

# Recombinant Allergens for Diagnosis and Immunotherapy of Allergic Disorders, with Emphasis on Cockroach Allergy

Kyoung Yong Jeong<sup>a,\*</sup>, Chein-Soo Hong<sup>b</sup> and Tai-Soon Yong<sup>a,\*</sup>

<sup>a</sup>Department of Parasitology and Institute of Tropical Medicine, <sup>b</sup>Department of Internal Medicine and Institute of Allergy, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea

**Abstract:** The prevalence of allergic disorders has increased over the past few decades and the quality of life has been significantly influenced at least for the allergic subjects. Allergen avoidance is thought to be the best way of preventing clinical manifestation of the disease, however, it is not possible for some allergens, and other pharmacological and/or immunological treatment has to be made. Repetitive injection of sensitized allergens to the patients (immunotherapy) is the only known curative approach to the disease even though the exact mechanism is not clear to date. Crude extract of allergens has lots of shortcomings which might arouse unexpected results. Genetic engineering and recombinant allergens are thought to be one of the alternative ways to overcome these limitations. Genetic engineering could facilitate the investigation of immune responses of the subjects especially on B cell and T cell epitopes, and produce the therapeutic allergens which might minimize the possible side effects. Furthermore, conjugation of immuno-modulatory molecules such as CpG-ODN, cytokines, or toxins which could act specifically to the given allergens, and maleylation of the allergens could maximize the prophylactic or therapeutic effect.

Immunotherapies for the pollen allergy and insect sting allergy have been thought to be successful. House dust mite allergy and cockroach allergy have been reported less beneficial by immunotherapeutic approaches. Cockroaches are one of the most important causes of asthma, and severe complications are often reported in the children in city dwellers with low-incomes. The studies of the biological functions of cockroach allergens and the use of recombinant allergens should allow understanding of mechanisms of cockroach-elicited allergic disorders and development of allergen-specific and sensitive diagnostics and tailored therapeutic approaches in the future.

**Keywords:** Allergen, asthma, cockroach, immunotherapy, recombinant allergen.

## INTRODUCTION

Allergic disorders are one of the most common diseases, affecting approximately 20-40% of the population in developed countries. More than 8 billion US dollars in the USA has been estimated to be spent for the health care of allergic disorders [1, 2, 3]. In the last few decades, the great progress has been achieved in the knowledge of allergy. Molecular and clinical data on allergens are growing exponentially these days [4, 5].

The onset of allergic disorders are thought to be affected both by genetic and environmental factors [6, 7, 8]. In a study using sera from 191 pairs of Australian twins, genetic control of IgE responses to rye grass pollen allergens was found to be limited and influenced largely by environmental factors [9]. Allergen-reactive Th2 cells are thought to play pathogenic roles in allergic disorders ('Th2 hypothesis') [10]. The increase in the prevalence of allergic disorders that has occurred over the past few decades is attributable to a reduced microbial burden during childhood, as a consequence of westernized lifestyle ('hygiene hypothesis') [11, 12]. The key elements affecting the production of IgE antibodies and

development of allergic diseases are thought to be the route of exposure, the allergen dose, age at sensitization, and the genes regulating host immune responses, rather than the intrinsic properties of the allergens [13].

Genetic factors contributing the onset of allergic diseases do not seem to be modified and/or modulated easily, however environmental factors more likely to be modulated. Allergens, antigens which induce IgE antibody responses in atopic individuals and elicit allergic reactions, are one of the reasonable targets to understand and get the allergic diseases under control.

## COCKROACH ALLERGY

Many people find cockroaches the most repulsive of all insects. This feeling arises because some species live in close association with people, inhabiting cupboard, sink cabinets, and other places where food is prepared or stored. Cockroaches may act as intermediate host of some parasites [14] and mechanically transmit many causative pathogens. Cockroaches found in the fossil record from 340 million years ago and over 3,500 cockroach species exist worldwide at the present time. Most of species live out of doors, but about 50 species live in or around human dwellings. Cockroaches can usually recognized by an oval shaped, a somewhat flattened, and usually brown or brownish-black body. The head is oriented downward and is barely visible from above because an

\*Address correspondence to these authors at the Department of Parasitology and Institute of Tropical Medicine, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Korea; Tel: 82-2-361-5290; Fax: 82-2-363-8676; E-mail: jeongky@paran.com; tsyong212@yumc.yonsei.ac.kr

extended pronotum covers most of it. Cockroaches feed on many kinds of plants and animal products, including human food, sewage, garbage, binding and sizing of books, and wallpaper paste. Reproduction occurs year-round. Most pest species are members of the families Blattidae and Blattellidae. The cockroaches of Blattidae are relatively large insects (most are 2.5 cm or more in length) with numerous spines on the ventroposterior margin of the femora. Oriental cockroach, *Blatta orientalis*, is about an inch long, dark brown, and broadly oval with short wings. This species is not widely distributed in Oriental regions, but in European regions. American cockroach, *Periplaneta americana*, is about 35-40 mm in length and reddish brown with well developed wings (Fig. 1A). Smokybrown cockroach (also called dusky brown cockroach), *P. fuliginosa*, are a uniform shiny dark tan to ebony with an unpatterned pronotum. Adults are 30-38 mm long and have a characteristic odor (Fig. 1B) [15]. These two species have a worldwide distribution. Japanese cockroach (also called Yamato cockroach), *P. japonica*, which is distributed in cool-temperature regions [16], is also found in Korean homes. Adults are dark to tan to ebony but not shiny and 20-25 mm long. Pronotum is uneven, and adult males have fully developed wings but females have short wings (Fig. 1C).

Family Blattellidae is a large group of small cockroaches, most of them 12 mm in length or less. German cockroach, *Blattella germanica*, is light brown with two longitudinal stripes on the pronotum (Fig. 1D), and shows most widely distribution in the world.

In Korean homes, four cockroach species have been found. The German, American, dusky brown, and Japanese cockroaches were found to infest 36.2% (63/174), 33.3% (58/174), 1.7% (3/174), and 1.1% (2/174) of homes in Seoul, respectively [17].

Sensitization to cockroach allergens has been recognized as an important cause of asthma since the first study of cockroach allergy by skin test (44% positive responses among 755 allergy clinic patients in New York) in 1964 [18]. A close association between cockroach allergy and low socioeconomic status has been reported [19, 20], and cockroach allergy is an important risk factor for asthma-related emergency room visits and hospital admission [21, 22, 23, 24, 25]. A history of perennial asthma, possibly worse in the winter is the most common clinical presentation of asthmatic patients with cockroach allergy [26]. Distinctive characteristics were identified from cockroach-allergic patients with asthma, when compared with those with ragweed asthmatics and asthmatics in general [25]. Patients with cockroach allergy had particularly high levels of serum IgE antibodies as compared to general asthmatics and ragweed allergic patients. Steroid dependency of the cockroach asthma was higher (32%) than those of general asthmatics ( $P < 0.05$ ) and ragweed asthma ( $P < 0.05$ ). Thus, cockroach asthma is a severe allergic asthma which needs more attention and intensive investigation.

Cockroach is also considered an important pest in day-care centers and elementary schools [27]. Appropriate care and measures should be undertaken, because early exposure to cockroach allergens is known found to be strongly associ-

ated with repeated wheeze during the first year of life [28, 29]. In particular, allergen avoidance in childhood is known to effectively reduce the prevalence of sensitization [30, 31]. Moreover, low-level cockroach exposure has been described as a risk factor of cockroach sensitization [32].

## COCKROACH INTERVENTION STUDIES

The avoidance of allergen exposure is thought to be a critical element for the prevention and management of allergic diseases [33]. Avoidance of dust mite allergen at high altitude was reported to lead to a decrease in airway inflammation and a consequent improvement in non-specific bronchial hyper-reactivity and symptoms. In addition, re-exposure resulted in a rapid relapse [34]. In particular, allergen avoidance in childhood is known to be effective to reduce the prevalence of sensitization [30, 31]. However, cockroach extermination alone was not reported to reduce the allergen levels in house dust [35, 36]. The effective approach combines the pesticide application, family education, and physical elimination of hiding places [37, 38, 39]. Recently, it was reported that it was possible to significantly reduce the cockroach infestation (allergen source) below the cockroach sensitization threshold (2 U/g) in bed and below the asthma morbidity threshold (8 U/g) on bedroom and living room floor/sofas, even in multifamily dwellings by integrated pest management combined with pesticide application, family education, and structural elimination of hiding places [40, 41]. The authors argued that intervention could provide cost benefit compared to a hospital stay for a patient with asthma. However, it was aided by entomologist or his staff throughout the study, especially for the insecticide applications and poison-bait placement. The level of expertise and effort that would be required to achieve significant cockroach extermination was not determined.

## STRATEGIES FOR THE ISOLATION OF CDNA CLONES ENCODING ALLERGENS

Molecular cloning of an allergen is the prerequisite for the production of recombinant proteins. IgE immunoscreening of expression cDNA libraries [42] or phage display [43, 44, 45] is one of the conventional ways to isolate cDNA encoding allergens. IgG1 of mouse immune serum, which is known to be produced by the influence of interleukin-4 could be used for the initial screening of cDNA library, and it has to be confirmed by human IgE antibodies. Reverse transcriptase-polymerase chain reaction (RT-PCR) is the simple and powerful method to detect and clone a specific RNA from a small number of cells [46, 47, 48]. Degenerate primers could be utilized when information on a partial amino acid sequence of the natural allergen or closely related protein are available. Full length sequence of cDNA from partial sequences could be obtained by rapid amplification of cDNA ends (RACE) [49, 50]. PCR amplification is particularly advantageous when only a limited amount of allergen-specific IgE is available, and multiple isoforms could be investigated at once. Cloning of PCR products was facilitated by the development of TA cloning for further manipulations [51]. The analysis of expressed sequence tags (ESTs) [52] from suspected organisms could be another choice for the identification of new allergens [53].



**Fig. (1).** Four species of domestic cockroaches found in Korea. (A) American cockroach (*Periplaneta americana*), (B) Dusky brown cockroach (*Periplaneta fuliginosa*), (C) Japanese cockroach (*Periplaneta japonica*), (D) German cockroach (*Blattella germanica*). Scale bars of 1 cm are included for comparison.

#### EXPRESSION SYSTEMS FOR THE PRODUCTION OF RECOMBINANT ALLERGENS

The allergen extracts may be contaminated with other allergens [54] or contain proteolytic enzymes that degrade other allergens [55]. It has been attempted to produce recombinant allergens similar to their native counterpart in antigenic reactivity, structure, and biological activity [56]. A variety of recombinant allergens have been expressed using both prokaryotic and eukaryotic systems [56, 57, 58, 59]. However, it is often difficult to obtain a recombinant allergen which contains the same post-translational modifications, as the native protein, as well as correct folding. In case of mite group 1 allergens, much effort has been made to produce the recombinant allergens which are active enzymatically as well as allergenically, and it was eventually re-

warded by using *P. pastoris* system [60, 61]. Carbohydrate epitope may be overcome by using peptide mimetics (peptide mimotopes) [62, 63], even though efforts have not been made in cockroach allergens. Fusion proteins may increase the solubility of recombinant proteins and facilitate protein purification and immobilization. Maltose binding protein could be used for these purposes [64], but glutathione-S-transferase (GST) and histidine tagging proteins are more commonly used fusion partners for the recombinant protein production. However, proteases could be used to cleave the fusion proteins. GST derived from *Schistosoma japonicum*, is one of the widely used choice of carrier protein, allowing the facilitated purification [65]. GST carrier has to be cleaved by digestion with site-specific proteases or removed by adsorption on glutathione-agarose because GST itself could have IgE-binding reactivity and allergenic [66]. Ex-

pression with a tagging protein of six consecutive histidines could also facilitate purification [67]. Six histidine tag allows the immobilization and efficient purification of the protein on metal-chelating surfaces such as nickel-nitrilotriacetic acid (Ni-NTA)-agarose. It can be applied to the other expression systems such as yeast and baculoviruses [68].

### (1) Prokaryotic Bacterial systems

*Escherichia coli* is one of the first choice and commonly utilized system for production of recombinant proteins, because it is easy to handle, and cost-effective [69, 70]. Incorrect folding and segregation into insoluble aggregates as inclusion bodies may lead to production of biologically inactive protein even after refolding. The use of molecular chaperones which help the proper folding and cellular targeting of proteins of interest is one of the ways to overcome its limitations [71]. Co-production of protein disulfide isomerases could increase disulfide bonding and influence on its folding process [72]. Different codon usages between *E. coli* and other organisms producing allergens may cause inefficient translation and low yield [73, 74].

### (2) Eukaryotic Expression Systems

The ability to perform many of the post-translational modifications is the main advantage of the eukaryotic system over prokaryotic one. Some allergens were produced in baker's yeast, *Saccharomyces cerevisiae* [75, 76]. However, recombinant Der p 1 expressed in *S. cerevisiae* has gone into the insoluble fraction. It showed high degree of antigenicity. However, clear differences with the native allergen were demonstrated [77]. Researchers tend to prefer the methylotrophic yeast *Pichia pastoris*. Recombinant proteins could be expressed in either intracellular or extracellular secreted forms [78]. The secreted foreign proteins could comprise the majority of the total protein in the medium, and recombinant proteins could be purified from the culture medium. Higher heterologous protein expression levels are another advantage over other eukaryotic expression systems as well as post-translational modification and fast and easy way of manipulation. Insect cells using baculovirus have proven to be one of good system for the overproduction of foreign proteins [79]. More facilities and skills are required for insect cell culture compared with yeast cell culture. It seems to have to think about the possible contaminations of similar proteins with the allergens from insect host cells if the allergen of interest is originated from insects or some arthropods. Nonetheless, the yield of recombinant allergens expressed in baculovirus systems is not as high as those achieved with *P. pastoris* system [80, 81, 82, 83, 84].

Per a 1, a major allergen of American cockroach, was expressed in mammalian system using Cos-1 cells, however the yield was not high compared to yeast systems [85]. Requirement of sophisticated equipment and skill for mammalian cell culture is another shortage of this system.

Plant system such as Chimeric tobacco mosaic virus could be utilized for the production of recombinant pollen allergens [86, 87, 88]. Post-translational modification could be expected, even though it requires elaborate purification methods and may lead to low yield.

## EVALUATION OF ALLERGENICITY

Allergenic extract or recombinant proteins could be used both for diagnosis and treatment. The allergen content of crude extract varies markedly for different source materials or different extraction procedures. Inconsistent protein contents and relative potencies have been reported even in the commercially available cockroach extracts [89]. Consequently, the problem of standardization is complicated, since currently no simple, completely satisfactory methods for the preparation of safe and effective allergen extracts are available. Allergen extract may contain non-allergenic and some toxic substances which are difficult to standardize. Severe IgE-mediated side effects of the treatment may occur during immunotherapy by the systemic administration of allergens. Consequently, it is often difficult to achieve therapeutically effective doses. Moreover, clinical diagnosis by case history and physical examination may often be subjective and susceptible to bias as exemplified by Williams et al [90]. In principle, the use of recombinant allergens is believed to overcome these limitations [91, 92, 93, 94, 95]. Currently, much effort is being made to standardize some of the main recombinant allergens in Europe (CREATE project) [96]. Before clinical use, it has to be tested whether it suitable for diagnosis (sensitivity and specificity) and useful for immunotherapy (safety and efficacy) in any way [97, 98]. Followings are description on different techniques of allergy diagnosis by evaluation of patients' reactivity (1-4), and assessment of allergen extracts or levels of allergen exposure (5).

### (1) Skin Tests

Skin prick test (SPT) is one of the commonly used methods because of its simplicity, safety, and inexpensiveness. The usual criterion for positive reaction is the mean wheal diameter of 3 mm, and it is the major diagnostic tool for defining sensitization to the allergen in question. Sometimes, the criterion using the 3 mm wheal diameter as the threshold for a positive SPT is not reliable enough to detect measurable levels of IgE antibodies [99, 100, 101].

Intradermal skin test titration protocol is thought to be one of the standards for the test of overall allergen potency. The dilution at which 50-mm skin test response (D50) to the allergen in question is determined in the target population (102, 103) as a measure of the allergen potency. Despite of some limitations, skin testing is the only *in vivo* test which could be carried out regularly in hospital.

### (2) IgE Reactivity

Since IgE was identified as the key molecule in mediating type 1 hypersensitivity reactions, assessment of specific IgE antibodies has been thought to be critical for identification of the causative agents in patients with allergic diseases [104, 105]. Inaccurate blood tests for specific IgE antibodies could result in an incorrect diagnosis [106, 107]. Therefore, a definitive clinical diagnosis on the results of any single diagnostic method, and combined with clinical history and physical examination are used to establish diagnosis of allergic diseases. Nevertheless, detection of IgE antibodies is widely used in laboratories due to its simplicity and reproducibility [108, 109, 110].

Radioallergosorbent test (RAST) which measures the presence of IgE antibodies directed against specific allergen was introduced as a diagnostic procedure [111]. Serum is mixed with allergen prior to its introduction into the test and the concentration of allergen required to produce 50% inhibition is a measure of the potency of the allergen [112]. Recently, it has been replaced by Pharmacia CAP system which is a fully automated solid-phase immunoassay [113]. Results are listed in kU/L, equilibrated against the World Health Organization standard for IgE. Enzyme-linked immunosorbent assay (ELISA) is one of the alternatives for the detection of allergen-specific IgE antibodies in laboratories. Accurate quantification of IgE antibodies to specific allergen is also possible by the development of chimeric ELISA [114]. Western blot analysis combined with SDS-polyacrylamide gel electrophoresis or 2-dimensional gel electrophoresis allows the detection of multiple allergens, and it is convenient for the initial characterization of the allergen extracts.

### (3) Histamine Release Assay

The ability of allergens to release histamines from peripheral leukocytes has been well documented [115]. The quantity of an allergen required to liberate 50% of the histamine from peripheral leukocytes (basophils) is an excellent measure of the relative potency [116, 117]. However, it is limited to research laboratories because of its difficulty and need for ready access to individuals sensitive to the given allergens. Immunohistochemistry is also useful for the animal experiments.

### (4) Challenge Test

Objective determination of airway responsiveness to histamine and methacholine is useful in the diagnosis of obstructive airway diseases such as asthma [118, 119]. The response of asthmatic patients to these agents ranges from 100 to 1000 times greater than that of normal subjects. A positive response to inhalation challenge is thought to be a much more reliable index of clinically significant allergies than skin test. Asthmatic patients are known to respond to small doses of methacholine, whereas allergic individuals without asthma respond only at much larger doses, and normals have little response at any dose [120]. Provocation with inhaled methacholine accompanied by serial measurement of FEV<sub>1</sub>, forced expiratory volume per second, as a means of demonstrating airway hyperreactivity [121]. A standardized bronchial inhalation challenge procedure was designed to resolve some of the problems of differences in interpretation arising out of differences in technique [122]. However, it involves some hazard and is limited to research purposes because of need for ready access to the subjects sensitive to the given allergens.

### (5) Two-Site ELISA

Monoclonal antibody based immunoassays have been widely used to quantitate or monitor specific allergen levels [123]. Quantitative analyses of specific or major allergenic components by ELISA assays are being performed to validate the allergenicity of various allergen extracts [124]. Mite group 1 [125] and mite group 2 [126] allergen measurements have been considered as a gold standard for monitoring mite

allergen extracts as well as dust mite allergen levels. Two-site ELISA have been developed using lateral flow technology, and can be used as rapid tests by patients themselves or by investigators, to make decisions about exposure and about allergen avoidance something like lateral flow test [127, 128]

## COCKROACH ALLERGENS

Cockroaches are known to produce potent aeroallergens which elicit the allergic diseases in genetically predisposed individuals [129], and sensitization to cockroach allergens is known to be strongly associated with asthma [130]. Cockroach allergens identified to the molecular level are summarized in Table 1. In case of mites, the proteolytic enzymes which are known to be contained in the feces are important causes of allergic reactions (enzyme hypothesis) [131]. Cockroach extract prepared from whole body or feces may contain digestive enzymes derived from their intestines or salivary glands and could elicit inflammatory reactions *in vitro* test. Serine proteases and their inhibitors are also known to be contained in the hemolymph of insects as a defense mechanism against the invading microorganisms [132, 133, 134]. Consequently, it is probable that protease-activated-receptors (PARs) may be activated and elicit the inflammatory reactions in a laboratory experiments or during immunotherapies. However, none of cockroach allergens cloned so far are proteolytic enzymes, implying that much caution is needed for the diagnosis and immunotherapies using cockroach extracts.

Cast-overs and excreted materials of cockroaches may also influence the development of allergic disorders, and much attention should be given to them.

### (1) Group 1 Allergens

Per a 1 (Cr-P11), which elicits skin reactivity in 93% among cockroach sensitive patients, has been purified from the crude extract of American cockroach [135]. The complete cDNA sequence of Per a 1.0103 coding for a novel Per a 1 isoallergen was reported by Yang et al., [136]. It is estimated that a polypeptide of 395 amino acids with a calculated molecular mass of 44.62 kDa and a predicted isoelectric point of 4.4 could be produced. No cysteine or any potential N-glycosylation site was found. Dotplot matrix analysis [137], in which repeated fragments appear as parallel lines above the diagonal, revealed four entire amino acid repeats of about 100 amino acids long, each of which containing two transmembrane regions. Pattern and profile searches indicated potential phosphorylation sites for cAMP- and cGMP-dependent protein kinase, protein kinase C, casein kinase II, and tyrosin kinase. A motif characteristic of mitochondrial energy transfer protein was also found [138], suggesting that the biological function of group 1 allergens may be modulated by phosphorylation. These sequence analyses have predicted that group 1 allergen is most likely directed to the exterior of the cells following the secretory pathway through endoplasmic reticulum or into the mitochondrial matrix. Per a 1 deletion mutants study has also shown that IgE binding epitopes are heterogeneously distributed among the different sera tested, and amino acid residues 78-85 and 267-274, containing internal repeats, were shown to be required for IgE binding [139]. Recombinant Per a 1

**Table 1. Immunochemical Properties of Cloned Cockroach Allergens**

| group | Biochemical identity       | Molecular weight (kDa) | IgE binding frequency (%) | Species (allergen)             | Expression systems for recombinant allergen |
|-------|----------------------------|------------------------|---------------------------|--------------------------------|---------------------------------------------|
| 1     | Unknown                    | 90                     | 77                        | <i>B. germanica</i> (Bla g 1)  | <i>E. coli</i> , <i>P. pastoris</i>         |
|       |                            | 20-25                  | 50                        | <i>P. americana</i> (Per a 1)  | <i>E. coli</i> , <i>P. pastoris</i> , COS   |
| 2     | inactive aspartic protease | 36                     | 60                        | <i>B. germanica</i> (Bla g 2)  | <i>E. coli</i> , <i>P. pastoris</i>         |
| 3     | Arylophorin/hemocyanin     | 72                     | 80                        | <i>P. americana</i> (Per a 3)  | <i>E. coli</i>                              |
| 4     | Lipocalin (calycin)        | 18                     | 40-60                     | <i>B. germanica</i> (Bla g 4)  | <i>E. coli</i> , <i>P. pastoris</i>         |
| 5     | Glutathione-S-transferase  | 23                     | 70                        | <i>B. germanica</i> (Bla g 5)  | <i>E. coli</i>                              |
| 6     | Troponin C                 | 18                     | 50                        | <i>B. germanica</i> (Bla g 6)  | <i>E. coli</i> , <i>P. pastoris</i>         |
| 7     | Tropomyosin                | 33                     | 50                        | <i>P. americana</i> (Per a 7)  | <i>E. coli</i> , <i>P. pastoris</i>         |
|       |                            | 33                     | 17                        | <i>B. germanica</i> (Bla g 7)  | <i>E. coli</i> , <i>P. pastoris</i>         |
|       |                            | 33                     | 27                        | <i>P. fuliginosa</i> (Per f 7) | <i>E. coli</i>                              |

expressed in mammalian Cos-1 cells showed significantly higher levels of IgE binding reactivity than recombinant Per a 1 expressed in *E. coli* probably due to the fact that *E. coli* system could not able to facilitate the post-translational modification [85]. In case of Per a 1, protein modifications are thought to be crucial to allergenicity as well as biological activity.

In German cockroach, Bla g 1, antigenically cross-reactive allergens with Per a 1, have been identified with immunochemistry [140]. Bla g 1 and Per a 1 constitute the group 1 cockroach allergens. Several isoforms and variants have been identified, and the nomenclature has been accepted by the WHO/IUIS Sub-committee on Allergen Nomenclature [141]. Bla g 1 and Per a 1 share about 70% of sequence identity and IgE-binding frequency in cockroach allergic patients of 30-50%. Up to seven amino acid repeats were identified by dotplot matrix analysis in Bla g 1 [142]. Amino acid sequence analysis of natural Bla g 1 and Per a 1 suggested that these allergens are cleaved by trypsin-like enzymes and secreted into digestive tract. High level of Bla g 1 expression was found in hindgut and proventriculus. A recent study have shown that Bla g 1 protein is most prevalent in the midgut, and the Bla g 1 gene is exclusively expressed by midgut cells [143]. Adult females produce and excrete more Bla g 1 in their feces than males or nymphs, probably because females process more food than other life stages of the German cockroach. Structural analysis suggested roles for the digestion or mitochondrial energy transfer, but the precise biological function of cockroach group 1

allergens still remain to be elucidated. Immunoblot analysis has shown that a single Bla g 1 duplex was sufficient for IgE binding [144]. Measurement of Bla g 1 levels has been used to estimate the exposure to cockroach allergens and exposure above 2 U/g dust is thought to be a strong risk factor for sensitization [145]. Exposure to Bla g 1 and Bla g 2 at 3 months of age was predicted lymphocyte proliferation at 2 years and repeated wheeze in the first year of life [146]. Per f 1 allergen from *P. fuliginosa* which is the most predominant cockroach species in homes in Tokyo area, Japan was detected in eight of ten homes [147], but no molecular or biochemical data is available. Standardized allergen extract preparations of *Blatta orientalis* which include Bla o 1 has approved for commercial distribution by the US FDA [148]. However, it's molecular or biochemical data is not available.

## (2) Group 2 Allergen

Bla g 2 was identified and purified by using monoclonal antibodies raised against German cockroach whole body extract [149]. Bla g 2 elicits IgE responses in 60 to 80% of cockroach allergic patients [130]. In a recent study using 118 sera from cockroach sensitized subjects, Bla g 2 was found to be the most important cockroach allergen, showing the highest prevalence of sensitization (54 to 71%) among five recombinant cockroach allergens tested (Bla g 1, Bla g 2, Bla g 4, Bla g 5 and Bla g 7) [150]. A cDNA encoding a 24 amino acid putative signal peptide and a 328 amino acid mature polypeptide, with a calculated molecular mass of 35.939 kDa was isolated by immunoscreening of *B. ger-*

*manica* cDNA library using mouse anti-Bla g 2 serum [151]. Bla g 2 shows primary sequence homology to aspartic proteinases. A molecular model of Bla g 2 showed that overall three-dimensional structure was similar to that of aspartic proteinases with a well-defined binding pocket [152]. However, amino acid substitutions in the catalytic triads of the molecule suggested that Bla g 2 is inactive (it was also confirmed by milk clotting microtiter plate assay) and homologous to mammalian pregnancy-associated glycoproteins. Crystal structure determination clearly showed amino acid substitutions in the catalytic triad and also found to be a zinc binding protein [153]. Its inactivity was confirmed using standard aspartic protease assays (milk clotting assay and hemoglobin assay) in a recent study [154]. High concentrations of Bla g 2 were found to be expressed in digestive organs (esophagus, gut and proventriculus) [151]. Three times more Bla g 2 concentrations were detected in feces, compared to whole body extract by two-site ELISA [155]. Cockroach allergen appeared to be particularly effective at sensitizing, with all atopic children exposed to more than 0.32 µg/g being sensitized [29]. The proposed threshold value of Bla g 2 is 0.08 µg/g dust (2 U/g), whereas those of mite group 1 and cat allergens are 2 µg/g dust and 8 µg/g dust, respectively [156]. Understanding of its biological functions and mechanism eliciting the allergic disorders may lead us to the better way to treat cockroach allergy.

### (3) Group 3 Allergen

Electroblotting analysis with SDS-PAGE revealed nine allergenic components from American cockroach, and two components of molecular weight 78 and 72 kDa were thought to be the major allergens among these proteins [135]. Subsequently, two cDNA clones compatible with molecular weight and isoelectrical point value (6.26 and 6.63) were isolated and named Per a 3 [157]. DNA sequence analysis revealed that two clones have significant degrees of identity with insect storage proteins (20.1-33.9%), insect juvenile hormone-suppressible proteins (30.9-36.4%), and arthropod hemocyanins (29.7-34.6%). No sequence similarity was found to other known allergens. Its recombinant allergen showed 47.45% (9/19) skin test reactivities on cockroach-sensitive asthmatic patients. Two additional members of Per a 3 allergen were isolated [158]. The calculated molecular weights were 56.2 and 46.7 kDa, and predicted values of isoelectrical point were 7.06 and 6.54, respectively. The recombinant allergens showed 26.3% (5/19) and 94.7% (18/19) skin reactivities on asthmatic patients. Sequence variations among isoforms resulted in a significant difference in skin reactivities. Per a 3 was also reported to induce interleukin-4 expression in peripheral blood mononuclear cells (PBMC) and T cell cultures of atopic patients, showing a good correlation with clinical symptoms, skin reactivity, specific IgE and proliferative responses [159]. No counterpart of Per a 3 was identified in German cockroach, and their biological functions are still unknown.

### (4) Group 4 Allergen

Using pooled sera from cockroach sensitized patient with asthma, cDNA encoding a polypeptide with estimated molecular weight of 20.904 kDa was isolated (160). From 40% to 60% cockroach allergic patients showed IgE reactivity to

its recombinant protein. Sequence similarity searches showed that Bla g 4 was a member of a ligand binding proteins or a calycin family of proteins, which include lipocalins and fatty acid binding proteins. This family contains several allergens eliciting IgE antibody responses, including mouse and rat urinary proteins, and other mammalian allergens [161]. Bla g 4 is speculated to bind pheromones, as the rodent calycin allergens are pheromone-binding proteins. The pheromone in the feces of *B. germanica* was found to cause the cockroach aggregations and significantly influence the growth of nymphs [162, 163]. Chemical communication signals are also spread by trophallaxis, the reciprocal exchange of liquid foods which are the regurgitated materials between nest mates [164]. Northern analyses and in situ hybridization showed that Bla g 4 is expressed only in the adult male reproductive system, and it was transferred to the female within the spermatophore during copulation [165]. Northern blot analysis also showed that Bla g 4 was not transcribed in *P. americana*. Recombinant protein was expressed in *P. pastoris* to improve yields (50 mg/L) with excellent allergenic activity [166], since recombinant Bla g 4 expressed in *E. coli* provided poor yield (0.25 mg/L).

### (5) Group 5 Allergen

Glutathione-S-transferase (EC 2.5.1.18; GST) was identified by the immunoscreening of *B. germanica* cDNA library using a serum pool from cockroach-allergic patients, and designated it as Bla g 5 [167]. Its estimated molecular mass was 23.176 kDa. By using glutathione affinity chromatography, natural GST was purified and recombinant protein was expressed in *E. coli*. Both natural and recombinant GST showed excellent IgE binding reactivity and demonstrated positive reactions in intradermal skin test among cockroach-allergic patients using as little as 3 µg of recombinant GST. GST was demonstrated to cause IgE responses in 70% of cockroach-allergic asthmatic patients. GST is known to be involved in the detoxification of toxic compounds, which are endogenous or exogenous, and found in most of multicellular eukaryotic animals [168, 169]. High levels of glutathione transferases are known to be produced for detoxification and it may be excreted with feces. Homologous GSTs have been identified as important allergens such as *Dermatophagoides pteronyssinus* [170], and *Schistosoma mansoni* [171]. A 25 kDa protein shared by several arthropod groups, such as mites, mosquitoes, and chironomids, was described in the serum of a patient who had only seasonal rhinoconjunctivitis and local hypersensitivity to mosquito bite. It also showed specific IgE against several arthropod species, and proposed as a panallergen involved in cross-reactivity among arthropod species [172]. Bla g 5 shows 28-50% of homology to the insect or mite GSTs and cross-reactivity among different species is to be determined [173]. Allergenicity and cross-reactivity of GSTs from different domiciliary cockroach species are needed to be investigated.

### (6) Group 6 Allergen

A cDNA clone encoding troponin was obtained from the *B. germanica* cDNA library by IgE Ab screening and designated Bla g 6 [174]. Molecular mass of Bla g 6 is 18 kDa and IgE binding frequency is 50% among cockroach-allergic patients. Troponin is known to play important roles in mus-

cle contraction as a calcium regulator [175]. Still, much effort is needed to characterize group 6 allergen.

### (7) Group 7 Allergens

Tropomyosin has been identified as an important allergen in invertebrate such as dust mites (Der f 10, Der p 10, Blo t 10, Lep d 10) [176, 177, 178, 179], chironomids (Chi k 10) [180], silverfish (Lep s 1) [181], as well as other crustacea [182, 183] and molluscs [184, 185, 186] (Fig. 2). However, none of vertebrate tropomyosins were found to be allergenic [187].

Tropomyosin was shown to be involved in cross-reactivity between mite, shrimp, and insects in shrimp-allergic patients [188, 189]. In a study using boiled Atlantic shrimp and German cockroach, IgE serum levels to both anti-shrimp and anti-cockroach allergens were found to be positive in 76 of the 89 (85.4%) tested sera and cross-reactivity presumably by tropomyosin was noticed [190]. Sensitization to a particular food allergen may lead to the sensitization to certain inhalant allergens [191] and some patients receiving immunotherapy for house dust mite allergy sensitized and produced IgE antibodies against snail

and shrimp [192, 193]. Tropomyosin has been suggested to be an invertebrate pan-allergen due to its cross-reactivity [194]. Sensitization to invertebrate tropomyosin often drives to the serious situations [195].

Tropomyosin from *P. americana* (Per a 7.0101) was cloned by RT-PCR using degenerate primers designed according to previously reported invertebrate tropomyosin sequences [196]. The deduced amino acid sequence encoded a polypeptide with a predicted molecular mass of 32.776 kDa and an isoelectric point of 4.69. Its recombinant protein was expressed in *E. coli* as a non-fusion protein and its IgE reactivity was compared with that of native counterpart. IgE-reacting antibodies to the purified tropomyosin were detected in 41% (12/29) of sera tested. Cross-reactive allergen, putative Bla o 7 from Oriental cockroach, was also identified by inhibition study. Interestingly, the deduced amino acid sequence of tropomyosin from *P. fuliginosa* (Per f 7), a peridomestic pest traveling worldwide [15], was identical with that of Per a 7, despite its nucleotide showed 98.5% identity [197]. Seven of 26 (26.9%) allergic sera had IgE specific for recombinant protein. An isoform of Per a 7 (Per a 7.0102) was identified, and plaque immunoassay showed that ap-

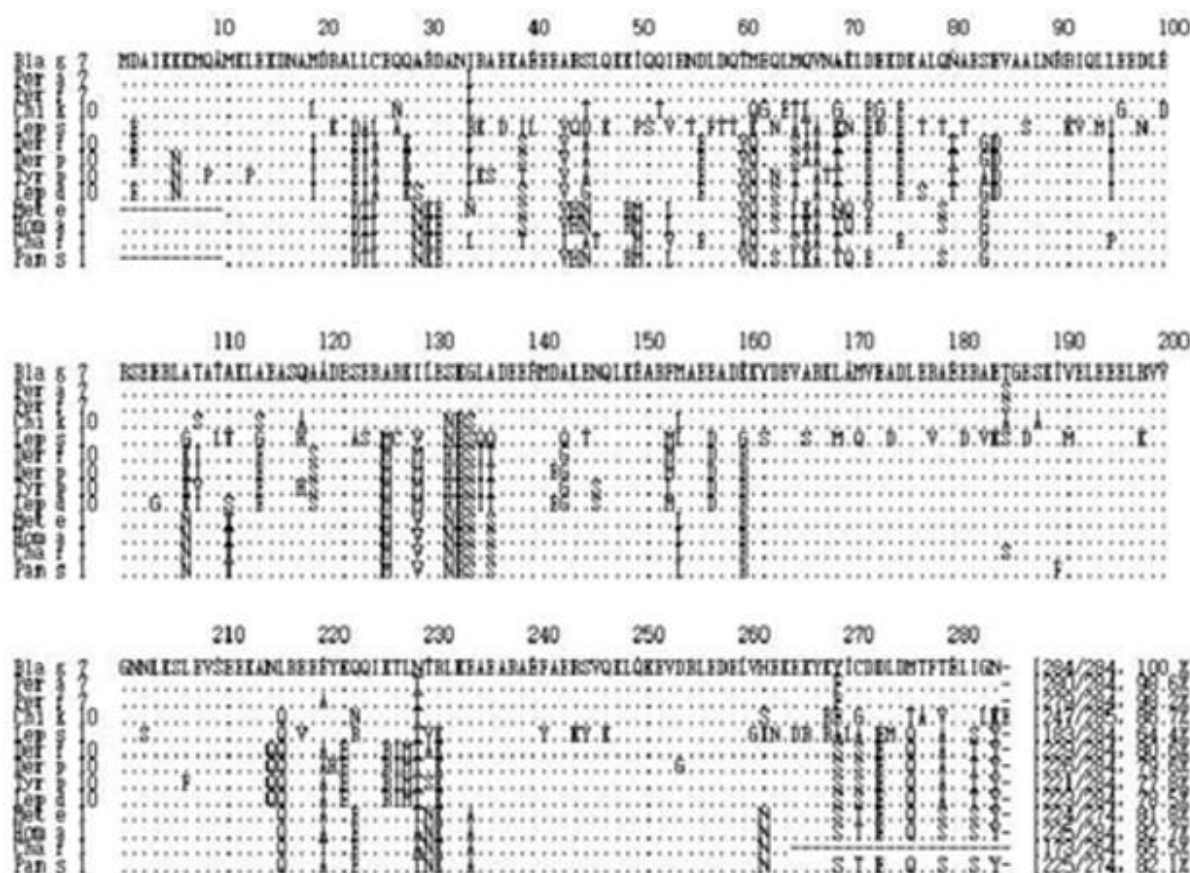


**Fig. (2).** Sequence alignment of Bla g 7 with other tropomyosins: Bla g 7 (*Blattella germanica*, AF260897), Per a 7 (*Periplaneta americana*, AF106961), Per f 7 (*Periplaneta fuliginosa*, AF454866), Chi k 10 (*Chironomus kiiensis*, AJ012184), Lep s 1 (*Lepisma saccharina*, AJ309202), Der f 10 (*Dermatophagoides farinae*, D17682), Der p 10 (*Dermatophagoides pteronyssinus*, Y14906), Tyr p 10 (*Tyrophagus putrescentiae*, AY623832), Lep d 10 (*Lepidoglyphus destructor*, AT250096), Met e 1 (*Metapenaeus ensis*, U08008), Hom a 1 (*Homarus americanus* slow muscle, AF034953), Cha f 1 (*Charybdis feriesus*, AF061783), Pan s 1 (*Panulirus stimpsoni*, AF030063). The percentage of each sequence

proximately 50% of the cockroach allergic patients have IgE to this allergen in Brazil [198]. German cockroach tropomyosin was cloned by RT-PCR using primers designed on the basis of known allergenic tropomyosins and designated Bla g 7 [199]. Its estimated molecular mass and isoelectrical point was 32.836 kDa and 4.57, respectively. IgE-binding reactivities of native and recombinant German cockroach tropomyosins (*E. coli*-expressed and *Pichia*-expressed) were compared, since the allergenicity of the recombinant tropomyosins derived from various arthropods is not coherent [200]. Native Bla g 7 showed 17.6% (6/34) IgE binding frequency to German cockroach sensitized sera, and *Pichia*-expressed recombinant Bla g 7 was able to inhibit 40.8% of IgE binding, whereas native Bla g 7 was able to inhibit 60.5% of IgE binding in the crude extract at an inhibitor concentration of 10  $\mu$ g/ml. IgE-binding epitopes were assessed by ELISA using recombinant fragments [201]. The seven tested sera revealed heterogeneous IgE-binding responses. This study demonstrated multiple IgE-binding epitope regions in a single molecule. A two-site ELISA was developed using monoclonal antibodies raised against recombinant Bla g 7. Monoclonal antibodies could able to detect not only cockroach tropomyosin but also mite tropomyosin. A two-site ELISA was applied to measure invertebrate tropomyosin level in the dusts from nine bedding samples and 13 kitchens. The tropomyosin was detected just in three samples from bedding (33.3%) and one of kitchen dust (7.7%). The concentration of the tropomyosin was estimated to be  $3.609 \pm 12.753 \mu\text{g/g}$  dust. The tropomyosin concentration in bedding samples was  $8.067 \pm 19.502 \mu\text{g/g}$  dust, while that of kitchen dust was  $0.523 \pm 1.813 \mu\text{g/g}$  dust. This observation was different from the previous study in Netherlands, which reported high levels of tropomyosin from 53 dust samples [202]. It was, however, in agreement with the results, a low level of sensitization to tropomyosin (17.6%), from studies using recombinant or native Bla g 7 in Korean populations [200], and it may reflect the regional and environmental differences. More tropomyosin levels in bedding samples may imply that the major source of tropomyosin is from dust mites. It is also noteworthy that bedding is heavily contaminated with cockroach allergens [40]. The proposed explanation was passive distribution of the cockroach allergen, with allergens carried into the bed on feet and clothes. It indicated that the allergens in the bedding samples were not derived only from dust mites but also contained the allergens derived from other sources and trapped in it. A recent study of IgE reactivity to shrimp tropomyosin in unexposed Orthodox Jews implied that the tropomyosin in dust could be a source of allergen sensitization and elicit the allergic reactions to the cross-reactive allergens [203].

Many species of arthropods are known as the sources of potent allergens that sensitize and elicit IgE-mediated allergic reactions in humans [204]. Tropomyosin level could directly reflect the rate of arthropod infestation because all invertebrate animals have the tropomyosin and it is not an excreted allergen such as mite group 1 [205] or group 2 [206], but it should be contained in dead debris. Tropomyosin level in house dust could indicate infestation not only of cockroach species but also of dust mites (house dust mite and storage mite) or other arthropods living indoors because of its cross-reactivity.

## (8) Other Possible Allergens

It is believed that there are yet more groups of cockroach allergens waiting to be investigated. An IgE-reactive protein with a molecular weight of 66 kDa on SDS-PAGE was purified by chromatography, however, its detailed molecular and biochemical properties have yet to be characterized [207].

Arginine kinase from the indianmeal moth, *Ploidia interpunctella* (Plo i 1), was identified using a panel of 102 sera from indoor allergen allergic patients [208]. IgE cross-reactive homologues in several invertebrate species such as mite, cockroach, lobster, king prawn, and mussel were demonstrated, and it was suggested as a new class of cross-reactive invertebrate pan-allergen. Arginine kinase was also identified in shrimp, *Penaeus monodon* (Pen m 2), by matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of the peptide digest [209]. Still, arginine kinase of cockroaches has not been studied yet.

Proteolytic activity of some allergens is reported to enhance their allergenic activity [210]. Protease-activated receptors (PARs) are one of the thoroughly investigated receptors which may contribute to the inflammatory reactions in airways [131]. Digestive proteases are enclosed by the peritrophic membrane or attached to it, and also concentrated in the fecal pellets [211, 212, 213, 214]. However, no proteolytic activity is found in hindgut in most insect [215], because a recycling mechanism acts to conserve luminal digestive enzymes [216, 217]. Crude extracts of insect could contain proteases and possibly affect on the laboratory experiments. One of the enzymes that may influence on the preparation of allergen extracts is the serine proteases and its inhibitors which found in arthropod hemolymph. These are known to function in protecting their host from infection parasites or regulating endogenous proteinases in coagulation, prophenol oxidase activation [132, 133, 134].

In some cases, sugar epitopes cause false-positive reactions in serodiagnosis [173, 218]. Studies on cockroach allergen to date are primarily focused on protein allergens. Attention on non-protein components may lead to the unexpected findings.

Some molecules which do not bind to IgE antibodies may influence the pathogenesis the allergic diseases. Chitin, found in the exoskeletons of arthropods, is the second most abundant polysaccharide in nature [219, 220]. Insect growth and metamorphosis are strictly dependent on the capability to remodel chitin-containing structures such as the cuticles of the epidermis and trachea as well as the peritrophic matrices lining the gut epithelium. Domestic arthropods should shed and cast over chitin coats, which are eventually to be mixed with house dust, during molting process. Interestingly, acidic mammalian chitinase have been described, although chitin does not exist in humans [221]. Acidic mammalian chitinase was also described to play important role in pathogenesis of Th2-dominated disorders such as asthma [222].

## FUTURE THERAPEUTIC AND DIAGNOSTIC APPROACHES OF RECOMBINANT ALLERGENS

Accurate diagnosis of allergy is dependent on the quality of allergen extracts. The presence of protease is one of complicating factors which influencing the quality of allergen

extracts. Some allergens have been known as proteolytic enzymes, which may degrade and influence on the stability of other allergens. Use of recombinant allergens is believed to eliminate these disadvantages inherent with the crude allergen extracts. The advent of microarray technology can facilitate multi-allergen tests using individual allergens which are well characterized, and allow the determination and monitoring of sensitization profile of patients [223]. Furthermore, recombinant allergens allow diagnosis of a patient's sensitivity to specific allergen and may aid in the tailored allergen specific immunotherapy by using genetically or chemically modified allergens which sensitize the subjects, not by using crude extract (summarized in Table 2). In case of cockroach allergy, a cocktail of Bla g 1, Bla g 2, Bla g 4, and Bla g 5 would diagnose 95% of patients with cockroach allergy by serologic analysis in the United States [224].

**Table 2. Allergen Modifications or the Tailored Allergen Specific Immunotherapy**

|                                                     |
|-----------------------------------------------------|
| <b>Chemically modified allergens (allergoids)</b>   |
| Formaldehyde polymerization of recombinant allergen |
| N-formyl-methionyl-leucyl-phenylalanine             |
| Maleylated allergens                                |
| CpG conjugated allergens                            |
| Toxin conjugated allergens                          |
|                                                     |
| <b>Genetically modified allergens</b>               |
| DNA vaccine                                         |
| Hypoallergen                                        |
| Allergen fragments or chimeric molecules            |
| Peptoides                                           |
| Shuffled allergens                                  |

Allergen-specific immunotherapy is an effective form of treatment and considered to be the only curative approach [225]. However, cockroach immunotherapy is not thought to have proven efficacy, although beneficial changes with immunotherapy with cockroach extract was reported in a study with 15 subjects with bronchial asthma due to cockroach hypersensitivity [226].

Many strategies for the production of allergens has been suggested and utilized for the therapeutic purposes [93, 227]. Chemical modification of recombinant allergens, which should reduce the allergenicity but retain the antigenicity, may improve the therapeutic efficacy. Beneficial effects may be expected by using 'allergoid' produced by formaldehyde polymerization of recombinant allergens [228] or recombinant allergens conjugated with N-formyl-methionyl-leucyl-

phenylalanine [229]. However, none of cockroach allergens was chemically modified and tried for immunotherapy. Maleylated shrimp tropomyosin showed a modulation of allergen-specific immune response by targeting to macrophage via scavenger receptor, even though it was found to have reduced allergenicity and antigenicity [230]. The use of a recombinant allergen-cytokine chimeric protein could promote polarization to Th1 cell responses and suppress allergic responses [231]. The principal cytokines which has potential to convert allergen-specific Th2 responses to Th1 responses are IFN- $\gamma$ , IL-12, and IL-18. Since DNA from bacteria was shown to activate NK cells and stimulate IFN- $\gamma$  production [232], CpG (unmethyl C-G dinucleotide) was extensively investigated as an adjuvant [233]. It was found that Toll-like receptor 9 was involved in the recognition of CpG [234]. However it is still largely unknown how molecular and cellular signaling cascades of CpG elicit protective effects in allergic inflammation and much remains to be elucidated [235]. Immunization with a plasmid containing cDNA encoding allergen resulted in Th1-like responses and genetic immunization turned out as a promising type of immunotherapy [236, 237]. Recently, effective short-course immunotherapy was reported using Amb a 1-immunostimulatory phosphorothioate oligonucleotide conjugate [238].

Some efforts have been made to construct the conjugates of allergens and toxins to provide the cytotoxic agent to be internalized and kill the cells, and eventually to induce immune tolerance or shift Th2 to Th1 cell responses. Anti-IgE with toxic A subunit of ricin [239], heat labile enterotoxin B subunit of *E. coli* [240], and cholera toxin B subunit [241], are examples of such attempts. Recently, ovalbumin-diphtheria toxin was demonstrated to protect OVA-sensitized mice from anaphylactic shock by binding to mast cells and destroy them [242]. However, caution has to be given for the use of toxins. For instance, the cross-reactivity between epitopes of the tetanus toxoid molecule and the  $\gamma$ -chain of high IgE receptor (Fc $\gamma$ RI) observed, and the presence of IgG antibodies against tetanus toxoid could induce mediator release from mast cell and Th2 cytokine production [243].

Analysis of B cell and T cell epitopes may allow manipulation of allergens and safer forms of specific immunotherapy with reduced allergenic molecules. Hypoallergen could be obtained by site-directed mutagenesis of IgE-binding epitope. Site-specific variants of the mite allergen Der p 2 showed 10 to 100-fold less potency on skin test, but one of them retained the ability to stimulate T cells from allergic individuals [244]. Allergen fragments or chimeric molecules consisted of a portion of allergen and a portion of homologous protein could be designed to disrupt conformational epitopes (B cell epitopes) and retain continuous epitopes (T cell epitopes). Interference with the formation of complexes involving the interaction of the MHC-peptide duplex may lead to the anergy or tolerance of T cells and suppression of Th2 type responses [245]. Certain structural analogs of allergen fragments which do not cause cross-linking of IgE and engage the T cell receptor (TCR) could be expected to lead to the allergen-specific anergy [246, 247]. Furthermore, appropriate chemical modification of allergen fragments (peptoids) may facilitate the induction of allergen-specific tolerance. These approaches require an understand-

ing of the relationship between structure and function/properties, especially for the B cell and T cell epitopes. However, DNA shuffling or molecular breeding that mimics natural evolution and can be performed without prior knowledge of structural and functional characteristics [248]. A large number of allergen chimeras having T cell epitopes with reduced anaphylactic potential could be efficiently produced and selected for allergen vaccination by DNA shuffling method.

The route of allergen administration for immunotherapy is another factor that influences the immune responses [249]. Subcutaneous injection is conventional way, but sublingual immunotherapy (SLIT) has become quite popular in Europe and local nasal immunotherapy (LNIT) is another alternative.

Recently, the use of purified allergens for the component resolved diagnosis was partly exemplified by house dust mite allergy [250]. The use of recombinant allergens will overcome the problems confronted with crude extracts and open the new era of tailored allergen-specific immunotherapy [251, 252]. A possible scheme for the diagnosis and allergen-specific immunotherapy was depicted in Fig. 3.

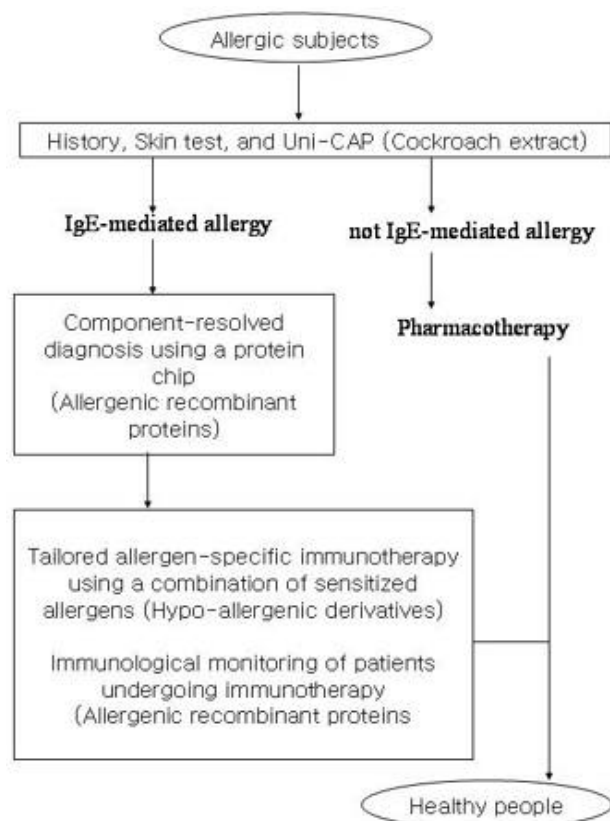


**Fig. (3).** Schematics of diagnosis and immunotherapy using recombinant allergens.

## CONCLUDING REMARKS

Pharmacotherapy of allergic diseases does not provide sustained relief of symptoms after completion of the course of medical treatment, and allergen-specific immunotherapy

is the only curative approach. The main limitations of allergen-specific immunotherapy are that it is time-consuming and entails a risk of anaphylaxis. Much effort is needed to overcome these problems. Even though cockroach allergy often causes serious problems, immunotherapeutic studies have been limited. Still, much remains to be studied and developed about cockroach allergy. It is believed that the use of recombinant allergens could facilitate the development of diagnostic and immunotherapeutic strategies. In addition, the development of two-site ELISA could aid allergen standardization, allergen avoidance procedure, the assessment of environmental allergen exposure, and the further characterization of allergens.

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