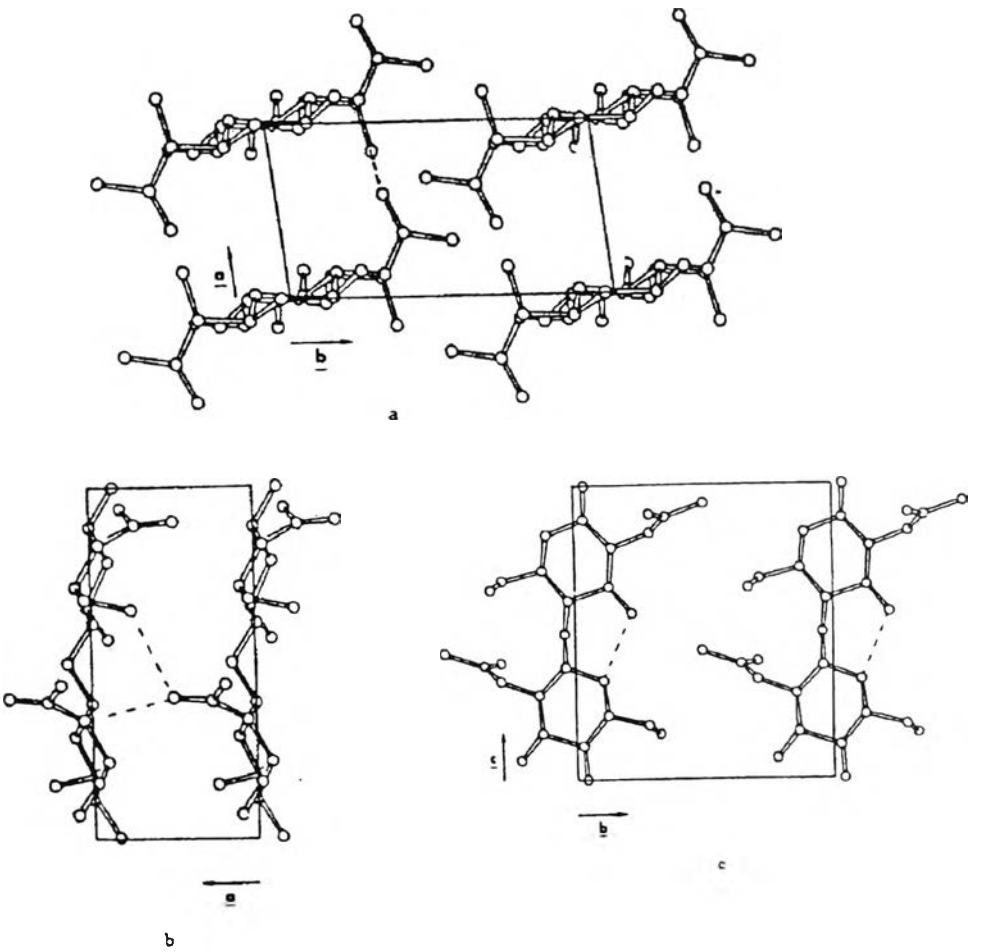


Figure 2.3 Structure of anhydrous β-chitin ; (a) *ab* projection, (b) *ac* projection, and (c) *bc* projection (Lotmar and Picken, 1950).

The α- and β-chitin can be distinguished by infrared spectroscopy due to their hydrogen bonding networks. The α-chitin exhibits a doublet at 1656 and 1621 cm^{-1} while the β-chitin exhibits a singlet at 1631 cm^{-1} (Rudall, 1963).

2.1.2.2 Degree of Deacetylation

The solubility of chitin-chitosan has been affected with the degree of deacetylation. The solubility will be improved when the degree of deacetylation increases or chitosan units increase. Due to the free amino group on chitosan unit protonated to form cationic amine group ($-NH^{3+}$), it can be dissolved in some organic solvents such as acetic acid, formic acid, and etc. Chitin-chitosan with degree of deacetylation (DD) more than 70% usually called "chitosan".

Chen *et al.* (1996) used chitin with DD 40% to prepared chitosan in different degree of deacetylation by 50% alkali treatment for twice times at 110°C for 2 h to get DD 87%. Then, the deacetylation was operated at 140°C for 3 h to get DD 90% chitosan.

Siraleartmukul *et al.* (1999) prepared chitosan with different degree of deacetylation from crab shell. The deacetylation was operated at ambient temperature with different time by using 50%(w/w) NaOH solution. The degree of deacetylation increased as the treatment time increased. Chitosan with DD 70% was obtained by deacetylation 7 days.

2.1.2.3 Molecular Weight

The molecular weight of native chitin is usually larger than one million while that of commercial chitosan product is between 100 000 and 1 200 000 (Goosen *et al.*, 1997).

Several methods are used to determine molecular weight; for example, light scattering, gel-permeation chromatography (GPC), high performance liquid chromatography (HPLC), and viscosity measurement. The viscometry method is one of the simplest and most rapid methods, which based on the well-known Mark-Houwink equation.

$$[\eta] = K \cdot M_w^a$$

Where; a and K are the constants.

$\overline{M}_w$ is the weight – average molecular weight

Since 1974 several reports have dealt with the determination of the values of K and a for the chitosan. However, each of these results was different although the determining conditions, such as solvents, and temperature, were very similar.

Wang *et al.* (1991) obtained the following equation to determine a and K.

$$K = 1.69 \times 10^{-30} \times DD^{14.0} \quad (r = 0.996)$$

$$a = -1.02 \times 10^{-2} \times DD + 1.82 \quad (r = 0.998)$$

2.1.2.4 Viscosity

The viscosity of chitosan in solution is influenced by many factors, such as the degree of deacetylation, molecular weight, concentration, ionic strength, pH, and temperature. In general, as the temperature or ionic strength rises, the viscosity of the polymer solution decreases. On the other hand, pH changing in the polymer solution may give different results depending on the type of acids used. For the degree of deacetylation, it might give the high intrinsic viscosity due to the coil expansion on dilute acid aqueous solution (Wang *et al.*, 1991).

2.1.2.5 Solubility

Chitin and chitosan degrade before melting, which is typical for polysaccharides with extensive hydrogen bonding. This physical property makes it difficult to dissolve in water, dilute acid, or alkali and other organic solvents under mild conditions. Chitin is reported to be soluble in *N,N*-dimethylformamide (DMF) containing 5% lithium chloride but the extent of solubility is dependent on the origins of chitin (Rutherford and Austin, 1978). In recent years, it has been found that chitosan can be dissolved in formic acid, methane sulfonic acid, hexafluoroisopropyl alcohol, hexafluoroacetone, and the mixture of 1,2-dichloroethane and trichloroacetic acid (35:65). Chitosan can be dissolved easier when the pH of the solution is less than 6 such as in acetic acid and formic acid. Other organic acids can be

used as a solvent such as adipic acid, citric acid, lactic acid, malic acid, malonic acid, oxalic acid, propionic acid, succinic acid, and tartaric acid.

2.1.3 Chemical modification

Chitin-chitosan has the specific properties as same as the cellulose which are bioactivity, biocompatibility, and biodegradability. It also has the potential reactive groups which are amino, primary, and secondary hydroxyl groups. Therefore, much attention has been paid to chemical modifications of chitin-chitosan not only to improve the solubility and overcome the rigid structure but also to obtain the property that can never be achieved in natural chitin-chitosan. Kurita *et al.* (1992) prepared tosylchitin by interfacial condensation at low temperature. The heterogenous reaction is a result in high molecular weight of chitosan. Up to now, less report concerned about the homogeneous reaction owing to the lack of solubility.

2.1.3.1 Chemical Modification of Chitin-Chitosan at N-position

Chitosan is N-deacetylation form of chitin that acetamide group is changed to amino group with alkali treatment. Much attention has been paid on modification of chitosan owing to the reactive amino group. In general, amino group can perform nucleophilic reaction, and react with another functional group such as acid, anhydride, and ester to form amide. Thus, chitosan has received much interest with these reactions.

Hirano *et al.* (1976) studied the selective N-acylation of chitosan by treatment of solution in aqueous methanolic acetic acid with carboxylic anhydrides at room temperature. The obtained N-acylchitosan was found to be effective as a selective aggregation of some cancer cells.

Kurita *et al.* (1988) reported that the N-acylation plays a role to loose hydrogen bonding and improve the properties, for example, metal absorption by the introduction of long chain alkyl group into the chitosan chain.

Nishimura *et al.* (1991) prepared the dissolvable chitosan derivatives, i.e., N-phthaloylchitosan, by phthalic anhydride in *N,N*-dimethylformamide (DMF) at 130°C. The obtained product was improved for the solubility in organic solvent such as *N,N*-dimethylformamide, pyridine, *N,N*-dimethylsulfoxide.

Kurita *et al.* (1992) used tosylchitin as a precursor for fully acetylation of free amino groups in tosylchitin under mild condition by using acetic anhydride.

2.1.3.2 Chemical Modification of Chitin-Chitosan at O-position

Similar to the N-position, primary and secondary hydroxyl groups at C-2, 3 and C-6 positions, respectively. The hydroxyl group can react with acid, anhydride, amide, acid halide, and ester to form ester bond. Thus, much attention has been focused on chitosan chain at hydroxyl group.

Tokura *et al.* (1983) studied the properties of water soluble chitin derivatives by modifying chitin into carboxymethyl-chitin (CM-chitin) and dihydroxypropyl-chitin (DHP-chitin). These derivatives were prepared successfully by simple procedures involving freezing and the addition of a detergent such as sodium dodecylsulfate (SDS).

Nishimura *et al.* (1986) reported the immunological of 6-O-carboxymethyl-chitin which could induce the cytotoxic macrophage and enhance the accessibity to lysozyme in mouse.

Several 6-O-substituted derivatives are prepared by using phthaloylchitosan as a starting material to react with some bulky groups, such as triphenylmethyl (trityl) and *p*-tolylsulfonyloxy (tosyloxy) groups in homogeneous solution under mild conditions. The obtained products are regioselectively reacted 3-O-acetylation to give better solubility (Nishimura *et al.*, 1991).

The interfacial reaction has been done successfully by Kurita (1992) to prepare 6-O-tosylchitin. It was used to be precursor for iodination to give more soluble in organic solvent.

2.1.4 Chitin-Chitosan for Drug Delivery System

Owing to its unique properties, i.e., bioactivity, biocompatibility, and biodegradability, chitin-chitosan has become an alternative polymer material for application on biosystems. Controlled release system or drug delivery system and the related prodrug by using chitin-chitosan derivatives have received much attention in recent years.

Sugano *et al.* (1980) found the hypocholesterolmic activity of chitosan by oral administration in rats. The safe oral dosage of chitosan as an additive for hens and broiler was < 1.4 g/kg of body weight per day, and < 0.8 g/kg of body weight per day in rabbits. In 1983, the United States Food and Drug Administration (USFDA) approved chitosan as a feed additive because of its hypocholesterolemic effect.

Hirano *et al.* (1990) reported that the serum cholesterol and triglycerol in rabbits, hens, and broilers was decreased by feeding 2% chitosan, while the feeding at 1% chitosan and 2% chitin didn't show the effect.

Chitosan forms micelles with cholesterol inside the digestive tract. Bacteria from the large intestines can digest these micelles and then excrete as free forms into faces without absorption in biosystem. Deuchi *et al.* (1994) proposed the solubilization of chitosan in the small intestine at pH 6-6.5. With the aggregation of polysaccharide chains, the oil droplets are entrapped in their matrices thereby passing through the lumen. Recently, Yoshioka *et al.* (1995) studied micelle properties of chitosan derivatives, sulfated N-acyl-chitosan (S-C_n-chitosan), to find the higher length of chains above C_{10} showing the micelle form.

Although chitin-chitosan is known to have a significant hypocholesterolmic activity in various experiment animals, its highly viscosity

restricts the use as a constituent of physiologically functional foods. The high molecular weight of chitin-chitosan and the high crystallinity lead to the low solubility in most organic solvents. The improvement of solubility, even it has been chemical modified into various derivatives, are still not satisfied. Therefore, many researchers proposed an alternative way to degrade the chain of polymer to be the oligomer by oligomerization via chemical treatment, photoirradiation, and enzymatic hydrolysis. Chemical treatment can be done by acid or base (Defaye *et al.*, 1989, and Allan *et al.*, 1997) to obtain dimer to decamer, while photoirradiation was achieved by using γ-ray, and UV (Ulanski *et al.* 1992, and Andrady *et al.*, 1996). The enzymatic hydrolysis (Hirano *et al.*, 1989, and Aiba *et al.*, 1994) was done by using chitinase from *Strepmyces griseus* or chitosanase from *Bacillus* sp. No. 7-M to obtain dimer to heptamer. Among these methods, enzymatic hydrolysis is the effective method under mild condition and does no harm to the environment. Moreover, it is more interesting pathway to maintain the structure of chitin-chitosan.

2.2 Chitinase Enzyme

A chitinase (EC 3.2.1.14) was described for the first time in 1911 by Bernard who found a thermosensitive and diffusable antifungal factor in orchid bulbs. In 1929, a similar factor from snails was reported by Karrer and Hoffman. Chitinases, an enzyme cleaving a bound between β-(1,4) glycosidic linkage of N-acetylglucosamine, are classified into 2 types based on the digestion at different position in the chitin chain, endochitinase and exochitinase (Flach *et al.*, 1992). Endochitinase is an enzyme cleaving β-(1,4) glycosidic linkage of N-acetylglucosamine within the chitin polymer whereas exochitinase is an enzyme cleaving at the end of chitin chain (Figure 2.4).

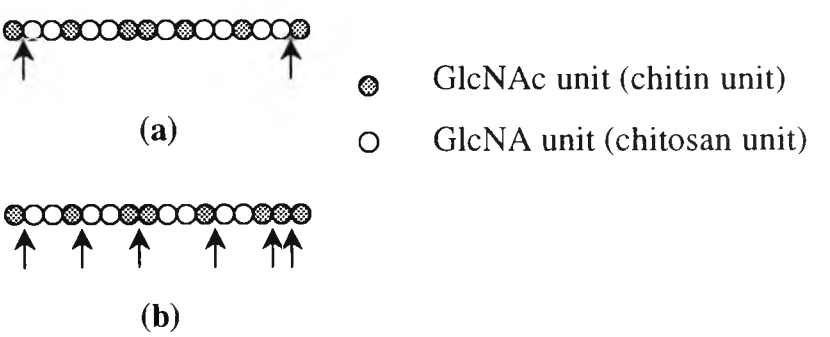


Figure 2.4 Action of enzyme (a) exochitinase, and (b) endochitinase.

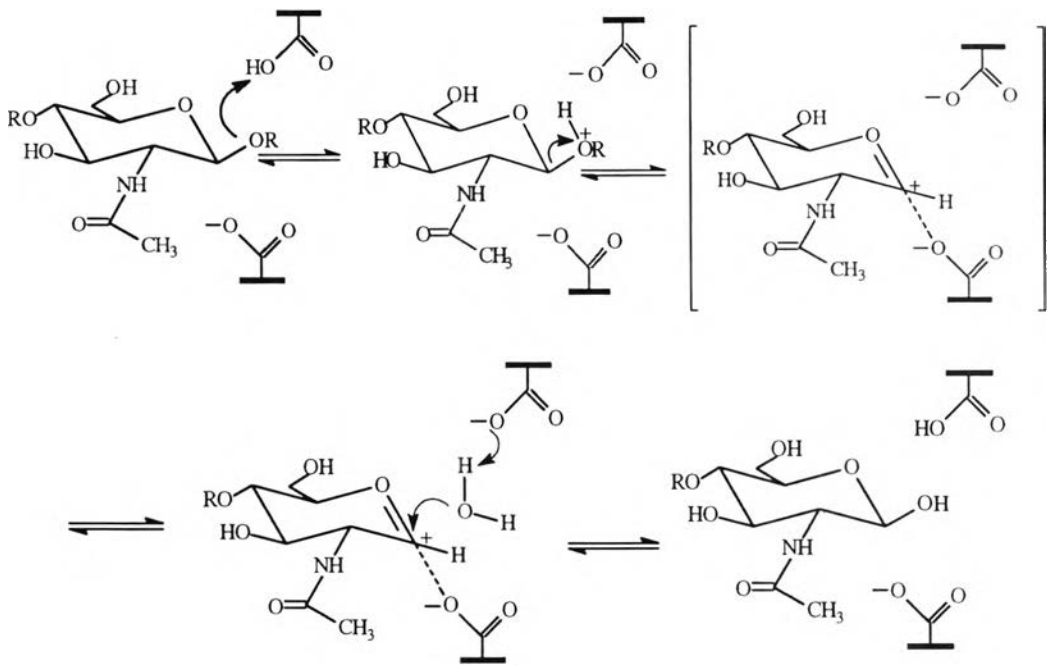
Chitinases are produced by a variety of microorganisms such as yeast, fungi, bacteria, and also plants. In microorganisms, these enzymes are critical for the normal life cycle functions of molting and cell division. In addition, plants produce chitinases as a defense against fungal pathogens (Felse *et al.*, 1999).

Chitinases were classified into 5 classes and grouped into 2 families different in structure and enzymatic degradation mechanism (Brameld *et al.*, 1998). Family 19 chitinases consisting of class I, II, and IV found only in plants which has mechanism like hen-egg white lysozyme (HEWL) (Scheme 2.2).

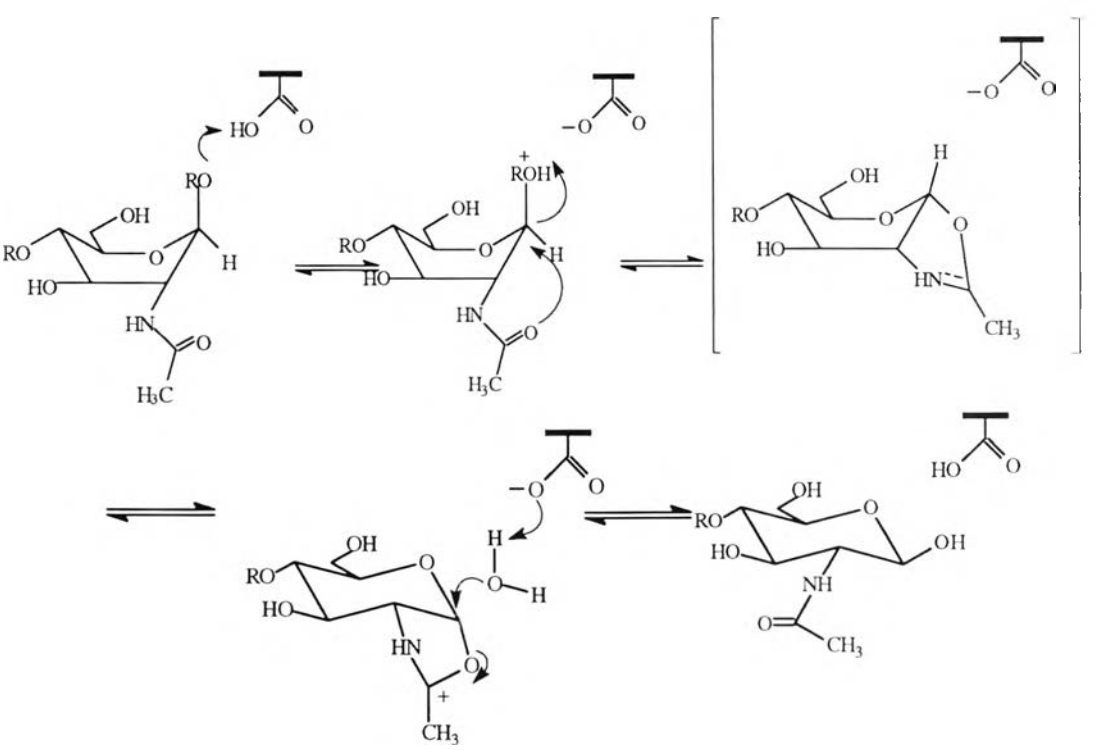
The mechanism of family 19 has been proposed by Hart (1995). In this mechanism, it requires two acidic residues to process hydrolysis while the product will be retained the initial anomeric configuration (equatorial form).

In contrast, family 18 chitinases have been isolated from a wide range of organisms including bacteria, fungi and animals consisting of class III and V. The mechanisms are believed to occur through anchimeric assistance by nieghboring N-acetyl group forming on oxazoline ion intermediate and substrate distortion into boat conformation (Scheme 2.3).

Scheme 2.2 Double-displacement hydrolysis mechanism



Scheme 2.3 Anchimeric stabilization hydrolysis mechanism



The chitinase activity from organisms can be determined by various methods such as viscosimetric assay, turbidimetric assay, and colorimetric assay (Aiba *et al.*, 1992). Although, chitinase can degrade chitin but chitin is not good substrate for measuring activity because of low surface area and solubility problem. Glycol-chitin, colloidal chitin and carboxymethylchitin are good substrates because they are soluble in solvents and have more surface area to be degraded.

There are reports described the effect of the degree of *N*-acetylation of chitosan substrates on the chitinase activities. Ohtakara *et al.* (1988) studied the action of microbial chitinases on moderately N-deacetylated chitosan (MDC) with different degree of N-acetylation in the homogenous solution state. It is clarified that all of chitinases hydrolyse MDC at faster rates as increasing the degree of *N*-acetylation. This indicates that chitinase has a specificity towards N-acetyl-D-glucosaminidic linkages in the chitosan chain. Hirano *et al.* (1989) reported that partially N-acetylated chitosans (PAC-H) with 40-80% acetyl content were hydrolyzed more than 100% N-acetylated chitosan by chitinases from *S. griseus*, *S. sp.*, and *S. antibioticus* in the suspension state at pH 6.8. Neugebauer *et al.* (1991) reported that partially *N*-acetylated chitosans with 50-95% acetyl content were digested more efficient than colloidal chitin in the suspension state at pH 4 by *Streptomyces lividans* chitinase.

Aiba *et al.* (1993) studied on the difference in substrate recognition by chitinase. It is reported that chitinase has higher activity than lysozyme on chitosan chain. The chitinases recognize only a GlcNAc residue in a chitosan chain while lysozyme recognizes GlcNAc sequences (more than 3 GlcNAc residues).

2.3 Oligochitin-Chiotsan

Recently, oligochitin-chitosan has received much interest in agriculture and medicine because its low molecular weight induces, high biodegradability, and high solubility in most organic solvent and water. Much attention has been paid on oligochitin-chitosan for practical applications *in vivo* system. Oligochitin-chitosan was reported for activities as elicitors, antibacterial agents, immuno-enhancers, and lysozyme inducers.

Hirano *et al.* (1992) reported about the effect of chitosan (MW 250 000) and its oligosaccharides (MW 304–1 306) on serum lysozyme activity by intravenous and oral administration in rabbits. It was found that chitosan oligosaccharides make the serum lysozyme activity enhanced about 3 times that of the control.

Oligosaccharides over the hexamer show high activity even at very low concentration. Roby *et al.* (1987) reported that hexamer to nanomer are the most efficient elicitors in melon plants to stimulate chitinase activity for defense fungal pathogen. Kendra *et al.* (1984) studied on chitosan oligomer for antifungal to *Fusarium solani*, which elicits pisatin formation in *Pisum sativum* and found that the heptamer unit was maximal in both antifungal activity and formation of pisatin.

Up to now, less report deals on the hypochloesterolmic activity of oligochitosan. Sugano *et al.* (1992) reported that the oligomer with molecular weight 6.0×10^2 did not have a hypocholesterolmic effect. They concluded that the cholesterolmic activity showed when the molecular weight of chitosan was at least 7×10^3.

2.4 The Potential of the Present Work

The enzymatic method by using chitinase from soil bacteria is an interesting way to prepare the oligomer. Owing to the warm climate of Thailand, there are many types of soil bacteria that can be easily obtained in nature. The present work proposed the use of *Staphylococcus species* strain TU005 (E) to digest chitosan oligochitin-chitosan. It is our interest to focus on the chemical modification of the oligochitosan, to get the reactive precursor oligochitosan. The present work will be the basic research for the oligochitosan derivatives designed for the hydrophobic micelle structured chitosan.