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EXPRESSION, PURIFICATION AND CHARACTERIZATION OF RECOMBINANT MOSQUITO SALIVARY ALLERGENS FOR THE DIAGNOSIS OF MOSQUITO ALLERGY

BY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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Abstract (1)

Recombinant Aed a 4

Accurate diagnosis of mosquito allergy has been hampered by the technical difficulty of obtaining mosquito salivary allergens. We wanted to express, purify and characterize a new 67 kDa recombinant *Aedes aegypti* salivary allergen Aed a 4, the alpha-glucosidase in the mosquito saliva, and to study its clinical relevance in the diagnosis of mosquito allergy. The recombinant Aed a 4 (rAed a 4) was expressed using a baculovirus/insect cell system. Homogeneity was achieved by a combination of anion- and cation-exchange chromatography. An enzyme-linked immunosorbent assay, immunoblotting and function measurement of alpha-glucosidase were used throughout the study. rAed a 4-specific IgE and IgG antibodies were also measured using ELISA in 13 mosquito allergic individuals who had severe reactions to mosquito bites and 18 controls who had a negative mosquito bites test. rAed a 4 bound to the IgE in mosquito allergic sera, as detected by ELISA and immunoblotting. Binding of rAed a 4 to IgE could be inhibited by addition of an *Aedes aegypti* head and thorax extract in a dose-dependent manner. The mean levels of rAed a 4-specific IgE and IgG were significantly higher in the allergic individuals than the controls. Using the mean of the controls plus 2 SD as a cut-off level, 46% of the 13 allergic individuals had a positive rAed a 4-specific IgE, while none of the controls was positive. rAed a 4 is identical to natural Aed a 4 and can be used for diagnosis of mosquito allergy.

Abstract (2)

Recombinant AFXa

To facilitate the studies of accurate diagnosis and immunological mechanisms of mosquito allergy, we expressed and tried to purify as well as identify a 54 kDa recombinant *Aedes aegypti* salivary protein, the anti-coagulant factor Xa (AFXa). The recombinant AFXa protein was expressed using a baculovirus/insect cell system. Factor Xa (FXa) inhibitory assays showed that as its natural counterpart, the recombinant AFXa had a biological function to inhibit FXa hydrolyzing chromozym X to release p-nitroaniline. The expression level of rAFXa is low as shown by SDS-PAGE and Western blot. There is a great possibility that AFXa is an allergen in the *Aedes aegypti* saliva as most secretory salivary proteins are infused into the host during the process of blood feeding. To date, all 4 proteins identified have demonstrated to be allergens. We have tried to purify the expressed recombinant protein from the culture medium using chromatography with DEAE-Sephacel, CM-Sepharose, and Mono S, but was not successful. Other methods such as chromatofocusing may be used in future experiments.

Abstract (3)

Recombinant Aed a 3

A 30 kDa recombinant *Aedes aegypti* salivary allergen Aed a 3, previously cloned, partially purified and identified in our lab, was expressed, purified and further studied its clinical relevance in the diagnosis of mosquito allergy. The recombinant Aed a 3 (rAed a 3) was expressed using a baculovirus/insect cell system and purified by an anion-exchange chromatography. rAed a 3 bound to the IgE in mosquito allergic sera, which was detected by ELISA and immunoblotting. Furthermore, the binding of rAed a 3 to IgE could be inhibited by the addition of an *Aedes aegypti* head and thorax extract in a dose-dependent manner. To investigate its clinical relevance, rAed a 3-specific IgE and IgG antibodies were measured, using ELISA in 13 mosquito allergic individuals who had severe reactions to mosquito bites and 18 controls who were negative to mosquito bite tests. The mean levels of rAed a 3-specific IgE and IgG were significantly higher in the allergic individuals than the controls. Using the mean of the controls plus 2 SD as a cut-off level, 46% of the 13 allergic individuals had a positive rAed a 3-specific IgE, while none of the controls were positive. We concluded that rAed a 3 may have identical immunogenicity to its natural counterpart and can be used for the diagnosis of mosquito allergy.

Abbreviations

AFXa:	anti activated factor X
BCIP:	5-bromo-4-chloro-3indoylphosphate p-toluidine salt
bp:	base pair
BSA:	bovine serum albumin
cDNA:	complementary deoxyribonucleic acid
cm:	centimetre
DMF:	N,N-Dimethylformamide
E. coli:	Escherichia coli
EDTA:	ethylenediamine - tetraacetic acid
ELISA:	enzyme linked immunosorbent assay
FBS:	fetal bovine serum
Fcϵ:	crystallized fragment of ϵ chain
FXa:	activated factor X
g:	gram
Ig:	immunoglobulin
IgE:	immunoglobulin E
IgG:	immunoglobulin G
IPTG:	isopropyl - β - thiogalactoside
kDa:	kilo dalton
L:	litre
LB broth:	Luria - Bertani broth medium

Mal I:	maltase-like I
mm:	millimetre
mM:	milli molar
MOI:	multiplicity of infection
mRNA:	messenger ribonucleic acid
NBT:	p-nitro blue tetrazolium chloride
ng:	nanogram
nm:	nano meter
OD:	optical density
ORF:	open reading frame
PBS:	phosphate buffered saline
PBS-T:	PBS Tween 20
PCR:	polymerase chain reaction
PEG 8000:	polyethylene glycol 8000
pI:	isoelectric point
pNPG:	p-nitrophenyl-α-D-glucopyranoside
r:	recombinant
RAST:	radioallergosorbent test
rpm:	rotations per minute
SDS - PAGE:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD:	standard deviation
Sf9:	Spodoptera frugiperda
TAE buffer:	Tris-acetate and EDTA buffer

TBST:	Tris, sodium chloride and Tween 20
TE buffer:	Tris and EDTA buffer
TNF:	tumor necrosis factor
Tris:	Tris (Hydroxymethyl) Aminomethane
µl:	micron litre
µm:	micron meter
µM:	micron molar
UV:	ultraviolet

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Introduction

1. General concepts of allergy

1. 1 Allergy and Allergens

Allergy is a disorder characterized by an increased ability of B lymphocytes to form IgE antibodies to certain groups of antigens that can activate the immune system after inhalation, digestion, or after penetration through the skin. Almost 42% of the Western world population suffers from allergies which can be considered a major health threat in industrialized

countries.[1, 2] The antigens which are capable of inducing synthesis of IgE antibodies at extremely low doses are called allergens. The known allergens include, but are not limited to, pollen allergens (grass pollen, tree pollen, weed and flower), mite allergens, animal allergens, fungal allergens, insect allergens, food allergens, etc.[3]

1. 2 Immunological mechanisms of allergy

Th1 and Th2 paradigms

According to the findings of Mosmann and Coffman, CD4+ Th cells are divided into two distinct subpopulations (Th1/Th2) on the basis of their cytokine production profiles.[4] Th1 cells produce INF-γ which are important for the clearance of intracellular pathogens but also mediate autoimmune pathology.[5] Th2 cells produce IL-4, IL-5, and IL-13 which play a role in the clearance of extracellular pathogens but are also implicated in allergic manifestation.[5] It became clear that IL-4 enhanced IgE

synthesis; IL-5 increased eosinophil growth and differentiation; and IL-13 increased mucus production and induced airway hyperreactivity. Since 1986, this Th1/Th2 paradigm has dominated our understanding of the pathophysiology of allergic diseases.[6]

Regulation of Th2 responses

The antigens that elicit IgE responses are mostly proteins or glycoproteins that have 5-80 kDa molecular weight.[7] However, structure and molecular analyses from many different allergen sources indicate that there are no apparent common structural motifs or biological properties that predict their allergenic nature. It is now becoming clear that the increased susceptibility of allergic individuals to mount IgE responses compared with non-allergic individuals is also controlled by genetic,[8-10] environmental and other factors.[11-14]

Specific IgE antibodies can be detected in the first few years after birth in allergic individuals, and seem to be induced by early, postnatal allergen contact.[15-17] This primary response to allergen is known as sensitization. Initial contact with minute amounts of intact, soluble allergen at the mucosal surface, might favor allergen uptake by immature antigen present cells (APCs including dendritic cell, B cell and macrophage), especially dendritic cell type 2.[18] Various stimuli such as recognition of characteristic patterns of pathogens,[19] inflammatory cytokines [20]and necrotic cells[21]trigger a complex maturation program that result in APCs migration from tissues to the draining lymph nodes.

In the process of migration, the APCs start to mature, specifically upregulating the surface expression of MHC molecules, cell-adhesion molecules(such as LAF-3, CD2

and DC-SIGN), and co-stimulatory molecules (for example B7.1 and B7.2 and B7h). The naive T cells that have specific allergenic TCRs sample the mature APCs. They interact with each other by cell-adhesion molecules first, then through TCR-MHC allergen complexes, CD28-B7 molecules and eventually an immunological synapase is formed between T cell and APC.[22] These membrane events translate into an early signaling pattern that is dependent on lck[23]and ltk[24] causing a low tyrosine phosphorylation of intracellular proteins, and favoring nuclear translocation of NF-AT (nuclear factor of activated T cells) and selective DNA demethylation and chromatin remodeling for GATA-3 (signal transducer and activator of transcription-3)-dependent genes such as IL-4 and IL-13.[25] All these events drive naive T cells to differentiate into Th2 cells that produce high amounts IL-4 and upregulate the expression of CD40 ligand, which further drive the T cell's differentiation into Th2 cells and clonal expansion.

Some data[26]show that IL-6 promotes Th2 differentiation by inducing the expression of IL-4 genes during activation of CD 4+ T cells, but other data suggests that the Th2 pathway may occur as the "default" in the absence of IL-12.[18] A recent paper[27] indicates that B7h-a costimulatory molecule on mature APCs and its ligand-inducible costimulatory (ICOS) molecules expressed on activated T cells are essential for Th2 cell activation, differentiation and effector cytokine expression. Other factors, such as low-affinity interactions between the TCR and its ligand, requiring longer times of T cell stimulation [28]and participation of CD4[29]often lead to Th2 differentiation.

During the above processes of Th2 cell activation, allergen specific B cells that upregulate the expressions of CD40 are also activated. Ligation of CD40 by CD40 ligand synergizes with the IL-4 receptor (IL-4R) signals to enhance the transcription of Cϵ

germline transcripts and activation-induced cytidine deaminase (AID), rearrangement of the IgE genomic locus and production of IgE antibodies.[30]IgE differs from other classes of Ig in that the majority of the antibodies are bound to high affinity IgE FcεRs that are mainly expressed on mast cells and basophils.

Allergic reactions (responses)

Allergic response can be characterized by a typical biphasic response consisting of an immediate reaction to an allergen, followed by a late-phase response. When allergic patients are exposed to allergens to which they are sensitized, the allergens cross-link receptor-bound IgE on the surface of mast cells, which triggers the degranulation and release of inflammatory mediators including histamines, prostaglandins, and other preformed or rapidly synthesized mediators that cause a rapid increase in vascular permeability and the contraction of smooth muscles.[31, 32] Additionally, the mast cell also produces TNF-β, IL-4, IL-5, IL-13 and other cytokines with various functions in the continuation and enhancement of the inflammation.[32, 33] They act by inducing sequential upregulation of adhesion molecules on endothelial cells in the blood vessels and by attracting leukocytes involved in the late-phase reaction, such as Th2 cells and eosinophils.[33, 34]The Th2 cell has a central role in maintaining the allergic late-phase inflammation. When memory Th cells of Th2 type are reactivated by allergens presented by local APCs, it is believed that high amounts of inflammatory-promoting cytokines including IL-4, IL-5 and IL-13 are synthesized. Eosinophils have an important role in the allergic late-phase reaction, to which they are recruited in high number.[35] The IL-5 production by local Th2 cells stimulates migration of eosinophils from the blood,[35, 36] it also activates the eosinophils, such that it primes them for degranulation and increases

their survival by inhibiting apoptosis.[37]The eosinophils express a number of Ig receptors including receptors for IgE.[38] Receptors that are probably involved in the activation and release of prestored granular basic mediators, such as major basic proteins (MBP) and eosinophil cationic protein (ECP). These proteins are toxic and are believed to be involved in the destruction of the epithelial cell layer that is characteristic for allergic asthma.[35, 38]

1. 3 Diagnosis and allergen-specific immunotherapy

Diagnosis

Diagnosis of allergy is based on either the detection of allergen-specific IgE antibodies in serum or other body fluids or on the elicitation of immediate symptoms by provocation testing with the relevant allergens (e.g. skin testing, nasal, oral or bronchial provocation).[39] The most frequently used methods include skin tests (*in vivo*) and *in vitro* measurement of allergen-specific IgE.

Skin tests are easy to perform and allow for a visualization of the response within a few minutes. They are performed by introducing a small quantity of allergens into the epidermis by pricking or scratching the skin or by intradermal injection, but research studies indicate that the prick/puncture skin test is the most useful technique with the most predictable results.[40] Most available assays for allergen-specific IgE antibodies utilized the principle of immunoabsorption. These assays include radioallergosorbent test (RAST),[41] enzyme-linked immunosorbent assays (ELISA),[42] and the Unicap test.[42] In these assays (including skin tests), an important factor is the quality of allergen.

Immunological mechanisms of allergen specific immunotherapy

Although many new and improved pharmacologic drugs have been introduced to reduce the symptoms of allergic diseases, antihistamines and steroids for instance, only allergen specific immunotherapy targets the natural cause of allergic diseases.[43, 44] Allergen specific immunotherapy is based on the administration of increasing doses of allergens to induce a state of allergen-specific non-responsiveness.[44] The efficacy of allergen specific immunotherapy has been proven in a number of studies involving patients allergic to tree, grass and weed pollen.[43, 45] It has also been demonstrated that allergen specific immunotherapy can prevent the progression of allergic disease from mild to severe manifestation of allergy.[46] Even though a lot of knowledge has been collected over the years, the mechanisms underlying successful allergen specific immunotherapy are not fully understood. The following factors may be involved in the effective allergen specific immunotherapy.

1. Induction of T cell anergy

Some data indicated markedly reduced T cell proliferative responses and a decline in the number of allergen specific T cell clones that could be derived during allergen specific immunotherapy.[47] Those allergen specific T cell clones that remained, showed deviation towards a Th1 type as compared with those before immunotherapy. An other study showed anergy of helper CD4 T cell clones were induced in vitro and this T cell unresponsiveness was allergen specific and reversible by addition of IL-2.[48] Furthermore, not only was T cell proliferation restored after addition of IL-2, but it was also accompanied by selective production of IL-2 and IFN-γ

without any production of IL-4, Il-5, or IL-13.[49] Some results denoted that allergen specific T cell anergy occurred in parallel to increase in IL-10 production.[50] IL-10 is not only an inhibitor of both Th1 and Th2 type T cell cytokine production and proliferation, but also an inhibitor of effector cells, such as mast cells, basophils, and eosinophils.[51]

2. Regulation of allergen specific IgE and IgG levels

Serum antigen-specific IgE levels increase during the early phase of allergen specific immunotherapy and then decrease after years of immunotherapy. The seasonal increase of IgE is reduced after immunotherapy.[52] Specific IgG (mainly IgG4 and IgG1) levels are increased during immunotherapy. The ratio of specific IgE to IgG4 decreased 10-fold within a few weeks.[49] Some data showed that the IgG4 antibody, induced by allergen specific immunotherapy as blocking antibodies inhibited the IgE-facilitated antigen presentation to T cells, which might reduce Th2 cell activation and the subsequent release of Th2 cytokines.[53] Other evidences supported that allergen specific immunotherapy induced IgG antibodies[54] compete with IgE for their binding sites on the allergens; this prevents the allergen-induced activation of basophils and, so, the release of biological mediators.[54, 55]

3. Effects on effector cells

In the early phase of allergen specific immunotherapy, decreases in histamine and sulfidoleukotriene release from basophils were documented,[56, 57] which can be attributed to suppression of cytokines in anergic T cells. There is clear evidence that effector cells of the allergic inflammation (mast cells, basophils and eosinophils) require T-cell cytokines for priming, survival, and activity. Moreover, IL-10 downregulates

eosinophil function and activity and suppresses IL-5 production by human Th2 clones.[58-60] It inhibits endogenous GM-CSF production and CD40 expression by activated eosinophils and enhances eosinophil cell death.[61, 62]

1. 4 Recombinant allergens in the diagnosis and immunotherapy of allergy

Diagnosis

Allergists have relied on natural allergenic products for the diagnosis and immunotherapy of allergic diseases since the turn of the last century. Both the quality and the standardization of natural allergen extracts have improved during the past 20 years. However, allergens prepared from natural source materials remain heterogeneous products, containing many nonallergenic proteins and other macromolecules that are apparently administered together with a few active components, the protein allergens. Allergens produced from natural source materials vary in allergen composition and content.[63] Natural products are also at risk of being contaminated with allergens from other sources and can contain proteolytic enzymes, which may be allergenic.[64] Attempts to isolate and purify the disease-eliciting allergens from the natural allergen sources for diagnostic and therapeutic purposes have proven too difficult and expensive for routine application.[65]

As a result, the disadvantages of allergen extract-based diagnosis are: 1) allergen extracts cannot identify the disease-eliciting allergens and; 2) false negative as well as false positive results are possible. However, with the introduction of recombinant DNA technology in the field of allergen characterization, an increasing number of recombinant allergens with immunological properties comparable to the natural allergens have

become available.[63, 65, 66] Diagnostic tests exclusively based on single recombinant allergens, allow for the precise measurement of levels of IgE antibodies to a particular allergen molecule. Thus, comparison of specific IgE levels with the allergenic potency of that determinant are available.[65, 67] This kind of diagnostic test will enable the precise selection of those specific molecules to which a patient is actually sensitized and as a logical next step, allergen-specific immunotherapy is being developed.[7]

One of the key advantages of recombinant allergens is that there is a high level of expressions of milligram or gram quantities of allergen that can be obtained in bacterial, yeast, or insect virus systems.[63] Recombinant allergens are effectively proteins that can be produced at will, under defined conditions, and purified with use of single-step procedures such as affinity chromatography. This has tremendous benefits in terms of quality control and standardization.[63] Another advantage of the recombinant allergens for diagnostic purposes is that they can be consistently produced at high purity. By careful allergen selection and formulation of the "cocktail", the allergenic activity of the natural product can be completely reproduced by recombinant allergens.[65, 68] With the co-application of recombinant allergens and microarray technology, new forms of multi-allergen tests which allow the determining and monitoring of complex sensitization profiles of allergic patients in single assays are being developed.[69]

Immunotherapy

The advent of recombinant allergens offers exciting new prospects for developing innovative allergen-specific immunotherapy in which the allergens are molecular entities and any substitutions, deletions, or modifications can be precisely defined at the level of

specific amino acids. For example, recombinant allergens can be engineered to produce "hypoallergens", that shows reduced binding to IgE antibodies, but retain T-cell epitopes. Hypoallergens have been developed for group 2 mite allergens, grass allergen Phl p 5, and peanut allergens Ara h 2 and Ara h 3.[70-74] Reduced immediate skin test reactivity to dimers and trimmers of recombinant Bet v 1 has been reported and natural isoforms of Bet v 1 with reduced IgE binding have also been identified.[47, 75] The rationale for using hypoallergens is that higher doses of allergen could be used for treatment with a reduced risk of adverse reactions.[63]

2. Mosquito Allergy

2. 1 Clinical manifestation

The term "mosquito allergy" should not be used to describe typical small local reactions to mosquito bites (wheals, flares, and delayed pruritic papules) which occur during natural sensitization and desensitization to mosquito saliva. Rather, it should be reserved for large or atypical (ecchymotic or vesiculating) local reactions,[76-78] or for systemic reactions such as anaphylaxis and generalized urticaria.[79-82] Inhalant allergy (rhinitis, conjunctivitis, and asthma) to the scales, hairs, and other emanations of mosquitoes have also been described.[83-85]

Large local reactions to mosquito bites consist of itchy, red and warm swellings appearing within minutes of being bitten, and itchy papules appearing 2-6 hours after the bites and persisting for days or weeks. Ecchymotic or vesiculated local reactions may also occur at the site of mosquito bites.[86] Sometimes, extremely large local reactions to

mosquito bites occur and may involve an entire body part such as the face, hand, arm, foot, or leg. These reactions are frequently misdiagnosed as cellulitis; however, by history, they develop within hours of mosquito bites, a time frame in which a bacterial infection is unlikely. Simons and Peng have named this clinical disorder "Skeeter Syndrome" and have documented the immunologic response to mosquito saliva in these patients.[76]

2. 2 Immunological mechanisms

The reactions to mosquito bites described above are mainly due to the effects of mosquito saliva infused during blood feeding. It was proved that mosquitoes whose salivary glands were removed could engorge blood without eliciting any reaction.[87] Reactions to mosquito bites are immunologic in nature and caused by specific sensitization to mosquito saliva. Initial exposure to species to which the subjects have not been previously exposed cause no reactions.[88] IgE-mediated type I and cell-mediated type III hypersensitivity are involved in mosquito allergy, IgG may also involved in severe local reactions, through a type III hypersensitivity such as mosquito bite-induced Arthur reactions.[89] Mosquito bite-induced immediate wheal and flare reactions correlate with both mosquito salivary gland-specific IgE and IgG levels, whereas bite-induced delayed indurations correlate with lymphocyte proliferation responses to mosquito allergens.[90] [91] Serum mosquito-specific IgE has been identified in humans by using passive cutaneous anaphylaxis transfer tests (Prausnitz-Kustner tests),[92] immunoassays such as ELISA[91, 93], RAST, [94] histamine release from

basophils,[92]and immunoblot techniques.[95-97] Mosquito salivary proteins are also involved in the hematophagy process and the transmission dynamics of pathogens.[98]

2. 3 Diagnosis.

A history of an allergic reaction which develops following a witnessed mosquito bite is helpful in diagnosing mosquito allergy; however, since most bites are painless and not directly observed, for example, when they occur during sleep or in infants and young children, a definite cause-and-effect relationship between the bite and the reaction is not often apparent. By the time the patient is seen in consultation by an allergist/immunologist or other specialist, symptoms and signs of the reaction have usually long disappeared.

Lack of readily available, specific, sensitive and safe assays for mosquito allergy is the major obstacle to accurate diagnosis. Currently, diagnosis of mosquito allergy is based on the following options: skin tests, mosquito bite challenge tests (not recommended) and *in vitro* tests. Skin tests performed with commercially available, nonstandardized mosquito whole body extracts cannot be relied upon for diagnosis. Because these extracts often result in false-negative tests due to the low content of the relevant mosquito salivary antigens, or false-positive tests due to the high content of the irritating nonallergic components.[99]

Mosquito bite challenge tests for diagnostic purposes are not recommended because some potential problems are associated with this method. For example, there is the risk of transmitting disease through the bite or the risk of causing anaphylaxis in a very sensitive patient.

The most widely available *in vitro* tests for mosquito allergy are the Unicap and RAST, in which a whole body extract of *Aedes communis* is used as the capture antigen. This test, recently evaluated in individuals documented as both with and without mosquito allergy, occasionally gives false-negative or false-positive results.[42] In a few medical centres with laboratories specializing in mosquito allergy studies, *in vitro* tests, in which pure mosquito saliva is used as the allergen are available for diagnostic purposes. These tests include sensitive, specific ELISAs for measurement of IgE and IgG,[91, 93] radioallergosorbent test (RAST),[94] immunoblotting,[97] and lymphocyte transformation tests.[90, 91]

2. 4 Immunotherapy

In individuals with a history of mosquito bite-induced anaphylaxis and delayed hypersensitivity, immunotherapy with whole body mosquito have been administered to individuals in uncontrolled, unblinded studies.[79, 80] Since immunotherapy with whole body mosquito extracts may result in severe side effects in certain patients,[80] immunotherapy with mosquito head and thorax extracts,[99] if available, should be used in preference, since they contain less body proteins and more salivary proteins than whole body extracts, and are relatively easy to prepare. Ideally, immunotherapy with pure mosquito salivary allergen preparations or pure recombinant mosquito salivary antigens should be given, but this is currently not feasible due to the lack of widespread availability and the prohibitive cost of these preparations. Proof of the concept of the usefulness of recombinant mosquito salivary antigens in immunotherapy is currently

being established in a mouse model of mosquito allergy[100-102] with the characterization of salivary allergens at the molecular level ongoing.

2. 5 Salivary proteins (allergens) and recombinant salivary allergens

The nearly fully sequenced genome of *Anopheles gambiae*[103] provides an unprecedented opportunity to study and compare the evolution of blood feeding in the Diptera *Anopheles* and *Drosophila.*[104] It also provides motivations for the study of the salivary gland transcriptome and proteome of other mosquitoes. Thirty-three full-length novel cDNAs were reported from *Anopheles stephensi* salivary glands.[105] Twenty-one novel *Anopheles gambiae* and thirty-one novel *Aedes aegypti* salivary gland cDNAs were found.[106, 107] Most of the above novel cDNA encoded proteins have not been expressed and identified.

Mosquito saliva contains many substances and according to their biological functions, these substances can be divided as below: (1) Anti-host homeostasis, which includes anticlotting, antiplatelet, and vasodilator. These substances presumably increase arthropod fitness by increasing the speed at which blood is found and imbibed and by decreasing the possibility of being killed by the host during feeding.[108, 109] (2) Substances that affect parasite transmission by arthropod vectors and thus may serve as vaccine targets against diseases.[110] [111, 112] (3) Some enzymes which are associated with sugar feeding [113, 114] as well as lysozyme, which may help to control bacterial growth in the sugar meal while stored in the mosquito crop.[115] (4) Substances that have immunomodulatory effects.[116-120] Some of the substances are allergens.[97, 121, 122]

Aedes aegypti saliva contains at least 31 proteins whose cDNA sequences have been deposited into the Genbank.[107] However, protein visualization techniques using gel electrophoresis and silver staining have only revealed about 20 peptides in the saliva of adult *Aedes aegypti* mosquito.[97, 123] These include α-amylase, anticoagulants, anti-TNF, apyrase, esterase, D7, α-glucosidase, and sialokinins.[98] Using SDS-PAGE and immunoblot techniques, at least 8 proteins have been identified as allergens which bind to the IgE of allergic subjects who have large local (skin) reactions to mosquito bites.[97] Seven proteins were already characterized by different researchers and 3 have been expressed, purified and characterized as allergens[114, 124-130](table 1). The three allergens are rAed a 1 (68 kDa), rAed a 2 (37 kDa) and rAed a 3 (30 kDa). All three recombinant allergens are found to be present in the saliva of *Aedes vexans* and other mosquito species[88, 97] and to have biological activity as shown in skin testing performed on 43 volunteers.[131] In the study, 43% of 28 mosquito bite test positive volunteers had a positive epicutaneous test to rAed a 1, 11% to rAed a 2 and 32% to rAed a 3, while none of 15 mosquito-bite test negative volunteers had a positive skin test to any of the recombinant antigens. Furthermore, a significant correlation between the sizes of recombinant allergen-induced skin reactions and mosquito bite-induced reactions was found in the study.[131]

3. Mosquito salivary Aed a 4 and AFXa proteins

In our laboratory, available cDNAs from *Aedes aegypti* salivary glands are Mal I (Aed a 4) and AFXa, which were cloned and provided by Dr. A. James, University of California, Irvine.

Table 1. *Aedes aegypti* salivary proteins whose cDNA have been cloned and sequenced

Proteins	Molecular Weight	cDNA sequenced	Biological function and allergenicity	References
α-Amylase 1997	81.5 kDa	Yes	Unknown	Grossman et al.,
Apyrase (Aed a 1)	68 kDa	Yes	Antiplatelet aggregation Allergen	Ribeiro et al., 1984 Peng et al., 1998
α-Glucosidase (Aed a 4) 1990	67 kDa	Yes	Sugar digestion Allergen	James et al., 1989 Marinotti et al., This study
Anticoagulant-factor Xa	54 kDa	Yes	Anticoagulant	1998 Stark and James,
D7 (Aed a 2)	37 kDa	Yes	Unknown Allergen	James et al., 1991 Peng et al, 1998
Aed a 3	30 kDa	Yes	Unknown Allergen	Xu et al., 1998
Sialokinins	1.4 kDa	Yes	Vasodilator	Ribeiro, 1992 Champagne and Ribeiro, 1994

.Table 1 lists Aedes aegypti salivary proteins whose cDNAs have been cloned and sequenced. Among them, Aed a 1, Aed a 2, Aed a 3 and Aed a 4 (this study) have been identified as allergens.

3. 1 Aed a 4 protein

James et al. (1989)[125] described a gene that is expressed specifically in the salivary glands of adult *Aedes aegypti*. Their data showed that the presumed protein, encoded by this gene, would be a secreted protein with a minimum molecular weight of 66.7 kDa. The deduced protein sequence was similar to the maltase from *Saccharomyces carlsbergensis* and thus this gene was named Maltase-like I (Mal I). Marinott and James (1990)[113] characterized an α-glucosidase from the adult salivary glands of *Aedes aegypti*. The α-glucosidase is a 67 kDa soluble glycoprotein that is secreted when mosquitoes take sugar and blood meals. It is expressed in both male and female *Aedes Aegypti* salivary glands. The characteristics of the α-glucosidase correlate well with the putative protein encoded by the Maltase-like I gene, therefore they proposed that the α-glucosidase present in the salivary glands was actually the final product coded by the Mal I gene. Its α-glucosidase activity can be determined by hydrolyzing p-nitrophenyl-α-D-glucopyranoside (pNPG) to release p-nitrophenolate, which produces a yellow color.

3. 2 AFXa protein

Stark and James (1995)[132] have described an anticoagulant that was only found in female salivary glands of *Aedes aegypti*. This anticoagulant delays both the prothrombin time and the activated partial thromboplastin time, but has no effect on the thrombin clotting time. It is a specific, reversible, noncompetitive, proteinaceous inhibitor of activated Factor X (FXa). The anticoagulant is secreted during blood meals. It's function can be detected by its ability to inhibit the function of FXa hydrolyzing chromozym X to release free p-nitroaniline or by inhibiting blood clot formation. Its

anticoagulant activity is destroyed by boiling for 10 minutes or by heating at 56 °C for 30 minutes, and has a 4 mM calcium optimum with no magnesium requirement, as well as a pH optimum of 8.0.

In 1998 , Stark and James[126] isolated the protein product and corresponding cDNA of a gene designated anticoagulant factor Xa (AFXa). The 1.88-kilobase (kb) pair cDNA is shown to encode a 415-amino acid conceptual translation product with a predicted molecular mass of 47.8 kDa. Primary amino acid sequence analysis shows that the AFXa gene product has similarities to the serpin superfamily of serine protease inhibitors. The recombinant protein expressed by the baculovirus/insect cells system has the appropriate molecular weight and expected activity in inhibiting FXa-directed coagulation.

Objectives

Purification or isolation of each of the saliva proteins is required to facilitate research in diagnosis and immunotherapy of mosquito allergy. However, pure mosquito saliva can be obtained only by dissecting the salivary glands out of the heads and thoraces of individual mosquitoes or by inserting the mosquito proboscises into capillary tubes, stimulating saliva secretion and collecting. These techniques are extremely labor-intensive and time-consuming, and the supply of mosquito saliva antigens for use in skin tests and in vitro tests is consequently limited. Utilization of molecular cloning techniques to clone and express mosquito salivary proteins will circumvent these problems. Therefore, the objectives of the present study are:

1. To express, purify and characterize a new mosquito salivary protein - Mal I protein (Aed a 4) and investigate its value in the diagnosis of mosquito allergy
2. To express, purify and characterize another new mosquito salivary protein - AFXa protein and investigate its value in the diagnosis of mosquito allergy
3. To express, purify and characterize a previously identified salivary allergen, rAed a 3, and further investigate its value in the diagnosis of mosquito allergy

Materials

1. Cell media, cell lines, and transfection kits

LB broth Base Tablets (1.1g /tablet, makes 50 ml) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), Grace's insect medium, Grace's insect medium (Supplemented), Express Five SFM medium, Top10, DH-5α and BL-21 competent *E. coli* cells, Sf9 cell line (originated from the IPLBSF-21 cell line, derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda*), High Five cell line (BTI-TN-5B1-4, originated from the ovarian cells of the cabbage looper, *Trichoplusia ni*) and Bac-N-Blue Transfection Kit (Version K) were purchased from Invitrogen (Sand Diego, CA, USA).

2. cDNAs , enzymes, vectors and recombinant viruses

Anticoagulant-factor Xa (AFXa) cDNA cloned in pBSKII as an EcoRI fragment [126] and Aed a 4 cDNA cloned as an EcoRI fragment into pEMBL plasmid [125] were kindly provided by Dr. A.A. James, University of California, Irvine. Restriction enzymes such as Bam HI, Bst 98I, EcoRI, EcoRV, T4 DNA ligase and Taq DNA polymerase were purchased from either Promega (Madison, WI, USA) or Invitrogen. Baculovirus transfer vector pVl393 was purchased from Invitrogen, pET-24b vector was obtained from Novagen (Madison, WI, USA)

rAed a 3 and rAed a 2 recombinant virus was previously prepared in our laboratory [128] [130] and stored at -70 °C.

3. Mosquito saliva extracts, mouse anti-serum and antibodies

Aedes Aegypti saliva (0.25 mg/ml) and salivary gland extracts (0.22 mg/ml) were prepared as described previously.[90, 133] Mouse anti-mosquito saliva serum[134] and mouse anti-rAed a 3 (prepared with an unpurified recombinant histidine-tagged Aed a 3) serum were prepared previously in our laboratory and stored at -70 °C freezer. Mouse anti-rAed a 4 serum and mouse anti-rAFXa serum were prepared as described in Methods.

Goat anti-human IgE was a gift from Dr. N.F. Adkinson, Jr., The John Hopkins Allergy and Asthma Centre (Baltimore, MD, USA). Monoclonal anti-human IgG was purchased from Pharmingen (San Diego, CA, USA). Alkaline phosphatase-conjugated rabbit anti-goat IgG (Fc Fragment Specific), alkaline phosphatase-conjugated $F(ab')_2$ fragment goat anti-rabbit IgG (H+L), alkaline phosphatase-conjugated goat anti-mouse IgG, Fcγ (Fragment Specific) were all purchased from Jackson ImmunoReasearch Lab Inc. (West Grave, PA, USA). Monoclonal anti-human IgE (Clone 7.12) was provided by Dr. Andrew Saxon, University of California, Los Angeles.

4. Mice

Female, 8 to 10 week old BALB/c mice were obtained from the Central Animal Care Services, University of Manitoba. All animals were kept under identical conditions in one room at the Service Facility. The experiments were approved by the University of Manitoba and the Animal Care and Use Committee. The investigators adhere to Canadian Council on Animal Care guidelines for human treatment of animals

5. Other materials and equipment

Bovine Factor Xa was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Chromozym X was acquired from Roche Diagnostics Canada (Laval, Quebec).

Chromatography media were bought from Pharmacia Biotech (Uppsala, Sweden). The rest of the chemicals were purchased from Sigma Chemical (St. Louis, MO, USA) or Bio-Rad (Richmond, CA, USA), unless otherwise specified. Diaflo ultrafiltration membranes and 200 ml Amicon stir cell were obtained from AMICON DIV., W.R. GRACE & CO. (MA, USA). QIAquick™ Gel Extraction Kit and QIAprep Spin Miniprep Kit were acquired from QIAGEN Inc. (ON, Canada). Labscale™ TFF System and Pellicon XL Filter membranes (BIOMAX TM 30 K and 10 K) were purchased from Millipore (MA, USA).

Methods

1. rAed a 4

1. 1 Preparation of media, culture of Sf9 and high five cells

Complete TNM-FH medium was prepared according to the instruction manual for insect cell lines. Briefly, fetal bovine serum (FBS) and gentamycin (10 mg/ml) were added into Grace's Insect Medium (supplemented) to 10 % and 10 μg/ml, respectively. The solution was filtered through a 0.22 μm filter into a sterile container and was stored at 4 °C. The Express Five SFM (serum-free) medium was filtered through 0.22 μm filter into sterile containers and 200 mM L-glutamine to 9% was added prior to use. The solution was stored at 4 °C.

Culture of Sf9 cells and high five cells were performed according to the instruction manual. Briefly, a vial of Sf9 cells was removed from liquid nitrogen and placed in a 37 °C water bath. The cells were thawed rapidly with gentle agitation and transferred to a 25 cm^2 cell culture flask, pre-wetted with a complete TNM-FH medium. After incubation for 30 minutes, the cells were fed with fresh TNM-FH medium. The cells were incubated until a confluent monolayer of cells were formed. After enough adherent cells were obtained, the cells were seeded into a 125 ml spinner flask, and incubated with constant stirring at 90 rpm, using a magnetic stir plate. The cells were subcultured into a 500 ml spinner flask when enough cells were grown. Culture of high five cells was the same as culture of Sf9 cells, except that the complete TNM-FH medium was changed to Express Five SFM medium.

During cell culture, cell counting and determination of cell viability were all performed according to the instruction manual with slight modifications (addition of 10 μl of 0.4% trypan blue solution to 0.1 ml of cell culture).

1. 2 Transformation of *E. coli* competent cells and miniprep plasmid DNA extraction.

The transformation of different strains of *E. coli* were performed according to the manufacture's instruction manual with some modifications. Briefly, a microcentrifuge tube containing 100 μl of the competent cells were thawed on ice and 20-40 ng of plasmid or 10 μl of ligation were added to the mixture. The reaction was incubated on ice for 30 minutes and heat-pulsed in a 42 °C water bath for 30 seconds, and was then incubated again on ice for 2 minutes.

After LB broth medium (preheated to room temperature) was added to the transformation reaction, the tube was incubated at 37 °C for 1 hour on a shaker. LB agar plate containing the appropriate antibiotic was spread with the culture and incubated at 37 °C overnight. A single colony was picked up for miniprep plasmid DNA preparation.

Miniprep plasmid DNA extraction was performed according to the protocol of Molecular Cloning[135] with some modifications. A single colony of bacteria was inoculated in 3-5 ml LB broth with appropriate antibiotic and was incubated overnight at 37 °C. The bacteria was collected by centrifugation, then suspended in 200 μl of Solution I (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA), and was put on ice. 400 μl of freshly prepared Solution II (0.2 M NaOH, 1% SDS) was added, mixed by inversion, and was kept on ice for 5 minutes. After 300 μl of 7.5 M ammonium, acetate (pH 7.5)

was added, the solution was mixed by inversion and kept on ice for 5 minutes. The solution was centrifuged at maximum speed for 20 minutes and the supernatant was transferred to a new tube. After addition of 0.8 volume of isopropanol, the solution was centrifuged at maximum speed for 15 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. Then the pellet was dissolved in TE buffer and stored at -20 °C.

For sequence purpose, all miniprep plasmid DNAs were prepared using the QIAprep Spin Miniprep Kit according to the protocols in the instruction manual. All DNA samples were sequenced by the Sequence Laboratory of the Manitoba Institute of Cell Biology.

1. 3 Large scale preparation of transfer vector pVL1393 containing Aed a 4 cDNA (pVL1393/Aed a 4) and purification using CsCl-ethidium bromide gradient centrifugation.

(1) Preparation

The large scale preparation and purification of pVL1393/Aed a 4 was performed according to the protocols of Molecular Cloning [135] with slight modifications. Briefly, a single Top10 *E. coli* colony carrying the pVL1393/Aed a 4 was inoculated in 5 ml of LB broth media, containing 100 μg/ml ampicillin and incubated at 37 °C for overnight on a shaker.

On the second day, 3 ml of the culture was inoculated to 500 ml LB broth containing 100 μg/ml ampicillin and incubated at 37 °C for overnight on a shaker.

On the third day, the bacterial cells were harvested by centrifugation at 4 °C, then suspended in 100 ml of ice-cold STE (0.1 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) and collected by centrifugation again. The washed pellet was resuspended in 18 ml of solution I (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0) and 2 ml of freshly prepared lysozyme (10 mg/ml in 10 mM Tris pH 8.0) was added. Then 40 ml of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added. The contents were thoroughly mixed and 20 ml of ice-cold Solution III (60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid, H_2O 28.5 ml) was added. After centrifugation, the supernatant was filtered through 4 layers of gauze into a 250-ml centrifuge bottle and 0.6 volume of isopropanol was added. The DNA was recovered by centrifugation, washed with 70% ethanol, and dissolved in 3 ml of TE (pH 8.0).

(2) Purification.

For every milliliter of the above DNA solution, 1 g of solid CsCl and 0.08 ml ethidium bromide solution (10 mg/ml) was added. After being well mixed, the solution was centrifuged at room temperature and the clear, red solution was transferred to a Beckman Quick-Seal tube. The density gradient was centrifuged at 60,000 rpm for 24 hours with a Ti 65 rotor. The lower band of DNA was collected by using an 18-gauge hypodermic needle and was put in microcentrifuge tubes. An equal volume of isoamyl alcohol was added to the DNA solution and the two phases were mixed by vortex. The mixture was centrifuged at room temperature and the lower, aqueous phase was transferred to a clean microcentrifuge tube. The extraction was repeated until all the pink color disappeared from both the aqueous phase and the organic phase. The DNA solution

was diluted with 3 times volume of water and the DNA was precipitated with 2 volumes of 100% ethanol for 30 minutes at 4 °C followed by centrifugation at 4 °C. The precipitated DNA was dissolved in TE (pH 8.0). The OD_{260} of the final DNA solution was measured using an Ultrospec 3100 pro UV/visible Spectrophotometer (Biochrom Ltd, Cambridge, UK). The DNA concentration was calculated and stored in aliquots at -30 °C.

1. 4 Construction of recombinant baculovirus transfer vector pVL1393/Aed a 4

The pEMBL plasmid containing Aed a 4 cDNA, cloned as an EcoRI fragment was excised with EcoRI. The DNA fragments were separated on 1% agarose gel. The 1.83 kb Aed a 4 cDNA fragment was eluted using the QIAquick™ Gel Extraction Kit. The purified cDNA was ligased with EcoRI digested pVL1393 transfer vector (Figure 1). Top10 competent *E. coli* was transformed with the ligation mixture. Single colonies were randomly picked up and miniprep plasmid DNA were prepared. Plasmid DNAs containing the cDNA fragments were cut with EcoRV to identify insertion direction. Correct and opposite insertion direction plasmid DNAs were used for Aed a 4 cDNA 5'-end and 3'-end sequences with Polyhedrin forward primer. We compared the two sequences with the Aed a 4 cDNA sequence which was deposited into Genbank to see if the 5'- and 3'-end of pVL1393/Aed a 4 were in open reading frame (ORF). The pVL1393 containing the correct insertion of Aed a 4 cDNA was named as pVL1393/Aed a 4 and the plasmid DNA was prepared on a large scale and purified, using CsCl-ethidium bromide gradient centrifugation.

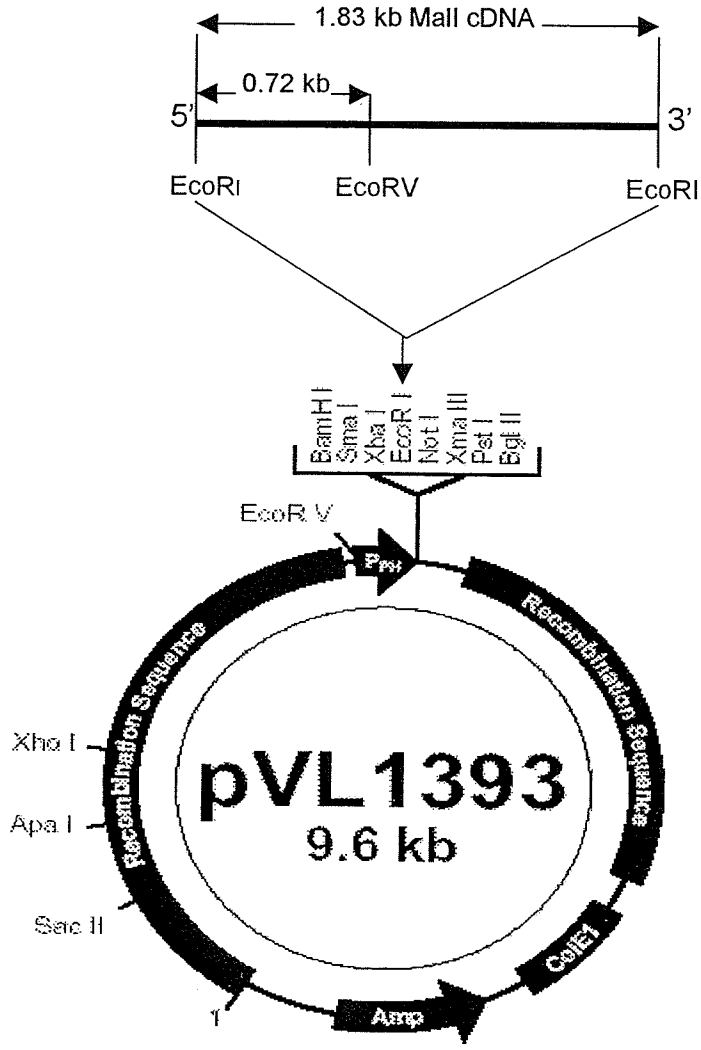


Figure 1. Construction of pVL1393/Aed a 4 vector
In pVL1393 vector, the fragment from EcoRV to EcoRI is 0.15 kb. If Aed a 4 cDNA was inserted in the correct direction, the EcoRV digested plasmid DNA should have a 0.87 kb fragment (0.15 + 0.72 = 0.87). Otherwise, it would have a 1.26 kb fragment (0.15 +1.11=1.26).

1. 5 Cotransfection of Sf9 cells with pVL1393/Aed a 4 and Bac-N-Blue ™ DNA

Log phase Sf9 cells (2 x 10^6) were seeded onto each of three 60 mm tissue culture plates and the plates were allowed to attach for 30 minutes. The transfection mixture was prepared by mixing 0.5 µg Bac-N-Blue ™ DNA, 4 µg of CsCl-ethidium bromide gradient centrifugation purified pVL1393/Aed a 4, 1 ml of Grace's Insect Medium (without FBS or supplements), 20µl of Cellfectin Reagent, and was incubated for 15 minutes at room temperature. After washing the culture plates twice with 2 ml of Grace's insect medium without supplements, the transfection mixture was added to the cotransfection plate, 1 ml of Grace's Insect Medium with 4 µg of pVL1393/Aed a 4 was added to the vector control plate, and 1 ml of Grace's Insect medium was added to the cell control plate. The three tissue culture plates were placed on a side/side rocker platform (Bello Glass Inc. NJ, USA) at room temperature with 2 sides/minute rocking for 4 hours. One ml of complete T.M.-F. media was added to each plate at the end of incubation, and the plates were sealed with paradigm, then incubated at 27°C for 72 hours. The cotransfection media were harvested for purification of recombinant viruses.

1. 6 Plaque assay and PCR analysis of recombinant virus

Log phase Sf9 cells were seeded at a density of 5 x 10^6 cells per plate and allowed to distribute evenly. The plates were infected with one ml of 10^{-2} or 10^{-3} or 10^{-4} dilutions of the cotransfection media for one hour at room temperature on a slowly side/side rocking platform. Then each plate was overlayed with 10 ml of baculovirus agarose-medium mixture. The overlayed plates were sealed and incubated at 27 ^{0}C.

7 days postinfection, the plates were scanned using a microscope. Any plaque which may be recombinant was circled. A sterilized Pasteur pipette was used to transfer the agarose plug containing the circled putative plaque to one well seeded with 5×10^5 Sf9 cell of 12-well microtiter plate.

Three days postinfection, wells were inspected and only wells in which cells were enlarged were selected. 0.75 ml media from each selected well was harvested and viral DNA was isolated according to the protocol in the instruction manual for transfection (Invitrogen) with slight modification. Briefly, the collected samples were centrifuged at room temperature to pellet the cell debris. The supernatant was transferred to a fresh tube and added to it was cold (4 °C) 20 % polyethylene glycol 8000 (PEG 8000) in 1 M NaCl. The tubes were inverted to mix and allowed to stand at room temperature for 30 minutes.

The tubes were centrifuged with maximum speed at room temperature. All medium was removed and 0.1 ml of sterile water was added to the pellet. After vortex and brief centrifugation, Proteinase K (10 mg/ml) was added, incubated at 50 °C for 1 hour, and was extracted with an equal volume of phenol-chloroform (1:1). DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2 volume of 100 % ethanol. After centrifugation with maximum speed at 4 °C, the pellet was washed with 70 % ethanol and was resuspend in 0.01 ml of sterile water.

Polyhedrin forward primer: 5'-TTTACTGTTTTCGTAACAGTTTTG-3' and polyhedrin reverse primer: 5'-CAACAACGCACAGAAATCTAGC-3' were used to perform following PCR reactions: one cycle of 94 °C for 2 minutes, 30 cycles of 94 °C for 15 seconds, 60 for 1 minute, 72 °C for 2 minutes 3 seconds and one cycle of 72 °C for

7 minutes. PCR products were analyzed on a 1% agarose gel and only pure recombinant viruses that contained the expected DNA fragment was selected as P1 stock.

1. 7 Preparation of high-titer viral stock and expression of rAed a 4 protein

After 8 days of using recombinant virus P1 stock to infect Sf9 cell, P2 stock was obtained. Two hundred and fifty ml Sf9 cell in 500 ml spinner flask was infected with P2 stock, after 95-100% of the cells were lysed , the supernatant was harvested as high titer P3 virus stock.

The P3 viral stock was diluted to 10^{-6}, 10^{-7} and 10^{-8} and a plaque assay was performed with the three dilutions using the method described above. Plaques on plates infected with same dilution were counted and their average values were used to calculate virus titer.

To increase the yield of expressed products, high five cells were used which were grown in Express Five SFM serum-free medium in spinner culture maintained at 27 °C with spin of 100 rpm. Two hundred fifty ml of suspension culture was infected with high titer rAed a 4 recombinant baculovirus P 3 stock in a 500 ml spinner flask. Cells were harvested by centrifuging at 5,000 *rpm* for 30 minutes at the time of 3 days postinfection. The supernatant was stored at -30 °C until purification could take place.

1. 8 Hydrolase assay

Aed a 4 is an α-glucosidase. The activity of α-glucosidase can be determined by the release of p-nitrophenolate from p-nitrophenyl-α-D-glucopyranoside (pNPG). The hydrolase assay was conducted at room temperature. Fifty µl of 0.1 M Na phosphate

buffer, pH 7.0, containing 10 μM pNPG, was added into each well of 96 well-flat bottom microplate. Then, 10 μl of test samples or negative controls were added and mixed. About 20 minutes later, the plate was read at 410 nm, using a POWERWAVE$_X$ Microplate Scanning Spectrophotometer (Bio-TEK Instruments, Inc. Highland Park, VT, USA)

1. 9 Purification of rAed a 4

Purification of the rAed a 4 was performed by a combination of anion exchange and cation exchange chromatography. The expressed rAed a 4 supernatant was concentrated about 10 times using a 200 ml Amicon stir cell, equipped with a Diaflo membrane (MW cut off 30,000) and then dialyzed against sodium phosphate buffer (20 mM, pH 8.0) at 4 °C. After centrifugation at 5,000 rpm for 30 minutes at 4 °C to remove any precipitation, the sample was loaded onto a DEAE-Sephacel column equilibrated with 10 times column volumes of the sodium phosphate buffer. The column was washed with 5 times column volume of the sodium phosphate buffer. The bound proteins were eluted with 10 times column volume of linear gradient NaCl from 0 to 0.65 M in sodium phosphate buffer with a flow rate of 1 ml/minute. One ml fractions were collected and assayed for protein concentration, using the Bio-Rad protein assay, and for glucosidase function using the hydrolase assay.

The fractions containing rAed a 4 (positive in the function assay) were pooled, dialyzed against sodium phosphate buffer (20 mM, pH 6.5), and centrifuged at 5,000 rpm for 30 minutes at 4 °C. The sample was then loaded onto a CM-Sepharose (fast flow) column equilibrated with sodium phosphate buffer. After washing with the buffer, the

bound proteins were eluted with a linear gradient NaCl from 0 to 0.65 M with a flow rate of 1 ml/ minute. One ml fractions were collected and assayed for protein concentration and glucosidase function as described previously. The fractions containing rAed a 4 were pooled and concentrated using a Labscale™ TFF System and Pellicon XL Filter membranes (BIOMAX TM 30 K). After measuring the protein concentration, the fraction was aliquoted and stored at -70 °C.

1. 10 Enzyme-Linked Immunosorbent Assay (ELISA) and ELISA inhibition test

An ELISA was developed to detect rAed a 4-specific IgE and IgG in human sera. Optimal conditions were chosen by checkerbroad titration. Microtiter plates were coated overnight at 4 °C with 0.2 μg/well of purified rAed a 4 in 0.05 M bicarbonate buffer, pH 9.6. The plates were washed three times with PBS-T and blocked with 2% BSA in PBS-T for 30 minutes at room temperature. 100 μl of the test serum (1:20 diluted for IgE and 1:200 diluted for IgG) were added to each well. The plates were incubated overnight at 4 ^{0}C. After washing, the bound specific IgE was detected by adding 100 μl of 1:2,000 diluted goat anti-human IgE, and the bound specific IgG was detected by adding 100 μl of 1:5,000 diluted monoclonal anti-human IgG. After incubation at 37 ^{0}C for 1.5 hour and a further three time washing, 100 μl of 1:3,000 diluted alkaline phosphatase conjugated rabbit anti-goat IgG was added for detecting IgE and 100 μl of 1:5,000 diluted alkaline phosphatase conjugated goat anti-mouse IgG was added for detecting IgG. The plates were incubated at 37 ^{0}C for 1.5 hour. Following another three time washing, 100 μl of the enzyme substrate solution (1 mg/ml of p-nitrophenylphosphate in diethanolamine buffer,

pH 9.8) was added. Optical absorbency at 410 nm was read using a POWERWAVE$_X$ Microplate Scanning Spectrophotometer (Bio-TEK Instruments, Inc. Highland Park, VT, USA). Each sample was tested in duplicate.

An inhibition test was performed to examine the inhibition of the binding of rAed a 4 to human IgE by the native allergen. Due to the difficulty in obtaining the large amount of mosquito saliva required to perform inhibition test, an *Aedes aegypti* head and thorax extract was used. The mosquito head and thorax extract was 10-fold diluted. Each dilution was mixed with an equal volume of 1:20 diluted mosquito-allergic serum and incubated at 4 °C for overnight. Incubation of PBS-T with the diluted serum served as a positive control. These incubations were then measured for rAed a 4-specific IgE by ELISA as described in ELISA.

1. 11 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a discontinuous system according to Laemmli [136] using a Bio-Rad mini slab gel apparatus to examine the expression or the purity of the recombinant proteins. Samples were prepared in SDS-PAGE loading buffer (0.0625 M Tris, pH 6.8, 2.5% SDS, 6% glycerol, 5% β - mercaptoethanol, 0.5% bromophenol blue) by boiling for 10 minutes, then loaded onto 10% or 12% polyacrylamise gel and separated by electrophoresis at 110 V for 2 hours, followed by staining with 0.25% Coomassie brilliant blue in 10% acetic acid and 45% methanol for 1 hour. Destaining in 30% methanol and 10% acetic acid solution and drying with BioDesignGelWrap™ (BioDesign Inc. New York, USA) at room temperature. Molecular weights of the

components were estimated with reference to the mobilities of prestained or natural unstained protein standards (Bio-Rad Laboratories, Mississauga, ON, Canada).

1. 12 Western blot

For the study of the binding of rAed a 4 or natural Aed a 4 to human IgE, electrophoretic transfer of proteins from gels to nitrocellulose membranes were performed at 140 V for 1.5 hour at 4 °C in a Trans-Blot cell (Bio-Rad). The transferred nitrocellulose membranes were blocked for 2 hours at room temperature with 5% BSA in TBST buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 0.1% Tween-20). The membranes were incubated for overnight at 4 °C with 1: 25 diluted pooled mosquito allergic sera. After three washes with TBST buffer, the membranes were incubated with 1:8,000 diluted monoclonal anti-human IgE for 1.5 hours at room temperature. After three washes, the membrane was incubated with 1:5,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG in TBST buffer for 1.5 hour at room temperature. After another three washes with TBST buffer, the membranes were incubated with the substrate, 1 ml BCIP [5-bromo-4-chloro-3indoylphosphate p-toluidine salt, 15 mg dissolved in 1 ml of N,N-Dimethylformamide (DMF)] and 1 ml NBT (p-nitro blue tetrazolium chloride, 30 mg dissolved in 1 ml 70% DMF) in 100 ml of color development solution (0.1 M Tris, 0.5 mM $MgCl_2$, pH 9.5) for 30 to 60 minutes. Prestained SDS-PAGE standards (Bio-Rad) were used to determine the relative molecular weights of the electrophoresed components.

1. 13 Electrophoresis of PCR products and digested DNA fragments on agarose gel

PCR products or DNA fragments digested by endonucleases were assessed by electrophoresis on agarose gel. Agarose gel at 1% was prepared by dissolving 1 g agarose in 100 ml TAE buffer (0.04 M Tris. and 0.001 M EDTA, Ph 8.0) using microwave, followed by adding ethidium bromide to a final concentration of 0.5 μg/ml, then pouring it onto a gel tray. After the gel solidified, the samples mixed in DNA loading buffer (10% glycerol, 7% sucrose, 0.025% bromophenol blue) were loaded into the wells. The electrophoresis was performed at 100 v for 40 minutes. After electrophoresis, gels were photographed under UV light.

1. 14 Production of mouse anti-rAed a 4

Four 8 week old female BALB/c mice were each immunized subcutaneously with 10 μg of purified rAed a 4 emulsified in complete Freund's adjuvant. This was repeated twice at 2 week intervals using incomplete Freund's adjuvant. Seven days after the last booster injection, the mice were bled and the sera were tested using an ELISA in which purified rAed a 4 was used as a capture antigen as described above. The four mice were sacrificed and sera from mice exhibiting a high titer were pooled and stored at -70 °C.

2. AFXa

2. 1 Construction of recombinant of recombinant baculovirus transfer vector pVL1393/AFXa1 and generation of recombinant virus

The pBlueScriptIISK(-) plasmid containing AFXa cDNA, cloned as an EcoRI fragment was excised with EcoRI and DNA fragments were separated on 1% agarose gel. The 1.8 kb AFXa cDNA fragment was eluted with the Mini spin kit. The purified cDNA was ligased with EcoRI digested pVL1393 transfer vector (Figure 2). Top10 competent *E. coli* was transformed with the ligation mixture. Single colonies were randomly picked up and miniprep plasmid DNAs were prepared. Plasmid DNAs containing the cDNA fragment were cut with EcoRV to identify insertion direction. Correct and opposite insertion direction plasmid DNAs were used for AFXa cDNA 5'-end and 3'-end sequence with Polyhedrin forward primer. We compared the two sequences with the AFXa cDNA sequence which was deposited into Genbank to see whether the 5'- and 3'-end of pVL1393/AFXa0 was in the open reading frame. The missing ATGTAT at the 5'-end of the pVl1393/AFXa0 was generated by nested gene amplification of part of pVL1393/AFXa0 using the primers 5'-CTCGGATCCATGTATCTGAAGATAGTAATATTAGTCACC-3' (forward) and 5'-CCTTTCGGATTAAACCTTAAGTAC-3' (reverse). The primers were designed to contain a BamHI site in the forward primer and a Bst 98I site in the reverse primer for directional cloning. The DNA polymerase (Invitrogen) was used in gene amplification reactions as follows: one cycle of 95 °C for 5 minutes, 30 cycles of 95 °C for 30 seconds, 54 °C for 45 seconds, 72 °C for 45 seconds and one cycle of 72 °C for 7 minutes. The amplified product was purified using DNA Gel Extraction kit and was digested with BamHI and Bst 98I and ligased with BamHI and Bst 98I digested pVL1393/AFXa0. DH5α competent cell was transformed using ligation mixture. Single colonies were picked up and miniprep plasmid DNA were prepared and excised with BamHI and

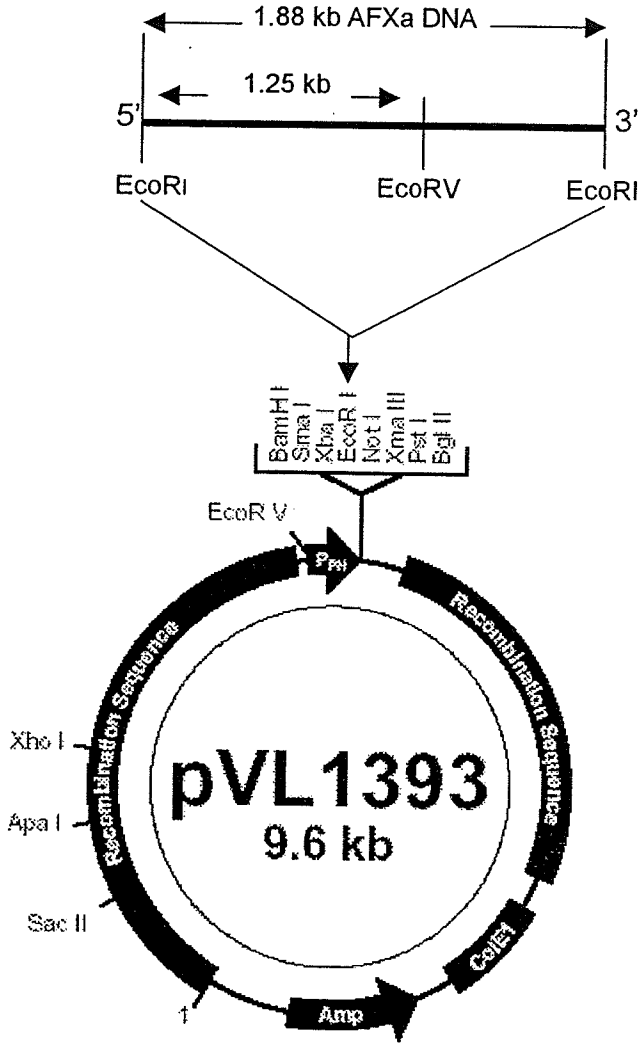


Figure 2. Construction of pVL1393/AFXa1 transfer vector
In pVL1393 vector, the fragment from EcoRV to EcoRI is 0.15 kb. If AFXa DNA was inserted in the correct direction, the EcoRV digested plasmid DNA should have a 1.4 kb fragment (0.15+1.25 = 1.4). Otherwise, it should have a 0.78 kb fragment (0.15 + 0.63 = 0.78).

Bst98I, the products were analyzed on agarose gel. DNA sequence was performed using polyhedrin forward primer to confirm the missing 6 bp was added into pVL1393/AFXa0 and the consequent plasmid was named as pVL1393/AFXa1. pVL1393/AFXa1 DNA was large scale prepared and purified using CsCl-ethidium bromide gradient centrifugation.

Cotransfection of Sf9 cells with pVL1393/AFXa1 and Bac-N-Blue ™ DNA, plaque assay, PCR analysis of recombinant virus, preparation of high titer viral stock and determination of virus titer were performed as described above.

2. 2 Cloning of AFXa cDNA without ATGTAT at 5' end into bacterial expression vector pET-24b

To express AFXa fusion protein in *E. coli*, the AFXa cDNA without the ATGTAT at the 5' end was subcloned into pET-24b vector as an EcoRI fragment to form an ORF with T7 Tag at the 5' end. After identification of inserter direction, DNA sequencing was performed to confirm that the newly cloned 5' end was in ORF. Then the plasmid was transformed into BL21 (DE3) *E. coli* competent cells and stored at -70 °C.

2. 3 Expression and partial purification of recombinant AFXa (rAFXa) protein in *E. coli*

A single BL21 colony carrying pET-24b/AFXa cDNA was picked from the LB agar plate, and was inoculated to 5 ml LB broth containing 30 μg/ml kanamycin. A single colony carrying pET-24b vector was also inoculated as control. The media was incubated at 37 °C overnight with constant shaking.

On the next day, the 5 ml medium was transferred to 500 ml LB broth containing 30 μg/ml kanamycin and continued to incubate for about 1 to 2 hours till the OD_{600} reached 0.6, then isopropyl thiogalavtopyranoside (IPTG) was added to 1 mM. One ml culture from the induced medium was taken at 1, 2, 3 and 4 hours after induction. Four hours after induction, the cells were harvested by centrifugation at 4 °C. The samples taken at 1, 2, 3 and 4 hours after the induction were analyzed by SDS-PAGE for the expression of the rAFXa protein with the induced vector as the control. The 500 ml bacterial pellet which had been induced to express rAFXa protein was suspended in PBS, sonicated (Sonics & Materials Inc. Danbury, CT, USA) for 1 minute, and centrifuged at 5,000 g for 5 minutes. 0.1 ml of the supernatant was taken for analysis to determine the purity of rAFXa by SDS-PAGE. The remaining supernatant was removed to a 15 ml tube. This step was repeated 7 times. During the first 2 times, the pellet was suspended in 10 ml of PBS; the 3rd and the 4th times pellet was suspended in 5 ml of PBS; and the last 3 times, the pellet was suspended in 2 ml of PBS.

2. 4 Production of mouse anti-rAFXa

Four 8 week old female BALB/c mice were each immunized subcutaneously with 10 μg of partially purified rAFXa (expressed in *E. coli*) emulsified in complete Freund's adjuvant. This was repeated twice, at 2 week intervals, using incomplete Freund's adjuvant. Seven days after the last booster injection, the mice were bled and the seras were tested using an ELISA in which partially purified rAFXa was used as capture antigens as described in ELISA. The four mice were sacrificed and the sera from mice that exhibited a high titer to rAFXa were pooled and stored at -70 °C.

2. 5 Time course study and expression of rAFXa in insect cells

50 ml of suspension culture of high five cells at 1.5 x 10^6 cells/ml were infected with the high titer rAFXa P 3 virus stock at multiplicity of infection (MOI) of 10 in a 125 ml spinner flask. 2 ml of culture was collected at 0 hours, 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, 144 hours and centrifuged at 6,000 rpm for 10 minutes. The supernatant was assayed for rAFXa protein expression using FXa chromogenic assay.

The expression of rAFXa was performed as described in the expression of rAed a 4, except that the infection of high five cells with P3 virus stock was at MOI of 10.

2. 6 Purification of rAFXa expressed in insect cells

(1). DEAE-Sephacel chromatography

After concentrating using a Labscale^{TM} TFF System and Pellicon XL Filter membranes (BIOMAX TM 30 K) and dialyzing against Tris buffer (20 mM Tris. pH 7.5), the culture supernatant was centrifuged at 5,000 rpm for 30 minutes at 4 °C and applied to a DEAE-Sephacel column equilibrated with 10 times column volumes of the Tris buffer at 0.5 ml/minute flow rate. The column was washed with the buffer until the baseline returned to 0. The bound proteins were eluted with a linear gradient of NaCl from 0 to 1 M in the Tris buffer. 2 ml-fractions were collected and monitored for protein

concentration using a Biological LP data view machine and for rAFXa content using the FXa chromogenic assay.

(2). CM-Sepharose chromatography with MES buffer or sodium phosphate buffer

The purification procedures were the same as described in DEAE-Sephacel chromatography. Two different buffers, 20 mM MES buffer pH 6.5 and 50 mM sodium phosphate buffer pH 7.5, were used. Since the FXa chromogenic assay can not be used when the samples are in phosphate buffer, the content of rAFXa was identified using Western blotting with mouse anti-rAFXa serum.

(3). Mono S chromatography

After concentrating and dialyzing against HEPES buffer (50 mM, pH 7.5), the culture supernatant was centrifuged (15,000 g for 40 minutes) and applied to a 1.0 ml Mono S column (kindly provided by Dr. A.A. James, University of California, Irvine.)(Pharmacia Biotech (Uppsala, Sweden) at 1.0 ml/minute flow rate. The column was washed with the same buffer until the base line returned to 0. The bound proteins were eluted with a 20 ml linear gradient of NaCl from 0 to 1 M in HEPES buffer. 1 ml-fractions were collected and monitored for protein concentration (OD 280) using a FPLC system accessory monitor (Pharmacia Biotech, Uppsala, Sweden). Fractions were assayed for the content of rAFXa, using both the FXa chromogenic assay and Western blotting with mouse anti-rAFXa serum.

2. 7 FXa chromogenic inhibition assay

All biochemical assays were performed in duplicate at 37 °C unless otherwise specified. Bovine FXa (1.1 ng) was incubated with expressed rAFXa supernatant, and cell supernatant as negative control in coagulation buffer (50 mM Tris, 250 mM NaCl, 0.1% polyethylene glycol 8000, 0.1% bovine serum albumin, pH 8.3) with 5 mM $CaCl_2$ for 15 minutes in a final volume of 0.2 ml.[126] Chromozym X (30 mM) was added and FXa activity was measured spectrophotometrically by following the release of free p-nitroaniline with a continuous change in absorbency at 410 nm using a $POWERWAVE_X$ Microplate Scanning Spectrophotometer.

30 mM chromozym X was added to each well and after 5, 10, 30, 60, and 120 minutes, the plates were read at OD 410 nm during each of the time intervals. The 30 minute reading results were used to calculate the inhibition values as follows: (mean of cell media control's OD value - mean of sample's OD value = sample's inhibition value.)

2. 8 Western blot

For rAFXa (expressed in insect cells) content, Western blot was performed as described above with some modifications. After blocking the membrane, it was incubated with 1: 5,000 diluted mouse anti-rAFXa serum, followed by incubation with 1:5,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG, then NBT and BCIP.

3. rAed a 3

3. 1 Expression of rAed a 3

Higher titer viral P3 virus stock was prepared and Plaque assays were carried out as described above. Time course study of rAed a 3 expression was performed. Fifty ml of suspension culture of high five cells at 1.5 X 10^6 cells/ml were infected with high titer rAed a 3 P 3 virus stock, at MOI of 5 in a 125 ml spinner flask. 2 ml of culture was collected at 0 hours, 24 hours, 48 hours, 72 hours, 96 hours, 120 hours and centrifuged at 6,000 rpm for 10 minutes. The supernatant was assayed for rAed a 3 expression amount with mouse anti-saliva IgG by Western blotting.

The expression of rAed a 3 was performed as described in the expression of rAed a 4 except that the infection of high five cells with P3 virus stock was at MOI of 5 and the supernatant was harvested at 4 days postinfection.

3. 2 Purification of rAed a 3

The expressed rAed a 3 supernatant was concentrated using a 200 ml Amicon stir cell equipped with a Diaflo membrane at 4 °C (cut off is 10,000 MW) and then dialyzed against Tris buffer (10 mM, pH 7.5). After centrifugation at 5,000 rpm for 30 minutes at 4 °C, to remove any precipitation, the sample was loaded onto a DEAE-Sephacel column equilibrated with 10 times column volumes of the same buffer. The column was washed with the buffer until the base line returned to 0, and the bound proteins were eluted with a linear gradient of NaCl from 0 to 0.6 M in the same buffer with a flow rate of 1 ml/minute. One ml fractions were collected and assayed for protein concentration using

the Bio-Rad protein assay. For rAed a 3 content, an ELISA was performed by coating 100 µl (1:5 diluted of each fraction) per well in 96 well microtiter plate. After washing and blocking, the plate was incubated with mouse anti-rAed a 3 serum, other steps were performed as described previously. Western blot was also performed to further confirm which fraction contained rAed a 3 using mouse anti-saliva serum . The fractions containing rAed a 3 were pooled, further concentrated using a Labscale™ TFF System, Pellicon XL Filter membranes: BIOMAX TM 10 K according to the manufacture's instructions, then aliquoted and stored at -70 °C.

3. 3 ELISA and ELISA inhibition tests

An ELISA was developed to detect rAed a 3-specific IgE and IgG in human sera. Optimal conditions were chosen by checkerbroad titration. Microtiter plates were coated overnight at 4 °C with 0.05 µg/well of purified rAed a 3 in 0.05 M bicarbonate buffer, pH 9.6. Other steps were performed as described in ELISA for rAed a 4.

An inhibition test was performed to examine the inhibiting of the binding of rAed a 3 to human IgE by the native Aed a 3. It was executed as described previously in the ELISA inhibition test for rAed a 4.

3. 4 Western blot

Western blots were performed as described above. For immunoblotting with mouse anti-saliva serum or mouse anti-rAed a 3 serum, the membranes were incubated overnight at 4 °C with mouse anti-saliva sera or mouse anti-rAed a 3-his fusion sera

diluted 1:5,000 in TBST Buffer, then incubated with 1:5,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG, and finally incubated with NBT and BCIP.

For the study of the binding of rAed a 3 to human IgE. The blocked membranes were incubated with 1:25 diluted human sera at 4 °C overnight and then incubated with monoclonal anti-human IgE (1:8,000) for 1.5 hour at room temperature followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (1:5,000 diluted). The membranes were finally incubated with NBT and BCIP.

Statistical analysis

Analysis of the data was performed using GraphPad Prism software. Unpaired t tests were used between group comparisons of IgE and IgG values. The chi square test was used to compare the positive percentages between groups.

Result

1. rAed a 4

1. 1 Construction of recombinant baculovirus transfer vector pVL1393/Aed a 4 and generation of recombinant virus

Thirteen single colonies were randomly picked. Miniprep DNAs were prepared, and digested with EcoRI. The digested products were analyzed on 1% agarose gel. Four colonies had the cDNA inserted (Figure 3). The four samples of DNAs were digested with EcoRV and the digested products were further analyzed on 1% agarose gel. Two colonies had the insertion correctly and the other two had the insertion in the opposite direction (Figure 4). Plasmid DNAs with correct and opposite insertions were used for Aed a 4 cDNA 5'-end and 3'-end sequencing with Polyhedrin forward primer, respectively. After being compared, the two sequences with the Aed a 4 cDNA sequence which was deposited into Genbank, showed that the 5'- and 3'-end of pVL1393/Aed a 4 were in ORF. The pVL1393/Aed a 4 DNA was prepared on a large scale, purified using CsCl-ethidium bromide gradient centrifugation and cotransfected into Sf9 cells with Bac-N-Blue ™ DNA.

Plaque assay was performed after cotransfection. Fourty four putative recombinant plaques were picked and viral DNAs were prepared. Three pure recombinant Aed a 4 virus plaques were confirmed by PCR analysis (Figure 5).

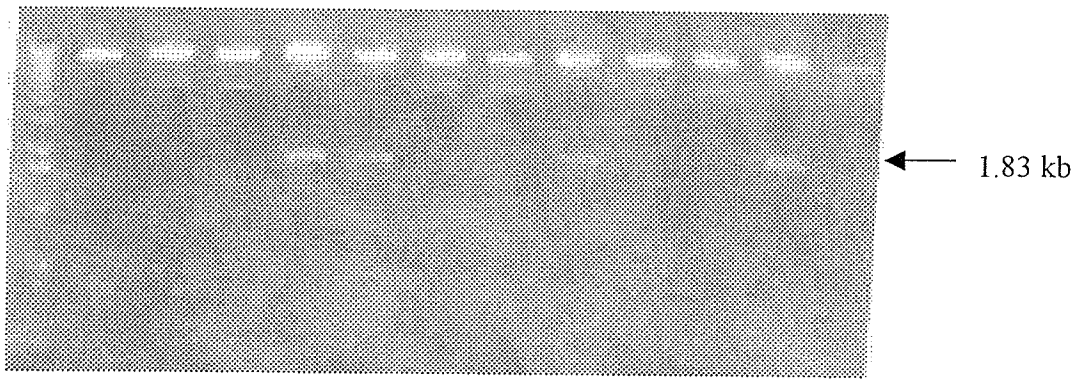


Figure 3. Selection of rAed a 4 cDNA inserter in pVL1393 transfer vector.

After transformation, thirteen single colonies were randomly picked up and miniprep DNAs were prepared. Five μl of DNA from each sample were digested with EcoRI and reaction products were separated on 1% agarose gel as described in the Methods. Four colonies had a 1.83 kb DNA inserter band.

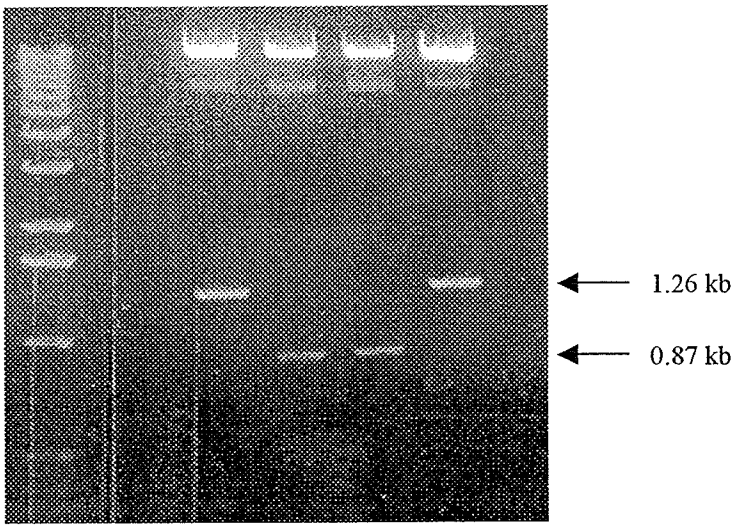


Figure 4. Identification of inserting direction of Aed a 4 cDNA in pVL1393 vector.

The four colonies containing the Aed a 4 cDNA were digested with EcoRV and the reaction products were separated on 1% agarose gel as described in the methods. Two colonies had a 0.87 kb DNA fragment (correct insertion) and other two had a 1.26 kb fragment (opposite insertion).

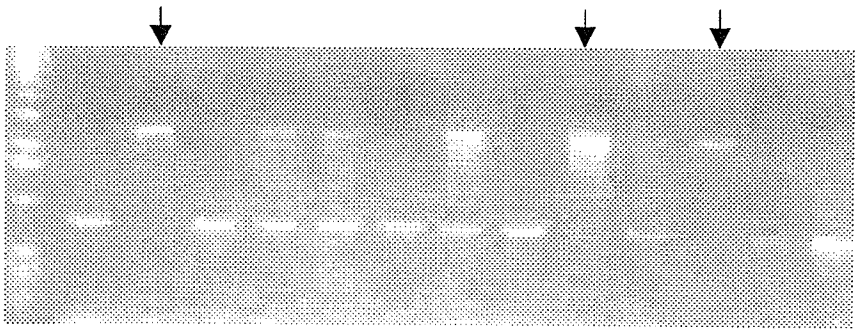


Figure 5. Analysis of PCR products for recombinant Aed a 4 virus. Fourty-four putative recombinant viral DNAs were prepared as described in the Methods. PCR reactions were performed and twenty μl of PCR product from each sample was analyzed on 1% agarose gel. The three arrows indicate the three pure recombinant virus plaques with the expected DNA bands.

1. 2 Generation of higher titer rAed a 4 P3 stock and expression of rAed a 4 protein

The expression of rAed a 4 protein in the cell culture supernatant by 3 plaques of rAed a 4 virus was proven by hydrolase assay. About 1,200 ml high titer rAed a 4 P3 virus stock was prepared. 250 ml of high five cells at a density of 1.5 X 10^6 cells/ml in 500 ml spinner flasks were infected with 50 ml of P3 stock. The supernatants of infected high-five cells were harvested three days after infection and stored at -30 °C. A total of 4,100 ml of supernatant was harvested.

1. 3 Purification of rAed a 4

The 4,100 ml of the supernatant containing rAed a 4 was concentrated to 400 ml using a 200 ml Amicon stir cell, equipped with a Diaflo membrane at 4 °C (cut off is 30,000 MW). rAed a 4 was purified from the concentrated supernatant by a combination of anion-exchange and cation-exchange chromatography. The first step was anion exchange using DEAE-Sephacel. rAed a 4 was adsorbed by the matrix and was eluted at 0.3 to 0.4 M NaCl with other proteins (figure 6). After this step, the pooled fraction containing rAed a 4 was still contaminated with some other proteins. Therefore, the second step, CM-Sepharose column (fast flow, kindly provided by Dr. Gilbert Arthur, Department of Biochemistry, University of Manitoba) was employed to further purify the rAed a 4. As shown in Figure 7 and Figure 8, almost all the contaminated proteins were excluded from the purified fraction, the α-glucosidase functional peak corresponded well with the protein peak. The rAed a 4 was eluted at 0.3 to 0.4 M NaCl. The fractions

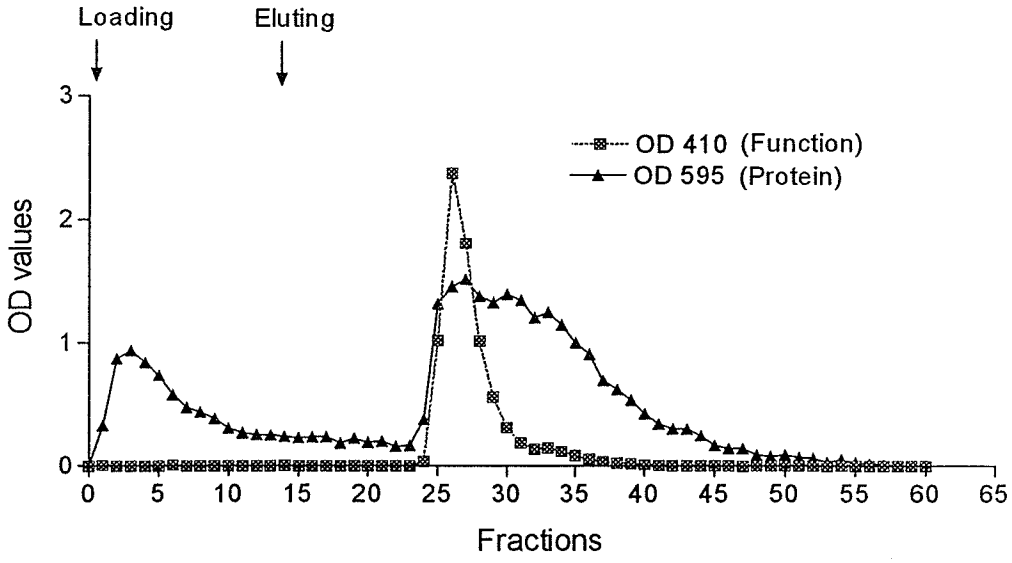


Figure 6. Step I purification of rAed a 4 (DEAE-Sephacel).
The concentrated rAed a 4 supernatant was dialyzed against sodium phosphate buffer (20 mM sodium phosphate, pH 8.0) and then loaded onto a DEAE-Sephacel column equilibrated with the same buffer. After washing the column with the same buffer, the bound proteins were eluted with a linear gradient of NaCl from 0 to 0.65 M in 20 mM sodium phosphate pH 8.0. Fractions were collected and assayed for proteins concentration using the Bio-Rad protein assay (OD 595 nm was read) and for rAed a 4 content using hydrolase assay (OD 410 nm was read).

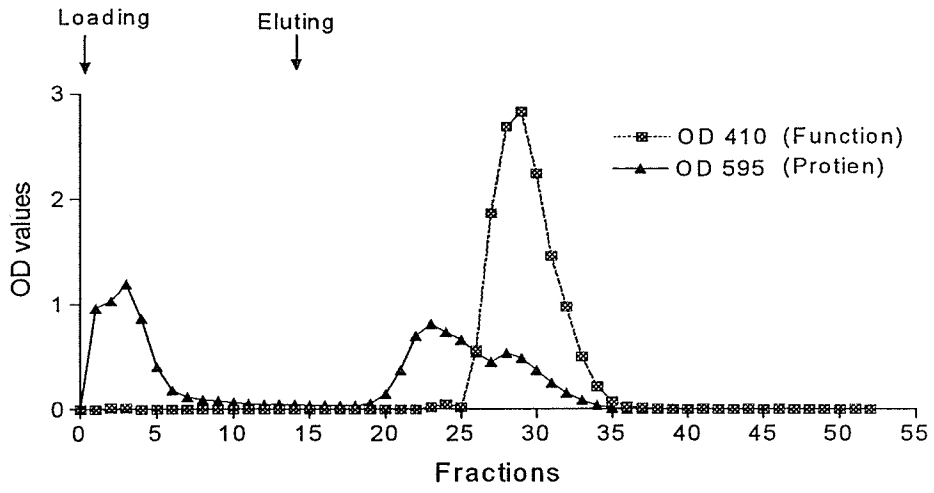


Figure 7. Step II purification of rAed a 4 (CM-Sepharose).
After the first step of purification, the fractions containing rAed a 4 were pooled and dialyzed against sodium phosphate buffer (20 mM sodium phosphate, pH 6.5) and then loaded onto a CM-Sepharose column equilibrated with the same buffer. After washing the column with the same buffer, the bound proteins were eluted with a linear gradient of NaCl from 0 to 0.65 M in 20 mM sodium phosphate pH 6.5. Fractions were collected and assayed for proteins concentration using the Bio-Rad protein assay (OD 595 nm was read) and for rAed a 4 content using hydrolase assay (OD 410 nm was read).

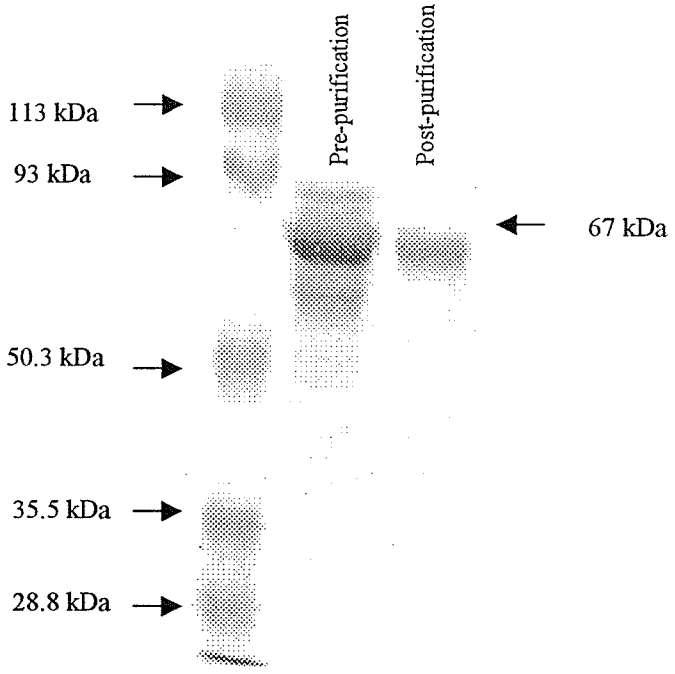


Figure 8. SDS-PAGE analysis for the purification of rAed a 4 Proteins, from rAed a 4 supernatant (before purification) and finally purified rAed, a 4 were separated on 10% SDS-PAGE as described in Methods. The finally purified rAed a 4 only had the 67 kDa band.

containing rAed a 4 were pooled and concentrated. Sixty five mg (2.5 mg/ml) of purified rAed a 4 was obtained from 4,100 ml of the supernatant.

1. 4 ELISA and ELISA inhibition test

As shown in Figure 9, the mean levels of rAed a 4-specific IgE and IgG are significantly higher in the allergic individuals (n = 13) than the controls (n = 18) ($p < 0.007$ for IgE and $p < 0.004$ for IgG). Using the mean of the controls plus 2 SD as a cut-off level, 46% of the 13 allergic individuals had a positive rAed a 4-specific IgE, while none of the controls was positive ($p < 0.001$) (Figure 10). Although the positive percentage of rAed a 4-specific IgG was higher in allergic individuals (7.7%) than that of the controls (0%), there was no statistically significance between the two groups ($p > 0.05$) (Figure 10).

The binding of serum Aed a 4-specific IgE to recombinant Aed a 4 is successfully inhibited by the addition of mosquito head and thorax extract in a dose-dependent manner as shown in Figure 11, indicating that the recombinant Aed a 4 and the native Aed a 4 in mosquito head and thorax extract have identical allergenicity and that the rAed a 4 capture-ELISA is specific for the detection of Aed a 4-specific IgE in human serum.

1. 5 Western blot

Western blot was performed to further characterize rAed a 4. As shown in Figure 12, both natural Aed a 4 in mosquito saliva and purified rAed a 4 bind to the IgE in the pooled human serum from mosquito allergic subjects, confirming that both natural and

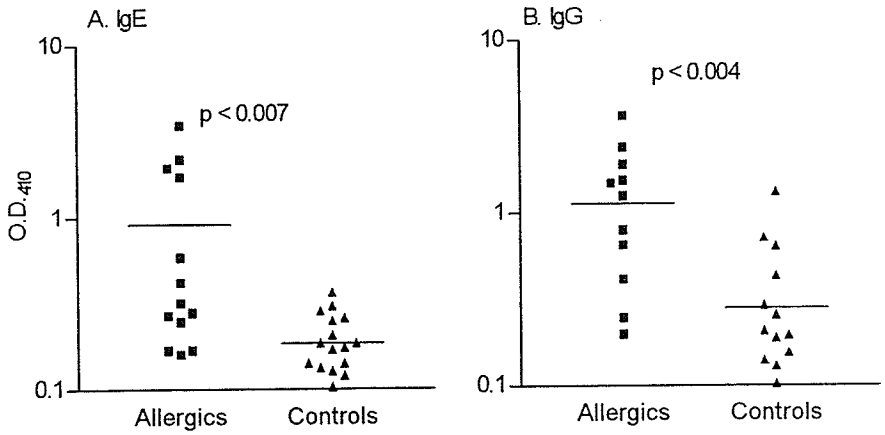


Figure 9. Binding of rAed a 4 to human IgE and IgG.
Serum rAed a 4-specific IgE and IgG levels were measured using ELISA in individuals allergic to mosquito bites (n = 13). Healthy volunteers with a negative mosquito bite test served as controls (n = 18).

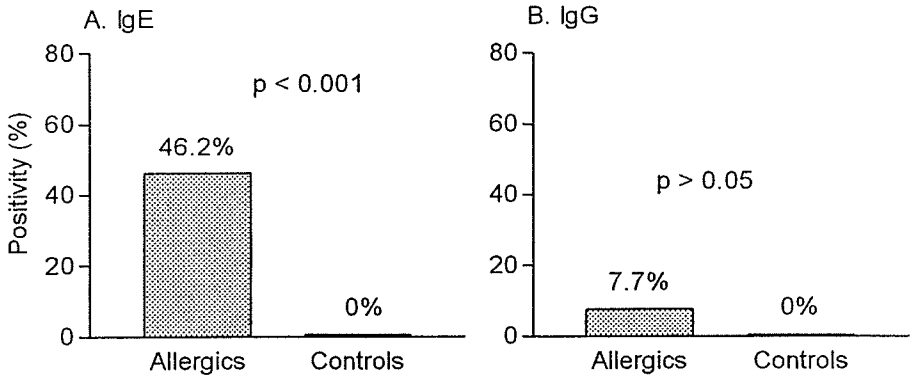


Figure 10. Positive percentage of rAed a 4-specific IgE and IgG in individuals allergic to mosquito bites (n = 13) and negative controls (n = 18). The geometric mean of the controls plus 2SD was used as a cut-off level.

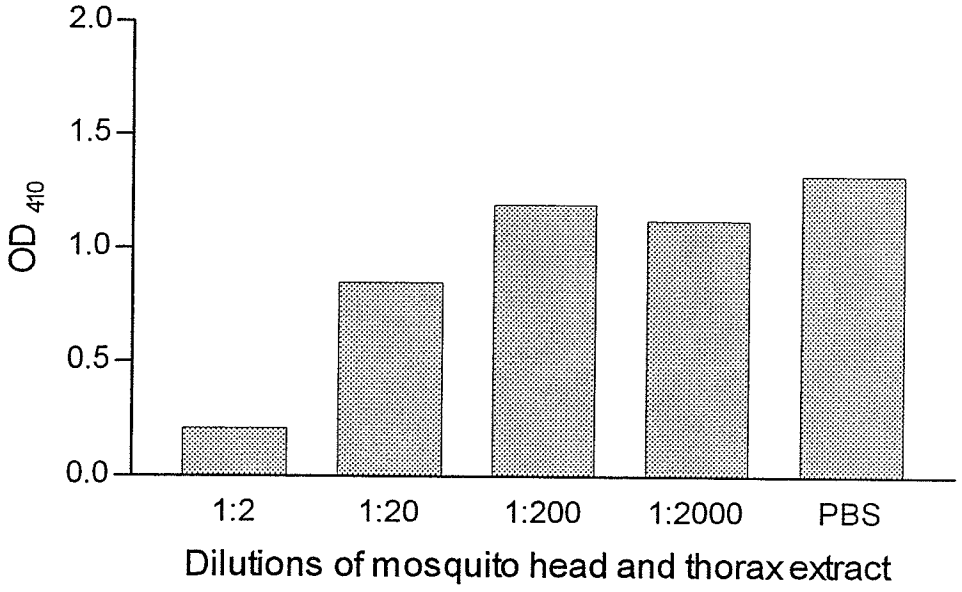


Figure 11. ELISA inhibition tests.
The mosquito head and thorax extracts were 10-fold diluted and each dilution was mixed with 1:40 diluted pooled mosquito-allergic human serum. Serum mixed with PBS buffer served as a positive control. After incubation at 4 °C overnight, the rAed a 4-specific IgE contained in the sera was measured by ELISA as described in the Methods.

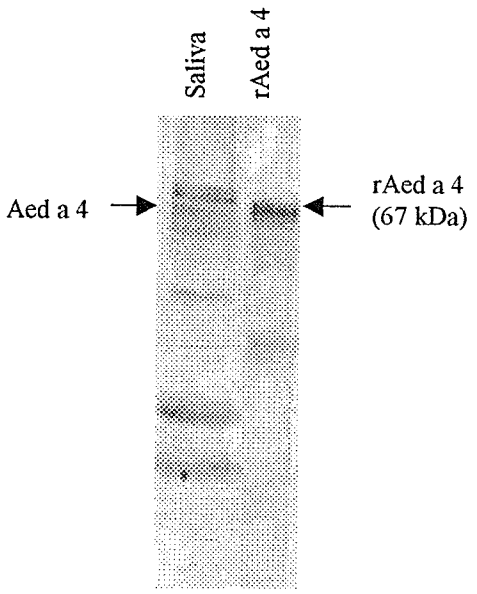


Figure 12. Western blot identification of rAed a 4
Proteins, in *Aedes aegypti* saliva and purified rAed a 4, were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was immunoblotted with a pooled mosquito allergic human serum followed by incubation with monoclonal anti-human IgE as described in Methods. Both native Aed a 4 in the saliva and rAed a 4 reacted with the IgE in the mosquito allergic human serum.

recombinant Aed a 4 have the same allergenicity. Also, recombinant Aed a 4 has the same molecular weight as natural Aed a 4 in mosquito saliva.

2. rAFXa

2. 1 Construction of recombinant baculovirus transfer vector pVL1393/AFXa1 and generation of recombinant virus

Thirty two single colonies were randomly picked up. Miniprep DNAs were prepared and digested with EcoRI. The digested products were analyzed on 1% agarose gel. Eight colonies had the insert (Figure 13) and then DNAs were digested with EcoRV. The digested products were further analyzed on 1% agarose gel. One colony had the correct insertion with the expected size of 1.4 kb DNA band and four had the opposite insertion (Figure 14). Plasmid DNAs with correct and opposite insertion were used for AFXa cDNA 5'-end and the 3'-end sequencing with Polyhedrin forward primer, respectively. After comparing the two sequences with the AFXa cDNA sequence which was deposited into Genbank, it showed that the 5'- end lacked the first 6 base pairs and could not form ORF, and I named this plasmid pVL1393/AFXa0. To express the secretory rAFXa in insect cells, forward and reverse primers were designed and PCR reactions were performed as described in the Method. The purified 260 bp PCR product was digested with BamHI and Bst 98I, and ligased with the two enzymes excised pVL1393/AFXa0. DH5α *E. coli* competent cells were transformed by the ligation mixture. Altogether, four single colonies were obtained. Miniprep DNAs from each samples were analyzed on

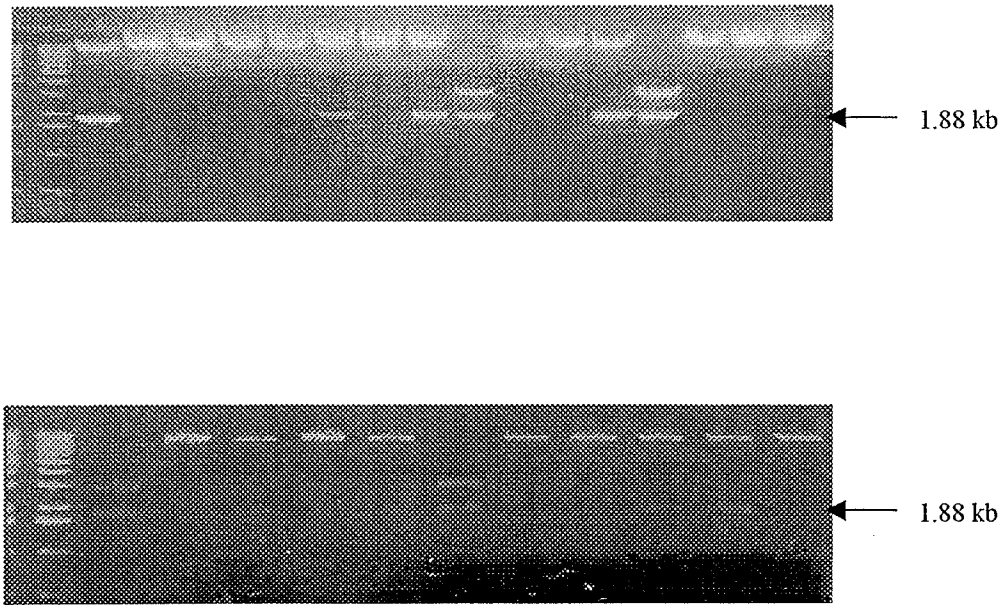


Figure 13. Selection of AFXa DNA inserter in pVL1393 transfer vector. After transformation, thirty-two single colonies were randomly picked up and miniprep DNAs were prepared. Five µl of DNA from each sample was digested with EcoRI and reaction products were analyzed on 1% agarose gel as described in the Methods. The 1.88 kb DNA inserter band was found in eight colonies.

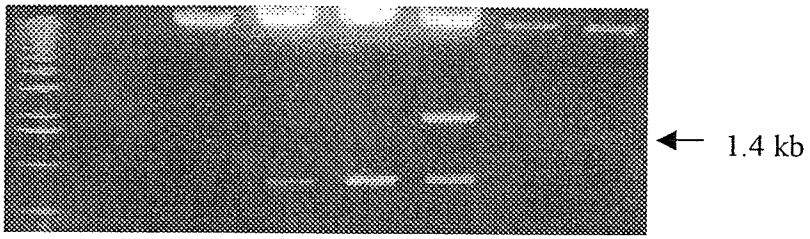


Figure 14. Identification of inserting direction of AFXa DNA in pVL1393 vector.
Eight colonies containing the AFXa cDNA were digested with EcoRV and the reaction products were separated on 1% agarose gel as described in the Methods. Only one colony had a very faint 1.4 kb DNA band indicated by an arrow, showing correct inserting direction.

agarose gel. The results showed that all the four colonies had the 260 bp inserter (Figure 15), and DNA sequencing confirmed the results. The consequent plasmid was named pVL1393/AFXa1 and was prepared on a large scale, and purified using CsCl-ethidium bromide gradient centrifugation and cotransfected into Sf9 cells with Bac-N-Blue ™ DNA.

Plaque assay was performed after cotransfection. Thirty four putative recombinant plaques were picked and viral DNAs were prepared. Five pure rAFXa virus plaques were confirmed by PCR analysis (Figure 16).

2. 2 Time course study and expression of rAFXa protein in insect cells

The expression of rAFXa protein in the cell culture supernatant by 5 plaques of rAFXa virus was confirmed by FXa chromogenic assay. 1,000 ml high titer rAFXa P3 virus stock was prepared. The expression of rAFXa was assayed by a FXa chromogenic assay. The result showed that the expression level of rAFXa peaked at 72 hours after infection with P3 virus stock at MOI of 10 and then decreased (Figure 17).

Two hundred and fifty ml of high five cell suspension at a density of 1.5 X 10^6 cells/ml in 500 ml spinner flasks was infected with rAFXa P3 virus stock at MOI of 10. The supernatant of infected high-five cells was harvested three days postinfection by centrifugation at 5,000 rpm for 30 minutes at 4 °C. 3,800 ml of the supernatant was harvested. The supernatant was concentrated to 400 ml using a Labscale™ TFF System with Pellicon XL Filter membranes (BIOMAX TM 30 K), and then stored at -30 °C.

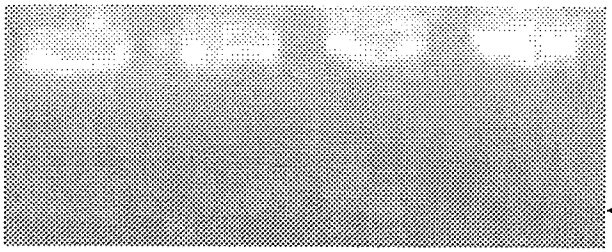


Figure 15. Selection of 260 bp DNA inserter in pVL1393/AFXa0 After transformation, four single colonies were picked up and miniprep DNAs were prepared. Ten µl of DNA from each sample was digested with BamHI and Bst 98I and reaction products were separated on 1% agarose gel as described in the Methods. All four colonies had the 260 bp band.

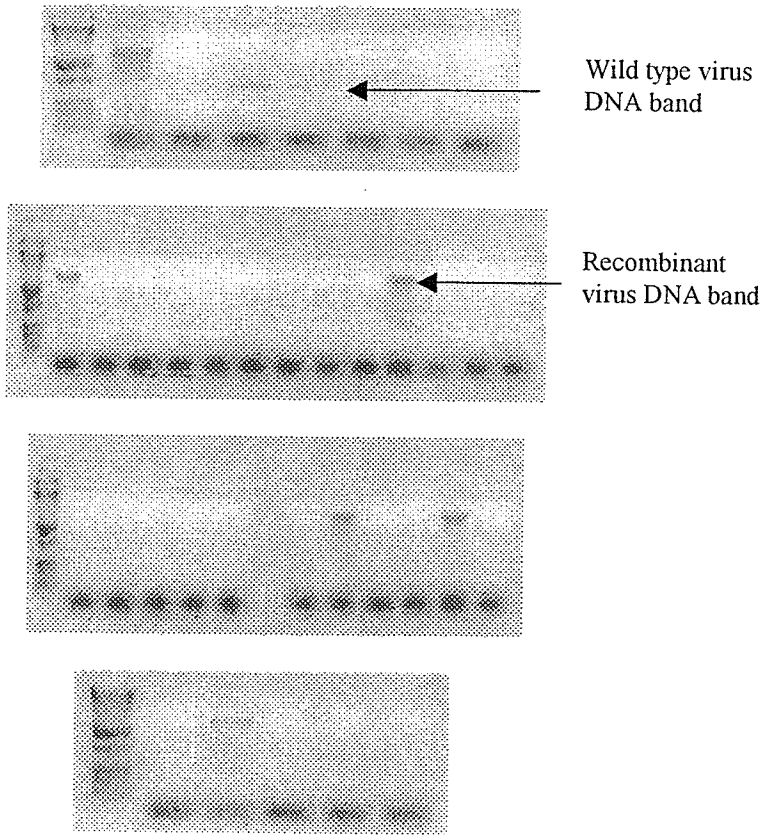


Figure 16. Analysis of PCR products for recombinant AFXa virus.

Thirty-four putative recombinant viral DNAs were prepared as described in the Methods. PCR reactions were performed and the PCR products from each sample were analyzed on 1% agarose gel. Five pure recombinant virus plaques were confirmed.

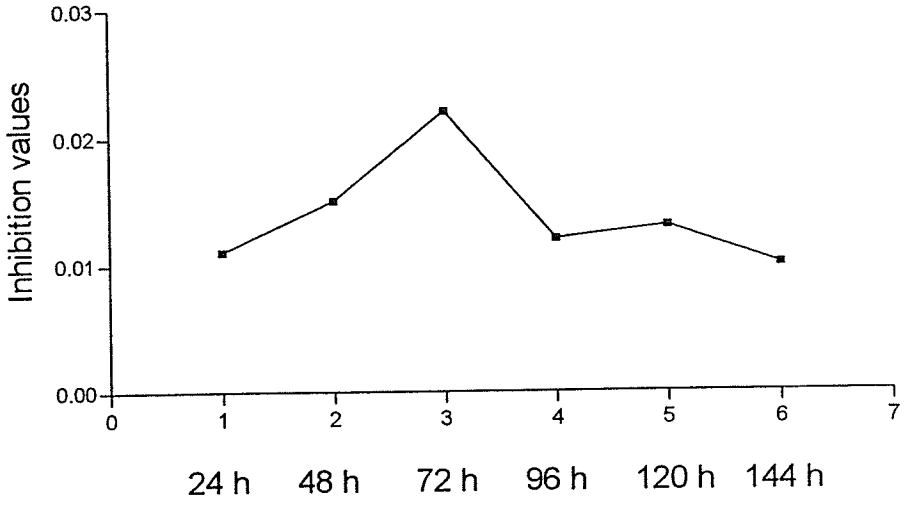


Figure 17. Time course study for the expression of rAFXa protein in insect cells.

Bovine FXa was incubated with expressed rAFXa supernatant and cell supernatant as negative control in coagulation buffer with 5 mM $CaCl_2$ for 15 minutes in a final volume of 0.2 ml. 30 mM Chromozym X was added and FXa activity was measured spectrophotometrically by following the release of free p-nitroaniline with a continuous change in absorbency at 410 nm.
Use the OD values (30 minutes after adding chromozym X) to calculate the inhibition values: sample's inhibition value = mean of cell media control's OD value - mean of sample's OD value. It showed that inhibition function of the supernatant reached maximum at 72 hours postinfection with P3 stock at MOI 10 and then decreased. The expressed rAFXa level should be same as the curve showed.

2. 3 Purification of rAFXa protein expressed in insect cells

(1). DEAE-Sephacel column

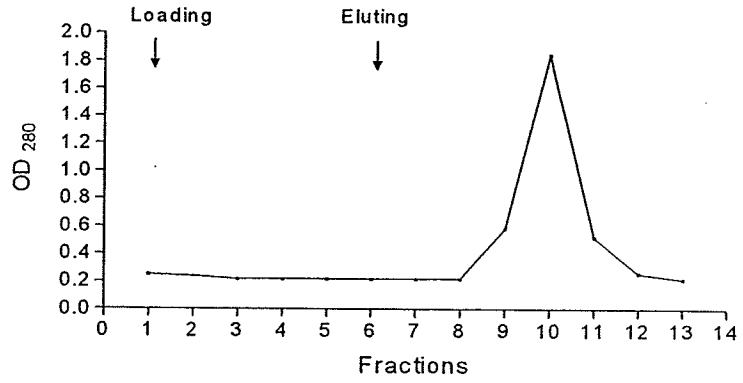
The procedures were described in the methods. 10 μl of sample from each fraction was used in a FXa chromogenic assay, to determine which fractions contained the rAFXa protein. As shown in Figure 18, all the proteins in the supernatant bind to the matrix, and are eluted together during NaCl linear gradient elution. The inhibition peak measured by the FXa chromogenic assay was paralleled with the large protein peak. Hence, rAFXa was not separated with other proteins using the DEAE-Sephacel column.

(2). CM-Sepharose chromatography with MES or sodium phosphate buffer

The procedures are described in Methods. After running the column using MES buffer, FXa chromogenic assay and Western blot were performed to determine which fraction contained the rAFXa protein. As shown in Figure 19 A, most proteins do not bind to the matrix. FXa chromogenic assay showed that the inhibition peak corresponded well with the large protein peak (Figure 19 B). Because the inhibition began to increase again, starting from fraction 7 to the end, Western blot was performed to confirm the inhibition assay results. As shown in Figure 19 C, no rAFXa protein band is found after fraction #5. Hence, the CM-Sepharose column with 20 mM MES buffer (pH 6.5) did not separate rAFXa from other proteins.

Because calcium will combine with the phosphate to form precipitate which will interfere with the FXa chromogenic assay, rAFXa protein in sodium phosphate can not be detected using the assay. Therefore, Western blot with mouse anti-rAFXa serum was performed to identify the presence of rAFXa. The results (not shown) were very similar

A.



B.

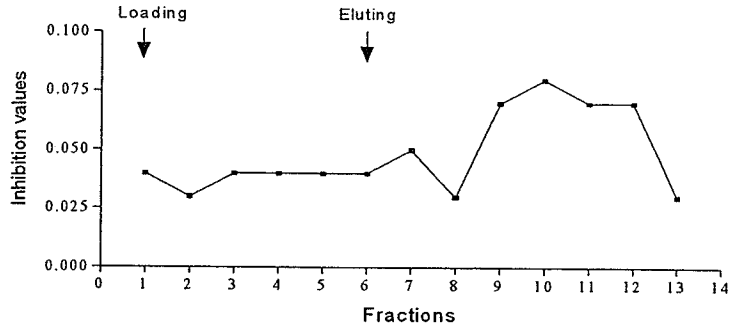


Figure 18 A. Purification of rAFXa using DEAE–Sephacel chromatography
The concentrated rAFXa supernatant was dialyzed against Tris buffer (20 mM pH 8.0), centrifuged and then loaded onto a DEAE-Sephacel column equilibrated with the same buffer. After washing the column with the same buffer, the bound proteins were eluted with a linear gradient of NaCl from 0 to 1 M in the Tris buffer. Fractions were collected and monitored for protein concentration using a Biological Data view machine (OD 280).
B. FXa chromogenic assay was performed for rAFXa content (OD 410). The inhibition peak overlapped with the protein peak. rAFXa was not purified by the DEAE-Sephacel column.

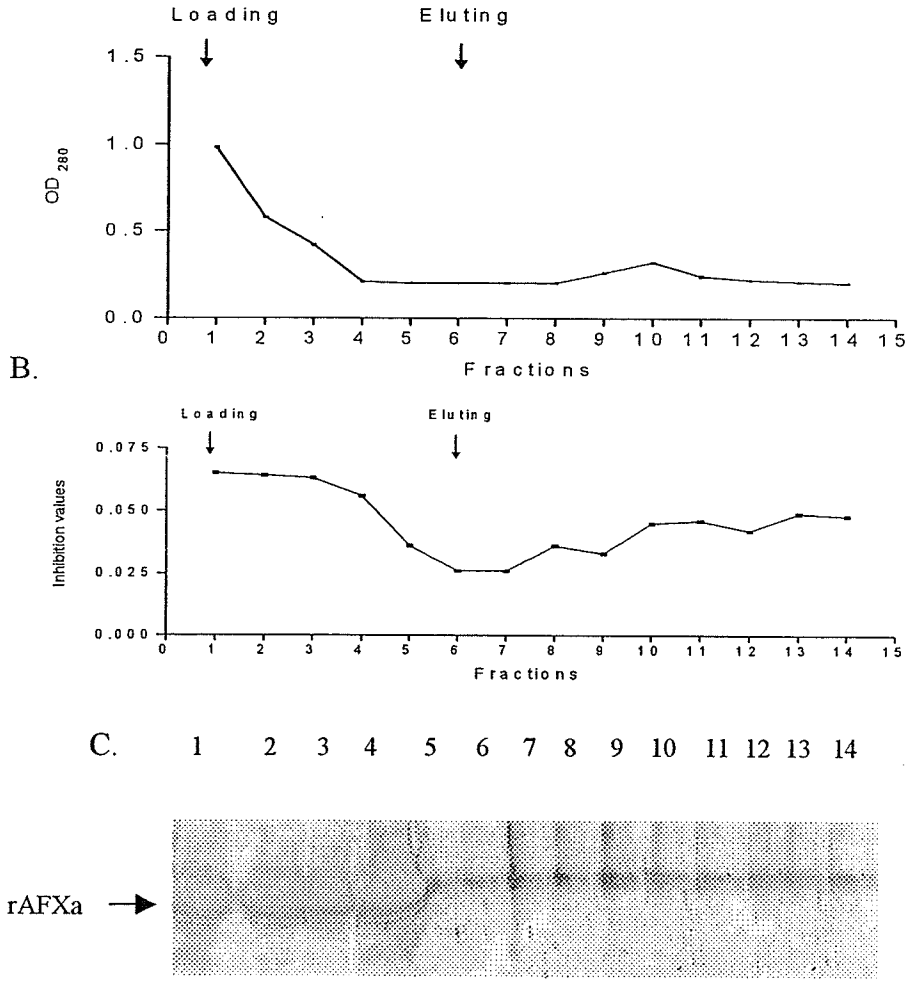


Figure 19. A. Purification of rAFXa using CM – Sepharose chromatography
The concentrated rAFXa supernatant was dialyzed against MES buffer (20 mM, pH 6.5), centrifuged, then loaded onto a CM-Sepharose column equilibrated with the same buffer. After washing the column with the same buffer, the bound proteins were eluted with a linear gradient NaCl from 0 to 1 M in the MES buffer (20 mM, pH6.5).
B. Fractions were collected and monitored for protein concentration using a Biological LP Data view machine (OD 280). FXa chromogenic assay was performed for rAFXa content (OD 410). The inhibition peak corresponded with the protein peak. rAFXa was not purified by the CM-Sepharose column.
C. Western blot was performed as described in Methods using sample from every fraction. Fractions containing rAFXa corresponded with the protein and inhibition peak, further confirming that rAFXa was not purified by CM-Sepharose column.

to those shown in Figure 19 A and C. Again, CM-Sepharose chromatography with 50 mM sodium phosphate (pH 7.5) did not separate the rAFXa protein from other proteins.

(3). Mono S column

Finally, a Mono S column was used to purify rAFXa protein. The procedures were described in detail in the Methods. As shown in Figure 20 A, most proteins do not bind to Mono S column, and almost no protein was eluted during the NaCl linear gradient elution process. Western blot analysis for rAFXa content showed that fractions 1 to 5 (the eluent) contained rAFXa protein band, and no rAFXa was detected in the eluted fractions (fractions 5 to 26) (Figure 20 B). The FXa chromogenic assay showed similar results (data not shown) to figure 19 B. Again, rAFXa protein was not separated from other proteins using Mono S chromatography.

2. 4 Expression and purification of rAFXa in *E. coli*

The detailed procedures were described in the Methods. As shown in Figure 21, 4 hours after induction with IPTG, the expression level peaked. There are extra induction bands at 1, 2, 3 and 4 hours post induction, compared with the corresponding vector controls.

Partial purification of rAFXa expressed in *E. coli* by centrifugation and sonication were performed as described in the Methods. As shown in Figure 22, after sonication and centrifugation 7 times, the supernatant showed a strong, clear rAFXa band, and most of the other proteins were removed.

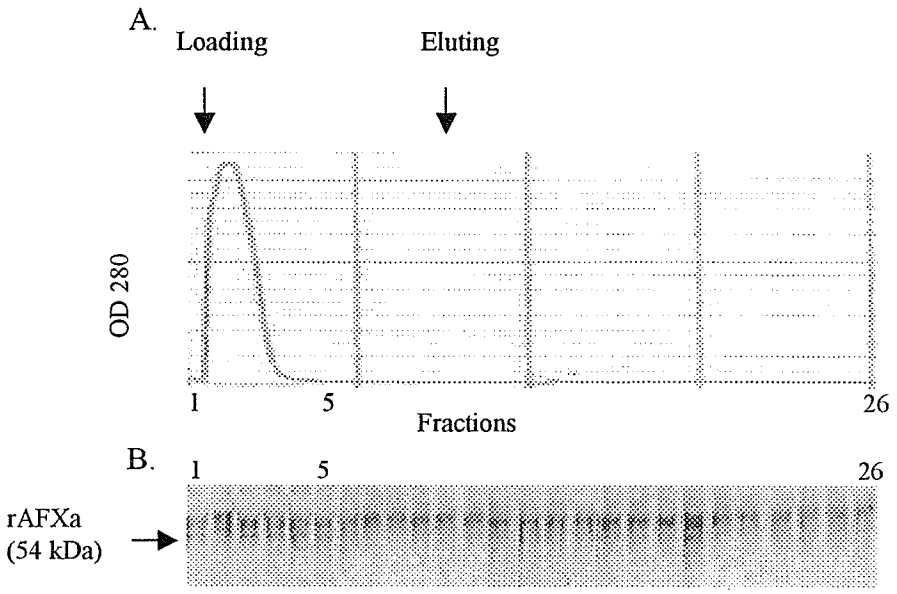


Figure 20 A. Purification of rAFXa using Mono S chromatography.
After concentrating and dialyzing against HEPES buffer (50 mM, pH 7.5), the culture supernatant was centrifuged and applied to a 1.0-ml Mono S column at 1.0 ml/minute flow rate. The column was washed with the same buffer until the base line returned to 0 and the bound proteins were eluted with a 20-ml linear gradient NaCl from 0 to 1 M in HEPES buffer (50 mM, pH 7.5). 1 ml fractions were collected and monitored for protein concentration (OD 280) using a FPLC system accessory monitor. Sample from each fraction was subjected to Western blotting with mouse anti-rAFXa sera for rAFXa content.
B. Western blot analysis.
Proteins in every fraction were separated on 10% SDS-PAGE, then transferred to nitrocellulose membrane and immunoblotted with mouse anti-mosquito saliva serum. Other steps were performed as described in Methods. The results showed that fractions (1 to 5) contained rAFXa and corresponded with the large protein peak (fraction 1 to 5). The rAFXa protein was not separated from the contaminated proteins.

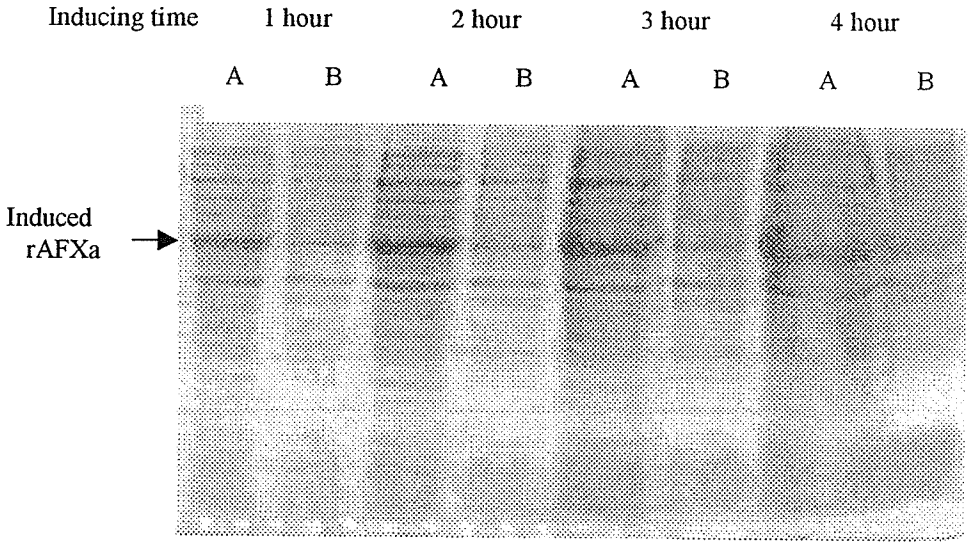


Figure 21. SDS-PAGE analysis of expression of rAFXa protein in E. coli. rAFXa protein in the bacterial lysates induced at various times (A) and control bacterial lysates (B) were subjected to SDS-PAGE analysis.

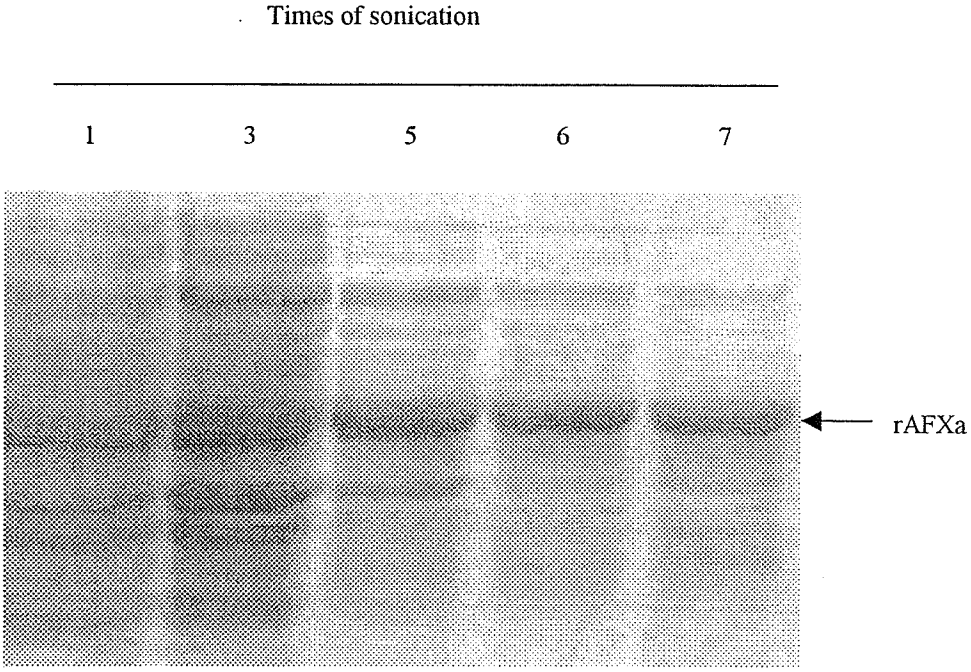


Figure 22. SDS-PAGE analysis of partially purified rAFXa protein expressed in E. coli.

After expression, the pellet was suspended in PBS, sonicated for one minute, then centrifuged at 5,000 X g for 5 minutes and the supernatant was kept. After sonication 7 times, the supernatant was analyzed by SDS-PAGE.

A DEAE-Sephacel chromatography with Tris buffer and a CM-Sepharose chromatography with sodium phosphate buffer were used to purify the rAFXa protein. Unfortunately, the results (not shown) were similar to the purification of rAFXa expressed in insect cells.

3. rAed a 3

3. 1 Expression of rAed a 3

A time course study was performed as described in the Methods. The expression amount of rAed a 3 was assayed with mouse anti-saliva IgG by immunoblotting. It showed (Figure 23) that the expression level of rAed a 3 peaked 96 hours after infection with P3 stock at MOI of 5, then the expression level decreased.

Two hundred fifty ml of high-five cells at a density of 1.5 X 10^6 cells/ml in 500 ml spinner flasks were infected with higher titer rAed a 3 virus P3 stock at MOI of 5. The supernatant of the infected high five cells were harvested 4 days postinfection by centrifugation at 5,000 rpm for 30 minutes and was stored at -30 °C. 2,400 ml supernatant was harvested totally. The supernatant was concentrated to 210 ml using a 200 ml Amicon stir cell equipped with a Diaflo membrane at 4 °C (cut off is 10,000 MW) before purification.

3. 2 Purification of rAed a 3

The purification process of rAed a 3 was performed as described in the Methods. Most proteins including rAed a 3 bound to the DEAE-Sephacel matrix in Tris buffer. The ELISA result showed two OD 410 nm peaks as shown in Figure 24 A. Samples, from the pooled second peak, the pooled first peak, and concentrated supernatant were analyzed

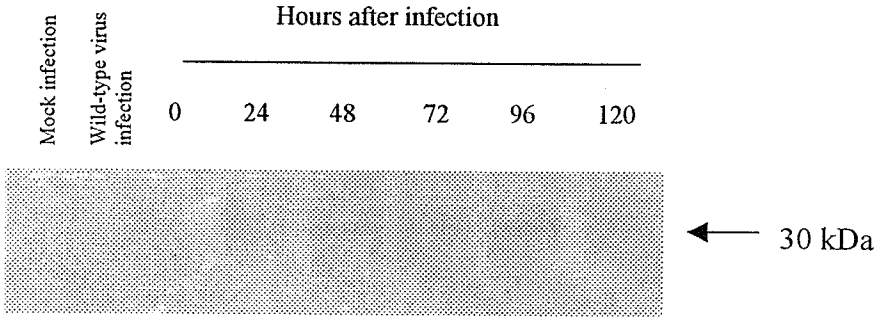


Figure 23. Time course study for the expression of rAed a 3. High Five cells were infected with high titer P3 virus stock at MOI of 5 and incubated at 27 °C. At 24 hour intervals after infection, culture medium was taken and analyzed for the presence of rAed a 3 by immunoblotting (see Methods). Cells infected with wild type virus and uninfected cells were used as negative controls.

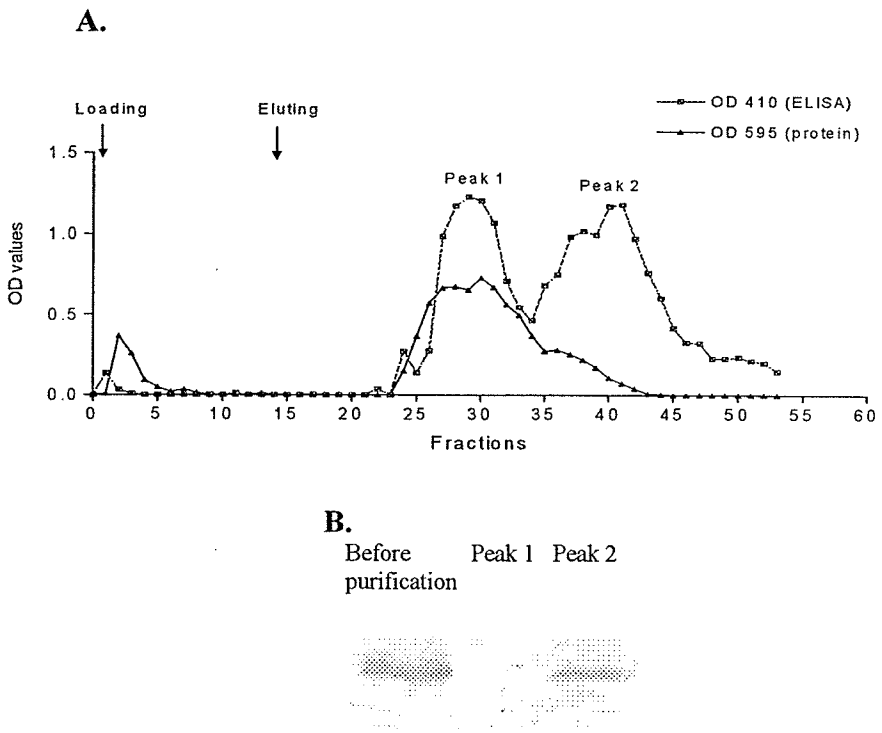


Figure 24 A. Purification of rAed a 3 (DEAE-Sephacel).
The concentrated rAed a 3 supernatant was dialyzed against Tris buffer (10 mM Tris. Buffer, pH 7.5) and then loaded onto a DEAE-Sephacel column equilibrated with Tris buffer. After washing the column with Tris buffer, the bound proteins were eluted with a linear gradient NaCl from 0 to 0.6 M in Tris buffer. Fractions were collected and assayed for proteins concentration using the Bio-Rad protein assay (OD 595 nm was read) and for rAed a 3 content using ELISA. In the ELISA, fractions (1:5) coated onto microplates were incubated with mouse anti-rAed a 3 serum (Previously prepared with an unpurified rAed a 3 fraction in our laboratory) followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG. Since the ELISA results showed two peaks after the gradient elution, Western blot was performed to see which peak contains rAed a 3.
B. Western blot analysis.
Proteins in the concentrated supernatant, peak 1 and peak 2, were separated on 12% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with mouse anti-mosquito saliva serum. Other steps were performed as described in Methods. The results showed that peak 2, not peak 1, contained rAed a 3.

by Western blot. As shown in Figure 24 B, the concentrated supernatant and the pooled second peak show a specific rAed a 3 (30 kDa) band, while the pooled first peak does not. Hence, rAed a 3 was eluted in the second peak at 0.4 to 0.6 M NaCl. The pooled second peak was further concentrated to 110 μg/ml, aliquoted and stored at -70 °C. Altogether, about 2.5 mg rAed a 3 was purified.

3. 3 ELISA and ELISA inhibition tests

As shown in Figure 25, the mean levels of rAed a 3-specific IgE and IgG are significantly higher in the allergic individuals (n = 13) than the controls (n = 18) ($p < 0.0006$ for IgE and $p < 0.05$ for IgG). Using the mean of the controls plus 2 SD as a cut-off level, 46% of the 13 allergic individuals had a positive rAed a 3-specific IgE, while none of the controls was positive ($p < 0.001$) (Figure 26). The positive percentages of rAed a 4-specific IgG were zero both in allergic individuals and the controls (Figure 26).

The binding of serum Aed a 3-specific IgE to recombinant Aed a 3 is successfully inhibited by the addition of mosquito head and thorax extract in a dose-dependent manner as shown in Figure 27, indicating that the recombinant Aed a 3 and the native Aed a 3 in mosquito head and thorax extract have identical allergenicity and that the rAed a 3 capture-ELISA is specific for the detection of Aed a 3-specific IgE in human serum.

3. 4 Western blot

Western blot was performed to further characterize rAed a 3. As shown in Figure 28, both natural Aed a 3 in mosquito saliva and purified rAed a 3 bind to the IgE in the

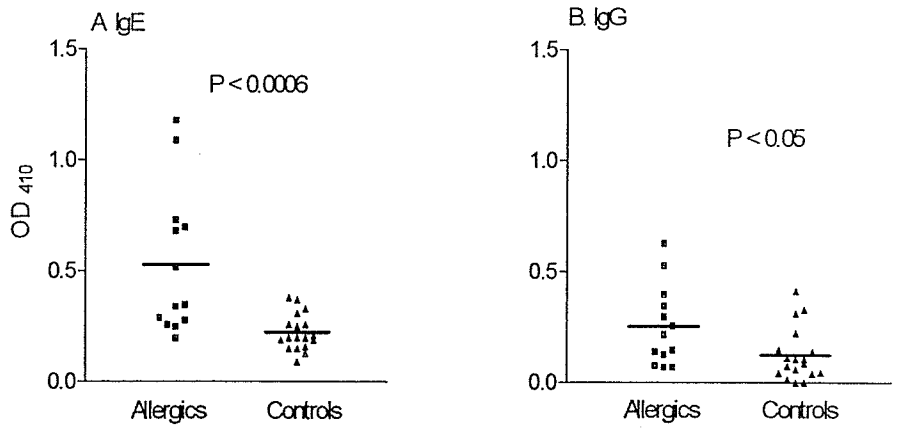


Figure 25. Binding of rAed a 3 to human IgE and IgG.

Serum rAed a 3-specific IgE and IgG levels were measured using ELISA in individuals allergic to mosquito bites (n = 13). Healthy volunteers with a negative mosquito bite test served as controls (n = 18).

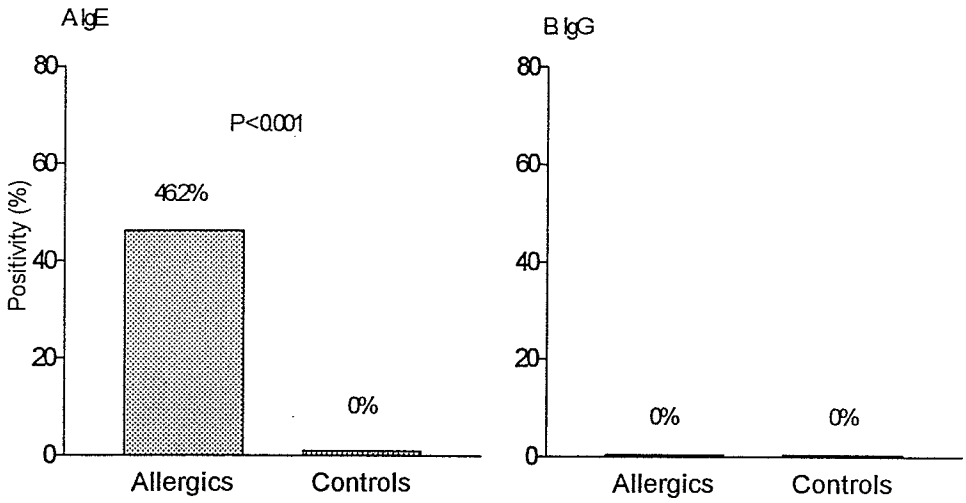


Figure 26. Positive percentage of rAed a 3-specific IgE and IgG in individuals allergic to mosquito bites (n = 13) and negative controls (n = 18). The geometric mean of the controls plus 2 SD was used as a cut-off level.

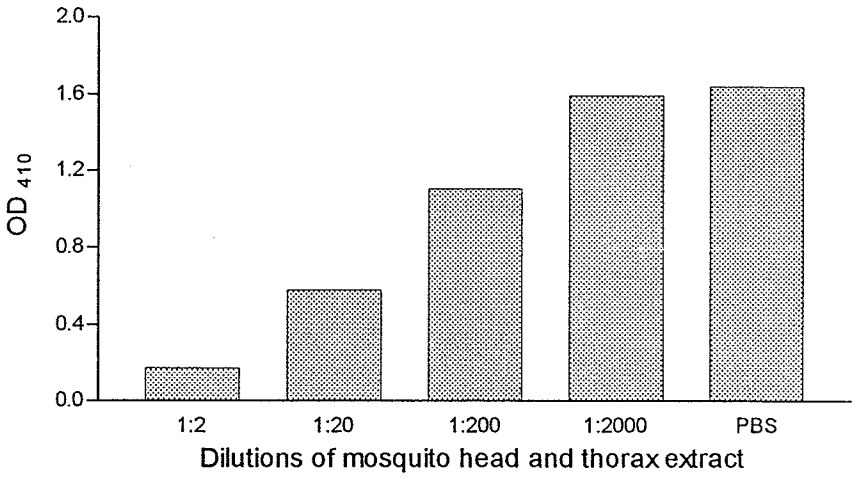


Figure 27. ELISA inhibition tests.
The mosquito head and thorax extract were 10-fold diluted and each dilution was mixed with 1:40 diluted pooled mosquito-allergic human serum. Serum mixed with PBS buffer served as a positive control. After incubation at 4 °C overnight, the rAed a 3-specific IgE contained in the sera was measured by ELISA as described in the Methods.

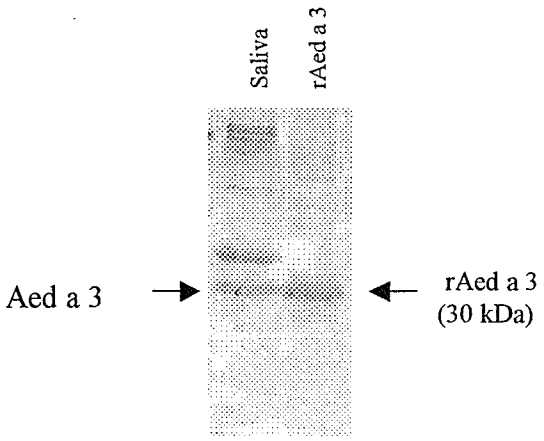


Figure 28. Western blot identification of rAed a 3.
Proteins in *Aedes aegypti* saliva and purified rAed a 3 were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was immunoblotted with a pooled mosquito allergic human serum and then incubated with monoclonal anti-human IgE as described in Methods. Both native Aed a 3 in the saliva and rAed a 3 reacted with the IgE in the mosquito-allergic human serum.

pooled human serum from mosquito allergic subjects, confirming that both natural and recombinant Aed a 3 have the same allergenicity. Also, recombinant Aed a 3 has the same molecular weight as natural Aed a 3 in mosquito saliva.

4. rAed a 3- and rAed a 4-specific IgE profiles in thirteen mosquito allergic subjects

Table 2 clearly indicates that among the 13 mosquito allergic subjects, IgE positivity to rAed a 3 and rAed a 4 are different. For example, subject #1 is positive to rAed a 3, but negative to rAed a 4; while subject #5 is positive to rAed a 4, but negative to rAed a 3; however, subject #2, #8 and #11 are positive to both rAed a 3 and rAed a 4. This demonstrates that each subject is sensitive to different allergens in the mosquito saliva. These results are similar to those reported in other kinds of recombinant allergens.[65, 137, 138] Subject #6, #7, #9 and #13 are sensitive to neither rAed a 3 nor rAed a 4, suggesting that these four subjects may be sensitive to other allergen components in the mosquito saliva. Table 2 also shows that if we use rAed a 3 or rAed a 4 separately to test the thirteen subjects, only 6 are positive (positivity is 46.2%); if we use a mixture of rAed a 3 and rAed a 4 to test these subjects, 9 should be positive (positivity is 69.2%).

The results indicate that the sensitivity of the diagnosis of mosquito allergy can be increased if multiple recombinant antigens are used, and that the mosquito saliva can be replaced with enough recombinant allergens that represent the allergen complexity of the mosquito saliva in the diagnosis of mosquito allergies.

Table 2. Positivity of thirteen diagnosed mosquito allergic subjects to rAed a 3 and rAed a 4

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12	13
rAed a 3	+	+	+	+	-	-	-	+	-	-	+	-	-
rAed a 4	-	+	-	-	+	-	-	+	-	+	+	+	-

Discussion

1. rAed a 4

In the past decade, a variety of recombinant allergens from plants, mites, molds, mammals, and insects have been expressed using various systems, both prokaryotic and eukaryotic.[139-142] These recombinant allergens, especially those expressed in the eukaryotic system, were similar to their native counterparts in structure, function, and immune reactivity. In-depth knowledge of molecular characteristics, including the three-dimensional structure and allergenic epitopes, can be more readily obtained using proteins produced by recombinant technology. The clinical use of these products may lead to improved diagnostic specificity and sensitivity[69, 141] as well as a safer and more effective immunotherapy.[143, 144]

Among prokaryotic expression systems, *E. coli* is the oldest and most commonly utilized system for producing recombinant proteins. Although a large amount of experience has been accumulated, the method of development for each allergen is still empirical and may present individual challenges.[145-147] The bacterial expression system is easy to handle, cost-effective, and can produce a high yield. However, overproduction of foreign proteins in the cytoplasm of *E. coli*, often occur simultaneously with misfolding and segregation into insoluble aggregates, such as inclusion bodies, which can lead to the production of a biologically inactive protein. Also, this system does not have the ability to perform post-translational modifications. Some recombinant allergens expressed by *E. coli* react well with IgE antibodies from allergic patients, but have little or none of the enzymatic or biological activity of the

natural protein,[142, 148] thus, allergens produced using *E. coli* are generally only used in *in vitro* assays.[149, 150]

The major advantage of an eukaryotic expression system over a bacterial one, is its ability to perform many of the post-translational modifications, including processing signal sequences, folding, disulfide bond formation, and adding lipids and carbohydrates. Proteins expressed using this system can be used in both *in vitro* and *in vivo* tests.[151, 152] A variety of eukaryotic expression systems are available; the most commonly used are yeast, baculovirus/insect cells, mammalian cells and plants. Insect cells have proven to be good hosts for the production of recombinant proteins especially insect recombinant proteins such as mosquito salivary proteins. It is an expression system with all the advantages of higher eukaryotic organisms. The viruses are usually derived from *Autographa californica* multiple capsid nucleopolyhedrovirus (AcMNPV), and the host cells are usually *Spodoptera frugiperda* ovarian cell lines Sf9 and 21 or the *Trichoplusia ni* egg-derived cell line High Five.

A number of recombinant allergens have been produced using the baculovirus/insect cell systems.[66, 151, 153-158] All of these recombinant allergens show IgE-binding capacity and behave with similar biological activities to their native counterparts. Cat allergen Fel d 1, when produced in *E. coli*, had to be refolded *in vitro* to become immunoreactive, but was fully reactive when expressed in insect cells.[148] Recombinant honey-bee venom hyaluronidase showed a comparable IgE reactivity when produced in *E. coli* and the baculovirus system. However, full enzymatic activity was found only in the recombinant protein produced in the baculovirus system.[155] The cat

flea allergen Cte f 1, produced in *E. coli* and refolded *in vitro*, only regained partial IgE-binding activity, while the one produced in insect cells, was fully IgE antibody-reactive.[157]

Aedes aegypti is a tropical and subtropical mosquito species with world-wide distribution. It is an efficient vector for the viruses that cause dengue and yellow fever.[125] Recent work[107] on a full-length cDNA library and salivary homogenates have revealed that *Aedes aegypti* salivary glands contain at least 31 secretory proteins, but, each pair of mosquito salivary glands usually have less than 3 μg of protein.[159] Identification and characterization of such compounds, are usually accomplished by tedious accumulations of over 1,000 pairs of dissected salivary glands as starting materials for fractionation and/or bioassay experiments.[107] In the saliva of *Aedes aegypti*, of the 20 peptides seen by SDS-PAGE,[123] there are at least 8 allergens.[97] Due to the technical challenges in obtaining saliva and in isolating each allergen from the saliva, production of recombinant proteins is a promising approach for investigating the role of the individual allergens in the saliva. It may eventually lead to successful mosquito allergy diagnosis and immunotherapy.

Adult male and female mosquitoes feed on sugar obtained mostly from flower nectar.[160] The sugar meal is an important source of energy for flight and survival, and contributes to the fertility of females.[161] It has been suggested that mosquito saliva helps in the ingestion of undissolved sugar.[162] The salivary glands of adult *Culex tarsalis* contain an enzyme capable of cleaving sucrose, the major oligosaccharide found in the nectar of many plants.[163] An α-glucosidase was detected in the adult salivary glands of *Aedes Aegypti* and *Aedes albopictus.* It showed the same molecular weight in

the two mosquito species.[113, 164] These data support the thesis that the α-glucosidase in the salivary glands help sugar digestion. α-glucosidase is expressed in different mosquito species and may be secreted into hosts during blood feedings.[123]

In the present study, the α-glucosidase is renamed as Aed a 4 according to allergen nomenclature,[165] rAed a 4 was expressed using a baculovirus/insect cell system. As discussed above, the systems have been used extensively for the production of large amounts of biologically-active recombinant proteins. Their ability to perform many of the post-translational modifications found in eukaryotic cells gives them a major advantage over prokaryotic expression systems, but the expressing efficiency of the system varies by approximately 1000-fold from gene to gene.[166] Most heterologous proteins are produced at amounts ranging from 1 mg to 100 mg per 10^9 cells (i.e. about one liter culture).[166, 167] The different expression vectors may also play a critical role in the expression levels of baculovirus system. In the present study, about 15 mg (Table 3) of recombinant Aed a 4 was obtained from 1 liter of culture medium by using small-scale batch fermentation in spinner cultures.

The rAed a 4 was purified from the culture medium using a combination of anion-exchange and cation-exchange chromatography that resulted in the recovery of a highly purified rAed a 4 as shown in the results. The purified rAed a 4 was shown to bind to the IgE in the sera of mosquito allergic subjects as measured by ELISA and by immunoblot analyses. The binding of rAed a 4 to the IgE could be inhibited by the addition of natural Aed a 4 present in the mosquito head and thorax extracts. The expressed recombinant Aed a 4 was able to hydrolyze the pNPG, releasing p-nitrophenolate to produce a yellow color as seen in the natural protein.[113] The rAed a 4 also possessed the same molecular

Table 3. Yield of recombinant mosquito salivary protein expressed using baculovirus/insect cell system

Recombinant mosquito allergen	Yield of purified protein (mg/L)	References
rAed a 1	10.0	Peng et al., 2001
rAed a 2	22.0	Present study
rAed a 3	1.0	Present study
rAed a 4	15.0	Present study

weight of 67 kDa, based on observations made by SDS-PAGE and immunoblotting. All of the above evidence indicates that the rAed a 4 expressed, using a baculovirus/insect cell system, has identical allergenicity and biological activity to its natural form. Besides, the mean levels of rAed a 4-specific IgE and IgG were significantly higher in allergic individuals than the controls. Using the mean of the controls plus 2 SD as a cut-off level, 46% of the 13 allergic individuals had a positive rAed a 4-specific IgE, while none of the controls were positive.

Aed a 4 may also be a cross-reactive allergen, as similar α-glucosidase was found in other mosquito species.[164] This is supported by the results of the present study, in which rAed a 4 bound to the IgE of mosquito-allergic subjects living in Manitoba where *Aedes aegypti* is not distributed.[97] All of above data support that Aed a 4 is an important allergen in the *Aedes aegypti* mosquito saliva.

2. rAFXa protein

Anticoagulants in mosquito salivary glands have been the subject of investigation since the early part of the last century.[168] Eight different mosquito species were tested for the presence of anticoagulants in salivary extracts by using *in vitro* clotting assays with plasma. The tests showed different activity profiles of anticoagulant in different mosquito species.[169] The *Aedes aegypti* anticoagulant has been shown to be a reversible, non-competitive, and non-covalent inhibitor of FXa, with no activity against thrombin and limited activity against trypsin.[132] Partial purification of this anticoagulant from salivary gland extracts yielded a basic, pI 9.7, 54 kDa protein, that exhibited the majority of the FXa-inhibitory activity.[168, 169] Recent data showed that

another cDNA, which has a domain signature of serpin (serine protease inhibitor) similar to AFXa, was sequenced from the *Aedes Aegypti* salivary cDNA library.[107] The anticoagulants in the saliva of mosquitoes help blood feedings and are secreted into hosts during the process.[123] Therefore, there is a great possibility that like other salivary allergens identified the anticoagulant in the saliva is an allergen.

The rAFXa protein was expressed using a baculovirus/insect cell system. The expressed recombinant protein could inhibit FXa activity by hydrolyzing chromozym X to release free p-nitroaniline as the natural AFXa in the mosquito saliva does.[132] It also had the same molecular weight of 54 kDa as the natural AFXa, based on the observation by immunoblotting with mouse anti-rAFXa. However, the rAFXa protein produced in *E. coli* had a lower molecular weight of about 47 kDa, the same molecular weight (47.8 kDa) as cDNA's conceptual translation product. This confirms that glycosylation accounts for the molecular weight difference among conceptual translation product, natural AFXa, and rAFXa produced in insect cells.[126]

We tried to use anion exchange, cation exchange and Mono S chromatography with different buffers to purify the rAFXa protein expressed in insect cells. However, the protein could not be separated from other proteins. We will try to purify rAFXa protein using other methods, such as chromatofocusing, in the future. The rAFXa protein expressed in *E. coli* was easily partially purified by repeated sonication and centrifugation, which may be used as an alternative approach to prepare rAFXa on a large scale. Mouse anti-rAFXa serum prepared using the partially purified fraction was able to react with the rAFXa expressed in the baculovirus system as shown by Western blot. Therefore, it is a suitable tool to use the fusing protein expressed in *E. coli* to

prepare mouse anti-target protein serum and use the serum to detect the target protein expressed in other expression system.

3. rAed a 3

rAed a 3 is a 30 kDa protein that elicited an IgE response in 32 % of 28 mosquito bite test-positive individuals and did not elicit any response in any of the 15 individuals with negative skin reactions to mosquito bites.[131] Its full-length cDNA (863 bp) encodes a putative protein of 254 amino acids, including 18 amino acids of a signal peptide. Its cDNA was cloned and sequenced in our laboratory.[128] Recent data showed that a protein sequence having high similarity to rAed a 3 was sequenced from *Aedes Aegypti* cDNA library.[107]

Using the baculovirus/insect cell system, rAed a 3 was expressed in the culture supernatant, confirming that the 18 amino acids at the 5' primer end are actually the signal peptides for the secretion of rAed a 3. rAed a 3 was purified with DEAE-Sephacel chromatography and the purity was high as shown by the Western blot results. The purified rAed a 3 bound to the serum IgE of mosquito allergic subjects as measured by ELISA and by immunoblot analyses. The binding of rAed a 3 to the IgE in the mixed mosquito-allergic serum could be inhibited by the addition of the natural Aed a 3 present in mosquito head and thorax extract. Analysis of the ELISA showed that the mean levels of rAed a 3-specific IgE and IgG were significantly higher in allergic individuals than the controls. Using the mean of the controls plus 2 SD as a cut-off level, 46% of the 13 allergic individuals had a positive rAed a 3-specific IgE, while none of the controls were positive. The Aed a 3 in the saliva has the same molecular weight and allergenicity as

rAed a 3 as shown by Western blot. Since rAed a 1 and rAed a 2 have cross-reactivity with the salivary protein in other mosquito species,[97, 107] we anticipate that rAed a 3 may also be a cross-reactive allergen. All of the evidence above indicates that the rAed a 3, expressed using a baculovirus/insect cell system, has identical allergenicity to its natural form in the saliva and support the idea that Aed a 3 is an important allergen in the *Aedes aegypti* mosquito saliva. Compared to other recombinant mosquito allergens expressed using baculovirus/insect cells, the yield of rAed a 3 is low as shown in table 3. It may be that the nature of the gene is the most important element in controlling the expression level of the protein.

4. The applications of rAed a 3 and rAed a 4

Immunological and biological investigations have shown that many recombinant allergens behave similarly to their natural counterparts.[170] Therefore, recombinant allergens can replace their natural counterparts and be used in the following aspects.

1. Cocktails of recombinant mosquito allergens matching the IgE epitope complexity present in mosquito saliva, can be used to replace the mosquito salivary extract in the diagnosis of mosquito allergy in both *in vivo* and *in vitro* tests. For tree and grass pollen allergies it was clearly demonstrated that mixtures of a few recombinant allergens can reproduce the allergenic complexity of natural allergen extracts required for *in vitro* allergy diagnosis,[171] and that these allergens contained most of the IgE epitopes present in the corresponding natural pollen extracts.[172, 173] So, it should be

feasible to replace mosquito saliva using a mixture of rAed a 1, rAed a 2, rAed a 3 and rAed a 4 for the *in vitro* and *in vivo* diagnosis of mosquito allergy.

2. Component-resolved diagnosis of mosquito allergy

There are at least 8 allergens in the saliva of *Aedes aegypti*. Mosquito allergic subjects may be sensitized to different allergens. As shown in Table 2, mosquito allergic subjects have different IgE sensitization profiles. For example, subject #1 is positive to rAed a 3, but negative to rAed a 4; while subject #5 is positive to rAed a 4, but negative to rAed a 3; however, subjects #2, #8 and #11 are positive to both rAed a 3 and rAed a 4. These clearly indicate that the sensitivity of each subject is different. These results are similar to those reported in other allergens.[54, 65] Diagnostic tests exclusively based on single recombinant allergens, allowing the precise measurement of levels of IgE antibodies to a particular allergen molecule and thus comparison of specific IgE levels with the allergenic potency of that determinant are available.[65, 67] Together with rAed a 1[66] and rAed a 2 which have been expressed previously in our lab, these four recombinant allergens when used singly in the diagnosis of mosquito allergy, will give us precise information about the individual subject's sensitization profile, the IgE-eliciting allergens, and as a logical next step, component-resolved forms of immunotherapy can be developed for mosquito allergy.

3. Component-resolved form of immunotherapy for mosquito allergy

Current forms of allergen specific immunotherapy are based on the administration of increasing doses of natural allergen-extract. All subjects showing allergic reactions to proteins present in a particular allergen source are treated with the same complex extract regardless of their individual sensitization profiles,[65] which cannot be subject-tailored

to include only the relevant allergens. The use of purified recombinant allergens will allow the production of reagents which contain only defined amounts of disease-eliciting allergens. It has been demonstrated that immunization of mice with purified recombinant allergens induced IgG antibodies which reacted with their natural counterparts, bound to epitopes recognized by human IgE and blocked human IgE binding to the allergens as well as allergen-induced histamine release from basophils. As shown in Table 2, after screening of mosquito allergic subjects using rAed a 3 and rAed a 4, we can use rAed a 3 to perform allergen specific immunotherapy for subject #1; use rAed a 4 for subject #5 and use both rAed a 3 and rAed a 4 to perform allergen specific immunotherapy for subject #2, #8 and #11.

5. Future directions

After investigating the values of recombinant mosquito allergens in the diagnosis of mosquito allergies, the next step is to explore the feasibility of using these recombinant n allergens in allergen specific immunotherapy. One main problem of the allergen specific immunotherapy is adverse side effects especially anaphylaxis after allergen injections. Some methodologies are being considered to generate hypoallergenic (modified) forms of allergens to overcome the problem. The native sequence and structure can be manipulated to generate hypoallergenic mutants that no longer bind IgE but retain T cell epitopes, ie loss of allergenicity with maintenance of immunogenicity.

Modern DNA technology allows the manipulation of the nucleotide coding the allergen sequence. Typically, substitution and deletions of nucleotide bases are used to

change the cDNA encoding an allergen leading to an altered amino acid sequence upon production of the recombinant protein. This modifies the tertiary structure and thereby the functional properties of the protein. Loss of native tertiary structure has a central role in loss of allergenicity as IgE epitopes are often discontinuous, being dependent on the juxtaposition in space of different parts of the molecule. In contrast, T cell epitopes being composed of a discrete string of amino acids, are generally not affected by changes in structure. There are a number of examples of the use of this technology. A Der f 2 mutant (C8/119S) that has lost an intramolecular disulfide bond leading to structural changes no longer binds IgE from patients' serum and instead induces a strong Th1 response.[174] Three major peanut allergens (Ara h1, h2 and h3) were cloned, characterized and subjected to site-directed mutagenesis.[175, 176] These mutants were generally poor competitors for binding of peanut-specific IgE compared to the wild type and binding by IgE from patient serum was reduced. The mutant allergens also retained the ability to stimulate proliferation by most patients and they, or their derivatives, may be suitable candidates for use in immunotherapy. These techniques can be used to produce recombinant hypoallergenic mosquito salivary allergens for allergen specific immunotherapy of people who have systemic allergic reactions to mosquito bites.

Conclusions

We conclude from the results of present study that

(1) rAed a 4, expressed by a baculovirus/insect cell system, is an allergen; it has identical allergenicity, molecular weight, and the same sugar digestion function as natural Aed a 4, and can be used in *in vitro* diagnosis of mosquito allergy.

(2) rAed a 3, expressed by a baculovirus/insect cell system, is an allergen; it has identical allergenicity, and molecular weight to its natural counterpart, and can be used in *in vitro* diagnosis of mosquito allergy.

(3) rAFXa, expressed by a baculovirus/insect cell system, has the same biological function as its natural form. Due to the difficulty of purifying rAFXa from the cell medium with the methods currently used, purification with other methods will be required in order to identify its allergenicity.

These recombinant mosquito salivary allergens will greatly facilitate the diagnosis of mosquito allergy and in the future, the immunotherapy of mosquito allergy.

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