CHAPTER II

MATERIALS AND METHODS

1. Biological materials

1.1 Crude papain

Papaya latex was collected by tapping from green fruits of papaya (Carica papaya). The collected latex was sieved by pouring through a muslin cloth to remove dirts, then dried by lyophilizer. The white powder of dried crude papain was obtained and stored at -20 ^{o}C in a refrigerator.

1.2 Chitin

Chitin(product code number C3387) was purchased from Sigma.

1.3 Fresh field rubber latex

Fresh field rubber latex, clone RRIM 600, was bought from plantation in Rayong province. After removal of dirts by pouring the latex through a muslin cloth, 15 ml of 25% ammonia solution and 70 ml of 10 % Tritin X-100 solution were added into 5 litre of fresh latex (final concentration of ammonia and Triton X-100 in latex are 0.075 % (v/v) and 0.14 % (w/v), respectively) as preservative and anticoagulant, respectively. The stabilized latice were kept in ice-box during delivery.

2. Chemicals

Casein hammarsten, L-cysteine hydrochloride, Hydroxylamine hydrochloride and thiourea (thiocarbamide) were obtained from BDH Laboratory Chemicals Division, England.

Ovalbumin grade V (code number A 5503) and glutaraldehyde were from Sigma Chemicals Co., U.S.A.

Sodium metabisulfite, copper sulfate pentahydrate were from M&B

Selenium powder, sulfuric acid AR and orthophosphoric acid were from E. Merck Ag. Darmstadt, Germany.

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

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

All of the chemical ingredients used in compound rubber formulation were provided by Banpan Research Laboratory Co. Ltd.

Other chemicals were analytical grade from Sigma Chemicals Co. U.S.A., BDH Laboratory Chemicals Division, England and E.Merck Ag. Darmstadt, Germany.

3. Apparatus

Magnetic stirer Nuova II, Barnstead / Thermolyne U.S.A. and IKAMAG, Janke & Kunkel GMBH & Co.KG Br.

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

Bench top centrifuge model H-103 N series, Kokusan Enshinki Co., Ltd., Japan.

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

Spectronic 20D, Bausch & Lomb, U.S.A.

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

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

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

Water bath, Heto Lab Equipment, Denmark and Charles Hearson Co., Ltd., England.

Peristaltic pump and fraction collector, LKB-Productor AB , Sweden.

Autoclave, Hirayama Manufacturing Cooperation, Japan.

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

Oven model UL-80, Memmert, Germany.

The apparatus as follows were kindly provided by the Rubber Quality Testing Unit at the Rubber Research Institute : Durometer hardness system (shore A) model 716(Instrument & MFG Co.,NY. U.S.A.), Hydraulic press (Apex construction, London), Micrometer model no.2046 (0.01 - 10 mm) (Mitutoyo, Japan), Mooney viscometer model SMV - 201 (Shimadzu, Japan), tensile tester model LR 5K (LLOYD Instrument), two-roll mill (Nam Lee Foundry).

Rheometer model EK-100H (EEKORNER IND Co., Ltd., Taiwan) was kindly provided by Banpan Research Laboratory Co. Ltd.

4. Assay of papain activity (FAO/WHO standard method , 1981)

4.1 Preparation of casein substrate solution

Disperse 1 g (moisture-free basis) of hammarsten casein in 50 ml of 0.05 M sodium phosphate solution, and heat at the temperature about 50°C for 30 minutes with occasional shaking. Cool to room temperature with continuous shaking, adjust to pH 6.0 $\pm$ 0.1 by the addition of 0.05 M of citric acid solution.

4.2 Determination of the proteolytic activity of papain

About 0.1-0.5 g (wet weight) of immobilized papain or 2 ml

of papain solution was incubated with 5 ml of 1 % casein substrate solution for 30 minutes in a water bath maintained at 40 ^{o}C. The reaction was stopped by adding 3 ml of cold 30 % TCA and mix immediately by swirling. Then all tubes were further incubated in the waterbath at the same temperature for another 30 min to allow complete coagulation of protein. Unhydrolyzed casein was removed by centrifugation at 3500 x g for 10 minutes (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 measured at 280 nm in a Shimadzu spectrophotometer, against its respective blank.

The blank tube was prepared by adding 2 ml of buffer instead of sample while the control tube was prepared by adding 3 ml of 30 % TCA in 2 ml of papain solution (or immobilized papain) before adding 5 ml of casein substrate.

Papain activity was reported as microgram tyrosine by comparing A_{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 of distilled water and then diluted to various concentrations (20-100 ug/ml). Papain activity was calculated in CDU (casein digestion unit) as follow :

$$\text{Papain activity} = \frac{\text{net A280} \times 10}{\text{slope} \times W \times t}$$

in which net A_{280} is the net A_{280} between sample and blank tube ,10 is the total volume in ml, slope of standard curve is μg / A_{280}, W is the wet weight in gram of immobilized papain on chitin (CDU/g), in

case of papain solution, W is mg of papain, which is dissolved in 2 ml aliquot of test solution added to the incubation mixture (CDU/mg) and t is the incubation time (minute).

By definition, 1 casein digestion unit (CDU) was defined as 1 ug of tyrosine liberated from casein digestion by 1 g (wet weight) of immobilized papain on chitin per minute under the assay condition.

5. Protein determination by Biuret method (ปาริชาติ ภู่สว่าง, 2530)

The biuret reaction for protein determination is one of the first colorimetric protein assay method developed and still widely used. Its sensitivity is in range of 1-10 mg protein. The nature of the colored complex involves complexation of copper in alkali solution with peptide linkage of proteins and also with tyrosine residues.

5.1 Preparation of biuret reagent

Dissolve 1.5 g of $CuSO_4.5H_2O$ and 6.0 g of sodium potassium tartrate ($NaKC_4O_6.4H_2O$) with 500 ml distilled water. Add 300 ml of 10 % (w/v) of NaOH, mix together and then bring the volume of liquid to 1 litre with distilled water. The biuret reagent should be deep blue in color. This solution may be stored indefinitely at 4°C until black precipitate is observed in the storage container.

5.2 Determination of protein by the biuret reaction

To 1 ml of solution containing 1-10 mg of standard protein (or papain solution), add 4 ml of biuret reagent. Mix well and allow the mixture to stand at room temperature for 20 min and then determine the absorbance at 570 nm in a spectrophotometer and determine the protein content (mg) from the standard curve.

Biuret standard curve (as shown in Appendix I), showing relationship between absorbance at 570 nm and concentration of standard protein (in this work using egg albumin as standard protein) was prepared by dissolving 0.25 g egg albumin in 25 ml of distilled water (the concentration is 10 mg / ml) and diluted to various concentrations (2-10 mg/ml), then assayed as the same method above.

6. Pretreatment of chitin (Stanley et al., 1975)

Chitin was moistened with distilled water and treated with 6 N hydrochloric acid at room temperature for 10 min and repeated washing for 3 times to remove calcium carbonate bound on chitin surface. Then, the chitin was washed with distilled water until pH test paper pressed on the moist surface gave a neutral test. To remove bound protein, the chitin was then heated on the waterbath ($50^{o}C$) for 3 hours with 5 N potassium hydroxide (50 g moist chitin in 300 ml of 5 N KOH). The chitin was again washed with distilled water until neutral and allowed to soak in warm ($50^{o}C$) 1% sodium chloride followed by warm 1% acetic acid to remove pigment and, finally, thoroughly washed with distilled water and dried at $60^{o}C$ overnight.

7. Immobilization of papain

7.1 Optimum conditions for papain immobilization by physical adsorption method (modified from Finley, et al, 1977)

Chitin, the carrier,was washed by acid-alkali treatment (as shown in Method 2.6). Pretreated chitin was fractionated into mesh sizes : 10-40, 40-80 and more than 80 mesh to determine the optimal

size for papain immobilization. The effect of papain concentration on physical adsorption was examined in the range of 1 - 10 mg/ml. The effect of buffer and pH range which is the most suitable were tested with 0.1 M acetate buffer (pH 5.5), 0.1 M phosphate buffer (pH 6.0-7.5), 0.1 M Tris buffer (pH 8.0-9.0). The proper reaction time was examined during 15 - 60 min. To strengthen the binding force, glutaraldehyde solution, ranging from 0.1 - 10 % was added with preincubation time ranging from 15 - 60 min before adding papain. Physical adsorbed papain on chitin was washed with distilled water to remove the unbound enzyme and excess glutaraldehyde. The optimal washings (1-4 times) and other proper conditions were selected.

7.2 Optimal conditions for papain immobilization by covalent-binding method (modified from Puvanakrishnan, 1980 and Ohmiya, 1978)

Covalent-binding method can be classified into 6 types as described by Chibata, 1978. In this research, chitin, the carrier is bound to the bi-functional reagent, glutaraldehyde. The effect of glutaraldehyde concentration, ranging from 0.1-1.0%, and pH of buffer (0.1 M acetate buffer pH 5.5, 0.1 M phosphate buffer pH 6.0-7.5, and 0.1 M Tris buffer pH 8.0-9.0) were tested together with the ratio of glutaraldehyde solution to buffer solution. The optimal reaction time for covalent-binding between chitin and glutaraldehyde in buffer solution was also examined. After that, glutaraldehyde-treated chitin was washed two times with distilled water to wash out excess glutaraldehyde solution. The effect of papain concentration, ranging from 1-10 mg/ml, in various pH of buffer, ranging from 5.5-9.0, were

investigated. The suitable reaction time for covalent-binding between glutaraldehyde-treated chitin and papain in buffer solution was examined during 15 - 60 min. Finally, immobilized papain by covalent binding method was washed with distilled water (1-4 times) to remove unbound papain. The best condition yielding maximum activity of immobilized papain was selected.

8. Properties of immobilized papain on chitin

8.1 Effect of pH and temperature on the activity of immobilized papain comparison to free papain solution

The effect of pH on the activity of papain was examined by using 1 % casein solution at various pH ranging from 5.5 - 9.0 as substrate at fixed temperature of 40 ^{o}C for 30 min.

The effect of temperature on the activity of papain was investigated by incubating immobilized papain or free papain with 1 % casein substrate pH 6.0 at various temperaturefor ranging from 30 - 90^{o}C for 30 min.

8.2 Effect of pH and temperature on the stability of immobilized papain comparing to free papain solution

The effect of pH on the stability of immobilized papain was examined by incubating immobilized papain in buffer at various pH ranging from 5.5-9.0 at 40^{o}C for 3 hrs before assaying for its retained activity. For free papain, the enzyme was dissolved in buffer solution at various pH, then incubated in a waterbath at 40 ^{o}C for 3 hrs and determined for its retained activity by using the same condition.

The effect of temperture on the stability of immobilized papain and free papain in phosphate-cysteine-EDTA buffer pH 7.5 was investigated by incubating immobilized papain or free papain solution in a waterbath at temperature ranging from 30 - 90 ^{o}C for 3 hrs and assayed for its retained activity.

8.3 Comparative Kinetics study of immobilized papain by using various substrate (Ohmiya,1978)

Kinetic study of immobilized papain was compared with corresponding free papain by using casein and ovalbumin which were in phosphate buffer pH 6.0 and rubber latex pH 7 - 8 as substrates. By varying substrate concentration and assay the enzyme activity, the Lineweaver-Burk plot was created in order to determine the V_{max} (maximum velocity) and the K_m (Michealis - Menten constant) for each substrate.

8.3.1 casein

The concentration of 0.25 %, 0.5 %, 1.0 %, 1.25 %, 2.0 %, 6.0 %, 8.0 % and 10.0 % in phosphate buffer pH 6.0 were used. The assay condition was at 40 ^{o}C for 30 min and the enzyme activity was expressed in CDU per mg protein.

8.3.2 Ovalbumin

The concentration of 0.5 %, 1.0 %, 2.0 % and 4.0 % in phosphate buffer pH 6.0 and the assay condition was at 40 ^{o}C for 30 min and the enzyme activity was expressed in CDU per mg protein.

8.3.3 Rubber latex

Rubber latex was diluted by distilled water from 32.32 % DRC to 30 %, 25 %, 20 % and 10 % DRC and adjusted pH to 7-8.

Each % DRC of rubber was examined for its protein content by semi-micro Kjeldahl method and percent total protein (6.25 xpercent N) was used as the substrate concentration. The assay condition of immobilized papain was at 40 ^{o}C for 3 hrs whereas the assay condition of free papain was at 50^{o}C for 2 hrs (Visessanguan, 1992). Retention of protein (%) was used to express the activity of immobilized papain and free papain.

9. Effect of temperature on storage stability :

Immobilized papains prepared by both methods and free papain were stored in 0.1 M phosphate - cysteine - EDTA pH 7.5 at room temperature (about 27-30 ^{o}C) for 48 hrs for short term storage and 4^{o}C for 3 months for long term storage. At time intervals, they were assayed for their remaining activities by using casein as substrate (Method 2.4.2).

10. Continuous operation

1% Casein in phosphate buffer pH 6.0 was used as substrate by continuous feeding of casein solution at the flow rate of 6 ml / hr with temperature-control at 40^{o}C into a 2.6 x 35 cm column containing about 200 g of covalently-immobilized papain. Operate this system for 7 days, the eluent collected at time intervals were assayed for the immobilized papain activities and the unbound papain was also determined by Method 2.4.2.

11. Preparation of latex deproteinization

11.1 Determination of dry rubber content (DRC)

(Tillekeratne et al. , 1987)

An aliquot of 10 ml of latex was pipetted into beaker or plate and coagulated with 20 ml of 5 % acetic acid in ethyl alcohol. After complete coagulation occured, the coagulum was then removed, washed with ethanol, creped through a two-roll mill and dried in a microwave oven at low power level for 10 minutes or in an oven at 60°C overnight. Weigh the dried coagulum and calculate DRC content by the equation below.

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

where W = weight of dry rubber (g)

V = volume of the latex taken (ml)

11.2 Preparation of 25 % DRC latex

Fresh field latex was diluted to 25 % DRC with water after 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 or by adding 0.01 M of phosphoric acid.

12. Determination of optimal conditions for latex deproteinization

12.1 Effect of pH , temperature , speed of shaking and enzyme concentration on latex deproteinization

Treatment of latex with immobilized papain was performed at various pH ranges : 6-7, 7-8 and 8-9, using fixed amount of immobilized papain at the concentration of 20 p.h.r. (1 gram wet weight : 5 g dry rubber) at 40°C in a shaking waterbath. Every 2 hrs, digested latex was removed and poured through a muslin cloth to separate immobilized papain from digested latex. Then digested latex was coagulated by using low power level of microwave for about 5 min. The coagulum was pressed by 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 examine the effect of temperature on latex deproteinization, field latex was diluted to 25% DRC, adjusted to the selected pH range and then treated with 20 p.h.r. of immobilized papain at various temperature (30°, 40° and 50 °C) in a shaking waterbath. At time intervals, latex sample was collected, dried and determined for % nitrogen content (Appendix III).

The immobilized papain concentration (10 - 40 p.h.r.) at suitable pH and temperature was selected using criteria that yield the maximum reduction in % nitrogen or protein content. After physical conditions were known, varying speed of shaking from 100, 120, 150 and 200 rpm under these optimal conditions were tried. The proper speed of shaking during enzyme treatment was selected from the maximum percent reduction in nitrogen content and maximum yield of

dried solid rubber.

12.2 Effect of viscosity-stabilizer,reducing agent and nonionic detergent on latex deproteinization

The viscosity-stabilizer, hydroxylamine hydrochloride, at the concentration of 0.10 p.h.r. or 0.15 p.h.r. was added into field latex before deproteinization under optimal conditions. The reducing agent, sodium metabisulfite, was tested at the concentration of 0.05 p.h.r. and the effect of nonionic detergent, Triton X-100 was tested at the concentration of 1.0, 1.1 and 1.2 p.h.r., respectively. The % nitrogen reduction of latex when each of chemicals was added was compared with control which is deproteinized rubber latex without each of these chemicals.

12.3 Effect of the activator, metal-chelating agent of papain on latex deproteinization

The activators of papain tested were the following : (1) thiourea 0.0020 p.h.r. or 0.0023 p.h.r.and (2) cysteine 0.0010 p.h.r. The effect of metal-chelating agent, EDTA at the concentration of 0.015 p.h.r. was also studied and compared the % nitrogen reduction with control.

12.4 Effect of latex dilution on before and after enzyme treatment on deproteinization efficiency

To test the effect of latex dilution before enzyme treatment, field latex was diluted with water to be 10% DRC, 20 % DRC and 25% DRC, respectively before adjusting to the selected conditions and addition of papain. To test the effect of latex dilution after enzyme treatment, digested latex (25 % DRC) was diluted with water at

different ratio (1:0.5, 1:1 and 1:2) before microwave coagulation. The % nitrogen reduction was determined (as shown in Appendix III) and compared with control which is 25 % DRC without dilution.

13. DPNR production

DPNR from immobilized papain on chitin was produced at the shaking flask level according to the selected optimal conditions (pH, temperature, speed of shaking, Triton X-100 concentration,immobilized papain concentration, hydroxylamine hydrochloride and sodium metabi sulfite concentration, thiourea concentration and latex dilution). The shortest time used for deproteinization is also selected. After deproteinization, the treated latex was coagulated in an aluminium tray by steam under pressure 15 lb / in^2 in an autoclave at 121 ^oC for 15 min and after that the coagulum was passed through a two-roll mill, washed and dried at 60 ^oC in a hot air oven. DPNR from free papain was produced according to its optimal conditions(Visessanguan, 1992). Control dried rubber was prepared by steam coagulation without any treatment.

14. Batch reusability of immobilized papain for producing DPNR

DPNR from immobilized papain was produced according to the optimal conditions. At the optimal deproteinization time, digested latex was removed and poured through a muslin cloth to separate immobilized papain from digested latex. Immobilized papain was reused to deproteinize latex and the digested latex was coagulated by using low power level of microwave oven for about 5 min. The coagulum was pressed by two-roll mill, washed with water and dried at 60 ^oC in

hot air oven. Latex samples of every batch were determined for % nitrogen content and weighed for determining the % DRC lost.

15. Raw rubber testing (RRIM, 1970)

Raw rubber properties of DPNR and its control were analyzed according to RRIM specifications (1970), which consist of the following contents : dirts; ash; volatile matter; nitrogen content; initial plasticity (Po); 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 in to approximate weight portions for each test. For Mooney viscosity, molecular weight and molecular weight distribution by gel permeation chromatography (GPC), the test procedure were carried out according to ASTM D1646 (1988) and ASTM D3536 (1980), respectively (Appendix III).

16. Vulcanized rubber testing

Due to rubber products are in vulcanizates, vulcanization test is important to determine the quality and performance of thier products. Before vulcanization test, the rubber was mixed with chemicals to form compound. The compound was determined cure characteristics (scorch time, cure time, cure rate and torque rise) by running on EEKORNER Rheometer model EK-100H at 155 ^{o}C for 7 min.

The vulcanizated samples were prepared by compression moulding at 155 ^{o}C for its optimal cure time indicated by Rheometer graph. The vulcanized rubbers were left for 24 hours and divided

into two groups. The vulcanizated samples of the first group were cut into test pieces for physical property measuring. The vulcanizated samples of the second group were ageing in a hot air oven at 70°C for 7 days and, after that they were also cut into test pieces for physical property determination. The physical properties tested are ; hardness (Shore A) (ASTM D1415, 1988) ; specific gravity (ASTM D3184, 1989) ; tensile strength, elongation at break and 300% modulus (ASTM D412, 1987) (Appendix III).