CHAPTER II

MATERIALS AND METHODS

2.1 Biological materials

2.1.1 Natural rubber latex

Fresh field latex from RRIM 600 clone was obtained from Pan Asia Biotechnology Co., Ltd. , was preserved with 0.3% (v/v) ammonia.

2.1.2 Alcalase

Alcalase (Purafect, 4000 E) was purchased from Lion Co. Ltd.

2.2 Chemicals

Most chemicals used in this investigation were analytical or reagent grade.

Acetone was purchased from Scharlua.

Acrylamide and N, N'- methylene- bis- acrylamide were products of Sigma.

Ammonium persulfate was product of BIO- RAD.

Copper sulfate was purchased from Fluka.

Normal-butyl acrylate was product of VIV Interchem Co., Ltd.

Selenium powder, Folin reagent, boric acid, potassium hydroxide, sodium hydroxide, methanol, *di*- sodium hydrogen phosphate anhydrous GR, Triton X- 100 were products of Merck.

Sulfuric acid and acetic acid were purchased from J.T. Baker.

Potassium sulfate anhydrous was purchased from May and Baker.

Sodium alginate and Brilliant Blue R 250 were purchased from Sigma.

Sodium chloride was a product from UNIVAR.

Normal saline solution was kindly provided from the Division of Dermatology, Department of Medicine, Chulalongkorn University.

Sodium dodecyl benzene sulfonate (SDBS) was industrial grade purchased from Lion Co. Ltd.

2.3 Apparatus

2.3.1 Apparatus in the Department of Biochemistry

pH meter model PHM-83 autocal, Radiometer, Denmark

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

Centrifuge, refrigerated centrifuge model J- 21 C, Beckman Instrument Inc, USA.

Centrifuge, microcentrifuge high speed model MC- 15 A, Tomy Seiko Co. Ltd., Japan

Incubator shaker model G 76D, New Brunswick Scientific Co., Inc. Edison, N. J. USA.

Magnetic stirrer and heater model IKAMA® GRH, Jane & Kunkel Gmbh & Co. KG, Japan

UV- Visible spectrophotometer model UV- 240, Shimadzu, Japan

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

2.3.2 Apparatus at National Research Council of Thailand

Elisa Reader, Multiskan Ex 200- 240 v, Labsystems

Total nitrogen analyzer, Gerhardt, Germany

SDS- PAGE, Hoefer mini VE set from Amersham Pharmacia, Sweden

Freeze dryer (Lyophilizer), Labconco Lyph 1 L, Labconco Corporation, USA.

2.3.3 Apparatus at Ban Pan Reasearch Co. Ltd.,

Centrifuge, (Alfa Laval, 7000xg) and 200- liter latex storage tank

2.3.4 Apparatus at Department of Nuclear Technology, Faculty of Engineering, Chulalongkorn University

Latex irradiator BSV-60, Institute of Isotope, Hungary.

2.3.5 Apparatus at Banpan research Bangkok Rubber Co.,

Micrometer model SM-114, Texlock Co., Japan

2.3.6 Apparatus at Prosthodontics Department, Faculty of Dentistry, Chulalongkorn University

Durometer (Shore A) model 471, Zwick, Germany

2.4 Assay of Alcalase specific activity

The enzyme activity was determined by measuring the absorbance of tyrosine (A280) liberated from casein substrate. This procedure was modified from Richardson and Te Whaiti's method (1978). By dissolving Alcalase in 0.05 M Tris- HCl buffer pH 7.6 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 before preincubation at 45 °C in a shaking water bath. At zero time, 1 ml of preincubated 0.5% casein substrate solution was added into the enzyme solution and incubated for exactly 20 minutes at 45 °C, then the reaction was stopped by adding 2 ml of 10 % trichloroacetic acid (TCA) solution and kept for another 30 minutes. Only clear solution was removed by centrifugation at 2500x g and measured for the optical density (OD) at 280 nm wavelength in a

spectrophotometer. Blank of test was prepared by adding 2 ml of 10 % TCA in enzyme solution before adding 1 ml of casein substrate solution and incubated at the same condition as sample. Tyrosine standard curve, 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 (20-160 μg/ ml). Alcalase specific activity was reported in casein digestion unit (CDU) according to the following calculation:

$$\text{Alcalase specific activity (CDU/ mg)} = \frac{\text{net } OD_{280} \times 4 \text{ (ml)}}{\text{Slope} \times w \text{ (mg)} \times 20 \text{ (min)}}$$

In which net OD_{280} is the difference between OD_{280} of sample and blank tube; 4 is the total volume, in ml, of the final incubation; slope is the slope of tyrosine standard curve; w is the weight in mg of original enzyme preparation in 0.1 ml aliquot of test solution added to the incubation mixture and 20 is the incubation time in minute. By definition 1 casein digestion unit (CDU) was defined as 1 μg of tyrosine liberated from casein digestion by enzyme 1 mg in one minute under the condition of the assay.

2.5 Deproteinization of natural rubber latex

Treatment of latex by Alcalase was performed with field latex which anti-coagulated and preserved with 0.3 % (v/ v) NH_3 . The ammoniated latex was determined total solid content (TSC), dry rubber content (DRC), volatile fatty acid (VFA), NH_3 and Mg content and then added diammoniumphosphate (DAP) to reduce Mg content to 50 ppm, maximum. The DAP- ammoniated latex was deproteinized

with Alacalase solution that contained 0.08% Alcalase, 0.4 % NH_3, 0.25% (w/ v) sodium dodecyl benzene sulfonate (SDBS) and 0.025 % (v/ v) and Triton X100 then left at room temperature for 1.30 hours. After that, added 0.015 phr sodium alginate and 0.20 phr KOH. The latex was adjusted to 25% DRC before centrifugation. After 2.5 hours incubation the latex was centrifuged at 7,000 x g and then adjusted to 60 % DRC and 0.6 % NH_3 as high ammonia deproteinized concentrated latex. The reaction size started at 800 ml of DAP- ammoniated latex and then scaled up to 200- 400 L of DAP- ammoniated latex.

2.6 Preparation of polysaccharide addition concentrated latex

Fresh field latex was anti-coagulated and preserved with only 0.4 % (v/ v) NH_3. The ammoniated latex was determined total solid content (TSC), dry rubber content (DRC), volatile fatty acid (VFA), NH_3 and Mg content and then added diammoniumphosphate (DAP) to reduce Mg content to 50 ppm, maximum. The DAP-ammoniated latex was added add 0.015 phr sodium alginate and 20% potassium hydroxide. The latex was adjusted to 25% DRC. After 3 hours incubation, the latex was centrifuged at 7,000 x g and then adjusted to 60 % DRC and 0.6 % NH_3 as high ammonia concentrated latex. The reaction size started at 200- 400 L of DAP-ammoniated latex.

2.7 Preparation of radiation vulcanized natural rubber latex

The fresh field latex, DPNRL and control concentrated latex was added n-butyl acrylate 5 phr and slow stirring continued for about 2 hours. Sample was irradiated at dose 10 kGy (dose rate 0.5 kGy/hour). After irradiation antioxidant that is Irganox 1520D was added 0.02 phr and stirred very gently.

2.8 Testing of concentrated latex specification (ISO- 2004- 1979 (E))

2.8.1 Determination of total solid content (TSC)

Weighed the empty petridish and record the empty pretidish weight. An aliquot of 5 g of latex was pipetted into a petridish and dried in an oven at 60 °C for 10-12 hours. Dried rubber in pretidish was weighed and calculated TSC content by the equation below.

$$\% \text{ TSC} = W_1 / W_0 \times 100$$

Where W_1 = weight of the dry rubber in pretidish (g)

W_0 = weight of the latex after subtraction the weight of pretidish (g)

2.8.2 Determination of dry rubber content (DRC)

An aliquot of 5 ml of latex was pipetted into a petridish and coagulated with 5% acetic acid in ethyl alcohol. After complete coagulation occurred, the coagulum was then removed, washed with water, creped and dried in an oven at 60 °C for 10-12 hours. Dried coagulum was weight and calculated DRC content by the equation below.

$$\% \text{ DRC} = W_1 / W_0 \times 100$$

Where W_1 = weight of the dry rubber (g)

W_0 = weight of the latex taken (g)

2.8.3 Determination of non rubber content (NR)

Non rubber content was calculated by the equation below.

TSC- DRC = (%) NR

2.8.4 Determination of volatile fatty acid (VFA)

Weighed 50 g of latex in a stainless beaker and added 50 ml of 30% $(NH_4)_2SO_4$. Heat on water bath at the temperature of 100 °C and squeeze for the serum. Filtrated the serum through Whatman number 1 filter paper. Pipetted 25 ml of serum into a flask containing 5 ml of 50% H_2SO_4. Passed steam through the steam jacketed distillation apparatus for at least 15 minutes with steam outlet open, introduce into the innertube 10 ml of acidified serum by pipette. Place a 100 ml graduated cylinder under the tip of condenser to obtain 100 ml of distilled serum. Degassed for 3 minutes and titrated with standard 0.01 N $Ba(OH)_2$ using phenolphthalene as indicator. The % VFA was calculated by the equation below.

$$\text{Volatile fatty acid, VFA(\%)} = \frac{[67.32 \times N \times V]}{m \times TSC} \times 50 + \frac{m(100 - DRC)}{100 \times P}$$

Where N: Normal of $Ba(OH)_2$ m: weight of latex (g)

V: Volume (ml) of $Ba(OH)_2$ used in titration TSC: % TSC of latex

P: Serum density = 1.02 megagram/ m^3 DRC: % DRC of latex

2.8.5 Determination of alkalinity (NH_3)

Weighed 10 g of latex into the beaker diluted with 200 ml distilled water and added non- ionic stabilizer such as Teric 16A. Titrated with standard 0.1 N $(NH_4)_2SO_4$ with methyl red indicated. Calculated in g NH_3 per 100 g latex.

2.8.6 Determination of Mg content (%Mg)

Weighed 10 g of latex into the beaker, added 10 ml of distilled water and 5 ml of 25% (v/v) acetic acid. Squeezed for the serum and poured on to the 80 mesh filter. Pipetted filtrated 10 ml serum into 50 ml beaker adjusted pH to 10.5 by NH4Cl or NH4OH then pipetted 4 ml of 4% KCN into the serum. Added 0.1 g of Erichrome Black T into the serum and the serum would turn violet. Titrated with 0.05 M EDTA. End point was the violet color turned blue.

Calculation: the Mg content was expressed in percent (w/w)

$$\text{Percentage Mg} = \frac{24.32 \times B \times D \times 10{,}000}{1{,}000 \quad 10 \quad C}$$

$$\text{Where B: EDTA factor} = \frac{\text{burette reading} \times (M)\ \text{EDTA}}{(v) \qquad (M)}$$

C: Value of solid in 10 g of latex (g) $C = \frac{A \times TSC}{100}$; A: Weight of latex (g)

D: Total volume of serum in sample (ml); D = (A- C) + 15

(15, 10 ml of water added + 5 ml of 25% acetic acid)

24.32 = Mw of Mg

2.8.7 Determination of mechanical stability time (MST; second)

Adjusted % DRC of concentrated latex to 55 % TSC with NH_3. Weighed 80 g of latex, warmed to 35 °C and spun with Klaxon machine at 14,000 rpm. Determined clotting time of rubber particles by dipping stirring rod into latex and dropped in water.

2.8.8 Determination of potassium hydroxide (KOH)

Added formaldehyde solution into 50 % TSC latex and diluted to 30 % TSC with water and titrated with standard KOH indicated end- point by pH meter. Plotted graph of 1) pH or 2) dpH/ dV or 3) d^2pH/ dv^2. V is volume of KOH at end- point.

2.8.9 Determination of nitrogen content (RRIM, 1992)

This specification is the rubber specification added to this research because of the requirement from glove manufacturers. The concentrated latex sample was dried as described on Methods 2.4.2. Rubber specimen was weighed accurately about 0.1-0.2 g into a micro Kjeldahl tube and 0.65 g of catalyst mixture (K_2SO_4 : Cu_2SO_4 .5HO : 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 followed by three washing with distilled water, then add indicators 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 0.01N H_2SO_4. Blank can be prepared by adding all the reagents but omitting the sample.

Calculation: Total nitrogen content was calculated 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)

2.9 Preparation of latex proteins

2.9.1 Extraction of water extractable proteins in solid rubber (ASTM D 5712-99)

The concentrated latex was poured onto the 20x 20 cm square plate then air-dried at room temperature for 8-12 hours. A piece of latex film was cut from each sample with 1x 1 cm square size, 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; extraction was at 37 °C and shook for 15 seconds after adding the water and again at 60 and 120 minutes. The extracted solution was filtered through filter paper (Whatman no.1) followed by centrifugation at 2,000 x g for 5 minutes. The filtrated-solution was lyophilized (Method 2.7.2).

The total protein concentrations in these samples were determined by modified Lowry method.

2.9.2 Lyophilization

The filtrated-solution was aliquated in plastic tube, 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 (Method 2.9).

2.9.3 Extraction of water extractable proteins in latex films (ISO/DIS 12243)

The concentrated latex was poured onto the 20x 20 cm square plate then air-dried at room temperature for 8-12 hours. A piece of latex film was cut from each sample with 1x 1 cm square size, weighed and transferred to a 200 ml flask and added 10 ml of phosphate buffered saline (PBS) per gram of specimen. The flask was sealed with sealing film; extraction was at 25 °C and shook for 15 seconds after adding the water and again at 120 minutes. After that decant off the extract and remove any particulate matter, by cenrifuging at 5,000 rpm for 15 minutes.The extract was precipitated and concentrated by method 2.8.

2.9.4 Latex protein extracts for skin prick test

The samples were weighted, cut into small strips (1 x 1 cm square) 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 result was defined as that with a wheal equal to or greater than that of a positive control.

2.10 Precipitation and concentration of protein (ISO DIS 12243)

Accurately transfer 4 ml each, of extractant (method 2.7.3), as a blank and the protein extracts to 10 ml polypropylene tubes. Add 0.4 ml of Deoxycholate (DOC),

mix and allow the stand for 10 minutes than add 0.4 ml of Trichoroacetic acid (TCA) and mix. Add 0.4 ml Phosphotungstric acid (PTA), mix and allow to stand for a further 30 min. After that centrifuge at 4500 rpm for 45 minutes. Decant the supernatant liquid and drain by inverting each centrifuge tube on an absorbent paper towel. Add 0.8 ml of 0.2 M sodium hydroxide solution to each tube, including the blank, to redissolve the precipitated protein.

2.11 Determination of water extractable proteins by modified Lowry method. (ASTM D 5712- 99)

The lyophilized protein samples (2.7.2) were resuspended with distilled water. The reaction was carried out with 160- 200 µl of 0.1 N sodium hydroxide and 2.5- 40 µl of protein solutions, then added 75 µl of alkaline copper sulfate, Reagent D, into each well of a flat bottom, 96-well, polystyrene microtiter plate, mixed and allowed to stand for 15 minutes at room temperature. The reaction was then added with 25 µl of dilute Folin solution, Reagent B, mixed thoroughly and allowed for 15 minutes at room temperature. Protein levels were evaluated against standard protein, ovalbumin, using a microplate reader (Multiskan Ex, Labstytems) at 750 nm wavelength.

2.12 Determination of water extractable protein by modified Lowry method (ISO DIS 12243)

0.8 ml of the redissolved protein solutions including the blank, add 0.3 ml alkaline copper sulfate, Reagent A, mix well. Add 0.1 ml of dilute Folin solution, Reagent B,mix and allow to stand at least 15 minutes and no longer than 1 hour before measuring the absorbance. Transfer the solutions to cuvettes and measure the absorbance at 750 nm.

2.13 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Slater et al. (1990), with a slight modification. Polyaclyamide gel at 15% was used as separating gel and 3% of the gel was used as stacking gel. Tris glycine (25 mM Tris, 192 mM glycine) buffer pH 8.3 containing 0.25% 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 the 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 Coomassie blue R-250.

2.14 Testing of the rubber vulcanizates (ASTM, D412, 1989)

2.14.1 Determination of tensile strength,300% modulus and Elogation at break (ASTM D412,1987)

The 5 dumbbell test pieces(figure 2.1) were cut out from the rubber vulcanized punching with a die using a single stroke of press. A reference of along the reference length by micrometer dial gauge.

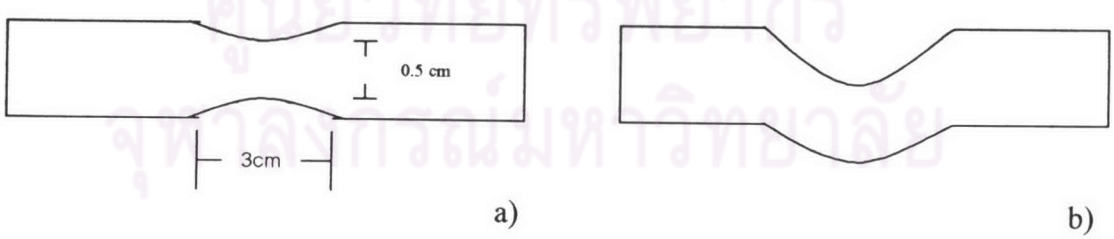

Figure 2.1 shape of dumbbell

a) For tensile strength ,% elongation at break and 300 % modulus test

b) For strength test

The two ends of the test piece were clamped into the two grips of the testing machine. The test piece was stretched at a constant rate of moving grips of 500 ± 50 mm the force required to stretch the sample to 300% of reference mark length and to breakage were recorded and calculated as follows:

$$\text{300\% modulus} = \frac{\text{Force at 25 cm. (kg)}}{\text{Cross-sectional area (cm}^2\text{)}}$$

$$\text{Tensile strength} = \frac{\text{Force at break (kg)}}{\text{Cross-sectional area (cm}^2\text{)}}$$

$$\text{\%Elongation at break} = \text{length of reference mark at break}$$

3.14.2 Tear strenght test (ASTM D624,2989)

Five test piece for tear resistant were cut out from vulcanized rubber by punching with die using a single stroke of press. The thickness of the test piece was measured by micrometer dial gauge. The highest force required to tear the test piece was recorded and calculated as follows:

$$\text{Tear strength} = \frac{\text{Highest force (kg)}}{\text{Thickness of test piece(cm)}}$$

3.14.3 Hardress test (ASTM D1415,1988)

The international hardness test is based on measurement of the penetration of a rigid ball into the rubber specimen under specified condition. Rubber vulcanized was prepared as flat and smooth sheet having thickens sufficient to fit the gap of type A

durometer. The plunger of durometer was pressed with the minor force on to the specimen; the scale was pointed and read as the hardness in shore A at room temperature. The median value of 5 different points distributed over the specimen was record.

2.15 Allergen detection by Skin Prick test (SPT)

Skin testing was performed by use of an epicutaneous method on forearms, using extracts made from control concentrated latex film, deproteinized concentrated latex film and saponified natural rubber (SAP- NR) prepared from ammoniated crumb. These samples were weighted, cut into small strips (1 x 1 cm^2) and then extracted in physiologic normal saline solution (NSS) at a 1:5 weight by volume for 15 minutes, at room temperature. There were 2 latex allergen solutions used in this research, the commercial latex allergen (Stallergenes, France) and the latex serum proteins. The latex serum proteins were prepared by method 2.7.3. The extracts, along with histamine phosphate; positive control and NSS; negative control were used for skin prick testing. The result was defined as that with a wheal equal to or greater than that of a positive control.