

# CHAPTER I

## INTRODUCTION

### 1. Introduction

#### 1.1 Background and Rationale

Latex of the rubber tree (*Hevea brasiliensis*) is used commercially for the production of natural rubber that is used industrially for various finished product, but in part of non-rubber constituents of natural rubber latex is not known. The major components of latex are rubber particles, cytosol and organelles called lutoids that both cytosol (C-serum) and lutoids (bottom fraction) are classified in non-rubber. *Hevea* latex or rubber latex is known to produce chitinases that these enzymes are isolated from the lutoid body of the rubber latex.

Chitin is a polysaccharide abundant in invertebrate exoskeletons including crustacean shells and fungal cell walls. A gigantic amount of chitin is processed as solid waste from seafood processing industry. The natural degradation of chitin presents an important feature, not only in the global recycling of carbon and nitrogen sources, but also in the production of useful chemical reagents. *N*-acetyl-*D*-glucosamine (GlcNAc) and glucosamine (GlcN), the monomer of chitin and chitosan, are therapeutic agents for osteoarthritis, inflammatory bowel diseases and gastritis. In nature, chitin-degrading enzymes are widely distributed among bacteria, arthropods, protozoans and plants. Nevertheless, commercial preparations of chitinases are inevitably expensive, especially, the purified chitinases.

Hence, in the present work illustrate that non-rubber constituent of *Hevea* latex from *H. brasiliensis* contained both endo- and exochitinase activities can potentially be an excellent source of low-cost chitinolytic enzymes, as it is regarded as a massive waste product that must be disposed of from rubber-processing plants.

## 1.2 Review of Literature

### 1.2.1 Proteins of rubber latex

Latex occurs in the plant kingdom in more than 12,000 species belonging to some 900 genera. Of these laticiferous plants, about 1000 species contain rubber. Commercial natural rubber is derived from only one cultivated plant: the rubber tree, *Hevea brasiliensis*, a plant that originates from Brazil but is cultivated mainly in Southeast Asia today. The terms rubber and latex are often used interchangeably in the literature and, hence, an early clarification is in order. Latex exudes from the rubber tree when it is tapped and its main constituent (other than water) is natural rubber, the polymeric hydrocarbon *cis*-polyisoprene. *Hevea* latex is regarded as the living cytoplasm of laticiferous cells and, accordingly, it is a rich blend of organic substances, including a mélange of proteins. A small number of these proteins have given rise to the problem of latex allergy.

Proteins make up about 1–2% fresh weight of *Hevea* latex (Archer *et al.*, 1961; Tata, 1980). Because natural rubber latex is not a homogeneous fluid, latex proteins are not homogeneously dispersed. Latex proteins are found in the latex sera are also associated with latex organelles that can be separated by high-speed centrifugation. About 70% of latex proteins are soluble, with the remaining being associated with membranes.

### 1.2.2 Tapped latex

The *Hevea* latex collected by regular tapping consists of the cytoplasm expelled from the latex vessels, and is similar to the latex in situ. Although Moir (1959) distinguished as many as nine fractions in *Hevea* latex following ultracentrifugation at 59,000g, there are basically three main fractions that are easily discerned (Cook and Sekhar, 1953). These are the rubber phase, the C-serum, and the bottom fraction (Fig. 1). The rubber phase comprises the rubber particles that are packed centripetally by centrifugation. With the C-serum in the interstices between rubber particles removed, two main proteins are extractable from the surface of the rubber particles. Whereas most of the C-serum and B-serum proteins are water-soluble, those of

the rubber particles are generally insoluble. The C-serum refers to the aqueous medium in which all the latex organelles are suspended. Being the cytoplasm of the laticifer, latex C-serum contains a large variety of proteins associated with cellular metabolism, as might be expected. For example, all the enzyme of the respiratory pathway is present.

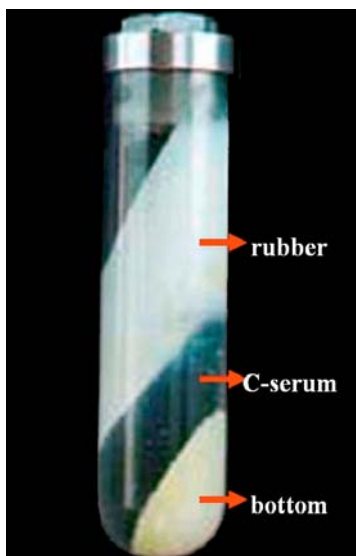


Figure 1. Fraction of fresh *Hevea* latex by ultracentrifugation into three major separated zones with the upper rubber layer, the aqueous phase C-serum and the bottom fraction containing luitoid particles.

Various enzyme specific to latex, such as the enzymes associated with the rubber biosynthesis pathway, are also found in the latex C-serum. The C-serum proteins are therefore numerous, probably in the hundreds. While the latex bottom fraction comprises mainly the luitoids, other minor organelles (e.g., ribosome, endoplasmic reticulum) are also present. Hence, although the B-serum is commonly thought of as the luitoidic serum, it also contains minute constituents derived from the other minor organelles deposited in the bottom fraction. The latex B-serum is obtained by repeated freezing and thawing of the bottom fraction of centrifuged latex. Compared with the C-serum, there are a smaller number of proteins (fewer than 20 major peptides), with a single protein, hevein, and making up 50–70% of the total B-serum soluble proteins (Tata, 1980; Archer *et al.*, 1969). Whereas the rubber particle proteins and practically all the C-serum proteins are generally acidic proteins, B-serum has a mixture of acidic and basic

proteins (Moir and Tata, 1960). A typical distribution of proteins in the rubber particles, the C-serum, the B-serum, and the lutoid membranes is shown in Table 1. These figures are comparable to those given by Tata (1980).

Table 1. Typical protein distribution in the centrifuged latex fractions

Latex fraction	Protein concentration (mg/ml)	%
Rubber particle membrane	3.5	25
C-serum	6.0	43
B-serum	3.6	26
Bottom fraction membranes	0.9	6
Total	14.0	100

Note: Protein in bottom fraction membranes based on Tata (1980). Other values from, H.Y. Yeang (unpublished).

### 1.2.3 Defense related proteins in higher plants

Higher plants respond to various physical, chemical and biological stresses in a variety of ways. These stress factors include wounding, exposure to heavy metals such as mercury, salinity, drought, cold, and invasion by fungi, bacteria and viruses. Plants defend themselves against such insults by physical strengthening of the cell wall through lignifications, suberization, and callose deposition; by synthesizing low molecular weight compounds known as phytoalexins which are toxic to the invading organisms; by producing various pathogenesis-related (PR) proteins such as chitinases,  $\beta$ -1,3-glucanases, and thaumatin-like proteins (Bowles, 1990). PR-proteins were first discovered, through advances in electrophoretic techniques, in tobacco plants hypersensitively reacting to tobacco mosaic virus (TMV) infection (Van Loon, 1970). Later, these proteins were found to be induced by bacterial and fungal infections in a

number of plant species (Redolfi, 1984). Observations such as induction of PR-genes and occurrence of systemic acquired resistance (SAR) in plants to subsequent infections after an initial necrotic infection (Kassanis *et al.*, 1974; Van Loon, 1975; Ward *et al.*, 1991), absence of the substrate for one of the PR proteins (chitinase) in plants but its presence in the cell wall of certain fungi, and the *in vitro* antifungal activity of chitinases (Mauch *et al.* 1988) suggested a defensive role for PR-proteins.

PR-proteins are currently grouped into eleven families (PR-1 through 11) based on their primary structures and immunological properties (Van Loon *et al.*, 1994). The PR-1 family comprises small proteins of unknown function that are induced upon pathogen invasion. The PR-2 family comprises  $\beta$ -1,3-glucanases, acting on  $\beta$ -1,3-glucan, a biopolymer found in plant and fungal cell walls. PR-3, 4, 8, and 11 families comprise plant chitinases belonging to various classes (I-VII). PR-5, 6, 7, 9, and 10 families comprise thaumatin-like proteins, protease-inhibitors, endoproteinases, peroxidases, and ribonuclease-like proteins, respectively (Van Loon, 1999). Proteins such as thionins and ribosome-inactivating proteins are also considered as PR-proteins. While the mode of action of many PR-proteins in plant-defense remains unclear, chitinase, a hydrolytic PR-protein, appears to exert its effect through degradation of the chitin-containing fungal cell wall and release of elicitor molecules. There is a growing body of evidence supporting the notion that higher chitinase activity helps plants resist fungal infection (Grison *et al.*, 1996; Terakawa *et al.*, 1997).

#### **1.2.4 Chitinase**

Chitinases can be classified in two major categories. Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating soluble low molecular mass multimers of *N*-acetylglucosamine (GlcNAc), such as chitotetraose, chitotriose and chitobiose (Sahai and Manocha, 1993). Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29) (Harman *et al.*, 1993), which catalyze the progressive release of chitobiose starting at the nonreducing end of the chitin microfibril; and 1,4- $\beta$ -*N*-acetylglucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases generating monomers of GlcNAc (Sahai and Manocha, 1993) (Fig. 2).

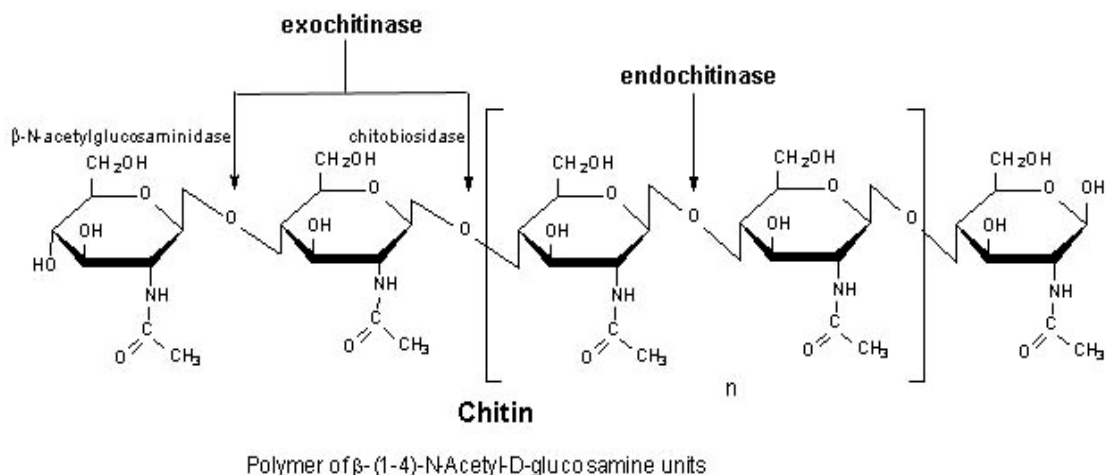


Figure 2. Enzymatic reaction catalyzed by endo- and exochitinase. Both chitinases are required for the degradation of chitin to generate a monomer of chitin or GlcNAc.

Chitinases are present in various organisms (Cohen-Kupiec and Chet, 1998). Depending on the organism of origin, these enzymes have different functions (Cohen-Kupiec and Chet, 1998). Bacterial chitinases are mainly involved in nutrition processes – they degrade chitin, delivering carbon and nitrogen to the cells (Patil *et al.*, 2000). In yeast and various fungi, these enzymes participate in morphogenesis – they take part in remodeling cell wall structure and daughter cell separation, and also in some pathogenesis processes (Shimono *et al.*, 2002). Chitinolytic activity was found in viruses, snails, fish, amphibians, mammals and also in gymnosperms and angiosperms, despite the fact that chitin is not present in these organisms. In plants, chitinases mainly play a role in the defense of the organism against pathogen attack (Hamel and Bellemare, 1995). A number of proteins demonstrating chitinolytic activity were identified in plants (Collinge *et al.*, 1993; Brunner *et al.*, 1998). It is possible to find them in all organs and plant tissues, in both the apoplast and the vacuole. These proteins present a large and diverse group of enzymes; they differ not only in spatial and temporal localization, but above all in their molecule structure and substrate specificity (Collinge *et al.*, 1993; Brunner *et al.*, 1998). Chitinases use two different hydrolytic mechanisms (Table 2) (Iseli *et al.*, 1996). Substrate-

assisted catalysis, characteristic for chitinases belonging to family 18, leads to retention of conformation at the anomeric carbon of the product (Van Aalten *et al.*, 2001). The reaction of hydrolysis carried out by chitinases from family 19, using the mechanism of acid catalysis, inverts the anomeric configuration (Brameld and Goddard, 1998).

Table 2. Differences between the chitinases of glycoside hydrolase family 18 and 19.

Glycosidase family	Class of chitinase	Catalytic mechanism	Intermediate	Anomeric Configuration of product	Inhibitor
18	III and V	substrate-assisted	oxazolinium ion	$\beta$	allosamidin
19	I, II, IV, VI and VII	acidic	oxocarbenium ion	$\alpha$	amidines amidrazones

Multiple chitinase isoforms and gene clusters have been detected in many plants analyzed to date. Plant chitinases are generally small proteins of molecular weight 25-40 kDa, with wide range of isoelectric points (3-10), and post-translational modifications such as glycosylation and prolyl-hydroxylation (Sticher *et al.*, 1992; Colinge *et al.*, 1993; Nielsen *et al.*, 1994). Chitinases generally show wide pH optima (4-9) for activity. Some chitinases, such as a yam class III chitinase, show two pH optima depending on the substrate used (Tsukamoto *et al.*, 1984). This particular chitinase is also stable at 80°C, whereas other plant chitinases show moderate temperature tolerance of up to 60°C. Chitinases contain several disulfide linkages through conserved cysteine residues in their tertiary structure. Crystal structures of Hevamine, a class III chitinase/lysozyme from rubber tree (*Hevea brasiliensis*) (Terwisscha van Scheltinga *et al.*, 1994 and 1995), and class II chitinases from barley (*Hordeum vulgare*) (Hart *et al.*, 1995) and jack bean (*Canavalia ensiformis*) (Hahn *et al.*, 2000) have been determined.

Based on their primary structures, plant chitinases have been classified into seven classes, class I through VII. Different chitinase classes have no apparent correlation to being present in a particular plant species and plant organ or tissue. However, certain chitinase

isoforms are sometimes induced by a particular elicitor. For example, in potato, a class I basic chitinase was strongly induced by ethylene and wounding whereas a class II acidic chitinase was induced by salicylic acid (Buchter *et al.*, 1997). Also, only particular isoforms show antifungal activities and certain isoforms have additional novel functions such as antifreeze activity (Sela-Buurlage *et al.*, 1993; Yeh *et al.*, 2000).

#### **1.2.4.1 Class I and II Chitinases**

Class I and II chitinases belong to the PR-3 family of pathogenesis-related proteins with the tobacco chitinases as the prototypical members. All members of the PR-3 family belong to family 19 of glycosyl-hydrolases, which catalyzes sugar hydrolysis with the inversion of configuration at the anomeric carbon.

Class I chitinases have an N-terminal cysteine-rich chitin-binding domain (CBD) that is homologous to hevein, a chitin-binding lectin from the rubber tree. CBD is separated from the catalytic domain by a proline- and glycine-rich hinge or spacer region, variable both in size and composition. For a tobacco class I chitinase, the deletion of CBD and the spacer region singly or in combination reduces the hydrolytic activity by 50%, whereas antifungal activity is reduced by 80% (Surarez *et al.*, 2001). Class I chitinases are synthesized as propeptides, directed to the secretory pathway and eventually directed to the vacuole by a short C-terminal signal sequence. Deletion of this C-terminal signal peptide redirects a class I chitinase to the apoplast while retaining the enzymatic activity (Grover *et al.*, 2001). Addition of this six amino acid signal (GLLVDTM) to an unrelated, usually secreted class III chitinase redirects this protein to the vacuole in tobacco (Neuhaus *et al.*, 1991). Class II chitinases are similar to class I but they lack the N-terminal CBD and the hinge region. In the catalytic domain they sometimes have a deletion as compared to class I chitinases. Class II chitinases are usually secreted to the apoplast as they lack the C-terminal vacuolar-targeting signal.

#### **1.2.4.2 Class III Chitinases**



Class III chitinases are unique in having a structure unrelated to any other class of plant chitinases. These chitinases belong to the PR-8 family and family 18 of glycosyl-hydrolases. Members of family 18 glycosyl-hydrolases catalyze sugar hydrolysis with the retention of configuration at the anomeric carbon. Class III chitinases generally have lysozyme activity and appear to be more closely related to the bacterial chitinases. A class III chitinase enzyme was purified from the seeds of *Benincasa hispida* (white gourd or wintermelon), a Chinese medicinal plant (Shih *et al.*, 2001). The enzyme is a 29 kDa protein with 27 amino acid N-terminal signal peptide (as deduced from N-terminal amino acid and genomic DNA sequences). The length of signal peptides and molecular weights are similar in other class III chitinases such as a pumpkin chitinase of 29 kDa with a 27-amino acid signal peptide (Kim *et al.*, 1999) and a sugar beet chitinase of 29 kDa with a 25-amino acid signal peptide (Nielsen *et al.*, 1993). The pumpkin class III chitinase was purified by chitin affinity chromatography, which showed strong retention of this protein. This is unusual for a class III chitinase since they do not have a chitin-binding domain. Class III chitinases show a wide range of isoelectric points, activity over a wide range of pH, and temperature stability at 60-70°C. The *B. hispida* chitinase has a pH optimum of 2 and retains approximately 50% activity at pH 8 (Shih *et al.*, 2001). Some class III chitinases, such as a yam enzyme, show two pH optima and heat stability at 80°C (Tsukamoto *et al.*, 1984). There is no post-translational modification reported for class III enzymes analyzed to date.

The three-dimensional structure of Hevamine, a chitinase/lysozyme from rubber tree, and its complex with the inhibitor allosamidin has been determined (Terwisscha van Scheltinga *et al.*, 1994, 1995, and 1996). The structure is an  $(\alpha/\beta)_8$  barrel similar to the bacterial family 18 glycosyl-hydrolases without significant sequence identity. These enzymes contain a substrate-binding cleft located at the C-terminal end of the  $\beta$ -strand in the barrel structure. The active site residue Glu127 of Hevamine is required for activity whereas Asp125 allows a wider pH range for catalysis. Hevamine requires chitopentose as minimum substrate. The catalysis occurs by retention of configuration at the anomeric carbon, and is substrate assisted. Generally class III chitinases also act as lysozymes. However, Bokma *et al.* (1997) showed that Hevamine hydrolyzes the glycosidic bond of the peptidoglycan between C-1 of *N*-acetylglucosamine and C-4 of *N*-acetylmuramic acid as opposed to lysozyme which catalyzes the hydrolysis of

peptidoglycan by cleavage of C-1 of *N*-acetylmuramic acid and C-4 of *N*-acetylglucosamine. Therefore, Hevamine and possibly other class III plant chitinases, are not strictly lysozymes. Some class III chitinases such as a sugar beet enzyme do not exhibit lysozyme activity (Nielsen *et al.*, 1993). A recent study shows kinetic constants of Hevamine by an improved assay method (Bokma *et al.*, 2000). The  $k_m$  and  $k_{cat}$  for *N*-acetylglucosamine-pentamer (GlcNAc)<sub>5</sub> and (GlcNAc)<sub>6</sub> were measured to be 13.8  $\mu$ M, 0.355/s and 3.2  $\mu$ M, 1.0/s, respectively. Allosamodin was found to be a competitive inhibitor with a  $K_i$  of 3.1  $\mu$ M.

#### 1.2.4.3 Class IV, V, VI, and VII Chitinases

Class IV, V, VI, and VII chitinases belong to the PR-3 family of pathogenesis-related proteins. Class IV chitinases, also identified mainly in dicotyledons, comprise a group of extracellular chitinase that share 41-47% sequence identity with class I chitinases in the catalytic domain and also contain cysteine-rich regions resembling chitin-binding domains, however, class IV chitinases are smaller because of deletions in both domains (Collinge *et al.*, 1993). Class V, VI, and VII chitinases have unique structures and are represented by one example each.

#### 1.2.4.4 Functions of plant chitinases

Plant chitinases have been known to be induced upon fungal infection and inhibit fungal growth in vitro, criteria initially used to implicate chitinases in plant defense (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988a and 1988b). The induction of chitinases was initially shown in pea plants infected with *Fusarium solani*, or challenged with other biotic or abiotic stress factors (Mauch *et al.*, 1988a). Protein extracts made from infected pea plants were able to inhibit growth of 15 of the 18 fungal species tested in vitro. Purified chitinase inhibited growth of only one fungal species whereas a combination of chitinase and another PR-protein,  $\beta$ -1,3-glucanase, inhibited the growth of all fungi tested showing a synergism in activities (Mauch *et al.*, 1988b). Subsequently, a number of studies verified these results in tobacco (Yun *et al.*, 1996), grapes (Derckel *et al.*, 1998), chickpea (Giri *et al.*, 1998), rice (Velazhahan *et al.*, 2000)

and other plants. The current view is that only specific isoforms are induced in response to a particular pathogen and only certain isoforms are able to inhibit specific fungi (Ji *et al.*, 2000; Sela-Buurlage *et al.*, 1993). For example, a class I chitinase from tobacco showed antifungal activity against *Fusarium solani*, but class II chitinases showed only a slight growth inhibitory effect when used with high concentrations of a  $\beta$ -1,3-glucanase (Jach *et al.*, 1995). Constitutive chitinase expression is higher and induction is stronger and quicker in the resistant varieties as compared to the susceptible varieties in some phyto-pathogen systems such as sugar beet (Nielsen *et al.*, 1993), wheat (Anguelova *et al.*, 2001) and tomato (Lawrence *et al.*, 2000). However, contrary data also exist showing no difference in the timing, induction, or intensity of PR-gene expression in susceptible and resistant cultivars, for example in cotton (McFadden *et al.*, 2001). Quick response in the resistant cultivars might affect the cell wall of germinating fungal spores, releasing elicitors leading to the expression of PR-genes and disease resistance. It was shown for *Alternaria solani* that a basic chitinase was only active on the germinating spores and not on the mature fungal cell wall for generation of elicitor molecules able to induce disease resistance (Lawrence *et al.*, 2000). The difference may be in the length of these fragments as it is known that 4-5 *N*-acetylglucosamine residues are necessary for defense elicitation. In an interesting study in potato, it was shown that chitinase and osmotin-like proteins interact with actin filaments (Takemoto, *et al.* 1997). It has been known that actin filaments show cytoplasmic aggregation at the site of fungal penetration. It was hypothesized that PR-proteins are translocated to the site of fungal penetration for effective blocking of the pathogen and/or for the release of elicitors.

Class IV chitinases are similar to class I, but they are smaller in size due to four deletions. A class IV chitinase was found to rescue a somatic embryo mutant of carrot unable to differentiate (De Jonge, 1992). It was found that a sugar beet class IV chitinase was not able to substitute, however a class I chitinase could. A possible substrate for this activity was found to be the arabinogalactan proteins (AGP) functioning in somatic embryogenesis in carrot (Van Hengel *et al.*, 2001). AGPs have a complex structure with a  $\beta$ -1,3-galactan backbone and highly heterogeneous side chains. These proteins are produced in somatic embryos and have half-lives of 10-15 min. Thus, it is thought that chitinases might act on these proteins for degradation, either destroying or generating signaling molecules. A specific chitinase isoform in *Pinus caribaea* was shown to interact with AGP present in embryogenic tissue but not with AGP from

non-embryogenic calli (Domon *et al.*, 2000). In Arabidopsis, a class IV chitinase was shown to be expressed only in seedpods and the promoter region fused with GUS showed expression in Arabidopsis and tobacco embryos (Gerhardt *et al.*, 1997). Albeit the fact that exact role of chitinases in embryogenesis is not clear, there is ample evidence that chitinases are important in this process and the nature of physiological chitinase substrates are beginning to emerge. In *Cichorium*, chitinase as well as  $\beta$ -1,3-glucanase and osmotin-like protein are synthesized at higher levels and secreted in the culture medium of somatic embryogenesis-competent cells (Helleboid *et al.*, 2000). It would be interesting to know if these enzymes exist in oligomeric complexes functioning in embryogenesis. Chitinases along with other PR-proteins have been implicated in freeze tolerance (see below) where they do form oligomeric complexes.

A class III chitinase gene from *Medicago truncatula* was shown to be specifically expressed in root cortical cells containing developing or mature arbuscules (Bonanomi *et al.*, 2001). This gene was not induced by phytopathogenic fungi and the expression was limited to cells harboring the arbuscules. Investigators hypothesize that this gene functions in suppression of the defense response in these cells by degrading fungal chitin elicitors. The hypothesis was substantiated by the fact that H<sub>2</sub>O<sub>2</sub> production, a hallmark of defense response, was absent from these cells (Salzer *et al.*, 1999) and that the other defense related proteins such as  $\beta$ -1,3-glucanases were present at low levels in these cells (Blee and Anderson, 2000). Another class III chitinase from *Sesbania rostrata* lacking the active site glutamic acid residue and devoid of chitinase activity was induced during nodule development. The active site glutamate is mutated to a lysine residue in this protein and therefore renders this protein a chitin binding lectin. This gene was induced within 4 h of inoculation with nodulation bacteria, and the protein was localized to the outer cell layer of nodules. Investigators suggest a role for this protein in Nod factor binding which would protect, concentrate, or facilitate its interaction with a receptor protein. Increased chitinase activity and induction of new isoforms have been observed in other plants, such as soybean, in symbiosis with nodulation bacteria (Xie *et al.*, 1999). Evidence for other physiological functions of chitinases in flowering, reproduction, germination, and plant growth are also beginning to emerge.

### 1.2.5 Chitin

Chitin can be found in a wide range of organisms. Chitin forms a polymer that allows it to function as a load-bearing component of the skeletal materials of many lower animals, for example the exoskeleton of arthropods (including insects and crustaceans). Chitin is also found in coelenterates, nematodes, protozoa, mollusks and the cell walls of many fungi. Chitin is almost always associated with other structural components like protein and glucans (Gooday, 1990). In the cuticle of crustaceans, chitin is also associated with calcium carbonate and pigments forming a complex armor-like matrix (Shimahara *et al.*, 1984). There are varying degrees of acetylation of chitin occurring in nature, providing a continuum from fully acetylated to completely deacetylated. Deacetylation may be involved in interaction with proteins (Blackwell, 1988).

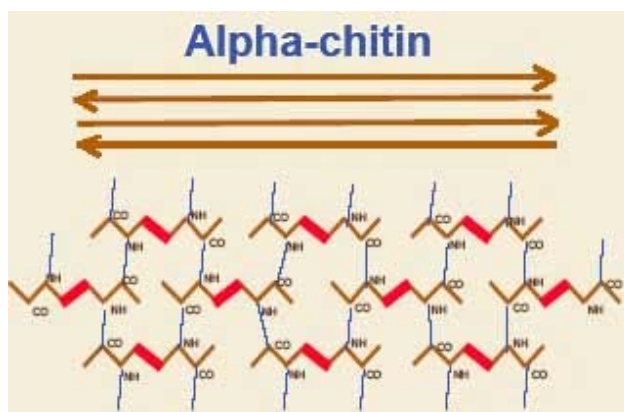


Figure 3. Crystalline structural molecule of  $\alpha$ -chitin.

Completely acetylated chitin can be found in the spines of certain marine diatoms. This highly crystalline material lacks a protein matrix. Chitin has an obvious structural similarity to cellulose: Chitin and cellulose chains have the same basic 21 helical conformation, in which two monomer residues repeat every 10.3-10.4 Å (Blackwell, 1988). Under the electron microscope, chitin has a fibrous, semi-crystalline morphology, with amorphous, less ordered

edges (Blackwell, 1988). If more than one in six monomers in chitin is *D*-glucosamine, the polymer would be considered a form of chitosan (Blackwell, 1988). Chitin occurs in two, possibly three, polymorphic forms based on crystalline packing : the  $\alpha$ -form (crustaceans, insects, fungi) is more common than the  $\beta$ -form, which is found exclusively in structures of aquatic organisms (e.g. squid pens, spines of polychaete *Aphrodite*, *Pogonophora* tubes, protective tubes of deep sea *vestimentiferan* worms (*Lamellibrachia satsuma*) located on hydrothermal vents on the east Pacific ridge). The possible third form,  $\gamma$ -chitin, has been suggested to compose the stomach lining of *Loligo* (Blackwell, 1988).  $\alpha$ -chitin (Figure 3) has an anti-parallel arrangement of polymer chains, while the configuration of  $\beta$ -chitin is parallel. This can be distinguished by infrared spectroscopy and X-ray diffraction spectra.  $\alpha$ -chitin forms hydrogen bonds between sheets,  $\beta$ -chitin does not.  $\beta$ -chitin swells readily in water, where it forms a series of crystalline hydrate structures. In the parallel chain structure, all chains have the same directionality. In the swollen state, water molecules are intercalated between the stacks of chains.  $\beta$ -chitin can be irreversibly converted to  $\alpha$ -chitin by treatment with acid (Blackwell, 1988). In this case, bundles of chains oriented in one direction meld with bundles parallel in the other direction. There is a strong similarity of chitin to cellulose: Native cellulose (Cellulose I) has parallel chains, and does not swell in water, but in alkali. Swelling and a regeneration from solution leads to the more stable cellulose II form with anti-parallel chain arrangement (Blackwell, 1988).

### 1.2.6 Measurement of endo- and exochitinase activities

For the estimation of randomly hydrolyzing endo-chitinase and exo-hydrolytic *N*-acetylglucosaminidase activities specific soluble and insoluble substrates are available. The most sensitive methods use  $^3\text{H}$ -chitin and fluorogenic substrates. Although radioactivity measurements are a sensitive method, the latter gives reproducible results. Soluble- and insoluble-chitin substrates labeled with dyes are also used to measure chitinase activity by estimating released dye spectrophotometrically. A carboxymethyl-substituted soluble chitin covalently linked with Remazol Brilliant Violet 5R is suitable for the screening of chitinolytic microorganisms and for detection of chitinase activity by plate-clearing assay. The method is essentially based on the precipitability of the non-hydrolyzed chitin by HCl. The absorbance in

the supernatant (containing lower oligomers) is used to measure the enzyme activity. However, exact quantitative measurement of chitinase activity is difficult because of nature and variable dye contents of the substrate.

From above, only fluorogenic substrates can determine difference between endo- and exochitinase activity. 4-Methylumbelliferyl-*N-N'-N''*-acetyl- $\beta$ -D-glucosaminide (4-MU- $\beta$ -(GlcNAc)<sub>3</sub>) and 4-Methylumbelliferyl-*N*-acetyl- $\beta$ -D-glucosaminide (4-MU- $\beta$ -GlcNAc), which are specific substrates, are used for assaying both chitinases, respectively (Fig. 4). These substrates are labeled with fluorophore (4-MU) tagging to the low molecular weight small molecules, therefore, whenever these specific substrates are hydrolyzed by endo- or exochitinase, it generates fluorophore.

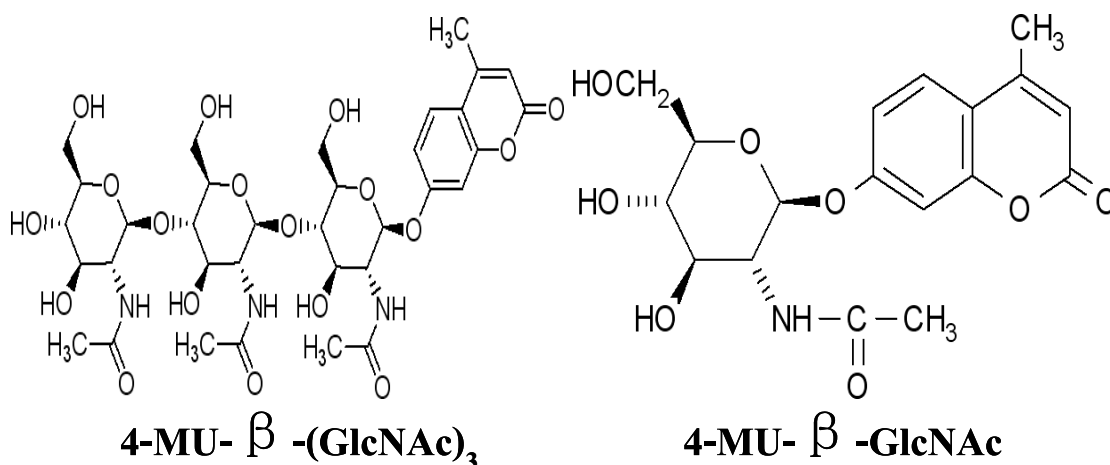


Figure 4. Specific substrates for assaying endo- and exochitinase activity. 4-Methylumbelliferyl-*N-N'-N''*-acetyl- $\beta$ -D-glucosaminide (4-MU- $\beta$ -(GlcNAc)<sub>3</sub>) and 4-Methylumbelliferyl-*N*-acetyl- $\beta$ -D-glucosaminide (4-MU- $\beta$ -GlcNAc) for assaying endo- and exochitinase, respectively.

### 1.3 Objective

In order to purify and characterize of endo- and exochitinase from BFM of *Hevea latex*.