CHAPTER I

INTRODUCTION

Chitin

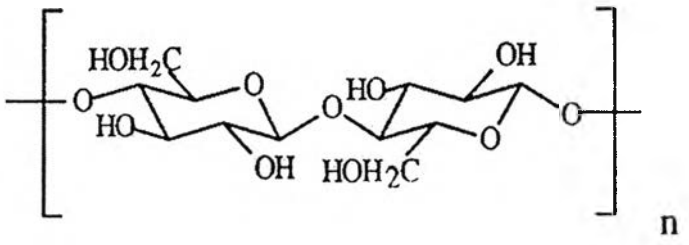
Chitin, a substrate of chitinase, is a straight chain homopolymer of β-1,4-linked-2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine, GlcNAc). The structure of chitin resembles cellulose with the C-2 hydroxyl group replaced by acetamido group, as shown in Figure 1.

In invertebrates, chitin occurs in a close association with water insoluble proteins which had to be remained in the production of chitin. Structure of chitin as determined by polarized light and electron microscope indicated that chains of chitin usually orientate in a high degree of order (Kramer and Koga, 1986). X-ray diffraction was the first to show the crystalline nature of chitin (Tracey, 1957). The chitin or polysaccharide chains are assembled into microfibrils forming into a crystalline structure via intramolecular hydrogen bond. A comparison of the X-ray data for chitin from different sources has revealed the existence, in nature, of three polymeric forms; α, β, and γ forms (Blackwell, 1988). Most chitin including those from insect, fungi and crustaceans are classified in the α-form. The rare second form known as β-chitin has been found in four sources: the spine of the polychaete *Aphrodite*, the pen of squid *Loligo*, the tubes of *Pogonophora*, and the spines of certain marine diatoms. The third form, γ-chitin, has been reported from the stomach lining of *Loligo* and probably in coelenterates (Tracey, 1957).

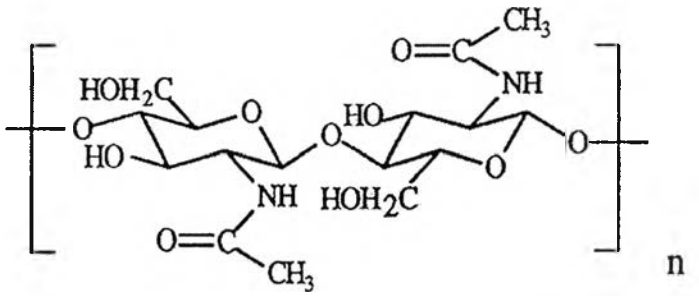
The structure of chitin

The structure refined for α-chitin either by X-ray diffraction (Minke and Blackwell, 1987) or linked atom least-square procedure (Blackwell, 1988) revealed an antiparallel of two adjacent polysaccharide chains. As shown in Figure 2, half of the hydroxyl groups of sugar ring are bonded to carbonyl groups within the same stack of chains and half are bonded to the hydroxyl groups between an adjacent stacks. The

a) cellulose



b) chitin



c) chitosan

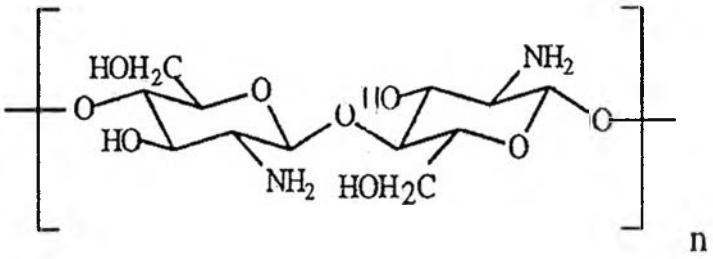
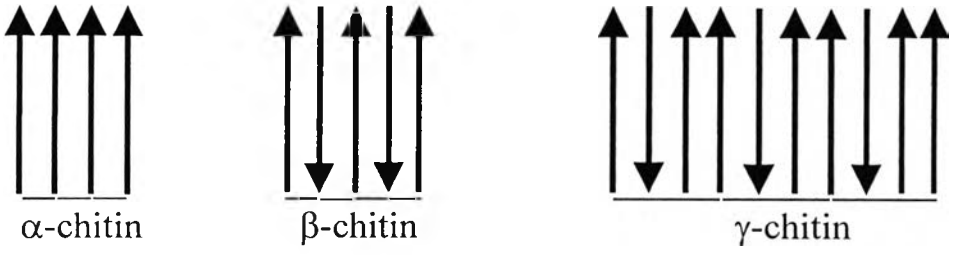


Figure 1. Chemical structure of cellulose (a), chitin (b), and chitosan (c)

A



B

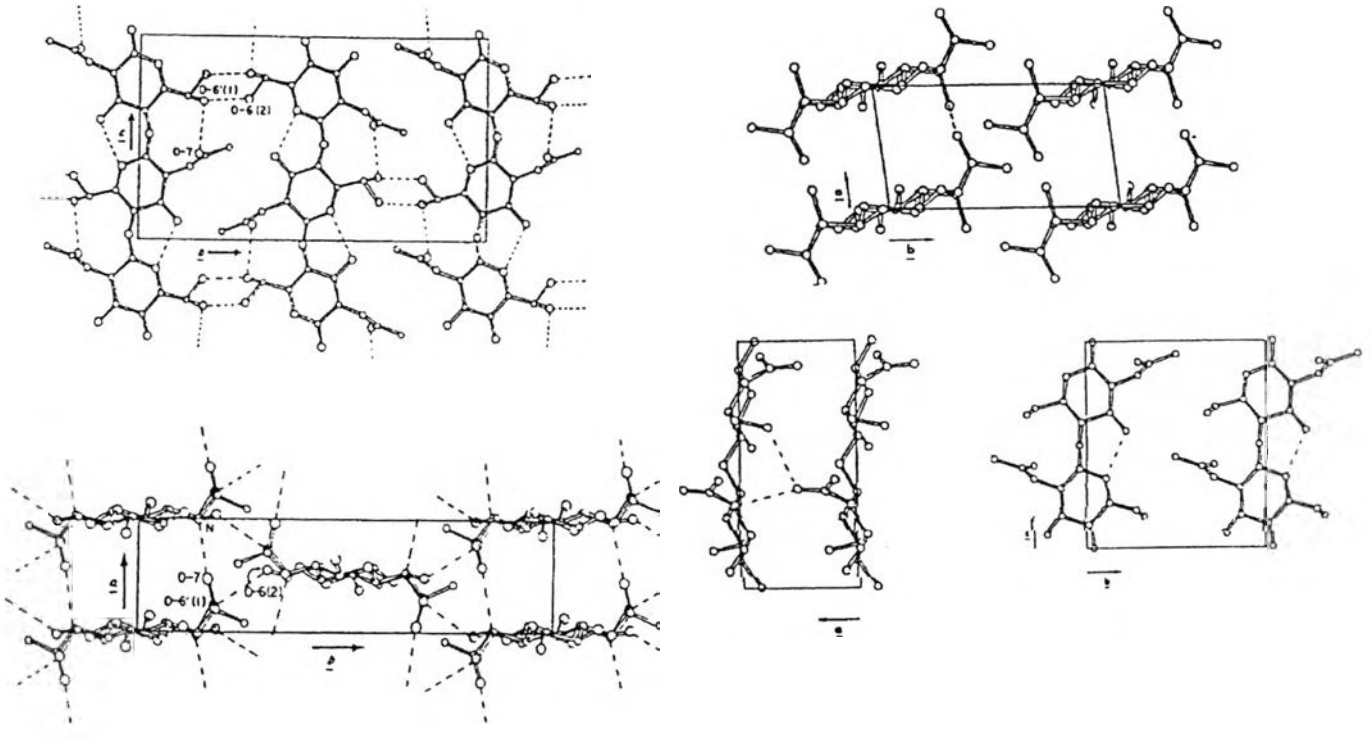


Figure 2. Crystalline structure of chitin. (A) Diagramatic illustration of three types of chitin with arrangements as antiparallel (α-chitin), parallel (β-chitin), and an alternative between two parallel and one antiparallel chains (γ-chitin). (B) Structure of α-and β-chitin and hydrogen bond linkage between to C=O…NH groups. (Minke and Blackwell, 1978)

existence of this intersheet bonding is probably responsible for the stability of the α-chitin structure specifically its inability to swell in water.

The β-chitin is characterized by a parallel arrangement of the polysaccharide chains as illustrated in Figure 2. (Rudall, 1963). In this arrangement, there are no hydrogen bonds between adjacent stacks. Thus, β-chitin is easily swollen by intercalation of water molecules between the stack of chitin chains. In this regard, it is interesting that β-chitin is found exclusively in aquatic organisms (Blackwell, 1988). Both α- and β-chitin are naturally occurred. Differences between two forms are slight, however, the α-form is more stable. β-chitin can be converted to the α-form by treatment with anhydrous formic acid or strong nitric acid. But there is no known means by which this transformation can be reversed (Tracey, 1957 and Blackwell, 1988). The infrared spectra of α- and β-chitin are also essentially similar. It is probable that the α- and β-forms do not differ in any essential chemical manner, since both are readily hydrolyzed by chitinase from a number of sources (Tracey, 1957). The paralled-β and antiparalled-α structures are analogous to the chain polarities found in the polymorphic form of cellulose I and cellulose II (Blackwell, 1988), respectively. Moreover, the viscosity of solution of chitin in nitric acid is the same order as that similar solution of cellulose. This indicates a degree of polymerization of more than a hundred of GlcNAc (Blackwell, 1988). The structure of γ-chitin was identified by Rudall (1963) in which a three-chain unit is arranged alternatively giving rise two parallel chains and one antiparallel chain. The diagrammatic structures of all forms of chitin are shown in Figure 2.

The applications of chitin and its derivertives

Chitin and chitosan and their respective monomers have significant potential applications in the pharmaceutical industry, medical, agricultural, cosmetics, water treatment and biotechnology. They can be used in making of contact lenses, artificial kidney membranes, antifungal preparations, biodegradable pharmaceutical carriers, blood anticoagglulants, microbiological media, wound healing accelerators and dressings from burns (Muzzarelli, 1977). In the agricultural industry, seeds can be protected from fungi by incapsulation in chitin and chitosan derivatives. The cosmetic

industry makes shampoos, gels, cream and even sponges with chitin and chitosan. In the food industry, they are used in the preparation of fruit juices or soluble coffee. Moreover, in the other industrial production, chitin and chitosan are used as chelating polymers for harmful metals (i.e., Hg, Pb and Cu), paper and textile additives, textile finishes, photographic products and processes, coaggulants for suspensions, water purifying systems and protein recovery processes (Muzzarelli, 1997). In addition, some shorter chitin oligosaccharides could have an inhibitory effect on the development of cancerous tumors (Flach et al., 1992) as shown in Table 1. Finally, a glycol chitin is used in the enzymatic assays which need a soluble substrate (Trudel and Asselin, 1989) whereas colloidal chitin is used in the sensitivity assays of enzymatic activity (Reissig et al., 1995; Boller et al., 1983).

Chitinolytic enzymes

Chitinolytic enzymes can be classified according to their hydrolytic activity into 2 main classes: chitinase (EC 3.2.1.14) and N-acetylglucosaminidase or N-acetylhexosaminidase (EC 3.2.1.52). However, a third term: chitodextrinase has recently been described in a marine bacterium (Bassler et al., 1991). Chitinase (EC 3.2.1.14) is a group of enzymes that hydrolyze chitin by cleaving the β-1,4 bond between two consecutive N-acetylglucosamine residues in the chitin chain into monomer, dimer, trimer and oligomer of N-acetylglucosamine (GlcNAc) residues.

Classification of chitinases according to their mode of action

Chitinase can be further divided into two types: endo-chitinase and exo-chitinase. Endochitinase hydrolyses β-1,4 glycosidic linkage randomly within the polymer chain giving rise N, N'-diacetyl-D-glucosamine [chitobiose, $(GlcNAc)_2$], as a major product together with some triacetylchitotriose and mixture of oligomers (Flach et al., 1992). Exochitinase attacks chitin molecules stepwise from the non-reducing end, releasing $(GlcNAc)_2$ or GlcNAc (Flach et al., 1992; Shaikh and Desphande 1993). Exochitinase can be divided into two subcategories: chitobiosidases, which catalyze the progressive release of $(GlcNAc)_2$ starting at the non-reducing end of the chitin, and 1-4, b-N-acetylglucosaminidases, which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Cohen-Kupiec

Table 1. Current practical uses of chitin, chitosan and their derivatives

Area of Application	Example
Agriculture	- Plant seed coating
	- Fertilizer
Food Industry	- Color stabilization
	- Emulsifying agent
	- Clarification and deacidification of fruits and beverages
	- Dietary fiber
	- Fiber-optic sensor for determination ethanol beverages and organic acid
Medical	- Dressing materials for the burns and skin lesions of humans and animals
	- Wound - dressings
	- Artificial limbs
	- Carrier-drug conjugates
Wastewater Treatment	- Recovery of metal ions, pesticides and phenols
	- Removal of dye
Cosmetics	- Moisturizer
	- Face, Hand and Body creams
Paper	- Surface Treatment
	- Photographic Paper
Biotechnology	- Enzyme immobilization
	- Chromatography
	- Ultrafiltration membranes

and Chet, 1998).Chitobiase, recently group together with N-acetylhexosaminidase, hydrolyses chitobiose resulting into 2 molecules of GlcNAc. Chitodextrinase is referred to the enzyme that selectively attacks low molecular weight chito-oligomers (Basssler et al., 1991). Hydrolytic activity of these enzymes may sometime depend upon the type of substrates. For example, the *Streptomyces* chitinase complex degrades pure crystallineβ-chitin from diatom spines into $(GlcNAc)_2$, but the enzyme degrades colloidal chitin into mixture of oligomers and $(GlcNAc)_2$ (Lindsay and Gooday, 1985). This suggests that a variation in the microenvironment of β-1,4-glycosidic linkage occuring in a natural chitin substrate may influence how the enzyme works, therefore, living organisms produces a family of different chitinases. Each chitinase has produced different substrate and product specificity (Shaikh and Deshpande, 1993). Transglycosylase activity associated with chitinase activity has also been detected (Usui et al., 1987).

Structural and functional relationship of chitinase

Chitinases share common structural features to other insoluble polysaccharide hydrolases, amylases, cellulases and xylanases, in the way that they posses discrete domains. The domains comprise catalytic and substrate binding or other domains such as flexible linker domains (Wilson et al., 1995). The chitin binding domain can be located C-terminal to the catalytic domain in some chitinases (Tsujibo et al., 1993a; Ueda et al., 1994; Watanabe et al., 1990a) or N-terminal in others (Blaak and Schrempf, 1995; Alam et al., 1996; Fuji and Miyashita, 1993; Watanabe et al., 1992). It appears that the binding domain of glycosyl hydrolases is usually joined to the catalytic domain by a flexible region (Gilkes et al., 1991). In many cases, these linker regions are easily recognized because they are rich in proline, threonine and serine, glutamine, and repeating sequences such as x-proline, x is any amino acid. The substrate-binding domain is responsible for the tight binding of the enzyme to insoluble polymeric substrate. The enzyme with deleted substrate binding domain shows less activity than that of full-length enzyme on insoluble substrate (Watanabe et al., 1994; Blaak and Schrempf, 1995). The hinge region may allow the catalytic domain to catalyze a number of cleavages due to its flexibility (Wilson et al., 1995). The third domain with amino acid sequence similarity to that of fibronectin type III

has been described for some bacterial chitinases (Blaak et al., 1993; Robbins et al., 1992; Watanabe et al., 1990b; Watanabe et al., 1993) and cellulase (Meink et al., 1991). However, the function of this domain remains unknown and most chitinases lack this domain (Blaak and Schrempf 1995, Flach et al., 1992).

Grouping of chitinase based on sequence homology within the catalytic domain

The catalytic domain of chitinase is the largest domain of the enzyme, which composes of 300 or more amino acid residues. Comparison of amino acid within this domain can be used to classify chitinases into 3 groups (A, B and C), as proposed by Watanabe et al., 1993. This classification is based on homology of amino acid sequences within the catalytic domain when they are compared to those of chitinase A1 and chitinase D of *B. circulans* WL-12. As shown in Figure 3, group A chitinase contains chitinases with amino acids of the catalytic domain homologous almost entirely to the catalytic domain of chitinase A1. Group B chitinase contains the catalytic domain homologous to almost all of the catalytic domain of chitinase D. Group C chitinase contains no sequence homology to the catalytic domains of chitinase A1 or chitinase D of *B. circulans* WL-12.

Chitinase group A has been found in different organisms including gram positive and gram negative bacteria, listed in Figure 3. Group B comprises three chitinases from *B. circulans* (chitinase D), *Streptomyces lividans* (chitinase A) and *S. lividans* (chitinase B). Group C consisting of two chitinases from *Streptomyces erythraeus* (Kamei et al., 1989), and chitinase II from *Aeromonase* sp. 10S-24 (Ueda et al., 1992) that has been reported to date.

Chitinolytic bacteria produce enzymes belonging to different groups. Conversely, chitinases belonging to the same group can often be found in widely different bacteria. This variability suggests that interspecific exchange of chitinase genes has occurred in the course of evolution. Chitinolytic bacteria might have developed by assimulating genes of various origins similar to that proposed for cellulase gene (Begiun, 1990).

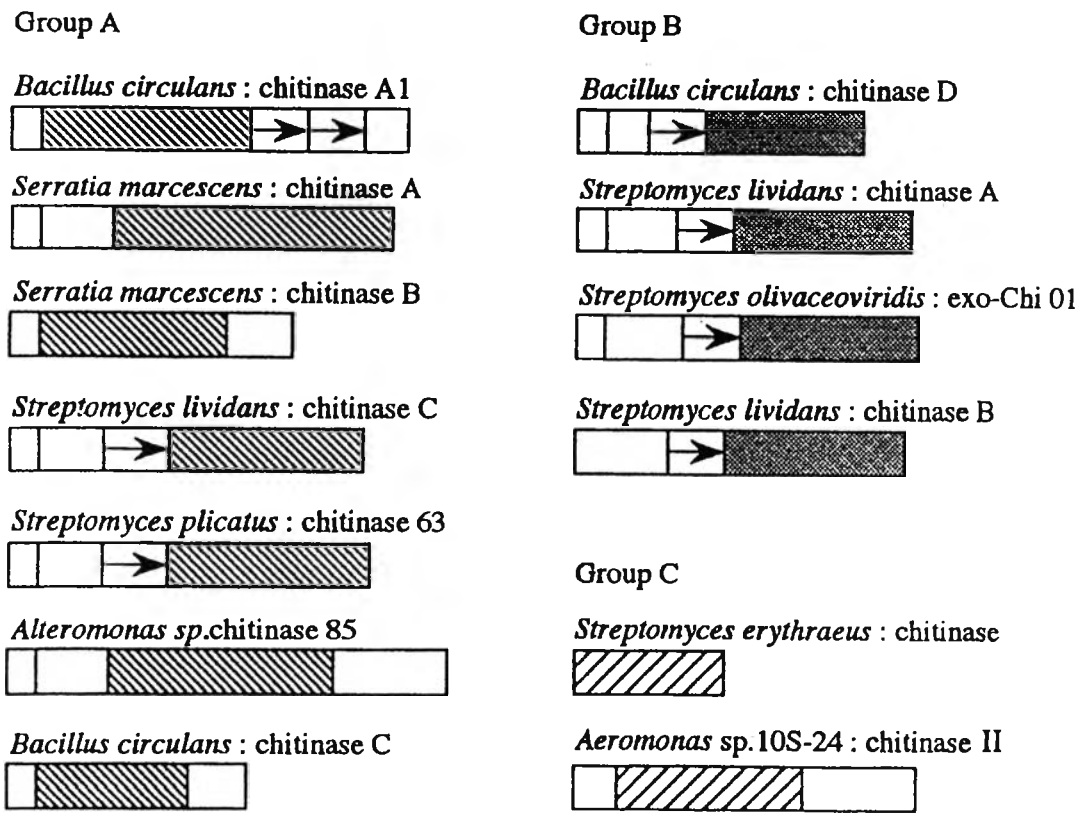


Figure 3. Classification of the bacterial chitinases based on the homology of similarity amino acid sequence of individual catalytic domains. Shadowed boxes indicate the homologous regions of individual chitinases to the catalytic domain of *B. circulans* chitinase A1; Group A, *B. circulans* chitinase D; Group B, or *Streptomyces erythraeus* chitinase; Group C. Arrows indicate fibronectin type III-like domain. From Hart et al., 1995). Amino acid similarity with in the catalytic domain of *B. circulans* chitinase was used to classify other bacterial chitinases into 3 groups. Group A similar with chitinase AI, Group B similar with chitinase D and Group C with no similarity with chitinases from *B. circulans*.

Three dimensional structure of chitinase

Currently, the nucleotide and amino acid sequences as well as 3D structures of several chitinases are known (Perrakis et al., 1994 and van Aaltem et al., 2000). Based on this amino acid sequence homology, chitinases can be categorized into 2 families of glycosyl hydrolase, comprising families 18 and 19 (Henrissat, 1991; Henrissat and Bairoch, 1993). The members classified within the same family have been proposed to share the same folding characteristic (Henrissat, 1991). Chitinases of family 19 has been found solely in plants and *Streptomyces* sp. They have a lysozyme-like fold as determined by X-ray structure of the chitinase from *Hordeum vulgare* L seeds (Hart et al., 1995). In contrast, family 18 chitinase has been found in a wide range of organisms, including bacteria, fungi, plants, insects, mammals, and viruses. Family 18 chitinases are conserved in two short sequence motifs. The two conserved regions are Kxxx(S/A)xGG and (F/L)DGxDxDxE, when x is any amino acid. One of these motifs contains 2 acidic residues essential for catalytic activity as shown by site-directed mutagenesis (Watanabe et al., 1993). To date, three dimentional structure of family 18 chitinases have been studied from bacteria, *Serratia marcescens* chitinase A and B (Perrakis et al., 1994) and plant (Hevamine from *Hevea brasiliensis*) (van Scheltinga et al., 1995).

The X-ray crystallography of chitinase A from *S. marcescens*, hevamine from *H. brasiliensis* and endo-β-N-acetylglucosaminidase F1 and H from *Flavobacterium meningosepticum* showed similar catalytic $(\alpha/\beta)_8$ barrel domains (Figure 4). In three enzymes, the substrate binding cleft is located at the carboxy-terminal of the β-strands in the $(\alpha/\beta)_8$ barrel. Structure determination of an enzyme-substrate complex of hevamine demonstrated that the substrate is tightly bound in the substrate-binding cleft, the O1 atom of GlcNAc moiety forms hydrogen bonding to the carboxylic group of a conserved Glu residue (van Scheltinga et al., 1995). The catalytic residues of this enzyme family was first reported by Watanabe et al. for chitinase A1 from *Bacillus circulans* WL-12 (Watanabe et al., 1993). Site-directed mutagenesis of Glu204 completely eliminated its activity. This residue was considered to be a proton donor in catalysis. From sequence comparison, the glutamic acid residue was found to be conserved in all chitinases in family 18. In *Serratia marcescens* chitinase A, the catalytic carboxylate corresponding to Glu204 of *B. circulans* chitinase A1 is Glu315.

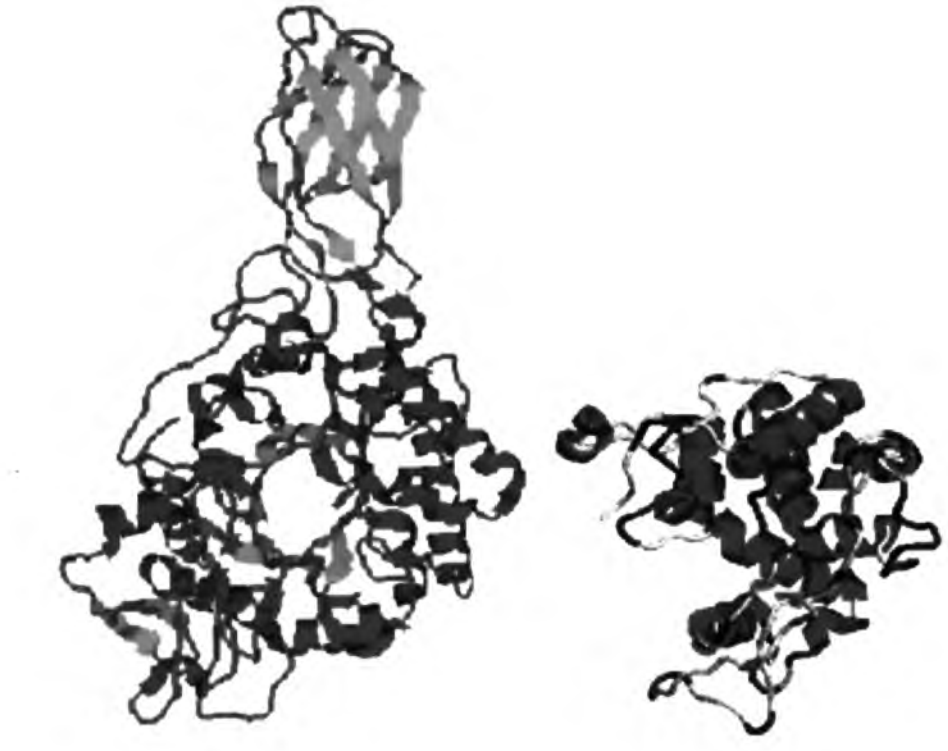


Figure 4. Three dimensional structure of family 18 chitinase and familly 19 chitinase. *S. marcescens* chitinase A (PDB number 1EDQ); Panel A and Barley chitinase (PDB number 1BBA); Panal B, α-helices are show in pink, and β-strands in yellow. This figure was created using RasMol 2.6.

B. circulans chitinase A1 produce β-anomer, hence is an enzyme that does not change the anomeric configuration of the C1 of the substrate, a retaining enzyme. Within retaining enzymes, the location of the second carboxylate is less than 5 A°. In the consensus region of the catalytic domain of family 18 chitinases, there are several conserved carboxylic amino acid residues, for example, Asp200 and Asp202 in chitinase A1 from *B. circulans*, Asp311 and Asp313 in chitinase A from *S. marcescens*. Site-directed mutagenesis of Asp200 and Asp202 in *B. circulans* chitinase A1 impaired the enzymatic activity, but did not completely eliminated the activity (Watanabe et al., 1993 and Watanabe et al, 1994). The location of these residues did not correspond to that of the carboxylate in lysozyme (Asp52) or in the family 19 barley chitinase (Glu89). Thus, the second carboxylate cannot be identified in any family 18 chitinase. The family 18 chitinases should have a different mechanism of catalysis. Recent studies on the family 18 family indicate that the catalytic reaction of the enzymes takes place through a substrate-assisted mechanism. As shown in Figure 5, a putative oxocarbonium ion intermediate is stabilized by an anchimeric assistance of the sugar N-acetyl group after donation of a proton from the catalytic carboxylate to the leaving group. Such a stabilization might occur either through a charge interaction between the C1 carbon and the carbonyl oxygen of the N-acetyl group or via an oxazoline intermediate with a covalent bond between C1 carbon and the carbonyl oxygen. The mechanism does not require the second carboxylate and can rationalize the anomer retaining reaction of the enzymes without the second carboxylate. This mechanism was first proposed for the spontaneous acid-catalyzed hydrolysis of 2-acetamido-substituted polysaccharides in solution (Piszkiewiez and Briuce, 1968), and applied to lysozyme mechanism (Lowe et al., 1967). Experimental evidence of substrate assistance in family 18 chitinase has been first provided by the crystal structure of the inhibitor allosamidin bound to chitinase from *Hevea brasilliensis* (Terwisscha et al., 1995). Allosamidin was found to strongly inhibit family 18 chitinase, but not hen egg white lysozyme nor human lysozyme (Koga et al., 1987). It consists of two β-1,4-linked N-acetylallosamine residues and an oxazoline derivative, allosamizoline (Figure 6). The strong inhibition by allosamidin suggests that the structure of allosamizoline residue is complementary to that of catalytic center. Thus, the allosamizoline structure is most likely to reflect the transition state

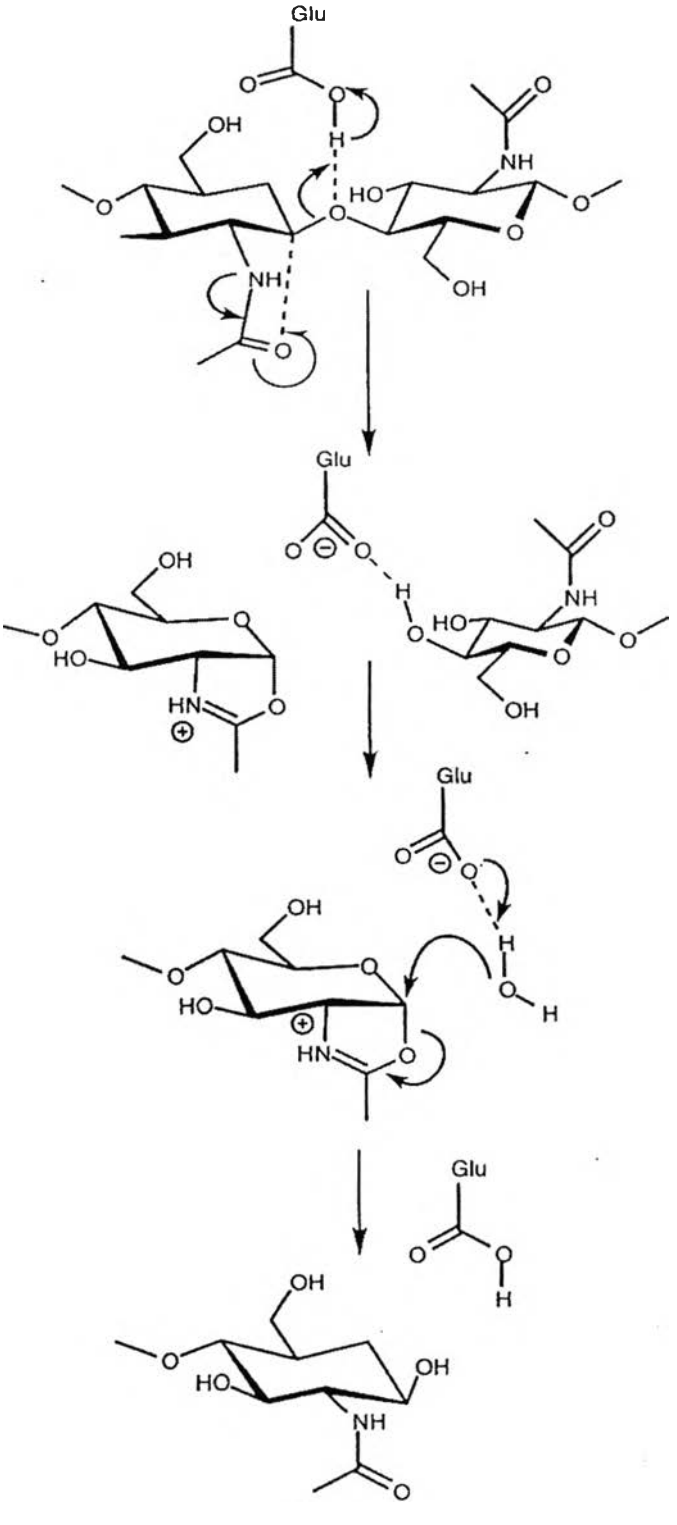


Figure 5. Mechanism of glycosyl hydrolysis catalyzed by family 18 chitinase following substrate-assisted catalysis. The oxocarbonium ion intermediate is stabilized by an anchimeric assistance of the sugar N-acetyl group after donating a proton from the catalytic carboxylate.

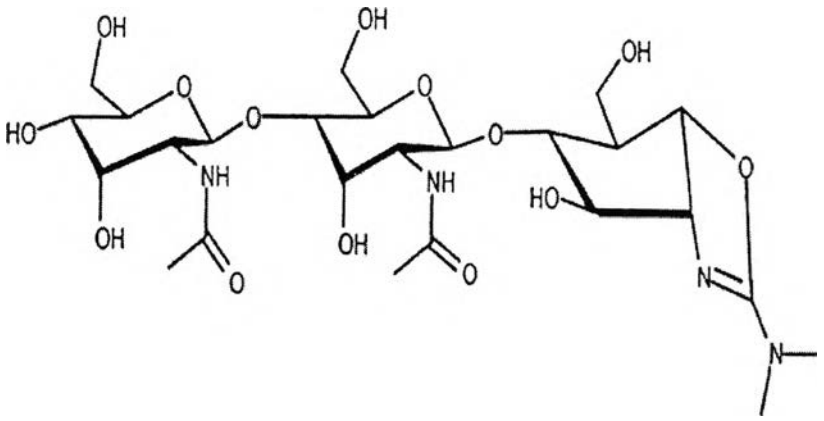


Figure 6. Structure of a strong inhibitor, allosmidin, for family 18 chitinases. The structure of allosamizoline residue is very similar to the transition state structure shown in Figure 5.

structure (Figure 5.). In fact Glu127 which acts as a proton donor in its catalysis was found to be very close to the oxazoline group in the structure of *H. brasiliensis* chitinase complexed with allosamidin; Oe2 atom of Glu127 is 4.3 A° away from the C1 atom. Recently studies by quantum mechanical calculation supported the substrate-assisted mechanism in family 18 chitinase (Brameld et al., 1998).

Chitinase assay

Chitinase activity can be assayed by a variety of 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 (Jeuniaux, 1966). The latter can be measured by colorimetric methods (Chen et al., 1982 and Reissig et al., 1955), chromatography, such as TLC (Zhu et al., 1992), HPLC (Hara et al., 1989 and Tsujibo et al., 1992), mass spectrometry (Lopatin et al., 1995) and radiochemical determination using regenerated [^{3}H] chitin (Molano et al., 1977). Activity of chitinase can also be detected directly on polycrylamide gel after electrophoresis either in non-denaturing or denaturing condition (Trudel and Asselin, 1989). Some of these methods will be described in the following.

Viscosimetric assay of chitinase

Various substrates have been used for chitinase assay by viscosimetric method. Such substrates are chitosan acetate, carboxymethyl chitin, glycol chitin and 6-O-hydroxypropylchitin (for review see Ohtakara, 1988). 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 (Ohtakara, 1988). 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 (Ohtakara, 1988). 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 (Jeuniaux, 1966).

Turbidimetric (nephelometric) method

The action of chitinase on colloidal chitin suspension results in a decrease in turbidity (Jeuniaux, 1966). The relative turbidity is measured immediately and 2 hr after mixing the enzyme and substrate. A 50% reduction in turbidity within 2 hr is corresponding to 10 nephelometric units (Jeuniaux, 1966). 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 enzyme activity (Jeuniaux, 1966).

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 N-acetylglucosamine released from colloidal chitin (Abeles et al., 1970), glycol chitin (Imoto and Yagishita, 1971) and partially N-acetylated chitosan (Aiba, 1992). 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 to the colorimetric detection (Jeuniaux, 1966). The GlcNAc is detected using ferric cyanide reagent after boiling in carbonate and ferricyanide (Imoto and Yagishita, 1971). The color product is measured by spectrometer. 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 chitinaes present in microorganisms, animals and plants (Jeuniaux, 1966). This technique is sensitive and comparable to radiochemical assay using regenerated [^{3}H] chitin (Molano et al., 1977). It has been shown that as little as 3×10^{-10} nmole of GlcNAc could be detected (Reissig et al., 1955). This assay is most useful when

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

Detection of chitinolytic activity in polyacrylamine 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 (Trudel and Asselin, 1989). 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 transilluminator. 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 for 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 (Trudel and Asselin, 1989). However the enzyme to be assayed must be renaturable after SDS-PAGE. The use of Triton X-100 was also described for renaturation of several enzymes (Lack and Springhorn, 1980).

Recently, a similar detection of enzyme activity in PAGE was developed (Tronsmo and Harman, 1993). However, the chromogenic substrate, 4-methyl-umbellifery (4-MU) derivatives of N-acetyl-β–D-glucosamine (short chain of oligomeric substrates) have been used in stead 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.

The applications of chitinases

Aggressive and defensive roles for chitinases

Chitinases are produced by a wide variety of pathogenic and parasitic microbes and invertebrates during their attack on chitin-containing organisms. Examples discussed include enzymes of insect and algal viruses, yeast killer toxin plasmids, bacterial and fungal pathogens of fungi and insects, and parasitic protozoa. These chitinases play roles in penetration of fungal cell walls, and exoskeletons and peritrophic membranes of arthropods. Salivas of some invertebrate predators have chitinolytic activity that may be involved in their attack on their prey. Chitinases play a major defensive role in all plants against attack by fungi, and perhaps also against attack by insects pests. The plant chitinases form a very large and diverse assemblage of enzymes from two families of glycosyl hydrolases. Some vertebrates, including fish and human, also may utilize chitinases in their defense against pathogenic fungi and some parasites (Gooday, 1999).

Biological control

The public concern over the harmful effects of chemical pesticides on the environment and human health has enhanced the search for safer, environmentally friendly control alternatives. Control of plant pests by the application of biological agents holds great promise as an alternative to the use chemicals. It is generally recognized that biological control agents are safer and more environmentally sound than is reliance on the use of high volumes of pesticides. Due to the importance of chitinolytic enzymes in insect, nematode, and fungal growth and development, they are receiving attention in regard to their development as biopecticides or chemical defense proteins in transgenic plants and microbial biocontrol agents. In this sense, biological control of some soil-borne fungal deseases has been correlated with chitinase production. Fungi- and bacteria-producing chitinases exhibit antagosism against fungi, and inhibition of fungal growth by plant chitinases has been demonstrated. Insect pathogenic fungi apparently overcome physical barriers of the host by producing multiple extracellular enzymes including chitinolytic enzymes, which help to penetrate the cuticle and facilitate infection (Esrella et al., 1999).

Preparation of chitooligosaccharide

There is a growing appreciation of the biologically active ologosaccarides. For example, chitohexaose and chitoheptaose show anti-tumour activity and are efficient elicitors of chitinase activity in melon plants. Due to, the conventional partial acid hydrolysis of chitin gives only low yields of the desired pentamers, hexamers and heptamers. *Bacillus* sp. chitosanase has been used for chitosan oligosaccharides production and give high yields more than 60% of oligomers (dimer to pentamers) (Izume and Ohtakara, 1987). Murao et al. (1992) reported a novel chitinase from *Vibrio alginolyticus*, which yielded chitotriose and chitopentaose from colloidal chitin.

Alternatively, an efficient enzymatic synthesis of oligosaccharides has been reported in the literature. Many of the carbohydrateses exhibit a transglycosylation reaction, with the formation of new glycosidic linkages. Nanjo et al. (1989) described the transglycosylation reaction of *Norcardia orientalis* chitinases. When tetramer or pentamer are incubated with the enzyme, accumulation of the hexamer or heptamer was observed, respectively. However, no chain elongation was obtained with the hexamer as the initial substrate, which may be due to its inability to function as an acceptor. A chitinase from *Trichoderma reesei* also showed efficient transglycosylation reaction with the tetramer, giving the hexamer and dimer as the major products (Usui et al., 1990). Usui et al. (1990) also observed chain elongation from dimer to hexamer and heptamer, using lysozyme catalysis in the presence of 30% ammonium sulphate in a buffer medium.

N-acetylglucosaminidase purified from *N. orientalis* also showed transglycosylation activity (Nanjo et al., 1990); the β-1, 6-linked disaccharide of GlcNAc and tri-N-acetylchitotriose were synthesized during the hydrolysis of di-N-acetyl chitobiose.

Occurrence of chitinases

Chitinolytic enzymes are commonly produced in chitin containing organisms, i.e., insect, crustaceans, marine invertebrates, nematodes, algae and fungi for using in their mophogenesis and exoskeleton development (Flach et al., 1992; Shaikh and

Deshpande, 1993). However, other organisms which do not contain chitin also produce chitinolytic enzymes; for example, a wide variety of bacteria, insectivorous animals and higher plants (Flach et al., 1992). The higher plants developing these enzymatic systems for defense mechanisms in response to pathogens and biotic stresses (Flach et al., 1992). Chitinases are secreted by the pancreas and gastric mucosa of insectivorous animals, i.e., fishes, amphibians and reptiles, as well as by the gastric mucosa of some insectivorous birds and mammals for using chitin as the nutrition source. Bacteria develop these enzymatic systems to utilize chitin as a carbon source (Flach et al., 1992; Shaikh and Deshpande, 1993).

Fungal chitinases

Fungal chitinolytic enzymes could be involved in the growth of the fungus itself. All types of chitinolytic enzyme activities are found in fungi; i.e., endochitinase, β-N-acetylglucosaminidase and exochitinase. Chitinase can be localized within the cell as soluble cytoplasmic protein, sequestered in microsomes or lyosomal vacuoles or bound to the membrane or cell wall. It can also be found secreted extracellularly.(Flach et al., 1992)

Plant chitinases

Plant chitinases are widely studied by several investigators. They are of interest partly due to the probable absence of chitin natural substrate in the plant itself. Chitinases are there for considered as a plant defense against pathogen (Flach et al., 1992). Chitinases are present either constitutively, specific steps of plant development or after induction. It has been shown that the latex contains large amounts of chitinase constitutively in *Hevea* (Martin, 1991). The induction factors of plant chitinase enzymes are numerous abiotic agents, i.e., ethylene treatment, salicylic acid, salt solutions, ozone, UV light, chemical induction or wounding and by abiotic factors such as fungi, bacteria, viruses, viroids, fungal cell wall components and oligosaccharides (Punja and Zhang, 1993). The mechanisms of the inductions of these factors are not completely elucidated (Flach et al., 1992).

These chitinase enzymes can be found in any organs of plant; i.e., leaf, root, seed and close contact site with fungal cells. In these organs, plant chitinases can be

found in any organells or fluid; i.e., vacuole, golgi cisternar, extracellular fluid, apoplastic compartments. Different classes of plant chitinases are distinguishable by molecular, biochemical and physiochemical criteria. Thus, plant chitinases may differ in substrate binding characteristics, localization within the cell and specific activities (Punja and Zhang, 1993). The plant chitinases are classified into five classes (class I-V) based on amino acid sequence features (Shinshi et al., 1990; Colling et al., 1993; Melchers et al., 1994). Class I chitinases are enzymes with and N-terminal cysteine-rich domain of about 40 amino acids and a highly conserved main structure. This class possess a leucine-rich or valine-rich signal peptide and can be acidic or basic proteins. Class II chitinases lack the N-terminal cysteine-rich domain but have high amino acid sequence identity to the main structure of class I chitinases. This class seem to be acidic proteins. Class III chitinases show no sequence similarity to enzymes in class I or II and can be acidic or basic protein. Class IV chitinases contain a cysteine-rich domain and a conserved main structure, which resemble those of class I chitnases but are significantly smaller due to the deletion. The amino acid sequence identity of class IV chitinases with class I chitinases is only 41-47% compared with 59-63% identity between the individual chitinases of class IV. The class I and II enzymes are serologically related, while class I and IV enzymes are serologically distinguishable. Class V chitinase show sequence homologous to exochitinases of *Bacillus circulans*, *Serratia marcescens* and *Streptomyces plicatus* bacteria with no sequence similarity to the previous classes.

Insect chitinases

In insect, chitinolytic activities were found principally in the integument, molting fluid, haemolymph and alimentary canal. In *Bombyx mori*, chitinase is synthesized as an inactive precursor that is activated by limited proteiolysis. Chitinase activities in insect molting fluid seem to be regulated by the molting ecdysteroid hormones, particularly during larval-pupal transformation (Flach et al., 1992). Several insect chitinases are glycoproteins (Funke et al., 1989; Kramerov et al., 1958). The molecular weights of insect chitinolytic enzymes usually range between 40,000 and 150,000, respectively (Flach et al., 1992).

Protozoan chitinases

Plasmodium gallinaceum ookinetes produce and secrete chitinase (Gilkes et al, 1991). Furturemore, malaria parasites (ookinetes) have been reported to digest the peritrophic membrane in the mosquito midgut during penetration (Flach et al., 1991).

Marine invertebrate chitinases

Chitinases have been characterized in marine invertebrates, molluses and crustaceans, such as oysters, prawns, lobsters and krills. In *Euphasia superba* and *Meganyciphanes norvegica*, a chitinase and a β-N-acetylglucosaminidase activity have been demonstrated. Both enzymes have broad pH optimum around 5.0 and temperature optima between 40 and 50 °C. Enzyme activities in the lower temperature range were still high, suggesting a functional adaptation to lower temperature of seawater (Flach et al., 1992).

Fish chitinases

Chitinase are also found in the digestive tract of some fish feeding on invertebrates such as in stomach of Japaneses eel (Kono et al., 1990). A chitinases were purified from the stomach of red sea bream, with molecular of 46,000, pI 8.3, optimal temperature of 50 °C and optimum pH of 5.5. Its activity was strongly inhibited by Hg^{2+}, Fe^{2+} and Sn^{2+}. Chitinase was also synthesized within the egg and could be induced during the larval period by the consumption of exogenous foods (Flach et al., 1992).

Bacterial chitinases

Bacterial chitinases are mostly found in organisms deposited in marine and soil (Clarke, 1956 and Herwig et al., 1988). The reason for which bacteria produce chitinase is to hydrolyze chitin and use as nutrient. Hence, bacteria play a major role in chitin mineralization (Herwig et al., 1988 and Yu et al., 1991a). This activity is in turn important for the maintenance of carbon flow in the carbon cycle and biomass turnover in nature. Bacterial chitnases were showed to be extracellular enzymes Several bacterial species produce chitinase; some of the best known, to produce chitinase includes the *Areomonas* sp., *Serratia* sp., *Myxobacter* sp., *Vibrio* sp.,

Streptomyces sp., and *Bacillus* species. Strain of *Serratia marcescens*, *Bacilllus* sp., and *Vibrio* sp. have been shown to produce a high level of chitinolytic enzymes.

Chitinase produced in *Bacillus circulans* and *S. macescens* are studied by many groups of scientist. In *B. circulans*, at least six major chitinases (A1, A2, B1, B2, C and D) have so far been found in the culture supernatant when induced with chitin (Watanabe et al., 1990a). Among these enzymes, the chitinase A1 has the most important role in degradation of chitin to chitobiose, $(GlcNAc)_2$. The *chiA* and *chiD* gene that encode the precursor of chitinase A1 and D, respectively, have been cloned and sequenced (Wantanabe et al., 1990b; Watanabe et al., 1992; Watanabe et al., 1994b). The action on partially N-acetylated chitosan and site directed mutagenesis of *B. circulans* chitinases enzymes were also studied (Watanabe et al., 1994a; Mitsutomi et al., 1995). The *S. macescensi* chitinase enzymes system is extracellular and is composed of five chitinolytic proteins with molecular weights of 21,000, 36,000, 48,000, 52,000 and 57,000, respectively (Flach et al., 1992). The activity types of enzymes are endochitinase and chitobiase (Monreal and Reese, 1969). The chitinase gene has been successfully transformed to some organisms such as *Lactococcus lactis* and *Lactobacillus plantarum* (Brurberg et al., 1994)

In addition to these bacteria, the ability to hydrolyze chitin is characteristic of the gram-positive *Streptomycetes* which are highly abundant in soil and known as important antibiotic producers. Though nearly all *Streptomycetes* species have been shown to be chitinolytic, and chitin has been successfully used to enrich predominantly *Streptomycetes* from soil, relative few studies on chitinolytic enzymes have been performed. Some of these enzymes have been enriched from culture filtrates of *Streptomycetes antibioticus*, *S. griseus*, *S. plicatus*, *S. erythreus*, *S. lividans* and two unclassified *Streptomycetes* strains (Ueno et al., 1990).

Purification, characterization and molecular cloning of chitinase

At the present time, various chitinases were isolated, purified and characterized from some microorganisms such as *Bacillus circulans* WL-12 (Wanatabe et al., 1990), *Serratia marcescens* (Nawani and Kapadnis, 2001) and an A*eromonas sp.*10S-24 (Ueda et al., 1992). One-step purification of chitinase has been reported by Roberts and Cabib (1982) using chitin affinity chromatography that is the

most specific method. It was used successfully for the purification of the chitinases from various organisms. However, this method is not always useful, since problems of the binding or the releasing chitinase occurs. Brurberg et al. (1994) purified chitinase from *E. coli* culture carrying *chiA* gene of *S. marcescens* using single step hydrophobic interaction chromatography. The gel filtration chromatography system was also developed for purification of chitinase from *S. marcescens* NK1 in a single step (Nawani and Kapadnis, 2001). Although, for most chitinase one step is generally inadequate for obtaining pure protein. Additional purification may be included. The research on purification of chitinases is summarized in Table 2. Wide range of molecular weight from 30 to 120 kDa are observed in bacteria and fungi. Some of these small chitinases may possibly be processed from a larger enzyme by limited proteolysis (Radwan et al., 1994). Most of the chitinases are active at a wide pH range of 4.0-8.0 and a temperature range of 40-55 °C.

In molecular cloning to achieve overproduction, changes in the induction pattern or changes in the localization (periplasmic/extracellular) of chitinases have been reported in many systems. Most chitinase genes was cloned, isolated, and expressed in *E. coli*, *Peudomonas* sp. or their natural hosts.

Cloning of the set of genes (*chi*A, *chi*B and *chi*C) that determine the expression of the chitinase complex and the regulatory genes (*chi*D and *chi*E) of *Serratia liquefaciens* has been reported (Joshi et al., 1988). Chitinase (Chi 63) encoding gene from *Streptomyces plicatus* has been sequenced and compared with the sequences of *S. marcescens chiA* and *B. circulans chiA1* genes reported earlier (Robbins et al., 1992); the three gene were strongly homologous. The binding and catalytic domains were adjacent to each other. Chitinase in yeast also posses these two domains. Moreover, Kuranda and Robbins (1991) observed a glycosylated 'hinge' between these two regions of the chitinase from *Saccharomyces cerevisiae*.

Several genes encoding chitinases were cloned and sequenced such as Chitinase A1 of *Bacillus circulans* WL-12 (Wanatabe et al., 1990), Chitinase C of *Serratia marcescens* (Suzuki et al., 1999), an A*eromonas caviae* (Sitrit et al., 1995) and an *Alteromonas sp.*strain O-7 (Tsujibo et al., 1992). A summary of cloning microbial chitinase genes is given in Table 3.

Bacillus licheniformis

Taxonomy and characterization

The genus Bacillus consists of a large number of diverse, rod-shaped Gram positive (or positive only in early stages of growth) bacteria which are capable of producing endospores that are resistant to adverse environmental conditions such as heat and desiccation (Claus and Berkeley, 1986). B. licheniformis is a ubiquitous, saprophytic, soil bacterium which is thought to contribute to nutrient cycling due to its ability to produce a wide variety of enzymes. Typically, the cells are motile by peritrichous flagella and are aerobic.

Although the genus *Bacillus* is rather heterogenous based on a wide range of DNA base ratios (32 to 69 mol% G + C) (Claus and Berkeley, 1986), the species *B. licheniformis* is rather homogeneous based on DNA-DNA hybridization studies. Historically, *B. licheniformis* and two closely related species, *B. subtilis*, and *B. pumilus*, were grouped taxonomically into what was known as the subtilis-group (Priest et al., 1988). However, recently methods have been developed that allow *B. licheniformis* to be differentiated from these other species (Gordon, 1973).

Human health hazards

B. licheniformis is not a frank human pathogen, but has on several occasions been isolated from human infections (Sugar and Johnson et al., 1983). Diseases attributed to *B. licheniformis* include bacteremia, opthalmitis following trauma, and there are reports of food poisoning based on circumstantial evidence (Tabbasa and Tarabay, 1979). However, the literature suggests that there must be immuno-suppression of the host, or there must be trauma (especially to the eye) followed by inoculation in high numbers of bacteria (Faner, 1963), before infection can occur (Claus and Berkeley, 1986). *B. licheniformis* does not produce significant quantities of extra-cellular enzymes or other factors that would predispose it to cause infection. Unlike several other species in the genus, *B. licheniformis* does not produce toxins. Overall, *B. licheniformis* has a low degree of virulence (Edberg, 1992). Although the possibility of human infection is not non-existent, it is low in the industrial setting where highly immunocompromised individuals would not be present. Infection might be a possibility following trauma, but in the industrial setting with the use of proper

Table 2. Comparison of the characteristics of purified chitinase from several microorganisms.

Source Organism	Molecular weight	Optimum pH	Optimum temp(°C)	pI	Reference
Aeromonas hydrophilia H-2330	62,000	5.0-8.0	40	4.0	Hiraga et al., 1997
Aeromonas sp. 10S-24					Ueda et al., 1992
Chitinase I	115,000	4.0	50	7.9	
Chitinase II	112,000	4.0	50	8.1	
Alteromonas sp. strain O-7					Tsujibo et al., 1992
Chitinase A	70,000	8.0	50	3.9	
Bacillus circulans WL-12					Wanatabe et al., 1990
Chitinase A1	74,000	5.0	N.D.	4.7	
Chitinase A2	69,000	N.D.	N.D.	4.5	
Chitinase B1	38,000	N.D.	N.D.	6.6	
Chitinase B1	38,000	N.D.	N.D.	5.9	
Chitinase C	39,000	N.D.	N.D.	8.5	
Chitinase D	52,000	N.D.	N.D.	3.9	
Clostridium paraputrificum					
Chitinase B	87,000	6.0	45	N.D.	Morimoto et al., 1997
Streptomyces erythraeus	30,000	5.0	N.D.	3.7	Hera et al., 1989

N.D. = not detectd

Table 2. (Continued)

Source Organism	Molecular weight	Optimum pH	Optimum temp(°C)	pI	Reference
Streptomyces thermoviolaceus OPC-250					Tsujibo et al., 2000
Chi30	30,000	4.0	60	3.8	
Streptomyces RC1071	70,000	8.0	40	N.D.	Gomes et al., 2001
Streptomyces sp. J13-3	31,000	6.0	45	3.9	Okasaki et al., 1995
Chitinase B1	38,000	N.D.	N.D.	5.9	
Serratia marcescens BJ2000					Brurberg et al., 1982
Chitinase A	61,000	4.0-7.0	N.D.	6.4	
Serratia marcescens NK1	57,000	6.2	47	N.D.	Nawani and Kapadnis, 2001
Serratia marcescens QMB1466					Roberta et al., 1982
Chitinase A	58,000	4.0-7.0	N.D.	6.4	
Xanthomonas sp. strain AK					Yamaoka et al., 1999
Chitinase A	64,000	4.0	35	N.D.	
Chitinase B	48,000	6.0	40	N.D.	

N.D. = not detectd

Table 3 Molecular cloning of chitinase genes

Source Organism	Host	Vector	DNA insert sizes (kb)	ORF (kb)	Reference
Aaphanocladium album	*E.coli*	pBL1	8.0	1.9	Blaiseau et al., 1992
Aeromonas hydrophilia	*E.coli*	pUC18	5.0	2.5	Tsujibo et al., 1992
Aeromonas caviae	*E.coli*	pBluescriptIISK	4.5	2.6	Sirit et al., 1995
Alteromonas sp. strain O-7	*E.coli*	pUC18	5.0	2.5	Tsujibo et al., 1992
Bacillus circulans WL-12					
Chitinase A1	*E.coli*	pKK223-3	4.0	2.1	Wanatabe et al., 1990
Chitinase C	*E.coli*	pUC19	2.8	1.5	Wanatabe et al., 1995
Clostridium paraputrificum					
Chitinase B	*E.coli*	pMW119	5.1	2.5	Morimoto et al., 1997
Enterobacter agglomerans	*E.coli*	pGEM-Teasy	2.2	1.7	Chernin et al., 1997
Streptomyces thermoviolaceus OPC-520					
Chi30	*E.coli*	pUWL219	3.4	1.0	Tsujibo et al., 2000
Serratia marcescens BJL200					
Chitinase B	*E.coli*	pBluescriptIISK	4.5	2.6	Sirit et al., 1995
Serratia liquefaciens					
Chitinase B	*E.coli*	pSJ12	4.6	1.5	Woytowich et al., 2000

N.D. = not detectd

safety precautions, good laboratory practices, and proper protective clothing and eyewear, the potential for infection of workers should be quite low.

Applications of *B. licheniformis*

The use of *B. licheniformis* for industrial production of enzymes should not pose environmental hazards. First, the number of microorganisms released from the fermentation facility is low. In addition, *B. licheniformis* is ubiquitous in the environment, and the releases expected from fermentation facilities operating under the conditions of this exemption will not significantly increase populations of this microorganism in the environment. Therefore, although *B. licheniformis* may be associated with livestock abortions, the use of this microorganism in fermentation facilities will not substantially increase the frequency of this occurrence, even if a scenario for high exposure to *B. licheniformis* released from the fermentation facility to livestock could be envisioned. This latter feature of the microorganism has been commercially exploited for over a decade. *B. licheniformis* has been used for industrial production of proteases, amylases, antibiotics, and specialty chemicals with no known reports of adverse effects to human health or the environment (Claus and Berkeley, 1986). The Agency has reviewed three submissions for production of enzymes using genetically modified *B. licheniformis*.

In conclusion, the use of *B. licheniformis* in fermentation facilities for production of enzymes or specialty chemicals presents low risk. Although not completely innocuous, *B. licheniformis* presents low risk of adverse effects to human health or the environment.

Thermotolerant bacteria

Thermotolerant microorganism is defined as those that can grow both at 30°C as well as 45°C or up to 60°C. Thermotolerant bacteria are the organisms that able to ferment lactose at 44-45 °C; the group includes genus *Escherichia* and some species of *Klebsiella*, *Enterobacter*, *Citrabacter* and *Bacillus*. Thermotolerant microbes can produce endospores which are resistant to high temperatures even though their cells may be sensitive example *Bacillus subtilis* is a mesophilic bacterium which produces

endospores resistant to 100 °C. We found that thermotolerant microbes can produce thermostable enzymes for example cellulase. The thermostable enzyme provides wide application in the food and sugar industry where high temperature processes such as pasteurization are used. Specifically, enzyme preparation could be used to clarify and extract fruit juices, in coffee and vanilla bean extraction, to reduce viscosity in concentrated foods to and, increase starch yields from various vegetables and grains. Other applications may be in paper, waste treatment, and agricultural industries to process cellulose-derived material.

The aims of this thesis

In Thailand, the chitinase from bacteria is widely studied in various bacterial due to the simple cultivation, large quantity of enzyme production simple enzyme purification. This thesis is aiming at screening, purification and characterization cloning of thermostable chitinase from thermotolerant bacteria. Therefore, an active thermostable chitinase was first screened and characterized. *Bacillus licheniformis* is the bacterium isolated which secreted, chitinase into the culture medium, and shows a visible clear zone when grown on colloidal chitin medium plate. This study will emphasize on characterization and partial purification of the chitinase enzyme(s) produce by *B. licheniformis*. Gene(s) encoding, for chitinase gene will also be cloned, sequenced and gene product characterized.