CHAPTER II
MATERIALS AND METHODS

2.1 Biological materials

2.1.1 Fresh field rubber latex from RRIM 600 clone was kindly provided by Rayong Bangkok Rubber Co., Ltd. Ammonia was sometimes used as preservative and anti-coagulant.

2.1.2 Papain product code number P3250, specified as crude extract was purchased from Sigma.

2.1.3 Alcalase T, dust-free granulate form of Alcalase was obtained from NoVo.

2.2 Chemicals

Casein hammarsten, hydrochloric acid, acetic acid, ethyl alcohol, copper sulfate, sodium lauryl sulfate and sodium bicarbonate were obtained from BDH Laboratory Chemicals Division, England.

Hydroxylamine hydrochloride, sodium metabisulfite, bromocresol green, ovalbumin, bovine serum albumin, glycine, tween 20, goat anti-human IgE combine with alkaline phosphatase and acrylamide were from Sigma Chemicals Co., U.S.A.

Trichloroacetic acids, sodium hydroxide, methyl red, sodium carbonate, bromophenol bule and methanol were from E. Merck Ag. Darmstadt, Germany.

Tris (hydroxymethyl)-aminomethane was from Carlo Erba.

Ammonia, sodium citrate, phosphotungstic acid, sodium chloride and potassium phosphate were from Carlo Erba.

Selenium powder and sodium carbonate were from APS Finechem.

Sulfuric acid, diethanolamine and Triton X-100 were from Fluka.

2.3 Apparatus

2.3.1 Apparatus in the Department of Biochemistry

pH meter model PHM83 AUTOCAL, Radiometer, Copenhagen, Denmark.

UV-Visible Spectrophotometer model UV-240, Shimadzu, Japan.

Vortex-Genie Mixer, Scientific industries Inc., U.S.A.

Shaking water bath, Heto Lab Equipment, Denmark and gyratory water bath shaker, Brunswick Scientific Co., Inc., Edison, U.S.A.

Freeze dryer (Lyophilizer), Eyela Tokyo Rikakikai Co., Ltd.

Autoclave, Hirayama Manufacturing Cooperation, Japan.

Oven model UL-80, Memmert, Germany.

Centrifuge Type H-11 N, Kokusan Ensinki Co., Ltd., Japan.

Microwave model EMO-900T, Sanyo Co., Ltd., Thailand.

Elisa Reader, Multiskan, Ex, Labsystems.

Total nitrogen analyzer, Gerhalt, Germany.

Gel electrophoresis apparatus, Mini-protein, Hoefer mini VE.

2.3.2 Apparatus kindly provided by Rayong Bangkok Rubber Co., Ltd.

Two-roll mill LRM 200, Lab. Tech. Engineering Co.Ltd.

Mooney viscometer model SMV 201, Shimadzu, Japan.

2.4 Assay of enzyme activity

2.4.1 Assay of papain activity (FAO/WHO Standard Method, 1981)

The enzyme activity was determined by measuring the optical density of tyrosine (OD 280) that liberated from casein substrate. Determination of the proteolytic activity of papain; papain 0.1-0.5 g (wet weight) or 2 ml of papain solution was incubated with 5 ml of 1% casein substrate solution for 30 minutes in waterbath (40°C). Stopped reaction by adds 3 ml of cold 30% trichloroacetic acid (TCA) and mix by swirling immediately. Incubated in waterbath (40°C) about 30 minutes for complete coagulation of protein. Removed unhydrolyzed casein by centrifugation at 3500xg for 10 minute (or filtered the mixture through a filter paper, Whatman No. 42). The subsequent filtrate must be perfectly clear. The absorbance of free tyrosine liberated was measure at 280 nm in spectrophotometer, against its respective blank.

The blank tube was prepared by adding 3 ml of 30% TCA in 2 ml of papain solution before adding 5 ml of casein substrate.

Papain activity was reported as microgram tyrosine by comparing OD 280 with tyrosine standard curve. The tyrosine standard curve (shown in Appendix I), showing relationship between absorbance at 280 nm and concentration of tyrosine was prepared by dissolving 0.01 g tyrosine in 100 ml distilled water and then diluted to various concentrations (20-100 μg/ml).

2.4.2 Assay of Alcalase activity (Richardson and Te Whaiti's,1978)

Dissolving Alcalase in 0.05 M Tris-HCL buffer pH 9.4-10 at the concentration of 0.02 g/100 ml and 0.1 ml of this enzyme solution was diluted with 0.9 ml Tris-HCL buffer before preincubation at 55 °C in a shaking waterbath about 5 minutes. At zero time, 1.0 ml of preincubated 0.5% casein substrate solution was added into the enzyme solution and incubated for 20 minutes at 55 °C, then the reaction was stopped by adding 2 ml of 10% TCA solution and kept for another 30 minutes. Only clear solution

was removed by centrifugation at 2500xg (or filtered the mixture through a filter paper, Whatman No. 42) and measured for OD 280 in a spectrophotometer. Blank of the test was prepared by adding 2 ml of 10% TCA in the enzyme solution before adding 1 ml of casein substrate solution and incubated at the same condition as sample.

Alcalase activity was reported in microgram tyrosine by comparing OD 280 with tyrosine standard curve. (Shown in Appendix I)

2.5 Preparation of latex for deproteinization

2.5.1 Determination of dry rubber content (%DRC)

Weighing about 5 g of latex in a petridish and coagulated with 5% acetic acid in ethylalcohol. After complete coagulation, the coagulum was removed and washed with water. The coagulum was sheeted out by steel rolling pin and thoroughly washed with water and dried in an oven at 60°C to a constant weight for 10-12 hours. Dried rubber sheet was weighted. The %DRC was calculated as formulated below.

$$\%DRC = W/V \times 100$$

Where W: Weight of dry rubber

V: Weight of latex

2.5.2 Preparation of 25% DRC latex

Fresh field latex was added hydroxylamine hydrochloride (0.15 p.h.r), sodium metabisulfite (0.05 p.h.r) and Wing Stay-L (0.1% v/v). Adjust pH to 7.6 (or pH to 9.4-10 for Alcalase) with 20% ammonia solution for papain, which is the optimal pH. Then diluted with water to 25% DRC. Hydroxylamine hydrochloride, sodium metabisulfite and Wing Stay-L was added to stabilize viscosity, control color and anti-oxidation respectively.

2.6 Deproteinization of natural rubber by protease and microwave energy

2.6.1 Determination of optimum condition of deproteinized natural rubber (DPNR)

2.6.1.1 To study the effect of enzyme concentration on deproteinization

An aliquot of 100 ml, 25%DRC, reaction mixture prepared as described in 2.5.2 was heated in a microwave (model EMO-900T, Sanyo) setting at 100°C for 5 minutes (5 liters of 20%DRC reaction mixture requires 13 minutes) until the temperature of the latex was 50-55°C. Papain 5% w/v stock solution was added with stirring to the concentration of 0.1,0.2,0.3,0.4,0.5 and 0.6 p.h.r. Alcalase concentration was adjusted to 0.03,0.06,0.1,0.2 and 0.3 p.h.r. After incubation at 50°C for 5 minutes, the latex was diluted with one fold of water at room temperature. The treated latex was coagulated with steam by autoclaving 10 minutes at 121°C under pressure 15 lb/in^2. The coagulum was pressed thrcugh a two-roll mill, washed with water and dried at 60°C in an air-circulating oven. Control rubber (CDPNR) for each set of enzyme treatment was subjected to microwave energy and steam coagulation. STR5L produced in the production line of Rayong Bangkok Rubber on the same day was used as "control production". Nitrogen content was determined by Kjeldahl method according to the Rubber Research Institute of Malaysia (RRIM) Standard Method, 1970. Protein concentration was determined by Modified Lowery method. The optimal enzyme concentration was selected from the lowest nitrogen content remained in dried solid rubber.

2.6.1.2 To study the effect of dilution on deproteinization

By using the optimal enzyme concentration obtained from 2.6.1.1, the latex samples were subjected to various water dilutions (0.5,1.0,1.5,2.0,2.5 and 3.0 fold). Steam coagulation was carried out by autoclaving 10 minutes at 121°C under pressure 15 lb/in^2. The coagulum was pressed through a two-roll mill, washed with water and dried at 60°C in an air-circulating oven. Nitrogen content was determined by Kjeldahl method according to the Rubber Research Institute of Malaysia (RRIM) Standard

Method, 1970. Protein concentration was determined by Modified Lowery method. The proper dilution was selected from the maximum per cent reduction in nitrogen content.

2.6.1.3 To study the effect of incubation time on deproteinization

By using the optimal enzyme concentration obtained from 2.6.1.1, the latex samples were subjected to incubation time (0,5,10,20,30,40 and 60 minutes). Diluted with suitable amount of water obtained from 2.6.1.2 and then steam coagulation was carried out by autoclaving 10 minutes at 121°C under pressure 15 lb/in^2. The coagulum was pressed through a two-roll mill, washed with water and dried at 60°C in an air-circulating oven. Nitrogen content was determined by Kjeldahl method according to the Rubber Research Institute of Malaysia (RRIM) Standard Method, 1970. Protein concentration was determined by Modified Lowery method. The optimal incubation time was selected from the lowest nitrogen content.

2.6.2 DPNR production

Deproteinization of natural rubber by using fresh field latex. The latex was added with 0.15 p.h.r hydroxylamine hydrochloride, 0.05 p.h.r sodium metabisulfite, 0.1% (v/v) Wing Stay-L and adjust pH to 7.6 with 20% ammonia solution. Treated by optimum condition for deproteinization. After deproteinization, the treated latex was coagulated by steam under pressure 15 lb/in^2 in an autoclave at 121°C for 10 minutes. The coagulum was passed through a two-roll mill, washed and dried at 60°C in an air-circulating oven. Control rubber (CDPNR) was prepared similarly to DPNR omitting enzyme treatment. DPNR was produced at approximately 5 kg of dry rubber per day starting from 15 liters of fresh field latex.

2.7 Testing of the properties of DPNR

2.7.1 Raw rubber testing (RRIM,1970)

The raw rubber test procedures for DPNR and its control were analyzes according to RRIM specifications, which consist of the following content: dirt, ash, nitrogen content, volatile matter, initial plasticity (Po), plasticity retention index (PRI), color index and mooney viscosity. Before testing, the rubber were homogenized by passed 6 times through a two-roll mill with the gap setting of 1.65 mm between the rolls at ambient temperature. The rubber was then cut into approximate weight portions for each test as follows:

2.7.1.1 Determination of dirt content

About 30 g of homogenized rubber was taken and passed twice through a cold mill with 0.33-mm nip setting. A test portion of approximately 10 g was accurately weight, cut into small strips and placed in a 500 ml. conical flask containing 250 ml of mineral turpentine and 1 ml of peptizing agent RPA No. 3. The flask with its content was heated at 125-130 $^{\circ}$ C by infrared lamps with occasional agitation until dissolution was complete (about 3 h). The hot rubber solution was filter through a previously weighed, clean and dry 45 μm sieve. The dirt in the flask was washed twice with 30-50 ml of hot mineral turpentine each time and filtered through the sieve. The dirt on the sieve was then washed again until free of rubber solution by hot mineral turpentine. The sieve with dirt was dried in an oven 90-100°C for one hour, cooled in a desiccator and weighed to the nearest 0.1 mg. The dirt content was expressed in percent (w/w).

$$\text{Percentage of dirt (\% dirt)} = \frac{\text{Weight of dirt (g)} \times 100}{\text{Weight of rubber specimen (g)}}$$

2.7.1.2 Determination of ash content

Weigh accurately 5-10 g each test portion of the homogenized rubber. Wrap in ashless filter paper and place in a crucible, which has been previously ignited and weighed. Introduce the crucible into a muffle furnace controlled at a temperature of 550±20°C until free from carbon (2-4 hours). When ashing was complete, allow the crucible to cool in a desiccator and then weigh it to the nearest 0.1 mg. The ash content was calculated as follows:

$$\text{Ash content (\%wt)} = \frac{\text{Weight of ash (g)} \times 100}{\text{Weight of test portion (g)}}$$

2.7.1.3 Determination of nitrogen content

Weigh accurately about 0.1-0.2 g of rubber portion into a micro Kjeldahl tube and 0.65 g of catalyst mixture (K_2SO_4 : $Cu_2SO_4.5H_2O$: SeO ; 30:4:1) and 2.5 ml of concentrated sulfuric acid were added. The mixture was boiled gently in the digestion unit until the solution becomes clear green or colorless with no yellow tint. Cool the digest and transfer to distillation unit follower by three washing with distilled water, which is the mixture of methyl red and bromocresol green into the receiving conical flask. Add about 10 ml of 67% sodium hydroxide solution to the distillation vessel, and pass steam through the distillation apparatus until the volume of distillate in the receiving flask reach 150 ml which take about 5 minutes. Immediately titrate the distillate with standardized 0.01 N H_2SO_4. Blank can be prepared by adding all the reagents but omitting the sample. Calculate the total nitrogen content as follows:

$$\text{\% Total nitrogen} = \frac{(V1 - V2) \times M \times 1.4}{W}$$

Where V1 = Volume of blank (ml)

V2 = Volume of titrant (ml)

M = concentration of H_2SO_4 (N)

W = weight of sample (g)

2.7.1.4 Determination of volatile matter

The homogenized rubber was weight approximately 11-12 g to the nearest 0.1 mg, passed through the cold mill roll with nip setting at 0.5 mm, then placed on aluminum tray and heated in an oven at 100±3°C for 4 hours. After heating, each test portion was kept in a polyethylene bag and hung on the rack to cool down for half an hour at 25°C then each of rubber sample was removed from the bag and weighed to the nearest 0.1 mg. The volatile matter was calculated as follows:

$$\text{Volatile matter (\%)} = \frac{A - B \times 100}{A}$$

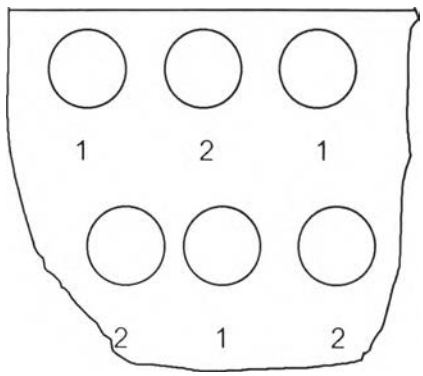
Where A: Weight of test portion before drying (g)

B: Weight of test portion after drying (g)

2.7.1.5 Determination of initial plasticity (Po) and plasticity retention index (PRI)

The plasticity retention index (PRI) test has been developed as a simple rapid method for measuring the resistance of raw rubber to oxidative breakdown on heating. The test involved measurement of the rapid plasticity of the rubber test pieces before and after a short period of heating in an air oven. The rapid plasticity is measured with the Wallace rapid plastimeter. A high value of PRI denotes high resistance to oxidative breakdown. A test portion of 20±5 g of the homogenized piece was passed twice through the rolls of a cool mill (doubling the sheet between passes) with nip

setting adjusted such that the final sheet thickness was 1.6-1.8 mm. The sheet that should be free from holds was immediately doubled and the two halves pressed lightly together by hand. Six test pellets were cut from the doubled sheet with the Wallace punch as illustrated below.



Punched pellets for

1) initial plasticity determination

2) aged plasticity determination

The test piece should be a disc of rubber of thickness between 3.2-3.6 mm and approximately 13 mm in diameter. The test pieces were divided into two sets each for plasticity determination before and after oven ageing. For ageing, the test pieces were heated in oven for 30 minutes at 140^{o}C. Then they were removed and allowed to cool to room temperature. The pellet piece was sandwiched between two pieces of thickness of 1 mm with constant compressive force of 10-0.1 kg_f for 15 seconds. The thickness at the end of this period was measured by Wallace rapid plastimeter. The median of the three unaged and the three aged test pieces were used to calculate the PRI as follow:

$$PRI = \frac{\text{Aged median plasticity value}}{\text{Unaged median plasticity value}} \times 100$$

2.7.1.6 Determination of color

The color of the raw rubber was compared and matched as closely as possible with that of standard colored glasses. The numerically higher index values have deeper color. A test portion of 20±5 g was taken from homogenized piece and prepared similarly to PRI test. The two test pieces were laminated together and pressed in the mould between two sheets of polyester or cellulose film using mould covers at not less than 3.5 MN/m^2 pressure on the cavity areas of the mould for 5 minutes at 150±3° C. The color of the test pieces was determined by matching as closely as possible to the appropriate color standard over a lighting box. The color was shown as index number of color glass.

2.7.1.7 Determination of Mooney viscosity (ASTM D1646,1988)

The viscosity of raw rubber was determined in a Mooney viscometer SMR 201 according to ASTM (D1646, 1988) standard procedures. Before loading the rubber, dies cavity and rotor should be heated up to the test temperature, 100°C. About 25 g of the homogenized rubber was cut into two portions. One portion was placed in the lower die cavity and the rotor was placed followed by another portion on the top of the rotor and the die was closed immediately. Preheat the specimen for 1 minute before starting the motor and set the running time with the motor on for 4 minutes. The viscosity was reported as Mooney unit, ML (1+4) at 100°C, the rotor size (L for large), the number of minutes for warming up in the machine (1 minute), the number of minutes of actual test (4 minute) and temperature (100°C).

2.8 Characterization and quantitation of protein in latex and raw rubber

2.8.1 Preparation of latex proteins

2.8.1.1 Extraction of water extractable protein in solid rubber

Each solid rubber specimen (STR5L, DPNR and CDPNR) or gloves was cut into small pieces, weighed about 1 g and transferred to a 200 ml flask. Distilled water was added: water: solid rubber = 10:1. The flask was sealed with parafilm; extracted at 37°C and shaked for 15 seconds after adding the water and shake again at 60 and 120 minutes. The extracted solution was filtered through filter paper (Whatman No.1), and centrifuged the filtrated solution at 2000xg for 5 minutes before lyophilization.

2.8.1.2 Lyophilization

The filtrated-solution was divided into 1-2 ml aliquots in plastic tubes, frozen at -80 °C, then lyophilized for 48 hours. Random sampling of lyophilized protein was resuspended with distilled water and assayed for water extractable protein (2.8.2).

2.8.2 Determination of water extractable protein by modified Lowry method

Solution for modified Lowry method

Solution A	:	Alkali copper sulfate (10 parts of C: 0.2 part of D)
Solution B	:	Dilute Folin reagent
Solution C	:	6% w/v of sodium carbonate
Solution D	:	1.5% w/v of copper sulfate in 3% w/v of sodium citrate
Solution DA	:	Alkali solution (10 parts of DC: 0.2 part of DD)
Solution DC	:	6% w/v of sodium carbonate
Solution DD	:	3% w/v of sodium citrate

2.8.2.1 Determination of water extractable protein by modified Lowry in the presence of $CuSO_4$

The lyophilized protein (2.8.1.2) was resuspended in 1.0 ml of distilled water. Each sample was assayed for total protein by modified Lowry method in a 96-well microtiter-plate. In each well solution A 200 μl was added, followed by 50 μl of protein solution, mixed and allowed for 15 min at room temperature. Then 50 μl of solution B was added. Protein concentration was determined by comparing OD 750 with known standard protein concentration, ovalbumin 0-50 μg, using a microplate reader (Multiskan Ex, Labstytem). Blanks are water.

2.8.2.2 *Determination of water extractable protein by modified Lowry in the absent of $CuSO_4$*

In order to correct the effect of $CuSO_4$, some of water extractable protein was also determined by using solution A in stead of DA (2.8.2).

Protein contents calculated from OD 750 with $CuSO_4$ substracted from OD 750 without $CuSO_4$ that evaluated from standard ovalbumin.

2.8.3 Characterization of water extractable protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Slater et al., with a slightly modification. Fifteen percent acrylamide-bis gel was used as the separating gel and 3% gel was used as the stacking gel. Tris glycine (25 mM Tris, 192 mM glycine) buffer pH 8.3 containing 0.1% w/v SDS was used as electrode buffer. Sample to be analyzed, was dissolved in Tris buffer, containing 60 mM Tris, 2% w/v SDS, 25% v/v glycerol, 14.4 mM 2-mercaptoethanol and 0.1% w/v bromophenol blue, and boiled for 5 minutes prior to application to gel. The electrophoresis was carried out at constant current of 15 mA, on a Mini-Protein (Hoefer mini VE) from cathode toward anode. When the electrophoresis was completed, the gel

was stained with 0.01% Coomassie blue R-250 in the mixture of 1:4.5 acetic acid / methanol.

2.8.4 Human serum samples

2.8.4.1 Control serum

A serum sample 10 ml was collected from a volunteer healthy person visiting at the Ramathibodi Hospital and Veterans Hospital for general check-up. Based on an interview these healthy persons have no symptom of latex allergy. No further demographic or medical information is available for the control's sera.

2.8.4.2 Serum from general allergic patients

The serum was collected from general at the Ramathibodi Hospital, Veterans Hospital and Faculty of Dentistry, Mahidol University. Based on an interview: "Do you have any allergic disease, allergic symptoms or family history of an allergic disease but not specify only rubber allergy?". If the patient answer "Yes", he or she will be requested to fill a questionnaire for further study on the prevalence of latex allergy and blood sample was collected on voluntary basis.

2.8.4.3 Serum from general allergic healthcare workers

The serum was collected from the population of healthcare workers at the Ramathibodi Hospital, Veterans Hospital and Faculty of Dentistry, Mahidol University, who has an allergic disease, allergic symptom or family history of an allergic disease but not specify only rubber allergy. Those who are willing to answer the questionnaire and donate blood sample were classified as general allergic healthcare worker.

2.8.5 Specific anti-latex IgE antibody detection

2.8.5.1 Enzyme allergosorbent test (EAST)

The latex enzyme allergosorbent test (EAST) with latex protein was the same as described by Czuppon et al., 1993 and Harncharoen, 1996 with some modification. The details are as follow:

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

A polystyrene 96 wells microtitre plate was filled up with 10 μg/well of latex protein solution (from 2.8.1.2) and incubated at 4 °C, in a moist chamber, overnight. The protein solutions were discard and washed three times with 500 μl 0.9% NaCl solution containing 0.1% Tween 20 (NSS / Tween20). Each well was blocked the unoccupied sited 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 of 100 μl were dispensed into each well, then incubated at room temparature for 180 minutes.

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

Goat anti-human IgE conjugated to alkaline phosphatase in blocking buffer (1:500, anti-IgE; blocking 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 microliter of 0.1% p-nitrophenyl phosphate in 10 mM diethanolamine and 0.5 mM $MgCl_2$, pH 9.5 was added and incubated at 37 °C for 30 minutes. An ELISA microplate reader measured absorbance reading at 405 nm.

2.8.6 Latex allergen detection by skin prick test (SPT)

The Human Rights and Ethics Committee of the Division of Dermatology Department of Medicine, Faculty of Medicine, Chulalongkorn Hospital approved the skin prick test (SPT) for this research. Assoc. Prof. Dr.Porntip Huiprasert made this study available. An epicutaneous method on forearms, using extracts made from three different brands of powdered latex gloves, STR5L, DPNR and CDPNR was performed with a patient showing Type I hypersensitivity. Each rubber specimen was weighed, cut into small pieces (1-2 cm^2) and then extracted in physiologic normal saline solution (NSS) at a 1:5 weight by volume for 15 minutes, at room temperature. The extracts, along with histamine phosphate; positive control and NSS; negative control were used for skin prick testing. The positive result was defined as that with a wheal equal to or greater than that of histamine phosphate used as positive control.