CHAPTER 2

Materials and Methods

2.1 Rice

Breeder seeds of Rice (*Oryza sativa* L.) used in the determination of lectin in embryo, seedlings, and for double gel immunodiffusion reaction were obtained from the Rice Germplasm Bank, Pathumthani Rice Research Centre, Department ofAgriculture, Ministry of Agariculture and Cooperatives. Stock seeds of rice cv RD 7 , RD 25used in purification of lectin were supplied by the RiceDivision, Department of Agriculture, Ministry of Agriculture andCoorperatives, Thailand. The international varieties; IR 36,IR 42,IR 50, IR 58, OS 4, HCCMM were obtained from the InternationalRice Research Institute (IRRI), the Philippines. Source and subspecies are summarized in table 2.1.

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2.2 Lectin

2.2.1 Wheat germ lectin or wheat germ aggluinin (WGA) was purchased from Sigma

Table 2.1 Rice varieties used in this research project

No.	Varieties	Source	Subspecies
1	RD 1	RGPB[*]	indica
2	RD 5	RGPB	indica
3	RD 6[G,A]	RGPB	indica
4	RD 7	RGPB	indica
5	RD 23	RGPB	indica
6	RD 25	RGPB	indica
7	Hantra 60[F] (HT 60)	RGPB	indica
8	Khaodokmali[A] 105 (KDML105)	RGPB	indica
9	Kaotakaeng 17 (KTH 17)	RGPB	indica
10	Lebmunang 111 (LMN 111)	RGPB	indica
12	Luangpratew 123 (LPT 123)	RGPB	indica
13	Khaoniawdam[G] (KND)	RGPB	indica
14	Niawkhiawngoo[G] (NKNg)	RGPB	indica
15	Niawsanpatong[G] (NSPT)	RGPB	indica
16	Nangmol S 4[A] (NMS 4)	RGPB	indica
17	Phaleaud 111 (PL 111)	RGPB	indica
18	Pattalung 60 (PTL 60)	RGPB	indica
19	Supanburi 60 (SPBR 60)	RGPB	indica

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Table 2.1 continued

No.	Varieties	Source	Suspecies
20	Sewmeajan[G] (SMJ)	RGPB	indica
21	Basmati 370[A] (BMT 370)	RGPB	indica
22	IR 36	IRRI[**]	indica
23	IR 42	IRRI	indica
24	IR 50	IRRI	indica
25	IR 58	IRRI	indica
26	OS 4	IRRI	indica
27	Khaolo (KL)	RGPB	javanica
28	Huachouchimomor (HCCMM)	IRRI	japonica
29	Himenomochi (HMNMC)	IRRI	japonica
30	Toyonishiki (TYNSK)	RGPB	japonica
31	_O. fatua_[w]	RGPB	-
32	_O. nivara_[w]	RGPB	-

[*] Rice Germplasm Bank, Prathumthani Rice Research Center

[**] International Rice Research Institute

[G] Glutinous rice, [A] Aroma rice, [F] Floating rice,

[w] Wild rice

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2.2.2 Lectins of rice RD 7 and RD 25 were prepared in the Depertment of Biochemistry as described in 2.3

2.3 Lectin purification

2.3.1 Embryo preparation

Rice hulls were removed by using a home made miller at the Department of Botany for small amount of sample, and at the Rice research Center for the large scale preparation. Rice caryopsis (brown rice) was excised manually with a scapel blade to separate embryo from endosperm

2.3.2 Extraction of Rice Lectin

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Embryoes (200 g) were ground to powder, and lipids were eliminated by chloroform-methanol 3:1 (V/V) as solvent. The defatted, and dried rice powder was then extracted in 10 volume of 0.01 M HCl containing 0.15 M NaCl, pH 2 and stirred overnight at 4 °C. The homogenate was blended, and insoluble material was pelletted by centrifugation at 10,000xg, 4 °C for 30 mins , the pellet was subjected to the second extraction under the same

condition. Both supernatant fractions were pooled and lectin was fractionated by ammonium sulfate precipitation up to 60% saturation. The precipitate was recovered by centrifugation at 10,000xg, 4 °C for 30 min and dissolved in PBS, then dialysed against 0.1 M phosphate buffer(pH 7.4) containing 0.15 M NaCl (PBS), and tested for hemagglutinating activity.

2.3.3 Lectin purification by column chromatography

Affinity chromatography was performed on a chitin column (thoroughly washed as decribed by Bloch and Burger, 1974, with distilled water, 0.15 M HCl, 2% $NaCO_3$ and 95% ethanol until the washed solution had an optical density less than 0.05 at a wavelenght of 280 nm, then equilibrated in 0.1 M PBS (as modified from Shen et al., 1984). The ammonium sulfate fractions (120 ml each) were applied to the chitin column (1.8x24 cm), the unbound proteins were washed out with PBS, then the bound lectin was eluted with 1% chitin hydrolysates (prepared according to Rupley, 1964 as shown in appendix I) in 0.05 M sodium acetate buffer,pH 3.8. Fractions (3 ml) were collected at a flow rate of 30 ml/h, absorbance at 280 nm and hemagglutinating titer were determined (as shown in 2.4) for each fraction after the removal of chitin hydrolysates by a Sephadex G-25 column packed in a 10

ml plastic syringe to the bed volume of 7 ml, by loading each fraction on the column and eluted with PBS. The void volume was collected.

Ion exchange chromatography on SP-Sephadex C-5Ø (Peumans et al,1982e) was prepared by swelling SP-SephadexC-5Ø in Ø.Ø5 M sodium acetate buffer, pH 3.8,containing 2 M NaCl, and extensively washed with Ø.Ø5 M sodium acetate buffer, pH 3.8, containing Ø.1 M NaCl, finally packed in a 1.8x24cm column, and equilibrated with the same buffer. The active fraction collected from chitin column was applied to the column, unbound proteins were washed out with equilibrating buffer. The column was then eluted with a linear gradient from Ø.1-1.Ø M NaCl in Ø.Ø5 M acetate buffer, pH 3.8. Fractions (3 ml each) were collected at a flow rate of 2Ø ml/h, and measured for the absorbance at 28Ø nm and hemagglutination activity. The hemagglutinating activity containing fractions were pooled and dialysed against Ø.15 M NaCl. Then, lectin was concentrated by ultrafiltration with Amicon membrane filter, MW cut off 1Ø,ØØØ.

2.4 Hemagglutination Assay

Blood taken from a New Zealand white rabbit was mixed with equal volume of PBS containing 1 % sodium citrate (Allen et al, 1973). The erythrocytes were pelleted by centrifuging at 200xg at 4 °C for 15 min and washed three times with PBS . Packed red cells were treated with equal volume of trypsin in PBS (1 mg/ml) at 37 °C for 30 min, then extensively washed at least 4 times with excess PBS. Finally, a 1% suspension of trypsin-treated cells in PBS was fixed by adding 25% glutaraldehyde to the final concentration of 0.25 %. This fixed erythrocytes suspension can be stored at 4 °C for at least 4 months. Agglutination assay was carried out in a 96 U-shape wells microtiterplate (Cooke Engineering Co.). The additions of the reagents were made in the following order: i) 25 ul of PBS, in each well; ii) 25 ul of purified lectin solution or crude lectin was serially diluted with 2-fold increments in each well iii) 25 ul of 1% fixed rabbit erythrocyte suspension. The plate was shaked vigorously and allowed to stand for 30 min. Positive and negative control were performed by adding and without adding of WGA respectively, starting from WGA (1 mg/ml) in PBS and made serially 2-fold dilution as mentioned previously. Agglutination was examined visually with the naked eyes. The agglutinated erythrocytes

formed a fuzzy mat on the surface of the well, whereas the nonagglutinated cells settled into a clearly circumscribed red dot. The hemagglutinating activity (HA Titer) was defined as the reciprocal of this end point dilution which showed complete agglutination. The minimum amount of lectin required to cause complete agglutination of erythrocytes was taken as 1 unit. Titer was defined as the reciprocal of the highest dilution that caused complete hemagglutination. The purity of lectin was compared by the specific hemagglutinating activity (HA) which was expressed as HA per milligram protein (HA/mg Protein).

2.5 Protein determination

Preparation of reagent for protein determination was performed according to Bradford (1976). Coomassie Brilliant Blue G-250 (100 g, purchased from Sigma) was dissolved in 50 ml 95% ethanol. To this solution 100 ml of 85% w/v (Merck) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter

Protein concentration in embryo, root, and leaf extracts were assayed by mixing 100 ul of samples to be mixed with 1 ml

protein reagent, the contents were mixed by inversion. The absorbance at 595 nm was measured after 2 min of incubation at room temperature, and before 1 h in a Spectronic 2000. Standard protein solution used in every assay contained 2-10 ug protein (BSA) in a volume made up to 100 ul with PBS.

2.6 Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was performed according to Reisfeld (1962). The slap gel consisting of two parts ;i) resolving gel solution of 15% acrylamide (Merck), 0.1% N,N'-methylene bisacrylamide, pH 4.3, ii)stacking gel solution of 3% acrylamide, 0.02% N,N'-methylene bisacrylamide, pH 6.7. The electrode buffer was 1.6 M beta-alanine-acetic acid pH4.5. Samples were diluted with electrode buffer containing a trace amount of sucrose and basic fuchin as tracking dye. The electrophoresis was run from the anode towards the cathode with constant current of 30 mA per slab gel at 4°C for 4-5 h, in a LKB 2001 vertical electrophoresis apparatus attached to a thermostat water-bath of LKB 2209 Multi Temp. The electrophoresis was terminated when the tracking dye comigrated to the lower edge. The gel was fixed and stained with 0.1% coomassie blue R in 50% methanol and 10% acetic

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acid overnight. Destaining was carried out by submerging the gel in aqueous solution of 5% methanol and 7% acetic acid, and changed the destaining solution at intervals until the protein bands appeared.

2.7 Preparation of the antisera against rice lectin (cv RD 7 &RD 25)

For primary injection, 1 mg of purified embryo lectin of rice cv RD 7 or RD 25 obtained from SP-Sephadex C 50 column was dissolved in 1ml of 0.15 M NaCl and mixed with equal volume of complete Freund's adjuvant (Ashford et al, 1982). The route of injection into 6 months old female New Zealand White rabbit was 0.5 ml by intramuscular injection (IM) in each hind leg at a dose of 1 ml per animal. The second injection was given one month later with equal dose of lectin mixed with incomplete Freund's adjuvant.The animal was bled 9-10 days after the booster injection. The antibody titer of serum (was defined as the reciprocal of the highest dilution that was able to react with lectin) was assayed by Ouchterlony method (Ouchterlony,1949). When the titer increased to 16, the rabbit was bled from the ear artery (10 ml) left standing at room temperature for 1 h and the

serum was collected, clarified by centrifugation (200xg at 4 °C) for 15 min. The clear serum (5 ml)was fractionated at 40% saturation with ammonium sulfate, the precipitate was pelleted by centrifugation in the same manner as serum collection. The pellet was resuspended in PBS (2.5 ml),and dialyzed against PBS at 4 °C overnight. The dialysate was preserved by adding thimerosal (Merck) at the final concentration of 0.01%, divided into small aliquots, each 1 ml, and stored at -15 °C for longterm use.

2.8 Immunidiffusion technique

The immunodiffusion plate was prepared in a small glass or plastic petri dish, 4 cm in diameter. The gel was prepared in the 0.04 M veronal buffer,pH 8.6, which composed of :

Sodium-Barbital (5,5'-diethyl barbiturate)	0.825	g
0.2 N HCl	3.82	ml
NaN_3	0.05	g
N-Acetylglucosamine (Sigma)	2.21	g
Noble agar (Difco)	1	g
Distilled water	100	ml

then, the mixture was boiled for 8 min.

A petri dish was filled with 3.5 ml of the molten gel solution, and allowed to set at room temperature. A hexagonal pattern of wells, 8 mm apart from edge to edge, 4 mm in diameter was cut using a metal cylinder. The gel plugs were removed with the help of a sharp-flat tip. The bottom of each well was sealed off with a drop of molten agar. To facilitate identification of antigens, the wells were labeled at the bottom of petri dish. The central well was filled with 20 ul of ammonium sulfate fractionated antiserum against RD 7, and 20 ul of crude extract lectin was applied to the appropriate well of a hexagonal pattern. The water saturated atmosphere was maintained by packing wet paper tissue in a plastic box, and covered with a tight plastic lid.The immunodiffusion plates were incubated in this unit at 4 °C for 24-48 h. Then, the unprecipitated proteins were washed off with excess PBS at room temperature (26-32 °C) overnight. The precipitin bands were visualized by staining with 0.5% coomassie brilliant blue R 250 dissolved in aqueous solution of 45% (V/V) methanol, and 10% (V/V) acetic acid, for 1 h or overnight at room temperature. Excess dye was destained with 20% (V/V) methanol, 10% (V/V) acetic acid. Photographs were taken when the sharp bands were apparent.

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2.9 Preparation of crude lectins for immunodiffusion

For each rice variety or cultivar, 50 rice seeds were used for crude lectin extraction. The hulls were manually removed, and embryos were excised, ground in a cold motar and extracted with 2 ml PBS. The homogenates were centrifuged, 48xg at 4 °C for 30 min in a microcentrifuge. Supernatant fraction was harvested and designated as crude extract of embryo lectin.

2.10 Immunofluorescent localization of rice lectin in leaf and root (Smith et al,1987; Diaz, et al, 1986)

Seeds of rice cv RD 7 were imbibed in tap water for 24 h, then imbibed seeds were germinated on two layers of moistened gauze cloth, placed on the aluminum sieve as a supporter in a glass chamber that contained water under the sieve, the chamber was covered with a plastic bag. Seeds were germinated for 4 days or 7 days at room temperature under 11-h photoperiod. The grolux fluorescent lamps served as light source. Roots and leaves were taken during day 4-7 after germination. Roots and leaves were rinsed with 10 mM phosphate buffer, pH 7.4 containing 0.15 M NaCl, 0.05% tween-20 and 0.02% NaN_3 (PTN) and left them submerged

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for 30 min at room temperature. Then root or leaf samples were incubated with the 1:10 dilution specific lectin antibody in 1 ml of PTN, containing 3% bovine serum albumin (BSA;fraction V; Sigma,) at room tempertature for 45 min. Excess antibody was rinsed by gentle agitation in PTN for 15 min. The second antibodies (goat antirabbit IgG-FITC conjugate;Sigma) dilution 1:500 in PTN containing 3% BSA were added, gently shaked at room temperature for 45 min. Finally the samples were extensively washed with PTN. Autofluorescence of the sample was masked with 0.01% Rhodamine 6G (Sigma) in PTN containing 3% BSA at room temperature for 10 min and washed with PTN. Mounting was performed with glycerol buffer (50% glycerol in PBS) on a glass slide, covered with coverslip and sealed with nail enamel. The control for autofluorescence was carried out by incubating with 1:40 dilution of nonimmune serum (collected before immunization in 3% BSA in PTN. Washings were carried out as described. After completion of the labeling procedure, the preparations were observed under a 20x or 40x objective lens of an epifluorescence microscope (Nikon, Optiphot) equipped with B filter cassette (excitation filter wavelength 420-485 nm and absorption filter of 520-580 nm). Photographs were taken with Nikon FX-35 A camera attached, using Kodak Extrachrome 400 ASA for diapositives.

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2.11 Development of ELISA procedure

2.11.1 Titer determination of the first antibody and optimum dilution of the first and the second antibodies (modified from Raikhel and Pratt,1987).

Ammonium sulfate fraction of rabbit antiserum was 5-fold diluted serially in PTN, starting from 1:200, each dilution (100 ul) was applied in a flat well of ELISA microtiter plate (Nunc, Denmark), precoated with 100 ul of pure lectin (100 ng/ml) in 0.1 M sodium carbonate buffer, pH 9.6 at $4^{\circ}C$ for at least 12 h. The microtiter plate was incubated for 45 min at $37^{\circ}C$ in a chamber with saturated humidity. Excess antibody was washed out with 200 ul PTN three times, and the second antibody of goat antirabbit IgG-alkaline phosphatase conjugate (Sigma) at the dilution 1:1500 in PTN was added (100 ul/ well). The reaction mixture was further incubated at $37^{\circ}C$ for 45 min, and the excess conjugates were washed out as described previously. The enzymatic reaction was performed by adding 100 ul of substrate solution of p-nitrophenylphosphate (1mg/ml) in 0.05 M sodium carbonate buffer,pH 9.6, containing 0.02% $MgCl_2$. After 1 h incubation at 37 $^{\circ}C$ in humid chamber, the reaction was stopped by adding 50 ul

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of 1 N NaOH, and the absorbance of the reaction mixture was measured at 405 nm in a Titertrek Multiskan Plus plate reader (Flow Laboratories). The titer of the first antibody was the maximum dilution that yield the absorbance at 405 nm 0.4 OD above the background(Banchuin et al.,1984).

The optimum dilution of the first and the second antibodies was determined by precoating the lectin ranging from 0-100 ng/well or 0-10 ng/well, and varying both dilutions of the first and the second antibodies. The time and temperature of incubation were the same as done in titer determination of the first antibody. The optimum dilution of the first and the second antibody that yield maximum absorbance approximately 1.5 were selected to use for the determination of lectin in the embryos, roots and leaves.

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2.11.2 Examination for the nonspecific binding of lectin to rabbit serum and to the second antibody conjugate.

The procedure was performed by incubating 100 ul of nonimmuneserum diluted in PTN (1:100,000) containing 100 mM GlcNAc or without to the lectin coated plate. The subsequent

procedures were the same as described in 2.11.1 . Nonspecific binding of lectin to the second antibody conjugate was done by omitting the first antibody, and directly incubated the lectin coated plate with the second antibody conjugate.

2.11.3 ELISA standard curve

Each well was coated with 100 ul of purified lectin at the concentration ranging from 10-100 ng/well, and left at 4 °C for at least 12 h in an humid chamber. Washing was done in the same manner as 2.11.1, followed by adding 100 ul per well of the first antibody (antilectin) at the dilution of 1:100,000 . The plate was incubated at 37 °C for 45 min and emptied. Then the washing step was carried out as described. The second antibody - alkaline phosphatase conjugate, at the dilution of 1:1,500 was added (100 ul per well) and followed by incubation at 37 °C for 45 min, then the washing step was repeated. The enzymatic reaction was carried out as described in 2.11.1. The reaction was stopped after 1 h incubation by adding 50 ul 1 N NaOH. Then, absorbance at 405 nm was measured.

2.11.4 Determination of embryo lectin in rice grain by ELISA method

Rice embryo preparation and crude lectin extraction were described previously in the preparation of crude lectin for double gel immunodiffusion, but in this experiment 0.1 M sodium carbonate buffer,pH 9.6 was used as extracting buffer. Supernatant fractions were harvested, protein concentration and lectin concentration were determined. The ELISA was performed by coating with the crude embryo extracts that were diluted to an appropriate dilution (e.g.1:40) in 0.1 M carbonate buffer,pH 9.6, at this dilution the absorbance should fit in the range of the standard curve. The later steps were similar to those described in 2.11.3.The standard curve using the first antibody (1:100,000) and the second antibody (1:1,500)was prepared simultaneously to determine the lectin concentration by reading the absorbance at 405 nm of the samples and compared with the standard curve.

2.11.5 Preparation of crude lectin extract in developing seedlings

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Rice seeds cv RD 7 were soaked in tap water at room temperature for 24 hours. The soaked seeds were germinated in the dark, or alternatively under 11-h photoperiod. Seeds were germinated in the same condition as described previously in the immunofluorescent localization of lectin. The other cultivars:

KDML 105, KTH 17, MNS 4, RD 23, RD 25, BMT 370, and SPBR 60 were germinated under dark condition. The roots and leaves (including leaf sheath) of seedlings were harvested (100 seedlings) from 4 days to 7 days old under dark and 11-h photoperiod. The other cv seedlings were harvested at 4 day old. All the root and leaf samples were frozen immediately at -15°C for an hour, and extracted with 1 ml 0.1 M PBS, pH 7.4. Homogenates were filtrated thruogh a gauze cloth, insoluble materials were spun at 48xg, 4°C for 30 min in a microcentrifuge.

2.11.6 Modification of ELISA method for the determination of lectin in vegetative tissues (modified according to Christensen et al, 1986)

Since very low absorbance was observed when crude lectin extract was directly coated on the microtiter plate, which indicated that the competition reaction occured between lectin and the other proteins in the crude extract, so that the chance of lectin to coat on the plate surface was diminished. In order to enhance the lectin binding on the plate, precoating of lectin receptor, 100 ul 0.2% of ovalbumin (a glycoprotein containing sugar moiety that serves as lectin receptor) in 0.1 M sodium carbonate buffer,pH 9.6 was performed at 4°C at least 12 h. A 100 ul aliquat of tissue extracts at an appropriate dilution

(leaf,1:10 in PBS; and root nondiluted) was added, and then incubated at 37 °C for 30 min, followed by washing and other consequences according to 2.11.3. The standard purified lectin was dissolved in 0.1 M PBS, pH 7.4. instead of carbonate buffer to prevent direct coating of lectin on the plate. Negative Control was performed by coating with 0.2% BSA instead of ovalbumin and proceed with similar conditions.