Production and Characterization of Hypoallergenic Chicken Eggs

By

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

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I am the author of the thesis entitled ‘Production and characterization of hypoallergenic chicken eggs’ submitted for the degree of Doctor of Philosophy

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Abbreviations

\( \Delta G \) – Change in free energy

\( ^\circ C \) – Degrees Celsius

\( \mu F \) – Microfarad

\( \mu g \) – Microgram

\( \mu L \) – Microliter

\( \mu M \) – Micromole

AAHL – Australian Animal Health Laboratory

APC – Antigen presenting cell

ATP – Adenosine triphosphate

BCIP – 5-bromo-4-chloro-3-indolyl-phosphate

BLAST – Basic Local Alignment Search Tool

bp – Base pairs

BSA – Bovine serum albumin

C – Cysteine

*Caenorhabditis elegans* – C. elegans

CD – Circular dichroism

CD-4 – Cluster of differentiation 4 glycoprotein

cDNA – Complementary DNA

CID – Collision induced activation

CO\(_2\) – Carbon dioxide

CRISPR-Cas9 – Clustered regularly interspaced short palindromic repeats-Cas9

CSIRO – Commonwealth Scientific and Industrial Research Organisation

CT – Cycle threshold

C-terminal/terminus – COOH (carboxy) terminal/terminus

dH\(_2\)O – Deionized water

DMEM – Dulbecco’s modified eagle’s serum medium

DNA – Deoxyribonucleic acid

dNTP – Deoxyribonucleotide triphosphate
dsRNA – Double stranded RNA

*E. coli* – *Escherichia coli*

EDTA – Ethylenediaminetetraacetic acid

FACS – Fluorescent-activated cell sorting

FAM – 6-carboxyfluorescein

Fc – Fragment crystallisable

FCS – Fetal calf serum

FceRI – High affinity IgE receptor

GE – Genome/genetic engineering

GFP – Green fluorescence protein

HDR – Homology-directed repair

HEPES – 4-2-hydroxyethyl-1-piperazineethanesulfonic acid

His – Histidine

**His-tagged** – Histidine tagged

HPLC – High-performance liquid chromatography

IFNγ – Interferon gamma

IgA – Immunoglobulin A

IgA₂ – Immunoglobulin A₂

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IL-4 – Interleukin 4

IL-5 – Interleukin 5

IL-12 – Interleukin 12

IL-13 – Interleukin 13

Indel – Insertion/deletion mutation

IPTG – Isopropyl β-D-1-thiogalactopyranoside

IU – International units

IUIS – International Union of Immunological Societies (allergen nomenclature)

kb – Kilobase

kcal – Kilocalorie
kDa – Kilodalton
kV – Kilovolt
LB – Lysogeny broth/Luria broth
LYS – Lysozyme
M-cells – Microfold cells
MCRI – Murdoch Childrens Research Institute
MFI – Mean fluorescence intensity
MgCl₂ – Magnesium chloride
MHC class II – Class II major histocompatibility complex
miRNA – MicroRNA
mL – Millilitre
mM – Millimole
mol – Mole
mRNA – Messenger ribonucleic acid
MW – Molecular weight
NaOH – Sodium hydroxide
NBT – Nitro blue tetrazolium
NCBI – National Center for Biotechnology Information
ng – Nanogram
NHEJ – Non-homologous end joining
Ni-NTA – Nickle-nitrilotriacetic acid
nmol – Nanomole
N-terminal/terminus – NH₂ (amine) terminal/terminus
OD – Optical density
OIT – Oral immunotherapy
Oligo – Oligonucleotide
Oligo-dT – Short sequence of deoxy-thymine nucleotides
OVAL – Ovalbumin
OVM – Ovomucoid
OVT – Ovotransferrin
PAGE – Polyacrylamide gel electrophoresis
PANK1 – Pantothenate kinase 1
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PGCs – Primordial germ cells
qPCR – Quantitative polymerase chain reaction
RAST – Radioallergosorbent test
RE – Restriction endonuclease/restriction enzyme
RISC – RNA-induced silencing complex
RNA – Ribonucleic acid
RNAi – RNA interference
RNase – Ribonuclease
rpm – Revolutions per minute
RT – Reverse transcriptase/reverse transcription
RT-PCR – Reverse transcription polymerase chain reaction
SC – Secretary component
SDS – Sodium dodecyl sulphate
SFM – Serum free medium
sgRNA – Small guide RNA
shRNA – Small hairpin RNA
siRNA – Small interfering RNA
SIT – Specific immunotherapy
SLIT – Sublingual immunotherapy
SPF – Specific pathogen free
SPT – skin prick test
SYBR Safe – cyanine dye
TAE – Tris-acetate ethylenediaminetetraacetic acid/Tris-acetate EDTA
TALENs – Transcription activator-like effector nucleases
Th0 – Naïve T-cells
Th1 – T-helper type 1 cells
Th2 – T-helper type 2 cells
Th3 – T-helper type 3 cells
Tol2 LHE – Tol2 left hand end
Tol2 RHE – Tol2 right hand end
Tris – 2-Amino-2-hydroxymethyl-propane-1,3-diol
UTR – Untranslated region
V – Constant voltage
YGP42 – Yolk glycoprotein 42
ZFN – Zinc finger nuclease
α livetin – serum albumin
Ω – Ohm
Abstract
Hypersensitivity to the chicken egg is a widespread disorder mainly affecting 0.5 – 2.5% of children worldwide. It is the second most common food allergy in children, next to cow’s milk allergy. Egg allergy is mainly caused by hypersensitivity to four allergens found in the egg white; ovomucoid, ovalbumin, ovotransferrin and lysozyme. In egg allergic individuals, these allergens cause conditions such as itching, atopic dermatitis, bronchial asthma, vomiting, rhinitis, conjunctivitis, laryngeal oedema and chronic urticaria, and anaphylaxis. Currently, there is no permanent cure for egg allergy. Upon positive diagnosis for egg allergy, strict dietary avoidance of eggs, and products containing traces of eggs, is the most effective way of avoiding future hypersensitivity reactions. However, it is difficult to fully avoid eggs since they are found in an extensive range of processed food products. Strict avoidance also poses nutritional disadvantages due to high nutritional value of eggs. In addition, allergen specific immunotherapy (SIT) is being pursued as a curative treatment, in which an allergic individual is gradually exposed to the allergen to induce tolerance. However, immunotherapy may cause unwanted immune reactions in individuals highly sensitive to eggs.

Use of recombinant proteins for immunotherapy has been beneficial due to the purity of the recombinant proteins compared to natural proteins. In Chapter 2 and Chapter 3 of this thesis, IgE reactive recombinant egg white proteins, which may be used as novel reagents for future immunotherapy, were produced. *Escherichia coli* was used as an expression system to produce these recombinant versions of ovomucoid, ovalbumin and ovotransferrin that showed IgE reactivity when tested.
against a pool of egg allergic patients’ sera. The IgE reactivity indicates that these recombinant proteins are capable of eliciting an immune response, thus being potential candidates for immunotherapy. The recombinant version of lysozyme did not show IgE reactivity for unknown reasons, which may be useful as a hypoallergen as is. The results also suggest that egg allergy in Australian populations may mainly be due to IgE reactivity to ovotransferrin and lysozyme, while ovomucoid showed higher IgE reactivity. The significance of this study is the potential use of the IgE reactive recombinant egg white proteins in immunotherapy to treat egg allergic patients.

Chapter 4 of this thesis aimed at producing a hypoallergenic variant of a major egg white allergen. Ovomucoid is the dominant allergenic protein in chicken egg white. Hypoallergenic variants of this allergen may be used in immunotherapy as an egg allergy treatment approach with reduced adverse effects. Two cysteine residues at C192 and C210 in domain III of the protein were mutated to alanine using site-directed mutagenesis, to disrupt two separate cysteine-cysteine bridges. Both mutated and non-mutated proteins were expressed in E. coli, which was confirmed by SDS-PAGE and immunoblotting. Immunoglobulin E (IgE) reactivity of the two proteins was analysed, by immunoblotting, with a pool of egg allergic patients’ sera. A pool of non-allergic patients’ sera was also used in a separate blot as a negative control. Mutant ovomucoid had significantly diminished IgE reactivity in the immunoblot, as demonstrated by lighter bands when compared to the non-mutated version, although there was more of the mutant protein immobilized on the membrane when compared to the wild-type protein. The non-allergic negative
control showed no bands indicating an absence of non-specific binding of secondary antibody. In conclusion, disruption of two cysteine bridges in domain III of ovomucoid reduces IgE reactivity. Following downstream laboratory and clinical testing, this mutant protein may be used in immunotherapy as an alternative to its natural counterpart to induce tolerance to ovomucoid with reduced adverse effects in egg allergy.

Potential use of genetic engineering to produce hypoallergenic chicken eggs was scrutinized in Chapter 5 of this thesis. A CRISPR-Cas9 driven knockout of ovomucoid in chicken fibroblast cells was attempted first, however successful results were not obtained. Possible ways of improving this approach is discussed in this chapter. Secondly, a RNAi driven knockdown of ovomucoid in chicken was conducted. Initial cell based assays confirmed the efficacy of the RNAi targets and mechanism. Gonad assays following micro-injection into chicken embryos further confirmed that the RNAi mechanism may be active in the transfected cells of the embryos. qPCR conducted using sperm of an adult rooster from the G0 generation predicted ~1.5% transgenesis in the G1 generation. As a future direction from this study, the eggs of the G1 generation can be tested for IgE reactivity. If the G1 generation and their eggs seem healthy after reduced ovomucoid expression, a complete knockout of the gene may be feasible to produce a significantly hypoallergenic chicken egg.

In conclusion, this PhD project aimed at developing recombinant allergenic and hypoallergenic variants of the egg white allergens that may be useful in treatment methods such as immunotherapy and diagnostic methods such skin prick tests. As a
novel approach for tackling egg allergy, genetic engineering tools were studied for their efficacy at producing hypoallergenic chicken eggs. The latter part of the project, although inconclusive, provided useful information for future research using genetic engineering in egg allergy. All approaches scrutinized in this project may, in the future, significantly improve egg allergy diagnosis, treatment and management.
CHAPTER 1

Literature Review

Cracking the egg: an insight into egg hypersensitivity

Publication:
1.1 Allergy

Allergy is a symptomatic and an abnormal overreaction by the immune system to innocuous environmental substances, such as grass pollen and eggs, known as allergens. Allergens are a type of antigen, which trigger a complex immune response upon contact with the immune system. Allergy is classified as a type 1 hypersensitivity reaction because of the immediate and inflammatory immune response that is characterized by the excessive production of the antibody IgE. An allergic response involves two reaction phases based on the contact of the allergen with the immune system; primary and secondary response. The primary response (upon initial contact with an allergen), stimulates the generation of IgE by B-lymphocytes. The secondary response (upon secondary contact with the same allergen) results in the degranulation of mast cells (mastocytes) and basophils (basophil granulocytes). The mediators (e.g. histamine) released by the degranulation process elicit clinical allergic symptoms such as bronchoconstriction, sneezing, rashes, itching or life threatening conditions such as anaphylaxis, just to name a few.  

Allergens, being proteins or chemical substances, originate from a variety of animals and plants and exist abundantly in the environment. In indoor environments, some of the most common allergens are dust mites, pet fur and dander, cockroach calyx, mould and wool. Outdoor allergens include, but not limited to, insect stings such as bees and wasps, grass pollen, weeds such as ragweed, and tree products such as birch sap and pollen. In addition to the above-mentioned sources, allergens can be found in many of the foods that humans consume including eggs, milk, legumes (e.g. peanuts), seafood, soy, tree nuts, and wheat (gluten). Many individuals are also
known to be allergic to artificial substances such as perfumes, latex and medications including penicillin and anaesthetics.\(^3\)

The most common allergic diseases include allergic rhinitis, asthma, hay fever, atopic eczema, atopic dermatitis and celiac disease.\(^2\) The term ‘atopy’ is often used to describe a number of allergic diseases. Atopy refers to the strong hereditary predisposition to produce increased amounts of IgE in response to common environmental allergens. Individuals with atopy have a tendency to possess one or more allergies.\(^2\) The combination of atopy and allergens may sometimes lead to severe hypersensitivity conditions such as anaphylaxis, in which allergen-induced release of mediators from mast cells and basophils results in a systematic and catastrophic physiological reaction that has the potential to prove fatal.\(^4\) Allergies that arise independent of IgE, such as contact dermatitis and hypersensitivity pneumonitis are classified as non-atopic conditions.\(^2\)

A marked increase in allergic diseases has been demonstrated in major industrialized countries such as Australia, United States and Europe over the last decade. In 2006, 50% of the population of the US has been identified as allergic.\(^5\) In Australia, 1 in 5 or 4.1 million people are affected by allergies, conferring a financial burden of over $7.8 billion per annum on the government. Prevalence of allergy in Australia is expected to rise by 70% (i.e. 7.7 million people) by 2050, provided that the current trend continues.\(^6\) The World Allergy Organisation has reported that 20-30% of the world population has been affected by allergy of some type in 2008.\(^7\)
1.2 Diagnosis of allergy

Scientific advances in delineation of antigen presentation and molecular signalling have provided insights into the underlying principles of the immune response to allergens and the subsequent synthesis of IgE, which leads to the clinical symptoms of allergy. This framework has assisted in developing the two widely used diagnostic approaches of allergy; skin tests and blood tests. A skin test involves the introduction of a small amount of a known concentrated allergen into the epidermis. If the individual is allergic to the introduced substance, a blister or a wheal (redness) takes place. The severity of the reaction is determined by measuring the diameter of the affected area. A blood test involves quantification of IgE both as total immunoglobulin concentration and as allergy-specific antibody concentration in the patient’s serum. The IgE levels are quantified mainly by using radioallergosorbent tests (RAST) in which radioisotope-labelled IgE binding antibodies are used to measure serum IgE levels. At present a similar, and commercial, method known as ImmunoCAP testing is used more frequently which is enzyme based rather than radioisotope based.

1.3 Treatment

The most common approach of allergy treatment focuses on allergen avoidance and pharmacotherapy to neutralize allergic symptoms. Pharmacotherapy exploits antagonistic drugs such as antihistamines, antileukotrienes, corticosteroids, cromolyn, methylxanthines, β-antagonists, muscarinic antagonists and mast cell stabilisers to block allergic mediators or to alleviate the degranulation process.
However, such therapeutics do not yield curative results due to their inability to inhibit IgE production. Furthermore, antagonistic drugs are capable of promoting immunosuppression or non-immunological effects.

Allergic desensitization through administration of appropriate concentrations of allergens has been conventionally used to improve immunological tolerance in allergic individuals. Allergen SIT is a desensitizing therapy that has been used practically for a century and represents the only remedial and allergen-specific treatment method. Allergen-SIT essentially involves weekly vaccination (subcutaneous) of increased doses of specific allergens into the patient with the aim of modifying Th2 lymphocytes into Th1 lymphocytes and therefore induce Immunoglobulin G (IgG) production instead of IgE. This method has been recognized as an effective treatment for rhinitis and asthma. Sublingual immunotherapy (SLIT) is another form of SIT, which involves administration of allergen doses via the sublingual mucosa. An analysis of multiple SLIT studies has confirmed the efficacy of SLIT on reducing rhinitis symptoms and the necessity of other medications. However, administration of increased amounts of allergens may cause excessive production of IgE and lead to therapy-induced anaphylaxis.
1.4 Allergic reaction

1.4.1 Cells involved in allergic reactions

Antigen presenting cells (APCs), also known as accessory cells, process and present foreign antigens to the class II major histocompatibility complex (MHC class II) and provide co-stimulatory signals necessary for T-cell activation (Figure 1.1) \(^{16-18}\). T-cells are a type of lymphocytes that are activated by APC signalling. T-helper cells (Th or CD4+T), a sub-type of T-cells, possess the T-cell receptor which specifically recognizes antigens bound to MHC class II complex, and express the CD4 surface protein \(^{19}\). When antigen-bound MHC class II complex interacts with naïve T-helper cells (Th0), they differentiate into Th1, Th2 or Th17 cells, secreting different types of cytokines, and prime as antigen specific memory T-cells capable of combating or reacting to future immune encounters. B-cells are another type of lymphocytes that exist as inactive non-dividing cells before the onset of an immune response. Cytokines released by T-cells induce B-cells to proliferate and differentiate into plasma cells secreting allergen specific antibodies \(^{20-23}\).

Mast cells and basophils perform a crucial role in allergy as effector cells. Derived from bone marrow, both types of cells possess cytoplasmic granules containing chemical mediators including histamine \(^{24}\). These cells constitutively express the high affinity receptor FceRI, which binds to the Fc portion of IgE \(^{25}\). Cross-linking of FceRI by immune complexes stimulate imperative host defence mechanisms including cell cytotoxicity, phagocytosis and regulation of immune response, and the release of inflammatory mediators which is the hallmark of allergic response \(^{25}\).
1.4.2 Non-allergic response

In non-allergic individuals, when the immune system encounters an allergen, the allergen is engulfed by APCs which migrate to peripheral lymphoid tissues, including lymph nodes. Here, the allergen is processed and presented to the MHC class II complex (Figure 1.1). Allergen bound MHC class II complex binds to the T-cell receptor of Th0 cells, stimulating Th0 differentiation into Th1 in the presence of cytokines Interleukin 12 (IL-12) and Interferon-γ (IFN-γ) (Figure 1.2)\(^{26,27}\). Pro-allergic IL-12 is produced by peripheral blood mononuclear cells and APCs, which in turn induce the production of IFN-γ by natural killer cells\(^ {28-32}\). Th1 lymphocytes then further secrete IFN-γ, which stimulates B-cells to proliferate and differentiate into plasma cells secreting the antibody IgG (Figure 1.2), a feature of non-allergic response\(^ {33}\). Such IgG antibodies, which bind to the allergens, are known as blocking antibodies.
Figure 1.1: The presentation process of an allergen by an antigen presenting cell. The allergen is engulfed by the cell and transported via an endocytic vesicle, in which the allergen is processed to produce peptides. The peptides then interact with and bind to MHC class II molecules released by the Golgi apparatus. Subsequently, the complex of MHC class II and allergen is transported by vesicles and displayed on the cell surface, ready to interact with a T-cell receptor.
In the non-allergic reaction, once the encountered allergen is processed and presented by APCs to the Th0 cells, the Th0 cells differentiate into Th1 cells. IFN-γ cytokine released by the Th1 cells induce B-cell differentiation into IgG producing plasma cells. The plasma cells then proliferate and produce large amounts of IgG, a characteristic of the non-allergic reaction.
1.4.3 Allergic response

The allergic reaction can be divided into two phases; the humoral (primary) response and the cell-mediated (secondary) response. The former refers to the initial encounter of the immune system with an allergen that triggers the production of IgE, and the latter refers to the secondary encounter of the immune system with the same (unprocessed) allergen, which triggers the release of chemical mediators responsible for clinical manifestation of allergy.

1.4.3.1 Allergic sensitization

Upon contact with the immune system, an allergen is engulfed and processed by antigen presenting cells (APCs) (Figure 1.1) and presented to naïve T-helper cells (Th0). This causes Th0 cells to differentiate into T-helper type 2 (Th2) cells in the presence of cytokines including Interleukin-4 (IL-4). The source of pro-allergic IL-4 is thought to be mast cells and basophils. Th2 cells then further secrete IL-4, IL-5 and IL-13, which induce B-cell differentiation into plasma cells secreting IgE. The IgE antibodies subsequently bind to the high affinity FcεRI receptors on mast cells and basophils, thus sensitizing the immune system to the allergen (Figure 1.3).

1.4.3.2 Allergic reaction

Upon secondary exposure to the same allergen, the allergen binds to the membrane bound IgE molecules on mast cells and basophils. Cross-linking of FcεRI occurs when multiple IgE molecules interact with the same intact allergen, triggering
degranulation of mast cells and basophils, consequently releasing granule-derived vasoactive chemicals such as histamine, serine protease tryptase, lipid mediators such as leukotrienes, prostaglandins and platelet activating factor, chemokines and cytokines IL-4, IL-5 and IL-13 (Figure 1.3). These mediators cause allergic symptoms such as airway smooth muscle constriction (bronchoconstriction), mucus hypersecretion, mucus cell metaplasia, redness, rashes, sneezing and anaphylaxis \(^2,38-42\).
Figure 1.3: Illustration of the allergic reaction. This figure outlines the steps of an allergic reaction. Once the initially encountered allergen is processed and presented by APCs to the Th0 cells, the Th0 cells differentiate into Th2 cells in allergic individuals. These Th2 cells then further secrete IL-4, IL-5 and IL-13, triggering B-cell differentiation into plasma cells. Subsequently, these plasma cells proliferate and produce excessive amounts of allergen specific IgE, which bind to the FcεRI receptors on mast cells and basophils, thus sensitizing the immune system to the allergen. Upon secondary encounter of the immune system with the same allergen, the allergen binds to multiple IgE held on mast cells and basophils. Cross linking of the FcεRI receptors, as a result of interaction between a single allergen and multiple IgE molecules, triggers the release of mediators including histamine, causing an allergic inflammation.
1.5 Food allergy

1.5.1 Food allergy overview

Food allergy is one of the major allergies in today’s society. In a double-blind placebo controlled food challenge study conducted by Bock et al.\textsuperscript{43}, 80% of the subject children were found to develop hypersensitivity symptoms to food allergens. In the developed world, 10% of children are affected by food allergies, while emerging or developing nations has a prevalence of 7%\textsuperscript{44}. Occurrence of food allergy, like other atopic diseases, is rising and remains a problem throughout the world. It is a major cause of anaphylaxis and treated worldwide in emergency departments and hospitals, incurring a massive financial burden on the governments and the public\textsuperscript{45}. Food hypersensitivities include abnormal overreactions caused by intolerance to food (non-allergic) and allergic responses caused by an antigen in the food. Intolerance to food is due to some physiological abnormality, such as the lack of lactase which causes intolerance to lactose, whereas the allergic food reactions are mediated by IgE or non-IgE immune responses\textsuperscript{45}. Food allergies arise through the interaction between the intestinal immune system and food allergens present in the food that we consume on a daily basis. Allergenic foods include, but not limited to, eggs, peanuts, fish and milk. Antigens (allergens) present in these foods are responsible for IgE-mediated hypersensitivity reactions. For example, people allergic to milk react to the milk whey protein allergen β-lactoglobulin\textsuperscript{46} and patients allergic to egg may be due to the egg white protein ovomucoid\textsuperscript{47}. Most of these allergens are glycoproteins that can withstand heat, acids and proteases\textsuperscript{45}, which explains why most allergenic foods remain allergenic even after cooking or processing.
1.5.2 Intestinal immune system

Food allergies arise through the involvement of the intestinal immune system, which is the largest, most complex and one of the most important parts of the immune system. The intestine plays a major role by distinguishing between harmless antigens and harmful antigens, pathogens and microorganisms. The intestine is exposed to different types of antigens and microorganisms than any other body part and this highlights the importance of having a powerful immune system to protect this tissue and the rest of the body. Non-allergic individuals demonstrate induction of intestinal local and systemic immunological tolerance to non-pathogenic antigens, a phenomenon known as oral tolerance. Antigens entering the intestine initially encounter microfold cells (M-cells) prior to being presented to APCs for processing. M-cells are a type of enterocytes found on the epithelium and they lack the mucus layer. APCs then present the antigen to Th0 cells. In areas such as the lamina propria of the intestine, MHC class II enterocytes act as APCs and present the antigens to Th0 cells. In both cases, the Th0 cells prime as intolerant cells, which causes B-cells to differentiate into IgA secreting plasma cells. A strong physical barrier, presence of antigen altering luminal enzymes, presence of specific regulatory T-cells in the lymphoid tissue of the gut, and the production and secretion of IgA capable of functioning in the hostile gut environment ensures immunosuppression of the intestinal immune system upon contact with an antigen and therefore causing antigen tolerance. Antigen tolerance is essential because eliciting an active immune response to harmless food antigens can be wasteful. Food intolerance or food allergy arises due to defects in one or more of the components responsible for
tolerance. As in many other allergies, genetic predisposition to produce IgE causes oral tolerance to fail or break down \(^{51}\).

1.5.3 Mucosal barrier of the intestinal immune system

Intestinal immune system functions mainly on suppressing immunity rather than promoting it, as a measure of protecting the host from unnecessary damage \(^{49}\). The mucosal barrier plays a major role in this immunosuppression. An understanding of the mucosal barrier is important for understanding the early phase of food allergy. The mucosal barrier comprises cellular and non-cellular components \(^{52,53}\). Enzymes and proteases present in the mouth and the intestine, combined with bile salts, lipases and carbohydrate breakdown enzymes facilitate digestion of food, and, at the same time make it almost impossible for an immune response to occur. However, many food antigens survive these harsh conditions and, as a defence mechanism, the intestinal immune system recognises potentially harmful antigens and elicits either a suppressive or an aggressive immune response, depending on the invasiveness/capabilities of the antigen. The goblet cells of the glandular epithelium secrete a range of mucin glycoproteins that line the outer epithelium of the entire digestive tract \(^{54}\). These mucin glycoproteins collectively form mucus, which traps antigens and stops them from gaining access to the underlying epithelium, a process known as non-immune exclusion. The mucus layer also harbours secretory IgA which prevents viral and bacterial binding to the epithelium. The maintenance and potential repairs of the epithelium are carried out by an association of secretory proteins known as trefoil factors \(^{55,56}\). Neonatal studies using rat and mouse have shown that
there is an increased permeability of the intestine in the neonate, allowing antigens to pass into the lamina propria of the gastrointestinal tract. The lamina propria harbours lymphocytes and therefore an antigen entering this layer can cause an active immune response. If a food antigen enters lamina propria, APCs may present the antigen to T-cells, causing antigen specific priming of B-cells. In such situations, intestinal immune system of an individual genetically predisposed to allergy produces antigen specific IgE. This highlights the importance of the mucus barrier in the developed gut at suppressing an immune response to antigens.

The epithelium is the other barrier that prevents an antigen from reaching the lamina propria to cause an immune response. Epithelial cell membranes are impermeable to macromolecules and each cell is joined to another by tight junctions, allowing only ions to pass through via ion channels. An abnormality in tight junctions can allow passage of antigens into the lamina propria and this permeability may be present in allergic patients. Furthermore, epithelial cells themselves can act as APCs, which present the antigens to T-cells in lamina propria. In a non-allergic individual, this antigen presentation activates regulatory T-cells, such as Th3, which secretes cytokines capable of suppressing an immune response. In an allergic individual, activation of immunosuppressing T-cells is defective and antigen presentation may activate Th2 cells involved in the production of cytokines involved in allergic reactions. In addition, trans-epithelial antigen transport has been shown to be carried out by cytokines such as IL-4, suggesting possible allergic reactions in sensitized individuals. This information highlights the importance of immunosuppressive activity of mucosal barrier in the intestinal immune system.
1.5.4 The role of IgA in the intestinal immunosuppression

Immunoglobulin A is an antibody that inhibits bacterial and viral attachment to the intestinal epithelium. IgA is the most abundant antibody in the body, however, unlike other antibodies, it does not involve itself in the complement system, which causes inflammation. IgA assists in trapping antigens in the mucus layer and it also facilitates expulsion of antigens from the body \(^{77-79}\). A glycoprotein known as the secretory component (SC) envelops the fragment crystallisable (Fc) region of IgA and covers the proteolytic cleavage sites, thus protecting IgA from proteases and other enzymes present in the intestine. It is reported that IgA deficiency cause high levels of food-antigen-specific antibodies in serum \(^{79}\). This further indicates the ability of IgA to suppress immune responses. The IgA system does not fully develop until the age of 4, therefore it can be suggested that, when coupled with defects in the mucosal barrier during development, priming of T-cells and B-cells to food allergens/antigens occur. This explains why food allergy is common among children than in adults.

1.5.5 Food allergens and types of food allergies

Food allergies arise through the interaction between the intestinal immune system and food allergens present in the food that we consume on a daily basis. Allergenic foods include, but not limited to, eggs, peanuts, fish and milk. Table 1.1 highlights the prevalence of different food allergies in the United States. Food allergies also seem to be the major cause for conditions such as asthma, eczema and respiratory allergies in children when compared to non-food allergies \(^{80}\). Antigens (allergens) present in these foods are responsible for IgE-mediated hypersensitivity reactions. For example,
people allergic to peanuts react to the peanut seed storage protein Ara h 1. These 
allergens are glycoproteins that can withstand heat, acids and proteases \textsuperscript{51}, which 
explains why most allergenic foods remain allergenic even after cooking or 
processing. A large number of plant and animal proteins responsible for food allergy 
have been identified and characterized. Majority of the plant food allergens belong 
to the cupin and prolamin superfamilies, profilin and plant defense proteins. Profilin 
allergens shows high cross-reactivity to pollen and other food allergens \textsuperscript{51}. When 
compared to plant allergens, animal food allergens are less diverse and have less 
cross-reactivity with other allergens. A few allergens include parvalbumin in fish, 
oveomucoid in eggs and alpha S1-casein in milk. A common component present in two 
or more allergens may cause cross-reactivity between the allergens. For example, 
serum albumin may sensitize an individual to both cow’s milk and beef, and 
potentially to other related meat products \textsuperscript{81}. An understanding of the structure and 
function of allergens themselves is important in many frontiers of allergy research. 
Allergens of interest for this project are reviewed and outlined further in this Chapter.
Table 1.1: Prevalence of food allergies in the United States (Table adapted from: Sampson 2004).

<table>
<thead>
<tr>
<th>Food</th>
<th>Young Children (%)</th>
<th>Adults (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>2.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Egg</td>
<td>1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Peanut</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Tree Nuts</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Fish</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Shellfish</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>Overall</td>
<td>6</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Figure 1.4: Percentage of children in the United States affected by asthma, eczema and respiratory allergy caused by food and non-food allergy. (Figure adapted from: Branum and Lukacs 2008.)
1.6. Egg allergy

1.6.1 Egg allergy overview

Hypersensitivity to the chicken (*Gallus gallus*) egg is a widespread disorder mainly affecting children, with a recent meta-analysis suggesting a prevalence of 0.5-2.5% of children. It is the second most common food allergy next to cow’s milk allergy and it is the most common allergy among children with atopic dermatitis. According to an Australian study conducted by the Murdoch Children’s Research Institute (MCRI), 8.9% of infants are allergic to eggs. However, egg allergy is not limited to children, with cases that have been reported of adult onsets. Children allergic to egg generally grow out of the condition by school age. Hypersensitivity to allergens present in the eggs causes conditions such as itching, atopic dermatitis, bronchial asthma, vomiting, angio-oedema, rhinitis, conjunctivitis, laryngeal oedema, chronic urticaria and allergic eosinophilic gastroenteritis. Egg allergic patients produce IgE specific to sequential (linear) and conformational epitopes of egg allergens. However, some sequential epitopes recognized by persistent egg allergic patients are not recognized by patients with transient egg allergy. This suggests a difference in severity of allergic reaction between persistent and transient egg allergic patients. Anaphylaxis can also occur in some individuals, however this depends on the processing of the egg. This may be due the reduction in IgE reactivity in heated egg, possibly due to the disruption of conformational IgE binding epitopes in the egg allergens. It has also been found that eggs heated on its own are more IgE reactive when compared to eggs heated in the presence of another substance such as wheat. This suggests that an egg allergic patient may show less reactivity to food items such as cakes or muffins than to fried or baked egg.
The prevalence of egg allergy and the aforementioned conditions highlight the importance of egg allergy research. An understanding of the different egg allergens, egg allergy diagnosis methods and treatment methods are imperative for future research.

1.6.2 Allergy to egg white

Egg allergy is mainly caused by 4 of the many different proteins that make up the egg white. However, these may not be the only allergenic egg white proteins, particularly in the wake of new discovery of a large number of minor proteins in the egg white. These allergens are namely ovomucoid, ovalbumin, ovotransferrin and lysozyme, with ovomucoid being the most allergenic of the four and ovalbumin being the most abundant (Table 1.2). A few studies have identified egg yolk allergens, such as α-livetin and YGB42, however these were not a focus of this project. The four aforementioned egg white allergens are produced by tubular gland cells, which comprise the majority of the magnum portion of the chicken oviduct. Induction of expression of these allergens are highly dependent on estrogens and this has been demonstrated in studies involving chicks, in which upon administration of estrogens the tubular gland cells started producing the allergenic proteins. Egg allergy can be caused by consumption of raw or cooked eggs, however a study conducted by Lemon-mule et al indicates that majority of the egg allergy patients are tolerant to heated eggs, although the allergenicity of proteins are not exclusively dependent on their resistance to heat and enzymes. The same study shows that continued exposure to the heated egg is associated with immunological changes similar to the
changes observed in developing tolerance to raw eggs. These immunological changes include increasing IgG₄ levels and a reduction in wheal sizes in egg white specific skin prick testing (SPT). IgA and IgA₂ may also be of importance in developing tolerance to egg white. However, more studies involving a larger number of subject individuals are necessary to confirm the effects of heating on egg white allergens and the resulting IgE reactivity in patients. Given that the structure of the proteins may play a crucial role in IgE binding and eliciting an allergic response, an understanding of the structure and function of each of the four allergens is essential for egg allergy research.

Table 1.2: The four main egg white allergens and their properties (Table adapted from: Caubet and Wang 2011⁸³).

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Egg white (%)</th>
<th>Molecular weight (kDa)</th>
<th>Carbohydrate (%)</th>
<th>IgE binding activity (Digestive)</th>
<th>Allergenic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Heat Treated</td>
<td>Enzyme Treated</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>11</td>
<td>28</td>
<td>25</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>54</td>
<td>45</td>
<td>3</td>
<td>Unstable</td>
<td>Unstable</td>
</tr>
<tr>
<td>Ovotransferrin</td>
<td>12</td>
<td>76.6</td>
<td>2.6</td>
<td>Unstable</td>
<td>Unstable</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>3.4</td>
<td>14.3</td>
<td>0</td>
<td>Unstable</td>
<td>Unstable</td>
</tr>
</tbody>
</table>
1.6.3 Allergens in the egg white

1.6.3.1 Ovomucoid (Gal d 1 or OVM)

Ovomucoid is the most allergenic and the dominant allergen found in the chicken egg white. Constituting 11\% of the total egg white, ovomucoid is a trypsin inhibitory glycoprotein with a molecular weight of 28 kDa\textsuperscript{109,110}. It is comprised of 186 amino acids arranged in three tandem domains, each approximately 60 amino acids in length, containing nine intra-domain disulfide bonds and five carbohydrate side chains. The trypsin inhibitory activity is limited to the second domain, in which a putative reactive site is present for trypsin inhibition\textsuperscript{109,111}. The arrangement of the three domains is also similar to the pancreatic trypsin inhibitor. Previous studies have indicated that allergenicity or IgE binding capacity of ovomucoid is not affected considerably by heat and proteinase activity\textsuperscript{94,112}. This suggests that the structure of the IgE binding epitopes of ovomucoid is mainly linear, however there are some conformational epitopes present in all domains\textsuperscript{110}. This suggests that cooking of eggs may have little or no effect on the allergenicity of ovomucoid. In addition, the third domain of ovomucoid shows more IgE binding activity when compared to the other two domains\textsuperscript{113}. It can be suggested that the third domain would be an ideal target for studies focusing on the production of hypoallergenic variants of ovomucoid that may be used for treatment strategies such as immunotherapy, which forms the overall aim of Chapter 4 of this thesis.
1.6.3.2 Ovalbumin (Gal d 2 or OVAL)

Ovalbumin is the most abundant (58%) protein in the egg white and it is considered to be the second most important allergen in egg hypersensitivity. Ovalbumin is a water soluble glycoprotein made up of 385 amino acids and has a molecular weight of 45 kDa. Mine and Zhang reported that carboxymethylation, urea treatment or heat denaturing at 95°C of ovalbumin does not significantly affect its IgE binding capacity. This suggests that anti-ovalbumin IgE primarily recognizes linear epitopes than conformational epitopes. Similar to ovomucoid, this indicates that cooking may not significantly affect the allergenicity of ovalbumin, however digestive enzymes may affect the allergenecity. The major function of ovalbumin in the chicken egg is currently unknown, however it is a member of the serpin family of proteins, although it is incapable of inhibiting protease activity. This lack of protein inhibitory activity is explained by the absence of an important serpin conformational change in the reactive centre loop of ovalbumin. Ovalbumin is also known to exhibit biological functions including antibacterial and immunomodulating activities. The antibacterial properties are however not exerted by intact ovalbumin, instead, proteolytic digestion of the protein has shown to produce peptide fragments capable of acting against organisms such as, but not limited to, Bacillus subtilis and Candida albicans. It is also believed that ovalbumin may act as a source of amino acids for embryonic development.
1.6.3.3 Ovotransferrin (Gal d 3 or OVT)

Transferrins are a group of iron-binding proteins found in vertebrates that has structural and functional similarities among each member. The molecular mass of these monomeric glycoproteins is about 76.6 kDa, and a molecule of transferrin can bind to two Fe$^{3+}$ with high affinity. Ovotransferrin is one of the members of transferrins expressed in the chicken oviduct. Expression of ovotransferrin in the oviduct is under steroid control and it differs from serum hen transferrin only by an attached carbohydrate$^{119,120}$. In vitro, ovotransferrin exhibits antimicrobial and immunomodulating activities$^{117}$. Ovotransferrin is capable of penetrating the outer membrane of *Escherichia coli*, allowing selective penetration of ions which causes decadence of electrical potential of the cell, which in turn halts the ability of the bacteria to reproduce (bacteriostatis)$^{121}$. Some research has been conducted to study the changes in allergenicity and conformational structure of ovotransferrin when heated. A study conducted by Tong *et al* $^{122}$ showed that the allergenicity of ovotransferrin correlated with the structural changes caused by heating. Unfolding of the structure caused an increase in allergenicity and cleavage and rearrangement of disulfide bonds lead to a decrease in allergenicity$^{122}$. This suggests that ovotransferrin is one of the allergens responsible for hypersensitivities caused by consumption of cooked eggs as well as raw eggs. Therefore, it is wise to suggest that research should be focused on ovotransferrin as much as on ovomucoid and ovalbumin.
1.6.3.4 Lysozyme (Gal d 4 or LYS)

Lysozyme, 14.3 kDa in size, is an enzyme that exhibits anti-microbial activity in the chicken egg, thus providing protection from potential microbial hazards. It is known to exert lytic activity on cell walls of microbes, therefore it is widely used in pharmaceutical products \(^{123}\). It is also used in various food products such as cheese \(^{90,124}\), which therefore poses a risk to egg allergic patients. Structurally, lysozyme contains conformational and sequential epitopes and it has \textit{in vitro} biological functions such as antibacterial, antiviral, immunomodulating and antitumor activities \(^{117,124}\). Although lysozyme is less allergenic than ovomucoid and ovalbumin \(^{83}\), the high occurrence in many food and pharmaceutical products makes it an important target in egg allergy research.

1.6.4 Allergy to egg yolk

As mentioned earlier, hypersensitivity to chicken’s egg is not only caused by the egg white. Chicken’s egg yolk is also responsible for causing IgE-mediated allergy, although with much lower prevalence than egg white hypersensitivity. Unlike allergy to egg white, which is commonly seen in young children with atopic dermatitis, allergy to egg yolk mostly affects during the adulthood. However, underlying immunological reactions to egg yolk allergens are mainly IgE-mediated, and usually produce symptoms such as urticaria, vomiting, abdominal pain and diarrhoea, as in allergy to egg white \(^{125-127}\). Egg yolk allergens include chicken serum albumin (\(\alpha\)-livetin or Gal d 5), yolk glycoprotein 42 (YGP42 or Gal d 6) and potential allergens known as apovitellenin I, apovitellenin VI and phosvitin \(^{92,128,129}\).
Egg yolk allergy is sometimes caused through an IgE-mediated hypersensitivity disorder known as the bird-egg syndrome, in which an individual is initially sensitized to inhalant avian antigens derived from sources such as bird’s blood serum, feathers, droppings and dander \textsuperscript{126} (Figure 1.5). This is different to allergen cross reactivity in which a patient allergic to a certain allergen is reactive to homologous or non-homologous allergens from the same or other species \textsuperscript{130,131}. Majority of patients with bird-egg syndrome claim to have regular exposure to pet birds or poultry where sensitization to airborne avian allergens take place, which then proceed to egg yolk allergy. Patients suffering from bird-egg syndrome suffer from both respiratory and gastrointestinal allergic symptoms such as asthma, rhinoconjunctivitis, edema, diarrhoea and vomiting. However, unlike allergy to egg white, which is predominantly seen in children with atopic dermatitis, bird-egg syndrome mainly affects adults with an obvious gender predisposition where majority of patients tend to be females \textsuperscript{126}. In addition, allergens produced by birds at workplace may also increase IgG levels in excess and can lead to allergic alveolitis. Currently, these IgG levels can be quantified and qualitatively evaluated using the UniCAP system, a type of ImmunoCAP \textsuperscript{132}.
Figure 1.5: An illustration of the Bird-Egg syndrome. An individual develops allergy to hen’s egg yolk following exposure to birds, where sensitization to inhalant avian allergens takes place resulting in respiratory allergy symptoms. The cross reacting allergen which is responsible for producing both respiratory and gastrointestinal allergy symptoms in bird-egg syndrome is identified as α-livetin (chicken serum albumin), a type of serum albumin present in birds and in the egg yolk as well.
1.6.5 Diagnosis of egg allergy

Different diagnostic techniques are used for successful diagnosis of egg allergy. Similar to other allergic conditions, the foremost step in egg allergy diagnosis is assessment of the medical history of the patient for potential evidence for clinical reactivity to eggs and physical examination. This helps to determine whether the patient has experienced adverse reactions to eggs and the potential involvement of the immune system. The medical history examination essentially takes into consideration the allergic conditions the patient has been previously exposed to and evaluates these information with the current symptoms, the type and the amount of food ingested and the timing of the reaction. The physical examination involves testing for gastrointestinal symptoms and detection of atopic features in the body.

The next step involves the use of in vitro or in vivo tests such as skin prick tests (SPT) and RAST/ImmunoCAP tests. These tests assist in determining if the patient possesses egg-allergen-specific antibodies in their immune system. Although these tests provide an estimation of allergen specific IgE in the patient, which positively correlates to the clinical reaction (wheal formation), they fail to fully ascertain diagnosis of clinical symptoms of egg allergy. Increasing levels of IgE may predict the presence of an allergy, however, there is no universal agreed level of IgE to confirm the condition as the IgE levels can differ between different individuals. This may also be due to the discrepancies between the testing method, testing criteria and prevalence of the allergy between studies. However, a recent study conducted by Sicherer et al. has further confirmed the significant predictive capacity of IgE as a marker in egg allergy. They also report of a web-based calculator that uses an algorithm, which is based on novel findings of egg specific IgE, that may allow
clinicians to predict the likelihood of the resolution or persistence of the disease. Supplementary studies on IgE, as a reliable egg allergy marker, may provide useful information for the development of effective diagnostic approaches.

In addition to SPTs and ImmunoCAP tests, elimination diets and oral food challenges can be used for the diagnosis of egg allergy. In an elimination diet diagnosis, once the patient is suspected of being allergic to eggs, a diet excluding eggs is followed to study the ability of the patient to maintain the diet without any hypersensitive reactions over a period of time. In an oral food challenge, the patient is asked to eat/ingest a small amount of egg to reproduce the hypersensitivity symptoms. It is also done in a dose dependant manner where the egg ingestion is increased until the symptoms are observable. Oral food challenge can be done openly, single-blinded, double-blinded and placebo controlled, with the latter being considered the gold standard in diagnosis of major food allergies because it provides information such as the amount of food needed to cause an allergic reaction and the extent of the patient’s reaction. However, the safety of oral food challenge has not been well established or scrutinized and may not be ideal if the patient is known to be anaphylactic to eggs, therefore much care should be taken when implementing this method. Overall, medical history and physical examination, SPTs or ImmunoCAP tests and oral food challenge combined together provides successful diagnosis of egg allergy (Figure 1.6), which can lead to the implementation of treatment strategies for the condition.
Figure 1.6: A typical diagnosis procedure of egg allergy. Once a suspected reaction to egg is reported, a patient undergoes the appropriate steps shown in this figure. (Figure adapted and modified from Eggesbo et al 2001 140).
1.6.6 Treatment of egg allergy

As for other food allergies, currently there is no temporary or permanent cure for egg allergy. Upon successful diagnosis for egg allergy, the most effective treatment is strict dietary avoidance of eggs and products containing traces of eggs. Eggs are an important source of nutrients and thus strict avoidance may cause malnutrition or eating disorders. The egg yolk is known to carry numerous health benefits, but since it is impossible to fully separate the egg yolk from the egg white without allergen contamination, complete avoidance of the egg is the only option, which results in reduced or no uptake of this important nutrient source. As a part of avoidance of eggs, it is important to educate patients how to identify potential egg allergens or traces of eggs in various food products such as cakes, biscuits, cereal and breads, as well as educate them about foods/supplements that can potentially replace eggs in their diet. This may include involvement of a dietician for patients with dietary limitations, such as vegetarians and multiple food allergic patients, to ensure proper intake of vital nutrients.

In addition to dietary avoidance, egg allergic patients are required to avoid vaccines and medications derived from eggs. For example, influenza vaccine is developed in chicken extra embryonic fluid and used extensively worldwide every year. Although the majority of egg allergic patients may not have adverse effects upon administration of influenza vaccine, it is important to take caution to avoid severe or anaphylactic reactions in the individuals that may well react to the egg allergens. These patients should receive the vaccine via a desensitization protocol upon diagnosis with a positive skin test. Yellow fever vaccine is another vaccine derived...
from chicken egg, with few reported cases of anaphylaxis upon administration. Moreover, there are reported cases of anaphylaxis due to the use of lipid emulsions and pharmaceutical products derived from eggs. Occurrence of egg allergens in our daily diet, vaccines and other pharmaceutical products complicates strict avoidance of eggs. Therefore, alternatives are required to treat patients in case of accidental exposure to the egg allergens.

In allergic conditions such as asthma, SIT is used to induce tolerance to the allergens. This method essentially involves administration of small amounts of an allergen to the patient, with slight increments, to induce tolerance to that particular allergen. A study conducted by Buchanan et al indicates that oral immunotherapy (OIT), a form of SIT, may provide a promising treatment strategy for patients without anaphylactic history to eggs. The authors suggest that OIT may even cause a shift in food allergy treatment paradigm from allergen avoidance to active immunotherapy. This is further supported by a recent study in which OIT showed successful desensitization to eggs, however tolerance was maintained only in 1/3 of the study population following discontinuation of the egg diet. However, extreme care should be taken during immunotherapy to avoid potential anaphylactic reactions. In case of an accidental exposure to egg allergens, and acute anaphylaxis, the most common treatment is administration of the hormone and neurotransmitter epinephrine. Upon administration of epinephrine at the right dosage and appropriate route, peripheral vasodilatory effects and bronchoconstriction caused by histamines and other chemical mediators released by allergic reactions are reversed, conditions such as urticaria, erythema and angioedema are reduced and further
release of chemical mediators are suppressed. Currently, epinephrine is available for self-administration by auto-injectors such as Epipen. In summary, allergen avoidance, SIT and administration of epinephrine provides temporary relief from egg allergic reactions. However, these methods can be costly and may have negative effects on the lifestyle of the patient. Therefore, it is imperative to investigate and research on permanent and affordable ways to treat egg allergy.

1.7 Future directions of egg allergy - Cracking the egg

1.7.1 Production of recombinant allergens and hypoallergens

Various research teams around the world are involved in finding answers or cures for different types of allergies. Among these, egg allergy research is no exception. Development of hypoallergenic variants of the egg allergens is given utmost priority by many researchers, because hypoallergens may be used in immunotherapy to desensitize egg allergic patients. In fact, a group from University of Guelph in Canada has studied the efficacy of an engineered ovomuoid third domain at desensitizing a murine model. This hypoallergenic third domain has been produced by directly targeting the IgE binding sites of that domain. A similar approach has been tested in 2004 by an Australian research team in which they successfully developed a T-cell reactive hypoallergenic variant of the latex allergen, Hev b 6.10, by targeting the disulphide bridges of that allergen, therefore it can be suggested that similar methods can be used for the development of hypoallergenic variants of egg allergens. A study conducted by Chen et al confirmed the efficacy of two house dust mite hypoallergens for immunotherapy and recommended one hypoallergen for vaccine
production. Another study conducted on the fish allergen parvalbumin has demonstrated that a hypoallergenic variant of the allergen can increase the production of IgG antibodies in the fish allergy patients, which binds to parvalbumin from various fish types, thus reducing binding of the allergens to IgE antibodies, which in turn reduced allergic reactivity \(^{156}\). In addition to the aforementioned methods, it has been recently reported that allergen conjugation with reducing sugars through the Maillard reaction may help attenuate allergic reactivity to allergens \(^{157}\). Production of IgE reactive recombinant variants of egg allergens is also important in diagnostic methods such as SPT and treatment methods such as SIT. A few hundred allergens have been expressed in recombinant systems, these include, but not limited to birch pollen, dust mites, peanuts and egg allergens \(^{98,158-161}\). Recombinant proteins offer accurate characterisation of the immunological properties of allergens, eliminate the contamination issues present in naturally isolated allergens and paves the way for development of immunotherapy and diagnosis methods with high pharmaceutical quality. Although the current results seem promising, all of the research conducted on hypoallergen and IgE reactive recombinant allergen production requires clinical trials before administration to patients.

### 1.7.2 Production of hypoallergenic chicken eggs

The focus of egg allergy research should not be limited to the aforementioned strategies and invention of drugs/vaccines. The advanced molecular and genetic techniques used in other medical research and life sciences can be utilised for tackling
egg allergy from the root of the cause. Genetic manipulation and protein engineering have become the most successful tools in producing disease free crops and animal products. A team in New Zealand has developed a transgenic calf lacking the milk allergen β-lactoglobulin using RNAi technology. The protein β-lactoglobulin causes mild to severe allergic reactions in individuals, especially children, sensitised to this protein. Using RNAi technology, the research team developed a transgenic calf that lacked the β-lactoglobulin protein in milk that it produced after hormonal induction. The lack of a highly effective treatment method for chicken egg allergy highlights the importance of utilisation of similar techniques to produce eggs that lack the allergic components. As in cow’s milk allergy project mentioned above, it is possible to simply knockdown the expression of the allergens in the chicken eggs using RNAi technology, however knockdown of the four allergens may have negative effects as these proteins comprise the majority of the egg white proteins and may reduce the nutritional value of the eggs. Knockdown of the allergen genes and introduction of hypoallergenic variants to replace the depleted natural allergens appear to be more realistic and a feasible approach to produce hypoallergenic eggs.

Production of hypoallergenic variants of proteins involves introduction of mutations to the IgE binding epitopes of the allergen and these mutations may alter the function of the protein. This is not an issue for hypoallergen production for SIT and SPT, however for the purpose of introducing hyapollergens into the genome of a chicken requires functional hypoallergens that can replace the normal allergens to produce functional eggs that can develop into hypoallergenic chicken. A study conducted by Resnik and Inga has demonstrated that mutagenesis on the transcription factor
Chapter 1

p53 has produced variants with different functions, however a large number of mutants retained their function. This is a concept known as conservative evolution in which protein function is retained with no detrimental effects on the organism after a mutation that changes an amino acid. This suggests that it is possible to alter the IgE binding epitopes of the egg white allergens and yet retain the overall protein function. A hypoallergenic variant of an egg white allergen can then be used to produce transgenic chicken. Production of transgenic chickens has been successfully done by some research groups. The methods used to produce transgenic chicken include gene gun technology, micro-injection, gene delivery to germ line and embryo and the use of nano-particles as gene carriers. Chicken primordial germ cells (PGCs), the precursors for sperm and eggs, are useful for introduction of exogenous genes into chickens and this technique is well established. It can be suggested that a germ line of PGCs carrying the hypoallergen may be introduced to a developing embryo to establish a transgenic model, and at the same time utilize RNAi to disrupt natural allergen expression.

RNAi is not the only option for the development of hypoallergenic chicken eggs. The advancement of techniques such as CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/Cas 9) based genome editing may also allow precision engineering of the chicken genome to edit/change the IgE binding epitopes to produce transgenic hens capable of producing hypoallergenic eggs. Establishment of a hypoallergenic lineage of chickens that can lay hypoallergenic eggs promises a solution for egg allergy patients struggling to completely avoid eggs and/or undergo immunotherapy. If any of the current research on egg allergy proves successful in
the future, a large number of families will benefit by being less apprehensive about eggs or egg contaminated products.

1.8 Synopsis and hypotheses

In summary, the prevalence of egg allergy and the lack of highly effective treatment and management methods demand more research being conducted to develop effective solutions for this condition. Such therapeutics or novel management strategies will significantly improve the quality of life of egg allergic patients while reducing the burden on the governments and societies. This formed the overall hypotheses and aims of subsequent chapters of this thesis, as outlined below.

Firstly, it was hypothesized that cloning and expression of cDNA of four major egg white allergens in *Escherichia coli* (*E.coli*) will allow production of IgE reactive recombinant versions that may be used in future diagnosis and treatment of egg allergy. Chapter 2 of this thesis aimed at cloning and expression of all four egg white allergens in *E.coli* to produce recombinant variants of the allergens and Chapter 3 was aimed at confirming the IgE reactivity of the recombinant allergens produced in Chapter 2 using egg allergic patients’ sera.

Secondly, it was hypothesized that disruption of cysteine-cysteine disulphide bridges using site-directed mutagenesis will allow production of a recombinant hypoallergenic variant of the major egg white allergen ovomucoid (Gal d 1) that may be used in immunotherapy for highly sensitive egg allergic patients. Chapter 4 of this
thesis aimed at disrupting cysteine-cysteine disulphide bridges in the third domain of ovomucoid to produce a hypoallergenic variant and elucidate its IgE reactivity using egg allergic patients’ sera.

Thirdly, it was hypothesized that genetic engineering and manipulation using CRISPR-Cas9 and RNAi will allow knockout/knockdown of ovomucoid in chicken as a hypoallergenic egg production strategy. The first aim of Chapter 5 of this thesis was to knockout ovomucoid from the genome of a chicken fibroblast cell line. The second aim of Chapter 5 was to knockdown ovomucoid in chicken embryos to develop chickens with reduced ovomucoid expression to scrutinize the feasibility of a complete knockout of the gene in chickens.

Chapter 6 of this thesis broadly discusses the importance of the findings presented in Chapters 2-5 in relation to current understanding and position of egg allergy. This chapter also provides future directions of the current project and implications for future egg allergy research, followed by concluding remarks.
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CHAPTER 2

Cloning and expression of major egg white allergens – from oviduct to recombinant protein

Publication:


(This publication includes findings from Chapter 2 & 3 of this thesis)
2.1 Introduction

Allergy to chicken (*Gallus gallus*) egg is a widespread condition affecting 0.5-2.5% of children worldwide \(^{82}\). It is known to be the predominant food allergy among children with atopic dermatitis and, among all children, it is the second most common food allergy \(^{83-85}\). A study conducted by the Murdoch Children’s Research Institute (MCRI) in Australia revealed that 8.9% of infants are allergic to eggs \(^{86}\). Symptoms and conditions caused by hypersensitivity to chicken egg include, but not limited to, atopic dermatitis, bronchial asthma, IgE-mediated egg allergy (with urticarial, angioedema, vomiting, diarrhoea and anaphylaxis), and allergic eosinophilic gastroenteritis (with pain, irritability, colic, and possibly oesophageal stricture and food impaction) \(^{90-93}\).

Egg allergy is mainly caused by 4 major proteins within the egg white \(^{97}\), namely ovomucoid (Gal d 1 or OVM), ovalbumin (Gal d 2 or OVAL), ovotransferrin (Gal d 3 or OVT) and lysozyme (Gal d 4 or LYS), with ovomucoid being the most allergenic of the four and ovalbumin being the most abundant. These proteins are produced in the magnum portion of the chicken oviduct, specifically by tubular gland cells \(^{100,101}\). Previous studies involving administration of oestrogen into chicks have shown that, post injection of oestrogen, the chicks produced the allergenic proteins, indicating that expression of the allergens present in the egg white are highly dependent on oestrogen \(^{102-104}\). Consumption of raw or cooked egg may cause allergic reactions, although the majority of patients with egg allergy are tolerant to cooked egg \(^{105}\).
Production of recombinant versions of natural allergenic proteins is an option for treating allergies since these recombinant proteins can be used in treatment methods such as allergen SIT and diagnostic methods such as skin prick tests (SPT). It is a way of producing allergens with high purity without contamination from other allergens since individual proteins are expressed separately in host systems. SIT essentially involves desensitization of an individual to an allergy by gradually administering the allergen to the patient. The first study of desensitization was reported in 1911 by Leonard Noon, in which grass pollen extract was used for desensitization of hay fever \textsuperscript{168}. This method has since then been used in SIT for other types of allergies \textsuperscript{169-172}. However, the use of crude extracts of allergen sources has shortcomings which lead to further complications, sometimes fatal, in the patient \textsuperscript{160}. These shortcomings include, but not limited to, contamination from other allergens or proteins and other non-allergenic material, underrepresentation of the allergen in the extract, changes in allergenic activities of the allergen and batch to batch variations during and after preparations \textsuperscript{160}. Therefore, recombinant production of allergens is an excellent option for SIT and SPT.

Production of recombinant proteins in host systems such as \textit{Escherichia coli} (\textit{E. coli}) has revolutionized biomedical science for many years. The need to utilize large amounts of animal or plant material to isolate small volumes of a single protein can be considered ancient, simply because of the myriad of advantages offered by recombinant protein expression. \textit{E. coli} has been an excellent choice for this purpose because of a number of reasons. It has extremely fast growth kinetics which is incomparable to other organisms, allowing it to propagate at a steady yet very fast
rate provided necessary media and ideal growth temperatures are given. *E. coli* also allows production of high density cultures for isolation of larger quantities of recombinant proteins. Media required to grow *E. coli* is also easy to prepare using inexpensive constituents. Different strains of *E. coli* also allow easy and efficient transformation of exogenous DNA. All of these features and the vast knowledge about the physiology of *E. coli* make it an excellent workhorse for recombinant protein production\textsuperscript{173,174}.

This chapter aimed at producing recombinant versions of the major egg white allergens OVM, OVAL, OVT and LYS using *E. coli* as a host system. For this purpose, it was necessary to isolate mRNA coding the allergens from a chicken oviduct and convert the mRNA into cDNA, which would be cloned into an expression vector. The allergen-vector constructs were transformed into *E. coli* for protein expression. The expressed proteins were then detected/identified using immunoassays.
2.2 Methods

2.2.1 mRNA extraction from oviduct

Animal experimentation/sampling was conducted under protocol AEC1496, approved by the Australian Animal Health Laboratory (CSIRO-AAHL) Animal Ethics Committee and in accordance with the Australian code of practice for the care and use of animals for scientific purposes.

A 6-month old egg laying hen was humanely euthanized by gassing with CO2 at the animal facility at CSIRO-AAHL. The oviduct was carefully dissected out from the hen (Figure 2.1). The magnum portion of the oviduct was incised and submerged in RNAlater and transported to the laboratory. Upon delivery, the magnum was snap frozen with liquid nitrogen and ground into a fine powder using a sterile, pre-chilled, mortar and pestle, making sure the powder does not thaw in due course. The powder was homogenised according the instructions on the Oligotex Direct mRNA kit (Qiagen, Cat No. 72202). Essentially, 700 mg of the powder was measured and mixed with 4 mL of buffer OL1 containing 30 μL/mL β-mercaptoethanol. 700 μL of the mix was loaded onto a QIAshredder spin column placed in a 2 mL collection tube and homogenised by centrifuging for 2 minutes at 13,000 rpm in a bench top microcentrifuge. 1.2 mL of buffer ODB was added to the homogenised lysate and centrifuged for 3 minutes at 13,000 rpm. The supernatant was transferred to a new RNase-free 2 mL tube and 120 μL of Oligotex suspension (pre-heated at 37°C, vortexed and cooled to room temperature) was added. The mix was incubated at room temperature for 10 minutes and centrifuged for 5 minutes at 13,000 rpm, and the supernatant was discarded. The pellet was resuspended in 100 μL of buffer OL1.
and 400 μL of buffer ODB was added and incubated at 70°C for 3 minutes and then placed at room temperature for 10 minutes. The Oligotex-mRNA complex was then pelleted by centrifuging at 13,000 rpm for 5 minutes and the supernatant was removed by pipetting. The pellet was resuspended in 350 μL of buffer OW1 by pipetting and the suspension was transferred into a small spin column and placed in a microcentrifuge tube. The tube was then centrifuged at 13,000 rpm for 1 minute and the flow-through was discarded. The spin column was transferred to a new RNase-free 2 mL tube and 350 μL of buffer OW2 was added, and centrifuged at 13,000 rpm for 1 minute and flow-through discarded. Residual buffer was removed by briefly re-centrifuging. The spin column was transferred into a fresh RNase-free tube and the resin was resuspended in 100 μL of hot (70°C) buffer OEB. The mRNA was eluted by centrifuging the microcentrifuge tube containing the column at 13,000 rpm for 1 minute. The mRNA concentration was measured on a Thermo Scientific Nano Drop 3300 spectrophotometer (hereafter referred to as Nano Drop). The mRNA was stored at -20°C.
Figure 2.1: The oviduct from an egg laying hen. The figure shows the different parts of a chicken oviduct. The highlighted magnum portion was used to isolate mRNA of OVM, OVAL, OVT and LYS for this project.
2.2.2 Primer design and preparation

The cDNA sequences of OVM, OVAL, OVT and LYS were obtained from the original published sequences to design the primers required to amplify the allergens 175-178. The primers were designed using the Sigma Aldrich DNA calculator. The primer sequences were designed to amplify the target sequence exactly from the start (forward primer or F) and the end (reverse primer or R) of the sequence coding for each allergen (Table 2.1). To make a working stock of 100 μM, the content in each primer tube was reconstituted in (number of nmol x 10) μL of nuclease free water.
Table 2.1: PCR primers for OVM, OVAL, OVT and LYS.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Restriction enzyme site</th>
<th>Sequence: 5’-3’ (Restriction enzyme site bolded)</th>
<th>TM* °C</th>
<th>Supplied nmol per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVM F</td>
<td>XhoI</td>
<td>CGCCTCGAGATGGCCATGGCAGGC</td>
<td>66</td>
<td>50.8</td>
</tr>
<tr>
<td>OVM R</td>
<td>HindIII</td>
<td>CGCAAGCTTTCAGCATTTTCCAAA</td>
<td>57</td>
<td>47.6</td>
</tr>
<tr>
<td>OVAL F</td>
<td>BamHI</td>
<td>CGCGGATCCATGGCTCCATCGGTGCA</td>
<td>69</td>
<td>32.4</td>
</tr>
<tr>
<td>OVAL R</td>
<td>EcoRI</td>
<td>CGCGAATTCTTAAGGGAAACACATCT</td>
<td>58</td>
<td>66.6</td>
</tr>
<tr>
<td>OVT F</td>
<td>XhoI</td>
<td>CGCCTCGAGATGAAGCTCATCCTCTGC</td>
<td>64</td>
<td>45.1</td>
</tr>
<tr>
<td>OVT R</td>
<td>HindIII</td>
<td>CGCAAGCTTTTACCTTGCCCTCAAGGAA</td>
<td>60</td>
<td>40.6</td>
</tr>
<tr>
<td>LYS F</td>
<td>BamHI</td>
<td>CGCGGATCCATGAGGTCTTTGCTAATC</td>
<td>61</td>
<td>39.1</td>
</tr>
<tr>
<td>LYS R</td>
<td>EcoRI</td>
<td>CGCGAATTCCTCACAGCCGGCAGCTCTT</td>
<td>66</td>
<td>38.8</td>
</tr>
</tbody>
</table>

*TM = Melting temperature
2.2.4 PCR of allergens

2.2.4.1 One step RT-PCR

To amplify the cDNA coding for the allergens, Qiagen OneStep RT-PCR (Qiagen, Cat.No. 210210) was used. Template RNA, primer solutions for OVM, OVAL and LYS, dNTP mix, 5x Qiagen OneStep RT-PCR buffer, RNase-free water were thawed, thoroughly mixed and placed on ice. The PCR master mix for each allergen was prepared on ice in a PCR tube according to the parameters in Table 2.2. Please note that template RNA was added after the PCR master mix was prepared and thoroughly mixed by pipetting. The PCR reaction was then carried out in a thermal cycler according to the parameters outlined in Table 2.3.
Table 2.2: Reaction composition for OneStep RT-PCR.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume in reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-free water</td>
<td>Variable (to total 50 μL)</td>
<td>-</td>
</tr>
<tr>
<td>5x Qiagen OneStep RT-PCR buffer</td>
<td>10 μL</td>
<td>1 x</td>
</tr>
<tr>
<td>dNTP mix (containing 10 mM of each dNTP)</td>
<td>2 μL</td>
<td>400 μM of each dNTP</td>
</tr>
<tr>
<td>Forward primer</td>
<td>Variable</td>
<td>0.6 μM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>Variable</td>
<td>0.6 μM</td>
</tr>
<tr>
<td>Qiagen OneStep RT-PCR enzyme mix</td>
<td>2 μL</td>
<td>-</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>Variable</td>
<td>5-10 units/reaction</td>
</tr>
<tr>
<td>Template RNA</td>
<td>Variable</td>
<td>1 pg- 2 μg/reaction</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 μL</strong></td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.3: PCR conditions for OneStep RT-PCR.

<table>
<thead>
<tr>
<th>Cycle step</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>30 minutes</td>
<td>50°C</td>
</tr>
<tr>
<td>Initial PCR activation step</td>
<td>15 minutes</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 minute</td>
<td>94°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>1 minute</td>
<td>50-68°C</td>
</tr>
<tr>
<td>Extension</td>
<td>1 minute</td>
<td>72°C</td>
</tr>
<tr>
<td>Repeat Denaturation-Annealing-Extension cycle for 35 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>10 minutes</td>
<td>72°C</td>
</tr>
</tbody>
</table>
2.2.4.2 Long Range PCR

To amplify the cDNA coding for OVT, a Qiagen LongRange 2-step RT-PCR was used (Qiagen, Cat.No. 206401). Initially, the mRNA was reverse transcribed; the template mRNA, oligo-dT, 5x LongRange RT buffer, dNTP mix and RNase-free water were thawed at room temperature, and stored on ice immediately after thawing. A master mix was prepared according to Table 2.4 in an Eppendorf tube. The tube was briefly spun to collect residual liquid from the tube walls. The tube was incubated at 42°C for 90 minutes. The reaction was then stopped by heating the tube at 85°C for 5 minutes. The reaction mix was stored on ice until the following PCR.

For the PCR, 10x LongRange PCR buffer, dNTP mix, primer solutions (OVT in Table 2.1) and RNase-free water were thawed and mixed thoroughly. A PCR master mix was prepared according to Table 2.5, along with cDNA prepared by reverse transcription. A second reaction was prepared with the PCR optimisation reagent Q-solution added. The PCR was carried out according to the parameters outlined in Table 2.6. The PCR products were then analysed by gel electrophoresis.
Table 2.4: Reverse transcription master mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LongRange RT buffer</td>
<td>4 μL</td>
<td>1x</td>
</tr>
<tr>
<td>dNTP mix (10 mM of each dNTP)</td>
<td>2 μL</td>
<td>1 mM of each dNTP</td>
</tr>
<tr>
<td>Oligo-dT (20 μM)</td>
<td>1 μL</td>
<td>1 μM</td>
</tr>
<tr>
<td>LongRange RNase inhibitor (4 U/μL)</td>
<td>0.2 μL</td>
<td>0.04 U/μL</td>
</tr>
<tr>
<td>LongRange Reverse Transcriptase</td>
<td>1 μL</td>
<td>1x</td>
</tr>
<tr>
<td>Template mRNA</td>
<td>11.8</td>
<td>875 ng</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20 μL</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5 LongRange PCR master mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume in reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LongRange PCR buffer with Mg$^{2+}$ 10x</td>
<td>5 μL</td>
<td>1x ; 2.5 mM Mg$^{2+}$</td>
</tr>
<tr>
<td>dNTP mix (10mM each)</td>
<td>2.5 μL</td>
<td>500 μM of each dNTP</td>
</tr>
<tr>
<td>OVT forward primer</td>
<td>Variable</td>
<td>0.4 μM</td>
</tr>
<tr>
<td>OVT reverse primer</td>
<td>Variable</td>
<td>0.4 μM</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>Variable</td>
<td></td>
</tr>
<tr>
<td>LongRange PCR enzyme mix</td>
<td>0.4 μL</td>
<td>2 units per 50 μL reaction</td>
</tr>
<tr>
<td>Template cDNA</td>
<td>6 μL</td>
<td>100 ng</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50 μL</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6 PCR conditions for LongRange PCR.

<table>
<thead>
<tr>
<th>Cycle step</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial activation step</td>
<td>3 minutes</td>
<td>93°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>15 seconds</td>
<td>93°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>30 seconds</td>
<td>58°C</td>
</tr>
<tr>
<td>Extension</td>
<td>3 minutes</td>
<td>68°C</td>
</tr>
<tr>
<td><strong>Repeat Denaturation-Annealing-Extension cycle for 35 times</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>End of PCR</td>
<td>4°C</td>
<td>Indefinite</td>
</tr>
</tbody>
</table>
2.2.5 Gel electrophoresis of PCR products

1 g of agarose was combined with 100 mL of TAE buffer (Tris-Acetate-EDTA) in an Erlenmeyer flask and warmed up in a microwave to melt the agarose. 5 μL of GelRed™ nucleic acid gel stain (Biotium, Cat.No. 41003) was added to the molten gel mixed thoroughly by swirling. The mixture was then poured into a gel cassette containing a gel comb and set at room temperature. The comb was removed and the gel was placed in an electrophoresis chamber and filled with TAE buffer until the gel was covered. In to the first well of the gel, 20 μL of 1 kb+ DNA ladder was added. 20 μL of each PCR product was then combined with 2 μL of sample loading dye and pipetted in to the remaining wells of the gel. The gel was then run at 100 V for 45 minutes. Upon completion, a gel image was captured using a Chemidoc system.

2.2.6 Gel purification

The PCR products run on the gel (Section 2.2.5) were purified using a QIAquick Gel Extraction kit (Qiagen, Cat.No. 28704). The gel bands containing the PCR products were excised using a clean, sharp scalpel blade and the gel slices were weighed. Each slice (one for each allergen) was put in an Eppendorf tube and 300 μL of buffer QG was added to each tube (1 volume of gel: 3 volumes of buffer). The tubes were incubated at 50°C for 10 minutes, with vortexing every 2-3 minutes, to dissolve the gel slices. Once the gel slices are completely dissolved, 100 μL of isopropanol was added to each tube and mixed. The tubes were placed in 2 mL collection tubes and centrifuged for 1 minute at maximum speed and the flow-through was discarded. The tubes were then washed with 500 μL of buffer QG by centrifugation and the flow-
through was discarded. Then the tubes were washed with 750 μL of Buffer PE, let the columns stand for 5 minutes, centrifuged for 1 minute to remove flow-through and re-centrifuged to remove residual wash buffer. The columns were placed in clean microcentrifuge tubes and the DNA was eluted by adding 50 μL of buffer EB and centrifuging. After adding buffer EB, the columns were let stand for 4 minutes before centrifugation to increase yield. The DNA concentrations were then measured using a Nano Drop.

2.2.7 Restriction enzyme digestion of PCR products

The four gel-purified PCR products (1 μg of each) were digested with the appropriate restriction enzymes (purchased from New England Biolabs); OVM and OVT with XhoI and HindIII & OVAL and LYS with BamHI and EcoRI. Separately, two 1 μg batches of pTrcHisA vector (Invitrogen, Cat.No. V360-20) were digested with XhoI and HindIII & BamHI and EcoRI (Please note that the pTrcHis expression vector comes in 3 different frames (A, B and C). The sequence of version A is in frame with allergen sequences, therefore pTrcHisA was used in this study). The restriction enzyme reactions were set up in microcentrifuge tubes as shown in Table 2.7, mixed thoroughly by pipetting, the tubes were briefly spun and incubated for 1 hour at 37°C. The digests were then gel purified using E-Gel Clonewell 0.8% SYBR Safe agarose gels and concentrations were measured.
Table 2.7: Restriction enzyme digestion of the allergens and the expression vector.

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Digestion Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ovomucoid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVM PCR product</td>
<td>1 µg</td>
<td>1 µL (10 units)</td>
</tr>
<tr>
<td>HindIII</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>Xhol</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>NE Buffer 2</td>
<td>5 µL (1x)</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>0.5 µL</td>
<td>Add to 50 µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>50 µL</td>
<td></td>
</tr>
<tr>
<td><strong>Ovalbumin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVAL PCR product</td>
<td>1 µg</td>
<td>1 µL (10 units)</td>
</tr>
<tr>
<td>EcoRI</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>BamHI</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>EcoRI Buffer (10x)</td>
<td>5 µL (1x)</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>0.5 µL</td>
<td>Add to 50 µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>50 µL</td>
<td></td>
</tr>
<tr>
<td><strong>Ovotransferrin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVT PCR product</td>
<td>1 µg</td>
<td>1 µL (10 units)</td>
</tr>
<tr>
<td>HindIII</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>Xhol</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>NE Buffer 2</td>
<td>5 µL (1x)</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>0.5 µL</td>
<td>Add to 50 µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>50 µL</td>
<td></td>
</tr>
<tr>
<td><strong>Lysozyme</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LYS PCR product</td>
<td>1 µg</td>
<td>1 µL (10 units)</td>
</tr>
<tr>
<td>EcoRI</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>BamHI</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>EcoRI Buffer (10x)</td>
<td>5 µL (1x)</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>0.5 µL</td>
<td>Add to 50 µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>50 µL</td>
<td></td>
</tr>
<tr>
<td><strong>pTrcHisA vector digest 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTrcHis A</td>
<td>2 µL (1 µg)</td>
<td>1 µL (10 units)</td>
</tr>
<tr>
<td>HindIII</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>Xhol</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>NE Buffer 2</td>
<td>5 µL (1x)</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>0.5 µL</td>
<td>Add to 50 µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>50 µL</td>
<td></td>
</tr>
<tr>
<td><strong>pTrcHisA vector digest 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTrcHis A</td>
<td>2 µL (1 µg)</td>
<td>1 µL (10 units)</td>
</tr>
<tr>
<td>HindIII</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>Xhol</td>
<td>1 µL (10 units)</td>
<td></td>
</tr>
<tr>
<td>NE Buffer 2</td>
<td>5 µL (1x)</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>0.5 µL</td>
<td>Add to 50 µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>50 µL</td>
<td></td>
</tr>
</tbody>
</table>
2.2.8 Ligation of PCR products into pTrcHisA expression vector

The four digested PCR fragments and the pTrcHisA vectors were then used in separate ligation reactions. The ligations were carried out with a vector:insert ratio of 1:1 (~20 ng of each) and T4 DNA ligase and ligase buffer with ATP (Promega, Cat.No.: 1801). Ligation reactions were incubated overnight at 4°C. All reactions (one for each allergen) were set up as follows:

- 20 ng of vector DNA (pTrcHisA)
- 20 ng of insert DNA (allergen)
- 1 μL of Ligase buffer
- 0.3 μL (0.9 Weiss units) of T4 DNA ligase
- X μL amount dH₂O to a total of 10 μL

2.2.9 Chemical transformation into E. coli TOP10 strain

The ligated constructs were then transformed into chemically competent E. coli TOP10 cells (Life Technologies, Cat.No. C4040-10). Essentially, 1 vial of frozen E. coli TOP 10 cells for each allergen construct was thawed on ice. 5 μL of ligation was added to each vial and mixed gently by tapping the tube 5-6 times. The vials were incubated on ice for 30 minutes. The cells were then heat-shocked on a heat-block for exactly 30 seconds at 42°C without any shaking or missing. The vials were placed on ice for 2 minutes. Then, 250 μL of pre-warmed LB media was added to each vial aseptically and the tightly capped vials were incubated at 37°C for 1 hour at 225 rpm. The cells from each vial was then plated on LB agar plates with 50 μg/mL ampicillin and incubated overnight at 37°C. Please note that content of each vial was plated on LB agar at different volumes to obtain well separated colonies; 20 μL, 50 μL and 100 μL.
2.2.10 Isolation of plasmid by mini-prep

Five transformant colonies of each allergen were grown separately overnight in LB media with 50 μg/mL ampicillin at 37°C with shaking 225 rpm. The next day, 2 mL of each overnight culture was centrifuged at maximum speed for 5 minutes to pellet the cells. Allergen-pTrcHisA plasmids from these cells were then isolated using a QIAprep Spin Miniprep kit (Qiagen, Cat.No. 27104). The cell pellets were resuspended in 250 μL of buffer P1 (with LyseBlue reagent added at a 1:1000 ratio to buffer P1). Then, to lyse the cells, 250 μL of buffer P2 was added to the resuspended cells and mixed thoroughly by inverting the tube 5-6 times until the mixture became completely blue. To stop the lysis reaction 350 μL of buffer N3 was added and mixed by inverting 5-6 times until the solution became colourless. The solutions were then centrifuged at 13,000 rpm for 10 minutes. The supernatant was applied to QIAprep spin columns by pipetting, centrifuged for 1 minute at maximum speed and flow-through was discarded. The spin columns were then washed with 500 μL of buffer PB, centrifuged and flow-through discarded. The columns were then washed with 750 μL of buffer PE and the spin columns were transferred into collection tubes. The columns were placed in fresh microfuge tubes and 50 μL of elution buffer was added and the let stand for 1 minute. The tubes were centrifuged at maximum speed for 1 minute to elute the plasmid DNA and the eluted DNA was measured using a Nano Drop.
2.2.11 Screening digests to confirm insert and sequencing

The isolated plasmids (5 clones from each allergen) were subjected to a screening restriction enzyme digestion to confirm insertion into pTrcHisA vector. The plasmids were digested according the method described in Section 2.2.7. OVM and OVT were digested with HindIII and XhoI while OVAL and LYS were digested with EcoRI and BamHI. The digested samples were then analysed by gel electrophoresis on 1% agarose gel following the procedure described in Section 2.2.5. Upon confirmation of insert, the clones were sequenced using pTrcHisA sequencing primers; Forward 5’-GAGGTATATATTAATGTATCG-3’ and Reverse 5’-GATTTAATCTGTATCAGG-3’. The sequencing was done by the Micromon DNA sequencing facility at Monash University (Clayton, Victoria, Australia). The sequences were compared with published allergen sequences on IUIS/NCBI by conducting a nucleotide BLAST search.

2.2.12 Pilot expressions - time course

For expression of the proteins, overnight cultures of E. coli TOP10 colonies containing each allergen construct (2 clones for each allergen) were grown separately in LB media with ampicillin (please note that OVAL expression was done by an undergraduate student as part of a honours project and only one clone was expressed). The overnight cultures were then re-inoculated in 10 mL of fresh LB media with ampicillin and grown to mid-log phase (OD600 0.4-0.6). Mid-log phase was reached after approximately 2 hours post inoculation and was measured using a spectrophotometer with an optical density set at OD600. A 1 mL sample of each culture was collected and pelleted and frozen for later analysis. Expression was
induced with 100 μL of 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to give a final concentration of 1 mM and grown for six hours at 37°C with shaking. Samples were collected, pelleted and frozen every 1-hour for determination of optimum expression time.

2.2.13 SDS-PAGE analysis

Each frozen cell pellet collected at different time points was resuspended and lysed with 400 μL of Cell Lytic B cell lysis reagent for ~10 minutes. Samples collected at all the time points were used for the analysis except for OVAL for which only the samples collected at hours 0, 2, 4, 5 & 6 were used. The lysed cells were then centrifuged at 13,000 rpm for 5 mins and the insoluble (pellet) and soluble (supernatant) fractions were separated. The insoluble fractions were resuspended in 100 μL of 2 x Tris-Glycine SDS sample loading buffer (Life Technologies, Cat. No. LC2676). 9μL of these resuspended samples were combined with 1 μL of NuPAGE reducing agent (10x) (Life Technologies, Cat. No. NP0004). The soluble samples (4 μL) were combined with 5 μL loading buffer and 1 μL reducing agent. All samples were then heated at 85°C for 2 minutes and loaded onto 4-20% gradient Novex Tris-Glycine gels (Life Technologies, Cat. No. EC60252BX5) along with the SeeBlue® Pre-stained protein standard (Life Technologies, Cat.No. LC5625). The gels were placed in a XCell II Blot™ Module (Life Technologies, Cat.No. EI0002) and tightened to create an inner and an outer chamber within the module. The inner chamber was filled with Novex® Tris-Glycine SDS running buffer (1x) (Life Technologies, Cat.No. LC2675) until the chamber was overflown. The outer chamber was then half filled with running buffer and the anode
and cathode were connected. The gel was run for ~1.5 hours at 125 V (constant voltage). Upon completion of the gel run, the gels were washed in dH2O to remove running buffer. The gels were then stained in SimplyBlue™ SafeStain (Life Technologies, Cat.No. LC6060) for one hour on a platform rocker. The stain was washed with dH2O and the gels were incubated in fresh dH2O overnight at room temperature with rocking. Next day the gels were dried using Gel-Dry™ drying solution (Life Technologies, Cat.No. LC4025) and the dried gel images were captured by scanning with a white background.

2.2.14 Western blot analysis

SDS-PAGE, as described in Section 2.2.13, was repeated again for 1 clone from each allergen. (Please note that western blot for OVAL was conducted by an undergraduate student as part of an honours project). Upon completion, the proteins on the gels were transferred onto nitrocellulose membranes on a XCell II™ Blot Module according to manufacturer’s guidelines. Essentially, a transfer sandwich was made in the following order: 2x blotting pad-filter paper-gel-nitrocellulose membrane-filter paper-2x blotting pad. The sandwich was then assembled into the module and the inner chamber was filled with Novex® Tris-Glycine transfer buffer (1x) (Life Technologies, Cat.No. LC3675). After connecting the anode and the cathode, the transfer was conducted at 25 V for 2 hours. The proteins on the nitrocellulose membranes were detected using WesternBreeze® chromogenic detection kit (Life Technologies, Cat. No. WB7103). First, the membranes were blocked in 10 mL of blocking buffer for 1 hour, followed by rinsing with dH2O twice (5 minutes each time).
The membranes were then incubated with 10 mL of the primary antibody prepared in blocking buffer for 1 hour. Anti-Xpress mouse monoclonal antibody (Life Technologies, Cat. No. R910-25) was used as the primary antibody at a concentration of 0.5 μg/mL. The unbound antibody was then washed 4 times (5 minutes each time) with 20 mL of antibody wash. The membranes were incubated with 10 mL of provided secondary antibody (anti-mouse IgG-alkaline phosphatase conjugated) for 30 minutes. The membranes were washed 5 times with antibody wash and rinsed 3 times with dH₂O. The membranes were then incubated with 5 mL of chromogenic substrate (5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT)) until purple bands developed on the membrane. When bands appeared, the reaction was stopped by rinsing the membranes 3 times with dH₂O. The western blot was repeated twice using two other primary antibodies: Tetra-His antibody (0.2 μg/mL) and Penta-His antibody (0.2 μg/mL). Images of all the developed blots were then captured on a Chemidoc.

2.2.15 Expression of the proteins in *E. coli* Express I² strain- time-course

The pTrcHisA vectors containing each allergen were transformed into Express I² chemically competent *E. coli* cells (New England BioLabs, Cat. No. C3037H), following manufacturer’s guidelines. Plasmids from the transformed clones were then isolated by mini-prep and were sequenced. Upon confirming the sequences, one clone from each allergen was subjected to a time-course expression to determine optimal expression time. An overnight culture for each allergen was prepared using a single colony. The next day, the overnight culture was used to inoculate 10 mL of fresh LB
media with ampicillin (50 μg/mL) and grown to mid-log phase at 37°C with shaking at 250 rpm. 1 mL of the culture was centrifuged and pelleted to be used as the non-induced control. Expression was induced by adding 36 μL of IPTG (0.4 mM final concentration) to the remaining 9 mL of mid-log phase culture and grown for 6 hours at 37°C with shaking. Every 1-hour, a 1 mL sample was collected and pelleted. All cell pellets were frozen at -20°C until further use.

### 2.2.16 SDS-PAGE and western blot analysis of the expressed proteins

Cell pellets collected (in Section 2.2.15) at time points 0, 2, 4, 5 & 6 were subjected to SDS-PAGE and western blot analysis following the method outlined in Sections 2.2.13 and 2.2.14. For western blotting, only anti-Xpress antibody (0.5 μg/mL) was used as the primary antibody and the images were captured using a camera instead of Chemidoc.

### 2.2.17 Mass-spectrometry analysis of the expressed proteins

Another SDS-PAGE was conducted using the corresponding expression fraction (soluble or insoluble) and the optimum time point of each allergen. For OVM and OVAL the soluble fraction at 2 hours was used, and for OVT and LYS the insoluble fraction at 2 hours was used. Once the gel run was complete, 4 bands conforming to the correct size of each allergen were incised using sterile blades and subjected to mass spectrometry analysis (16 gel bands in total). The gel bands were in-gel-digested with trypsin and incubated overnight at 37°C to digest the proteins into peptides. In-
gel-digest samples were analysed on a LTQ Orbitrap Elite (Thermo Scientific) coupled to an Ultimate 3000 RSLC (rapid separation liquid chromatography) nanosystem (Dionex). The nano-RSLC system was equipped with an Acclaim Pepmap nano-trap column and an Acclaim Pepmap analytical column. 2 μl of the peptide mix (i.e. each digested band) was loaded onto the trap column of 3% CH₃CN containing 0.1% formic acid for 5 minutes before the enrichment column is switched in-line with the analytical column. The LTQ Orbitrap Elite mass spectrometer was operated in the data-dependent mode, whereby spectra were acquired first in positive mode followed by collision induced activation (CID). Ten of the most intense peptide ions with charge states ≥2 were isolated and fragmented using normalized collision energy of 35 and activation Q of 0.25 (CID). Data analysis was carried out using Proteome Discoverer (Thermo Scientific version 1.4) with Mascot against the BIRDS database. Search parameters were precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.6 Da (CID). Carbamidomethyl of cysteine was set as fixed modification and oxidised methionine as a variable modification. Trypsin with maximum of 1 missed cleavage was used as the cleavage enzyme.

2.2.18 Purification of recombinant proteins

Recombinant proteins expressed in both TOP10 and Express Iq E. coli strains were subjected to a number of protein purification procedures using Qiagen Ni-NTA based protein purification kits. However these did not yield satisfactory results, therefore were discontinued.
2.3 Results

2.3.1 PCR amplification of the egg white allergen cDNA

The mRNA isolated from chicken oviduct were used to PCR amplify egg white allergens (OVM, OVAL, OVT & LYS) using one-step PCR method. For Gal d 3, a long-range PCR, that utilises the traditional two-step method, was used. The one-step PCR gel result showed a band for OVM at 800 bp (expected 630 bp), OVAL between 800-2000 bp (expected 1158 bp), no band for OVT (expected 2115) and Gal d 4 at above 400 bp (expected 441) (Figure 2.2). The long-range PCR conducted for OVT with and without Q-solution both produced bands at the expected range band, however the band of the sample without Q-solution was brighter and clearer than the sample with Q-solution (Figure 2.3).
**Figure 2.2: One-step PCR of allergens.** Allergens were PCR amplified using mRNA isolated from a chicken oviduct. The cDNA synthesis and the PCR amplification was done in a single reaction tube in a one-step method. Allergen specific primers with restriction enzyme sites inserted were used for the PCR. Bands were present in OVM (627 bp), OVAL (1163 bp) & LYS (444 bp) lanes while OVT did not have a band at the expected range. The arrows indicate the location of the three PCR products on the gel.
Figure 2.3: Long-range PCR of OVT. This long-range PCR method was conducted with and without the optimisation reagent Q-solution to amplify OVT, using specific primers with restriction enzymes. Both lanes showed a band at the expected size (2137 bp), with the PCR without Q-solution showing the prominent band. The arrow indicates the location of the PCR product on the gel.
2.3.2 Cloning of the PCR amplified egg white allergens into pTrcHisA expression vector

The gel-purified PCR products (allergens) and pTrcHisA vector were digested by restriction enzymes, which showed bands at the corresponding size when analysed by gel electrophoresis post-digestion (Figure 2.4). Undigested pTrcHisA vector appeared at a lower size when compared to the digested samples (Figure 2.4). The digested PCR products were then ligated into their respective digested pTrcHis vector. The ligations were transformed into E. coli TOP10 cells, plasmids isolated and were subjected to a screening digest. The screening digest results showed that 4/5 OVM clones, 5/5 OVAL clones, 5/5 OVT clones and 4/5 LYS clones carried the insert (Figure 2.5). One clone carrying the insert from each allergen was then sequenced. When the obtained sequences were analysed on NCBI BLAST, all sequences showed ≥ 98 % similarity to the published sequences: OVM 99 % (Figure 2.6), OVAL 99 % (Figure 2.7), OVT 98 % (Figure 2.8) and LYS 99 % (Figure 2.9).
Figure 2.4: Restriction enzyme digested PCR products and pTrcHisA vector. OVM & OVT were digested with XhoI & HindIII and OVAL & LYS were digested with BamHI and EcoRI (A). Two lots of pTrcHisA vector were also digested with the same restriction enzyme combinations; XhoI & HindIII and BamHI & EcoRI (B).
Isolated plasmids from five clones of each allergen-pTrcHisA constructs were digested with their respective restriction enzymes. The arrows show the inserts digested out of the allergen-pTrcHisA constructs. OVM (A), OVAL (B), OVT (C) & LYS (D).
Figure 2.6: IUIS/NCBI BLAST search result conducted using the sequence obtained from OVM cDNA cloned into pTrcHisA. When the sequence of OVM (Query) was BLAST searched, Gallus gallus serine peptidase inhibitor SPINK7 (synonym for ovomucoid) mRNA was identified (Sbjct).
**Figure 2.7: IUIS/NCBI BLAST search result conducted using the sequence obtained from OVAL cDNA cloned into pTrcHisA.** When the sequence of OVAL (Query) was BLAST searched, Gallus gallus ovalbumin mRNA was identified (Sbjct).
Gallus gallus TFEW mRNA for ovotransferrin BB type, complete cds
Sequence ID: dbj|AB215094.1  Length: 2118  Number of Matches: 1
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*Figure 2.8: Continued on next page*
Figure 2.8: IUIS/NCBI BLAST search result conducted using the sequence obtained from OVT cDNA cloned into pTrcHisA. When the sequence of OVT (Query) was BLAST searched, Gallus gallus ovotransferrin mRNA was identified (Sbjct).
**Figure 2.9: IUIS/NCBI BLAST search result conducted using the sequence obtained from LYS cDNA cloned into pTrcHisA.** When the sequence of LYS (Query) was BLAST searched, *Gallus gallus* lysozyme mRNA was identified (Sbjct).
2.3.3 Time-course expression, SDS-PAGE analysis and western blot analysis of allergens

Expression of the allergen sequences in *E. coli* was induced with IPTG and samples were collected every one-hour for six hours. The samples collected at hours 0, 2, 4, 5 & 6 were then analysed by SDS-PAGE and western blot. Two clones of each allergen was analysed by SDS-PAGE. The soluble fraction of OVM clone 1 showed an increasing pattern (increasing band intensity with time) at the expected molecular weight of 28 kDa. The insoluble fraction of the same clone or the soluble and insoluble fraction of the second clone did not show a distinct pattern (Figure 2.10). Both clones of OVT showed an increasing pattern of expression with time in both clones (Figure 2.11). LYS, however, did not show a distinct pattern of expression in either of the clones when analysed by SDS-PAGE (Figure 2.12).

When OVM was analysed by western blot using 3 different antibodies, both soluble and insoluble fractions showed expression. The soluble fraction had clearer and distinct bands when compared to the inconsistent pattern of the insoluble fraction. In the soluble fraction of OVM, 5-hours band appeared to be clearer and darker than the other bands (Figure 2.13). Western blot of OVT showed that it was expressed only in the insoluble fraction, with multiple bands appearing throughout the blot, including one at the expected molecular weight (76 kDa) (Figure 2.14). Western blot of LYS showed that the expression is limited to the insoluble fraction at the expected molecular weight (14.7 kDa). The band at 5-hours appeared to be darker than the other bands (Figure 2.15).
As mentioned earlier, SDS-PAGE and western blot for OVAL was done by an undergraduate student as a part of an honours research project. The results obtained from that project are included in this chapter (Figure 2.16). The soluble fraction on the SDS-PAGE and western blot showed bands appearing at the expected molecular weight (45 kDa). The insoluble fraction in the western blot showed distinct bands, whereas the insoluble fraction in the SDS-PAGE did not show distinct bands.
Figure 2.10: SDS-PAGE analysis of two OVM clones after time-course expression.

The cell pellets containing expressed OVM collected at different time intervals of the time-course were lysed and the insoluble and soluble fractions were separated. The insoluble fractions were resuspended in 100 μL of 2 x Tris-Glycine SDS sample loading buffer. 9 μL of these resuspended samples were combined with 1 μL of NuPAGE reducing agent (10x). The soluble samples (4 μL) were combined with 5 μL loading buffer and 1 μL reducing agent. All samples were then heated at 85°C for 2 minutes and analysed on a 4-20% gradient Novex Tris-Glycine gel. A molecular weight marker was included in the first lane of both gels. The gels were stained in SimplyBlue™ SafeStain. (A)-clone 1, (B)-clone 2. The arrows and the boxes show the expected molecular weight of OVM and its location on the gel.
Figure 2.11: SDS-PAGE analysis of two OVT clones after time-course expression. The cell pellets containing expressed OVT collected at different time intervals of the time-course were lysed and the insoluble and soluble fractions were separated. The insoluble fractions were resuspended in 100 μL of 2 x Tris-Glycine SDS sample loading buffer. 9 μL of these resuspended samples were combined with 1 μL of NuPAGE reducing agent (10x). The soluble samples (4 μL) were combined with 5 μL loading buffer and 1 μL reducing agent. All samples were then heated at 85°C for 2 minutes and analysed on a 4-20% gradient Novex Tris-Glycine gel. A molecular weight marker was included in the first lane of both gels. The gels were stained in SimplyBlue™ SafeStain. (A)-clone 1, (B)-clone 2. The arrows and the boxes show the expected molecular weight of OVT and its location on the gel.
Chapter 2

Figure 2.12: SDS-PAGE analysis of two LYS clones after time-course expression. The cell pellets containing expressed LYS collected at different time intervals of the time-course were lysed and the insoluble and soluble fractions were separated. The insoluble fractions were resuspended in 100 μL of 2 x Tris-Glycine SDS sample loading buffer. 9μL of these resuspended samples were combined with 1 μL of NuPAGE reducing agent (10x). The soluble samples (4 μL) were combined with 5 μL loading buffer and 1 μL reducing agent. All samples were then heated at 85°C for 2 minutes and analysed on a 4-20% gradient Novex Tris-Glycine gel. A molecular weight marker was included in the first lane of both gels. The gels were stained in SimplyBlue™ SafeStain. (A)-clone 1, (B)-clone 2. The arrows and the boxes show the expected molecular weight of LYS and its location on the gel.
Figure 2.13: Western blot analysis of OVM time-course expression. Following expression of OVM in E. coli TOP10, cell pellets collected at hours 0, 2, 4, 5 & 6 were lysed and the insoluble and soluble fractions were separated. The samples (time intervals) of both samples were run on 4-20% gradient Novex Tris-Glycine gels and were transferred on to nitrocellulose membranes. The three membranes were incubated with three separate primary antibodies produced in mouse: Anti-Xpress (A), Tetra-His (B) & Penta-His (C) (arrows = expected size of OVM). The blots were incubated with anti-mouse IgG-alkaline phosphatase conjugate (as a secondary antibody) and the bands were detected using a chromogenic substrate (BCIP/NBT). Please note that the 6th hour of insoluble fraction is not included on the blot.
Figure 2.14: Western blot analysis of OVT time-course expression. Following expression of OVT in E. coli TOP10, cell pellets collected at hours 0, 2, 4, 5 & 6 were lysed and the insoluble and soluble fractions were separated. The samples (time intervals) of both samples were run on 4-20% gradient Novex Tris-Glycine gels and were transferred on to nitrocellulose membranes. The three membranes were incubated with three separate primary antibodies produced in mouse: Anti-Xpress (A), Tetra-His (B) & Penta-His (C) (arrows = expected size of OVT). The blots were incubated with anti-mouse IgG-alkaline phosphatase conjugate (as a secondary antibody) and the bands were detected using a chromogenic substrate (BCIP/NBT). Please note that the 6th hour of insoluble fraction is not included on the blot.
Figure 2.15: Western blot analysis of LYS time-course expression. Following expression of LYS in E. coli TOP10, cell pellets collected at hours 0, 2, 4, 5 & 6 were lysed and the insoluble and soluble fractions were separated. The samples (time intervals) of both samples were run on 4-20% gradient Novex Tris-Glycine gels and were transferred on to nitrocellulose membranes. The three membranes were incubated with three separate primary antibodies produced in mouse: Anti-Xpress (A), Tetra-His (B) & Penta-His (C) (arrows = expected size of LYS). The blots were incubated with anti-mouse IgG-alkaline phosphatase conjugate (as a secondary antibody) and the bands were detected using a chromogenic substrate (BCIP/NBT). Please note that the 6th hour of insoluble fraction is not included on the blot.
Figure 2.16: SDS-PAGE and western blot of OVAL time-course expression. (A)- SDS-PAGE of insoluble and soluble fractions of E. coli TOP10 after time-course expression. Fractions collected at 0, 2, 4, 5 & 6 hours were analysed on a 4-20% gradient Novex Tris-Glycine gel. (B)- Western blot analysis of the insoluble and soluble fractions of E. coli TOP10 after time-course expression. Fractions collected at 0, 1, 2, 3, 4, 5 & 6 were run on a 4-20% gradient Novex Tris-Glycine gel and transferred to a nitrocellulose membrane. The blot was incubated with monoclonal anti-ovalbumin antibody produced in mouse as the primary antibody and was then incubated with anti-mouse IgG-alkaline phosphatase conjugate as the secondary antibody. The bands on the blot were detected using a chromogenic substrate (BCIP/NBT). Figure and description adopted from: Chamika De Silva 2012.
2.3.4 Expression of allergens in *Express lq E. coli*

The allergen-pTrcHisA constructs were transformed into *Express lq E. coli* cells and the transformed plasmids were sequenced. The sequencing showed that OVM and LYS were 100% similar to the *E. coli* TOP10 propagated plasmids while OVAL was 98% similar and OVT was 99% similar to the TOP10 sequences (sequencing results not shown). SDS-PAGE and western blot analysis of the expressed proteins showed that OVM and OVAL are expressed in both soluble and insoluble fractions while OVT and LYS were limited to the insoluble fraction (Figure 2.17). OVM showed, in the soluble fraction, no band at 0 hours, a dark band at 2 hours and from thereon diminishing bands with increasing time. The insoluble fraction of OVM showed a dark and large band at the expected range of 28 kDa along with multiple bands of different sizes for all time points, except 0 hours where there were no bands present. OVAL showed a faint band at 0 hours with intensifying bands with time, in the soluble fraction. The insoluble fraction of OVAL showed no band at 0 hours and intensifying bands with increasing time. Both fractions of OVAL showed multiple bands at different sizes, including dark and large bands at the expected size of 45 kDa. OVT showed multiple bands for all time points in the insoluble fraction, including at the expected size of 76 kDa, except for 0 hours where there were no bands. LYS showed a single band at the expected size of 14.7 kDa for all time points except 0 hours in the insoluble fraction, with 2 and 6 hours showing darker bands than 4 and 5 hours.
Figure 2.17: Expression of recombinant allergens in Express I™ E. coli to determine the optimum time point of expression and the fraction where the expressed proteins are found. The transformed Express I™ E. coli cells (for each allergen) were induced with IPTG and grown for 6 hours. Samples were collected and pelleted every 1-hour, including before IPTG induction (0 hours). The pellets representing the time points 0, 2, 4, 5 and 6 were lysed and the soluble and insoluble fractions were run on SDS gels. The gels were then blotted onto nitrocellulose and incubated with Anti-Xpress antibody for detection purposes. The blots were then detected using a chromogenic substrate (BCIP/NBT). (A) OVM, (B) OVAL, (C) OVT, (D) LYS. The arrows indicate the band of interest for each protein.
2.3.5 Mass spectrometry analysis of the expressed proteins

A SDS-PAGE analysis was conducted using the expressed protein fractions. This gel allowed incision of 4 bands from the expected size range of each allergen that was subjected to mass spectrometry analysis (Figure 2.18). Each gel slice was numbered for identification purposes. The mass spectrometry analysis of the recombinant protein bands showed that OVAL, OVT and LYS are positively identified (Table 2.8). The analysis showed no indication of presence of OVM in the protein bands 1-4, that were incised from the gel run with OVM expressed *E. coli* extract. Bands 5-8 incised from the OVAL gel were identified as ovalbumin, 9-12 incised from OVT gel were identified as ovotransferrin, and bands 13-16 incised from LYS gel were identified as lysozyme (Table 2.8).
Figure 2.18: Chosen slices of gels for mass spectrometry analysis following SDS-PAGE of all recombinant allergens. Four slices of gel were incised from the area of the gel each allergen is expressed. Each was numbered according to the figure and subjected to trypsin in-gel digestion and subsequent mass spectrometry analysis.

Table 2.8: Mass spectrometry results of the recombinant allergens.

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<th>Database</th>
<th>Gel section</th>
<th>Identified protein name</th>
<th>Protein score</th>
<th>Sequence coverage (%)</th>
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<tr>
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<td>1</td>
<td>Ovomucoid</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Birds</td>
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<td>Ovomucoid</td>
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</tr>
<tr>
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<td>-</td>
</tr>
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<td>Lysozyme</td>
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2.4 Discussion

Egg white allergy is a widespread disorder mainly affecting children. The wide use of chicken eggs in various food and pharmaceutical products complicates avoidance of egg white, which is the only current management strategy available for egg allergic patients. However, strict avoidance of egg allergens may be very difficult to achieve due to its extensive use in processed foods and pharmaceutical products. Avoidance of eggs may also have nutritional disadvantages to the individual \(^{83,181}\). Allergen specific-immunotherapy (SIT) is currently being pursued as a promising curative treatment for food allergy, including egg allergy. SIT involves the administration of an allergen starting at very small amounts and increasing to a higher maintenance dosing, to promote tolerance by the immune system \(^{12}\). Egg allergic patients react to each allergen at different intensities; therefore, it will be important to administer the allergens separately in SIT to better manage adverse reactions. However, isolating egg white allergens without contamination from the remaining allergens is extremely difficult, time consuming and expensive. Production of recombinant variants of allergens is an effective alternative to natural allergens, since the allergen is produced independently of the others.

*E. coli* is an excellent host organism for expression of recombinant proteins because it offers many advantages compared to other host systems including, but not limited to, easy propagation, production of large culture volumes and easy transformation of exogenous DNA \(^{173,174}\). This chapter aimed at isolating the cDNA of the major egg white allergens, cloning them into an expression vector and expressing the recombinant proteins in *E. coli*.
Isolation of mRNA from an animal tissue can often be a problematic task due the
tendency of mRNA, or RNA, to degrade easily. In this study, the oviduct was obtained
immediately after the hen was euthanized, chopped in to small pieces and preserved
in RNAlater reagent to avoid such degradation. This was crucial to avoid incorrect
cDNA being produced. The success of the mRNA isolation was evident by the
successful PCR amplification of the allergen cDNA. It was necessary to utilize a one-
step PCR method, in which cDNA synthesis and subsequent PCR is done in a single
step, to amplify the cDNA because the conventional PCR using a separately prepared
cDNA library did not yield any results for unknown reasons. The one-step PCR showed
that OVM, OVAL and LYS were successfully amplified, evident by bands appearing at
the expected sizes. OVT was not amplified in the one-step PCR possibly due to its
larger size when compared to the other three allergens. However, this was easily
overcome by using a long-range PCR method, which is designed to amplify large DNA
fragments. In this long-range PCR, an optimisation reagent, Q-solution, was used in
order to see if the quality and the quantity of the DNA could be improved, however
the results indicated that amplification of OVT was better without the Q-solution.

The successful insertion of the PCR products into the pTrcHisA expression vector was
shown by the screening digests that were performed after the PCR products were
inserted into the vector using restriction enzymes. A screening digest is often useful
prior to sequencing to avoid unnecessary costs associated with sequencing. The
sequencing results finally established that the correct allergen sequences were PCR
amplified. The minute discrepancies in the obtained sequences and the published
sequences can be attributed to PCR errors or sequencing errors, however, the extent
of these discrepancies were not deemed a problem for this study. It is important to point out that most of the published sequences of egg allergens are extracted from chickens in countries other than Australia. This study used DNA from a hen from Australia, therefore it is wise to suggest that the minute differences in the sequence may well be due to genetic changes that naturally exist between chickens from different continents.

The proteins were expressed using pTrcHisA for a number of reasons. This vector is designed for efficient and high level expression of recombinant proteins using a trc (trp-lac) promoter \(^ {182}\). This vector also carries lac repressor gene lacI\(^q\) which allows controlled induction of protein expression using IPTG, even in hosts with or without the lacI\(^q\) gene \(^ {183}\). The proteins expressed using this vector are expressed as N-terminal 6x His tagged proteins, which allows detection using anti-His antibodies and purification using immobilized metal ions such as nickel, copper and cobalt in the presence of chelating agent such as nitrilotriacetic acid (NTA). In addition, this vector adds an Xpress epitope to the N-terminus, which allows detection of the expressed proteins using anti-Xpress antibodies. All of these features made this vector an excellent choice for the purpose of this study.

A pilot time-course expression of the proteins was done to determine the optimum time \(E.\ coli\) TOP10 needs to express each protein without causing too much degradation. SDS-PAGE analysis provided a protein profile of all the proteins in the cell extracts, while the western blots assisted in detecting the protein of interest. Although some of the SDS-PAGE results showed a pattern in protein expression,
particularly in OVM and OVT, the gels provided inconclusive results due to the large number of other proteins that seemed to show similar expression patterns. However, the successive western blot results provided important information about how each protein is expressed in *E. coli* TOP10. OVM and OVAL were expressed as soluble and insoluble proteins while OVT and LYS were expressed strictly as insoluble proteins.

When exogenous or endogenous proteins are overexpressed in *E. coli*, these proteins are often accumulated in inclusion bodies of the host cells. When disulphide bonded proteins are expressed in the cytosol of the cells, the reducing environment of the cytosol forces misfolding or unfolding of the proteins, thus causing aggregation of the protein as inclusion bodies \(^{184,185}\). However, a fraction of the proteins containing disulphide bridges may sometimes be appropriately folded and end up as a soluble protein in *E. coli* \(^{186}\). This provides a possible explanation to the expression of OVM and OVAL in both the soluble and insoluble fractions and the restriction of OVT and LYS to the insoluble fraction. Nevertheless, this may not be completely accurate as all four proteins were expressed under similar conditions, hence if overexpression was the sole cause, all proteins should have shown similar results. Biological functions of proteins or chemicals may cause changes in host systems, just as humans react to foreign bodies such as pathogens. Therefore, it’s wise to suggest that formation of inclusion bodies can possibly, or at least partly, be ascribed to the response of the host to the function of the proteins.

Another noticeable feature of the SDS-PAGE and western blot results is the appearance of bands of different sizes in the insoluble fractions. This may well be due
to protein degradation. *E. coli* is known to contain mechanisms of degrading abnormal proteins \(^{187,188}\), which may explain why OVM, OVAL and OVT showed breaking down of the protein by showing multiple bands. It is quite intriguing that LYS is not affected by protein degradation machinery, possibly because the antibacterial properties of LYS counteracts the cell mechanism to degrade it, which is an important characteristic for an antibacterial agent.

Expression of non-degraded recombinant proteins in the soluble fraction of *E. coli* is often advantageous for downstream applications such as protein purification. Availability of soluble proteins eliminates the use of costly refolding experiments post purification. To reduce protein degradation and to potentially express OVT and LYS in the soluble fraction in this study, the allergen-vector constructs were transformed into *Express Iq E. coli* strain. This strain is derived from the highly versatile *E. coli* BL21 strain and is designed for expression of toxic or unfavourable proteins \(^{189}\). The subsequent SDS-PAGE and western blot analysis, following time-course expression using the freshly transformed cells, noticeably indicated a significant reduction in protein degradation, which was evident by appearance of less bands in the insoluble fractions of all four proteins in contrast to the results obtained using the TOP10 strain. OVT and LYS were not solubilized even in this strain, however the yield of OVM and OVAL were significantly increased in both fractions as shown by appearance of prominent bands.

Expression of OVM resulted in an interesting pattern in the soluble fraction, in which the amount of soluble protein was higher at 2 hours than the subsequent hours. OVM
is a trypsin inhibitor and availability of high concentrations of this protein may be toxic for the host cells. It can be suggested that host defence machinery may have limited the expression of soluble, correctly folded and functional OVM with increasing time. This statement can be further supported by the expression of OVAL, which has increased with time, in contrast to OVM. OVAL is considered to be a storage protein with no known adverse effects or toxicity, which may explain why the host cells have allowed excessive production of soluble OVAL as evident by the presence of larger bands in the western blot. Unlike the OVM, OVT or LYS, OVAL has expressed more soluble protein than the insoluble proteins. This can be explained by the conformational structure of OVAL; it contains only one intrachain disulphide bond which is not essential for its native conformation\(^{190}\). Disruption of this disulphide bond still preserves OVAL’s native-like form, although it makes the protein more susceptible to proteolytic digestion due to its fluctuating conformation\(^{190}\). Therefore, expression of this protein in the reducing environment of the cell cytosol does not entirely affect its natural conformation, significantly reducing protein aggregation. The formation of cell aggregates, or inclusion bodies, in a lesser extent may well be due to the unstable conformation in a fraction of the expressed protein resulting in damaged proteins. This feature of OVAL, combined with its reputation as a neutral protein, has allowed expression of a higher amount of soluble protein.

OVT was limited to the insoluble fraction, similar to the *E. coli* TOP10 cells, possibly due the instability caused by the deformation of disulphide bonds. It is known that disruption of a single disulphide bond of OVT makes it less stable compared to its native form, while altering its iron binding properties\(^{191}\). LYS was also limited to the
insoluble fraction, however, with no protein degradation, similar to what was observed when expressed in *E. coli* TOP10. As aforementioned, LYS’s antibacterial properties may have prevented it from being degraded. However, how can the protein be limited to the insoluble fraction, which results from misfolding or unfolding of the protein, and still be functional? Native LYS has four disulphide bonds that are independent of one another 192. Reduced forms LYS is known to refold rapidly (<4 milliseconds) back to its native form, for which none of the disulphide bonds are indispensable 192,193. Therefore, it can be suggested that the reduced forms of LYS are aggregated as soon as the proteins are overexpressed in *E. coli* and later refolding protects the protein from host defences due to the protein’s retained stability and function as an antibacterial.

The mass spectrometry analysis, although successfully identified OVAL, OVT and LYS, failed to identify OVM. Trypsin in-gel digestion was used for the digestion/preparation of the gel slices. The trypsin inhibitory activity of OVM may have affected the results indicating that the recombinant protein is partially or fully functional. An ideal suggestion to confirm the identity of recombinant OVM would be to either use OVM specific antibodies or *N*-terminal amino acid sequencing. However, the appearance of the protein in Figure 2.17 near the molecular weight of 30 kDa corresponds to the expected molecular weight of 28 kDa of OVM. For this mass spectrometry analysis, it was decided to analyse four gel bands per protein from where the protein of interest is expressed on the gel. This was done to ensure that the correct band is identified. However, the results indicated that all four bands were representatives of each allergen, suggesting that the smaller bands may be degraded
proteins while the larger bands may be aggregates. It was only necessary to confirm that the expressed proteins are indeed the allergen of interest, therefore the result of this mass spectrometry analysis was sufficient for this study.

Purification of the expressed recombinant proteins were attempted, however did not yield satisfactory results. Further purifications were not attempted due time and resource limitations. It was decided to use the crude E. coli extracts after protein expression for the successive chapters of this thesis. As a future direction, these recombinant proteins can be purified using a number of methods. All the proteins were expressed with N-terminal 6xHis-tags, therefore Ni-NTA affinity purification followed by high-performance liquid chromatography (HPLC) can be used to purify the proteins for downstream applications including animal based studies or clinical testing. A circular dichroism (CD) spectroscopy analysis of the purified recombinant allergens can be used to obtain detailed structural information, especially for OVM which was not identified by mass spectrometry in this study. The most important characteristic of the produced recombinant allergens is that they are not contaminated with other egg white proteins, which can potentially be used in SIT for patients allergic to a particular/specific egg white allergen. However, the allergenecity, or IgE reactivity, of these recombinant allergens must first be established and is addressed in Chapter 3 of this thesis.
Chapter 2

2.5 Conclusion

Allergy to chicken egg white is a disorder of the immune system mainly affecting children. Pure individual egg white allergens will be beneficial for treatment methods, such as immunotherapy, and diagnostics, such as skin prick tests. Recombinant protein production has been utilized to produce a range of other types of allergens. This study aimed at producing recombinant versions of the 4 major egg white allergens; OVM, OVAL, OVT and LYS. *E. coli* was used as the host system of choice due to the myriad of benefits it offers for recombinant protein production. The results showed that all four proteins were successfully expressed in *E. coli*. These proteins have the potential to be used in downstream applications and potentially be used in animal trials and clinical testing aimed at treating egg allergy.
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CHAPTER 3

Immunological comparison of recombinant allergens with natural eggwhite allergens

Publication:


(This publication includes findings from Chapter 2 & 3 of this thesis)
3.1 Introduction

Food allergy is the major cause of conditions such as asthma, eczema and respiratory allergies in children \(^{80}\). Egg allergy is no exception; it is second only to cow’s milk allergy in the spectrum of food allergies and it affects mainly children \(^{51}\). It is also the most common allergy among children with atopic dermatitis \(^{83,84}\). Egg allergy also affects adults \(^{87}\), however in a smaller scale when compared to the number of affected children. Children with egg allergy normally grow out of the condition by school age \(^{88,89}\), however, due to the severity of the condition which may cause life-threatening anaphylaxis, it remains an immense problem in many societies around the world.

The pathogenesis of egg allergy directly involves the intestinal immune system. The mucosal barrier of the intestine provides the first line of defence against potentially harmful or non-harmful environmental substances including allergens. The intestinal immune system often promotes immune-suppression instead of immune-promotion to avoid unnecessary harm to the individual \(^{49}\). The digestive enzymes, proteases, bile salts and lipases also plays a key role against foreign substances by creating a harsh environment \(^{52}\). However, all of these obstacles are overcome by egg white allergens in individuals with egg allergy; partly due to the rigidity of the egg allergens and partly due to abnormalities in the intestinal immune system \(^{62}\). The abnormalities in the intestinal immune system may be due to genetic predisposition. In egg allergic patients, upon contact with an egg allergen, antigen presenting cells (APCs) found mainly in the lamina propria of the intestine present the allergen/antigen to T-cells. Differentiation of these T-cells causes antigen specific priming of B-cells to produce
excessive amounts of allergen specific IgE. The specific IgE molecules are directly responsible for the release of chemical mediators that cause clinical symptoms of egg allergy.²²⁰

Currently there is no permanent cure for egg allergy. The most advisable management method is strict dietary avoidance, however the occurrence of egg and traces of eggs in a range of food products, pharmaceuticals such as vaccines and other consumables makes it almost impossible to completely avoid egg allergens.⁸³ The most successful egg allergy treatment strategy in the clinic is allergen SIT. Being a food allergy, egg allergy is often treated with OIT, a type of SIT, and has been the most promising method. OIT aims to induce tolerance to an individual allergic to a particular food allergen by orally administering the allergen.¹⁴⁹,¹⁹⁴ Immunotherapy takes into account the role of IgE during the adverse immune reactions and aims to promote stepwise reduction in the production of allergen specific IgE, a process known as allergen desensitization.¹⁹⁵ This immune rehabilitation process may have drawbacks, especially in highly sensitive individuals who may be prone to severe conditions such as anaphylaxis.

During OIT, it is important to consider the allergic reactivity of each patient to specific allergens in the chicken egg. Egg allergy is mainly caused by the four main allergens in the egg white; ovomucoid (OVM), ovalbumin (OVAL), ovotransferrin (OVT) and lysozyme (LYS). Some patients may react to one allergen at higher intensities than to the other allergens. This complicates the process of OIT because natural allergen extracts used in OIT are often not pure, contaminated with other allergens. It is also
difficult to control the amount of natural egg allergens to be administered to the patient. Due the difficulties faced with natural allergens in OIT, production of recombinant allergens has been pursued as an excellent alternative for many allergies. Recombinant allergens are purer than their natural counterparts and free from contamination with other allergens and substances. These recombinant allergens can also be used in safer practice of diagnostic methods such as skin prick tests (SPT).

This Chapter aimed at scrutinizing the IgE reactivity of recombinant OVM, OVAL, OVT and LYS produced in Chapter 2 of this thesis. These recombinant allergens were analysed for their IgE reactivity using egg allergic non-allergic patients’ sera from a well-defined Australian population. Initially, it was necessary to immunologically analyse natural egg white with each allergic sera sample. A pool of egg allergic sera was then made using the most reactive sera samples, which was then used to analyse the IgE reactivity of the recombinant allergens.

3.2 Methods

3.2.1 Preparation of natural egg whites

Commercially available caged and free-range eggs were purchased from a supermarket. Each type of egg was cracked and the contents were put into a sterile porcelain bowl. The egg yolks were carefully removed by sucking the yolks using an empty plastic bottle, ensuring the yolk is not damaged and completely removed. The protein concentration of each egg white was measured using a Nano Drop. 1 volume
of each egg white was then combined with 3 volumes of dH$_2$O and the protein concentrations were measured again.

### 3.2.2 SDS-PAGE of the natural egg whites to determine protein concentration

10 $\mu$g and 20 $\mu$g of each egg white (caged and free-range) was then analysed by SDS-PAGE. 1 $\mu$L (for 10 $\mu$g) and 2 $\mu$L (for 20 $\mu$g) of the egg white preparations were combined with 20 $\mu$L of 2 x Tris-Glycine SDS sample loading buffer (Life Technologies, Cat. No. LC2676). All samples were then heated at 85°C for 2 minutes and loaded onto 4-20% gradient Novex Tris-Glycine gels (Life Technologies, Cat. No. EC60252BX5). The gels were placed in a XCell II Blot™ Module (Life Technologies, Cat.No. EI0002) and tightened to create an inner and an outer chamber within the module. The inner chamber was filled with Novex® Tris-Glycine SDS running buffer (1x) (Life Technologies, Cat.No. LC2675) until the chamber was overflown. The outer chamber was then half filled with running buffer and the anode and cathode were connected. The gel was run for ~1.5 hours at 125 V (constant voltage). Upon completion of the gel run, the gels were washed in dH$_2$O to remove running buffer. The gels were then stained in SimplyBlue™ SafeStain (Life Technologies, Cat.No. LC6060) for one hour on a platform rocker. The stain was washed with dH$_2$O and the gels were incubated in fresh dH$_2$O overnight at room temperature with rocking. Next day the gels were dried using Gel-Dry™ drying solution (Life Technologies, Cat.No. LC4025) and the dried gel images were captured by scanning with a white background.
3.2.3 Western blot analysis of natural egg white to test for non-specific binding of secondary antibody

A SDS-PAGE was conducted using 10 μg of each egg white which were run on two lanes next to each other. SeeBlue® Pre-stained protein standard (Life Technologies, Cat.No. LC5625) was also included in another lane. Upon completion, the proteins on the gels were transferred onto nitrocellulose membranes on a XCell II™ Blot Module. Essentially, a transfer sandwich was made in the following order: 2 x blotting pad-filter paper-gel-nitrocellulose membrane-filter paper-2x blotting pad. The sandwich was then assembled into the module and the inner chamber was filled with Novex® Tris-Glycine transfer buffer (1x) (Life Technologies, Cat.No. LC3675). After connecting the anode and the cathode, the transfer was conducted at 25 V for 2 hours. The membrane was then stained in 10 mL of Ponceau S (Sigma-Aldrich, Cat.No. P7170) for 5 minutes and the stain was washed with dH2O. The membrane was visualised and then destained by washing the blot in 10 mL of 0.1M NaOH until the colour washed off. The blot was then washed 3-4 times with dH2O before proceeding to the next step.

The proteins on the nitrocellulose membranes were detected using WesternBreeze® chromogenic detection kit (Life Technologies, Cat. No. WB7103). First, the membrane was blocked in 10 mL of blocking buffer (hammerstein casein solution in buffered saline- supplied in WesternBreeze® Chromogenic kit) for 1 hour, followed by rinsing with dH2O twice (5 minutes each time). The membrane was incubated with 10 mL of monoclonal anti-human IgE-alkaline phosphatase conjugated-antibody (Sigma-Aldrich, Cat.No. A3076) at a concentration of 1:1000 for 1 hour with agitation. The
membrane was washed 5 times with prepared antibody wash and rinsed 3 times with dH₂O. The membrane was then incubated with 5 mL of chromogenic substrate (5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT)) until purple bands developed on the membrane. When bands appeared, the reaction was stopped by rinsing the membrane 3 times with dH₂O. The developed blot was then captured on a Chemidoc.

### 3.2.4 Immunological analysis of natural egg white against egg allergic patients’ sera

Experimentation involving patients’ sera was conducted in compliance with the National Statement on Ethical Conduct in Human Research (2007) with approval from Deakin University Faculty of Science, Engineering and Built Environment Human Ethics Advisory Group (HEAG), with a project approval number of STEC-34-2013-DHANAPALA.

Egg white from a freshly laid egg was separated from the egg yolk and 1 volume of the egg white was diluted with 3 volumes of dH₂O. 10 μg of the diluted egg white (per well) was run on 4-20% gradient Tris-Glycine gels and blotted on to nitrocellulose as previously discussed in Section 3.2.2 and Section 3.2.3. The Poceau S stained blots were then carefully cut into individual lane strips using sterile scissors, a total of 36 strips were obtained of which 3 contained the molecular weight marker. The strips were then destained by washing with 0.1M NaOH to remove Ponceau S stain. Lastly,
the strips were blocked using the provided blocking buffer for 1 hour and the blocking buffer was washed twice with dH₂O for two minutes.

Allergic and non-allergic patients’ sera were obtained from the Murdoch Childrens Research Institute of the Royal Children’s Hospital (Melbourne, Australia): 25 egg allergic, 5 other allergies, and 10 non-allergic. 100μL of each allergic serum sample was diluted with 900 μL of blocking buffer. The nitrocellulose strips with immobilised egg white proteins were then incubated with each of the diluted serum sample overnight at 4°C (i.e. 1 strip per patient). Next, the strips were incubated with anti-human IgE (alkaline phosphatase conjugated) secondary antibody produced in mouse. Three nitrocellulose strips were incubated with only the secondary antibody as negative controls. The detection was carried out using the WesternBreeze® Chromogenic kit as previously mentioned in Section 3.2.3.

3.2.5 Mass spectrometry analysis of the natural proteins

The 4 bands that appeared to be the 4 allergens were incised from a freshly run gel with egg white and a mass spectrometry analysis was conducted to confirm the identity of the proteins. The gel bands were in-gel-digested with trypsin and incubated overnight at 37°C to digest the proteins into peptides. In-gel-digest samples were analysed on a LTQ Orbitrap Elite (Thermo Scientific) coupled to an Ultimate 3000 RSLC nanosystem (Dionex), as described in section 2.2.17 of this thesis. Data analysis was carried out using Proteome Discoverer (Thermo Scientific version 1.4) with Mascot against the BIRDS database. Search parameters were precursor
mass tolerance of 10 ppm, fragment mass tolerance of 0.6 Da (CID). Carbamidomethyl of cysteine was set as fixed modification and oxidised methionine as a variable modification. Trypsin with maximum of 1 missed cleavage was used as the cleavage enzyme.

### 3.2.6 Immunoassay of recombinant proteins against allergic patients’ sera

The 4 recombinant egg white allergens produced in Chapter 2 were freshly expressed in *E. coli* and 5 mL cultures were pelleted after 2 hours post induction with IPTG. The cell pellets were lysed using Cell Lytic B and the fractions of *E. coli* that showed expression of each allergen were run on SDS-PAGE and blotted onto a nitrocellulose membrane. For OVM and OVAL, the soluble fraction was used and for OVT and LYS, the insoluble fraction was used. This was done in duplicate, one to be used with allergic sera and the other with non-allergic control sera. Another nitrocellulose blot was prepared by immobilising the soluble and insoluble fractions of *E. coli* extract (i.e. *E. coli* containing the pTrcHisA vector without an insert was IPTG induced for 2 hrs and soluble and insoluble fractions were collected).

A pool of egg allergic patients’ sera was prepared by combining 50 μL sera of patients 4, 5, 6 & 7 with 1800 μL of blocking buffer. The 2 mL allergic patients’ serum pool was first pre-incubated with the nitrocellulose membrane containing the *E. coli* extracts for 2 hrs at room temperature. Then one of the nitrocellulose membranes with the recombinant proteins was incubated with the same *E. coli* depleted serum pool overnight at 4°C with agitation. The remaining recombinant protein membrane was
incubated in a pool of non-allergic patients’ sera pool prepared by combining 50 μL of 10 non-allergic sera samples with 1500 μL blocking buffer. All the blots were then incubated with anti-human IgE-alkaline phosphatase conjugated-secondary antibody produced in mouse (1:1000), and detected using the chromogenic substrate, as described in Section 3.2.3.

The above-mentioned western blot was repeated using anti-human-IgE-alkaline phosphatase conjugated-secondary antibody produced in goat (1:1000). In this case, an extra western blot was conducted using the recombinant proteins with anti-Xpress antibody as the primary antibody and anti-mouse IgG-alkaline phosphatase conjugated-antibody as the secondary antibody.

3.3 Results

3.3.1 Preparation of egg white

Freshly laid caged and free-range egg whites were analysed by SDS-PAGE at two different concentrations; 10 μg and 20 μg. There was no visible difference in the protein profile of the two types of eggs. Lanes loaded with 10 μg of egg white showed clearer bands than the lanes loaded with 20 μg (Figure 3.1). The caged and free-range egg whites were then analysed by western blotting using anti-human-IgE antibody. There were no visible bands in the western blot at the beginning of chromogenic development. After 4.5 minutes of chromogenic substrate incubation, faint bands appeared at two different molecular weights (Figure 3.2).
Figure 3.1: SDS-PAGE of egg whites to determine the best amount to use for successive immunological analysis. Egg whites from freshly laid caged and free-ranged eggs were separated from the egg yolks. The egg whites were diluted in 3 volumes of dH$_2$O. 10 μg and 20 μg of each type of egg white was then analysed by SDS-PAGE on a 4-20% gradient Tris-Glycine gel.
Chapter 3

Figure 3.2: Western blot analysis of natural egg whites to detect possible non-specific binding of anti-human-IgE antibody. 10 μg of prepared egg white from caged and free-range eggs were run on a 4-20% gradient Novex Tris-Glycine gel along with the SeeBlue® pre-stained protein standard (MW). Upon completion, the proteins on the gel was transferred onto a nitrocellulose membrane and the protein bands were visualised with Ponceau S solution. After an image of the protein profile was captured, the blot was destained using 0.1 M NaOH and washed with dH₂O. The blot was then incubated with anti-human-IgE-alkaline phosphatase conjugated-antibody and the blot was detected using a chromogenic substrate (BCIP/NBT).
3.3.2 Immunological analysis of natural egg white

Prior to testing of the recombinant proteins, natural egg white was tested against a population of egg allergic and other allergic sera to test for IgE reactivity (allergenicity). The western blots conducted shows that 10 patients were allergic to OVM, 4 to OVAL, 14 to OVT and 18 to LYS (Figure 3.3 and Figure 3.4). A number of individuals showed reactivity to more than 1 allergen as depicted in Table 3.1. Patients who showed reactivity to all 4 allergens had higher egg specific serum IgE levels when compared to the others (Table 3.1). The mass spectrometry analysis conducted on the 4 bands, identified as the allergens, confirmed the identities of the proteins, except for OVM (Table 3.2).
**Figure 3.3: Western blot analysis of chicken egg white against egg allergic patients’ sera.** Natural egg whites were immobilized on nitrocellulose strips membranes and were incubated with egg allergic patients’ sera. The strips were then incubated with anti-human-IgE secondary antibody and bands were detected using a chromogenic substrate (BCIP/NBT). Control strips were incubated only with the secondary antibody.
Figure 3.4: Number of individuals allergic to each natural egg white allergen in an Australian population. This graph shows the number of patients allergic to each allergen as determined from the western blot analysis shown in Figure 3.3.
Table 3.1: The number of allergens each patient is allergic to (as extracted from the western blots on Figure 3.3) and their corresponding egg specific serum IgE levels.

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<th>OVAL</th>
<th>OVT</th>
<th>LYS</th>
<th>Other allergies</th>
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Table 3.2 Mass spectrometry analysis of the natural egg white allergens.

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<th>Protein score</th>
<th>Sequence coverage (%)</th>
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<td>Birds</td>
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<td>Lysozyme</td>
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3.3.3 Immunological analysis of recombinant egg white allergens

The recombinant proteins were then blotted onto nitrocellulose membranes and tested against egg allergic sera and non-allergic sera as the primary antibodies and anti-human-IgE antibody produced in mouse as the secondary antibody. The allergic sera was first pre-incubated with *E. coli* extract, this blot did not appear to have any bands post incubation with secondary antibody and chromogenic development (Figure 3.5). The blot incubated with allergic sera had multiple bands appearing for all allergens at the expected molecular weights, except LYS. OVM had two bands, OVAL had a doublet band and OVT had a prominent band at the expected molecular weight with some faint smeared bands at lower molecular weights (Figure 3.5). The blot incubated with non-allergic sera had bands appearing for OVAL and OVT (Figure 3.5).

A second western blot was conducted using a different secondary antibody; anti-human-IgE antibody produced in goat. Another western blot was simultaneously conducted using anti-Xpress antibody as the primary antibody and anti-mouse-IgG antibody as the secondary antibody. The western conducted with the anti-Xpress antibody showed multiple bands for all allergens at the expected molecular weights, with multiple bands appearing for OVM, OVAL and OVT. OVAL in this blot showed a large band at the expected molecular weight with an excessive number of bands of different molecular weights (Figure 3.6). The blot incubated with allergic patients’ sera showed bands for OVM, OVAL and OVT at the expected molecular weights, with multiple bands appearing at different molecular weights (Figure 3.6). The blot incubated with non-allergic patients’ sera did not have any bands (Figure 3.6).
Figure 3.5: Immunological analysis of recombinant proteins against allergic patients’ sera and non-allergic sera using secondary antibody produced in mouse. The allergic serum pool was pre-incubated with E. coli extract (A). Then two blots were prepared, one was incubated with the pre-incubated allergic sera pool (B) and the other was incubated with the non-allergic sera pool (C). All blots were then incubated with anti-human IgE secondary antibody produced in mouse and developed with a chromogenic substrate (BCIP/NBT). The arrows indicate the band of interest for each allergen.
Figure 3.6: Immunological analysis of recombinant proteins against allergic patients’ sera and non-allergic sera using secondary antibody produced in goat. The allergens were first detected using anti-Xpress antibody to re-affirm the expression of all proteins (A). Then two blots were prepared, one was incubated with an allergic sera pool pre-incubated with E. coli extract (B) and the other was incubated with a non-allergic sera pool (C). Blots (B) and (C) were then incubated with anti-human IgE secondary antibody produced in goat and developed with a chromogenic substrate (BCIP/NBT). The arrows indicate the band of interest for each allergen.
3.4 Discussion

Hypersensitivity to chicken egg is a disorder of the immune system mainly affecting children. As for many other food allergies, strict avoidance of eggs is the most efficient management strategy. However, this approach is rather impractical due to the extensive use of eggs in a range of food products, vaccines and other consumables\(^8^3\). The existence of eggs or traces of eggs in these products increases the risk of accidental exposure of the patient’s immune system to egg allergens, possibly causing unwanted clinical symptoms such as itching, rashes, urticaria, vomiting, and in the worst case, immune reactions such as anaphylaxis. In such instances, use of epinephrine auto-injectors such as the EpiPen may provide temporary relief, however severe reactions may not be relieved \(^1^5^1\).

Immunotherapy is the only curative method pursued by clinicians to treat many types of allergies, including egg allergies. Allergen SIT involves the administration of small doses of an allergen to the patient to induced tolerance to that particular allergen\(^1^4^9\). These allergens are administered subcutaneously, sublingually or orally. OIT is often used for treating food allergies in countries such as Australia and Europe. In this process, patients are orally given extracts of the relevant allergen with increasing doses \(^1^9^4\). OIT is advantageous for food allergic treatment because it takes into account the normal route a food allergen enters the body; the gastrointestinal tract. However, there are drawbacks with OIT, and other types of immunotherapy, due to the unavailability of pure uncontaminated allergens. Recombinant protein production allows production of recombinant allergens with high purity independent of other allergens from the same source. This chapter analysed the IgE reactivity of
the recombinant egg white allergens produced in Chapter 2 of this thesis. The recombinant proteins were analysed using egg allergic and non-allergic patients’ sera.

Prior to testing the produced recombinant allergens against allergic patients’ sera for IgE reactivity, it was necessary to test the obtained patient sera against natural egg white to choose the most allergic serum samples. The Western blot shown in Figure 3.3 indicates that the obtained serum population did not react to all of the allergens at similar intensities, with some samples showing reactivity to only one allergen. This result suggests that egg allergy in Australian populations may mainly be due to allergic reactivity of OVT and LYS, although individuals allergic to OVM showed higher intensity of IgE reactivity. Patients that showed reactivity to all 4 allergens had higher egg specific serum IgE levels, suggesting a possible correlation between the high serum IgE levels and multi-reactivity to different allergens. However, this correlation cannot be confirmed without a larger number of samples to show statistical significance. It should be noted that some individuals who had egg specific serum IgE did not react to any of the allergens, which can be attributed to allergy to egg yolk in these patients, an aspect not addressed in this study. The blot also shows that OVM has appeared at a higher molecular weight when compared to the recombinant protein, possibly due to the heavy glycosylation of natural OVM in egg white. The mass spectrometry analysis showed that OVM was not identified, possibly due to the trypsin inhibitory activity of OVM. The gel slices were trypsin digested for mass spectrometry analysis, therefore OVM may have affected this process thus hindering
the result. The same result was also observed in Chapter 2 when the recombinant proteins were analysed by mass spectrometry analysis.

The 4 serum samples that showed most IgE reactivity to natural egg white were then used to make a serum pool that showed reactivity to all 4 allergens, which was used to test the recombinant allergens for IgE reactivity. The results shown in Figure 3.5 indicate that 3 of the 4 allergens were IgE reactive; with only LYS not showing reactivity. However, non-allergic sera blot also showed bands for OVAL and OVT indicating that the secondary antibody non-specifically binds to these two recombinant proteins. Therefore, it was decided to repeat the western blot using a secondary antibody produced in goat instead of mouse. The blot shows that OVM and OVT had more reactivity when compared to OVAL, consistent with the result seen in Figure 3.3 in which natural OVM and OVT were more allergenic to the 4 chosen serum samples. The serum pool was pre-incubated with \textit{E. coli} extract to deplete the pool of any \textit{E. coli} specific IgE, which could have given a false positive in the experimental blots. However, the results indicate that there were no \textit{E. coli} specific IgE present in the serum pool. The successful use of crude extract shows that it is not vital to purify the natural egg proteins for preliminary IgE reactivity studies like the current study. In addition, the results also show that heating of the protein samples at 85°C for 2 minutes before SDS-PAGE does not appear to affect the IgE reactivity of the proteins. The blot conducted using anti-Xpress antibody confirmed that all proteins are expressed and present on the blot.
The western blots of the recombinant allergens showed the presence of multiple bands for OVM, OVAL and OVT. This is probably due to breakdown of the proteins during expression. Some of the breakdown proteins were IgE reactive; these may have been breakdown products containing IgE binding epitopes of the proteins. Regardless of the protein breakdown, all three proteins had a band at the expected molecular weight. As mentioned earlier, LYS was not found to be IgE reactive for an unexplainable reason. However, it can be suggested that competition from the other three allergens, in particular highly IgE reactive OVM and OVT, may have contributed to the diminished or loss of IgE reactivity of LYS. This may be overcome by conducting the western blot of the allergens separately and independently of one another.

The production of IgE reactive recombinant counterparts of natural egg allergens opens doors for downstream applications, including SIT. IgE reactive OVM has been previously produced by Rupa and Mine \(^{159}\), however the current study focuses on an Australian population, which has not been tested previously. Other research groups have expressed OVAL, OVT and LYS in various expression systems, however their IgE reactivity has not been tested simultaneously against egg allergic patients’ sera, or, poorly studied. The clinical significance of this study is the potential use of purified recombinant proteins for SIT and for diagnostic methods such as SPT. Currently, SPT utilizes whole egg extracts or components isolated from natural eggs for diagnosis\(^{196,197}\). The recombinant proteins produced in this study, due to the lack of contamination with other allergens, may be used in SPT to pinpoint which allergen a patient might be allergic to. Another important application of the produced recombinant proteins is OIT, a type of SIT, in which heat denatured proteins can be
administered orally to induce tolerance. OVM and OVT are known to contain conformational and linear IgE binding epitopes, making them IgE reactive even after heat denaturing, with OVT showing an increase in IgE reactivity after heat denaturing\textsuperscript{122,198}. These properties make these two proteins ideal candidates for OIT. For patients allergic to more than one allergen, the recombinant proteins can be used in a step-wise manner in SIT to induce tolerance without causing an augmented immune response.

3.5 Conclusion

In summary, this study assessed the relative IgE reactivity of four recombinant egg white allergens in a well-defined Australian patient group. The patients’ sera were first tested against natural egg white, which revealed that the patient population is mostly affected by allergenicity to OVT and LYS while OVM showed higher reactivity. The immunoassays conducted to scrutinize the recombinant allergens showed that OVM, OVAL and OVT were IgE reactive while LYS was not IgE reactive. The availability of purified preparations of these IgE reactive recombinant allergens promises to contribute significantly to egg white allergy diagnostics and therapeutics in the future.
CHAPTER 4

Hypoallergenic variant of the major egg white allergen ovomucoid produced by disruption of cysteine bridges

Publication:
Dhanapala P, Doran T, Tang M, Suphioglu C: Hypoallergenic variant of the major egg white allergen Gal d 1 produced by disruption of cysteine bridges: International Archives of Allergy and Immunology (submitted for review).
4.1 Introduction

Hypersensitivity to chicken egg is caused by allergens present in the egg white and egg yolk. Among these, OVM (ovomucoid) is known to be the most allergenic and predominant allergen and it is found in the chicken egg white. This 28 kDa glycoprotein accounts for approximately 11% of the total egg white protein. The tertiary structure of OVM is composed of 186 amino acids which form three domains, with each domain containing approximately 60 amino acids. The tertiary structure is robustly supported by 9 intra-domain cysteine-cysteine disulphide bridges and 5 oligosaccharide side chains. The function of OVM is known to be a trypsin inhibitor, however the trypsin inhibitory activity is limited to the second domain. Hypersensitivity to OVM occurs because of its ability to efficiently bind to IgE. It has 8 IgE binding epitopes, of which some are linear while others are conformational. The highly IgE reactive epitopes present in the third domain make it the most allergenic domain of the three. The presence of linear IgE binding epitopes in OVM makes it resistant to conditions such as heat and/or proteolytic digestion. Since egg allergic patients are often allergic to cooked egg, it can be suggested that OVM plays a crucial role in cooked egg allergy due to its rigidity. These specific features of OVM make it the prime allergen when compared to other allergens in chicken egg and an ideal target for development of egg allergy treatment strategies.

There is no long term cure for egg allergy. Strict avoidance of egg is the currently recommended management strategy, however avoidance is difficult and may cause malnutrition in children, especially in financially disadvantaged families where procurement of more expensive nutritional supplements or food that can replace...
eggs may be difficult. It is also problematic to completely avoid eggs because of the presence of components or traces of egg in various food products, pharmaceutical products and vaccines. Allergen specific OIT offers a potential treatment strategy, not only for egg allergy but also for other type of food allergies. OIT essentially involves the gradual oral feeding of an allergen to the patient in order to induce tolerance. However, OIT can be perilous for some patients, primarily because of the high allergenicity of some allergens and the sensitivity of the patient, which may cause adverse conditions such as anaphylaxis that can even lead to death. Adverse reactions to OIT are currently a potential barrier to clinical application. Therefore, production of less allergenic versions, or hypoallergens, of allergens has been the focus of many research groups, since these hypoallergens can offer improved safety of OIT.

Production of hypoallergenic OVM can be achieved by using mutagenesis as a tool in two different strategies; one, by mutating the sequences of the IgE binding epitopes, two, by targeting the secondary structure of the proteins. Drew et al (2004) successfully produced a hypoallergenic variant of the major latex allergen Hev b 6.10 by disrupting the cysteine-cysteine bonds of the protein to reduce its IgE reactivity. In this study, it was aimed to produce a hypoallergenic variant of OVM by targeting cysteine-cysteine disulphide bridges using site-directed mutagenesis.
4.2 Methods

4.2.1 Site-directed mutagenesis of OVM

The cDNA of OVM was cloned into pTrcHisA expression vector as discussed in Dhanapala et al 2015\textsuperscript{161}. This construct was used for site-directed mutagenesis of nucleotides coding two cysteine residues, using QuickChange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technologies Cat. No. 210513). Two TGC triplicates coding for cysteine 192 and 210 (Figure 4.1) were targeted in order to disrupt two different cysteine-cysteine bridges located in domain III of OVM (Figure 4.2). The TGC codons were changed to GCC codons that code for alanine. Initially, mutagenic primer pairs were designed according to the mutagenesis kit guidelines. The two pairs were named PM7 and PM9, because the mutations were targeting the 7\textsuperscript{th} and the 9\textsuperscript{th} cysteine-cysteine bridges, respectively. The primers are as follows;

PM7 forward 5'-GGCAACAAGTGCAACTTC\textbf{GCC}AATGCAGTCGTGGAAAG-3'
PM7 reverse 5'-CTTTCCACGACTGCATTGGCGAAGTTGCACTTGTTGCC-3'
PM9 forward 5'-ACTCTCACTTTAAGCCATTTTGGAAAA\textbf{GCCC}TGAAGCTTGGCTGT-3'
PM9 reverse 5'-ACAGCCAAGCTTTCAAGGCTTTTCCAAATGGGCTAAAGTGAGAT-3'

The bolded and underlined \textbf{GCC} on forward primers show the mutations. To mutate the OVM cDNA in pTrcHisA vector, the above-mentioned primers and the cDNA constructs (as template DNA) were subjected to a polymerase chain reaction (PCR). The PCR reaction was set up according to Table 4.1. The PCR was then run according to the cycling parameters outlined in Table 4.2. Following the PCR, the reaction was digested with \textit{DpnI} for 5 minutes at 37°C, to digest the non-mutated template DNA. Figure 4.3 outlines the site-directed mutagenesis process.
Figure 4.1: The nucleotide and amino acid sequence of OVM. The squared cysteine (C) residues at positions C192 and C210 are the targeted residues. These were replaced with alanine by mutating the nucleotides to GCC.
Figure 4.2: The secondary structure of OVM showing the total number of cysteine bridges. The two arrows show the two cysteine bridges that would be destroyed by the mutations shown in Figure 1. (Figure adapted from Kato et al 1987).
Table 4.1: Mutagenic PCR master mix components.

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Table 4.2: Mutagenic PCR conditions.

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</tbody>
</table>
Figure 4.3: An outline of the site-directed mutagenesis process. (Figure adapted from QuickChange Lightning Multi Site-Directed Mutagenesis kit instructions manual.)
4.2.2 Chemical transformation into *E. coli*

The mutated plasmids were then transformed into XL10-Gold ultracompetent *E. coli* cells. Essentially, 2 μL of the provided β-mercaptoethanol mix was added to 45 μL of XL10-Gold cells in an Eppendorf and incubated on ice for 10 minutes, with gentle swirling every 2 minutes. Then, 1.5 μL of the *DpnI*-treated mutagenesis reaction was added to the *E. coli* cells, gently mixed and incubated on ice for 30 minutes. The mix was then heat-pulsed for exactly 30 seconds at 42°C on a heat block and incubated on ice for 2 minutes. The reaction was incubated with 0.5 mL of pre-heated LB media at 37°C for 1 hour at 250 rpm. The transformant was then spread-plated on LB agar with 50 μg/mL ampicillin and incubated overnight at 37°C. The next day, 6 clones were grown in fresh LB media with ampicillin and grown overnight. The cells in overnight cultures were pelleted by centrifuging at 13,000 rpm for 5 minutes and subjected to a mini-prep (Qiagen Cat. No. 27104) to isolate the plasmid constructs according to the method outlined in section 2.2.10. The isolated plasmids of the 6 clones were sequenced to confirm the mutations. The sequences were aligned and compared with wild type OVM using the NCBI BLAST tool. The clones that had the correct sequence and the mutations were then transformed into *Express Iq* chemically competent *E. coli* cells (New England BioLabs, Cat.No.: C3037H) following manufacturer’s guidelines. The transformants were plated on LB agar with ampicillin and incubated overnight at 37°C. In addition to the mutant transformants plate, a sample of glycerol-stocked *E. coli* containing the wild-type ovomucoid construct was also plated on LB agar with ampicillin.
4.2.3 Time-course expression of mutant OVM to determine optimum expression time

A single colony of the mutant OVM was grown overnight in LB media with 50 μg/mL ampicillin. The overnight culture was then subcultured in 10 mL of fresh LB media and grown to mid-log phase (OD$_{600}$ 0.4-0.6). A 1 mL sample of the cells was pelleted to be used as the unexpressed control (0 hour) of the time-course expression. Expression was then induced with 40 μL of IPTG and the cells were incubated for 6 hours at 37°C with shaking at 250 rpm. A 1 mL sample was collected every one hour for a 6 hour period. The pellets collected at time points 0, 2, 4, 5 and 6 were lysed using 400 μL of Cell Lytic B (Sigma Aldrich, Cat.No: B7435) lysis reagent and centrifuged at 13,000 rpm for 5 minutes to separate the pellet (insoluble fraction) and the supernatant (soluble fraction). The two fractions were analysed by SDS-PAGE and western blot according to the methods described in Sections 2.2.13 and 2.2.14.

4.2.4 Expression and immunoblotting of wild type and mutant OVM using three different detection antibodies

The wild-type and mutant OVM were expressed in *E. coli* to their optimum time points as determined by the time-course expressions (wild-type OVM optimum time was determined in Chapter 2). Cells were pelleted and lysed using Cell Lytic B as previously described. The soluble fractions of both proteins were run on SDS-PAGE in equal amounts (15 μL), along with a molecular weight marker. A gap lane was left between the two proteins to avoid any cross-contamination between the two variants. The SDS gel was then transferred on to a nitrocellulose membrane to be
used for western blotting. A total of 5 nitrocellulose membranes were prepared this way, of which 2 would be used in the analysis described in Section 4.2.5. Three prepared nitrocellulose membranes were subjected to western blotting using 3 different antibodies that can detect the expressed protein (e.g. anti-Xpress antibody, tetra-His antibody and penta-His antibody).

4.2.5 Immunological analysis of wild type vs. mutant OVM using western blot

Experimentation involving patients’ sera was conducted in compliance with the National Statement on Ethical Conduct in Human Research (2007) with approval from Deakin University Faculty of Science, Engineering and Built Environment Human Ethics Advisory Group (HEAG), with a project approval number of STEC-34-2013-DHANAPALA.

The two remaining nitrocellulose membranes from Section 4.2.4 were used for immunoblotting using egg allergic and non-allergic patients’ sera to test for IgE reactivity. In Chapter 3, a pool of egg allergic patients’ sera and a pool of non-allergic patients’ sera for immunological analysis of recombinant egg white proteins were used. In this study, the same pooled serum preparations were used and incubated one membrane with allergic patients’ sera and the other with non-allergic patient’s sera, and incubated overnight at 4°C. The blots were then incubated with anti-human IgE (alkaline phosphatase conjugated) secondary antibody produced in goat at a dilution of 1:1000. The bands were detected using a chromogenic substrate as used in the western blots described in Section 2.2.14.
4.3 Results

4.3.1 Mutagenesis of OVM

Following site-directed mutagenesis to alter C192 and C210, 6 clones were sequenced to confirm the mutations. Five of the 6 clones had only one mutation present (Figure 4.4). One clone had both of the mutations at the expected locations of the sequence (Figure 4.5). When the wild-type OVM sequence was aligned with the mutant OVM sequence on NCBI BLAST, it was seen that the TGC codons (cysteine) for C192 and C210 had been changed to GCC, which in turn codes for alanine.

4.3.2 Time-course expression of mutant OVM to determine optimum expression time

The mutant OVM protein was expressed in E. coli following IPTG induction for 6 hours, and pellets were collected every one hour, including one before IPTG induction. The pellets from time points 0, 2, 4, 5 and 6 were lysed and the soluble and insoluble fractions were analysed using SDS-PAGE and western blot. The results show that the optimum expression time point for mutant OVM is 5 hours (Figure 4.6), as compared to 2 hours for wild-type OVM (Figure 4.6). It can also be seen that the expression level of mutant OVM decreases after 5 hours.
Figure 4.4: Sequence alignment of wild-type OVM (query) and mutant OVM (Sbjct) showing only one mutation. The outlined region shows the mutation following site-directed mutagenesis. The mutation is at C192, which changed the codon TGC to GCC.
Figure 4.5: Sequence alignment of wild-type OVM (query) and mutant OVM (Sbjct) showing both mutations. The two outlined regions show the two mutations following site-directed mutagenesis. The two mutations are at C192 and C210, which changed the codon TGC to GCC.
Figure 4.6: Time-course expression of the mutant OVM. The mutant OVM (B) was subjected to a time-course expression to determine its optimal expression time and conditions. This was then compared with the time-course expression of the wild-type OVM (A) as previously shown in Chapter 2. The arrows indicate the expected molecular weight of OVM.
4.3.3 Expression and immunoblotting of wild type and mutant OVM using three different detection antibodies

The wild-type and mutant recombinant OVM proteins were expressed in LB till their respective optimum time points by inducing with IPTG. The proteins were analysed by SDS-PAGE and western blotting using three different antibodies (anti-Xpress, Tetra-His and Penta-His antibodies). The SDS-PAGE shows that similar amounts of both proteins are loaded on to the gel (Figure 4.7). The western blots show that there is a slightly higher amount of mutant protein present on the nitrocellulose membrane (Figure 4.7).

4.3.4 Immunological analysis of wild type vs. mutant OVM using western blot

Two nitrocellulose membranes were prepared using the same samples used for the blots shown in Figure 4.7. The two membranes were subjected to western blotting using egg allergic patients’ sera and non-allergic sera. The egg allergic patients’ sera blot shows reduced binding (lighter colouration) for the mutant OVM lane when compared to the wild-type OVM (Figure 4.8). The non-allergic sera blot showed no detectable bands in either of the lanes representing wild-type or mutant OVM (Figure 4.8).
Figure 4.7: Immunoblot comparison of the wild-type and mutant OVM immobilized on nitrocellulose. Three western blots were conducted using His-tag specific antibodies (Tetra-His & Penta-His) and anti-Xpress antibody to compare the expression level of wild-type and mutant (PM7/9) OVM. The arrow indicates the expected molecular weight of OVM.
**Figure 4.8: Immunological comparison of IgE reactivity of wild-type and mutant OVM.** Western blots were conducted, with exactly the same amount of proteins loaded, with egg allergic and non-allergic patients’ sera. Anti-human IgE produced in goat was used as the secondary antibody. Non-allergic controls were used to test for any non-specific binding of secondary antibody. The blots show a loss of IgE reactivity in the mutant PM7/9. The arrows indicate the expected molecular weight of OVM.
4.4 Discussion

Hypersensitivity to chicken egg white is mainly caused by 4 major egg white allergens. Of these, OVM is known to be the most allergenic protein. OVM is known to cause hypersensitivity in its natural or cooked form. This may primarily be due to its rigid tertiary structure that allows it to withstand harsh conditions such as heat and stomach/digestive acids. Due to the lack of an effective curative treatment, strict avoidance is currently the standard method of managing egg allergy. However, this strategy is not feasible due to the difficulty in achieving complete egg avoidance and the high nutritional value of eggs in a balanced diet, especially for children. Induction of tolerance to allergens is a well-established strategy for treatment of different types of allergies such as insect venom or pollen allergy. Immunotherapy, specifically OIT, which is a type of allergen SIT, has been explored for the induction of tolerance to food allergens. OIT involves feeding a patient increasing amounts of raw or cooked versions of the allergen source, in order to induce desensitization or long-lasting tolerance to the allergen \(^{194}\). One barrier to implementation of OIT in the clinical setting is the high rate of adverse reactions necessitating discontinuation of therapy, which primarily involve immediate allergic reactions to the allergen \(^{210,211}\). Recombinant versions of allergens offer an approach to reduce adverse reactions, thereby allowing improved effectiveness \(^{160}\). These recombinant allergens are purer and free from contamination from other allergens of the food source, thus may also be useful for diagnosis of allergy (e.g. skin prick tests or immunoassay). Food allergies, including allergy to chicken egg, may sometimes cause severe reactions such as anaphylaxis. In such patients, use of natural or recombinant allergens for diagnosis or immunotherapy may be associated with unwanted allergic reactions. Therefore,
hypoallergenic, or less allergenic, versions of allergens would be useful in such patients with severe allergic reactions. Production of hypoallergenic variants has been rigorously pursued in allergy research. For example, the production of a hypoallergenic variant of the major latex allergen Hev b 6.01 by site-directed mutagenesis by Drew et al 2004\textsuperscript{154}, and development of a vaccine using hypoallergenic derivatives of the birch pollen allergen Bet V 1 by Niederberger et al 2004\textsuperscript{215}. In this study, a hypoallergenic variant of the major egg white allergen OVM (OVM) that showed reduced IgE reactivity when compared to its wild-type counterpart was developed.

For mutagenesis, it was decided to use alanine as a replacement for cysteine residues at C192 and C210 because it is the most common amino acid that does not have extreme electrostatic or steric effects on the conformation of the protein\textsuperscript{216}. The sequencing result of the 6 clones post mutagenesis showed that 5 clones had only one of the desired mutations present. The mutagenesis kit used in this study allowed introducing multiple mutations in a single reaction. Therefore, the low efficiency can be attributed to factors such as the quality of the template DNA or efficiency of the mutagenic primers. Nevertheless, one clone had both of the desired mutations at C192 and C210, replacing TGC codons (cysteine) with GCC (alanine). OVM secondary structure is made up of three tandem domains (I-III), with domain III showing high IgE reactivity\textsuperscript{113}. By targeting C192 and C210, it was aimed to destroy two cysteine-cysteine disulphide bridges in domain III, thus altering its conformation. It was hypothesized that altering the conformation of domain III may have a significant effect on IgE reactivity of the whole protein.
The mutant OVM was successfully expressed in *E. coli*. A time-course expression was conducted to determine the optimum time point for expression of the mutant protein. It is known that the wild-type recombinant OVM was best expressed at 2 hours post induction with IPTG \(^{161}\). However, the expression pattern of the mutant protein was different to that of the wild-type, as shown in Figure 4. The mutant protein’s expression level increased with time up until 5 hours, as opposed to the wild-type protein’s which showed a reduction in expression after 2 hours. Similar to the wild-type, the mutant was highly expressed in the insoluble fraction, indicating that the expression of the protein causes formation of inclusion bodies in *E. coli*. Nonetheless, the amount expressed in the soluble fraction was sufficient for the remainder of this study.

When analysing two proteins on an immunoblot to compare their reactivity for an antibody, it is essential to immobilize similar amounts of the two proteins. When comparing recombinant proteins, it is crucial that the proteins are purified to allow loading of similar amounts of proteins to a gel to be transferred on to a nitrocellulose membrane. In this study, purified recombinant versions of the wild-type or mutant OVM was not available due to time and resource constraints. Therefore, after inducing expression till the optimum time point of each variant, similar volumes of the crude *E. coli* extracts were loaded onto gels, transferred on to nitrocellulose and subjected to detection using different antibodies to confirm that both proteins are expressed and loaded at similar quantities. When analysed on SDS-PAGE and western blotted using anti-Xpress, Tetra-His and Penta-His antibodies, it was evident that
there was more of the mutant protein immobilized on the nitrocellulose membrane when compared to the wild-type protein. This was not a significant issue as the aim was to test the IgE reactivity of the mutant against the wild-type OVM. It was only vital to ensure that the wild-type protein did not exceed the amount of mutant protein on the membrane. Following the aforementioned immunoassays, the two proteins were compared against each other for IgE reactivity using egg allergic patients’ sera. The blot in Figure 4.8 clearly shows that there is a significantly visible reduction of IgE reactivity in the mutant protein, although there is more of the mutant protein immobilized on the membrane. One may argue that the IgE in the sera may have attached/reacted to \textit{E. coli} protein, however the results in Chapter 3 shows that the IgE in the egg allergic sera used do not react to \textit{E. coli} proteins \textsuperscript{161}. The membrane incubated with non-allergic sera showed no bands, indicating that the secondary antibody, anti-human IgE produced in goat, does not non-specifically bind to the recombinant proteins.

This study shows that disruption of only 2 out of 9 cysteine-cysteine bridges in OVM by targeting C192 and C210, significantly reduces its reactivity to egg-specific IgE. The result also suggests that the structure of the protein plays a crucial role in its allergenicity. This mutant OVM has the potential to be used in safer egg OIT. This study provides preliminary results for future research involving production of hypoallergenic variants of egg allergens, in particular OVM. The result obtained from this study should be followed by further \textit{in vitro} and \textit{in vivo} experimentation. The foremost next step is purification of the protein from the soluble fraction of \textit{E. coli}. The protein was expressed with a 6x histidine tag, therefore nickel affinity
purification techniques can be utilized for this purpose. The purified protein can then be used in B-cell and T-cell activation tests/assays. T-lymphocytes (T-cells) are known to be important in allergic desensitization \(^{217,218}\), therefore it is imperative to test the ability of the hypoallergenic OVM produced in this study to stimulate T-cells. Animal models also play a pivotal role in food allergen research \(^{219}\), therefore it should be suggested that the hypoallergenic OVM produced in this study should undergo animal model based experimentation prior to clinical testing.

4.5 Conclusion

Egg allergy is a wide spread disorder affecting mainly children. Natural and recombinant egg allergens are used in SIT. However, these have disadvantages when dealing with patients with high reactivity to the allergens. In this study, a hypoallergenic variant of the major egg white allergen OVM was produced by disrupting two cysteine-cysteine bridges using site-directed mutagenesis. This hypoallergenic variant, upon purification and further immunological analysis, may be used as an excellent constituent in future immunotherapy vaccines for egg allergy.
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CHAPTER 5

Development of genome engineering (GE) approaches to hypoallergenic egg production
5.1 Introduction

Hypersensitivity to chicken egg is a condition affecting mainly children. Currently, dietary avoidance and allergen SIT are pursued as egg allergy management strategies. Production of recombinant variants of egg allergens and production of hypoallergenic variants of the egg allergens has been the focus of egg allergy research in order to assist in diagnostic methods such as skin prick tests (SPT) and treatment methods such as SIT. However, egg allergy research should not be limited to clinical improvements. The abundantly available knowledge and resources in advanced molecular and genetic techniques used in other medical research and life sciences may be utilised for tackling egg allergy from the root of the cause.

Genetic manipulation and engineering have become successful strategies in producing disease free crops and animal products. A team in New Zealand has developed a transgenic calf lacking the milk allergen β-lactoglobulin using RNAi technology. The protein β-lactoglobulin causes mild to severe allergic reactions in individuals sensitised to this protein. Using RNAi technology, the research team developed a transgenic calf that lacked the β-lactoglobulin protein in milk post hormonal induction. The lack of a highly effective treatment method for chicken egg allergy provides the opportunity to utilize similar techniques to produce eggs that lack the allergic components. It may be possible to simply knockdown the expression of the allergens in the chicken eggs using RNAi technology, however knockdown of the four allergens may have negative effects as these proteins comprises the majority of the egg white proteins and may reduce the reproductive viability and the nutritional value of the eggs. However, a simple knockdown or knockout of certain
allergens may be possible due to the presence of functionally similar homologous proteins in the egg white. It is also imperative to study the importance of these proteins to the developing eggs, which can also be scrutinized by knocking down/out the individual genes.

Fire et al\textsuperscript{220} first published work on RNAi in *Caenorhabditis elegans* (*C.elegans*) which earned them a Nobel Prize in 2006. This mechanism targets exogenous double-stranded RNA (dsRNA), which may be an evolutionarily significant mechanism to target pathogens such as viruses. RNAi may also be involved in regulation of gene expression, in which excessive translation of mRNA coding a specific protein is inhibited. A ribonuclease known as DICER cleaves dsRNA into small non-coding RNAs; microRNA (miRNA) or small interfering RNA (siRNA). These small non-coding RNAs then associates with RNA-induced silencing complex (RISC) to initiate targeting and degradation of exogenous dsRNA\textsuperscript{220-222}. The use of RNAi for human advantage essentially involves “fooling” of the host defence mechanisms. miRNAs or siRNAs complementary to mRNA of a targeted gene can be introduced to the host to promote silencing or knockdown of the targeted gene expression \textsuperscript{46,222,223}. This method can be effectively used for knockdown of allergen genes in chickens to either study the importance of the proteins or to produce hypoallergenic eggs.

RNAi is not the only option for the development of hypoallergenic chicken eggs. The advancement of techniques such as CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-Cas 9)\textsuperscript{167} based genome editing may also allow precision engineering of the chicken genome to edit/change the IgE binding epitopes to
produce transgenic hens capable of producing hypoallergenic eggs. This method can also be used for functional analysis of the allergens by targeted gene knockouts. CRISPR-Cas9 mechanism is used against exogenous DNA elements in bacteria and archaea as a part of their adaptive immune system. Cas9 is a small RNAs guided nuclease which allows genome editing through double-stranded breaks in the targeted gene loci. Non-homologous end joining (NHEJ) or homology-directed repair (HDR), both DNA damage repair pathways, typically repairs the site cleaved by Cas9. NHEJ caused by Cas9 can be harvested to efficiently introduce insertion/deletion (indels) mutations, which can cause a frameshift in the mRNA reading frame, thus resulting in a premature termination of translation. On the other hand, HDR can be utilized to introduce point mutations or to insert specific sequences to the targeted gene loci via recombination using donor DNA templates that are supplied exogenously. NHEJ may be a useful tool in knocking out allergen genes in the chicken genome, while HDR can be used to mutate IgE binding epitopes of the allergens or to introduce sequences for a hypoallergenic variant of the selected allergen into the genome167,224.

Egg white allergy is caused by allergens present in both the egg white and yolk. Of these egg white allergens are considered to be the predominant allergens due to their high IgE reactivity, abundance and structural stability. These allergens are namely ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozyme (Gal d 4)97,98. Ovomucoid, hereafter referred to as OVM, is the most allergenic and the most structurally stable allergen of all. The rigid properties of OVM protect it and its IgE binding properties from cooking and the harsh environment in
the gut, therefore playing a crucial role in egg hypersensitivity caused by both raw and cooked egg\textsuperscript{94,112}. These properties make OVM an excellent target for production of hypoallergenic chicken eggs.

In this chapter, it was aimed to investigate genome engineering strategies to knockout and knockdown OVM. The first aim of this chapter was to utilize CRISPR-Cas9 to knockout OVM in chicken DF-1 fibroblast cells. The second aim was to utilize RNAi to knockdown OVM in chicken to study the feasibility of a complete knockout in chicken.

5.2 Methods

Animal experimentation/sampling was conducted under protocol AEC1496, approved by the Australian Animal Health Laboratory (CSIRO-AAHL) Animal Ethics Committee and in accordance with the Australian code of practice for the care and use of animals for scientific purposes.

Please note that all experiments in this chapter utilized Promega restriction enzymes as opposed to New England Biolabs enzymes used in previous chapters.

5.2.1 CRISPR-Cas9 knockout of OVM in DF-1 cells

For all CRISPR-Cas9 related experimental procedures, a protocol by Ran et al 2013\textsuperscript{224} was used as a reference.
5.2.1.1 Preparation of small guide RNA (sgRNA) expression constructs

DNA sequence of exon 1 of OVM gene, located on chromosome 13 (NCBI accession number: NC_006100), was used to design two small guide RNAs (sgRNAs). The exon 1 sequence shown below was submitted to an online CRISPR Design Tool (http://tools.genome-engineering.org) and two sgRNAs with the highest quality score and the lowest number of off-targets in genes were chosen.

**OVM exon 1:**
ATGGCCATGGCAGGCGTCTTCGTGCTGTTCTCTTTCGTGCTTTGTGGCTTCCTCCCAG

**sgRNA 1:**
CAGCACGAAGACGCCTGCCA (quality score: 87, off-targets in genes: 0)

**sgRNA 2:**
CTGTTCTCTTTCTGCTTTT (quality score: 75, off-targets in genes: 0)

The sgRNAs were then custom made, with a top strand and a bottom strand (reverse complement) for each sgRNA. Both strands had additional nucleic acids in addition to the sgRNA sequence, as shown below;

**sgRNA-top:** CACCGNNNNNNNNNNNNN (N denotes the sgRNA sequence)

**sgRNA-bottom:** AAACNNNNNNNNNNNNNC (N denotes the reverse complemented sgRNA sequence)

Phosphorylation and annealing of the top and bottom strands of sgRNAs and the subsequent ligation into the pSpCas9 (BB)-2A-GFP vector were done according the protocols on Ran et al 2013\(^{224}\).
5.2.1.2 Transformation of sgRNA-pSpCas9 (BB)-2A-GFP constructs into E.coli

The sgRNA-pSpCas9 (BB)-2A-GFP constructs were transformed into electro-competent DH5-α E.coli cells using electroporation. The reactions were set up in microfuge tubes on ice as follows;

- 30 μL of 10% glycerol
- 7 μL of electro-competent E.coli
- 2 μL of sgRNA- pSpCas9 (BB)-2A-GFP construct

The reactions were transferred into chilled electrode gap cuvettes and were pulsed in an E.coli pulser at 2.25 kV with electrical capacitance set at 25 μF and electrical resistance set at 200 Ω. The transformants were then plated on LB agar with ampicillin (100 μg/mL) and incubated overnight at 37°C. The following day, a single colony representing each sgRNA was grown overnight in LB media with ampicillin. Plasmids were isolated from these cultures following the mini-prep procedure outlined in section 2.2.10 of this thesis. The insertion of sgRNA in to pSpCas9 (BB)-2A-GFP was confirmed by sequencing with a U6 promoter forward primer: 5’-GAGGGCCTATTTCCCATGATTCC-3’.

5.2.1.3 Cell culture of DF-1 cells and transfections with sgRNA

Chicken fibroblast cells, DF-1 (ATCC Cat.No. CRL-12203), were cultured in a Corning® 75 cm² cell culture flask in Dulbecco’s modified eagle’s serum media (DMEM) containing 10 % fetal calf serum (FCS), 1% HEPES (4-2-hydroxyethyl-1-
piperazineethanesulfonic acid), 5.6% sodium bicarbonate, 2 mM L-glutamine and 100 μg/mL of each of penicillin and streptomycin antibiotics. After 3 days, the cells in the flask were washed with phosphate buffered saline (PBS-pH 7.4) and were separated from the flask wall and collected by incubating with 2 mL Trypsin for 5 minutes. The cells were then counted using a haemocytometer. The cells were then re-cultured in DMEM in a 24-well plate (100,000 cells per well) according to the plate plan on Table 5.1 and were grown to 90% confluency (approximately 15 hours). The cells were then transfected with the sgRNA constructs in triplicates as shown in Table 1. For transfection of each well 500 ng of sgRNA construct was combined with 100 μL of Opti-MEM® (Life Technologies Cat.No. 51985091) and incubated at room temperature for 5 minutes. At the same time, 2 μL of Lipofectamine® 2000 was combined with 100 μL of Opti-MEM® and incubated for 5 minutes. The two reactions were then combined in a single tube and incubated for 20 minutes at room temperature. The total reaction was then added to the well to transfect the cells.

Please note that before transfections, fresh DMEM were added to each well containing the cultured cells. The transfected cells were grown for approximately 36 hours and transfection efficiency was confirmed by observing under fluorescence microscopy (Leica DMLB) to detect green fluorescence protein (GFP).

Table 5.1: Plate plan of DF-1 cells to be transfected with sgRNA constructs.

<table>
<thead>
<tr>
<th>Untransfected</th>
<th>Untransfected</th>
<th>Untransfected</th>
<th>sgRNA1</th>
<th>sgRNA1</th>
<th>sgRNA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>sgRNA2</td>
<td>sgRNA2</td>
<td>sgRNA2</td>
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5.2.1.4 Enrichment of GFP positive cells by FACS

Fluorescent-activated cell sorting (FACS) media was prepared by filtering phenol red-free DMEM through a 0.22 μM filter to remove antibiotics. The cells in the transfected wells were dissociated from the wells using trypsin and were collected in 15 mL Falcon tubes. The cells were centrifuged at 1350 rpm (200 g) for 5 minutes. The cells were resuspended in 200 μL of FACS media and the cells were filtered through a mesh cap into a strainer tube. The cells expressing GFP were then sorted in a FACSCalibur (BD-Biosciences) flow cytometer. The sorted cells were cultured in DMEM in a 24-well plate and were allowed to expand for 2 weeks. DMEM were changed every 3 days by removing 100 μL of old media and adding 100 μL of fresh media.

5.2.1.5 Functional analysis of sgRNA by SURVEYOR assay

Genomic DNA from the sorted and cultured cells from section 5.2.14 were isolated using a Blood & Cell Culture DNA Mini kit (Qiagen, Cat. No. 69581) following manufacturer’s instructions (two samples prepared: sgRNA1 and sgRNA2). Mutations caused by sgRNAs were detected using a SURVEYOR® Mutation Detection kit (Transgenomic® Cat.No. 706020). Initially, a forward and a reverse primer were designed to amplify the targeted region of OVM exon 1 by the two sgRNAs:

Forward: 5’-CGCGGACCTCAATAAATATTACCAGCCT-3’
A gradient PCR was conducted in a thermocycler using GoTaq® Green Master Mix (Promega Cat.No. M712) according to the reaction and PCR conditions shown in Table 5.2 to determine optimum PCR conditions. For this purpose genomic DNA from sgRNA1 sample was used as template and the PCR products were analysed by agarose gel electrophoresis as described in section 2.2.5. Then another PCR was conducted using the determined optimum temperature to amplify the targeted regions from genomic DNA of sgRNA1, sgRNA2 and wild-type samples. 5μL of each PCR product was analysed by gel electrophoresis. The sgRNA PCR product (test) and wild-type PCR product (reference) were then hybridized to form heteroduplexes and homoduplexes. Essentially, 5μL of PCR products of sgRNA1 & wild-type, sgRNA2 & wild-type, and wild-type & wild-type (reference) were separately mixed in PCR tubes and were hybridized in a thermocycler with the conditions outlined in Table 5.3. The hybridized samples were then digested with SURVEYOR nuclease according the following reaction:

- 400 ng of hybridized DNA
- 0.15 M MgCl2
- 1 μL SURVEYOR Enhancer S
- 1 μL SURVEYOR Nuclease S

The reactions were gently mixed by pipetting and incubated at 42°C for 6 minutes. The SURVEYOR nuclease treated samples were then analysed on a 1% agarose gel.
Table 5.2: Reaction and PCR conditions using GoTaq® Green Master Mix.

<table>
<thead>
<tr>
<th><strong>Reaction Mix</strong></th>
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<tbody>
<tr>
<td><strong>Component</strong></td>
<td><strong>Volume</strong></td>
<td><strong>Final concentration</strong></td>
</tr>
<tr>
<td>GoTaq® Green Master Mix</td>
<td>25 μL</td>
<td>1 x</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1 μL</td>
<td>1 μM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1 μL</td>
<td>1 μM</td>
</tr>
<tr>
<td>Genomic DNA template</td>
<td>1 μL</td>
<td>100 ng</td>
</tr>
<tr>
<td>dH₂O</td>
<td>22 μL</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
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<td>-</td>
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<table>
<thead>
<tr>
<th><strong>PCR conditions</strong></th>
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<tr>
<td><strong>Step</strong></td>
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<tr>
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<td>Annealing (Gradient)</td>
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<td>30 seconds</td>
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<td>Extension</td>
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<td>Final extension</td>
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Table 5.3: DNA hybridization conditions for a thermocycler.

<table>
<thead>
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<th>Temperature</th>
<th>Time</th>
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<tbody>
<tr>
<td>95°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>95°C to 85°C</td>
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<tr>
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<td>4°C</td>
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5.2.2 RNAi knockdown of ovomucoid

5.2.2.1 shRNA design

Two small interfering RNA molecules (siRNAs) targeting chicken ovomucoid gene were designed using the Dharmacon siRNA design tool. They were 18-19 nucleotides in length with a total GC content of less than 50%. To generate effective shRNAs, siRNA sequences were further screened using the criteria previously outlined by Taxman et al 2010 225. Briefly, potential siRNAs were scored for the stability of the duplex formed between nucleotides 6 – 11 on the sense and 14 – 9 on the antisense strands. Those siRNAs with a central duplex ΔG of >-12.9 kcal /mol were discarded. ΔG scores were calculated as described in Freier et al 1986 226 (Figure 5.1). The chosen two sense siRNA sequences are as follows;

\( \text{siRNA1} \) – GAACCAATATCAGCAAAGA (19 nucleotides)
\( \text{siRNA2} \) – GATGTATTGGTTGCAAC (18 nucleotides)
| Candidate Sequences | Start pos or ID | F1 at A or T | F2 at A or T | F3 at A or T | F4 at A or T | A1 at GATC | A2 at GATC | T1 at TGAC | T2 at TGAC | F5 at GATT | F6 at GATT | F7 at GATT | F8 at GATT | F9 at GATT | F10 at GATT | F11 at GATT | F12 at GATT | F13 at GATT | F14 at GATT | F15 at GATT | F16 at GATT | F17 at GATT | F18 at GATT | F19 at GATT | F20 at GATT | Score ≤ 35% of X | TTTT ≤ 4 | TTTTT ≤ 4 | TTTTTT ≤ 4 | TTTTTTT ≤ 4 | TTTTTTTT ≤ 4 | Total Score (max 8) |
|---------------------|----------------|-------------|-------------|-------------|-------------|------------|------------|-----------|------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| 2 GAACCAATATCACGCAAAGA | 1 1 1 | -9.7 | 1 | yes | 2 | 5 |
| 3 GGCCAAATGATGGTGGTTT | 1 1 2 | -10.8 | 1 | yes | X | 5 |
| 4 GGCCAAATGATGGTGGTTT | 1 1 2 | -10.8 | 1 | yes | X | 5 |
| 5 GATGATTGGTTTGGCAACA | 1 1 1 | -10.6 | 1 | yes | 4 |
| 6 CAGACATGGGAAAGGCAAAGA | 1 1 1 | -12.3 | 1 | yes | 4 |
| 7 GAGGATGTGATGTGGATGT | 1 1 1 | -12.4 | 1 | yes | 4 |
| 8 CAGGAGAGATGGCGAGAAA | 1 1 1 | -12.8 | 1 | yes | 4 |
| 9 GAGAATGCGCAAGCACCTT | 1 1 2 | -13.4 | 0 | yes | 4 |
| 10 GAGGAGGCTGGGGAACTTTA | 1 1 2 | -13.9 | 0 | yes | 4 |
| 11 GCCTGGGCGAGCTGGTTGA | 1 1 2 | -13.9 | 0 | yes | 4 |
| 12 AGGGGACGATATGTTTTT | 1 1 2 | -16.6 | 1 | yes | X | 3 |
| 13 AAGATGATATATGCTGCTTC | 1 1 2 | -11.5 | 1 | yes | 3 |
| 14 CTCGCGATGGGCTGGCAA | 1 1 1 | -13.5 | 0 | yes | 3 |
| 15 TACTGCGAGAATGCGACCTA | 1 1 1 | -13.7 | 0 | yes | 3 |
| 16 CGTCTGCTGACTGATGCTG | 1 1 1 | -13.9 | 0 | yes | 3 |
| 17 CTTCCCGAGATGTGCTGCTTT | 1 1 2 | -14.8 | -1 | yes | X | 3 |
| 18 TACCGGACGTGGCGTGGTTT | 1 1 2 | -14.8 | -1 | yes | X | 3 |
| 19 CCTGGGCGAGATGGCTGCTG | 1 1 2 | -16.2 | -1 | yes | X | 3 |

**Figure 5.1: Screening of siRNA selected by Dharmacon algorithm for suitability.** The highlighted siRNAs were chosen as these met all the requirements and had a higher score than other candidates. Please note that only 19/50 candidates are shown here.
5.2.2.2 U6 promoter driven RNAi knockdown of OVM in DF-1 cells using the shRNAs

In this section, the chosen siRNAs were used in a cell culture based experiment to knockdown OVM.

5.2.2.2.1 Preparation of shRNA driven by chicken U6 promoter.

The two siRNAs designed in section 5.2.2.1 were then used to produce two short hairpin RNA (shRNA) expression constructs via a one-step PCR method;

\[
\text{shRNA1 (named shOvm1)-}
\]

CTCGAGTTCCAAAAAAAGACCAATATCAGAAAGATCTTTGCTGATATTGCTTAAACCCCAGTGTCTCTCGGA

\[
\text{shRNA2 (named shOvm2)-}
\]

CTCGAGTTCCAAAAAAAGATGTTATGGTTGGAACATCTTTGCAATGTGCAACCACCATCAAAACCCCAGTGTCTCTCGGA

The shRNA sequences shown above essentially have the following components in the given order: Terminator-Sense siRNA (bolded)-Loop-Anti sense siRNA (bolded)-Part of chicken U6-4 promoter. The reverse sequence of the two shRNAs were custom made as oligos and were used in a PCR reaction to combine the hairpin to a chicken U6 promoter. The PCR was carried out as a gradient PCR using GoTaq® Green Master Mix (see section 5.2.1.5) with the following temperatures: 59.1°C, 62.6°C, 64.3°C, 66.1°C, 69.4°C and 72°C. The desired PCR products of each shRNA (+ U6-4 promoter) were gel purified according the method described in section 2.2.6. The gel purified PCR products were then ligated into pGEM®-T Easy (Promega Cat.No. A1360) which
does not require restriction enzyme (RE) digestion prior to insertion. The ligations were then transformed into electro-competent *E. coli* as described in section 5.2.1.2. These cells were propagated and plasmids were isolated by mini-prep as described in section 2.2.10. To confirm the insertion of shRNAs (including U6 promoter), the plasmids were digested with EcoRI (this site is present either side of the multiple cloning site of pGEM®-T Easy) and analysed by agarose gel electrophoresis. Vectors containing the insert were then sequenced to confirm sequence validity of shRNAs and U6 promoter using the pUC/M13 forward sequencing primer: 5’-CGCCAGGTTTTCCCAGTCACGAC-3’. The constructed vectors were named pU6-4shOvm1 and pU6-4shOvm2.

### 5.2.2.2 Insertion of OVM target region into pEGFP_C vector

The next task was to insert the region of OVM targeted by the shRNA into pEGFP_C vector which expresses proteins with a C-terminal fusion to green fluorescence protein (GFP). Initially, two primers were designed with a RE added to each primer;

**Forward-5’** CGCAGATCTGCTGAGGT GGACTGCAGT-3’ RE site: BglII  
**Reverse-5’-** CGCAAGCTTTCAGCATTTTCCAGGACTGCAGT-3’ RE site: HindIII

A PCR was conducted using the above primers to amplify the targeted OVM region from OVM cDNA inserted into pTrcHisA vector used in Chapters 2 & 3. Then the PCR product was ligated into pGEM®-T Easy vector using T4 DNA ligase as previously discussed in section 2.2.8 and the ligation was transformed into electro-competent
*E. coli*. Vectors were re-isolated from 10 transformant clones by mini-prep and the vectors were subjected to a screening digest with EcoRI to confirm successful insertion. Six clones bearing the insert were then sequenced to confirm the validity of the sequences. The obtained sequences were searched on NCBI/BLAST to confirm it matches with the sequence of OVM. A sample of pGEM®-T Easy containing the correct insert was digested with REs BgIII and HindIII with the relevant buffers to cut out the OVM fragment. A sample of pEGFP_C was also digested with the same enzymes. The digested pGEM®-T Easy and pEGFP_C vector were run on a 1% agarose gel and the bands of interest were gel purified. The OVM fragment was then ligated into pEGFP_C with T4 DNA ligase. The ligation was transformed into electro-competent *E. coli*, propagated and plasmids were isolated by mini-prep. The constructed plasmid was named pEGFP_C_OVM.

### 5.2.2.2.3 Cell culture and transfection

DF-1 chicken fibroblast cells were cultured in a 75 cm² flask as described in section 5.2.1.3. The cells were then subcultured into a 24-well plate as shown in Table 5.4. The cells were grown to 80% confluency and were co-transfected with pEGFP_C_OVM containing the targeted OVM sequence and pU6-4shOvm1 or pU6-4shOvm2 containing the shRNA. The transfections were done as described in section 5.2.1.3, however 500 ng of pEGFP_C_OVM and 500 ng of pU6-4shOvm1 or pU6-4shOvm2 (total of 1 μg) were transfected. As a control, some cells were left untransfected. In addition to the untransfected cells, two negative controls were done: pEGFP_C_OVM only and pEGFP_C_OVM + Irrelevant shRNA. The positive
control cells were transfected with shEGFP which specifically targets EGFP in pEGFP_C_OVM vector. All the cells were then grown for 48 hours, visualised under fluorescence microscopy, and measured for mean fluorescence intensity using FACS. The mean fluorescence intensities of each treatment were then blotted against each other in a bar graph.

Table 5.4: Plate plan of DF-1 cells to be transfected with shRNA constructs

<table>
<thead>
<tr>
<th>Untransfected</th>
<th>Untransfected</th>
<th>Untransfected</th>
<th>pEGFP_C_OVM Only</th>
<th>pEGFP_C_OVM Only</th>
<th>pEGFP_C_OVM Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEGFP_C_OVM + Irrelevant shRNA</td>
<td>pEGFP_C_OVM + Irrelevant shRNA</td>
<td>pEGFP_C_OVM + Irrelevant shRNA</td>
<td>pEGFP_C_OVM + shEGFP</td>
<td>pEGFP_C_OVM + shEGFP</td>
<td>pEGFP_C_OVM + shEGFP</td>
</tr>
<tr>
<td>pEGFP_C_OVM + pU6-4shOvm1</td>
<td>pEGFP_C_OVM + pU6-4shOvm1</td>
<td>pEGFP_C_OVM + pU6-4shOvm1</td>
<td>pEGFP_C_OVM + pU6-4shOvm2</td>
<td>pEGFP_C_OVM + pU6-4shOvm2</td>
<td>pEGFP_C_OVM + pU6-4shOvm2</td>
</tr>
<tr>
<td>-</td>
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</tbody>
</table>

5.2.2.3 Preparation of mini-Tol2 transposon and microinjection into chicken embryos

In this section, the same siRNAs used in section 5.2.2.2 were used to design two hairpin microRNAs (miRNA) mimicking natural mir-30 miRNA structure to target OVM in chicken.
5.2.2.3.1 miRNA design

The two siRNAs designed using the Dharmacon tool were inserted into the native structure of gene expression regulatory microRNA known as miR-30. The native structure of miR-30 is as follows;

```
A UC G GAAGC
GCG CUGUAAACAUCC GACUGGAAGCU U A
CGU GACGUUUUGUAGG CUGACUUUCGG G G
C -- -- G UAGAC
```

The two strands highlighted in red and blue were replaced with the sequences of the two siRNAs. The top strand highlighted in red is the sense strand and the bottom strand highlighted in blue is the anti-sense strand of the relevant siRNA. Once the siRNA were inserted into the miR-30 structure, they were named miOvm1 and miOvm2;

**miOvm1:**

```
A GU G GAAGC
GCG GAACCAAUAUCA GCAAAGAGCCU U A
CGU CUUGGUUAUAGU CGUUUCUCGGG G G
C -- -- G UAGAC
```

**miOvm2:**

```
A AG G GAAGC
GCG AUGUAUUGGUUU GCAACAGGACU U A
CGU UACAUAACCCAAA CGUUGUCCUGG G G
C -- -- G UAGAC
```
Please note that for miOvm1 a GC was added at the 3’ end of the siRNA to make the two strands 22 bp long and miOvm2 was added with a GGA at the 3’ end. The miRNA structure was then predicted using ViennaRNA (University of Vienna) online tool to confirm the structure. The miRNAs were then custom made as oligonucleotides. The oligos were synthesised as two separate strands; a sense sequence (forward) and an antisense sequence (reverse). To the forward sequence of each miRNA, a BamHI overhang (GATC) was added to the 5’ end and a KpnI overhang (GTAC) was added to the 3’end. The synthesised oligonucleotides are as follows;

miOvm1 Forward (RE sites **bolded and italicized in black**):

\[ \text{GAT} \text{CGAGAAACAATATCAGTGAAGAGCCTGTAAGCAGCAGATGGGGCTCTTTGCTGATATTGGTTCTCGAC} \]

miOvm1 Reverse:

\[ \text{GAATACATACATCAGCAAAGAGCCTGCACCTGCTGCTTCACAGGCCTCTTTGCACTGATATTGGTTTCGC} \]

miOvm2 Forward (RE sites **bolded and italicized in black**):

\[ \text{GAT} \text{CGAGAATGTATTGGTTTAGCAACAGGACTGTGAAGCAGCAGATGGGGCTCTTTGCAACATACATCTCGAC} \]

miOvm2 Reverse:

\[ \text{GCAAGAATACATCGAAAGGACCTGCACCTGCTGCTTCACAGGCCTCTTTGCACTGATATTGGTTTCGC} \]

Please note that the sense strand is denoted by **red** and antisense strand is denoted by **blue**.
The oligonucleotides of each miRNA (forward and reverse) were then annealed to each other by combining 2 μg of each oligonucleotide with 46 μL of annealing buffer and incubating at 90°C for 3 minutes. The reaction was then cooled down to room temperature and 5 μL of each reaction was diluted in 45 μL of dH2O.

5.2.2.3.2 Construction of mini-Tol2 transposon for micro-injection

The next step was to construct a vector harbouring the mini-Tol2 transposon for delivery of the designed miRNAs into chick embryos (Figure 5.2). As the first step, it was necessary to insert the miRNAs into intron 5 of PANK1 (pantothenate kinase 1) gene (hereafter referred to as Intron) to effectively induce RNAi mechanism. The Intron has been previously inserted into a pEGFP_N1 vector, which has the sequence needed for expression of GFP (eGFP). This vector was known as pEGFP_N1Intron. Insertion of the Intron has divided the eGFP sequence into 2 sections flanking the inserted Intron. To insert the miRNAs into Intron in pEGFP_N1Intron vector, the available vector was digested with REs BamHI and KpnI by incubating the following reaction at 37°C for 3 hours;

1 μL BamHI
1 μL KpnI
2 μL MC buffer
1 μg (1 μL) of pEGFP_N1Intron plasmid
15 μL of dH2O to make up to 20 μL
Figure 5.2: Construction of the vector harbouring mini-Tol2 transposon for delivery of miRNAs targeting OVM in to chicken embryos. The first task was to insert “eGFP-Intron-miRNA-Intron-eGFP” fragment from pEGFP_NiOvm1 or pEGFP_NiOvm2 vector into the base vector pT2-009 which harbours the sequence of mini-Tol2 transposon (Tol2 LHE & Tol2 RHE as shown in the figure) and pCAGGS promoter. The second task was to PCR amplify PolyA fragment from pEGFP_N1Intron vector and insert in to pT2-009 downstream of second eGFP fragment. The final vector was named Tol2Ovm1 or Tol2Ovm2.
The digested and undigested sample (control) were run on a 1% agarose and the digested sample was gel purified. This purified sample was then used in a ligation reaction with the diluted miRNAs from section 5.2.2.3.1. Please note that the ligations for miOvm1 and miOvm2 were done separately according to the following reaction;

1 μL of the diluted annealed oligonucleotides (miOvm1 OR miOvm2)
1 μL of Ligase buffer
1 μL T4 DNA ligase
100 ng (5 μL) of BamHI and KpnI digested pEGFP_N1Intron
2 μL of dH2O to make up to 10 μL

A control ligation was also performed with only 5 μL of digested pEGFP_N1Intron and 5 μL of dH2O. All reactions were incubated at 4°C overnight. The ligations were then transformed into electro-competent E.coli and were propagated. The transformants were plated on LB agar with kanamycin (50 μg/mL) and incubated overnight at 37°C. 8 colonies representing each miRNA were grown overnight in LB media with kanamycin and plasmids were isolated by mini-prep. 5 samples of each construct were then sequenced to confirm the insertion of miRNA into pEGFP. The constructed vectors were named pEGFP_NiOvm1 and pEGFP_NiOvm2.

The next task was to cut out the “eGFP-Intron-miRNA-Intron-eGFP” fragment from pEGFP_NiOvm1 and pEGFP_NiOvm2 and insert it into pT2-009 vector which encodes the mini-Tol2 transposon and pCAGGS promoter. For this purpose the constructed pEGFP_NiOvm1 and pEGFP_NiOvm2 vectors were digested with REs NotI and XhoI to separate the “eGFP-Intron-miRNA-Intron-eGFP” from the pEGFP_N1 vector.
backbone. The reaction was further digested with FspI to digest the pEGFP_N1Intron backbone to smaller fragments. The digests were then analysed by gel electrophoresis, and the fragment of interest was gel purified. Parallel to this, two samples of pT2-009 plasmid were digested with REs NotI and XhoI. One sample was digested with high fidelity NotI while the other was digested with standard NotI for quality testing purposes. The digested and undigested pT2-009 were also analysed by gel electrophoresis and the gel band of interest was gel purified. The gel purified “eGFP-Intron-miRNA-Intron-eGFP” fragments (containing miOvm1 & miOvm2) and the RE digested pT2-009 were then ligated separately using T4 DNA ligase. The ligations were transformed into electro-competent E.coli and propagated. Plasmids were isolated from 6 transformant clones of each miRNA and were subjected to a screening digest using NotI and XhoI to confirm insert.

The final task of this section was to insert a PolyA signal to the pT2-009 vector, downstream of the already inserted “eGFP-Intron-miRNA-Intron-eGFP” fragment. The PolyA sequence was PCR amplified from pEGFP_N1Intron vector by gradient PCR according to the method and conditions described in section 5.2.1.5 and Table 5.2. The gradient temperatures used were: 50.2°C, 54.9°C, 59.5°C and 62°C. The primers used for this PCR were;

PolyA Forward- 5’-ACTGCGGCCGCGACTCTAGATCA-3’
Poly A Reverse- 5’-ACTGAATTCCAGCTTACAAATTACGC-3’
The PCR product was then ligated into pGEM®-T Easy vector as described in section 5.2.2.2.1 and were subjected to a screening digest using EcoRI to confirm insert. To confirm the validity of the sequence, a single clone with the insert was sequenced using pUC/M13 forward sequencing primer: 5’-CGCCAGGGTTTTCCCAGTCACGAC-3’. Afterwards, the constructed pT2-009 (containing miOvm1 or miOvm2) and pGEM®-T Easy containing PolyA were digested with NotI. The digests were analysed by gel electrophoresis and the required bands were gel purified. The purified PolyA was then ligated into the pT2-009 harbouring miOvm1 and miOvm2 using T4 DNA ligase. The final vectors were named Tol2Ovm1 and Tol2Ovm2. These vectors were transformed into electro-competent *E. coli* and were propagated. The transformants were plated on LB agar with ampicillin and one clone representing each of Tol2Ovm1 and Tol2Ovm2 were sequenced to confirm the final product.

### 5.2.2.3.3 Micro-injection of mini-Tol2-miRNA constructs into chick embryos

*E. coli* harbouring the constructed mini-Tol2-miRNA vectors (Tol2Ovm1 and Tol2Ovm2) were grown in LB media with ampicillin. Plasmids were then isolated using a Qiagen Plasmid Maxi kit (Qiagen, Cat. No. 12163) following manufacturer’s guidelines. Then, 0.6 μg of Tol2Ovm1 or Tol2Ovm2 was combined with 1.2 μg of pT2-002a plasmid (which codes for transposase) and 45 μL of OptiPro™ (Life Technologies, Cat.No. 12309-019) serum free media (SFM) and incubated at room temperature for 5 minutes. At the same time, 3 μL Lipofectamine™ 2000 and 45 μL of OptiPro™ SFM were combined and incubated at room temperature for 5 minutes. Afterwards, both
mixes were combined and incubated at room temperature for 20 minutes. This final mixture is the transfection complex.

For micro-injections, 100 fertilized eggs (50 for Tol2Ovm1 and 50 for Tol2Ovm2) were obtained from specific pathogen free (SPF) hens. These eggs were incubated to 2.5 days (15-16 Hamburger-Hamilton stages). A small opening was made on the pointy end of each recipient egg by carefully crack opening the shell with a sterile metal forceps. The embryos were observed under a microscope for viability and 1-2 μL of the transfection complex was micro-injected into the dorsal aorta of the embryo. The opening on the shell was then re-sealed with 2 layers of sterile paraffin film and the eggs were returned to the incubator. Of the dedicated 50 eggs for each mini-Tol2-miRNA construct, 29 eggs were micro-injected and 21 were discarded for Tol2Ovm1, while 36 eggs were micro-injected and 14 were discarded for Tol2Ovm2. The discarded eggs were due to incorrect staging of eggs, infertility of eggs or early death of embryos.

5.2.2.3.4 Gonad assays of micro-injected embryos

At 14 days of embryonic development of the micro-injected eggs, 5 eggs of each mini-Tol2-miRNA construct were subjected to gonad assays. The gonads of the embryos were dissected out of the body and were placed on a glass microscopy slide. The gonads were then visualised under green fluorescence microscopy. The remainder of the eggs that were not opened for gonad assays were incubated till hatching and the
hatchlings were grown to sexual maturity. Chicks injected with Tol2Ovm1 were named M1 chicks and the chicks injected with Tol2Ovm2 were named M2 chicks.

5.2.2.3.5 Semen screening by qPCR

The hatched chicks were grown to sexual maturity and semen samples from roosters were obtained to screen for the presence of mini-Tol2-miRNA (miOvm1 or miOvm2) transgene. DNA from the semen samples were extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Cat. No. 69506) according to manufacturer’s guidelines. The isolated genomic DNA was diluted 1/100 in sterile nuclease free dH2O and was subjected to quantitative PCR (qPCR) on a Mastercycler® Ep Realplex (Eppendorf) following manufacturer’s guidelines. The qPCR reaction mix was prepared according to Table 5.5. Two reactions were set up for each DNA sample; experimental reaction using specific mini-Tol2 primers (Forward primer: 5’ CAGTCAAAAAGTACTTATTGAGATCACT 3’; Reverse primer: 5’ GGGCATCAGCGCAATTCA ATT 3’) and a detection probe (5’ ATAGCAAGGGAAAATAG 3’); control reaction using primers specific for a genomic control region (Forward primer 5’ GATGGGAAAACCTGAACCTC 3’; Reverse primer 5’ CAACCTGCTAGAGAAGATGAGAAGAG 3’) and a detection probe (5’ CTGCACTGAATGGAC 3’). The qPCR was carried out according to the conditions outlined in Table 5.6. Each rooster was tested at least twice and was classified positive if a CT (cycle threshold) value of less than 36 was obtained for experimental reactions. A CT of less than 32 for control reactions was used to indicate the presence of sufficient DNA in the sample tested. The relative levels of integrated mini-Tol2-
miRNA DNA in semen were calculated by comparing the mean CT values from genomic and mini-Tol2 qPCR from semen samples of each bird.

**Table 5.5 qPCR reaction for semen screening**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taqman 2x universal master mix</td>
<td>10 μL</td>
</tr>
<tr>
<td>20x FAM labelled assay mix</td>
<td>1 μL</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1 μL</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1 μL</td>
</tr>
<tr>
<td>Genomic DNA template</td>
<td>9 μL</td>
</tr>
</tbody>
</table>

**Table 5.6 qPCR reaction conditions for semen screening**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Step 1</td>
<td>95°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td>Step 2</td>
<td>60°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Repeat step 1 &amp; 2 45 times</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3 Results

5.3.1 CRISPR knockout of ovomucoid in DF-1 cells

Two sgRNAs targeting exon 1 of OVM were designed. The chosen sgRNAs had the highest quality score and no off-targets in other genes of the chicken genome. The two sgRNAs (each sgRNA had a top and a bottom strand) were then cloned into pSpCas9 (BB)-2A-GFP vector. The sequencing results show that both sgRNAs has been successfully inserted into the vector (Figure 5.3). Confluent DF-1 cells were then transfected with the constructed pSpCas9 (BB)-2A-GFP containing the sgRNAs. When observed under bright-field microscopy, there were no visible differences between the cells of each treatment (Figure 5.4). When observed under green fluorescence, the un-transfected cells did not show any green colour while the sgRNA1 and sgRNA2 transfected cells had green fluorescing cells (Figure 5.4). The green fluorescence cells in the transfected populations were then sorted using FACS.

Genomic DNA was isolated from the sorted and expanded cells, which was subjected to SURVEYOR analysis. The gradient PCR conducted to determine the best annealing temperature of the primers designed to amplify the targeted genomic region showed clear bands at ~500 bp for all the temperatures, with 55.7°C yielding the most prominent band (Figure 5.5). PCR to amplify the targeted genomic sequence from wild-type cells, with the determined optimum annealing temperature, yielded a clear band at ~500 bp (Figure 5.5). Appearance of bands at ~500 bp indicates successful amplification of the targeted DNA from genomic DNA.
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The PCR amplified targeted DNA from cells treated with sgRNA (sgRNA1 & sgRNA2) were the hybridized with DNA from wild-type cells. The hybridized DNA were then digested with SURVEYOR nuclease. Gel electrophoresis of the digested products showed one band at ~500 bp for all treatments, indicating no mutations are present (Figure 5.5).
Figure 5.3: Sequence of sgRNA1 and sgRNA2 inserted into pSpCas9 (BB)-2A-GFP vector. The sequence of the top strand of sgRNA1 is shown in (A), while the bottom strand is shown in (B). Top strand of sgRNA2 is shown in (C) and bottom strand is shown in (D). The sequence highlighted and bolded in red are the sgRNA sequences. Please note that for the sequences shown for the bottom strands of both sgRNAs, the obtained sequence was reverse complemented.
Figure 5.4: Fluorescence microscopy of DF-1 cells transfected with pSpCas9 (BB)-2A-GFP containing sgRNAs. For each experimental treatment, 500 ng of sgRNA combined with Lipofectamine® 2000 was added to the well. As a control, some cells were left un-transfected.
Figure 5.5: SURVEYOR analysis to detect mutations. Gradient PCR of the target region done using genomic DNA isolated from cells targeted by sgRNA1 to determine the optimum PCR conditions (A). PCR, with the determined optimum annealing temperature, of the target region of genomic DNA isolated from wild-type cells (B). PCR of target region of genomic DNA of sgRNA1 hybridized with wild-type, sgRNA2 hybridized with wild-type and wild-type only reference after digestion with SURVEYOR nuclease (C).
5.3.2 RNAi knockdown of ovomucoid

Two siRNAs targeting OVM were designed using the Dharmacon algorithm. The sequences of these siRNAs were then used in cell-based and animal (chicken) based experimentation to knockdown OVM. For the reader’s convenience, a flowchart of the major steps taken in these experiments is provided below in Figure 5.6.

**Figure 5.6: Flowchart outlining the major steps taken to study RNAi knockdown of OVM**
5.3.2.1 Knockdown of ovomucoid in DF-1 cells

The two siRNAs that target OVM mRNA were used to design shRNAs (shOvm1 & shOvm2) driven by the chicken U6-4 promoter. The gradient PCR conducted using the reverse complemented sequences of shOvm1 and shOvm2, to combine the shRNAs with the U6 promoter, showed a band at ~300 bp for all temperatures (except for 72°C for shOvm2 where there was no band). For shOvm1 66.1°C yielded the most prominent band and for shOvm2 62.6°C yielded the most prominent band (Figure 5.7). Primer dimers (smaller band) were also visible in all lanes. The U6 + shRNA PCR products were then ligated into pGEM®-T Easy vector (pU6-4shOvm1 & pU6-4shOvm2). The screening digests showed that 5/6 clones of pU6-4shOvm1 contained the insert and 6/6 clones of pU6-4shOvm2 contained the insert (Figure 5.7). The sequencing result confirmed that both the sequence of U6 promoter and shRNA had been combined and inserted in to pGEM®-T Easy (Figure 5.8).

The next task was to insert OVM mRNA sequence targeted by the two shRNAs in to pEGFP_C vector. The PCR conducted to amplify the OVM sequence showed a prominent band at ~500 bp (Figure 5.9). Primer dimers were also visible. The PCR product was then ligated into pGEM®-T Easy and the screening digest conducted post ligation showed a band at ~600 bp (insert) in 9/10 clones in addition to a larger band at ~7000 bp (vector backbone) in all clones (Figure 5.9). Sequencing and NCBI/BLAST confirmed that DNA sequence of interest was successfully amplified from OVM cDNA (Figure 5.10). The OVM fragment was then cut out of pGEM®-T Easy and inserted in to pEGFP by RE digestion and ligation (Figure 5.9).
Figure 5.7: Construction of shRNAs driven by a U6 promoter. A reverse complemented sequence of each shRNA was used as a primer to amplify the sequence of chicken U6 promoter from a donor vector (A). A gradient PCR was conducted to amplify the U6-4 promoter using the shOvm1 and shOvm2 primers (B). The desired PCR product is shown by an arrow. The PCR amplified U6-4 + shRNA constructs were then ligated into pGEM®-T Easy vector (pU6-4shOvm1 & pU6-4shOvm2). Plasmids from 6 transformant clones of each of pU6-4shOvm1 and pU6-4shOvm2 were subjected to a screening digest using EcoRI to confirm insert (C). The arrow shows the U6 + shRNA fragment digested out of the vector by EcoRI.
**Figure 5.8: Sequence results of the U6 + shRNA after insertion in pGEM®-T Easy.** The known sequence of chicken U6-4 promoter is shown in (A). The reverse sequence of shOvm1 is shown in (B) and shOvm2 in (C). Sequencing result for pU6-4shOvm1 is shown in (D) and pU6-4shOvm2 is shown in (E). The chicken U6-4 sequence is shown in red and shRNA sequences are shown in blue.

| (A) | GAATTGTGGAGCAGCGGAAAGAGGCTTCCGCCGGCCCTATATTGAAATCGAGAGACTTCGGG 
CGTGCAGCGCGGCGCAATCGGAAGAGATTTGGAAGATCTCGAGACCCCAAAATCT 
TGGAAAGAAAGAATATCTACTGGCAGAATATGTAGGATTTTTGGAAGAGGTGG 
AAGAAATAGTGAATACGCGCAATCGGCATCTACCGCTACTTTATAATCTAGTA 
TATAGGCTTAAATACGTCGAGACACTGGGAGTGTAGTTGGAACACATTCCA 
| (B) | TCCGAGAGACACTGGGAGTTGGAACCAAATATCAGCAAAGAATTCAAGAGATCTTTTG 
GCTGATATTGGTCTTTTGGAAACGAG | (C) | TCCGAGAGACACTGGGAGTTGGAACCAAATATCAGCAAAGAATTCAAGAGATCTTTTG 
GCTGATATTGGTCTTTTGGAAACGAG | (D) | CGTCGACATGTCGCCCCGGCCATGGACGCACCTGGGAGTTCGGACGAATTTGGAAGAG 
GAAGAAGGCTTCCGCCGGCCCTATATTGAAATCGAGAGACTTCGGGAGTGT 
TGGAAAGAAAGAATATCTACTGGCAGAATATGTAGGATTTTTGGAAGAGGTGG 
AAGAAATAGTGAATACGCGCAATCGGCATCTACCGCTACTTTATAATCTAGTA 
TATAGGCTTAAATACGTCGAGACACTGGGAGTGTAGTTGGAACACATTCCA 
| (E) | CTATGCATCCAAAGCGCGTGGAGCTTCCGCCATATGAGCTGCACTGGGAGCGGCG 
GCAATCCGCCGCGACCGCGCAATCGGAAGAGATTTGGAAGATCTCGAGACCCCA 
AAATCTTCTGCGAAAAAAGAATATCTACTGGCAGAATATGTAGGATTTTTGGA 
AGAAATAGTGAATACGCGCAATCGGCATCTACCGCTACTTTATAATCTAGTA 
TATAGGCTTAAATACGTCGAGACACTGGGAGTGTAGTTGGAACACATTCCA 

*Note: The sequence representation may contain OCR errors and is provided for reference only.*
Figure 5.9: Insertion of OVM target region in to pEGFP_C. The region of OVM targeted by the two shRNAs was amplified from OVM cDNA (A). The PCR product was then ligated in to pGEM\textsuperscript{®}-T Easy vector. The ligated vector was transformed in to E.coli and vectors isolated from 10 transformant clones were subjected to a screening digest using EcoRI to confirm insert (B). The arrow shows the insert digested out by EcoRI. A clone with the correct insert was digested with BgIII and HindIII (C). At the same time, pEGFP_C vector was digested with BgIII and HindIII (D). The digested OVM target sequence was ligated in to digested pEGFP_C using T4 DNA ligase to produce the pEGFP_C\_OVM vector.
Figure 5.10: PCR amplified OVM target region. The sequence highlighted in red was PCR amplified from OVM cDNA. The amplified sequence was then inserted into pEGFP_C vector to produce the pEGFP_C_OVM vector.
Confluent DF-1 cells were then transfected with pEGFP_C_OVM containing targeted OVM and pU6-4shOvm1 or pU6-4shOvm2 harbouring the U6-4 driven shRNAs, along with negative and positive controls. Some cells were left untransfected as an additional negative control. The transfected cells were observed under fluorescence microscopy 48 hours post transfection. Under bright-field, cell of all treatments looked normal and no discrepancies were visible (Figure 5.11). Under green fluorescence light, no cells were visible in the untransfected sample, while the others had cells emitting green fluorescence. Cells transfected with pEGFP_C_OVM only and pEGFP_C_OVM + Irrelevant shRNA showed more green fluorescing cells when compared to cells transfected with pEGFP_C_OVM + shEGFP, pEGFP_C_OVM + pU6-4shOvm1 or pEGFP_C_OVM + pU6-4shOvm2 (Figure 5.11).

The mean fluorescence intensity (MFI) of untransfected and transfected cells were measured by FACS. pEGFP_C_OVM + shEGFP, pEGFP_C_OVM + pU6-4shOvm1 and pEGFP_C_OVM + pU6-4shOvm2 treated cells had a lower MFI than pEGFP_C_OVM only and pEGFP_C_OVM + Irrelevant shRNA treated cells (Table 5.7 and Figure 5.12). pEGFP_C_OVM + pU6-4shOvm1 and pEGFP_C_OVM + pU6-4shOvm2 treated cells had a lower MFI than the positive control pEGFP_C_OVM + shEGFP. Untransfected cells had little or no MFI.
Figure 5.11: RNAi Knockdown of OVM in DF-1 cells. Confluent DF-1 cells were co-transfected with pEGFP_C_OVM containing the targeted OVM fused to GFP and pU6-4shOvm1 or pU6-4shOvm1 containing shRNAs. For each treatment, 500 ng of pEGFP_C_OVM and 500 ng of vectors containing shRNA (total of 1 μg) were transfected. The cells were then grown for 48 hours, visualised under fluorescence microscopy. In addition to the un-transfected cells, two negative controls were done: pEGFP_C_OVM only and pEGFP_C_OVM + Irrelevant shRNA. The positive control cells were transfected with shEGFP which specifically targets EGFP in pEGFP_C_OVM vector.
Figure 5.12: Mean fluorescence intensity of DF-cells treated with shRNAs measured by FACS. Untransfected and transfected cells were collected 2 days after transfections by dissociating from the wells with trypsin. The cells were prepared in FACS media and fluorescence intensity was measured. The negative control with pEGFP_C + Irrelevant shRNA was used as 100% mean fluorescence intensity for comparison with other treatments.

Table 5.7 Mean fluorescence intensities (MFI) of DF-1 cells treated with shRNAs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransfected</td>
<td>43</td>
</tr>
<tr>
<td>pEGFP_C_OVM Only</td>
<td>35, 344</td>
</tr>
<tr>
<td>pEGFP_C_OVM + Irrelevant shRNA</td>
<td>31, 530</td>
</tr>
<tr>
<td>pEGFP_C_OVM + shEGFP</td>
<td>18, 698</td>
</tr>
<tr>
<td>pEGFP_C_OVM + pU6-4shOvm1</td>
<td>13, 038</td>
</tr>
<tr>
<td>pEGFP_C_OVM + pU6-4shOvm2</td>
<td>11, 098</td>
</tr>
</tbody>
</table>
5.3.2.2 RNAi knockdown of ovomucoid in chicken

In vitro analysis showed that both shRNAs would efficiently silence OVM-GFP fusion, therefore the next step was to use the same siRNA sequences, used to design the aforementioned shRNAs, to design two miRNAs mimicking the structure of miR-30. When the sequences of two miRNAs (miOvm1 & miOvm2) were submitted to ViennaRNA online tool, the expected structure was predicted (Figure 5.13). The two miRNAs were then successfully inserted into the Intron (intron 5 of PANK1 gene) within pEGFP_N1Intron plasmid by RE digestion (Figure 5.14). Sequencing further confirmed the successful insertion of the miRNAs (Figure 5.14).
Figure 5.13: Predicted secondary structure of the two miRNAs. The secondary structure was predicted by submitting the miRNA sequences to ViennaRNA online tool. (A) miOvm1, (B) miOvm2.
Figure 5.14: Insertion of miRNAs in to the Intron contained in the pEGFP_N1Intron vector. pEGFP_N1Intron plasmid containing the PANK1 Intron was digested with Kpnl and BamHI (A). These two sites are located in the intron where the miRNA was to be inserted. The annealed miRNAs did not need to be digested because they were designed with overhang RE sequences at the ends. The miRNAs were ligated into the vector using T4 DNA ligase. The ligated plasmids were sequenced to confirm the insert: (B) pEGFP_NiOvm1, (C) pEGFP_NiOvm2. The insert is highlighted in red in (B) & (C).
The next task was to insert the “eGFP-Intron-miRNA-Intron-eGFP” fragment into pT2-009 vector which encodes the mini-Tol2 transposon. Triple RE digestion of pEGFP_NiOvm1 and pEGFP_NiOvm1 containing “eGFP-Intron-miRNA-Intron-eGFP” resulted in 4 bands on the agarose gel and the double digest resulted in 2 bands (Figure 5.15). pT2-009 was also digested with REs; the sample digested with standard NotI yielded a prominent band than the sample digested with high fidelity NotI (Figure 5.15). The screening digest conducted upon ligation of “eGFP-Intron-miRNA-Intron-eGFP” into pT2-009 confirmed 1/6 clones of pT2-009 containing miOvm1 had the insert while 3/6 clones of pT2-009 containing miOvm2 had the insert.

It was then necessary to insert PolyA into pT2-009, downstream of “eGFP-Intron-miRNA-Intron-eGFP” fragment. Gradient PCR conducted to amplify PolyA from pEGFP_N1Intron resulted in prominent bands at ~200 bp in all temperatures (Figure 5.16). Primer dimers were also visible in this gel. The PCR product was then inserted into pGEM®-T Easy and the screening digest confirmed successful insertion in all digested clones by showing a band at ~200 bp (Figure 5.16). Sequencing and subsequent NCBI/BLAST search showed that the amplified sequence is 100% similar to the known PolyA sequence (Figure 5.17). The PolyA was then successfully digested out of pGEM®-T Easy and inserted into RE digested pT2-009 to produce Tol2Ovm1 and Tol2Ovm2 (Figure 5.16).
Figure 5.15: Insertion of “eGFP-Intron-Hairpin-Intron-eGFP” fragment from pEGFP_NiOvm1 & pEGFP_NiOvm2 into pT2-009. The “eGFP-Intron-Hairpin-Intron-eGFP” fragments from pEGFP_NiOvm1 & pEGFP_NiOvm2 were isolated by RE digestion (A). The band shown by the arrow in triple digest was gel purified and used. Then pT2-009 was digested with REs (B). After ligation of the “eGFP-Intron-Hairpin-Intron-eGFP” into pT2-009, a screening digest was performed to confirm insertion (C). 6 clones of pT2-009 containing miOvm1 and 6 clones of pT2-009 containing miOvm2 were subjected to this screening digest. The arrows on (C) shows the insert (“eGFP-Intron-Hairpin-Intron-eGFP”) which harbours either miOvm1 or miOvm2.
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Figure 5.16: Insertion of PolyA into pT2-009. A gradient PCR was conducted to amplify the PolyA sequence from pEGFP_N1Intron (A). Arrow indicates the PCR product of interest. The PCR product was gel purified and cloned into pGEM®-T Easy vector. Eight clones of transformant pGEM®-T Easy were subjected to a screening digest with EcoRI to confirm insertion of PolyA (B). Then PolyA was cut out of pGEM®-T Easy by digesting with NotI and was gel purified (C). Arrow indicates the PolyA insert cut out of pGEM®-T Easy. The pT2-009 vectors containing the miRNAs (miOvm1 or miOvm2) were also digested with NotI to allow insertion of the NotI digested PolyA (D). The bands indicated by the arrow in (D) were gel purified and used for the ligations with the insert (PolyA). The final vectors were named Tol2Ovm1 and Tol2Ovm2.
### Figure 5.17: Comparison of PCR amplified PolyA sequence with the known PolyA sequence on NCBI/BLAST.

**Query**: obtained sequence after cloning and sequencing,  
**Sbjct**: known PolyA sequence.

<table>
<thead>
<tr>
<th>Range</th>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
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<td>422 bits(228)</td>
<td>2e-122</td>
<td>228/228(100%)</td>
<td>0/228(0%)</td>
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Constructed Tol2Ovm1 and Tol2Ovm2 along with pT2-002a which encodes transposase were successfully micro-injected into 2.5 days old chicken embryos (Figure 5.18). Ten eggs at 15 days (5 for each of Tol2Ovm1 and Tol2Ovm2) were then subjected to gonad assays. Of the 5 Tol2Ovm1 injected embryos, 3 were female and 2 were male, whereas of the Tol2Ovm2 injected embryos, 2 were female and 3 were male. Gonads from all eggs were observed under bright-field and fluorescence microscopy. When observed under fluorescence, all gonads had large numbers of cells emitting green fluorescence (Figure 5.19 & Figure 5.20). Please note that only selected male gonads are shown in this chapter (Please refer to the Appendix A for images of all gonads).

Of the remaining eggs that were incubated for hatching 9 chicks hatched and they were named according to the corresponding mini-Tol2-miRNA construct they were injected with; M1 & M2. Of the hatched chicks, there were 3 M1 males, 1 M1 female, 3 M2 males and 2 M2 females. 2 M1 males, 2 M2 males and 1 M2 female survived to sexual maturity. One M1 male bird was subjected to semen screening by qPCR to confirm the presence of mini-Tol2-miRNA (miOvm1) transgene. The qPCR yielded a CT (cycle threshold) value of 31 for mini-Tol2-miRNA and 26 for genomic DNA. This predicts that 0.605% of this rooster’s sperm carries the mini-Tol2-miRNA transgene, which translates to ~1.5% of G1 chicks being fully transgenic, as determined by previous experiments done at CSIRO-AAHL. Please note that semen collection from the remaining roosters has been difficult, therefore semen screening results of these roosters were not available at the time of submission of this thesis.
Figure 5.18: Micro-injection of Tol2Ovm1 and Tol2Ovm2 in to chicken embryo. These images show the micro-injection of Tol2Ovm1 or Tol2Ovm2 along with pT2-002a transposase in to a 2.5 days old chicken embryo. For the purpose of this image, the injected material contained a dye, Bromophenol blue, showing distribution of the material throughout the embryo as shown in the images above.
Figure 5.19: Gonad analysis of embryos micro-injected with Tol2Ovm1 and pT2-002a. The gonads were observed under bright-field and green fluorescence light at x40 and x100 magnification. Both gonads of each bird were observed. Only male gonads are shown in this figure.
Figure 5.20: Gonad analysis of embryos micro-injected with Tol2Ovm2 and pT2-002a. The gonads were observed under bright-field and green fluorescence light at x40 and x100 magnification. Both gonads of each bird were observed. Only male gonads are shown in this figure.
5.4 Discussion

Chicken egg allergy is a common disorder affecting mainly children worldwide. Like in many other food allergies, the most efficient way of egg allergy management is strict dietary avoidance. Dietary avoidance of eggs may however result in malnutrition in developing/growing children, as eggs contain essential vitamins and minerals. Complicating the situation further, eggs or traces of eggs are present in a range of food products and vaccines, making it difficult to completely avoid eggs. SIT is currently being pursued as the only curative treatment strategy for egg allergy and other food allergies. Production of recombinant variants and hypoallergenic variants of egg and other food allergens has been the focus of many researchers, which may be useful in well-controlled SIT and diagnostic techniques such as SPT. However, even SIT has limitations. The success of SIT primarily depends on the sensitivity of the patient to a given allergen. Administration of allergens may sometimes result in undesirable adverse reactions such as anaphylaxis, which has the potential to lead to death.

The lack of highly effective egg allergy management strategies leads to the utilization of unconventional strategies to answer questions in egg allergy. Almost all research on egg allergy has overlooked tackling egg allergy at the root of the cause, away from the human immune system, the allergen source itself. It may well be possible to change the allergenic properties of chicken egg into a hypoallergenic form using well established methods and techniques used elsewhere in biological/biomedical research, including RNAi and CRISPR-Cas9. Ovomucoid (OVM) is the most allergenic protein in the chicken egg white. It is structurally rigid and contains multiple IgE
binding epitopes, allowing it to retain its allergenic properties even after heating (cooking) and/or proteolytic digestion. Therefore, in this study, OVM was chosen as a candidate for knockout and knockdown studies.

It was hypothesized that knockout/knockdown of OVM may not have adverse effects on the chicken or the developing eggs due to the presence of a natural homologue present in the egg white known as ovoinhibitor (OVH). Both OVM and OVH are serine protease inhibitors characterized by three well-conserved intra-domain disulphide bridges known as the Kazal domain\textsuperscript{227,228}. OVM expression is limited to the egg white, whereas OVH is expressed in almost all components of the egg. It is poorly understood why OVM, being functionally similar to OVH yet less potent, has evolved as a separate protein in the egg white. It can be suggested that OVM evolved, in wild birds, to provide extra protection against trypsin during egg formation inside the oviduct or against bacterial proteases exogenously introduced to the egg. Today, commercial egg laying hens are raised in highly controlled and cleaner environments and therefore face foreign pathogens in a lesser extent when compared to their wild counterparts. Therefore, in this study, it was suggested that knockdown or knockout of OVM may not have a significant effects in chickens' development or health.

CRISPR-Cas9 based precision genome engineering has been utilized as a potent tool in many disciplines across biological and biomedical sciences. One of the goals of this chapter was to design two guide RNAs (sgRNAs) capable of knocking out OVM in DF-1 chicken fibroblast cells. Although there is no preferred region of the coding sequence of a gene to be targeted for a simple gene knockout, exon 1 of OVM gene
in chromosome 13 was chosen as a starting point for this experiment. It is essential to choose sgRNAs with a high score given by the online CRISPR design tool to increase chances of efficient gene knockouts, therefore, for this study, the sgRNAs with the highest scores were selected. When DF-1 cells were transfected with the pSpCas9 (BB)-2A-GFP carrying either of the sgRNA, some cells produced green fluorescence. This was an indication that the pSpCas9 (BB)-2A-GFP has been successfully transfected into these cells. The cells were FACS sorted and re-cultured to produce a fully transfected cell population, allowing isolation of only targeted genomic DNA. The DNA samples isolated from cells targeted with sgRNAs were hybridized with wild-type DNA to produce heterocomplexes, which allows SURVEYOR nuclease to detect mismatches and subsequent digestion of the DNA strand. However, the gel analysis of SURVEYOR nuclease digested heteroduplexes did not yield multiple bands indicating a failed knockout of OVM gene by either of the sgRNAs. It is unclear why the two sgRNAs, having high scores, failed to mutate the targeted gene. The next step would be to design new sgRNAs to target other exons of the OVM gene to scrutinize a gene knockout. This was not attempted in this study due to time limitations.

Parallel to the CRISPR-Cas9 knockout of OVM in DF-1 cells, a RNAi knockdown study was also conducted. The aim of this study was to design two miRNAs capable of knocking down OVM in chickens. As the foremost step, 50 candidate siRNAs were designed using the Dharmacon tool and an algorithm was used to choose the best two siRNAs targeting the OVM mRNA sequence. It was then necessary to study the efficacy of these two siRNAs at knocking down OVM. Wise et al. 2007\textsuperscript{229} describes a mechanism of RNAi using shRNAs driven by a chicken U6 promoter in DF-1 cells. A
similar approach was followed in this study, in which the two designed siRNAs were inserted into a known hairpin structure to produce two shRNAs driven by the chicken U6-4 promoter. pGEM®-T Easy was the vector of choice for expression of the shRNAs because this work did not require directional cloning as the shRNAs were fused to their own U6-4 promoters. Upon ligation of the U6-4 promoter + shRNAs in to pGEM®-T Easy (which were named pU6-4shOvm1 & pU6-4shOvm2), a screening digest was done for selected clones to confirm that the insert had been successfully ligated. Next, the targeted OVM was cloned into pEGFP_C vector, which expresses GFP with a C-terminal fusion of the inserted sequence/protein. It was expected that if either or both of the shRNAs targeted OVM, a reduction of GFP expression will be observed in transfected DF-1 cells. PCR amplification of the targeted OVM fragment from OVM cDNA was successful as evident by the screening digest and the sequencing results. It was not necessary to perform another screening digests after inserting the PCR product from pGEM®-T Easy into pEGFP_C vector because directional cloning using two different REs was used.

The pEGFP_C containing the targeted OVM fragment, which was named pEGFP_C_OVM, was co-transfected with the pU6-4shOvm1 or pU6-4shOVM2 into DF-1 cells. As expected, both U6-4shOvm1 and pU6-4shOVM2 showed significant knockdown of GFP, indicating the two shRNAs are efficiently targeting OVM via RNAi. The observation of healthy and similar cell populations under bright-field microscopy in all treatments, including controls, shows that the study was well controlled with minimal or no external influence. The untransfected cells did not yield any GFP signal, indicating that the cells used for this study was completely free of GFP. The
pEGFP_C_OVM only transfected cells showed the highest GFP expression as this sample did not have any shRNAs targeting the GFP sequence. The cells transfected with pEGFP_C_OVM and an irrelevant shRNA however showed slightly less GFP expression when compared to pEGFP_C_OVM only control. This can be attributed to the total amount of DNA affecting the transfection efficiency, therefore pEGFP_C_OVM + irrelevant shRNA was used as the true negative control. The positive control (pEGFP_C_OVM + shEGFP) contained shRNA capable of targeting the GFP sequence in pEGFP_C_OVM. As expected, this control showed significant knockdown of GFP when compared to the negative control. It should be noted that pU6-4shOvm1 and pU6-4shOVM2 transfected cells showed higher knockdown of GFP signals when compared to the positive control. This was not expected because similar amounts of DNA were added to both the positive control and the experimental cells. This may possibly be due the ability of the designed hairpins to target OVM more efficiently when compared to shEGFP targeting the GFP sequence. The result of this experiment showed that either of the two chosen siRNAs may be adequate to silence OVM in chicken by RNAi, however both were used for the follow up experiments.

The chicken U6-4 promoter is a Polymerase III type promoter\textsuperscript{229} which, although known to be effective \textit{in vitro}, may be toxic \textit{in vivo} possibly due to the excessive expression of shRNAs saturating and impeding the RNAi mechanisms\textsuperscript{230}. Therefore the two siRNAs, used as shRNAs in the earlier experiment, were then used to produce miRNAs mimicking the structure of miR-30. It was hypothesized that hitch-hiking the miR-30 like miRNAs into the PANK1 Intron will allow a more natural, promoter-less, expression of the siRNAs \textit{in vivo}. MicroRNAs (miRNAs) are short (~22 nucleotides)
molecules that function as regulators of gene expression and mRNA stabilizers. These molecules, expressed in multicellular organisms, leave the nucleus as ~60 nucleotide molecules known as pre-miRNAs. At the cytoplasm, the pre-miRNAs are further cleaved by the enzyme Dicer to produce the mature miRNA (~22 nucleotides). The mature miRNAs can then recognize and bind to the 3’ end of the untranslated region (UTR) of target mRNAs using the RNA-induced silencing complex (RISC) to induce mRNA regulation including inhibition or degradation\textsuperscript{231-233}. The model miRNA chosen for this study, miR-30, is from a family of miRNAs involved in transcriptional regulation and can be used for expression of siRNAs\textsuperscript{231,234}.

When the structure of the two miRNAs in this experiment, miOvm1 and miOvm2, were predicted using ViennaRNA online tool, the structures were similar to that of miR-30. The miRNAs were then inserted into the PANK1 Intron that had already been cloned in to a pEGFP_N1Intron plasmid by directional cloning. The sequencing result further confirmed that the miRNAs were successfully cloned into the Intron. The “eGFP-Intron-Hairpin-Intron-eGFP” from pEGFP_N1Intron was then digested out by a triple RE digest. It was necessary to conduct a triple digest because the pEGFP_N1Intron backbone and “eGFP-Intron-Hairpin-Intron-eGFP” were of similar sizes and would have been difficult to separate by gel electrophoresis. The RE FspI simply cleaved the pEGFP_N1Intron backbone into two smaller fragments. The “eGFP-Intron-Hairpin-Intron-eGFP” was then successfully inserted into pT2-009 vector by directional cloning. The PolyA sequence was added in to the pT2-009, downstream of “eGFP-Intron-Hairpin-Intron-eGFP” fragment, as it is essential for gene expression. A gradient PCR was done to amplify the PolyA sequence from pEGFP
to choose the best PCR conditions, however all temperatures were successful. When the obtained sequence was compared to the known sequence of PolyA, a 100% similarity was observed. The insertion of PolyA into pT2-009 transposon concluded the construction of the vectors for delivery of miRNAs into the chicken embryos, which were named Tol2Ovm1 and Tol2Ovm2.

The constructed Tol2Ovm1 and Tol2Ovm2 plasmids were microinjected into 2.5 days old chicken embryos. Each of these vectors, which encodes a transposon and miRNA, was delivered along with pT2-002a which encodes a transposase. The Tol2Ovm1 or Tol2Ovm2 and pT2-002a carry the mini-Tol2 transposon and transposase respectively, which allow transposition of at least 10,000 bp of DNA fragments into a targeted host\textsuperscript{235}. This was the best option for the insertion of “eGFP-Intron-Hairpin-Intron-eGFP” cassette, which is >4000 bp in size, into the genome of cells in the micro-injected embryos. Following micro-injections, gonads were observed from embryos at day 14. The presence of large numbers of GFP positive cells in all observed gonads indicates that miRNAs are being effectively processed by RNAi mechanisms, thus fusing the two fragments of eGFP to produce GFP. This indicates that successful transposition has occurred, followed by expression, intron splicing and miRNA processing. Essentially, the results of this experiment show that the two miRNAs may successfully knockdown OVM in transgenic chicken (G1 generation).

The remainder of the embryos were allowed to hatch and the chicks were raised to sexual maturity. This generation is the G0 generation. The next step was to screen sperm samples from adult males for the presence of the transgene. Results from one
M1 male bird indicates that ~1.5% G1 offspring from this rooster will be fully transgenic. In addition, the presence of the mini-Tol2-miRNA transgene in sperm cells indicates that primordial germ cells (PGCs) has been transfected when the transfection complex was microinjected into the embryos. This rooster, and other remaining roosters upon semen screening, can be mated with wild-type females to produce transgenic chicks that will have knocked-down OVM levels. From this point, important observations can be made about the importance of OVM for the developing chicken. The level of OVM knockdown can be measured while measuring the expression levels of OVH, in comparison to wild-type chicks. If significant knockdown of OVM is achieved, it may even be possible to tests eggs from the G1 generation against egg allergic patients’ sera for IgE reactivity. Although not completely hypoallergenic, egg white with reduced OVM may play a crucial role in egg allergy treatment methods such as immunotherapy and diagnostic methods such as skin prick tests for highly reactive patients.

The current results of this study demonstrate that RNAi may be used as an important tool to knockdown allergenic proteins in the chicken egg. The impending results of this study will provide much more important information on feasibility of knocking out OVM from the chicken genome to produce significantly hypoallergenic chicken eggs using CRISPR-Cas9, or, other techniques such as TALENs (transcription activator-like effector nuclease) and ZFN (zinc finger nuclease) based genome engineering. These techniques, along with the understanding of IgE binding epitopes of egg allergens, may also allow precision genome engineering to alter the IgE binding epitopes of all the allergens to produce a chicken lineage capable of laying...
hypoallergenic chicken eggs. Such achievement in egg allergy may in fact cause a paradigm shift in food allergy research as a whole.

5.5 Conclusion

Egg allergy is a wide-spread disorder mainly affecting children. Currently, strict dietary avoidance and immunotherapy are pursued as the most effective management and treatment strategies. Traditionally, egg and other food allergy research has been focussing mainly on improving the immune system of the patients. Tackling the problem at the root of the cause has been overlooked by many, especially at a time when genetic engineering is being efficiently used to answer important questions in biomedical science, environmental science and agriculture. In this study, a knockout of the most important egg white allergen OVM was attempted in DF-1 chicken fibroblast cells, with no satisfactory results. As a second approach, RNAi knockdown of OVM was attempted using two siRNAs. The DF-1 cell culture based experiment showed that the two siRNAs can efficiently knockdown a GFP-OVM fusion, suggesting that OVM would be efficiently silenced in vivo. The subsequent delivery of the siRNAs, in the form of miRNAs, in to chicken embryos showed successful transgene integration and that the designed miRNAs are being effectively processed by RNAi mechanisms, promising the development of OVM knocked-down transgenic hens. This was further supported by the semen screening result of one male bird, which indicated that ~1.5% of G1 chicks will be fully transgenic with knocked-down OVM. The current results and results from follow up studies will
provide important information necessary for development of hypoallergenic chicken eggs using genome engineering strategies.
CHAPTER 6

General discussion and future perspectives
6.1 Discussion

The chicken egg has been a part of the human diet for centuries. It is virtually a part of daily diets in non-vegan individuals due to its wide-range use. In the culinary world alone, eggs are used in food products including, but not limited to, cakes, hamburgers, stocks, battered foods, pasta, mayonnaise, breads, and custard or on its own in forms such as omelettes. This wide use is primarily due to the high availability, affordability and nutritional value of eggs. Inclusion of chicken egg provides many health benefits, especially for growing children, due to the presence of proteins, a mix of fats, minerals/elements such as magnesium, iron, calcium and potassium, and vitamins such as thiamin (B1), riboflavin (B2), vitamins B6, B12, D and E, folate and biotin, just to name a few. Other food products may also carry traces of eggs carried on by food production machinery or utensils. Apart from these, traces of eggs are also found in pharmaceutical products including vaccines such as influenza. The allantoic or amniotic fluids of fertilized eggs are known to be effective and cheap for culturing of viruses required for production of vaccines. Isolation of egg-cultured viruses often causes carry-over of traces of egg proteins into the final product. However, not all individuals can enjoy the above-mentioned benefits from eggs or egg products. Today, 0.5-2.5% of children are affected by hypersensitivity to the chicken egg. With no permeant cure available, strict dietary avoidance is the best egg allergy management strategy. The wide use of eggs in a range of aforementioned products however, complicates strict avoidance and increases the chances of accidental exposure, which may result in adverse allergic reactions such as anaphylaxis. Eggs are cheap and readily available in both developed and underdeveloped societies. Therefore, strict avoidance of eggs may be nutritionally
disadvantageous for egg allergic children coming from financially disadvantaged families. Apart from dietary avoidance, egg allergen SIT is currently being pursued as a therapeutic measure. SIT includes OIT, a widely used method for treating food allergies, which involves oral administration of small amounts of the allergen to the individual in a dose-increasing manner to induce immunological tolerance.

Currently, crude egg extracts or allergens isolated from natural egg white are used in OIT to treat egg allergy and this poses some complications. Crude egg extracts contain a mix of proteins, including a mix of allergens. This increases the risk of unwanted immune responses when administered to patients during OIT, especially in highly egg allergic patients. These extracts are also used in diagnostic methods such as skin prick testing (SPT), which may also cause severe allergic reactions during diagnosis. The presence of multiple allergens in the extract also undermines the recognition of causative egg allergen during diagnosis. The crude allergen extracts may also contain contaminant proteins and other elements which may impede proper diagnosis or treatment of egg allergy. Allergens isolated from natural egg white may contain traces of other egg allergens which can cause unwanted immune responses. The high occurrence of egg allergy and absence of highly effective treatment and management methods laid the foundation for the development of the hypotheses and aims of this thesis. There were three main aims for this project: production of IgE reactive recombinant versions of 4 major egg white allergens; production of a hypoallergenic variant of the major egg white allergen ovomucoid; and investigation of genetic engineering approaches to produce hypoallergenic chicken eggs.
IgE reactive recombinant variants of allergens have been used in SIT for treatment of a number of allergies. Individual recombinant allergens are purer and less (or not) contaminated with other proteins or materials when compared to their natural counterparts, thus offering an excellent option for SIT including OIT. In egg allergy, the use of recombinant proteins for OIT is beneficial because of the shortcomings of the allergens extracted from natural egg white, which can trigger unwanted immune responses. For highly sensitive individuals, the separate recombinant proteins can be administered separately to better manage the desensitization process. The recombinant proteins can also be used in SPT which may help accurately identify which allergen a patient is allergic to, without causing unwanted immune reactions that may be caused by all the allergens/traces of other allergens present in natural egg white extracts.

Chapter 2 of this thesis focussed on production of recombinant variants of the major egg white allergens: ovomucoid (OVM), ovalbumin (OVAL), ovotransferrin (OVT) and lysozyme (LYS). cDNA of all four allergens were successfully isolated from an oviduct of an egg laying hen. The cDNA were expressed in pTrcHisA vector, which fuses a 6xHis tag and an Xpress epitope to the expressed protein which allows identification and purification of the proteins. The recombinant proteins were first expressed in *E. coli* TOP10 strain, however breaking down of the proteins was visible when analysed by SDS-PAGE and western blotting. Therefore, it was decided to express the protein in more versatile *E. coli* Express I*®* strain. The results evidently showed an improvement of protein expression by showing less degradation when compared to the proteins expressed in *E. coli* TOP10. Although detection by anti-His antibodies
and anti-Xpress antibody assisted in confirming the correct protein has been expressed, it was decided to further analyse the proteins by mass-spectrometry, which also confirmed the identity of the proteins, except for OVM. It can be suggested that the trypsin inhibitory activity of OVM may have disrupted the in-gel trypsin digestion of the proteins in preparation for mass spectrometry analysis, thus causing the failure to successfully identify OVM. However, earlier detection by different antibodies at the correct molecular weight and cDNA sequencing result was sufficient to conclude that OVM in fact has been successfully expressed.

The formation of inclusion bodies during protein expression in \( E. \text{coli} \) restricted the expression of OVT and LYS to the insoluble fraction, while OVM and OVAL were found in both soluble and insoluble fraction. This was not considered an issue for this experiment because it was decided to use crude \( E. \text{coli} \) extract with the expressed proteins for downstream applications. Ni-NTA based protein purification was attempted, however the purified proteins did not show high purity when analysed by SDS-PAGE. Follow-up nickel-affinity chromatography or size exclusion chromatography is necessary to isolate the expressed protein with high purity, however, this was not attempted due to time and resource constraints.

In Chapter 3, the recombinant proteins produced in Chapter 2 were further studied for their IgE reactivity using allergic patients’ sera. The immunological analysis of allergic patients’ sera against natural egg white suggested that egg allergy in Australian populations may be due to hypersensitivity to OVT and LYS. The result also preserved the status of OVM being the most reactive egg allergen by showing dark
bands of intense IgE binding in the western immunoblots for the patients that were allergic to OVM. However, a much larger sera population is needed to draw any conclusions. When the recombinant proteins were tested against a pool of egg allergic patients’ sera OVM, OVAL and OVT were found to be IgE reactive while LYS did not show any reactivity. The most probable explanation for the absence of IgE reactivity of LYS is competition by the other allergens during incubation with the sera. This may be overcome by testing each recombinant allergen with egg allergic sera independent of one another, nonetheless this was not attempted due to the unavailability of sufficient amounts of blood sera. The produced IgE reactive OVM, OVAL and OVT, due to the lack of contamination from other allergens or each other, has the potential to be used in OIT and SPT upon purification and further animal based and clinical experimentation. Purified preparations of these recombinant proteins may carry trace amounts of *E. coli* proteins, however this should not present a problem as *E. coli* is part of human gut microbiota.

As mentioned earlier, IgE reactive recombinant allergens may improve OIT. However, highly sensitive patients may still excessively react to these recombinant proteins during OIT, potentially causing life-threatening conditions such as anaphylaxis. OIT is of course done in a clinical setting under the watchful eyes of healthcare professionals, but hypersensitivity overreactions sometimes may still lead to unfavourable situations and can be costly. In such instances the use of less allergenic, or hypoallergenic, variants of allergens may prove to be extremely beneficial. Hypoallergens have been previously produced in studies involving allergies caused by allergens from different sources. These include, but not limited to, the production of
a hypoallergenic variant of the latex allergen Hev b 6.01 by Drew *et al* 2004, production of a vaccine using hypoallergenic derivatives of the birch pollen allergen Bet v 1 by Niederberger *et al* 2004, pre-clinical testing of a vaccine developed with a combination of hypoallergenic dust mite allergens Der p 1 and Der p 2 by Wai *et al* 2012 and, more recently, pre-clinical characterization of hypoallergenic carp allergen Cyp c 1 (parvalbumin) by Zuidmeer-Jongejan *et al* 2015. There is however lack of studies conducted on production of hypoallergenic recombinant variants of egg allergens with the focus been given to development of hypoallergenic natural egg white by different treatment methods such as heating, enzymatic digestion, radiation and ethanol precipitation. These preparations may still be less versatile than recombinant hypoallergens due to contamination from other allergens present in the egg white.

Chapter 4 of this thesis addressed production of a hypoallergenic variant of the dominant egg white allergen OVM. There are 9 cysteine-cysteine disulphide bridges in OVM, giving it structural stability even in the presence of heat and proteases. It has both linear and conformational IgE binding epitopes which, combined with structural stability, manages to retain its IgE reactivity thus potentially contributing to cooked egg allergic reactions. The presence of conformational epitopes in OVM highlights the possibility of making structural changes to reduce IgE reactivity. This was exploited in this chapter by mutating 2 cysteine-cysteine disulphide bridges using site-directed mutagenesis, similar to the development of Hev b 6.01 by Drew *et al* 2004. It was decided to target 2 disulphide bridges as a starting point and mutate more bridges if necessary. However, the disruption of just two disulphide
bridges significantly reduced IgE reactivity, as shown by the immunoblotting conducted using egg allergic patients’ sera. It should be noted that the observed reduction in IgE binding to the mutant was in comparison to the recombinant wild-type protein which was loaded on to the gel (and transferred on to nitrocellulose) at a higher concentration than the mutant, which essentially indicated a significant reduction of IgE reactivity. This hypoallergenic variant of OVM too has the potential to be used in OIT following animal and clinical trials. Nevertheless, unlike wild-type recombinant allergens, this hypoallergen cannot be used in diagnostic approaches such as SPT because of potential misdiagnosis of less sensitive patients.

The recombinant allergens and hypoallergenic OVM produced in Chapters 2-4 essentially may assist in improving the patient’s immune system against the allergen, which is the ultimate goal of OIT. Some research has also been conducted on disrupting the allergic cascade by reducing the activity of cytokines, such as IL-4 and IL-13, involved in allergic reactions\(^{239}\), which is again targeting the patient’s immune system. In the vast arena of egg allergy research, there is a lack of, more likely an absence of, research focussing on the root of the course which is the chicken itself. Methods such as RNAi and CRISPR-Cas9 are widely used in a range of research to answer questions in biomedicine and agriculture. A prime example of these is the development of a calf producing milk lacking $\beta$-lactoglobulin using RNAi by Jabel et al 2012\(^{46}\). These methods have the potential to be used in egg allergy research.
Chapter 5 of this thesis aimed at using CRISPR-Cas9 to knockout OVM in a chicken fibroblast cell line and using RNAi to knockdown OVM in the chicken. OVM was chosen as the targeted allergen due to its dominance as an allergen over the other egg white allergens. The CRISPR-Cas9 driven knockout however did not yield satisfactory results due to unknown reasons. In this experiment, only exon 1 of OVM genomic DNA was targeted by two small guide RNAs (sgRNAs). It can be suggested that sgRNAs targeting different exons of the gene should be scrutinized to potentially and effectively knockout the gene.

RNAi knockdown of OVM in the chicken, on the other hand, provided satisfactory preliminary data that may be useful in studying the importance of OVM for the developing chick and for egg laying. This experiment aimed to produce two short hairpin RNAs (shRNAs) capable of knocking down OVM expression. When the two shRNAs were tested in a chicken fibroblast cell line, it was evident that both shRNAs were effective at significantly knocking down OVM through RNAi. These shRNAs were then microinjected, in the form of micro RNAs (miRNAs), into chicken embryos in order to produce OVM knocked-down transgenic chickens. Preliminary gonad assays showed that both miRNA constructs are being targeted by the host RNAi machinery, thus indicating that OVM will be knocked-down to a certain extent in the transgenic chicken. Follow up semen screening of a male from the G0 generation male chickens predicted that ~1.5% of the G1 offspring will be transgenic and may have OVM knocked-down as a result.
The knockdown of OVM via RNAi will provide information about the importance of the protein for the chicken, however transgenic G1 generation hens and their eggs are necessary for further analysis and to draw conclusions. Only male birds from the G0 generation were selected because their semen will be used to produce the G1 transgenic generation. Thus far, the selected males (2 representing each miRNA) seem healthy, somewhat providing assurance towards the production of the transgenic generation. Although a hypoallergenic egg was not decisively produced within the time-frame of this project, the RNAi knockdown data highlights the potential of genetic manipulation/engineering in egg allergy research, opening a brand-new frontier in the fight against this wide-spread condition.

It is poorly understood why the human immune system recognizes some proteins in the egg as allergens and the others not. This is true for not just egg allergy, but also for other types of allergies. It is widely suggested that genetic predisposition and environmental factors may cause allergy\textsuperscript{240-242}, however it is still unclear why any changes in the immune system can consider certain substances as allergens. For instance, cancer patients carry mutations in their genome that cause unfavourable conditions such as reduction in tumour suppression\textsuperscript{243}. Any such mutations are not known to be responsible for allergies which may cause the immune system to recognize harmless environmental substances as harmful ones. It is suggested that a gene responsible for the skin barrier disease ichthyosis vulgaris, known as filaggrin (FLG), may play a crucial role in allergic contact dermatitis\textsuperscript{244}, yet again it is not conclusive.
The lack of understanding of the exact biological cause of allergy has led to extensive scrutiny of the immune response and the properties of allergens. Current broad understanding of the changes that occur in innate and adaptive immunity during allergic reactions has permitted the development and effective utilization of methods such as SIT to treat allergies. The understanding of the properties and IgE reactive epitopes of different allergens has allowed production of recombinant allergens and hypoallergens, which in turn target improvement of immunotherapy and allergy diagnosis. The scarcity of such recombinant allergens and hypoallergens for egg allergy treatment and diagnosis was addressed in this thesis, leading to the successful production of IgE reactive OVM, OVAL, OVT and LYS and a hypoallergenic variant of OVM.

As mentioned earlier, genetic engineering tools such as RNAi and CRISPR were used in this PhD project as a novel frontier in egg allergy research. These techniques should not be limited to egg allergy research as they have substantial potential to be used in other food allergies such as peanuts, tree nuts, wheat, soy and seafood. Allergens involved in these food allergies are widely-studied and therefore genetic engineering may be useful in developing transgenic crops and animals without such allergenic traits. Such advanced techniques may not however be useful in other allergies such as house dust mites and grass pollen, simply because it is impossible to replace all dust mites and grasses in the environment. However, it is wise to state that tackling of food allergies alone can improve quality of life of many individuals while reducing the burden on medical industries and governments by saving millions-billions of dollars spent annually on food allergy management. Being the second most
common food allergy in children and affecting 0.5-2.5% of children worldwide, a cure or a solution for egg allergy sure will be a significant contributing factor for such improvement of health and wellbeing of the society.

6.2 Future perspectives

The IgE reactive allergens and OVM hypoallergen produced in this study can be further studied using B-cell and T-cell activity assays. For successful immunotherapy using these recombinant proteins it is important to validate their ability to retain T-cell reactivity, a feature necessary for immunotherapy\textsuperscript{253}. In addition, the hypollergenic OVM protein should be able to demonstrate reduced B-cell reactivity while retaining T-cell reactivity. To conduct either of the above mentioned tests, it is important to purify the protein from \textit{E. coli} extract. All proteins were expressed with a 6xHis tag therefore nickel-affinity resin based purification can be used for initial purification, followed by nickel-affinity chromatography for a highly pure product. Size-exclusion and ion-exchange chromatography may also be used for purification of such proteins. It will also be interesting to study the efficacy of these recombinant proteins at desensitization using sensitized animal models such as mice or sheep. These animal models, especially mice, are widely used in biomedical research and can be easily sensitized using wild-type allergens \textsuperscript{254}. The animal testing can then be followed by clinical testing prior to be used in a regular basis for egg allergic patient treatment.
In the future, the transgenic hens that will be produced from the G0 generation of chickens produced in this project can be studied for the level of expression of OVM and possible upregulation/downregulation of other proteins. Their eggs can also be tested for their IgE reactivity with egg allergic patients’ sera, in comparison to wild-type eggs. If the chickens seem healthy and are capable of laying eggs after knockdown of OVM, a total knockout of the gene may be attainable using methods such as CRISPR-Cas9, ZFN or TALENs. A successful knockout of OVM will allow production of a significantly hypoallergenic chicken egg that may be used for immunotherapy and even human consumption for patients allergic only to OVM. If total knockout of OVM is not feasible, the hypoallergenic variant produced in this study can be knocked-in using the same genome engineering methods.

An alternative for knockdown or knockout of the allergens is changing the known sequences of IgE binding epitopes of the egg allergens using precision genome engineering methods such as CRISPR-Cas9. This can be implemented for addressing highly abundant allergens such as OVAL because a knockdown or a knockout of such proteins may prove detrimental to the chicken or the egg while reducing the quality of the eggs. It will also be fascinating to scrutinize cross-species gene swapping to produce hypoallergenic eggs. For instance, the human lysozyme sequence can possibly be knocked-in in place of the chicken lysozyme in the chicken genome as human lysozyme is similar to its chicken counterpart in molecular size, have similar functions and, most importantly, humans will not be allergic to human proteins. However, such cross-species genetic engineering should carefully address the ethical
nature of the research to avoid negativity about such research in the society. A final hypoallergenic egg should then be studied using animal models and clinical testing.

6.3 Conclusion

Hypersensitivity to the chicken egg is a wide-spread condition affecting mainly children. It affects 0.5-2.5% of children world-wide and 8.9% in Australia. Currently, avoidance of eggs or egg-related products are pursued as the most effective management strategy. However, this is extremely difficult and places a massive burden on affected family simply because of the abundance of egg-related food and pharmaceutical products. As a treatment method, immunotherapy is widely used in food allergies, including egg allergy. This too has limitations due to the unavailability of pure individual egg allergens and unwanted immune responses caused by the use of crude egg extracts or contaminated individual natural allergens for immunotherapy. The research conducted in this PhD project successfully produced IgE reactive versions of egg white allergens. In addition, a hypoallergenic variant of the major egg white allergen OVM was also produced. All of these proteins qualify to be used in pre-clinical and clinical testing following B-cell and T-cell activity testing. As a separate avenue for tackling egg allergy, this PhD project also scrutinized the used of genetic engineering to produce hypoallergenic chicken eggs. RNAi was used to deliver an OVM knockdown mechanism to developing chicken embryos, whose gonads demonstrated successful delivery and potential activity of the RNAi machinery. At the conclusion of this project, the parental generation (G0) of male birds seem healthy and predicted 1.5% transgenesis in the next generation (G1). The
G1 generation will provide important information required for use of precision genome engineering to develop hypoallergenic chicken eggs. In conclusion, this project provided useful results, reagents and novel information necessary for the development of a solution to treat chicken egg white allergy.

-The End-
References

4. Peavy RD, Metcalfe DD. Understanding the mechanisms of anaphylaxis. Current Opinion in Allergy and Clinical Immunology 2008;8:310-5.
21. Croft M, Bradley LM, Swain SL. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. Journal of Immunology 1994;152:2675-85.


de Maat-Bleeker F, van Dijk AG, Berrens L. Allergy to egg yolk possibly induced by sensitization to bird serum antigens. Annals of Allergy Asthma & Immunology 1985;54:245-8.


Chinoy B, Yee E, Bahna SL. Skin testing versus radioallergosorbent testing for indoor allergens. Clinical and Molecular Allergy 2005;3:4-.


Sicherer SH. Elimination Diets and Oral Food Challenges. Food Allergy: John Wiley & Sons Ltd; 2013:296-305.


151. Waserman S, Watson W. Food allergy. Allergy, Asthma, and Clinical Immunology 2011;7:S7-S.


197. Schoos A-MM, Chawes BLK, Følsgaard N, Samandari N, Bønnelykke K, Bisgaard H. Disagreement Between Skin Prick Test and Specific IgE in Young Children. Allergy 2014:n/a-n/a.


Appendices

Appendix A – RNAi knockdown of OVM – Gonad assays supplementary figures

Appendix B – Publications
Appendix A

RNAi knockdown of OVM

Gonad assays supplementary figures
Figure A1: Gonads of Tol2Ovm1 micro-injected Male 1 under bright-field and fluorescence microscopy.
Figure A2: Gonads of Tol2Ovm1 micro-injected Male 2 under bright-field and fluorescence microscopy.
Figure A3: Gonads of Tol2Ovm1 micro-injected Female 1 under bright-field and fluorescence microscopy.
Figure A4: Gonads of TolZ0v1 micro-injected Female 2 under bright-field and fluorescence microscopy.
Figure A5: Gonads of Tol2Ovm1 micro-injected Female 3 under bright-field and fluorescence microscopy.
Figure A6: Gonads of Tol2Ovm2 micro-injected Male 1 under bright-field and fluorescence microscopy.
Figure A7: Gonads of Tol2Ovm2 micro-injected Male 2 under bright-field and fluorescence microscopy.
Figure A8: Gonads of Tol2Ovm2 micro-injected Male 3 under bright-field and fluorescence microscopy.
Figure A9: Gonads of Tol2Ovm2 micro-injected Female 1 under bright-field and fluorescence microscopy.
Figure A10: Gonads of Tol2Ovm2 micro-injected Female 2 under bright-field and fluorescence microscopy.
Appendix B

Publications
Review
Cracking the egg: An insight into egg hypersensitivity
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Abstract
Hypersensitivity to the chicken egg is a widespread disorder mainly affecting 1–2% of children worldwide. It is the second most common food allergy in children, next to cow’s milk allergy. Egg allergy is mainly caused by hypersensitivity to four allergens found in the egg white: ovomucoid, ovalbumin, ovotransferrin and lysozyme. However, some research suggests the involvement of allergens exclusively found in the egg yolk such as chicken serum albumin and YGP42, which may play a crucial role in the overall reaction. In egg allergic individuals, these allergens cause conditions such as itching, atopic dermatitis, bronchial asthma, vomiting, rhinitis, conjunctivitis, laryngeal oedema and chronic urticaria, and anaphylaxis. Currently there is no permanent cure for egg allergy. Upon positive diagnosis for egg allergy, strict dietary avoidance of eggs and products containing traces of eggs is the most effective way of avoiding future hypersensitivity reactions. However, it is difficult to fully avoid eggs since they are found in a range of processed food products. An understanding of the mechanisms of allergic reactions, egg allergens and their prevalence, egg allergy diagnosis and current treatment strategies are important for future studies. This review addresses these topics and discusses both egg white and egg yolk allergy as a whole.

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1. Allergy

Allergy is a symptomatic and an abnormal overreaction by the immune system to innocuous environmental substances, such as grass pollen and eggs, known as allergens. Allergens are a type of antigen, which trigger a complex immune response upon contact with the immune system. Allergy is classified as a type 1 hypersensitivity reaction because of the immediate and inflammatory immune response that is characterised by the excessive production of the antibody Immunoglobulin E (IgE). An allergic response involves two reaction phases based on the contact of the allergen with the immune system; primary and secondary response. The primary response (upon initial contact with allergen), stimulates the generation of IgE by T-lymphocytes. The secondary response (upon secondary contact with the same allergen) results in the degranulation of mast cells (mastocytes) and basophils (basophil granulocytes). The mediators (e.g. histamine) released by the degranulation process elicit clinical allergic symptoms such as bronchoconstriction, sneezing, rashes, itching or life threatening conditions such as anaphylaxis, just to name a few (Janeway and Traverse, 1994; Kay, 2001).

2. Allergic response

2.1. Allergic sensitisation

Upon contact with the immune system, an allergen is engulfed and processed by antigen presenting cells (APCs) and presented to naïve T-helper cells (Th0). This causes Th0 cells to differentiate into T-helper type 2 (Th2) cells in the presence of cytokines including Interleukin-4 (IL-4). Th2 cells then further secrete IL-4, IL-5 and IL-13, which induce B-cell differentiation into plasma cells secreting IgE. The IgE antibodies subsequently bind to the high affinity FcεRI receptors on mast cells and basophils, thus sensitizing the immune system to the allergen (Kay, 2001) (Fig. 1).

Abbreviations: OIT, oral immunotherapy; SIT, specific immunotherapy; SPT, skin prick test.
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2.2. Allergic reaction

Upon secondary exposure to the same allergen (unprocessed by APCs), the allergen binds to the membrane bound IgE molecules on mast cells and basophils. Cross-linking of FceRI occurs when multiple IgE molecules interact with the same intact allergen, triggering degranulation of mast cells and basophils, consequently releasing granule-derived vasoactive chemicals such as histamine, serotonin, proteases, and cytokines IL-4, IL-5 and IL-13 (Fig. 1). These mediators cause allergic symptoms such as airway smooth muscle constriction (bronchoconstriction), mucus hypersecretion, mucus cell metaplasia, redness, rashes, sneezing and anaphylaxis (Kay, 2001).

3. Food allergy

Food allergy is one of the major allergies in today’s society. In a double-blind placebo controlled food challenge study conducted by Allan Bock et al. (1988), 80% of the subject children were found to develop hypersensitivity symptoms to food allergens. In the developed world, 10% of children are affected by food allergies, while emerging or developing nations has a prevalence of 7% (Prescott et al., 2013). Occurrence of food allergy, like other atopic diseases, is rising and remains a problem throughout the world. It is a major cause of anaphylaxis, especially by nuts, and treated worldwide in emergency departments and hospitals, incurring a massive financial burden on the governments and the public (Bock et al., 2001; Hugh, 2004). Food hypersensitivities include abnormal overreactions caused by intolerance to food (non-allergic) and allergic responses caused by an antigen in the food. Intolerance to food is due to some physiological abnormality, such as the lack of lactase which causes intolerance to lactose, whereas the allergic food reactions are mediated by IgE or non-IgE immune responses (Hugh, 2004). Food allergies arise through the interaction between the intestinal immune system and food allergens present in the food that we consume on a daily basis. Allergenic foods include, but not limited to, eggs, peanuts, fish and milk. Antigens (allergens) present in these foods are responsible for IgE mediated hypersensitivity reactions. For example, people allergic to milk react to the milk whey protein allergen β-lactoglobulin (Jabed et al., 2012) and patients allergic to egg may be due to the egg white protein ovomucoid (Ando et al., 2008). Most of these allergens are glycoproteins that can withstand heat, acids and proteases (Hugh, 2004), this explains why most allergenic foods remain allergenic even after cooking or processing.

4. Egg allergy

Hypersensitivity to the chicken (Gallus gallus) egg is a widespread disorder mainly affecting children, with a recent meta-analysis suggesting a prevalence of 0.5–2.5% of children (Rona et al., 2007). It is the second most common food allergy next to cow’s milk allergy and it is the most common allergy among children with atopic dermatitis (Caubet and Wang, 2011; Langeland, 1985; Sampson, 1983). According to an Australian study conducted by the Murdoch Children’s Research Institute (MCRI), 8.9% of infants are allergic to eggs (Osborne et al., 2011). However, egg allergy is not limited to children, with cases that have been reported of adult onsets (Unsel et al., 2013). Children allergic to egg generally grow out of the condition by school age (Boyano-Martínez et al., 2002; Venter et al., 2008). Hypersensitivity to allergens present in the eggs causes conditions such as itching, atopic dermatitis, bronchial asthma, vomiting, angio-oedema, rhinitis, conjunctivitis, laryngeal oedema, chronic urticaria and allergic eosinophilic gastroenteritis (Eigenmann, 2000; Fremont et al., 1997; Jaffe et al., 1994; Quirce et al., 2001). Egg allergic patients produce IgE specific to sequential (linear) and conformational epitopes of egg allergens. However some sequential epitopes recognised by persistent egg allergic patients are not recognised by patients with transient egg allergy (Järvinen et al., 2007). This suggests a difference in severity of allergic reaction between persistent and transient egg allergic patients. Anaphylaxis may also occur in some individuals, however this depends on the processing of the egg. This may be due...
the reduction in IgE reactivity in heated egg, possibly due to the disruption of conformational IgE binding epitopes in the egg allergens. It has also been found that eggs heated on its own are more IgE reactive when compared to eggs heated in the presence of another substance such as wheat (Bloom et al., 2014; Watanabe et al., 2014). This suggests that an egg allergic patient may show less reactivity to food items such as cakes or muffins than to fried or baked egg. The prevalence of egg allergy and the aforementioned conditions highlight the importance of egg allergy research. An understanding of the different egg allergens, egg allergy diagnosis methods and treatment methods are imperative for future research.

5. Allergy to egg white

Egg allergy is mainly caused by 4 of the many different proteins that make up the egg white (Leduc et al., 1999; Rupa and Mine, 2003). However, these may not be the only allergenic egg white proteins, particularly in the wake of new discovery of a large number of other proteins in the egg white (Mann and Mann, 2011). These allergens are namely ovomucoid, ovalbumin, ovotransferrin and lysozyme, with ovomucoid being the most allergenic of the four and ovalbumin being the most abundant (Fig. 2). A few studies have identified egg yolk allergens, such as chicken serum albumin and YGB 42 (Fig. 2). The four aforementioned egg white allergens are produced by tubular gland cells which comprise the majority of the magnum portion of the chicken oviduct (Kohler et al., 1968; Palmiter et al., 1970). Induction of expression of these allergens are highly dependent on estrogens and this has been demonstrated in studies involving chicks in which upon administration of estrogens the tubular gland cells started producing the allergenic proteins (Kohler et al., 1969; Oka and Schimke, 1969; Palmiter and Wrenn, 1971). Egg allergy can be caused by consumption of raw or cooked eggs, however a study conducted by Lemon-Mulé et al. (2008) indicates that majority of the egg allergy patients are tolerant to heated eggs, although the allergenicity of proteins are not exclusively dependent on their resistance to heat and enzymes (Astwood et al., 1996). The same study shows that continued exposure to the heated egg is associated with immunological changes similar to the changes observed in developing tolerance to raw eggs. These immunological changes include increasing IgG4 levels and a reduction in wheal sizes in egg white specific skin prick testing (SPT). IgA and IgA2 may also be of importance in developing tolerance to egg white (Konstantinou et al., 2014). However, more studies involving a larger number of subject individuals are necessary to confirm the effects of heating on egg white allergens, resulting IgG reactivity and other immunological changes in patients. Given that the structure of the proteins may play a crucial role in IgE binding and eliciting an allergic response, an understanding of the structure and function of each of the four allergens is essential for future studies.

6. Allergens in the egg white

6.1. Ovomucoid (Gal d 1)

Ovomucoid is the most allergenic and the dominant allergen found in the chicken egg white. Constituting 11% of the total egg white, ovomucoid is a trypsin inhibitory glycoprotein with a molecular weight of 28 kDa (Kato et al., 1987; Mine and Zhang, 2002a). It is comprised of 186 amino acids arranged in three tandem domains, each approximately 60 amino acids in length, containing nine intra-domain disulfide bonds and five carbohydrate side chains. The trypsin inhibitory activity is limited to the second domain, in which a putative reactive site is present for trypsin inhibition (Abdou et al., 2013; Kato et al., 1987). The arrangement of the three domains is also similar to the pancreatic trypsin inhibitor. Previous studies have indicated that allergenicity or IgE binding capacity of ovomucoid is not considerably affected by heat and proteinase activity (Jarvinen et al., 2007; Konishi et al., 1985). This suggests that the structure of the IgE binding epitopes of ovomucoid is mainly linear, however there are some conformational epitopes present in all domains (Mine and Zhang, 2002a). This suggests that cooking of eggs may have little or no effect on the allergenicity of ovomucoid.

In addition, the third domain of ovomucoid shows more IgE binding activity when compared to the other two domains (Zhang and Mine, 1998). It can be suggested that the third domain would be an ideal target for studies focusing on the production of hypoallergenic variants of ovomucoid that may be used for treatment strategies such as immunotherapy.

6.2. Ovalbumin (Gal d 2)

Ovalbumin is the most abundant (58%) protein in the egg white and it is considered to be the second most important allergen in egg hypersensitivity. Ovalbumin is a water soluble glycoprotein made up of 385 amino acids and has a molecular weight of 45 kDa (Nisbet et al., 1981). Mine and Zhang (2002b) reported that carboxymethylxyl, urea treatment or heat denaturing at 95 °C of ovalbumin does not significantly affect its IgE binding capacity. This suggests that anti-ovalbumin IgE primarily recognises linear epitopes than conformational epitopes. Similar to ovomucoid, this indicates that cooking may not significantly affect the allergenicity of ovalbumin, however digestive enzymes may affect the allergenicity. The major function of ovalbumin in the chicken egg is currently unknown, however it is a member of the serpin family of proteins although it is incapable of inhibiting protease activity. This lack of protein inhibitory activity is explained by the absence of an important serpin conformational change in the reactive centre loop of ovalbumin (Huntington and Stein, 2001). Ovalbumin is also known to exhibit biological functions including antibacterial and immunomodulating activities (Kovacs-Nolan et al., 2005). The antibacterial properties are however not exerted by intact ovalbumin, instead, proteolytic digestion of the protein has shown to produce peptide fragments capable of acting against organisms such as, but not limited to, Bacillus subtilis and Candida albicans (Pellegrini et al., 2004). It is also believed that ovalbumin may act as a source of amino acids for embryonic development (Abdou et al., 2013).

6.3. Ovotransferrin (Gal d 3)

Transferins are a group of iron-binding proteins found in vertebrates that has structural and functional similarities among each member. The molecular mass of these monomeric glycoproteins is about 76.6 kDa, and a molecule of transferrin can bind to two Fe3+ with high affinity. Ovotransferrin is one of the members of transferrins expressed in the chicken oviduct. Expression of ovotransferrin in the oviduct is under steroid control and it differs from serum hen transferrin only by an attached carbohydrate (Kurokawa et al., 1995; Williams et al., 1982). In vitro, ovotransferrin exhibits antimicrobial and immunomodulating activities (Kovacs-Nolan et al., 2005). Ovotransferrin is capable of penetrating the outer membrane of Escherichia coli, allowing selective penetration of ions which causes decayence of electrical potential of the cell, which in turn halts the ability of the bacteria to reproduce (bacteriostasis) (Aguilera et al., 2003). Some research has been conducted to study the changes in allergenicity and conformational structure of ovotransferrin when heated. A study conducted by Tong et al. (2012) showed that the allergenicity of ovotransferrin correlated with the structural changes caused by heating. Unfolding of the structure caused an increase in allergenicity and cleavage and rearrangement of disulfide bonds lead to a decrease in allergenicity.
(Tong et al., 2012). This suggests that ovotransferrin is one of the allergens responsible for hypersensitivities caused by consumption of cooked eggs as well as raw eggs. Therefore, it is wise to suggest that research should be focused on ovotransferrin as much as on ovomucoid and ovalbumin.

6.4. Lysozyme (Gal d 4)

Lysozyme, 14.3 kDa in size, is an enzyme that exhibits antimicrobial activity in the chicken egg, thus providing protection from potential microbial hazards. It is known to exert lytic activity on cell walls of microbes, therefore it is widely used in pharmaceutical products (Perez-Calderon et al., 2007). It is also used in various food products such as cheese (Fremont et al., 1997; Weber et al., 2009), which therefore poses a risk to egg allergic patients. Structurally, lysozyme contains conformational and sequential epitopes and it has in vitro biological functions such as antibacterial, antiviral, immunomodulating and antitumor activities (Kovacs-Nolan et al., 2005; Weber et al., 2009). Although lysozyme is less allergenic than ovomucoid and ovalbumin (Caubet and Wang, 2011), the high occurrence in many food and pharmaceutical products makes it an important target in egg allergy research.

7. Allergy to egg yolk

Chicken’s egg yolk is also responsible for causing IgE-mediated allergy, although with much lower prevalence than egg white hypersensitivity. Unlike allergy to egg white, which is commonly seen in young children with atopic dermatitis, allergy to egg yolk mostly affects during the adulthood. However, underlying immunological reactions to egg yolk allergens are mainly IgE-mediated, and usually produce symptoms such as urticaria, vomiting, abdominal pain and diarrhoea, as in allergy to egg white (de Maat-Bleeker et al., 1985; Mandallaz et al., 1988; Szepfalusi et al., 1994).

8. Allergens in the egg yolk

8.1. Chicken serum albumin (Gal d 5 or α-livetin)

Chicken serum albumin is the first allergen identified from the hen’s egg yolk. It is a water soluble globular glycoprotein with a molecular weight of 69 kDa. The main function of this protein is to transport metals in the blood stream (Predki et al., 1992). Serum albumin had been confirmed as an inhalant allergen as well as a food allergen through bronchial provocation and oral challenge tests, respectively. The main route of sensitisation for serum albumin is through the respiratory tract as seen in bird-egg syndrome. However, the vice versa of bird to egg allergy is also true, where initial sensitisation to egg yolk proteins pave the way to respiratory allergic symptoms in egg-bird syndrome. Serum albumin is a partially heat resistant allergen, thus some patients are able to tolerate extensively heated egg yolk, but not raw egg yolk (Quirce et al., 2001).

8.2. YGP42 (Gal d 6)

Yolk glycoprotein 42 (YGP42) is the second allergen identified from the hen’s egg yolk. It is a glycoprotein with a molecular weight of 35 kDa. According to the homology search conducted by BLAST, YGP42 is a fragment of the vitelligenin-1 (VTG-1) precursor. YGP42 contains three glycosylation signals at positions: 1662, 1698 and 1703. The peroxidate oxidation treatment of YGP42 did not abolish the IgE binding capacity indicating that sugar residues are not related to allergenicity. Furthermore, heat treatment and reduction did not affect allergenicity of YGP42 at all, while simulated gastric acid digestion completely eliminated its IgE reactivity. YGP42 was also investigated for any implication in bird-egg syndrome (Amo et al., 2010).

8.3. Other IgE binding proteins in the egg yolk

Apart from the recently discovered egg yolk allergens Gal d 5 and Gal d 6, few other proteins from the hen’s egg yolk proteome have exhibited a possible allergenic potential (Anet et al., 1985; Walsh et al., 1988). Three abundant egg yolk proteins from the egg yolk known as apovitellenin I, VI and phosvitin have demonstrated substantial IgE binding capacity in egg allergic patients’ sera. Furthermore, all the proteins were found to be effective at binding of egg yolk specific IgE. However, the clinical relevance of these proteins to egg yolk allergy have not been established to date, and therefore their status as allergens is uncertain (Walsh et al., 1988).
9. The bird-egg syndrome

Bird-egg syndrome is an IgE-mediated hypersensitivity disorder where a human develops an allergy to egg yolk, specifically to serum albumin, following sensitisation to inhalant avian antigens derived from sources such as bird's blood serum, feathers, droppings and dander (Mandalaz et al., 1988) (Fig. 3). This is different to allergen cross reactivity in which a patient allergic to a certain allergen is reactive to homologous or non-homologous allergens from the same or other species (Bublin et al., 2013; Rhijn et al., 2013). Majority of patients with bird-egg syndrome claim to have regular exposure to pet birds or poultry where sensitisation to airborne avian allergens take place, which then proceed to egg yolk allergy. Patients suffering from bird-egg syndrome suffer from both respiratory and gastrointestinal allergic symptoms such as asthma, rhinoconjunctivitis, edema, diarrhoea and vomiting. However, unlike allergy to egg white, which is predominantly seen in children with atopic dermatitis, bird-egg syndrome mainly affects adults with an observed gender predominance where majority of patients tend to be females. In addition, allergens produced by birds at workplace may also increase IgE levels in excess and can lead to allergic alveolitis. Currently, these IgE levels can be quantified and qualitatively evaluated using the UniCAP system, a type of ImmunoCAP (Lopata et al., 2004).

The common antigen present in hen's egg yolk and other avian antigens responsible for bird-egg syndrome is recognised as α-livetin, which corresponds to chicken serum albumin (Szepefalusi et al., 1994; Williams, 1962). Serum albumin belongs to a group of protein fraction known as livetins in the egg yolk. Livetines are water soluble glycoproteins that are derived from blood serum proteins of the hen. Furthermore, radioallergosorbent (RAST) test inhibition experiments had demonstrated inhibition of IgE binding to livetins of hen's egg yolk by avian antigens from different bird species. This result indicates that serum proteins from different bird species have highly conserved epitopes which allow specific IgE antibodies to cross react (Mandalaz et al., 1988). The clinical relevance of serum albumin in bird-egg syndrome has been established by immunological methods in order to validated it as a food allergen designating the allergen name Gal d 5 (Quirce et al., 2001).

An individual develops allergy to hen's egg yolk following exposure to birds, where sensitisation to inhalant avian allergens takes place, resulting in respiratory allergy symptoms. The cross reactive allergen which is responsible for producing both respiratory and gastrointestinal allergy symptoms in bird-egg syndrome is identified as chicken serum albumin present in bird antigens and in the egg yolk as well.

10. Diagnosis of egg allergy

Different diagnostic techniques are used for successful diagnosis of egg allergy. Similar to other allergic conditions, the foremost step in egg allergy diagnosis is assessment of the medical history of the patient for potential evidence for clinical reactivity to eggs and physical examination. This helps to determine whether the patient has experienced adverse reactions to eggs and the potential involvement of the immune system (Metcalf and Sampson, 1990; Sicherer, 1999). The medical history examination essentially takes into consideration the allergic conditions the patient's parents are/were exposed to and evaluates this information with the current symptoms, the type and the amount of food ingested and the timing of the reaction. The physical examination involves testing for gastrointestinal symptoms and detection of atopic features in the body. The next step involves the use of in vitro or in vivo tests such as SPT and ImmunoCAP specific IgE blood tests (formerly known as radioallergosorbent tests or RAST) (Bock et al., 1977; Chinyo et al., 2005). These tests assist in determining if the patient possesses egg allergen specific antibodies in their immune system. Although these tests provide an estimation of allergen specific IgE in the patient, which positively correlates to the clinical reaction (wheal formation), they fail to fully ascertain diagnosis of clinical symptoms of egg allergy. Increasing levels of IgE may predict the presence of an allergy, however, there is no universal agreed level of IgE to confirm the condition as the IgE levels can differ between different individuals. This may also be due to the discrepancies between the testing method, testing criteria and prevalence of the allergy between studies. However, a recent study conducted by Sicherer et al. (2014) has further confirmed the significant predictive capacity of IgE as a marker in egg allergy. They also report of a web-based calculator that uses an algorithm, which is based on novel findings on egg specific IgE, that may allow clinicians to predict the likelihood of the resolution or persistence of the disease. Supplementary studies on IgE as a reliable egg allergy marker may provide useful information for the development of effective diagnostic approaches.

In addition to SPTs and ImmunoCAP tests, elimination diets and oral food challenges can be used for the diagnosis of egg allergy. In an elimination diet diagnosis, once the patient is suspected of being allergic to eggs, a diet excluding eggs is followed to study the ability of the patient to maintain the diet without any hypersensitive reactions over a period of time (Sicherer, 2013). In an oral food challenge, the patient is asked to eat/ingest a small amount of egg to reproduce the hypersensitivity symptoms. It is also done in a dose dependant manner where the egg ingestion is increased until the symptoms are observable. Oral food challenge can be done openly, single-blinded, double-blinded placebo controlled, with the latter being considered the gold standard in diagnosis of major food allergies because it provides information such as the amount of food needed to cause an allergic reaction and the extent of the patient's reaction (Rance et al., 2009). However, the safety of oral food challenge has not been well established or scrutinised and may not be ideal if the patient is known to be anaphylactic to eggs, therefore much care should be taken when implementing this method. Overall, medical history and physical examination, SPTs or ImmunoCAP tests and oral food challenge combined together provides successful diagnosis of egg allergy (Fig. 4), which can lead to the implementation of treatment strategies for the condition.

11. Treatment of egg allergy

As for other food allergies, currently there is no temporary or permanent cure for egg allergy. Upon successful diagnosis for egg allergy, the most effective treatment is strict dietary avoidance of eggs and products containing traces of eggs. Eggs are an important source of nutrients and thus strict avoidance may cause malnutrition or eating disorders. The egg yolk is known to carry numerous health benefits, but since it is impossible to fully separate the egg yolk from the egg white without allergen contamination, complete avoidance of the egg is the only option, which results in reduced or no uptake of this important nutrient source (David et al., 1984; Friedman, 1996). As a part of avoidance of eggs, it is important to educate patients how to identify potential egg allergens or traces of eggs in various food products such as cakes, biscuits, cereal and breads, as well as educate them about foods/supplements that can potentially replace eggs in their diet. This may include involvement of a dietician for patients with dietary limitations, such as vegetarians and multiple food allergic patients, to ensure proper intake of vital nutrients.
Fig. 3. An illustration of the bird-egg syndrome.

Fig. 4. A typical diagnosis procedure of egg allergy. Once a suspected a reaction to egg is reported a patient undergoes the appropriate steps shown in this figure. Figure adapted and modified from Eggesbo et al. (2001).
In addition to dietary avoidance, egg allergic patients are required to avoid vaccines and medications derived from eggs. For example, influenza vaccine is developed in chicken extra embryonic fluid and used extensively worldwide every year (Caubet and Wang, 2011). Although the majority of egg allergic patients may not have adverse effects upon administration of influenza vaccine (Chung et al., 2010), it is important to take caution to avoid severe or anaphylactic reactions in the individuals that may well react to the egg allergens (Zeiger, 2002). These patients should receive the vaccine via a desensitisation protocol upon diagnosis with a positive skin test. Yellow fever vaccine is another vaccine derived from chicken egg, with few reported cases of anaphylaxis upon administration (Kelso et al., 1999). Moreover, there are reported cases of anaphylaxis due to the use of lipid emulsions and pharmaceutical products derived from eggs (Artesani et al., 2008; Buchman and Ament, 1991; Hofer et al., 2003). Occurrence of egg allergens in our daily diet, vaccines and other pharmaceutical products complicates strict avoidance of eggs. Therefore, alternatives are required to treat patients in case of accidental exposure to these allergens.

In allergic conditions such as asthma, specific immunotherapy (SIT) is used to induce tolerance to the allergens. This method essentially involves administration of small amounts of allergens to patients in, with slight increments, to induce tolerance to that particular allergen. A study conducted by Buchanan et al. (2007) indicates that oral immunotherapy (OIT), a form of SIT, may provide a promising treatment strategy for patients without anaphylactic history to eggs. The authors suggest that OIT may even cause a shift in food allergy treatment paradigm from allergen avoidance to active immunotherapy. This is further supported by a recent study in which OIT showed successful desensitisation to eggs, however tolerance was maintained only in 1/3 of the study population following discontinuation of the egg diet (Caminiti et al., 2015). However, extreme care should be taken during immunotherapy to avoid potential anaphylactic reactions. In case of an accidental exposure to egg allergens, and acute anaphylaxis, the most common treatment is administration of the hormone and neurotransmitter epinephrine (Kemp et al., 2008; Sampson, 2004; Waserman and Watson, 2011). Upon administration of epinephrine at the right dosage and appropriate route, peripheral vasodilatory effects and bronchoconstriction caused by histamines and other chemical mediators released by allergic reactions are reversed, conditions such as urticaria, erythema and angioedema are reduced and further release of chemical mediators are suppressed. Currently, epinephrine is available for self-administration by auto-injectors such as Epipen. In summary, allergen avoidance, SIT and administration of epinephrine provide temporary relief from egg allergic reactions. However, these methods can be costly and may have negative effects on the lifestyle of the patient. Therefore, it is imperative to investigate and research on permanent and affordable ways to treat egg allergy.

12. Future perspectives in egg allergy research – cracking the egg problem

Various research teams around the world are involved in finding answers or cures for different types of allergies. Development of hypoallergenic variants of the egg allergens is given utmost priority by many egg allergy researchers, because hypoallergens may be used in immunotherapy to desensitise egg allergic patients. In fact, a group from University of Guelph in Canada has studied the efficacy of an engineered ovomucoid third domain at desensitizing a murine model (Rupa and Mine, 2006). This hypoallergenic third domain has been produced by directly targeting the IgE binding sites of that domain. A similar approach has been tested in 2004 by an Australian research team in which they successfully developed a T-cell reactive hypoallergenic variant of the latex allergen, Hev b 6.10, by targeting the disulphide bridges of that allergen (Drew et al., 2004), therefore it can be suggested that similar methods can be used for the development of hypoallergenic variants of egg allergens. In addition to the aforementioned methods, it has been recently reported that allergen conjugation with reducing sugars through the Maillard reaction may help attenuate allergic reactivity to allergens (Rupa et al., 2014). Production of IgE reactive recombinant variants of egg allergens is also important in diagnostic methods such as SPT and treatment methods such as SIT (Dhanapala et al., 2015; Valenta et al., 2011). Although the current results seem promising, all of the research conducted on hypoallergen and IgE reactive recombinant allergen production requires clinical trials before administration to patients.

The focus of egg allergy research should not be limited to the aforementioned strategies and invention of drugs/vaccines. The advanced molecular and genetic techniques used in other medical research and life sciences can be utilised for tackling egg allergy from the root of the cause. A team in New Zealand has developed a transgenic calf lacking the milk allergen β-lactoglobulin using RNAI technology (Jabed et al., 2012). Similar methods can be used in egg allergy research providing promising methods of egg allergy treatment or management. The advancement of techniques such as CRISPR (clustered regularly interspaced short palindromic repeats) (Sander and Joung, 2014) based genome editing may also allow precision engineering of the chicken genome to edit/change the IgE binding epitopes to produce transgenic hens capable of producing hypoallergenic eggs. If any of the current research on egg allergy proves successful in the future, a large number of families will benefit by being less apprehensive about eggs or egg contaminated products.

Conflict of interest

There is no conflict of interest

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References


Kovacs-Nolan, Kelso, Kato, Jaffe, Hofer, Friedman, Eigenmann, Chung, Chinoy, 382
Maat-Bleeker, reactions for (1), induced Allergy components expression emulsions. Evidence (2), in Am.
Enzyme allergy: statement of the European Society for Clinical Immunology and Allergy. J. Allergy Clin.
Immunol. 86 (3), 624–626.
Production and immunological analysis of IgE reactive recombinant egg white allergens expressed in Escherichia coli

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ABSTRACT

IgE-mediated allergy to chicken egg affects a large number of children and adults worldwide. The current management strategy for egg allergy is strict avoidance, however this is impractical due to the presence of eggs in a range of foods and pharmaceutical products including vaccines. Strict avoidance also poses nutritional disadvantages due to high nutritional value of eggs. Allergen specific immunotherapy is being pursued as a curative treatment, in which an allergic individual is gradually exposed to the allergen to induce tolerance. Use of recombinant proteins for immunotherapy has been beneficial due to the purity of the recombinant proteins compared to natural proteins. In this study, we produced IgE reactive recombinant egg white proteins that can be used for future immunotherapy. Using E. coli as an expression system, we successfully produced recombinant versions of Gal d 1, 2 and 3, that were IgE reactive when tested against a pool of egg allergic patients’ sera. The IgE reactivity indicates that these recombinant proteins are capable of eliciting an immune response, thus being potential candidates for immunotherapy. We have, for the first time, attempted to produce recombinant versions of all 4 major egg white allergens in E. coli, and successfully produced 3, with only Gal d 4 showing loss of IgE reactivity in the recombinant version. The results suggest that egg allergy in Australian populations may mainly be due to IgE reactivity to Gal d 3 and 4, while Gal d 1 shows higher IgE reactivity. This is the first report of a collective and comparative immunological analysis of all 4 egg white allergens. The significance of this study is the potential use of the IgE reactive recombinant egg white proteins in immunotherapy to treat egg allergic patients.

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1. Introduction

Allergy to chicken (Gallus gallus) egg is a widespread condition affecting 0.5–2.5% of children worldwide (Rona et al., 2007). It is known to be the predominant food allergy among children with atopic dermatitis and, among all children, it is the second most common food allergy (Sampson, 1983, Langeland, 1985, Caubet and Wang, 2011). A study conducted by the Murdoch Children’s Research Institute (MCRI) in Australia revealed that 8.9% of infants are allergic to eggs (Osborne et al., 2011). Symptoms and conditions caused by hypersensitivity to chicken egg include, but not limited to, atopic dermatitis, bronchial asthma, IgE mediated egg allergy (with urticarial, angioedema, vomiting, diarrhea and anaphylaxis), and allergic eosinophilic gastroenteritis (with pain, irritability, colic, and possibly oesophageal stricture and food impaction) (Eigenmann, 2000; Jaffe et al., 1994, Fremont et al., 1997, Quirce et al., 2001).

Egg allergy is mainly caused by 4 major proteins within the egg white (Leduc et al., 1999), namely Gal d 1 (ovomucoid), Gal d 2 (ovalbumin), Gal d 3 (ovotransferrin) and Gal d 4 (lysozyme), with Gal d 1 being the most allergenic of the four and Gal d 2 being
The most abundant. These proteins are produced in the magnification portion of the chicken oviduct, specifically by tubular gland cells (Kohler et al., 1968, Palmer et al., 1970). Previous studies involving administration of oestrogen into chicks have shown that, post injection of oestrogen, the chicks produced the allergenic proteins, indicating that expression of the allergens present in the egg white are highly dependent on oestrogen (Palmer and Wrenn, 1971, Kohler et al., 1969, Oka and Schimke, 1969). Consumption of raw or cooked egg may cause allergic reactions, although the majority of patients with egg allergy are tolerant to cooked egg (Lemon-Mulé et al., 2008). Egg allergy can also be caused by allergens in the egg yolk (de Maat-Bleeker et al., 1985), however these are not addressed in this study.

Production of recombinant versions of natural allergenic proteins is an option for treating allergies since these recombinant proteins can be used in treatment methods such as allergen specific immunotherapy (SIT) and diagnostic methods such as skin prick tests (SPT). It is a way of producing allergens with high purity without contamination from other allergens since individual proteins are expressed separately in host systems. Valenta and colleagues (Valenta et al., 2011) broadly discuss the importance of using recombinant allergens for immunotherapy and production of safer vaccines to better manage allergies. In this study, we have produced IgE reactive recombinant egg allergens and compared them with their natural counterparts. For the first time, we have performed collective and comparative immunological analysis of the natural and recombinant proteins of all 4 egg white allergens against an Australian population, which forms the overall aim of this study.

2. Methods

2.1. PCR of allergen coding sequences from oviduct total mRNA

Animal experimentation/sampling was conducted under protocol AEC1496, approved by the Australian Animal Health Laboratory (CSIRO-AAHL) Animal Ethics Committee and in accordance with the Australian code of practice for the care and use of animals for scientific purposes.

The magnification portion of a fresh oviduct was obtained from an egg laying hen. Upon extraction from the hen, the oviduct was chopped into ~5 mm pieces and stabilised in RNAlater RNA stabilising reagent. Total mRNA was then extracted from 180 mg of oviduct using a Oligotex Direct mRNA extraction kit (Qiagen, Cat.No.:72022), following the manufacturer’s guidelines. Gel d 1, 2 & 4 coding sequences were isolated directly from the total mRNA using Qiagen OneStep RT-PCR kit (Qiagen, Cat.No.: 210210). The forward (F) and the reverse (R) primers for each allergen were (bold letters represent inserted restriction sites): Gel d 1 F with Xhol 5'-CGCTCGAGATGGCATGGCAGCAGGC-3', Gel d 1 R with HindIll 5'-CGGAAGCTTCCAGATTTCTCAAAATGTC-3', Gel d 2 F with BamHI 5'-CCGGATCCATGATGCTCTACGTCG-3', Gel d 2 R with EcoRI 5'-GGGATCCGAGCTTAAACCGAAACTACACCTCTTAC-3', Gel d 4 F with BamHI 5'-CCGGATCCGTCGTTGCTGTACACCTTT-3', Gel d 4 R with EcoRI 5'-CCGGATCCGTCGTTGCTGTACACCTTT-3', for PCR amplification of Gel d 3, the total RNA was used to produce a cDNA library using Omniscript Reverse Transcription kit (Qiagen, Cat.No.: 205110). Qiagen LongRange PCR kit (Qiagen, Cat.No.: 206401), which uses the PCR optimising reagent Q-solution, was used to amplify the coding sequence from the cDNA library. The primers used for amplification of Gel d 3 were: Gel d 3 F with Xhol 5'-CGCTCGAGATGGCATGGCAGCAGGC-3' and Gel d 3 R with HindIll 5'-CGGAAGCTTCCAGATTTCTCAAAATGTC-3'. The PCR products were run on a 1% agarose gel and purified using a MinElute gel extraction kit (Qiagen, Cat.No.: 28604).

2.2. Cloning of allergens into pTrcHisA vector and expression in E. coli

The four gel purified PCR products (1 μg of each) were digested with the appropriate restriction enzymes; Gel d 1 and Gel d 3 with Xhol and HindIll & Gel d 2 and Gel d 4 with BamHI and EcoRL. Separately, two 1 μg batches of pTrcHisA vector (Invitrogen, Cat.No.: V360-20) were digested with Xhol and HindIll & BamHI and EcoRL. The digests were then gel purified using E-Gel Clonewell 0.8% SYBR Safe agarose gels and concentrations were measured. The four digested PCR fragments and the pTrcHisA vectors were then used in separate ligation reactions. The ligation reactions were carried out overnight at 4°C. The four ligation reactions, conducted for each PCR product, were then transformed into Express F chemically competent E. coli cells (New England BioLabs, Cat.No.: C3057H) following manufacturer’s guidelines. The transformants were plated on LB-agar (one plate for each allergen) with 50 μg/mL ampicillin and incubated overnight at 37°C. Next day, single colonies from each LB agar plate were picked and grown overnight in LB+ ampicillin at 37°C. Afterwards, 2 mL of each culture was centrifuged at 13,000 rpm to pellet the cells, and supernatant discarded. These cell pellets were used to isolate the pTrcHisA vectors containing the inserts using QIAprep Miniprep kit (Qiagen, Cat.No.: 27104) following manufacturer’s guidelines. The isolated plasmids were sequenced to confirm insert of the PCR product (allergen) using pTrcHisA forward and reverse primers: F5-GAGGTATATATTAATGTATCG-3' and R5-GATTTAATCTGTATCAGG-3'. The sequences were compared with published allergen sequences on IUIS/NCBI.

For expression of the proteins, overnight cultures of E. coli colonies containing each allergen construct were grown separately in LB media with ampicillin. The overnight cultures were then re-inoculated in 10 mL of fresh LB media and grown to mid-log phase (OD600 0.4–0.6). A 1 mL sample of each culture was collected and pelleted and frozen for later analysis. Expression was induced with 40 μL of 100 mM IPTG to give a final concentration of 0.4 mM and grown for six hours at 37°C with shaking. Samples were collected, pelleted and frozen every 1 hour for determination of optimum expression time.

2.3. SDS-PAGE and Western blotting of the expressed proteins

Each frozen cell pellet collected at different time points was resuspended and lysed with 400 μL of Cell Lytic B cell lysis reagent for ~10 min. Only the samples collected at 0, 2, 4, 5, 6 h were used for the analysis. The lysed cells were then centrifuged at 13,000 x g for 5 min and the insoluble (pellet) and soluble (supernatant) fractions were separated. The insoluble fractions were resuspended in 100 μL of 2X Tris-Glycine SDS sample loading buffer (Life Technologies, Cat. No.: LC2676). 9 μL of these resuspended samples were combined with 1 μL of NuPAGE reducing agent (10X) (Life Technologies, Cat. No.: NP0004). The soluble samples (4 μL) were combined with 5 μL loading buffer and 1 μL reducing agent. All samples were then heated at 85°C for 2 min and loaded onto 4–20% gradient Novex Tris-Glycine gels (Life Technologies, Cat. No.: ECG0252BX5). The gel was run for ~1.5 hrs at 125 constant voltage. Upon completion, the proteins on the gels were transferred onto nitrocellulose membranes on a XCell II™ Blot Module (Life Technologies, Cat. No.: E00002) according to manufacturer’s guidelines. The proteins on the nitrocellulose membranes were detected using WesternBreeze® chromogenic detection kit (Life Technologies, Cat. No.: WB7103) according to the manufacturer’s instructions using Anti-Xpress mouse monoclonal antibody (Life
2.4. Mass spectrometry analysis of the recombinant proteins

Selected gel bands of the optimum time points of each allergen (2 h) were incised and subjected to mass spectrometry analysis. The gel bands were in-gel-digested with trypsin and incubated overnight at 37 °C to digest the proteins into peptides. In-gel-digested samples were analysed on a LTQ Orbitrap Elite (Thermo Scientific) coupled to an Ultimate 3000 RSLC nanosystem ( Dionex). The nanoLC system was equipped with an Acclaim Pepmap nano-trap column and an Acclaim Pepmap analytical column. 2 µL of the peptide mix (i.e. each digested band) was loaded onto the trap column of 3% CH3CN containing 0.1% formic acid for 5 min before the enrichment column is switched in-line with the analytical column. The LTQ Orbitrap Elite mass spectrometer was operated in the data-dependent mode, whereby spectra were acquired first in positive mode followed by collision induced activation (CID). Ten of the most intense peptide ions with charge states ≥2 were isolated and fragmented using normalized collision energy of 35 and activation Q of 0.25 (CID). Data analysis was carried out using Proteome Discoverer (Thermo Scientific version 1.4) with Mascot against the BIRDS database. Search parameters were precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.6 Da (CID). Carbamidomethyl of cysteine was set as fixed modification and oxidised methionine as a variable modification. Trypsin with maximum of 1 missed cleavage was used as the cleavage enzyme.

2.5. Immunoassay of natural egg white using serum from allergic patients

Experimentation involving patients’ sera was conducted in compliance with the National Statement on Ethical Conduct in Human Research (2007) with approval from Deakin University Faculty of Science, Engineering and Built Environment Human Ethics Advisory Group (HEAG), with a project approval number of STEC-34-2013-DHANAPALA.

Egg white from a freshly laid egg was separated from the egg yolk and 1 volume of the egg white was diluted with 3 volumes of dH2O. 10 µg of the diluted egg white (per well) was run on gels and blotted onto nitrocellulose as previously discussed. The blots were then cut into individual lane strips, a total of 36 strips were obtained. Allergic patients’ sera were obtained from the Royal Children’s Hospital (Melbourne, Australia): 25 egg allergic and 5 other allergies. 100 µL of each serum sample was diluted with 900 µL of blocking buffer (Hammerstein casein solution in buffered saline-supplied in WesternBreeze® Chromogenic kit). The nitrocellulose strips with immobilised egg white proteins were then incubated with each of the diluted serum sample overnight at 4 °C (i.e. 1 strip per patient). Next, the strips were incubated with anti-human IgE (alkaline phosphatase conjugated) secondary antibody produced in mouse. The detection was carried out using the WesternBreeze® Chromogenic kit as previously mentioned. 3 nitrocellulose strips were incubated with only the secondary antibody as a negative control.

2.6. Mass spectrometry analysis of the natural proteins

The 4 bands that appeared to be the 4 allergens were incised and a mass spectrometry analysis was conducted to confirm the identity of the proteins, according to the method described in Section 2.4.

2.7. Immunoassay of recombinant proteins against allergic patients sera

The recombinant proteins were freshly expressed and 5 mL cultures were pelleted after 2 h post induction with IPTG. The cell pellets were lysed as previously discussed and the fraction that shows expression were run on SDS-PAGE and blotted onto a nitrocellulose membrane. For Gal d 1 and Gal d 2, the soluble fraction was used and for Gal d 3 and Gal d 4, the insoluble fraction was used. This was done in duplicate, one to be used with allergic sera and the other with non-allergic control sera. Another nitrocellulose blot was prepared by immobilising the soluble and insoluble fractions of E. coli extract (i.e. E. coli containing the vector without an insert were IPTG induced for 2 h and soluble and insoluble fractions were collected).

A pool of egg allergic patients’ sera was prepared by combining 50 µL of patients 4, 5, 6 & 7 with 1800 µL of blocking buffer. The 2 mL allergic patients’ serum pool was first pre-incubated with the nitrocellulose membrane containing the appropriate E. coli extract for 2 h at room temperature. Then one of the nitrocellulose membranes with the recombinant proteins was incubated with the same E. coli depleted serum pool overnight at 4 °C. The remaining recombinant protein membranes was incubated in a pool of non-allergic patients sera prepared by combining 50 µL of 10 non-allergic serum pool with 1500 µL blocking buffer. All the blots were then incubated with anti-human IgE (alkaline phosphatase conjugated) secondary antibody produced in goat (1:1000), and detected using the chromogenic substrate, as described above.

3. Results

3.1. PCR amplification of the egg white allergen cDNA

The mRNA isolated from chicken oviduct were used to PCR amplify egg white allergens Gal d 1, 2 and 4 using one-step PCR method. For Gal d 3, a long-range PCR, that utilises the traditional two-step method, was used. The PCR gel results showed a band for Gal d 1 at 800 bp (expected 630 bp), Gal d 2 between 800–2000 bp (expected 1158 bp), Gal d 3 at 2000 bp (expected 2115) and Gal d 4 at above 400 bp (expected 441) (Fig. 1). The long-range PCR conducted for Gal d 3 produced a brighter band when used without Q-solution (Fig. 1).

3.2. Cloning and expression of recombinant egg white allergens

The amplified PCR products were then digested by restriction enzymes and ligated into pTrcHisA vector that adds a 6xHis tag to the expressed proteins. The vector-insert constructs were transformed into Express P® chemically competent E. coli cells and expression induced with IPTG. Expression was conducted for 6 h and samples were collected every 1 h to determine the optimum expression time for each allergen. The collected samples were pelleted, lysed and the soluble and insoluble fractions were run on SDS gels for analysis. These gels were then western blotted, incubated with detection antibody and visualised with a chromogenic substrate. For Gal d 1 and 2, the western blot analysis showed that the proteins are found in both soluble and insoluble fractions with an optimum expression time of 2 h. Gal d 2 shows increase in expression every hour, at the same time showing an increase of breakdown of the protein. Gal d 3 and 4 showed expression only in the insoluble fraction, with an optimum expression time of 2 h. Gal d 3 showed breaking down of the protein throughout the expression period, while Gal d 4 showed a decrease in the amount of protein after 2 h and increasing again in 5–6 h (Fig. 2).
Fig. 1. PCR amplification of the egg white allergens. The allergens were isolated from mRNA using one-step (A) and long-range (B) PCR methods. For the long-range method, the PCR was conducted with and without the optimisation reagent Q-solution (this method was used to amplify Gal d 3). For all 4 allergens, bands appeared at approximately the expected size.

Fig. 2. Expression of recombinant allergens to determine the optimum time point of expression and the fraction where the expressed proteins are found. The transformed Express E. coli cells (for each allergen) were induced with IPTG and grown for 6 h. Samples were collected and pelleted every 1 hour, including before IPTG induction (0 hours). The pellets representing the time points 0, 2, 4, 5 and 6 were lysed and the soluble and insoluble fractions were run on SDS gels. The gels were then blotted onto nitrocellulose and incubated with anti-Xpress antibody for detection purposes. The blots were then detected using a chromogenic substrate. (A) Gal d 1, (B) Gal d 2, (C) Gal d 3, (D) Gal d 4.
3.3. Mass spectrometry analysis of recombinant egg white allergens

The mass spectrometry analysis of the recombinant protein bands (Fig. 3) shows that Gal d 2, 4 and 4 are positively identified (Table 1). The analysis showed no indication of presence of Gal d 1 in the protein bands 1–4, that were incised from the gel run with Gal d 1 expressed E. coli extract. Bands 5–8 incised from the Gal d 2 gel were identified as ovalbumin, 9–12 incised from Gal d 3 gel were identified as ovotransferrin, and bands 13–16 incised from Gal d 4 gel were identified as lysozyme.

3.4. Immunological analysis of natural and recombinant egg white allergens

Prior to testing of the recombinant proteins, natural egg white was tested against a population of egg allergic and other allergic sera to test for IgE reactivity (allergenicity). The western blots conducted shows that 10 patients were allergic to Gal d 1, 4 to Gal d 2, 14 to Gal d 3 and 18 to Gal d 4 (Fig. 4). A number of individuals showed reactivity to more than 1 allergen as depicted in Table 2. Patients who showed reactivity to all 4 allergens had higher egg specific serum IgE levels when compared to the others (Table 2). The mass spectrometry analysis conducted on the 4 bands, identified as the allergens, confirmed the identities, except for Gal d 1 (Table 3).

The recombinant proteins were then blotted onto nitrocellulose membranes and tested against anti-egg allergic sera and non-allergic sera. The allergic sera was first pre-incubated with E. coli extract, this blot did not appear to have any bands post incubation with secondary antibody and chromogen development. The blot incubated with allergic sera had multiple bands appearing for all allergens, except Gal d 4. The blot incubated with non-allergic sera did not have any bands.

4. Discussion

Egg white allergy is a widespread disorder mainly affecting children. The wide use of chicken eggs in various food and pharmaceutical products complicates avoidance of egg white, which is the only current management available for egg allergic patients. However, strict avoidance of egg allergens may be very difficult to achieve due to its extensive use in processed foods and pharmaceutical products. Avoidance of eggs may also have nutritional disadvantages to the individual (Mofidi, 2003, Caubet and Wang, 2011). Allergen specific-immunotherapy (SIT) is currently being pursued as a promising curative treatment for food allergy, including egg allergy. SIT involves the administration of allergen starting at very small amounts and increasing to a higher maintenance dosing, to the patients to promote tolerance by the immune system (Akdis and Akdis, 2011). Egg allergic patients react to each allergen at different intensities; therefore, it will be important to administer the allergens separately in SIT to better manage adverse reactions. However, isolating egg white allergens without contamination from the remaining allergens is extremely difficult, time consuming and expensive. Production of recombinant variants of allergens is an effective alternative to natural allergens, since the allergen is produced independently of the others. In this study, we aimed to produce recombinant egg white allergens and compared their IgE reactivity within a defined Australian patient group. The recombinant versions of Gal d 1, 2 and 3 that we produced showed IgE reactivity when tested against allergic patients’ sera.

When PCR amplifying cDNA sequences for cloning purposes, it is vital to use methods that generate minimal errors. Gal d 1, 2 and 4 were successfully amplified using the standard PCR methods, however for Gal d 3 a long-range PCR was used to amplify its longer length. The PCR products shown in Fig. 1 did not appear on the gel exactly at the right molecular weight. This can be attributed to the conditions of gel run including the percentage of agarose in the gel. The amount of DNA loaded onto the gel may also have affected the predicted size. The subsequent cloning in pTrcHisA vector and sequencing (results not shown) confirmed that the PCR amplification of cDNA was successful. Gal d 2 and Gal d 3 showed 99% similarity to the published sequences on IU.IS/NCBI, this may be attributed to PCR or sequencing error due to their larger number of nucleotides compared to Gal d 1 and Gal d 4 which showed 100% similarity to the published sequences. The Express™ E. coli strain was chosen for expression of the allergens because this BL21 derivative is tolerant to toxic proteins. Gal d 1 is a trypsin inhibitor and Gal d 4 is an antibacterial agent, therefore this strain of E. coli was ideal for protein expression.

Expression of all four proteins in insoluble fraction indicates formation of inclusion bodies when the proteins are expressed. This is common in E. coli since the introduced proteins are deemed foreign inside the host. However, Gal d 1 and 2 were also found in the soluble fraction, which explains that the functional properties of the two proteins may not have had a significant effect on the host cells. One may argue that over-expression of the proteins may have caused the formation of inclusion bodies, but the expression was carried out under the same conditions for all four proteins, therefore formation of inclusion bodies can likely be ascribed to the response of the host to the function of the proteins. E. coli is known to contain mechanisms of degrading abnormal proteins (Goldberg, 1972), which may explain why Gal d 1, 2 and 3 shows breaking

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down of the protein by showing multiple bands in Fig. 2. It is quite intriguing that Gal d 4 (lysozyme) is not affected by protein degradation machinery, possibly because the antibacterial properties of Gal d 4 counteracts the cell mechanism to degrade it, which is an important characteristic for an antibacterial agent. The localisation of the proteins in different fractions did not affect this study since we used the crude extracts for immunological analysis. The mass spectrometry analysis, although successfully identified Gal d 2, 3 and 4, failed to identify Gal d 1. Trypsin in-gel digestion was used for the digestion/preparation of the gel slices. The trypsin inhibitory activity of Gal d 1 may have affected the results indicating that the recombinant protein is partially or fully functional. An ideal suggestion to confirm the identity of recombinant Gal d 1 would be to either use Gal d 1 specific antibodies or N-terminal amino

Fig. 3. Recombinant egg white allergen gel slices for mass spectrometry. Four slices of gel were incised from the area of the gel each allergen is expressed. Each was numbered according to the figure and subjected to trypsin in-gel digestion and subsequent mass spectrometry analysis.

Fig. 4. Immunological analysis of chicken egg white against egg allergic patients' sera. (A) Western blots of natural egg white conducted against patient’s sera. Control strips were incubated with only the secondary antibody to study for non-specific binding of the secondary antibody. (B) Number of individuals allergic to each allergen as extracted from the Western blots.
acid sequencing. However, the appearance of the protein in Fig. 2 near the molecular weight of 30 kDa corresponds to the expected molecular weight of 28 kDa of Gal d 1.

Prior to testing the produced recombinant allergens against allergic patients’ sera for IgE reactivity, it was necessary to test the obtained patient sera against natural egg white to choose the most allergic serum samples. The Western blot shown in Fig. 4 indicates that the serum population we obtained did not react to all of the allergens at similar intensities, with some samples showing reactivity to only one allergen. This result suggests that egg allergy in Australian populations may be mainly due to allergenic reactivity of Gal d 3 and 4, although individuals allergic to Gal d 1 show higher reactivity. Patients that showed reactivity to all 4 allergens had higher egg specific serum IgE levels, suggesting a possible correlation between the high serum IgE levels and multi-reactivity to different allergens. However, this correlation cannot be confirmed without a larger number of samples to show statistical significance. It should be noted that some individuals with serum IgE levels of more than 0 IU/mL did not react to any of the allergens, which can be attributed to allergy to egg yolk in these patients, an aspect not addressed in this study. A considerable number of individuals in the egg allergy population we used were also allergic to peanut, and some were allergic to rye grass, however a correlation cannot be established due to the lack of literature reporting on any possible cross reactivity between egg allergens, peanut allergens and/or rye grass allergens. The blot also shows that Gal d 1 has appeared at a higher molecular weight when compared to the recombinant protein, possibly due to the heavy glycosylation of natural Gal d 1 in egg white (Mine and Zhang, 2002). Similarly to the recombinant protein, mass spectrometry analysis failed to identify Gal d 1 in natural egg white, which further supports our aforementioned suggestion that the recombinant Gal d 1 may be biologically functional.

The 4 serum samples that showed most IgE reactivity to natural egg white were then used to make a serum pool that showed reactivity to all 4 allergens, which was used to test the recombinant allergens against IgE reactivity. The results shown in Fig. 5 indicate that 3 of the 4 allergens were IgE reactive; with only Gal d 4 not showing reactivity. The blot shows that Gal d 1 and 3 had more reactivity when compared to Gal d 2, consistent with the result seen in Fig. 4 in which natural Gal d 1 and 3 were more allergenic to the 4 chosen serum samples. We pre-incubated the serum pool with E. coli extract to deplete the pool of any E. coli specific IgE, which could have given a false positive in the experimental blots. However, the results indicate that there were no E. coli specific IgE present in the serum pool. The successful use of crude extract shows that it is not vital to purify the natural egg proteins for preliminary IgE reactivity studies like the current study. In addition, the results also show that heating of the protein samples at 85 °C for 2 min before SDS-PAGE does not appear to affect the IgE reactivity of the proteins.

The production of IgE reactive recombinant counterparts of natural egg allergens opens doors for downstream applications,
including SIT. IgE reactive Gal d 1 has been previously produced by Rupa and Mine (Rupa and Mine, 2003), however the current study focuses on an Australian population, which has not been tested previously. Other research groups have expressed Gal d 2, 3 and 4 in various expression systems, however their IgE reactivity has not been tested simultaneously against egg allergic patients’ sera, or, poorly studied. The clinical significance of this study is the potential use of purified recombinant proteins for SIT and for diagnostic methods such as skin prick tests (SPT). Currently, SPT utilizes whole egg extracts or components isolated from natural eggs for diagnosis (Cortot et al., 2012, Schoos et al., 2014). The recombinant proteins we produced, due to the lack of contamination with other allergens, may be used in SPT to pinpoint which allergen a patient might be allergic to. Another important application of the produced recombinant proteins is oral immunotherapy (OIT), a type of SIT, in which heat denatured proteins can be administered orally to induce tolerance. Gal d 1 and Gal d 3 are known to contain conformational and linear IgE binding epitopes, making them IgE reactive even after heat denaturing, with Gal d 3 showing an increase in IgE reactivity after heat denaturing (Cooke and Sampson, 1997, Tong et al., 2012). These properties make these two proteins ideal candidates for OIT.

All of the proteins were simultaneously expressed with N-terminal 6xHexa-His tags, therefore Ni-NTA affinity purification followed by HPLC can be used to purify the proteins for downstream animal based studies or clinical testing. A circular dichroism (CD) spectroscopy analysis of the purified recombinant allergens can be used to obtain detailed structural information, especially for Gal d 1 which was not identified by mass spectrometry in this study. In addition, a purified version of the produced Gal d 4 can be used in a refolding experiment which may assist in retaining its IgE reactivity. The most important characteristic of the produced recombinant allergens is that they are not contaminated with other egg white proteins, which can potentially be used in SIT for patients allergic to a particular/specific egg white allergen. For patients allergic to more than one allergen, the recombinant proteins can be used in a step-wise manner in SIT to induce tolerance without causing an augmented immune response. In summary, this is the first study to simultaneously express all 4 egg white allergens in the same expression system in order to assess their relative IgE reactivity in a well-defined Australian patient group. The availability of purified preparations of such recombinant allergens promises to contribute significantly to egg white allergy diagnostics and therapeutics in the near future.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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References


