Cloning, Expression and Characterisation of Novel Chicken Egg Yolk Allergens

by

Sarukkalige Chamika De Silva
B. Biomed (Hons)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

December, 2016
I certify the following about the thesis entitled 'Cloning, Expression and Characterization of Novel Chicken Egg Yolk Allergens' submitted for the degree of Doctor of Philosophy

a. I am the creator of all or part of the whole work(s) (including content and layout) and that where reference is made to the work of others, due acknowledgment is given.

b. The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

c. That if the work(s) have been commissioned, sponsored or supported by any organisation, I have fulfilled all of the obligations required by such contract or agreement.

d. That any material in the thesis which has been accepted for a degree or diploma by any university or institution is identified in the text.

e. All research integrity requirements have been complied with.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: SARUKKALIGE CHAMIKA DE SILVA

Signed: Signature Redacted by Library

Date: 24th December 2016
I am the author of the thesis entitled ‘Cloning, Expression and Characterization of Novel Chicken Egg Yolk Allergens’ submitted for the degree of Doctor of Philosophy

This thesis may be made available for consultation, loan and limited copying in accordance with the Copyright Act 1968.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: SARUKKALIGE CHAMIKA DE SILVA

Signed: Signature Redacted by Library

Date: 24th December 2016
Acknowledgements

This project would not have been possible without the guidance and support of several individuals who in many ways provided their assistance in the preparation and completion of this study. First and foremost, I would like to thank my supervisor Associate Professor Cenk Suphioglu for giving me the opportunity to carry out this PhD project and providing valuable guidance throughout the research. Thank you very much Cenk for providing unreserved support all the time throughout these years which helped me complete this PhD project.

Thank you Professor Mimi Tang and Professor Tim Doran for providing samples that were necessary to carry out my PhD project. I want to thank Poultry CRC for their generosity in providing research funding to carry out the PhD. I specially want to thank Professor Mingan Choct, Liz Roan, Mick Warner and Professor Patrick Blackwell for providing support for my PhD.

Thanks to all the fellow students at NeuroAllergy Research Laboratory especially Pathum for providing me support whenever I needed. Thanks to my friends Pathum, Damitha, Ciaran and Colm for all the help and good times we had. Thank you to my girlfriend Dilusha, for all the love and support you gave during a significant stage of my PhD work. Finally, I would like to thank my family for all the support, encouragement and love you gave throughout my life. Thank you to my brother Piu for everything you have done for me. Thank you to my parents for all the sacrifices you have done for me. Thank you again to my family for providing me with love and support all the time.
# Table of Contents

**Acknowledgements** ........................................................................................................ i

**Publications** .................................................................................................................... vii

**List of Figures** ................................................................................................................ vili

**List of Tables** .................................................................................................................. xi

**Abbreviations** ................................................................................................................ xii

**Abstract** ........................................................................................................................... xvii

**Chapter 1: Literature review** ......................................................................................... 1

1.1 An insight into allergy: Classification and nomenclature............................................. 2

1.2 Molecular and cellular basis of allergy...................................................................... 4

1.2.1 The role of T cell subsets in allergy and health ..................................................... 4

1.2.2 Pathogenesis of IgE-mediated allergy................................................................... 9

1.2.2.1 Sensitisation phase ....................................................................................... 9

1.2.2.2 Activation phase ....................................................................................... 10

1.2.3 Pathogenesis of non-IgE mediated allergy........................................................ 12

1.3 Allergens....................................................................................................................... 13

1.4 Risk factors of allergic diseases................................................................................ 16

1.4.1 The genetic basis of allergic diseases ................................................................ 16

1.4.2 The environmental basis of allergic diseases.................................................... 18

1.4.2.1 Cellular and molecular basis of hygiene hypothesis ............................... 19

1.4.2.2 Population based studies on hygiene hypothesis ................................. 20

1.5 Egg allergy................................................................................................................... 21

1.5.1 Allergy to egg yolk ......................................................................................... 22

1.5.2 Allergens in the hen’s egg Yolk.......................................................................... 23

1.5.2.1 Chicken serum albumin (Gal d 5) ............................................................ 23

1.5.2.2 The bird-egg syndrome............................................................................ 24
Chapter 2: Immunological analysis of egg yolk proteins from hen, quail and emu and production of recombinant Gal d 6 in E. coli.... 35

2.1 Introduction........................................................................................................... 36

2.2 Materials & methods............................................................................................ 38

2.2.1 Immunological analysis of hen, quail and emu egg yolk extracts .......... 38

2.2.1.1 Human patients’ sera ............................................................................. 38

2.2.1.2 Preparation of crude egg yolk extracts (CEY) from hen, quail and emu eggs................................................................. 40

2.2.1.3 SDS-PAGE analysis of CEYs of hen, quail and emu eggs................. 40

2.2.1.4 Western immunoassay of CEYs........................................................... 41

2.2.1.4.1 Western immunoassay of hen’s CEY with individual patients’ sera ................................................................. 41

2.2.1.4.2 Western immunoassay of hen, quail and emu CEYs with pooled patients’ sera .......................................................... 42

2.2.1.5 Proteomic analysis of IgE reactive proteins from CEYs.................... 42

2.2.2 Cloning and expression of Gal d 6 in E. coli expression system .......... 43

2.2.2.1 Strains, vectors and growth conditions.............................................. 43

2.2.2.2 Extraction of Poly A+ mRNA from chicken liver .............................. 43

2.2.2.3 Reverse transcription polymerase chain reaction (RT-PCR) amplification of Gal d 6................................................................. 45

2.2.2.4 Analysis and gel purification of PCR amplified Gal d 6 gene .......... 46
Chapter 3: Immunological comparison of native and recombinant hen’s egg yolk allergen α-livetin (Gal d 5)
Chapter 4: Production of hypoallergenic variant of recombinant hen’s egg yolk allergen chicken serum albumin (Gal d 5) .......... 107

4.1 Introduction........................................................................................................... 108

4.2 Materials and methods.......................................................................................... 110

4.2.1 Site-directed mutagenesis of Gal d 5 ................................................................. 110

4.2.2 Transformation of XL10-Gold ultracompetent cells ........................................... 111

4.2.3 Identifying colonies harbouring pTrcHis A-Gal d 5196/205-388/397 construct .............. 112

4.2.4 Cloning and expression of rGal d 5196/205-388/397 in K. lactis.......................... 112

4.2.4.1 Cloning of Gal d 5196/205-388/397 in K. lactis................................................. 112

4.2.4.2 Detection of secreted rGal d 5196/205-388/397 by transformed K. lactis . 113
4.2.4.3 Cloning and expression of rGal d $5^{196/205-388/397}$ in *E. coli* .......... 114
4.2.5 Immunological analysis of rGal d 5 and mutant rGal d $5^{196/205-388/397}$ .... 114
4.3 Results............................................................................................................. 115
4.3.1 Recombinant production of Gal d $5^{196/205-388/397}$ in *K. lactis* ............ 115
4.3.2 Recombinant production of rGal d $5^{196/205-388/397}$ in *E. coli* ............... 124
4.3.3 Immunological analysis of non-mutant and mutant rGal d 5 ................. 125
4.4 Discussion....................................................................................................... 127

**Chapter 5: General discussion**................................................................. 132

**References**.................................................................................................. 142
Publications

**Peer-reviewed journals**

1. **De Silva C**, Dhanapala P, Doran T, Tang MLK & Suphioglu C (2016): Molecular and immunological analysis of hen’s egg yolk allergens with a focus on YGP42 (Gal d 6), Molecular Immunology: 71: 152-160.


**Conference papers**


**Publications under preparation**

1. **De Silva C**, Dhanapala P, Doran T, Tang MLK & Suphioglu C: Immunological analysis of natural and recombinant hen’s egg yolk allergen chicken serum albumin.
List of Figures

Figure 1.1: The steps involved in the IgE-mediated allergic reaction……………….11

Figure 2.1: SDS-PAGE analysis of CEYs under reducing and denaturing conditions……………………………………………………………………………53

Figure 2.2: Western immunoassay conducted using individual patients’ sera against hen’s CEY…………………………………………………………………………...55

Figure 2.3: Western immunoblots conducted using pooled patients’ sera against all CEYs…………………………………………………………………………...55

Figure 2.4: Percentage of patients with IgE reactivity to proteins (P1 to P7) of hen’s egg yolk, as analysed from the western immunoblot in Figure 2.2 and Table 2.2…57

Figure 2.5: RT-PCR of Gal d 6 gene………………………………………………..61

Figure 2.6: Restriction enzyme digest screening for E. coli colonies harbouring pTrcHis A-Gal d 6 construct………………………………………………………61

Figure 2.7: Sequence alignment comparison of DNA sequence derived for pTrcHis A-Gal d 6 construct using NCBI Nucleotide BLAST tool………………………….62

Figure 2.8: Time-course of expression of soluble and insoluble E. coli cell culture fractions harbouring rGal d 6………………………………………………………..64

Figure 2.9: Purification of 6×His tagged rGal d 6…………………………………..64

Figure 2.10: Inhibition ELISA experiments………………………………………….65

Figure 3.1: RT-PCR of Gal d 5 gene. The PCR product was electrophoresed on a 1% (W/V) agarose gel………………………………………………………………….90

Figure 3.2: Restriction enzyme digest screening for the colonies harbouring the pTrcHis A-Gal d 5 construct………………………………………………………….90

Figure 3.3: Sequence alignment comparison of DNA sequence derived for pTrcHis A-Gal d 5 construct using NCBI Nucleotide BLAST tool………………………….91

Figure 3.4: Time-course of expression of soluble and insoluble E. coli cell culture fractions harbouring rGal d 5………………………………………………………..92
Figure 3.5: PCR amplification of Gal d 5 gene. The PCR product was electrophoresed on E-Gel® CloneWell™ agarose gel with 0.8% SYBR Safe™ DNA Gel stain.................................................................93

Figure 3.6: Restriction enzyme digest screening for the colonies harbouring the pKLAC2-Gal d 5 construct.................................................................93

Figure 3.7: Sequence alignment comparison of DNA sequence derived for pKLAC2-Gal d 5 construct using NCBI Nucleotide BLAST tool.................................94

Figure 3.8: SacII digest of the pKLAC2-Gal d 5 construct. The digested plasmid was electrophoresed on a 1% (w/v) agarose gel.................................................95

Figure 3.9: Whole cell PCR analysis for screening of strains containing multi-copy integrants.................................................................................96

Figure 3.10: Time-course of expression of K. lactis culture supernatants containing secreted rGal d 5.................................................................97

Figure 3.11. Analysis of immunoprecipitation purified rGal d 5 (A) SDS-PAGE and (B) western immunoassay of purified rGal d 5 fractions...............................98

Figure 3.12. Testing of patients allergic to hen’s egg white described in Table 1 for IgE reactivity with dot-blotted nGal d 5.................................................................99

Figure 3.13. Testing of patients sensitised to nGal d 5 for IgE reactivity with dot-blotted rGal d 5.........................................................................................99

Figure 3.14. Comparison of the binding activity of human specific IgE against recombinant and natural Gal d 5.................................................................100

Figure 4.1: Nucleotide BLAST search result of the pTrcHis A-Gal d 5^{196/205-388/397} DNA sequence (plus strand) obtained from pTrcHis forward primer..............117

Figure 4.2: Nucleotide BLAST search result of the pTrcHis A-Gal d 5^{196/205-388/397} DNA sequence (minus strand) obtained from pTrcHis reverse primer............118

Figure 4.3: (A) PCR of Gal d 5^{196/205-388/397}. The PCR product was electrophoresed on E-Gel® CloneWell™ agarose gel with 0.8% SYBR Safe™ DNA Gel stain........119
Figure 4.4: Nucleotide BLAST search result of the pKLAC2- Gal d 5$^{196/205-388/397}$ DNA sequence (plus strand) obtained from pTrcHis forward primer…………………120

Figure 4.5: Nucleotide BLAST search result of the pKLAC2-Gal d 5$^{196/205-388/397}$ DNA sequence (minus strand) obtained from pTrcHis reverse primer……………121

Figure 4.6: SacII digest of the pKLAC2-Gal d 5$^{196/205-388/397}$ construct………………123

Figure 4.7: Whole cell PCR analysis for screening of K. lactis strains containing multi-copy integrants………………………………………………………………123

Figure 4.8: SDS-PAGE analysis of spent culture media from K. lactis strains harbouring rGal d 5$^{196/205-388/397}$ …………………………………………………124

Figure 4.9: Time-course of expression of soluble E. coli cell culture fractions harbouring rGal d 5$^{196/205-388/397}$ ………………………………………125

Figure 4.10: Western blot analysis of non-mutant rGal d 5 expressed by K. lactis and E. coli and mutant rGal d 5$^{196/205-388/397}$ produced by E. coli………………126

Figure 4.11: Western immunoblots conducted using nGal d 5 sensitised patients’ sera against non-mutant rGal d 5 expressed by K. lactis and E. coli and mutant rGal d 5$^{196/205-388/397}$ produced by E. coli……………………………………126
List of Tables

Table 1.1: Gell and Coomb’s classification of hypersensitivity disorders of the immune system

Table 1.2: The six main allergens from the hen’s egg white and the yolk

Table 2.1: The levels of allergen specific IgE antibodies against egg white determined by ImmunoCAP (Phadia)

Table 2.2: Qualitative IgE binding data from western immunoassay based on Figure 2.2

Table 2.3: Protein identities revealed from mass spectrometry analysis, their approximate molecular weights on SDS-PAGE, score and percentage sequence coverage for each protein

Table 3.1: The levels of allergen specific IgE antibodies against egg white determined by ImmunoCAP (Phadia)

Table 4.1: Mutagenic primers used for disruption of disulfide bridges in Gal d 5 (CSA). Substitutions of Cys by Ala residues are indicated by the codon GCC bolded and underlined

Table 4.2: Thermal cycling conditions for mutant strand synthesis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAHL</td>
<td>Australian Animal Health Laboratory</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>amds</td>
<td>Acetamidase gene</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Apo B</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>BBC</td>
<td>Boston Birth Cohort</td>
</tr>
<tr>
<td>BCIP/NBT</td>
<td>5-bromo-4-chloro-3’indolyphosphate p toluidine/nitro-blue tetrazolium chloride</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster differentiation 4</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster differentiation 8</td>
</tr>
<tr>
<td>CD25</td>
<td>Cluster differentiation 25</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CEY</td>
<td>Crude egg yolk extract</td>
</tr>
<tr>
<td>CMP</td>
<td>Cow’s milk protein</td>
</tr>
<tr>
<td>CSA</td>
<td>Chicken serum albumin</td>
</tr>
<tr>
<td>CSIRO</td>
<td>Commonwealth Scientific and Industrial Research Organisation</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxyl terminal</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DB</td>
<td>Disulfide bond</td>
</tr>
<tr>
<td>DBPCFC</td>
<td>Double blind placebo controlled food challenge</td>
</tr>
<tr>
<td>dH2O</td>
<td>Deionized water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>ds-DNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EAACI</td>
<td>European Academy of Allergy and Clinical Immunology</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked-immunosorbant assay</td>
</tr>
<tr>
<td>FcεRI</td>
<td>High affinity IgE receptor</td>
</tr>
<tr>
<td>FPE</td>
<td>Food protein induced enteropathy</td>
</tr>
<tr>
<td>FPIAP</td>
<td>Food protein induced proctocolitis</td>
</tr>
<tr>
<td>FPIES</td>
<td>Food protein induced enterocolitis syndrome</td>
</tr>
<tr>
<td>GOS</td>
<td>Galacto-oligosacharide</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HEAG</td>
<td>Human Ethics Advisory Group</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human Leukocyte Antigen-antigen D Related</td>
</tr>
<tr>
<td>IB</td>
<td>Inclusion body</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgG1</td>
<td>immunoglobulin G-1</td>
</tr>
<tr>
<td>IgG3</td>
<td>immunoglobulin G-3</td>
</tr>
<tr>
<td>IgG4</td>
<td>immunoglobulin G-4</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IL-5</td>
<td>Interleukin-5</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-9</td>
<td>Interleukin-9</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IL-13</td>
<td>Interleukin-13</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>JAK1</td>
<td>Janus kinases 1</td>
</tr>
<tr>
<td>JAK3</td>
<td>Janus kinases 3</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMR</td>
<td>Measles, mumps and rubella</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>NFA</td>
<td>Non-IgE mediated food allergies</td>
</tr>
<tr>
<td>nGal d 5</td>
<td>Natural Gal d 5</td>
</tr>
<tr>
<td>nGal d 6</td>
<td>Natural Gal d 6</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Ni²⁺-Nitrilotriacetic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OFC</td>
<td>Oral food challenges</td>
</tr>
<tr>
<td>OTI</td>
<td>Oral tolerance induction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pNPP</td>
<td>Alkaline phosphatase yellow</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PPV</td>
<td>Poor positive predictive value</td>
</tr>
<tr>
<td>RAST</td>
<td>Radioallergosorbent test</td>
</tr>
<tr>
<td>rGal d 5</td>
<td>Recombinant Gal d 5</td>
</tr>
<tr>
<td>rGal d 6</td>
<td>Recombinant Gal d 6</td>
</tr>
<tr>
<td>RORγt</td>
<td>RAR-related orphan receptor gamma</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDM</td>
<td>Site directed mutagenesis</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIT</td>
<td>Specific immunotherapy</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
</tr>
<tr>
<td>SOC</td>
<td>Super optimal broth with catabolite repression</td>
</tr>
<tr>
<td>SPT</td>
<td>Skin prick test</td>
</tr>
<tr>
<td>ss-DNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription 3</td>
</tr>
<tr>
<td>STAT4</td>
<td>Signal Transducer and Activator of Transcription 4</td>
</tr>
<tr>
<td>STAT5</td>
<td>Signal Transducer and Activator of Transcription 5</td>
</tr>
<tr>
<td>STAT6</td>
<td>Signal Transducer and Activator of Transcription 6</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Th0</td>
<td>Naive T helper cell</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1 cell</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2 cell</td>
</tr>
<tr>
<td>Th9</td>
<td>T helper 22 cell</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17 cell</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>TNF-β</td>
<td>Tumour necrosis factor-β</td>
</tr>
<tr>
<td>Tr</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>Tr1</td>
<td>T regulatory 1 cell</td>
</tr>
<tr>
<td>VTG-1</td>
<td>Vitellogenin-1</td>
</tr>
<tr>
<td>VTG-2</td>
<td>Vitellogenin-2</td>
</tr>
<tr>
<td>YCB</td>
<td>Yeast carbon base</td>
</tr>
<tr>
<td>YGP42</td>
<td>Yolk glycoprotein 42</td>
</tr>
<tr>
<td>α-MF</td>
<td>α-mating factor</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
</tbody>
</table>
Abstract

Allergy to hen’s egg white is one of the most common forms of food allergies and, as a result, has been well investigated. However, allergy to hen’s egg yolk has been overlooked by many studies, despite evidence of causing important immunological hypersensitivity in humans. Furthermore, the use of recombinant allergens in diagnosis and treatment of allergy has been very popular due to their excellent pharmaceutical qualities and standardised content. Therefore, immunological studies of hen’s egg yolk and production of recombinant versions of egg yolk allergens is of great interest. My work involves investigating hen’s egg yolk proteome in relation to food allergy using molecular, proteomic and immunological methods. The major objectives of this study were three fold. First objective was to investigate sensitisation to egg yolk proteins in patients diagnosed with allergy to egg white. The second objective involved producing immunologically active recombinant versions of hen’s egg yolk allergens; chicken serum albumin and yolk glycoprotein 42 (YGP42). The final objective was to develop a recombinant based mutant variant of chicken serum albumin with reduced immunoglobulin E (IgE) antibody reactivity. In order to investigate sensitisation to egg yolk proteins, I conducted Western immunoassays using sera from 25 patients with allergy to hen’s egg white (Chapter 2). The results showed that 36% of the patients tested had concomitant sensitisation to egg yolk proteins indicating a possible link between allergy to egg white and allergy to egg yolk. The proteomic analysis identified vitellogenin-1, vitellogenin-2 and apolipoprotein B as major IgE-reactive proteins. Then, the production of IgE-reactive recombinant YGP42 and chicken serum albumin in Escherichia coli and Kluyveromyces lactis, respectively, are presented in Chapters 2 and 3. We were able to confirm that recombinant chicken serum albumin to have similar IgE-reactivity to
its natural counterpart. Finally, this thesis presents the successful production of a mutant variant of recombinant chicken serum albumin using site directed mutagenesis and showed that it completely lacks IgE-reactivity (Chapter 4). The work presented in this study confirms hen’s egg yolk as an important source of food allergens. The recombinant allergens produced in this study may be useful in the preparation of standardised reagents for the diagnosis and immunotherapy of hen’s egg yolk allergy in the future.
Chapter 1: Literature review

Publication:
1.1 An insight into allergy: Classification and nomenclature

Allergy is a term used to describe a collection of diseases manifesting in many different ways such as, food allergy (gastroenteritis), asthma, urticaria, anaphylaxis and rhinitis [1, 2]. Allergies mainly occur in young children. However, they can affect individuals from any age group, from infancy to adulthood to elderly [1]. In technical terms, allergy is a hypersensitivity disorder of the immune system caused by non-pathogenic environmental substances resulting in self-inflammatory damage. Antigens that trigger allergic reactions are termed as allergens. Hypersensitivity reactions of non-immunological nature are not categorized as allergies. For example, food intolerance due to enzymatic defects or pharmacological activity and side effects to drugs belong to non-allergic hypersensitivity disorders [2-4].

Traditionally, allergic diseases were classified under Type I hypersensitivity according to Gell and Coomb’s classification of hyper-immune diseases (Table 1.1) [5]. Mutually exclusive and distinct pathophysiological mechanisms responsible for disease progression and symptoms were used as the basis for this classification [4-6]. Since the conception of Gell and Coomb’s classification about 40 years ago, numerous patho-physiologies and patho-mechanisms underlying allergic diseases have been discovered. Although, the framework provided by Gell and Coomb’s classification is not inaccurate, it is now considered outdated [3, 4, 6, 7]. Therefore, Gell and Coomb’s classification of hypersensitive diseases is no longer used in relation to allergic diseases. Nevertheless, it is still used by some authors occasionally when describing hypersensitivity diseases including allergies [4, 6].
Table 1.1: Gell and Coomb’s classification of hypersensitivity disorders of the immune system [5, 8].

<table>
<thead>
<tr>
<th>Category</th>
<th>Disease name</th>
<th>Mediator</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Allergy</td>
<td>IgE</td>
<td>Allergens</td>
</tr>
<tr>
<td>Type II</td>
<td>Cytotoxic hypersensitivity</td>
<td>IgG, IgM, complement</td>
<td>Individual’s own cells, drugs, haptnes</td>
</tr>
<tr>
<td>Type III</td>
<td>Immune complex hypersensitivity</td>
<td>IgG, IgM</td>
<td>Foreign sera, therapeutic antibodies</td>
</tr>
<tr>
<td>Type IV</td>
<td>Delayed-type (cellular) hypersensitivity</td>
<td>T lymphocytes, macrophages</td>
<td><em>Mycobacterium tuberculosis</em>, heavy metals (Ni)</td>
</tr>
</tbody>
</table>

A revised classification for allergic diseases is formulated by the European Academy of Allergy and Clinical Immunology (EAACI) nomenclature task force. According to EAACI position paper, allergic diseases are separated into two categories, based on its immuno-pathological mechanisms known as antibody-mediated and non-antibody mediated [3]. Majority of patients with allergies exhibit antibody-mediated allergic diseases. In antibody-mediated allergic diseases, the immune system produces antibodies of immunoglobulin E (IgE) isotype against allergens. Consequently, these patients are said to be suffering from IgE-mediated allergy. By contrast, patients suffering from non-antibody mediated allergy, do not exhibit raised levels of allergen specific IgE. Nevertheless, those patients also exhibit and suffer from typical allergic symptoms, as in IgE-mediated allergy. Little is known about the underlying mechanism responsible for non-IgE mediated allergies. However, it is believed that a cellular mechanism, independent from IgE antibodies, is responsible for non-antibody mediated allergic diseases [3, 7].

The term atopy was introduced by Coca & Cooke in 1926 to describe the inherited hypersensitivity towards allergens in man [9]. Over the years, many physicians and researchers used the term atopy, simply to describe IgE-mediated allergic diseases.
However, at the same time, paediatricians used the term atopy to describe the genetic predisposition to develop IgE-mediated allergies due to the strong familial basis [4]. Because of this inconsistent use of the terminology, much confusion was created among readers. Therefore, EAACI position paper re-defined atopy as the genetic predisposition to produce IgE antibodies against allergens and develop typical symptoms of allergies. Therefore, the term atopy is no longer used to describe an IgE-mediated hypersensitivity [3, 7].

1.2 Molecular and cellular basis of allergy

1.2.1 The role of T cell subsets in allergy and health

Cluster differentiation 4 (CD4+) T helper (Th) lymphocytes are the main effector cells involved in pathogenesis of allergic diseases [10, 11]. Functional Th cell subsets include Th1, Th2, Th9, Th17, Th22, regulatory T (Tr) and follicular helper cells based on their overall cytokine secretion profile. It has been shown that all T helper cell subsets are derived from the same precursor Th cell known as undifferentiated or naive T helper (Th0) cell. Polarization into a committed Th cell subset from Th0 cells is driven under the influence of a combination of strong micro-environmental signals and genetic factors acting at the level of antigen presentation. Initiation of a particular Th cell subset is directed through factors such as specific cytokines, cytokine receptors, and transcription factors at the time Th0 cells engage with the antigens [12]. Dose of antigen, route of antigen exposure, physical form of immunogen and the type of adjuvant are some of the environmental determinants proposed. However, the genetic basis of Th cell polarization still remains poorly understood [13-15]. In the context of allergies Th cell subsets, Th1, Th2, Tr cell and recently identified Th17 subsets are of particular important in understanding the underlying mechanism governing pathogenesis of allergies [13-15].
Th1 lymphocytes produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β) and other cytokines responsible for macrophage activation and cell mediated immunity against intracellular pathogens such as bacteria and viruses. Th1 cell differentiation is stimulated by IL-12 and IFN-γ, and mediated by Signal Transducer and Activator of Transcription 4 (STAT4) and T-bet [12]. The signature Th1 cytokine, IFN-γ is known to activate macrophages and stimulate B lymphocytes to produce several subclasses of immunoglobulin G (IgG) antibodies, such as IgG1 and IgG3. IgG antibodies are capable of binding to high-affinity Fcγ receptors and complement proteins resulting in opsonisation and phagocytosis of particulate microbes [13]. Furthermore, Th1 cells are known to play a down-regulatory function on Th2 activity by inhibiting IgE production by B cells [16].

On the other hand, Th2 cells produce cytokines such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 that promote antibody mediated defence against multicellular parasites such as gastro intestinal nematodes. Th2 cell differentiation is stimulated by IL-4 mediated activation of Janus kinases 1 (JAK1) and JAK3 along with recruitment of transcription factors STAT3, STAT5, STAT6 and GATA3 [12]. IL-4 and IL-13 are known to stimulate IgE antibody production from B cells while IL-5 recruits and differentiates eosinophils. The main effector function of Th2 type cytokines are production of antigen specific IgE antibodies leading to mast cell and eosinophil mediated inflammation. Furthermore, an immunosuppressive role for Th2 lymphocytes have been described since IL-4, IL-10 and IL-13 together are known to inhibit several macrophage functions. [16-19]. In the event of prolonged Th1 response due to sustained intracellular microbial infection a switch to Th2 response may occur. This is considered as an important protective mechanism of Th2 immune response which results in downgrading the effects of prolonged cell mediated immunity of Th1 response which could damage the host [16, 20].
The IgE-mediated allergic hypersensitivity is considered to arise as a result of strong Th2 immune response against common environmental allergens in predisposed individuals. Cytokine profile of T cell clones derived from majority of allergic patients exhibit a Th2 phenotype with high production of IL-4, IL-5 and little or no production of IFN-γ [16, 20]. In healthy subjects, the concentration of serum IgE is the lowest compared to other 5 types of antibodies. The production of IgE is very low at birth and gradually increases until the age of 10 to 15 years. However, patients with allergies exhibit earlier and sharper IgE production indicating a predominant Th2 immune response [21].

Furthermore, Th2 lymphocytes are known to preferentially induce production of IgG4 antibodies through the help of IL-10. The normal immune response of IgG4 is mainly limited to non-microbial antigens. The accompanying involvement of IgG4 in allergy has a protective role. In IgE-mediated allergies, increased production of IgG4 antibodies is associated with a decrease in symptoms. In healthy individuals, the immune system reacts to allergens with increased allergen specific IgG4 antibodies without detectable IgE antibodies. This immune response is termed as the modified Th2 response, which represents the typical healthy immune response to allergens. However, it is quite common to find both allergen specific IgG4 and IgE antibodies together in affected individuals. Well known examples of this phenotype can be seen in allergy to bee venom, rodent proteins and mammalian serum albumins. Furthermore, IgG isotype (IgG1 and/or IgG4) is more common in subjects with IgE-mediated allergy, than in IgE-negative subjects as in allergy to pollen and mites. Some allergens elicit the classical IgE-mediated immune response without any IgG antibody productions, whereas other allergens produce an IgG (IgG1 and/or IgG4) response without any detectable IgE. This difference in the type of immune response suggests that not all allergens are equal [22].
Two mechanisms have been suggested to explain the protective role of IgG4 antibodies in relation to allergy. Firstly, it has been proposed that IgG4 antibodies act as blocking antibodies by preventing the interaction between IgE and allergens. On the other hand, IgG4 is considered as a marker for tolerance induction. A positive correlation has been observed between increments in IgG4 concentration, and reduction in allergic symptoms in follow up studies. However, the exact mechanism of IgG4 dependent tolerance induction is not clear. IgG4 is generally regarded as a non-pathogenic antibody. However, IgG4 has been implicated in both allergic and non-allergic diseases as well. For example BanLec1, a lecithin from banana is a known potent allergen that induces IgG4 resulting in allergies. However, the involvement of IgG4 antibodies as the sole food allergy mediator is not fully resolved. According to EAAIC taskforce report food specific IgG4 does not indicates food allergy. Furthermore, IgG4 antibodies are also involved in non-allergic diseases such as pemphigus and sclerosing autoimmune pancreatitis [22].

In allergic individuals the underlying mechanism governing the preferential activation of allergen specific Th2 cells is not fully elucidated. The presence of IL-4 in the immunological milieu of the patient during the initial allergen exposure is identified as a crucial factor driving the polarization of Th2 response. In vitro studies conducted using both human and murine T cells clones have demonstrated IL-4 as an important requirement for the development of Th2 immune response [23, 24]. Furthermore, T cells from IL-4 deficient mouse models failed to stimulate a Th2 response following in vitro stimulation [25]. However, despite these results it is still not clear what is the source of initial IL-4 production which is required for polarization of Th0 into active Th2 lymphocytes [16].
Tr cells are another subset of T lymphocytes described in humans responsible for suppression and regulation of overall immune responses against antigens. Majority of Tr cells exists as CD4+CD25+ T regulatory 1 (Tr1) type, secreting cytokines IL-10 and transforming growth factor-β (TGF-β) (Figure 1.1) [26-28]. Tr1 cells play an important role in maintaining immunological tolerance to self and innocuous environmental substances by suppressing Th2 cells. Tr1 secreted IL-10 and TGF-β has shown to suppress IgE production and favour production of non-inflammatory antibodies such as IgG4 and immunoglobulin A (IgA). Therefore, a weak Tr1 immune function would lead to a stronger Th2 polarized immune response leading to allergy [26, 27]. Healthy and allergic individuals both represent Tr1, Th1 and Th2 cells in different proportions. It has been proposed that the ratio all three T cell subsets to be a crucial factor determining health or allergy where patients suffering from allergy represent a dominant Th2 response against allergens while healthy individuals exhibit a dominant Tr1 response against allergens [27].

The newly discovered Th cell subset, Th17 has been reported to implicate in development of allergic asthma and atopic dermatitis. Development of Th17 cells is stimulated by TGF-β and IL-6 or IL-21, and is mediated by STAT3 and RAR-related orphan receptor gamma (RORγt). Th17 cells are characterised by the production of IL-17, IL-17F, IL-22 and other cytokines responsible for inducing autoimmunity. Studies conducted on mice have shown that IL-17 and IL-22 are capable of triggering inflammation. It has been shown that elevated levels of circulating Th17 cells, plasma IL-17 and IL-22 are important characteristics of allergic asthma. However, the role of Th17 cells in development of allergic asthma hasn’t been completely elucidated. Further investigations at population level focusing on different classifications of asthma, and experiments on the cellular basis are required [12, 29].
1.2.2 Pathogenesis of IgE-mediated allergy

The IgE-mediated immune response occurs in a cascade fashion from initial allergen exposure to the ultimate tissue inflammation. For simplification, the IgE-mediated allergic reaction is described in two phases known as sensitisation phase and activation phase (Figure 1.1) [2, 10, 15, 30].

1.2.2.1 Sensitisation phase

Immunological sensitisation starts during the initial encounter between an allergen and the immune system. Infiltrated allergens are captured by antigen presenting cells (APC) such as dendritic cells, macrophages and granulocytes, most likely from the site of entry. The epitopes of internalized allergens are then processed and presented to Th0 cells through class II major histocompatibility complex (MHC) molecules of APCs. Following engagement with the presented allergens, Th0 cells differentiate into an active cytokine secreting Th2 state. Polarization into Th2 lymphocytes is further aided by the presence of IL-4 in the patient’s immunological milieu. Th2 cells produce cytokines such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 that provide optimum help for IgE production, and activation of cellular components of the allergic cascade [2, 4, 13, 15, 31].

Cytokines IL-4, IL-5 and IL-13 are recognized as the signature cytokines of Th2 lymphocyte profile. IL-4 is the main cytokine responsible for inducing B cells to produce allergen specific IgE antibodies and further enhances the development of additional Th2 cells. Newly synthesized IgE antibodies then bind with high affinity IgE receptors (FceRI) expressed on the surfaces of mast cells and basophils. IgE bound mast cells and basophils are now said to be sensitised, since they can be activated by subsequent allergen exposures. IL-5 has a role in development and
activation of eosinophils, which produce high levels of inflammatory mediators. IL-13 further enhances IgE production and stimulates mucus secretion by epithelial cells [2, 4, 10, 13, 15, 31-33].

1.2.2.2 Activation phase

When a sensitised immune system is exposed to the same allergen, or a different cross-reactive allergen with the same IgE-binding epitopes, they bind and cross-link IgE immobilised on mast cells and basophils. Cross-linking cell bound IgE results in explosive degranulation of mast cells and basophils, releasing chemical mediators such as histamines, prostaglandins, cytokines and leukotrienes, which are responsible for the typical symptoms of allergy [10, 32].
Figure 1.1: The steps involved in the IgE-mediated allergic reaction. At the initial allergen exposure, APCs capture and present the allergens to Th0 cells. With the aid of IL-4 in the patient’s immunological milieu Th0 cells differentiate into an active Th2 type, secreting cytokines such as IL-4, IL-5 and IL-13. IL-4 produced by Th2 cells then activates B cell to produce allergen specific IgE antibodies and further enhances the differentiation of Th0 into Th2. At the end of the sensitisation phase IgE antibodies bind to FcεRI on mast cells and basophils. During the secondary and subsequent exposures to the same offending allergen they bind and cross-link IgE immobilised on mast cells and basophils. Cross-linking IgE antibodies result in explosive degranulation releasing variety of chemical mediators causing inflammation.
1.2.3 Pathogenesis of non-IgE mediated allergy

Non-IgE mediated allergies are less commonly encountered than IgE-mediated allergic diseases. The underlying mechanism responsible for pathogenesis of non-IgE mediated allergies is believed to be cause by mechanisms independent from IgE antibodies. However, the pathophysiological mechanism responsible for non-IgE mediated allergic reaction is poorly understood. Non-IgE mediated allergies are mainly caused by food proteins, especially cow’s milk proteins (CMP) and soy proteins. Therefore, non-IgE mediated allergies are often referred to as non-IgE mediated food allergies (NFA). Some common manifestations of NFAs include food protein-induced enterocolitis syndrome (FPIES), food protein-induced allergic proctocolitis (FPIAP), and food protein-induced enteropathy (FPE) [34-36].

Typically, patients suffering from NFAs do not exhibit allergen specific IgE antibodies in serology based diagnostic tests. However, some patients with NFAs were found to have low levels of IgE against offending food allergens. For example, some patients suffering from FPIES are reported to exhibit detectable levels of allergen specific IgE antibodies. Nevertheless, the clinical relevance between allergen specific IgE antibodies and FPIES is still not clear [37, 38].

Only a limited number of studies have addressed the underlying immunological mechanism of NFAs. TNF-α is known to play an important role in pathogenesis of NFA. It has been shown that in patients with cow’s milk allergy, TNF-α production by mononuclear cells increases following CMP challenge leading to increased intestinal permeability [39]. A central role for TNF-α in pathogenesis of NFAs is further supported by studies presenting evidence of increased amounts of faecal TNF-α and the presence of TNF-α in the duodenal biopsy specimens in patients with FPIES. Furthermore, it has been shown that intact CMP holds a superior capability to
stimulate PBMCs from patients with FPIES to produce TNF-α than processed CMP. The authors also reported a substantial drop in TNF-α production following avoidance of CMP in FPIES patients [40-42]. The Th lymphocyte response in NFAs is also predominantly directed towards Th2 type as in IgE-mediated allergies however, without detectable allergen specific IgE antibodies. Morita et al., demonstrated that Th2 cytokines, namely IL-3, IL-5 and IL-13, were significantly produced by PBMCs from patients with NFAs when stimulated with CMP [43]. The increased production of TNF-α and the polarization of Th0 cells into Th2 state can be considered as important steps in pathogenesis of NFAs [38, 43-45].

1.3 Allergens

Antigens that stimulate IgE-mediated or non-IgE mediated allergic hypersensitivity disorders are defined as allergens [3]. Existing studies on molecular, biological and immunological basis of allergens are primarily investigated in the context of IgE-mediated allergy. In this thesis, the role of allergens will be discussed in relation to IgE-mediated hypersensitivity only. Allergens are derived from numerous sources such as foods, animal dander, chemical additives, drugs, fungi, insect stings and pollen [19].

Most of the well-known allergens are glycoproteins with molecular masses ranging from 5 kDa to 70 kDa. The antigenic determinants of those allergenic glycoproteins are commonly found on their protein components [46]. Traditionally, it was thought that carbohydrates are not capable of serving as epitopes resulting in the formation of specific IgE antibodies. However, this assumption has been proven false by studies presenting evidence on carbohydrates containing allergenic epitopes. For example, allergy to cetuximab, (chimeric mouse-human IgG1 monoclonal antibody against epidermal growth factor receptor) arises as a result of producing specific IgE
antibodies against oligosaccharide galactose-α-1,3-galactose on the Fab portion of
the heavy chain [47, 48]. Furthermore, pure carbohydrates are also reported to cause
IgE-mediated hypersensitivity as in allergy to galacto-oligosacharides (GOS) [48].

Significant proportion of studies has been dedicated to investigate the role of food
allergens in causing IgE-mediated hypersensitivity. Most common IgE-mediated
food allergies in children are caused by peanuts, milk and hen’s egg proteins.
Majority of the food allergies seen in adults are attributed to allergens derived from
peanuts, tree nuts, fish and shellfish. All of these food sources contain thousands of
proteins and yet only a handful of them cause allergy [49].

Availability of high amounts of allergenic proteins in the food source, resistance to
processing, digestion and existence of multiple linear IgE binding epitopes are some
characteristics of food allergens [46, 49]. The presence of proteins in high quantities,
as a requirement to act as an allergen, is demonstrated by the increased prevalence of
codfish allergy among Norwegians. The increased consumption of codfish coincides
with the high frequency of codfish allergy in Norway. Consequently, allergy to fish
is not prominent in Texas where beef consumption is high [46]. A single allergen is
considered to carry at least two IgE-binding epitopes in order to cross-link mast cell
bound IgE. Two types of IgE-binding epitopes are described, known as linear and
conformational. Linear or sequential epitopes only require the primary amino acid
sequence of the protein for IgE binding. On the other hand, conformational epitopes
require secondary or tertiary structures in addition to the primary amino acid
sequences for IgE binding. Conformational epitopes are mainly seen in aeroallergens
where allergens are presented to the immune system in their native form. On the
other hand, linear epitopes are of particular importance to food allergens. Food
allergens are presented to the immune system after partial digestion or denaturation
in the gastrointestinal tract, which reveal their linear epitopes. Study by Vila et al.,
showed that patients with milk allergy have higher specific IgE antibodies to linear
epitopes than to native α and β casein proteins [50].

Resistance to digestion and denaturation is considered a distinctive character of food
allergens. The ability of food allergens to withstand digestion in the gastrointestinal
tract increases the probability of encountering the immune system in their intact
form. Numerous studies have reported that food allergens hold a superior capability
to resist in vitro pepsin digestion compared to non-food allergens and non-allergenic
proteins. However, these in vitro digestion models do not fully represent the human
digestion. Therefore, further studies are required to evaluate the relationship between
allergenicity and resistance to gastric acid digestion [46, 49].

Three-dimensional structures of some allergens have been studied using X-ray
diffraction and nuclear magnetic resonance (NMR) in order to find a unifying link
among allergens. Birch pollen allergens Bet v 1 and Bet v 2 and timothy grass pollen
allergen Phl p 2 are some of the allergens subjected to structural studies. These
studies found that the shape and the dimensions of the allergens to be a common
trait. The average shortest and longest dimensions of the allergens were 3.4 nm and
4.4 nm, respectively, giving them a spherical shape. However, structures of many
more allergens need to be analysed to obtain a better understanding of the molecular
basis of allergenicity [46].

Obviously, not all proteins with above-mentioned characteristics are allergens.
Therefore, it is still not clear why some proteins act as allergens while others do not.
Development of an allergic disease is multifactorial. An allergen must be presented
to a genetically or environmentally predisposed individual in order to induce an
allergic reaction [19]. In order to find the answer to the question “What makes a
protein an allergen?”, genetic and environmental factors must be taken into account as well, along with their biochemistry.

1.4 Risk factors of allergic diseases

1.4.1 The genetic basis of allergic diseases

Pathogenesis of allergic diseases is considered to have a strong hereditary factor. The risk of developing allergic diseases is higher in individuals with a family history. For example, a positive family history is found to be a strong risk factor for both asthma and rhinitis [51, 52]. An epidemiological study from Sweden showed that the risk of developing asthma and rhinitis increases up to four-fold and six-fold, respectively, in individuals with a family history [53]. Another population-based study from Germany investigated the risk of developing asthma and rhinitis in children with affected parents. This study estimated that if one parent had asthma, the odds ratio of a child inheriting asthma to be 2.6. The odds ratio reported for rhinitis was 3.6. However, the same study reported that 22% of children without a positive family history to be suffering from allergic diseases as well, indicating the influence of environmental factors [54].

Although, there is compelling evidence of familial basis of allergic diseases, identification of culprit genes had been problematic. The presences of multiple markers, vaguely described phenotypes and possible involvement of multiple genes have all contributed to difficulties in conducting genetic studies. Multiple chromosome regions are implicated with numerous phenotypes of allergic diseases, indicating the genetic heterogeneity of allergies. Candidate gene approach and positional cloning are some of the techniques used to identify genes related to allergic diseases. Candidate gene approach is used to identify disease genes through polymorphisms in known genes. On the other hand, positional cloning involves
linking suspected chromosomal regions with a disease through genetic markers [55, 56].

Several loci have been linked with IgE-mediated allergy by candidate gene approach. IgE-mediated allergy to short ragweed pollen allergen Ra5 has been reported to be involved with an allele of the HLA-DR (Human Leukocyte Antigen-antigen D Related) locus [57]. Furthermore, polymorphisms of the β-chain of the FceRI receptor and IL-4 family of cytokine genes on chromosome 5 have been linked with IgE-mediated allergy [58, 59]. However, candidate gene studies had yielded some conflicting reports as well. For example, some alleles found to be linked with asthma were independent of raised IgE levels and other markers of IgE-mediated allergy [60].

Positional cloning studies have linked several chromosomes and chromosomal regions with allergic phenotypes, in particular with asthma and rhinitis. Diverse populations have been used for positional cloning studies. However, majority of the studies have included predominantly Caucasian populations. Some recently reported allergy related genes are ADAM33 on chromosome 20p, SPRINK5 on 5q31-35, IRAKM on 12q, DPP10 on 2q14-32, GPRA on 7p, HLA-G on 6p21 and PHF11 on 13q14 [61-67]. A common finding in many studies was the existence of multiple susceptible loci in many linkage regions of chromosomes. Positions 31 to 35 on the long arm of chromosome 5 have shown to harbour multiple genes related to pathological mechanisms of allergy. Genes regulating Th1/Th2 polarization and IgE production (i.e. IL-4, IL-13, TIM1 and TIM3), innate immunity (i.e. CD14) and genes improving T cell adhesion (i.e. CYFIP2) are some examples for susceptible genes on chromosome 5 with links to allergy [56, 59].

Study conducted by Tripathi et al., using Boston Birth Cohort (BBC) investigated the link between allergic sensitisation to common environmental allergens, and
cockroach allergens with single-nucleotide polymorphisms (SNP) in known candidate genes in the pathogenesis of allergic diseases. It was shown that SNPs near or in JAK2, CNOT6, MAML1, CD40, IL-4R, and IL-5RA genes were associated with allergic sensitisation. SNPs near or in JAK1, JAK3, IL-5RA, FCER1A, and ADAM33 genes were associated with cockroach sensitisation. SNP rs6665683 in FCER1A was shown to have a strong association with cockroach sensitisation and high levels of specific IgE to cockroach allergens. Also SNP rs7851969 in JAK2 showed consistent association with sensitisation to house dust mite, and cockroach allergens. Further validation to these findings can be brought by analysing the same genes on different populations [68].

Large number of susceptibility genes for allergies have been uncovered by Genome-wide association studies (GWAS). According to the Catalogue of Published Genome-Wide Association Studies, there are about 40 GWAS for asthma and 3 GWAS for atopy been conducted along with a plethora of susceptible genes and loci. However, association of a gene to allergic disease does not certainly imply biological functionality. Therefore, follow-up studies are required to translate findings of GWAS into the biological functionality, which will then enhance diagnosis and therapeutics of allergies [69].

1.4.2 The environmental basis of allergic diseases

The rising incidence of allergic diseases cannot be justified by genetics alone. The history of environmental exposure and life style of affected individuals are also important risk factors of allergies. This dimension of the allergic diseases was conceived from epidemiological studies, which illustrate the association between human health and adverse environmental conditions. The change in the environment could mean an addition of a risk factor such as infectious agents resulting in plagues.
However, the opposite is also true where an alteration in the environment results in removal of a protective factor leading to diseases [70]. Based on this model, epidemiologist David Strachan conceived the idea of hygiene hypothesis in his study that used the 1958 British birth cohort [71]. Hygiene hypothesis regards infections in early childhood as protective, which may prevent the development of allergies. Hygiene hypothesis also states that this benefit of infections is lacking or limited in highly developed regions of the world when compared to the rest of the world due to sanitary living standards and advanced health care. Excessive cleanliness, vaccinations and widespread use of antibiotics commonly seen in the urban Western lifestyle, are considered strong risk factors for development of allergies [70-72]. Even though, hygiene hypothesis is a well-accepted theory, there are evidence that do not agree with it. In Japan, where hygiene standards are high, asthma levels are much lower than in the United States or Australia. Also in the United States asthma is reported to be high among children who live in very poor housing [73].

1.4.2.1 Cellular and molecular basis of hygiene hypothesis

The framework required to support hygiene hypothesis is laid down by both the Th1/Th2 imbalance theory and early life events leading to allergic sensitisation. The capacity to mount an immune response against common environmental allergens is acquired even before birth. During the gestation period, the foetal immune cells normally assume a Th2 and Tr1 type response. Therefore, foetus is exposed to high concentrations of Th2 and Tr1 type cytokines. This is believed to be an adaptation by the foetus to down-regulate maternal Th1 immune response against foeto-paternal antigens, which may lead to foetal rejection. In addition, studies have demonstrated that foetus is often exposed to allergens that cross the placenta from the maternal
circulation. Therefore, *in-utero* priming of T lymphocytes by allergens and allergen sensitisation is possible [55, 74, 75].

However, after birth it has been suggested that a normal individual’s immune system gradually changes to a Th1 type immune response against common environmental allergens. By contrast, in potentially allergic individuals, further upregulation of Th2 type immunity takes place. It has been proposed that in healthy subjects the shift from Th2 to Th1 immunity against allergens is achieved through the aid of infections. Macrophages when engulfing microbes such as *Mycobacteria* and *Lactobacilli* secrete IL-12, which is a known potent stimulator of Th1 lymphocytes. Therefore, it is said that IL-12 rich environment drives the development of Th1 lymphocytes leading to down-regulation of Th2 lymphocytes [55, 74, 75].

### 1.4.2.2 Population based studies on hygiene hypothesis

Comparative studies conducted at the population level have provided some widespread support to hygiene hypothesis [19, 55]. One of the most informative studies ever performed was the investigation of former East and West German populations following the fall of Berlin wall in 1989. Unification of Germany allowed researches to study genetically equal populations, which used to live under very different environmental conditions for about four decades. Allergies such as hay fever in particular were less common in East Germany than West Germany before unification. However, after unification an increase in hay fever among children from former East Germany was reported, with the advent of Western life style [76]. Furthermore, an inverse relationship between allergies and infectious diseases was shown by serological studies conducted on hepatitis A, *Helicobacter pylori*, *Toxoplasma gondii*, childhood measles and *Mycobacterium tuberculosis*. During infections with such microbes, macrophages become active and secrete IL-12, which
promotes Th1 type immunity leading to down-regulation of Th2 immune response. These findings explain why populations, particularly in Western Europe, North America and Australia, exhibit a high degree of allergies due to the lack of infections caused by high sanitary living conditions [19, 55].

1.5 Egg allergy

Allergy to hen’s (*Gallus domesticus*) egg is one of the most common forms of childhood allergies seen in industrialised countries. The prevalence of egg allergy sufferers in industrialised countries ranges from 1.6% to 3.2% [77, 78]. The development of egg allergy generally occurs during infancy and resolves before the school age. Sensitisation to egg allergens can occur even before birth *in utero* and during breast-feeding. As a consequence, clinical reactions to eggs, even without direct exposure, can occur in infants sensitised in such ways [79]. Egg allergens are known to cause both IgE-mediated and non-IgE mediated allergic reactions. However, the most common form of egg hypersensitivity is due to IgE-mediated allergy. Clinical manifestations to egg allergy occur in various organ systems such as the skin, the respiratory system and the gastrointestinal system. Some of the common forms of clinical signs and symptoms of egg allergy are urticaria, angioedema, asthma and vomiting. In infants, the most common clinical manifestation is atopic dermatitis. Severe anaphylaxis due to egg allergy is possible, especially in patients with acute IgE-mediated sensitivity to hen’s egg. For example, 7% of severe anaphylactic reactions were reported due to egg ingestion in infants according to a survey from Germany. Generally, fatal reactions due to eggs are uncommon, but have been reported [78, 80].

Six allergenic proteins from both the albumen and the yolk have been identified from the hen’s egg. The egg white contains four well known allergens, namely ovomucoid
(Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozyme (Gal d 4) [78].

The egg yolk consists of two minor allergens known as α-livetin (Gal d 5) and yolk glycoprotein 42 or YGP42 (Gal d 6) [81, 82]. The immunogenic properties of egg white allergens have been extensively studied over the years [78]. Some of the earliest studies reported the degree of allergenicity of egg white allergens to be in the following order: lysozyme, ovomucin, ovalbumin and ovomucoid, based on skin prick test (SPT) diagnosis. However, further studies demonstrated that ovomucoid to be the dominant allergen followed by ovalbumin, ovotransferrin and finally lysozyme [83-87].

Egg yolk proteome was not a popular subject for immunological studies like the egg white [78]. However, a limited number of studies have confirmed egg yolk as a source of allergenic proteins [78, 81, 82]. Currently, there could be many knowledge gaps in regards to the allergenicity of the hen’s egg yolk. For example, the frequency of recognition of egg yolk allergens in sensitised patients, T and B cell epitopes relevant to yolk allergens and the association of egg white and egg yolk allergies still remain to be investigated.

1.5.1 Allergy to egg yolk

Allergy to egg yolk is present in a considerable percentage of individuals however, with much less prevalence and potency than allergy to egg white. Unlike egg white allergy, which is commonly seen in young children with atopic dermatitis, egg yolk allergy mostly affects during the adulthood. The underlying immunological mechanism responsible for egg yolk allergy is IgE-mediated according currently available reports, and usually produce symptoms such as urticaria, vomiting, abdominal pain and diarrhoea [88-90].
The true existence of allergy to egg yolk was highly doubtful in the past despite well-investigated reports on anaphylactogenic properties of egg white constituents. Although sensitisation to egg yolk had been reported before, its actual existence were not confirmed and the general notion was that allergy to egg yolk cannot occur without the existence of egg white allergy [91].

However, Rubin SS., for the first time presented a case of a genuine sensitisation to egg yolk independent of egg white allergy [91]. The report describes the case of a 19-year-old soldier, who was diagnosed with angioneurotic oedema following typhus fever and yellow fever vaccines. According to the patient’s medical history, he was not able to tolerate eggs but was able to consume hard-boiled egg white without any complications. SPTs on the patient were positive for egg yolk and negative for egg white, chicken feathers and chicken meat [91].

Yellow fever and typhus fever vaccines are cultured in chick embryos and yolk sacs, respectively. It had been shown that those vaccines contain egg yolk constituents. Considering the patient’s medical history and SPT results, the author was able to conclude definitively that the allergic manifestations were due to egg yolk components in the administered vaccines. As a result, this case report confirmed the fact that genuine sensitisation to egg yolk exists and that it can occur without concomitant egg white sensitivity [91].

1.5.2 Allergens in the hen’s egg Yolk

1.5.2.1 Chicken serum albumin (Gal d 5)

Chicken serum albumin (CSA) also known as α-livetin, is Gal d 5 the first allergen identified from the hen’s egg yolk. Gal d 5 is synthesised in the liver of laying hens and present in both the blood circulation and the egg yolk [82, 92]. It is a water-
soluble globular glycoprotein with a molecular weight of 69 kDa. Gal d 5 had been confirmed as an inhalant allergen as well as a food allergen through bronchial provocation and oral challenge tests, respectively. Gal d 5 is a partially heat resistant allergen, thus some patients are able to tolerate extensively heated egg yolk, but not raw egg yolk. The main route of sensitisation for Gal d 5 is through the respiratory tract as seen in bird-egg syndrome [82].

Table 1.2: The six main allergens from the hen’s egg white and the yolk (allergen data obtained from www.allergen.org)

<table>
<thead>
<tr>
<th>Allergen name</th>
<th>Source of the allergen</th>
<th>Biochemical name(s)</th>
<th>MW(SDS-PAGE) kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal d 1</td>
<td>Egg white</td>
<td>Ovomucoid</td>
<td>28</td>
</tr>
<tr>
<td>Gal d 2</td>
<td>Egg white</td>
<td>Ovalbumin</td>
<td>44</td>
</tr>
<tr>
<td>Gal d 3</td>
<td>Egg white</td>
<td>Ovotransferrin</td>
<td>78</td>
</tr>
<tr>
<td>Gal d 4</td>
<td>Egg white</td>
<td>Lysozyme</td>
<td>14</td>
</tr>
<tr>
<td>Gal d 5</td>
<td>Egg yolk</td>
<td>Chicken serum albumin/α-livetin</td>
<td>69</td>
</tr>
<tr>
<td>Gal d 6</td>
<td>Egg yolk</td>
<td>YGP42</td>
<td>35</td>
</tr>
</tbody>
</table>

1.5.2.2 The bird-egg syndrome

Bird-egg syndrome is an IgE-mediated hypersensitivity disorder. In bird-egg syndrome, allergy to egg yolk develops, following sensitisation to inhalant avian antigens derived from sources such as bird’s blood serum, feathers, droppings and dander [89]. This is different to cross reactivity, where a patient allergic to a certain allergen reacts to homologous or non-homologous allergens from the same or different species [93, 94]. In bird-egg syndrome, sensitisation and allergy to egg yolk occurs due to a common allergen present in both avian antigens and the egg yolk. The common allergen present in hen’s egg yolk and other avian antigens is Gal d 5 [90, 92].

Most patients with bird-egg syndrome have regular exposure to pet birds or poultry where sensitisation to inhalant avian antigens occurs. Patients with bird-egg
syndrome suffer from both respiratory and gastrointestinal allergic symptoms such as asthma, rhinoconjunctivities, oedema, diarrhoea and vomiting. 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 [89, 90]. The higher number of females reported in these studies may just relate to higher number of females in the poultry industry. However, in the study conducted by Quirce et al., majority of the patients with bird-egg syndrome was males [82]. Furthermore, 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 [95]. Furthermore, radioallergosorbent test (RAST) 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 [89].

1.5.2.3 YGP42 (Gal d 6)

Gal d 6 is the second allergen identified from the hen’s egg yolk. It is a glycoprotein with a molecular weight of 35 kDa. Gal d 6 is the C-terminal fragment of vitellogenin-1 (VTG-1) precursor, which is a serum lipoprotein specific for laying hens. VTG-1 is produced in the liver of laying hens and transported into the growing oocyte by receptor-mediated endocytosis. Following delivery into the oocyte, VTG-1 precursor is enzymatically cleaved into several fragments giving rise to mature proteins lipovitellin-1, phosvitin, lipovitellin-2 and YGP42 [81, 96].

Gal d 6 contains three glycosylation signals at positions 1662, 1698 and 1703. The periodate oxidation treatment of Gal d 6 does not abolish the IgE binding capacity,
indicating that sugar residues are not related to allergenicity. Furthermore, heat treatment and reduction does not affect the allergenicity of Gal d 6. However, simulated gastric acid digestion completely eliminated its IgE reactivity. Gal d 6 was also investigated for any implication in bird-egg syndrome. The inhibition immunobloting experiments did not demonstrate any degree of inhibition of allergens from chicken meat and feather extracts. Therefore, Gal d 6 is not considered to be involved in bird-egg syndrome [81].

1.5.2.4 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 [97, 98]. Three abundant egg yolk proteins from the egg yolk known as apovitellenin I, apovitellenin VI and phosvitin have demonstrated substantial IgE binding capacity among egg allergic patients. Furthermore, all of these proteins were found to effectively inhibit binding of egg yolk specific IgE. However, the clinical relevance of these proteins in relation to food allergy has not been established so far. Therefore, their status as allergens still remains uncertain [98].

1.5.3 Diagnosis of egg allergy

Several diagnostic tools must be used to confirm egg allergy in an individual. Diagnosis of egg allergy usually commences with an investigation of medical history and physical examination looking for evidence of clinical reactivity to eggs. Next steps may involve in vitro and in vivo diagnostic tests. Some of the commonly used diagnostic tests are the measurement of allergen-specific IgE antibodies, SPT, atopy patch tests, diagnostic elimination diets and oral food challenges (OFC) [80]. SPT involves introducing allergens into the skin using a lancet to detect egg specific IgE
antibodies [99]. The reliability of SPT depends on the quality of the allergen extract used. A wheal size greater than or equal to 3 mm is considered positive for egg allergy when a clear clinical history is taken into account. However, SPTs have a poor positive predictive value (PPV). Therefore, a positive SPT result without a clinical history may not indicate egg allergy. A negative SPT essentially rule out IgE-mediated egg allergy since it has a high negative predictive value (NPV) [80, 100, 101].

Egg allergen specific IgE antibodies can be quantified using standardized in vitro IgE assays. As in SPT, the accuracy of the result heavily depends on the quality of the allergen extracts used. The predictive values for egg specific IgE for diagnosis reported by various study groups are not consistent. These variations are mainly due to the different parameters used in the studies. Therefore, it has been recommended to consider the relevant variables when determining cut-off levels in patient populations [102, 103].

To date, the gold standard for diagnosis of all food allergies is double blind placebo controlled food challenge (DBPCFC). The major drawback of challenge tests is that it can trigger potentially life threatening allergic reactions. Therefore, challenge tests are only conducted by trained health care personal with adequate facilities. DBPCFC are widely used in diagnosis of egg and milk allergy however, requires substantial improvements for implication in everyday practice [102].

1.5.4 Management of egg allergy

Currently, there is no cure for allergies. Therefore, patients are required to undergo management according to the guidelines prepared by dieticians and clinicians. Egg avoidance is essential in managing egg allergy. Avoidance of eggs when they are
prepared in a recognizable form can be easily achieved. However, avoiding eggs in manufactured food products and contaminated food can be challenging. Furthermore, mothers nursing infants, who are known to be sensitised to eggs, must also take extra care since breast milk can contain egg allergens derived from the diet. Therefore, mothers may have to depend on an egg free diet if infants develop clinical reactions following breast feeding [104]. An egg free diet does not impose the risk of nutritional deficiency unless there are additional dietary limitations such as vegetarian diet or multiple food allergies. If such situation arises, a dietician should be consulted [101].

Apart from dietary elimination of eggs, patients may have to avoid vaccines derived from chicken eggs. Measles, mumps and rubella (MMR), influenza and yellow fever vaccines are cultured in chicken eggs. Influenza and yellow fever vaccines both contain measurable quantities of egg proteins both from the albumen and the yolk and patients with egg allergy have reacted adversely following administration of these vaccines. Patients with mild egg allergy are capable of receiving the vaccination with minimal or no side effects. However, for patients with severe egg allergy, administration of vaccines is not generally recommended. However, MMR vaccine is deemed safe to administer to egg allergic patients. A large scale study that vaccinated egg allergic children showed no risk of severe allergic reactions [100, 101, 105].

In egg allergy, provision of emergency medication commonly involves the use of antihistamines and adrenaline. Antihistamines are administered orally and are recommended to treat mild reactions. Adrenaline is available as a self-injectable auto injector and is recommended in the event of severe allergic reactions. Both
medications provide symptomatic relief by reversing the effects of chemical mediators released during the allergic reaction [100, 101, 105].

As for treatment strategies at experimental level, some progress has been made in increasing tolerance to eggs by means of oral immunotherapy, also known as oral tolerance induction (OTI). OIT has demonstrated successful results in peanut allergy. It has been shown that early consumption of peanuts in infancy to result in reduction of peanut allergy. These findings indicate that consumption of peanuts during infancy may result in prevention of developing peanut allergy [106]. Although some patients were able to tolerate certain doses of egg proteins, none of them were able to tolerate the complete dose. The efficiency of OTI is still questionable since it is not clear whether partial tolerance is resulted by OTI or due to spontaneous allergy resolution and therefore, it requires further investigations [104, 105, 107]. Based on the current results, OTI is still not recommended for use in routine clinical practice [105].

1.6 A potential cure for allergies: Allergen-specific immunotherapy

Allergen-specific immunotherapy (SIT) is the only treatment strategy that has demonstrated a curative potential in allergies [108]. SIT involves gradually administering allergen extracts to the patient in order to increase the capacity of the immune system to tolerate allergens [109, 110]. The allergen extracts used in immunotherapy are now known as allergen vaccines since they have the capacity to act as immune modifiers [111]. The traditional route of administration of immunotherapy is subcutaneously and known as injection immunotherapy. Injection immunotherapy is known as the most efficient form of immunotherapy. Nasal, oral, intra-lymphatic and sublingual are some other forms of immunotherapy. [109-111].
Injection immunotherapy is proven to work well with insect venom allergy, especially for *Hymenoptera* venom allergy. In fact, venom immunotherapy is now approved as the standard care for *Hymenoptera* venom allergy. Furthermore, some clinical trials have demonstrated promising results with allergen SIT for pollen allergy and house dust mite allergy [111, 112]. Injection immunotherapy also had limited success in reducing clinical reactivity in patients allergic to fruits, vegetables and tree nuts [107]. Despite these successes, immunotherapy for food allergies is still deemed unsafe. The last injection immunotherapy trial conducted to treat peanut allergy resulted in failure. Majority of patients in the test group produced severe adverse reactions following injection immunotherapy. Therefore, injection immunotherapy for food allergies is currently abandoned [111, 113, 114].

The mechanism on how tolerance is achieved during immunotherapy is not well understood. Recent studies have shown that immunotherapy causes a change in the overall Th cell response by increasing Th1 differentiation and reversing Th2 into Th0. As a result of this immune deviation, the immune system increases the production of IgG1 and IgG4, while decreasing the production of allergen specific IgE [115]. Further observations during immunotherapy include an increase in CD8+ cells and reduction in inflammatory cell recruitment, activation and mediator secretion [111, 115].

1.7 The application of recombinant DNA technology in allergy diagnosis and SIT

Commercial allergen extracts, derived from natural sources, have many drawbacks that cannot be resolved by any practical methods currently available. Natural allergen products are often contaminated with various allergenic and non-allergenic substances. In some cases, the allergen of interest is very low or absent. Therefore,
patients undergoing treatment with natural allergen extracts face the risk of developing new sensitisations. Furthermore, precise diagnosis of allergic diseases is difficult with the use of natural allergen preparations due to the contamination issue [115-118].

For successful diagnosis and SIT of allergic diseases, the purity and the definition of the allergen extracts are crucial. The above-mentioned difficulties associated with commercial allergen extracts can be successfully overcome with the use of recombinant DNA technology. With the use of recombinant DNA technology, recombinant allergens can be harvested in unlimited amounts with properly defined immunological, biological and molecular features. Recombinant allergens can be produced to mirror the IgE-reactivity of natural allergens for diagnosis and are already in use for clinical diagnosis [115-118].

Furthermore, recombinant allergens can be genetically engineered to produce hypoallergens with reduced IgE-binding activity. Advantages of using recombinant based hypoallergens over natural allergens in SIT are two-fold. Firstly, hypoallergens offer improved safety due to low IgE-reactivity. Secondly, the dose that can be safely administered to patients can be increased. Therefore, the efficacy of hypoallergen based SIT can also rises with the dose increments [116]. The first injection immunotherapy trial conducted with recombinant based hypoallergenic variants of the major birch pollen allergen Bet v 1 was highly successful. Patients that underwent immunotherapy showed strong IgG antibody response against the Bet v 1 allergen and exhibited reduced clinical reactivity during subsequent birch pollen seasons [116, 119].
1.8 Aims of this study

As described above, very little is known about allergy to egg yolk. The existence of allergy to hen’s egg yolk was highly doubted by many in the past. Recent discovery of allergens Gal d 5 and Gal d 6 has spurred much interest in hen’s egg yolk allergy. However, in depth investigations involving hen’s egg yolk in relation to food allergy has been rarely performed. Therefore, allergy to hen’s egg yolk is still a developing area. The use of recombinant allergens has been a very popular approach in the field of allergy for both diagnosis and therapy due to the many advantages they offer. However, to date, production of recombinant versions of hen’s egg yolk allergens Gal d 5 and Gal d 6 have not been reported in the literature. Thus, the main focus of this PhD project was to investigate allergy to hen’s egg yolk in humans, mainly at molecular level, and to produce recombinant versions of hen’s egg yolk allergens. The work of this thesis was conducted in order to provide a better understanding and fill research gaps in knowledge regarding allergy to hen’s egg yolk. Below is a brief description of the research hypotheses addressed and the manner in which they were investigated in this PhD project.

In Chapter 2, I investigated the in vitro IgE-reactivity of hen’s egg yolk proteins using molecular and proteomic methods. Furthermore, in addition to hen’s egg yolk I included egg yolks from emu and quail in some of the work conducted. The first aim of this study was to investigate the existence of sensitisation to egg yolk proteins from hen, emu and quail in patients with allergy to hen’s egg white. It was hypothesised that majority of patients diagnosed with allergy to hen’s egg white would have concomitant sensitisation to egg yolk proteins. Here, Western immunoblot was used as a rapid method of identifying IgE reactive proteins using sera from a cohort of 25 egg white allergy patients. The second aim of this chapter
involved identifying the IgE-reactive egg yolk proteins from all of the bird species used in the Western immunoassays. The third and final aim of Chapter 2 was to produce a recombinant version of hen’s egg yolk allergen Gal d 6 as a soluble fraction in an *E. coli* expression system. It was hypothesised that cloning and expression of complementary DNA (cDNA) coding for Gal d 6 in *E. coli* would allow production of an IgE-reactive recombinant Gal d 6 as a soluble fraction.

In Chapter 3, the main objective was to produce an IgE reactive recombinant version of hen’s egg yolk allergen Gal d 5 as a soluble fraction. Firstly, an *E. coli* expression system was used to clone and express recombinant Gal d 5. It was hypothesised that cloning and expression of cDNA coding for Gal d 5 in *E. coli* would allow production of an IgE-reactive recombinant Gal d 5 as a soluble fraction. However, the hypothesis was rejected as 100% of the recombinant Gal d 5 was expressed as an insoluble fraction by *E. coli*. Therefore, as an alternative method I used an eukaryotic protein expression system *Kluyveromyces lactis* (*K. lactis*) yeast strain. Here, I hypothesised that the cloning of cDNA coding for mature Gal d 5 in frame with the yeast secretion signal would allow production of recombinant Gal d 5 as a secreted protein. Finally, in this chapter, we comparatively analysed the IgE-binding reactivity of recombinant and natural Gal d 5 using sera from egg white allergy patients.

In Chapter 4, the main objective was to produce a recombinant based mutant variant of Gal d 5 with reduced IgE binding capacity in order to develop a hypoallergen for potential use in immunotherapy in the future. In this Chapter, site directed mutagenesis (SDM) technique was used to disrupt disulfide bonds of Gal d 5 to synthesize a mutant Gal d 5 DNA. Then cDNA coding for mutant Gal d 5 was cloned
into *K. lactis* for recombinant protein expression. It was hypothesised that recombinant Gal d 5 with a modified conformation resulted by disulfide bond disruption would reduce or completely impair interaction with IgE antibodies. However, *K. lactis* yeast expression system failed to express the mutant Gal d 5 despite successful cloning. Therefore, as an alternative approach I expressed mutant Gal d 5 as a soluble fraction in an *E. coli* expression system. The recombinant based mutant Gal d 5 was then comparatively analysed with wild-type recombinant Gal d 5 to evaluate the IgE-binding capacity, which successfully demonstrated reduction in IgE binding.

The Chapter 5, which is the final chapter of this PhD thesis, discuss the findings presented in Chapters 2-4. All the findings are summarised and presented in Chapter 5 and explains how the work of this PhD project has contributed to improve knowledge regarding allergy to hen’s egg yolk. Furthermore, this chapter also provides future directions of the current project.
Chapter 2: Immunological analysis of egg yolk proteins from hen, quail and emu and production of recombinant Gal d 6 in *E. coli*

Publication:
*De Silva C*, Dhanapala P, Doran T, Tang MLK & Suphioglu C (2016): Molecular and immunological analysis of hen’s egg yolk allergens with a focus on YGP42 (Gal d 6), Molecular Immunology: 71: 152-160.
2.1 Introduction

Hen’s egg has been a very popular diet in most regions of the world since ancient times [120]. Nevertheless, immunological hypersensitivity to hen’s egg is present in a significant percentage of children [121, 122]. The major allergens are found in the egg white. On the other hand, egg yolk is also reported to exhibit low level allergenicity in humans [78]. Food allergy can be divided into primary and secondary food allergies based on the sensitisation process. Allergic hypersensitivity reactions independent of sensitisation to offending food source is defined as primary food allergies. Secondary food allergy is defined as allergic hypersensitivity reactions arising as a result of initial sensitisation to offending food source. A study has demonstrated that approximately 9.4% and 5.5% of the population are affected by primary and secondary food allergies, respectively [123]. In adults, secondary food allergy often follows primary food allergy caused by inhalant or contact allergens. Secondary food allergy is mainly attributed to cross-reactive IgE antibodies that recognize homologous sequences among allergens. On the other hand, primary food allergies are mainly seen in children caused by the offending food allergens [123, 124].

Clinical studies have shown that sensitisation to egg white allergens precede onset of clinical reactivity to hen’s egg yolk. Development of egg allergy starts with sensitisation to Gal d 1 and Gal d 2. However, during the course of the disease, patients tend to produce IgE antibodies against other egg white allergens (Gal d 3 and Gal d 4) and finally against egg yolk allergens. Furthermore, IgE reactivity to Gal d 5 has been identified as a marker for egg yolk sensitisation. Nevertheless, not all egg yolk allergic patients react with Gal d 5 and therefore, Gal d 6 or other
potential egg yolk allergens, such as apolipoproteins, may play a role in development of allergy to egg yolk [98, 124].

Patients suffering from allergy to hen’s egg face significant challenges in terms of quality of life. Due to the lack of cure for egg allergy, avoidance of eggs and egg derived food products represents the sole strategy to manage the condition. As a consequence, there is a current trend towards consuming eggs from different bird species such as quail, duck, turkey (*Meleagris gallopavo*), sea-gull and emu (*Dromaius novaehollandiae*), as an alternative to hen’s egg. Nevertheless, clinical and serological cross-reactivity between hen’s egg allergens and proteins from other bird eggs such as turkey, duck, goose and sea-gull have been reported [125]. Additionally, allergy to quail, duck and goose eggs, independent from allergy to hen’s egg, have been reported as well [120, 126].

Current data regarding hypersensitivity to bird eggs suggest that diagnosis of egg allergy should include discrimination of egg white and the yolk proteomes at molecular level. For proper patient management, in detail diagnosis, based on molecular techniques, may equip clinicians to provide better dietary advice. As a result, patients can avoid unnecessary dietary restrictions leading to improvements in quality of life. An important requirement for successful molecular based diagnosis is the availability of well-defined standardized allergen preparations. A popular approach for developing allergen preparations for diagnosis is the use of recombinant DNA technology to produce recombinant allergens that mimic the IgE-reactivity of their natural counterparts [116].

The overall objective of this chapter was to subject egg yolk proteins of hen, emu and quail using immunological, proteomic and molecular methods in order to shed light on egg yolk allergy. The first aim of this chapter involved investigating the
sensitisation profiles of egg white allergic patients and compared \textit{in vitro} IgE reactivity of egg yolk proteins from hen, emu and quail eggs. Furthermore, we discriminately analysed free range and caged chicken eggs in order to investigate if different environmental conditions of laying hens would influence the allergenicity of the egg yolk proteins. In this chapter, we used a cohort of 25 patients diagnosed with allergy to hen’s egg white using \textit{in vitro} immunological methods.

The second objective of this chapter involved producing an immunologically active recombinant hen’s egg yolk allergen Gal d 6 (rGal d 6). In this chapter, we present the successful expression of rGal d 6 as a soluble fraction in \textit{E. coli}. rGal d 6 was then subjected to \textit{in vitro} immunological analysis using patients’ serum sensitised to natural Gal d 6 (nGal d 6) to compare its’ allergenicity with crude hen’s egg yolk extract.

\textbf{2.2 Materials & methods}

\textbf{2.2.1 Immunological analysis of hen, quail and emu egg yolk extracts}

\textbf{2.2.1.1 Human patients’ sera}

Sera from 25 patients allergic to hen’s egg white and non-allergic individuals as healthy controls were obtained from the Royal Children’s Hospital (Melbourne, VIC, Australia). The egg white specific IgE levels of patients are shown in Table 2.1. 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.
Table 2.1: The levels of allergen specific IgE antibodies against egg white determined by ImmunoCAP (Phadia)

<table>
<thead>
<tr>
<th>Patient number</th>
<th>IgE level (IU/mL) against hen’s egg white</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>93.50</td>
</tr>
<tr>
<td>2</td>
<td>28.80</td>
</tr>
<tr>
<td>3</td>
<td>13.60</td>
</tr>
<tr>
<td>4</td>
<td>9.26</td>
</tr>
<tr>
<td>5</td>
<td>13.20</td>
</tr>
<tr>
<td>6</td>
<td>8.63</td>
</tr>
<tr>
<td>7</td>
<td>6.89</td>
</tr>
<tr>
<td>8</td>
<td>18.50</td>
</tr>
<tr>
<td>9</td>
<td>16.50</td>
</tr>
<tr>
<td>10</td>
<td>4.13</td>
</tr>
<tr>
<td>11</td>
<td>3.62</td>
</tr>
<tr>
<td>12</td>
<td>1.02</td>
</tr>
<tr>
<td>13</td>
<td>1.21</td>
</tr>
<tr>
<td>14</td>
<td>1.32</td>
</tr>
<tr>
<td>15</td>
<td>1.72</td>
</tr>
<tr>
<td>16</td>
<td>2.08</td>
</tr>
<tr>
<td>17</td>
<td>2.21</td>
</tr>
<tr>
<td>18</td>
<td>2.58</td>
</tr>
<tr>
<td>19</td>
<td>1.78</td>
</tr>
<tr>
<td>20</td>
<td>1.19</td>
</tr>
<tr>
<td>21</td>
<td>1.25</td>
</tr>
<tr>
<td>22</td>
<td>1.05</td>
</tr>
<tr>
<td>23</td>
<td>0.32</td>
</tr>
<tr>
<td>24</td>
<td>0.18</td>
</tr>
<tr>
<td>Patient number</td>
<td>IgE level (IU/mL) against hen’s egg white</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>25</td>
<td>0.47</td>
</tr>
</tbody>
</table>

2.2.1.2 Preparation of crude egg yolk extracts (CEY) from hen, quail and emu eggs

Chicken (White Leghorns) and emu eggs were provided by the Australian Animal Health Laboratory (AAHL, Geelong, VIC, Australia). Caged chicken eggs, free-range chicken eggs and quail eggs were bought from a local market. Shelling and separation of the egg yolk from the albumen was performed manually. In order to minimize egg white contamination, intact yolks were rinsed thoroughly with deionized water (dH₂O) and rolled on a filter paper to remove remaining residual egg white as much as possible. The vitelline membrane was pierced using a pair of tweezers and the yolk constituents were extracted with a pipette. The solubilisation of egg yolk samples was based on the method published by Guilmineau et al., [127]. One volume of egg yolk was mixed with 12 volumes of 0.1 M glycine/NaOH buffer (pH 9; 0.56 NaCl) and kept overnight at 4°C to equilibrate.

2.2.1.3 SDS-PAGE analysis of CEYs of hen, quail and emu eggs

Novex 4-20% Tris-Glycine gels (Thermo Fisher Scientific®, Waltham, MA, USA) were used for all sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and denaturing conditions. Approximately 25 μg of protein was applied per lane. The CEY samples were prepared by dissolving in Novex Tris-Glycine SDS sample buffer (2×) (Thermo Fisher Scientific®, Waltham, MA, USA), NuPAGE sample reducing agent (10×) (Thermo Fisher Scientific®, Waltham, MA, USA) and dH₂O. The egg yolk samples were mixed by pipetting and heated at 85°C for 2 minutes prior electrophoresis. SeeBlue pre-stained protein standard (Thermo

40
Fisher Scientific®, Waltham, MA, USA) was used as the molecular weight marker. Electrophoretic separation was performed using an X-Cell SureLock Mini-Cell system (Thermo Fisher Scientific®, Waltham, MA, USA) applying a constant voltage of 125 V for 90 minutes. The protein bands were then stained for visualization using SimplyBlue SafeStain (Thermo Fisher Scientific®, Waltham, MA, USA).

2.2.1.4 Western immunoassay of CEYs

2.2.1.4.1 Western immunoassay of hen’s CEY with individual patients’ sera

For the immunoassay with individual patients’ sera, only CEY prepared from hen’s eggs provided by AAHL was used. Following SDS-PAGE, proteins were electro-transferred on to nitrocellulose membranes using X-Cell II Blot Module system (Thermo Fisher Scientific®, Waltham, MA, USA). The egg yolk immobilised nitrocellulose membranes were developed using WesternBreeze® Chromogenic Western Blot Immunodetection Kit according to manufacturer’s instructions (Thermo Fisher Scientific®, Waltham, MA, USA). Following transfer, the nitrocellulose membrane was stained with Ponceau S solution (Sigma-Aldrich, MO, USA) for visualization of proteins. Lanes containing the CEY profiles were cut into individual strips. Then all nitrocellulose membrane strips were destained with 0.1 M NaOH and immediately blocked using the blocking solution (hammersten casein solution) provided in the kit. Then, egg yolk immobilised strips were individually incubated overnight at 4°C with patients’ sera (patient no. 1 to 25) (diluted 1:10 in blocking solution) and 5 non-allergic subjects’ sera (diluted 1:10 in blocking solution) as negative controls. Then, unbound antibodies were removed by washing the serum-incubated nitrocellulose strips four times in the antibody wash solution provided in the kit for 5 minutes. Following washing, all serum-incubated nitrocellulose strips were incubated with monoclonal anti-human IgE mouse
antibodies labelled with alkaline phosphatase (diluted 1:1000 in blocking solution) (Sigma-Aldrich, MO, USA) for 2 hours at room temperature. CEY immobilised strips without any serum incubations, (“no serum” negative controls) were incubated for 2 hours at room temperature with anti-human IgE mouse antibodies labelled with alkaline phosphatase (diluted 1:1000 in blocking solution). Then all the nitrocellulose strips were washed with antibody wash solution. The signal was developed by adding 5-bromo-4-chloro-3’indolyphosphate p toluidine/nitro-blue tetrazolium chloride (BCIP/NBT) chromogenic substrate. This immunoassay was repeated one more time to ensure the reproducibility. The blot IgE reactivity for each IgE-reactive protein was then analysed by eye.

2.2.1.4.2 Western immunoassay of hen, quail and emu CEYs with pooled patients’ sera

A second Western immunoassay was conducted using a pool of sera (diluted 1:10 in blocking solution) composed from patients 1, 3, 10, 14, 15, 16 and 25, against all CEYs. SDS-PAGE of CEY samples was conducted according to method mentioned in section 2.2.1.3. Then, separated CEYs were electro-transferred on to nitrocellulose membranes and developed according to the method mentioned in Section 2.2.1.4.1. Another Western immunoassay was carried out using a pool of non-allergic individuals’ sera (diluted 1:10 in blocking solution) as a negative control. This immunoassay was repeated one more time to ensure the reproducibility.

2.2.1.5 Proteomic analysis of IgE reactive proteins from CEYs

The IgE reactive protein bands were excised from SDS-PAGE gels and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS). The proteins were in-gel digested with trypsin and incubated overnight at 37°C. Then, analysis of digested peptide mixtures was carried out at Bio21 Institute (Melbourne, VIC,
Australia) on a LTQ Orbitrap Elite (Thermo Fisher Scientific®, Waltham, MA, USA) coupled to an Ultimate 3000 RSLC nanosystem (Dionex, Sunnyvale, CA). Analysis of data was done using a Proteome Discoverer (Thermo Fisher Scientific® version 1.4) with a MASCOT search engine against the BIRDS database. Carbamidomethylation of cysteine was set as the fixed modification while oxidized methionine was set as the variable modification. The precursor mass tolerance was set at 10 ppm and fragment mass tolerance was set at 0.6 Da. One missed cleavage was allowed.

2.2.2 Cloning and expression of Gal d 6 in E. coli expression system

2.2.2.1 Strains, vectors and growth conditions

NEB Express Iq Competent E. coli cells (New England Biolabs (NEB), Ipswich, MA, USA) were used as the cloning and expression host. E. coli cells were grown in lysogeny broth (LB) supplemented with 50 µg/mL ampicillin at 37°C. pTrcHis A expression vector (Thermo Fisher Scientific®, Waltham, MA, USA) contains the ampicillin resistance gene (β-lactamase), which allows growth on media containing ampicillin. The selection of E. coli cells transformed with pTrcHis A vectors were performed by growing on LB agar medium supplemented with 50 µg/mL ampicillin at 37°C.

2.2.2.2 Extraction of Poly A+ mRNA from chicken liver

Animal experimentation/sampling was conducted under protocol AEC1496, approved by the Australian Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organisation (AAHL-CSIRO) Animal Ethics Committee and in accordance with the Australian code of practice for the care and use of animals for scientific purposes.
Total messenger ribonucleic acid (mRNA) from chicken liver was extracted using Oligotex Direct mRNA kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. All the centrifugation steps were carried out at 13,000 rpm. A freshly harvested liver from an egg laying hen was stabilized in RNA stabilization reagent RNA later, which was provided by AAHL (Geelong, VIC, Australia). The liver tissue was immediately frozen in liquid nitrogen upon removal from RNA later and ground until a fine powder was formed using a sterile mortar and pestle. 186 mg of tissue powder was measured in a sterile liquid nitrogen cooled 15 mL tube and mixed with 2 mL of buffer OL1 containing 0.42 M β-mercaptoethanol (β-ME). 700 µL of the resulting lysate was transferred into a QIAshredder spin column, contained in a 2 mL collection tube and centrifuged for 2 minutes. 1.2 mL of buffer ODB was added to the lysate and mixed by pipetting prior centrifugation for 3 minutes. The supernatant was transferred into a sterile microcentrifuge tube and 165 µL of Oligotex suspension was added to the sample and mixed by vortexing. The mixture was incubated at room temperature for 10 minutes and centrifuged for 5 minutes to pellet the Oligotex: mRNA complex. The supernatant was discarded. Oligotex: mRNA pellet was then resuspended in 200 µL of buffer OL1 containing 0.42 M β-ME and mixed by vortexing. Then, 400 µL of buffer ODB was added to the sample and incubated at 70°C for 3 minutes and placed at room temperature for 10 minutes. Oligotex: mRNA complex was then pelleted by centrifugation for 5 minutes. The supernatant was discarded and the pellet was resuspended in 400 µL of buffer OW1 by vortexing. The lysate was then transferred into a small spin column placed in a sterile microcentrifuge tube and centrifuged for 1 minute. The flow-through was discarded. The spin column was centrifuged again for 1 minute and the flow-through was discarded. Then, 600 µL of buffer OW2 was added on to the spin column and centrifuged for 1 minute in a new sterile microcentrifuge tube. The flow-
through was discarded. The spin column was centrifuged again for 1 minute and the flow-through was discarded. The spin column was then transferred into a new RNase-free microcentrifuge tube and 200 µL of hot (70°C) buffer OEB was loaded on to the column. The resin was resuspended in buffer OEB by pipetting and centrifuged for 1 minute to elute mRNA. The concentration of the collected mRNA sample was measured using a Thermo Fisher Scientific® NanoDrop™ 1000 spectrophotometer.

2.2.2.3 Reverse transcription polymerase chain reaction (RT-PCR) amplification of Gal d 6

The RT-PCR amplification of Gal d 6 was performed using OneStep RT-PCR kit (QIAGEN, Hilden, Germany) according to manufacturer’s instructions. Oligonucleotide primers used for amplification of Gal d 6 gene were based on the sequence published for VTG-1 precursor in Genbank (Genbank accession number: D89547). The sequence that code for Gal d 6 of the VTG-1 gene was amplified with oligonucleotide forward primer 5’-CGCGGATCCCTGAAATAGCTTCACAAATGCAC-3’ and oligonucleotide reverse primer 5’-CGCGAATTCTTAACTACAGTCACCAGTGCAG-3’. *Bam*HI and *Eco*RI restriction sites were incorporated into 5’ ends of the forward and reverse primers, respectively for cloning (restriction sites underlined in the primer sequences). Furthermore, a 5’ CGC extension sequence was added to each primer in order to increase the cleavage efficiency of the restriction enzymes. The RT-PCR master mix was prepared by mixing 10 µL of 5× QIAGEN One-Step RT-PCR buffer, 2 µL of 10 mM dNTP mix, 10 µL of 5× Q-solution, 1 µL of 1 µM forward primer, 1 µL of 1 µM reverse primer, QIAGEN One-Step RT-PCR enzyme mix and 170.82 ng of mRNA in a 50 µL total reaction volume in a sterile PCR tube. The thermal cycler conditions used for RT step were 30 minutes at 50°C
for RT and 15 minutes at 95°C for activation of HotStart DNA polymerase. The parameters used for the 3 step PCR cycling step were 35 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 53°C, 2 minute extension at 72°C and a final 10 minute extension at 72°C.

2.2.2.4 Analysis and gel purification of PCR amplified Gal d 6 gene

The PCR product was electrophoretically analysed on 1% (W/V) agarose gel stained with GelRed™ nucleic acid stain (Biotium, Hayward, CA, USA). The electrophoresis was performed at 100 V for 45 minutes. Following electrophoresis, the PCR product was purified using MinElute Gel Extraction kit (QIAGEN, Hilden, Germany) according to manufacturer’s instructions. The gel section containing the PCR fragment was excised using a sterile scalpel and weighed. 3 volumes of buffer QG was added to 1 volume of gel (100 mg gel ~ 100 µL). The tube was incubated at 50°C for 10 minutes to dissolve the gel slice. Then, 1 gel volume of isopropanol was added to the sample and mixed. The sample was applied to a MinElute column placed in a 2 mL collection tube and centrifuged for 1 minute. The flow-through was discarded. 500 µL of buffer QG was added to the tube and centrifuged for 1 minute. The flow-through was discarded. Then, 750 µL of Buffer PE was added to the tube and allowed to stand for 5 minutes prior centrifugation for 1 minute to wash. The flow-through was discarded and the tube was centrifuged again for 1 minute to remove residual wash buffer. The column was placed in a clean microcentrifuge tube. Then, 50 µL of buffer EB was added to the centre of the MinElute column and allowed to stand for 1 minute. The DNA was then eluted by centrifuging for 1 minute. The concentration of gel purified PCR sample was measured using a NanoDrop™ spectrophotometer and stored at -20°C until needed.
2.2.2.5 Synthesis of pTrcHis A-Gal d 5 construct

The restriction enzyme digestion of PCR amplified Gal d 6 and pTrcHis A plasmid was conducted according to New England Biolabs (NEB) Time-Saver™ protocol. 1 μg of DNA was digested with 1 μL of BamHI, 1 μL EcoRI and 5 μL of 10× NEBuffer 3.1 in a 50 μL total reaction volume. As a negative control, 1 μg of pTrcHis A was mixed with 5 μL of 10× NEBuffer 3.1 in a 50 μL total reaction volume without restrictions enzymes. All samples were then incubated at 37°C for 1 hour. Then, all three samples were electrophoretically analysed on 1% (W/V) agarose gel stained with GelRed™ nucleic acid stain. The DNA samples were gel purified using QIAquick Gel Extraction kit as mentioned in Section 2.2.2.3. The concentrations of gel purified DNA samples were measured using a NanoDrop™ spectrophotometer and stored at -20°C until needed.

The ligation reaction was performed under standard conditions [23]. Equal amounts (~30 ng) of digested Gal d 6 gene and linearized pTrcHis A were ligated with 1 μL of T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and 10× T4 DNA ligase reaction buffer (New England Biolabs, Ipswich, MA, USA) in a 10 μL total reaction volume. As a negative control, approximately 30 ng of undigested pTrcHis A plasmid vector was mixed with T4 DNA ligase and 10× T4 DNA ligase buffer in a 10 μL total reaction volume. Both samples were then incubated overnight at 4°C.

2.2.2.6 Chemical transformation of E. coli

The ligation reaction was chemically transformed into competent E. coli according to NEB high efficiency transformation protocol. A tube of chemically competent E. coli cells was thawed on ice for 10 minutes. 5 μL of ligation reaction was added to the cell mixture and mixed by flicking the tube 5 times. The tube was placed on ice for 30 minutes. Then, heat shock was performed at 42°C for 20 seconds. The tube was
again placed on ice for 5 minutes. Then, 950 µL of room temperature super optimal broth with catabolite repression (SOC) media was added to the tube and incubated at 37°C with shaking at 250 rpm for 1 hour. 100 µL and 200 µL of transformation mixture were plated on LB agar and incubated overnight at 37°C.

2.2.2.7 Growth of transformant colonies and plasmid isolation

Five individual colonies from the overnight transformation plates were picked using sterile pipette tips and inoculated into fresh LB and allowed to grow overnight at 37°C with shaking at 250 rpm. Then, plasmid isolation from overnight bacterial cultures was performed using QIAprep Spin Miniprep kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. All centrifugation steps were performed at 13, 300 rpm at room temperature. 1.5 mL of overnight bacterial culture was centrifuged for 3 minutes and the supernatant was discarded. Then 250 µL of buffer P2 was added to the suspension and mixed by inverting the tube. 350 µL of buffer N3 was added to the cell suspension and mixed by inverting the tube. The tube was then centrifuged for 10 minutes. Then, 800 µL of the supernatant was added to the QIAprep 2.0 spin column and centrifuged for 1 minute. The flow-through was discarded. The spin column was washed by adding 750 µL of buffer PE and centrifuging for 1 minute. The flow-through was discarded and the spin column was centrifuged for 1 minute to remove residual wash buffer. The spin column was then placed in a new microcentrifuge tube and 50 µL of buffer EB was applied to the centre of the column and allowed to stand for 1 minute, prior centrifuging for 1 minute to elute DNA. The concentrations of isolated plasmid DNA samples were measured using a NanoDrop™ spectrophotometer and stored at -20°C.
2.2.2.8 Restriction enzyme digest screening for colonies harbouring the pTrcHis A-Gal d 5 construct

The restriction enzyme digestions of isolated plasmids were performed according to the NEB Time-Saver™ protocol (Section 2.2.2.5) using restriction enzymes *Bam*HI and *Eco*RI. All digested samples were electrophoretically analysed on 1% (W/V) agarose gel stained with GelRed™ nucleic acid stain. For all the positive clones, glycerol stocks were made by mixing 0.5 mL overnight bacterial culture with 0.5 mL of sterile 80% (v/v) glycerol and stored at -80°C until needed. The plasmids harbouring the insert were then sequenced using pTrcHis A forward primer 5’-GAGGTATATATTAATGTATCG-3’ and pTrcHis A reverse primer 5’-GATTTAATCTGTATCAGG-3’ (Micromon-Monash University Clayton, VIC, Australia). The DNA sequences were then compared against the nucleotide sequence database of National Centre for Biotechnology Information (NCBI) web site using the nucleotide Basic Local Alignment Search Tool (BLAST).

2.2.2.9 Time course of expression and analysis of recombinant protein expression levels

The selected transformant clone was streaked for single colonies on LB agar media from the glycerol stock and incubated overnight at 37°C. Following incubation, a single colony was picked using a sterile pipette tip and inoculated into 10 mL of LB media and allowed to grow overnight at 37°C with shaking at 250 rpm. The overnight culture was then re-inoculated into fresh LB media and allowed to grow at 37°C with shaking at 250 rpm until optical density reached 0.4 - 0.6 at 600 nm. Then, expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to give a final concentration of 1 mM. The culture was allowed to grow for 5 hours and cell pellets were collected every hour by centrifugation.
All cell pellets were resuspended in 400 µL of CellLytic™ B bacterial cell lysis reagent (Sigma-Aldrich, MO, USA) and briefly vortexed. The resultant cell lysate was mixed using a tube mixer for 10 minutes. The cell lysate was then centrifuged at 13,000 rpm for 5 minutes to separate soluble and insoluble fractions. The insoluble fractions were further resuspended in 100 µL of Novex® Tris-Glycine SDS-Sample Buffer (2×). The expression levels were examined on SDS-PAGE gels according to the protocol mentioned in Section 2.2.1.3. Un-induced culture fractions were run as controls. The authenticity of the recombinant protein was confirmed by Western immunoblotting. Separated proteins were electro-transferred on to a nitrocellulose membrane and developed according to the protocol mentioned in Section 2.2.1.4.1 with the following changes. Monoclonal Anti-Xpress™ antibody produced in mouse (Thermo Fisher Scientific®, MA, USA) (diluted 1:1000 in blocking solution) was used as the primary antibody while alkaline phosphatase-conjugated anti mouse IgG antibody (Thermo Fisher Scientific®, MA, USA) was used as the secondary antibody.

### 2.2.2.10 Purification of rGal d 6

rGal d 6 fused to a 6×His affinity tag was purified using Ni²⁺-Nitrilotriacetic acid (Ni-NTA) resin using Ni-NTA spin kit (Qiagen, Hilden, Germany) under native conditions according to manufacturer’s instructions. In brief, a pellet derived from a 5 mL cell culture volume was resuspended in 630 µL of lysis buffer (50mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). After centrifugation, the cleared lysate containing 6×His tagged rGal d 6 protein was loaded on to the Ni-NTA spin column pre-equilibrated with lysis buffer. The resin was then washed thrice with 600 µL wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). The recombinant protein was eluted using 200 µL of elution buffer (50mM
NaH$_2$PO$_4$, 300 mM NaCl, 500 mM imidazole, pH 8.0). The collected fractions were then analysed on SDS-PAGE and Western immunoblotting according to the protocol mentioned in Section 2.2.2.9.

2.2.3 Immunological analysis of rGal d 6

2.2.3.1 Inhibition enzyme-linked immunosorbent assay (ELISA)

In brief, 10 µg of crude egg yolk in 50 µL of coating buffer (60 mM NaHCO$_3$, 30 mM Na$_2$CO$_3$, pH 9.3) was used to coat each well of an ELISA microtiter plate. The proteins were allowed to coat the wells for 2 hours at 37°C. Each sample was done in triplicates. After triple washing the wells with 0.05% phosphate buffered saline with Tween 20 (PBST), each well was blocked with 50 µL of 1% (w/v) bovine serum albumin (BSA) in 1× PBS for 2 hours at 37°C. The serum pool from patients 3, 5, 10, 16 and 25 were used since they had the highest reactivity against Gal d 6 and due to the limited availability of sera. The wells were triple washed with 0.05% PBST. Then, all wells were incubated with 50 µL control serum pool (diluted 1:10 in 0.05% BSA in 1× PBS) and 50 µL serum pools which were pre-incubated with different amounts (10 µg and 50 µg) of rGal d 6 for 1 hour at room temperature. Following triple washing with 0.05% PBST, all wells were incubated with 50 µL of monoclonal anti-human IgE mouse antibodies labelled with alkaline phosphatase (diluted 1:2000 in 0.05% BSA in 1× PBS) for 1 hour at 37°C. After triple washing the wells with 0.05% PBST, 50 µL of alkaline phosphatase yellow (pNPP) liquid substrate (Sigma-Aldrich, MO, USA) was added to each well and incubated at 37°C. After the colour development, the reaction was terminated by adding 13 µL of 3 M NaOH, and absorbance was read at 405 nm. Percentage of inhibition was calculated using the following formula.
% Inhibition = 100 – (absorbance value with inhibitor/absorbance value without inhibitor × 100)

A second ELISA was also done using CEY to inhibit IgE binding to coated rGal d 6 according to the same method described above. Wells of the microtiter plate was coated with 10 µg of rGal d 6 in 50 µL of coating buffer. The test serum pool was pre-incubated with 10 µg or 50 µg of CEY. The absorbance was measured at 405 nm and the percentage of inhibition was calculated.

2.3 Results

2.3.1 Immunological and mass spectrometry analysis of CEY fractions from hen, emu and quail eggs

The complexity of CEY profiles of different bird species is evident as seen on SDS-PAGE (Figure 2.1). The resulting protein profiles of various CEYs show a large number of protein bands. Although there are some overlapping bands, most proteins could be clearly distinguished. Approximately 13, 7 and 11 different proteins ranging from 10-250 kDa could be distinguished in the hen (Figure 2.1, A), emu (Figure 2.1, B) and quail (Figure 2.1, C) egg yolks, respectively. No differences were observed for the protein profiles generated from caged chicken eggs, free-range chicken eggs and hen’s eggs obtained from AAHL. However, a high molecular weight protein just under 250 kDa marker can be observed in the hen’s egg yolk from free range eggs. This high molecular weight protein was absent in both the caged eggs and eggs obtained from AAHL. Considerable number of proteins from all three avian species had similar migration levels on the SDS-PAGE indicating potential homology among proteins. Nevertheless, all three egg yolk samples from different bird species were clearly distinguishable from each other based on their protein profiles on SDS-PAGE.
Western immunoassay performed with individual patients’ sera (Figure 2.2) against hen’s CEY extract revealed multiple IgE reactive proteins. The repeated immunoassay also reproduced the same result. 56% (14 out of 25) of the egg white allergy patients tested in this study had IgE reactivity to multiple egg yolk proteins with varying degrees of intensities. A second Western immunoblot was conducted with a pool of sera composed of patients seropositive for hen’s egg yolk against CEYs prepared from all bird eggs (Figure 2.3). The repeated immunoassay also reproduced the same result. Immunoblots conducted with pooled sera against hen, emu and quail CEYs showed multiple IgE reactive proteins. IgE reactive proteins from various CEYs were assigned with an identification code. The serum pool
detected seven different proteins (P1 to P7) (Figure 2.3, A) from hen’s CEY, which were also detected in the Western blot performed with individual patients’ sera (Figure 2.2). When analysing the immunoblots of hen’s CEY, it was clear that not all hen’s egg yolk proteins had genuine IgE reactivity (Figure 2.2 and Figure 2.3, A). Hen’s egg yolk proteins P6 and P7 had reactivity in negative control no serum and non-allergic serum lanes indicating non-specific binding with the secondary antibody. Furthermore, all patient serum lanes also had reactivity with P6 and P7. This result indicates that reactivity of proteins P6 and P7 in patients are due to non-specific binding with the detection antibody and not due to genuine sensitisation. In this study, proteins P6 and P7 were not considered as proteins with potential allergenic capacity. As a result, patients 6, 12, 13, 20 and 21 that have only reacted with proteins P6 and P7 were considered non-sensitised towards CEY. Therefore, the percentage of patients with genuine IgE-reactivity to hen’s CEY is 36% (9 out of 25) after the exclusion of patients with false positives. The percentage of patients reacted to each protein is shown in Figure 2.4. Table 2.2 shows the IgE binding data of hen’s egg yolk sensitised patients.

Emu CEY showed six IgE reactive proteins (X1 to X6) (Figure 2.3, B). Quail CEY showed five IgE reactive proteins (Y1 to Y5) (Figure 2.3, C). The band intensities of IgE reactive proteins from hen’s CEYs were very similar. However, in CEY from caged chicken eggs, the protein P5 had slightly higher IgE-reactivity when compared to CEYs from AAHL and free-range eggs.
Figure 2.2: Western immunoassay conducted using individual patients’ sera against hen’s CEY. Lanes 1-25: CEY immobilised nitrocellulose strip 1 to 25 incubated with patient serum 1 to 25, respectively, Lanes 26-29: CEY immobilised nitrocellulose strips incubated with sera of non-allergic subjects, N: Negative control strips incubated with secondary antibody only, MW: molecular weight marker in kDa (SeeBlue pre-stained protein standard).

Figure 2.3: Western immunoblots conducted using pooled patients’ sera against all CEYs. (A) Immunoblot of hen’s CEYs. Lane 1: CEY from chicken eggs obtained from AAHL, Lane 2: CEY from caged chicken eggs, Lane 3: CEY from free-range chicken eggs. (B) Immunoblot of CEY from emu eggs. (C) Immunoblot of CEY from quail eggs. MW: molecular weight marker in kDa (SeeBlue pre-stained protein standard). Arrows indicate IgE reactive egg yolk proteins.
Table 2.2: Qualitative IgE binding data from Western immunoassay based on Figure 2.2. The type of egg yolk proteins each patient had reacted with and the degree of intensity with their corresponding clinical data. + mild reactivity, ++ moderate reactivity, +++ very strong reactivity.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>IgE level (IU/mL)</th>
<th>Blot IgE sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Egg white</td>
<td>Rye</td>
</tr>
<tr>
<td>1</td>
<td>93.50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13.60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>13.20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8.63</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>16.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4.13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.02</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.21</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.32</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.72</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2.08</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.19</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>1.25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient number</td>
<td>IgE level (IU/mL)</td>
<td>Blot IgE sensitivity</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td></td>
<td>Egg white</td>
<td>Rye</td>
</tr>
<tr>
<td>25</td>
<td>0.47</td>
<td>88.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.4: Percentage of patients with IgE reactivity to proteins (P1 to P7) of hen’s egg yolk, as analysed from the Western immunoblot in Figure 2.2 and Table 2.2.

In order to determine the identities of the IgE reactive proteins of various CEYs, they were subjected to proteomic analysis using LC-MS/MS. The identity of each protein band was determined from critically analysing both the mass spectrometry data and molecular weights of each protein based on SDS-PAGE. The identities revealed for all the IgE reactive proteins were previously known hen’s egg yolk proteins. The identities of proteins analysed from the LC-MS/MS and their approximate molecular weights on SDS-PAGE are listed in the Table 2.3. The mass spectrometry result confirmed that all the IgE reactive proteins of hen’s CEYs are fragments derived
from well-known precursor proteins of the hen; VTG-1, VTG-2 and Apo B. The proteomic analysis of hen’s egg yolk proteins identified P4 as Gal d 5 and P5 as Gal d 6 (Table 2.3). According to the Western immunoassay conducted with individual patients’ sera 16% of the patients tested have recognized Gal d 5 (P4) while 28% of subjects have recognized Gal d 6 (P5) (Table 2.2). The degree of IgE reactivity in both allergens ranged from very strong to mild in sensitised patients. The identities of IgE reactive proteins from emu and quail CEYs are listed in Table 2.3. Here also majority of the proteins were found to be fragments of precursor proteins as in the IgE reactive hen’s egg yolk proteins. However, the mascot BIRD database is not complete with all the bird species. Therefore, it was clear that the majority of emu and quail proteins had matches to proteins with close homologies from other bird species such as chicken, duck and turkey. Some of the IgE-reactive proteins in the emu and quail egg yolks came up as egg white proteins such as ovalbumin and ovotransferrin indicating minor contamination with the egg white. However, mass spectrometry analysis did not reveal any contamination with human proteins.
Table 2.3: Protein identities revealed from mass spectrometry analysis, their approximate molecular weights on SDS-PAGE, score and percentage sequence coverage for each protein

<table>
<thead>
<tr>
<th>Protein band</th>
<th>Molecular weight</th>
<th>Identity</th>
<th>Species</th>
<th>Score</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>120</td>
<td>Vitellogenin II</td>
<td>Chicken</td>
<td>13575</td>
<td>52</td>
</tr>
<tr>
<td>P2</td>
<td>90</td>
<td>Apolipoprotein B</td>
<td>Chicken</td>
<td>8412</td>
<td>30</td>
</tr>
<tr>
<td>P3</td>
<td>72</td>
<td>Apolipoprotein B</td>
<td>Chicken</td>
<td>523037</td>
<td>30</td>
</tr>
<tr>
<td>P4 (Gal d 5)</td>
<td>65</td>
<td>Chicken serum albumin</td>
<td>Chicken</td>
<td>3490</td>
<td>54</td>
</tr>
<tr>
<td>P5 (Gal d 6)</td>
<td>42</td>
<td>Vitellogenin I</td>
<td>Chicken</td>
<td>5438</td>
<td>35</td>
</tr>
<tr>
<td>P6</td>
<td>35</td>
<td>Vitellogenin I</td>
<td>Chicken</td>
<td>5500</td>
<td>28</td>
</tr>
<tr>
<td>P7</td>
<td>30</td>
<td>Vitellogenin II</td>
<td>Chicken</td>
<td>5294</td>
<td>37</td>
</tr>
<tr>
<td>X1</td>
<td>120</td>
<td>Apolipoprotein B</td>
<td>Chicken</td>
<td>524</td>
<td>14</td>
</tr>
<tr>
<td>X2</td>
<td>72</td>
<td>Ovotransferrin</td>
<td>Emu</td>
<td>943</td>
<td>46</td>
</tr>
<tr>
<td>X3</td>
<td>65</td>
<td>Ovalbumin</td>
<td>Emu</td>
<td>455</td>
<td>21</td>
</tr>
<tr>
<td>X4</td>
<td>52</td>
<td>Apolipoprotein B</td>
<td>Chicken</td>
<td>723</td>
<td>15</td>
</tr>
<tr>
<td>X5</td>
<td>42</td>
<td>Fibrinogen beta chain</td>
<td>Zebra finch</td>
<td>520</td>
<td>16</td>
</tr>
<tr>
<td>X6</td>
<td>35</td>
<td>Vitellogenin II variant 1</td>
<td>Emu</td>
<td>548</td>
<td>14</td>
</tr>
<tr>
<td>Y1</td>
<td>90</td>
<td>Transferrin receptor protein I-like</td>
<td>Turkey</td>
<td>574</td>
<td>13</td>
</tr>
<tr>
<td>Y2</td>
<td>68</td>
<td>Serum albumin</td>
<td>Domestic duck</td>
<td>2971</td>
<td>29</td>
</tr>
<tr>
<td>Y3</td>
<td>42</td>
<td>Ovalbumin</td>
<td>Common quail</td>
<td>1111</td>
<td>43</td>
</tr>
<tr>
<td>Y4</td>
<td>34</td>
<td>Apolipoprotein A-I</td>
<td>Japanese quail</td>
<td>795</td>
<td>70</td>
</tr>
<tr>
<td>Y5</td>
<td>31</td>
<td>Apolipoprotein A-I</td>
<td>Japanese quail</td>
<td>2298</td>
<td>86</td>
</tr>
</tbody>
</table>
2.3.2 Expression of rGal d 6 in *E. coli*

Analysis of RT-PCR product on 1% (W/V) agarose gel showed a predominant band approximately in between 500 bp and 1000 bp markers (expected size: 876 bp) (Figure 2.5). *E. coli* cells transformed with pTrcHis A-Gal d 6 construct had growth when incubated on LB agar under ampicillin resistance. When *Bam*HI and *Eco*RI digested plasmids of transformant clones were electrophoresed on 1% (W/V) agarose gel, all clones showed the insert approximately at 1000 bp marker (expected size: 876 bp) (Figure 2.6). Sequencing analysis of plasmids harbouring the insert confirmed the in-frame ligation of the Gal d 6 into the pTrcHis A vector within the multiple cloning site (MCS). The NCBI BLAST search of the derived sequence from pTrcHis A-Gal d 6 construct produced *Gallus gallus* vitellogenin 1 (VTG1) (sequence ID: ref\_NM_0010044008.2) as the top match with a 99% similarity corresponding to the mature peptide YGP42 (Gal d 6) of the C-terminal part of the VTG-1 gene (Figure 2.7).
Figure 2.5: RT-PCR of Gal d 6 gene. The RT-PCR product was electrophoresed on a 1% (W/V) agarose gel. Lane 1: RT-PCR product of Gal d 6 gene. Lane 2: negative control RT-PCR without template mRNA. MW, molecular weight marker GeneRuler 1kb Plus DNA ladder in bp. Arrow indicates the RT-PCR amplified Gal d 6 gene.

Figure 2.6: Restriction enzyme digest screening for E. coli colonies harbouring pTrcHis A-Gal d 6 construct. The digested plasmids were electrophoresed on a 1% (W/V) agarose gel. Lanes 1-5: BamHI and EcoRI digested plasmids of five individual E. coli colonies grown on transformation plate with antibiotic. MW: molecular weight marker GeneRuler 1kb Plus DNA ladder in bp. Arrow indicates the Gal d 6 gene.
Figure 2.7: Sequence alignment comparison of DNA sequence derived for pTrcHis A-Gal d 6 construct using NCBI Nucleotide BLAST tool. The DNA sequence represents the mature peptide YGP42 (Gal d 6) of the C-terminal part of the VTG-1 gene.
The Western immunoblot of time-course of expression experiment showed a predominant protein band approximately at 42 kDa region when probed with Anti-Xpress antibody. *E. coli* cells harbouring the construct have expressed majority of the rGal d 6 as an insoluble fraction. However, a reasonable amount of soluble rGal d 6 was also expressed by the *E. coli* cells, with an optimum time of 5-hour post IPTG induction (Figure 2.8).

The production of a recombinant protein fused to a short polypeptide such as 6×His tag permits the purification of the fusion peptide using a metal chelating resin (Figure 2.9). The SDS-PAGE analysis (Figure 2.9, A) of the purified soluble rGal d 6 using the Ni-NTA resin revealed a predominant band approximately at 42 kDa region. However, several *E. coli* derived proteins were also present in the purified fraction. The Western immunoblot of purified protein fraction probed with Anti-Xpress antibody showed a clear band approximately at 42 kDa region corresponding to the protein band in SDS-PAGE (Figure 2.9, B).
Figure 2.8: Time-course of expression of soluble and insoluble *E. coli* cell culture fractions harbouring rGal d 6. Lane 1: un-induced culture fractions. Lanes 2-5: IPTG induced culture fractions at time points 2nd, 3rd, 4th and 5th hours, respectively, analysed by (A) SDS-PAGE and (B) Western immunoassay. Arrows indicate rGal d 6 in the soluble fraction at 42 kDa. MW: molecular weight marker in kDa (SeeBlue pre-stained protein standard).

Figure 2.9: Purification of 6×His tagged rGal d 6. Lane 1: cleared lysate, Lane 2: flow-through, Lane 3-5: 1st, 2nd and 3rd wash, respectively. Lane 6: elution, analysed by (A) SDS-PAGE and (B) Western immunoassay. MW: molecular weight marker in kDa (SeeBlue pre-stained protein standard). Arrows indicate purified rGal d 6 at 42 kDa.
2.3.3 Inhibition ELISA experiments

The results of inhibition ELISA experiments are shown in Figure 2.10. The results shown in Figure 2.10 demonstrate that rGal d 6 is able to inhibit IgE binding to CEY by up to 30%. The amount of rGal d 6 required to achieve maximum IgE binding inhibition of 30% was 50 μg. On the other hand, CEY was able to inhibit IgE binding to rGal d 6 by up to 31% as shown in Figure 2.10. Further, inhibitions with increasing amounts of proteins were not achieved in both cases. The amount of CEY required to achieve maximum IgE binding inhibition of 31% was 50 μg. However, inhibitions with increasing amounts of proteins reached a plateau at 50 μg in both cases. This experiment was only conducted once due to the limited availability of sera.

Figure 2.10: Inhibition ELISA experiments. When rGal d 6 was used as an inhibitor, CEY was immobilised on to the wells of the microtiter plate. When CEY was used as an inhibitor, rGal d 6 was immobilised on to the wells of the microtiter plate. The IgE antibody binding of selected seven patients reactive towards CEY proteins was detected calorimetrically at OD 405 nm.
2.4 Discussion

Hen’s egg is one of the most well-known sources of allergens. Egg white allergens have much stronger allergenic potential than the yolk proteins. Hen’s egg yolk allergy is still a developing area within the domain of food allergy. Allergy to egg white and egg yolk has been often investigated separately. However, concomitant sensitisation to egg white and yolk proteins due to cross reactive proteins has been reported previously [98]. Nevertheless, the association between egg white and egg yolk allergies have been overlooked by many researchers. Egg white and egg yolk allergens have a similar origin and therefore, occurrence of cross sensitisation or co-sensitisation in egg allergic patients would be a high possibility.

CEY extracts were made according to the method reported by Guilmineau et al., [127]. The protein profiles of CEYs were investigated on SDS-PAGE without delipidation. Most studies have used delipidated egg yolk fractions in order to reduce the smearing effect caused by the high lipid content. Chemicals used during delipidation process can significantly alter the nature of proteins and thereby affecting the result of immunoassays. Therefore, we used the whole egg yolk extract without subjecting to delipidation throughout the study. The SDS-PAGE profile of the un-delipidated CEYs obtained offered good resolution of protein bands, indicating that most of the egg yolk proteins have been solubilized in the 0.1 M glycine/NaOH buffer (pH 9; 0.56 NaCl). An important advantage of the approach used here is that egg yolk proteins are presented in the same format as would be encountered by an individual during the sensitisation process. The egg yolk allergens Gal d 5 and Gal d 6 are both glycoproteins, which were identified using fractioned yolk extracts [81, 82]. In theory, any protein can act as an IgE reactive allergen.
Therefore, it is imperative to bring the whole egg yolk under investigation to reveal allergenicity of other yolk proteins.

The protein profiles generated from caged chicken eggs, free-range chicken eggs and hen’s eggs obtained from AAHL were similar. However, a high molecular weight protein just under 250 kDa marker was observed in the hen’s egg yolk from free range eggs. This could be a marker of free-range condition. However, further proteomic analyses are required to investigate if the presence of high molecular weight protein in free-range eggs is due to the environmental factors. In this study, we employed Western immunoblotting as a rapid method of identifying IgE reactive proteins in various CEYs. The sera we used in this study came from a cohort of patients diagnosed with allergy to hen’s egg white. However, no clinical data were available regarding sensitisation or allergic reactivity to hen’s egg yolk in those patients. Therefore, these patients may or may not have clinical reactivity to hen’s egg yolk. First major aim of this chapter was to investigate the sensitisation profiles of egg white allergic patients and compare in vitro IgE reactivates of egg yolks from hen, emu and quail. Sensitisation to a certain allergen does not necessarily imply clinical allergy [3, 7]. However, it is well known that majority of patients with sensitisation develops clinical symptoms upon ingestion of the offending food. Therefore, the major epitopes relevant to sensitisation are of great interest.

Western immunoassay results showed that multiple egg yolk proteins from hen, emu and quail have reacted with IgE antibodies of many patients. Sensitisation to egg yolk proteins could be a result of direct exposure to yolk proteins during consumption or due to the existence of similar IgE binding epitopes present in egg white and yolk proteins. In such cases, both types of allergens are responsible for overall pathogenesis and pathophysiology of egg allergy. Therefore, it is imperative
that clinical diagnosis of egg allergy include both the egg white and egg yolk and determine appropriate management and treatment strategies based on a patient’s total sensitisation profile. However, proteins P6 and P7 were considered as false positives due to the influence of the secondary antibodies. A possible reason behind this could be the presence of hen’s egg proteins in the diet fed to mouse. As a consequence the mouse immune system may have generated IgG antibodies against egg yolk proteins P6 and P7. Production of IgG antibodies against non-pathogenic proteins is considered as a normal process of a healthy immune system [22]. However, it is not possible to elucidate why the mouse immune system only produced IgG antibodies against only those two egg yolk proteins.

Furthermore, sensitised patients against hen’s CEY were also able to recognize a number of proteins from the yolk proteomes of emu and quail. Here also, cross sensitisation to egg yolk proteins from different bird species could be a result of direct exposure to respective bird eggs or due to the existence of similar IgE binding epitopes among different egg proteins. If sensitisation is due to cross-reactive IgE antibodies, allergic reactions to emu and quail egg proteins can occur during the first exposure even without the initial sensitisation phase. Therefore, for such patients, emu and quail eggs may not be suitable to use as an alternative to hen’s eggs.

Sensitisation to certain allergens is not a definitive marker of allergy. This is because not all sensitised individuals develop clinical symptoms of allergic hypersensitivity when exposed to relevant allergens. Therefore, this result does not confirm allergy to egg yolk in patients identified with sensitisation to egg yolk proteins. Nevertheless, presence of reactive antibodies is considered a risk factor for allergy. Furthermore, it has been shown that sensitisation to allergens can be used to predict the onset of clinical allergy. For example, presence of hen’s egg white specific IgE antibodies
even without clinical symptoms can be used to predict the development of allergy before 7-10 years of age [3].

IgE reactive egg yolk proteins from all CEYs were subjected to proteomic analysis in order to reveal their identities. According to the mass spectrometry results, all IgE reactive hen’s egg yolk proteins belongs to major precursor protein families VTG-1, VTG-2 and apolipoprotein B (Apo B). VTG-1 precursor is already implicated in allergy. The allergen Gal d 6 is the C-terminal part of VTG-1 precursor [81]. Already known hen’s egg yolk allergens Gal d 5 and Gal d 6 were able to identify through proteomic analysis. Gal d 5 is a protein with a molecular weight of 69 kDa. However, on the SDS-PAGE analysis it was seen approximately at 65 kDa region. This may be due to the limited solubility resulted by the presence of high lipid content or due to the alteration of three dimensional structure caused by method used for CEY preparation.

Furthermore, Walsh et al., reported apovitellenin I and VI to be significantly allergenic in some patients with egg allergy. Therefore, this result is consistent with our result since apovitellenin I is a fragment derived from Apo B [98]. Both VTG-1 and VTG-2 are collectively known as vitellogenins. During egg formation, vitellogenins are transported into developing oocyte through a process known as receptor mediator endocytosis. Once they are transported into the egg yolk, mature proteins are produced as a result of enzymatic cleavage. On the other hand, Apo B is also produced in liver and transported into growing oocytes the same way as vitellogenins, resulting in the formation of mature Apo B fragments through enzymatic activity [96, 128, 129]. The MASCOT bird database is not complete with proteins from all bird species. Therefore, most proteins from emu and quail had matches to proteins with closest homologies from other bird species such as hen.
Inability to identify the exact mature proteins derived from vitellogenins or Apo B precursors is a limitation of this study. Egg yolk is a more complex system than the albumen, with a significant proportion of lipids. The presence of high amounts of lipids has made identification of allergens from the egg yolk very challenging and may have thus contributed to fewer studies reported in the literature. Nevertheless, this study had narrowed down the search for allergens out of hundreds of different proteins for just three classes of precursor proteins. In order to identify IgE reactive proteins, each mature protein derived from precursors should be purified and subjected to immunological testing using sera from patients allergic to hen’s egg. Furthermore, we performed the immunological analysis of egg yolk using sera from patients allergic to hen’s egg white. This also can be considered as another limitation of this study since clinical reactivity to yolk proteins may not exist in those patients, even though sensitisation is present. Therefore, in order to establish the clinical relevance of those three egg yolk precursor proteins, it is imperative to subject those proteins to both in vivo and in vitro studies using patients with clinical reactivity to hen’s egg yolk.

Comparison of immunoblot results with the patients’ clinical data showed that the majority of patients that reacted strongly with the egg yolk had high IgE levels against hen’s egg white. However, this was not always the case since some patients with low IgE also had strong reactivity against egg yolk proteins, suggesting presence of clinically unique allergens in the egg yolk. In this study, more patients had reacted with Gal d 6 (28%) than Gal d 5 (16%). It was previously suggested that Gal d 6 is a minor allergen implicated in egg allergy. However, our results indicate that Gal d 6 may be more allergenic and affects more patients than originally thought.
The cDNA coding for the mature egg yolk allergen Gal d 6 was selectively amplified from the VTG-1 precursor using liver tissue from an egg-laying hen. The sequencing result obtained for Gal d 6 gene showed several insertion mutations, especially at the end of the sequence. However, this does not affect the validity of the construct. These errors are mainly attributed to the introduction of artefactual mutations, rather than due to true mutations resulted during the PCR amplification. The Gal d 6 was cloned into pTrcHis A plasmid vector where the trc promoter leads the translation of the fusion peptide with the 6×His tag on the N-terminal side. The soluble and insoluble fractions of IPTG induced and un-induced E. coli fractions were subjected to Western immunoassay and probed with anti-Xpress antibody specific to the fusion peptide. The developed immunoblot revealed a clear band at 42 kDa, which is the expected size of natural Gal d 6.

This result is in excellent agreement with studies conducted by Mann & Mann., and Yamamura et al., where they reported the size of natural Gal d 6 as 42 kDa [96, 130]. Furthermore, our proteomic analysis of CEY proteins also indicated that the protein P5 approximately at 42 kDa region to be YGP42 or Gal d 6. However, the theoretical molecular weight of Gal d 6 is 31.45 kDa as calculated by ExPASy ProtParam tool. On the other hand, the study by Amo et al., which confirmed YGP42 as an allergen, reported a molecular weight of 35 kDa from SDS-PAGE [81]. The discrepancies observed regarding the molecular weight on SDS-PAGE could be attributed to the different sample preparation methods used.

The solubility of recombinant proteins produced by bacteria is a major concern in the production process. Although bacterial expression systems provide a convenient way for recombinant protein expression, many foreign proteins expressed in bacterial hosts undergo partial or irregular folding resulting in insoluble and refractile
aggregates known as inclusion bodies (IB) [131]. Therefore, to retain the biomedical value, it is essential to produce the recombinant protein as a soluble fraction in its native form. However, it is not possible to predict the solubility of a foreign protein before the actual expression [131, 132]. In this study, majority of the rGal d 6 has been produced as an insoluble protein as seen by the Western immunoassay result. However, there were reasonable amount of soluble rGal d 6 produced by the E. coli cells that could be used for purification and other downstream applications. Therefore, enhancement of bacterial cells to increase production of rGal d 6 as a soluble fraction was not necessary.

The production of a recombinant protein fused to a short polypeptide such as 6×His tag permits the purification of the fusion peptide using a metal chelating resin. The SDS-PAGE analysis of the purified soluble rGal d 6 using the Ni-NTA resin revealed a predominant band at around 42 kDa. However, the final purified rGal d 6 preparation was present with several potential contamination E. coli proteins. The authenticity of the rGal d 6 was determined by probing the purified protein with Anti-Xpress antibody. Furthermore, most water soluble proteins are highly susceptible to proteolytic degradation [133]. However, in this study no such degradation was observed.

Obtaining a recombinant protein as a soluble fraction in sufficient amounts is highly desirable. Although bacteria have the capacity to produce protein with the correct amino acid sequence based on the gene, they lack the machinery for post-translational modification. Recombinant allergens produced in bacteria are structurally different than their wild type counterparts [134]. Therefore, analysing the immunological potency of recombinant allergen in comparison to natural allergen is a crucial step in recombinant allergy research [116]. In this study, we analysed the
immunological relevance of rGal d 6 through inhibition ELISA technique and showed that it has cross reactivity with CEY. rGal d 6 showed clear IgE reactivity by inhibiting IgE binding to CEY. Similarly, CEY was able to inhibit IgE binding to rGal d 6. It is usually expected that the percentage of inhibition could reach nearly 100% due to the abundant existence of Gal d 6 in the CEY extract. However, the inhibition of rGal d 6 due to CEY was only 31%. This may be because of the three dimensional structures of proteins often altered when immobilised on ELISA plates. The alteration of structure of proteins can also change the IgE binding epitopes and therefore, reduce inhibition. These results indicate that rGal d 6 is immunologically reactive and allergenic. Therefore, it is clear that IgE binding epitopes are present in rGal d 6. However, these results are not sufficient to establish the immunological equivalence in comparison to natural Gal d 6 (nGal d 6). In order to overcome this limitation, rGal d 6 must be compared with purified nGal d 6 through RAST using a panel of sera from patients diagnosed with allergy to hen’s egg yolk. However, purification of nGal d 6 proves to be a challenge and thus purified rGal d 6 provides a more logical option.

In conclusion, results of this chapter suggest that some patients with allergy to hen’s egg white are sensitised against egg yolk proteins. Furthermore, our results indicated that sensitisation is not limited to hen’s egg yolk. It was shown that a number of yolk proteins from emu and quail were also able to react with egg white allergy patients’ serum. Therefore, in those patients’ development of allergy can be attributed to allergens derived from both the albumen and yolk. Furthermore, these results suggest that there are potentially undiscovered allergens within the egg yolks of hen, emu and quail. Mass spectrometry results revealed that IgE reactive yolk proteins derived from VTG-1, VTG-2 and Apo B precursor families. In addition, we report the successful production of rGal d 6 as a soluble fraction in E. coli. The inhibition
ELISA immunoassays showed clear IgE binding to rGal d 6 and indicated a similar level of allergenicity to natural CEY. However, it is important to analyse the exact level of allergenicity of rGal d 6 in comparison to purified nGal d 6 in future studies. Knowledge on exact allergenicity of rGal d 6 would be highly valuable to researchers where it would facilitate decision-making when subjecting it to further molecular studies such as epitope analysis, production of hypoallergenic mutants and determining structural and functional basis of allergenicity. Indeed, our results highlight the significance of Gal d 6 (YGP42) as an important allergen of the egg yolk, and paves the way for the preparation of recombinant allergens for potential use in future diagnostics and therapeutics of egg yolk allergy.
Chapter 3: Immunological comparison of native and recombinant hen’s egg yolk allergen α-livetin (Gal d 5)

Publication under preparation:
De Silva C, Dhanapala P, Doran T, Tang MLK & Suphioglu C: Immunological analysis of natural and recombinant hen’s egg yolk allergen chicken serum albumin.
3.1 Introduction

Gal d 5 is a partially heat-labile allergen from the egg yolk of domestic chicken. Gal d 5 is synthesised in the liver of laying hens, and is present in both the blood circulation and the egg yolk. It is considered as the most common avian allergenic serum albumin and is the causative allergen implicated in bird-egg syndrome [82, 135]. Gal d 5, like most other serum albumins, can act as both a food and an inhalant allergen resulting in respiratory and food allergy symptoms, respectively. In bird-egg syndrome, sensitisation to Gal d 5 usually starts with the exposure to airborne chicken serum through the respiratory tract. Development of clinical allergy then takes place when the immune system encounters Gal d 5 derived from the egg yolk or chicken serum [82, 92].

Studies on IgE sensitisation to Gal d 5 is very limited when compared to egg white allergens, and other allergenic serum albumins from different species [92]. A cross-sectional survey on IgE reactivity to various allergens reported that 0.14% of subjects had IgE sensitisation to Gal d 5 [136]. However, another study reported the percentage of IgE sensitisation to Gal d 5 to be high as 20% [137]. Furthermore, to the best of our knowledge there are no reports of production of a recombinant Gal d 5 (rGal d 5). The use of recombinant allergens over natural allergen extracts in allergy diagnosis, treatment, and research offer many advantages due to their superior pharmaceutical quality as discussed in Chapter 1. Current applications and future potential developments of recombinant allergen use in clinical and research settings are extensively discussed in the scientific literature [115, 116, 118, 133, 138, 139].

The first aim of this chapter was to produce a recombinant version of Gal d 5 as a soluble fraction. We first used NEB Express™ Competent E. coli to produce rGal d
In this study, the *E. coli* strain was not successful at producing rGal d 5 as a soluble fraction despite successful expression of rGal d 6 in Chapter 2. Our pilot expression study showed that 100% of the rGal d 5 was produced as IBs indicating the unpredictability and challenging nature of recombinant protein production. However, in the event of IB formation, manipulation of microbiological growth conditions may result in induction of soluble protein production. These include changing culture media composition, growth temperature, production rate and the availability of heat-shock chaperones. However, implicating such changes do not guarantee the minimization of IB formation. Therefore, we employed a different recombinant protein production platform as an alternative approach to express rGal d 5 as a soluble fraction in a eukaryotic expression system [131, 132].

In this chapter, we present the successful cloning and expression of rGal d 5 as a soluble fraction using *Kluyveromyces lactis* (*K. lactis*) yeast strain. Then, in this study, we immunologically compared rGal d 5 and natural Gal d 5 (nGal d 5) using serum from patients allergic to hen’s egg white. The IgE-mediated allergic reaction is the most common form of food allergy. Therefore, we comparatively analysed specific IgE binding capacity of rGal d 5 with nGal d 5 using ELISA and Western dot blot analysis.

### 3.2 Materials and methods

#### 3.2.1 Human patients’ sera

Sera from 21 patients allergic to hen’s egg white were obtained from the Royal Children’s Hospital (Melbourne, VIC, Australia). The egg white specific IgE levels of patients are shown in Table 2.1. 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.

Table 3.1: The levels of allergen specific IgE antibodies against egg white determined by ImmunoCAP (Phadia)

<table>
<thead>
<tr>
<th>Patient number</th>
<th>IgE level (IU/mL) against hen’s egg white</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>93.50</td>
</tr>
<tr>
<td>2</td>
<td>28.80</td>
</tr>
<tr>
<td>3</td>
<td>13.60</td>
</tr>
<tr>
<td>4</td>
<td>9.26</td>
</tr>
<tr>
<td>7</td>
<td>6.89</td>
</tr>
<tr>
<td>8</td>
<td>18.50</td>
</tr>
<tr>
<td>9</td>
<td>16.50</td>
</tr>
<tr>
<td>10</td>
<td>4.13</td>
</tr>
<tr>
<td>11</td>
<td>3.62</td>
</tr>
<tr>
<td>13</td>
<td>1.21</td>
</tr>
<tr>
<td>14</td>
<td>1.32</td>
</tr>
<tr>
<td>15</td>
<td>1.72</td>
</tr>
<tr>
<td>16</td>
<td>2.08</td>
</tr>
<tr>
<td>17</td>
<td>2.21</td>
</tr>
<tr>
<td>18</td>
<td>2.58</td>
</tr>
<tr>
<td>19</td>
<td>1.78</td>
</tr>
<tr>
<td>21</td>
<td>1.25</td>
</tr>
<tr>
<td>22</td>
<td>1.05</td>
</tr>
<tr>
<td>23</td>
<td>0.32</td>
</tr>
<tr>
<td>24</td>
<td>0.18</td>
</tr>
<tr>
<td>25</td>
<td>0.47</td>
</tr>
</tbody>
</table>
3.2.1 Cloning and expression of Gal d 5 in E. coli expression system

3.2.1.1 RT-PCR amplification of Gal d 5

RT-PCR amplification of Gal d 5 was performed using OneStep RT-PCR kit according to the manufacturer’s instructions. The oligonucleotide primers required for amplification of mature Gal d 5 gene were based on the nucleotide sequence published on NCBI (NCBI reference number: NM_205261.2). The oligonucleotide forward primer 5’-CGCGGATCCCTTTTGCTCGATGACAGACG-3’ and reverse primer 5’-CGCGAATTCTTAAGCACCAATTCCTAATGTGGC-3’ were synthesised at Micromon-Monash University (Clayton, VIC, Australia). BamHI and EcoRI restriction enzyme sites were incorporated into 5’ ends of forward and reverse primers, respectively to facilitate cloning of Gal d 5 gene (restriction sites are underlined in the primer sequences). Furthermore, a 5’ CGC extension was added to each primer in order to increase the cleavage efficiency of the restriction enzymes. Total mRNA extracted from the chicken liver in Section 2.2.2.2 was used as the template for RT-PCR. The RT-PCR master mix was prepared by mixing 10 μL of 5× QIAGEN One-Step RT-PCR buffer, 2 μL of 10 mM dNTP mix, 10 μL of 5× Q-solution, 1 μL of 1 μM forward primer, 1 μL of 1 μM reverse primer, QIAGEN One-Step RT-PCR enzyme mix and 170.82 ng of mRNA in a 50 μL total reaction volume in a sterile PCR tube. The thermal cycler conditions used for RT step were 30 minutes at 50°C for RT and 15 minutes at 95°C for activation of HotStart DNA polymerase. The parameters used for the 3 step PCR cycling step were 35 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 53°C, 2 minute extension at 72°C and a final 10 minute extension at 72°C. The RT-PCR product was then analysed on 1% (W/V) agarose gel and purified according to the protocol mentioned in Section 2.2.2.4.
3.2.1.2 Synthesis of pTrcHis A-Gal d 5 construct and expression of rGal d 5 in *E. coli*

Restriction enzyme digestion and cloning of Gal d 5 gene into pTrcHis A plasmid vector was performed according to the protocol mentioned in Section 2.2.2.5. The ligation reaction was transformed into chemically competent NEB Express 
Competent *E. coli* according to the protocol mentioned in Section 2.2.2.6. Five transformant clones were picked using sterile pipette tips and inoculated into fresh LB media containing 50 μg/mL ampicillin and allowed to grow overnight at 37°C with shaking at 250 rpm. Then, plasmid isolation of all five overnight bacterial cultures was performed according to the protocol outlined in Section 2.2.2.7. Restriction enzyme digest screening for clones harbouring pTrcHis A-Gal d 5 construct and sequencing of the constructs were done according to the protocol mentioned in Section 2.2.2.8. Time course of expression analysis of rGal d 5 was carried out according to the method mentioned in Section 2.2.2.9. *E. coli* culture fractions were collected at 2nd, 3rd and 5th hourly time points.

3.2.2 Cloning and expression of Gal d 5 in *K. lactis* eukaryotic protein expression system

3.2.2.1 Natural allergen extract

Lyophilized chicken serum albumin (nGal d 5) powder (chicken serum albumin, CSA62-0001) available at the highest purification level (≥ 96% purity) was purchased from Equitech-Bio, Inc. (Kerrville, TX, USA) and prepared at a concentration of 10 mg/mL in 1× PBS.
3.2.2.2 Strains, vectors and growth conditions

*E. coli* strain NEB® 5-alpha F’ F′ (New England Biolabs Inc., MA, USA) was used as the cloning host and was grown in LB medium supplemented with 50 µg/mL ampicillin at 37°C. *K. lactis* strain GG799 (New England Biolabs Inc., MA, USA) was used as the host strain for the secretion of rGal d 5. *K. lactis* were grown in either YPGal medium (1% (W/V) yeast extract, 2% (W/V) peptone, 2% (W/V) galactose) or YPGlu medium (1% (W/V) yeast extract, 2% (W/V) peptone, 2% (W/V) glucose) at 30°C. The *K. lactis* integrative expression vector pKLAC2 (New England Biolabs Inc., MA, USA) contains the *Aspergillus nidulans* acetamidase gene (*amdS*), which allows growth on nitrogen-free minimal medium containing acetamide. The selection of *K. lactis* cells transformed with pKLAC2 vectors were grown on yeast carbon base (YCB) agar medium supplemented with 5 mM acetamide at 30°C.

3.2.2.3 PCR amplification of Gal d 5 gene with a C-terminal hemagglutinin (HA) epitope tag

Gal d 5 gene was amplified by PCR technique using the QIAGEN Fast Cycling PCR kit (Qiagen, Hilden, Germany). pTrcHis A-Gal d 5 expression vector was used as the template DNA (constructed in Section 3.2.1.2). Primers used for amplification of Gal d 5 gene were based on the sequence published on NCBI (NCBI accession number: NM_205261.2).

The sequence coding for the mature Gal d 5 was amplified with the forward primer 5’-CGCCTCGAGAAAAGATTTTGCTCGTGATGACAGACAAAGAG-3’ and reverse primer 5’-CGCGCGGCGGCTTTATGCATAATCTGGAACATCATATGG
ATAAGCACCAATTCTAATGTGGCTCTGC-3’. *XhoI* and *NotI* restriction enzyme sites were incorporated into 5’ sides of forward and reverse primers, respectively (restriction sites underlined in the primer sequences). Forward primer contains a Kex protease cleavage site immediately downstream of the *XhoI* restriction site (nucleotides in italics). The reverse primer contains sequences for C-terminal hemagglutinin (HA) epitope (nucleotides in bold) and a TTA stop codon immediately downstream the *NotI* restriction site. Both primers contain CGC extensions at the 5’ ends. The PCR reaction mix was prepared by mixing 10 µL of QIAGEN PCR Fast Cycling Master Mix, 4 µL of 5×Q-Solution, 2.5 µL of forward primer to a final concentration of 0.5 µM, 1.82 µL of reverse primer to a final concentration of 0.5 µM and 1.82 µL of template DNA (<300 ng) in a 20 µL total reaction volume. The thermal cycler conditions were 5 minutes at 95°C for activation of HotStart DNA polymerase, 35 cycles of 5 seconds denaturation at 96°C, 5 seconds annealing at 60°C, 5.34 minutes of extension at 68°C and a final extension for 1 minute at 72°C. The PCR product was then analysed on E-Gel® CloneWell™ agarose gel with 0.8% SYBR Safe™ DNA Gel stain (Thermo Fisher Scientific®, MA, USA), powered through E-Gel® iBase Power System (Thermo Fisher Scientific®, MA, USA). The PCR product was then extracted from the bottom collection wells of the precast gel by pipetting. The concentration of the gel-purified PCR product was then measured by NanoDrop™ spectrophotometer and stored at -20°C until needed.

### 3.2.2.4 Construction of pKLAC2-Gal d 5 expression vector

The mature Gal d 5 gene was cloned into the *XhoI* and *NotI* sites, in frame with the α-mating factor (α-MF) secretion leader sequence of the pKLAC2 vector, to yield pKLAC2-Gal d 5 construct. The mature Gal d 5 gene and the pKLAC2 vector were digested with restriction enzymes *XhoI* and *NotI* according to NEB Time-Saver™
protocol (protocol mentioned in Section 2.2.2.5). Ligation of the digested products was conducted under standard conditions with a 1:6 vector to insert ratio keeping the total DNA under 10 ng. The amounts of vector and insert DNA were calculated using the following formula.

\[
\text{ng Insert} = \frac{\text{ng Vector} \times \text{bp Insert}}{\text{bp Insert}}
\]

4 ng of digested pKLAC2 vector and 4.80 ng of linearized Gal d 5 gene were ligated using 1 µL of T4 DNA ligase and 10× T4 DNA ligase reaction buffer in a 10 µL total reaction volume. The ligation reaction was incubated overnight at 4°C. The ligation reaction was chemically transformed into NEB 5-alpha F’ Φ Competent E. coli according to NEB high efficiency transformation protocol (protocol mentioned in Section 2.2.2.6). Transformed cells were grown overnight on LB agar medium at 37°C. Screening for E. coli colonies harbouring pKLAC2-Gal d 5 construct was performed by restriction enzyme digestion of isolated plasmids with XhoI and NotI (Protocol outlined in Section 2.2.2.8). The plasmids harbouring the insert were then sequenced using #S1274 LAC4mt promoter sequencing forward primer 5’-GCGGATAACAAGCTCAAC-3’ (Micromon-Monash University, Clayton, VIC, Australia). The DNA sequences obtained from sequencing were then subjected to nucleotide BLAST search using the NCBI online tool.

3.2.2.5 Production of linearized pKLAC2 expression cassette

pKLAC2-Gal d 5 construct was digested with SacII restriction enzyme in order to produce the linear expression cassette. 1 µg of pKLAC2-Gal d 5 construct was digested with 1 µL of SacII and 5 µL of NEBuffer 3.1 in a 50 µL total reaction
volume. The sample was then incubated at 37°C for 1 hour. The digested DNA was then desalted using QIAquick® PCR purification kit (QIAGEN, Hilden, Germany) according to manufacturer’s instructions. All centrifugation steps were carried out at 13,000 rpm at room temperature. 5 volumes of Buffer PB were mixed with 1 volume of linearized DNA. Then, 10 µL of 3 M sodium acetate (C₂H₃NaO₂) (pH 5.0) was added to the sample to adjust the pH. To bind DNA, two SacII digested pKLAC2-Gal d 5 reactions (100 µL of total volume) were run through a single QIAquick column placed in a 2 mL collection tube by centrifugation for 1 minute. The flow-through was discarded. Then, 750 µL of Buffer PE was added to the QIAquick column and centrifuged for 1 minute to wash. The flow-through was discarded. The QIAquick column was centrifuged for an additional 1 minute and the flow-through was discarded. The QIAquick column was placed in a clean 1.5 mL microcentrifuge tube. To elute DNA, 50 µL of Buffer EB was added to the centre of the QIAquick membrane and allowed to stand for 1 minute, prior centrifugation for 1 minute. The concentration of purified DNA was measured using a NanoDrop™ spectrophotometer.

3.2.2.6 Transformation of K. lactis

Linearized expression cassette was introduced into K. lactis GG799 competent cells by chemical transformation according to the manufacturer’s instructions. A tube of K. lactis competent cells was thawed on ice. Then, 620 µL of NEB Yeast Transformation Reagent was added to the cells and mixed by inverting the tube until the solution was homogeneous. 1 µg of linearized pKLAC2 expression cassette was added to the cell mixture in a volume less than 15 µL and mixed by inverting the tube several times. The mixture was incubated at 30°C for 30 minutes. Then, the cells were heat shocked by incubating at 37°C for 1 hour in a water bath. The cells
were pelleted by centrifugation at 7000 rpm for 2 minutes. The supernatant was discarded. The cell pellet was resuspended in 1 mL sterile YPGlu medium and centrifuged at 7000 rpm for 2 minutes. The supernatant was discarded. The cell pellet was resuspended again in YPGlu media in a sterile 1.5 mL tube and incubated at 30°C for 3.5 hours with shaking at 250 rpm. The cell mixture was then transferred into a new microcentrifuge tube. The cells were pelleted by centrifugation at 7000 rpm for 2 minutes and the supernatant was discarded. The cell pellet was resuspended in 1 mL of sterile 1× PBS. Then, 50 µL and 100 µL aliquots were plated on YCB agar medium containing 5 mM acetamide and incubated at 30°C for 3.5 days until colonies formed.

### 3.2.2.7 Identification of multi-copy integrants

*K. lactis* transformants with multi-copy integrants were identified through whole cell PCR analysis. Individual *K. lactis* colonies were patched onto fresh YCB agar medium plates containing 5 mM acetamide and incubated at 30°C for 1-2 days. Cells from an area approximately 1 mm² were harvested by scraping with a sterile pipette tip and resuspended in 25 µL 1 M sorbitol (Sigma-Aldrich, MO, USA) containing 10 mg/mL lyticase (Sigma-Aldrich, MO, USA). The cells were mixed by vortexing and incubated at 30°C for 1 hour. Lyticase-treated cells were lysed in a thermocycler by incubating at 98°C for 10 minutes. Then, PCR reaction was made by adding 10 µL of 10× integration primer 2 (5’-ATCATCCTTGTGCGAAGC-3’), 10 µL of 10× integration primer 3(5’-ACCTGAAGATAGCTTCTAA-3’), 10 µL of 2 mM dNTPs, 10 µL of 10× ThermoPol Buffer, 1 µL of Taq DNA Polymerase and 34 µL of deionised water to lyticase-treated cells in a final reaction volume of 100 µL. The thermal cycler conditions were: 30 cycles at 30 seconds at 94°C, 30 seconds at 50°C and 3 minutes at 72°C and a final incubation at 72°C for 10 minutes. The whole cell
PCR products were analysed on E-Gel® CloneWell™ agarose gel with 0.8% SYBR®
Safe DNA gel stain.

3.2.2.8 Time course of expression and detection of secreted rGal d 5

Transformed *K. lactis* strain containing multi-copy integrants were screened for its’
ability to secrete rGal d 5 in YPGal medium. A small aliquot of frozen *K. lactis* cells
was revived on YCB agar medium containing 5 mM acetamide by incubating at
30°C for 1.5 days. For expression, a single *K. lactis* colony was grown in 5 mL of
YPGal medium at 30°C with shaking at 250 rpm for 7 days. Culture supernatants
were harvested by centrifugation daily starting from 2<sup>nd</sup> day to 7<sup>th</sup> day during the
time course of expression. Collected culture supernatants were then analysed by
SDS-PAGE (protocol mentioned in Section 2.2.1.3) and Western blotting (protocol
mentioned in Section 2.2.1.4.1). Here, monoclonal anti-HA antibody produced in
mouse (diluted 1:1000 in blocking solution) (Sigma-Aldrich, MO, USA) was used as
the primary antibody, while alkaline phosphatase-conjugated anti-mouse IgG
antibody was used as the secondary antibody.

3.2.2.9 Purification of rGal d 5

Purification of rGal d 5 was performed using Pierce Magnetic HA-Tag IP/Co-IP kit
(Thermo Fisher Scientific®, MA, USA) according to the manufacturer’s instructions.
25 µL of Pierce anti-HA Magnetic Beads were mixed with 175 µL of IP Lysis/Wash
Buffer by gently vortexing in a microcentrifuge tube. The tube was placed into a
magnetic stand to collect the beads. The supernatant was discarded. 1 mL of IP
Lysis/Wash Buffer was added to the microcentrifuge tube and gently vortexed for 1
minute. The magnetic beads were collected using a magnetic stand. The supernatant
was discarded. 300 µL of culture supernatant sample containing HA-tagged rGal d 5
from 6th day was added to the pre-washed magnetic beads and incubated at room temperature for 30 minutes with mixing. Then magnetic beads were collected with a magnetic stand and the unbound sample was removed and saved for analysis. 300 µL of IP Lysis/Wash Buffer was added to the tube and mixed gently. The beads were collected with a magnetic stand and the supernatant was discarded. This washing step was repeated twice. Then, 300 µL of DI water was added to the tube and gently mixed. The beads were collected with a magnetic stand and the supernatant was discarded. 100 µL of Elution Buffer was added to the magnetic beads and incubated at room temperature with mixing for 10 minutes. Then, magnetic beads were separated on a magnetic stand and the supernatant containing the recombinant protein was collected for analysis. All the fractions were then analysed on SDS-PAGE and Western blotting as mentioned in the Section 3.2.2.8.

3.2.2.10 Identification of patients sensitized to nGal d 5

Western dot blot analysis was conducted using the Bio-Dot Microfiltration apparatus (Bio-Rad, Hercules, CA, USA) with the buffers provided in WesternBreeze Chromogenic immunodetection system. 3 µg of nGal d 5 in a total volume of 100 µL of 1× PBS, was spotted on to a nitrocellulose membrane. Following blocking using the bocking buffer, nGal d 5 spots were incubated with 21 individual patients’ sera (diluted 1:10 in blocking buffer) allergic to hen’s egg white and 2 non-allergic subjects’ sera (diluted 1:10) overnight with gentle agitation at room temperature. After washing, all serum pre-incubated spots were incubated with monoclonal anti-human IgE antibody produced in goat and labelled with alkaline phosphatase (diluted 1:2000 in blocking buffer). The signal was developed by incubating the nitrocellulose membrane with BCIP/NBT chromogenic substrate.
3.2.2.11 Dot-blot analysis of rGal d 5

3 µg of rGal d 5 was spotted on to nitrocellulose membrane using the Bio-Dot Microfiltration apparatus according to the protocol mentioned in Section 3.2.2.10 above. rGal d 5-spotted membrane was probed with pooled sera (diluted 1:5 in blocking buffer) composed of patients sensitised to nGal d 5 (patients 3, 10, 15, 16, 17, 19, 24 and 25). As a negative control, two rGal d 5 spots were probed with sera from 2 non-allergic individuals. After washing, all serum pre-incubated spots were incubated with monoclonal anti-human IgE antibody produced in goat labelled with alkaline phosphatase (diluted 1:2000 in blocking buffer). The signal was developed by incubating the nitrocellulose membrane with BCIP/NBT chromogenic substrate.

3.2.2.12 Indirect ELISA for quantification of specific IgE against rGal d 5 and nGal d 5

In brief, 1 µg of nGal d 5 and rGal d 5 in 100 µL of coating buffer (100 mM bicarbonate/carbonate coating buffer, pH 9.6) were allowed to coat wells of an ELISA microtiter plate. The proteins were allowed to coat the microtiter wells overnight at 4°C. After triple washing with 0.05% PBST, all protein coated wells were blocked with 1% (W/V) non-fat dry milk in 1× PBS for 2 hours at room temperature. The plates were triple washed with 0.05% PBST. nGal d 5 and rGal d 5 coated wells were then incubated with patient sera (diluted 1:10 in 1% (W/V) non-fat dry milk in 1× PBS) 3, 10, 15, 16, 17, 19, 24, 25 and one non-allergic sera (diluted 1:10 in 1% (W/V) non-fat dry milk in 1× PBS) overnight at 4°C. Each sample was done in triplicates. After triple washing the wells with 0.05% PBST, all wells were incubated with monoclonal anti-human IgE antibody produced in goat and labelled with alkaline phosphatase (diluted 1:1000 in 1% non-fat dry milk in 1× PBS) for 2 hours at room temperature. Following triple washing with 0.05% PBST, 200 µL of
alkaline phosphatase Yellow (pNPP) liquid substrate was added to the wells and incubated at 37°C. The colour development was terminated by adding 3 M NaOH (50 µL per well) and the plate was read at 405 nm using a plate reader.

3.3 Results

3.3.1 Expression of rGal d 5 in E. coli

Analysis of RT-PCR product on 1% (W/V) agarose gel showed a predominant band between 1500 bp and 2000 bp markers (expected size: 1827 bp) (Figure 3.1). E. coli cells transformed with pTrcHisA-Gal d 5 construct had growth when grown on LB agar under ampicillin resistance. When BamHI and EcoRI digested plasmids of selected transformant clones were electrophoresed on 1% (W/V) agarose gel, 4 out of 5 clones showed the insert approximately at 2000 bp marker (expected size: 1827 bp) (Figure 3.2). Sequencing analysis of plasmids harbouring the insert confirmed the in-frame ligation of the Gal d 5 gene within the MCS of pTrcHisA vector. The NCBI BLAST search of the derived sequence produced Gallus gallus albumin (sequence ID: refINM 205261.2) as the top match with a 96% sequence similarity (Figure 3.3).
Figure 3.1: RT-PCR of Gal d 5 gene. The PCR product was electrophoresed on a 1% (W/V) agarose gel. Lane 1: RT-PCR product of chicken Gal d 5 gene. Lane 2: negative control RT-PCR without template mRNA. MW: molecular weight marker GeneRuler 1kb Plus DNA ladder in bp. The arrow indicates the RT-PCR amplified Gal d 5 gene.

Figure 3.2: Restriction enzyme digest screening for the colonies harbouring the pTrcHis A-Gal d 5 construct. The digested plasmids were electrophoresed on a 1% (W/V) agarose gel. Lanes 1-5: BamHI and EcoRI digested plasmids of 5 individual bacterial colonies grown on transformation plate with antibiotic. MW: molecular weight marker GeneRuler 1kb Plus DNA ladder in bp. The arrow indicates the Gal d 5 gene.
Figure 3.3: Sequence alignment comparison of DNA sequence derived for pTrcHis A-Gal d 5 construct using NCBI Nucleotide BLAST tool.

SDS-PAGE Analysis of transformed *E. coli* culture fractions of the pilot expression study showed multiple protein bands in both the soluble and insoluble fractions (Figure 3.4, A). The Western blot analysis of *E. coli* culture fractions showed a predominant band in between 64 kDa and 98 kDa markers on the insoluble culture fractions (expected size: 69 kDa) (Figure 3.4, B). The highest band intensity was observed on 5-hour post IPTG induction. No bands were detected in between 64 kDa and 98 kDa markers on the soluble fraction of the Western blot. However, several low molecular weight breakdown products of rGal d 5 can be seen in both the soluble and insoluble fractions.
3.3.2 Expression of Gal d 5 in K. lactis

The full-length cDNA coding for the mature Gal d 5 gene was amplified in a single step using QIAGEN Fast Cycling PCR kit. A single predominant band was visible just below 2000 bp marker (expected size: 1827 bp) when electrophoresed on E-Gel® CloneWell™ agarose gel (Figure 3.5). E. coli cells transformed with newly synthesised pKLAC2-Gal d 5 construct had growth when grown on LB agar under ampicillin resistance. When XhoI and NotI digested plasmids of selected transformant clones were electrophoresed on 1% agarose gel, 6 out of 7 clones showed the insert approximately at 2000 bp marker (expected size: 1827 bp) (Figure 3.6). Sequencing analysis pKLAC2-Gal d 5 constructs confirmed the ligation of Gal d 5 gene in-frame with the α-MF secretion signal of the pKLAC2 vector. The NCBI BLAST search of the derived sequence produced Gallus gallus albumin (sequence ID: refINM 205261.2) as the top match with a 98% sequence similarity (Figure 3.7).
Figure 3.5: PCR amplification of Gal d 5 gene. The PCR product was electrophoresed on E-Gel® CloneWell™ agarose gel with 0.8% SYBR Safe™ DNA Gel stain. Lane 1: PCR amplified Gal d 5 gene. MW: molecular weight marker in bp (E-Gel® 96 High Range DNA marker). The arrow indicates the PCR amplified Gal d 5 gene.

Figure 3.6: Restriction enzyme digest screening for the colonies harbouring the pKLAC2-Gal d 5 construct. The digested plasmids were electrophoresed on a 1% (W/V) agarose gel. Lanes 1-7: XhoI and NotI digested plasmids of 7 individual bacterial colonies grown on transformation plate under ampicillin resistance. MW: molecular weight marker in bp (E-Gel® 96 High Range DNA marker). The arrow indicates the Gal d 5 gene.
Figure 3.7: Sequence alignment comparison of DNA sequence derived for pKLAC2-Gal d 5 construct using NCBI Nucleotide BLAST tool.
pKLAC2-Gal d 5 construct isolated from the selected clone was digested with SacII to generate the linearized expression cassette in order to facilitate integrative transformation of *K. lactis*. Electrophoresis of SacII digested pKLAC2-Gal d 5 construct on 1% (w/v) agarose gel confirmed successful digestion of the construct (Figure 3.8). There are 2 SacII restriction enzyme sites in the pKLAC2 vector. The SacII digested construct showed a fragment of >6.2 kb which contains P_{LAC4-PBI}, the cloned Gal d 5 gene and the *amdS* selection gene, and another fragment with an approximate size of 2.8 kb containing the remainder of the pKLAC2 vector. Following introduction of linearized expression cassette into *K. lactis* cells, transformants were selected by growth on YCB agar minimal media containing 5 mM acetamide. A randomly selected *K. lactis* transformant was then subjected to whole cell PCR to confirm the multiple integration of expression cassettes. Analysis of whole cell PCR product showed an amplification product approximately at 2.3 kb when electrophoresed on 1% (w/v) agarose gel (Figure 3.9).

![Figure 3.8: SacII digest of the pKLAC2-Gal d 5 construct. The digested plasmid was electrophoresed on a 1% (w/v) agarose gel. Lane 1: SacII digest of the pKLAC2-Gal d 5 construct. Arrow a, indicates the expression cassette containing the Gal d 5 gene. Arrow b, indicates the remainder of the pKLAC2 vector. MW: molecular weight marker in bp (E-Gel® 96 High Range DNA marker).](image-url)
A time course of expression was performed to confirm the secretion of rGal d 5 and to determine the optimum recombinant protein expression conditions of *K. lactis* cells harbouring rGal d 5. The culture supernatant samples were analysed by SDS-PAGE (Figure 3.10, A) and Western blot (Figure 3.10, B). SDS-PAGE analysis of culture supernatants showed a single predominant band in between 64 kDa and 98 kDa markers in all the culture fractions (expected size: 1827 bp). The Western blot showed a predominant band approximately in between 64 kDa and 98 kDa markers corresponding to the protein on SDS-PAGE confirming the authenticity of rGal d 5. SDS-PAGE analysis of nGal d 5 also showed a predominant band in between 64 kDa and 98 kDa markers and was comparable to the SDS-PAGE of rGal d 5 (Figure 3.10, C). The SDS-PAGE analysis of the time course of expression showed the highest recombinant protein production on the 6th day.
Figure 3.10: Time-course of expression of *K. lactis* culture supernatants containing secreted rGal d 5 and SDS-PAGE of nGal d 5. Lanes 1-6: culture supernatants from day 2 to 7, respectively analysed by (A) SDS-PAGE and (B) Western immunoassay. (C) SDS-PAGE of nGal d 5. MW: molecular weight marker in kDa (SeeBlue Pre-Stained protein standard). Arrows indicates rGal d 5 approximately at 69 kDa region.

Expression of a recombinant protein fused to an epitope tag such as HA epitope provides the means to purify the fusion peptide using immunoprecipitation. The SDS-PAGE analysis of immunoprecipitation purified rGal d 5 showed a predominant band approximately at 69 kDa with no visible contaminant *K. lactis* proteins (Figure 3.11, A). The Western immunoblot of immunoprecipitation-purified rGal d 5 probed with monoclonal anti-HA antibody produced in mouse showed a predominant band approximately at 69 kDa corresponding to the protein band on SDS-PAGE (Figure
Furthermore, no proteolytic degradation of rGal d 5 was observed in all SDS-PAGE and Western immunoblot analysis of *K. lactis* culture supernatants.

![Figure 3.11: Analysis of immunoprecipitation purified rGal d 5. (A) SDS-PAGE and (B) Western immunoassay of purified rGal d 5 fractions. Lane 1: unbound sample. Lane 2: elution containing rGal d 5. MW: molecular weight marker in kDa (SeeBlue Pre-Stained protein standard). Arrows indicate rGal d 5.](image)

### 3.3.3 Immunological analysis of nGal d 5 and rGal d 5

Sera from 21 patients’ allergic to hen’s egg white were tested with dot-blot analysis to examine the frequency and degree of IgE sensitisation against nGal d 5. The dot-blot analysis was performed only once due to the limited availability of patients’ sera. The dot-blot analysis (Figure 3.12) showed that 38% (8 out of 21) of egg white allergy patients to have IgE reactivity to nGal d 5. Out of the all sensitised patients, patient 3 exhibited significantly high IgE reactivity against nGal d 5 when compared to others. Other sensitised patients exhibited mild IgE reactivity to Gal d 5. The ability to recognize rGal d 5 by the immune systems of nGal d 5 sensitised patients was demonstrated by Western dot blot (Figure 3.13). rGal d 5 showed clear IgE reactivity when probed with pooled patients’ sera. rGal d 5 showed negligible level of IgE reactivity when probed with non-allergic sera.

An indirect ELISA assay was performed to compare the degree of IgE reactivity of nGal d 5 and rGal d 5 (Figure 3.14). The result shows that patient 3 to have the
highest IgE reactivity against both nGal d 5 and rGal d 5. The remaining patients showed mild reactivity to nGal d 5 and rGal d 5. Furthermore, in every patient the amount of IgE reactivity directed at nGal d 5 and rGal d 5 were very similar.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>21</td>
<td>9</td>
<td>7</td>
<td>19</td>
<td>25</td>
<td>17</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>B</td>
<td>23</td>
<td>10</td>
<td>15</td>
<td>1</td>
<td>8</td>
<td>24</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>C</td>
<td>22</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>14</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.12: Testing of patients allergic to hen’s egg white described in Table 1 for IgE reactivity with dot-blotted nGal d 5. The grid above shows the identity of the serum used corresponding to each spot. nGal d 5 sensitised patients are highlighted in the grid. Dots denoted by N are non-allergic serum.

Figure 3.13: Testing of patients sensitised to nGal d 5 for IgE reactivity with dot-blotted rGal d 5. Dots denoted by N are sera from non-allergic subjects.
Figure 3.14: Comparison of the binding activity of human specific IgE against recombinant and natural Gal d 5. Blue boxes represent IgE reactivity against nGal d 5 and red boxes represent IgE reactivity against rGal d 5. N denotes the non-allergic serum and numbers denotes different patients’ sera. Data are average of triplicate measurements SE ±.

3.4 Discussion

The main objective of Chapter 3 was to produce an IgE-reactive recombinant version of hen’s egg yolk allergen Gal d 5 and analyse its’ IgE binding capacity in comparison to nGal d 5. The first challenge of this study was to successfully clone and express sufficient amounts of rGal d 5 as a soluble fraction. Variety of recombinant production platforms are being designed in order to meet these demands. However, there is no universal protein expression system or a predictive tool, which can guarantee the desired outcome. This is mainly attributed to the biochemistry of each protein and its effects on the host expression system [140].

Due to the success with expressing rGal d 6 in *E. coli*, it was decided to clone and express rGal d 5 in the same strain. Analysis of RT-PCR result indicated that cDNA coding for the mature egg yolk allergen Gal d 5 was selectively amplified from total
mRNA extracted from liver tissue. Analysis of SDS-PAGE and Western blot results of IPTG induced culture fractions of *E. coli* harbouring pTrcHis A-Gal d 5 confirmed the successful expression of rGal d 5. These data suggest that the PCR amplified Gal d 5 gene has been successfully integrated to the MCS of the pTrcHis A vector. Therefore, translation of rGal d 5 fusion peptide with a *N*-terminal 6×his tag has been successfully lead by the *P* _trc_ resulting in high levels of recombinant protein yield. However, complete lack of soluble rGal d 5 indicates that rGal d 5 has undergone irregular folding resulting in insoluble and refractile IBs.

Even though, *E. coli* host expression systems are well known for their versatility in recombinant protein production, they also contain few drawbacks. For example production of recombinant proteins, which contains large amounts of disulphide bonds (DBs) has always been challenging. DBs are very common in proteins such as mammalian proteins, hormones and secreted proteins [141]. Gal d 5 is a secreted protein with 17 DBs stabilizing it [142]. DBs are crucial for correct protein folding, stability and functionality. In general, the cytoplasm of *E. coli* cells does not offer a favourable environment for DB formation due to the presence of numerous DB reductases, such as thioredoxins and glutaredoxins. These DB reductases quickly reduce DBs formed between two cysteine groups back to its thiolate state. Failure to form DBs in the recombinant proteins results in it being formed as inactive IBs. Therefore, it is highly possible that the large number of DBs present in Gal d 5 haven’t been formed in the *E. coli* cytoplasm and have undergone IB formation [141, 143]. On the other hand it is clear why Gal d 6 protein production by *E. coli* as a soluble fraction had relative success when compared to Gal d 5 protein production. It is evident that lack of DBs in Gal d 6 [144] may have contributed to undergo successful expression of it as a soluble fraction.
In order to retain the biomedical value of a biomolecule, it is essential that the recombinant protein is produced in a soluble fraction, resembling as much as possible to its native form. Therefore, in the event of IB formation, a number of strategies have been recommended to promote the production of recombinant proteins as a soluble fraction in the literature. If IB formation is due to lack of DB formation, \textit{in vitro} refolding can be used to obtain a functional protein. However, it is well known that refolding in IBs is often unpredictable and challenging. Furthermore, \textit{in vitro} refolding is highly time consuming and require a large amounts of reagents [143]. Majority of the mentioned implications are aimed at changing growth conditions of the host strain to induce soluble protein expression. Nevertheless, introducing changes in growth conditions do not always guarantee successful expression of soluble recombinant protein [131, 132]. Therefore, overall generation of the soluble protein is the preferred choice [143].

As an alternative approach, we decided to use a different host organism to express rGal \textit{d} 5 as a soluble fraction. In this chapter, we used the eukaryote yeast strain \textit{K. lactis} to produce rGal \textit{d} 5 as a secreted protein. Our results confirmed that rGal \textit{d} 5 produced by \textit{K. lactis} is IgE-reactive and has very similar IgE binding levels to nGal \textit{d} 5. Furthermore, \textit{in vitro} immunoassay results indicate that patients with allergy to hen’s egg white can have reactive IgE antibodies against Gal \textit{d} 5, indicating potential allergy to hen’s egg yolk.

Analysis of PCR results indicated that cDNA coding for the mature egg yolk allergen Gal \textit{d} 5 was selectively amplified from the pTrcHis A-Gal \textit{d} 5 construct. The randomly selected \textit{K. lactis} transformant strain was confirmed as a multi-copy integrant using the whole cell PCR technique. In this strategy, only strains containing multiple copies of pKLAC2 expression cassette will produce an amplicon.
with a size of 2.3 kb. Strains harbouring multiple copy inserts may have higher recombinant protein expression levels than single copy integrants. However, this technique is unable to indicate the number of integrated expression vector fragments in the cells [145]. *K. lactis* transformant selection is based on acetamide selection instead of traditional antibiotic selection. It has been reported that acetamide selection enriches transformants with multi-copy integrants over transformants with single copy integrants. As a result, majority of the strains contain multiple copies of the expression fragment [145, 146]. Therefore, we were able to obtain a strain with multi-copy integrants with relative ease.

Analysis of galactose-induced *K. lactis* culture supernatants with SDS-PAGE and Western immunoassay confirmed the presence of rGal d 5 at the expected size. These data suggests that the pKLAC2 containing Gal d 5 gene has been successfully integrated into the *K. lactis* genome at the *LAC4* locus, which drives protein expression. Furthermore, this result indicates that cloning of Gal d 5 gene into pKLAC2 expression vector had been done in-frame with the α-MF secretion leader sequence. The α-MF domain directs the fusion protein to be efficiently transported through yeast secretory pathway. Furthermore, there were not any visible *K. lactis* derived proteins in the culture supernatants. Therefore, lack of detectable amounts of native host proteins serves as the first step of purification. Lack of native of *K. lactis* proteins in the spent culture media indicates that those proteins haven’t been secreted efficiently through the yeast secretory pathway. Multiple integration of Gal d 5 gene may have resulted in overexpression of the recombinant protein. Therefore, a high amount of recombinant Gal d 5 can enter the *K. lactis* secretory pathway preventing or reducing the secretion of native proteins.
The expression of fusion protein with a HA-epitope tag provides the means to selectively purify proteins through immunoprecipitation in addition to serving as an identification tag. The rGal d 5 was purified to homogeneity by immunoprecipitation using anti-HA magnetic beads. There were no visible *K. lactis* derived proteins on SDS-PAGE indicating a high level of purity. The Western immunoassay of purified rGal d 5 indicated that the majority of the anti-HA magnetic beads were able to capture the target rGal d 5 protein without any cross reactivity with native *K. lactis* host proteins. A major concern in recombinant protein production is protein degradation due to proteolysis [133]. Our results confirmed that there was no proteolytic degradation of rGal d 5.

The dot-blot immunoassay showed that 38% (8 out of 21 patients) of egg white patients to have IgE sensitisation directed against nGal d 5. However, only one patient (patient number 3) out of the 8 IgE-reactive patients had significant reactivity against nGal d 5 when compared to non-allergic controls. The amount of specific IgE antibodies directed against a certain allergen can vary from person to person influenced by a combination of genetic and environmental factors. However, exact determinants responsible for producing such high amounts of specific IgE against nGal d 5 in our experiment cannot be deduced without further investigations. Furthermore, the remaining seven patients which have sensitisation against nGal d 5 had very minor reactivity. This could be attributed to the fact that Gal d 5 is a minor allergen. A limitation in this study is the use of sera from patients diagnosed with egg white allergy rather than from patients diagnosed with egg yolk allergy. At the present time, allergy to hen’s egg yolk is not commonly tested in hospitals which forced us to use sera from egg white allergy patients. The patients sensitized towards nGal d 5 may or may not develop symptoms of allergy from Gal d 5. Nevertheless, presence of such antibodies is considered a risk factor for allergy and to have a
predictive value. For example, it has been shown that presence of hen’s egg white specific IgE antibodies even without clinical symptoms can be used to predict the development of allergy before 7-10 years of age [3].

IgE binding capacity between recombinant and their corresponding natural allergens may be different. Therefore, in this study we comparatively analysed the degree of IgE binding of rGal d 5 and nGal d 5. The ELISA experiment showed that both recombinant and natural Gal d 5 to exhibit very similar levels of IgE binding. Furthermore, the dot-blot analysis of rGal d 5 confirmed that it is IgE reactive. These results together suggest that rGal d 5 produced by *K. lactis* is recognizable by the human immune system and bears similar epitopes to that of nGal d 5.

In conclusion, Chapter 3 presents the successful production of IgE-reactive rGal d 5 using *K. lactis* yeast strain. We have shown that rGal d 5 has similar allergenicity to nGal d 5 by comparing IgE binding activity of both using sera from egg white allergy patients. Furthermore, we were able to isolate rGal d 5 with a high level purity with relative ease. Therefore, rGal d 5 produced in this chapter is a potential candidate to be used in *in vitro* allergy diagnosis in the future. However, it is also important to further subject rGal d 5 into immunological investigations using sera from patients allergic to hen’s egg yolk. Immunological analysis of rGal d 5 in comparison to nGal d 5 using patients with allergy to hen’s egg yolk may provide some in depth validation to the results presented in this chapter. Furthermore, availability of IgE reactive recombinant allergens opens the way to develop recombinant based hypoallergenic variants with the aim to reduce IgE-mediated side effects during immunotherapy. The efficacy of immunotherapy combined with recombinant based hypoallergenic allergens has demonstrated promising results. For example, subcutaneous immunotherapy trials conducted using recombinant hypoallergenic
variants of grass and birch pollen allergens showed that recombinant vaccines have vaccination characteristics and is clinically effective [116]. Therefore, availability of an IgE reactive recombinant hen’s egg yolk allergen Gal d 5 provide the means for development of hypoallergenic derivatives using techniques such as site-directed mutagenesis (SDM) [147]. Consequently, availability of hypoallergenic rGal d 5 variants in the future may open new treatment strategies to cure egg allergy.
Chapter 4: Production of hypoallergenic variant of recombinant hen’s egg yolk allergen chicken serum albumin (Gal d 5)
4.1 Introduction

The use of genetically engineered recombinant based hypoallergenic variants in SIT has proved its therapeutic supremacy over crude allergen extracts during SIT [116]. Furthermore, the reduced risk of side effects in patients undergoing SIT with hypoallergens has been well commended [116, 119]. For example, recombinant based hypoallergenic variants of peanut and birch pollen allergens has been shown to perform SIT without the risk of severe allergic reactions in patients [119, 148]. In Chapter 3, we produced an immunologically active recombinant version of Gal d 5 with similar IgE reactivity to its wild type counterpart, which may be useful as standardised reagents in diagnostics in the future. In addition, rGal d 5 produced in the previous chapter can be used as a platform to develop hypoallergenic variants with reduced IgE binding capacity for potential use in SIT for patients suffering from hen’s egg yolk allergy.

The availability of information regarding IgE binding allergenic epitopes can immensely benefit the production of hypoallergens. IgE binding epitopes could be either sequential or conformational. Reducing or complete elimination of IgE binding can be achieved by introducing a number of mutations targeting the allergenic epitopes using techniques such as SDM. Furthermore, in addition to serving as a tool for introducing mutations to a DNA sequence, SDM can also be used to successfully map epitopes of allergens, especially conformational epitopes [147, 148].

A common approach to develop hypoallergenic mutants is by changing the overall conformation of the allergen by disrupting disulfide bonds (DB) by using the SDM technique. DBs can be deleted by substituting corresponding cysteine residues that form DBs with a different amino acid by introducing appropriate mutations to the DNA sequence. Systematic disruption of DBs allows researchers to evaluate the
relationship between the allergen confirmation and the degree of IgE-mediated immune response. Gal d 5 is stabilized by 17 DBs [142]. In this chapter, our primary aim was to develop a mutant variant of Gal d 5 with reduced IgE binding capacity, which could be used as a SIT hypoallergen in the future. However, due to the lack of data regarding IgE binding antigenic epitopes of Gal d 5, an alternative approach was required to develop a mutant with reduced IgE binding reactivity.

In this chapter, we first developed a mutant variant of Gal d 5 by simultaneously disrupting two randomly selected DBs, by substituting cysteine (Cys) with alanine (Ala). We hypothesised that removal of DBs would result in a mutant variant of Gal d 5 with reduced or no IgE-binding reactivity at all. In order to test the hypothesis, we comparatively analysed the IgE binding capacity of recombinant based mutant Gal d 5 and native Gal d 5 using patients’ sera. The mutant variant of Gal d 5 was developed by deleting two DBs at Cys196-Cys205 and Cys388-Cys397 by replacing cysteine residues at positions 205 and 397 with alanine, using SDM. The mutant Gal d 5 (Gal d 5\[^{196/205-388/397}\]) DNA sequence was generated using the pThcHis A-Gal d 5 construct as the template from Chapter 3. The Gal d 5\[^{196/205-388/397}\] DNA was selectively amplified and subcloned into pKLAC2 expression vector to synthesise pKLAC2- Gal d 5\[^{196/205-388/397}\] construct for expression in *K. lactis* yeast strain. However, recombinant Gal d 5\[^{196/205-388/397}\] (rGal d 5\[^{196/205-388/397}\]) was not expressed by *K. lactis* despite successful cloning.

Therefore, we decided to express Gal d 5\[^{196/205-388/397}\] cDNA in an *E. coli* expression system. The mutant plasmid pThcHis A-Gal d 5\[^{196/205-388/397}\] produced by SDM was used to transform *E. coli* cells for expression of rGal d 5\[^{196/205-388/397}\]. Here, expression of rGal d 5\[^{196/205-388/397}\] as a soluble fraction by *E. coli* was successful. The contribution of the combined effect of disrupting Cys196-Cys205 and Cys388-Cys397 of Gal d 5 to human IgE binding was comparatively analysed with native
Gal d 5 expressed by *E. coli* and *K. lactis* in the previous chapters. The immunoassay results showed that IgE binding capacity of rGal d 5<sup>196/205-388/397</sup> has been completely abolished due to disruption of DBs. Therefore, rGal d 5<sup>196/205-388/397</sup> may contain a therapeutic value as a hypoallergen. However, lack of IgE reactivity of a mutant variant based on Western immunoassay does not necessarily confirm it as a hypoallergen. Even with reduced IgE binding capacity, the allergenicity of some mutant variants of allergens are known to be unaffected [148]. Therefore, the true allergenicity of the mutant Gal d 5 produced in this chapter must be assessed using techniques such as mediator release assays and T cell proliferation assays in order to confirm its immune reactivity.

4.2 Materials and methods

4.2.1 Site-directed mutagenesis of Gal d 5

Gal d 5<sup>196/205-388/397</sup> variant, with two Cys-Ala substitutions leading to the disruption of the corresponding DB, were generated by using the QuickChange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The construct pTrcHis A-Gal d 5 synthesised in Section 3.2.1.4 was used as the template for mutant strand synthesis. Mutagenic primers were designed using the web-based QuickChange Primer Design Program available online at [www.agilent.com/genomics/qcpd](http://www.agilent.com/genomics/qcpd). Two TGC codons corresponding for cysteine residues at 205 and 397 (UniProt identifier #P19121) were changed to GCC codons for alanine using primers Cys205 and Cys397 and their corresponding reverse primers (Table 4.1).
Table 4.1: Mutagenic primers used for disruption of disulfide bridges in Gal d 5 (CSA). Substitutions of Cys by Ala residues are indicated by the codon GCC bolded and underlined.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Position of disulfide bond</th>
<th>Targeted cysteine residue</th>
<th>Mutagenic primers (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys205</td>
<td>196-205</td>
<td>205</td>
<td>GAGTGATGTCGGTGGCTGCCCTGGACACCAAGG</td>
</tr>
<tr>
<td>Cys397</td>
<td>388-397</td>
<td>397</td>
<td>AACTGATAAACCCTGCTGAGGCCCTACGCAAATGCTCAAGAG</td>
</tr>
</tbody>
</table>

Site-directed mutagenesis reaction was prepared by mixing 2.5 µL of 10× QuickChange Lighting Multi reaction buffer, 16.08 µL of double-distilled water, 0.75 µL of QuikSolution, 100 ng of template DNA, 100 ng of each mutagenic primer, 1 µL of dNTP and 1 µL of QuikChange Lighting Multi enzyme blend in a 25 µL total reaction volume in a sterile PCR tube. The site-directed mutagenesis reaction was then subjected to PCR according to the cycling parameters outlined in Table 4.2. Following temperature cycling, the reaction was placed on ice for 2 minutes to cool below 37°C. Then 1 µL of Dpn I restriction enzyme was added to the amplification reaction and incubated at 37°C for 5 minutes to digest the parental non-mutated double stranded DNA (ds-DNA).

Table 4.2: Thermal cycling conditions for mutant strand synthesis

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>95°C</td>
<td>20 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65°C</td>
<td>30 seconds/kb of plasmid length</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>65°C</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

4.2.2 Transformation of XL10-Gold ultracompetent cells

Following digestion of parental ds-DNA, mutated single stranded DNA (ssDNA) was transformed into XL10-Gold ultracompetent cells (Agilent Technologies, Santa
Clara, CA, USA). First, a tube of XL10-Gold ultracompetent cells was thawed on ice. Then, 45 µL of ultracompetent cells were mixed with 2 µL of β-ME provided with the kit in a pre-chilled Eppendorf tube and mixed by swirling. The tube was incubated on ice for 10 minutes, swirling gently every 2 minutes. Then 1.5 µL of the Dpn I treated DNA was transferred into the aliquot of ultracompetent cells and gently mixed by swirling. The transformation reaction was incubated on ice for 30 minutes. Following incubation, the tube was heat-pulsed at 42°C for 30 seconds in a heat-block and incubated on ice for 2 minutes. Then, 0.5 mL of preheated (42°C) NZY+ broth was added to the tube and incubated at 37°C for 1 hour with shaking at 250 rpm. The transformation reaction was then spread on LB agar plates containing 50 µg/mL ampicillin and incubated overnight at 37°C.

4.2.3 Identifying colonies harbouring pTrcHis A-Gal d 5196/205-388/397 construct

E. coli colonies grown on LB agar plates were propagated in fresh LB containing 50 µg/mL ampicillin and plasmids were isolated according to the method outlined in Section 2.2.2.6. Then, colonies containing pTrcHis A-Gal d 5196/205-388/397 were verified by plasmid DNA sequencing using pTrcHis forward primer (5’-GAGGTATATATTAATGTATCG-3’) and pTrcHis reverse primer (5’-GATTTAATCTGTATCAGG-3’). The sequencing was carried out at Micromon, Monash University.

4.2.4 Cloning and expression of rGal d 5196/205-388/397 in K. lactis

4.2.4.1 Cloning of Gal d 5196/205-388/397 in K. lactis

The Gal d 5196/205-388/397 gene was amplified using PCR technique with Xhol and NotI restriction sites incorporated on 5’ and 3’ ends, respectively with a C-terminal HA epitope tag to facilitate cloning into the pKLAC2 expression vector. The pTrcHis A-
Gal d$^{5196/205-388/397}$ construct was used as the template. The PCR was done using QIAGEN Fast Cycling PCR kit according to the method outlined in Section 3.2.2.3. The PCR amplified Gal d$^{5196/205-388/397}$ gene was cloned into the XhoI and NotI sites in frame with the $\alpha$-MF secretion leader sequence of the pKLAC2 vector to yield pKLAC2-Gal d$^{5196/205-388/397}$ construct according to the method outlined in Section 3.2.2.4. The pKLAC2-Gal d$^{5196/205-388/397}$ was then digested using SacII restriction enzyme to produce the linearized expression cassette and desalted according to the method outlined in Section 3.2.2.5. Then, linearized expression cassette containing Gal d$^{5196/205-388/397}$ was chemically transformed into competent K. lactis cells according to the method outlined in Section 3.2.2.6. The transformed K. lactis cells were selected by growing on YCB agar medium containing 5 mM acetamide and incubated at 30°C for 3.5 days until colonies formed. Then, K. lactis strains harbouring tandem copies of expression cassette were scored by whole cell PCR technique according to the method outlined in Section 3.2.2.7.

**4.2.4.2 Detection of secreted rGal d$^{5196/205-388/397}$ by transformed K. lactis**

Transformed K. lactis strains containing multi-copy integrants were screened for its ability to secrete rGal d$^{5196/205-388/397}$ in YPGal medium. A small aliquot of frozen K. lactis cells was revived on YCB agar medium containing 5 mM acetamide by incubating at 30°C for 2 days. For expression, individual K. lactis colonies were grown in 5 mL of YPGal medium at 30°C with shaking at 250 rpm for 2 days. Then, culture supernatants were harvested by centrifugation and analysed by SDS-PAGE and Western blot technique for the presence of secreted rGal d$^{5196/205-388/397}$ according to the method outlined in Section 3.2.2.8.
4.2.4.3 Cloning and expression of rGal d 5\textsuperscript{196/205-388/397} in \textit{E. coli}

pTrcHis A- Gal d 5\textsuperscript{196/205-388/397} construct synthesised previously was chemically transformed into NEB 5-alpha F' Competent \textit{E. coli} cells according to NEB high efficiency transformation protocol mentioned in Section 2.2.2.6. \textit{E. coli} colonies harbouring the pTrcHis A-Gal d 5\textsuperscript{196/205-388/397} construct were grown on LB agar under ampicillin resistance. Then \textit{E. coli} transformants were subjected to a time course of expression study to determine the optimum recombinant protein expression conditions. The time course of expression analysis was conducted through SDS-PAGE and Western immunoblot technique according to the method outlined in Section 2.2.2.9. Here, only soluble fractions of \textit{E. coli} culture fractions were analysed by SDS-PAGE and Western blot for the presence of rGal d 5\textsuperscript{196/205-388/397}. For the Western blot monoclonal Anti-Xpress\textsuperscript{TM} antibody produced in mouse was used as the detection antibody.

4.2.5 Immunological analysis of rGal d 5 and mutant rGal d 5\textsuperscript{196/205-388/397}

A Western immunoassay was conducted to comparatively analyse the level of egg white allergic patients’ IgE binding to rGal d 5 produced by \textit{K. lactis} in Chapter 3, rGal d 5 produced by \textit{E. coli} as an insoluble fraction in Chapter 3 and mutant rGal d 5\textsuperscript{196/205-388/397} produced by \textit{E. coli} in Chapter 4. Crude \textit{E. coli} extracts containing rGal d 5, mutant rGal d 5\textsuperscript{196/205-388/397} and purified \textit{K. lactis} derived rGal d 5 were electroblotted to a nitrocellulose membrane according to the method outlined in Section 2.2.1.4.1. The identities of each rGal d 5 proteins were again verified through Western blot technique using the relevant primary antibodies prior immunoassay using the method outlined in Section 2.2.1.4.1. The protein immobilised nitrocellulose membrane was stained with Ponceau S solution for visualization of
proteins. The regions containing rGal d 5 proteins were separated by cutting with a scissor. The rGal d 5 immobilised nitrocellulose membranes were destained with 0.1 M NaOH and blocked with blocking solution. Protein immobilised membranes were individually incubated overnight with pooled sera (diluted 1:10 in blocking solution) from patients sensitized to nGal d 5 (patients 3, 10, 15, 16, 17, 19, 24 and 25) at 4°C. As a negative control, all rGal d 5 immobilised membranes were incubated with non-allergic patients’ sera (diluted 1:10 in blocking solution) as well. Then, all serum pre-incubated nitrocellulose membranes were incubated with monoclonal anti-human IgE antibody produced in goat labelled with alkaline phosphatase (diluted 1:1000 in blocking solution) for 1 hour at room temperature. The signal was developed by adding BCIP/NBT chromogenic substrate.

4.3 Results

4.3.1 Recombinant production of Gal d 5<sup>196/205-388/397</sup> in K. lactis

Transformed XL10-Gold ultracompetent cells with pTrcHis A-Gal d 5<sup>196/205-388/397</sup> construct following SDM had growth under ampicillin resistance. Transformant colonies were screened for plasmids bearing the mutation Cys to Ala at positions 205 and 397 by plasmid DNA sequence analysis. The Nucleotide BLAST search of the derived plasmid DNA sequences produced Gallus gallus albumin (sequence ID: refINM 205261.2) as the top match. Furthermore, sequence alignment comparison of the mutant and non-mutant Gal d 5 DNA confirmed the successful mutations of native sequence TGC into GCC at Cys 205 (Figure 4.1) and Cys397 (Figure 4.2).

The full-length Gal d 5<sup>196/205-388/397</sup> gene amplified with restriction sites Xhol and NotI and a C-terminal HA tag showed a single predominant band at approximately 2000 bp marker (expected size: 1827 bp) when electrophoresed on agarose gel (Figure 4.3, A). NEB Express F' Competent E. coli cells transformed with newly
synthesised pKLAC2-Gal d 5^{196/205-388/397} construct had growth when grown on LB agar under ampicillin resistance. However, only one colony was able to grow on transformation plates. When XhoI and NotI digested plasmid of the transformant clone were electrophoresed on agarose gel, an insert at approximately 2000 bp marker confirming the successful construction of pKLAC2-Gal d 5^{196/205-388/397} (expected size: 1827 bp) (Figure 4.3, B). Sequencing analysis of plasmids harbouring the Gal d 5^{196/205-388/397} insert confirmed the ligation of Gal d 5^{196/205-388/397} in-frame with the α-MF secretion signal of the pKLAC2 vector. The NCBI BLAST search of the derived sequence produced Gallus gallus albumin (sequence ID: refINM 205261.2) as the top match with a 97% sequence similarity. Furthermore, sequence alignment comparison of the mutant and non-mutant Gal d 5 DNA confirmed the presence of mutations introduced to pTrcHis A- Gal d 5^{196/205-388/397} (Figure 4.4 and Figure 4.5).
Figure 4.1: Nucleotide BLAST search result of the pTrcHis A-Gal d 5\textsuperscript{196}/205-388/397 DNA sequence (plus strand) obtained from pTrcHis forward primer. The sequence in the box shows the nucleotide substitution that resulted by SDM on the plus strand. Nucleotides T and G of the codon TGC (Cys 205) substituted by nucleotides G and C resulting in codon GCC (Ala 205).
Figure 4.2: Nucleotide BLAST search result of the pTrcHis A-Gal d 5'196/205-388/397 DNA sequence (minus strand) obtained from pTrcHis reverse primer. The sequence in the box shows the mutations resulted by SDM on the minus strand. Nucleotides C and A of the anti-codon GCA (codon TGC: Cys 397) substituted by nucleotides G and C resulting in anti-codon GGC (codon GCC: Ala 397).
Figure 4.3: (A) PCR of Gal d 5196/205-388/397. The PCR product was electrophoresed on E-Gel® CloneWell™ agarose gel with 0.8% SYBR Safe™ DNA Gel stain. Lane 1: PCR amplified Gal d 5196/205-388/397. The arrow indicates the PCR amplified Gal d 5196/205-388/397. (B) Restriction enzyme digest screening of the colony harbouring the pKLAC2- Gal d 5196/205-388/397 construct. Lane 1: XhoI and NotI digested pKLAC2- Gal d 5196/205-388/397 construct of the transformant clone. The arrow indicates the Gal d 5196/205-388/397. MW: molecular weight marker (Quick-Load Purple 2-Log DNA ladder).
Figure 4.4: Nucleotide BLAST search result of the pKLAC2- Gal d 5196/205-388/397 DNA sequence (plus strand) obtained from pTrcHis forward primer. The sequence in the box shows the nucleotide substitution that resulted by SDM on the plus strand. Nucleotides T and G of the codon TGC (Cys 205) substituted by nucleotides G and C resulting in codon GCC (Ala 205).
Figure 4.5: Nucleotide BLAST search result of the pKLAC2-Gal d 5\textsuperscript{196/205-388/397} DNA sequence (minus strand) obtained from pTrcHis reverse primer. The sequence in the box shows the mutations that resulted by SDM on the minus strand. Nucleotides C and A of the anti-codon GCA (codon TGC: Cys 397) substituted by nucleotides G and C resulting in anti-codon GGC (codon GCC: Ala 397).
The pKLAC2-Gal d 5^{196/205-388/397} construct was digested with SacII restriction enzyme in order to facilitate insertion of the expression cassette into the K. lactis genome by integrative transformation. Electrophoresis of SacII digested pKLAC2-Gal d 5^{196/205-388/397} construct on 1% (w/v) agarose gel showed two bands approximately at 2.8 kb and the other with a size of >6.2 kb confirming the successful digestion of the plasmid (Figure 4.6). In this study, we only scored for K. lactis strains harbouring multiple copies of the expression fragment by whole cell PCR. Analysis of whole cell PCR products showed an amplification product approximately at 2.3 kb in K. lactis clones when electrophoresed on 1% (w/v) agarose gel (Figure 4.7). Only strains containing tandem copies of expression fragment amplifies a PCR product of 2.3 kb. Our results show that the 87.5% (7 out of 8) of the K. lactis transformant population to bear multi-copy integrants.

All the K. lactis strains bearing multi copy integrants were screened their ability to secrete rGal d 5^{196/205-388/397} in YPGal medium. Analysis of culture supernatant samples on SDS-PAGE showed multiple secreted proteins by K. lactis strains (Figure 4.8). The Western blot analysis of the corresponding SDS-PAGE produced a negative result (result not shown). Western blot analysis confirmed that all the K. lactis strains tested did not secrete the rGal d 5^{196/205-388/397} into the culture media.
Figure 4.6: *SacII* digest of the pKLAC2-Gal d 5<sup>196/205-388/397</sup> construct. The digested plasmid was electrophoresed on E-Gel<sup>®</sup> CloneWell™ agarose gel with 0.8% SYBR Safe™ DNA Gel stain. Lane 1: *SacII* digest of the pKLAC2- Gal d 5<sup>196/205-388/397</sup> construct. Lane MW: molecular weight marker in kb (Quick-Load Purple 2-Log DNA ladder). Arrow a indicates the expression cassette containing the Gal d 5 gene. Arrow b indicates the remainder of the pKLAC2 vector.

Figure 4.7: Whole cell PCR analysis for screening of *K. lactis* strains containing multi-copy integrants. The PCR product was electrophoresed on E-Gel<sup>®</sup> CloneWell™ agarose gel with 0.8% SYBR Safe™ DNA Gel stain. Lane 1-7: *K. lactis* test strains harbouring multi-copy integrants. Arrow indicates the amplicon at 2.3 kb. Lane 8: *K. lactis* test strain without multi-copy integrants. MW: molecular weight marker (Quick-Load Purple 2-Log DNA ladder).
4.3.2 Recombinant production of rGal d 5^{196/205-388/397} in *E. coli*

*E. coli* cells transformed with pTrcHisA-Gal d 5^{196/205-388/397} construct had growth when grown on LB agar under ampicillin resistance. Pilot expression study confirmed the expression of rGal d 5^{196/205-388/397} as a soluble fraction. The SDS-PAGE analysis of soluble *E. coli* culture fractions showed multiple proteins (Figure 4.9, A). The corresponding Western blot analysis of soluble *E. coli* culture fractions showed a predominant band in between 64 kDa and 98 kDa markers (expected size: 69 kDa) (Figure 4.9, B). The optimum recombinant protein production was seen at 4-hour post IPTG induction.
Figure 4.9: Time-course of expression of soluble *E. coli* cell culture fractions harbouring rGal d $5^{196/205-388/397}$. Lane 1: un-induced culture fractions. Lanes 2-4: IPTG induced culture fractions at time points 3rd, 4th and 5th hours, respectively analysed by (A) SDS-PAGE and (B) Western immunoblot probed with monoclonal Anti-Xpress™ antibody produced in mouse. MW: molecular weight marker in kDa (SeeBlue Pre-Stained protein standard). Arrows indicate rGal d $5^{196/205-388/397}$.

### 4.3.3 Immunological analysis of non-mutant and mutant rGal d 5

The identities of each rGal d 5 were confirmed prior immunoassay through Western blot technique. The recombinant proteins on Ponceau S stained membrane and antibody-probed membranes were comparable (Figure 4.10). Immunological reactivity of rGal d 5 produced by *K. lactis*, insoluble rGal d 5 produced by *E. coli* and mutant rGal d $5^{196/205-388/397}$ produced by *E. coli* were comparatively analysed in terms of IgE binding capacity through Western immunoblot (Figure 4.11). Western immunoblot performed with pooled patients’ sera showed that rGal d 5 expressed by *K. lactis* to have the highest IgE reactivity. The insoluble rGal d 5 also had detectable IgE reactivity however, at a very low level. Western immunoblot of rGal d $5^{196/205-388/397}$ showed no IgE reactivity at all. The control Western immunoblots conducted with non-allergic individuals’ sera did not show IgE reactivity against any rGal d 5.
Figure 4.10: Western blot analysis of non-mutant rGal d 5 expressed by *K. lactis* and *E. coli* and mutant rGal d 5$^{196/205-388/397}$ produced by *E. coli*. (A) Ponceau S stained blot: Lane 1: purified rGal d 5 expressed by *K. lactis*. Lane 2: non-mutant rGal d 5 produced by *E. coli* as an insoluble fraction (crude cell culture extract). Lane 3: mutant rGal d 5$^{196/205-388/397}$ produced by *E. coli* as a soluble fraction (crude cell culture extract). (B) Western blot of rGal d 5 expressed by *K. lactis* probed with monoclonal anti-HA antibody produced in mouse, Lane 1: immunoprecipitation purified rGal d 5. (C) Western blot of rGal d 5 expressed by *E. coli*. Lane 1: non-mutant rGal d 5 expressed by *E. coli* as an insoluble fraction (crude cell culture extract). Lane 2: mutant rGal d 5$^{196/205-388/397}$ produced by *E. coli* as a soluble fraction (crude cell culture extract) probed with monoclonal Anti-Xpress™ antibody produced in mouse. MW: molecular weight marker in kDa (PageRuler Plus Prestained Protein Ladder).

Figure 4.11: Western immunoblots conducted using nGal d 5 sensitised patients’ sera against non-mutant rGal d 5 expressed by *K. lactis* and *E. coli* and mutant rGal d 5$^{196/205-388/397}$ produced by *E. coli*. Lane 1: non-mutant rGal d 5 expressed by *K. Lactis*. Lane 2: non-mutant rGal d 5 expressed as an insoluble fraction by *E. coli*. Lane 3: mutant rGal d 5$^{196/205-388/397}$ produced by *E. coli* as a soluble fraction. (A) Western immunoblot incubated with nGal d 5 sensitized patients’ sera. (B) Negative control Western immunoblot incubated with non-allergic sera. MW: molecular weight marker in kDa (PageRuler Plus Prestained Protein Ladder).
4.4 Discussion

In Chapter 3, we successfully cloned the cDNA for Gal d 5 and demonstrated that the recombinant protein retained the human IgE binding reactivity. Furthermore, our results in Chapter 3 confirmed that rGal d 5 retained the antigenic structure of the native allergen, by quantitative IgE binding assays using sera from sensitised patients. Therefore, these results indicate that the rGal d 5 is a suitable model system for producing hypoallergenic variants and for investigating the antigenic determinants of Gal d 5. The main objective of Chapter 4 was to produce a mutant variant of Gal d 5 with reduced IgE binding capacity for development of a hypoallergen, for potential future use in SIT.

The first challenge of this study was to successfully introduce mutations to the cDNA sequence of Gal d 5, in order to reduce the IgE binding reactivity. However, linear or conformational IgE-binding epitopes of Gal d 5 are not yet described. Therefore, we simultaneously disrupted two randomly selected DBs via SDM. In certain allergens the disruption of intramolecular DBs has been shown to induce protein unfolding and decrease of allergenicity [148]. Using our mutagenesis strategy, one cysteine from each pair of 2 selected DBs was replaced with alanine, preventing the disulfide bond from forming at that position. When Cys196-Cys205 and Cys388-Cys397 lacking Gal d 5 cDNA was cloned into the K. lactis, no secreted recombinant protein was detected in the spent culture media. However, our results confirmed that transformed K. lactis colonies grown under acetamide selection had multiple copies of expression fragments containing rGal d 5^196/205-388/397.

There could be several reasons why a recombinant protein is poorly expressed or not expressing at all. Toxicity of the protein to the host cell, insolubility or mRNA secondary structure preventing interactions with the cellular machinery and lack of
DB formation are some reasons causing difficulties with recombinant protein expression [143, 149]. The mutant Gal d 5 cDNA was cloned in frame with α-MF secretion leader sequence in order to facilitate secretion of the recombinant protein. If protein expression was achieved, the α-MF domain directs the fusion protein to be efficiently transported through the *K. lactis* secretory pathway into the growth medium. Complete lack of secreted recombinant protein indicates that expression of the fusion peptide was not directed by the *K. lactis* protein production machinery. The most likely reason for this is that the mutated mRNA Gal d 5 secondary structures may be blocking interactions with the cellular machinery and thereby preventing translation of the recombinant protein. Furthermore, the mutant Gal d 5 proteins may have undergone incorrect folding due to DB disruption, and thereby not being able to secrete into the growth media. If this is the case, the improperly folded mutant Gal d 5 may exist in yeast cell pellets. The main goal of the project is to produce a biologically active mutant Gal d 5 protein lacking IgE-reactivity. If mutant Gal d 5 has been produced as IBs it is more likely to be non-functional and offer very little or no biomedical value for experiments conducted in this PhD project and future applications suggested [131, 132]. As a result, we did not attempt to extract mutant Gal d 5 proteins from yeast cell pellets.

In order to achieve rGal d 5^{196/205-388/397} expression, pTrcHis A-Gal d 5^{196/205-388/397} construct generated from SDM was transformed into NEB 5-alpha F’ Competent *E. coli* strain. Here, the rGal d 5^{196/205-388/397} was expressed as fusion peptide with a C-terminal 6×His tag. The Western blot of the IPTG induced soluble culture fractions confirmed that the rGal d 5^{196/205-388/397} was expressed under the direction of *Ptrc* as a soluble fraction. To retain the biomedical value, it is essential to produce the recombinant protein as a soluble fraction in its native form [131, 132]. Therefore,
we did not analyse the insoluble culture fraction and therefore, our result does not indicate the expression levels of insoluble rGal d 5\textsuperscript{196/205-388/397}.

Previously \textit{E. coli} failed to express wild type Gal d 5 as a soluble fraction. However, \textit{E. coli} cells were able to express mutant Gal d 5 as a soluble fraction. This again proves the unpredictable nature of protein production host platforms [140]. A possible explanation for this double nature of the \textit{E. coli} host expression system may attributed to the presence or absence of DBs. \textit{E. coli} cells were only successful at expressing mutant Gal d 5 which lacked two DBs as a soluble fraction. It is well known that the cytoplasm of \textit{E. coli} cells have a reducing effect on DBs, and thereby preventing the formation of DBs resulting in IBs [143]. The lack of DBs in mutant Gal d 5 may have contributed to undergo proper folding in the \textit{E. coli} cytoplasm.

The Western immunoassay results confirmed that simultaneous deletion of Cys196-Cys205 and Cys388-Cys397 DBs resulted in complete elimination of IgE reactivity. As per the non-mutant recombinant Gal d 5, we analysed \textit{K. lactis} and \textit{E. coli} expressed proteins as well. As expected, native rGal d 5 expressed by \textit{K. lactis} as a soluble fraction had very strong IgE reactivity when compared to \textit{E. coli} derived insoluble rGal d 5 and mutant rGal d 5\textsuperscript{196/205-388/397}. This is mainly due to the fact that \textit{K. lactis} expressed native rGal d 5 had maintained the antigenic structure which contributes towards IgE binding better than the latter two. Unlike \textit{E. coli}, eukaryotic systems like \textit{K. lactis} may result in posttranslational modifications leading to better conservation of the native structure of Gal d 5. Furthermore, expression of rGal d 5 as a soluble fraction by \textit{K. lactis} guarantees the proper folding of the protein again resulting higher structural similarity to the native protein.

Furthermore, this result validates the dot-blot immunoassay conducted with rGal d 5 in Chapter 3 where rGal d 5 was shown to be IgE reactive. Insoluble rGal d 5 expressed by \textit{E. coli} had very low but detectable IgE reactivity. This confirmed that
the native rGal d 5 expressed as an IB has not completely lost its IgE reactivity. However, when compared with non-mutant rGal d 5 expressed by K. lactis, it was clear that significant level of IgE reactivity of E. coli derived rGal d 5 was lost due to improper folding.

The elimination of IgE binding reactivity can be directly attributed to the disruption of the two DBs. The disruption of two DBs may have altered the confirmation of native Gal d 5 and lead to protein unfolding. Therefore, it is clear that the Cys196-Cys205 and Cys388-Cys397 DBs together have a significant contribution to maintaining the IgE binding structure of Gal d 5. However, this result does not reject the existence and contribution of linear IgE binding epitopes to the IgE binding reactivity. Sequential IgE binding epitopes may also require a certain structural context for proper interactions with IgE antibodies. As a result, disruption of DBs may have reduced or completely impaired access to sequential IgE binding epitopes due to the modified confirmation [148]. However, another contributing factor leading to lack of IgE-reactivity of mutant Gal d 5 could be the availability of less mutant protein immobilized on the nitrocellulose membrane, compared to the natural Gal d 5. Reduced amount of mutant Gal d 5 may have resulted in less IgE-reactivity being detected. According to the visual analysis of the Western blot there were reasonable amount of mutant Gal d 5 available for IgE-reactivity. As a consequence, complete lack of IgE-reactivity of shown by this experiment can still be considered reliable. However, complete lack of IgE-reactivity may attribute to the combined effect of the mutation along with reduced amount of mutant Gal d 5 on the nitrocellulose membrane.

Although rGal d 5^{196/205-388/397} produced in this chapter confirms complete lack of IgE reactivity, our results are not sufficient to verify it as a hypoallergen. Previous studies
have shown discrepancies between IgE-binding reactivity and allergenic potency. Even with reduced IgE binding reactivity following DB elimination, some recombinant mutants retained the allergenicity [148]. These results confirmed that the disruption of DBs have no effect on the allergenic potency. Therefore, it is important to further extend investigation of rGal d 5 196/205-388/397 in order to evaluate its allergenicity in comparison to its native counterparts. Analysis of allergenicity can be conveniently performed by subjecting rGal d 5 196/205-388/397 into mediator release assays using cells expressing human FcεRI for IgE. Determination of levels of mediator release induced by mutant rGal d 5 proteins can provide a better understanding of the effect of deleting DBs on the allergenic nature. In addition, it is important to show that such hypoallergens maintain their ability to proliferate T cells so that they can modulate the non-allergenic (Th1 and Tr1) immune pathway.

In conclusion, our results provide some understanding of the contribution of Cys196-Cys205 and Cys388-Cys397 to the IgE binding reactivity. The initial step of producing a hypoallergen is successfully performed in this chapter. Therefore, the mutant Gal d 5 designed in this chapter is a potential candidate for specific immunotherapy applications. It would now be of great interest to further extend this work to confirm the hypoallergenic nature of the rGal d 5 196/205-388/397.
Chapter 5: General discussion
Allergy to hen’s egg is one of the most common forms of food allergies affecting humans. Research conducted regarding food allergies generally investigates the whole food source in order to understand the relationship between disease and the causative allergens. Surprisingly, in allergy to hen’s egg, only the egg white proteome has been subjected to investigation, leaving egg yolk proteome untouched by vast majority of studies. The egg yolk was mainly excluded in research because initially it was not found to cause severe allergic reactions like allergens from the egg white. Therefore, knowledge regarding allergy to egg yolk did not have much progression like egg white allergy. As a result, misunderstandings regarding hen’s egg yolk allergy may have occurred especially with clinicians and other health care workers. It is widely believed that allergy to egg yolk does not exist or at least allergy to egg white is required in order to experience hypersensitivity reactions against egg yolk proteins. The main scope of this PhD thesis was to subject hen’s egg yolk proteome into immunological studies in relation to food allergy. In this PhD project, various proteomic and molecular methods were employed in order to shed light on to IgE-mediated hypersensitivity to hen’s egg yolk proteins. Furthermore, the use of recombinant DNA technology has been very popular in the field of allergy. Recombinant allergens have been successfully used in both diagnosis and treatment of allergies. In addition, recombinant DNA technology has been a powerful tool to investigate allergy at molecular level revealing many facts [116, 118]. As per our knowledge, there are no known published reports regarding recombinant production of Gal d 5 and Gal d 6, the two major allergens of hen’s egg yolk. Therefore, a significant amount of knowledge can be uncovered with the application of recombinant DNA technology to egg yolk allergens.

In Chapter 2, we first investigated if patients allergic to hen’s egg white would have sensitisations to egg yolk proteins from hen, emu and quail. Western immunoassay
result confirmed that many patients tested had genuine sensitisation against multiple egg yolk proteins from all the bird eggs. Interestingly, this finding is contrary to the popular belief that reactivity to egg white is not associated with sensitisation to egg yolk. Currently, there is no available literature regarding immunological studies on any type of bird eggs other than hen’s egg. This result suggests that patients with hen’s egg white allergy can have sensitisations to bird eggs from different species as well. In such patients, sensitisation to those egg yolks may occur as a result of direct exposure or due to common epitopes present among proteins due to high degree of homology. As a result, those patients may develop IgE-mediated hypersensitivity reactions in the event of exposure to those eggs. Therefore, such patients will not be able to consider emu and quail eggs as an alternative to chicken eggs.

Sensitisation to allergens does not necessarily imply clinical hypersensitivity and therefore, our result is unable to confirm clinical allergy in those patients. However, sensitisation is found to be a risk factor for allergy and can be used to predict the development of allergy as in allergy to egg white [3]. The actual state of clinical reactivity to egg yolk in those patients is not known since egg yolk is not used for \textit{in vitro} or \textit{in vivo} diagnosis of allergy. Findings of this result confirm that concomitant sensitisation to egg white and egg yolk is not uncommon and emphasise hen’s egg yolk as an important source of allergens. Therefore, our result suggests that including both the egg white and the egg yolk during diagnosis of egg allergy is very important. As a result, knowledge obtained by using both the egg white and the yolk at diagnosis stage may help avoid unnecessary dietary restrictions and immensely benefit managing egg allergy better.

A limitation of this study was the inability to identify the IgE-reactive egg yolk proteins conclusively due to the use of crude egg yolk preparations. In order to identify proteins precisely the use of purified proteins is essential. However, through
proteomic analysis we were able to identify that most IgE-reactive proteins belong to major precursor proteins of the hen’s egg yolk namely; VTG-1, VTG-2 and Apo B. YGP42 (Gal d 6), which is the C-terminal part of VTG-1, is already confirmed as an allergen. Apovitellenin I and apovitellenin VI that are derived from Apo B are reported to exhibit significant allergenicity in some patients [81, 96, 98, 150, 151]. However, those proteins are not yet confirmed as food allergens. A large number of mature protein fragments derived from those precursors exist in the egg yolk, which may have significant allergenic potential. Therefore, it would be of great interest to subject each mature protein in its purified form to immunological analysis in order to reveal their allergenicity.

The second major aim of this PhD project was to produce IgE-reactive recombinant proteins of both Gal d 5 and Gal d 6. This PhD thesis for the first time presents the successful production of recombinant versions of Gal d 5 and Gal d 6. In Chapter 2, we first successfully cloned and expressed Gal d 6 in *E. coli* as a soluble fraction. Producing recombinant proteins as a soluble fraction in its native form is a common challenge faced by researchers very often. Majority of the rGal d 6 has been produced as IBs. However, there were reasonable amount of soluble rGal d 6 produced by the *E. coli* cells that could be used for purification and other downstream applications. Therefore, enhancement of bacterial cells to increase production of rGal d 6 as a soluble fraction was not necessary.

A crucial stage in recombinant allergy research is the analysis the IgE-reactivity profile of recombinant proteins in comparison to natural counterparts in a given population. Here, we showed that rGal d 6 is IgE-reactive by inhibition ELISA experiments. rGal d 6 and CEY of hen was able to inhibit binding of IgE to each other in similar amounts (approximately 30%). However, it is not possible to
determine the exact IgE binding level of rGal d 6 with this experiment. In order to determine the precise IgE-reactivity it is essential to compare the IgE-reactivity in comparison to purified natural Gal d 6. However, our work confirms that rGal d 6 produced by *E. coli* is IgE-reactive and therefore, can be considered for *in vitro* and *in vivo* diagnosis of egg yolk allergy after further validation of its immune reactivity. Furthermore, rGal d 6 produced here can be used as a platform to develop hypoallergenic variants with reduced IgE binding capacity for potential use in SIT for patients suffering from hen’s egg yolk allergy.

In Chapter 3, we successfully produced an IgE-reactive version of Gal d 5 as a soluble fraction. However, our first attempt to produce rGal d 5 as a soluble fraction using NEB Express I* Competent* E. Coli failed. Although, rGal d 5 was expressed in sufficient amounts, 100% of the recombinant protein was expressed as insoluble IBs. Formation of IBs indicates irregular folding of the recombinant protein losing its native conformation. As a result, rGal d 5 produced by *E. coli* may have lost its immunological potency due to irregular folding, making it less useful for other downstream applications. Gal d 5 is a secreted protein with 17 DBs stabilizing it [142]. DBs are crucial for correct protein folding, stability and functionality. In general, the cytoplasm of *E. coli* cells does not contain a favourable environment for DB formation due to the presence of numerous DB reductases such as thioredoxins and glutaredoxins. These DB reductases quickly reduce DBs formed between two cysteine groups back to its thiolate state. Failure to form DBs in the recombinant proteins, results in it being formed as inactive IBs. Therefore, it is highly possible that the large number of DBs present in Gal d 5 haven’t been formed in the *E. coli* cytoplasm and have undergone IB formation [141, 143].

As an alternative approach, we decided to use a different host strain to express rGal d 5 as a soluble fraction. In Chapter 3, we used the yeast strain *K. lactis* to produce
rGal d 5 as a secreted protein. Here, we successfully expressed rGal d 5 as a secreted protein and isolated with high-level purity and confirmed it as IgE-reactive. Furthermore, ELISA experiment conducted with patients’ sera confirmed that both the nGal d 5 and rGal d 5 have very similar IgE-reactivity. These results suggest that rGal d 5 produced by *K. lactis* is recognizable by the human immune system and bears similar epitopes to that of nGal d 5. As a result, rGal d 5 presented in this PhD project may be an excellent reagent for the use of *in vitro* and *in vivo* diagnosis of allergy to egg yolk. Further confirmation of the results presented in Chapter 3 can be accomplished by subjecting rGal d 5 to immunoassays with sera from patients diagnosed with allergy to hen’s egg yolk.

rGal d 5 produced in Chapter 3 is a suitable model system for producing hypoallergenic variants and for investigating the antigenic determinants of Gal d 5. A large number of recombinant based hypoallergenic variants have been designed by many researchers globally [116, 118, 147, 148]. However, to the best of our knowledge, there are no known published reports on the production of a hypoallergenic variant of Gal d 5. Therefore, in Chapter 4, the main objective was to produce a recombinant-based mutant variant of Gal d 5 for the potential development of a hypoallergen. Here, our mutagenesis strategy involved producing a mutant variant of Gal d 5 by disruption of two DBs simultaneously. Usually, selection of DBs for disruption is based on the IgE binding epitopes of the native allergen in order to impair IgE-binding capacity. However, such information regarding Gal d 5 is not yet available. Therefore, we disrupted two randomly selected DBs in order to induce change in the overall conformation. At this point, advantages of this approach are two-fold. Potential reduction of IgE-reactivity due to DB disruption also indicates the contribution of the targeted DBs to the maintenance of the antigenic structure of
Gal d 5. Previous studies have successfully used disruption of DBs to map conformational epitopes of allergens [147].

In Chapter 4, our initial intention was to express mutant Gal d 5 as a secreted protein using *K. lactis*. Unexpectedly, *K. lactis* cells failed to express and secrete the mutant Gal d 5 despite successful cloning. Successful expression of a given protein is dependent on the nature of the protein and the host system used and there is no universal protein expression system or a predictive tool, which can guarantee the desired outcome. Therefore, this result indicates that the change caused by the mutation had resulted in blocking expression or secretion of the protein since the non-mutant Gal d 5 DNA sequence was successfully expressed and secreted by *K. lactis*. Isolating the exact reasons behind failure to express the mutant Gal d 5 in *K. lactis* without further experimentation is difficult as there may be multiple factors involved. The most likely reason for this may be that the mutated mRNA Gal d 5 secondary structures blocking interactions with the cellular machinery and thereby preventing translation of the recombinant protein. Furthermore, the mutant Gal d 5 proteins may have undergone incorrect folding due to DB disruption, and thereby not being able to secrete into the growth media. If this is the case the improperly folded mutant Gal d 5 may exist in yeast cell pellets. The main goal of the project was to produce a biologically active mutant Gal d 5 protein lacking IgE-reactivity. If mutant Gal d 5 has been produced as IBs, it is more likely to be non-functional and offer very little or no biomedical value for experiments conducted in this PhD project, and future applications suggested in this thesis [131, 132].

As an alternative approach, we decided to express the mutant variant of Gal d 5 in NEB 5-alpha F’ F’ Competent *E. coli* strain as a soluble fraction. Here, the cloning and expression of the mutant Gal d 5 as a soluble fraction was successfully achieved by the *E. coli* protein production machinery. Previously *E. coli* failed to express wild
type Gal d 5 as a soluble fraction. However, *E. coli* cells were able to express mutant Gal d 5 as a soluble fraction. This again proves the unpredictable nature of protein production host platforms [140]. A possible explanation for this double nature of the *E. coli* host expression system may attributed to the presence or absence of DBs. *E. coli* cells were only successful at expressing mutant Gal d 5 which lacked two DBs as a soluble fraction. It is well known that the cytoplasm of *E. coli* cells have a reducing effect on DBs, and thereby preventing the formation of DBs resulting in IBs [143]. The lack of DBs in mutant Gal d 5 may have contributed to undergo proper folding in the *E. coli* cytoplasm.

The Western immunoblot conducted to assess the IgE-binding capacity confirmed that the disruption of DBs have completely abolished the IgE-reactivity of mutant Gal d 5 expressed in *E. coli*. Furthermore, this result confirms that major antigenic epitopes of Gla d 5 are conformational and DBs Cys196-Cys205 and Cys388-Cys397 together are highly important in maintaining the IgE-binding structure of Gal d 5.

The ultimate goal of producing mutant variants with reduced IgE-reactivity is to develop a hypoallergen for successful use in specific immunotherapy. Many research groups have developed mutant variants of allergens with reduced IgE-reactivity using the same strategy as described in this PhD project [147, 148]. However, reduction in IgE-binding capacity does not always imply that the mutant variant is a hypoallergen. This is due to the fact that not all mutants with reduced IgE-reactivity are reported to exhibit reduced allergenicity in reality [148]. Therefore, the mutant Gal d 5 produced in this PhD project must be subjected to further investigations such as mediator release assays and T cell reactivity assays in order to confirm whether it is a true hypoallergen.

In conclusion, the work presented in this PhD thesis provides some valuable insight into IgE-mediated hypersensitivity to hen’s egg yolk, which is one of the least
investigated food allergies. Our initial investigation in Chapter 1 successfully set the stage for entire PhD project by showing that sensitisation to hen’s egg yolk proteins exists in many patients diagnosed with allergy to hen’s egg white. Most IgE reactive proteins were identified as fragments of VTG-1, VTG-2 and Apo B precursors. This shows that there could be potentially undiscovered allergens within the hen’s egg yolk. The rGal d 6 expressed as a soluble fraction was shown to be IgE reactive. However, in future work, it is important to subject rGal d 6 to IgE-binding assays in comparison to natural Gal d 6 in order to confirm the exact level of IgE-reactivity. The rGal d 5 produced as a secreted protein showed very similar IgE-binding reactivity to nGal d 5. Therefore, rGal d 5 can be considered as an excellent reagent for diagnosis of egg yolk allergy. For both of the recombinant allergens produced in this PhD project, further validation for their IgE-reactivity profiles can be brought by conducting immunoassays using sera from patients diagnosed with clinical allergy to hen’s egg yolk. Finally, we produced a recombinant-based mutant variant of Gal d 5 with completely impaired IgE-reactivity. Therefore, mutant Gal d 5 may serve as an excellent therapeutic agent for SIT as a hypoallergen. In order to validate mutant Gal d 5 as a hypoallergen, it is essential to conduct further investigations such as mediator release assays. Not using sera from patients’ allergic to hen’s egg yolk in the immunoassays is a limitation in this PhD project. However, results of this PhD project strongly suggest that allergy to hen’s egg yolk is more common than previously thought and showed that allergy to egg yolk is equally important as allergy to hen’s egg white. As a result, patients with IgE-mediated hypersensitivity to hen’s egg must ideally undergo clinical examinations focusing both on the egg yolk and the egg white, which would lead to better understanding and management of egg allergy. Our work in this PhD has shown the importance of egg yolk as an important
source of food allergens. However, yet egg yolk allergy research can be considered as a developing field and therefore further investigations on the subject is warranted.
References


53. Lundback, B., Epidemiology of rhinitis and asthma. Clinical and Experimental Allergy, 1998. 2: p. 3-10.


57. Marsh, D.G., et al., HLA-Dw2: a genetic marker for human immune response to short ragweed pollen allergen Ra5. I. Response resulting primarily from


126. Caro Contreras, F.J., et al., Allergy to quail's egg without allergy to chicken's egg. case report. Allergologia Et Immunopathologia.


149. NEW ENGLAND BioLabs Inc. Bypassing Common Obstacles in Protein Expression. 2017 [cited 2017; Available from: https://www.neb.com/tools-