Structure and function studies of recombinant clusterin

Yvonne T. Gonzalez Cendales
University of Wollongong

Structure and Function Studies of Recombinant Clusterin

A thesis submitted in (partial) fulfillment of the requirements for the award of the degree of
MASTERS OF SCIENCE
Research

From

UNIVERSITY OF WOLLONGONG

By Yvonne Teresa González Cendales

Supervisor MARK R. WILSON

Department of Biological Sciences
December 2007

DECLARATION

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilling the requirements for the degree Master of Biotechnology - Research. It does not incorporate any material previously published or written by any person except where due reference is made in the text. The experimental work described in this thesis is original work and has not been previously submitted for a degree or diploma at any university.

Yvonne Teresa González Cendales

December 13, 2007.

ACKNOWLEDGEMENTS

I wish to acknowledge with thanks the support, guidance and patience of Professor Mark Wilson, Department of Biological Sciences, University of Wollongong, who has instructed me, given me invaluable advice and reviewed this thesis from its start to finish and without whom this project would not have been accomplished.

I also would like to thank the people in Professor Wilson's laboratory, Amy Wyatt, Elise Steward and Justin Yerbury for their advice and assistance; also to Rebecca Dabbs, who helped me to get started. And especially to Steven Poon, who has guided me through the project.

Many thanks to the people from the University of Wollongong for their continuous support and guidance, especially Mark Dowton and Ren Zhang, Department of Biological Sciences, and James Atkinson and Liz Benny from Campus East.

And finally a big thanks to the people who have helped me through these last two years, without whose support, patience and love this accomplishment would not have been possible. Thank you with all my heart, Kris Nunney, Teresa Cendales de González, Janet Thompson, Jose Francisco González, Timothy Walsh, Angela Bromley and all the Nunney family.

Yvonne Teresa González Cendales
December, 2007.

ABSTRACT

Clusterin is a ubiquitous secreted glycoprotein that acts as an extracellular chaperone. It effectively inhibits stress-induced protein aggregation by an ATP independent mechanism, binding to partially unfolded proteins and forming soluble high molecular weight complexes with them (Wilson and Easterbrook-Smith, 2000). A model proposed by Yerbury *et al.* (2005) suggested that if clusterin mediates the clearance and degradation of misfolded extracellular proteins *in vivo* and this action is anti-inflammatory, a recombinant form of clusterin might be therapeutically useful to clear protein debris from sites of disease and inflammation and in this way suppress pathology. The expression of recombinant mammalian proteins is often performed using mammalian or insect cells because some post-translational processes (glycosylation, phosphorylation, oligomerization, specific protein cleavage and disulfide bond formation) are not performed by bacterial cells (Sambrook *et al*., 1989). However, since Stewart *et al*. (2007) showed that clusterin retains its chaperone function when deglycosylated and that deglycosylation did not induce major structural changes in the molecule, the possibility of producing chaperone-active human clusterin in bacteria became apparent. This thesis describes an attempt to produce a chaperone-active recombinant clusterin expressed in bacteria to provide a large scale supply of protein useful in therapeutic applications. In addition, two different commercially available recombinant clusterin proteins were compared in structure and chaperone function with human serum clusterin to provide additional insights into any functional limitations of recombinantly expressed clusterin proteins and to supply the information to the manufacturer.

Firstly, the expression of clusterin - MBP fusion protein was attempted in *E. coli* TBI but full length recombinant clusterin - MBP fusion protein was only detected as a minor species; a large part of the product was cleaved from MBP during expression and most of the MBP-clusterin fusion protein was degraded with time. Thus, because it was not possible to obtain a source of stable clusterin - MBP fusion protein, the expression of clusterin – 6xHis tag fusion protein was attempted.

Gateway® cloning technology was used for cloning and expressing clusterin – 6xHis tag fusion protein in *E. coli*. After expression, the protein was extracted and purified under denaturing conditions. The purified protein was subsequently refolded in 50 mM NaH_2PO_4, 300 mM NaCl, 1 mM DTT, 10% glycerol, 0.1% azide, alkylated with 50 mM IAA and dialyzed prior to developing functional and structural analyses. After expression in *E. coli* TBI, clusterin – 6xHis Tag fusion protein was detected along with disulfide-bonded high molecular weight aggregates and putative truncated products. While structural studies by CD revealed that the

predicted secondary structure content of clusterin – 6xHis tag fusion protein and plasma-derived clusterin were similar, functional analysis revealed that the former had little if any chaperone activity (its limited effects on the heat-induced precipitation of fibrinogen were not dose-dependent).

Structural (SEC, CD) and functional analyses (chaperone assays and IgG binding test) of Flag-Tagged full-length recombinant human clusterin (expressed in human embryonic kidney cells) and His-Tagged partial-length recombinant rat clusterin (expressed in *E. coli,* not post translationally processed, thus not cleaved into α and β chains and not glycosilated) (both commercial proteins from BioVendor- Laboratory Medicine, Inc.) indicated that the recombinant proteins tested had (i) an increased proportion of high molecular weight species when compared with plasma-derived clusterin, (ii) predicted secondary structure content similar to that of plasma-derived clusterin, (iii) chaperone activity but were less effective than plasma-derived clusterin (recombinant human 28%, recombinant rat 48% less effective) and (iv) were able to bind IgG, the IgG binding activity was dose-dependent and similar to that of plasma-derived clusterin. Furthermore, the results obtained for recombinant rat clusterin suggested that the expression of chaperone active clusterin in bacteria is possible and that cleavage into α and β chains and the C-terminus of the beta chain (residues 22-145) are not required for chaperone or IgG binding activity. Further studies will be required to optimize production of a chaperone active form of human clusterin in bacteria.

.

TABLE OF CONTENTS

Title page	i
Declaration	ii
Acknowledgements	iii
Abstract	iv
Table of Contents	vi
Abbreviations	x
List of Tables	xi
List of figures	xii
1. Introduction	1
1.1 Clusterin Distribution	2
1.2 Clusterin structure	3
1.3 Clusterin function	4
1.3.1 Clusterin as an extracellular chaperone	5
1.3.2 Similarities between clusterin and the small heat shock proteins	6
1.3.3 Role of clusterin in extracellular quality control of protein folding	8
1.3.4 Protein folding diseases and role of clusterin	10
1.4 Aims of the project	12
2. Materials and Methods	14
2.1 General buffers and solutions	14
2.1.1 Buffers	14
2.1.2 Human Clusterin	14
2.2 Expression of clusterin - Maltose Binding Protein (MBP) fusion protein by induction of *E. coli* TBI pMal-c2x-clusterin clones.	14
2.2.1 Bacterial culture media	14
2.2.2 Reagents	15
2.2.3 Protein expression from pMal™ vectors	15
2.2.3.1 Analysis of plasmids for the presence of clusterin-encoding insert by restriction enzyme digestion.	16
2.2.3.2 Protein expression	16
2.2.4 Protein extraction.	16
2.2.4.1 Protocol for small scale protein extraction	17
2.2.4.2 Protocol for large scale protein extraction	17

2.3 Expression of clusterin - 6xHisTag fusion protein using an *E. coli* expression system with Gateway® cloning technology. 17
2.3.1 *att*B-Flanked clusterin-encoding PCR product 19
2.3.1.1 Agarose gel elecrophoresis. 20
2.3.2 Creation of entry clones from attB-flanked PCR products via the BP reaction. 20
2.3.2.1 Transformation of the recombination reaction products into competent cells. 20
2.3.2.2 Analysis of plasmids for the presence of clusterin insert by restriction enzyme digestion. 21
2.3.3 Creation of expression clones by recombination of an entry clone and a destination vector via the LR reaction. 21
2.3.3.1 Transformation of expression vector into competent cells 21
2.3.3.2 Analysis of putative expression plasmids for the presence of clusterin-encoding insert by restriction enzyme digestion 21
2.3.4 Protein expression from Gateway® expression clones 21
2.3.5 Protein extraction. 22
2.3.5.1 Small scale protein extraction 22
2.3.5.2 Large scale protein extraction. 22
2.4 Purification of clusterin using Immunoaffinity chromatography 23
2.4.1 Immunoaffinity chromatography using anti-clusterin monoclonal antibody coupled immunoaffinity columns for purification of clusterin – MBP fusion protein. 23
2.4.2 Immunoaffinity chromatography using Ni-NTA immunoaffinity column for purification of clusterin - 6xHisTag fusion protein 24
2.4.3 Refolding and alkylation of clusterin - 6xHis Tag fusion protein 24
2.4.4 Concentration of protein by ultrafiltration and estimation of protein concentration by absorbance at 280 nm 25
2.5 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie Blue Stain and Western Blot 25
2.5.1 Buffers and reagents 25
2.5.2 SDS-PAGE gel composition 25
2.5.3 SDS-PAGE of recombinant forms and plasma-derived clusterin 26
2.5.4 Western Blot 26
2.5.4.1 Transfer of proteins from SDS-polyacrylamide gels to nitrocellulose. 26
2.5.4.2 Immunodetection of clusterin. 26
2.5.4.3 Immunodetection of MBP fusion protein. 27
2.6 Protein precipitation assays to measure chaperone activity 27
2.7 Analyses of clusterin structure 27
2.7.1 Size exclusion chromatography (SEC) 27
2.7.1.1 SEC to evaluate the structure of clusterin-MBP fusion protein, flag-tagged recombinant human clusterin and His-tagged recombinant rat clusterin. 27
2.7.1.2 SEC to purify clusterin - 6x His Tag fusion protein. 28

2.7.2 Circular dichroism spectroscopy 28
2.7.2.1 CD to evaluate the structure of clusterin - 6x His Tag fusion protein. 28
2.7.2.2 CD to evaluate the structure of flag-tagged recombinant human clusterin and His-tagged recombinant rat clusterin (BioVendor-Laboratory Medicine, Inc). 29
2.8 IgG Binding Assays 30
2.8.1 Evaluating binding of clusterin to human IgG by ELISA. 30

3. Expression, purification and characterization of clusterin - Maltose binding protein (MBP) fusion protein 31
3.1 Introduction 31
3.2 Methods 31
3.2.1 Restriction digestion of purified plasmids to test for the presence of the clusterin encoding insert. 31
3.2.2 Expression and purification of clusterin - MBP fusion protein. 31
3.2.3 Analysis of recombinant clusterin structure and chaperone action. 32
3.3 Results 32
3.3.1 Restriction digestion of purified plasmids to test for the presence of the clusterin encoding insert. 32
3.3.2 Expression of recombinant clusterin protein. 33
3.3.3 Extraction and purification of clusterin – MBP fusion protein. 35
3.3.4 Large scale purification of clusterin – MBP fusion protein. 36
3.3.5 Analysis of the recombinant product structure 37
3.4 Discussion 40

4. Expression, purification and characterization of clusterin - 6xHis tag fusion protein 42
4.1 Introduction 42
4.2 Methods 42
4.2.1 Construction of an *att*B-flanked clusterin-encoding PCR product. 42
4.2.2 Creation of an entry clone from attB-flanked PCR products via the BP reaction. 42
4.2.3 Creation of expression clones by recombination of clusterin-pDONR™201 and the destination pET-DEST42 vector via the LR reaction. 43
4.2.4 Clusterin - 6xHis Tag fusion protein expression from Gateway® expression clones and purification by Ni-NTA affinity chromatography. 43
4.2.5 Analysis of clusterin - 6xHis tag fusion protein structure and chaperone action. 43
4.3 Results 43
4.3.1 Analysis of the *att*B-flanked clusterin-encoding PCR product 43
4.3.2 Creation of an entry clones from attB-flanked PCR products via the BP reaction. 44
4.3.3 Creation of expression clones by recombination of clusterin-pDONR™201 and pET-DEST™42 destination vector via the LR reaction. 46

4.3.4 Protein expression from Gateway® expression clones. 48
4.3.5 Analysis of the structure of the recombinant products. 49
4.3.5.1 SDS-PAGE. 49
4.3.5.2 Circular dichroism spectroscopy. 52
4.3.6 Analysis of the chaperon action of clusterin – 6xHis tag fusion product. 54
4.4 Discussion 55

5. Comparative studies of human plasma-derived clusterin and recombinant human and rat clusterin 57
5.1 Introduction 57
5.2 Methods 58
5.3 Results 58
5.3.1 Analysis of the structure of the recombinant products 58
5.3.1.1 SDS-PAGE 58
5.3.1.2 Size exclusion chromatography. 59
5.3.1.3 Circular dichroism spectroscopy. 61
5.3.2 Analysis of the function of the recombinant products. 62
5.3.2.1 Protein precipitation assays 62
5.3.2.2 IgG binding ELISA assay. 63
5.4 Discussion 64

6. Conclusions 66

References 70

ABBREVIATIONS

Bovine serum albumin	BSA
Citrate synthase	CS
Dithiothreitol	DTT
Iodoacetamide	IAA
Heat-denatured casein	HDC
Immunoglobulin G	IgG
Phosphate buffered saline	PBS
Tris EDTA	TE
Maltose Binding Protein	MBP
Lauria Bertani	LB
Isopropylthio-β-galactoside	IPTG
Tris Acetate EDTA	TAE
PBS/0.1% sodium azide	PBS/Az
Sodium dodecylsulphate polyacrylamide gel electrophoresis	SDS-PAGE
Size Exclusion Chromatography	SEC
Circular dichroism spectroscopy	CD
Millidegrees	mdeg
mean residue ellipticity	θ_{MRE}

LIST OF TABLES

Table 1	Band patterns expected and detected in the agarose gel showed in Figure 9 of *pMal*-c2x-clusterin plasmid pMal-c2x-clusterin after digestion after digestion with *Bgl*I, *Sma*I and *Xba*I	33
Table 2	Band patterns expected and detected in the agarose gel shown in Figure 19 resulting from restriction digestion of entry clone clusterin-pDONR™201 plasmid with *EcoR*I and *Bgl*II.	45
Table 3	Band patterns expected and detected in the agarose gel showed in Figure 20 of expression clone clusterin-pET-DEST™42 after digestion with *Sfi*I.	47
Table 4	Predicted percentages of secondary structural features for clusterin – 6xHis Tag fusion protein and plasma-derived clusterin based on Far-UV CD data and predicted by computational analyses using the prediction program ContinLL	53
Table 5	Predicted percentages of secondary structural features for plasma-derived clusterin, recombinant human clusterin and recombinant rat clusterin based on Far-UV CD data and predicted by computational analyses using the prediction program ContinLL.	61
Table 6	Comparison of characteristics of recombinant human and rat clusterin expressed in *E. coli*.	68

LIST OF FIGURES

Figure 1	Predicted structure of human clusterin	3
Figure 2	Theoretical model for extracellular quality control of non-native extracellular proteins by chaperones	8
Figure 3	pMal™ vector series c2x	15
Figure 4	Integration-excision reaction between *E. coli* and λ bacteriophage in the switch between the lytic and lysogenic pathway	17
Figure 5	Outline of the BP and LR reactions in the Gateway® cloning technology	18
Figure 6	Antibody affinity chromatography	23
Figure 7	CD spectra shown by three common motifs of secondary structure	29
Figure 8	Diagram illustrating clusterin-encoding sequence inserted downstream from the malE gene of *E. coli* TB1in the pMal-c2x vector.	32
Figure 9	Image of an ethidium bromide stained 1% agarose gel showing the results of restriction digestion of pMal-c2x-clusterin plasmids.	33
Figure 10	Image of anti-clusterin Western blot probing for expressed clusterin - MBP fusion protein in non-reduced whole cell lysates of *E. coli* TBI pMal-c2x-clusterin clones.	34
Figure 11	Image of anti-MBP Western Blot probing for clusterin - MBP fusion protein in non-reduced whole cell lysates of *E. coli* TBI pMal-c2x-clusterin clones.	35
Figure 12	Image of anti-clusterin Western blot analysis of non-reduced soluble and insoluble fractions prepared from induced *E. coli* TBI pMal-c2x-clusterin.	36
Figure 13	Image of anti-clusterin Western Blot analysis of non-reduced soluble extract from large scale culture of induced *E. coli* TBI pMal-c2x-clusterin	37
Figure 14	Absorbance (280 nm) traces for size exclusion chromatographic analysis of plasma-derived clusterin and recombinant human clusterin	38
Figure 15	Line of best fit for protein mass versus the elution volume for protein standards after SEC	39
Figure 16	Images of SDS-PAGE (a) and Immunoblot (b) of recombinant and plasma-derived clusterin	39

Figure17	Image of an ethidium bromide stained 1% agarose gel showing attB-clusterin PCR product.	44
Figure 18	Diagram illustrating the entry clone (clusterin-pDONR™201).	44
Figure19	Image of an ethidium bromide stained 1% agarose gels showing Entry clone (pDONR™201+ *att*B Clusterin) before and after restriction digestion with *EcoR*I (a) and with *Hind*II (b)	45
Figure 20a	Figure 20a. Diagram illustrating the resulting expression clone (clusterin - pET-DEST™42).	47
Figure 20b	Image of an ethidium bromide stained 0.8% agarose gel showing putative clusterin - pET-Dest™42 expression plasmids before and after restriction digestion with *Sfi*I.	48
Figure 21	Image of Western Blot analysis using anti-clusterin antibodies of samples of the extracted protein fractions (soluble protein fraction and the inclusion bodies) to corroborate the presence of clusterin – 6xHis Tag fusion protein.	49
Figure 22	Image of a reducing SDS-PAGE gel stained with Coomassie Blue showing analysis of clusterin - 6xHis Tag fusion protein after Ni-NTA affinity chromatography.	49
Figure 23	Diagram illustrating the elution fractions obtained by SEC (a) and image of SDS-PAGE gel stained with Coomassie Blue showing analysis of the eluted fractions (b).	50
Figure 24	Image of reducing SDS-PAGE gel stained with Coomassie Blue showing analysis of clusterin - 6xHis Tag fusion protein purifed by Ni-NTA chromatography.	51
Figure 25	Image of Western Blot analysis of preparations of clusterin - 6xHis tag fusion protein using anti-clusterin antibodies.	52
Figure 26	CD spectra of clusterin - 6xHis tag fusion protein and plasma-derived clusterin	53
Figure 27	Influence of clusterin proteins on the heat-induced aggregation of fibrinogen	54
Figure 28	Image of SDS-PAGE gel stained with Coomassie Blue showing analysis of recombinant human clusterin, recombinant rat clusterin and plasma-derived human clusterin	58
Figure 29	Absorbance (280 nm) traces for size exclusion chromatographic analyses of plasma-derived clusterin, recombinant human clusterin and recombinant rat clusterin.	59
Figure 30	Line of best fit for protein mass versus the elution volume for protein standards after SEC.	60

Figure 31	CD spectra of recombinant proteins and plasma-derived clusterin. The relation between θ_{MRE} and wavelength were plotted for plasma-derived clusterin, recombinant human and rat clusterin proteins.	61
Figure 32	Influence of clusterin proteins on heat-stressed fibrinogen aggregation.	63
Figure 33	Binding of plasma-derived clusterin, recombinant rat and recombinant human clusterin to human IgG	64

1. INTRODUCTION

Clusterin is a ubiquitous secreted glycoprotein of 75–80 kDa that acts as an extracellular chaperone. It effectively inhibits stress-induced protein aggregation by an ATP independent mechanism, binding to partially unfolded proteins and forming soluble high molecular weight complexes with them (Wilson and Easterbrook-Smith, 2000). Although clusterin can not refold proteins itself, it may interact with other chaperones to achieve this (Poon *et al.*, 2000).

Molecular chaperones are a diverse group of proteins that assist the non-covalent assembly/disassembly of other macromolecular structures; however, chaperones are not permanent components of these structures when they are performing their normal biological functions. Assembly refers to intracellular processes like folding of nascent polypeptide chains, unfolding and refolding of polypeptides during their transfer across membranes and to the association of polypeptides to form complexes. Disassembly processes include partial unfolding and dissociation of protein subunits during normal functions or degradation (Henderson and Pockley, 2005).

Chaperones prevent protein misfolding or facilitate proper protein folding by recognizing and non-covalently binding to exposed hydrophobic surfaces of proteins that are incompletely folded or partially unfolded because of stress (Wilson and Easterbrook-Smith, 2000) or because of amino-acid substitutions (Yerbury *et al.*, 2005). Stress refers to factors like extremes of pH and temperature, oxidative stress, macromolecular crowding, or inappropriate ionic strength that may destabilize normal protein folding (Yerbury *et al.*, 2005) and result in protein aggregation (Henderson and Pockley, 2005).

In aqueous environments hydrophobic surfaces stick together while charged surfaces bind surfaces with opposite charge. Thus, protein aggregation results from the interaction of exposed charged or hydrophobic surfaces on proteins. Such exposure can occur during the synthesis of nascent polypeptide chains, the translocation of proteins across membranes and also following physical or chemical stresses (Henderson and Pockley, 2005). All cells need chaperone functions to prevent and reverse inappropriate protein-protein interactions. Folded proteins are much less susceptible to aggregation than unfolded or partially folded proteins. The aggregation of non-native proteins can lead to a failure in cellular function and subsequent disease. A great number of diseases are associated with the failure of proteins to remain properly folded under physiological conditions. Examples of these diseases are cystic fibrosis, brittle bone disease, some types of cancer and the amyloidoses (Pain, 2000).

In cystic fibrosis, abnormal secretion is the result of an improperly folded mutant chloride-channel protein. Some connective tissue diseases resulting from mutated collagens are also characterized by defective protein folding. An example is brittle bone disease or osteogenesis imperfecta which is associated with defective mineralization of bones resulting from a mutation in the type I collagen triple-helix that changes a glycine to another residue, breaking the tripeptide pattern sequence. This mutation slows down the rate of collagen folding and produces an abnormal conformation. Some cancers are associated with mutation in the protein p53 which plays a role in cell cycle control. Tumor-associated p53 mutants have a folding defect that compromises the stability of the native p53 tetramer that binds to DNA. Finally, in the amyloidoses, proteins aggregate to form insoluble fibrils (amyloid) that accumulate in the tissues. Alzheimer's disease, Parkinson's disease, bovine spongiform encephalopathy and Creutzfeldt-Jakob disease are associated with the production of amyloid fibrils (Pain, 2000).

Some studies have shown a positive correlation between clusterin expression and the diseases cited above or pathological stresses like pressure or ischemic kidney insult (Wilson and Easterbrook-Smith, 2000). In general, clusterin levels are up regulated in response to stress or disease (Bjork and Sistonen, 2006).

1.1 Clusterin Distribution

In animal tissues clusterin mRNA is ubiquitous, being found in many different tissues like rat prostate gland and quail neuroretinal cells. Clusterin protein is present in most human physiological fluids (Wilson and Easterbrook-Smith, 2000) but the expression is limited to particular cell types (Bjork and Sistonen, 2006). The physiological fluids in which clusterin is present include plasma, serum, cerebrospinal fluid, breast milk and urine. It is abundant in epithelial cells, mainly in testis, epidermis, liver, stomach and brain. It is also found in platelets at high concentration because it is synthesized by megakaryocytes (Rosenberg and Silkensen, 1995). Animal studies have found developmental variation in clusterin expression (Rosenberg and Silkensen, 1995).

Rosenberg and Silkensen (1995) refer to clusterin circulating as a high density lipoprotein bound to apolipoprotein A-1 (apo A-1). However, some studies suggest that apo A-1 is not necessary for clusterin to circulate because clusterin levels are normal in patients deficient in apo A-1, apolipoprotein A-11 and high density lipoprotein (Choi-Miura *et al*., 1993). Although clusterin is generally secreted by mammalian cells, it can be retained intracellularly under stress conditions (Wilson and Easterbrook-Smith, 2000). Reddy *et al.* (1996) claimed to demonstrate a truncated intracellular nuclear form of clusterin in transforming growth factor β (TGF- β) treated cells. Also, Mahon *et al*. (1999) reported intracellular clusterin in chickens, which migrates at 70 kDa on SDS-PAGE and appears not to be intracellularly cleaved. Mahon

et al. (1999) suggest that the lack of cleavage could be the reason why avian clusterin is not secreted.

1.2 Clusterin Structure

Clusterin is encoded by a single gene located on chromosome 8p21-p12 that encodes an mRNA of approximately 2 kb. Clusterin mRNA is translated on membrane-bound ribosomes to give a 449 amino acid primary polypeptide chain (Jones and Jomary, 2002). During translation, the polypeptide is translocated into the lumen of the endoplasmic reticulum where a proteolytic process removes the secretory signal sequence, and the product is internally cleaved at its Arg227–Ser228 bond, to produce two subunits, the α (residues 23–227) and β (residues 228–449) chains that form the mature protein. Five disulfide bonds are formed which stabilize the two chains in an anti-parallel arrangement. The mature protein is glycosylated and secreted from the cell (Wilson and Easterbrook-Smith, 2000). In SDS-PAGE performed under non reducing conditions, clusterin is detected as a single band at about 70-80 kDa; under reducing conditions bands at 35-40 kDa are found, representing the α and β subunits of clusterin (Wilson and Easterbrook-Smith, 2000).

Sequence analyses predict that clusterin contains a disulfide-linked core region flanked by sections of the α and β subunits that contain three amphipathic α helices, and two coiled-coil α helices (Wilson and Easterbrook-Smith, 2000) (Figure 1).

Please see print copy for figure 1

Figure 1. Predicted structure of human clusterin (Jones and Jomary, 2002). The upper part of the figure is a representation of the precursor polypeptide chain. After cleavage of this polypeptide, the secretory signal peptide (magenta) is removed and the α and β chains are generated and assembled in an anti-parallel arrangement. The cysteine-rich centers (red) in the molecule are linked by five disulfide bridges (red ellipses) and are flanked by two predicted coiled-coil α-helices (green) and three predicted amphipathic α-helices (dark blue). The N-linked glycosylation sites are represented as yellow spots (Jones and Jomary, 2002).

Clusterin is heavily glycosylated; it is estimated that 20-25% of the total mass of the mature molecule consists of carbohydrate. A study by Kapron *et al*. (1997) used matrix-assisted laser desorption ionization mass spectrometry to reveal two molecular weight species of holoclusterin (58505 ± 250 and 63507 ± 200 Da), which probably represent two major glycoforms. There are six N-linked glycosylation sites in clusterin; three N-linked glycosylation sites in the alpha chain (α64N, α81N and α123N) and three in the beta chain (β64N, β127N and β147N). Seven types of oligosaccharide structure (monosialobiantennary structure, bisialobiantennary structure without or with one fucose, trisialotriantennary structure without or with one fucose and trisialotriantennary structure with two fucose and/or tetrasialotriantennary structure) were detected in analyses of human serum clusterin (Kapron *et al*., 1997).

In addition to the secreted form of clusterin, Reddy *et al*. (1996) claim to have observed an intracellular nuclear form of clusterin with apparent molecular weight of 43 kDa (estimated by SDS-PAGE) accumulated in the nucleus of HepG2 and CCL64 cell lines after treatment with transforming growth factor β (TGFβ). Reddy *et al*. (1996) demonstrated that a 43 kDa form of the protein can be translated *in vitro* from mRNA lacking the sequence encoding the N-terminal 33 amino acids of clusterin (including the hydrophobic signal sequence). If this happens *in vivo*, it would prevent the protein being imported into the endoplasmic reticulum and thus its glycosylation and secretion.

1.3 Clusterin Function

The biological distribution of clusterin is wide, and across species clusterin maintains a high level of sequence homology (70–80% between mammals). These characteristics suggest that clusterin performs one or more essential biological functions (Wilson and Easterbrook-Smith, 2000). Many putative functions have been attributed to clusterin; however most of these have not been substantiated. The fact that clusterin interacts with a wide range of molecules, and the ubiquitous up regulation of clusterin expression in developmental remodeling, apoptosis, and in response to stress, make it difficult to clarify clusterin functions (Jones and Jomary, 2002). It has been reported that clusterin binds lipids, amyloid proteins, components of the complement membrane attack complex and immunoglobins (Jones and Jomary, 2002). Wilson and Easterbrook-Smith (1992) reported that clusterin binds human IgM and IgA, and all the isotypes of human IgG; also, they suggest that clusterin contains binding sites for the Fc and Fab regions of IgG. Since many of clusterin's biological ligands are hydrophobic, it has been proposed by Humphreys *el al*. (1999) that clusterin has a tendency to bind hydrophobic molecules regardless of their specific functions. Furthermore, Humphreys *el al*. (1999) has proposed that the three predicted amphipathic α helices in clusterin may be crucial for these types of interactions.

1.3.1 Clusterin as an extracellular chaperone

In vitro, extracellular chaperones inhibit the aggregation of purified proteins subjected to chemical and physical stresses by forming stable non-covalent complexes with them. Humphreys *et al*. (1999) demonstrated that clusterin protects glutathione S-transferase (GST), catalase, ovotransferrin and IgG from precipitation after stress induced by heat, and also that clusterin is able to protect α–lactalbumin and bovine serum albumin (BSA) from precipitation induced by reduction with dithiothreitol (DTT). In studies by Humphreys *et al*. (1999), clusterin was shown to bind preferentially to stressed proteins and, after size exclusion chromatography and SDS-PAGE, a high molecular weight (HMW) fraction was detected confirming the formation of complexes between clusterin and the stress-induced protein. In addition, Humphreys *et al.* (1999) showed that clusterin was able to interact with and stabilize a greater number of smaller versus larger stressed protein molecules, and that the efficiency of clusterin as a chaperone was inversely proportional to the subunit molecular mass of the target protein. However, because the relationship was not uniform, it was suggested that the mechanism of action of clusterin was different for heat stressed proteins and reduced proteins. Furthermore, Poon *et al*. (2000) showed that proteins in whole human serum are more susceptible to aggregation and precipitation when clusterin had been selectively depleted by immunoaffinity chromatogrpahy. Poon *et al.* (2000) suggest that the chaperone action of clusterin depends on conformational and kinetic factors. In a study that examined the interaction between clusterin and intermediately folded forms of α-lactalbumin, Poon *et al.* (2002) observed that clusterin does not interact as a chaperone with stable, non-aggregating intermediate states of proteins, or states of proteins undergoing very rapid aggregation. Instead, clusterin interacted with slowly aggregating, long-lived and precipitation-bound intermediate states of proteins that are present on an irreversible, off-folding pathway to form soluble complexes and thereby prevent precipitation.

Humphreys *et al.* (1999) tested GST and catalase activity when exposed to heat in the presence of clusterin; they found that clusterin was unable to protect the enzymes against their loss of activity. Poon *et al.* (2000) demonstrated that regardless of the presence or absence of ATP, clusterin did not promote the recovery of enzyme activity of alcohol dehydrogenase (ADH) or catalase following heat stress. Furthermore, it was shown that clusterin stabilized the heat-stressed enzymes by forming clusterin-ADH and clusterin-catalase complexes that preserved the enzymes in a state competent for subsequent ATP-dependent refolding by heat-shock chaperone 70 to recover enzyme activity. Thus, like the small heat-shock proteins (sHSPs), a family of intracellular chaperones, clusterin can not refold heat-stressed enzymes but can preserve them in a state that is later able to undergo ATP-dependent refolding. Collectively, all these findings suggest that clusterin inhibits stress-induced protein aggregation by binding to partly unfolded proteins in an ATP-independent way to form soluble, high-molecular-weight complexes with them but is unable to independently promote protein refolding.

In aqueous solution at physiological pH, clusterin is found as a mixture of individual α-β heterodimers, together with aggregates of these structures (predominantly in the forms of (α-β_{2-4}) (Humphreys *et al*., 1999). The studies of Poon *et al.* (2000) suggest that the chaperone active form of clusterin is the 80 kDa heterodimer. Aggregation of the α-β heterodimer is reversible; mildly acidic pH favours dissociation of the aggregates into individual α-β heterodimers, increasing the level of exposed hydrophobic regions on the molecule and coincidentally increasing its binding to a wide variety of ligands. Consequently, the chaperone action of clusterin is enhanced at lower pH (Poon *et al*., 2002). This may have important physiological relevance because during tissue damage or inflammation (e.g. cardiac ischemia, infarcted brain, Alzheimer's disease) the local pH decreases to <6 (local acidosis occurs) (Poon *et al*., 2002). It is under these conditions, which would favour protein unfolding, that clusterin will exert an enhanced chaperone action.

Studies by Stewart *et al*. (2007) showed that clusterin retains its chaperone function when deglycosylated and that deglycosylation did not induce major structural changes in the molecule. However, deglycosylation did increase the level of exposed hydrophobicity on the molecule, its binding to a variety of ligands and its tendency to aggregate in solution. These studies suggest that conjugated sugars may sterically "hinder" ligand binding sites on clusterin and that their removal provides ligands with enhanced access to binding sites.

1.3.2 Similarities between clusterin and the small heat shock proteins

Clusterin, like mammalian small Heat Shock Proteins (sHSPs), exists in solution as heterogeneous aggregates of high molecular mass; however, in contrast to the sHSPs, dissociation of aggregated forms of clusterin is not stimulated by increased temperature (Poon *et al*., 2002). The expression of mammalian sHSPs and clusterin are both controlled by sHSF1 (heat shock factor 1), a transcriptional activator (Wilson and Easterbrook-Smith, 2000). Moreover, clusterin and sHSPs have some sequence similarity; clusterin and β-crystallin display 25% amino acid sequence similarity (Wilson and Easterbrook-Smith, 2000). In addition, clusterin and sHSPs are dynamic proteins that contain amino acids sections with conformational flexibility and disordered structure; clusterin is predicted to have a greater degree of disorder (Carver *et al*., 2003). Also, clusterin is generally found extracellularly, in contrast, sHsps are ubiquitous intracellular proteins (Carver *et al*., 2003).

Related to clusterin function, there are many similarities between clusterin and the sHSPs. The sHSPs and clusterin can inhibit protein precipitation induced by stress (Poon et al., 2002). Clusterin expressed during cellular stress, like the sHSPs, acts in a chaperone-like manner and binds to hydrophobic regions of partly unfolded, stressed proteins, to prevent protein precipitation (Humphreys *et al*., 1999). The sHSPs and clusterin only bind to long lived, intermediate states of proteins that slowly aggregate as they progress along their off-folding pathway (Poon *et al*., 2002). Carver et al. (2003) state that, the conformational

dynamism and aggregated state of clusterin and sHSP may be crucial for their chaperone function. Mammalian sHSPs and clusterin are both found as heterogeneous aggregates, so, the subunit exchange may be significant in regulating the chaperone action of these proteins (Carver *et al*., 2003). Moreover, as stated above, clusterin, like the sHSPs, does not protect enzymes from stress-induced loss of activity; and they both exert their chaperone action without the need for hydrolysis of ATP (Carver *et al*., 2003).

The sHSPs and clusterin can protect cells from stress (Wilson and Easterbrook-Smith, 2000). For example, Narberhaus (2002) state that α-crystallin-type proteins, a diverse protein family included in the sHSPs that exist in most, but not all, animals, plants, bacteria and archaea, have a specialized role in the maintenance of clear vision but are also involved in a large number of cellular activities. Some of these activities include thermotolerance, resistance to apoptosis, protection against oxidative stress, desiccation tolerance, protection of the photosynthetic apparatus or of mitochondrial NADH:ubiquinone oxidoreductase (complex I) and protection against acid shock. Rogalla *et al*. (1999) showed that the ability of HSP27, a sHSP, to confer resistance against oxidative stress when overexpressed in L929 cells and immortalized rat neuroblasts 13.S.1.24 cells. Furthermore, a study of Wehmeyer and Vierling (2000) on the function and regulation of sHSPs during seed development found a correlation between decreased HSP17.4 protein levels and the inability of the seed to survive desiccation, suggesting that sHSPs have a general protective role throughout the seed.

Similar protective effects have also been shown for clusterin. A study by Humphreys *et al*. (1997) exposed transfected murine L929 cells expressing human clusterin to cytotoxic agents in order to examine the effects of the agents on the survival of these cells and on the biosynthesis and subcellular localization of clusterin. The agents tested were TNFα, TGFβ, colchicine, staurosporine, and azide. Humphreys *et al.* (1997) observed, in response to cytotoxin treatment, a novel clusterin molecule that appeared as two bands about 36 and 38.5 kDa when analysed under reducing conditions by SDS-PAGE and immunoblotting. They suggest that the molecule may result from toxin-induced disruption of processes of normal cellular protein production. The study also reported that the overexpression of clusterin in the cells provided them with resistance to TNFα-mediated cytotoxicity when compared with control L929 cells that did not express human clusterin. Also, as other studies have done, they reported that exposure of L929 cells to TGFβ protected the cells from cytotoxicity associated with subsequent exposure to TNFα (Humphreys *et al*., 1997). Moreover, Sensibar *et al.* (1995) showed that overexpression of clusterin in LNCaP cells protected them from apoptosis induced by TNFα. LNCaP is an androgen-sensitive, human prostatic cancer cell line that responds to TNFα in culture by undergoing programmed cell death (Sensibar *et al*., 1995). Furthermore, Sensibar *et al*. (1995) showed that anti-sense clusterin mRNA enhanced the sensitivity of LNCaP cells to TNFα accompanied by a reduction in clusterin biosynthesis and, in addition, that only higher levels of clusterin expression provided protection against

TNFα. A transfected clone expressing low levels of clusterin was not protected, while a clone that expressed higher levels was given complete protection from TNFα.

As one last example of clusterin protecting cells from stress, a study by So *et al.* (2005) evaluated clusterin expression levels in human breast cancer to determine whether antisense oligonucleotides or double-stranded small interfering RNAs (siRNA) targeting the clusterin gene enhanced apoptosis induced by paclitaxel. The data from So *et al.* (2005) confirmed that clusterin is present in > 60% of invasive breast cancers, and showed that clusterin expression is rapidly up-regulated in various tissues undergoing apoptosis, including normal and malignant breast tissues (protecting them from cellular death). Because tumour progression and drug resistance results, in part, from increased expression of cell survival genes that collectively regulate the apoptotic ‘‘switch’’ of cancer cells, So *et al.* (2005) identified clusterin as a valid therapeutic target in strategies employing gene down-regulation therapy for patients with advanced cancer.

1.3.3 Role of clusterin in extracellular quality control of protein folding

Yerbury *et al.* (2005) suggest that clusterin forms complexes with misfolded proteins *in vivo.* Yerbury *et al.* (2005) propose the theoretical model shown in Figure 2, in which clusterin, as an extracellular chaperone, can mediate the uptake of misfolded protein ligands via cell-surface receptors and direct them towards intracellular degradation. They suggest that clusterin’s key role in this model is its ability to interact with extracellular non-native proteins and cell surface receptors. Clusterin’s ability to recognize and interact with non native extracellular proteins that expose increased hydrophobicity allows the formation of complexes; the complexes may then be selectively endocytosed via receptors and subsequently degraded intracellularly in lysosomes (Yerbury *et al.*, 2005).

Please see print copy for figure 2

Figure 2. Theoretical model for extracellular quality control of non-native (NN) extracellular proteins by chaperones. The binding of extracellular chaperones to NN proteins allows the selective endocytosis of NN protein-chaperone complexes via receptors expressed on liver cells and the reticuloendothelial system. The complexes are internalized and moved by vesicular transport to lysosomes where intracellular degradation occurs (Yerbury *et al.*, 2005).

The suggestions of Yerbury *et al*. (2005) are based on studies that support the role of clusterin in receptor-mediated endocytosis and intracellular degradation of non native proteins. Examples of these studies follow. Kounnas *et al*. (1995) identified clusterin as a ligand of a specific cell surface receptor, glycoprotein 330 (gp330)/megalin, which is member of the low density lipoprotein receptor gene family. Kounnas *et al*. (1995) demonstrated that uptake and degradation of clusterin is mediated by the gp330/megalin endocytic receptor; furthermore, they suggest that clusterin would function to target lipoproteins for clearance. Hammad *et al*. (1997) showed that complexes formed *in vivo* in Alzheimer's disease between clusterin and the amyloid-β (Aβ) peptide are taken up by neural epithelial cells by way of megalin, and are subsequently degraded in lysosomes. Likewise, Zlokovic *et al*. (1996) used a guinea pig brain model to show that clusterin facilitates the transport of a soluble form of amyloid β-peptide (sAβ) complexes across the blood-brain and blood-cerebrospinal fluid barriers, and they suggest that gp330/megalin mediates cellular uptake and transport, acting as a receptor for clusterin. This soluble form of Aβ could be the immediate precursor of Aβ which becomes the principal constituent of amyloid deposits in Alzheimer's disease; however, this has not yet been established (Zlokovic *et al*., 1996).

As a last example, Bartl *et al*. (2001) analyzed the endocytic activity of vital fibroblasts and epithelial cells exposed to cellular debris and membrane remnants. They reported a mechanism for the clearance of cellular debris which appears to operate into fibroblast and epithelial cells but not in macrophages, and involves clusterin. Clusterin mediates binding of the debris to the lipoprotein receptors megalin and low density lipoprotein receptor-related protein (LRP). Bartl *et al*. (2001) suggest that clusterin might act as a molecular adaptor ensuring the interaction between specific endocytosis receptors and a broad range of hydrophobic, modified, denatured, or aggregated molecules, to promote their disposal by cellular uptake and degradation.

Amongst the studies suggesting a role for clusterin in disease are those using clusterin-knockout mice. Rosenberg *et al*. (2002) established a protective role for clusterin against chronic glomerular kidney disease after showing that the kidneys of clusterin deficient mice developed a progressive glomerulopathy with age, characterized by mesangial expansion and accumulation of insoluble protein deposits containing immunoglobulin (IgG, IgM, IgA) and complement (C1q, C3 and C9) complexes. Rosenberg *et al*. (2002) also suggest that clusterin has a direct role in the processing of immunocomplexes.

A study by De Mattos *et al*. (2002) in which clusterin knock out mice were crossed with a transgenic mouse expressing in the brain an Alzheimer's disease-associated mutant form of the human amyloid precursor protein (APP) suggested that clusterin has a role in influencing amyloid deposition and the associated neuritic toxicity. One of the key factors in Alzheimer's disease may be the conversion in the brain of the normally soluble amyloid-β (Aβ) peptide to insoluble forms with a high β-sheet content (DeMattos *et al*., 2004). De Mattos *et al*. (2002)

suggest that clusterin expression facilitates the conversion of soluble Aβ into forms with a high β-sheet content (amyloid fibrils) and also that the absence of clusterin was associated with alterations in the levels of soluble brain Aβ. In contrast, in a subsequent study by De Mattos *et al*. (2004), a protective role for clusterin against Alzheimer's disease was suggested using double-knockout mice (ablated expression of both clusterin and ApoE genes). This study concluded that clusterin was involved in regulating the process of Aβ aggregation *in vivo* and *in vitro*, acting as an Aβ chaperone and (cooperatively with ApoE) suppressing Aβ deposition (DeMattos *et al*., 2004).

1.3.4 Protein folding diseases and role of clusterin

As has been mentioned previously, the aggregation of non-native proteins can lead to a failure in cellular function and subsequent disease. Also, because folded proteins are much less susceptible to aggregation than unfolded or partially folded proteins, all cells need chaperone functions to prevent and reverse inappropriate protein-protein interactions and to prevent protein misfolding or facilitate proper protein folding (Henderson and Pockley, 2005). Protein misfolding diseases are diseases that have heterogeneous etiologies, but with which pathology is associated, in some way, with abnormal protein conformation because of the failure of the protein to achieve or to maintain its native conformation (Pain, 2000).

Amyloidosis is a general term that describes a range of diseases characterized by the deposition of insoluble fibrillar aggregates of proteins in different organs, leading to cell damage, organ dysfunction and death (Calero *et al*., 2000). Amyloid fibrils form from non-native or partially unfolded proteins. Some amyloid disorders are Alzheimer's disease, Parkinson's disease, senile systemic amyloidosis, familial amyloid polyneuropathy, bovine spongiform encephalopathy and Creutzfeldt-Jakob disease (Pain, 2000). As discussed above, De Mattos *et al*. (2004) concluded that clusterin was involved in regulating the process of Aβ aggregation *in vivo* and *in vitro*, acting as an Aβ chaperone and (cooperatively with ApoE) suppressing Aβ deposition (DeMattos *et al*., 2004) in Alzheimer's disease. Concurring with this, Calero *et al*. (2000) proposed that clusterin has a neuroprotectant role in Alzheimer's disease by maintaining Aβ solubility, preventing Aβ aggregation and fibrillization and modulating its blood-brain barrier transport at the cerebrovascular endothelium. Furthermore, Choi-Miura and Oda (1996) found clusterin noncovalently complexed with soluble Aβ in normal cerebrospinal fluid, and in Aβ deposits in diffuse and compact plaques in Alzheimer's disease. They showed that clusterin slowed the aggregation of Aβ *in vitro* and suggested that clusterin could exert a neuroprotective action *in vivo*. They also showed that cytokines such as TGFβ and interleukin 1, which are known to colocalize with Aβ deposit in the senile plaques, enhanced the expression of clusterin, which may link clusterin to inflammatory mechanisms in Alzheimer's disease.

α-Synuclein is a neuronal protein generally located in the nuclear envelope of neurons and presynaptic nerve terminals. It has been reported that α-synuclein itself can assemble into

fibrils *in vitro* and that this behaviour is associated with disease (Sasaki et al., 2002). Sasaki et al. (2002) investigated clusterin expression in cases with "α-synucleinopathies", such as Parkinson's disease, dementia with Lewy bodies and multiple system atrophy; clusterin was found in about 50% of the cortical Lewy bodies in cases with dementia, and in 10% of the glial cytoplasmic inclusions in cases with multiple system atrophy. Lewy bodies and glial cytoplasmic inclusions are inclusion bodies formed by α-synuclein. Also, immunohistochemical analysis carried out for Sasaki et al. (2002) found Lewy bodies with strong immunoreactivity for clusterin but with weak immunoreactivity for α-synuclein, and suggested clusterin as an anti-amyloidogenic molecule that modifies the formation of α-synuclein inclusion bodies and prevents the pathological aggregation of α-synuclein due to its chaperone activity.

Prion diseases are sporadic, transmissible, infectious or inherited neurodegenerative diseases in which the normal trans-membrane prion protein (PrP^{C}) that localizes in presynaptic membranes is replaced by a protease-resistant, β-sheet-containing isoform (PrP^{RES}) that is pathogenic. PrP has the property of aggregating into amyloid fibrils and being deposited in the brains in cases with transmissible encephalopathies (TSEs), when PrP^{C} is converted into PrP^{RES}. Prion diseases or transmissible encephalopathies include Creutzfeldt-Jakob disease, fatal familial insomnia, Gerstmann-Straussler-Scheinker syndrome and kuru in humans (Freixes *et al.*, 2004). Freixes *et al.* (2004) studied the association of clusterin with PrP^{RES} deposits in the frontal cortex and cerebellum in patients with sporadic Creutzfeldt-Jakob disease - they demonstrated the capacity of clusterin to interact and form aggregates with PrP^{RES}, and suggested that clusterin modifies PrP toxicity in Creutzfeldt-Jakob disease by participating in PrP clustering and sequestration.

Mallory bodies are cytoplasmic protein deposits, formed from abnormally folded keratins, mainly keratin 8, and stress proteins like HSP70, HSP25, and p62. They are mainly found in hepatocytes of patients with alcoholic and non-alcoholic steatohepatitis, in various other chronic toxic, metabolic and cholestatic liver disorders and in certain hepatocellular carcinomas (Janig et al., 2005). Janig et al. (2005) investigated possible interaction of clusterin with misfolded and aggregated keratins in transfected cell models and found clusterin associated with misfolded keratin but only when its signal peptide was deleted and its secretion inhibited. Furthermore, they suggested that the ability of clusterin to bind to misfolded proteins, including keratins, is restricted to the extracellular space. The suggestion was made because clusterin was found associated with Mallory bodies in the extracellular space (due to cell lysis) but not in cytoplasmic Mallory bodies.

1.4 Aims of the Project

The working hypothesis of this project is based on the model proposed by Yerbury *et al.* (2005). If clusterin mediates the clearance and degradation of misfolded extracellular proteins *in vivo* and this action is anti-inflammatory, then making a recombinant form of clusterin might be therapeutically useful to clear protein debris from sites of disease and inflammation and in this way suppress pathology.

Expression of recombinant mammalian proteins is often performed using mammalian or insect cells because the expression of eukaryotic proteins in prokaryotic cells can be problematic. Some post-translational processes like glycosylation, phosphorylation, oligomerization, specific protein cleavage and disulfide bond formation are not performed by bacterial cells (Sambrook *et al.*, 1989).

However, recombinant protein expression using mammalian cell cultures are very expensive in both time and money - even thought such cell culture yields biologically active proteins with all the required posttranslational modifications, low yields of the recombinant protein, long cultivation times and requirements for expensive bioreactors and medium components are typical (Vallejo and Rinas, 2004). Bacterial expression, on the contrary, results in higher yields - bacteria grow rapidly in inexpensive media in which high cell concentrations can be obtained (Vallejo and Rinas, 2004).

Recombinant human clusterin had been expressed before by using yeast (Lakins *et al.*, 2002) and insect cells. However, since Stewart *et al.* (2007) recently showed that clusterin retains its chaperone function when deglycosylated and also, that deglycosylation did not induce major structural changes in the molecule, the possibility of inexpensively producing large quantities of chaperone-active clusterin in bacteria became apparent.

Since recombinant clusterin expressed in bacteria could provide a large scale supply of chaperone-active protein useful in therapeutic applications, a primary aim of this project was to express clusterin in *E. coli* and evaluate its chaperone activity. If it were possible to express a chaperone-active form of clusterin in *E. coli*, this product could be used as a clearance agent and/or inhibitor of protein aggregation *in vivo* and become a promising new treatment for serious human diseases related to protein aggregation and inflammation, such as Alzheimer's disease, Parkinson's disease, senile systemic amyloidosis, Creutzfeldt-Jakob disease, and chronic liver diseases.

Furthermore, as a secondary aim of this project, two different commercially available recombinant clusterin proteins were compared in structure and chaperone function with human serum clusterin. The tested products were Flag-Tagged recombinant human clusterin expressed in human embryonic kidney cells (BioVendor- Laboratory Medicine, Inc) and His-Tagged recombinant rat clusterin from *E. coli* (BioVendor- Laboratory Medicine, Inc). As a

result of its expression in bacteria, the recombinant rat clusterin, unlike the human serum clusterin and the Flag-Tagged recombinant human clusterin, consists of a single polypeptide (not cleaved into α and β chains). These comparisons were performed to provide additional insights into any functional limitations of recombinantly expressed clusterin proteins.

2. MATERIALS AND METHODS

2.1 General buffers and solutions

General buffers and solutions used are shown below. Other less commonly used buffers and solutions are listed in the respective chapters.

2.1.1 Buffers

- Phosphate Buffered Saline (PBS), pH 7.4 (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 1.4 mM Na_2HPO_4 anhydrous).
- 1% (w/v) Heat Denatured Casein (HDC) in PBS, pH 7.4 (1% (w/v) Heat Denatured Casein from Bovine milk, 0.01% Thimerosal in PBS).
- Tris EDTA (TE), pH 8.0 (10 mM Tris-Cl, 1 mM EDTA).
- 0.1% (v/v) Triton X-100 in PBS

2.1.2 Human Clusterin

Plasma-derived clusterin and deglycosylated clusterin were supplied by Mark's Wilson laboratory (University of Wollongong, Australia) to be used as a reference. Clusterin was purified from human plasma by immunoaffinity chromatography as described in Wilson and Easterbrook-Smith (1992). Deglycosylation of clusterin was performed by enzymatic digestion with *N-glycosydase F* as described in Stewart *et al*. (2007). Clusterin proteins were stored at -20^{o}C until use.

2.2 Expression of clusterin - Maltose Binding Protein (MBP) fusion protein by induction of *E. coli* TBI pMal-c2x-clusterin clones.

The bacterial culture media, reagents and methods used in the present project for the expression of recombinant clusterin protein are described below.

2.2.1 Bacterial Culture Media

- Lauria Bertani (LB) broth, pH 7.0 (Bacto tryptone (Bacto Laboratories) 10 g/l, Bacto yeast extract (Bacto Laboratories) 5 g/l, NaCl 10 g/l, MilliQ Water 1 l).
- Lauria Bertani (LB) agar, pH 7.0 (Bacto tryptone (Bacto Laboratories) 10 g/l, Bacto yeast extract (Bacto Laboratories) 5 g/l, NaCl 10 g/l, agar-agar 15 g/l, milliQ Water 1l).

- SOC medium, pH 7.0 (Bacto tryptone (Bacto Laboratories) 20 g/l, Bacto yeast extract (Bacto Laboratories) 5 g/l, NaCl 0.5 g/l, KCl 250 mM 10 ml/l, milliQ Water 1 l, filter sterilized glucose 1M 20 ml/l).

2.2.2 Reagents

- 100 mM IPTG (Isopropylthio-β-galactoside) in dH_2O

2.2.3 Protein expression from pMal™ vectors

The pMal™ vectors provide a method for expressing and purifying a protein produced from a cloned gene or open reading frame. The cloned gene is inserted downstream from the *malE* gene of *E. coli*, which encodes maltose binding protein (MBP), resulting in the expression of a MBP fusion protein. In order to give high level expression of the cloned sequences, the method uses the *tac* promoter and the *MalE* translation initiation signals. The vector express the *MalE* gene fused to the *LacZα* gene and restriction sites for inserting the coding sequence of interest are available between the *MalE* and *LacZα* genes. The pMal-c2 series (Figure 3) has a deleted signal sequence leading to cytoplasmic expression of the fusion protein. Fusion proteins expressed from pMal-c2 plasmid often constitute 20-40% of the total cellular protein (BioLabs New England Instruction Manual, 2006).

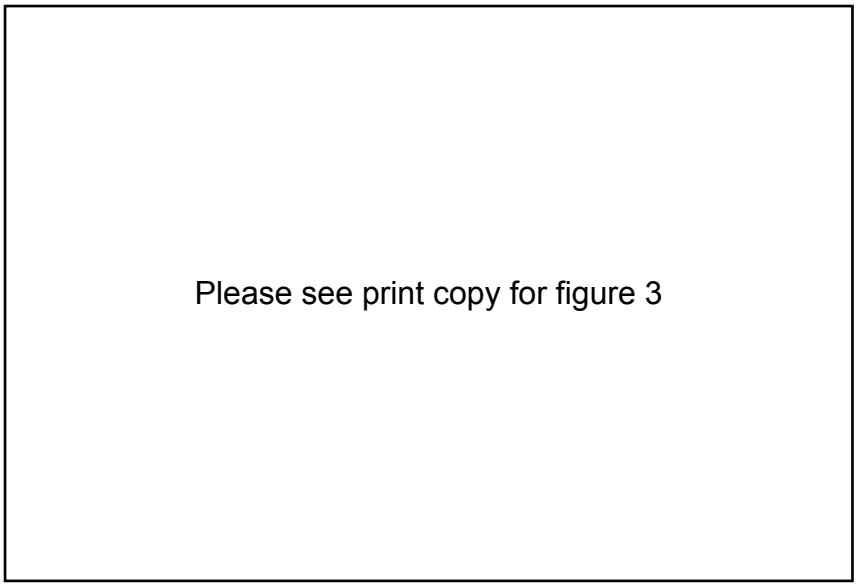


Figure 3. pMal™ vector series c2x. The pMal™ vector for expressing fusion proteins uses the *tac* promoter and the *MalE* translation initiation signals. The pMal™ vectors express the *malE* gene fused to the lacZα gene. Restriction sites (polylinker) between malE™ and lacZα are available for inserting the coding sequence of interest (BioLabs New England Instruction Manual, 2006).

The strain used as host for the pMal™ vector was *E. coli* TB1 following the recommendation of the BioLabs New England pMal™ Protein fusion and purification system protocol (2006). This strain has given the best results when considering plasmid stability, expression and purification. Isopropylthio-β-D-galactoside (IPTG) is a synthetic analog of lactose that induces the activity of β-galactosidase by binding and inhibiting the Lac repressor. In cloning experiments the *LacZ* gene is replaced by the gene of interest and in this way IPTG induces gene expression (Sambrook *et al*., 1989).

2.2.3.1 Analysis of plasmids for the presence of clusterin-encoding insert by restriction enzyme digestion. Plasmids from clones of *E. coli* TBI transformed with pMal-c2x-clusterin, supplied by Mark Wilson's laboratory, were extracted using the Wizard® Plus minipreps DNA purification system (Promega, Sydney) and analysed by 1% agarose gel electrophoresis. In addition, samples of the plasmids were digested with the restriction enzymes *Bgl*II, *Sma*I and *Xba*I (Fermentas, Life Science) for 1.5 hours at 37°C prior to electrophoresis. *Bgl*II has recognition sites in both the clusterin sequence and the pMal-c2x plasmid sequence. *Sma*I has a single restriction site only in the clusterin sequence. *Xba*I has a single restriction site in the pMal-c2x plasmid.

Restriction digests were performed by adding the components below to an Eppendorf tube and incubating for 1.5 h at 37 °C.

- Water, nuclease free 16 µl
- 10X recommended buffer for restriction enzyme 2 µl
- Substrate DNA 2 µl
- Restriction enzyme 0.5 – 1 µl (5-10 u)

2.2.3.2 Protein expression. *E. coli* TBI pMal-c2x-clusterin, supplied by Mark Wilson's laboratory, was grown in 1-5 ml LB broth at 37°C to $2x10^8$ cells/ml (A_{600} of ~0.5); subsequently, the culture was diluted into LB broth with 100 µg/ml of amplicillin (volume of 5 ml for small scale preliminary assays, and 2 liters for large scale assays) and grown at 37°C to $2x10^8$ cells/ml. The bacteria were induced to express the clusterin – MBP fusion protein by adding 0.3 mM IPTG and incubating at 25°C overnight. After protein induction the bacteria were harvested by centrifugation at 3440 x g in a Beckman J2-MC (4300 rpm using a 10JA Beckman Rotor) for 10 min, the supernatant was removed and the pellet collected for protein extraction.

2.2.4 Protein extraction.

The protocol for protein extraction using the Complete lysis-β (2x), EDTA free reagent set (Roche Applied Science Cat # 04719 948 001) was performed. Complete Lysis-β (2×) EDTA-free allows rapid cell lysis of bacteria cells with simultaneous inhibition of protease activity in the cell lysate. Complete Lysis-β (2×) EDTA-free contains a mild, non-ionic detergent in 20

mM Tris/HCl (pH 7.5) that allows efficient and gentle extraction of proteins, especially recombinant proteins. Also, the Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablets supplied in the kit allow the inhibition of a broad spectrum of proteases, like serine and cysteine proteases, and in this way provide protection of proteins isolated from bacteria (Roche Product Data Sheet 2007).

2.2.4.1 Protocol for small scale protein extraction. A preliminary small scale assay for protein extraction, from a 5 ml bacterial culture, was performed. The harvested *E. coli* TBI pMal-c2x-clusterin culture was resuspended in 500 µl of Lysis-β reagent, vortexed until homogenized and shaken at 600 rpm for 1 min at room temperature in a MS1 Minishaker (IKA, Sweden). The suspension was centrifuged at 27000 xg for 5 min. The supernatant, containing the soluble protein fraction, was collected. Cell debris and insoluble proteins were contained in the pellet. Purification of inclusion bodies was carried out by resuspending the pellet into 400 µg/ml lysozyme, vortexing for 1 min, adding 3.3 ml of 1:20 diluted Lysis-β reagent, vortexing 1 min, and centrifuging at 13000 rpm for 10 min. Subsequently, the pellet was resuspended again in 3.3 ml of 1:20 diluted Lysis-β reagent, vortexed for 1 min, and centrifuged at 13000 rpm for 10 min. The latter process was performed one more time. Finally, the inclusion body pellet was resuspended in 300 µl of sterile water.

2.2.4.2 Protocol for large scale protein extraction. The harvested *E. coli* TBI pMal-c2x-clusterin from 2 liters culture was resuspended in 40 ml of Lysis-β reagent, vortexed until homogenized and shaken at 600 rpm for 10 min at room temperature in a MS1 Minishaker (IKA, Sweden). The suspension was centrifuged at 27000 xg for 15 min. The supernatant, containing the soluble protein fraction, was collected. The preliminary small scale assay described in 2.2.4.1, showed clusterin present in the soluble protein fraction.

2.3 Expression of clusterin - 6xHisTag fusion protein using an *E. coli* expression system with Gateway® cloning technology.

The Gateway® system is a method to clone and sub clone DNA sequences; its main benefit is the fact that the DNA sequence of interest has to be cloned only one time to create an entry clone, and after that the DNA fragment can be transferred to any Gateway expression plasmid in one simple reaction (Invitrogen User Manual, 2003). The technology exploits the integration/excision reaction between *E. coli* and λ bacteriophage in the switch between the lytic and lysogenic pathway (Figure 4). The bacteria possess a stretch of DNA called *att*B; also, in phage there is a stretch of DNA called *att*P. When the phage infects a bacterium, the injected lambda DNA recombines with the bacterial DNA and is integrated into the bacterial genome. The process is made via the *att* sites in the presence of integration-specific enzymes (bacteriophage λ Integrase and Excisionase proteins, and the *E. coli* Integration Host Factor). Once integrated, the hybrid recombination sites are called *att*L and *att*R (L stands for left, R

stands for right). Furthermore, when the phage undergoes the lytic phase, the phage DNA can excise itself from the bacterial DNA, using a different set of recombination and excision enzymes, and the *att*L site recombines with the *att*R site, coincident with separation of phage DNA from the bacterial genome (Invitrogen User Manual, 2003) (Figure 4).

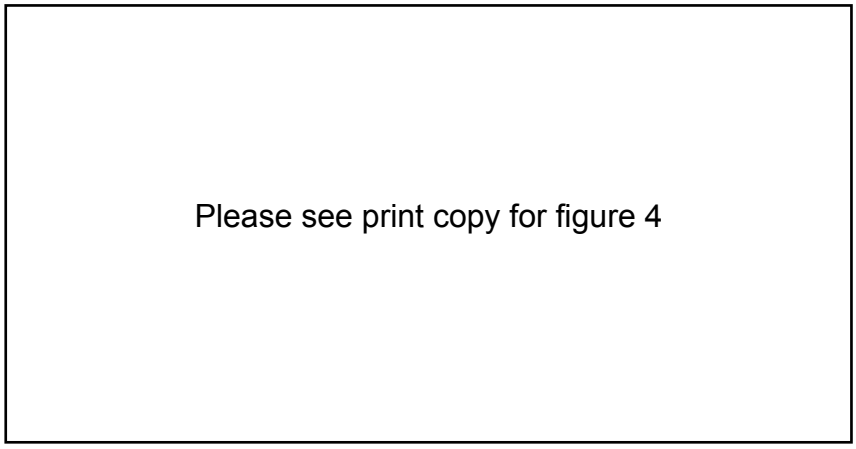


Figure 4. Integration-excision reaction between *E. coli* and λ bacteriophage in the switch between the lytic and lysogenic pathway (Invitrogen User Manual, 2003).

The Gateway® system relies on artificial recombination sites or *att* sequences, *att*P, *att*B, *att*L and *att*R. The reaction is conservative; there is no synthesis or loss of nucleotides, the DNA segments that flank the recombination sites are simply switched. The specificity of the system is given by 7 nucleotides of the core sequence that allow minimal cross reactivity among *att* sequences. In the BP reaction, the *att*B site reacts with the *att*P site to create *att*L and *att*R sites. The *att*B1 and *att*B2 sequences must be added to specific primers that amplify the target gene. Then, the DNA fragment is combined with a donor vector that contains *att*P1 and *att*P2 sequences and a *ccd*B gene. Next, the addition of BP Clonase™ generates an entry clone and a by-product fragment containing *ccd*B. The presence of the *ccd*B gene allows negative selection of unwanted by-product plasmids after recombination. The *ccdB* gene code for *ccdB* protein which inhibits the growth of most *E. coli* strains because it interferes with *E. coli* DNA gyrase. When recombination occurs, the *ccd*B gene is replaced by the gene of interest, consequently, cells that take up unreacted vectors carrying the *ccd*B gene or by-product molecules retaining the *ccd*B gene will fail to grow. The entry clones are selected on plates containing kanamycin because the donor vector possesses the kanamycin resistance gene. Subsequently, the recombination of the entry clone with a destination vector that has the *att*R1 and *att*R2 sequences and the *ccd*B marker produces the expression clone. The expression clone is generated along with a by-product plasmid containing *ccd*B, due to the addition of LR Clonase™. The expression clones are selected on plates containing ampicillin because of the ampicillin resistance gene in the destination vector (Invitrogen User Manual, 2003) (Figure 5).

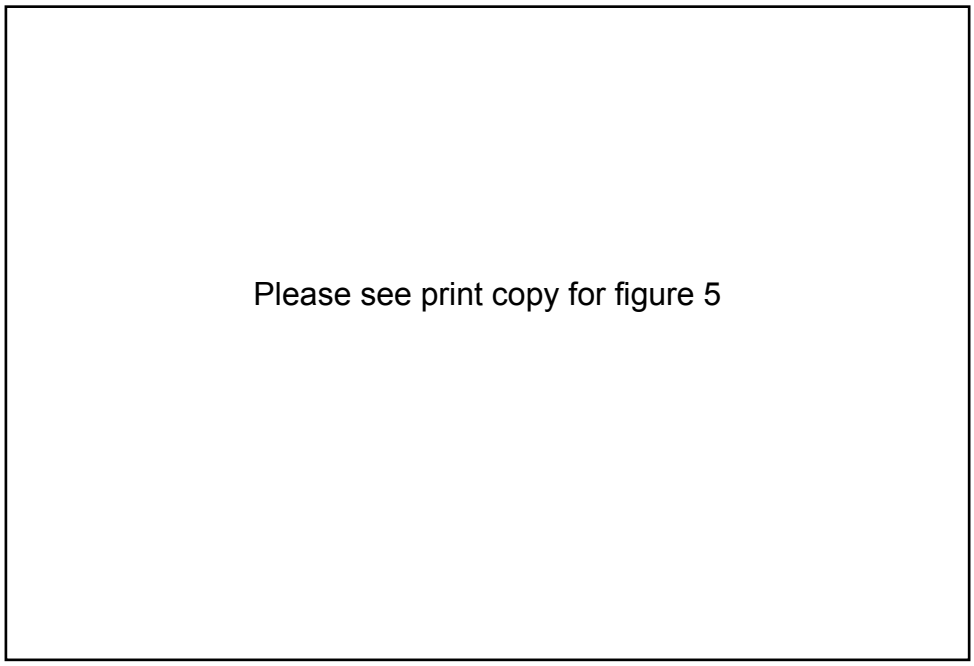
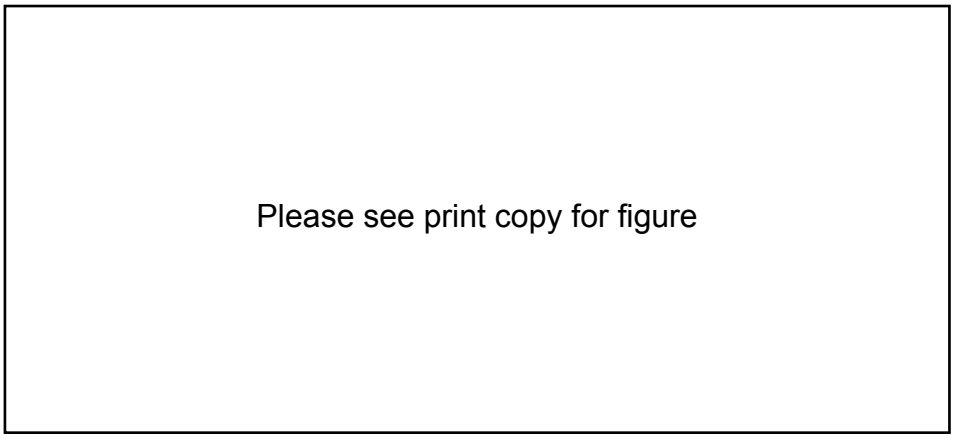


Figure 5. Outline of the BP and LR reactions in the Gateway® cloning technology (Invitrogen User Manual, 2003) .

2.3.1 *att*B-Flanked clusterin-encoding PCR product

A polymerase chain reaction (PCR) product encoding human clusterin lacking the signal peptide was generated using primers designed to incorporate 5`-terminal *att*B sequences, which subsequently allowed insertion of the PCR product into a Donor vector via a Gateway reaction. The addition of the specific *att*B sequences conferred directionality to the insertion of the PCR product.

The primers used had the sequences shown below:



For the PCR reaction, a volume of 20 µl of PCR mixture contained 1x of *Pfu* DNA polymerase buffer with 20 mM $MgSO_4$ (Promega, Sydney), 1 Unit *Pfu* polymerase (Promega, Sydney),

200 ng clusterin pRC HT7 plasmid (88 ng/µl, supplied by Mark Wilson's laboratory) as PCR template, 200 µM of dNTPs, 500 nM of *attB*1 forward primer and *attB*2 reverse primer, and sterilized distilled water. The PCR reaction was performed using an Eppendorf Mastercycler programmed to: 95 °C for 30 seconds, 10 cycles of 95 °C for 30 seconds, 45 °C for 30 seconds, 68 °C for 3 minutes, 20 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and finally 68 °C for 3 minutes. Subsequently, the PCR product was analyzed by agarose gel electrophoresis to assess the yield and purity of the product and, also, to estimate the amount of DNA obtained.

2.3.1.1 Agarose gel elecrophoresis. The following reagents were used to perform Agarose gel elecrophoresis

- TAE (Tris Acetate EDTA), pH 8.0 (0.04 M Tris-acetate, 0.01 M EDTA)
- Agarose Gel 1% in TAE, pH 8.0
- Ethidium Bromide 0.5 ug/ml in water

Agarose gel electrophoresis was carried out using a 1% agarose gel, TAE as the electrophoresis buffer and applying a constant voltage of 60 V. A 1 kb DNA Step Ladder (Promega, Sydney) was used as a marker. After electrophoresis, the gel was immersed in water containing 0.5 µg/ml of ethidium bromide and allowed to stain for 10 min. Subsequently, the gel was immersed in water for 20 min to destain.

2.3.2 Creation of entry clones from attB-flanked PCR products via the BP reaction.

A volume of 20 µl of BP recombination reaction contained 1.12 ng of attB-flanked clusterin-encoding PCR product, 300 ng pDONR™201 vector (Invitrogen, Sydney), 1x BP Clonase™ Reaction buffer (Invitrogen, Sydney), TE Buffer pH 8.0, and 4 µl BP Clonase™ enzyme mix (Invitrogen, Sydney). The reaction was mixed by vortexing briefly and incubated at 25°C for 1 hour. Subsequently, 4 µg of proteinase K was added and the reaction incubated at 37°C for 10 minutes. Lastly, 1 µl of the reaction was used to transform *E. coli* TOP10 electro-competent cells.

2.3.2.1 Transformation of the recombination reaction products into competent cells. BP recombination reaction (1 µl) was used to transform 40 µl of *E. coli* TOP10 electro - competent cells by electroporation. The electroporation was carried out in a Gene pulser apparatus (BioRad, Sydney) set at resistance (OHMS) of 200, capacitance (uFD) 25 and 2.5 kV. After electroporation the cells were added to 450 µl SOC medium, incubated for 1 hour at 37°C, and 100 µl subsequently plated onto LB plates containing 50 µg/ml kanamycin. After incubation at 37°C overnight, plates were examined and samples of the colonies were taken and grown independently in LB broth containing 50 µg/ml kanamycin in order to carry out plasmid purification to verify the presence of the desired plasmid, and in this way select the expression clones.

2.3.2.2 Analysis of plasmids for the presence of clusterin insert by restriction enzyme digestion. Plasmids from transformants grown on kanamycin plates were extracted using the Wizard® Plus minipreps DNA purification system (Promega, Sydney) and analysed by 1% agarose gel electrophoresis. Samples of the plasmids were also digested with the restriction enzymes EcoRI (Fermentas, Life Sciences) or *Hind*II (Fermentas, Life Science) for 1.5 hours at 37°C prior to electrophoresis. *EcoR*I has a cut position at 817 bp in the clusterin-encoding sequence and a cut position at 1744 bp in the pDONR™201 vector. *Hind*II has cut positions at 150 bp and 1010 bp in the clusterin-encoding sequence and does not cut the pDONR™201 vector. Restriction digestions were performed as in 2.2.3.1

2.3.3 Creation of expression clones by recombination of an entry clone and a destination vector via the LR reaction.

The LR reaction involves the reaction of the entry clone (clusterin-pDONR™201) with a destination vector (pET-DEST™42) to create an expression clone. A volume of 10 µl of BP recombination reaction contained 100 ng entry clone (clusterin-pDONR™201), 150 ng destination pET-DEST™42 vector (Invitrogen, Sydney), TE Buffer pH 8.0 and 1x Gateway LR Clonase II enzyme mix (Invitrogen, Sydney). The reaction was mixed by vortexing briefly and incubated at 25°C for 2 hour. Subsequently, 1 µg of proteinase K (Invitrogen, Sydney) was added and the reaction incubated at 37°C for 10 minutes. Finally, the reaction was used to transform competent cells.

2.3.3.1 Transformation of expression vector into competent cells. An aliquot of 1 µl of the LR reaction was transformed into 50 µl of *E. coli* BL21 by heat-shocking the cells at 42°C for 40 seconds after 30 minutes of incubation on ice. After heat-shock, the cells were added to 250 µl SOC medium and incubated for 1 hour at 37°C. Lastly, 100 µl of the cells were plated on LB plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. Plates were subsequently examined and samples of colonies taken and grown independently in a volume of 1 ml of LB broth containing 100 µg/ml ampicillin.

2.3.3.2 Analysis of putative expression plasmids for the presence of clusterin-encoding insert by restriction enzyme digestion. Plasmids were extracted from transformants using the Wizard® Plus minipreps DNA purification system (Promega, Sydney) and analysed by 1% agarose gel electrophoresis. Using the protocol described in 2.2.3.1, samples of the plasmid were separately digested with *Sfi*I (Fermentas, Life Sciences). *Sfi*I has a cut position at 407 in the clusterin-encoding sequence.

2.3.4 Protein expression from Gateway® expression clones

After confirming the presence of the expression vector, a small scale protein expression assay was performed. *E. coli* BL21 clusterin pET-DEST™42 was grown in 5 ml LB broth at 37°C to $2x10^8$ cells/ml; subsequently, the culture was diluted in 10 ml of LB broth containing 100 µg/ml ampicillin and grown at 37°C to $2x10^8$ cells/ml. The bacteria were induced to

express clusterin – 6x His Tag fusion protein by adding 0.3 mM IPTG and incubating them overnight at 25°C. The bacteria were subsequently harvested by centrifugation in a Beckman J2-MC at 3440 x g for 10 min; the supernatant was removed and the pellet collected for protein extraction.

For large scale protein expression of clusterin – 6xHis Tag fusion, *E. coli* BL21 clusterin pET-DEST™42 was grown in 5 ml LB broth at 37°C to $2x10^8$ cells/ml and then diluted in 250 ml of LB broth containing 100 µg/ml ampicillin and grown as described in the small scale assay. The induction was made by addition of 0.3 mM IPTG and incubation overnight at 37°C. The bacteria were subsequently harvested (as described above) and the pellet collected for protein extraction.

2.3.5 Protein extraction.

The protocol for protein extraction using the Complete lysis-β (2x), EDTA free reagent set (Roche, Sydney) was performed. The EDTA-free lysis buffer leaves the stability and function of metal-dependent proteins unaffected and the affinity purification of poly-his tagged fusion proteins via Immobilized metal affinity chromatography is facilitated. (Roche Product Data Sheet 2007).

2.3.5.1 Small scale protein extraction. A preliminary small scale assay for protein extraction from a 10 ml bacterial culture was performed as described in 2.2.4.1. The soluble protein fraction and the inclusion bodies were collected and analyzed by Western Blot using anti-clusterin antibodies. The preliminary small scale assay showed most of the clusterin – 6xHis Tag fusion protein present in the inclusion bodies.

2.3.5.2 Large scale protein extraction. The protocol for large scale protein extraction was performed as described in the data Sheet of Complete lysis-β (2x), EDTA free reagent set (Roche Product Data Sheet 2007) to purify the inclusion bodies in order to extract the 6xHis-Tag clusterin fusion protein. For each 250 ml of harvested *E. coli* BL21 culture expressing 6xHis-Tag clusterin fusion protein, 10 ml of Lysis-β reagent was added to resuspend the cells. The cells were vortexed until homogenized and shaken at 600 rpm for 10 min at room temperature in a MS1 Minishaker (IKA, Sweden). The suspension was centrifuged at 27000 xg for 15 min. The supernatant, containing the soluble protein fraction, was collected. For purification of inclusion bodies, the pellet containing the cell debris and insoluble proteins was resuspended into 7 ml of Lysis-β reagent, supplemented with 400 µg/ml lysozyme and 0.5 mg/ml DNase I (Roche, Sydney), and incubated at room temperature for 30 min. Subsequently 100 ml of 1:20 diluted Lysis-β reagent was added and the mix vortexed briefly and centrifuged at 27000 xg for 15 min. The pellet obtained was resuspended again in 100 ml of 1:20 diluted Lysis-β reagent, vortexed briefly and centrifuged at 27000 xg for 15 min. The latter process was performed two more times. Finally, the inclusion body pellet was

resuspended in 10 ml 8 M urea, 50 mM phosphate, 0.3 M NaCl, 10 mM DTT and vortexed at 200 rpm overnight at 4°C in a MS1 Minishaker (IKA, Sweden). Finally, the suspension was centrifuged at 2000 rpm for 30 min and the supernatant collected and stored at -20 °C.

2.4 Purification of Clusterin using Immunoaffinity Chromatography

Affinity chromatography includes purification methods based on a specific affinity between a protein and its matrix-bound ligand that can be biologically specific (peptide, antibody, nucleic acid) or can exploit nonspecific interactions (with lectins or dyes) (Bollag et al., 1996). In antibody affinity chromatography, a column is packed with beads to which a specific antibody is covalently attached. The antibody column retains the specific protein that reacts with the immobilized antibody while all other proteins pass through the column. After the binding step, the bound protein is eluted by adding an excess of ligand or by changing the salt concentration or pH (Figure 6) (Lodish et al., 2000).

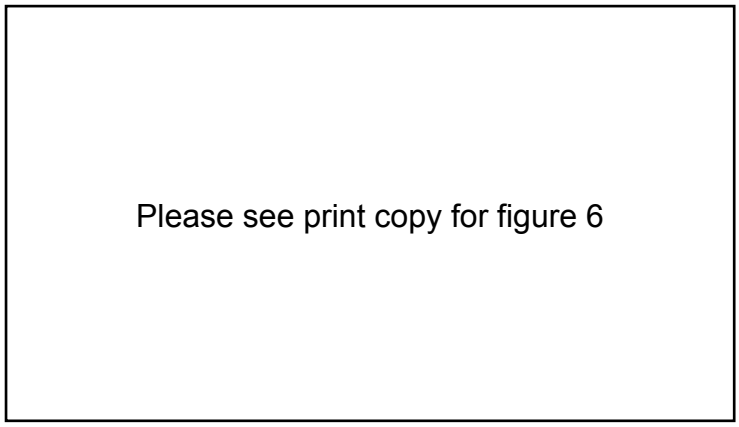


Figure 6. Antibody affinity chromatography. Only protein with high affinity for the antibody is retained by the column. Unbound proteins flow through the column (tube 1). The desired protein, bound to the column, is subsequently eluted (tube 3) by disruption of the antigen-antibody complexes (Lodish *et al*., 2000).

2.4.1 Immunoaffinity chromatography using anti-clusterin monoclonal antibody coupled immunoaffinity columns for purification of clusterin – MBP fusion protein.

Immunoaffinity chromatography has previously been used successfully to purify clusterin from human serum (Wilson and Easterbrook-Smith, 1992). Two Sepharose-based immunoaffinity columns, bearing immobilized G7 and 41D anti-clusterin antibodies, were supplied for purification of clusterin (Mark Wilson's laboratory).

The soluble fraction obtained after protein extraction was suction filtered through a Whatman 70 mm diameter glass microfibre filter. Subsequently, in order to capture the clusterin present in the filtered protein fraction, it was passed through the anti-clusterin immunoaffinity columns

(connected in tandem) using a BioRad EconoPump Model CP-1 set to a flow rate of 0.5 ml/min. After clusterin binding, the columns were washed with 60 ml of PBS/0.1% sodium azide (PBS/Az), and then 30 ml of 0.5% (v/v) Triton X-100 in PBS/Az to remove any bound apolipoprotein A-I (which is known to co-purify with clusterin) (Rosenberg and Silkensen, 1995). The columns were next washed with a further 30 ml of PBS/Az and then clusterin was eluted using 2 M guanidine hydrochloride/PBS (pH 8.0). The eluate was dialyzed overnight at 4^{o}C against 3 L of PBS to remove the guanidine hydrochloride present and finally concentrated (as described in 2.4.4) to a final volume of about 0.4 ml, and stored at -20^{o}C.

2.4.2 Immunoaffinity chromatography using Ni-NTA immunoaffinity column for purification of clusterin - 6xHis Tag fusion protein

Ni-NTA matrices are used for purifying 6xHis-tagged proteins in a liquid chromatography system; a Ni-NTA Superflow 1 ml cartridge was used to purify clusterin – 6xHis Tag fusion protein. A Ni-NTA Superflow 1 ml cartridge (6.7 mm x 28.0 mm) (Qiagen, Australia) was equilibrated with 8 M urea 0.1 M NaH_2PO_4, 0.1 M Tris at pH 8.0 at room temperature before clusterin – 6xHisTag fusion protein in 8 M urea, 50 mM NaH_2PO_4, 0.3 M NaCl, 1 M DTT, pH 8.0 was loaded. Protein was passed over the column at a flow rate of 1 ml/min using an AKTA $_{FPLC}$ (GE Healthcare, Sydney). Clusterin – 6xHis Tag fusion protein bound to the column; the protein fraction that did not bind to the column was collected. After binding of clusterin – 6xHis tag fusion protein, the column was washed with 8 M urea, 0.1 M NaH_2PO_4, 0.1 M Tris at pH 8.0. Clusterin– 6xHis Tag fusion protein was eluted using 8 M urea, 0.1 M NaH_2PO_4, 0.1 M Tris at pH 4.5 and the absorbance at 280 nm of the eluate continuously quantified. The eluate was collected and concentrated to 1 ml (as described in 2.4.4).

2.4.3 Refolding and alkylation of clusterin - 6xHis Tag fusion protein

The production of recombinant proteins in bacteria frequently yields an inactive protein, aggregated in the form of inclusion bodies. Proteins found in the inclusion bodies require solubilization and refolding procedures after extraction. Solubilization could be carried out by using high concentrations of denaturing agents (such as urea) and low molecular weight reagents such as DTT or 2-mercaptoethanol, which reduce inter- and intramolecular disulfide bonds and keep cysteines in their reduced state. Refolding techniques include direct dilution that consists of diluting the concentrated protein-denaturant solution into a refolding buffer that allows the protein to regain its native structure (Vallejo and Rinas, 2004).

Because the eluted clusterin – 6xHis Tag fusion protein was eluted in a buffer containing high concentrations of a denaturing agent (8 M urea, 50 mM phosphate, 0.3 M NaCl, 10 mM DTT) a refolding process was necessary. The protein was refolded by direct dilution, adding a volume of 1 ml of clusterin – 6xHis Tag fusion protein into the refolding buffer, 50 mM NaH_2PO_4, 300 mM NaCl, 1 mM DTT, 10% glycerol, 0.1% azide. The protein was added drop by drop and the solution continually stirred. After refolding, the clusterin – 6xHis Tag fusion protein was alkylated with 50 mM iodoacetamide (IAA) (Sigma, USA). Alkylation of the protein

avoids the formation of disulfide-bonded high molecular weight aggregates. Subsequently the protein was concentrated to 1 ml (as described in 2.4.4) and stored at 4°C. For functional and structural analysis of clusterin – 6xHis Tag fusion protein, the refolded protein was dialyzed overnight at 4°C against 2 L of PBS, pH 7.5 (for chaperone and SEC assays) or 5 mM Na_2HPO_4 , pH 8.5 (for CD assays).

2.4.4 Concentration of protein by ultrafiltration and estimation of protein concentration by absorbance at 280 nm

Proteins in the eluate were concentrated by ultrafiltration using Millipore Amicon Ultra-15 centrifugal filter units (30000 Da Molecular Weight Cut Off) and a Megafuge 1.0R Heraus centrifuge operating at 4°C and 2500 rpm.

Recombinant clusterin concentration was estimated by measuring the protein absorbance at 280 nm. Absorbance measurements were made in a WPA Biowave S2100 Diode Array Spectrophotometer using quartz cuvettes. For the purpose of these estimates, it was assumed that a 1 cm light path through a 1 mg/ml solution of clusterin would give an absorbance at 280 nm of 1.0.

2.5 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie Blue Stain and Western Blot

2.5.1 Buffers and reagents

- SDS gel loading buffer (50 mM Tris-HCL pH 6.8, 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol)
- Tris-glycine electrophoresis buffer (0.5 mM Tris, 50 mM glycine pH 8.3, 0.1% (w/v) SDS)
- Western Transfer buffer (48 mM Tris base, 39 mM glycine, 20% (v/v) methanol, 0.037% (w/v) SDS)
- Coomassie Blue R-250 (methanol 50% (v/v), acetic acid 10% (v/v), Coomassie Blue R-250 0.25% (w/v)).
- Destain solution for Coomassie Blue R-250 (methanol 50% (v/v), acetic acid 10% (v/v)).

2.5.2 SDS-PAGE gel composition

- 10% SDS-PAGE Acrylamide resolving gel (10% acrylamide mix (29% w/v acrylamide and 1% w/v N,N'-methylenebisacrylamide), 1.5 M Tris pH 8.8, 10% (w/v) SDS, 10% (w/v) ammonium persulfate, 0.04% (v/v) TEMED (N,N,N',N'-tetramethylenediamine)).
- 5% SDS-PAGE Stacking gel (5% acrylamide mix (29% w/v Acrylamide and 1% w/v N,N'-methylenebisacrylamide), 1.0 M Tris pH 6.8, 10% (w/v) SDS, 10% (w/v), ammonium persulfate, 0.1% (v/v) TEMED).

2.5.3 SDS-PAGE of recombinant forms and plasma-derived clusterin

Recombinant forms of clusterin and plasma-derived clusterin samples were analysed by SDS-PAGE under both reducing and non-reducing conditions. Samples were reduced by adding 1 µl of β-mercaptoethanol to 20 µl of sample buffer containing protein before boiling for 5 min. After boiling, the samples were centrifuged briefly at 13000 rpm (13800 x g) (to remove insolubles) and subsequently resolved on a 1.5 mm thick 10% SDS-PAGE gel.

SDS PAGE gels were placed in a mini-vertical gel electrophoresis unit (SE 250, Amersham Biosciences). Samples and a protein standard (Precision Plus Protein Standard, BioRad) were loaded into sample wells and electrophoresis performed using a constant 120 V. Proteins separated by SDS-PAGE were stained with Coomassie Brilliant Blue (see above). Excess dye was removed by immersing the stained gel in destain solution overnight. In some cases, after electrophoresis, proteins in the gel were electrophoretically transferred onto nitrocellulose membrane in order to perform a Western Blot.

2.5.4 Western Blot

2.5.4.1 Transfer of proteins from SDS-polyacrylamide gels to nitrocellulose. A Nitrocellulose BioTrace NT Membrane (PALL Gelman Laboratory) was equilibrated in Western Transfer Buffer for 30 min. Filter paper and a gauze pad which were used in the transfer were previously wet in the transfer buffer. Subsequently, the gel sandwich was prepared in the gel holder cassette; one gauze pad was placed on the base (cathode) of an electrode cassette (gray side), followed by three layers of filter paper, then the gel and the nitrocellulose membrane, and finally three layers of filter paper and a second gauze pad. Air bubbles that could be present were removed by rolling a glass tube. The gel holder cassette was placed in a BioRad electroblotting Mini Trans-Blot Electrophoretic transfer cell. The transfer was carried out at 100 V for 1 hour at 4^{o}C. A stirring bar was used to help maintain the buffer temperature and ion distribution in the buffer tank (BioRad Instruction Manual, 2007). After the transfer, the membrane was blocked by placing it into 1% HDC/PBS/ 0.01% thimerosal overnight at 4^{o}C.

2.5.4.2 Immunodetection of clusterin. A cocktail of anti-clusterin antibodies was prepared with 10 µg/ml each of 41D and G7 anti-clusterin antibodies in 3 ml of 1% HDC/PBS/0.01% thimerosal. The blocked membrane was incubated in this solution with rocking for 2 hours at room temperature. Subsequently, the membrane was washed three times with PBS for 10 minutes each wash. A secondary antibody was prepared by diluting 4 µl of sheep anti-mouse-HRP and 0.2 µl Precision StrepTactin-HRP conjugate (BioRad Cat # 161-0380) in 2 ml 1% HDC/PBS/0.01% thimerosal. The washed membrane was incubated with secondary antibody at room temperature for 1 hour. Finally, the membrane was washed repeatedly with distilled water and then with 0.1% (v/v) Triton X-100/PBS (5 min each wash with shaking) and developed using a SuperSignal West Pico Chemiluminescence (ECL) detection kit (Pierce, USA) following the manufacturer's instructions.

2.5.4.3 Immunodetection of MBP fusion protein. To detect fusion proteins containing MBP, blocked nitrocellulose membrane was incubated with rabbit anti-maltose binding protein (MBP) antibody (diluted 1 in 2000 in 1% HDC/PBS/0.01% thimerosal) for 2 hours at room temperature with rocking. Subsequently the membrane was washed 3 x 10 min with PBS. A secondary antibody was prepared by diluting 4 µl of sheep anti-rabbit IgG-HRP and 0.2 µl Precision StrepTactin-HRP conjugate in 2 ml 1% HDC/PBS/0.01% thimerosal. The washed membrane was incubated with secondary antibody at room temperature for 1 hour with rocking. Finally the membrane was washed repeatedly with distilled water and then with 0.1% Triton X-100/PBS (3 x 5 min) before being developed by ECL as described above.

2.6 Protein precipitation assays to measure chaperone activity

The abilities of clusterin – 6xHis Tag fusion protein and plasma-derived clusterin to inhibit the heat-induced aggregation and precipitation of fibrinogen were compared. Individual solutions of fibrinogen (0.3 mg/ml) or clusterin (plasma-derived and clusterin – 6xHis Tag fusion protein, all at 0.05 mg/ml), and mixtures of fibrinogen with each of these proteins (at 0.05 mg/ml and 0.1 mg/ml) were prepared in PBS and heated at 55°C.

In the same way, the abilities of the following proteins to inhibit the heat-induced aggregation and precipitation of fibrinogen were compared: human plasma clusterin, flag-tagged full-length recombinant human clusterin (expressed in human embryonic kidney cells) and His-tagged partial-length recombinant rat clusterin (amino acid 146-360 of the rat clusterin sequence niProtKB/Swiss-Protein entry P05371, expressed in *E. coli,* not post translationally processed, not cleaved into α and β chains and not glycosylated) (both commercial proteins from BioVendor- Laboratory Medicine, Inc.). As above, individual solutions of fibrinogen (0.3 mg/ml) or clusterin (all at 0.02 mg/ml), and mixtures of fibrinogen with each of these proteins (all at the same final concentrations) were prepared. The experiments were performed in the wells of 96 well plates (Greiner, Germany). Aggregation of fibrinogen was monitored by measuring light scattering (turbidity) at 360 nm in a FLUOStar Optima (BMG Labtech, Melbourne) microplate reader. Three replicates of all samples were assayed.

2.7 Analyses of clusterin structure

2.7.1 Size Exclusion Chromatography

2.7.1.1 SEC to evaluate the structure of clusterin–MBP fusion protein, flag-tagged recombinant human clusterin and His-tagged recombinant rat clusterin. The solution size/oligomeric state of plasma-derived and recombinant forms of clusterin were compared by size exclusion chromatography (SEC) using an $AKTA_{FPLC}$ system (G E Healthcare, Sydney), with UNICORN™ software and a Bio™-SEC-S4000, silica-based gel filtration column (Phenomenex, Sydney). The Bio™-SEC-S4000 column is capable of resolving proteins of

high molecular mass ranging in size from 66 to 670 kDa. The column was equilibrated in PBS containing 0.02% (w/v) azide, pH 7.4. Aldolase (150 µg; 170 kDa), BSA (50 µg; 67 kDa), chymotrypsinogen A (15 µg; 25 kDa), Blue dextran (50 µg; 2000 kDa) and ferritin (50 µg; 460 kDa) were used as molecular mass standards. In addition, 50 µg aliquots of the recombinant forms of clusterin and plasma-derived clusterin were analyzed. Separations were performed at a flow rate of 0.2 ml/min and the absorbance at 280 nm of eluted proteins was continuously recorded.

2.7.1.2 SEC to purify clusterin – 6xHis Tag fusion protein. SEC chromatography using a Superose-6 column (GE Healthcare, Sydney) was performed subsequent to Ni-NTA affinity chromatography (2.4.2) in order to further purify clusterin – 6xHis Tag fusion protein present in the flow through fraction. A 24 ml bed volume Superose™ 10/300 GL column (GE Healthcare, Sydney) was equilibrated with 8 M urea, 50 mM NaH_2PO_4, 0.3 M NaCl, 2 mM DTT, pH 8.0 at room temperature. The protein fraction that did not bind to the Ni-NTA column (2.4.2) was reduced with 10 mM DTT and loaded onto the Superose-6 column. Protein was passed over the column at a flow rate of 0.2 ml/min and the absorbance at 280 nm of the eluted fractions continuously quantified using an $AKTA_{FPLC}$ (GE Healthcare, Sydney). The eluted fractions were collected separately and analysed by SDS-PAGE. Finally, the fraction containing clusterin – 6xHis Tag fusion protein was refolded and alkylated as described in 2.4.3.

2.7.2 Circular dichroism spectroscopy

Circular dichroism spectroscopy (CD) is defined as the differential absorbance of the left circularly polarized light (LCPL) and right circularly polarized light (RCPL) and provides information about the secondary and tertiary structures of proteins. Increased relative absorption of left polarized light results in a positive CD signal, and when there is an increment of relative absorption of right polarized light, it results in a negative CD signal. Secondary and tertiary structure of protein can be estimated using CD spectra. Also, changes in the CD signal reflect changes in protein structure. Secondary motifs exhibit distinctive CD spectra in the far ultraviolet region (170-260 nm) while tertiary structure can be predicted in the near ultraviolet region (260-320 nm) (Boxrud, 2007). Alpha-helix, beta sheet, and random coil motifs of secondary structure exhibit the characteristic CD spectra showed in Figure 7.

2.7.2.1 CD to evaluate the structure of clusterin – 6xHis Tag fusion protein. The secondary structure of clusterin – 6xHis Tag fusion protein and plasma-derived clusterin were compared using circular dichroism spectroscopy. Far ultraviolet spectra (190-250 nm) were acquired using a 1.0 mm path length cell and a Jasco J-810 spectropolarimeter (Jasco, Canada) linked to a CDF-426S/L Peltier system (Jasco, Canada) with Jasco-SpectraManager software. Spectra were acquired at 100 millidegree sensitivity, scanning continuously at speed 50 nm/min and accumulated over 2 scans.

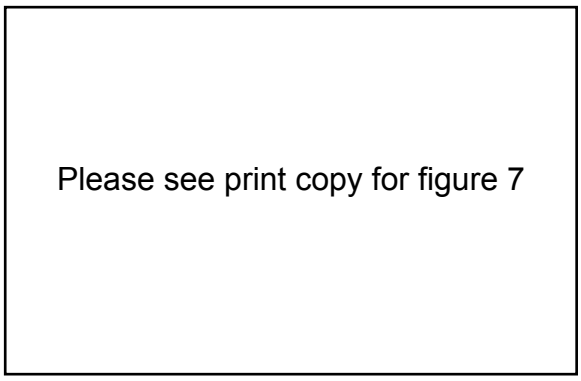


Figure 7. CD spectra shown by three common motifs of secondary structure (Boxrud, 2007).

Clusterin – 6xHis Tag fusion protein and plasma-derived clusterin (30 µg of protein) were prepared in a total volume of 300 µl 5 mM Na_2HPO_4 buffer, pH 7.4, and analyzed in the spectropolarimeter. Results were reported in units of ellipticity or millidegrees (mdeg), and the raw data were expressed as mean residue ellipticity (θ_{MRE}), correcting the molar ellipticity for concentration and the number of amino acid residues in the protein. Values were calculated assuming that (i) plasma-derived clusterin contains 427 residues and has an average molecular mass of 61 kDa (Stewart *et al.*, 2007), and (ii) clusterin – 6xHis tag fusion protein contains 433 residues and has a molecular mass of 50 kDa (estimated by SDS-PAGE). Estimation of the α-helical, β-sheet and unordered secondary structure content were made by deconvolution of the θ_{MRE} data using the program CONTIN-LL (Provencher and Glochner, 1981) (Whitmore and Wallace, 2004).

2.7.2.2 CD to evaluate the structure of flag-tagged recombinant human clusterin and His-tagged recombinant rat clusterin (BioVendor-Laboratory Medicine, Inc). The secondary structure of recombinant forms and plasma-derived clusterin were compared using circular dichroism spectroscopy as described in 2.7.2.1. Recombinant forms and plasma-derived clusterin (20 µg of protein) were prepared in a total volume of 300 µl 5 mM Na_2HPO_4 buffer, pH 7.4, and analyzed in the spectropolarimeter. Results were reported in mdeg and the raw data were expressed as θ_{MRE}, correcting the molar ellipticity for concentration and the number of amino acid residues in the protein.

Values were calculated assuming that (i) plasma-derived clusterin contains 427 residues and has an average molecular mass of 61 kDa (Stewart *et al.*, 2007), (ii) flag-tagged recombinant human clusterin contains 438 residues and has a molecular mass of 51,27 kDa (BioVendor Product Data Sheet, 2007), and (iii) His-tagged recombinant rat clusterin contains 215 residues and has a molecular mass of 26.5 kDa (BioVendor Product Data Sheet, 2007). Estimation of the α-helical, β-sheet and unordered secondary structure content were made as in 2.7.2.1.

2.8 IgG Binding Assays

Studies by Wilson and Easterbrook-Smith (1992) showed that clusterin binds to human IgM and IgA, and to all the isotypes of human IgG, and also that clusterin has binding sites for the Fc and Fab regions of IgG. The binding affinity of clusterin for the human immunoglobulin isotypes was estimated by ELISA and ranked as follows: $IgG_3 > IgG_4 > IgM > IgG_1 > IgG_2 >$ IgA. Also, in this study they suggest that clusterin contains multiple immunoglobulin binding sites because of its enhanced binding to aggregated IgG (Wilson and Easterbrook-Smith, 1992).

2.8.1 Evaluating binding of clusterin to human IgG by ELISA.

The wells of a 96-well ELISA plate (Greiner, Germany) were coated with 100 µl of purified human IgG (diluted to 20 µg/ml in PBS) then incubated for 1 hour at 37°C with 1% (w/v) heat-denatured casein in PBS (HDC/PBS). After washing the plate with PBS, biotinylated plasma-derived and recombinant forms of clusterin, at various concentrations diluted in PBS, were incubated in the wells for 1 hour at 37°C. Also, a negative control protein (a-lactoalbumin) was included at various concentrations diluted in PBS in the assay, to confirm that the signal generated was specific to the protein concerned. Next, three washes were performed with PBS. Wells were subsequently incubated 1 hour at 37°C with a volume of 50 µl of steptavidin/biotinylated horseraddish peroxidase (each at 2.5 µg/ml) solution (SA/HRP) in HDC/PBS. The tray was then washed again with PBS, 0.1% Triton X-100 in PBS, and finally PBS. Lastly, the substrate solution (2.5 mg/ml *o*-phenylenediamine dihydrochloride in 0.05 M citrate, 0.1 M phosphate pH 5.0, containing 0.03% (w/v) hydrogen peroxide) was added to all wells. The reaction was stopped with 50 □l/well 1 M HCl and the absorbance measured at 450 nm with a SpectraMax Plus microplate reader (Molecular Devices, USA). Negative (two wells coated with a-lactoalbumin and added with biotinalated a-lactoalbumin) and positive controls (two wells coated with IgG were added with rabbit anti- human IgG and wells coated with biotinilated clusterin) were also included in the assay (data not shown).

3. EXPRESSION, PURIFICATION AND CHARACTERIZATION OF CLUSTERIN - MALTOSE BINDING PROTEIN (MBP) FUSION PROTEIN

3.1 Introduction

The expression of eukaryotic proteins in prokaryotic cells can be problematic, especially concerning the expression of functional membrane or secretory proteins, because some post-translational processes (glycosylation, phosphorylation, oligomerization, specific protein cleavage, ordered disulfide bond formation) are not performed by bacterial cells (Sambrook *et al*., 1989). However, the demonstrations by Stewart et al. (2007) that clusterin retains its chaperone function when deglycosylated and that deglycosylation did not induce major structural changes in the molecule, raised the possibility to inexpensively produce large quantities of chaperone-active clusterin in bacteria.

Recombinant clusterin expressed in bacteria will consist of a single polypeptide (not cleaved into α and β chains). It was hoped that this lack of cleavage would not substantially compromise the chaperone action of the molecule. Recombinant clusterin was expressed as a fusion protein with MBP to allow its rapid purification. The desired outcome was that the results of these studies indicate that the recombinant protein is similar in chaperone function to the wild type protein purified from human plasma.

3.2 Methods

3.2.1 Restriction digestion of purified plasmids to test for the presence of the clusterin encoding insert.

Restriction digestion (with *Bgl*II, *Sma*I and *Xba*I; as described in 2.2.3.1) of purified pMal-c2x-Clusterin plasmids supplied by Rebecca Dabbs (who worked as a Research Assistant in Mark Wilson's laboratory) followed by 1% agarose gel electrophoresis were carried out to confirm the presence of the clusterin-encoding insert.

3.2.2 Expression and purification of clusterin - MBP fusion protein.

The clusterin - MBP fusion protein was expressed in *E. coli* TBI pMal-c2x as described in 2.2.3.2 and samples of uninduced and induced bacteria were collected. In order to confirm the expression of clusterin – MBP fusion protein by *E. coli* TBI pMal-c2x after induction, SDS-PAGE and Western Blot analysis were completed as in 2.5. After confirming the presence of recombinant clusterin in the samples, large scale expression (2.2.3.2), protein extraction (2.2.4) and immunoaffinity chromatography (using G7 and 41D anti-clusterin monoclonal antibodies) (2.4.1) were performed.

3.2.3 Analysis of recombinant clusterin structure.

The purified protein was concentrated by ultrafiltration (2.4.3) and structurally compared with plasma derived clusterin. SDS-PAGE (2.5.3) and size exclusion chromatography (2.7.1.1) were performed.

3.3 Results

3.3.1 Restriction digestion of purified plasmids to test for the presence of the clusterin encoding insert.

A pMal-c2x vector was used for expressing clusterin. Clusterin was inserted downstream from the *malE* gene of *E. coli* TB1, which encodes maltose-binding protein (MBP) resulting in the expression of clusterin - MBP fusion protein (figure 8). The pMal-c2x plasmid has a size of 6646 bp (BioLabs New England Instruction Manual, 2006) while the clusterin insert fragment is 1279 bp. The expected and actual band patterns detected after restriction digestion of the plasmid with *Bgl*I, *Sma*I and *Xba*I are shown below (Table 1).

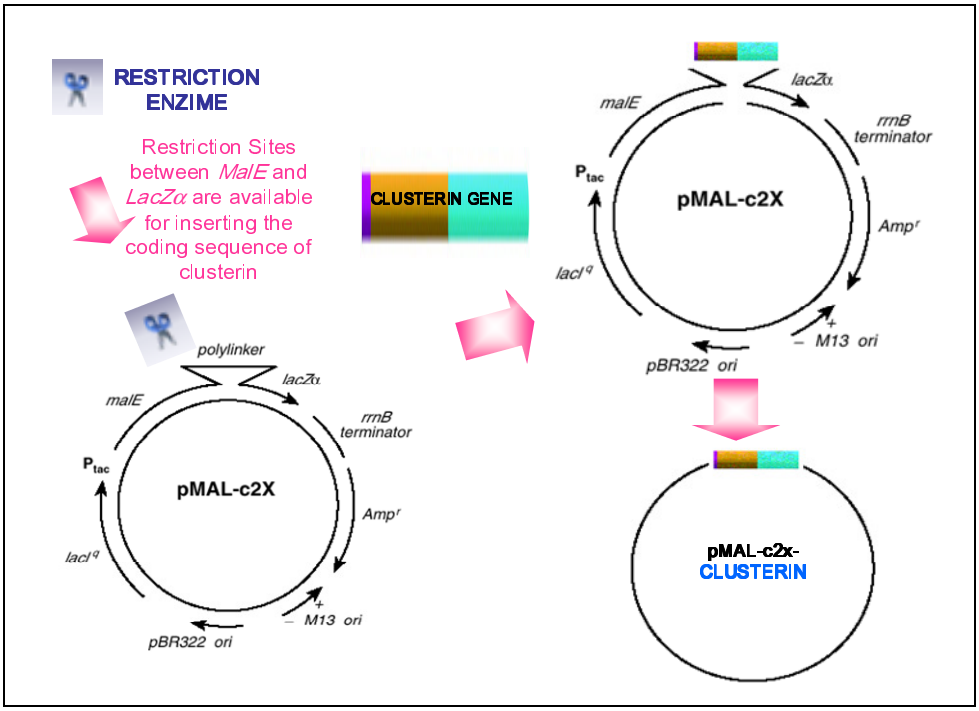


Figure 8. Diagram illustrating clusterin-encoding sequence inserted downstream from the malE gene of *E. coli* TB1in the pMal-c2x vector.

The findings in Figure 9 confirmed the presence of the clusterin encoding insert in the plasmid vector. The two different plasmids tested, from *E.coli* TB1 pMal-c2x-clusterin conserved in glycerol strain and from *E.coli* TB1 pMal-c2x-clusterin conserved in LB agar plate strain, cut with *Bgl*I, *Sma*I and *Xba*I, showed the expected band pattern in the agarose gel (Figure 9).

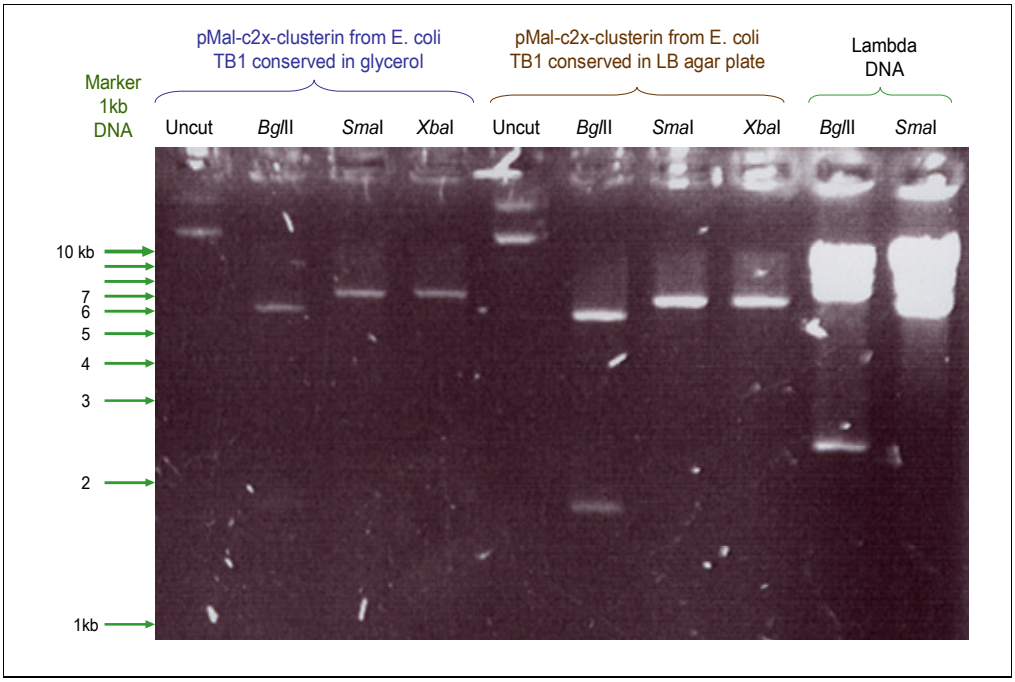


Figure 9. Image of an ethidium bromide stained 1% agarose gel showing the results of restriction digestion of pMal-c2x-clusterin plasmids. Plasmid samples prepared from two colonies of *E. coli* TB1 pMal-c2x-clusterin (one grown from a glycerol stock and another taken from an LB agar plate stored at 4°C) were digested with *Bgl*II, *Sma*I and *Xba*I. The positions of molecular size markers are indicated at the left of the image.

Table 1. Band patterns expected and detected in the agarose gel showed in Figure 9 of pMal-c2x-clusterin plasmid after digestion with *Bgl*I, *Sma*I and *Xba*I

Restriction enzyme	Cut position in Clusterin (bp)	Cut position in pMAL-c2x (bp)	Expected band pattern pMal-c2x-clusterin after digestion	Detected band pattern pMal-c2x-clusterin after digestion
*Bgl*II	920	1987	1640 and 6285	~ 1.6 and ~ 6 kb
*Sma*I	592	-	Linear 7925	~ 7 kb
*Xba*I	-	2707	Linear 7925	~7 kb

3.3.2 Expression of recombinant clusterin protein.

The expression of recombinant clusterin by induction of a small scale culture (5 ml) of *E. coli* TBI pMal-c2x-clusterin with 0.3 mM IPTG incubated at 37°C (overnight) or 25°C (6 hours or overnight), was assessed using SDS-PAGE and Western Blot analysis. After Western Blot with anti-clusterin antibody and under non reduced conditions, the samples showed the band pattern displayed in Figure 10. Clusterin protein was not detected in lysates of uninduced *E. coli* TBI pMal-c2x-clusterin clones. The expected molecular mass for the clusterin - MBP

fusion product is ~ 92 kDa; but unexpectedly, bands at about 50-60 kDa were detected on the immunoblot in lanes corresponding to lysates of induced *E. coli* TBI pMal-c2x-clusterin. The bands detected at 50-60 kDa correspond to degraded fusion protein or free recombinant clusterin protein. Also, the most intense band detected corresponded to overnight IPTG induction at 25℃, suggesting that maximum protein expression occurred under these conditions.

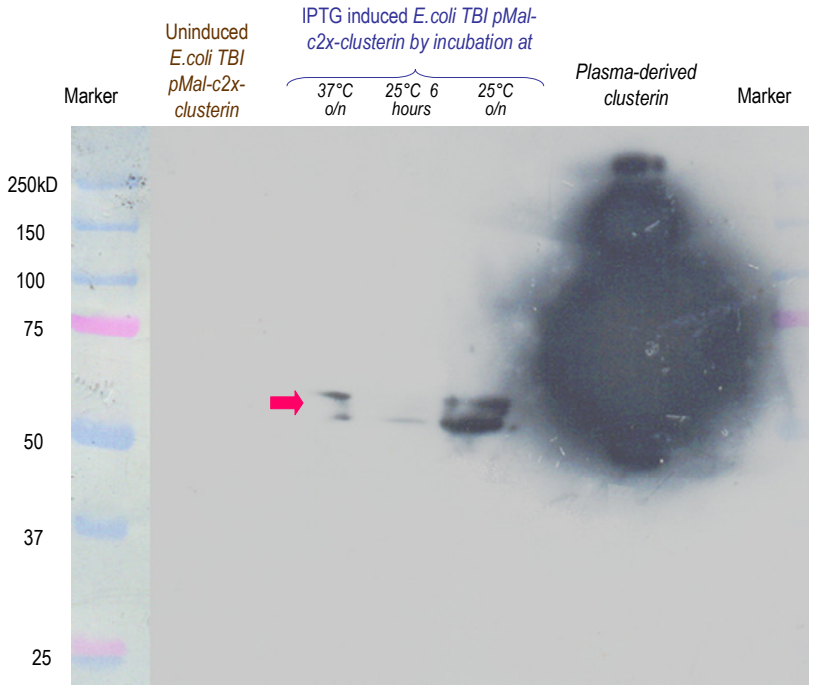


Figure 10. Image of anti-clusterin Western blot probing for expressed clusterin – MBP fusion protein in non-reduced whole cell lysates of *E. coli* TBI pMal-c2x-clusterin clones**.** The red arrow indicates the position of bands detected in lysates of induced clones. The position of molecular mass standards is shown to the left of the image. The identity of samples analysed is indicated above each lane.

Samples taken after IPTG induction were also analyzed by Western Blot using anti-MBP antibody. In the sample corresponding to overnight induction at 25℃, a band of ~ 90 kDa was detected (Figure 11), indicating the presence of intact clusterin - MBP fusion protein (which would be expected to have a molecular mass of about 92 kDa). Both samples also showed a strong band near ~ 42 kDa corresponding to free MBP. This suggests that the fusion protein is degraded; one of the causes of this could be host proteases (BioLabs New England Instruction Manual, 2006).

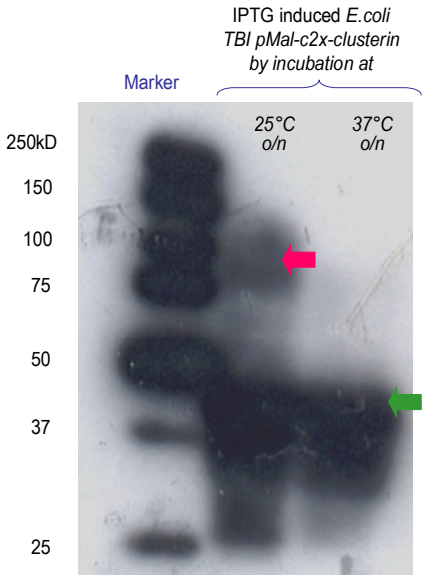


Figure 11. Image of anti-MBP Western Blot probing for clusterin - MBP fusion protein in non-reduced whole cell lysates of *E. coli* TBI pMal-c2x-clusterin clones**.** The position of molecular mass standards is shown to the left of the image. The identity of samples analysed is indicated above each lane. The red arrow indicates the position of intact clusterin - MBP fusion protein. The green arrow indicates the position of free MBP.

Evidence that clusterin - MBP fusion protein is degraded during expression/purification is clear in Figures 10 and 11, where it is clear that most of the recombinant product migrates at less than the mass expected of the intact clusterin-MBP fusion protein. It is uncertain why a band at about 90 kDa, representing intact clusterin-MBP fusion protein, was detected in Figure 11 but not Figure 10, but this may reflect the differential abilities of the anti-MBP and anti-clusterin antibodies to detect this species. The bands at 50-60 kDa detected by anti-clusterin antibody (Figure 10) were not detected by anti-MBP antibody (Figure 11) - this suggests that much of the clusterin-MBP fusion protein has been cleaved free of MBP.

3.3.3 Extraction and purification of clusterin – MBP fusion protein.

Because the expression of recombinant proteins in bacteria often results in incorrectly folded proteins that become incorporated into inclusion bodies (Roche Product Data Sheet 2007), the complete lysis-B (2x), EDTA free Reagent set from Roche (Sydney, Australia) was used to extract soluble and insoluble proteins. After induction and protein extraction, samples of the soluble protein fraction and insoluble protein fraction (inclusion bodies) were collected and analyzed by SDS-PAGE and Western blot using anti-clusterin antibodies in order to discern the protein fraction(s) in which clusterin protein was present. A band at ~ 40 kDa was detected in the sample corresponding to the soluble fraction on the immunoblot (Figure 12) which suggests the presence of degraded fusion protein. A less intense band was also detected at about ~100, which could correspond to intact clusterin – MBP fusion protein

(expected to have a molecular mass of about 92 kDa). Clusterin was not detected in the inclusion bodies sample (insoluble protein fraction).

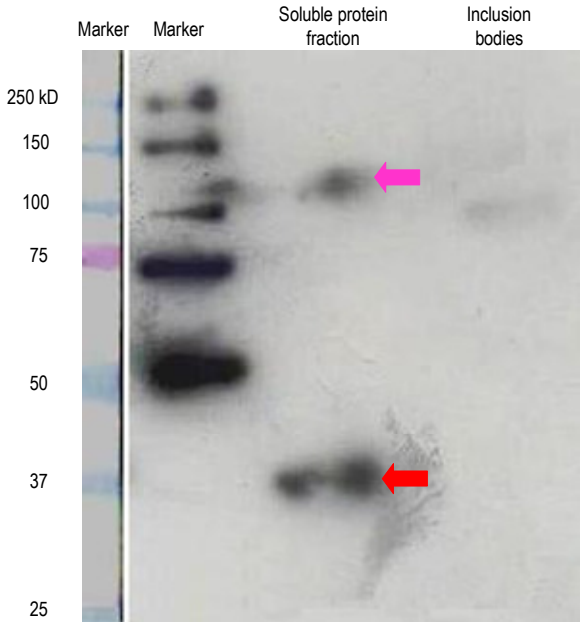


Figure 12. Image of anti-clusterin Western blot analysis of non-reduced soluble and insoluble fractions prepared from induced *E. coli* TBI pMal-c2x-clusterin. The position of molecular mass standards is shown to the left of the image. The identity of samples analysed is indicated above each lane. The red arrow indicates the position of degraded fusion protein. The pink arrow indicates the position of clusterin – MBP fusion protein.

3.3.4 Large scale purification of clusterin – MBP fusion protein.

Small scale expression of clusterin – MBP fusion protein only yielded the full length (~ 92 kDa) fusion protein as a minor product; immunoblotting suggested that most of the fusion protein was degraded during expression/purification. For this reason, a large scale expression was carried out in an attempt to obtain a sufficient quantity of full length clusterin - MBP fusion protein.

Subsequent to IPTG induction of a large scale culture (2 litres) of *E. coli* TBI pMal-c2x-clusterin, clusterin-containing protein was extracted by rapid lysis and purified by immunoaffinity chromatography. After purification, the protein was concentrated by ultrafiltration; the concentration of purified recombinant clusterin was estimated by absorbance at 280 nm as being 0.90 mg/ml (a volume of 1.45 ml was obtained). Western Blot analysis was performed to assess the presence of clusterin – MBP fusion protein (Figure 13).

Western blot analysis of the purified sample did not detect the full length fusion protein (expected at ~ 92 kDa), but detected several bands of lower apparent molecular mass (at ~ 70 kDa, ~ 60 kDa, ~ 50 kDa and ~ 37 kDa). This result suggests that the fusion protein had undergone extensive proteolytic degradation during expression/purification.

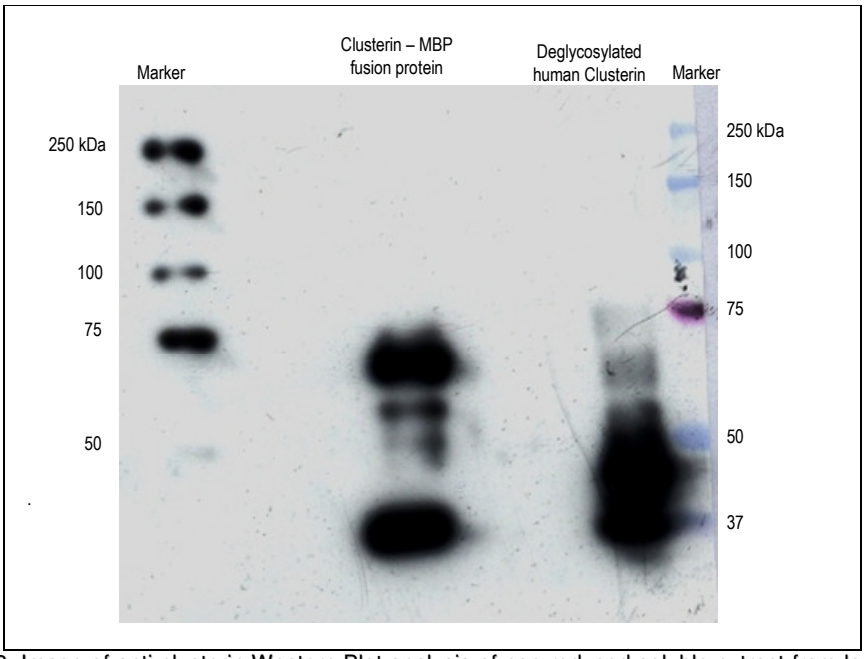


Figure 13. Image of anti-clusterin Western Blot analysis of non-reduced soluble extract from large scale culture of induced *E. coli TBI* pMal-c2x-clusterin. The position of molecular mass standards is shown to the left and right of the image. The identity of samples analysed is indicated above each lane. Deglycosylated human clusterin (2.1.2) was used as a positive control for the blot.

3.3.5 Analysis of the recombinant product structure

Protein immunoaffinity purified from the large scale culture of *E. coli* TBI pMal-c2x-clusterin did not contain the expected full length recombinant protein. SEC was performed to further characterise the product. The SEC was performed two months after the protein was obtained.

Recombinant clusterin and clusterin from human plasma were analyzed by SEC, under two different pH conditions. In aqueous solution at physiological pH, clusterin is found as a mixture of individual α-β heterodimers, together with aggregates of these structures (predominantly in the forms of (α-β)2-4) (Humphreys et al., 1999). A Bio™-SEC-S4000 column was equilibrated with PBS pH 7.5 and clusterin samples were supplemented with 5 µl of HCl 0.05 M to adjust the pH to 5.5. Clusterin samples were incubated at room temperature for 15 min before chromatography in order to allow pH-dependent equilibration of clusterin oligomerization.

In Figure 14, the difference between the elution profiles of the proteins tested is shown. The first absorbance peak at around 2.6 ml corresponds to Blue dextran (2000 kDa). The next absorbance peak is present at around 3.9 ml and the last one at around 4.3 ml; these two peaks correspond to BSA (67 kDa) and chymotrypsinogen A (25 kDa), respectively. The absorbance peak corresponding to aldolase is not visible due to the broad shoulder generated by the blue dextran.

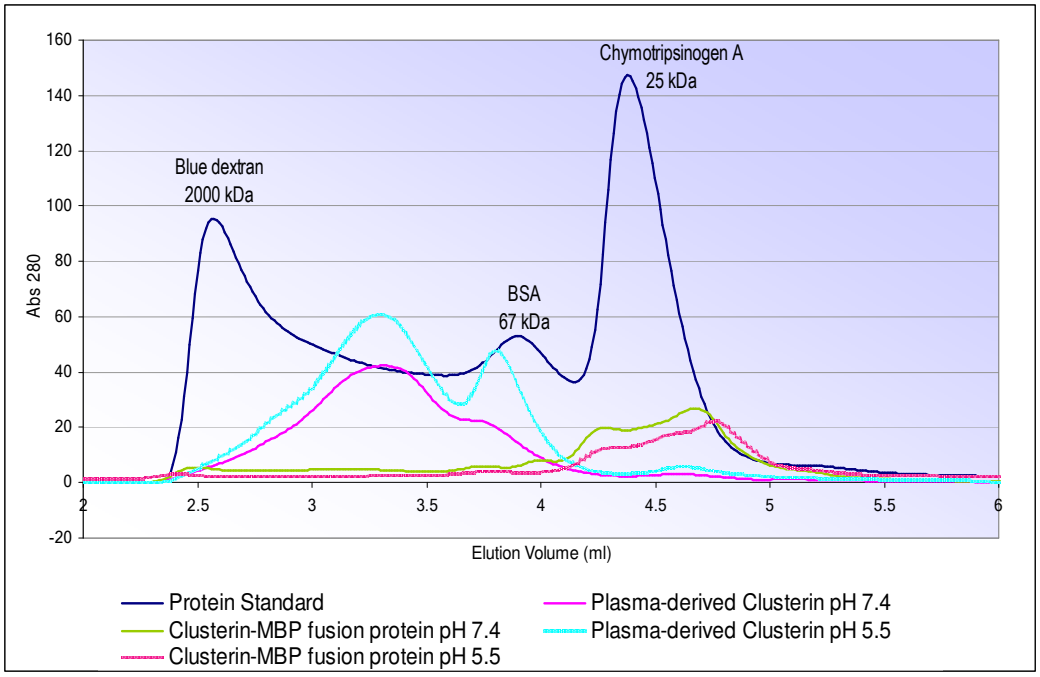


Figure 14. Absorbance (280 nm) traces for size exclusion chromatographic analysis of plasma-derived clusterin and recombinant human clusterin. Proteins (50 µg) were loaded onto a Bio™-SEC-S4000 column using an AKTA$_{FPLC}$ system. Separations were performed at a flow rate of 0.2 ml/min. Protein standard mix composed by Blue dextran (2000 kDa), aldolase (170 kDa), BSA (67 kDa) and chymotrypsinogen A (25 kDa) is displayed as a blue line. Elution profiles formed by recombinant clusterin at pH 7.4 (green line) and pH 5.5 (red line); and elution profiles of clusterin from plasma at pH 7.4 (pink line) and pH 5.5 (blue line) are displayed.

As shown in the chromatogram in Figure 14, analysis of human plasma clusterin at pH 7.4 detected a broad major peak at around 3.3 ml suggesting a mixture of species, mainly aggregates of α-β heterodimers. When the same sample was analysed at pH 5.5, in addition to the same broad peak at about 3.3 ml, a minor peak at 3.8 ml was also detected; this observation suggests that lowering pH induces dissociation of clusterin oligomers. SEC analyses of recombinant clusterin at pH 7.4 and 5.5 detected major absorbance peaks at around 4.67 ml and 4.76 respectively; representing molecules of low molecular weight (smaller than 25 kDa) and suggesting that the recombinant protein has been degraded.

The mass of proteins analysed were estimated using the equation shown on Figure 15, derived by a line of best fit to the data gathered using the molecular mass standards.The protein size calculated for plasma-derived clusterin suggests that at pH 7.4 this protein is found as tetramers (HMW species of about 314 kDa); and when the pH is decreased (pH 5.5) tetramers and dimers of heterodimer molecules are found (HMW species of ~264 and ~138 kDa, respectively). The sizes for recombinant clusterin at pH 7.4 and pH 5.5 were not estimated because some of the peaks were outside the range of the standard proteins.

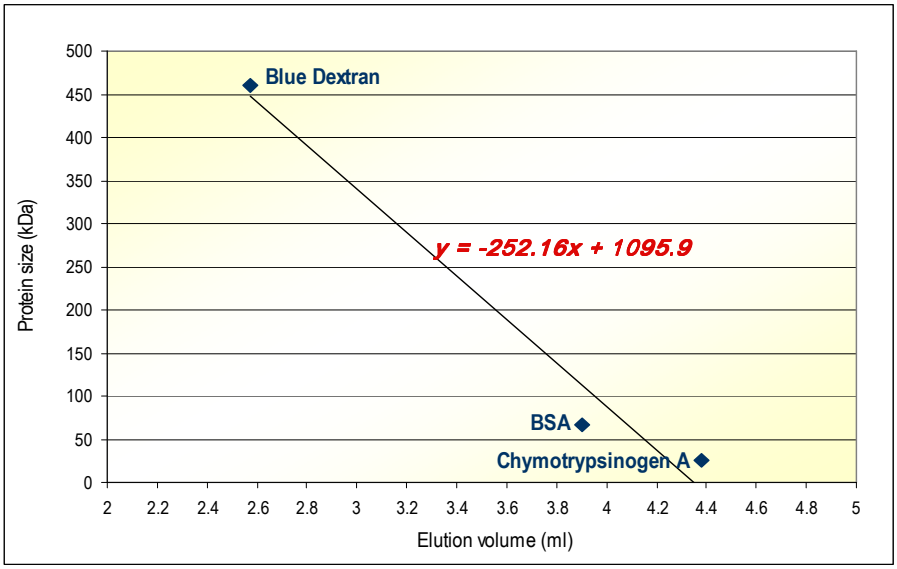


Figure 15. Line of best fit for protein mass versus the elution volume for protein standards after SEC.

The SEC analyses suggested that the recombinant protein was migrating at a position consistent with a mass of less than 25 kDa, consistent with proteolytic degradation of the product. Four months after the initial purification of the product, further SDS-PAGE and Western Blot analyses were performed (Figure 16).

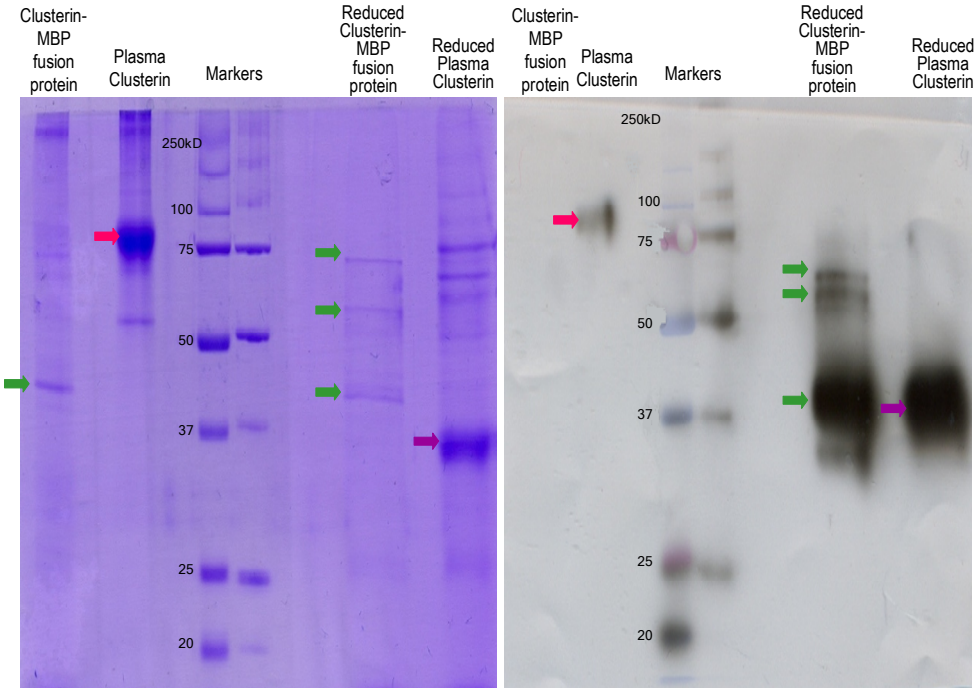


Figure 16. Images of SDS-PAGE (a) and Immunoblot (b) of recombinant and plasma-derived clusterin. The position of molecular mass standards is indicated on each image. The identity of samples analysed is indicated above each lane. The pink arrows indicate the position of plasma-derived clusterin under non-reducing conditions. The purple arrows indicate the position of plasma-derived clusterin under reducing conditions. The green arrows indicate position of degraded fusion protein.

The Coomassie Blue stained SDS-PAGE gel and a corresponding Western Blot are shown in Figure 16. Plasma-derived clusterin was detected as a band at ~ 80 kDa under non reducing conditions and as a band at about ~ 35 kDa under reducing conditions (representing the two co-migrating clusterin subunits). In the Coomassie Blue stained gel, under non-reducing conditions, in addition to the broad band at about 80 kDa (representing glycosylated plasma clusterin) other bands at ~ 250 kDa and ~ 60 kDa were detected. These probably represent SDS-resistant clusterin aggregates and incompletely glycosylated clusterin, respectively. When analysed under either reducing or non-reducing conditions, the recombinant clusterin sample contained multiple bands with no evidence of an abundant 90 kDa species expected for intact clusterin - MBP. The several bands detected in the recombinant clusterin sample of lower apparent molecular mass than expected (at ~ 60 kDa, ~ 55 kDa and ~ 40 kDa) suggest again, that the fusion protein had undergone extensive proteolytic degradation during expression/purification. In the Western blot recombinant clusterin was strangely not detected under non-reducing conditions but was detected under reducing conditions; a possible explanation could be insufficient concentration of the protein loaded in this well (as a mistake).

Also, In Figure 16, the SDS-PAGE under reduced conditions detected plasma -derived clusterin and recombinant clusterin at different molecular mass; plasma -derived clusterin was found running at about 35 kDa while clusterin-MBP protein was running at about 40 kDa. On the other hand, the immunoblot in figure 16 showed the samples, plasma-derived clusterin and recombinant clusterin under reduced conditions, running at around the same molecular mass (~40 kDa). This may result from the anti-clusterin antibody detecting a minor species in the clusterin-MBP sample (not clearly visible on the gel).

The results of the SDS-PAGE and immunoblotting agreed with those from SEC and suggested that no intact clusterin - MBP was obtained and that the product underwent time-dependent degradation (even when stored frozen at -20 ^{o}C).

3.4 Discussion

The expression of recombinant clusterin - MBP fusion protein was attempted in *E. coli* TBI pMal-c2x-clusterin clones. Protein species reactive with anti-clusterin antibodies were detected in the soluble fraction prepared from cell lysates. Unexpectedly, full length (~ 92kDa) recombinant clusterin - MBP fusion protein was only detected as a minor species following overnight induction of expression at 25^{o}C.

The preliminary induction in small scale culture expressed species reacting with anti-clusterin antibodies that corresponded to free recombinant clusterin; and species reacting with anti-MBP antibodies that corresponded to free MBP. In these preliminary tests, recombinant clusterin - MBP fusion protein was detected reacting only with anti-MBP antibodies. The fact

that the intact clusterin - MBP fusion protein was not visualized reacting with clusterin antibodies suggested the presence of intact fusion protein species in a very low concentration. Subsequently, protein extraction was performed and both free recombinant clusterin and clusterin - MBP fusion protein were found in the soluble fraction of the cell lysates. However, when a large scale culture was subsequently used, western blot analysis of the purified sample did not detect the full length fusion protein, but instead several degraded products.

The results of immunoblotting suggested that most of the clusterin - MBP fusion protein is degraded during expression/purification. Production of intact clusterin - MBP fusion protein would have allowed rapid purification of the recombinant product on an amylose resin (which binds MBP). Although initial SDS-PAGE and immunoblotting analyses indicated the possibility that intact clusterin - MBP fusion protein might have been obtained, subsequent SEC and further SDS-PAGE and immunoblotting analyses showed that the product (whatever its precise composition) was being degraded with time (even when stored frozen at -20 ^{o}C).

Thus, the results obtained clearly indicated that it would not be possible to use the current plasmid and bacterial strain to obtain a source of stable clusterin – MBP fusion protein for use in therapeutic or other applications. On the other hand, further identification and characterization of the degraded clusterin – MBP fusion protein products would provide an indication of the region at which the protein is truncated / degraded and therefore provided clues as the protease(s) involved, so broaden analysis are recommended for future studies.

Degradation of recombinant proteins during storage has been shown before. A study by Mironova *et al*. (2005) reported data on the degradation of full length recombinant human interferon gamma (rhIFN-g) after purification and storage at 4^{o}C in a protease free medium (0.4 M NaCl, 20 mM Tris-HCl, pH 8.2, sterile solution). In this study SDS-PAGE analysis revealed that samples taken soon after the commencement of protein storage predominantly contained full-length rhIFN-g, whereas samples taken at later time points also contained truncated forms. Furthermore, a study by Chang *et al*. (1996) characterized the degradation products of recombinant human interleukin-1 receptor antagonist (rhIL-1ra) formed during storage at 30°C in aqueous solution. They observed that degradation was greater as the storage temperature and storage time were increased; purification and characterization of the degraded products revealed that in the absence of stabilizers the protein degraded chemically by deamidation and physically by precipitation.

Since the primary aim of this project was to express clusterin in *E. coli* as a fusion protein to allow rapid purification of the product for therapeutic applications, it became necessary to reclone clusterin cDNA into an alternative vector for protein expression. The subsequent vector was chosen because it encoded an N-terminal 6xHis tag to allow affinity purification of recombinant fusion protein using a nickel-chelating resin.

4. EXPRESSION, PURIFICATION AND CHARACTERIZATION OF CLUSTERIN - 6HIS TAG FUSION PROTEIN

4.1 Introduction

Recombinant clusterin expressed in bacteria will consist of a single polypeptide (not cleaved into α and β chains). It is hoped that this lack of cleavage will not substantially compromise the chaperone action of the molecule. If recombinant clusterin were to be used as a therapeutic, it would be necessary to have a method to allow rapid purification of the product. For this reason, recombinant clusterin was initially expressed as a fusion protein with MBP in order to allow its purification by amylose affinity chromatography. However, preliminary work showed that most of the recombinant clusterin protein was cleaved from MBP during expression in *E. coli* and it became necessary to reclone clusterin cDNA into alternative vectors for protein expression.

To clone and subclone the clusterin DNA sequence, and for the expression of clusterin - polyhistidine (6xHis) tag fusion protein, an *E. coli* expression system with Gateway® cloning technology was used.

4.2 Methods

4.2.1 Construction of an *att*B-flanked clusterin-encoding PCR product.

An *att*B-flanked clusterin-encoding PCR product was created by using primers (provided by Mark Wilson) designed to incorporate 5`-terminal *att*B sequences in a PCR as described in 2.3.1, and the PCR product analyzed to assess its yield and purity by agarose gel electrophoresis (described in 2.3.1.1).

4.2.2 Creation of an entry clone from *att*B-flanked PCR products via the BP reaction.

After verifying the presence and purity of the *att*B-flanked clusterin-encoding PCR product, and estimating its concentration, the DNA fragment was combined with the donor vector pDONR™201 by BP reaction as described in 2.3.2. Subsequently, 1 µl of the BP reaction was used to transform *E. coli* TOP10 electro-competent cells by electroporation (described in 2.3.2.1). Finally, plasmids were extracted and analysed for the presence of clusterin insert by restriction digestion with *EcoR*I and *Hind*II as described in 2.3.2.2.

4.2.3 Creation of expression clones by recombination of clusterin-pDONR™201 and the destination pET-DEST42 vector via the LR reaction.

The LR reaction to create an expression clone was carried out as described in 2.3.3. A volume of 1 µl of the LR reaction was used to transform *E. coli* BL21 competent cells by heat-shocking (described in 2.3.3.1). The presence of clusterin-encoding insert in the destination vector was confirmed by restriction digestion with *Sfi*I as described in 2.3.3.2.

4.2.4 Clusterin - 6xHis Tag fusion protein expression from Gateway® expression clones and purification by Ni-NTA affinity chromatography.

After confirming the identity of the expression vector, small and large scale protein expression assays were performed as described in 2.3.4. Following expression of clusterin – 6xHis Tag fusion protein by *E. coli* BL21, small (2.3.5.1) and large scale (2.3.5.2) protein extractions were performed. The extracted protein fractions (soluble protein fraction and the inclusion bodies) were collected and analyzed by Western Blot using anti-clusterin antibodies (2.5.4). Subsequent to verifying the expression of the protein, a Ni-NTA Superflow Cartridge (Qiagen, Australia) was used to purify clusterin – 6xHis Tag fusion protein as described in 2.4.2 from the large scale culture. The fraction that did not bind to the Ni-NTA column was also collected and analysed by SDS-PAGE. The analysis revealed clusterin – 6xHis Tag fusion protein in this fraction, thus this fraction was further purified by SEC chromatography using a Superose-6 column (GE Healthcare, Sydney) (as described in 2.7.1.2). Finally, the protein fractions obtained from both purification methods were concentrated, refolded, alkylated and dialyzed (as described in 2.4.3) before functional and structural analyses. Analyses were performed using a pooled-mix of the two protein fractions obtained.

4.2.5 Analysis of clusterin - 6xHis Tag fusion protein structure and chaperone action.

The purified clusterin – 6xHis Tag fusion protein was structurally and functionally compared with plasma derived clusterin. SDS-PAGE (2.5.3), Western blot (2.5.4), CD (2.7.2.1) and chaperone assays (2.6) were performed.

4.3 Results

4.3.1 Analysis of the *att*B-flanked clusterin-encoding PCR product

Because the Gateway® system relies on artificial recombination sites or *att* sequences (*att*P, *att*B, *att*L and *att*R), the *att*B1 and *att*B2 sequences were added to specific primers used to amplify the clusterin gene. After amplification by PCR, the *att*B-flanked clusterin-encoding PCR product was analyzed by agarose gel electrophoresis (Figure 17). The PCR product was expected to be a 1279 bp DNA fragment and was found migrating as a single band to a position at about 1.3 Kb. The concentration of DNA in the *att*B-flanked clusterin-encoding PCR product was estimated by comparison with the DNA marker as about 25 ng/µl. The production of the *att*B-flanked clusterin-encoding PCR product allowed insertion of the clusterin gene into a Donor vector via a Gateway reaction.

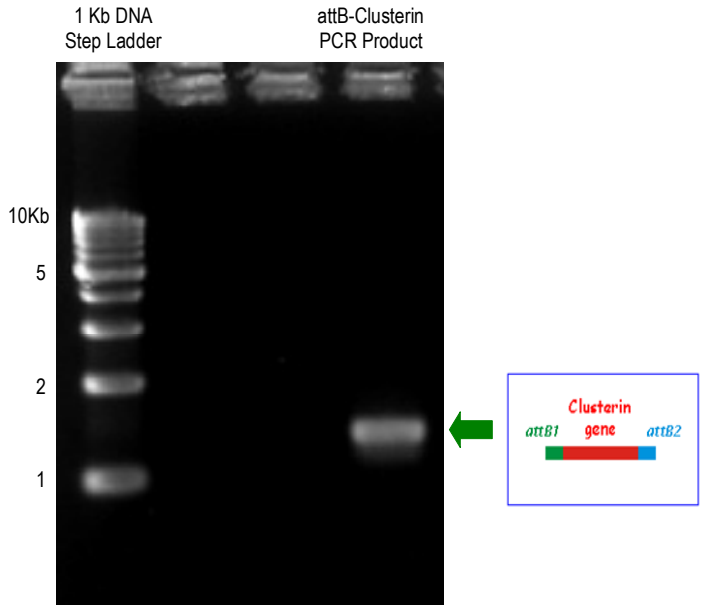


Figure17. Image of an ethidium bromide stained 1% agarose gel showing *att*B-clusterin PCR product. The identity of samples analysed is indicated above each lane. The green arrow indicates the position of the *att*B-clusterin product obtained by PCR. The positions of molecular size markers are indicated at the left of the image.

4.3.2 Creation of an entry clone from *att*B-flanked PCR product via the BP reaction.

The *att*B-flanked clusterin-encoding PCR product was combined with pDONR™201 donor vector (Invitrogen, Sydney) that contains *att*P1 and *att*P2 sequences and a ccdB gene. The addition of BP Clonase™ enzyme mix (Invitrogen, Sydney) generated an entry clone (Figure 18) when the DNA from the *att*B-clusterin PCR product replaced the region between 111 bp and 2352 bp in the pDONR™201. The resulting entry clone clusterin-pDONR™201 is a plasmid of 3508 bp. Plasmids from transformants grown in the presence of kanamycin were extracted and the entry clone was transformed into *E. coli* TOP10 electro - competent cells. Transformants were selected on LB plates containing kanamycin and extracted plasmid DNA analysed by restriction digestion with *EcoR*I (Fermentas, Life Sciences) and *Hind*II (Fermentas, Life Science).

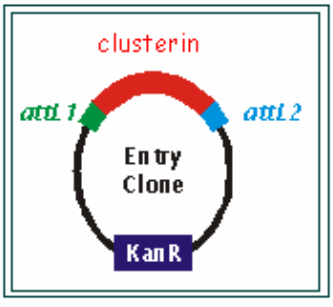


Figure 18. Diagram illustrating the entry clone (clusterin-pDONR™201).

The expected and actual band patterns detected after restriction digestion of the entry clone clusterin- pDONR™201 plasmid with *EcoR*I and *Hind*II are shown below (Table 2).

Table 2. Band patterns expected and detected in the agarose gel shown in Figure 19 resulting from restriction digestion of entry clone clusterin- pDONR™201 plasmid with *EcoR*I and *Bgl*II.

Restriction enzyme	Cut position in clusterin (bp)	Cut position in pDONR™201 (bp) without 111-2352 region	Expected band pattern clusterin- pDONR™201 after digestion	Detected band pattern clusterin- pDONR™201 after digestion
*EcoR*I	817	-	Linear 3508	~ 3.5 kb
*Hind*II	150 and 1010	2510	426, 860 and 2220	Two bands <1 kb consistent with 426 bp & 860 bp and one band smaller than 3 kb

These findings suggested that the clusterin encoding sequence had been successfully inserted into the plasmid vector.

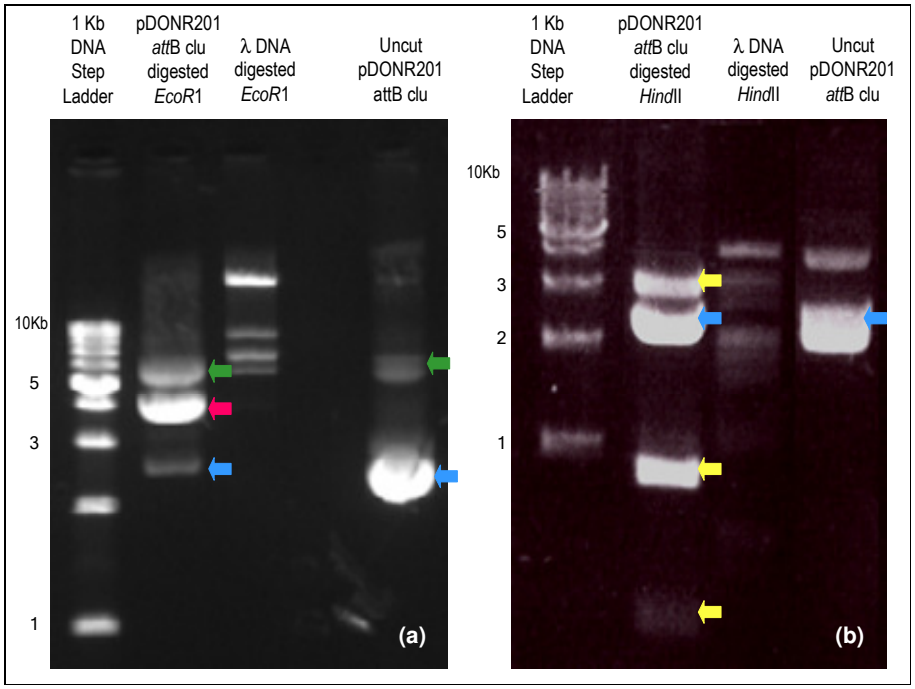


Figure19. Image of ethidium bromide stained 1% agarose gels showing Entry clone (pDONR™201+ *att*B clusterin) before and after restriction digestion with *EcoR*I (a) and *Hind*II (b). The identity of samples analysed is indicated above each lane. The pink arrow indicates the position of the entry clone in its linear form (3508 bp). Digestion of the entry clone with *Hind*II (in b) produces the fragments indicated by the yellow arrows. The super coiled (blue arrow) and nicked forms (green arrow) are also indicated. The lambda DNA showed the expected cutting pattern with the two restriction enzymes. The positions of molecular size markers are indicated at the left of each image.

4.3.3 Creation of expression clones by recombination of clusterin-pDONR™201 and pET-DEST™42 via the LR reaction.

Recombination of the clusterin-pDONR™201 entry clone with a destination vector that has the *att*R1 and *att*R2 sequences and the *ccd*B marker was carried out to produce an expression clone. After recombination, the *ccd*B gene was replaced by the gene of interest in the expression clone. The *ccdB* gene codes for *ccdB* protein that interferes with *E. coli* DNA gyrase and thus inhibits the growth of most *E. coli* strains (Figure 5) (Invitrogen User Manual, 2003). The expression clones were selected on plates containing ampicillin.

The pDEST™17 plasmid (Invitrogen, Sydney) is an N-terminal fusion vector that contains an ATG initiation codon upstream of a 6x His tag; the presence of the N-terminal 6xHis tag allows affinity purification of recombinant fusion protein using a nickel-chelating resin. Preliminary assays performed using the destination vector pDEST™17 showed, after transformation of *E. coli* BL21 competent cells by heat-shocking, a large number of growing colonies in the absence of the entry clone (negative control). The possibility of contaminating the solutions with bacteria carrying a plasmid encoding ampicillin resistance or another plasmid carrying the same antibiotic resistance was tested. Bacterial contamination was tested by plating an aliquot of each solution onto LB plates containing ampicillin, but under these conditions no colonies grew. Plasmid contamination was tested by transforming *E. coli* BL21 with aliquots of each separate solution used in the LR reaction. Except for the plate corresponding to cells transformed with pDEST™17 no colonies grew on the plates. The findings suggested deletion (full or partial) of the *ccd*B gene from pDEST™17. Also, because plasmids from six different clones of cells obtained after transformation were extracted and analysed by restriction digestion with *Age*I (which has a single cut site at 1337 bp in the clusterin gene) and none of them showed the expected band pattern after digestion, the results indicated that the clusterin gene was not inserted in the plasmids present in the transformed cells and the use of another destination vector was required.

Subsequently, the creation of an expression clone was attempted by recombination of clusterin-pDONR™201 and the destination pET-DEST™42 vector via the LR reaction. The pET-DEST™42 vector is a plasmid of 7440 bp, with *att*R1 and *att*R2 sites, ampicillin resistance gene, T7 promoter (allows high level IPTG-inducible expression of the recombinant protein) and C-terminal polyhistidine tag (which allows rapid purification of the protein product). The LR reaction generated an entry clone when the DNA from the *att*B-clusterin PCR product replaced the region between bases 409 and 2092 in the pET-DEST™42. The resulting expression clone clusterin-pET-DEST™42 was a plasmid of 7036 bp (Figure 20a).

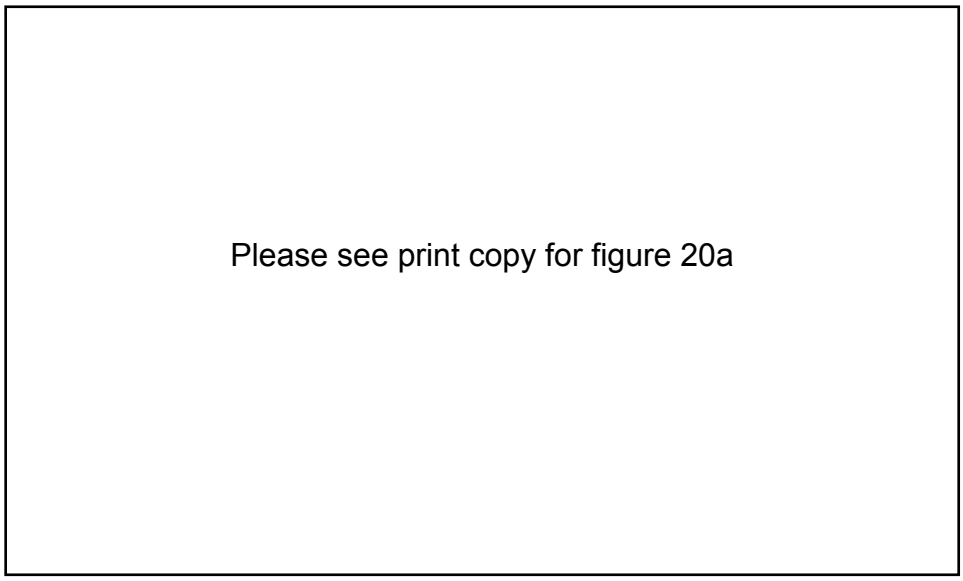


Figure 20a. Diagram illustrating the resulting expression clone (clusterin - pET-DEST™42). The diagram illustrate the elements contained in pET-DEST42: a T7 *lac* promoter*, the* recombination sites, shadow area, *att*R1 (bases 402-526) and *att*R2 (bases 1982-2106) for recombination cloning of the clusterin gene from an entry clone*, c*hloramphenicol resistance gene, *ccd*B gene, V5 epitope and 6xHis tag, ampicillin resistance gene*,* pBR322 origin for low-copy replication and maintenance of the plasmid in *E. coli* and *lac*I gene encoding the lac repressor to reduce basal transcription from the T7*lac* promoter (Invitrogen User Manual, 2003).

The expression clone was transformed into *E. coli* BL21 and then selected on LB plates containing ampicillin. Plasmids from 6 transformants were extracted and individually analysed by restriction digestion with *Sfi*I (Fermentas, Life Sciences). The expected and actual band patterns detected after restriction digestion of the extracted putative clusterin-pET-DEST™42 plasmids are shown below (Table 3).

*Sfi*I cut once in the clusterin gene (cut site at 404 bp) but did not cut pET-Dest™42; thus, digestion of the expression clone with *Sfi*I produced a linear fragment of 7036 bp. The findings in Figure 20 confirmed the presence of the clusterin encoding insert in the plasmid from clones 1, 2, 3 and 4. Clone 4 was chosen for protein expression.

Table 3. Band patterns expected and detected in the agarose gel shown in Figure 20 for *SfiI* digestion of clusterin-pET-DEST™42 plasmids.

Restriction enzyme	Cut position in clusterin (bp)	Cut position in pET-DEST™42 (bp)	Expected band pattern clusterin-pET-DEST™42 after digestion	Detected band pattern clusterin-pET-DEST™42 after digestion
*Sfi*I	404	-	Linear 7036 kb	~ 7 kb in clones 1,2,3 and 4 Multiple bands in clone 5 ~ 5 kb and >10 kb in clone 6

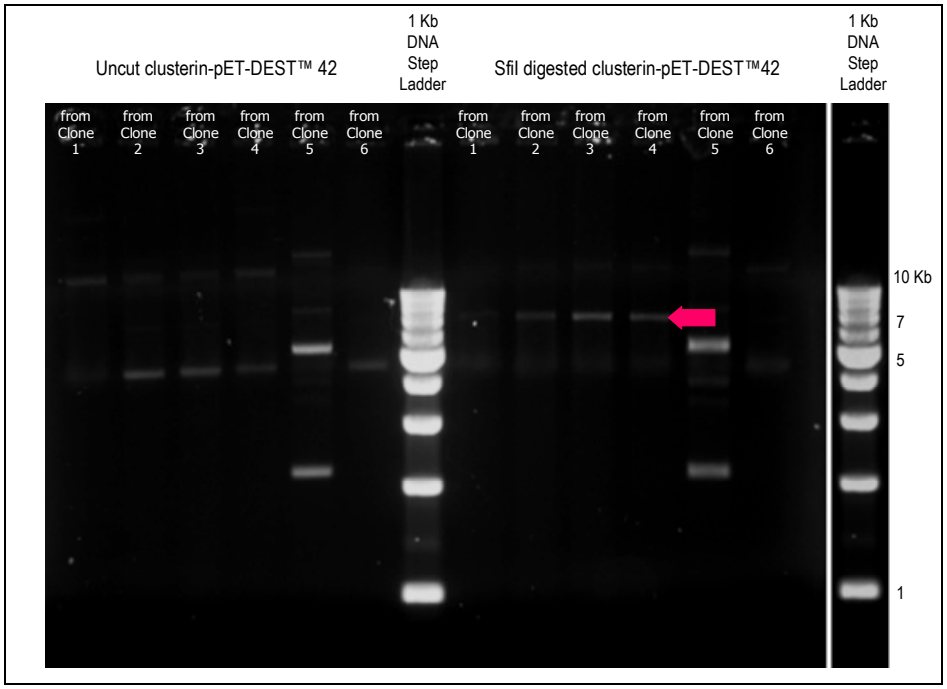


Figure 20b. Image of an ethidium bromide stained 0.8% agarose gel showing putative clusterin - pET-Dest™42 expression plasmids before and after restriction digestion with *Sfi*I. The identity of samples analysed is indicated above each lane. The pink arrow indicates the position of the expression clone clusterin - pET-Dest™42 in its linear form (7036 bp). The positions of molecular size markers are indicated at the right of the image.

4.3.4 Protein expression from Gateway® expression clones.

Clusterin – 6xHis Tag fusion protein was inducibly expressed in *E. coli* BL21 clusterin-pET-DEST™42. The analysis of the presence of the recombinant product in samples of the extracted protein fractions (soluble protein fraction and the inclusion bodies) was performed by Western Blot analysis using anti-clusterin antibodies. The samples were analyzed under both reducing and non-reducing conditions; an image of the Western blot is displayed in Figure 21.

Bands representing the presence of clusterin were absent in the soluble protein sample under both reducing and non-reducing conditions. When inclusion bodies samples were analyzed, under reducing and non-reducing conditions, bands migrating to positions of about ~120, ~50 and ~30 kDa reacted with anti-clusterin antibodies, indicating that most of the clusterin - 6xHis tag fusion protein was present in the inclusion bodies. Under reducing conditions, analysis of the inclusion bodies sample detected a lesser amount of material of about 120 kDa; this residual material probably represents SDS-resistant clusterin aggregates. Major bands were also detected corresponding to monomeric clusterin – 6xHis tag fusion protein (~50 kDa) and putative truncated product (~30 kDa).

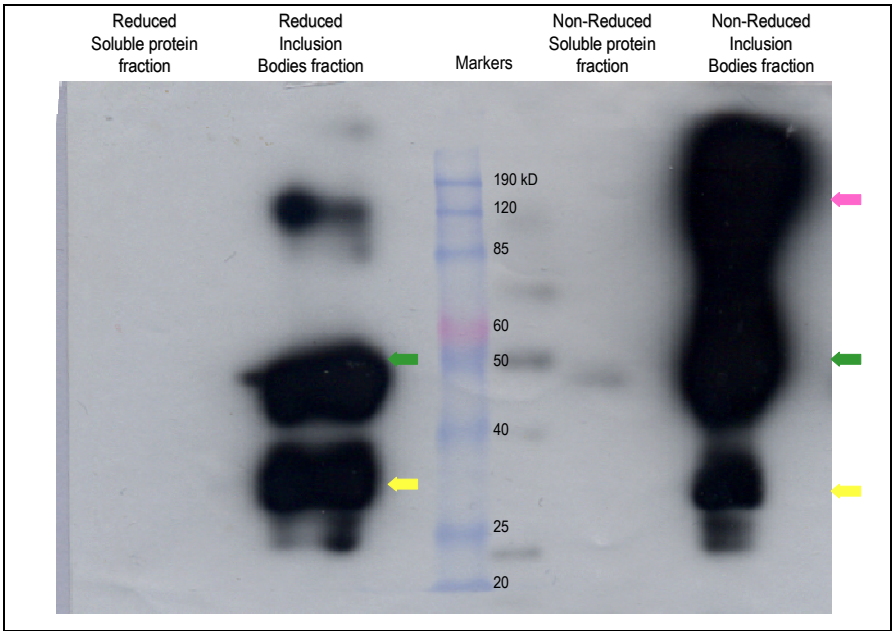


Figure 21. Image of Western Blot analysis using anti-clusterin antibodies of samples of the extracted protein fractions (soluble protein fraction and inclusion bodies) to verify the presence of clusterin – 6xHis Tag fusion protein. The identity of samples analysed is indicated above each lane. Pink arrow indicates the position of disulfide-bonded high molecular weight aggregates. Green arrows indicate the position of monomeric clusterin – 6xHis Tag fusion protein and yellow arrows indicate the position of a presumptive truncated product. The position of molecular mass standards is indicated on the image.

4.3.5 Analysis of the structure of the recombinant products.

4.3.5.1 SDS-PAGE. After purification by Ni-NTA affinity chromatography, clusterin – 6xHis tag fusion protein was analysed by SDS-PAGE and stained with Coomassie Blue. The fraction that did not bind to the Ni-NTA column was analysed as well. The samples were analyzed under reducing conditions; an image of the SDS-PAGE is displayed in Figure 22.

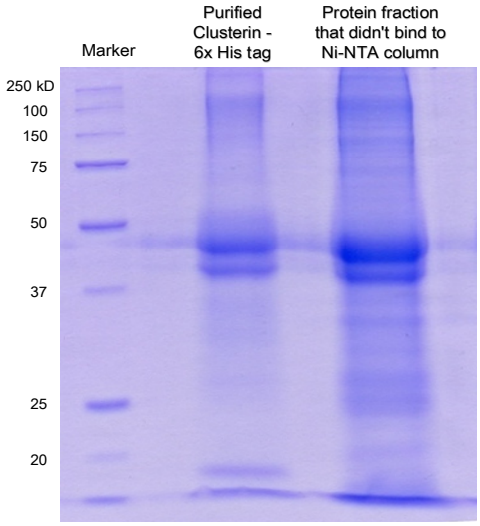


Figure 22. Image of a reducing SDS-PAGE gel stained with Coomassie Blue showing analysis of clusterin - 6xHis Tag fusion protein after Ni-NTA affinity chromatography. The position of molecular mass standards is indicated at the left of the image. The identity of samples analysed is indicated above each lane.

Clusterin - 6xHis Tag fusion protein migrated essentially as two bands to positions of about ~ 40 and ~ 50 kDa, suggesting that the eluted protein from the column was a mixture comprised of intact clusterin - 6xHis Tag fusion protein and putative truncation product. The protein fraction that did not bind to the Ni-NTA column migrated as two major bands at about ~40 - 50 kDa and also contained lesser amounts of material of various molecular masses. This finding suggested that there was clusterin - 6xHis Tag protein present in the fraction that did not bind to the column and that an additional purification step was required.

The reason(s) why not all of the fusion protein bound to the Ni-NTA column are unclear but may include steric factors in which the 6-his tag may have been "buried" within the three dimensional fold of the recombinant clusterin. Alternatively, or in addition, the column may have been overloaded.

SEC chromatography using a Superose-6 column (GE Healthcare, Sydney) was performed subsequent to Ni-NTA affinity chromatography in order to purify clusterin - 6xHis Tag fusion protein that had not bound to the Ni-NTA column. The protein was passed over the column, the absorbance at 280 nm of the eluted fractions continuously quantified and fractions collected separately and analysed by SDS-PAGE under reducing conditions (Figure 23).

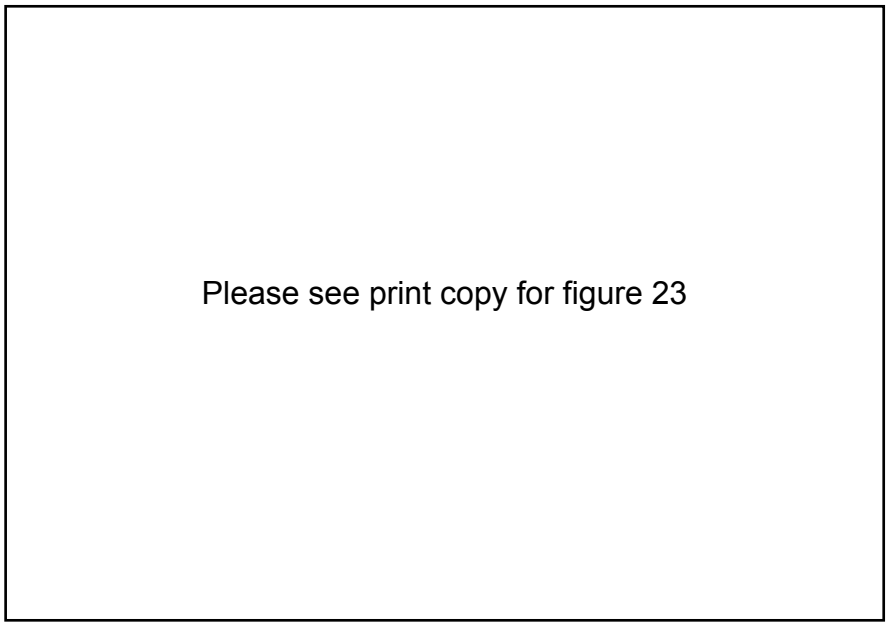


Figure 23. Diagram illustrating the eluted fractions obtained by SEC (a) and image of SDS-PAGE gel stained with Coomassie Blue showing analysis of these fractions (b). Superose™ 10/300 GL column (GE Healthcare, Sydney) was equilibrated with 8 M urea, 50 mM NaH_2PO_4, 0.3 M NaCl, 2 mM DTT. The absorbance peaks of the eluted fraction collected after SEC are identified above each peak (in a). The pink arrows (in b) indicate the positions of intact clusterin – 6xHis Tag fusion protein (upper band) and putative truncation product (lower band). The position of molecular mass standards is indicated on the image (in b). The identity of samples analysed is indicated above each lane.

The eluted fraction 3 migrated mostly as two bands centred at a position of about 50 kDa, probably representing clusterin – 6xHis tag fusion protein and putative truncation product. In fraction 3, less intensely stained bands were also evident in the range 40 - 45 kDa. Fraction 2 migrated as less intensely stained bands at about 120, 100, and two bands centred at a position of about 50 kDa. No bands were detected in fraction 1 probably because the quantity of protein loaded was too low to be detected.

The clusterin – 6xHis tag fusion protein obtained by Ni-NTA affinity chromatography and that obtained by SEC chromatography were refolded in 50 mM NaH_2PO_4, 300 mM NaCl, 1 mM DTT, 10% glycerol, 0.1% azide, alkylated with 50 mM IAA and then analysed by SDS-PAGE and Western blot. Before performing functional and structural assays the proteins were dialyzed against 2 L of PBS pH 7.5 (for chaperone assays) or 5 mM Na_2HPO_4 pH pH 8.5 (for CD assays). An image of the SDS-PAGE analysis of clusterin – 6xHis tag fusion protein obtained by Ni-NTA affinity chromatography is displayed in Figure 24. The fusion protein migrated essentially as two bands centred at a position of about 50 kDa. A Western Blot analysis of clusterin – 6xHis tag fusion protein obtained by Ni-NTA affinity chromatography and that obtained by SEC chromatography is displayed in Figure 25.

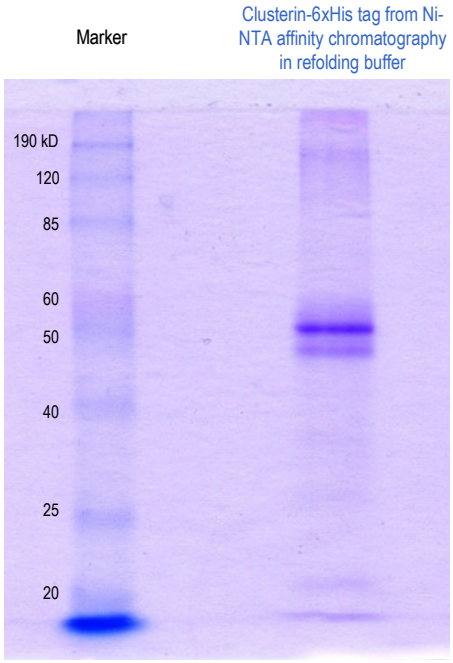


Figure 24. Image of reducing SDS-PAGE gel stained with Coomassie Blue showing analysis of clusterin - 6xHis Tag fusion protein purifed by Ni-NTA chromatography. The position of molecular mass standards is indicated at the left of the image. The identity of samples analysed is indicated above each lane.

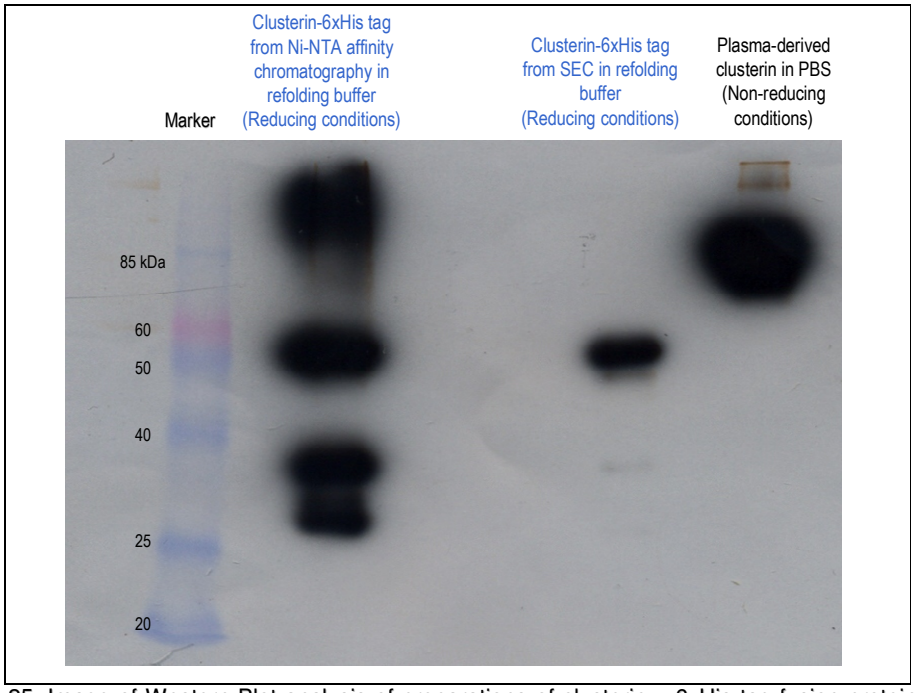


Figure 25. Image of Western Blot analysis of preparations of clusterin – 6xHis tag fusion protein using anti-clusterin antibodies. The identity of samples analysed is indicated above each lane. The position of molecular mass standards is indicated at the left of the image. Plasma-derived clusterin was included as a control.

Western blot analysis of clusterin – 6xHis tag fusion protein obtained by Ni-NTA affinity chromatography detected a number of bands reactive with anti-clusterin antibodies: (i) a high molecular mass band at greater than 85 kDa which probably represents SDS-resistant clusterin aggregates, (ii) a band slightly above 50 kDa corresponding to monomeric clusterin – 6xHis tag fusion protein, and (iii) bands in the range 25-40 kDa that probably represent truncated products. Clusterin – 6xHis Tag fusion protein obtained by SEC chromatography migrated as a single major band at a position slightly above 50 kDa. As expected, plasma-derived clusterin migrated as a single major band to a position of about ~ 75-80 kDa.

4.3.5.2 Circular dichroism spectroscopy. The structures of clusterin – 6xHis tag fusion protein and plasma-derived clusterin were compared by CD. A plot of θ_{MRE} versus wavelength is shown in Figure 26.

Plasma-derived clusterin displayed a peak minimum at 209 nm of -9702.7 $\theta_{MRE,}$ and clusterin - 6xHis tag fusion protein displayed a minimum at 208 nm with an intensity of -6772.3. The data indicate that the overall secondary structure content of the analysed proteins exhibited the characteristic CD spectra of alpha-helix motifs (Figure 7) (Boxrud, 2007). Deconvolution of these spectra using the program ContinLL (Provencher and Glochner, 1981) gave the predicted secondary structure content shown in Table 4. The qualities of the fits of the calculated secondary structures to the experimental data were determined using the normalized root-mean-square deviation (NRMSD) parameter (Mao *et al.*, 1982). NRMSD

values of less than 0.1 usually mean that the calculated secondary structure corresponds well with the actual structure of the protein (Brahms and Brahms, 1980)

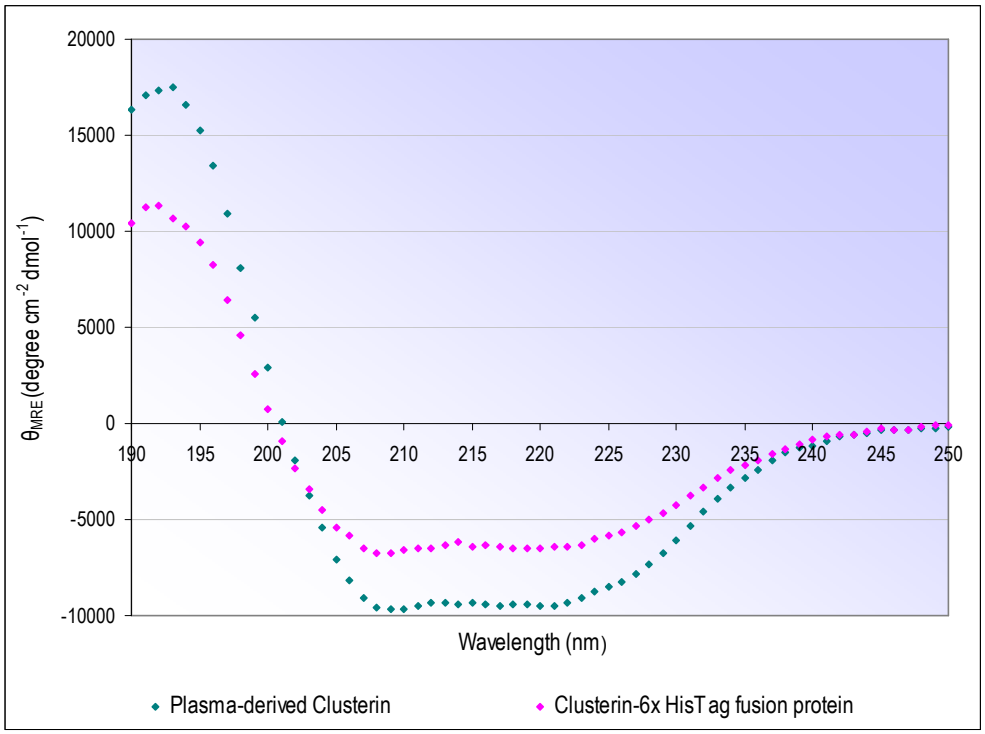


Figure 26. CD spectra of clusterin – 6xHis tag fusion protein and plasma-derived clusterin. The θ_{MRE} is plotted against wavelength. The identity of each plot is indicated in the key.

Table 4. Predicted percentages of secondary structural features for clusterin – 6xHis Tag fusion protein and plasma-derived clusterin based on Far-UV CD data and predicted by computational analyses using the prediction program ContinLL (Provencher and Glochner, 1981). NRMSD is a parameter indicating goodness-of-fit of the calculated structure to the experimental data.

Protein	α-helix (%)	β-sheet (%)	Turns (%)	Unordered (%)	NRMSD
Plasma-derived clusterin	29.6	22.3	19.2	28.9	0.025
Clusterin–6xHis tag fusion protein	21.5	27.5	20.8	30.2	0.083

The CD spectra and calculated secondary structure were similar to those previously reported for human clusterin (Bailey *et al.*, 2001) (Hochgrebe *et al.*, 2000).

The CD spectra obtained for both proteins were similar, their local minima at 220 and 209 nm (plasma-derived clusterin), and, 218 and 208 nm (clusterin – 6xHis Tag fusion protein) suggested that both proteins contain substantial amounts of α-helix. Deconvolution of these spectra indicated that clusterin – 6xHis Tag fusion protein and plasma-derived clusterin contained 20-30% of each of unordered, α-helix and β-sheet content. The analysis showed NRMSD values were well below 0.1 indicating that the predicted secondary structure content corresponds well with the actual structure of the protein.

4.3.6 Analysis of the chaperone action of clusterin – 6xHis tag fusion product.

The chaperone actions of clusterin – 6xHis Tag fusion protein and plasma-derived clusterin were compared by measuring their abilities to inhibit the heat-induced aggregation and precipitation of fibrinogen. Individual solutions of fibrinogen (0.3 mg/ml), plasma-derived clusterin (0.05 mg/ml) and clusterin – 6xHis tag fusion protein (0.05 mg/ml); and mixtures of fibrinogen with these proteins at 0.05 mg/ml and 0.1 mg/ml were prepared in PBS. The assay was performed in the wells of 96 well plates, and the precipitation induced by heating at 55°C and determined by measuring the light scattering of the solution at 360 nm every 2 minutes for 1 hour in a FLUOstar Optima micro plate reader (BMG Labtech, Australia).

Fibrinogen underwent extensive precipitation after being heated alone at 55°C. No clusterin - 6xHis Tag fusion protein or plasma-derived clusterin precipitated when heated in the absence of fibrinogen. Plasma-derived clusterin at 0.05 mg/ml inhibited the precipitation of fibrinogen by about 80%, and by about 84% when at 0.1 mg/ml, these results confirm its known chaperone action. Clusterin – 6xHis Tag fusion protein at 0.05 and 0.1 mg/ml inhibited the precipitation of fibrinogen by about 23%; thus, this recombinant clusterin was about 72% less effective than plasma derived clusterin. The results are shown in Figure 27.

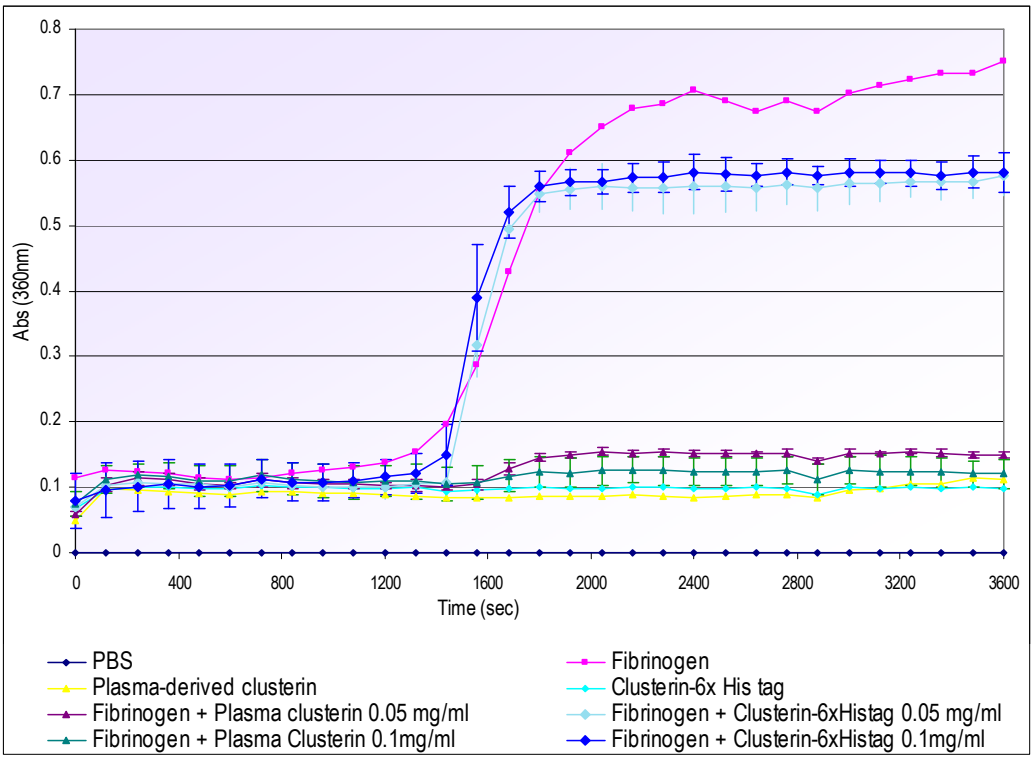


Figure 27. Influence of clusterin proteins on the heat-induced aggregation of fibrinogen. Fibrinogen (0.3 mg/ml) was heated at 55°C in the absence or presence of plasma-derived clusterin or clusterin – 6xHis Tag fusion protein (at 0.05 and 0.1 mg/ml). Protein precipitation was measured as light scattering at 360 nm. Each data point represents the mean of triplicate determinations, and the error bars are standard errors of the mean in each case. The identity of each plot is indicated in the key.

4.4 Discussion

Clusterin – 6xHis tag fusion protein was expressed in *E. coli* TBI clusterin-pET-DEST™42 using Gateway® cloning technology. Protein species reactive with anti-clusterin antibodies were detected by Western Blot in the inclusion bodies fraction prepared from cell lysates. Monomeric clusterin – 6xHis tag fusion protein along with presumptive truncated products and SDS-resistant clusterin aggregates were detected (Figure 21). Affinity chromatography using a Ni-NTA Superflow Cartridge (Qiagen, Australia) allowed purification of clusterin – 6xHis tag fusion protein, but additional SEC chromatography using a Superose-6 column (GE Healthcare, Sydney) was required in order to further purify fusion protein that had not bound to the Ni-NTA column.

After purification, clusterin – 6xHis tag fusion protein obtained by Ni-NTA affinity chromatography and that obtained by SEC chromatography were in 8 M urea, 0.1 M NaH_2PO_4, 0.1 M Tris. Before functional and structural analyses were performed, the proteins were refolded in 50 mM NaH_2PO_4, 300 mM NaCl, 1 mM DTT, 10% glycerol, 0.1% azide, alkylated with 50 mM IAA and dialyzed. Subsequent SDS-PAGE and immunoblotting analyses suggested that the refolding and alkylation processes did not produce changes in the molecular mass or secondary structure of clusterin – 6xHis tag fusion protein.

The presence of truncated products along with monomeric recombinant clusterin suggests that future studies should consider the use SEC after Ni-NTA affinity chromatography in order to purifying the full-length protein from the degradation products

CD spectra suggested that clusterin – 6xHis tag fusion protein and plasma-derived clusterin have similar secondary structure. Also, deconvolution of these spectra indicated that both, clusterin – 6xHis Tag fusion protein and plasma-derived clusterin, contained 20-30% of each of unordered, α-helix and β-sheet content. The presence of a slightly larger percentage of β-sheet content in clusterin – 6xHis tag fusion protein suggested that the lack of cleavage could cause variations in the structure of this recombinant form.

Chaperone assays indicated that the clusterin – 6xHis Tag fusion protein had little if any chaperone activity; the fusion protein was 72% less effective than plasma derived clusterin at inhibiting the heat-induced precipitation of fibrinogen and its effects were not dose-dependent. Although SDS-PAGE and immunoblotting analyses suggested that the refolding and alkylation processes did not produce substantive changes in the molecular mass of clusterin – 6xHis tag fusion protein, these effects may warrant closer examination. Size exclusion chromatographic analyses or mass spectrometry analysis could be used to study the effects of these processes on clusterin structure.

In SDS-PAGE analyses under non-reducing conditions, monomeric clusterin – 6xHis tag fusion protein was found along with a large amount of disulfide-bonded high molecular weight

aggregates. It is thought that the chaperone active form of clusterin is the 80 kDa heterodimer and that dissociation of clusterin oligomers into individual α-β heterodimers increases its chaperone action (Poon *et al.*, 2002). Therefore, additional analysis (e.g. chaperone assays at mildy acidic pH or in the presence of reducing agent(s)) are required to clarify whether the presence of these disulfide-bonded high molecular weight aggregates is a reason for the little chaperone activity of this recombinant protein.

To conclude, a mixture of intact clusterin–6xHis Tag fusion protein and putative truncation product was obtained. It would not be possible to obtain a source of chaperone-active clusterin with the protein produced as described in this study. However, because recombinant rat clusterin expressed in *E. coli* is chaperone active (Chpt 5), it is clearly possible to produce chaperone active clusterin in bacteria (lacking cleavage into α and β chains). Therefore, to optimize production of a chaperone active form of human clusterin in bacteria, future studies should

i. examine the effect of denaturation, reduction and alkylation processes on the activity of recombinant clusterin
ii. examine the effect of the 6 His tag on the chaperone activity of the protein by cleaving off the tag and then performing chaperone assays.

5. COMPARATIVE STUDIES OF HUMAN PLASMA-DERIVED CLUSTERIN AND RECOMBINANT HUMAN AND RAT CLUSTERIN

5.1 Introduction

Mammalian proteins are usually expressed in mammalian, yeast or insect cell systems to avoid the problems of the expression of eukaryotic proteins in prokaryotic cells. In order to provide additional insights into any functional limitations of recombinantly expressed clusterin proteins, two commercially available recombinant clusterin proteins were compared in structure and chaperone function with plasma-derived clusterin. The recombinant products tested were Flag-Tagged recombinant human clusterin (expressed in human embryonic kidney cells, HEK293) and His-Tagged partial-length recombinant rat clusterin (expressed in *E. coli*). Both products were supplied by BioVendor- Laboratory Medicine, Inc.

Flag-Tagged recombinant human clusterin is a 51.27 kDa protein that was purified by anti-flag affinity chromatography, filtered (0.4 micron) and lyophilized in PBS, pH 7.5 (BioVendor Product Data Sheet, 2007). The product has a total of 438 amino acid; the amino acid sequence (displayed below) from 1 to 427 is identical to Swiss-Prot-P10909 (amino acid 23-449, secreted human clusterin) and also comprises a C-terminal Flag tag of 11 amino acids (underlined) (BioVendor Product Data Sheet, 2007).

DQTVSDNELQ EMSNQGSKYV NKEIQNAVNG VKQIKTLIEK TNEERKTLLS NLEEAKKKKE
DALNETRESE TKLKELPGVC NETMMALWEE CKPCLKQTCM KFYARVCRSG SGLVGRQLEE
FLNQSSPFYF WMNGDRIDSL LENDRQQTHM LDVMQDHFSR ASSIIDELFQ DRFFTREPQD
TYHYLPFSLP HRRPHFFFPK SRIVRSLMPF SPYEPLNFHA MFQPFLEMIH EAQQAMDIHF
HSPAFQHPPT EFIREGDDDR TVCREIRHNS TGCLRMKDQC DKCREILSVD CSTNNPSQAK
LRRELDESLQ VAERLTRKYN ELLKSYQWKM LNTSSLLEQL NEQFNWVSRL ANLTQGEDQY
YLRVTTVASH TSDSDVPSGV TEVVVKLFDS DPITVTVPVE VSRKNPKFME TVAEKALQEY
RKKHREE**AAA DYKDDDDK**

His-Tagged recombinant rat clusterin was purified by Ni-NTA chromatography, filtered (0.4 micron) and lyophilized in 0.01 M Tris, pH 7.2. The recombinant rat clusterin is a 26 kDa protein that consists of a single polypeptide (not post translationally processed, thus not cleaved into α and β chains). The product is comprised of 240 amino acids (displayed below), 215 amino acid residues (146-360) of the rat clusterin sequence (UniProtKB/Swiss- Protein entry P05371), 16 amino acids at the N-terminus of T7-Tag (underlined), and 9 amino acids at the C-terminus of His-Tag (underlined) (BioVendor Product Data Sheet, 2007).

MASMTGGQQM **GRDP**NSSSPF YFWMNGDRID SLLESDRQQS QVLDAMQDSF TRASGIIDTL
FQDRFFTHEP QDIHHFSPMG FPHKRPHLLY PKSRLVRSLM PLSHYGPLSF HNMFQPFFDM
IHQAQQAMDV QLHSPALQFP DVDFLKEGED DRTVCKEIRH NSTGCLKMKG QCEKCQEILS
VDCSTNNPAQ ANLRQELNDS LQVAERLTQQ YNELLHSLQS KMLNTSSLLE Q**ALEHHHHHH**

5.2 Methods

Flag-Tagged recombinant human clusterin and His-Tagged recombinant rat clusterin were compared in structure and chaperone function with human plasma clusterin. SDS-PAGE (2.5.3), size exclusion chromatography (2.7.1.1), circular dichroism spectroscopy (2.7.2.2) and chaperone assays (2.6) were performed.

5.3 Results

5.3.1 Analysis of the structure of the recombinant products.

5.3.1.1 SDS-PAGE. Recombinant forms of clusterin and plasma-derived clusterin samples were analysed by SDS-PAGE. The samples were analyzed under both reducing and non-reducing conditions; an image of the SDS-PAGE is displayed in Figure 28. Under non-reducing conditions, Flag-tagged recombinant human clusterin, like plasma-derived clusterin, migrated as a single major band to a position of about ~75-80 kDa. In both samples there was also a lesser amount of material near the top of the gel which may represent SDS-resistant clusterin aggregates. Under reducing conditions, both samples migrated as a single major band at about ~35 kDa and also showed minor bands in the range 50 -100 kDa. Under non-reducing conditions, His-Tagged recombinant rat clusterin migrated as a major band to a position of about 27 kDa and other bands were also evident in the range 60-75 kDa and at apparent masses greater than 150 kDa. Under reducing conditions, the higher molecular mass bands were absent and essentially all the protein migrated to a position of about 27 kDa.

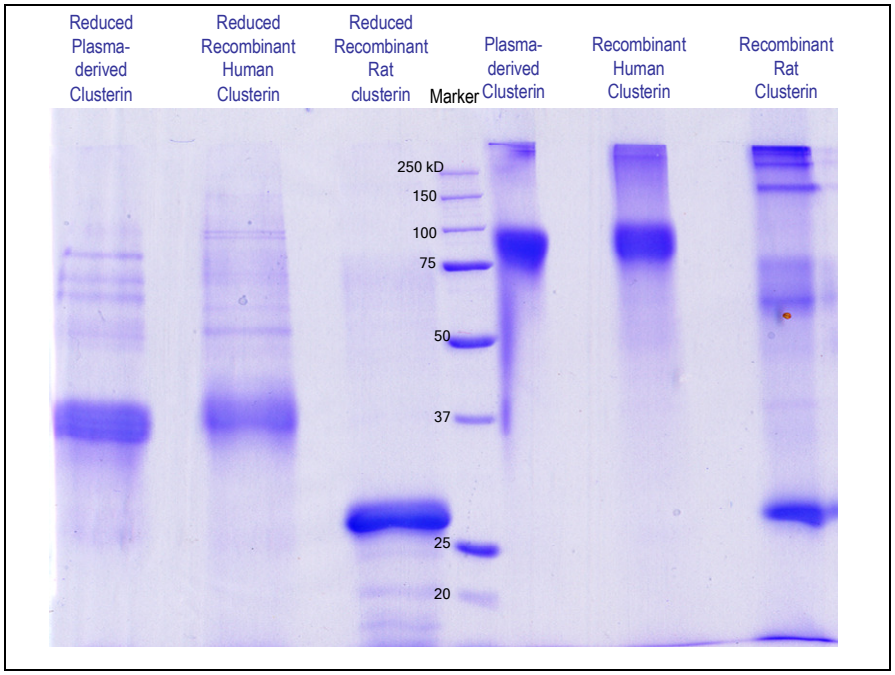


Figure 28. Image of SDS-PAGE gel stained with Coomassie Blue showing analysis of recombinant human clusterin, recombinant rat clusterin and plasma-derived human clusterin. The position of molecular mass standards is indicated on the image. The identity of samples analysed is indicated above each lane.

5.3.1.2 Size exclusion chromatography. Because proteins can be separated and purified on the basis of size using SEC, the absorbance (280 nm) traces for SEC analyses of plasma-derived clusterin, flag-Tagged recombinant human clusterin, His-Tagged recombinant rat clusterin and protein standards were compared.

Two mixtures of protein standards were used. One protein standard mix consisted of ferritin (50 µg, 460 kDa) and BSA (67 kDa, 50 µg), the other comprised aldolase (150 µg, 170 kDa) and chymotrypsinogen A (15 µg, 25 kDa). The different amounts of proteins used in the mix of aldolase and chymotrypsinogen A is due to the strong absorbance at 280 nm of chymotrypsinogen A compared with aldolase. The absorbance of a protein depends on its content of tyrosine, phenylalanine and tryptophan residues (Bollag *et al.*, 1996). An amount of 50 µg of the clusterin samples was loaded onto a Bio™-SEC-S4000 column using an $AKTA_{FPLC}$ system equilibrated in PBS containing 0.02% (w/v) azide. The separations were performed at a flow rate of 0.2 ml/min.

In Figure 29 the difference between the elution profiles of the proteins tested is shown. As expected, the elution profile of the standard proteins showed ferritin eluting first and displaying an absorbance peak at around 3.5 ml. Subsequently aldolase showed an absorbance peak at around 3.8 ml. BSA and chymotrypsinogen A had absorbance peaks at 4 ml and 4.4 ml, respectively. The major absorbance peaks for recombinant clusterin proteins and clusterin from plasma are indicated by thick, solid vertical arrows. The unfilled vertical arrows indicate HMW species of protein eluting at the exclusion limit of the column (> 2000 kDa).

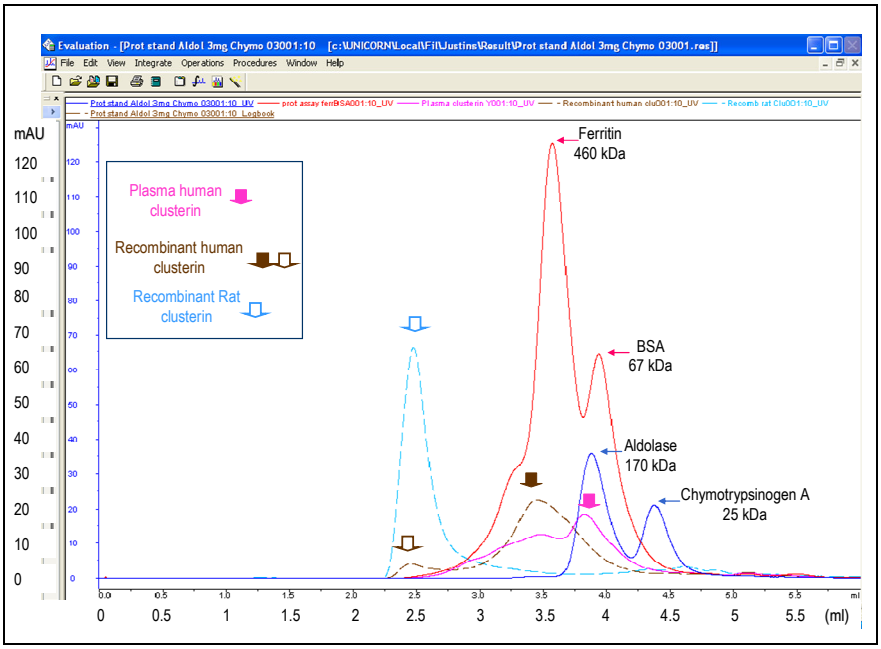


Figure 29. Absorbance (280 nm) traces for size exclusion chromatographic analyses of plasma-derived clusterin, recombinant human clusterin and recombinant rat clusterin. Proteins (100 µl, 0.5 mg/ml) were loaded onto a Bio™-SEC-S4000 column using an $AKTA_{FPLC}$ system. Separations were performed at a flow rate of 0.2 ml/min. The arrows indicate absorbance peaks of the proteins. Unfilled arrows indicate HMW species of the protein.

Plasma derived clusterin displayed a broad major peak at around 3.8 ml and a minor peak at around 3.5 ml suggesting that the plasma-derived clusterin sample was comprised of a mixture of oligomeric species, in accordance with observations made by Humphreys *et al.* (1999). Relative to plasma-derived clusterin, the recombinant forms of clusterin tested had a greater proportion of high molecular weight (HMW) species. The HMW species of the recombinant proteins eluted near the exclusion limit of the column. Human recombinant clusterin eluted in a broad major peak at around 3.5 ml and a minor peak at around 2.5 ml suggesting that it consisted of a mixture of oligomeric species with an average mass greater than plasma-derived clusterin. The recombinant rat clusterin eluted in a broad peak at 2.5 ml suggesting that it consisted primarily of HMW species.

The mass of proteins analysed were estimated using the equation shown in Figure 30, derived by a line of best fit to the data gathered using the molecular mass standards.

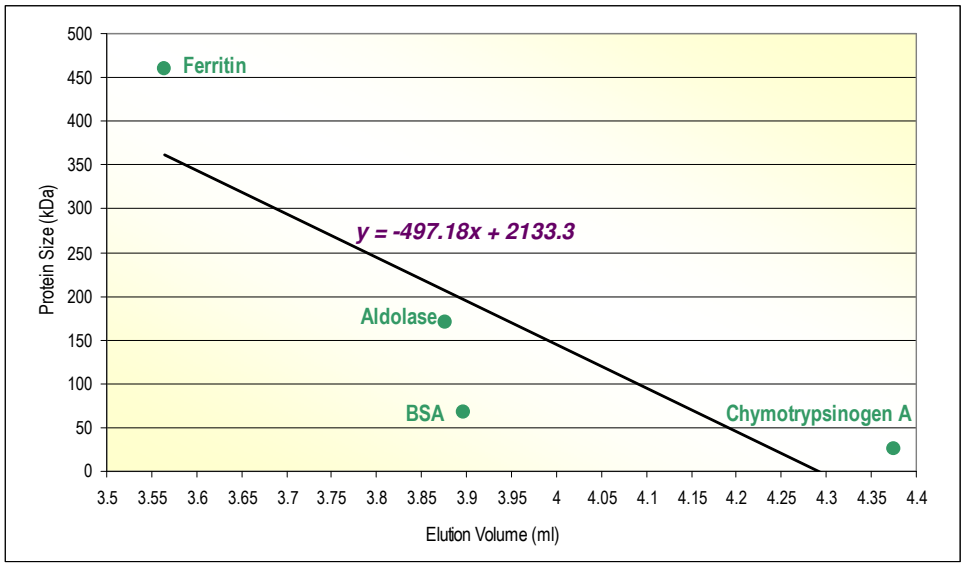


Figure 30. Line of best fit for protein mass versus the elution volume for SEC protein standards.

The mass estimated for plasma-derived clusterin suggests that it was present as a mixture of trimers and pentamers (HMW species of about 235 and 402 kDa). The masses of the HMW forms of recombinant human clusterin were estimated as about 421 and 917 kDa, representing octamers and oligomers formed by groups of 18 molecules. The mass estimated for the recombinant rat clusterin suggests that this protein was present as a mixture of oligomers formed by groups of 35 molecules (HMW species of 904 kDa). However, it is important to note that the HMW species of the recombinant proteins were eluting near the exclusion limit of the column (> 2000 kDa) and outside the range of the standard proteins; thus, the estimates of mass for these very large species may involve considerable error.

5.3.1.3 Circular dichroism spectroscopy. The structures of flag-tagged recombinant human clusterin and His-tagged recombinant rat clusterin were compared with the structure of plasma-derived clusterin by circular dichroism spectroscopy. A plot of θ_{MRE} versus wavelength is shown in Figure 31.

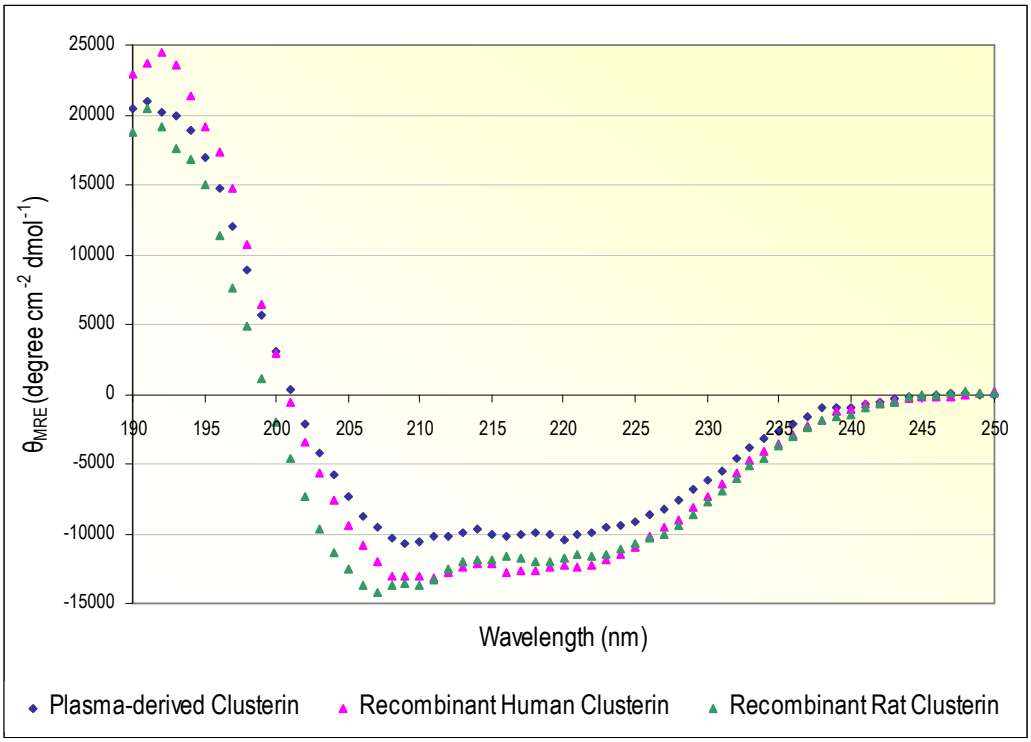


Figure 31. CD spectra of recombinant proteins and plasma-derived clusterin. The relation between θ_{MRE} and wavelength were plotted for plasma-derived clusterin, recombinant human and rat clusterin proteins. The identity of each plot is indicated in the key.

Plasma-derived clusterin displayed a peak minimum at 209 nm of -10654.8 θ_{MRE}. Recombinant human clusterin displayed a minimum of -13155.2 θ_{MRE} at 209 nm, and recombinant rat clusterin displayed a minimum at 207 nm with an intensity of -14232.5. Deconvolution of these spectra using the program ContinLL (Provencher and Glochner, 1981) gave the predicted secondary structure content shown in Table 5.

Table 5. Predicted percentages of secondary structural features for plasma-derived clusterin, recombinant human clusterin and recombinant rat clusterin based on Far-UV CD data and predicted by computational analyses using the prediction program ContinLL. NRMSD is a parameter indicating goodness-of-fit of the calculated structure to the experimental data.

Protein	α-helix (%)	β-sheet (%)	Turns (%)	Unordered (%)	NRMSD
Plasma-derived clusterin	30.4	21.9	18	29.7	0.024
Recombinant human clusterin	35.1	18.5	18.2	28.2	0.040
Recombinant rat clusterin	37.4	14.9	19.2	28.6	0.038

The secondary structure predictions of plasma-derived clusterin, Flag-tagged recombinant human clusterin and His-Tagged recombinant rat clusterin were generally similar. The CD spectra and calculated secondary structure content were similar to those previously reported for human clusterin (Bailey *et al.*, 2001) (Hochgrebe *et al.*, 2000) (Stewart *et al.*, 2007). Deconvolution of these spectra indicated that the predicted secondary structure of all the proteins tested contained 30-38% α-helix content, 18-22% β-sheet content and 28-20% unordered structure. The analysis showed NRMSD values were well below 0.1 indicating that the predicted secondary structure content corresponds well with the actual structure of the protein (Brahms and Brahms, 1980). Furthermore, the finding that the predicted secondary structure of His-Tagged recombinant rat clusterin was similar to that of plasma-derived clusterin suggests that the lack of cleavage into α and β chains and the C-terminal truncation did not lead to major changes in the secondary structure of the protein.

5.3.2 Analysis of the function of the recombinant products.

5.3.2.1 Protein precipitation assays. The chaperone actions of plasma-derived and recombinant clusterin were compared by measuring their relative abilities to inhibit the heat-induced aggregation and precipitation of fibrinogen. The results are shown in Figure 32. Fibrinogen underwent extensive precipitation after being heated alone at 55°C. In contrast, the addition of plasma-derived clusterin inhibited the final extent of precipitation of fibrinogen by about 64%. Recombinant human and recombinant rat clusterin were also able to inhibit the heat-induced aggregation and precipitation of fibrinogen. Recombinant human clusterin and recombinant rat clusterin inhibited the final extent of precipitation of fibrinogen by about 46% and 33%, respectively. None of plasma-derived clusterin, recombinant human or recombinant rat clusterin precipitated when heated in the absence of fibrinogen.

The ability of plasma-derived clusterin to inhibit fibrinogen precipitation confirms its known chaperone action. Recombinant human clusterin was about 28% less effective than plasma derived clusterin while recombinant rat clusterin was about 48% less effective than plasma-derived clusterin. However, it is important to consider the presence of a large amount of HMW species, especially pronounced in the recombinant rat clusterin sample revealed by SEC analyses. It has been demonstrated that the dissociation of the clusterin oligomers enhances the chaperone action of clusterin (Poon et al., 2002). Furthermore, the results suggested that the lack of cleavage of the recombinant rat clusterin into α and β chains and the fact that it is a C-terminally truncated product did not ablate the chaperone action. These differences may however account for the reduced chaperone activity relative to plasma-derived clusterin.

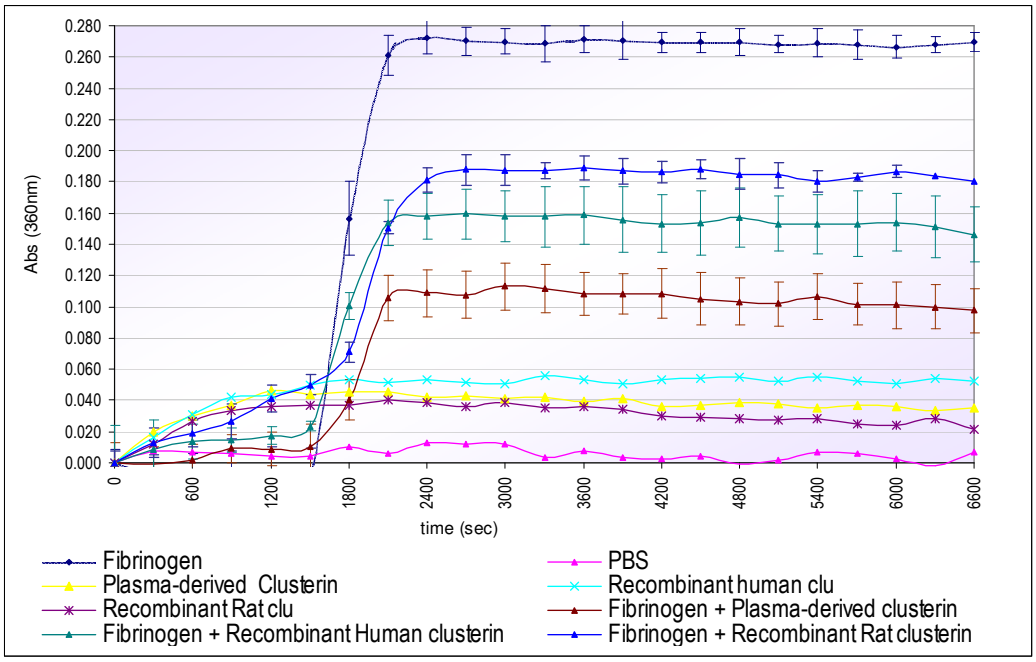


Figure 32. Influence of clusterin proteins on heat-stressed fibrinogen aggregation. Fibrinogen (0.3 mg/ml) was heated at 55^{3}C in absence and presence of plasma-derived clusterin, human recombinant clusterin or rat recombinant clusterin (all at 0.02 mg/ml). Protein precipitation was measured as light scattering at 360 nm. Each data point represents the mean of triplicate determinations, and the error bars are standard errors of the mean in each case. The identity of each plot is indicated in the key.

5.3.2.2 IgG binding ELISA assay. Lakins *et al.* (2002) proposed that clusterin has independent binding sites for native protein ligands (IgG, poli-C9, $A\beta_{1\text{-}40}$), stressed protein ligands and megalin. They also provided evidence that the binding site(s) for IgG are contained in the C-terminus of the α-chain and/or the N-terminus of the β-chain of clusterin. The IgG binding activity of recombinant human clusterin and recombinant rat clusterin were compared with that of plasma-derived clusterin. All the clusterin proteins showed similar dose-dependent binding to human IgG (Figure 33). Analysis with protein concentrations of 10 µg/ml and 20 µg/ml showed recombinant human clusterin IgG binding activity slightly less than that of plasma-derived clusterin. Rat recombinant clusterin displayed slightly greater binding than the other two proteins at concentrations of 5 µg/ml and 10 µg/ml. When analysed at 20 µg/ml, recombinant rat clusterin IgG bound slightly less than plasma-derived clusterin but slightly more than recombinant human clusterin.

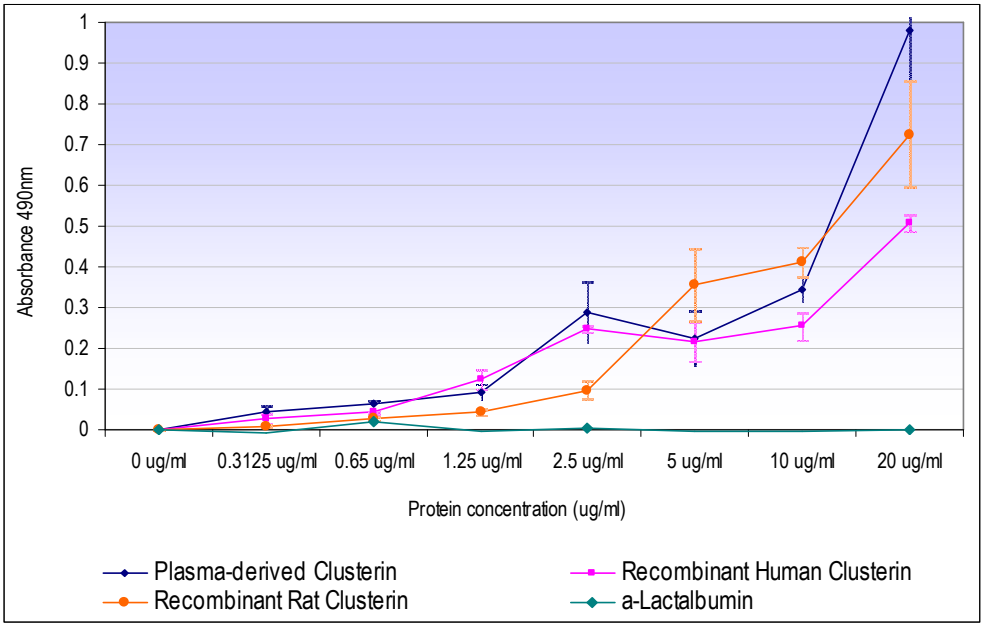


Figure 33. Binding of plasma-derived clusterin, recombinant rat and recombinant human clusterin to human IgG. Each data point represents the mean of triplicate determinations, and the error bars are standard errors of the mean in each case. The identity of each plot is indicated in the key

5.4 Discussion

The structure and chaperone function of Flag-tagged recombinant human clusterin expressed in human embryonic kidney cells and His-tagged C-terminally truncated recombinant rat clusterin expressed in *E. coli* were compared with plasma-derived clusterin to provide insight into any important differences between the recombinant products and the naturally occurring molecule. SDS-PAGE under non-reducing conditions revealed Flag-tagged recombinant human clusterin migrating as a single major band to a position of about ~75-80 kDa along with SDS-resistant clusterin aggregates. Under reducing conditions, it migrated as a single major band at about ~35 kDa and also showed minor bands in the range 50-100 kDa. These results were similar to the ones displayed for plasma-derived clusterin.

His-Tagged recombinant rat clusterin migrated as a major band to a position of about 27 kDa together with other bands in the range 60-75 kDa and at apparent masses greater than 150 kDa under non-reducing conditions. Under reducing conditions, all the protein migrated to a position of about 27 kDa.

SEC analyses showed that the recombinant proteins tested had a great proportion of high molecular weight species. Furthermore, SEC suggests that recombinant rat clusterin consisted primarily of HMW species and had greater proportion of HMW species when compared with recombinant human clusterin and plasma-derived clusterin.

The CD spectra obtained for all proteins were similar. Deconvolution of these spectra indicated that the predicted secondary structure content of all the proteins tested contained 30-38% of α-helix content, 18-22% of β-sheet content and 28-20% of unordered structures. Furthermore, the finding that the predicted secondary structure of His-Tagged recombinant rat clusterin was similar to that of plasma-derived clusterin suggests that the lack of cleavage into α and β chains and the C-terminal truncation did not lead to major changes in the secondary structure of the protein.

Chaperone assays indicated that recombinant human clusterin and recombinant rat clusterin possess chaperone action. The chaperone action of both recombinant proteins tested was less effective than plasma derived clusterin at inhibiting the heat-induced precipitation of fibrinogen. IgG binding ELISA assays indicated that recombinant human clusterin and recombinant rat clusterin are able to bind to IgG and that their dose-dependent IgG binding activity was very similar to that of plasma-derived clusterin.

Lastly, the results obtained for recombinant rat clusterin indicate that the expression of chaperone active clusterin in bacteria is possible and more importantly that C-terminally truncated clusterin lacking of cleavage into α and β chains works as a chaperone. However, whether the lack of cleavage and the C-terminal truncation (residues 22-145) are possible reasons for reduced chaperone activity should be considered.

6. CONCLUSIONS

Previous studies have suggested that clusterin mediates the clearance and degradation of misfolded extracellular proteins *in vivo* and that this action is anti-inflammatory (Yerbury *et al*., 2005). Therefore, a recombinant form of clusterin might be therapeutically useful to clear protein debris from sites of disease and inflammation. Furthermore, since Stewart *et al*. (2007) showed that clusterin retains its chaperone function when deglycosylated and also that deglycosylation did not induce major structural changes in the molecule, the possibility of inexpensively producing large quantities of chaperone-active clusterin in bacteria became apparent. Expression of a chaperone-active human clusterin in bacteria has not been reported before the present work. As stated before, some post-translational processes are not performed by bacterial cells; therefore the expression of clusterin in *E. coli* could produce recombinant protein with a different structure to that of the plasma-derived form. The absence of cleavage between the α and β chains, and potentially inappropriate disulfide bonding, are two possible factors that could contribute to differences in structure. Even small variations in structure could be cause reduced chaperone activity.

Firstly, the expression of clusterin - MBP fusion protein was attempted in *E. coli* to produce a recombinant product that could be rapidly purified using an amylose resin. Results from the current study demonstrate that:

i. Full length recombinant MBP-clusterin fusion protein was only detected as a minor species.
ii. Recombinant clusterin was cleaved from MBP during expression.
iii. Most of the MBP-clusterin fusion protein was degraded with time.

Thus, the results obtained indicated that it would not be possible to use the pMal-c2x-clusterin plasmid and the bacterial strain tested to obtain a source of stable clusterin - MBP fusion protein.

Secondly, the expression of clusterin – 6xHis tag fusion protein was attempted in *E. coli* to produce a recombinant product that could be rapidly purified using a nickel resin. The results presented here demonstrate that:

i. Clusterin - 6xHis Tag fusion protein was detected after expression along with disulfide-bonded high molecular weight aggregates and putative truncated products.

ii. The predicted secondary structure content of clusterin - 6xHis tag fusion protein and plasma-derived clusterin were similar.
iii. The clusterin – 6xHis Tag fusion protein had little if any chaperone activity and the effect of the fusion protein on heat-induced precipitation of fibrinogen was not dose-dependent.

Clusterin – 6xHis tag fusion protein was extracted and purified under denaturing conditions in 8 M urea, 0.1 M NaH_2PO_4, 0.1 M Tris. The fusion protein was subsequently refolded in 50 mM NaH_2PO_4, 300 mM NaCl, 1 mM DTT, 10% glycerol, 0.1% azide, alkylated with 50 mM IAA and dialyzed prior to developing functional and structural analyses. Although SDS-PAGE and immunoblotting analyses suggested that the refolding and alkylation processes did not produce substantive changes in the molecular mass of clusterin – 6xHis tag fusion protein, these effects may warrant closer examination. It would not be possible to use the clusterin - 6xHis Tag fusion protein produced as described in this study to provide a source of chaperone-active clusterin. Potential solutions to the problem are discussed below.

In order to provide additional insights into any functional limitations of recombinantly expressed clusterin proteins, two commercially available recombinant clusterin proteins were compared in structure and chaperone function with plasma-derived clusterin. The results demonstrate that the recombinant proteins tested:

i. Had an increased proportion of high molecular weight species when compared with plasma-derived clusterin.
ii. Had predicted secondary structure content similar to that of plasma-derived clusterin.
iii. Possess chaperone activity but were less effective than plasma-derived clusterin (recombinant human and recombinant rat forms were 28% and 48% less effective, respectively).
iv. Were able to bind IgG - the IgG binding activity was dose-dependent and similar to that of plasma-derived clusterin.

More importantly, the results obtained for recombinant rat clusterin suggested that:

i. The expression of chaperone active clusterin in bacteria is possible.
ii. A C-terminally truncated clusterin product lacking cleavage into α and β chains is chaperone-active.

Unexpectedly, clusterin – 6xHis tag fusion protein produced as described in this study was not a chaperone active product. Thus, in order to identify a reason for the little chaperone activity of clusterin – 6xHis tag fusion protein, the findings obtained for this protein and recombinant rat clusterin were compared (Table 6).

Table 6. Comparison of characteristics of recombinant human and rat clusterin expressed in *E. coli*.

	Clusterin – 6xHis tag fusion protein	**His-Tagged recombinant rat clusterin**
Structure	single polypeptide	single polypeptide
Molecular mass	~50 kDa monomeric clusterin–6xHistag fusion protein ~30 kDa putative truncated product	26 kDa protein Great proportion of HMW species (SEC)
Purification process	Solubilization of IB and Ni-NTA chromatography purification by using high concentrations of denaturing (urea) and reducing (DTT) agents. Refolding, alkylation, concentration, dialyzed	Ni-NTA chromatography filtered (0.4 micron) lyophilized in 0.01 M Tris, pH 7.2
Amino acid content	438 amino acid ✓ 427 amino acid residues (23-449) of secreted human clusterin sequence ✓ 6 amino acid of His tag	240 amino acids ✓ 215 amino acid residues (146-360) of rat clusterin sequence ✓ 16 amino acid of T7 tag ✓ 9 amino acid of His tag
Chaperone assays	no significant chaperone activity effect on precipitation of fibrinogen was not dose-dependent	inhibits heat-induced aggregation of fibrinogen

The above comparison identifies the following major differences between the two proteins which may be relevant to the measured difference in chaperone activity: (i) the rat protein migrates in SEC as almost exclusively very high molecular weight species, while the human protein (analysed by SDS-PAGE and immunoblotting) consisted of a mixture of monomeric fusion protein, high molecular weight aggregates and putative truncation product, (ii) the human product was denatured in 8 M urea and refolded, while the conditions used to purify the rat protein are not provided by the manufacturer and (iii) the rat protein was (presumably) not reduced or alkylated while the human protein was both reduced and alkylated. Dissociation of clusterin oligomers into individual α-β heterodimers increases its chaperone action (Poon *et al.*, 2002). Thus, the nature of the oligomerization of each of the two proteins in solution may impact on their ability to act as chaperones. SEC analyses of reduced and alkylated clusterin – 6x His tag fusion protein are needed to further characterise the solution size of this recombinant product. It is possible that denaturation of the human clusterin product during purification compromised its chaperone activity. However, arguing against this is the observation that human plasma derived clusterin is denatured in 2 M GdHCl during purification yet still remains chaperone-active. Lastly, in itself, reduction does not inhibit the chaperone action of clusterin (Humphreys et al., 1999), however the effect of alkylation of the reduced -SH groups on the chaperone action is unknown. Therefore, this is another factor that needs to be examined if a chaperone-active human clusterin product is to be produced in *E. coli*.

Therefore, in summary, future directions to optimize production of a chaperone active form of human clusterin in bacteria should include:

i. Examination of the effect of denaturation, reduction and alkylation processes on the activity of recombinant clusterin.
ii. Studies should be performed to determine the optimum pH conditions for solubilization of clusterin with urea. Inclusion bodies that are solubilized by high concentrations of urea, as a chaotropic agent, may contain and spontaneously produce cyanate (Hagel *et al.*, 1971), which can carbamylate the amino groups of the protein; also, solubilization by urea is pH dependent (Estapé and Rinas, 1996) .
iii. Inclusion bodies could be solubilized with guanidinium hydrochloride, detergents or low concentrations of denaturants other than urea.
iv. In the present study, direct dilution was used as a technique for protein refolding because of its simplicity; but other techniques could be tried such as dialysis and diafiltration, SEC with a column previously equilibrated with the refolding buffer, matrix-assisted protein refolding or hydrophobic interaction chromatography (Vallejo and Rinas, 2004).

Hopefully these approaches will identify a route to express and purify chaperone-active recombinant clusterin from bacteria which can then be trialed as a potential therapeutic in animal models.

In conclusion, this project is relevant to the hypothesis that:

- clusterin mediates the *in vivo* clearance and degradation of misfolded extracellular proteins (Yerbury *et al.*, 2005)
- clusterin may have anti-inflammatory action (Yerbury *et al.*, 2005)

Therefore, if it is possible to produce recombinant clusterin with good chaperone activity, various pathologies associated with one or more misfolded extracellular proteins could potentially be treated with clusterin, for example:

- acute inflammatory conditions (e.g. rheumatoid arthritis) where local injection of recombinant clusterin could help dispose of pro-inflammatory antibody aggregates.
- Systemic amyloidoses like light chain amyloidosis where intravenous administration of clusterin could inhibit the generation of pathological extracellular protein aggregates.

REFERENCES

Bailey, R. W., Dunker, A. K., Brown, C. J., Garner, E. C. and Griswold, M. D. (2001). *Clusterin, a binding protein with a molten globule-like region*. Biochemistry 40: 11828-40.

Bartl, M. M., Luckenbach, T., Bergner, O., Ullrich, O. and Koch-Brandt, C. (2001). *Multiple Receptors Mediate apoJ-Dependent Clearance of Cellular Debris into Nonprofessional Phagocytes*. Experimental Cell Research 271: 130-141.

BioLabs New England Instruction Manual, I. (2006). "pMAL Protein Fusion and Purification System. ." Expression and Purification of Proteins and Cloned Genes Version 5.1. Retrieved July 30, 2007, 2007, from http://www.neb.com/nebecomm/products_intl/productE8000.asp.

BioRad Instruction Manual, L. (2007). "Mini Trans-Blot Electroforetic Transfer Cell Instruction Manual Catalog number 170-3930." Retrieved July 31, 2007, 2007, from http://www.bio-rad.com/cmc_upload/0/000/013/280/M1703930E.pdf.

BioVendor Product Data Sheet, L. M., Inc. (2007, 09 / 30). "Human Clusterin (Apo-J, Complement cytolysis inhibitor, TRPM-2, Complement-associated protein SP-40, 40) Flag-Tagged Recombinant Protein. Product Data Sheet." Retrieved November 1, 2007, from www.biovendor.com.

BioVendor Product Data Sheet, L. M., Inc. (2007, 09/30). "Rat Clusterin (Apo-J, Complement cytolysis inhibitor, TRPM-2, Complement-associated protein S-40-,40) His-Tagged Recombinant Protein." Retrieved November 1, 2007, from www.biovendor.com.

Bjork, J. K. and Sistonen, L. (2006). *Clustering of heat-shock factors.* Biochemical Journal 395: e5-6.

Bollag, D. M., Rozycki, M. D. and Edelstein, S. J. (1996). *Protein methods*. New York, Wiley-Liss.

Boxrud, P. (2007). "Practical CD (circular Dichroism) guide: A practical guide to useing the Olis CD." Retrieved May 17, 2007, from www. olisweb.com.

Brahms, S. and Brahms, J. (1980). *Determination of protein secondary structure in solution by vacuum ultraviolet circular dichroism*. Journal of Molecular Biology 138: 149-178.

Calero, M., Agueda, R., Etsuro, M., Berislav, Z., Blas, F. and Jorge, G. (2000). *Apolipoprotein J (clusterin) and Alzheimer's disease*. Microscopy Research and Technique 50: 305-315.

Carver, J. A., Rekas, A., Thorn, D. C. and Wilson, M. R. (2003). *Small heat-shock proteins and clusterin: intra- and extracellular molecular chaperones with a common mechanism of action and function?* IUBMB Life 55: 661-8.

Chang, B. S., Reeder, G. and Carpenter, J. F. (1996). *Development of a stable freeze-dried formulation of recombinant human interleukin-1 receptor antagonist*. Pharmaceutical Research 13: 243-9.

Choi-Miura, N. H. and Oda, T. (1996). *Relationship between multifunctional protein "clusterin" and Alzheimer disease*. Neurobiology of Aging 17: 717-22.

Choi-Miura, N. H., Sakamoto, T., Ohtaki, S., Nakamura, H., Ishizawa, S., Takagi, Y., Gomi, K. and Tomita, M. (1993). *Elevated complement activities of sera from patients with high density lipoprotein deficiency (Tangier disease): the presence of normal level of clusterin and the possible implication in the atherosclerosis*. Clinical & Experimental Immunology 93: 242-7.

DeMattos, R. B., Cirrito, J. R., Parsadanian, M., May, P. C., O'Dell, M. A., Taylor, J. W., Harmony, J. A. K., Aronow, B. J., Bales, K. R., Paul, S. M. and Holtzman, D. M. (2004). *ApoE and Clusterin Cooperatively Suppress Aβ Levels and Deposition: Evidence that ApoE Regulates Extracellular Aβ Metabolism In Vivo*. Neuron 41: 193-202.

DeMattos, R. B., O'Dell, M. A., Parsadanian, M., Taylor, J. W., Harmony, J. A. K., Bales, K. R., Paul, S. M., Aronow, B. J. and Holtzman, D. M. (2002). *Clusterin promotes amyloid plaque formation and is critical for neuritic toxicity in a mouse model of Alzheimer's disease*. Proceedings of the National Academy of Sciences of the United States of America 99: 10843-8.

Estapé, D. and Rinas, U. (1996). *Optimized procedures for purification and solubilization of basic fibroblast growth factor inclusion bodies*. Biotechnology Techniques 10: 481-484.

Freixes, M., Puig, B., Rodriguez, A., Torrejon-Escribano, B., Blanco, R. and Ferrer, I. (2004). *Clusterin solubility and aggregation in Creutzfeldt-Jakob disease*. Acta Neuropathologica 108: 295-301.

Hagel, P., Gerding, J., Fieggen, W. and Bloemendal, H. (1971). *Cyanate formation in solutions of urea. I. Calculation of cyanate concentrations at different temperature and pH.* Biochim Biophys Acta 243: 366-73.

Hammad, S. M., Ranganathan, S., Loukinova, E., Twal, W. O. and Argraves, W. S. (1997). *Interaction of apolipoprotein J-amyloid beta-peptide complex with low density lipoprotein receptor-related protein-2/megalin. A mechanism to prevent pathological accumulation of amyloid beta-peptide*. Journal of Biological Chemistry 272: 18644-9.

Henderson, B. and Pockley, G., Eds. (2005). *Molecular chaperones and cell signalling*. Cambridge, UK, Cambridge University Press.

Hochgrebe, T., Pankhurst, G. J., Wilce, J. and Easterbrook-Smith, S. B. (2000). *pH-dependent changes in the in vitro ligand-binding properties and structure of human clusterin*. Biochemistry 39: 1411-9.

Humphreys, D., Hochgrebe, T. T., Easterbrook-Smith, S. B., Tenniswood, M. P. and Wilson, M. R. (1997). *Effects of clusterin overexpression on TNFα- and TGFβ-mediated death of L929 cells*. Biochemistry 36: 15233-43.

Humphreys, D. T., Carver, J. A., Easterbrook-Smith, S. B. and Wilson, M. R. (1999). *Clusterin has chaperone-like activity similar to that of small heat shock proteins*. Journal of Biological Chemistry 274: 6875-81.

Invitrogen User Manual, C. (2003, Version E, 22 September 2003, 25-0522). "Gateway® Technology. A universal technology to clone DNA sequences for functional analysis and expression in multiple systems." Catalog nos. 12535-019 and 12535-027. Retrieved August 6, 2007, 2007, from www.invitrogen.com.

Janig, E., Stumptner, C., Fuchsbichler, A., Denk, H. and Zatloukal, K. (2005). *Interaction of stress proteins with misfolded keratins*. European Journal of Cell Biology 84: 329-339.

Jones, S. E. and Jomary, C. (2002). *Clusterin*. The International Journal of Biochemistry & Cell Biology 34: 427-431.

Kapron, J. T., Hilliard, G. M., Lakins, J. N., Tenniswood, M. P., West, K. A., Carr, S. A. and Crabb, J. W. (1997). *Identification and characterization of glycosylation sites in human serum clusterin*. Protein Science 6: 2120-33.

Kounnas, M. Z., Loukinova, E. B., Stefansson, S., Harmony, J. A., Brewer, B. H., Strickland, D. K. and Argraves, W. S. (1995). *Identification of glycoprotein 330 as an endocytic receptor for apolipoprotein J/clusterin.[erratum appears in J Biol Chem 1995 Sep 29;270(39):23234]*. Journal of Biological Chemistry 270: 13070-5.

Lakins, J. N., Poon, S., Easterbrook-Smith, S. B., Carver, J. A., Tenniswood, M. P. R. and Wilson, M. R. (2002). *Evidence that clusterin has discrete chaperone and ligand binding sites*. Biochemistry 41: 282-91.

Lodish, H., Berk, A., Zipursky, L. S., Matsudaira, P., Baltimore, D. and Darnell, J. (2000). *Molecular cell biology*. New York Basingstoke, W. H. Freeman ; Macmillan.

Mahon, M. G., Lindstedt, K. A., Hermann, M., Nimpf, J. and Schneider, W. J. (1999). *Multiple Involvement of Clusterin in Chicken Ovarian Follicle Development. Binding to two oocyte-specific members of the low density lipoprotein receptor gene family*.

Mao, D., Wachter, E. and Wallace, B. A. (1982). *Folding of the H+-ATPase Proteolipid in Phospholipid Vesicles*. Biochemistry 21: 4960-4968.

Mironova, R., Niwa, T., Handzhiyski, Y., Sredovska, A. and Ivanov, I. (2005). *Evidence for non-enzymatic glycosylation of Escherichia coli chromosomal DNA*. Molecular Microbiology 55: 1801-11.

Narberhaus, F. (2002). *Alpha-crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network*. Microbiology & Molecular Biology Reviews 66: 64-93; table of contents.

Pain, R., Ed. (2000). *Mechanisms of protein folding*. Oxford, Oxford University Press.

Poon, S., Easterbrook-Smith, S. B., Rybchyn, M. S., Carver, J. A. and Wilson, M. R. (2000). *Clusterin is an ATP-independent chaperone with very broad substrate specificity that stabilizes stressed proteins in a folding-competent state*. Biochemistry 39: 15953-60.

Poon, S., Rybchyn, M. S., Easterbrook-Smith, S. B., Carver, J. A., Pankhurst, G. J. and Wilson, M. R. (2002). *Mildly acidic pH activates the extracellular molecular chaperone clusterin*. Journal of Biological Chemistry 277: 39532-40.

Poon, S., Treweek, T. M., Wilson, M. R., Easterbrook-Smith, S. B. and Carver, J. A. (2002). *Clusterin is an extracellular chaperone that specifically interacts with slowly aggregating proteins on their off-folding pathway*. FEBS Letters 513: 259-266.

Provencher, S. M. and Glochner, J. (1981). *Estimation of protein secondary structure from circular dichroism*. Biochemistry 20: 33-37.

Reddy, K. B., Jin, G., Karode, M. C., Harmony, J. A. and Howe, P. H. (1996). *Transforming growth factor beta (TGFβ)-induced nuclear localization of apolipoprotein J/clusterin in epithelial cells*. Biochemistry 35: 6157-63.

Roche Product Data Sheet , D. (2007). "Complete lysis-B (2x), EDTA free reagent set." Retrieved http://www.roche-applied-science.com/proddata/intnl/3_2_7_1_45_1.html April 4, 2007.

Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A. P., Buchner, J. and Gaestel, M. (1999). *Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation*. Journal of Biological Chemistry 274: 18947-56.

Rosenberg, M. E., Girton, R., Finkel, D., Chmielewski, D., Barrie, A., 3rd, Witte, D. P., Zhu, G., Bissler, J. J., Harmony, J. A. K. and Aronow, B. J. (2002). *Apolipoprotein J/clusterin prevents a progressive glomerulopathy of aging*. Molecular & Cellular Biology 22: 1893-902.

Rosenberg, M. E. and Silkensen, J. (1995). *Clusterin: Physiologic and pathophysiologic considerations*. The International Journal of Biochemistry & Cell Biology 27: 633-645.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989). *Molecular cloning : a laboratory manual*. Cold Spring Harbor, N.Y. 8805, Cold Spring Harbor Laboratory Press.

Sasaki, K., Doh-ura, K., Wakisaka, Y. and Iwaki, T. (2002). *Clusterin/apolipoprotein J is associated with cortical Lewy bodies: immunohistochemical study in cases with alpha-synucleinopathies*. Acta Neuropathologica 104: 225-30.

Sensibar, J. A., Sutkowski, D. M., Raffo, A., Buttyan, R., Griswold, M. D., Sylvester, S. R., Kozlowski, J. M. and Lee, C. (1995). *Prevention of cell death induced by tumor necrosis factor alpha in LNCaP cells by overexpression of sulfated glycoprotein-2 (clusterin)*. Cancer Research 55: 2431-7.

So, A., Sinnemann, S., Huntsman, D., Fazli, L. and Gleave, M. (2005). *Knockdown of the cytoprotective chaperone, clusterin, chemosensitizes human breast cancer cells both in vitro and in vivo*. Molecular Cancer Therapeutics 4: 1837-49.

Stewart, E. M., Aquilina, J. A., Easterbrook-Smith, S. B., Murphy-Durland, D., Jacobsen, C., Moestrup, S. and Wilson, M. R. (2007). *Effects of glycosylation on the structure and function of the extracellular chaperone clusterin*. Biochemistry 46: 1412-22.

Vallejo, L. and Rinas, U. (2004). *Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins*.

Wehmeyer, N. and Vierling, E. (2000). *The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance*. Plant Physiology 122: 1099-108.

Whitmore, L. and Wallace, B. A. (2004). *DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data*.

Wilson, M. R. and Easterbrook-Smith, S. B. (1992). *Clusterin binds by a multivalent mechanism to the Fc and Fab regions of IgG*. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1159: 319-326.

Wilson, M. R. and Easterbrook-Smith, S. B. (2000). *Clusterin is a secreted mammalian chaperone*. Trends in Biochemical Sciences 25: 95-98.

Yerbury, J. J., Stewart, E. M., Wyatt, A. R. and Wilson, M. R. (2005). *Quality control of protein folding in extracellular space*. EMBO Reports 6: 1131-6.

Zlokovic, B. V., Martel, C. L., Matsubara, E., McComb, J. G., Zheng, G., McCluskey, R. T., Frangione, B. and Ghiso, J. (1996). *Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers*. Proceedings of the National Academy of Sciences of the United States of America 93: 4229-34.