Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Serum samples

All sera from Group (I) – (V) were locally obtained while sera of Group (VI) were obtained from Taiwanese asthmatics (kindly provided by Dr Chua K.Y. from National University of Singapore **(Table 2.1).** Patients in Group (I) were those visiting the ENT Department of University Malaya Medical Centre (UMMC) from mid-1996 to 1998 while Group (II) were patients who visited the Chest Clinic between 1996 to 1999. All were subjected to SPT and saliva samples were obtained from them too. Group (III) were asthmatic children visiting the Paediatrics Clinic in UMMC from Jan 1999 to 2001. These children were, however, not subjected to SPT. Only a maximum of 3 ml of blood was taken from each of them whenever possible. Group (IV) consisted of normal healthy subjects without any history of allergy, were recruited as control group in this study. Similarly, these subjects also underwent SPT and about 5 ml of blood was taken from them. Lastly, sera from 60 Taiwanese asthmatic patients (kindly provided by Dr Chua K.Y, NUS) were also included in this study for comparison with Malaysian asthmatics.

Table 2.1: Background information of study population

Study group	No. of subjects	Mean age and range (years)
I. Patients with rhinitis	291	32 (17 - 58)
II. Patients with asthma	298	44 (17 - 65)
III. Children with asthma	150	8 (2 - 15)
IV. Negative controls	30	25 (23 - 58)
V. Taiwanese patients with asthma	60	(Not available)

2.1.2 Questionnaire

The questionnaire that was used in this study was designed in the simplest form to obtain sufficient clinical history from the patients of Groups (I), (II) and (IV) (**Appendix 1**). Information derived was later analysed with Microsoft Excel programme.

2.1.3 Allergen extracts

Allergen extracts (**Table 2.2**) were obtained from Meridian (USA), in concentrations of 10,000 Allergenic Units or AU/ml and preserved in 50% glycerol. The choice of allergens was based on the current knowledge of sources of allergens, as reported in journals, including some inhalant allergens as recommended by Lee, 1990. *B. tropicalis* extract was obtained from Dr. Caraballo L. from Columbia and was used in the SPT as recommended, i.e. at 1 μg/ml concentration. Plant aeroallergens from the local fauna were selected on the advice of Prof. Lim A.L. from the Botany Department in University of Malaya. Aliquots of 60 μl were brought to the clinic each time, kept at 4°C and were used before their expiry dates.

Table 2.2: List of aeroallergens and food allergens used in SPT

Sources of aeroallergens	Food allergens
HDMs: *D. pteronyssinus* , *D. farinae* and *B. tropicalis*	Oyster (*Ostrea* spp.)
House dust	Shrimp (*Crago* sp.)
Cat hair (*Felis domesticus*)	Crab (*Xiphosurus sowerbyl*)
Dog hair	Soybean (*Glycine soja*)
Mold mix	Chili pepper (*Capsicum frutescens*)
Grass mix	Egg white
Cockroach (*Periplaneta americana*)	Chicken meat (*Gallus* sp.)
Acacia spp.	Beef (*Bovine* sp.)
Melaleuca sp.	
Aspergillus fumigatus	
Bahia spp.	
Rough pigweed	

2.1.4 Indoor dust sampling requirements

A vacuum cleaner of 1000-Watts power and a collection device (ALK, Denmark), were used. Each dust sample was kept in a foil and a plastic sealbag. Alcohol was needed to wipe the device after each round of sampling.

2.1.5 Molecular cloning vectors

The cloning vector for *Escherichia coli* expression system, pGEX-4T-1 (**Fig. 2.1**) [Pharmacia, (USA)] expresses a cloned gene as a fusion protein to a fusion partner, glutathione-*S*-transferase (GST). It contains an open reading frame encoding GST, followed by unique restriction endonuclease sites for *Bam*H I, *Sma* I and *Eco*R I and termination codons in all three frames. The *lac* repressor binds to the p_{tac} promoter, repressing the expression of GST fusion protein. Upon induction with Isopropyl-β-thiogalactopyranoside (IPTG), derepression occurs and therefore GST fusion protein is expressed. The polylinker of pGEX-4T-1 also contains protease cleavage sites enabling cloned protein to be released from the GST moiety.

The cloning vector used in *Pichia pastoris* expression system is the pPICZ B (3328 bp) (**Fig. 2.2**) [Invitrogen Corporation (USA)]. The 5'*AOX1* promoter region (bases 1-942) allows methanol-inducible, high-level expression in *Pichia,* targetting plasmid integration to the *AOX1* locus. The multiple cloning site has 10 unique restriction sites enabling the insertion of the gene of interest into the expression vector. The C-terminal myc epitope tag - (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn) permits detection of the fusion protein by the anti-C-myc antibody while the C-terminal polyhistidine tag encodes six histidine residues that form a metal binding site for affinity purification of the recombinant protein. The *TEF1* and EM7 promoters drive expression of the *Sh ble* gene (*Streptoalloteichus hindustanus ble* gene) in *Pichia* to confer Zeocin™ resistance. Linearization at the *AOX1* locus is permitted at *Sac* I, *Pme* I or *Bst* X I unique restriction sites.

2.1.6 *Escherichia coli* strains

- **BL21 (DE3) trxB**: F^-, *ompT*, (r_B^-,m_B^-), (*imm*21, *lac*I , *lac*UV5, T7 *pol*, int$^-$), trxB$^-$.
- **TOP10F'**:{*pro*AB, *lacI*^{q}, *lacZ*ΔM15, Tn*10*(Tet^R)] *mrc*A, Δ(*mrr-hsdRMS-mcr*BC), Δ*lac*X74, ϕ80*lacZ*ΔM15, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L(StrR), *end*A1, *nup*Gλ$^-$.

2.1.7 *Pichia pastoris* strain X33

The genotype of *Pichia* strain used in this study was the X33, a wild-type *Pichia* strain that is useful for selection on Zeocin™ and large-scale growth. The growth temperature is 28-30°C for liquid cultures, plates and slants. Growth above 32°C during induction is detrimental to protein expression and can lead to cell death. Doubling time of log phase *Pichia* strains is about 2 hours and normal growth can be measured up to one OD_{600} = ~5 $X10^7$ cells/ml.

2.1.8 Media for cloning and expression

(a) *Media for E. coli expression*

All the media needed for *E. coli* expression such as Luria-Bertani (LB) broth, SOB and SOC liquid media were prepared as described in Sambrook *et al.* (1989). After autoclaving, the SOB medium (pH 7.0) was allowed to cool down to just below 60°C and then 10 ml of sterilized 2M $MgSO_4$ was added. The SOC liquid medium had an extra addition of 10 ml of sterilised 50% glucose.

(b) Media for Pichia expression

Medium (1) and (2) were prepared as recommended by manufacturer; and allowed to cool to at least 55°C before adding Zeocin™ to a final concentration of 25 μg/ml. Agar plates were stored at 4°C in the dark; stable for 1-2 weeks.

(1) Yeast Extract Peptone Dextrose Medium [YPD (+Zeocin™)]

(2) Buffered Methanol-complex Medium (BMMY)

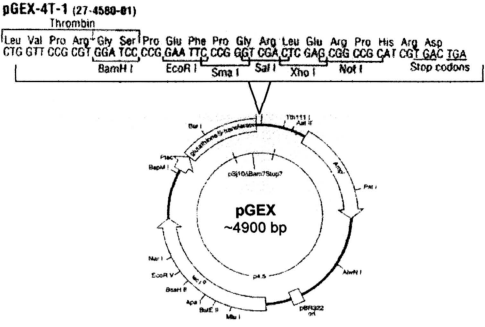

Fig. 2.1: The features of cloning vector, pGEX-4T-1

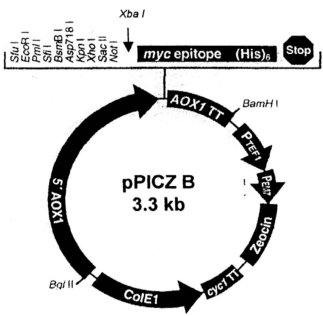

Fig. 2.2: The features of the cloning vector, pPICZ B

2.1.9 Antibiotics as selection markers

Ampicillin, is a derivative of penicillin that kills growing cells by interfering with a terminal reaction in bacterial cell wall synthesis. The mechanism of resistance is provided by the resistance gene (*bla*) specifying for a periplasmic enzyme, β-lactamase, which cleaves the β-lactam ring of the antibiotic. In this study, ampicillin was used at a working concentration of 50-125 μg/ml.

Zeocin™ from the phleomycin family of antibiotics is a basic, water-soluble, copper-chelated glyco-peptide, which shows strong toxicity against bacteria, fungi (including yeast), plants and mammalian cell lines. When Zeocin™ enters the cell, the Copper cations are reduced from Cu^{2+} to Cu^{1+} and removed by sulfhydryl compounds in the cell. This activates Zeocin™ to bind DNA and cleave it, causing cell death. However, activity of Zeocin™ is inhibited by extreme pH and high ionic strength. As such, to optimise selection in *Pichia* expression system, salt concentration must be <110 mM and pH must be 7.5.

2.1.10 Reagents for bacterial expression and post-expression analysis

Common chemicals and reagents were mostly obtained from Sigma (USA), BDH, Merck or Fluka, unless otherwise stated. Reagents for GST-Der p 10 *E. coli* expression (**i-iii**), for purification involving affinity chromatography (**iv-v**) and for protein analysis, (**vi-xi**); were prepared as described in Sambrook *et al.* (1989).

i. Isopropyl-β-thiogalactopyranoside (IPTG) stock solution
ii. Phenylmethylsulfonyl fluoride (PMSF) solution
iii. Ampicillin stock solution
iv. Tris-buffered saline (TBS)
v. Glutathione elution buffer
vi. 5X Acrylamide/bisacrylamide (38:2) [30% acrylamide stock solution]
vii. SDS sample dye (5x)
viii. Gel Separating Buffer (5x)
ix. Transfer buffer

2.1.11 Reagents for yeast expression and post-expression analysis

Most reagents used for the *E. coli* expression system were similarly used in the *Pichia* expression system too. Based on the polyhistidine (6xHis) sequence in the *Pichia*-produced rDer p 10, the ProBond™ Purification System was used to facilitate purification. This resin has been pre-charged to bind to a considerable amount (1-5 mg) of recombinant protein per 1 ml. Buffers needed were prepared according to the purification protocol from the manufacturer.

2.1.12 Peptide synthesis requirements

Multipin Peptide Synthesis kits were purchased from Chiron (Victoria, Australia), comprising of a software programme - PepMaker; 20 Fmoc-protected amino acids (10 g/bottle); and a set of synthesis kit (**Fig. 2.3**). Each synthesis kit consisted of 96 polyethylene pins with gears attached to each pin, a holder, reaction trays (polypropylene 96-well microtiter plates), duplicates of positive and negative control pins with synthesized peptides (PLAQ and GLAQ), and antibody for testing; all of which were kept in a sealable container. These items were stored at 2°- 8°C and away from direct sunlight.

PepMaker programme - The PepMaker application enabled the preparation of schedules for synthesis allowing the choice of synthesis type, selection of coupling chemistry and most importantly to set up the parameters of the synthesis. Synthesis details included well volume; amino acid concentration; number of copies, peptide design and also the day-to-day synthesis schedule [**Appendix 3**].

Amino acids - The amino acids (AA) recommended for use with this kit had α-amino groups protected with the 9-fluorenylmethoxy-carbonyl (Fmoc) group and the following side chain protecting groups: *t*-butyl ether (Bu^t) for serine, threonine and tyrosine; *t*-butyl ester (OBu^t) for aspartic acid and glutamic acid; *t*-butoxycarbonyl (BOC) for lysine, histidine and tryptophan; trityl (Trt) for cysteine and 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl (Pmc) for arginine. These amino acids were stored at 2°- 8°C.

Block, pins and reaction trays - A block describes the complete unit of pin holder, with 96 pins attached to an 8 x 12 matrix and 5 legs to prop it up. A pin (**Fig. 2.4**) is described as polyethylene rods of about 2.5 cm in length. The important part of a pin is the 'gear' attached to the stem, which is the exact site of peptide synthesis. The polypropylene reaction tray resembles the common microtitre plate of 96-wells, differing only in the numbering system of the wells i.e. referred as having 12 rows and 8 columns; unlike the commonly used 8 x 12 configuration.

Solvents and solutions - Most of the chemicals and reagents used in peptide synthesis were obtained from Sigma Chemical Co. (USA); BDH Ltd. (England); Auspep (Australia) and Fluka Chemika (Switzerland); unless otherwise stated. All reagents were of AR standard, stored appropriately as required. Besides common chemicals such as methanol, ethanol, etc., two main groups of chemicals were the solvents and chemicals required in the peptide synthesis procedure such as N,N-dimethylformamide (DMF), piperidine, acetic anhydrate, trifluoroacetic-acid (TFA), ethanedithiol, diisopropylcarbodiimide (DIC), 1-hydroxybenzo-triazole (HOBt) and anisole. As these chemicals were toxic, flammable, corrosive or carcinogenic, appropriate precautions (i.e. wearing gloves, lab-coat, facial mask, etc.) were heeded and all syntheses were carried out in a fully functional and operating fume hood.

Reagents were used at a recommended molarity of 1.00:1.00:1.20 for AA:DIC:HOBt. DIC was the activator and HOBt acted as an additive. For a satisfactory volume of activator:amino acid; solution was prepared at 20:80. Thus if 1 ml of activated amino acid was needed at a final concentration of 100 mM in the activated mixture, 0.8 ml of 150 mM HOBt solution in DMF was prepared. Into this, enough of the Fmoc protected amino acid would be dissolved to give a 125 mM solution. Then the activator solution was prepared; 0.2 ml of 500 mM DIC in DMF. Finally just before dispensing, both solutions were mixed together giving a solution of activated amino acid that contained 100 mM amino acid, 100 mM activator DIC and 120 mM HOBt.

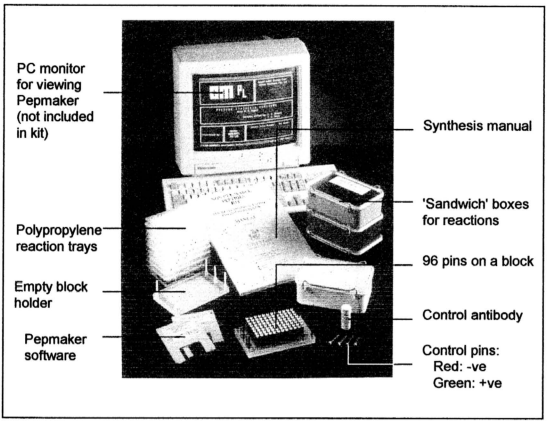

Fig. 2.3: Multipin Peptide Synthesis kit from Chiron Mimotope Peptide System.

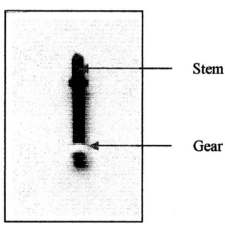

Fig. 2.4: A pin showing the gear, which is the site of peptide synthesis

2.1.12 General materials

Restriction endonucleases were obtained either from Promega (USA), New England Biolab or Boeringher Manheim. *Taq* polymerase and dNTP mix were obtained from Promega while the DNA markers were from BRL. Oligonucleotides used as primers in the PCR reaction of the bacterial expression system was obtained from the DNA Peptide Synthesis Service of National University of Singapore (NUMI) while Operon Technologies Inc., prepared the primers for the yeast expression system. Pre-stained markers were used, obtained from Bio-Rad (USA). Rabbit peroxidase-conjugated anti-human antibodies were purchased from DAKO (Denmark) or Kirkegaard and Perry Laboratories (KPL, USA) while anti-GST antibody was obtained from Pierce and anti-C-myc antibody was provided by Dr Tan C.S. of MARDI. The colorimetric substrates used depended on the enzyme conjugated to each secondary antibody. *O*–phenylene diamine (OPD), 3',3',5',5'-Tetramethylbenzidine (TMB), 2.2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and ρ–Nitrophenyl phosphate (ρNPP) were prepared and used as described in Sambrook *et al.*, (1989).

2.2 Skin prick test

Skin prick test (SPT) was carried out at the ENT Clinic and the Chest Clinic of University Hospital, Kuala Lumpur. Upon clinical examination and getting the patients' consent, questions were posed followed by SPT. Six µl of various allergen extracts was placed 2 cm apart on the volar surface of the forearm of patients. These extracts were introduced into the skin subcutaneously, by using a sterile syringe needle. Histamine (1mg/ml) and phosphate buffered saline (PBS) were included as positive and negative controls respectively. PBS was used as control because it represents the natural human fluid. Sizes of resulting wheals for each allergen were measured after 15 min and recorded. Positivity was determined on the basis of wheal size exceeding that of the negative control.

2.3 Serum and saliva sampling

a) ***Serum samples*** - Samples served to provide primary antibodies *in vitro* studies involving ELISA. SPT was performed upon patient's consent and 5 ml of blood was taken from patient. Blood samples were centrifuged at 3000 rpm for 5 min to collect the sera that were then stored in tubes and kept at -20°C until needed.

b) ***Salivary samples*** - Uninduced salivary samples were obtained from patients and the control group by absorption of dental swabs (Walterollen, Germany), which was then spun in a special tube called salivette (Sarstedt, Germany) (**Fig. 2.5**) at 1500 rpm for 5 min. Similarly, samples were kept at -20°C. Not all patients contributed significant amount of saliva from their swabs.

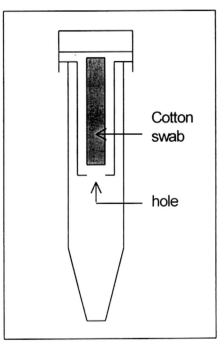

Fig. 2.5: Salivette

N.B. A two stage test tube for collecting saliva samples. Salivary flow was stimulated mechanically by introducing the cotton swab into the subject's mouth for one to two minutes. The saliva-soaked cotton was then placed into the upper compartment. Salivette was centrifuged at 1500 rpm for 5 minutes. The saliva collected at the bottom of the tube was then frozen until needed.

2.4 Method of dust sampling

The homes of 36 ten-year old children living in the vicinity of Kuala Lumpur and Petaling Jaya (Selangor) were involved in this study. Dust was collected and handled as recommended by the New Zealand collaborators. Using the vacuum cleaner and the collection device, two dust samples were obtained from each household. Sample 1 was obtained from the floor of the main living room. A two metre square (2 m x 1 m) area of a carpeted floor was vacuumed for 2 min while uncarpeted floor was vacuumed at a rate of 1 min/m^2. Sample 2 was obtained from the child's mattress, vacuumed for 2 min. Samples were kept in the freezer and sent by air to be analysed in University of Otago, New Zealand. The dust was later sieved (425 μm) with the fine dust used to estimate Der p 1 and Der f 1 levels using a double monoclonal antibody ELISA as described by Luczynska *et al.*, 1989.

2.5 Expression of Der p 10 in *Escherichia coli*

The various methods used for cloning and manipulating DNA fragments such as the extraction of DNA, quantification of DNA and RNA, small-scale or large-scale of plasmid DNA isolation, restriction enzyme digestion, DNA agarose electrophoresis, preparation of blunt-ended DNA fragments, the ligation of DNA fragments; were mostly carried out according to laboratory manual by Sambrook *et al.*, (1989). Therefore only steps involving modifications are described in the production of rDer p 10.

2.5.1 Construction of pGEX-Der p 10

Amplification of Der p 10 cDNA

The polymerase chain reaction (PCR) was carried out to amplify cDNA fragments of Der p 10 (kindly provided by Dr Thomas W.R., through Dr Chua K.Y.) using downstream and upstream complementary sequence primers. In a standard 25 μl PCR reaction, 50-100 ng of DNA template was mixed with 50 pmol of upstream primer, 50

pmol of downstream primer, 25 nmol each of dNTP's, 1 Unit of *Taq* polymerase and PCR buffer containing 50 mM Tris (pH 8.8), 10 mM ammonium sulphate, 4 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.1 mg/ml of BSA. PCR reaction was carried out on the Perkin-Elmer GeneAmp PCR machine according to schedule of first denaturing the template DNA at 94°C for 3 min, followed by 25 reaction cycles of denaturation at 94°C for 1 min; annealing at 55°C for 45 sec and extension at 72°C for 1 min; with a final extended reaction at 72°C for 5 min. Upon completion, 5 µl of the product was analysed by gel electrophoresis [1% (w/v) agarose (Seakem, USA), dissolved in 1x TAE (pH 8.5)]. DNA samples were mixed with 6x BPB loading buffer (at 5:1 ratio) loaded into the gel and run at 90V. The gel was visualised with ethidium bromide staining under UV illumination.

Restriction enzyme digestion

Plasmid DNAs were digested with appropriate restriction enzymes to excise inserted DNA fragments for 1-2 h. Each digestion was carried out in a sterile 1.5 ml Eppendorf vial in final reaction volumes of 30 to 50 µl. containing 0.5 to 100 µg of DNA and 10 µl of restriction endonuclease in appropriate buffer. Double digestions with *Sma* I and *Bam*HI were carried out in assay buffer sequentially, first with *Sma* I at 25°C for 1 hour and then with *Bam*HI at 37°C for another hour and further incubated at 37°C for 2 hrs for complete digestion. The cut DNA was then electrophoresed on agarose gels containing ethidium bromide and was visualised under UV.

Ligation of insert and vector

Ligation mixture containing one part vector and five parts insert [vector:insert=1:5] amounting to 50 µl was then prepared in an Eppendorf tube and a "vector only" preparation for self-ligation, was also prepared as a control (**Table 2.3**). A competent cell control was also prepared as a systematic control. The ligation was performed overnight at 16°C in the water bath.

Table 2.3: The reagents for ligation. "Vector only" served as the control for the ligation assay.

Reagents	"Insert + Vector" Volume (µl)	"Vector only" Volume (µl)
Insert (Der p 10 cDNA)	25.0	-
Vector (pGEX-4T-1)	5.0	5.0
T4 DNA ligase	1.0	1.0
10x ligase buffer	5.0	5.0
sdH_2O	14.0	39.0
Total	50.0	50.0

Another restriction endonuclease digestion was carried out after this ligation process to determine correct insertion of the insert into vector, before being the transformation of the construct into the competent cells. The plasmid DNA construct containing pGEX-4T-1 and the Der p 10 gene was therefore designated **pGEX-Dp10.**

2.5.2 Competent cells preparation

The procedures to prepare competent cells were as described by Nishimura *et al.* (1990) with slight modification. TOP10F' bacteria were grown in 50 ml culture medium (LB media supplemented with filtered 10 mM $MgSO_4.7H_2O$ and 0.2% glucose) to the mid-logarithmic phase (OD_{600}=0.4-0.7) in a shaking incubator at 37°C before the cells were harvested by centrifugation at 2500xg for 5 min at 4°C. The cell pellet was then resuspended gently in 0.5 ml pre-chilled culture medium and then mixed without being vortexed, with 2.5 ml of storage medium [36% glycerol, 12% PEG (MW 6000), 12 mM $MgSO_4$ in LB and sterilised by filtration]. The competent cells were divided into aliquots of 100 µl each in microcentrifuge tubes and stored at -80°C.

2.5.3 DNA transformation of *E. coli*

Following a successful ligation procedure producing the pGEX-Dp 10 construct, *E. coli* transformation was carried out according to the "heat shock" method described by Nishimura *et al.* (1990) with slight modifications. Frozen competent cells (200 µl) were thawed, mixed immediately with a maximum of 10 µl of ligation mixture, and incubated on ice for 30 min. The mixture was then subjected to a heat pulse at 42°C for 90 sec, and then chilled on ice for 1 to 2 min. A volume of 1 ml of pre-chilled culture medium was then added followed by incubation at 37°C in a shaking bath for at least 30 minutes. The mixture (from 10 to 200 µl, depending on the DNA amounts used in transformation) was then plated on agar plate containing ampicillin to allow selection for transformants and then incubated at 37°C overnight.

2.5.4 Bacterial colony screening

Amp^R transformants were selected and raised in minipreps containing 2 ml LB (+Amp), shaken at 37°C overnight. On the following day, 1.5 ml of the broth was removed and added to 4.5 ml of fresh LB broth with ampicillin before being shaken for another 1 hour. One of these tubes were selected, from which 100 µl of its content was removed and placed in an Eppendorf tube and centrifuged at 14 000xg for 5 min. The pellet was retained and promptly kept at 4°C, to serve as the "non-induced sample" in the SDS-PAGE analysis. Then into all the rest of the broth in the tubes, 25 µg/ml of the inducer IPTG was added and shaking was resumed for another 2 hours (37°C, 200 rpm). Then 0.1 ml of culture from each miniprep was pelleted and added with 10 µl of gel loading sample buffer, to be boiled for 10 minutes before subjected to a SDS-PAGE analysis.

2.5.5 Expression of GST-Der p 10

An overnight small-scale culture of *E. coli* BL21, transformed with the putative pGEX-Dp 10 construct, was diluted 1:100 in 200 ml LB broth containing 2% glucose and 0.1 mg/ml ampicillin. Culture was grown at 37°C with vigorous shaking to an OD_{600}=0.5 before adding IPTG to a final concentration of 0.1 mM to induce expression. Induction was allowed only for 2 hours to avoid inclusion bodies formation.

2.5.6 Purification of GST-Der p 10

Upon verification of GST-Dp 10, a scaled-up continuous 1-litre culture was carried out. After induction for 2 hours, the cells were pelleted and resuspended in 10 ml of ice-cold 1X PBS. Once homogenous, PMSF (1mM), 20 µg/ml of DNAse I and 25 µg/ml of lysozyme were added to the cell suspension. All subsequent steps were carried out at 4°C. *E. coli* cells were lysed by using sonicator [Heat systems-Ultrasonic Inc. USA] consisting of 6 times of 30 sec/burst. Cell suspension was kept cold and sonication was done with intervals. Frothing in the flask was also minimized to avoid rapid degradation of proteins. Lysate was then centrifuged at 15,000 rpm, 4°C for 15 min. Both supernatant and pellet were collected for analysis on SDS-PAGE and further purification steps.

Column preparation and protein adsorption on beads

Seventy mg of Glutathione Sepharose® 4B resin was hydrated in TBS for 2 hours to form approximately 200 µl of washed bead suspension. Beads were poured into a 15 ml chromatography column and allowed to settle. This volume of 200 µl will henceforth be referred to as 1 bed volume. Washing of beads was done with TBS until OD_{280} = 0.00 was reached. Purification started with washing the loaded column with 20 bed volumes of 1x PBS containing 10% glycerol and 1 mM PMSF. Supernatant was added into the column at 4°C. A 10-min incubation was allowed for adsorption before supernatant was collected and after repeating the procedure, another 10-min was

allowed for higher adsorption. The beads were washed in the column with TBS with the same amount as the supernatant. The flowthrough was drained away and washed continuously until OD_{280} = 0.00, showing that the GST-protein had effectively bound onto the beads and residual contents in the supernatant had been discarded.

Elution of the GST protein

Two ml of glutathione elution buffer per ml of matrix was added and the flowthrough from the column was collected in 1.5 ml Eppendorf tubes in consecutive 0.5 ml volumes. A method of stepwise collection rather than one whole flowthrough of 10 ml of elution buffer was found to give a better yield. The protein in these eluates was then quantified at OD_{280}. Elution was carried out three times with 1 x 300 µl and 2 x 200 µl glutathione elution buffer before being pooled. The success of elution was indicated by the differences indicated by the OD_{600} of 1 ml-elution fractions. The collected fractions were later analysed to ascertain successful expression. The whole method described has been summarised in a flowchart shown in **Fig. 2.6.**

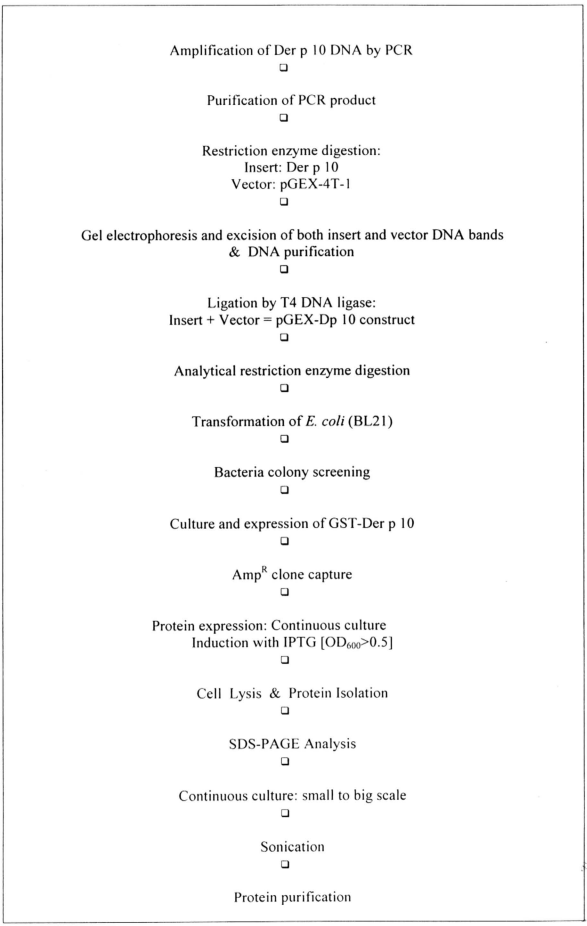

Fig. 2.6: Flowchart of cloning and expression procedures of GST-Der p 10 in *E. coli*

2.6 Expression of Der p 10 in *Pichia pastoris*

2.6.1 Construction of pPICZ Dp10

Amplification of Der p 10 cDNA

As in the bacterial system, PCR was carried out to amplify DNA fragments from DNA templates using a different set of primers. PCR reaction was carried out by first denaturing the template DNA at 94°C for 3 min, followed by 25 reaction cycles of denaturation at 94°C for 1 min; annealing at 55°C for 45 sec and extension at 72°C for 1 min; with finally an extended reaction at 72°C for 5 min. Similarly, upon completion, 5µl of the product was analysed by gel electrophoresis and UV illumination.

Propagation of the pPICZ B plasmid

The vector pPICZ B that was supplied in a lyophilised form once reconstituted, was maintained at -20°C. Propagation of the vector was then carried out by transformation into a *rec*A, *end*A1 *E. coli* strain, Top 10F'. The gene of interest (Der p 10) was ligated into this vector, in-frame with the C-terminal tag. Transformation was carried out appropriately in *E. coli* and transformants were selected on low LB plates containing 25 µl/ml Zeocin™. Analysis of 10 to 20 transformants by restriction mapping was carried out to confirm in-frame fusion of the gene with C-terminal tag and the His-tag. Purification and linearization of the recombinant plasmid for transformation were carried out before transformation into *P. pastoris*.

Restriction enzyme digestion

Restriction enzyme reactions were prepared in two separate tubes as in **Table 2.4.** Incubation of these reaction tubes at 37°C was carried out for 1 hour. Then, after adding 5 µl of gel loading buffer, the digests were resolved in a 1% agarose gel electrophoresis run at 110V. The DNA bands were then excised from the gel, with the correct sizes for pPICZ B [3.3 kb] and Dp 10 (~800bp) and kept separately in new microcentrifuge tubes.

Table 2.4: Two tubes containing the insert and vector for restriction enzyme digestion

Tube 1: Insert Der p 10

Reagents	Volume (µl)
Sterile deionised water	7.0
10x Multi-core buffer	2.0
Der p 10 PCR product	10.0
*Eco*RI	0.5
*Xba*I	0.5
Total	20.0

Tube 2: Vector pPICZ B

Reagents	Volume (µl)
Sterile deionised water	7.0
10x Multi-core buffer	2.0
Plasmid pPICZ B (0.2 µg/µl)	10.0
*Eco*RI	0.5
*Xba*I	0.5
Total	20.0

Ligation of Der p 10 fragment into pPICZ B vector

The ligation mixture (**Table 2.5**) was prepared as follows and ligation process was carried out at 4°C overnight.

Table 2.5: Ligation mixture preparation

Reagents	Volume (µl)
Ligation DNA	7
5x ligation buffer	2
T4 ligase (1U/µl)	1
Total	10

Transformation in E. coli (Top 10F')

The heat shock method was used for the transformation. All 10 µl of ligation mixture was transferred into a 1.5 ml microcentrifuge tube containing 100 µl of *E. coli* (Top10F') competent cells and incubated on ice for 30 minutes. The tube was given a heat shock treatment at 42°C for 1 min and was returned to the ice for another 2 min. Then 1 ml of SOC medium was added and further incubated at 37°C for 1 hour. The cells were then spun at medium speed (6500 rpm) for 1 min and the supernatant was removed. The pellet was re-washed with another 1 ml of SOC medium, spun, and again re-pelleted. With 100 µl of SOC medium, cells were resuspended and plated on LLB agar containing 25 µg/ml Zeocin™ and incubated at 37°C overnight.

After incubation, LLB+Zeocin™ agar plates were infected with 50 µl from tube i, 100 µl from tubes i and ii and also another for the 'self-ligation' or 'vector only' control. A competent cell control was also prepared as a systematic control. Transformants were later isolated and analysed for the presence and orientation of insert.

PCR screening of bacteria transformant colonies

This step was carried out to confirm the presence of required DNA plasmid in the bacterial colonies. Ten bacterial transformant colonies of on the overnight agar plate after the transformation with the recombinant DNA, were picked with sterile yellow pipette tips and transferred individually into 0.5ml thin-walled PCR tubes and the same tip were tandemly inoculated onto a new LSLB-Zeocin agar master plate and incubated at 37°C for future recovery. For the PCR, 150 µl of reaction mixture was prepared as shown in **Table 2.6**. Into every tube containing the transferred colonies, 14 µl reaction mixture was dispensed followed by a 1-min centrifugation before PCR.

Table 2.6: PCR reaction mixture prepared for PCR screening of bacteria transformant colonies

Reagents	Volume (µl)
Sterile deionised water	121.5
10x PCR buffer containing $MgCl_2$ (20 mM) and dNTP mix (5 mM)	15
Primer 1: 5' AOX, 25 pmol/µl	6
Primer 2: 3' Dp 10, 25 pmol/µl	6
Taq Polymerase, 5 U/µl	1.5
Total	150

PCR schedule consisted of initial denaturation at 94°C for 3 min, and 25 cycles of further denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec and finally extension at 72°C for 1 min. Upon completion, 5 µl of the PCR products were then analysed by 1% agarose gel electrophoresis to identify positive bacteria.

2.6.2 Transformation of *P. pastoris*

Preparation of yeast competent cells

A single colony of *P. pastoris* strain (X33 wild type) was selected and seeded into 5 ml of YPD medium in a 50 ml conical tube. Incubation was carried out in a shaking incubator at 30°C overnight. Fifty mililitres of fresh YPD medium in a 250 ml flask was inoculated with 0.25 ml of overnight yeast culture and growth was continued in the shaking incubator at 30°C for another 5 hours. The culture was then transferred to a 50 ml centrifuge tube and centrifuged at medium speed of 6500 rpm for 5 min. The pellet was washed with 50 ml of ice-cold water, spun and re-pelleted. This step was repeated, followed by washing the pellet twice again with ice-cold 1M sorbitol and spun. Finally the pellet containing yeast competent cells was resuspended with 200 µl of 1M sorbitol and kept on ice, ready for transformation.

Plasmid linearisation

The 5-hour incubation for growth of yeast culture was used for the linearisation of the plasmid containing the construct pPICZDp10. The reaction mixture as shown in **Table 2.7** was prepared:

Table 2.7: Reaction mixture prepared for plasmid linearisation

Reagents	Volume (µl)
Sterile deionised water	60.0
10x Multi-core buffer	9.0
Plasmid pPICZDp10 (0.2 µg/µl)	20.0
*Sac*I (Invitrogen, USA)	1.0
Total	90.0

The tube containing this mixture was incubated at 37°C for at least 1 hour. Then 10 µl 3M sodium acetate and 100 µl of isopropanol were added and centrifuged at high speed for 5 min. The pellet was resuspended in 0.5 ml ethanol and re-spun. The pellet was at last dried under vacuum for 5 min.

Yeast transformation by electroporation

The vacuum-dried linearised pPICZDp10 construct was first re-dissolved in 5 µl of sterile deionised water and mixed with 80 µl of yeast competent cells in a pre-chilled 0.2 cm electroporation cuvette (Bio-Rad, USA) and further incubated for 15 min on ice. Transformation was performed using the Genepulser (Bio-Rad) whereby the yeast cells were pulsed at voltage of 1500V, with a capacitance of 25 µF and the resistance of 200 Ω. This was followed by the immediate addition of 1 ml 1M sorbitol and a further 1-hr incubation at room temperature.

The transformed yeast cells were then transferred into a 1.5 ml microcentrifuge tube, spun and re-pelleted. The cell pellet was then resuspended in 1 ml of YPD medium

and then incubated at 30°C for another 1 hour. Tube was again spun at medium speed for 1 min and supernatant was carefully removed. The pellet was then resuspended in 100 µl YPD medium and plated out on a YPD agar plate containing 100 µg/ml Zeocin™. Plate was further incubated at 30°C for the next two days.

Screening of yeast transformant colonies

As was done in screening the bacterial colonies, this procedure involving PCR was also used to confirm a correct insertion of the pPICZ-Dp10 construct in the yeast genome. Yeast colonies on the overnight YPD agar plate were picked with sterile pipette tips and transferred individually into clean 0.5 ml thin-walled PCR tubes and tandemly patched onto a new gridded YPD+Zeocin™ agar plate for future recovery. The transformants in each of the 43 tubes were then mixed with 14 µl of standard PCR reaction mixture (**Table 2.8**).

Table 2.8: PCR reaction mixture prepared for the screening for transformed yeast colonies.

Reagents	Volume (µl)
Sterile deionised water	429
10x PCR buffer containing $MgCl_2$ (20 mM) and dNTP mix (5mM)	65
Primer 1: 5' AOX, 25 pmol/µl	25
Primer 2: 3' Dp 10, 25 pmol/µl	25
Taq polymerase, 5U/µl	6
Total	650

PCR amplification schedule consisted of the initial 1-cycle denaturation at 94°C for 4 min, and 25 cycles of amplification at 94° C for 45 sec; annealing at 55°C for 45 sec; extension at 72° C for 1 min; ending with a final cycle of extension at 72° C for 5 min.

Upon completion, 5 µl of gel loading buffer was added into each PCR product and analysed by electrophoresis to identify positive yeast transformant colonies.

2.6.3 Expression of recombinant Der p 10 in yeast

Expression was initiated by inoculating 25 ml of BMGY medium with single colony of pPICZ-Dp10/X33 recombinant yeast in a 250 ml flask, carried out at 30°C in a 250 rpm shaking incubator to reach OD_{600} = 2-6. Cells were harvested by centrifugation at 6500 rpm for 5 min and half of the cell pellet was resuspended with 50 ml of BMMY medium to an OD_{600}=1.00 in a 500 ml flask. Growth of yeast was resumed in the shaking incubator with the addition of 100% methanol to a final concentration of 0.5% (v/v) every 24 hr. A sequence of 1.0 ml culture was collected in 1.5 ml centrifuge tubes at different time points i.e. at 0 hour, 8 hr, 24 hr, 48 hr, 72 hr and 96 hr. When collected, each tube was spun at medium speed for 1 min. Supernatant was discarded and the pellet was kept and for a further collective expression profile study.

2.6.4 Purification of yeast-expressed rDer p 10

Yeast cells were pelleted by using high-speed centrifugation, 15 000 rpm at 4°C. Then 2 ml of PBS was added and cells were resuspended and poured into a mortar; ground with 1 mg of quartz sand (Merck). Mortar, pestle and sand had been sterilised by autoclaving. This physical grinding of the whole cell extract, taking care not to generate extreme heat, was carried out in lieu of sonication. The whole content was rinsed into a new centrifuge tube followed by addition of sufficient 10% (w/v) SDS, adjusted to 1% (w/v) in the whole mixture. The cell extract was then boiled for 15 min. This mixture was brought down to 4°C before being spun at 10 000 x g for 30 min; producing supernatant and pellet.

I) Dialysis of supernatant containing recombinant protein.

This supernatant was carefully loaded into a dialysis tube, securely tied at both ends with double knots and left to suspend in a 1-liter beaker containing distilled water, kept at 4°C overnight. Although suspended, the tube was made fully submerged vertically in the water to ensure effective dialysis. This was followed by another round of dialysis, this time submerging the tube in a Pro-Bond buffer at pH 7.8. This dialysis procedure served to remove the SDS from the supernatant; before being subjected to undergo further purification steps.

II) Column preparation

The pre-equilibration, loading and washing were all performed in "batch mode" – where processes were completed in the columns provided with the snap-off cap at the bottom of the column still intact. Elution steps were performed by gravity flow column chromatography. Four ml of resin was loaded into the column and left to settle, making sure that no air-bubbles were trapped in the column. Batch method purification was carried out. Volume between 2 to 3 ml Pro-Bond buffer (pH 6.0) containing 3 ml of 500 mM imidazole was added into column and allowed to flow through the resin followed by flushing out impurities with Pro-Bond buffer (pH 7.8).

III) Purification procedure

The dialysis tube was carefully opened and its content (which will be referred as dialysate) was spun down for 5 min before being poured into the column. After washing, 500mM imidazole (pH 6.0) was added; 0.5 ml at a time, and the elution was collected consecutively in Eppendorf tubes. The flow-through was collected, labelled and kept at 4°C for analysis.

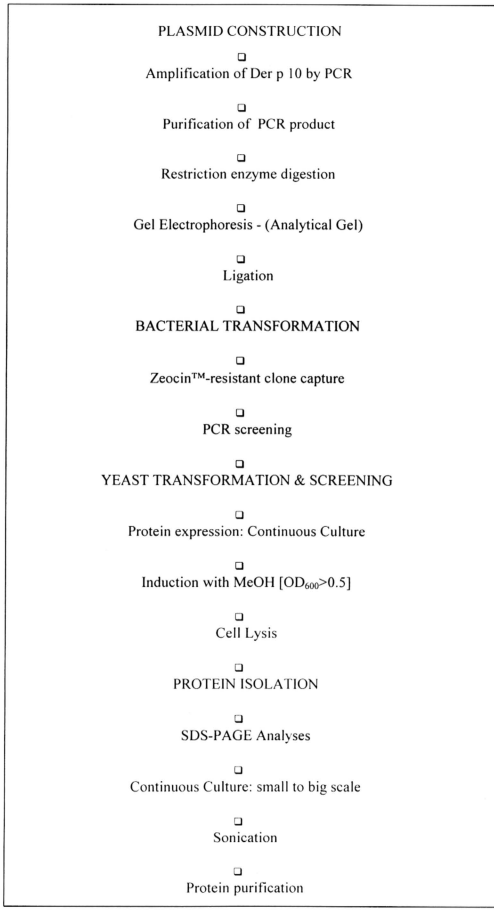

Fig. 2.7: Flowchart of cloning and expression procedures of Der p 10 in *P. pastoris*

2.7 Protein Analysis and Immunochemistry

2.7.1 Quantification of proteins

Proteins were quantified by using Bradford reagents (Amersham, USA) as described by Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.7.2 Verification of proteins

Polyacrylamide gel electrophoresis of proteins

The discontinuous, denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was routinely used for the separation of denatured proteins, as described by Laemmli (1970). The acrylamide/bis-acrylamide gel was assembled using a Hoefer protein mini-gel kit. Protein samples were first mixed with 1.5 volume of 5X sample dye containing 500mM Tris (pH 6.8), 10% SDS, 15% glycerol, 5% and 0.05% bromophenol blue. The samples were then immersed in boiling water bath for 5 min, after which a short burst spin was carried out. Only the supernatants were loaded onto gel. Electrophoresis was carried out at 55V through the stacking gel and doubled to 110V upon reaching the resolving gel, until the bromophenol blue dye reached the bottom of the gel.

Coomassie staining

To visualise the separated proteins after electrophoresis, Coomassie staining of the SDS-PAGE gel was carried out by immersing the gel in a staining solution containing 0.1% Coomassie Blue R250 (Sigma), 50% methanol and 10% acetic acid for 30 min. The gel was then washed overnight with a destaining solution containing 10% methanol and 10% acetic acid on a shaker platform at room temperature until clear bands were obtained and observed against the pre-stained molecular weight markers.

Western Blot and Immunodetection

Proteins were transferred from SDS-PAGE gels to polyvinylidene diflouride (PVDF) [Millipore] membranes essentially as described by Towbin *et al.* (1979) and then probed with immunodetection systems. The Bio-Rad TransBlot electroblotting system was used to prepare the Western Blots and was manipulated according to protocols provided by the manufacturer.

Immunodetection through enzymic colorimetric system was utilised. The membrane was first placed in blocking buffer, consisting of 4% (w/v) non-fat milk powder (Graduate, Nestle) in TBST (TBS with 0.05% [v/v] Tween-20), and agitated at room temperature for 30 min. The membrane was then exposed to the primary antibody dilution of a specific antibody targeting the protein of interest in TBST with 0.5% (w/v) milk powder. Incubation of the membrane in the solution was carried out with agitation, at room temperature for two hours or overnight at 4°C. The membrane was then washed three times on the shaker platform for 10 min each, with TBST, followed by 1-hr incubation in secondary antibody solution containing an appropriate dilution of enzyme-conjugated antibody in TBST and 0.5% (w/v) milk powder. After four washes with TBST of 10 min each, the membrane was finally rinsed with distilled water. For protein detection, BCIP/NBT were used in the substrate solution since the secondary antibody was alkaline phosphatase-conjugated, producing a purple product. Reaction was terminated by rinsing the membrane with water for about 10 min.

2.7.3 Enzyme Linked Immunosorbent Assay (ELISA)

Indirect ELISA was used mostly for detection of specific antibodies in the patient's serum and saliva. Allergen extracts used for SPT, synthetic peptides and the recombinant proteins served as antigens in these assays. The immunoglobulins such as IgE, IgG and IgA found in the serum and saliva were traced as primary antibodies and the antigenicity was evaluated in terms of the Ig-binding performance.

The antigen was diluted in PBS in appropriate concentrations such as (1:50 dilutions etc.) and then filled 50 µl/well of the microtitre plates (Nunc, Denmark) and was incubated at 4°C overnight. The unbound antigens were then removed and washed with washing buffer containing PBS and Tw20 (0.1%). Uncoated surfaces in the wells were then blocked with 100 µl/well with 2% (w/v) bovine serum albumin (BSA) in PBS for 2 hours at room temperature, followed by yet another two washes. The test samples containing the primary antibodies in a solution of 2% BSA/ PBS added with 0.05% (v/v) Tw20, were then placed into the wells and incubated at either room temperature or at 37°C for 1-6 hours. This solution was discarded, followed by four washes. An appropriate amount of enzyme-labelled secondary antibody (commonly used: rabbit HRP-conjugated anti-human antibodies) diluted in the same solution was later added 50 µl/well and incubated for a further one hour at room temperature. This solution was then drained away and given crucial 5X-washes in preparation for the final colorimetric reaction. The enzymatic reaction was carried out by adding 50 µl/well substrate solution containing 0.5 mg/ml *O*-phenylenediamine (OPD), 0.01% (v/v) H_2O_2, 50mM sodium citrate and 150mM sodium phosphate (pH 5.6). This enzymatic reaction was carried out away from the light and was terminated after 15 min by adding 50 µl/well with 4M H_2SO_4. Absorbance at 490 nm was measured by using the ELISA reader (Dynatech, USA).

2.7.4 Immunoblotting assays

Immunoblotting and immunochemistry were used to check the specificity of antibodies towards the antigens or proteins of interest. The standard assay was conducted using recombinant Der p 10 (5 µg/well) coated on microtitre plate and 50µl/well of diluted antiserum was used as first antibody. Alkaline phosphatase-conjugated anti-mouse IgG was used as the secondary antibody. In an immunoblotting assay, the recombinant Der p 10 was separated by 12% SDS-PAGE, transferred onto PVDF membrane and then probed with

serum of HDM-allergic patient. Horseradish peroxidase-conjugated IgG was used as secondary antibody, and binding on membrane was best visualised by using chromogenic agent diaminobenzidine (DNB).

2.7.5 Mouse antiserum preparation

The immunisation was carried out by initially emulsifying purified rDer p 10 (150 μg) in Freund's complete adjuvant and then injected subcutaneously into 10-week old BALB/cJ mouse at multiple sites. Boosters of rDer p 10 emulsified in Freund's incomplete adjuvant were then given after every 2 weeks. Bleeds from the tail was done a week after the booster injection.

Partially purified mouse IgG was obtained by precipitating the mice antiserum in a final concentration of 50% of ammonium sulphate. The addition of highly charged ions such as ammonium and sulphate to the protein solution causes a disruption of the hydrogen bonds between protein and water, resulting in precipitation of proteins from the solution. Immunoglobulins precipitate at low salt concentrations.

2.8 Peptide synthesis

2.8.1 Generation of the synthesis schedule

The Der f 10 and Blo t 5 were synthesized in this study as 8-mer and 10-mer peptides. The amino acid sequence was first entered into the PepMaker application and selection was made for concentration of amino acids for this synthesis (60mM); well volume (150μl); peptide length (10-mer); number of overlapping amino acid and number of copies (duplicates). A schedule was produced, carrying information on number of blocks and pins needed and the amount of individual amino acid and the other chemicals i.e.; DMF, DIC and the HOBt required for the synthesis. Together with the amounts were the well positions to be added with appropriate activated amino acid mixtures for the coupling procedure.

2.8.2 Pre-synthesis preparation

The following preparatory steps were needed prior to the actual procedures of peptides synthesis;

i. Preparation of the block and pins for synthesis: The number of pins to be used was left on the block according to the synthesis schedule and the unneeded ones were removed using a clean pair of forceps, avoiding contact especially on the gears, which might lead to dislodging or contamination. The word 'block' will hereafter be referring to the prepared block with pins attached to it.

ii. Weighing amino acids and activating chemicals: Careful and precise weighing of Fmoc-protected amino acids, HOBt (Chiron); DIC (Millipore, USA) based on the generated schedule; was performed prior to the act of synthesis. Chemicals were placed in thoroughly cleaned and labelled universal glass bottles and tightly sealed with parafilm, stored at 4°C and was opened only on the day of synthesis. The caps and inserts of these bottles were inert to any of the reagents or the solvents used. Cross-contamination was avoided while weighing these different amino acids by rinsing spatula with ethanol and drying it thoroughly. DIC (in liquid form) was however weighed last or immediately before the coupling procedure to avoid evaporation.

iii. Purification of N,N-Dimethylformamide (DMF) : DMF is the preferred solvent needed in Fmoc chemistry, being compatible with various stages of synthesis. DMF should be pure and must not contain free amines as DMF, at room temperature in the presence of light, slowly decomposes and liberates dimethylamine, a secondary base that cleaves the highly labile Fmoc-protecting group. This premature cleavage of both α-amino protecting group and any side-chain protecting groups by bases must not happen at the wrong time during the synthesis cycle and it is thus imperative to use freshly purified DMF. A distinct 'fishy' smell would mean that the DMF contains amines. Therefore, a simple but effective purification method was used whereby the DMF was filtered through a freshly packed column (30 cm in height; 1.5 cm in diameter) containing a mixture of aluminium oxide

(alumina) acidic pH 4.5: basic pH 9.5 at the ratio of 2:1; with the basic alumina placed on top. This column was wrapped up with foil and propped up by a retort stand in the fume hood. DMF was poured through the packed column and collected in a dark bottle, to be sealed up tightly and used within two days.

2.8.3 Fmoc-deprotection and washing of synthesis pins

Although pins in the kit were supplied ready for use, they must be Fmoc-deprotected prior to the commencement of synthesis. Fmoc-deprotection is the act of removing the Fmoc-protecting group before the synthesis and the coupling of subsequent amino acids. Being base-labile, this protecting group is readily removed by treatment with piperidine in DMF.

The block was first placed in a bath (polyethylene sandwich box) containing about 200 ml of 20% (v/v) piperidine ($C_5H_{11}N$) in purified DMF for 20 min at room temperature, enough to cover the pins up to at least half their height. Then block was removed from the solution and excess liquid shaken off, before being placed in a DMF bath for 2 min. Then the block was again immersed completely in a methanol bath for 2 min and washed in a second methanol bath, this time with agitation. This washing step was repeated twice with fresh methanol up to at least half the pin height, before the block was finally removed and allowed to be air-dried for at least 30 min.

2.8.4 Coupling the N-α-Fmoc-protected amino acids

This important procedure in peptide synthesis describes the actual appending of the first amino acid and subsequent amino acids, to each Fmoc-deprotected pin to form the peptide.

i. Coupling the first amino acid to the pin: The weighted amino acids for coupling procedure for the day were taken out of storage and allowed to reach room temperature. Both HOBt and DIC were dissolved in correct volumes of freshly distilled DMF, as stated

in the schedule. Then specified volumes of HOBt/DMF solution were added to the first amino acid in the universal bottle as indicated by the daily synthesis schedule to be dissolved completely by vortexing, before adding the activator i.e. DIC/DMF solution. This activated amino acid solution was then dispensed into appropriate wells of the reaction tray as instructed in the daily synthesis schedule for the day's couplings stringently. Subsequently, the rest of the amino acids were activated and dispensed appropriately. Due to the instability of the activated amino acids leading to their deterioration over time, a specific order of amino acids dispensing was followed as recommended by the manufacturer; A-D-E-F-G-I-L-M-P-S-T-V-Y-W-Q-N-K-C-H-R. The air-dried block was placed in fresh DMF bath covering the gears, for 5 minute. Excess DMF was shaken off followed by careful positioning of pins in the wells containing the dispensed activated amino acid solutions. Finally the block, correctly oriented on the reaction tray, was placed in a sealable container and left overnight for incubation at room temperature. This marked the first coupling of the whole synthesis.

ii. Processing the block after first coupling: After the overnight incubation to couple the first amino acid, the block of pin was taken off the reaction tray to stop the coupling reaction and was further processed in the following steps. The block was again thoroughly washed as in the steps described in **section 2.3.3**, leaving out the step involving piperidine. Again, the block of pins was allowed to air-dry for at least 30 min.

iii. Second and subsequent couplings: Upon drying, the pin-bound amino acids were deprotected again and the next amino acid was coupled as indicated in the daily synthesis schedule using the method described in **section 2.9.4** *(i)*. The Fmoc-deprotection, washing, coupling and washing steps were repeated until all amino acids were coupled to the required length of peptides to be synthesized on all pins.

2.8.5 Acetylation of terminal amino groups

Once the synthesis was completed, the N-terminus of each peptide was routinely acetylated in order to remove the charge associated with its free terminal amino group. The block of pins was removed from the reaction tray in which the last amino acid was coupled and washed as mentioned in **section 2.9.4.** The pins were Fmoc-deprotected the final time, washed and then placed into reaction tray containing 150 µl/well of the following acetylation mixture:

DMF : acetic anhydride : N-ethyldiisopropylamine ;

193 : 6 : 1 (v/v/v)

The block and tray containing the above volatile mixture were placed in sealable container and reaction was allowed to proceed for 90 min at room temperature. Then the block of pins was removed and washed in a methanol bath for 15 min, followed by air-drying for at least 15 min. The pin-bound peptides were then ready for side-chain deprotection.

2.8.6 Side-chain deprotection

All protecting groups used to protect side-chain functionalities during the synthesis must be removed from the synthesized peptides prior to ELISA testing. This was accomplished by treating the pins with 200 ml of side-chain deprotection or cleavage mixture of trifluoroacetic acid : ethanedithiol : anisole prepared at 38:1:1 (v/v/v) in a polypropylene box for 2.5 hours in the fume hood.

Upon removal from the side-chain deprotection mixture, the block was immediately immersed in methanol for 10 min. Excess methanol was shaken off followed by soaking the block in 0.5% acetic acid (0.5 ml glacial acetic acid per 100 ml) in methanol/distilled water mixed at 1:1 (v/v) for 60 min. Then the pins were again immersed twice in fresh methanol for 2 min, and finally allowed to air-dry overnight. The block of

pins with completed synthesis was then stored in a sealable container with desiccant at 4°C until needed in the subsequent assay, the pinELISA. In summary, the general scheme for synthesizing overlapping peptides is as shown in **Fig. 2.8.**

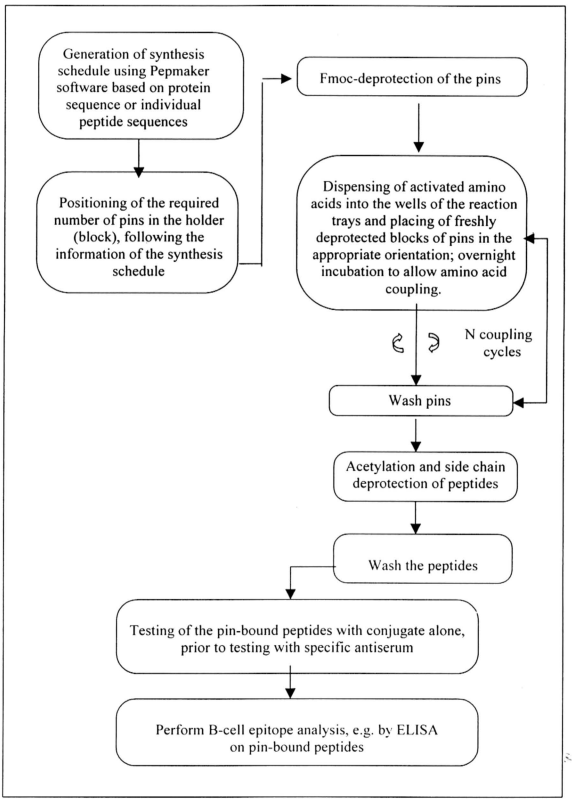

Fig. 2.8: General scheme for synthesizing overlapping Fmoc-peptides based on the amino acid sequence of Der f 10; using the Multipin Peptide Synthesis approach.

2.9 Synthesis of conformational peptides

2.9.1 Synthesis strategy

The strategy of synthesizing conformational peptides was acquired from Queensland Institute of Medical Research, with the kind guidance of Dr Cooper J. A., The basic technique of synthesizing conformational peptides does not differ much from the linear peptide synthesis except that it involves the sequence called 'flanking heptads', synthesized at both ends of the peptide of interest (serving as an 'insert'). These flanking heptads, each representing coils, would then render the insert, a propensity to form a coiled peptide. In this synthesis, peptides were designed based on the Der f 10 sequence too, by fragmenting 16 residues as the insert for the first peptide, and the next peptide was then made of another 16 residues from the main sequence, 'offset' by 7 amino acids from the main protein sequence. Therefore based on the example below, insert #2 differs from insert #1 by 7 amino acids, yet bearing similar 9 amino acids; giving the idea of an overlap of every 9 residues of each peptide (**Fig. 2.9**). The synthesis involved appending foreign fragments (flanking heptads) to the putative fragment (insert) which results in a conformational peptide. This explains why these peptides are sometimes called chimeric peptides (**Fig. 2.10**).

2.9.2 Method of conformational peptide synthesis

Similarly done in the production of linear peptides, the main steps involved in conformational peptides synthesis were;

I. Synthesis of the C-terminus flanking heptads, Q – K – V- K – D –E – L

Double portions of these amino acids were weighed to obtain 120 mM of 150 µl/well in the reaction tray. First coupling was carried out by allowing incubation for 4 hours in a preparation containing activated glutamine (**Q**+HOBt+DIC+DMF) for all the pins. The pins were removed from reaction tray, flicked gently to remove previous coupling mixture, rinsed in the methanol for 2 min, dried and placed again in a freshly prepared

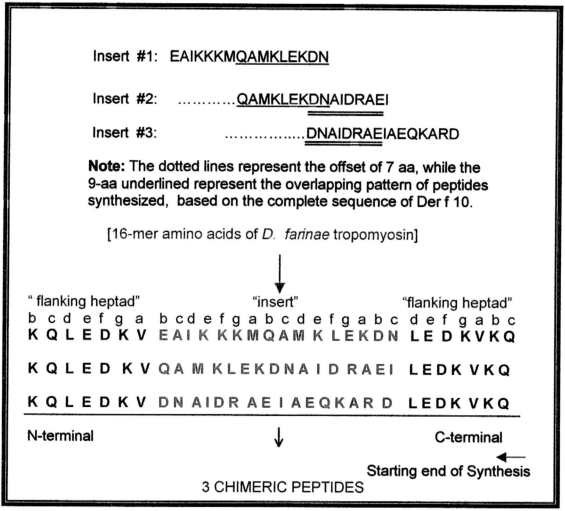

Fig. 2.9: The structure plan of conformational (chimeric) peptides. The seven amino acids segment in the helical coiled coil is symbolically represented by the [a-b-c-d-e-f-g] formulation.

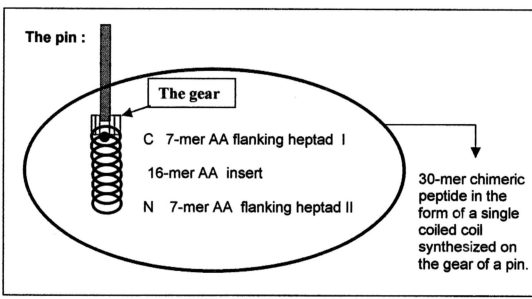

Fig. 2.10: The pictorial depiction of the 30-mer conformational peptide synthesis on the pin, or chimeric peptide, consisting of two flanking heptads and the 16-mer insert.

reaction tray of activated glutamine. This is known as "double coupling", carried out to increase the percentage of binding from [>95%] to be 9025% i.e. $(95\%)^2$. Washing was carried out followed by air-drying. Fmoc-deprotection of pins was repeated, followed by the double coupling of the next amino acid, K. These steps were repeated until the last residue of the flanking heptad, leucine (L).

II. Synthesis of the 'inserts'

The coupling of 16 residues of amino acids on each pin, was carried out similarly as synthesizing linear peptides. Since the synthesis was carried out at a higher concentration of amino acid (120mM), a 4-hour incubation for the coupling procedure was deemed sufficient. Other procedures; deprotection, washing and drying were similarly done till the 16^{th} residue for each pin. Double coupling was not done for this construction of inserts. Acetylation and side-chain deprotection too were not done, pending the synthesis of the N-terminus flanking heptad.

III. Synthesis of the N-terminus flanking heptads, V- K - D - E - L- Q - K

This third part of coupling was carried out in a similar manner with the synthesis of the first flanking heptad described earlier, this time starting with amino acid, valine (V) first and ending with the last amino acid, lysine (K). Washing and Fmoc-deprotection as described before were duly done before each coupling and double coupling was carried out too for each amino acid as for the first flanking heptad.

IV. Acetylation and side-chain deprotection

As with linear peptides, after the last coupling, washing and Fmoc-deprotection; acetylation and side-chain deprotection were finally carried out for these chimeric peptides as described in section **2.9.5** and **2.9.6.**

2.10 PinELISA

ELISA was also the recommended method in detecting the interaction between an antibody and its specific epitope(s) on the synthesised peptides, as part of the PEPSCAN method.

2.10.1 *Pre-coating of pins*

The first step in the assay was to pre-coat the pins with a pre-coating buffer (also called "blocking buffer") [0.01M PBS pH 7.2; 2% (w/v) bovine serum albumin (BSA); 0.1% (v/v) Tween 20; 0.1% (w/v) sodium azide]. The wells were filled up to 175 µL/well, slightly higher than the usual level of 150 µl/well to allow effective blocking. This step was needed to reduce non-specific binding and would therefore give a better signal-to-background ratio in the whole assay.

2.10.2 Conjugate Test

The pin-bound peptides were challenged against antibodies in the human serum i.e. the primary antibodies. The secondary antibody or the conjugate used in this pinELISA was anti-human antibodies that could react directly and specifically with pin-bound peptides. This reaction could mask or be mistaken as binding of primary antibodies to the peptide(s). Therefore, reactivity of the peptides against conjugate was checked before involving a primary antibody. Optimisation was done to find out the optimum concentration of the conjugate to be used in the assays. The conjugate was the rabbit peroxidase-labeled anti-human antibodies (DAKO, Denmark), (IgG, IgE or IgA) diluted at 1:1000 in conjugate diluent [1% (v/v) rabbit serum; 0.1% (v/v) Tween 20; 2.0% (w/v) sodium caseinate; 0.01M PBS pH 7.2] and 150 µl/well was dispensed into the microtitre plates. This conjugate incubation was then allowed to incubate at room temperature for 60 min with slight agitation on the shaker.

2.10.3 Primary antibody incubation

Serum from allergic patients served as the primary antibody and was used at different dilutions in the assay according to the type of immunoglobulin to be detected. For IgG antibody detection the serum used were at 1:500 while for IgE detection, the serum dilution was decreased to 1:100 due to the scarcity of the antibody in the human serum. A few samples of saliva were also tested with the pins to detect salivary IgA antibody using a dilution of 1:100. Patient's serum or saliva was diluted in pre-coat buffer and was dispensed (150 µl/well) into microtitre plates. Excess pre-coat buffer was flicked off the pins at the end of the blocking step and placed in the wells containing the diluted serum/saliva and were incubated overnight at 4°C.

2.10.4 Secondary antibody incubation

After the overnight incubation, the pins were removed from the diluted serum preparation and washed (4X) in a bath of freshly prepared 0.01M PBS (pH 7.2), with agitation on the shaker at room temperature. Then the block was placed in a reaction tray containing (150 µl/well) conjugate preparation (secondary antibody). As was used in the conjugate test, the procedure for this second antibody incubation was carried out as described in section **2.1.2** after the serum incubation.

2.10.5 Substrate reaction

This is the final step where the Ag-Ab binding is visualised with the aid of a colorimetric reagent. Two solutions were prepared for this reaction.

(i) Substrate buffer solution

The substrate buffer solution was prepared using 17.8 g $Na_2HPO_4\,2H_2O$ and 16.8 g citric acid monohydrate dissolved in 1 l of distilled water at room temperature. The pH was adjusted to 4.0 with 1.0M Na_2HPO_4 solution or 0.8M citric acid when necessary.

Storage is at 4°C to avoid contamination and required aliquots were brought to room temperature before use.

(ii) Colorimetric substrate solution

Colorimetric reagent used was ABTS (2,2'-azino-di[3-ethyl-benzthiazoline sulfonate]) (Sigma, USA) completely dissolved 0.5 mg/ml in substrate buffer solution at room temperature. Another reagent, OPD (*o*-phenylenediamine) was sometimes used, with the pH of substrate buffer changed to 5.6 before use. Hydrogen peroxide (30% v/v) (BDH, UK) was added at 1 µl/ml into substrate buffer just immediately before the substrate reaction.

Procedure: The substrate solution consisting of the substrate buffer, colorimetric reagent (ABTS or OPD) and hydrogen peroxide; was prepared immediately before use and dispensed 150 µl/well into clear, flat-bottomed ELISA microtitre reading plates (NUNC Denmark). Excess PBS from the last wash was flicked off the pins and was carefully placed in the wells containing the substrate solution ensuring correct labelling and orientation. This reaction was allowed to carry out at room temperature for less than 45 min with agitation, watching over colour development, before stopping the reaction by removing the pins from the wells. Absorbance of the resulting substrate solution in the microtitre plate was read promptly at wavelengths of 405 nm using an ELISA plate reader (Dynatech, USA) when using ABTS in the substrate solution; and of 490 nm when using OPD in the substrate solution.

2.10.6 Removal of bound antibodies from pin-bound peptides

A major benefit of the Multipin system is that the covalently coupled peptides on the pins can be reused repeatedly for the ELISA testing after a harsh but careful treatment using a sonicator bath containing detergent or chaotropic agents such as sodium dodecyl sulphate (SDS), to remove pin-bound antibodies. The sonicator bath (Nylex, USA) was

filled with 2 liters of sonication solution [0.1M PBS; 1% (w/v) SDS] at pH 7.2 adjusted with 50% w/v NaOH. The solution was first heated up to reach within 55-60°C followed by the addition of 2-mercaptoethanol (Sigma) to a final concentration of 0.1% (v/v), referred to as "disruption buffer". The harmful mercaptoethanol vapour required that this disruption process be carried out in a fully functional fume-hood. The block was placed afloat in the bath, with the pins pointing downward, and sonication was carried out for 10 min; then rinsed twice in hot distilled water (60°C) for 30 sec before placing it in a belly-dancer water bath at 60°C for 30 min. The block was then washed with hot methanol (60°C) for at least 15 sec. Finally; the block was air-dried for at least 15 min before reuse. When not used immediately, the block was stored in a sealed container with some desiccant, at 4°C. In summary, the whole process of pinELISA can be described in a flowchart as shown in **Fig. 2.11**.

2.11 Analysis of Pepscan results

Absorbance as shown by Pepscan of both linear and conformational the peptides, was represented by graphs showing the antibody binding reactivities of the patients. The Microsoft Excel programme was used in the construction of graphs and also in carrying out some statistical calculations. Further analyses were also carried out by comparison of results through online programmes in the Internet.

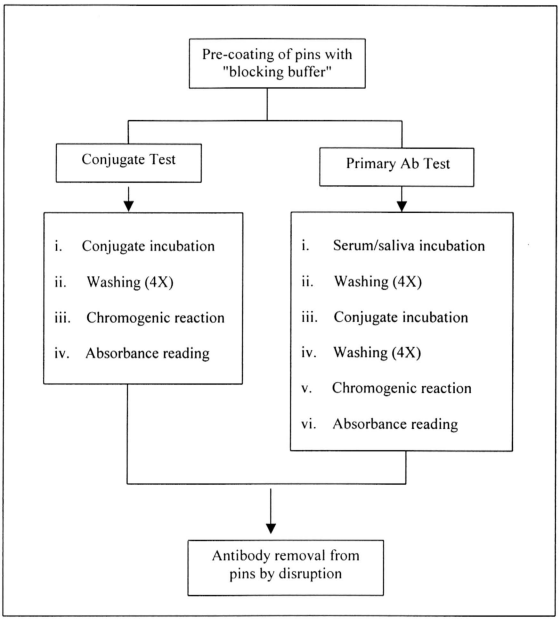

Fig. 2.11: Summary of the pinELISA.
N.B. The conjugate test is a test to check for non-specific binding prior to the primary antibody test. It was also carried out to check for complete removal of antibody after a primary antibody assay.