Chapter 5

RESULTS AND DISCUSSION OF SERUM ANTIBODIES TO rDer p 10 AND OTHER RECOMBINANT MITE ALLERGENS

5.1 Serum antibodies to the recombinants of Der p 10

The recombinant Der p 10 produced from the two gene expressions as described in Chapter 4 were tested for antigenicity in the indirect in-house ELISA. Of importance here is the presence of specific IgE antibodies to the rDer p 10 to determine properties such as antigenicity and allergenicity in the serum of allergic patients and controls. The measure of success in production of rDer p 10 is the accuracy of the recombinant proteins mimicking the native protein in terms of its structure, thereby recognised by the specific antibodies. The yields of recombinant mite tropomyosins from 1 liter of culture ranged from 0.1-1.5 mg, showing that the recovery varied and was also dependent on the efficiency of the purification. However, amounts of each recombinant protein did not vary much from batch to batch, although lower, when compared with those produced by Aki *et al.* (1995) and Asturias *et al.* (1998). These recombinants of mite tropomyosin were also included in assays together with a few other recombinant allergens from the mites, *D. pteronyssinus* and *B. tropicalis.*

Table 5.1: Characteristics of mite tropomyosins (Der p 10) cloned and expressed in this study

Expression System Host	Fusion partners	Calculated molecular mass (kDa) of rDer p 10	Amount of pure recombinant protein/ litre of culture
E. coli	GST	58 kDa	1.5 mg/l
P. pastoris	C-myc & 6xHis	36 kDa	0.54 mg/l

In the immunoblotting assay, rDer p 10 was found to show specific IgG-binding activity in human serum with HDM allergy, therefore indicating the existence of B-cell epitopes in the rDer p 10 (**Fig. 5.1**).

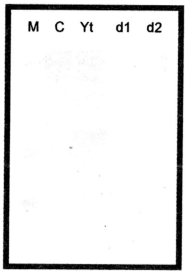


Fig. 5.1: Immunoblot of specific-IgG reactivity of an allergic rhinitis patient with HDM allergy to different proteins. [C, control of normal yeast cells; Yt, total yeast lysate; d1 and d2, 10 µl and 20 µl of affinity purified rDer p 10, respectively]

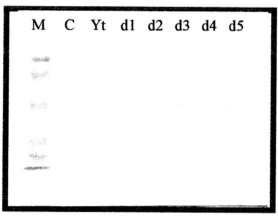


Fig. 5.2: Immunoblot of specific-IgE reactivity of an allergic rhinitis patient with HDM allergy to different proteins. [C, control of normal yeast cells (20 µl); Yt, total yeast lysate (20 µl); d1 (5 µl); and d2 (10 µl); d3 (15 µl); d4 (20 µl) and d5 (25 µl) of affinity purified rDer p 10, respectively. M=molecular weight marker]

5.1.1 IgE binding reactivities to the recombinant mite tropomyosins, GST-Der p 10 and GST- Blo t 10 in the sera of Malaysian subjects.

The prevalence of allergy to tropomyosins of the two main HDM species, *D. pteronyssinus* (Der p 10) and *B. tropicalis* (Blo t 10) was investigated. The recombinant produced in this study, GST-Der p 10 was compared with GST-Blo t 10 (provided by Dr Chua K.Y). Appropriately GST protein from *S. japonicum* (26 kDa) was used as control. Sera from 100 adult asthmatic patients, 100 allergic rhinitis patients, 30 normal healthy subjects and 12 negative controls, were subjected to ELISA using biotinylated anti-human IgE, followed by the use of avidin and ρNPP in the substrate. IgE bound to avidin-biotin complex was visualised at 405 nm wavelength.

Concomitant incidence of IgE binding to both recombinant mite tropomyosin (GST-Der p 10 and GST-Blo t 10) in sera of the positive cases could be seen in **Fig. 5.3** and **Fig. 5.4**. However, in **Fig. 5.5** concomitant binding to all three antigens; GST, GST-Der p 10 and GST-Blo t 10 was also observed in 3 of the sera of the normal healthy subjects. A generally low binding reactivity was observed among the sera from the non-allergic subjects, with most of the dOD not exceeding 0.1. However, reactions that showed a higher binding to GST and not to both GST-Der p 10 and GST-Blo t 10, were observed in some subjects. None of the 12 negative controls showed an elevated binding reactivity (OD>0.2) (**Fig. 5.6**).

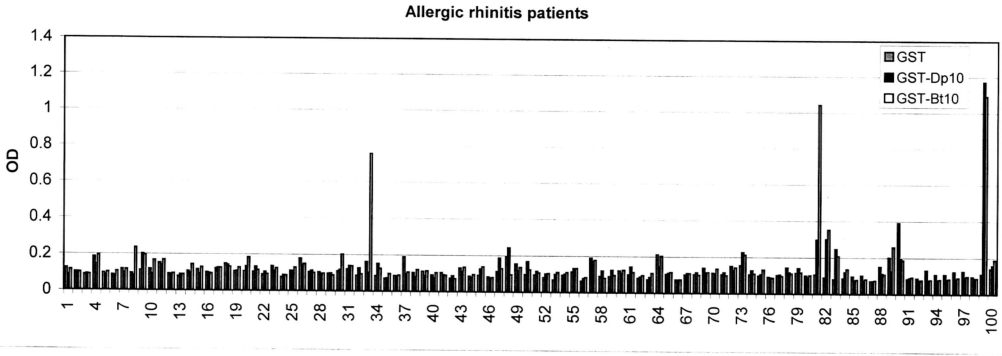


Fig 5.3: The absorbance (OD at 405 nm) representing IgE-binding reactivities of 100 allergic rhinitis patients. The GST protein (*Sj* 26) served as control antigen as both the recombinant mite tropomyosins were produced as fusion proteins

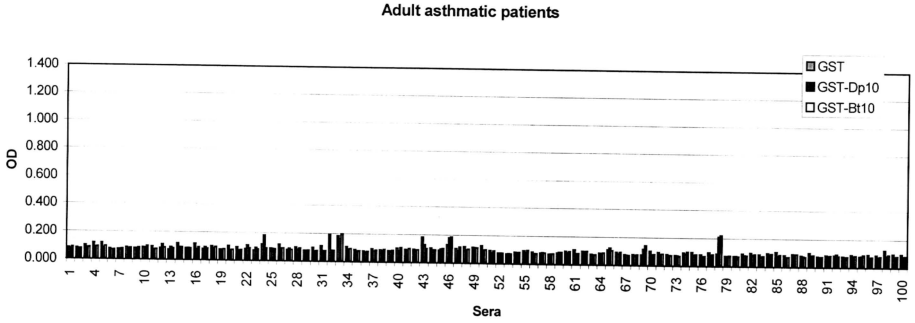


Fig 5.4: The absorbance (OD at 405 nm) representing IgE-binding reactivities of 100 asthmatic patients. The GST protein (*Sj* 26) served as control antigen as both the recombinant mite tropomyosins were produced as fusion proteins

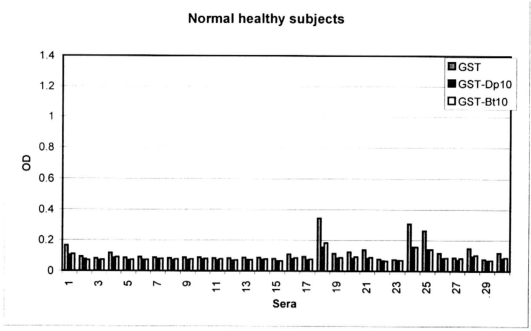


Fig 5.5: The absorbance (OD at 405 nm) representing IgE-binding reactivities of 30 normal healthy subjects. The GST protein (Sj 26) served as control antigen as both the recombinant mite tropomyosins were produced as fusion proteins.

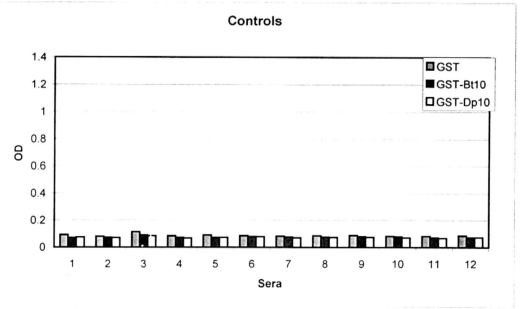


Fig 5.6: The absorbance (OD at 405 nm) representing the IgE-binding reactivities of 12 non-allergic subjects with negative-SPTs, serving as negative controls.

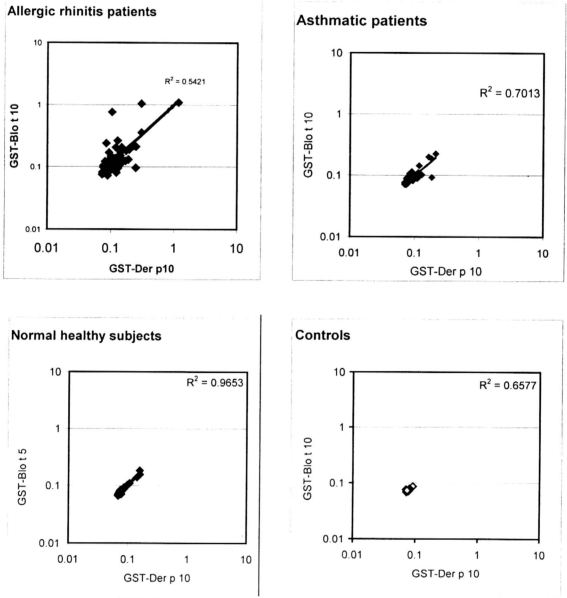


Fig. 5.7: The correlation analysis of anti-Der p 10 and anti-Blo t 10 IgE antibody titres (in logarithmic values) in 100 allergic rhinitis, 100 asthmatics, 30 normal healthy subjects (NHS) and 12 SPT-negative controls. R^2 is the squared-correlation value.

Table 5.2: The percentage of the IgE reactivities to mite tropomyosins GST-Der p 10 and GST-Blo t 10 among Malaysians

	N	GST-Der p 10	GST-Blo t 10	GST*
Allergic rhinitis patients	100	17 (17%)	17 (17%)	4 (4%)
Adult asthmatic patients	100	6 (6%)	9 (9%)	3 (3%)
Normal healthy subjects	30	2 (7%)	4 (13%)	4 (13%)
Negative SPT subjects	12	0	0	0

*GST is the glutathione-*S*-transferase protein, acting as the control.

5.1.2 IgE binding reactivities of 60 Taiwanese sera to the recombinant mite tropomyosins, GST-Der p 10 and GST- Blo t 10.

Sera from 60 asthmatic patients from Taiwan were also tested with two recombinants; GST-Der p 10 and GST-Blo t 10. GST protein from *S. japonicum* (26 kDa) was used as control. The longitudinal profile of IgE binding reactivities is shown in **Fig. 5.8**. The mean IgE binding reactivity to GST (dOD=0.089), added with the standard deviation (OD=0.027) was taken as the cut-off value to decide for positive IgE binding to the two mite tropomyosins. Therefore, with the cut-off value of 0.116, 15 (25%) of the patients had IgE to the GST-Blo t 10 while 13 (22%) had positive IgE-binding to GST-Der p 10. It was also found that 5 (8%) of these patients had IgE binding above the cut-off value for GST. Four of the patients showed reactivity to GST and to one or both of the mite tropomyosins. Only one of these patients had elevated IgE titre for GST without showing reactivity to the GST-fused tropomyosins. This strongly suggests that the patient had an actual allergy to the GST protein *per se* and not to the tropomyosins. Indeed glutathione allergen in the mite has been reported as an allergen, designated as the mite allergen of

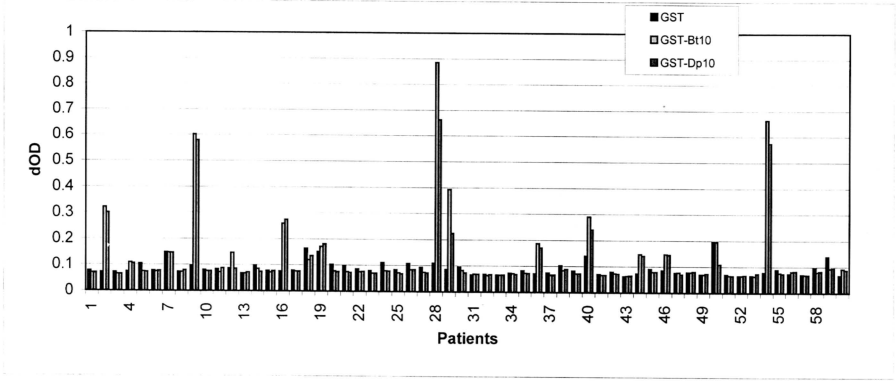


Fig 5.8: The absorbance (OD at 405 nm) representing IgE-binding reactivities of 60 Taiwanese asthmatic patients. The GST protein (*Sj* 26) served as control antigen as both the recombinant mite tropomyosins were produced as fusion proteins

group 8 (O'Neill, 1994) This result however is an important evidence to fend any suspicion that the IgE antibodies may have recognised the GST portion of the fusion proteins, GST-Der p 10 and GST-Blo t 10. Therefore it can be safely argued that GST protein portion in a GST-tagged recombinant allergen does not offer itself as a convenient IgE binding site, which would otherwise produce false positive results in both ELISA and SPTs. Subsequently, the other four patients with elevated IgE to GST and to both or one of the GST-fusion proteins, suggesting that patients have allergy to either or both of the mite tropomyosins as well as to GST. **Fig. 5.9** also further illustrates that there is a concomitant incidence of allergenicity to the two mite tropomyosin recombinants; thus confirming the IgE reactivities to the same group of allergen, although from two different HDM species.

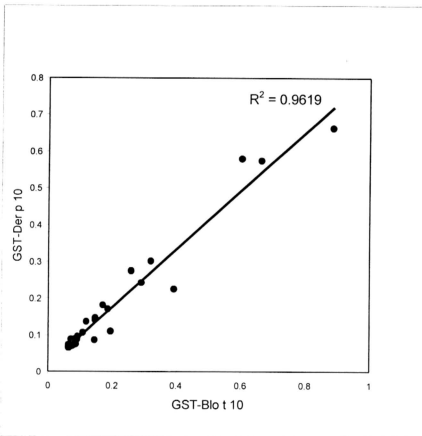


Fig. 5.9: IgE binding reactivities of 60 Taiwanese asthmatic patients to two recombinant mite tropomyosins, GST-Der p 10 and GST-Blo t 10. Statistical analysis produced the correlation coefficient, R^2 = 0.9619 and p=0.027.

The IgE-binding reactivities to the recombinant mite tropomyosins of two different species, GST-Der p 10 and GST-Blo t 10 showed excellent correlation (R^2 = 0.9619, $p<0.05$) although low frequency (i.e. only 22-23%) of positive cases was observed (**Fig. 5.9**). All but one (2 %) of the positive patients showed binding for both allergens. The ability of the serum IgE antibodies to show a near identical binding capacity to these tropomyosins from two different species of mites is very striking, affirming a possible case of crossreactivity. This is however not surprising as tropomyosins of invertebrate origin share a high percentage of homology (60-80%). Therefore significant homology of Der p 10 to other invertebrate tropomyosins and to the IgE-binding epitopes could explain the high cross-reactivity found between arthropods (insects and crustacean) and mollusks (gastropods and cephalopods) (van Ree *et al.*, 1996; Leung *et al.*, 1996; Martinez *et al.*, 1997) and supports the idea that tropomyosin might represent a pan-allergen (Baldo & Panzani, 1988).

Indirectly too, this data shows that the asthmatic patients in Taiwan have specific IgE antibodies that recognise the same site or folding on the Group 10 of mite antigens; suggesting antibody recognition and subsequent binding based on indiscriminate structure of an antigen. Another assumption which can be drawn from this binding profile is the possibility of a co-existence of these two mites in the environment, thus their concomitant occurrence and interestingly, at almost equivalent quantities of specific IgE as shown in each positive serum.

5.2 IgE binding reactivities to recombinant *D. pteronyssinus* allergens.

5.2.1 IgE binding reactivities in sera of allergic rhinitis patients to recombinant Der p allergens

Sera from 40 allergic rhinitic patients were also tested against crude extract and recombinants of *D. pteronyssinus* allergens; GST-Der p 1, GST-Der p 2, GST-Der p 5, GST-Der p 10 and Der p 10 produced by *P. pastoris;* for specific IgE through ELISA. Glutathione-*S*-transferase protein from *Schistosoma japonicum* (26 kDa) was used as control. **Fig. 5.10** shows the IgE binding to the seven groups of antigens.

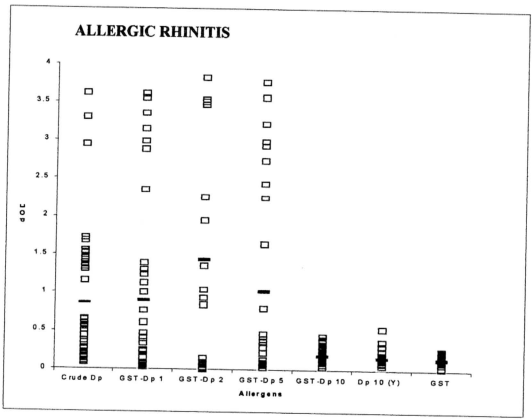


Fig. 5.10: The IgE binding reactivities of 40 allergic rhinitis patients to 5 recombinant allergens of *D. pteronyssinus* through ELISA. The antigens were recombinant allergens fused to GST. For comparison, each serum (in 1:10 dilution) was also tested with crude extract of *D. pteronyssinus* and Der p 10 produced by the yeast *P. pastoris* [Dp 10 (Y)]. Glutathione-*S*-transferase, GST, from *Schistosoma japonicum* (Sj-26 kDa) served as a control. Blue open boxes represent IgE binding of positive-SPT patients while red open boxes represent negative-SPT patients. Red line represents the mean values.

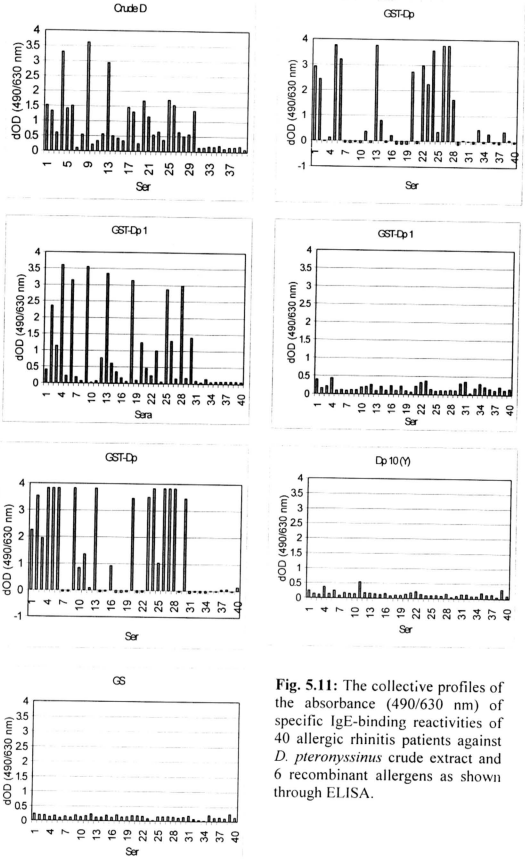


Fig. 5.11: The collective profiles of the absorbance (490/630 nm) of specific IgE-binding reactivities of 40 allergic rhinitis patients against *D. pteronyssinus* crude extract and 6 recombinant allergens as shown through ELISA.

5.2.2 IgE binding reactivities in sera of asthmatic patients to recombinant Der p allergens

Similarly, sera from 40 asthmatic patients were also tested against crude extract and recombinants of *D. pteronyssinus* allergens for specific IgE through ELISA. Glutathione *S*-transferase protein from *S. japonicum* (26 kDa) was used as control. **Fig. 5.11** below shows the IgE binding reactivities of the sera to the recombinant Der p allergens and the binding to individual recombinant allergens are shown in **Fig. 5.12**.

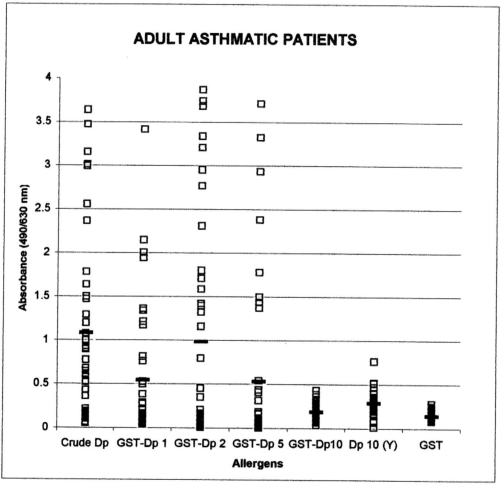


Fig. 5.12: The IgE binding reactivities of 40 adult asthmatic patients to 5 recombinant allergens of *D. pteronyssinus* through ELISA. The antigens were recombinant allergens fused to GST. For comparison, each serum (in 1:10 dilution) was also tested with crude extract of *D. pteronyssinus* and Der p 10 produced by the yeast *P. pastoris* [Dp 10 (Y)]. Glutathione-*S*-transferase (Sj-26 kDa) from *Schistosoma japonicum* served as a control.

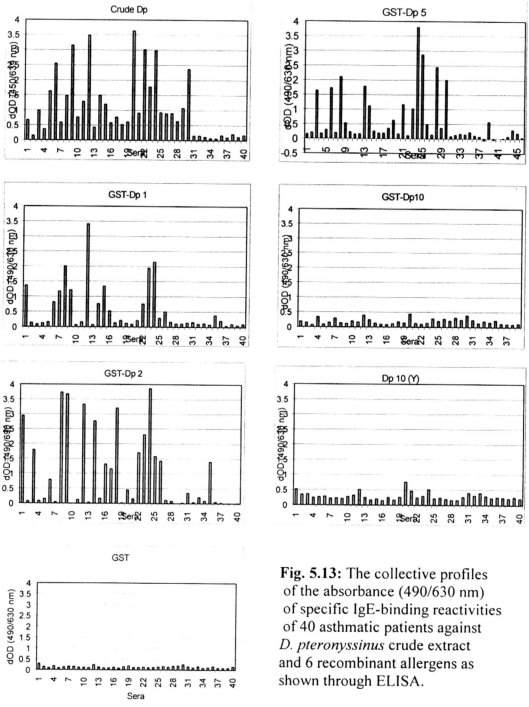


Fig. 5.13: The collective profiles of the absorbance (490/630 nm) of specific IgE-binding reactivities of 40 asthmatic patients against *D. pteronyssinus* crude extract and 6 recombinant allergens as shown through ELISA.

5.3 IgE binding reactivities to recombinant *B. tropicalis* allergens.

5.3.1 IgE binding reactivities in sera of allergic rhinitis patients to rBlo t allergens.

To further investigate the recombinants of *B. tropicalis,* a similar assay was carried out, this time with a bigger array of Blo t recombinants, which were GST fusion proteins, and some recombinants produced from *Pichia* expression system. Another group of 30 allergic rhinitis patients were pre-selected, based on positive responses to the crude Blo t extract. Another ten sera were which showed low responses to the crude Blo t extract were selected too, as controls. Results of the assay are shown in two graphs, **Fig. 5.14** to show the collective spectrum of the IgE binding responses to the recombinants for ease of comparison, while **Fig. 5.15** are the individual absorbance of the sera to each of the recombinant.

These recombinants serving as antigens in the ELISA assays were also subjected to the other three main groups, namely the asthmatics, the child asthmatics and the normal healthy subjects. Forty sera from each group were used in the assay.

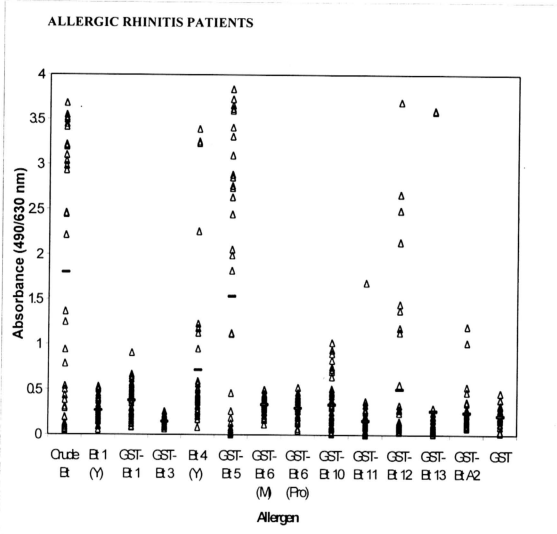


Fig. 5.14: The IgE-binding reactivities of 40 allergic rhinitis patients to differen groups of allergens in *Blomia tropicalis* mite, as detected by ELISA. The firs allergen is the crude extract of *B. tropicalis.* The rest of the allergens wer recombinant allergens: four allergens [Bt 1 (Y), Bt 4(Y) and Bt 6 (Pro & M)] wer produced by yeast (*P. pastoris)* while the rest were produced as fusion through th GST gene expression system by *E. coli.* The GST protein in the last assay serve as control.

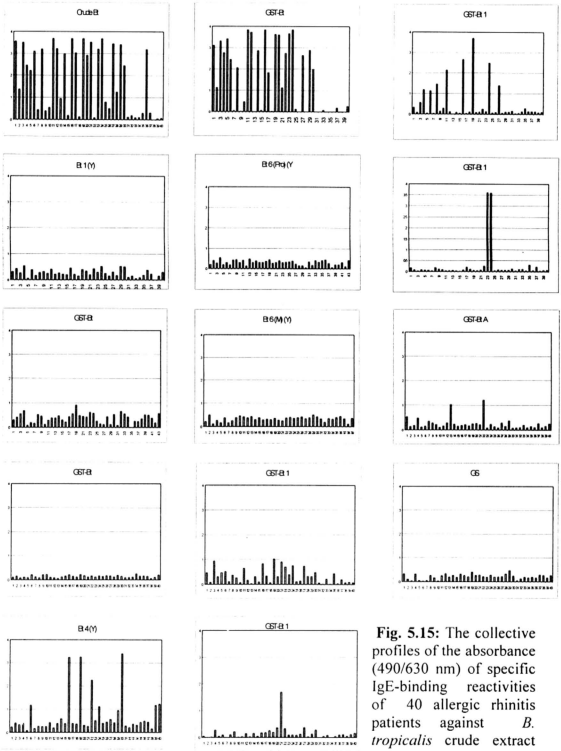
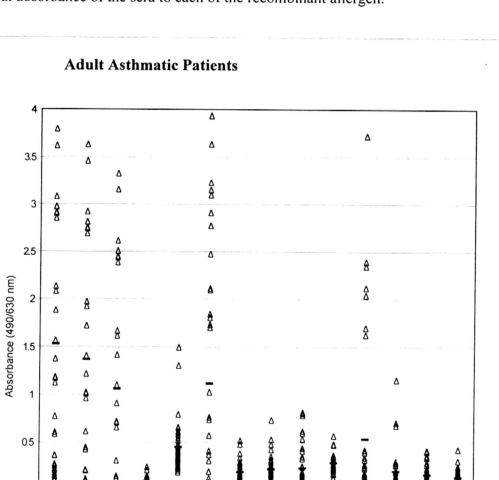


Fig. 5.15: The collective profiles of the absorbance (490/630 nm) of specific IgE-binding reactivities of 40 allergic rhinitis patients against *B. tropicalis* crude extract and 12 recombinant allergens, as shown by

5.3.2 IgE binding reactivities in sera of asthmatic patients to recombinant Blo t allergens.

The same recombinants of *B. tropicalis* described in **section 5.3.1**, were also put to test among adult asthmatics. A sub-sample group of 30 asthmatic patients with positive SPT to crude Blo t extract were pre-selected for this experiment. Another ten sera, which showed low responses to the crude Blo t extract, were selected too, as controls. Results of the assay are shown in two graphs, **Fig. 5.16** to show the collective spectrum of the IgE binding responses to the recombinants for ease of comparison, while **Fig. 5.17** are the individual absorbance of the sera to each of the recombinant allergen.



5.16: The IgE-binding reactivities of 40 asthmatic patients to different groups of allergens in *Blomia tropicalis* mite, as detected by ELISA. The first allergen is the crude extract of *B. tropicalis*.The rest of the allergens were recombinant allergens : four allergens [Bt1 (Y), Bt 4 (Y) and Bt 6 (Pro & M) were produced by yeast (p. pastoris) while the rest were produced as fusion through the GST gene expression system by *E. coli*. The GSTprotein in the last essay served as control.

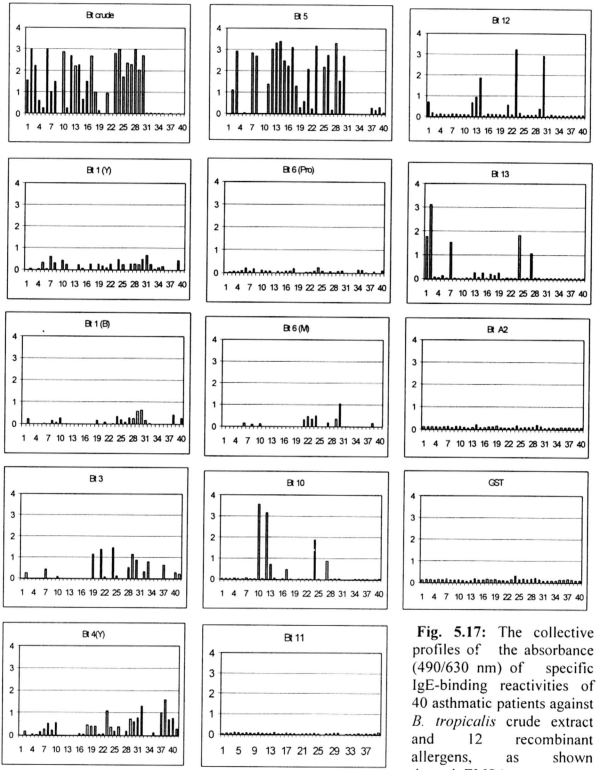


Fig. 5.17: The collective profiles of the absorbance (490/630 nm) of specific IgE-binding reactivities of 40 asthmatic patients against *B. tropicalis* crude extract and 12 recombinant allergens, as shown through ELISA.

5.4 IgE binding reactivities in sera of asthmatic children to recombinant *D. pteronyssinus* and *B. tropicalis* allergens.

5.4.1 IgE binding reactivities in sera of asthmatic children to recombinant Der p allergens

Sera from 40 asthmatic children (age ranging from 2-15, mean 8.3) from the Paediatric Clinic in UMMC were also tested against recombinant allergens of *D. pteronyssinus*; GST-Der p 1, GST-Der p 2, GST-Der p 5, GST-Der p 10 and Der p 10 produced by *P. pastoris* [Der p 10 (Y)], for specific IgE through ELISA (**Fig. 5.18**). Glutathione-*S*-transferase protein from *S. japonicum* (26 kDa) was used as control as all these recombinants except Der p 10 (Y) were fusion proteins of GST.

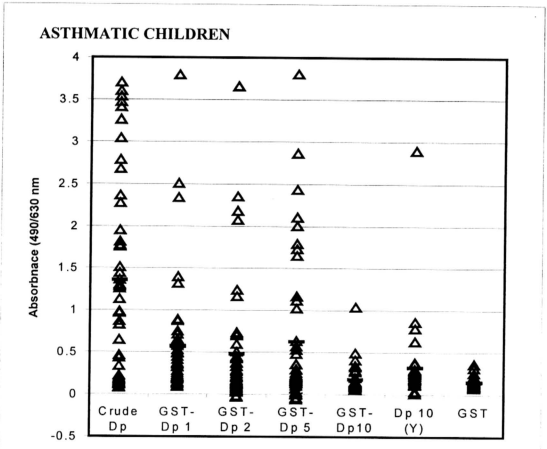


Fig. 5.18: The IgE binding reactivities to recombinant allergens of *D. pteronyssinus* in 40 asthmatic children. The recombinants were fusion proteins, GST-Der p 1 (GST-Dp 1), GST-Dp 2, GST-Dp 5 and GST Dp 10. Dp 10(Y) was the recombinant mite tropomyosin expressed in the yeast *P. pastoris* and glutathione-S-transferase (GST) was the control.

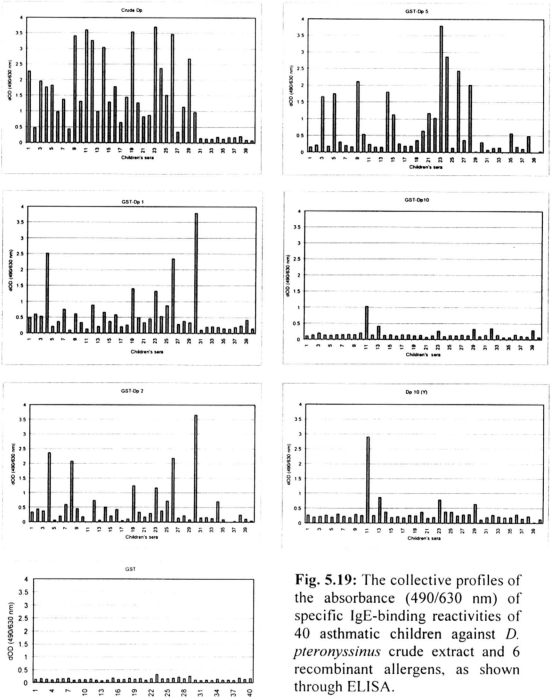


Fig. 5.19: The collective profiles of the absorbance (490/630 nm) of specific IgE-binding reactivities of 40 asthmatic children against *D. pteronyssinus* crude extract and 6 recombinant allergens, as shown through ELISA.

As shown in **Fig. 5.19**, most of the positive-SPT sera had specific-Der p 1 and specific-Der p 2 IgE. The cut-off point was taken at 0.200, based on the mean dOD of IgE binding to the GST. As expected, there were a higher number of responders to the two major allergens, Der p 1 and Der p 2. The young patients clearly showed high response to GST-Der p 1 with 90% of the 30 patients showing elevated values of above 0.2 in the ELISA. However in the 10 patients who did not show binding to the crude extract, 3 of them showed elevated IgE binding response to recombinant Der p 1. Twenty (67%) of the children reacted to GST-Der p 2 while 73% showed binding reactivity to it. The recombinant mite tropomyosin did not show high binding reactivtiy to IgE antibodies in the patients' sera.

Some sera also showed high IgE reactivity to Der p 5, with some higher than the titres shown towards Der p 1 and Der p 2. A generally low response towards both the recombinants produced in this study, GST-Der p 10 and Der p 10 (Y) was observed with the general dOD not exceeding the value 1.000. However the result was concomitant for both these mite tropomyosin recombinants.

5.4.2 IgE binding reactivities in sera of asthmatic children to recombinant Blo t allergens.

The same recombinants of *B. tropicalis* described in **section 6.4** were again tested against the sera of the asthmatic children. A sub-sample group of 30 asthmatic patients with positive SPT to Blo t crude extract were pre-selected for this experiment. Another ten sera, which showed low responses to the crude Blo t extract, were selected too, as controls. Results of the assay are shown in two graphs; **Fig. 5.20** shows the collective spectrum of the IgE binding responses to the recombinants for ease of comparison, while **Fig. 5.21** is the individual absorbance of the sera to each of the recombinant allergen.

Similar to the pattern of allergenicity of the adults, the children also showed reactivities to the first two groups of HDM, Der p 1 & Blo t 1 and Der p 2 & Blo t 2. Although both Blo t 1 and Blo t 2 allergens showed higher allergenicity in terms of frequency as in the Der p 1 and Der p 2, lower absorbance was noted, suggesting that the strength of IgE binding to Blo t 1 & 2 was lower than that of Der p 1 and Der p 2. This shows that although allergens of similar groups may succeed in attracting IgE to bind, their strengths of binding to IgE differ. This implies the difference and perhaps the size of epitopes present on the allergens from the two species.

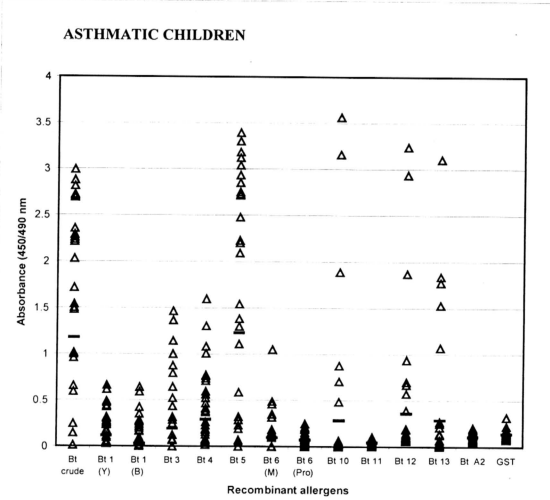


Fig. 5.20: The IgE-binding reactivities of 40 asthmatic children to different groups of allergens in *B. tropicalis* mite, as detected by ELISA. The first allergen is the crude extract of *B. tropicalis.* The rest of the allergens were recombinant allergens: [Bt 1 (Y), Bt 4(Y) and Bt 6 (M) and Bt 6 (Pro)] were produced by yeast (*P. pastoris)* while the rest were produced as fusions through the GST gene expression system by *E. coli*. The GST protein served as control.

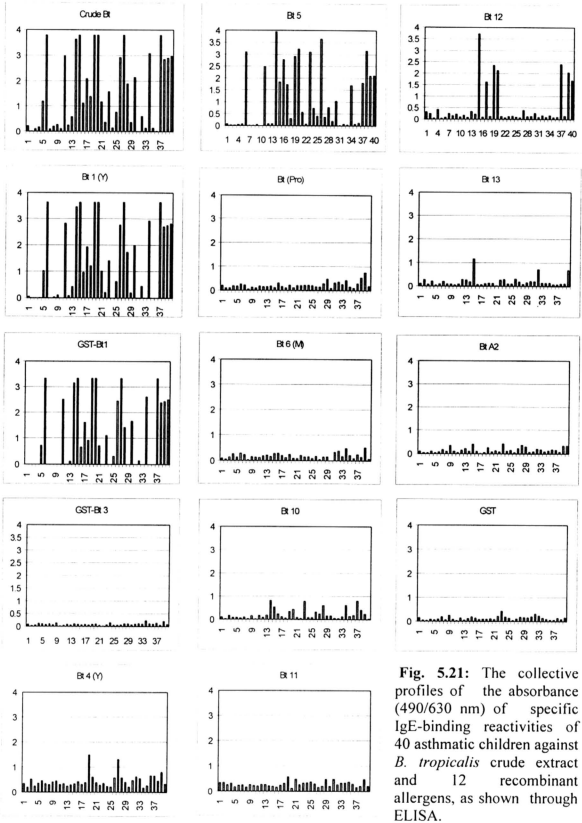


Fig. 5.21: The collective profiles of the absorbance (490/630 nm) of specific IgE-binding reactivities of 40 asthmatic children against *B. tropicalis* crude extract and 12 recombinant allergens, as shown through ELISA.

5.5 Comparison of reactivity to the recombinants of Der p allergens

Table 5.3 shows the combined IgE reactivities of the different groups of patients and controls, to the Der p recombinants. Crude extract of Der p was used while the other recombinants were GST-fusion proteins.

Table 5.3: The percentage of positive IgE binding reactivity in atopic patients to crude extract of *D. pteronyssinus* (Der p) and a few recombinant Der p allergens

	N	CrDer p	Der p 1	Der p 2	Der p 5	Der p 10	GST
Allergic rhinitis	40	29 (73%)	20 (50%)	19 (48%)	19 (48%)	15 (38%)	4 (10%)
Asthma patients	40	30 (75%)	17 (43%)	19 (48%)	12 (30%)	13 (33%)	3 (8%)
Asthmatic children	46	34 (74%)	33 (72%)	24 (52%)	22 (48%)	9 (20%)	4 (9%)
Controls	6	0	2 (33%)	0	0	1 (17%)	0

CrDer p = crude Der p

GST = Glutathione-S-transferase protein

Almost half of the allergic rhinitis patients showed reaction to each Der p 1, Der p 2 and Der p 5 while 15 out of the 40 patients reacted to Der p 10. Strong positive reactions were obtained in ELISA against Der p 1 and Der p 5 as seen in **Fig. 5.11, Fig. 5.13** and **Fig. 5.18**, confirming the importance of the two allergens. Der p 2 allergen showed IgE reactivity in almost half the population tested, but most of the positive responses were not as strong as those shown for Der p 1. Interestingly, as was shown by Asturias *et al.*, (1998) the Der p 10 was able to bind to only 15 (38%) of the allergic rhinitis patients tested. This is in contrast to the results of IgE binding of GST-Der p 10 that was found by Aki *et al.*, 1995, who found 80% allergenicity to it.

Table 5.3 shows the summarised results of the ELISA using the recombinant Der p allergens. Out of the 30 **asthmatic patients** who reacted positively to the crude Der p extract, 27 (90%) had specific IgE to the GST-Der p 1 allergen, 20 % to Der p 2, % to Der p 5 and % to GST-Der p 10. In the case of rDer p 10 produced in yeast, an almost equivalent percentage of patients reacted to it, suggesting that the specific IgE in the each patient could recognise both recombinants.

Adult asthmatics showed different IgE reactivities from the asthmatic children. Out of the 40 **asthmatic** adults, IgE binding reactivities to Der p crude extract was detected in 30 (75%) while and only 17 (43%) and 19 (48%) reacted to rDer p 1 and rDer p 2 respectively. rDer 5 produced 30% reactivity and similarly, rDer p 10 produced 33% reactivity.

Among the 46 **asthmatic children**, Der p 1 showed equivalent percentage of IgE binding reactivities with that of the crude Der p. Thirty-four (74%) of the children showed reactivity to the crude extracts while 33 (72 %) also showed reactivity to the Der p 1 allergen. Both rDer p 2 and rDer p 5 had positive reactions from 24 (52%) and 22 (48%) patients respectively while against rDer 10, only 9 (20%) had IgE-binding reactivity to it.

Within the group of 6 **controls**, only rDer p 1 and rDer p 10 were recognised. However with the crude extract of Der p, weak reactions were obtained (dOD < 0.2), and the frequency of binding was low, with only 2 (33%) and 1 (17%) for each recombinant. The number of controls was limited due to insufficient antigen. Specific IgE antibodies to the other recombinants however were not detected. This surprising outcome could actually be explained by the fact that the crude extract used, might have had lower concentration of component allergens when compared to the single recombinant allergen preparation and therefore would understandably elicit stronger reaction in IgE binding.

Strong positive reactions were obtained in ELISA against Der p 1 and Der p 5 as seen in graphs, **Fig. 5.10-13**, **Fig. 5.18** and **Fig. 5.19** confirming the importance of the two allergens. Der p 2 allergen showed IgE reactivity in almost 48%, i.e., half the population tested, but most of the positive responses were not as strong as those shown for Der p 1. Der p 10 was able to bind to only 15 (38%) of the allergic rhinitis patients tested. As these were selected patients who had initially shown very high IgE binding reaction to the crude extract, the subsequent high percentage of binding to rDer p 10 suggests that, when Der p 10 protein alone was used in the assay, it is presented in such a way that precise IgE antibody binding could take place without much hindrance from other components as would have happened in the crude extract assay. The lack of competition for binding this time around therefore could have contributed to the higher percentage of binding when using the recombinants as antigens.

As in the adult group, the young asthmatics also showed higher response to GST-Der p 1 with 90% of the 30 patients showing elevated absorbance values of above 0.2 in the ELISA. Twenty (67%) of them showed reactivity to GST-Der p 2 and 22 (73%) reacted to GST-Der p 5. Only 5 (17%) showed IgE reactivity to GST-Der p10 while 4 of them showed reactivity to GST. However in the 10 patients who didn't show any IgE reactivity to the crude extract of Der p, 3 (30%) showed elevated IgE response to GST-Der p 1 whereas two (20%) reacted to GST-Der p 2. GST-Der p 5 and GST-Der p 10 was each recognised by IgE in one patient but none of these patients reacted to GST. GST was included as a control as the recombinant proteins were produced as fusion proteins carrying the GST protein. In the case of IgE-binding reaction, 11 of the atopic patients had reactions to GST. As a control, GST did not bind to the IgE in sera of the patients that reacted to the Der p crude extract. It is therefore strongly suspected that the two patients, who showed IgE binding to crude extract as well as to GST, had actually shown a probable case of cross-reactivity of IgE recognising the mite glutathione-*S*-transferase.

5.6 Comparison of reactivity to the recombinants of Blo t allergens

Greater binding capacity to Der p allergens than to Blo t allergens was observed. However, the number of positivity remains highly significant among the Blo t responders, surpassing that of Der p. The sera from 80 allergic patients; 40 with asthma and 40 with allergic rhinitis were tested for specific IgE against 10 different *B. tropicalis* recombinant allergens. These allergens represented almost all the identified allergens found in a *B. tropicalis* mites; Blo t 1 (Y), Blo t 1, Blo t 3, Blo t 4, Blo t 5, Blo t 6, Blo t 10, Blo t 11, Blo t 12, Blo t 13, and Blo t 13. Blo t A2 is a novel enzymatic allergen. Blo t 1 (Y) and Blo t 4 (Y) were produced by yeast (*P. pastoris*) expression system while the rest of the allergens were fusion protein produced by the GST-expression system by *E. coli*. Combined results are as shown in **Table 5.4.**

Table 5.4: Table showing the percentage of positive IgE binding reactivity in atopic patients to crude extract of *Blomia tropicalis* (Blo t) and a few recombinant Blo t allergens.

	N	CBt	Bt1*	Bt1	Bt3	Bt4*	Bt5	Bt6(P)*	Bt6(M)*	Bt10	Bt11	Bt12	Bt13	BtA2	GST
Allergic rhinitis patients	40	30 (75%)	26 (65%)	29 (73%)	5 (13%)	12 (30%)	22 (55%)	10 (25%)	8 (20%)	12 (30%)	8 (20%)	13 (33%)	4 (10%)	5 (13%)	7 (18%)
Asthmatic adults	40	29 (73%)	26 (65%)	21 (53%)	5 (13%)	17 (43%)	21 (53%)	19 (48%)	15 (38%)	13 (33%)	19 (48%)	16 (40%)	11 (28%)	10 (25%)	5 (13%)
Asthmatic children	40	27 (68%)	20 (50%)	12 (30%)	12 (30%)	17 (43%)	20 (50%)	9 (23%)	8 (20%)	6 (15%)	6 (15%)	8 (20%)	9 (23%)	8 (20%)	4 (10%)

CBt - Crude *B. tropicalis* extract

Bt* - recombinants expressed in *Pichia* yeast

Bt1,3,5,10,11,12,13,A2 were GST-fusion recombinants

BtA2 - novel protein of *B. tropicalis*

Bt6(P) - Proenzyme form of Bt 6

Bt6(M) - Mature form of Bt 6

GST- Glutathione-*S*-transferase protein

5.7 Studies of mite and seafood tropomyosins antigens

Mice were immunised with Der p 10 recombinant allergen produced in the *Pichia* expression system to determine the ability to induce epitope specificity response. The affinity purified recombinant Der p 10 was found to be immunogenic in mice. A titre of 5000X dilution of antiserum binding against recombinant Der p 10 was observed in the antiserum of a nine-week immunized mouse (**Fig. 5.22**). Antiserum raised in mice showed specific IgG-binding reactivity to mite tropomyosin. With the existence of B-cell epitopes shared by the recombinant and the native mite tropomyosin, it is confirmed that *Pichia*-expressed Der p 10 has similar immunogenic activity with that of the native mite tropomyosin.

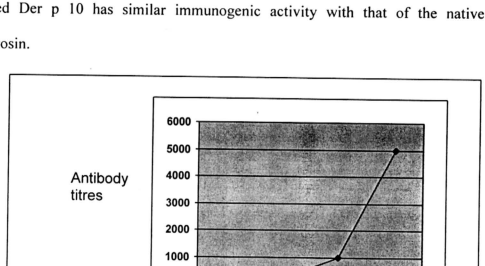
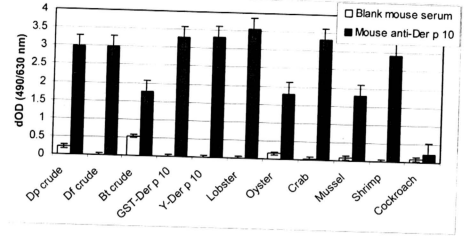


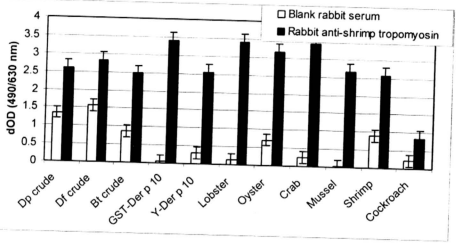
Fig. 5.22: Antibody titres of specific IgG observed in the antiserum of a nine-week immunized mouse.

This antiserum raised against rDer p 10 subsequently recognised both Der p 10 recombinants expressed in *E. coli* (GST-Der p 10) and in *Pichia* yeast (rDp 10) in ELISA (**Fig. 5.23**). A high absorbance was also obtained when the antiserum was challenged against crude HDM extract, suggesting that the antibodies recognised the native Der p 10 too. The clear contrast in anti-mouse IgG binding reactivity of the antiserum and the blank serum of an unimmunised mouse clearly shows the ability of the recombinant mite tropomyosin to elicit an immunogenic response in the mouse.

MOUSE ANTI-DER P 10 (MITE TROPOMYOSIN)



RABBIT ANTI-SHRIMP TROPOMYOSIN



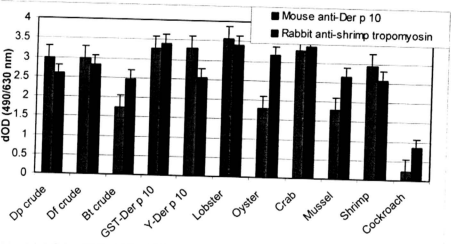


Fig. 5.23: ELISA results of antisera binding to various tropomyosin containing allergen extracts. Rabbit antiserum was raised agains shrimp tropomyosin while mouse antiserum was raised against th yeast-produced Der p 10. For controls, the blank sera from bot uninduced rabbit and mouse were used.

If immunogenicity reflects the potential of a protein to induce IgE antibodies, whereas cross-reactivity is the reactivity of (usually pre-existing) IgE antibodies with the target protein, then the Der p 10 produced in the yeast system is therefore considered immunogenic. This antiserum was also subsequently used in the ELISA for binding to seafood antigens. With the knowledge that a lot of seafood organisms (lobster, oyster, mussel, shrimp and crab) contain similar contents of tropomyosin with that of the mite, comparison was made between the antibody binding to mite tropomyosin and to seafood tropomyosins. Cockroach extract was included in this assay on the basis that its tropomyosin content has been proven allergenic (Rizzo *et al.*, 1999; Pomés *et al.* 2001). The above-mentioned extracts, acting as antigens were coated onto the plate wells and serum from Y-Der p 10 immunized mice, rabbit antiserum against shrimp tropomyosin (kindly provided by Dr J.A. Asturias, Spain) and blank sera from uninduced or unimmunised mouse and rabbit (as negative controls) provided the primary antibodies, while the alkaline phosphatase conjugated anti-mouse IgG and anti-rabbit IgG antibodies served as the secondary antibodies.

The first graph in **Fig. 5.23** shows the IgG binding of the rabbit antiserum raised against shrimp tropomyosin to the various antigens while the second shows the IgG binding reactivity of the serum of Der p 10-immunised mouse. Both antisera were compared with blank sera from unimmunised mouse and rabbit as controls. The last graph was produced by combining the results of the IgG binding of both antisera to the same antigen, side by side, for further comparison. All but the antigens from the cockroach showed high binding of the IgG in the antisera from immunized mouse and rabbit to the various antigens. Unlike the blank serum, the immunised rabbit antiserum containing IgG antibodies to shrimp tropomyosin seemed to recognize the epitopes on the various antigens, suggesting that these seafood antigens possessed binding sites, which were recognized by the antibodies, initially raised against the mite and shrimp tropomyosins. The high antibody binding

capacity of the rabbit antiserum to these antigens suggests that these seafood antigens contained B-cell epitopes recognizable to the IgG in the antiserum. This also indirectly confirms the similarity in the tropomyosin proteins present in the seafood and the mite extracts. In addition to that, the fact that shrimp-tropomyosin specific antibodies recognized recombinants of Der p 10 produced in this study is proof that both recombinants had sustained characteristics resembling closely to those of the native tropomyosin protein.

Therefore this part of the study indirectly indicates that the Der p 10 recombinants were able to mimic most of the epitopes present in these various sources native tropomyosins. The fact that the shrimp tropomyosin antiserum could recognize the rDer p 10 shows that the recombinant products bear almost identical resemblance to native protein. In fact, looking at the last graph, both recombinant tropomyosins showed high binding reactivity when tested with both antiserum from the mouse and rabbit; an indicator that the recombinants carried almost similar epitopes as those found on native tropomyosins, both in the mite and the shrimp. Of interest here is the lower absorbance shown by the antisera to the crude extract of cockroach as compared to the other crude extracts containing tropomyosins, especially when it was reported that amino acid sequences in *D. pteronyssinus* tropomyosin showed 80% identity to cockroach (*Periplaneta americana*) tropomyosin through sequence alignment (Asturias *et al.*, 1998). This low absorbance could also reflect a low concentration of cockroach tropomyosin protein in the crude extract, a common characteristic showing the inconsistencies of contents in extract preparations.

5.8 *In vivo* SPT reactivity to Der p 10 recombinants

Fig. 5.24 shows the SPT results on the arm of a volunteer (Mr Teh Ooi Kock of University Malaya). Extracts tested included histamine, PBS and crude extracts and some seafood extracts; crab, oyster, mussel, shrimp and lobster (from Bencard, UK). Although not of equal sizes, wheals produced by both recombinants of Der p 10 in the SPT suggest that these recombinants of Der p 10 were able to elicit immune reactions, showing proof that both recombinants had allergenic and immunogenic properties. GST-Blo t 10 produced an almost equivalent reaction as the GST-Der p 10. Extracts from shrimp and lobster were the only seafood extracts giving positive SPT reactions while the rest of the seafood extracts gave negative SPT results.

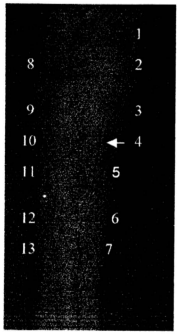


Fig. 5.24: SPT results on the arm of a volunteer.

Extracts tested are as listed below.

1.	Histamine	8.	GST-Blo t 10
2.	PBS	9.	Crab extract
3.	Der p extract	10.	Oyster extract
4.	Der f extract	11.	Mussel extract
5.	Blo t extract	12.	Shrimp extract
6.	GST-Der p 10	13.	Lobster extract
7.	Y-Der p 10		

5.9 Discussion

Use of recombinant allergens in allergy diagnosis

Advances in protein chemistry have since produced improved and more consistent natural allergenic products for diagnosis and treatment of allergic diseases. However, these allergens prepared from natural source materials remain heterogeneous products that may still contain many non-allergenic macromolecules. In vegetable extracts, high amounts of pigments, carbohydrate, and low-solubility materials can hamper the analysis of the allergenic contents. Minor but potent protein allergenic components can also be masked by the presence of major contributors. For example, in a study involving allergens from olive, it was found that the existence of major allergens could hide other strong but scarce allergens (Tejera *et al.*, 1999). These natural allergens also risk being contaminated with allergens from other sources and can contain proteolytic enzymes (Van Der Veen *et al.*, 1996). These enzymes, allergenic or non-allergenic can cause degradation and loss of potency if administered together with other allergens during immunotherapy. Furthermore, due to the mixture of multiple major and minor allergens, natural allergenic extract is difficult to prepare as a standardised mixture, suitable and safe for use in SPT and also immunotherapy.

In this study, from the array of ELISA, the trends of IgE binding to the recombinants were observed to distinguish the allergenicity of the different allergens in the two species of HDMs, *D. pteronyssinus* and *B. tropicalis*. The ELISA involving the allergens of the former species showed higher reactivities than that shown by the Blo t. However, the larger array of Blo t allergens was able to show a wider picture of allergenicity among the patients, not only in terms of frequency but also in the strength of IgE-binding. The recombinant proteins were mostly produced in fusion with GST and polyhistidine $[His]_6$ tags to facilitate purification and detection of the putative proteins from the other cellular host proteins. However tags in a fusion protein can sometimes be

problematic especially when this portion of the recombinant might be an alternative target for antibody binding and therefore contribute to "false positive" results. In a few cases IgE binding reactivity to GST control was detected; which could be explained by the fact that Der p 8 was indeed a mite glutathione transferase (O' Neill *et al.*, 1994). This produced a fear that these fusions might have affected the outcome of the ELISA by producing "false positives". Fortunately, generally low binding observed in the assays involving GST-fusion proteins indicated that allergen-specific IgE did not seem to 'favour' the GST. Furthermore, GST control in all assays confirmed its impartial role as a fusion to the recombinants. There was also no indication that $[His]_6$ tag interfered with the reactivity of rDer p 10, as confirmed by Crameri *et al.* (1998) who showed that the short $[His]_6$ tag in their recombinant *Aspergillus fumigatus* allergens did not interact with sera of allergic patients, therefore, allowing allergens with $[His]_6$ tag to be used in a straightforward way for *in vitro* and *in vivo* applications.

Evaluation of crude extracts and recombinants in allergy diagnosis

Recombinant allergens serve as very useful representatives of the repertoire of allergens in an organism. The aim of achieving precise knowledge or information of an allergy leading on to the useful manipulation in immunotherapy has therefore highlighted the importance of an initial step of carrying out component-resolved diagnostics facilitated by high quality production of recombinant allergenic components. The crude extract, serving as the full challenge, is expected to show highest IgE binding response (if any) while GST, the control should, in turn, show the lowest. In this study, 12 recombinant allergens all produced by means of two expression systems, i.e. by *E. coli* or yeast *P. pastoris*, at times showed higher reactivities than the crude extract. While crude HDM extract is deemed to contain all allergens and therefore warrants a maximum response, this phenomenon need not be true. A few reasons inherent to the crude extracts may explain this unusual response. Firstly, the crude extracts are composed of a mixture of allergens of

various kinds, from enzymes like proteases and inhibitors that would disrupt the presence of other components including certain allergens when kept in storage for too long. For example, group 1 mite allergens are cysteine proteases while group 9 are collagenolytic serine proteases. Secondly, the crude extract may not be 'complete' as some allergens may be absent if certain component of an organism is inadvertently excluded in the preparation, for example fecal pellets of a mite or the pollen of grasses. Inconsistencies also exist in terms of the proportions of allergens in commercial crude extracts (Ortolani *et al.* 1994; Meyer *et al.*, 1989). Crude extracts may also be contaminated with other major allergens giving a false positive result. For example, in a study of dog extract in SPT reactions, positive results were obtained but through RAST, dog-specific antibodies failed to be detected. It was later revealed that the wheal was actually caused by the presence of Der p 1 contamination in the dog extract (Meyer *et al.*, 1989).

There are two probable reasons when interpreting the low binding reactivities in the results of ELISA involving recombinant allergens. A recombinant allergen may have failed to produce the right folding as in the native and therefore failed to be recognised by antibodies. However, in this study, a comparison to the native mite tropomyosin could not be carried out, thus forcing mite allergenicity to be deduced indirectly only from comparison and interpretation of results obtained. Another reason could be due to the fact that the allergen is only a minor one in the array of allergens in a mite. As a rule, a major allergen can induce an allergic reaction in more than 90% of patients allergic to the protein while minor allergen is only important for a small number of people, typically 30 to 70% (Baranink, 1988). A low response to a recombinant allergen therefore should be interpreted with caution so as not to make a wrong conclusion about its allergenicity. Therefore, it is deemed best to do a comparison of a recombinant allergen against that of its native form for a better evaluation of the allergen. A notably large difference in binding of antibodies to a native allergen and its recombinant form would tentatively imply the substandard quality

of recombinant production, perhaps stemming from incorrect sequence expression, incorrect folding or the absence of post-translational modifications.

Evaluation of recombinant Der p 10

Cloning of cDNA encoding mite tropomyosin (Der p 10) and expression by *E. coli* bacteria and *P. pastoris* yeast produced recombinant proteins in a quantity of 1-2 mg/liter culture; with which subsequent assays proved to be recognizable to almost 25% of the population tested. This does not reflect the failure of the recombinants to emulate the native allergen, but is a result of low positivity to Der p 10 in the population tested, especially that it had proven itself to be antigenic and immunogenic in the *in vivo* evaluation (**Fig. 5.24**). The combined use of rDer p 10 and other components of house dust mite allergens in the form of recombinant allergens has enabled the determination of exact high responders in the house dust mite allergy in Malaysian patients. Initial low frequency of response in the GST-Der p 10 antigenic evaluation; and relating it to the absence of post-translational modification in bacteria systems led on to the selection of yeast system to produce Der p 10 for further comparison. This underlines the difference of production of proteins with and without the post-translational modifications as offered by the respective hosts, in relation to its allergenicity. Schistosome derived-GST serving as the control in the assays confirmed its impartial role, neither enhancing nor impairing the performance of the recombinants as antigens in the ELISA assays. This was the basis of using uncleaved GST-Der p 10 in subsequent assays.

Antigenicity and allergenicity of mite tropomyosins (Der p 10 and Blo t 10)

The mite tropomyosins (Der p 10) used in this study, was produced as recombinant proteins from the expression systems of bacteria (*E. coli*) and yeast (*P. pastoris*) as described in **Chapter 4**. Evaluation of these two recombinants of mite tropomyosins faced a shortcoming, as the native mite tropomyosin could not be obtained for comparison.

Therefore the effects of each variation upon, for example, IgE antibody binding reflecting their antigenicity, or the structural stability, was difficult to analyse without the native tropomyosin. However, as there were two clones to be compared, confirmation based on each other antigenic properties can be taken for consideration. For instance, both recombinants were able to present themselves for antibody binding. In 40 allergic rhinitis patients, there was comparable binding of IgE to both recombinants. To be noted here is the low IgE binding strength shown by both recombinants with absorbance of <0.5 for GST-Der p 10 and <0.6 for the *Pichia*-produced Der p 10. Majority of the patients seemed to show higher binding to the GST-Der p 10, although most did not exceed OD 0.4 .

Contrary to the report by Aki *et al.*, (1995), [that HDM tropomyosin showed 80% allergenicity by means of testing 31 sera for IgE binding reactivity initiated that mite], this study found recombinant mite tropomyosins (rDer p 10), expressed in prokaryotic and eukaryotic systems, did not match this description. Only about 20-30% of the test population showed IgE binding to these recombinants. The low prevalence of allergenicity to these recombinants suggests that among Malaysian patients, Der p 10 is not a consistently high responder in the array of allergens of *D. pteronyssinus*. In our neighbouring country, Singapore, low response to Group 10 mite allergens was also reported (Yi *et al.*, 2002), proving that group 10 mite allergen is not a major allergen in this part of the region. In the evaluation of *in vitro* diagnostic value of recombinant mite tropomyosin allergens using sera from 100 allergic rhinitis patients and 100 asthmatics, comparable IgE binding results were obtained in comparison to another recombinant mite tropomyosin Blo t 10. This is expected, considering that Blo t 10 and Der p 10 shares a homology of >70%. The serological investigation involving 60 asthmatic Taiwanese sera, also showed similar trend of low absorbance for IgE antibody binding. Both results also showed a tendency to agree with Asturias *et al.*, (1998) that only 20% of sera from monosensitised Spanish patients showed positive results with the rDer p 10 in ELISA. This discrepancy may be due to the different pools of sera (i.e. Malaysian, Taiwanese versus

Japanese), suggesting that there are some inherent factors in the sera that might have contributed to the differences. Therefore too, had rDer p 10 been a major allergen, it would by right be more appropriate if the monosensitised sera were to show a higher and precise reactivity to Der p 10. Thus, in polysensitised patients, higher prevalence of Der p 10 reactivity could have been a result of cross-reactivity to different tropomyosins. Because most tropomyosins share a high percentage of homology among different seafood species, it is justifiable to link the high reactivity (80%) to cross-reactivity of a polysensitised population.

Comparison of mite tropomyosins (Der p 10 and Blo t 10) to other tropomyosin-containing extracts

Investigators such as Lehrer *et al.* (1990); Daul *et al.* (1994); Shanti *et al.* (1993), Leung *et al.* (1994) studied allergenic activities of shrimps as a model food allergen. Daul *et al.* (1994) discovered a 36kD allergen *Pen a* I, which reacted with 28/34 (82%) of sera from shrimp-sensitive, SPT-positive and RAST-positive, individuals. Endoproteinase Lys-C digestion on *Pen a* I yielded a 21-residue peptide which strongly suggests that *Pen a* I is a shrimp tropomyosin. Through immunoblot and RAST inhibition, its presence in other crustaceans was demonstrated; leading to the proposal that '*Probably, tropomyosin is a major crustacean allergen present in crab, shrimp and crawfish*' (Daul *et al.*, 1994). Similarly, Witteman *et al.* (1994) identified a cross-reactive allergen, presumably tropomyosin, in shrimp, mite and insects. Although homology is very high within tropomyosins from arthropods with 80-75% identity, it was found to decrease as phylogenetic distances increased: nematodes (72-69%) and mollusks (65%). Tropomyosins from vertebrates (human, chicken, mouse) contain less than 56% identical amino acids, although the high similarity (74%) reflects a similar three-dimensional structure.

In this study, mouse antisera containing anti-mite tropomyosin antibodies and rabbit antisera raised against shrimp tropomyosin, both recognised the conserved epitopes

of other arthropod tropomyosins (**Fig. 5.23**). This indirectly suggests a case of interspecies cross-reactivity where the antibodies are produced from the same antigenic response to the tropomyosins. This situation has been observed also in the antibody response of rabbit immmunised with tree (Ipsen *et al.*, 1985) and grass (Standring *et al.*, 1987) allergens, thus showing that cross-reactivity is not limited to the human immune responses. Therefore shared allergenic epitopes in cross-reactive allergens too can lead to IgE recognition reactivities causing clinical manifestation, i.e. when a special threshold level of specific IgE antibody is reached (Ventas, 1991). However, there have been similar reports of reduced reactivity of the recombinants when compared with the native protein. Tsai *et al.* (2000) reported that while 80% (55/67) of mite sensitive sera showed positive *in vitro* IgE reactivity with native Der p 11, only 62% of the sera tested reacted positively with the recombinant Der p 11 (designated rDf642 protein); to which they attributed the reason being that the N- and C-terminal truncations in the rDf642 may have resulted in lower frequency of *in vitro* IgE.

Allergenicity of mite and seafood tropomyosins

Recent works in shrimp tropomyosins reported six IgE-reactive peptides (Reese *et al.*, 2001) with different degrees of homology to Der p 10. The sequences of the peptides E2, E3, E4, and E6, obtained from *Penaeus aztecus* (brown shrimp) tropomyosin, are highly homologous to Der p 10 (with 100, 77, 81, and 93% identity, respectively), while peptide sequences P6 and P, from *P. indicus* (Indian shrimp), have a 65% homology to Der p 10. An important fact that differentiates mite tropomyosin from seafood tropomyosins is that the latter would have undergone heating upon cooking before being consumed. The heat might have affected the structure and therefore their antigenic determinants too.

Despite the presence of tropomyosin in humans and in dietary meats (chicken, pork, beef and mutton) the lack of allergenic reactivity between invertebrate and

mammalian tropomyosins is striking. IgE responses are selective for cockroach, mite, snails, and seafood tropomyosins which share ~80% sequence homology. These tropomyosins show ~45% homology with human and meat tropomyosins, which presumably explains the lack of allergenic cross-reactivity. The presence of IgE epitopes in the part of molecules that do not share homology with human and meat tropomyosin could explain the lack of sensitization to meat and human endogenous tropomyosins (Pomés *et al.*, 2001).

Assembling complete panels of allergens for specific allergy diagnosis or monitoring

The progress in characterising HDM allergens has given new scope for recombinant or modified allergens to be used for more effective immunological treatment as well as for studying the induction of sensitization and disease (Thomas *et al.*, 1998). The recombinant allergen molecules can be used to establish multi-allergen test systems (for example, allergen microarrays) that facilitate the determination of the reactivity profile of an allergic patient. On the basis of this information, it is possible to discriminate between those allergic patients who are sensitized against a few allergen components (oligosensitized patients) and those who react against many allergen components (polysensitized patients). Antigen-targeted immunotherapy of established allergy can be applied to oligosensitized patients, according to their reactivity profiles, whereas polysensitized patients might benefit more from symptomatic treatment. Furthermore, with relevant hypoallergenic allergen sources, it will be possible to assemble prophylactic allergy vaccines. Shibasaki *et al.*, (1994) showed that infants first produce IgE to the Der p 2 and then the response diversifies. This concurs with the finding that IgE binding of most children is predominantly restricted to Der p 1 and Der p 2 while the binding in adult patients becomes diverse (O'Brien and Thomas, 1994). Cord blood cells can mount proliferative responses to purified Der p 1 and Der p 2, suggesting that an early sensitization to these allergens (Holt *et al.*, 1995). Some allergens are labile (King *et al.*,

1996) and potent, inducing very effective IgE responses even though they are low in concentration in extracts but. Allergen imbalances could also affect the efficacy of desensitization, where side effects of allergens in high dose would hinder the ability to each therapeutic dose of the other allergens. The same difficulty would be encountered during monitoring by skin test whereby HDM extracts may have important limitations for measuring both qualitative and quantitative differences in sensitization (Thomas *et al.*, 1998). For instance, Der p 1 and 2 at up to 10 - 30% of the proteins in extracts are abundant proteins (Heymann *et al.*, 1989). Therefore, subjects with high anti-Der p 1 IgE titres and few other specificities react strongly with HDM extracts while those binding to 3-4 other allergens present in low concentrations are low responders.

On the whole, the observed trends of IgE binding to recombinant allergens were able to reveal the allergenicity of allergens in *D. pteronyssinus* and *B. tropicalis*. This study showed that HDM allergenicity occurs at component level and not to the whole total extract. Results from this work show most of these allergens are important hypersensitising agents, although their specific incidences seem to be dependent on different factors, such as the geographic area of the patients or the origin of the samples used for immunologic assays. This approach of identifying potential responders among the recombinants on the basis of the initial positive response to the crude extracts of the HDM is deemed appropriate to obtain a patient's profile of allergy. Although these recombinants used in this study were not the complete array of the allergenic components in the native HDM allergens, but they represent almost all major allergens in the mites. Therefore, subsequent to the production, usage of these recombinants is a step towards refining their qualities for their appropriate application. The diagnostic value of recombinant allergens suggests that a selection of a few allergens from an allergenic source might be sufficient for diagnosis of sensitised subjects. In all these aims, recombinant allergens must be made to present themselves strategically and correctly, for recognition and then for binding to antibodies.