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# Identification of a Novel Hydrophobic Allergen from *Hevea brasiliensis* Bottom Fraction Membrane

NOVEL RUBBER LATEX ALLERGEN

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## SUMMARY

A hydrophobic allergen was extracted from *Hevea brasiliensis* via a bottom fraction membrane (BFM). The extracted BFM proteins were used to detect specific human IgE and IgG. Serum samples from latex glove factory workers (LGWs) had noticeably higher levels of both immunoglobulin (Ig) isotypes in comparison to control groups (health care workers, coated allergen particle positive ($CAP^+$) and negative ($CAP^-$)). Modified co-immunoprecipitation was applied in order to isolate the molecules recognized by specific IgE in a subject. The analysis identified a reactive 55 kDa conformational epitope. We suggest that this molecule should be incorporated in the specific IgE immunoassay for screening of rubber latex allergy.

KEYWORDS: latex, allergen, IgG, IgE, immunoprecipitation

## INTRODUCTION

Latex is the milky sap produced by the tropical rubber tree *Hevea brasiliensis* (Euphorbiacea). Proteins from *Hevea* latex in products such as gloves, condoms, and surgical aids can cause a hypersensitivity reaction.[1] Latex allergy has become a serious problem worldwide due to the increasing use of natural rubber latex (NRL) products.[2] Proteins present in various NRL products have been implicated in causing allergenic responses in specific risk groups.[3] One risk group of particular interest consists of health care workers (HCWs) exposed to powdered NRL gloves.[4] Other important risk groups are workers in industries in which latex products are manufactured, including those collecting and processing latex from rubber trees. Patients with congenital anomalies such as spina bifida, those undergoing multiple surgeries, and patients with atopy are more susceptible to latex protein-induced allergy and asthma.[5] It has been reported that 2.8-8.8% of all HCWs and 29-72% of patients suffering from spina bifida are allergic to *Hevea* latex proteins.[6-7]

As described by Moir,[8] when fresh latex is centrifuged, it separates into three layers, with the rubber cream on the top, the bottom fraction at the bottom and the C-serum in between. The bottom fraction consists mainly of lutoid particles and Frey-wyssling complexes. These particles are membrane bound. Lutoids are spherical in shape, vary in size from 0.5-3 μm, and are bound by a single osmosensitive membrane about 8 nm thick.[9] The bottom fraction is subjected to repeated freeze-thraw cycles and recentrifuged so that the supernatant and pellet are separated. The supernatant is the B-serum and the pellet is a mixture of bottom fraction membrane and other co-sedimenting materials. There is evidence suggesting that the water extractable proteins in latex are the cause of the immediate allergy (hypersensitivity type I reaction).[10] Thus, previous studies have concentrated on soluble proteins located in B-

serum and C-serum (reviewed by Nel and Gujuluva).[11] However, some allergens have been found in membrane-bound particles such as Hev b 1 (Rubber Elongation Factor).[12] At present, a total of 13 latex allergens, Hev b1-13[13], have been designated by the International Allergen Nomenclature Committee. According to the latex glove manufacturing processes, pre-vulcanisation and post-vulcanisation leaching usually includes extensive washing steps with water. Thus, water-soluble proteins from C-serum and B-serum can be leached out by washing when the latex is processed.[14] However, hydrophobic proteins still remain in the latex products. Consequently, they may induce the allergic responses in certain glove-users, i.e. HCWs.

Previous studies of latex allergy have focused on the B-serum as the source of allergenic proteins, whereas the residue or pellets have been omitted. The present study aims to isolate proteins from the bottom fraction membrane (BFM) in order to gain intact antigens that have conformational epitopes for allergenicity by focusing on specific binding of IgE and IgG in sera. As a consequence, identification of the novel immunoreactive molecules against IgE will benefit the improvement of latex allergic screening kit.

## MATERIALS AND METHODS

### Serum samples

Serum samples were obtained from 170 latex glove factory workers (LGWs) residing in Songkhla province, Thailand; 35 health care workers (HCWs) samples were generously provided by Dr. Porntip Puvabunditsin, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; 31 coated allergen particle positive ($CAP^+$) and 22 coated allergen particle negative ($CAP^-$) samples were generously provided by Dr. Robert G. Hamilton, Johns Hopkins University School of Medicine, Baltimore, USA. The medical ethics committees of Songklanakarin Hospital, King Chulalongkorn Memorial Hospital and Johns Hopkins Hospital approved the study protocol, and subjects gave written consent for their participation in the study.

### Allergic skin test

A Skin prick test was performed using the bottom fraction membrane proteins preparation as described below. A 50% glycerol in saline solution (diluent) served as a negative control, whereas the positive control was histamine hydrochloride (1 mg/ml in diluent). The Skin test response was assessed as positive if the maximum wheal diameter was at least 3 mm greater than observed for the negative control, and the skin index was greater than 0.6. The skin index was calculated as the ratio of the diameter of the allergen wheal to the histamine wheal.

### ImmunoCAP

Latex ImmunoCAP (Pharmacia Biotech Inc., Sanfancisco, CA) was used to test for latex specific IgE in the sera of patients and controls according to the instruction of the

manufacturer. The protocol of the manufacturer was followed, and a value of 0.35 U/ml or more was considered positive.

**Statistics**

Statistical analysis was performed with GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). Probability (P) values (paired t-tests) less than 0.05 were considered significant. All results were expressed as mean ± SD.

**Collection of latex from rubber trees**

Fresh *Hevea* latex used throughout this study was obtained from regularly tapped trees from clone RRIM 600, which was grown at Songkhla Rubber Research Center, Songkhla, Thailand. The trees were tapped starting at 06.00 hours, and the exuded latex was collected in beakers and chilled on ice. Latex was collected for sixty minutes at each tapping.

**Preparation of BFM proteins**

The chilled latex was filtered through 4 layers of cheesecloth to remove the particulate materials and bark tissue debris. The filtrate was collected and centrifuged at 59,000 × *g* for 45 minutes at 4°C in an ultracentrifuge (Beckman, Model: L 8-70M). 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. The bottom fraction was isolated and washed three times by suspending in 5 volumes of Tris-buffered saline, (TBS: 50 mM Tris-HCl, pH 7.4, containing 0.9% NaCl), stirred for 30 minutes at 4°C and recovered by centrifugation at 30,000 × *g* for 30 minutes. It was then subjected to bursting by alternative freezing and thawing (4 times) and the bottom fraction membrane (BFM) pellet was separated and washed 3 times with TBS. The washed BFM was suspended in 10 volumes of extracting

buffer (50 mM Tris-HCl, pH 7.4, containing 0.2% Triton X-100) and extracted by stirring overnight at 4°C. The supernatant containing extracted BFM proteins was separated after centrifugation at 10,000 × *g* for 25 minutes and incubated with SM2 absorbent (1:10 w/v) for 15 minutes to remove residual Triton X-100.

**Verification of IgE and IgG in sera by indirect enzyme-linked immunosorbent assay (ELISA)**

To analyze the response of IgE in serum samples, each well of a 96-well immunomaxi high binding plate (TTP, Stafa-Zurich, Switzerland) was coated with 50 µl of 10 µg/ml of BFM proteins at 4°C for 18 hours. Unbound antigens were removed from the plate by washing five times with washing buffer (0.05% tween 20/PBS, pH 7.2). The coated plate was filled with 200 µl blocking solution (2% skim milk/PBS, pH 7.2) and incubated at room temperature for 2 hours to block unoccupied sites on the plate. After washing five times with washing buffer, 50 µl of the sera (diluted 1:5 in 2% skim milk/PBS) were added to each well, and incubated at room temperature for 1 hour. Then, the wells were washed and further incubated with 50 µl of horseradish peroxidase (HRP) conjugated goat anti-human immunoglobulin E (IgE)(Sigma Chemical Co., St. Louis, MO). After incubation for 1 hour at room temperature, all wells were washed as above, 100 µl of the substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB), (KPL, Inc., Gaithersburg, ML) were added and incubated in the dark at room temperature for the optimal period. The enzymatic reaction was stopped with 100 µl of 1N HCl and the optical density (OD) was measured at a wavelength of 450 nm using a microtiter plate reader.

A similar procedure for detecting IgG was applied as described previously. The dilution of individual serum used was 1:1,000 in 2% skim milk/PBS. The HRP conjugated

mouse monoclonal anti-human IgG (generously provided by Dr. Robert G. Hamilton) was added to monitor the immune-complex captured in the ELISA well.

**Biotinylation of BFM proteins and co-immunoprecipitation**

BFM proteins were biotinylated with 10 mM Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL) at 4°C for 2 hours. The biotinylation was quenched on ice for 2 hours. The binding/wash buffer (modified PBS buffer pH 7.4 (0.14 M NaCl, 8 mM sodium phosphate, 2 mM potassium phosphate and 0.01 M KCl)) was exchanged to remove excess biotin reagent using a desalting column with binding/wash buffer; this was done five times in a microcentrifuge at 4,000 × *g* for 1 minute. Co-immunoprecipitation was performed using a Seize X Immunoprecipitation Kit (Pierce Biotechnology). The Seize X Protein A gel was conjugated with $IgG_{2a}$ subclass monoclonal antibody (mAb) to human Fcε (Biodesign International, Saco, ME) using disuccinimidyl suberate (DSS) cross-linker and incubated at room temperature for 1 hour. After washing five times with washing buffer, 350 µl of serum sample were added to mAb to human Fcε conjugated protein A bead, and washed with binding/wash buffer five times in a microcentrifuge at 4,000 × *g* for 1 minute. Subsequently, the labeled BFM proteins were added to the washed beads and rotated for 1 hour at room temperature. After incubation, mixtures were washed five times, and the biotinylated BFM proteins were dissociated from protein A beads by elution buffer (pH 2.8). The immuno-precipitated proteins were resolved by 15% SDS-PAGE under reducing conditions and subsequently transferred to a PVDF membrane. Occupation of the free membrane surface was performed in 5% skim milk/PBS at 4°C, overnight. The blocked membrane was incubated for 1 hour at room temperature with HRP-conjugated streptavidin. The biotinylated proteins were visualized by chemiluminescence with the ECL western blotting analysis system (Amersham Health, Buckinghamshire, UK), then exposed to Kodak X-ray film.

**Indirect ELISA for validation of biotinylated BFM proteins**

To validate IgE in sera by indirect ELISA, each well of a 96-well polystyrene microtiter plate (Corning, Inc.) was coated with 50 µl of 100 µg/ml egg white avidin (Sigma Chemical Co.) at 4°C for 18 hours. Unbound antigens were removed from the plate by washing five times with washing buffer (0.05% Tween 20/PBS, pH 7.2). The coated plate was filled with 200 µl blocking solution (2% skim milk/PBS, pH 7.2) and incubated at room temperature for 2 hours to block unoccupied sites on the plate. After washing five times with washing buffer, 50 µl of the labeled BFM proteins (diluted 1 µg/ml, 10 µg/ml) were added to each well, and the plates were incubated at room temperature for 1 hour. After washing five times with washing buffer, 50 µl of the sera (diluted 1:5 in 2% skim milk/PBS) were added to each well, and incubated at room temperature for 1 hour. The wells were washed and further incubated with 50 µl of HRP conjugated goat anti-human IgE (Sigma Chemical Co.). After incubation for 1 hour at room temperature, all wells were washed as above and 100 µl of the TMB, (KPL, Inc.) were added. The plates were incubated in the dark at room temperature for the optimal period. The enzymatic reaction was stopped with 100 µl of 1N HCl and the OD was measured at a wavelength of 450 nm using a microtiter plate reader.

**Competitive ELISA for immune-complex detection**

Competitive ELISA was carried out in order to demonstrate the occurrence of IgE specifically interacting with the biotinylated BFM proteins in the eluted fraction collected from the co-immunoprecipitation process. The eluted fraction was preincubated with 1.2 µg of unlabelled BFM proteins at 37°C for 1 hour. The ELISA well was precoated with 50 µl of 100 µg/ml egg white avidin (Sigma Chemical Co.). Unbound egg white avidin was removed from the plate by washing five times. The well was filled with 200 µl blocking solution (2% BSA/PBS, pH 7.2) and incubated at room temperature for 2 hours. After extensive washing,

50 µl of the reaction mixture were applied into the egg white avidin-coated well and incubated at room temperature for 1 hour. Then, the wells were washed and 50 µl of HRP conjugated goat anti-human IgE (Sigma Chemical Co.) were added. After incubation for 1 hour at room temperature, all wells were washed as above and 100 µl of the chromogenic substrate TMB (KPL, Inc.) were added. The plates were incubated in the dark at room temperature for the optimal period. The enzymatic reaction was stopped with 100 µl of 1N HCl and the OD was measured at a wavelength of 450 nm using a microtiter plate reader. The competitive result was determined in comparison with the control treatment in which the eluted fraction was not preincubated with the unlabelled BFM proteins. The % inhibition value of the sample was calculated from the formula % inhibition = ([OD of no inhibitor – OD of inhibitor]× 100/ OD of no inhibitor).

## RESULTS

### Verification of serum specific IgE against BFM proteins

Four sample groups were used to evaluate the level of specific IgE against BFM proteins (Fig. 1). The mean value of specific IgE from 170 serum samples from LGWs was 0.10 ± 0.26 OD. Only 38 individuals had a specific IgE level above the mean value. The mean value obtained from 31 $CAP^+$ subjects was equal to that of the LGW group. Nine $CAP^+$ samples had the specific IgE level above the mean value whereas 11 out of 22 $CAP^-$ samples exhibited specific IgE level higher than the mean value (0.02 ± 0.02 OD). Seven sera from the HCW group (n = 35) had an OD greater than the mean value (0.04 ± 0.13 OD). The cut-off value was arbitrarily defined as the mean + 2 SD of $CAP^-$ group (0.02 + 0.04). Regarding the decision line of 0.06 there were 56, 11, 1 and 5 samples in LGWs, $CAP^+$, $CAP^-$, and HCWs classified as positive for IgE against BFM proteins. The three highest levels of specific IgE in LGW group from sera no. 4, 248, and 253 were 2.71, 1.40, and 0.78 OD, respectively.

There were no significant differences in mean between LGWs and the others ($CAP^+$, $CAP^-$, and HCWs; $P = 0.50$, 0.13, and 0.19 respectively, paired t-tests).

**Verification of serum specific IgG against BFM proteins**

In addition to the level of specific IgE against BFM proteins, we determined the level of the specific IgG in individual groups detailed above (Fig. 2). The analyzed mean values of LGWs, $CAP^+$, $CAP^-$, and HCWs were $0.20 \pm 0.34$, $0.09 \pm 0.13$, $0.02 \pm 0.04$, and $0.03 \pm 0.04$ OD, respectively. The numbers of samples in each group with an OD higher than referring mean values were 50, 9, 7, and 15. The cut-off value was calculated from the mean and SD obtained from the $CAP^-$ group as described earlier. According to the arbitrarily defined cut-off value from mean + 2 SD of $CAP^-$ group ($0.02 + 0.07 = 0.09$), there were 86, 9, 1, and 3 subjects classified as IgG against BFM proteins. Comparison of $CAP^+$, $CAP^-$, and HCWs means against that of LGWs mean showed significant differences with the latter a groups ($P$=0.07, $P$=0.02*, and $P$=0.01*, paired t-tests).

**Correlation of specific IgE and IgG**

To correlate the levels of IgE and IgG, the ODs of each sample were plotted in scatter format (Fig. 3). The sera from samples 4, 248, and 253 had the highest levels of specific IgE with the ODs specific for IgG of 2.64, 1.16, and 1.53 respectively (Table 1). These LGW subjects were selected for further validation of clinical symptoms and for the skin prick test. Clinical symptoms i.e. asthma, eczema, and conjunctivitis suggested that only subject no. 4 was allergic to rubber latex components. Skin prick tests were negative for all subjects.

**Validation of efficiency in biotinylation process of BFM proteins**

The quality of labeled BFM proteins was analyzed using the avidin capturing system (Fig. 4). The biotinylated BFM proteins at 10 μg/ml showed positive immunoreactivity with serum from LGW no. 4 when HRP conjugated goat anti-human IgE was used as a secondary antibody (OD 450 nm = 1.95). This specific formation of the immune-complex was demonstrated by dilution effect when 1 μg/ml biotinylated BFM proteins were introduced (OD 450 nm = 0.39).

**SDS-PAGE and co-immunoprecipitation**

The BFM proteins separated by 15% SDS-PAGE under reducing conditions contained two major bands at 30 and 34 kDa by Comassie Brilliant Blue 250 (Fig. 5B). The remaining proteins were in the range 10 to 70 kDa. The segregated polypeptides were transferred to a PVDF membrane and probed with serum no. 4, which had the strongest specific IgE reactivity against BFM proteins in indirect ELISA. No reactive band could be observed by Western immunoblotting (data not shown). Co-immunoprecipitation was subsequently performed to the specific IgE anti-BFM components in native conformation. Serum no. 4 was blended with Protein A gel covalently linked with mAb ($IgG_{2a}$) anti- human IgE. After extensive washes, the biotinylated BFM proteins were mixed with IgE-trapped gel. The precipitated fraction was eluted from the gel and further subjected to SDS-PAGE under reducing conditions. The proteins were blotted to a PVDF membrane and probed with avidin-HRP. A 55 kDa band was observed using the ECL chemiluminescence visualization system (Fig. 5A). No binding activity was found in $CAP^+$ and $CAP^-$ samples with negative OD for specific IgE.

**The competitive ELISA for detecting immune-complex**

To confirm the presence of anti-BFM IgE-protein complex in the purified eluate from the co-immunoprecipitation process, avidin-biotin capture inhibition ELISA was performed (Fig. 6). After neutralizing the eluate with 2% BSA/PBS pH 7.4 to promote the rebinding of immune-complexes, the mixture was added into the ELISA well coated with egg-white avidin. The captured complex containing IgE specifically unite to biotinylated BFM proteins were observed using HRP conjugated goat anti-human IgE. In parallel, the specific binding of IgE antibodies was verified by mixing the neutralized eluate with unlabelled BFM proteins. The OD diminished 26.89% compared to the non-inhibition well, demonstrating the competition between biotinylated and unlabelled BFM proteins for specific IgE.

## DISCUSSION

The incidence of immediate hypersensitivity to natural rubber latex has increased since the early 1980s.[2] This increase has been caused in part by the institution of mandatory universal precautions for handling bodily fluids. Prevalence of latex sensitization and allergy is reported to be 3% to 17% in HCWs, 11% in LGWs and 1% to 6.5% in the general population.[15]

In addition to presenting symptoms, several immunological assays have been developed, e.g. specific IgE determination by CAP-Rast, basophil histamine release assay, ELISA and immunoblotting to diagnose latex sensitization. Allergy to NRL involves sensitization to multiple constituent proteins; therefore different groups of patients respond to specific latex proteins in various ways. For this reason, having pure identified allergens is necessary to make the standard for immunologic diagnosis. Currently, thirteen Hev b proteins have been recognized by the International Union of Immunological Societies (IUIS) as latex

allergens.[13] Whereas most of the C-serum and B-serum proteins are water soluble, those of the rubber particles are in generally insoluble, i.e. Hev b 1.[16] The Hev b 2, 4, 6 and 10 were isolated from B-serum while allergens residing in the BFM have never been reported. Although in the glove manufacturing process rubber latex has to pass through extensive washing, there are still cases reported of allergic reaction to rubber gloves.[14] Therefore, we investigated whether the causative allergens are less water-soluble and whether the novel allergens are hydrophobic.

Considering the arbitrarily defined cut-off values in indirect ELISA for specific-IgE and -IgG against BFM proteins, the number of LGW group was higher than HCW group (Fig. 1, 2). In addition, the $CAP^+$ group showed comparatively low response to BFM proteins. This result supported the suggestion that the different rubber components which these study groups experience give rise to different diagnostic patterns. This finding refers that LGWs are often exposed to BFM proteins in the latex glove production process.

Recently, Kraft et al[17] have described the influence of IgG level on the anti-allergic response. If the FcγRIIB becomes co-aggregated with FcεRI through the allergen cross-linking via IgG and IgE, the pro-allergenic signal will be diminished. Interestingly, only LGW number 4 showed clinical symptoms (Table 1), although the corresponding IgG level was high. The optimal ratio of IgG/IgE should be further examined to predict the patient's status as described elesewhere.[18] However, the BFM proteins are prepared in crude form; the IgG level probably does not reflect the specific binding to the same allergic molecules recognized by the elevated IgE.

Considering the results of the skin prick test, a number of investigators have also reported a lack of correlation between *in vitro* (ELISA, RAST, and immunoblotting) and *in vivo* (skin test) measures of IgE using double-blind, placebo-controlled, food challenges (DBPCFCs; the standard for food allergy diagnosis).[19,20] Moreover, the quality of these methods is difficult to assess. Physicians should realize that a positive result from allergen-specific IgE does not always indicate allergy. The standard for the diagnosis of allergic disease remains a combination of positive double-blind challenge, the presence of specific IgE, and demonstration that the symptoms are the result of IgE-mediated inflammation.[21] Accordingly, identification of allergic molecules presented in BFM will support the development of immunodiagnosis.

The $CAP^+$ sample which showed the high level of CAP unit contained no specific IgE for allergic components in biotinylated BFM proteins, suggesting the absence of BFM proteins in the CAP assay. Accordingly, combining of the purified 55 kDa protein (Fig. 5A) with BFM proteins in the CAP assay should be assessed for its diagnostic value.

The major finding of the study is the isolation of the molecule captured by specific IgE in LGW no. 4. Individual protein allergens can be complicated in structure, with various antibody recognition sites (epitopes). Epitopes can have either linear form or a more complex conformational structure, and glycoproteins may contain or be influenced by sugar moieties.[22] This suggests the recognition of conformational epitopes by specific IgE, which has been previously reported in soybean profilin[23] and grass pollen[24] allergens. The 55 kDa protein located is within the range of Hev b 4 molecular sizes.[13] However, Hev b 4 was isolated from B-serum in water-soluble form.[16] The 55 kDa proteins, thus may belong to a novel allergenic class. To confirm the novelty of this molecule, gene cloning and protein expression will be

further performed. The negative result obtained from the CAP assay in latex-sensitized patients thus follows from the 55 kDa protein being absent. Including this molecule in the assay panel will improve the diagnostic value of screening for the risk of latex allergy.

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**Table 1** Characteristics of seven selected samples from LGW group demonstrating high level of specific IgE and/or IgG against BFM proteins. Clinical symptoms and skin prick test are included.

| Latex glove factory workers (no.) | Sex | Age (years) | Working period (months) | Symptom | Skin prick test | IgE ELISA OD 450 nm | IgG ELISA OD 450 nm |
|---|---|---|---|---|---|---|---|
| 2 | female | 30 | 24 | no | negative | 0.44 | 1.92 |
| 4 | female | 51 | 22 | asthma, eczema, conjunctivitis | negative | 2.71 | 2.64 |
| 144 | female | 30 | 20 | no | negative | 0.82 | 0.02 |
| 248 | female | 29 | 24 | no | negative | 0.78 | 1.16 |
| 252 | female | 38 | 120 | no | negative | 0.23 | 1.30 |
| 253 | female | 34 | 120 | no | negative | 1.40 | 1.53 |
| 255 | female | 35 | 60 | no | negative | 0.15 | 1.58 |

## FIGURE LEGENDS

**Figure 1** Relative comparison of specific IgE level from 31 $CAP^+$, 22 $CAP^-$, 170 LGW and 35 HCW samples by indirect ELISA. Each data point represents the specific IgE level in relation to the absorbance value at OD 450 nm. Solid horizontal lines represent the means of individual categories. These data are representative of three independent experiments.

**Figure 2** The IgG level from 31 $CAP^+$, 22 $CAP^-$, 170 LGW and 35 HCW samples was assayed by indirect ELISA. Solid horizontal lines represent the means of individual categories. These data are the representative of three independent experiments.

**Figure 3** Correlation of specific IgE and IgG levels obtained from indirect ELISA (Fig 1 and 2, respectively). Each data point represents an individual sample from 31 $CAP^+$, 22 $CAP^-$, 170 LGW and 35 HCW groups. LGWs no. 4, 248, 253 are shown in the ellipse area.

**Figure 4** The efficiency of the biotinylation process was validated using avidin capturing ELISA. This data is the representative of three consecutive experiments.

**Figure 5** Identification of immunoreactive allergen by co- immunoprecipitation assay (A). Protein mixtures of biotinylated BFM proteins are shown in lane 1. Fractions collected from incubation with LGW sample no. 4, $CAP^+$ sample no. P1396 and $CAP^-$ sample no. P2136 are shown in lanes 2, 3, and 4. Whole protein extract is shown by Coomassie Blue R250 staining in polyacrylamide gel under denaturing condition (B). BFM proteins were shown in lane 1.

**Figure 6** The competitive ELISA for detecting immune-complex in the eluted fraction of co-immunoprecipitation experiment. The eluted fraction was added to egg white avidin-coated wells (A). The eluted fraction was blended with unlabelled BFM proteins before applying into the egg white avidin-coated well (B). These data are the representative of three independent experiments.

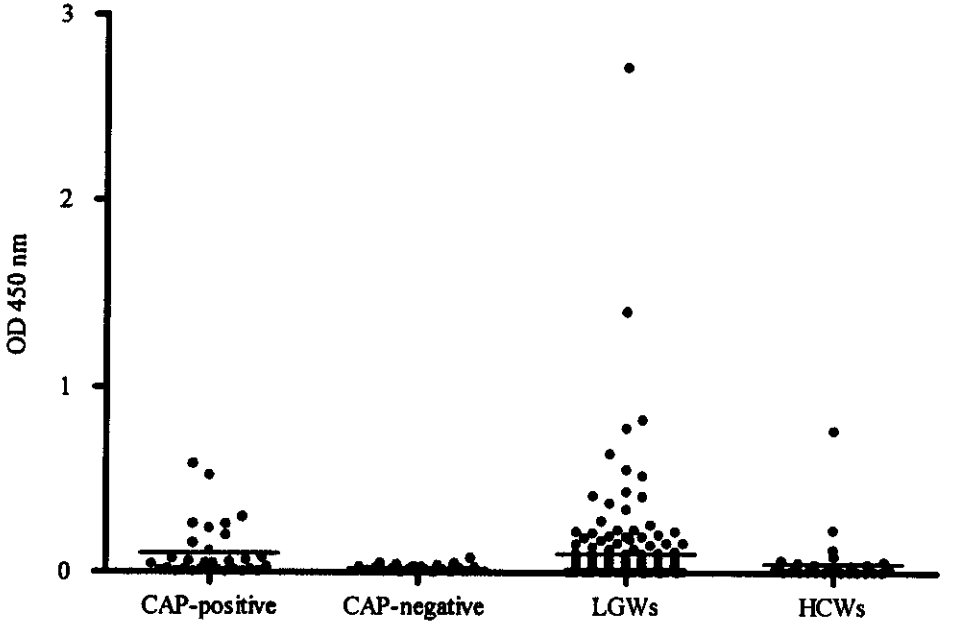


**Figure 1**

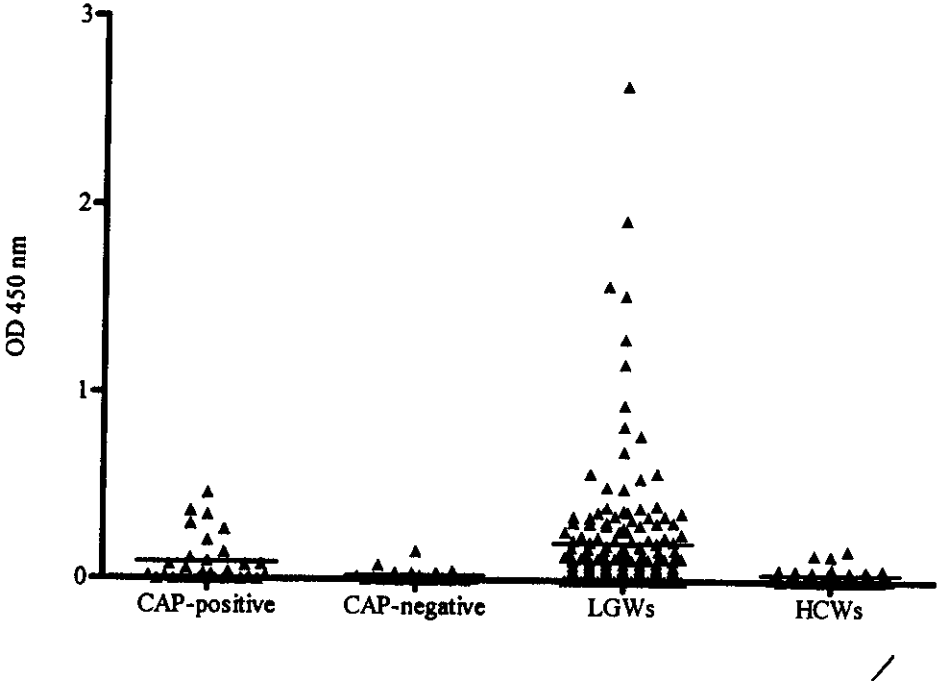


**Figure 2**

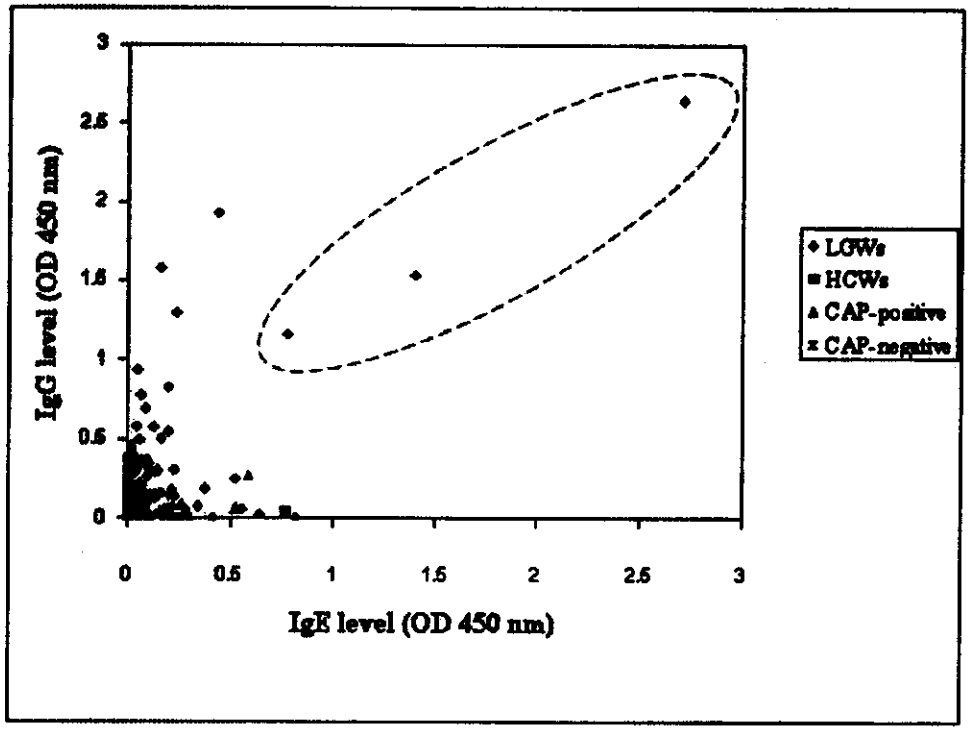
IgG level (OD 450 nm)
IgE level (OD 450 nm)
0
0.5
1
1.5
2
2.5
3
LGWs
HCWs
CAP-positive
CAP-negative

Figure 3

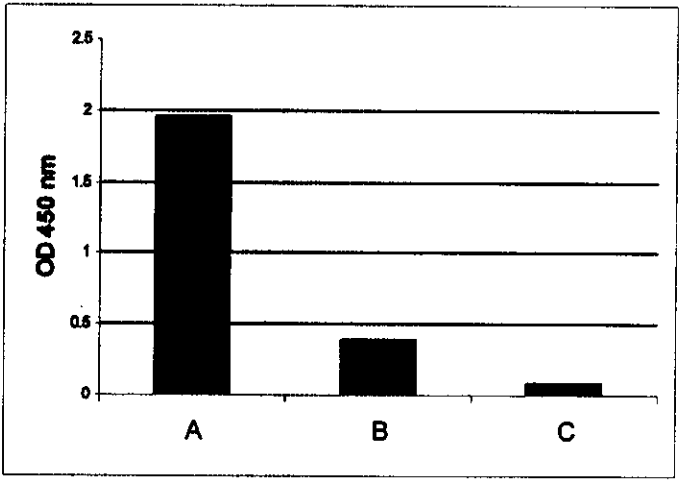


**Figure 4**

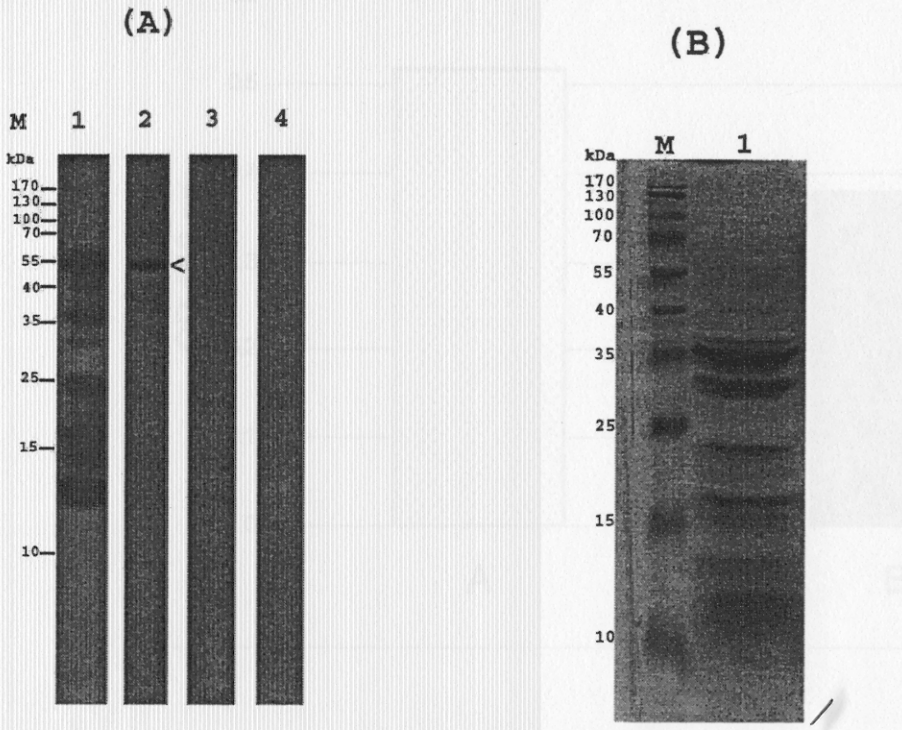
(A)
M
1
2
3
4
kDa
170
130
100
70
55
40
35
25
15
10
(B)
M
1
kDa
170
130
100
70
55
40
35
25
15
10

**Figure 5**

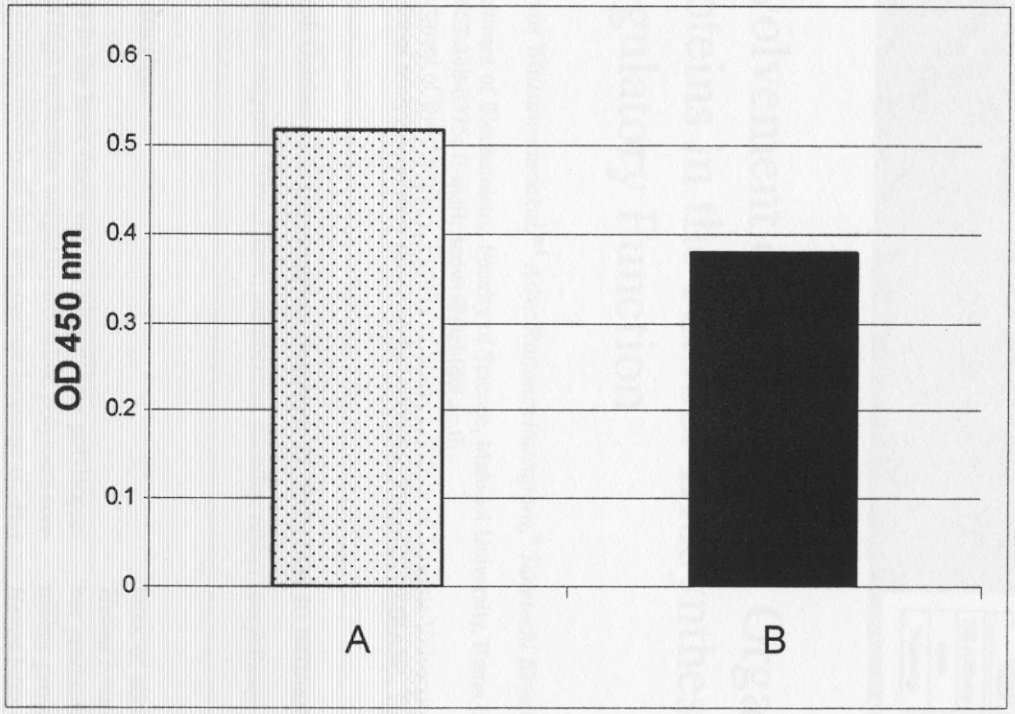


**Figure 6**