Chapter 2

Materials and Methods

Materials

1. Microorganisms

A total of 49 microbial strains, potential pathogens of the oral cavity and respiratory tract, were tested in this study. These were *Porphyromonas gingivalis* W 50, *P. gingivalis* 381, *P. gingivalis* ATCC 33277, *Prevotella intermedia* 25611, *Tannerella forsythia* ATCC 43037, *Aggregatibacter actinomycetemcomitans* ATCC 33384, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus mutans* ATCC 25175, *Lactobacillus fermentum* ATCC 14931, *Candida albicans* ATCC 90028, *C. albicans* ATCC 10231 and 28 clinical isolates. *Candida glabrata* ATCC 90030, *C. glabrata* ATCC 66032, *Candida tropicalis* ATCC 750, *C. tropicalis* ATCC 13803, *C. tropicalis* ATCC 66029, *Candida krusei* ATCC 6258, *C. krusei* ATCC 34135, *Cryptococcus neoformans* ATCC 90112, *C. neoformans* ATCC 90113.

2. Chemicals

Chemicals and enzymes were obtained as follows: acrylamide, 2-mercaptoethanol, Folin-Ciocalteau reagent, ammonium sulfate, ammonium persulfate, sodium chloride, $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 2H_2O$ were from Merck Ltd. (Thailand). N,N′-methylene-bisacrylamide, N,N,N′N′-tetramethylethylenediamine (TEMED), Coomassie Brilliant Blue R-250 and ammonium bicarbonate were from Fluka (service via Sigma-Aldrich distribution network). Ethylenediamine-tetraacetic acid disodium salt-2-hydrate (EDTA) and sodium acetate were from Riedel-deHaën (service via Sigma-Aldrich distribution network), Sodium dodecyl sulfate (SDS), Tris-HCl, glycine, aprotinin from bovine lung (6.5 kD marker for SDS-PAGE), bovine serum albumin (BSA), soybean trypsin inhibitor, Bromophenol blue, Serva blue G

(equivalent to Coomassie Brilliant Blue G-250), dimethyl sulfoxide (DMSO), molecular weight marker for SDS-PAGE Ultra-low range (product no. M3546, molecular weight range 1,060-26,600 Daltons), amphotericin B (Sigma-aldrich), CAPS, morpholinepropanesulfonic acid (MOPS) and chitinase were from Sigma-Aldrich (St. Louis, MO, USA). Low molecular weight protein marker (LMW calibration kit, molecular weight range 14-94 kDa), DEAE-Sepharose Fast Flow, Peptide marker kit (Code number 80-1129-83, molecular weight range 2.5-16.9 kDa) were from Pharmacia Biotech (Amersham Biosciences, at present). Acetic acid, acetone, chloroform, methanol were from Labscan Asia Co., Ltd. Dithiothreitol (DTT) was from Pierce Biotechnology Inc. (Rockford, IL, USA). Sodium bicarbonate, Sodium hydroxide, Copper sulfate, Sodium tetratartate, glycerol, Tricine, Bio-Rad protein assay, RPMI-1640 medium (with L-glutamine, without sodium bicarbonate), Sabouraud dextrose agar (SDA) from Difco Laboratories, SM2 (Bio-Rad), Triton X-100, , calcium chloride, Magnesium sulfate, yeast extract, peptone, glucose, GelCode® Glycoprotein Staining Kit from Pierce (Rockford, IL, USA), N, N′, N″-triacetylchitotriose (chitotriose) from Sigma-Aldrich.

Methods

1. Collection of latex from rubber trees

All experiments were carried out with fresh latex from rubber trees of *Hevea brasiliensis* (clone RRIM 600) age about 20 years grown at Songkhla Rubber Research Center, Hat Yai, Songkhla. The trees were tapped regularly on a half-spiral, alternate-day tapping system. Fresh latex was collected into ice-chilled plastic containers under the collecting tube of latex drops for 30 min per tapping tree. The pooled latex was kept in an ice container, brought to the laboratory and separated latex fractions at the same day.

2. Separation of latex by ultracentrifugation

The chilled latex was filtered through 4 layers of cheesecloth to remove the particulate materials and tissue debris. The filtrate was collected and centrifuged at 59,000 g for 45 min at 4°C in an ultracentrifuge (Beckman model L8-70M). After being centrifuged, the latex was then separated into 4 distinct layers depend on different density of compounds containing in the latex. The top layer was a white creamy layer of rubber, the next layer underneath was a

yellowish called Frey-Wyssling, the middle layer was a clear solution called C-serum and the pellet was bottom fraction which mainly comprise with lutoid particles as shown in Fig. 3A.

3. Preparation of C-serum, B-serum and rubber particles

3.1 C-serum

A spatula was used to make an opening in the rubber cream layer of the ultracentrifuged latex. The milky top layer of the serum phase was carefully sucked out and discarded while the clear serum fraction was collected through a Pasteur pipette.

3.2 B-serum

A spatula was used to make an opening in the rubber cream layer, small volume of aqueous phase was discarded, and the rubber layer was them scooped out. A spatula was inserted to collected the pellet (bottom fraction) and lightly rinsed with 50 mM Tris-HCl, pH 7.5 containing 0.9% (w/v) NaCl to remove other parts of latex components. The bottom fractions were pooled and then subjected to freezing and thawing until most of particles were lysed. The yellow aqueous called B-serum was collected after ultracentrifugation on a Beckman model L8-70 M ultracentrifuge at 59,000 x g for 45 min at 4°C.

3.3 Preparation of rubber particles

Most of the small rubber particles (SRP) are isolated from Moir's zone 2 rubber layer of the ultracentrifuged latex (Fig. 3B) (Mior, 1959). The entire rubber layer (zone 1 and zone 2 together) was scooped out and zone 2 rubber translucent cream at the bottom of rubber layer (facing C-serum) was skimmed off and collected with a spatula. This SRP cream was resuspended in 10x volume by weight of Tris-buffer saline (TBS) containing 0.9% NaCl in 50 mM Tris-HCl buffer. The washed SRP solution was diluted to give an absorbance at 600 nm of about 0.8-0.9. Rubber particles suspensions were freshly prepared and kept on ice for daily use.

4. Preparation of rubber particle surface proteins

The small rubber particle proteins (SRPP) were isolated by modifying methods used for the extraction of proteolipids, previously described by Folch and Lees (1951) and Hasma (1987). The washed rubber particle suspension was extracted with five volumes of a continuously stirred chloroform-methanol mixture (2:1; v/v) for 15 min. The solution was filtered through filter

paper and the filtrate was covered with large volume of 0.6% (w/v) NaCl solution overnight. A whitish fluffy proteolipid layer formed at the interphase was separated and dried under vacuum before dissolved in either 0.2% (v/v) Triton X-100 for yeast aggregation analysis, after the removal of the Triton X-100 by SM2 absorbent ,or 10% dimethyl sulfoxide (DMSO) for study on their effect on the AMB activity.

5. Phase partitioning of tryptic SRPP-derived peptides

The tryptic SRPP-derived peptides (3 ml), obtained by treating SRPP (400 μg/ml with trypsin (0.1 mg/ml) for 15 min, was mixed with 3 ml of chloroform/methanol (2:1, v/v) mixture tube, shook for 2 min and allowed to settle. Equilibration of solution between the organic and aqueous phases was complete within a few minutes, and a period of 2 h shaking was found satisfactory to ensure full attainment of equilibrium. The upper and lower phases in a new centrifuge tube were separated, collected and dried under vacuum. Each fraction was dissolved in 1 ml of 0.2% Triton X-100® in TBS. Triton X-100® was removed by incubated with SM2 absorbent (1:10, w/v) for 1 h to remove residual Triton X-100® before further used (i.e. in the checkerboard titration to determine their effects on the MIC_{80}s of AMB).

6. Purification of *Hevea* protease inhibitor (HPI)

The C-serum was fractionated step-wise by addition of acetone in increments of 0-20%, 20-40%, 40-60%, 60-80% and 80-95%. The total *Hevea* protease inhibitor present in 80-95% fraction (Sritanyarat *et al*., 2006) was used in studying its enhancing effect on the antifungal activity of hevein.

Note: The amount of acetone to be added to the solution to give desire final concentration was showed in Table 2.

Table 2 The amount of acetone to be added to the solution (Scopes, 1994).

Volume of Miscible Solvent, ml to be added to 1 Liter

From C_1 % \ To C_2 % →	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
0	52	111	176	250	333	428	538	666	818	1,000	1,222	1,500	1,857	2,333	3,000	4,000	5,666	9,000	19,000
5		55	117	187	266	357	461	583	727	900	1,111	1,375	1,714	2,166	2,800	3,750	5,333	8,500	18,000
10			58	125	200	285	384	500	636	800	1,000	1,250	1,571	2,000	2,600	3,500	5,000	8,000	17,000
15				62	133	214	307	416	545	700	888	1,125	1,428	1,833	2,400	3,250	4,666	7,500	16,000
20					66	142	230	333	454	600	777	1,000	1,285	1,666	2,200	3,000	4,333	7,000	15,000
25						71	153	250	363	500	666	875	1,142	1,500	2,000	2,750	4,000	6,500	14,000
30							76	166	272	400	555	750	1,000	1,333	1,800	2,500	3,666	6,000	13,000
35								83	181	300	444	625	857	1,166	1,600	2,250	3,333	5,500	12,000
40									90	200	333	500	714	1,000	1,400	2,000	3,000	5,000	11,000
45										100	222	375	571	833	1,200	1,750	2,666	4,500	10,000
50											111	250	428	666	1,000	1,500	2,333	4,000	9,000
55												125	285	500	800	1,250	2,000	3,500	8,000
60													142	333	600	1,000	1,666	3,000	7,000
65														166	400	750	1,333	2,500	6,000
70															200	500	1,000	2,000	5,000
75																250	666	1,500	4,000
80																	333	1,000	3,000
85																		500	2,000
90																			1,000

7. Purification of antifungal protein

B-serum was fractionated step-wise by addition of acetone in increments of 0-20%, 20-40%, 40-60% and 60-80% with stirring in ice bath for 10 min. The pellet of 60-80% acetone fraction was dissolved in 20 mM Tris-HCl, pH 7.4 and submitted to anion exchange chromatography on a DEAE-Sepharose Fast Flow column equilibrated in 50 mM Tris-HCl, pH 7.4 and washed with 50 mM Tris-HCl, pH 7.4 until the A_{280} fell below 0.01. Fractions of 4 ml were collected. The bound protein was eluted with a gradient of 0-0.5 M NaCl in the same buffer. Each fraction was assayed for antifungal activity according to broth microdilution method. The peak fractions containing antifungal activity were pooled, dialyzed against water, lyophilized and analyzed the molecular weight by MALDI-TOP mass spectrometer.

8. Antifungal protein identifications

8.1 Mass spectrometric analysis

Matrix-Assisted Laser Desorption Ionization with Time of Flight ((MALDI-TOF) mass spectra were recorded on a Bruker Biflex III mass spectrometer. Hevein was dissolved in 50 μl of 5% ACN solution containing 0.5% TFA to a saturated solution. Two μl protein solution was mixed with 2 μl of Sinapinic acid-saturated matrix solution. Two μl of this mixture was deposited on the target and dried. Then the target was subjected to MALDI-TOF mass spectrometry analysis.

8.2 Amino acid sequence analysis

The first NH-terminal 11 amino acids were determined by Edman degradation with Applied Biosystems-Procise HT Instrument (Mayo Proteomic Research, Minneapolis, MN).

9. Thermal stability study of antifungal protein-hevein

For studying the effect of heat treatment on the antifungal activity of hevein, hevein was incubated at various temperatures at 4, 50, 60, 70, 80, 90°C and boiled for 0.5, 1 and 1.5 h. The remaining activity of each treatment was assayed for antifungal activity by broth microdilution method. Hevein (kept in 4°C before assayed) was used as 100% remaining antifungal activity control and expressed the results as percentages of the control.

10. pH stability study of antifungal protein-hevein

Effect of pH on the antifungal activity of hevein was studies by preincubating hevein at different pH from 2 to 9 for 24 h at 4°C and then readjusted to pH 7.4 before assay for the remaining antifungal activity. All buffers were 50 mM and contained: Glycine-HCl buffer (pH 2-3), acetate buffer (pH 4-5), phosphate buffer (pH 6) and Tris-HCl buffer (pH 7-9). The results were expressed as the percentage of remaining inhibitory activity when compared to the control which were in double distilled water and kept at 4°C before being assayed.

11. Determination of the MICs by broth microdilution method

MICs were performed by broth microdilution modification of National Committee for Clinical Laboratory Standards (NCCLS) method M27-A with RPMI 1640 medium (with L-glutamine, without sodium bicarbonate) and buffered with 0.165 M morpholinepropanesulfonic acid (MOPS). The pH of the medium was adjusted to 7. The yeasts were grown on Sabouraud dextrose agar (SDA) at 35°C for 24 h. The inoculum was prepared by picking five colonies from these cultures and suspended in RPMI 1640 medium, the cell density adjusted to the density of a 0.5 McFarland standard at 530 nm, resulting in a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells/ml. A working suspension was prepared by diluted 1: 50, and further diluted 1: 20 with RPMI 1640 medium to obtain 1 x 10^3 to 5 x 10^3 CFU/ml. The broth microdilution test was done by using 96 well plastic flat bottom plates with covers. Then, 50 µl of hevein was added and serially diluted two fold dilution to appropriate concentrations. Control was included for the determination of the growth of each *Candida* species without the presence of hevein. Fifty µl of working inoculum was added to 100 µl final volume. After inoculation, plate was incubated at 35°C for 24 h. MIC_{80}s were measured with microplate reader (Biotrak II Visible Plate Reader) at optical density 620 nm. The ODs of the blank wells were subtracted from the ODs of the inoculated wells. Throughout this study the MIC was determined from the concentrations of the antifungal agent required for 80% inhibition of growth (MIC_{80}).

12. Disk diffusion assay

This was done as described previously (Roberts *et al*., 1990) with several modifications. SDA was autoclaved and cooled to 45°C. A suspension of *C. albicans* in RPMI

1640 medium was added to a concentration of $1x10^4$ organisms/ml and 20 ml portions of the liquid agar suspension were poured to Petri dish. Sterile paper discs were placed on the solidified agar, 40 µl of diluted hevein preparations were added to each disc, and the plates were incubated overnight at 37°C. Clear zones of fungal growth were visualized around disc containing inhibitory concentrations of hevein.

13. The enhancing effect of *Hevea* protease inhibitor (HPI) on the antifungal activity of hevein

The disk diffusion assay was similarly employed for investigating the effect of a *Hevea* protease inhibitor (HPI) on the antifungal activity of hevein. The HPI was partially purified, as an 80-95% acetone precipitated fraction from the C-serum isolated from ultracentrifuged fresh latex, according to the method described by Sritanyarat *et al*., 2006. Hevein at 40 µg was mixed with either 200 or 400 µg HPI before adding it to each paper disc. The resulting clear zones of fungal growth were compared to those of the controls containing either hevein (40 µg) or HPI (400 µg) alone.

14. Analysis of yeast cell aggregation induced by hevein

The experiments were done following the method as described previously (Allen *et al.,* 2001). Briefly, the yeast cells were suspended in calcium binding buffer (CBB); 20 mM Tris-HCl, pH 6.75, 0.5 mM $CaCl_2$, 5 mM $MgSO_4$, 0.1 mM DTT, 154 mM NaCl (or CBB containing 10 mM EDTA) and diluted to the required absorbance at 620 nm (A_{620}). Appropriate concentration of hevein or buffer was added to the final volume of 800 µl for all samples. After protein supplement, the A_{620} of each sample was measured every minute for 2 h. Aggregation is recorded by decreasing of A_{620} higher than the negative control (without protein addition) as the aggregated material sediments to the bottom of the assay tube.

15. Antifungal agent preparation

Amphotericin B was obtained as powder. Amphotericin B (AMB) was dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. Serial twofold dilutions of AMB were diluted in DMSO at concentration ranged from 3.13 to 200 µg/ml and were stored at -80°C until

use. Working solutions of AMB were diluted in RPMI 1640 medium to concentration ranged from 0.0625 to 4 μg/ml.

16. Enhancing effects of SRPP on MIC_{80}s of AMB

The enhancing effect of small rubber particle proteins (SRPP) on MIC_{80}s of AMB against *Candida* species were examined under the same experimental conditions used for determination of the MICs by a modified checkerboard broth microdilution method. AMB was dissolved in DMSO to make stock solution and kept at -80°C until used. RP and SRPP were dissolved in 10% DMSO. In one dimension, a two-fold serial dilution series of AMB in MOPS-buffered RPMI was prepared; in the second dimension a two-fold dilution series of SRPP in the same broth was added at a volume of 25 μl of each dimension per well. The final concentration of AMB ranged from 0.156 to 1 μg/ml, that of SRPP from 0.02 to 50 μg/ml. Aliquots of 25 μl of AMB and SRPP (and in the case of the single-drug control, 25 μl of that drug and 25 μl of medium with the drug solvent at the final concentration) at a concentration four times the targeted final concentration were dispensed in the wells in order to obtain a two-dimensional checkerboard. Plates were inoculated with 50 μl of inoculum containing 1 x 10^3 to 5 x 10^3CFU/ml. After 24 h incubation at 35°C, MIC_{80}s were measured with microplate reader (Biotrak II Visible Plate Reader) at optical density 620 nm. The ODs of the blank wells were subtracted from the ODs of the inoculated wells.

17. Specific dye binding assay of yeast cell aggregation

This method was modified from the assay of rubber particle aggregation induced by *Hevea* latex lectin described previously (Wititsuwannakul *et al*., 2007). The yeast cells were adjusted to the density of 0.1 at absorbance 600 nm (OD_{600}) and used as working suspension of yeast cell. Yeast cell suspension (25 μl) was mixed with 25 μl of solution containing SRP, SRPP or BSA and incubated at room temperature for 30 min. After staining by mixing with 10 μl of 0.1% w/v basic fuchsin, the mixture was loaded into a hematocrit tube by means of capillary suction. The mixture-containing end was plugged by pressing it into the Seal-ease from Clay Adams Co. or modeling clay. The yeast cell aggregate, separated into the top layer after centrifuged for 5 min in a micro-hematocrit centrifuge, was examined microscopically.

Aggregation and autoaggregation controls were respectively carried out by mixing either SRP or SRPP with TBS and yeast cell suspension with TBS.

18. Assay of induced yeast cell agglutination

Agglutination of yeast cells induced by SRPP and trypsinized SRPP were tested by adding 50 μl of yeast cells suspension (adjust density to 0.3 at OD 600 nm) to a two fold dilution series of SRPP or trypsinized SRPP at concentration range from 0.05-100 μg/ml in the wells of round-bottom microtiter plates. Plates were then shaken and cells allowed settling at room temperature. Visible aggregations were observed in the bottom of wells. Negative control well containing 50 μl of yeast cells with TBS was run with each microtiter plate. Testing was repeated twice. Evaluation of results was determined by grading system. (+): maximum visual aggregation in the bottom of wells, (0): no visible aggregation or settling, suspension remain turbid. One drop of mixture was then placed on slide and covered with a cover slip. The agglutination was examined with a light microscope with dark-field illumination.

19. Mass spectrometric analysis of 14 kDa and 24 kDa of SRPP

19.1 Preparation and washing of excised 14 and 24 kDa gel fragments

20 μl of each SRPP sample was run in 12% SDS-PAGE and stained in Coomassie Brilliant Blue R-250. The 14 kDa and 24 kDa protein bands were separately excised from a stained polyacrylamide gel. Cut each gel piece into small particles (~1 mm^2) using a scalpel, and placed into a 0.5 ml siliconized tube. Added ~100 μl of 25 mM NH_4HCO_3/50% acetonitrile (or enough to immerse the gel particles) and vortexed for 35-40 min on a low setting (more like shaking). Used gel loading pipet tips to remove the solution (pale blue in the case of Coomassie staining) and discarded. Then, repeated this wash/dehydration step up to ~3 times. Once all Coomassie has been removed, dehydrated gels with acetonitrile (100 μl). At this point the gel pieces should shrink and became an opaque-white color. If not, removed the acetonitrile and replaced with a fresh one. Then, removed the acetonitrile with SpeedVac for 3-5 min. Added 30 μl of the 10 mM DTT solution to cover the gel pieces, and reduced for 30-45 min at room temperature. Replaced the DTT solution with roughly the same volume of 55 mM iodoacetamide (30 μl). Incubated for 45 min at room temperature in the dark. Removed the iodoacetamide

solution and washed the gel pieces with ~100 µl of 25 mM NH_4HCO_3 pH 8, for 10 min while vortexing. The gel was removed washed and dehydrated with ~100 µl acetonitrile. Removed the acetonitrile and dried the gel pieces in a vacuum centrifuge for 3-5 min.

19.2 Trypsin digestion of the 14 and 24 kDa proteins

Rehydrated gel particles in 25 µl trypsin (20 ng/µl trypsin; sequence grade; Promega, Pierce) in 25 mM NH_4HCO_3, pH 8 and placed on ice for 10-15 min. Remove excess trypsin solution and overlaid the rehydrated gel particles with 30 µl of 25 mM NH_4HCO_3 to keep them immersed throughout digestion. Incubated 12 to 16 h at 37°C.

19.3 Tryptic peptide recovery using a Zip-Tip

Add 5 µl of 5% aqueous TFA to halt the digestion and shake the tubes containing gel pieces for about 10 min and centrifuged briefly to bring the liquid to the bottom of the tube. Prepare a saturated solution of HCCA in 1:1 acetonitrile : acidified water (0.1% TFA) and dilute this matrix solution by a factor of 2 and place 3 µl in as many tubes as needed (one for each digestion). Set pipettor to 10 µl and aspirate 1:1 acetonitrile : 0.1% TFA through the Zip-Tip, dispense to waste. Then, repeat twice with fresh solution. Aspirate 10 µl of 0.1% TFA and dispensed to waste. Then, repeated twice with fresh 0.1% TFA. Bind peptides by performing 3-10 cycles of aspirating and dispensing the digest solution through the Zip-Tip. Wash the Zip-Tip by aspirating with 10 µl of 0.1% TFA and dispensing to waste and repeat once with fresh solution. Set pipettor to 3 µl and elute peptides using 3 cycles of aspirating and dispensing the matrix solutions through the Zip-Tip. On the fourth aspiration, 1 µl was dispensed directly to the MALDI plate, allowed to dry before subjected to MALDI-TOF mass spectrometry analysis.

20. Gel electrophoresis

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

SDS-PAGE was performed according to the modified method of Laemmli, 1976. The separation slab gel (10 x 8 x 0.1 cm) was cast in 12% of acrylamide concentration using a standards 2 chambered plexigels gradient mixer and containing 3% (w/v) acrylamide of staking gel (10 x 2 x 0.1 cm), the gel dimension for small plate of “ATTO” Dual mini slab series, model AE-6530. The compositions of both separation and stacking gels were summarized in Table 3

20.1.1 Preparation of sample for SDS-PAGE

The protein samples were mixed with sample buffer (with final concentration of 0.0627 M Tris-HCl buffer pH 6.8, 10% (v/v) glycerol, 1% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) 2-mercaptoethanol and 0.0005% (w/v) bromophenol blue as the dye (Ausebel, 1987), heated sample for 10 min in boiling water, spun down protein solution for a minute and loaded into the wells of SDS-PAGE.

20.1.2 Running condition

The running electrophoresis buffer (pH 8.3) contained 0.025 M Tris-HCl, 0.192 M Glycine and 0.1% (w/v) SDS. The electrophoresis was performed at room temperature with the anode in the lower chamber, 14 mA of constant current per slab gel was applied until the tracking dye was approached the bottom of the separating gel.

Table 3 Composition of reagents for SDS-PAGE.

Stock solutions	3% Stacking gel (µl)	12% Separating gel (µl)
30% Acrylamide + 0.8% Bisacrylamide	300	2,400
1.5 M Tris-HCl pH 8.9	-	1,500
0.5 M Tris-HCl pH 6.8	750	-
10% SDS	30	60
Distilled water	1,745	1,880
0.2 M EDTA	20	-
1% Ammonium persulfate*	150	150
TEMED*	5	10
Total volume (ml)	3,000	6,000

*Added to chemically the gel within 10 min.

20.1.3 Coomassie staining

The electrophoresis gel was fixed and stained with staining solution [0.2% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid] for 1 h. Destaining of excess dye was performed by repeating changes of destining solution [20% (v/v) methanol and 10% (v/v) acetic acid] until the background was clear. Low molecular weight protein markers (LMW calibration kit) from Pharmacia consisted of phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α- lactalbumin (14 kDa) from Sigma were used as a standard protein.

20.2 Tricine-SDS-PAGE

Tricine-SDS-PAGE, polyacrylamide gel (16.5%T, 3%C) was prepared using the method as described by Schägger and von Jagow, 1987 with the following modifications for the gel dimension of the small plate of "ATTO" Dual mini slab series, model AE-6530.

Determination of %T and %C for acrylamide gels:

$$\%\ T = \left[\frac{(\text{acrylamide} + \text{bisacrylamide})\ g}{100\ ml} \right] \times 100$$

$$\%\ C = \left[\frac{(\text{bisacrylamide})\ g}{(\text{acrylamide} + \text{bisacrylamide})\ g} \right] \times 100$$

Where %T is percent total acrylamide

%C is the ratio of cross-linker to acrylamide monomer

Table 4 Composition of reagents for Tricine-SDS-PAGE.

Stock solutions	Separating gel 16.5%T, 3%C (μl)	Spacer gel 10%T, 3%C (μl)	Stacking gel 4%T, 3%C (μl)
Distilled water	1,500	675	1,140
Gel buffer (3 M Tris, pH 8.45)	2,330	500	190
48% Acrylamide + 1.5% Bisacrylamide	2,330	305	120
Glycerol	750	-	-
10% SDS	70	15	15
0.2 M EDTA	-	-	15
10% Ammonium persulfate	12	5	15
TEMED	1.2	0.5	0.75
Total volume (ml)	7 ml	1.5 ml	1.5 ml

Note : Spacer gel ~ 0.1 cm, stacking gel ~0.8 cm (from bottom well to spacer gel).

20.2.1 Preparation of sample for Tricine-SDS-PAGE

The protein samples were mixed with (3x) sample buffer [with final concentration of 50 mM Tris-HCl buffer pH 6.8, 12% (v/v) glycerol, 4% (w/v) sodium dodecyl sulfate (SDS), 2% (v/v) 2-mercaptoethanol and 0.01% (w/v) Serva blue G as the dye] and heated for 10 min in boiling water, spun down protein solution for a minute and loaded into the wells.

20.2.2 Running condition

Electrophoresis was performed at 4°C with the anode buffer in the lower chamber and cathode buffer in the upper chamber. The constant voltage (30 volts) per slab gel was applied until the tracking dye had completely entered the separating gel and 20 mA of

constant current per slab gel was further applied until the tracking dye was approached the bottom of the separating gel.

Cathode buffer (top), ~80 ml	Anode buffer (bottom), ~250 ml
0.1 M Tris-HCl	0.2 M Tris-HCl
0.1 M Tricine	(Adjust to pH 8.9 with HCl)
0.1 % SDS	
pH 8.25	

20.2.3 Serva blue G staining

The electrophoresis gel was fixed in fixing solution [50% (v/v) methanol and 10% (v/v) acetic acid] for 1 h and stained with staining solution [0.025% (w/v) Serva blue G and 10% (v/v) acetic acid]. The stained gel was then destained with several changes of destaining solution [10% (v/v) acetic acid] until the background was clear. Peptide Marker Kit from Amersham Biosciences consisted of horse myoglobin peptides (2512, 6214, 8159, 10 700, 14 404 and 16 969 Da) were used as a standard protein.

21. Glycoprotein staining

The carbohydrates of rubber particle-bound proteins (SRPP) were stained by using Gelcode® Glycoprotein Staining Kit. The staining was performed using the protocol suggested by the manufacturer's instruction.

SDS-PAGE of proteins samples and glycoprotein standard (5 μg) were run on a 12% slab gel. After electrophoresis, gel was fixed by completely immersing it in 100 ml of 50% methanol for 30 min. The gel was then washed by gently agitation with 100 ml of 3% acetic acid for 10 min twice. It was then transferred into 25 ml of Oxidizing Solution and gently agitated for 15 min and washed gel by gently agitating with 100 ml of 3% acetic acid for 5 min three times. The gel was stained in 25 ml of GelCode® Glycoprotein Staining Reagent and gently agitated for 15 min and then transferred gel to 25 ml of Reducing Solution and agitated for 5 min. The gel was washed extensively with 3% acetic acid and then with ultrapure water. Glycoproteins appeared as magenta bands and stored gel in 3% acetic acid.

22. Protein determination

Protein was assayed using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories) based on the method of Bradford (Bradford, 1976) with bovine serum albumin as standard. This method is a simple and accurate procedure for determining concentration of solubilized protein. It involves the addition of an acidic dye to protein solution, and subsequent measurement at 595 nm with a spectrophotometer or microplate reader. Comparison to a standard curve provides a relative measurement of protein concentration.

The principle of the Bio-Rad Protein Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie® Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine. Spector found that the extinction coefficient of a dye-albumin complex solution was constant over a 10-fold concentration range. Thus, Beer's law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration.

To prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized (DDI) water. Filter through Whatman #1 filter (or equivalent) to remove particulates. This diluted reagent may be used for approximately 2 weeks when kept at room temperature. Then, prepare five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of the assay for BSA is 0.2-0.9 mg/ml. Pipette 100 µl of each standard and sample solution into a clean, dry test tube. Protein solutions are normally assayed in duplicate or triplicate. Add 5.0 ml of diluted dye reagent to each tube and vortex. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour and measure absorbance at 595 nm.