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**INVESTIGATION OF
LIPOPOLYSACCHARIDE BINDING PROTEIN
IN THE BOVINE MAMMARY GLAND**

A thesis submitted in partial fulfilment
of the requirements for the Degree of

Master of Science in Biology



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Tē Whare Wānanga o Waikato

by
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ABSTRACT

This study investigated the presence of LBP in the bovine mammary gland during varying stages of development and infection. The project was initiated by the discovery of an EST in a mastitic mammary cDNA library which bore homology to lipopolysaccharide binding protein (LBP). The results add to the understanding of the innate immune defences of the gland and the biological regulation of LBP.

The expression profile of LBP in bovine mammary tissue was characterised by northern blot analysis which showed that LBP levels were increased in infected and involuted tissues. This is supported by the results from *in situ* hybridisation which detected local expression of LBP in epithelia surrounding ducts and alveoli in infected tissue. LBP was also shown to be increased in mammary secretions of whey and skim milk from infected and involuted glands by western blotting with an antibody that was raised against a peptide of LBP. These results were quantified by ELISA. The LPS-binding activity of the native protein was confirmed by ligand blotting, immunoprecipitation and ELISA. Attempts were made to investigate the immuno-stimulatory activity of LBP using a recombinant protein in a cytokine induction assay. Due to problems encountered with the expression system the results from this can not be accurately analysed and further optimisation is required.

This is the first report of local expression of LBP in the mammary gland and taken together, these results suggest an important role for LBP in the local innate defence of the gland.

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TABLE OF CONTENTS

<i>Abstract</i>	ii
<i>Acknowledgements</i>	iii
<i>Table of Contents</i>	v
<i>List of Figures</i>	xii
<i>List of Tables</i>	xiv
<i>List of Abbreviations</i>	xvi

Chapter One	Introduction	1
1.1	The Innate Immune Response.....	1
1.1.1	The Acute Phase Response.....	1
1.1.2	Immune Recognition of Pathogens.....	2
1.1.2.1	Recognition of Bacterial Toxins	3
1.1.2.2	Pattern Recognition Receptors for Bacterial Toxins: The Toll-like Receptors	4
1.1.3	Complications arising from Over-stimulation of the Immune Response	4
1.2	Lipopolysaccharide Binding Protein.....	5
1.2.1	The LBP Protein Family	6
1.2.2	Detection of LBP Homologues.....	6
1.2.3	The Structure of LBP	8
1.2.4	The Function of LBP	10
1.2.4.1	The CD14 Receptor and the LBP Signalling Pathway	12

1.2.4.2 Action of LBP against Gram Positive Bacteria and Fungi.....	15
1.2.5 Expression Patterns of LBP	16
1.2.6 Detection of LBP in Milk	18
1.3 Aims of this Thesis	20
Chapter Two Materials and Methods	21
2.1 Background Experiments	21
2.2 Full length Sequencing of Bovine Mammary LBP	21
2.3 Northern Blotting	23
2.3.1 RNA Isolation and Preparation.....	23
2.3.2 Preparation of LBP and CD14 Probes	25
2.3.2.1 Subcloning.....	25
2.3.2.1.1 Alkaline Lysis Extraction of Plasmid DNA	26
2.3.2.2 Restriction Digests.....	27
2.3.2.3 Radioactive Labelling of DNA Probes	28
2.3.2.4 Probe Purification and Incorporation Check	28
2.3.3 Northern Blotting.....	29
2.3.3.1 Prehybridisation	30
2.3.3.2 Hybridisation.....	31
2.4 <i>In situ</i> Hybridisation.....	31
2.4.1 Preparation of Slides.....	32
2.4.2 Preparation of Probes.....	33
2.4.2.1 α -lactalbumin Probes	33

2.4.2.2 LBP Probes.....	33
2.4.2.2.1 PCR Reaction for Subcloning.....	34
2.4.2.2.2 Preparation of the pT-LBP Construct	34
2.4.2.3 Restriction Digests	35
2.4.2.4 Radioactive Labelling of DNA Probes	36
2.4.2.5 Incorporation Check.....	36
2.4.2.6 Integrity Check.....	37
2.4.3 Hybridisation	37
2.4.4 Slide washing.....	38
2.4.5 Emulsion.....	38
2.4.6 Developing	39
2.4.7 Staining.....	39
2.5 Western Blotting	40
2.5.1 Sample Preparation.....	40
2.5.1.1 Protein Concentration Assay.....	41
2.5.2 Western Blot Analysis.....	41
2.5.3 Stripping Western Blots.....	42
2.5.4 Antibody Detection.....	42
2.6 Production of an Anti-LBP Antibody	43
2.6.1 Peptide Design	43
2.6.2 Peptide Conjugation.....	45
2.6.2.1 Activation of Carrier Proteins	45
2.6.2.2 Conjugation of Carrier Proteins to the Peptide	46
2.6.3 Inoculation	46

2.6.4 IgG Purification of Anti-sera	47
2.7 Immunoprecipitation with the LBP-N Antibody	48
2.7.1 Biotinylation of the LBP-N Antibody	49
2.8 ELISA	50
2.8.1 Spike Recovery	52
2.9 Recombinant Protein Expression	53
2.9.1 Recombinant Protein Expression in Bacteria	53
2.9.1.1 PCR Reaction for Subcloning of LBP	53
2.9.1.2 Preparation of the pET-LBP Construct	54
2.9.1.3 Induction of Protein Expression	55
2.9.1.4 Mini-extractions	56
2.9.2 Recombinant LBP Expression in Mammalian Cells	57
2.9.2.1 Preparation of the G-LBP Construct	57
2.9.2.1.1 PCR Reaction	59
2.9.2.1.2 The BP Reaction	60
2.9.2.1.3 Transformation	61
2.9.2.1.4 The LR Reaction	62
2.9.2.1.5 Transformation	62
2.9.2.2 Preparation of Mammalian Cells	63
2.9.2.3 Mini transfection	63
2.9.2.4 Protein and RNA Extraction	64
2.9.2.4.1 RNA Precipitation	64
2.9.2.4.2 Protein Extraction	65
2.9.2.5 Large Scale Transfection	66

2.10 Purification of Recombinant LBP	67
2.10.1 Purification in Native Conditions	68
2.10.1.1. Preparation of Bacterial Cell Lysate	68
2.10.1.2 Preparation of Mammalian Cell Lysate	68
2.10.1.3 Protein Purification	69
2.10.2 Purification in Denaturing Conditions.....	69
2.10.2.1 Preparation of Bacterial Cell Lysate	69
2.10.2.2 Protein Purification	69
2.10.3 Regeneration of Protein Purification Columns.....	70
2.10.4 Purification with Vivaspin Columns	70
2.10.5 Detection of Purified Protein	70
2.11 LBP Activity	71
2.11.1 ELISA	71
2.11.2 Immunoprecipitation with <i>E. coli</i> Proteins.....	71
2.11.3 Ligand Blotting with LPS.....	72
2.11.3.1 Biotinylation of LPS.....	72
2.11.4 Cytokine Induction Assay.....	73
2.11.4.1 Production of Recombinant LBP Protein.....	74
2.11.4.2 Stimulation of TNF α Production in Monocytes	74
2.11.4.3 Cell Proliferation Assay	75
Chapter Three Results	77
3.1 Full length Sequencing of Bovine Mammary LBP	77

3.2 Northern Blotting	80
3.3 <i>In situ</i> Hybridisation.....	83
3.4 Production of an Anti-LBP Antibody	84
3.4.1 Activation of Carrier Proteins.....	84
3.4.2 Peptide Conjugation.....	85
3.4.3 IgG purification of anti-sera	86
3.5 Western Blotting	86
3.6 Immunoprecipitation with the LBP-N Antibody	88
3.7 ELISA	89
3.7.1 Spike Recovery.....	90
3.8 Recombinant Protein Expression	92
3.8.1 Recombinant LBP Expression in Bacteria	92
3.8.2 Recombinant LBP Expression in Mammalian Cells	94
3.8.2.1 Northern Blotting	94
3.8.2.2 Western Blotting	95
3.9 Purification of Recombinant LBP	97
3.10 LBP Activity	98
3.10.1 ELISA	98
3.10.2 Immunoprecipitation with <i>E. coli</i> Proteins.....	98
3.10.3 Ligand Blotting with LPS.....	99
3.10.4 Cytokine Induction Assay.....	101
3.10.4.1 Cell Lysate Preparation	102
3.10.4.2 Cell Proliferation Assay	103

Chapter Four	Discussion	105
4.1	Full length Sequencing.....	106
4.2	Northern Blotting	106
4.3	<i>In situ</i> Hybridisation.....	107
4.4	Western Blotting	109
4.5	Immunoprecipitation	110
4.6	ELISA	110
4.7	Recombinant Protein Expression	112
4.8	Purification of Recombinant Protein.....	114
4.9	LPS Binding Activity.....	115
4.10	Cytokine Induction.....	116
Chapter Five	Summary and Conclusions	117
Appendix		121
References		132

LIST OF FIGURES

Chapter One

Figure 1.1: Diagram of BPI structure	9
Figure 1.2: The LBP signalling pathways.....	14

Chapter Two

Figure 2.1: Northern blotting system	30
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Chapter Three

Figure 3.1: Northern blot analysis of LBP mRNA expression in bovine tissues....	81
Figure 3.2: Northern blot analysis of CD14 mRNA expression in bovine tissues	82
Figure 3.3: <i>In situ</i> hybridisation of LBP in mastitic bovine mammary tissue	83
Figure 3.4: Sephadex purification of carrier proteins	85
Figure 3.5: Western blot analysis of LBP in bovine serum and whey samples.....	87
Figure 3.6: Western blot analysis of LBP in bovine whey and skim milk samples	87
Figure 3.7: Western blot analysis of immunoprecipitation of LBP	88
Figure 3.8: Representative standard curve of human LBP from ELISA	89
Figure 3.9: ELISA spike recovery method one.....	90
Figure 3.10: ELISA spike recovery method two	91
Figure 3.11: Detection of recombinant LBP expression in <i>E. coli</i> cells using an anti-histidine antibody.....	93

Figure 3.12: Detection of recombinant LBP expression in *E. coli* cells
using an anti-LBP antibody 93

Figure 3.13: Detection of recombinant LBP mRNA expression in cos7 cells 94

Figure 3.14: Detection of recombinant LBP protein expression in cos7 cells
with an anti-LBP antibody..... 95

Figure 3.15: Detection of recombinant LBP protein expression in cos7 cells
with an anti-histidine antibody 96

Figure 3.16: Detection of recombinant stat5b expression in cos7 cells..... 96

Figure 3.17: Western blot analysis of immunoprecipitation with
E. coli proteins..... 99

Figure 3.18: Ligand blot of bovine serum and whey milk samples 100

Figure 3.19: Ligand blot of bovine skim and whey milk samples 101

Figure 3.20: Detection of recombinant LBP in mammalian cell lysates 102

Figure 3.21: Standard curve of TNF α concentration..... 103

Figure 3.22: Effects of LBP and LPS on WEHI cell proliferation assay..... 104

LIST OF TABLES

Chapter One

Table 1.1: Alignment of LBP N-terminal sequences 7

Chapter Two

Table 2.1: Primers for full length sequencing of bovine mammary LBP 22

Table 2.2: RNA samples used for northern blot analysis..... 24

Table 2.3: Bovine mammary tissues used for *in situ* hybridisation analysis 32

Table 2.4: PCR primers for cloning of bovine mammary LBP
into the pGEM-T Easy vector 33

Table 2.5: Preparation of probes for *in situ* hybridisation..... 35

Table 2.6: Milk samples analysed for LBP by western blot analysis 40

Table 2.7: Alignment of the C-terminal peptide used for antibody production..... 43

Table 2.8: Alignment of the N-terminal peptide used for antibody production..... 44

Table 2.9: Bovine milk and serum samples analysed by ELISA 51

Table 2.10: PCR primers designed for cloning of bovine mammary LBP
into the pET-23b expression vector 54

Table 2.11: PCR primer sequences for cloning of bovine mammary LBP
into the Gateway expression system (Invitrogen)..... 58

Table 2.12: Volumes used in transfection reactions for recombinant expression
in mammalian cos7 cells..... 64

Table 2.13: Buffers used for ProBond protein purification system 67

Table 2.14: Plate design for cytokine induction assay 75

Chapter Three

Table 3.1: Full length sequence of bovine mammary LBP 77

Table 3.2: Conceptual protein translation of bovine mammary LBP 78

Table 3.3: Clustal W alignment of LBP homologues 79

Table 3.4: Clustal W alignment of bovine BPI and bovine mammary LBP 80

Table 3.5: LBP concentration ranges for bovine samples as determined
by ELISA..... 89

Table 3.6: Percent recovery from spike recovery method one..... 91

Table 3.7: Percent recovery from spike recovery method two 91

LIST OF ABBREVIATIONS

aa	amino acid
ATCC	American type culture collection
BME	β -mercaptoethanol
BPB	bromophenol blue
BPI	bactericidal permeability increasing protein
BSA	bovine serum albumin
CD14	differentiation antigen of monocytes
CETP	cholesteryl ester transfer protein
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO	chinese hamster ovary
CMV	cytomegalovirus
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescent
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
EST	expressed sequence tag
EtBr	ethidium bromide
FCS	foetal calf serum
f.s.	filter sterilised
<i>g</i>	gravitational constant
G-ve	gram negative bacteria
G+ve	gram positive bacteria
GPI	glycosyl-phosphatidylinositol
HBT	Hycult biotechnology
HDL	high density lipoprotein
HEPES	2-[4-(2-hydroxyethyl)-piperazine] ethanesulfonic acid
HRP	horse radish peroxidase

IGEPAL	polyethyleneglycolmono[4-(1, 1, 3, 3-tetramethylbutyl)-phenyl]ether
IgG	immunoglobulin G
IL-1	interleukin 1
IL-6	interleukin 6
IPTG	isopropylthio- β -D-galactoside
Kb	kilo base pairs
kDa	kilodaltons
KLH	keyhole limpet hemocyanin
LB	luria base
LBON	LB media with no salt
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LUNX / PLUNC	lung specific X protein / palate, lung and nasal epithelial clone
MBS	3-maleimidobenzoic acid N-hydroxy-succinimide ester
mCD14	membrane bound CD14 receptor
MOPS	3-(N-morpholino) propanesulfonic acid
MQ	Millipore Q grade water
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MW	molecular weight
NCBI	National centre for biotechnology information
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCW	pneumococcal cell wall
PLTP	phospholipid transfer protein
PMSF	phenylmethylsulfonyl fluoride
PSP	parotid secretory protein
PVP	polyvinylpyrrolidone

CHAPTER ONE INTRODUCTION

1.1 THE INNATE IMMUNE RESPONSE

There are two main divisions of the immune response to microbial invasion, the first being the ‘innate response’ which is followed by the more specific ‘acquired immune response’. The acquired immune response is mediated by lymphocytes with specific antigen receptors, which are produced after exposure to the pathogen, making the response highly specific, but also slower in producing a reaction (Kimbrell and Beutler, 2001). The initial response is from the innate immune system which is rapid and non-specific, relying on phagocytic cells and serum proteins to offer a broad range of protection against a variety of pathogens. This response acts to quickly clear toxins and foreign bodies from the site and stimulate the mediators involved in inflammation to repair the damage (Nairn and Helbert, 2002).

1.1.1 THE ACUTE PHASE RESPONSE

The body’s first response to the invasion of pathogens is the ‘acute phase’ response. This involves proteins and mediators which are usually present at basal levels in the circulation and are rapidly induced at the first signs of infection. The main role of acute phase proteins is to keep infection at bay while signalling to other innate immune molecules to stimulate the inflammatory response. Most proteins involved in the acute phase response are produced by the liver and circulated in the plasma. As the first molecules on the scene, they stimulate production of cytokines in order to recruit phagocytic cells such as monocytes and macrophages to the site of infection. The phagocytic cells can then act to clear the

area of toxins by engulfing the bacteria, which results in inflammation (Nairn and Helbert, 2002).

A delicate balance is required to maintain the system, which requires a high level of regulation, as there can be fatal consequences if the response is too great or too little. In order to maintain such an essential role there are often a number of alternative pathways involved in eliciting the response required.

1.1.2 IMMUNE RECOGNITION OF PATHOGENS

Different pathogens release a range of toxins, making it essential that any immune response released in the first instance is non-specific and therefore able to counter a wide variety of molecules. However, as more is understood about the different proteins and mediators involved in this response, it is noted that these often selectively react to a specific set of pathogenic components, raising the debate that the system is not entirely non-specific. There are two main theories of immune signalling, one being that microbes contain 'pathogenic patterns' which are recognised by receptors on immune cells and cause stimulation of the immune response (Mushegian and Medzhitov, 2001; Medzhitov and Janeway, 2002). The other theory is that host cells, damaged by the invading organisms, release chemical signals such as uric acid crystals which stimulate dendritic cells, initiating the acquired immune response (Heath and Carbone, 2003; Shi *et al.*, 2003). It is most likely that both of these pathways are occurring and complement each other in aiding the sensitivity and specificity of the immune system. The pathogenic pattern theory is the best described so far, and will be the focus for the rest of this section.

Pathogenic patterns are usually essential microbial elements, with high levels of conservation making it easy for the proteins involved to target these regions (Mushegian and Medzhitov, 2001). Proteins involved in detecting these patterns are sometimes grouped under the term 'pattern recognition receptors'. The binding of a microbial 'pattern' to a receptor on immune cells leads to a signalling cascade resulting in the activation of transcription factors which control expression of the immune response genes and cause release of cytokines and other effector molecules (Kimbrell and Beutler, 2001; Mushegian and Medzhitov, 2001; Bochud and Calandra, 2003).

1.1.2.1 Recognition of Bacterial Toxins

Pattern recognition receptors targeting bacteria often recognize toxic components of the bacterial cell wall. In gram negative (G-ve) bacteria the main toxin is lipopolysaccharide (LPS), which is essential for the viability of the cells. During cell division, lysis and death, LPS is released from the cell wall and the toxic moiety, lipid A, is exposed to immune cells (Van Amersfoort *et al.*, 2003). Due to its conservation and its high immunogenicity, LPS and more specifically lipid A, is a prime target for pattern recognition receptors. Similarly, there are pattern recognition receptors which target components of gram positive (G+ve) bacterial cell walls which are comprised of peptidoglycan and lipoteichoic acid (LTA) (Van Amersfoort *et al.*, 2003). While the recognition patterns are still not well understood, it seems that there are receptors which are more specific, recognising a pattern such as LPS, while others act more broadly and are able to recognise components from both types of bacteria, for instance LPS and LTA.

1.1.2.2 Pattern Recognition Receptors for Bacterial Toxins:

The Toll-like Receptors

Toll-like receptors (TLR) are transmembrane proteins which are expressed on a range of cells, including monocytes. The Toll receptors were first discovered in *Drosophila* where they were shown to play a role in defence against fungi and G+ve bacteria. A number of protein homologues have been found in other species, with ten identified in humans to date (reviewed in Aderem and Ulevitch, 2000; Akira, 2000). The human TLR genes have been characterised and seven of them found to be specific pattern recognition receptors which interact with different microbial patterns. For instance, the TLR2 protein detects components of G+ve bacteria, while TLR4 has been shown to be essential for recognition of LPS from G-ve bacteria (Tapping *et al.*, 2000), although levels of both TLR4 and TLR2 may be increased during infection (Goldammer *et al.*, 2004). Activation of TLRs leads to intracellular signalling which activates transcription factors such as NF- κ B, causing the induction of cytokines and expression of immune related genes (Akira, 2000; Mushegian and Medzhitov, 2001). These include the acute phase proteins which act to produce an inflammatory response to eliminate the infection.

1.1.3 COMPLICATIONS ARISING FROM OVER-STIMULATION OF THE

IMMUNE RESPONSE

Over-stimulation of the immune response is of particular concern when targeting new medical applications of immuno-stimulatory molecules. One of the key threats with these is the delicate balance of the innate immune system. The consequences of tipping this balance to one side can be seen in cases of persistent infection which leads to chronic inflammation. In these cases the immune system is being constantly stimulated by the toxins in the bloodstream causing the levels

of inflammatory cells to remain high. This can eventually lead to conditions such as endotoxemia and septic shock, with damage occurring due to the large numbers of phagocytic cells endeavouring to cope with the onslaught of toxins by producing large quantities of reactive oxygen species (Oh, 1998; Bochud and Calandra, 2003). Sepsis related conditions cause breathing rate to increase and become abnormal, and hypoperfusion of the liver and central nervous system, leading to a high level of fatalities (Oh, 1998).

1.2 LIPOPOLYSACCHARIDE BINDING PROTEIN

The focus of this study was the investigation of lipopolysaccharide binding protein (LBP), which is an acute phase protein that is known to bind to the LPS component of G-ve bacterial cell walls (Tobias *et al.*, 1988; Tobias *et al.*, 1989). Like most acute phase proteins it is produced by the liver and is present in the circulation at basal levels which rise rapidly in response to infection. In humans there is a 100-fold increase, with plasma levels rising from approximately 0.5 µg/ml to 50 µg/ml during infection (Schumann *et al.*, 1990). The regulation of LBP expression has been shown to be dependent on the presence of cytokines, tumour necrosis factor (TNF α) and interleukins 1 and 6 (IL-1, IL-6), which have a synergistic effect on the increase in expression (Grube *et al.*, 1994). This regulation is characteristic of a class I acute phase protein (Schumann *et al.*, 1996). It is also interesting to note that LBP induces expression of TNF α and IL-6, suggesting that there may be a cyclic effect, with LBP acting as an auto-inducer (Grube *et al.*, 1994).

1.2.1 THE LBP PROTEIN FAMILY

LBP belongs to a family of proteins which were originally based on the common function of lipid binding and transport and comprised of three other main members: bactericidal permeability increasing protein (BPI), cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) (Tobias *et al.*, 1988; Hailman *et al.*, 1996; Kirschning *et al.*, 1997). Of these BPI is the best characterised and is the only family member whose crystal structure has been elucidated. Based on this structural information it was found that BPI contains two unique domains, one at each terminus of the protein (Beamer *et al.*, 1998). Recent studies have discovered more proteins possessing at least one of the BPI domains, extending the BPI-like protein family to include at least 13 proteins (Bingle and Craven, 2002; Mulero *et al.*, 2002; Wheeler *et al.*, 2002). The activities and functions of these new family members are yet to be defined, but it is thought that some of them are related to the immune response. This is based on observations of their expression profiles which show increased levels during inflammation and infection. The idea that they comprise similar functions is supported by genetic mapping analyses which have located the genes for 11 of the 13 known family members on a cluster on human chromosome 20q11.2 (Gray *et al.*, 1993; Mulero *et al.*, 2002; Wheeler *et al.*, 2002; Wheeler *et al.*, 2003).

1.2.2 DETECTION OF LBP HOMOLOGUES

LBP was first characterised from human and rabbit serum by Schumann *et al.* (1990). Since then, homologues have been identified in a range of mammals including mice (Gallay *et al.*, 1993a), cows (Khemlani *et al.*, 1994) and baboons (Haudek *et al.*, 2000). Alignments of the published full length or partial N-terminal sequences of these proteins show a high degree of conservation

(Table 1.1). Those that have been characterised further have been found to have molecular weights of approximately 60 kDa. Khemlani *et al.* (1994) were the first to identify and characterise LBP in bovine serum, noting that in the purified samples from bovine serum there were often several bands corresponding to the approximate MW of LBP at 65, 60 and 51 kDa. This has not been reported in other species, but may occur as the result of different glycosylation patterns. Methods for purification of LBP from bovine and human serum have been developed (Schletter *et al.*, 1994; Horadagoda *et al.*, 1995; Bochsler *et al.*, 1996), enabling their further characterisation.

Table 1.1: Alignment of LBP N-terminal sequences

Identical residues are shown in red and conserved residues which match the majority of the other sequences shown in blue. The conceptual translation from the sequence from this study is in bold and is named bLBP. The bovine LBP sequence shown is from Khemlani *et al.* (1994) and the baboon sequence is from Haudek *et al.* (2000). The NCBI accession numbers for the other LBP sequences shown below are as follows; Human LBP: NP_004130, Rabbit LBP: B35843, Mouse LBP: NP_032515, Human BPI: NP_00176, Bovine BPI: NP_776320.

bLBP	N P R I F G T R G D Q G L E Y V A Q E E L
Bovine LBP	A N P G L V V R I T D Q G L E Y V A Q E E L
Human LBP	A N P G L V A R I T D K G L Q Y A A Q E Q L
Rabbit LBP	T N P G L I T R I T D K G L E Y A A R E Q L
Mouse LBP	T N P G L V T R I T
Baboon LBP	N P G L V A R T T N K G L E Y S A Q E
Human BPI	V N P G V V V R I S Q K G L D Y A S Q Q Q T
Bovine BPI	T N P G I V A R I T Q K G L D Y A C Q Q G V

1.2.3 THE STRUCTURE OF LBP

LBP is synthesized in hepatocytes as a 50 kDa polypeptide and is glycosylated to give a mature protein of approximately 60 kDa (Ramadori *et al.*, 1990). The full length sequence includes at least two cysteine residues which form a disulfide bond that is highly conserved in BPI family members (Beamer *et al.*, 1998). The number of potential glycosylation sites varies depending on the species (Schumann *et al.*, 1996).

Structural information for LBP and other members of the BPI protein family is predominantly based on the x-ray crystallographic structure of BPI (Beamer *et al.*, 1997; Kleiger *et al.*, 2000), as it is the only family member to date to have had its crystal structure determined. LBP is thought to have a very similar structure to BPI as in humans they share 45% sequence similarity, with the highest levels of conservation surrounding important structural components (Beamer *et al.*, 1998). Both BPI and LBP are known to bind to the lipid A portion of LPS (Tobias *et al.*, 1989; Marra *et al.*, 1992; Mathison *et al.*, 1992), while CETP and PLTP are more involved in binding and transporting phospholipids. Two lipid binding sites have been located in apolar pockets (Beamer *et al.*, 1997; Beamer *et al.*, 1999), which could allow lipids to be transported through aqueous environments, and multiple sequence alignments of other family members show that the most highly conserved regions are clustered close to these pockets, in the centre of the protein (Beamer *et al.*, 1998). This is consistent with the characteristic function of the main family members in lipid transfer, and although lipid A is not actually a phospholipid these pockets may be involved in LPS binding and transport also.

The structure of BPI is defined by the two BPI domains, known as the N-terminal domain and the C-terminal domain (Figure 1.1). These form a unique structure called the BPI fold. The N-terminal and C-terminal domains have similar secondary and tertiary structures despite sharing less than 20% sequence similarity (Beamer *et al.*, 1999). Both domains are composed of approximately 200 amino acids which form a 'barrel' of two α -helices and a twisted β -sheet. A proline-rich linker joins the two, forming the distinct 'boomerang' shape (Beamer *et al.*, 1997).

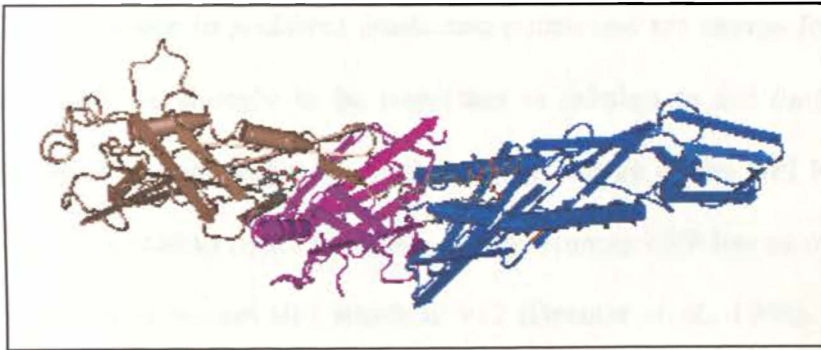


Figure 1.1: Diagram of BPI structure

The N-terminal domain is shown in blue and the C-terminal domain is in brown. The linker region is shown in pink. Source: NCBI structure database 1BPI mmdb: 6490 (Beamer *et al.*, 1997).

Despite the structural similarity of the domains, the low sequence similarities between BPI and LBP could explain the difference in their functional properties. In BPI, the N-terminal domain is essential for the bactericidal properties of the protein while both domains are involved in LPS binding and neutralisation (Iovine *et al.*, 1997; Iovine *et al.*, 2002). LBP, however, is not bactericidal and experiments with truncated peptides of LBP identified an LPS binding site in the N-terminal of the protein (residues 1 – 197) (Han *et al.*, 1994; Theofan *et al.*, 1994). The C-terminal domain has been shown to be responsible for transfer of

LPS to macrophage cells and the resulting cytokine induction (Abrahamson *et al.*, 1997; Iovine *et al.*, 2002). As the N-terminal domain is predominantly involved in LPS or lipid binding, it is more highly conserved in different family members compared to the C-terminal domain. One of the observations in line with this is the presence of a number of positive residues at the tip of the N-terminal domain which are conserved in LBP and BPI but do not appear to have a structural role. It is thought that they may be involved in electrostatic interactions involved in LPS binding (Beamer *et al.*, 1998).

The difference in predicted isoelectric points and net charge for the proteins BPI and LBP are thought to be important in relation to the functional differences displayed. In particular, the high positive charge of the BPI N-terminal domain may be related to its bactericidal activity. Human LBP has an overall charge of -4 compared to human BPI which is +12 (Beamer *et al.*, 1998). Despite this, both proteins bind with high affinity to LPS, which is negatively charged.

1.2.4 THE FUNCTION OF LBP

Although human BPI and LBP share approximately 50% sequence similarity and are predicted to have almost identical tertiary structures, they play opposing roles in the immune response. Both proteins bind to LPS, but BPI acts to diminish the immune/inflammatory response by sequestering LPS and clearing it from the circulation, preventing its immune stimulating effect (Marra *et al.*, 1992; Iovine *et al.*, 1997). On the other hand, LBP is suggested to transfer LPS to a receptor on monocytes which results in stimulation of pro-inflammatory cytokines and the induction of the inflammatory response. The purpose of these two proteins being

activated upon the same stimulus but having antagonistic effects may be to maintain a critical balance and prevent over or under-stimulation.

The presence of endotoxin (LPS) in the bloodstream causes an increase in production of inflammatory cytokines. This response is increased in the presence of LBP as it enhances LPS binding to monocytes, leading to stimulation of cells involved in the inflammatory response and increased expression of key cytokines such as $\text{TNF}\alpha$, and IL-6 (Mathison *et al.*, 1992; Roy *et al.*, 2001). LBP enhances this response and increases the sensitivity by lowering the activation threshold. This was shown by Gallay *et al.* (1993b), with LBP deficient mice, and also by Wurfel *et al.* (1997) who observed that whole blood from LBP deficient mice was 1000 times less responsive to LPS. However, *in vivo* responses to LPS inoculation in these mice did not show the same pattern (Jack *et al.*, 1997), suggesting that alternative pathways are involved. This is not unexpected as essential immune responses such as this often have alternative pathways. While Jack *et al.*, found that LBP is not essential for response to bacteria and clearance of LPS, it was shown to be necessary for survival of infection in mice by *Salmonella typhimurium* (Bernheiden *et al.*, 2001; Heinrich *et al.*, 2001).

It has been shown that both LBP and BPI compete for the binding of LPS (Dentener *et al.*, 1993; Wilde *et al.*, 1994), with the presence of BPI inhibiting LBP binding significantly, suggesting that both proteins bind LPS at a similar site on the molecule. However, it was also noted that the LBP binding to LPS was never totally suppressed even with much higher concentrations of BPI. This lends more evidence to the role these two proteins have in maintaining a balance in the body's reaction towards invasion of infectious bacteria. The presence of other

LPS binding proteins such as lactoferrin and cationic antimicrobial peptides also must be taken into account in maintaining the delicate balance, as these molecules are known to compete with BPI and LBP for LPS binding (Elass-Rochard *et al.*, 1998; Scott *et al.*, 2000).

1.2.4.1 The CD14 Receptor and the LBP Signalling Pathway

The CD14 receptor is a differentiation antigen of monocytes. It was first discovered by Wright *et al.* (1990), and is usually found as a glycosyl-phosphatidylinositol (GPI)-anchored receptor on macrophage membranes (mCD14). The receptor binds to LPS causing the reaction pathway to occur which leads to the production of cytokines involved in inflammation, mainly TNF α , IL-6 and IL-1 (Lee *et al.*, 1993; Leeuwenberg *et al.*, 1994) (Figure 1.2). The pathway of activation that occurs once LBP has facilitated LPS binding to CD14 is one which is still not well understood. As CD14 is not a transmembrane protein, other proteins or factors must be associated in order for monocytes to be activated. It has been found that TLR4 (Tapping *et al.*, 2000; Bernheiden *et al.*, 2001), and its association protein, MD-2 are essential for cellular activation by LPS (Poltorak *et al.*, 1998; Muta and Takeshige, 2001; Viriyakosol *et al.*, 2001; Nagai *et al.*, 2002). It has also been found that the LBP-LPS complex is internalised after binding to mCD14, with inhibition by different antibodies showing that the internalisation process and signal transduction were two separate reactions (Gegner *et al.*, 1995).

While CD14 is able to bind LPS independently of LBP, the presence of LBP increases the sensitivity of the system (Kirkland *et al.*, 1993). It has been shown that these proteins form a trimolecular complex which allows monocytes to detect

LPS at concentrations as low as 10 pg/ml (Mathison *et al.*, 1992; Thomas *et al.*, 2002). This enables the system to act against endotoxin invasion quicker and more effectively, preventing high LPS concentrations occurring which could cause damage.

A soluble form of the CD14 receptor also exists (sCD14), and is produced by the shedding of mCD14 (Leeuwenberg *et al.*, 1994). This acts in a similar way, by binding LBP-LPS complexes (Tobias *et al.*, 1995), but has also been found to be involved in an immuno-inhibitory action involving the transfer of LPS to plasma lipoproteins such as high density lipoprotein (HDL) (Figure 1.2). The transfer of LPS to HDL leads to clearance from the circulation, effectively neutralising the LPS induced immune response (Wurfel *et al.*, 1995; Wurfel *et al.*, 1997). In this way LBP can act to both increase and decrease the immune response to LPS. Wurfel *et al.* (1995), and Wurfel and Wright (1997), showed that LBP could transfer LPS directly to HDL particles, or alternatively use a two-step process via sCD14. The two-step process is favoured, with LBP and sCD14 acting synergistically to increase the rate of neutralisation. One molecule of LBP was able to move multiple LPS molecules, and the presence of LBP reduces the concentration of HDL required for complete neutralisation of LPS-sCD14 complexes.

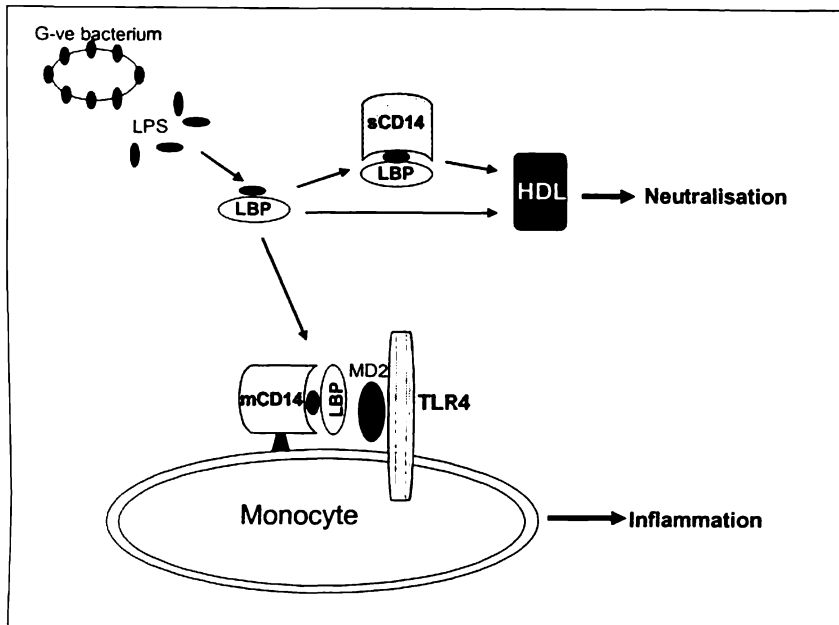


Figure 1.2: The LBP signalling pathways

Lipopolysaccharide binding protein (LBP) binds to lipopolysaccharide (LPS) released from gram negative bacterial membranes. The LBP-LPS complex interacts with the CD14 receptor which is either soluble (sCD14) or bound to the membrane of monocytes (mCD14). LBP can cause neutralisation of the LPS toxin by transferring it to lipoproteins (HDL) either directly or via the sCD14 receptor. Alternatively, LBP can transfer LPS to the mCD14 receptor which activates a signalling pathway via the toll-like receptor 4 (TLR4) and its association protein (MD-2), causing induction of effector molecules involved in the inflammatory response.

The factors determining the response of LBP to LPS are not well defined, but it has been shown that the concentration of LBP present can alter the response, with high concentrations inhibiting monocyte responses to LPS by causing dissociation of LPS from mCD14. It is thought that this causes inhibition by preventing transfer of LPS from CD14 to TLR4 (Thompson *et al.*, 2003). It has also been discovered that the speed of transfer between LPS and sCD14 is dictated by the lipid composition of the membrane, with phosphatidylcholine being an essential requirement for LPS neutralisation (Wurfel and Wright, 1997).

1.2.4.2 Action of LBP against Gram Positive Bacteria and Fungi

While it has been well known for some time that LBP was an important mediator in G-ve bacterial infection, more recently it has been shown that it actually plays a broader role in immune defence mechanisms, acting in response to G+ve bacteria and fungi. Blairon *et al.* (2003), found that serum levels of LBP were increased in patients suffering sepsis with similar concentrations in those infected with fungi or with either type of bacteria.

It has been discovered that LBP is able to bind to an important cell wall component of G+ve bacteria, namely LTA, as noted by Fan *et al.* (1999) who found that the conformation of a G+ve bacterium, *Bacillus subtilis*, was altered after being exposed to serum, and this change was essential for LBP binding to occur. It has also been demonstrated that LBP can bind to LTA of other G+ve bacteria including *Streptococcus pneumoniae* and *Staphylococcus aureus* (Schroder *et al.*, 2003). Weber *et al.* (2003), also discovered that LBP plays an essential role in the immune response to G+ve pneumococci and was shown to bind to the pneumococcal cell wall (PCW) which is the major inflammatory component comprised of both LTA and peptidoglycan.

The LBP response against G+ve bacteria involves CD14 as well, as observed by Dziarski *et al.* (1998) and Schroder *et al.* (2003). From these findings it was suggested that LBP and CD14 may act as pattern recognition receptors and are able to detect a structural or conformational similarity between LPS and LTA. *In vitro* experiments helped to further define the pathway as a TLR2-dependent (not TLR4 nor MD-2) response to LTA in CHO cells (Schroder *et al.*, 2003).

These findings are particularly interesting to this study as other research on LBP in the mammary gland has looked at the increase during mastitic infection caused by LPS (Bannerman *et al.*, 2003), whereas in New Zealand, bovine mastitis is predominantly caused by *Streptococcus uberis*, a G+ve bacterium. The finding that LBP is up-regulated in infections caused by a range of different microbes suggests that it is involved in a more broad range role in the immune response than initially proposed. This also has significant implications regarding the applications of LBP in treatments and the monitoring of diseases.

1.2.5 EXPRESSION PATTERNS OF LBP

In most species studied to date, LBP is predominantly produced in the liver and is found as a plasma protein (Schumann *et al.*, 1990), while BPI is expressed in the secretory granules of leukocytes (Weiss *et al.*, 1978). Other members of the family appear to have limited spatial expression also, being found mostly in localised regions of high immune response factors. This includes parotid secretory protein (PSP), which has been identified in salivary glands (Owerbach and Hjorth, 1980), and LUNX/PLUNC which was discovered in airway epithelia (Bingle and Craven, 2002; Wheeler *et al.*, 2003). However, recent discoveries have shown that there is still much to be understood about the regulation and expression patterns of this family of proteins.

Extra-hepatic expression of other acute phase proteins has been identified in a number of tissues, some examples can be found in Molenaar *et al.* (1996), Urieli-Shoval *et al.* (1998), Vreugdenhil *et al.* (1999) and Jabs *et al.* (2003), but the regulation and roles of the locally expressed proteins are not well understood. It is surmised that locally expressed immune proteins may play a role specific to

the site of expression which may be different to the role they have at other sites. Alternatively the proteins could be expressed to give increased protection to the specific locations which are predominantly sites of entry for pathogens, such as epithelial mucosal membranes in airways.

There are few published cases of extra-hepatic LBP expression. Su *et al.* (1994), measured expression patterns of mRNA in rats by PCR and northern blot analysis after stimulation of the acute phase response and identified extra-hepatic expression of LBP mRNA in lung, kidney and heart which showed an increase upon stimulation of the immune response. This was compared to samples from spleen and stomach which showed no LBP expression occurring. LBP mRNA has also been detected in human intestinal epithelial cells *in vitro* using RT-PCR on cells that had been stimulated with glucocorticoids and cytokines (Vreugdenhil *et al.*, 1999). Other studies have detected LBP expression in respiratory epithelial cells from human lung tissue (Dentener *et al.*, 2000), and in alveolar macrophages in neonatal rats (Lee *et al.*, 2000). In the latter case it was interesting that the local production of LBP by alveolar macrophages decreased during maturation, with minimal expression occurring in adult tissues. It is suggested that the protein plays a compensatory role during a critical time of development, while the specific immune system is still maturing. This is also supported by results from a study of LBP levels in neonatal sepsis which found a greatly increased response compared to similar cases in adults (Berner *et al.*, 2002).

1.2.6 DETECTION OF LBP IN MILK

The mammary gland is a tissue rich in immunological factors especially during colostrogenesis, when important immune enhancing factors are transferred to the young neonate, and also at times of mammary stress such as during mastitis and involution. Some of these proteins are produced locally within the gland, while others are transported into the gland along with the macrophages, neutrophils and other factors essential to the immune response (Pfaffl *et al.*, 2003).

Many of these immune-related proteins, particularly the acute phase proteins, are current targets of research due to their large, rapid inducibility making them ideal markers for detection of onset of disease or infection, as well as being potential treatments for a variety of inflammatory diseases. The current search for proteins such as these in milk is driven by a number of factors including the potential applications of these proteins as medical markers and treatments, and also the degree of conservation between homologues suggests that the bovine forms may also be active in humans. Milk is an easy and convenient source, as it is already collected and processed in large quantities, so potentially large amounts of the proteins could be extracted and purified.

The detection of LBP in milk is a very recent discovery and no methods for purification from milk have been published to date. A study by Bannerman *et al.* (2003), monitored changes in levels of LBP in bovine whey milk during mastitic infection induced by LPS inoculation. This showed an increase in milk LBP during infection which paralleled the increase in plasma LBP, but was consistently much lower, ranging between 6 and 34 µg/ml compared with serum

which ranged from 38 to 137 $\mu\text{g/ml}$. LBP has also been detected in human whey, and was reported to be up to 1,000-fold lower than serum levels (Labeta *et al.*, 2000). The sCD14 receptor has been detected in bovine colostrum and milk at 100 to 1,000 times the concentration of that in serum (Filipp *et al.*, 2001). Labeta *et al.* (2000) also detected sCD14 in human breast milk, with concentrations 20 times higher than in serum. It was found that the level in milk was highest just after parturition with a gradual decline over the first three months, and a slight rise seen after six months.

The reason for the presence of LBP in milk is currently not well understood. During colostrogenesis many immune proteins are recruited into the gland to pass onto the young neonate. A similar process may also occur during times of infection or involution, with LBP being transported in from the circulation, along with other immune related factors, such as the white blood cells and lymphocytes, to boost defence in the gland. Alternatively, it may leak into the gland at times of infection or inflammation. Normally the barrier between blood and milk is strictly controlled by tight junctions, but during inflammation, infection or involution these junctions can become leaky, allowing exchange of plasma proteins and milk proteins (Stelwagen *et al.*, 1997). However both of these scenarios do not explain the presence of LBP in normal milk, which supports the other hypothesis: that the gland itself may be producing the protein. The data presented in this study suggest that the mammary gland may be involved in local expression of LBP, although further investigation is required to fully resolve this issue.

1.3 AIMS OF THIS THESIS

LBP plays an important role in the immune response against microbial toxins, with the ability to either stimulate the inflammatory response or aid in neutralising the toxin. The protein, or derivatives of it, has potential to be used as a therapeutic treatment in a number of different situations as it has been shown to be present at high levels in diseases caused by a range of different bacteria as well as fungi. The presence of LBP in bovine milk could be a convenient source to purify LBP in sufficient quantities for commercial production. The purpose of this study was to investigate the presence of LBP in the bovine mammary gland and in mammary secretions.

A temporal expression profile of LBP in the bovine mammary gland was determined using northern blotting, and *in situ* hybridisation analyses were used to detect if the gene was being expressed locally. An antibody was produced for western blot analysis which allowed detection of LBP protein in a range of mammary secretions and bovine serum samples and the amount of LBP present in these samples was quantified using a commercially available ELISA kit. Bio-activity assays were performed to confirm the LPS binding and immuno-stimulatory activity of the bovine LBP protein using either whey fractions containing the native protein, or a recombinantly expressed protein.

CHAPTER TWO MATERIALS AND METHODS

2.1 BACKGROUND EXPERIMENTS

The expressed sequence tag (EST) for bovine mammary LBP was discovered in a cDNA library derived from mastitic mammary tissue. The library was constructed using the expression vector ZAP express λ pBKCMV (Stratagene, California, USA) as a partnership between AgResearch (Ruakura, Hamilton, NZ) and Genesis Research & Development Corporation Limited (Auckland, New Zealand). The EST clone was transfected into *Escherichia coli* (*E. coli*) cells, from which the EST insert region was amplified by PCR using generic vector primers and the resulting product sequenced in one direction. The sequence of the EST showed homology to LBP and was selected for further analysis as a potential antimicrobial protein in milk.

2.2 FULL LENGTH SEQUENCING OF BOVINE MAMMARY LBP

In order for a more thorough sequence analysis to be performed, a 15 ml culture was grown from the glycerol stock of the initial culture. This was grown overnight at 37°C in Luria base (LB) broth (Invitrogen, California, USA) with 50 μ g/ml kanamycin. The cells were harvested by centrifuging at 2,000 x *g* for 10 minutes. The media was removed and the plasmid DNA was extracted from the cells using the Concert Rapid Midiprep system (Invitrogen) according to the manufacturers' instructions. The DNA was then precipitated with 0.3 M sodium acetate pH 5.2, and 800 μ l isopropanol. This was centrifuged at 20,000 x *g* for 30 minutes and the pellet washed in 1 ml 75% ethanol before resuspending in 50 μ l filter sterilised (f.s.) Millipore Q grade (MQ) water.

The purified DNA was analysed by spectrophotometry at an absorbance of $\lambda=260$ nm, indicating a nucleic acid concentration of 580 ng/ μ l. The presence and integrity of the insert was confirmed by restriction digest using the enzyme *Pst* I (Roche Applied Science, Basel, Switzerland), which cuts within the insert. The restriction digest was performed in a 10 μ l solution comprised of 1 μ g DNA, 2 μ l acetylated bovine serum albumin (BSA), 5 U enzyme, and 1X restriction enzyme buffer. The digest was incubated for one hour at 37°C and the products observed following agarose gel electrophoresis.

The purified plasmid was sent to the University of Waikato DNA sequencing facility (Hamilton, NZ) for sequencing using primers for both T7 and T3 DNA promoter sequences, as these sites flank the insertion region in the vector (Table 2.1). From these results, internal primers were produced (Invitrogen, Auckland, NZ), in order to obtain high quality full length sequence (Table 2.1), as the length of the clone is approximately 1700 bp.

Table 2.1: Primers for full length sequencing of bovine mammary LBP

T7	TAA TAC GAC TCA CTA TA
T3	AAT TAA CCC TCA CTA AAG GG
<u>Internal primers:</u>	
Forward: GAG CTA CAG CCT TAT CTT CAA ACT	
Reverse: TTG GGG TAA AAG TTG TTG AGA A	

The sequences were aligned with the initial contig sequence on the AgResearch EST database and edited by examining the resulting electropherograms. This final edited sequence was then used in DNA and conceptual translation protein alignments with other published sequences of LBP from different species.

2.3 NORTHERN BLOTTING

2.3.1 RNA ISOLATION AND PREPARATION

Northern blotting analysis was performed on a variety of mammary tissues at different stages of development, lactation and infection (Table 2.2). Liver samples were used as positive controls of LBP expression, also allowing a comparison between samples from healthy animals and from cows induced with mastitis. A sample of mRNA from somatic cells taken from mastitic milk was also included to indicate whether LBP was being recruited to the gland with these cells, as this would contain a large proportion of white blood cells that have been transported into the milk during infection as well as some mammary epithelial cells which have been sloughed off.

Table 2.2: RNA samples used for northern blot analysis

RNA was extracted from ground tissue samples using the Trizol method (Invitrogen) for northern blot analysis. The tissues were collected from cows at various stages of mammary development or infection. The infected tissues are from cows that had mastitic infections which had either been induced by inoculation of *Streptococcus uberis* or were opportunistic infections. Cows which had induced infections had been inoculated in one quarter, with the diagonally opposite quarter treated as the control. Of these cows, one became mastitic (high responding), while another resisted infection, remaining normal (non responding).

Bovine Tissue type	Description	Bacteriology
Normal mammary		
pregnant alveolar	85 days postpartum	
colostrum alveolar	day 1	
lactating alveolar	6 hours post partum	
involuting alveolar	72 hour post milking	
involuting alveolar	8 day post milking	
intestinal composite	stomach and intestine	
epithelial composite	skin, trachea, bladder, lung	
Opportunistic mammary infection		
control alveolar	uninfected cow	
subclinical alveolar	infected mastitic	<i>Corynebacterium</i> sp
chronic alveolar	infected mastitic	<i>Corynebacterium</i> sp
acute alveolar	infected mastitic	<i>Staphylococcus aureus</i>
control/subclinical cisternal	uninfected quarter	<i>Streptococcus uberis</i>
chronic cisternal	infected mastitic	<i>Staphylococcus</i> sp
acute cisternal	infected mastitic	<i>Staphylococcus aureus</i>
Induced mammary infection		<i>Streptococcus uberis</i>
control alveolar	uninfected quarter	high responding cow
mastitic alveolar	infected quarter	high responding cow
mastitic cisternal	infected quarter	high responding cow
control alveolar	uninfected quarter	non responding cow
mastitic alveolar	infected quarter	non responding cow
Other		
liver control	uninfected	
liver infected	mastitic	
milk cells	mastitic	

RNA isolation was performed according to the protocol for Trizol reagent (Invitrogen). RNA was isolated from various tissue samples which had previously been collected, frozen in liquid nitrogen and crushed to a fine powder (Table 2.2). These were stored at -80°C until required. For isolation of total RNA for northern blot analysis, approximately 0.12 g of ground tissue was weighed into a cold tube

and 1.5 ml of Trizol reagent added. The tissue was homogenized and centrifuged at 12,000 x *g* for 10 minutes at 4°C. The resulting supernatant was collected into a new tube and 0.3 ml of chloroform was added. This was then mixed and centrifuged at 400 x *g* for five minutes. The aqueous phase was transferred to a new tube and 0.7 ml of isopropanol was added. This was mixed and incubated at room temperature (RT) for 30 minutes to precipitate the RNA. After the incubation, the RNA was pelleted by centrifugation at 10,000 x *g* for 15 minutes and the supernatant was discarded. The resulting pellet was washed in 1 ml of 75% ethanol, then the ethanol was removed and the pellet was air-dried for 10 minutes. The RNA pellet was dissolved in 200 µl diethylpyrocarbonate (DEPC) treated MQ water and the concentration determined by spectrophotometry at $\lambda = 260$ nm. The integrity of the RNA was examined by electrophoresis on a 1.2% RNA agarose gel in 1X MOPS running buffer, in northern loading buffer containing ethidium bromide (EtBr) which was photographed under UV light.

2.3.2 PREPARATION OF LBP AND CD14 PROBES

2.3.2.1 Subcloning

Probes for northern blotting were prepared from purified plasmid DNA for LBP and CD14. The midiprep DNA described in Section 2.2 was used for LBP. For CD14, clones for two ESTs were received from Genesis Research & Development and were reconstituted in 5 µl f.s. MQ water. A transformation was done into DH5 α subcloning efficiency *E. coli* cells (Invitrogen) using 1 µl CD14 DNA and 25 µl cells. These were incubated on ice for 45 minutes, heat shocked at 42°C for 45 seconds and replaced on ice. The cells were plated onto LB agar plates with

50 µg/ml kanamycin, and incubated overnight at 37°C. Six of the resulting colonies were used to inoculate 5 ml LB broth containing 50 µg/ml kanamycin. These were grown overnight at 37°C, shaking at 300 rpm.

2.3.2.1.1 Alkaline Lysis Extraction of Plasmid DNA

The plasmid DNA was extracted by alkaline lysis (Sambrook and Russell, 2001). For this, the cells were harvested from the overnight culture by centrifuging at 3,000 x g for five minutes. The supernatant was removed and the pellet resuspended in 100 µl of chilled solution I (10 mM ethylenediaminetetraacetate (EDTA), 50 mM glucose, 25 mM Tris pH 8) and vortexed to ensure complete dispersion. The cells were lysed by adding 200 µl solution II (1% sodium dodecylsulfate (SDS), 0.2 N NaOH) and mixed by inversion before adding 150 µl chilled solution III (3 M K, 5 M acetate). The solution was then incubated on ice for five minutes, after which the cellular debris was removed by centrifugation at 20,000 x g for 10 minutes.

The DNA was extracted and purified by phenol chloroform extraction. This was achieved by transferring the supernatant to a new tube and adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen), vortexing, and separating the phases by centrifuging for five minutes at 20,000 x g. The aqueous phase was retained, an equal volume of chloroform added, and the phases separated by mixing and centrifuging as before. The aqueous phase was retained from this and the DNA precipitated by adding two volumes of 100% ethanol, mixing, and centrifuging at 20,000 x g for 30 minutes. The pellet was then washed in 1 ml of 80% ethanol and air-dried before finally resuspending in 50 µl T.1E

buffer (10 mM Tris pH 8, 0.1 mM EDTA). This buffer was used in preference to TE buffer (10 mM Tris pH 8, 1 mM EDTA) as it has a lower concentration of EDTA to minimise chelation of magnesium ions which are important in sequencing and restriction digests.

To ensure that no RNAses were present the DNA was further purified using a PCR purification column (Qiagen, California, USA) following the manufacturers instructions. The presence of the insert was confirmed by restriction digest with *Xho* I and *Eco*R I (Roche Applied Science), and verified by sequencing (University of Waikato DNA sequencing facility).

2.3.2.2 Restriction Digests

The coding region for the genes was excised from the plasmid by restriction digest. A 10 µl restriction digest reaction was performed for each probe which included approximately 5 µg purified plasmid DNA, 1 µl restriction enzyme buffer, 5 U of each enzyme and 4 mM spermidine, made up to the final volume with MQ water. CD14 inserts were cut with enzymes *Xho* I and *Eco*R I, and LBP was cut with *Xho* I and *Nde* I. The reaction was incubated at 37°C for one hour and 2 µl DNA loading dye was added to each sample, which was then analysed by agarose gel electrophoresis. The resulting insert band was cut out of the gel and the DNA extracted by freeze-squeezing. This is done by placing the gel slice in a folded piece of parafilm and freezing between two freezer packs. The band is then thawed under pressure between thumb and forefinger and the extracted liquid collected by a pipette (Dr Dave Musgrave, personal communication).

2.3.2.3 Radioactive Labelling of DNA Probes

Based on estimates from the band intensity on the gel of the restriction digest, 2 μ l (approximately 20 ng DNA) of the freeze-squeezed samples was used to make northern probes. This was added to 45.5 μ l TE buffer and denatured by boiling for five minutes. The solution was cooled on ice for five minutes and added to a tube of Rediprime II (Amersham Biosciences, Amersham, England) which contained a buffered solution of dATP, dGTP, and dTTP, Klenow enzyme and random primers. This was gently mixed and 2.5 μ l α^{32} P-labelled dCTP (3,000 Ci/mmol; Amersham Biosciences) was added before incubating for one hour at 37°C.

2.3.2.4 Probe Purification and Incorporation Check

The unincorporated nucleotides were removed from the probe by a Mini Quick spin DNA column (Roche Applied Science). This was prepared by flicking the tube to resuspend the sephadex matrix, removing the cap and snapping off the tip. The column was then placed in a 1.5 ml eppendorf tube and centrifuged at 1,000 x g for one minute to remove the eluted buffer. The column was transferred to a fresh tube and the probe added. The purified probe was eluted by centrifuging at 1,000 x g for four minutes and the incorporation of radioactivity measured.

To check the incorporation of the radioactive nucleotide, a sample of the probe was taken before and after purification and the incorporation measured on a scintillation counter. For this, samples of 1 μ l probe before and after purification were added to eppendorf tubes containing 1 ml BCS scintillation fluid (Amersham Biosciences). These were measured on a 1409 DSA liquid scintillation counter (PerkinElmer Massachusetts, USA) and the percentage of incorporation was

calculated from the resulting cpm values. The probes indicated 30% to 40% incorporation which is within the range required for an adequate signal. The probe was then denatured by adding 5 μ l of 3 M NaOH and incubating for five minutes at RT before adding 2.5 μ l 1 M Tris pH 7.2 and 5 μ l 3 M HCl.

2.3.3 NORTHERN BLOTTING

The RNA samples were prepared in northern loading buffer to give a final concentration of 5 μ g RNA per well. These were denatured at 65°C for 15 minutes, before running on a 1.2% RNA agarose gel in 1X MOPS buffer at approximately 120 V for one hour with an RNA standard ladder (Invitrogen).

The northern blot procedure was carried out according to the protocol in Sambrook and Russell (2001). Before blotting, the gel and nylon Hybond N+ membrane (Amersham Biosciences) were prepared by washing in 10X sodium citrate/chloride buffer (SSC) for approximately 30 minutes at RT. The blotting system (Figure 2.1) was prepared by placing the gel former upside down in a tray containing at least 400 ml 10X SSC. Two pieces of wet 3MM filter paper were placed across this and submerged in the buffer to form the wicks. The gel was placed on top of these, with the wells face-down, and the membrane was placed on the gel. Any air bubbles were removed by gently rolling a plastic pipette along the length of the gel, and gladwrap was wrapped around the sides to cover the gap between the membrane and the tray to prevent the buffer evaporating. Two pieces of wet 3MM paper were placed on top of the membrane followed by two pieces of dry filter paper and a stack of paper towels. These were held down with a piece of glass, and left overnight to blot.

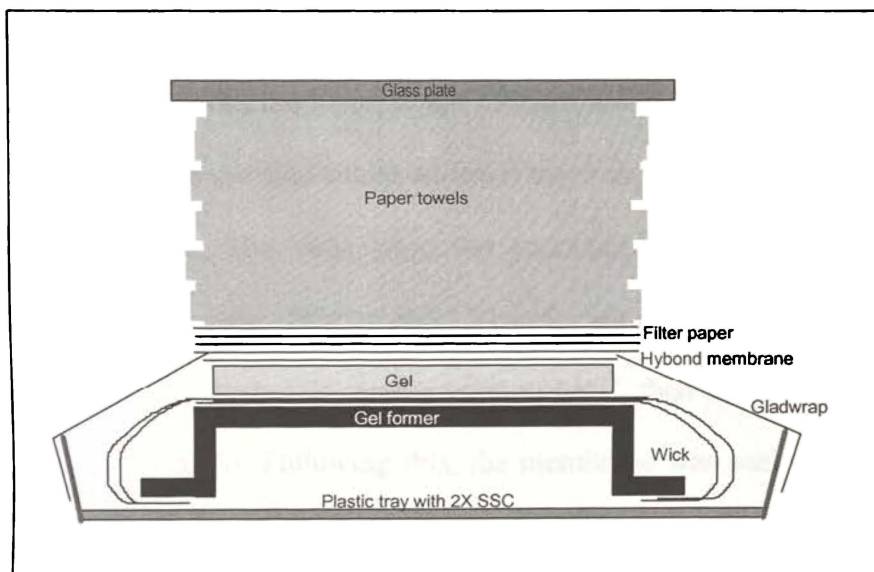


Figure 2.1: Northern blotting system

RNA samples were separated on an agarose gel and transferred onto a Hybond N+ membrane using the system shown.

After blotting, the membrane was rinsed in 2X SSC for two minutes and the RNA cross-linked to the membrane by irradiating for 1200 μ joules in a UV stratalinker 1800 (Stratagene). The membrane was then stained with methylene blue stain for 5 to 10 minutes and destained with MQ water. The membrane was dried prior to probing.

2.3.3.1 Prehybridisation

Before hybridising the membrane was placed in a hybaidd bottle (Thermohybaidd, Middlesex, UK) and incubated at 65°C for half an hour. The membrane was then equilibrated in 10 ml of warm Church and Gilbert hybridisation buffer (Church and Gilbert, 1984) at 65°C for one hour before hybridising with the probe.

2.3.3.2 Hybridisation

For hybridisation, the Church and Gilbert buffer was replaced with 10 ml fresh buffer and the purified probe added. This was then incubated at 65°C overnight with rotation. The next day, the membrane was washed to remove any unhybridised probe. This involved a brief wash in solution I (2X SSC, 0.1% SDS), followed by a further 20 minute wash at 65°C, then 20 minutes in solution II (1X SSC, 0.1% SDS). Following this, the membrane was sealed in a plastic bag and exposed to XAR-5 film (Eastman Kodak Company, New York, USA) overnight at -80°C. The resulting picture showed high background so more stringent washes were performed, first for 20 minutes at 65°C in solution III (0.5X SSC, 0.1% SDS), then for another 20 minutes in solution IV (0.1X SSC, 0.1% SDS). The membrane was again sealed and exposed to film overnight. This gave a much cleaner background so a second exposure was done for three days at -80°C.

2.4 *IN SITU* HYBRIDISATION

In situ hybridisation was performed on a small range of mastitic and healthy bovine mammary tissues (Table 2.3) to determine whether LBP mRNA is being expressed by cells in the gland or whether it is associated with white blood cells in the milk. Probes specific for α -lactalbumin were included as a comparison since this is a normal milk protein known to be expressed by the alveolar epithelial cells (Molenaar *et al.*, 1992). *In situ* hybridisation was performed according to the method of Ohlsson *et al.* (1989).

Table 2.3: Bovine mammary tissues used for *in situ* hybridisation analysis

Tissues from infected or control quarters were analysed. The tissue type, infection state and predominant bacterial species present in the samples are shown.

Infection state	Tissue	Bacteriology
Mastitic	Cisternal	<i>Staphylococcus</i>
Control	Cisternal	
Mastitic	Teat	<i>Corynebacterium</i>
Mastitic - subclinical	Cisternal / Alveolar Junction	<i>Staphylococcus</i>

2.4.1 PREPARATION OF SLIDES

The slides of tissue sections used for *in situ* hybridisation were from a stock which had been made previously. The preparation of these involved fresh tissue samples which were cut into thin strips of approximately 30 mm² and fixed overnight in 10% formalin. The tissues were then washed briefly in 70% ethanol and placed in Tissue Tek cassettes (Miles Australia Ltd., Victoria, Australia) for embedding. They were processed in an increasing ethanol gradient of 70%, 80% and 95%, and soaked in toluene before being embedded in paramat wax (BDH Laboratory Supplies, Dorset, England). The tissues were cut into approximately 7 µm sections and placed onto polysine coated microscope slides (Biolab Scientific, Auckland, NZ). They were then dried at 37°C to 50°C and stored at RT.

The paraffin was removed with a series of washes beginning with three 10 minute washes in xylene, followed by two washes of five minutes in bulk 100% ethanol and a five minute wash in 100% analar grade ethanol. The slides were dried before pre-treating to increase permeabilisation. For pre-treatment, the slides were washed for 10 minutes in 0.2 M HCl, followed by a 30 minute wash in 2X SSC and 15 minutes at 37°C in proteinase K solution (0.2 M Tris pH 7.2, 2 mM CaCl₂, 2 µg/ml proteinase K). They were then washed for five minutes in 100 ml of

triethanolamine buffer with 700 µl acetic anhydride, repeating the wash after adding a further 700 µl acetic anhydride. The slides were dehydrated with a series of five minute washes in 70% ethanol, 95% bulk ethanol and 95% analar grade ethanol. After drying, the sections were outlined with a PAP pen (Daido Sangyo Co. Ltd, Tokyo, Japan), to form a water-repellent barrier around each section which prevents the probe solutions mixing.

2.4.2 PREPARATION OF PROBES

2.4.2.1 α-lactalbumin Probes

Probes for α-lactalbumin were prepared from stock constructs that had been produced by Dr Phil L’Huillier (AgResearch, Ruakura).

2.4.2.2 LBP Probes

A construct of LBP in the pGEM-T Easy vector (Promega, Wisconsin, USA) (pT-LBP) was prepared by PCR amplification using primers designed for recombinant protein expression, which allowed the same construct to be used (Table 2.4).

Table 2.4: PCR primers for cloning of bovine mammary LBP into the pGEM-T Easy vector

LBP forward: CAT ATG AAT CCA AGA ATT TTC GGC ACG
LBP reverse: CTC GAG TCA AAC TCT CAG GTA CCG

2.4.2.2.1 PCR Reaction for Subcloning

PCR was performed using the original construct of LBP in the λ pBKCMV vector as template DNA. The reactions were performed in 15 μ l volumes containing 0.5 μ g template DNA, 10 μ M of each primer, 1.5 μ l PCR buffer with MgCl_2 , 1 μ M dNTPs, and 1 U Taq DNA polymerase. A negative control with no template DNA was also included. The PCR reaction had an initial denaturing step for five minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at the annealing temperature and two minutes at 72°C, with a final extension incubation at 72°C for 10 minutes. The extension times were longer than usual as it was recommended that this improves results with longer constructs (Dr Craig Smith, personal communication).

Initially a gradient PCR was performed with the annealing temperatures ranging between 50°C and 70°C. This showed the optimum temperature for both primer sets was between 50°C and 53°C. The PCR products were visualised on a 1% agarose gel with EtBr, under UV light. The resulting product bands were cut from the gel and the DNA obtained by freeze-squeezing.

2.4.2.2.2 Preparation of the pT-LBP Construct

The DNA was ligated into the pGEM-T Easy vector which utilises the A-overhangs created by Taq polymerase during the PCR reaction. The ligation reaction used 1 μ l DNA (PCR product), 1 μ l pGEM-T Easy vector, 5 μ l ligase buffer and 1 μ l ligase enzyme in a total volume of 10 μ l made up with MQ water. The reaction was incubated at RT for three hours. The pT-LBP construct was then transformed into DH5 α *E. coli* cells (Invitrogen) and grown on LB agar plates

with 100 µg/ml ampicillin. For this, the ligation reaction was added to 25 µl cells and this was incubated on ice for 20 minutes, heat shocked at 42°C for 45 seconds and replaced on ice for a further two minutes. The bacteria were then incubated in 0.9 ml LB broth for one hour, while shaking at 37°C. From this, 100 µl was plated onto an LB agar plate with ampicillin and grown overnight at 37°C, shaking at 300 rpm. A colony was chosen from the plate and grown in 5 ml LB media overnight. The plasmid DNA was then extracted by alkaline lysis as described in Section 2.3.2.1.1, and resuspended in 50 µl T.1E buffer.

2.4.2.3 Restriction Digests

The α-lactalbumin and pT-LBP constructs were cut with single enzymes to allow transcription with either T7 or SP6 RNA polymerase, creating sense and anti-sense probes. It is important for this that the restriction enzymes cut only once and do not create a 3' overhang. The plasmids and enzymes used are shown in Table 2.5.

Table 2.5: Preparation of probes for *in situ* hybridisation

Constructs of LBP and α-lactalbumin were used as probes for *in situ* hybridisation on healthy and mastitic bovine mammary tissues. Details of the probes are shown including the restriction enzyme used to linearise the construct and the polymerase used to create a sense or anti-sense probe.

Probe	Plasmid	Enzyme	Polymerase	Orientation
LBP	pGEMT-easy	<i>Sal</i> I	T7	sense
LBP	pGEMT-easy	<i>Nco</i> I	SP6	anti-sense
α-lactalbumin	pRc/CMV	<i>Xba</i> I	T7	sense
α-lactalbumin	pTF-29/pTF7-5	<i>Eco</i> R V	T7	anti-sense

Digests were performed in 20 µl reactions with 10 µg DNA, 2 µl restriction enzyme buffer, 2 µl enzyme, made up to the final volume with MQ water. A

reaction with 2 µg DNA and both enzymes was also performed as a control. The reactions were incubated at 37°C for one hour and the DNA then precipitated by adding 10 µl 7.5 M ammonium acetate and 66 µl 95% ethanol. This was left at RT for five minutes before the DNA was collected by centrifugation at 15,000 x g for 10 minutes. The pellet was washed twice in 70% ethanol, then air-dried and resuspended in 25 µl T.1E buffer. The success of the digests was visualised by running 2 µl of the precipitated DNA in DNA loading dye on a 1% agarose gel. This was run in 1X tetraacetythylenediamine (TAE) buffer with EtBr to allow the DNA to be visualised on the gel under UV light.

2.4.2.4 Radioactive Labelling of DNA Probes

The probes were prepared by adding approximately 3 µg DNA template, 4 µl transcription buffer, 0.01 M dithiothreitol (DTT), 0.8 µl ribonuclease inhibitor (RNasin; Promega), 4 µl rAGC, 2.5 µl $\alpha^{35}\text{S}$ -labelled UTP (1,000 Ci/mmol) (Amersham Biosciences), 0.5 µl SP6 or T7 RNA polymerase (Epicentre, Wisconsin, USA) and 3.2 µl DEPC treated water. The solution was mixed and spun briefly before incubating at 37°C for one hour.

2.4.2.5 Incorporation Check

The incorporation of radioactivity was confirmed initially by dotting 5 µl of probe onto two pieces of filter paper. One piece was then washed twice in trichloroacetic acid (TCA) for five minutes each. Both papers were dried and checked with a Geiger counter. The total incorporation count was measured on a scintillation counter using 0.5 µl probe in 400 µl scintillation fluid. This gave results of 240,000 and 410,000 cpm for LBP anti-sense and sense probes, respectively. The

corresponding figures for α -lactalbumin were 1,310,000 and 770,000 cpm. The probes were incubated briefly on ice before precipitating by adding 50 μ g yeast tRNA, 2.05 μ l 3 M sodium acetate and 50 μ l 95% ethanol. The solution was mixed and centrifuged at 18,000 $\times g$ for 20 minutes. The resulting pellet was washed twice in 70% ethanol, and resuspended in 20 μ l formamide/10 mM DTT (Chomczynski, 1992).

2.4.2.6 Integrity Check

The integrity of the probes was visualised by doing a mini northern blot (Molenaar and Wilkins, 1991). The samples for this were prepared by adding 0.5 μ l probe to 5 μ l sequenase buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue (BPB), 0.05% xylene cyanol). This was loaded onto a 1.5% RNA agarose gel and run in 1X MOPS running buffer at 25 to 50 mA, 100 V for approximately 1.5 hours. A mini blotting system was prepared by placing the gel upside down on a piece of plastic then laying a piece of Hybond N+ membrane on top along with two pieces of filter paper and approximately 5 cm of paper towels. These were held down by a light weight and were left overnight to blot. The membrane was then dried and exposed to film for 20 minutes.

2.4.3 HYBRIDISATION

Ultraspeed solution (Ambion, Texas, USA) was melted at 65°C and prepared by adding 1 M DTT at a ratio of 1:10. The hybridisation solution consisted of a volume of probe to give approximately 30,000 cpm per section and Ultraspeed solution to a final volume of 30 μ l per section. The solution was spread onto each section using a bent pipette tip and the slides were placed in a rack in a humidity

chamber containing a solution of 2X SSC in 50% formamide. This was incubated at 55°C overnight.

2.4.4 SLIDE WASHING

The slides were washed twice in a 1:1 solution of 2X SSC/50% formamide at 55°C, first for five minutes, then for 20 minutes. They were then washed four times in wash solution I (0.2X SSC, 20 mM β -mercaptoethanol (BME)) for five minutes each, followed by five minutes in wash solution II (2X SSC, 20 mM BME). The slides were washed again for 30 minutes in wash solution II to which 100 μ g/ml of RNase A and 1 μ g/ml of T1 RNase was added. This was followed by another 10 minutes in wash solution II and four 15 minute washes in wash solution I. The slides were then dehydrated as before, with five minute incubations in 70% ethanol, 95% bulk ethanol and 95% analar ethanol. After drying, the slides were exposed to film overnight. Because the initial exposure showed high backgrounds, they were washed again in 2X SSC/50% formamide at 50°C overnight, followed by four brief washes in wash solution I at RT and five minutes in wash solution II. The slides were dehydrated, re-dried and exposed to film for four days. The results from this showed a cleaner background, with a potential signal on one of the slides.

2.4.5 EMULSION

Due to the light sensitivity of the emulsion solution the following procedure was carried out using a Kodak orange safelight with a 10-watt bulb. The LM-1 emulsion solution (Amersham Biosciences) was melted in a 42°C waterbath in the dark. Approximately 200 μ l of emulsion solution was spread onto each slide using

a plastic pipette tip. Two blank slides were also prepared as controls. After spreading the emulsion solution, the slides were stood vertically in a dark box and left to dry for two hours. The slides were then placed in a light-proof box containing desiccant. The box was wrapped in tinfoil and gladwrap to avoid both moisture and light and stored at 4°C for two months before developing.

2.4.6 DEVELOPING

Due to the light sensitivity the following procedure was also performed using the orange safelight. The developing reagents were tested using a blank slide which had been exposed to light, while the signal intensity was observed by developing a test slide. Once these were found to be satisfactory the remaining slides were developed by gently washing in D19 developer (Eastman Kodak Company) for four minutes, followed by 30 seconds in 0.1% acetic acid. They were then fixed with Ilford film fixer solution (Ilford Imaging, Cheshire, England) for five minutes and washed in running tap water for one hour, with a quick rinse in distilled water before air-drying.

2.4.7 STAINING

The slides were placed in distilled water for five minutes before staining in progressive Gill's haematoxylin for three minutes. Excess stain was rinsed off in a running waterbath for five minutes before counter-staining in eosin for approximately 20 seconds. After another five minute rinse in running water the slides were dehydrated by quick rinses in 70% ethanol, 100% bulk ethanol, and twice in xylene. Coverslips were mounted on the slides using DPX mountant (BDH Laboratory Supplies) and left to dry overnight. The slides were then

examined and photographed using an Olympus BH2 microscope (Olympus Optical Company, Inc., Tokyo, Japan) and a ProgRes C14 digital camera (Jenoptik, Jena, Germany).

2.5 WESTERN BLOTTING

2.5.1 SAMPLE PREPARATION

A range of skim and whey milk samples were used from different stages of mammary secretion (Table 2.6). The skim fraction was obtained by centrifuging chilled whole milk at 1,500 x g for 15 to 20 minutes at 4°C and collecting the skim fraction by piercing the tube above the cell pellet with a soldering iron. Whey was prepared from the skim milk by ultracentrifuging at 100,000 x g for one hour at 4°C and collecting the middle layer. Serum samples were also obtained which had been collected from whole blood samples of healthy and infected cows. All of the samples were stored in aliquots at -20°C.

Table 2.6: Milk samples analysed for LBP by western blot analysis

A range of whey and skim milk samples were collected at different stages of lactation, as described in the table. These were analysed for the presence of LBP by western blotting.

Whey milk samples	Description
Human	mid-lactation
Bovine normal	control quarter from a mastitic cow
Bovine colostrum	day one secretion
Bovine mastitic	<i>Streptococcus uberis</i> induced infection
Bovine 8 day involution	8 day post milking (during mid-lactation)
Bovine late lactation involution	3 day post drying off (late lactation)
Skim milk samples	
Bovine 72 hour involution	72 hour post milking (during mid-lactation)
Bovine 8 day involution	8 day post milking (during mid-lactation)
Bovine subclinical mastitic	opportunistic infection

2.5.1.1 Protein Concentration Assay

A Bradford assay was performed on the different milk and serum samples (Table 2.6), to determine the total protein concentration based on a standard curve of BSA. Milk and serum samples were prepared by diluting at 1:100 or 1:1,000 from which 10 μ l was added to 0.9 ml Bradford reagent (Sigma, Missouri, USA). The solutions were incubated at RT for 10 minutes, and protein concentration was determined by spectrophotometry at an absorbance of $\lambda = 595$ nm. Based on the concentrations from this, the samples were diluted to 1 μ g/ μ l in 3X Laemmli buffer and stored at -20°C.

2.5.2 WESTERN BLOT ANALYSIS

The presence of LBP in milk and serum samples was detected by western blot analysis. Whey and skim milk samples were prepared in 3X Laemmli buffer and denatured by boiling for five minutes. Approximately 5 to 10 μ g of protein was loaded per well, and the samples electrophoresed on an SDS-PAGE gel using the Invitrogen NuPAGE western blotting system. The samples were run on 10% Bis-Tris gels in NuPAGE MOPS running buffer at 200 V for approximately 30 minutes. A prestained molecular weight protein marker was run on each gel, either Benchmark protein ladder or SeeBlue Plus2, both of which were purchased from Invitrogen. The gel was transferred onto a BioTrace NT 0.45 μ m nitrocellulose transfer membrane (Pall Gelman Sciences, Michigan, USA) at 30 V for one hour. The membrane was then stained with ponceau S and destained with MQ water.

Western blot analysis was performed by incubating the membrane in blocking solution (1% polyvinylpyrrolidone (PVP), 0.1% BSA) for at least two hours at RT, or at 4°C overnight. It was then incubated in primary antibody diluted in 1X Tris buffered saline (TBS)-IGEPAL, pH 7.6 for two hours at RT. The membrane was washed three times in 1X TBS-IGEPAL for at least five minutes each before incubating in the diluted secondary peroxidase (HRP)-conjugate for at least one hour. After a further three washes it was covered in enhanced-chemiluminescent (ECL) solution for detection by luminescence on XAR-5 film.

2.5.3 STRIPPING WESTERN BLOTS

In some cases it was necessary to reuse a western blot for detection using a different probe. For this, membranes were stripped by submerging in stripping buffer (50 mM Tris, 2% SDS pH 7.6, 0.7% BME), at 50°C for 30 minutes. They were then washed three times in 1X TBS-IGEPAL for 15 to 30 minutes per wash. The membrane could then be re-blocked and probed.

2.5.4 ANTIBODY DETECTION

Initially a goat anti-human LBP antibody made against an N-terminal peptide of the human LBP sequence was purchased from Santa Cruz Biotechnologies (California, USA). Due to the homology of the bovine and human sequences it was thought that the antibody would be able to cross react with the bovine protein and be used to detect the presence of LBP in various milk samples. The antibody was used to probe western blots at a dilution of 1:5,000. This was detected by a HRP-labelled rabbit anti-goat IgG antibody (Dako, California, USA) at a dilution of 1:100,000. However, even at this high dilution there was still non-specific

binding, and the primary antibody gave highly variable results. It is possible that the goat IgG is similar to the bovine and cross-reactivity was the cause of the non-specific binding. Because of the difficulties in optimising this system, antibodies specific to the bovine sequence were developed in rabbits.

2.6 PRODUCTION OF AN ANTI-LBP ANTIBODY

2.6.1 PEPTIDE DESIGN

The predicted translation of the bovine mammary LBP DNA sequence was used in protein alignments with published protein sequences for human, rabbit, rat and mouse. Based on this a 15 amino acid (aa) peptide (LBP-C) was designed which incorporated a conserved region on the C-terminus, and should result in the production of an antibody that would cross react with LBP from other species (Table 2.7). Based on the BPI crystal structure, this region was found to lie in the linker between the two main arms of the boomerang. This peptide was synthesized by Auspep (Victoria, Australia).

Table 2.7: Alignment of the C-terminal peptide used for antibody production

Identical residues are shown in red and conserved residues which match the majority of the other sequences are shown in blue. The sequence from this study is named bLBP and is shown in bold. The NCBI accession numbers for the other proteins shown are as follows; Human LBP: NP_004130, Mouse LBP: NP_032515, Rabbit LBP: B35843, Rat LBP: NP_058904.

bLBP	Q I H K D F L F L G T N V R Y
Human LBP	Q I H K D F L F L G A N V Q Y
Mouse LBP	Q I R K D F L Y L G A N V Q Y
Rabbit LBP	Q T H E N F L L V G A N I Q Y
Rat LBP	Q I H K N F L Y L G A N V Q Y

However, due to a high degree of hydrophobicity, the peptide was insoluble in a number of solvents. Despite this, antibody production with the peptide was attempted but unsuccessful.

Due to the insolubility of the first peptide and the uncertainty of the outcome, a second peptide (LBP-N) was designed which encompassed a conserved region on the N-terminus (Table 2.8). Based on the BPI 3D structure it was in a nonstructured region at the end of the N-terminal arm. This suggested that the antibody would be more successful as external, non-structured regions are more easily accessible for antibody binding.

Table 2.8: Alignment of the N-terminal peptide used for antibody production

Identical residues are shown in red and conserved residues which match the majority of the other sequences are shown in blue. The sequence from this study is in bold and is named bLBP. The NCBI accession numbers for the other proteins shown are as follows; Human LBP: NP_004130, Mouse LBP: NP_032515, Rabbit LBP: B35843, Rat LBP: NP_058904.

bLBP	P P D S S I R L N T K S F R A
Human LBP	P P D S N I R L T T K S F R P
Mouse LBP	P H D S G I R L N T K A F R P
Rabbit LBP	P A D L N I R R T T K S F R P
Rat LBP	P P D S N I R L N T K A F R P

A cysteine residue was added to the N-terminus to allow for coupling between the cysteine and the free amino group on the carrier protein. Because of this it was important that there were no other cysteines present in the peptide, to avoid the formation of disulfide bridges.

2.6.2 PEPTIDE CONJUGATION

The carrier proteins were activated by the linker reagent 3-maleimidobenzoic acid N-hydroxy-succinimide ester (MBS) (Sigma) using a method based on Harlow and Lane (1988). The peptide was conjugated to keyhole limpet hemocyanin (KLH), which is a common carrier protein that is large and has low immunogenicity. These two characteristics are important as they ensure that the peptide is retained in the animal long enough to elicit an antibody response, while also ensuring that the majority of the antibodies produced are against the peptide and not the carrier protein. The peptide was also conjugated to ovalbumin so that it could be detected on a gel by western blot analysis to test the antibody's reactivity. The conjugation process involved two steps; the activation of KLH and ovalbumin with MBS, followed by conjugation of these proteins to the peptide.

2.6.2.1 Activation of Carrier Proteins

Stock solutions of KLH (Sigma) and ovalbumin (Sigma) were prepared at 10 mg/ml concentrations in 1X phosphate buffered saline (PBS). MBS was prepared by dissolving 5 mg in 0.2 ml dimethylfluoride. For the activation, 10 μ l of MBS was added dropwise to 150 μ l KLH, with frequent mixing. This was then incubated on a rotator at RT for 30 minutes. The same procedure was followed for ovalbumin activation using a half reaction. The solutions were run through 10 ml G10 Sephadex columns (Amersham Biosciences) which had been prepared by washing with 1X PBS. The activated proteins were washed through the column with 1X PBS and the eluant was collected in 1 ml fractions. The protein content of

each fraction was tested by a Bradford assay and the fractions which contained the most protein were pooled.

2.6.2.2 Conjugation of Carrier Proteins to the Peptide

The peptide was dissolved in 1X PBS to give a final concentration of 10 mM in a volume of 300 μ l. This solution was added to the activated ovalbumin and KLH, with 150 μ l added to KLH and 75 μ l added to the ovalbumin. This was then rotated at RT for three hours and dialysed overnight at 4°C against 2 L of 1X PBS, 1% phenylmethylsulfonyl fluoride (PMSF). The remaining dialysate was then tested for protein content with a Bradford assay.

2.6.3 INOCULATION

The protein aliquots were used to inoculate two rabbits. The inoculations were performed by Ric Broadhurst (AgResearch, Ruakura). The initial inoculation mixture included 200 μ g protein and Freund's complete adjuvant at a ratio of 1:1. The adjuvant is a water-in-oil emulsion which contains killed (*Mycobacterium tuberculosis*). This acts to incite the immune response and prolong the life of the antigen. Subsequent booster shots were given after four weeks and then at intervals of 2 to 3 weeks using a 1:1 ratio of 100 μ g of protein in Freund's incomplete adjuvant, which does not contain the killed bacteria.

Approximately one week after the second booster shot a test bleed was taken and the full rabbit serum was used to probe a preliminary western blot to observe the estimated specificity of the antibody by comparing the immune and pre-immune serums. The blood was collected from the animals approximately a week later,

and left to clot at RT. The serum was collected, pooled and centrifuged at 4,000 x g for five minutes to remove any remaining red blood cells and debris. It was then aliquoted into 10 ml fractions and stored at -80°C.

2.6.4 IgG PURIFICATION OF ANTI-SERA

A protein A column was used to purify IgG from the serum using a method based on Harlow and Lane (1988). First an ammonium sulfate precipitation was performed by diluting an aliquot of serum 1:1 with 100 mM Tris pH 8. The solution was then placed on ice and 0.52 g ammonium sulfate was added slowly, while mixing to avoid high local concentrations of salt. Once mixed, the solution was incubated on ice for 30 minutes. The protein was removed by centrifuging at 1,000 x g for five minutes at 4°C and the supernatant collected. The supernatant was then made up to 50% ammonium sulfate by adding a further 5.3 g slowly, with mixing as before. This was again incubated on ice for 30 minutes and centrifuged. The pellet was resolubilised in 10 ml 100 mM Tris pH 8, and loaded onto the protein A column which was regenerated by washing with approximately 40 ml of 100 mM Tris pH 8.

The IgG was eluted with 10 ml 100 mM glycine pH 4, followed by 10 ml 100 mM glycine pH 3. This was collected in fractions of 0.5 ml into tubes containing 100 µl 1 M Tris pH 8. These were immediately mixed and stored on ice. The column was then washed through with 20% ethanol and stored at 4°C. The protein content of each eluted fraction was measured by a Bradford assay and those containing the most protein were pooled. These were dialysed overnight at 4°C against 2 L of a solution containing 1X TBS, 1% PMSF. The recovered IgG from

dialysis was again measured by a Bradford assay and stored in aliquots at -80°C, with a working aliquot at 4°C.

The purified antibody was characterised using a range of concentrations in western blot analysis in order to determine the optimal concentration. These were used in conjunction with a goat anti-rabbit IgG-HRP antibody (Sigma) at a dilution of 1:10,000.

2.7 IMMUNOPRECIPITATION WITH THE LBP-N ANTIBODY

Immunoprecipitation was performed to determine if the antibody is able to bind the LBP protein in its native state. This was performed using a milk sample of late lactation involuted whey secretion, following the protocol described in Harlow and Lane (1988). This method involved first removing most of the other IgG proteins in milk with protein A agarose beads, then adding the LBP-N antibody to bind to any LBP present in the whey sample. More protein A agarose beads were then added to bind to the antibody, and the bound LBP was released by adding 3X Laemmli buffer.

All the solutions used contained a protease inhibitor cocktail (Sigma) to prevent degradation of the protein. A slurry of protein A agarose (Sigma) was prepared by washing the agarose three times in RIPA buffer (1% NP40 (Sigma), 50 mM Tris pH 7.5, 50 mM NaF, 1 mM EDTA) and resuspending in a 50% slurry containing approximately 0.5 ml agarose. The milk solution contained 50 µl involuted whey, made up to 1 ml in RIPA buffer with 1X protease inhibitor cocktail (Sigma). This solution was mixed for 30 minutes at 4°C with 10 µl of protein A slurry. The

agarose beads were pelleted by centrifuging at 400 x g for one minute and the supernatant transferred to a fresh tube. To this, 180 µg of LBP-N antibody was added and the solution mixed overnight by rotation at 4°C. Following this, 15 µl of freshly washed protein A agarose slurry was added to the tube and this was mixed by rotation at 4°C for two hours. The beads were then pelleted by centrifuging and the supernatant removed. The beads were then washed twice in 1 ml RIPA buffer and three times in 1X PBS before the supernatant was removed and the beads resuspended in 20 µl 3X Laemmli buffer. This was then incubated in a boiling waterbath for three minutes and pelleted. The supernatant was collected and loaded on an SDS-PAGE gel for western blotting analysis.

2.7.1 BIOTINYLATION OF THE LBP-N ANTIBODY

The LBP-N antibody was biotinylated for detection of the immunoprecipitation. This allowed a direct detection of LBP using the biotin-LBP-N antibody and a streptavidin-HRP conjugate (Biogenex, California, USA). Although it is possible to use the unbiotinylated antibody directly, problems may arise with this as the detection would then be with the goat anti-rabbit IgG conjugate which can also detect other IgG from the milk that have bound to the protein A agarose, giving a less specific result.

Biotinylation was performed using EZ-link NHS-LC-Biotin (Pierce, Illinois, USA). The biotin was made up to 1 mg/ml in dimethyl sulfoxide (DMSO), (Sigma) and a 1 ml solution of 1 mg/ml antibody was made in coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.4) and cooled on ice. While on ice, 40 µl of biotin solution was added to the antibody and this was left to incubate on ice for

two hours. Any unreacted biotin was then removed using Vivaspin columns (Vivascience, Hannover, Germany) by spinning down 0.5 ml solution at a time at 1,000 x g for 30 minutes each. This was followed by three washes of 0.05 M phosphate buffer (0.05 M NaH₂PO₄, 0.05 M Na₂HPO₄, pH 7.1), before finally resuspending the remaining solution in 0.5 ml phosphate buffer. The antibody was stored in aliquots at -80°C, with a working stock at 4°C. It was characterised by western blot analysis and found to give an optimal signal at a dilution of 1:1,000.

2.8 ELISA

An ELISA kit for LBP detection (Hycult Biotechnology (HBT), Uden, The Netherlands) was used to determine the concentration of LBP in a range of milk and serum samples (Table 2.9). The ELISA used an antibody which was cross-reactive for a number of species, including bovine. It was intended for use as a measure of LBP concentrations in serum samples although a recent study had also used it to determine LBP concentrations in whey milk (Bannerman *et al.*, 2003). The kit was based on the sandwich ELISA method using a cross-reactive human antibody to LBP for initial sample binding, followed by binding of biotinylated LPS. This was then detected with a streptavidin-HRP conjugate. The ELISA was performed following the instructions provided by the manufacturer. The final step involved detection by adding tetramethylbenzidine (TMB) solution. The TMB solution provides a colour reaction allowing the amount of LBP present to be determined. The plate was wrapped in tinfoil to prevent light interfering with the reaction and the colour allowed to develop for 15 to 30 minutes. The reaction was stopped by adding 100 µl 2 M citric acid and the plate was read on a ELx800 plate reader (BioTek Inc., Vermont, USA) at an absorbance of $\lambda = 450$ nm. All of

the samples were tested in duplicate, and based on the resulting (optical density) OD values the averages were calculated and a standard curve was plotted of absorbance against concentration. The resulting regression equation was used to calculate sample concentrations in ng/well. This value was then used to determine the total concentration in $\mu\text{g/ml}$ by taking into account the dilution factor used.

Initially, the dilutions suggested in the manufacturer's guide were followed, with serum samples being measured at a dilution of 1:100, and milk samples tested in a 10-fold dilution series ranging from 1 to 1:1,000. However, it was found that the detection system was very sensitive and most of these results were outside of the standard concentration range. A number of dilutions of the different samples were tested in order to get consistent results and over the course of the experiments the optimal dilution for the various samples was determined (Table 2.9).

Table 2.9: Bovine milk and serum samples analysed by ELISA

The samples were tested using an ELISA kit from HBT, and were within the standard curve at the range of dilutions shown in the table.

Whey	Dilution range
Normal	1:100 - 1:800
Colostrum	1:500 - 1:1000
Mastitic	1:2000 - 1:8000
Late lactation involution	1:1000 - 1:4000
Serum	
Normal	1:1000 - 1:3000
Toxic infection	1:1000 - 1:3000

Problems were encountered with the standard curve, as the highest concentration often gave a signal that was too high and could not be included, therefore decreasing the measurable range considerably. In some experiments the standard

concentrations were halved to try to get a more linear standard curve, but often the top standard was still out of range.

2.8.1 SPIKE RECOVERY

Serial dilutions of normal and mastitic whey were tested to investigate any potential interference that may be occurring. Two methods of spike recovery were also used to test for linearity and interference. The first method used the standard human LBP, provided with the ELISA kit, to add 20 ng of LBP into serially diluted samples of mastitic whey. The degree of linearity and the observed concentration difference between spiked and unspiked samples would give an indication of interference occurring in detection of LBP in whey samples. The results from this showed reasonable linearity but replication of the duplicates was poor. Because there were difficulties getting a linear curve with mastitic whey a different method was also tested.

Any interference occurring would be caused by other factors in the milk preventing LBP binding to the plate, either by sequestering LBP or competitively binding to LPS. This could not be accurately observed from the first method of spike recovery as the additional LBP was added from a purified buffered solution, so how this interacted with the LBP and other factors present in the mastitic whey sample is not known. In order to have a more accurate measure of interference it was decided to use mastitic whey samples to spike normal whey. Having milk as a 'base' in both solutions ensures that there should not be any interaction problems with the spiked LBP and LBP already present in the sample. Therefore this method should highlight any interference being caused by other milk factors.

Normal whey gave consistent results within each plate so a dilution series of this could be expected to give a linear curve. This series was spiked with approximately 10 ng LBP, which was estimated from previous concentration results for mastitic whey.

2.9 RECOMBINANT PROTEIN EXPRESSION

In order to obtain pure LBP protein, which could be used in bio-activity assays, recombinant protein expression was performed in both bacterial and mammalian cells. Although LBP is a glycoprotein it is possible that the glycans may not be required for activity so bacterial recombinant expression was attempted since it is also easier to perform and scale up. The protein was expressed in *E. coli* cells using the pET system (Novagen, Wisconsin, USA). However, in order to produce a protein which was more similar to the native form, recombinant expression was also performed in mammalian cells using the Gateway expression system (Invitrogen).

2.9.1 RECOMBINANT PROTEIN EXPRESSION IN BACTERIA

2.9.1.1 PCR Reaction for Subcloning of LBP

Primers were designed for the PCR amplification of bovine mammary LBP DNA which incorporated restriction enzyme sites at either end of the coding sequence to ensure the insert is ligated in the correct orientation. Two reverse primers were designed to code for different constructs, one coding for the native protein sequence alone and the other allowing the addition of six histidine residues on the C-terminal end of the protein as encoded in the pET-23b expression vector (Table 2.10).

Table 2.10: PCR primers designed for cloning of bovine mammary LBP into the pET-23b expression vector

The no stop primer allows the addition of a six histidine residue tag on the end of the construct, whereas the stop primer codes for the protein only. The stop codon is underlined. The primers also encode a restriction enzyme sequence to allow the construct to be excised from the vector. The enzyme cleavage sequence is in bold and the enzyme coded for is shown in brackets.

LBP forward (<i>Nde</i> I):	CAT ATG AAT CCA AGA ATT TTC GGC ACG
LBP reverse (<i>Xho</i> I) No stop:	CTC GAG AAC TCT CAG GTA CCG AAC
LBP reverse (<i>Xho</i> I) Stop:	CTC GAG <u>TCA</u> AAC TCT CAG GTA CCG

PCR was performed using these primers and the resulting PCR products were then ligated into the pGEM-T Easy transition vector to produce the pT-LBP construct, as described in Sections 2.4.2.1 and 2.4.2.2.

2.9.1.2 Preparation of the pET-LBP Construct

The insert was cut out of the plasmid using the restriction enzymes *Xho* I and *Nde* I whose cleavage sites are encoded by the PCR primers. The insert was then ligated into the expression vector, pET-23b, which had been cut with the same enzymes. The construct (pET-LBP) was first cloned into DH5α *E. coli* cells and grown on an LB agar plate with 100 µg/ml ampicillin. Three of the resulting colonies were used to inoculate 5 ml cultures and the plasmid extracted from these by the miniboil extraction method (Sambrook and Russell, 2001). For this, the cells were harvested from the overnight culture by centrifuging at 3,000 x g for five minutes and the pellet resuspended in 700 µl STET buffer (0.1 M NaCl, 10 mM Tris pH 8, 1 mM EDTA pH 8, 5% Triton X-100) with lysozyme to a final concentration of 0.6 µg/µl. This was incubated in a boiling waterbath for

70 seconds and pelleted at 15,000 x *g* for 10 minutes. The pellet was discarded and 750 μ l isopropanol added to the supernatant. The DNA was precipitated by centrifuging at 15,000 x *g* for 15 minutes. The resulting pellet was washed in 80% ethanol and air-dried before being resuspended in 50 μ l T.1E buffer.

The presence of the insert was confirmed by restriction digest with *Xho* I and *Nde* I, with approximately 5 μ g DNA, 5 U of each enzyme, 1 μ l restriction buffer and 4 mM spermidine in a 10 μ l reaction volume. This was incubated at 37°C for one hour and the result observed on a 1% agarose gel with EtBr.

2.9.1.3 Induction of Protein Expression

The constructs that tested positive for the presence of the insert were then transformed into BL21 salt-inducible (SI) *E. coli* cells (Invitrogen), which allow protein expression to be induced by the addition of salt. BL21-SI cells contain the *proU* promoter and T7 RNA polymerase genes. Expression of these genes is induced upon addition of NaCl into the culture. This system was used as it has a tighter control of expression compared to the usual isopropylthio- β -D-galactoside (IPTG) induction, which can be leaky. This is important when producing proteins which could be toxic to the bacterium.

The cells were grown on LB agarose which contains no salt (LBON) and had been inoculated with 100 μ g/ml ampicillin. Colonies from this were used to inoculate 5 ml cultures of LBON media and were grown overnight at 30°C. Once again, the presence of the insert was confirmed by a miniboil plasmid extraction (Section 2.9.1.2) and the positive cultures used to inoculate 50 ml LBON media containing

100 µg/ml ampicillin. These were grown at 30°C until the OD at $\lambda = 600$ nm was approximately 0.5. Protein expression was induced by adding NaCl to a final concentration of 0.3 M, and allowed to continue for three hours. The cells were then harvested by centrifuging at 3,000 x *g* for five minutes.

2.9.1.4 Mini-extractions

To confirm the presence of the protein, 1 ml aliquots were taken from the culture before and after the induction. The cells were harvested from this by centrifuging at 3,000 x *g* for five minutes. Whole cell extracts from the induced and non-induced cultures were prepared by adding 50 µl cracking buffer (6 M urea, 0.1 M Tris pH 6.8, 4% SDS) along with 50 µl 3X Laemmli buffer. These were boiled for five minutes and 20 µl of each was loaded onto a SDS-PAGE gel.

Cell samples from the soluble and insoluble fractions were also prepared from the induced cultures by adding 100 µl sonication buffer (25 mM HEPES pH 7.7, 0.1 M KCl, 12.5 mM MgCl₂, 20% glycerol, 0.1% IGEPAL). This was sonicated and centrifuged at 10,000 x *g* for 10 minutes. The soluble fraction is contained in the supernatant, so 50 µl of this was collected and added to 50 µl 3X Laemmli buffer. The pellet containing the insoluble fraction was resuspended in 50 µl cracking buffer with 50 µl of 3X Laemmli buffer. These were boiled for five minutes and 20 µl loaded on a gel. The presence of the recombinant protein was analysed by western blotting as described in Section 2.5.2. These were either probed with the LBP-N antibody at a dilution of 1:5,000 or with an anti-histidine (anti-His)-HRP antibody (Roche Applied Science) at a 1:10,000 dilution, which detects the histidine tag on the recombinant protein.

2.9.2 RECOMBINANT LBP EXPRESSION IN MAMMALIAN CELLS

Recombinant protein expression was performed in cos7 fibroblast cells obtained from American Type Culture Collection (ATCC) (USA), which are derived from a primate kidney line (*Cercopithecus aethiops*). These were chosen as they are easy to grow and transfect and optimum transfection ratios using Fugene transfection reagent (Roche Applied Science) had previously been determined.

2.9.2.1 Preparation of the G-LBP Construct

The Gateway system is based on the lambda recombination mechanisms for integration into *E. coli*. It was developed to allow a construct to be cloned into a generic entry vector from which the gene could then be inserted into a variety of expression systems including mammalian, baculovirus and bacterial. In this case the mammalian system was used as it would give a recombinant protein which most closely resembled the native protein. Ligation into the vectors is achieved by the inclusion of recombination sequences (*att* regions) which allow cloning into the entry vector, and from the entry vector into the expression vector, by using the vector specific clonase enzyme mix. A human cytomegalovirus (CMV) promoter is incorporated into the vector to control expression of the protein of interest.

The G-LBP construct was prepared using the Invitrogen Gateway system. Primers were designed for PCR amplification for cloning into the Gateway entry vector pDONR221. The expression vector pDEST26 was used as it includes a tag of histidine residues at the N-terminus, allowing the resulting protein to be purified by metal chelation chromatography. However, as the histidine tag may interfere

with activity a cleavage site for tobacco etch virus (TEV) protease was incorporated to allow for cleavage of the histidine tag if necessary (Table 2.11).

Table 2.11: PCR primer sequences for cloning of bovine mammary LBP into the Gateway expression system (Invitrogen)

A TEV protease cleavage site (bold) is encoded into the forward primer to allow cleavage of a six histidine residue tag encoded by the vector. Due to the length of the primers, a two step PCR reaction was performed using the nested forward primers shown.

Forward primer 1:

GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTA **GAG AAC CTG TAC TTC CAG TCC**

Forward primer 2:

GAG AAC CTG TAC TTC CAG TCC AAT CCA AGA ATT TTC GGC ACG

Reverse primer:

GGGG AC CAC TTT GTA CAA GAA AGC TGG G TTA TTA AAC TCT CAG GTA CCG AAC GTT

Because the Gateway system requires the *att* sites for cloning into the vectors, the resulting primers are much greater than the usual 20 bases long. Long primers can cause problems in a PCR reaction as they bind at lower temperatures and can form hairpin loops more easily. Because a whole cleavage sequence encoding seven amino acids also had to be incorporated, a two step PCR process was designed using two forward primers. The first PCR reaction was performed using the forward primer which included the gene specific region and the TEV cleavage site. The PCR product from this was then used as the template in the next PCR reaction using the second forward primer which coded for the TEV cleavage site and included the *att* sites as specified in the Gateway manual. The reverse primer was used in both reactions and included a stop codon at the end of the gene specific region, followed by the *att* site.

For expression of recombinant proteins in mammalian cells it was important to ensure the construct included a start codon and a 5' UTR. The Gateway construct for LBP had a start codon encoded in the vector at the beginning of the insert. A 5' UTR was not included in the sequence as the presence of the histidine tag and the TEV site should have been sufficient.

TEV protease was chosen as it is a plant protease with high specificity. It was important to ensure that endogenous forms of the protease would not be present in the cells and media being used as this could cause premature cleavage of the histidine tag and affect the yield of the protein available to be purified from the samples. TEV protease has a recognition sequence of seven amino acids (Glu Asn Leu Try Phe Gln Gly) with cleavage occurring between Gln and Gly. This results in an extra glycine remaining on the end of the protein but as LBP is a large protein it was thought that this would not have a significant effect on the folding or activity of the recombinant protein. Although a spacer sequence is recommended to improve efficiency of TEV cleavage when using poly-histidine tag vectors (Invitrogen), it was thought that this was unnecessary due to the length of the *att* sequence between the cleavage site and the gene coding region.

2.9.2.1.1 PCR Reaction

A two step PCR amplification reaction was required to incorporate the full TEV protease cleavage site onto the construct. The PCR reaction contained 1.5 µl 10X buffer containing MgCl₂, 0.3 mM dNTPs, 1 U Taq polymerase, 0.5 µl (approximately 0.5 µg) DNA template, 0.6 µM each primer Forward 1 and Reverse (Table 2.11) in a final volume of 15 µl. A temperature gradient reaction

was carried out to determine the optimum annealing temperature for the primers. The reaction consisted of an initial denaturing step for five minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at the annealing temperature which ranged from 50°C to 70°C, and two minutes at 72°C, with a final extension incubation at 72°C for 10 minutes.

The result was visualised by agarose gel electrophoresis with EtBr staining, showing that the optimum annealing temperature was between 50°C and 53°C. The resulting bands were cut from the gel and the DNA extracted by freeze-squeezing, for use as the template DNA for the second PCR reaction.

The second PCR reaction was performed in a similar manner, excepting that in this reaction the primers used were Forward 2 and Reverse (Table 2.11). The reaction was run on a 1% agarose gel and the bands cut out and freeze-squeezed to extract the DNA.

2.9.2.1.2 The BP Reaction

The BP reaction for cloning the PCR product into the Gateway entry vector was performed according to the manufacturers' instructions however, the volumes were decreased as it has been found previously that a half reaction still gives successful results (Dr Roger Collin, personal communication). For each reaction the total volume was 6 µl composed of 1 µl BP reaction buffer, 2 µl DNA, 1 µl vector pDONR221, 1 µl BP clonase enzyme mix and TE buffer. A positive control was included using the control DNA (pENTR-GUS) from the cloning kit, and a negative control with no DNA was also included. For the other reactions the

PCR product was used. The reaction was allowed to proceed for two hours at RT and was stopped with the addition of 1 μ l proteinase K (2 μ g/ μ l) to each tube and incubated at 37°C for 10 minutes. It was then stored at -20°C.

2.9.2.1.3 Transformation

For the transformation, 1 μ l of the entry clone reaction was added to 50 μ l DH5 α library efficiency cells while on ice. This was incubated on ice for 30 minutes and heat shocked at 42°C for 45 seconds before returning to ice for two minutes. After this, 450 μ l SOC medium (Invitrogen) was added and the culture was incubated at 37°C for one hour, shaking at 250 rpm. The culture was plated at dilutions of 100 μ l and 20 μ l onto LB agar plates inoculated with 50 μ g/ml kanamycin. The plates were then incubated overnight at 37°C.

From these, single colonies were used to inoculate three cultures of 5 ml Terrific broth (TB), (Invitrogen) containing 50 μ g/ml kanamycin. These were grown at 37°C overnight, with shaking at 250 rpm. The plasmid was extracted by a multi miniboil extraction method as described in Section 2.9.1.2, and the resulting DNA resuspended in 50 μ l T.1E buffer.

Because there are no suitable restriction enzymes sites close to the insertion region, the presence of the insert was confirmed by PCR. This was performed in a 15 μ l PCR reaction which used the same quantities as described earlier, with the outer primers and an annealing temperature of 50°C.

2.9.2.1.4 The LR Reaction

The G-LBP construct was produced by cloning into the pDEST26 mammalian expression vector. The LR reaction for cloning into the expression vector was carried out according to the instructions provided but the quantities were halved. The final reaction mix contained 1 µl DNA, 1 µl pDEST26 vector, 2 µl LR reaction buffer and 2 µl LR clonase enzyme mix, made up to a final volume of 10 µl with TE buffer. The reaction was left to proceed for two hours at RT, then 1 µl proteinase K was added and the solution was incubated at 37°C for 10 minutes.

2.9.2.1.5 Transformation

The plasmid was transformed as described in Section 2.9.2.1.3. The cultures were then plated onto LB agar plates with 100 µg/ml ampicillin, using 100 µl culture. The plates were incubated at 37°C overnight.

Colonies were used from these to inoculate cultures of 5 ml TB broth containing 100 µg/ml ampicillin. These were grown at 37°C overnight with shaking. The G-LBP DNA was extracted by alkaline lysis extraction (Section 2.3.2.1.1). The presence of the insert was confirmed by PCR analysis and also was sent for sequencing at the University of Waikato DNA sequencing facility using the same primers as used in the PCR reaction.

2.9.2.2 Preparation of Mammalian Cells

Recombinant protein expression was performed in mammalian cos7 cells in an attempt to produce a protein with more similarity to the native form. Cos7 cells are adherent cells and were grown on 10 cm cell culture dishes in cos7 growth media containing 1X Dulbecco's modified eagle medium (DMEM), (Invitrogen) and 10% foetal calf serum (FCS) (Invitrogen). The cells were grown in normal cell culture conditions of 37°C, 5% CO₂. They were passaged twice weekly by removing the media and washing the cells in 1X PBS, then incubating for three minutes at 37°C in cos7 trypsin solution (1 ml trypsin, 300 µl 20% EDTA, 8.7 ml 1X PBS) to dislodge the cells. Growth media was added to neutralise the trypsin and the cell solution was spun down at 180 x g for three minutes. The cell pellet was resuspended in 5 ml growth media and 0.5 ml of this was added to a new dish containing 9.5 ml growth media, to give a 1:10 dilution.

2.9.2.3 Mini transfection

Initially a small scale transfection was done. The cells were seeded at 1:8 into six well plates and grown to approximately 50% – 70% confluency. The transfection reaction solution was prepared by adding 3 µg DNA and 6 µl Fugene transfection reagent made up to a total volume of 100 µl per well with 1X DMEM (with no FCS) (Table 2.12). The transfection solution was prepared and incubated at RT for 15 minutes. The growth media was removed from the cells and they were washed in 1X PBS before adding 0.9 ml 1X DMEM to each well. After the transfection reaction solution was added the cells were incubated at 37°C, 5% CO₂ for five hours, after which 1 ml growth media was added per well to give a final concentration of 5% FCS. Expression was then allowed to proceed for two

days before harvesting. A negative control was included using cells transfected with the empty pDEST26 vector (vector alone), and a positive control performed using a stat5b DNA construct which had been prepared previously by Judi McCracken (AgResearch, Ruakura) in the pCS2 vector.

Table 2.12: Volumes used in transfection reactions for recombinant expression in mammalian cos7 cells

	DNA (μg)	Fugene (μl)	Total volume (μl)
6-well plate	3	6	100
60 mm dish	7.5	15	250
10 cm dish	12	24	400

2.9.2.4 Protein and RNA Extraction

The protein and RNA was extracted from the cells for detection of the LBP construct by northern and western blotting analysis. This was carried out according to the method given in the product insert instructions for Trizol reagent.

The media was removed from the cells and retained at -20°C for further analysis. The cells were then washed off by adding 1 ml Trizol. The mixture was then transferred to fresh tubes and incubated for five minutes at RT. To this, 200 μl of chloroform was added and inverted to mix. This was incubated for three minutes and centrifuged at 12,000 x g for 15 minutes.

2.9.2.4.1 RNA Precipitation

The upper aqueous phase was transferred to a new tube while the remaining lower phase was kept for protein extraction. To the upper phase, 0.5 ml isopropanol was added and this was incubated for 10 minutes. The RNA was precipitated by

centrifuging at 12,000 x g for 10 minutes and the resulting pellet was washed in 75% ethanol. The ethanol was removed after centrifuging at 7,500 x g for five minutes. The pellet was air-dried for 10 minutes and resuspended in 50 µl DEPC water. The integrity of the RNA samples was tested by running 1 µg on a 1.2% RNA agarose gel in loading dye containing EtBr. The RNA samples were then used in a northern blot analysis by running 5 µg RNA on a 1.2% RNA agarose gel in loading dye and probing with an LBP probe (Section 2.3.2). The northern blot was carried out according to the method described in Section 2.3.3.

2.9.2.4.2 Protein Extraction

The lower layer containing the protein was mixed with 0.3 ml 100% ethanol and incubated for three minutes. The DNA was removed by centrifuging at 2,000 x g for five minutes and the resulting supernatant was transferred to a new tube. Protein was extracted from this by adding 1.5 ml isopropanol and incubating for five minutes at RT. The protein was precipitated by centrifuging at 12,000 x g for 10 minutes. The protein pellet was then washed three times in 2 ml 0.3 M guanidine hydrochloride in 95% ethanol for 20 minutes each time. After the final wash, 2 ml of ethanol was added to the pellet, vortexed and incubated at RT for 20 minutes. This was then centrifuged at 7,500 x g for five minutes, the ethanol removed and the pellet air-dried. Initially, the pellet was incubated at 50°C in 50 µl of 1% SDS to dissolve, but due to low solubility, 100 µl 3X Laemmli buffer and 150 µl 2D sample buffer (8 M urea, 4% CHAPS, 3 M thiourea, and 40 mM DTT) were also added and the solution vortexed until the pellet had completely dissolved. The samples were run on a 10% Bis-Tris NuPAGE gel, with 20 µl loaded per well. Western blots were performed on these using the LBP-N

antibody and the anti-His antibody following the western protocol described in Section 2.5.2. The presence of stat5b protein in the positive control samples was also detected by western blotting using a rabbit anti-stat5b antibody (Santa Cruz Biotechnologies) at a 1:5,000 dilution.

2.9.2.5 Large Scale Transfection

To produce enough protein for histidine tag purification in the ProBond system (Invitrogen), approximately 5 to 10 million cells are required. To determine the number of cells in a 10 cm dish, the cos7 cells were grown to approximately 90% confluency and trypsinised. To count them, 100 μ l of the new cell suspension was added to 900 μ l trypan blue stain (Invitrogen), vortexed, and the solution applied by pipette to a haemocytometer. The number of cells present in four corners of the grid was counted and from this the total number of cells was calculated.

Based on this, three 10 cm plates were used for the transfection in order to get sufficient protein for purification. Cos7 cells were grown in these until they were approximately 60% confluent. The transfection mixture was made up according to the ratios shown in Table 2.12, in a final volume of 400 μ l per plate. After incubating the cells with the transfection solution for five hours, 4 ml of growth media was added to each plate and they were left for two days.

After two days, the cells were trypsinised and the pellets from all three plates were combined and resuspended in 8 ml native binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.4), containing 1X protease inhibitor

cocktail (Sigma). The cells were counted as described above, giving a total cell count of 6.6×10^6 cells/ml.

2.10 PURIFICATION OF RECOMBINANT LBP

The recombinant His-tag protein was purified by metal chelation chromatography using the ProBond system which utilises Ni^{2+} - Histidine binding affinity to purify His-tag proteins. Proteins were eluted in a pH gradient in either native or denaturing conditions. For this, 10X stock solutions of A (200 mM NaH_2PO_4 , 5 M NaCl) and B (200 mM Na_2HPO_4 , 5 M NaCl) were prepared and the buffers were made by combining different ratios of these as shown in Table 2.13.

Table 2.13: Buffers used for ProBond protein purification system

Buffers were prepared from stock solutions of 10X A (200 mM NaH_2PO_4 , 5 M NaCl), and 10X B (200 mM Na_2HPO_4 , 5 M NaCl). For native buffers these stocks were diluted to a 1X concentration. All buffers have a final composition of (20 mM sodium phosphate, 500 mM sodium chloride). Volumes shown are for a final solution volume of 100 ml.

Native Buffers				
Buffer	1X A (ml)	1X B (ml)	Final pH	
Binding	5.8	94.2	7.2 - 7.6	
Wash 1	74	26	6	
Wash 2	87	13	5.5	
Elution	100	0	4	
Denaturing buffers				
Buffer	10X A (ml)	10X B (ml)	Other	Final pH
Lysis	0.58	9.42	6M GnHCl	7.8
Binding	0.58	9.42	8 M Urea	7.8
Wash 1	7.38	2.62	8 M Urea	6
Wash 2	9.71	0.83	8 M Urea	5.3
Elution	10	0	8 M Urea	4

Approximately 2 ml of ProBond nickel resin (Invitrogen) was loaded into a 15 ml tube and the supernatant removed after centrifuging at 800 x *g* for two minutes. The resin was washed twice in 7 ml MQ water and three times in 7 ml binding buffer with spinning and removal of the supernatant between each wash.

2.10.1 PURIFICATION IN NATIVE CONDITIONS

2.10.1.1. Preparation of Bacterial Cell Lysate

Purification of the native protein was performed by first resuspending the cell pellet in 10 ml native binding buffer and adding lysozyme to give a final concentration of 100 µg/ml. The solution was then incubated on ice for 15 minutes. The cells were lysed by sonicating with three short 10 second bursts, then four freeze/thaw cycles in a dry ice/ethanol bath and a 37°C waterbath. The solution was then centrifuged at 3,000 x *g* for 15 minutes and filtered through a 0.2 µm filter before loading on the column.

2.10.1.2 Preparation of Mammalian Cell Lysate

Cells from mammalian cell culture were prepared for His-tag purification by collecting the cells after treating with trypsin and resuspending the cell pellet in 8 ml native binding buffer containing protease inhibitors. The cells were lysed by rapid freeze/thaw twice in a dry/ice ethanol bath and a 42°C waterbath. The solution was then passed through an 18 gauge needle four times and centrifuged at 3,000 x *g* for 15 minutes.

2.10.1.3 Protein Purification

For purification of protein by the native procedure the cell lysate was rocked with the prepared resin for one hour at RT. This was loaded onto a column and washed three times with 4 ml native binding buffer, followed by three washes in native wash buffer pH 6 and two washes in native wash buffer pH 5.5. The protein was eluted with 5 ml native elution buffer pH 4 and collected in 1 ml fractions.

2.10.2 PURIFICATION IN DENATURING CONDITIONS

2.10.2.1 Preparation of Bacterial Cell Lysate

For purification of the protein in denaturing conditions the cell pellet was resuspended in 10 ml guanidine lysis buffer which had been warmed to 37°C. This was rocked at RT for 5 to 10 minutes before lysing the cells with three short bursts of sonication. The cell debris was removed by centrifuging at 3,000 x g for 15 minutes. The resulting supernatant was then stored on ice while the column was prepared.

2.10.2.2 Protein Purification

The lysate was batch bound to the resin by adding 5 ml lysate to the resin and rocking for approximately 10 minutes. The resin was allowed to settle and the supernatant removed. This was repeated until all the lysate had been put through, then the resin was washed twice with 8 ml denaturing binding buffer, twice with 8 ml denaturing wash buffer pH 6 and twice with 8 ml denaturing wash buffer pH 5.3. For each wash the resin was rocked for two minutes then centrifuged at 800 x g for two minutes and the supernatant removed. On the last wash the resin was loaded into a column and the remaining buffer allowed to flow through. The

protein was then eluted with 10 ml denaturing elution buffer pH 4 and the eluant collected in 1 ml fractions.

2.10.3 REGENERATION OF PROTEIN PURIFICATION COLUMNS

Following elution, the column was stripped of bound ions with the following series of washes; 16 ml 50 mM EDTA, 16 ml 0.5 M NaOH, 16 ml MQ water. The column was then recharged with 16 ml $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (5 mg/ml) and washed again in 16 ml MQ water. The column was then stored in 20% ethanol at 4°C.

2.10.4 PURIFICATION WITH VIVASPIN COLUMNS

In another attempt to His-tag purify the mammalian recombinant protein a mini His-column was used. Cos7 cells grown in 60 mm dishes were transfected as described in Section 2.9.2.3, with the G-LBP construct using the ratios shown in Table 2.12. After two days, the cell lysates were collected in 0.5 ml native binding buffer and lysed by sonication. The lysate was then purified using a Vivapure metal chelate mini spin column (Vivascience), following the instructions provided by the manufacturer. After loading the lysate, all of the flow through and washes were collected for later analysis by western blotting.

2.10.5 DETECTION OF PURIFIED PROTEIN

The protein content of the fractions collected was measured using a Bradford assay and also observed by western blot analysis with the LBP-N antibody and the anti-His antibody as described in Section 2.5.2.

2.11 LBP ACTIVITY

LBP has two major functions, the first being the ability to bind lipopolysaccharide which was confirmed by three different methods. The second is the immuno-stimulatory activity, which was tested by the ability to induce TNF α production in monocytes.

2.11.1 ELISA

An ELISA kit (HBT) was used to measure LBP concentration by initial binding to a cross-reactive LBP antibody, followed by binding to biotinylated LPS. This was then detected using a streptavidin-HRP conjugate. The second binding step allows detection of the LPS binding ability of the native protein in milk. A number of different whey samples were tested on the ELISA as described in Section 2.8.

2.11.2 IMMUNOPRECIPITATION WITH *E. COLI* PROTEINS

The ability of the native LBP protein to bind to LPS was also seen in an immunoprecipitation experiment with a whey fraction from a late lactation involuted mammary secretion. *E. coli* proteins in agarose were obtained from Sigma, and the method for the protein A agarose immunoprecipitation described in Section 2.7 was performed with some modifications.

First, a 1 ml solution was made containing 50 μ l whey in RIPA buffer. To this, 15 μ l of the slurry of *E. coli* proteins in agarose was added and this was rotated overnight at 4°C. Following this, a further 15 μ l agarose slurry was added and the solution was rotated for two hours at 4°C. The beads were pelleted by centrifuging at 400 x g for one minute, and the supernatant retained for later analysis. The

pellet of beads was resuspended in 20 μ l 3X Laemmli buffer and boiled for three minutes before re-pelleting by centrifugation. The supernatant from this was also collected and these samples were run on an SDS-PAGE gel for analysis by western blot with the biotinylated LBP-N antibody.

2.11.3 LIGAND BLOTTING WITH LPS

The ability of the denatured protein to bind to LPS was seen in western blot analysis of milk samples separated by SDS-PAGE. Initially western blots on a range of whey and skim milk samples were probed with the biotinylated-LPS from the ELISA kit. The reconstituted sample was diluted in western washing buffer at 1:100 and the signal detected with the streptavidin-HRP conjugate at 1:80,000 using the western procedure described in Section 2.5.2. Due to the limited shelf life of this sample, it was worthwhile to produce a similar stock.

2.11.3.1 Biotinylation of LPS

Most of the western blot analysis on LPS binding was performed using the biotinylated LPS included with the ELISA kit (Section 2.8). As this was limited in quantity, LPS from *E. coli* strain O55:B5 (Sigma) was biotinylated for further investigations with LBP and other LPS binding proteins. The biotinylation of LPS was done according to the method in Luk *et al.* (1995). For this, 1 mg LPS was dissolved in 0.9 ml of 0.1 M sodium acetate pH 5. A solution of 100 mM sodium metaperiodate (Sigma) was also made up in 0.1 M sodium acetate and cooled on ice before adding 0.1 ml to the LPS solution. The oxidation reaction was then allowed to proceed on ice for 30 minutes in the dark. The reaction was stopped by adding 2 μ l glycerol and left on ice for five minutes. The unreacted biotin was

removed by dialysis overnight at 4°C in 1X PBS. Biotin-LC-hydrazide (Pierce) was prepared as a 50 mM solution in DMSO and 0.1 ml added to the oxidised LPS sample. This was rotated at RT for two hours and dialysed overnight in 1X PBS at 4°C. The reactivity and specificity of the biotinylated LPS was tested on western blots and the optimal concentration for specific detection was determined to be 1:40,000. This was then used to probe western blots of milk samples, as described in Section 2.5.2, using the streptavidin-HRP conjugate at 1:80,000.

2.11.4 CYTOKINE INDUCTION ASSAY

The immuno-stimulatory activity of the recombinant LBP protein from mammalian cos7 cells was investigated using an assay to measure TNF α production. This was achieved by transfecting mammalian cos7 cells to produce recombinant LBP protein and using this to stimulate TNF α production in monocytes. The supernatant was collected from these cells and used to treat a TNF α susceptible cell line, allowing the amount of TNF α produced to be determined by measuring cell death.

Because the recombinant protein has an N-terminal tag of histidine residues and a TEV protease cleavage site, it was initially attempted to purify the protein and cleave the His-tag. However, a number of attempts at purifying the recombinant protein using metal chelation chromatography resulted in very little to almost no protein recovered. Because of this the whole cell lysate was used on the assay, with a negative control of cells transfected with vector only as a comparison. Problems were also encountered with this however, as western blots were used to verify the presence of LBP in the transfected cells and the absence of it in the

negative controls. But the results from these were ambiguous, with bands present at the approximated molecular weight in both positive and negative samples. In spite of this a trial assay was performed as it was thought that an effect may still be observable.

2.11.4.1 Production of Recombinant LBP Protein

Cos7 cells were grown and transfected as described in Section 2.9.2.2. After incubating for two days, the media was collected and stored at -20°C for further analysis. The cells were collected with trypsin as described Section 2.9.2.3 and the cell pellet resuspended in 0.5 ml native binding buffer. The cells were kept on ice while they were lysed by four brief bursts of sonication and the resulting lysate was stored at -80°C. A sample of the lysate was taken for western blot analysis, and was stored in 3X Laemmli buffer at -20°C.

2.11.4.2 Stimulation of TNF α Production in Monocytes

For this, the human THP-1 monocyte cell line (ATCC) was grown in THP-1 growth media containing 1X RPMI 1640 (Invitrogen) base, 10% FCS, 10 mM HEPES, 1X sodium pyruvate, 10X mercaptoethanol, 100 U penicillin and 100 μ g/ml streptomycin. Because these cells grow in suspension they were split twice weekly at a 1:20 dilution by transferring 0.5 ml cell suspension to a new plate containing 9.5 ml growth media.

All samples were prepared in assay media which was the same as the growth media except that it contained only 3% FCS and actinomycin D (Calbiochem, California, USA) at a ratio of 5 μ l actinomycin D to 10 ml assay media. LPS from

E. coli strain O55:B5 (Sigma) and standard human LBP from the ELISA kit (HBT) (Section 2.8) were used as controls. A five-fold dilution series of each sample was plated out across the rows, with all samples assayed in duplicate. Two plates were tested using cell lysates from different transfection experiments. The plate design can be seen in Table 2.14. Ten µg/ml LPS was added to each of the wells containing the cell lysates and the standard LBP. THP-1 cells were added to each well at a density of 2×10^4 , and the plates were incubated at 37°C for four hours. The plates were then centrifuged at 200 x g for five minutes and the supernatants assayed for TNFα concentration.

Table 2.14: Plate design for cytokine induction assay

The ability of recombinant bovine LBP to induce TNFα production in monocytes was measured in a cell proliferation assay on WEHI-13VAR cells. Lysates were collected from cells transfected with an LBP construct (rLBP +) or a vector only control (rLBP -). The amount of LBP in these was not quantified so only dilutions are shown. A standard human LBP (HBT) was included. All of these samples also contained 10 ng/ml LPS. Standard dilutions of LPS and TNFα were also included as shown.

LPS 100 ng/ml	LPS 20	LPS 4	LPS 0.8	LPS 0.16	LPS 0.032	LPS 0.0064	LPS 0.00128	LPS 0.000256	LPS 0	TNFα	1 ng/ml
rLBP + neat	rLBP + 1:5	rLBP + 1:25	rLBP + 1:125	rLBP + 1:625	rLBP + 1:3125	rLBP + 1:15625	rLBP + 1:78125	rLBP + 1:390625	rLBP + 0	TNFα	0.2
rLBP - neat	rLBP - 1:5	rLBP - 1:25	rLBP - 1:125	rLBP - 1:625	rLBP - 1:3125	rLBP - 1:15625	rLBP - 1:78125	rLBP - 1:390625	rLBP - 0	TNFα	0.04
LBP std 400 ng/ml	LBP std 80	LBP std 16	LBP std 3.2	LBP std 0.64	LBP std 0.128	LBP std 0.0256	LBP std 0.00512	LBP std 0.001024	LBP std 0	TNFα	0.008
										TNFα	0.0016
										TNFα	0.00032
										TNFα	0.000064
										TNFα	0

2.11.4.3 Cell Proliferation Assay

Quantification of TNFα produced by the THP-1 cells was performed using a cell proliferation assay (Espevik and Nissen-Meyer, 1986), with WEHI-13VAR cells (ATCC) which are susceptible to TNFα. The WEHI-13VAR cells are derived

from a mouse fibrosarcoma line and are a mixture of suspension and adherent cells. These were grown in the growth media described for the THP-1 cells and were passaged at 1:20 twice weekly. Passaging was performed by collecting the media, then washing the adherent cells in 1X PBS and incubating in 2 ml WEHI trypsin solution (1 ml trypsin, 300 μ l 1% EDTA, 8.7 ml PBS) for three minutes at 37°C. The trypsin was neutralised by adding 3 ml growth media and this was added to the rest of the media and the cells collected by centrifugation at 200 x g for five minutes. The supernatant was discarded and the cells resuspended in 10 ml growth media. For the assay, the cells were seeded into 96-well plates at a density of 2×10^5 cells/ml, with 100 μ l cells plated per well. The cells were grown at 37°C for two days before assaying.

The supernatants from the wells of the THP-1 plates were transferred to the WEHI cell plates and were incubated at 37°C for two days. The WEHI cell plates also included a five-fold dilution series of TNF α with an initial concentration of 1 ng/ml. After the incubation the assay was developed by adding 10 μ l MTT reagent (Roche Applied Science) to each well and incubating for four hours at 37°C. Enzymes present in viable cells are able to convert the MTT reagent to blue tetrazolium crystals, which are then solubilised by addition of 100 μ l 10% SDS in 0.01 M HCl per well. After addition of SDS the plates were incubated at 37°C overnight and read the following day on an ELx800 plate reader (BioTek) at 490 nm. The protocol suggests reading them at 570 nm, but that was not an option on this particular plate reader and it was suggested that this absorbance should be suitable (Nikki Harcourt, personal communication).

CHAPTER THREE RESULTS

3.1 FULL LENGTH SEQUENCING OF BOVINE MAMMARY LBP

The full length sequence for the mammary derived LBP clone gave a sequence of 1368 base pairs (Table 3.1), with a predicted amino acid sequence of 456 residues (Table 3.2). This coded for the mature protein without the signal peptide and contained both of the BPI domains characteristic of the LBP/BPI family members. The sequence includes five cysteine residues, three of which are conserved in the published LBP sequences, with the other two being unique to this LBP sequence. There are also five potential N-glycosylation sites (Asn-X-Ser/Thr) present in the sequence (Table 3.2).

Table 3.1: Full length sequence of bovine mammary LBP

AATCCAAGAA	TTTTTCGGCAC	GAGGGGGGAC	CAGGGCTTGG	AGTACGTGGC	CCAAGAGGAG
CTGTTGGCTC	TGCAGAGTAA	GCTGCACAAA	GTCACACTGC	CCGACTTCAA	CGGGGACGTC
AGGATCAAAC	ATTTTGGCAG	TGTGGACTAT	AGATTCCACA	GCCTGAACAT	TCAGAGCTGT
AAGCTGCTTG	GCTCCGCTCT	GAAGCTCCTC	CCCAATCAGG	GCCTGCATTT	CTCTATCTCC
GACTCCTTCA	TCCAGGTCAC	GGGCGACTGG	AAGGTGCGCA	AGAGGATACT	GAGACTAGAC
GGCTCCTTTG	ACGTGAAGGT	CAAGGGCATC	ACCATTTTCAG	TCAATCTTCT	CTTGGACAGT
GAACCCTCTG	GGAGACCCAA	AGTGGCTGTC	TCCAGCTGCA	GTAGCCACAT	CCGTGATGTG
GAGGTGCACA	TATCAGGAGA	TTTGGGGTGG	CTGCTGAATC	TCTTCCATAA	CCAGATCGAG
TCCAGGTTCC	GAAGGGTCTT	GGAGAGCAAG	ATTTGCGAAA	TTATTGAGGA	CTCGGTGACC
TCTGAGCTAC	AGCCTTATCT	TCAAACCTCTG	CCAGTCACAA	CAGAGATTGA	TCATCTCGCT
GGCCTTGATT	ACAGCTTAAT	GGGGGCCCCCT	CAAGCAACAG	CCCAAATGCT	GGATGTGATG
TTCAAGGGTG	AAATTTTTAG	CCGAGATGAC	CGCTTCCCAG	TTGCTTTTCCT	TGCTCCTGTC
ATGAACCTTC	CTGAGGAACA	CAGCCGAATG	GTCTACTTTG	CCATCTCTGA	TTATGCCTTC
AACACCGCCA	GCCTAGTTTA	TCATAAAGCG	GGATTCCCTGA	ACTTTACTAT	CACGGATGTG
ATTCCGCCTG	ATTCTAGCAT	TCGACTGAAC	ACCAAGTCCT	TCCGCGCCTT	CGTTCCCAGG
ATAGCCAGAC	TGTACCCCAA	CACGAACTTG	GAGCTCCAGG	GAGCAGTGAT	CTCTGCCCCG
TGCCTGAACT	TCAGCCCTGG	AAATCTGTCT	ACAGCTGCCC	AGATGGAGAT	CGAAGCCTTT
GTGCTCCTGC	CCAACCTCTGT	CAAGGAGCCT	GTCTTCCGGC	TCAGTGTTGGC	CACTAACGTG
TCTGCCATGT	TGACCTTCAA	CACCAGCAAG	ATCACTGGAT	TCTTGGAGCC	AGGAAAGATA
CAAGTGGAAC	TGAAAGAATC	TAAAGTTGGA	CGATTCAATG	TGGAGCTGTT	GGAGGCACTG
CTCAATTATT	ACCTTCTCAA	CAACTTTTAC	CCCAAGGTCA	ATGATAACGT	TGGCAGAAGG
CTTCCCCCCT	CCCTCTGCTA	CGGAAAAATT	CAGCTCTACG	ACCCCATTTCT	CCAGATCCAC
AAGGACTTCC	TGTTTTTTGGG	GACCAACGTT	CGGTACCTGA	GAGTTTGA	

Table 3.2: Conceptual protein translation of bovine mammary LBP

The potential N-glycosylation sites are shown in red.

NPRIFGTRGD	QGLEYVAQEE	LLALQSKLHK	VTLPDFNGDV	RIKHFGSVDY	RFHSLNIQSC
KLLGSALKLL	PNQGLHFSIS	DSFIQVTGDW	KVRKRILRLD	GSFDVKVKGI	TISVNLLLDS
EPSGRPKVAV	SSCSSHIRDV	EVHISGDLGW	LLNLFHNQIE	SRFRRVLESK	ICEIIEDSVT
SELQPYLQTL	PVTTEIDHLA	GLDYSLMGAP	QATAQMLDVM	FKGEIFSRDD	RFPVAFLAPV
MNLPEEHSRM	VYFAISDYAF	NTASLVYHKA	GFLNFTITDD	VIPPDSSIRL	NTKSFRAFVP
RIARLYPNTN	LELQGAVISA	PCLNFSPGNL	STAAQMEIEA	FVLLPNSVKE	PVFRLSVATN
VSAMLTFN ^{TS}	KITGFLEPGK	IQVELKESKV	GRFNVELLEA	LLNYLLNNF	YPKVNDNVGR
RLPPSLCYGK	IQLYDPILQI	HKDFLFLGTN	VRYL RV		

The nucleotide and predicted protein sequences were aligned against other published LBP sequences from different species and showed good sequence similarity (Table 3.3). There was also a high degree of similarity between the bovine LBP sequence and the bovine BPI sequence (Table 3.4).

Table 3.3: Clustal W alignment of LBP homologues

Identical residues are shown in red, and conserved residues which match the majority of the other sequences are shown in blue. The conceptual translation of LBP from this study is named bLBP.

NCBI accession numbers for the other sequences are; Human LBP: NP_004130, Rabbit LBP: B35843, Mouse LBP: NP_032515 and Rat LBP: NP_058904.

bLBP	(1)	-----NPRIFGTRGDQGLEYYVAQEELLALQSKLHKVTLPLDFNG	65
Human LBP	(1)	MGALARALP-SILLALLLTSTPEALGANPGLVARITDKGLQYAAQEGLLALQSELRLITLPLDFTG	
Rabbit LBP	(1)	MGTWARALLGSTLLSLLAAAPGALGTNPGLITRITDKGLEYYAAREGLLALQRLKLEVTLPDSDG	
Mouse LBP	(1)	-MKAGTGPLLSTLLGLLFLSIQGTGCVPNGVVARITDKGLAYAAKEGLVALKRELKYLITLPLDFSG	
Rat LBP	(1)	-MKSATGPLLPTLLGLLLLSIPRTQGVNPAMVVRITDKGLEYYAAKEGLLSLQRELKYLITLPLDFSG	
Consensus	(1)	A A L STLLG L L L S P G NPGLVARITDKGLEYYAAKEGLLALQRELYKITLPLDFSG	
bLBP	(39)	DVRIKHFGSVDYRFHSLNIQSKCLLGSALKLLPNQGLHFSISDSFIQVTDGWKVRKRILRLDGSF	130
Human LBP	(65)	DLRI PHVGRGRYEFHSLNIHSCCELLHSALRPVPGQGLSLSIDSSIRVQGRWKVKRSFFKLQGSF	
Rabbit LBP	(66)	DFRIKHFGRAQYKFYSLKI PRFELLRGTLRPLPGQGLSLDISDAYIHVRGSKVKRKAFLRLKNSE	
Mouse LBP	(65)	DFKIKAVGRGQYEFHSLNIQNCCLRGSSSLKLLPGQGLRLAISDSSIGVRGKWKVKRSFSLKLHGSF	
Rat LBP	(65)	DFKIKAVGRGQYEFHSLNIQSCQLRGSSSLKPLPGRGLSLSIDSSISVRGKWKVKRSFVSLHGSF	
Consensus	(66)	DFRIKHVGRGQYEFHSL IQSCCELLGSALKPLPGQGLSLSIDSSI VRGKWKVKRSFSLKL GSF	
bLBP	(104)	DVKVKGITISVNLLEDSEPSGRPKVAVSSCSSHIRDVEVHISGDLGWLNLNLFHNQIESRFRVLE	195
Human LBP	(130)	DVSVKGISISVNLLEDSESSGRPTGYCLSCSSDIADVEVDMSGDSGWLNLNLFHNQIESKFQKVLE	
Rabbit LBP	(131)	DLYVKGITISVHLVLGSESSGRPTVTSSCSSDIQNVELDIEGDEELNLLQSQIDARLREVLE	
Mouse LBP	(130)	DLDVKGVITISVDLLLGMDPSGRPTVSASGCSRI CDLDVHISGNVGNLNLNLFHNQIESKLQKVLE	
Rat LBP	(130)	DLDVKSVTISVDLLLGVDPSERPTVTASGCSNRI RDLELHVSGNVGNLNLNLFHNQIESKLQKVLE	
Consensus	(131)	DL VKGITISV LLLGSEPSGRPTVT SSCSS I DVEVHISGDLGWLNLNLFHNQIESKLQKVLE	
bLBP	(169)	SKICEIIEDSVTSELQPYLQTLPTVTEIDHLAGLDYSLMGAPQATAQMLDVMFKGEIFSRDDRFP	260
Human LBP	(195)	SRICEMI QKSVSSDLQPYLQTLPTVTEIDSFADIDYSLVEAPRATAQMLEVMFKGEIFHRNHRSP	
Rabbit LBP	(196)	SKICRQIEEAVTAHLQPYLQTLPTVTQIDSFAGIDYSLMEAPRATAGMLDVMFKGEIFPLDHRSP	
Mouse LBP	(195)	NKVCEMI QKSVTSDLPYLQTLPTVTEIDNVLGIDYSLVAAPQAKAQLDVMFKGEIFNRNHRSP	
Rat LBP	(195)	SKICEMI QKSVTSDLPYLQTLPTVADIDITILGIDYSLVAAPQAKAQLDVMFKGEIFNRNHRSP	
Consensus	(196)	SKICEMI QKSVTSDLPYLQTLPTVTEIDSIAGIDYSLVAAPQATAQMLDVMFKGEIF RNHRSP	
bLBP	(234)	VAF LAPVMNLP EEHSRMVYFAISDYAFNTASLVYHKAGFLNFTITD-VIPDPSSIRLNTKSFRAF	325
Human LBP	(260)	VTLLA---AAEHNKMVYFAISDYVFNTASLVYHEEGYLNFSITDDMIPPSNIRLNTTKSFRPF	
Rabbit LBP	(261)	VDFLAPAMNLP EAHSMVYFSAISDYVFNTASLAYHKSGYWNFSITDAMVPADLNIRRTTKSFRPF	
Mouse LBP	(260)	VATPTPTMSLPEDSKQMVYFAISDHAFNIASRVYHQAGYLNFSITDDMLPHDSGIRLNTKAFRPF	
Rat LBP	(260)	VTTPTPTMSLPEDSKQMVYFAISDQAFNIATRVYHQAGYLNFTITDDMLPPDSNIRLNTKAFRPF	
Consensus	(261)	V LAP M LPEDH RMVYFAISDYAFNTASLVYH AGYLNFSITDDMIPPSNIRLNTKSFRRPF	
bLBP	(298)	VPRIARLYPNTNLELQGAVISAPCLNFS PGNLSTAAQMEIEAFVLLPNSVKEPVFRLSVATNVSA	390
Human LBP	(321)	VPRLARLYPNNMNLQGSVPAPLNFSPGNLSVDPYMEIDAFVLLPSSSKEPVFRLSVATNVSA	
Rabbit LBP	(326)	VPLLANLYPNNMNLQGTVNSEQLVNLSTENLLEEPMDIEALVVLPSAREPVFRLGVATNVSA	
Mouse LBP	(325)	TPQIYKKYPDMKLELLRTTVSAPILNVSPGNLSLAPQMEIEGFVILPSSARESVFRLGVVTVNVFA	
Rat LBP	(325)	TPLITRKYPDMNLELLGTTVSAPILNVSPGNLSLAPQMEIEGFVILPSSARESVFRLGVVTVNVFA	
Consensus	(326)	VP IARLYPNNMNLQGTTVSAPILNVSPGNLSLAPQMEIEAFVILPSSAREPVFRLSVATNVSA	
bLBP	(363)	MLTFNTSKITGFLPGKIQVELKESKVGFRNVLELLEALLNYYLLNNFYPKVNDNVGRRLPSPSLCY	455
Human LBP	(386)	TLTFNTSKITGFLKPGKVKVELKESKVGFLNAELLEALLNYYLLNTLYPKFNDKLAEGFPLPLK	
Rabbit LBP	(391)	TLTNLNRKITGFLKPGRLQVELKESKVGFRNVLELLEALLNYYLLNNLYPKVNEKLHRFPLPLR	
Mouse LBP	(390)	SLTFNTRKVTGMLHPDKAQVRLIESKVGIFNVNLFQAFNYYLLNSLYPDVNAELAQGFPLPLPR	
Rat LBP	(390)	SLTFDNSKVTGMLHPEKAQVRLIESKVGMFNVNLFQAFNYYLLNSLYPDVNDLAKGFPLPLR	
Consensus	(391)	SLTFNTSKITGFL PGKIQVELKESKVGIFNVLELLEALLNYYLLNSLYPKVND LA GFPLPL R	
bLBP	(428)	GKIQLYDPILQIHKDFLFLGTNVRYLRV-	484
Human LBP	(451)	-RVQLYDLGLQIHKDFLFLGANVQYMRV-	
Rabbit LBP	(456)	-HIQLYDLLQTHENFLLVGANIQYRRV-	
Mouse LBP	(455)	-HIQLHDLDFQIRKDFLYLGANVQYMRV-	
Rat LBP	(455)	-RIKLHDLDFQIHKDFLYLGANVQYMRV-	
Consensus	(456)	RIQLYDL LQIHKDFLFLGANVQYMRV	

Identical residues are shown in red. The conceptual translation of LBP from this study is named bLBP and the NCBI accession number for bovine BPI is P17453.

	1		130
bLBP	(1)	-----NPRI FGTRGDQGL EYVAQ EELLALQ SKLHK VTLPDFNG	
Bovine BPI	(1)	MARGPDTARRWATLVVLAALGTA VTTNP GI VARITQKGLDYACQ QGVLTQ KELEK IT IPNFSG	
Consensus	(1)	NP I A GLDY Q LL LQ L KITIP F G	
	66		130
bLBP	(39)	DVRIKHFG SVDY RFHS LNIQ SCKL LGS ALKLLP NQGLHFSI SD SFI QVTG DWK VRK RILRLD GSF	
Bovine BPI	(66)	NFKIK YLG KGQY SF FSMVIQ GFNL PNS QIRPLP DKGLDL SI RDASI KIRG KWKARK NF IKLGGNF	
Consensus	(66)	KIKH G Y F SL IQ L S IK LP GL SI DA I I G WK RK IKL G F	
	213		195
bLBP	(104)	DVKV KGI TI SVNLLDSEPS -GRPKAVSSCSSHI RDV EVHISG -DLGWL LNL FHNQIESRFRRV	
Bovine BPI	(131)	DL SV EGI SI LAGLNLGYDPASGHSTVTCSSSGINTVRIHISG SSLGWL IQLF RKRIES LLQKS	
Consensus	(131)	DL V GISI L L DPA G V SSCSS I V IHISG LGWLINLF IES K	
	219		260
bLBP	(167)	LESKICE IIEDSVTSELQPYLQTLPTVTTEIDHLAGLDYSLMGAPQATA QMLDVMFKGE IFSRDDR	
Bovine BPI	(196)	MTRKICEVVTSTVSSKLQPYQTLPTVTTKLDKVAGVDYSLVAPP RATANNDWLLKGEFFSLAHR	
Consensus	(196)	L KICEII SVSS LQPY QTLPTVT ID LAGLDYSLMA P ATAN LD L KGE FS R	
	261		325
bLBP	(232)	FPVAF LAPVMNLP EEH SRMVYFAISDYAFNTASLVYHKAGFLNFTITDVI PP -DS SIRLNTK SFR	
Bovine BPI	(261)	SPPPF APPALAFP SDH DRMVYLGISEYFFNTAGFVYQKAGALNLT LRDDMIP KES KFRLTTK FFG	
Consensus	(261)	P F P L P DH RMVY AISDY FNTA VY KAG LN TI D I P DS RL TK F	
	326		390
bLBP	(296)	AFVPRIARLYPNTNLELQGAVISAPCLNFSGNLSTAAQMEIEAFVLLPNSVKEPVFLRLSVATNV	
Bovine BPI	(326)	ILIPQVAKMFPDMQMLFIWASLPPKL TMKPS SLDLIFVLDTQAFALPNS SLDPFLLEMNINL	
Consensus	(326)	IP IAKLFP NL L P L P L LD AF ILPNS DPLF L M NL	
	391		455
bLBP	(361)	SAMLTFTNTSKITGFL EPGK IQVELKESKVGRFNVELLEALINYYLLNNFYPKVNDNVGRRLP PSL	
Bovine BPI	(391)	SVVVGAKS DRLIGELRLDKLLELKHS DIGPF SVE SLQSVINYYVMPTIVLPVIN KKLQKGFP LPL	
Consensus	(391)	S ML S KI G L KI LEK S IG F VE L ALIN Y L P IN L K P L	
	456		486
bLBP	(426)	CYGKIQLYDPILQ IHKDFLFLGTNVRVLRV-	
Bovine BPI	(456)	P-AYIELFNLTLQPYQDFL LFGADVQYS---	
Consensus	(456)	A I LF LQ H DFL G V Y	

The expression profile of LBP in the bovine mammary gland was observed by northern blot analysis on RNA samples from bovine mammary tissues at different stages of development and infection (Table 2.2). The methylene blue stained membrane shows appropriate RNA levels and intensities, except in lanes 12 and 13 which have very faint ribosomal bands suggesting the RNA has been degraded. (Figures 3.1 and 3.2)

The membrane was hybridised overnight with an $\alpha^{32}\text{P}$ -labelled DNA probe specific for either LBP or CD14. The results with the LBP probe showed a single band running at approximately 1.35 kb in relation to the RNA marker (Figure 3.1), while the results with the CD14 probe showed a band running at approximately 2.37 kb (Figure 3.2).

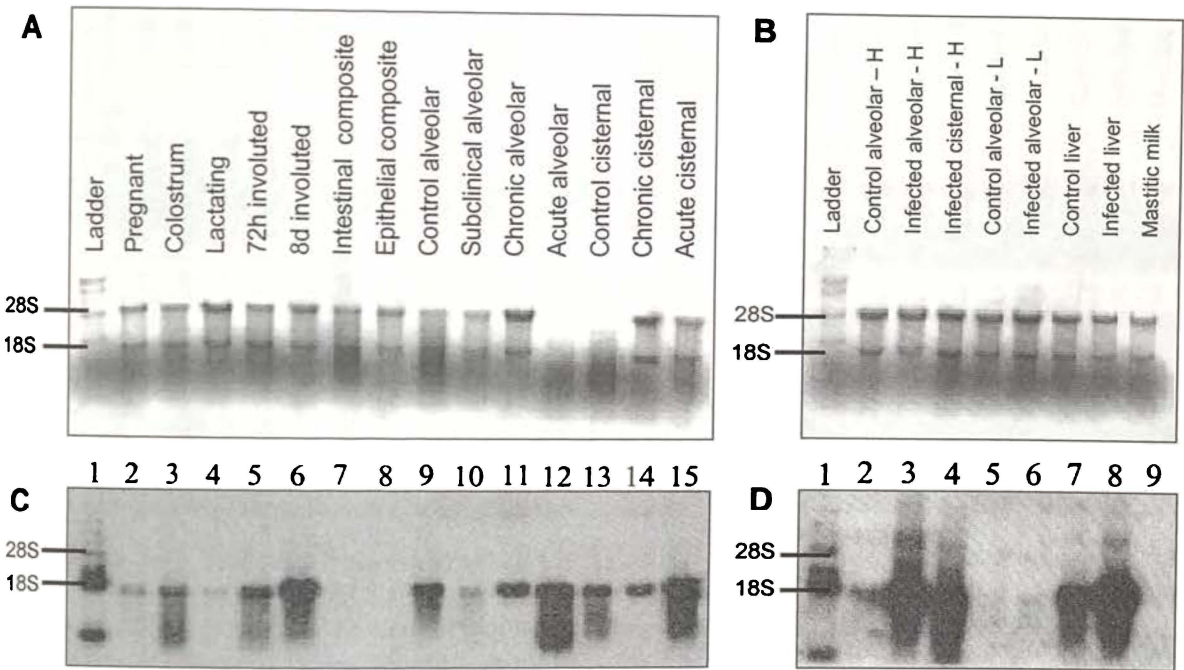


Figure 3.1: Northern blot analysis of LBP mRNA expression in bovine tissues

(A) and (B) methylene blue stain of northern membranes. (C) and (D) three day exposures of northern blot on XAR film after hybridisation with an $\alpha^{32}\text{P}$ -labelled DNA probe for bovine mammary LBP. (A) and (C) include mammary tissue from a range of developmental stages as shown, as well as mastitic tissues at different stages of infection in lanes 9 – 15. Lanes 7 and 8 have composites of intestinal and epithelial tissues respectively. (B) and (D) show samples of mammary tissue from an induced mastitic infection, with H referring to a high responding cow and L referring to a non responding cow. RNA samples from liver and mastitic milk cells are also shown on this blot.

The expression profile of LBP mRNA across the tissues shows a stronger signal in infected and involuted tissues compared with the control and normal lactation samples (Figure 3.1).

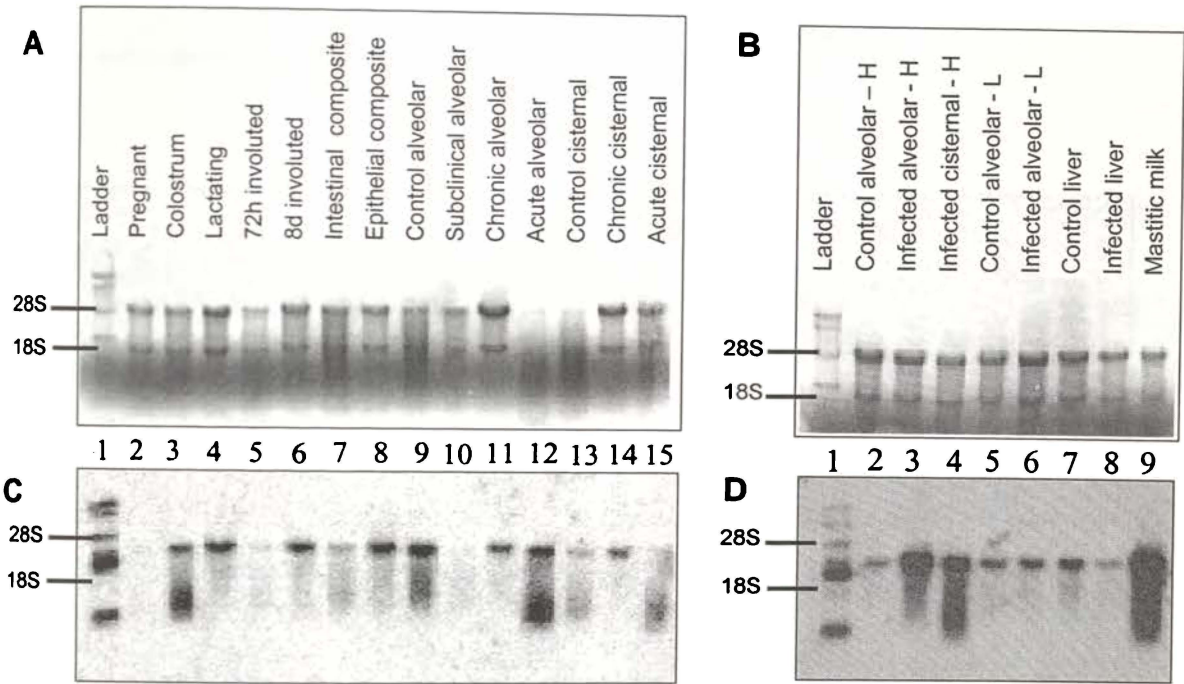


Figure 3.2: Northern blot analysis of CD14 mRNA expression in bovine tissues

(A) and (B) methylene blue stain of northern membranes. (C) and (D) three day exposures of northern blot on XAR film after hybridisation with an $\alpha^{32}\text{P}$ -labelled DNA probe for CD14. (A) and (C) include mammary tissue from a range of developmental stages as shown, as well as mastitic tissues at different stages of infection in lanes 9 – 15. Lanes 7 and 8 have composites of intestinal and epithelial tissues respectively. (B) and (D) show samples of mammary tissue from an induced mastitic infection, with H referring to a high responding cow and L referring to a non responding cow. RNA samples from liver and mastitic milk cells are also shown on this blot.

CD14 mRNA is highly expressed during mastitic infection of the mammary gland, but is only present at low levels at other stages, including during involution. The highest signal is detected in the sample of RNA collected from cells in mastitic milk (Figure 3.2).

3.3 *IN SITU* HYBRIDISATION

In situ hybridisation was performed on a small range of mastitic bovine mammary tissues (Table 2.3). The two near identical sections on each slide were probed with either a sense or anti-sense $\alpha^{35}\text{S}$ -labelled probes for LBP and α -lactalbumin, with the sense probes acting as negative internal controls.

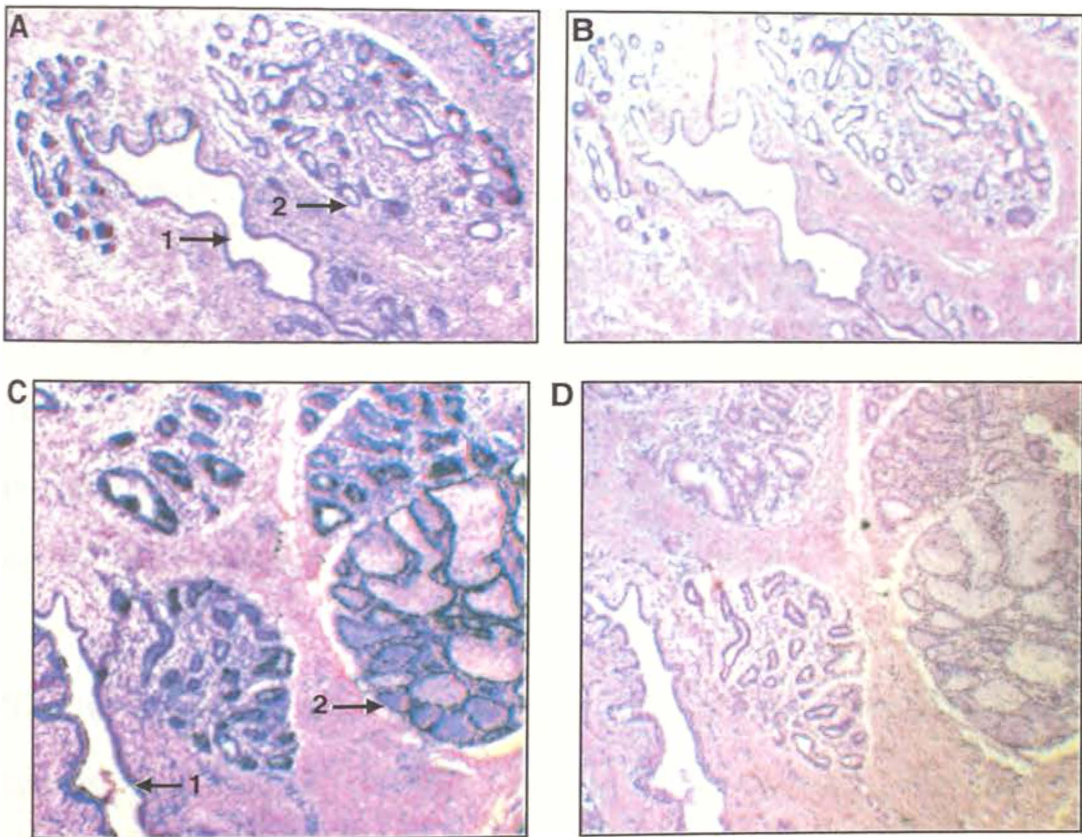


Figure 3.3: *In situ* hybridisation of LBP in mastitic bovine mammary tissue

Bovine mammary tissue was sectioned for *in situ* hybridisation and probed with an $\alpha^{35}\text{S}$ -labelled LBP probe. The tissue shown is from a section of cisternal/alveolar junction from a cow with an opportunistic subclinical mastitic infection. (A) shows expression of LBP mRNA detected in epithelial cells surrounding ductal tissue (arrow 1) and involuting alveoli (arrow 2), this is also shown in (C) with alveoli at varying degrees of involution expressing LBP. (C) and (D) show neighbouring sections probed with the sense construct as a negative control. 40X magnification.

Figure 3.3 shows a section of tissue from a mammary cisternal/alveolar junction taken from a cow suffering a subclinical mastitic infection where the predominant pathogen is *Staphylococcus aureus*. A clear, specific signal is evident in the epithelial cells surrounding ducts and infected alveoli. The corresponding section probed with the sense strand showed no signal. No signal was seen on any of the other slides including those probed with α -lactalbumin (results not shown).

3.4 PRODUCTION OF AN ANTI-LBP ANTIBODY

Antibodies were produced against synthesised peptides based on a conceptual translation of the LBP DNA sequence obtained in this study (Table 3.2). Initially a 15 aa peptide was designed against a conserved portion of the C-terminus (Table 2.7). However, the LBP-C peptide had a high proportion of hydrophobic residues and was found to be insoluble in a number of solvents. The resulting antibody was non specific so another peptide was designed.

The LBP-N antibody was produced from a 15 aa peptide designed from a conserved N-terminal region on the translated protein sequence (Table 2.8). This region corresponded to an unstructured region on the N-terminus of the BPI structure. With a much lower hydrophobicity ratio, no problems were encountered with the solubility of this peptide.

3.4.1 ACTIVATION OF CARRIER PROTEINS

The first step of the conjugation was activation of the carrier proteins KLH and ovalbumin. This was accomplished by incubation with MBS and purified by running through a sephadex column. The eluted fractions were measured for

protein content by a Bradford assay and those within the peak were pooled (Figure 3.4).

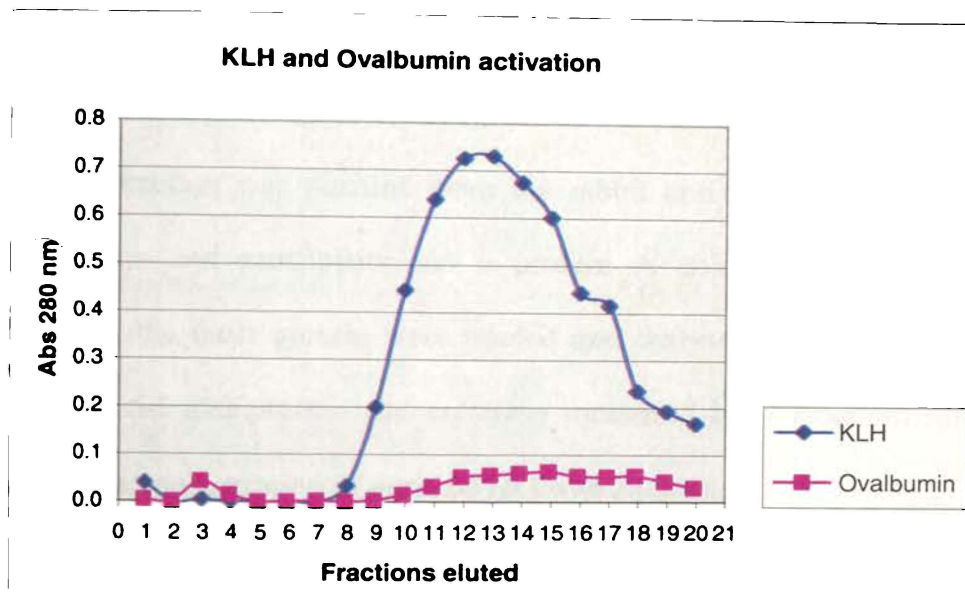


Figure 3.4: Sephadex purification of carrier proteins

The carrier proteins, hemocyanin (KLH) and ovalbumin, were activated with a linking reagent and purified through a sephadex column. The resulting fractions were tested by a Bradford assay and the fractions within the peaks were pooled (fractions 10-17 for KLH, and 12-18 for ovalbumin).

A Bradford assay was carried out to measure protein content in the pooled samples. This gave a total concentration of activated protein of $0.177 \mu\text{g}/\mu\text{l}$ for KLH, and $0.03 \mu\text{g}/\mu\text{l}$ for ovalbumin.

3.4.2 PEPTIDE CONJUGATION

The peptide was conjugated to the activated carrier proteins and any unconjugated peptide removed by dialysis. The solutions were collected in a final volume of 2 ml for ovalbumin and 4.5 ml for KLH. A Bradford assay was performed on these to determine the final protein concentration of conjugated peptide. Protein

concentration was 0.09 $\mu\text{g}/\mu\text{l}$ for ovalbumin-LBP and 0.26 $\mu\text{g}/\mu\text{l}$ for KLH-LBP. The KLH-LBP solution was then aliquoted into appropriate concentrations for the inoculation of two rabbits.

3.4.3 IgG PURIFICATION OF ANTI-SERA

The IgG fraction was purified from the rabbit anti-sera by ammonium sulfate precipitation and purification on a protein A column. The eluted fractions containing the most protein were pooled and dialysed overnight. The dialysate was collected and protein concentration measured by a Bradford assay which showed the concentration of purified IgG was 2 $\mu\text{g}/\mu\text{l}$.

3.5 WESTERN BLOTTING

LBP was detected in a variety of milk fractions from different stages of lactation by western blot analysis with the LBP-N antibody (Figures 3.5 and 3.6).

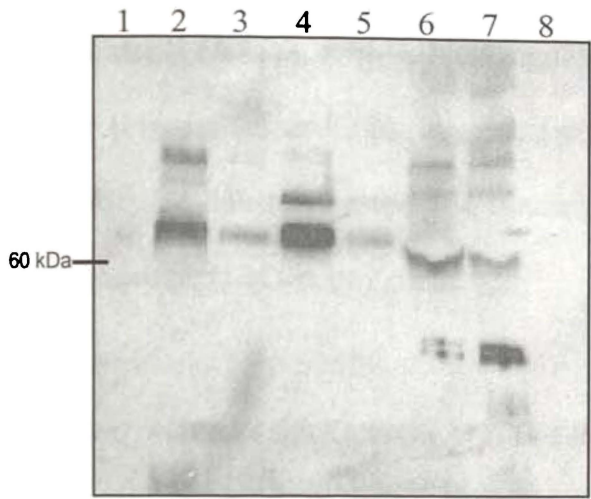


Figure 3.5: Western blot analysis of LBP in bovine serum and whey samples

Bovine whey and serum samples were tested for the presence of LBP by Western blot analysis with an anti-LBP antibody. Lanes are as follows; 1: normal lactation whey, 2: day one colostrum whey, 3: mastitic whey, 4: eight day involuted whey, 5: late lactation involuted whey, along with bovine serum samples from a healthy cow (lane 6) and a cow suffering a toxic infection (lane 7). Bovine serum albumin was run as a negative control (lane 8). A molecular weight marker (Invitrogen) was run and the position of the 60 kDa band is shown. Five minute exposure.

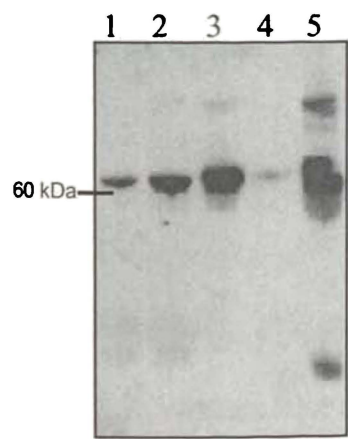


Figure 3.6: Western blot analysis of LBP in bovine whey and skim milk samples

Bovine milk fractions were tested for the presence of LBP by Western blot analysis with an anti-LBP antibody. Lanes are as follows: 1: subclinical mastitic skim milk, 2: 72 hour involuted skim milk, 3: eight day involuted skim milk, 4: late lactation involuted whey, 5: day one colostrum whey. A molecular weight marker (Invitrogen) was run and the position of the 60 kDa band is shown. Two minute exposure.

The results with the LBP-N antibody showed a clear immunoreactive band at approximately 60 kDa, with a high signal in colostrum, mastitic and involuted samples (Figures 3.5 and 3.6). In some samples other MW bands are also present which may be due to non-specific binding or could be different glycoforms or cleavage products of the protein.

3.6 IMMUNOPRECIPITATION WITH THE LBP-N ANTIBODY

Immunoprecipitation was performed to determine if the LBP-N antibody was able to bind the native form of the protein, as this would be useful for the development of a purification method of LBP from milk. This was done using protein A agarose and whey fraction from a late lactation involuted mammary secretion. The resulting pellet and supernatant were tested for the presence of LBP by western blotting with the biotinylated LBP-N antibody (Figure 3.7).

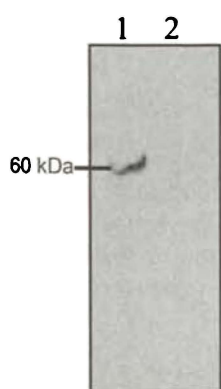


Figure 3.7: Western blot analysis of immunoprecipitation of LBP

Immunoprecipitation was performed by binding an anti-LBP antibody to protein A agarose beads and incubating these with a sample of involuted bovine whey. The beads were spun down and the supernatant collected (lane 2). Denaturing buffer was then added to release the bound LBP and this was also collected (lane 1). A protein marker (Invitrogen) was run and the position of the 60 kDa band is shown. Presence of LBP was detected by western blot analysis with a biotinylated anti-LBP antibody. Five minute exposure.

The resulting western blot showed a clear band at an approximate MW of 60 kDa in the sample from the pelleted protein A agarose, with nothing visible in the supernatant fraction (Figure 3.7). This shows that the antibody is able to bind the native form of LBP.

3.7 ELISA

The ELISA was used to quantify the amount of LBP present in a range of whey and serum samples based on a standard curve of human LBP (Figure 3.8). Over the course of the experiments the samples were found to give a range of concentrations (Table 3.5).

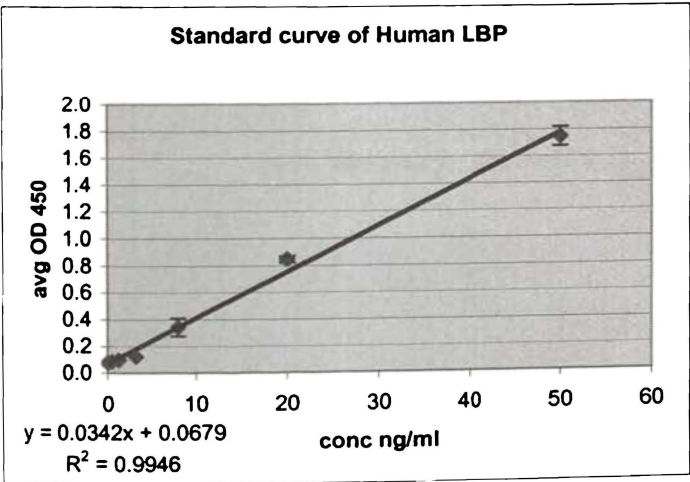


Figure 3.8: Representative standard curve of human LBP from ELISA

Table 3.5: LBP concentration ranges for bovine samples as determined by ELISA

Whey	Avg conc range (µg/ml)
Normal	0.5 - 3
Colostrum	9
Mastitic	13 - 20
Late lactation Involution	7 - 20
Serum	
Normal	6
Sick	26

3.7.1 SPIKE RECOVERY

Two spike recovery methods were used to determine the degree of specificity and accuracy of the system, and detect if any interference was occurring. The first method was to inoculate a linear dilution of mastitic whey samples with 20 ng of the standard human LBP. Based on the results from this it was concluded that the mastitic whey dilutions may have been too low, in which case the results seen could be due to interfering substances. Despite this, an increase is seen in the spiked samples (Figure 3.9), although it is larger than expected, and the percent recoveries calculated from the results show values greater than 100% (Table 3.6), suggesting interference or an underestimation of the spiked amount. Due to poor replication of the spiked dilutions the average concentrations could not be accurately determined meaning there is some difficulty in interpreting this data.

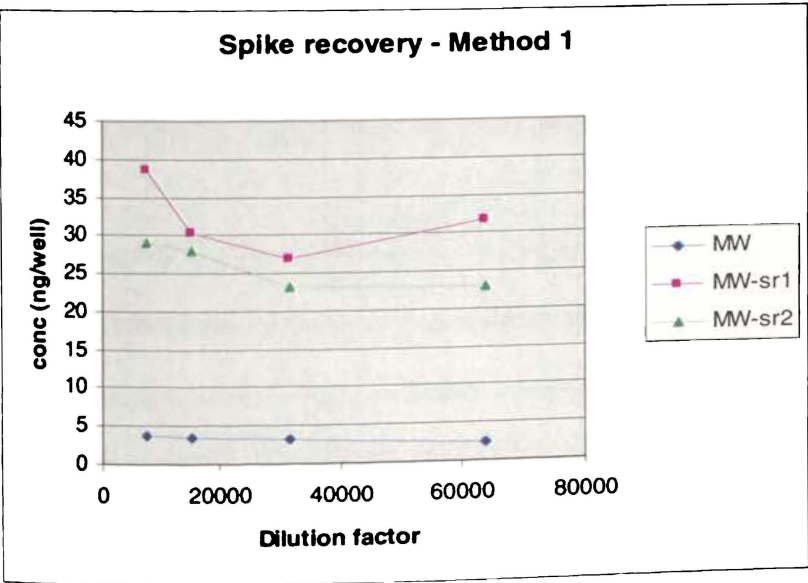


Figure 3.9: ELISA spike recovery method one

This method measured amount of LBP in bovine mastitic milk samples which had been spiked with 20 ng of standard human LBP. Key: MW is the average from the mastitic whey sample, MW-sr1 and MW-sr2 are the duplicate mastitic milk samples that were spiked with 20 ng.

Table 3.6: Percent recovery from spike recovery method one

Dilution	%R spike1	%R spike2
1:8000	161.99	122.25
1:16000	129.09	119.54
1:32000	116.39	100.46
1:64000	142.17	102.30

To more accurately detect any interference that could be occurring, a second spike recovery method was performed with mastitic whey being used to spike a dilution series of normal whey.

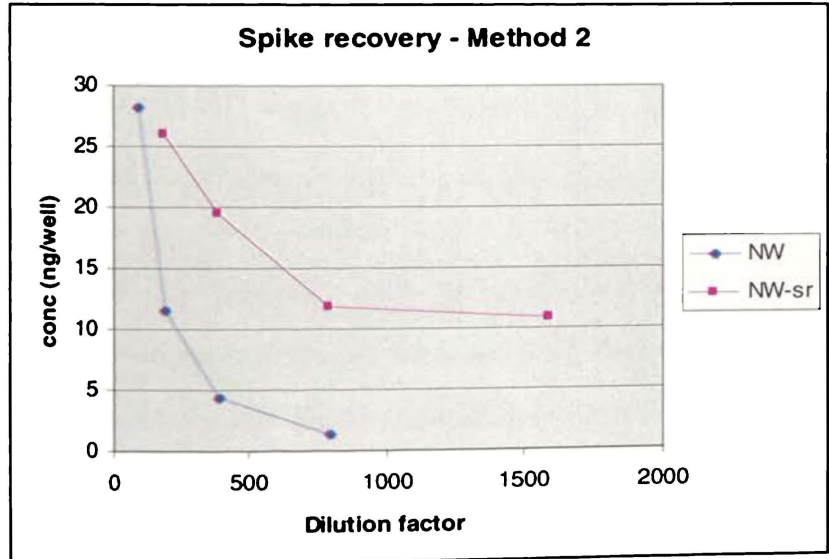


Figure 3.10: ELISA spike recovery method two

This method measured the amount of LBP in normal bovine whey samples which had been spiked with mastitic bovine whey containing approximately 10 ng of LBP. Key: NW is the serial dilution series of normal whey, NW-sr is the normal whey dilution series spiked with 10 ng LBP.

Table 3.7: Percent recovery from spike recovery method two

Dilution	%R
1:200	120.92
1:400	136.08
1:800	104.50

Results presented in Figure 3.10 show a definite increase in spiked samples compared to unspiked. The percent recovery values in Table 3.7 show similar results to those in Table 3.6, with values greater than 100%. This may indicate the presence of interfering factors, or could be due to inaccuracy in determining the concentration of LBP in the spiked volume.

Results indicate there was some discrepancy between the linear dilution and spike recoveries, which suggests that some interference is occurring. It was also observed that results differed between plates, making accurate determination of total concentrations difficult. The decrease in concentration seen in normal whey across experiments suggests that LBP may be being degraded or losing activity over time. Although multiple freeze-thawing was avoided by aliquoting the samples into small volumes, the storage conditions may not have been optimal.

3.8 RECOMBINANT PROTEIN EXPRESSION

3.8.1 RECOMBINANT LBP EXPRESSION IN BACTERIA

In order to produce a pure sample of LBP for activity analyses a recombinant protein was expressed in *E. coli* using the pET-LBP construct. Cell fractions were collected after induction of protein expression and the presence of LBP detected by western blot analysis with the anti-His and LBP-N antibodies.

The western blot probed with the anti-His antibody showed a clear immunoreactive band at approximately 60 kDa in the induced whole cell and insoluble fractions, which was not present in the non induced fraction or in the induced soluble fraction (Figure 3.11).

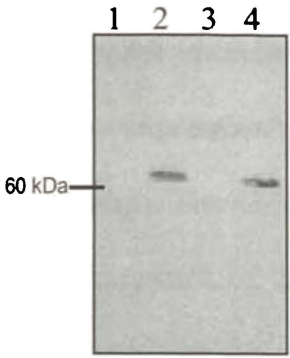


Figure 3.11: Detection of recombinant LBP expression in *E. coli* cells using an anti-histidine antibody

Recombinant bovine mammary LBP was expressed in BL21-SI *E. coli* cells. Cell extracts were analysed by western blotting using an anti-histidine antibody (Roche). The lanes are as follows; 1: non-induced whole cell extract, 2: induced whole cell extract, 3: induced soluble fraction, 4: induced insoluble fraction. The position of the 60 kDa band is shown. Five minute exposure.

The results of the western blot probed with LBP-N antibody show large smears in the induced whole cell and insoluble fractions (Figure 3.12).

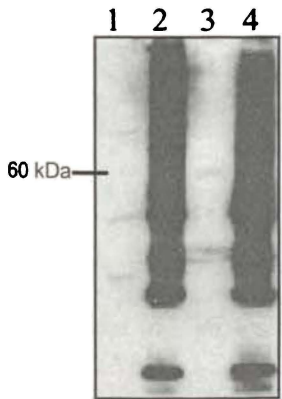


Figure 3.12: Detection of recombinant LBP expression in *E. coli* cells using an anti-LBP antibody

Recombinant bovine mammary LBP was expressed in BL21-SI *E. coli* cells. Cell extracts were analysed by western blotting using an anti-LBP antibody. The lanes are as follows; 1: non-induced whole cell extract, 2: induced whole cell extract, 3: induced soluble fraction, 4: induced insoluble fraction. The position of the 60 kDa band is shown. Thirty second exposure.

3.8.2 RECOMBINANT LBP EXPRESSION IN MAMMALIAN CELLS

Recombinant expression was also performed in mammalian cells using the Gateway expression system to produce a protein with greater similarity to the native form. The G-LBP construct was transfected into cos7 cells, along with a negative control of the pDEST26 vector only, and a positive control of stat5b. After three days the media was collected, the cells were lysed, and the RNA and protein extracted for analysis.

3.8.2.1 Northern Blotting

The RNA collected from the cells was analysed by northern blot analysis by probing with LBP (Figure 3.13).

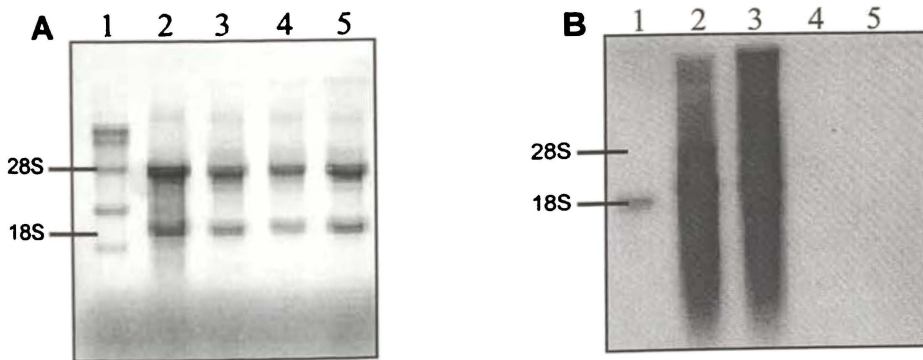


Figure 3.13: Detection of recombinant LBP mRNA expression in cos7 cells

Cos7 cells were transfected and the RNA collected after two days for detection of LBP expression by northern blot analysis. (A) Methylene blue stain of northern membrane. (B) Thirty minute exposure of northern blot on XAR film after hybridisation with an $\alpha^{32}\text{P}$ -labelled DNA probe specific to LBP. An RNA marker is shown in lane 1. Lanes 2 and 3 contain the RNA from cells transfected with the LBP construct, while lanes 4 and 5 represent the negative control of RNA from cells transfected with vector only.

Northern blot analysis exhibited a strong signal in the RNA from cells transfected with the LBP construct, indicating that mRNA for LBP is being expressed in these cells. In contrast, no signal is seen in cells transfected with the negative control RNA (Figure 3.13).

3.8.2.2 Western Blotting

The protein samples were tested by western blot analysis using the LBP-N antibody and anti-His antibodies to detect the presence of LBP (Figures 3.14 and 3.15) and an anti-stat5b antibody for the positive control (Figure 3.16).

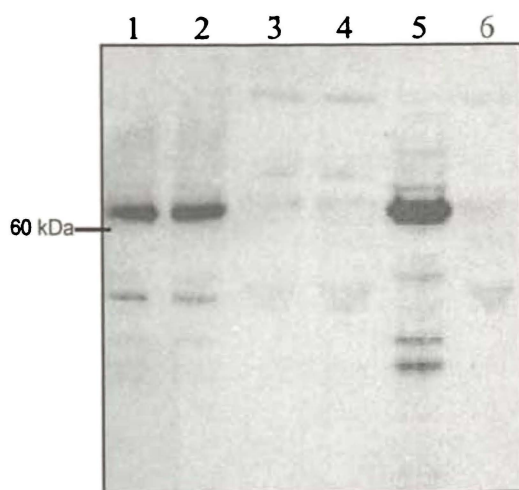


Figure 3.14: Detection of recombinant LBP protein expression in cos7 cells with an anti-LBP antibody

Cos7 cells were transfected with an LBP construct produced by the Gateway system (Invitrogen) (lanes 1 and 2). A transfection control was done using vector only (lane 5). The cell growth media was also collected from each fraction and tested (lanes 3 and 4: LBP construct, lane 6: vector only). Protein was harvested from the cells and the presence of LBP detected by western blot analysis with an anti-LBP antibody. A protein marker (Invitrogen) was also run and the position of the 60 kDa band is shown. Two minute exposure.

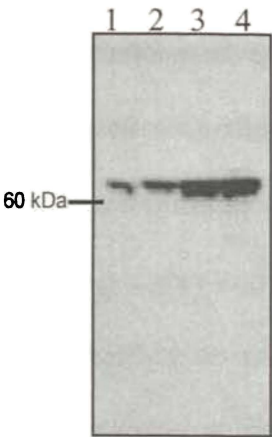


Figure 3.15: Detection of recombinant LBP protein expression in cos7 cells with an anti-histidine antibody

Cos7 cells were transfected with a histidine-tagged LBP construct produced by the Gateway system (Invitrogen) (lanes 1 and 2). Controls were also done with cells transfected with vector only (lanes 3 and 4). Protein was harvested from the cells and the presence of LBP detected by western blot analysis with an anti-histidine antibody. The position of the 60 kDa band is shown. Two minute exposure.



Figure 3.16: Detection of recombinant stat5b expression in cos7 cells

Cos7 cells were transfected with a construct of stat5b as a positive transfection control. Protein was collected from the cells and analysed by western blotting with a stat5b antibody. The position of the 85 kDa band on the molecular weight marker is shown. Two minute exposure.

Westerns probed with the LBP-N antibody and the anti-His antibody both show an immunoreactive band at approximately 60 kDa in samples from cells transfected with the LBP construct. However, in the negative controls of cells transfected with vector only there is also a band at this level, which often appears to have a higher signal than the positive samples and is present when probed with either LBP-N or anti-His antibodies (Figures 3.14 and 3.15). This result was surprising and a number of westerns on different transfection samples were carried out to determine the cause of this but as yet it is unknown and further investigation is required to resolve these issues.

3.9 PURIFICATION OF RECOMBINANT LBP

To obtain a sample of pure LBP the recombinant protein was purified by nickel affinity chromatography with the ProBond system (Invitrogen). The eluted fractions were run on an SDS-PAGE gel along with fractions from the flow through and washing steps. The presence of the protein was detected by western blot analysis using the LBP-N and anti-His antibodies. These showed no bands in the purified samples from native or denaturing conditions (results not shown).

Because of the low and sometimes undetectable yields from the ProBond purification, a Vivaspin mini His column (Vivascience) was used to attempt to purify recombinant protein from mammalian cell lysates. The fractions from this were tested on a western blot as described above, however these were also unsuccessful in identifying bands corresponding to the recombinant protein in the purified samples (results not shown).

3.10 LBP ACTIVITY

LBP activity was assessed by measuring LPS binding and cytokine inducing abilities.

3.10.1 ELISA

The ELISA results described in Section 3.7 demonstrated the ability of native LBP in whey samples to bind to LPS. The ELISA is based on two steps, the first being the binding of LBP to an antibody specific for LBP, followed by detection of bound protein by LPS binding. The first step means that although some interference was seen in the ELISA experiments, the results can be predominantly attributed to the presence of LBP binding in the milk, rather than other LPS binding proteins.

3.10.2 IMMUNOPRECIPITATION WITH *E. COLI* PROTEINS

LPS binding was also assessed by immunoprecipitation using a whey fraction from a late lactation involuted mammary secretion with *E. coli* proteins in agarose. The western blot showed a clear band in the pelleted fraction at an apparent MW of 60 kDa (Figure 3.17). The supernatant fraction showed no immunoreactivity with biotinylated LBP antibody. The same result was seen when the blot was stripped and reprobed with biotinylated-LPS (Figure 3.17).

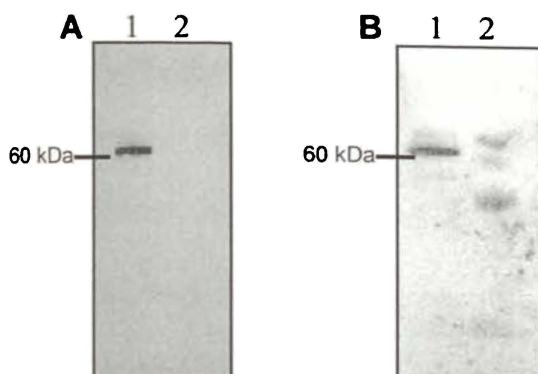


Figure 3.17: Western blot analysis of immunoprecipitation with *E. coli* proteins

Immunoprecipitation was performed by incubating a sample of late lactation involuted whey with *E. coli* proteins in agarose (Sigma). The agarose beads were spun down and the supernatant collected (lane 2). Denaturing buffer was added to cause release of bound LBP and this is shown in lane 1. Presence of LBP was detected by western blot analysis with an anti-LBP antibody shown in (A), or with a biotinylated LPS conjugate (B). The position of the 60 kDa band is shown. Five minute exposure.

The results shown in Figure 3.17 indicate that factors present in the whey sample are able to bind to *E. coli* proteins. The presence of one immunoreactive band at the correct MW in both blots suggests the band present is LBP.

3.10.3 LIGAND BLOTting WITH LPS

Ligand blotting was also performed using a biotinylated LPS conjugate to investigate LPS binding ability on a range of milk and serum samples. The results shown here are from blots probed with the LPS conjugate produced as described in Section 2.11.3.1.

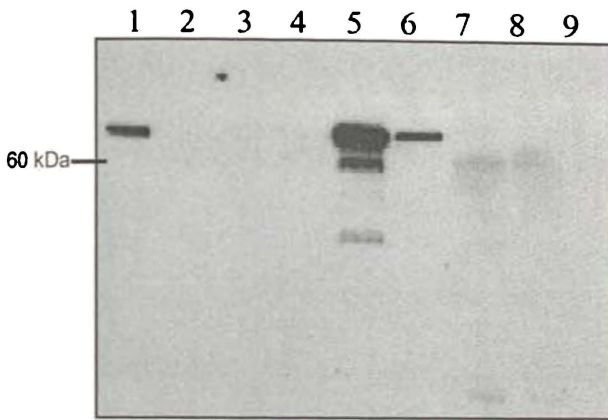


Figure 3.18: Ligand blot of bovine serum and whey milk samples

Protein samples were tested by western blotting with a biotinylated LPS conjugate. Bovine whey samples were run as shown in lanes; 2: normal lactation, 3: day one colostrum, 4: mastitic, 5: eight day involuted, 6: late lactation involuted. A sample of human normal lactation whey is shown in lane 1, and bovine serum samples from a healthy cow and a cow suffering a toxic infection are shown in lanes 7 and 8 respectively. Bovine serum albumin was run as a negative control (lane 9). The position of the 60 kDa band is shown. Five minute exposure.

Figure 3.18 shows a high signal in both involuted whey samples and in human normal lactation whey, a faint signal was also present in mastitic whey. Faint bands are also present in both serum samples, with a lower molecular weight band at approximately 30 kDa also present.

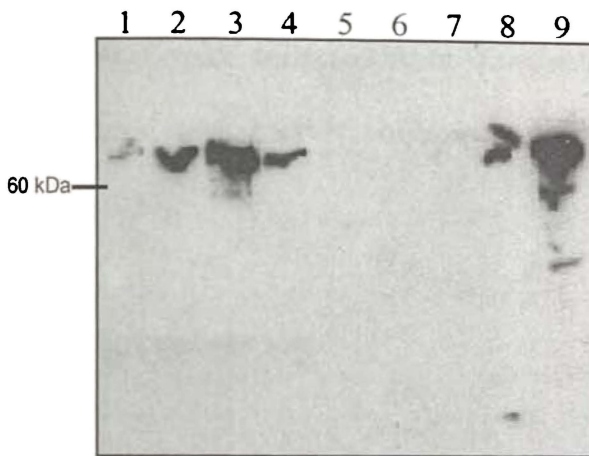


Figure 3.19: Ligand blot of bovine skim and whey milk samples

Protein samples were tested by western blotting with a biotinylated LPS conjugate. Bovine milk samples were run as shown in lanes; 1: subclinical mastitic skim, 2: 72 hour involuted skim, 3: eight day involuted skim, 4: once daily milking skim, 5: normal lactation whey, 6: day one colostrum whey, 7: mastitic whey, 8: late lactation involution whey, 9: eight day involuted whey. The 60 kDa band is shown. Five minute exposure.

Figure 3.19 shows high intensity bands in involuted whey and skim samples, with faint bands visible in the mastitic milk samples also.

These results are slightly different to those seen in blots probed with the LBP-N antibody, which show high levels of LBP in colostrum and mastitic milks as well as involuted samples (Figures 3.5 and 3.6). However, these results do verify that the protein being detected is able to bind to LPS.

3.10.4 CYTOKINE INDUCTION ASSAY

The ability of the mammalian recombinant LBP protein to induce cytokine production was measured by stimulating $\text{TNF}\alpha$ production in human monocytes and quantifying the amount of $\text{TNF}\alpha$ produced using a cell proliferation assay.

3.10.4.1 Cell Lysate Preparation

Cell lysates were collected from transfected cos7 cells and tested on a western blot probed with LBP-N antibody to detect the presence of recombinant LBP (Figure 3.20).

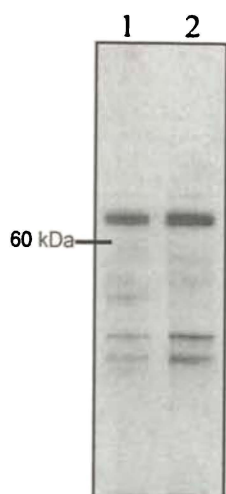


Figure 3.20: Detection of recombinant LBP in mammalian cell lysates

Cos7 cells were transfected with either an LBP construct produced by the Gateway system (Invitrogen), (lane 1), or a negative control of vector alone (lane 2). The presence of LBP was detected by western blotting with an anti-LBP antibody. The position of the 60 kDa band is shown. Two minute exposure.

The results from the western blots show a range of bands, with a clear band just above the position of the 60 kDa marker (Figure 3.20). The slight increase in MW may be due to the addition of extra residues coding for the TEV protease cleavage site and the histidine tag. The presence of the band in both the positive and negative samples is similar to that seen in Figure 3.14 and may be due to non-specific binding or errors in the transfection procedure. Because this result is unexpected the experiment was repeated, but gave similar results each time, so it was decided to continue with the assay anyway as it was possible that a difference in effect may be seen between the samples.

3.10.4.2 Cell Proliferation Assay

The amount of $\text{TNF}\alpha$ produced by human monocytes after stimulation with recombinant LBP and LPS was measured on a cell proliferation assay using WEHI-13VAR cells. A standard curve of $\text{TNF}\alpha$ was prepared and is shown in Figure 3.21.

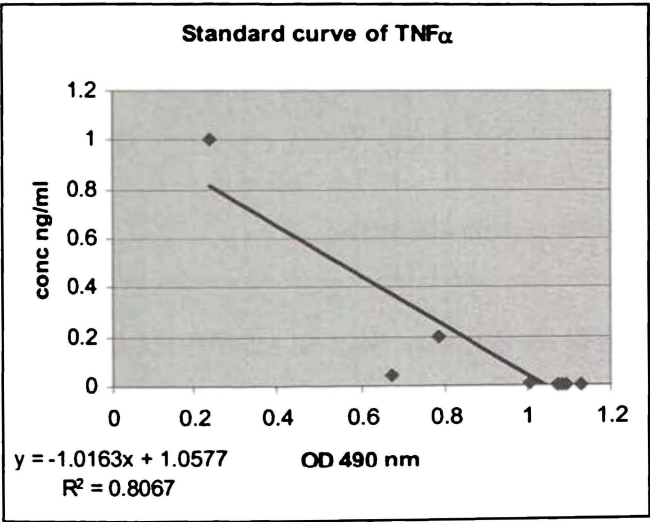


Figure 3.21: Standard curve of $\text{TNF}\alpha$ concentration

Figure 3.21 shows the concentration range of $\text{TNF}\alpha$, with increasing concentration and OD being equivalent to increased mortality of the WEHI-13VAR cells. The range of concentrations measured was too low to produce a linear standard curve, and because of this the concentrations of the samples could not be accurately determined. However, visual observations of the plates showed a definite colour gradient and some analysis was performed on the optical densities measured.

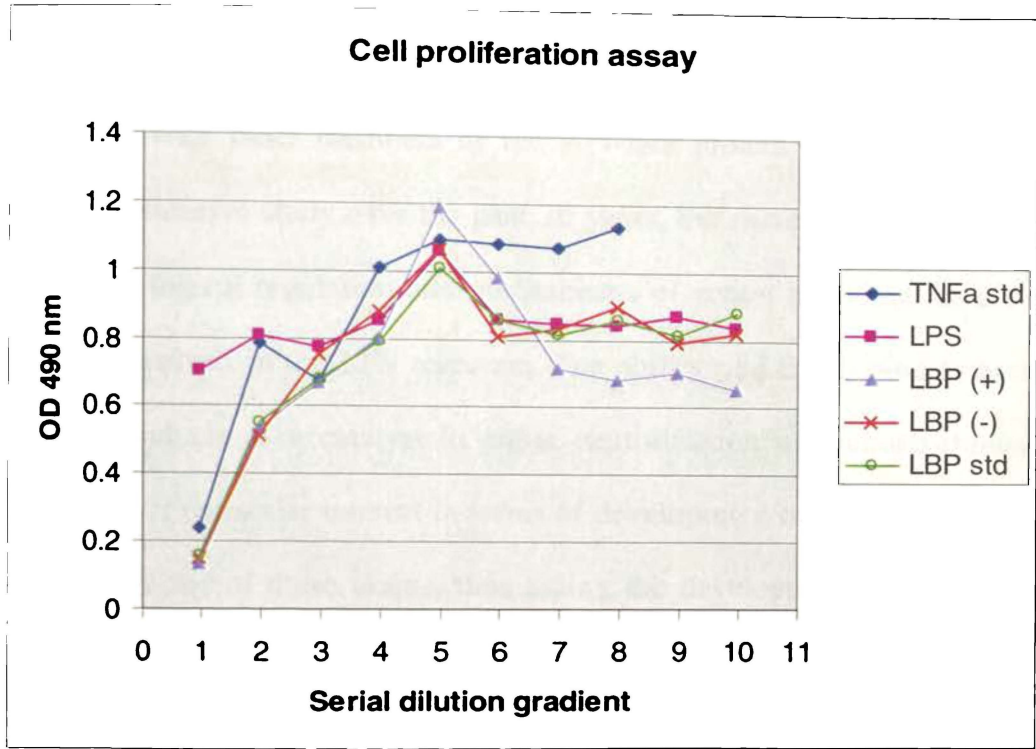


Figure 3.22: Effects of LBP and LPS on WEHI cell proliferation assay

Samples containing recombinant LBP with 10 ng LPS were used to induce TNFα production in monocytes, which was quantified by a WEHI cell proliferation assay. Cell lysates from cos7 cells transfected with LBP (LBP +) or vector only (LBP -) were tested along with standard human LBP (LBP std) from HBT. Standard dilutions of LPS and TNFα were also included. All samples were diluted in a five-fold serial gradient.

The cell proliferation results show a similar pattern occurring in the samples from transfected cells and the standard human LBP sample, which is surprising as this includes the negative control cell lysate. These samples also show a difference to the LPS standard curve which is more defined at higher concentrations (Figure 3.22). Further investigation will have to be undertaken in order to assess the activity of LBP more accurately.

CHAPTER 4 DISCUSSION

LBP, along with other members of the BPI-like protein family, has been the subject of extensive study over the past 20 years, but there is still much to learn about its biological regulation and mechanisms of action including the different pathways involved in the LPS response. The ability of LBP to bind to bacterial endotoxin and act as a catalyst in either neutralisation or immuno-stimulatory pathways is of particular interest in terms of developing a better understanding of the pathogenicity of these toxins, thus aiding the development of more specific and efficient treatments and detection systems. This is particularly important for diseases such as sepsis and endotoxemia which have high mortality rates and are difficult to treat (Oh, 1998; Bochud and Calandra, 2003). The discovery that LBP also has a role in the response against toxins from G+ve bacteria and fungi (Fan *et al.*, 1999; Blairon *et al.*, 2003; Weber *et al.*, 2003), has widened its proposed role in the immune response, identifying it as a broad range pattern recognition receptor.

The identification of an EST from a mastitic mammary cDNA library which had sequence similarity to LBP initiated this project, as the presence of bioactive molecules in the bovine mammary gland and particularly in milk fractions, is of considerable interest for the development of nutraceuticals, diagnostic kits or medical treatments. The ability to extract and purify the protein from milk offers an alternative from other sources such as serum or synthetic processes.

4.1 FULL LENGTH SEQUENCING

Full length sequencing and alignments with other published LBP sequences showed a high degree of sequence similarity between all sequences. This includes the highly conserved cysteine residues and five potential glycosylation sites. Although only an N-terminal fragment of bovine LBP has been published (Khemlani *et al.*, 1994), this was also included in alignments with the translated protein sequence. The two bovine LBP sequences were identical apart from seven consecutive residues. Based on the alignments with other LBP N-terminal sequences from different species, this region is highly conserved so it may be that discrepancies in this sequence are due to errors in the sequencing reaction or the interpretation of the data. Confirmation of this result would allow the protein to be identified accurately as either the same as bovine LBP from serum, or a mammary derived isoform of the protein.

4.2 NORTHERN BLOTTING

Northern blotting analysis was used to observe the expression patterns of LBP mRNA over a range of mammary tissues at various stages. The expression profile showed high levels of expression in involuted and infected tissues, indicating that LBP levels in the mammary gland are increased at times when the defences of the gland are stimulated to either pass on immune factors to the growing neonate, or to protect the gland during stress or infection. This is consistent with the finding that levels of plasma LBP are raised soon after the onset of infection (Schumann *et al.*, 1990). Expression was high in both cisternal and alveolar sections of mastitic mammary tissue, suggesting a major role for LBP in the innate immune defence of the gland against pathogenic attack.

These results were compared with northern blots probed with CD14, the receptor required for the LBP-mediated response. This showed a similar pattern of expression to LBP, with high levels found in infected tissues. The main observation from this, however, is that the highest signal was observed in the RNA sample from mastitic milk cells, whereas there was no expression of LBP detected in this sample. This sample would contain a mixture of immune cells such as lymphocytes and neutrophils as well as epithelial cells which have sloughed off. These results confirm the finding of Wright *et al.* (1990) that CD14 is entering the gland with the phagocytic cells, whereas LBP must be entering the gland by another means. It has been suggested that LBP could enter the gland in a similar way to BSA, via increased vascular permeability at times of infection (Bannerman *et al.*, 2003). The alternative hypothesis is based on the discovery of extra-hepatic expression in a range of epithelial tissues including the intestine and respiratory tract (Vreugdenhil *et al.*, 1999; Dentener *et al.*, 2000), offering the possibility that the mammary gland may also locally produce LBP.

4.3 *IN SITU* HYBRIDISATION

Local expression of LBP mRNA in the mammary gland was tested directly by *in situ* hybridisation on a small set of mastitic and control bovine mammary tissues. Expression of LBP was detected in epithelial cells surrounding ducts and alveoli in a tissue with a subclinical infection. This finding is significant as it is the first published result showing local expression of LBP mRNA in the mammary gland and also because it shows the induction of LBP expression during an infection where the predominant pathogen is a G⁺ve bacterium (*Staphylococcus aureus*). It has previously been shown that serum levels of LBP increase during G⁺ve sepsis

(Blairon *et al.*, 2003), and also that LBP is able to bind to elements of the G+ve bacterial cell wall (Weber *et al.*, 2003). The induction of LBP expression confirms what was seen in the northern blot results which showed expression of LBP mRNA was increased in mastitic tissue and was not present in RNA isolated from mastitic milk. These findings suggest that locally expressed LBP may play an important role in the innate defence of the bovine mammary gland.

LBP expression was not detected in the other mammary tissues tested, which included mastitic and control cisternal tissues and a sample of infected teat. No signal was detected in the sections probed with α -lactalbumin. This is unusual since α -lactalbumin is a well known mammary expressed protein (Molenaar *et al.*, 1992), and may reflect the age of the probes or the possibility that degradation has occurred which would account for the lack of signal. Alternatively, something may have occurred during the preparation process which resulted in deficient α -lactalbumin probes. Because of this, it is not possible to accurately predict if the lack of signal on the other tissues probed with LBP was due to a lack of expression. Due to time constraints, the experiment was limited to a single investigation on a small range of tissues and in the light of the findings here it is reasonable to assume that further optimisation may reveal a more definitive expression profile. Examination of the expression of LBP in the mammary gland in a wider range of tissues would allow for a greater understanding of the local regulation of LBP expression and the role it plays in defence of the mammary gland.

4.4 WESTERN BLOTTING

In order to detect the presence of the LBP protein in milk an antibody was raised against a peptide covering a conserved region of the translated protein sequence. Initially, an antibody was raised against a C-terminal peptide, but this was unsuccessful due to a high proportion of hydrophobicity rendering it too insoluble to produce an active antibody. Because of this a second peptide from the N-terminal region was used to produce an antibody in rabbits, from which the serum was collected and the IgG fraction purified. After characterisation, the antibody was used to detect LBP in milk samples using western blot analysis. These showed a similar expression pattern to that seen in the northern blots, with the highest levels of LBP being detected in colostrum, mastitic and involuted secretions. Expression was also seen to increase with the duration of involution, with eight day samples showing a much higher signal than milk collected 72 hours after the onset of involution.

The presence of LBP in mastitic bovine whey has recently been detected by ELISA (Bannerman *et al.*, 2003). The results from the present study demonstrate that LBP is also present in bovine whey from colostrum and involuted secretions, as well as skim milk secretions from infected and involuted tissues. These results lend further support to the hypothesis that LBP plays a role in the innate immune defence of the mammary gland. Some variability was seen in banding patterns on the western blots which has previously been observed by Khemlani *et al.* (1994), and could be attributed to the presence of different glycoforms of LBP.

4.5 IMMUNOPRECIPITATION

Immunoprecipitation of late lactation involuted whey with the LBP-N antibody shows the ability of the antibody to bind the protein in its native state. This finding is useful for the development of a purification method to extract LBP from milk samples allowing a pure native protein to be used in further investigations. Purification methods have been described for bovine and human serum based on ion exchange chromatography (Schletter *et al.*, 1994; Horadagoda *et al.*, 1995; Bochsler *et al.*, 1996). The ability to purify LBP from milk samples has the potential to be scaled up for commercial extraction for nutraceutical and/or pharmaceutical applications.

4.6 ELISA

Based on the results from the western blot analyses, the amount of LBP present in normal, colostrum, mastitic and involuted whey secretions was quantified with a commercial ELISA kit. The amount of LBP was increased in all samples compared with normal lactation whey, and increased during infection or involution at a similar level to LBP in serum.

These results differ slightly from those published by Bannerman *et al.* (2003), which measured changes in levels of LBP in bovine milk during nonpathogenic mastitic infection induced by inoculation with LPS, and gave slightly higher amounts of LBP compared with those shown here. The present study detected similar levels of LBP in serum and whey samples, whereas Bannerman *et al.*, found that the LBP levels in whey were lower than in serum. This difference could be due to different infection states, as the mastitic sample used in this study

had been induced by inoculation with a whole bacterium (*Streptococcus uberis*) compared with inoculation of purified LPS. It is unknown what effect this could have on LBP expression, but factors such as the status and type of infection, combined with other factors such as the cattle breed, age, farm management and nutrition may also influence the concentration of LBP in the milk (Stelwagen, 2002a, b).

Two spike recovery methods were used to test for any interfering substances in the ELISA reaction and the results from these suggested that there was some interference occurring. The first method used the standard LBP provided to spike a linear dilution series of mastitic whey. The percent recovery from this shows interference was occurring, causing the LBP concentrations to be overestimated. It may be that the standard LBP interacts differently with the detection system compared with the endogenous LBP in the milk sample. To account for this, a second method was tested using an estimated concentration of mastitic whey to spike a linear dilution series of normal whey. The percent recovery from this also shows interference occurring and it is thought that because of the difficulties in quantifying the amount of LBP in mastitic whey the spiked amount may not have been accurately estimated. There are a number of other possible factors which may account for some of the interference detected by these methods. These include the possibility of endogenous LPS present in the sample binding to LBP and leading to an underestimation of the actual concentration of LBP in the milk, and non specific binding by the antibody involved in the detection system. If the antibody was binding non-specifically to other LPS binding proteins present in the

samples, or had different binding efficiencies for the standard LBP compared to native LBP in milk, this could also give overestimated values.

The results also showed that the concentration of LBP gradually decreased over the course of the experiments, which could be due to degradation of the protein. Further investigation into addressing these issues would allow for a more accurate quantification of the amount of LBP present in milk.

4.7 RECOMBINANT PROTEIN EXPRESSION

A recombinant protein was produced in both bacterial and mammalian cells for use in activity assays. Expression in bacteria was performed using the pET system (Novagen), and salt-inducible BL21 *E. coli* cells. This involved producing a construct with restriction enzyme sites at either end to ensure uni-directional cloning, and a two stage cloning procedure into the pET expression vector via the transitional pGEM-T Easy vector. Two constructs were produced, one which encoded the mature protein sequence and another which allowed for the addition of a C-terminal histidine tag. The presence of the protein was confirmed by western blot analysis using both the LBP-N antibody and also an antibody which recognised the histidine-tag.

Western blot results detected LBP in the insoluble cell fraction, which is common for proteins that are large and potentially toxic to the cell. Due to its size and ability to bind to the LPS component on the cell wall of the bacteria it is most likely that the recombinant LBP protein would be packaged into inclusion bodies, which make up the insoluble fraction. This can make it more difficult for

recombinant protein production and purification as the proteins packaged in this way are often not correctly folded, and also need harsher chemicals to be released from the inclusion bodies.

The western blot probed with LBP-N antibody showed large smears which could be due to the antibody binding non-specifically, degradation of the protein by cellular enzymes, or the protein may be binding to aggregates of LPS from the bacterial cell wall. If it is the latter, this would indicate that LPS binding activity does not require glycosylation for activity. Optimisation of the expression system and further tests to determine the cause of these results would aid in a greater understanding of the structure/binding relationship between LBP and LPS. Further tests using site directed mutagenesis on potential glycosylation sequences could also be used in determining the requirements of glycosylation for activity of LBP.

Expression was also performed in mammalian cos7 cells in order to produce a protein more similar to the native form. A construct was prepared using the Gateway system (Invitrogen) for expression in the pDEST26 vector, which includes an N-terminal histidine tag, allowing the resulting protein to be purified by metal chelation chromatography. A TEV protease cleavage site was encoded before the string of histidine residues to allow removal of the His-tag if necessary. Results from the northern blots showed that the mRNA for LBP was being expressed in LBP transfected cells, but the western results did not show any difference in band pattern between cells transfected with LBP or vector alone. Western blots of the stat5b transfection which were probed with the LBP-N antibody did not show these bands, suggesting that something is changing during

the protein translation/expression stages which may be related to the Gateway pDEST26 vector. There are a number of possibilities for this result including that there is some kind of interference occurring with the pDEST26 vector, the antibody is binding non-specifically, or there may have been handling errors in the transfection procedure. Further investigation is required to fully determine the cause of the problem and better optimise the system for protein expression. This could include using a wider range of controls such as untransfected cells as well as trialling other vectors, cell types and expression systems.

4.8 PURIFICATION OF RECOMBINANT PROTEIN

The constructs produced for recombinant protein expression in the pET and Gateway expression systems allowed the addition of a tag of histidine residues onto the end of the protein. The His-tag allows the protein to be purified from the cell extracts using a nickel affinity column with the ProBond purification system (Invitrogen) or Vivaspin mini-His columns (Vivascience). Both the native and denaturing purifying conditions were trialled with the ProBond system, with very low yields collected. This could be due to the problems with the expression systems or the protein becoming degraded. Because LBP is such a large protein it is possible that the histidine tag was obscured during folding leading to a lower binding capacity or the elution step may not have been sufficient to release bound protein. The optimisation of the expression system is required in order to better define where the problems in purification are occurring. The development of affinity purification techniques using the LBP-N antibody could be a useful alternative for protein purification.

4.9 LPS BINDING ACTIVITY

The LPS binding ability of the LBP in milk samples was tested by three different methods which confirmed binding ability and also identified LBP as the major LPS binding protein in milk. This is interesting, as a number of proteins which are present in milk are also known to bind to LPS, such as lactoferrin and cationic peptides (Elass-Rochard *et al.*, 1998; Scott *et al.*, 2000). However, ligand blot analysis with biotinylated LPS showed very few bands present, with the predominant bands in involuted milk samples matching those seen in western blots with the LBP-N antibody. Immunoprecipitation with *E. coli* proteins in agarose also gives an alternative possibility for purification of LBP from milk samples using an LPS affinity column.

It is interesting to note that the ligand blot analysis also showed the ability of LBP in milk samples to bind to LPS after being denatured on an SDS-PAGE gel. The LPS binding site is known to cover approximately 200 residues in the N-terminal domain, (Han *et al.*, 1994; Theofan *et al.*, 1994), in which case these must have retained their binding ability despite being subjected to denaturing conditions. It may be that the experimental conditions allowed the proteins to renature sufficiently for LPS binding to occur. It is also possible that the LBP in the other samples detected with the LBP-N antibody, such as colostrum and mastitic whey may not have retained LPS binding ability after being denatured. Alternatively the degradation of the LBP protein in these samples as seen in the ELISA may have affected the ability of LPS binding to be detected.

4.10 CYTOKINE INDUCTION

The immuno-stimulatory activity of LBP was investigated using an assay which measures the induction of TNF α production in monocytes. This involved the stimulation of TNF α production by incubating monocytes with varying concentrations of LBP and a standard amount of LPS. The amount of TNF α produced was quantified in a cell proliferation assay using WEHI-13VAR cells. The results from this showed a difference between the standard curve of LPS and the other samples. A similar pattern was observed in all the LBP samples which included standard human LBP and cell lysates from cells transfected with LBP or with vector alone. This was unexpected, as the negative control (vector alone cell lysate) showed a similar pattern to the samples containing LBP, making it difficult to interpret the results. There were also problems with the concentration ranges of TNF α and LPS used and optimisation of these is necessary for a more accurate analysis. Due to the problems encountered with the recombinant expression system it would also be desirable to use a sample of purified native protein from milk. Other experiments could also be performed to look at inhibition of the response using anti-LBP antibodies, which would verify that it is LBP causing the response.

CHAPTER FIVE SUMMARY AND CONCLUSIONS

The aim of this project was to investigate the presence of LBP in the bovine mammary gland for the purpose of determining the potential of LBP as a nutraceutical product from milk. This included characterising LBP expression in tissue and milk samples and producing a recombinant protein to examine immune related activity.

Although LBP has been detected in bovine and human milk, the source of the protein was unknown, with most researchers assuming that the protein was being transported into the gland along with the neutrophils and other immune factors. The results from this study are the first to prove that local expression of LBP occurs in the bovine mammary gland. This was shown by northern blot and *in situ* hybridisation analyses which identified the epithelial cells surrounding the ducts and alveoli as a site of LBP mRNA expression. Further evidence to support these results and define the expression patterns would be useful in understanding the regulation of mammary produced LBP. Local expression of LBP in the mammary gland suggests that it plays an important role in the innate immune defence systems of the gland, while also possibly playing a role that is specific to the site of expression. These findings are significant in the development of LBP as a nutraceutical product, as a better understanding of the regulation and expression means that LBP can be extracted from mammary secretions with higher quantities, and could also allow manipulation of the system to produce more LBP.

The production of an antibody specific to an N-terminal region of LBP (LBP-N) allowed the protein to be detected by western blotting in a range of milk and

serum samples. These results were quantified with a commercial ELISA which showed that the relative concentrations of LBP were increased in secretions from infected and involuted glands, supporting the findings from the northern and western blots. Because some interference was detected in the ELISA, further optimisation may be required to identify interfering substances and provide a more accurate quantification of LBP concentrations in milk samples. However, the results do indicate that LBP is present in mammary secretions at concentrations which are appropriate for extraction and purification for use as a nutraceutical or nutritional additive.

The antibody was also shown to bind to the native protein by immunoprecipitation with protein A agarose and a whey sample from an involuted gland. This result is important in the development of a purification system for extraction of LBP from milk. As previous methods for extraction of LBP have only been developed for serum, a protocol for milk would be useful as it is a much more efficient and convenient source for commercial applications.

The LPS binding ability of the LBP protein in milk was tested by ligand blotting, ELISA and immunoprecipitation. These results matched those seen with the LBP-N antibody, confirming the presence of an active LBP protein in bovine milk. The results also identified that LBP was the major LPS binding protein in milk, with very few other bands detected in ligand blots. This finding indicates an alternative possibility for LBP purification from milk, using an LPS binding column. This could also be used for extraction of other LPS binding proteins

which may be of interest in other investigations of potential bioactive proteins from milk.

In order to investigate the activity of LBP further, a recombinant protein was produced in bacteria and mammalian cells. The detection of the bacterially expressed protein with the LBP-N antibody gave results which could suggest that glycosylation is not required for LPS binding activity. Structural investigations using site directed mutagenesis of potential glycosylation sites could be useful in developing a better understanding of the mechanisms of LBP activity. This knowledge could also be used in the development of derivatives of LBP which may be targeted to particular areas or actions for specific treatments.

Mammalian expression of the recombinant LBP protein also requires more specific optimisation and characterisation as the presence of the protein was difficult to verify by western blotting. The development of a recombinant protein would be useful in further investigations of the mechanisms and activity of LBP including the effects of LBP on a range of major pathogens including fungi and G⁺ve bacteria in both *in vitro* and *in vivo* assays.

The ability to bind and transfer LPS, triggering either neutralising or immunostimulatory pathways is a key target for the development of specialised nutraceutical products or therapeutic treatments. Derivatives of the protein may be useful for specifically targeting one of these pathways, or act by sequestering LPS and preventing its toxicity. This would be particularly useful in cases such as sepsis or endotoxemia where the immune response is over-stimulated, as LBP or

derivatives of the protein may be able to restore the balance by helping to clear the system via the neutralising pathway.

The results achieved during the course of this research have met the aims of the thesis by characterising the expression and activity of LBP derived from the bovine mammary gland. The detection and quantification of LBP in milk has shown the great potential of milk as a source for the protein and the experiments showing its ability to bind to LPS and stimulate cytokine induction are useful in the investigations of LBP as a potential nutraceutical/therapeutic treatment in humans as well as animals. The discovery that LBP is produced by secretory epithelial cells within the mammary gland is also important as it gives a greater insight into the immune defences of the mammary gland, and further investigation will be able to improve understanding of the regulation of LBP and the mechanisms of its actions.

APPENDIX REAGENTS

1% Agarose gel

1 g Agarose

100 ml 1X TAE buffer (with EtBr)

Microwave to dissolve

Alkaline lysis solution I

10 mM EDTA

50 mM Glucose

25 mM Tris pH 8

MQ water

Alkaline lysis solution II

1% SDS

0.1 N NaOH

MQ water

Alkaline lysis solution III

3 M Potassium 29.442 g Potassium acetate

5 M Acetate 11.5 ml Glacial acetic acid

to 100 ml with MQ water

Church & Gilbert buffer

500 mM Na₂HPO₄ pH 7.2

1 mM EDTA

7% SDS

DEPC treated MQ water

Dissolve overnight at 65°C, then store at 37°C

Cos7 growth media

1X DMEM

10% FCS

MQ water

Cos7 trypsin solution

1 ml Trypsin

300 µl 20% EDTA

8.7 ml 1X PBS

Coupling buffer

0.1 M NaHCO₃

0.5 M NaCl

MQ water

pH to 8.4

Cracking buffer

6 M Urea

4% SDS

0.1 M Tris pH 6.8

MQ water

Denaturing binding buffer

8 M Urea

20 mM Sodium phosphate

500 mM NaCl

pH to 7.8

Denaturing elution buffer

8 M Urea

20 mM Sodium phosphate

500 mM NaCl

pH to 4.0

Denaturing wash buffer pH 5.3

8 M Urea

20 mM Sodium phosphate

500 mM NaCl

pH to 5.3

Denaturing wash buffer pH 6

8 M Urea

20 mM Sodium phosphate

500 mM NaCl

pH to 6.0

DNA loading dye

0.25% BPB

0.25% Xylene cyanol

30% Glycerol

MQ water

ECL solution A

5.5 mg Luminol (5-amino 2,3-dihydro-1,4-phthalazinedione), Sigma A-8511

60 µl DMSO

ECL solution B

2.8 mg p-Coumaric acid, Sigma C-9008

100 µl DMSO

ECL working solution

2 ml 0.1 M Tris pH 8.6

4.8 µl Solution A

0.8 µl Solution B

0.6 µl 30% H₂O₂

Gill's Hematoxylin

4 g Hematoxylin

0.4 g Sodium iodate

35.2 g Aluminium sulfate

250 ml Ethylene glycol

40 ml Glacial acetic acid

710 ml MQ water

Guanidine lysis buffer

6 M Guanidine hydrochloride

20 mM Sodium phosphate

500 ml NaCl

pH to 7.8

3X Laemmli loading buffer

30% Glycerol

15% β -mercaptoethanol

3% SDS

0.1875 M Tris pH 6.8

A few grains of BPB

LB agar

6.4 g LB agar base

200 ml MQ water

Microwave to dissolve

Autoclave

LB broth

4 g LB broth base

200 ml MQ water

Microwave to dissolve

Autoclave

LBON agar

Add 15 g bactoagar per litre of LBON media before autoclaving.

LBON media

2 g Bactotryptone

1 g Yeast extract

200 ml MQ water

pH to 7

Autoclave

Methylene blue stain

0.1 g Methylene blue

500 ml 0.3 M Sodium acetate

10X MOPS

0.2 M MOPS

80 mM Sodium acetate

10 mM EDTA

Store at 4°C.

1X MOPS running buffer (Northern)

1X MOPS

18 ml/L Formaldehyde

Native binding buffer

20 mM Sodium phosphate

500 mM NaCl

pH to 7.2 – 7.6

Native wash buffer pH 5.5

20 mM Sodium phosphate

500 mM NaCl

pH to 5.5

Native wash buffer pH 6

20 mM Sodium phosphate

500 mM NaCl

pH to 6

Native elution buffer

20 mM Sodium phosphate

500 mM NaCl

pH to 4

Northern BPB/XC dye

0.25% BPB

0.25% Xylene cyanol

1 mM EDTA pH 8

50% Glycerol

DEPC treated MQ water

Northern loading buffer

10 µl Formamide

3.5 µl Formaldehyde

2 µl 10X MOPS

2 µl BPB/XC dye

2.5 µl DEPC treated MQ water

Northern wash solution I

2X SSC

0.1% SDS

Northern wash solution II

1X SSC

0.1% SDS

Northern wash solution III

0.5X SSC

0.1% SDS

Northern wash solution IV

0.1X SSC

0.1% SDS

NuPAGE MOPS running buffer (Westerns) (catalogue number NP0001)

1 M MOPS

1 M Tris

69.3 mM SDS

20.5 mM EDTA

MQ water

NuPAGE Transfer buffer (catalogue number NP0006)

500 mM Bicine

500 mM Bis-Tris

20.5 mM EDTA

1 mM Chlorobutanol

MQ water

1X PBS

137 mM NaCl

2.7 mM KCl

4.3 mM Na₂HPO₄·7H₂O

1.4 mM KH₂PO₄

pH to 7.4

0.05 M Phosphate buffer

0.05 M NaH₂PO₄

0.05 M Na₂HPO₄

pH to 7.1

Ponceau S stain

3 ml Glacial acetic acid

0.3 g Ponceau S

30 ml MQ water

ProBond 10X solution A

200 mM NaH₂PO₄

5 M NaCl

ProBond 10X solution B

200 mM Na₂HPO₄

5 M NaCl

Proteinase K solution (ISH)

0.2 M Tris pH 7.2

2 M CaCl₂

2 µg/ml Proteinase K

RIPA buffer

1% NP40 (Sigma)

50 mM Tris pH 7.5

50 mM NaF

1 mM EDTA

RNA agarose gel

1.2 g Seakem LE agarose (for a 1.2% gel) / 1.5 g for a 1.5% gel

1.8 ml Formaldehyde

10 ml 10X MOPS

88 ml DEPC treated MQ water

RPMI 1640 base (2X) (catalogue number: 31800-022)

1 sachet RPMI 1640 powder (with L-glutamine and no sodium bicarbonate)

500 ml MQ

Add 2 g/L sodium bicarbonate

Filter sterilise

Store at 4°C

20X SSC

3 M NaCl

0.3 M Sodium citrate

pH to 7

Sequenase buffer (RNA)

0.05% BPB

0.05% Xylene cyanol

20 mM EDTA

95% Formamide

Slide washing (ISH) solution I

0.2X SSC

20 mM BME

Slide washing (ISH) solution II

2X SSC

20 mM BME

Sonication buffer:

25 mM HEPES pH 7.7

0.1 M KCl

12.5 mM MgCl₂

20% Glycerol

0.1% Nonidet P40

STET buffer

0.1 M NaCl

10 mM Tris pH 8

1 mM EDTA pH 8

5% Triton X-100

1X TAE buffer (with EtBr)

200 ml 10X TAE

1800 ml MQ water

100 µl EtBr

10X TBS

40 g NaCl
12.1 g Tris
500 ml MQ water
pH to 7.6

1X TBS-IGEPAL

1X TBS
0.5% IGEPAL
pH 7.6

2DE sample buffer

8 M Urea
4% CHAPS
3 M Thiourea
40 mM DTT

TE buffer

10 mM Tris pH 8.0
1 mM EDTA pH 8.0
MQ water

T.1E buffer

1 M Tris pH 8.0
0.5 M EDTA pH 8.0
MQ water

Terrific broth:

9.4 g Terrific broth base
200 ml Milli-Q water
Microwave to dissolve
Autoclave

THP-1 and WEHI cell growth media

1X RPMI 1640 base

10% FCS

10 mM HEPES

1X Sodium pyruvate

10X Mercaptoethanol

100 U Penicillin

100 µg/ml Streptomycin

pH to ~ 7.5

Triethanolamine buffer

13.2 ml Triethanolamine

1 L DEPC treated MQ water

pH 8

WEHI trypsin solution

1 ml Trypsin

300 µl 1% EDTA

8.7 ml 1X PBS

Western blocking solution

1% PVP

0.1% BSA

1X TBS-IGEPAL

Western stripping buffer

50 mM Tris

2% SDS pH 7.6

Add 0.7% BME to working solution

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