# Chapter 1

# LITERATURE REVIEW

## 1.1 Allergy

### 1.1.1 Definition of allergy

The term '**hypersensitivity**' is applied when an aberrant yet adaptive immune response occurs. Such reactions provoked by antigens called allergens, are the result of normally beneficial immune responses acting inappropriately, sometimes causing inflammatory reactions and tissue damage. Hypersensitivity is not manifested on first contact with the antigen, but on subsequent contact and the cause of hypersensitivity reaction varies from one individual to the next. Coombs and Gell described four types of hypersensitivity reaction (Type I, II, III and IV). The first three types are antibody-mediated while the Type IV is mediated mainly by T-cells and macrophages. Briefly, Type I (Immediate) hypersensitivity occurs when an immunoglobulin E (IgE) antibody response is directed against environmental allergens such as house dust mites (HDM), pollen or animal dander. In the case of food allergies, Type I reactions are most common among these four types (Kaminogawa, 1996). The resulting release of pharmacologic mediators by IgE-sensitised mast cells produces acute inflammatory reactions such as in asthma and rhinitis. Type II occurs when antibody, usually IgG binds to either self antigen of foreign antigen on cells, leading to phagocytosis, killer cell activity or complement-mediated lysis. Type III hypersensitivity develops when large quantities immune complexes are formed and cannot be cleared adequately by the reticuloendothelial system, leading to serum-sickness type of reactions. Type IV or delayed type hypersensitivity (DTH) is mostly manifested when antigens (for example, those on the tubercle bacilli) are trapped in a macrophage, stimulating T cells to produce cytokines to mediate a range of inflammatory responses. As this study looks into the effect of HDM allergy, emphasis will be given to the first type.

Therefore, the term '**allergy**', originally coined in 1906 by von Pirquet, means 'changed reactivity' of the host when meeting an 'agent' on a second or subsequent occasion. Another term '**atopy**', described by Coca and Cooke in 1923, refers to clinical manifestations of Type I hypersensitivity, which include asthma, eczema, hay fever, urticaria and food allergy. These usually occur in subjects with a family history of similar conditions, and who also show immediate wheal-and-flare skin reactions to common environmental allergens. Therefore, allergy or hypersensitivity denotes a disease or reaction caused by an immune response to one or more allergens, resulting in tissue inflammation and organ dysfunction. Although remote, allergy can be fatal when it manifests itself in a severe form involving anaphylaxis. General distress, discomfort and debility affecting the 'quality of life' of the allergic sufferers are the main reasons patients seek treatment. These chronic or acute allergic manifestations are work reducing in terms of job hours lost, work performance and productivity. Furthermore, allergy involves an economic predicament for its expensive treatment, including prescription of drugs and over-the-counter medicines, thus making allergy an 'expensive health handicap'.

### 1.1.2 Causes and manifestations of allergy

The cause of allergy is multifactorial, depending on the interaction, in genetically susceptible (atopic) persons, between the time and amount of allergen exposure and the presence of non-specific "adjuvant" factors, including air pollution (Bjorksten, 1994). Additional risk factors in allergy are family history and serum IgE levels - the higher the serum IgE concentration, the greater the chance of developing atopy (Roitt, 1997). The combination of genetic susceptibility and environmental factors induce allergic sensitization and subsequently local inflammation, resulting in atopic manifestations (Laan *et al.*, 2000). Atopic individuals often express disease in more than one organ due to the ability of IgE to sensitize mast cells anywhere in the body (Holgate, 2000). To develop an allergy, a person has to be exposed to the allergen. Four categories of allergens: inhalants (e.g. pollen), ingestants (e.g. food), contactants ( e.g. cosmetics) and injectants (e.g. bee

sting) cause adverse effects on specific target areas such as the upper and lower airways, the gastrointestinal tract and the skin. Of particular interest in this study are the inhalants that include common entities (aeroallergens) breathed in through the respiratory system such as dust and HDM, pollens, moulds, feathers and animal dander (small scales from animal skin). Inhalants start their invasion by eliciting immunologic effects as soon as they succeed in attaching themselves to the mucous membranes.

***Manifestations of allergy:***

i. Asthma

Asthma is often characterised by episodes of wheezy breathlessness due to airway narrowing, which is partially or totally irreversible, almost invariably accompanied by airway hyperresponsiveness. Causes of asthma depend on the interplay between genetic factors, the environment and several specific and non-specific triggers. Majority of asthma patients are atopic although a small number have non-atopic or intrinsic asthma that often has a later onset. However, Humbert *et al.* (1999) found more similarities than differences in the airway abnormalities of atopic and non-atopic patients. For instance, both variants are characterized by tissue infiltration by eosinophils and activated T cells and increased production of interleukin-4 (IL-4), IL-5, IL-13 and chemokines. Furthermore, both types are found to have similar numbers of bronchial mucosal cells that contain mRNA for the ε germ-line transcript (Iε) and ε heavy chain of IgE (Cε), suggesting that intrinsic asthma may be associated with local production of IgE against unknown antigens.

ii. Allergic rhinitis

Rhinitis affects about 25% of the population (Sibbald and Rink, 1991). Rhinitis has been less investigated than asthma, perhaps because it is rarely life threatening. Allergic rhinitis comprises episodes of sneezing, itching of eyes and nose, rhinorrhea and nasal obstruction. Seasonal rhinitis occurs when pollen and other plant allergens are released into the atmosphere especially in spring, whereas perennial allergens are present in a patient's

local environment virtually all year round, e.g. HDMs. Other forms of rhinitis are the non-allergic, idiopathic ("vasomotor") rhinitis, hormonal rhinitis, drug-induced rhinitis and food-induced rhinitis. Diagnosis is usually established by using clinical history, skin prick tests and specific IgE. When diagnosis is uncertain, challenges with allergens may be performed. Established methods such as the peak nasal inspiratory flow and rhinomanometry are used to evaluate these nasal provocation tests with allergens.

The treatments of allergic rhinitis, as with other allergic diseases consists of allergen avoidance (whenever possible and practical), anti-allergic medication, and immunotherapy for specific allergens (also known as desensitization or hypo-sensitization). Drugs currently used to treat allergic rhinitis include symptom-relieving antihistamines, anticholinergic agents and topical corticosteroids to suppress allergic inflammation (Holgate, 2000).

iii. Atopic dermatitis, urticaria, angioedema and anaphylaxis.

Atopic dermatitis caused by HDM is exacerbated presumably by both inhalation and skin contact (Larsen 1993, Adinoff *et al.*, 1998). Atopic dermatitis or atopic eczema is characterized by itchy red rash, consisting of tiny papules, sometimes with urticarial component, which may form confluent red sheets. Tupker *et al.* (1996) stressed that respiratory route may be relevant in the induction and exacerbation of dermatitis in patients. These patients had early bronchial reaction after HDM inhalation, a history of asthma, and an elevated total IgE level in the blood. IgE-mediated acute urticaria is characterised by widespread, itchy wheals or hives; often associated with sensitivity to foods, certain drugs and latex. Like atopic dermatitis, most effective step in evading urticaria is allergen avoidance. The systemic release of histamine and other pharmacologic mediators caused by IgE-mediated sensitivity to foods (e.g., peanuts, nuts, fish, shellfish and dairy products), drugs, bee and wasp stings and latex may result in anaphylaxis; a severe and sometimes fatal, systemic allergic reaction. Anaphylaxis comprises of symptoms that may be serious such as laryngeal oedema, lower-airway obstruction and

hypotension, which require prompt treatment of epinephrine administration for the reversal of histamine actions, followed by $H_1$-receptor-antagonist and corticosteroids.

### 1.1.3 Allergy and the immune system

Following the discovery of elevated IgE concentration in the sera of atopic persons by Ishizaka and Ishizaka (1978), atopy was re-defined as 'that form of immunological reactivity of the subject in which IgE antibody is readily produced in response to ordinary exposure to the common allergens of the subject's environment' (Pepys, 1994). Three components involved in an allergic reaction are the allergen, allergen-specific antibody and effector cells. Typically, allergens are multivalent antigens while the antibody component is the immunoglobulin E (IgE). The effector cells are mast cells or basophils, which constitutively express high-affinity IgE receptors (the FcεR I) on their surface. The first step in the pathogenesis of an allergic disease is the production of the IgE response to an allergen. The B cells of an allergic individual produce IgE directed against environmental allergens, whereas a non-allergic individual does not. The basis of the immunological mechanisms eliciting allergy is the Type I Immediate Hypersensitivity (**Fig. 1.1**). Copious amounts of antibodies, predominantly IgE, are produced after contact with low levels of environmental allergens recognised as antigens. IgE binds to mast cells via specific receptors (FcεRI) and this interaction of this bound IgE with allergen in turn leads to the release of mast cells mediators (e.g. autocoids, cytokines) that produce the clinical symptoms of allergy (Roitt, 1997). The early phase reaction appears within minutes after allergen challenge; characterised by itching, sneezing and/or nasal blockage. Coughing, wheezing and breathlessness are also symptoms from the lower airways associated with early response.

Each receptor and the corresponding antibody only binds to a small area of the whole antigenic molecule called epitope, which is about the size of a hexasaccharide or hexapeptide (Anon., 1994). An antigen may have many epitopes, and thus may induce

many B-cells to respond specifically. In some instances, although affinity may be different, two epitopes may be structurally related and antibodies specific to one epitope may also bind the other, resulting in 'cross-reactivity'.

Exposure to the allergen sources is an important event in the development of allergic disease. This means that antigen-presenting cells (APCs) are likely to be the initiators of the ensuing allergic response. The APCs are cells of the reticulo-endothelial lineage, e.g. Langerhans cells of the skin and follicular dendritic cells of lymph nodes. By endocytosis they take up incoming materials like allergenic protein molecules and break down these molecules enzymatically. The fragmented allergen will bind HLA class II molecules that line the luminal side of fused vacuoles inside the APC. These class II molecules carrying the short stretches of allergens, typicallly peptides of 12-17 amino acids will be exposed on the cell surface after the vesicle has fused with the cell membrane.

The functional outcome of the selective T-cell activation and subsequent proliferation is dependent on the phenotype of the individual T-cell. Th-1-like cells will primarily secrete IFN-γ and IL-2 whereas TH-2-like cell will produce large quantities of IL-4 and IL-5. Studies of allergen-specific T-cell clones indicate that atopic persons respond to allergen molecules with TH-2-like pattern. Non-atopic persons exhibit a TH-1-like response.

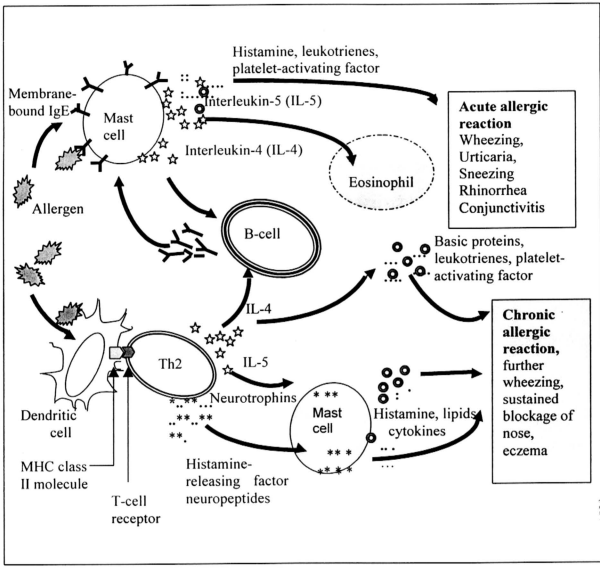


**Fig 1.1**: Pathways leading to acute and chronic allergic reactions. Acute allergic reactions are due to the antigen-induced release of histamine and lipid mediators from mast cells. In the skin and upper airways, basophils (not shown) may also participate in allergic tissue reactions. Chronic allergic reactions, including the late-phase reaction, may depend on a combination of pathways, including the recruitment of eosinophils, the liberation of mast-cell products by histamine-releasing factors and neurogenic inflammation involving neurotrophin and neuropeptides. MHC denotes major histocompatibility complex. (Adapted from Paul, 1991)

### 1.1.4 Serum immunoglobulins in allergy reactions

Atopic patients produce a higher proportion of antibodies to common aeroallergens following natural exposure and in particular, to allergen(s) they are sensitive to. Antibodies (or immunoglobulins) in the serum are pivotal in causing destruction of invading antigens, be it a protein or microbe. The N-terminal ends of the antibodies bind the antigen immediately upon gaining entry into the host. Therefore, the sequence variability within the variable domains from one antibody to another accounts for the antibody specificity for a particular epitope. Other molecules in the serum, such as IL-1, IL-6 and TNF play important roles in inflammatory diseases, while in the case of allergy, the IL-4 in rodents has been proven to be central to the production of antibodies that cause allergic responses (antibodies of the IgE class). Based on the finding that neutralising of IL-4 prevents IgE responses, there are suggestions that blocking the IL-4 action or production in humans might be useful to prevent or treat allergies (Paul, 1991).

Of all 5 major classes (isotypes) of antibodies in human, IgG, IgE and IgA are given emphasis in this study. The commonest in serum, IgG, represents about 75% of the total serum pool, has a molecular weight of 146 kDa, with 4 sub-classes ($IgG_{1-4}$), each bearing about 90% homology to each other. IgE, also known previously as 'reaginic antibody', has a molecular weight of 190 kDa, circulating in the blood as well as on the surfaces of tissue mast cells; at extremely low levels. Described as monomeric, IgE has no hinge region, making it a very rigid molecule without much flexibility. IgA is the second most common antibody in the serum and its main function is to protect the external mucosal surfaces. IgA found in the external body secretions is a dimer called sIgA, which has extra features in the form of a J-chain, and an additional 70kDa secretory component. This component transports B-cell-produced sIgA from the submucosa across the epithelial cells to the mucosal surface.

## 1.2 Epidemiology of allergy in Malaysia

The **frequency** of disease is expressed in terms of its **incidence** (the number of new cases per head of population occurring within a given period) and its **prevalence** (the number of cases of condition per unit of population at one point of time). In recent years, several epidemiological studies have indicated that prevalence of allergic diseases is increasing worldwide. This prevalence statement however may not be correct, as the increase may have been the result of better diagnosis as more determining assays have now been made available. Therefore, based on a century's report, prevalence of allergies in the population seems to have **increased** from 1-2% in the year 1900, to almost 30% today, with nearly 10% of them suffering allergies involving localised IgE-mediated anaphylactic reactions to extrinsic allergens such as grass pollens, animal dander, HDM faeces, etc. (Roitt, 1997).

Allergy research in Malaysia started in the late 70's. Thomas *et al.* (1978) first reported allergy related to *D. pteronyssinus* and house-dust, among Malaysian rhinitic patients. Among 774 allergic rhinitis patients of General Hospital, Kuala Lumpur; Imran and Hamimah (1983) detected skin sensitivity responses to *D. pteronyssinus* (52%), house dust (43%) and shrimp (24%). Then, by using the radio-allergosorbent technique (RAST), elevated IgE was detected in the sera of 70% of Malaysian atopic patients (Gan and Rajagopalan, 1987). In 1993, Jaafar and Pettit reported that 3.7% of 14 342 patients at the skin clinic of National University of Malaysia and a private dermatology practice; suffered from atopic dermatitis. This is probably the only publication investigating atopic dermatitis in Malaysia, reflecting the scarcity of studies on atopic dermatitis in this country. In another study involving 314 patients with clinically suspected allergic rhinitis, Ho *et al.* (1995a) found that allergy to multiple allergens is common among rhinitis patients in Malaysia. Similarly, Sam and Lee (1995) found that 92% of allergic rhinitis patients had elevated IgE for at least one out of 10 allergens challenged, and that 86% of the patients

had elevated IgE against either *D. pteronyssinus* or *D. farinae*. A screening of aeroallergens in ASEAN regions found that most Malaysians were indeed sensitised to HDMs (Lee, 1990). In the Kelantan state, Quah and co-workers (1997) studied the prevalence and severity of asthma, rhinitis and eczema in 7055 (5-14 year old) schoolchildren, as part of an international study of epidemiology of asthma and allergic diseases in childhood (ISAAC). Through questionnaires, overall prevalence of rhinitis and eczema symptoms was found to be 27% and 12% respectively. Asthma prevalence as characterised by the terms 'ever wheezed'; 'wheezing in last 12 months' and 'ever diagnosed with asthma' were 9.4%, 6.0% and 9.4% respectively. Rhinitis was more prevalent in the 12 to 14-yr age group (38.2%) than the younger 5 to 7-yr age group (18.2%). Contrary to that, eczema affected the younger children more (at 13.7%) than in the 12-14 age group (9.9%) (Quah *et al.*, 1997). A short study on food allergy in Malaysian children was also reported by Chan *et al.* (1999) who found no differences in the mean age, age of onset of asthma, sex and racial distribution, breastfeeding, and family history of asthma between Malaysian children (aged between 5-15 years) who have food-related symptoms and those without. The Malaysian Institute for Medical Research in Kuala Lumpur also conducted ecological studies since 1970's, to identify HDM in Malaysian homes. Besides HDM prevalence, Ho *et al.* (1995b) also reported the seasonal prevalence of air-borne pollen and spores in Kuala Lumpur.

### 1.3 Assessment of asthma and allergic rhinitis

Some allergy symptoms may not be IgE-mediated. Hyper-reactivity, for instance, is a state of general hyper-responsiveness to common stimuli and irritants such as dust, tobacco smoke, cold air, milk, eggs, perfume, preservatives and pets, and the abnormal reactions often characterised as "an allergy". These complaints need further tests to determine whether they are due to allergy (i.e. IgE-mediated inflammation/atopy) or are caused by other reasons such as intolerance or toxicity.

The diagnosis of an IgE-mediated allergy is based on three conditions; symptoms from target organs; positive provocation with the allergen at the target organ and, the occurrence/ aggravation of symptoms due to natural exposure to the allergen source in the patient's daily lifestyle. However, more practical and convenient procedures, for allergy diagnosis are needed due to the difficulty of an extended symptom registration over a long period and also the impracticality of performing bronchial provocation test or food challenge with large number of suspected allergens. Therefore a common but systematic method of allergy in evaluating the IgE sensitivity and allergen exposure is as shown in **Fig. 1.2.** Diagnosis of allergy is primarily dependent upon the positive clinical history of patients. For example, a patient with rhinitic symptoms (persistent runny nose, frequent sneezing, itchy nose and/or blocked nose) may be suffering from either vasomotor rhinitis or allergic rhinitis; or other nasal-related disorders. Therefore, confirmation of the presence of specific IgE antibodies directed towards the inhalant(s) or food(s) implicated in the history is necessary.

Diagnosis of allergy is carried out in various forms; through provocation tests such as epidermal (skin prick) (Turkeltaub *et al.*, 1982; Dreborg, 1989; Van Metre *et al.*, 1990; Hamburger *et al.* 1991) or unilateral nasal challenge (Wagenmann *et al.*, 1996); intradermal tests, double-blind placebo-controlled food challenge (DBPCFC) test for food allergy (Metcalfe and Sampson, 1990; Anderson, 1994). Skin prick test (SPT) is the commoner of two important tests which target the blood; the other being intracutaneous/intradermal test. SPT is often preferred for initial testing because it is cost-effective, simple and rapid, less painful (i.e. performed percutaneously), compatible with testing more allergens per session and most importantly, being less apt to give rise to systemic reactions (Nelson, 1983). A positive SPT giving immediate wheal-and-flare skin reaction against relevant allergen(s) is instrumental in supporting clinical diagnosis made by physicians. In a study performed with recombinant pollen allergen, Niederberger *et al.*,

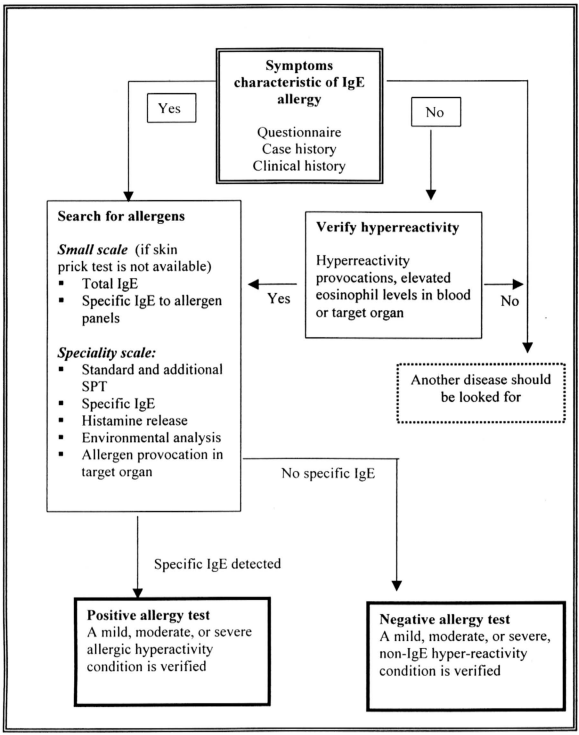


**Fig. 1.2**: The strategy for allergy testing

(2001) showed that skin prick tests correlated better with immediate type respiratory symptoms compared to in-vitro specific IgE tests. The other form of skin testing, the intradermal injection, is considered more uniform as antigens are delivered in the superficial layers of the skin to obtain quantitative results. However, reproducible accuracy still eludes this test, showing varying dimensions of wheals and flare upon a second intradermal test (King, 1990).

In addition, *in vitro* serological investigations such as radioimmunoassay or RAST (Gleich *et al.*, 1971, Gan and Rajagopalan, 1987;Daul *et al.*, 1994), enzymatic immunoassays (EIA) and immunoblotting techniques (Baldo *et al.*, 1989; Lavaud *et al.*, 1994) are useful tools to detect and quantitate elevated specific-immunoglobulins and other allergy-related entities in the serum. The enzyme-linked immunosorbent assay (ELISA) is a common immunoassay for the semiquantitative and qualitative determination of antibodies in human serum or plasma. Briefly, in an ELISA, the wells of a microtitre plate are first coated with antigens. Specific antibodies present in the patient's sample bind to the antigens upon recognition to form an Ag-Ab complex. Then, in a second step, the enzyme labelled secondary antibody (enzyme conjugate) binds to the Ag-Ab complex leading to the formation of an enzyme-labeled Ag-Ab sandwich complex. The enzyme-labeled Ag-Ab complex then converts the added substrate that catalyzes the formation of coloured substance. The rate of colour formation of the chromogen (e.g. ortho-phenylenediamine), is a function of the amount of enzyme conjugate complexed with the bound antibody and is thus proportional to the concentration of the respective antibodies in the patient's sample. Radioallergosorbent assay (RAST) detects circulating IgE to immobilised specific allergen determinants through the use of the secondary antibody, which is complexed to a radioisotope (usually $^{125}I$). Measurement of bound radiolabel by gamma counters thus indicates directly the amount of circulating IgE in the serum sample, and thus, the degree of allergenicity. However, problems in disposing the radioactive wastes from this assay can make its use very limited.

## 1.4 Aetiological agents of allergy

### 1.4.1 General aspects of allergens

"*What makes an allergen an allergen*?" is the most important question in allergy studies as there is no unifying theory as to why some proteins are allergenic and others are not. Allergens must be able to strategically reach target sites, and thereafter interact and stimulate the immune cells and mast cells in the immune system. Aalberse (2000) stated that any protein might be allergenic, particularly if it succeeds in evading activation of the Th2 suppressive mechanisms. The presence of adjuvant also influences the outcome of an immune response to a certain protein and may drive the response towards a Th1-like response (Jahn-Schmid *et al.*, 1997).

Allergens are generally found to be soluble proteins or glycoproteins, that function in their natural state as enzymes, for instance, by inducing proteolysis (Kay, 2001). In addition, most allergenic properties related to the enzymatic activity lead to increased mucosal permeability. The first evidence that the biological function of an allergen influences its ability to induce IgE responses was provided through the demonstration that enzymatically active Der p 1 cleaves the low-affinity receptor for IgE, CD23 (Hewitt *et al.*, 1995). This led on to the discovery that the proteolytic activities of Der p 1 and other mite proteinases can be linked to allergenicity; thereby highlighting 3 main lines of evidence, that, (1) Der p 1 directly promotes IgE synthesis; (2) allergens damage lung epithelium and increases bronchial permeability, and (3) Der p 1 causes release of pro-inflammatory cytokines from bronchial epithelial cells, mast cells and basophils (Gough *et al.*, 1999).

While emphasis has been given to mite allergens for having enzymatic activities, which may enhance their allergenic properties; many other allergens show no homologies with enzymes. For example, Can f 1, a major allergen from dog, is a protease inhibitor and many major allergens such as the cod allergen Gal d 1 and the olive allergen Ole e 1, show enzyme-inhibiting properties. Ironically too, many allergenic proteins show close homology with cytoskeleton proteins, regulatory proteins (Stewart and Thompson, 1996) or

motility-associated proteins (Aki *et al.*, 1995; Asturias *et al.*, 1998). It should also be noted that several major allergens (e.g. the lipocalins derived from rodents, horse and cockroach; or Der p 2 and Fel d 1) do not show any evidence that they are enzymes. Platts-Mills *et al.*, (1998) had the opinion that diverse foreign proteins can be allergens and that, not surprisingly, many of these proteins show homology with enzymes. However, enzymatic activities should not be considered as a common feature of allergens. Through a study of allergen structures of dust mites, cockroach and cat; Pomés *et al.* (2001), claimed that the ability of an allergen to induce an IgE response is largely independent of its biological functions. Rather, they suggested critical elements such as allergen dose, route of exposure and the genetic predisposition of the host and particularly the allergen- processing mechanism (resulting in Th2 response albeit still unknown); as important facts controlling allergenicity. It was also asserted in earlier studies of conformational stability of B cell epitopes of group 1 and group 2 of *Dermatophagoides* spp. allergens by Lombardero *et al.* (1990), that physical properties of allergens (low molecular weight and solubility), limiting low dose exposure (1 to 10 ng/day), host genetics and immunoregulatory processes; are more important than gross structural features of allergens, in the induction and maintenance of IgE antibody responses. Nearly all those studies stress that allergens have diverse biological functions for example, as enzymes, enzyme inhibitors, lipocalins or structural proteins) and yet as a rule, the allergen function is unrelated to its ability to cause IgE responses (Chapman *et al.*, 2000).

***Nomenclature system for allergens***

A nomenclature system has been established for allergens which cause IgE-mediated atopic allergies in humans by the WHO/IUIS Allergen Nomenclature Subcommittee; King T.P., Hoffmann D., Loewenstein H., Marsh D.G., Platts-Mills T.A.E. and Thomas W.R. was published in World Health Organisation Bulletin 72:797-806 (1994). It is defined by a designation that is composed of the first three letters of the genus; a space; the first letter of the species name; a space and an Arabic number. In the event of

two species names having identical designations, they are discriminated from one another by adding one or more letters (as necessary) to each species designation. For example, Der p 1 signifies one of the various allergens isolated from the HDM *Dermatophagoides pteronyssinus*. Fragments of allergens prepared by recombinant technology or by chemical synthesis are also designated a system of nomenclature closely related to the native allergens. To differentiate from the natural (n) allergen, the prefix of 'r' is added.

***Common aeroallergens***

Allergens (usually 10-100 kDa in size), which possess aerodynamic properties, are termed aeroallergens. Aeroallergens include pollens, molds, and animal (e.g. mammals, mites, birds, and insects) particles such as hair, saliva, dander, urine, feces, and body parts. An aeroallergen must be sensitizing and must be present in the ambient air in ample quantities to cause symptoms in an allergic individual. They are easily dissolved from the source particle (e.g. pollen grains or mite faecal pellets) and are highly stable. Most importantly, aeroallergens express allergenicity in low doses.

### 1.4.2 House dust mites

As early as 1921, Kern first suggested the occurrence of allergens in house dust as a causal factor for allergic rhinitis and asthmatic symptoms. However this 'allergenic factor' remained unsolved until Voorhorst *et al.* (1964) found important clues, which strongly suggested a mite to be responsible for the house-dust allergenicity; based on the similar skin reactions of atopic patients to this mite and those caused by house-dust. Fain (1966) identified this mite as *Dermatophagoides pteronyssinus* (Trouessart) (**Fig. 1.3**) and subsequent studies confirmed the predominance of this species in house dust in many parts of the world. These members of the Pyroglyphididae mites belong to the order Astigmata of the Acari, appropriately named based on the absence of stigmata on the idiosoma. Then in 1968, Miyamoto and his co-workers established *D. farinae* and house dust as causative antigens in bronchial asthma. Other mite species have since been implicated in house dust

allergies especially from the family Pyroglyphididae too, such as *Euroglyphus maynei* (Cooreman) and *E. longior* (Trouessart). Sensitisation to storage mites initially appeared to be restricted to occupational exposure, mostly observed in grain workers and farmers. However, in the late 70's storage mites began to draw attention as important contributors of allergens when traced in house dust too. In West Malaysia, like many regions, *D. pteronyssinus* has been found to be the predominant allergen, which causes allergic rhinitis (Thomas *et al.*, 1978) although a recent study by the Institute for Medical Research of Malaysia found that the amount of *D. farinae* in Malaysian homes exceeded the amount of *D. pteronyssinus* found (0.19 mg/g dust to 0.09 mg/g dust).

Another species of dust mite, *Blomia tropicalis* **(Fig. 1.4)** from the family of Glycyphagidae has been described as the most numerous non-pyroglyphid mite in the house dust in tropical and subtropical areas in the world, ranging at 100 to 3000 mites per gram (Hurtado and Parini, 1987) In regions including Central and South America, Singapore, Taiwan, Hong Kong, India and Egypt, both *B. tropicalis* and *D. pteronyssinus* were found with high frequencies and at high levels of infestation in houses (van Hage-Hamsten *et al.*, 1990; Arruda and Chapman, 1992; Arlian *et al.*, 2002) RAST inhibition assays demonstrated minimal to moderate cross-reactivity with *D. pteronyssinus* and *D. farinae*. Group 1 and 2 allergens from the *Dermatophagoides* spp. were not among the common allergenic component with *B. tropicalis*. It was therefore concluded that *B. tropicalis* is an important source of unique as well as cross-reacting allergens (Fernandez-Caldas *et al.*, 1993).

**Fig. 1.3**: *Dermatophagoides pteronyssinus* as seen under the electronmicroscope (400X)

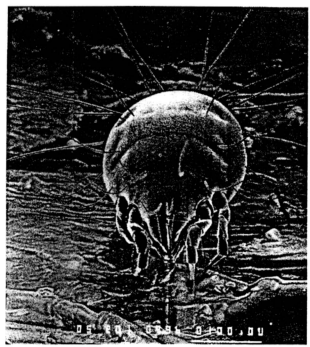

**Fig. 1.4**: *Blomia tropicalis* as seen under the electronmicroscope (200X)

Developing from egg through larval and nymphal stages to adulthood, all within a month, a mature mite may reach a size of about 200 to 300 μm, which is just beyond normal vision resolution (Wharton, 1976). These HDMs are free-living and non-parasitic, feeding on fungi, human and animal dander and organic debris; and absorb water hygroscopically from ambient humidity (Ledford, 1994). Unlike the pyroglyphids, some non-pyroglyphid species have a hypopial stage within the nymphal stage when the mites can survive for a long time without food. Depending on environmental conditions, an adult mite can live for a few weeks up to several months. Dust mites thrive best in favourable conditions such as in a temperature of 23-30°C and 80-90% relative humidity. As a mite can produce 200 to 300 eggs within two months after maturity, a small mite population may accumulate to several thousand mites per gram of household dust under normal conditions!

In Malaysia, 80% of houses surveyed were positive for the mite, with densities ranging from 2 to 50 mites per gram of dust (Mariana *et al.*, 2000). Colonies of the species have also been successfully established in the lab and 63 out of 85 (74%) suspected allergic rhinitis patients demonstrated positive reactions in ELISA using extracts from the spent materials. From the survey, the Division of Acarology of the Malaysian Institute for Medical Research thus reported an update on the HDM fauna in the most populated place in Malaysia, i.e. the Klang Valley, based on the dust samples collected from 20 houses from March 1994 to February 1995. Twenty-two species from 9 families of HDMs were identified, with all houses infested with at least 6 different HDM species. *B. tropicalis was* discovered to be most common; densely found at an average of 8934 mites/g of dust, followed by *D. pteronyssinus* and then *Malayoglyphus intermedius*. Another report of the HDM fauna in Singaporean homes also showed a great disparity of the predominance of *B. tropicalis* (62% of total mites) compared to *D. pteronyssinus* (16% of total mites) in the 50 houses evaluated (Chew *et al.*, 1999).

### 1.4.3 General aspects of mite allergens

By 1990, 7 groups of allergens from *D. pteronyssinus* had been identified. The first mite allergen purified and characterized was a faecal protein designated Der p I (Tovey *et al.*, 1987). This 25 kDa cysteine protease, was considered a major allergen as it provoked a reactivity in at least 80% of sera (Chua *et al.*, 1988). Other groups of HDM allergens include Der II, 14 kDa, (Chua *et al.*, 1990a & b, Trudinger *et al.*, 1991) which is more resistant to temperature and pH changes; Der III, a 30 kDa serine protease; Der IV, a chymotrypsin-like amylase; Der V (Tovey *et al.*, 1989) a 15 kDa protein which was found to react to 50% of allergic sera, particularly asthma patients; Der VI and Der VII, defined by cDNA clones, reacting with 40% of allergic sera. These allergens were later renamed according to the 1994 Allergen Nomenclature System (as in section **1.3.2**). Most of the major or important allergens in mite body and faeces extracts are found in a molecular weight range of 10 - 70 kDa. In some cases, larger allergens (>110 kDa) could be detected by immunoelectrophoresis and/or immunoblotting, but they have not been fully characterised (Colloff *et al.*, 1992). A large number of allergenic components have been identified in extracts of mite culture (**Table 1.1**), although the exact number of reported components varies from 4 to 26 and from 5 to 19 in mite bodies and faeces, respectively (Arlian *et al.*, 1987a and 1987b). Among them, antigens in groups of Der 1 (25 kDa), Der 2 (14 kDa), Der 3 (28-30 kDa), Der 4 (60 kDa), Der 6 (25 kDa) and Der 7 (22-28 kDa) have been characterised as major or important allergens (King *et al.*, 1994). Japanese researchers have identified other *D. farinae* antigens, such as Mag 1 (39 kDa), Mag 29 (68 kDa) and Mag 44 (37 kDa) as the corresponding allergens (Aki *et al.*, 1994a, 1994b, 1995) and additional low-molecular-weight antigens, which appear to be asthma-inducible (Yamaguchi *et al.*, 1994).

**Table 1.1:** Characteristics of **mite allergens**

| Allergen | E.g. | Molecular Mass (kDa) | Function | IgE-binding (% patients) | References |
|---|---|---|---|---|---|
| Group 1 | Der p 1<br>Der f 1 | 25 | Cysteine protease | 70-90 | Chua *et al.*, 1988; Dilworth *et al.*, 1991; Smith *et al.*, 1999. |
| Group 2 | Der p 2<br>Der f 2 | 14 | ? (Epididymal protein homologue) | 80 | Chua *et al.*, 1990; Trudinger *et al.*, 1991; Smith *et al.*, 1999; Gafvelin *et al.*, 2001. |
| Group 3 | Der p 3 | 25 | Trypsin | 80 | Smith *et al.*, 1994; Nishiyama *et al.*, 1995. |
| Group 4 | Der p 4 | 60 | α-Amylase | 45 | Lake *et al.*, 1991; Mills *et al.*, 1999. |
| Group 5 | Der p 5 | 15 | ? (Fatty acid binding protein) | 40-50 | Tovey *et al.*, 1989; Lin *et al.*, 1994; Caraballo *et al.*, 1996; Arruda *et al.*, 1997. |
| Group 6 | Der p 5 | 25 | Chymotrypsin | 40 | Yasueda *et al.*, 1993, Bennet and Thomas, 1996; Kawamoto *et al.*, 1999. |
| Group 7 | Der p 7 | 22-31 | ? | 50 | Shen *et al.*, 1993; 1995. |
| Group 8 | Der p 8 | 26 | Glutathione-*S*-transferase | 40 | O' Neill *et al.*, 1994. |
| Group 9 | Der p 9 | 25 | Collagenolytic serine protease | 80 | Stewart *et al.*, 1994; King *et al.*, 1996. |
| Group 10 | Der p 10<br>Der f 10 | 35 | Tropomyosin | 5-95 | Aki *et al.*, 1995; Asturias *et al.*, 1998 |
| Group 11 | Der p 11 | 39 | Paramyosin | 30 | Tsai *et al.*, 1998; 2000; Ramos *et al.*, 2001. |
| Group 12 | Der p 12 | 14 | ? | 50 | Puerta *et al.*, 1996 |
| Group 13 | Der p 13 | 15 | Fatty acid binding protein | 10 | Caraballo *et al.*, 1997 Puerta *et al.*, 1999, |
| Group 14 | Der p 14 | 190 | Apolipoprotein | | Epton *et al.*, 1999 |
| Mag 29 | | 70 | Heat shock protein (hsp70) | 17 | Aki *et al.*, 1994 |

[N.B. (?) Some functions of the allergens are yet to be ascertained or confirmed]

### 1.4.4 Mite tropomyosins

Generally, tropomyosins, which have been highly conserved during the process of evolution, present themselves as a ubiquitous protein in many organisms, with distinct isoforms found in muscle (skeletal, cardiac, and smooth), brain, and non-muscular tissues. Structurally, tropomyosin is the smallest and simplest of the fibrous proteins, consisting two α-helical chains of 33 kDa that are parallel and arranged in a coiled-coil structure. The coiled structure is based on a repeated pattern of seven amino acids with hydrophobic residues at its first and fourth positions. An amino acid sequence deduced from cDNA revealed significant homology with tropomyosins conserved in a wide range of animals. In association with the troponin complex, tropomyosin plays a central role in the calcium dependent regulation of muscle contraction. Appropriately, Witteman *et al.* (1994) found that mite body extract contains more tropomyosin than spent medium extract. This actin-binding protein is also a relevant allergen in various extracts from invertebrates such as crustaceans, mites, insects and mollusks. Shanti *et al.* (1993) first isolated the major heat-stable shrimp allergen (designated Sa-II) characterised as a 34 kDa heat stable protein, equivocally identified as tropomyosin. Then Aki *et al.*, (1994a) reported native mite tropomyosin as well as purified recombinant protein encoding mite tropomyosin (Mag44) both showed IgE binding responses which are significant in frequency and capacity, implying that both possess IgE-binding determinants, thus showing its allergenicity. This native tropomyosin reacted with specific IgE in the 31 sera tested at a high frequency (80.6%), comparable to that of Der f 1 (90.3%) and Der f 2 (74.2%). However, Asturias *et al.* (1998) reported that the IgE-binding reactivity to the recombinant he produced was not able to match the allergenicity described by Aki *et al.* (1995). He further suspected the discrepancy to have been caused by the nature of sera used; i.e. a monosensitised population in his assay against that of a polysensitised population in Aki's assay (Asturias J.A., personal communication).

Investigations into other organisms such as shrimps, or insects and even human tropomyosin for sequence homology identity could account for cross-reactivity or high anti-tropomyosin IgE titres in tropomyosin-allergic individuals (Witteman *et al.* 1994). Competitive IgE binding inhibition assays subsequently showed a high degree of cross-reactivity of *Paneus indicus* shrimp tropomyosin with tropomyosins from other crustaceans and even *Drosophila melanogaster*. However, despite what has been described for crossreactivity between shrimp and mites, van Ree *et al.* (1996) found out that tropomyosin played only a minor role as a cross-reactive allergen in 28 HDM allergic patients who developed asthma after consuming snails. Similarly, Ayuso *et al.*, (1999) claimed that the muscle protein tropomyosin is not an important vertebrate meat allergen.

***What does tropomyosin do as an allergen?*** This is an unresolved question (Aki T, personal communication). The fact that invertebrate tropomyosins are allergenic (Witteman *et al.*, 1994; Leung *et al.*, 1996; Asturias *et al.*, 1998; Santos *et al.*, 1999) while tropomyosins from vertebrates are non-allergenic; adds mystery to this question.

## 1.5 Recombinant Protein Expression

One of the challenges in allergy studies is in obtaining allergens that will induce immune responses. For example, the native mite tropomyosin was purified from an extract of purified mite body with a recovery of only about 0.03%(w/w). Therefore, about 3 kg of mite culture or 3 g of purified mite bodies is needed to obtain 1 mg of native tropomyosin (nDer p 10), depending on conditions of cultivation and biochemical purification techniques (Aki T., personal communication). Thus this problematic isolation of mite tropomyosin lends significance to the alternative method of mite tropomyosin production i.e. through a heterologous expression system as a recombinant form, rDer p 10.

### 1.5.1 Recombinant allergens

Advances of protein chemistry have produced natural allergenic products for diagnosis and treatment of allergic diseases. These natural allergens not only vary in contents and allergen composition, but are also at risk of 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 (Valenta *et al.*, 1995; 1999, Reese *et al.*, 1996).

Therefore the use of recombinant proteins is seen here as a way to improve the various problems when using natural allergens. Furthermore, sequence homology searches have vastly facilitated the identification and knowledge of putative biological functions of allergens and scaled-up production of these allergens is made possible with the use of various systems such as in bacteria, yeast and insect viruses.

### 1.5.2 Prokaryotic expression system

The expression of heterologous proteins in bacteria is by far the most widely used approach for the production of cloned gene products, both for fundamental studies or commercial purposes. Expression of any foreign gene in *E. coli* begins with the insertion of a cDNA copy of the gene of interest into an expression vector, often combined with a DNA sequence encoding the tag such as GST tag, C-myc tag (Glockshuber *et al.*, 1990) and *Strep* tag (Schmidt and Skerra, 1994). These tag sequences subsequently facilitate the recombinant protein detection or purification.

### 1.5.3 Yeast as a eukaryotic expression system

Yeast offers the ease of microbial growth and gene manipulation found in bacteria along with the eukaryotic environment and ability to perform many eukaryotic-specific post-translation modifications, such as proteolytic processing, folding, disulfide bridge formation and glycosylation (Eckart and Bussineau, 1996). Such post-translational modifications are not carried out in bacteria.

Methylotroph yeasts such as *Pichia pastoris* have been utilized to produce almost 300 foreign proteins (Higgins *et al.*, 1998) since 1984. Factors contributing to the popularity of this system include the use of alcohol oxidase I (*AOX1*) promoter; the ability to stably integrate expression plasmids at specific sites in the yeast genome in either single or multicopy; and the ability to culture strains in high density fermenters.

## 1.6 Epitopes and epitope mapping

### 1.6.1 Definition of an epitope

Epitopes or antigenic determinants of an antigenic macromolecule is defined as; any region of 6-12 amino acid or carbohydrate residues against which antibodies can bind and thus have the ability or potential to elicit, and combine with specific antibody. Determinants exposed on the surface of the macromolecule are likely to be immunodominant, i.e. more immunogenic than other (immunorecessive) determinants which are less exposed, while some (i.e. those within the molecule) are non-immunogenic (immunosilent) (Anon., 1994). There are two types of epitopes: continuous and discontinuous. A continuous epitope is composed of a contiguous stretch of residues in a protein while a discontinuous epitope consists of a group of residues that are not contiguous in the sequence, but are brought together by the folding of the polypeptide chain, or by the juxtaposition of two separate peptide chains.

Schou (1995) wrote a review on the B- and T-cell epitopes of allergen molecules underlining some important facts to differentiate them. B-cell epitopes are entities recognised by antibodies, which usually require three-dimensional 'intactness' to be recognised by antibodies. However, T-cell epitopes do not have the same conformational constraints as B-cells epitopes to be recognised by the T-cell receptor (TCR) in the context of Major Histocompatibility Complex (MHC) class II molecules. Although all allergens studied display multiple T-cell epitopes, major allergens are found in many isoforms that display differences in epitope structure. T-cell epitopes are scattered along the entire amino acid sequence of all allergens and the recognition pattern varies from one patient to another. Van Regenmortel (1989) first postulated that, in order to mimic a protein epitope with small synthetic peptides, it is important to choose a sequence that is hydrophilic, surface-oriented, and flexible. To this he added that most naturally occurring proteins found in physiological solutions have their hydrophilic residues on the surface and their hydrophobic residues buried, suggesting that antibodies bind to epitopes on the surface of naturally occurring proteins.

### 1.6.2 Epitope mapping

Epitope mapping is the identification of the regions of interaction between an antigen and an antibody. The information derived from epitope mapping, even if approximate, may help in the designing of experiments to precisely map the residues involved in the interface that can elicit an antibody response, with which eventually the researcher might claim to have produced an "epitope map". Epitope mapping can further contribute to the designing of peptides, which are able to mimic the interacting surface of the antigen, and in understanding where important regions of an antigen are located in its three-dimensional structure. Therefore, interest in epitope mapping is shared by biologists from a wide range of disciplines in which antibodies are used as molecular reagents.

Epitope mapping is seemingly confined to protein antigens although simple chemical molecules, nucleic acids and carbohydrates can also act as antigens.

Many methods of epitope mapping have been reported. Historically, empirical epitope mapping of protein antigens relied upon either enzymology i.e. through enzyme digestion; or cynogen bromide cleavage into successively small fragments retaining epitopic specificity. The advent of molecular biotechnology and advances in peptide synthesis technology has currently brought epitope mapping into focus. Recombinant DNA technology used to map dominant epitopes of the major mite allergens Der p I and Der p II showed that the recombinant polypeptides, which are known to possess conformation-dependent epitopes (Lombardero *et al.*, 1990); maintained most (Der p II) or part (Der p 1) of the IgE binding activity of the natural allergens (Greene and Thomas, 1992; Chua *et al.*, 1990b). Glutathione transferase fusion proteins, obtained by cloning Der p 1 cDNA fragments in the vector pGEX, had IgE binding activity only if the expressed fragment consisted of more than 30 amino acid residues (Greene *et al.*, 1991; Greene and Thomas, 1992): with evidence that the five IgE binding sites identified formed a larger discontinuous epitope. To scan for epitopes, there are problems in interpretation of the resulting data. Commonly reported when using human sera are the multiple weak reactivities, a result of the poor signal to background ratios, reflecting the lack of antibodies recognising linear epitope(s) tested. Very often, this problem cannot be improved by increasing the concentration of the test sera. Based on the conclusion drawn from the similarity of the signals to those obtained when testing a monoclonal antibody known to recognise a discontinuous epitope, many or all of the small peaks present are believed to be due to segments of discontinuous determinants. However, another approach to extracting a meaningful data from these scans on a set of sera is to analyse them from the strongest and the most common reactivities by making a "consensus plot" and further comparing them with epitope mapping predictions.

### 1.6.3 Epitope mapping of allergens

The common characteristics found in these allergenic molecules include having a general molecular size ranging from 5 to 40 kDa; all being proteins of which some may be glycosylated; and being able to dissolve readily under normal conditions of the human mucosal membranes (Schou, 1995).

Methods of epitope mapping also vary over the years, ranging from gene fragmentation to hydrogen exchange nuclear magnetic resonance spectroscopy mapping of antibody epitopes on the HDM allergen Der p 2 (Mueller *et al.*, 2001). The use of overlapping synthetic peptides is not the only mode for epitope mapping of allergens. Antigenic and allergenic epitopes of Lol p VB were localized using gene fragmentation (Ong *et al.*, 1995) and the epitope of major peanut allergen Ara h II, were defined by murine monoclonal antibodies specific to it (Burks *et al.*, 1995). However, overlapping synthetic peptides have played a significant role in IgE epitope mapping of allergens such as cat (*Felis domesticus*) major allergen Fel d I (van Milligen *et al.*, 1994) and the latex allergen, Hev b 1 (Chen *et al.*, 1996).

By means of monoclonal antibodies and human IgE an extensive range of common as well as species specific B-cell epitopes have been demonstrated on Der p 1, Der f 1 and Der m 1 (from *D. microceras*) (Lind *et al.*, 1987). This is in line with the clinical observations that the majority of HDM allergic patients react with more than one species. Der p 1 (having 222 amino acids encoded by a single gene) have been analysed for B-cell epitopes by means of short synthetic peptides as well as larger fragments expressed from rDNA constructs. Recombinant fragments p53-99 and p98-140 were shown to bind to IgE from HDM allergic patients (Greene *et al.*, 1991) so these stretches of polypeptide apparently fold up in partially correct configurations. A different approach demonstrated that rabbit antibodies to synthetic peptides p52-71 and p117-133 bound the intact Der p 1 and Der f 1 too (Jeannin *et al.*, 1993).

## 1.7 Epitope mapping by Pepscan

Epitope mapping by Pepscan enables peptides to be synthesized on a solid support and can be tested for binding to antibody receptors, while still permanently attached to the solid support. Peptide synthesis usually starts with the selection of regions for synthesis by using computer programmes such as MacDNASIS and other programmes. This system has been utilized in many areas of epitope mapping, such as of myohemerythrin (Geysen *et al.*, 1987a), HIV proteins (Meloen *et al.*, 1989) and the outer structural protein of Foot-and-Mouth Disease virus (Geysen *et al.*, 1984; Meloen and Barteling, 1986) and even in cases concerning allergens such as ovalbumin (Gal d II) by Elsayed and Stavseng (1994). Advantages of peptide synthesis employed for epitope mapping include the requirement of minimum basic skills in organic chemistry to produce more than 1000 peptides (octapeptides) per 10 working days. Minimal chemical laboratory space is needed; only requiring a fume hood as method does not involve the use of highly hazardous compounds. Most importantly, on solid phase support, peptides are produced conveniently and are re-testable.

### 1.7.1 Overview of the chemistry of peptide synthesis

Solid Phase Peptide Synthesis Chemistry (SPPS Chemistry) pioneered by Merrifield (1963), involves the sequential addition of amino acids to create a linear peptide chain. The three main steps are: ***Immobilization*** where the C-terminal amino acid of the growing peptide chain is anchored covalently to a solid support during synthesis; ***Chain assembly*** in which the C-terminus of the growing peptide is covalently bound to an insoluble or solid support such as a resin, during synthesis. Each amino acid is added to the chain by successive cycles of deprotection, activation and coupling; ***Cleavage*** involves the removal of peptides from the resin or the solid support upon completion of synthesis. Non-cleavable synthetic peptides however are simply left on the support, to be tested out in subsequent assay.

The raw materials in a peptide synthesis are protected-amino acids that prevent unwanted reactions at their α-amino and side-chain functionalities. The three chemical reactions involved in chain assembly are: **Deprotection** is the removal of the N-terminal Fmoc protecting group after coupling by piperidine (a secondary base) to make the α-amino group on the end of the peptide chain accessible for the addition of the next amino acid. The C-terminus of the incoming amino acid needs a chemical activation before it can be coupled to the growing peptide chain. **Activation** converts the next amino acid to be added to an active ester and during coupling; this active ester forms an amide bond with the deprotected α-amino group on the end of the peptide chain. **Coupling** is the formation of an amide bond between the carboxyl of an incoming, activated amino acid and the amino acid terminal of the growing, resin-bound peptide chain. After coupling, a new cycle of synthesis begins with the next deprotection. The objective in peptide synthesis is to obtain the highest coupling efficiency possible, preferably greater than 99.5%.

### 1.7.2 Rationale for design of chimeric coiled-coil peptide

In 1972, Hodges *et al.* found out that tropomyosin is a coiled coil of two α-helical with a 7-residue repeat that places two large hydrophobic residues at positions 1 and 4 where they provide a packing surface. To study such α-helical coiled coil, Hodges *et al.* (1981) later synthesized several models; such as $(LEALEGK)_5$. Subsequently, Lau *et al.* (1984) used the stepwise solid-phase synthesis with which they constructed a series of peptides, Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-$NH_2$ where n=1-5. The resulting 29- and 36- residue peptides were large enough to form the two-stranded α-helical coiled coil (which are parallel and in register) structures as found in tropomyosin and thus confirmed the secondary and tertiary structures. The first heptad contains the sequence: MKQLEDK that includes several of the features found in the stable coiled-coil heptad repeat motif: *[a-b-c-d-e-f-g]* (Cohen and Parry, 1990). A consensus valine was found in the 'a' position and when V and L occupy positions *'a'* and *'d'*, a coiled dimer is favoured (Harbury *et al.*,

1994). From these consensus features, the stable model heptad repeat VKQLEDK was derived, shown to have the potential to form a α-helical coil (Ellenberger *et al.*, 1992). Therefore this heptad, when used as flanks on both sides of a peptide of interest, forms the 'framework peptide', which has the propensity to aid or force the insert (peptide of interest) to form a coil.