CHAPTER II
MATERIALS AND METHODS

2.1 Biological materials

2.1.1 Rubber latex

Three types of rubber latex were used in this research;

2.1.1.1 Fresh field latex from RRIM 600 clone was obtained from Rayong Bangkok Rubber Co., Ltd. Ammonia was sometimes used as preservative and anti-coagulant.

2.1.1.2 Ammoniated latex (AL) was preserved and stabilized with 0.3% ammonia and 0.025% w/v TMTD/ZnO.

2.1.1.3 Skim latex was the serum fraction after centrifugation of ammoniated latex containing about 4-6 % dry rubber content (DRC), kindly provided by Rayong Bangkok Rubber Co., Ltd.

2.1.2 Chemicals

Most chemicals used in the present investigation were of analytical or reagent grade. Phenol reagent was prepared in the Department of Biochemistry's laboratory by the method of Ciocalteau (1927).

Hydroxylamine hydrochloride, copper sulfate were purchased from Fluka.

Selenium powder, potassium hydroxide, sodium hydroxide, and methanol were products of Merck.

Sulfuric acid, acetic acid purchased from J.T. Baker.

Phosphoric acid, sodium metabisulfite were products from Mallinckrodt.

Potassium sulfate anhydrous was from May and Baker.

All of chemicals used in compound rubber formulation namely Hisil 233s(precipitated silica), Zinc oxide active (ZnO), 2,2 methylene bis-4-methy-6-p-butyl phenol (22 cp 46), wax, stearic acid, diethylene glycol, Shellflex, sulfur, MBTS (2-2 dibenzothiazyl disulfide), and tetramethy thiuram monosulfide (TMTM) were kindly provided by Banpan Research Laboratory Co., Ltd.

2.2 Apparatus

2.2.1 Apparatus in the Department of Biochemistry

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

pH meter model PHM-83 autocal, Radiometer, Denmark.

Gel Permeation Chromatography (GPC); model 510 Waters, Millipore.

2.2.2 Apparatus funded by National Research Council of Thailand

Elisa Reader, Multiskan, Ex, Labsystems

Total nitrogen analyzer, Gerhalt, Germany

2.2.3 Apparatus kindly provided by Banpan research Bangkok Rubber Co.,

Mooney viscometer model SMV 201, Shimadzu, Japan

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

Hydraulic press model TEE 120, Dahtyan Hydraulic Machine Industrial Co., Ltd., Taiwan

Micrometer model SM-114, Texlock Co., Japan

Durometer (Shore A) model 7206, Zwick, Germany

Instron Testing Machine model 1101 (U.S.A), Rheometer model 100S, Monsanto, U.K.

2.2.4 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

2.3 Method for preparation of latex for deproteinization

2.3.1 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 oven at 60 °C for 10-12 hours. Dried coagulum was weighted and calculated for DRC content by the equation below.

$$\% \text{ DRC} = W/V \times 100$$

Where W = weight of the dry rubber (g)

V = weight of the latex taken (g)

2.3.2 Preparation of 30%DRC latex

Fresh field latex was diluted to 30 %DRC with water before adding hydorxylamime hydrochloride to 0.15 parts per hundred of rubber (p.h.r), sodium metabisulfite to 0.05 p.h.r. and 0.1% v/v of Wing Stay-L were added as viscosity stabilize, color control and anti-oxidation respectively.

2.3.3 Preparation of solid crumb

2.3.3.1 Preparation of solid crumb (AL-crumb)from ammoniated latex

Ammoniated latex was diluted to 15 %DRC with water before adding hydroxylamine hydrochloride to 0.15 p.h.r of rubber, and sodium metabisulfite to 0.05 p.h.r. and Wing Stay-L to 0.1% v/v, then coagulated with 2% formic acid. After the completion of coagulation after 12-15 hrs, the coagulum was then collected, washed with water, pressed through a shredder and used as ammoniated solid crumb (AL-crumb) for saponification.

2.3.3.2 Preparation of solid crumb latex (SK-crumb) from skim latex

Skim latex was incubated at 60 °C with stirring to remove ammonia before adding hydroxylamine hydrochloride to 0.15 p.h.r, sodium metabisulfite to 0.05 p.h.r. and Wing Stay-L to 0.1 %v/v. This skim latex was coagulated by adding concentrated sulfuric acid. The coagulum was washed with water and pressed.

2.4 Saponification of latex

Treatment of latex by saponification was performed at various concentrations of potassium hydroxide at fixed isopropanol concentration of 5 %v/v at 70 °C for 3 hrs. The saponified latex was diluted with one-volume of water and coagulated with 2% formic acid. 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 institute Research of Malaysia (RRIM) standard method, 1970.

To investigate the effect of isopropanol concentration on deproteinization, field latex was prepared in the optimal potassium hydroxide concentration and then treated with isopropanol at various concentrations (0, 1, 2, 5 %v/v). After the

reaction the latex was coagulated and determined for % nitrogen as described previously.

By varying temperature from 40 °C to 80 °C at the optimal potassium hydroxide and optimal isopropanol concentrations. The proper temperature was selected from the maximum percent reduction in nitrogen content.

After saponification, latex was diluted with water at different ratios (1:0, 1:1.5, 1:2, 1:3 and 1: 6 %v/v) and then coagulated with 2% formic acid. The optimal dilution was selected from the lowest nitrogen content.

2.5 Saponification of solid crumb

By using the optimal conditions for the saponification of latex investigated solid crumbs, AL-crumb and SK-crumb, under similar optimal conditions observed for latex. The alkali solution was reused several cycles until the total nitrogen content could not be significantly reduced.

2.6 Production of saponified natural rubber (SAP-NR)

SAP-NR was produced at approximate 1 kg of dry rubber from fresh field latex according to the selected optimal conditions. Control dried rubber was prepared by formic coagulation without any treatment.

2.6.2 Saponification of solid rubber crumb

Saponification of solid rubber crumb by using AL-crumb and SK-crumb was performed at the optimal conditions for the saponifcation of latex. Raw rubber properties were determined according to the testing methods of ASTM.

2.7 Raw rubber testing (RRIM, 1970)

Raw rubber properties of SAP-NR and its control were analyzed according to RRIM specifications, which consist of the following content: dirt, ash, volatile matter, nitrogen content, plasticity (P_0) and plasticity retention index (PRI), color index and Mooney viscosity. For every test, the rubber was 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 Determination of ash content

Weigh accurately 5-10 g test portion of the homogenized rubber. Wrap in ashless filter paper and place in a crucible, which was previously ignited and weighed. Introduce the crucible into a muffle furnace controlled at a temperature of 550±20 °C until free from carbon. When ashing was complete, allow the crucible to cool in a desiccator and then weigh it to the nearest 0.1mg.

Calculation: the ash content was calculated as follows:

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

2.7.2 Determination of volatile matter

The test portion was cut approximately 11-12 g from the homogenized rubber, and then weighed to the nearest 0.1 mg, passed through the cold mill, 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 weighed to the nearest 0.1 mg.

Calculation: 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)

2.7.3 Determination of dirt content

About 30 g of homogenized rubber was 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 °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 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, dried in an oven 90-100 °C for one hour, cooled in a desiccator and weighed to the nearest 0.1\mg.

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

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

2.7.4 Determination of nitrogen content

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 : $Cu_2SO_4 .5H_2O$: 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 0.01N 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)

2.7.5 Determination of color index

The test piece of homogenized rubber was prepared by weighing 20 ±5 g of homogenized pieces then passed twice a two-roll mill with nip setting to the final sheet thickness 1.6-1.8 mm, then immediately doubled and pressed tightly

together by hand. Two disc-shaped pellets were put together and pressed in the mold between two sheets of polyester or cellulose film using mold covers at not less than 3.5 MN/m^2 pressure on the test piece was determined as Lovibond index by matching as closely as possible to the approximate color standard over lighting box. The result was shown as index number of color glass.

2.7.6 Determination of plasticity retention index (PRI)

The plasticity retention index (PRI) test has been developed as a simple and rapid method for measuring the resistance of raw rubber to oxidative breakdown upon heating at high temperature in short period of time. The test piece of homogenized rubber was prepared by the same procedure as color test. Six test pellets were cut from the doubled sheet with the Wallace punch as illustrated below, and divided into two sets. One set of test pellet was for initial plasticity determination and another for aged plasticity after aging in an oven at 140 °C for 30 minutes. The test pellet was removed from the oven and allowed to cool at room temperature before measurement.

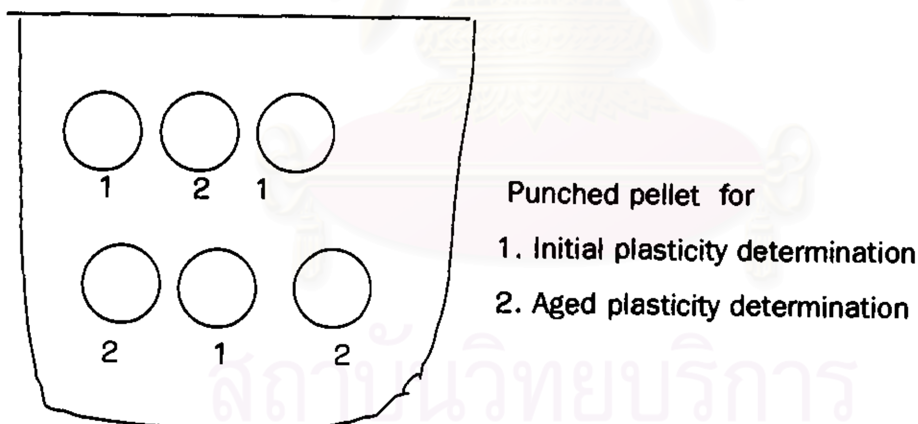

Figure 2.1 Illustration of test piece punching from rubber sheet.

The test pellet was sandwiched between 2 pieces of aluminum sheet and pressed between the two parallel plate to fixed thickness of 1 mm with compressive force of 10±0.1 kgf for 15 seconds of Wallace rapid plastimeter.

Calculation: The median values of three unaged and three aged testpieces were used to calculate the PRI as follows:

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

2.7.7 Determination of the 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, die cavity and rotor should be heated up to the test temperature, 100°C About 25 g. of the homogenized rubber was cut into halves. One half was placed in the lower die cavity and the rotor was placed followed by another half on the top of the rotor and the die was closed immediately. Preheat the specimen for 1 min 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.

2.8 Molecular characteristics of NR by Gel Permeation Chromatography (GPC)

The molecular weight distribution (MWD) was determined in a GPC model 510;Waters, using two columns in series, packed with styrene-divinylbenzene copolymer having exclusion limits of 10^7 and 10^5. The rubber sample was prepared at a concentration of 0.01%w/v in THF and was filtered through a Millipore filter 0.2 μm before injection. The measurements were made using THF as eluent, with a flow rate of 1.0 ml/min at 35 °C, monitoring with reflective index (RI) detector.

2.9 Determination of non-rubber content of NR

2.9.1 Measurement of gel content

The gel content was determined by dissolving approximately 0.5 g. of the rubber in toluene, which was kept in the dark for 1 week at room temperature. The solution was centrifuged at 3,500 rpm. for 1 hour to separate the gel from the sol fraction. The separated gel fraction was dried in vacuum and weighted to estimate the gel content.

2.9.2 Measurement of acetone soluble content

Rubber sample was allowed to dissolve in acetone at 0.5%w/ v for 16 hours in the dark. The acetone solution was then centrifuged at 3,500 rpm for 1 hour. The sol fraction was separated and the rubber was dried under vacuum to constant weight and weighted to calculate the acetone soluble content.

2.10 Fourier Transform Infrared (FT-IR) analysis of amino groups and ester groups in NR

The rubber samples for FT-IR analysis were prepared by casting 1% of the rubber solution in chloroform on a KBr disk placed on activated silica gel and drying under a stream of nitrogen gas to form a round transparent film of about 1.5 cm in diameter. The film was scanned with a Perkin Elmer 2000 FT-IR spectrometer at a resolution of 6 cm^{-1}. The spectrum obtained from the average of 200 scans.

2.11 Scanning electron microscope (SEM) analysis of surface area of rubber

The rubber samples for SEM analysis were prepared by cutting the rubber into thin film and fixed on the sample holder. The rubber was coated with gold and scanning with Scanning microscope, JEOL, JSM-6400 of 100X.

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

2.12.1 Compounding

Rubber specimen either SNR or control was mixed with compounding additives according to the formulation shown in Table 2.1 in order to compare SNR and its control rubber with respect to its cure characteristic and physical properties of rubber vulcanizate.

Table 2.1 The compounding formulation chosen for assessing the cure behavior of natural rubber

TTR 5L	15.0
BR	70.0
NBR	15.0
Zinc oxide active	3.0
Stearic acid	1.0
Hisil 233s	50.0
Dalex	3.0
PEG 4000	2.5
Wax	1.0
22 cp 46	0.5
MBTS	1.35
TMTM	0.08
Sulfur	1.70

Here Hisil 233s (precipitated silica) is reinforcing filler: Zinc oxide active and stearic acid form the activators of vulcanization: MBTS (2-2 dibenzothiazyl disulfide) and TMTM (tetramethy thiuram monosulfide) are accelerators of vulcanization, 22cp46 (2,2 methylene bis-4-methy-6-p-butyl phenol) and wax are antioxidants; PEG (ethylene glycol) is a depressor of surface active absorption and Dalex is processing oil. Sulfur is the widely used crosslinking agent.

Compounding was carried out on a smooth two-roll mill at room temperature by adding the chemicals as follows:

1. Homogenize rubber for 5-10 minutes
2. Add Hisil 233s+PEG+Dalex+Stearic acid and mix for 7 minutes
3. Add wax+22cp46+ZnO active and mix for 5 minutes
4. Add MBTS+TMTM and mix for 3 minutes
5. Finally add sulfur and mix for 2 minutes

The compound was kept at room temperature for 1 day before determination of its cure characteristic.

2.12.2 Cure characteristic of the compound rubber

Cure characteristic of the compound rubber was run on a Rheometer Model 100s (Mosanto, UK.) for 10 minutes at 155 °C with disc oscillating at 3° arc. From the rheometer curve recorded, all the necessary reading was determined and reported.

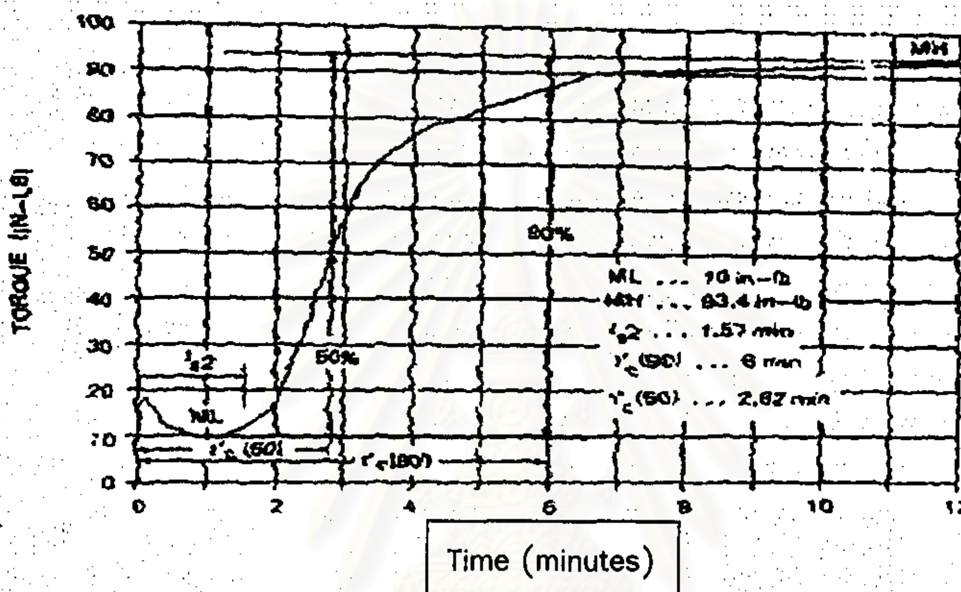

Figure 2.2 Rheometer curing curve

1-minimus modulus (ML); 2-scorch point (1 torque unit rise above minimum viscosity; Ts-scorch time; 3-maximum modulus (MH); 4-50% crosslinks; T_{50} - time to 50% crosslinks; 5- optimum cure (90% crosslinks); T_{90}- time to optimum cure

2.12.3 Test piece preparation for measuring physical properties

The compounding samples were vulcanized in a compression mold at 155 °C for a period of time to reach optimum cure indicated by rheometer graph obtained in 24 h and left for 24 h before cutting in test piece.

The physical properties usually measured are Hardness (Shore A type), 300% Modulus, Elongation at break, Tensile strength, and specific gravity.

2.12.3.1 Specific gravity (ASTM D3184, 1989)

This test method was based on water replacement concept. The weight of rubber vulcanized test piece was weighted in the air and recorded as Wa, and the weighed again in the water by tieing with a copper wire, recorded as Ww. The specific gravity of rubber vulcanized was determined by equation as below

$$\text{Specific gravity} = \frac{Wa\ (g)}{Ww\ (g)}$$

2.12.3.2 Determination of Tensile strength, 300% Modulus and Elongation at break (ASTM D412, 1987)

The 5 dumbbell test pieces (figure 2.4) were cut out from the rubber vulcanized by punching with a die using a single stroke of a press. A reference of length 2cm was marked and the thickness of the dumbbell test piece measured along the reference length by a micrometer dial gauge.

Figure 2.3 Shape of a 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:

$$\text{300\% modulus} = \frac{\text{Force at 60 cm. (kg)}}{\text{Cross-sectional area (cm}^2\text{)}}$$

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

% Elongation at break = (length of reference mark at break−2) x 50

2.12.3.3 Tear strength test (ASTM D624, 2989)

Five test piece for tear resistant were cut out form 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:

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

2.12.3.4 Hardness 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 thickness 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 recorded.

2.13 Preparation of latex proteins

2.13.1 Extraction of water extractable proteins in solid rubber

A piece of solid rubber (SAP-NR and its control) was cut from each sample, 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 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) followed by centrifugation at 2,000 G for 5 minutes. The filtrated-solution was lyophilized (2.13.2)

The total protein concentration in this sample was determined by modified Lowery method.

2.13.2 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 (2.13.3).

2.13.3 Determination of water extractable proteins by modified Lowery method

Solution for modified Lowery 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 sulfate (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

Measurement of water extractable protein by Modified Lowry in the preset of $CuSO_4$

The lyophilized protein (2.13.2) was resuspended with distilled water. These samples were assayed by the micro -plate modified Lowry method as followed: solution A 200 µl was added to 50 µl of sample solution in each wells of micro titer plate, mixed and allowed for 15 min at room temperature. Then 50 µl of solution B was added. Protein levels were evaluated against standard protein, ovalbumin, using a microplate reader (Multiskan Ex, Labstytem) at 750 nm wavelength at 750 nm after 15-20 min. The $O.D_{750}$ was subtracting from the blank, which was water. Protein contents were evaluated from standard ovalbumin. The standard protein was done like this method except 50 µl of 0-50 µg ovalbumin was used instead of sample solution.

Measurement of water extractable protein by Modified Lowry in the absent of $CuSO_4$

Protein samples were assayed by the micro -plate modified Lowry method as followed: solution DA 200 µl was added to 50 µl of sample solution in each wells of micro titer plate, mixed and allowed for 15 min at room temperature. Then 50 µl of solution DB was added and measured for absorbance at 750 nm after

15-20 min. The $O.D_{750}$ was subtracting from the blank, which was water. Protein contents were evaluated from standard ovalbumin. The standard protein was done like this method except 50 μl of 0-50 μg ovalbumin was used instead of sample solution.

Proteins contents calculated from $O.D_{750}$ with $CuSO_4$ substracted from $O.D_{750}$ without $CuSO_4$ that evaluated from standard ovalbumin.

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

SDS-PAGE was carried out according to Slater et al. (1990), with a slight modification. Fifteen percent acrylamide-bis gel 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 0.1%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 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/ 1:4.5 acetic acid/methanol.

2.14 Human serum samples

2.14.1 Control's sera

Control's sera were collected from Thai Red Cross. All Samples were based on assumption that they came from healthy persons who have no symptoms of allergy to latex. No demographic or medical information is available on the control's sera.

2.14.2 Sera from patients with latex allergy

The sera were collected from atopic persons who have been working at Chulalongkorn Hospital and had experienced clinical symptoms of allergy to latex products. The reference serum sample was kindly provided from a person who works at the Health care Department, who has clinical symptoms of allergy to latex. And showed positive skin prick test (SPT) with extracts from natural products (2.16) Others serum sample were collected from patient who came to Chulalongkorn Hospital on general purpose.

2.15 Specific anti-latex IgE antibody detection

2.15.1 Enzyme allergosorbent (EAST) test

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 follows:

The lyophilized latex protein was 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 filed up with 10μg/well of latex protein solution and incubated at 4 °C, in a moist chamber, overnight. The protein solutions were discard and washed three times with 500μl 0.9% NaCl solution containing 0.1% Tween 20(NSS/Tween20). 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 of 100 μl were dispensed into each well, then incubated at room temperature for 180 minutes.

Serum was discard and the wells were washed three times 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. Absorbance reading at 405 nm was measured by an ELISA microplate reader.

2.16 Allergen detection by Skin Prick test (SPT)

The Human Rights and Ethics Committee of dermal clinic at Srinakharinwirot University, Prasarnmitr campus approved this study. Skin testing was made available by Dr. Suwirakorn Ophaswongse that performed by use of an epicutaneous method on forearms, using extracts made from two different brands

of powdered latex gloves, non-treated rubber and SAP-NR rubber prepared from ammoniated crumb and skim crumb. These sample were weighed, cut into small strips (1 to 2 cm) 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.

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