CHAPTER IV

DISCUSSION

4.1 Compared measuring methods of chitinase activities

Plants produced chitinases (Cohen-Kupiec and Chet, 1998) in many plant–pathogen combinations. Chitinolytic enzymes were therefore of foremost importance to many living organisms. Bacteria, fungi, invertebrates and vertebrates used the chitinolytic enzyme system to digest chitin. This system consisted of endo- and exochitinase. In this research, we proposed a method for the determination of endo- and exochitinase activity using 4-MU-β-$(GlcNAc)_3$ and 4-MU-β-GlcNAc as substrates for assaying in both chitinases, respectively. This method was more sensitive methods that had been put forward based upon chromogenic (Vergauwen and Van Laere, 1998) or fluorescent chitooligosaccharides (Cottaz *et al*., 2000). In fact, these substrates could be digested by both exochitinases and endochitinases (Ren *et al*., 2000). On the other hand, the most sensitive method for the determination of chitinolytic enzymes was a radiochemical method based on the formation of soluble chitooligosaccharides from tritiated chitin prepared by reacetylation of chitosan with labeled acetic anhydride (Molano *et al*., 1977). Unfortunately, the use of this method was restricted to laboratories with special equipment. The most widely used methods for the detection of chitinolytic enzyme activity were based upon the colorimetric estimation of the reducing saccharides obtained after chitinase action towards its substrate (Miller, 1959; Reissig *et al*., 1955). These methods lacked sensitivity. Previously described methods for endochitinase determination included reduction of turbidity (turbidimetric method) of a fine colloidal chitin suspension (Broadway *et al*., 1995; Jeuniaux, 1966; Trosmo and Harman, 1993) or reduction of viscosity (viscosimetric method) of chitosan, glycolchitin or carboxymethylchitin solutions (Jeuniaux, 1966; Ohtakara, 1988). Turbidimetric method was rapid and accurate, but was suitable only for the estimation of relatively high activities, in limpid and poorly colored solutions (Jeuniaux, 1966). Viscosimetric method of endochitinase activity determination was somewhat troublesome and too time-consuming when numerous samples must

be examined (Ohtakara, 1988). However, all the above mentioned substrates, which were widely used for the determination of chitinolytic activity, were unable to distinguish between the different types of chitinolytic enzymes that chitin-degrading organisms produce, except in chromogenic or fluorescent method.

2. Presence of endochitinase and exochitinase in BFM of *Hevea* latex

Although chitinases have been studied extensively, the nomenclature of chitinolytic enzymes was still confused. However, most enzymes can be characterized either as endochitinases (splitting randomly within the chitin polymer), exochitinases could be divided into two subcategories: chitobiosidases (EC 3.2.1.29) (Harman *et al*., 1993), which catalyzed the progressive release of chitobiose starting at the nonreducing end of the chitin microfibril; and 1,4-β-*N*-acetylglucosaminidases (EC 3.2.1.30), which cleaved the oligomeric products of endochitinases and chitibiosidases generating monomers of GlcNAc. As proposed by Harman *et al*. chitinases from *Trichoderma harzianum* were classified into endochitinases, which cleaved randomly and required at least the tetramer of chitin for activity, chitobiosidases, which released chitobiose from chitin and required the trimer, and glucosaminidases, which released monomeric units and required at least the dimer (Harman *et al*., 1993). The chitobiosidase they proposed, indeed, was usually called exochitinase. Therefore, according to the nomenclature proposed by Harman *et al*. the difference between endo- and exochitinase was that endochitinase required at least tetramer and exochitinase required at least trimer as substrate. All types of chitin-degrading activities had been found in fungi, such as an endochitinase and a 1,4-β-*N*-acetylglucosaminidase from *Aspergillus nidulans*, and an exochitinase from *Mucor rouxii* (Flach *et al*., 1992).

In prelim study, we could detect both endo- and exochitinase activity in BFM of in different *Hevea* latex clones in order to show the common presence of enzyme by tapping induction. The result indeed displayed clonal variations on the chitinases as shown in Table 3. It was found that RRIT 251 clone had the highest of both endo- and exochitinase, with the lowest in BPM-24 clone (Table 3). These data suggested that both endo- and exochitinase were detected for BFM of all the *Hevea* rubber clones, might have active role participating in the plant defense mechanism against pathogens. The rubber trees were prone for opportunistic pathogen attacks

from tapping wounds, presence of the two chitinase forms might provide a combined or active synergistic protection. Besides, the variations might give a clue as to the difference in disease resistant among these rubber clones. In addition, these results were correlated with the report of Chen *et al*. (1996) that both exo- and endochitinase activity were detected in *Xenorhabdus nematophilus*, *Xenorhabdus bovienii* and *Photorhabdus luminescens* by using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-β-D-*N*-*N*'-*N*"-triacetylchitotriose, respectively, as substrates. Also, Kang *et al*. (1999) demonstrated that 60 kDa of purified chitinase from *Metarhizium anisopliae* were composed of both endo- and exochitinase activity. Recently, Xia *et al*. (2001) observed that purified chitinase from *Aspergillus fumigatus* having molecular mass of 46 kDa classified as a semiprocessive enzyme with both endo- and exochitinase activities, which may act processively at the ends of chitin following an initial random attack.

3. Purification of endo- and exochitinase

From previous study, we found that the activity of endo- and exochitinase in RRIT251 clone was higher than that in other clones (PB311, RRIM600 and BPM-24). However, the latex of RRIM600 clone was chosen for all experiments of purification as it's the common clone in rubber plantation that produced latex in high yield.

Endo- and exochitinase from bottom fraction membrane (BFM) of *Hevea* latex were purified by using Triton X-100 extraction, following 2 steps of column chromatography (ion-exchange and gel filtration) and IEF analysis, in the finally. BFM of *Hevea* latex, which was integral membrane proteins, were characterized by a hydrophobic domain that interacted with the hydrophobic core of the lipid bilayer (Tanford and Reynolds, 1976). In order to prepare sample before loading onto the first column, nonionic detergent (Triton X-100) were widely used for the solubilization and extraction of these proteins (Tanford and Reynolds, 1976; Helenius *et al*., 1979).

After loading sample onto cation-exchange column, the elution profile of CM-Sepharose column was shown in Figure 5 with both eluted protein and chitinase activity peaks. Both chitinase (endo-and exo-) activities were found for BFM (Fig. 5). The results showed that the two enzyme forms were eluted in the same protein peak, indicating their ionic properties are

very similar. However, the activity levels of the two enzyme forms were quite much different. Exochitinase was only a small fraction of the overall activities, while the endochitinase was several folds much higher using substrates specific for the two forms for distinction of enzymes. This is not surprising or unexpected, as the chitinolytic activity is mainly the function of endochitinase for generation of oligochitin small fragments for further degradation to the basic monomers as end products. It thus was quite clear that both have very similar properties but only with different catalytic activities for the glycosidic bonds of chitin. The two enzyme forms as eluted from CM-Sepharose column was not yet in the purified forms as evidenced by other co-eluted proteins as indicated in Figure 7 on SDS-PAGE analyses. They need to be further purified so that the homogeneous enzyme forms could be analyzed for properties, on both kinetic parameters and sequence determinations. Characterization in some specific nature of the two enzyme forms is also needed so that their roles in physiological function on the defense can be designated. The 2 enzyme forms as eluted were then subjected to further purify by Sephadex G-75. The G-75 results showed the enzyme solution was separated as eluted major protein peaks of four fractions in this step (Fig. 6). Of the four eluted peaks of protein, only one protein peak was found containing the activity of both endo- and exochitinase (Fig. 6). Elution of both enzyme forms in the same eluted protein peak indicated that they are of very similar or the same molecular weigh. The activity ratio was again found similar to that observed with the ion-exchange column results, with very much higher endochitinase and exochitinase was only a small fraction of the overall activities eluted under the same protein peak. These results indicated that the two enzyme forms are similar or of the same properties, but are only different specific catalytic activities. Similar rationale implied the chitinolytic activity is the main function of endochitinase in generating oligochitin and exochitinase has a minor role in further degradation to end product monomers.

All of these results indicated that could not yet separate the two enzymes from each other physically. Therefore, attempt to use other methods was employed. Other chromatographic (hydrophobic interaction and affinity chromatography) techniques to isolate them were carried out, but was not succeed. Eventually, we tried and discovered the most suitable method in separating these two enzyme forms. The effective method for separate the two chitinase forms was based on the pI differences of the enzymes using the technique of isoelectric

focusing (IEF). From the results of IEF analysis (Fig. 9) coupled with specific assay substrates, it was clearly found that the pH 9.3 protein was identified as exochitinase and the pH 9.5 protein was endochitinase. This is the first report on the clear separation of the two enzyme forms, as never reported before by any investigator. Moreover, the results clearly implicated the prominent role of endochitinase in the chitinolytic process, which was understandable or commonly conceived considering the polymeric nature of the chitin as of the gigantic structure. To complete the chitinolytic process, exochitinase is only needed as a secondary to the endochitinase action upon oligochitin available for complete degradation to the monomer end products. Moreover, all results from purification steps were similar to be Hevamine or endochitinase A and B, in other previous reports (Martin, 1991; Archer, 1976; Tata *et al*., 1983; Jekel *et al*., 1991). It was previously reported that B-serum of *Hevea* latex contained 2 isozymes of chitinase called Hevamine A and Hevamine B. While, Martin (1991) reported seven isoforms of chitinase found in B-serum from which six of them had both lysozyme and chitinase activities.

4. Characterization of endo- and exochitinase

Purified *Hevea* endo- and exochitinase, which separated from IEF analysis, were studied about the properties (pH, temperature and kinetics parameters).

With optimal pH, it was found that at pH 5, both enzymes were with the highest activity levels (Fig. 11). The relationship between activity of both enzymes and the pH of the assay systems was shown in the Figure 11. The results showed that the extent of these chitinases reaction was within the pH narrow ranges. Maximum activity was between pH 4-6 and then it sharply drops outside this range with the peak activity at pH 5, whereas a bean chitinase retained 50% activity at pH 3 and 9 with an optimum at 6.5 (Boller *et al*., 1983). The same optimal pH for endo- and exochitinase as reported in these findings might be of significant in term of the enzyme function. Complete degradation of natural chitin substrate requires both enzyme forms working together in a manner of assembly sequential actions. Both endo- and exochitinase work in a concerted fashion in chitinolytic process under the same mixture, so it would be natural for both to function maximally under the same pH of the common setting. While prominent role of

endochitinase was of key important as clearly conceived in the earlier finding on activity ratio of 12 folds higher, but still the need for exochitinase is also essential for complete chitin degradation. Therefore, it's quite common to expect both the enzyme forms to have the same optimum pH, not only for each own but also the combined maximum activity. In addition, pH stability of the 2 purified enzymes as shown in Figure 12 indicated that both purified enzymes were quite stable over a broad pH range of 3-7 and then declined at pH above 7. Activity as remained at pH 8-9 was lower than those at pH 3-7. However, they both were quite stable in the pH ranges 3-9 as tested in this study as about 80% activity of both enzymes still remained at pH 9. The results indicated and clearly showed that they were more stable in acidic condition and become slightly less stable at alkaline pH above 7. But yet they displayed unique property of pH stability between pH ranges 3-9.

For optimal temperature, the results in Figure 13 demonstrated that both purified enzymes were quite sensitive to the temperature effect. Maximum activity was observed for both enzymes in the range of 30-45°C. The activity was then sharply dropped at above 45°C with hardly any activity detected at 55°C and above. In the other hand, carrot chitinase retained 65% activity after treatment at 60°C (Zhang *et al*., 1996), wintermelon chitinase remained approximately 75% activity after 70°C exposure (Shih *et al*., 2001) and a class III yam chitinase was shown to be resistant to temperatures of 80°C (Tsukomoto *et al*., 1984). Behavior of the 2 purified enzymes was almost identical as seen by superimposition of the 2 curves for both purified enzymes. These results thus clearly indicated that these two purified enzymes exert maximum activity only in the 30-45°C ranges. Moreover, thermal stability of the 2 purified enzymes as shown in Figure 14 revealed that the two separated purified enzymes were quite heat stable at up to 75°C. Nearly full remaining activity was observed at 75°C. The thermal stability of both endo- and exochitinase was from 37-75°C with almost no loss of the activity for both the separated purified enzyme forms. These results thus indicated that both chitinases were quite a heat stable protein.

To determine the kinetics parameters (Km and Vmax) of endo- and exochitinase, enzymes saturation curves need to be characterized first. The results obtained in the study (Figure 15-16) indicated the endochitinase was much more active than the exochitinase, based on the maximum activity attained earlier at much lower enzyme concentration. The difference was

almost 3 folds lower for endochitinase reaching the Vmax (at 15 μg) as compared to 40 μg for exochitinase. This might be taken as a common understanding and logical rationale considering the tremendous chitin molecular size with numerous endo-glycosidic bonds required to be hydrolyzed before the exochitinase to act upon small oligochitin with fewer bonds.

The Km and Vmax values of endochitinase were calculated from double reciprocal plots (Lineweaver-Burk plots) to be 99.73 μM and 666 unit/ml of 4-MU-β-$(GlcNAc)_3$, respectively (Fig. 17), while the Km and Vmax values of exochitinase was found to be 0.61 mM and 526 unit/ml of 4-MU-β-GlcNAc, respectively (Fig. 18). These Km value when compared with those obtained for endochitinase in the previous experiment showed quite a large difference, which showed that endochitinase was more sensitive than exochitinase about 6 times while Vmax value of both enzymes was not quite different. It should be noted that the kinetic parameters (Km and Vmax) as determined in this study was carried out with the artificial synthetic substrates. These substrates are labeled with fluorophore (4-MU) tagging to the low molecular weigh small molecules as 4-MU-β-$(GlcNAc)_3$ for endochitinase and 4-MU-β-GlcNAc for exochitinase. The fluorophore (4-MU) tagging is for the sensitivity purpose in the assay that might not be reflecting the real values for the natural chitin substrates. It's very likely that the kinetic parameters (Km and Vmax) for the two enzymes might be different from these values in the assays and determined with the chitin natural substrates. Therefore, the chitin natural substrate assays should be carried out and compared.

5. Analysis of the N-terminal sequence of purified endo- and exochitinase

N-terminal sequencing of their purified chitinases yielded 10 amino acid residuals as shown in Figure 19. The comparison of N-terminal amino acid sequencing between endo- and exochitinase (Fig. 19) showed that they did not similar sequence homology with each other. Also, N-terminal sequencing of both chitinases were corresponding with pI of them that indicating pI of endochitinase was higher than that in exochitinase. On the basis of these alignments we believed that both chitinases should be classified in family 18 due to their enzymes could hydrolyze these chromogenic substrates (4-MU-β-$(GlcNAc)_3$ and 4-MU-β-GlcNAc for endo- and exochitinase, respectively). The analysis of amino acid composition in endochitinase

showed that this enzyme was rich in basic amino acids (Arg), which was not identical to other reported in chitinases, whereas amino acid composition of exochitinase was less basic than that in endochitinase as shown in Figure 9, which not like to previous reports in chitinases, but similar sequence homology to Hevamine (*endochitinase* in *Hevea* latex) about 50%. From all results, we could summarize that both enzymes, which had the chitinase enzyme properties as verified by the enzyme activity assays and unique characteristics as reported with several detailed criteria in this study, might be a new type in the large family of the chitinases.