Chapter 4

Discussion

Part A: Antifungal protein from *Hevea* latex

The tapping system in latex collection involves multiple wounds over a period of times that create easy and dangerous entry sites for pests and pathogens. Plants respond to wounding by activating genes that play key roles in wound healing and defense against both herbivores and pathogens (Bowles, 1990). Latex has been implicated to play a role in defense (Dussourd and Eisner, 1987; Farrell *et al*., 1991). Han *et al*. (2000) reported expressed sequence tag (EST) analysis of the latex of *Hevea* rubber tree, and showed that the genes coding for rubber particle-associated proteins (REF and SRPP) were the most abundantly expressed among the 125 ESTs studied, along with defense-related genes. Kush *et al*. (1990) also observed that the transcript levels of plant defense- or stress-related genes were 10- to 50- fold higher in latex than leaves. Hydrolytic enzymes such as β-1,3-glucanase (Chye and Cheung, 1995), cellulose, and polygalacturonase (Kush *et al*., 1990) were also highly expressed in laticifers. In addition, the *in vitro* antifungal activity of latex has been demonstrated for several species including *Lactuca sativa, Asclepias curassavica, Carica papaya,* and *C. albicans* (Moulin-Traffort *et al*., 1990; Giordani *et al*., 1991, 1996, 1999). Hevein has been described as an antifungal protein (Van Parijs *et al*., 1991; Koo *et al*., 1998). Hevein comprised a high proportion in the EST pool and was accumulated more than 10- fold in the latex when compared to the leaf tissues (Ko *et al*., 2003). Screening for antifungal activity in the latex of rubber trees (*H. brasiliensis*) has been done and revealed that the B-serum from lutoid fraction contained antifungal activities. Our further search for the antimicrobial proteins was carried out in the B-serum.

It was found that acetone fractionation of the B-serum was very efficient and suitable for the purposed study. The antifungal activities distribution among the 4 proteins fractions (0-40%, 40-60%, 60-70% and 70-80% acetone) but the 70-80% acetone fraction showed

the strongest antifungal activity. The observed cumulative results pointed out some interesting points. Firstly, the B-serum proteins, antifungal activity could only be precipitated out at high acetone level, which makes it simpler and easier to prepare and separate it from the total B-serum proteins for further purification. This could be accomplished by ridding of other contaminant B-serum proteins with the low acetone content (up to 60%) and subsequently utilizing the 60-80% acetone fraction in the serial acetone fractionation process. Secondly, these observations suggested that the antifungal activity was very likely to be small molecules of low MW protein and lastly, these observed results also hinted that the B-serum antifungal protein might probably be not only a small protein but also with high degree on the property of hydrophobicity in its structure and molecular nature. These were then characterized and verified on the antifungal protein properties.

A rather homogeneous antifungal protein was obtained in the 70-80% acetone fractionation. Upon further purification by ion-exchange chromatography DEAE-Sepharose Fast Flow column, the purified protein has identical N-terminal sequence and amino acid composition as those of hevein (Walujono *et al*., 1975). Molecular weight estimation of the antifungal protein by SDS-PAGE and Tricine-SDS-PAGE under denaturing conditions indicated a M_r value between 9 and 10 kDa. This appears to be similar to of hevein behavior, as previously observed by Tata (1975). Analysis by SDS-PAGE yielded a somewhat higher value (9 kDa) but overestimation is commonly observed by this method with proteins rich in cysteine and glycine. Based on the protein identification by peptide mass fingerprinting data obtained, the purified antifungal protein was confirmed to be the same protein formerly known as hevein. The electrophoretic behavior of heat-denatured hevein in the presence of SDS (but without reducing agents) showed that a considerable part of the protein is present as an oligomer of molecular mass much higher than that of monomeric hevein, when a reducing agent was added, the amount of oligomer decreased somewhat. This evidence indicated that some intermolecular disulfide bonds can be formed in the unfolded state of hevein; however, other mechanisms of covalent oligomerization could also be taking place (Hernandez-Arana *et al*., 1995).

The results on effect of temperature and pH on antifungal activity of hevein suggested that it is stable to heat and a wide pH range. The result indicated that hevein is a very thermostable protein, probably the most stable small protein in B-serum of the latex. Accordingly,

the four disulfide bridges had been suggested to play an important role in the stabilization of the native hevein state (Hernandez-Arana *et al*., 1995).

Hevein is one of the major proteins in lutoids, which are small vacuole-derived organelles (Archer *et al*., 1969). Lutoids contain proteins encoded by defense- or stress- related genes (Ko *et al*., 2003). This indicates that defense is one of the functions of laticifers. Hevein is accumulated more than 10- fold in the latex when compared to the leaf tissues (Kush *et al*., 1990). Mature hevein (Van Parijs *et al*., 1991) and chitinases (Schlumbaum *et al*., 1986; Broekaert *et al*., 1988) are known inhibitors of fungal growth *in vitro*. Hence, it appears that in a broad sense this class of related chitin-binding proteins may serve to protect plants from attack by a wide range of potential pathogens. Hevein has previously been shown to have antifungal activity against various plant pathogenic fungi (Van Parijs *et al*., 1991). In our study, this is the first testing of its activity against various commensals and potential pathogens of the oral cavity and respiratory tract. The inhibitory effect of a purified hevein sample obtained by a new procedure, that was far more simple than the previously described methods (Van Parijs *et al*., 1991), showed strong inhibition against *Candida* spp. (MIC range 12-190 μg/ml) but less for *C. krusei* ATCC 34135 and *C. glabrata* ATCC 90030 with MICs (>190 μg/ml). It was much less active against oral Gram-negative species (MIC 896 μg/ml) and inactive against Gram-positive species at concentrations of 1.8 mg/ml. The antifungal property of hevein has been investigated against potential human pathogenic fungi including *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata*. The structural components of the cell wall of these fungi are mainly chitin, (1,3)β-D-glucan, (1,6)β-glucans, lipids, and peptides embedded in a protein matrix (Selitrennikoff, 2001). Hevein is a small chitin-binding protein that targets the chitin of the pathogenic fungal cell wall which could inhibit by the specific inhibitor chitotriose. An estimate of the fungal wall pore size predicts that proteins of larger than 15 to 20 kDa will not pass through the fungal wall (Money, 1990). Thus, hevein with a molecular size of 4.7 kDa could penetrate the fungal cell wall matrix. As can be seen from the aggregation experiment, within 2 h of mixing, hevein caused a Ca^{2+}-dependent aggregation of *C. tropicalis*. Aggregation of the fungal cell was inhibited by EDTA. We assume that aggregation was due to the penetration of hevein through the protein matrix of the cell wall to bind to the chitin embedded in the protein matrix in the presence of Ca^{2+}. This binding then led to a modification of the wall to induce aggregation and finally death of the fungus within 24 h. This

study showed that hevein increased yeast cell permeability and membrane fluidity. Hevein at concentration higher than 100 μg/ml is thought to induce alterations in cell permeability by inserting between the membrane lipid bilayers, causing changes to membrane properties and functions.

Our quantitative analyses of the growth-inhibitory effect of hevein gave some inconsistent results as shown by the broad distribution of MICs obtained against *C. albicans*, ranging from 12-95 μg/ml for strain ATCC 10231 and from 23-190 μg/ml for strain ATCC 90028 using different isolation and testing procedures (data not shown). For the disk diffusion assay, a higher concentration of hevein was required for growth inhibition (Fig. 15). The differences observed could be due to the different times of sample preparation. Different cultivation conditions, different protocols, or the different strains used had effects on the MICs values, for example, the test by the disc diffusion assay yielded higher MICs than those obtained by the broth microdilution method with the same material. MIC values are therefore dependent on the choice of protocol.

A *Hevea* protease inhibitor (HPI) has recently been purified and characterized from *Hevea* latex in our laboratory (Sritanyarat *et al*., 2006). The assay of HPI, isolated from an 80-95% acetone fraction of the C-serum, against several proteases, including serine and aspartic proteases, revealed that it inhibited only subtilisin A and trypsin. A protease inhibitor cocktail has been shown to enhance the activity of the antifungal peptides, Mucin MUC7 12-Mer-L and 12-Mer-D Peptides, by preventing them from protease degradation (Wei and Bobek, 2005). It was therefore interesting to see if HPI had any effect on the antifungal activity of the latex hevein peptide. The HPI by itself was found to possess no antifungal activity, thus supporting the earlier finding on its ineffectiveness against aspartic protease (Sritanyarat *et al*., 2006), a virulence factor secreted by *C. albicans* (Pichová *et al*., 2001; Skrbec and Romeo, 2002). However, it was shown to enhance the antifungal activity of the hevein. The HPI may help to prevent hevein degradation by inhibiting the intracellular proteases produced after the fungi are killed. Hence, it may be possible for the HPI acting as a natural protease inhibitor to be a candidate for inclusion in the protease inhibitor cocktail used to enhance the effectiveness of antifungal peptides in their therapeutic applications

C. albicans is part of the normal microbial flora of the host and can be found in the digestive, oral cavity and vaginal tracts (Shepherd, 1986) and is a major human opportunistic pathogen, causing both mucosal and systemic infections called candidiasis, especially in immunocompromised patients (Odds, 1988; Odds, 1994). However, an increased prevalence of candidiasis has been attributed to the widespread use of antibiotics and immunosuppressive agents (Gadea *et al*., 1997). At present synthetic antifungal drugs are available for the treatment of *Candida* infections some having very serious side effects to immunocompromised and AIDS patients who suffer from immune dysfunction. In the future it may be necessary to seek out and test naturally occurring antifungal substances in order to treat these patients. However as hevein is a known allergen (Hev b 6.02), its therapeutic application may be limited. Further work will be required to investigate this possibility. An ability to modify the hevein to eliminate its allergenicity without loosing its antifungal activity would obviously create more opportunities for the use of this biologically active 'waste latex protein'.

Part B: Small rubber particles and their enhancing effect on the antifungal drug

Rubber particles (RP), comprising 30-45% of the whole latex volume, are colloidal components in the natural rubber latex. Particle size of the RP in fresh latex varies over wide ranges, from 0.02 to 3 μm (Southorn and Yip, 1968; Gomez and Hamzah, 1989). The 0.1 μm particle size had been observed and recorded to be with maximum distribution frequency (van den Tempel, 1952; Gomez and Moir, 1979). The highly abundant small RP (SRP), mainly those in the zone 2 used in this study, accounts for *ca* 95% of the total RP population in *Hevea* latex (van den Tempel, 1952; Gomez and Hamzah, 1989). Our results in this study showed the SRP was able to strongly induce the yeast cells aggregation. This might probably be through a specific recognition or interaction with N-acetylglucosamine sugar on the 24 kDa glycoprotein of the SRPP. This is deduced from the results on chitinase treated 24 kDa SRPP that was failed to induce the yeast cells aggregation. The chitinase treated SRPP was thus abolished of the 24 kDa glycoprotein active function thru removal of the carbohydrate moiety. Based on this finding, the SRP may thus function as colloidal barriers against the fungal invasion of the rubber trees. Aggregation of yeast cells was, however, not a prerequisite required for the SRPP effect in reducing or lowering the MIC_{80}s of AMB tested against *Candida* species. The positive results on

reduced MIC_{80}s effect seem to be attributed by and directly related to the hydrophobic nature of the SRPP. The higher hydrophobic degree of the tryptic SRPP-derived peptides might probably give rise to the better enhancing effect obtained for lowering the MIC_{80}s, as was supported by result obtained upon lipid solvent fractionation. The trypsin treated study also showed the yeast cell aggregation was not a required factor for the SRPP exerting the enhancing effects on the reduced MIC_{80}s of AMB on *Candida* species. The two effects seem to be of different mechanisms and unrelated.

In fact, the effect pointed out as directly correlated to the hydrophobic nature and properties of the tryptic peptides digest of the SRP proteins. The higher level of the hydrophobic degrees give the better enhancing effect obtained on the MIC_{80}s, as evidence by the solvent fractionation. The tryptic peptides derived from the 14 kDa-SRPP are with molecular weight ranges of 1,045-1,621 and those of 24 kDa are 812-2,270 Daltons. They are all of small amphiphilic heteropeptides, as similar to the AMB molecule of molecular weight 924 Daltons with two distinct hydrophilic and hydrophobic moieties. The compared similar properties may facilitate the formation composite entity via the hydrophobic interactions between AMB and the peptides, so that the potential antifungal action of AMB is enhanced thru lowering of the MIC_{80}s. AMB has been used as the standard treatment for invasive aspergillosis, particularly for severe and life-threatening (Steven *et al.*, 2000). While the AMB is associated with a number of adverse effects or reactions, in many cases the efficacy of the drug outweighs the risk. Further investigation on the enhancing effect of the SRPP may help to reduce the incidence of serious adverse effects of AMB therapy. The lower dose with the higher efficacy was thus documented in these experiments.

The tryptic peptides of the 14 kDa SRP protein seems more attractive for further study and investigation on their potential therapeutic application in enhancing AMB activity, based on the nature and properties of this protein. Since it does not contain any of the known Hev b-1 allergen epitopes, hence the minimum risk on associated allergenic effects. On the contrary, the tryptic peptides of the 24 kDa SRP protein contains one Hev b-3 allergen epitope, making it less attractive to be of potential therapeutic uses. The associated allergenic effect was thus undesirable and needed to be removed, if ever be of potential application, to be used alone or in combination with the peptides of the 14 kDa SRP protein.