CHAPTER 4

DISCUSSION

4.1 Study on surface agglutinin of angled loofah

Lectins in plants were proposed to play important role in the defense mechanism of plant which includes interaction with pathogenic organism and the peroxidase-like or superoxide dismutase-like activities. Recently, lectin on surface of cucumber seedling has been reported which displayed the mentioned properties (Skubatz & Kessler, 1984). Its localization on the surface and its biological and enzyme properties seems to fit well with the suggested role of lectin in one of the step in plant defense mechanism. Primary screening of local cucurbit plants showed agglutinin activity in angled loofah which was observed to be a plant which is highly resistant to fungi causing diseases. In view of the reports mentioned above, study was carried out on surface agglutinin of both the pericarp of angled loofah fruit which contained highest hemagglutinating activity and the seedling of the same plant. As our study will concentrate on lectin with similar properties to that reported by Skubatz & Kessler (1984) and test on pathogenic organism will be focused on fungi , emphasis of the experiments was on chitin-specific lectin. Chitin and chitotriose,the oligomers of N-acetyl-D-glucosamine were one of the constituents of fungal cell wall (Neter, 1956).

In the attempt to identify the lectin of interest , namely chitin-specific lectin , sugar inhibition tests were caried out for the fraction obtained from pericarp shown to contain hemagglutinating activity. However, the sugar inhibition tests carried out on many

carbohydrates including chitin turned out negative. However, when these fractions was subjected to ND-PAGE and identification of protein bands with chitin-specific hemagglutinating activity was carried out on 0.4 cm gel slices , the lectin could be identified in the crude extract, AS-35 and DEAE-Cellulose fraction. Other protein bands on the gel also showed HA, but the HA observed were not stable in comparison with chitin-specific band. DEAE-Cellulose pooled fractions also showed positive sugar inhibition test on hemagglutinating activity by conventional lectin assay. It seemed that in the crude and AS-35 fractions, some endogeneous contaminants hindered the lectin binding to sugars. These contaminants apparently could be removed by DEAE-Cellulose column and gel electrophoresis. This problem could be the obstruction which resulted in the failure in using affinity chromatography in the purification of the pericarp lectin which will be discussed later.

Chitin is the oligomers of N-acetyl-D-glucosamine. However, the pericarp lectin only showed specificity to chitin and chitotriose (a trimer of N-acetyl-D-glucosamine , NAG) but not N-acetyl-D-glucosamine itself. Probably, the lectin may be specific to oligomer of NAG from three sugars unit onward. In our experiment , chitin solution used was prepared in our own laboratory by hydrolysis of ground chitin. The exact content and unit of NAG on the chitin oligomers that were effective on the lectin could not be determined. The amount reported was the weight of total chitin powder mechanically measured, thus, do not represent the real amount of chitin bound to the lectin and appeared rather high. Many of the lectins from cucurbit plant showed the preference for the oligomers with varying number of NAG units *e.g.* lectin from *Cucurbita pepo* prefered dimer and tetramer of NAG was very potent being better than the monomer at 250 and

5000 times respectively (Allen, 1979). Lectin from the exudate of ridge guard (*Luffa acutangula*) was also most specific to tetramer of NAG (Anantharam *et al*, 1986). It is interesting that only lectin from 9-day old seedling root surface was shown to be specific to chitin and the specificity was observed only with surface washing not the root homogenate. The difference was further confirmed by the difference in ND-PAGE protein patterns of both fractions in which the chitin-specific band was not presence in the homogenate (data not shown). Root surface extract of 12 day old seedling was specific to chitosan and galactose (data not shown). This result implied existance of different lectins on the root surface. However, chitin-specific lectin on root surface of day 9 seedling root was selected for further study due to its contained highest hemagglutinating activity.

4.2 Purification of lectins from fruit pericarp and seedling root surface of angled loofah

Since crude extract of fruit pericarp of angled loofah contained a lot of proteins, ammonium sulfate precipitation was applied as the first step of purification. The lectin of interest was identified to be in the precipitate of 30-50% ammonium sulfate fraction (Table 3 and Fig.5). Although, this step removed large amount of proteins, it did not give good yield or satisfactory purification.

Affinity chromatography is widely used for purification of lectins due to the high specificity and good yield which could reduce a few steps from purification procedures.

However, when chitin and chitotriose affinity columns was used with the same purpose on pericarp and seedling root

surface lectins, the attempts were unsuccessful (section 3.4). There were reports that affinity chromatography could not be applied for purification of some lectins. For instance, the isolation of *Dolichose biflorus* lectin with N-acetyl-galactosamine immobilized to sepharose was not successful and was later proved to be caused by substitution of the binding site at C-6 hydroxyl group of the carbohydrate in the matrix. The better way to solve this problem was affinity electrophoresis which was a combination of affinity chromatography, hog blood group A+H as an affinity substance and conventional electrophoresis (Borrebacek and Etzler, 1980). Another example was the lectin from ground elder (*Aegopodium podagraria*) rhizomes which also could not be purified by Gal-NAG sepharose. An affinity chromatography of erythrocyte membrane protein immobilized on cross-linked agarose was used instead (Peuman *et al*, 1985). The reason of failure in using affinity column to purify both pericarp and seedling roots surface lectin may be the same as the negative result obtained in conventional sugar inhibition tests of fraction from the purification steps. The chitin column used was the un-hydrolysed powder form ; thus , containing large oligomers of NAG. The failure of lectin to bind the chitin column was most probably due to the carbohydrate binding site of the lectin cannot accommmodate large molecule of chitin. As Anantharam *et al* (1986) reported for the isolation of lectin from the exudate of ridge gaurd (*Luffa acutangula*), it could be purified by affinity column of soybean glycoprotein on Sepharose 6B which had suitable ligands corresponded to number of NAG units of chitin shown positive sugar inhibition test. This could be confirmed by sugar inhibition test of two of angled loofah lectins on oligomers of varying NAG units. On the other hand, if the number

of NAG units did not affect the binding, there may be some other explanation which has not yet been investigated. The possibility of the presence of chitinase activity in the same was suggested ; the chitinase may bind the column and at the same time hydrolysed the chitin and released itself from the column. Some other methods had been attempted in oder to overcome the problem. Those methods were :

1) Chemical stabilization of the lectin

Many lectins in cucurbitaceae family were reported to contain disulfide bridge in their structures (Sabnis & Hart ,1978 and Read & Northcote, 1983) and caused characteristic gelling of cucurbit exudate when reduced. This may cause obstructions in the purification process. Either dithiothreitol or β-mercaptoethanol was used as antioxidant agent in the extraction and purification steps. In comparison of treated and nontreated β-mercaptoethanol with fruit pericarp in purification step , no differences was observed. Since gelling characteristic of fruit pericarp and seedling root surface was not observed and β-mercaptoethanol had no effect on them , disulfide bond may not be available on these lectins.

Phenolic compounds may be other interfering factor in the purification process. Addition of polyvinylpyrorydone (PVP) (Loomis & Battaile,1966) or sodium metabisulfite which prevent oxidation of phenols to quinones, although improved the extraction process did not help improve any binding to any columns.

Kochibe and Matta (1989) reported that glycerol could reduce aggregation in extract of mushroom (*Psathyrella velulina*) and enable purification of the lectin by chitin column. However,

application on the pericarp extract did not facilitate the purification process.

2) Chromatofocusing column

Based on pI values, chromatofocusing column had been successfully used to purify lectin from peanut (*Arachis hypogaea*) (Miller,1983) and potato (*Solanum tuberosum*) (McCuurrach & Kilpatrick, 1986). However, when AS-35 fraction of pericarp extract was applied to chromatofocusing column at all possible pI ranges, no protein peak could be identified with hemagglutinating activity. In the later experiment, when purified pericarp lectin was subjected to isoelectric focusing gel for pI determination, a pI value of 6.25 was reported. Looking back at the chromatographic profile of chromatofocusing column (Fig.5), there was a protein peak eluted at this pI value. It is possible that the lectin may be actually separated on the column, but some components in the chromatofocusing system hindered the detection of hemagglutinating activity.

3) DEAE-Cellulose column

Bloch and Burgur (1974) reported sucessful used of DEAE-Cellulose column for isolation of WGA in wheat germ extract. This column was also applied for purification of pericarp lectin. Several conditions have been tried without satisfactory results (data not shown). The condition which was most promising, giving a protein peak with hemagglutinating activity specific to chitin was using phosphate buffer pH 7.4 and elution with NaCl gradient 0-0.6 M (section 2.2.8.3, Fig.6). However, comparison of the purification

fold and the yield of the protein eluted from gel slice of ND-PAGE, it was dicided to bypass this method (section 3.6.2.3). Therefore, the elution of the lectin from protein band in ND-PAGE was the main purification step employed for purification of pericarp lectin.

For the purification of seedling root surface lectin which could not be detected on any columns used , possibly with the same reason as pericarp lectin or the minute amount of the protein extracted ; formalinized rabbit erythrocytes was used to adsorb the lectin on the principle that lectin are generally identified by the ability to agglutinate erythrocytes of an appropriate type and released by addition of the appropriate sugar. However, the erythrocytes to be used as affinity adsorbant should be tough in hypotonic and hypertonic conditions and could be prepared by formaldehyde treatment (Reitherman *et al*,1974). When the method was applied to root surface lectin, it resulted into 2.1 folds of purification of root surface extract of loofah seedlings with about 84% recovery of activity. Lectin from jack bean meal , *Ulex europeus* seeds and lima bean could be recovered from 50 to 100% by the same technique. Although, the seedling root surface lectin obtained by this technique did not show up as discrete band on ND-PAGE ,it appeared as one band in SDS-PAGE.

4.3 Characteristics of lectins from fruit pericarp and seedling root surface

Having purified fruit pericarp and seedling root surface lectins, it was nescessary to characterize their physicochemical

and biological properties.

Molecular weight of pericarp lectin as determined by gel filtration on Sepharose 6B column was 105,000 dalton when 5% chitin was included in the phosphate buffer pH 7.4 used as eluent (Fig.9 and 10). When the column was eluted with phosphate buffer alone, the molecular weight of the protein was determined as 18,000 dalton (data not shown). Read and Northcote (1983) also observed similar result with PP1 , phloem proteins of *Cucurbita maxima* (pumpkin) and suggested that the lectin was retarded on the gel filtration resin due to the steric effect of the carbohydrate part on the molecule of the lectin which was a glycoprotein. This effect can be overcome by filling sugar binding sites on the lectin with soluble ligand. This suggestion was sucessfully applied to pericarp lectin as shown in Figure 9. When lectin was subjected to SDS-PAGE, the lectin appeared as three bands (Fig.11) with apparent MW of 41,680 , 31,620 and 26,300 daltons , respectively (Fig.12). The sum of the molecular weight of these three protein bands on SDS-PAGE was 99,600 which was within 5% error when compared to the molecular weight of the lectin on Sepharose 6B (105,000). Therefore, it is proposed that the pericarp lectin has a native molecular weight of 105,000 and contain three nonidentical subunits with MW of 41,680 , 31,620 and 26,300. Anatharam *et al* (1986) found a lectin from exudate of fruit of ridge gaurd (*Luffa acutangula*) with molecular weight of 48,000 as a dimer of identical subunits of 24,000 and it is not a glycoprotein. Although most of the reported characteristics of the exudate lectin were rather different from pericarp, they resemble in their sugar specificity of NAG oligomers.

The molecular weight of seedling root surface lectin could not be determined by method of gel filtration due to the minute amount

of this purified fraction. Therefore, SDS-PAGE was the only method for MW determination of the root lectin which was apparently 28,000 (Fig.14). In the SDS-PAGE pattern of crude extract of the root lectin , there were protein bands the molecular weight of which correspond well to the three subunits of pericarp lectin. However, when the root lectin was subjected to purification by formalinized-trypsinized rabbit erythrocytes , only the band with molecular weight 28,000 appear on the SDS-PAGE. This also corresponds closely to one of the subunit of pericarp lectin (26,300). It could be postulated that the root lectin may be the same protein as pericarp lectin *i.e.* containing three subunits but dissociated in the process of purification with formalinized-trypsinized rabbit reticulocytes and only the 28,000 subunit bound to the red cells as detected on SDS-PAGE. Alternatively, the root lectin may actually be the protein with MW of 28,000 or an aggregate of this single subunit and did not contain other non-identical subunit at all. These could be proved by determination of the native molecular weight of root lectin by a method sensitive to minute amount of protein such as gel filtration on FPLC. On the other hand, antibody raised from either of the lectin could be applied to cross react with the other lectin to confirm the hypothesis. It has been postulated that plant genome may contain several genes , each encoding a lectin that cross-react with antibodies raised against lectin purified from different tissues of the same plant. It is likely that the expression of these genes is developmentally regulated ; one gene may encode a lectin expressed in seeds and another may code for protein active in roots or leave (Goldbuge *et al*, 1983 ; Vodkin *et al*, 1983). These lectins may be formed from different combination of a few peptide subunits, resulting in some related properties and certain variations such

as observed for *Phaseolus vulgaris* lectins (Bauman *et al*, 1979 ; Pasztai *et al*, 1981).

Lectins from cucurbit plants were usually found in monomer and dimer forms. Lectin from exudate of fruit of *Cucurbita pepo* was revealed as monomer with MW of 20,000 dalton and was not glycoprotein (Anthony,1979) whereas phloem exudate lectin from *Cucurbita maxima* also had the MW of 20,000 and exist as monomer (Sabnis & Hart, 1978). Phloem lectins from *Cucurbita maxima* ; PP1 and PP2 were a monomer of 12,600 and dimer of 46,000, respectively (Read & Northcote, 1983). Except for the cucumber surface agglutinin which was apparently consisted of identical subunit with MW of 18,000 dalton , whereas the aggregate form was about 140,000 dalton (Kessler, 1988). Etzler (1985) suggested that the presence of lectins in different tissues of the same plant with some similarity may indicated that during evolution , lectin may have become adapted for unique function in different tissues.

Many other characteristics of the pericarp and root lectins , although different were somehow closely related ; *e.g.* the pI's of pericarp lectin was 6.25 while that of root lectin was 6.15. Both are glycoproteins as determined by anthrone reaction ; the pericarp lectin contained 43% (w/w) and 12% (w/w) for root lectin (Table 7). The differences in pI or carbohydrate content of both lectins may be the consequence of the presence of other two subunits in pericarp lectin (MW 41,680 and 31,620) but not in the purified root lectin.

The heat stability profiles of both lectins resemble each other closely ; while in varying pH's , the pericarp lectin seemed to be more sensitive to pH changes (Fig. 17 and 18). At pH's from 8 onwards, the HA of pericarp lectin dropped by 50%. An interesting result was observed at pH 8. When phosphate buffer was observed at

pH 8. When phosphate buffer was used at pH 8 the HA was still remained at 100% but dropped to 50% when Tris-HCl was used. Thus, the dropped in HA pH 8.0 may either be an artifact of the nature of the buffer or may affect the subunit interaction of the lectin itself. However, such changes were not observed on root lectin ; therefore , the latter suggestion was more appropriate.

In view that both lectins are specific to chitin and chitotriose and can inhibit fungal growth quite well (Table 8), perhaps the chitin binding domain may exist in the 28,000 subunit. From the result in Table 8, seedling root lectin inhibited the growth of all fungi tested to a high degree and the inhibition could not be observed in presence of chitin (data not shown). This evidence supported that it was the chitin-specific lectin which acted upon fungi. This is not surprising, considering the fact that fungal cell wall contain chitin and the proposal that the chitin binding domain exist in the 28,000 subunit. Dixon (1986) with the postulated that inhibition of fungal growth by WGA (Mirelman, 1975) may be contaminated by chitinase, the most potent fungal growth inhibitory enzyme. However, Broekaert *et al* (1989) showed that the action of chitin-specific lectin from stinging nettle rhizome and tobacco chitinase on fungi resulted in different physiology of fungal spore, with the latter resulted in spore lysis. Spore lysis was observed with action of the chitin-specific lectin from fruit pericarp and and seedling root surface of angled loofah. This may weaken the suggestion of chitinase activity in the lectin preparations. However, this would be best confirmed by direct assay of chitinase activity in the samples. Plant chitinase has MW of 30,000 (Jeuniaux, 1966) which is quite close to the molecular weight of seedling root lectin and the smallest subunit of fruit pericarp lectin. However,

these two lectins were reported with acedic pH's whereas most plant chitinases are basic proteins. Moreover,the optimum temperature and pH of chitinase are 40°C and pH 5.0 respectively which differed from the observed temperature and pH resistance of both angled loofah lectins. These properties in molecular indicated further that the chitin-specific lectins from angled loofah are not chitinase.

4.4 Biological roles of lectins from angled loofah

With regard to the localization of the lectin being studied *i.e.* on the surface of the seedling root and on the pericarp of the loofah fruit , a role of the lectins in defense mechanism of the plant seems logical. Phathogenic microorganism is one of the threatening factor for plants. Investigation of the ability of these two proteins to inhibit the growth of some pathogenic fungi were carried out. The five species of fungi tested were known to be affected by chitin specific lectins. Seedling root surface was more effective in inhibiting growth of all fungi tested , in most cases it caused complete growth inhibition. However, pericarp lectin affected the fungi tested to a lesser degree. If the proposed structures of both lectins as discussed in the section 4.3 are correct , the higher fungistatic effect of root lectin is not a surprise. The association of three nonidentical subunits in the pericarp may hinder the chitin-binding domain in the 28,000 subunit ; hence , binding of the lectin to fungal spores is not effective as the single subunit root lectin. To further support this explanation, it was observed that clumping of the non-germinated spores of most fungi occured in all the experiment with root lectin except the case of *C.kikuchii* and *F.oxysporum*. this may be related to the shape

of the spores. The spores of these two species are large and not round ; thus , cell agglutination or clumping by lectin as multivalent binding ligand is difficult.

When superoxide dismutase activity was assayed both by spectrophotometric method and activity stain of ND-PAGE (section 3.9. 6.2), significant SOD-like activity was found associated with the fractions of purified lectins and the lectin band on the gel. SOD has been accepted as plant defense tool against free radicals. Kessler (1988) also reported the presence of lectin on the surface of cotyledon of cucumber seedlings. The lectin was chitin-specific lectin , heat and acid-base stable, fungistatic and contain SOD-like activity.

From all the results obtained together with recent findings and hypotheses as dicussed so far, it is reasonable to propose a role for the seedling root lectin in the primary defense against pathogens and free radicals for the growing seedlings. However, the role of the pericarp lectin cannot be proposed exactly as such. Explanation is still required for the existence of the pericarp lectin as complex structure of three subunits.

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SUMMARY

1. Lectin from angled loofah fruit pericarp was purified by ammonium sulfate precipitation at 30-50% saturation and protein elution from gel slice at the identified lectin band on ND-PAGE with 3.11 folds of purification and 45.7% recovery. Characterization of this lectin revealed that it had native molecular weight of 105,000 with three nonidentical subunits (41,680 , 31,600 and 26,300) and its pI was 6.25. It was a glycoprotein with 43% (w/w) of carbohydrate.

2. Lectin from angled loofah seedling was found highest on the root surface of 9-day old seedling and could be purified by elution of lectin bound to formalinize-trypsinized rabbit erythrocytes with chitin. The lectin was purified at 2.1 folds with 8.4% recovery. The molecular properties of this lectin revealed that it had molecular weight of 28,000 with 12% (w/w) of carbohydrate composition and pI's value of 6.15.

3. Both lectins were specific to chitin , stable to heat and wide pH range.

4. The two lectins exhibited their biological properties in growth inhibition of some fungi and showed superoxide dismutase activity.

5. It was proposed that root lectin may be the same protein as the smallest subunit of pericarp (MW 26,300). This subunit may act as chitin binding domain and function in the defense mechanism of angled loofah seedling against pathogenic fungi.