# CHAPTER I

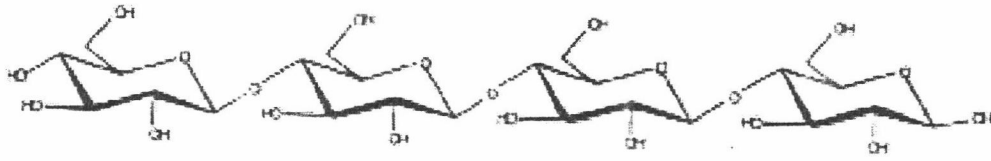
# INTRODUCTION

## CHITIN

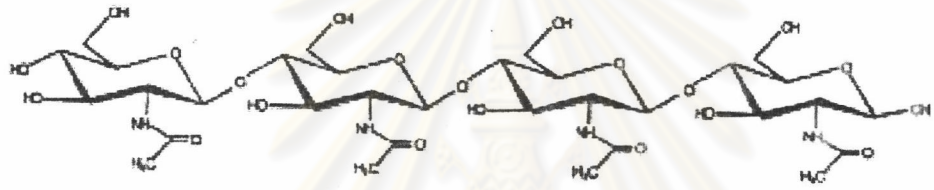
Chitin is one of the three most abundant polysaccharide in nature, in addition to cellulose and starch. It ranks second to cellulose as the most plentiful organic compound on earth. At least 10 gigatons of chitin are synthesized and degraded each year in the biosphere. Chitin is a polymer of unbranched chains of β-1,4-linked aminosugar, *N*-acetyl glucosamine (GlcNAc) molecules, which is partially deacetylated. This polysaccharide is found in the marine environment and is an important nutrient and carbon source for the ecosystem in the sea [1]. Chemically, cellulose and chitin are polysaccharide polymers, or large molecules consisting of smaller sugar molecules strung together, like pearls on a strand (Figure 1). The chemical structure of chitin is similar to cellulose with monomers attached via β(1→4) linkages, monomers of cellulose and chitin are glucose and 2-acetamido-2-deoxy-β-D-Glucose (GlcNAc), respectively. Chitosan is the deacetylated form of chitin in varying degrees of deacetylation and is composed primarily of glucosamine, 2-amino-2-deoxy-D-Glucose (GlcN)[2] (Figure 2).

Chitin is found naturally in the shells of crustaceans, such as crawfishes, crabs shrimps, and lobsters. It is the major structural component of exoskeleton of marine zooplankton, including coral and jellyfish [3, 4, 5, 6]. Insect, such as ladybugs and butterflies, have chitin in their wings. The cell wall of yeast, mushrooms, and other fungi also contain this natural substance [7]. Chitin can be synthesized by some unicellular organisms, such as diatom and protozoa. The chitin synthesized is illustrated in Figure 3.

(A) cellulose



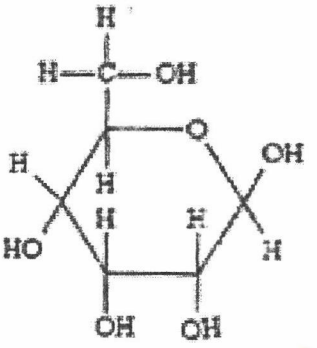
(B) chitin



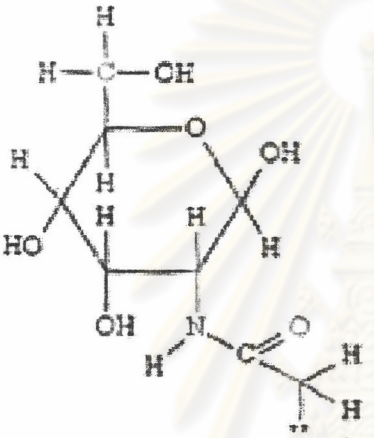
(C) chitosan



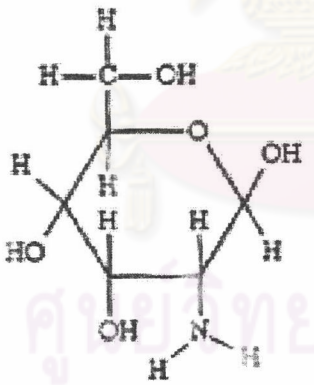
**Figure 1 Polymer of (A) cellulose, (B) chitin, and (C) chitosan.**



**(A) monomer of cellulose:**
Glucose



**(B) monomer of chitin:**
2-acetamido-2-deoxy-β-D-Glucose (NAG)



**(C) monomer of chitosan:**
2-amino-2-deoxy-D-Glucose (glucosamine)



**Figure 2 Monomer of (A) cellulose, (B) chitin and (C) chitosan.**

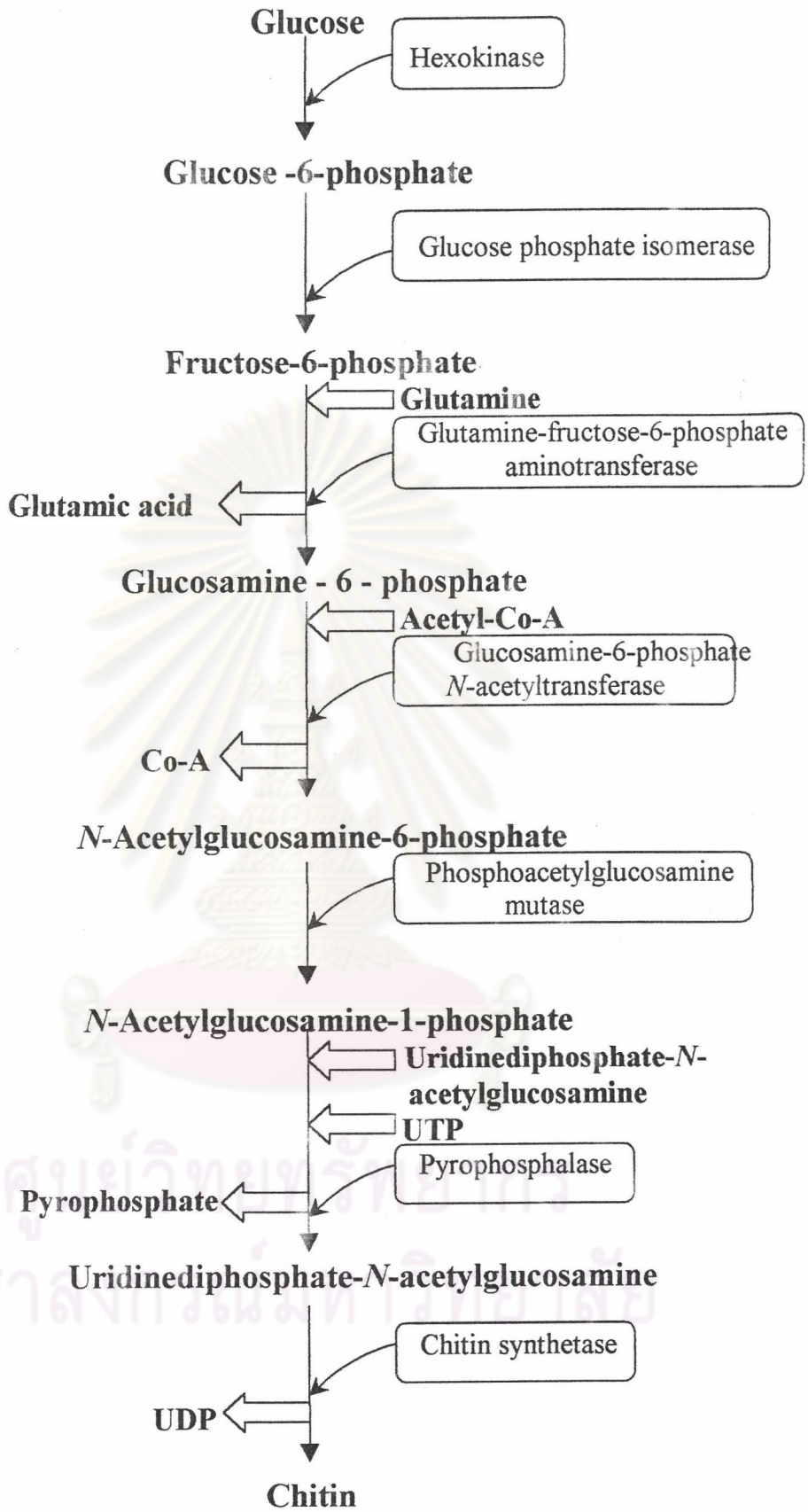



**Figure 3 Chitin synthesis.**

In most organisms chitin is modified by forming linkages with other polymers like glucans, proteins, etc. However, some centric diatoms, such as *Thalassiosira fluviatilis* produce radiating spines that are the purest form of chitin known in nature as they are fully acetylated and unlinked with other extracellular components [8].

Electron microscopy shows that chitin is the crystalline that has a fibrous morphology [9]. From comparison of X-ray data of chitin from different sources, chitin is classified into three groups by the molecular conformation in nature α-chitin, β-chitin and, γ-chitin [41]. The α-chitin and β-chitin are arranged in antiparallel and parallel form, respectively. The γ-chitin is a mixed orientation of strand form of α-chitin and β-chitin (Figure 4). Most of chitin found in nature are in the α-form, such as chitin from crustaceans, insects, and fungi. However, we can find β-chitin in the spines of polychaete *Aphrodite*, the pen of the squid *Loligo*, the tube of *Pogonophor*, and the spines of certain marine diatoms. The γ-chitin has been reported from the stomach lining of *Loligo*.

Chitin and its derivatives are of interest because they have various biological activities and broad range of industrial application [6, 10]. [Table 1]

Many countries already used chitin and chitosan in a variety of products, yet a whole array of possible materials could be made from these compounds. Unlike most polysaccharides, chitosan has a strong positive charge that allows it to bind to negatively charged surfaces such as hair and skin. This makes it useful as an ingredient in skin and hair care products. Several studies indicate that chitosan's charge also helps it bind to fats and cholesterol and initiate clotting of red blood cells. In Japan, chitosan was first used for wastewater treatment because of its metal-binding properties, but today chitin and chitosan are found in many products from antibiotics and surgical sutures to dietary supplements, foods, and cosmetics. It also can be found in pet foods and is used to make cloth for undergarments and socks.

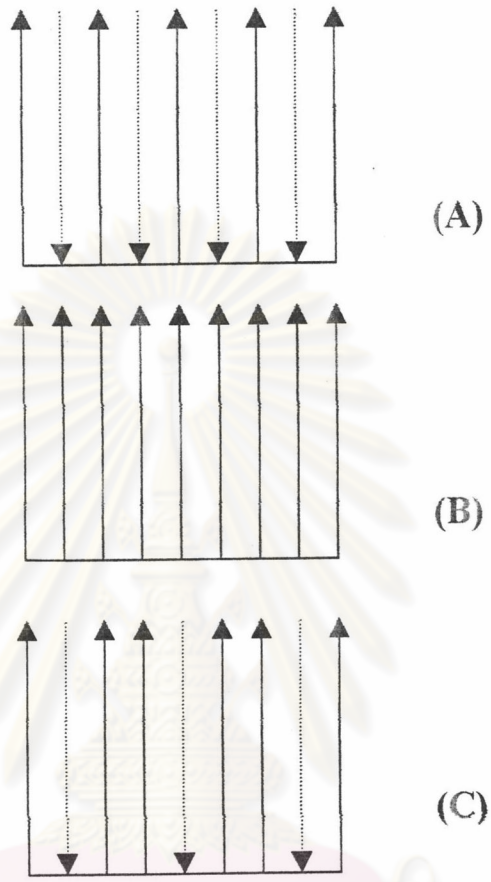



**Figure 4 Diagrammatic illustration of three types of chitin with arrangements as (A) α-chitin, (B) β-chitin and (C) γ-chitin. Arrows show the direction of chitin strands.**

**Table 1 Application of chitin and its derivatives.**

| Area of Application | Example |
|---|---|
| Nutrition | Dietary supplement and dietary fiber<br>Cholesterol reducer<br>Fiber source<br>Bifidobacteria promoter<br>Lactose intolerance aid<br>Weight-loss aid<br>Reduction of lipid absorption<br>Infant feed ingredient |
| Food | Nutraceutical<br>Food film<br>Flavor preservative<br>Flavor enhancer<br>Texture-enhancing agent |
| Biomedical | Wound healing and wound dressing<br>Burn healing<br>Bone healing<br>Intraocular and contact lenses<br>Eye and gum disease treatment<br>Plaque reducer and other dental care treatment<br>Skin irritation relief<br>Sutures<br>Anti-tumor agent<br>Osteoarthritis treatment<br>AIDS inhibitor<br>Skin grafts |
| Skin and Hair care | Moisturizing creams and lotion<br>Hair care product |
| Environment and Agriculture | Water treatment<br>Seed treatment<br>Insecticide |

**Table 1 (continued)**

| Area of Application | Example |
|---|---|
| Antimicrobial agent | Bactericidal<br>Fungicidal<br>Measure of mold contamination in agriculture commodities |
| Edible film industry | Controlled moisture transfer between food and surrounding environment<br>Controlled release of antimicrobial substances<br>Controlled release of antioxidants<br>Controlled release of nutrients flavors and drugs<br>Reduction of oxygen partial pressure<br>Controlled rate of respiration<br>Temperature control<br>Controlled enzymatic broening in fruits<br>Reverse osmosis membranes |
| Additive | Clarification and deacidification of fruits and beverages<br>Natural flavor extender<br>Texture controlling agent<br>Emulsifying agent<br>Thickening and stabilizing agent<br>Color stabilization |
| Purification of water | Recovery of metal ions, pesticides, phenols, and PCB's<br>Removal of dyes |
| Other applications | Enzyme immobilization<br>Encapsulation of nutraceuticals<br>Chromatography<br>Analytical reagents |

In the U.S., chitin and chitosan are being used in seed treatment, animal feed supplementation, and water purification, as well as in hair care products and dietary supplements. Natural substances such as chitin and chitosan demand a closer look at a time when synthetic compounds are losing their appeal.

Chitin can be processed into many derivative, such as chitosan, chitin oligosaccharide and chitosan oligosaccharide. The most readily available is being chitosan, which is formed when chitin is heated with concentrated NaOH solution. The natural chitin was processed into oligomer and monomer as show in Figure 5.

Different amount of chitin was found in different organisms as shown in Table 2 (Muzzarelli: All numbers are approximate).

Chitooligosaccharides are prepared by chemical hydrolysis, including alkaline hydrolysis and acid hydrolysis, or enzymatic hydrolysis. Chemical hydrolysis gives products that have low degree of polymerization, varying from monomer to trimer. Differ from chemical hydrolysis, enzymatic hydrolysis gives high degree of polymerization, ranging from tetramer to heptamer that can used for different applications [9] and the hydrolysis products were specific for each enzyme (Figure 6).

The complete enzymatic hydrolysis of chitin to free GlcNAc is performed by a chitinolytic system, the action of which is known to be synergistic and consecutive [2, 12].

## CHITINASE

Different organisms produce a wide variety of hydrolytic enzymes that exhibit different substrate specificities and other properties useful for various functions. Chitinase (EC. 3.2.1.14) is a group of enzymes, glycosyl hydrolase, capable of degrading chitin directly to low molecular weight products.

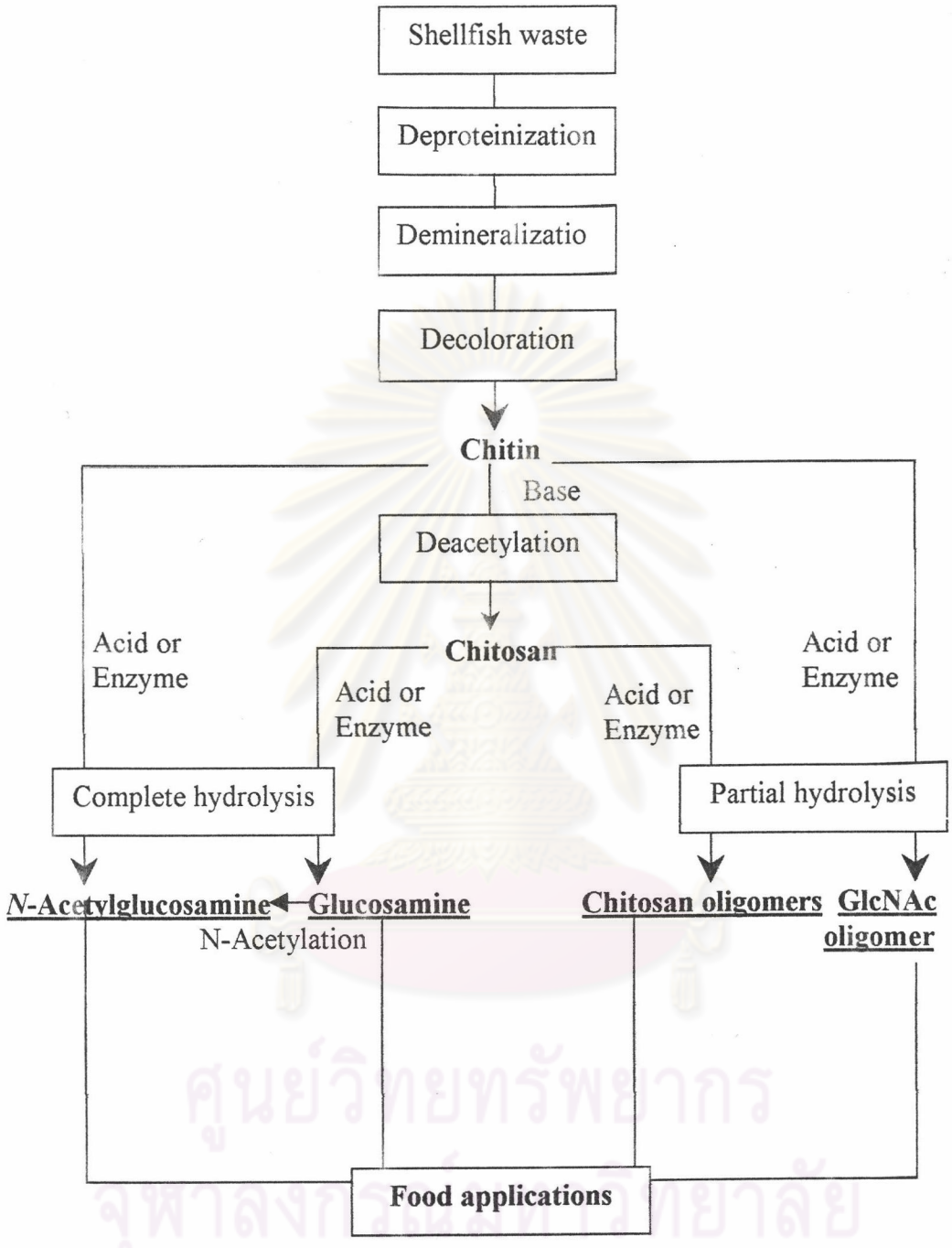



**Figure 5 Simplified flowchart for preparation of chitin, chitosan, their oligomers and monomers from shellfish waste [11].**

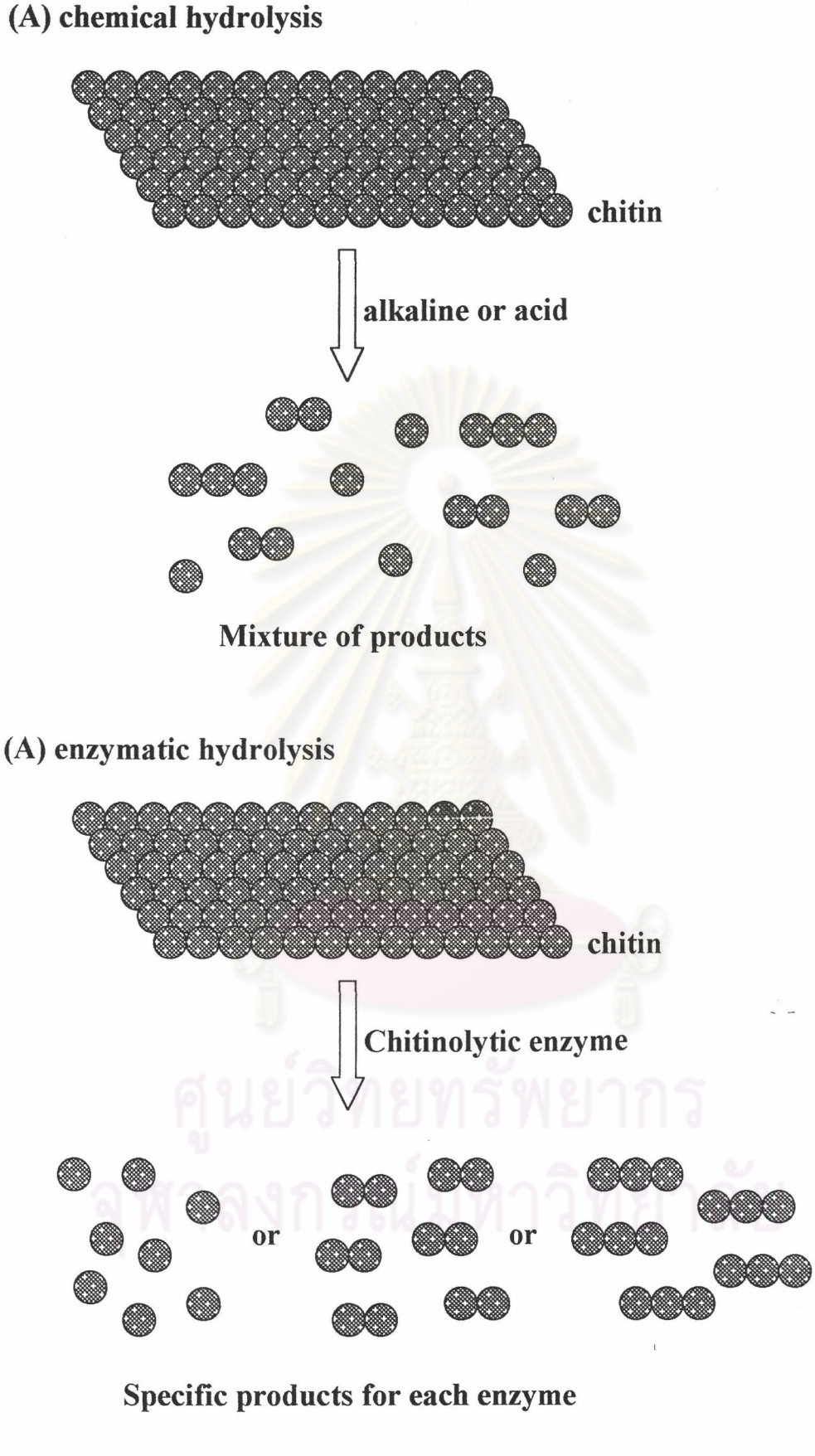



**Figure 6 Hydrolysis of chitin.** (A) chemical hydrolysis (B) enzymatic hydrolysis. = N-acetyl-D-glucosamine.

**Table 2 Percentage of chitin in different sources.**

| Source of chitin | Percentage of chitin |
|---|---|
| Fungi | 5-20% |
| Worms | 20-38% |
| Squids/Octopus | 3-20% |
| Scorpions | 30% |
| Spiders | 38% |
| Cockroaches | 35% |
| Water Beetle | 37% |
| Silk Worm | 44% |
| Hermit Crab | 69% |
| Edible Crab | 70% |

Chitinases have been found in a wide range of organisms, including viruses, bacteria, yeast and fungi, invertebrates, insects, crustaceans, plants, and animals. In microorganisms, chitinases play roles in nutrition, parasitism, and cell wall morphogenesis [13] whereas it is also required for cell separation during growth in yeast. In fungi and protozoa, they are also involved in morphogenesis. In plants and bacteria, chitinolytic enzymes as well as chitosanolytic enzymes and glucanase could be used as natural antifungal agent, they express these enzymes to protect themselves against fungal attack. Especially in higher plants, chitinases were thought to be a self-defense related protein for protection against fungal pathogen [14, 15]. Baculoviruses, which are used for biological control of insect pests, also produce chitinases for pathogenesis [1]. Chitinase activity in human serum has recently been described. The possible role suggested is a defense against fungal pathogens [16, 17] enzyme, chitotriosidase is used as a marker of Gaucher disease, a lysosomal storage disorder [17].

Chitinases can be classified in two major categories, including endochitinase and exochitinase. Endochitinase cleave chitin randomly at internal sites, generating soluble low molecular mass multimers of *N*-acetyl glucosamine (GlcNAc) molecules, such as chitotetraose, chitotriose, and the dimer, di-acetylchitobiose [18]. Exochitinase can be divided into two subcategories: chitobiosidases (EC. 3.2.1.29) [19], which catalyze the progressive release of diacetylchitobiose starting at the nonreducing end of chitin and β-1,4-*N*-acetyl glucosaminidase (EC. 3.2.1.30); which cleave the oligomeric products of endochitinase and chitobiosidases generating monomers of GlcNAc [18]. Based on amino acid sequence similarity of chitinases from various organisms, five classes of chitinases have been proposed. These classes can be grouped into two different families of glycosylhydrolase, families 18 and 19 [20, 21] based on the amino acid sequence similarity of their catalytic domains. The catalytic domains of family 18 chitinases have $(\beta/\alpha)_8$ barrel folds [22-26], whereas those of family 19 chitinases have high α-helical content and share the bilobal α+β folding motif of lysozyme, which form a

well-defined substrate binding cleft between the lobes (Figure 7). Chitinolytic bacteria generally produce multiple chitinases derived from different genes. Many chitinolytic bacteria produce only family 18 chitinases, while some other bacteria such as *Streptomyces* species also produce family 19 chitinases [27]. Family 18 chitinases are classified into three subfamilies, subfamilies A, B, and C [28]. The classification is based on homology of amino acid sequence inside the catalytic domain, the largest domain of enzyme which composes of at least 300 amino acid residues, compared with chitinase A1 and chitinase D of *Bacillus circulans* WL-12 as shown in Figure 8. Chitinase subfamily A contains amino acid residues in their catalytic domain homologous almost entirely to the catalytic domain of chitinase A1. Chitinase subfamily B contains catalytic domain homologous to the catalytic domain of chitinase D and chitinase subfamily C contain amino acid sequences in their catalytic domain that has no homology to the catalytic domain of chitinase A1 nor D. More than that, chitinase in subfamily A have an insertion domain between the seventh and eighth β-strands of the $(\beta/\alpha)_8$ barrel basic structure, which is missing in chitinase of subfamilies B and C.

## Mechanism of chitinases

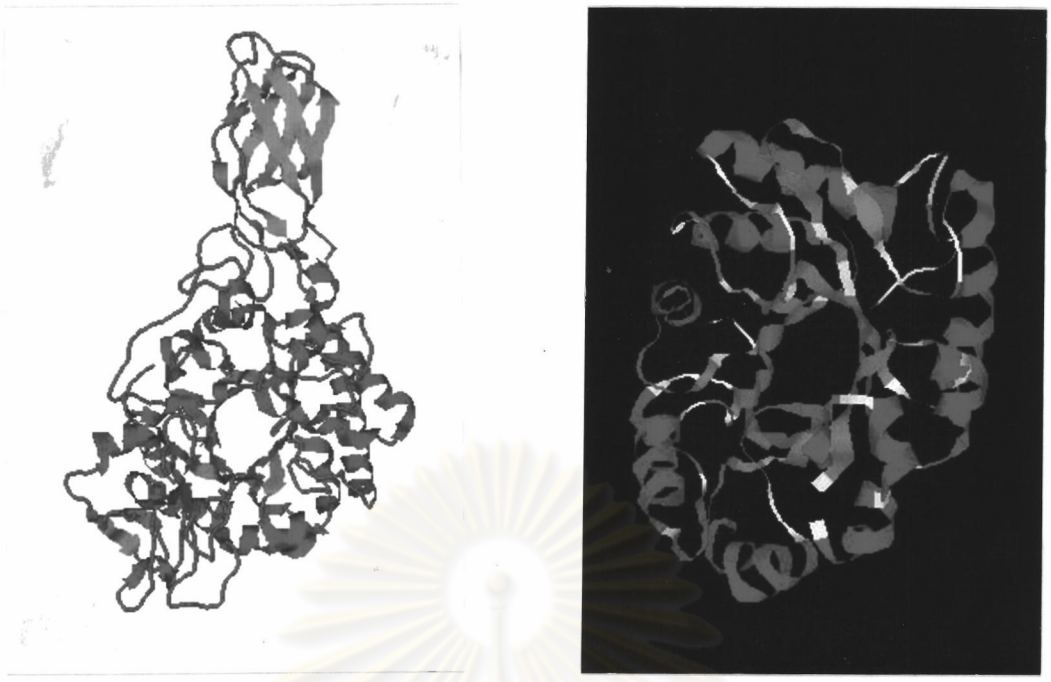
Mechanism of chitinases was cleaving the β-1,4-linkages between GlcNAc residues. There are two general mechanistic pathways for glycosyl hydrolysis, one is the hydrolysis with retention of the anomeric configuration in products, and the other with the invertion.

Extensive studies of the mechanism of hen egg white lysozyme (HEWL) illustrated that, glycoside hydrolysis required two acid residues (Glu36 and Asp52), one of which is protonate.

In family 18, the catalytic residue was first reported by Watanabe et. al., for *Bacillus circulans* WL-12. Site-directed mutagenesis of Glu204 completely eliminated chitinase activity. This result indicated that Glu is a proton donor in catalytic reaction. From amino acid sequence comparison in

this group, the glutamic acid residue was found as a conserved residue in all chitinase family 18. In chitinase A of *Serratia marcescens*, the catalytic carboxylate that responding to Glu204 of *Bacillus circulans* chitinase A is Glu315. *Bacillus circulans* chitinase A1 produce β-anomer, indicated that enzyme dose not change the anomeric configuration of C1 of the substrate, a retaining enzyme. In consensus region of the catalytic domain of family 18 chitinases, there are several other conserved carboxylic amino acid residues, such as Asp200 and Asp202 in chitinase A from *Bacillus circulans*, Asp311 and Asp313 in chitinase A from *Serratia marcescens*. Unlike Glu, site-directed mutagenesis of Asp200 and Asp202 in chitinase A from *Bacillus circulans* decreased activity of enzyme, but did not completely eliminated chitinase activity [29, 30]. The location of these residues did not correspond to carboxylate in lysozyme (Asp52) or in family 19 barley chitinase (Glu89).

The family 18 chitinases have two types of different catalytic mechanisms. Recent studies on the family 18 chitinase indicated that the catalytic reaction of the enzymes take place through a substrate-assisted mechanism. As shown in Figure 9, a putative oxocarbonium ion intermediate is stabilized by an anchimeric assistance of the sugar-N-acetyl group after proton donation from the catalytic carboxylate to the leaving group. Such a stabilization might occur either through a charge interaction between the C1 carbon and oxygen carbonyl on acetyl group or via an oxazoline intermediate with a covalent bond between C1 carbon and the oxygen of carbonyl group. This mechanism does not require the second carboxylate and the prodes can rationalise the anomer retaining reaction of enzymes without the second carboxylate.



A. B.

C.



**Figure 7 Three dimensional structure of family 18 chitinase and family 19 chitinase.**

Panel A: Family 18 chitinase from *Serratia marcescens* (chitinase A)
Panel B: Family 18 chitinase from Hevamine
Panel C: Family 19 chitinase from Barley
α-helices were shown in pink, and β-strands were shown in yellow.
(create by RasMol 2.6)

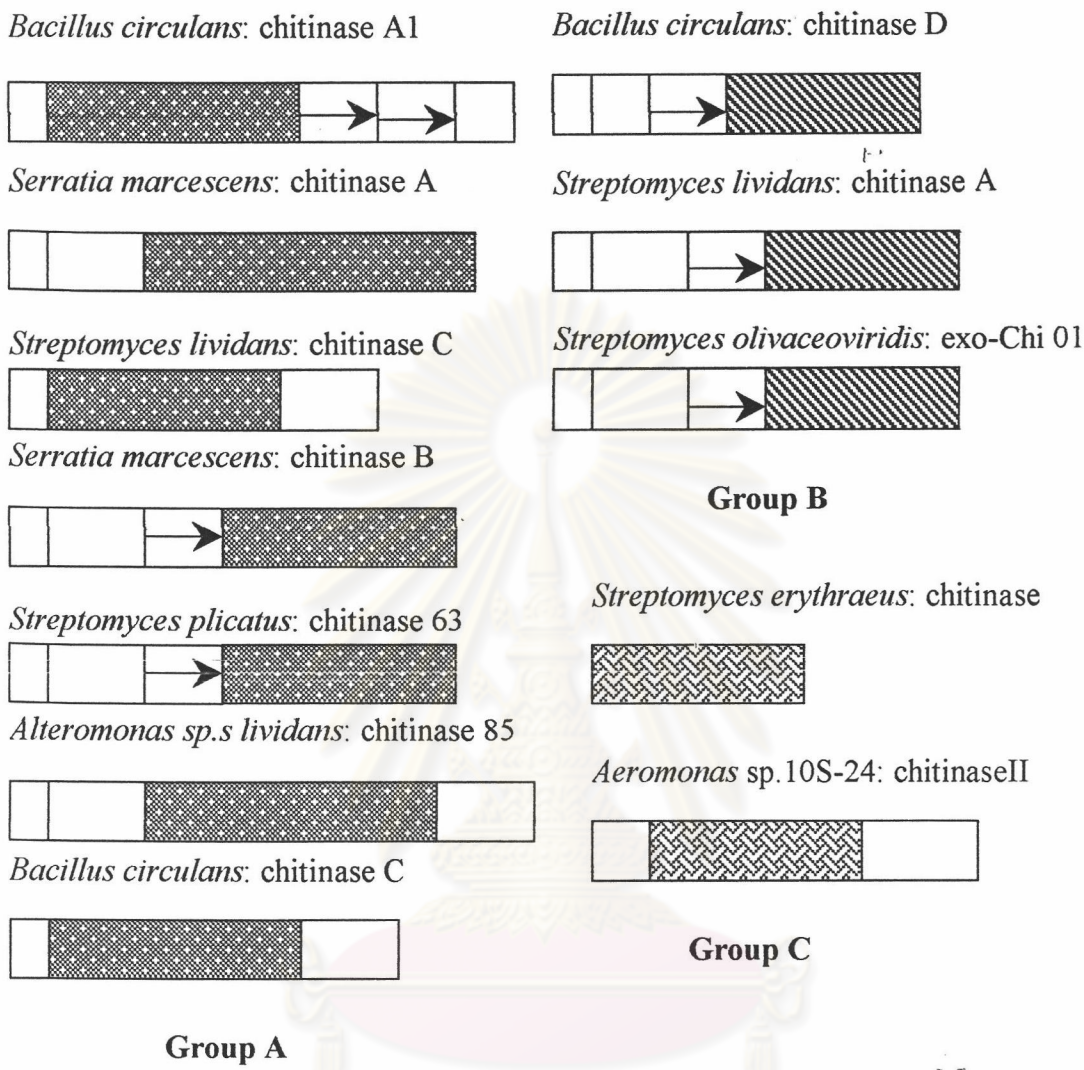



**Figure 8 Classification of bacterial chitinases based on the homology of amino acid sequence of individual catalytic domains.** The homologous regions of individual chitinase were showed as shadowed boxes. Group A ▨, the catalytic domain similarity to chitinase A1 from *Bacillus circulans* , Group B ▨, the catalytic domain similarity to chitinase D from *Bacillus circulans*, and Group C ▨, the catalytic domain not similarity to chitinase from *Bacillus circulans*. Arrows indicated fibronectin type III-like

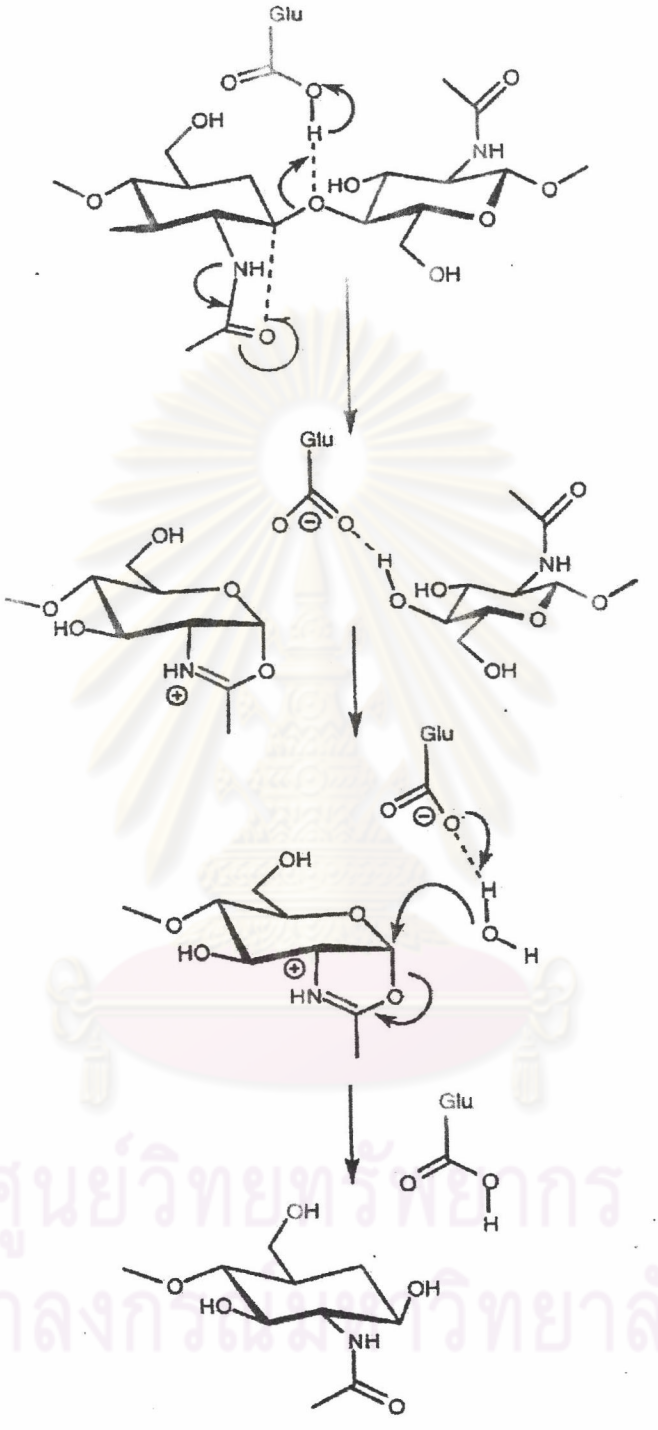



**Figure 9 Mechanism of glycosyl hydrolysis catalyzed by family 18 chitinase following substrate-assisted catalysis**. The oxocarbonium ion intermediate is stabilized by an anchimeric assistance of the sugar N-acetyl group after donating a proton from the catalytic carboxylate.

## Chitinase assay

Chitinase activity can be assayed by various procedures. These include the measurement of reducing in size of substrate and detection of chitooligosaccharide or GlcNAc generated from the hydrolytic reaction. The former is determined by viscosimetric or turbidimetric detection [31]. The latter can be measured by colorimetric methods [32, 33], chromatography, such as TLC [34], HPLC [35, 36], mass spectrometry and radiochemical determination using regenerated [$^3$H] chitin [37]. Activity of chitinase can also be detected directly on polyacrylamide gel after electrophoresis either in non-denaturing or denaturing condition [38]. Some of these methods will be described in the following.

### 1. Viscosimatric assay of chitinase

In this method, various substrates have been used for chitinase assay. Such substrates are chitosan acetate, carboxymethyl chitin, glycol chitin and 6-O-hydroxypropylchitin. The assay is based on the measurement of the viscosity of a substrate solution, which is reduced by the action of chitinase in an Ostwald viscosimeter [39]. The flow time of reaction mixture (substrate-enzyme) is measured at different time intervals using various dilutions of enzyme. The rate of which is a function of enzyme concentration.

The assay technique was claimed to be sensitive and effective to detect a slight activity [39]. However, it is inconvenience due to its tedious procedure and time consuming especially with numerous samples. Another consideration is the absolute rate of decreasing in viscosity may vary between batchs of substrate preparation i.e., degree of polymerization. Thus, it appears to be hardly useful for absolute standardization of chitinase measurement [31].

**2. Turbidimetric (nephelometric) method**

The action of chitinase on colloidal suspension results in a decrease in turbidity. The relative turbidity is measured immediately and 2 hr after mixing the enzyme and substrate. A 50% reduction in turbidity within 2 hours is corresponding to 10 nephelometric units. Thus the amount of enzyme is expressed in term of nephelometric unit. This method is rapid and accurate. However, it is suitable only for the estimation of a relative high activity of enzyme [31].

**3. Colorimetric method**

The colorimetric assay for chitinase is based on the determination of reducing sugars. The one that most widely used is based on the determination of monomeric GlcNAc released from colloidal chitin, glycol chitin, and partially N-acetylated chitosan during chitinolytic degradation. This method is suitable for chitinase with exoenzyme activity. However, an accurate determination of endochitinase can also be accomplished by the enzymatic hydrolysis of the reaction products to monomeric GlcNAc prior the colorimetric detection [31]. The GlcNAc is detected using ferric cyanide reagent after boiling in carbonate and ferric cyanide [40]. The color product is measured by spectrophotometry. One unit of chitinase is defined as the amount of enzyme that releases 1 μmole of GlcNAc in 1 min under the assay condition. However, most bacterial chitinases release dimer GlcNAc, therefore, μmole equivalent of GlcNAc could be used in unit definition

The colorimetric assay is specific for GlcNAc and is applicable to various types of chitinase present in microorganisms, animals and plants [31]. This technique is sensitive and comparable to radiochemical assay using regenerated [$^3$H] chitin. It has been shown that as little as $3x10^{-10}$ nmol of GlcNAc could be detected [33]. This assay is most useful when native chitin

or chitin containing materials like fungal cell wall are to be employed as substrate.

**4. Detection of chitinolytic activity in polyacrylamide gel electrophoresis (PAGE)**

The technique of activity staining of chitinase in polyacrylamide gel is first described for directly detecting chitinase activity after resolving under native or denaturing condition [38]. Following PAGE, the enzyme is allowed to react with a glycol chitin substrate embedded in the resolving gel (SDS-PAGE, after replacing SDS with Triton X-100) or in another substrate gel (nondenaturing PAGE). This reaction creates lytic zone(s) corresponding to the bands of resolved chitinases. The lytic band(s) is made visible by staining the reacted substrate gel with Calcofluor white M2R, a fluorescent brightener. The Calcofluor white has affinity to intact chitin and gives fluorescent on a UV transsilluminator. The digested glycol chitin losses the ability to bind the fluorescent dye. Thus, the digested glycol chitin can be viewed as non-fluorescent band(s). Detection of enzyme activity on the gel facilitates the identification of chitinase in a crude enzyme preparation. It is also useful monitoring the purity of enzyme in process of purification. This method also combines the advantageous of high resolution and molecular weight determination by SDS-PAGE. Thus, the molecular weight of chitinolytic enzyme can be directly estimated. However, the enzyme to be assayed must be renaturable.

Recently, a similar detection of enzyme activity in PAGE was developed. However, the chromogenic substrate, 4-methyl-umbellifery (4-MU), derivatives of N-acetyl-β-D-glucosamine (short chain of oligomeric substrates) have been used instead of glycol chitin. Thus, a band of enzyme is revealed as a bright fluorescence. Enzymes with different substrate specificity can be determined using various 4-MU-oligomeric substrates.

## Application of chitinase

Chitinases catalyze the hydrolysis of chitin. In recent years, soil-borne microorganisms that produce chitinases are considered as potential biocontrol agents against fungi and nematodes, which cause diseases of agricultural crops. Chitinases also play an important physiological and ecological role in ecosystem as recyclers of chitin, by generating carbon and nitrogen sources [59].

Chitinases have many industrial and agricultural applications [42, 43] that require different types of preparations.

### Chitinases in biocontrol of plant pathogenic fungi and insects.

In many plant species, local invasion of the pathogen induces production of PR-protein like chitinases, β-1,3-glucanases, proteinases, proteinase inhibitors, etc. [44]. As pathogenic fungi and insects contain chitin in their protective covers, induction of chitinases in plants is the main defense responses. Most of these chitinases are induced in vegetative plant organs by infection but some are also present in seeds. Hadwiger and Beckman [45] demonstrated that extracts of the pea endocarp contain chitinase and chitobiase activity. In fact, there is no other better proof for the contribution of plants chitinases in self defense than the formation of chitosan in the cell wall of a bean rust fungus, *Uromyces viciae-fabae* to combat with the chitinase activity. Most of chitinase preferentially cleave highly acetylated substrates and the activities decrease in the degree of deacetylation. Therefore, increase in the deacetylation level on the surface of hyphae may be useful for the fungus to resist plant chitinases. And the presence of chitin deacetylase activity during the formation of infection structures supported this hypothesis.

Several species of fungi are very potent biocontrol agents of plant pathogenic fungi and insects. The mycoparasitic and entomopathogenic fungi produce chitinase for invasion and as one of the host-killing component. A

most studied mycoparasitic fungus, were found to be inhibitory to a wider range of deleterious fungi than similar enzymes from other sources

To control pests, such as longhorn beetles and aphids, the enzymatic treatment before or simultancously along with the entomopathogenic fungus, itself was successfully tried.

**Mosquito control**

*M. verrucaria*, a saprophytic fungus, produces a total complex of an insect cuticle degrading enzymes [42]. It has been seen that both first (I) and fourth (IV) instar larvae of a mosquito, *A. aegypli*, can be killed within 48 hours with the help of the crude preparation from *M. verrucaria*. However, the time period was found to be decrease correspondingly to 24 and 48 hours when the purified chitinase was supplemented with lipolytic activity.

**Chitinase as a target for biopesticides**

Chitin is present in exoskeleton and gut lining of insects. The insect molting enzyme, chitinase has been described from *Bombyx mori*, *Manduca sexta*, and several other species. Similarity, chitinases have been implicated in different morphogenetic events in fungi. The pseudotrisaccharide, allosamidin (Figure 10) is a potent inhibitor of chitinases from most of the sources. The allosamidin was found to be inhibitory after ingestion to the growth of mite, *Telranychus urticae* and a larva of the housefly, *M. domestica*. However, there is no report for the inhibition of lepidopteran insects by oral or topical application. Nevertheless, chitinase inhibitors can be explored as potential biopesticides.

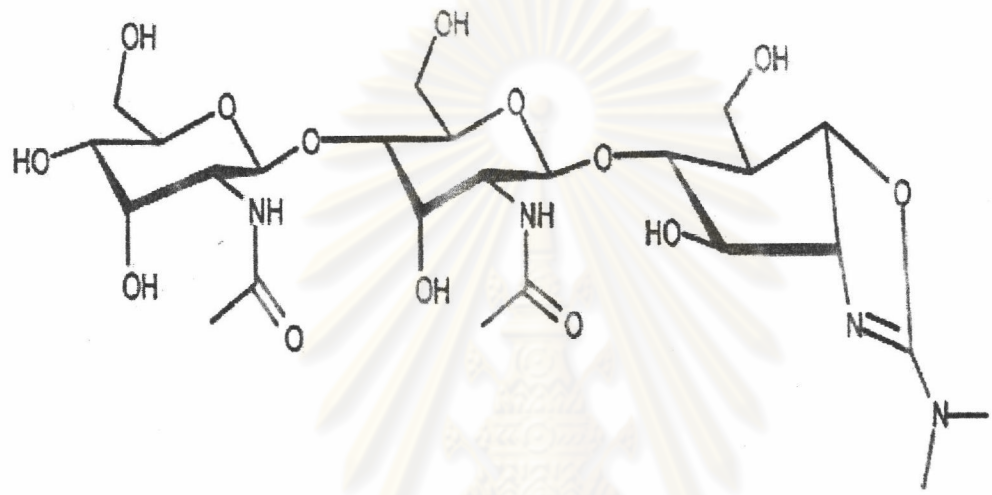



**Figure 10 Structure of strong inhibitor for family 18 chitinase, allosamidin.** Structure of allosamidin is similar to structure of the transition state in mechanism of family 18 chitinase (as shown in Figure 9).

**Production of chitooligosaccharides**

Chitooligosaccharide can be prepared, as shown in Figure 5, there is a growing appreciation of the potential of biologically active chitooligosaccharides. They act as clicitors of plants defense, involved in the signaling for root nodule formation, and are potentially useful in a human medicine, too. For example, chitohexaose and chitoheptaose show anti-tumor activity. A chitinase from *Vibrio diginolyticus* was used to prepare chitopentaose and chitotriose from colloidal chitin. *N,N'*-Diacetylchitobiose has been widely used as a starting material for systhesis of biologically active compound. A chitinase preparation from *S. griseus* was used for the enzymatic hydrolysis of colloidal chitin. The chitobiose produced was subjected to chemical modifications to give novel disaccharide derivatives of 2-acetamido-2-deoxy-D-allopyranose moieties that are potential intermediates for the synthesis of an enzyme inhibitor.

## Plant chitinases

The proposed role of plant chitinases is a defense mechanism against chitin-containing organisms. It has been observed that purified barley chitinases inhibit the growth of fungal hyphae [60]. Heterologous chitinase gene expression is used in various plants to enhance their defense mechanisms against fungal pathogens [61].

Plant chitinases use two different hydrolytic mechanisms. Chitinases offamily19, such as the 26 kDa endochitinase (class II) from *Horde volgare* L. (barley), invert the anomeric configuration of the hydrolyzed GlcNAc residue. Chitinase of family 18, such as the 29 kDa hevamine (class III) from rubber tree, retain the anomeric configuration of the hydrolyzed residue.

Most of studies on plant chitinases have focused on their role as pathogenesis-related (PR) proteins. In other causes, however, chitinases and other enzymes produced by plants as part of hypersensitive response are for the

propose of generating mycorrhizal associations with symbiotic fungi. Spruce (*Picea ables*) cells respond to elicitors from ectomycorrhizal fungi with an array of physiological phenomena. Salzer *et al.* demonstrated that two class I exochitinases of 28 and 36 kDa, which are produced by spruce cells, can inactivate fungal elicitors. The inactivation of the elicitors by chitinases is most probably caused by cleavage of elicitor-active GlcNAc oligomers to inactive monomers. This indicates that constitutively expressed chitinases, which are localized in the apoplastic space of the host root, could degrade part of chitinous elicitors on their way across the plant cell wall before they reach their receptors in the plant plasma membrane. Thus, inactivation of fungal chitin-derived elicitors might be one of many instrumental preconditions required to create a compatible interaction between plant and fungus in the mycorrhiza.

**Bacterial chitinase**

Chitinase producing bacteria were found in both marine and soil. In bacteria, chitinases play roles in nutrition and parasitism whereas in fungi, protozoa, and invertibrates they are also involved in morphogenesis. The bacterial chitinase activities were important for maintenance of carbon flow in the carbon cycle and biomass turnover in nature. Bacterial were produced extracellular chitinase and secreted out of cell to degraded chitin, for example, *Bacillus* sp., *Serratia* sp., *Vibrio* sp., *Streptomyces* sp., *Arthrobacter* sp., etc. Bacteria, including *Serratia marcescens*, *Bacillus* sp., and *Vibrio* sp. were produced a high level of chitinolytic enzyme.

Bacteria produce chitinase to meet nutritional needs. They usually produce several chitinases, probably to hydrolyze the diversity of chitins found in nature. Bacterial chitinases belong to family 18 of glycosyl hydrolases. These enzymes operate by the mechanism leading to overall retention of the anomeric configuration of the hydrolyzed residue. It has been demonstrated that conserved glutamic acid and aspartic residues (four amino acids apart) of chitinase A1 of *Bacillus circulans* WL-12 were essential for the hydrolysis of

chitin. These residues are also conserved in ChiA of *Serratia marcescens*. However, the determination of the crystal structure of ChiA demonstrated that the same glutamic acid residue but a more distant aspartic acid residue (76 amino acids distant) are the most likely amino acid residues to constitute the active site of the enzyme.

Chitinase produced from *Bacillus circulans* and *Serratia marcescens* were studied. In *Bacillus circulans*, at least 6 major chitinase, including chitinase A1, A2, B1, B2, C, and D, were found in culture medium when induced by chitin. Chitinase A1 plays role in degradation of chitin to chitobiose, $(GlcNAc)_2$. The *chiA* and *chiD* encoded the precursor of chitinase A1 and D have been cloned and sequenced. Both of *chiA* and *chiD* were used for classified family 18 chitinase into group A, B, and C by compared homology of amino acid sequence inside the catalytic domain.

## *Microbacterium* sp.

*Microbacterium* sp. is gram-positive bacteria with a small diphtheroid rod with the round ends in shape; angular and palisade arrangements of cell masses are typical. *Microbacterium* sp. was classified in genus *Microbacterium*, Family Microbacteriaceae. It is non-motile and non-spore forming. It can grow on surface of solid media at 30 °C with aerobic condition the best. It can produced weak acid (chiefly L(+)-lactic acid) from carbohydrates fermentation and contained catalase activity. Optimum growth temperature is 30 °C; limited or variation growth at 15 and 35 °C; no growth at 10 and 40 °C; survives 72 ° C for 15 minutes or more in skim milk. This genus has morphological change during growth: long chain of regular rods in exponential phase, coccoid form in stationary phase, and aberrant form sometimes seen. This genus has relatively low activity of glycolytic enzyme.

*Microbacterium* sp. was found in dairy products, presumably as a result of improper cleansing of dairy equipment. Some of *Microbacterium* sp. was

bacterial pathogen, for example *Microbacterium nematophilum*, it can induce morphological change in the nematode, *C. elegans* [62].

There is a report show that *Microbacterium liquefaciens* can immobilize in polyvinyl alcohol and reduce chromate in wastewater from modern industries, such as stainless steel manufacture, leather tanning, textile manufacture, electroplating and alloy preparation [63].

In this study, *Microbacterium* sp. TU05 was found to produce high chitinase activity. Chitinase production and some properties of crude chitinase such as optimum pH, optimum temperature and substrate specificity was determined. Crude chitinase from *Microbacterium* sp. TU05 was partially purified and molecular weight of chitinase was estimated by SDS-PAGE followed by activity staining. Cloning of DNA fragment containing chitinase gene from *Microbacterium sp.* TU05 was focused on chromosomal DNA. The chromosomal DNA of *Microbacterium* sp. TU05 was digested with restriction enzyme and cloned into *E. coli* JM109 using pBluescript II $SK^-$ as a cloning vector. Phenotype screening was used to screen for colonies of transformant containing chitinase gene. The partial sequence of chitinase gene was determined by PCR amplification using primers, designed from conserve sequences of family 18 chitinase gene of *Bacillus* sp..