CHAPTER I

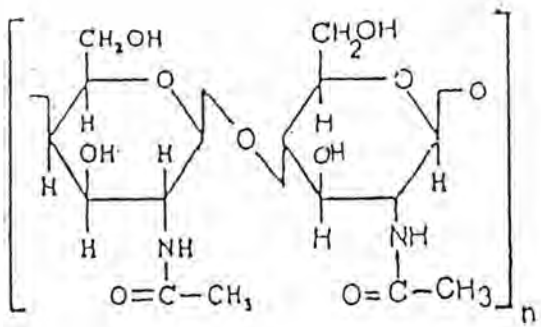
INTRODUCTION

1.1 Chitin

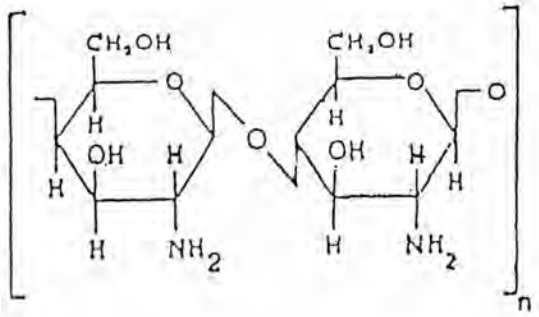
Chitin, a cellulose-like biopolymer, was discovered by Braconnot in 1811 from mushroom by treating *Agaricus valvaceus* with diluted warm alkali and then isolating (Muzzarelli, 1977). Chitin is an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine (NAG) (Figure 1) which are arranged in an antiparallel (α-chain) or parallel (β-chain) (Blackwell, 1988). It is one of the most abundant polysaccharides in nature. Chitin is a common constituents of the exoskeletons of arthropods found associated with proteins, and as a cell wall of molds and fungi by associated with other polysaccharides (Muzzarelli, 1977). Other sources of chitin include various forms of marine life, protozoa , nematodes and insects. It has been estimated that several million tons of chitin are associated with planktonic crustacea alone.

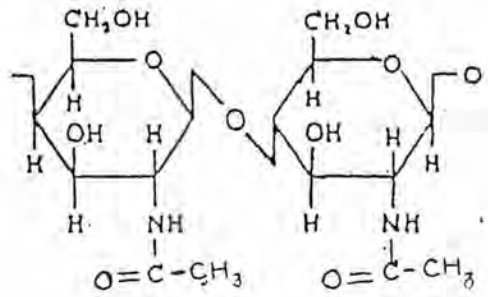
Chitin and chitosan are copolymer of NAG and glucosamine. The copolymer is called chitin when it contains less than 7% nitrogen and chitosan when the nitrogen content exceeds 7% (Davies and Hayes, 1988). The difference between chitin and chitosan is the degree of acetylation of D-glucosamine residue. Usually those of chitin are acetylated more than 60% and those of chitosan are acetylated less than 40%. The enzyme which hydrolyzes chitin is chitinase, and for chitosan is chitosanase. The difference of the two enzymes are not well clarified

chitin



chitosan





chitobiose

Figure 1 Chitin, chitobiose and chitosan structures.

at the molecular level (Ando, Noguchi, Yanagi *et al.*, 1992). Only zygomycetous fungi have cell walls composed of both chitin and chitosan (Yabuki *et al.*,1988). Furthermore, chitin can be thought of as a naturally occuring cellulose derivative since it is composed of *N*-acetyl-2-amino-2-deoxy-β-D-glucopyranosyl residues joined by β-1,4-glucosidic bonds. Like cellulose, chitin chains have an extended conformation and form insoluble and crystalline microfibrils. In nature, chitin is quite insoluble in water but partial acid hydrolysis yields a series of soluble β-1,4-linked oligomers that are useful substrates for study on the enzymes. The preparation procedure consists of the dissolution of chitin into and the reprecipitation from concentrated hydrochloric acid and the dispension of the precipitated chitin into water. Colloidal chitin serves as a substrate of chitinase and a carbon and nitrogen source of chitinolytic microorganisms (Shimahara and Takiguchi, 1988). It is prepared by treatment of chitin with aqueous sodium hydroxide solution to give alkali chitin (sodium alkoxides of chitin). The alkali chitin can be reacted with ethylene chlorohydrin (2-chloroethanol) to give glycol [O-(2-hydroxyethyl)] chitin, and reacted with chloroacetic acid (or its salt) to give carboxymethyl [O-carboxymethyl (CM)] chitin. Glycol chitin and CM-chitin are soluble in water and hydrolyzed by chitinase (EC 3.2.1.14) and lysozyme (EC 3.2.1.17) (Hirano *et al.*,1988).

Enzymatic hydrolysis of chitin to its constituent monomer is performed by the binary chitinase enzyme system. Chitin is first hydrolyzed by chitinase to low-molecular weight multimer of NAG, the dimer *N*,*N*'-diacetylchitobiose (chitobiose) being predominant.

Chitobiase hydrolyzes chitobiose to NAG (Fukamizo *et al.*,1986, Jeuniaux, 1966 and Jannatipour *et al.*, 1987).

1.2 Chitinases

Chitinase was described for the first time in 1911 by Bernard who found a thermosensitive and diffusable antifungal factor in orchid bulbs (Flach *et al.*,1992).

Chitinases (poly-β-1,4-(2-acetamido-2-deoxy)-D-glucoside glycanohydrolases, EC 3.2.1.14) are defined as enzymes cleaving the bond between the C1 and C4 of two consecutive *N*-acetylglucosamines of chitin. Endochitinases, exochitinases (EC 3.2.1.14), β-*N*-acetylglucosaminidases and chitobiases (EC 3.2.1.30) have been characterized. Usually β-*N*-acetylglucosaminidases is defined as an enzyme releasing *N*-acetylglucosamine monomers from chitin. Exochitinase is an enzyme releasing chitobiose and endochitinase as an enzyme splitting within the chitin polymer. Chitobiase hydrolyses chitobiose to *N*-acetylglucosamine (Figure 2).

Some chitinases also display more or less pronounced lysozyme activity (EC 3.2.1.17) corresponding to the cleavage of glycosidic bond between the C1 of *N*-acetylmuramic acid (Mur-NAc) and the C4 of *N*-acetylglucosamine in the bacterial peptidoglycan (Jolles P.& Joller J., 1984).

Chitinases are commonly found in a wide range of organisms including bacteria, fungi, insects, crustaceans, some vertebrates and higher plants. All organisms that contain chitin also contain chitinases

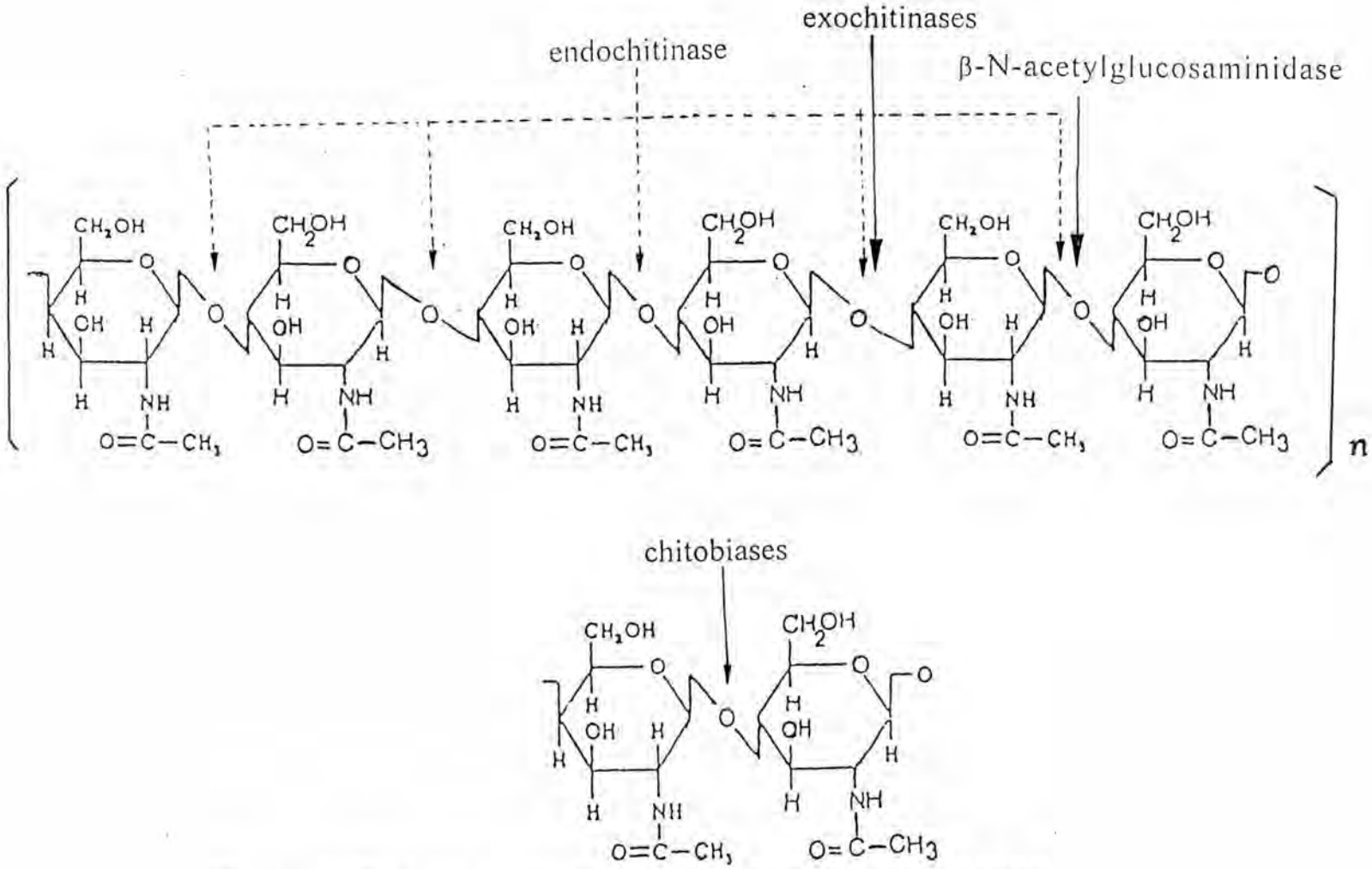


Figure 2 Chitinases and related enzymes action on chitin chain.

which are presumably required for morphogenesis of cell wall and exoskeletons. Other organisms that do not contain chitin may produce chitinase to degrade the polymer for food , e.g. soil bacteria that secrete chitinases in response to chitin in their environment (Oranusi and Trinci, 1985) and the enzyme found in digestive tract of fish. Plants also have been found to contain chitinase, often after enzyme synthesis has been induced by microbial infections or other injuries (Pegg and Young,1982; Boller *et al.*,1983; Boller,1985). Since plants do not contain chitin in their cell walls, it has been postulated that they produce chitinase to protect themselves from chitin-containing parasites (fungi, insects) (Abeles *et al.*, 1970), but direct evidence supporting this hypothesis has been lacking (Roberts and Selitrennikoff, 1988). Chitinases in vertebrates are synthesized in and secreted by the pancreas and gastic mucosa of insectivorous fishs, amphibians and reptiles. In some insectivorrous birds and mammals, the enzyme is secreted by the gastric mucosa. In higher plant, chitinases can be divided into at least three classes based on their amino acid sequences (Shinshi *et al.*,1990).

Class I chitinases. Class I chitinases contain an amino terminal cysteine-rich domain of about 40 amino acids. They usually have a basic isoelectric point and are located in the vacuole.

Class II chitinases. Class II chitinases are similar to class I enzymes, but the cysteine-rich domain and the proline-rich small region that follows are missing (Leah *et al.*,1991). Class II chitinases seem to be acidic proteins,e.g., PR-P and PR-Q proteins from tobacco (Flach *et al.*,1992).

Class III chitinases. Class III chitinases have no sequence similarities with class I and class II chitinases. They can be acidic or basic proteins.

1.3 Assay methods of chitinase enzyme

Chitinase activity has been assayed by a veriety of procedures, including the monitoring of changes in the molecular size of substrate and the determination of oligosaccharides or *N*-acetylglucosamine liberated in the reaction.

1.3.1 Viscosimetric assay for chitinase

Viscosity measurements for chitinase activity monitor the changes in the molecular size of substrate. Insoluble compounds such as colloidal chitin and chitin are used as substrates in these assay procedures. The assay of chitinase activity by the viscosimetric method was first carried out using the solution of chitosan acetate. Carboxymethylchitin has been reported to be a water-soluble substrate suitable for the assay of chitinase activity. However, both compounds have the disadvantage that the viscosity was markedly affected by ionic strength and pH. On the contrary, glycol chitin does not have such disadvantage and is a useful substrate for the viscosimetric assay.

A unit of chitinase activity is defined as the amount of the enzyme which is required to attain a half-life time in 30 min at 30°C and

optimum pH. Thus, the viscosimetric unit for chitinase can be obtained by dividing 30 min by half-life time (Ohtakara, 1988).

1.3.2 Tritiated chitin assay for chitinase

The radiochemical assay uses [acetyl-^{3}H]chitin as a substrate. The assay, based on the formation of soluble oligosacchrides from [^{3}H] chitin, is the most sensitive, because of the possibility of using substrate of very high specific activity. It is suitable for both endo- and exochitinases as it obviates the need for an auxiliary β-*N*-acetylhexosaminidase, and is extremely simple to carry out (Cabib, 1988 (b)).

A unit of chitinase activity by this method is defined as that amount of enzyme which catalyzes the release of 1 μ mol of soluble product (calculated as *N*-acetylglucosamine, NAG) in 1 min at 30°C (Cabib, 1988 (a)).

1.3.3 Colorimetric assay for chitinase

The colorimetric assay described here is applicable to various types of chitinase present in microorganisms, animals and plants. The most widely used colorimetric assay for plant chitinases has been an exochitinase assay, based on the determination of monomeric *N*-acetylglucosamine (NAG) released from colloidal chitin. However, plant chitinases generally are endochitinases which produce chitooligosaccharides as principle products (Boller *et al.*, 1983).

Therefore, measurements of plant chitinases with the exochitinase assay should be viewed with caution. For accurate determination, it is essential to measurement the chitooligosaccharides produced in the assay. This can be accomplished by the enzymatic hydrolysis of the reaction products to monomeric NAG prior to the colorimetric measurement.

Endochitinase forms soluble chitooligosaccharides from soluble chitin. After removal of the undigested substrate by centrifugation, the chitooligosaccharides are completely hydrolyzed to monomeric NAG by incubation with snail gut enzyme. The monomeric NAG is then determined with ρ-dimethylaminobenzaldehyde (DMAB) (Boller and Mauch, 1988).

One unit of chitinase is defined as the amount that catalyzes the release of soluble chitooligosaccharides containing 1 μmol of NAG in 1 minute.

Other colour reagent such as ferricyanide have been employed for assaying the product of chitinase. However, these reagents are not suitable for detecting the products of plant chitinase because the method for this colour reagent was to measure the reducing of colour of ferricyanide and the colour in the extract of plant was interfere in the procedure.

Comparing the method discussed above, the viscosimetric assay has the advantages of using a soluble substrate (which facilitates kinetic analyses) and of being more specific for endochitinase than other assays. Disadvantages are the requirement for an artificial substrate and, in the absence of specialized equipment, its rather time consuming and tedious procedure. The radiochemical assay is more rapid and simpler than the

colorimetric assay but hazadous. Therefore, the use of the radiochemical assay is recommended for routine work. The colorimetric assay is most useful when the radioactive substrate is not available, or when native chitin or chitin-containing materials like fungal cell walls are employed as substrates (Boller and Mauch, 1988).

1.4 Occurrence of chitinases

1.4.1 Fungal chitinases

All types of chitin-degrading activities were found in fungi. For example, an endochitinases and β-N-acetylglucosaminidase in *Aspergillus nidulans* (Flach *et al.*,1992) and an endochitinase in *Mucor rouxii* (Pedraza-Reyes and Lopez-Romero, 1986). Fungal wall degrading enzymes could be involved in the growth of the fungus itself.

The hypomycete vascular wilt fungus *Verticillium albo-atrum* produces a constitutive chitinase in culture which has been implicated in the *in vitro* and *in vivo* lysis of fungal mycelium (Pegg, 1988). Chitinases seem to play a physiological role related to the mycoparasitic activity displayed by several species of fungi, such as the genus *Trichoderma*. Some species of *Trichoderma* have been used as biological control agents against fungal pathogen. The degradation and further assimilation of phytopathogenic fungi, namely mycoparatism, has been proposed as the major mechanism accounting for the antagonistic activity against phytopathogenic fungi displayed by *Trichoderma*

(Siwayaprahm,1997). Table 1 shows some fungl chitinases and some properties.

1.4.2 Bacterial chitinases

Bacteria play a large role in chitin mineralisation, for example in marine waters and sediments, but not all species are able to hydrolyze chitin. Distribution of chitinase and chitobiase in strains of *Bacillus* was studied and found that *B. licheniformis* X-7u produced thermostable chitinases (Flach *et al.*,1991).

Bacterial chitinases were shown to be extracellular enzymes. *Serratia mrcescens* (Roberts and Cabib, 1982 and Vorgias *et al.*,1992), growing on chitin as a carbon source, secretes into the medium a chitinase of high specific activity. Because of its stability, wide pH optimum and linear kinetics over a greater range than that of other chitinases, the enzymes are well studied for analytical use. For these reasons, they are also a good candidate for the large scale degradation of chitin for industrial purposes (Cabib,1988 (a)). A laboratory isolate *Aeromonas hydrophila* sub sp. *Anaerogenes A 52* inducibly produced chitinase and concomitantly chitobiase (EC 3.2.1.30) in its culture broth.

B.circulans WL-12 is one of the bacteria that secretes chitinases into the culture medium. When the bacterium was grown in the medium containing chitin as an inducer substrate, six distint chitinases were detected in the culture supernatant. One of the six chitinases, chitinase A1, showed strong affinity to chitin and was suggested to play a major role in the degradation of chitin in the chitinase system of *B. circulans*

Table 1 Fungal chitinases and some properties.

Source of chitinase (reference)	Methods of purification	MW (kDa)	pH (optimum)	pI
Trichoderma harzianum (De La Cruz *et al.*, 1992)	- 0-80% amm. sulfate precipitation - chitin digestion - chromatofocusing			
- chitinase 33	- Sephacryl S-200 HR column	42.0		
37		33.0		
42		37.0		
Penicillium oxalicum (Rodriguez *et al.*, 1995)	- 0-70% amm. sulfate precipitation - anion and cation exchanger - Sephacryl S-200	21.5	4.5	
Vibrio sp. (Ohtakara *et al.*, 1979)	- 0-70% amm. sulfate precipitation - DEAE-Sephadex A-25 column - Hydroxyapatite column - Sephadex G-100	63.0	6-8	3.7

WL-12 (Watanabe *et al.*, 1990). Table 2 shows some bacterial chitinases and some properties.

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 *Streptomyces* species have been shown to be chitinolyic, and chitin has been successfully used to enrich predominantly *Streptomyces* from soil, relatively few studies on chitinolytic enzymes have been performed. Some of these enzymes have been enriched from culture filtrates of *Streptomyces antibioticus, Streptomyces griseus, Streptomyces plicatus, Streptomyces erythreus, Streptomyces lividans* and two unclassified *Streptomyces* strains (Ueno *et al.*, 1990).

1.4.3 Insect chitinases

In insects, chitinolytic activities were found principally in the integument, moulting fluid, haemolymph and alimentary canal. In *Bombyx mori*, chitinase is synthesized as an inactive precursor which is activated by limited proteolysis. Chitinase activities in insect moulting fluid seem to be regulated by the moulting ecdysteroid hormones, particularly during larval-pupal transformation (Flach *et al.*, 1992).

1.4.4 Marine invertebrate chitinases

Chitinases have been characterized in marine invertebrates, molluses and crustaceans, such as oysters, prawns, lobsters and krills. In

Table 2 Bacterial chitinases and some properties.

Source of chitinase (reference)	Methods of purification	MW (kDa)	pH (optimum)	pI
Bacillus circulans WL-12 (Watanabe *et al.*, 1990)	- 0-40% amm. sulfate precipitation - Chitin-chitinase complex - Sephadex G-100			
- chitinase A1		74.0		4.7
A2		69.0		4.5
B1		38.0		6.6
B2		38.0		5.9
C		39.0		8.5
D		52.0		5.2
Bacillus licheniformis X-7U (Takayanagi *et al.*, 1991)	- Butyl-Toyoperarl 650 M column - Q-Sepharose column - Sephacryl S-200 column			
- chitinase I		89.0	6.0	
II		76.0	6.0	
III		66.0	5.0	
IV		59.0	5.0	
Serratia marcescens (Roberts & Cabib, 1982)	- Chitin affinity column			
- chitinase I		52.0	4-7	
II		58.0		

Table 2 (continued)

Source of chitinase (reference)	Methods of purification	MW (kDa)	pH (optimum)	pI
Streptomyces erythraeus (Hara *et al.*, 1989)	- DEAE-cellulose column pH 7.5 and 4.0 - Bio-Gel P-60 - DEAE-Sephadex	30.0	5	3.7
Streptomyces albidoflavus (Broadway *et al.*, 1995)	- 0-95% amm. sulfate precipitation - DEAE column			
- chitinase I		59.0	5.1	
II		45.0	5.3	
III		38.5	5.75	
IV		27.0	5.8	
V		25.5	5.9	

Euphasia superba and *Meganyciphanes norvegica*, a chitinase and a β-N-acetylglucosaminidase have been demonstrated. Both enzymes have broad pH optima 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).

1.4.5 Fish chitinases

Chitinases have also been described in the digestive tract of some fishes feeding on skrill or in Dover sole. A chitinase was purified from the stomach of red sea bream, with molecular weight 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+}. The chitinase was synthesized within the egg and could be induced during the larval period by the consumption of exogenous foods (Flach *et al.*, 1992).

1.4.6 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).

1.4.7 Plant chitinases

Plant chitinases were described reported in almond emulsin, bean and other seeds. Abeles *et al.* (1970) discovered that chitinase is induced in bean leaves by the plant hormone, ethylene, in parallel with β-1,3-glucanase. Since chitin and β-1,3-glucans are important cell wall components of fungal, therefore chitinase and β-1,3-glucanase have an antifungal function.

Plant chitinases are also found in the higher plants such as forest trees, wheat germ, barley grain, tomato leaves, yam root, rubber tree latex, bean seeds etc. However, chitin is not synthesized by vascular plants, but *N*-acetylglucosamine as a constituent of plant polymers, e.g., as a glycoprotein, is widespread in many spermatophytes, especially in seeds, including tomato (Pegg,1988). Treatment of peas with *Fusarium solani* spores enhanced synthesis of chitinase and β-1,3-glucanase which are the enzymes for hydrolysis of the components of fungal cell wall (Watanabe *et al.*, 1990). The increased in enzyme activities observed in infected plants was due to the induction of the plant enzymes. It has been suggested that plant chitinases might function as an important defense against potential pathogens (Huynh *et al.*, 1992). Table 3 shows some plant chitinases and some properties.

Plant chitinases are present either constitutively or after induction. The induction mechanisms are not completely elucidated. Infection with pathogens, treatment with chitooligosaccharides and other fungal or bacterial extracts, physical or chemical stresses and wounding can all influence chitinase production.

Table 3 Plant chitinases and some properties.

Source of chitinase (reference)	Methods of purification	MW (kDa)	pH (optimum)	pI
Zea mays Maize	- 0-80% amm. sulfate precipitation			
(Nasser *et al.*, 1988)	- Sephadex G-500	25.0		
	- Electroeluted 12% PAGE	25.0		
	- DEAE-cellulose	29.0		
		34.5		
Wheat germ (Molano	- Acetic acid pH 4.5 precipitation	33.0		7.5-
et al., 1979)	- Chitin affinity column			9.2
	- Sephadex G-50			
Pisum sativum Pea	- 0-90%amm. sulfate precipitation	33.1		
(Mauch *et al.*, 1990)	- Trisacryl-DEAE	36.2		
	- Chromatofocusing column	32.0		
Cucurbita sp.	- Sepharose 6B column	29.8		
Pumpkin (Esaka *et al.*, 1990)				
Nicotiana tabacum	- Sephadex G-25			
tobaco (Sela-Buurlage	- Sepharose column			
et al., 1993)	- Chitin affinity column			
- chitinase Ia	- Superdex-75 gel filtration column	32.0		
Ib	- Mono S column	34.0		
	- S-Sepharose column			

Table 3 (continued)

Source of chitinase (reference)	Methods of purification	MW (kDa)	pH (optimum)	pI
Phaseolus vulgaris leaves (Boller *et al.*, 1988)	- Crude extract - **Heat treatment** - Amm. sulfate precipitation - Chitin affinity column	32.5	6.5	9.4
Lycopersicon esculentum Tomato(Pegg,1988)	- Crude stem extract - DEAE-cellulose column - Amm. sulfate precipitation - IEF (pH 3.5-10.0) - Hydroxyapatite - IEF (pH 3.5-10.0) - Bio-Gel P-100 column	31.0	5.1	

1.4.7.1 Constitutive plant chitinases

Chitinases are sometimes observed constitutively in plants. In *Hevea*, the latex contains large amounts of chitinase. Chitinases can also be produced during specific steps of plant development, e.g., chitinase was expressed in tobacco explants during flower formation and in apical leaves (Flach,1992).

1.4.7.2 Ethylene treatment

Chitinases can be induced by ethylene treatment. Abeles *et al.* (1970) and Boller *et al.* (1983) showed that chitinase activity in bean seedlings increased 30-fold after exposure to exogenous ethylene. In carrot cell cultures, four chitinases were induced by ethylene (Kurosaki *et al.*,1989).

1.4.7.3 Viral infection

Some chitinases can be induced by virus infection, e.g. tobacco leaves infected with TMV (tobacco mosaic virus). Legrand *et al.* (1987) found two additional chitinases in cucumber, also induced chitinase by TMV infection.

1.4.7.4 Infection by microorganism, wounding and fungal elicitors

Elicitors caused a very rapid activation of chitinase transcription with 10-fold stimulation after 5 minutes. Chitinase transcripts were also greatly accumulated in wounded and infected hypocotyls. Flach *et al.* (1992) also studied the relationships between oligosaccharide size and elicitor efficacity : for the colorimetric assay, the hexamer of chitin was the most efficient elicitor, whereas the heptamer was the most convenient one for the radiochemical assay.

1.4.7.5 Chemical induction

Chitinase activity can be induced by treatments with salicylate or mercuric chloride but, as for most other induction means, the effects of chemical treatments are not specific because other defence mechanisms are simutaneously induced.

Plants do not contain an immune system and thus are vulnerable to pathogens. In order to protect themselves from pathogens, plants have evolved a number of defense responses that are elicited during their life cycle in response to developmental signals and pathogen attack. Plant chitinase has been speculated to play a crucial role in plant defense against fungal pathogens because of its ability to digest chitin, a major constituent of the cell walls of a number of fungal pathogens. Purified plant chitinases display strong antifungal activity against nonpathogenic

plant fungi such as *Trichoderma reesei* and *Trichoderma hamatum* but show relatively weak antifungal activity on phytopathogenic fungi (Huynh *et al.*, 1992). Roberts and Selitrennikoff (1988) were purified chitinases from three grains, barley, maize and wheat and all three grain chitinases inhibited hyphal extension of Trichoderma reesei and Phycomyces blakesleeanus.

Chtinases can be found in vacuole (e.g. chitinase from ethylene-treated bean leaves), Golgi apparatus, intracellualr fluid, leaf protoplast, apoplastic compartment (e.g., chitinases from tobacco, oat leaves and wheat germ) In maize chitinases (PRm 3, 4, 5 and 7) are extracellular enzymes. In table 3 shows some properties of some plant chitinases, the isoelectric point of plant chitinases generally basic or very acid. Some chitinases were described as basic proteins, *Rubus* and *Parthenocissus* have isoelectric point of 9.0 and 9.9 respectively. Tobacco chitinases (PR-P and PR-Q) have a acidic isoelectric point of 4.9 and 3.8 respectively. All plant chitinases investigated so far are small proteins with molecular weight varied between 25,000 and 40,000 (Flach *et al.*, 1992).

The aims of this thesis are

1.To screen for chitinases in some local crops.

2.To purify and characterize chitinase from angled loofah fruit pericarp.