Chapter 2

Materials and Methods

2.1 Natural latex specimen

2.1.1 Collection

The fresh field latex specimens were collected from the *H. brasiliensis* trees: clones RRIM600, GT1, PB5/51 and KRS165. The rubber latices from clones RRIM600, GT1, PB5/51 were from a plantation in Rayong Province, while the clone KRS were kindly provided by the Pong Rad Rubber Research Station at Chantaburi Province. Tapping in private plantations usually started about 04.00 am, whereas tapping at the Rubber Research Station started at 08.00 am. The latex that flowed down into the collection cup was pooled about 2 hours later.

2.1.2 Preservation and transportation

Fresh latex specimens were filtered through three layers of muslin cloth and preserved in 0.1 M phosphate buffer, by added 0.4 M phosphate buffer pH 7.4 into field latex at the ratio of 1:3 v/v.

The containers were closed and sealed with sealing film (Whatman Lab Sealing) and stored in an ice box during transportation to Bangkok. The latex specimens were stored in a cold room at 4-10 ^{o}C until the proteins preparation was begun.

2.2 Preparation of latex proteins

2.2.1 Clear serum(C-serum) separation

Preserved latex specimens were centrifuged at 20,000 G, for 30 minutes at temperature 25°C in a Beckman L8-70 centrifuge (Beckman Instruments, Inc., California, USA. The latex specimens were separated into three phases, rubber particle and bottom fraction were discarded. C-serum was drawn by syringe 20 ml and long needle guage no. 15. The C-serum was assay for total protein by micro Bradford's method (Bradford, 1976) and stored at 0-4°C.

2.2.2 Acetone precipitation

The C-serum was gently poured into cold acetone, -20°C, at the ratio 1:2 (serum:acetone) The precipitated protein was collected by centrifuging at 2,000 x g for 5 min at 25°C, the supernatant was discarded and the pellet was redissolved in an equal volume of distilled water before precipitation. The protein concentration was determined by micro Bradford assay.

2.2.3 Dialysis

The dialysis bags, which is 1,000 Dalton molecular weight cutoff, were filled with the redissolved latex C-serum protein (from 2.2.2, about 200 ml/bag), then dialyzed against four litters of distilled water for 48 hours at 4-10°C, with replacement of water every 12 hours period.

2.2.4 Lyophilization

The dialyzed protein solution was aliquated in 12x75 plastic tubes, 3 ml/tube, frozen at -20 ^{o} C, then lyophilized for 48 hours. Random sampling of lyophilized protein was resuspended with distilled water and assayed for protein.

2.2.5 Sodium dodecyl sulfate-polyacylamide gel electrophoresis

Sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) was performed according to Slater et al (1990), with slightly modification. Fifteen percent acrylamide-bis gel was used as the separating gel and 3% of the gel was used as the stacking gel. Tris-glycine (0.025 M Tris, 0.192 M glycine) buffer pH 8.8 containing 0.1% SDS was used as electrode buffer. Sample to be analysed, was dissolved in Tris buffer, containing 0.0625 M Tris, 0.2% SDS, 10% glycerol, 0.5% 2-mercaptoethanol, and 0.01% Bromphenol blue, and boiled for 3 minutes prior to application to the gel. The electrophoresis was carried out at constant current of 40 mA, on a Mini-PROTEAN II Cell (Bio-Rad Laboratories, Inc., New York, USA) from cathode toward anode. When the electrophoresis was completed, the gel was stained with Coomassie blue G-250 or transferred the protein-bands to nitrocellulose membrane in a Mini Trans-Blot Cell (Bio-Rad Laboratories, Inc., New York, USA).

2.2.7 Extraction of proteins from rubber gloves and tires

A piece of thin rubber specimen (5x5 cm) was cut from each glove sample at a palm part, weighed and transferred to a 200 ml flask and added 10 ml of distilled water per gram of specimen. The flask was sealed with sealing film, and shake for 15 seconds after adding the water and again at 60 and 120 minutes The extracted solution was centrifuged at 2,000 G for 5 minutes, followed by filtering it through filter paper

(Whatman no.3). The filtered-solution was dialyzed in a dialysis tubing against water (2.2.3) and lyophilized (2.2.4).

A moderate used rubber tires were collected from garages, washed and cut to small pieces, weighed for 5 gram / sample, transferred to a 200 ml flask and followed the similar procedure for extraction, dialyzation and lyophilization as used with the glove sample.

The total protein concentration in these samples were determined by micro Bradford assay.

2.3 Human serum samples

2.3.1 Blood donor's sera

Donor's sera were collected from residual serum side tubes of 352 Red Cross volunteer blood donors, who had no known history of any allergy. All donations were from workplace mobile collection sites and the Red Cross Prapinklao Hospital Branch, at Bukalo Thonburi, Bangkok. To obtain a sample broadly representative of the population, no more than 10 samples per day were taken from a single mobile unit.

2.3.2 General allergic adult's sera

Patients who came to Allergic Clinic, Prapinklao Hospital and physical examination showed one of the general allergic symptoms, were interviewed about their histories concerning the latex allergy and asked for blood collection. No other question was asked except the question like "Do you have any experience about the natural rubber goods allergy?".

2.3.3 General allergic children's sera

Most of general allergic children sera were from the allergic children whose bloods were collected for other proposes, and no one had history of natural rubber allergy. These children were admitted in the Children Hospital, Phyathai, Bangkok. Some of the sera were obtained from allergic children in Prapinklao Hospital.

2.4 Specific anti-latex IgE antibody detection

2.4.1 **Enzyme allergosorbent test**

The latex enzyme allergosorbent test (EAST) with latex protein was the same as described by Czuppon et al (1993) with some modification. The details are as follows:

The lyophilized latex protein were resuspended in sodium carbonate-bicarbonate buffer, 0.1 M, at pH 9.6. The final concentration of protein was 50 mg/ml.

A polystyrene 96 wells microtitre plate was filled up with 200 µl/well of latex protein solution (from 2.4.1.1) and incubated at 4°C, in a moist chamber, overnight. The protein solution were discarded and washed three time with 500 µl of 0.9% NaCl solution containing 0.1% Tween 20 (NSS/Tween 20). Each well was blocked the unoccupied sites with 200 µl of phosphate-buffer saline containing 3% bovine serum albumin (BSA), 0.05% Tween 20 and 0.01% sodium azide (blocking buffer).

Test serum samples were diluted at 1:10, 1:100, 1:200, 1:400 and 1:800 with 0.9 % NaCl solution (serum : solution) and 100 µl were dispensed into well, then incubated at room temperature for 30, 60, 120 and 180 minutes.

Serum were discarded and the wells were washed three times with 500 µl of NSS/Tween 20.

Goat anti-human IgE conjugated to alkaline phosphatase (Sigma Chemical Company, St. Louis, USA) in blocking buffer (1:1,000, anti-IgE: buffer) 100 µl was added and then incubated at 37° C, in moist chamber for two hours.

The anti-human IgE was discarded and the wells were washed three times with 500 µl of NSS/Tween 20.

One hundred microlitter of 0.1% p-nitrophenyl phosphate (Zymed Laboratories, San Francisco, USA) in 10 mM diethanolamine and 0.5 mM $MgCl_2$, pH 9.5 was added and incubated at 37°C for 30 minutes. Absorbance reading at 405 nm was measured by an ELISA microplate reader Titertek Multiskan, Labsystem and Flow Laboratory, Finland).

2.4.2 **Immunostain of antigen**

This procedure followed Slater et al (1990) with some modifications (Figure 2.1). The acetone-precipitated field latex proteins, separated by SDS-PAGE (2.2.5) were transferred to a nitrocellulose membrane (Micron Separations Inc., USA) at 240 mA for 2 hours in 15.6 mM Tris, 120 mM glycine buffer, pH 8.3. The membrane was cut into strips for immunostaining. Each strip was immersed in blocking buffer for 2 hours, and incubated at 4°C overnight in the diluted human EAST-positive sera (1:10), then washed with excess amount of NSS/Tween 20 and incubated with goat anti-human IgE conjugated to alkaline phosphatase for 2 hours at room temperature, then washed with excess amount of NSS/Tween 20. Color was developed with 20 ml 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Zymed Laboratories, San Francisco, USA).

Immunostaining Diagram

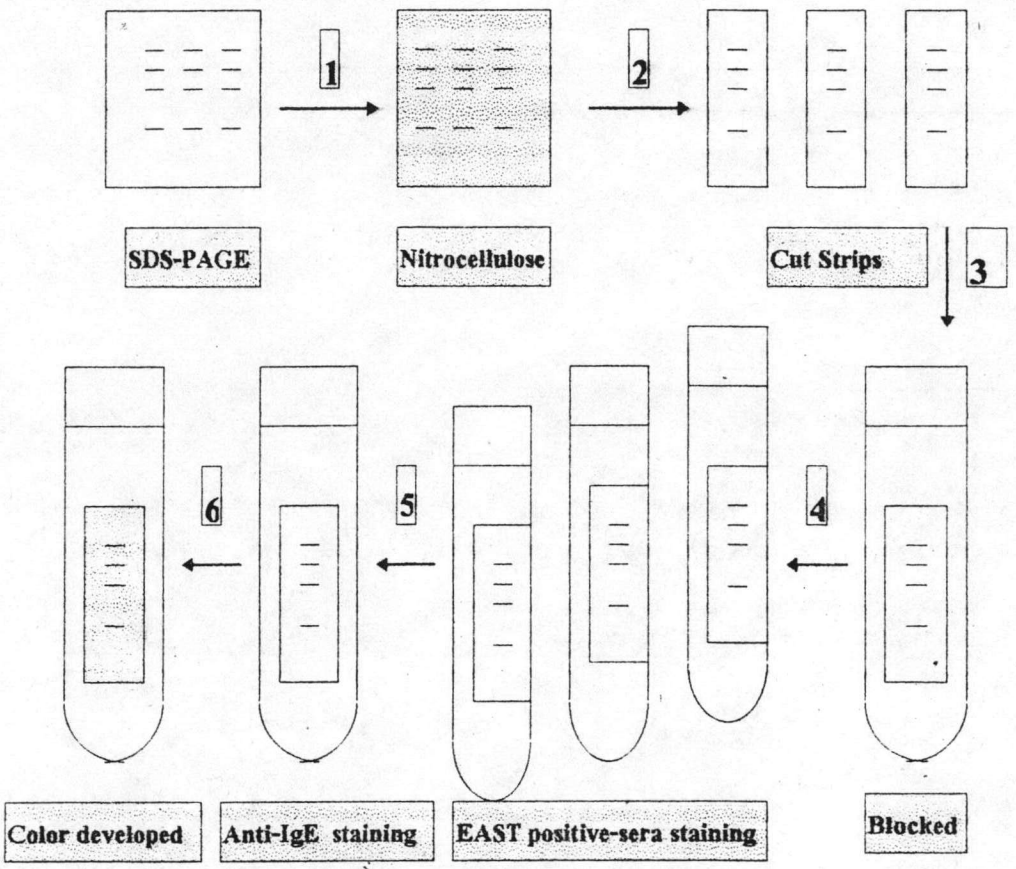

Figure 2.1 Immunostaining procedure: (1) polyacrylamide with separated C-serum latex proteins were electrotransferred onto nitrocellulose membrane, (2) the membrane was cut into strips, (3) the strips were blocked in blocking buffer for 2 hours, (4) the strips were washed and incubated in each EAST positive-human serum, (5) the strips were wash and incubated in goat anti-human IgE for 2 hours, (6) Color was developed.

2.5 Rabbit IgG against latex allergens preparation

2.5.1 Gel filtration

Bio-Gel P-100 (Bio-Rad Laboratories, Inc., New York, USA), 50 g was swollen in 100 ml of water and transferred to 0.5 M HCl. After acid treatment, the gel was washed several times with water then treated with 0.5 M NaOH followed by washing several times. The gel was washed in phosphate buffer pH 7.4 and then packed into column (3 x 100 cm) and equilibrated in the same buffer at flow rate of 30 ml/h.

Suspension of lyophilized latex protein (1.0 g/ml) 5 ml with Blue dextran and potassium dicromate, was loaded onto the column. Fraction of 3 ml were collected at the same time of A_{280} was measured by Econo System (Bio-Rad Laboratories, Inc., New York, USA). Each fraction was coated in the 96 well microtitre plate (followed 2.4.1). The fractions that showed positive result with pool positive EAST sera, were pooled, dialyzed, and lyophilized.

2.5.2 Rabbit immunization

Latex proteins separated by gel filtration were immunized to four rabbits. The rabbit were divided into two groups for two different doses of immunization i.e. 0.5 and 1.0 mg per rabbit. The pool latex proteins, 0.5 or 1.0 mg., were dissolved in 0.5 ml sterile 0.85 % NaCl and mixed with equal volume of Freund's complete adjuvant (FCA) by double hub needle method. The primary immunization was performed subcutaneously into 8-10 sites along rabbits' flanks. After primary injection the rabbits were boosted with 0.15 or 0.30 mg in 0.5 ml of the proteins which was emulsified with 0.5 ml of Freund's incomplete adjuvant (FIA). Injections were done alternately on both sides of rabbits' flanks.

For testing antibodies titer, 1 ml of rabbit's blood were drawn from ear vein after immunization every other week. The antibodies titer was determined by gel double diffusion. When the antibody titer of the serum increased at least 1:32, 5 ml of blood were collected.

Rabbit sera were collected after blood clotting at room temperature. The cloudy sera were decanted and centrifuged at 300 rpm for 15 minutes. Sera obtained were frozen at -80°C in 1 ml aliquots.

2.5.3 Double gel diffusion method

Primary antibody titer determination was measured by double gel diffusion according to Ouchterlony method (Ouchterlony, 1980). Three and half ml of 1.0 % noble agar (BBL) in 25 nM Tris, 50 nM NaCl pH 7.5 was poured into a 5 cm diameter Petri dish. After solidifying, 4 mm radius holes were punched. Protein antigens, 0.4 μg were added in the center well. The serum sample were prepared in serial double dilution 1:2, 1:2^{2}, 1:2^{3}, 1:2^{4}, 1:2^{5} and 1:2^{6}. Ten microliter of each dilution was added to the surrounding wells. The plate was then incubated in a humid chamber at room temperature for 24 hours before precipitate line was observed. Titer of the anti-serum was the highest dilution which still showed the precipitin line.

2.5.4 ELISA determination of rabbit antibody titer

Indirect Enzyme-linked Immunosorbent Assay (indirect ELISA) was used to determine the serum titer. Titer of the anti-serum was the highest dilution which still showed the yellow-color. The method was modified from the general protocol (Rose et al., 1986; Catty and Raykundalia, 1990) as illustrated in Figure 2.2.

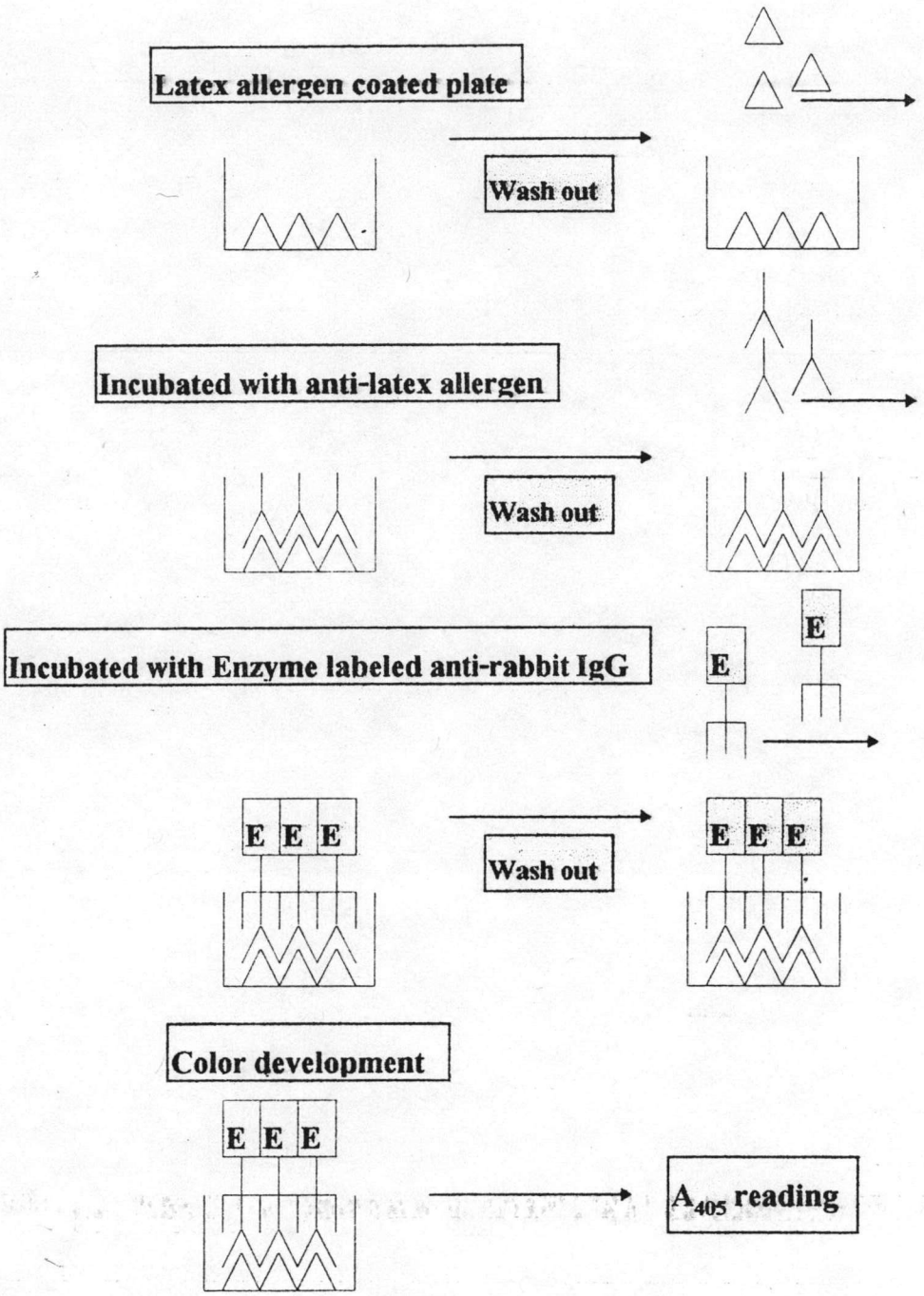

Figure 2.2 Illustration of indirect anti-latex allergen ELISA

△ = purified latex allergens, ⅄ = rabbit anti-latex allergens

E = goat anti-rabbit IgG (alkaline phosphatase conjugate)

2.6 Allergens detection with rabbit IgG

2.6.1 Indirect competitive ELISA determination of latex allergen

The rabbit anti-latex protein serum, diluted 1: 1,000 was competed with various concentration of protein antigens (from 2.5.1): 0.01, 0.5, 0.10, 0.50, 1.00 and 10.00 μg, in a 96-well microtiter plate coated with 1 μg/well of antigen. The neutralized-serum was incubated at 37°C, in a moist chamber for two hours. The serum was discarded and washed with 500 μl of NSS/Tween 20. Then, nonspecific binding was prevented by adding 200 μl of blocking buffer and washed 3 times with 500 μl NSS/Tween 20. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Chemical Company, St.Louis, USA) in blocking buffer 100 μl was added. After 2 hours of incubation at 37°C, the well was washed 3 times with NSS/Tween 20. Substrate, 0.1% p-nitrophenyl phosphate (Zymed Laboratories, San Francisco, USA) in 10 mM diethanolamine and 0.5 mM $MgCl_2$, pH 9.5, was added and product was measured spectrophotometrically at 405 nm after incubation at 37°C for 30 min by ELISA microplate reader (Titertek Multiskan, Labsystem and Flow Laboratory, Finland).

Instead of the protein allergen, bovine gamma globulin (Bio-Rad Laboratory, Inc. California, USA), ribonuclease A, lysozyme (chicken egg white), bovine serum albumin, and α-chymotrypsin (Sigma Chemical Co., St. Louis, USA) were used to compete with coated latex allergen, for negative control.

2.6.2 Latex protein dot blot

This method followed Beezhold et al (Beezhold et al., 1995). A (3 x10 cm) strip was cut from each glove sample. The strips were rinsed to remove powder and loosely-bound proteins, before being placed (outer-surface face-up) in the plastic

apparatus (Figure 2.2), forming test wells with the rubber film being the bottom of each well. The wells were then treated with diluted (1: 10) rabbit anti-latex protein serum containing 1% BSA to prevent nonspecific binding of antibody to the film. The well were washed and goat anti-rabbit IgG conjugated to alkaline phosphatase in blocking buffer 100 µl was added. After 2 hours of incubation at 37°C, the well was washed 3 times with NSS/Tween 20. Substrate, 100 µl of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Zymed Laboratories, San Francisco, USA), was added and color product was detected.

2.6.3 **Latex-protein dot blot modification**

The rubber glove strips (2.6.3) or dried filter paper that saturated with 5 µg/ml of latex protein allergens (positive control) were padded or electrical protein transferred onto nitrocellulose membrane (2.4.3). Then the nitrocellulose membrane strips were used instead of rubber strips in 2.6.3.

Negative control had been done by using dried filter paper that saturated with 5 µg/ml of bovine gamma globulin, ribonuclease A, lysozyme (chicken egg white), bovine serum albumin or α-chymotrypsin.

2.6.4 **Latex particles agglutination inhibition technique**

Preserved latex from 2.1.2 was diluted to be 10% DRC. The diluted latex was gently mixed with 0.5% (v/v) glutaladehyde to fix the protein associated on surface of the rubber particle. After 30 minutes of fixing, rubber was centrifuged at 2,000x g and the supernatant discarded. The fixed rubber particles were washed three times with 0.9% NaCl solution and resuspended in 0.9% NaCl, the final concentration was varied from 1 to 10% DRC. Anti-latex protein serum, was serially two-fold diluted with

0.9% NaCl, and each dilution was mixed with and equal volume of 10% DRC fixed-rubber particle. Agglutination was observed within 5 minutes.

The extracted protein from the natural rubber products (from 2.2.7), latex antigens (from 2.5.1) and 1 % BSA solution were used to mixed with the rabbit anti-serum at the same volume and the mixed sera were mixed with the 10% DRC fixed-rubber particle then agglutination was observed after 5 minutes.

Bovine gamma globulin, ribonuclease A, lysozyme (chicken egg white), bovine serum albumin or α-chymotrypsin were used for negative control.