CHAPTER II

MATERIALS AND METHODS

2.1 Bacteria

Klebsiella sp. R15 was isolated from rhizosphere of rice grown in Thailand at Rangsit Rice Experimental Station by Poontariga (1981).

E.coli K12 was obtained from the unit cell of Genetic Engineering, Department of Biochemistry Faculty of Science, Chulalongkorn University.

Klebsiella pneumoniae M5a1 was kindly provided from Dr.C.Kenedy and maintained at the Department of Biochemistry Faculty of Science, Chulalongkorn University.

2.2 Rice

Foundation seeds of *Oryza sativa* CV. RD7 were obtained from the Department of Rice Research, Ministry of Agriculture and Cooperatives, Thailand.

2.3 Media and growth condition

2.3.1 Bacteria

Luria broth medium(LB), (Luria et al., 1960)

Tryptone	10.0 g/l
NaCl	10.0 g/l
Yeast Extract	5.0 g/l

Adjust pH to 7.0 with 0.1 N NaOH and 15 g/l agar was added for solid medium.

Nitrogen free medium(NF),(Dobereiner, 1977)

K_2HPO_4	0.05	g/l
KH_2PO_4	0.15	g/l
$CaCl_2 \cdot 2H_2O$	0.01	g/l
$MgSO_4 \cdot 7H_2O$	0.02	g/l
$NaMoO_4 \cdot 2H_2O$	0.002	g/l
$FeCl_3$	0.01	g/l
$NaHCO_3$	0.01	g/l
Glucose	20.0	g/l

Adjust pH to 7.0 with 0.1 N NaOH or 0.1 N HCl.

Media for *E.coli* and *K. pneumoniae* M5a1 (Shapiro and Stadtman, 1967)

K_2HPO_4	17.41	g/l
KH_2PO_4	13.60	g/l
Glucose	1.98	g/l
NH_4Cl	0.2140	g/l
$MgSO_4 \cdot H_2O$	0.4184	g/l
K_2SO_4	2.4921	g/l
NaCl	2.5128	g/l

Adjust pH to 7.0 with 0.1 N NaOH or 0.1 N HCl.

2.3.1.1 Maintenance of bacterial cultures

All bacterial cultures was maintained by adding equal volume of media culture in the sterile glycerol and kept in a deep-freezer at -75 °C for 1-2 years.

2.3.1.2 Cultivation of bacteria

A single colony of bacteria from LB plate was

inoculated into 100 ml of LB broth in a 500 ml Erlenmeyer flask and incubated at 30 °C with shaking in an orbital shaker until OD_{420} reached 0.8-1.0 , cells were then transferred into a 1 l Erlenmeyer flask containing 250 ml NF medium(*Klebsiella* R15) or medium for *E.coli* using 3 % inoculum and incubated until OD_{420} reached 0.6 - 0.7. Cells were harvested by centrifugation at 7,000 g, 4 °C 20 min, washed once with 0.85 % NaCl and suspended in 0.85 % NaCl before they were inoculated into rice seedlings. For GS purification, cells were washed three times with 50 mM Tris-HCl buffer pH 7.5.

2.3.2 Bacteria-Rice association

Rice seeds were dehulled by hand and surface sterilized by soaking in 95 % ethanol 30 sec and immediately washed off with sterile distilled water containing Tween 20 (5-6 drops /100 ml) several times then soaking in 20 %, 15 % and 10 % Clorox which contained Tween 20 (5-6 drops /100 ml) for 20, 15 and 10 min respectively and washed off with excess amount of sterile distilled water. Seeds were germinated on 0.5 % agar in Petri dishes about 20 seeds per plate and placed in the dark for 4 days. On the fifth day 10 rice seedlings which were contamination-free and approximately equal size were selected and transferred to a 130 ml test tube containing 7.0 ml sterile distilled water. Seedlings were placed in a controlled temperature room at 25 °C, and under a 11/13 light/dark condition. Illumination was provided by plant-growth fluorescent lamps.

The experiment was designed to measure nitrogenase and GS activity in the same lot of rice seedlings as shown in Figure 2.1. For nitrogenase assay 6 tubes were used for each treatment, and using 15 tubes for assay of GS activity in each treatment, control tubes containing the same amount of *Klebsiella* R15 or rice seedlings only were

Figure 2.1 Protocols of *Klebsiella* R15 inoculation in rice(RD7)

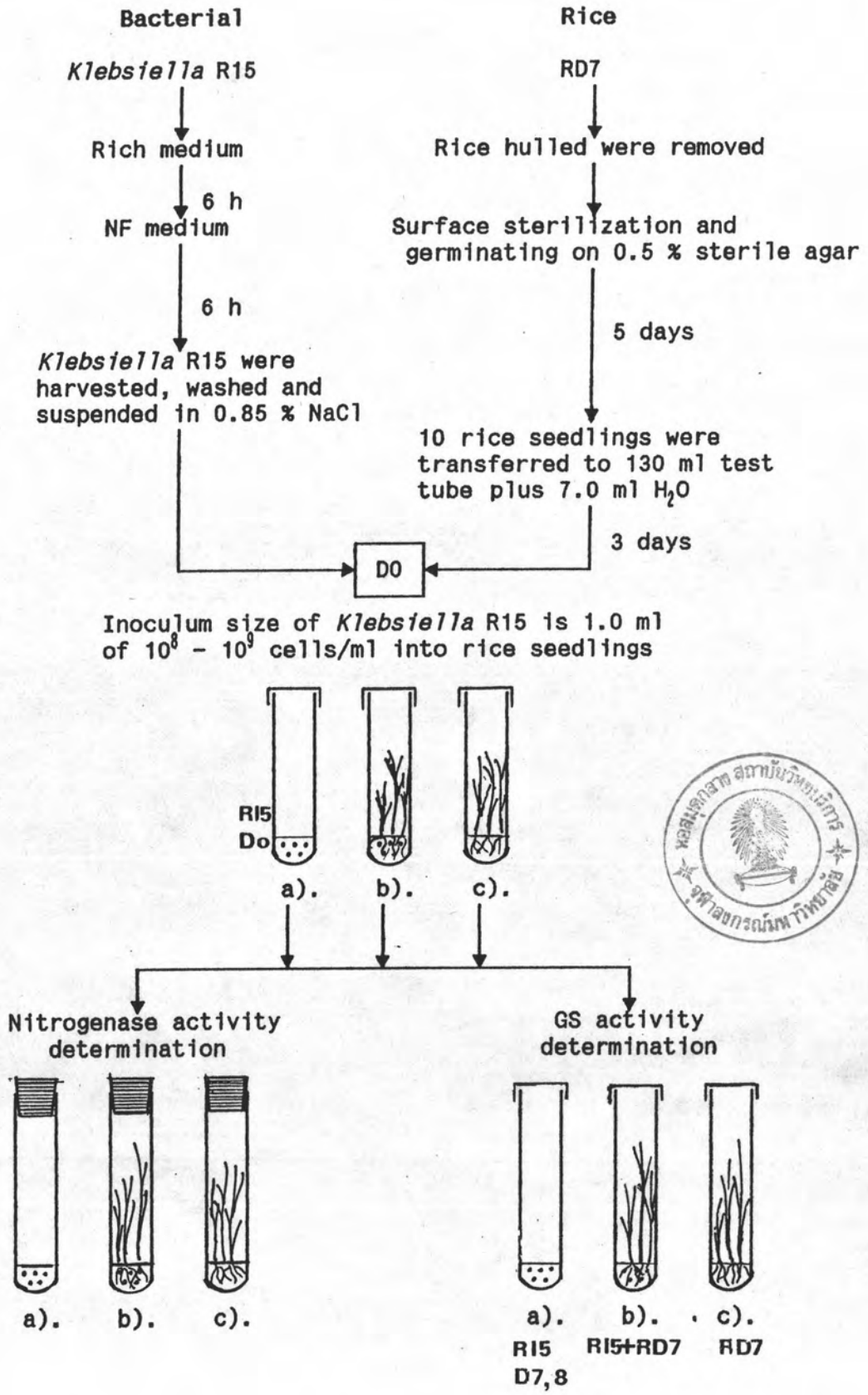


Figure 2.2 Rice seedlings were grown in test tube with 7 ml of sterile water for nitrogenase and GS assay.

performed similarly as the experimental tubes containing rice seedlings and *Klebsiella* R15.

The number of viable bacterial were determined before and after inoculation by spread plate method(Ronald et al., 1984). The serial dilution of *Klebsiella* R15 in 0.85 % NaCl were spread on to the surface of NF agar plate in volume of 0.10 ml. And then the number of colonies that grown were count after incubating at 30 °C 48 h.

2.4 Purification of GS

For each lot of GS purification (modified from Boontariga, 1988) washed cells from 2-3 l of NF culture were frozen overnight and the crude enzyme solution was prepared by disrupting cells in 20 ml of 50 mM Tris-HCl buffer, pH 7.5 in a sonicator(Heat System Ultrasonic Inc Model W.375) with pulsed input, 50 % duty cycle until cells were broken completely. Cell debris was removed by centrifugation at 30,000 g 30 min. Crude enzyme solution was loaded onto a Reactive Blue Sepharose CL-6B column (7.0 X 1.8 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH 7.5 (containing 1 mM $MnCl_2$) at flow rate of 9.0 ml min^{-1}. Then the column was washed with equilibrating buffer until no significant absorbance at 280 nm was observed. GS was eluted from the column with 2 mM ADP in the same buffer. Fractions containing GS activity were pooled, and concentrated in a Centrifugal Ultra Free Unit (Millipore) The concentrated solution was loaded onto a Sepharose-4B column(50.0 x 1.8 cm) : pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.5 at constant flow rate of 11.0 ml min^{-1}. Fraction of 2.0 ml were collected and assayed for GS activity, those fractions showing GS activity were pooled and concentrated.

2.5 Determination of GS Activity by Transferase Assay

2.5.1 GS in bacteria

The transferase activity of GS was assayed essentially according to Farden and Robertson(1980) in which the reaction mixture was freshly prepared before use by mixing 1.0 ml 0.5 mM imidazole-HCl buffer, pH 7.0, 2.0 ml 0.15 M glutamine, 0.3 ml o.1 M $MnSO_4$, 0.4 ml 0.01 M ADP, 0.2 ml 1.0 M Sodium arsenate pH 7.0 and 1.1 ml distilled water to make 5.0 ml of reaction mixture. For standard curve 125 µl of this reaction mixture was mixed with 100 µl of increasing concentration of γ-glutamyl hydroxamate. For unknown samples, 125 µl of reaction mixture was mixed with 100 µl test solution to give a total volume of 225 ul and preincubated at room temperature(25 °C) for 5 min. The reaction was started by adding 25 µl hydroxylamine solution (prepared by mixing 1.0 M NH_2OH-HCl:1.0 M NaOH = 1:1 just before use). After incubation for 15 min at room temperature the reaction was stopped by adding 0.5 ml $FeCl_3$ reagent(prepared by mixing 10 % $FeCl_3$ $6H_2O$: 24 % TCA:6 M HCl: distilled water = 8:2:1:13). Control assays were performed by minus ADP, and sodium arsenate. Assay mixture was centrifuged at 5,000 g for 20 min and the absorbance (A_{540}) of γ-glutamyl hydroxamate formed was determined in a Spectronic 2000 spectrophotometer. Standard curve of γ-glutamyl hydroxamate(0.1-1.0 µmol) was prepared in every assay. The specific activity unit of GS transferase activity was expressed as (µmol γ-glutamyl hydroxamate formed)·(mg protein)$^{-1}$·min^{-1}.

Protein was determined by the method of Bradford (1976) (Appendix III) using BSA in the range of 0-10 µg/100 µl as standard.

2.5.2 GS in bacteria-rice association

To determine GS activity, 3 sets of 15 test tubes were used (a). rice seedlings only (b). rice seedlings + *Klebsiella* R15 and (c). *Klebsiella* R15 only. Each tube was closed with aluminum foil instead of rubber stopper. GS was assayed when nitrogenase activity in another set of tubes increased to the maximal level(7-8 days after inoculation), 150 rice seedlings from 15 tubes/set were pooled, rice roots were cut and washed with 50 mM Tris-HCl buffer, pH 7.5 until the washing was cleared. Bacterial cells (*Klebsiella* R15) in the outer rhizosphere were washed off from rice root into 50 mM Tris-HCl buffer, pH 7.5 3-5 times, the wash off were pooled and centrifuged at 8,000 g for 30 min at 4 °C. The pelleted cells were frozen overnight at -20 °C. Assay for GS activity was performed as described previously (2.6.1).

The rhizoplan bacteria firmly attached to the epidermal cells and root hairs of rice roots refered to as "endorhizospheric bacteria", were isolated by grinding the rice roots(150 rice seedlings) in precooled mortar with 2.0 ml 100 mM Tris-HCl buffer, pH 7.6 (containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 1 mM $MgCl_2$), TBEM(Appendix II), or alternatively rice roots frozen in liquid nitrogen for at least 1 h were used. The root homogenate was filtered through a lawn sieve and washed with 100 mM Tris-HCl buffer, pH 7.6 several times. Root homogenate was then centrifuged at 60,000 g 30 min at 4 °C, the supernatant fraction was concentrated with Centrifugal Ultra Free Unit (Millipore). The GS activity residing in rice roots supernatant fraction was considered as rice GS, and the GS activity belonging to the pellet fraction was considered as mixture of bacterial GS and rice GS.

2.6 Determination of Nitrogenase activity

Nitrogenase acctivity was determined by acetylene reduction assay(ARA) according to Elkan(1987), using 3 sets of 6 replication tubes, each set contains: (a). rice seedlings only (b). rice seedlings + *Klebsiella* R15, and (c). *Klebsiella* R15 only. Each tube was closed with a serum stopper, the head space was changed to 10 % acetylene by removing air and replacing with equal volume of acetylene(Ratanachoke, Engineering Ltd.). At various time intervals 300-400 µl of the gaseous phase was injected in a gas chromatograph(Packard Model 437A) under the following conditions:

Column packing	Porapak N
Column temperature	90 °C
Injector temperature	110 °C
Detector temperature	150 °C
Flow rate of N_2	30 $ml \cdot min^{-1}$
H_2	25 $ml \cdot min^{-1}$
air	250 $ml \cdot min^{-1}$

The assay was performed daily after inoculation of *Klebsiella* R15 for 6-7 days or until the nitrogenase activity was plateau. The standard pure ethylene gas (99.9 %)(Thai Industrial Gas Ltd.) was diluted by air and injectd at increasing volume as standard before every assay.

2.7 Polyacrylamide Gel Electrophoresis

2.7.1 Nondenaturing polyacrylamide gel electrophoresis (PAGE).

Electrophoresis was performed on 7.5 % polyacrylamide gel

(Davis, 1964) of 0.5 mm thick slab gel(10.2 x 8.2 cm) with Tris-glycine buffer pH 8.3(Appendix II). The electrophoresis was run from cathode towards the anode with constant current of 20 mA per slab gel at 4 °C for 1-2 h, in a Midget Electrophoresis apparatus (LKB) attached to a thermostat water bath of LKB 2209 Multi Temp. The protein bands were stained with Coomassie brilliant blue R 250 (Appendix II). The GS activity was stained by incubating the gel in the transferase assay mixture for 20 min at room temperature and color was developed by dipping gel in the $FeCl_3$ reagent as described in 2.5.1.

2.7.2 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE).

This was carried out in polyacrylamide gel of 12 % T according to Laemmli (1970). Preparation of stock solutions and gels were described in Appendix III . The electrophoresis was performed as described above(2.7.1), but the running temperature was 15 °C.

2.8 Preparation of the Antiserum Against GS (Ashford et al., 1982)

2.8.1 Immunization

Primary immunization was performed by dissolving 180 µg of purified GS protein in 0.6 ml of sterile 0.85 % NaCl and mixed with equal volume of Freund's complete adjuvant(FCA) and injected via intradermal route into 6-8 sites on the rabbit's back (0.2 ml /site). For boosting, subcutaneous injection at 2-3 weeks intervals after primary injection were performed using 50-200 µg GS protein in 0.6 ml of sterile 0.85 % NaCl and mixed with equal volume of Freund's incomplete adjuvant (FIA).

For test bleeds, 1.0 ml of blood were drawn from ear vein in order to determine the serum titer by Ouchterlony method

(Ouchterlony, 1953) using 1.0 % agar(Difco) in 50 mM Tris-HCl buffer pH 7.5 , 0.15 M NaCl, 0.02 % NaN_3 pored into a Petri dish about 3.5 ml per plate. When the agar was hard, the pattern consists of hexagonal wells with a central hole were cut using a metal cylinder of 4 mm diameter. Fill the central well with 1 µg GS protein and 20 µl antiserum was applied to each well of the hexagonal pattern in sequential dilutions and incubated the plate in a plastic box with water saturated atmosphere at room temperature for 24-28 h then the unprecipitated proteins were washed off with 0.85 % NaCl at room temperature overnight. The precipitin bands were visualized by staining with 0.25 % Coomassie brilliant blue R 250 dissolved in 1:1 solution of 95 % ethanol and 10 % acetic acid for 1-6 h and destained with 38 % ethanol, 3 % acetic acid for 1 h. Finally the gel was destained with 28 % ethanol, 3.5 % acetic acid. When the antibody titer of serum(the reciprocal of the highest dilution that was able to form precipitin band with GS) increased to 32, 10 ml blood was collected for further purification of GS antibody.

2.8.2 Purification of GS Antibody

Rabbit antiserum 2.0 ml was diluted with equal volume of 0.1 M PBS1(Appendix II) and loaded on an affinity chromatography column (Immobilized Protein A 2.5 ml) previously equilibrated with 0.1 M PBS1 pH 7.5 and eluted with the same buffer at the flow rate 12 ml min^{-1} until no significant absorbance at 280 nm was observed. Then the antibody against GS was eluted from column with 0.1 M glycine-HCl buffer pH 2.8. Fractions containing antibody against GS were pooled, neutralized to pH 7.0 and concentrated to 2.0 ml with a Centrifugal Ultra Free Unit (Millipore). Desalting was performed on an excellulose GF-5 desalting column. For storage GS antibody was dissolved in 0.1 M PBS1 and divided

into 0.5 ml aliquot and kept frozen at -20 °C until use.

2.9 Western blot analysis of GS Protein by GS Antibody

For the determination of the sensitivity of GS antibody towards homologous antigen GS of *Klebsiella* R15 and the cross reactivity of GS antibody with other heterologous antigens by Western blot analysis, the procedures are as follows.

2.9.1 Electrophoresis of the separated peptide from gel to membrane filter.

After SDS-PAGE has been done protein bands on slab gel were transferred to a nitrocellulose membrane(LKB) as described by Lin and Kasamatsu(1983). Transfer buffer used was 25 mM Tris-HCl and 150 mM glycine buffer pH 8.3.

The eletroblotting was performed at 0.6-1.2 mA, 4 °C for 1 h. Then the transferred membrane was washed briefly with distilled water and 50 mM Tris-HCl buffer saline pH 7.5, TBS(Appendix II) and non specific binding was blocked by incubating the membrane overnight in TBS-milk (TBS, 1 % skim milk) with gentle shaking at room temperature.

2.9.2 Antibody-antigen reaction on the membrane filter.

Transfer the nitrocellulose membrane to a plastic tray containing 30 ml fresh TBS-milk and add 37.5 µl antibody against GS protein (dilution 1:800). After incubation with gentle shaking at room temperature for 1 h, then the membranes were washed three times with 40 ml TBS- Tween 20) each for 5-15 min. After that, 30 ml of fresh TBS-Tween 20 containing 10 µl second antibody (horseradishperoxidase-goat anti-rabbit IgG;dilution 1:3,000) was added to the membrane and incubated for 1 h with gentle shaking. Decant away the second antibody

solution and washed as described previously.

2.9.3 Detection of antibody-binding polypeptides.

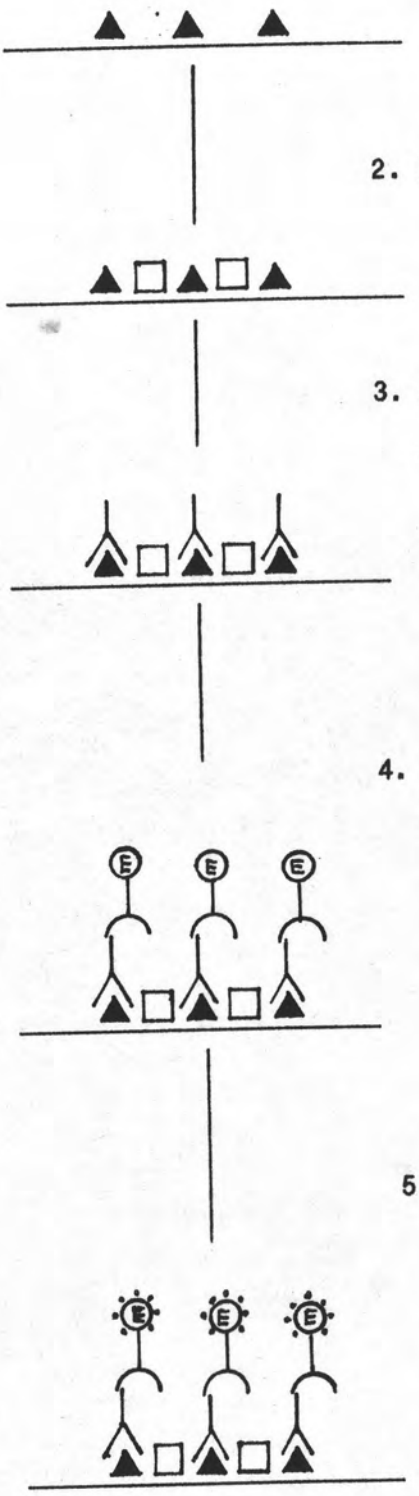
Transfer the washed membrane to a clean tray containing 10 ml 0.03 % 3,3'-diaminobenzidine tetrahydrochloride (DAB) in TBS and add 10 µl of 30 % H_2O_2. (freshly prepared according to Walk, 1984). Wait for about 10-30 min, then stop reaction by changing the solution to distilled water when the brown color of protein bands reached the desired intensity.

2.10 Determination of GS Protein by ELISA

The ELISA procedure were modified from Chung(1984) and Joseph and Meek (1987) as shown in Figure 2.3.

The pure GS antigen or GS protein in samples extract were diluted serially in 0.1 M phosphate buffer saline pH 7.4, PBS2 (Appendix II). These diluted solutions(50 µl) were coated on microtiterplate(Nunc) and kept in humid chamber at 4 °C for 36 h. Then uncoated buffer solutions were removed by inversion and flicking, followed by washing 3 times with 3 x 100 µl PBS2-Tween(PBS2 containing 0.05 %Tween 20)into each well and immediately removed at the first, and for the two final washings PBS2-Tween was allowed to stand for 2-5 min. Blocking of nonspecific binding was done by adding 100 µl of the washing buffer containing 2 % BSA to block the unsaturated plastic surface and incubated in humid chamber at room temperature for 1 h. Then the the excess BSA solution was removed and the plate was washed 3 times as described above. The first antibody(purified antibody against GS) was diluted in PBS2(1:25,000) and 50 µl was added into each well, incubated for 2 h at room temperature in humid chamber. The excess first antibody

Figure 2.3 Protocol of non-competitive ELISA



1. Immobilized GS antigen(▲) on solid phase and washed.

2. Blocked unsaturated solid phase by 2 % BSA.(□)

3. Antibody against GS(⅄) bind to antigen and unboundantibody are removed by washing.

4. Horseradishperoxidase-goat Anti-rabbit IgG(Ⓔ) bind to antibody against GS.

5. Following addition of ABTS the amount of colored product form is proportional to standard and test sample levels.

was removed and the plate was washed as described above. The second antibody (horseradishperoxidase-goat Anti-Rabbit IgG) diluted in PBS2 (1:1,000) was added 50 µl/well and incubated 1 h followed by washing. The enzymatic reaction was initiated by adding 50 µl of 2,2'-azino-di(3-ethylbenzthiazoline) sulfonic acid(ABTS) 0.5 mg ml^{-1} in citric acid buffer, pH 4.2 with 1 µl of 30 % H_2O_2 ml^{-1} (Appendix II). The colored product of reaction on the plate was monitored at 405 nm with a Titertek Multiskan Plus plate reader(Flow Laboratories) after 60 min.

Standard curve was prepared by plotting the rate of product formation against the known amounts of GS purified from *Klebsiella* R15 (0.5-5.0 ng/well).

Examination for the nonspecific binding of the GS antibody to GS protein was performed by using preimmuneserum in place of antiserum against GS at the dilution of 1:10,000, after precoating with GS antigen, and after that the subsequence procedures were the same as described previously. Nonspecific binding of GS protein to the second antibody was checked by omitting the first antibody, and directly incubated the GS antigen coated plate with the second antibody.