Chapter 1

Introduction

Introduction

There are as many as 12,500 species, belonging to 900 genera, of latex-producing plants. *Hevea brasiliensis* is a tropical rubber tree belonging to the family Euphorbiaceae. It is extensively cultivated in Southeast Asia for the production of natural rubber. Several biological functions of plant latex had been suggested including a form of stored food, excretory waste deposits as well as plant defense. Invaders that attack latex-producing plants are normally faced with a combination of physical and chemical defenses provided by the latex. In the case of injuries, protective barrier resulted from latex drying will prevent entry of fungi and bacteria. Various exuded latices are known to contain proteins involved in plant defense such as glycosidases (Giordani and Lafon, 1993), proteases (Lynn and Clevette-Radford, 1986), acid phosphatases (Lynn and Clevette-Radford, 1987a), amylases (Lynn and Clevette-Radford, 1987a), chitinases (Jekel *et al*., 1991), hevein (Van Parijs *et al*., 1991), proteinase inhibitors (Archer, 1983, Lin and Lu, 1994), β-1,3-glucanase (Chye and Cheung, 1995) and several pathogenesis related (PR) proteins (Stintzi *et al*., 1993).

For the *Hevea* latex, rubber particles (RP) make up 30-45% of the whole latex volume. The two major RP proteins of 14.6 and 24 kDa had been classified as proteolipids and demonstrated in washed RP obtained from ultracentrifuged fresh latex (Hasma, 1987; Wititsuwannakul *et al*., 2004). The genes encoding these two major proteins were found to be most abundant in the analysis of the *Hevea* laticifer transcriptome, comprising 29% of the total ESTs (Ko *et al*., 2003). The second most abundant transcripts were defense- or stress-related genes, suggesting that defense is one of the functions of laticifers (Ko *et al*., 2003). A deduced amino acid sequence obtained from a full-length cDNA encoding the 24 kDa was shown to have a high homology to that of the 14.6 kDa protein (Oh *et al*., 1999). At present, the biological

function of the RP proteins remains obscure. Although, the involvement of the RP proteins in rubber biosynthesis had been suggested (Dennis and Light, 1989; Oh *et al*., 1999), a later study in micromorphology and RP protein characterization has indicated that the core proteins were not necessary for the rubber biosynthesis (Singh *et al*., 2002). However, the studies related to antifungal action of *H. brasiliensis* latex (Giordani *et al*., 1999 and Giordani *et al*., 2002) suggested possible association of antifungal factor with the rubber particles in plant defense. The *Hevea* latex contains numerous other non-rubber constituents including proteins which are presently overlooked and discarded as waste in the rubber industry. In our laboratory had already purified and characterized several biological-active molecules present in non-rubber constituents from latex such as β-1,3-glucanase isozymes (Churngchow *et al*., 1995), a polyphenol oxidase (Wititsuwannakul *et al*., 2002), NAD(P)H quinone reductase (Chareonthiphakorn *et al*., 2002) and a protease inhibitor (Sritanyarat *et al*., 2006). These proteins involved in defense mechanism of rubber tree. Since the rubber trees are tapped or wounded almost everyday for latex collection it has been suggested that the rubber tree must be well equipped with antimicrobial compounds in order to protect itself from any invaders. Previous report (Van Parijs *et al*., 1991) showed that one substance with antifungal activity was hevein, a major protein component of the B-serum derived from the bottom fraction of centrifuged fresh latex. Hevein was active against several phytopathogenic fungi included *Botrytis cinerea, Fusarium culmorum, Fusarium oxysporum, Phycomyces blakesleeanus, Pyrenophora tritici-repentis, Pyricularia oryzae, Septoria nodorum* and *Trichoderma hamatum* (Van Parijs *et al*., 1991). Hence, the B-serum normally treated as waste may be a great biotechnological resource due to the large quantity produced and its potential for possible use as an antimicrobial agent, a value added product. In this study we determined the antimicrobial activity of freshly prepared latex serum against various potentially pathogenic oral microorganisms, identify the active compound that possesses antimicrobial activity. Enhancing effects of proteins from C-serum and rubber particles on antifungal activity of effective compounds, amphotericin B, were also reported.

Review of literatures

1. Para rubber tree (*Hevea brasiliensis*)

There are very many plants capable of producing latex. They belong to several different families but are mainly of the Dicotyledon. Latex is the fluid, generally milky in appearance, which exudes from these plants after the slightest wound. The latex is produced and stored in parenchyma cells or, more generally found, in the tube structures known as laticifers. Of some 12,500 species of laticiferous plants, approximately 7,000 produce polyisoprene. In most cases the polyisoprene is present with mixed resins, making the latex difficult to use when the content of the latter is high. Only a limited number of rubber-producing plants can be cultivated and have economic importance. Amongst them, *Hevea* (*Hevea brasiliensis*) which grows in the hot humid intertropical regions was revealed to be the best rubber producer. The secondary laticiferous vessels of the trunk are exploited by tapping the bark. The tree releases a large amount of latex at each tapping and can be tapped for a few decades (de Faÿ and Jacob, 1989). Para rubber trees (*Hevea brasiliensis*) are classified in the division Magnoliophyta, class Magnoliopsida, order Euphorbiales, family Euphorbiaceae (the spurge family).

1.1 Laticifers (latex vessels) in *Hevea*

The type of laticifers is characteristic of the plant species. The complex types of laticiferous system in plant are classified as articulated and non-articulated according to the mode of origin. Both are tubular structures and described as latex vessels. Articulated laticifers are compound in origin comprising a series of cells, either remaining blunt or becoming continuous tubular structures due to partial or complete dissolution of end walls. Depending on the presence or absence of lateral connections they are further categorized as anastomosing and non-anastomosing respectively. The non-articulated laticifers are simpler in structure. They may remain as single unbranched cells or branched structures extending throughout the shoot and root system (Premakumari and Panikka, 1992).

Hevea laticifers are present in all organs except wood although occasional presence of plugged vessels having latex has been found. Xiuqian (1987) reported that the primary laticifers found in the seed (cotyledon), leaf, flower, root and young stem are non-articulated which show intrusive growth into the intercellular spaces of the primary phloem and

cortex (Xiuqian, 1987). The commercially exploited part of *Hevea* for its latex is the main trunk, where the secondary laticifers are distributed in the bark. The laticifers form concentric rings almost alternating with layer of other phloem tissue. After the formation of cambium, this type of laticifers is periodically differentiated by the activity of vascular cambium (in the secondary phloem of the tree) by a method similar to the way the vessels are initiated and hence called "latex vessels". The secondary laticifers are articulated and belong to the anastomosing, coenocytic type. They are anastomosed as a result of partial hydrolysis of adjacent walls, and thus from a tube-like network or paracirculatory system through the plants. Inside the network, the laticiferous vessels run up, in a slightly inclined path to the long axis of the tree, in counterclockwise direction. The degree of inclination varies from 2-7° depending on clone (Premakumari and Panikka, 1992; de Faÿ and Jacob, 1989). The structure of mature *Hevea* bark is showed in Fig. 1. In the soft bark region the laticifers are continuous, while most of them in the hard bark region are discontinuous and hence non-functional. In the hard bark region the latex vessels become crushed and broken due to the push and pull exerted by the surrounding tissue, especially the stone cell, during radial growth (Premakumari and Panikka, 1992). The truly productive tissue in bark is thus the inner soft bark which lies between the cambium and the hard bark.

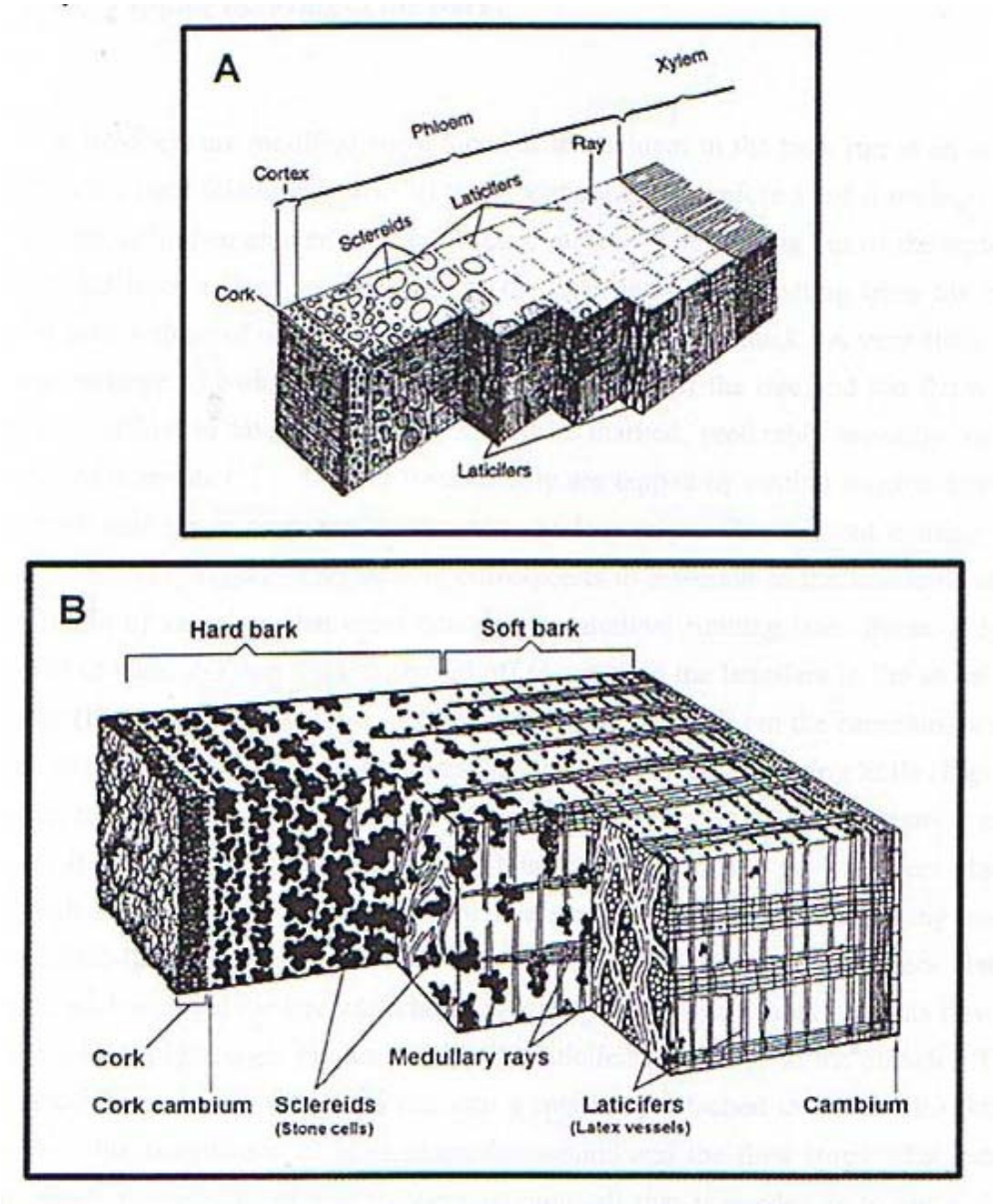


Fig. 1 Mature bark of *Hevea brasiliensis* (a three dimensional view).

The figure 1 A and B depict arrangement of laticifers in the secondary phloem. Cortex cells produce the cork cambium and then cork cambium produces cork. Medullary rays are bundles of cells that radiate from the center of a tree like the spokes of a wheel (they are most easily seen in the cross section of a tree trunk). They store food and transport it horizontally within the tree (Vicher, 1923; Fahn, 1990; Premakumari and Panikka, 1992).

1.2 Tapping (Shaving of the Bark)

The laticifers are modified sieve tubes of the phloem in the bark that run at an angle of 2-7° to the right (counterclockwise) of the vertical and therefore a cut from high left to low right will open greater number of latex vessels. The tapping cut should have a slope of about 30° to the horizontal. Rubber trees are usually tapped by cutting a spiral groove in the bark halfway or more around the stem, as deep as possible but without injuring the tree's cambium growth. The tapping corresponds to a wound in the tree bark, only a thin slice of bark, 2-3 mm thick is shaved off to cut open the laticifers in the so called soft bark (Fig. 2). The groove cuts almost into the cambium but the cambium is not injured in this process. The tapper needs great skill with special tapping knife as the tree is easily damaged if the bark is cut too deep. Latex which is the cytoplasm of the specialized cells known as laticifers (latex vessels), gushes out of the tree when laticifers are severed during tapping. This flow is due to the very high turgor pressure inside the laticifers compared to the outside. The latex is collected by allowing it to run into a small cup attached to the trunk. After some time the coagulation of latex plugs this wound and the flow stops. To restart flow from a tapping cut in a subsequent tapping, all that is needed is to cut a thin shaving of the bark along with which the plugs of coagulated latex are also removed and release the latex upward into the new cut (Premakumari and Panikka, 1992; John, 1992; Kush, 1994).

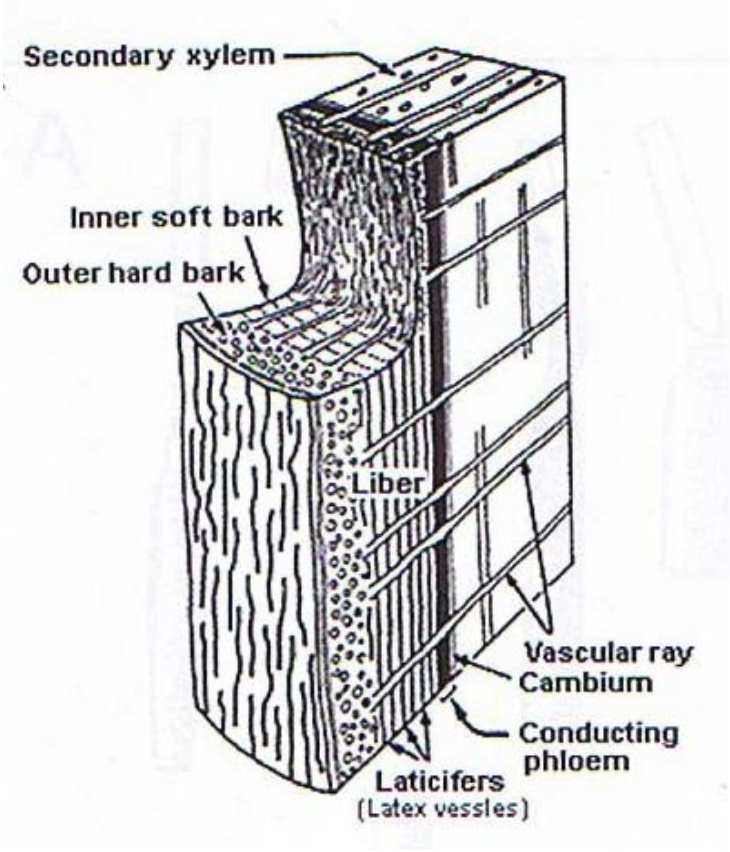


Fig. 2 General organization of *Hevea brasiliensis* bark at tapping cut level.

1.3 Function and constituents of laticifers

The laticifers system is developed by the dissolution of the cross walls of cells which possess all the usual constituents of cytoplasmic organelles such as nucleus, ribosome, endoplasmic reticulum, Golgi apparatus, mitochondria proplastids, and which very early differentiate characteristic organelles (lutoids and special plastids such as Frey-Wyssling particles) and rubber particles. The nucleus of a laticifer initial retains central position and during the course of ontogeny the cytoplasm forms a peripheral layer where numerous nuclei are embedded in mature latex vessels laticifers. As the vessels mature the lumen becomes increasingly occupied by numerous rubber particles so that the original cytoplasm and its organelles become confined to the side of the vessels. According to the laticiferous system these coenocytic laticifers are multinucleate; nuclei and mitochondria are located parietally and most are not expelled during tapping. Wounding stimulates metabolic activity in the phloem, and the ribosome, mitochondria, enzymes, and rubber particles lost in the outflow are quickly regenerated (de Faÿ and Jacob, 1989; John, 1992; Kozlowski and Pallardy, 1997).

A number of functions have been ascribed to laticifers. It has been suggested that they form a secretory tissue, but the advantage to the plant of the latex stored is doubtful. The enormous amount of energy involved in the synthesis of rubber or of other terpenes does not seem to be recoverable. Possibly the most popular justification of laticifer presence has been a presumed protective role. The credibility of the proposal that latex offers protection from attacks by fungi and insect pests has been increased by the characterization in *Hevea brasiliensis* latex of the chitin binding protein, hevein, and of chitinase activity, both of which are found in the lutoid organelle. However, such protective proteins are not peculiar to latices (hevein shows considerable homology to wheat germ agglutinin) and even the presence of rubber does not seem to offer protection by deterring herbivore feeding, insect attack or the incidence of fungal disease. In conclusion, it must be admitted that during the twentieth century little advance has been achieved in our understanding of the biological functions or advantages of laticifers to the plant (Farrell, 1991).

1.4 Major constituents of latex

Fresh *Hevea* latex is a polydisperse system in which negatively charged particles of various types are suspended in an ambient serum (C-serum). The two main particulate phases

contained in *Hevea* latex are rubber particles constituting 20-45% by volume of fresh latex and lutoid particles (10-20%). The third type, on a quantum basis, is the Frey-Wyssling complexes (1-3%) (d' Auzac and Jacob, 1989; Premakumari and Panikka, 1992).

The fresh latex can be separated into 4 main fractions after subjected on high-speed centrifugation (Fig. 3). The top fraction consists almost entirely of rubbers which lie above the orange-colored layer containing Frey-Wyssling particles, the middle zone is colorless which made up of the aqueous phase of the latex (C-serum), and yellowish bottom fraction consists mainly of membrane organelles called lutoid particles. Details of these fractions were described in the following part.

1.4.1 Rubber Particles

Rubber hydrocarbon is the major component of *Hevea* latex. The dry rubber content of latex may vary from 25 to 45%. The number average molecular weight ranges from 200 to 600 kDa. The rubber molecules are found as particles in the latex. The particles consist mainly of rubber (90%) associated with lipophilic molecules, mainly lipids and proteins, forming the film enclosing rubber particles (Ho *et al*., 1976). This film carries negative charges and is responsible for the stability of rubber particles suspended in aqueous serum. The particles size ranges from 5 nm to 3 μm with spherical shape. They also show plasticity as having polygonal shape in mature laticifers where the particles are numerous. The size distribution as determined by the electron microscope showed maximum distribution of 0.1 μm particles (Gomez and Moir, 1979). The 0.1 μm particles may contain several hundred of rubber molecules. Molecular weight analyses using gel permeation chromatography showed the bimodal distribution of rubber for low and high molecular weights, with the average values of 100-200 kDa and 1,000-2,500 kDa, respectively (Subramaniam, 1976). Three distinct zones with RP have been separated by means of ultracentrifugation of fresh latex (Moir, 1959). The largest rubber particles are found in the uppermost Moir's zone 1 (Southorn 1969; Yeang *et al*., 1995). Due to a large area occupied by zone 1, its RP diameter of 1.55 μm, as well as those as large as 3 μm to 6 μm, had been reported (Dickenson, 1969). In Moir's zone 2, the size of RP varies from 0.05 to 0.25 μm and those in Moir's zone 3 are of a lower average size (0.035-0.2 μm) (Hamzah and Gomez, 1982). The other main component of rubber particles is the enclosing membrane consisting of lipids, proteins, and

enzymes. These components contribute colloidal charge to the rubber particle and their stability in the latex (Wititsuwannakul and Wititsuwannakul, 2001).

1.4.1.1 Rubber particles membrane

The rubber particles are commonly found in association with lipids, which is thought to be of membrane nature. Microscopically, the particles appear to have a uniform structure, with the rubber molecules enclosed by a thin film (Southorn, 1961). When examined by electron microscopy, the rubber particles appear homogeneous, and have a uniform internal structure, but are surrounded by a film that is more opaque than the polyisoprenes contained inside (Lau *et al*., 1986). Analyses of the nature of the film enclosing the rubber particles show the presence of phospholipids and proteins, together with neutral lipids similar to the membrane structure. (d' Auzac and Jacob, 1989), and the composition of the membrane of rubber particles separated and purified by ultracentrifugation analyzed. The membrane components comprise lipids, proteins, enzymes, and charges (Wititsuwannakul and Wititsuwannakul, 2001).

1.4.1.1.1 Rubber particle lipids

Analyses of rubber particles purified by ultracentrifugation showed them to contain up to 3.2% total lipids, of which ~2.1% are neutral lipids expressed as rubber weight (Ho *et al*., 1976). Separation of neutral lipid showed it is composed of at least 14 different substances. Triglycerides were the most abundant, accounting for almost 45% of the neutral lipids, while sterols, sterol esters and fatty acid esters constituted about 40%. Other neutral lipids present in trace amount were diglycerides, monoglycerides, and free fatty acids. In addition, tocotrienols and some phenolic substances were also found to be associated with rubber particles (Ho *et al*., 1976). Phospholipids are important components of the rubber particles. Analyses of the phospholipids consistently showed three spots on the thin-layer chromatography (TLC) separation: these were identified as a considerable quantity of phosphatidylcholine and smaller quantities of phosphatidylethanolamine and phosphatidylglycerol. Phosphatidic acid was found to be predominant in the membrane of lutoids, but was not detected on the rubber particles (Dupont *et al*., 1976). In addition, the presence of sphingolipids and glycolipids has also been reported. The stability of rubber particles suspension in latex is dependent on the negative charges film of proteins and phospholipids (Philpott and Wesgarth, 1953).

1.4.1.1.2 Rubber particle proteins

Proteins are found as indigenous components of the film enclosing the rubber particle. Together with lipids, these proteins form the membrane of particles which contribute to their stability. The pI of these proteins ranges from 3.0 to 5.0 which is characteristic for surface proteins. In an electric field, particles will move toward the anode, indicating that they have net negative charge on the surface (Verhaar, 1959). Anionic soaps do not affect the particles' colloidal stability, but cationic soaps cause flocculation, probably due to neutralization of the surface charge. These proteins can be considered as intrinsic or peripheral, depending on their binding and affinity with rubber particles. One of the most plentiful proteins in latex is α-globulin with pI of 4.5. It was found both in the cytosol and adsorbed onto the particles' surface, and might contribute to their colloidal stability in latex (Archer *et al*., 1963a). A protein group of hydrophobic nature was also found in rubber particles, and proteolipids have been isolated and characterized (Hasma, 1987). This protein was suggested to be a component of the polar lipid backbone that forms part of the membrane of rubber particles. The protein content of rubber particles was recently refined for a more accurate quantitative analysis (Yeang *et al*., 1995) in light of the concern over rubber protein allergens. Other proteins with enzyme activity have also been described. One interesting and well-characterized enzyme is rubber transferase, which was first detected on the washed rubber particle surface (Audley and Archer, 1988). This enzyme is involved in the synthesis and formation of rubber molecules on the particles' surface, and was found to be distributed between the cytosol and rubber particles, similar to the case of α-globulin. It has been isolated and purified from latex C-serum, but was active only when adsorbed onto particles for the chain elongation process of rubber molecules (Light and Dennis, 1989). Another important protein found to be actively involved in rubber synthesis was a 14 kDa protein referred to as rubber elongation factor (REF). The amino acid sequence of REF has been determined, and the molecular cloning of the *REF* gene also carried out (Attanyaka *et al*., 1991). More recently, molecular cloning of a protein that is tightly bound onto the small rubber particles has been reported (Oh *et al*., 1999). The cloned cDNA encodes a 24 kDa protein which is tightly bound onto the rubber particles. This protein was suggested to be active in the synthesis of rubber, together with the REF. The 24 kDa protein has been reported previously as a potent latex allergen, and is always found together with REF, bound so tightly to particles that they cannot be

removed even by extensive washing. In addition to these tightly bound and well-characterized proteins, some peripheral proteins have also been described, though their function is unknown (Wititsuwannakul and Wititsuwannakul, 2001).

1.4.1.1.3 Rubber particle enzymes

The formation of rubber molecules, at least in the terms of the elongation steps, occurs at the particles' surface (Archer *et al*., 1982), and rubber transferase is the enzyme responsible for this process. This enzyme was also found in the C-serum, and is most likely distributed between the two fractions (McMullen and McSweeney, 1966). It has been isolated from the C-serum and purified for enzyme characterization (Archer and Audley, 1967). The enzyme was without activity in the absence of washed rubber particles, remaining inactive while not adsorbed onto the particles, even when the latter have been purified by gel filtration and washed repeatedly. The reaction catalyzed by rubber transferase appears to be essentially chain extension or elongation of the pre-existing rubber molecule, although a role in the formation of new rubber molecules has also been suggested (Lynen, 1969).

1.4.1.1.4 Rubber particle charges

The colloidal stability of latex is attributed to the presence of surface charges on the rubber particles. The film or membrane surrounding the particles provide them with a negative charge, as shown by surface potential or zeta potential (Southorn and Yip, 1968). The particle membrane composition of proteins, phospholipids, and other substances has been described (Wititsuwannakul and Wititsuwannakul, 2001), and shown to be of a negatively charged nature (Ho et al., 1976). The reduction of phospholipid content of a cloned latex known for its instability has also been noted, and this observation was subsequently extended to show that the lipid content of rubber particles correlated positively with colloidal stability of the latex (Sherief and Sethuraj, 1978). The colloidal stability was reduced by magnesium released from the damaged or ruptured lutoids (Philpott and Wesgarth, 1953) as the surface charges were neutralized. The effect of inorganic cations was investigated in relation to flocculation of the rubber particles and to plugging of the latex vessels, with a negative effect on latex flow (Yip and Gomez, 1984).

1.4.2. Lutoid particles and B-serum

The lutoid particle (so called because of the incorrect original attribution of the yellow color of the fraction) is the vacuolar tonoplast of the latex and is found in the sediment or bottom fraction obtained on centrifuging latex, in addition to some Frey-Wyssling particles that actually cause the yellow color. Lutoid particles are the most numerous of the larger organelles in the latex next to the rubber particle, since they form 10-20% by volume of fresh latex, whereas rubber particles form 30-45%. This highly osmosensitive organelle are membrane-bound vesicles, which are fairly spherical in shape with 0.5-3.0 μm in diameter, bounded by a semi-permeable membrane about 8 nm thick, consists mainly of phosphatidic acid in which unsaturated and saturated fatty acids are present in equal proportions (Webster and Baulkwil, 1989; Dupont *et al*., 1976)

The lutoids have a liquid content called B-serum. The B-serum has a pH of about 5.5 which consists of an acid serum enriched with divalent cations (Mg^{2+}, Ca^{2+}) and positively charged proteins which may neutralize the negatively charged of rubber particles and resulted in destabilization of the colloidal solution which is latex. Release of protein components from lutoid particles of exuded latex lyses as a result of contact with rainwater can cause cessation of latex flow, due to coagulation, during tapping of rubber trees (Webster and Baulkwil, 1989). In addition, lutoids contain a wide range of hydrolytic enzymes and some of their enzymatic properties are analogous to those of lysosomes of animal cells (Dupont *et al*., 1976). Approximately 20% of the dry matter in the lutoids is water-soluble protein, of which about 70% is hevein. This substance is anionic protein, has a very low molecular weight of about 5,000 Da and showed to contain no less than 5% sulfur, all as cystine (Webster and Baulkwil, 1989). In the light of its low molecular weight and high disulfide content, Archer considered that it could be a protease inhibitor but several test carried out by Walujono *et al.* do not appear to confirm this hypothesis (d' Auzac and Jacob, 1989; Archer, 1960; Walujono *et al*., 1975).

1.4.3 Frey-Wyssling particles

On ultracentrifugation of fresh latex the Frey-Wyssling particles may sediment centrifugally or rise centripetally to form a zone beneath the rubber particles. These particles are mainly composed of lipid material and are yellow or orange in color due to the presence of carotenoids. Yellow globules, in clusters in tapped latex were first noted by Frey-Wyssling (Frey-

Wyssling, 1929). Using electron microscopy revealed the structural complexity of these organelles which consists of one or more inclusions containing the lipid-carotenoid complex accompanied by another type of inclusion (a single one in general) with a complex system of branching single-membrane tubules associated with several concentric double-membrane lamellae (d' Auzac and Jacob, 1989; Premakumari and Panikka, 1992). The complex structure of Frey-Wyssling complexes has been elaborated by Dickenson who described a series of concentric lamellae of the double unit membrane and the system of tubules and also highly folded invaginations of the inner membrane (Dickenson, 1969).

The Frey-Wyssling complexes are more or less spherical in shape in a size range of 3-6 μm (diameter) and are bounded by a double membrane. Within the membrane there are two types of particles-large osmiophilic globules in variable numbers and a system of rope-like tubules of about 750 Å diameter, usually embedded in a membrane bound matrix of osmiophilic nature. The Frey-Wyssling complexes are considered to have vital role in metabolic activities. Though Dickenson (1969) opined that these structures may be possible sites of rubber biosynthesis, the double membrane and presence of carotene and polyphenol oxidase in the Frey-Wyssling complexes led to a tentative suggestion that it is a type of plastid (Premakumari and Panikka, 1992). Polyphenol oxidase and β-carotene are the classic markers of the Frey-Wyssling complexes. However, the Frey-Wyssling complex is still the organelle in the laticiferous cell whose physiological role remains the most mysterious.

1.4.4 C-serum

The aqueous phase of the laticiferous cytoplasm contains most of soluble compounds normally found in plant cells such as inositols, carbohydrates, amino acids, proteins, inorganic anions and metal ions, together with the enzymes and intermediates of various biochemical process, including rubber biosynthesis. C-serum is the most non rubber part in the latex and contains proteins approximately 60% of total proteins in latex. A great number of proteins have low isoelectric point which is anionic at the normal pH (6.9) of the serum (Webster and Baulkwil, 1989).

Natural rubber latex consists of approximately: 34% rubber cis-1,4-polyisoprene, 1% proteins, 0.1-0.5% sterol glycosides, 1.5-3.5% resins, 0.5-1% ash, 1-2% sugars and 55-65% water (Cacioli, 1997). For the part of proteins, approximately 20% is adsorbed on rubber particles,

20% associated with the sedimentable fraction and thus essentially with the lutoids, the remaining 60% is reported as being cytosolic or the serum fraction (d' Auzac and Jacob, 1989). Bearing in mind the great variability of latex, these organic non-rubber constituents may vary both in composition and concentration, depending on various physiological and physical parameters (Pakianathan *et al.*, 1992).

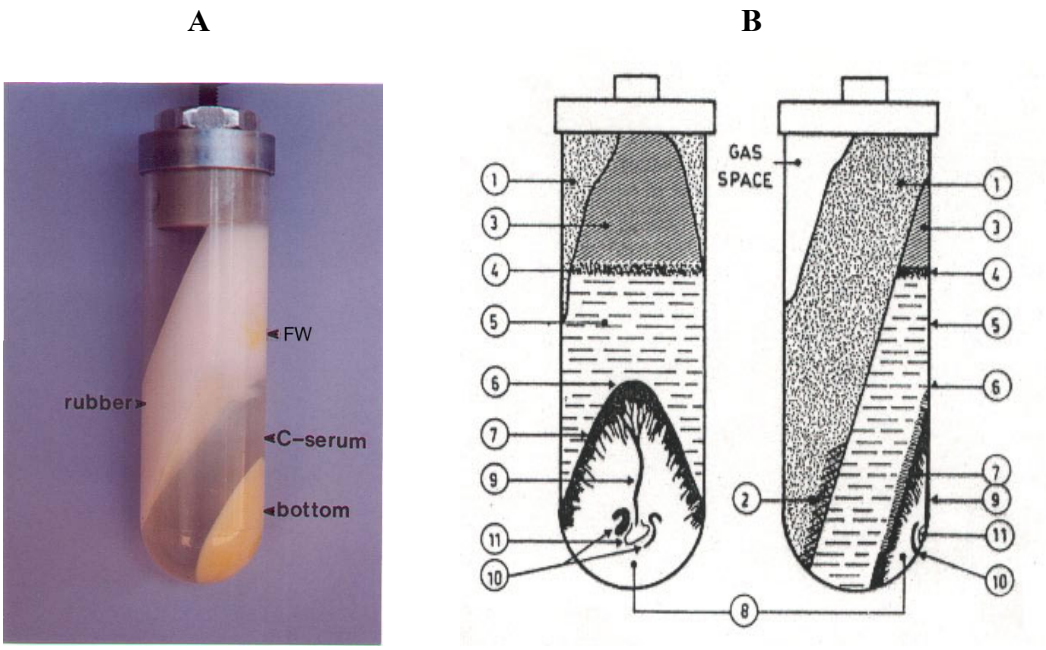


Fig. 3 Ultracentrifuged freshly collected latex.

(A) Fresh *Hevea* latex was subjected to ultracentrifugation at 59,000 x g for 45 min at 4°C and 4 main layers depend on different density of compounds containing in the latex were obtained. FW means Frey-Wyssling, Bottom means bottom fraction consisting mainly of lutoid particles (Moir, 1959).

(B) Fractions 1-3 correspond to the white rubber phase. Fraction 4 is a yellow-orange constituted by Frey-Wyssling particles. Fraction 5 is an almost clear serum (C-serum) corresponding to the latex cytosol. Fractions 6 to11 constitute the "bottom fraction" in which fraction 8, quantitatively the more important, is the lutoid fraction intensely pink-colored after neutral absorbtion (d' Auzac and Jacob, 1989).

2. Plant defense

Throughout their life cycle, plants have to react to various threats coming from the outside environment. They have developed a broad range of strategies, collectively known as "defense" or "stress" responses, to protect themselves against biotic and abiotic stresses and the proteins actively synthesized in accordance with this reaction are called "defense-related proteins". Plants react to wounding and pathogen attack by activation a set of genes, most of which play a role in wound healing and prevention of a subsequent pathogen invasion. Wounding sites are known to be important entry points for many pathogens, when a plant is infected with a pathogen to which it is susceptible (disease), the pathogen replicates and frequently spreads throughout the plant, often causing considerable damage and even death of the host (Klessing, 1994; Peña-Cortés, 1995; Wojtaszek, 1997). The outcomes of plant-mediated interactions are variable, and only a few provisional patterns can be identified at present. In higher plants, the consequences of wounding from insect herbivory and infection by pathogens can be different, with differences manifested at the levels of gene expression, induced chemistry, and host resistance to further challenge (Fidantsef, 1999).

Plants recognize and resist many invading phytopathogens by inducing a rapid defense response, termed the hypersensitive response (HR). The HR often associated with changes in the composition and physical properties of cell walls, the biosynthesis of secondary metabolites that serve to isolate and limit the spread of the invading pathogen and necrotic lesion of cell/tissue at the site of invasion. This local HR often also triggers a rise in salicylic acid (SA) and production of an unknown systemic, phloem-mobile signal. SA then induces a number of proteins that combine to provide nonspecific resistance, a general state of immunity against a broad range of pathogens, throughout the plant; a phenomenon known as systemic acquired resistance (SAR). These proteins include a class of proteins called pathogenesis-related (PR or P4) proteins (Table 1), and phytoalexins (antimicrobial compounds). Once triggered, SAR provides resistance to a wide range of pathogens for days (Baker, 1997; Hopkins, 1999).

However, through research on defense responses, it is now recognized that local events in the immediate zone of injury or pathogen invasion also trigger systemic events. These systemic events can be extremely rapid and generally involve a massive amplification in the response, since local changes occurring within one cell or small group of cells produce systemic

changes throughout an entire organ system(s). Some classes of defense-related products accumulate only at the local sites, whereas others accumulate locally and systemically (Bowles, 1990).

There is good evidence that both local and systemic responses involve the coordinate regulation of gene expression: i.e. different defense genes are activated simultaneously within the same cell. Sometimes these encode a set of enzymes that function within a specific metabolic pathway, but genes encoding structurally and functionally diverse products can also be activated. Comparison of the kinetics of local expression of defense genes in susceptible and resistant plant cultivars suggests that a major determinant of success in a resistance strategy lies in its speed. But there is also evidence that different classes of genes, even in a resistance strategy, are expressed sequentially and the chronology of expression may be common to many pathogens invading a diverse array of plant species. An intensive research effort has focused on the study of local responses. By contrast, systemic responses are only just beginning to be investigated at the molecular level, but they are already known to underlie the phenomenon of acquired resistance: the means by which an interaction with one pathogen/symbiont can influence the outcome of a subsequent challenge by the same or a different organism (Bowles, 1990).

2.1 Antifungal proteins

During the last two decades, the incidence of human fungal infections, especially involving immunocompromised patients, has dramatically increased (Cox and Perfect, 1993; Fox, 1993; Herbrecht, 1996). This is in part due to the tremendous advances in medicine that permit the saving of patients with neoplastic and immunocompromised diseases who would otherwise not have survived. Encouragingly, naturally occurring antifungal proteins and peptides, as well as synthetic derivatives, have the potential to be very interesting clinical leads (Selitrennikoff, 2001). Fungi are an extremely diverse group of organisms, with about 250,000 species widely distributed in essentially every ecosystem. Humans and other animals are exposed to fungi from the moment of birth. Fortunately, only 200 or so species are pathogenic to mammals, although many nonpathogenic fungi cause allergy symptoms (Ainsworth, 1973). The majority of fungal exposures and infections are self-limiting in intact animal hosts (Khardori, 1989). However, in patients with compromised immune systems, infections even by fungal organisms with low virulence can be life threatening; for example, systemic fungal infections of leukemia patients

account for 50% of fatalities (Levitz, 1992; Pfaller and Wenzel, 1992). Nosocomial bloodstream infections have a similar fatality rate (Lyman and Walsh, 1992).

Plant also exposed to a large number of pathogenic fungi; although they do not have an immune system, plants have evolved a variety of potent defense mechanisms, including the synthesis of low-molecular-weight compounds, proteins, and peptides that have antifungal activity. Similarly, bacteria, insects, mollusks, fungi, and mammals synthesize a number of proteins and peptides that are antifungal. These proteins appear to be involved in either constitutive or induced resistance to fungal attack. It is a testament to the efficacy of these defenses that plants and animals, including humans, do so well against pathogenic fungi (Selitrennikoff, 2001).

There are hundreds of antifungal peptides and proteins known, with more being discovered almost daily. This brief review will focus on proteins with molecular masses of greater than ~5 kDa, about 50 amino acids in length.

2.1.1 PR proteins

Plants when exposed to pathogens such as fungi and viruses produce low-molecular-weight antimicrobial compounds called phytoalexins, antimicrobial peptides, and small proteins [e.g., thionins (Bloch *et al*., 1998; Florach and Stiekema, 1994), defensins (Broekaert *et al*., 1995), hevein-like proteins, and knottin-like peptides (Segura *et al*., 1993)] and up-regulate a number of antimicrobial proteins. These plant proteins, called pathogenesis-related (PR) proteins, have been classically divided into 17 groups based on serological and amino acid sequence analyses (Table 1).

Table 1 Recommended classifications of pathogenesis-related proteins (PRs) (Van Loon and Van Strien, 1999)

Families	Type member	Properties	Gene symbol
PR-1	Tobacco PR-1a	antifungal	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr2, [Gns2('Glb')]*
PR-3	Tobacco P, Q	chitinase type I, II, IV, V, VI, VII	*Ypr3, Chia*
PR-4	Tobacco 'R'	chitinase type I,II	*Ypr4, Chid*
PR-5	Tobacco S	thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	proteinase-inhibitor	*Ypr6, Pis ('Pin')*
PR-7	Tomato P_{69}	endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	chitinase type III	*Ypr8, Chib*
PR-9	Tobacco'lignin-forming peroxidase	peroxidase	*Ypr9, Prx*
PR-10	Parsley 'PR1'	'ribonuclease-like'	*Ypr10*
PR-11	Tobacco 'classV'chitinase	chitinase, type I	*Ypr11, Chic*
PR-12	Radish Rs-AFP3	defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	thionin	*Ypr13, Thi*
PR-14	Barley LTP4	lipid-transfer protein	*Ypr14, Ltp*
PR-15	Barley OxOa (germin)	oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	'oxalate oxidase-like'	*Ypr16*
PR-17	Tobacco PRp27	unknown	*Ypr17*

2.1.2 Defensins

Defensins are a diverse group of low-molecular mass cysteine-rich proteins found in mammals, fungi (Lacadena *et al*., 1995), insects (Lamberty *et al*., 1999), and plants (Broekaert *et al*., 1995; Bull *et al*., 1992). The insect and mammalian defensins are quite small (3 to 5 kDa) and form voltage-dependent ion channels in plasma membranes (Landon *et al*., 1997; Landon *et al*., 2000; Thevissen *et al*., 1996). Thionins are also small (3 to 5 kDa) cysteine-rich peptides that are toxic to fungi (Thevissen *et al*., 1996). Plant and fungal defensins are cysteine-rich proteins ranging from 45-54 amino acids, are positively charged, and in most cases contain four disulfide bonds that stabilize each protein in solution (Selitrennikoff, 2001).

Hevein is one of the major proteins in the lutoid bodies of rubber tree latex (Archer, 1960). Hevein is a small (4.7 kDa) single-chain protein of 43 amino acid unusually rich in cysteine and glycine (Walujono *et al*., 1975). Recently, hevein has been shown to be bind chitin and to inhibit the growth of several chitin-containing fungi (Van Parijs *et al*., 1990). Therefore, it has been suggested that hevein plays a role in the protection of wound sites from fungal attack (Van Parijs *et al*., 1990). Various classes of chitin-binding proteins have been reported to contain polypeptide domains homologous to the hevein sequence. The lectins from the monocotyledonous species wheat, barley, and rice are composed of four repetitive hevein-like domains (Wright *et al*., 1984; Raikhel and Wilkins, 1987; Lerner and Raikhel, 1989; Wilkins and Raikhel, 1989), whereas a related lectin from the dicotyledonous *Urtica dioica* is thought to be composed of two such domains arranged in tandem (Chapot *et al*., 1986). Broekaert *et al*., 1990 have determined its cDNA sequence and found that it is synthesized as a precursor with a signal sequence of 17 residues, followed by the hevein domain of 43 residues and a C-terminal region of 144 residues. The C-terminal domain and the precursor consisting of the hevein and the C-terminal domain are also present in the lutoid-body fraction of latex (Lee *et al*., 1991). Analysis by ion-spray mass spectrometry of hevein showed that minor components with one and two additional C-terminal amino acids occur (Soedjanaatmadja *et al*., 1994), indicating that processing probably occurs by cleavage at the N-terminal of the C-terminal domain, followed by removal of 4-6 residues from the C-terminus of hevein (Soedjanaatmadja *et al*., 1994).

2.1.3 Cyclophilin-like protein

Cyclophillins are a highly conserved group of proteins that are the intracellular receptors for cyclosporine; they have been found in a wide variety of organisms, including bacteria, plants, animals, and fungi (Ostoa-Saloma *et al*., 2000). Recently, an 18-kDa protein was isolated from mung bean (*Phaseolus mungo*) with activity against *R. solani, F. oxysporum, B. cinerea,* and *Coprinus comatus* (Ye and Ng, 2000). This protein, called mungin, showed significant homology to cyclophilins and inhibited α- and β- glucosidases in vitro. However, the antifungal mechanism of action of mungin is not known.

2.1.4 Glycine/histidine-rich proteins

Insects synthesize a number of glycine/histidine-rich antifungal proteins and polypeptides, including those from *Holotrichia diomphalia* larvae [holotrichin, 84 amino acids (Lee *et al*., 1995)], *Sarcophaga peregrina* [fresh fly, AFP, 67 amino acids (Iijima *et al*., 1993)] and *Tenebrio molitor* [tenecin, 49 amino acids (Dae-Hee *et al*., 1998; Lee *et al*., 1999; Lee *et al*., 1996; Lee *et al*., 1999)]. They are extremely rich in glycine and histidine, which comprise as much as 80% of the amino acids. Importantly, fungi inhibited included *C. albicans*, the most common human pathogen. The mechanism of action of these proteins is not understood.

2.1.5 Ribosome-inactivating proteins (RIPs)

RIPs are RNA *N*-glycosidases that depurinate rRNA, resulting in the arrest of protein synthesis due to ribosome damage. Plant RIPs inhibit mammalian, bacterial, fungal, and plant protein syntheses *in vitro* and *in vivo* (Iglesias *et al*., 1993). RIPs have been classified into three groups. Type 1 RIPs are single-chain *N*- glycosidases with molecular masses of 11 to 30 kDa. Type 2 RIPs contain two chains, a cell-binding lectin (B chain) and an *N*-glucosidase (A chain), with molecular masses of ~60 kDa (Zhang *et al*., 1999); type 2 RIPs include toxic members such as ricin and nontoxic members such as ebulin 1 (Girbes *et al*., 1993) and nigrin b. Type 3 RIPs consist of four chains organized as two dimmers of type 2 RIPs.

2.1.6 Lipid-transfer proteins (LTPs)

LTPs have the ability to transfer phospholipids between membranes. LTPs are small proteins (~8.7 kDa) of ~90 amino acids stabilized by four disulfide bonds with a central tunnel-like hydrophobic cavity. They have been isolated from a number of sources, including mammals, plants, fungi, and bacteria, and may play several in vivo roles, including lipid

exchange between cytoplasmic organelles and, importantly, defense against pathogenics (Guerbette *et al*., 1999). LTPs are active *in vitro* against a number of bacteria and fungi; however, the mechanism of action is not known. It may be that these proteins insert themselves into the fungal cell membrane, and the central hydrophobic cavity forms a pore, allowing efflux of intracellular ions and thus leading to fungal cell death. How this is related to their lipid transfer function is not clear (Selitrennikoff, 2001).

2.1.7 Protease inhibitors

Protein inhibitors of serine (e.g. trypsin and chymotrypsin) and cysteine proteases have emerged as a class of antifungal proteins that have potent activity against plant and animal pathogens. Cysteine protease inhibitors have been isolated from a number of plants and form a fourth group of cystatins, the phytocystatins, which are single polypeptides of 10 to 12 kDa and share common structural motifs. Although phytocystatins are active against plant pathogens such as *Fusarium solani* (MIC of 20 μg/disk in and disk agar diffusion assay) and *Trichoderma reesei* (250 μg/disk) (Joshi *et al*., 1998). The mechanism of antifungal activity is not understood.

2.1.8 Other proteins

New proteins that have antifungal activity but do not neatly fall into any of the above classes are being discovered at a rapid pace. Viridin, a novel protein isolated from the culture medium of Trichoderma viride, has a molecular mass of 65 kDa and is active against sensitive fungi at 6 μM (Hao *et al*., 1999; Hao *et al*., 2000). Snakin-1 isolated from potato has a molecular mass of 6.9 kDa and is active at 10 μM (Segura *et al*., 1999). A 30-kDa protein with very potent antifungal activity (50 ng/disk in an agar diffusion assay) was isolated from Englemann's daisy (*Engelmannia pinnatifida*); this protein showed 35 to 50% identity to self-incompatibility glycoproteins, not previously known to be antifungal (Huynh *et al.,* 1996). The mechanism of action of none of these proteins is known.

3. *Candida albicans*

C. albicans which is part of the normal microbial flora of the host and can be found in the digestive, oral cavity and vaginal tracts (Shepherd, 1986). It is a major human opportunistic pathogen, causing both mucosal and systemic infections so-called candidiasis, in

immunocompromised patients (Odds, 1988; Odds, 1994). However, an increased prevalence of candidiasis has been assigned to the widespread use of antibiotics and immunosuppressive agents (Gadea, 1997). Various systemic and local factors such as malnutrition, immunodeficiencies, endocrine disorders, malignant diseases, radiation therapy, xerostomia, and denture wearing can predispose humans to *Candida* infections (Odds, 1988; Oksala, 1990; Scully *et al*., 1994).

C. albicans is a serious agent of infection, particularly in immunocompromised patients. The delicate balance between the host and this otherwise normal commensal fungus may turn into a parasitic relationship, resulting in the development of infection, called candidiasis. The nature and extent of the impairment of normal host defense influence the manifestation and severity of infection. In general, superficial mucocutaneous candidiasis is frequent in patients with T-cell deficiencies, such as AIDS patients. The more serious, life-threatening, deep-seated or disseminated candidiasis is normally found in a spectrum of severely immunocompromised patients (Bodey, 1993; Odds, 1988). The fungus is not a mere passive participant in the infections process, and a hypothetical set of virulence factors for *C. albicans* has been proposed and supported by various studies. These fungal attributes include the production of secreted hydrolytic enzymes, dimorphic transition (morphogenetic conversion from budding yeast to the filamentous growth form or hypha), antigenic variability, the ability to switch between different cell phenotypes, adhesion to inert and biological substrates, and immunomodulation of host defense mechanisms (Cutler, 1991).

Initially, the cell wall was considered an almost inert structure that supplies rigidity and protection to the protoplast. Today, the cell wall is well established as being essential to almost every aspect of the biology and pathogenicity of *C. albicans* (Cassone, 1989). The cell wall acts as a permeability barrier and is the structure that maintains the characteristic shape of the fungus. Also, as the most external part of the cell, the wall mediates the initial physical interaction between the microorganism and the environment, including the host. For these reasons, the cell wall of *C. albicans* is the focus of study by numerous research groups (Chaffin *et al*., 1998).

3.1 Cell wall and morphology

Although the terms “dimorphism” and “dimorphic fungus”, i.e., existing in two

morphological forms, are well established and commonly accepted when referring to *C. albicans*, strictly speaking this fungus has the ability to adopt a spectrum of morphologies and thus *C. albicans* could be considered a "polymorphic" or "pleomorphic" organism (Kerridge, 1993; Odds, 1988). Since changes in the cell wall determine the shape of the whole fungal cell, the cell wall is the structure ultimately responsible for a given morphology. *C. albicans* can reproduce by budding, giving rise to the formation of yeast cells (also designated blastospores or blastoconidia). The production of germ tubes results in the conversion to a filamentous growth phase or hypha, also called the mycelial form. The formation of pseudohyphae occurs by polarized cell division when yeast cells growing by budding have elongated without detaching from adjacent cells. Under certain nonoptimal growing conditions, *C. albicans* can undergo the formation of chlamydospores, which are round, retractile spores with a thick cell wall. These morphological transitions often represent a response of the fungus to changing environmental conditions and may permit the fungus to adapt to different biological niches. The transition from a commensal to a pathogenic lifestyle may also involve changes in environmental conditions and dispersion within the human host (Chaffin *et al*., 1998). The ultrastructure, composition, and biological properties of the cell wall are affected by these morphological changes (Cassone, 1989). Although progress has been achieved in the recent years, the molecular mechanisms governing these morphogenetic conversions are still not fully understood, partly due to the difficulty of genetic manipulations in this fungus (Kurtz *et al*., 1990; Kurtz *et al*., 1988; Scherer and Magee, 1990). Recent reports that may herald rapid advances in this area have identified transcriptional regulatory genes, a general transcriptional repressor *TUP1* (Braun and Johnson, 1997), a putative transcriptional factor *RBF1* (Ishii *et al*., 1997), and a *myc*-like transcriptional factor *EFG1* (Stoldt *et al*., 1997) that effect cellular morphology when their expression is altered. Most of the observations from these studies have been incorporated by Magee, 1997 into a model for the regulation of pseudohyphal growth.

3.1.1 Cell wall and interactions with the host

Two major aspects of the host-parasite interactions are the adhesion of *C. albicans* cells to host cells and tissues and the immunomodulation of the host immune response (Chaffin *et al*., 1998). Adhesion is a prerequisite for colonization and an essential step in the establishment of infection. *C. albicans* adheres to epithelial cells, endothelial cells, soluble factors,

extracellular matrix, and inert materials implanted in the body of the host. Multiple adherence mechanisms appear to be used by *C. albicans* cells. Physical interactions of this fungus with the host are mediated at the cell surface, and cell wall constituents implicated in binding have been designated adhesins (Calderone, 1993). The large repertoire of adhesins displayed by this fungus may reflect the variety of host sites that it can invade. Another important aspect of interactions with the host, with direct implications for pathogenesis, is the potential of this fungus to modulate the immune response mounted by the host (Cassone, 1989; Cutler, 1991; Domer, 1989). The capacity of cell wall constituents, including glucan, chitin, and mannoproteins, to modulate (activate of depress) the immune response is well documented (Ashman and Papadimitriou, 1995; Cassone, 1989). Mannans and mannoproteins display the most potent immunomodulatory activity, being able to regulate the action of virtually all arms of the immune system (natural killer cells, phagocytic cells, cell-mediated immunity, and humoral mechanisms). Although individual cell wall moieties with immunomodulatory properties are described below (Chaffin *et al*., 1998).

3.1.2 Cell wall composition and organization

3.1.2.1 Composition

Approximately 80 to 90% of the cell wall of *C. albicans* is carbohydrate. Three basic constituents represent the major polysaccharides of the cell wall: (i) branched polymers of glucose containing β-1,3 and β-1,6 linkages (β-glucans); (ii) unbranched polymers of *N*-acetyl-D-glucosamine (GlcNAc) containing β-1,4 bonds (chitin); and (iii) polymers of mannose (mannan) covalently associated with proteins (glyco[manno]-proteins). In addition, cell walls contain proteins (6 to 25%) and minor amounts of lipid (1 to 7%) (Chaffin *et al*., 1998). The microfibrillar polymers (β-glucans and chitin) represent the structural components of the wall. They form a rigid skeleton that provides strong physical properties to the cell. From a quantitative point of view, β-glucans are the main constituent, accounting for 47 to 60% by weight of the cell wall. Chitin is a minor (0.6 to 9%) but important component of the *C. albicans* wall, particularly of the septa between independent cell compartments, budding scars, and the ring around the constriction between mother cell and bud (Elorza *et al*., 1983; Molano *et al.,* 1980). On the other hand, mannose polymers (mannan), which do not exist as such but are found in covalent association with proteins (mannoproteins), represent about 40% of the total cell wall polysaccharide and are the main material of the cell wall matrix. The term “mannan” has been

used also to refer to the main soluble immunodominant component present in the outer cell wall layer of *C. albicans*, called phosphomannoprotein or phosphopeptidomannan complex. This cell wall fraction contains homopolymers of D-mannose (as the main component), 3 to 5% protein, and 1 to 2% phosphate (Reiss, 1993). The general features of cell wall mannoproteins in *C. albicans* are basically identical to those found for *Saccharomyces cerevisiae*, one of the most thoroughly investigated yeasts in this regard. Several studies have resulted in a detailed knowledge of the structure of this cell wall constituent in *C. albicans*. Thus, mannose polymers are linked to the protein moiety through asparagines [by N-glycosidic bonds through two GlcNAc (di-*N*-acetylchitobiose) residues] and threonine or serine (by O-glycosidic, alkali-labile linkages) residues. The N-glycosidically linked carbohydrate is composed of backbone chains of α-1,6-linked mannopyranosyl residues to which oligosaccharides side chains are attached. The side chain mannopyranosyl residues contain α-1,2, α-1,3, β-1,2, β-1,6 and phosphodiester linkages as well as branches (α-1,6) that are oversynthesized under acidic growth conditions. The O-glycosidically-linked sugar component consists of single mannose residues and short, unbranched mannose oligosaccharides. Several studies raise the question of additional sugars present in cell wall constituents. These observations include the following: (i) not all proteinaceous moieties present in cell wall extracts from this fungus react with concanavalin A, a lectin recognizing α-mannosylpyranose, or with polyclonal and monoclonal antibodies that recognize other mannan epitopes, such as factor 6, a mannooligosaccharide that confers serotype A specificity; (ii) differences in glycosylation and in sensitivity to neuraminidase have been detected in candidal receptors for complement; and (iii) treatment with neuraminidase affects the electrostatic surface properties of *C. albicans* as detected with a fluorescent probe. As suggested in these studies, the observations raise the possibility that additional sugars are cell wall constituents. However, the observations could reflect the existence of contaminating proteases in the glycosidase preparation. Sugar residues other than mannose may define either additional functional or antigenic motifs or both in cell wall glycoproteins. The percent composition of walls from yeast cells and filamentous forms are similar, although the relative amounts of β-glucans, chitin, and mannan vary according to the *C. albicans* growth form considered. Hyphal cells contain at least three times as much chitin as yeast cells do. Chitin is the first polymer to appear in regenerating protoplasts. Although the ratio of β-1,3- to β-1,6-glucan in the soluble fraction is similar in yeast and hyphal cells, the

insoluble glucan in the initial period of germ tube formation contains considerably more β-1,3 linkages than that found in yeast and mature hyphal cells (Chaffin *et al*., 1998).

3.1.2.2 Organization

The different cell wall components interact with each other to give rise to the overall architecture of the cell wall. Besides hydrogen and hydrophobic bonds, there is also experimental evidence for the presence of covalent linkages between different components (Ruiz-Herrera, 1992; Sentandreu *et. al*., 1994). Surarit *et al*., 1988 reported the presence of glycosidic linkages between glucan and chitin in the nascent wall of *C. albicans*. Recent evidence indicates that mannoproteins may also establish covalent associations with β-glucans. It is suggested that β-1,3- and β-1,6-glucans are linked to proteins by phosphodiester linkages, a process that may involve the participation of a GPI (glycosyl phosphatidylinositol) anchor (Kapteyn *et al*., 1995). Protein and mannoprotein species that are released only after digestion of the glucan cell wall network with β-glucanases may play a key role in configuring the final cell wall structure characteristic of each growth form (yeast and mycelium) of *C. albicans.* Interactions between glyco(manno) proteins and chitin also appear to exist in the wall of *C. albicans* cells as deduced from two lines of evidence: (i) chitinase treatment of isolated cell walls solubilizes protein moieties, and (ii) the kinetics of incorporation of protein and mannoprotein constituents into the walls of regenerating protoplasts is altered in the presence of nikkomycin, an antibiotic that blocks chitin synthesis (Chaffin *et al*., 1998).

Cell wall architecture has been studied most extensively in *S. cerevisiae* and is likely to be a model for *C. albicans* since there are some similar observations, in particular sensitivity to enzymatic digestion, glucan-mannoprotein linkages, and candidate proteins, that fit the same model. In recent study, Kollar *et al*., 1997 detected the presence of material containing all four major cell wall components, β-1,3-glucan, β-1,6-glucan, chitin, and mannoprotein. Their analysis indicated that β-1,6-glucan has some β-1,3-glucan branches that may be linked to the reducing end of chitin. The β-1,6-glucan and mannoprotein are attached through a remnant of the mannoprotein GPI anchor. Reducing ends of β-1,6-glucan may also be attached to the nonreducing end of β-1,3-glucan. The proportion of cell wall polysaccharide involved in this type of structure is not clear. The following cell wall building block, where the linkages are indicated by the long dashes, is proposed (Klis *et al*., 1997; Kollár *et al*., 1997): Mannoprotein--GPI

remnant-- β-1,6-glucan-- β-1,3-glucan--chitin. The authors point out that these linkages are likely to the formed in the periplasmic space as a common end of the individual biosynthetic pathways. Chitin and β-1,3-glucan are synthesized at the plasma membrane and extruded into the periplasm, mannoprotein is synthesized in the cytoplasm and transported through the secretory pathway, and β-1,6-glucan synthesis may occur partially in the endoplasmic reticulum or Golgi complex (Kollár *et al*., 1997). Not all components are necessarily present in a complex; therefore, the authors suggest that more chitin may be present in inner cell wall layers and more mannoprotein may be present in the outer layers (Chaffin *et al*., 1998).

3.1.2.3 Layering

Since polysaccharides are poorly reactive to the ordinary fixatives and stains used for transmission electron microscopy, only a few well-defined ultrastructure details are obtained by conventional protocols (Fig. 4A and B). However, transmission electron microscopy studies performed with more special techniques or with cytochemical stains and contrasting agents show several layers in the cell wall of *C. albicans* (Fig. 4C to F). The appearance of these layers is variable and seems to be related to the strain examined, growth conditions, morphology, and preparation of the specimens. Thus, there is no consensus about the number of layers present in the cell wall. Different authors have reported the presence of three to eight different layers. The outer cell wall layer appears as a dense network with a fibrillar or flocculent aspect, whereas the inner wall layer appears contiguous with the plasmalemma with extensive membrane invaginations involved in anchoring of the cell wall to the membrane. The microfibrillar polysaccharides glucan and chitin, the components that supply rigidity to the overall wall structure, appear to be more concentrated in the inner cell wall layer, adjacent to the plasma membrane. In contrast, proteins and mannoproteins appear to be dominant in the outermost cell wall layer (Fig. 4B), although they are also present through the entire wall and at the inner regions of the cell wall. Some of the later proteins may be covalently associated with glucans. Evidence from several cytochemical and cytological studies indicate that the cell wall layering may be due to the distribution of mannoproteins at various levels within the wall structure (Cassone, 1989). In any case, it seems clear that layering may be the result of quantitative differences in the proportions of the individual wall components (β-glucans, chitin and mannoproteins) in each layer rather than of qualitative differences (Chaffin *et al*., 1998).

3.1.2.4 Fimbriae

The outer cell wall layer that is composed mainly of mannoproteins appears as a dense network of radially projecting fibrils (Bobichon *et al*., 1994; Cassone, 1989), designated fimbriae. These fibrils extend for 100 to 300 nm and are approximately 5 nm in diameter (Bobichon *et al*., 1994). Both filamentous forms and blastospores exhibit this characteristic feature (Bobichon *et al*., 1994). *C. albicans* fimbriae consist of many subunits assembled through noncovalent hydrophobic interactions (Yu *et al*., 1994). The major structural subunit of fimbriae is a glycoprotein with an apparent molecular mass of 66 kDa, while the unglycosylated protein has an approximate molecular mass of 8.64 kDa (Yu *et al*., 1994). In crude extracts, in addition to the 66-kDa moiety, components migrating with an electrophoretic mobility equivalent to proteins of 54, 47, and 39 kDa reacted with monoclonal antibodies (MAbs) raised against purified fimbriae, suggesting the presence of species with differing degrees of glycosylation (Yu *et al*., 1994). The hydrophobic status of the cells profoundly affects fimbriae structure. Hydrophilic cells have long, compact, evenly distributed fibrils, while hydrophobic cells have short, blunt fibrils (Fig. 4E and F). The overall hydrophilic status may be due to masking of hydrophobic components by hydrophilic surface fibrils. Fimbrial components mediate the adherence of *C. albicans* to glycosphingolipid receptors on human epithelial cells (Chaffin *et al*., 1998).

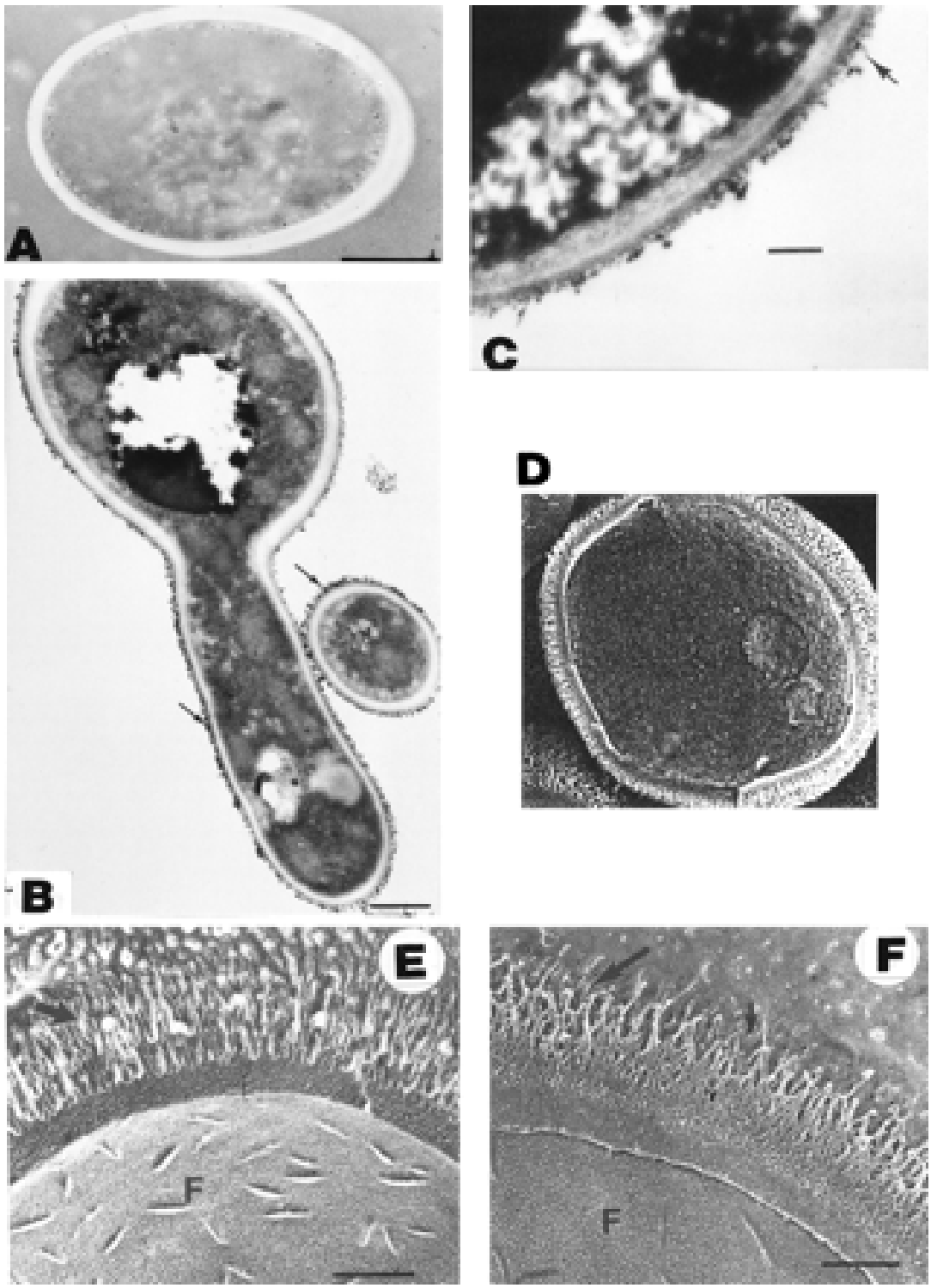


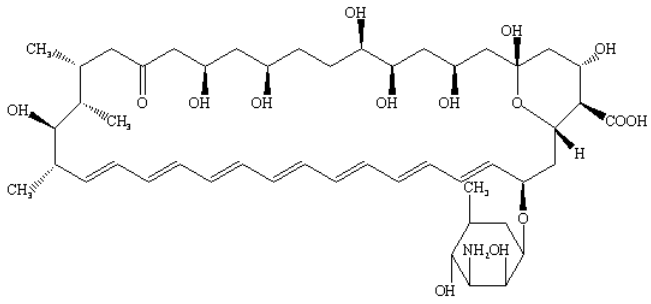
Fig. 4 Cell wall structure.

(A) Transmission electron micrograph of a section of a *C. albicans* cell prepared by freeze-substitution, showing the cell wall as a thick, electron-dense, homogeneous structure. The presence of distinct layers was not evident in this preparation. Bar, 1 μm. (B) Thin sections of cells treated with gold-conjugated concanavalin A, showing an intense labeling with gold particles of the external wall surface. The surface exhibits a fibrillar appearance (arrows), suggesting that concanavalin A-reactive cell wall components, i.e., mannoproteins, are particularly abundant at the most external wall layers. The remaining wall structure also appeared as a homogenous structure in this transmission electron micrograph. Bar, 0.5 μm. (C) Other procedures for transmission electron microscopy examination of thin sections of *C. albicans* cells revealed more clearly the presence of an outer floccular layer (arrow) and showed that the remaining cell wall structure is not homogeneous and that some layering exists. Bar, 200 nm. (D to F) Complexity of the wall ultrastructure and presence of distinct layers in the cell wall of *C. albicans* as revealed by different scanning electron microscopy-based procedures such as cryo-scanning electron microscopy (D) and freeze-fracture, freeze-etch analysis (E and F). The presence of well-ordered, regularly arranged, radiating fibrils in the outer layer is particularly evident in the micrographs shown in panels E (hydrophilic cells) and F (hydrophobic cells). Bar, 0.3 μm in both panels (Chaffin *et al*., 1998).

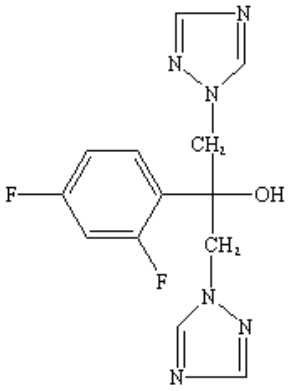
4. Antifungal agents

The past decade has witnessed a significant increase in the prevalence of resistance to antibacterial and antifungal agents. Resistance to antimicrobial agents has important implications for morbidity, mortality and health care costs in U.S. hospitals, as well as in the community. Hence, substantial attention has been focused on developing a more detailed understanding of the mechanisms of antimicrobial resistance, improved methods to detect resistance when it occurs, new antimicrobial options for the treatment of infections caused by resistant organisms, and methods to prevent the emergence and spread of resistance in the first place. The annual death rate due to candidiasis was steady between 1950 and about 1970. Since 1970, this rate increased significantly in association with several changes in medical practice, including more widespread use of therapies that depress the immune system, the frequent and often indiscriminate use of broad-spectrum antibacterial agents, the common use of indwelling intravenous devices, and the advent of chronic immunosuppressive viral infections such as AIDS. These developments and the associated increase in fungal infections intensified the search for new, safer, and more efficacious agents to combat serious fungal infections (Ghannoum and Rice, 1999).

For nearly 30 years, amphotericin B (Fig. 5), which is known to cause significant nephrotoxicity, was the sole drug available to control serious fungal infections. The approval of the imidazoles and triazoles in late 1980s and early 1990s were major advances in our ability to safely and effectively treat local and systemic fungal infections. The high safety profile of triazoles, in particular fluconazole (Fig. 5), has led to their extensive use. Fluconazole has been used to treat in excess of 16 million patients, including over 300,000 AIDS patients, in the United States alone since the launch of this drug. Concomitant with this widespread use, there have been increasing reports of antifungal resistance. Hope to make an understanding of antifungal resistance mechanisms accessible to those who use these agents clinically, as well as those who may wish to study them in the future (Ghannoum and Rice, 1999).



Amphotericin B



Fluconazole

Fig. 5 Structures of representative antifungal agents.

(http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8/antibiotics/antifungals.html)

4.1 Polyene: Amphotericin B

Amphotericin B was isolated from a strain of *Streptomyces nodosus* recovered from a soil sample obtained at Tembladora on the Orinoco River in Venezuela by Gold and colleagues. It has broad-spectrum antifungal activity, is fungicidal and has excellent in-vitro activity against *Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, Paracoccidioides brasiliensis, Sporotrichium spp.* and *Torulopsis (Candida) glabrata.* It also has excellent activity against *Candida albicans* and most other *Candida* spp., except for *Candida lusitaniae*. It has variable activity against *Aspergillus* spp. and zygomycetes (e.g. *Mucor* spp.), whereas *Fusarium* and *Trichosporon* spp. and *Pseudoallescheria boydii* are often resistant (Andriole, 1998; Georgopapadakou and Walsh, 1996; Andriole and Bodey, 1994). Although this drug is not well absorbed after oral administration, an oral preparation is available for treatment of oral mucosal candidiasis. Topical preparations are also available. Intravenous amphotericin B has been the mainstay of effective therapy for invasive fungal infections. It is recommended by most clinical mycologists as the drug of choice for severe blastomycosis, coccidioidomycosis (pulmonary, meningeal and disseminated), paracoccodioidomycosis, histoplasmosis, fusariosis, severe and moderate cryptococcal meningitis, candidiasis (including candidaemia, disseminated candidiasis, endophthalmitis, endocarditis, peritonitis and candida infections of the central nervous system) and for all forms of invasive aspergillosis and mucormycosis (Andriol, 1999).

Nephrotoxicity is the most serious side effect of amphotericin B therapy. Almost every patient develops some abnormality in renal function. A number of methods have been used to attempt to reduce this serious side effect, most notably, the use of liposomal preparations of this drug (Hiemenz and Walsh, 1996).

4.1.1 Mechanism of action

Amphotericin B represented the standard of therapy for systemic fungal infections. There is an association between polyene susceptibility and the presence of sterols in the plasma membrane of the cells. All organisms susceptible to polyenes, e.g., yeasts, algae, and protozoa, contain sterols in their outer membrane, while resistant organisms do not. The importance of membrane sterols for polyene activity is also supported by earlier studies, where it was shown that fungi can be protected from the inhibitory action of certain polyene by the

addition of sterol to the growth medium. It was suggested that this effect is due to a physicochemical interaction between added sterols and the polyene, which prevents the drug from binding with the cellular sterols. It has been proposed that the interaction of the antifungal with membrane sterol results in the production of aqueous pores consisting of an annulus of eight amphotericin B molecules linked hydrophobically to the membrane sterols (Fig. 6). This configuration gives rise to a pore in which the polyene hydroxyl residues face inward, leading to altered permeability, leakage of vital cytoplasmic components, and death of the organism. Although amphotericin B is the most effective antifungal drug available, its narrow therapeutic index continues to limit its clinical utility. To reduce untoward effects, amphotericin B has been formulated in liposome to allow the transfer of higher doses of amphotericin B with less toxicity to mammalian cells. Several amphotericin B liposomal preparations have been developed, including ABELCET, Amphoteck, and AmBisome. It is hypothesized that once amphotericin B is incorporated into liposomes, it may participate in a selective transfer mechanism, which involves its transfer from the “donor” liposome to the ergosterol-containing “target” in the fungal cell membrane aided by the fungal and/or host phospholipases (Ghannoum and Rice, 1999).

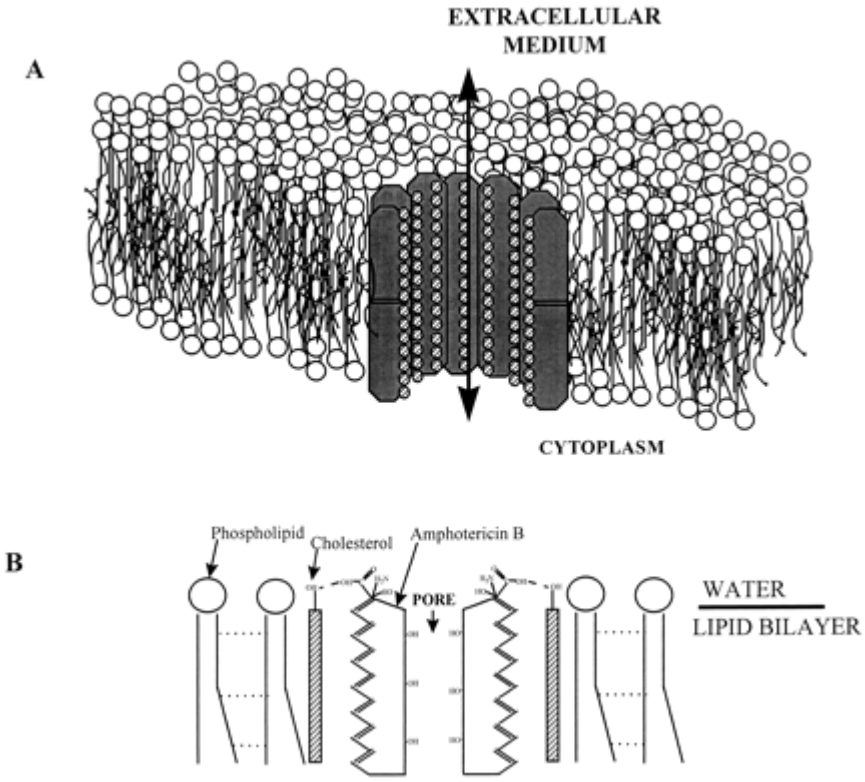


Fig. 6 Schematic representation of the interaction between amphotericin B and cholesterol in a phospholipids bilayer.

(A) The conducting pore is formed by the end-to-end union of two wells or half pores. (B) Molecular orientation in an amphotericin B-cholesterol pore. The dotted lines between the hydrocarbon chains of phospholipids represent short-range London-van der Waals forces. The dashed lines represent hydrogen bonds formed between amphotericin B and cholesterol molecules (Ghannoum and Rice, 1999).

Objectives

1. To screen for antimicrobial activity present in the latex.

2. To purify and characterize the properties of *Hevea* latex antifungal proteins.

3. To investigate the properties of antifungal proteins against *Candida* spp.

4. To characterize potency of antifungal proteins.

5. To evaluate the defense role attributed by antifungal proteins and rubber particles in the *Hevea* latex.

6. To study of effect of rubber particle in inducing yeast cell aggregation and lowering the MIC of amphotericin B.

7. To characterize the rubber particle proteins involved in yeast cell aggregation and lowering the MIC of amphotericin B.