Induction of Auto-antibodies to Cathepsin B

by

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PREFACE

The experimental work described in this thesis was carried out in the School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg, from January 1998 to December 1999 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.

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ABSTRACT

Because tumours are comprised of "self" cells and antigens, they escape recognition by the immune system, which discriminates between "self" and "non-self". One such antigen is cathepsin B, a lysosomal cysteine proteinase, that has been implicated as one of the proteolytic enzymes involved in tumour invasion and metastasis. Cathepsin B autoantibodies could open possibilities which may be useful in cancer immunotherapy. In this study generation of cathepsin B autoantibodies was attempted by manipulating the immune system into recognising and responding to cathepsin B in complex with a "foreign" protein, bovine serum albumin (BSA).

Cathepsin B was isolated from rabbit liver using the three phase partitioning (TPP) method, modified by adding t-butanol in the homogenisation buffer. Isolation of cathepsin B and cathepsin L, using this novel method, minimised the formation of artefacts such as a covalent cathepsin L-stefin B complex and produced higher yields of enzyme.

Pure rabbit liver cathepsin B was conjugated to BSA, using glutaraldehyde as coupling agent, and administered intramuscularly into rabbits. Another three inoculation protocols, which functioned as controls were: i) free cathepsin B administered intramuscularly, ii) complexed cathepsin B administered intravenously, and iii) free cathepsin B administered intravenously.

IgGs isolated from inoculated rabbits' serum were assayed by a three layer ELISA system, immunoinhibition assays and dot blots. The anti-complex (intramuscular) antibodies showed the highest recognition for cathepsin B and were the only antibodies that were immunoinhibitory. This suggests that the immune system was, to some extend, successfully manipulated into recognising the complexed "self" cathepsin B.

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LIST OF ABBREVIATIONS

A_{280}	absorbance at 280 nm
Ab	antibody
ABTS	2,2'-azino-di-(3-ethyl)-benzthioline sulfonic acid
Ag	antigen
APC	antigen presenting cell
bis	*N,N'*-methylenebisacrylamide
BSA	bovine serum albumin
BSA-TBS	bovine serum albumin dissolved in Tris-buffered saline
t-butanol	2-methylpropan-2-ol
Bz	benzoyl
CD	cluster of differentiation
C-terminal	carboxy terminal
CTL	cytotoxic T lymphocyte
dist.H_2O	distilled water
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E	extinction coefficient
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBL	Friend virus-induced erythroleukemia
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
g	relative centrifugal force

h	hour(s)
HLA	human leukocyte antigen
HRPO	horseradish peroxidase
hsp	heat shock protein
IFN-γ	γ-interferon
IgG	immunoglobulin G
IL	interleukin
Igs	Immunoglobulins
K_{AV}	availability constant
kDa	kilodalton(s)
α_2M	α_2-macroglobulin
MHC	major histocompatibility complex
mIg	membrane immunoglobulin
min	minute(s)
M_r	relative molecular weight
MW	molecular weight
NHMec	7-amino-4-methyl coumarin
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS-Tween	Tween 20 diluted in phosphate-buffered saline
PEG	polyethylene glycol
RT	room temperature
s	second(s)

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TCA	trichloracetic acid
TCR	T cell receptor
TEMED	*N, N, N', N'*-tetramethylethylenediamine
Th (cells)	T helper (cells)
TIL	tumour infiltrating lymphocytes
TPP	three phase partitioning
tricine	*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Z	benzoyloxycarbonyl

CHAPTER 1
INTRODUCTION

A cancer cell is a cell that has lost control of growth and position. Normally, cells undergo carefully controlled proliferation, so that cell division is balanced by cell death and loss. Tumours or neoplasms develop as a result of the tumour cells escaping this fine balance between cell growth and death.

A tumour can either be benign or malignant. Benign tumours remain localised to the tissue from which they arise, growing by expansion (Burn and Meyrick, 1977). Malignant tumours grow by expansion as well as by infiltration. The malignant tumour cells become detached from the main mass, extend through the adjacent tissues, penetrate into body cavities and invade the neighbouring blood vessels and lymphatics. At the latter stage, tumour cells can be carried away by the lymph to local lymph nodes and from there to more distant lymph nodes and to organs such as the liver or lungs (Burn and Meyrick, 1977). At the distant sites, the tumour cells can settle, grow and give rise to a secondary or metastatic tumour. The sequence of events whereby a secondary tumour arises is called metastasis. The word "cancer", refers to malignant, metastasing tumours. The metastatic ability of cancers is what makes them so dangerous and difficult to treat. Before a cancer can metastase, i.e. spread to a distant site and form a secondary tumour, it must be able to invade neighbouring areas. It does this by breaking down the normal barriers that keep the tissue's cells in place. One such barrier is the basement basal lamella. Cancer cells commonly attack constituents of the extracellular matrix, the "glue" that binds cells together (Buckman, 1997). This attack on the extracellular matrix is carried out by a variety of enzymes, secreted by the cancer cells, that can break down specific components of the matrix.

One such class of enzymes, the lysosomal cathepsins, have been implicated in malignant progression of tumours through the observation that their activity and secretion or membrane association is increased in cancer cells. The cathepsins are normally found intracellularly, in the endolysosomal system, and play a variety of digestive and processing roles to maintain normal cellular metabolism (Yan *et al.*, 1998). Various studies have shown a correlation between secretion of cathepsins and pathological conditions. For example, cathepsins B and L are thought to be involved in arthritis, cathepsins B, D and S in Alzheimer's disease (reviewed by Yan *et al.*, 1998) and cathepsins B, D and L in cancer progression (Berquin and Sloane, 1996).

Cathepsin B [EC 3.4.22.1.], a lysosomal cysteine proteinase, has been more extensively studied than the other cathepsins, probably because of its implication in a variety of human

tumours. It was also the first lysosomal cysteine proteinase to be purified to homogeneity (Sloane *et al.*, 1990). Overexpression of cathepsin B activity has been reported in human tumour types, including carcinoma of the colon and rectum, breast, bladder, pancreas, lung, liver, oesophagus, stomach, cervix and ovary (reviewed by Sloane *et al.*, 1990). This overexpression has been correlated with malignant progression. Cells at the invasive edge of various tumours express the highest cathepsin B activity (Berquin and Sloane, 1996), and it was shown that cathepsin B activity in the plasma membrane/endosomal fractions of metastatic tumours is increased in parallel with metastatic capability (reviewed by Sloane *et al.*, 1990). Both normal and tumour cathepsin B can degrade the extracellular matrix proteins laminin, fibronectin and type IV collagen at neutral and acidic pH (Lah *et al.*, 1989; Buck *et al.*, 1992). These are components of the basement membrane of blood vessels, a structure which forms a barrier to metastasising tumour cells (Berquin and Sloane, 1996). Because the metastatic tumour cells move through extracellular matrices containing collagens, glycoproteins and proteoglycans, Sloane *et al.* (1990) hypothesised that the presence of lysosomal proteinases like cathepsin B and L and lysosomal glycosidases like β-hexosaminidase adjacent to the tumour cell surface may enable tumour cells to establish a local microenvironment in which focal degradation of connective tissue is enhanced. Dehrmann *et al.* (1995; 1996), however, showed that cathepsin B and L are active in the normal extracellular milieu, so a special microenvironment may not be necessary. Cathepsin B has also been implicated in the invasive processes of angiogenesis through the observation of its presence in endothelial cells of blood vessels irrigating tumours (Berquin and Sloane, 1996).

Alterations in the activity of cathepsin B in tumours may reflect changes in its synthesis, in activation and processing, in intracellular trafficking and delivery and in the endogenous inhibitors or cystatins (reviewed by Sloane *et al.*, 1990). Sloane *et al.* (1990), suggested that, because cathepsin B is released from tumour cells both in its active and latent forms, and because of its presence in membrane fractions as both the active and latent forms, more than one mechanism may be responsible for the redistribution and release of cathepsin B in malignant cells. Most of the reports on cathepsin B expression in tumours only assess activity and, therefore, the levels of regulation that are altered in each case is unknown (Berquin and Sloane, 1996).

These questions on the regulation of cathepsin B in tumours might be answered if one could follow the tissue and cellular distribution of the enzyme by immunocytochemistry. Antisera against lysosomal hydrolases are powerful tools to investigate the role of lysosomes in cell and tissue physiopathology, and an antibody that binds specifically to native and active cathepsin B would be a useful probe for both *in vivo* and *in vitro* studies where one requires only native enzyme to be recognised. Immunocytochemistry for the purpose of studying the role of cathepsin B in malignancy thus ideally requires antibodies against native cathepsin B.

In previous studies on the raising of antibodies specific for cathepsin B in an experimental animal (Pierart-Gallois *et al.*, 1977; Schwartz and Barrett, 1980; Graf *et al.*, 1981; Mort *et al.*, 1981; Moin *et al.*, 1992), cathepsin B, isolated from a different species from that of the experimental animal, was inoculated. Although the properties of cathepsin B vary very little between species, even a slight difference in amino acid composition may trigger an immune response if cathepsin B from one species is inoculated into a different animal species.

The primary objective of the project reported here was to investigate the possibility of raising auto-antibodies specific for cathepsin B, i.e., antibodies raised in rabbits against cathepsin B isolated from rabbit liver. Auto-antibodies against cathepsin B may be useful in cancer immunotherapy. In order for auto-antibodies to be raised, the immune system must be able to recognise the "self" antigen. This may be problematic, because of the immune system's ability to differentiate between "foreign" material (non-self) and the body's own tissue (self), causes the immune system to only recognise non-self antigens. In other words, a rabbit's immune system would normally fail to recognise inoculated native rabbit cathepsin B. However, we hypothesised that it may be possible to induce the formation of autoantibodies by complexing the enzyme to a foreign carrier protein. By complexing cathepsin B to a carrier protein such as bovine serum albumin (BSA), it was hypothesised that the immune system might recognise the cathepsin B-BSA complex, producing antibodies against the complex and simultaneously against the cathepsin B epitopes.

In order for the hypothesis to be fully understood, some basic theory on the humoral response will be described. Upon binding of an antigen to the surface "advertisement" immunoglobulin on B lymphocytes, which serves as the antigen receptor, a signal is transmitted to the cell interior. The bound antigen is internalised and returned to the cell surface as peptides bound to major histocompatibility complex (MHC) class II molecules. This peptide:MHC complex is recognised by helper T cells which deliver activating signals to the B cell. The B cells then differentiate into antibody-secreting plasma cells. In such thymus-dependent antibody responses, recognition of the same antigen by the helper T cell and the B cell, is called linked recognition. As a result of linked recognition, $CD4^+$ T cells that are specific for peptides of the antigen must first be activated to produce appropriate armed helper T cells. The armed helper T cells will in turn help only B cells whose receptors bind to the same antigen or one of its proteins. Although the epitope recognised by the armed helper T cell must be linked to that recognised by the B cell, the two cells need not recognise identical epitopes (Janeway and Travers, 1994).

Linked recognition plays a very important role in the regulation and manipulation of the humoral immune response. It ensures self tolerance. A B cell that binds a self antigen will present peptides derived from it on self MHC class II molecules. T cells that recognise peptides derived from self proteins are either eliminated in the thymus or inactivated in the

periphery, in the early neonatal period and, therefore, no T cell help will be available to activate such B cells. T cell tolerance therefore provides the mechanism for preventing the production of self-reactive antibodies (Janeway and Travers, 1994).

According to the hypothesis tested in the present study, it may be possible to circumvent T cell tolerance and generate auto-antibodies specific for cathepsin B, by linking the enzyme to BSA. In this way, the T cells may recognise the BSA portion of the cathepsin B-BSA complex as foreign, enabling them to activate B cells that recognise cathepsin B epitopes, causing these to produce antibodies against cathepsin B. A similar concept has been successfully used in the immunisation of infants against *Hemophilus influenzae* B, a bacterial pathogen that infects the lining of the brain and causes meningitis and, in severe cases, neurological damage or death (reviewed by Janeway and Travers, 1994). Protective immunity against this pathogen is mediated by antibodies to its capsular polysaccharide. Because T cell independent responses are weak in the immune system of infants, an effective vaccine was created by chemically linking tetanus toxoid, a foreign protein, to the polysaccharide. B cells that bind the polysaccharide component of the vaccine were activated by helper T cells specific for peptides of the linked toxoid protein.

A major problem found in previous studies (Graf *et al.*, 1981; Mort *et al.*, 1981; Wardale *et al.*, 1986; Moin *et al.*, 1992), was that the antibodies raised against cathepsin B recognised only the denatured form of the enzyme. This might be due to the fact that isolated cathepsin B is proteolytically inactive, as the active site cysteine becomes oxidised by atmospheric oxygen in the oxidative environment obtaining *in vitro* (Dehrmann *et al.*, 1996). This isolated, inactive cathepsin B, which is usually inoculated intramuscularly (Graf *et al.*, 1981; Mort *et al.*, 1981; Moin *et al.*, 1992) would survive in its native conformation for only a very short period of time under the extracellular conditions (Dehrmann *et al.*, 1996).

Cathepsin B activity is connected with the existence of an ion pair between the active site Cys-29 (pKa 3.6) and His-199 (pKa 8.6) called a thiolate imidazolium ($-S/^{+}H-$) ion pair (Meloun *et al.*, 1988; Hasnain *et al.*, 1992; Turk *et al.*, 1994). These two residues are parts of two different domains and the active site is formed only when the two domains are held together closely (Turk *et al.*, 1994). Turk *et al.* (1994) described the active site as a zipper-like conformation, kept together by hydrogen bonding, electrostatic and Van der Waal's forces, in which the active site $-S^{-}/H^{+}Im-$ion pair forms a stabilising "hook and eye".

Turk *et al.* (1994) showed that inactivation of cathepsin B in alkaline medium, is caused by the deprotonation of the imidazole moiety of the $-S^{-}/^{+}HIm-$ ion pair. This causes the enzyme to be destabilised and leads to unfolding. The inactive form of cathepsin B is stable under extracellular physiological conditions for only a very short period in its native form before it denatures (Dehrmann *et al.*, 1996). However, it was discovered by Dehrmann *et al.* (1996),

that by reducing the active site cysteine of the lysosomal cysteine proteinases, cathepsins B and L, the enzymes were not only activated, but their stability was markedly increased. Reductive activation is effected by removal of the group blocking the active site thiol. The consequent reconstitution of the thiolate-imidazolium ion pair leads to enhanced enzyme stability (Dehrmann *et al.*, 1996). Because the activated form of the enzyme is stable for longer periods of time, when injecting pre-activated cathepsin B into a test animal, there might be a greater chance of eliciting antibodies against native cathepsin B. Dehrmann *et al.* (1996) also suggested that an alternative route for eliciting anti-cathepsin B antibodies may be to inoculate activated cathepsin B directly into the bloodstream. This activated enzyme would not only be more stable, but it would be immediately inhibited and stabilised by cystatins (Turk *et al.*, 1993). Cathepsin B might be presented in this form or may be passed on to α_2-macroglobulin (Chu *et al.*, 1994) for immune presentation. This formed a second hypothesis explored in the present study.

α_2-Macroglobulin (α_2M) is a plasma proteinase inhibitor and inhibits endopeptidases of almost any class or specificity (Barrett and Starkey, 1973). It is composed of two noncovalently bonded pairs of identical subunits joined by disulfide bonds (Barrett and Starkey, 1973; Sottrup-Jensen *et al.*, 1983). Each subunit contains a specific sequence of amino acids termed the "bait region", which is highly susceptible to proteolytic cleavage (Barrett and Starkey, 1973). When this region is cleaved by a proteinase, a conformational change occurs in the inhibitor, resulting in irreversible binding of the proteinase (Barrett and Starkey, 1973). The proteinase is apparently inhibited, but retains activity against small substrates (Barrett and Starkey, 1973; Salvesen *et al.*, 1981; Chu and Pizzo, 1993). It is therefore more sterically shielded than inhibited.

Once the proteinase-α_2M complex is formed, it can undergo receptor-mediated endocytosis into macrophages and other cells bearing the α_2M receptor (Chu *et al.*, 1994). Chu *et al.* (1994) demonstrated that complexing antigen (Ag) with α_2M does enhance both the rate and efficiency of Ag uptake and presentation by macrophages. Therefore, coming back to the second hypothesis, if activated cathepsin B is injected straight into the bloodstream of a rabbit, it may encounter α_2M, cleave its "bait region", form a complex with the inhibitor and be taken up by antigen presenting cells (APCs). This may in turn cause an enhancement of the humoral response against cathepsin B. Cathepsin B would need to be reductively activated with cysteine, immediately before inoculation, for it to be stable for longer and in order for the enzyme to be able to cleave the α_2M "bait region".

In order to produce antibodies to rabbit cathepsin B, a source of the enzyme was required. It was necessary to isolate the enzyme "in-house", as rabbit tissue is not a usual source of this enzyme, and this required developing a new method. At the same time, this provided an opportunity to explore new approaches to the isolation of cathepsin B.

The aims of this study were thus, i) to generate auto-antibodies in rabbits against cathepsin B, by complexing cathepsin B to the carrier protein BSA, ii) to generate antibodies specific for the native active form of cathepsin B by reductively activating cathepsin B, before injecting it straight into the bloodstream. Both of these aims required the prior isolation of cathepsin B from rabbit liver. A third aim was to increase the yield of isolated cathepsin B, which we hypothesised might be achieved by the incorporation of t-butanol in the homogenisation buffer.

CHAPTER 2

MATERIALS AND METHODS

In this chapter methods will be described that are considered fundamental and were used routinely throughout this study. Also described in this chapter are methods which are considered to be relatively specialised but would hinder the intended structure of the relevant chapter. Methods that are considered to be more specialised will be described in the relevant chapters. In this chapter only reagents that were prepared before use are described, while reagents that were used "as-is" are referred to at the relevant point in the procedure.

2.1 Materials

Most common chemicals used were from BDH, Merck or Boehringer Mannheim and were of the highest purity available (AR grade). Coomassie brilliant blue R-250 was from Merck. Dialysis tubing, haemoglobin, 7-amino-4-methyl coumarin, dithiothreitol (DTT), S-Sepharose, Sephacryl S-100, Sephadex G-75 and Sephadex G-25 were from Sigma Chemicals Co., St. Louis, Mo. USA. Acrylamide and N,N'-methylenebisacrylamide were from BDH. N,N,N',N'-tetramethylethylenediamine (TEMED) was from Bio-Rad. BSA (Fraction V) and 2,2'-azino-di-(3-ethyl)-benzthiozoline sulfonic acid (ABTS) were from Boehringer Mannheim, SA. Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were from Cambridge Research Chemicals, UK. Freund's complete and incomplete adjuvants were from Difco, Mi., USA. Sheep anti-rabbit IgG was from NII (The Natal Institute of Immunology), Pinetown, South Africa. ELISA plates were Nunc-Immuno Maxisorp F96 plates from Weil Organisation, SA.

2.2 Protein assays

In protein purifications, protein quantitation after each step in the process is necessary. A method of protein determination that is rapid, reproducible and uses small amounts of sample is therefore advantageous. Thus, the Bradford dye-binding protein assay (Bradford, 1976), as modified by Read and Northcote (1981) was used in the present study.

2.2.1 Bradford dye-binding assay

The Bradford dye-binding assay involves the binding of Coomassie brilliant blue G-250 to protein. This dye exists in two different colour forms, the cationic red form, and the anionic blue form. The red form is converted to the blue form upon binding to protein, shifting the absorption maximum of the dye from 465 to 595 nm. The protein can thus be quantified spectrophotometrically at 595 nm. The protein-dye complex has a high extinction coefficient at 595 nm, leading to great sensitivity (Bradford, 1976).

Other than being reproducible, rapid, sensitive and linear, the Bradford dye-binding assay has the advantage of being insensitive to magnesium chloride, potassium chloride, sodium chloride, ethanol and ammonium sulfate, which are all commonly used chemicals. However, small amounts (less than 1%) of Tris, acetic acid, 2-mercaptoethanol, sucrose, glycerol, EDTA, and trace amounts of Triton X-100, sodium dodecyl sulfate (SDS) and Hemosol have small effects, but these can be eliminated by running a proper control with the assay (Bradford, 1976).

The major disadvantage of the Bradford-dye binding assay is the wide variation in colour yield of different individual proteins. Read and Northcote (1981) stated that because of this variation, it is important to choose a protein standard suitable for the application used. They also found that variation in the response of different proteins in the assay was greatly reduced by increasing the concentration of dye and modifying the acid/alcohol ratios. These modifications increased the colour yield to values much closer to that of the standard protein. Instead of using Coomassie brilliant blue G-250 dye, Read and Northcote (1981) used the more pure Serva blue G dye. The sensitivity of the assay was increased so that as little as 0.1 µg of protein (2 µg/ml in 50 µl) could be accurately determined.

The two protein assays that have been established are the macroassay with a working range of 5-25 µg of protein (Bradford, 1976) and the microassay with a working range of 1-5 µg protein (Read and Northcote, 1981).

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. Visual checks for precipitation were made prior to use and, if precipitation was observed, the reagent was filtered and re-calibrated or a new batch was made up.

Standard protein solution. A 0.1 mg/ml ovalbumin solution was made up in dist.H_2O.

2.2.1.2 Procedure

Protein samples were diluted to 50 µl with dist.H_2O, dye reagent (950 µl) was added, the solution was mixed and left to stand for 2 min to allow colour development. The absorbance was read at 595 nm, in plastic microcuvettes (1 ml), against an appropriate blank consisting of dist.H_2O (50 µl) and dye reagent (950 µl). A standard curve, from 0-50 µl (0-5 µg) of the standard protein solution (BSA) was constructed. Protein concentration was determined from the equation:

$$\text{Absorbance} = \text{slope} \times \text{protein concentration} + \text{y-intercept}$$

generated by linear regression analysis of the standard curve data.

2.3 Concentration of samples by dialysis against sucrose or polyethylene glycol

In the present study, dilute protein often needed to be concentrated, for example, for inoculation purposes.

Dialysis tubing with a M_r cut-off of 12 000 was used to concentrate samples against sucrose or polyethylene glycol (PEG), of M_r 20 000. The sucrose and PEG have a high osmotic pressure when in solution. A concentration gradient is established between the solvent (water) in the dialysis bag, and the solution (containing sucrose or PEG) at the exterior surface of the tubing membrane which causes water and buffer ions to move out of the dialysis bag, dissolving and diluting the solute, while proteins with a M_r larger than 12 000 (cut-off limit of tubing) are retained in the bag. The end result is a concentrated protein solution within the bag. Although sucrose is much less expensive than PEG, it has the disadvantage in that it is small enough to diffuse into the dialysis bag. High levels of sucrose in a sample may interfere with certain assays or applications. Therefore, when sucrose-free samples were required, they were dialysed against PEG (M_r 20 000).

2.4 SDS-PAGE

The purity of protein samples during isolation was followed using SDS-PAGE. This technique was also used to monitor antibody specificity with western blotting.

The most popular medium used for electrophoresis is polyacrylamide gel. This gel results from the polymerisation of acrylamide monomer into long chains and the cross-linking of these by bifunctional compounds such as *N,N'*-methylene bisacrylamide. This reaction creates a porous matrix in which the pore size is of the same order as protein molecules. When placed in an electric field, during electrophoresis, proteins are subject to molecular sieving which slows down the migration of larger proteins relative to smaller proteins. In the Ornstein-Davis polyacrylamide gel electrophoresis (PAGE) system, two different buffer systems are used, with two different gel porosities. This system was developed by Ornstein (1964) and Davis (1964) to obtain starting bands as thin as possible, and to minimise the diffusion of the protein bands in the gel.

In the Ornstein-Davis system, the sample and stacking gels contain Tris-HCl buffer and the electrode buffers are Tris-glycine. The sample and stacking gel pH is 6.8. At this pH, glycine, in the electrode buffer, has a small negative charge with a resultant low mobility relative to the mobility of the chloride ions in the sample and stacking gel. The proteins move at an intermediate mobility between the glycine (trailing) and chloride (leading) ions. The voltage gradient in front of the interface is low, in contrast to the steep voltage gradient behind the interface, and proteins in the lower voltage gradient will have lower mobility than the chloride

ions, while proteins in the high voltage gradient will have higher mobility than the glycine ions. As a result, all proteins stack at a sharp interface between the leading and trailing ions. The large pore stacking gel serves to control convection which would otherwise occur due to the high density of the concentrated protein bands.

When the protein stack reaches the resolving gel, the increased pH of the resolving gel causes increased dissociation, and hence increased mobility of glycine ions. The glycine ions then overtake the proteins, and form a front with the chloride ions and tracking dye. The destacked proteins are left to migrate in a uniform voltage gradient at a constant pH. The pore size of the resolving gel is smaller than that of the stacking gel, and hence molecular sieving of the proteins occurs. Proteins are therefore separated on the basis of their size, shape and intrinsic charge at the pH of the resolving gel.

The use of the anionic detergent, SDS (sodium dodecyl sulfate), in PAGE allows for the molecular weights of proteins to be estimated (Shapiro *et al.*, 1967). For SDS-PAGE, the protein mixture is denatured by boiling in the presence of excess SDS and thiol reagent such as 2-mercaptoethanol (to cleave disulfide bonds). Under these conditions, most polypeptides bind SDS in a constant weight ratio of 1.4 g SDS per gram of polypeptide (Reynolds and Tanford, 1970). During SDS-PAGE, the intrinsic charges of the polypeptides are insignificant compared with the negative charges provided by the bound detergent. As a result, the SDS-polypeptide complexes have identical charge densities. This allows them to differentially migrate in the polyacrylamide gel, of the correct porosity, according to polypeptide size and not charge. Since the distance migrated by a polypeptide is inversely proportional to its size, the protein of interest can be identified in the gel profile if the molecular weight of its constituent polypeptides is known or, conversely, the molecular weight of individual polypeptides can be determined. The estimation of the molecular weight of a sample polypeptide, requires a standard curve of $\log_{10}$ (polypeptide molecular weight) versus distance migrated for each of the standard molecular weight markers, relative to the migration of the bromophenol blue dye. From its relative mobility, the molecular weight of an unknown can then be read off the curve.

2.4.1 Tris-Tricine SDS-PAGE

The Tris-Tricine system (Schägger and von Jagow, 1987), a modification of the Laemmli (1970) method, was used in the current study. In this system, lower MW proteins can be resolved from the SDS micelles due to the decreased mobility of protein relative to the trailing ion arising from the use of a higher pH and Tricine as a trailing ion in the stacking phase (Schägger and von Jagow, 1987). When this system is used, with the recommended three sets of gel porosities, the separation of proteins of MW 1 000-100 000 can be achieved on a single gel (Schägger and von Jagow, 1987).

2.4.1.1 Reagents

Solution A: Gel buffer [3 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris (72.7 g) was dissolved in approximately 180 ml of dist.H_2O, SDS [6 ml of a 10% (m/v) solution] was added, and the solution was adjusted to pH 8.45 with HCl, and made up to 250 ml.

Solution B: Monomer solution [49.5% (m/v) acrylamide, 3% (m/v) Bis-acrylamide]. Acrylamide (48 g) and Bis-acrylamide (3 g) were dissolved and made up to 100 ml with dist.H_2O and stored in an amber coloured bottle at 4°C.

Solution C [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.2 g) was made up to 2 ml in dist.H_2O just before use.

Anode buffer [200 mM Tris-HCl, pH 8.9]. Tris (24.22 g) was dissolved in approximately 950 ml of dist.H_2O, adjusted to pH 8.9 with HCl and made up to 1 litre.

Cathode buffer [100 mM Tris-HCl, 100 mM Tricine, 0.1% (m/v) SDS, pH 8.25]. Tris (12.2 g) and Tricine (17.9 g) were dissolved in approximately 950 ml of dist.H_2O, SDS [10 ml of a 10% (m/v) solution] was added, and the solution was adjusted to pH 8.25 with HCl and made up to 1 litre.

Solution D (500 mM Tris-HCl, pH 6.8). Tris (3 g) was dissolved in 40 ml dist.H_2O, adjusted to pH 6.8 with HCl and made up to 50 ml. This buffer was made up weekly, because of its poor buffering capacity at 2.1 pH units below its pKa at 4°C.

Solution E [10% (m/v) SDS]. SDS (10 g) was dissolved in 100 ml dist.H_2O with gentle heating if necessary.

Solution F: Reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Buffer D (2.5 ml), 10% SDS (4 ml) (solution E), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with dist.H_2O. The marker dye, bromophenol blue was added (5 µl) before used.

Solution G: Non-reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, pH 6.8]. Buffer D (2.5 ml), 10% SDS (4 ml) (solution E) and glycerol (2 ml) were made up to 10 ml with dist.H_2O. The marker dye, bromophenol blue was added (5 µl) was added before used.

Stain stock solution [1% (m/v) Coomassie blue R-250]. Coomassie blue R-250 (1 g) was dissolved in 100 ml of dist.H_2O by magnetic stirring for 1 h at room temperature. The solution was 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 (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml), and made up to 500 ml with dist.H_2O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) was mixed with acetic acid (100 ml) and made up to 1 litre with dist.H_2O.

Destaining solution II [7% (v/v) acetic acid, 5% (v/v) methanol]. Acetic acid (70 ml) was mixed with methanol (50 ml), and made up to 1 litre with dist.H_2O.

2.4.1.2 Procedure

Table 1. Preparation of Tricine-Tricine running and stacking gels.

Reagents	Running gel	Stacking gel
Gel buffer (solution A)	6 ml	1.5 ml
Monomer (solution B)	3.6 ml	0.5 ml
dist.H_2O	8.4 ml	4 ml
Ammonium persulfate (solution C)	60 µl	30 µl
TEMED	6 µl	12 µl

For SDS-PAGE, the Hoefer SE 250 Mighty Small II vertical slab electrophoresis unit was assembled as described in the manufacturer's manual. For each of the two sides of the apparatus, one notched aluminium plate and one glass plate were cleaned with 70% ethanol. These were clamped with two 1.5 mm polyethylene spacers separating them at the edges. The bottom of the sandwich chamber was plugged with 1% molten agarose before the running gel solution was run into the space between the plates, to a depth 3 cm from the top of the glass plate. The running gel solution was immediately overlayed with dist.H_2O to allow for even polymerisation. Once the gel had set (appearance of interface between gel solution and water, usually about 1 h), the water was removed by inversion. Stacking gel solution was poured in, up to the notch of the aluminium plate, and a 10- or 15-well comb was inserted to form the sample application wells. Once the gel had set (about 30 min), the comb was removed and the wells were rinsed with dist.H_2O.

Cathode buffer was poured into the top electrode compartment and anode buffer into the bottom compartment. For reducing SDS-PAGE, samples were combined with an equal volume of reducing treatment buffer (solution F) and incubated in a boiling waterbath for 90 s, before they were placed on ice until loaded onto the gel. Samples for non-reducing SDS-PAGE were combined with half their volume of non-reducing treatment buffer (solution G), before loading. A marker dye, bromophenol blue (5 µl), which migrates with the buffer front, was added to each sample before loading onto the gels. Samples were applied

into the wells, using a Hamilton microsyringe, at a concentration of at least 1 µg for Coomassie blue R-250, and 250 ng per band for silver staining. The gel was connected to a power pack and run at 80 V (for two gels), with unlimited mA, until the tracking dye had entered the running gel, after which the voltage was increased to 100 V. The gel was run until the tracking dye was about 0.5 cm from the bottom of the running gel. The gel was disconnected from the power supply, and the plates removed. The gels were removed and placed in Coomassie blue R-250 staining solution for 4 h. Following rinsing with dist.H_2O, the gel was placed into destain I overnight and into destain II to effect complete destaining. Gels were stored in polythene zip-seal bags and kept well hydrated until photographed or scanned.

2.5 Silver staining of electrophoretic gels

In the present study, diluted protein fractions, often in nanogram amounts, needed to be detected on electrophoretic gels. Silver staining is 50-100 times more sensitive than Coomassie blue staining, and is comparable in sensitivity to autoradiography (Nielsen and Brown, 1984).

The colour formation is due to silver complexes with ionic or charged amino acid side chains in the proteins. This process is dependent on formaldehyde being a strong reducing agent under alkaline conditions which is necessary for visualisation of the silver-amino acid complex (Nielsen and Brown, 1984). Image development requires a pH change which causes the formation of insoluble silver salts. Such precipitates attach onto the gel surface and impair the contrast, i.e. causing non-specific backstaining (Blum *et al.*, 1987). Pre-treatment of gels with sodium thiosulfate increased sensitivity of the stain due to the thiosulfate chemically dissolving the silver salts by complexation (Blum *et al.*, 1987). This technique therefore creates greater contrast, due to less backstaining, without impairing the high sensitivity of staining.

2.5.1 Reagents

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.2% (v/v) formaldehyde]. Methanol (100 ml), and glacial acetic acid (24 ml), were made up to 200 ml with dist.H_2O. Just before use, 37% formaldehyde was added (25 µl/50 ml).

Wash solution [50% (v/v) ethanol]. Ethanol (100 ml) was made up to 200 ml with dist.H_2O.

Pre-treatment solution [0.02% (m/v) $Na_2S_2O_3.5H_2O$]. $Na_2S_2O_3.5H_2O$ (0.1 g) was made up to 500 ml with dist.H_2O.

Impregnation solution [0.2% (m/v) $AgNO_3$, 0.03% (m/v) formaldehyde]. $AgNO_3$ (0.4 g) was made up to 500 ml with dist.H_2O. Just before use, 37% formaldehyde was added (15 µl/50 ml).

Development solution [6% (m/v) Na_2CO_3, 0.0004% (m/v) $Na_2S_2O_3.5H_2O$, 0.02% (v/v) formaldehyde]. Na_2CO_3 (12 g), pre-treatment solution (4 ml) was made up to 200 ml with dist.H_2O. Just before use, 37% formaldehyde was added (25 µl/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 Procedure

All steps were carried out on a shaker, at RT in clean glass containers rinsed with 70% ethanol. Immediately after electrophoresis, the gel was soaked in fixing solution (1 h or overnight), followed by wash solution (3 x 20 min) to neutralise the gel and allow for subsequent treatment with the acid labile $Na_2S_2O_3$. The gel was treated with pre-treatment solution (1 min), rinsed with dist.H_2O (3 x 20 s), and soaked in impregnation solution (20 min). The gel was rinsed with dist.H_2O (3 x 20 s) to remove excess $AgNO_3$ from the gel surface, and immersed in development solution until bands were evident against a lightly stained background (< 10 min). The gel was rinsed in dist.H_2O (2 x 2 min), treated with stop solution (10 min), and again washed in wash solution (2 x 2 min). The gel was stored and sealed in a plastic bag, in the dark at 4°C, until photographed or scanned.

2.6 Protein fractionation using three phase partitioning (TPP)

Three phase partitioning (TPP) uses tertiary butanol (t-butanol) and ammonium sulfate to precipitate enzymes and proteins from aqueous solutions and can be used upstream with crude samples and downstream where a scaleable simple step is needed (Dennison and Lovrien, 1997). t-Butanol is usually completely miscible with water, but upon the addition of enough salt, such as ammonium sulfate, the solution separates into two phases, a lower aqueous phase and an upper, t-butanol phase. If protein is present in the original aqueous phase it may separate into a third phase, intermediate between the lower aqueous and upper t-butanol phase (Dennison and Lovrien, 1997). TPP was used in the present study, to isolate cathepsin B from rabbit liver and cathepsin L from sheep liver. TPP is discussed in detail in Chapter 3.

2.6.1 Procedure

TPP followed after homogenisation and acid precipitation at pH 4.2. It was effected by the addition and mixing in of 2-methylpropan-2-ol (t-butanol) to 30% (v/v of final volume of pH 4.2 supernatant). Solid $(NH_4)_2SO_4$ [20% (m/v) based on the volume of the pH 4.2 supernatant and t-butanol] was added and dissolved with gentle stirring. The mixture was centrifuged (13 000 x g, 10 min, 4°C), the interfacial protein precipitate was discarded and the supernatant t-butanol and subnatant aqueous phases decanted. Further $(NH_4)_2SO_4$ was added

to the supernatant and subnatant to bring the concentration to 35% (m/v) (based on the volume of pH 4.2 supernatant and t-butanol) and dissolved by gentle stirring. The solution was centrifuged in a swing-out rotor, the supernatant decanted and discarded and the interfacial (precipitated protein layer collected. This precipitate was redissolved in the appropriate column buffer, at one-tenth of the volume of the pH 4.2 supernatant. The solution was centrifuged (15 000 x g, 10 min, 4°C) and filtered through Whatman No. 4 filter paper to remove undissolved protein before it was loaded onto a S-Sepharose column.

2.7 Generation and isolation of antibodies

2.7.1 Generation of antibodies

In the present study, different protocols were attempted to generate antibodies in rabbits. Antigen was inoculated intravenously, directly into the peripheral ear vein, and intramuscularly into the hind leg muscle. Antigen, inoculated intramuscularly, was emulsified with an adjuvant which facilitates the slow release of the antigen as the emulsion breaks down. This slow release prolongs the exposure of the antigen to the rabbit's immune system. Freund's complete adjuvant (FCA) additionally contains killed *Mycobacterium tuberculosis* cells which attract neutrophils and macrophages to the inoculation site and hence enhances the primary response.

2.7.1.1 Reagents

Antigen (cathepsin B). Rabbit liver cathepsin B was isolated as described in Section 3.2.4.2.

2.7.1.2 Procedure

FCA was used in the first inoculation and in subsequent intramuscular inoculations the antigen was emulsified in Freund's incomplete adjuvant (FIA) to further stimulate the selected B cell clone, which produces antibodies specific to the antigen. For intramuscular inoculations, rabbits were inoculated once weekly for 3 weeks, followed by a 3 week rest period, and by another series of one inoculation per week for 3 weeks. Because adjuvants could not be used when the antigen was administered intravenously, more inoculations, at shorter time intervals in-between, needed to be done. Therefore, for intravenous inoculations, the rabbits were inoculated twice weekly for 3 weeks, followed by a 3 week rest period, followed by a repeat of the 3 week series of inoculations.

2.7.2 Isolation of IgG from rabbit serum

IgG was purified from rabbit serum by a simple, convenient method which takes advantage of the protein precipitating properties of the high molecular weight, linear polymer, polyethylene glycol (PEG) (Polson *et al.*, 1964). PEG is a water-soluble polymer which precipitates

proteins at room temperature without denaturation (Polson *et al.*, 1964). It operates on a steric exclusion mechanism, whereby proteins are concentrated in the extrapolymer space, until they exceed their solubility limit. The ability to precipitate a protein is an inverse function of the molecular weight of the PEG molecular species employed (Polson *et al.*, 1964). Polson *et al.* (1964) also found that the concentration of polymer required to precipitate a protein is a function of the nett charge on the molecule, and hence is dependent on the pH of the medium in which the protein is suspended.

2.7.2.1 Reagents

Borate buffered saline, pH 8.6. A sodium borate solution was prepared by dissolving boric acid (2.16 g), NaCl (2.19 g), NaOH (0.7 g) and 37% HCl (0.26 ml) in 950 ml dist.H_2O, checking and adjusting the pH (if necessary) with HCl or NaOH, and making the solution up to 1 litre with dist.H_2O.

100 mM Na-phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. $NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, titrated to pH 7.6 using NaOH, and made up to 1 litre with dist.H_2O.

2.7.2.2 Procedure

Rabbits were bled at 0, 4, 8 and 10 weeks from the marginal ear vein into 10 ml glass test tubes. The blood was allowed to clot overnight at 4°C whereafter the serum was drawn off and diluted 1:2 with borate buffer. Solid polyethylene glycol (M_r 6 000) was added to the diluted serum to 14% (m/v), dissolved by gentle stirring, and the solution was centrifuged (12 000 x g, 10 min, RT). The pellet was redissolved in the original serum volume in sodium phosphate buffer. PEG was added to a concentration of 14% (m/v), dissolved by gentle stirring, and centrifuged (12 000 x g, 10 min, RT). The pellet was redissolved in half the initial serum volume in sodium phosphate buffer containing 60% glycerol, and stored in sterile containers at -20°C. The A_{280} of a 1:40 dilution of IgG solution in 100 mM phosphate buffer was determined and the concentration of IgG in the undiluted solution was calculated from the extinction coefficient of IgG, E_{280} (1 mg/ml) = 1.43 (Hudson and Hay, 1980).

CHAPTER 3
ISOLATION OF CATHEPSINS B AND L

3.1 Introduction

Protein purification normally consists of two main steps, the first step being fractionation by salt precipitation and the second step being purification by chromatography. After fractionation, the protein is only partially purified from unwanted cellular compounds. Conventional salting out uses ammonium sulfate and takes advantage of sulfate being the leading Hofmeister kosmotrope and anionic "water structure promoter" (Dennison and Lovrien, 1997). A kosmotrope is an anion which tends to stabilise protein structure. Three phase partitioning is related to conventional salting out in this aspect but additionally makes use of the C4 alcohol, t-butanol, an organic solvent which tends not to denature proteins (Dennison and Lovrien, 1997).

Because protein folding and solubility depend on hydration, the kosmotropes t-butanol and sulfate, which cause the ordering of water, are used. The opposite of a "kosmotrope" is a "chaotrope", which disrupts water structure. Ordering of water molecules minimises the surface area of a protein in solution and stabilises the protein. Tertiary butanol is completely miscible with water, but can separate into two phases upon the addition of enough salt (ammonium sulfate), to form the lower, aqueous phase, and upper, t-butanol phase. If protein is present, it may form a third phase, intermediate between the aqueous and t-butanol phases, consisting of concentrated and dewatered protein (Pike and Dennison, 1989a). During TPP, the top layer can first be drawn off, the midlayer pierced to draw off the bottom layer, leaving the precipitated protein behind.

The salt, ammonium sulfate, is used due to important physiochemical characteristics of the sulfate, SO_4^{2-}. During precipitation, the macromolecules are forced into conformational tightening, displacing water molecules out of the macromolecular domain (Dennison and Lovrien, 1997). This "conformational tightening" is made possible by pushing and pulling by the sulfate. One SO_4^{2-} aq. ion is very large in its hydrated form and carries up to 14 water molecules. As a result, in conventional salting out, a major portion of the water in solution is occupied in increasing the size of SO_4^{2-} aq. The resultant large $SO_4^{2-}.(H_2O)_n$ therefore crowds loosely conformed peptide, pushes it, and forces conformational tightening (Dennison and Lovrien, 1997). In "pulling", SO_4^{2-} binds to cationic groups on the protein molecule by electrostatic interaction. This results in drawing the protein conformation inward, decreasing protein net hydration.

Four major features of the sulfate ion enable it to act as a pushing and pulling agent: (i) Its divalency, enabling electrostatic binding to cationic side chains, (ii) its tetrahedrality, giving it the capacity to use all four of its oxygen atoms in hydrogen bonding, (iii) its large hydration, and (iv) its hardness (Dennison and Lovrien, 1997).

Like sulfate, t-butanol is a water structure promotor and because of it's size and "bushy" structure, it does not easily penetrate folded proteins. Hence it acts as a differentiating solvent and cosolvent (reviewed by Dennison and Lovrien, 1997).

During conventional salting out, the precipitated protein sinks. In contrast, in the presence of t-butanol, in TPP, the protein floats upon the aqueous phase (Pike and Dennison, 1989a). Tertiary butanol binds to some proteins, and therefore probably helps the proteins to float due to the proteins now having an exposed nonpolar character (Dennison and Lovrien, 1997). The t-butanol/protein complex has a lower density than the water/protein complex, causing it to float (Pike and Dennison, 1989a). If it is true that the hydrophobic character of the protein is increased by t-butanol, it could lead to the protein being more easily salted out, i.e. less ammonium sulfate is needed (Pike and Dennison, 1989a).

An advantage of TPP is that contaminants that are soluble in organic solvents are removed in the t-butanol layer, extracting unwanted lower molecular weight compounds such as lipids, phenolic compounds, and some detergents (Dennison and Lovrien, 1997). Protein complexing agents such as tannins and phenolics as well as other enzyme inhibitors are apparently removed (Pike and Dennison, 1989a). Also, multimeric proteins in which the subunits are linked by hydrophobic bonds, such as haemoglobin, tend to be denatured by the TPP process (Jacobs *et al.*, 1989), which is advantageous as haemoglobin is a problematic contaminant in the isolation of protein from animal tissue. Since TPP is a concentrating or "dewatering" step, due to the kosmotropes that tighten and stabilise the protein conformation, the proteins are precipitated in a protected form. After conventional ammonium sulfate precipitation, the sample must first be dialysed to rid it of excess salt before it can be applied to chromatography steps. This is not necessary after TPP, however, as the sample contains no, or very little, salt (Pike and Dennison, 1989b).

TPP has been very successful and superior to conventional ammonium sulfate precipitation in the isolation of cathepsin L (Pike and Dennison, 1989b), cathepsin D (Jacobs *et al*, 1989) trypanosome proteinases (Troeberg *et al*, 1996) and cathepsin B (Meinesz, 1996).

During any isolation process, a certain percentage of the wanted protein (enzyme) is lost. Although this is expected and cannot be prevented, the quest is to obtain the highest possible yield of enzyme. Not only the concentration of enzyme, but also its activity is important. In the present study, the "lysosomal" cysteine proteinases, cathepsins B and L were isolated. In the intact cell, these enzymes occur within the endolysosomal system. This

compartmentation facilitates the establishment of a low pH, reducing environment within the endolysosomal system, needed for the efficient activity of these enzymes. Also, by compartmentation, the cell deploys these enzymes economically and stores them, while protecting itself as these enzymes' actions would prove fatal or damaging, were they to be released into the cytoplasm (Holtzman, 1989). As a further protection, inhibitors of these proteinases exist in the cytoplasm. The stefins are cysteine proteinase inhibitors that occur in the cytoplasm, while kininogen and α_2-macroglobulin occur in the plasma, and type I cystatins are primarily extracellular (Barrett, 1987).

The first step in protein isolation is usually homogenisation in an appropriate buffer. Homogenisation causes the breakdown of the cellular compartmentation, causing the proteinases and their inhibitors to mix in the homogenate. Stefin B is the endogenous inhibitor of the proteinases of interest in this study, cathepsin B and L. The cysteine cathepsin/stefin B interaction is tight but non-covalent. However, the formation of an artifactual, covalent complex of cathepsin L and stefin B has been found, which might be catalysed by a factor present in liver homogenates (Pike *et al.*, 1992; Coetzer *et al.*, 1995). Inhibition, protein/protein interactions, proteolysis and autolysis, can seriously affect the yield of proteinase obtained. This problem was addressed in the current study, by adding t-butanol to the homogenisation buffer.

Tertiary butanol stabilises proteins rather than denaturing them, as do other common organic solvents. This may be because t-butanol may be too large to gain access to the protein interior. Most proteins have surface hydrophobic patches, however, constituting up to 50% of the surface area (Pain, 1982), and it appears that t-butanol may bind to these patches. In the present study, it was found that t-butanol reversibly inhibits the activity of cathepsin B and many other enzymes. Although this inhibiting action of t-butanol is not yet fully understood, it has been found that in the presence of t-butanol, some proteins undergo conformational changes, favouring an increased proportion of α-helices (Pike and Dennison, 1989a).

Since t-butanol appears to stabilise and reversibly inhibit enzymes, it was hypothesised that it may be beneficial to add t-butanol to the homogenisation buffer in order to inhibit proteolysis and enzyme/inhibitor interactions. With the t-butanol already present, the homogenate supernatant would then be poised for fractionation by TPP. This hypothesis was explored in the present study.

3.2 Cathepsin B

Cathepsin B [EC 3.4.22.1] is a lysosomal cysteine proteinase, originally defined as the enzyme in bovine spleen deaminating benzoylarginine amide in the presence of cysteine (reviewed by Katunuma and Kominami, 1983). Cathepsin B is the most abundant lysosomal

cysteine proteinase, and was the first lysosomal cysteine proteinase purified to homogeneity and consequently has been more thoroughly studied than other cysteine proteinases (Sloane *et al.*, 1990). Cathepsin B plays a role in physiological processes such as bone resorption (Delaisse *et al*, 1991), antigen processing (Guagliardi *et al.*, 1990), cartilage proteoglycan breakdown (Buttle and Saklatvala, 1992), and intracellular protein turnover (Barrett and Kirschke, 1981). It is one of the proteolytic enzymes that has been implicated in human tumour invasion and metastasis (Sloane *et al.*, 1990) and has been linked to tumour progression through the observation that its activity, secretion or membrane association are increased during tumour progression (Berquin and Sloane, 1996). It has also been implicated in other diseases such as arthritis and muscular dystrophy (Mort *et al.*, 1984).

The molecular weight of cathepsin B from various organs and tissues is in the range of 24 000 - 28 000 daltons. Depending on species and tissue of origin, mature cathepsin B exists in a single chain form, a double chain form, or as both the single and double chain forms (reviewed by Sloane *et al.*, 1990).

Evident from X-ray crystallography (at a 2.15 Å level), cathepsin B is a bilobal enzyme with two globular domains (Musil *et al.*, 1991). The active site and substrate binding cleft are located between the two domains. Cathepsin B activity requires the existence of an ion pair between the active site Cys-29 (pKa 3.6) and His-199 (pKa 8.6) (Meloun *et al.*, 1988; Hasnain *et al.*, 1992; Turk *et al.*, 1994). The thiol and imidazole side-chains of Cys-29 and His-199 form an ion-pair over the pH range 4.0-8.5 (Mort and Buttle, 1997). Substrate peptide bond cleavage is mediated by nucleophilic attack by S^- from Cys-29 on the carbonyl carbon atom, followed by proton donation from His-199 (Mort and Buttle, 1997).

These two residues are situated on different domains, and the active site is formed only when the two domains are held close together by hydrogen bonding, electrostatic, and Van der Waal's forces (Turk *et al.*, 1994).

Cathepsin B is an endopeptidase and unlike other lysosomal cysteine proteinases, can also accept an arginine, two residues N-terminal to the scissile bond, i.e. in the P_2 pocket. This is made possible by the presence of a glutamic acid (Glu-245) at the base of the P_2 pocket, which acts as an acceptor for the positive charge on the arginine side-chain (Hasnain *et al.*, 1993).

Cathepsin B also acts as an exopeptidase (carboxydipeptidase), predominantly at acidic pH (Takahashi *et al.*, 1986). This activity is made possible by the occluding loop that partially blocks the end of the active site cleft and positions a positively charged imidazole group of His-111 to accept the negative charge at the C-terminus of the substrate (Musil *et al.*, 1991). It has been shown that the occluding loop can adopt a conformation that no longer blocks the binding cleft, allowing the enzyme to also act as an endopeptidase (Cygler *et al.*, 1996).

It was originally thought that cathepsin B acts optimally against most substrates in slightly acidic media (Barrett and Kirschke, 1981), and that the sharp fall in activity at pH-values above 7.0 is due to irreversible inactivation (Barrett, 1973). However, it was shown by Buck *et al.* (1992) that above pH 7.0, human cathepsin B undergoes a time-dependent autodegradation, which is slowed by the presence of substrates such as fibronectin and blocked by the addition of the irreversible inhibitor, E-64. This finding argues that cathepsin B is not irreversibly denatured at a pH above 7.0. It was found in other studies that the pH optimum for cathepsin B is in the range 7-8.0 (Dehrmann *et al.*, 1996; Willenbrock and Brocklehurst, 1985). Not only pH, but various other factors play a role in cathepsin B activity and stability. Dehrmann *et al.* (1995; 1996) showed that the activity of cathepsin B and cathepsin L is affected by specific buffer ions as well as pH, ionic strength and the presence of protein substrates. Stability of the native, active conformation of cathepsin B depends on an active site thiolate-imidazolium pair that holds the two domains of the enzyme together (Turk *et al.*, 1994). If this ion pair is disrupted, either by deprotonation of the imidazole moiety at high pH, and/or by ionic protonation, the two cathepsin B domains "unzip" along the active site groove, causing inactivation (Turk *et al.*, 1994). Dehrmann *et al.* (1996) also found that the activation state of the enzyme markedly affects the stability of the enzyme.

3.2.1 Cathepsin B assay

Cathepsin B was previously assayed with Bz-DL-Arg-NPhNO_2 or Bz-Arg-2-NNap as substrates (Barrett and Kirschke, 1981). However, these two substrates are insensitive and are also susceptible to cathepsin H. Substrates containing larger peptide sequences are more selective and specific than the blocked amino acid derivatives, as has been found with the serine proteinases (Barrett and Kirschke, 1981). Therefore, the naphthylamide substrate containing the -Arg-Arg- sequence is extremely selective for cathepsin B (reviewed by Barrett and Kirschke, 1981). The most sensitive, safe and convenient leaving group for substrates of the cysteine proteinases is 7-amino-4-methylcoumarin. Barrett and Kirschke (1981) report that Z-Arg-Arg-NHMec is sensitive and specific for cathepsin B, and this substrate was consequently used for cathepsin B assays in the present study.

Dehrmann *et al.* (1995) modified the enzyme assay method devised by Barrett and Kirschke (1981) in order to minimise the dilution of the buffer. In the present study, a modified version of the method of Dehrmann *et al.* (1995) was used for the macroassay.

3.2.1.1 Reagents

Buffer/activator [0.1 M Na-phosphate, 4 mM Na_2EDTA, 0.02% NaN_3, 5 mM dithiothreitol, pH 6.0]. NaH_2PO_4 (6.90 g), Na_2EDTA (0.93 g) and NaN_3 (0.1 g) were dissolved in 450 ml

dist.H_2O, adjusted to pH 6.0 with NaOH and made up to 500 ml. Immediately before use, dithiothreitol was added to 5 mM (i.e. 0.51 g/500 ml).

1 mM Z-Arg-Arg-NHMec substrate stock solution. Z-Arg-Arg-NHMec (1.1 mg) was dissolved in DMSO (1.5 ml), divided into 100 µl aliquots and stored at -4°C.

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

1 mM 7-amino-4-methylcoumarin standard. 7-amino-4-methylcoumarin (1.8 mg) was dissolved in DMSO (10 ml).

385 mM Dithiothreitol (DTT). DTT (0.06 g) was dissolved in 1 ml dist. H_2O.

3.2.1.2 Procedure

Microassay. Enzyme sample (10 µl) and buffer/activator (75 µl) were added to wells of a white Fluoronunc maxisorp microtitre plate and preincubated for 2 min at 37°C. Thereafter, 40 µM substrate solution (25 µl) was added and incubated at 37°C for 10 min, and the fluorescence of the free aminomethylcoumarin determined in a microplate fluorescence reader (Cambridge Technology, Model 7620) with excitation at 370 nm and emission at 460 nm.

For monitoring the effluents from chromatography columns, the fluorescence readings obtained from the fluorometer were used directly and expressed in terms of "arbitrary fluorescence units"

Standard curves of different aminomethylcoumarin concentrations were constructed for quantitation of product. Three different aminomethylcoumarin concentration ranges were used: 2-10 µM, 10-100 µM and 100-1000 µM. Product concentration could be calculated from the curves as follows:

$$\text{Product concentration} = \frac{\text{Fluorescence intensity - y-intercept}}{\text{Slope}}$$

where the product concentration would be pmoles/110µl.

Macroassay. All solutions were equilibrated to 37°C prior to use, except the enzyme solution which was kept on ice. Enzyme sample, diluted to 10 µl, buffer (800 µl), and 385 mM DTT solution (65 µl) were mixed and preincubated for 2 min at 37°C. In the final mix, the concentration of DTT is 25 mM, which was found by Pillay (1999) to activate cathepsin B optimally. After activation, 40 µM Z-Arg-Arg-NHMec (125 µl) was added. The fluorescence of the aminomethylcoumarin liberated was monitored continuously for 5 or

10 min in a Hitachi F-2000 spectrofluorometer with excitation at 370 nm and emission at 460 nm.

3.2.2 The effect of t-butanol on cathepsin B activity.

t-Butanol has been sucessfully used as an agent in isolating proteinases in the protein fractionation method, three phase partitioning (TPP). However, the effect that t-butanol has on the structure and activity of these enzymes is unknown. The effect of t-butanol on the activity of cathepsin B was investigated in the present study.

3.2.2.1 Reagents

Buffer [0.1 M Na-phosphate, 4 mM Na_2EDTA, 0.02% NaN_3, pH 6.0]. NaH_2PO_4 (6.90 g), Na_2EDTA (0.93 g) and NaN_3 (0.1 g) were dissolved in 450 ml dist.H_2O, adjusted to pH 6.0 with NaOH and made up to 500 ml.

40 µM Z-Arg-Arg-NHMec solution. As described in Section 3.2.1.1..

385 mM Dithiothreitol (DTT). As described in Section 3.2.1.1.

3.2.2.2 Procedure

The macroassay, as described in Section 3.2.1.2, was adapted to include t-butanol in the reaction mixture. Tertiary butanol at different percentages (0%, 10%, 20% and 30%) was added to the assay buffer and substrate. In order to keep the buffer ionic strength constant between assays containing different percentages of t-butanol, 504 µl of buffer was used and the rest of the 800 µl made up of t-butanol (volume depending on % used) and dist.H_2O. In each case, the fluorescence of the liberated aminomethylcoumarin was monitored continuously for 10 min. Data was sampled at 10 s intervals and the activity at each t-butanol percentage was determined from the slope of the plot of fluorescence intensity versus time.

3.2.2.3 Results

The activity of cathepsin B at 0% t-butanol was taken as 100% and the activities at 10%, 20% and 30% t-butanol were expressed relative to this. Relative activity decreased sharply with increasing concentration of t-butanol and was essentially zero at 30% t-butanol (Fig. 3.1).

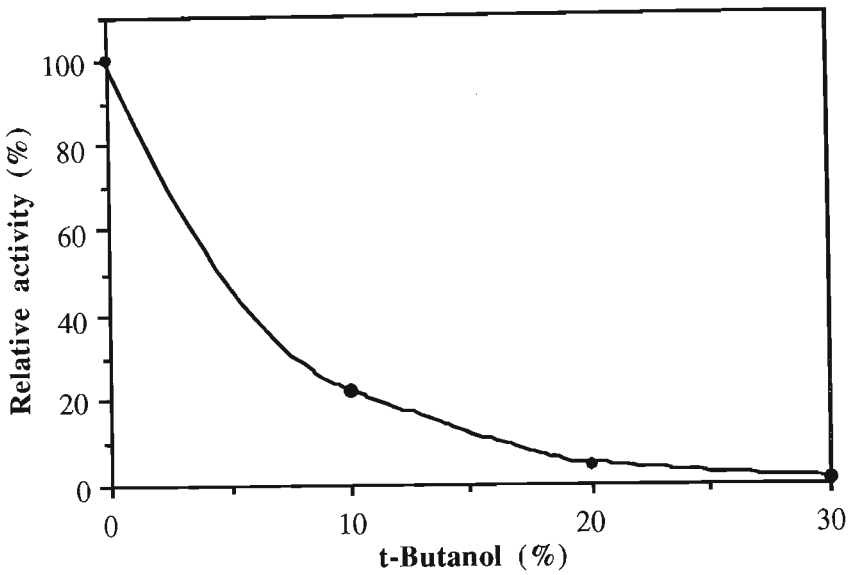

Figure 3.1. The effect of t-butanol concentration upon the activity of cathepsin B

3.2.3 Recovery of cathepsin B activity upon removal of t-butanol by dialysis

3.2.3.1 Reagents

Buffer [0.1 M Na-phosphate, 4 mM Na_2EDTA, 0.02% NaN_3, pH 6.0]. As described in Section 3.2.2.1.

40 µM Z-Arg-Arg-NHMec solution. As described in Section 3.2.1.1.

385 mM Dithiothreitol (DTT). As described in Section 3.2.1.1.

3.2.3.2 Procedure

Enzyme sample, diluted to 10 µl, assay buffer (504 µl), and t-butanol (260 µl) (giving 30% t-butanol in the final mix) was dialysed in several changes of assay buffer for 72 h at 4°C, in dialysis tubing with a M_r cut-off of 12 000. The activity of cathepsin B in the sample containing 30% t-butanol, before dialysis, and in the dialysed sample was assayed as described in Section 3.2.1.2. The control sample consisted of 10 µl diluted enzyme sample, 504 µl assay buffer and 260 µl dist.H_2O. The control sample was also dialysed for 72 h at 4°C, to allow for possible enzyme loss during the 3 day dialysis period. No allowance was made for possible differential volume changes, during dialysis, between the sample and the control.

3.2.3.3 Results

For each sample, the fluorescence of the liberated aminomethylcoumarin was monitored continuously for 10 min. Data was sampled at 10 s intervals. Activity was determined from

the slopes of the plots of fluorescence intensity versus time and expressed in terms of relative activity (Fig. 3.2). The activity of the control sample, containing no t-butanol, was taken to be 100%. About 80% of cathepsin B activity was recovered by dialysis for 3 days (Fig. 3.2).

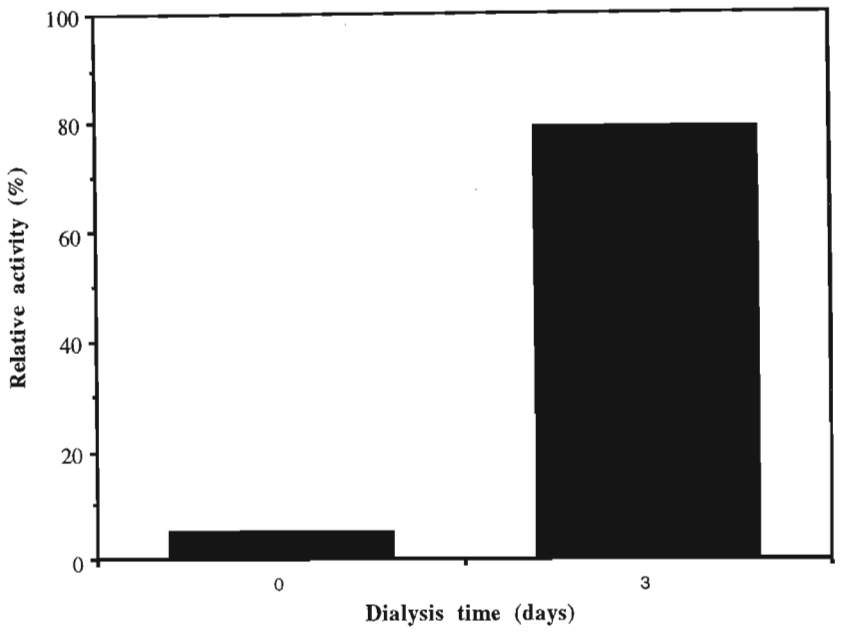

Figure 3.2. Recovery of cathepsin B activity upon removal of t-butanol by dialysis.

3.2.4 Purification of cathepsin B using the conventional method

Past methods for the isolation of cathepsin B have relied on techniques such as covalent chromatography, using an aminophenylmercuric acetate coupled to Sepharose (Barrett and Kirschke, 1981), or affinity chromatography using a specialised affinity resin, Agarose-Ahx-Gly-Phe-Gly-Sc (Rich *et al.*, 1986). Rich *et al.* (1986) found that the cathepsin B that they isolated was 80% active. This method therefore seems to be very successful and quick, but the resin is currently not available. Meinesz (1996) developed a quick, cost effective method for the isolation of cathepsin B from bovine spleen, where TPP was followed by chromatography on S-Sepharose. This method was used to isolate cathepsin B from rabbit liver in the present study. Although successful, the yield from this method was low, especially for the purposes of the present study where large quantities of cathepsin B were needed. Consequently, it was decided to explore the possibility of increasing the yield of cathepsin B by homogenising the tissue in the presence of t-butanol.

3.2.4.1 Reagents

Homogenisation buffer [50 mM Na-acetate, 150 mM NaCl, 1 mM Na_2EDTA, pH 4.5]. Glacial acetic acid (2.86 ml), NaCl (8.77 g) and Na_2EDTA (0.37 g) were dissolved in 950 ml dist.H_2O, adjusted to pH 4.5 with diluted NaOH, and made up to 1 litre.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.0]. Glacial acetic acid (2.29 ml), Na_2EDTA (0.74 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres of dist.H_2O, adjusted to pH 5.0 with diluted NaOH, and made up to 2 litres.

Buffer B [100 mM Na-acetate, 500 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.5]. Glacial acetic acid (11.44 ml), NaCl (58.44 g), Na_2EDTA (0.74 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres of dist.H_2O, adjusted to pH 5.5 with diluted NaOH, and made up to 2 litres.

S-Sepharose fast flow. S-Sepharose (115 ml slurry) was mixed with Buffer A (250 ml). The resulting slurry was packed into a glass column. Before use, the column bed was regenerated with two column volumes of 2 M NaCl in buffer A, followed by equilibration with five column volumes of 70 mM NaCl in buffer A. The column was regenerated with one column volume of 2 M NaCl in buffer A in between purifications, to elute any proteins that remained bound to the bed after the salt gradient in the previous purification. Regeneration was followed by equilibration with five column volumes of buffer A or buffer A containing 70 mM NaCl, depending on the salt gradient used.

Sephacryl S-100 HR. Sephacryl S-100 was prepared by diluting 400 ml of the supplied hydrated gel in 800 ml of buffer B. The resulting slurry was packed into a glass column under gravity. Initially, the column was equilibrated with five column volumes of buffer B, before use. In between purifications, the column was re-equilibrated with two column volumes of buffer B.

Rabbit liver. Fresh rabbit liver was obtained from Muskin Farms outside Pietermaritzburg, and Kia-ora Farms in Cato Ridge. The fresh liver was immediately diced into cubes and frozen at -70°C for at least 72 hours before use.

3.2.4.2 Procedure

Rabbit liver was processed essentially according to Kirschke *et al.* (1989). The rest of the purification was carried out according to Meinesz (1996), with slight modifications. Frozen rabbit liver (915 g) was thawed overnight at 4°C and homogenised at a ratio of 1:1 (liver mass to homogenisation buffer volume) with homogenisation buffer in a Waring blendor for 2 min. The homogenate was centrifuged (9 000 x g, 20 min, 4°C). The supernatant was decanted and adjusted to pH 4.2 with diluted glacial acetic acid and again centrifuged (15 000 x g, 20 min, 4°C) to remove precipitated material. The supernatant was used for TPP fractionation.

The ammonium sulfate concentrations required for the TPP step were first optimised. Fractions, obtained by 5% increments in ammonium sulfate saturation, were assayed for protein content and cathepsin B activity. The fraction, obtained by a cut from 20-35% saturation, was found to be optimal.

TPP was effected on the pH 4.2 supernatant by adding 30% (v/v of final pH 4.2 supernatant volume) of t-butanol. Crushed, solid $(NH_4)_2SO_4$ [20% (m/v) based on the volume of the pH 4.2 supernatant and t-butanol] was added and dissolved with gentle stirring. The mixture was centrifuged (13 000 x g, 10 min, 4°C), the interfacial protein precipitate discarded and the supernatant t-butanol and subnatant aqueous phases decanted. Further $(NH_4)_2SO_4$ was added to the supernatant and subnatant to bring the concentration to 35% (m/v) (based on the volume of the pH 4.2 supernatant and t-butanol) and dissolved by gentle stirring. The solution was centrifuged (9 000 x g, 10 min, 4°C) in a swing-out rotor in order to harvest the precipitate completely. The supernatant and subnatant were discarded and the precipitate collected. The precipitate was redissolved in buffer A at one-tenth of the volume of the pH 4.2 supernatant. The solution was centrifuged (15 000 x g, 10 min, 4°C) to remove insoluble material and the supernatant filtered through Whatman No. 4 filter paper. The clarified sample was applied to a column of S-Sepharose (2.5 x 22.6 cm = 111 ml), pre-equilibrated with 70 mM NaCl in buffer A. Unbound material was eluted with two column volumes of 70 mM NaCl in buffer A. Bound material was eluted with a 70-300 mM NaCl gradient in buffer A in *ca.* 5 column volumes, all at 50 ml/h. The active fractions were pooled and concentrated by dialysis against sucrose to 1-5% of the volume of the molecular exclusion column and applied to a column of Sephacryl S-100 (2.5 x 80.5 cm = 395.3 ml) pre-equilibrated with buffer B, and eluted at 25 ml/h. The active fractions of *ca.* M_r 28 000 were pooled and concentrated by dialysis against PEG 20 000.

3.2.4.3 Results

The purification of rabbit liver cathepsin B, using the conventional TPP method, is summarised in Table 3.1.

Table 3.1. The purification of rabbit liver cathepsin B, using the conventional TPP method.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	1 300	451 814	2 260	0.005	(1)	(100)
Supernatant (pH 4.2)	610	26 608	1 719	0.065	13	76.05
TPP (20-40%)	118	2 070	1 093	0.53	106	48.34
S-Sepharose	54	645	797	1.24	248	35.27
Sephacryl S-100	39	3	218	71.75	14 350	9.66

During TPP, a large amount of contaminating protein is removed in the first TPP cut [0-20% $(NH_4)_2SO_4$] while cathepsin B precipitates at a higher salt concentration (30%). However, the precipitation of cathepsin H, L and S overlaps with that of cathepsin B. Cathepsin H and L are removed in the ion exchange step, as they elute after cathepsin B (Meinesz, 1996). Cathepsin B eluted from the S-Sepharose column at 86-212 mM NaCl (Fig. 3.3). After each chromatography step, the active peak was determined by testing fractions for cathepsin B activity using the microassay in Section 3.2.1.2. Before the active fractions were pooled, they were run on SDS-PAGE to establish their purity.

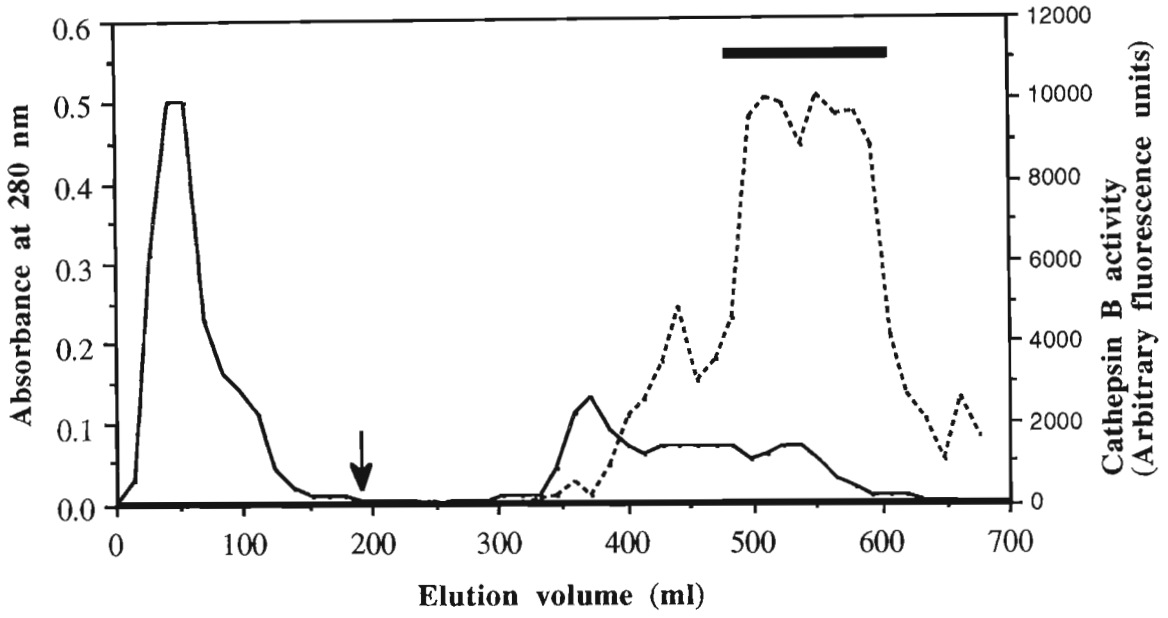

Figure 3.3. Chromatography of the 20-35% pH 4.2 TPP fraction on S-Sepharose (conventional method).

Column, 2.5 x 22.6 cm (111 ml bed volume), equilibrated with 20 mM Na-acetate, pH 5.0, containing 70 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, and eluted with a 70-300 mM NaCl gradient, in 5 column volumes of the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 6.9 ml (8.5 min). (—) A_{280}, (...) cathepsin B activity against Z-Arg-Arg-NHMec, (▬) pooled fractions.

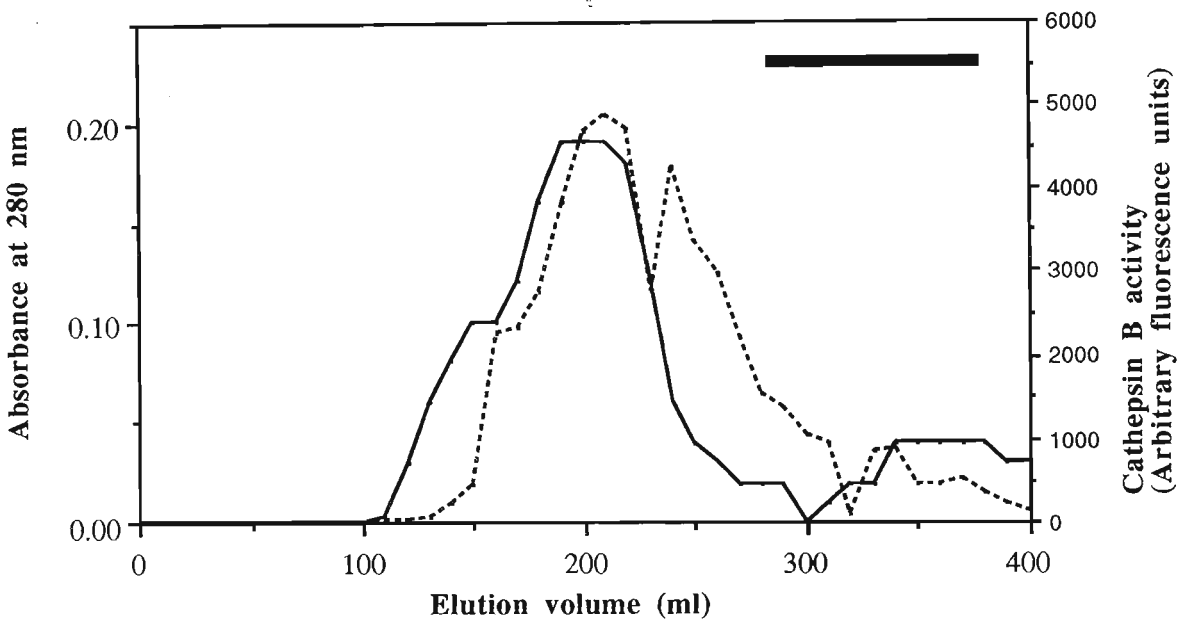

Figure 3.4. Chromatography of the pH 5.0 S-Sepharose fraction of cathepsin B on Sephacryl S-100 (conventional method).

Column, 2.5 x 80.5 cm (395 ml bed volume) equilibrated and run in buffer B [100 mM Na-acetate, pH 5.5, containing 500 mM NaCl, 1 mM Na_2EDTA. 0.02% NaN_3 (Section 3.2.3.1)]; flow rate, 25 ml/h (5 cm/h); fractions, 5 ml (12 min). (—) A_{280}, (...) cathepsin B activity against Z-Arg-Arg-NHMec, (▬) pooled fraction.

The overall progression of the isolation was analysed by reducing Tris-Tricine SDS-PAGE (Fig. 3.5). It is evident from the 28 kDa protein band, seen in lane f (Fig. 3.5), that cathepsin B eluted from Sephacryl S-100 as a pure fraction. A yield of 9.7% of cathepsin B was obtained (Table 3.1).

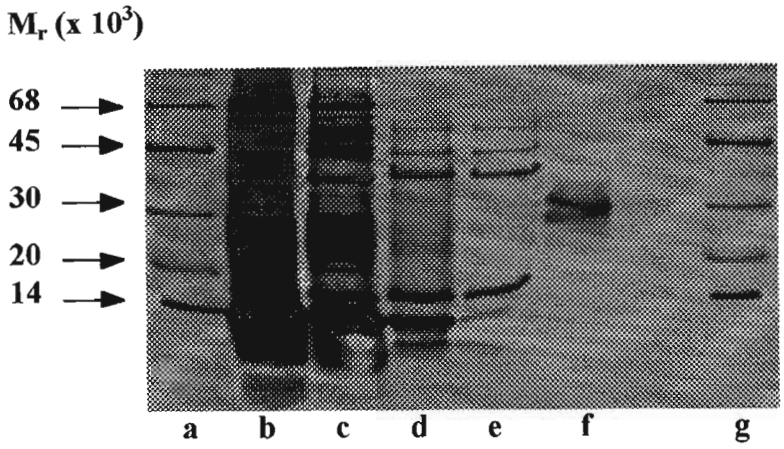

Figure 3.5. Reducing Tris-Tricine SDS-PAGE of cathepsin B isolated from rabbit liver by the conventional method.

Samples were boiled in reducing treatment buffer and loaded onto a 10% Tris-Tricine gel. (b) liver homogenate fraction; (c) pH 4.2 acid precipitate supernatant; (d) TPP fraction; (e) S-Sepharose fraction; (f) Sephacryl S-100 fraction; (a,g) MW markers (BSA, M_r, 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; lysozyme, M_r 14 000. Visualised using silver staining.

3.2.5 Control method for isolation of rabbit liver cathepsin B

The control method for isolation of rabbit liver cathepsin B, was carried out exactly as the conventional method (Section 3.2.3.1), except that the pH of the homogenisation buffer was lowered to pH 4.0. This lower pH, instead of pH 4.5, as in the conventional method, was used to counter the neutral pH buffering capacity of the homogenate, giving a final homogenate pH of *ca.* pH 4.5. Also, in the conventional isolation, homogenisation was carried out at a ratio of 1:1 (liver mass to homogenisation buffer volume). To further buffer the pH of the homogenised tissue, a liver mass to homogenisation buffer volume ratio of 1:2 was used in the control method.

3.2.5.1 Reagents

Homogenisation buffer [150 mM Na-acetate, 1 mM Na_2EDTA, pH 4.0]. Glacial acetic acid (17.16 ml) and Na_2EDTA (0.74 g) were dissolved in 1.9 litres of dist.H_2O, adjusted to pH 4.0 with diluted NaOH, and made up to 2 litres with dist.H_2O.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.0]. As described in Section 3.2.4.1.

Buffer B [100 mM Na-acetate, 500 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.5]. As described in Section 3.2.4.1.

S-Sepharose fast flow. As described in Section 3.2.4.1.

Sephacryl S-100 HR. As described in Section 3.2.4.1.

Rabbit liver. As described in Section 3.2.4.1.

3.2.5.2 Procedure

Rabbit liver was processed as described in Section 3.2.4.1. Frozen rabbit liver (299.7 g) was thawed overnight at 4°C, homogenised for 3 min at a ratio of 1:2 (liver mass to homogenisation buffer volume) with homogenisation buffer in a Waring blendor and centrifuged (9 000 x g, 20 min, 4°C). The supernatant was decanted and adjusted to pH 4.2 with diluted glacial acetic acid and again centrifuged (15 000 x g, 20 min, 4°C) to remove precipitated material. The supernatant was used for TPP fractionation.

TPP was effected on the pH 4.2 supernatant as described in Section 3.2.4.2., except that an optimal TPP cut of 20-40% ammonium sulfate, instead of 20-35%, was taken. Chromatography steps were as described in Section 3.2.4.2, except that the S-Sepharose column (2.5 x 18.3 cm = 89.9 ml) was equilibrated with buffer A, containing no salt, and the bound material was eluted with a gradient of 0-300 mM NaCl in buffer A in *ca.* 6 column volumes. The active fractions were pooled, concentrated by dialysis against sucrose to less than 5% of the volume of the molecular exclusion column and applied to a column of Sephacryl S-100 (2.5 x 73 cm = 358.5 ml) equilibrated in Buffer B (flow rate 5 cm/h). The active fraction was pooled and re-concentrated by dialysis against PEG 20 000.

3.2.5.3 Results

The purification of rabbit liver cathepsin B, using the control method, is summarised in Table 3.2.

Table 3.2. The purification of rabbit liver cathepsin B, using the control TPP method.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purifi-cation (fold)	Yield (%)
Homogenate	642	20897	1362183	65	(1)	(100)
TPP (20-40%)	98	4763	228194	479	7.4	16.8
S-Sepharose	21	63	55020	874	13.4	4.0
Sephacryl S-100	5	16	17259	1061	16.3	1.3

Because the homogenisation buffer was lowered to pH 4.0, and the liver mass to buffer volume ratio was changed to 1:2, the homogenate supernatant pH was lower than that of the supernatant in the conventional method. As a result, a very small amount of glacial acetic acid was required to change the pH to 4.2.

Through optimisation, it was found that in the control method, the 20-40% TPP cut contained optimal cathepsin B activity (results not shown). Cathepsin B eluted from the S-Sepharose column at 138-190 mM NaCl (Fig. 3.6). Cathepsin B eluted from the Sephacryl S-100 column at an elution volume of 220-234 ml (Kav = 0.3) (Fig. 3.7).

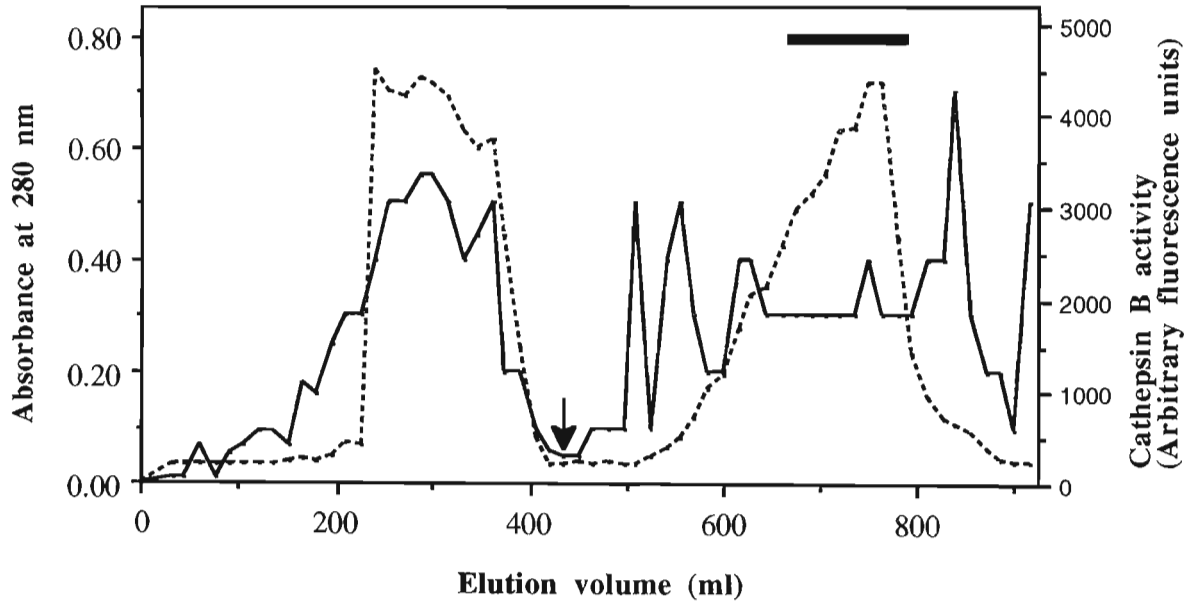

Figure 3.6. Chromatography of the 20-40% pH 4.2 TPP fraction on S-Sepharose (control method).

Column, 2.5 x 18.3 cm (89.9 ml bed volume), equilibrated in buffer A [20 mM Na-acetate, pH 5.0, containing 1 mM Na_2EDTA, 0.02% NaN_3], and eluted with a 0-300 mM NaCl gradient in 5 column volumes of the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 15 ml (18 min). (—) A_{280}, (...) cathepsin B activity (arbitrary) against Z-Arg-Arg-NHMec, (▬) pooled fractions.

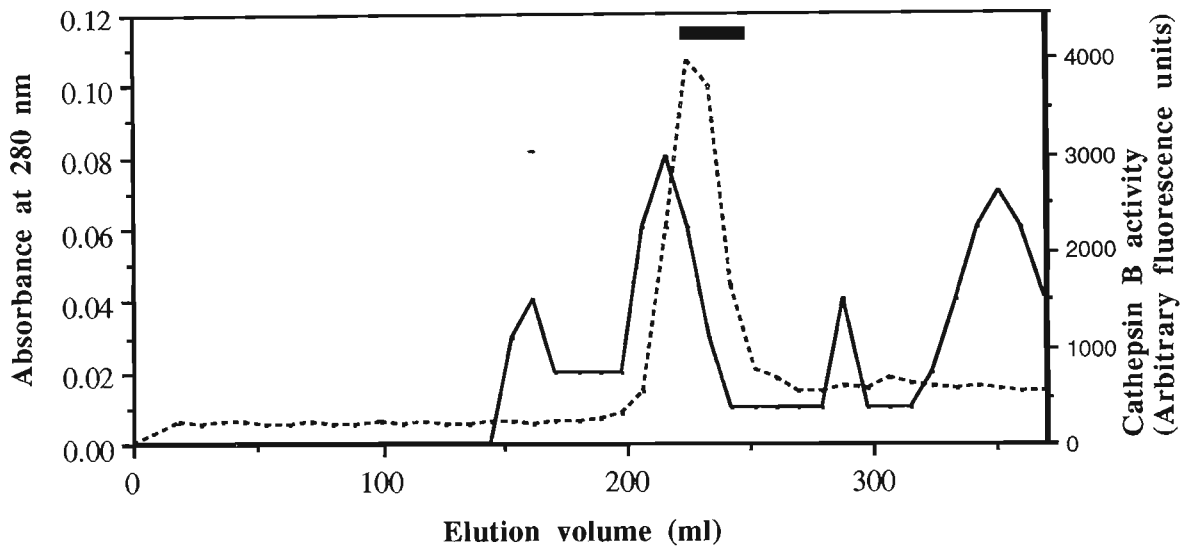

Figure 3.7. Chromatography of the pH 5.0 S-Sepharose fraction of cathepsin B on Sephacryl S-100 (control method).

Column, 2.5 x 73 cm (358.5 ml bed volume) equilibrated and run in buffer B [100 mM Na-acetate, pH 5.5, containing 500 mM NaCl, 1 mM Na_2EDTA. 0.02% NaN_3]; flow rate, 25 ml/h (5 cm/h); fractions, 4.5 ml (10.8 min). (—) A_{280}, (...) cathepsin B activity against Z-Arg-Arg-NHMec, (▬) pooled fraction.

The overall progression of the isolation was analysed by reducing and non-reducing Tris-Tricine SDS-PAGE (Figs. 3.8 and 3.9). In Fig. 3.8, the pure cathepsin B (lane f), appears as two bands as a result of reduction. In Fig. 3.9 cathepsin B appears as one 28 kDa protein band, confirming purity of cathepsin B. The two bands appearing in Fig. 3.8 might represent the single chain form and the heavy chain of the double chain form of cathepsin B. A yield of 1.3% of cathepsin B was obtained (Table 3.2).

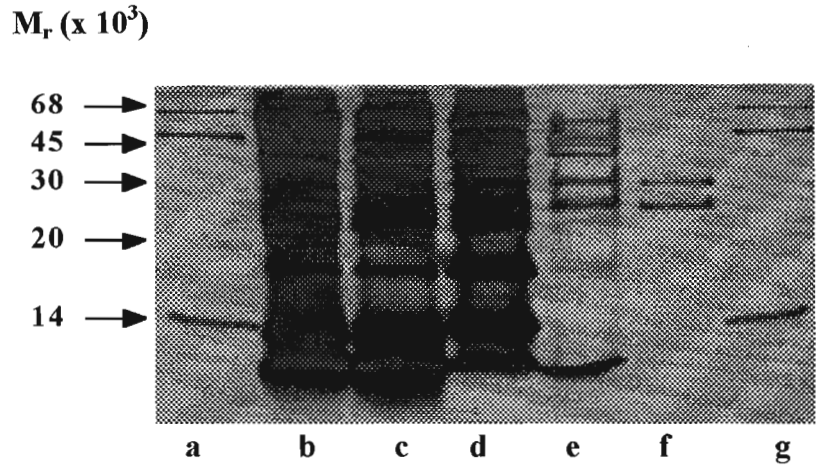

Figure 3.8. Reducing Tris-Tricine SDS-PAGE of cathepsin B isolated from rabbit liver by the control method.

Samples were boiled in reducing treatment buffer and loaded onto a 10% Tris-Tricine gel. (b) liver homogenate fraction; (c) pH 4.2 acid precipitate supernatant; (d) TPP fraction; (e) S-Sepharose fraction; (f) Sephacryl S-100 fraction; (a,g) MW markers (BSA, M_r, 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; lysozyme, M_r 14 000. Visualised using silver staining.

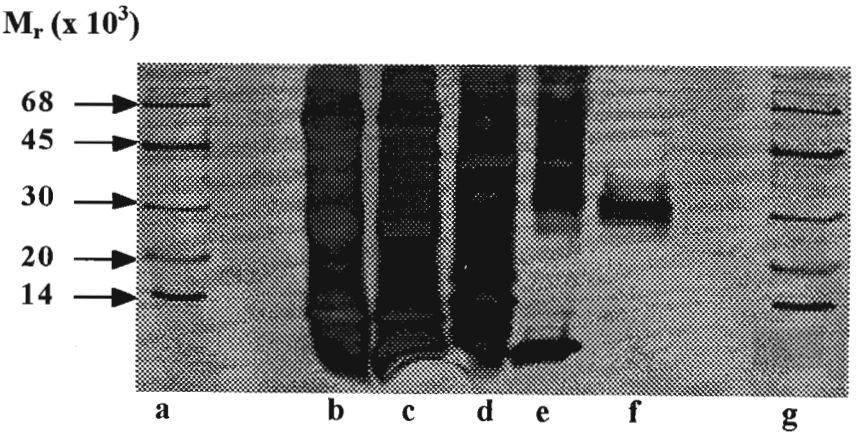

Figure 3.9. Non-reducing Tris-Tricine SDS-PAGE of cathepsin B isolated from rabbit liver by the control method.

Samples were boiled in non-reducing treatment buffer and loaded onto a 10% Tris-Tricine gel. (b) liver homogenate fraction; (c) pH 4.2 acid precipitate supernatant; (d) TPP fraction; (e) S-Sepharose fraction; (f) Sephacryl S-100 fraction; (a,g) MW markers (BSA, M_r, 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; lysozyme, M_r 14 000. Visualised using silver staining.

3.2.6 Isolation of rabbit liver cathepsin B using a novel method with t-butanol in the homogenisation buffer

3.2.6.1 Reagents

Homogenisation buffer [150 mM Na-acetate, 1 mM Na_2EDTA, pH 4.0]. As described in Section 3.2.5.1.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.0]. As described in Section 3.2.4.1.

Buffer B [100 mM Na-acetate, 500 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.5]. As described in Section 3.2.4.1.

S-Sepharose fast flow. As described in Section 3.2.4.1.

Sephacryl S-100 HR. As described in Section 3.2.4.1.

Rabbit liver. As described in Section 3.2.4.1.

3.2.6.2 Procedure

Rabbit liver was processed as described in Section 3.2.4.1. Frozen rabbit liver (312 g) was thawed overnight at 4°C and homogenised as described in Section 3.2.5.2, but in a mixture of two parts of homogenisation buffer and sufficient t-butanol to give 30% (w/v) t-butanol in the homogenate. The homogenate was centrifuged (9 000 x g, 20 min, 4°C), the supernatant adjusted to pH 4.2 with dilute acetic acid and again centrifuged (15 000 x g, 20 min, 4°C). A 20-40% ammonium sulfate TPP cut was taken as described in Section 3.2.5.2, without the further addition of t-butanol. Chromatography steps were as described in Section 3.2.5.2.

3.2.6.3 Results

The purification of rabbit liver cathepsin B, using the modified TPP method with t-butanol in the homogenisation buffer, is summarised in Table 3.3.

Table 3.3. The purification of rabbit liver cathepsin B, using the novel TPP method.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purifi-cation (fold)	Yield (%)
Homogenate	842	6155	813322	132	(1)	(100)
TPP cut (20-40%)	79	507	639585	1261	9.6	78.6
S-Sepharose	15	93	198557	2142	16.2	24.4
Sephacryl S-100	5	36	115210	3215	24.3	14.2

Cathepsin B eluted from the S-Sepharose column at 113-158 mM NaCl (Fig. 3.10). The enzyme eluted from the Sephacryl S-100 column at an elution volume of 223 ml (Kav = 0.25) (Fig. 3.11).

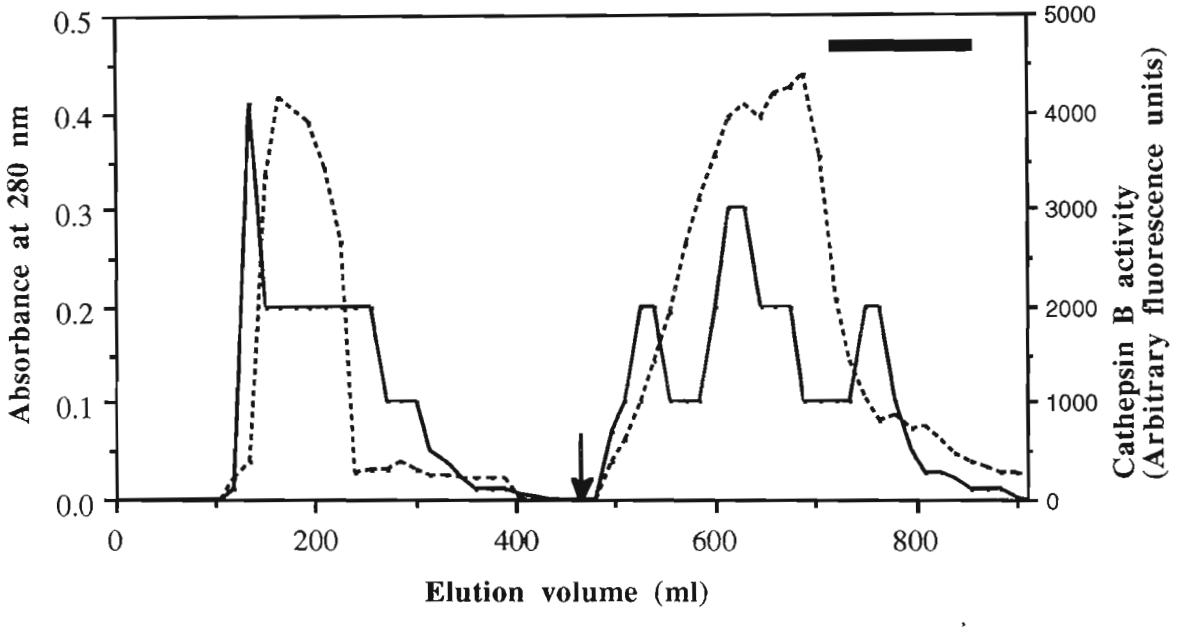

Figure 3.10. Chromatography of the 20-40% pH 4.2 TPP fraction on S-Sepharose (novel method).

Column, 2.5 x 18.3 cm (89.9 ml bed volume), equilibrated in buffer A [20 mM Na-acetate, pH 5.0, containing 1 mM Na_2EDTA, 0.02% NaN_3], and eluted with a 0-300 mM NaCl gradient in 5 column volumes of the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 15 ml (18 min). (—) A_{280}, (...) cathepsin B activity against Z-Arg-Arg-NHMec, (▬) pooled fractions.

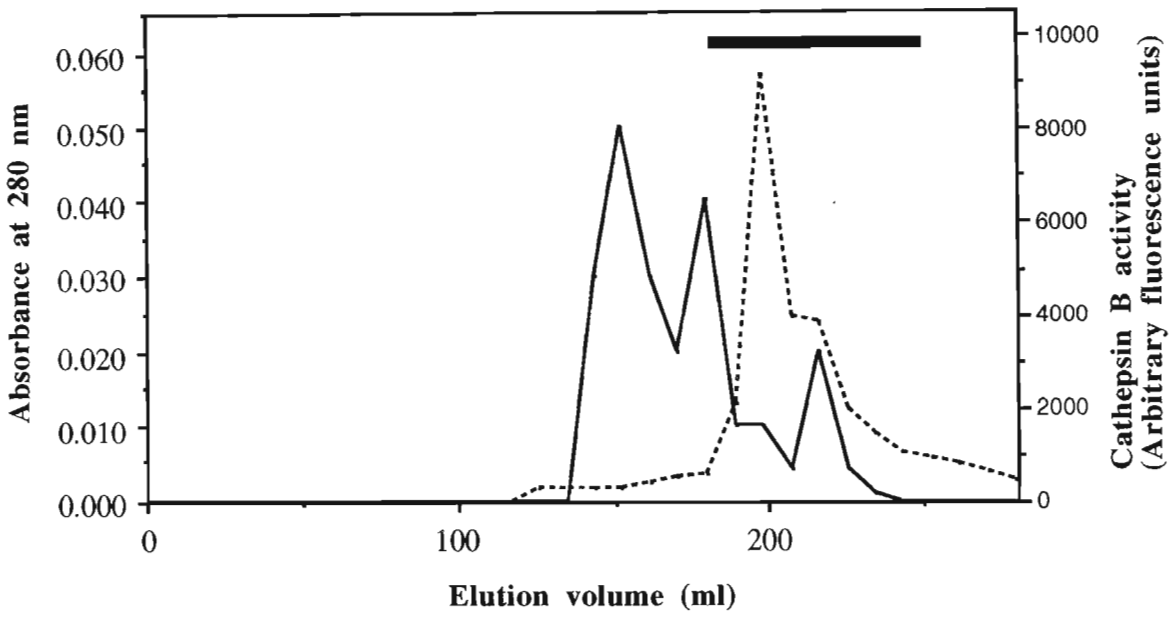

Figure 3.11. Chromatography of the pH 5.0 S-Sepharose fraction of cathepsin B on Sephacryl S-100 (novel method).

Column, 2.5 x 73 cm (358.5 ml bed volume) equilibrated and ran in buffer B [100 mM Na-acetate, pH 5.5, containing 500 mM NaCl, 1 mM Na_2EDTA. 0.02% NaN_3]; flow rate, 25 ml/h (5 cm/h); fractions, 4.5 ml (10.8 min). (—) A_{280}, (...) cathepsin B activity (arbitrary fluorescence units) against Z-Arg-Arg-NHMec, (▬) pooled fraction.

The overall progression of the isolation was analysed by reducing and non-reducing Tris-Tricine SDS-PAGE (Fig. 3.12 and 3.13). Purity of cathepsin B was confirmed by these gels (lanes f). A yield of 14.2% was obtained (Table 3.3).

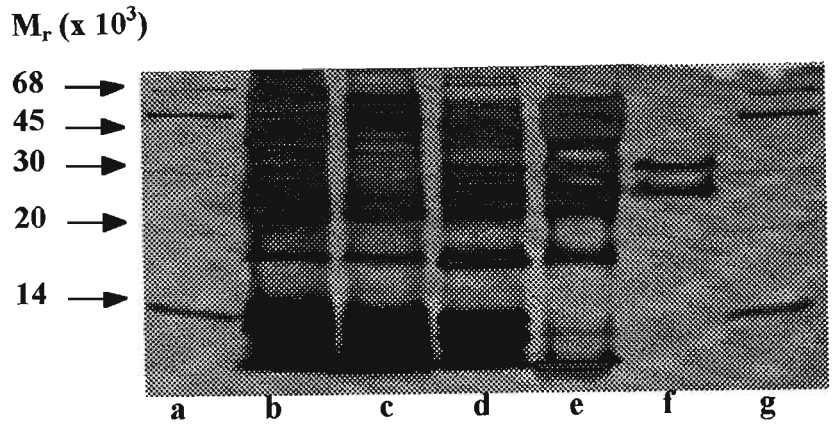

Figure 3.12. Reducing Tris-Tricine SDS-PAGE of cathepsin B isolated from rabbit liver by the novel method.

Samples were boiled in reducing treatment buffer and loaded onto a 10% Tris-Tricine gel. (b) liver homogenate fraction; (c) pH 4.2 acid precipitate supernatant; (d) TPP fraction; (e) S-Sepharose fraction; (f) Sephacryl S-100 fraction; (a,g) MW markers (BSA, M_r, 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; lysozyme, M_r 14 000. Visualised using silver staining.

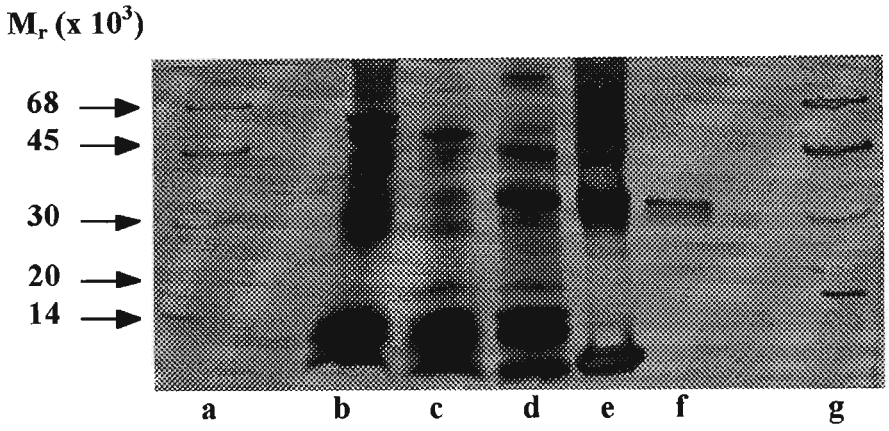

Figure 3.13. Non-reducing Tris-Tricine SDS-PAGE of cathepsin B isolated from rabbit liver by the novel method.

Samples were boiled in non-reducing treatment buffer and loaded onto a 10% Tris-Tricine gel. (b) liver homogenate fraction; (c) pH 4.2 acid precipitate supernatant; (d) TPP fraction; (e) S-Sepharose fraction; (f) Sephacryl S-100 fraction; (a,g) MW markers (BSA, M_r, 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; lysozyme, M_r 14 000. Visualised using silver staining.

3.3 Cathepsin L

Cathepsin L [EC 3.4.22.15] is a lysosomal cysteine proteinase, playing its main role in the degradation of proteins in lysosomes but which is sometimes secreted in a precursor form (Kirschke *et al.*, 1998). Cathepsin L is also involved in processes such as activation of

proplasminogen activator (urokinase type) thereby initiating a proteinase cascade, in degradation of matrix proteins, inhibition of normal antigen processing and promotion of proliferation processes (reviewed by Kirschke *et al.*, 1998).

Cathepsin L is synthesised as a preproenzyme, after which the signal peptide is cotranslationally removed in the endoplasmic reticulum. An ability of the propeptide to act as an inhibitor of mature cathepsin L was suggested by studying its crystallographic structure (reviewed by Kirschke *et al.*, 1998). The molecular masses of rat, mouse and chicken cathepsin L, as determined by SDS-PAGE, are M_r of *ca.* 38 000 for procathepsin L and 28 000 for cathepsin L in the single chain form. The double chain form has a heavy chain with a M_r of *ca.* 24 000 and a light chain with a M_r of *ca.* 4 000 (reviewed by Kirschke *et al.*, 1998).

The isoelectric point for mature cathepsin L is in the range of 5.0-6.3 (reviewed Kirschke *et al.*, 1998). It is stable in the pH range 4.5-6.0 and is catalytically active in the presence of thiol compounds and EDTA at pH 3.5-7.0 (reviewed by Kirschke *et al.*, 1998). It was also shown that the stability of cathepsin L is affected by ionic strenth and reductive agents (Dehrmann *et al.*, 1995; 1996).

3.3.1 Cathepsin L assay

Cathepsin L has a much more restricted activity on synthetic substrates than cathepsin B (Barrett and Kirschke, 1981). The synthetic substrates, Bz-Arg-NH_2, Z-Lys-$OPhNO_2$, and Z-Phe-Arg-NHMec have been used for cathepsin L. These are also susceptible to cathepsin B, but are more sensitive to cathepsin L (Barrett and Kirschke, 1981). Therefore, when assaying pure cathepsin L, these substrates can be used. However, when assaying a sample containing cathepsin B, problems arise, as all the activity observed cannot be assigned to cathepsin L. Dehrmann (1998), showed that at pH 5.5, the optimal pH for activity and stability of cathepsin L, 0.5 M urea causes an increase in cathepsin L activity. This was in contrast to goat spleen cathepsin B, which is not activated by urea at 0.5 M, but is completely, though reversibly, denatured by 1.5 M urea. Cathepsin L was consequently assayed with Z-Phe-Arg-NHMec, the most commonly used substrate for cathepsin L, with the presence of 3 M urea in the assay buffer, to inhibit cathepsin B activity.

3.3.1.1 Reagents

Buffer/activator [400 mM Na-acetate, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 8 mM DTT, pH 5.5]. Glacial acetic acid (23 ml), Na_2EDTA (1.5 g) and NaN_3 (0.2 g) were dissolved in 950 ml dist.H_2O, adjusted to pH 5.5 with NaOH and made up to 1 litre with dist.H_2O. DTT was added to a concentration of 8 mM (0.00671g DTT/5 ml buffer) immediately before use.

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

20 µM substrate solution. Substrate stock solution (0.1 ml) was diluted to 5 ml with dist.H_2O.

8 mM Dithiothreitol (DTT). DTT (1.2 mg) was dissolved in 1 ml dist.H_2O.

3.3.1.2 Procedure

Microassay. Enzyme sample (10 µl), and buffer/activator containing 8 mM DTT (75 µl), was added to the wells of a white Fluoronunc maxisorp microtitre plate and preincubated for 2 min at 37°C. Thereafter, 20 µM substrate solution (25 µl) was added and incubated at 37°C for 10 min, and the fluorescence of the liberated aminomethylcoumarin determined in a fluorescence microplate reader (Cambridge Technology Model 7620) with excitation at 370 nm, and emission at 460 nm. The standard curve was constructed as described in Section 3.2.1.2.

Macroassay. The macroassay was according to Barrett and Kirschke (1981), with modifications according to Dehrmann *et al.* (1995). All solutions were equilibrated at 37°C prior to use, except the enzyme solution which was kept on ice. Enzyme sample, diluted to 10 µl, buffer (800 µl), and 8 mM DTT solution (65 µl) were mixed and preincubated for 2 min at 37°C. After activation, 20 µM Z-Phe-Arg-NHMec solution (125 µl) was added. The fluorescence of the liberated aminomethylcoumarin was immediately monitored continuously for 5 or 10 min in a Hitachi F-2000 spectrofluorometer with excitation at 370 nm and emission at 460 nm.

3.3.2 Effect of increased t-butanol concentration on cathepsin L activity

3.3.2.1 Procedure

The method was as described in Section 3.2.2.2.

3.3.2.2 Results

The activity of cathepsin L at 0% t-butanol was taken as 100%, and the activity at other t-butanol concentrations, was expressed relative to this (Fig. 3.14). Relative activity declined with increasing t-butanol concentration and was essentially zero at 30% t-butanol (Fig. 3.14).

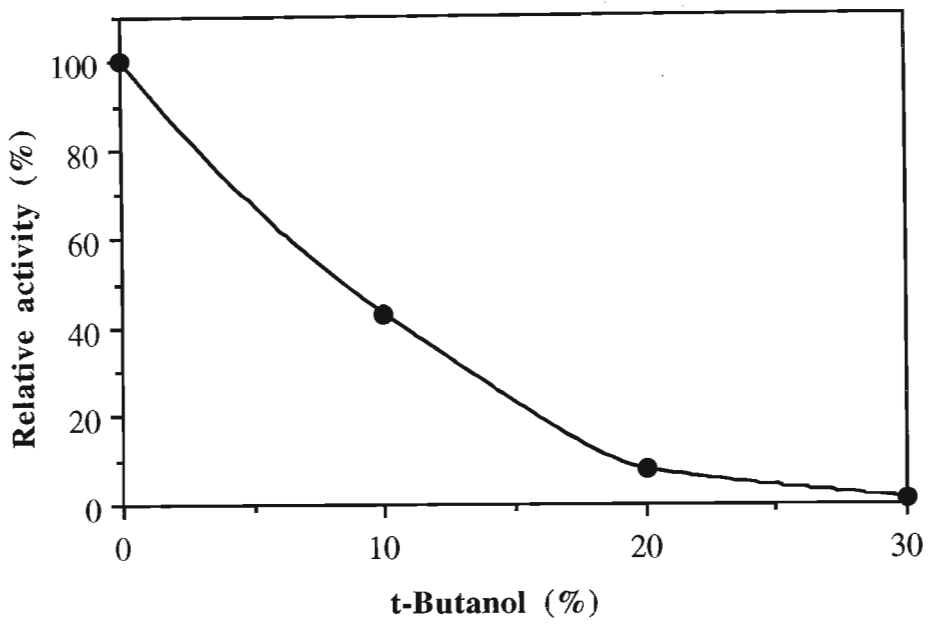

Figure 3.14. The effect of t-butanol on cathepsin L activity.

3.3.3 Control method for the isolation of sheep liver cathepsin L

Cathepsin L has been purified from various sources, including rat liver, rat kidney, rabbit liver (reviewed by Kirschke *et al.*, 1989), human and sheep liver (Coetzer, 1992), baboon liver (Coetzer, 1992; Coetzer *et al.*, 1995) and bovine liver (Mason, 1986). Mason (1986) found that sheep's liver gave the highest yield of cathepsin L.

Mature cathepsin L can exist as a single-chain form, with M_r 28 000, or as a two-chain form, consisting of a heavy chain, M_r 24 000 and a light chain, M_r 4 000 (Kirschke *et al.*, 1998). Different cathepsin L isolation protocols from different source tissues, gave different forms of cathepsin L, as either the two-chain form (reviewed by Kirschke *et al.*, 1998), the single-chain form (Pike and Dennison, 1989b; Coetzer, 1992) or as both the two- and single-chain forms (reviewed by Kirschke *et al.*, 1998). Pike and Dennison (1989b) stated that the occurrence of the two-chain form might be correlated with the length of the isolation procedure. They isolated single-chain cathepsin L from sheep liver by a rapid procedure involving TPP. This procedure, by Pike and Dennison (1989b), was used in the present study, with modifications described by Pike *et al* (1992).

During the development of the TPP method for the isolation of cathepsin L from sheep liver, Pike (1990) observed a proteolytically active, covalent, complex of stefin B, the cytoplasmic inhibitor of cathepsin L, and cathepsin L. This complex also occurred after isolations from human and baboon liver (Coetzer *et al.*, 1995). It was proved by Pike *et al.* (1992) that the complexes were not only generated by the TPP method, as they also occurred after isolation

using conventional ammonium sulfate precipitation. This suggested that the complexes may be present in tissue or may be formed at a pre-salting out stage, i.e. in the homogenate. The formation of this complex compromises the yield of free cathepsin L (Pike *et al.*, 1992; Coetzer *et al.*, 1995). The link in the complex, between stefin B and cathepsin L, appears to be a thioester bond between Cys-3 of the stefin B with Asp-71 of cathepsin L (Dehrmann, 1998). The formation of the complex may be catalysed by a factor in the homogenate (Pike *et al.*, 1992; Coetzer *et al.*, 1995). It was shown by Pike and Dennison (1989a), that in the presence of t-butanol, some proteins undergo conformational changes, favouring an increased proportion of α-helices. By adding t-butanol during homogenisation, cathepsin L may be reversibly inactivated due to conformational change brought about by the t-butanol, which in turn could prevent complex formation with stefin B. This hypothesis was tested in this study by firstly observing the effect of t-butanol on cathepsin L activity, and secondly, by adding t-butanol during homogenisation in the isolation procedure.

3.3.3.1. Reagents

Homogenisation buffer [100 mM Na-acetate, 0.1% (m/v) Na_2EDTA, 2% (v/v) n-butanol, pH4.0]. Glacial acetic acid (11.44 ml) and Na_2EDTA (2.0 g) were dissolved in 1.9 litres dist.H_2O and n-butanol (40 ml) was added, and the solution adjusted to pH 4.0 with NaOH and made up to 2 litres with dist.H_2O.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (2.29 ml), Na_2EDTA (0.74 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres dist.H_2O, adjusted to pH 5.5 with NaOH and made up to 2 litres with dist.H_2O.

S-Sepharose fast flow. As described in Section 3.2.4.1.

Sephadex G-75. Sephadex G-75 was prepared by adding 31.3 g of dry Sephadex G-75 to 380 ml of buffer A. The mix was boiled and allowed to stand overnight at 4°C. The resulting slurry was packed into a glass column under gravity. The column bed was washed with five column volumes of buffer A before use.

Sheep liver. Fresh sheep livers were collected from the Cato Ridge abattoir, cut into cubes and immediately frozen (-70°C) for a minimum of 3 days before use.

3.3.3.2 Procedure

Cathepsin L was isolated from sheep liver as described by Pike *et al.* (1992), with slight modifications. Frozen sheep liver (180 g) was thawed overnight at 4°C and homogenised at a ratio of 1:2 (liver mass to homogenisation buffer volume) with homogenisation buffer in a Waring blendor for 2 min. The homogenate was centrifuged (6500 x g, 30 min, 4°C) and the supernatant adjusted to pH 4.2 with 1 M HCl and stirred. The pH 4.2 supernatant was

centrifuged (6000 x g, 20 min, 4°C). The resultant supernatant was collected and used for TPP fractionation.

TPP was effected on the pH 4.2 supernatant by adding 30% (v/v of final pH 4.2 supernatant volume) of t-butanol. Crushed, solid $(NH_4)_sSO_4$ [20% (m/v) based on the volume of the pH 4.2 supernatant and t-butanol] was added and dissolved with gentle stirring. The solution was centrifuged (6000 x g, 15 min, 4°C) in a swing-out rotor. The precipitate was discarded, and to the collected supernatant and subnatant, a further amount of $(NH_4)_2SO_4$ was added to bring the final concentration to 45% (m/v) (based on the volume of the pH 4.2 supernatant and t-butanol). This mixture was centrifuged (6000 x g, 15 min, 4°C). The supernatant was discarded and the precipitate collected and redissolved in buffer A at one fifth of the volume of the pH 4.2 supernatant volume. The pH was adjusted to pH 5.5 with NaOH, and the resuspended material was centrifuged (27000 x g, 10 min, 4°C) to remove any insoluble material. The clarified sample was applied onto a column of S-Sepharose (2.5 x 23 cm = 113 ml), pre-equilibrated with 200 mM NaCl in buffer A. Unbound material was eluted with two column volumes of 200 mM NaCl in buffer A. Bound material was eluted with a 200-600 mM NaCl gradient in buffer A in *ca.* 5 column volumes, at 50 ml/h. The active fractions, corresponding to the active peak, were pooled and concentrated by dialysis against PEG 20 000.

3.3.3.3 Results

The purification of sheep liver cathepsin L, using the control TPP method, is summarised in Table 3.4.

Table 3.4. The purification of sheep liver cathepsin L, using the control TPP method.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	345	4630	12537	2.7	(1)	(100)
pH 4.2 supernatant	350	3455	12733	3.7	1.4	101.6
TPP cut (20-45%)	42	181	3270	18.1	6.7	26.1
S-Sepharose	33	0.8	2133	2585.5	954.0	17.0

Cathepsin L eluted from the S-Sepharose column at 458-552 mM NaCl (Fig. 3.15).

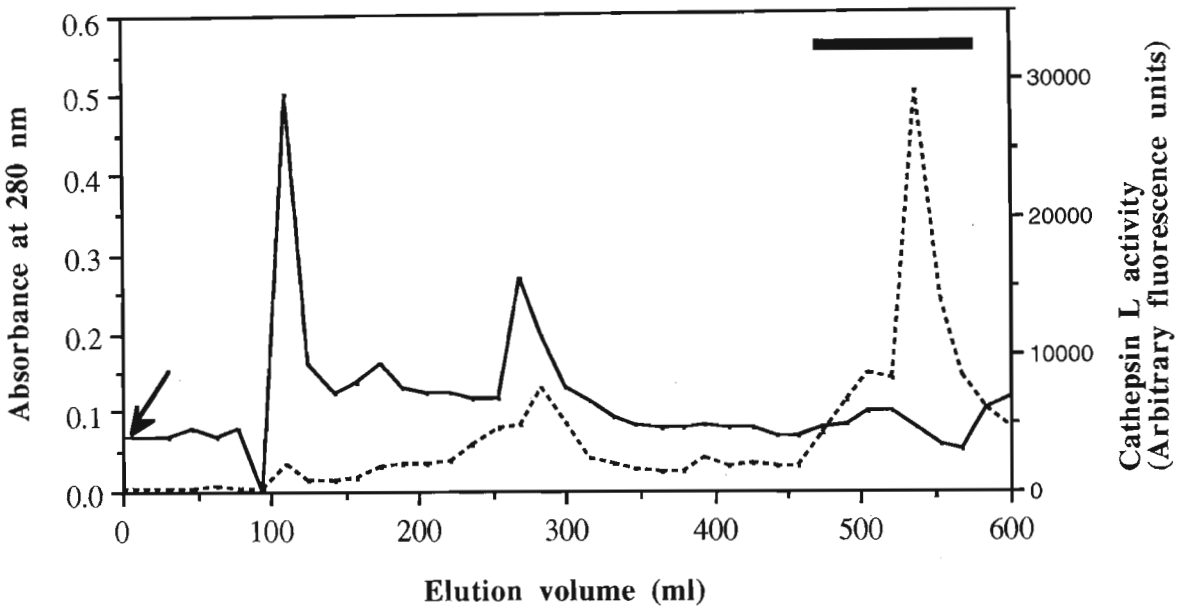

Figure 3.15. Chromatography of the 20-45% pH 4.2 TPP fraction of cathepsin L on S-Sepharose (control method).

Column, 2.5 x 23 cm (113 ml bed volume), equilibrated in 20 mM Na-acetate, pH 5.5, containing 200 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, and eluted with a 200-600 mM NaCl gradient in the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 7.9 ml (9.62 min). (—) A_{280}, (...) cathepsin L activity (arbitrary fluerescence units) against Z-Phe-Arg-NHMec, (▬) pooled fractions.

Analysis of the active peak on reducing SDS-PAGE showed two bands of M_r of about 25 000 and below 14 000 (Fig 3.16, lane e). This suggests that the cathepsin L-stefin B complex was isolated and the band below 14 000 is stefin B. The overall progression of the isolation up to the S-Sepharose stage was analysed by reducing Tris-Tricine SDS-PAGE (Fig. 3.16). A yield of 17.0% cathepsin L was obtained (Table 3.4).

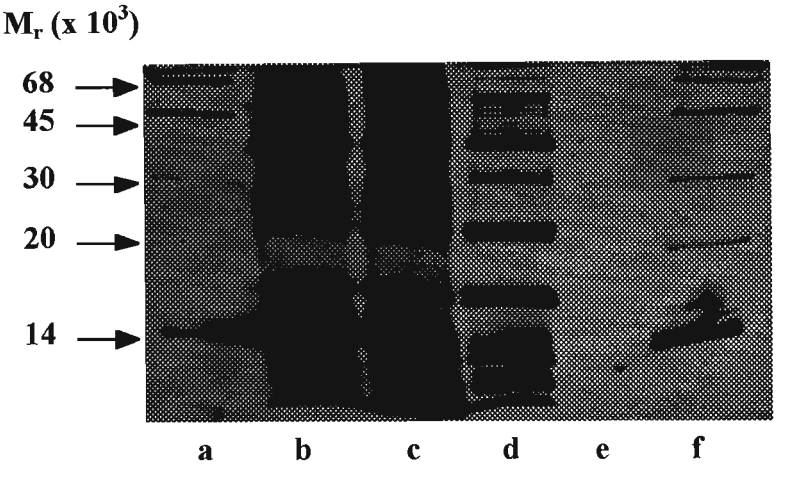

Figure 3.16. Reducing Tris-Tricine SDS-PAGE of cathepsin L isolated from sheep liver by the control method.

Samples were boiled in reducing treatment buffer and loaded onto a 10% Tris-Tricine gel. (b) liver homogenate fraction; (c) pH 4.2 acid precipitate supernatant; (d) TPP fraction; (e) S-Sepharose fraction; (a,f) MW markers (BSA, M_r, 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; lysozyme, M_r 14 000). Visualised using silver staining.

3.3.4 Isolation of sheep liver cathepsin L using a novel method with t-butanol in the homogenisation buffer

3.3.4.1 Reagents

Homogenisation buffer [100 mM Na-acetate, 0.1% (m/v) Na_2EDTA, 2% (v/v) n-butanol, pH4.0]. As described in Section 3.3.3.1.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. As described in Section 3.3.3.1.

S-Sepharose fast flow. As described in Section 3.2.4.1.

Sephadex G-75. As described in Section 3.3.3.1.

Sheep liver. As described in Section 3.3.3.1.

3.3.4.2 Procedure

Frozen sheep liver (180 g) was thawed overnight at 4°C and homogenised in a mixture of two parts of homogenisation buffer and sufficient t-butanol to give 30% (w/v) t-butanol in the homogenate. The homogenate was centrifuged (6 500 x g, 30 min, 4°C) and supernatant adjusted to pH 4.2 with 1 M HCl and stirred. The pH 4.2 supernatant was centrifuged (6 000 x g, 20 min, 4°C). A 20-45% ammonium sulfate TPP cut was taken as described in Section 3.3.3.2., without the addition of further t-butanol. The clarified TPP cut was applied to a column of S-Sepharose (2.5 x 23.5 cm = 115.4 ml bed volume), pre-equilibrated with 200 mM NaCl in buffer A. A gradient of 200-600 mM NaCl in buffer A in *ca.* five column volumes was used to elute the bound material after unbound material was eluted with 200 mM NaCl in buffer A, at 50 ml/h. The fractions, corresponding to the active peak were pooled and dialysed against sucrose to 1-5% of the volume of the molecular exclusion chromatography column.. This fraction was applied to a column of Sephadex G-75 (2.5 x 76.5 cm = 375.7 ml bed volume) pre-equilibrated with 200 mM NaCl in buffer A and eluted at 25 ml/h. The active fractions, of *ca.* M_r 25 000 were pooled and dialysed against PEG 20 000.

3.3.4.3 Results

The purification of sheep liver cathepsin L, using the novel TPP method with t-butanol in the homogenisation buffer, is summarised in Table 3.5.

Table 3.5. The purification of sheep liver cathepsin L, with t-butanol in the homogenisation buffer.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purifi-cation (fold)	Yield (%)
Homogenate	520	6682	27981	4.2	(1)	(100)
pH 4.2 supernatant	500	3390	25140	7.4	1.8	89.9
TPP cut (20-45%)	44	707	3339	4.7	1.1	6.0
S-Sepharose	35	0.9	2983	3278.0	782.3	10.7
Sephadex G-75	7	0.13	173	1330.8	317.6	0.62

Peak activity of cathepsin L eluted from the S-Sepharose column at 435-525 mM NaCl (Fig. 3.17). Analysis of this peak by non-reducing SDS-PAGE showed a band of *ca.* M_r 25 000 (Fig. 3.20).

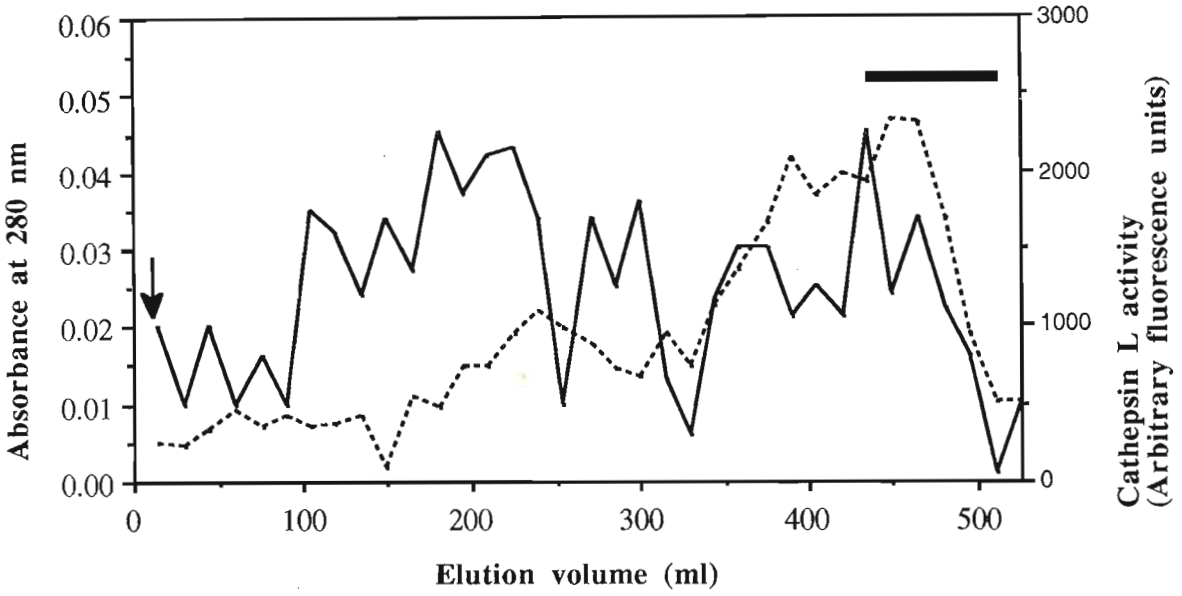

Figure 3.17. Chromatography of the 20-45% pH 4.2 TPP fraction of cathepsin L on S-Sepharose (novel method).

Column, 2.5 x 23.5 cm (115.4 ml bed volume), equilibrated in 20 mM Na-acetate, pH 5.5, containing 200 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, and eluted with a 200-600 mM NaCl gradient (231 ml) in the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 7.9 ml (9.62 min). (—) A_{280}, (...) cathepsin L activity against Z-Phe-Arg-NHMec, (▬) pooled fractions.

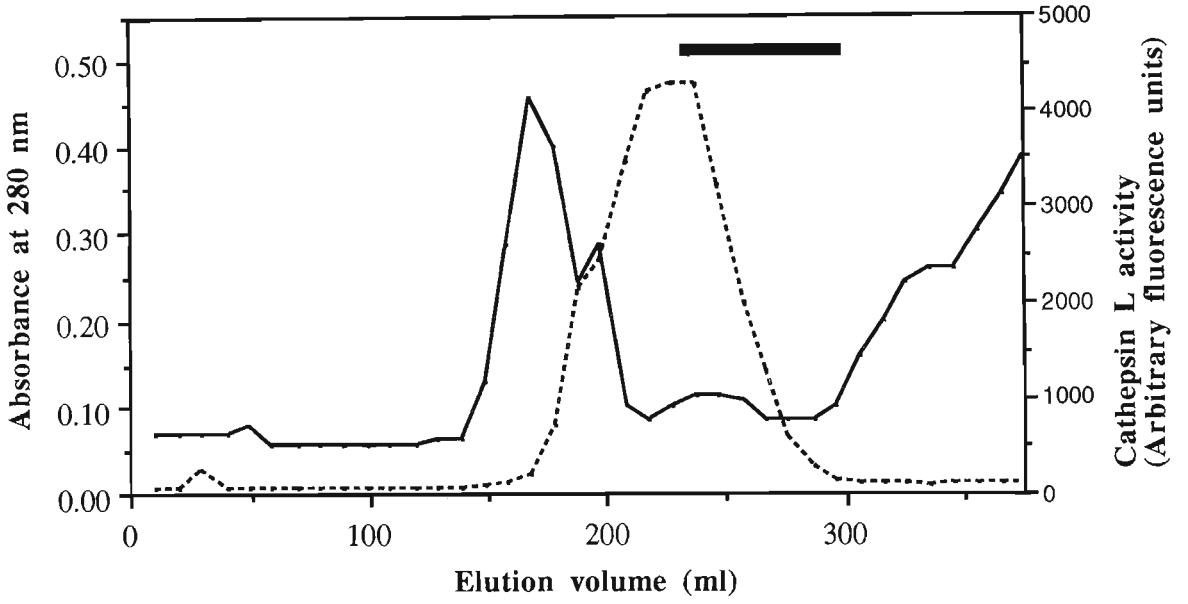

Figure 3.18. Chromatography of the pH 5.5 S-Sepharose fraction of cathepsin L on Sephadex G-75 (novel method).

Column, 2.5 x 76.5 cm (375.7 ml bed volume) equilibrated and ran in 20 mM Na-acetate, pH 5.5, containing 200 mM NaCl, 1 mM Na_2EDTA. 0.02% NaN_3; flow rate, 25 ml/h (5 cm/h); fractions, 4.94 ml (12.35 min). (—) A_{280}, (...) cathepsin L activity against Z-Phe-Arg-NHMec, (▬) pooled fraction.

The pooled cathepsin L fraction from S-Sepharose was chromatographed on Sephadex G-75 column (Fig 3.18). The eluted fractions were analysed on SDS-PAGE to test the purity of cathepsin L (results not shown) and the pooled fraction (Fig. 3.19, lane e) was chosen on this basis. A yield of 0.62% cathepsin L was obtained (Table 3.5).

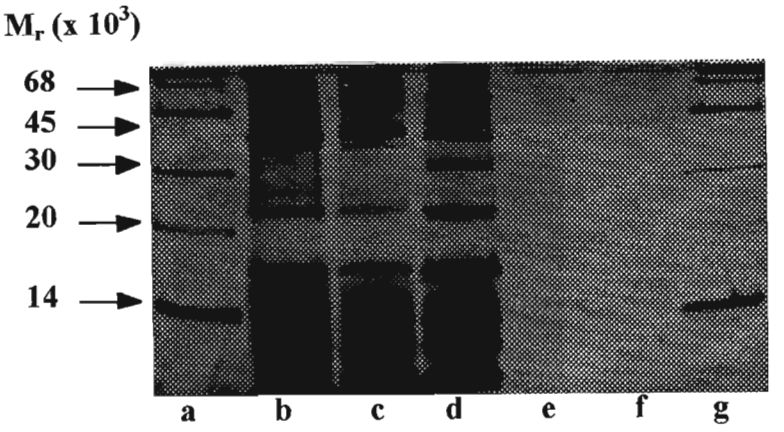

Figure 3.19. Reducing Tris-Tricine SDS-PAGE of cathepsin L isolated from sheep liver by the novel method.

Samples were boiled in reducing treatment buffer and loaded onto a 10% Tris-Tricine gel. (b) liver homogenate fraction; (c) pH 4.2 acid precipitate supernatant; (d) TPP fraction; (e) S-Sepharose fraction; (f) Sephadex G-75 fraction; (a,g) MW markers (BSA, M_r, 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; lysozyme, M_r 14 000). Visualised using silver staining.

No bands are evident in lane e in Fig. 3.19. However, a band can be seen in lane f (Fig. 3.19) and it is therefore concluded that the S-Sepharose sample loaded in lane e was not concentrated enough.

3.4 Discussion

Cathepsin B was isolated in the present study for further studies on generating autoantibodies specific for the enzyme (Chapter 4). Large quantities of cathepsin B were needed for these purposes. The isolation method as described by Meinesz (1996), referred to as the conventional isolation method in this chapter, was modified in the upstream stages in order to attempt to increase the yield of cathepsin B. The conventional method (Meinesz, 1996) for cathepsin B isolation, makes use of a sequence of homogenisation → acid precipitation → TPP → chromatography.

A closer examination was made of the effect of t-butanol on cathepsin B and cathepsin L activity. An increased concentration of t-butanol depressed the activity of cathepsin B and at 30% t-butanol, cathepsin B activity was essentially extinguished (Fig 3.1). t-Butanol had the same effect on cathepsin L activity (Fig. 3.14). It was observed, however, that t-butanol causes reversible inactivation, as activity of cathepsin B was restored by dialysing out the t-butanol (Fig. 3.2). After a 3-day long dialysis period at 4°C of a sample of cathepsin B containing 30% t-butanol, the activity of cathepsin B increased by 69%. Loss of enzyme activity, due to autocatalysis, was compensated for by keeping a sample of cathepsin B

containing no t-butanol in identical conditions, for the same period of time. The activity of the enzyme in the sample containing no t-butanol, after 3 days of dialysis, was taken as 100% activity. Similar effects of t-butanol have been reported by Vaillancourt *et al.* (1998) who reported that t-butanol inhibits the activity of 2,3 dihydroxybiphenyl 1,2-dioxygenase. Although the inhibiting action of t-butanol is not yet understood, it has been found that in the presence of t-butanol, some proteins undergo conformational changes, favouring an increased proportion of α-helices (Pike and Dennison, 1989a).

In the conventional isolation methods for cathepsin B and L, 30% t-butanol is added at the TPP step, after homogenisation and acid precipitation. Due to the insights on t-butanol's effect on cathepsin B and L, it was hypothesised that by adding t-butanol straight to the homogenisation buffer, final yields may be increased. Before carrying out enzyme activity assays, for monitoring enzyme purity by calculation of specific activity and total activity, t-butanol was removed by dialysis.

3.4.1 Isolation of cathepsin B

Three different isolations were carried out, differing in the TPP and homogenisation steps. The control isolation differed from the conventional isolation (Meinesz, 1996) only in the homogenisation buffer used. The homogenisation buffer in the conventional isolation was a 50 mM Na-acetate buffer containing 150 mM NaCl and 1 mM Na_2EDTA with a pH of 4.5. The homogenisation buffer used in the control and novel isolations methods were changed to be a 150 mM Na-acetate buffer containing no NaCl, and the pH reduced to pH 4.0. This reduction in pH was to increase the buffering capacity of the homogenisation buffer during homogenisation. To further buffer the change in pH during homogenisation, the homogenisation buffer volume to liver mass ratio was changed from 1:1 (as used in the conventional isolation) to 2:1 for the control and novel isolations methods.

The efficiency of the isolation of cathepsin B, with t-butanol added to the homogenisation buffer (novel isolation method), was measured by comparing it to the control isolation method, which followed the conventional isolation method as described by Meinesz (1996). The results obtained by the novel isolation method could not be compared directly to that of the conventional isolation method due to the difference in the homogenisation buffers used. Also, in the conventional isolation method, a different mass of rabbit liver was used, and the ratio of liver mass to homogenisation buffer volume differed, compared to the control and novel isolations methods.

It is interesting to note that the total activity in the homogenate of the conventional isolation (Table 3.1), is much lower than those of the homogenates of the control and novel isolations (Tables 3.2 and 3.3), in spite of the fact that a much higher mass of rabbit liver was used. The

higher pH and presence of NaCl in the homogenisation buffer, used in the conventional method, might have contributed to the lower cathepsin B activity. The pH and ionic strength of a buffer plays an important role on cathepsin B a cathepsin L activity. It was reported by Dennison *et al.* (1992) that the activity of cathepsin L is decreased by an increase in ionic strength. Regarding cathepsin B, the effect of pH and ionic strength on its activity has been explained by Turk *et al.* (1994) in a model showing the deprotonation of the imidazole moiety of the active site -$S^-/^+$HIm ion pair. Deprotonation of the imidazole moiety of the ion pair, caused by increased pH and ionic strength leads to the "unzipping" of the molecule (Chapter 1).

The pH 4.2 supernatant sample was not taken for assays for the control and novel isolations, as only a minimal amount of acetic acid was used to adjust the pH to 4.2. This might have been due to the higher buffering capacity caused by the lowered pH used for the homogenisation buffer in these isolations. In the conventional isolation a much higher quantity of acetic acid was needed to adjust the pH of the homogenate to pH 4.2, causing acid precipitation.

Because t-butanol reversibly inactivates cathepsin B activity, it was necessary to dialyse samples containing t-butanol before assaying for activity. But, even after dialysis, some t-butanol may still remain. Also, if the homogenate sample containing t-butanol was dialysed for too long, autocatalysis and proteolysis may occur. A catch-22 situation is therefore created where the activity of the enzyme is depressed unless all the t-butanol is dialysed out, but, if dialysed too long, activity may be lost via autocatalysis and proteolysis. This might explain why the activity of the homogenate containing t-butanol (novel isolation) (Table 3.3) is lower than that of the homogenate of the control isolation, containing no t-butanol (Table 3.2). If this hypothesis is correct, the activity values in the purification tables might be misleading. As a result, specific activity and yield values would also be misleading. Another possible explanation for the low activity in the homogenate containing t-butanol, is the presence of inhibitors that may bind more readily with cathepsin B after the t-butanol has been dialysed out. Because TPP removes most of the t-butanol, the activity of cathepsin B, from the homogenate containing t-butanol, is suppressed until the TPP step.

From the results in Tables 3.2 and 3.3, it can be concluded that homogenisation in the presence of 30% t-butanol is more efficient and gives an 11-fold increase in final yield and a 3-fold increase in final specific activity, compared to homogenisation in the absence of t-butanol.

3.4.2 Isolation of cathepsin L

Proteolytically active, covalent complexes of stefin B and cathepsin L, which compromise the yield of free cathepsin L, have been reported after isolations (Coetzer *et al.*, 1995; Pike *et al.*,

1992). It has been hypothesised that the formation of this complex may be catalysed by a factor in the homogenate (Dehrmann, 1998) which implies the involvement of an enzyme. But, 30% t-butanol extinguishes all enzyme activity. The addition of 30% t-butanol to the homogenate during homogenisation could therefore suppress the formation of the stefin B-cathepsin L complex. Also, if the presence of t-butanol causes conformational change, favouring increased proportions of α-helices in enzymes, as Pike and Dennison (1989a) found, complex formation of stefin B and cathepsin L may be prevented.

Cathepsin L was found to be pure after the S-Sepharose step for the homogenate containing no t-butanol (control isolation) and the homogenate containing 30% t-butanol (novel isolation). However, a complex of cathepsin L and stefin B was isolated in the control isolation (Fig. 3.16). This complex was dissociated by reducing SDS-PAGE, evident in Fig. 3.16 (lane e), where the bands of cathepsin L (25 kDa) and stefin B (14 kDa) can be seen. For the novel isolation, only one band of molecular weight of 25 kDa is evident on a reducing Tris-Tricine gel (Fig. 3.19) which suggests that only the single chain form of cathepsin L was isolated. In the novel isolation method there is no evidence that the complex of stefin B and cathepsin L was isolated.

The homogenisation buffer used for the control and novel methods differed from that used in the method described by Pike *et al.* (1992) in that the pH was lower. This might have contributed to the suppression of complex formation. Coetzer *et al.* (1995) found that low pH homogenisation decreased the proportion of complex but led to isolation of two-chain cathepsin L. The enzyme cleaving the cathepsin L to the two-chain form may have been inhibited by the 30% t-butanol.

The yield difference between isolations of the homogenate containing 30% t-butanol and the homogenate containing no t-butanol, is less marked than for cathepsin B. An increase in yield of about 1.4-fold was finally obtained (Table 3.5).

CHAPTER 4
ANTIBODIES

4.1 Introduction

The immune system has evolved to combat infection by developing a series of defence mechanisms that are equal to a diverse array of infectious agents in its effectiveness and ingenuity. The success of the immune system depends on its ability to differentiate between "foreign" material (non-self) and the body's own tissue (self) which causes the immune system to recognise only non-self material or antigens.

Not only the T cell-mediated arms of the immune response have been explored for cancer therapy. In carcinoma patients treated with a monoclonal antibody (MAb1) against epithelial cell adhesion molecule (EPCAM), a cell surface glycoprotein overexpressed by colorectal carcinoma cells, micrometastasis was prevented (Riethmüller *et al.*, 1994). When the monoclonal antibody was administered together with granulocyte-macrophage colony-stimulating factor (GM-CSF), complete remission resulted (Riethmüller *et al.*, 1994).

For autoantibodies to be generated against tumour antigens, the immune system must be able to recognise the "self" antigen. Self-reacting B cells which can develop into antibody-producing plasma cells can only produce antibodies in the presence of the appropriate T helper cells. A B cell that binds a self antigen will present peptides derived from it on self MHC class II molecules. T cells that recognise peptides derived from self proteins are either eliminated in the thymus or inactivated in the periphery in early neonatal life and, therefore, no T cell help will be available to activate such B cells. It is this T cell tolerance that prevents the production of self-reactive antibodies (Chapter 1). Therefore, success of the humoral response against "self" antigens starts with activation of T cells.

Tumours seem to be able to escape in the face of fully capable T cells, causing tolerance of the immune system to cancer cells. It is interesting that some patients have cancer despite an immune response to tumour antigens. It is possible that the tumour may establish its own microenvironment by altering the expression of MHC molecules and the levels of other activities affecting immune induction, such as peptide transporter molecules, preventing the presentation of peptide determinants on their cell surface (Nanda and Sercarz, 1995).

A large set of antigenic determinants of the self actually do not induce self-tolerance (reviewed by Sercarz *et al.*, 1993). These peptide determinants therefore furnish target structures for autoimmune attack (reviewed by Lanzavecchia, 1995). In the same way, such antigenic determinants may cause immune responses directed against tumours. Examples are the

self-proteins specific to the melanocyte lineage, tyrosinase (gp 100), and Melan-A-MART-1, recognised by T cells, are found in a majority of melanoma patients (Pardoll, 1999; Pieper *et al.*, 1999; Chaux *et al.*, 1999). It is thought that only well expressed self-determinants efficiently induce tolerance. On every self-antigen there are sequestered determinants that are normally unsuccessful in inducing tolerance, but under severe inflammation with its cytokine milieu, it can be displayed to the immune system (Nanda and Sercarz, 1995). Self-reactive tumour-specific T cells that evade negative selection can be mobilised when MHC molecules, surface adherence molecules, and costimulators are up-regulated to become available for killing interactions with newly displayed, previously "cryptic" self-antigenic determinants (reviewed by Sercarz *et al.*, 1993; Lanzavecchia, 1995). The density of the up-regulated peptide-MHC complexes and the expression of costimulatory signals may be critical in activating otherwise silent tumour-specific T cells (Nanda and Sercarz, 1995). Therefore, to induce an efficient immune response against tumours, these signals must be defined and it must be learned how to manipulate them. In other words, the system has to be manipulated in order to signal "danger" to the immune system for it to react.

In the present study, it was hypothesised that a "danger" signal could be induced to activate the immune system into producing autoantibodies recognising the lysosomal cysteine proteinase, cathepsin B, a "self-antigen", by conjugating the proteinase to BSA. In this way the immune system may be "tricked" into producing auto-antibodies against cathepsin B due to the recognition of the foreign BSA molecule in the conjugate. The cathepsin B-BSA conjugate bound to surface immunoglobulins on B-cells would be internalised and returned to the cell surface as peptides bound to MHC class II molecules. The peptide:MHC complex may then be recognised by helper T cells which deliver activating signals to the B cell. The B cells may then, in turn, differentiate into antibody-secreting cells.

Cathepsin B, a lysosomal cysteine proteinase, has been implicated in a variety of human tumours (reviewed by Sloane *et al.*, 1990). It causes the malignant progression of tumours, as a result of its increased activity and secretion or membrane association in cancer cells (Yan *et al.*, 1998). Although cathepsin B occurs on the surface of cancer cells, it occurs intracellularly in normal cells. The expression of the enzyme on the surface of cancer cells, may result in a difference in the conformation of the enzyme, compared to when expressed intracellularly. This may increase immunity against cathepsin B, but may not be high enough to pose a "danger" signal to initiate an immune response. Antibodies directed against cathepsin B in cancer patients may stop the progression of tumours due to the neutralisation of cathepsin B activity. Also, opsonisation of tumour cells by the adherent antibodies may lead to their subsequent elimination by other elements of the immune system, such as macrophages.

In the present study, an attempt was made to raise autoantibodies in rabbits against cathepsin B, isolated from rabbit liver. It was anticipated that these autoantibodies would not only

recognise cathepsin B, but may also inhibit its activity. In a study by Pierart-Gallois *et al.* (1977), antibodies generated against rat liver cathepsin B in rabbits, not only recognised, but also inhibited rat liver cathepsin B activity. The rat liver cathepsin B these investigators injected, was conjugated to bovine serum albumin (BSA) with glutaraldehyde as the coupling agent. They attempted, successfully, to couple cathepsin B to albumin while avoiding direct contact of cathepsin B with glutaraldehyde, as direct contact with glutaraldehyde completely inactivates cathepsin B. According to Pierart-Gallois *et al.* (1977), the conjugated cathepsin B was active against BANA. It is important to note, however, that the cathepsin B preparation used for conjugation was only partially purified and that the substrate, BANA, is not specific for cathepsin B. It might be possible, therefore, that a contaminating proteinase, other than cathepsin B, could have contributed to the hydrolysis of BANA. However, because Pierart-Gallois *et al.* (1977) obtained good results, with the antibodies recognising and neutralising cathepsin B, the same conjugation protocol was used in the present study, but with pure cathepsin B.

Different inoculation protocols were used in the present study in an attempt to generate autoantibodies against cathepsin B in rabbits. Conjugated cathepsin B was injected intramuscularly, using adjuvants, and intravenously, without the use of adjuvants. Free cathepsin B was administered in the same way, intramuscularly and intravenously. However, free cathepsin B, injected intravenously, was activated with cysteine, immediately before inoculation, to increase the possibility of the enzyme being complexed with α_2-macroglobulin (Chapter 1).

4.2 Conjugation of cathepsin B to BSA using glutaraldehyde

Cathepsin B was conjugated to BSA using the bifunctional coupling agent, glutaraldehyde, according to the method of Pierart-Gallois *et al.* (1977). Pierart-Gallois *et al.* (1977) did not record any buffers used or the molecular ratios of BSA, enzyme or glutaraldehyde. As a result, the method outline described by Pierart-Gallois *et al.* (1977) was used as a basis, with the details being adapted from a method described by Bulinski and Gunderson (1986).

4.2.1 Reagents

Cathepsin B. Rabbit liver cathepsin B was isolated as described in Section 3.2.4.2.

Conjugation buffer [200 mM Na-acetate, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (11.43 ml) and NaN_3 (0.2 g) were dissolved in 850 ml of dist.H_2O, titrated to pH 5.5 with NaOH, and made up to 1 litre with dist.H_2O.

Bovine serum albumin solution. BSA (2.5 mg, 0.037 µmoles, assuming an M_r of 68 000) was dissolved in 1 ml conjugation buffer by gentle stirring.

0.2 M Glycine. Glycine (0.9 g) was dissolved in 5 ml of conjugation buffer.

Sephadex G-25. Sephadex G-25 was prepared by hydrating 16 g of dry Sephadex G-25 overnight in 80 ml conjugation buffer, followed by boiling and allowing to stand overnight at 4°C. The resulting slurry was packed into a glass column under gravity. The column bed was washed with five column volumes of conjugation buffer before use.

Sephadex G-100. Sephadex G-100 was prepared by adding 27.3 g of dry gel powder to 410 ml of conjugation buffer. The mix was boiled and allowed to stand overnight at 4°C. The resulting slurry was packed into a glass column under gravity. The column bed was washed with five column volumes of conjugation buffer.

4.2.2 Procedure

Glutaraldehyde (148 µl of a 25% solution, 3,7 µmoles) was added, dropwise with stirring, to the BSA solution (1 ml), and gently stirred overnight at room temperature. The molar ratio of BSA to glutaraldehyde was therefore 1:100. The solution was loaded onto a Sephadex G-25 column (2.5 x 25 cm = 123.0 ml), and eluted with conjugation buffer, to remove unreacted glutaraldehyde. Pure cathepsin B (1 mg, 0.037 µmoles), purified as described in Section 3.2.4.2, was immediately mixed with the "activated" serum albumin, which eluted at Vo, by gentle stirring, overnight at 4°C. Unreacted glutaraldehyde in the conjugate was quenched with 0.2 M glycine, in conjugation buffer. The quenched conjugate solution was loaded onto a Sephadex G-100 column (2.5 x 83.5 cm = 410.0 ml), and eluted with conjugation buffer, at a flow rate of 49.1 ml/h (10 cm/h) to separate conjugated from free cathepsin B. The amount of cathepsin B conjugated to the carrier protein, BSA, was estimated as described in Section 4.2.3.

4.2.3 Estimation of coupling yield

Each elution fraction was run on a reducing and non-reducing Tris-Tricine gel and visualised by silver staining. The fractions containing protein complexes of apparent molecular weight of 150 kDa or larger, eluted at Vo (143-180 ml). These fractions were assumed to be complexed cathepsin B (Pierart-Gallois *et al.*, 1977). These fractions were pooled, concentrated and stored at -20°C.

The free enzyme peak, eluted from the Sephadex G-100 column, was determined by testing peaks for cathepsin B activity, as described in Section 3.2.1.2, using Z-Arg-Arg-NHMec. This peak was pooled and the enzyme concentration determined using the Bradford-dye binding assay as described in Section 2.2.1. This free enzyme amount was subtracted from the original amount of enzyme used for conjugation, to give the amount of cathepsin B conjugated to BSA. This figure was used to calculate the amount of conjugate required for inoculation.

4.3 Production of autoantibodies against cathepsin B in rabbits

Attempts were made to raise antibodies in rabbits against cathepsin B, isolated from rabbit liver, using four different protocols differing in the site of inoculation and on whether cathepsin B was inoculated as a free or conjugated enzyme.

Two male rabbits were inoculated intramuscularly with rabbit liver cathepsin B conjugated to BSA. A total of 25 µg cathepsin B per animal was administered once a week for 3 weeks. After a 3 week rest period, another series of 3 injections of conjugated cathepsin B were administered at weekly intervals. For the first injection of each 3 week inoculation series, the cathepsin B preparation was emulsified in a 1:1 (v/v) ratio with Freund's complete adjuvant (FCA). Thereafter, for the last two injections of each series, cathepsin B was emulsified in a 1:1 (v/v) ratio with Freund's incomplete adjuvant (FIA).

Two male rabbits were inoculated intramuscularly with free rabbit liver cathepsin B using the same protocol as described above for the intramuscular inoculation of cathepsin B conjugated to BSA.

Two male rabbits were inoculated intravenously into the peripheral ear vein with cathepsin B conjugated to BSA. Because no adjuvants are used for intravenous inoculations, a total of 25 µg cathepsin B, per animal, was administered twice a week for 3 weeks. After a 3 week rest period, another series of 2 injections per week for 3 weeks followed.

Two male rabbits were inoculated intravenously with free cathepsin B. The same protocol was used as described above for the intravenous inoculation of conjugated cathepsin B. However, in this protocol, cathepsin B in the inoculum was activated by adding 50 mM cysteine, immediately before inoculation.

Rabbits were bled from the marginal ear vein at 4 and 8 weeks and by cardiac punture at 10 weeks. The progress of the antibody response to cathepsin B was followed by ELISA analysis of the serum samples, as described in Section 4.4.

4.4 Enzyme-linked immunosorbent assay (ELISA).

The enzyme-linked immunosorbent assay (ELISA), introduced by Engvall and Perlmann (1971), is based on the labelling of an antibody or antigen, with a chemically conjugated enzyme, to allow the detection of immune complexes formed on a solid phase. In the three layer or non-competitive ELISA system, the simplest and most commonly used ELISA system, antigen is coated to the plastic surface of wells of polystyrene microtitre plates. The primary antibody is quantified by allowing it to form a complex with the immobilised antigen. After excess antibody has been washed away, the degree or amount of reactivity is quantified with an enzyme conjugated to a secondary antibody. The secondary antibody will recognise

the primary antibody bound to the immobilised antigen, and the enzyme reacts with a substrate to yield a coloured product which can be measured spectrophotometrically.

This three layer ELISA system was used in the present study to evaluate the progress of polyclonal antibody production during the immunisation of rabbits with cathepsin B and cathepsin B-BSA complex. Usually, a blocking agent of 0.5% (m/v) bovine serum albumin-PBS is used. Because the antigen immobilised here was BSA in complex with cathepisn B, this blocking agent could not be used, as primary antibodies against BSA might cause misleading results. Various blocking agents were tested, and 5% milk-PBS proved successful (results not shown). Milk's suitability as a blocking agent was tested in an ELISA using cytochrome C as an antigen, chicken anti-cytochrome C IgY as primary antibody, and rabbit anti-chicken IgG as secondary antibody (results not shown).

4.4.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 1 litre of dist.H_2O.

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

0.1% PBS-Tween. Tween 20 (1 ml) was made up to 1 litre in PBS.

0.15 M citrate-phosphate buffer, pH 5.0. A solution of citric acid.H_2O (21.0 g/l) was titrated with a solution of $Na_2HPO_4.2H_2O$ (35.6 g/l) to pH 5.0.

Substrate solution [0.05% (m/v) 2,2'-azino-di(3-ethyl)-benzthiozoline sulphonic acid (ABTS) and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer]. ABTS (7.5 mg) and H_2O_2 (7.5 µl) were dissolved in citrate-phosphate buffer, pH 5.0 (15 ml), for one ELISA plate.

Stopping buffer [citrate-phosphate-0.1% (m/v) NaN_3]. NaN_3 (0.1 g) was made up to 100 ml in citrate-phosphate buffer.

4.4.2 Procedure

Wells of microtitre plates (Nunc Immunoplate) were coated with antigen (150 µl) at a predetermined dilution (1 µg/ml as determined by a checkerboard ELISA) in PBS overnight at room temperature. Wells were blocked with milk-PBS (200 µl) for 1 h at 37°C and washed 3 times with PBS-Tween. Serial two fold dilutions of the primary rabbit IgG in milk-PBS (1 mg/ml) were added (100 µl), incubated for 1 h at 37°C and excess antibody washed out 3 times with PBS-Tween. A 1:450 dilution of sheep anti-rabbit IgG-horseradish peroxidase (HRPO) conjugate in milk-PBS, was added (120 µl) and incubated for 1 h at 37°C. The ABTS substrate (150 µl) was added and incubated in the dark for optimal colour development

(usually 10-20 min). The enzyme reaction was stopped by the addition of 50 µl of 0.1% (m/v) NaN_3 in citrate-phosphate buffer and the absorbance read at 405 nm in a Bio-Tek EL307 ELISA plate reader.

4.4.3 Results

Sera of 4, 8 and 10 week bleeds, from each rabbit, were tested using a chequerboard ELISA coated with cathepsin B (0.5-10 µg/ml) The results, in each case, showed no antibody titre, even with the 10 week sera. To enhance the sensitivity of the ELISA, IgG was isolated from the pre-immune as well as the 4, 8 and 10 week sera, as described in Section 2.6.2, and the IgG rather than the sera used for ELISAs. Chequerboard ELISAs were used as with the sera. No antibody titres were obtained, even with the 10 week IgG and a cathepsin B coat concentration of up to 10 µg/ml. It was therefore decided to collect blood from each rabbit by cardiac puncture at 10 weeks. IgG was isolated as described in Section 2.6.2, and stored at -20°C. Cardiac puncture produces a much larger quantity of blood, and therefore the IgG isolated from the 10 week sera, obtained by cardiac puncture, was used to further characterise the antibodies.

4.5 Immunoinhibition assays using a synthetic substrate

4.5.1 Macroassay

4.5.1.1 Reagents

Cathepsin B. Rabbit liver cathepsin B was isolated as described in Section 3.2.4.2.

Buffer/activator [0.1 M Na-phosphate, 4 mM Na_2EDTA, 0.1% (v/v) Tween 20, 5 mM dithiothreitol, pH 6.0]. NaH_2PO_4 (6.90 g), Na_2EDTA (0.93 g) and Tween 20 (500 µl) were dissolved in 450 ml dist.H_2O, adjusted to pH 6.0 with NaOH and made up to 500 ml with dist.H_2O. This buffer was made up fresh before use, as it contained no NaN_3.

1 mM Z-Arg-Arg-NHMec substrate stock solution. As described in Section 3.2.1.1.

40 µM Z-Arg-Arg-NHMec substrate solution. As described in Section 3.2.1.1.

5 mM Dithiothreitol. DTT (0.0051 g) was dissolved in dist.H_2O (5 ml).

4.5.1.2 Procedure

For the immunoinhibition assay, the enzyme macroassay devised by Barrett and Kirschke (1981) was adapted as described by Dehrmann *et al.* (1995) in order to minimise the dilution of the buffer. Cathepsin B (750 ng) was diluted in assay buffer (10 µl). To the enzyme solution, IgG, at 1 mg/ml in assay buffer, was added (700 µl). This mixture was incubated for 15 min at 30°C. After incubation, DTT at a concentration of 5 mM was added (165 µl) and

activation allowed for 2 min before the substrate, Z-Arg-Arg-NHMec, at a concentration of 40 μM was added (125 μl). Immediately after addition of the substrate, the fluorescence of the liberated aminomethylcoumarin was monitored for 10 min in a Hitachi F-2000 spectrofluorometer with excitation at 370 nm and emission at 460 nm.

4.5.1.3 Results

No difference was evident in the enzyme activity in the presence of non-immune IgG compared with enzyme activity in the presence of the test IgG solutions. In other words, no immunoinhibition was observed (results not shown).

4.5.2 Microassay

For the purpose of testing immunoinhibition across a range of antibody concentrations, the microassay was used.

4.5.2.1 Reagents

Cathepsin B. Rabbit liver cathepsin B was isolated as described in Section 3.2..2.

Assay buffer [0.1 M Na-phosphate, 4 mM Na_2EDTA, 0.1% (v/v) Tween 20, pH 6.0]. NaH_2PO_4 (6.90 g), Na_2EDTA (0.93 g) and Tween 20 (500 μl) were dissolved in 450 ml dist.H_2O, adjusted to pH 6.0 with NaOH and made up to 500 ml with dist.H_2O.

Activation buffer [0.1 M Na-phosphate, 4 mM Na_2EDTA, 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, adjusted to pH 6.0 with NaOH and made up to 500 ml with dist.H_2O. Immediately before use, dithiothreitol was added to 5 mM (i.e. 0.51 g/500 ml).

1 mM Z-Arg-Arg-NHMec substrate stock solution. As described in Section 3.2.1.1.

40 μM Z-Arg-Arg-NHMec substrate solution. As described in Section 3.2.1.1.

4.5.2.2 Procedure

Serial-two-fold dilutions of IgG (25 μl), with a starting concentration of 10 mg/ml, in assay buffer, were prepared in eppendorf tubes. To each of the dilutions, cathepsin B (500 ng), made up in 0.1% Brij 35, was added (25 μl). This mixture was incubated for 15 min at 30°C. After incubation, 25 μl of the incubated mixture was added to wells of a white Fluoronunc maxisorp microtitre plate, followed by the addition of activation buffer (60 μl). Activation was allowed for 2 min at 30°C before the substrate, Z-Arg-Arg-NHMec, at a concentration of 40 μM was added (25 μl). The assay solution was allowed to incubate for 10 min at 37°C whereafter the liberated aminomethylcoumarin was determined in a fluorescence microplate

reader (Cambridge Technology, Model 7620) with excitation at 370 nm and emission at 460 nm.

The results were analysed using the student's *t* test (Samuels, 1989), based on the null hypothesis written as,

$$H_O: \mu_1 = \mu_2$$

which asserts that μ_1 and μ_2 are equal, where μ_1 represents the enzyme activity in the presence of non-immune IgG and μ_2 represents the enzyme activity in the presence of the respective IgG preparations. Inhibition was accepted when H_O was rejected, i.e. when $\mu_1 \neq \mu_2$, and no inhibition (no effect) accepted when H_O proven, i.e. when $\mu_1 = \mu_2$. This choice was made by analysing the significant difference, statistically, at the 0.1% level.

4.5.2.3 Results

The effect of different IgG preparations on cathepsin B activity is summarised in Table 4.2.

Table 4.2 Effect of different preparations of antibodies, each at four-fold dilution concentrations, on cathepsin B activity against Z-Arg-Arg-NHMec.

Antibody (IgG)	Inoculation site	Effect of IgG preparations, at serial-four-fold dilution concentrations, on cathepsin B activity.			
		10 mg/ml	2.5 mg/ml	0.625 mg/ml	0.156 mg/ml
IgG anti- (free cathepsin B)	Intramuscular	0	0	0	0
IgG anti- (conjugate)	Intramuscular	0	0	0	0
IgG anti- (free cathepsin B)	Intravenous	0	0	0	0
IgG anti- (conjugate)	Intravenous	0	0	0	0

Note: 0, = no inhibition

According to the student's *t* test, with a 0.1% confidence interval, none of the IgG preparations had any effect on cathepsin B activity. However, a very high confidence interval (0.1%) was used, the reason being that the sample sizes were small. Also, in previous reports the antibodys' inhibitory effects on proteinases were significant enough without the need to

analyse results statistically using a *t* test. This resulted in a high confidence that no inhibition took place, i.e. $\mu_1 = \mu_2$.

For each inoculation protocol, two male rabbits were inoculated (Section 4.3). For the *t* test in this section, the mean of all the sample readings of both rabbits together were calculated. However, for surety, each individual rabbit's sample means were taken to compare to non-immune (results not shown). At a 0.1% confidence interval, none of the IgG of any of the rabbits had any effect on cathepsin B activity (results not shown).

4.6 Immunoinhibition using haemoglobin as substrate

The traditional haemoglobin assay (Anson, 1939) has been used to assay cathepsin D activity in this laboratoy (Pillay, 1999; Fortgens, 1996). The hydrolysis of haemoglobin by a protease generates TCA-soluble peptides which can be measured spectrophotometrically. It has been shown that cathepsin B hydrolyses haemoglobin (Pillay, 1999).

4.6.1 Reagents

Cathepsin B. Rabbit liver cathepsin B was isolated as described in Section 3.2.3.2.

Buffer/activator [0.1 M Na-phosphate, 4 mM Na_2EDTA, 0.1% (v/v) Tween 20, 5 mM DTT, pH 6.0. As described in Section 4.5.1.1.

5% (m/v) Haemoglobin substrate. Bovine haemoglobin powder (5 g) was dissolved in dist.H_2O (100 ml) with gentle magnetic stirring.

Diluent [0.1% Brij]. Brij 35 (0.1 g) was dissolved in 95 ml dist.H_2O and made up to 100 ml.

TCA-stop solution [10% (m/v)]. TCA (50 g) was made up to 500 ml with dist.H_2O.

4.6.2 Procedure

Cathepsin B at a concentration of 500 ng (10 µl) was made up in 0.1% Brij 35 (90 µl). Cathepsin B was preincubated with immune or non-immune antibody (1 mg/ml) in assay buffer in the absence of DTT (total volume 100 µl, 30 min, 37°C). Haemoglobin substrate solution (1 ml) was added, followed by the addition of assay buffer containing 5 mM DTT (1 ml). For a zero-time control, a sample (1 ml) was removed and mixed with TCA (1 ml) immediately after addition of the enzyme. After incubation at 37°C (2 h), TCA (1 ml) was added to the reaction mixtures. The precipitated protein in both reaction mixtures and blanks, was spun to a pellet in a benchtop centrifuge. The supernatants were filtrated through Whatman No. 4 filter paper. The absorbance of the reaction mixture supernatant was read

against the blank supernatant in a 3 ml quartz cuvette at 280 nm. Triplicate assays were carried out for both samples and controls.

No inhibition was evident when the inhibition of enzyme activity by the different IgG preparations were expressed as a percentage of the activity in the presence of non-immune IgG. The results were therefore analysed using the student's *t* test as described in Section 4.4.2.2.

4.6.3 Results

The effect of different IgG preparations on cathepsin B activity, using haemoglobin is summarised in Table 4.3.

Table 4.3 Effect of different IgG preparations on cathepsin B activity, using haemoglobin.

Antibody (IgG)	Inoculation site	Effect of IgG on cathepsin B activity using haemoglobin
IgG anti-(free cathepsin B)	Intramuscular	0
IgG anti-(complex)	Intramuscular	0 (+)
IgG anti-(free cathepsin B)	Intravenous	0
IgG anti-(complex)	Intravenous	0

Note: 0 = no inhibition; + = activity inhibited.

According to the *t* test, none of the IgG preparations had any effect on cathepsin B activity, at a 0.1% confidence level. However, when results were analysed statistically of IgG preparations from each individual rabbit (as described in Section 4.4.2.3), for surety, one rabbit's IgG had a statistically significant effect on cathepsin B activity. This rabbit was inoculated intramuscularly with complex (Cathepsin B-BSA), and its anti-(complex)-IgG inhibited cathepsin B activity. All the other IgGs from individual rabbits had no effect, statistically, at the 0.1% confidence level (results not shown).

4.7 Dot blotting

Dot blotting is the analysis of macromolecules applied directly to the immobilising matrix as opposed to transferring them from a gel. The use of dot blots as a first step preceeding gel blotting, is a common practice. In such dot blot assays, droplets of samples are directly applied to an immobilising matrix, which is then processed through quenching, probing, and detection.

In the present study, the dot blot assay was used to characterise the rabbit antibody reaction with purified cathepsin B (antigen). Because the complex, cathepsin B-BSA, was also blotted as an antigen, BSA-TBS, usually used to dilute the antigen and primary and secondary antibodies, could not be used. Instead, 5% (m/v) milk-TBS was used as the diluent and the blocking agent.

4.7.1 Reagents

Tris buffered saline (TBS; 20 mM Tris, 200 mM NaCl, pH 7.4). Tris (4.84 g) and NaCl (23.38 g) were dissolved in 1950 ml of dist.H_2O, adjusted to pH 7.4 with HCl, and made up to 1 litre.

5% (m/v) milk-TBS. Fat free milk powder (5 g) was dissolved in about 90 ml of TBS and made up to 100 ml.

Alkaline phosphatase buffer (100 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 9.5). Tris (12.1 g) and $MgCl_2.H_2O$ (0.233 g) were dissolved in 800 ml dist.H_2O, adjusted to pH 9.5 with HCl and made up to 1 litre with dist.H_2O.

Nitroblue tetrazolium substrate solution [0.003% nitro-blue tetrazolium]. Nitro-blue tetrazolium (0.03 g) was dissolved in 70% (v/v) dimethyl formamide (1 ml).

Bromo-chloro-indolyl phosphate substrate solution (0.0015% bromo-chloro-indolyl phosphate). Bromo-chloro-indolyl phosphate (0.015 g) was dissolved in dimethyl formamide (1 ml). Immediately prior to use, the nitro-blue tetrazolium solution (1 ml) and bromo-chloro-indolyl phosphate solution (1 ml) were diluted to 100 ml with alkaline phosphatase buffer.

4.7.2 Procedure

Aliquots (2 µl) of cathepsin B and BSA were dotted onto a nitrocellulose membrane, all at a concentration of 10 µg/2 µl, using a micropipette.

The nitrocellulose membrane was blocked for 1 h with 5% (m/v) fat-free milk powder in TBS and washed in TBS (3 x 5 min). Incubation followed for 2 h with primary antibody in 5% milk-TBS and, after incubation, washing in TBS (2 x 5 min). The membrane was incubated in alkaline phosphatase-linked secondary antibody in 5% milk-TBS for 1 h, and again washed in TBS (3 x 5 min). It was immersed in substrate solution and reacted in the dark until bands were clearly evident against a lightly stained background. The membrane strips were removed from the substrate solution, washed in dist.H_2O, and dried between filter paper, for good preservation of the bands.

4.7.3 Results

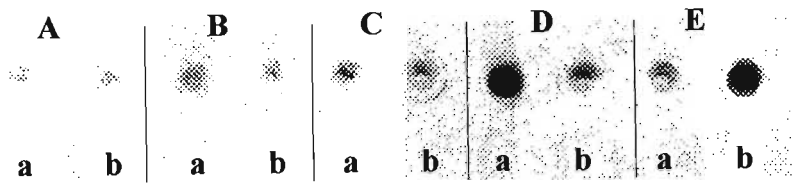

Figure 4.1. Dot blot analysis of autoantibodies against pure rabbit liver cathepsin B.

(a) and (b) represent IgG from two individual rabbits, that were inoculated using the same inoculation protocol.

A: Rabbit non-immune, 10 µg.

B: Rabbit anti-free cathepsin B, injected intravenously, 10 µg.

C: Rabbit anti-complex, injected intravenously, 10 µg.

D: Rabbit anti-complex, injected intramuscularly, 10 µg.

E: Rabbit anti-free cathepsin B, injected intramuscularly, 10 µg.

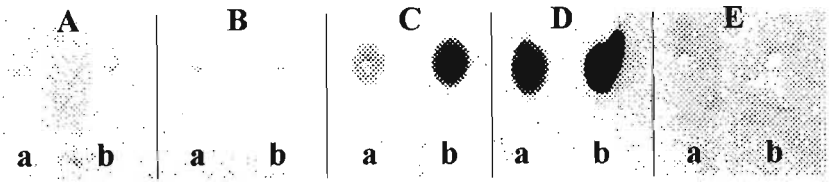

Figure 4.2. Dot blot analysis of antibodies against bovine serum albumin (BSA).

(a) and (b) represent IgG from two individual rabbits, that were inoculated using the same inoculation protocol.

A: Rabbit non-immune, 10 µg.

B: Rabbit anti-free cathepsin B, injected intravenously, 10 µg.

C: Rabbit anti-complex, injected intravenously, 10 µg.

D: Rabbit anti-complex, injected intramuscularly, 10 µg.

E: Rabbit anti-free cathepsin B, injected intramuscularly, 10 µg.

The degree of recognition of cathepsin B (Fig. 4.1) and BSA (Fig. 4.2) by antibodies were measured in terms of the darkness of the dot formed on the nitrocellulose membrane. Highest to lowest recognition corresponded with dark to lighter dots.

Cathepsin B was recognised by the following antibodies, in sequence from highest to lowest recognition : Rabbit anti-complex (intramuscular), rabbit anti-free cathepsin B (intramuscular), rabbit anti-complex (intravenous) and lastly, rabbit anti-free cathepsin B (intravenous) (Fig. 4.1).

The rabbit IgG from rabbits inoculated with free cathepsin B did not recognise BSA, whereas the IgG from rabbits inoculated with complex (cathepsin B-BSA) did recognise BSA (Fig. 4.2).

4.8 Discussion

The main aim of the present study was to "trick" the immune system into generating autoantibodies specific for cathepsin B by linking the enzyme to a foreign protein, BSA. Different immunisation protocols were used with two varying factors, namely, i) the site of inoculation and ii) whether cathepsin was inoculated as a free or complexed enzyme.

The immunisation protocol on which the hypothesis was based involved administering complexed cathepsin B intramuscularly. A further three inoculation protocols which functioned as controls were as follows: i) Free cathepsin B administered intramuscularly, ii) complexed cathepsin B administered intravenously, and iii) free cathepsin B administered intravenously.

Usually the progress of antibody production, during the term of inoculation, is evaluated by the three layer ELISA system. Once the antibody response peaks, the rabbits are bled by cardiac puncturing. However, in the present study the ELISA results (not shown) indicated no antibody titres. It was thought, however, that maybe the three layer ELISA system may not have been the best to use as the cathepsin B is partially denatured upon binding to the ELISA plate surface during coating. If the antibodies were generated against native cathepsin B, they might not have been able to recognise the modified (partially denatured) conformation of the immobilised enzyme. It might also be possible that the antibody response was very weak, and the antibody titre therefore too low and the ELISA used not sensitive enough to accommodate this. It was decided to bleed by cardiac puncturing at 10 weeks as done by Pierart-Gallois *et al.* (1977). Cardiac puncturing produces greater volumes of serum which allows for more trial-and-error assays. Also the greater volume produced by pucturing allows for easier isolation of IgG to sensitise the immunoassays. All the rest of the immunoassays were performed using the IgG isolated from the 10 week sera.

A chequerboard of the three layer ELISA was done with the 10 week IgG and cathepsin B (results not shown). Still no antibody titre was obtained with any of the IgG preparations.

It was decided to do immunoinhibition assays, where both the IgG and cathepsin B would be in solution, i.e. cathepsin B in its native conformation. No immunoinhibition was observed from the results of the immunoinhibition macroassay using the synthetic substrate, Z-Arg-Arg-NHMec (Section 4.5.1). The fact that a new starting solution has to be made up for each macroassay for each antibody concentration and the long duration of the macroassay, made it impractical to use this assay to test immunoinhibition across an antibody range. Only one antibody concentration was therefore used in the macroassay to test immunoinhibition of cathepsin B.

For the purpose of testing the immunoinhibition of cathepsin B across a range of antibody concentrations, the microassay was used. This assay uses the microplate reader which allows the different antibody concentration controls to be read simultaneously. This was not possible with the Hitachi F-2000 spectrofluorometer used for the macroassay. Therefore, immunoinhibition across a range of antibody concentrations was assayed using a serial 2-fold dilution of antibody. The microassay was done, as in the macroassay, using the synthetic substrate, Z-Arg-Arg-NHMec. No significant inhibition was observed when the enzyme activity, exposed to different IgG preparations, was expressed as a percentage of the activity in the presence of non-immune IgG. The results were therefore analysed using the student's *t* test, based on the null hypothesis (Section 4.5.2.2). With a 0.1% confidence interval, none of the IgG preparations had any effect on cathepsin B activity.

Concerning immunoinhibition assays, the size of the substrate used might influence the result (Richmond, 1977). Upon binding of antibodies to an active site, the active site is firstly occluded or partially occluded by the antibody, and secondly a conformational change is induced in the active site (Richmond, 1977). It might be possible that a small substrate, such as the synthetic substrate, Z-ArgArg-NHMec, used as described in Sections 4.4.1 and 4.4.2, may be able to gain access to the antibody-occluded cathepsin B active site, and therefore be hydrolysed. Results obtained in this case would, therefore, be misleading.

This problem may be solved by using a larger substrate, too large to enter the active sites of already "blocked" enzymes. Haemoglobin was used as the larger substrate in the present study. Another advantage in using haemoglobin is that it is more representative of the *in vivo* situation than a synthetic substrate such as Z-Arg-Arg-NHMec. According to the results (Section 4.6.3), analysed by using the student's *t* test, none of the IgG preparations inhibited cathepsin B activity. These results were statistically analysed by pooling the results of the two rabbits that were inoculated by the same inoculation protocol. However, when the results of each rabbit was analysed separately, one rabbit's IgG exhibited immunoinhibition. This was one of two rabbits that were inoculated intramuscularly with complexed cathepsin B.

To further evaluate the antibodies, dot blots were performed using cathepsin B and BSA as the immobilised antigen. Cathepsin B was recognised to some extend by all the IgG from all the rabbits. The highest recognition of cathepsin B, in terms of the darkest dots formed, was by the antibodies from a rabbit inoculated intramuscularly with complexed cathepsin B. This was the same IgG that showed inmunoinhibition with haemoglobin (Section 4.6). The next highest recognition of cathepsin B was by the antibodies of the rabbits inoculated intramuscularly with free cathepsin B. From the darkness of the dots, it seems that the recognition of cathepsin B by the anti-free cathepsin B (intramuscular) was almost as high as the recognition by the antibodies produced by the anti-complex (intramuscular). However,

only the anti-complex (intramuscular) antibodies showed immunoinhibition in the assay using haemoglobin. The third highest recognition of cathepsin B was by the anti-complex (intravenous) antibodies, followed by the anti-free cathepsin B (intravenous) antibodies. The antibodies from all the rabbits inoculated with complexed cathepsin B recognised the BSA, whereas the antibodies from rabbits inoculated with free cathepsin B did not recognise the BSA on the blot.

The fact that the anti-complex (intramuscular) antibodies showed the highest recognition for cathepsin B and showed slight immunoinhibition, suggests that the immune system was to some extent successfully tricked into reacting to the cathepsin B-BSA complex, resulting in the production of cathepsin B autoantibodies. Also, immunoinhibition indicates the recognition of the enzyme in is native conformation. Although the anti-free cathepsin B (intramuscular) antibodies showed almost equally high recognition of cathepsin B on the dot blot, it did not show any signs of immunoinhibition. These antibodies could therefore possibly have been generated to denatured cathepsin B. Also, cathepsin B in complex with BSA is "protected" and therefore less prone to degradation compared to free cathepsin B in the extracellular physiological environment.

The anti-free cathepsin B (intravenous) and anti-complex cathepsin B (intravenous) showed almost equal recognition of cathepsin B on the dot blot, but not as high as the recognition by the antibodies from rabbits injected intramuscularly. It was hypothesised that if cathepsin B is injected in its active form straight into the bloodstream, it might be taken up by the circulating α_2M. The proteinase-α_2M complex would then undergo receptor-mediated endocytosis into macrophages and other cells bearing the α_2M receptor, which would enhance the rate of presentation by macrophages (Chapter 1). Once peptides of the Ag, in this case, cathepsin B, are presented on MHC class II on the APC (macrophage), the T helper (Th) cell would recognise them and "signal" the B cells to produce antibodies to the Ag. However, the free cathepsin B inoculated intravenously into rabbits, was isolated from rabbits. Therefore, if endocytosed and presented by the macrophages, it may not necessarily be recognised by the Th cells. This might explain the lower recognition on the blots compared to the anti-complex (intramuscular) antibodies. Also, the antibodies made against the free cathepsin B (intravenous) might recognise denatured cathepsin B. But, why did the anti-complex (intravenous) antibodies have a weaker recognition too? The reason for this might be explained by the fact that, as already said, once injected into the bloodstream (intravenous), it was assumed that it would be complexed to α_2M. For a proteinase to be complexed by α_2M, the plasma proteinase inhibitor's "bait region" must be cleaved by the proteinase. That is the reason for activating cathepsin B first, before inoculating it intravenously. The complexed cathepsin B, however, was inactive even after "activation" with DTT or cysteine (results not

shown). The complexed cathepsin B therefore had to rely on being internalised directly by macrophages, without the help of α_2M.

Another variable in the inoculation protocols was that adjuvants were used when inoculating intramuscularly, but not for intravenous inoculations. When inoculating intravenously, adjuvants may not be used, as they may cause harmful reactions in the experimental animal. This may explain the lower cathepsin B recognition of the antibodies produced intravenously. Adjuvants such as FCA are effective in that they improve the humoral response by assuring the effective participation of the B and T cells and APCs. The emulsion causes the slow release of the immunogen into the extracellular environment. The down-side in using adjuvants when inoculating cathepsin B is that it can increase denaturation of cathepsin B and therefore antibodies would be produced that recognise denatured cathepsin B. This denaturation may be caused by the paraffin oil and mannide mono oleate present in the adjuvant, or by the vigorous mixing of the Ag with the adjuvant when preparing the water-in-oil emulsion.

From the results it can be concluded that only the anti-complex cathepsin B (intramuscular) antibodies showed immunoinhibition to cathepsin B and that all the antibodies from all four different protocols recognised cathepsin B immobilised on the nitrocellulose membrane in the dot blot analysis. Cathepsin B is partially denatured upon the binding to the nitrocellulose membrane. The results can, therefore, not distinguish if the antibodies were made against native or denatured cathepsin B, except for the anti-complex cathepsin B (intramuscular) antibodies which inhibited cathepsin B, i.e. recognised native (active) cathepsin B. Different ELISA systems may be used to determine which form of cathepsin B the antibodies recognise. But, the amount of cathepsin B available was too limited to permit further exploration.

The aim of the present study, however, was to determine if one could "trick" the immune response into generating autoantibodies to cathepsin B by complexing it to BSA. This hypothesis was supported by the fact that the highest recognition of cathepsin B on a dot blot was by anti-complex cathepsin B (intramuscular) and that this same antibody was the only one inhibiting cathepsin B activity. The immunoinhibition by the anti-complex cathepsin B (intramuscular) antibodies was, however, very weak.

For future research on this topic, the conjugation process of cathepsin B to BSA should be optimised in order for conjugated cathepsin B to be active. If the enzyme could be activated in the complexed form, the active site would be is exposed and, as a result, there would be a chance for antibodies to be generated to epitopes situated in the active site area. The immunogenicity of cathepsin B should be questioned as it is a low molecular weight protein with a single epitope. Enzymes that are more immunogenic should be considered.

CHAPTER 5
GENERAL DISCUSSION

Both benign and malignant tumours are characterised by uncontrolled cell division. Benign tumours, however, remain localised and are amenable to surgical intervention or localised radiotherapy. By contrast, malignant tumours have the additional property of protease-mediated invasion of surrounding tissue. Invasion of blood or lymph vessels results in malignant cancer cells becoming widely disseminated, forming secondary tumours at distant sites. This can happen early, often before the primary tumour is diagnosed, so that malignant cancer is essentially a systemic disease, for which a systemic therapy is required. Currently, chemotherapy is the only available systemic therapy. However, chemotherapy, although very effective in cases such as childhood leukemias, target all dividing cells and, in consequence, has severe side-effects. Also, chemotherapy is largely ineffective against tumours of solid tissue, mainly because it selects for cells having overexpressed membrane transporters which rapidly exports the chemotherapeutics from the cells. As a result, malignant tumours rapidly evolve into chemotherapy-resistant, and consequently lethal, cancers.

The immune system is capable of mounting systemic responses to systemic infections by invading foreign organisms, but the immune system largely ignores tumours because these are comprised of "self" cells. However, in a small number of cases, a phenomenon known as "spontaneous remission" occurs. In these cases the immune system spontaneously becomes responsive to the cancer cells and eliminates them, demonstrating that the immune system, when appropriately activated, indeed has the capacity to selectively kill cancer cells. The key question, in terms of cancer therapy, is how may the immune system be "appropriately activated", i.e. is it possible to have engineered remission, rather than spontaneous remission. Before addressing this question, it is interesting to note that chemotherapy, because it suspends cell division, also suppresses the immune system and thus essentially eliminates the possibility of spontaneous remissions occurring.

5.1 The "danger" concept and the two signal model

The present study addresses the possibility of cancer immunotherapy by generating autoantibodies against cathepsin B. But cathepsin B is a self-protein, and the conventional viewpoint is that the immune system distinguishes between self and non-self, only responding to non-self. However, there is a new perspective that the immune system is more concerned with "danger" than with the distinction between self and non-self (Matzinger, 1994). According to Matzinger (1994), this is not a new theory, but a different way of looking at immunity. If the immune system does indeed only discriminate between self and non-self, the

sheer number of things to which it must be tolerant would be overwhelming (Matzinger, 1994).

Cohen (1992) addressed this issue by asking: "why has evolution been forced to accommodate natural autoimmunity?". Some immunologists claim that natural autoimmunity is a mistake, due to a leakiness in thymic clonal deletion and anergy. Anergy is a potentially reversible specific immunological tolerance in which the lymphocytes becomes functionally non-responsive (Roitt, 1994). But Cohen (1992) proposes that natural autoimmunity evolved as a result of the molecular conservation of the biosphere. Some critical and highly conserved molecules play an important role in all cellular life, meaning that the conserved molecular self of a human cannot be totally different from the conserved molecules of parasites (Cohen, 1992). The immune system is constantly bombarded by self-like foreign molecules, such as the heat shock protein, hsp 65, which arrive with parasites in the context of infection, and the immune system is capable of using autoimmunity to an hsp molecule to help reject an invading microbe (reviewed by Cohen, 1992). It was therefore proposed that autoimmunity is organised and regulated naturally to conserved molecules such as hsp 65. Autoimmunity can thus be used to fight infections, reject tumour cells, or repair tissue damage without the damage caused by chronic, progressive autoimmune disease.

Another problem that arises with the self-non-self discrimination theory, is that scientists have to identify what exactly self is and what non-self is, with the answers varying greatly (reviewed by Matzinger, 1994). According to Matzinger (1994), distinguishing dangerous from harmless structures would be far more efficient and would make more evolutionary sense than discriminating between self and non-self. But what distinguishes dangerous from harmless foreign structures? Matzinger (1994) proposed that the answer be found in taking the emphasis away from recognition and specificity of individual lymphocytes, and to shift it towards the interaction between lymphocytes. This concept brings us to the two signal models.

In 1970 Bretscher and Cohn were the first to propose the concept of a second signal, and later a slightly different model was proposed by Lafferty and Cunningham (1975) (reviewed by Matzinger, 1994). The two models agreed that signal one occurs when a lymphocyte's antigen-specific receptor (Ab or TCR) contacts the appropriate antigen, i.e. native structures for B cells and peptide/MHC complexes for T cells. Both the models also agree that signal one is not enough for activation, which requires a second signal. The main difference between the models is that where Bretscher and Cohn's model suggests that signal two is supplied by an antigen responsive cell (the T helper), a process named help, Lafferty and Cunningham's model suggests that the second signal comes from an antigen presenting cell, a process named costimulation (reviewed by Matzinger, 1994).

According to Matzinger (1994), Bretscher and Cohn's model can function to maintain tolerance because signal two comes from an antigen specific cell (lymphocyte), not an antigen presenting cell, because APCs would present antigens non-specifically. Regarding the question: "who helps the first helpers?", Matzinger (1994) suggests that T helper cells are driven by the Lafferty-Cunningham type of co-stimulation, while B cells need the Bretscher-Cohn type derived from helper T cells. In other words, signal two would be help from Th cells for B cells and co-stimulation from APC's for T cells (Matzinger, 1994).

But how does this two signal model fit in with autoimmunity and tolerance, with regard to autoantibody production? Although the normal view is that a B cell can act as an APC, according to Matzinger (1994), only experienced T cells, but not virgin T cells, can be activated by B cells. Because B cells can capture antigens $10\text{-}10^4$ fold more effectively than other APCs (Lanzavecchia, 1985), due to their surface Igs, unstoppable autoimmune responses would be initiated if B cells could activate virgin T cells (Matzinger, 1994). Matzinger (1994) suggested that in order to prevent such autoaggression, virgin T cells need be activated first by a professional APC before being able to interact with a B cell. Therefore, because T cells are tolerant to the normal concentrations of self-proteins presented by the APCs, no T helper cells will be activated, and autoreactive B cells would die for lack of help, i.e. signal two (Matzinger, 1994). Lassila *et al.* (1988) tested this theory by using allogeneic chicken chimeras with MHC^a bodies and MHC^b B cells and found that the B cells were able to accept T cell help but could not initiate the process that led to activation of the help they needed. The virgin T cells in the MHC^a chimeras needed first to be primed by professional MHC^b APCs. Therefore, it seems that it is the state of the T cell and not that of the B cell that matters where tolerance is concerned. Therefore, in order to induce an autoimmune response, i.e. autoantibodies, for cancer immunotherapy, T cells must be manipulated as it is the state of the T cells that determines tolerance. A better understanding of $CD4^+$ T cells is therefore a prerequisite to the planning of schemes for manipulating autoimmune responses.

5.2 $CD4^+$ T cells

5.2.1 The function and importance of T cells in inducing autoimmunity/ anticancer immunity

The fact that most tumours are MHC class I positive, but MHC class II negative, and the ability of $CD8^+$ CTLs to lyse tumour cells directly upon recognition of peptide-MHC class I complexes expressed by a tumour, therefore enabling them to eradicate large tumour masses *in vivo* resulted in much attention being given to the role of $CD8^+$ CTLs in cancer immunity (reviewed by Pardoll and Topalian, 1998). Also, the list of tumour antigens identified by tumour-reactive $CD8^+$ CTLs is increasing. Less attention has been given to $CD4^+$ Th cells in this regard.

Reports on cytokine-secreting tumour cell vaccines gave concrete evidence for the participation of T helper (Th) cells in generating and maintaining anti-tumour immunity (reviewed by Topalian, 1994). Overwijk *et al.* (1999), highlighted the importance of the role of $CD4^+$ T cells in generating autoantibodies against autoantigens for vaccination of cancers. In their experiment, vaccination with rVVm TRP-1 induced both autoimmunity and tumour protection in a manner that depended on both antibody and $CD4^+$ T cells. It has also been shown that the specific tolerisation of autoreactive $CD4^+$ T cells can completely abrogate autoimmune disease, which further highlights the important role for $CD4^+$ T cells in the immunity to self-antigens (reviewed by Overwijk *et al.*, 1999). It has been shown that immune responses to less immunogenic proteins display more pronounced dependency on "help" from $CD4^+$ T cells (reviewed by Overwijk *et al.*, 1999). Also, the fact that multiple antigens expressed by a certain tumour elicit an immune response in the autologous cancer patient, has made it evident that the recognition of tumour antigens is not the limiting step in immune responses against tumours. Rather, it seems to be the effector arm (cytokine milieu) of the immune system that is responsible for the failure of the cancer patient's immune system to prevent or control cancer (Sahin *et al.*, 1997).

During the early stages of tumour development, inflammatory-like responses cause the infiltration of macrophages and polymorphonuclear cells which in turn produce chemokines and cytokines. These stimulants induce the accumulation and expansion of T lymphocytes at the tumour site, resulting in either tumour eradication or progression (Goedegebuure and Eberlein, 1995). Many solid tumours are characterised by a T cell infiltrate consisting of $CD4^+$ and $CD8^+$ T cells (reviewed by Goedegebuure and Eberlein, 1995). These tumour-infiltrating lymphocytes (TILs) can be divided into tumour-specific and tumour-nonspecific T cells. While a small proportion of tumour-specific $CD4^+$ TILs directly lyse tumour cells in an HLA class I- or II-restricted manner, the majority recognise tumour antigens presented on HLA class II molecules by APCs. Tumour-nonspecific $CD4^+$ TILs recognise antigens that are being expressed in the tumour environment, presented by HLA class II as a result of stress, for example the heat shock protein, hsp 70.

Tumour-nonspecific TILs have specificity for other cells, other than tumour cells, in the tumour environment. The functional traits of these cells are unknown but could potentially affect the functional activity of tumour-specific T cells (Goedegebuure and Eberlein, 1995). $CD4^+$ T cells can be functionally distinguished into Th0, Th1, and Th2. $CD4^+$ Th1 cells produce IL-2 and IFN-γ, and consequently support the development of a cellular immune response and Th2 cells produce IL-4, IL-5 and IL-10 and are critical for the development of a humoral response (reviewed by Goedegebuure and Eberlein, 1995). The efficacy of an antitumor immune response is codetermined by the net effect of stimulatory and inhibitory responses. Therefore, an understanding of the developmental pathways of $CD4^+$ TIL is very

important regarding immunotherapy of cancer (Goedegebuure and Eberlein, 1995). The diagram in Fig. 5.1 describes the integrated immune response to tumour cells, and explains the importance of the $CD4^+$ T cells in lending help to CTLs, IgG responses and maintaining immune memory.

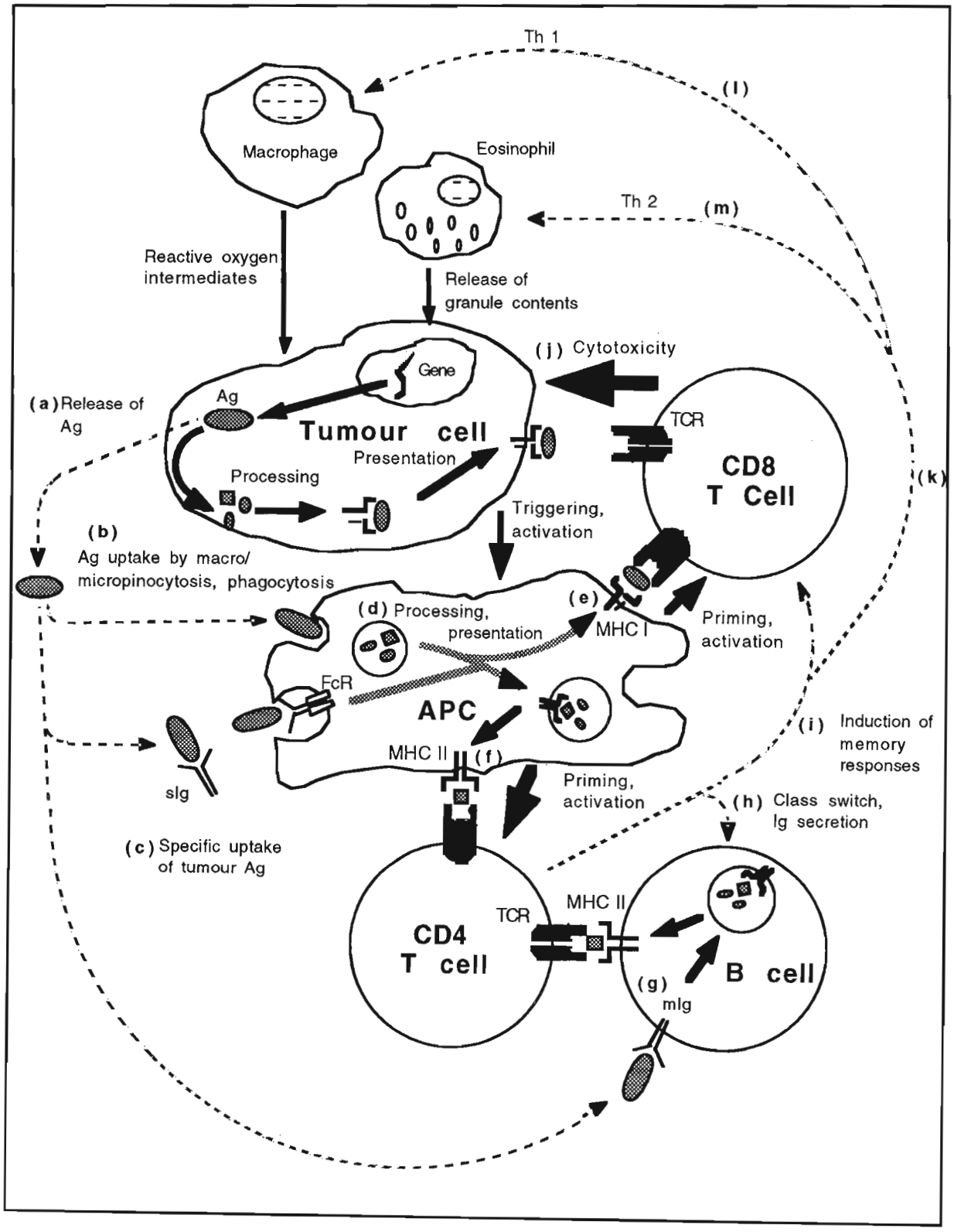

Figure 5.1 The integrated specific immune response to human tumour antigens, involving $CD4^+$, $CD8^+$ T cells, B cells and APCs.

(a) Tumour antigens (Ags) are released by secretion, shedding, or tumour cell lysis, and are captured by APCs, by either **(b)** macro/micropinocytosis, phagocytosis or **(c)** via Fc-receptor mediated endocytosis of soluble immunoglobulin (sIg)-antigen complexes. Uptaken antigens are **(d)** processed and presented by either **(e)** MHC class I (MHC I) or **(f)** MHC class II (MHC II) molecules for priming and activation of $CD8^+$ and $CD4^+$ T cells respectively. **(g)** Uptake of antigen by B cells also occurs and is driven by membrane Ig (mIg) leading to MHC class II antigen presentation to $CD4^+$ T cells. Antigen presentation to $CD4^+$ T cells by both (f) APCs and (g) B cells is critical for the immune response as it induces **(h)** class switching and the production and secretion of IgG and IgA antibodies by B cells and **(i)** long lasting T cell memory responses. In addition, (e) priming and activation of $CD8^+$ T cells by the APC, triggers **(j)** the cytotoxic activity of $CD8^+$ T cells inducing tumour cell lysis and augmentation of the immune response. **(k)** Primed $CD4^+$ T cells at sites of tumour metastasis, orchestrate effector function, involving, **(l)** Th 1 cells that activate macrophages to produce reactive oxygen intermediates and **(m)** Th 2 cells that activate eosinophils to release their granule contents (Adapted from Sahin *et al.*, 1997, and Pardoll and Topalian, 1998).

From this it can be concluded that, although many vaccines have so far been designed to generate $CD8^+$ T cell responses, the importance of $CD4^+$ T cells should not be ignored, as they lend help to CTLs, induce IgG responses, maintain immune memory, as well as being able to directly kill target cells via lysis or cytokine secretion (Pardoll and Topalian, 1998).

5.2.2 Tolerisation of T cells.

Tumour cells release antigens by secretion, shedding or necrosis, which are captured by professional APCs that process and finally present epitopes of these antigens for T and B cell priming (Huang *et al.*, 1994) (Fig. 5.1). But why do certain tumours, such as melanomas, still grow despite the expression of highly immunogenic tumour antigens (reviewed by Toes *et al.*, 1999)? According to Toes *et al.* (1999), the mechanism of tolerance induction, regarding $CD4^+$ T cells, might mimic tolerance induction to peripheral tissue antigens. Peripheral tolerance induction of antigen-specific $CD4^+$ and $CD8^+$ T cells to antigens expressed outside the lymphoid system is mediated by cross-presentation of the antigen on bone marrow-derived APCs (reviewed by Toes *et al.*, 1999). The immune system receives no inflammatory stress during the initial growth of a tumour, and Toes *et al.* (1999), therefore suggested that the

antigen derived from the tumour might be shunted in the same cross-tolerising pathway as reported for peripheral tissue antigens (Van Parijs *et al.*, 1996).

How, then, could the immune system be manipulated to induce an autoimmune response, involving the humoral immune system? We have already established that the state of the T cells determines the outcome of the humoral response (Overwijk *et al.*, 1999), from which it follows that the tolerisation of Th cells could cause a problem concerning immune intervention schemes based on the induction of humoral and/or T cell immune responses against tumours or self-proteins. So, the next question is: "How could tolerance of the Th cells be broken?" This question puts a focus on T cell recognition of the MHC/peptide complex and the mechanisms predetermining it. This involves the uptake, processing and presentation of an antigen or self-antigen and, to a deeper level, crypticity of an epitope. How can these factors be manipulated to increase "danger" to the immune system?

5.3 Factors influencing the danger signal

5.3.1 The critical density of MHC-peptide complex

A large set of antigenic determinants of the self have been reported not to induce self-tolerance (reviewed by Sercarz *et al.*, 1993). The important linkage between cancer immunology and autoimmunity was recognised by Coulie *et al.* (1994) after they discovered that the target for a melanoma-specific $CD8^+$ T cell clone grown from a melanoma patient was wild-type tyrosinase, a melanosomal enzyme selectively expressed in melanocytes and responsible for one of the steps in melanin biosynthesis (reviewed by Pardoll, 1999). A number of reports followed with the same results, where melanoma-specific $CD8^+$ T cells recognised melanocyte-specific (tissue specific) antigens, rather than melanoma-specific antigens (reviewed by Pardoll, 1999). This opens more possibilities for cancer immunotherapy, as these self-peptide determinants could provide potential targets for immune responses against tumours (i.e. autoimmune responses against cancer). This could eliminate the effort of having to discriminate between self and non-self or neoself-antigens expressed by the tumour, therefore widening the posibilities and choices regarding cancer immunotherapy.

But then, why do some patients still have cancer despite the immune responses to tumour antigens (reviewed by Sercarz *et al.*, 1993; Nanda and Sercarz, 1995)? The answer might lie in looking at the broader picture, in the fact that a tumour establishes its own microenvironment. It does so by altering the expression of MHC molecules or the level of other activities affecting immune induction, such as peptide tranporter molecules, and preventing the display of peptide determinants for T cell recognition (reviewed by Nanda and Sercarz, 1995). Because the immune system gets propelled only upon ligand recognition in a context of heightened expression of costimulatory molecules, adhesion molecules, and HLA molecules on

APCs, Nanda and Sercarz (1995), hypothesised that the density of the up-regulated peptide-MHC complexes and the expression of costimulatory signals that influence activation of the silent tumour-specific T cell repertoire, may be critical in the cancer patient. This would all take place in the context of "danger", the concept explained by Matzinger (1994).

A study done by Hu *et al.* (1993) on an envelope (env) protein of Friend murine leukemia virus, that is expressed at high levels by a Friend virus-induced erythroleukemia (FBL) of B6 origin, showed that *env*-transgenic mice were tolerant to the (whole) env protein. But, adoptive transfer of antigen-specific T cells (derived from nontransgenic mice) into the transgenic mice that harboured FBL resulted in complete eradication of FBL without any sign of autoimmune injury in any of the lymphoid or other tissues expressing env (Hu *et al.*, 1993). According to Nanda and Sercarz (1995), this selective tumour-specific destruction reported by Hu *et al.* (1993), may be a result of the increased level of expression of the immunogenic determinants in the tumour cells and the unique inflammatory conditions existing at the tumour site. Nanda and Sercarz (1995) went a step further by suggesting that from this data it can be concluded that both the critical density of MHC-peptide complexes as well as a critical set of costimulatory/accessory molecules are necessary to activate T cells.

5.3.2 The antigen

An important feature of either spontaneous or experimentally induced autoimmune disease is that the ability to break tolerance and induce autoimmunity is highly dependent on the particular autoantigen (Pardoll, 1999). Therefore, it follows that attempts to induce autoimmune responses against tissue-specific self-antigens as a strategy for cancer immunotherapy would depend significantly on which antigen was chosen (Pardoll, 1999; Overwijk *et al.*, 1999). Nanda and Sercarz (1995) suggested that a peptide determinant should be used that is recognised by T cells that are readily isolated from the cancer patient. This would ensure that the peptide binds to the MHC and that the peptide-specific T cell repertoire exists in the individual.

It was hypothesised by Kumar and Sercarz (1995) that tolerance is broken by autoantigens that represent cryptic epitopes, presented at higher density, i.e. a "danger" signal. This fact supports the principle that the ability to break tolerance and induce autoimmunity (i.e. autoimmune responses against tissue-specific self-antigens for cancer immunotherapy), is highly dependent on the particular autoantigen used. This fact is proven by experiments done by Overwijk *et al.* (1999) where a panel of recombinant vaccinia, incorporating the murine homologues of each of the defined melanocyte-specific differentiation antigens targeted by T cells from human melanoma patients were used. All the variables in the system were being held constant while the melanocyte-specific antigen incorporated into the vaccine was varied. Each of the recombinant vaccinia was analysed for their ability to induce vitiligo in C 57 BL6

mice as a measure of induction of tissue-specific autoimmunity. They showed that only recombinant vaccinia expressing TRP-1 caused protection against B16 melanoma challenge.

5.3.2.1 Crypticity of an epitope

The mechanisms responsible for generation of cryptic epitopes fall into three general categories (reviewed by Lanzavecchia, 1995). The first is increased antigen delivery to the processing compartment, when surface receptors are down-regulated by ligands such as antibodies. Here, membrane Ig on B cells or soluble IgG antibodies may drive antigen capture by FcR^+ APC.

The second mechanism is the modulation of antigen processing, occurring when antigen is bound to antibodies, or when subtle changes in the processing machinery occurs. Another point in this second category, is that APCs may express slightly different sets of proteases, causing differences in antigen processing.

The third mechanism is an increase in MHC class II synthesis or in expression of adhesion and costimulatory molecules, resulting in increased yield of cryptic epitopes and T cell stimulatory capacity.

According to Lanzavecchia (1995), these three mechanisms may act synergistically: Antibodies can increase uptake, induce down-regulation, and modify processing. T or B cell activation may induce receptor down-regulation and increased synthesis of class II and costimulatory molecules. Cytokines can increase synthesis of class II molecules and proteases. Thus, this process is catalytic in the sense that, after an initial trigger, the response may become self-sustaining through the reciprocal stimulatory effect of T and B cells, antibodies and cytokines, a fact that may explain the spreading of the autoimmune response.

Three possible initiating events that could trigger the "autoimmune spiral" have been suggested (reviewed by Lanzavecchia, 1995). The first suggestion is that induction of an autoimmune response happens the same way as during the induction of an immune response to foreign antigens, presented on dendritic cells. The second suggestion or possibility is that the cryptic epitopes are presented on non-professional APCs, such as resting B cells or epithelial cells. Although T cells should be tolerised in this case, according to Lanzavecchia (1995), there may be cases where T cells can be activated even if the antigen is presented on nonprofessional APCs, occurring at inflammatory sites or in the microenvironment of a lymph node where costimulation could be provided in a bystander fashion. T cells might also be primed by recognition of a cross-reactive antigen presented by professional APCs as a result of molecular mimicry or at the presence of a second TCR with a different antigen specificity. The third possibility is that the initiating event is the activation of autoreactive B cells, which may occur in the absence of T cells specific for the self-antigen. For example, autoreactive B cells could

take up a self-antigen complexed with a foreign antigen and be stimulated by T cells specific for the foreign antigen. Alternatively, autoreactive B cells may take up a foreign antigen that cross-reacts with a self-antigen at the B cell level, but contains different T cell epitopes. Another possibility here is that B cells might be directly activated by highly organised self antigens, where autoreactive T cells may be primed by these B cells that take up and present self-antigen, or by dendritic cells that take up self-antigen complexed to IgG antibodies.

5.4 Generating autoantibodies against cathepsin B.

The main objective of the present study was to generate autoantibodes against cathepsin B. We have already established the importance of the $CD4^+$ T helper cells regarding the humoral immune response, and the fact that it is the state of the T cell that determines tolerance, and breaking of tolerance for that matter. Following this fact, the importance of the processes leading up to presentation of an antigen epitope on MHC class II for $CD4^+$ T cell recognition has also been established. After taking Matzinger's (1994) "danger" concept into consideration, it is realised that in order to generate an autoimmune response, the immune system must receive a "danger" signal.

There are several ways in which the immune system could be manipulated by incorporating "danger" in order to generate immune responses. One way of transforming a weak stimulus into a "danger" stimulus, is to use adjuvants, as dangerous stimuli produce a costimulatory milieu. A reason for this might be its penchant for inducing macrophages to produce interleukin-12 (IL-12), a cytokine that is a potent activator of T helper type 1 (Th 1) cells (reviewed by Nanda and Sercarz, 1995) and cytotoxic $CD8^+$ T lymphocytes (CTLs). Noguchi *et al.* (1995) showed that a mutant peptide of p 53 injected along with IL-12 destroyed a tumour in a tumour-bearing host, whereas the same peptide or IL-12 alone did not. But with regard to generating autoantibodies against cathepsin B, the use of only adjuvants would not be successful, because, although the mIg (membrane immunoglobulin), on the surfaces of B cells, would recognise cathepsin B, no specific help would be rendered available and hence no antibodies would be generated.

So, how did we go about tricking the immune system into generating autoantibodies against cathepsin B? We used the third possible initiating event that could trigger the "autoimmune spiral", as described by Lanzavecchia (1995) in Section 5.3.2.1, by complexing cathepsin B, a self-protein ("S"), to BSA, a foreign protein ("F"). Since T and B cells must recognise linked, but not necessarily identical, determinants (Janeway and Travers, 1994), helpers specific for the "F" part (BSA) of an antigen (in this case the cathepsin B-BSA complex), should be able to help B cells specific for "F" as well as B cells specific for a shared self-determinant, "S" (cathepsin B) (Matzinger, 1994). Both B cells could capture the antigen, process it, and present the various processed peptides to activated T helpers, which would not be able to

distinguish between them. T cells would recognise the "F" region presented on MHC class II on APCs, signal one, and receive signal two from the APC and be activated. The T helpers would also recognise the "F" peptide on the surface of both types of B cells and offer help (signal two for B cells). Both B cells should be activated, multiply and secrete antibodies (Fig. 5.2).

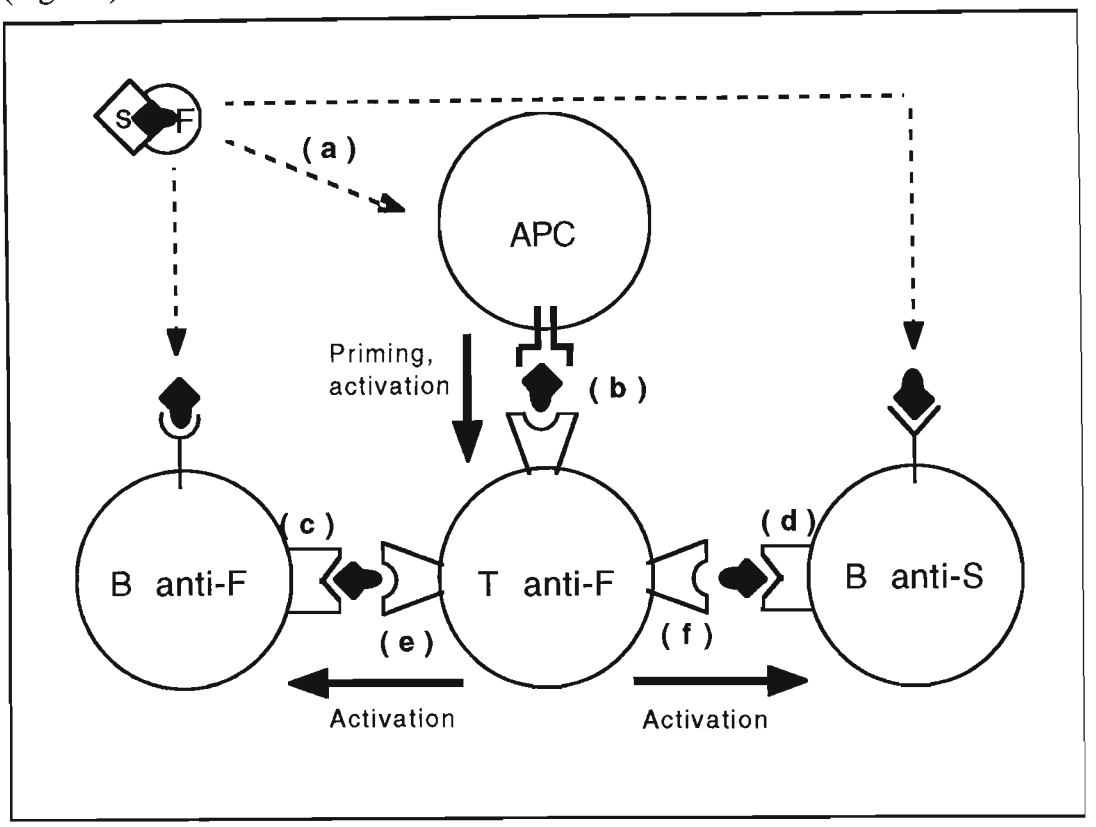

Figure 5.2. Helper T cells and B cells recognise the same antigen, i.e. linked recognition.

(a) An antigen with a foreign ("F") and self-determinant ("S") part, is taken up by an APC and **(b)** presented on MHC class II (MHC II) for priming and activation of T helper cells (T anti-F). Both **(c)** B cells specific for the "F" part (B anti-F) and **(d)** B cells for the "S" part (B anti-S) of the antigen capture, process and present the various processed peptides to activated T helpers (T anti-F). **(e,f)** The T helpers recognise the "F" peptide on the surface of both types of B cells and offer help (signal two) for avtivation of B cells (adapted from Matzinger, 1994).

The goal of the present study was not only to generate autoantibodies recognising native cathepsin B but also, preferably autoantibodies that would neutralise cathepsin B activity. However, the antibodies obtained did not neutralise cathepsin B activity. The assays used to test immunoinhibition may not have been sensitive enough though. A higher immune response (antibody recognition of cathepsin B), was obtained from sera of rabbits injected with cathepsin B-BSA complex as evident on dot blots (Figs. 4.1 and 4.2). This proves that the

immune system could indeed be "tricked" by incorporating a danger signal, in this case, a foreign protein, BSA.

It has been shown by Guagliardi *et al.* (1990) that cathepsin B and D and class II molecules coexist in the same endosomal particles in B cells. These proteinases have been implicated as being involved in professional antigen processing, particularly for MHC class II presentation, by comparing peptides that had been digested *in vitro* with known T cell determinants and by inhibition or enhancement of antigen presentation with protease inhibitors (reviewed by Sercarz *et al.*, 1993). Cathepsin E, a pepsin-like protease and plasma membrane-associated endopeptidase, has also been implicated (reviewed by Sercarz *et al.*, 1993). The determinants emerging from native protein digestion may have experienced a series of protease cleavages which could differ among particular cells. The site of cleavage may have special relationships to certain MHC molecules per se as well as to the variable lifetime of processing intermediates in different subcellular compartments (reviewed by Sercarz *et al.*, 1993).

Cathepsin B's involvement in antigen processing could possibly hamper processing and therefore presentation, where cathepsin B is the antigen in question, as in the case of the present study. Cathepsin B might therefore not have been an ideal choice as self-antigen against which to induce autoimmune responses. A protein involved in tumour metastasis, but not in antigen processing , might be a better choice.

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PUBLICATIONS

Use of 2-Methylpropan-2-ol to Inhibit Proteolysis and Other Protein Interactions in a Tissue Homogenate: An Illustrative Application to the Extraction of Cathepsins B and L from Liver Tissue

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A typical protein isolation from tissue consists of upstream methods of homogenization/extraction and concentration and crude fractionation, where yield is the most important criterion, and more discriminating downstream stages such as chromatography, where purification is the more important criterion. Three-phase partitioning (TPP[2]) (using *t*-butanol and ammonium sulfate) is an upstream procedure used for concentration and crude fractionation early in a protein isolation procedure. It has been applied to the isolation of a number of different proteins, including cathepsins (1, 2), and the principles of the method have been explored (3, 4).

A common property of all agents that stabilize protein structure is that they are excluded from the protein domain (5). *t*-Butanol is unique among common water-soluble organic solvents in that it tends to not denature proteins, suggesting that it too may be too large to gain access to the protein interior (4). However, at a concentration of ca. 30%, *t*-butanol appears to generally inhibit the activity of enzymes (Fig. 1 and Ref. 6). In the case of cathepsins B and L, and presumably all proteins which have been isolated by TPP (see Table 1, Ref. 4), these changes are reversible and activity is restored upon removal of the *t*-butanol.

Homogenization is often the first step in a protein isolation and many unwanted molecular interactions may occur in a homogenate, e.g., proteolysis (e.g., Ref. 7) or proteinase/inhibitor interactions (e.g., Ref. 8), leading to the generation of artifacts and perhaps compromising yield. In previous cathepsin isolations, we have used a sequence of homogenization → pH 4.2 precipitation → TPP. Because *t*-butanol reversibly inactivates many enzymes, including proteases (Fig. 1), it was considered that these three steps may be profitably combined, by incorporating 30% *t*-butanol directly in a low-pH homog-

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[2] Abbreviations used: *t*-butanol, 2-methylpropan-2-ol; TPP, three-phase partitioning; Cbz, carbobenzoxy; AMC, 7-amido-4-methylcoumarin.

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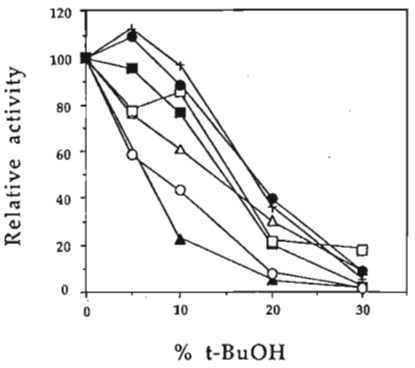

IG. 1. Effect of *t*-butanol concentration on the activity of some nzymes: (▲) cathepsin B; (○) cathepsin L; (□) cathepsin D; (■) -glucosidase; (△) fruit bromelain; (●) trypsin; (+) chymotrypsin.

nization buffer. Here we report on the application of this pproach to the isolation of cathepsins B and L from abbit liver and sheep liver, respectively.

Cathepsins B and L were extracted from liver tissue y established methods (8), which served as controls, nd by a method incorporating *t*-butanol in the homogenization buffer. Fresh livers from each species were eparately diced and separated into two portions, containing equal amounts from each liver, which were eparately frozen at −70°C and stored for at least 48 h ut not more than 2 weeks.

In the control method for cathepsin B, thawed rabbit ver (originally 300 g, i.e., before being frozen) was omogenized for 3 min in two parts of homogenization uffer (150 mM Na acetate, 1 mM EDTA, pH 4.0) in a Waring Blendor and centrifuged (9000*g*, 20 min, 4°C). The supernatant was adjusted to pH 4.2 with dilute cetic acid and centrifuged (15,100*g*, 20 min, 4°C). -Butanol was added to constitute 30% of the total ombined volume of *t*-butanol plus supernatant and a 0–40% ammonium sulfate TPP cut was taken, with centrifuging (15,100*g*, 10 min, 4°C), after each precipitation. The 20% precipitate was discarded and the 20–40% precipitate was redissolved, in one-tenth of the volume of the acid supernatant, in Buffer A (20 mM Na acetate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.0). The redissolved solution was centrifuged (15,100*g*, 10 min, 4°C) to remove any insoluble material. The clarified solution was applied to a column of S-Sepharose (2.5 × 18.3 cm) and unbound material was eluted with Buffer A. Bound material was eluted with a gradient of 0–300 mM NaCl in Buffer A in ca. six column volumes. Fractions active against Cbz-Arg-Arg-AMC were pooled.

In the novel method using *t*-butanol in the homogenate, rabbit liver was homogenized as before, but in a mixture of two parts of homogenization buffer and sufficient *t*-butanol to give 30% (v/v) *t*-butanol in the homogenate. The homogenate was centrifuged (9000*g*, 20 min, 4°C), the supernatant adjusted to pH 4.2 with dilute acetic acid, and the mixture again centrifuged (15,100*g*, 20 min, 4°C). A 20–40% ammonium sulfate TPP cut was taken, as before, and chromatography was as described above.

A similar approach was used with cathepsin L, except that the homogenization "buffer" in the control consisted of 2% *n*-butanol–1% NaCl–0.1% EDTA and a 20–45% TPP cut was taken (8). In the novel method, liver was homogenized in a mixture of two parts of homogenization buffer (150 mM Na acetate, 1 mM EDTA, pH 4.0) and sufficient *t*-butanol to give 30% (v/v) *t*-butanol in the homogenate.

The results for cathepsin B (Table 1), which were essentially reproducible in repeat isolations, reveal very much less activity in the homogenate obtained in the presence of *t*-butanol, despite the fact that the assays were done after removal of the *t*-butanol. However, there is a relatively large increase in activity after the TPP step (2.8-fold) in the case of the homog-

TABLE 1

Extraction and Upstream Fractionation of Cathepsin B from 300 g of Rabbit Liver

	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
			Control			
Icmogenate (supernatant)	642	20,897	1,362,183	65	(1)	(100)
TPP cut (20–40%)	98	4,763	228,194	479	7.4	16.8
-Sepharose	21	63	55,020	874	13.4	4.0
			Homogenization in 30% *t*-butanol			
Icmogenate (supernatant)	842	6,155	813,322	132	(1)	(100)
TPP cut (20–40%)	78.5	507	639,585	1261	9.6	78.6 *(47)*[a]
-Sepharose	14.6	93	198,557	2142	16.2	24.4 *(15)*
mprovement			3.6-fold	2.5-fold		

[a] Figures shown in italics represent yield based on the control homogenate without *t*-butanol.

TABLE 2

Extraction and Upstream Fractionation of Cathepsin L from 180 g of Sheep Liver

	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
			Control			
Homogenate (supernatant)	345	4630	12,537	2.7	(1)	(100)
TPP cut (20–45%)	42	181	3,270	18.1	6.7	26.1
S-Sepharose	33	0.8	2,133	2585.5	954.0	17.0
			Homogenization in 30% *t*-butanol			
Homogenate (supernatant)	520	6682	27,981	4.2	(1)	(100)
TPP cut (20–45%)	44	707	3,339	4.7	1.1	6.0 *(13.3)*[a]
S-Sepharose	35	0.9	2,983	3278.0	782.3	10.7 *(23.8)*
Improvement			1.4-fold	1.3-fold		

[a] Figures shown in italics represent yield based on homogenate without *t*-butanol.

enate containing *t*-butanol. The TPP step removes most of the *t*-butanol, as this is salted out to form a separate phase, while selectively fractionating the proteins. It appears that, in the presence of *t*-butanol, cathepsin B is more effectively extracted but that most of its activity is latent until made patent by the TPP step; by contrast, in the control isolation, there is a relatively large loss of activity during TPP. Here we have reported only on a novel approach to the *extraction* of cathepsin B from liver: for final isolation of the enzyme, a choice of downstream methods is available, including affinity chromatography on Sepharose-Ahx-Gly-Phe-Gly-Sc (9).

In the case of cathepsin L, higher apparent yields were obtained at all steps in the extraction using 30% *t*-butanol, with an increase in yield of about 1.4-fold being finally obtained after S-Sepharose chromatography (Table 2). In previous isolations of cathepsin L from sheep (8), human, and baboon liver (10), we have consistently obtained the enzyme in two forms: as the ca. 24-kDa free enzyme and as a ca. 35-kDa, proteolytically active, covalent complex of cathepsin L with stefin B. In the present study, after homogenization in 30% *t*-butanol, no evidence of this complex was found, supporting the contention that it may be an isolation artifact. Presumably, its formation in the homogenate is inhibited by 30% *t*-butanol.

Although *t*-butanol is not generally denaturing, it does irreversibly denature hemoglobin. This has been used to advantage (e.g., see Refs. 1, 11) but the denaturing effect on hemoglobin does not apply to all proteins with a quaternary structure (11). In general, it appears that homogenization in 30% *t*-butanol gives a "cleaner" extract which is easier to handle in centrifugation steps. This may reflect the fact that *t*-butanol denatures hemoglobin and solubilizes hydrophobic compounds.

We attribute the improved yields of cathepsins B and L partly to the ability of *t*-butanol to inhibit enzyme activity (including proteolysis) and prevent unwanted protein–protein interactions. Homogenization in 30% *t*-butanol is probably not limited in its usefulness only to the isolation of cathepsins as described here and we believe that it may be a generally useful upstream method in the extraction of proteins from animal, plant, and microbial tissues.

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t-Butanol: nature's gift for protein isolation

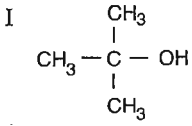
C. Dennison*, L. Moolman*, C.S. Pillay* and R.E. Meinesz*

Homogenization in 30% t-butanol is a method with potential for wide application in the isolation of proteins from animal, plant or microbial sources. Unusually, for an organic solvent, t-butanol stabilizes protein structure. It also inhibits enzyme activities and protein/protein interactions. By inhibiting proteolysis and obviating unwanted protein/protein interactions, the presence of approximately 30% t-butanol in a homogenization buffer generally minimizes the formation of artifacts and gives a higher yield. The presence of t-butanol in the homogenate leads naturally on to concentration and fractionation of proteins by three-phase partitioning, simply by the addition of increments of ammonium sulfate. In turn, fractions from three-phase partitioning have a low salt content and are thus poised for ion-exchange chromatography.

Because proteins must be in solution to be separated and purified, protein isolation usually starts with homogenization of the tissue from which the protein is to be isolated.[1] However, the breakdown of cellular compartmentation, concomitant with homogenization, can lead to unwanted protein/protein interactions and the consequent formation of homogenization artifacts. A major problem is proteolysis, caused by the release of proteases from the compartments where they are usually sequestered, and for this reason protease inhibitors are often added to a homogenization buffer. This is less appropriate, however, when cellular proteases themselves are the subject of interest. In the intact cell, proteases and their endogenous inhibitors often occur in different compartments. For example, the 'lysosomal' cysteine proteinases, cathepsins B, L, H, S, etc., occur in the endosomal system whereas stefin B, their endogenous inhibitor, occurs in the cytoplasm. Normally, the cysteine cathepsin/stefin B interaction is a tight, but non-covalent, interaction. However, we have found that formation of an artifactual, covalent, complex of cathepsin L and stefin B is apparently catalysed by a factor concurrently present in liver homogenates.[2,3] The possibility exists that many other similar, artifactual protein/protein interactions may occur in a homogenate and any method that can inhibit both proteolysis and other protein/protein interactions in a homogenate is therefore to be welcomed.

t-Butanol (I) is a reagent used in the established method for protein fractionation known as three-phase partitioning (TPP).[4]

I



In this method, t-butanol is added to about 20–30% (volume ratio) to a protein solution. Subsequent addition of sufficient ammonium sulfate causes the solution to divide into three phases, an upper t-butanol phase (which contains any non-polar solutes), a lower aqueous phase (containing the ammonium sulfate) and a third, intermediate, phase of precipitated (and concentrated) protein. The extent to which protein precipitates into the third phase is a function of the ammonium sulfate concentration and TPP can thus also be used for the fractionation of proteins.

TPP has some advantages over conventional salting out. First, with conventional salting out, the solution becomes increasingly dense with increasing ammonium sulfate concentration, until the stage is reached where it becomes difficult to sediment the precipitated protein. In TPP, however, the precipitated protein floats on the aqueous layer and so, as this becomes ever denser with increasing ammonium sulfate concentration, the precipitated protein floats more and more easily. Secondly, the precipitated protein from conventional salting out generally has a high salt concentration, so salting out must be followed by a desalting step, or by hydrophobic interaction chromatography, which can tolerate high salt concentration. By contrast, the precipitate from TPP has a low salt concentration and so TPP can be followed immediately by ion-exchange chromatography, for example.

Uniquely among common organic solvents, t-butanol stabilizes proteins rather than denaturing them.[4] A common property of all agents that stabilize protein structure is that they are excluded from the protein interior.[5] For example, the sulfate ion, the principal agent in salting out of proteins from water or water/t-butanol co-solvents, stabilizes protein structure

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because the sulfate ion accretes to itself more than 14 water molecules and so the hydrated ion is very large and is unable to access the protein interior.[4] The fact that t-butanol stabilizes rather than denatures proteins may be due to the fact that it too may be too large to gain access to the protein interior. However, most proteins have surface hydrophobic patches, constituting up to 50% of the surface area,[6] and t-butanol appears to bind to these patches, which accounts for the fact that proteins float in TPP.

All enzymes that have been tested are inhibited by t-butanol, with approximately 30% t-butanol being required to completely extinguish activity.[7] In most cases, for instance in all of the many proteins that have been isolated by TPP,[4] this loss of activity may be reversed by removing the t-butanol, for example by dialysis or by phase separation in TPP. The only exception found thus far is haemoglobin, which is denatured by t-butanol, probably by irreversible changes to the inter-subunit interactions. This is not disadvantageous, however, because in many cases haemoglobin is a problematic contaminant in the isolation of proteins from animal tissue. The basis for the inhibiting action of t-butanol is not yet clear, but it has been noted that in the presence of t-butanol some proteins undergo conformational changes, favouring an increased proportion of α-helices.[8] Not only are enzyme activities extinguished by t-butanol, but it appears that protein/protein interactions are also inhibited.[7]

The fact that t-butanol can inhibit proteolysis or enzyme/inhibitor interactions suggests that there may be merit in using t-butanol right at the outset of a protein isolation, that is, the tissue being homogenized in the presence of 30% t-butanol. This concept was tried in the isolation of the lysosomal cysteine proteinase, cathepsin B, from liver tissue, with a resulting 6-fold increase in final yield. A problem attending the isolation from liver of the related lysosomal cysteine proteinase, cathepsin L, is the formation in the homogenate of a proteolytically active, covalent, complex of cathepsin L with the cytoplasmic inhibitor, stefin B, which compromises the yield of the free enzyme.[2,3] Homogenization in 30% t-butanol obviated formation of the complex, resulting in a 40% increase in yield of the free enzyme.[7] With homogenization in 30% t-butanol, the homogenate is poised for fractionation by TPP, simply by adding increments of ammonium sulfate. Since the sulfate ion is more effective at low pH,[4] homogenization in a low pH buffer should be the first choice. In turn, the low-salt active fraction from TPP is poised for fractionation by ion-exchange or affinity chromatography. For the latter, removal of residual t-butanol, for example by dialysis, may be advantageous.

It would appear that in the properties of t-butanol, nature has provided proteinologists with the gift of a uniquely useful tool. We believe that t-butanol will have widespread application in the isolation of proteins from animal, plant and microbial sources, when its fundamental difference from other organic solvents, such as ethanol and acetone, is fully appreciated.

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In Brief...

Conservation in crisis?

Papers presented at the most recent symposium of the Wildlife Management Association of Southern Africa, held at George, expressed concern about threats to South Africa's biodiversity. It was reported that 'the indigenous freshwater fishes of the Western and Eastern Cape are rapidly approaching widespread and final extinctions'. Dean Impson of Cape Nature Conservation showed that of the 19 indigenous freshwater fishes of the Western Cape rivers (of which 16 are found only there), 11 species are now critically rare or endangered in terms of Red Data Book criteria. Of these fish, only one species was endangered in 1977, three in 1987, nine in 1996 and 11 species in 1999.

Moreover, the provincial conservation agencies have been losing large numbers of staff as long-serving personnel have been given severance packages and then not replaced. In the Western Cape, there were eight staff members involved in freshwater conservation in the provincial conservation authority in 1993. By last year this number had decreased to two. Kas Hamman, the director of Cape Nature Conservation, reported that there had been a 30% loss of conservation staff from the agency in the last three years. At the same time, funding for nature conservation management and research had been reduced throughout the country, it was said. A common sentiment at the symposium was that if current trends in staffing and funding cuts continued, the likelihood of South Africa maintaining its natural resource base was 'minimal'.

Bleek Collection honoured by UNESCO

The remarkable archive of documents recording the language and folklore of a group of San (Bushman) people, held jointly by the University of Cape Town and the National Library, has been formally listed in UNESCO's Memory of the World Register. The papers, compiled over many years by Dr Wilhelm Bleek, his daughter Dorothea and sister-in-law Lucy Lloyd, starting in Cape Town in the 1870s, include nearly 12 000 pages of verbatim accounts of Bushman life, ritual and myth. Bleek was a German linguist who worked originally with /Xam convicts who had been sent to work on a breakwater in Table Bay. The /Xam language is now extinct. These records played an important part in revealing the meaning of San rock art as pioneered by David Lewis-Williams at the University of the Witwatersrand.

The Memory of the World programme, of which the register is an integral part, is to 'guard against collective amnesia', and aims to preserve valuable archives throughout the world.

Healthy funding for sports science

Discovery Health will donate millions of rands for sports science research at the University of Cape Town over the next 10 years. The healthcare company's generosity will be used variously on behalf of the Bioenergetics of Exercise Research Unit, to fund the chair of acclaimed sports scientist Tim Noakes, and the High Performance Centre at the Sports Science Institute. The investment should eventually be worth well over R20 million.

Professor Noakes was recently honoured as one of the 22 founding members of the International Olympic Committee's new Olympic Academy of Science. Although the role of the academy is still undecided, Noakes considers that it should coordinate research to understand how the human body responds to the demands of exercise.

Recognition for water research

The University of Pretoria has established the African Water Issues Research Unit in the Department of Political Sciences, with Anthony Turton, a political scientist who has specialized in the politics of water in southern Africa, as its head. The unit plans to develop a scientific understanding of the role of water in terms of social and economic stability, and to forge strong international links. More information about AWIRA plans is available on the university's website (http://www.up.ac.za/academic/libarts/polsci/awira) or via e-mail (awira@postino.up.ac.za).

The university has also formed a 'strategic alliance' with the CSIR in water research, one of a number of such initiatives between the two institutions, to promote research and training.