CHAPTER 3

RESULTS

3.1 Distribution of lectin in angled loofah fruit

Angled loofah fruit was separated into three parts : pericarp, cortex and seed. Agglutinin was extracted with PBS as described in section 2.2.1. Each extract was tested for hemagglutinating activity according to section 2.2.5. The highest specific hemagglutinating activity was found in pericarp extract, cortex and seed, respectively as shown in Table 2.

Since the highest specific hemagglutinating activity of pericarp, the extract was chosen for further purification and study.

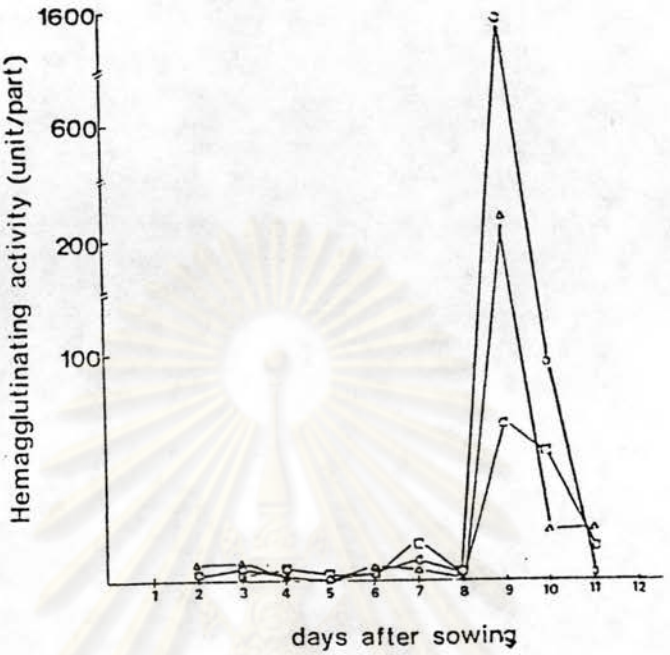
3.2 Distribution of lectin in angled loofah seedlings

Angled loofah seedlings in several stages of development were prepared and separated into three parts : cotyledon, hypocotyl and root and surface agglutinin extracted as described in section 2.2.2. Each extract was tested for hemagglutinating activity as mentioned in section 2.2.5. The profile of the specific hemagglutination activity was shown in Figure 2A. Agglutinin was not detectable in all parts up to day 8, reached a peak at day 9 and declined afterward. In addition, the hemagglutinating activity of homogenates of the seedlings after surface extraction were also determined. The profile of hemagglutinating in homogenate fractions were shown in Figure 2B. High hemagglutinating activity was observed in homogenate of cotyledon while only low level detected in root homogenate.

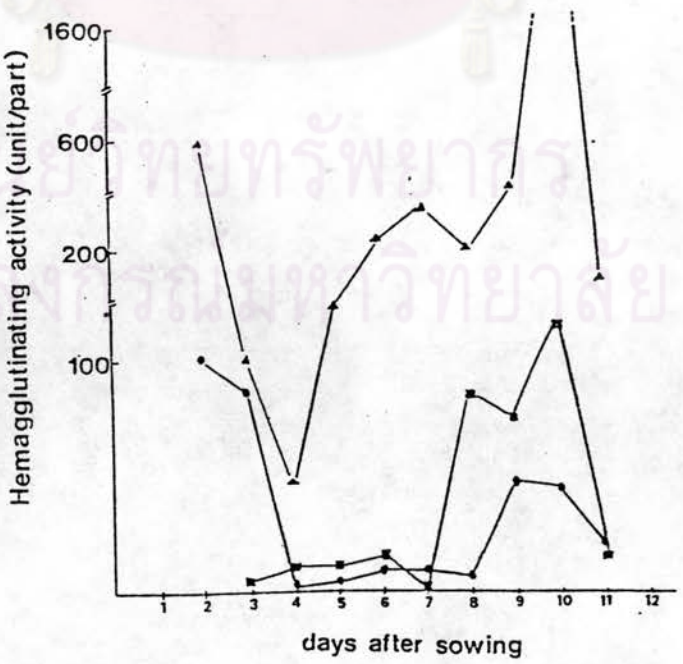
Table 2 Hemagglutinating activity in different parts of loofah fruit.

Agglutinin extract (*Luffa actutangula* Roxb.)	specific activity (HA unit/mg protein)
pericarp	919.6
cortex	300.1
seed	34.6

A)


1600
600
200
100
Hemagglutinating activity (unit/part)
1 2 3 4 5 6 7 8 9 10 11 12
days after sowing

B)


1600
600
200
100
Hemagglutinating activity (unit/part)
1 2 3 4 5 6 7 8 9 10 11 12
days after sowing

The highest specific hemagglutinating activity was found on the root surface of day-9 seedling. Hence, surface extract from root of 9 day-old seedling was selected for further study.

3.3 Release of seedling root surface agglutinin

To determine the most suitable time to extract agglutinin from root surface of 9 day-old seedling, an experiment was carried out as described in section 2.2.3 and found that agglutinin could be extracted even at 1 minute immersion in water (fraction I), but higher when left for 30 minutes (fraction II). However, the HA declined when left immersed for the second and the third 30 min cycles (fraction III,IV). When one step immersion of root in water for 91 min (fraction V), it was shown that the agglutinin extracted was comparable to the sum of agglutinin in fraction I to IV (14 and 13 unit/root respectively). The profile of the surface agglutinin released in each fraction were presented in Figure 3.

3.4 Sugar inhibition test of pericarp extract

Sugar specificity of agglutinin is normally demonstrated by ability of the sugar in inhibiting hemagglutinating activity. Many sugars including monosaccharides, disaccharides and polysaccharides (Table 3) at a concentration up to 500 mM were used in the inhibition test as described in section 2.2.6. None of the sugars showed inhibition on the agglutination of trypsinized rabbit erythrocytes induced by crude extract or 30-50% ammonium sulfate (AS-35) fraction. This could be that the agglutinin in the sample has no sugar specificity or there is certain factor in the crude extract that

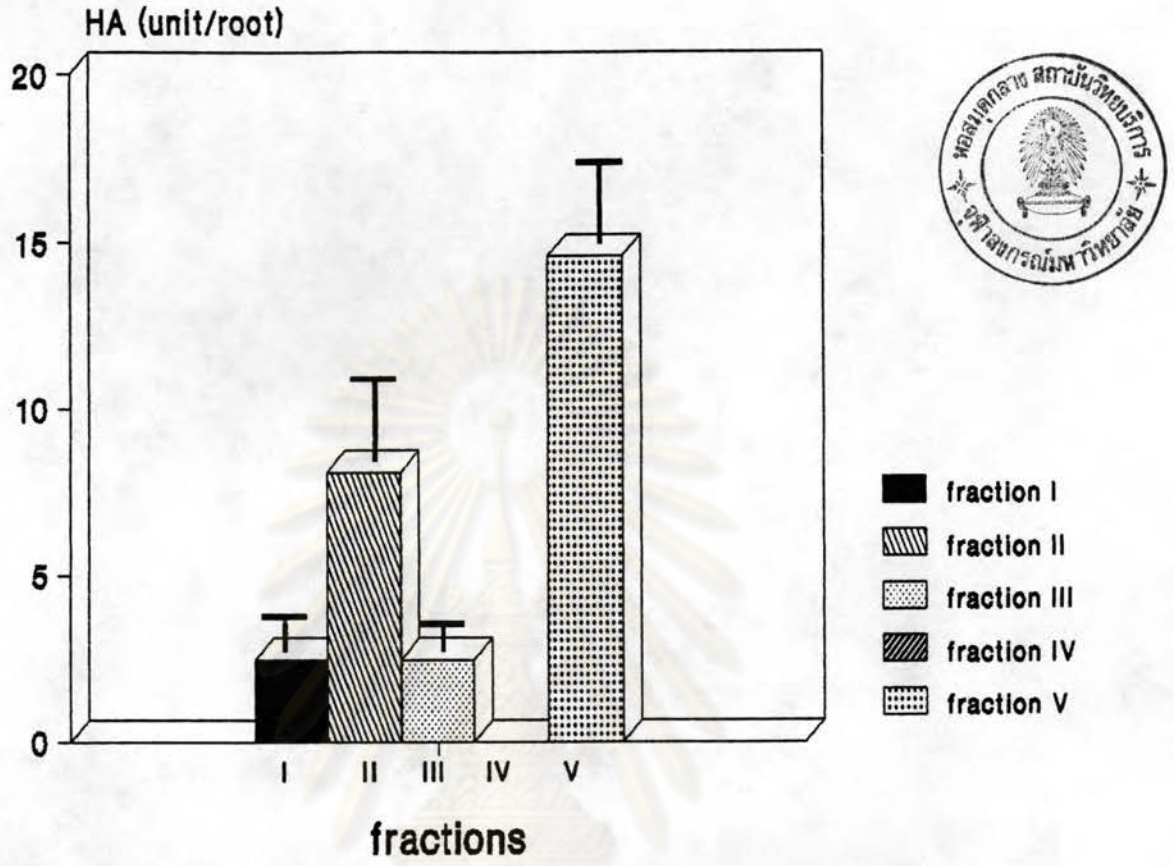


Figure 3 Release of agglutinin from seeedling root surface.

Hemagglutinating activity of surface agglutinin extracted by immersion in water for different period were investigated according to section 2.2.3.

fraction I	1 min-immersion.
fraction II-IV	three sucessive washes for 30 min each.
fraction V	one step 91 min immersion
fraction VI	homogenate from fraction I-IV and V

(note: ⊤ at the top of the bars showed standard deviation of each fraction.)

Table 3 List of carbohydrate used in sugar inhibition test of lectin from *Luffa acutangula* Roxb.

Monosaccharide	Disaccharide	Polysaccharide
glucose	sucrose	chitin*
galactose	maltose	chitosan
fucose	lactose	chitotriose
xylose	-	starch
mannose		glycogen
arabinose		
mannosamine		
glucosamine		
galactosamine		
manitol		
sorbitol		
ribose		
2-deoxyglucose		
nueraminic acid		
D-galacturonic acid		
N-acetyl-D-glucosamine		
N-acetyl-D-galactosamine		

Note: All those sugars were D-configuration.
Concentration of the sugar used were 3.9-500 mM.
* chitin hydrolysate

prevent successful sugar inhibition test. However, when hemagglutinating activity and sugar inhibition test were carried out (see section 2.2.9.2) on gel slices obtained from nondenaturing polyacrylamide gel electrophoresis (section 2.2.9.1) of crude extract, AS-35, AS-57 and DEAE-Cellulose, interesting result was observed. As the identification of lectin and its specific sugar by this method is rather tedious, only a few appropriate sugars were selected for the test. As most lectins in the family Cucurbitaceae including lectin from cucumber surface (Kessler,1984) were reported to be specific to the monomer and polymer of N-acetyl-D-glucosamine and its polymers such as chitotriose and chitin, they were chosen for the sugar inhibition test. Only the protein bands at position 0.4 cm from the top of the gel (Fig.4) showed HA with specificity for chitin and chitotriose at the concentration 83 μg chitin/ml and 0.52 μg chitotriose/ml .

3.5 Sugar inhibition test on surface extract of root seedling

Several carbohydrates (Table 3) were tested for inhibition of hemagglutinating activity of surface extract from 9 day-old seedlings as described in section 2.2.6. It was found that only chitin can specifically inhibit the hemagglutinating activity of root surface extract at 41.6 μg chitin/ml.

3.6 Purification of chitin specific lectin from pericarp extract

3.6.1 Ammonium sulfate precipitation

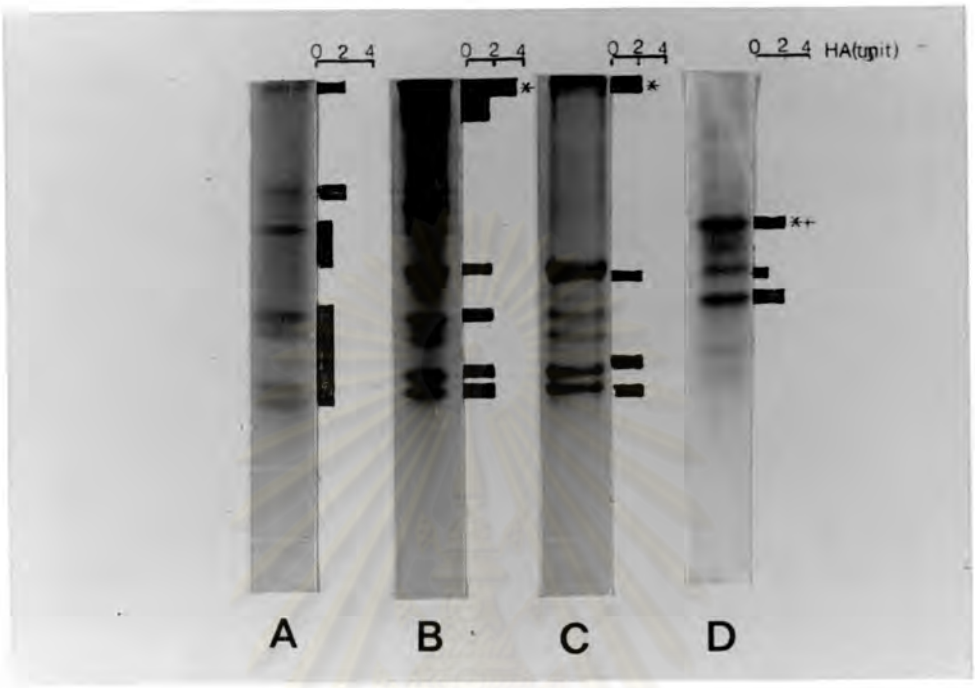


Figure 4 Localization of lectin on ND-PAGE

Samples of pericarp extractfrom different purification steps (crude, AS-35, DEAE-Cellulose, AS-57) were subjected to 7.5% ND-PAGE. Each gel was cut into 0.2 cm-slice as described in section 2.2.9.1 after electrophoresis.Then, each slice was tested for hemagglutinating activity and sugar specificity (section 2.2.5 and 2.2..5.6). The hemagglutinating activity of each slice was displayed in unit at the right side of each lane and *,** indicate chitin and N-acetyl-D-glucosamine specificity, respectively.

Lane A crude 100 μg

B AS-35 100 μg

C DEAE-Cellulose 100 μg

D AS-57 100 μg

Purification of lectin from fruit pericarp started with conventional step of ammonium sulfate precipitation. To determine the appropriate concentration for precipitating the agglutinin from fruit pericarp extract, serial precipitation of proteins by increasing concentration of ammonium sulfate was performed as described in section 2.2.7. Fruit pericarp extract was prepared according to section 2.2.1 and fractionated by gradual addition of solid ammonium sulfate with stepwise increase of percent saturation from 0-70 %. The precipitate at each step was collected by centifugation, resuspended in PBS and dialysed overnight in excess PBS. Hemagglutinating activity against trypsinized rabbit erythrocytes and protein content were determined. It was shown that hemagglutinating activity was highest in the 30-50 % ammonium sulfate fraction as shown in Table 4. Again, test of sugar specificity was negative in these fractions and identification of the fraction with chitin specific lectin was performed on gel slice (section 2.2.9.2). The 30-50 % fraction was shown to contain lectin of interest (Fig.4). This fraction was, thus, name AS-35 and used in the next step of purification.

3.6.2 Purification by column chromatographies

3.6.2.1 Chitin and chitotriose affinity column

As chitin and chitotriose showed specific inhibition on hemagglutinating activity of pericarp lectin, chitin and chitotriose were chosen as the appropriate affinity columns for next step of purification. Dialysed AS-35 was loaded to chitin or chitotriose column prepared as described in section 2.2.8.1 and 2.2.8.2, respectively and eluted with 1% chitin hydrolysate. Chitin

Table 4 Hemagglutinating activity in different fractions of ammonium sulfate precipitation of pericarp extract.

fraction	specific HA (unit/mg protein)
crude	612
0-30%	606
30-50% *	1613
50-70%	727
supernatant	470

* shown to be chitin specific in section 3.4

hydrolysate was then removed by passing the eluate through sephadex G-25 column or millipore prior to assay for hemagglutinating activity. As the results, there was nonspecific binding in chitin column and no binding in chitotriose column.

3.6.2.2 Chromatofocusing column

Attempts to use affinity columns as one step of purification were not successful, separation of protein according to its isoelectric point by chromatofocusing column was tried. Polybuffer exchanger (PBE 94), an anion exchanger, was used as column resin and eluted with polybuffer 74, follwed by 25 mM and 1M NaCl, respectively. The protein peaks were pooled and protein recovered as described in section 2.8.2.4. The chromatographic profile was shown in Figure 5. Hemagglutinating activity cannot be detected in any fractions eluted from the column, therefore this technique was not included in the purification step.

3.6.2.3 DEAE-Cellulose column

The DEAE-Cellulose was prepared as described in section 2.8.2.3. Dialysed AS-35 fraction of pericarp was loaded to DEAE-Cellulose column, washed with 0.02 M phosphate buffer pH 7.4 until A_{280} of the eluted fraction is negligible. The elution was done by gradient of 0-0.6 M in phosphate buffer. After that, the remaining protein of the column was washed with 1M NaCl. Hemagglutinating activity was tested in every fractions with A_{280} and the fractions of the peaks with hemagglutinating activity was pooled and tested for chitin specificity. The peak which was chitin

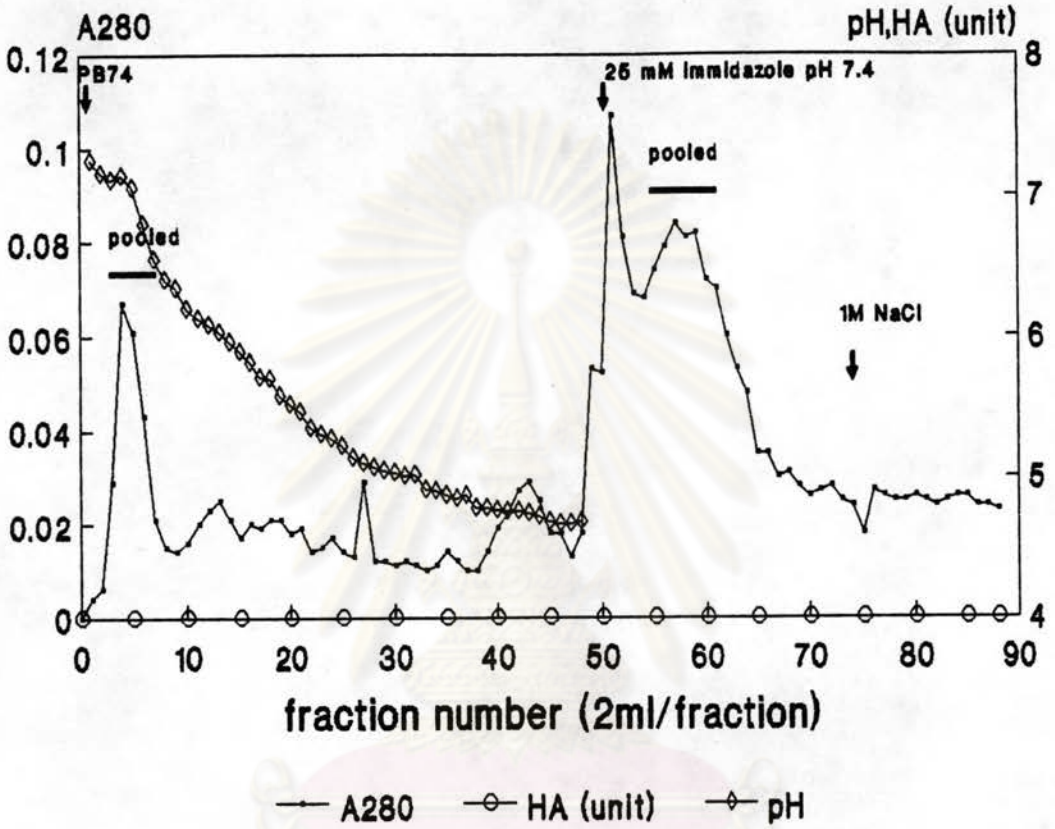


Figure 5 Separation of angled loofah fruit pericarp lectin by chromatofocusing column.

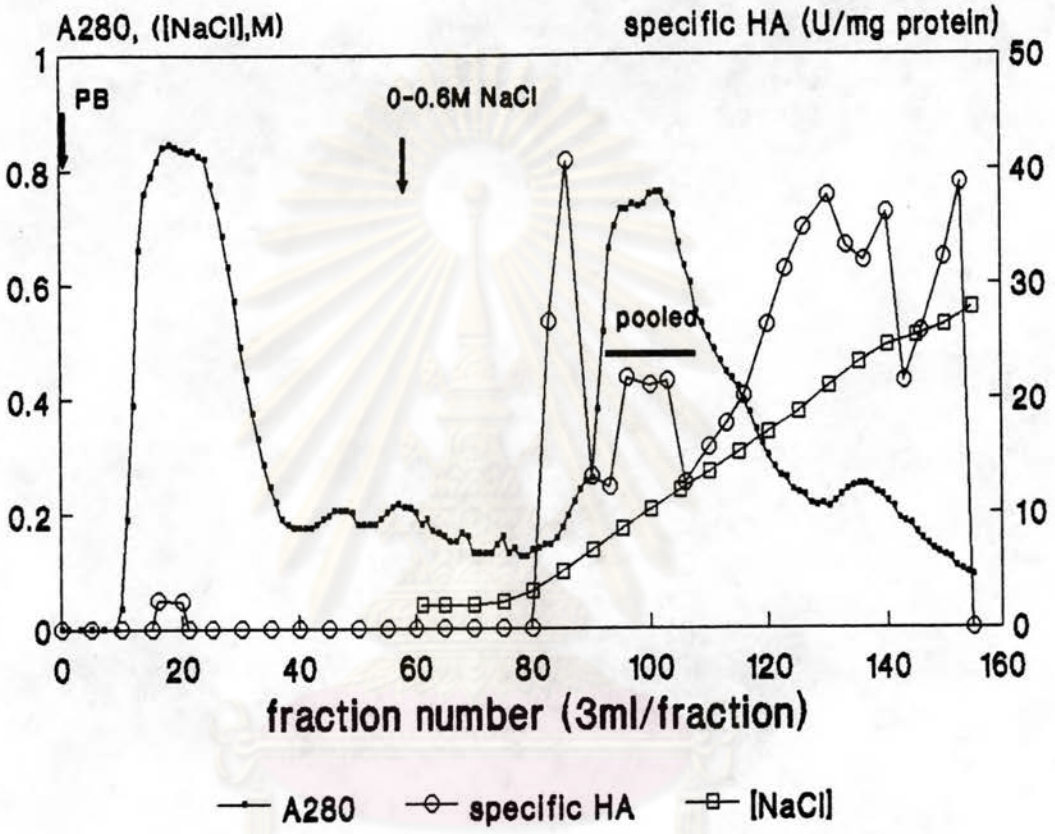


Figure 6 Chromatographic profile of angled loofah pericarp lectin by DEAE-Celllose column.

specific was collected for further purification. Figure 6 showed chromatographic profile of the AS-35 fraction on DEAE-Cellulose column. Fractions 91-115 which were identified as chitin specific lectin were eluted at the salt concentration of 0.20 M. The pooled fractions was specific to chitin at 125 μg chitin/ml and was concentrated by ultrafiltration and kept for further study. This step gave only 0.7% recovery and 1.37 folds of purification which seemed to be much lesser than the step of ammonium sulfate to protein elution as described in the following section. Therefore,this step would bypass.

3.6.3 Purification by protein elution from ND-PAGE

After the attempt to use the affinity chromatography and chromatofocusing column to purify chitin-specific lectin had failed. Next, ND-PAGE had been tried for the isolation. Identification was performed according to section 2.2.9.1 and protein at the identified site was eluted as described in section 2.2.9.2. The electrophoretic pattern was shown in Figures 4 and 7 and purification table was shown in Table 5. The gel elution method yielded 47% of hemagglutinating activity with 3.1 folds of purification comparing to ammonium sulfate and DEAE-Cellulose column.

3.7 Purification of seedling root surface lectin by fixed erythrocytes

Due to the minute amount of lectin obtained from surface washing, conventional method of purification was not applicable. To overcome this problem, formalinized rabbit erythrocytes was used as the affinity adsorbant as described in section 2.2.13. The result is

Table 5 Purification table of pericarp lectin.

fraction	total protein (mg)	total HA (unit)	sp.HA (unit/mg protein)	purification fold	%recovery
crude	840	896,000	1,067	1	100
AS-35	284.4	317,440	1,116	1.06	35.4
gel slice	125.4	409,600	3,265	3.11	45.7

Table 6 Purification of seedling root surface lectin by adsorption to formalinized trypsinized rabbit erythrocytes.

fraction	protein (mg)	total HA (unit)	SP.HA (unit/mg protein)	purification fold	%recovery
crude	0.10	664.3	2643	1	100
purified-lectin	0.01	54.7	5575	2.1	8.4

shown in Table 6. The recovery of hemagglutinating activity was small with 2.1 folds of purification.

3.8 Monitoring of purification of pericarp and seedling root surface lectins

Purifications of pericarp and seedling root surface lectins were monitored through ND-PAGE as described in section 2.2.9.3. The protein patterns of each purification steps were shown in Figures 7 and 8, respectively.

For pericarp lectin, the AS-35 fraction did not remove much of the proteins from crude extract (lane B). The protein in crude extract (lane A) did not show clearly in the gel. Probably, there is certain interfering factor in the crude extract that affect either protein determination or protein staining. However, when AS-35 was passed through DEAE-Cellulose column, lots of proteins were removed (lane C). When the protein purified by elution from gel slice (section 2.2.9.2) was rechecked on ND-PAGE (lane D), satisfactory purity is obtained.

Figure 8 showed the results of purification of root surface lectin by adsorbant on formalinized trypsinized rabbit erythrocytes. Most proteins were remove by this method. However, the fraction eluted from the erythrocytes did not show discrete band.

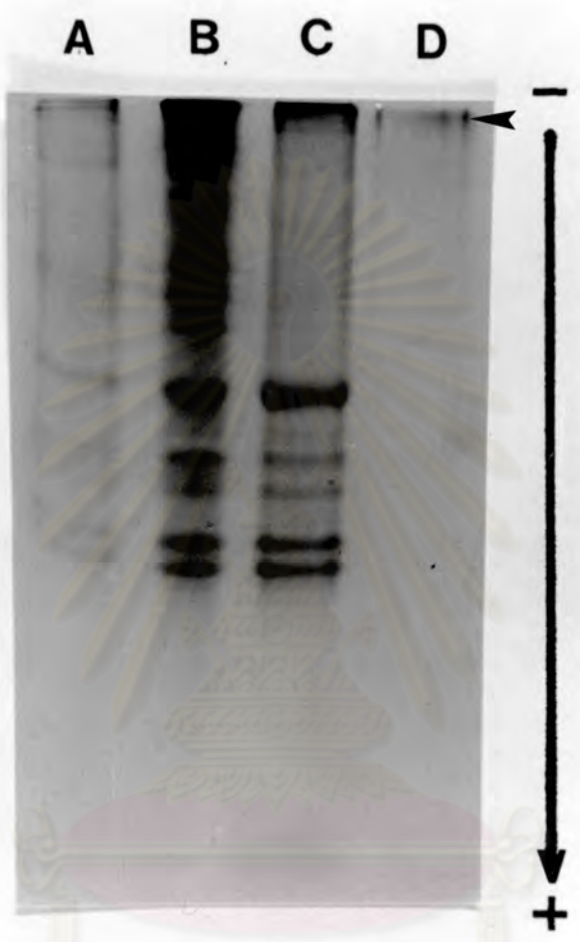


Figure 7 Protein pattern of ND-PAGE of pericarp lectin.

Nondenaturing polyacrylamide gel electrophoresis (ND-PAGE) was performed as described in section 2.2.9.3 and based on 20 μg protein.

Lane A crude extract

B AS-35

C DEAE-Cellulose fraction

D Gel slice

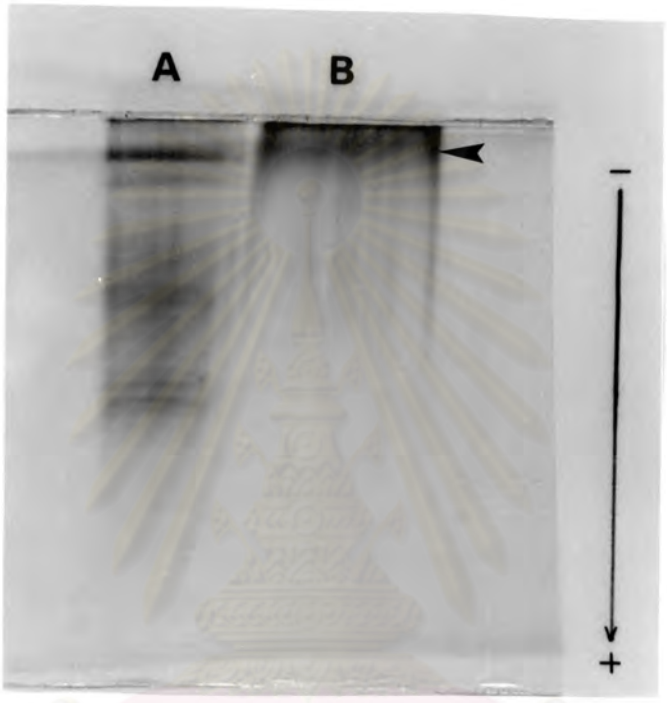


Figure 8 Nondenaturing polyacrylamide gel electrophoresis of seedling root surface lectin.

Lane A crude seedling root surface lectin (80 μg)

B purified seedling root lectin (20 μg)

3.9 Characterization of purified chitin specific lectin from loofah fruit pericarp and seedling root surface

3.9.1 Molecular weight determination

3.9.1.1 Molecular weight determination of pericarp lectin by gel filtration

Molecular weight of pericarp lectin was determined by gel filtration using sepharose 6B column as described in section 2.2.14. The column was preequilibrated with 0.02 M phosphate buffer pH 7.4 containing 5 mg/ml chitin before loaded and eluted with the same solution, only one peak was eluted from the column (Fig.9). The protein peak cannot be detected when the sample was eluted with phosphate buffer without chitin. The molecular weight of eluted peak was determined to be 105,000 from the calibration curve of the standard proteins of known molecular weight (Fig 10).

3.9.1.2 Molecular weight determination of pericarp lectin by SDS-PAGE

Purified pericarp lectin was subjected to SDS-PAGE under reducing condition according to section 2.2.10 (Fig.11). The protein appeared as 3 bands when stained with coomassie blue R. The molecular weight of the three bands determined from molecular weight calibration curve were 41,680, 31,600 and 26,300, respectively (Fig.12). The sum of the molecular weight of these bands is 99,580 which corresponded closely to the molecular weight of the native protein obtained from Sepharose 6B column (105,000). Thus,the

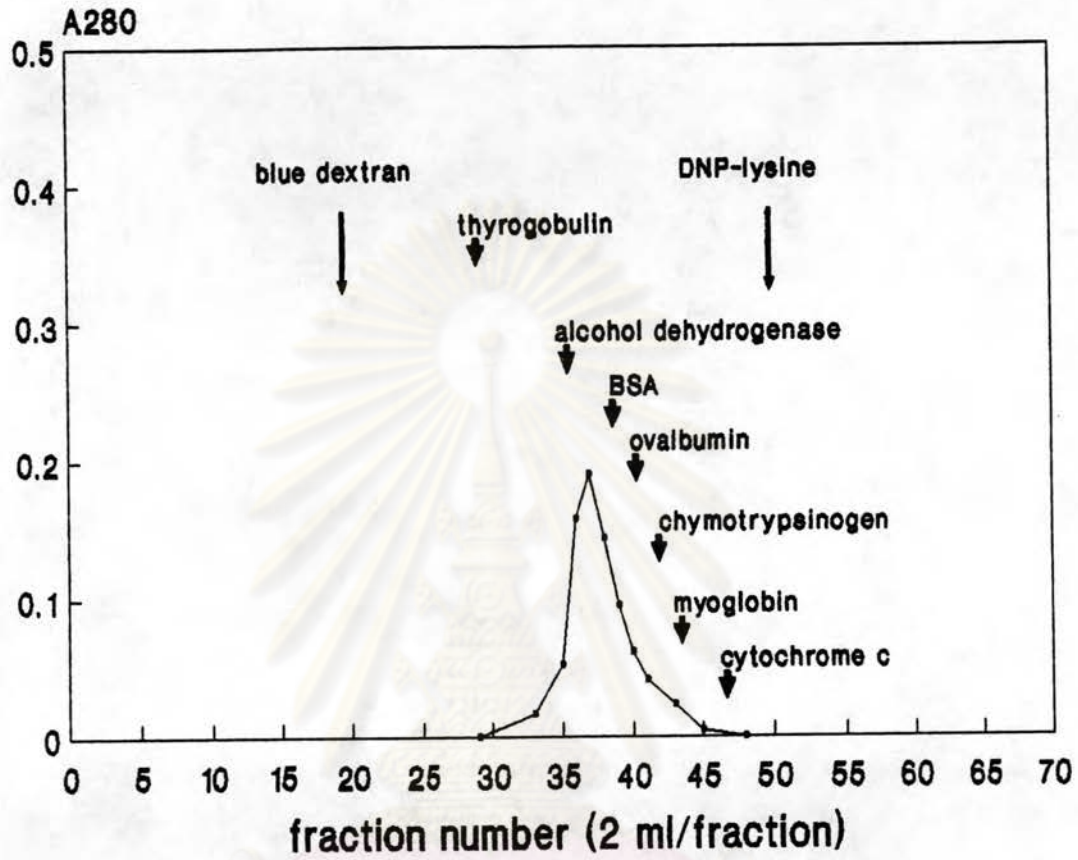


Figure 9 Elution profile of purified pericarp lectin on sepharose 6B column.

Purified lectin from fruit pericarp (1 ml of 0.5 mg) was loaded on sepharose 6B column equilibrated and eluted as described in section 2.2.14. This column was calibrated with blue dextran (2,000,000), DNP-lysine (366) and the following standard proteins : thyroglobulin (669,000) , alcohol dehydrogenase (150,000), BSA (68,000), ovalbumin (43,000), chymotrypsinogen (23,240), myoglobin (17,000), cytochrome C (12,380).

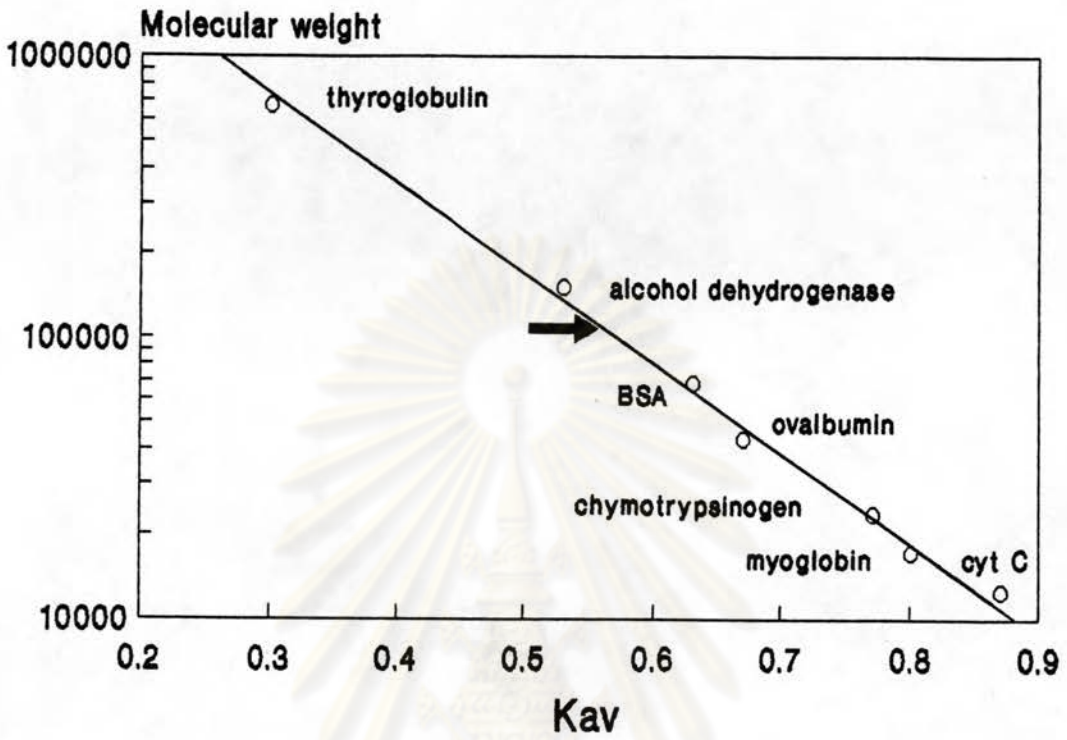


Figure 10 Molecular weight calibration curve for determination of molecular weight by gel filtration on sepharose 6B.

The K_{av} of each standard protein was calculated from Figure 9 and were plotted against its molecular weight. The K_{av} of purified pericarp lectin was indicated by the arrow which corresponded to molecular weight of 105,000. Molecular weight of the standard proteins were presented in Figure 9.

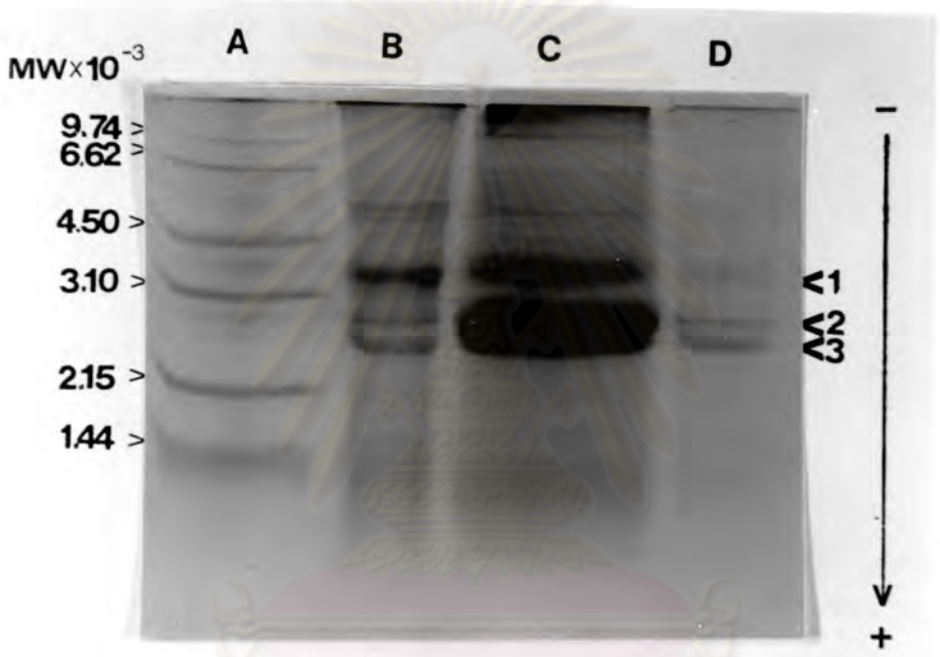


Figure 11 Molecular weight determination of pericarp lectin by SDS-PAGE.

SDS-PAGE was performed as described in section 2.2.10.

Lane A standard proteins: phosphorylase b (9.74 kD), BSA (6.62 kD), ovalbumin (4.50 kD), carbonic anhydrase (3.10 kD), trypsin inhibitor (2.15 kD) and lysozyme (1.44 kD).

B AS-35 80 μg

C DEAE-Cellulose fraction 80 μg

D purified lectin from gel slice 80 μg

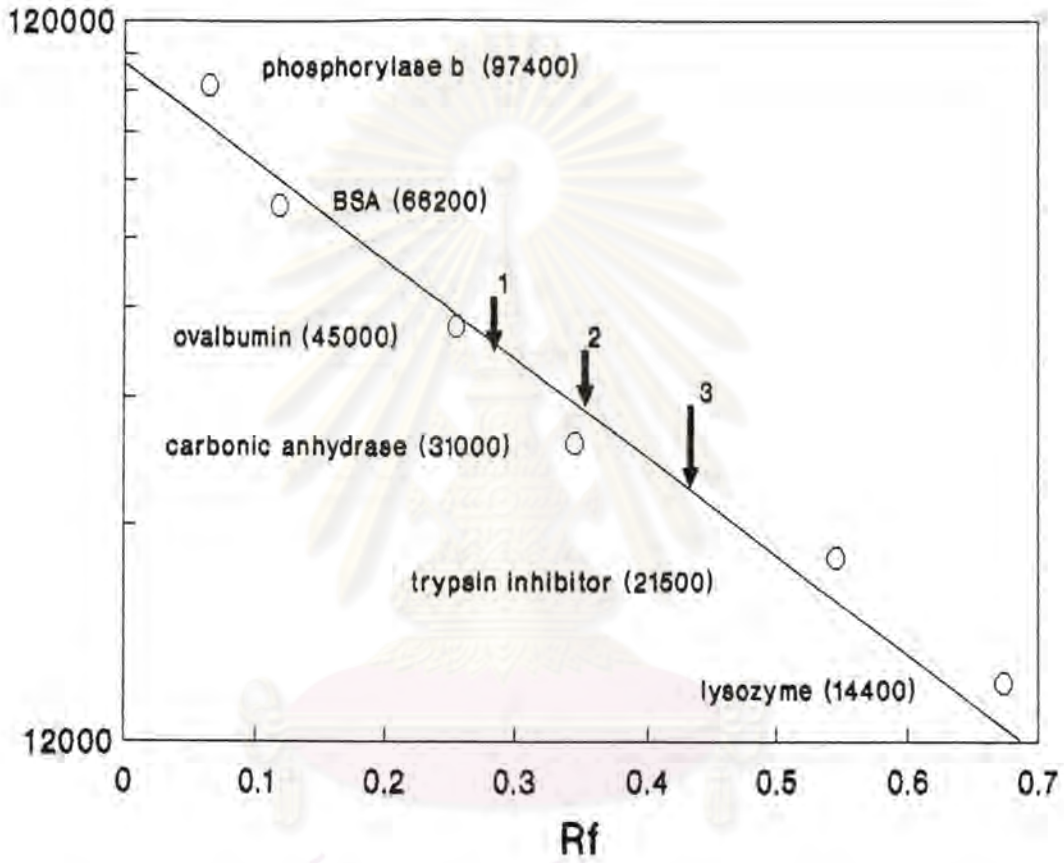


Figure 12 Molecular weight calibration curve of standard proteins separated on 12.5% SDS-PAGE.

Molecular weight of standard markers (as shown in the figure) were plotted on semilog scale against their relative mobility on 12.5 % SDS-PAGE. The arrows indicated subunits of pericarp lectin which corresponded to 41,680,31,600 and 26,300, respectively.

pericarp lectin seems to be consisting of 3 nonidentical subunits.

3.9.1.3 Molecular weight determination of root lectin by SDS-PAGE

The lectin of seedling root surface purified by fixed red cells was subjected to SDS-PAGE as described in section 2.2.10 under reducing condition (Fig.13). The molecular weight was 28,000 based on a standard curve of protein markers (Fig.14). Since the amount of purified root lectin obtained by purification with fixed red cells was minute, determination of molecular wieght by gel filtration was not possible. Therefore, only SDS-PAGE was employed for the purpose.

3.9.2 pI determination of pericarp lectin and seedling root lectin

Purified pericarp lectin and seedling root surface lectin were analysed for their pI by isoelectricfocusing on polyacrylamide gel (IEF) as described in section 2.2.15. The range of the pI used was 4-6 but the standard pI used were mixture of proteins with pI 4.6-9.6. Therefore, the protein with pI at the both ends were not well separated. The protein staining of the IEF gel was shown in Figure 15. Each protein showed up as one single band and the pI's read out from the pI calibration curve (Fig.16) was 6.25 for pericarp lectin and 6.15 for seedling root surface lectin.

3.9.3 Carbohydrate content in pericarp and seedling root lectin

Carbohydrate content of each purified lectin was also determined in comparison of glucose as standard in anthrone reaction as

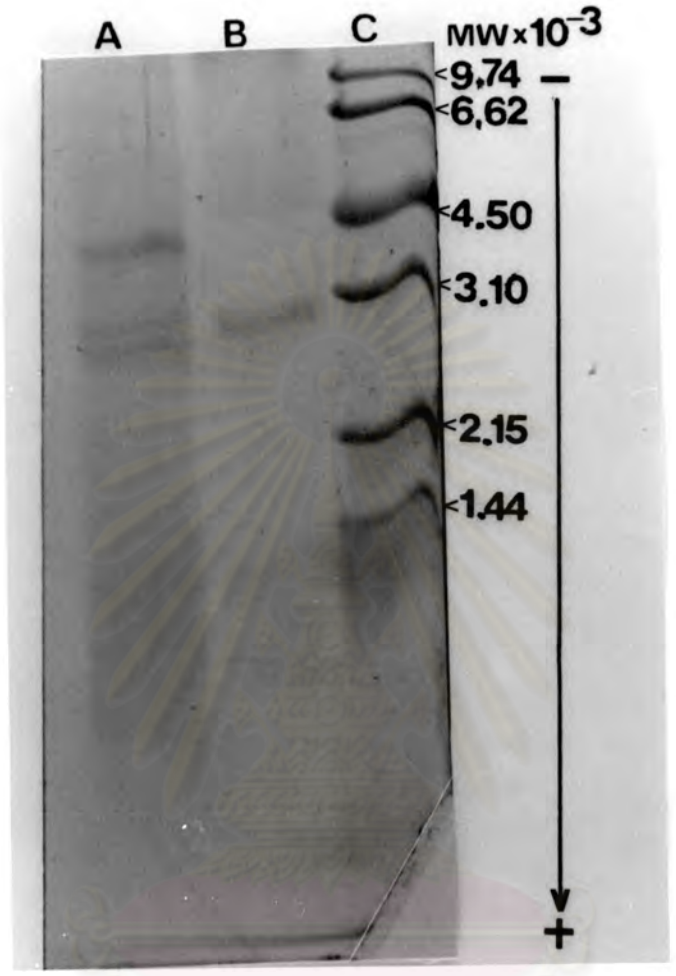


Figure 13 Molecular weight determination of purified root lectin by SDS-PAGE.

Purified root lectin was determined for its molecular weight by SDS-PAGE as mentioned in section 2.2.10.

Lane A crude 20 μg

B purified fraction 20 μg

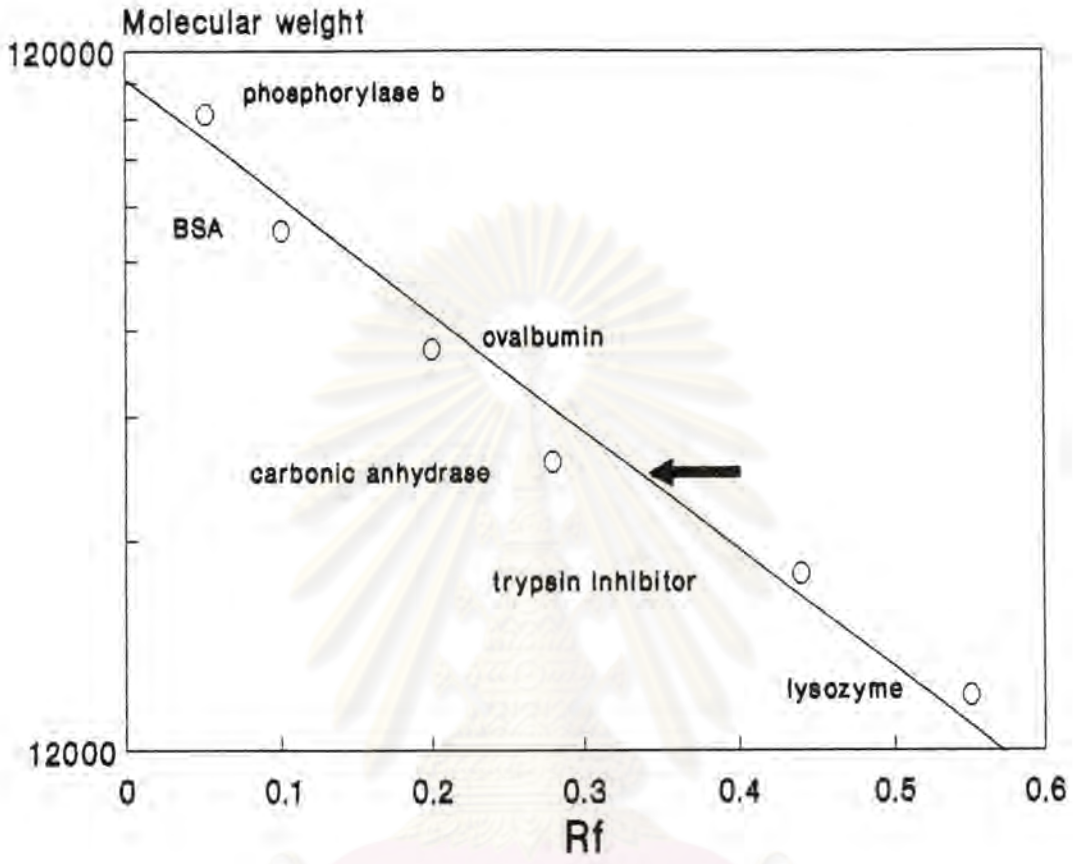


Figure 14 Standard curve for determination of molecular weight of purified seedling root lectin by SDS-PAGE.

12.5% gel SDS in SDS-PAGE was performed as described in section 2.2.10. The relative mobility (Rf) of each standard proteins (showed in the figure) was calculated and plotted against its molecular weight on semilog scale. The arrow indicated band position purified lectin.

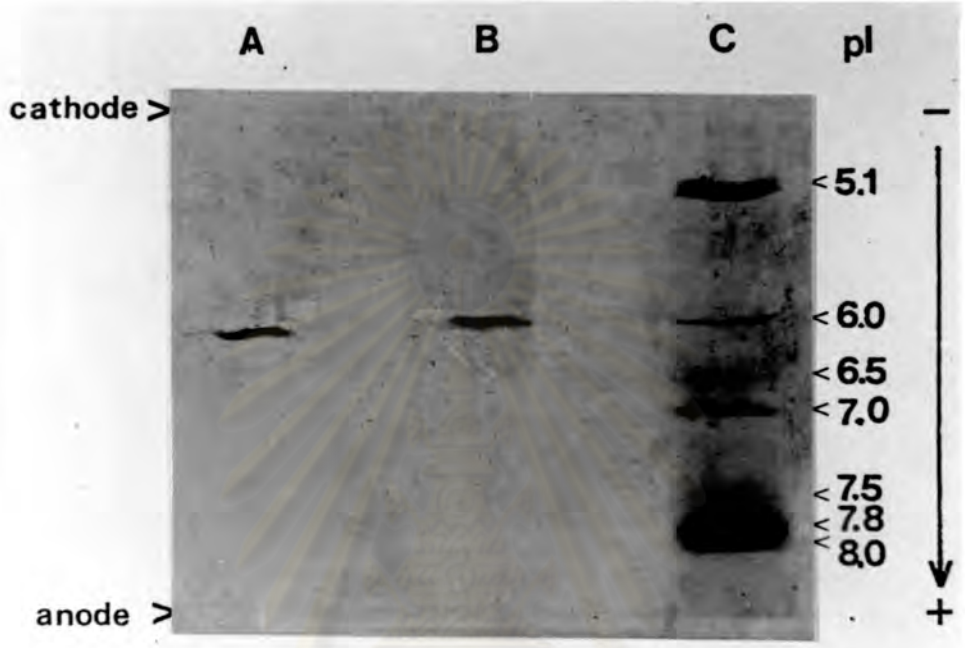


Figure 15 Isoelectric focusing polyacrylamide gel electrophoresis of purified pericarp and seedling root surface lectins.

Isoelectric focusing (IEF) polyacrylamide gel electrophoresis was used for pI determination of purified lectins from pericarp and root according to the method in section 2.2.15 with simultaneous run of pI standard markers: basic lentil lectin (pI 8.2), acidic lentil lectin (pI 7.8), human hemoglobin (pI 6.5), bovine carbonic anhydrase (pI 6.0), β-lactoglobulin B (pI 5.1).

Lane A pericarp lectin

B root surface lectin

C standard markers

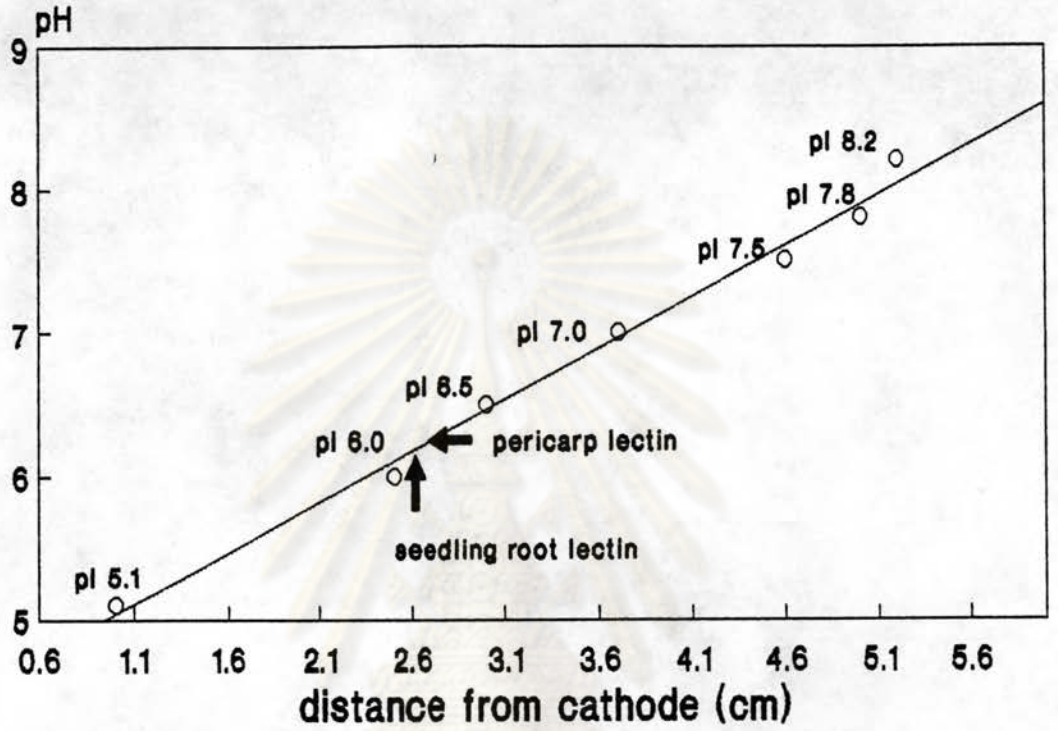


Figure 16 Calibration curve of standard pI markers for determination of isoelectric pH of pericarp lectin and seedling root lectin.

The migration distance from cathode of the pI standard markers (ascribed in Figure 15) were measured and plotted against pI's. Band position of the two purified lectins were indicated by arrows.

described in section 2.2.21 as shown in Table 7,the carbohydrate content of pericarp lectin and root lectin were 43 % and 12 % respectively.

3.9.4 Thermostability test

An aliquot of each purified lectin was incubated at various temperature from 0-120°C as described in section 2.2.16. Hemagglutinating activity were tested with trypsinized rabbit erythrocyte. Hemagglutinating activity of both lectins were fully maintained after incubation at 0-60°C for 20 minutes (Fig. 17). The lectins were partailly inactivated after heating at 70°C. Complete inactivation occured after heating pericarp lectin at 90°C for 20 minutes. However, root lectin still maintained part of its activity at 90°C but was completely inactivated at 100°C.

3.9.5 pH stability test

An aliquot of each purified lectin in distilled water was lyophylised and dissolved in different pH's from 3-10 as described in section 2.2.17. After two folds dilution of each kind of buffer with PBS,the pH could bring back to 7.4. The hemagglutination activity test was performed with formalinized trypsinized rabbit erythrocytes since they were proved to be more susceptible to wide pH range with several kinds of buffer. The seedling root surface lectin was rather stable in pH range 3-9 and its activity dropped to 50% at pH 10 (Fig.18). The activity of pericarp lectin was most stable at pH 7-8 and less stable at pH values above 8 and below 7. It was note that Tris-HCl buffer pH 8 could reduce HA to 50% whereas phosphate buffer

Table 7 Carbohydrate content in loofah lectins.

Source of lectin from *Luffa acutangula* Roxb.	Content of carbohydrate (%w/w)
fruit pericarp	43
seedling root surface	12

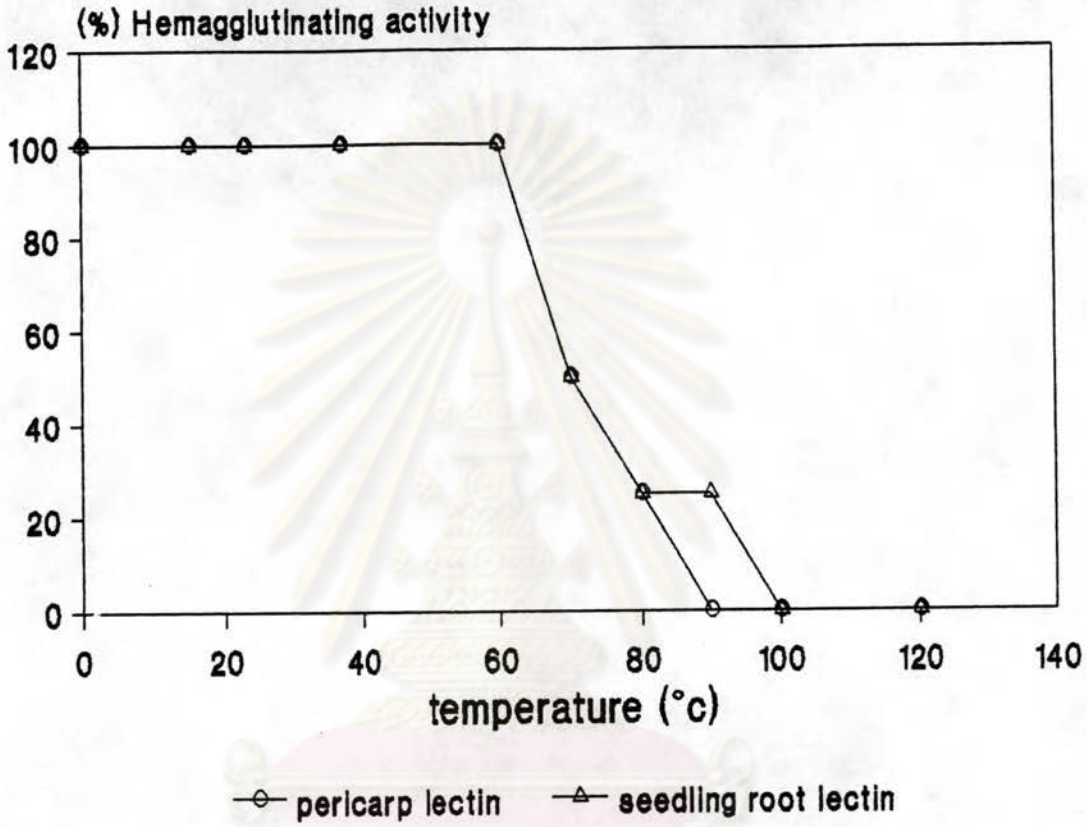


Figure 17 Thermostability of the purified pericarp and seedling root lectin from angled loofah.

An aliqiot of each lectin was cooled and heated at different temperature from 0-120°C for 20 min and rapidly cooled in ice. Hemagglutinating activity was determined using trypsinized rabbit erythrocyte. Results were expressed as percentage of agglutination activity in comparison to the control which was lectin kept in ice.

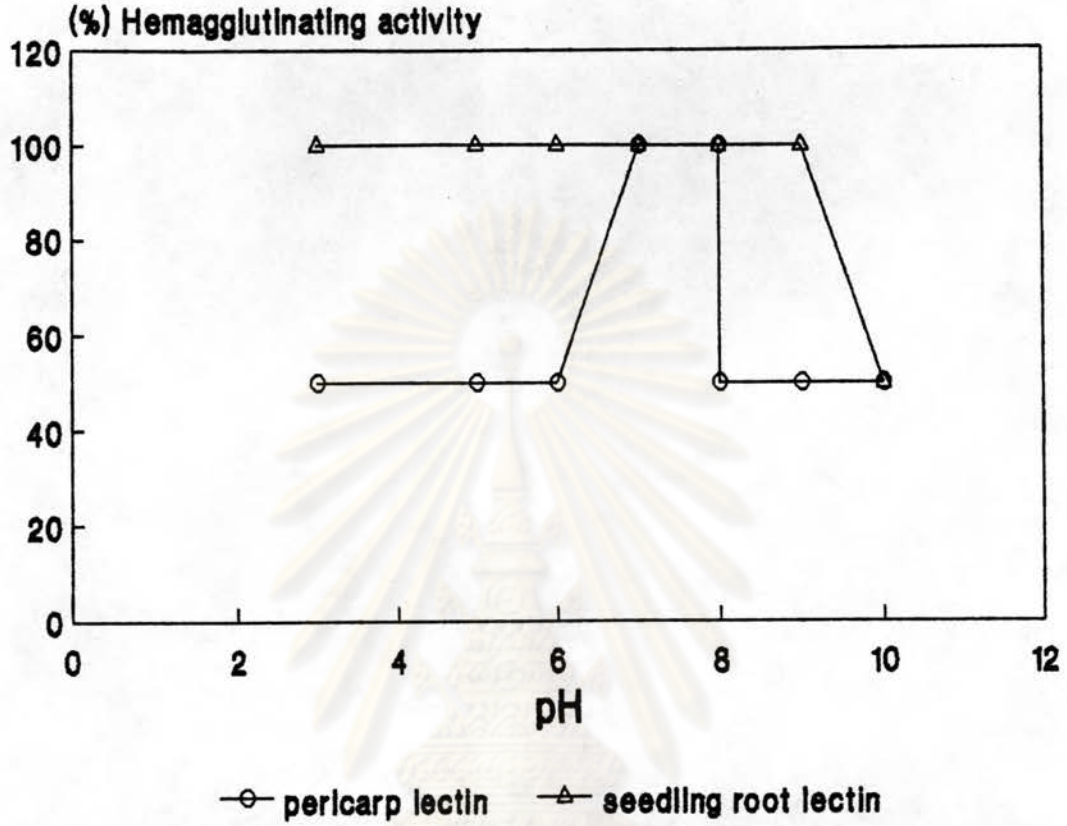


Figure 18 pH stability profile of purified pericarp and root lectins from angled loofah.

Each purified lectin was subjected to tested of stability at difference pH's as described in section 2.2.17. At pH 8.0, experiments were performed on two kind of buffers : pH 8.0 phosphate buffer pH 8.0 and Tris-HCl pH 8.0.

at the same buffer could not.

3.9.6 Some biological properties of purified lectins

The purified lectins from pericarp and root were tested for their biological properties in terms of certain defense mechanism such as effect on plant pathogens or test of superoxide dismutase (SOD)-like activity.

3.9.5.1 Effect on fungal growth

Lectins from several fractions of purification step of both fruit pericarp and seedling root surface were tested for effect on fungal growth as described in section 2.2.18. Five species of fungi which were known to be inhibited by chitin-specific lectin or being pathogenic to plants were used in the tests. It was found that lectins from those fractions could inhibit fungal growth.

As shown in Table 8, pericarp extracts from all steps of purification except crude fraction can completely inhibit growth of *A.flavus*. For other fungi tested, the most purified fraction *i.e.* the lectin eluted from gel slice showed the highest inhibitory effect when compared to other partially purified fractions , even the amount of protein used in the test was ten times lower.

For seedling root lectin, either the crude or purified fraction appeared very effective in growth inhibition of most fungi. Complete inhibition by both fractions were observed in *T.viride*, *A.flavus* and *C.kikuchii* ; while nearly 90 % inhibition was observed for *F.oxysporum*. Only *C.lunata* seemed to be least affected by these two fractions (54% inhibition) even the test

concentration were raise up to 0.35 µg f or crude extract and 0.21 µg for purified fraction. It was noted that, for seedling root extract, both the crude and purified fraction gave similar effect on the spore germination.

In the cases where high percentage of inhibition on fungal growth were observed , spore clumping was almost the consequence. As shown in Figure 19 , for *T.viride* and *A.flavus* where more than 80 % inhibition was reported for all fractions (A & B c,d,e,f,g), spore clumping was observed . Similar result was also shown for the effect of seedling root lectin on *C.kikuchii* (D f,g).

Table 8 Fungal growth inhibition test

Various fractions from fruit pericarp and seedling root surface lectins were tested for inhibition of fungal growth as described in section 2.2.18. The results were expressed as percentage of nongerminated spores to total spores in the same field.

Number in parenthesis showed protein concentration in μg unit that caused inhibition.

Test fraction (μg protein)	(%) Inhibition of spore germination				
	T.viride	*A.flavus*	*C.lunata*	*C.kikuchii*	*F.oxysporum*
control (distilled water)	2	0	19	9	14
pericarp lectin					
crude(3.18)	0	0	2	0	5
AS35(0.42)	66	100	98	38	86
DEAE-cellulose (0.04)	84	100	20	52	52
gel slice(0.03)	100	100	52 (0.09)	90	86
seedling root lectin					
crude(0.14)	100	100	54 (0.35)	100	88
purified lectin (0.07)	100	100	54 (0.21)	100	87

Figure 19 Effect of lectin fractions from pericarp and seedling root of angle loofah on fungal growth.

Fraction from each step of purification of pericarp lectin of angled loofah was tested for effect on spore germination of several fungi.

Test fungi

A = *Trichoderma viride*

B = *Aspergillus flavus*

C = *Curvularia lunata*

D = *Cercospora kikuchii*

E = *Fusarium oxysporum*

Test material

a = germination in distilled water

b = "————————" crude extract of pericarp (3.18 μg)

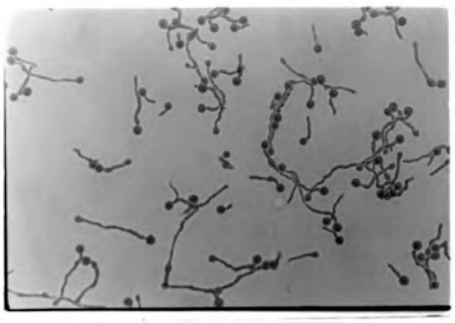
c = "————————" AS-35 "————————" (0.42 μg)

d = "————————" DEAE-Cellulose fraction of pericarp (0.04 μg)

e = "————————" gel slice "————————" (0.03 μg)

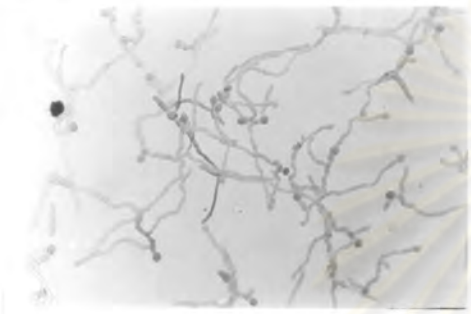
f = "————————" crude extract of seedling root (0.35 μg)

g = "————————" purified seedling root lectin (o.21 μg)



(a)

A. *Tricoderma viride*



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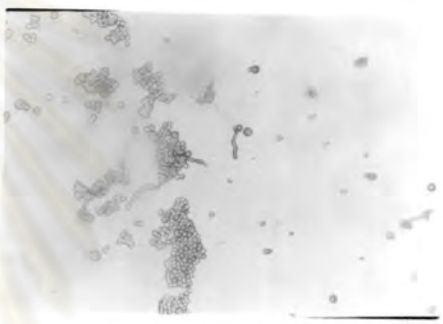

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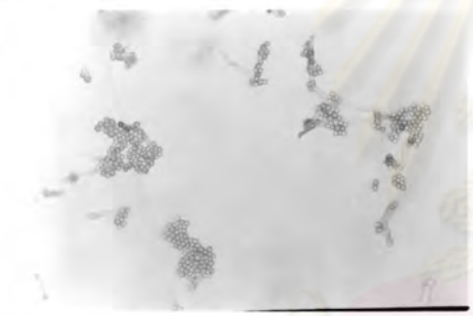

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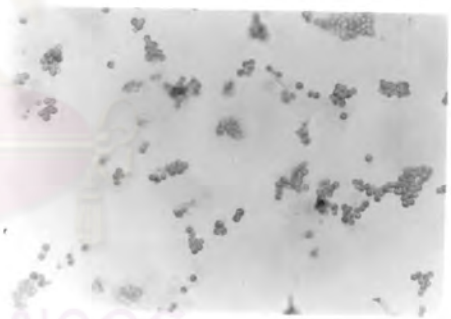

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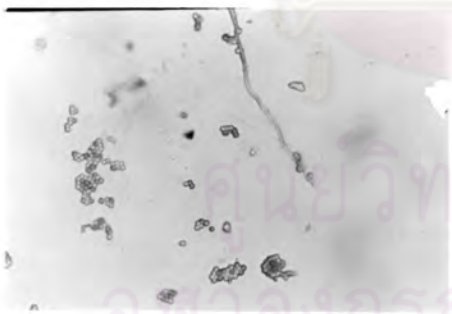

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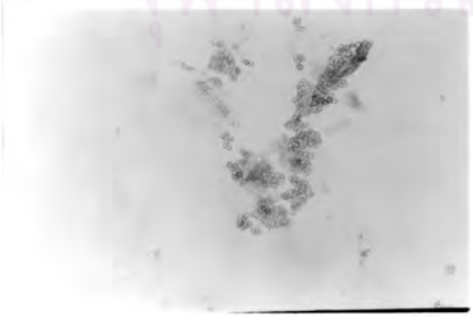

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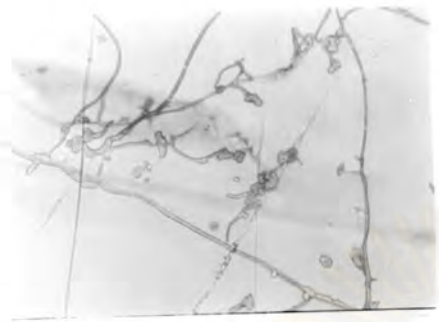

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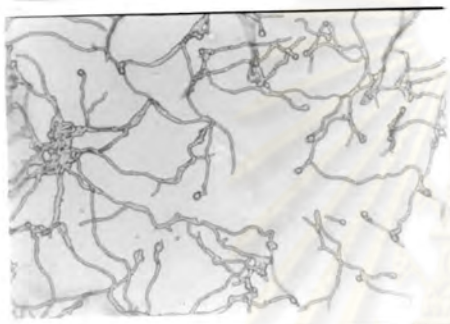
B. *Aspergillus flavus*



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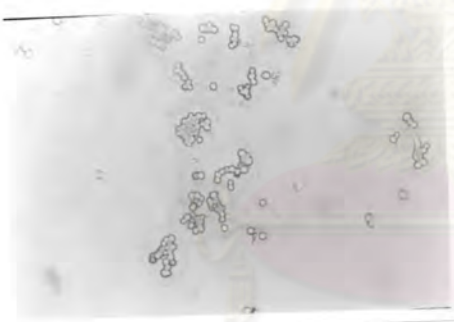

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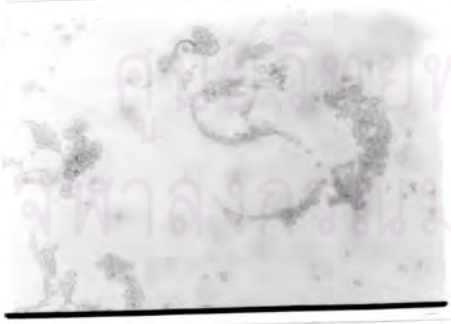

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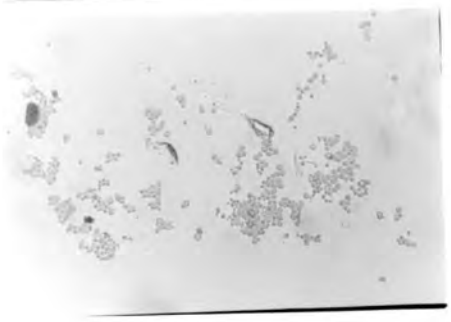

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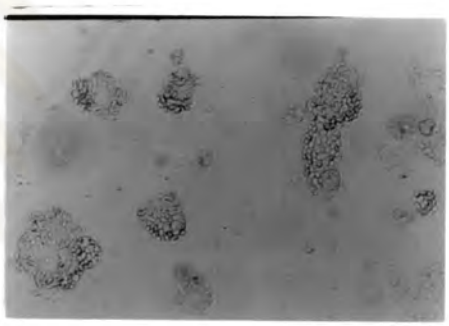

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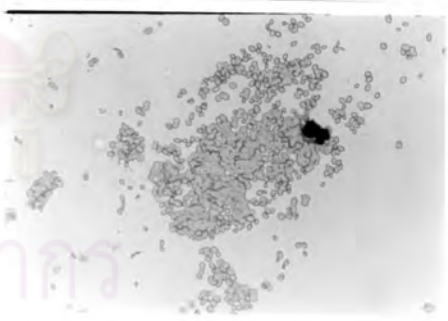

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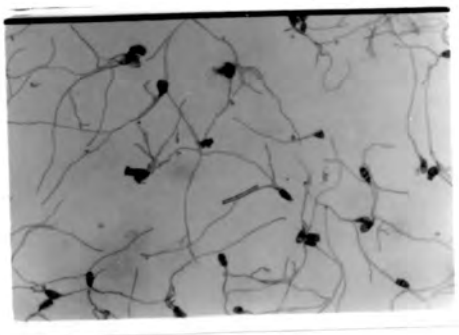
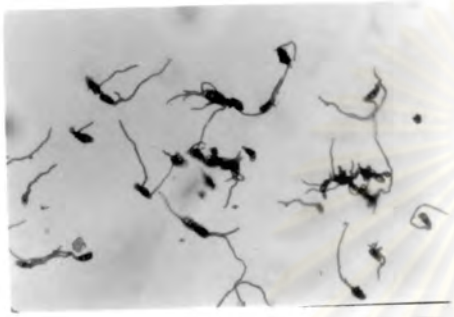


(a)

C. *Curvularia lunata*



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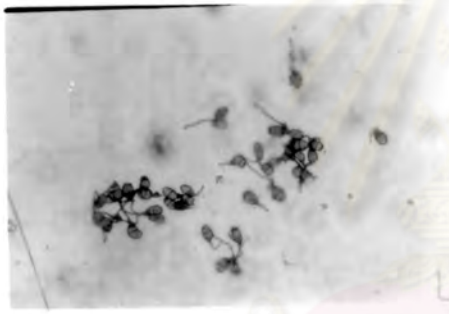

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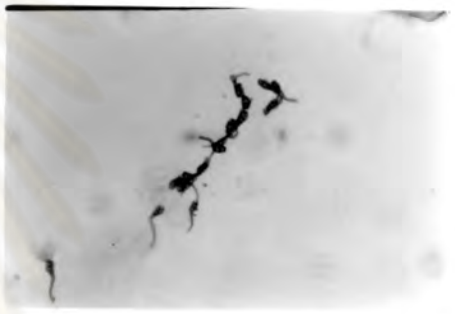

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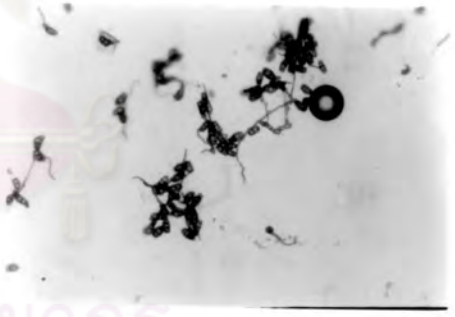

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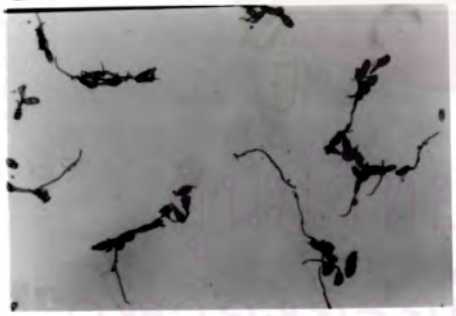

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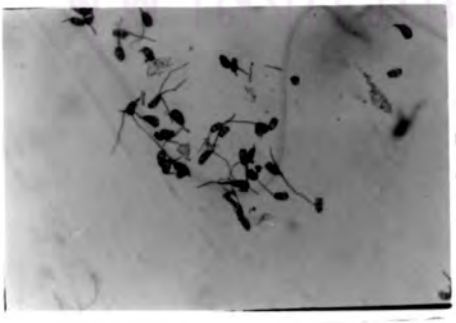

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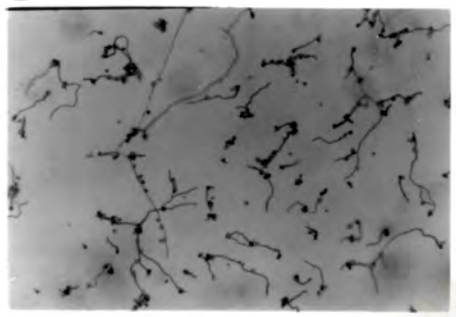
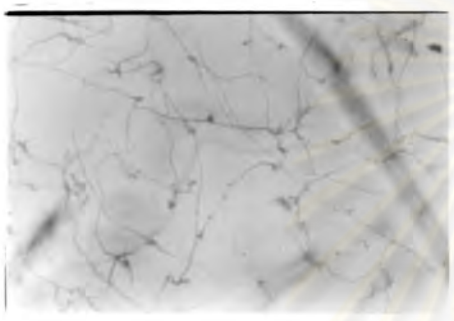


(a)

D. *Cercospora kikuchii*



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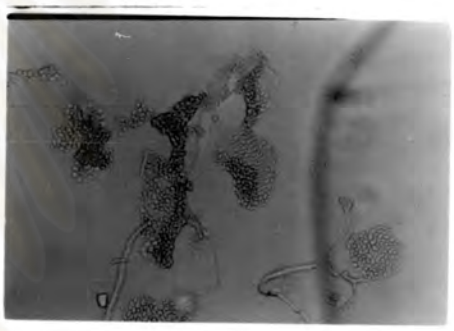

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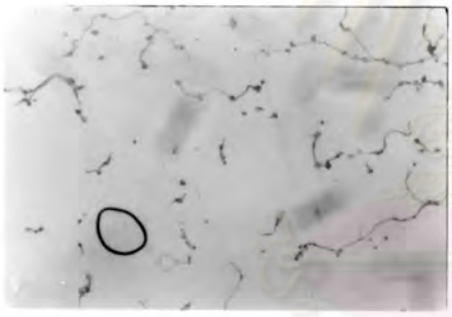

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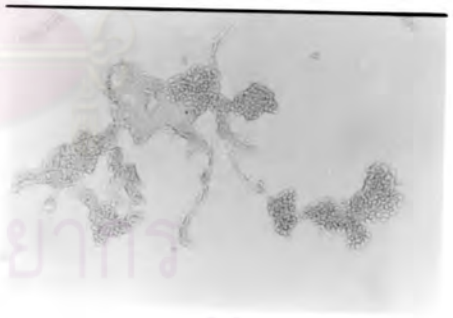

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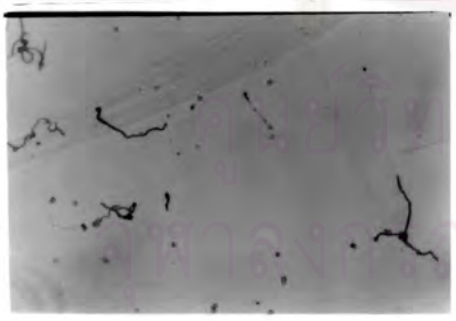

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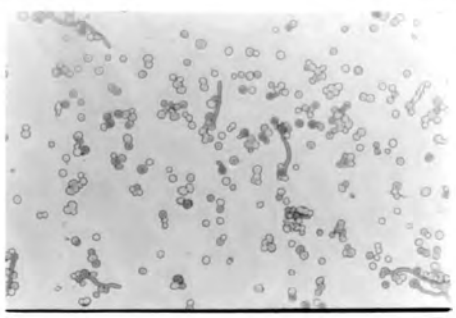
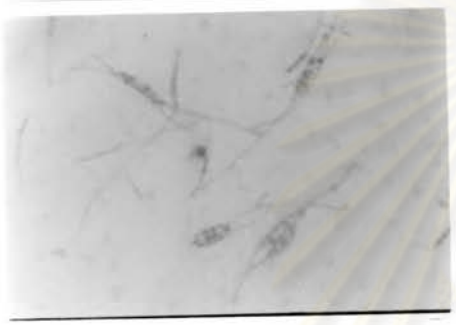


(e)

a

E. *Fusarium oxysporum*



b

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g

3.9.6.2 SOD-like activity

Fractions from each purification step of fruit pericarp and seedling root surface were tested for SOD activity by xanthine-xanthine oxidase system (section 2.2.19.1). The activity detected by this method was reported in Table 9. High SOD activity was found in crude extracts of pericarp and seedling root surface : 140 and 173 units of SOD/mg protein, respectively. Purification steps of fruit pericarp lectin removed significant amount of SOD especially the DEAE-Cellulose fractions in which SOD was decreased by 80 %. The gel slice fraction retained comparable SOD activity to the AS-35 fraction (35%). SOD activity in crude extract of seedling root surface was comparable to crude extract of pericarp (173.25 and 140.06 unit/mg protein, respectively). One step purification of seedling root lectin removed apprximately 50% SOD activity.

When each fractions of pericarp extract were subjected to ND-PAGE and stained for SOD according to the method described in section 2.2.19.2. Several SOD bands could be observed in the crude or partially purified fraction. However, the band identified to be chitin specific lectin at the top of the gel also showed staining for SOD. (Fig.20)

When SOD activity in the surface extract of cotyledon, hypocotyl and root of seedling at different ages were measured (Fig.21) , high SOD activity was observed in the root in comparison to other parts. A peak of SOD was observed at day 5 and the level of SOD increased again from day 7 onward.

Table 9 SOD activity in fruit pericarp and seedling root surface.

fraction		SOD specific activity (unit/mg protein)
pericarp:	crude	140.06
	AS-35	52.48
	DEAE-Cellulose	29.07
	gel slice	43.26
seedling:	crude	173.25
	purified lectin	83.33

Note: The SOD activity was detected by xanthine-xanthine oxidase method as described in section 2.2.19.1.

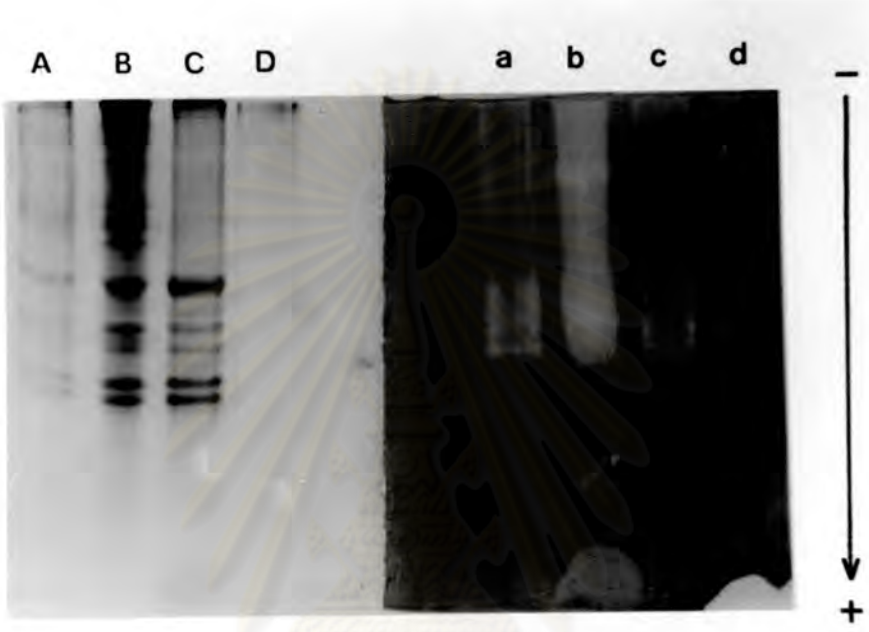

Figure 20 Superoxide dismutase activity stain of pericarp extract on ND-PAGE.

SOD-activity stain was performed as described in section 2.2.19.2 (displayed in printed letter), in comparison with Coomassie blue R protein stain (shown in capital letter).

Lane A,a crude extract

B,b AS-35

C,c DEAE-Cellulose partial purified fraction

D,d gel slice purified fraction

Note : All fraction based on 20 μg protein.

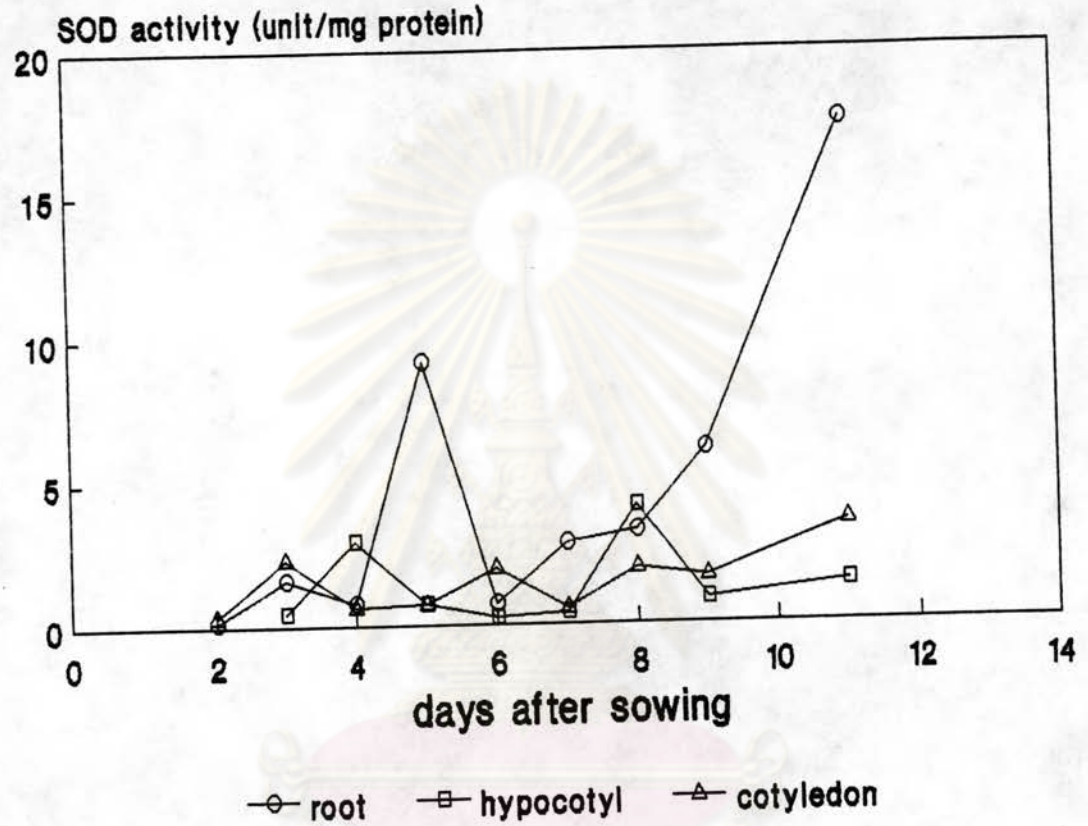

Figure 21 SOD activity of surface extracts of parts from seedlings.

Extracts from each part of seedling in developing stage were tested for SOD-activity which determined by xanthine-xanthine oxidase system according to section 2.2.19.1.