CHAPTER II

MATERIALS AND METHODS

2.1 Biological materials

2.1.1 Rubber latex

Two types of rubber latex were used in this research;

2.1.1.1 Commercial concentrated latex 60% was obtained from Bangkok Rubber Co. Ltd. Normally it was preserved and stabilized with high ammonia and TMTD/ZnO stabilized system.

2.1.1.2 Fresh field latex from 3 different clones of rubber, RRIM 600, GT 1 and PB 5/51 were bought from the rubber plantation in Rayong Province. Ammonia was sometimes used as preservative and anticoagulant.

2.1.2 Enzymes

Two types of commercial protease were used in this study;

2.1.2.1 Papain product code number P3375, specified as crude extract, was purchased from Sigma.

2.1.2.2 Alcalase T, dust-free granulate form of alcalase, was obtained from Novo.

2.2 Chemicals

L-cysteine hydrochloride, hydroxylamine hydrochloride and thiourea (thiocarbamide) were from BDH.

Sodium metabisulfite, anhydrous potassium sulfate, copper sulfate pentahydrate ($CuSO_4$. $5H_2O$) and phosphorus pentaoxide were from M&B.

Selenium powder, sulfuric acid AR (S.G.1.84) and ortho-phosphoric acid were from Merck.

Ammonia solution C.G.25% NH_3, RG was from Riedel-de Haën.

Triton X-100 (Analyzed reagent) was from Packard Instrument Company Inc.

Mineral turpentine (high aromatic white spirit) b.p. 155-196°C and RPA No.3 xylyl mercaptan (36%) were supplied by the Rubber Quality Testing Unit at the Rubber Research Institute, Department of Agriculture, Bangkok.

All of chemical ingredients in compound rubber formulation namely Hisil 233S, ZnO active, 22 cp 46, wax, stearic acid, diethylene glycol, Shellflex, sulfur, MBTS, MBT and TMTM were provided by Banpan Research Laboratory Co. Ltd.

Other chemicals were reagent grade or unless specified.

2.3 Apparatus

Autoclave model HA-30, Hirayama Manufacturing Co., Japan.

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

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

Psychrotherm, controlled environment incubator shaker, New Brunswick Scientific Co., Inc., Edison, U.S.A.

Oven model UL-80, Memmert, Germany.

Spectrophotometer model Spectronic 2000, Bosch & Lomb, U.S.A.

Other apparatus and materials for raw rubber testing were kindly provided by the Rubber Quality Testing Unit at the Rubber Research Institute.

Mooney viscometer model SMV 201 (Shimadzu, Japan), Two-roll mill model LRM 200 (Lab. Tech. Engineering Co. Ltd.), Hydaulic press model TEE120 (Dahtyan hydraulic machine industrial Co. Ltd., Taiwan), Micrometer model SM-114 (Teclock Co., Japan), Durometer (Shore A) model 7206 (Zwick, Germany) and Instron testing machine model 1101 (U.S.A.) were kindly provided by Banpan Research Laboratory Co. Ltd.

Rheometer model 100S (Monsanto, U.K.) was kindly provided by the Rubber Research Unit, Department of Chemistry, Mahidol University.

2.4 Assay of Alcalase activity

The enzyme activity was determined by measuring the absorbance of tyrosine (A_{280}) 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 buffer before preincubation at 45°C in a shaking waterbath. At zero time, 1.0 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 2500 x g and measured for A_{280} in a Spectronic 2000. 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. 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 activity was reported in casein digestion unit (CDU) according to the following calculation :

$$\text{Alcalase activity (CDU/mg)} = \frac{\text{net } A_{280} \times 4}{\text{slope} \times W \times 20}$$

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

2.5 Optimum condition for Alcalase activity

pH optimum of Alcalase was examined by measuring tyrosine liberation in CDU as described before, using 0.025 M Tris-HCl buffer for pH 7.2-9.0 and 0.025 M glycine buffer for pH 8.6-10.0. Temperature optimum was examined by varying the temperature of incubation in the range of 25-70°C.

2.6 Assay of Papain activity (FAO/WHO standard method, 1972)

The enzyme solution was prepared by dissolving 0.002 g papain in 100 ml of phosphate-cysteine buffer, pH 6.0, and 2 ml of this solution was incubated with 5 ml of casein substrate solution at 40°C in a shaking waterbath for 60 minutes. The reaction was stopped by adding 3 ml of cold 30% TCA and left at room temperature for 30 minutes. Unhydrolyzed casein was removed by centrifugation at 2500 x g for 5 minutes, and the supernatant fraction was measured for absorbance of free tyrosine at 280 nm in a Spectronic 2000, where blank tube was prepared by adding 3 ml of 30% TCA in 2 ml of enzyme solution before adding 5 ml of casein substrate. Papain activity was reported as microgram tyrosine by comparing A_{280} with tyrosine standard curve as described previously. Papain activity was calculated in CDU as follows:

$$\text{Papain activity (CDU/mg)} = \frac{\text{net } A_{280} \times 10}{\text{slope} \times W \times 60}$$

in which net A_{280} is the net A_{280} between sample and blank tube; 10 is the total volume in ml; slope is slope of tyrosine standard curve; W is the weight in mg of original enzyme preparation in the 2 ml aliquot of test solution added to the incubation mixture and 60 is the incubation time in min.

2.7 Optimum condition for Papain

The effect of pH on papain activity was examined by the same assay method at various pH ranging from 5.7-9.0 at 45°C for exactly 20 minutes. The effect of temperature was investigated by incubating the

enzyme solution with casein substrate at various temperatures ranging from 30-70°C for 20 minutes.

2.8 Preparation of latex for deproteinization

2.8.1 Determination of dry rubber content (DRC)
(Tillekeratne et al., 1987)

An aliquot of 10 ml of latex was pipetted into a beaker and coagulated with 5% acetic acid in ethyl alcohol. After complete coagulation occurred, the coagulum was then removed, washed with ethanol, creped through a two-roll mill and dried in a microwave oven at medium low power level for 10 minutes. Weigh the dried coagulum and calculate DRC content by the equation below.

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

where W = weight of the dry rubber (g)

V = volume of the latex taken (ml)

2.8.2 Preparation of 25% DRC latex

Concentrated latex 60% was diluted to 25% DRC with water before adding hydroxylamine hydrochloride to 0.15 parts per hundred of rubber (p.h.r.), and sodium metabisulfite to 0.05 p.h.r. The pH of latex was adjusted to the desired pH-range by adding ammonia solution or removing ammonia by evaporation at 60°C.

For fresh field latex, clarification was firstly performed by pouring the latex through a sieve or muslin cloth in order to remove some contaminants such as barks, stones etc.

2.8.3 Preparation of enzyme solution

The calculated amount of enzyme, required in p.h.r. unit, was weighed and dissolved in distilled water and undissolved particles were removed, only clarified solution of enzyme was used.

2.9 Deproteinization of latex (modified from Chang et al., 1977)

Treatment of latex with both enzymes was performed at various pH at fixed enzyme concentration of 0.3 p.h.r. at 50°C in a shaking waterbath, and 50-100 ml aliquot of digested latex was withdrawn at time intervals. For concentrated latex 60%, aliquot was further diluted with ten-volume of water and coagulated with 2% composite mixture of sulfuric acid and phosphoric acid (1:1). In case of fresh field latex, the digested latex was coagulated with steam in an autoclave for 3 minutes at 121°C under pressure of 15 lb/inch^2. The coagulum was pressed by a two-roll mill, washed with water and dried at 60°C in an air-circulating oven. Nitrogen content was determined by Semi-micro Kjeldahl method according to RRIM standard method (1970).

To investigate the effect of temperature on deproteinization, field latex was prepared in the optimal pH range and then treated with enzyme 0.3 p.h.r. at various temperature (40, 50 and 60°C) in a shaking waterbath. At time intervals latex sample was collected and determined for % nitrogen content as described previously.

By varying the enzyme concentration from 0.1 to 0.4 p.h.r. at the optimal pH and temperature. The proper enzyme concentration was selected from the maximum percent reduction in nitrogen content.

2.10   Optimum processing for DPNR production

    2.10.1   Effect of ammonia evaporation on raw rubber properties

        Concentrated latex 60% was evaporated in a shaking waterbath at different temperatures (50, 60 and 70°C). The pH of latex was recorded every 20 minutes until the final pH was approximately 9. An aliquot of 5 ml latex was poured into a petridish for preparation of total solid film. Raw rubber properties were determined by the testing methods of ASTM.
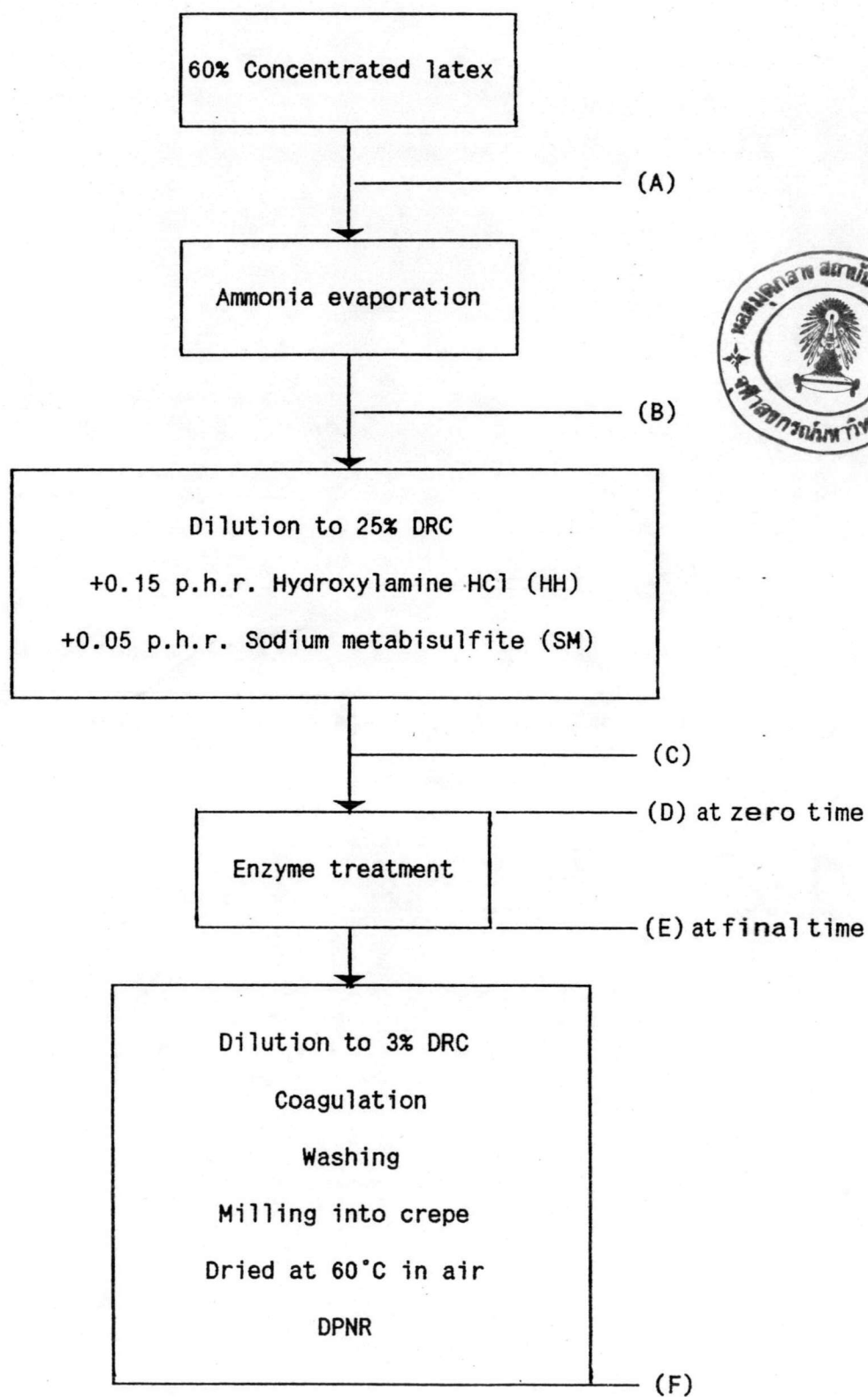
    2.10.2   Effect of dilution and coagulant on raw rubber properties

        After enzyme treatment, latex was diluted with water at different ratio (1:2, 1:5 and 1:10 v/v) and then coagulated with 2 different coagulants, (a) 2% formic acid and (b) 2% composite mixture of sulfuric acid and phosphoric acid 1:1. When coagulation was completed, the coagulum was washed, milled in a two-roll mill into crepe, and dried at 60°C in an oven. Raw rubber properties were determined by the testing methods of ASTM. The effect of thiourea treatment was monitored by dipping the rubber coagulum into 0.2% thiourea solution before drying.

    2.10.3   Effect of each step in DPNR production on raw rubber properties

        The latex was sampled at each step of the production scheme (Figure 2.1) and its properties were analyzed by the testing methods of ASTM.

Figure 2.1 DPNR production scheme


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        ┌─────────────────────────────┐
        │   60% Concentrated latex    │
        └─────────────────────────────┘
                      │ ───────────────────── (A)
                      ▼
        ┌─────────────────────────────┐
        │    Ammonia evaporation      │
        └─────────────────────────────┘
                      │ ───────────────────── (B)
                      ▼
   ┌──────────────────────────────────────────┐
   │            Dilution to 25% DRC            │
   │  +0.15 p.h.r. Hydroxylamine HCl (HH)      │
   │  +0.05 p.h.r. Sodium metabisulfite (SM)   │
   └──────────────────────────────────────────┘
                      │ ───────────────────── (C)
                      │ ───────────────────── (D) at zero time
                      ▼
        ┌─────────────────────────────┐
        │      Enzyme treatment       │ ──────── (E) at final time
        └─────────────────────────────┘
                      ▼
   ┌──────────────────────────────────────────┐
   │            Dilution to 3% DRC             │
   │               Coagulation                 │
   │                 Washing                   │
   │            Milling into crepe             │
   │          Dried at 60°C in air             │
   │                  DPNR                     │
   └──────────────────────────────────────────┘ ──── (F)
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2.11 DPNR production

DPNR was produced at the shaking flasks level and pooled to 250 g of dry rubber from both concentrated latex 60%, and field latex according to the selected optimal condition and processing. Control dried rubber of each latex source was prepared by either acid coagulation or steam coagulation without any treatment.

2.12 Raw rubber testing (RRIM, 1970)

Raw rubber properties of DPNR and its control were analyzed according to RRIM specifications, which consist of the following contents : dirt, ash, volatile matter, nitrogen, plasticity (Po) and plasticity retention index (PRI), color index and Mooney viscosity. For every test, the rubber sample 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 sheet was then cut into approximate weight portions for each test as follows :

2.12.1 Determination of dirt content

About 30 g of homogenized rubber was passed twice through a cold mill with 0.330 mm nip setting. A test portion of approximately 10 g was accurately weighed, 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 contents was heated at 125-130°C by infrared lamps with occasional agitation until dissolution was complete (about 3 h). The hot rubber solution was filtered 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 at 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 per cent (w/w).

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

2.12.2 Determination of ash content

Weigh accurately 5-10 g test portion of the homogenized rubber (to the nearest 0.1 mg). 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 0.1 mg.

Calculation : the ash content was calculated as follows:

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

2.12.3 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. Pass 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 h. After heating, each test portion was kept in a polythene 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 :

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

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

    B : Weight of test portion after drying (g)

2.12.4 Determination of nitrogen content

Rubber specimen was weighed accurately about 0.2-0.3 g into a micro-Kjeldahl tube and 0.65 g of catalyst mixture (K_2SO_4 : $CuSO_4$: $5H_2O$: SeO = 30:4:1) and 7-8 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 the distillation unit followed by three washing with distilled water. Add 5 ml of boric acid solution and 2-3 drops of indicator solution, which is the mixture of methyl red and bromcresol green into the receiving conical flask, then place at the end of the condenser. Add about 10 ml of 40% NaOH solution to the distillation vessel, and pass steam through the distillation apparatus until the volume of distillate in the receiving flask reaches 150 ml and then lower the receiver and wash the end of the condenser with water. Immediately titrate the distillate with standardized 0.01 M H_2SO_4. Blank can be prepared by adding all the reagents but omitting the sample.

Calculation : Total nitrogen content was calculated as follows :

$$\%\text{Total nitrogen} = \frac{28 \times (V_S - V_B) \ M}{W}$$

where V_S = ml of H_2SO_4 required for titration of the contents of the
   receiving flask

V_B = ml of H_2SO_4 required for titration of the blank

M   =   Molarity of the H_2SO_4

W   =   the weight of sample used (g)

2.12.5   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. A test portion of 20 ± 5 g of the homogenized piece was passed twice through 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. 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 ageing 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.
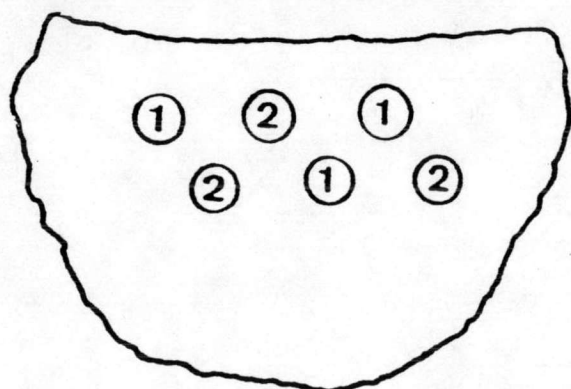
Punched pellet for

1. initial plasticity determination

2. aged plasticity determination

Figure 2.2   Illustration of testpiece punching from rubber sheet.

The test pellet was sandwiched between 2 pieces of aluminum sheet and pressed between the two parallel plate to a 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 :

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

2.12.6 Determination of color index

The testpiece of homogenized rubber was prepared by the same procedure as PRI test. 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 cavity areas of the mold for 5 min at 150±3°C. The color of testpiece 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.

2.12.7 Determination of the Mooney viscosity (ASTM D1646, 1988)

The viscosity of raw rubber was determined in a Mooney viscometer SMV 201 according to ASTM (D1646, 1988) standard procedure. Before loading the rubber, die cavity and roter should be heated up to the test temperature, 100°C. About 25 g of the homogenized rubber was cut into 2 halves. One half was placed in the lower die cavity and the rotor was placed followed by another half on 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.

2.12.8 Characterization of Viscosity-stabilized rubber

The accelerated – storage hardening test (ASHT) provides an indication of natural solid rubber with respect to its increased viscosity during storage as a result of the formation of crosslink between the rubber molecules. This test involves the measurement the Wallace plasticity of standard testpiece before and after a short period of storage over phosphorus pentaoxide at atmospheric pressure at 60°C for 24 h. The testpiece was prepared by the same method as for PRI test. One set of 3 pellets was placed on the platform in a weighing bottle containing P_2O_5 about 6-8 g, and placed in an oven at 60°C for 24 h, whereas another set was in normal atmosphere at room temperature. After 24 h the plasticity of all testpieces were determined by Wallace plastimeter as well as PRI test. The median value of each set was reported and ΔP or the difference of median plasticity between unaged and aged sample was calculated as follows :

$$\Delta P = P_H - P_0$$

where P_H = median plasticity value of 3 storage-hardened testpieces

P_0 = median plasticity value of 3 non-storage hardened testpiece

2.13 <u>Testing of the rubber valcanizates</u> (ASTM D412, 1989)

2.13.1 <u>Compounding</u>

Rubber specimen either DPNR or control was mixed with compounding additives according to the formulation shown in Table 2.1 in order to compare DPNR produced from each clone, and its control rubber with respect to its cure characteristic and physical properties of rubber vulcanized.

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

Natural rubber	100.0 g
Hisil 233 S	45.0
Zinc oxide active	3.0
22 cp 46	0.3
Wax	1.0
Stearic acid	1.2
DEG	3.5
Shellflex	1.5
Sulfur	2.0
MBTS	0.78
MBT	0.2
TMTM	0.12

in which Hisil 233S (precipitated silica) is reinforcing filler; ZnO active, stearic acid form the activators of vulcanization; MBTS (2-2 di-benzothiazyl disulfide), MBT (2-mercaptobenzothiazole), TMTM (tetramethyl thiuram monosulfide are accelerators of vulcanization,

22cp46 (2,2 methylene bis-4-methyl-6-p-butyl phenol)and wax are antioxidants ; DEG (diethylene glycol) is a depressor of surface active absorption and Shellflex is processing oil; and 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 3 minutes

2. Add Hisil 233S + DEG + Shellflex + Stearic acid and mix for 7 minutes

3. Add wax + 22 cp 46 +ZnO active and mix for 3 minutes and then leave until the compound cool down to room temperature

4. Add MBTS + MBT + TMTM and mix for 3 min

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.

2.13.2 Cure characteristic of the compound rubber was run on a Rheometer Model 100S (Monsanto, 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.
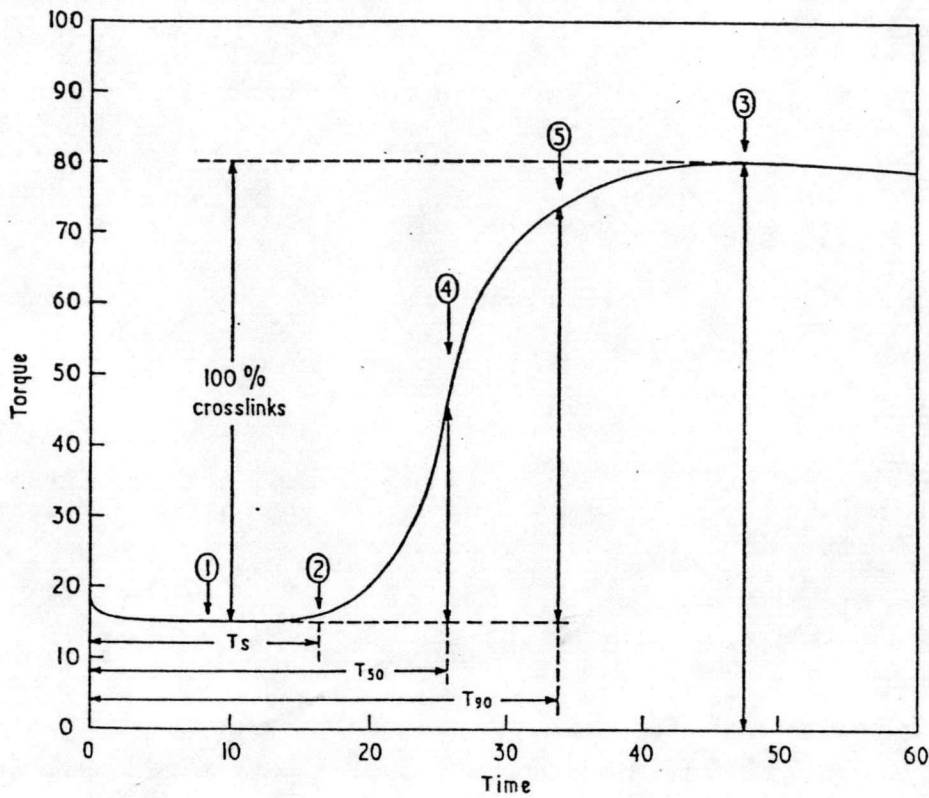
Figure 2.3 Rheometer curing curve

1 – minimum modulus (ML) ; 2 – scorch point (1 torque unit rise above minimum viscosity; T_S – 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

2.13.3 <u>Testpiece preparation for measuring physical properties</u>

The compound 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 into testpiece.

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

2.13.3.1 <u>Hardness</u> (Shore A) (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 a 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 International Rubber Hardness Degrees (IRHD) at room temperature.; The median value of 3 or 5 different points distributed over the specimen was recorded.

2.13.3.2 <u>Specific gravity</u> (ASTM D3184, 1989)

This test method was based on water replacement concept. The weight of rubber vulcanized testpiece was weighed in the air and recorded as Wa, and then 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

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

2.13.3.3 <u>Determination of Tensile strength, 300% Modulus and Elongation at break</u> (ASTM D412, 1987)

The 3 dumbbell testpieces (Fig. 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 2 cm. was marked and the thickness of the dumbbell testpiece measured along the reference length by a micrometer dial gauge.
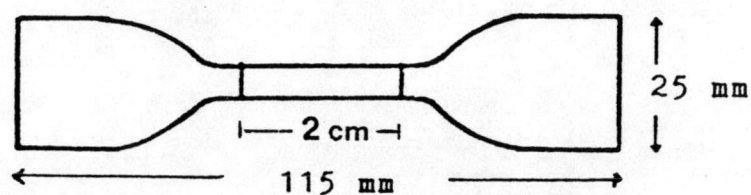
Figure 2.4 Shape of a dumbbell testpiece

Two ends of the dumbbell testpiece were clamped into the two grips of the testing machine, Instron M1011. The testpiece was then stretched at a constant rate of moving grips of 500 ±50 mm/min. The force required to stretch the sample to 300% of reference mark length and to breakage were automatically recorded and calculated over cross-section area of testpiece as 300% modulus and tensile strength (stress at break) respectively. The maximum stretching of reference mark was recorded for calculation of Elongation at break (%) as follows :

$$\text{Elongation at break} = \frac{(\text{Maximum stretch (in cm)}-2)}{2} \times 100$$
(in percent)