CHAPTER IV

DISCUSSION

Chitinases have been reported to be present in wide range of living organisms especially bacteria, fungi and higher plants. However, application of chitinases for commercial uses focused mainly on bacterial and fungal enzymes. Although chitinases in plants have been constantly reported, there is no serious consideration of extracting plant chitinase for commercial applications. This may be due to many plant chitinases occurred by induction and those reported to be constitutive enzymes occurred in plants with commercial values such as *Hevea* latex. In this study, we tried to screen for the presence of chitinases in the organs of the crops which are of low commercial values such as leaves and pericarp.

4.1 Screening of chitinases in local crops

4.1.1 Methods of screening

In the experiments, these procedures for detecting chitinase activities were employed.

4.1.1.1 Chitin-agar Plate Assay for chitinase

Roberts and Selitrennikoff (1988) reported a method for detecting chitinase using agar plate in which colloidal chitin was included with bacto nutriens. A paper disc soaked with sample was placed on the agar and clear zone around the disc observed after incubation. In our experiment colloidal chitin was suspended in the agar without the bacto nutrients since it caused contamination from bacteria, and sample wells was punched in the agar (0.5 cm. in diameter) instead of using paper disc. This allowed application of higher volume of chitinase sample, hence possible detection of chitinase in some diluted samples. This method, however, is for preliminary screening and is not as sensitive compared to other methods such as activity staining in gel as mentioned in section 2.3.3.

4.1.1.2 Chitinase activity stain on polyacrylamide gel

Trudel and Asselin (1989) reported the method to detect chitinase both on SDS and nondenaturing polyacrylamide gel. They reported that in the SDS-PAGE, 0.01 % glycol chitin, the substrate for chitinase, can be pre-mixed in the 12% separating gel before electrophoresis. After electrophoresis, the gel was incubated with purified triton X-100 to remove SDS and other macro molecules from the gel before staining with Calcofluor white M2R which will stain for undigested chitin. The area at the chitinase band would not be stained

with the dye because most chitinases can digest glycol chitin (Molano *et al.* 1979).

Trudel and Asselin (1989) reported that 0.1 % glycol chitin can not be pre-mixed on the gel of non-denaturing PAGE before electrophoresis because the substrate will diffuse during electrophoresis causing smearing not discrete bands. They reported a more tedious procedure of using overlaying gel with 0.01 % glycol chitin on top of the ND-PAGE after electrophoresis, incubated and stained with the dye as for SDS-PAGE. However, in our experiments, we performed the ND-PAGE in the same way as SDS- PAGE by incorporating glycol chitin in the separating gel. The chitinase staining turned out to be discrete bands and more sensitive than agar plate assay (Figure 4). Commercial chitinase (*Serratia marcescens*) was also loaded on the gel as a positive control of the activity stain.

4.1.1.3 Quantitative colorimetric assay of chitinase

As mentioned in section 2.3.2, there are many colorimetric methods for assaying chitinase activity, but the most suitable one for plant chitinase is *p*-dimethylaminobenzaldehyde. In this method, plant chitinase which is endochitinase will produce short chain oligosaccharides which needed to be digested to monomeric NAG by snail gut enzyme before forming color complex with DMAB (*p*-dimethylaminobenzaldehyde). Therefore, the amount of products detected by the dye is not the real products of the chitinase. Corrections

for the chitinase activity was performed using correction factors as shown in section 2.3.2.

4.1.2 Chitinase in leaves of local crops

Leaves of seven local crops were extracted with 100 mM sodium citrate buffer pH 5.0 and screened for chitinase using chitin-agar plate technique. The leaves of star gooseberry, ivygourd, papaya, jackfruit, tamarind, roseapple and aztec kuamochill were extracted as such without any induction procedure. Only the extract of leaves of papaya showed positive clear zones on the agar plate. However, when all the samples were subjected to chitinase activity stain in ND-PAGE (data not shown), the extract of papaya showed positive chitinase bands in addition to leaves of aztec kuamochill. This indicates that chitinase activity stain on the gel is more sensitive than the agar plate technique. The chitinase of these leaves are constitutive since the plants were not exposed to any inducers.

4.1.3 Chitin oligosaccharides as elicitor of chitinase activity in bean seeds

Plant chitinases are present either constitutively or by induction. Fungi, virus, wounding, chemical and plant hormone (ethylene) could induce chitinases in plants. Nasser *et al.* (1988) induced chitinase in maize seeds (*Zea mays cv.* INRA 258) by treatment with 0.2% mercuric chloride and inoculated with a suspension of Brome mosaic

virus. They found eight pathogenesis-related proteins accumulated in maize leaves after treatment. Kragh *et al.* (1990) studied chitinase in susceptible and resistant primary leaves of barley and found chitinase activity to be induced five-folds and three-folds respectively, at 7 day after inoculation with the powdery mildew fungus (*Eryiphe graminis* f. sp. nordei). Several glycosidic components from the cell wall of pathogenic fungi have been implicated in host-pathogen interaction. These components called "elicitor", have been shown to trigger in plants a variety of defense mechanism. Since chitin is an important component of most fungal cell walls, it is possible that chitin oligomers may be potent elicitors of defense mechanisms, and particularly of chitinase activity. Rody *et al.* (1978) tested this hypothesis in the melon using chemically synthesized chitin oligomers and found that the smallest active chitin oligomers, the hexamer and heptamer, were the most efficient elicitors of chitinase activities. In our study, we induced chitinase in bean seeds by incubating bean seeds in medium with colloidal chitin (0.5, 1.0 and 2.0%). The medium with 0.5% colloidal chitin could induce chitinase after incubation for 36 hr (data not shown), 1.0 and 2.0% colloidal chitin could induce chitinase after incubation for 30 and 36 hr, respectively. Therefore colloidal chitin can induce chitinases in bean seeds. Wheat germ agglutinin was found to inhibit growth of some fungi, this lectin binds very strongly to chitin. Molano *et al.*(1979) suggested the possibility that its effect on growth might be mediated by an inhibition of a chitin synthetase. They prepared agglutinin by affinity adsorption on chitin, after this protein was purified, it was discovered that it was not a true inhibitor of the

chitin synthetase but the decrease in chitin accumulation observed resulted from destruction of the polysaccharide as it was formed, indicating a chitinase activity associated with the lectin. Pongdontri (1993) purified and characterized a chitin-specific lectin from fruit pericarp of angled loofah which has an anti-fungal growth effect. It was speculated that the lectin may also posses chitinase activity. When the extract of pericarps of angled loofah prepared by the method of Pongdontri (1993) was assayed for chitinase activity by both agar plate and 12% ND-PAGE, it was found that there were two chitinase activity bands present in the extract, one was at the top half of the gel, the other appeared in the lower half of the gel.

4.2 Purification of chitinase from fruit pericarp of angled loofah

Since, it was possible to detect chitinase activity in the pericarp of angled loofah, it was decided that further study will be concentrated on the extract. The pericarp of angled loofah was of no commercial value, easy to obtained and can be obtained in large amount. Purification of the chitinase was carried out before further characterization.

4.2.1 Ammonium sulfate precipitation

The most convenient way of removing contaminating proteins from crude sample was ammonium sulfate fractionation. The crude extract of the angled loofah was fractionated at various range of

ammonium sulfate concentrations and detected for chitinase activity. It was found that chitinase was detected in the fractionation range 30-50 and 50-70% ammonium sulfate (Figure 7). To harvest as much of the chitinase as possible, it was decided to keep the 30-70% ammonium sulfate precipitate as the result of the first step of purification. Although high activity was collected in this fraction, the purification is only 6 folds with 87.9% yield (Table 4). Further step of purification was carried out.

4.2.2 DEAE-cellulose column chromatography

The dialysed 30-70% ammonium sulfate fraction in 0.02 M sodium phosphate buffer (pH 7.4) was applied to DEAE-cellulose column. In the first few runs, the sample which contained a brown colour resulted in binding of the color to the resin and interfere with protein staining of the polyacrylamide gel electrophoresis. These fractions with brown colour gave blue background which could not be destained, so the protein pattern of 30-70% ammonium sulfate precipitation was not clear. This problem was solved by adding of polyvinypolypyrorydone (PVPP) into the crude plant extract to remove the phenolic compound which gave the brown colour. After successful removal of the brown colour, chitinase was purified further by the DEAE-cellulose column. The enzyme bound to the column and can be eluted with 0.18-0.2 M NaCl. The ND-PAGE protein pattern showed only a few bands of proteins in the fraction with 2 bands showing positive chitinase stain. The same sample subjected to SDS-PAGE

showed only a few bands with only one band positive for chitinase (Figure 11). It seemed that peak III from the DEAE-cellulose column contained mostly chitinase contaminated with only minute amount of other proteins (Figure 10). The enzyme yield at this step was low at 6.7% although the purity was quite high 18.9 (Table 4). Therefore, it was decided that the enzyme would not be further purified since there was little activity left and only slightly contaminated.

4.3 Characterization of the partial-purified chitinase

4.3.1 Molecular weight determination

The chitinase was determined for its molecular weight by two methods.

4.3.1.1 Molecular weight determination on SDS-PAGE

Since the pooled fraction from DEAE-cellulose column was slightly contaminated with other protein bands, it was decided to cut the chitinase-positive protein band in ND-PAGE, eluted and determined its molecular weight on SDS-PAGE. Two protein bands (a and b) with chitinase activity in ND-PAGE were cut and reapplied to SDS-PAGE. Protein band a stained as two protein bands in SDS-PAGE with only the upper band was positive to chitinase stain. Protein band b stained as one protein in SDS-PAGE which was positive to chitinase stain (Figure 12). Therefore, it seems that both are chitinases which contain the one

identical subunit separated on SDS-PAGE with the molecular weight of 29,000. The slow-migrating band (protein band a) had two subunits with molecular weight 29,000 and 25,000, with the 29,000 subunit containing the chitinase activity.

4.3.1.2 Molecular weight determination on Sephadex G-100

Attempt has been made on identifying the moleculat weight of the angled loofah chitinase on Sephadex G-100 using the pooled fractions from DEAE-cellulose column but the protein in the sample was too minute and cannot be detected in the column. Therefore, the 30-70% precipitate was used to apply on the Sephadex G-100 and the enzyme peak identified by its activity stain on polyacrylamide gel. A few protein peaks were separated (Figure 14) with peak I containing the chitinase activity as identified by ND-PAGE and SDS-PAGE (Figure 15). However, the activity stain of the fraction at the top of the peak still showed the chitinase bands a and b. From the results in section 3.3.1 and 3.3.2 which was interpreted as the presence of two chitinases bands : band (a) with 2 subunits with molecular weight of 25,000 and 29,000 while band (b) contained only the 29,000 subunit. Both chitinases bands a and b were co-eluted in DEAE-cellulose and Sephadex G-100. The fractions from the top of the peaks from both columns yielded 2 chitinase bands on the ND-PAGE gel and one band on SDS-PAGE gel. It can be speculated that there existed two chitinases one with 2 subunits with molecular weight of 25,000 and 29,000 while the other

contained one subunit with molecular weight of 29,000. When peak I in the Sephadex G-100 was analyzed further, it covered the molecular weight range of 53,000-22,500. Therefore, it is possible that both chitinases were co-eluted in Sephadex G-100, with the smaller chitinase eluted at the fraction at the top of the peak which corresponded to the molecular weight of 30,000.

4.3.1.3 Some characteristics of the angled loofah chitinase

The partial purified chitinase from DEAE-cellulose column was investigated on some of its biochemical properties such as pI, optimum pH, optimum temperature and its substrate specificity. The pI of the enzyme was determined to be 5.4 from isoelectric focusing gel electrophoresis. Other plant chitinases were reported to have pI's in the more basic range of 7.5-9.4 (Table 3). Its pH optimum of 3.5 is also more acidic than those reported in some plant chitinases studied. The angled loofah lectin reported by Pongdontri (1993) was purified from the 30-50% ammonium sulfate precipitate of the fruit pericarp which is within the range we used to purify the chitinase (30-70%). The lectin was also reported to bind to DEAE-cellulose column and could be eluted at 0.2 M NaCl the same salt concentration as our chitinase. Polyacrylamide gel electrophoresis in native state showed 2 bands with haemagglutinating activity one in the 30-50% precipitation and another

in 50-70% precipitate. Their relative migration distance in the gel were comparable to the two chitinase bands detected in the ND-PAGE gel of the 30-70% precipitate in our experiment. However, the chitin-specific lectin reported by Pongdontri (1993) was the slow-migrating band on top of the gel whose molecular weight in gel filtration column was 105,000 and molecular weight of subunits on SDS-PAGE were 41,680, 31,620 and 26,300. Our chitinases, however, consist of both slow and fast migrating bands which were co-purified either in DEAE-cellulose or Sephadex G-100 column with a reported molecular weight of 30,000 on Sephadex G-100 column and 54,000 (slow migrating) and 29,000 (fast migrating) on SDS-PAGE. Therefore, it seemed that the angled loofah chitin specific lectin and the chitinase may be different proteins. Other possibility is that one of the subunit with molecular weight of 31,620 in the chitin specific lectin, contains the chitinase activity. This subunit has similar molecular weight to the chitinase found in this work. If this is the case, this subunit should also exist as an individual protein functioning as chitinase only and can also associate with two other peptides to form the chitin-specific lectin. Since the chitin-specific lectin was reported to have antifungal effect, it may act as a defense mechanism of the plant by binding to the chitin constituent of the fungal cell wall and the chitinase subunit acts to hydrolyse the chitin. This hypothesis must be further work out by isolating the 3 subunits of the chitin-specific lectin and investigate their haemagglutinating and chitinase activity.