Induction of autoantibodies to cathepsin L as a step towards an anti-cancer vaccine

by

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PREFACE

The experimental work described in this thesis was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, from January 2003 to November 2005 under the supervision of Professor Clive Dennison.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

Karabo Motsamai

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ABSTRACT

Cancer is a disease that is caused by mutations in somatic cells. Metastasis is the major cause of death from cancer and often complicates treatment. Malignant tumours secrete degradative enzymes such as cathepsin L which degrade the extracellular matrix to facilitate tumour invasion and metastasis.

The immune system does not normally recognize and eradicate tumours because they arise from self tissues to which the immune system is tolerant. Self antigens are poorly immunogenic because they lack T cell help. In this study, a foreign α-glucosidase was conjugated to self rabbit cathepsin L using glutaraldehyde to specifically provide T helper cell epitopes. The conjugate was used to immunise two male rabbits. A second pair of rabbits (male and female), was primed with sheep cathepsin L (to induce T helper cell activation) and received rabbit cathepsin L boosters. A third pair of rabbits which served as a control was immunised with sheep cathepsin L. The two pairs of test rabbits made high avidity antibodies against rabbit cathepsin L, showing a similar response to control rabbits when antibodies were tested in an ELISA. Western blot analysis showed that these anti-cathepsin L autoantibodies were specific for rabbit cathepsin L.

Rabbits which were immunised with the conjugate were inoculated with sheep cathepsin L nine weeks after the final inoculation with the conjugate. Analysis of antibodies in an ELISA showed that antibody responses against rabbit cathepsin L were augmented in a manner that is characteristic of memory responses. Low titre antibodies against sheep cathepsin L were also produced.

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LIST OF ABBREVIATIONS

A_{280}	absorbance at 280 nm
Ab	antibody
AFP	α-fetoprotein
Ag	antigen
APC	antigen presenting cell
BAFF	B cell activating factor
BCR	B cell receptor
bis	N,N`-methylenebisacrylamide
BSA	bovine serum albumin
BSA-PBS	bovine serum albumin dissolved in phosphate-buffered saline
t-butanol	2-methylpropan-2-ol
Bz	benzoyl
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CLIP	class II-associated invariant chain peptide
C-terminal	carboxy terminal
CTLA-4	cytotoxic T lymphocyte antigen-4
CTL	cytotoxic T lymphocyte
DC	dendritic cell
dist.H_2O	distilled water
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E	extinction coefficient
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
g	relative centripetal acceleration
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour(s)
HLA	human leukocyte antigen
IDO	indoleamine-2,3-dioxygenase
IFN-γ	γ-interferon
IgG	immunoglobulin G
Ii	invariant chain
IL	interleukin
Igs	Immunoglobulins
kDa	kilodalton(s)
MHC	major histocompatibility complex
min	minute(s)
MMP	matrix metalloproteinases
M_r	relative molecular weight

mRNA	messenger ribonucleic acid
M6P/IGF2R	mannose 6-phosohate/insulin-like growth factor II receptor
MW	molecular weight
NHMec	7-amino-4-methyl coumarin
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen associated molecular patterns
PBS	phosphate-buffered saline
PBS-Tween	Tween 20 diluted in phosphate-buffered saline
PEG	polyethylene glycol
p-NPP	p-nitrophenylphosphate
RAG	recombinant activator gene
RT	room temperature
s	second(s)
SCC	squamous carcinoma cell
SDS	sodium dodecyl sulfate[1]
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCR	T cell receptor
TEMED	N,N,N`,N`-tetramethylethylenediamine
TGF-β	transforming growth factor-β
Th (cells)	T helper (cells)
TIL	tumour infiltrating lymphocytes
TLRs	toll-like receptors
TNF-α	tumour necrosis factor-α
TPP	three phase partitioning
Tregs	T regulatory cells
UPA	urokinase-type plasminogen activator
V(D)J	variable diversity junction
VLPs	Viral-like particles
Z	benzoyloxycarbonyl

[1] In this dissertation, "sulfate" is spelled with an "f", in accordance with the IUPAC convention [see D.P. Kelly (1995) Arch. Microbiol. **163**, 157-158].

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CHAPTER 1
INTRODUCTION

Cancer is a disease caused by gene mutations in somatic cells, resulting in loss of control of cell division (Evans, 1991) and the development of tumours. In benign tumours, although there is loss of control of cell division, the tumour mass remains *in situ* and is thus amenable to surgical intervention. Malignant tumours, by contrast, additionally release proteolytic enzymes that degrade the extracellular matrix (ECM) and thus facilitate invasion into the surrounding tissue and spread to distant organs (Nicolson, 1986). At a distant site, the tumour cells may lodge in a capillary bed and extravasate to form a secondary tumour, or metastasis. Development of metastases often complicates therapy and the prognosis is generally poor. Furthermore, treatment is often complicated by the fact that, by the time a primary tumour is discovered, it has often already spread to distant sites (Sherbet, 1982).

Currently, cancer is predominantly treated by surgery (for solid tumours), radiation or chemotherapy but these are often unsuccessful. Surgery and radiation are focused methods and are thus unsuitable for treatment of disseminated metastatic cancer. Chemotherapy is a systemic method but it targets all dividing cells, non-specifically. Severe side effects thus limit the dosages to levels that may not kill all cancer cells. The surviving cells also usually have a greater than normal resistance to xerobiotics, such as the chemotherapeutic agents used, so that these eventually become ineffective. However, in rare cases, patients with disseminated metastatic cancer miraculously recover, a phenomenon known as spontaneous remission. It appears that in these cases the immune system spontaneously becomes "aware" of the cancer and specifically eliminates all of the cancer cells. Properly alerted, therefore, the immune system has the capacity to "cure" cancer and this has engendered much interest in the possibilities of immunotherapy. However, immunotherapy in cancer treatment is limited by the fact that cancer cells are "self", rather than foreign, and the immune system is generally tolerant to its own tissues and does not normally elicit a response to self antigenic determinants (Evans, 1991). However, it has been demonstrated that tolerance is not absolute, so that self-reactive lymphocytes could be exploited for cancer cell rejection (Mapara and Sykes, 2004).

The present study was part of a larger study aimed at exploring the possibility of *engineering* cancer remission in a manner analogous to the chance spontaneous remissions which sometimes occur. This larger study has two principal aims; i) exploring ways of temporarily breaking immune self tolerance and ii) finding appropriate target antigens. The present study formed part of the studies aimed at the first objective. Pending the outcome of studies aimed at the second objective, cathepsin L, a protease purported to be involved in cancer invasion, was arbitrarily chosen as a target antigen in the present study. Cathepsin L is normally involved in intracellular proteolysis, within the endolysosomal system (Pillay and Dennison, 2002) but previous studies in this laboratory have demonstrated that it could play an extracellular role (Dehrmann *et al.*, 1995).

1.2 The biology of cancer

The following review will highlight the key factors that lead to metastasis and how immunotherapy may be a promising form of cancer treatment.

Multicellular organisms are organised into interdependent systems that regulate bodily functions. Cellular processes are tightly controlled, so that a fine balance is maintained between cell division, differentiation, growth and ultimately, death. When the regulatory mechanisms are lost, cell division and metabolic processes become disrupted, leading to organ failure and disease.

The underlying cause of cancer is gene mutation in somatic cells, which may be triggered by external factors such as radiation, chemicals or viruses or could be a consequence of shifts in gene expression (epigenetic events) (Sherbet, 1982). A cell that has undergone such mutations is said to be transformed and hence develops into a tumour. Tumours are biologically categorised into two groups, benign and malignant (Evans, 1991). Benign tumours are often encapsulated, remain at their site of origin and are rarely fatal. Malignant tumours, on the other hand, generally invade the surrounding tissue and spread throughout the body, causing complications both in disease management and treatment (Evans, 1991).

Tumours vary widely in terms of their behaviour and histologic appearance (Sherbet, 1982). The principal feature of a tumour cell is loss of response to homeostatic processes that regulate cell division (Nowell, 2002). When a normal cell is transformed into a tumour, it undergoes progressive change and finally acquires a new

unstable phenotype (Nicolson, 1986). Although tumours are believed to arise from a single transformed cell (monoclonal origin), they often show phenotypic diversity, and so cells within the same tumour mass will display different features (Evans, 1991). Most malignant tumour cells generally display a combination of the following features (Nicolson 1986; Evans, 1991; Nowell, 2002):

- Acquisition of new or modified cell surface antigens, some tumours displaying proteins that are normally found in embryonic tissue, e.g. carcinoembryonic antigen (CEA) and α-fetoprotein (AFP).
- Overexpression of degradative enzymes such as endolysosomal cysteine proteases, matrix metalloproteases (MMPs), serine and aspartyl proteases.
- Altered expression of MHC antigens
- Morphological and metabolic alterations
- Decrease or disappearance of specialised organelles or metabolic functions.

The phenotype of a tumour cell is not outstandingly different from that of a normal cell, however, and therapies targeted at tumour cells are often harmful to normal cells.

The major cause of death from cancer is metastasis: the formation of multiple tumour colonies that have detached from the primary tumour and spread via the circulatory system to distant sites (Nicolson, 1986; Woodhouse *et al.*, 1997). Metastasis is a multistep process that involves sequential interlinked processes such as angiogenesis, local invasion, detachment from the primary tumour, invasion of the vascular system, transport within the circulatory system, lodgement at a distant site, extravasation and growth (Rusciano and Burger, 1992; Woodhouse *et al.*, 1997; Nowell, 2002). The process depicted in Fig. 1.1 is a simplified summary of these steps. Tumours often grow to a certain limiting size before invading the surrounding tissue (Evans, 1991). Tumour growth is facilitated by angiogenesis (generation of new blood vessels), which allows the tumour to get nutrients and excrete waste substances (Baker, 2001).

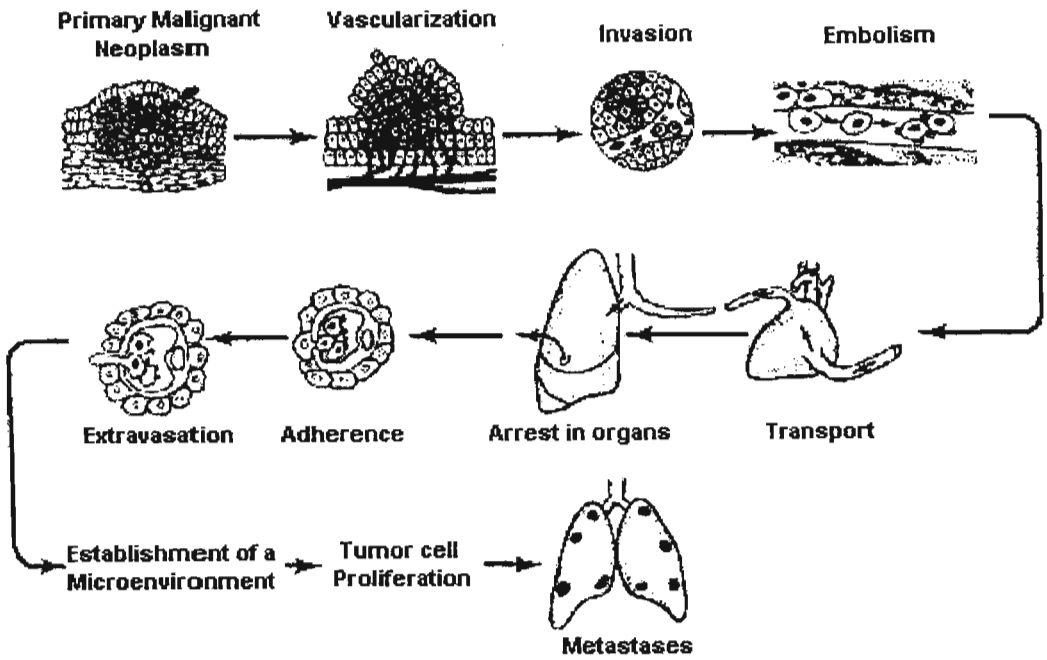

Figure 1.1 Steps in the formation of melanoma metastases.
The process of metastasis is sequential and requires the completion of several highly selective steps. Adapted from Fidler (1995).

As the primary tumour mass expands, malignant cells extend from the original site and invade the surrounding normal tissue by releasing proteolytic enzymes that degrade the exracellular matrix and basement membrane (Goldfarb and Brunson, 1989). Individual cells or tumour emboli (group of malignant cells) that penetrate through blood or lymph vessels are transported to sites distant from the primary tumour where they arrest in specific organs (Nicolson, 1986). Extravasation and invasion of the adjacent tissue result in establishment of secondary colonies that eventually grow and may metastasise again (Nowell, 2002). Rusciano and Burger (1992) pointed out that certain tumours metastasise to specific organs, a phenomenon called "seed and soil". This predicts that, despite the fact that tumour cells that enter the circulation could be taken anywhere, tumour cells will only grow in organs that best support their growth (Fidler, 1995). A number of strategies have been explored to inhibit the metastatic process, including enzyme inhibitors, antiangiogenic therapy, radiation, chemotherapy and immunotherapy. Of interest in the present study is the involvement of cathepsin L, an endolysosomal cysteine proteinase, in the metastatic process and how it may be used to label cancer cells for immune destruction.

1.3 Cathepsin L and its role in tumour invasion

Cathepsin L is a lysosomal cysteine proteinase that is ubiquitously expressed in many species (Turk *et al.*, 2001). Cathepsin L is synthesised as the preproenzyme, processed to the proenzyme in the ER and subsequently targeted to the endolysosomal system by the mannose 6-phosphate recognition marker (Sahagian and Gottesman, 1982).

The principal role of cathepsin L in the endolysosomal system is a general one, mainly protein degradation and turnover (Bond and Butler, 1987). More specialised roles have been identified, such as hormone processing (Dunn *et al.*, 1991) and antigen processing (Maric *et al.*, 1994; Nakagawa *et al.*, 1998; Hsieh *et al.*, 2002). Cathepsin L has a dual role in antigen processing. On one hand it degrades endocytosed proteins to produce peptides that are loaded into the binding groove of MHC class II molecules (Roche and Cresswell, 1991; Wolf and Ploegh, 1995). On the other hand, it degrades the invariant chain (Ii) progressively leaving class II-associated invariant chain peptide (CLIP) associated with MHC class II molecule (Nakagawa *et al.*, 1998). Honey *et al.* (2002) proposed a role for cathepsin L in T cell positive selection that is independent of Ii processing. In cortical thymic epithelial cells, cathepsin L regulates the selection of $CD4^+$ T cells by directly generating MHC class II epitopes thus eliciting positive selection (Honey *et al.*, 2002).

Other than being versatile in physiologic processes, cathepsin L also plays a major role in pathologies, such as arthritis (Tao *et al.*, 1994) and tumour progression and metastasis (Dohchin *et al.*, 2000; Fröhlich *et al.*, 2001; Troy *et al.*, 2004). The involvement of cathepsin L in tumour progression and metastasis is of particular interest in this study because it is an attractive target for immunotherapy, whereby production of anti-cathepsin L autoantibodies could neutralise cathepsin L and may lead to elimination of cancer cells, secreting cathepsin L, by the immune system.

Control of proteolytic activity in cells is achieved at various levels including expression, synthesis, secretion, maturation, compartmentalisation, turnover and control by proteinase inhibitors (Rzychon *et al.*, 2004). Chauhan *et al.* (1998) showed that in transformed cells, secreted cathepsin L carried a mannose 6-phosphate marker and should be targeted to the lysosomes. Two possible explanations were provided to account for secretion of cathepsin L in transformed cells. First, Lazzarino and Gabel

(1990) pointed out that the efficacy of cathepsin L delivery to the "lysosomes"[2] is greatly reduced, because the rate at which cathepsin L is expressed exceeds its rate of delivery to the "lysosome". As a result, mannose 6-phosphate receptor levels become limiting at high levels of cathepsin L due to saturation of the cation-independent mannose 6-phosphate receptor (Gal *et al.*, 1985), so that "excess" enzyme is secreted. Secondly, Lorenzo *et al.* (2000) showed that in murine squamous carcinoma cells (SCC-VII), secretion of procathepsin L into the medium was due to a deficiency in mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R). Since the M6P/IGF2R is essential for lysosomal maturation, SCC-VII cells lack functional lysosomes (Ludwig *et al.*, 1994); as a consequence of the M6P/IGF2R deficiency, lysosomal enzymes are temporarily stored in endosomes/prelysosomes before being secreted (Lorenzo *et al.*, 2000).

Cathepsins are synthesised as inactive precursors and are activated by proteolytic removal of the N-terminal peptide once they reach the endolysosmal system (zymogen activation) (Turk *et al.*, 2001). This prevents unwanted proteolysis that could be deleterious to the cell and ensures efficient delivery of protease to the relevant cellular compartment. Besides zymogen activation, endogenous inhibitors of cathepsins, cystatins, also control proteolysis of mature active cathepsins in extra-endolysosomal compartments (Rzychon *et al.*, 2004). The splice variant of MHC class II associated invariant chain p41 controls the activity of extracellular cathepsin L by binding to its active site, thus serving as both a chaperone and an inhibitor (Gunčar *et al.*, 1999; Lennon-Duménil *et al.*, 2001). Imbalance in the extracellular protease inhibitor levels may result in uncontrolled proteolysis, which facilitates tumour invasion (Fröhlich *et al.*, 1999).

Most malignant cells express cathepsin L at elevated levels compared to their normal cell counterparts (Chauhan *et al.*, 1991; Plebani *et al.*, 1995; Park *et al.*, 1996). Overexpression of cathepsin L leads to its secretion (Gal *et al.*, 1985) where it degrades the extracellular matrix (ECM) and basement membrane to facilitate tumour

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[2] See Pillay and Dennison (2002) for comments on the confusion in the literature regarding the naming of organelles of the endolysosomal system.

invasion and metastasis (Turk *et al.*, 2002). In murine and human melanoma models, active cathepsin L was found to be associated with plasma membrane fractions (Rozhin *et al.*, 1989; Fröhlich *et al.*, 2001). Immunolocalisation of cathepsins B and L in normal and malignant gastric tissues showed that tumours that overexpressed both cathepsins had a high invasive potential in the early stages of tumour progression (Dohchin *et al.*, 2000). Herszènyi *et al.*, (1999) showed that high levels of cathepsin L in patients with colorectal cancer were associated with poor prognosis. The involvement of cathepsin L in the early stages of colorectal cancer was also demonstrated by Troy *et al.* (2004). Plebani *et al.* (1995) highlighted the importance of cathepsins B and L in tumour invasion showing that gastric tissue samples from patients with liver metastasis had significantly higher levels of the cathepsins than samples from patients without metastases.

The mechanisms used by tumour cells to invade the surrounding tissue are similar to other physiologic processes such as nerve growth and inflammation (Woodhouse *et al.*, 1997). Monocyte-derived macrophages secrete cathepsin L which degrades the surrounding tissue during an inflammatory response (Reddy *et al.*, 1995). The challenge, therefore, is to design forms of therapy that will be limited to the tumour microenvironment or which will be temporaneously limited, so that the cancer might be eliminated without permanently harming the patient in other ways.

Cathepsin L is optimally active in slightly acidic reducing conditions found in the endolysosomal system (Turk *et al.*, 2001). Its involvement in extracellular roles such as the inflammatory response and tumour invasion and metastasis raised questions regarding its activity profile, because it had been reported to be largely inactive at physiological pH (Barrett and Kirschke, 1981). However, Dehrmann *et al.* (1995) showed that by taking account of the effects of ionic strength on the activity profile of cathepsin L, substantial activity at physiological pH could be demonstrated and, therefore, its involvement in extracellular roles is indeed possible. In addition, the association of cathepsin L with p41 has been shown to prevent pH induced inactivation of cathepsin L in the extracellular milieu of antigen presenting cells, thus maintaining a pool of mature enzyme in the microenvironment of antigen presenting cells (Fiebiger *et al.*, 2002).

Cathepsin L is, therefore, an attractive target for cancer immunotherapy. However, it is not the sole candidate because other proteolytic enzymes, such as the MMPs and urokinase-type plasminogen activator (UPA), have also been implicated in cancer invasion and metastasis.

1.4 Aspects of immunologic tolerance

It is important for the immune system to be largely unresponsive to self-proteins, because a strong reactivity towards self results in autoimmune disease. Effective immune responses against tumours are a rarity because of the phenomenon of immunologic tolerance. Under normal circumstances, the immune system does not mount a sustainable destructive response towards self proteins, because the effector cells of the immune system can make a distinction between self and non-self entities (Janeway *et al.*, 2005). An understanding of how the immune system maintains tolerance towards self proteins would be beneficial in identifying the key elements that can be exploited to induce responses against tumours or to treat autoimmune disease.

The mammalian immune system is organised into two main parts, innate and adaptive immunity (Janeway *et al.*, 2005), which interact to fight infections. These two arms of the immune system use distinctly different mechanisms to distinguish between self and nonself. The innate immune system forms the first line of defence against invading pathogens and uses non-specific mechanisms of recognition. The receptors that are used to recognise pathogens are encoded in the germline and expressed by cells of a given type (Medzhitov and Janeway, 2000). As pointed out by Medzhitov and Janeway (2000), the innate immune system is never involved in autoreactivity and, therefore, tolerisation of the adaptive immune system is of greater importance.

Recognition in the adaptive immune system depends on two types of receptors, B cell receptors (BCR) and T cell receptors (TCR) (Nossal, 1991; Goodnow *et al.*, 2005). These receptors have the ability to bind an unlimited variety of ligands, not only pathogen derived, but also self proteins (Janeway *et al.*, 2005). Mechanisms that lead to immune tolerance are complex and not rigorous as exemplified by several autoimmune diseases. However, self-reactive clones are restricted from developing into fully reactive lymphocytes by the following mechanisms (Goodnow *et al.*, 2005):

- Clonal deletion
- Receptor editing by further V(D)J recombination
- Clonal anergy

These mechanisms are operative in both B and T cell clones with minor differences that arise as a result of anatomical restrictions imposed on each lymphocyte (*i.e.* B cells develop mainly in the bone marrow while T cells develop in the thymus; therefore, the microenvironment of each organ shapes the development of the lymphocyte in question). Induction and maintenance of tolerance are influenced by expression patterns, location and immunogenicity of antigens (Garza *et al.*, 2000), as will be described in subsequent sections. Lymphocyte tolerance mechanisms are summarised in Fig. 1.2.

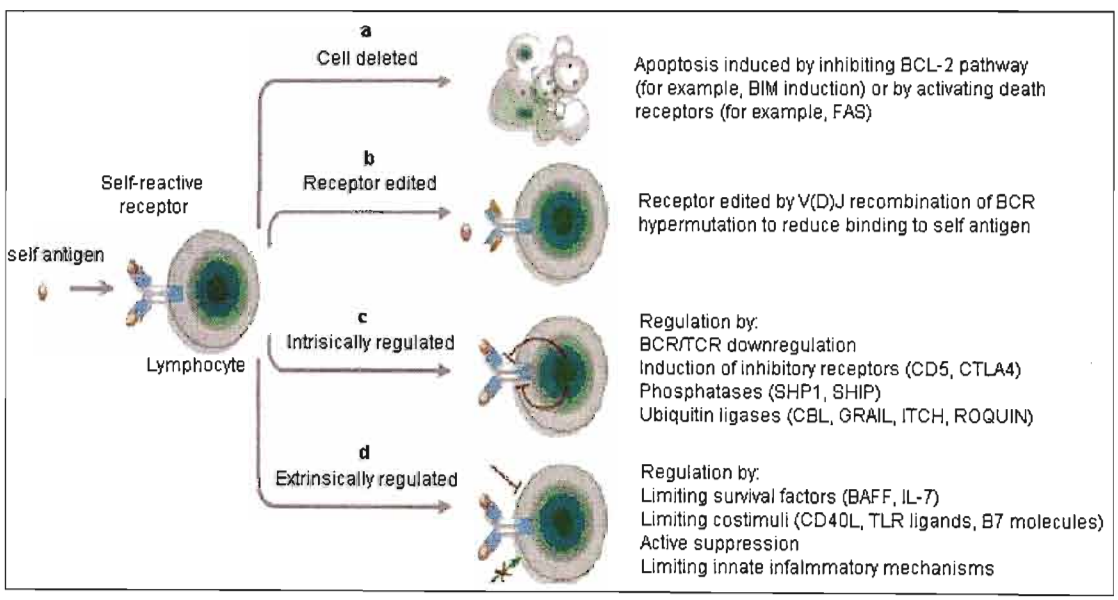

Figure 1.2 Regulation of self-reactive receptors at different points during B- and T-cell differentiation.
a, The cell is deleted through induction of cell death. **b**, The receptor is edited to one that is less self-reactive. **c**, Biochemical or gene-expression changes intrisically dampen the self-reactive receptor's ability to activate the cell. **d**, The ability of self-reactive cells or antibody to cause autoimmunity is limited by using extrinsic suppression and limiting essential growth factors, co-stimuli and inflammatory mediators. Adapted from Goodnow *et al.* (2005).

1.4.1 T cell tolerance

T cells develop in the thymus where they continually encounter a wide variety of antigens, including self antigens (Janeway *et al.*, 2005). In the thymus, development of T cells starts in the cortex and as T cells mature they migrate to the medulla (Palmer, 2003). Immature T cells that are reactive towards self antigens expressed in the thymus are induced to undergo apoptotic cell death and are thus deleted in a

process called negative selection (Ribas *et al.,* 2003; Nossal, 1991; Janeway *et al.*, 2005). Deletion of self-reactive clones is triggered when T cells encounter self-peptide/MHC complexes displayed on the surface of cortical thymic epithelial cells, whereby TCRs that bind these ligands trigger maturation signals that inhibit recombinant activator gene (RAG) expression and induce apoptosis (Palmer, 2003). Kappler *et al.* (1987) showed that in mice that expressed the allelic forms of the class II MHC molecule, i.e. T cells bearing vβ17a^{+} receptors, were depleted in the periphery, but occurred at normal levels among immature thymocytes. This indicates that self-reactive T cells are deleted in the thymus during the transition of $CD4^{+}8^{+}$ (double positive) cells into mature $CD4^{+}8^{-}$ and $CD4^{-}8^{+}$ cells (Mapara and Sykes, 2004). However, T cells that have a low affinity for self MHC/peptide do not undergo apoptosis; rather the signal provided by TCR ligation results in positive selection (Mapara and Sykes, 2004). Autoreactive mature T cells have also been shown to be eliminated in the thymus by negative selection (Tian *et al.*, 2004).

Negative selection is the predominant process in the thymus that deletes autoreactive T cells from the T cell repertoire. However, a fraction of autoreactive T cells is salvaged from apoptotic cell death by receptor editing (Nemazee and Hogquist, 2003). During T cell differentiation in the thymus, V(D)J recombination assembles unique TCR genes from the variable (V), diversity (D) and joining (J) gene segments (Goodnow *et al.*, 2005). TCR is composed of two chains, α and β (or γ and δ) (Medzhitov and Janeway, 2000). When T cells receive a negative signal, the alpha chain undergoes further rearrangement to replace the offending receptor (Nemazee and Hogquist, 2003). McGargill *et al.* (2000) demonstrated that T cells that edit their receptors expressed high levels of mRNA for receptor activator genes (RAG). In addition, new TCR α chains were produced preferentially in the thymic environment that had been manipulated to predominantly express a self antigen (McGargill *et al.*, 2000).

Specific antigen recognition requires two signals; the first signal is engagement of TCR with peptide/MHC complex which induces the second signal; ligation of co-stimulatory molecules, CD28 and CTLA-4 (cytotoxic T lymphocyte antigen 4) receptors on T cells and B7 molecules on antigen presenting cells (Macián *et al.*, 2004). However in the absence of co-stimulation, T cells become inactivated and are said to be in a state of anergy (Macián *et al.*, 2004; Janeway *et al.*, 2005; Goodnow *et*

al., 2005). When anergic T cells encounter antigen, they are prevented from proliferating and differentiating into effector cells because they do not produce IL-2 (Otten and Germain, 1991; Goodnow *et al.*, 2005). Dendritic cells (DCs) are important in priming naïve T cells (Macián *et al.*, 2004) and, therefore, can induce and maintain tolerance (Ohashi and DeFranco, 2002). In the thymus, mature DCs are essential for the deletion of newly generated T cells that have a high affinity for self peptides (Banchereau and Palucka, 2005). In the periphery, immature DCs deliver negative signals to the T cells, because they bear low levels of co-stimulatory molecules and MHC molecules (Schwartz, 2003). Steinman *et al.* (2000) pointed out that such tolerising DCs remain in their immature state and, therefore, tolerise self-reactive T cells.

Tissue specific antigens are rarely expressed in the thymus. Potentially autoreactive clones are, therefore, released into the periphery. Here they could cause massive tissue destruction if not eliminated (Rocha and von Boehmer, 1991). In addition some autoreactive T cells escape thymic deletion (Herndon *et al.*, 2005). Unlike thymic negative selection, binding of autoreactive T cells to antigens results in initial expansion of the T cell clone, followed by subsequent downregulation of the receptor or cell death (Rocha and von Boehmer, 1991). Herndon *et al.* (2005) showed that autoreactive T cells in the periphery can also be eliminated by $CD8^+$ cytototoxic mediated lysis.

1.4.2 B cell tolerance

B cells develop in the bone marrow where they undergo a series of developmental stages until they mature and migrate to secondary lymphoid organs (Nemazee and Hogquist, 2003). The heavy chain locus of the surface immunoglobulin (Ig) which is the B cell receptor (BCR) is rearranged to produce variable (V), diversity (D) and junction (J) segments in the pro-B cell stage (Kouskoff and Nemazee, 2001). The B cells progress to the pre-B cell stage where the light chain locus is rearranged by the assembly of V and J segments such that each B cell has one unique BCR (Kouskoff and Nemazee, 2001). The resulting immature B cells test their receptors for autoreactivity as they migrate to the spleen (Nemazee and Hogquist, 2003).

In the bone marrow, immature autoreactive B cells undergo negative selection through inactivation or deletion (Gay *et al.*, 1993). If the surface Ig molecule binds

strongly to a self antigen, the B cell rapidly internalises the forbidden receptor and maturation stops temporarily (Hartley *et al.*, 1993). Maturation arrest results in receptors such as CD62 ligands (CD62L), required for the B cell to progress into secondary lymphoid organs, not being expressed (Hartley *et al.*, 1993). Secondly, receptors for B cell activating factor (BAFF), a cytokine that is essential for peripheral B cell survival, are weakly expressed (Gavin *et al.*, 2003). However, RAG 1 and RAG 2, which encode the core enzymes for V(D)J recombination, continue to be expressed, which allows the BCRs to be edited by rearranging a replacement BCR light chain (Tiegs *et al.*, 1993; Kouskoff and Nemazee, 2001; Nemazee and Hogquist, 2003). If the forbidden receptor persists, the B cell dies within 1-2 days in the bone marrow or shortly after arriving in the spleen (Hartley *et al.*, 1993).

Mature B cells are exported to secondary lymphoid organs (Tiegs *et al.*, 1993). In the periphery, selection of B cells occurs after interactions with antigens (Russell *et al.*, 1991). Julien *et al.* (2002) showed that binding of a soluble low-affinity self antigen by selected clones of B cells elicits positive selection. The significance of such autoreactive B cells has not yet been established fully, but it has been suggested that they may be important in the initial stages of responses to bacteria and viruses (Ochsenbein *et al.*, 1999). The presence of autoreactive B cells in the B cell repertoire has largely been attributed to non-rigorous tolerance mechanisms, because positive selection of such low-affinity self-reactive B cell clones had not been demonstrated *in vivo*. As pointed out by Nossal (1991), a consequence of B cell positive selection may be that individuals possess a repertoire of B cells that are reactive towards a wide variety of self soluble protein determinants.

B cells require two signals for effective antibody production. Signal one is provided by binding of the antigen to the BCR and signal two is provided by T helper cells (Goodnow *et al.*, 2005). Ligation of CD40 ligand (CD40L) on T cells by B cell costimulatory molecules induces secretion of cytokines such as, IL-2, IL-4, IL-5 and IL-21 which are required by the B cells to proliferate and differentiate into plasma cells (Janeway *et al.*, 2005). High-affinity B cells that have a potential to bind self protein determinants remain tolerant because they lack T cell help (Goodnow *et al.*, 2005). Furthermore, B cells that present self antigens to $CD4^+$ T cells deliver a death signal via the Fas ligand on T cells, and are thus eliminated from the periphery by apoptosis (Vignaux and Golstein, 1994). However, autoreactive B cells are often

generated during a response to foreign antigens (Nossal, 1991). For this reason B cells in germinal centres undergo V(D)J rearrangement (Han *et al.*, 1997). This has a dual role resulting in 1) homing of receptors towards foreign antigens and 2) elimination of self-reactive clones (Rice *et al.*, 2005).

1.5 Tumour immunology

The observation that some cancer patients undergo spontaneous remission shows that the immune system is capable of recognising and eradicating tumours (Espinoza-Delgado, 2002). Immunity to cancer is complex, because it does not conform to the self/nonself paradigm (Houghton and Guevara-Patiño, 2004). A majority of tumour associated antigens are self or mutated self proteins of which the immune system is tolerant (Mapara and Sykes, 2004). Therefore, the focus of tumour immunology is to break tolerance towards self tissues by converting tolerant lymphocytes (T and B cells) into tumour killing effector cells (Espinoza-Delgado, 2002). The greatest challenge, however, is to overcome possible deleterious effects of an autoimmune disease that may be induced concomitantly with anti-tumour responses. A number of tumour vaccine strategies have been devised and tested both in pre-clinical and clinical studies. The immunological basis of such strategies will be discussed below.

Induction of a tumour killing response is further complicated by tumour counterattack, whereby tumours employ several mechanisms to evade the immune system (Ribas *et al.*, 2003). Such mechanisms have well been documented (Espinoza-Delgado, 2002; Ribas *et al.*, 2003; Mapara and Sykes, 2004; Munn and Mellor, 2004) and include the following:

- Tumours downregulate antigen expression or processing and presentation by MHC molecules.
- Tumour derived cytokines such as IL-10, transforming growth factor β (TGF-β) and prostaglandins block APC maturation and differentiation. In addition, decreased expression of co-stimulatory molecules on APCs results in suboptimal presentation of antigens.
- Tumour cells produce factors that interfere with lymphocyte function (Fas ligand).

- Expression of the immunosuppressive enzyme, indoleamine-2,3-dioxygenase (IDO) protects tumours against rejection, because T cells are unable to proliferate in the presence of IDO.
- Finally, suppression of lymphocytes by regulatory T cells

In general tumours create a microenvironment that is antagonistic to immunological processes that would otherwise clear out "infections".

1.5.1 Tumour vaccine strategies

A number of infectious diseases have been eradicated by vaccinations with attenuated forms of disease-causing microorganisms. The success of this approach is attributed to the ability of the immune system to form long-lived memory cells that are more effective in clearing out infections than naïve cells (Janeway *et al.*, 2005). Immunotherapy has been centred on creating an environment that enhances antigen presentation to naïve T cells (King, 2004).

Irradiated tumour cells (autologous or allogeneic) have been used in combination with strong adjuvants, so that tumour cells may be presented in an inflammatory context to attract host APCs (Ribas *et al.*, 2003). Using whole tumour cells delivers all the relevant tumour antigens to the immune system, so that it can mount up an effective antitumour response (Espinoza-Delagado, 2002). This type of approach has failed to eradicate tumours in clinical trials and it has been limited further by the fact that tumour samples are difficult to obtain (autologous vaccines) and that not all tumours express the same profile of antigens (allogeneic vaccines) (King, 2004). Berd (2001; 2003) described a vaccine strategy using hapten modified intact autologous tumour cells. Hapten modification of antigens has been shown to augment a response towards weakly immunogenic antigens (Weigle, 1965). In combination with a strong adjuvant, this type of approach has a capacity to induce a tumour killing response, but, in clinical settings, tumour regression was not significant (Berd, 2001; 2003).

Whole cell tumour vaccines have been improved by transfecting tumour cells with cytokine genes (Berzofsky *et al.*, 2004). Dranoff *et al.* (1993) showed that irradiated tumour cells expressing GM-CSF (granulocyte-macrophage colony stimulating factor), protected mice against tumour challenge with the same tumour type. The expression of GM-CSF or other cytokines such as IL-2 or interferon-γ (INF-γ), recruits DCs and other host APCs which effectively prime naïve T cells (Berzofsky *et*

al., 2004). The vaccine was tested in a phase I trial in patients with pancreatic cancer, where three out of fourteen patients remained disease free at least 25 months after diagnosis (Jaffee *et al.*, 2001).

Professional APCs such as DCs are powerful stimulators of naïve T cells (Janeway *et al.*, 2005). Berzofsky *et al.* (2004) pointed out that in tumour-bearing hosts, DCs show maturation and functional defects. *In vitro* generated DCs loaded with tumour protein extracts (Paglia *et al.*, 1996; Zitvogel *et al.*, 1996), synthetic peptide tumour epitopes (Mayordomo *et al.*, 1996) or DCs fused with irradiated tumour cells (Berd, 2001) have been found to successfully prime cytotoxic T lymphocytes that lyse target cells. In phase I and phase II clinical trials, vaccination with DCs loaded with melanoma antigens resulted in 9.5% tumour regression, where 11 out of 116 patients responded (Rosenberg *et al.*, 2004).

Induction of specific $CD8^+$ T cells has been shown to be crucial in anti-tumour responses (Tsomides and Eisen, 1994; Gollob *et al.*, 1998; Pardoll, 1999), because tumours are generally MHC class I^+ and class II^- (Gerloni and Zanetti, 2005). In murine models, activation of anergic $CD8^+$ T cells that escaped thymic deletion protected mice against tumour challenge, but did not result in autoimmunity (Morgan *et al.*, 1998; Cordaro *et al.*, 2002). However, these responses depend on interactions with other cell types such as $CD4^+$ T cells and APCs (Rosenberg *et al.*, 2004). Marzo *et al.* (2000) proposed that $CD4^+$ T cells are important in maintaining the CTL pool and function through the CD40L on $CD4^+$ T cells which cross-links the CD40 receptor on APCs.

Humoral responses in tumour immunotherapy have received less attention. These responses are limited by the fact that target proteins need to be expressed at the cell surface (Banchereau and Palucka, 2005). Generation of specific self reactive antibodies has been demonstrated in several models (Weigle, 1965; Lin *et al.*, 1991; Dalum *et al.*, 1996; Chakerian *et al.*, 2001). However, it has not been demonstrated how antibodies generated in this manner could effect tumour regression. Monoclonal antibodies have been used alone or used as vehicles that deliver conjugated toxins or other tumour killing agents to the tumour site (Davis *et al.*, 2003). Monoclonal antibodies that are used alone are mostly directed at cell surface targets where they induce antibody dependent cytotoxic cell lysis (Davis *et al*, 2003). In a melanoma

model, antibodies generated against MCAM/MUC18, a cell surface molecule expressed by malignant melanoma cells inhibited tumour invasion and metastasis in mice bearing melanoma cells (Mills *et al.*, 2002).

Tumours create for themselves a microenvironment that supports and propagates their growth. The success or failure of cancer vaccines is determined by the cellular interactions that occur within the tumour microenvironment. Tumour progression is associated with chronic inflammation, recruiting neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes to the site of the tumour (Coussens and Werb, 2002). It has been observed that many malignancies arise from areas of infection and inflammation, which occur as part of protective mechanisms against pathogens (Ben-Baruch, 2005). Inflammatory cells induce DNA damage and cell transformation, leading to cancer (Coussens and Werb, 2002). Normal inflammation, however, is self-limiting because the production of pro-inflammatory cytokines is immediately followed by anti-inflammatory cytokines, but dysregulation of any of the associated factors can lead to pathogenesis (Coussen and Werb, 2002). Tumour progression is often associated with immunosuppression; therefore, effective tumour vaccines should modify the tumour microenvironment in favour of tumour-killing responses.

1.5.2 The link between innate and adaptive immunity

Historically, a clear distinction was made between innate and acquired immunity. In immunological studies, these two arms of the immune system have been largely treated as independent entities. However, recent research has shown that innate and adaptive responses are tightly interwoven. Adaptive immunity has a very high specificity for antigens, but the initiation of a response is largely dependent upon innate immune recognition. This dependence is a consequence of a need for antigen presentation, a function that is performed by antigen presenting cells (APCs) (Hoebe *et al.*, 2004).

The innate immune system forms the first line of defence against invading pathogens and in most cases these responses effectively clear the infection without the adaptive immune response being triggered. However, in some instances, the innate immune system becomes overpowered and as the pathogen multiplies within the host, the adaptive immune effectors are deployed to clear the infection. Effective immune

responses thus require both arms of the immune system. There are a number of ways in which the innate and adaptive immune systems interact to mount a response against invading pathogens.

1.5.3 Requirement for adjuvants

Successful priming of adaptive responses requires initial co-inoculation of the immunogen with heat killed microbial elements (Freund's complete adjuvant) (Schnare *et al.*, 2001). The importance of innate immune signalling for activation of adaptive immune responses is now recognised. Toll-like receptors provide an essential link between the innate and adaptive responses mainly by making antigen presentation effective.

The most important phase in generating antibody responses against poorly immunogenic antigens such as self antigens is the development of antigen-specific T cell help (McHeyzer-Williams *et al.*, 2003). Dendritic cells (DCs) play a central role in T cell activation and differentiation into T helper (Th) and cytotoxic T lymphocytes (CTL) (Bancehereau and Steinman, 1998).

Toll-like receptors (TLRs) are a family of transmembrane receptors which belong to the interleukin-1 receptor superfamily (Medzhitov *et al.*, 1997). TLRs recognise pathogen-associated molecular patterns (PAMPs) which are highly conserved components of microbial origin that are essential for microbial survival (Xu *et al.*, 2005). Different subsets of dendritic cells (DC) express TLRs (Iwasaki and Medzhitov, 2004), which upon binding to pathogens, induce DC maturation. DCs capture microbial antigens in peripheral tissues and migrate to the draining lymph nodes to present the processed peptides to naïve T cells in the context of MHC molecules (Iwasaki and Medzhitov, 2004).

T cells require two signals from DCs for optimal activation; the first signal is antigen-specific and is provided by binding of T cell receptor (TCR) to the peptide presented by the MHC molecule, while the second signal is delivered through ligation of co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86) on DCs with CD28 on T cells (Janeway *et al.*, 2005). Resting DCs do not have the capacity to activate T cells; they acquire this ability after undergoing a maturation process (Iwasaki and Medzhitov, 2004). Therefore, binding of TLR ligands on DCs induces up-regulation of both MHC and co-stimulatory molecules, which lead to DC maturation and

cytokine expression. DCs are also important in overcoming suppression mediated by regulatory T (Treg) cells (Pasare and Medzhitov, 2004a; 2004b). The mechanism by which DCs block T regulatory cell immune suppression is mediated by IL-6, which is produced by DCs in response to TLR ligation and helps pathogen-specific T cells to overcome the suppressive effects of T regulatory cells (Pasare and Medzhitov, 2003b). IL-6 does not affect the function of T regulatory cells, which is necessary to prevent bystander T cell activation (Pasare and Medzhitov, 2003a). However, if high affinity self peptides are presented at the same time on DCs so that they come in contact with self reactive T cells, suppression is overcome because the presence of DC-derived cytokines favour activation and hence autoimmunity develops (Pasare and Medzhitov, 2003a).

1.5.4 Effector cells of the immune system

One of the biggest challenges facing the development of cancer vaccines is the type of response for which vaccines should aim. CTL responses were regarded to be superior to any other type of response, because most tumours were found to be infiltrated with tumour infiltrating lymphocytes (TILs). Furthermore, it was argued that solid tumours are sequestered from the action of neutralising antibody and target antigens should be localised to the cell surface (Tsomides and Eisen, 1994). It is well established that $CD4^+$ T cells augment and sustain the effector mechanisms of $CD8^+$ T cells. However, the direct tumour killing effect of $CD4^+$ T cells is now being revealed (Corthay *et al.*, 2005).

Vaccines mainly target adaptive immune responses, because of the diverse range of antigens that can be recognised. However, these responses require initial signalling that is provided by the innate immune system and is often induced by including adjuvants which serve to elicit inflammatory responses that recruit innate immune cells including APCs. The cells of the innate immune system detect pathogens by germline encoded receptors called pattern recognition receptors (PRRs) (Iwasaki and Medzhitov, 2004), which can recognise a limited number of antigens. Effective immune responses result from recruitment, activation and coordination of relevant immune effector cells. With regard to cancer, NK cells and T lymphocytes have been considered to be potent effectors of tumour regression (Tsan, 2005).

1.5.4.1 T lymphocytes

Many human cancers are infiltrated with T cells (tumour infiltrating lymphocytes; TILs) (Tsomides and Eisen, 1994). Two major T cell subsets, $CD8^+$ T cells and $CD4^+$ helper T cells comprise a population of TILs within the tumour mass (Chiou *et al.*, 2005). Although TILs are found in large numbers in the tumour microenvironment, they fail to control tumour growth and spread (Chiou *et al.*, 2005). Moretta (2005) pointed out that T cell function may be more relevant than T cell numbers in effecting tumour regression and, therefore, it may be important to understand the mechanisms that underlie T cell function in order to design effective tumour vaccines. Coulie and Connerotte (2005) reviewed a number of clinical studies and observed that in most instances the frequency of tumour-specific T cells is correlated with therapeutic efficacy of the vaccine. However, some patients have strong anti-vaccine T cell responses without detectable clinical benefit, which may suggest that other factors are important in initiating tumour rejection (Coulie and Connerotte, 2005). Lonchay *et al.* (2004) observed that some patients display tumour regression with no detectable anti-vaccine T cells, which implies that the vaccine may modify an immunosuppressive environment within the tumour, creating conditions that favour activation and proliferation of T cells directed against other tumour antigens (Coulie and Connerotte, 2005).

Since most tumours are MHC class I positive and MHC class II negative, the role of T cells in tumour immunity has largely been attributed to $CD8^+$ T cells (Gerloni and Zanetti, 2005). Activated $CD8^+$ T cells differentiate into effector cytotoxic lymphocytes (CTL) which induce lysis of target cells (Janeway *et al.*, 2005) or employ indirect killing mechanisms that involve IFN-γ (Schüler and Blankenstein, 2003). The direct tumour killing effects of $CD4^+$ helper T (Th) cells are not well characterised, but it has become evident that the role of Th cells extends beyond providing help for $CD8^+$ T cells (Knutston and Disis, 2005). Upon activation, $CD4^+$ T cells differentiate into two major subsets depending on the signal they receive from APCs, mainly T helper 1 (Th1) and Th2 cells (Janeway *et al.*, 2005). Th1 cells produce cytokines such as IL-2, IL-12 and IFN-γ and direct CTL type of responses, while Th2 cells produce IL-4 and IL-5 which induce production of IgE antibodies and the IgG_1 subclass (McHeyzer-Williams *et al.*, 2003). Hung *et al.* (1998) proposed that $CD4^+$ T cell responses against tumours could be mediated by delayed-type

hypersensitivity (DTH)-like reactions, attracting inflammatory cells like macrophages, granulocytes, eosinophils or NK cells to the vicinity of the tumour. This type of response was also demonstrated by Corthay *et al.* (2005).

$CD4^+$ T cells interact with tumour-specific CTL in a number of ways that enhance tumour-killing properties of CTL. Using a mouse model, Marzo *et al.* (2000) showed that $CD4^+$ T cells are important in maintaining the CTL pool and function, and allowing $CD8^+$ T cells to infiltrate the tumour. Cytokines such as IFN-γ, produced by Th cells, alter tumour expression of key MHC molecules and also activate APCs to upregulate MHC class I molecules thus contributing to increased antigen presentation to $CD8^+$ (Früh and Yang, 1999; Surman *et al.*, 2000). Th1 cells induce the production of opsonising antibodies that enhance the uptake of tumour cells by APCs (Hung *et al.*, 1998). In addition to maintaining the CTL pool in the initial effector phase, antigen-specific Th cells are required for reactivation of memory $CD8^+$ T cells (Gao *et al.*, 2002). In general Th cells can modulate anti-tumour responses (Fig. 5.1).

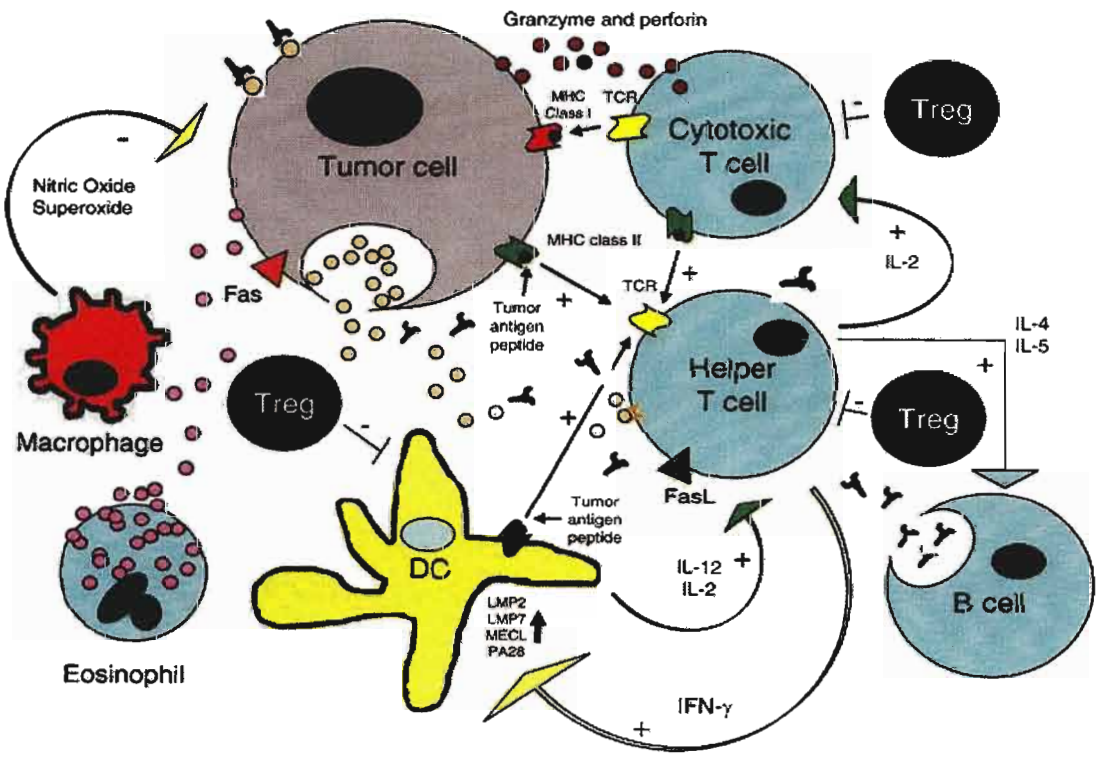

Figure 1.3 The role of T helper cell in tumour responses.
The Th cell plays an extensive role and is able to interact with the tumour cell and a number of immune effectors through contact dependent or contact independent mechanisms. This simplified diagram omits many of the Th cell roles including several types of co-stimulatory molecules. A *plus sign* indicates a potentially positive impact on the antitumour response while a *negative sign* indicates a detrimental effect. T regulatory cell (Treg). (Knutson and Disis, 2005).

$CD4^+$ T cells also direct B cell responses by inducing their differentiation into Ig secreting plasma cells (Janeway *et al.*, 2005). Antigen activated APCs prime naïve Th cells, which in turn expand and differentiate into effector cells that regulate the differentiation and functional status of antigen primed B cells (McHeyzer-Williams *et al.*, 2003). Development of plasma cells and memory B cells has been described as a serial synapsis model, which involves three major phases (McHeyzer-Williams *et al.*, 2003) as shown in Fig. 5.2. Phase I is particularly important, because the interactions that occur there can impact on the quality of subsequent B cell responses (McHeyzer-Williams, 2005). This implies that in breaking tolerance towards self proteins, the type of T cell antigen can determine the outcome of the response.

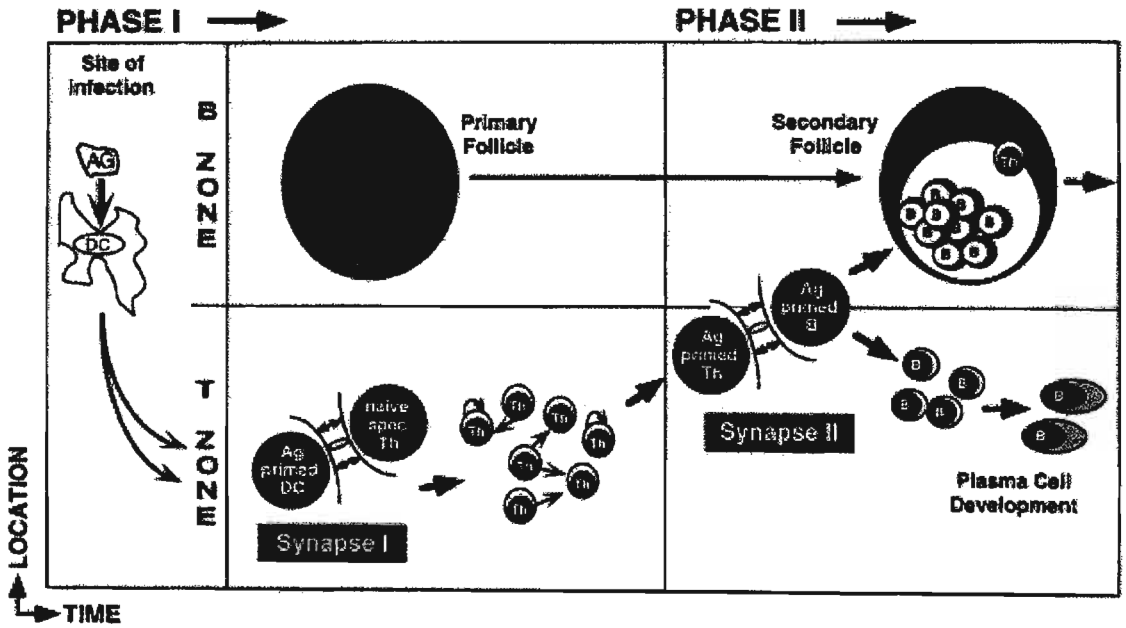

Figure 1.4 The serial synapsis model, phases I and II.
Phase I begins at the site of antigen entry with activation of local APCs (DCs as the most efficient local APCs), antigen uptake, processing and presentation of antigenic peptides by these DCs and migration to the T-cell zones of draining secondary lymph node (LN) tissue. Synapse I represents the first contact between the activated DCs and naïve Th cells with specific TCRs. Subsequent clonal expansion and differentiation of the Th cells is critically influenced by the cell surface phenotype of the activated DCs and the soluble cytokines it produces. Autocrine and paracrine effects of Th cell-derived cytokines can also influence commitment to function among the antigen-specific Th cells. Migration of expanded Th cells to the follicular borders of the LNs signifies the end of Phase I prior to Th-B-cell interactions. Antigen-specific B cells must encounter antigen, process and present antigenic peptides in the context of MHC class II to initiate cognate T-cell help in Synapse II and progress the immune response to Phase II. Multiple Th cell subsets with differing cell surface phenotype and cytokine-producing profiles can influence the cellular outcome of Synapse II. B cells in the non-GC plasma cell pathway remain in the T-cell zones, can undergo isotype-switch but do not somatically diversify their B cell receptors (BCRs). B cells that move into the follicular areas rapidly expand to form secondary lymphoid follicles (McHeyzer-Williams *et al.*, 2003).

Induction of immune responses against self antigens is further hampered by immunosuppressive activities of T regulatory cells (Tregs). About 5-10% of $CD4^+$ T lymphocytes ($CD25^+CD4^+$ T regulatory cells) suppress T cell activation and expansion of potentially self-reactive T cells normally present in the immune system (Sakaguchi, 2005). Tregs are produced in the thymus as functionally distinct and mature T cell subpopulation (Sagaguchi, 2004). Mechanisms of Tregs suppression are poorly understood, but it has been postulated that T cell receptor (TCR) stimulation and IL-2 (Thornton, 2004) are essential for their suppressor function. An elevated proportion of $CD25^+CD4^+$ Treg cells in the total $CD4^+$ T cell population has been demonstrated in different cancers (Woo *et al.*, 2001; Liyanage *et al.*, 2002; Curiel *et al.*, 2004). In tumour bearing mice depletion of $CD25^+CD4^+$ T cells was shown to elicit effective immune responses to syngeneic tumours in otherwise nonresponding mice (Shimizu *et al.*, 1999).

Effective anti-tumour responses should, therefore, overcome suppressive effects of T regulatory cells. In immune responses against pathogens, Tregs suppress activation of bystander T cells (Fehérvari and Sakaguchi, 2004). Depletion of Tregs in normal mice resulted in various autoimmune diseases (Wang and Wang, 2005) indicating that these cells have an essential role in controlling immune responses. With regard to B cell responses, Lim *et al.* (2004) demonstrated that Tregs can migrate to follicles where they suppress germinal center T helper cells, resulting in reduced Ig production. Janssens *et al.* (2003) also showed that $CD25^+CD4^+$ T cells can directly inhibit B cell responses by killing antingen presenting B cells via Fas mediated lysis. Dendritic cells (DCs) have been shown to produce cytokines that help T cells overcome suppressive effects of Tregs (Sakaguchi, 2005). Presenting self antigens to the immune system in the presence of strong adjuvants such Freund's complete adjuvant creates an environment that favours DC maturation and expression of costimulatory molecules, which increase their capacity to activate naïve T cells. To further augment responses towards self antigens, cytokines or chemokines that are capable of reversing the suppressive function of Tregs can be used as adjuvants (Wang and Wang, 2005).

1.5.5 Should the immune system elicit a response against tumours?

One of the longstanding immunological paradigms is the self/nonself hypothesis, which envisages that the immune system is tolerant to 'self', but responds to foreign entities (nonself) (Janeway *et al.*, 2005). In the context of cancer, the immune surveillance hypothesis proposed that tumours arise with similar frequency to infections and that the immune system is constantly activated by tumour-associated antigens and eliminates these tumours (Pardoll, 2003). This view was challenged when studies done in mice revealed that immunosuppressed individuals were not predisposed to tumour development (Pardoll, 2003). Tumours arise from normal body cells and, therefore, should not trigger an immune response because they are a component of self (Anderson and Matzinger, 2000). However, it could be argued that because some tumours express new antigens, they should trigger a response, which will be contrary to the natural processes of life, where new proteins are expressed as the body develops into puberty and eventually ages (Matzinger, 2001).

Janeway (1992) proposed that the main task of the immune system is to discriminate infectious self from non-infectious self. According to Medzhitov and Janeway (2000) the innate effector cells are endowed with pathogen recognition receptors (PRRs) which distinguish non-infectious self from infectious nonself. This innate recognition signals infection to the adaptive immune system by presenting pathogen antigens to lymphocytes which have more specific receptors and thus ensure destruction of the pathogen and protection of host cells (Medzhitov and Janeway, 2000). In light of this view, tumours need not trigger a response because there are no infectious elements that can activate resting APCs (Matzinger, 1998).

The view that the immune system responds to antigens on the basis of foreignness was challenged when Matzinger (1994) proposed the danger model; suggesting that the immune system is more concerned with damage and destruction than with foreignness. It is well established that B cells require two signals for activation, signal one being antigen recognition by the BCR and signal two being T cell help (McHeyzer-Williams *et al.*, 2003). In addition, Th cells are stimulated by recognition of antigen in association with MHC molecules (signal one) and co-stimulation from APCs (signal two) (Tsomides and Eisen, 1994). Anderson and Matzinger (2000) argued that if APCs have such a vital role in priming the effector cells of the adaptive immune system, how do they distinguish self from nonself? Janeway (1992)

proposed that APCs express toll-like receptors (TLRs) which bind pathogen associated molecular patterns (PAMPs) thus inducing APC maturation. Contrary to this view, Matzinger (1994) proposed that APCs are activated by endogenous cellular alarm signals from distressed or injured cells.

Therefore, on the basis of Matzinger's danger model, the immune system should not elicit a response against tumours, because cancer cells do not send any alarm signals to the resting APCs (Fuchs and Matzinger, 1996). Furthermore, Matzinger (1998) pointed out that as tumours grow they induce tolerance to themselves in the way that normal tissues do, by offering signal 1 without 2. Therefore, by the time tumour cells migrate and cause damage, the relevant T cells have been tolerised (Pardoll, 2003).
The basis of the danger model is that the ultimate controlling signals that drive immunological responses are endogenous rather than exogenous; they are the alarm signals delivered by stressed or injured tissues (Matzinger, 2001). This paradigm shift may have several implications for anti-tumour vaccine design. Matzinger (1998) pointed out that a response against antigen is sustained only when the alarm signal continues. In the case of infections the response will be sustained until the pathogen is cleared, but with cancer, as damage caused by the vaccine heals, the response eventually wanes, whether or not the tumour has been cleared (Kowalczyk, 2002). Therefore, in breaking tolerance towards 'self' proteins, the deleterious effects of immune induced tissue damage will be short-lived.

1.6 Study design

Mechanisms that render B and T cells tolerant to self proteins are not rigorous, so that potentially autoreactive cells form part of the lymphocyte repertoire. Thymus-dependent antibody responses require activation of B cells by T helper cells that respond to the same antigen (Goodnow, 1996). This mechanism, called linked recognition is crucial in antibody responses because it augments such responses and helps maintain self-tolerance (Janeway *et al.*, 2005). In the absence of T cell help, B cell stimulation is a negative signal for B cells and leads to Fas mediated cell death (Gavin *et al.*, 2003). B cells fail to elicit a response against haptens but if T cell help is provided in the form of additional antigenic determinants conjugated to the hapten, antibodies against the hapten can be generated (Janeway *et al.*, 2005). For self proteins, T cell help has been provided in different ways. Weigle (1965) induced a

sustainable response against thyroglobulin in rabbits by immunisation with diazonium-coupled thyroglobulin. In another study, mouse ubiquitin was modified by replacing a small segment of the protein with a foreign T cell epitope (OVA 325-336). This modified ubiquitin induced a sustainable response against native ubiquitin in mice (Dalum *et al.*, 1996). Chackerian *et al.* (2001) generated high avidity autoantibodies against mouse TNF-α that was arrayed on papillomavirus viral-like particles (VLPs).

During an immune response to foreign antigens, B cells hypermutate the V genes in order to improve on their specificity (Nossal, 1991). One consequence of somatic hypermutation is that, by chance, the anti-foreign specificity may be lost, but reactivity to a self antigen may be gained by accident (Nossal, 1991). As demonstrated by Lin *et al.* (1991), in their model study using cytochrome c, the chances of gaining reactivity to a self antigen increase if there is antibody cross-reactivity between the two proteins (also demonstrated by Weigle, 1965).

The aim of this study was to break tolerance to a lysosomal cysteine proteinase, cathepsin L, and generate high avidity long-lasting antibodies in rabbits. Six rabbits were immunised in the following manner: Two rabbits were injected with a (rabbit) cathepsin L-(bovine) α-glucosidase conjugate, two other rabbits were primed with sheep cathepsin L followed by booster injections with rabbit cathepsin L, and two control rabbits were immunised with sheep cathepsin L throughout the duration of the protocol. Rabbits that had been immunised with the conjugate were injected with sheep cathepsin L nine weeks after the final booster injection. In this study T cell help was provided mainly by bovine α-glucosidase and sheep cathepsin L, but presented to the immune system in three different ways. Anti-cathepsin L antibodies generated in this manner may inhibit tumour invasion and metastasis and this will have to be tested in future studies. In this laboratory, previous attempts to raise autoantibodies against cathepsin B in rabbits were not successful (Moolman, 2000).

CHAPTER 2
MATERIALS AND METHODS

In this chapter fundamental biochemical methods that were routinely used throughout the study are described. Specialised methods are described in appropriate chapters. Included here are techniques considered to be more general and which would otherwise negatively impact the structure of the relevant chapter.

2.1 Materials

Most common chemicals used in this study were of the highest purity available and were obtained from BDH, Merck or Sigma. Dialysis tubing, dithiothreitol (DTT), S-Sepharose slurry, Sephadex G-25, Sephadex G-75, Sephadex G-100, cysteine.HCl, Coomassie brilliant blue R-250, 7-amino-4-methyl coumarin, Z-Phe-Arg-NHMec, Z-Arg-Arg-NHMec, 4-methylumbelliferone, 4-methylumbelliferyl-α-glucoside, N, N^{l}-methylbisacrylamide, Freund's complete and incomplete adjuvants, goat anti-rabbit IgG alkaline phosphatase conjugate were from Sigma (St. Louis, Mo. USA). Gluataraldehyde was from Fluka (Buchs, Switzerland). Bovine serum albumin (BSA) was from Boehringer Mannheim. Acrylamide and N, N, N′, N′-tetramethylethylenediamine (TEMED) were from BDH. Imidazole was from Merck (Germany). Serva blue G dye was from Serva (Germany). Nunc-immunosorp F96 plates were from AEC Amersham (SA). NBT and BCIP were from Roche diagnostics (Mannheim, Germany). p-Nitrophenylphosphate was from BDH.

2.2 Protein assays

Routine quantification of protein samples was necessary throughout the study to assess purification efficiency. The Bradford dye-binding assay (Bradford, 1976) is a rapid and sensitive technique that is resistant to interference by most common laboratory chemicals. The procedure modified by Read and Northcote (1981) was used in this study.

2.2.1 Bradford dye-binding assay

The Bradford dye-binding assay is based on the binding of Coomassie blue dye to basic amino acid side chains. Coomassie blue G-250 is a triphenylmethane dye (Sapan *et al.*, 1999) which exists in three forms at acid pH, the cationic red form, an anionic blue form and a neutral green form (Zor and Selinger, 1996). The anionic blue form of the dye binds to proteins to form a complex that absorbs light intensely

at 595 nm (Chial and Splittgerber, 1993; Congdon *et al.*, 1993). Dye binding is effected by electrostatic interactions that occur between positively charged arginine and lysine side chains (Congdon *et al.*, 1993; Chial and Splittgerber, 1993) and the dye's sulfonic acid groups (Compton and Jones, 1985). However, hydrophobic interactions of the dye with adjacent tyrosine, tryptophan, and phenylalanine residues have also been implicated (Tal *et al.*, 1985).

Selective binding of dye to arginine and lysine side chains causes colour variability among different proteins (Compton and Jones, 1985), because the arginine and lysine contents of proteins vary. This colour variability is the major limitation of the Bradford assay. Several modifications of the original Bradford method have been published. Read and Northcote (1981) used Serva blue in place of Coomassie blue and changed the dye, acid and ethanol concentrations. This modification brought the response curves of other proteins closer to that of the BSA standard. Zor and Selinger (1996) suggested that the ratio of the absorbances at 590 nm and 450 nm improves the linearity of the assay system, and, therefore, enhances the assay sensitivity. This approach gives a linear response in the range 0-20 µg when using BSA as standard. However, taking readings at two different wavelengths is tedious and time consuming, while for most applications a single reading at 595 nm suffices.

Each modification of the Bradford assay does not perfectly reduce the variability of protein response. Therefore, a protein standard that gives a colour yield that is similar to that of test proteins can be used for any given application. Despite this major limitation, the Bradford assay is convenient and provides a good estimation of protein quantity in crude and purified samples. In addition, it is economical (only small amounts of sample are used), rapid, simple to perform and essentially resistant to interference by most laboratory reagents, with the exception of high concentrations ($\geq$1%) of detergents.

2.2.1.1 Reagents

Dye reagent. Serva blue G dye (50 mg) was dissolved in 88% phosphoric acid (50 ml) and 99.5% ethanol (23.5 ml). The solution was made up to 500 ml with dist.H_2O and stirred for 30 min. The resulting solution was filtered through Whatman No. 1 filter paper and stored in an amber-coloured glass bottle. The solution was checked

for precipitation before use, and if precipitation was observed a new batch of reagent was made up.

Standard protein solution. A 0.1 mg/ml ovalbumin solution was made up in dist.H_2O. This solution was diluted to100 μg/ml for the micro-assay.

2.2.1.2 Procedure

Micro-assay. Protein standard or samples were diluted to 50 µl with dist.H_2O or buffer, respectively, in 1.5 ml microfuge tubes. Dye reagent was added and the solution was mixed by inversion of the tube and colour was developed for 2 min. The absorbance was read at 595 nm in 1 ml plastic cuvettes against appropriate blanks. A standard curve from 0-50 µl (0-5 µg) of the standard protein solution was constructed. Assays were carried out in triplicate and the amount of protein was calculated from the equation generated by linear regression analysis of the standard curve from 0-50 μl (0-5 μg) of the standard protein.

2.3 Concentration of protein samples by dialysis against polyethylene glycol

A consequence of column chromatography is dilution of protein samples. Dialysis of samples against polyethylene glycol (PEG, Mr 20 000) is a simple inexpensive method of concentrating large volumes of dilute protein samples. Protein solution was placed in a dialysis tubing with a molecular weight cut-off of 12-kDa and dialysed against PEG. PEG has a high osmotic pressure when in solution, which results in a movement of water and buffer ions down their concentration gradient established between the protein solution within the dialysis tubing and PEG at the exterior surface of the membrane. The net effect is gradual reduction of water inside the tubing, thus concentrating the protein retained in the bag. When the desired volume of protein solution has been reached, the process can be stopped.

Alternatively, sucrose can be used in place of PEG. However, a disadvantage of sucrose is that it is small enough to diffuse into the dialysis tubing, contaminating the protein sample. However, for many applications, this is of no moment.

2.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis describes the migration of charged molecules in an electric field. The size and purity of protein samples were assessed by SDS-PAGE under reducing and non-reducing conditions.

The use of polyacrylamide as a medium for gel electrophoresis was introduced by Ornstein (1964). The gel is formed by free radical polymerisation of acrylamide monomer units cross-linked with bisacrylamide, resulting in a uniform pore size. Cross-linking gives the gel its mechanical stability. The pore size of the gel can be varied by changing the proportions of acrylamide and bisacrylamide. Ornstein (1964) and Davis (1964) developed a discontinuous gel system consisting of two gels of different porosities, a non-restrictive large pore gel (stacking gel) and a smaller pore separating gel (resolving gel). The two buffer system employed consists of chloride ions in the sample and stacking gel, and glycine in the electrode buffers. At pH 6.8, glycine (trailing ion) has a slight negative charge and its mobility is low compared to that of the chloride ion (leading ion). The sample protein has an intermediate mobility and is sandwiched between the two ions, generating a sharp interface of proteins between the leading ions and the trailing ions according to Kohlrausch's regulatory function (Blackshear, 1984). Once the proteins reach the resolving gel, the glycine ions move faster, because of the increased pH, and hence overtake the proteins to form a front with chloride ions and the tracking dye. Proteins are left to migrate in a uniform voltage where they are separated according to size, charge and shape.

The use of sodium dodecyl sulfate (SDS), an anionic detergent, in PAGE allows for molecular weight determination (Shapiro *et al.*, 1967). Protein molecules bind SDS at a constant weight ratio of 1.4 g to 1 g of protein, resulting in rod-like complexes that have a net negative charge imparted by the SDS (Reynolds and Tanford, 1970). These complexes have the same charge to mass ratio and are, therefore, separated on the basis of size only in an electric field. Protein subunits can be resolved by boiling the sample in excess of SDS, together with a reducing agent such as 2-mercaptoethanol which breaks the disulfide bonds and destroys tertiary and quaternary structure. There is a linear relationship between the $\log_{10}$ of the molecular weight of SDS/protein complex and their R_m (ratio of the distance migrated by the protein to that migrated by a marker dye). By constructing a standard curve of log MW vs R_m of each protein standard, the molecular weight of the unknown protein can be determined by interpolation.

In this study, SDS-PAGE was conducted using modifications of the methods of Laemmli (1970), Ornstein (1964) and Davis (1964). After electrophoresis, gels were stained by methods described in Section 2.5.

2.4.1 Reagents

Solution A: Monomer solution [30% (m/v) acrylamide, 2.7% (m/v) bis-acrylamide]. Acrylamide (14.6 g) and bis-acrylamide (0.4 g) were dissolved in 40 ml dist.H_2O, adjusted to pH 8.8 with HCl and made up to 50 ml with dist.H_2O.

Solution B: 4 x running gel buffer [1.5 M Tris-HCl, pH 8.8]. Tris (9.07 g) was dissolved in approximately 40 ml dist.H_2O, adjusted to pH 8.8 with HCl and made up to 50 ml with dist.H_2O.

Solution C: 4 x stacking gel buffer [500 mM Tris-HCl, pH 6.8]. Tris (3.0 g) was dissolved in 40 ml dist.H_2O, adjusted to pH 6.8 with HCl and made up to 50 ml with dist.H_2O and stored at 4°C. A fresh solution was made up every two weeks.

Solution D [10% (m/v) SDS]. SDS (10 g) was dissolved in 100 ml dist.H_2O.

Solution E: Initiator [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was dissolved in 1 ml dist.H_2O just before use.

Solution F: Tank buffer [250 mM Tris, 192 mM glycine, 0.1% (m/v) SDS, pH 8.3]. Tris (3.0 g) and glycine (14.4 g) were dissolved in 1 L dist.H_2O. Just before use, SDS stock (solution D) (2.5 ml) was added to 250 ml of the buffer.

Solution G: Reducing treatment buffer [125 mM Tris-HCl buffer, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.02% bromophenol blue, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4 ml) (solution D), glycerol (2.0 ml), 2-mercaptoethanol (1 ml) and bromophenol blue (0.002 g) were made up to 10 ml with dist.H_2O.

Solution H: Non-reducing treatment buffer [125 mM Tris-HCl buffer, 4% (m/v) SDS, 20% (m/v) glycerol, 0.02% bromophenol blue, pH 6.8,]. Buffer C (2.5 ml), 10% SDS (4 ml), glycerol (2.0 ml) and bromophenol blue (0.002 g) were made up to 10 ml with dist.H_2O.

2.4.2 Procedure

The Hoefer SE 250 Mighty Small II vertical slab electrophoresis unit was assembled as described in the manufacturer's manual. For each gel sandwich, the notched

aluminium plate, glass plate, 1.5 mm spacers and combs were washed thoroughly with detergent, rinsed with water and cleaned with 70% ethanol. The notched aluminium plate and glass plate were clamped together with two 1.5 mm polyethylene spacers separating them at the edges.

The running gel solution (prepared as described in Table 2.1) was poured slowly into the space between the plates, to a depth of 3 cm from the top of the glass plate and overlayered with dist.H_2O. An interface became visible between the gel solution and water once the gel had set (about 1 h). The water was removed by inversion and stacking gel solution (Table 2.1) was poured up to the notch of the aluminium plate. Immediately, a 10 or 15 well comb was inserted to form sample wells. Once the gel had set (about 30 min), the comb was removed and the wells were rinsed with dist.H_2O. The gel sandwich was transferred to a 250/260 SE mighty small gel electrophoresis unit. Tank buffer was poured into both the upper and lower chambers of the unit.

Samples were combined with an equal volume of treatment buffer, boiled for about 2 min in a water bath and placed on ice until loaded onto the gel. For non-denaturing SDS-PAGE, samples were mixed with an equal volume of non-reducing treatment buffer before loading onto the gel. Specified amounts of samples were applied into the wells. The gel unit was connected to a power supply and run at 18 mA per gel and 400 V until the bromophenol dye front was about 5 mm from the bottom of the gel. At this point the gel unit was disconnected from the power supply, the plates were removed and the gel was rinsed with dist.H_2O before staining the gel using an appropriate method described in Section 2.5.

Table 2.1 Preparation of Laemmli running and stacking gels.

	Volume (ml)	
	Running gel (12.5%)	Stacking gel (4.0%)
Solution A	6.25	0.94
Solution B	3.75	-
Solution C	-	1.75
Solution D	0.15	0.07
Solution E	0.075	0.035
Dist.H_2O	4.75	4.3
TEMED	0.01	0.015

2.5 Staining of electrophoretic gels

Electrophoretic gels require visualisation of protein bands to provide information regarding composition and purity of samples. Staining procedures vary greatly in terms of their sensitivity, rapidity and simplicity. A given staining technique may not be entirely perfect for any one application, because each technique has its own merits and limitations. While a single technique may be sufficient for most purposes, a second technique can be used to complement the first, should it be found lacking in some way. For example, Coomassie blue stained gels can also be stained using imidazole-SDS-zinc reverse staining to visualise proteins present in very low amounts (nanogram range) or acidic proteins that have low affinity for the dye (Fernandez-Patron *et al.*, 1995). Gillespie and Elliott (2005) did a comparative analysis of four commonly used staining procedures, Coomassie brilliant blue (CBB) staining, ruthenium-complex-based SYPRO Ruby fluorescent staining, silver staining and imidazole-SDS-zinc reverse staining. The methods were compared in terms of sensitivity, simplicity, rapidity, cost efficiency, interprotein variability and compatibility with downstream analysis (Gillespie and Elliott, 2005). While CBB, SYPRO Ruby and silver staining procedures may be suited for selected applications, imidazole-SDS-zinc reverse staining was found to have many advantages as judged by the criteria mentioned above (Gillespie and Elliott, 2005). In this study, three techniques were used namely Coomassie blue staining, silver staining and imidazole-SDS-reverse staining.

2.5.1 Coomassie blue R-250 staining

Coomassie blue binds to proteins to form a dye-protein complex that has an intense blue colour. The standard Coomassie blue staining of Laemmli gels described here is time consuming because it involves a period of incubation with the dye solution and subsequent destaining steps to remove the excess dye. It was observed in this study that Coomassie blue stain is superior to amido black in staining zymograms. However, it is important to avoid overstaining which reduces the sensitivity of the assay. Leber and Balkwill (1997) proposed a staining method that merged the conventional staining and destaining steps, and was faster and more sensitive than the separate staining and destaining steps. Coomassie blue stain was used for staining of

zymograms, normal Laemmli gels were stained using methods described in subsequent sections.

2.5.1.2 Reagents

Stain stock solution [1% (m/v) Coomassie blue R-250]. Coomassie blue R-250 (1.0 g) was dissolved in 100 ml dist.H_2O by magnetic stirring for 1 h at room temperature and filtered through Whatman No. 1 filter paper.

Staining solution [0.125% (m/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock (12.5 ml), methanol (50 ml) and acetic acid (10 ml) were made up to 100 ml with dist.H_2O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (250 ml) and acetic acid (50 ml) were made up to 500 ml with dist.H_2O.

Destaining solution II [7% (v/v) acetic acid, 5% (v/v) methanol]. Acetic acid (35 ml) and methanol (25 ml) were made up to 500 ml with dist.H_2O.

2.5.1.3 Procedure

All steps were carried out in clean plastic containers at room temperature on a shaker. After gel electrophoresis, the gel was removed using gloves and immersed in staining solution for 4 h or overnight. After rinsing with dist.H_2O, the gel was placed in several changes of destain I and destain II. Gels were photographed and stored in sealed plastic bags at 4°C.

2.5.2 Silver staining

The silver staining technique of Blum *et al.* (1987) is highly sensitive, detecting as low as nanogram amounts of protein. Silver ions form complexes with charged amino acid side chains, which can be visualised by reduction with formaldehyde under reducing conditions (Nielson and Brown, 1984). Image development requires the formation of insoluble silver salts; however, these salts attach onto the gel surface and cause non-specific backstaining (Blum *et al.*, 1987). Treatment of gels with sodium thiosulfate complexes the silver salts and dissolves them, reducing background staining (Blum *et al.*, 1987). This technique is 50-100 times more sensitive than Coomassie blue staining and comparable to imidazole-SDS-reverse staining.

2.5.2.1 Reagents

Milli-Q ultra pure water was used for rinse steps and preparation of all reagents and is referred to as dist.H_2O.

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.05% (v/v) formaldehyde]. Methanol (50 ml) and glacial acetic acid (12 ml) were made up to 100 ml with dist.H_2O. Just before use, 37% formaldehyde was added (25 µl in 50 ml).

Wash solution ["50%" (v/v) ethanol]. Ethanol (96%) (250 ml) was made up to 500 ml with dist.H_2O.

Pre-treatment solution [0.02% (m/v) $Na_2S_2O_3.5H_2O$]. $Na_2S_2O_3.5H_2O$ (0.04 g) was dissolved in 200 ml dist.H_2O.

Impregnation solution [0.2% (m/v) $AgNO_3$, 0.03% (v/v) formaldehyde]. $AgNO_3$ (0.2 g) was dissolved in 100 ml dist.H_2O and filtered through Whatman No. 1 filter paper. Just before use, 37% formaldehyde (15 µl in 50 ml) was added.

Developing solution [6% (m/v) Na_2CO_3, 0.0004% (m/v) $Na_2S_2O_3.5H_2O$, 0.05% (v/v) formaldehyde]. Na_2CO_3 (12 g) was dissolved in 190 ml dist.H_2O, pre-treatment solution was added (4 ml) and the solution was made up to 200 ml with dist.H_2O. Just before use, 37% formaldehyde was added (25 µl in 50 ml).

Stop solution [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and glacial acetic acid (12 ml) were made up to 100 ml with dist.H_2O.

2.5.2.2 Procedure

All steps were carried out at room temperature on a shaker in meticulously clean glass containers. After electrophoresis, gels were rinsed briefly with dist.H_2O and soaked in fixing solution for 1 h or overnight. After fixation, gels were treated with wash solution (3 x 20 min), followed by pre-treatment solution (1 min) and subsequently soaked in impregnation solution (20 min). After a brief rinse in dist.H_2O (3 x 20 s), gels were immersed in development solution and visually monitored until bands became clearly visible against a slightly yellow background. Development was stopped by rinsing gels with dist.H_2O and incubating with stop solution (10 min). After a final rinse with wash solution (2 x 2 min), gels were photographed and stored in sealed plastic bags at 4°C.

2.5.3 Imidazole-SDS-zinc reverse staining

Heavy metal salts such as zinc and copper can form precipitates on the gel matrix, leaving proteins unstained and transparent (Dzandu *et al.*, 1988). Staining of gels is done in two steps; proteins are loaded with SDS and subsequent soaking in zinc solution causes deposition of an insoluble complex on the gel surface, staining it white and leaving protein bands unstained (Fernandez-Patron *et al.*, 1992; Ortiz *et al.*, 1992). Imidazole-SDS-zinc reverse staining is simpler and faster than silver staining and allows for protein detection on gels only 20 min after electrophoresis. In this study, the method described by Fernandez-Patron *et al.* (1995) was used.

2.5.3.1 Reagents

Solution A [200 mM imidazole, 0.1% (m/v) SDS]. Imidazole (0.68 g) and SDS (0.05 g) were dissolved in 50 ml dist.H_2O.

Solution B [200 mM zinc sulfate]. Zinc sulfate (2.89 g) was dissolved in 50 ml dist.H_2O.

2.5.3.2 Procedure

Following electrophoresis, gels were washed briefly in distilled water and incubated in 50 ml of solution A with gentle shaking for 15 min. After discarding this solution, gels were rinsed briefly with dist.H_2O (30 s), soaked in solution B (50 ml) and agitated (15-60 s) over a dark surface to monitor the extent of development. Development was stopped by discarding solution B and rapidly rinsing under running dist.H_2O (10-15 s).

2.6 Substrate gel electrophoresis

Zymography is a simple, sensitive and inexpensive assay for analysing proteolytic activity (Leber and Balkwill, 1997). Incorporation of a substrate, particularly gelatin, into non-reducing SDS-PAGE gels allows for detection of proteolytic activity and gives estimates of the molecular weights of proteolytic enzymes (Leber and Balkwill, 1997). Since SDS denatures proteins, proteases must be renatured by removal of SDS with Triton X-100 (Heussen and Dowdle, 1980). Incubation of the gel in appropriate assay buffer containing required activators ensures specific enzyme activity, which can be visualised after staining as clear bands against a dark background. A variation of the standard zymography method has been reported by García-Carreño *et al.*

(1993), where the gel is incubated in substrate solution after electrophoresis rather than the substrate being copolymerised with the gel.

2.6.1 Reagents

1% (m/v) gelatin. Gelatin (0.05 g) was dissolved in 5 ml of solution B (Section 2.4.1) with gentle heating.

2.5% (v/v) Triton X-100. Triton X-100 (5.0 ml) was made up to 200 ml with dist.H_2O.

Assay buffer [100 mM Na-acetate buffer, 1 mM Na_2EDTA, 40 mM cysteine, 0.02% NaN_3, pH 5.0]. Glacial acetic acid (2.86 ml) and Na_2EDTA (0.186 g) were dissolved in 450 ml dist.H_2O and adjusted to pH 5.0 with NaOH. NaN_3[3] (0.1 g) was added and the pH was measured and adjusted if necessary. The solution was made up to 500 ml with dist.H_2O. Cysteine.HCl (0.35 g) was added to 50 ml buffer immediately before use.

Staining solution. As described in Section 2.5.1.

Destaining solution. As described in Section 2.5.1.

2.6.2 Procedure

SDS-PAGE was carried out as described in Section 2.4.2, except that 0.1% (m/v) gelatin was incorporated into the running gel.

After electrophoresis, gels were washed with two changes of 2.5% (v/v) Triton X-100 (50 ml) over 1 h at room temperature. Following this, gels were rinsed with dist.H_2O, incubated in assay buffer (50 ml, 37°C) for 3 h and stained as described in Section 2.5.1.

2.7 Protein fractionation using three-phase partitioning (TPP)

Three-phase partitioning is a technique that selectively concentrates proteins from aqueous and t-butanol mixtures using ammonium sulfate. Tertiary-butanol is a C_4 alcohol that is completely miscible with water. Addition of an optimum concentration of ammonium sulfate to protein solutions containing t-butanol effects a phase

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[3] NaN_3 is added after titration as exposure of NaN_3 to acidic solutions liberates the highly toxic gas, HN_3.

separation, where the protein forms an interface between an upper t-butanol phase and a lower aqueous phase (Pike and Dennison, 1989a).

Dennison and Lovrien (1997) have given a detailed account of how ammonium sulfate and t-butanol co-operatively precipitate proteins. The sulfate ion (SO_4^{2-}) uses a combination of six interdependent mechanisms to salt out proteins: ionic strength effects, kosmotropy, cavity surface tension enhancement, dehydration, exclusion-crowding and interaction with cationic groups on the protein. The sulfate ion binds to cationic groups on the protein at a pH where proteins have a net positive charge, Z_H^+, which results in conformation shrinkage and contraction of the protein. Electrostatic interactions between the sulfate ion and protein are optimal at low ammonium sulphate concentrations, in the range 0.1-0.2 M. At higher concentrations, 0.4-4 M, the effects of kosmotropy etc. begin to operate and reinforce one another not only to precipitate (or co-crystallise) the protein, but to stabilise it by tightening its conformation.

The properties of ammonium sulphate are enhanced by t-butanol. Unlike most common organic solvents, t-butanol stabilises proteins and promotes conformational changes that favour an increased proportion of α-helices (Dennison and Lovrien, 1997; Pike and Dennison, 1989a). This conformational change is not always favourable, however, because oligomeric proteins such as haemoglobin are denatured (Pike and Dennison, 1989a). In the TPP technique, t-butanol acts as a co-solvent and partially binds to protein and forms a complex that has a low density, causing it to float on the aqueous phase (Pike and Dennison, 1989b).

TPP was favoured over conventional salting out because the protein precipitate is essentially devoid of salt and can be applied directly to an ion-exchange column without prior desalting (Pike and Dennison, 1989a). In protein isolation, homogenisation disrupts cellular compartmentalisation which often leads to the generation of isolation artefacts. It has been found that t-butanol inhibits enzymes reversibly and reduces protein-protein interactions (Dennison *et al.*, 2000), a property which was exploited in protein isolation to inhibit unwanted proteolysis. In protein purification, TPP is mostly used upstream, but it can also be useful downstream (Dennison and Lovrien, 1997; Singh *et al.*, 2001).

2.7.1 Procedure

Generally TPP is performed at ambient temperature but t-butanol crystallises below 25°C and may require prior warming to about 30°C. For isolation of proteins from tissue samples, t-butanol was mixed with homogenisation buffer to a final volume of 30% (v/v). The volume of t-butanol required was calculated as follows:

$$y = (0.3/0.7)\, x$$

Where y = volume of t-butanol

x = volume of buffer

Tissue sample was homogenised in the buffer-t-butanol solution and centrifuged. The homogenate was adjusted to pH 4.2 with 1 M HCl or dilute acetic acid and centrifuged as before. The optimal ammonium sulphate sulfate cut was established by performing cuts at specified increments of ammonium sulfate dissolved in 10 ml aliquots of the acid supernatant. After centrifugation (8 000 x g, 10 min, 4°C) in a swing-out rotor, the precipitate from each cut was either redissolved in the appropriate buffer or discarded before increasing the % of ammonium sulfate in the acid supernatant. All cuts and the final pellet were redissolved in buffer and assayed for protein content and enzyme activity.

2.8 Isolation of IgG from rabbit serum

IgG was isolated from rabbit serum by precipitation with polyethylene glycol, a high molecular weight linear polymer that is soluble in water (Polson *et al.*, 1964). In solution, PEG occupies a very large space and, due to steric exclusion, proteins become concentrated in the extrapolymer space, until they exceed their solubility limit. This precipitation is dependent on the pH of the protein solution because the concentration of PEG required to precipitate a protein is a function of the net charge of the protein (Polson *et al.*, 1964).

2.8.1 Reagents

Borate buffered saline, pH 6.8. Boric acid (2.16 g) NaCl (2.19 g), NaOH (0.7 g) and 37% HCl (0.26 ml) were dissolved in 950 ml dist.H_2O. The pH was measured and adjusted as required with HCl or NaOH and the solution was made up to 1 L.

100 mM Na-phosphate buffer, 0.02% NaN_3 (m/v), pH 7.6. $NaH_2PO_4.H_2O$ (13.8 g) was dissolved in 950 ml dist.H_2O and adjusted to pH 7.6 with NaOH. NaN_3 (0.2 g)

was added, the pH was checked and adjusted if necessary and the solution was made up to 1 L.

2.8.2 Procedure

Rabbits were bled from the marginal ear vein into glass tubes at weeks 0, 4 and 8. Blood was allowed to clot overnight, supernatant serum was drawn off the clot into 1.5 ml microcentrifuge tubes and the remaining serum was recovered by centrifugation (3 000 x g, 10 min, RT) of the clot. One volume of rabbit serum was diluted with two volumes of borate buffered saline. Solid polyethylene glycol (PEG) (Mr 6 000) [14% (m/v)] was dissolved in dilute serum by gentle stirring and centrifuged (12 000 x g, 10 min, RT). The supernatant was decanted and the pellet was redissolved in 100 mM Na-phosphate buffer in the original serum volume. 14% (m/v) PEG was dissolved again and the solution was centrifuged as before. The final pellet was redissolved in 100 mM Na-phosphate buffer containing 60% (v/v) glycerol and stored at -20°C. IgG concentration was determined by measuring the absorbance at 280 nm of a 1/40 dilution of IgG in phosphate buffer, and IgG concentration was calculated from the extinction coefficient of IgG, $E_{280}^{1mg/ml} = 1.43$ (Hudson and Hay, 1980).

CHAPTER 3
Isolation of cathepsin L and acid α-glucosidase

3.1 Introduction

Protein isolation from whole tissue homogenates has been considered to be superior to methods that require subcellular fractionation (Mason *et al.*, 1984). However, tissue homogenisation destroys cellular compartmentation, which often leads to uncontrolled proteolysis and protein/protein interactions and consequent generation of isolation artefacts (Dennison, 2003).

One way of controlling proteolysis in the homogenate is to add specific and non-specific inhibitors (Dennison *et al.*, 2000). Although this is effective in controlling non-specific proteolysis, it makes it difficult to isolate the enzymes being inhibited (Dennison *et al.*, 2000). t-Butanol is a C_4 alcohol and, although it reversibly inhibits most enzymes, unlike most organic solvents it does not denature proteins, except oligomeric types such as haemoglobin (Dennison and Lovrien, 1997). Dennison *et al.* (2000) showed that incorporation of 30% t-butanol in the homogenisation buffer inhibited proteolysis and protein/protein interactions. The clarified homogenate can subsequently be used directly for TPP fractionation.

In TPP, proteins are precipitated at ammonium sulfate concentrations that are less than that required for conventional salting out, and protein fractions can be applied directly onto ion exchange columns without prior desalting as protein is precipitated out of the aqueous phase containing the salt (Pike and Dennison, 1989b). TPP fractionation was used in this study in the rapid isolation of cathepsin L and acid α-glucosidase. It has also been used in the isolation of other proteins such as cellulases (Dennison and Lovrien, 1997) and cathepsin D (Jacobs *et al.*, 1989). Roy and Gupta (2002) coupled TPP with metal-affinity-based separation in the isolation of soybean trypsin inhibitor and showed that the modification improved the specificity of TPP. Protein isolation in this study was performed in a few simple steps (Fig. 3.1).

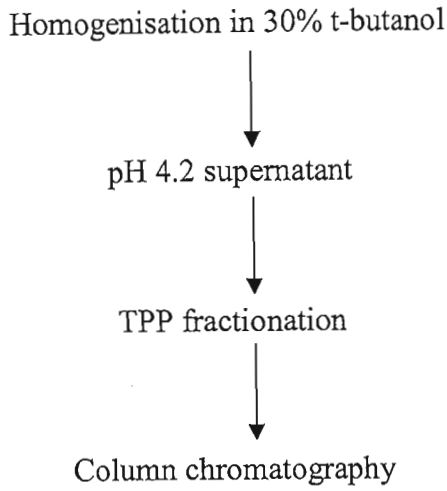


Figure 3.1 Scheme of cathepsin L and acid α-glucosidase isolations.

3.2 Cathepsin L

Cathepsin L (EC 3.4.22.15) is the most active of the endolysosomal cysteine proteinases in the degradation of substrates such as azocasein (Barrett and Kirschke, 1981). The primary function of cathepsin L in the endolysosomal system is protein turnover (Kominami *et al.*, 1988), but it has been shown to have more specialized roles (discussed in Chapter 1).

Structurally, cathepsin L is very similar to papain in terms of amino acid sequence and folds (Turk *et al.*, 2001). Fujishima *et al.* (1997) presented the crystal structure of mature cathepsin L (Fig. 3.2). Briefly, mature cathepsin L comprises two domains separated by a V-shaped active site cleft (Turk *et al.*, 2002). The catalytic Cys-25 (papain numbering) is located at the top of the central α-helix in the left domain and forms an ion pair with His-159 positioned in the β-barrel domain on the opposite site of the active site cleft (Turk *et al.*, 2001; Turk *et al.*, 2002). Substrate binding sites consist mainly of S2, S1, S1′, S2′ and S3 (Turk *et al.*, 2002). S2, S1 and S1′ are well defined substrate binding sites and basically determine the specificity of the enzyme (Turk *et al.*, 2001). However, the S2 subsite is deep and hydrophobic, and it is the major specificity determinant (Fujishima *et al.*, 1997).

Cathepsin L is an endopeptidase and it has generally been reported to exhibit maximum activity toward synthetic substrates at pH 5.5 (Turk *et al.*, 1993). However, Dehrmann *et al.* (1995) showed that an error had been made in using buffers of constant molarity in determining the pH optimum. Barrett and Kirschke (1981) reported that cathepsin L is unstable at neutral pH and in slightly alkaline conditions.

At both acid and alkaline pH, inactivation is accompanied by a loss of α-helical content and thus the enzyme becomes irreversibly denatured (Dufour *et al.*, 1988; Turk *et al.*, 1999). Turk *et al.* (1993), however, showed that the rate of enzyme inactivation decreases with substrate concentration, which could suggest that the substrate somehow protects the enzyme from inactivation.

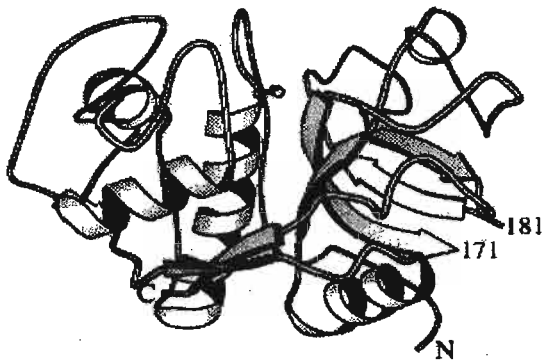


Figure 3.2 Structure of human cathepsin L.
The catalytic Cys-25 positioned at the top of the central α-helix is presented using the ball-and -stick model. N and C represent the amino and carboxyl termini respectively (Fujishima *et al.*, 1997).

Various sources of cathepsin L have been used including rabbit liver (Mason *et al.*, 1984), human liver (Mason *et al.*, 1985), sheep liver (Pike and Dennison, 1989a) and baboon liver (Coetzer, 1995). Sheep liver was found to yield the highest amount of purified cathepsin L compared to human, rabbit, bovine or rat livers (Mason, 1986). The choice of enzyme source, however, is largely influenced by the intended purpose for which the enzyme is required. In this study, cathepsin L was required for generation of antibodies in rabbits in an attempt to break self tolerance towards the rabbit enzyme (Chapter 4). Therefore, rabbit livers were used as the source of enzyme.

3.2.1 Cathepsin L assay

Cathepsin L was assayed using a synthetic substrate, Z-Phe-Arg-NHMec. To date a synthetic substrate that is specific for cathepsin L has not been described. Substrates such as Bz-Arg-NH_2 and Z-Lys-$OPhNO_2$ have also been used for cathepsin L, although their use is limited by the fact that they are also sensitive to cathepsin B, which makes it difficult to distinguish the two enzymes in isolation procedures

(Barrett and Kirschke, 1981). Z-Phe-Arg-NHMec is also susceptible to cathepsin B, but it is more sensitive to cathepsin L (Barrett and Kirschke, 1981). Dehrmann (1998) showed that the presence of 3 M urea in the assay buffer (pH 5.5) partially inhibits the activity of cathepsin B against Z-Phe-Arg-NHMec, so that an increase in the Z-Phe-Arg-NHMec/Z-Arg-Arg-NHMec ratio can be taken as a measure of increase in cathepsin L activity.

3.2.1.1 Reagents

Buffer/Activator [400 mM Na-acetate buffer, 4 mM Na_2EDTA, 3 M Urea, 0.02% (m/v) NaN_3, 8 mM DTT, pH 5.5]. Glacial acetic acid (23.0 ml), Na_2EDTA (1.5 g) and urea (180.18 g) were dissolved in 950 ml dist.H_2O and adjusted to pH 5.5 with NaOH. NaN_3 (0.2 g) was added; the pH was measured and adjusted if necessary and the solution was made up to 1 L with dist.H_2O. Just before use, DTT (6.71 mg in 5 ml assay buffer) was added.

1 mM substrate stock solution. Z-Phe-Arg-NHMec (1.0 mg) was dissolved in 1.5 ml DMSO and stored at 4°C.

20 μM substrate solution. Substrate stock solution (20 μl) was made up to 1.0 ml with dist.H_2O.

3.2.1.2 Procedure

Enzyme sample (25 μl) was added to 75 μl of buffer/activator in the wells of a white fluoronunc maxisorp microtitre plate and preincubated at 37°C for 2 min, in a temperature-controlled cell of a microplate fluorescence reader (Cambridge Technology model 7620). Substrate solution was added, mixed and the plate was incubated for 10 min. The fluorescence of the liberated aminomethyl coumarin was measured at 2 min intervals in the microplate fluorescence reader, with excitation at 360 nm and emission at 460 nm.

Standard curves were constructed using aminomethylcoumarin concentrations in the ranges, 2-10 μM, 10-100 μM and 100-1000 μM. Product concentration was calculated from the curve using equation 3.1.

$$[Product] = Fluorescence\ intensity - y\text{-}intercept/Slope \quad \ldots\ldots 3.1$$

Elution profiles were constructed using the fluorescence readings obtained directly from the fluorometer, which were designated "arbitrary fluorescence units."

3.2.2 Cathepsin B assay

Cathepsin B coprecipitates with cathepsin L in TPP and Z-Phe-Arg-NHMec, which is used for cathepsin L, is also sensitive to cathepsin B. Therefore, a specific assay for cathepsin B was required to correct for cathepsin B activity in the fractions. Z-Arg-Arg-NHMec is highly specific for cathepsin B and is insensitive to both cathepsins H and L (Barrett and Kirschke, 1981).

3.2.2.1 Reagents

Buffer/Activator [100 mM Na-phosphate buffer, 4 mM Na_2EDTA, 0.02% NaN_3, 5 mM DTT, pH 6.0]. NaH_2PO_4 (6.90 g) and Na_2EDTA (0.93 g) were dissolved in 450 ml dist.H_2O and adjusted to pH 6.0 with NaOH. NaN_3 (0.1 g) was added, the pH checked and adjusted if necessary and the solution was up to 500 ml with dist.H_2O. Just before use, DTT (0.0102 g) was added to 10 ml buffer.

1 mM Z-Arg-Arg-NHMec substrate stock solution. Z-Arg-Arg-NHMec (1.1 mg) was dissolved in 1.5 ml DMSO and stored at 4°C.

40 µM Z-Arg-Arg-NHMec substrate solution. Substrate stock solution (40 µl) was made up to 1 ml with dist.H_2O.

3.2.2.2 Procedure

The method was as described in Section 3.2.1.2.

3.2.3 Purification of cathepsin L from rabbit liver

3.2.3.1 Reagents

Homogenisation buffer [100 mM Na-acetate buffer, 0.1% (m/v) Na_2EDTA, 30% (v/v) t-butanol, pH 4.0]. Glacial acetic acid (5.72 ml) and Na_2EDTA (1.0 g) were dissolved in 950 ml dist.H_2O, adjusted to pH 4.0 with NaOH and made up to 1 L with dist.H_2O. Just before use, t-butanol was added to the appropriate volume of buffer to constitute 30% of the mixture.

Buffer A [20 mM Na-acetate buffer, 1 mM Na_2EDTA, 50 mM NaCl, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (2.29 ml), Na_2EDTA (0.74 g) and NaCl (5.84 g) were dissolved in 1.9 L dist.H_2O and adjusted to pH 5.5 with NaOH. NaN_3 (0.4 g) was added, the pH was measured and adjusted if necessary and the solution was made up to 2 L with dist.H_2O.

Buffer B [20 mM Na-acetate buffer, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (2.29 ml) and Na_2EDTA (0.74 g) were dissolved in 1.9 L dist.H_2O and adjusted to pH 5.5 with NaOH. NaN_3 was added, the pH was measured and adjusted if necessary and the solution was made up to 2 L with dist.H_2O.

S-Sepharose fast flow. S-Sepharose slurry (120 ml) was mixed with buffer A (250 ml). The resulting slurry was packed into a glass column under gravity and equilibrated with five column volumes of buffer A before use. In between purifications, the matrix was regenerated with one column volume of 2 M NaCl in buffer B.

Rabbit liver. Fresh rabbit livers were obtained from Crafty Duck animal farm in Pietermaritzburg and immediately diced into small cubes and stored at -70°C for at least three days before use.

3.2.3.2 Procedure

The method was essentially that described by Moolman (2001) for the purification of sheep liver cathepsin L. Frozen liver (100 g) was thawed overnight at 4°C and homogenised in a Waring blendor for 2 min in a 1:2 ratio (liver mass to homogenisation buffer volume). The homogenate was centrifuged (6500 x g for 30 min, 4°C, in a JA-10 rotor). The supernatant was decanted into a clean container, adjusted to pH 4.2 with 1 M HCl and centrifuged as before.

TPP was preformed on the pH 4.2 supernatant (the pH 4.2 supernatant already contains t-butanol and subsequent mention of it takes note of this fact) by adding 20% (m/v) of solid ammonium sulfate (based on the volume of the pH 4.2 supernatant). The solution was centrifuged (6000 x g, 15 min, 4°C) in a swing-out rotor. The interfacial pellet was discarded and the % of ammonium sulfate was increased to 40% (m/v) (based on the total volume of the collected supernatant and subnatant). The solution was centrifuged as before and the final pellet was redissolved in buffer A at one fifth of the volume of the pH 4.2 supernatant. The protein solution was adjusted to pH 5.5 with NaOH, dialysed overnight at 4°C against buffer A and finally centrifuged (10 000 x g, 15 min, 4°C) to remove insoluble material.

The clarified TPP fraction was loaded onto a column of S-Sepharose (2.5 x 23 cm = 115 ml bed volume; flow rate = 50 ml/h), pre-equilibrated with buffer A. The gel bed was washed with two column volumes of buffer A and the bound protein was eluted

with a 50-600 mM NaCl linear gradient in buffer A, in six column volumes, at 50 ml/h. Fractions with significantly high Z-Phe-Arg-NHMec activity and low Z-Arg-Arg-NHMec activity were pooled and concentrated by dialysis against PEG 20 000. This fraction was dialysed against buffer B and rerun on the S-Sepharose column (2.5 x 23 cm = 115 ml bed volume), pre-equilibrated with buffer B. Unbound material was washed with two column volumes of buffer B and the bound protein was eluted with a linear gradient of 0-400 mM NaCl in buffer B, in six column volumes, at 50 ml/h. Z-Phe-Arg-NHMec active fractions were pooled and concentrated by dialysis against PEG 20 000.

3.2.3.3 Results

The purification procedure used in this study was rapid and generated appreciable yields of protein (Table 3.1). The first linear gradient of 50-600 mM NaCl separated cathepsin L from cathepsin B (Fig. 3.3), while the second gradient of 0-400 mM NaCl (Fig. 3.4) removed other contaminating proteins and generated pure cathepsin L (Fig. 3.5), although at a high cost in yield (Table 3.1).

Table 3.1 Purification of cathepsin L from rabbit liver.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	210	2541	11192	4.4	(1)	(100)
pH 4.2 supernatant	210	1207	10056	8.3	1.9	90
TPP cut (20-40%)	83	367.5	8758	12.1	2.7	78
1st S-Sepharose	60	37.18	1193	44.4	10.1	10.7
2nd S-Sepharose	3.6	1.2	96.2	57.7	13.1	0.62

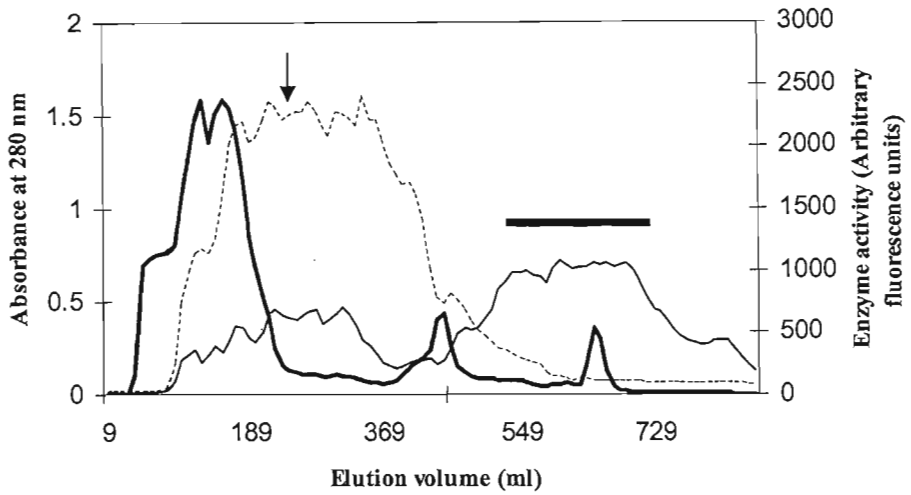


Figure 3.3 Chromatography of 20-40% TPP fraction on S-Sepharose. Column, 2.5 x 23 cm (115 ml bed volume), equilibrated in buffer B [20 mM Na-acetate buffer, pH 5.5, containing 1 mM Na_2EDTA, 0.02% NaN_3], and eluted with a 50-600 mM NaCl gradient in 6 column volumes of the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 9 ml (10.8 min). (—) A_{280}, (----) Z-Phe-Arg-NHMec activity, (—) Z-Arg-Arg-NHMec activity, (▬) pooled fractions.

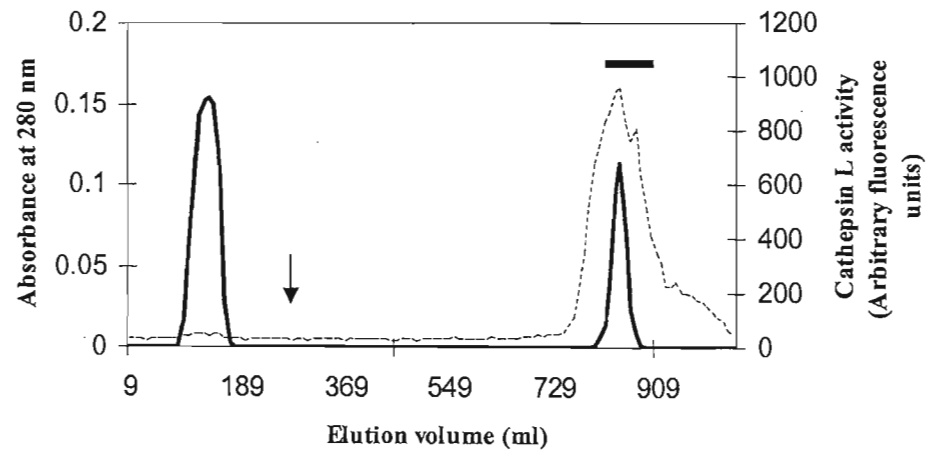


Figure 3.4 Rechromatography of the S-Sepharose fraction on S-Sepharose. Column, 2.5 x 23 cm (115 ml bed volume), equilibrated in buffer B [20 mM Na-acetate buffer, pH 5.5, containing 1 mM Na_2EDTA, 0.02% NaN_3], and eluted with a 0-400 mM NaCl gradient in 6 column volumes of the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 9 ml (10.8 min). (—) A_{280}, (----) Z-Phe-Arg-NHMec activity, (▬) pooled fractions.

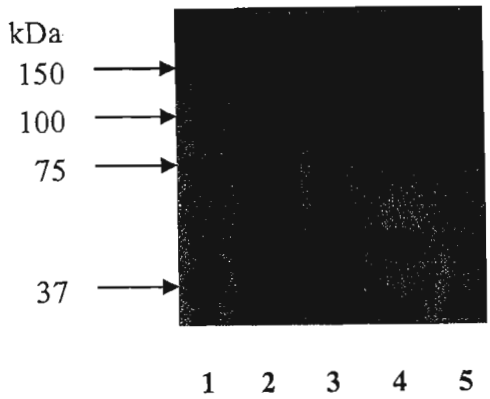


Figure 3.5 Reducing SDS-PAGE of samples taken at different stages during purification of rabbit liver cathepsin L.
Samples were boiled in reducing treatment buffer and loaded onto 12.5% polyacrylamide gel. Lane 1, Molecular weight markers (Precision Plus Protein Kaleidoscope standards ranging from 250-10 kDa, Bio-Rad), lane 2 homogenate, lane 3, 20-40% TPP fraction, lane 4, S-Sepharose fraction, lane 5, pure rabbit cathepsin L. Visualised using imidazole-SDS-zinc reverse staining.

3.2.4 Purification of cathepsin L from sheep liver

3.2.4.1 Reagents

Homogenisation buffer [100 mM Na-acetate buffer, 0.1% (m/v) Na_2EDTA, 30% (v/v) t-butanol, pH 4.0]. As described in Section 3.2.2.1.

Buffer B [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. As described in Section 3.2.2.1.

S-Sepharose fast flow. As described in Section 3.2.2.1.

Sheep liver. Fresh sheep liver was obtained from Cato Ridge abattoir, and immediately cut into cubes and stored at -70°C for at least three days before use.

3.2.4.2 Procedure

TPP fractionation was as described in Section 3.2.2.2. The clarified TPP fraction was applied to a column of S-Sepharose (2.5 x 23 cm = 115 ml bed volume), pre-equilibrated with five column volumes of buffer B. Unbound material was washed with two column volumes of buffer B and the bound protein was eluted with a linear gradient of 0-200 mM NaCl in buffer B. Z-Phe-Arg-NHMec active fractions were pooled and concentrated by dialysis against PEG 20 000.

3.2.4.3 Results

Purification of sheep liver cathepsin L is shown in Figs 3.6 and 3.7 and summarised in Table 3.2

Table 3.2 Summary of purification of sheep liver cathepsin L.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	320	3872	17143	4.4	(1)	(100)
pH 4.2 supernatant	305	1754	10000	5.7	1.3	58
TPP cut (20-40%)	86	752.5	6219	8.3	1.9	36
S-Sepharose	8	1.8	1412	178	178	8

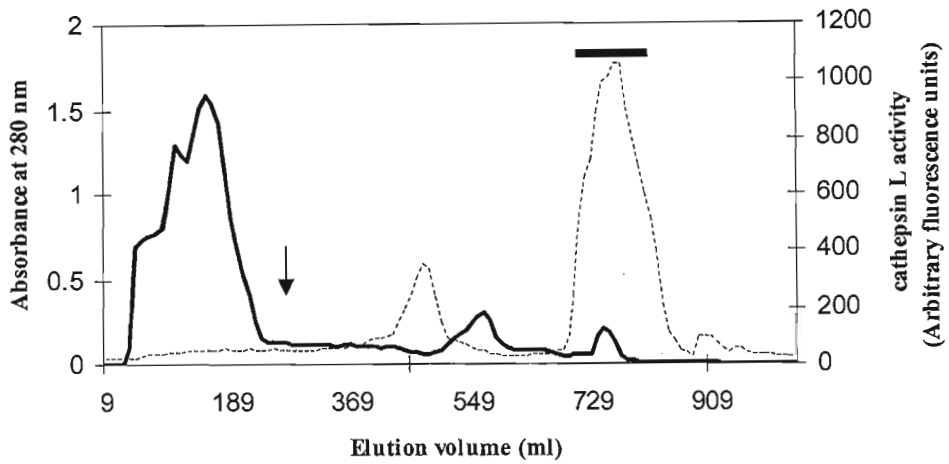


Figure 3.6 Chromatography of the 20-40% TPP fraction of sheep liver cathepsin L on S-Sepharose.

Column, 2.5 x 23 cm (115 ml bed volume), equilibrated with buffer A [20 mM Na-acetate buffer, pH 5.5, containing 50 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3], and eluted with a 0-200 mM NaCl gradient in 6 column volumes in the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 9 ml (10.8 min). (—) A_{280}, (----) Z-Phe-Arg-NHMec, (▬) pooled fractions.

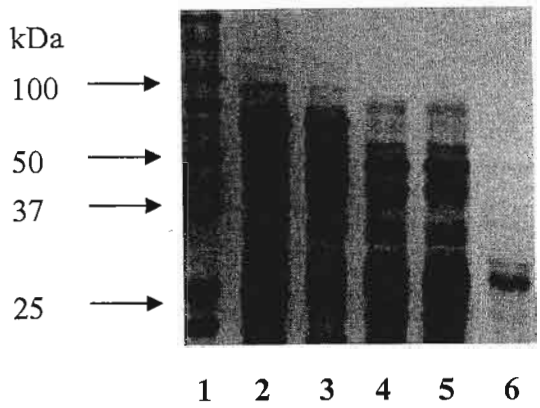


Figure 3.7 Reducing SDS-PAGE of samples taken at different stages during purification of sheep liver cathepsin L.
Samples were boiled in reducing treatment buffer and loaded onto 12.5% polyacrylamide gel. Lane 1, Molecular weight markers (Precision Plus Protein Kaleidoscope standards ranging from 250-10 kDa, Bio-Rad), lane 2 homogenate, lane 3, pH 4.2 supernatant, lanes 4 and 5, 20-40% TPP fraction, lane 6, pure sheep cathepsin L. Visualised using silver staining.

3.3 Acid α-glucosidase

α-Glucosidases are broadly categorised into two large groups based on their activity pH optima; pH 6-7 for neutral α-glucosidase and pH 4-5 for acid α-glucosidase. Acid α-glucosidase (EC 3.2.1.3) is an endolysosomal enzyme that hydrolyses α-1,4 and α-1,6 glucosidic bonds in maltose, glycogen and some oligosaccharides to give free glucose (Tashiro *et al.*, 1986). The absence of acid α-glucosidase leads to excessive storage of glycogen inside lysosomes, a condition known as type II glycogenosis (Pompe's disease) which is fatal in infancy (Mehrani and Storey, 1993).

The active site of acid α-glucosidase has been the subject of debate. One view was that acid α-glucosidase possesses two active sites, one that hydrolyses low molecular weight substrates such as maltose and the other that binds high molecular weight substrates such as glycogen (Jeffrey *et al.*, 1970; Palmer 1971). Contrary to this view, Onodera *et al.* (1989) performed a kinetic analysis of rabbit liver acid α-glucosidase using maltose and shellfish glycogen as substrates and showed that the enzyme exhibited a single active site mechanism. Hermans *et al.* (1991) identified one ionisable group (Asp-518) in the catalytic site of human α-glucosidase and suggested that hydrolysis of both low and high molecular weight substrates occurs at a single active site. However, Chadalavada and Sivakami (1997) proposed a kinetic model indicating a bifunctional active site consisting of a small maltose hydrolyzing subsite that can cleave only the α-1,4 bonds of maltose, located within a large glycogen hydrolyzing site that can cleave both α-1,4 and α-1,6 bonds.

Acid α-glucosidase has been purified from a number of sources including trout liver (Mehrani and Storey, 1993), rabbit liver (Onodera *et al.*, 1989), cattle liver (Bruni *et al.*, 1969), pig liver (Tashiro *et al.*, 1986; Nakasone *et al.*, 1991) and human placenta (Hermans *et al.*, 1991). The enzyme has a high affinity for dextran-type gel filtration media, which act as a substrate analogue of the natural substrates such as glycogen (Nakasone *et al.*, 1991). In this study, purification of α-glucosidase was performed in two simple steps, fractionation using TPP and affinity chromatography on Sephadex G-100.

3.3.1 Acid α-glucosidase assay

A synthetic substrate, 4-methylumbelliferyl-α-glucoside was used for α-glucosidase activity assays. The substrate liberates 4-methylumbelliferone, a weakly fluorescent group. The addition of glycine-NaOH (pH 10.5) converts 4-methylumbelliferone to a more fluorescent anion and stops further hydrolysis of the substrate (Daniels and Glew, 1984).

3.3.1.1 Reagents

Assay buffer [100 mM Na-acetate buffer, pH 4.5]. Gacial acetic acid (2.86 ml) was dissolved in 450 ml dist.H_2O, adjusted to pH 4.5 with NaOH and made up to 500 ml with dist.H_2O.

Substrate stock solution [20 mM 4-methylumbelliferyl-α-glucoside]. 4-Methyl-umbelliferyl-α-glucoside (6.7 mg) was dissolved in 1.0 ml DMSO and stored at 4°C.

Substrate solution. The substrate stock solution was diluted to a working strength of 0.2 mM with dist.H_2O (10 µl was made up to 1 ml dist.H_2O) before use.

Stopping reagent [50 mM glycinate buffer, pH 10.5]. Glycine (3.75 g) was dissolved in 450 ml dist.H_2O, adjusted to pH 10.5 with NaOH and made up to 500 ml with dist.H_2O.

10 mM 4-Methylumbelliferone standard. 4-methylumbelliferone sodium salt (198.2 mg) was dissolved in 10 ml DMSO. For construction of a standard curve this standard solution was diluted 1:1000 with dist.H_2O; 5 to 100 µl (0.05 to 1 nmol) and stopping reagent (200 µl) were added to the wells of a white fluoronunc microtitre plate and the fluorescence was measured in a microplate fluorescence reader

(Cambridge Technology model 7620), with excitation at 360 nm and emission at 460 nm.

3.3.1.2 Procedure

The enzyme sample (25 µl) was added to 75 µl of assay buffer followed by 25 µl of substrate solution, in the wells of a white fluoronunc microtitre plate, mixed thoroughly and incubated at 37°C for 10 min. Stopping reagent (175 µl) was added and the solution was mixed thoroughly. The fluorescence of the free 4-methylumbelliferone was measured in a microplate fluorescence reader (Cambridge Technology model 7620), with excitation at 360 nm and emission at 460 nm and 4-methylumbelliferone was quantitated by reference to the standard curve.

3.3.2 Purification of acid α-glucosidase from bovine liver

3.3.2.1 Reagents

Homogenisation solution [1% NaCl, 0.1% Na_2EDTA, 30% t-butanol]. NaCl (10 g) and Na_2EDTA (1.0g) were dissolved in 1 L dist. H_2O. Just before use, t-butanol was added to constitute 30% (v/v) of the final volume.

Equilibration buffer [20 mM Na-acetate buffer, 0.02% NaN_3, pH 5.8]. Glacial acetic acid (2.29 ml) was dissolved in 1.9 L of dist. H_2O and adjusted to pH 5.8 with NaOH. NaN_3 (0.4g) was added, the pH was measured and adjusted if necessary and the solution was made up to 2 L with dist. H_2O.

Sephadex G-100. Sephadex G-100 was prepared by adding 30 g of the xerogel to 500 ml of dist.H_2O. The mixture was boiled in a water bath for 1 h and allowed to cool to room temperature overnight. The resulting slurry was packed into a glass column under gravity. The column bed was subsequently equilibrated with five column volumes of equilibration buffer before use. The column bed was regenerated with one column volume of 0.25% maltose in equilibration buffer before the next purification step.

Bovine liver. Fresh bovine liver was obtained from Cato Ridge abattoir, diced into small cubes and stored at -70°C for at least three days before use.

3.3.2.2 Procedure

The purification procedure was essentially according to Meinesz (1996). Frozen liver (100 g) was thawed overnight at 4°C and homogenised in a Waring blendor for 3 min in homogenisation solution using a 1:2 ratio (liver mass to homogenization buffer). The homogenate was centrifuged (6000 x g, 30 min, 4°C, in a JA-10 rotor). The supernatant was decanted into a clean container, adjusted to pH 4.2 with dilute glacial acetic acid and centrifuged as before.

Fractionation by three-phase partitioning was effected on the pH 4.2 supernatant by adding 15% (m/v) of solid ammonium sulfate (based on the volume of the pH 4.2 supernatant) and dissolving by gentle stirring. The solution was centrifuged (8000 x g, 10 min, 4°C) in a swing-out rotor. The precipitate was discarded and the % of ammonium sulfate was increased to 25% (m/v) (based on the total volume of supernatant and subnatant), dissolved and centrifuged as before. The final pellet was redissolved in equilibration buffer at one tenth of the volume of the acid supernatant.

The protein sample obtained from TPP was dialysed overnight at 4°C against equilibration buffer and centrifuged (10 000 x g, 15 min, 4°C) in a JA-10 rotor to remove insoluble material. The clarified fraction was applied to a Sephadex G-100 column (5 x 17 cm = 334 ml bed volume; flow rate = 75 ml/h), pre-equilibrated with equilibration buffer. The gel bed was washed with one column volume of equilibration buffer and the bound protein was eluted with 0.25% maltose in the same buffer. Fractions with activity on 4-methylumbelliferyl-α-glucoside were pooled and concentrated by dialysis against PEG 20 000.

3.3.2.3 Results

The combination of TPP and affinity chromatography on Sephadex G-100 (Fig. 3.8) was rapid and the recovery of pure α-glucosidase (Fig. 3.9) was 3.4% (Table 3.3).

Table 3.3 Purification of acid α-glucosidase from bovine liver.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	150	2730	838	0.307	(1)	(100)
pH 4.2 super.	144	698	456	0.654	2.13	54
TPP cut (20-30%)	23	77.28	247	3.2	10.4	29
Sephadex G-100	3.6	1.48	28.6	19.3	63.03	3.4

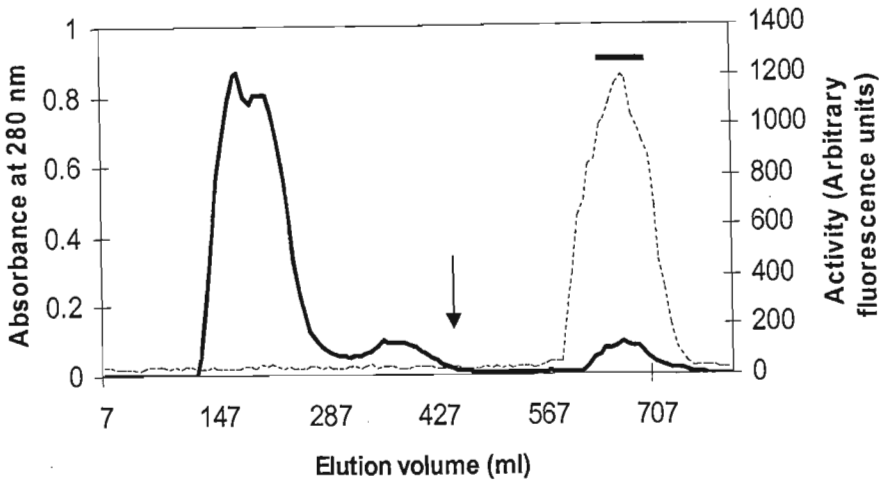


Figure 3.8 Affinity chromatography of the 15-25% TPP fraction of bovine liver acid α-glucosidase on Sephadex G-100.

Column, 5 x17 cm (334 ml bed volume), equilibrated with 20 mM Na-acetate buffer, pH 5.8, containing 0.02% NaN_3, and eluted with 20 mM Na-acetate, pH 5.8, containing 0.25% maltose, 0.02% NaN_3 applied at ↓; flow rate, 75 ml/h (3.8 cm/h); fractions, 7 ml (5.6 min). (—) A_{280}, (----) acid α-glucosidase activity, (▬) pooled fractions.

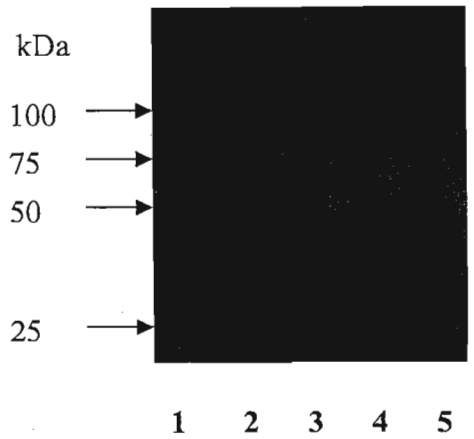


Figure 3.9 Reducing SDS-PAGE of samples taken at different stages during purification of bovine α-glucosidase cathepsin L.

Samples were boiled in reducing treatment buffer and loaded onto 12.5% polyacrylamide gel. Lane 1, Molecular weight markers (Precision Plus Protein Kaleidoscope standards ranging from 250-10 kDa, Bio-Rad), lane 2 homogenate, lane 3, pH 4.2 supernatant, lane 4, 15-25% TPP fraction, lane 5, acid α-glucosidase after Sephadex G-100 step. Visualised using imidazole-SDS-zinc reverse staining.

3.4 Discussion

Cathepsin L and α-glucosidase isolated in this study were required for generation of cathepsin L autoantibodies in rabbits (Chapter 4). Rapid, high yield methods were required to generate quantities of enzyme sufficient for conjugation, inoculation and immunological assays. TPP fractionation followed by chromatography was found to be superior to procedures previously described for the isolation of rabbit cathepsin L (Mason *et al.*, 1984) and α-glucosidase (Tashiro *et al.*, 1986). These previous methods employed several chromatography steps after ammonium sulfate precipitation that take a long time and may compromise protein yields.

3.4.1 Isolation of cathepsin L

Mason *et al.* (1984) purified rabbit liver cathepsin L with a molecular weight of 29 kDa. A method described by Pike and Dennison (1989a) for the isolation of sheep liver cathepsin L generated high yields of enzyme in relatively few steps. Cathepsin L generally binds tightly to cation exchangers and, therefore, elutes at higher salt concentrations than other lysosomal cathepsins such as cathepsins B and H (Barrett and Kirschke, 1981). The rabbit liver enzyme, however, has been reported to elute at lower salt concentrations than that from sheep, human or rat liver (Mason, 1986). In this study, rabbit liver cathepsin L did not bind to S-Sepharose at 200 mM NaCl, the initial concentration that had worked well for the sheep liver enzyme (Pike and Dennison, 1989a). High resolution is achieved if the protein of interest binds to the ion exchange matrix relatively tighter than contaminating proteins. The fact that rabbit liver cathepsin L binds less tightly to S-Sepharose than sheep liver cathepsin L meant that more than one chromatography step was required to purify the enzyme, whereas a single step on S-Sepharose was sufficient to purify the sheep liver enzyme.

Rabbit liver cathepsin L was isolated in a single chain form with a molecular weight of 30 kDa. Cathepsin L isolated from various sources has been reported to comprise two chains (Mason *et al.*, 1985; Mason, 1986). The preprocathepsin L, is processed to procathepsin L with a molecular mass of 39 kDa and converted to a mature single chain form with a molecular mass of 30 kDa and a two chain form with molecular masses of 25 kDa and 5 kDa (Kominami *et al.*, 1988; Ishido *et al.*, 1998). However, it has been argued that the two chain form of cathepsin L may be an isolation artefact, resulting from long procedures that lead to autolysis (Dehrmann, 1998).

Lysosomal cysteine proteinases coexist with their endogenous inhibitors, the cystatins, which are localised in extralysosomal compartments (Rzychon *et al.*, 2004). It has been demonstrated that cathepsin L forms a complex *in vitro* with its inhibitor, stefin B, in a non-inhibitory mode, which compromises the yield of free cathepsin L from a homogenate (Pike *et al.*, 1992). Since t-butanol inhibits protein/protein interactions, it has been proposed that its inclusion in the homogenisation buffer may inhibit the formation of cathepsin L/stefin B complex (Dennison *et al.*, 2000), leading to a higher yield of the free enzyme.

3.4.2 Isolation of α-glucosidase

Bruni *et al.* (1969) purified acid α-glucosidase from bovine liver using Sephadex G-100. To obtain a pure enzyme, the Sephadex G-100 step was done twice, since the first run gave a contaminated product that was further purified by Bio-gel P60 chromatography before running it again on Sephadex G-100. In the procedure followed in this laboratory TPP fractionation followed by a single run on Sephadex G-100 was sufficient to produce a pure enzyme.

Acid α-glucosidase from various sources has been consistently purified in at least two active forms (Bruni *et al.*, 1969; Tashiro *et al.*, 1986; Onodera *et al.*, 1989; Nakasone *et al.*, 1991; Mehrani and Storey, 1993). Two forms of α-glucosidase with molecular masses of 76 kDa and 66 kDa were purified from bovine liver in this study. There has been speculation as to whether the different forms are artifacts or simply different forms of α-glucosidase that exist *in vivo*. Acid α-glucosidase is synthesised as a 110 kDa precursor which is processed to a 95 kDa intermediate and subsequently converted to a mature form of 76 kDa (Wisselaar *et al.*, 1993). Characterisation of their catalytic properties indicated that these two enzyme forms are not varieties of a single enzyme molecule but different species of acid α-glucosidase. Tsuji and Suzuki (1987) compared the molecular structures of the two species and observed differences in the enzymes' amino acid composition, content of total hexose and secondary structure, while the carboxy-terminal sequences were identical. Whether these differences have any significance in determining the molecular weight of the processed enzyme is not clear.

Moreland *et al.* (2005) proposed a model for maturation of acid α-glucosidase. Once in the "lysosomes", the 95 kDa intermediate undergoes proteolytic processing both at

the carboxy-terminal and amino-terminal to yield a 76 kDa species (Wisselaar *et al.*, 1993). Further proteolytic cleavage at the amino-terminal of the 76 kDa species produces a 70 kDa polypeptide complex consisting of three polypeptides of 19.4 kDa, 10.3 kDa and 3.9 kDa (Moreland *et al.*, 2005). Martiniuk *et al.* (1984) had previously reported the association of low molecular weight polypeptides of 25 kDa and 21 kDa with the two major forms of acid α-glucosidase. The significance of a multimeric enzyme has not been established. However, Martiniuk *et al.* (1984) showed that all the forms of acid α-glucosidase are deficient in glycogenosis type II patients. In the present study, a major band of 95 kDa, and several lower MW bands, was found (Figure 3.9).

CHAPTER 4
GENERATION OF ANTI-CATHEPSIN L AUTOANTIBODIES

4.1 Introduction

The immune system has the ability to elicit a response against a wide variety of antigens, but maintains tolerance towards self antigens. Several mechanisms are employed by the immune system to prevent autoimmunity (Chapter 1). However, tolerance is not absolute, so that self-reactive B and T cell clones form part of the circulating B and T cell repertoire (Nossal, 1991).

There are several challenges in attempts to induce autoimmunity to treat cancer. First, tumours arise from healthy body tissues to which tolerance has been established, and as the tumour grows it induces tolerance towards itself in the same manner as normal tissues (Matzinger, 1998). Therefore, the immune system does not destroy tumours even when they express new or mutated antigens. In addition, tumours continually lose antigen expression because of a high frequency of mutations that occur in individual cells (Evans, 1991). Thus, the first step towards successful immunotherapy is to break tolerance towards tumour antigens. Second, the number of lymphocyte clones in a given immune repertoire that can recognise tumour antigens may be relatively small, so that the rapidly growing tumour may outrun the response (Mapara and Sykes, 2004). Third, ensuing autoimmune responses may be deleterious to the host, especially if the targeted antigens are shared with normal cells. Finally, tumours create a microenvironment that is antagonistic towards lymphocyte function and viability, so that tumour infiltration is poor or the tumour environment induces lymphocyte dysfunction (Ribas *et al.*, 2003).

A majority of cancer vaccines mainly target T cell responses, because tumour antigens may not necessarily be displayed on the cell surface or be secreted (Espinoza-Delgado, 2002). This does not mean that B cell responses are completely irrelevant in this regard, because some tumour associated antigens are expressed on the cell surface. In addition, the metastatic process is multifaceted so that immunotherapy can target any of the key phases in a manner that will inhibit the process. For instance, as tumours grow and progress they secrete degradative enzymes which degrade the ECM to facilitate tumour invasion and metastasis

(Nowell, 2002). Antibody responses directed against these degradative enzymes may lead to tumour regression. Cathepsin L has been implicated in tumour invasion and metastasis (Park *et al.*, 1996; Dohchin *et al.*, 2000; Fröhlich *et al.*, 2001), thus it was hoped that anti-cathepsin L antibodies generated in a manner described in this study may lead to immune elimination of cathepsin L-secreting cancer cells. Rousselet *et al.* (2004) generated an anti-cathepsin L ScFv fragment, which was shown to inhibit tumour growth and metastasis in nude mice when transfected into melanoma cells. The ability of anti-cathepsin L ScFv fragment to reduce tumour growth and metastasis was mainly due to the strong inhibition of procathepsin L secretion (Guillaume-Rousselet *et al.*, 2002). Mills *et al.* (2002) demonstrated that fully human anti-MUC18 antibodies significantly reduced tumour growth in nude mice when delivered after a tumour challenge.

Antibody production is a well-regulated process that requires a balance between specific variables including adjuvants, antigen dose, delivery time and localisation (Zinkernagel, 2000). Adjuvants are particularly important as they improve immune reactivity towards antigens. Bacterial components in Freund's complete adjuvant deliver danger signals of infection by engaging toll-like receptors (TLRs) on host immune cells (Chapter 5). Adjuvants induce an inflammatory response, recruiting macrophages and APCs to the injection site (Roitt and Delves, 2001). APCs take up antigen and migrate to the draining lymph nodes where they present antigen to T cells. APCs provide accessory costimulatory signals that favour immune responsiveness over tolerance induction (Roitt and Delves, 2001). Adjuvants also prevent immediate degradation of antigen by forming a depot from which antigen is released slowly over a period (Zinkernagel, 2000).

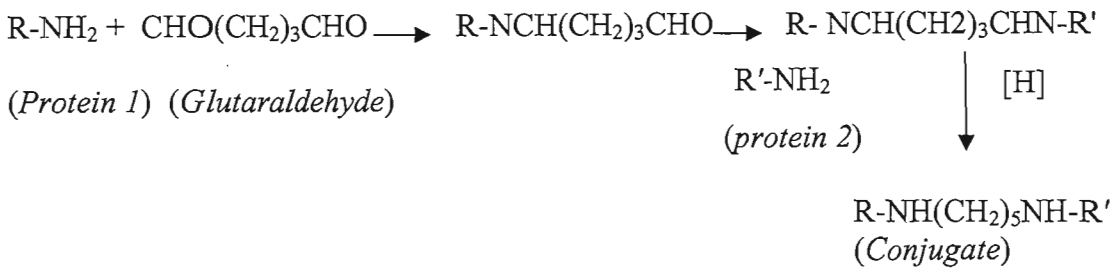
At the molecular level the primary immune response begins at the site of antigen entry where activated APCs migrate to the draining lymph node and begin a cascade of lymphocyte activation (McHeyzer-Williams *et al.*, 2003). Mature APCs are very efficient in priming antigen specific T cells because they deliver essential costimulatory molecules. Activated T cells provide help to B cells in the form of cytokines which direct B cell proliferation and differentiation into plasma cells. In the absence of T cell help, B cell activation is a negative signal, which induces tolerance towards the antigen (Jacquemin *et al.*, 2001). Activation of T cell-dependent B cell responses induces short-lived plasma cell differentiation and the germinal centre (GC)

development of affinity-matured, long-lived plasma cells and memory B cells (McHeyzer-Williams, 2003). The serial synapse model envisages that the primary immune response develops in three phases beginning at the site of antigen entry, progresses to lymphocyte activation and finally to the development of long term memory (McHeyzer-Williams *et al.*, 2003). B cells undergo somatic hypermutation in germinal centres, where antibody variants affinity-mature (McHeyzer-Williams *et al.*, 2001). Somatic hypermutation in germinal centres has a dual role; 1) it improves the affinity of antibody towards its antigen, 2) it deletes self reactive antibody variants that may arise due to loss of antibody specificity for its antigen (Nossal, 1991).

Sheep cathepsin L is sufficiently immunogenic in rabbits and chickens because, upon innoculation, both produced antibodies of high titre (Coetzer *et al.*, 1992). However, in the present study, rabbit cathepsin L (self antigen) was used to immunise rabbits, and normally self antigens fail to elicit B cell responses because T cell help is not available. Therefore, bovine α-glucosidase was coupled to rabbit cathepsin L to provide T helper cell epitopes. Two main protocols were used to break tolerance towards rabbit cathepsin L. First, two rabbits were immunised with a (foreign) α-glucosidase-(self)cathepsin L conjugate. Second, another pair of rabbits was primed with sheep cathepsin L and received rabbit cathepsin L boosters. A third pair of rabbits served as a control and were immunised with sheep cathepsin L. Rabbits that were initially immunised with the conjugate were inoculated with sheep cathepsin L nine weeks after the final immunisation with the conjugate to compare the nature of the response when a self protein rather than a foreign protein was used to prime the immune system.

4.2 Conjugation of bovine α-glucosidase to rabbit cathepsin L

Bovine acid α-glucosidase was chemically linked to rabbit cathepsin L using glutaraldehyde, a homobifunctional linker. Cathepsin L is optimally active at around pH 5.5, and as the pH increases beyond 7 it becomes irreversibly denatured (Turk *et al.*, 1999). Glutaraldehyde reacts principally with the amines of lysine groups and N-terminal residues (Scheme 4.1) (Hudson and Hay, 1980).



Scheme 4.1 Reaction of glutaraldehyde with two proteins.
The first CHO group reacts with NH_2 groups in the first protein and the second protein is coupled to the first by reacting with the second CHO group of glutaraldehyde. Adapted from Hudson and Hay (1980).

NH_2 groups are dominant at high pH, where cathepsin L is denatured, so that it would be impossible to assay for its activity in the conjugate. A trade off was, therefore, made, whereby the reaction was carried out at pH 6.5, using a 50 molar excess of glutaraldehyde over cathepsin L over a period of 4 h. The use of a two-enzyme system provides a reliable assay for assessing the success of conjugation. Sheep cathepsin L is sufficiently immunogenic in rabbits to not require coupling to a carrier protein (Coetzer, 1992). However, rabbit cathepsin L is not immunogenic in rabbits because of the absence of T cell help. Bovine α-glucosidase was coupled to cathepsin L to specifically provide T cell epitopes which would prime T helper cells to augment anti-cathepsin L B cell responses.

4.2.1 Reagents

Rabbit cathepsin L. Rabbit liver cathepsin L was isolated as described in Section 3.2.2.

Bovine α-glucosidase. Bovine liver α-glucosidase was purified as described in Section 3.3.2.

Conjugation buffer [0.15 M phosphate buffer, pH 6.5]. NaH_2PO_4 (41.4 g) was dissolved in 1.9 L dist.H_2O, adjusted to pH 6.5 with NaOH and made up to 2 L with dist.H_2O.

Elution buffer [0.15 M phosphate buffer, 0.25 M glycine, 0.25% maltose, pH 6.5]. NaH_2PO_4 (41.4 g), glycine (37.5 g) and maltose (5.0 g) were dissolved in 1.9 L dist.H_2O, adjusted to pH 6.5 with NaOH and made up to 2 L with dist.H_2O.

4.2.1.1 Procedure

The method used was essentially that described by Hudson and Hay (1980) for conjugation of alkaline phosphatase to IgG. Bovine α-glucosidase (1 mg, 0.013 μmoles) was added to a solution of rabbit cathepsin L (1.2 mg, 0.033 μmoles) and dialysed overnight at 4°C against conjugation buffer. The solution was equilibrated to room temperature and glutaraldehyde (66 μl of a 25% solution, 1.65 μmoles, 50 fold molar excess over cathepsin L) was added dropwise with gentle stirring. The reaction was allowed to continue for 4 h at room temperature, with periodic stirring. Free glutaraldehyde in the conjugate was quenched with elution buffer, and the solution was dialysed against the same buffer before loading it onto a Sephadex G-100 column (2.5 x 78 cm = 383 ml bed volume). The column was eluted with elution buffer (flow rate = 50 ml/h; 10 cm/h). Individual fractions were evaluated by zymography and non-reducing SDS-PAGE gels (12.5%) visualised by imidazole-SDS-zinc reverse staining (Section 2.5.3.1). Cathepsin L and α-gluosidase activities assays were performed as described in Section 3.2.1 and Section 3.3.1, respectively. Fractions corresponding to both cathepsin L and α-glucosidase activities (Fig. 4.1) were pooled and concentrated by dialysis against PEG 20 000.

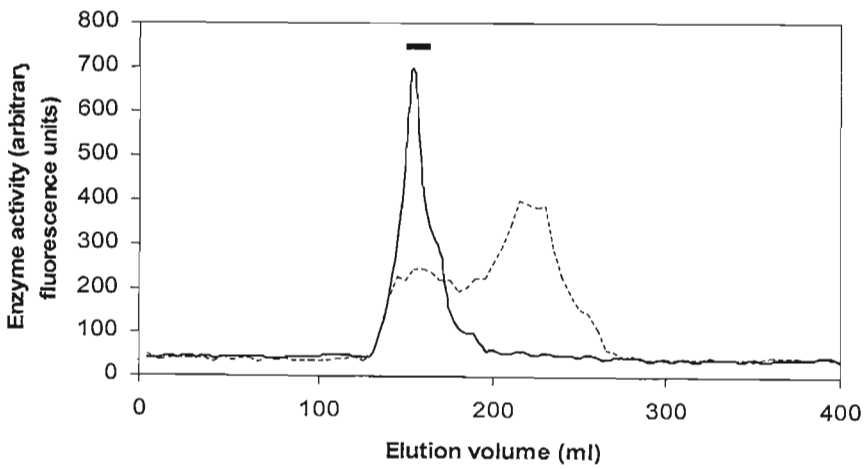


Figure 4.1 Chromatography of the reaction mixture on Sephadex G-100. Column, 2.5 x 78 cm (383 ml bed volume), was equilibrated with elution buffer [0.15 M phosphate buffer, 0.25 M glycine, 0.25% maltose, pH 6.5], and eluted with the same buffer; flow rate, 50 ml/h; (----) cathepsin L activity (arbitrary fluorescence units) against Z-Phe-Arg-NHMec, α-glucosidase activity (arbitrary fluorescence units) against (—) 4-Methyl-umbelliferyl-α-glucoside, (▬) pooled fractions.

4.3 Production of anti-cathepsin L antibodies in rabbits

Cathepsin L antibodies were raised in rabbits in two ways. In the first, two rabbits (designated R1 and R2) were immunised with the α-glucosidase-cathepsin L conjugate (a total of 24 μg) emulsified with Freund's complete adjuvant (FCA) (for the first inoculation) in a 1:1 ratio by trituration using a syringe. A total of 1 ml was injected subcutaneously on the back of each rabbit at several sites. Subsequent inoculations were performed in a similar manner at two, four and six weeks, but the immunogen was emulsified with Freund's incomplete adjuvant (FIA).

In the second, six rabbits were immunized with a total of 50 μg rabbit or sheep cathepsin L. Two test rabbits (designated RT1 and RT2) were primed with sheep cathepsin L emulsified with FCA, followed by boosters with rabbit cathepsin L emulsified with FIA. Control rabbits (designated RC1 and RC2) were immunized with sheep cathepsin L for the entire immunsation period. The two rabbits (in this instance designated RR1 and RR2 for antibody identification) that had previously received the conjugate were inoculated with sheep cathepsin L emulsified in FIA.

Rabbits were bled from the marginal ear vein before the first inoculation and at four and eight weeks post immunisation. Serum was separated from the clots and the IgG was isolated as described in Section 2.8, and stored at -20°C. Antibody production was tested using ELISA and antibody specificity was determined by western blots.

4.4 Enzyme linked immunosorbent assay (ELISA)

Antibody production was monitored using ELISA, a technique that is based on specific antigen/antibody interactions. A three layer system (indirect ELISA) is the most commonly used. In this system, antigen is immobilised on a microtitre plate. Unoccupied sites are blocked with an inert protein to reduce non-specific adsorption of detection molecules. The specific antibodies to be quantified (the primary antibody) are added and after washing excess antibody, formation of the antigen-antibody complex is detected by anti-species enzyme-linked antibody (secondary antibody). The enzyme reacts with a substrate that yields a coloured product upon hydrolysis, which can be quantified spectrophotometrically.

Antibodies raised against the conjugate were tested against pure rabbit cathepsin L rather than the conjugate because antibodies against the linked protein are also produced during a response towards the target protein.

4.4.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. NaCl (8.0 g), KCl (0.2 g), $Na_2HPO_4.H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 1 L dist.H_2O.

0.5% (m/v) Bovine serum albumin-PBS (BSA-PBS). BSA (0.5 g) was dissolved in 100 ml PBS.

0.1% PBS-Tween. Tween 20 (1 ml) was made up to 1 L with PBS.

Substrate buffer [10% (w/w) diethanolamine, 0.01% (m/v) $MgCl_2$, pH 9.8]. Diethanolamine (97 ml) was dissolved in approximately 1 L of a 0.01% $MgCl_2$ solution and adjusted to pH 9.8 with HCl. The solution was made up to 1 L with dist.H_2O.

Substrate solution [1.0 mg/ml p-nitrophenylphosphate (p-NPP)]. p-Nitrophenylphosphate (0.015g) was dissolved in 15 ml substrate buffer (for one ELISA plate).

Stopping solution [2 M sodium carbonate]. Sodium carbonate (42.2 g) was dissolved in 200 ml dist.H_2O.

Carbonate coating buffer. $NaHCO_3$ (0.21 g) was dissolved in 45 ml dist.H_2O, titrated to pH 6.0 with HCl and made up to 50 ml with dist.H_2O.

4.4.2 Procedure

Wells of a microtitre plate (Nunc Immunoplate) were coated with antigen (150 µl) at a concentration of 1 µg/ml in carbonate coating buffer overnight at room temperature. Wells were blocked with BSA-PBS (200 µl) for 1 h at 37°C to prevent non-specific binding of Ab, and washed three times with PBS-Tween (Tween is a non-ionic detergent which blocks hydrophobic interactions). Serial two-fold dilutions of the primary IgG in BSA-PBS (starting at 250 µg/ml as determined by checkerboard ELISA) were added (100 µl) and incubated for 1 h at 37°C. Excess antibody was washed three times with PBS-Tween. A 1/30000 dilution of goat anti-rabbit IgG-AP conjugate in BSA-PBS was added (120 µl) to each well and incubated for 1 h at 37°C, and the plates were washed three times with PBS-Tween. The p-NPP substrate

solution (150 µl) was added and the colour was allowed to develop in the dark against the background of the negative controls. The enzyme reaction was stopped by addition of sodium carbonate (150 µl) and the absorbance was read at 405 nm in a Bio-Tek EL 307 ELISA plate reader.

4.5 Competition ELISA

Rabbits that were inoculated with both sheep and rabbit cathepsin L (RT1, RT2, RR1 and RR2) could produce antibodies against both species variants. To test the specificity of anti-cathepsin L autoantibodies produced in these rabbits, a form of competition ELISA was necessary.

4.5.1 Reagents

As described in Section 4.4.1.

4.5.2 Procedure

Wells of a microtitre plate (Nunc Immunoplate) were coated with rabbit cathepsin L (wells A to F) and sheep cathepsin L (wells G and H) (150 µl) at a concentration of 1 µg/ml in carbonate coating buffer overnight at room temperature. Wells were blocked with BSA-PBS (200 µl) for 1 h at 37°C to prevent non-specific binding of Ab, and washed three times with PBS-Tween. In microfuge tubes, serial two-fold dilutions of the primary IgG in BSA-PBS (starting at 250 µg/ml) were prepared (100 µl) and sheep cathepsin L was added (1µg/ml) and incubated for 15 min at 37°C. Long periods of incubation were avoided because the reactivity between the two proteins is high which could lead to false results. Aliquots of 100 µl were added to the wells of the microtitre plate and incubated for 1 h at 37°C. Subsequent steps were performed as described in Section 4.4.2.

4.6 Measurement of antibody avidity

Avidity is a measure of the overall binding constant, which is the functional affinity for all the antibody molecules in an antiserum (Roitt and Delves, 2001). Antibody/antigen interactions follow the law of mass action, with antibody avidity determining the overall rate of the reaction (Whicher and Perry, 1984). Antibodies form non-covalent bonds with antigens and the reaction depends upon "closeness of fit", which results in continuous association and dissociation that occurs with rearrangement of binding sites (Whicher and Perry, 1984). High avidity is mostly

favoured over low avidity, because it is associated with a wide variety of functions *in vivo*, including immune elimination of antigen, virus neutralisation and a protective role against bacteria (Roitt and Delves, 1984). Therefore, if high avidity antibodies could be produced against self cathepsin L, they may inactivate cathepsin L *in vivo* and subsequently lead to tumour regression. The method used for determining antibody avidity in this study was that of Chakerian *et al.* (2001), as described below.

4.6.1 Reagents

Urea, 8 M. Urea (14.4 g) was dissolved in 30 ml PBS.

All other reagents were as described in Section 4.4.2.

4.6.2 Procedure

Wells of a microtitre plate (Nunc Immunoplate) were coated with rabbit or sheep cathepsin L (150 µl) at a concentration of 1 µg/ml in carbonate coating buffer overnight at room temperature. Wells were blocked with BSA-PBS (200 µl) for 1 h at 37°C to prevent non-specific binding of Ab, and washed three times with PBS-Tween. Appropriate antibody dilutions (depending on the titre of each antibody) of the primary IgG in BSA-PBS (100 µl) were added and incubated for 1 h at 37°C. Excess antibody was washed off three times with PBS-Tween. Triplicate wells were treated with either PBS or 8 M urea for 5 min. Wells were washed three times with PBS-Tween and the ELISA was performed as described in Section 4.4.2. The avidity index was calculated as the ratio of the mean absorbance values of urea-treated wells to PBS control wells multiplied by 100.

(Urea-washed wells/PBS-washed wells) x 100 .....*4.1*

where values x $\geq$ 50%, 50% > x > 30%, and x $\leq$ 30%, were defined as high, intermediate, and low avidity respectively (Chakerian *et al.*, 2001).

4.7 Western blotting

Western blotting involves electrophoretic transfer of proteins separated on SDS-PAGE gels onto a support matrix where they are available for further analysis (Towbin *et al.*, 1979). In immunological studies, this technique usually complements ELISA to assess the specificity of an antibody for its antigen.

Proteins are transferred from a polyacrylamide gel to a nitrocellulose filter. Nitrocellulose membrane has a high affinity for protein molecules. However, the

success of protein transfer requires a balance between experimental conditions that favour mobility of proteins out of the gel and binding to the nitrocellulose. SDS gives proteins a high mobility, but it disrupts hydrophobic interactions between protein and nitrocellulose, which compromises the effectiveness of binding. On the other hand, omission of SDS compromises protein transfer. Including methanol in the blotting buffer improves adsorption of proteins onto nitrocellulose.

Once the protein is successfully transferred to the membrane, it becomes available for a variety of analytical procedures, such as sequencing or characterisation of antigen/antibody interactions. In this study the specificity of antibodies was assessed by probing the transferred proteins with relevant antibody solutions. Transfer is often confirmed by staining the proteins, preferably with a reversible stain such as Ponceau S. Once the stain is removed the nitrocellulose is blocked with inert protein, usually non-fat milk, to prevent non-specific binding of protein to the nitrocellulose. The membrane is incubated first with the test antibody (primary antibody) followed by a secondary antibody conjugated to an enzyme which hydrolyses a substrate to yield a product which precipitates onto the membrane.

4.7.1 Reagents

Phosphate buffered saline. As described in Section 4.4.1.

5% (m/v) milk-PBS. Fat free milk powder (5 g) was dissolved in 100 ml of PBS.

Alkaline phosphatase buffer [100 mM Tris-HCl buffer, 0.5 mM $MgCl_2$, pH 9.5]. Tris (12.1 g) and $MgCl_2$ (0.233 g) were dissolved in 900 ml dist.H_2O, adjusted to pH 9.5 with HCl and made up to 1 L with dist.H_2O.

5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) alkaline phosphatase substrate. NBT (0.03 g) was dissolved in 70% (v/v) dimethyl formamide (DMF) (1 ml) and BCIP (0.015 g) was dissolved in 70% (v/v) DMF (1 ml). Immediately before use, BCIP (1 ml) and NBT (1 ml) were diluted to 100 ml with alkaline phosphatase buffer.

4.7.2 Procedure

After separation of proteins on SDS-PAGE (Section 2.4.2), on duplicate gels, one gel was used for blotting and the other was stained. The gel was washed in blotting buffer for 15 min. The transfer cassette, three pieces of blotting paper placed on fibre

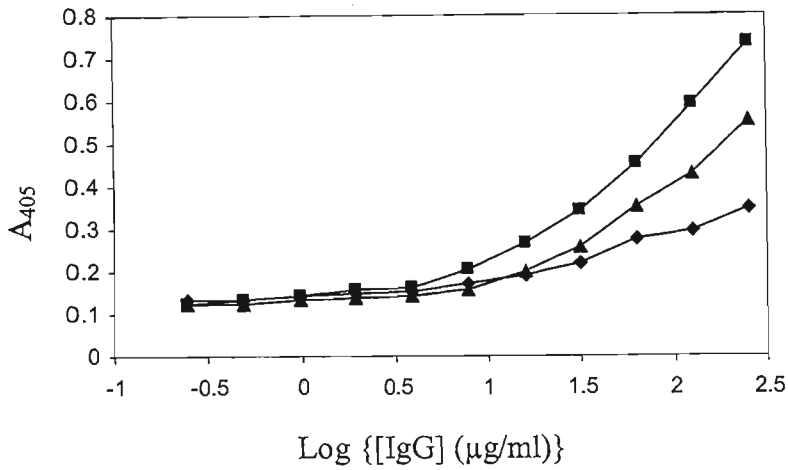
pads on either site of the transfer cassette and the nitrocellulose membrane were soaked in blotting buffer. The gel was placed on top of three layers of wetted blotting paper and the nitrocellulose membrane (cut to fit the dimensions of the gel) was placed on the gel and air bubbles were dislodged by rolling a 5 ml pipette over the nitrocellulose. Three more layers of wetted blotting paper were placed over the nitrocellulose and the transfer cassette was closed. The sandwich was transferred to a half full blotting chamber (Hoefer TE Transphor unit) and inserted between the two electrodes such that the nitrocellulose was on the anodal side. The tank was filled with blotting buffer and the blotting chamber was attached to a power supply. Electroblotting was performed at 200 mA for 16 h, the system being cooled to about 4°C by a circulating water bath.

Following transfer, the nitrocellulose was air dried for about 1.5 h. Protein bands were visualised with ponceau S stain and the nitrocellulose was cut into strips corresponding to the number of samples. The stain was washed off and the nitrocellulose strips were blocked with non-fat milk in PBS for 1 h. The membranes were washed with PBS (3 x 5 min) and antibody samples diluted in BSA-PBS were added and incubated for 2 h. Following washing with PBS (3 x 5 min), the strips were incubated in goat anti-rabbit IgG-alkaline phosphatase conjugate at a dilution of 1/50 000 in BSA-PBS (1 h). The strips were washed with PBS (3 x 5 min) and subsequently incubated in BCIP/NBT substrate and reacted in the dark until bands were visible against the background of the non-immune control. The reaction was stopped by washing the strips in distilled water. The membrane strips were dried between pieces of filter paper to preserve the bands.

4.8 Results

Anti-cathepsin L autoantibodies were produced in two male rabbits (R1 and R2) that were immunised with the α-glucosidase-(rabbit) cathepsin L conjugate (Fig. 4.2). Rabbit 1 responded more than rabbit 2.

A



B

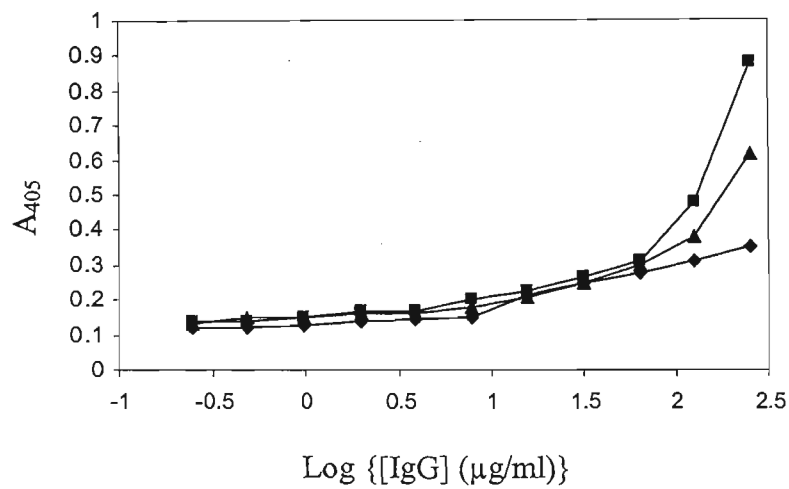


Figure 4.2 Analysis of antibody production in rabbits immunised with α-glucosidase-cathepsin L conjugate.

Rabbit cathepsin L was coated onto the ELISA plates at a concentration of 1 µg/ml, and incubated with serial two fold dilutions of anti-cathepsin L antibodies at weeks 4 and 8. Binding was visualised by goat anti-rabbit IgG-alkaline phosphatase conjugate. (♦) week zero, (▲) week 4, (■) week 8. Each point is the mean of duplicate A_{405} values. R1 (panel A), R2 (panel B).

Antibodies were found to be specific for rabbit cathepsin L when tested in western blots (Fig. 4.3).

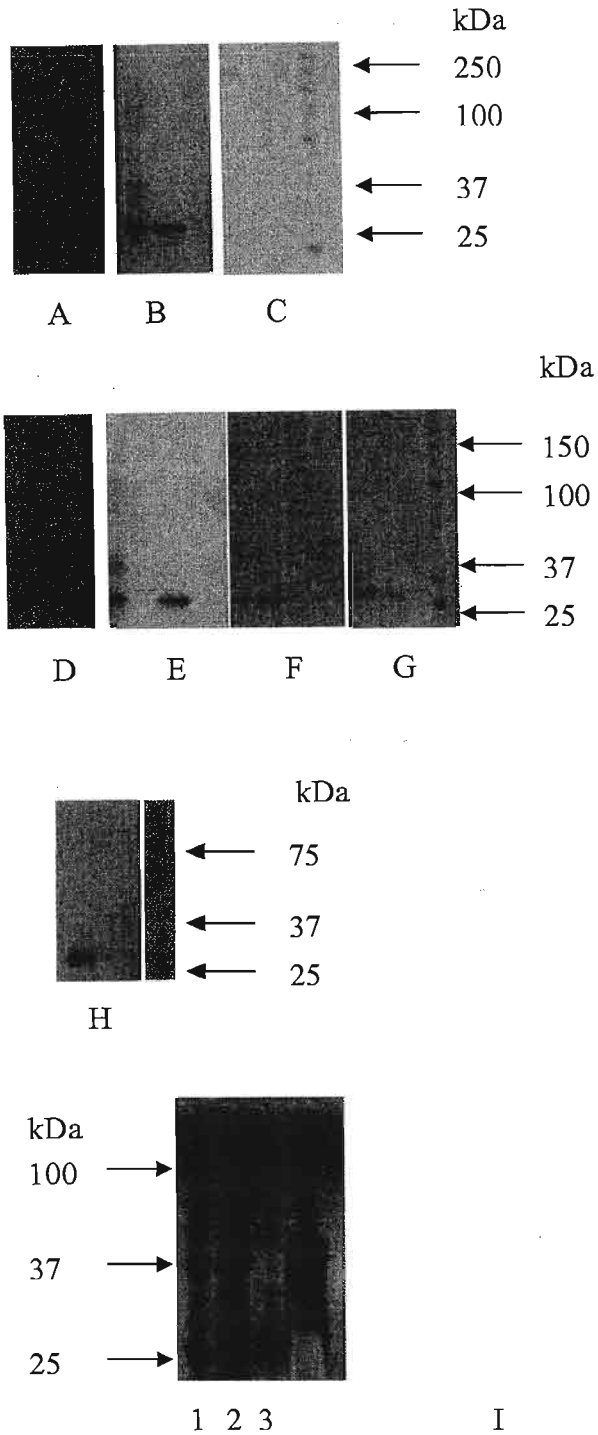


Figure 4.3 Western blot analysis of anti-rabbit cathepsin L autoantibody specificity. Molecular weight markers (Precision plus protein standards) recombinant protein markers ranging from 250-10 kDa. Template gel (I) used for blotting, Lane 1 (MWM), lane 2 (20-40% TPP fraction from rabbit cathepsin L isolation), lane 3 (pure sheep cathepsin L). Blots: A (R1) and B (R2) were probed with anti-cathepsin L autoantibodies, C was probed with non-immune IgG. D (RR1), E (RR2), F (RT1), G (RT2), H (RC1).

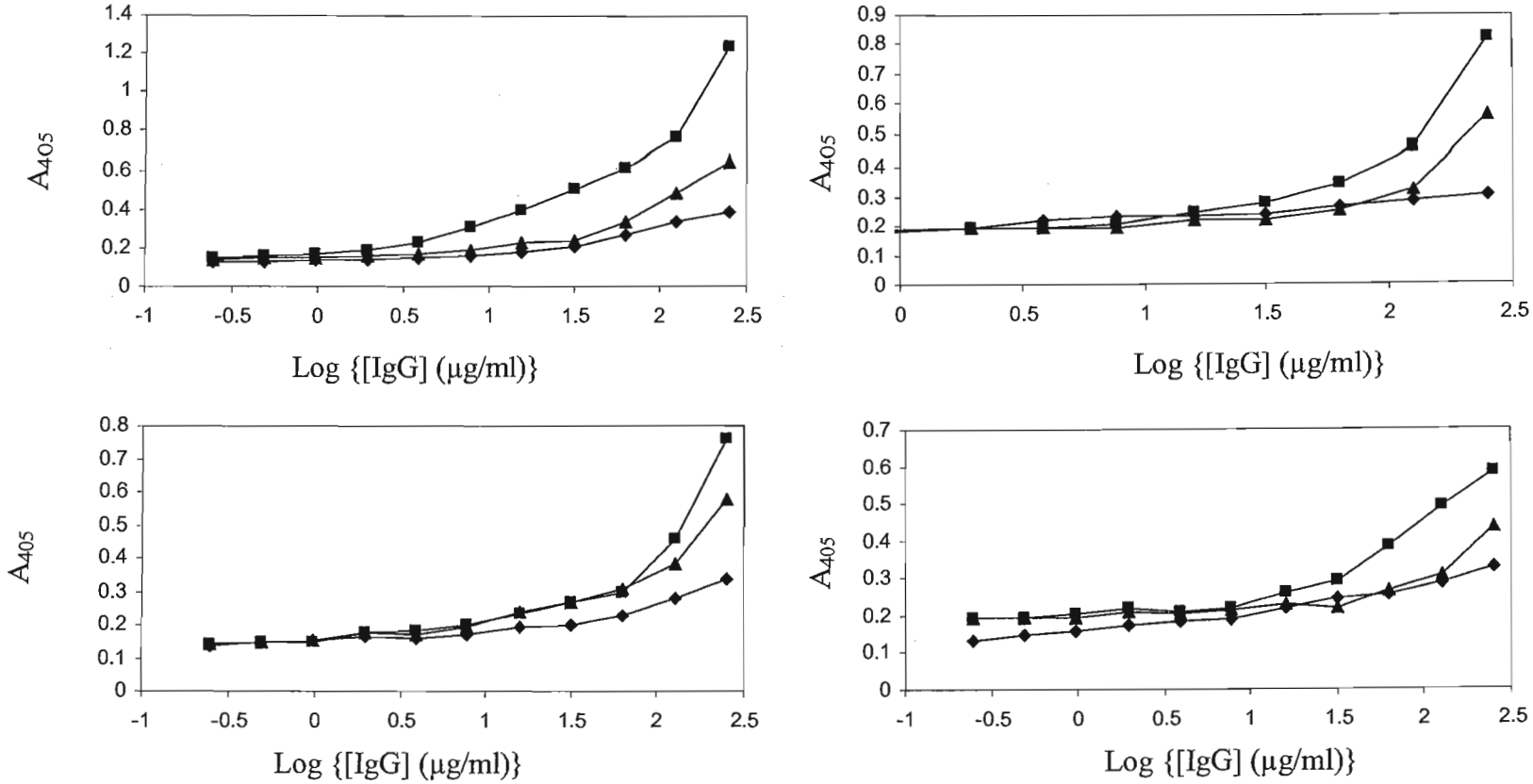


Figure 4.4 Analysis of antibody production in rabbits inoculated with conjugate and boosted with sheep cathepsin L.
Rabbit cathepsin L (A and C) or sheep cathepsin L (B and D) was coated onto the ELISA plates at a concentration of 1 µg/ml, and incubated with serial two fold dilutions of IgG preparations (starting at 250 µg/ml) obtained at weeks 4 and 8 from rabbits inoculated with the conjugate and boosted with sheep cathepsinL (RR1 for A and C and RR2 for B and D). Binding was visualised by goat anti-rabbit IgG-alkaline phosphatase conjugate. (♦) week zero, (▲) week 15, (■) week 20. Each point is the mean of duplicate A_{405} values.

Nine weeks after the final inoculation with the conjugate, rabbits were injected with sheep cathepsin L (RR1 and RR2). Antibody analysis in ELISA at week 15 and week 20 showed a high response against rabbit cathepsin L. These antibodies appeared to be of higher titre when tested against rabbit cathepsin L than when tested against sheep cathepsin L (Fig. 4.4). Analysis on western blots indicated that the antibody preparation comprised both rabbit anti-cathepsin L autoantibodies and anti-sheep cathepsin L antibodies (Fig. 4.3). Further characterisation of antibodies in a form of competition ELISA indicated that antibodies against rabbit cathepsin L were produced following inoculation with sheep cathepsin L (Fig. 4.5). The validity of this method was tested with anti-cathepsin L antibodies produced in control rabbits that were immunised with sheep cathepsin L (RC1). The response was similar to the non-immune preparation, showing that antibodies specific to sheep cathepsin L formed complexes with cathepsin L in solution, thus making them unavailable for binding to immobilised sheep cathepsin L.

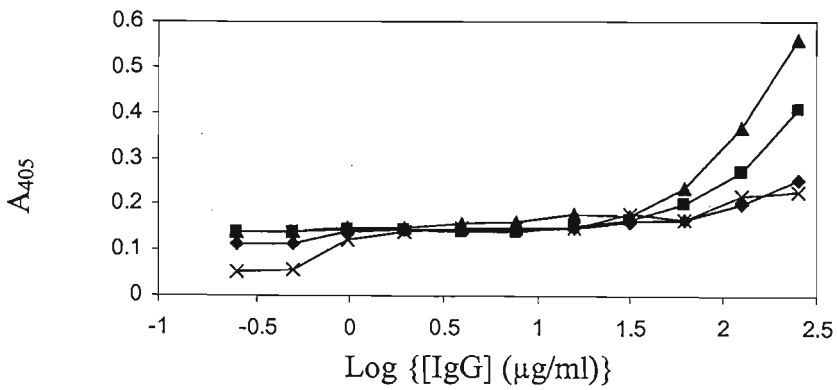


Figure 4.5 Analysis of antibody production in rabbits inoculated with sheep cathepsin L and boosted with rabbit cathepsin L, in a competition ELISA.

Plates were coated with rabbit cathepsin L. Serial dilutions of the primary antibody (week 4, starting at 250 µg/ml) were pre-mixed with pure sheep cathepsin L (1 µg/ml solution) for 15 min and thereafter added to the wells and incubated for 1 h. Binding was visualised by goat anti-rabbit IgG-alkaline phosphatase conjugate. Non-immune (♦), RT1 (▲), RT2 (■), control rabbit RC1 (x). Each point is the mean of duplicate A_{405} values.

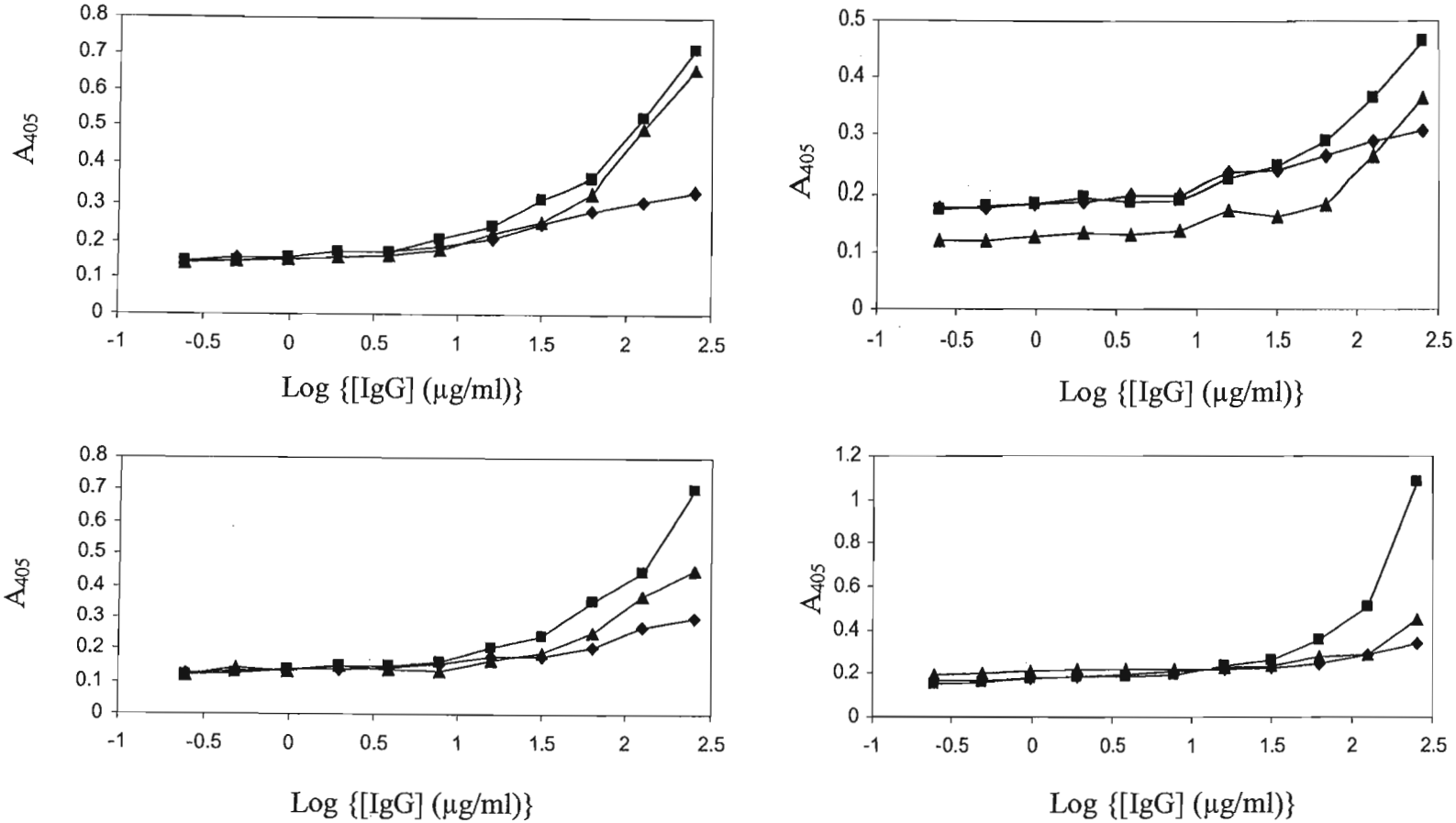


Figure 4.6 Reactivity of antibody produced in rabbits primed with sheep cathepsin L and boosted with rabbit cathepsin L. Rabbit cathepsin L (A and C) or sheep cathepsin L (B and D) was coated onto the ELISA plates at a concentration of 1 µg/ml, and incubated with serial two fold dilutions of IgG preparations (starting at 250 µg/ml) obtained at weeks 4 and 8 from rabbits RT1 (A and C) and RT2 (B and D). Binding was visualised by goat anti-rabbit IgG-alkaline phosphatase conjugate. (♦) week zero, (▲) week 4, (■) week 8. Each point is the mean of duplicate A_{405} values.

Rabbits that were primed with sheep cathepsin L and thereafter received rabbit cathepsin L boosters (RT1 and RT2) produced antibodies against both rabbit and sheep cathepsin L (Fig. 4.6). Western blots showed that anti-cathepsin L autoantibodies were specific for rabbit cathepsin L, while a certain proportion of the antibodies targeted sheep cathepsin L, indicating that the IgG preparation consisted of anti-cathepsin L autoantibodies and anti-sheep cathepsin L antibodies (Fig. 4.3). This was also confirmed in a competition ELISA, whereby effective binding of anti-cathepsin L autoantibodies was demonstrated after removing anti-sheep cathepsin L specific antibodies (Fig. 4.7).

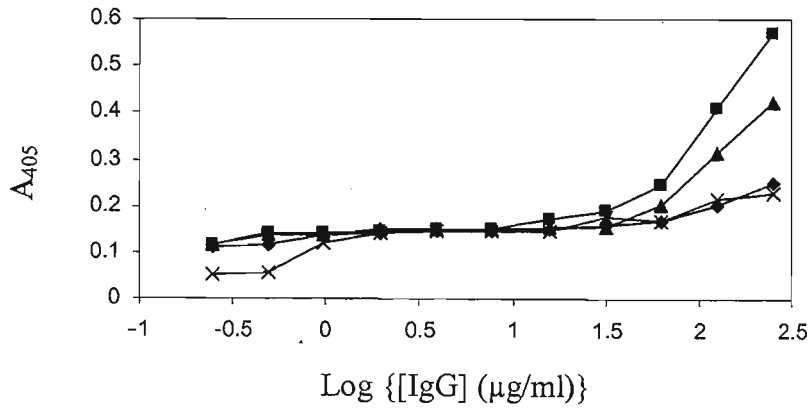


Figure 4.7 Binding of anti-cathepsin L specific antibodies (generated in rabbits that were inoculated with sheep cathepsin L nine weeks after the final immunisation with the conjugate) to purified rabbit cathepsin L in a competition ELISA.

Plates were coated with rabbit cathepsin L. Serial dilutions of the primary antibody (week 4, starting at 250 µg/ml) were pre-mixed with pure sheep cathepsin L (1µg/ml solution) for 15 min and thereafter added to the wells and incubated for 1 h. Binding was visualised by goat anti-rabbit IgG-alkaline phosphatase conjugate. Non-immune (♦), RR1 (▲), RR2 (■), control rabbit (RC1) (x). Each point is the mean of duplicate A_{405} values.

Control rabbits (RC1 and RC2) produced anti-sheep-cathepsin L antibodies satisfactorily as expected. The response in both rabbits was similar so results for only one rabbit (RC1) are shown (Fig. 4.8).

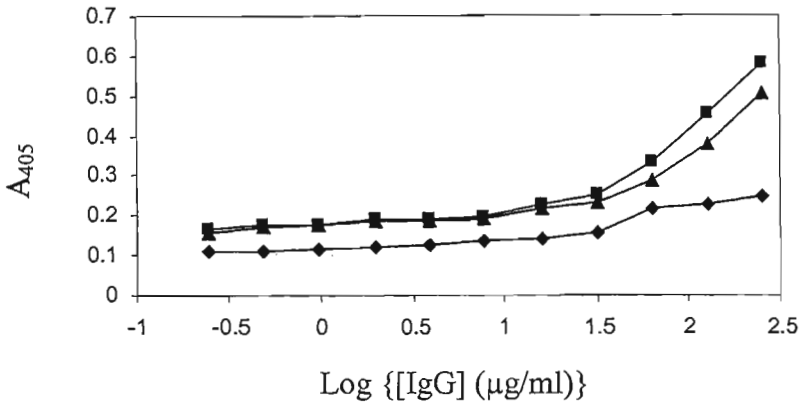


Figure 4.8 Analysis of antibody production in rabbits immunised with sheep cathepsin L. Sheep cathepsin L was coated onto the ELISA plates at a concentration of 1 µg/ml, and incubated with serial two fold dilutions of rabbit anti-(sheep) cathepsin L antibodies starting at 250 µg/ml at weeks 4 and 8. Binding was visualised by goat anti-rabbit IgG-alkaline phosphatase conjugate. (♦) week zero, (■) week 4, (▲) week 8. Each point is the mean of duplicate A_{405} values.

All antibody preparations were of high avidity when tested against both rabbit and sheep cathepsin L (Table 4.1). Control rabbits which were immunised with sheep cathepsin L produced antibodies that were of high avidity when tested against rabbit cathepsin L indicating that antibodies produced against both rabbit and sheep cathepsin L recognised very similar epitopes.

Table 4.1 Avidity measurements of anti-cathepsin L autoantibodies.

"Antibody identification"	Avidity index (%) of antibody tested against	
	Rabbit cathepsin L	Sheep cathepsin L
R1[a]	91	87
R2	93	82
RR1	92	81
RR2	99	84
RT1	92	94
RT2	82	77
RC1	86	95

[a]Antibodies were isolated from sera collected (week 4 after the first inoculation) from rabbits that were immunised with α-glucosidase-cathepsin L conjugate (R1 and R2), sheep cathepsin L nine weeks after the final inoculation with the conjugate (RR1 and RR2), primed with sheep cathepsin L and received rabbit cathepsin L boosters (RT1 and RT2) and sheep cathepsin L (RC1), respectively.

4.9 Summary of results

The ELISA results presented in this chapter are summarised in Table 4.2

Table 4.2 Summary of ELISA analyses of antibodies produced.

Figure	Immunogen		Auto-Abs to rabbit cathepsin L	Abs to sheep cathepsin L
	Primer	Booster		
4.2	Rabbit cathepsin L - bovine α-glucosidase conjugate	Rabbit cathepsin L	+++	NA
4.4	Rabbit cathepsin L - bovine α-glucosidase conjugate	Sheep cathepsin L	+++	++
4.5	Sheep cathepsin L	Rabbit cathepsin L	++[1]	NA
4.6	Sheep cathepsin L	Rabbit cathepsin L	++	++
4.7	Rabbit cathepsin L - bovine α-glucosidase conjugate	Sheep cathepsin L	+++[1]	NA
4.8	Sheep cathepsin L	Sheep cathepsin L		

[1]Competition ELISA – rabbit cathepsin L (adsorbed to plate) in competition with soluble sheep cathepsin L (reveals rabbit cathepsin L-specific Abs).

The results suggest that autoantibodies to cathepsin L may be elicited in different ways: either by conjugation to an allogeneic protein, which provides non-self T cell epitopes, or by simply priming with allogeneic cathepsin L and boosting with syngeneic cathepsin L. A slightly higher titre of rabbit cathepsin L autoantibodies is obtained, however, when the conjugate is used for priming, compared to priming with allogeneic (sheep) cathepsin L. When the conjugate is used for priming, it appears to make little difference whether boosting is effected with allogeneic or syngeneic cathepsin L.

Both competition ELISAs (Figs. 4.5 and 4.7 and Table 4.2) and western blotting (Fig.4.3) confirmed that antibodies specific to rabbit cathepsin L had been elicited.

CHAPTER 5
GENERAL DISCUSSION

The objective of the present study was to break self tolerance towards rabbit cathepsin L and induce auto-antibody responses. Katz *et al.* (1970) showed that non-immunogenic haptens can be modified by conjugation to immunogenic proteins (carriers) that provide cognate T cell help to the hapten specific B cells, so that antibodies against the hapten can be produced. Haptens are non-immunogenic because they lack repetitive epitopes and, therefore, require cognate T cell help to induce B cell responses (McHeyzer-Williams *et al.*, 2003). However, self proteins require T cell help to induce antibody responses because antigen specific T cells have been tolerised and, therefore, T cell help is not available.

It was proposed that, by conjugating a self protein to a strong T cell antigen, the required T cell help may be provided. This was demonstrated in this study whereby rabbits injected with the α-glucosidase-cathepsin L conjugate made antibodies against rabbit cathepsin L, indicating that tolerance towards cathepsin L may not be absolute. The antibodies produced were specific for cathepsin L as indicated by the western blots. Antibodies against α-glucosidase were also produced, although they were of lower titre than the anti-cathepsin L antibodies. Rabbits did not show any signs of autoimmune disease and remained healthy throughout the immunisation period.

Why must the carrier molecule be joined to the poorly immunogenic antigen as opposed to the two proteins being co-inoculated? T cell-dependent antibody responses require coordination between antigen specific T and B cells, which is termed linked recognition (Janeway *et al.*, 2005). This means that B and T cells recognise the same antigen, but not necessarily identical epitopes (Janeway *et al.*, 2005). B cells bind an antigen, internalise, process and present it to the T helper cell in the context of MHC class II molecules. Activated T cells in turn produce cytokines that induce B cell differentiation into antibody secreting plasma cells. However, if the two proteins were to be co-inoculated there would simply be competition between the two proteins, so that a protein with dominant epitopes will induce a strong response (Roitt and Delves, 2001). Alternatively, the two proteins may induce antibody responses independently of each other, as happens with cocktail vaccine preparations.

Bovine acid α-glucosidase in a conjugate was able to prime helper T cells necessary to direct B cell responses against rabbit cathepsin L, thus breaking T cell tolerance.

Rabbits that were primed with sheep cathepsin L and given rabbit cathepsin L boosters also produced antibodies against rabbit cathepsin L. But what purpose does priming rabbits with sheep cathepsin L serve in inducing anti-cathepsin L autoantibodies? According to the serial synapsis model, the development of T cell-dependent B cell primary immune response begins with recruitment, expansion and differentiation of antigen specific T helper cells (McHeyzer-Williams *et al.*, 2003). Priming rabbits with sheep cathepsin L was, therefore, necessary to deploy T cell help, meaning that when rabbit cathepsin L was inoculated, T cell help for rabbit cathepsin L specific B cells was already available. However, this does not mean that linked recognition is irrelevant in this regard because, as pointed out by Lin *et al.* (1991), antibody production towards the self antigen is most probable when the priming antigen shares similar epitopes with the self protein. Cross-reactivity between rabbit and sheep cathepsin L was demonstrated in this study by western blot analysis. Cross-reactivity between species variants of cathepsin L was also demonstrated by Mason (1986), whereby antibodies, raised against the human enzyme, recognised sheep, rabbit and rat enzymes.

In the initial stages of a primary immune response the antibody repertoire produced is quite diverse and generally not very specific (Roitt and Delves, 2001). Antibody molecules start to affinity-mature in germinal centres where they undergo somatic hypermutation (McHeyzer-Williams, 2003). This means that the affinity of the antibody improves with subsequent immunisations (Nossal, 1991). Initially, antibodies produced against sheep cathepsin L are of low affinity and by the time they reach germinal centres and start to hypermutate their V genes, they encounter rabbit cathepsin L and, therefore, the specificity towards sheep cathepsin L is lost while that towards rabbit cathepsin L is gained.

When rabbits that were initially immunised with the conjugate received sheep cathepsin L boosters nine weeks post-immunisation, they produced antibodies against rabbit cathepsin L in a manner that resembles secondary immune responses. Why would boosters with sheep cathepsin L improve responses against rabbit cathepsin L? In autoimmune diseases, such as multiple sclerosis and systemic lupus erythematosus,

reactivity from a single epitope spreads to other epitopes derived from the same antigen or other antigens derived from the same tissue, generating immune diversification termed determinant spreading (Ribas *et al.*, 2003). A similar phenomenon may have taken place in this case, although the mechanisms involved and the response conditions may be different. Sheep cathepsin L was mixed with Freund's incomplete adjuvant, which means that antibody production towards sheep cathepsin L may have been weak to begin with, but because rabbit and sheep cathepsin L share quite similar epitopes, circulating memory B cells may have been activated by this fact. T cell help is essential for memory responses (McHeyzer-Williams and McHeyzer-Williams, 2005), and the fact that these rabbits produced such high titre antibodies may indicate that long-term memory T helper cells were fully developed. It would have been useful to deliver rabbit cathepsin L boosters in rabbits that were immunised with the conjugate and compare their responses with the ones that received sheep cathepsin L.

T cell help can be provided in a number of ways. Chakerian *et al.* (2001) conjugated mouse tumour necrosis factor-α (TNF-α) to viral like particles (VLPs) so that it formed repetitive structures on the VLP surface. Inoculation of this conjugate in mice induced long-lasting high titre antibodies against mouse TNF-α (Chakerian *et al.*, 2001). Dalum *et al.* (1996) replaced a small segment of mouse ubiquitin with immunodominant T cell epitopes from OVA. Mice that were immunised with modified ubiquitin produced anti-ubiquitin autoantibodies of high titre.

In this study, it was demonstrated that tolerance towards a self protein can be broken and high-avidity anti-self antibody responses can be induced. The fact that rabbits in this study produced high avidity antibodies towards their own cathepsin L indicates that self tolerance may not be absolute. This opens a door for further studies: for example, it would be useful to study the effects of anti-cathepsin L autoantibodies on tumour-bearing rabbits. Tumours abberantly secrete (syngeneic) cathepsin L, so the results of the present study - especially that autoantibodies may be elicited by priming with allogeneic cathepsin L and boosting with syngeneic cathepsin L - raises the intriguing possibility that all that may be necessary to engineer cancer remission might be to inoculate with allogeneic cathepsin L, since the tumour itself would provide a continuous boost with syngeneic cathepsin L.

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