CHAPTER III

RESULT

Natural rubber and other polyisoprenoids obtained from plants are high molecular weight hydrocarbon polymers consist almost entirely of the 5 carbon isoprene units, C_5H_8. These polyisoprenoids (esp. rubber) are the major components of the latex, natural rubber latex (NRL), synthesized by specially differentiated cells of the plants and other living organisms. The detailed studies on structure of these polymers have shown that the double bonds in the rubber from *Hevea* and also guayule are in the *cis* configuration, *cis*-1,4-polyisoprene, and those from *gutta percha* and *chicle* are in the *trans* configuration, *trans*-1,4-polyisoprene. The natural polyisoprenes are synthesized by enzyme-catalyzed polymerization of isoprene units to various different degrees resulting in a wide range of the molecular weights, depending on the sources which they are derived from. *Hevea* rubber is a typical high molecular weight *cis*-polyisoprene with a very wide range of molecular weights distribution. *Gutta percha* and *chicle* are *trans*-polyisoprenes with relatively much lower molecular weight than the *Hevea* rubber (Tanaka, 1991). In general, the molecular weight of these different polyisoprenes in the latex can range from a few thousand daltons up to several million daltons.

Numerous plants belonging to several different families can form and containing latex. The rubber is hight molecular weight polyisoprene produced in the latex of about 300 genera of Angiosperms. The milky latex fluid will flow from these plants with a slight incision of the tissues. In guayule (*Parthenium argentatum*), the latex is produced and stored in parenchyma cells (Backhaus and Walsh, 1983). But more commonly in most plants, the latex is produced and stored in the tubular structure known as laticifers. The most prominent and well studied among these is *Hevea brasiliensis*. The laticiferous system of the rubber tree (*Hevea*) has been extensively reviewed (de Fay *et al*., 1989).

The latex composition is quite varied among the different plant species. The polymers in the suspension of which it is formed may contain varying proportion of rubber and other different compounds. Of some 12,500 species of laticiferous plants, about 7,000 are found to produce polyisoprenes. In most case the polyisoprene is with mixed resin, making the latex

difficult to use when the content of resin is high. A limited number of rubber-producing plants can be suitably utilized and only a few species are cultivated and have economic importance. Among them, *Hevea brasiliensis* is proved to be the best rubber producer. A few hundred ml of latex can be obtained from each tree by simply incising the bark, the common practice of tapping the rubber trees.

Hevea brasiliensis is used commercially for production of the natural rubber that is used industrially for various finished products. High-yielding clones of *Hevea* have been selected and developed leading to the high productivity of the cultivars in rubber plantations, especially in Southeast Asia. The plant breeders have bred out to a great extent that the regulatory mechanism which control of the carbon sources derived from photosynthesis are activity channeling into rubber formation. This has thus resulted in far more rubber being synthesized than would be produced in normal or the native clones of the *Hevea.* The discovery that latex production in *Hevea* can be stimulated by plant hormone, ethylene, is obviously of advantage to rubber planters. This has led to a great deal of research for understanding hormonal stimulation mechanism. Today, ethylene generator (Ethephon) is commonly used to stimulate the *Hevea* latex production. The effect of high rubber production on growth is quite considerable in rubber trees of high-yielding clones, as evidenced in the reduction in girth increment of the trunk of rubber trees.

The latex that contains the rubber particles are accumulated in the very specialized cells or vessels known as laticifers. In *Hevea*, the rubbers are formed and stored in rings of laticifers in the bark. Anatomoses between adjacent vessels in the rings allow the latex from a large area of cortex to drain upon tapping. The opening of the latex vessels from tapping cuts causes latex to flow out due to the high inside turgor pressure. Flowing of latex will continue for a certain length of time and subsequently stop due to rubber particle coagulation and flocs formation and plugging of the vessel ends. The latex is specialized cytoplasm containing several different organelles in addition to rubber particles. Organelles include nucleus, mitochondria, endoplasmic reticulum fragments and ribosomes. In addition to these minor components, there exist two important major specialized particles which are unique characteristics of the *Hevea* latex, namely the lutoids and Frey-Wyssling particles.

Lutoids, a major component of latex, are osmotically active vescicles of 1-3 μm in diameter. They are grey in color and bounded by a single unit membrane. Characterization and biochemical analyses were studied and clarified to some extents. Lipids of the lutoids membrane are quite rich in phosphatidic acids and saturated fatty acyl residues. The name lutoid (= yellow) turned out to be a misnomer. The lutoids as originally isolated were coexisted and mixed or contaminated with the yellow Frey-Wyssling particles which contain a high content of β-carotene. The Frey-Wyssling particles represent a minor component of the latex vesicles. The particles are bounded by a double membrane containing many membrane tubular structures and β-carotene giving it characteristic orange to yellow color layer upon fractionation by ultracentrifugation of the fresh *Hevea* latex.

Generally, the latex can be fractionated into 3 distinct zones as the top rubber layer, the middle fraction aqueous phase of latex called C serum and the relatively heavy bottom fraction consists mainly of the lutoids. Separation of the latex into three major zones is by ultracentrifugation, on which their chemical compositions can then be determined. Bottom fraction (BF) can be studied by repeat freezing and thawing of lutoids. In this manner membrane of the lutoids are ruptured and the liquid content, referred to as B serum, can be analyzed. The B serum has been found to contain several proteins and enzymes as well as metal ions. It can thus be visualized that *Hevea* latex is a cytoplasmic system consisting of various particles (rubber and BF membrane organelles) dispersed in the aqueous serum phase. Several enzymes in the lutoid B-serum have been extensive studied (Wititsuwannakul, 2001), but the BF membrane (BFM) associated enzyme are yet to be studied. It's therefore investigated and determined in this study.

The BF membrane (BFM) associated enzyme or as the membrane bound enzyme was investigated for chitinolytic activity, namely the function of chitinase enzymes. We have previously studied the presence and activity of chitinase in the B-serum (Punya, 1994). However, reports on the findings and presence of membrane bound chitinases in fungi (Balasubramanian and Manocha, 1992; Sakurada *et al*., 1998) has prompted us to investigate the possible existence of *Hevea* membrane bound chitinases in the BF organelles. Lutoids derived from the centrifuged fresh latex BF was employed for collection of membrane in this study. The membrane as BFM was washed clean by ultracentrifugation of the lutoids homogenate pellet prior to assay for chitinase activity. Both the membrane bound and solubilized forms of the enzyme were

determined and found quite active. For analyses of the enzyme properties, the solubilized form is presented. The detailed findings in this investigative study are as described below as to the results obtained. Characterization and biochemical analyses on the enzyme properties on various aspects strongly pointed out the importance and possibly significant role of this new found form of chitinase enzyme activity as will be subsequently described and discussed further.

Chitinase is enzyme for hydrolytic digestion of chitin collectively called chitinolytic process. However, it's difficult to distinguish the hydrolytic types using the common chitin substrate as the enzymes are of two forms being endo-chitinase or exo- chitinase. However, with the advent of new fluorophore labeled substrate has made it possible to clearly differentiate and determine the two enzyme forms for detailed characterization.

The BFM was interestingly found comprising the two forms of enzymes, both endo- and exochitinase, in this study. This is the first clear finding in plants that was earlier difficult to delineate the two chitinolytic types.

These BFM enzymes were probably related to wounding response of the rubber trees from tapping cuts as the wound induced enzyme in defense mechanism against attacks by fungus and other microorganisms. Induced chitinases occurred in a number of higher plants catalyzing the hydrolysis of chitin. Since chitin is not present in plants, the enzyme had no apparent function in the plant primary metabolism. Indirect evidence suggested a defense role against fungal pathogens since chitin was the key important component of fungal cell wall. Chitinases had also been identified in the laticifers of numerous latex-containing species.

Hevea brasiliensis was one of the well-known latex-containing plants that had previously been reported to contain chitinase in the latex. Studies on *Hevea* chitinase or Hevamine were reported with enzyme characterization and its antifungal property (Martin, 1991; Archer, 1976; Tata *et al*., 1983; Jekel *et al*., 1991), but as yet no clear indication for the two enzyme forms nor the exact chitinolytic types indicated in the past reports. With new substrates available for assaying the two enzyme forms, distinct characteristic on properties and kinetic parameters can be clearly presented. In this study we reported that the enzymes, containing both endo- and exochitinase activity was present and found in the latex. They were purified and characterized for the enzyme properties in various aspects for a better understanding of the

chitinase. Potential application in the uses of enzymes producing *N*-acetylglucosamine is also discussed.

1. Clonal variations of the *Hevea* latex chitinases

The *Hevea* latex bottom fraction (mainly lutoids) from centrifuged fresh latex contains several pathogenesis-related (PR) proteins as the induced enzymes in the wounding response. These induced PR enzymes include chitinases, β-1,3-glucanase and hevein (Churngchow *et al*., 1995, Subroto *et al*., 1996, Van Parijs *et al*., 1991). The membrane chitinase found with the BFM was presumed to be one of those pathogenesis-related proteins. The accumulation of PR proteins in the lutoids was evidently the wound response from tapping cuts or injury of rubber trees for harvesting latex. Previous reports on *Hevea* latex chitinases could not show the enzymes were either endo- or exochitinase and might be both in the assays. With new substrates available (4-MU-β-$(GlcNAc)_3$ for endo- and 4-MU-β-GlcNAc for exo-) we are the first to the two enzyme forms in *Hevea* latex.

In the first experiment, detection of chitinase activity in different rubber clones was determined in order to show the common presence of enzyme by tapping induction. The study was also to show the different levels of enzyme and variations of rubber clones (RRIM600, RRIT251, PB311 and BPM-24). 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. With this data, it might be of use in the clonal selection process for superior rubber clones as to disease resistant based on better defense mechanism and high-yielding criteria.

Table 3. Comparison of endo- and exochitinase activity in BFM of four rubber clones.

Rubber clone	Total activity (unit) / Fresh latex (Liter)	
	Endochitinase	Exochitinase
RRIM600	141	132
RRIT251	446	336
PB-311	381	284
BPM-24	121	113

2. Purification of the BFM chitinases

As earlier mentioned, the membrane derived from the BF organelles was found with the two forms of the enzyme, both endo- and exochitinase as shown in Table 3. Since they were of different activity levels, it would be of interest to further characterize the two enzyme forms. Purification was then attempted in order to separate the two by employing the protocols as previously used for the B-serum by stepped column chromatography.

Fresh latex could be fractionated and separated by centrifugation into 3 fractions as previously described, and the sediment fresh BF was isolated for the membrane (BFM). Archer (1976) had isolated two major B-serum basic proteins with very similar amino acid compositions from lutoid as called Hevamine A and B, respectively. Tata *et al*. (1983), in using a similar isolation procedure, isolated the same protein and demonstrated that they were chitinase by comparing the N-terminal sequences of Hevamine to the chitinase of *Parthenocissus* and cucumber showing them for very similar sequence. Jekel *et al*. (1991) had also confirmed these results by the primary structures characterization. Martin (1991), using a different method isolating seven isozymes of chitinase in lutoid solution and characterized enzymic physical properties of the *Hevea* chitinase. Punya (1994) reported that *Hevea* chitinases had three isozymes which had the same molecular weight of 26 kDa. In these reports, they demonstrated that chitinase or Hevamine could be detected in B-serum of the latex, but none has shown the existence or presence of BF membrane associated as a different enzyme form.

In this study, we found the membrane bound form of chitinase as has not been previously detected or reported. This is in spite of the reports (Balasubramanian and Manocha, 1992; Sakurada *et al*., 1998) on membrane bound chitinase in fungi and other microbes. We therefore initiate the study in this research using BFM of the centrifuged fresh latex to purify and characterize the membrane bound chitinase as outlined here.

2.1 Isolation of endo-, exochitinase by ion exchange chromatography

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. The solubilized BFM was treated by anion exchange chromatography as the first step using CM-Sepharose column. This was based on the enzyme nature as basic proteins. Testing with a cation (DEAE) exchange column showed that it was unbound, but was highly retained in anion (CM) one.)The BFM of lutoid particles prepared by repeat (4-5 x) freeze-thawing at -20 and 37°C and be washed clean by centrifugation. The BFM pellet was washed five times (50 mM Tris-HCl buffer at pH 7.4 with 0.9% NaCl) to remove contaminants. Washed BFM was then extracted with 0.2% Triton x-100 (called BFM-X). Before loading sample on CM-Sepharose column, Triton x-100 was eliminated by SM2 (polystyrene) beads as in Methods.

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.

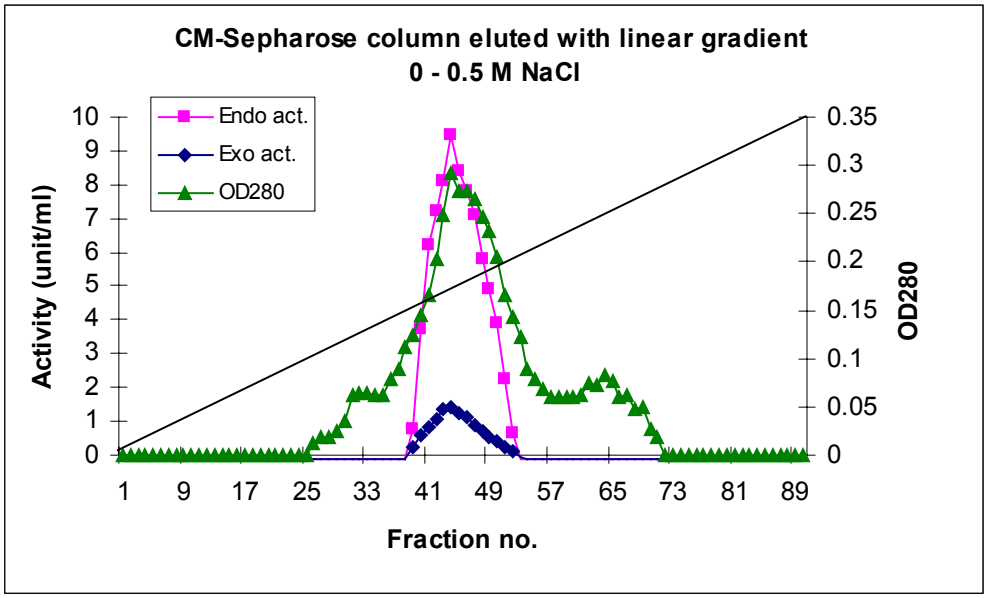

Figure 5. Elution profile of endo- and exochitinase activity and OD_{280} of the fraction from CM-Sepharose column chromatography. Concentrated BFM-X of *Hevea* latex was loaded onto the column equilibrated with 50 mM Acetate buffer pH 5.0 and eluted with linear gradient of sodium chloride 0-0.5 M in same buffer. Fraction sizes of 2 ml were collected at the flow-rate of 0.5 ml/min.

2.2 Endo-, exochitinase purified by size exclusion chromatography

To further purify both chitinase forms and remove other impurities, the size exclusion chromatography was employed as the next step. Sephadex G-75 was chosen as the suitable gel filtration chromatography, based on satisfactory results obtained for the B-serum chitinases. The enzyme from eluted ion-exchange chromatography was concentrated to a small volume as described in the Methods. The concentrated fraction contained activity of both endo- and exochitinase was with a bit higher specific activity, due to the more stable and removal of dilution effect on enzyme solution.

The concentrated enzyme solution from ion-exchange column was loaded onto Sephadex G-75, equilibrated with the same buffer. 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.

The resulted Sephadex G-75 purification step was found very satisfactory as revealed by a single protein band in the SDS-PAGE analyses (Fig. 7). This is a much improvement in the purification protocol compared to the reports by others. The two enzyme forms were then quite suitable for the more detailed characterization and analyses of properties in the later part. Purification protocol for the nature of two enzyme forms were compared and summarized as detailed in Table 4.

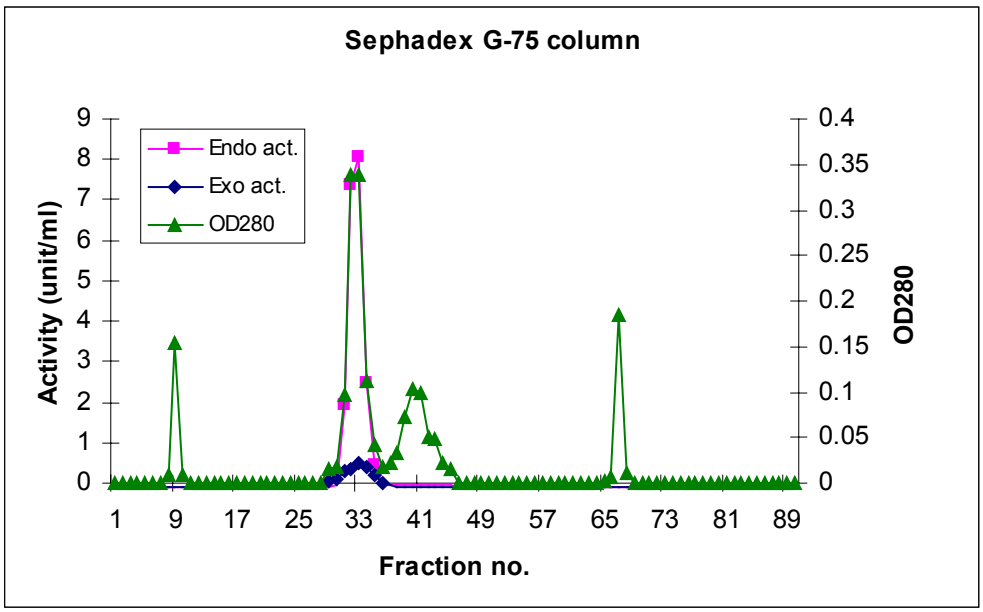

Figure 6. The elution profile of endo- and exochitinase activity and OD_{280} of the fraction from Sephadex G-75 column chromatography. Concentrated protein, contained endo- and exochitinase activity, were applied to a Sephadex G-75 column equilibrated with 50 mM acetate buffer pH 5.0. Eluted proteins from the column were monitored at 280 nm and 2 ml fractions were collected at the flow-rate of 0.5 ml/min.

2.3 Comparison on endo- and exochitinase in the purification steps

Summary on purification results as reported for the two enzyme forms as in Table 4 was for comparison on the activity ratio and their distribution. CM-Sepharose chromatography showed only one protein peak contained endo- and exochitinase activities. These results suggested that both were basic proteins by virtue of binding to cation exchange column at pH 5.0. In this purification step, endochitinase activity was 61 % recovered, while exochitinase activity yielded only 4%. Specific activity of 29.04 and 1.24 unit/mg proteins was found for endo- and exochitinase, respectively.

The concentrated enzyme of ion-exchange eluted protein was loaded onto Sephadex G-75 column equilibrated with the same buffer was eluted into four major protein peaks. Only one protein peak contained the activity of both endo- and exochitinase. In this step,

endo- and exochitinase activity recovery was 19% and 2% with final specific activity of 38.84 and 3.34 unit/mg protein, respectively. The results were as summarized in Table 4.

Table 4. The purification protocol of endo- and exochitinase from BFM of *Hevea* latex.

Step	Total protein (mg)	Total activity (unit)		Specific activity (unit/mg)		Recovery (%)	
		endo	exo	endo	exo	endo	exo
BFM-X	51.85	77.25	54.30	1.49	1.05	100	100
CM-Sepharose	1.61	46.76	2.00	29.04	1.24	61	4
Sephadex G-75	0.38	14.76	1.27	38.84	3.34	19	2

3. Analyses of endo- and exochitinase by gel electrophoresis

The endo- and exochitinase derived from BFM extraction was measured for the activity by using the specific substrate (+ chromophore) to identify and distinguish the two enzyme forms. Upon their purification as outlined in the preceding parts, they were then analyzed by SDS-PAGE for criteria of homogeneity. Zymogram analysis was performed to identify activity of the separated protein bands. In addition, analysis by isoelectric focusing (IEF) was also carried out for determining pI of the two enzyme forms. The results thus obtained will then enable us to differentiate the enzymes and better characterization on their unique properties.

3.1 SDS-PAGE analyses of the purified enzymes

Electrophoresis analysis was performed on SDS-PAGE with 12% gel in order to verify the homogeneity of the purified enzymes in each step of the protocols. Information on the molecular mass data was also obtained for molecular weigh of the two enzyme forms in the same analyses. The protein patterns revealed by SDS-PAGE analysis was shown in Figure 7. The

solubilized BFM crude enzyme homogenate displayed approximately 5 major protein bands (Lane 2) with some minor faint bands. After being purified by CM-Sepharose step, major protein band of 30 kDa was clearly observed (Lane 3) together with small amounts of some other proteins as present in the starting homogenate. Further purified of the enzyme by gel filtration chromatography (Sephadex G-75), both enzyme forms showed only single protein band as revealed by the one major protein of 30 kDa on SDS-PAGE (Lane 4). The results clearly indicated that the two forms of enzyme were both of the same molecular weigh as 30 kDa protein.

To justify the purity and homogeneity of the two enzymes, we increased the amount of protein from 10 to 20 μg to confirm no other contaminant protein was detected at 20 μg level. The same single 30 kDa protein band was clearly demonstrated as shown on SDS-PAGE (Lane 5), but with a higher intensity of the band staining. The result was thus as confirmation on the enzyme homogeneity for both enzyme forms.

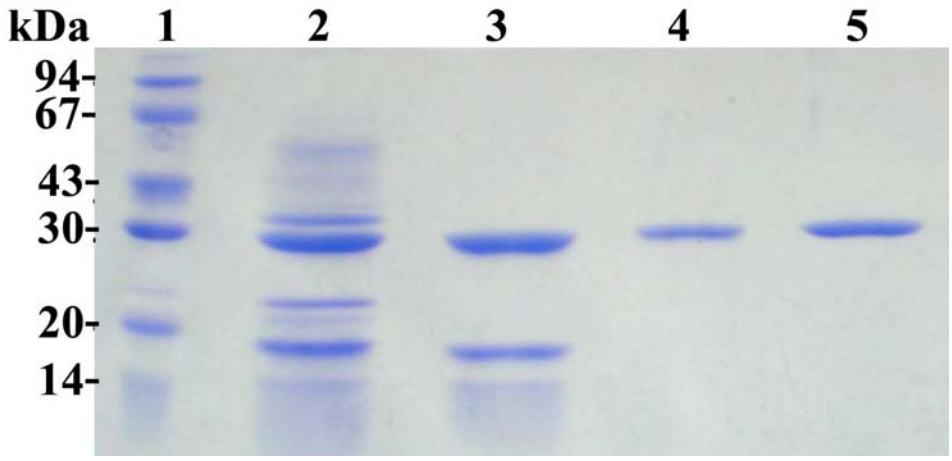

Figure 7. Analysis of *Hevea* latex endo- and exochitinase in BFM on 12% (w/v) of polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE). Proteins were stained with Coomassie blue R 250. Lane 1: Standard protein marker, Lane 2: Bottom fraction membrane extracted with 0.2% Triton x-100 (BFM-X, 10 μg), Lane 3: Bound chitinase in CM-Sepharose (10 μg), Lane 4 and 5: Purified chitinase from Sephadex G-75 (10 and 20 μg, respectively).

3.2 Zymogram analyses of the purified enzymes

As shown under SDS-PAGE analyses (Fig. 7), one single band of the purified protein indicated that endo- and exochitinase were of the same molecular weigh of 30 kDa as calibrated from Figure 7. In order to make sure that the purified protein was really the chitinase enzyme, the activity staining of the 30 kDa protein band analyzed by zymogram technique as described in details in the Methods.

To visualize the chitinolytic activity of the two enzyme forms, zymogram analysis on SDS-PAGE was carried out. The purified 30 kDa protein was loaded onto 12% (w/v) polyacrylamide gels which was incorporated with glycol chitin used as substrate for detecting the activity of chitinase . Both protein staining and activity staining were done in the same SDS-PAGE. The result shown in Figure 8 indicated that the 30 kDa protein did indeed have the chitinolytic activity. The position of the activity staining was at the same of the 30 kDa protein position. The results (Fig. 8) confirmed that the activity staining or zymogram analysis of the purified chitinases was the 30 kDa protein. The homogeneous 30 kDa protein was composed of endo- and exochitinase, as revealed for the one single band staining of both protein (Lane 2, 3) and chitinase activity staining of the zymogram analysis (Lane 4) as visualized and shown in Figure 8.

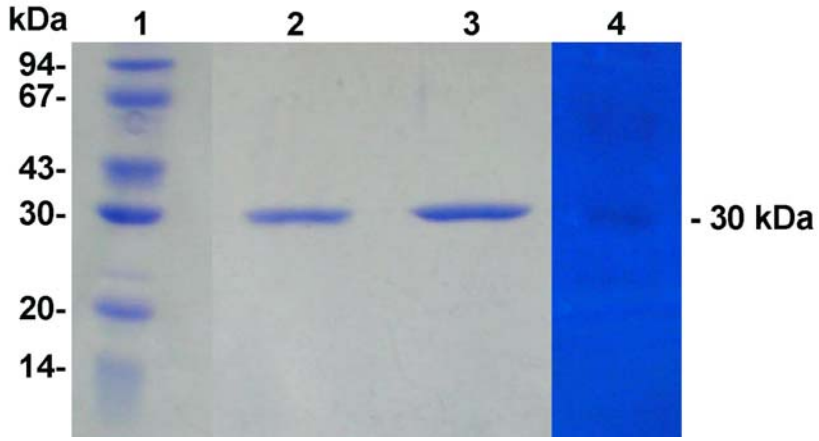

Figure 8. SDS-PAGE and zymogram analysis (chitinase activity staining) in 12% (w/v) polyacrylamide gel. Lane 1: Standard protein marker, Lane 2 and 3: Purified chitinase from Sephadex G-75 (10 and 20 μg, respectively), Lane 4: Chitinase activity staining after SDS-PAGE in gel containing 0.01% (w/v) glycol chitin of purified chitinase from Sephadex G-75.

3.3 Isoelectric focusing (IEF) analyses of the purified enzymes

Isolation and purification of the two enzyme forms as precede outlined in the protocols was by far success to a certain extent for identifying endo- and exochitinase by substrate specificity criteria. As demonstrated from the results, several steps in the purification still showed both chitinases as the co-migrated proteins, be it by chromatography or SDS-PAGE.

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).

IEF in agarose gel was performed in the pH ranges of 3-10. The purified enzymes containing endo- and exochitinase activity was subjected to IEF. After isoelectric

focusing was completed, supporting gel was cut into two parts. The first part of gel was placed in a plastic box containing 50 mM acetate buffer pH 5.0, while the second part of gel was fixed by the fixing solution (10% TCA). This was followed by staining for the protein using Coomassie blue G 250 to locate the isolated protein bands according to the differences in pI of the enzymes. The proteins stained as separated by IEF were found to be composed of two distinct bands at the pH of 9.3 and 9.5. The focused separated two distinct protein bands clearly indicated the two enzyme forms have the different pI and could be separated from each other physically. To identify and differentiate the enzyme forms, the two positioned proteins were eluted from the IEF gel and differential assayed for identifying the enzyme form activity (endochitinase or exochitinase) based on the specific substrates for assay determination as described.

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. It was thus quite suitable and satisfactory for separating the two chitinase forms using IEF, based on the pI differences and the specific differential substrates assay. The results shown in Figure 9 indicated that the endochitinase pI was 9.5 and of exochitinase were 9.3 that can be clearly separated by IEF as demonstrated in this study. It was also of noteworthy that they were similar basic proteins of high pI (9.3 and 9.5), but with different catalytic specificity for the chitin substrate.

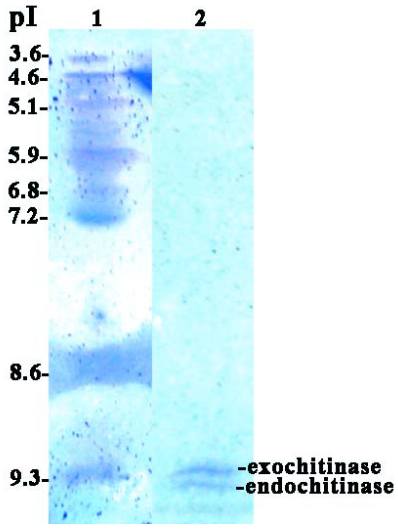

Figure 9. Isoelectric focusing gel electrophoresis of endo- and exochitinase stained with Coomassie blue R 250. Lane 1: Standard protein marker, Lane 2: Purified chitinase from Sephadex G-75.

3.4 SDS-PAGE analyses of the IEF purified enzymes

The extracted and gel eluted IEF purified enzymes as described in the preceding part was concentrated for SDS-PAGE analyses for molecular weigh determinations as the two distinct separated purified enzymes. This is to compliment the earlier analyzed results and to verify that they are of the same molecular weigh. The results (Fig. 10) clearly showed that the pI 9.5 endochitinase (Lane 2) and pI 9.3 exochitinase (Lane 3) displayed the single protein band exactly the same molecular weigh of 30 kDa. The Figure 10 results thus served as supporting evidence for the single protein band seen for the two enzyme forms in Figure 7 and Figure 8 zymogram. In addition, the protein profiles in Figure 10 and 11 showed homogeneity of the two enzyme forms highly suitable for more detailed characterization.

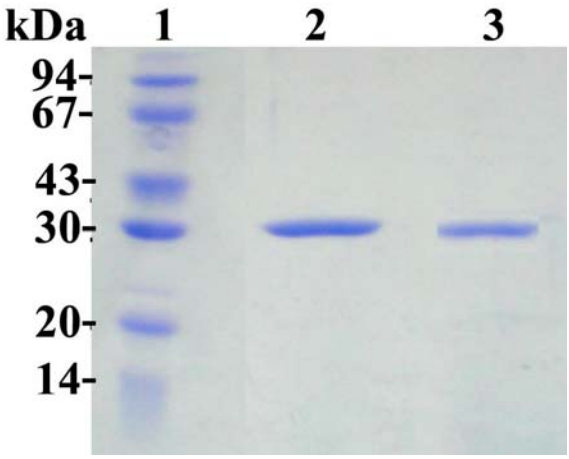

Figure 10. Analysis of *Hevea* latex endo- and exochitinase from IEF preparation on 12% (w/v) of polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE). Proteins were stained with Coomassie blue R 250. Lane 1: Standard protein marker, Lane 2: Endochitinase fraction derived from IEF, Lane 3: Exochitinase fraction derived from IEF.

3.5 IEF purified enzymes and properties

The properties of the IEF purified enzymes were briefly summarized to differentiate the two enzyme forms in Table 5. As preceding indicated, the endochitinase of pI 9.5 could be clearly separated from exochitinase of pI 9.3, but they were of the same molecular weigh as 30 kDa protein. The IEF purified enzymes showed similar specific activity of 38.84 and 3.34 unit/mg protein for the endo- and exochitinase, respectively as in the Table 5. The endochitinase was almost 12 folds higher on activity ratio as to the exochitinase. The results thus 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.

Table 5. Physical and enzymic properties of endo- and exochitinase in BFM.

Fractions	Molecular mass (kDa)	pI	Specific activity (unit/mg)
Endochitinase	30	9.5	38.84
Exochitinase	30	9.3	3.34

4. Characterization of the purified BFM chitinase

Having a success in separation of the purified two enzyme forms of the chitinase together with the basic properties studied, we're now ready for more characterization of the separated enzymes. Both endochitinase and exochitinase will be investigated as distinct entity in this study, so that a specific nature and unique enzyme behavior or property can be assigned to each of the two forms. Since this is the first and pioneer study on the purified two enzyme forms, the data obtained would be valuable in the future subsequent study and better understanding of the chitinase roles in plant system for both the endo- and exo- on chitinolytic catalysis. Several criteria will be characterized, including the kinetic study on the separated enzyme forms for differentiation using kinetic parameters.

4.1 Optimum pH study for the two separated purified enzymes

This study was on determinations of pH optimum for maximum activity. To investigate the optimal pH of endo- and exochitinase, the two purified enzyme forms (endo- or exochitinase) obtained from IEF (1 μg) were for assays using specific substrates (4-MU-β-$(GlcNAc)_3$ for endo- and 4-MU-β-GlcNAc for exochitinase) in vary pH ranges of 3-9. The optimal pH for the two purified enzyme was shown in Figure 11 on the maximum activity determined in the pH ranges 3-9. It was found that at pH 5, both enzymes were with the highest activity levels.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. 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.

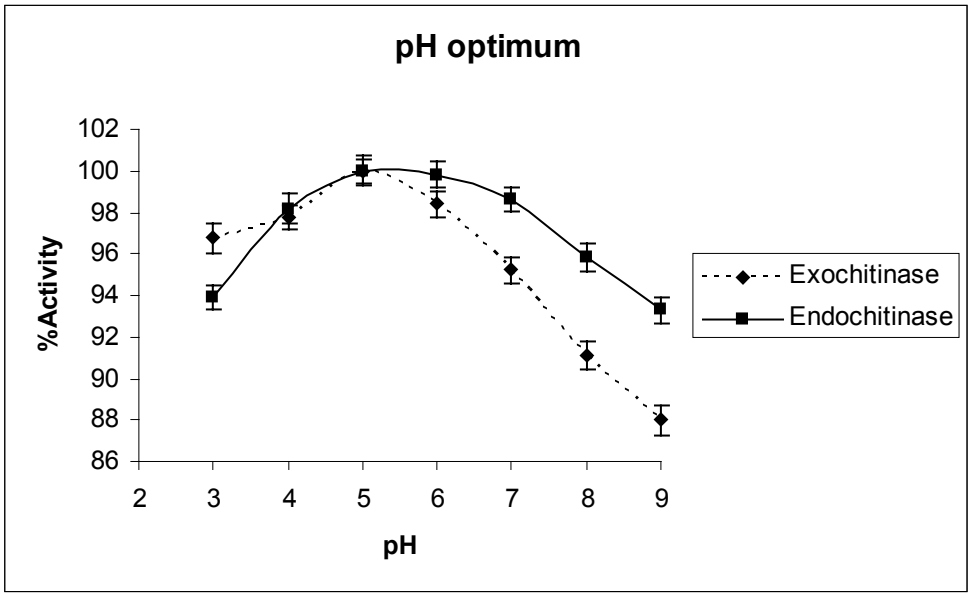

Figure 11. Effect of pH on endo- and exochitinase activity. One microgram of the purified enzymes (endochitinase or exochitinase) obtained from IEF was assayed in a various pH values from pH 3-10. The assayed mixture was incubated at 37°C for 20 min. The activities of both chitinases were plotted against the final pH of each reaction.

4.2 pH stability of the two separated purified enzymes

Stability of the two separated purified enzymes under different pH was investigated in this study. To study the pH stability of the two purified enzyme forms in this experiment, the optimum assay condition at pH 5 was chosen to monitor the enzyme activity remained after preincubated under different ranges of pH 3-9. This is in order to see how well they can withstand the different pH environment prior to the optimal pH 5 assay.

The experiment was carried out by preincubating the 2 purified enzymes obtained by IEF (1 μg) at various pH ranges of 3-9 and then readjusted to pH 5 for determination of the remained chitinases activity. The results 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.

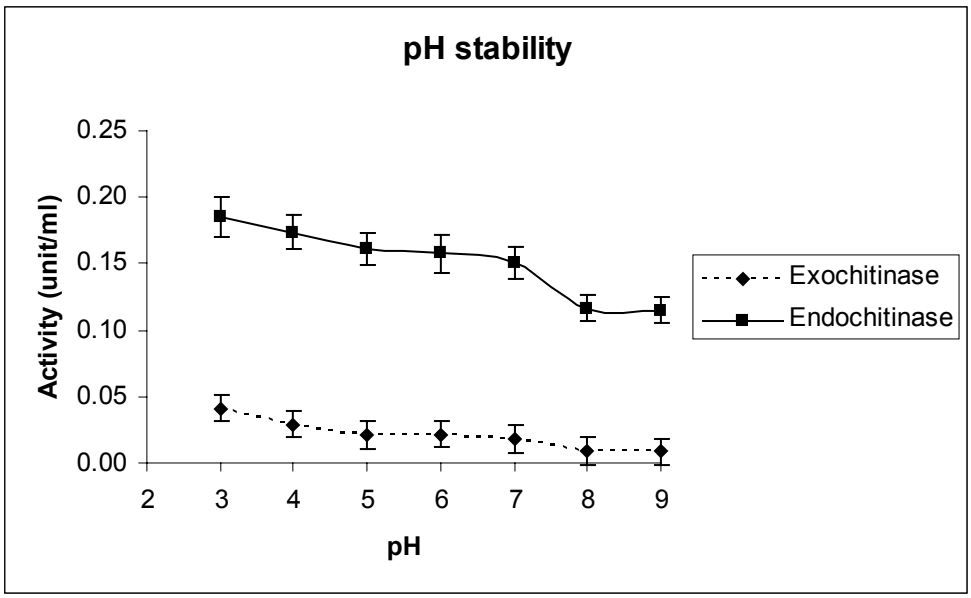

Figure 12. pH stability of endo- and exochitinase. The purified enzymes (endochitinase or exochitinase) obtained from IEF were preincubated for 20 min at various pH (3-9) and then adjusted to pH 5.0. An aliquot of 1 μg enzyme (endochitinase or exochitinase) was assayed for the remained activity in 50 mM sodium acetate buffer pH 5. The reaction mixture was incubated at 37°C for 20 min. The percentages of the activity compared to the control were plotted against pH.

4.3 Optimum temperature for the two separated purified enzymes

Temperature has a key role in enzyme functions on activity and stability. To determine the optimum temperature for chitinases assay, both enzymes were determined at various temperature ranges from 30-70°C. 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. 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.

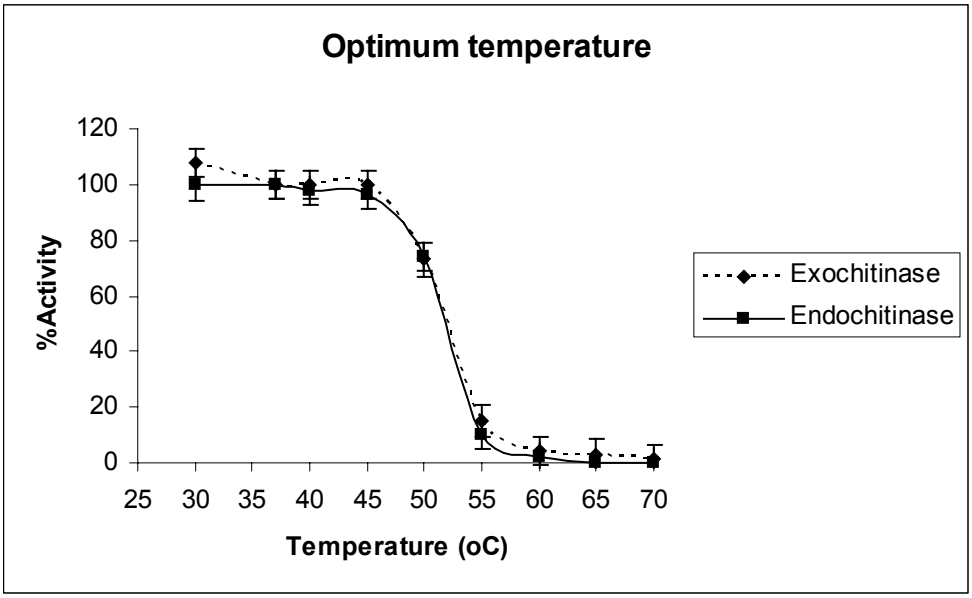

Figure 13. Effect of temperature on endo- and exochitinase activity. One microgram of the purified enzymes (endochitinase or exochitinase) obtained from IEF was assayed for enzyme activity at different temperature from 30°C to 70°C. The reaction mixture was incubated for 20 min in 50 mM sodium acetate buffer pH 5.0. The activities were plotted against the incubation temperatures.

4.4 Temperature stability of the two separated purified enzymes

The temperature effect on stability of the two separated purified enzymes was tested in this experiment. This test was carried out by preincubating the purified enzymes (endochitinase or exochitinase) obtained from IEF (1 μg) at various temperatures from 37-75°C for 20 min before subjecting to the optimal assay conditions. The enzyme mixture was kept in ice bath immediately after vary temperature preincubation before determining the remained activity. The results 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.

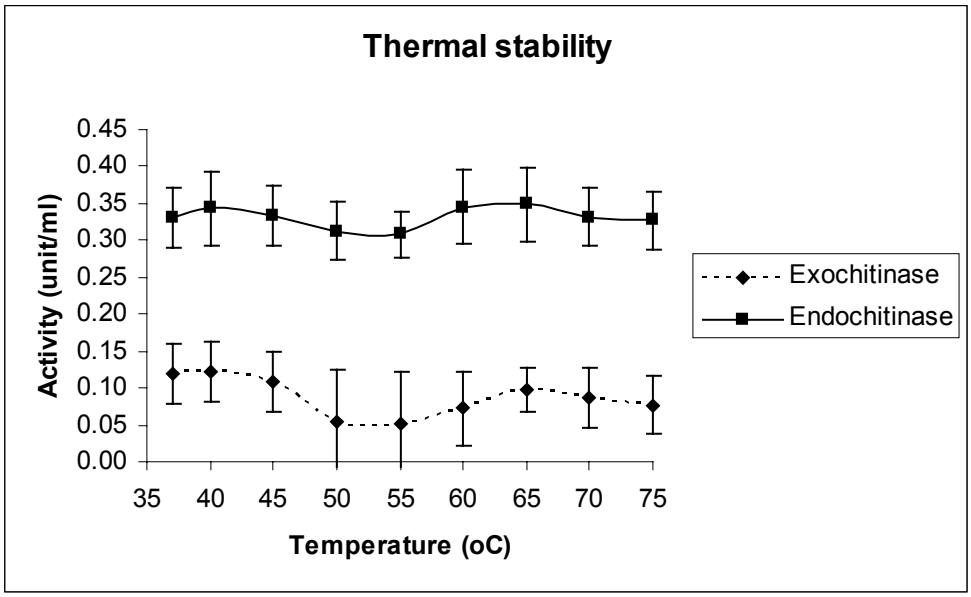

Figure 14. Thermal stability of endo- and exochitinase. The purified enzymes (endochitinase or exochitinase) obtained from IEF were incubated at various temperature from 37°C to 75°C for 20 min and cooled in ice bath before detecting the remained activity. An aliquot of 1 μg purified enzymes was assayed for the remained activity in 50 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated at 37°C for 20 min. The percentage of activities was plotted against the temperatures.

5. Kinetic study of the two separated purified enzymes

The success in obtaining the homogeneous chitinase of the two separated purified enzyme forms makes it suitable to study the kinetic properties as no interference from other contaminants. The kinetic parameters obtained for the two purified enzyme forms will be useful determinants not only on the separated enzyme properties, but also the enzyme specificity and their proportion on chitinolytic role as to the enzymes' turnover number. These criteria are important for understanding on their physiological function as deduced from the kinetic parameters as markers for the activity sharing as contributed by the enzyme forms in the concerted chitinolytic process.

5.1 Basic kinetic properties of the two separated purified enzymes

Before determining the kinetics parameters (Km and Vmax) of endo- and exochitinase, enzyme saturation curves need to be characterized first. The concentration of enzyme for maximum activity (Vmax) in saturation curve will be the primary parameter differentiating the two enzyme forms. Assay on calibration of varying amount of purified proteins and enzyme activity levels were shown in Figure 15 (endochitinase) and Figure 16 (exochitinase). The purified endochitinase (50 μg protein) and exochitinase (70 μg) were obtained from IEF in the incubation assays for Vmax.

The results revealed that the formation of reaction products was a linear curve up to 10 μg and the saturation was observed at 15 μg for endochitinase (Fig. 15). For exochitinase, the linear curve was up around 30 μg and the activity saturation was observed to begin at around 40 μg (Fig. 16). For subsequent kinetic studies 15 μg endochitinase and 40 μg exochitinase were chosen in determination of the kinetics parameters (Km, Vmax), as optimal enzyme concentrations. The 2 enzyme levels were at the beginning of the highest activity for the two enzyme forms as shown in these assays. The results obtained in the study (Fig. 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.

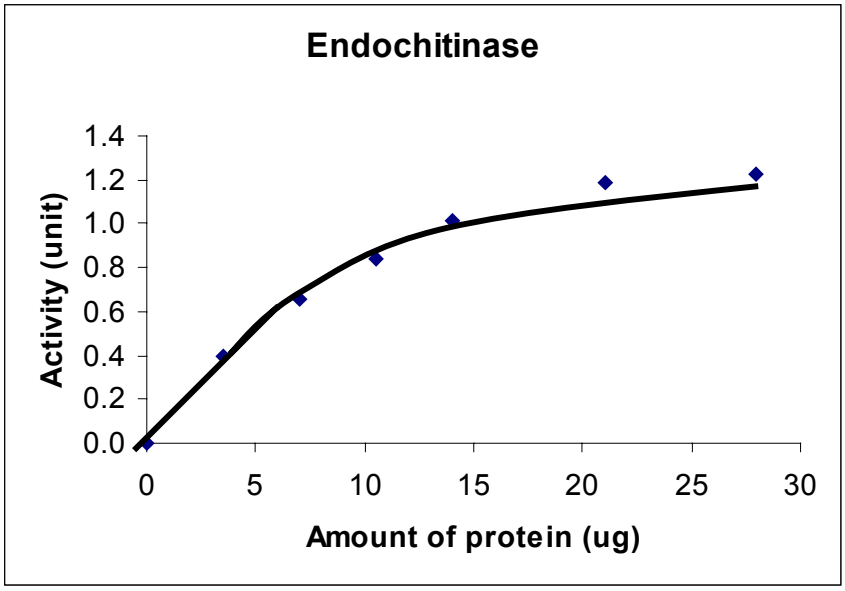

Figure 15. The fluorimetric assay of endochitinase. The assay was performed with varying amount of the purified endochitinase obtained from IEF (0-50 μg protein). The enzyme was incubated in 50 mM acetate buffer pH 5.0 containing 1 mM of 4-MU-β-$(GlcNAc)_3$ as substrate at 37°C for 20 min. The rate of reaction form each assay was plotted against amount of protein.

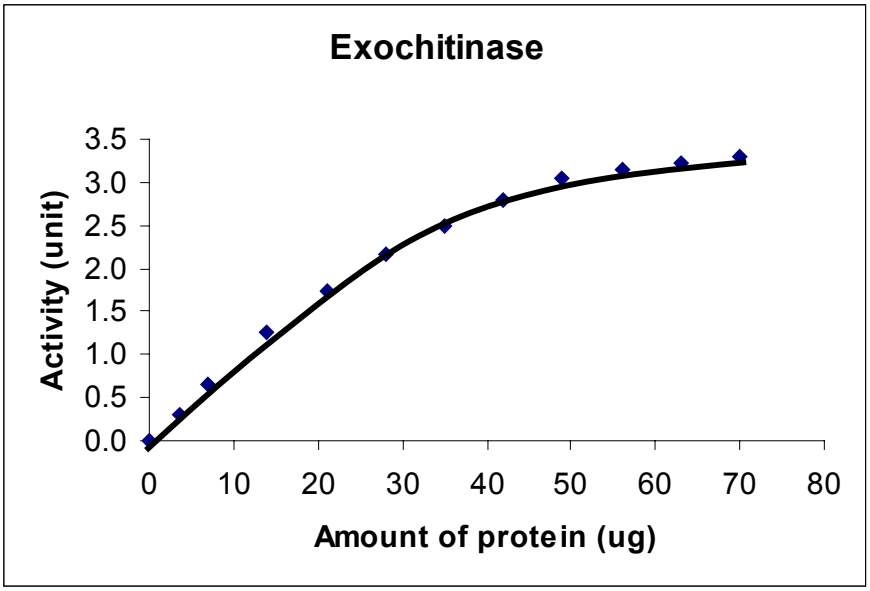

Figure 16. The fluorimetric assay of exochitinase. The assay was performed with varying amount of the purified exochitinase obtained from IEF (0-70 μg protein). The enzyme was incubated in 50 mM acetate buffer pH 5.0 containing 1 mM of 4-MU-β-GlcNAc as substrate at 37°C for 20 min. The rate of reaction form each assay was plotted against amount of protein.

5.2 Km and Vmax determinations for the endochitinase

Determinations of Km and Vmax for the purified endochitinase were done in the next two experiments, and the results were as shown in Figure 17 (A and B). The Km and Vmax of endochitinase were determined by employing the specific substrate 4-MU-β-$(GlcNAc)_3$ as described in the Methods that was very sensitive and specific. It was different from the other substrate which was specific only for exochitinase. The results in Figure 17 A showed the endochitinase activity at varying substrate concentrations. The plot of initial velocity of endochitinase showed a typical Michaelis-Menten kinetics, as displaying saturation curve at increasing 4-MU-β-$(GlcNAc)_3$ concentrations.

Double reciprocal plots (Lineweaver-Burk plot) were then carried out as calculated from the results in Figure 17 A. The resulted double reciprocal plots showed the perfect linear line as shown in Figure 17 B. Intercepts of the plot was used for Km and Vmax

determinations of the endochitinase. It was found that the purified enzyme has a Km value of 99.73 μM 4-MU-β-$(GlcNAc)_3$. The calculated Vmax with this substrate was 666 unit/ml for 4-MU-β-$(GlcNAc)_3$. These Km and Vmax wil then be compared with those obtained for exochitinase in the next experiment.

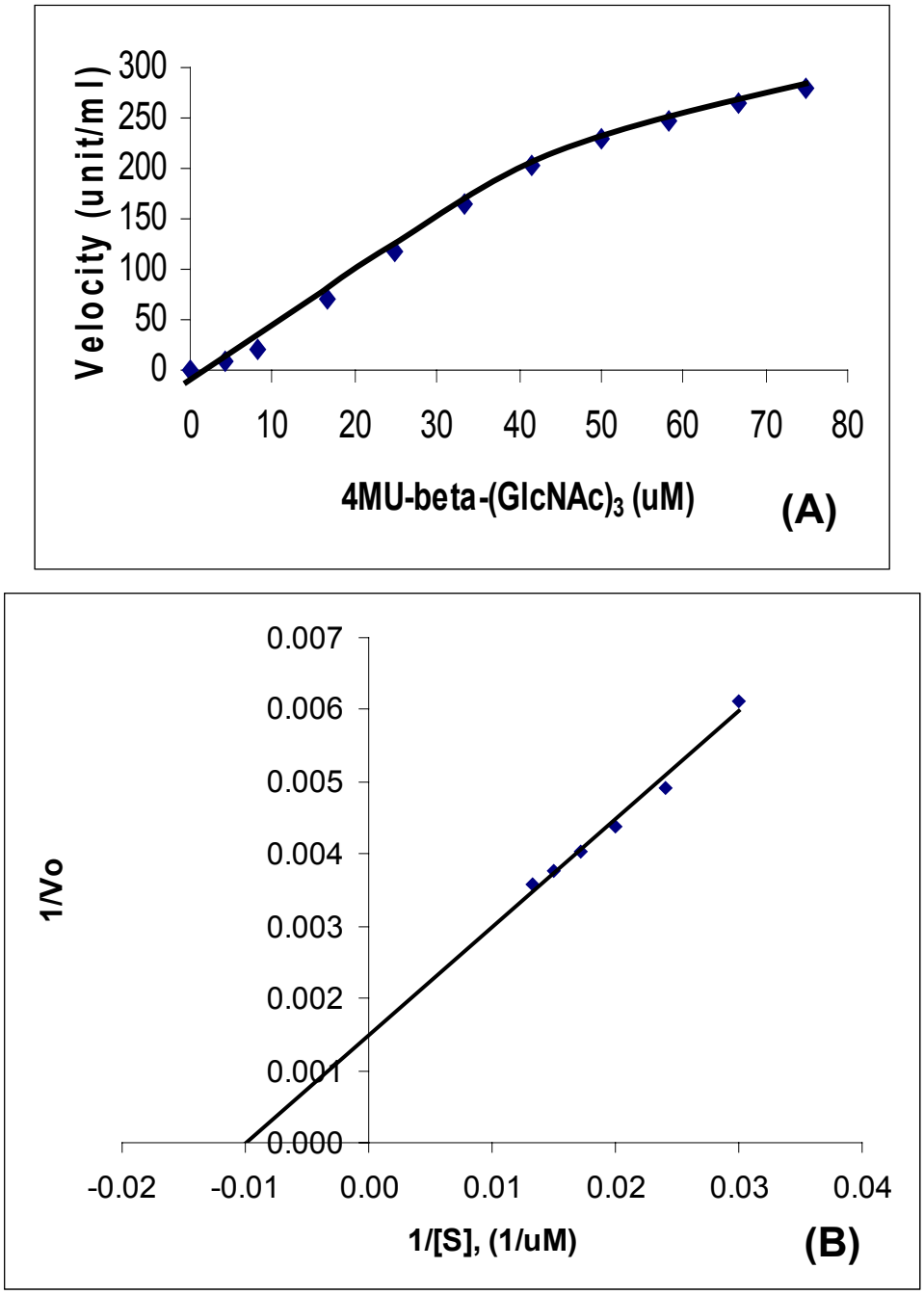

Figure 17. Determination of Km and Vmax values of the purified endochitinase obtained from IEF for 4-MU-β-$(GlcNAc)_3$. Fourteen microgram of purified endochitinase was tested for activity in 50 mM sodium acetate buffer pH 5.0 contain various concentrations of 4-MU-β-$(GlcNAc)_3$ (0-75 μM). The reaction mixture was incubated at 37°C for 20 min. The plot between the initial velocity and the concentration of 4-MU-β-$(GlcNAc)_3$ showed in A. The reciprocal activities were plotted against the reciprocal concentration of 4-MU-β-$(GlcNAc)_3$ as showed in B.

5.3 Km and Vmax determinations for the exochitinase

Determination of Km and Vmax for the purified exochitinase were done similar to that of the purified endochitinase with the major different on the substrate employed. The Km and Vmax of exochitinase determined by employing the specific substrate 4-MU-β-GlcNAc as was described in the Methods. That substrate was for exochitinase assay, very sensitive and specific for the exochitinase only. The results in Figure 18 A showed the enzyme activity at varying concentrations of 4-MU-β-GlcNAc substrate. The plot on the enzyme initial velocity showed a typical Michaelis-Menten kinetics, as displaying saturation curve at increasing 4-MU-β-GlcNAc concentrations.

Lineweaver-Burk plot or the double reciprocal plots were then carried out as calculated from the results in Figure 18 A. The resulted Lineweaver-Burk double reciprocal plots showed the perfect linear line as shown in Figure 18 B. Intercepts of the plot was used for determinations on Km and Vmax of the exochitinase. It was found that the purified enzyme has a Km value of 0.61 mM 4-MU-β-GlcNAc. The calculated Vmax with this substrate was 526 unit/ml for 4-MU-β-GlcNAc. 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.

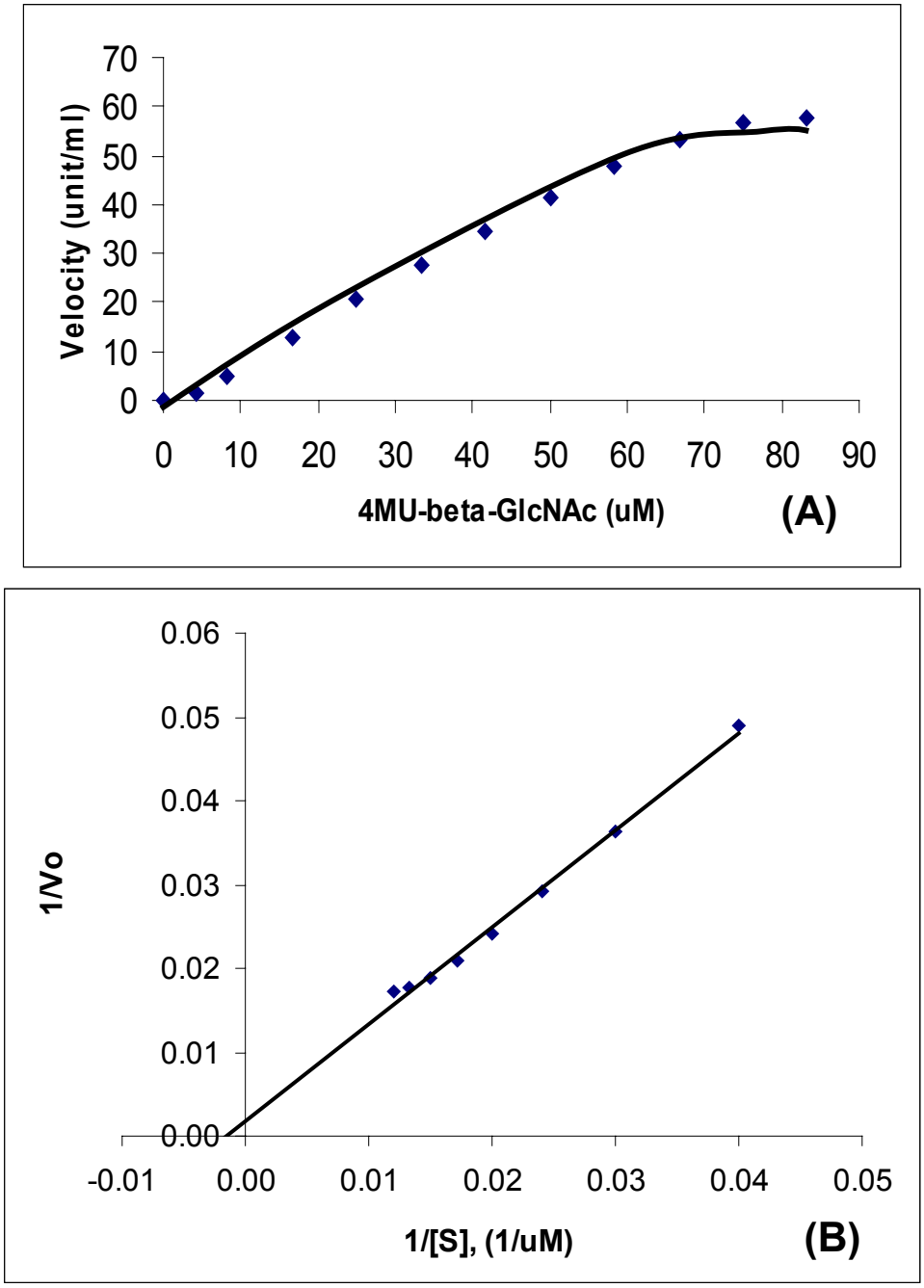

Figure 18. Determination of Km and Vmax values of the purified exochitinase obtained from IEF for 4-MU-β-GlcNAc. Forty two microgram of purified exochitinase was tested for activity in 50 mM sodium acetate buffer pH 5.0 contain various concentrations of 4-MU-β-GlcNAc (0-85 μ M). The reaction mixture was incubated at 37°C for 20 min. The plot between the initial velocity and the concentration of 4-MU-β-GlcNAc showed in A. The reciprocal activities were plotted against the reciprocal concentration of 4-MU-β-GlcNAc as showed in B.

6. N-terminal sequence Analysis of the endo- and exochitinase

To detect N-terminal protein sequence, the purified endo- or exochitinase solution obtained from IEF preparation were checked N-terminal amino acid sequencing using the Edman-degradation method as performed by Scientific Equipment Center (Prince of Songkla university). N-terminal sequencing of their purified chitinases yielded 10 amino acid residuals (Fig. 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. From former result, we could conclude that endochitinase was a basic than that in exochitinase. Besides, the current study could clearly separate these chitinases by the IEF preparation and a search of several databases (Genbank, PDB, SWISS-PROT, PRF and PIR) using the BLASTP program revealed that endo-and exochitinase did not conserve homology with other chitinases, except exochitinase was similar sequence homology to Hevamine or endochitinase in *Hevea brasiliensis* about 50% whereas endochitinase did not. 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.

(A)

Endochitinase	NH_2-KEESRRRRHR
Oryza sativa	67 E ESRRRGRHR 76
Ustilago maydis 521	309 KEERRRRRHN 318
Mus musculus	27 KEETRRRRRR 36
Hevamine	1 GGI AIYWGQN 10

(B)

Exochitinase	NH_2-HDYAIYFGTN
Theileria annulata	219 VNYAI FFGTN 228
Yarrowia lipolytica	834 LDYTTYFGTN 843
Emiliania huxleyi virus 86	295 HEYAIYFIPY 304
Hevamine	1 GG I AIYWGQN 10

Figure 19. Alignments of the N-terminal amino acid sequences of endochitinase (A) and exochitinase (B). Hypothetical protein [*Oryza sativa* (*japonica cultivar group*)] (Genbank accession number BAD45978), Hypothetical protein UM04821.1 [*Ustilago maydis* 521] (Genbank accession number XP760968), Unnamed protein product [*Mus musculus*] (Genbank accession number BAE42930), Coatomer alpha subunit, putative [*Theileria annulata*] (Genbank accession number CAI74823), Hypothetical protein [*Yarrowia lipolytica*] (Genbank accession number XP501038), Putative serine protease [*Emiliania huxleyi virus* 86] (Genbank accession number YP293913) and Hevamine [*Hevea brasiliensis*, Peptide Partial, 273 aa] (Genbank accession number AAB19633). Amino acids identical to the N-terminal sequence of endo- and exochitinase are shown in black background.