Chapter 3

Results

3.1 Natural latex specimen

3.1.1 Collection

Fresh field latex from all rubber clones was adjusted with 0.4 M phosphate buffer pH 7.4 (latex : buffer = 3:1) to the final concentration of 0.1 M phosphate, and stored at 4° C in an ice box without other preservative substance. For clone RRIM600, fresh latex was collected in a 2 l bottle from a plantation in Ampur Pluak Dang, Rayong Province, 170 km from Bangkok. Latices from clone GT1 and PB5/51 were collected from a plantation in Ampur Ban Khai, Rayong Province, 190 km from Bangkok. These specimens were collected in series of 50 ml plastic screw-cap tubes filled up to 40 ml level, of total volume 1,200 ml. Latex of clone KRS165, was collected from Pong Rad Rubber Research Station in Chantaburi Province, as 2x900 ml bottles. The container, the volume, and transportation time of latex specimens from each rubber clone were different, due to the difference in location of plantation, distance and time of specimens collection. Latices from clones RRIM600 and KRS165 were collected and stored overnight before protein separation on the next day. For clones GT1 and PB5/51, specimens were transported by bus to Bangkok and processed within 6 hours after collection. Most of the previous published data on rubber proteins were from latices preserved in ammonia or frozen and thaw just before protein separation. In this study the transportation time from collection sites to the Biochemistry laboratory, Chulalongkorn University, varied from 4-12 hours.

The dry rubber content (DRC) was determined before use with correction for the dilution by phosphate buffer after tapping and the results were shown in Table 3.1. The DRC of these latex specimens were in the range of 26.9-34.3 %.

3.2 Preparation of latex proteins from clear serum (C-serum)

3.2.1 **C-serum separation**

After centrifugation, the latex was separated in three phases, rubber phase, C-serum, and the bottom fraction. The C-serum was harvested by a syringe attached to a long needle. From one litter of diluted latex from each clone the C-sera obtained were 732, 824, 719 and 736 ml respectively. It was about 70% of the latex volume (after correction with diluting factor). The soluble protein concentration in the C-serum varied from clone to clone, but the soluble protein concentrations per total latex volume are more or less the same (Table 3.2).

3.2.2 **Yield after acetone precipitation and dialysis**

Cold acetone precipitated C-serum protein was harvested by centrifugation. Pellet was dissolved to initial volume with distilled water before protein determination. The yield of soluble protein of each rubber clone is shown in Table 3.3 ranging from 3.9 to 4.1 mg/ml. Protein recovery was approximately 90 %.

3.2.3 **Dialysis of protein**

After dialysis against distilled water the protein was measured and shows in Table 3.3. The recovery was about 80 %.

Table 3.1 DRC of latex specimens and collection dates.

Clone	Date	%DRC
RRIM600	9/Jan/96	34.3
GT1	14/May/96	26.9
PB5/50	14/May/96	36.1
KRS165	2/May/96	33.8

Table 3.2 Soluble protein concentration in C-sera of four-rubber clones: RRIM600; GT1; PB5/51 and KRS165.

Latex protein	RRIM600	GT1	PB5/51	KRS165
C-serum	732	824	719	736
Latex volume (ml)	750	750	750	750
Buffer volume (ml)	250	250	250	250
Corrected C-serum volume (ml)	492.75	548.25	479.25	496.50
Protein concentration (mg/ml of C-serum)	6.7	6.1	6.7	6.6
Soluble protein conc. (mg/ml of latex)	4.4	4.5	4.3	4.4

Table 3.3 C-serum protein recovery after acetone precipitation and dialysis.

Clone	Soluble protein concentration (mg/ml)			
	C-serum	After acetone precipitation	After dialysis	% recovery
RRIM600	4.4	4.1	3.5	79.5
GT1	4.2	3.9	3.4	80.9
PB5/51	4.4	4.0	3.5	79.5
KRS165	4.4	4.0	3.5	79.5

3.2.4 Distribution pattern of soluble protein in SDS PAGE

Separation of C-serum proteins by SDS gel electrophoresis demonstrated similar distribution patterns of latex proteins from 4 clones: RRIM600; GT1; PB5/51 and KRS 165 (Figure 3.1). The range of molecular weight from 14 to 66 kD were detected in all rubber clones studied, with major bands located at 36, 45, and 66 kD.

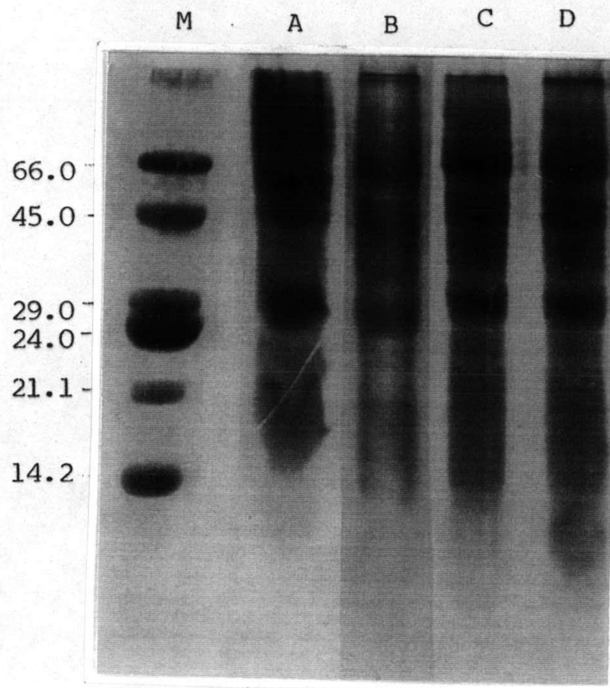

Figure 3.1 SDS-PAGE analysis of C-serum proteins in 15 % polyacrylamide gel. Lane M = molecular weight markers, lane A = clone RRIM600, lane B = clone GT1, lane C = clone PB5/51 and lane D = clone KRS165. Each lane was loaded with latex proteins 1 mg in 20 μl.

3.3 Human serum samples

2.3.1 **Blood donor's sera**

The control sera were from the healthy adults who has donated blood regularly at the Red Cross, Pinklao Hospital Branch and has reported no history of any allergy. The control sera include 176 males and 176 females with age ranging from 18 to 50 years old and with no historical symptom of rubber allergy. Distribution of occupations among 372 donors are 110 students, 30 college teachers, 15 civil officers, 50 factorial workers (other than rubber industry), 25 merchants, 25 office employees, 4 nurses, 43 naval officers and 50 sailors(Table 3.4).

2.3.2 **General allergy patient's sera**

Samples of general allergic patient's sera in this study can be divided into two groups, adults (100 cases) and children (100 cases), occupation data of the adult patients was shown in Table 3.5. These allergic cases can be categorized by three general allergic symptoms into: 1) ulticaria, 93 cases; 2) Rhinitis, 60 cases; and 3) conjunctivitis 47 cases (Table 3.6). These allergic groups are 37 men, 56 women, 56 boys and 44 girls.

3.4 Latex allergens

3.4.1 **Enzyme allergosorbent test**

Enzyme Allergosorbent Test (EAST) showed positive results evident by the yellow-color wells (Figure 3.2). Total positive EAST results were 16 cases among 372 donor's sera or 4.5%. In 200 general allergic patient's sera, EAST was positive 22 cases (11%) shown in Table 3.6.

3.4.2 **Identification of latex protein allergens by immuno stain**

Sera from individuals with positive EAST were used for immuno staining of latex proteins that were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The IgE antibodies from positive EAST sera bound in a heterogeneous manner to 6 latex polypeptides of different molecular weights: 14, 18, 25.5, 30, 32, 38 and 52 kD (Figure 3.3 and Table 3.7). The number of latex polypeptides that reacted with specific IgE in the sera of atopic cases and control group are varied, this study showed the maximum number of 3 polypeptides can be found in one serum specimen (Figure 3.3 and Table 3.8).The result of immunoblot test that was considered positive if the binding of IgE to at least one polypeptide was demonstrated. Using this criterion positive immunoblot was detected most frequently against a 30 kD antigen which bound IgE antibodies in 12 of 22 (54%) patient sera, and 5 of 16 control sera. The 25.5 kD antigen was placed at second most frequently detected antigen followed by the 18 kD polypeptide. It is concluded that the major latex protein allergens are polypeptide of the MW range 14-52 kD.

Table 3.4 Donor's occupational information.

Occupation	Male	Female
Student	50	60
Teacher	12	18
Civil Officer	7	8
Industrial Worker	10	40
Office Employee	14	11
Merchant	13	12
Nurse	-	4
Naval Personnel	70	23

Table 3.5 Occupational information of general allergic adults patients.

Occupation	Male	Female
Student	5	8
Teacher	-	3
Civil Officer	6	5
Industrial Worker	9	10
Office Employee	3	4
Merchant	10	12
Naval Personnel	4	8
Housewife	-	13

Table 3.6 Distribution of positive EAST results for anti-latex IgE in atopic and control 352 study subjects by age and sex.

Sample group	Sex (no.)	Age ± SD	Symptom			Positive EAST(%)
			Ulticaria	Rhinitis	Conjunctivitis	
Atopic	Male (37)	31.7 ± 7.3	22	8	7	4 (10.8)
Adult	Female (63)	30.2 ± 6.7	34	19	10	8 (12.6)
Children	Male (56)	8.3 ± 5.1	20	18	18	6 (10.7)
	Female (44)	7.6 ± 4.3	17	15	12	4 (9.1)
Total	200		93	60	47	22 (11.0)
Control						
Blood donors	Male (176)	37.5 ± 5.9	-	-	-	7 (3.9)
	Female (176)	29.8 ± 6.4	-	-	-	9 (5.1)
Total	352					16 (4.5)

Note The relative risk of latex allergy in atopic / control group = 2.6 base on a case-control study fourfold table and significantly different by chi-square test ($X^2 = 8.289$, $p < 0.05$). The difference of prevalence between sex and age in each groups is not significantly different ($p > 0.05$).

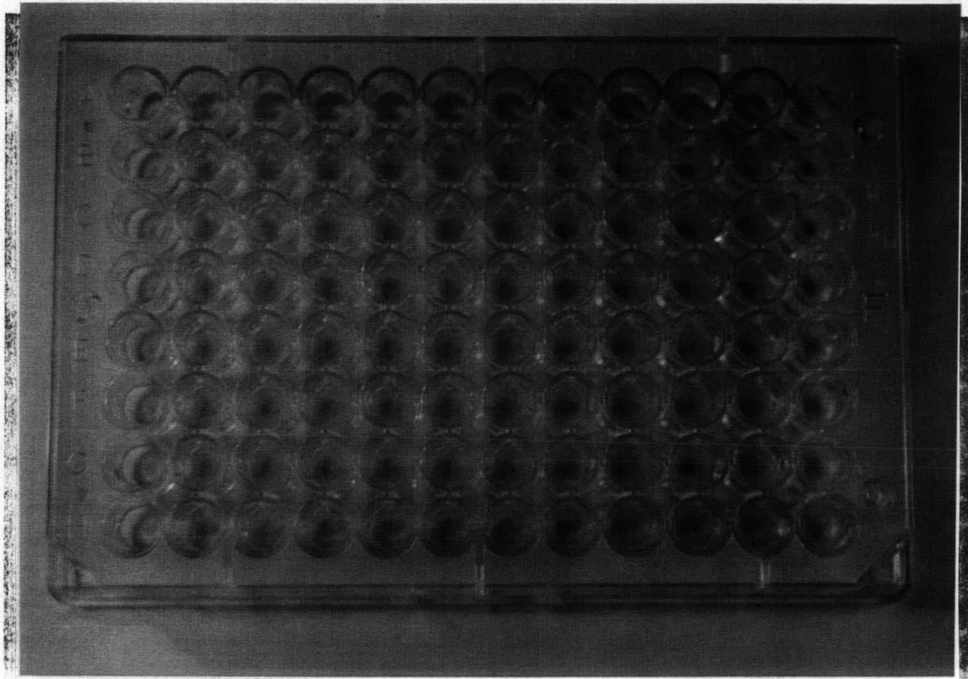

Figure 3.2 Positive EAST of the test sera: the serum that gave positive results were seen as yellow-color wells C3, D6, B10 and G10.

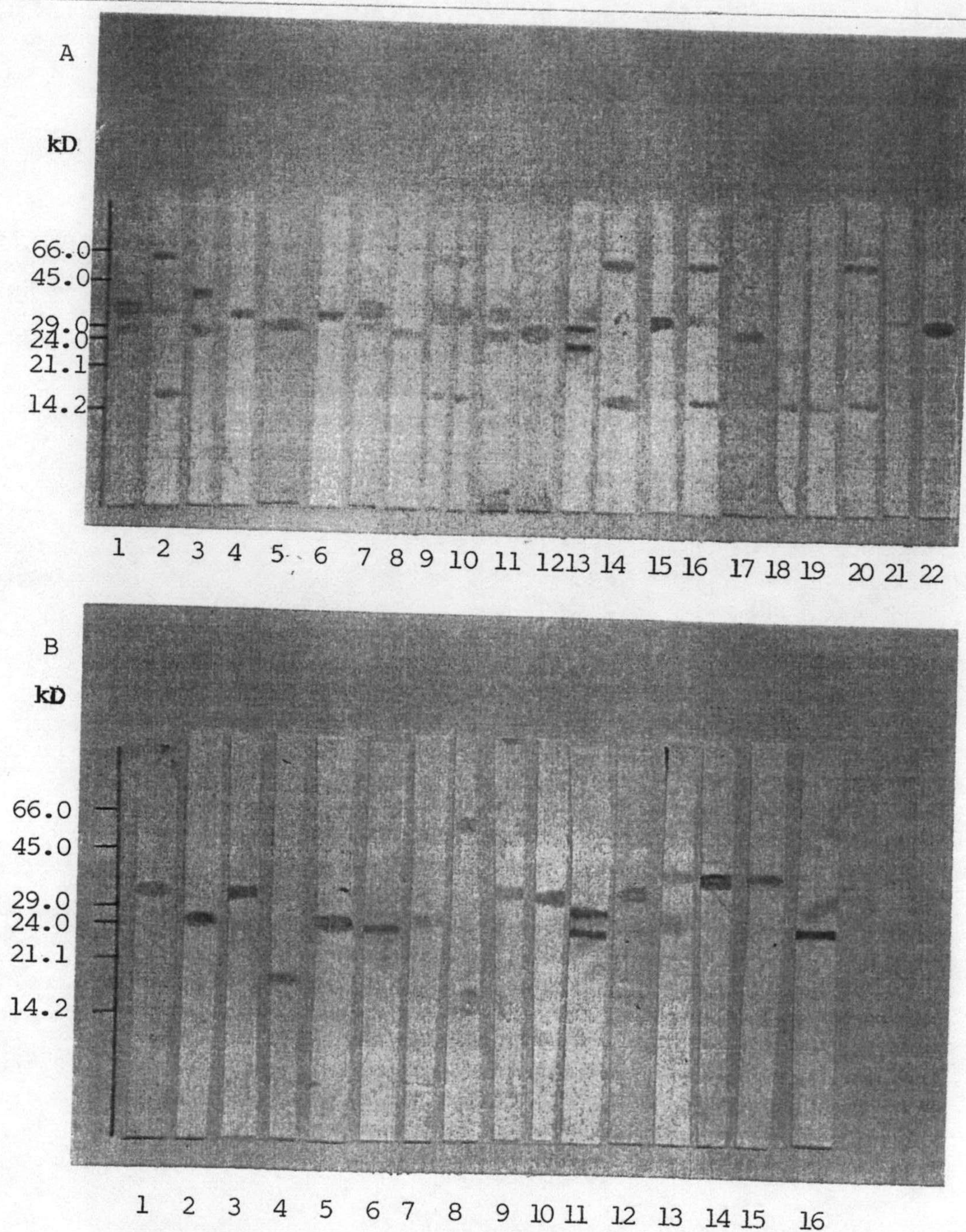

Figure 3.3 IgE immuno blots of acetone precipitated latex proteins:

A, binding of specific IgE from the general allergic sera, strip 1-12 are adults' 13-22 are children's sera. B, specific binding of the IgE from blood donor control.

Table 3.7 The frequency of latex protein antigens that react with the specific IgE in atopic and control sera.

MW of latex protein antigen (kD)	Atopic sera (no.)	Control sera (no.)	Total (no.)
14	-	1	1
18	8	1	9
25.5	7	7	14
30	12	5	17
38	3	1	4
52	6	1	5
Total	22	16	38

Table 3.8 Number of latex protein antigens that react with the specific IgE in sera from atopic and control groups.

No. of latex allergen bound to IgE	Atopic group	Control group
1	11	12
2	7	4
3	4	-
Total	22	16

3.4.3 Prevalence of latex allergens and relative risk

3.4.3.1 Prevalence of latex-specific IgE antibodies

Enzyme Allergosorbent Test showed positive results 11.0% in the allergic sera, these were 4 men, 8 women, 6 boys and 4 girls. In the control sera, there were 16 persons, 7 males and 9 females who showed positive EAST test (4.5%). This prevalence was confirmed by the result of immunoblot (Table 3.6 and Figure 3.3)

3.4.3.2 Risk of NRL-allergy in Thailand.

The probability risk of atopic patient to get anaphylactic hypersensitivity, is estimated by the case-control fourfold table (Mausner and Bahn, 1974). Statistical calculation, chi-square test, showed that atopic patients are considered high risk group as evident by the statistical significant of 2.6 fold higher than the healthyblood donors population, which is calculated $X^2 = 8.289$ and probability is 3.989×10^{-3} ($p<0.05$).

3.4.3.3 Trends of occupation risk

Table 3.9 showed that in blood donor group: industrial workers; office employee; merchant; and nurse have higher risk than the average prevalence of the group (4.5%). In atopic patients office employee and industrial worker showed obviously high risk comparing to the mean of the group of 11.0 %.

3.4.3.4 Relationship of the clinical symptoms and prevalence

Among these 200 atopic cases, of three categories of allergic symptoms there is no significant major clinical symptom related to the anti-latex antibodies positive patients.

Table 3.9 EAST positive results classified by occupation.

Occupation	Atopic Adult	Donor
Student	2 (15%)	4 (4%)
Teacher	-	1 (3%)
Civil Officer & Naval personnel	3 (13%)	1 (1%)
Industrial Worker	3 (16%)	4 (8%)
Office Employee	2 (29%)	3 (12%)
Merchant	1 (4%)	2 (13%)
Nurse	-	1 (25%)
Housewife	1 (8%)	-

Table 3.10 EAST positive results classified by allergic symptom.

Symptom	No. EAST Positive
Ulticaria	11 (12%)
Rhinitis	7 (12%)
Conjunctivitis	4 (8%)

3.5 Rabbit IgG against latex allergens

3.5.1 Gel filtration profiles of latex proteins

Latex protein fraction were separated on a gel filtration chromatography, Bio-Gel P-100. The protein profiles were shown in Figure 3.4. Latex protein from clones RRIM600, GT1, PB5/51 and KRS165 exhibit the same pattern corresponding to the SDS-PAGE (Figure 3.1). The fractions that showed positive EAST with pooled positive EAST serum were those with molecular weight between 14-52 kD. These fractions were pooled and immunized into 4 rabbits (Method 2.5.2).

3.5.2 Rabbit IgG against latex allergens

After immunization, rabbit blood samples were collected every week intervals. The double gel diffusion method (Figure 3.5) was performed and the appearance of rabbit IgG titer had been observed in the second week after the first immunization (Figure 3.6). Maximum titer (8-16) was observed 3 weeks after the second booster and declined. Sera were pooled, aliquoted and stored at -20°C.

3.6 Specific detection of rubber allergens in NR product

3.6.1 Indirect competitive ELISA with rabbit anti-latex allergens

3.6.1.1 Optimization of anti-serum

Preliminary study had been done to determine the appropriate dilution of the first and second antibody in the indirect ELISA technique. Ten microgram per microliter of latex pool allergens were coated on to microtiter wells and twofold serial dilution of the rabbit anti-sera with 1/1,000, 1/5,000, 1/10,000 goat anti-rabbit IgG and A_{405} was shown in Figure 3.7. The appropriate concentration of antisera was 1/1,000.

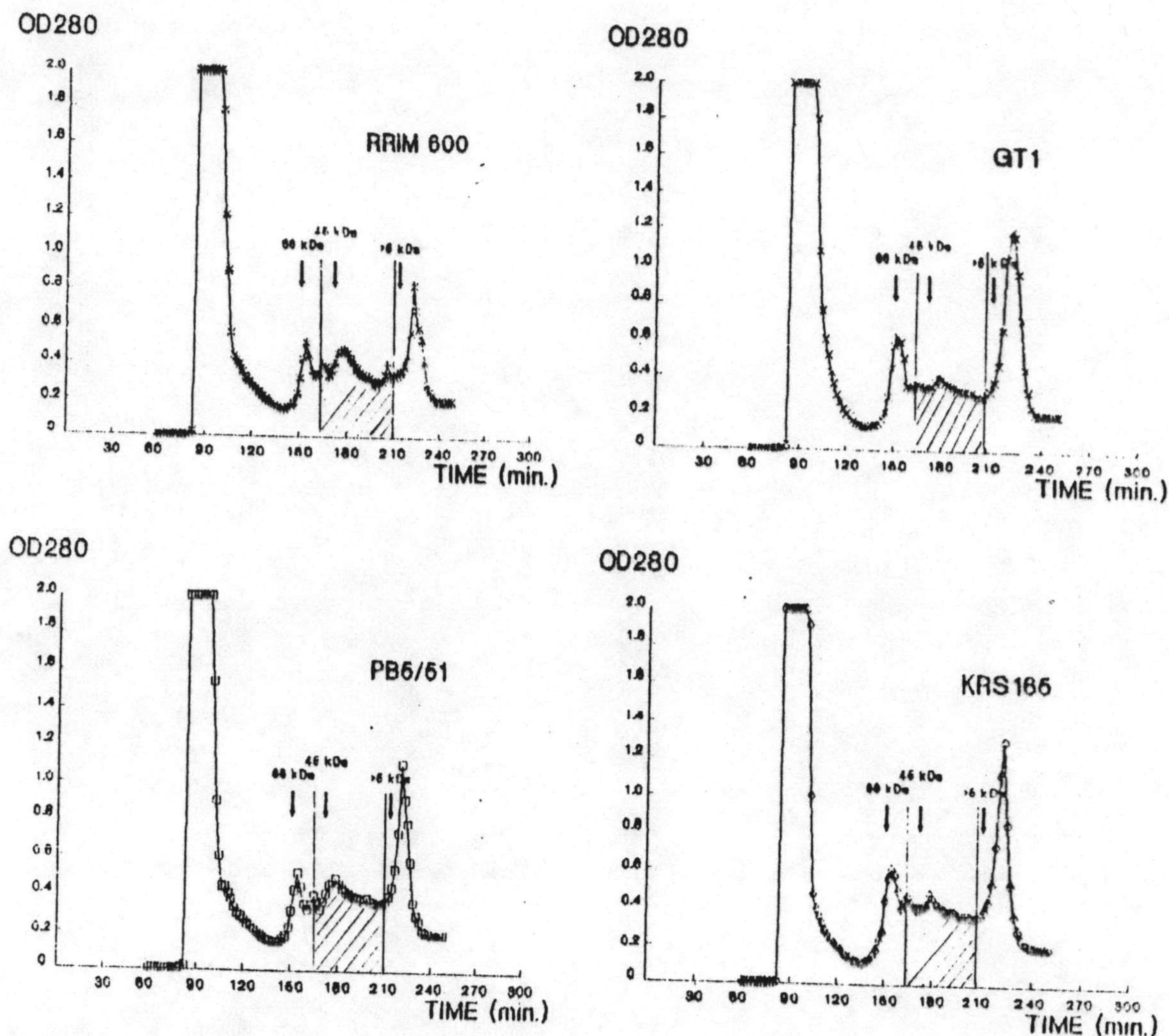

Figure 3.4 Comparison of the distribution of C-serum latex protein fractions from a Bio-Gel P100 column:

Absorbance of fractions at 280 nm, A = rubber clone RRIM600, B = GT1, C = PB5/51, and D = KRS165. Standard MW. markers 66, 45 and 6 kD were used as markers (arrows). Fractions with positive EAST [14-52] were pooled and used as mixture of antigens for immunization.

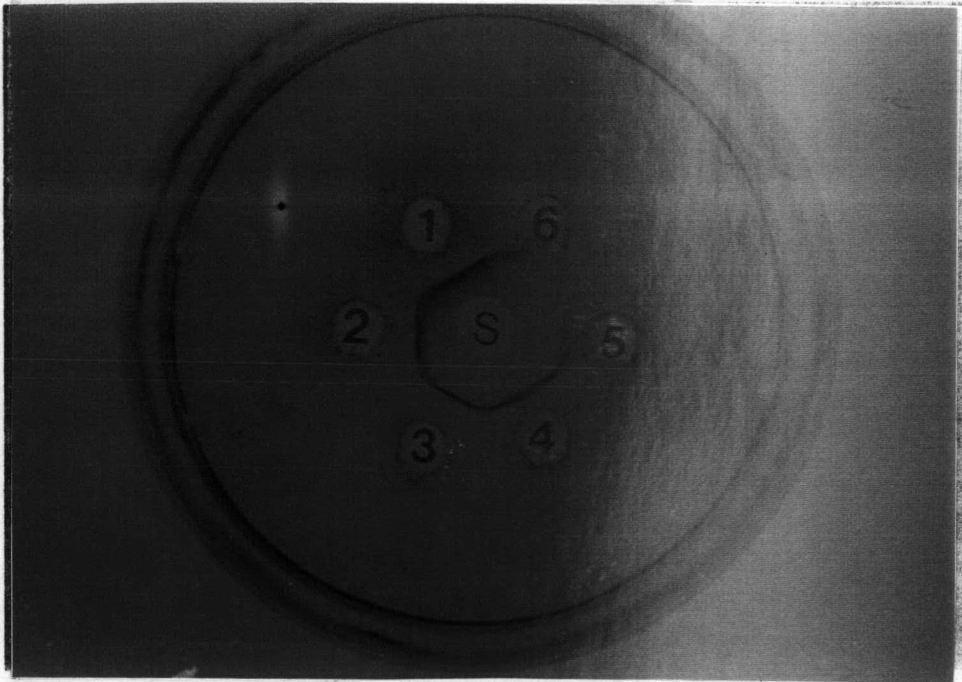

Figure 3.5 Double diffusion gel of rabbit anti-latex allergen:

According to Ouchterlony (1980) method, S = allergen solution ; 1 = 1:2; 2 = diluted $1{:}2^2$ rabbit serum; 3 = diluted $1{:}2^3$ rabbit serum; 4 = diluted $1{:}2^4$ rabbit serum; 5 = diluted $1{:}2^5$ rabbit serum; and 6 = diluted $1{:}2^6$ rabbit serum.

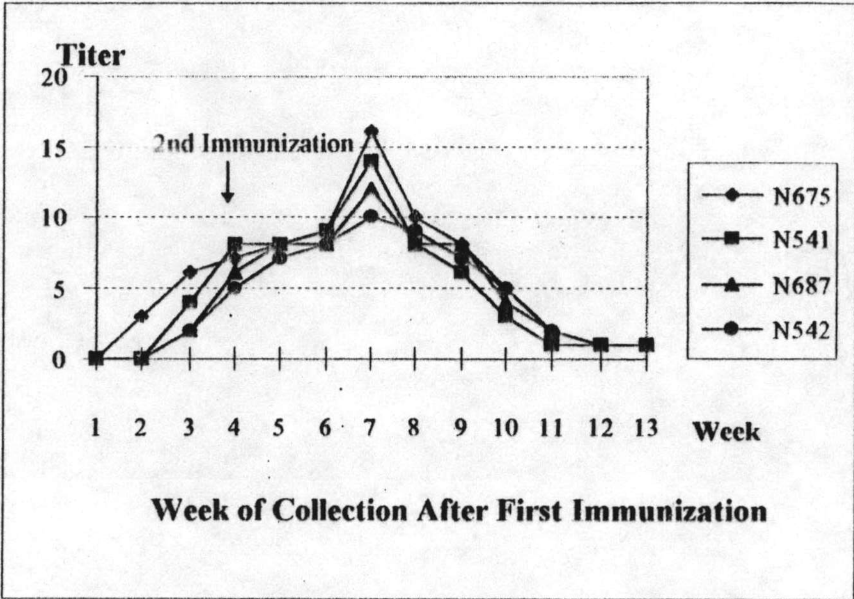

Figure 3.6 Development of anti-latex sera in four rabbits immunized with latex allergens. Antibody titer in the rabbit sera following immunization.

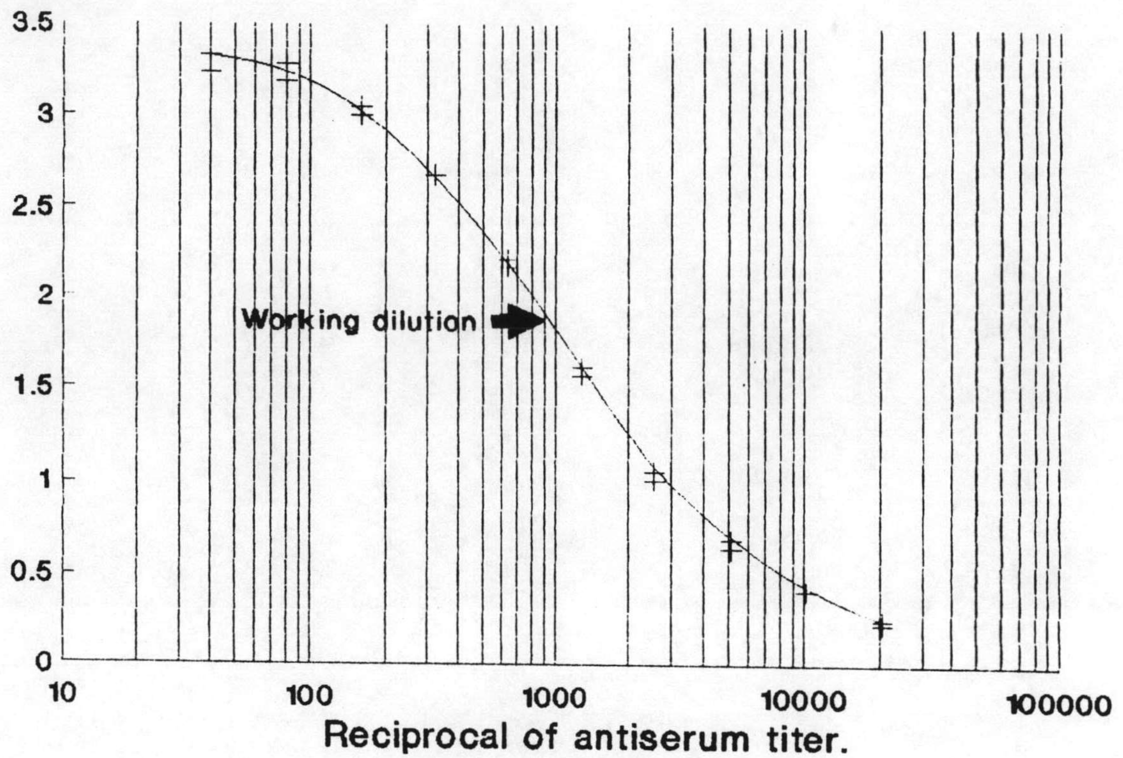

Figure 3.7 Optimization of anti-latex allergen IgG antibody:

Rabbit anti-serum was diluted in two-fold from 1:10 in 96-well microtiter plate. The appropriate concentration of 1:1,000 was selected, which yield optical density (at 405 nm) about 1.8.

3.6.1.2 Allergen detection in NR products

Two types of natural rubber products: gloves and tires, representing products made from concentrated latex and solid rubbers were used to validate the rabbit sera. Antigen inhibition immunoassay was used to quantify latex allergen in samples of gloves from 3 commercial brands, and samples of used tires of used tires from 4 commercial brands. Using latex allergens coated microtitre plate, the rabbit anti-latex protein serum was incubated at the same time with rubber latex proteins allergens or extracted soluble proteins from the rubber gloves and tires. The curve of pooled allergen was a plott of added amount of allergen and % inhibition (Figure 3.8) The amount of allergen/total protein in the unknow samples were determine from plots of % completition versus total protein added (Figure 3.9, 3.10). The range of allergen concentration that can be detected with this method was between 0.01-0.1 μg or 10-100 ng of allergen in each well. C-serum protein allergens in the four rubber clones were detected and showed in Table 3.11, ranging from 675-849 μg/ml of latex. The extracted protein from rubber examination gloves of three brands: C-Rubber Co.Ltd., Union Rubber Gloves Co.Ltd., and WA Rubber mate that were used in Department of Biochemistry, Faculty of Science, Chulalongkorn University and Immunological Laboratory, Phrapinklao Hospital, had been tested and the content of protein allergens measured with this method was 84.4, 62.8, and 17.5 μg/g of glove respectively (Table 3.12). Used rubber tires of four with brand names: Firestone, Bridgestone, Michelin and Siam Tire were extracted for protein and the allergen quantity was measured. It has been found that the allergen concentrations were 4.7, 4.4, 3.8, and 5.2 μg/g of tire respectively (Table 3.13).

3.6.1.3 **Specificity and precision**

The specificity of indirect competitive ELISA was tested by using non-allergen protein: bovine gamma globulin (Bio-Rad Laboratory, Inc. California, USA), ribonuclease A, lysozyme (chicken egg white), bovine serum albumin, and α-chymotrypsin (Sigma Chemical Co., St. Louis, USA) instead of free latex allergen to inhibit the immunoreaction. These proteins do not cross-react with rabbit anti-latex antisera (Table 3.13). The precision of the indirect competitive ELISA were done by both intra- and inter-assays and the results are shown in Table 3.14. The coefficient of variation (cv) is lower than 5 % in the intra-assay and lower than 10% in inter-assay.

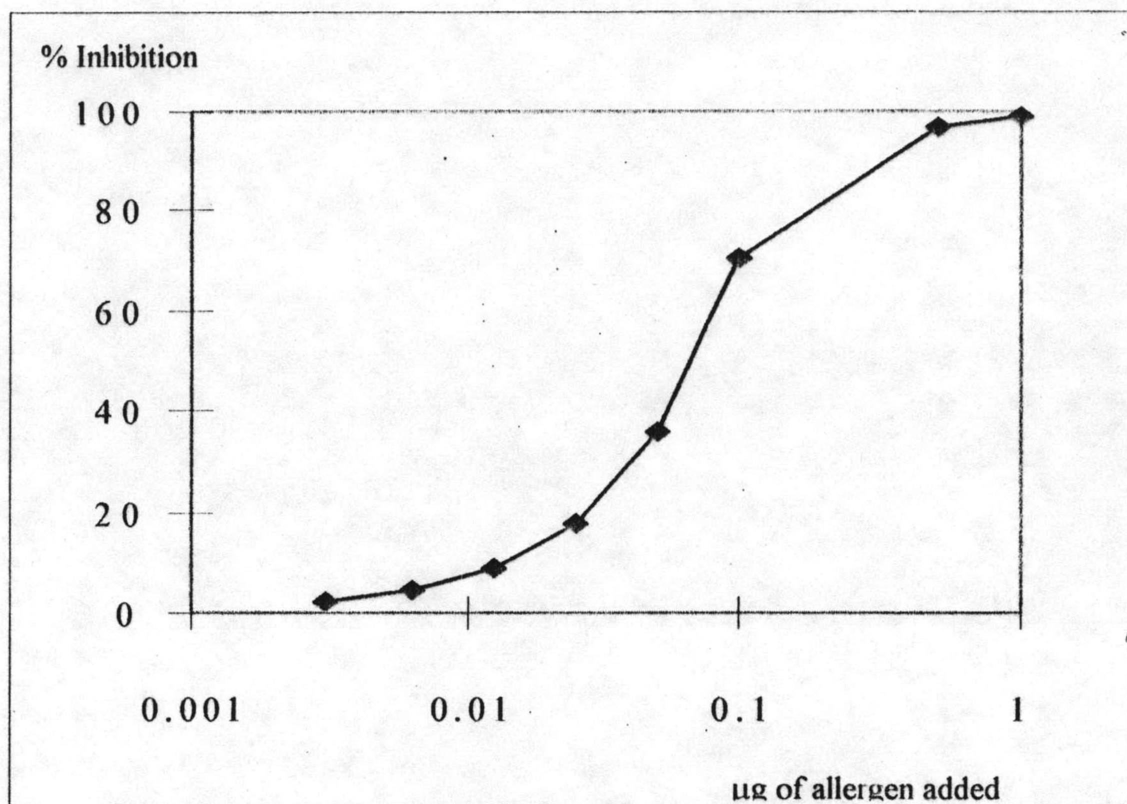

Figure 3.8 A pooled allergen curve of indirect competitive ELISA:

Bound latex allergen in microtiter was reacted with rabitt anti-latex protein serum (1:1,000) in the presence of increasing amount of free latex allergen (0.003125-1.0 µg). The per cent completition correctes with added free allergens.

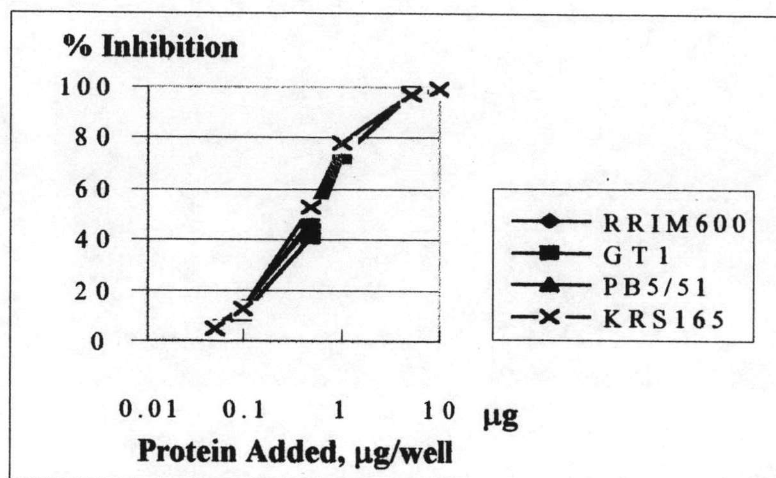

Figure 3.9 Correlation between % inhibition of indirect competitive ELISA and latex total protein. Latex protein allergen was bound to microtiter wells. After wells were washed, rabbit pool sera containing rubber latex allergens-specific IgG was added together with increasing amount of total C-serum protein (0 - 10 µg) from each rubber clone.

Table 3.11 Allergens detection in natural latex : clone RRIM600, GT1, PB5/51 and KRS165.

Specimen	Soluble Protein* (mg/ml)	Allergen (μg/ml)	% of allergen in the total protein
RRIM600	4.4	849	19.3
GT1	4.5	675	15.0
PB5/51	4.3	765	17.8
KRS165	4.4	770	17.5

*Total protein concentration was determined according to Bradford (1976).

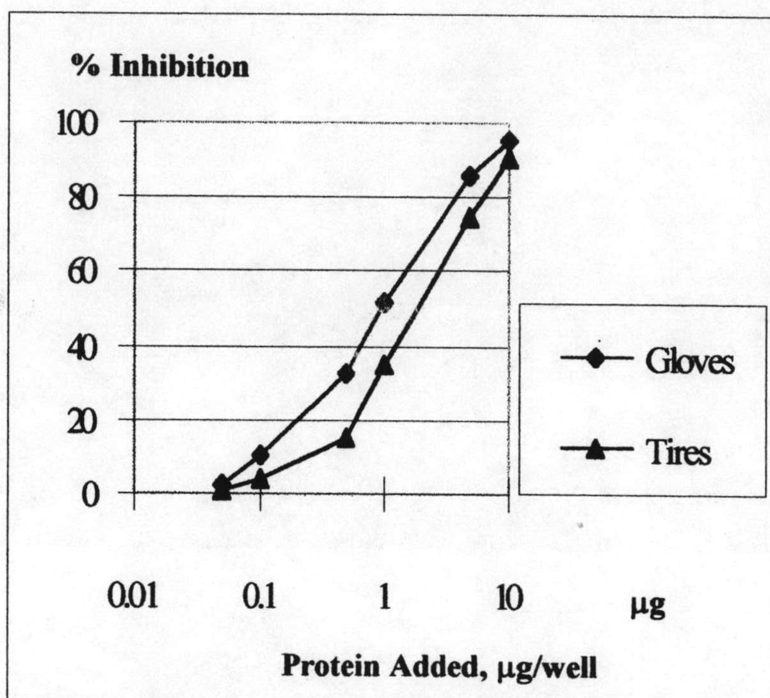

Figure 3.10 Correlation between % inhibition of indirect competitive ELISA and protein extracted from gloves and used tires. Latex protein allergens was bound to microtiter wells. After wells were washed, rabbit pool sera containing rubber latex allergens-specific IgG was added together with increasing amount of protein (0 and 10 µg) rubber gloves or tires extraction proteins.

Table 3.12 Amount of allergens detected in samples of natural latex examination gloves and used-rubber tires by anti-latex allergen rabbit sera produced in this experiment.

Specimen	n	Extracted Protein* (μg/g)	Allergen (μg/g)	% of allergen in the total extracted protein
Glove A	5	418.8	84.2	20.1
Glove B	5	317.2	62.8	19.8
Glove C	5	93.7	17.4	18.6
Firestone	3	1,110.7	4.7	0.4
Bridgestone	3	2,120.6	4.4	0.2
Michelin	3	2,515.2	3.8	0.1
Siam tire	3	3,117.9	5.2	0.2

*Total protein concentration was determined according to Bradford (1976).

Table 3.13 Specificity of indirect competitive ELISA.

Allergen added (μg)	0.003125	0.00625	0.0125	0.025	0.05	0.1	0.5	1.0
% inhibition	2.20	4.41	8.82	17.73	35.70	70.20	96.90	99.10
bovine gamma globulin	0.003125	0.00625	0.0125	0.025	0.05	0.1	0.5	1.0
% inhibition	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ribonuclease A	0.003125	0.00625	0.0125	0.025	0.05	0.1	0.5	1.0
% inhibition	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
lysozyme	0.003125	0.00625	0.0125	0.025	0.05	0.1	0.5	1.0
% inhibition	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BSA	0.003125	0.00625	0.0125	0.025	0.05	0.1	0.5	1.0
% inhibition	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
α-chymotrypsin	0.003125	0.00625	0.0125	0.025	0.05	0.1	0.5	1.0
% inhibition	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 3.14 Precision of the indirect competitive ELISA:

Allergen ranging from 0.3625-100 ng/well showed the percentage of coefficient of variation (% cv) in the intra-assay lower than 5% and inter-assay of ELISA lower than 10%.

	Latex antigen (ng)							
Variance	0.3625	0.625	1.25	2.5	5	10	50	100
intra-assay								
mean	0.364	0.627	1.29	2.5	5.1	9.9	50	98
SD	0.005	0.007	0.02	0.03	0.1	0.2	0.7	2.0
n	11	11	11	11	11	11	11	11
%cv	1.3	1.1	1.5	1.2	1.9	2.0	1.4	4.1
inter-assay								
mean	.362	0.624	1.26	2.5	5.2	10.3	50.4	100
SD	0.011	0.005	0.03	0.13	0.2	0.1	0.7	1.5
n	11	11	11	11	11	11	11	11
%cv	3.0	0.8	2.3	5.2	3.8	0.9	1.4	1.5

3.6.2 **Latex protein dot blot**

The three brands of examination glove were used in dot blot (Figure 3.11) and visual grading can be done roughly. Suitable concentration of the rabbit anti-sera used in this test starting with the dilution of 1:5,000 (serum : phosphate buffer). Sample C has lowest allergen than the others because of the lowest intensity of color. Four spots in each rubber glove sample are replicates of the same procedure, but show variation in color intensity.

3.6.3 **Modified dot blot**

Different background color of rubber gloves made it difficult to compare or grade the quantity of latex upon the surface of latex gloves. Electro-transport of protein onto the nitrocellulose membrane can eliminate the background variation of the specimens. For the three brands of the latex examination gloves the quantities of protein can be estimated by this method (Figure 3.12). Allergenic protein, dropped on the filter paper shows a distinctive visible color at 0.1 - 1.0 μg level (Figure 3.13), but non-allergen protein: bovine gamma globulin, ribonuclease A, lysozyme (chicken egg white), bovine serum albumin, and α-chymotrypsin used as negative control show negative results, similar to the base line color, when no-protein was applied (Figure 3.14).

3.6.4 **Latex particle agglutination inhibition technique**

Glutaladehyde fixed latex particles were serially diluted for a checker box test with the diluted rabbit serum. Condition that provides a good result for reading with naked eyes is 3% DRC glutaladehyde fixed-latex particle and 1:10 dilution of rabbit serum (Figure 3.15). Agglutination could be observed against a dark background. The

bovine gamma globulin, ribonuclease A, lysozyme (chicken egg white), bovine serum albumin or α-chymotrypsin are unable to inhibit agglutination (Figure 3.16).

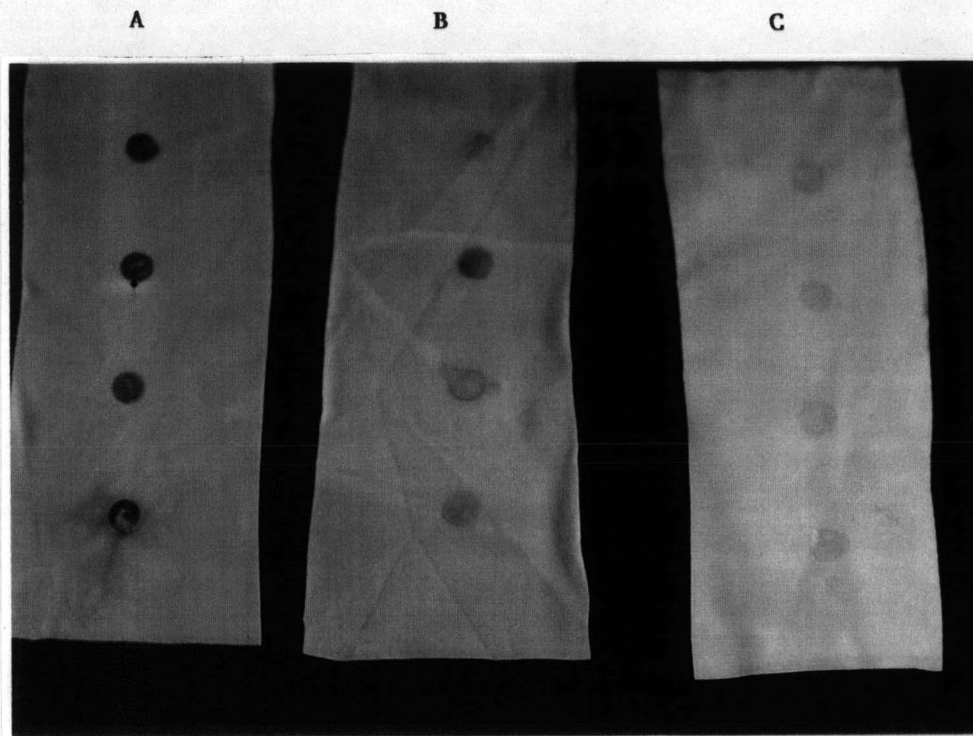

Figure 3.11 The latex dot blot protein assay. This method was modified from Beezhold et al. (1995). Antigen that bound on surface of examination gloves was detected by the latex specific IgG from rabbit.The alkaline phosphatase conjugated anti-IgG was stained and followed by color development: A = glove sample A; B = glove sample B; and C = glove sample C. (Spots in each sample are 4 replicates of the same condition.)

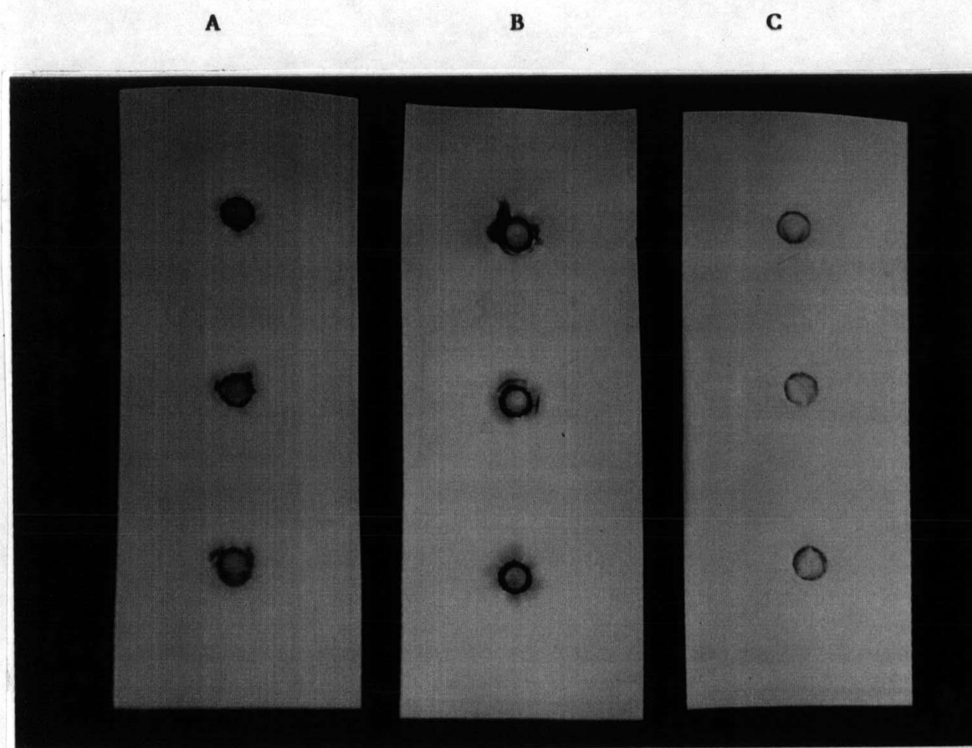

Figure 3.12 Modified dot blot assay. The latex sheet of dot blot (Beezhold, et al. 1995) was electro-transferred onto the nitrocellulose membrane then antibody was stained and the second antibody was introduced followed by color development.

A = protein transferred from glove brand A;

B = protein transferred from glove brand B;

C = protein transferred from glove brand C.

(Spots in each sample are replicates of the same condition.)

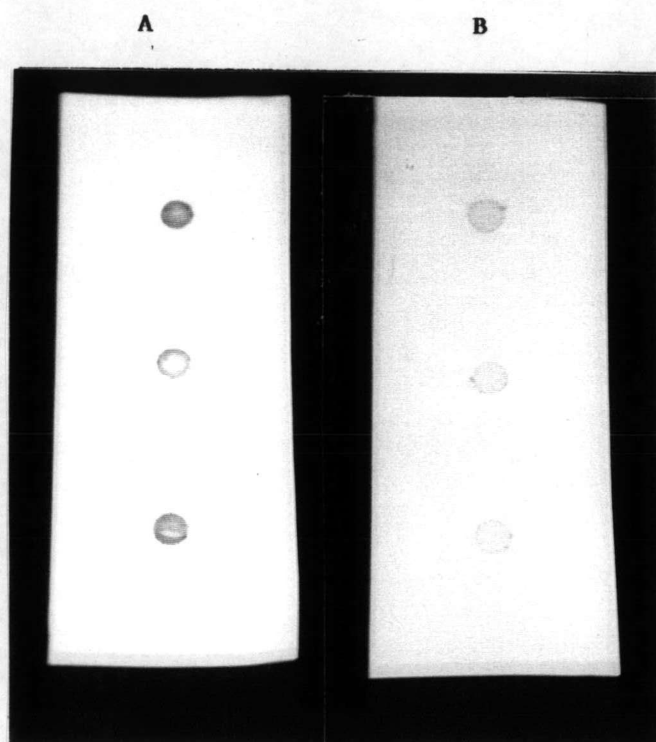

Figure 3.13 Filter papers saturated with 1.0 or 0.1 μg/ml of rubber latex allergen were air-dried before allergen was electro-transferred on to nitrocelulose then stained with specific rabbit IgG, counter stained with anti-rabbit IgG, conjugated with alkaline phosphatase followed by color development. A = allergen concentration 1 μg/ml; B = allergen concentration 0.1 μg/ml. (Spots in each sample are the same condition.)

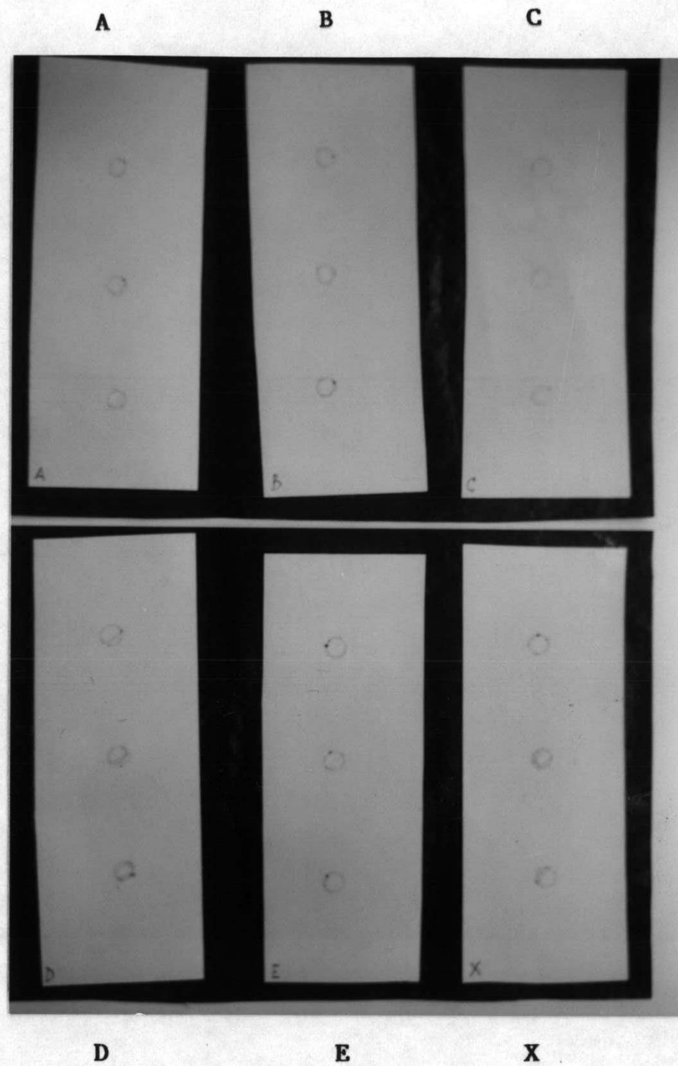

Figure 3.14 Modified-dot blot specificity. Filter paper saturated with 1.0 μg/ml of bovine gamma globulin, ribonuclease A, lysozyme (chicken egg white), bovine serum albumin and α-chymotrypsin were air-dried before the allergen was electro-transferred on to nitrocelulose, stained with specific rabbit IgG, counter stained with anti-rabbit IgG, conjugated with alkaline phosphatase followed by color development. A = bovine gamma globulin; B = ribonuclease A; C = lysozyme (chicken egg white);
D = bovine serum albumin; E = α-chymotrypsin; X = no-protein, blank.

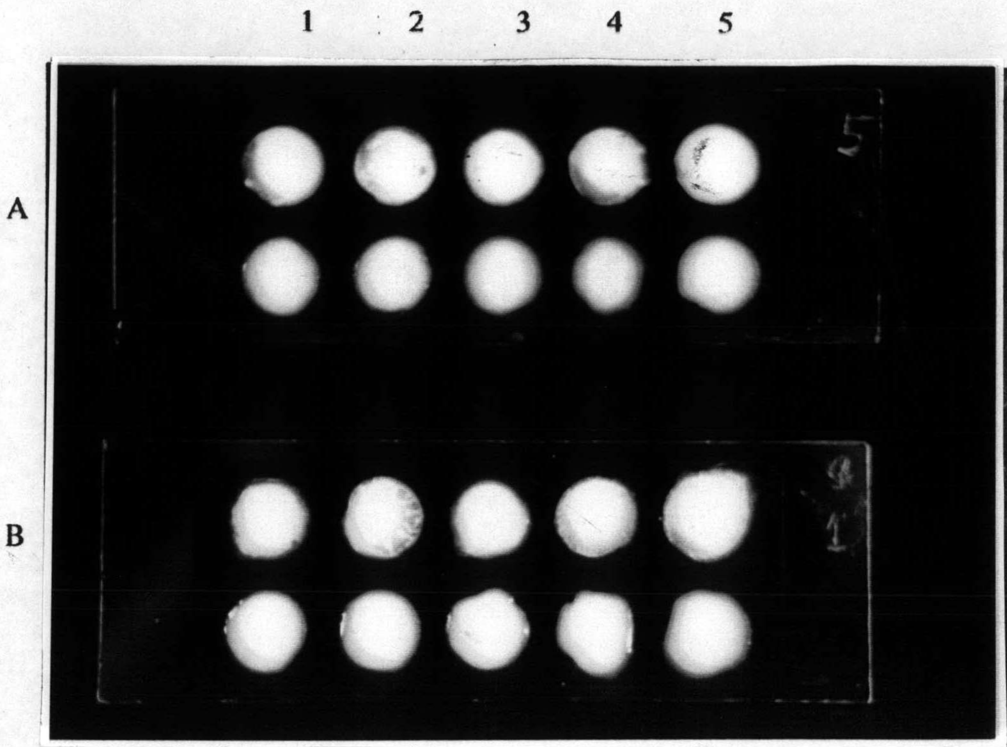

Figure 3.15 In each well of slide A and B, one drop of specific rabbit IgG was applied, followed by glutaraldehyde-fixed latex particle (LP) at reducing % DRC, in the absence free allergen in the upper rows of each slide, or in the presence of 0.1 μg free allergen in the lower rows. Agglutination is visible in upper rows but inhibited by free allergen in the lower rows.

	Slide A	Slide B
Upper row wells	1 2 3 4 5	1 2 3 4 5
IgG	+ + + + +	+ + + + +
LP (%DRC)	10 9 8 7 6	5 4 3 2 1
Lower row wells		
IgG	+ + + + +	+ + + + +
allergen	+ + + + +	+ + + + +
LP (%DRC)	10 9 8 7 6	5 4 3 2 1

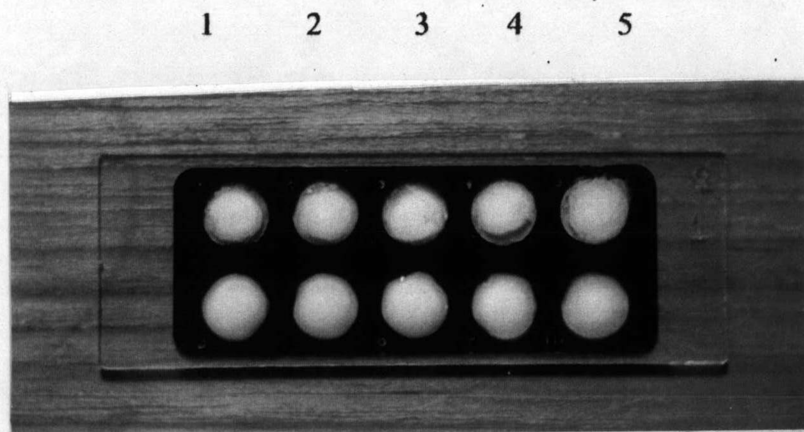

Figure 3.16 Specificity of latex particles agglutination inhibition technique.

No competition can be observed when non allergen proteins were used : Upper row, well 1 = bovine gamma globulin, well 2 = ribonuclease A, well 3 = lysozyme (chicken egg white), well 4 = bovine serum albumin and well 5 = α-chymotrypsin do not inhibit agglutination of the latex particle after adding mixture of antibody to these proteins

: Lower row, positive agglutination when 0.1 μg allergen was added.