CHAPTER 3

Results

3.1 Purification of rice lectin (cv RD 7 and RD 25)

Lipids from 200 g of embryo was removed by organic solvent, because interference of lipids would occur if this step was omitted. The acid extract was carried out in 0.01 M HCl, 0.15 M NaCl,pH 2. The protein extract contained specific hemagglutinating actvity (HA/mg protein) as shown in Table 3.1. Further lectin purification was achieved by ammoniumsulfate fractionation at 60 % saturation. The total activity was increased to almost the same extent in both cultivars, RD 7 and RD 25 in this step. The lectin containing dialysate from ammoniumsulfate fraction was applied on the chitin column, and the other proteins were eluted (Fig 3.1 a & b). At neutral pH rice lectin was tightly bound to chitin, so that it was not eluted with 0.2 M GlcNAc, but efficiently eluted with 1 % W/V chitin hydrolysate (oligomer of chitin) at pH 3.8. The elution profile of rice lectin either cv RD 7 or RD 25 exhibited only one symmetrical protein peak, but the hemagglutinating activity (HA) of RD 25 was broader than RD 7(Fig 3.1 b and a). Table 3.1 showed significant removal of non chitin binding proteins during the

Table 3.1 Purification of rice lectin from embryo of rice (Oryza sativa L., cv RD 7 and Rd 25)

Procedure	Protein (mg/ml)	Total Protein (mg)	HA* Titer	Total HA (unit) $\times 10^5$	Activity % Yield	Specific Activity (unit/mg Protein)	Purification (Fold)
RD 7							
Acid Extracts	0.86	1703.40	8	6.39	100	9.35	1
$(NH_4)_2SO_4$	7.31	1023.40	128	7.16	112	17.51	2
Chitin	0.07	3.58	256	5.63	88	3932.40	420
SP-Seph -dex C-50	0.01	1.05	128	5.22	81	15311.00	1,637
RD 25							
Acid Extracts	0.55	753.18	8	4.35	100	14.46	1
$(NH_4)_2SO_4$	3.89	467.40	128	6.140	140	32.86	2
Chitin	0.06	6.48	128	6.02	138	2321.37	160
SP-Sepha -dex C-50	0.03	0.53	512	4.20	96	19715.05	1,362

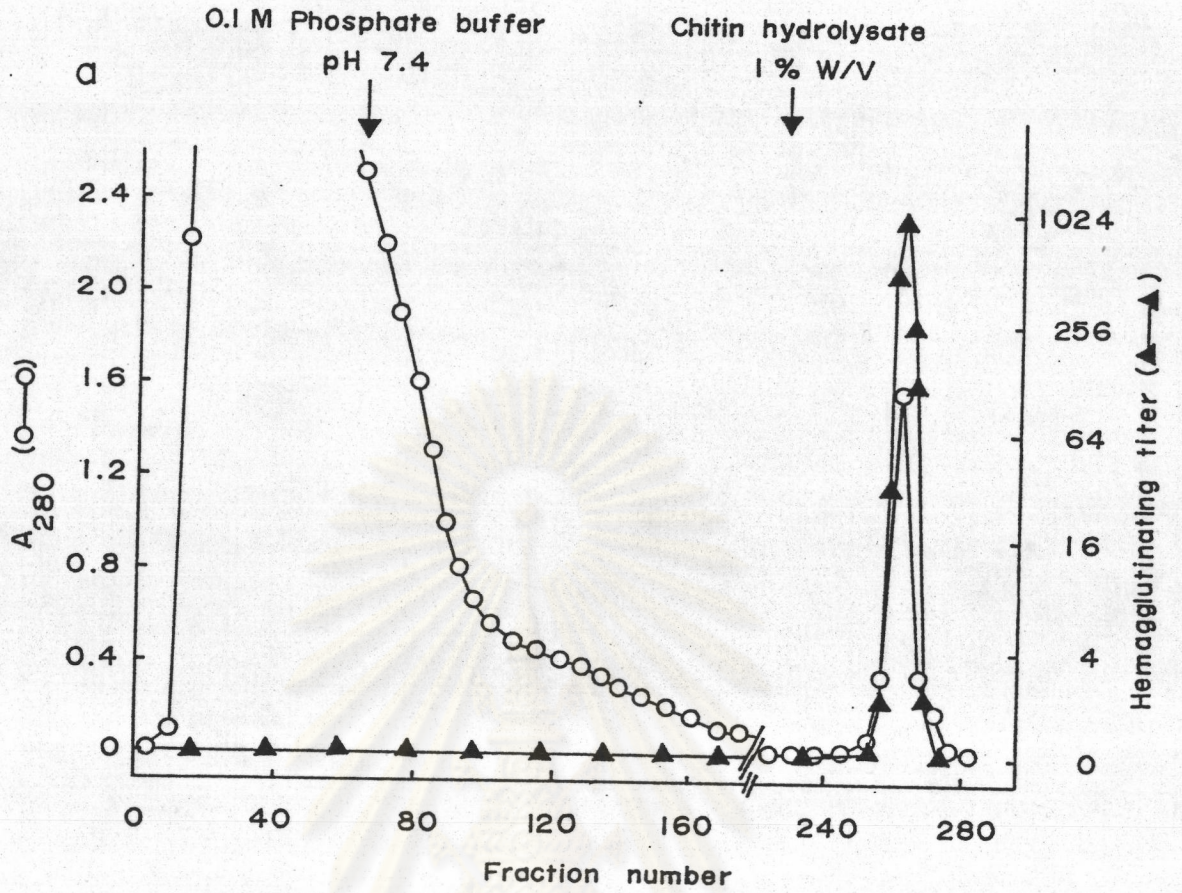
* Hemagglutinating Activity

Figure 3.1 Separation of rice lectin on chitin column.

The lectin containing dialysate from ammoniumsulfate fraction was applied to a column (1.8x24 cm) of chitin. The column was eluted with 600 ml of 0.1 M of PBS pH 7.4, and 300 ml of 1% chitin hydrolysate in 0.05 M acetate buffer pH 3.8. Fractions of 3 ml were collected. The symbol are : o——o , absorbance at 280 nm; ▲——▲ , hemagglutinating activity

a) RD 7 lectin, total volume loaded 140 ml

b) RD 25 lectin, total volume loaded 117 ml


a
0.1 M Phosphate buffer
pH 7.4
Chitin hydrolysate
1 % W/V
A_{280} (O—O)
Hemagglutinating titer (▲—▲)
2.4
2.0
1.6
1.2
0.8
0.4
0
1024
256
64
16
4
0
0
40
80
120
160
240
280
Fraction number

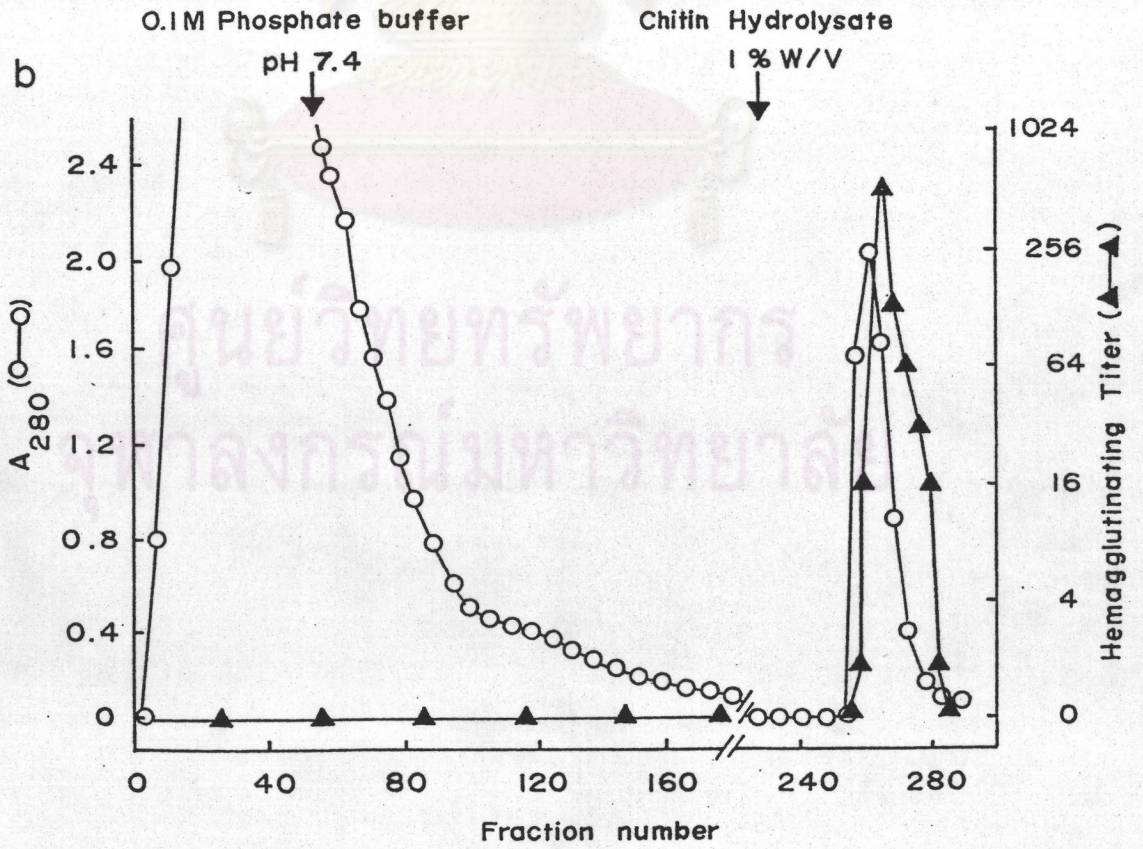
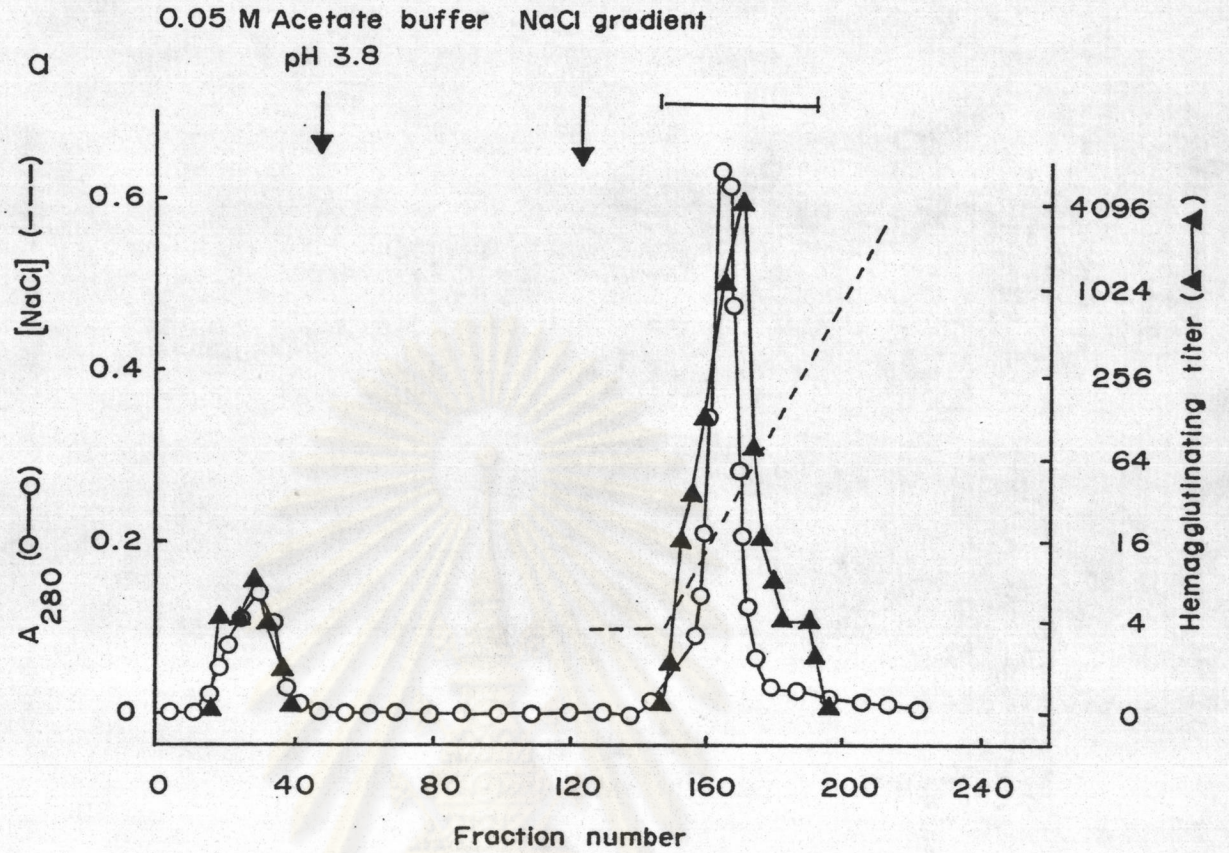

b
0.1M Phosphate buffer
pH 7.4
Chitin Hydrolysate
1 % W/V
A_{280} (O—O)
Hemagglutinating Titer (▲—▲)
2.4
2.0
1.6
1.2
0.8
0.4
0
1024
256
64
16
4
0
0
40
80
120
160
240
280
Fraction number

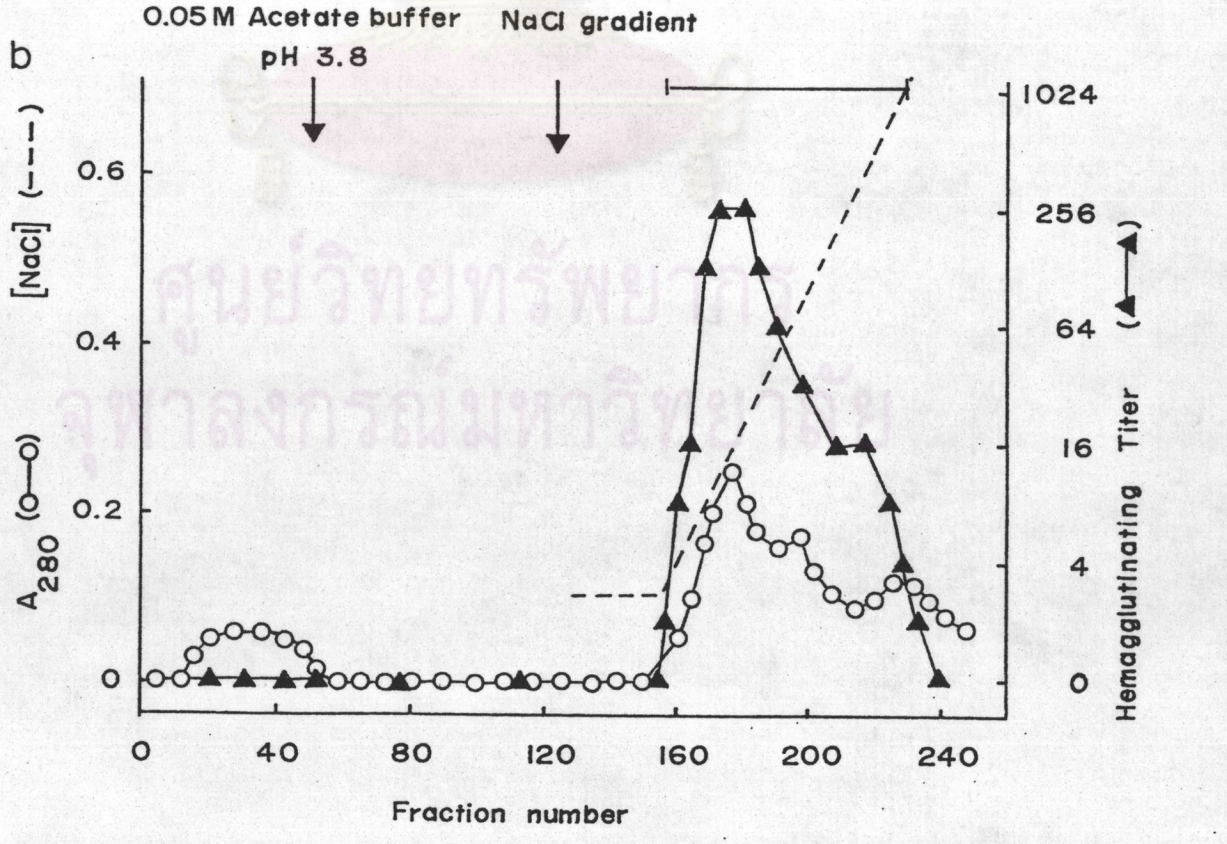
Figure 3.2 The elution profiles of lectin on SP-Sephadex ion exchange column.

The SP-Sephadex C 50 column was maintained in 0.05 M sodium acetate buffer, containing 0.1 M NaCl. The rice lectin fractions obtained from chitin column were pooled and applied onto the column and eluted by a linear gradient of NaCl (400ml) from 0.1-1.0 M (-----). Frctions of 3 ml were collected, and determined for absorbance at 280 nm (o——o) and hemagglutinating activity (▲——▲) of lectin.

a) RD 7 lectin (3.58 mg protein/55 ml)

b) RD 25 lectin (6.48 mg protein/120 ml)


a
0.05 M Acetate buffer
pH 3.8
NaCl gradient
[NaCl] (---)
A_{280} (o—o)
Hemagglutinating titer (▲—▲)
0.6
0.4
0.2
0
4096
1024
256
64
16
4
0
0
40
80
120
160
200
240
Fraction number


b
0.05 M Acetate buffer
pH 3.8
NaCl gradient
[NaCl] (---)
A_{280} (o—o)
Hemagglutinating Titer (▲—▲)
0.6
0.4
0.2
0
1024
256
64
16
4
0
0
40
80
120
160
200
240
Fraction number

step of chitin affinity column. The activity of lectins from both cultivars RD 7 and RD 25 was highly recovered at the step of ammonium sulfate fractionation (112 and 140%). The actvity yield of RD 7 lectin eluted from the chitin column was less than RD 25. The final step of lectin purification was performed on a cation exchange column, SP-Sephadex C 50 (Sulfopropyl Sephadex C 50), where the negatively charged or neutral proteins at pH 3.8 were eluted with acetate buffer, and positively charged proteins were retained and desorbed with linear gradient of 0.1-1.0 M NaCl. The major activity peak of both lectins were eluted from SP-Sephadex column at 0.25 M NaCl, but RD 7 lectin showed a sharp symmetrical protein peak (Fig 3.2 a) with one small peak of unbound protein that contained hemagglutinating activity. Fig 3.2 b showed the elution profile of RD 25 lectin, which was different from RD 7 (Fig 3.2 a) by the absence of the first peak with hemagglutinating activity, and the very broad lectin peaks that extended beyond 0.7 M NaCl. The purity of lectin, considered from the specific activity (HA/mg protein) was increased significantly after the chitin affinity column to 420 and 160 fold for RD 7 and RD 25 respectively, and after ion exchange column to 1,637 and 1,362 fold for RD 7 and RD 25 respectively. The activity yield of lectin from the final purification step was more than 80% for RD 7 and 90% for RD 25. The percentage of lectin in total

proteins of the crude extract was about the same (Ø.6-Ø.7%) in both rice cultivars.

3.2 Homogeneity of rice lectin

The lectin of rice RD 7 and RD 25 were found to be homogeneous by polyacrylamide gel electrophoresis (PAGE) at pH 4.3, with 15% acrylamide. A nearly single homogeneuos lectin band was observed after separation on chitin column as shown in Fig 3.3, lane 3 & 6 for RD 7 and RD 25 respectively. The lectin obtained from SP-Sephadex column also displayed one single band in PAGE (Fig 3.3 lane 4 for RD 25 and lane 5 for RD 7). Lectins from both rice cultivars either from affinity column or ion exchange column migrated at the same position (lane 3,6,4 and 5). Lectin was a minor protein in embryo crude extract, judging from the density of protein bands in lane 1 and lane 8.

Figure 3.3 Polycryamide gel elctrophorogram of rice lectin RD 7 and RD 25

lane 1 Acid extract RD 25, 55 ug

lane 2 Ammoniumsulfate fraction RD 25, 80 ug

lane 3 Chitin column RD 25, 30 ug

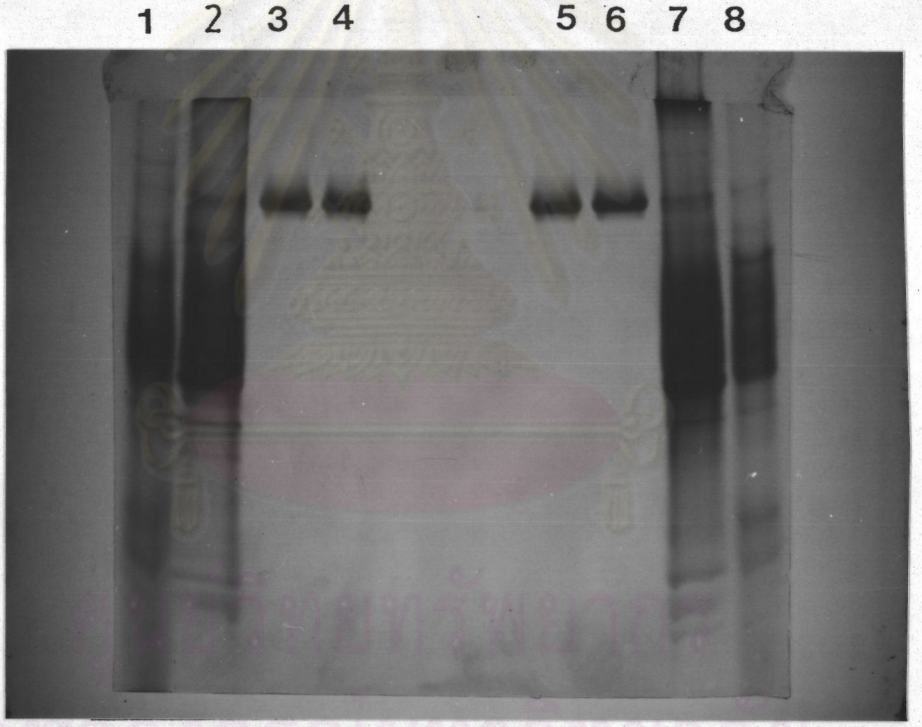
lane 4 SP-Sephadex column RD 25, 30 ug

lane 5 SP-Sephadex column RD 7, 30ug

lane 6 Chitin column RD 7, 30ug

lane 7 Ammonium sulfate fraction RD 7, 100ug

lane 8 Acid extract RD 7, 50ug


1 2 3 4 5 6 7 8

3.3 Antibody production

3.3.1 Titer of antiserum

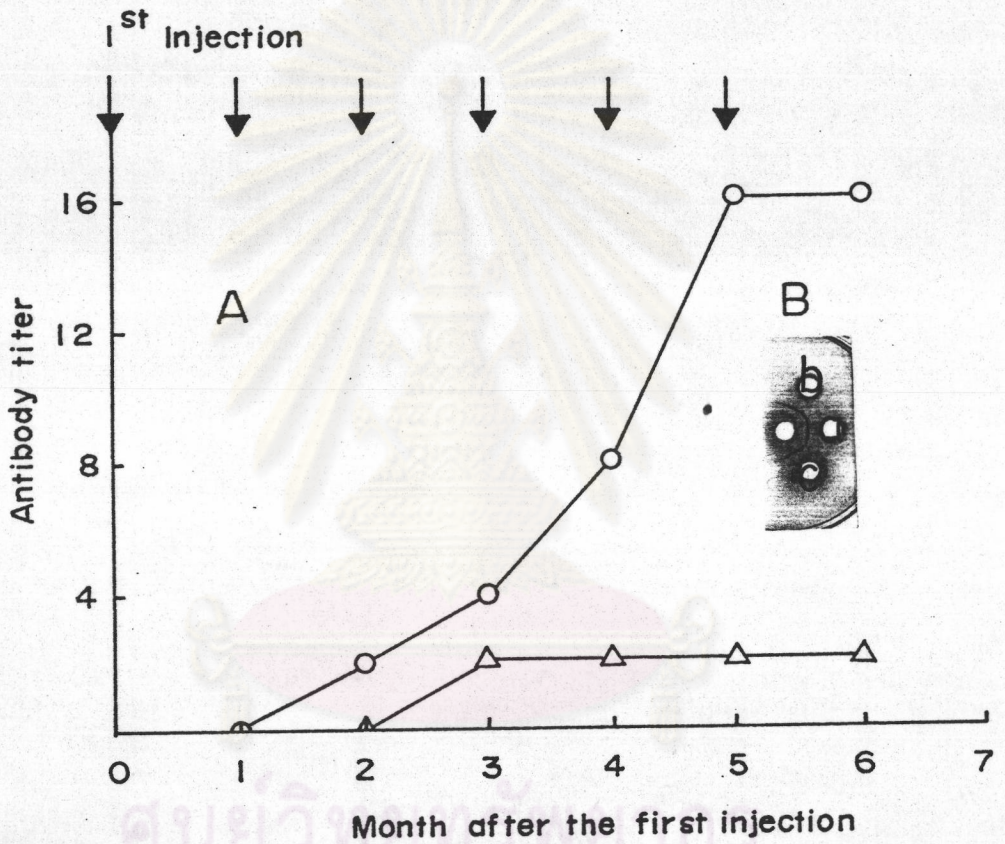
Antilectin (cv RD 7 and RD 25) was prepared in female New Zealand White rabbit. Two animals were injected with lectin (each cultivar). One month after the first injection rabbits were bled and determined the antibody titer. No reactive precipitin line were observed in four animals, 10 days after the first injection rabbits were bled, and antibody titer measurment was repeated, the titer was zero. Antibody against cv RD 7 was observed in two months after the first injection (titer 2), but RD 25 maintained zero(Fig. 3.4 A). The booster and bleeding series were repeated over a period of 5 months. Three months later the antibody titer against RD 7 and RD 25 raised to 4 and 2 respectively. The other two animals did not repond to lectin and no antibody titer was detected over six months of immunization. The titer of antilectin RD 25 was increased to two and maintained at two over six months, whereas the titer of antilectin RD 7 assessed constantly at 16 after the fifth month of immunization. Cross reaction of antilectin RD 7 with RD 25 was tested. Antiserum against rice lectin RD 7 cross-reacted with rice lectin RD 25 (Fig.3.4 (B). Large blood volume was collected when the serum titer reached 16

Figure 3.4 Immunization scheme and antibody titer against lectin RD 7 and RD 25

Antibody titer was determined by double imuunodiffusion in gel.

A) Immunization record and titer of rabbit antiserum against lectin RD 7 (○——○) and RD 25(△——△) , booster (⬇)

B) Cross-reactivity between lectin cv RD 7 and RD 25, well (a) lectin RD 25, well (b) RD 7, well (c) RD 7 (d) central well antilectin RD 7


1st Injection
16
12
8
4
Antibody titer
A
B
b
0
1
2
3
4
5
6
7
Month after the first injection

and immunoglobulins were partialy purified.

3.3.2 Partial purification of immunoglobulins.

Five milliter of antiserum against lectin of rice RD 7 was repeatedly fractionated by ammoniumsulfate at 40%, 31%, 31% saturation, which eliminated some serum proteins. The white precipitate of partially purified immunoglobulin was recovered and rapidly solubilized in PBS (2.5 ml). After one step purification, the titer of lectin antibody was increased to 32 . Since rice lectin cv RD7 and RD 25 were antigenically related to eack other as shown in Fig 3.4 (B). Only partially purified immunoglobulins against lectin RD 7 was used for the dertermination of lectin in other varieties of rice in embryo, root and leaf by enzyme-linked immunosorbent assay (ELISA).

3.4 Lectin distribution in root and leaf of rice

The fluorescent antibody technique was a sensitive method applicable to plant and animal tissues. However plants tissues express autofluorescence, because of the polyphenolic compounds in the cell wall, so that,using of fluorescent antibody

in plant samples was faced with more problems than animal samples. Autofluorescence in plant tissues can be eliminated by masking with rhodamine or its derivatives such as tetramethyl rhodamine conjugated BSA. In this experiment, the indirect fluorescent antibody method was chosen, in which the commercial FITC conjugated goat antirabbit immunoglobulin was the second antibody. The more brilliant light was observed in samples resulting from amplification of the second antibody. Lectins distribution and localization in rice tissue were observed by comparing fluorescent intensity between sample preincubated in lectin antibody, control samples preincubated in non immune serum (Fig 3.5,Fig 3.6). Fluorescent intensity specific to lectin on 4 day old seedlings root were observed in the root cap especially the muci gel(Fig 3.5 A) and the root hair tip (Fig 3.5 E). Lectins distribution on the surface of epidermal cells were less dense than in the root cap regions. Lectin was able to be detected by the fluorescent antibody in the root cap of 7 day old seedling root (data not shown). In the flowering stage, lectin was observed only in the root tip(Fig 3.5 C), but not in the roots'epidermal cells. In the control root samples (Fig 3.5 B,D,F) lectins' fluorescent was not distinguishable as compared to those samples treated with lectin antibody. Root lectin was more abundant in seedling stage, and a few in flowering stage.

Figure 3.5 Distrbution of lectin on the root surface of rice RD 7

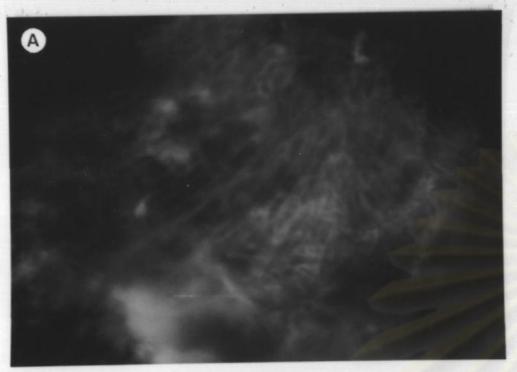
The root samples were incubated with antilectin followed by FITC-cojugated goat antirabbit immunoglobulin as decribed in method 2.10 .

A,B root cap of 4 day old seedling (x25)

C,D root tip of adventious root in flowering stage (x25)

E,F root hair of 4 day old seedling (x25)

B,D,F) control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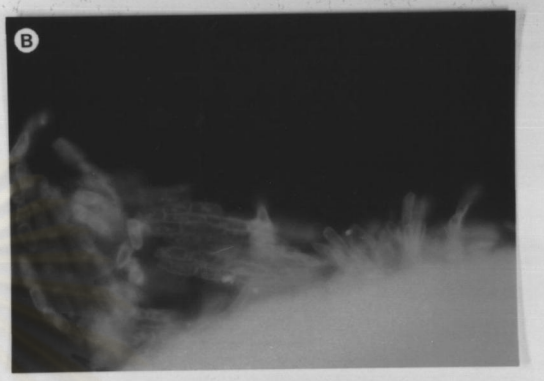
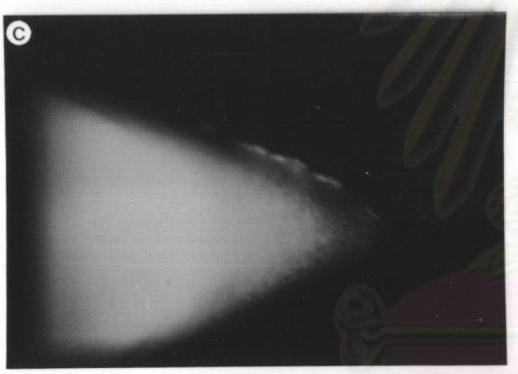
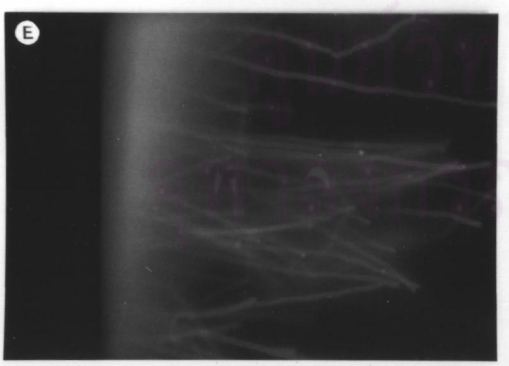
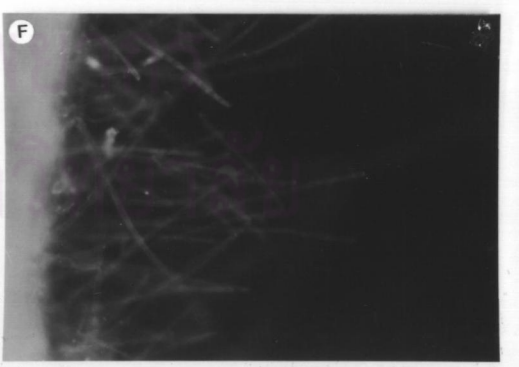

B


C


D

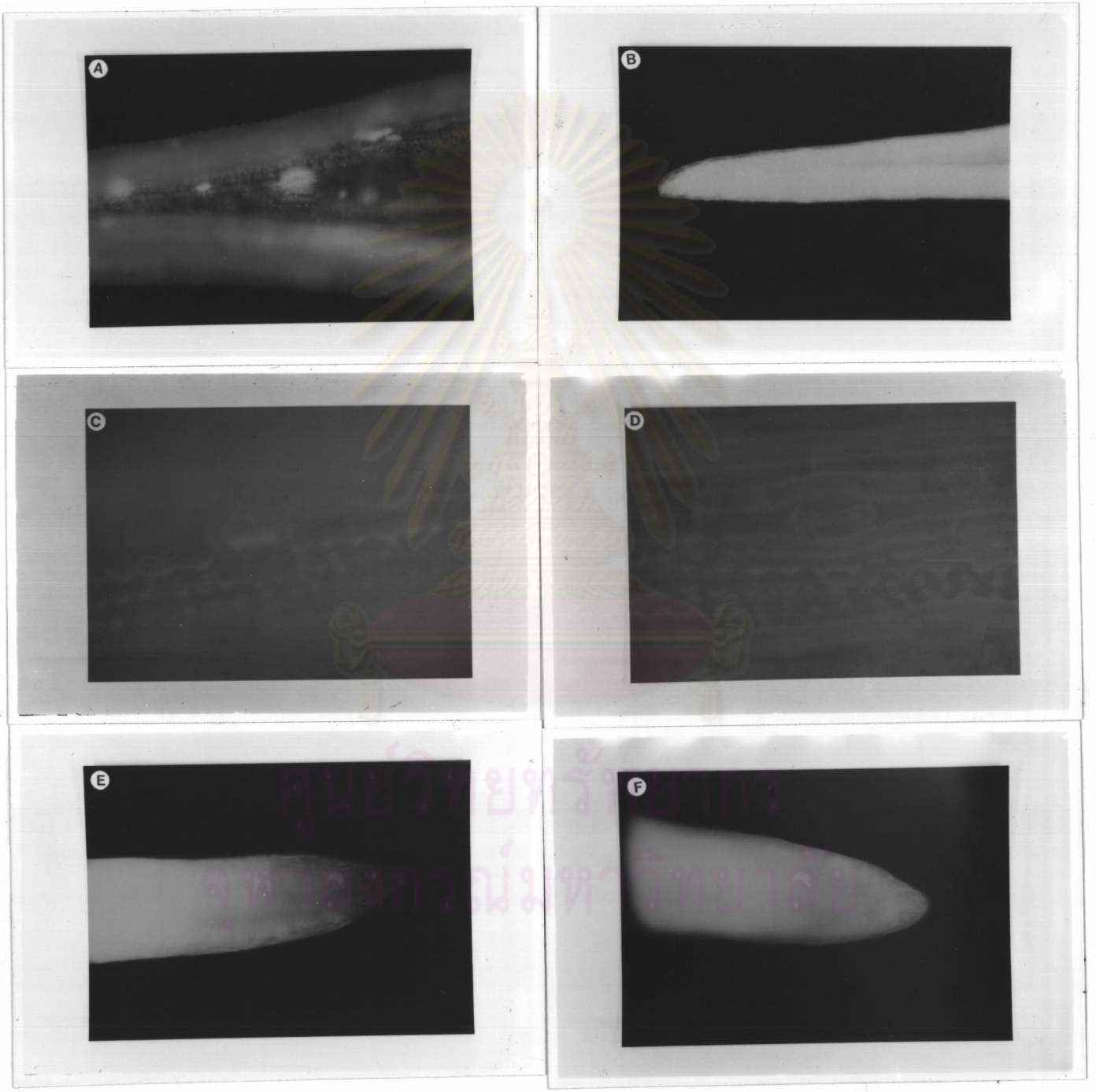

E


F

In the areal parts of rice, localization of lectin was performed in leaf samples, because leaf epidermal cells consist of cutin and trichome which posses hydrophobic property, therefore the aqueous immunological reagents could not touch the leaf surface. Addition of the surfectant such as Tween-20 to the solution was helpful, and immunological reagents were allowed to contact with epidermal lining cells. The lectin distribution on the leaf surface concentrated on hydathode as shown in Fig 3.6 A. Control samples treated with nonimmune serum showing only background fluorescent (Fig 3.6 B,D,F). Besides, lectin was localized at the opening stoma (Fig 3.6 C) as compared with corresponding control (Fig 3.6 D). In the flowering stage of rice plants, lectin was not observed in leaf(Fig 3.6 E). In general autofluorescent in leaf did not show adversely affect as in root, the background fluorescent was in red or yellow shade, which contrasted with the brilliant green area of lectin's fluorescence.

3.5 Relationship among lectins of different rice varieties

Double gel immunodiffusion was the best method for studying relationship among antigens (rice lectin). The resultant bands from antigen-antibody complex were displayed in different

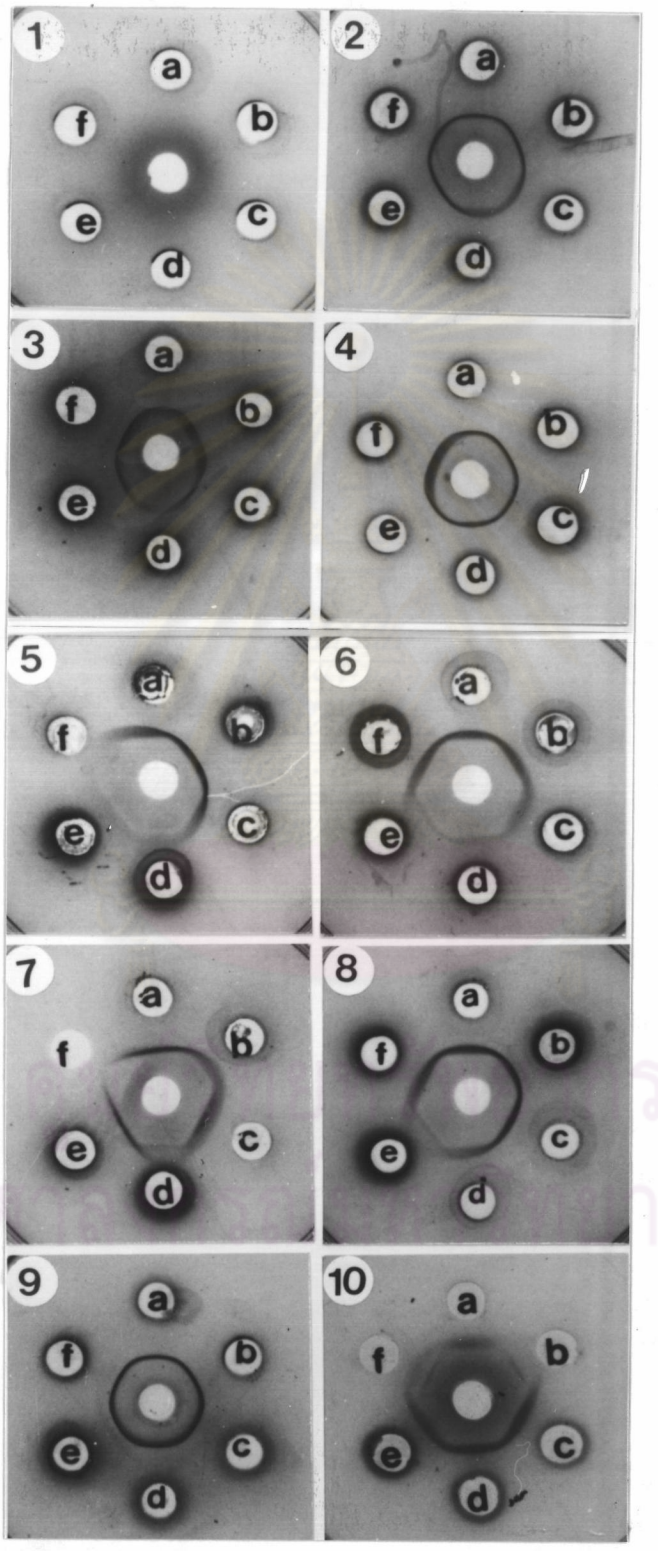

A
B
C
D
E
F

patterns, which depending upon antigen properties. The amount and number of bands refer to antigenic form (isoform), and the position of the band would encounter for its similarity or difference. The cross reactivity of antibody against lectin from rice RD 7 and embryo lectin from other 32 varieties of rice were developed according to Table 3.2 using equal volume(20ul) of crude lectin extract from equal number of 50 embryos. Antiserum of RD 7 was applied to the central well of each plate, and respective lectins in the peripheral wells as listed in Table 3.2.The rice lectin of all three subspecies of O. sativa L.namely indica, japonica,and javanica; and two species of wild rice and WGA were cross-reacted with antisera against RD 7. The precipitin bands were obtained in different patterns (Fig 3.7), including diffusive band, sharp band of different position or multibands. Figure 3.7 showed cross reaction among local cultivars, exotic cultivars and two species of wild rice. Except the pricipitin band devolped by WGA (Triticum aestivum) which was distinguishable obviously by different position, the precipitin bands observed in the Genus Oryza were :

i) sharp and non diffusive bands of homologous antigens were observed in 21 cultivars of local and exotic origins namely RD 7(2a-10a),RD 1 (2b), RD 5 (2c), RD 6 (2d), RD 23 (2f), RD 25

Table 3.2 The list of rice varieties, species and WGA circumscribed wells as shown in Fig 3.9

Plate NO.	a	b	c	d	e	f
1	RD 7	RD 7	RD 7	RD 7	RD 7	RD 7
2	RD 7	RD 1	RD 5	RD 6	RD25	RD 23
3	RD 7	NSPT	LPT 123	KTH 17	LMN 111	NMS 4
4	RD 7	PL 111	KND	SMJ	NKNg	NSPT
5	RD 7	NSPT	PTL 60	KTH 17	KDML 105	HT 60
6	RD 7	RD 25	BMT 370	KDML 105	IR 58	IR 50
7	RD 7	HCCMM	IR 36	IR 58	IR 50	WGA
8	RD 7	OS 4	IR 36	IR 42	IR 58	IR 50
9	RD 7	IR 36	TYNSK	HMNMC	KL	RD 25
10	RD 7	O. nivara	TYNCK	HMNMS	KL	O. fatua


1
a
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4
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b
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d
e
f
9
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b
c
d
e
f
10
a
b
c
d
e
f

(2e,6b,9f), LPT 123 (3c), NMS4 (3f), KND (4c), NKNg (4e), SMJ (4d), PL111 (4b), LMN 111 (3e), OS4 (8b), IR 36 (7c,8c,9b), IR 42 (8d), HCCMM (7b), TYNSK (9c,10c), HMNMC (9d,10d), KL (9e,10e),and IR 50 (6f,7e,8f.

ii) broaden or diffusive bands were developed by 4 cultivars; KTH 17 (3d), BMT 370 (6c), PTL 60 (5c), HT 60 (5f),).

iii) very condense bands, this pattern was unique for only NSPT as shown in Fig 3.9 (3b,4f).

iv) different position of very faint and unclear bands and unfused with other types as mentioned in i)-iii), as observed in KDML 105 (5e,6d),IR 58 (6e,7d,8e), O. fatua (10f)and O. nivara (10b). In general, lectins among cultivated RD and IR varieties (O. sativa subspecies indica) were similar to each other and to other exotic cultivars, either the subspecies japonica or javanica. Although lectin from KDML 105 was antigenically more closely related to IR 58, but very low in contents. The wild rice ,O. fatua (10f), contained two forms of lectin, one was common with RD 7 and the other lectin band of O. fatua was similar to another species of wild rice, O. nivara . The extra

band of NMS4(3f) was not similar position to any other varieties, but it also cross-reacted with antibody against RD 7.

3.6 Development of ELISA method

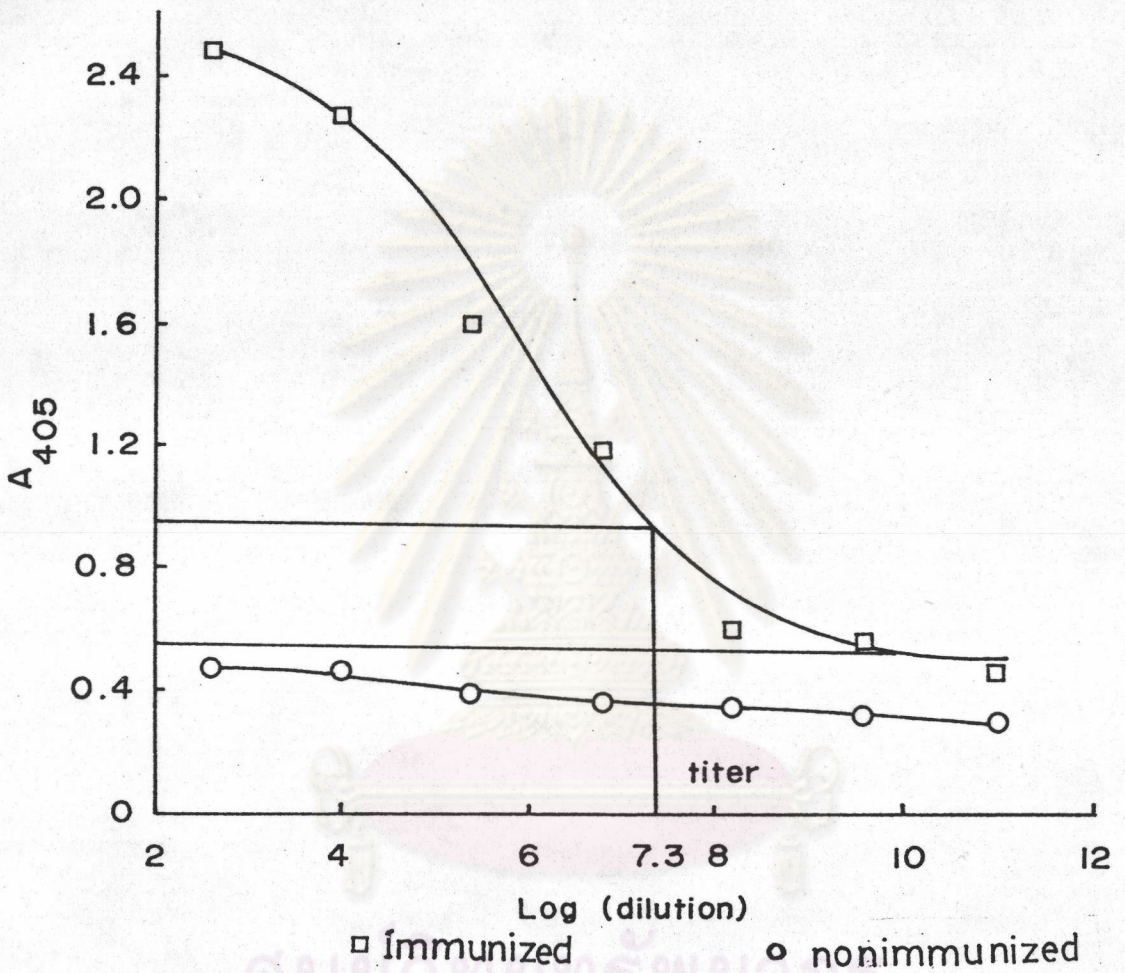
Indirect ELISA scheme for lectin determination was developed by coating lectin to polystryrene ELISA plate, and antibody against lectin, the first antibody generated in rabbit was added, followed by incubating with alkaline phosphatase conjugated goat antirabbit immunoglobulin, which is the second antibody, the lectin quantity corresponded to the absorbance value of the product, resulting from the labelled enzymed on the second antibody.

3.6.1 Determination of titer and optimum dilution of the first antibody.

Lectin antibody was serially diluted by 5-fold increment of PTN. The dilution of the second conjugated antibody was fixed at 1:1,500 , and the amount of lectin was fixed at 10 ng/well during this experiment. The maximum dilution or titer of the lectin antibody estimated from Fig 3.8 was 2.14×10^{7}, which was the maximum dilution that yield the absorbance (405 nm) 0.4 OD

Figure 3.8 Titer determination of the first antibody against lectin.

The plate was coated with 10 ng lectin/well, the first antibody was serially diluted 5-fold incremant in PTN , and the dilution of the second antibody was fixed at 1:1,500, incubation time of the first and second antibody were 45 min, and the absorbance at 405 nm was read after 1 h of enzymatic reaction.

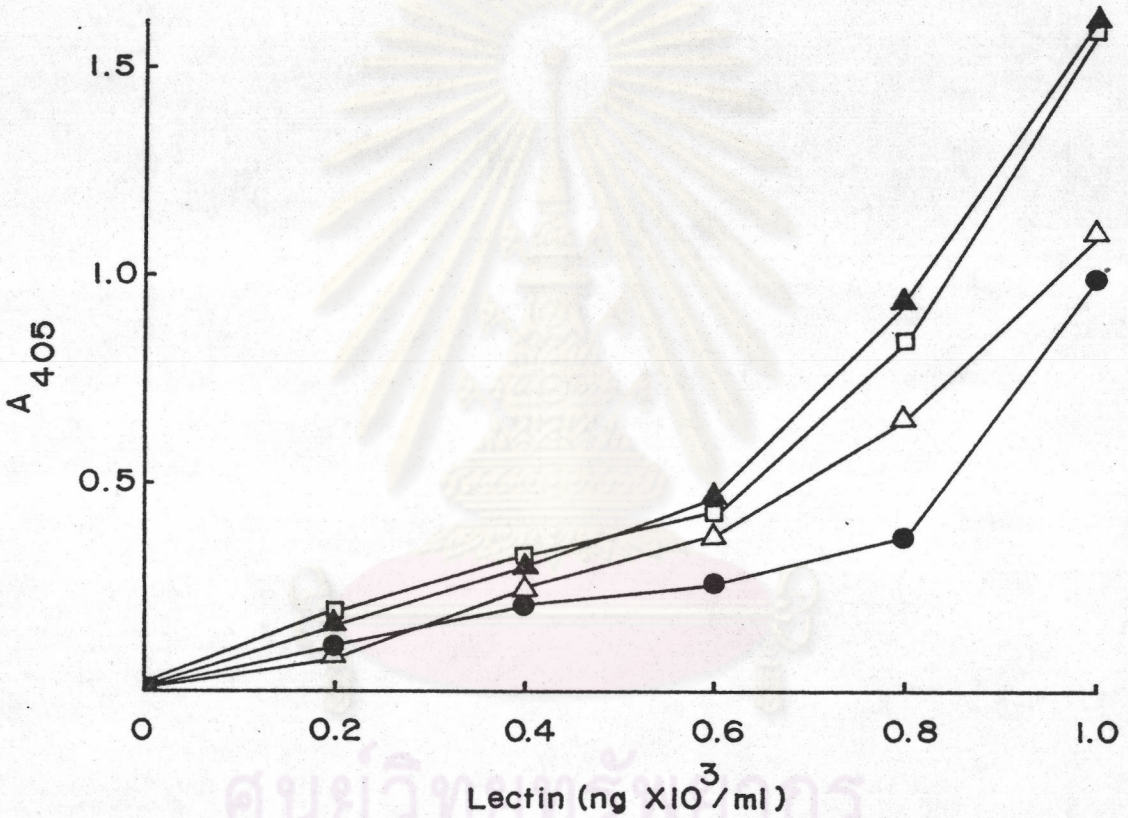


above background. The titer value from ELISA was so high when compared to the agar gel method. However the appropriate dilution of the first antibody for determination of lectin in plant samples was tested as followed.

Various concentrations of lectin ranging from 0-100 ng/well or (0-1,000 ng/ml) were coated to the wells (100 ul) followed by varying the first and second antibody. Fig 3.9 showed dilution curve resulted from various dilutions of both antibodies which resulted in different ranges of absorbance, but the concave patterns were similar. The optimum dilution of the first and second antibody at 400,000 and 6,000 was selected for saving the second antibody, The dilution of the first antibody at 800,000 and the second antibody at 3,000 and 6,000 was not suitable, because absorbance yield was too low. Lectin in embryo was determined using the optimum condition (first/second antibody; 400,000/6,000), but given low absorbance at 405 nm, and the lectin quantity could not be estimated. Therefore, lectin concentration range in the standard curve was reduced to 0-100 ng/ml or 1-10 ng/well, and the optimum dilution of the first and second antibodies were again varied. Fig 3.10 a,b showed 8 dilution curves resulted from various dilutions of the first and the second antibody. The optimum dilution of the first and the

Figure 3.9 Optimum dilution curves for lectin ranging from Ø-1ØØ ng/well.

The plate was coated with lectin ranging from Ø-1ØØ ng/well and incubated with the first and second antibody for 45 min at 37 °C, and the absorbance at 4Ø5 nm was read after 1 h of ezymatic reaction, the dilution ratio of the first/the second antibodies were, 4ØØ,ØØØ/3,ØØØ (▲——▲), 4ØØ,ØØØ/6,ØØØ (□——□), 8ØØ,ØØØ/3,ØØØ (△——△), and 8ØØ,ØØØ/6,ØØØ (●——●)



second antibodies were 100,000 and 1,500 respectiveiy. This condition was selected for the determination of lectin in embryo, the sensitivity of the method to detect the lectin was 10 ng/ml, and the suitable range was 10-60 ng/ml, because the curve of lectin concentration from 60-100 ng/ml was flat (Fig 3.10 a). The low absorbance yield obtained with of the first antibody dilution of 400,000 and 800,000, and the second antibody of 1,500 and 3,m00 (Fig 3.10 b) were not suitable for low lectin assay.

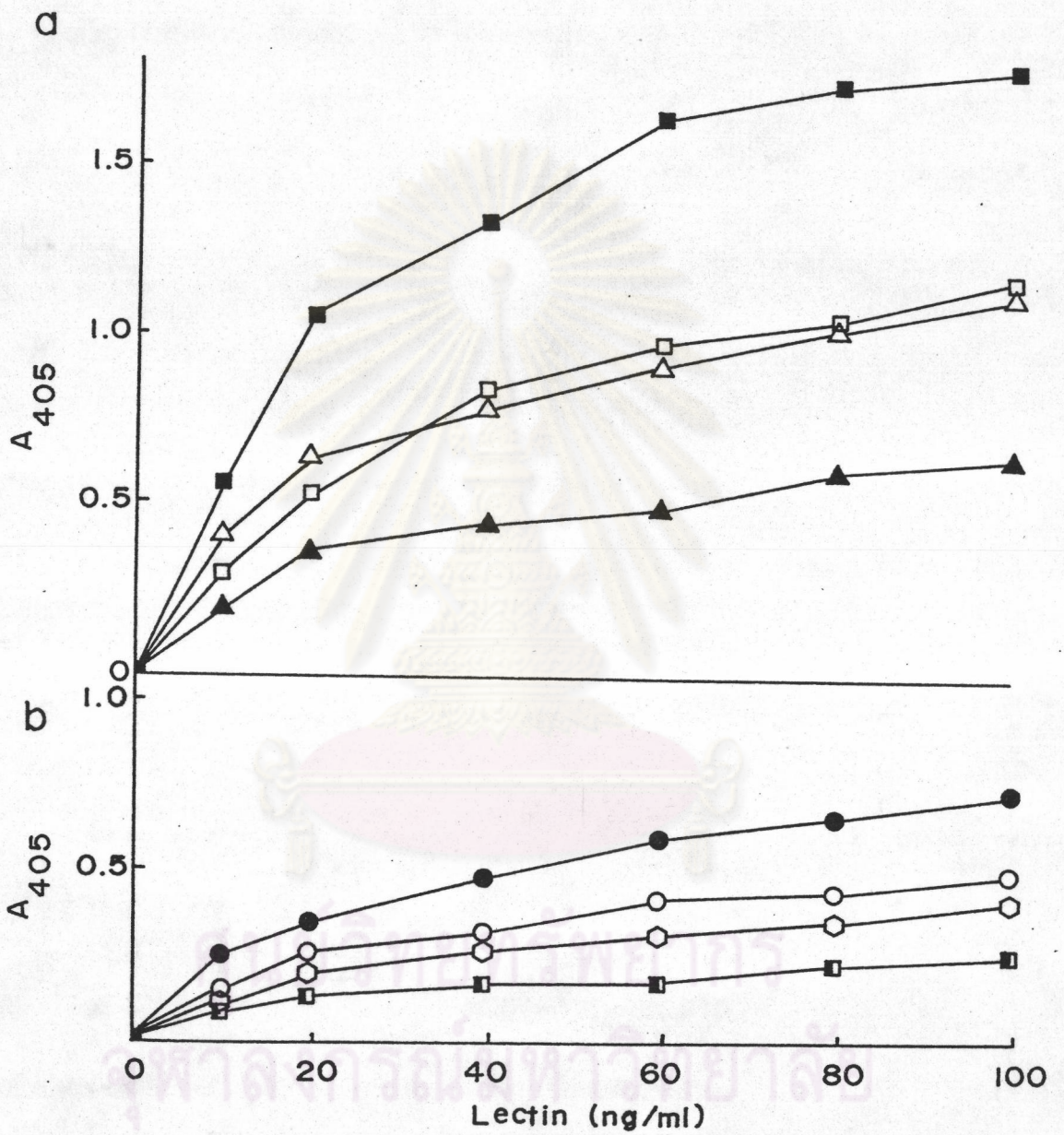
3.6.2 Screening for nonspecific binding between lectin and antibodies (first and second)

The nonimmume serum was diluted to 1:100,000 , and the second antibody dilution was 1,500 and lectin concentration from 10-100 ng/ml was coated, the binding between lectin binding site to carbohydrate moiety of antibody molecule and other glycoprotein did not affect absorbance. The presence or the abscence of hapten sugar (0.1 M GlcNAc) resulted in zero values of the absorbance at 405 nm, whereas the immune serum yielded absorbance at 405 nm.

Many factors affected the ELISA curve,such as the dilution of first and second antibodies, time and temperature of

Figure 3.10 Optimum dilution curves for lectin ranging from 0-10 ng/well.

The plate was precoated with lectin ranging from 0-10 ng/well and incubated with the first and the second antibody for 45 min at 37 °C, and the absorbance at 405 nm was read after 1 h of enzymatic reaction. The ratio of the first and the second antibody dilution was ; a) 100,000/1,500(■——■), 100,000/3,000 (□——□), 200,000/1,500 (△——△), 200,000/3,000 (▲——▲) ; b) 400,000/1,500 (●——●), 800,000/1,500 (○——○), 400,000/3,000 (⬡——⬡), and 800,000/3,000 (◧——◧)


a
b
A_{405}
A_{405}
0
0.5
1.0
1.5
0
20
40
60
80
100
Lectin (ng/ml)

incubation. The important step was the incubation time of enzymatic reaction which was never ignored, it was accurate stopped at 1 h.

3.6.3 Precision and accuracy of the ELISA

The standard method was consistence as indicated by % coefficience of variation (CV) . The degree of distribution in values depended upon binding capacity, each well maintained the free surface areas which was unoccupied by lectin, those areas were blocked with BSA which covered these left areas, and prevented the attachment of other molecules. Table 3.3 showed the distribution of absorbance values which exhibited variation of the standard deviation between absorbance values observed from blocked and nonblocked wells. Comparison of % CV, it was not significantly different. The percentage error in intra-assay was less than 7 %, whereas in inter-assay was more than 13 % (range 2-15 %). The same % CV was obtained from modified ELISA method, where only two points were more than 1Ø % in intra-assay, and 2-13% in inter-assay were repeated. The accuracy of ELISA was done by adding the known amount of pure lectin into samples, from Table 3.4 the high accuracy of this method was indicated by the percent recovery of92-98 % .

Table 3.3 The precision of the ELISA method.

Lectin ranging from Ø-1ØØ ng/ml, showed the percentage coefficience of variation (% CV) in intra-assay and interassay of both normal and modified methods as described in 2.11.3 and 2.11.6 respectively.

Variance	Lectin concentration (ng/ml)					
	1Ø	2Ø	4Ø	6Ø	8Ø	1ØØ
Intra-assay						
Mean A_{405}	Ø.45	Ø.62	Ø.88	1.Ø8	1.21	1.52
S.D.	Ø.Ø3	Ø.Ø2	Ø.Ø5	Ø.Ø2	Ø.Ø8	Ø.Ø8
n	6	6	6	6	6	6
% CV	6.6	3.2	5.6	1.8	6.6	5.3
Inter-assay						
Mean A_{405}	Ø.41	Ø.6Ø	Ø.81	1.Ø6	1.16	1.26
S.D.	Ø.Ø3	Ø.Ø1	Ø.11	Ø.Ø9	Ø.11	Ø.14
n	6	6	6	6	6	6
% CV	8.5	2.2	13.8	9.3	9.9	11.3
Intra-assay[M]						
Mean A_{405}	Ø.38	Ø.6Ø	Ø.93	1.16	1.26	1.37
S.D.	Ø.Ø3	Ø.Ø6	Ø.Ø8	Ø.Ø9	Ø.Ø9	Ø.Ø5
n	5	5	5	5	5	5
% CV	9.9	1Ø.7	8.8	8.4	7.5	3.9
Inter-assay[M]						
Mean A_{405}	Ø.42	Ø.62	Ø.93	1.16	1.26	1.35
S.D.	Ø.Ø5	Ø.Ø4	Ø.Ø6	Ø.Ø5	Ø.Ø7	Ø.Ø3
n	5	5	5	5	5	5
% CV	13.Ø	6.7	7.Ø	4.6	5.7	2.2

[M] Measurement by modified method

Table 3.4 The accuracy of the ELISA method.

Lectin was determined by ELISA, where known amount of pure lectin was added into the sample, which had been determined for existing content.

Lectin (ng/ml)		Lectin measured (ng/ml)	% Recovery
sample	standard lectin added		
25	20	44±1	97.8
25	40	60±4	92.3
25	60	82±5	96.5

Values presented are mean of 6 measurements

3.7 Lectin contents in embryo of various cultivars.

The lectin contents in embryo determined by ELISA (2.11.3) were in the nanogram quantities. Total lectin per 50 embryos and lectin concentration (ng/mg protein) were listed in Table 3.5 . The four varieties contained high amount of lectin (>Mean+SD) were TYNSK, NSPT, PL 111 and NMS 4. Five varieties contained slightly low amount of lectin were IR 58, HT 60, KTH 17, BMT 370 and KND. The high amount (3560-5173 ng/100 plants) of lectin content were observed in the glutinous rice varieties of NKNg, SMJ, NSPT, TYNSK, and HMNMC. Considering the lectin concentration (ng/mg protein), the two varieties; KDML 105 and IR 58 contained very low concentration of lectin 89-95 ng/mg protein comparing to the mean of the tested samples.

3.8 Lectin in vegetative tissues of developmental rice seedling cv RD 7

Determination of lectin in root and leaf of rice by using the same procedure as used in embryo was not achieved, because lectin concentration was very low as shown in Table 3.6.

Tabel 3.5 Lectin contents in embryo of rice

Varieties	ng Lectin/mg protein	Total lectin/50 embryo
NKNg	1442±31	4269±93
IR 36	1421±69	4208±204
IR 42	1272±44	4186±144
SMJ	1222±51	4331±179
NMS4	1206±129	4571±489
RD 7	1184±92	3632±281
NSPT	1089±36	5173±170
PTL 60	1041±132	3523±447
RD 25	1003±61	3590±216
RD 23	1002±76	4035±305
TYNSK	944±84	5232±464
LPT 123	921±37	4051±165
PL 111	905±54	4709±283
RD 1	882±45	3785±193
RD 5	865±96	2700±298
HCCMM	820±37	2238±101
IR 50	746±21	1729±50
LMN 111	722±23	3182±101
OS 4	637±28	3642±157
HMNMC	623±24	3560±136
RD 6	551±38	2647±181
KL	541±20	4178±152
HT 60	521±14	1664±45
KTH 17	473±13	1593±43
BMT 370	415±13	1806±56
KND	205±10	1259±60
KDML 105	95±2	336±7
IR 58	89±3	279±9
Mean ± SD	815±362	3218±1350

Values presented are mean of 6 measurements

Table 3.6 Comparison of normal and modified procedures for lectin determination in vegetative tissues of rice seedlings (RD 7)

Rice (RD 7) seedlings grown under dark and light condition were assayed for lectin by normal and modified ELISA procedure.

Time (day)	Lectin concentration (ng/ml)							
	Light				Dark			
	Leaf		Root		Leaf		Root	
	N	M	N	M	N	M	N	M
4	31	703±63	<10	34±1	40	1345±267	11	48±5
5	<10	289±6	<10	24±1	24	654±63	14	28±2
6	<10	246±26	<10	25±1	<10	286±23	15	19±1
7	<10	61±10,	<10	15±1	<10	88±1	<10	23±4

N : Determination by ELISA method previously used with embryos.

M : Determination by modified ELISA method

Values presented are mean of 6 measurements

Other proteins in the plant samples could compete with lectin for the surface area, so that lectin failed to coat to the well, therefore the ELISA procedure was modified by precoating the well with Ø.2 % ovalbumin in sodium carbonate buffer pH 9.6, to enhanced lectin binding to its receptor which was the carbohydrate moiety in ovalbumin molecule and followed by the normal procedure. Fig 3.11 is a typical standard curve of the modified ELISA procedure showing in the sensitivity of 1Ø ng/ml and suitable analytical range 1Ø-1ØØ ng/ml, lectin content in 7 rice cultivars were redetermined by the modified ELISA as Shown in Table 3.6/1 . The percent errors were 2-13 which was the same as % CV. Lectin content in seedlings of rice cv RD 7, grown under dark and light conditions was determined during 4-7 days after germination. The concentration of lectin was presented in terms of ng lectin/mg protein, and ng total lectin per 1ØØ plants. Different amount of lectin in developmental seedling of rice (RD 7) were summarized in Table 3.7. The comparison of total lectin per 1ØØ plants as shown in Table 3.7 indicates that lectin is present in leaf of 4 day old of dark grown seedlings about 2.5 fold of the light-grown seedlings which was significantly different, statistical analysis by t-test value at 95% confidence. The level of leaf lectin in dark and light grown seedlings which gradually decreased on day 5-7. The root lectin

Figure 3.11 Standard curve of modified ELISA

The plate precoated with 0.2% ovalbumin and lectin was applied in the 0-100 ng/ml, The first and second antibodies dilutions were 1:100,000 and 1:1,500 respectively, the plate was incubated at 37 °C for 45 min. A 405 nm was read after 1 h of ezymatic reaction.

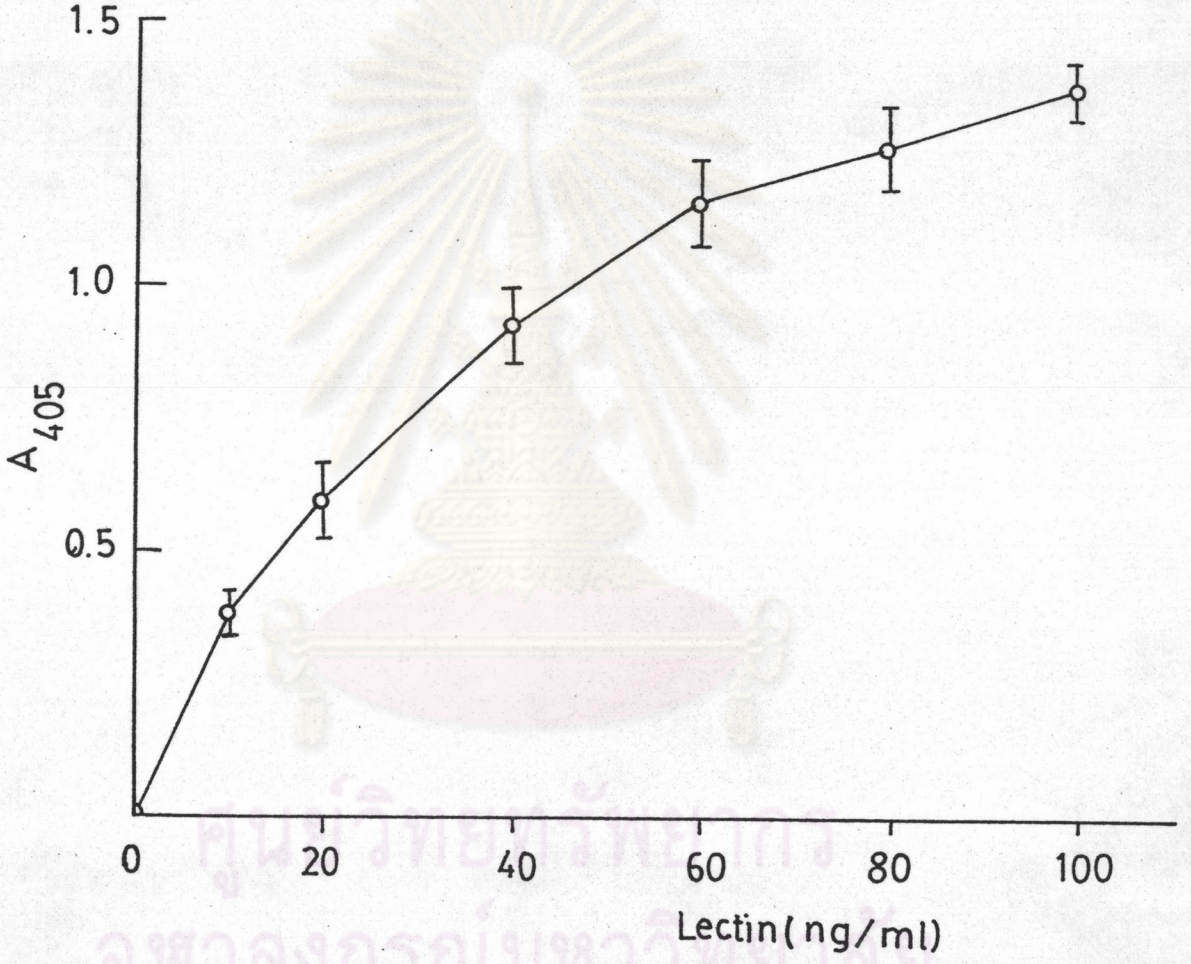

1.5
1.0
0.5
A_{405}
0
20
40
60
80
100
Lectin (ng/ml)

Table 3.7 Lectin content in embryo of rice, determined by both ELISA methods.

Lectin in embryos of 7 rice cultivars, compared to Table 3.5 which were determined by ELISA method, without ovalbumin precoating.

Varieties	ng lectin/ mg protein		Total lectin (ng/ 50 embryo)	
	N	M	N	M
RD 7	1184±92	1278±49 (>8%)	3632±281	3926±151 (>8%)
NKNg	1442±31	1344±27 (<7%)	4269±93	3826±83 (<11%)
PTL 60	1041±131	985±26 (<6%)	3523±247	3138±84 (<11%)
HT 60	521±14	580±20 (>11%)	1164±45	1808±62 (>8%)
KTH 17	473±13	420±16 (<11%)	1593±43	1560±60 (<2%)
KDML 105	95±2	105±3 (>10%)	336±7	380±10 (>13%)
IR 58	89±3	81±3 (<9%)	279±9	300±10 (>7%)

N determined by ELISA M determined by modified ELISA

Values presented are mean of 6 measurements

Table 3.8 Lectin distribution in root and leaf of rice (RD 7) seedlings under light and dark conditions.

Time (day)	ng lectin/ mg protein				Total lectin(ng)/100 plants			
	Light		Dark		Light		Dark	
	leaf	root	leaf	root	leaf	root	leaf	root
4	109±10*	17±1	210±41*	15±2	893±80*	44±1*	2628±522*	58±6*
5	40±1	14±1	127±12	19±1	461±9	32±2	1367±132	38±2
6	39±4	16±1	51±4	11±1	501±53	39±2	603±49	15±1
7	9±2	12±1	17±2	12±2	175±44	23±2	153±20	28±5

Values presented are mean of 6 measurements

* Significant different between light and dark condition.

Statistical analysis by t-test at 95% confidence, t-value from table = ± 1.96.

root lectin/100 plants in dark and light grown seedlings on day 4 were lower than leaf about 45 and 20 fold respectively, lectin in root grown under dark condition on day 4 was singificantly higher than root grown under light condition. Lectin in root on day 5-7 (dark and light condition) fluctuate abount 15-39 ng/100 plants.

The lectin concentration (ng lectin/mg protein) is the parameter which indicates the amount of lectin per total protein. The leaf of dark grown seedlings on day 4 and 5, contained lectin concentration significantly higher than the light grown seedlings 2-fold and 3-fold respectively(Table 3.8). On the day 6 and 7 lectin concentration in leaf was not different between dark and light grown conditions. The concentration of leaf lectin gradually decreased during development from day 4-7,but the concentration of root lectin remained unchanged along development under dark or light grown condition.

3.9 Comparison of lectin in vegetative tissues among varieties of rice

The comparative study of lectin was conducted in 4 day seedlings in 8 varieties of rice grown under dark environment as

shown in Table 3.9. Total amount of lectin in leaf(100 seedlings) was compared among 8 cultivars. Lectin of NMS 4 was significantly higher(>Mean+2SD) than the other cultivars about 2-fold of mean (Table 3.9), whereas RD 7, RD 23, and SPBR 60 contained moderately (1176-2628 ng/100 plants), and BMT 370, and KTH 17 contained low amount of lectin about 0.5 fold of the mean. The leaf of KDML 105 contained the lowest amount of lectin (<Mean+2SD) among 8 cultivars. Seedlings' root of NMS 4 also contained significantly high lectin (>Mean+2SD) about 2.5 fold of the mean, six cultivars of RD 7, RD 23, SPBR 60, KTH 17, BMT 370, and RD 25 contained moderate amount (17-58 ng/100 plants), and lectin content of KDML 105 was the lowest amount (<Mean-2SD).

When considered lectin concentration (ng/mg protein) in root and leaf as shown in Table 3.9. Lectin in leaf of RD 23 was significantly high about 2-fold of the mean, other 5 cultivars contained moderate values (115-220 ng/mg protein), two cultivars of KDML 105 and KTH 17 was low in lectin concentration (32-59 ng/mg protein). In root, lectin concentration of NMS 4 and RD 23 were sinificantly high about 2 fold of the mean, whereas RD 7, SPBR 60, BMT 370, RD 25 were moderate (10-19 ng/mg protein). The root lectin concentration of KDML 105 and KTH 17 were very low (6-8 ng/mg protein) about 2-fold of the mean.

Table 3.8 Lectin content in root and leaf of 8 rice cultivars, All cultivars were germinated under dark condition for 4-7 days, root and leaf were harvested, and measured the lectin concentration by modified method of ELISA.

Varieties	ng lectin/ mg protein		Total lectin (ng/100 plants)	
	leaf	root	leaf	root
NMS4	228±61	31±7	4230±1135	92±20
RD 7	210±42	15±2	2628±522	58±6
RD 23	307±29	29±2	2405±224	34±2
SPBR 60	151±19	13±1	1776±228	33±3
KTH 17	32±2	8±1	859±150	31±2
BMT 470	136±14	19±1	773±80	24±1
RD 25	115±17	10±1	711±104	17±1
KDML 105	59±15	6	598±151	5
Mean ± SD	155±85	16±9	1785±1167	36±25

Values presented are mean of 6 measurements

3.10 Effect of exogenous nitrogen on lectin content

Total lectin content in leaf tissue were not significantly decreased, when seedlings were grown under 2 and 20 mM NH_4Cl, but the lectin level in the root was significantly different only at 20 mM NH_4Cl (Table 3.10). The total lectin in root grown in the absence of NH_4Cl or under the presence of 2 mM NH_4Cl was about 2-fold higher than in seedling root under the presence of 20 mM NH_4Cl. In terms of concentration (ng lectin /mg protein), the lectin was about 2-fold decreased (24/13) when grown under 20 mM NH_4Cl, but in leaf the value was only 1.2 fold significantly different. The result suggested that lectin biosynthesis in seedling root and leaf tissuesmight be repressed in the presence of exogenous NH_4Cl (20 mM).

Table 3.1Ø Effect of NH_4 ion on lectin content in root and leaf. Rice seedlings were grown in aqueous solution that contained NH_4Cl 2 and 2Ø mM for 4 days under dark condition, and lectin were measured by modified ELISA method.

[NH_4Cl] (mM)	ng lectin/ mg protein		Total lectin (ng/1ØØ plants)	
	leaf	root	leaf	root
Ø	228±61	31±7	2628±522	92±2Ø
2	212±5	24±1	1681±57*	1Ø2±4
2Ø	173±1 *	13±1 *	1623±16*	49±2 *

Values presented are mean of 6 measurements

* Significant different of total lectin and lectin concentration between Ø, 2 and 2Ø mM NH_4Cl.

Statistical analysis by t-test at 95% confidence, t-value from table = ± 1.96