Materials and Methods

Materials

1. Chemicals

1.1 Buffers and additives

Citric acid, disodium hydrogenphosphate, glycine, hydrochloric acid, methanol, potassium chloride, potassium dihydrogen orthophosphate, sodium carbonate, sodium chloride, sodium dodecyl sulfate, sodium hydrogen carbonate, Tris (hydroxymethylaminomethane), Triton X-100, Tween 20

1.2 Electrophoresis reagents

Acetic acid, acrylamide, ammonium persulfate, ampholytes, bromphenol blue, coomassie brilliant blue, ethylene diaminetetraacetate (EDTA), glycerol, glycine, methanol, 2-mercaptoethanol, N,N´-methylene bis-acrylamide, N,N,N´,N´-tetramethylenediamine (TEMED), riboflavin, tricine

1.3 Protein determination reagents

Bovine serum albumin, copper sulfate, folin-ciocalteu's phenol reagent, sodium carbonate, sodium hydroxide, sodium potassium tartate

1.4 Immunochemicals

Alkaline phosphatase conjugate substrate (Bio-Rad), biotinylated anti-human IgE antibody (Bio-Rad), bovine serum albumin, cobalt chloride, 3,3´-diaminobenzidene tetrahydrochloride (DAB), o-dianisidine, hydrogen peroxide, peroxidase conjugated goat anti-human IgE antibody (Sigma), peroxidase conjugated goat anti-rabbit IgG antibody (Sigma), o-phenylenediamine

dihydrochloride, ponceau–S, streptavidin-alkaline phosphatase conjugate (Bio-Rad)

2. Human sera

Seventeen sera of adult patients with latex allergy (P1-P11 and S1-S6) were provided by two hospitals in Thailand, Songklanagarind Hospital (Hat-Yai) and Siriraj Hospital (Bangkok). These patients were diagnosed by clinical history and showed a positive skin prick test (SPT) response to fresh latex and glove extracts except one female patient (S6) who had been diagnosed with latex allergy based on only her clinical history. The results of SPT obtained from these patients are summerized in Table 4 and 5. Four sera of individuals with negative SPT to fresh latex extract (N1- N4) and one pool serum of cord blood (CB) obtained from Songklanagarind Hospital were also used as negative control. All sera were stored frozen at -20°C until used.

3. Instruments

3.1 Automated Microplate Reader (Bio-Tek, Model: ELx 808)

3.2 Centrifuge (Beckman, Model: Avanti™ 30, J-30I)

3.3 Deep-Freeze Refrigurator

3.4 Electrophoresis Cell (ATTO, Dual Mini Slab)

3.5 Mini IEF Cell (Bio-Rad, Model: 111)

3.6 Orbital Shaker

3.7 Power Supply (Bio-Rad, Model: Power Pac 300)

3.8 Power Supply (Bio-Rad, Model: 1000/500)

3.9 Protein Blotting Cell (Bio-Rad, Mini Trans-Blot)

3.10 Spectrophotometer (Beckman, Model: DU 650 i)

3.11 Ultracentrifuge (Beckman, Model: L8-70M)

Table 4 Intradermal skin test reactivity in subjects with fresh latex extracts.

Subject code	RP	C-serum	B-serum	BFM 1	BFM 2
P1	+	-	-	-	-
P2	-	+	-	-	-
P3	-	-	-	-	+
P4	-	-	-	-	+
P5	-	+	-	-	-
P6	-	-	-	+	-
P7	+	-	-	-	-
P8	-	-	-	+	-
P9	-	+	+	-	+
P10	-	+	+	+	+
P11	+	-	-	-	-
S1	+	+	+	+	+
S2	-	+	+	-	+
S3	-	+	+	-	+
S4	-	+	+	-	+
S5	-	+	+	-	+

BFM 1 = Partial purified 17 kDa protein from bottom fraction membrane
BFM 2 = Triton X-100-extracted proteins from bottom fraction membrane
P1-P11 = Patients from Songklanagarind Hospital
S1-S5 = Patients from Siriraj Hospital
+ = Positive intradermal skin test
- = Negative intradermal skin test

Table 5 Intradermal skin test reactivity in subjects with glove extracts.

Subject code	Unison	Doctor Hand	Ansell	Unknown	Mala
P1	-	-	-	-	-
P2	-	-	-	-	-
P3	-	-	-	-	-
P4	-	-	-	-	-
P5	-	-	-	-	-
P6	+	-	-	-	-
P7	-	+	-	-	-
P8	-	+	-	-	+
P9	+	-	-	+	-
P10	+	-	+	-	+
P11	+	-	-	-	-
S1					+
S2					+
S3					+
S4					+
S5					+

P1-P11 = Patients from Songklanagarind Hospital

S1-S5 = Patients from Siriraj Hospital

\+ = Positive intradermal skin test

\- = Negative intradermal skin test

Methods

1. Collection of latex from rubber tree

The fresh *Hevea* latex used througout this study was obtained from the clone RRIM 600 which grown at Songkhla Rubber Research Center, Hat Yai, Songkhla. The trees were tapped by "jebong" knife on panel BO-1 under ½ S d/2 tapping system. Tapping starts about 06.00 hours and the exuded latex was collected in beakers which were chilled on ice (Fig. 9). Latex collection period of sixty minutes was done on each tapping.

2. Preparation of rubber particles, C-serum and bottom fraction

The chilled latex was filtered through 4 layers of cheese cloth to remove the particulated materials and bark 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 centrifugation, the latex was separated into three distinct layers with the rubber cream and Frey Wyssling complexes on the top, the bottom fraction at the bottom and C-serum in between (Fig. 10). By this separation, the bottom fraction consists mainly of lutoid particles and some heavy fraction of Frey-wyssling complexes as described by Gomez and Samsidar Hamzah (1989).

3. Preparation of lutoid particles

Separation of purified lutoid particles from fresh latex was carried out according to Dupong *et al.* (1976) which obtained by centrifugation on

Fig. 9 Collection of fresh latex from rubber tree.

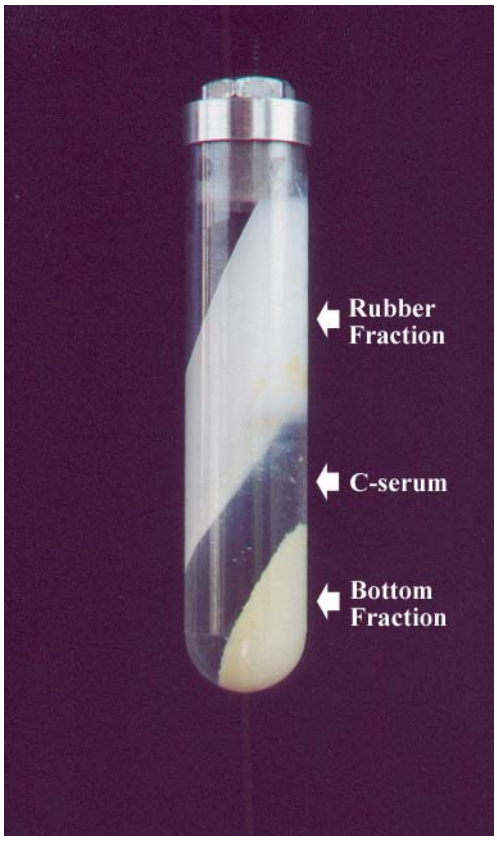

Fig. 10 Ultracentrifugation of fresh latex.

discontinuous sucrose density gradients. First, chilled latex was centrifuged at 8,000 rpm for 15 min at 10°C in a Beckman J-30I centrifuge. After centrifugation, the rubber cream was scooped off and discarded. The bottom fraction was laid on the top of 3 layers of 0.8, 1.2 and 1.8 M sucrose solution. An input of 2.5 ml of bottom fraction was used for each 12 ml centrifuged tube. The tubes were centrifuged in a swinging bucket rotor at 35,000 g for 180 min at 4°C in a Beckman L8-70M ultracentrifuge. Only two fractions of lutoid particles, a light one (from the 0.8/1.2 M interface) and a heavy one (from the 1.2/1.8 M interface) were obtained (Fig. 11). The fraction arrested at the interface 1.2/1.8 M was collected using a fraction collector. This fraction as reported by Dickenson (1965) and Moreau *et al.* (1975) is not contaminated with mitochondria, microsomes or plastid-like Frey-Wyssling particles.

4. Preparation of bottom fraction membrane (BFM)

4.1 Method A

The intact bottom fraction separated from ultracentrifuged latex was washed 2 times to remove the contaminated C-serum and rubber particles by suspending the bottom fraction in five volumes of isotonic buffer and recovered by centrifugation at 59,000 g for 45 min. The washed bottom fraction was then suspended in ten volumes of distilled water (DW) with overnight stirring at 4°C. The remaining insoluble BFM was collected by centrifugation at 10,000 g for 30 min and further washed 3 times with isotonic buffer to eliminate residual B-serum.

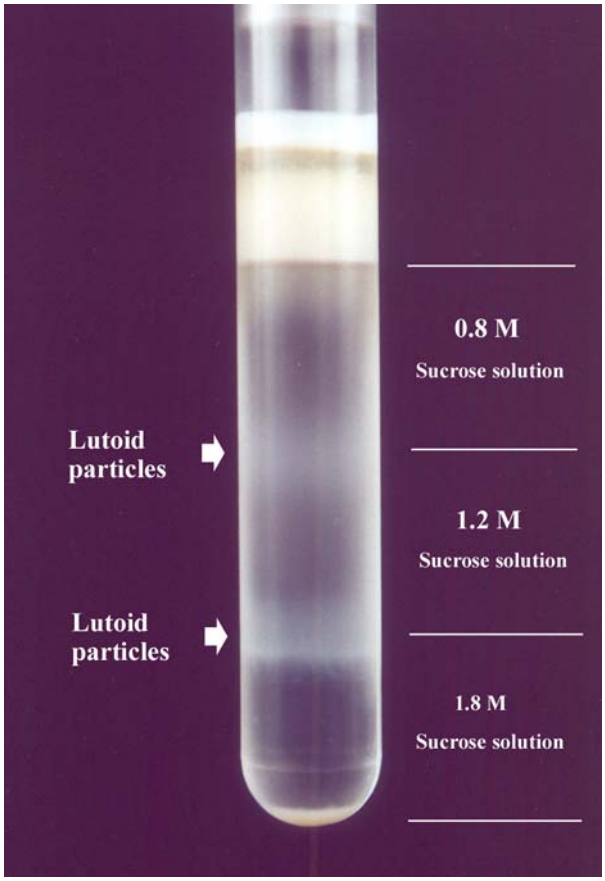

Fig. 11 Separation of purified lutoid particles from fresh latex by centrifugation on discontinuous sucrose density gradients.

4.2 Method B

The washed bottom fraction was suspended in ten volumes of distilled water containing 0.7% ammonium hydroxide with overnight stirring at 4°C. The remaining insoluble BFM was collected by centrifugation at 10,000 g for 30 min and further washed 3 times with isotonic buffer to eliminate residual B-serum.

4.3 Method C

Preparation of BFM was carried out according to Cornish and Backhaus (1990). The bottom fraction was suspended in ten volumes of extraction buffer containing 100mM Tris-HCl, pH 7.5, 50 mM KF, 1% ascorbic acid, 5 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulphonyl fluoride (PMSF). The mixture was overnight stirred at 4°C and the remaining insoluble BFM was collected by centrifugation at 10,000 g for 30 min. The BFM was scooped from the centrifuged tubes and suspended in ten volumes of ice-cold washing buffer containing 100 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$ and 10 mM 1,4-dithiothreitol (DTT). The BFM was again washed for 2 times, 30 min each with continuous stirring and recovered by centrifugation at 10,000 g for 30 min.

5. Extraction of proteins from various fractions of fresh latex

5.1 Rubber particles (RP)

The rubber cream (zone 1 and 2) separated from ultracentrifuged latex was washed 3 times by suspending in a five volumes of isotonic buffer (50 mM Tris-HCl, pH 7.4 containing 0.9% NaCl) with gently stirring for 30 min each at 4˚C and recovered by centrifugation at 59,000 g for 45 min.The proteins which associated within the interfacial layer surrounding the RP were extracted by

suspending the washed RP in five volumes of 50 mM Tris-HCl, pH 7.4 containing 0.2% Triton X-100 with overnight stirring at 4°C. The extract was recovered by centrifugation as before and proteins in the extract were precipitated with cold acetone (80% saturation). The acetone precipitates were frozen in aliquots at -20˚C until used. When use, the precipitate was dissolved in 50 mM Tris-HCl, pH 7.4 containing 0.2% Triton X-100.

5.2 C-serum

The C-serum separated from ultracentrifuged latex was recentrifuged at 59,000 g for 45 min to obtained clear serum. The C-serum was then freeze-dried and redissolved in distilled water.

5.3 B-serum

The bottom fraction separated from ultracentrifuged latex was washed 2 times to remove the contaminated C-serum and rubber particles by suspending in five volumes of isotonic buffer and recovered by centrifugation at 59,000 g for 45 min. The washed bottom fraction was then subjected to alternated freezing (-20˚ C) and thawing (RT) for 4 times to released its B-serum contents. The B-serum was separated from the remaining insoluble bottom fraction membrane by centrifugation at 30,000 g for 30 min. The B-serum was further freeze-dried and redissolved in distilled water.

5.4 Bottom fraction membrane (BFM)

The intrinsic proteins of washed BFM obtained from different preparation were extracted by suspending the BFM in 50 mM Tris-HCl, pH 7.4 containing 0.2% Triton X-100 with overnight stirring at 4°C. The extract was recovered by centrifugation at 10,000 g for 30 min and the proteins were precipitated with

cold acetone (80% saturation). The acetone precipitates were dissolved with 50 mM Tris-HCl, pH 7.4 containing 0.2% Triton X-100.

5.5 Lutoid membrane (LM)

Lutoid particles which obtained by centrifugation on discontinuous sucrose density gradients (from the 1.2/1.8 M interface) were bursted by suspending the lutoid particles in distilled water with overnight stirring at 4°C. The LM was recovered by centrifugation at 10,000 g for 30 min and then washed 3 times with isotonic buffer to eliminated residual B-serum. The intrinsic proteins of LM were extracted by suspending washed LM in 50mM Tris-HCl, pH 7.4 containing 0.2% Triton X-100 with overnight stirring at 4°C. The extract was recovered, precipitated with cold acetone and dissolved in solubilizing buffer similar to those described for BFM proteins.

6. Isolation of proteolipids from BFM

Isolation of proteolipids from washed BFM or LM was carried out according to methods of Folch and Lees (1951) and Hasma (1987). An aliquot containing BFM or LM was extracted with five volumes of a continuously stirred chloroform-methanol mixture (2:1; v/v) for 15 min. The extract was separated and filtered. A flask was filled to the brim with extract and completely immersed in an enamel porcelain pot nine-tenths filled with distilled water containing 0.6% NaCl. The mouth of the flask was 2 inches below the surface of the water. After standing for 48 h, the flask was taken out the water. It contained a lower transparent chloroform phase, an upper water phase and a whitish “fluff” at the interphase (Fig. 12). A much water as possible was removed without disturbing

the fluff and the flask was placed at -20°C. After 6 h the fluff had frozen, while the chloroform remained liquid. Fluff and chloroform were separated by filtration through a filter paper. The fluff on filter paper was transfer to centrifuge tube and centrifuged at 4,500 rpm at 4°C for 30 min. At the end of the centrifugation run the chloroform at the bottom of the tube was removed by means of pipetting. The centrifugation was repeated and some additional chloroform was further removed. The fluff was precipitated with cold acetone (90% saturation) and recovered by centrifugation. The dry acetone fluff precipitate was frozen at -20°C until used.

7. Preparation of 17 and 30-35 kDa BFM proteolipids

The preparation of the two main BFM proteolipids, 17 and 30-35 kDa proteins was carried out as described by Summers and Szewezyk (1996). The proteolipids were extracted by mixing the dry fluff precipitate with DW containing 2% SDS. The solubilized supernatant was separated by centrifugation at 10,000 g for 10 min. The mixture of isolated BFM proteolipids was resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.2 μm pore size Bio-Rad polyvinylidene fluoride (PVDF) membrane. The targeted 17 and 30-35 kDa protein bands on the membrane were excised and eluted from the membrane in 50 mM Tris-HCl, pH 9.5 containing 2% SDS and 1% Triton X-100. After vortexing the membrane in eluant for 10 min, the supernatant was collected and proteins in the extract were precipitated with cold acetone (80% saturation) by keeping at -20°C for 2h. The acetone

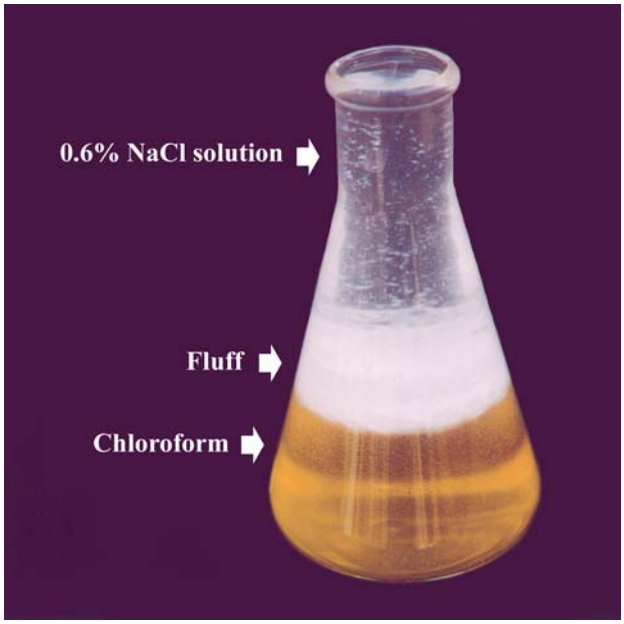

Fig. 12 BFM fluff after washing with distilled water containing 0.6% NaCl for 48 h.

precipitates were dissolved in solubilizing buffer before subjecting to analytical SDS-PAGE and stained to ascertain their purity before used as immunogens.

8. Alkaline stability studies

8.1 Fractionated fresh latex

Alkalinization of B- and C-serum was carried out by direct addition of ammonium hydroxide into the serum solutions to reach 0.7% (v/v). For the insoluble protein fractions of washed RP, BFM and BFM proteolipids-fluff, five volumes of water was first added and followed by ammonium hydroxide to 0.7% (v/v) with an overnight stirring. The alkaline stability of proteins in the respective ammoniated solutions was performed by incubation at room temperature for various durations as indicated.

The alkaline hydrolysates of B- and C-serum mixtures were separated into corresponding clear supernatant fractions by centrifugation after 15, 30, 60, 120 and 180 days of incubation. They were dialysed against frequent changes of cold distilled water and freeze-dried. The dry powder was dissolved with distilled water before subject to SDS-PAGE. Similarly, the alkaline suspension mixture of the RP, BFM and BFM proteolipids were separated by centrifugation into corresponding soluble supernatant and remaining insoluble pellet fraction after 15, 30, 60 and 120 days of incubation. The supernatant of fractions were separately dialysed against frequent changes of cold distilled water before precipitate with acetone. The acetone precipitates were dissolved with solubilizing buffer before subjecting to SDS-PAGE. The respective isolated pellet fractions were rinsed with 50mM Tris-HCl, pH 7.4 buffer and extracted

with Tris-HCl buffer containing either 2% SDS or 0.2% Triton X-100 before resolved under SDS-PAGE.

8.2 The mixture of C-serum and bottom fraction

The C-serum and bottom fraction separated from ultracentrifuged fresh latex was mixed together and ammoniated to 0.7% (v/v) by direct addition of ammonium hydroxide. The mixture was subjected to an overnight stirring at 4°C for complete bursting of lutoid particles. After 15, 30, 60 and 120 days of incubation at room temperature, the alkaline suspension mixture was separated by centrifugation into corresponding soluble supernatant (mixed B- and C-serum) and remaining insoluble pellet fractions. The supernatant fractions were separately dialysed against frequently changes of cold distilled water before precipitated with acetone (90% saturation). The acetone precipitates were dissolved with solubilizing buffer before subjecting to SDS-PAGE. The respective isolated pellet fractions were rinsed with Tris-HCL buffer and extracted with buffer containing 0.2% Triton X-100 before resolved under SDS-PAGE.

9. Preparation of ammoniated latex

Ammoniation of latex was carried out by direct addition of ammonium hydroxide into fresh latex to reach 0.7% (v/v) with an overnight stirring. The ammoniated latex was incubated at room temperature for 60 days with weekly stirring.

10. Extraction of proteins from ammoniated latex

The 60-day-ammoniated latex was separated into corresponding serum and rubber fractions by centrifugation at 50,000 g for 1 h. The serum was separately stirred for 48 h to eliminated the residual ammonia before freeze-dried. The dry powder was then dissolved in distilled water before subjecting to analytical SDS-PAGE or used as immunogens.

11. Preparation of latex concentrate

Field latex of various rubber clones grown at Songkhla Rubber Research Center was collected and sieved through a eighty-mesh sieve into large receptor tanks. Immediate addition of ammonia to reach 0.4% (v/v) was performed and then followed by ZnO_2 (0.125%, w/w) and tetramethylthiuram disulphide (0.125%, w/w) as the secondary preservatives. The ammoniated latex is then transferred to the settling tanks and kept for an overnight to allow settlement of the sludge. The bottom sediment sludge was discarded and upper latex bulk subjected to centrifugation by de Larval centrifuge (type 410A-79M/3786) (Fig. 13). During centrifugation, the concentrate and skim latex were let through separate channels into two stationary gullies where they were collected individually at the same time. Latex concentrate of about 60% dry rubber content was further ammoniated to 0.7% and stored at room temperature for about 1-2 month duration to reach maturation with proper level of mechanical stability time (MST).

Fig. 13 de Larval centrifuge (type 410A-79M/3786).

12. Identification of latex composition during processing the field latex to latex concentrate

A sample of field latex prior to ammoniation, ammoniated latex prior to centrifugation, latex concentrate and skim latex after centrifugation was fractionated by ultracentrifugation at 59,000 g for 1 h. In addition, the discarded sludge which obtained at the bottom of settling tank prior to centrifugation, the remaining serum and sludge within the bowl casing after centrifugation (Fig. 14) were also sampled and ultracentrifuged as above.

13. Extraction of proteins from latex concentrate

High ammonia (HA) latex concentrate was separated into corresponding serum and RP fractions by centrifugation at 59,000 g for 1 h after 4, 37, 101 and 145 days of storage. The serum fraction was isolated and recentrifuged as above to obtained clear serum. The clear serum was dialysed with frequently changes of cold distilled water for 48 h and freeze-dried. The dry powder was dissolved with distilled water before subjecting to SDS-PAGE. The respective isolated RP was rinsed twice with isotonic buffer and then suspended in extracting buffer (50 mM Tris-HCl, pH 7.4 containing 2% SDS) for 24 h. The extract was recovered by centrifugation as before and precipitated with cold acetone (80% saturation). The acetone precipitates were dissolved with 50 mM Tris-HCl, pH 7.4 containing 2% SDS before subjecting to SDS-PAGE.

Fig. 14 Remaining latex serum (A) and sludge (Band C) within the bowl casing of centrifuge.

14. Extraction of proteins and proteolipids from sludge

The pellet separated from ultracentrifuged sludge was washed 3 times by suspending in excess volume of isotonic buffer and recovered by centrifugation at 30,000 g for 30 min. The washed pellet was then resuspended in 2 volumes of 50 mM Tris-HCl,pH 7.4 containing 0.2% Triton X-100 or 2% SDS with overnight stirring. The extract was recovered by centrifugation at 30,000 g for 30 min and precipitated with cold acetone (80% saturation). The protein precipitate was dissolved in 50 mM Tris-HCl, pH 7.4 containing 0.2% Triton X-100 or 2% SDS. For proteolipid extraction, the washed pellet was extracted with 5 volumes of chloroform-methanol mixture (2:1, v/v) under continuously stirred and washed with 0.6% NaCl solution as previously described.

15. Preparation of glove proteins

To study protein contents in various latex gloves, protein extracts were prepared from three brands of non-sterile examination gloves (Unison, Safe Skin and Mala), two brands of sterile surgical glove (Doctor Hand and Unknown) and one brand of household glove (Ansell). Pieces (1x1cm) of gloves weighting 1 g from each brand were extracted with 10 ml of deionized water at 37°C for 2 h. The eluate was centrifuged at 500 g for 20 min to remove insoluble matter before measurement of protein concentration by the Lowry micro assay method (ASTM, D5712-95). The eluates were concentrated by Speedvac concentrator for suitable concentration before subjecting to SDS-PAGE analysis.

To raised polyclonal antibodies against glove proteins, glove protein eluate was prepared from a set of non-sterile examination, ultra-low protein gloves

produced from nine manufactures. Pieces (1x1cm) of gloves weighing 1 g from each brand were extracted overnight with 10 ml of deionized water at room temperature. The eluates were pooled and centrifuged in a Beckman L8-70M centrifuge for 15 min at 10,000 g to remove insoluble matter before freeze-drying. The freeze-dried powder was dissolved in distilled water and recentrifuged as above to obtain clear supernatant and used as immunogen.

16. Determination of protein concentration

16.1 Lowry method (Lowry *et al.,* 1951)

The assay mixture contained 100 ml of protein sample solution and 3 ml of freshly prepared alkaline copper solution (the mixture of 2% $Na_2 CO_3$ in 0.1 N NaOH, 1% potassium tartate and 0.5% $CuSO_4$. 5 H_2O at the ratio of 100:1:1). The mixture was mixed and left to stand at room temperature for 10 min. 0.1 Folin-Ciocalteu's phenol reagent (0.3 ml) was then added, mixed thoroughly and left to stand at room temperature for 30 min. Absorbance reading, recorded at 650 nm, were calibrated against standard bovine serum albumin (BSA). Results were expressed in μg/ml of extracts.

16.2 Lowry micro assay method (ASTM, D 5712-95)

This method provides an analytical test for determination on the amount of total water extractable protein associated with latex gloves.

Glove proteins were extracted in distilled water at 37°C for 2 h as previous described. The proteins in extract were precipitated by using deoxycholate, trichloroacetic and phosphotungstic acids. Sedimentation of the protein pellet was carried out by centrifugation at 6,000 g for 15 min. The

precipitated proteins were dissolved in 0.1 N sodium hydroxide and their concentration determined according to procedure set out in the Bio-Rad DC protein kit. Absorbance was recorded at 750 nm and readings calibrated against standard ovalbumin. Results were presented in μg/g of gloves.

17. Preparation of antibodies

Triton X-100 extracted BFM proteins, BFM proteolipids with MW of 17 kDa and 30-35 kDa, ammoniated latex and glove proteins were used as immunogens. Preparation of these immunogens were as previously described under headings 5.4 (Method A), 6, 10 and 15, respectively. Each immunogen (200 μg) in 0.5 ml phosphate buffered saline (PBS) was mixed with an equal volume of complete Freunds' adjuvant and injected intracutaneously and subcutaneously on the back of two rabbits as described by Dunbar and Schwoebel (1990). Two booster doses, each containing 100 μg of proteins in 0.5 ml PBS with an equal volume of incomplete Freunds' adjuvand, were administrated at three week intervals. Blood was withdrawn from the rabbit and the prepared serum was frozen in aliquots at -20°C until used.

18. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with the Dual Mini Slab (ATTO corporation, Tokyo) and carried out according to the method of Leammli (1970). The separating gel (10x8x0.1cm) containing 7-15% gradient or 12% of acrylamide and the stacking gel (10x2x0.1cm) containing 3 or 4% of acrylamide. The composition of both gels is given in Table 1 and 2.

A protein sample, approximately 30 μg, was treated with solubilizing buffer with final concentration of 0.0625 M Tris-HCl buffer, pH 6.8, 2% SDS, 10% glycerol (w/v), 5% mercaptoethanol (v/v) and 0.001% bromophenol blue as the dye. Before applying the sample, the proteins were dissociated by immersing the sample in boiling water for 5 min.

The standard molecular weight markers (Pharmacia, Sweden) used were phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

The SDS-PAGE was performed at room temperature with the electrode buffer pH 8.3 which consisted of 0.025 M Tris-HCl, 0.193 M glycine and 0.1% SDS. A constant electrical current of 14-20 mA per one slab gel was applied until the tracking dye marker approached the bottom of gel.

After the electrophoresis was completed, the protein bands were fixed and stained for 2 h with 0.2% Coomassie brilliant blue R in 50% methanol and 7% acetic acid at room temperature. Destaining of excess dye was achieved by repetitive changing of destaining solution containing 20% methanol and 10% acetic acid until the clear background was obtained.

Table 6. The composition of separating gel.

Reagents	7%	12%	15%
Distilled water (ml)	5.02	3.35	2.35
1.5 M Tris-HCl, pH 8.8 (ml)	2.50	2.50	2.50
10% (w/v) SDS (µl)	100	100	100
Acrylamide/bis (ml) (30% T, 2.67% C)	2.33	4.00	5.0
10% Ammonium persulfate (µl)	50	50	50
TEMED (µl)	5	5	5
Total monomer (ml)	10	10	10

Table 7. The composition of stacking gel.

Reagents	3%	4%
Distilled water (ml)	6.35	6.1
0.5 M Tris-HCL, pH6.8 (ml)	2.5	2.5
10% (w/v) SDS (µl)	100	100
acrylamide/bis (ml) (30% T, 2.67% C)	1.00	1.33
10% Ammonium persulfate (µl)	50	50
TEMED (µl)	10	10
Total monomer (ml)	10	10

The molecular weight of each protein sample was determined from a plot of relative migration values (R_f) against the log MW of standard makers. R_f was defined as:

$$R_f = \frac{\text{Distance protein has migrated from origin}}{\text{Distance from origin to reference point (the tracking dye)}}$$

19. Tricine-SDS-PAGE

Sample preparation and Tricine-SDS-PAGE were performed according to Schagger and von Jagow (1987). The slab gel (0.1 cm thickness) consisted of three sections: a 7 cm separating gel (16.5% T, 3% C); a 1 cm spacer gel (10% T, 3% C) and a 0.8 cm stacking gel (4% T, 3% C). T denotes the total percentage concentration of both monomers (acrylamide and bisacrylamide). C denotes the percentage concentration of the cross-linker relative to the total concentration T. The composition of separating, spacer and stacking gels is given in Table 3.

The protein samples were incubated for 10 min at 90°C in 4% SDS, 12% glycerol (w/v), 50 mM tris, 2% mercaptoethanol (v/v), 0.01% Serva blue G adjusted with HCl to pH 6.8.

The standard MW markers (Phamacia, Sweden) used were globin III (2.5 kDa), globin II (6.2 kDa), globin I (8.1 kDa), globin I+III (10.7 kDa), globin I+II (14.4 kDa) and globin (16.9 kDa).

Table 8. The composition of separating, spacer and stacking gel (μl).

Reagents	Separating gel (16.5% T, 3% C)	Spacer gel (10% T, 3% C)	Stacking gel (4% T, 3% C)
Distilled water	1,500	1,350	1,140
Gel buffer (3 M Tris, pH 8.45)	2,300	1,000	190
Acrylamide-bis (49.5% T, 3% C)	2,300	610	120
Glycerol	750	-	-
10% SDS	70	30	15
0.2 M EDTA	-	-	15
10% APS	2.4	10	15
TEMED	2.4	1.0	1.0
Total monomer	7 ml	3 ml	15 ml

The electrophoresis was performed at room temperature with the cathode buffer, pH 8.25 (0.1 M Tris, 0.1 M Tricine and 0.1 M SDS) at the upper chamber and anode buffer, pH 8.9 (0.2 M Tris) at the lower chamber. The electrophoresis run started at 30 V. After about 30 min, when the sample had completely enter the stacking gel, the running condition was set at 40 mA at 4°C.

After the electrophoresis was completed, the protein bands were fixed in a solution containing 50% methanol and 10% acetic acid for 30 min, before they were stained with 0.025% Serva blue G in 10% acetic acid for 2 h. A complete background destaining was achieved by shaking the gels in 10% acetic acid for 2 h; the destaining solution was renewed every 30 min.

20. Polyacrylamide gel isoelectric focusing

Isoelectric focusing was performed with the Bio-Rad Mini IEF cell (Model 111) and carried out according to the instruction manual. The polyacrylamide slab gel (3.16 ml) containing 0.625 ml of monomer concentrated stock solution (25% acrylamide and 0.07% bis), 0.625 ml of 25% (v/v) glycerol, 0.16 ml of Bio-lyte ampholytes 3/10 and 1.72 ml of deionized water. The gel mixture was degassed for 5 min before it was initially polymerized by adding 10 µl of 0.02% ammonium persulfate, 15 ml of 0.1 riboflavin and 5 µl of TEMED and immediately poured into the casting tray. The gel was photopolymerized for 45 min at room temperature. After the gel had set completely, the casting tray was removed. The samples and standard markers were directly applied to the gel surface and left to diffuse into the gel for 5 min. Focusing was carried out at constant voltage in a stepwised manner at 100 V for 15 min, 200 V for 15 min

and finally 450 V for 60 min. After focusing was completed, the glass plate was removed from the gel supporting film. The protein bands in IEF gel were visualized by staining with solution containing 0.04% (w/v) Coomassie Brilliant blue G 250, 27% (v/v) isopropanol and 1% (v/v) acetic acid and followed by destaining with solution containing 25% (v/v) isopropanol and 7% (v/v) acetic acid until the background was clear.

The IEF markers (Phamacia, Sweden) used for determination of pI value were amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.6), β- lactoglubulin (pI 5.1), cabonic anhydrase II (pI 5.9), cabonic anhydrase I (pI 6.6), myoglobulin (pI 6.8 and 7.2), lentil lectin (pI 8.6 and 8.8) and trypsinogen (pI 9.3).

21. Thermal stability of ammonia-treated BFM proteins

The released BFM proteins in solution media of the 60-day alkaline treated BFM were tested for thermal stability by incubating the solution media at the temperature of latex prevulcanization (70°C, 2 h) in water bath. After incubation, the solution was centrifuged at 15,000 g for 15 min to precipitate the denatured proteins and the thermal stable proteins remaining in the supernatant were resolved under SDS-PAGE.

22. Electrophoretic blotting and immunological detection

22.1 Electrophoretic blotting

Electrophoretic blotting was performed basically to Towbin (1979). Proteins were first subjected to electrophoresis and then transferred to nitrocellulose sheets as follows.

Nitrocellulose (0.45 μm pore size) was immersed in a blotting buffer (25 mM Tris, 192 mM glycine, pH 8.3). Upon completion of electrophoresis, the gel, on a layer of wet Whatman 3 MM paper, was placed on a Scotch-Brite pad. The transfer membrane was put on the top of the gel, making sure that no air bubbles were trapped between the membrane and the gel. The membrane was covered with two sheets of Whatman 3 MM paper and a second Scotch-Brite pad. The assembly was mounted in a gel holder which was then inserted, with the membrane toward the anode, into a Mini Trans Blot Cell (Bio-Rad laboratories, Richmond, Califonia) containing about 0.75 litre of cold transfer buffer. Protein transfer was carried out at 100V for 1 h at 4°C.

After blotting, the membrane was stained with 0.5% Ponceau S red in 1% acetic acid solution to demonstrated successful transfer of proteins and then destained with distilled water.

22.2 Latex-specific IgE antibodies

22.2.1 Horseradish Peroxidase System

The sheets or strips (0.4 cm width) of blotted membrane were soaked overnight at room temperature in blocking buffer containing 1% BSA in PBS. After blocking, human sera diluted 1:10 in blocking buffer were added and incubated for 18 h at 4°C with continuous shaking. The sheets or strips of cellulose membrane were then washed four times each with PBS. Peroxidase conjugated goat anti-human diluted 1:1000 in blocking buffer was then added and the sheets or strips were incubated for 2 h at room temperature with continuous shaking. After washing four times for ten min each with PBS, the sheets or strips were placed in a DAB substrate solution.

22.2.2 Amplified detection system

The blotted membrane was rinsed with Tris buffered saline (TBS) and then soaked overnight at room temperature with blocking buffer containing 1% BSA in TBS. After blocking, human sera diluted 1:10 in TBS containing 1% BSA and 0.05% Tween 20 were added and the membrane was incubated overnight at 4°C with continuous shaking. The membrane was then washed 3 times for 10 min each with TBS containing 0.05% Tween-20 (TTBS). Biotinylated anti-human IgE (Vector Laboratories, Burhingame, CA) dilution 1:2,000 in TTBS was added and the membrane was incubated for 1 h at room temperature. After washing as before, the membrane was incubated with a 1:5,000 dilution in TBS of streptavidin-alkaline phosphatase conjugate (Bio-Rad Laboratory, CA) for 30 min at room temperature. After washing, the Bio-Rad substrate kit containing nitro blue tetrazolium (NBT) and 5-bromo-4 chloro-3 indolyl phosphate (BCIP) was added. IgE binding was recognized by the appearance of a purple color on the membrane.

23. Indirect ELISA

Standard published methods were used for optimising the ELISA (Avrameas and Guilbert, 1971; Engvall and Perlman, 1971). Flat bottomed, Maxi Sorp (Nunc, Denmark) were coated using 100µl of protein dilutions in carbonate-bicarbonate buffer, pH 9.6. The coated proteins were extracted from RP, BFM, B- and C-serum. Proteins were allowed to bind overnight at 4°C and the following day the plate was blocked for 1 h at 37°C using a solution containing 1% BSA and 0.05% Tween-20 in PBS. The plate was then washed 3 times with a

1 min soak time between washes. The wash buffer used was PBS containing 0.05% Tween-20 (PBS-T).

Serial dilutions of rabbit antiserum were made in PBS-T. 100 µl was loaded into the wells of antigen coated microtitre plate and incubated for 1 h at 37°C after which unbound antisera was removed using the same washing protocol as described above. The conjugate, goat anti-rabbit IgG-HRP (Sigma Chemical Co.), was diluted 1: 5000 in PBS-T and 100 µl dispensed into each well. After incubation for 1 h at 37°C, the plate was washed as described above and 100µl of a substrate solution (hydrogen peroxide and O-phenylenediamine dihydrochloride) was added. Color was allowed to develop for 30 min in the dark before the reaction was stopped with 50 µl of 2 M sulphuric acid. Absorbance was read using a spectrophotometric plate reader (Bio-Tex, EL_x 808) with a single wavelength at 492 nm.