Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author’s right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author’s permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage. [http://researchspace.auckland.ac.nz/feedback](http://researchspace.auckland.ac.nz/feedback)

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.

Note: Masters Theses

The digital copy of a masters thesis is as submitted for examination and contains no corrections. The print copy, usually available in the University Library, may contain corrections made by hand, which have been requested by the supervisor.
DEVELOPMENT AND EVALUATION OF A β-GLUCAN BIOPOLYMER FORMULATION OF LACTOFERRIN PRODUCED USING A NOVEL CRYOMILLING TECHNIQUE

HEMANT KUMAR

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy,

The University of Auckland, New Zealand

2010
Abstract

Transformation of therapeutic proteins from the new compound stage to a marketed product for clinical use depends on the development of an appropriate formulation, using technologies that avoid the underlying causes of protein degradation. Although synthetic biodegradable polymers have been extensively studied in the search for better protein delivery formulation development, the use of natural biodegradable polymers in drug delivery continues to be an area of active research. In the present study, a natural polymer, β-glucan, was investigated as a protein carrier. A novel methodology was developed to produce microparticles encapsulating bovine lactoferrin (bLF), a whey protein from milk. β-glucan was investigated as a protein delivery candidate for the first time in this study, the formulation demonstrated a significant improvement in in vivo activity, which suggested improved bioavailability of bLF when formulated using β-glucan.

β-glucan is a polysaccharide obtained from a natural source, barley, it is extensively used as a food additive and is well known for its potential nutritional and biological applications. The physicochemical properties (rheological behaviour, mechanical strength, stress studies, molecular weight and density) of the β-glucan were investigated. Simulated gastric pH condition was found to have no effect on molecular weight and viscosity of β-glucan at all tested concentrations.

The bLF was characterised for its mass, purity and amino acid sequence, bLFCin was generated by pepsin digestion, purified by ion exchange adsorptive membrane technique and characterised. Biological activity of bLF and bLFCin was evaluated.

β-glucan films containing bLF (1 and 10% w/w) were cast with or without hydrophobic and hydrophilic excipients. Films were milled and optimised at different milling conditions and particle size analysis was conducted. The entrapment efficiency of bLF in films with hydrophobic excipients was found to be higher than films without excipients. A High pressure liquid chromatography (HPLC) method was developed and validated to measure the concentrations of bLF in the formulations.

Complete recovery of bLF from films and particles could not be achieved and further studies were conducted to investigate the stability of bLF and interactions between bLF and β-glucan. The release of bLF from films was higher than from particles. Particles without excipients showed lower release. Solid state characterisation
showed that there was no change in the structure when bLF was milled alone but minor changes were observed when bLF in β-glucan films were milled to form particles. These changes were due to interactions between β-glucan and bLF, which were investigated by surface plasmon resonance (SPR) studies. The stability evaluation of bLF and β-glucan was carried out using liquid chromatography-mass spectrometry (LC-MS/MS) and size exclusion chromatography-multi-angular laser light scattering (SEC-MALLS) respectively.

In vitro evaluation of biological activity of bLF showed that there was no change in osteoblast proliferation activity after milling but reduction in activity was seen when bLF from the particles was tested. In vivo evaluation using a mouse model demonstrated a considerable improvement in bioavailability with particles encapsulating bLF and bone mineral density gain increased significantly.

This study demonstrated the potential of β-glucan as an encapsulating agent for the oral delivery of therapeutic proteins.
Acknowledgements

I would like to express my sincere gratitude to my supervisor Dr Jingyuan Wen for her continuous support, guidance, encouragement and trust throughout my PhD. Her passion for protein research always inspired me.

I am grateful and would like to express my sincere gratitude to Dr Craig Bunt, both as my supervisor (first year) and later as my co-supervisor for his overall direction, ongoing support, innovative ideas, encouragement and guidance throughout my PhD. Without him this project would not have been possible.

I would like to thank my other co-supervisors Associate Professor Sanjay Garg for his support and Professor John Shaw for his support and thorough review of my thesis and very helpful feedback.

I am grateful to Professor Jillian Cornish, my project advisor from the Bone Group, Department of Medicine, for her support in bone related work and critical scientific inputs. Professor Cornish was instrumental in arranging in vivo studies in France. My sincere thanks to Maureen Watson and Karen Collon for their support in carrying out osteoblast studies.

My sincere thanks goes to Professor Subhash Chand, IIT, Delhi for giving me an opportunity to learn different techniques of protein and peptide purification during my visit to his lab for four months.

I would like to thank Dr Anne Blais, INRA-Agro Paristech, France for carrying out animal studies.

I would like to thank Associate Professor Alan Easteal, Department of Chemistry for giving me his precious time to discuss DSC and IR results.

I also thank Associate Professor Tom Britain for his help in using the CD instrument and Professor Heather Baker for her time to share her knowledge about lactoferrin.

My special thanks goes to Michelle Nieuwoudt at Department of Chemistry for her help in spectroscopic studies.
My special thanks to Dr Christina Buchannan, School of Biological Sciences for helping me in the HPLC assay. I also thank Martin Middleditch for his help in mass spectrometry.

Would like to acknowledge the help of Fiona Clow in surface plasmon resonance studies.

I would like to thank all the staff at the School of Pharmacy for their support. My special thanks to Lesley and Vivien who helped me to fix the ageing cryomilling machine a number of times during this project.

I am grateful to the University of Auckland for providing me with a doctoral scholarship during my PhD and Education, New Zealand for providing funding support to work in IIT, Delhi for four months. My special thanks for conference funding support from the School of Pharmacy.

I would like to pay my regards to my mentor through the CRS programme, Professor Ian Tucker for overall mentoring and teaching me the critical aspect of the research.

My special thanks to all my buddies in PhD – Shane, Srinivas, Puneet, Mridula, Ilva, Judy, Darren, Alvin and Thilini for their companionship throughout this journey. I would also thank my buddies at IIT, Delhi for their help in various ways – Rashi, Asif and Alok.

I would like to thank Laura Clunie for proofreading this thesis.

My special thanks to my parents and parents in-laws for their blessings.

I would like to thank with a special mention for the love and support of my wife, Usha whose support, patience and encouragement made this thesis come to this stage. My two beautiful daughters, Ekta and Tanya deserve a special mention for bearing with me especially during last phase of my PhD, to whom I always promised a long holidays and gifts after my PhD.
Publications

Publications arising from this thesis


Refereed Full Conference Papers


Refereed Conference Abstracts


Conference Abstracts


Poster

Patents

## Contents

**Abstract** .......................................................................................................................... ii
**Acknowledgements** ........................................................................................................ iv
**Publications** ....................................................................................................................... vi
**Contents** ............................................................................................................................. ix
**List of Figures** ..................................................................................................................... xiv
**List of Tables** ...................................................................................................................... xx
**List of Abbreviations** .......................................................................................................... xxii

### CHAPTER 1 General Introduction .................................................................................. 1
1.1 Introduction ..................................................................................................................... 2
1.2 Literature Review ............................................................................................................. 4
  1.2.1 Gastrointestinal tract (GIT) ..................................................................................... 4
    1.2.1.1 Protein digestion and absorption ................................................................. 7
  1.2.2 Oral delivery of proteins .......................................................................................... 8
  1.2.3 Potential oral formulations for proteins ................................................................. 10
  1.2.4 Protein stability ....................................................................................................... 15
  1.2.5 Formulation approaches to improve protein stability ........................................... 17
  1.2.6 Characterisation techniques for proteins ............................................................ 20
  1.2.7 Techniques for monitoring protein instability ...................................................... 21
  1.2.8 Protein selected for this study .............................................................................. 21
  1.2.9 Biopolymer selected for this study ...................................................................... 22
1.3 Aim and specific objectives ............................................................................................ 22
1.4 Thesis organisation ......................................................................................................... 23

### CHAPTER 2 Characterisation of β-glucan ..................................................................... 25
2.1 Introduction ..................................................................................................................... 26
  2.1.1 Beta Glucan (β-glucan) ........................................................................................ 26
    2.1.1.1 β-glucan structure ........................................................................................ 26
    2.1.1.2 Extraction and purification of barley β-glucan .............................................. 29
    2.1.1.3 Determination of molecular weight of β-glucan ......................................... 30
    2.1.1.4 Applications of β-glucan ............................................................................ 32
  2.2 Characterisation of β-glucan ....................................................................................... 35
  2.3 Rheological properties of β-glucan ............................................................................ 36
    2.3.1 Materials ............................................................................................................. 37
    2.3.2 Methods ............................................................................................................. 37
      2.3.2.1 Microscopic characterisation of β-glucan .................................................... 37
      2.3.2.2 Preparation of samples ............................................................................. 37
      2.3.2.3 Rheological measurements ................................................................... 37
  2.4 Mechanical characterisation of β-glucan gel ............................................................ 38
    2.4.1 Methods ............................................................................................................. 38
      2.4.1.1 Preparation of sample .............................................................................. 38
      2.4.1.2 Gel strength measurement .................................................................. 39
  2.5 Molecular weight distribution of β-glucan ............................................................... 39
    2.5.1.1 Average particle size of β-glucan in different solvent systems ................. 40
    2.5.1.2 Density measurement of β-glucan ............................................................. 40
  2.5.2 Methods ................................................................................................................. 40
    2.5.2.1 Chromatographic conditions .................................................................. 40
    2.5.2.2 Preparation of samples ............................................................................. 41
CHAPTER 3  Lactoferrin Characterisation, Derivitisation and Purification of Lactoferricin  

3.1  Introduction .................................................................................................................. 67
   3.1.1  Milk proteins ........................................................................................................... 67
   3.1.1.1  Whey ................................................................................................................ 71
   3.1.1.2  Whey proteins ................................................................................................. 72
   3.1.1.3  Lactoferrin (LF) ............................................................................................... 74

3.2  Characterisation of bLF and bLFcin ......................................................................... 77

3.3  Materials ..................................................................................................................... 80

3.4  Methods ....................................................................................................................... 80
   3.4.1  Physical characterisation of unmilled and milled bLF .......................................... 80
   3.4.2  Total Protein determination in bLF sample by Biuret method ................................ 81
   3.4.3  Mass determination and identification of bLF ....................................................... 81
   3.4.4  Mass determination by Matrix-assisted laser ionization (MALDI) ......................... 83
   3.4.5  Enzymatic derivitisation of bLF into bLFcin and its purification ......................... 83
      3.4.5.1  Ultrafiltration .................................................................................................. 83
      3.4.5.2  Derivitisation/Digestion of bLF ..................................................................... 84
      3.4.5.3  Tricine-SDS-PAGE ....................................................................................... 85
   3.4.6  Purification of bLFcin ........................................................................................... 87
      3.4.6.1  Gel filtration ................................................................................................... 87
      3.4.6.2  Preparatory ion exchange chromatography ..................................................... 87
      3.4.6.3  Fast protein liquid chromatography (FPLC) .................................................. 88
      3.4.6.4  Ion exchange using microporous membrane ................................................ 88
      3.4.6.5  Sample preparation for mass spectrometry .................................................... 89
      3.4.6.6  Biological activity of bLF and bLFcin ............................................................. 89

3.5  Results and discussion ............................................................................................... 90
   3.5.1  Physical characterisation of bLF ......................................................................... 90
   3.5.2  Protein estimation, molecular weight determination, and evaluation of purity of bLF ................................................................. 91
      3.5.2.1  Protein estimation of bLF ............................................................................ 91
      3.5.2.2  Molecular weight and purity determination .................................................. 92
      3.5.2.3  Mass spectrometric analysis ........................................................................ 93
      3.5.2.4  Identification of bLF by LC-MS/MS ............................................................. 94
   3.5.3  Enzymatic derivitisation of bLF ......................................................................... 95
CHAPTER 4  Formulation Development and Characterisation .......... 109

4.1 Introduction ................................................................. 110
4.2 Materials ..................................................................... 111
4.3 Methods ...................................................................... 111
  4.3.1 Analytical method ..................................................... 111
    4.3.1.1 Chromatographic conditions ................................. 111
    4.3.1.2 Preparation of standard solution ............................ 112
    4.3.1.3 Linearity ................................................................. 112
    4.3.1.4 Method validation .................................................. 113
    4.3.1.5 Specificity ............................................................... 113
    4.3.1.6 Sensitivity ............................................................... 113
    4.3.1.7 Recovery ................................................................. 113
    4.3.1.8 Accuracy and Precision ........................................... 113
    4.3.1.9 Stability of bLF ....................................................... 114
  4.3.2 Preformulation studies .............................................. 114
  4.3.3 Formulation .............................................................. 114
    4.3.3.1 Casting of films ..................................................... 115
    4.3.3.2 Staining of films to investigate bLF entrapment ......... 115
    4.3.3.3 Milling of films ...................................................... 116
    4.3.3.4 Process optimisation .............................................. 116
  4.3.4 Formulation Characterisation ........................................ 118
    4.3.4.1 Physical appearance .............................................. 118
    4.3.4.2 Evaluation of particle size distribution and zeta potential .... 118
    4.3.4.3 Morphology ........................................................... 119
    4.3.4.4 Evaluation of drug entrapment efficiency (DEE) .......... 119
  4.3.5 Validation of extraction method .................................... 120
    4.3.5.1 Recovery of bLF by enzyme treatment ..................... 120
    4.3.5.2 Evaluation of interactions of bLF with β-glucan2 and sucrose .... 120
  4.3.6 In vitro release of bLF from the films and microparticles .......... 121
  4.3.7 Stability of formulation ............................................. 121
    4.3.7.1 Physical appearance .............................................. 121
    4.3.7.2 Molecular weight of β-glucan ................................ 122
    4.3.7.3 Stability of bLF .................................................... 122
    4.3.7.4 FTIR spectroscopy ................................................ 122
    4.3.7.5 Raman spectroscopy ............................................. 123
    4.3.7.6 Modulated temperature differential scanning calorimetry (MTDSC) .... 123
    4.3.7.7 Thermo gravimetric analysis (TGA) .......................... 123
    4.3.7.8 X-ray diffraction studies ....................................... 123
    4.3.7.9 HPLC-UV and LC-MS/MS studies of bLF and bLF extract from β-glucan2 bLF milled particles .......... 124
4.3.7.10 Circular dichroism spectroscopy ...................................................... 125
4.3.8 Investigation of the interaction between β-glucan2 and bLF .............. 125
4.4 Results and discussion ............................................................................. 126
4.4.1 Analytical method .................................................................................. 126
4.4.1.1 Chromatographic conditions ............................................................... 126
4.4.1.2 Method validation .............................................................................. 127
4.4.2 Casting of films ...................................................................................... 129
4.4.2.1 Visual and SEM evaluation of films ................................................... 129
4.4.2.2 Yield and moisture content of films and microparticles ..................... 129
4.4.2.3 Staining of the film .......................................................................... 132
4.4.3 Optimisation of milling Process ............................................................... 133
4.4.3.1 Milling parameters .......................................................................... 133
4.4.3.2 Zeta potential of different formulations ............................................ 138
4.4.4 Drug entrapment efficiency ................................................................... 140
4.4.5 Extraction efficiency validation .............................................................. 141
4.4.5.1 Effect of duration on extraction of bLF from β-glucan2 bLF films and
β-glucan2 bLF milled particles .................................................................. 141
4.4.5.2 Effect of pH and volume on the extraction of bLF from β-glucan2 bLF films and β-glucan2 bLF milled particles ......................... 142
4.4.5.3 Recovery of bLF from β-glucan2 bLF milled particles after enzyme
treatment ................................................................................................. 144
4.4.5.4 Evaluation of interactions between bLF and β-glucan2 or sucrose... 144
4.4.6 In vitro release of bLF ........................................................................... 145
4.4.7 Stability of bLF and β-glucan2 bLF milled particles ......................... 147
4.4.7.1 Stability of bLF ............................................................................... 147
4.4.7.2 Long term stability of formulation ................................................... 148
4.4.7.3 Spectroscopic analysis of fresh unmilled and milled samples..... 151
4.4.7.4 Raman spectroscopy ....................................................................... 156
4.4.7.5 DSC studies .................................................................................... 157
4.4.7.6 XRD studies ................................................................................... 166
4.4.7.7 MALDI TOF analysis of fresh samples and stability samples ..... 168
4.4.7.8 FTIR studies of stability samples of bLF and β-glucan2 bLF milled particles ................................................................. 171
4.4.7.9 Stability of bLF and bLF from particles by HPLC-UV and LC-MS/MS 174
4.4.7.10 Circular dichroism (CD) studies .................................................... 175
4.4.8 Interaction between β-glucan and bLF .................................................. 176
4.5 Conclusion ................................................................................................. 180

CHAPTER 5 Evaluation of Formulation using Cell Culture and Animal Model .................................................. 182
5.1 Introduction ............................................................................................... 183
5.2 Materials .................................................................................................. 183
5.3 Methods .................................................................................................... 184
5.3.1 Osteogenic activity of bLF .................................................................. 184
5.3.2 Reproducibility of ELISA method ...................................................... 184
5.3.3 Evaluation of uptake of bLF from β-glucan2 bLF milled particles using
Caco-2 cell monolayer .............................................................................. 185
5.3.4 In vivo evaluation of the formulation .................................................... 188
5.4 Results and discussion ............................................................................. 190
5.4.1 ELISA standard curve .................................................................................. 190
5.4.2 Investigation of biological activity of formulation ................................. 191
5.4.3 Uptake studies evaluation ....................................................................... 194
5.4.4 In vivo studies of β-glucan2 bLF milled particles ................................. 196
5.5 Conclusion .................................................................................................. 198

CHAPTER 6 General Discussion and Future Directions .................... 200
References ....................................................................................................... 210
List of Figures

Figure 1.1  Schematic diagram of the human gastrointestinal system. Adapted from (30). ................................................................. 6
Figure 1.2  Schematic diagram of digestion of proteins and peptides ................................................................. 7
Figure 1.3  Schematic tranverse section of intestinal epithelium and follicle associated epithelium (FAE) depicting M cell transport of particles or pathogens. (I) Passive transcellular transport. (II) Paracellular transport between adjacent cells. (III) Particles can be absorbed by M cells of FAE found in Peyer’s patches. Adapted from (25) with permission. ........................................................................ 11
Figure 1.4  Schematic diagram showing method of entrapment of proteins. Adapted from (97) with permission. ........................................... 18

Figure 2.1  Structure of barley β-glucan A: simplified representation (G–glucosyl residues), (3 and 4 represents β-(1→3) and (1→4) linkages and Cellotriosyl and Cellotetraosyl residues are shown as G4G4G or G4G4G4G. B: Linear unbranched β-(1→3) and (1→4)-D-glucopyranose units in a non-repeating but non random order .................................................................... 28
Figure 2.2  Light microscope pictures of top lit β-glucan1 (top, bar=50 µm) and back lit β-glucan2 (bottom, bar=100 µm)........................................................... 43
Figure 2.3  Shear rate vs viscosity of β-glucan1 solution (1% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3. ....................... 45
Figure 2.4  Shear rate vs viscosity of β-glucan2 solution (1% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3. ....................... 45
Figure 2.5  Shear rate vs viscosity of β-glucan1 solution (4% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3. ....................... 46
Figure 2.6  Shear rate vs viscosity of β-glucan2 solution (4% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3. ....................... 46
Figure 2.7  Shear rate vs viscosity of β-glucan1 solution (7% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3. ....................... 47
Figure 2.8  Shear rate vs viscosity of β-glucan2 solution (7% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3. ....................... 47
Figure 2.9  Shear rate vs viscosity of β-glucan1 solution (10% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bar are ± SEM, n=3. ................................................. 48
Figure 2.10  Shear rate vs viscosity of β-glucan2 solution (10% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3. ....................... 48
Figure 2.11  Temperature vs viscosity plot of β-glucan1 solution at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●) at 25, 37 and 70˚C. Error bars are ± SEM, n=3. ................................................................................. 51
Figure 2.12  Temperature vs viscosity plot of β-glucan2 solution at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●) at 25, 37 and 70˚C. Error bars are ± SEM, n=3. ................................................................................. 51
Figure 2.13  Shear rate vs viscosity of β-glucan1 solution (1% w/w) at 25˚C (♦), 37˚C (■) and 70˚C (▲). Error bars are ± SEM, n=3. .............................................. 52
Figure 2.14  Shear rate vs viscosity of β-glucan2 solution (1% w/w) at 25˚C (♦), 37˚C (■) and 70˚C (▲). Error bars are ± SEM, n=3. .............................................. 52
Figure 2.15  Shear rate vs viscosity of β-glucan1 solution (4% w/w) at 25˚C (♦), 37˚C (■) and 70˚C (▲). Error bars are ± SEM, n=3. .............................................. 53
Figure 2.16 Shear rate vs viscosity of β-glucan2 solution (4% w/w) at 25˚C (♦), 37˚C (■) and 70˚C (▲). Error bars are ± SEM, n=3 ........................................... 53

Figure 2.17 Shear rate vs viscosity of β-glucan1 solution (7% w/w) at 25˚C (♦), 37˚C (■) and 70˚C (▲). Error bars are ± SEM, n=3 ........................................... 54

Figure 2.18 Shear rate vs viscosity of β-glucan2 solution (7% w/w) at 25˚C (♦), 37˚C (■) and 70˚C (▲). Error bars are ± SEM, n=3 ........................................... 54

Figure 2.19 Shear rate vs viscosity of β-glucan1 solution (10% w/w) at 25˚C (♦), 37˚C (■) and 70˚C (▲). Error bars are ± SEM, n=3 ........................................... 55

Figure 2.20 Shear rate vs viscosity of β-glucan2 solution (10% w/w) at 25˚C (♦), 37˚C (■) and 70˚C (▲). Error bars are ± SEM, n=3 ........................................... 55

Figure 2.21 Average gel strength profile of β-glucan1 (♦) and β-glucan2 (●) gel at different concentrations (1, 2, 3, 4, 7 and 10% w/w) at 25˚C. Error bars are ± SEM, n=3 ........................................... 57

Figure 2.22 Representative chromatogram of β-glucan2 showing scattering light signal (red) and refractive index detector signal (blue) .......................................... 60

Figure 2.23 Average particle size of β-glucan1 and β-glucan2 in different solvent systems. Error bars are ± SEM, n=100 ................................................................. 61

Figure 2.24 Representative chromatograms of β-glucan1 and β-glucan2 showing scattering light signal (red) and refractive index detector signal (blue); (A – 0.2 M HCL, B – 2 M HCL ) .................................................................................. 63

Figure 2.25 Representative chromatograms of β-glucan2 showing scattering light detector signal (red) and refractive index detector signal (blue); (C - 6 M HCL, D - 0.1 M HCL at 120˚C) .................................................................................. 64

Figure 3.1 Schematic diagram of the composition of milk showing the relative amounts of water and solids. The solids consist of various components including protein which comprises whey protein (20%) and casein (80%). .................................................................. 68

Figure 3.2 Schematic diagram of acid and rennet mediated cheese making. Adapted from (151) with permission ................................................................. 71

Figure 3.3 Ribbon diagram of bLF showing the four sub-lobes and secondary structural features β-sheets (blue), α-helices (orange and red) and two ferric ions bound to each lobe. Green represents the LFcin containing region of the N-terminal. Modified from (172) .................................................. 76

Figure 3.4 Summary of biological functions of LF. Modified from (173). ........................................................................ 77

Figure 3.5 Ultrafiltration cell assembly with 50 kDa cut off membrane used to remove the minor contaminant. ........................................................................ 84

Figure 3.6 Unmilled bLF as received (top left), milled bLF (top right) for 4 min using cryomill. SEM of bLF (bottom). ........................................................................ 90

Figure 3.7 BSA concentration vs absorbance at 520 nm calibration. Error bars are ± SD (n=2). ............................................................................................... 91

Figure 3.8 SDS- PAGE (12%) gel stained with Coomassie brilliant blue: reading left to right, lane 1, molecular weight marker; lane 2, bLF (5 µg); lane 3, bLF (10 µg); lane 4, bLF (15 µg); lane 5, b LF (20 µg); lane 6, bLF (25 µg). ....................................................................................... 92

Figure 3.9 Molecular mass profile of bLF determined by MALDI-TOF. BSA was used as an external calibrant. ........................................................................ 93

Figure 3.10 SDS-PAGE (Laemmli) of bLF stained with Coomassie brilliant blue; reading left to right, lane 1, molecular weight markers, lane 2, bLF before ultrafiltration or control; lane 3, bLF after ultrafiltration ...................................................................... 95
Figure 3.11  Tricine-SDS-PAGE profile of peptides generated by pepsin hydrolysis of bLF stained with Coomassie brilliant blue; reading left to right, lane 1, undigested bLF; lane 2, digested bLF at 0.5 hour; lane 3, digested bLF at 1 hour; lane 4, molecular mass markers; lane 5, digested bLF at 2 hours; lane 6, digested bLF at 3 hours; lane 7, digested bLF at 4 hours; lane 8, digested bLF at 5 hours. ..............................97

Figure 3.12  Tricine-SDS-PAGE profile of peptides generated by pepsin hydrolysis of bLF stained with Coomassie Brilliant blue; reading left to right, lane 1, molecular mass markers; lane 2, undigested bLF; lane 3, digested bLF at 15 mg/g of protein; lane 4, digested bLF at 20 mg/g of protein; lane 5, digested bLF at 25 mg/g of protein; lane 6, digested bLF at 30 mg/g of protein; lane 7, digested bLF at 35 mg/g of protein ..............................................................................................98

Figure 3.13  Fraction number vs absorbance at 280 nm after gel filtration of bLF hydrolysate. ..........................................................................................................................99

Figure 3.14  Fraction number vs absorbance at 280 nm of the fractions collected after ion exchange chromatography of bLF hydrolysate. ...........................................100

Figure 3.15  FPLC chromatogram showing elution of peptides with different buffer conditions. Fractions collected are shown in red. .................................................101

Figure 3.16  Fraction number vs absorbance at 250 nm of eluted fractions with adsorptive membrane chromatography. .................................................................102

Figure 3.17  Tricine SDS-PAGE profile of fractions eluted from membrane adsorber membrane stained with Coomassie brilliant blue; reading left to right, lane 1, low molecular mass markers; lane 2, fraction 1 with 2 M NH₄ Cl; lane 3, fraction 8; lane 4, fraction 9; lane 5, fraction 21; lane 6, fraction 22; lane 7, control-bLF hydrolysate .................................................103

Figure 3.18  Mass spectrum of fraction 9 after adsorption membrane purification. .................................................................................................................................105

Figure 3.19  Thymidine incorporation by osteoblasts after treatment with various concentrations of control, synthetic bLFcin from Auspep (Australia) and purified bLFcin. Error bars are ± SEM, n=6, * p<0.05 ......................................................................................................................107

Figure 3.20  Thymidine incorporation by osteoblasts after treatment with various concentrations of control and bLF. Error bars are ± SEM, n=6, * p<0.05. ......................................................................................................................107

Figure 4.1  Assembly used for milling of films to produce microparticles and optimisation of formulation. Freezer mill used for milling films (A), stainless steel end plugs (B) used to seal the polycarbonate tubes (D), the sample was placed in polycarbonate tubes (D), stainless steel impactor (C) is put along the sample in polycarbonate tube. Polycarbonate tube along with sample was placed in a slot (E) and a basket containing liquid nitrogen (F) ..................................................................................117

Figure 4.2  A representative HPLC chromatogram of bLF (50 µg/ml) at 214 nm. The elution time was 24.55 min. .................................................................................................128

Figure 4.3  SEM micrograph of β-glucan2 film (top) and a cross section of β-glucan2 film (bottom). .............................................................................................................130

Figure 4.4  SEM micrograph of β-glucan2 bLF film (top) and a cross section of β-glucan2 bLF film (bottom). .............................................................................................................131

Figure 4.5  Light microscope pictures of β-glucan2 film (top) and β-glucan2 bLF film after staining with Prussian Blue. Circled areas indicate regions stained blue (bottom). .............................................................................................................132
Figure 4.6  SEM micrograph of unmilled β-glucan2 (top) and with higher magnification (bottom). ................................................................. 135

Figure 4.7  SEM micrograph of milled β-glucan2 (top) and β-glucan2 bLF milled particles (bottom). ......................................................... 136

Figure 4.8  Effect of various milling conditions upon average particle size. Key: #, #, # milling time in min, cycle in numbers, bLF loading in %. Error bars are ± SEM, n=3. ..................................................... 137

Figure 4.9  Average release of bLF from β-glucan2 bLF milled particles (△) or films (▲) with PEG 2000 particles (○), with PEG 2000 films (●), with Kollicoat particles (□) or with Kollicoat films (■). Release assessment media PBS (pH 7.4). Error bars are ± SEM, n=3. 146

Figure 4.10  Average initial (white) stability of bLF at different temperatures (4, 25 and 37˚C) and after 24 hours (dark). Error bars are ± SEM, n=3. 147

Figure 4.11  Unmilled β-glucan2 (top left), fresh β-glucan2 bLF milled particles (top right), β-glucan2 bLF milled particles after storage for 6 months at 25˚C/60% RH (bottom left) and 40˚C/70% RH (bottom right). ................................................................................................................ 149

Figure 4.12  Average β-glucan2 molecular weight over 6 months at 25˚C/60% RH (open) and 40˚C/75% RH (closed). Error bars are ± SEM, n=3. 150

Figure 4.13  IR spectra of unmilled bLF (blue) and milled bLF (red). ............................................................... 152

Figure 4.14  IR spectra (expanded region) of unmilled (red) and milled bLF (blue). ............................................................... 152

Figure 4.15  IR spectra of unmilled (blue) and milled β-glucan2 (red). ............................................................... 153

Figure 4.16  IR spectra (expanded region) of unmilled (blue) and milled β-glucan2 (red). ............................................................... 153

Figure 4.17  IR spectra of β-glucan2 bLF milled particles (red) and addition of 90% β-glucan2 and 10% bLF result (blue), the circled regions show changes observed in the spectra following cryomilling. 154

Figure 4.18  IR spectra (expanded) of β-glucan2 bLF milled particles (red) and addition result of 90% β-glucan2 and 10% bLF (blue). 155

Figure 4.19  Raman spectra of unmilled β-glucan2 (red), unmilled bLF (blue) and β-glucan2 bLF milled particles (green). ............................................................... 156

Figure 4.20  DSC thermograms of bLF unmilled (a, solid line) and milled (b, dash line). ............................................................... 161

Figure 4.21  DSC thermograms of (a) unmilled β-glucan2, (b) milled β-glucan2 and (c) β-glucan2 bLF milled particles. ............................................................... 162

Figure 4.22  DSC thermograms of unmilled β-glucan2 (top) and β-glucan2 bLF milled particles (bottom) heated to 150˚C at 10˚C/min, cooled at 5˚C and re-heated to 300 and 350˚C respectively. ............................................................... 163

Figure 4.23  DSC thermograms of (a) unmilled bLF and (b) milled bLF after vacuum drying at 30˚C for 24 hours. ............................................................... 164

Figure 4.24  DSC thermograms of (a) unmilled β-glucan2, (b) milled β-glucan2 and (c) β-glucan2 bLF milled particles after vacuum drying at 30˚C for 24 hours. ............................................................... 164

Figure 4.25  TGA traces of bLF unmilled (black) and milled (red) showing water loss with temperature increase. ............................................................... 165

Figure 4.26  TGA traces of unmilled β-glucan2 (green), milled β-glucan2 (red) and β-glucan2 bLF milled particles (black). ............................................................... 165
Figure 4.27 XRD patterns of: (a) unmilled bLF, (b) milled bLF, (c) unmilled β-glucan2, (d) β-glucan2 and bLF physical mixture without milling and (e) β-glucan2 bLF milled particles. .......................................................... 166

Figure 4.28 XRD pattern of bLF (a) unmilled and (b) milled. .............................................. 167

Figure 4.29 Mass spectra of fresh bLF samples: unmilled (A), milled (B) and extracted from β-glucan2 bLF milled particles (C). .......................................... 169

Figure 4.30 Mass spectra of bLF samples after storage at 25˚C/60 RH for 4 months: unmilled (a) milled (b) and extracted from β-glucan2 bLF milled particles (c). .................................................................................. 170

Figure 4.31 IR spectra of unmilled bLF after storage at 25˚C/60% RH (blue) and 40˚C/70% RH (red) [top] and unmilled β-glucan2 after storage at 25˚C/60% RH (blue) and 40˚C/70% RH (red) [bottom] for 6 months. .......................................................... 172

Figure 4.32 IR spectra of β-glucan2 bLF milled particles after storage at 25˚C/60% RH (top) and unmilled β-glucan2 after storage at 40˚C/70% RH (bottom) for 6 months. .............................................................. 173

Figure 4.33 CD spectra of fresh samples of unmilled bLF (solid line), milled bLF (broken line) and bLF extracted from β-glucan2 bLF milled particles (dash line).......................................................... 175

Figure 4.34 SPR sensorgram of β-glucan2 (1 mg/ml) interacting with bLF. Stage 1: baseline signal of HBS buffer followed by injection of β-glucan2; stage 2: steady state of interaction; stage 3: A high proportion of the molecule interacting with bLF remains bound following the end of the injection. .................................................................................. 178

Figure 4.35 SPR sensorgram of β-glucan2 (1mg/ml) interacting with bLF after a number of regeneration steps. .............................................................. 178

Figure 4.36 SDS-PAGE (10%) scan of samples. Lanes 1, 3 and 5: bLF with Lichenase enzyme; Lanes 2, 4 and 6: Lichenase enzyme; lane 7: bLF milled; Lane 8: supernatant after bLF extraction; Lane 9: supernatant after lichenase treatment; Lane 10: Lichenase treated insoluble pellet of β-glucan2 bLF particles; Lane 11: β-glucan2 only; Lane 12: bLF and β-glucan2 physical mixture. Std LF represents bLF. ................................................................................................ 179

Figure 5.1 Standard curve of bLF from the kit (▲) and bLF from Fonterra (♦). .......................... 190

Figure 5.2 Osteoblast mitogenic assay of bLF: From left to right: control; bLF unmilled; bLF milled for 4 min using cryomill; bLF extracted from β-glucan2 milled particles for 3 hours and freeze dried. Error bars represent ± SEM, n=6, *p<0.05. ................................................................. 192

Figure 5.3 Osteoblast mitogenic assay of β-glucan2 at different concentrations (top) and bLF at different concentrations (bottom). From left to right (top): control; β-glucan2 at 1, 10 and 100 µg/ml; control; β-glucan2 at 0.01, 0.1 and 1 µg/ml. Bottom: control; bLF std; bLF extract at 10 µg/ml; control; bLF std and bLF extract at 100 µg/ml. Error bars represent ± SEM, n=6, *p<0.05. .................................................................................. 193

Figure 5.4 Average bLF uptake by Caco-2 cells at different concentrations: 25 µg/ml (▲), 50 µg/ml (♦), 100 µg/ml (▲). Error bars are the range between samples, n=2 .............................................................................................. 195

Figure 5.5 Average bLF uptake from β-glucan2 bLF milled particles by Caco-2 cells at different concentrations: 25 µg/ml (▲), 50 µg/ml (♦), 100 µg/ml (▲). Error bars are the range between samples, n=2: .................................................................................. 195
Figure 5.6  Average bLF uptake from β-glucan2 bLF milled particles by Caco-2 cells at concentrations: 25 µg/ml (▲), 50 µg/ml (●), 100 µg/ml (■). Error bars are the range between samples, n=2. .......................................................... 196

Figure 5.7  Average BMD gain of mice fed with diet supplemented with different concentrations of bLF. The Ovx or sham mice were fed for 4 months with either control diet for sham and Ovx control or a diet including Sham 10 (Sham 10), 5 (Ovx 5) and 10g/kg (Ovx 10) bLF. F1 (Ovx F1) and F10 (Ovx F10) are the formulations containing 1 g and 10 g of particles, encapsulating 100 mg and 1 g of bLF respectively. Error bars are ± SEM, n=10. .......................................................... 197

Figure 5.8  Average immunoreactive bLF in the plasma of mice fed with diet supplemented with different concentration of bLF. The Ovx or sham mice were fed for 4 months with either control diet for Sham and Ovx control (Ovx C) or a diet including Sham 10 (Sham 10), 5 (Ovx 5) and 10g/kg (Ovx 10) bLF. F1 (Ovx F1) and F10 (Ovx F10) are the formulations containing 1 g and 10 g of particles, encapsulating 100 mg and 1 g of bLF respectively. Error bars are ± SEM, n=10. .......................................................... 198
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Recent industrial technologies for oral protein/peptide delivery. Adapted from (79) with permission.</td>
</tr>
<tr>
<td>Table 1.2</td>
<td>Common mechanisms and causes of protein instability. Adapted from (29) with permission.</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Biologically active glucans and their sources. Modified from (113) with permission.</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Flow behaviour index of β-glucan1 (1) and β-glucan2 (2) solution (1, 4, 7 and 10% w/w) at 25, 37 and 70°C.</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>Area and force maxima of β-glucan1 and β-glucan2 gels at different concentrations.</td>
</tr>
<tr>
<td>Table 2.4</td>
<td>Average molecular weight and polydispersity index of β-glucan1 and β-glucan2.</td>
</tr>
<tr>
<td>Table 2.5</td>
<td>Average molecular weight of β-glucan2 at given stress conditions.</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Typical composition of bovine milk and whey from (140) with permission.</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Composition and physiological functions of major milk and whey proteins from (137) with permission.</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Protein profile of whey. Adapted from (151) with permission.</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>Composition of separating gel (10 ml) and stacking gel (5 ml).</td>
</tr>
<tr>
<td>Table 3.5</td>
<td>Composition of resolving gel, spacer and stacking gel for Tricine-SDS-PAGE.</td>
</tr>
<tr>
<td>Table 3.6</td>
<td>Peptides identified and amino acid sequencing in trypsin digests of bLF by LC-MS/MS.</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>HPLC gradient conditions for bLF elution.</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Milling time x cycles x bLF loading (2 x 3 x 2) factorial design to investigate optimisation of the cryo-milling method for producing β-glucan2 milled particles.</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>Inter- and intra-day accuracy and precision results for the determination of bLF by the HPLC method. Replicate samples (n=3) of each concentration of bLF were assayed.</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Effect of pH or simulated biological fluids upon zeta potential of β-glucan2 only or β-glucan2 bLF milled particles with or without various excipients. Replicate (n=3) zeta potential (mV) along with standard deviations.</td>
</tr>
<tr>
<td>Table 4.5</td>
<td>Average (n=3) entrapment efficiency of different β-glucan2 bLF films or β-glucan2 bLF milled particles.</td>
</tr>
<tr>
<td>Table 4.6</td>
<td>Average (n=3) bLF recovery from β-glucan2 bLF film or β-glucan2 bLF milled particle.</td>
</tr>
<tr>
<td>Table 4.7</td>
<td>Average (n=3) bLF recovery from β-glucan2 bLF films or β-glucan2 bLF milled particles at pH 4.0, 5.5 and volume 10 and 20 ml for 3 hours in phosphate buffer (pH 7.4).</td>
</tr>
<tr>
<td>Table 4.8</td>
<td>Average recovery (n=3) of bLF from different combinations of carbohydrates and bLF.</td>
</tr>
<tr>
<td>Table 4.9</td>
<td>Thermodynamic parameters for unmilled bLF and milled bLF derived from MTDSC measurements without vacuum drying.</td>
</tr>
</tbody>
</table>
Table 4.10  Thermodynamic parameters for bLF and milled bLF derived from MTDSC measurements with vacuum drying at 30°C for 24 hours.  159
Table 4.11  Thermodynamic parameters for unmilled β-glucan2, milled β-glucan2 and β-glucan2 bLF milled particles derived from MTDSC measurements without vacuum drying.  160
Table 4.12  Thermodynamic parameters for unmilled β-glucan2, milled β-glucan2 and β-glucan2 bLF milled particles derived from MTDSC measurements vacuum drying at 30°C for 24 hours.  160
Table 4.12  Molecular mass of bLF and bLF extracted from β-glucan2 bLF milled particles stored at different storage conditions for 4 and 6 months using MALDI-TOF.  168
Table 5.1  Composition of cell lysis buffer.  187
Table 5.2  Composition of diet fed to mice with bLF and β-glucan2 bLF milled particles.  189
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin 1 converting enzyme</td>
</tr>
<tr>
<td>AA2P</td>
<td>L-ascorbic acid-2-phosphate (AA2P)</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>Analytical reagent</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated total reflection</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>AB</td>
<td>Acrylamide bisacrylamide</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type cell culture</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Beta Glucan</td>
<td>β-glucan</td>
</tr>
<tr>
<td>bLF</td>
<td>Bovine Lactoferrin</td>
</tr>
<tr>
<td>bLFcin</td>
<td>Bovine Lactoferrin</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>CAGR</td>
<td>Cumulative annual growth rate</td>
</tr>
<tr>
<td>CPPs</td>
<td>Cell penetrating peptides</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Colon carcinoma cancer cells</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>C*</td>
<td>Critical concentration</td>
</tr>
<tr>
<td>CAP</td>
<td>Cellulose acetate phthalate</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug delivery systems</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DEE</td>
<td>Drug encapsulation efficiency</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>dn/dc</td>
<td>Differential refractive index</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>ED</td>
<td>Electrodialysis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electro spray ionisation</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immune sorbent assay</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut associated lymphoid system</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally regarded as safe</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid)</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodo acetic acid</td>
</tr>
<tr>
<td>IAM</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonization</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared spectroscopy</td>
</tr>
<tr>
<td>J/g</td>
<td>Joules/gram</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LF</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td>LFcin</td>
<td>Lactoferricin</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>MF</td>
<td>Microfiltration</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix assisted laser desorption ionisation- time of flight</td>
</tr>
<tr>
<td>MTDSC</td>
<td>Modulated temperature differential scanning calorimetry</td>
</tr>
<tr>
<td>ME</td>
<td>Microemulsion</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectroscopy</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MQ</td>
<td>Milli Q</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>M/Z</td>
<td>Mass/charge</td>
</tr>
<tr>
<td>mV</td>
<td>Milli volt</td>
</tr>
<tr>
<td>NF</td>
<td>Nanofiltration</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>O/W</td>
<td>Oil in water</td>
</tr>
<tr>
<td>Ovx</td>
<td>Ovariectomised mice</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Polylactic glycolic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PACA</td>
<td>Polyalkylycyanocrylate</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PEG2000</td>
<td>Polyethylene glycol 2000</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethacrylate</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Sham</td>
<td>Sham operated mice</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC-MALLS</td>
<td>Size exclusion chromatography connected to multi angular laser light scattering</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethyl ethylenediamine</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting transition/ melting tempertaure</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluro acetic acid</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermo gravimetric analysis</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl benzidine</td>
</tr>
<tr>
<td>Tricine</td>
<td>N-(Tri(hydroxymethyl)methyl)glycine</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USFDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>UF</td>
<td>Ultrafiltration</td>
</tr>
<tr>
<td>W/O</td>
<td>Water in oil</td>
</tr>
<tr>
<td>WPI</td>
<td>Whey protein isolate</td>
</tr>
<tr>
<td>WPC</td>
<td>Whey protein concentrate</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
<tr>
<td>$\eta_0$</td>
<td>Zero shear viscosity</td>
</tr>
</tbody>
</table>
CHAPTER 1
General Introduction
1.1 Introduction

Significant advancement in biotechnology and separation technology has led to purification of new biomolecules and has resulted in the availability of a large number of therapeutic proteins and peptides in the last two decades (1, 2). As therapeutic proteins have become available with multiple biological functions, such as antibodies, enzymes, receptors and ligands, the importance and growth in biopharmaceutical research in this area has become more evident (3). Essentially, proteins are high molecular weight macromolecules composed of several amino acids joined together by peptide bonds. Proteins engineered in the laboratory for pharmaceutical use are known as therapeutic proteins. A large number of therapeutic proteins, such as interferons, growth factors and vaccines are on the market and many more are in different stages of clinical trials. In 2005, the Pharmaceutical Business Review reported that the protein therapeutics market could be poised to grow at a cumulative annual growth rate (CAGR) of 10.5% from 2003 to 2010, in other words, to almost double in terms of value. Features like high specificity, low toxicity, high potency, biological and chemical diversity make protein pharmaceuticals very valuable in treating human diseases.

Major pharmaceutical companies across the world are actively pursuing protein drug discovery or acquiring small biological companies to ensure their presence in this emerging market (4).

Although protein and peptide drugs are attracting great interest, particularly in treating chronic diseases, their delivery still poses a great challenge because of low oral bioavailability. This results from their poor stability to proteolytic and hydrolytic degradation, short biological half life and low permeability across gastrointestinal tract (GIT) barriers due to their poor diffusivity and low partition coefficient in the lipid membranes (5, 6). As a result the protein drugs are rapidly cleared from the systemic circulation and the necessary dose frequency is high. The major routes of administration for proteins and peptides are parenteral (7). Almost 95% of the protein therapeutics approved by the Food and Drug Administration (FDA) today are injectable products. Although parenteral routes are used to achieve good therapeutic effects, they are poorly accepted by patients due to the pain associated with administration and inconvenience, which leads to non-compliance that might compromise the efficacy of therapy (8). Apart from this, injectable formulations have a high cost of production (1). Out of many alternative routes of administration that are
available such as nasal, buccal, rectal, vaginal, pulmonary, ocular and transdermal. However, the oral route of drug delivery is the most popular, convenient and acceptable (9, 10) and the compliance rate with the oral route is maximal.

Research into drug delivery is a fundamental aspect of the drug development process and offers various excellent delivery systems that are designed to provide protection to protein therapeutics that are otherwise susceptible to degradation (11). This can improve their stability, absorption and pharmacokinetic profile at the target site. The key factors in considering specific drug delivery systems (DDS) are safety, stability and efficacy.

A number of approaches have been reported to improve the oral bioavailability of therapeutic proteins, such as co-administration of enzyme inhibitors (12) and/or absorption enhancers (12-14), encapsulation in liposomes (15-17), microemulsions (18, 19), or biodegradable particles (20-22) and chemical modification of proteins (23). All these approaches have their advantages and disadvantages. From the point of view of stability, particulate delivery systems such as polymeric microparticles have been investigated extensively. Other colloidal carriers, such as microemulsions and liposomes (24), have the limitation of poor stability in the GIT on oral administration (25). Rieux et al (2006) summarised the major advantages with particulate delivery systems (26):

1. Polymeric particles are more stable in the GIT compared to other colloidal carriers.

2. The surface of the particles could be modified to change physicochemical characteristics of the particles as required.

3. Particles could be transported across the mucosa if the size of the particles is below 10 µm.

4. Favourable surface phenomena like zeta potential and hydrophobicity could be achieved by surface modification using various polymeric materials.
There are a number of ways to prepare biodegradable polymeric microparticles as carriers for protein therapeutics and other drug substances. The most popular techniques are double emulsion, solvent evaporation, organic phase separation and spray drying (25). Although all these techniques have their advantages and specific disadvantages, all techniques have one common disadvantage: the need for organic solvents in at least one of the production steps (27), which might expose the protein to a potential degradation pathway. Various polymers, natural, semi-synthetic and synthetic, have been extensively investigated for drug delivery. The most commonly used natural polymers are chitosan, alginate, albumin, dextran, gelatin and casein. Polyester family synthetic biodegradable polymers, such as polylactic acid (PLA) and polylactic glycolic acid (PLGA), are amongst the most investigated polymers, especially in microparticulate systems. Apart from the requirement for organic solvents, the PLGA based delivery system, however, may result in a sharp rise in acidity as the polymer degraded into glycolic acid and lactic acid, leading to protein aggregation (28).

Transformation of therapeutic proteins from the new compound stage to a marketed product for clinical use is dependent on the development of an appropriate formulation and technologies that avoid the underlying causes of protein degradation (29). Although synthetic biodegradable polymers have been extensively studied in the search for better formulation development for therapeutics, the use of natural biodegradable polymers in drug delivery continues to be an area of active research (25). In the present study, a natural polymer, β-glucan, was investigated as a protein carrier. A novel methodology was developed to produce microparticles encapsulating bLF, a whey protein from milk, has been developed. The project aims to investigate β-glucan as a potential polymer for encapsulating this protein in microparticles as an oral protein delivery system.

1.2 Literature Review

1.2.1 Gastrointestinal tract (GIT)

The human digestive system is composed of different organs: the mouth or oral cavity, oesophagus, stomach, small intestine and large intestine (Figure 1.1). Broadly the entire system is divided into two main segments based on their anatomical locations, the upper GIT and the lower GIT. The upper GIT consists of the mouth, pharynx, oesophagus, stomach and duodenum. The lower GIT consists of the two
distal parts of the small intestine, which are the jejunum and ileum, the large intestine, which has three important parts: the caecum, colon and rectum, and the anus (30). There are associated organs which are important for the GIT system to work properly and are situated outside the GIT. These include the salivary gland, which produces saliva, the exocrine pancreas, which secretes pancreatic juice, and the liver, which produces bile, an important substance for fat digestion and absorption.

The major physiological role of the digestive system is digestion, absorption of food nutrients, secretion and excretion. Food is ingested as large pieces. To absorb food, it has to be broken down to lower molecular weight molecules, as larger molecules are unable to cross the membrane of the GIT. The associated organs of the digestive system secrete various enzymes, water, ions and other substances and their release is stimulated by the presence of a meal in the GIT. Secretions and other juices help to convert large molecules into smaller molecular weight products that are suitable for absorption. This entire process of breakdown starts from the oral cavity, where saliva is secreted that contains α-amylase, a major digestive enzyme that breaks starch, a polysaccharide into monosaccharides. The pH in the mouth is slightly alkaline, which is conducive for amylase to act on starch. Other enzymes present in saliva include lysozyme and sialoperoxidase. Some absorption of low molecular weight molecules from food takes place in the oral cavity. This route of absorption is often used for drug absorption, particularly those drugs that are not stable at lower pH and when response is required immediately, e.g. glyceryl trinitrate, which is used in angina attacks.

The breakdown of protein starts in the stomach wherein the pH is highly acidic (around 2.0). Proteolytic enzymes are secreted in the stomach and act on proteins, which leads to their hydrolysis. Not many substances are absorbed from stomach. It has been reported that aspirin and alcohol are the main substances that are absorbed from the stomach. Pancreatic juice consists of precursors of proteolytic enzymes - trypsin, chymotrypsin and carboxypeptidases. Lipase, α-amylase, ribonuclease and deoxyribonuclease are secreted as active enzymes. Most of the digested products are absorbed in the proximal small intestine. One of the reasons for major absorption from the small intestine is its vast surface area due to the presence of mucosal folds, villi and microvilli. Villi are the tiny projections on the mucosa of the small intestine. Water and some electrolytes are absorbed in the large intestine.
Figure 1.1 Schematic diagram of the human gastrointestinal system. Adapted from (30).
1.2.1.1 Protein digestion and absorption

Protein digestion starts in the stomach due to the presence of pepsin and other proteases. About 15% of the dietary protein is digested in the stomach. In the small intestine, pancreatic juices containing trypsin, chymotrypsin and elastase act on proteins and convert them into oligopeptides, as shown in Figure 1.2. Oligopeptides are further degraded into di- and tri-peptides by the action of oligopeptidase and amino-peptidase. Finally di- and tri-peptides are converted into amino acids, which are transported across the mucosal membranes of the small intestine by various mechanisms, such as passive diffusion, facilitated diffusion or active transport. Di- and tri- peptides are transported by secondary active transport. Large macromolecules such as proteins are also absorbed by the process known as endocytosis. The macromolecules usually attach to the specific receptors on the mucosa and the entire molecule is engulfed.

![Figure 1.2 Schematic diagram of digestion of proteins and peptides.](image-url)
1.2.2 Oral delivery of proteins

The application of proteins and peptides as therapeutic agents is hampered by the lack of an effective method and route of delivery. As briefly discussed in the Introduction, the oral route of drug delivery is considered the most clinically acceptable. Although parenteral (intravenous, intramuscular, and subcutaneous) is the most common route of therapeutic protein delivery, it is not the preferred route, especially not for long term treatment and in certain disease conditions. The other routes that have been under active investigation or have shown some level of success are nasal (31, 32), transdermal (33), buccal (34), pulmonary (35, 36), rectal (37), vaginal (38) and ocular (39).

Because of the large size of the proteins and their vulnerability to proteolytic enzymatic degradation, low lipophilicity and subsequent poor intestine permeability, short plasma life and stability concerns (40, 41), it has always been a challenge to develop an effective delivery system. There are a few protein-based formulations for cyclosporine A and desmopressin that are available in oral dosage forms. Several approaches have been used to improve the oral bioavailability of therapeutic proteins, such as chemical modification of proteins, addition of protease inhibitors (42), absorption or permeation enhancers (43) and formulation carriers (44). Chemical modification of the protein is aimed to (i) improve the stability against enzymatic degradation, and (ii) increase the lipophilicity (12, 45) of the molecule so that it can partition into the epithelial membrane and be absorbed transcellularly (46). Moreover, chemical modification also decreases the immunogenicity. Chemical modification is effected either by directly altering the side chains of the protein or the sugar part attached to the protein, especially in the case of glycoproteins. Prodrug and analogue formation provides protection from different proteases, leading to availability of higher amounts for absorption (47). Although protease inhibitors do improve the absorption of proteins, they have limitations in long term therapy and are not a favourite with clinicians as unwanted proteins also get absorbed into the system (42). It has been reported that frequent use of protease inhibitors might lead to hyperplasia of the pancreas and even cause carcinoma (48).

The epithelial cells in the intestinal mucosa are connected by tight junctions with a space in between the cells called the ‘paracellular space,’ which is in the order of 10-50 Å. This indicates that a molecule or particle with a radius greater than 15 Å, equivalent to approximately 3.5 kDa, is not suitable for transport via the paracellular
route under normal conditions (49). Considerable research has been carried out on improving the paracellular transport of proteins by tight junction modification (50, 51). Chitosan (52), and thiolated polymers (53) have been used to improve the absorption of therapeutic proteins considerably with this approach but it also might lead to absorption of potentially unwanted substances present at the site of absorption along with the desired macromolecules (43, 50). Alkyl glycosides, surfactant type excipients and calcium chelators like ethylene diamine tetra acetic acid (EDTA) have been reported to improve the bioavailability of several peptides (54). The mechanism of opening the tight junctions and transcellular route by increasing membrane fluidity and solubilising cell membranes has also been reported (55).

The possibility of improving bioavailability without interfering with intestinal epithelial physiology or proteolytic enzyme activity lies in using pharmaceutical technologies that involve protection from gastric acid and pepsin, temporarily lowering the pH in the small intestine to inactivate the intestinal enzymes, or adhesion to mucous membranes. Protection from gastric acid and pepsin is achieved by enteric coating (56-58) of the product or drug directly, so the drug is delivered at a specific site in the small intestine. Bioavailability of insulin has been improved to close to 10% by using smart polymer microparticles (59). Mucoadhesive agents prolong the residence time by attaching to a mucous membrane and improving the absorption of biologicals susceptible to proteolytic enzymes in the intestine (60-62).

Using particulate delivery systems for oral delivery of macromolecules is the foremost strategy that is being investigated. Particulate delivery systems protect the protein and peptide drugs from enzymatic degradation and help in transfer of drugs across the epithelial mucosa. With nanoparticles, depending upon their particle size, surface charge can be taken up by M cells across mucosal epithelium (63). Particles can also be taken up by lymphoid tissue in Peyer’s patches. Peyer’s patches are follicles of lymphoid tissue, part of the gut-associated lymphoid system (GALT), which is involved in the development of the mucosal immune system. The common polymeric delivery systems are based on hydrogels (64), nano/micro particles and lipid based delivery systems. Solid polymeric particles have shown greater stability in the GIT as well as on storage compared to lipid based systems such as microemulsions and liposomes (65). The polymeric carriers that are used extensively are PLA, PLGA (66), chitosan (67), polymethacrylate (PMMA) and polyalkylcyanoacrylate (PACA).
A strategy involving receptor-mediated endocytosis has also been shown to improve the absorption of therapeutic proteins. There is no limitation with regard to the size of the macromolecule with those drugs that are transported by endogenous cellular transport systems. There are many known receptor recognizable ligands, such as lectins, toxins, transferrin, and vitamins (vitamin B12, folate, riboflavin, and biotin), which can be used to deliver protein drugs to specific target cells (68-70). Vitamin B12 conjugates (68) have been successfully used to deliver therapeutic proteins orally. The epithelial membrane in the GIT is considered to be the most prominent barrier to the absorption of macromolecules. Some cell-penetrating peptides (CPPs) have been used to internalise bioactives into the cells (71, 72) without causing any harm to the membranes (72). CPPs are a class of short peptides that confer a targeting ability to protein drugs by conjugating with the protein drug and delivering it to the specific cells. Some of the known CPPs are TAT (73), penetratin and oligoarginine. It has been demonstrated that CPPs are able to deliver small molecules, liposomes, and nanoparticles, as well as macromolecules into cells and tissues. The strategy has shown promising results to improve the permeability of macromolecules but the mechanism of delivery is nonspecific and strategy lacks sufficient in vivo data.

1.2.3 Potential oral formulations for proteins

It is estimated that close to 900 protein based compounds are in various stages of clinical studies for their potential therapeutic effects, and 30-40% of the total drug approvals nowadays are in the biological category, which includes antibodies, proteins and peptides (74). To translate the huge variety of potential therapeutic compounds into stable and effective products, a suitable formulation technology is required to protect the integrity of proteins from the harsh GIT environment. Whether a delivery system is invasive or non-invasive, the formulation of therapeutic proteins is always a challenge because of their complex structure and stability concerns. Systemic absorption of intact therapeutic protein and peptide drugs through the epithelium is a prerequisite for a successful delivery system and is one of the major challenges to effective protein and peptide delivery. Absorption of drugs through the epithelium takes place either by passive or active mechanisms, through the cells (transcellular) or through the spaces between the cells (paracellular). See Figure 1.3. Lipophilic drug molecules generally take the transcellular route and as proteins and peptides are hydrophilic and have a high molecular weight, the paracellular route is also not feasible (75).
Figure 1.3 Schematic tranverse section of intestinal epithelium and follicle associated epithelium (FAE) depicting M cell transport of particles or pathogens. (I) Passive transcellular transport. (II) Paracellular transport between adjacent cells. (III) Particles can be absorbed by M cells of FAE found in Peyer’s patches. Adapted from (25) with permission.
Oral formulation of insulin for diabetes has been the focus of research for more than a decade. Calcitonin, a peptide secreted by the parathyroid gland in humans and used to treat Paget’s disease, and parathyroid hormone (PTH) are the two potential candidates that are under active investigation for the treatment of osteoporosis using an oral formulation with new formulation technologies (76). Other important therapeutic potential candidates being explored for oral delivery are interferons, human growth hormone, gonadotrophin-releasing hormone, encephalin, vaccines, enzymes, hormone analogue and peptide-based enzyme inhibitors (42). Some of the commercial technologies that are used for oral formulation are summarised in Table 1.1. The other important examples of polypeptides that are available as oral dosage forms are cyclosporine A and desmopressin (77). One of the reasons for cyclosporine absorption through the GIT is its high lipophilicity (77) and its metabolic stability (78). This clearly indicates that the design has to be on a case by case basis as every protein or peptide candidate has different physicochemical properties.
Table 1.1 Recent industrial technologies for oral protein/peptide delivery. Adapted from (79) with permission.

<table>
<thead>
<tr>
<th>Developer</th>
<th>Peptide</th>
<th>Product name</th>
<th>Technology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emisphere Technology Inc.</td>
<td>Calcitonin,</td>
<td>n.a</td>
<td>Eligen Technology, permeation enhancers based on modified carboxylic acids</td>
</tr>
<tr>
<td></td>
<td>GLP-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biocon</td>
<td>Insulin</td>
<td>IN-105</td>
<td>Insulin-polymer conjugate</td>
</tr>
<tr>
<td>Thiomatrix</td>
<td>Insulin,</td>
<td>n.a</td>
<td>Thiemer technology, novel multifunctional polymers</td>
</tr>
<tr>
<td></td>
<td>Calcitonin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetology Ltd.</td>
<td>Insulin</td>
<td>Capsulin™</td>
<td>Enteric capsule containing a mixture of absorption enhancers and solubilizers</td>
</tr>
<tr>
<td></td>
<td>GLP-1</td>
<td>combulin™</td>
<td>Capsulin™ including insulin senstizer</td>
</tr>
<tr>
<td>Apollo Life Sciences</td>
<td>Insulin</td>
<td>Oradel™</td>
<td>Gastric protection and permeation improvement using molecular transporters</td>
</tr>
<tr>
<td>Unigene</td>
<td>GLP-1</td>
<td>n.a</td>
<td>The formulation is based on enteric coated dosage form and contains an organic acid to lower intestinal pH as well as permeation enhancers</td>
</tr>
<tr>
<td></td>
<td>PTH analogs</td>
<td>n.a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soloman calcitonin</td>
<td>n.a</td>
<td></td>
</tr>
<tr>
<td>Oramed Pharmaceuticals Inc.</td>
<td>Insulin</td>
<td>ORMD-0801</td>
<td>Enteric capsule containing a mixture of absorption enhancers and protease inhibitors in omega -3 fatty acids</td>
</tr>
<tr>
<td></td>
<td>GLP-1</td>
<td>ORMD-0901</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Influenza vaccine</td>
<td>ORMD-1001</td>
<td></td>
</tr>
</tbody>
</table>
To develop a stable and effective oral formulation it is very important to understand the critical physicochemical properties of proteins and peptides, such as molecular weight, PI (isoelectric point), solubility, lipophilicity, stability profile, charge and receptor affinities. Different delivery systems that are being used in oral delivery of proteins and peptides are microemulsions (ME), liposomes and particulate-based systems.

Generally, formulations of protein-based drugs intended for oral delivery have been developed in enteric coated tablets and capsules. Enteric coating protects the proteins from enzymatic degradation and also prevents the dilution of the protein drugs and helps in release of drug at the site where most of the absorption takes place.

ME are clear, spontaneously forming single phase colloidal dispersions and are thermodynamically stable, isotopic systems where two immiscible liquids are present as macroscopically one phase. It is achieved with the help of surfactants and co-surfactants. There are two types of ME depending on the continuous phase: oil-in-water (o/w) and water-in-oil (w/o). The fundamental difference between normal emulsions and microemulsions is the particle size and stability. Emulsions are kinetically stable but ME are thermodynamically stable. The advantages of ME in oral delivery are prevention of degradation of proteins and peptides in the GIT and enhancing their permeability (80). W/o ME systems have been demonstrated to improve chemical stability of horseradish peroxidase after storage. W/o ME have also been used to immobilise water soluble enzymes such as lipase (81, 82). It has been reported that oral bioavailability of a number of bioactive peptides was increased by lipid-based ME (83).

Liposomes were discovered by a British haematologist named Dr Bangham in 1961. These are small vesicles with a lipid bilayer of phospholipids. They can entrap hydrophilic drugs in their internal aqueous core and lipid soluble drugs in their membranes. The phospholipids are amphipathic, having both a hydrophilic or polar head and a hydrophobic or non-polar tail. Many investigators have demonstrated liposomes to be one of the potential drug delivery systems, especially in cancer therapy, where liposomal formulations are available commercially, and gene delivery (84). They are often named based on their size and the number of the bilayers as large unilamellar vesicles (LUV), small unilamellar vesicles (SUV) and multilamellar vesicles (MLV). To provide more stability to liposomes in vivo, coating with
polyethylene glycol is employed and such liposomes are called ‘stealth liposomes’. By doing this they escape recognition by the immune system of the body. However their use in oral delivery systems is very limited because of their instability in the GIT environment (25, 85). The improvement of intestinal absorption of insulin and calcitonin by coating liposomes with mucoadhesive polymers like chitosan and carbopol have been demonstrated in the past (85). The particulate delivery system for oral delivery is discussed in detail under formulation approaches for proteins in Section 1.2.5.

1.2.4 Protein stability

Proteins are highly complex but organised entities, which are prone to various degradations, physical or chemical. These degradations might occur for environmental reasons or during different manufacturing processes. Whether it is a solid or liquid dosage form, it is important to understand the various degradation pathways in order to develop a clinically viable formulation. The processes that expose the proteins to various degradations include aqueous/organic interfaces, elevated temperatures, vigorous agitation, hydrophobic surfaces and detergents (86). In order to maintain their functional properties, proteins must maintain their structural and chemical integrity. To achieve this it is imperative to understand the causes and mechanism of degradation of the protein of interest. Several investigators have reported the different pathways (87), mechanisms and prevention (88, 89) of degradation and various methods of stabilizing proteins in aqueous or solid dosage formulations (90). The fact that certain natural organisms such as anaerobic, methanogenic or sulphur-reducing bacteria can grow comfortably at extreme temperatures close to 100°C (91, 92) has provided valuable information for stabilizing protein pharmaceuticals. The thermophillic proteins that showed higher stability in their molecular structure include increased hydrophobic interactions, greater molecular packing, more hydrogen bonds, more salt bridging (93, 94) and high cellular content of sugars, salts and other osmolytes such as glutamate and di-myoinositol-phosphate and its isomer. Major mechanisms of physical and chemical instability are given in Table 1.2.
Table 1.2 Common mechanisms and causes of protein instability. Adapted from (29) with permission.

<table>
<thead>
<tr>
<th>Instability</th>
<th>Potential causes/ protein moiety involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregation</td>
<td>Intermolecular associations</td>
</tr>
<tr>
<td>Deamidation</td>
<td>Asparagine and glutamine residues</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Cystine and methionine residues</td>
</tr>
<tr>
<td>Disulfide bond reshuffling</td>
<td>Cystine residues</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>Asparagine - Y peptide bond</td>
</tr>
<tr>
<td>Succinimidation</td>
<td>Iso-aspartate</td>
</tr>
<tr>
<td>Non- disulphide Crosslinking</td>
<td>Dimers and polymers</td>
</tr>
<tr>
<td>Maillard reaction</td>
<td>Reducing sugars – amino group</td>
</tr>
</tbody>
</table>
Not much has been studied about the chemical instability of proteins compared to the chemical instability of small molecules. Exposing the therapeutic proteins to elevated temperatures during manufacturing or storage generally leads to decreased chemical stability by accelerating almost all chemical degradation reactions. Residual moisture in the protein formulation is also considered to be responsible for chemical instability. Aspartic acid-hexapeptide degradation increases with an increase in the moisture content in the vial (73). Pikal et al (1992) reported that the oxidation of lyophilized human growth hormone significantly changes with the decrease in moisture content but not by increasing moisture content (95). Other factors that affect protein stability are formulation pH, adsorption, salts, metal ions, chelating agents, shear stresses, non-aqueous solvents and protein concentration.

1.2.5 Formulation approaches to improve protein stability

Advantages of particulate-based delivery systems particularly for protein and peptide drugs were mentioned in Section 1.1. There are commercial products on the market such as Lupron\textsuperscript{®} Depot\textsuperscript{®}, Zoladex\textsuperscript{®}, Decapeptyl\textsuperscript{®}, Eligard\textsuperscript{®}, Enantone\textsuperscript{®}, Trenantone\textsuperscript{®}, Profact\textsuperscript{®} that have resulted from different encapsulation technologies (96). The encapsulation or entrapment of hydrophilic drug can principally be achieved in two ways (Figure 1.4):

1. Addition of the drug prior to the polymerisation process of monomers of the carrier results in the encapsulation of drug within the particle.

2. Addition of drug after the polymerisation, wherein the drug is adsorbed on the surface of the particle. This process is known as sorption and the mechanism is believed to be due to ionic interactions.

Various polymeric materials have been extensively investigated for encapsulation and many have found applications in commercially available products. The important criteria in selecting a polymer are its biocompatibility, degradation and the ease of straightforward production. The degradation pathway and the physicochemical properties of the polymers determine the mechanism and profile of drug release. The choice of the method to prepare nano-/micro-particles depends on the polymer and on the solubility and stability profile of the drug. Most of the encapsulation methods are based on three basic techniques: solvent extraction/evaporation, phase separation (coacervation) and spray drying. Commonly used methods for
encapsulation are precipitation, solvent evaporation, salting out, desolvation and ionic gelation.

![Diagram](image)

**Figure 1.4** Schematic diagram showing method of entrapment of proteins. Adapted from (97) with permission.

The solvent evaporation and precipitation method involves dispersion of polymer in a water immiscible organic solvent such as dichloromethane, chloroform or ethyl acetate. This is followed by emulsification of organic solvent in the aqueous phase containing a stabiliser, such as polyvinyl alcohol (PVA), and the hydrophobic drug forming an o/w emulsion. Finally, the organic phase is evaporated. In the case of hydrophilic drugs such as proteins and peptides, the aqueous phase containing drug is emulsified in the organic phase containing polymer and an outer aqueous phase containing surfactant is added to form a w/o/w emulsion. Evaporation of the organic phase is carried out by stirring until most of the organic solvent evaporates. The resultant particles are washed several times, centrifuged and lyophilised (98). This method is extensively used for encapsulation and PLA and PLGA are the polymers used most frequently. A similar method with a small variation was reported by Leroux et al (1995), known as the emulsion-diffusion method wherein an o/w emulsion is prepared with a partially water soluble solvent, containing polymers and an aqueous solution containing stabiliser. A large volume of water is added, which causes the diffusion of the partially water soluble solvent into the outer phase and produces the
particle. The biggest disadvantage of the phase separation technique is residual solvents and coacervation agents in the final product.

Ionic gelation and coacervation methods are mild methods and have the advantage of an organic solvent free process. In the coacervation method, oppositely charged polyelectrolytes with a relatively low charge density are mixed at a particular temperature, pH and concentration and a liquid polyelectrolyte complex is formed. Sodium alginate forms a gel structure in water in the presence of cations such as calcium, zinc and barium. Alginate particles are prepared by dropwise addition of sodium alginate solution into calcium chloride solution.

Recent trends in formulation processes for protein pharmaceuticals are inclined towards solid formulation processes as they provide better storage stability. One of the alternative techniques to freeze drying to produce solid particles is spray drying. Spray drying is a popular technique to produce solid particles, powders or granules from a mixture of drug and excipients in solutions or suspensions. The method involves drying of atomised droplets in a continuous flow of hot air or nitrogen gas. Atomisation results in formation of small droplets, from which solvent evaporates instantaneously, leading to the formation of free flowing particles. The final particle size depends on the size of nozzle, spray flow rate, atomisation pressure and inlet air temperature. Although spray drying is a one-step drying method to produce dry powder from liquid, it is also considered a harsh method as the exposure of the protein surface to the drying temperature may have a detrimental effect on the protein stability. Some excipients like trehalose are added to protect the protein from degradation during spray drying.

A spray freeze drying method that is also quite common in the pharmaceutical industry is a multistep cryogenic process used in the manufacture of a commercial product, Nutropin Depot™, a controlled release formulation of human growth hormone (99).

Agnihotri et al (2004) have developed and discussed a novel method known as the sieving method to manufacture chitosan microparticles. The particles were prepared by crosslinking chitosan and the hydrogel formed was passed through sieve. Though the particles produced were irregular and large, they resulted in slow release of small molecules (100).
1.2.6 Characterisation techniques for proteins

Various physicochemical parameters, such as total protein content, molecular weight determination and amino acid sequencing are evaluated by using different techniques as a preformulation step. These characterisation techniques are well established and are widely used in the pharmaceutical industry.

Total protein concentration is measured by various methods such as the Bradford, Biuret or Lowery methods, which are based on dye. A direct method can also be used for comparatively pure proteins by measuring the absorption of a protein solution at one more wavelength in the near UV region (260-280 nm). The Bradford method is widely used as it is more sensitive and less affected by other common biochemicals.

Electrophoresis techniques are used to evaluate molecular weight and purity of proteins and peptides. Electrophoresis is the migration of charged molecules in a solution in response to an electric field. The rate of migration depends on net charge, size, shape of the molecule and strength of the field. SDS is added to the sample preparation and running buffer. SDS interacts with the protein, unfolding it and adding multiple charges to the molecule from the sulphate group. Reducing agents are added to completely unfold the protein and proteins are separated on the basis of molecular weight. Electrophoresis is also used to evaluate the presence of aggregates.

MS is another technique to characterise proteins and peptides for their mass and is a very efficient technique to accurately measure the mass of a protein. MS is based on mass to charge ratio. It works by bombarding the protein molecules with a stream of high energy electrons and converting some of the molecules to ions that are accelerated in an electric field. These accelerated ions are separated according to their mass to charge ratios in an electric field, detected by a detector and amplified to a recorder. The trace from the recorder is a mass spectrum. There are two main methods for ionisation of whole proteins: electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI). MALDI has an advantage over ESI in the ease with which the samples are prepared and analysed. This technique is more tolerant of salts, buffers and denaturants in the sample. MS is also used to determine amino acid sequencing of a protein or peptide. It is generally done by digesting the protein by a proteolytic enzyme.
1.2.7 Techniques for monitoring protein instability

Proteins undergo various stresses during different formulation steps that might result in the degradation of protein. Many physical techniques are available to monitor protein instability. Differential scanning calorimetry (DSC) and circular dichroism are generally used to study unfolding, whereas fluorescence, infrared spectroscopy (IR), optical rotation, UV and DSC are good techniques to monitor protein aggregation. In DSC, protein unfolding gives an endothermic peak whereas aggregation is shown as an exothermic peak. Sometimes aggregation and unfolding happen at the same time. In that case the modulated DSC instrument is a good choice due to its higher resolution (101). T_m determination is dependent on several factors, one of the most important of which is temperature ramping rate. It is reported that lowering the temperature ramping rate reduces the T_m. There is not a significant effect of protein concentration on the T_m. IR and capillary electrophoresis (CE) have been used for Tm determinations (102). The IR technique is also used for determination of secondary structure of proteins and any change in the secondary structure. Interaction with other excipients during formulation processing can be evaluated by looking at the amide I region (1620-1690 cm\(^{-1}\)). Another method that is in use for monitoring aggregation is SDS-PAGE. Although size exclusion HPLC (SEC-HPLC) is a technique of choice, if a protein has a carbohydrate moiety or interacts with the column, this may change the elution profile and give incorrect molecular weight results. This issue can be resolved by coupling the HPLC with a light scattering detector along with refractive index detectors (103).

Reverse phase HPLC (RP-HPLC) is also used for quantification and separation of proteins and protein fragments, but is not capable of identifying protein degradation products completely, therefore other techniques like liquid-chromatography-mass spectrometry (LC-MS) and amino acid sequencing are used to further supplement the findings (104).

1.2.8 Protein selected for this study

Although many proteins present in whey have different biological functions, most of the applications of whey are due to the presence of LF. It is considered as a minor protein present in milk, as discussed in Section 3.1.1.3, but is associated with various biological applications. bLF is being used in commercial products such as milk based infant formulas for boosting the immune system against infections, mimicking human
breast milk, health supplements, functional food drinks, cosmetics and in chewing gums as an oral care product. bLF is being extracted from milk in large quantities and is extensively studied nowadays for its various potential effects, both industrial and biological. Recently, a patented activated LF has been used to protect fresh meat against pathogenic bacteria in USA. In this study, bLF is considered to be a potential candidate for development of an oral formulation to be used either as a nutraceutical or pharmaceutical dosage form.

1.2.9 Biopolymer selected for this study

β-glucans are unbranched polysaccharides obtained from different natural sources, are known for their health benefits, are used in by food industry and is discussed in detail in Section 2.1. Very limited information is available on their applications in drug delivery especially the delivery of therapeutic proteins.

1.3 Aim and specific objectives

The main aim of this work was to investigate the potential of barley β-glucan as a particulate carrier for oral delivery of a whey protein, bovine Lactoferrin, (bLF) and its bioactive peptide, bovine Lactoferricin (bLFcin). To achieve this aim the following specific objectives were envisaged:

1. To evaluate the physicochemical properties of β-glucan and to characterise bLF.

2. To develop and optimise a novel method for microparticle production of β-glucan encapsulating bLF and to evaluate the stability of β-glucan, bLF and particles.

3. To derivitise bLF into bLFcin with a proteolytic enzyme, pepsin followed by purification of bLFcin, characterisation of bLFcin and evaluation of its in vitro activity.

4. To evaluate in vitro and in vivo bioavailability of the bLF from microparticles encapsulating bLF.
1.4 Thesis organisation

Chapter 2 presents the results obtained after evaluating physicochemical properties of two grades of β-glucan, designated as β-glucan1 and β-glucan2, such as molecular weight, rheological behaviour of freshly prepared different concentrations at different parameters and gel strength studies. Stress studies were carried out on β-glucan2 as this grade was further used for casting films and particle production. A concentration of β-glucan at which it does not form gel and is independent of change in pH was selected for casting films and particle production.

Chapter 3 includes characterisation of bLF (molecular weight, mass spectrometry and peptide identification) and enzymatic derivitisation and optimisation of derivitisation into its bioactive peptide bLFcin. Purification was carried out by various techniques and purified bLFcin was characterised for its molecular weight, peptide identification and sequencing. The purified bLFcin was evaluated for its biological activity on the osteoblast cells.

Chapter 4 presents the details of a novel cryomilling technique and procedures of casting films and producing particles. The effect of different milling conditions on the average particle size was investigated. A HPLC method was developed and validated to determine the concentration of bLF in the particles. Solid state studies were carried out using FTIR and DSC to evaluate any structural change during the process. The formulation that gave minimum average particle size with high loading was selected for further studies. Different excipients were included to evaluate their effects on the sustained release action of the particles and films. The results from these studies were a prompt to undertake the interaction evaluation of bLF with β-glucan using surface plasmon resonance studies, which showed strong interactions with the protein. Stability studies of bLF were carried out by FTIR and MS after extraction of bLF from the particles. Stability studies of β-glucan were carried out by assessing average molecular mass for six months at different storage conditions.

Chapter 5 presents the in vitro and in vivo studies of the microparticles. The biological activity of bLF after extraction was evaluated using osteoblast proliferation assays. Once the biological activity was noticed, uptake of bLF and bLF from the particles were evaluated by Caco-2 cell monolayers. In vivo studies were carried out to assess the improvement in bLF bioavailability and BMD gain after mice were fed a
diet supplemented with bLF or particles encapsulating bLF, compared with a control diet.

Chapter 6 presents the general discussion and future directions arising from this work.

In the entire thesis two grades of β-glucan are represented as β-glucan1 and β-glucan2. The films of both the grades were designated as β-glucan1 films and β-glucan2 films with either 1 or 10% w/w of loaded bLF. In all subsequent chapters β-glucan2 bLF film represents bLF loaded 10% β-glucan2 films and β-glucan2 bLF milled particle represents bLF (10% w/w) loaded β-glucan2 milled particles unless specified. bLF represents bLF and LF represents LF from all known sources.
CHAPTER 2
Characterisation of β-glucan
2.1 Introduction

2.1.1 Beta Glucan (β-glucan)

β-glucan is the chemical name of polymers of β-glucose differing in glycosidic bond position. Although they are chemically heterogeneous, they are known by the common name ‘β-glucan’. The β-glucan that is used in this project is sourced from barley and comes under the common name of cereal β-glucan. Cereal beta glucans are non starch polysaccharides of glucose residues interlinked via β-(1→3) and β-(1→4)–glucosidic linkages and are found as a predominant cell wall component of cereal grains, such as barley, oats and wheat (105, 106). They are concentrated in the cell walls of the endosperm and the aleurone layer of barley, oats and wheat. Aleurone is the outermost layer of the endosperm and is found to be multilayered in barley. They are found exclusively in the cell walls of plants belonging to the Poaceae family. Other β-glucans are found in the cell walls of some species of fungi, algae and horsetail (107). β-glucans have gained a lot of interest over recent years because of their wide range of potential applications in the health sector and industrial applications. This major thrust is solely due to their various reported health benefits.

2.1.1.1 β-glucan structure

β-glucans are linear, unbranched polysaccharides containing monomeric β-D-glucopyranosyl residues with two types of linkages, (1→6)-O-linked β-D-glucopyranosyl units and (1→3)-O-linked-β-D-glucopyranosyl units. Cereal β-glucan chains have around 70% (1→4) and 30% (1→3) linkages. Large numbers of (1→4)-linkages are present in groups of 2 or 3 separated by single-(1→6)-linkages in the barley β-glucan chain (Figure 2.1). Barley cell walls consist of about 70% β-glucan and barley aleurone has 26% β-glucan, while other polysaccharides found in the barley are arabinoxylan, cellulose and glucomannan. The content of β-glucan in barley is in the range of 3-11% and β-glucan content is 3-7%, 1-2% and less than 1% in oats, rye and wheat respectively (108).

Apart from cereal cell walls of the Poacea family of grasses, β-glucans are produced by prokaryote and eukaryote organisms and are divided into several classes depending on their structural features. β-(1→3)-D glucans are the most abundant of all the β-glucans and are found in mushrooms, seaweed and yeast. They have a common backbone of β-(1→3)-linked β-D-glucopyranosyl units along with randomly dispersed side chains of β-D-glucopyranosyl units attached by (1→6) linkages.
Curdlan, a polysaccharide extracted from different species of *Agrobacterium* strains, consists of linear β-(1→3)-D-glucans, generally consisting of 12000 glucose units, insoluble in water, alcohols and most organic solvents, but soluble in dilute bases and dimethyl sulphoxide (DMSO). It forms a weak gel upon heating above 55°C and cooling but the gel strength increases on further heating to 80-100°C. This property has helped Curdlan find most of its applications in the food industry such as in pasta noodles and bean-starch vermicelli. In Japan, where its use in food is permitted, it is being used to improve the texture of fish, pastas and tofu (109).

β-(1→3)(1→6)-D glucans extracted from several fungal and yeast species are high molecular weight polymers with highly ordered helical structures consisting of a main chain of β-(1→3) linked glucose residues branched with β-(1→6)-D-glucosyl units. The solubility of these polymers depends upon the frequency and length of side chains. Soluble varieties found in *Sclerotium rolfsii* result in viscous solutions and their viscosity does not change over a wide range of temperature. Scleroglucans are neutral polysaccharides and β-glucan extracted from spent brewer’s yeast has emulsion stabilising effects that could be exploited.
Figure 2.1 Structure of barley β-glucan A: simplified representation (G-glucosyl residues), (3 and 4 represents β-(1→3) and (1→4) linkages and Cellotriosyl and Cellotetraosyl residues are shown as G4G4G or G4G4G4G. B: Linear unbranched β-(1→3) and (1→4)-D-glucopyranose units in a non-repeating but non random order.
2.1.1.2 Extraction and purification of barley β-glucan

Several methods have been developed to extract and improve the yield of β-glucan from different cereal sources. There are several steps involved in the extraction and purification. Generally three basic steps are followed in all the processes to extract β-glucan. The first step is inactivation of endogenous β-glucanases, which are responsible for the degradation of β-glucan that results in low molecular weight of the polymer and can alter its physicochemical properties. The second step involves extraction of β-glucan, followed by precipitation as the third step.

Bhatty and MacGregor (1993) reported a method involving several steps such as refluxing barley flour in aqueous ethanol and treating the flour with dilute acid to deactivate endogenous enzymes. β-glucan is then concentrated by precipitation from the supernatant using a water miscible organic solvent or inorganic salt like ammonium sulphate solution (110). Subsequently, Bhatty (1993b) reported extraction of 80% of β-glucan with 4% sodium hydroxide (111). The final product after purification contained 60% of β-glucan. Morgan and Ofman (1998) developed a method using hot water extraction followed by filtering and two freeze-thaw cycles and achieved β-glucan with purity of more than 90%. The heating temperature used in this process was 25-55°C. Supernatant was frozen at -10°C and thawing was carried out at room temperature. The fibrous material present in the thawed solution was recovered by filtration and washed with water before being dried at 50°C. The freeze-thaw step was repeated after the purification. The final product was dried at 50°C (112). This method was considered more efficient and did not involve any organic solvent. The extraction at lower temperature resulted in low extraction of polysaccharide and it is reported that extraction at 55°C for two and a half hours gives maximum yield. The product that is extracted with this new two step process is named Glucagel™. It forms soft translucent gels at a low concentration.

Recently extraction methods involving enzymes and other techniques have been employed to improve the purity and yield of β-glucan. The extraction cost was quite high with the previous methods, particularly for the food industry. Techniques such as ultrasonication are used after water extraction or high pressure homogenisation before enzyme digestion helps to get better extraction of β-glucan. β-glucans that are extracted by different methods give products of varying molecular weight and chain lengths.
Extraction of β-glucan from fungi is generally done by breaking the hydrogen bonding in cell walls by treating with hot water followed by extraction under strong alkaline conditions. Enzyme hydrolysis is sometime employed in order to form a water soluble β-glucan that can easily be recovered. Extraction and purification process parameters like temperature and time of extraction greatly influence β-glucan properties. For instance, mild extraction conditions may not deactivate endogenous enzymes and result in increased depolymerisation of the β-glucan.

2.1.1.3 Determination of molecular weight of β-glucan

The molecular weight and other physicochemical properties of β-glucan depend upon the source, cultivar, and extraction method, which again depend upon several parameters such as temperature of extraction and extraction solvent. β-glucans of different molecular weight and chain lengths are obtained. Therefore molecular weight determination is an important characterisation technique for every batch of the polysaccharide extracted. Fincher and Stone (1986) found the range of molecular weight of isolated mixed link of β-(1→3) and β-(1→4) glucan to be $2.0 \times 10^4$ – $40.0 \times 10^6$ g/mol. Although these linkages are found in all cereals of the Poaceae family, the molecular weight variability is also reported between cereals, with oat β-glucan generally having a range of 0.065–3 $\times 10^6$ g/mol and barley β-glucan having a range of 0.15-2.5 $\times 10^6$ g/mol (109). Morgan et al (1999) extracted different fractions of β-glucan with a range of 14000–560 000 g/mol. The large variation in the molecular weight of β-glucan from different sources is due to variation in cell wall structure, different extraction and isolation methods.

Size exclusion chromatography (SEC) is a liquid chromatography method used to determine molecular weight of macromolecules based on their molecular size. SEC is also known as gel permeation chromatography (GPC) or gel filtration chromatography (GFC). SEC involves separation of molecules when a liquid mobile phase is passed through the stationary phase (column) at a fixed flow rate and polymer solution is injected into the column. The larger molecules elute first as they cannot enter the small pore size of the packing material of the column and stay in the interstitial space, whereas smaller molecules enter the smaller pores and elute later. A differential refractive detector responds to the elution by generating a peak for each band as it passes through, the size of which is proportional to the concentration. To improve separation, high pressure pumps capable of operating at high pressure, generally up to 6000 pound force per square inch (psi), are used. An appropriate mobile phase is
selected so that the sample polymer should be completely dissolved and the viscosity of the sample should be low. Some polymers like polyesters and polyolefins dissolve only at high temperatures. Stationary phases are selected based on the sample to be analysed and should not interact chemically with the sample. They should be stable at a wide temperature range and have an adequate range of pore sizes and sufficient pore volume to efficiently resolve the sample's molecular weight distribution. Generally, semi-rigid polymeric gels are used for high pressure SEC (HPSEC). The typical available pore size of the columns ranges from 60 to 4000 Å and the particle size of the packing material is usually in the range of 5-10 µm. The sample volume depends on the number of columns and generally ranges from 25-200 µl. The sample concentration is selected depending upon the molecular size of the sample and its viscosity. Generally, dilute solutions are preferred as high concentrations of the sample can lead to peak shifts towards lower retention volumes and band broadening due to viscous fingering, believed to be due to polymer chain entanglements. Optimum sample concentrations usually range from 0.1% for high molecular weight samples to 1% for low molecular weight samples. Flow rates of 0.5-1.0 ml/min are generally employed depending upon number of columns. For single columns, 0.5 ml/min is preferred and 1.0 ml/min is preferred for multiple columns.

For determining the molecular weight distribution of a polymer, calibration is carried out using narrowly distributed standards (different molecular weights) of the polymer under investigation. Retention volume of each standard is equated with its stated molecular weight. The main limitation with this method is the lack of availability of different polymer standard types. Commercially available standards include polystyrene, polyethylene, poly (methyl methacrylate), polyethylene oxide, dextran and pullulan. For direct measurement of molecular weight without the need for external standards, a second detector is installed to an SEC system such as light scattering detectors. These detectors are sensitive to molecular weight.

One of the fastest and most versatile techniques for determination of absolute molar mass of the macromolecule in solution is light scattering, which covers a wide range of molar mass (100–10^5 g/mol). When the determination is made over the entire range of angles, it is called multi-angle light scattering. The first commercial light scattering photometer using a laser was introduced by Wyatt and Philips in 1970. Light scattering depends on two fundamental principles. Firstly, the intensity of light scattered by the sample is directly proportional to the product of the molar mass and concentrations. Secondly, variation of the scattered light intensity with angle is
proportional to the average size of the molecules. The light scattering technique is considered ideal for studies involving aggregation and associations, which are very important in the stability studies in polymer-based drug delivery systems.

### 2.1.1.4 Applications of β-glucan

β-glucans from all sources have a wide range of potential applications in various industries, especially in the food industry, and wide therapeutic applications as well. However, parameters like molecular weight, degree of branching, number of substituents and the presence of single and triple helix are considered to contribute to their biological roles. The United States Food and Drug Administration (USFDA) recommends a daily intake of 3 g of oat β-glucan as a part of a healthy diet. Curdlan is used in food in Japan. β-glucans are designated as Generally Recognised As Safe (GRAS) by the US FDA. The European Communities Commission have permitted soluble β-glucans for the treatment of oral mucositis in head and neck cancer patients (113). Some of the examples of biologically active β-glucans are given in Table 2.1. The applications of D-glucans are considered to be increasing because chemical additives are becoming less attractive among consumers. Apart from their application as thickening and gelling agents, β-glucans from different sources have been used to produce polymeric films that are stable at higher temperatures and have high tensile strength. Curdlan could be used to coat gelatin capsules for greater heat resistance and storage. The ability of curdlan to form an impermeable film provides another potential application in preserving meat products. Similarly the film of the gel is used to reduce water loss from the food product where water is required.

Cereal β-glucans are soluble dietary fibres and are finding applications as stabilisers in the production of low fat products such as ice creams, yoghurts and salad dressings. Generally, β-glucans are used in thermo-reversible form in pasta noodles, bean-starch vermicelli and molded products. β-glucan solutions are reported to increase the elasticity and yield of curd when added to milk. This effect is believed to be due to the gel forming ability of β-glucans and formation of a structured complex of elastic casein-protein-glucan matrix. Several investigators have reported the use of cereal β-glucans as fat replacers in cheese, cookies and sauces. Addition of oat β-glucans to cheese reduces the hardness (109).

Although all classes of β-glucans have applications in industry, especially the food industry, cereal β-glucans are better known for their biological applications. Cereal β-
glucans have applications as a functional dietary fibre and are known to reduce serum cholesterol and liver low-density lipoproteins. This helps in reducing cardiovascular diseases (114), hyperglycaemia and hyperinsulinemia. β-glucans extracted from bacteria, yeasts and fungi with a β-(1→3)-D backbone have immunomodulatory applications and are better known as biological response modifiers (BRM) (115, 116). BRM from fungi, yeasts and seaweed function as immunostimulants against infectious diseases and certain types of tumours. Several studies have demonstrated that β-glucans from yeasts absorb mycotoxins, probably through hydrogen bonding and Van der Waals forces, and protect against protozoal infections. β-glucans activate microglial cells, which have a role in binding to debris from brain cells, and are therefore reported to have a positive role in Alzheimer’s disease, AIDS and multiple sclerosis. They bind to free radicals and have an antioxidant effect, which is responsible for their role in healing wounds. The mechanism of all the reported effects of β-glucans are probably due to activation of the immune system. Amati et al (2005) reported the presence of major receptors for β-glucan, termed dectin-1, on the antigen presenting cells, which supports the mechanism of an immunobiological role of β-glucans (117). Curdlan and its derivatives are reported to have anti-HIV and anti-tumour activities (118). Lentinan from *Lentinus edodes*, a mushroom, has shown anti-carcinogenic activity in animals and humans (109).
Table 2.1 Biologically active glucans and their sources. Modified from (113) with permission.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Main Glycosidic linkages</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley glucan</td>
<td>β-(1→3), β-(1→4)</td>
<td><em>Hordeum vulgare</em> (barley)</td>
</tr>
<tr>
<td>Curdlan</td>
<td>β-(1→3)</td>
<td><em>Alcaligenes faecalis</em> var.</td>
</tr>
<tr>
<td>Glucan phosphate</td>
<td>β-(1→6)</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>Sceleroglucan</td>
<td>β-(1→3), β-(1→6)</td>
<td><em>Sclerotium glucanicum</em></td>
</tr>
<tr>
<td>Pustulan</td>
<td>β-(1→6)</td>
<td><em>Penicillium allahabadense</em></td>
</tr>
<tr>
<td>Schizophyllan</td>
<td>β-(1→3)</td>
<td><em>Schizophyllum commune</em></td>
</tr>
<tr>
<td>Lentinan</td>
<td>β-(1→3), β-(1→6)</td>
<td><em>Lentinus edodes</em></td>
</tr>
</tbody>
</table>
The application of β-glucans in drug delivery systems has not been extensively explored and very limited information is available on their applications in drug delivery. The lack of use of β-glucans in this area is perhaps due to significant variability in their physicochemical properties, which depends on the source of the β-glucan, extraction process temperature, which determines purity, and molecular weight. The majority of their applications are in the food industry, where they are used in food as a bioactive agent. There have been few attempts to use β-glucans as a controlled release delivery system. Konke et al (1992) used curdlan to prepare tablets containing theophylline in which one of the formulations was prepared by spray drying particles of curdlan and theophylline solution. A controlled release profile of the drug was shown (119). Schizophyllan, a β-(1→3)-D-glucan is reported to form a complex with nucleic acid. It is used as a carrier and is resistant to nuclease (120).

### 2.2 Characterisation of β-glucan

β-glucans from various sources are used in the food industry for their wide positive health benefits as discussed in Section 2.1.1.4. Recently there has been a growing interest in β-glucan for its potential role as a delivery system, especially for oral delivery (119, 121, 122).

Hydrophilic polymers of natural origins are being extensively explored for encapsulation of proteins, peptides (79) and other pharmaceutical agents (123). β-glucans have several advantages as potential candidates for protein drug delivery, such as high stability at various temperatures and pH levels, biocompatibility, lack of known toxicity or allergenicity and formation of translucent gels at low concentrations. They are classified as soluble dietary fibre but are reported to be partially degraded in the colon by β-glucanases in the local flora of human colon (113). As humans lack β-hydrolases, β-glucans remain in the blood stream and lymphatic system for a long time without any structural change (113).

Cereal β-glucans show variability between different cereals and also among the same species. They have been extracted by different methods and as a result show variability in molecular weight (0.15-2.5x10^6 g/mol), rheological behaviour and gelling properties. Both the β-glucans characterised in this study were extracted using the non solvent method.
There were two batches of β-glucan sourced from the same company and designated as β-glucan1 and β-glucan2. The initial physicochemical studies were carried out on both β-glucan1 and β-glucan2. β-glucan2 was the main polysaccharide for all subsequent studies.

The specific objectives of this chapter are:

1. To investigate the rheological properties of the β-glucan1 and β-glucan2 in aqueous solutions as a function of pH, ionic strength and concentrations.

2. To evaluate the mechanical properties of β-glucan1 and β-glucan2 at different concentrations.

3. To determine the molecular weights of β-glucan1 and β-glucan2.

4. To evaluate stress studies of β-glucan2.

Rheological behavior was studied at different parameters with the aim of determining the optimum concentration of β-glucan, so that the protein can be dissolved in the aqueous solution of β-glucan at a lower temperature before it sets as a gel. Gel strength was evaluated at different pH and temperatures to optimise the concentrations at which the gel set in. Molecular weight determination was done as there is a wide variability in the molecular weight of β-glucan obtained from barley and the gelling behaviour depends on the molecular weight of the β-glucan. Stress studies were done to investigate the fate of β-glucan in acidic conditions that mimic those of the stomach. Two batches of β-glucan were purchased from the same source and both batches were extracted by different proprietary methods, which were based on aqueous extraction.

2.3 Rheological properties of β-glucan

The rheological behavior of cereal β-glucan is largely dependent on its molecular and structural features. β-glucans are extracted from cereals using different methods, leading to variability in their molecular weight and structural arrangements and conformations. It has been reported in the literature that changes in the molecular weight and conformation affect the rheological properties of β-glucan (124), an important factor that can influence compounding properties. Parameters such as
tempetature and ionic strength may also play an important role influencing rheological properties and are therefore potential parameters to evaluate.

### 2.3.1 Materials

An initial sample of β-glucan was kindly donated (designated β-glucan1) and a later sample was purchased (designated β-glucan2) from GraceLinc Limited (New Zealand). Sodium nitrate, sodium azide and dextran were purchased from Sharlau Chemie (Spain). Dextran was purchased from Sigma (USA). All other chemicals used in the study were at least reagent grade. Water used in the preparation of buffers was Milli Q water obtained by reverse osmosis (Millipore, USA).

### 2.3.2 Methods

#### 2.3.2.1 Microscopic characterisation of β-glucan

The microscopic examination of β-glucan1 and β-glucan2 were carried out using Olympus SZX12 with camera DP12 (Japan). Lighting conditions were selected in order to distinguish surface and internal characteristics of the powder.

#### 2.3.2.2 Preparation of samples

Different concentrations (1, 4, 7 and 10 % w/w) of β-glucan1 and β-glucan2 solutions were prepared in a conical flask by mixing β-glucan powder in preheated Milli Q (MQ) water and heating the mixture to 80°C in a temperature controlled water bath (Heto, SBD-50, Joan Nordic A/S Denmark) for 30 min. The duration of 30 min was chosen to complete the hydration of β-glucan1 and 2 (125). The mixture was cooled to room temperature with constant slow stirring. To avoid any water loss at 80°C, the mouth of the conical flask was covered with aluminum foil. All samples were prepared in triplicate.

#### 2.3.2.3 Rheological measurements

The flow behavior of β-glucan1 and β-glucan2 was studied by measuring apparent viscosity (η) over a range of shear rates. The relationship between shear rate and shear stress was assessed using Brookfield DV-III+ programmable cone and plate rheometer geometry along with CP-40 and CP-52 cone spindle (Brookfield Engineering Laboratories Inc. USA). The temperature was controlled by a jacketed steel cup, which was attached to a temperature controlled water bath.
The instrument was controlled and data were recorded using the Brookfield Rheocalc 3.2 software. All the samples prepared were measured for their flow properties at temperatures of 25, 37 and 70°C and pH of 1.5, 4.0, 7.0 and 10.0. Ionic strength of 0.2 and 0.5 M phosphate buffer was used to study the effect of ions on the flow behaviour. All the measurements were carried out at 25°C unless specified. The data were analysed by generating rheograms of shear rates against viscosity. Pseudoplastic behaviour of β-glucan was described by the power law (Equation 2.1).

\[ F^N = \eta' G \quad \text{Equation 2.1} \]

This equation can be expressed in logarithmic form as:

\[ \log G = N \log F - \log \eta' \quad \text{Equation 2.2} \]

In the equations above, F is shear stress, G is shear rate, \( \eta' \) is apparent viscosity and N is the flow behaviour index or index of deviation from Newtonian flow behaviour. When N=1, it indicates Newtonian flow behavior, whereas N<1 corresponds to pseudoplastic flow and N>1 indicates dilatant flow. Flow behaviour index is determined from the linear regression equation obtained by plotting log G as a function of log F (126).

### 2.4 Mechanical characterisation of β-glucan gel

Mechanical properties of the different concentrations of β-glucan1 and β-glucan2 in gel were investigated using a texture analyzer with the aim of identifying the concentration at which β-glucan forms a structured gel.

#### 2.4.1 Methods

**2.4.1.1 Preparation of sample**

Samples were prepared as described in Section 2.3.2.2 and kept at room temperature for 16 hours for gel stabilisation. The sample concentrations were 1, 2, 3, 4, 7 and 10% w/w.
2.4.1.2 Gel strength measurement

All the measurements were carried out using a TA-XT2i instrument (Stable Microsystems, UK). Samples (50 g) were prepared in 5 cm tall plastic jars. Each sample jar was placed at the centre of the stage under the probe. Tests were performed in compression mode using a 10 mm diameter cylindrical probe (Delrin P/10). The crosshead speed was set at a constant speed of 1 mm/sec and the probe was set to travel 15 mm into the test samples. The maximum force required for the probe to penetrate into the sample, the set distance and force required to remove the probe from the test sample were measured. The measurement was performed in triplicate. All the measurements were carried out at room temperature. Data were recorded and analysed with T32 software.

2.5 Molecular weight distribution of β-glucan

As discussed in Section 2.1.1.3, β-glucans show a great variability in molecular weight, which is largely dependent on the source, geographical parameters and method of extraction. There are many methods that have been reported for molecular weight determination of β-glucan as discussed in Section 2.1.1.3. SEC or GPC, is commonly used for determining molecular weight distribution. After a thorough literature search, a SEC method with multi angle laser light scattering (SEC-MALLS), which can detect aggregates and gives accurate molecular weight, was chosen to determine molecular weight distribution of β-glucan1 and β-glucan2.

SEC-MALLS works on the basic light scattering principle, that the amount of light scattered is proportional to the product of the molar mass, concentration and specific refractive index increment and is represented as follows:

\[ I \propto M c \left( \frac{dn}{dc} \right)^2 \]

Equation 2.3

In this equation, M represents molarity, c represents concentration and \( \frac{dn}{dc} \) represents specific refractive index.

The angular variation of the scattered light is directly related to the size of the molecule and \( \frac{dn}{dc} \) is critical for accurate molecular weight calculations.
2.5.1.1 Average particle size of β-glucan in different solvent systems

Average particle sizes of β-glucan1 and β-glucan2 in different solvent systems were determined using an optical microscopy technique as explained in Section 4.3.4.2. The results are presented as an average of 100 particles measured at random.

2.5.1.2 Density measurement of β-glucan

The density of both β-glucan1 and β-glucan2 was determined using a pycnometer at room temperature using a liquid displacement method with methanol as a displacement media (126).

2.5.2 Methods

2.5.2.1 Chromatographic conditions

The SEC system was comprised of a HPLC pump (waters 515), a Degassex DG-4400 on-line degasser connected to a series of three columns [Ultrahydrogel 2000 pore size 2000Å, Ultrahydrogel 250 pore size 250Å (Waters) and a PL aquagel-OH - mixed pore size] with a PL aquagel guard column and a 0.5 µm in-line filter, a Rheodyne manual injector and a Waters column oven. The mobile phase was 0.1 M sodium nitrate with 0.02% w/v sodium azide, which was used at a flow rate of 0.5 ml/min to elute the polymer. The mobile phase was filtered through 0.22 µm cellulose filter paper (Millipore USA). An injection volume of 200 µl was used. The SEC system was connected to a DAWN-DSP MALLS detector (Wyatt Technologies Corporation, USA) with laser wavelength of 632.8 nm and a Waters 2410 differential Refractometer. The columns and refractive index (RI) detector were maintained at 40°C. A dextran standard of M_w 25,000 and polydispersity of 1.03 was used for normalisation of the light scattering detectors. The concentration of the dextran used was 3 mg/ml.

Data acquisition and processing were performed using ASTRA 4 software (Wyatt Technologies Corporation, USA). Calculations of weight-average molecular weight (M_w), polydispersity index M_w/M_n and z-average-root-mean square (RMS) were performed by ASTRA 4 software. A Brookhaven BI-DNDCW Differential Refractometer was used for determining dn/dc.
2.5.2.2 Preparation of samples

β-glucan1 and β-glucan2 samples were prepared in the mobile phase (0.1 M sodium nitrate solution containing 0.02% w/v sodium azide). Five concentrations of β-glucan1 and 2 (0.5, 1.0, 2.0, 3.0 and 5.0 mg/ml) were prepared in mobile phase by heating the solution at 80°C for 30 min, followed by slow stirring for 15 min and cooling down to room temperature. All sample solutions were filtered through 0.45 µm nylon syringe filters (Millipore, USA) before injection.

Measurement of dn/dc was carried out by preparing five different concentrations (0.5, 1.0, 2.0, 3.0 and 5.0 mg/ml) of β-glucan1 and 2 in the mobile phase and recording the data by using the Brookhaven BI-DNDCW Differential Refractometer at 535 nm in static mode. An average of two measurements was taken to calculate dn/dc.

2.6 Stress studies of β-glucan

The aim of this study was to evaluate degradation of β-glucan2 when exposed to various concentrations of inorganic acids and at various temperatures. It is reported in the literature that β-glucan from oats undergoes total hydrolysis at a very low pH and high temperature (120°C) (127), but there is a very little information about the barley β-glucan used in this project. Moreover, formulations for oral delivery require knowledge of the stability of β-glucan at lower pH. This study was carried out on β-glucan2 only because the manufacturer ceased production of β-glucan1.

2.6.1 Methods

2.6.1.1 Sample preparation

β-glucan2 samples were prepared in 0.2, 2.0 and 6.0 M HCl as 1% w/v solutions and were incubated for six hours at room temperature. Additionally a 1% w/v solution was prepared with 0.1 M HCl and incubated at 120°C for one hour. All the samples were neutralised with sodium bicarbonate to pH 6.0. All the samples were further diluted to a final concentration of 2.5 mg/ml for analysis. All the samples were prepared in triplicate and results were reported as mean ± SD.
2.6.1.2 Molecular weight measurement

All the samples were measured for their molecular weight by SEC-MALLS as described in Section 2.5.2.1

2.7 Results and Discussion

2.7.1 Visual and microscopic examination of β-glucan

β-glucan1 was a white, light (wool like) fibrous material, which disperses easily in cold water (Figure 2.2-top). β-glucan2 was off-white powder, was dense and sticky and did not disperse easily in cold water (Figure 2.2-bottom).
Figure 2.2 Light microscope pictures of top lit β-glucan1 (top, bar=50 µm) and back lit β-glucan2 (bottom, bar=100 µm).
2.7.2 Rheological properties of β-glucan1 and β-glucan2

The initial assessment of concentrations of β-glucan in aqueous solution at which bLF dissolves easily were determined by evaluating change in viscosity at different pH, temperature and ionic strengths. Maria et al (2004) reported that at low concentrations, β-glucan from barley follows the Newtonian law of flow and it shows pseudoplastic behavior at higher concentrations (128). Moreover, rheological characterisation is important as barley β-glucans exhibited large differences in their flow properties because of large variations in their molecular weight, which further depended on source and extraction method (129).

2.7.2.1 Effect of pH on different concentrations of β-glucan1 and 2

The flow curves were generated between shear rate and viscosity and data are presented in Figures 2.3, 2.5, 2.7 and 2.9 for β-glucan1 and 2.4, 2.6, 2.8 and 2.10 for β-glucan2 solutions. The flow curves in Figures 2.3 and 2.4 show that there was no difference in viscosity over a shear rate of 375-525 s⁻¹, At a concentration of 1% of β-glucan1 and 2, the viscosity was negligible, which might be because the polymer did not form any gel structure at low concentrations. There was no change in viscosity seen over a range of tested shear rates. With increase in pH from 1.5 to 4, the η₀ increased but remained the same as the shear rate increased. The η₀ was similar at pH 4.0, 7.0 and 10.0. One of the reasons for low viscosity at pH 1.5 could be partial hydrolysis, which can result in low viscosity at lower shear rates, as reported in previous studies (131). β-glucan2 solution (1%) at pH 4.0, 7.0 and 10.0 showed a slight increase in viscosity initially and then showed Newtonian-like regions with constant viscosity (108), whereas β-glucan1 solution at pH 7.0 and 10.0 showed a slight decrease in viscosity initially followed by Newtonian behaviour. The slight increase in viscosity at higher pH might be due to partial entanglement of chains.

Bohm and Kulicke (1999) reported a sharp increase in η₀ at concentrations above the critical concentration (c*) in cereal β-glucan, which was also observed in our study. β-glucan1 7% solution showed no effect of pH on viscosity until pH 7.0 and showed higher zero shear viscosity at pH 10.0, whereas β-glucan2 showed a large difference
in zero viscosity at all pH values and the difference in viscosity narrowed as the shear rate increased. β-glucan1 and β-glucan2 10% solutions showed higher viscosity at lower shear rates and then a steep drop in viscosity with an increase in shear rate (Figure 2.9 and 2.10). The increase in $\eta_0$ at lower shear rates at pH 1.5 is probably due to aggregation of β-glucan at low pH. The sharp increase in $\eta_0$ at high concentrations of β-glucan is due to the onset of chain entanglement (130).

![Figure 2.3](image1.png)

Figure 2.3 Shear rate vs viscosity of β-glucan1 solution (1% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3.

![Figure 2.4](image2.png)

Figure 2.4 Shear rate vs viscosity of β-glucan2 solution (1% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3.
Figure 2.5 Shear rate vs viscosity of β-glucan1 solution (4% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3.

Figure 2.6 Shear rate vs viscosity of β-glucan2 solution (4% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3.
Figure 2.7 Shear rate vs viscosity of β-glucan1 solution (7% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3.

Figure 2.8 Shear rate vs viscosity of β-glucan2 solution (7% w/w) at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3.
Figure 2.9 Shear rate vs viscosity of β-glucan1 solution (10% w/w) at pH: 1.5 (+), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bar are ± SEM, n=3.

Figure 2.10 Shear rate vs viscosity of β-glucan2 solution (10% w/w) at pH: 1.5 (+), 4.0 (■), 7.0 (▲) and 10.0 (●). Error bars are ± SEM, n=3.
2.7.2.2 **Effect of temperature on viscosity of different concentrations of β-glucan1 and 2**

The effect of temperature on the viscosity at 25, 37 and 70°C is shown in Figures 2.11 and 2.12. The data presented show a progressive decrease in viscosity with the increase in temperature for all concentrations (1, 4, 7 and 10% w/w) of the samples tested at a constant shear rate, which follows the Arrhenius equation. Figures 2.13 and 2.14 show the average viscosity of β-glucan1 and β-glucan2 solutions at different concentrations (1%, 4%, 7% and 10% w/w) as a function of shear rate at temperatures of 25, 37 and 70°C.

In previous studies, Burkus and Temelli (2005) demonstrated the decrease in viscosity of barley β-glucan gel with increase in temperature over a wide range of temperatures (125). Contrary to this, Gomez et al (1997) found high viscosity at lower shear rates at 70°C (132). Similar results of reducing viscosity of oat β-glucan with increase in temperature were also reported by Dawkins and Nnanna (1995) (133). The flow behaviour index of β-glucan2 (1, 4 and 7%) in the temperature range of 25, 37 and 70°C was higher than 0.950, indicating nearly Newtonian behavior, whereas for the 10% solution, the flow behaviour index decreased from 0.5124 to 0.0709, indicating pseudoplastic flow behaviour (Table 2.2).

Freshly prepared β-glucan1 and β-glucan2 solutions with concentrations of 1 and 4% w/w did not show any change in viscosity with increase in shear rate at all temperatures (Figures 2.13, 2.14, 2.15 and 2.16). This indicates Newtonian flow behavior, in which viscosity is independent of shear rate. At lower shear rates and at 70°C the viscosity was lower, which is similar to the results reported by Burkus and Temelli (2005). A shear thinning effect was observed with 7% β-glucan1 (Figure 2.17) at 25 and 37°C, whereas Newtonian behaviour was observed at 70°C. 7% β-glucan2 (Figure 2.18) showed regimes of Newtonian and non Newtonian flow. At lower shear rates of 10-20 s⁻¹ and at 25°C, it showed Newtonian behavior, with a transition to pseudoplastic behaviour at higher shear rates. However, at higher temperatures (37 and 70°C) the pattern was nearly Newtonian at all shear rates. The concentration of 10% w/w β-glucan2 solution (Figure 2.20) demonstrated Newtonian behaviour at low shear rates and then experienced a drop in viscosity with increase in shear rate at all temperatures. This drop was comparatively less at 70°C whereas β-glucan1 at 10%
showed pseudoplastic behaviour at lower temperatures and Newtonian behaviour at 70°C. The flow behaviour index of β-glucan1 solution at 1% increased from 0.2887 at 25°C to 0.9834 at 70°C, demonstrating a transition from pseudoplastic to nearly Newtonian flow. The same was observed with the 7% solution (Figure 2.19).

**Table 2.2** Flow behaviour index of β-glucan1 (1) and β-glucan2 (2) solution (1, 4, 7 and 10% w/w) at 25, 37 and 70°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>1% (w/w)</th>
<th>4% (w/w)</th>
<th>7% (w/w)</th>
<th>10% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1)</td>
<td>(2)</td>
<td>(1)</td>
<td>(2)</td>
</tr>
<tr>
<td>25</td>
<td>0.288</td>
<td>0.959</td>
<td>1.070</td>
<td>0.958</td>
</tr>
<tr>
<td>37</td>
<td>0.685</td>
<td>0.999</td>
<td>0.566</td>
<td>0.951</td>
</tr>
<tr>
<td>70</td>
<td>0.983</td>
<td>0.951</td>
<td>0.844</td>
<td>0.986</td>
</tr>
</tbody>
</table>
Figure 2.11 Temperature vs viscosity plot of β-glucan1 solution at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●) at 25, 37 and 70°C. Error bars are ± SEM, n=3.

Figure 2.12 Temperature vs viscosity plot of β-glucan2 solution at pH: 1.5 (♦), 4.0 (■), 7.0 (▲) and 10.0 (●) at 25, 37 and 70°C. Error bars are ± SEM, n=3.
Figure 2.13 Shear rate vs viscosity of β-glucan1 solution (1% w/w) at 25°C (♦), 37°C (■) and 70°C (▲). Error bars are ± SEM, n=3.

Figure 2.14 Shear rate vs viscosity of β-glucan2 solution (1% w/w) at 25°C (♦), 37°C (■) and 70°C (▲). Error bars are ± SEM, n=3.
Figure 2.15 Shear rate vs viscosity of β-glucan1 solution (4% w/w) at 25°C (♦), 37°C (■) and 70°C (▲). Error bars are ± SEM, n=3.

Figure 2.16 Shear rate vs viscosity of β-glucan2 solution (4% w/w) at 25°C (♦), 37°C (■) and 70°C (▲). Error bars are ± SEM, n=3.
Figure 2.17 Shear rate vs viscosity of β-glucan1 solution (7% w/w) at 25°C (♦), 37°C (■) and 70°C (▲). Error bars are ± SEM, n=3.

Figure 2.18 Shear rate vs viscosity of β-glucan2 solution (7% w/w) at 25°C (♦), 37°C (■) and 70°C (▲). Error bars are ± SEM, n=3
Figure 2.19 Shear rate vs viscosity of β-glucan1 solution (10% w/w) at 25°C (♦), 37°C (■) and 70°C (▲). Error bars are ± SEM, n=3.

Figure 2.20 Shear rate vs viscosity of β-glucan2 solution (10% w/w) at 25°C (♦), 37°C (■) and 70°C (▲). Error bars are ± SEM, n=3.
2.7.3 Mechanical characterisation of β-glucan1 and 2

The data obtained from the gel strength studies showed that with the increase in concentration of β-glucan1 and 2 gel, the area increased and force maxima required to break the gel increased (Table 2.3 and Figure 2.21). This result indicates that there is no gel formation at lower concentrations. There was no force maxima recordable at the concentrations of 1, 2 and 3%, and the area recorded with 1 and 2 % in β-glucan1 and 2 was similar and also very small, suggesting no setting of gel structure, even after 16 hours of storage. There was a sharp increase in area and force maxima at a concentration of 4% and further increased with increase in concentration. The force maxima of β-glucan2 was lower compared to β-glucan1, which might be because the firmness of the gel decreased with the increase in molecular weight as observed in previous studies (128).

<table>
<thead>
<tr>
<th>β-glucan Gel concentration (%) w/w</th>
<th>Area (N.mm)</th>
<th>Standard deviation ± SD, n=3</th>
<th>Force maxima (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.117</td>
<td>0.123</td>
<td>0.0001/0.025</td>
</tr>
<tr>
<td>2</td>
<td>0.134</td>
<td>0.139</td>
<td>0.008/0.005</td>
</tr>
<tr>
<td>3</td>
<td>0.266</td>
<td>0.185</td>
<td>0.138/0.085</td>
</tr>
<tr>
<td>4</td>
<td>2.085</td>
<td>0.875</td>
<td>1.101/0.607</td>
</tr>
<tr>
<td>7</td>
<td>9.060</td>
<td>6.325</td>
<td>0.820/1.354</td>
</tr>
<tr>
<td>10</td>
<td>25.855</td>
<td>20.891</td>
<td>0.623/1.320</td>
</tr>
</tbody>
</table>
Figure 2.21 Average gel strength profile of β-glucan1 (♦) and β-glucan2 (●) gel at different concentrations (1, 2, 3, 4, 7 and 10% w/w) at 25°C. Error bars are ± SEM, n=3.
2.7.4 Molecular weight determination of β-glucan1 and 2

The average molecular weight of the β-glucan1 and β-glucan2 and polydispersity index is shown in Table 2.4 and the representative chromatogram is shown in Figure 2.22. β-glucan1 showed a lower molecular weight with an average molecular weight of 90,632 g/mol compared to 241,705 g/mol for β-glucan2. An average dn/dc was calculated from five concentrations and was found to be 0.1401 for β-glucan1 and 0.1079 for β-glucan2.

A direct method to measure the molecular weight of β-glucons was employed because of the lack of commercially available β-glucan standards. Although high performance size exclusion chromatography is a commonly used method for measuring the molecular weight of polymers, it is based on retention volume, which is dependent on hydrodynamic volume rather than molecular weight only. For more reliable results, reference standards should be of similar chemical structure, as the hydrodynamic volume is directly related to the type of glycosidic linkages, branching, and to a lesser extent the constituent sugars. Use of commercial standards like pullulan and dextran can lead to unreliable molecular weight estimation as they are not suitable for polysaccharides with extended chains such as β-glucan (134). HPSEC with laser scattering detector does not need any calibrating standards and the method is used frequently to get reliable results (129, 135).

The molecular weight of both the β-glucons was found to be within the reported values in the literature: 14000–560 000 g/mol (136). The range of molecular weights is very large as it depends on the method of extraction and the source. β-glucons from different Greek barley cultivars were reported to be between 0.45 x 10^6 ~1.32 x 10^6 g/mol, when extracted by using ethanol (129).
Table 2.4 Average molecular weight and polydispersity index of β-glucan1 and β-glucan2.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>β-glucan1 Average molecular weight (g/mol)</th>
<th>Polydispersity index</th>
<th>β-glucan2 Average molecular weight (g/mol)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90,824</td>
<td>1.46</td>
<td>241,700</td>
<td>1.43</td>
</tr>
<tr>
<td>2</td>
<td>92,524</td>
<td>1.45</td>
<td>243,319</td>
<td>1.44</td>
</tr>
<tr>
<td>3</td>
<td>88,548</td>
<td>1.45</td>
<td>240,096</td>
<td>1.43</td>
</tr>
<tr>
<td>Average</td>
<td>90,632</td>
<td>1.453</td>
<td>241,705</td>
<td>1.433</td>
</tr>
<tr>
<td>± SEM</td>
<td>1153.14</td>
<td>0.838</td>
<td>930.43</td>
<td>0.827</td>
</tr>
</tbody>
</table>
Figure 2.22 Representative chromatogram of β-glucan2 showing scattering light signal (red) and refractive index detector signal (blue).

2.7.4.1 Density measurement of β-glucan1 and 2

The density of β-glucan1 and β-glucan1 at 20°C was found to be 1.04 and 1.40 respectively with a SD of 0.18 and 0.06 respectively.

2.7.4.2 Particle size determination of β-glucan1 and 2

The average particle size analysis of β-glucan1 and β-glucan2 in different solvent systems showed that β-glucan1 had a high average particle size compared to β-glucan2 in all the solvent systems (Figure 2.23). The average particle size of β-glucan1 and β-glucan2 without any solvent was around 38 µm and 16 µm respectively.
Figure 2.23 Average particle size of β-glucan1 and β-glucan2 in different solvent systems. Error bars are ± SEM, n=100.
2.7.5 Stress studies of β-glucan2

The average molecular mass of β-glucan2 at different conditions is given in Table 2.5 and representative chromatograms are shown in Figure 2.24 and Figure 2.25. Johansson et al (2006) reported that carbohydrates do not undergo hydrolysis at pH 1.0-1.5 and a temperature of 37°C, the usual conditions in the human stomach (127). The average mass of β-glucan2 determined by SEC-MALLS (Table 2.4) was 241,700 g/mol, with a polydispersity of 1.43, which is similar to other natural polymers such as guar gum. The sample that was treated with 0.2 M HCl (Figure 2.24-A) showed the average mass increased to 297,700 g/mol, which was perhaps caused by aggregation due to intermolecular hydrogen bonds (135). The extra peak after the eluent peak at 29.9 ml was probably due to residual HCl and excess NaHCO₃ used for pH adjustment.

The samples treated with 2.0 M HCl (Figure 2.24-B) showed the average molecular weight increased but degraded when treated with 6 M HCl (Figure 2.25-C). Heating at 120°C at pH 1 (Figure 2.25-D) led to marked degradation, with an average molecular weight of 5,917 g/mol and polydispersity of 1.38. The overlaid chromatogram of stress samples for β-glucan2 is shown in Appendix 1.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Average molecular weight of β-glucan2 (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>241,700 ± 2405.05</td>
</tr>
<tr>
<td>0.2 M HCl</td>
<td>297,700 ± 618.49</td>
</tr>
<tr>
<td>2 M HCl</td>
<td>348,200 ± 797.84</td>
</tr>
<tr>
<td>6 M HCl</td>
<td>88,750 ± 262.67</td>
</tr>
<tr>
<td>0.1 M HCl @120°C</td>
<td>5,917 ± 20.91</td>
</tr>
</tbody>
</table>

Values represent ± SEM, n=3.
Figure 2.24 Representative chromatograms of β-glucan showing scattering light signal (red) and refractive index detector signal (blue); (A – 0.2 M HCL, B – 2 M HCL)
Figure 2.25 Representative chromatograms of β-glucan2 showing scattering light detector signal (red) and refractive index detector signal (blue); (C - 6 M HCL, D - 0.1 M HCL at 120°C)
2.8 Conclusion

β-glucan1 and β-glucan2 were characterised and compared for their rheological behaviour, molecular weight and mechanical strength. Stress studies were carried out on β-glucan2. At lower concentrations, both showed Newtonian flow behaviour and they showed shear thinning properties at higher concentrations. Rheologically, β-glucan1 and β-glucan2 exhibited random coil type chain polysaccharide behaviour. There was no effect of pH and ionic strength on the flow properties of both the β-glucans at lower concentrations. There was a large variation in the $\eta_0$ at lower shear rates in β-glucan2 probably due to the coil overlap phenomenon at lower concentrations. The average molecular mass of β-glucan1 was found to be lower than β-glucan2 but β-glucan1 had higher gel strength at higher concentrations. Gel structure was not formed below 3% w/w in both the grades even after 19 hours storage at room temperature. Stress studies on β-glucan2 indicated that there was no degradation of the β-glucan2 at gastric conditions of low pH which suggests that β-glucan2 can protect the protein in gastric conditions. The average molecular mass of β-glucan2 was higher and less dispersed than β-glucan1, therefore β-glucan2 was used for further formulations.
CHAPTER 3
Lactoferrin Characterisation, Derivitisation and Purification of Lactoferricin
3.1 Introduction

3.1.1 Milk proteins

The use of milk as a food component dates back to the domestication of animals. The biological functions of milk in preventing infection have been recognized for thousands of years, but due to the systematic research on milk proteins started in early nineteenth century, they are now probably the best characterised food protein system. Milk is a complex fluid with several molecular entities, but the principal constituents are water, lipids, sugar (lactose) and proteins. Milk is a complete food for newborn mammals, as it provides complete nutrition during early stages of rapid development, due to the presence of following ingredients: lactose (5.0%), proteins (3.2%), lipids (4.0%) and mineral salts (0.7%).

The major components of milk that are responsible for immunity and infection in newborns are immunoglobulins. Similarly colostrum (initial milk produced after parturition) is considered important for newborn mammals as it provides an initial concentrated source of those immunoglobulins necessary for immunity against infection (137). Milk protein is composed of two major fractions, a micellular (casein) and soluble (whey) fraction. The content and composition of casein and whey protein in milk differs among species. Casein is the main protein compound of bovine milk, constituting about 80% of the total protein fraction. Several investigators have reported that dietary proteins provide a rich source of physiologically active peptides and these peptides are produced through different pathways such as hydrolysis by digestive enzymes, hydrolysis by proteolytic microorganisms (138) and/or through proteolytic enzymes derived from microorganisms or plants (139). Recombinant DNA techniques are also in use nowadays for the production of specific peptides or their precursors in microorganisms. The typical composition of bovine milk and whey is given in Table 3.1 and Figure 3.1.
Table 3.1 Typical composition of bovine milk and whey from (140) with permission.

<table>
<thead>
<tr>
<th>Component</th>
<th>Milk (% w/v)</th>
<th>Whey (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>2.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Whey Proteins</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Fat</td>
<td>3.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Ash</td>
<td>0.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Total solids</td>
<td>12.8</td>
<td>6.35</td>
</tr>
</tbody>
</table>

Figure 3.1 Schematic diagram of the composition of milk showing the relative amounts of water and solids. The solids consist of various components including protein which comprises whey protein (20%) and casein (80%).
The flexible structure of caseins renders them susceptible to proteolysis, which facilitates their natural function as a source of amino acids. The lack of stable tertiary structure indicates that caseins are non-denaturable and extremely heat stable. Whey proteins, especially β-lactoglobulin, are quite resistant to proteolysis.

Neither casein nor individual casein fractions have any established physiological role, except carrying ions like Ca$^{2+}$, PO$_4^{3-}$, Fe$^{2+}$, Zn$^{2+}$, Cu$^{2+}$ and acting as precursors of bioactive peptides. However, peptides derived from casein have been shown to possess various biological properties. These bioactive peptides are hidden in an inactive state inside the polypeptide chain of caseins. Bovine casein consists of four components: αs1-casein, αs2-casein, β-casein and k-casein, which are released either through hydrolysis by digestive enzymes or through proteolytic enzymes derived from plants or microorganisms (139). These properties make them a vital component of health-promoting foods called functional foods. Various physiological functions of milk and whey proteins are listed in Table 3.2

By definition, bioactive peptides are specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (141). Moreover, milk proteins are considered the most important source of bioactive peptides and large numbers of bioactive peptides have been reported in milk protein hydrolysates and fermented dairy products (142-145).

The various beneficial health effects produced by the milk peptides are antibacterial, antioxidative, antithrombotic, antihypertensive, immunomodulatory and nervous system regulatory effects (146-148). Some peptides show antihypertensive activity and these peptides are referred to as casokinins. The angiotensin I converting enzyme (ACE) hydrolyses the largely inactive angiotensin I to actapeptide angiotensin II, which increases blood pressure. Thus milk derivatives that act as ACE inhibitors are antihypertensive peptides, and other antihypertensive peptides are located in the primary sequence of bovine β-lactoglobulin (β-lactorphin) and human β- and kappa caseins (k-casein).
Table 3.2 Composition and physiological functions of major milk and whey proteins from (137) with permission.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (g/l)</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total caseins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α -Casein</td>
<td>13.0</td>
<td>Ion carrier (Ca, PO₄, Fe, Zn, Cu)</td>
</tr>
<tr>
<td>β-casein</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>κ-casein</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Total whey protein</td>
<td>6.3</td>
<td>67.3</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>3.2</td>
<td>Retinol carrier, binding fatty acids, possible antioxidant</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>1.2</td>
<td>Lactose synthesis in mammary gland</td>
</tr>
<tr>
<td>Immunoglobulins (A,M and G)</td>
<td>0.7</td>
<td>Immune protection</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.1</td>
<td>Antimicrobial, antioxidant, iron absorption, immunomodulation</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>0.03</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.0004</td>
<td>Antimicrobial, synergistic effect with LF</td>
</tr>
<tr>
<td>Proteose-peptone</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Glycomacropeptide</td>
<td>1.2</td>
<td>Antiviral, bifidogenic</td>
</tr>
</tbody>
</table>

In vitro activity of immunomodulating peptides resulting from tryptic and chymotryptic hydrolysis of αs1- and β-casein has been reported to stimulate macrophage activity against red blood cells. Casein derived immunopeptides have been shown to stimulate the phagocytic activity of murine and human macrophages and to protect against Klebsiella pneumoniae infection in mice. The peptides may stimulate the proliferation and maturation of T cells and natural killer (NK) cells for the defence of the newborn against a large number of bacteria, particularly enteric bacteria. Injection of casein or α lactalbumin peptides has been found to have direct immunomodulating activity against Klebsiella pneumoniae in rats. Lahov and Regelson (1996) (149) reported antibacterial activity of isracidin, the 1-23 fragments of αs1 casein obtained from the action of chymosin, against Staphylococcus aureus and Candida albicans. The injection of isracidin into the udders of sheep and cows gave protection against mastitis.

In order to function physiologically in the human body, peptides must be absorbed from the intestine in an active form. However, there is no evidence that these peptides can be absorbed from the intestine in adults and the proposed properties remain to be proven. Di- and tripeptides can be absorbed by the intestine, but it is not clear that larger bioactive peptides containing more than three amino acids are absorbed from the intestine and reach the target organ. Yamamoto reported
absorption of two antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, from sour milk (150).

3.1.1.1 Whey

Whey, a yellow-green liquid, is a by-product of cheese or casein manufacture from milk. It is also sometimes referred to as milk plasma. It is one of the components that separate from milk after curdling, when rennet or an acidic substance is added. There are two types of whey, sweet whey and sour/acid whey (Figure 3.2). Sweet whey is obtained while manufacturing rennet types of hard cheese like cheddar. Sour or acid whey is obtained during production of acid cheese like cottage cheese. Previously, whey was considered to be a waste by-product as its biological benefits were not fully known. It was disposed of in rivers, causing heavy water pollution. However, it had been in use for centuries for various functions, including as an aphrodisiac and for burns. Hippocrates recorded the health benefits of whey in ancient Greece (151). Other uses and products of whey reported are whey butter and whey porridge. The advent of two decades of research particularly in separation technology and a better understanding of its biological functions has led to an increased interest in whey. It is being produced in large quantities - approximately 9 litres of whey is produced per kilogram of cheese manufactured - and therefore the output of whey from a large cheese making plant can be over 1 million litres daily (152).

Figure 3.2 Schematic diagram of acid and rennet mediated cheese making. Adapted from (151) with permission.
Whey processing, an important step to isolate solids responsible for health benefits, started in the early 1900s and involved hot roller milk driers, concentrating whey by heating and cooling to solidification then extruding in a tunnel. This was followed by two stage steam heating and rotary drum and spray drying. The process was eventually abandoned due to its high cost. Other processes reported involved long tube multiple-effect evaporators. The final product was available as a water insoluble, yellowish powder with limited applications. Modern techniques like membrane filtration, including microfiltration (MF), ultrafiltration (UF), electrodialysis (ED), nanofiltration (NF) and reverse osmosis, are employed for separation and fractionation of whey proteins. Spray drying is then used to obtain the products with different protein contents.

3.1.1.2 Whey proteins

Whey proteins are a mixture of globular proteins fractionated from whey using different methods: MF, UF, ED and NF. The principal proteins in whey are listed in Table 3.3. Various whey protein-rich commercial products are produced, and are termed whey protein concentrate (WPC) and whey protein isolates (WPI), containing 30-85% and approximately 95% protein respectively. WPC is produced by ultrafiltration of whey to remove any remaining lactose, followed by spray drying (153). WPI is produced by ion-exchange chromatography with membrane filtration and spray drying (154).

Most applications of WPC and WPI are found in baking, processed dairy products such as ice cream, and the meat and seafood industries. Medicinal and nutritional products are obtained through fractionation of whey by filtration into various proteins (155). The concentrations and composition of whey proteins depend on many variables like the source of milk, season, type of feed and the stage of lactation.

β-Lactoglobulin (β-LG) is a major whey protein with a molecular weight (MW) of 18 kilo Daltons (kDa). It constitutes approximately 50% of the total whey proteins (156) and is present in the milk of buffalo, cow, sheep and goat. It has been reported that human, rat, mouse, pig and camel milk does not contain β-LG protein, whereas these animals have α-Lactalbumin (α-LA) as the principal whey protein in their milk. β-LG is quite stable at low gastric pH and is resistant to proteolysis. However, β-LG is considered to be the most allergenic protein in bovine milk, and therefore is not used in human infant formulas.
α-LA, a calcium binding protein, is the major protein in human milk with a MW of 14 kDa. It is used in infant formula. Bovine serum albumin (BSA) is present in the milk, probably as a result of leakage from blood, and has no biological function in the milk. De Wit, (1998) reported that BSA transports insoluble free fatty acids. Immunoglobulins (Ig) are the largest group of proteins in whey with a molecular weight of 150 kDa. IgG₁ is the principal Ig in bovine, caprine or ovine milk, with lower amounts of IgG₂, IgA and IgM. IgA is the principal Ig in human milk. The significance of these proteins as physiologically active components in the diet is now being increasingly acknowledged. A number of proteins that occur naturally in raw food materials exert their physiological actions either directly or upon enzymatic hydrolysis in vitro and in vivo.

**Table 3.3** Protein profile of whey. Adapted from (151) with permission.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (g/l)</th>
<th>Molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactoglobulin</td>
<td>2-4</td>
<td>18</td>
</tr>
<tr>
<td>α-Lactoalbumin</td>
<td>1.2-1.5</td>
<td>14</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>0.6-0.9</td>
<td>150-1000</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>0.3-0.6</td>
<td>69</td>
</tr>
<tr>
<td>Lactoferrin (LF)</td>
<td>0.02-0.2</td>
<td>78-92</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>0.02-0.05</td>
<td>78-89</td>
</tr>
<tr>
<td>Glycomacropeptide</td>
<td>1-1.2</td>
<td>7</td>
</tr>
</tbody>
</table>

There are many proteins in milk that are present in very low or trace amounts and have biological activities. Minor whey proteins, which are constituted mainly of LF and lactoperoxidase, are currently attracting considerable attention because of their increasing role as nutraceuticals. New ways are being developed industrially to purify these minor whey proteins, in order to enhance the value of milk proteins. Andersson and Mattiasson (2006) reported an extraction method involving simulated moving bed chromatographic technology on a large scale to raise the productivity of LF (157).
3.1.1.3 Lactoferrin (LF)

LF is one of the minor whey proteins but is now considered as a prominent protein in milk as it is associated with various biological functions. LF is a monomeric, 80 kDa glycoprotein, with a single polypeptide chain of about 690 amino acids (158, 159). It belongs to the same family as transferrin (TF), an iron binding protein in the serum, due to its amino acid sequence relationship. LF has a strong affinity for iron and is therefore also considered an iron binding protein. It was first discovered in 1938 as a red protein (160), and first isolated in 1960 from human and cow’s milk. The industrial scale production of bovine LF (bLF) began in 1985. The first commercial production of human recombinant LF in the United States of America (USA) started in 1995 and LF was first used as a food additive in yoghurt in Japan in 2001.

Apart from milk, LF is present in various secretory fluids such as saliva, tears, seminal fluid and mucous secretions of mammals: human, pig, horse, cow, buffalo, sheep, goat, camel and mouse. It is also present endogenously within specific granules of neutrophils. LF is synthesised in the neutrophils during the maturation process. After saturated with iron, LF has a salmon pink colour (161). The intensity of the pink colour of LF depends on the degree of iron saturation. Commonly iron saturation in LF is 15-20% in its natural form. LF with less than 5% iron saturation is termed apolactoferrin (apo LF) and LF saturated with iron is referred to as hololactoferrin (holo LF). LF from human breast milk contains less than 5% iron, so as referred to as apo LF (161).

The three dimensional structure of LF from most of the species known is well defined. Crystal structures of LF from cow (162), human (163, 164), buffalo (165), horse and camel (166) have similar overall structural organisation. LF has two homologous globular N- and C- lobes also referred to as N- and C- terminal halves. Each lobe is further composed of two sub-lobes known as domains, which form a cleft that has a ferric (Fe$^{3+}$) ion binding site. These four sub-lobes or domains are represented as N1, N2, C1 and C2 respectively (Figure 3.3). Each molecule of LF has two Fe$^{3+}$ ions tightly attached to it. Bicarbonate ions act synergistically in iron binding in both the lobes. LFs from buffalo, cow, goat and sheep have over 90% of their amino acid sequence identical to each other, while human LF and mouse LF share sequence identity close to 70%. LF has a net positive charge, making it highly basic with an isoelectric point (PI) of around 9.
The iron binding property and its close resemblance to TF has led to the establishment of the role of LF in iron absorption in the GIT, antimicrobial activity, modulation of iron metabolism during inflammation (167, 168) cellular growth and differentiation, host defence against microbial infection, anti inflammatory activity and cancer protection (Figure 3.4). There are two mechanisms that are considered responsible for the antimicrobial role of LF. It has a very high affinity for iron and depletes the microbe of iron, which is believed to be important for microbial metabolism (158). LF has a positive charge and possibly binds to and even penetrates the cell membrane of microbes, thereby interfering with the cell wall synthesis, which is another mechanism of inhibiting the growth of microbes (169). The antibacterial effect is more pronounced in the peptides purified from the N-terminal lobe of LF, which are known as lactoferricins (LFcin) (170, 171)
Figure 3.3 Ribbon diagram of bLF showing the four sub-lobes and secondary structural features β-sheets (blue), α-helices (orange and red) and two ferric ions bound to each lobe. Green represents the LFcin containing region of the N-terminal. Modified from (172)
Omata et al (2001) suggested that basic peptides from LF can disrupt the parasite membranes and interfere with the integrity of the parasite membrane (174). The anti-inflammatory activity of LF has been widely reported by binding to bacterial endotoxin, a lipopolysaccharide (175). Other mechanisms include sequestration of free iron at the inflammatory site, e.g. rheumatoid joints (176) and inhibiting the migration of Langerhans cells, which are responsible for inflammation in cutaneous tissues (177). Several reports based on animal studies suggest that LF can inhibit the development of tumours (178, 179) through various mechanisms. Immunomodulation may be the most probable mechanism that has been suggested for LF’s anti-cancer activity. LF stimulates production of immune cells, including NK cells and lymphocytes (180, 181).

### 3.2 Characterisation of bLF and bLFcin

Proteins are macromolecules consisting of well defined and regular primary, secondary, tertiary and quaternary three dimensional structures. The therapeutic application of a protein is largely dependent upon its molecular structure, conformation and purity. Very small structural changes during formulation, processing, handling and storage can have a significant impact on the protein’s
biological activity either by modifications of the active site or by changing the protein conformation. Therefore characterisation of proteins is considered very important for successful formulation. There are various techniques that are available to characterise individual proteins, as discussed in Section 1.2.6. SDS-PAGE is a technique that is used for protein separation based on their molecular size. The most commonly used electrophoretic technique for protein separation is Laemmli-SDS-PAGE (182), which is commonly used for characterisation of protein and peptides.

Gel filtration separates the proteins based on differences in size and is a widely used technique for separation of proteins, since it is a simple technique and proteins are separated in their native conformations. This technique was developed in the 1950s using cross-linked dextrans and involves partitioning of molecules between the volume of mobile phase and the accessible volume contained within the stationary porous beads (183). Protein molecules percolate through and around the porous beads in the column and are separated according to their mass and shape. Depending on the fractionation range of the gel matrix, large proteins are excluded from the volume of the beads and elute first. Smaller proteins occupy the volume of liquid surrounding the beads and the total volume contained within the porous beads and are eluted later.

The basic principle of ion exchange is that separation is achieved on the basis of the charges carried by solute molecules. Ion exchange is capable of separating molecules with very small differences in charge and is therefore a technique of very high resolving power and is very useful in preparative experimental techniques. The separation in ion exchange chromatography is obtained by reversible adsorption. Most ion exchange techniques are performed in two main stages. The first stage is sample application and adsorption. Unbound substances can be washed out from the exchanger bed using a few column volumes of starting buffer. In the second stage, substances are eluted from the column and separated from each other. The separation occurs because different substances have different affinities for the ion exchanger due to differences in their charge. These affinities can be controlled by varying conditions such as ionic strength and pH. The differences in charge properties of biological compounds are often considerable. Since ion exchange chromatography is capable of separating species with very minor differences in properties, such as two proteins differing by only one amino acid, it is a powerful separation technique. An ion exchanger consists of an insoluble matrix with charged groups that have been covalently bound. The charged groups are associated with
mobile counter ions. These counter ions can be reversibly exchanged with other ions of the same charge without altering the matrix. The choice of the ion exchanger matrix was made carefully after taking into account the stability of the sample and molecular size of the sample.

Fast protein liquid chromatography (FPLC) is a form of column chromatography used to separate or purify proteins from complex mixtures. It is very commonly used in biochemistry and enzymology. Columns used with an FPLC can separate macromolecules based on size, charge distribution, hydrophobicity, or biorecognition as with affinity chromatography.

Microporous ion exchangers are composed of synthetic microporous membranes that have functional groups attached covalently to the inner surface of the membranes. Pressure applied forces the liquid through the micropores of the membrane, bringing target substances into direct contact with binding sites. This direct convection to the binding sites minimises diffusion limitation of mass transfer without sacrificing capacity. The major application of this technique is in the separation of biomolecules. By choosing the appropriate conditions, the target substances are selectively bound, whereas contaminants pass through the membrane or vice versa. Bound biomolecules can also be separated by step or gradient elution. For LF derivatives, a strong cationic exchanger with a sulphonic acid functional group (−R−CH₂−SO₃⁻) has been used. The advantages with this technique are units that are ready to use, easy handling and short processing time.

It has been reported that pepsin digested LF has stronger antimicrobial activity than the native protein (184). The bioactive peptides are found in the N terminus of bovine LF and human LF and are reported to be more active than parent LF in antimicrobial activity. The main objectives of this chapter are:

1. Protein estimation, mass determination and evaluation of purity of bLF.

2. Mass determination and peptide identification of bLF.

3. Enzymatic derivitisation of bLF to bLFcin and purification using various techniques.
4. Characterisation of bLFcin: mass determination by mass spectrometry and amino acid sequencing.

5. Evaluation of biological activity of bLF and purified bLFcin.

3.3 Materials

Bovine LF was kindly donated by Fonterra (New Zealand), pepsin (1:3000) was purchased from Sisco Research Laboratories (SRL) (India), bovine serum albumin (BSA) was purchased from Sigma Chemicals (USA) and ultrafiltration membrane with a cut off of 50 kDa was purchased from Millipore (USA). Electrophoretic assembly was purchased from Bio-Rad (USA). Acrylamide, bisacrylamide, SDS, (Tri (hydroxymethyl) methyl) glycine (Tricine) and Tetramethyl ethylenediamine (TEMED) were purchased from Sigma (USA). Tris (hydroxymethyl) aminomethane (Tris) and Coomasive brilliant blue R 250 were purchased from SRL (India). Coomasie blue G-250 molecular weight marker and ammonium persulphate (APS) were purchased from Sigma (USA). Sample buffer and urea were purchased from BDH (USA) and Mercaptoethanol from Sigma (USA). Acrylamide and bisacrylamide were purchased from SRL (India). The ultra low range marker, M3546-1VL, was purchased from Sigma (USA). Sephadex G-25 was purchased from Pharmacia (USA). Minimum Essential Medium (MEM), fetal bovine serum (FBS), fetal calf serum (FCS) and Dulbecco’s Modified Eagles Medium (DMEM) were purchased from Invitrogen (New Zealand). Sodium potassium tartrate, copper sulphate and potassium iodide were purchased from BDH (USA). Standard bLF for biological activity was donated by Lactopharma, New Zealand. All other chemicals used in the study were at least of reagent grade. All the reagents and buffers used in protein estimation were of analytical grade. Water used in the preparation of all solutions and buffers was obtained by reverse osmosis by the Milli Q unit from Millipore (USA)

3.4 Methods

3.4.1 Physical characterisation of unmilled and milled bLF

Physical characterisation of bLF unmilled and milled was done by taking pictures using a Panasonic DMC-FZ25 camera (Panasonic, Japan). The morphology of bLF was evaluated with a scanning electron microscope (SEM) (Phillips XL30S FEG, Netherlands). The sample for SEM was prepared by placing a small amount of bLF...
powder on the stubs, which were then coated with gold-palladium and micrographs were recorded.

### 3.4.2 Total Protein determination in bLF sample by Biuret method

The Biuret method of protein analysis is based on the principle that, under alkaline conditions, proteins containing two or more peptide bonds form a purple coloured complex with copper salts in the reagent. This method is similar to the Lowery method but involves a single incubation of 20 min. The Biuret reagent is composed of (per litre final volume) sodium potassium tartrate (9 g), copper sulphate (3 g) and potassium iodide (5 g), all dissolved in the order listed in 500 ml of 0.2 M sodium hydroxide (NaOH) before making the volume to 1.0 l.

The Biuret reagent was prepared by dissolving 2.25 g sodium potassium tartrate, 0.75 g copper sulphate (CuSO₄·5H₂O) and 1.25 g potassium iodide, in that order, in 125 ml of 0.2 M NaOH. Finally the volume of the solution was made up to 250 ml with Milli Q (MQ) water. BSA stock solution (5 mg/ml) was prepared and further diluted with MQ water to 0.125, 0.250, 0.500, 0.750, 1.0 and 1.25 mg/ml in duplicate and controls were prepared in duplicate. Stock solution of bLF (5 mg/ml) was prepared and three concentrations in duplicate were diluted to 1.0, 2.5 and 5.0 mg/ml within the standard range. Six ml of Biuret reagent was added to 4 ml of sample, then solutions were mixed well and incubated at 37°C for 10 min, during which time a purple colour developed. Absorbance was measured at 520 nm and a standard curve was generated by plotting absorbance versus concentration of BSA. Concentrations of the samples were determined from the standard curve.

### 3.4.3 Mass determination and identification of bLF

Stock solution of 30% acrylamide and 0.8% bisacrylamide was prepared in MQ water and stored at 4°C. Stock solution of 1.5 M Tris HCl (pH 8.8) and 0.4% SDS was stored at room temperature. Stock solution of 0.5 M Tris HCl (pH 6.8) and 0.4% SDS was also stored at room temperature. Ammonium persulphate (APS) (10%) was prepared and stored at 4°C.

The gel plates of the assembly were cleaned, dried, arranged and sealed with 1% w/v agar solution and leak tested to confirm sealing of the two gel plates. A spacer was placed between the plates before sealing. The composition of separating and stacking gel is outlined in Table 3.4.
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Separating gel (ml)</th>
<th>Stacking gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris HCL, pH 8.8</td>
<td>2.50</td>
<td>0.630</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>0.10</td>
<td>0.050</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>3.30</td>
<td>0.830</td>
</tr>
<tr>
<td>0.8% bis Acrylamide</td>
<td>4.00</td>
<td>3.400</td>
</tr>
<tr>
<td>MQ water</td>
<td>4.00</td>
<td>3.400</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.100</td>
<td>0.050</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Separating gel was prepared first by adding the ingredients as listed in Table 3.4 without APS and TEMED. After adding appropriate quantities of APS and TEMED, the separating gel was poured immediately into the space between the two plates gently with a pipette, leaving at least 1.5 cm from the top for stacking gel. 100-200 µl of MQ water was added on top of the separating gel to allow the gel surface to remain flat. The gel was left to polymerise for 10-15 min, after which the water was removed. Stacking gel solution was prepared as per Table 3.4, then APS and TEMED were added, the gel was mixed and poured over the separating gel and the comb was inserted carefully. Stacking gel was allowed to polymerise at room temperature for about 10 min, after which the comb was removed from the gel. The plate assembly was placed in the electrophoresis tank filled with 1x running buffer. Stock solution of bLF (1 mg/ml) was prepared in MQ water and 5, 10, 15 and 25 µl were placed in Eppendorf tubes. Then 5 µl of sample buffer was added to each and heated to boiling temperature for 5 min. The protein solution was briefly centrifuged and then loaded onto the gel. The assembly was connected to power and the gel was run at 50 V for two and a half hours. Staining solution was prepared, composed of methanol (30% v/v), acetic acid (10% v/v) and Commassie blue R (0.1% v/v). Destaining solution was composed of 30% v/v methanol, 10% v/v of acetic acid and 60% v/v MQ water. Molecular mass was determined by comparing gel scans of the samples with those of molecular mass markers.
3.4.4 Mass determination by Matrix-assisted laser ionization (MALDI)

A bLF sample was prepared by dissolving 1 mg of bLF in 1 ml MQ water and a solution of matrix (sinapinic acid) was prepared by mixing acetonitrile, water and trifluoro acetic acid (50:50:0.1). The matrix solution was mixed with the analyte and spotted onto a MALDI plate. External calibration was performed using a calibration solution of BSA before sample analysis. Mass spectra were recorded on a Voyager DE-pro MALDI-TOF mass spectrometer (Applied Biosystems, USA) operating in linear mode using positive ionisation. Accelerating voltage was 25000 V, grid voltage 90%, guide wire 0.15%, extraction delay was 750 ns and the mass range set to 10000-100000 m/z.

Protein identification by MS/MS was carried out commercially.

3.4.5 Enzymatic derivitisation of bLF into bLFcin and its purification

3.4.5.1 Ultrafiltration

Before enzymatic derivitisation of bLF, a solution of bLF was purified using a stirred ultrafiltration cell, Model 8200 (Millipore, USA). A membrane with a 50 kDa molecular weight cut off (Mx 50) was fixed into the chamber and the sealant gasket was carefully placed over the membrane. The membrane was washed for 30 min with MQ water under nitrogen purging. 150 ml of 1% w/w bLF solution was poured into the cell apparatus (Figure 3.5) and the stirring assembly was fitted into the cell body. This cell body was slided into the retaining stand. The pressure of nitrogen was adjusted to 410 mm Hg. The apparatus was operated for 3 hours. The process yield was 50 ml of retentate and 100 ml of permeate. The retentate was loaded onto the SDS-PAGE to assess the purity of bLF. Four batches of 150 ml each were purified and total of 200 ml of retentate was obtained. The protein content was measured from the standard curve generated by plotting the absorbance versus concentrations of BSA at 280 nm.
3.4.5.2 Derivitisation/Digestion of bLF

Digestion of purified bLF was carried out as per the method described previously by Plate et al. (2006) (185). Stock solutions of NaOH (0.2 N) and HCl (0.2 N) were prepared. 2 ml of 5% w/v purified bLF solution was acidified with 0.2 N HCl to pH 2.5 and incubated with 3 mg of pepsin (30 mg/g protein) at 37°C for 5 hours. A sample was withdrawn after every hour of incubation. The reaction was stopped by boiling the solution for 15 min and the pH was adjusted to 7.0 with 0.2 N NaOH. The samples were centrifuged at 12000 rpm for 10 min. The supernatant was stored at -20°C. All the samples were in duplicate and the control was treated in the same way.

Optimisation of digestion was carried out by incubating the purified bLF sample (2 ml of 5% w/v) with different concentrations of pepsin (15, 20, 25, 30 and 35 mg/g of protein basis), containing 1.5, 2.0, 2.5, 3.0 and 3.5 mg of pepsin respectively. Optimisation of digestion with respect to time was carried out at different time intervals (15, 20, 30 and 45 min) at constant concentration of the pepsin (30 mg/g of protein). Further processing was carried out as described above in this section.
3.4.5.3 **Tricine-SDS-PAGE**

To obtain a high resolution of peptides of low molecular weight in electrophoresis, a Tricine-SDS-PAGE method reported by Schagger (2006) (186) was followed. The method involved a gel composition of 16% Total (T), 6% Cross linker (C) with 6 M urea. Urea was added to further increase the resolution of low Mw peptides. Acrylamide-Bisacrylamide (AB) stock solutions were prepared: AB3 (49.5% T, 3% C): 48 g acrylamide and 1.5 g bisacrylamide in 100 ml MQ water, and AB6 (49.5% T, 6% C): 46.5 g acrylamide and 3 g bisacrylamide in 100 ml MQ water. Stock solutions were stored at 7-10°C. Stock solution of 3x gel buffer was prepared by mixing 72.66 g of Tris, 0.6 g of SDS and 17.24 ml of conc. HCl and volume was made up to 200 ml with MQ water. Stock solutions of 4% stacking gel were prepared by mixing 1 ml of AB-3 and 3 ml of 3x gel buffer and volume was made up to 12 ml with MQ water. The composition of resolving, spacer and stacking gels is presented in Table 3.5. The incubation buffer or sample buffer consisted of 3% w/w SDS, 6% β-mercaptoethanol, 30% glycerol w/v, 0.05% Coomassie blue G-250 and 150 mM Tris HCl (pH 7.0). Fixing solution consisted of 40% methanol and 10% acetic acid. Staining solution consisted of 0.025% Coomassie blue G and 10% acetic acid. Destaining solution was prepared by mixing 10% v/v of acetic acid in MQ water. The buffers prepared for running the gel were 10x cathode buffer, which consists of 1 M Tris, 1 M Tricine and 1% SDS, (pH 8.25), 10x anode buffer, which consists of 1 M Tris and 0.225 M HCl, (pH 8.9). The molecular weight range of the marker was 1.060-26.600 kDa. The marker was diluted 1:20 with sample buffer.

The gel plates were sealed as mentioned above with 1% agar aqueous solution. Resolving gel was prepared by adding the different solutions as described in Table 3.5 and 10 ml was poured in between the plates to fill up to 11 cm height. 5 ml of spacer gel (10%) was prepared and laid over the resolving gel to 1 cm. 8 ml of stacking gel was prepared and poured on top of the spacer gel. While preparing the gels, APS and TEMED were added at the end, as they were the catalyst for gel polymerisation. All the digested samples were diluted with MQ water to get the final concentrations of the protein to approximately 1 mg/ml. Out of this stock, 10 µl of sample was mixed with 10 µl of sample buffer and after a short spin, was incubated at 37°C for 15 min. Molecular weight ladder 2.5 µl was mixed with 15 µl of dye and 10 µl of this mixture was loaded along the samples. The gels were mounted into the vertical electrophoresis apparatus. Anode buffer was added as the lower electrode buffer and cathode buffer as the upper electrode buffer. 10 µl of the sample was...
loaded under the cathode buffer. The running conditions were set as follows: Initial voltage 30 V, mid voltage 200 V, and final voltage 300 V. The total time of running the gel was approximately three hours.

After carefully removing the gel from the electrophoresis assembly, the gel was incubated with fixing solution for 30 min on a gel shaker followed by staining with Coomassie dye for one hour. Gel was destained with destaining solution twice for 20 min incubation each. Gels were finally washed with water, transferred between two transparent plastic sheets and scanned using the scanner.

Table 3.5 Composition of resolving gel, spacer and stacking gel for Tricine-SDS-PAGE.

<table>
<thead>
<tr>
<th>Material</th>
<th>Resolving gel (16%+6M Urea)</th>
<th>Spacer gel (10%)</th>
<th>Stacking gel (4 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB-6</td>
<td>5.0 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AB-3</td>
<td>-</td>
<td>0.8 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Gel buffer (3x)</td>
<td>5.0 ml</td>
<td>1.333 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Urea</td>
<td>5.4 g</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MQ water to final volume</td>
<td>15.0 ml</td>
<td>1.866 ml</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>50 µl</td>
<td>20.0 µl</td>
<td>45.0 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5.0 µl</td>
<td>3.0 µl</td>
<td>6.0 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>15.0 ml</td>
<td>4.0 ml</td>
<td>6.0 ml</td>
</tr>
</tbody>
</table>
3.4.6 Purification of bLFcin

The target peptides, which comprised of the amino acid sequence from 17-41 of the N-terminal region of the bLF molecule, have a high proportion of basic amino acid residues and has high PI (8.5-9.0) (185). Various techniques were evaluated to purify bLFcin from the hydrolysate, which are mentioned below.

3.4.6.1 Gel filtration

Sephadex G-25 (100 g) was soaked in 500 ml of distilled water for one hour, boiled for 10 min to remove any trapped air and soaked overnight at room temperature to swell. The suspension was packed gently in a glass column of 1.8 cm internal diameter and 20 cm height. The column was equilibrated for one hour with 0.1 M phosphate buffer, (pH 7.4) at a flow rate of 0.6 ml/min. 500 µl of the bLF hydrolysate (17 mg/ml) was added to the column and fractions were collected after discarding the initial fractions. The absorbance was recorded at 280 nm for each fraction using a spectrophotometer and the concentration was calculated from the standard curve prepared with BSA. Fractions that showed higher protein concentrations were pooled and concentrated using a speed vac (SpeedVac® Plus, Thermo Savant, USA). Concentrated samples were run on SDS-PAGE to determine the approximate molecular weight.

3.4.6.2 Preparatory ion exchange chromatography

Sephadex SP G-25 (100 g), a strong cationic exchanger matrix, was boiled for 3 hours in 0.1 M phosphate buffer (pH 7.0) and allowed to swell for 5 hours. The mixture was poured gently into the glass column (21 cm x 3 cm). The column was equilibrated with 0.1 M phosphate buffer at a flow rate of 0.4ml/min overnight. Equilibration was stopped when the pH of the inlet buffer and outlet was same. bLF hydrolysate solution (17 mg/ml) was loaded onto the column and the unbound proteins were washed with 0.1 M phosphate buffer. Absorbance was recorded at 280 nm using a spectrophotometer after every 4 hours. When the absorbance was negligible, the gradient method with 20 mM phosphate buffer was run followed by 2 M ammonium chloride buffer. The fractions from 20 mM phosphate buffer and 2 M ammonium chloride were collected. Each fraction was of 5 ml and a total of 120 fractions were collected. Absorbance was recorded at 280 nm and the concentrations were calculated from the standard curve generated with BSA. The fractions with high
concentrations were further concentrated and Tricine SDS-PAGE was run to evaluate the molecular weight of the fractions before further characterisation.

3.4.6.3 Fast protein liquid chromatography (FPLC)

The column (Mono S™ 5 H/R from Amersham Pharmacia Biotech AB, Sweden), a strong cationic exchanger with a particle size of 10 µm and a charged group (–CH₂-SO₃), was connected to a FPLC machine (GE healthcare, USA). Unicorn software (version 5.0) was used to control the system. The column was equilibrated with low ionic buffer (20 mM phosphate buffer) for 10 min and then equilibrated with high ionic buffer (1 M NaCl) for another 10 min. After further equilibration with the 20 mM phosphate buffer, 2 ml of the sample (bLF hydrolysate 10 kDa permeate) of 14 mg/ml of hydrolysate was injected at a flow rate of 1.0 ml/min. Unbound fractions were collected and a NaCl step gradient was run with concentrations of 0.1-0.4 M NaCl. A gradient with ammonium chloride (2 M) was run and fractions were collected using a fraction collector (Frac-900 from GE Healthcare, USA). Protein concentrations were determined at 280 nm and Tricine SDS-PAGE was run with major fractions for molecular weight and purity evaluation.

3.4.6.4 Ion exchange using microporous membrane

Purification of bLFcin from bLF hydrolysate was carried out using a method described by Plate et al (2006) (185). Sartobind exchange MA units (MA 15) from Sartorius (Germany) were rinsed with 10 ml of 20 mM sodium phosphate buffer (pH 7.0) using a 10 ml disposable syringe for equilibration. Moderate pressure was applied to the syringe plunger. bLF hydrolysate solution was diluted with 20 mM phosphate buffer to 10 mg/ml and the final pH was adjusted to 7.0. The unit was washed with 15 ml of 20 mM phosphate buffer until the effluent showed negligible absorbance at 280 nm. Protein hydrolysate solution was diluted to a final concentration of 10 mg/ml with phosphate buffer and filtered using a 0.45 µm cartridge filter (Millipore, USA). This filtered sample solution was then loaded. The unit was washed with equilibration buffer (20 mM phosphate buffer) until the outlet buffer absorbance was negligible. A step gradient of sodium chloride (5 ml of each concentration; 0.1 M-1.0 M) was used to elute impurities bound to the adsorber membrane, followed by 2 M ammonium chloride (5 ml). Fractions of 1 ml were collected and protein concentration was determined by a spectrophotometer, recording the absorbance at 280 nm.
The 2 M ammonium chloride fractions were desalted by using dialysis tubing (benzoylated, average flat width 32 mm from Sigma, USA). Tubing was submerged in a solution of 2% sodium bicarbonate and 0.05% sodium EDTA and boiled for 10 min on a water bath. After boiling, this solution was discarded and the tubing was boiled in water twice for 10 min each. One end of the tubing was sealed with dialysis clips and solution of the fraction was poured into the tubing bag. The other end of the tubing was sealed and the tubing bag was carefully hung in a large volume of MQ water. The water contained a magnetic bead and was placed on the magnetic stirrer and allowed to agitate. The entire assembly was placed in a temperature controlled chamber (-4°C). The protein content of the sample was then determined.

3.4.6.5 Sample preparation for mass spectrometry

Samples from the purified fraction were prepared in diluents consisting of water and acetonitrile (1:1), and 0.1% formic acid was added. The concentration of the final solution was 10 mg/l. The data were generated using the Q star ESI quadruple time-of-flight mass spectrometer (Applied Biosystems, USA).

3.4.6.6 Biological activity of bLF and bLFcin

The biological activity of bLF and bLFcin was assessed on the osteoblast cells from foetal rat calvariae. The primary osteoblasts were prepared as previously described by Cornish et al (1998) (187). In brief, osteoblasts were isolated by collagenase digestion from 20 day foetal rat calvariae. The calvariae of the foetal rat were excised and the frontal and parietal bones free of suture and periosteal tissue were collected. The calvariae were sequentially digested using collagenase and digests 3-4 were pooled and grown in T-75 flasks in DMEM/10% FBS/ 5 µg/ml - L-ascorbic acid-2-phosphate (AA2P). Cells were grown to 90% confluence and then trypsinised and seeded into 24 well plates (50,000 cells/ml, 0.5 ml/well) in MEM/5% FBS/5 µg/ml AA2P and incubated for 24 hours. Cells were then serum starved in MEM/0.1% BSA/5 µg/ml AA2P for 24 hours. Medium was replaced, growth substances added and cells incubated a further 24 hours. [³H]-thymidine was added 6 hours prior to the end of this incubation period. Cells were washed and then processed for thymidine incorporation. Thymidine incorporation was assessed using a Trilux counter (Wallac, 1450 Microbeta, USA). There were six wells in each group and statistical significance was determined by one-way analysis of variance with post hoc examination of
significance using the method of Dunnett. Desalted fraction 9 (1.46 mg/ml) was used for evaluating biological activity.

3.5 Results and discussion

3.5.1 Physical characterisation of bLF

Bovine LF is a solid with a salmon pink colour. The crystals were irregular in shape and size with sharp edges (Figure 3.6). After milling the colour changed to light pink as a result of particle size reduction of bLF.

Figure 3.6 Unmilled bLF as received (top left), milled bLF (top right) for 4 min using cryomill. SEM of bLF (bottom).
3.5.2 Protein estimation, molecular weight determination, and evaluation of purity of bLF

3.5.2.1 Protein estimation of bLF

The standard curve generated using the Biuret assay maintained a good linearity (Figure 3.7) with a linear equation $y = 0.0371x + 0.0014$, and the correlation coefficient ($r^2$) was 0.996. The average protein concentration of three bLF solutions (1.0, 2.5 and 5.0 mg/ml) was calculated to be 1.09, 2.52 and 5.45 mg/ml respectively.

![Figure 3.7 BSA concentration vs absorbance at 520 nm calibration. Error bars are ± SD (n=2).](image-url)
3.5.2.2 Molecular weight and purity determination

The SDS-PAGE analysis shown in Figure 3.8 shows that the molecular weight of native bLF is approximately 80 kDa. The intensity of the band increased as the concentration of the bLF increased. The SDS-PAGE profile of bLF indicated that the sample was pure, apart from a light band at around 35 kDa, which probably represents a contaminant protein.

![Figure 3.8 SDS-PAGE (12%) gel stained with Coomassie brilliant blue: reading left to right, lane 1, molecular weight marker; lane 2, bLF (5 µg); lane 3, bLF (10 µg); lane 4, bLF (15 µg); lane 5, bLF (20 µg); lane 6, bLF (25 µg).](image)
3.5.2.3 Mass spectrometric analysis

The exact molecular mass of bLF was determined by MS (Figure 3.9) and was found to be 82,591 Da, which is similar to what has been reported previously (161).

![Figure 3.9](image-url) Molecular mass profile of bLF determined by MALDI-TOF. BSA was used as an external calibrant.
3.5.2.4 Identification of bLF by LC-MS/MS

Four peptides were identified in trypsin digests of bLF with 7% sequence coverage of the whole sequence as shown in Table 3.6. This sequence was carried out commercially and we acknowledge the help provided in identification of bLF.

<table>
<thead>
<tr>
<th>Peptides detected</th>
<th>Amino acid sequence (Single letter code)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27-37</td>
<td>WCTISQPEWFK</td>
</tr>
<tr>
<td>58-66</td>
<td>RAFALECIR</td>
</tr>
<tr>
<td>256-277</td>
<td>APVDAFKECHLAQVPSPHAVVAR</td>
</tr>
<tr>
<td>304-315</td>
<td>SFQLFGSPPGQR</td>
</tr>
</tbody>
</table>
3.5.3 Enzymatic derivitisation of bLF

3.5.3.1 Ultrafiltration

As noticed in Figure 3.8, probable contamination was minor but was important to remove before enzymatic digestion of bLF. Figure 3.10 shows the distinctive band of bLF in the retentate after the ultrafiltration using a 50 kDa cut off membrane, indicating the removal of probable contamination. The process resulted in 50 ml of retentate and 100 ml of permeate.

![Image of SDS-PAGE with molecular weight markers and bands for LF (C) and LF (R)]

*Figure 3.10* SDS-PAGE (Laemmli) of bLF stained with Coomassie brilliant blue; reading left to right, lane 1, molecular weight markers, lane 2, bLF before ultrafiltration or control; lane 3, bLF after ultrafiltration
3.5.3.2 Derivitisation of bLF and process optimisation

Pepsin has been used for controlled hydrolysis of LF from different sources to produce bioactive peptides. Although other proteases such as trypsin, chymotrypsin and plant and microbial proteases have been reported, the antimicrobial effect of the resulting peptides was much weaker than observed with pepsin (188). The probable reason for this might be the cleavage site specificity of pepsin, which preferentially cleaves at the carboxyl terminus of phenylalanine and leucine residues in the substrate.

With the increase in time of digestion, the intensity of bands of the lower molecular weight peptides was more evident, as observed in Figure 3.11. It has been reported previously that bovine and human LF are readily digested by gastric pepsin under acidic conditions (188). Furthermore, it has also been reported that the antibacterial effect of the peptides after 30 min and after 4 hours was found to be same. Recio and Visser (1999) found that by increasing the concentration of pepsin or increasing the time of digestion, the yield of cationic peptides can be improved (189). Our studies showed that with increase in time the intensity of lower molecular weight peptides increased and showed similar results to those reported by Recio and Visser (1999). It appeared from the SDS-PAGE profile that the intensity of lower molecular mass peptide bands increased with time. Therefore optimisation of digestion was carried out.
Figure 3.11 Tricine-SDS-PAGE profile of peptides generated by pepsin hydrolysis of bLF stained with Coomassie brilliant blue; reading left to right, lane 1, undigested bLF; lane 2, digested bLF at 0.5 hour; lane 3, digested bLF at 1 hour; lane 4, molecular mass markers; lane 5, digested bLF at 2 hours; lane 6, digested bLF at 3 hours; lane 7, digested bLF at 4 hours; lane 8, digested bLF at 5 hours.
Figure 3.12 shows band intensity at different concentrations of pepsin and digestion at a constant time (5 hours). The band intensity at all concentrations appeared similar under the testing conditions, suggesting that the concentration of enzyme does not have a marked effect on the digestion. Most investigators have used a pepsin concentration of 30 mg/g of protein and 4-5 hours of digestion.

Figure 3.12 Tricine-SDS-PAGE profile of peptides generated by pepsin hydrolysis of bLF stained with Coomassie Brilliant blue; reading left to right, lane 1, molecular mass markers; lane 2, undigested bLF; lane 3, digested bLF at 15 mg/g of protein; lane 4, digested bLF at 20 mg/g of protein; lane 5, digested bLF at 25 mg/g of protein; lane 6, digested bLF at 30 mg/g of protein; lane 7, digested bLF at 35 mg/g of protein
3.5.3.3 Gel filtration and preparatory ion exchange chromatography

All the fractions 25 to 40 were pooled (Figure 3.13) and concentrated using a speed vac (SpeedVac® Plus, Thermo Savant USA), then Tricine-SDS-PAGE was run. No bands corresponding to the desired molecular weight of bLFcin were observed. The gel filtration profile of the fractions is shown in Figure 3.13.

The profile of fractions collected with 2 M ammonium chloride is shown in Figure 3.14. The protein concentration of the major fractions (Fraction 31) was found to be 3.2 mg/ml. The fractions numbered 26 to 33 showed higher absorbance and were found to be not pure when evaluated by Tricine-SDS-PAGE.

![Figure 3.13](image-url) Fraction number vs absorbance at 280 nm after gel filtration of bLF hydrolysate.
Figure 3.14 Fraction number vs absorbance at 280 nm of the fractions collected after ion exchange chromatography of bLF hydrolysate.
3.5.3.4 Fast protein liquid chromatography

The protein concentrations of the major fractions eluted were as follows:
Fraction eluted with 0.4 M sodium chloride: 1.8 mg/ml and 1.5 mg/ml.
Fraction eluted with 2 M ammonium chloride: 0.380 mg/ml.
After evaluating their purity and removing the salt, the peptides were characterised by mass spectrometry. The peptides that were eluted with 0.4 M sodium chloride contained peptides less than 3000 Da. The fraction with 2 M ammonium chloride was found to have peptides in the range of 4000-4300 Da. The FPLC profile is shown in Figure 3.15.

Figure 3.15 FPLC chromatogram showing elution of peptides with different buffer conditions. Fractions collected are shown in red.
3.5.3.5 Ion exchange using microporous membrane

Ion exchange chromatography using microporous membrane is also called adsorptive membrane chromatography. This technique is particularly useful to separate cationic proteins and peptides of high value. Short cycle times, ease in scale up and customisation to specific needs are some of the other major advantages of membrane adsorber technology. These strongly acidic membranes trap proteins in their anionic form. The absorption profile of the fractions eluted from adsorption membrane and their Tricine-SDS-PAGE profile are shown in Figure 3.16 and 3.17 respectively. The bands were quite distinct and indicate that the fractions were quite pure and the mass was also close to the target peptide. The protein contents of the fractions 1, 8, 9, 21 and 22 were 0.277, 0.346, 1.46, 0.175 and 0.247 mg/ml respectively.

Fractions 1, 9 and 22 were further characterised for exact mass and amino acid sequencing.

![Figure 3.16](image-url)  
**Figure 3.16** Fraction number vs absorbance at 250 nm of eluted fractions with adsorptive membrane chromatography.
Figure 3.17 Tricine SDS-PAGE profile of fractions eluted from membrane adsorber membrane stained with Coomassie brilliant blue; reading left to right, lane 1, low molecular mass markers; lane 2, fraction 1 with 2 M NH₄Cl; lane 3, fraction 8; lane 4, fraction 9; lane 5, fraction 21; lane 6, fraction 22; lane 7, control-bLF hydrolysate.
3.5.3.6 Characterisation of purified peptide

Fraction 9 was reduced with DTT and alkylated with iodoacetamide (IAA), then desalted using a pipette tip filled with C\textsubscript{18} material (StageTip). The sample was introduced into the Q-STAR mass spectrometer using a direct infusion borosilicate needle.

The MS spectrum (Figure 3.18) showed a charge series of peaks, corresponding to the (552.98 6+), (662.78 5+), (828.22 4+) and (1103.95 3+) states of the peptide. These all correspond to an uncharged monoisotopic mass of 3308.8 Da.

The different charge states were selected for fragmentation (MS/MS). The MS/MS spectra were combined into a single spectrum, which was deconvoluted to the 1+ charge state and submitted for database searching (Mascot). A search of the complete NCBInr database returned a single hit for bLF, which matched the predicted bLFcin peptide plus one additional amino acid, alanine.

The identified peptide contained two cysteine residues, which in some fractions were linked with a disulfide (SS) bridge. Fraction 9 peptide, with a sequence FKCRRWQWRMKKLGAPSITCVRAFA and without a SS bridge, had a theoretical average mass of 3196.87 Da and theoretical monoisotopic mass of 3194.72 Da. Fraction 22 peptide, with a sequence FKCRRWQWRMKKLGAPSITCVRAFA and a SS bridge, had a theoretical average mass of 3194.85 Da and a theoretical monoisotopic mass of 3192.70 Da. Fraction 1 peptide, with a sequence FKCRRWQWRMKKLGAPSITCVRAFA after reduction and alkylation, had a theoretical average mass of 3310.97 Da and a theoretical monoisotopic mass of 3308.76 Da. The complete list of amino acids with names and one letter codes is enclosed in Appendix 2.

Fractions 9 and 22 were alkylated without prior reduction and submitted for mass measurement. Both samples returned a monoisotopic mass of 3192.75 Da, which indicated that both had an intact disulfide bond.

De novo sequencing was partially successful in the middle part of the peptide. Due to the high peptide mass and the non-tryptic nature of the peptide, fragmentation parameters could not be optimised for efficient fragmentation of the N- and C-terminal parts, hence sequencing of these parts was not possible. However, the full sequence
was annotated on the MS/MS spectrum. This gave an extremely good fit in the central part of the peptide (800-2300 Da) and, because of the reasons stated above, a poorer fit in the terminal regions. Isobaric amino acids leucine and isoleucine (L / I) and semi-isobaric amino acids glutamine and lysine (Q / K) cannot be distinguished in de novo sequencing.

![Figure 3.18 Mass spectrum of fraction 9 after adsorption membrane purification.](image)
3.5.3.7 Biological activity of bLF and bLFcin

The bLFcin produced as a result of pepsin digestion consisted of a positively charged looped peptide containing residues 17 to 41 of the bLF molecule. They have shown activities including broad-spectrum activity against Gram-positive and Gram-negative bacteria. They are also reported to have antifungal, antiviral and antitumour activities (190, 191). The antibacterial activity of pepsin-digested bLF hydrolysate is higher than the native bLF molecule (188). The focus of this study was to characterise bLFcin peptide (17-41) purified in the lab and investigate its osteogenic activity on osteoblast cells. There was a previous published report in which synthetic LF peptides (192) with a sequence (20-30), molecular weight 1544.9 Da and (17-30), molecular weight 1993 Da were compared with the full length LF.

Figure 3.19 shows the effect of bLFcin on osteoblast cell proliferation. The effect was compared with the control and standard synthetic LFcin (Auspep, Australia), with a similar amino acid sequence as that of bLFcin (FKCRRWGRMKKLGAPSITCVRRAFA), the molecular weight of Auspep was 3197 Da. The purified bLFcin did not show osteogenic activity at 0.1 µg/ml compared to control. At 1 µg/ml the osteogenic activity was found to increase compared to control. At 10 µg/ml Auspep showed significant osteogenic activity whereas activity was reduced with purified bLFcin as compared to control. Auspep at 50 µg/ml showed no significant osteogenic activity as compared to control and osteogenic activity was reduced significantly with purified bLFcin. At higher concentrations of bLFcin it was observed that cells lifted from the culture plates, suggesting that the purified sample of bLFcin might have traces of ammonium chloride that had a toxic effect on the cells.

Figure 3.20 shows the effect of bLF on the proliferation of osteoblast cells. There was a significant increase in the osteogenic activity at 10 and 100 µg/ml of bLF std and bLF compared to control, which strongly suggests that the whole bLF molecule has far higher osteogenic activity compared to bLFcin. This was also observed in previous studies with the peptides mentioned above (192).
Figure 3.19 Thymidine incorporation by osteoblasts after treatment with various concentrations of control, synthetic bLFcin from Auspep (Australia) and purified bLFcin. Error bars are ± SEM, n=6. * p<0.05

Figure 3.20 Thymidine incorporation by osteoblasts after treatment with various concentrations of control and bLF. Error bars are ± SEM, n=6, * p<0.05.
3.6 Conclusion

Bovine LF was characterised and purified with well established techniques, including electrophoresis, mass spectrometry and amino acid sequencing. The bLF was found to be considerably pure and a minor contaminant was removed by ultra filtration for enzyme digestion.

The purified protein was digested with pepsin enzyme and different purification techniques were employed to achieve purification of bLFcin. The adsorber membrane technique of purification was found to be simple and bLFcin was isolated in higher purity with this technique. The purified LFcin was found to have a mass of 3192.75 Da. The technique was found to be fast, reliable and efficient and resulted in pure bLFcin as characterized by MS/MS.

Osteoblast apoptosis is considered to be an important process in bone formation and skeletal integrity in general and bLF has been reported to promote osteoblast survival (193). bLF was found to have a more pronounced proliferative effect on osteoblast cells compared to bLFcin when used in equimolar concentrations. Higher concentrations of bLFcin produced by AUSPEP did have a modest proliferative effect. bLFcin produced in this study also showed significant proliferative effects at 1 µg/ml, but at higher concentration the osteogenic activity reduced.

From this experiment, it is apparent that although bLFcin has been reported to have high antibacterial activity compared to bLF, it has lower osteogenic activity. Therefore, bLF was selected for further studies.
CHAPTER 4
Formulation Development and Characterisation
4.1 Introduction

During the past decade the application potential of LF as a therapeutic protein and as a food additive has increased tremendously because of the discovery of its various biological actions. LF is commercially available from a number of sources, with the biggest being from bovine milk. Recombinant human LF is also produced from *Aspergillus niger*, transgenic cows and rice (194). Despite LF’s potential applications in many disease states (195), its poor oral bioavailability and instability in the GIT are major obstacles in achieving maximum therapeutic efficacy. The practical approach would be formulation of such native proteins into particles that are resistant to organic solvents, moisture, acidic environments and/or enzymatic degradation (196), which maintain the integrity of proteins at the same time. Due to the advantages offered by polymeric particulate based drug delivery systems, encapsulation of bLF in a polymeric carrier has the potential to meet these requirements.

The specific aims of this chapter are:

1. To develop and validate a suitable analytical method that can be used on a routine basis for quantification of bLF in the formulation.

2. To prepare bLF-loaded microparticles with either 1 or 10% w/w bLF using the cryomilling method.

3. To optimise milling conditions to achieve minimum average particle size with highest loading.

4. To characterise the optimised formulation.

5. To evaluate the stability of bLF and the β-glucan2 bLF milled particles.

6. To evaluate the interaction between bLF and β-glucan2.

Different milling condition parameters (time of milling, cycles and loading %) were studied with the goal of preparing an optimised formulation with an average particle size of less than 10 µm, high entrapment efficiency of bLF and low initial burst effect without the loss of biological activity of bLF. The particle size of less than 10 µm has
been reported to show some success in crossing the GIT via M cells in the GALT (10).

4.2 Materials

Bovine LF (bLF) was kindly donated by Fonterra (New Zealand), β-glucan2 was purchased from GraceLinc Limited (New Zealand) and standard bLF was purchased from Sigma Aldrich (USA). Trifluoro acetic acid (TFA) (protein sequence grade R3, lot no.0305094) was purchased from Applied Biosystems (USA). HPLC grade acetonitrile was purchased from Merck (USA). Cellulose acetate phthalate (CAP) and Kollicoat® MPE 100 P (Kollicoat) were purchased from BASF (Germany). Polyethylene glycol 2000 (PEG2000) was purchased from BDH (USA). EDC (1-ethyl-(3-dimethylaminopropyl)-carbonimide and NHS (N-hydroxsuccinimide) were purchased from GE healthcare (USA) All other chemicals used in this study were at least of reagent grade. Water used in the preparation of all solutions and buffers was obtained by reverse osmosis by the Milli Q unit from Millipore, (USA).

4.3 Methods

4.3.1 Analytical method

A new HPLC method was developed and validated for the quantification of bLF in the formulation. This is described below.

4.3.1.1 Chromatographic conditions

The HPLC system included a micro flow pump unit (Beckman Coulter, USA), System Gold 128 Solvent System Module, an auto sampler injector (Perkin Elmer series 200) and a diode array UV detector (System Gold 166 detector) set at 214 nm. Data was processed with 32 karat software (Beckman Coulter, USA). Different separating columns (C4, C5, C8, C18 and Mono S) were tried to get optimum separation. Separation and quantification of bLF was achieved with a C5 micro column (10 cm x 1.0 mm, particle size 3 µm, pore size 300 Å, Sigma, USA) equipped with a 1 cm x 1.0 mm guard column of the same packing material. The C5 column was subsequently used for the rest of the quantification studies.

All samples were analysed under gradient elution at a flow rate of 0.05 to 0.2 ml/min and the injection volume was 20 µl. The column was maintained at a running
The temperature of 37°C. The temperature of the auto sampler was kept at 4°C. Before sample injection, the column was preconditioned by running a blank gradient and further equilibrated in buffer A and B (65:35). The gradient conditions are summarised in Table 4.1. Detection was recorded at 214 nm. The retention time of bLF was 24.55 min. Mobile phase A consisted of 0.1% TFA in MQ water. Mobile phase B consisted of 0.1% TFA v/v in water and Acetonitrile (10:90 v/v)

Table 4.1 HPLC gradient conditions for bLF elution.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase B (%)</th>
<th>Flow rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>35</td>
<td>0.05</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>0.05</td>
</tr>
<tr>
<td>16</td>
<td>45</td>
<td>0.05</td>
</tr>
<tr>
<td>19</td>
<td>50</td>
<td>0.20</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>0.20</td>
</tr>
<tr>
<td>24</td>
<td>70</td>
<td>0.20</td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>0.20</td>
</tr>
<tr>
<td>26</td>
<td>100</td>
<td>0.20</td>
</tr>
<tr>
<td>27</td>
<td>35</td>
<td>0.20</td>
</tr>
<tr>
<td>47</td>
<td>35</td>
<td>0.05</td>
</tr>
</tbody>
</table>

4.3.1.1 Preparation of standard solution

A stock solution of standard bLF was prepared by accurately weighing appropriate amounts on a microbalance (Mettler Toledo, USA) and dissolving in MQ water to give a final concentration of 1.0 mg/ml. Working standard solutions of bLF (6.25, 12.5, 25, 50, 75 and 100 µg/ml) were prepared by further diluting the stock solution with buffer A.

4.3.1.2 Linearity

A calibration curve was prepared with six concentrations of the standard bLF solution (n=3) in the range of 6.25-100 µg/ml. Linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.
4.3.1.3 Method validation

The method was validated according to International Conference on Harmonisation (ICH) guidelines for industry on validation of analytical procedures. The parameters evaluated were specificity, sensitivity, linearity, precision, accuracy and repeatability.

4.3.1.4 Specificity

The specificity of the method was demonstrated by injecting the $\beta$-glucan2 solution that was used for encapsulation both with and without bLF. Mass spectrometry was used to differentiate and identify the peak.

4.3.1.5 Sensitivity

The lowest standard (6.25 µg/ml) on the calibration curve was identified as the lower limit of quantification (LOQ), where the analyte peak retained, resolved and was reproducible.

4.3.1.6 Repeatability

Injection repeatability was investigated by injecting six sample solutions of bLF (50 µg/ml) during the same day.

4.3.1.7 Recovery

Recovery of bLF was determined by preparing three bLF concentrations (final concentration 10, 60 and 100 µg/ml), which were spiked with 2% w/w of freshly prepared $\beta$-glucan2 solution, then centrifuged at 14000 rpm for 10 min at 4°C. The samples were analysed and the percentage recovery was calculated.

4.3.1.8 Accuracy and Precision

Intra-day accuracy and precision were determined by analysis of three replicates of high, medium and low concentrations (100, 60 and 10 µg/ml) on the same day. Inter-day accuracy and precision of the method were determined by the analysis of samples of the same concentration on three different days. The overall precision of the method was expressed as relative standard deviation (% RSD). Accuracy of the method was determined by comparing the means of measured concentrations to their true concentrations.
4.3.1.9 Stability of bLF

In order to evaluate the stability of bLF (1 mg/ml) solution during analysis, short term stability was evaluated by storing 50 µg/ml samples at 4°C, 25°C and 37°C for 24 hours. The peak shape and retention time were closely evaluated periodically for any degradation (197). The data were analysed statistically using Mini Tab version 12. P-values were used to declare significance.

For evaluation of stability of bLF and the stability of bLF extracted from the β-glucan bLF milled particles, other methods such as SDS-PAGE, mass spectrometry, DSC and FTIR (198) were used, because the HPLC method has its limitations in evaluating complete stability of proteins (104, 197). The DSC technique is very useful in evaluating unfolding of proteins, in which it shows an exothermic peak in place of an endothermic peak. FTIR is an extensively used technique for characterisation of the secondary structure of a protein and detection of any change in the structure. This technique was employed to evaluate short term and long term stability of bLF.

A more efficient technique, LC-MS/MS, was employed to monitor any degradation products of bLF during processing in order to measure stability of bLF. The stability of bLF and bLF extracted from the particles was evaluated by injecting the samples into HPLC, then the HPLC fractions were collected from the peak and peptide identification was done to investigate any change in protein backbone.

4.3.2 Preformulation studies

The physicochemical properties of the polysaccharide β-glucan2, including rheological behaviour, mechanical strength, average molecular mass and stress studies, were evaluated as described in Chapter 2. Density of β-glucan1 and 2 was evaluated in water and methanol. bLF purity, molecular weight, peptide identification and total protein content were evaluated as described in Chapter 3.

4.3.3 Formulation

The formulation involved two major steps: casting films of β-glucan2 encapsulating bLF and milling the dried films to produce microparticles.
4.3.3.1 Casting of films

9.0 g or 9.9 g of β-glucan2 powder was accurately weighed and dispersed in 490 or 545 g respectively of preheated MQ water and kept at 80°C for 30 min in a temperature controlled shaking water bath (SBD50 Bio Maxi Shake, Heto, Denmark) with constant slow stirring. The mixture was cooled down to 25°C with constant slow stirring until the solution was free of any lumps. 1.0 g or 0.1 g of bLF was accurately weighed and dissolved in the β-glucan2 solution with slow stirring. 10 ml of this solution was poured into each petri dish (8.5 cm diameter). The petri dishes containing the mixture were dried overnight at 25°C in the drying oven (Binder, USA). The dried films were carefully peeled off from the petri dish. The weight of the films was measured to establish the yield of the films. The moisture content of the films was measured by halogen moisture analyser (HG63, Mettler Toledo, USA).

Other formulations of the films were cast with excipients such as CAP (2% w/w of β-glucan2), Kollicoat (2% w/w of β-glucan2) and PEG2000 (2% w/w of β-glucan2) to evaluate the effect of hydrophilic and hydrophobic excipients on the in vitro drug release from films and particles.

4.3.3.2 Staining of films to investigate bLF entrapment

A modified method of staining the films was used to investigate the entrapment of bLF within the casted film. It was evaluated by screening the surface of the casted film for the presence of bLF. The method is called the Perls Prussian Blue reaction method (199). It is based on the principle that dilute HCl hydrolysates the protein and releases the ferric ions from bLF, which in the presence of ferrocyanide ions are precipitated as an insoluble blue coloured complex. The Prussian Blue reaction is commonly used as a histopathology stain to detect the presence of iron in a biopsy specimen. bLF has iron bound to it, so this dye was expected to hydrolyse bLF present on the surface and react with the iron to give an intense blue colour. This test was done as an indicative test to investigate whether bLF was present on the surface of the film. Staining solution was prepared by mixing equal parts of aqueous solution of potassium ferrocyanide (10% w/v) and HCl (10% v/v). The samples of the films were fixed on a glass slide and flooded with the above solution for 15 min, then washed with distilled water. The films were observed under the microscope and pictures were taken using a Nikon Coolpix 4500 camera (Nikon Co, Japan)
### 4.3.3.3 Milling of films

The films were cut into smaller films with scissors. 500 mg of the film was weighed accurately and loaded onto the polycarbonate tube, which was placed in the freezer mill assembly (SPEX 6750 freezer mill, USA) and milled at different settings to establish the optimal mill setting.

The freezer mill assembly (Figure 4.1) consisted of a tub (F) containing liquid nitrogen (-195.8°C) that cooled down the entire internal assembly including the polycarbonate tube (D) containing the film sample. The polycarbonate tube/center cylinder was placed in a slot (E) in the internal assembly of the machine. A rod shaped steel impactor (C) was placed along the sample in the polycarbonate tube, or sample vial. The sample vial was sealed on both sides using stainless steel plugs (B). The entire internal assembly was immersed in the liquid nitrogen bath inside the mill. The mill was programmed for operation with optimised milling settings. The sample, embrittled by liquid nitrogen, was milled with the stainless steel impactor, which was magnetically driven back and forth against the stationery end plugs. The only moving part was the steel impactor. Pre-cooling was done to bring the temperature of the internal assembly and the sample tube to cryogenic temperatures, i.e. below -80°C, with liquid nitrogen. According to the machine specifications, anything between 10 and 30 min of pre-cooling time was considered sufficient, therefore pre-cooling of 15 min was selected. During the operation some heat may have been produced while milling, so cooling was scheduled between each cycle for 2 min. An impact rate of 10 was used, which is the maximum that the freezer mill model 6750 can achieve. The final parameters of the milling operation were a precooling time of 15 min, cooling between each cycle for 2 min, an impact rate of 10 and sample weight of 500 mg.

### 4.3.3.4 Process optimisation

Three process parameters were optimised to achieve a formulation with an average particle size of less than 10 µm. These were number of cycles, time of each cycle and bLF loading. After formulation optimisation for optimum particle size, attempts were made to add other excipients including CAP, Kollicoat and PEG2000 to evaluate the effects of these excipients on the release profile of the microparticles.

Two loading concentrations of bLF (1% and 10% w/w of β-glucan2) were selected. To optimise the milling conditions, films were milled for different durations and number of cycles and characterised for particle size distribution. All the milling conditions were
carried out in triplicate (Table 4.2). The process yield was evaluated by weighing the particles produced at the end of the process. The moisture content of the particles was recorded after every batch using the halogen moisture analyser. The formulation with the lowest particle size distribution and the highest entrapment efficiency was selected for further study.

Figure 4.1 Assembly used for milling of films to produce microparticles and optimisation of formulation. Freezer mill used for milling films (A), stainless steel end plugs (B) used to seal the polycarbonate tubes (D), the sample was placed in polycarbonate tubes (D), stainless steel impactor (C) is put along the sample in polycarbonate tube. Polycarbonate tube along with sample was placed in a slot (E) and a basket containing liquid nitrogen (F).
Table 4.2 Milling time x cycles x bLF loading (2 x 3 x 2) factorial design to investigate optimisation of the cryo-milling method for producing $\beta$-glucan2 milled particles.

<table>
<thead>
<tr>
<th>Milling time (min)</th>
<th>Cycles</th>
<th>bLF loading (%w/w)</th>
<th>Batch number and order of production</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1, 23, 19</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1</td>
<td>24, 18, 11</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>9, 2, 20</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
<td>4, 21, 17</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>10</td>
<td>25, 26, 27</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>10</td>
<td>5, 16, 22</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>10</td>
<td>3, 8, 14</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>10</td>
<td>15, 13, 7</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>10</td>
<td>12, 6, 10</td>
</tr>
</tbody>
</table>

4.3.4 Formulation Characterisation

4.3.4.1 Physical appearance

After milling, the particles were observed for any change in colour or physical characteristics. Pictures were taken using a Panasonic DMC-FZ25 camera (Panasonic, Japan).

4.3.4.2 Evaluation of particle size distribution and zeta potential

Particle size analysis of the formulations was carried out by optical microscopy (200, 201). Determination of particle size was done either directly from microscope images or from a captured image. In both the methods a reference scale was used to measure the particle size. Optical microscopy was selected because other techniques were not suitable for particle size analysis. This is because particles agglomerate or swell in aqueous media during analysis, giving variable results because of the hydrophilic nature of the polymer. The particles were mounted on a slide and placed on a light microscope stage (Leica, GmbH, Germany). The microscope eyepiece was fitted with a micrometer for size measurement of individual particles. The diameter of the particles was measured at two points and the mean of both dimensions was taken as the particles were of various shapes. The reading of the micrometer depended on
the magnification of objectives and was calculated as follows: At 100x, each division on the micrometer is equal to 10 µm and at 200x, 400x and 1000x, each division is equal to 5 µm, 2.5 µm and 1 µm respectively. Measurement was carried out on a series of randomly selected fields after mounting the sample on the glass slide. To minimise the error of sample size, 300 particles were measured in each formulation (202). The analysis was carried out in triplicate and an average of the three measurements was taken. Statistical significance of the milling conditions was analysed using a general linear model (Minitab 15.1.0.0, Minitab Inc. USA).

For the measurement of zeta potential, triplicate samples of β-glucan2 (unmilled and milled) and the β-glucan2 bLF milled particles with 10% bLF loading were diluted in solutions of simulated gastric and simulated intestinal fluids (pH 1.2 and 7.4). Each sample was placed in the electrophoretic cell of the Zetasizer (Malvern Instruments, UK) and measurements were recorded. The values were recorded as the mean of three determinations. Simulated gastric fluid and simulated intestinal fluid were prepared according to the method described in United States Pharmacopeia (USP).

4.3.4.3 Morphology

The shape and surface texture of films and microparticles were evaluated with a scanning electron microscope (SEM) (Phillips XL30S FEG, Netherlands). The sample for SEM was prepared by placing a small amount of microparticles on the stubs, which were then coated with gold-palladium and micrographs were recorded. For films, a small portion was cut in liquid nitrogen and placed on the edge of the stub and fixed with adhesive tape.

4.3.4.4 Evaluation of drug entrapment efficiency (DEE)

An indirect method was used to determine DEE in all the formulations (97). In this method, percentage entrapment of bLF was determined from the difference between the total amount of the bLF loaded or added initially to the formulation and the unentrapped amount detected in the supernatant. Particles (10 mg) in triplicate were accurately weighed and dispersed in 10 ml phosphate buffered saline (PBS) at pH 7.4 and stirred slowly for 10 min before centrifugation at 14000 rpm for 10 min at 4°C. The supernatant was aspirated and the samples were analysed using a validated HPLC method to determine the bLF in the supernatant. The results are presented as percentage entrapment efficiency.
4.3.5 Validation of extraction method

A good extraction method is required to recover the full amount of bLF entrapped from films and microparticles. Our initial studies indicated that the full amount of entrapped bLF could not be recovered in the first 3 hours in phosphate buffer (pH 7.4) and MQ water at room temperature, so extraction was carried out for 6, 12 and 24 hours. Other parameters such as pH of the media (4.0 and 5.5) and volume of the extraction media (10 and 20 ml) were adjusted and the extraction of bLF was evaluated for first 3 hours. For each variable, 10 mg of sample in triplicate was weighed and dispersed in 10 ml PBS at both pH 4.0 and 5.5 and kept for 3 hours with constant stirring. Solutions were then centrifuged at 14000 rpm at 4°C for 10 min and supernatant was analysed for bLF using a validated HPLC method. The two volumes used were 10 and 20 ml for 10 mg of particles for 3 hours. The data are presented as a mean percentage recovery ± SD, n=3.

4.3.5.1 Recovery of bLF by enzyme treatment

β-glucan2 bLF milled particles (10 mg) were accurately weighed and dispersed in 10 ml MQ water with constant stirring, then allowed to stand for 12 hours at room temperature with slow stirring. The dispersion was centrifuged at 14000 rpm for 10 min at 4°C and supernatant was collected. The supernatant was first analysed for bLF concentration and then incubated with 10 µl lichenase enzyme (E-LICHN, Lot 70801, Megazyme International, Ireland) with an activity of 336 U/mg for 3 hours at 30°C. The insoluble pellet was resuspended in 2 ml MQ water and incubated with 10 µl Lichenase enzyme for 3 hours at 30°C. All the samples were analysed for concentration of bLF using an ELISA kit. All the samples were in triplicate and the percentage recovery was calculated from an average of three.

4.3.5.2 Evaluation of interactions of bLF with β-glucan2 and sucrose

It was important to investigate whether bLF was undergoing degradation or interactions with β-glucan2 while milling. Therefore the following investigations were conducted:

- β-glucan2 bLF milled particles (10 mg) were dispersed in 5 ml MQ water, then the dispersion was heated to 80°C for 5 min and centrifuged at 14000 rpm for 10 min. The supernatant was then analysed by HPLC.
• β-glucan2 particles (10 mg) were mixed with 1 mg bLF, 5 ml MQ water was added, then the dispersion was mixed and heated to 80°C for 5 min and centrifuged at 14000 rpm for 10 min, and the supernatant was analysed.

• Sucrose (45 mg) was dissolved in MQ water by heating to 80°C for 30 min, then cooled down and bLF (5 mg) was added and dissolved. The solution was analysed using HPLC. Sucrose was used in the same ratio as β-glucan2.

• A solution of bLF (1 mg/ml) was prepared in MQ water and heated to 80°C in a water bath for 5 min, centrifuged and analysed by HPLC.

4.3.6 In vitro release of bLF from the films and microparticles

The *in vitro* release study was carried out using Franz diffusion cell instrument (Logan Instruments Corporation, USA). The assembly consists of an upper donor compartment and a lower receptor compartment. β-glucan2 bLF (10 mg) milled particles were weighed and dispersed in 12 ml PBS (pH 7.4) using the Franz cell apparatus without a donor chamber. The temperature of the bath was kept at 37°C. Samples (500 µl) were withdrawn at predetermined time points (5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420 and 480 min) and the same volume was replaced with PBS. Filter cartridges (0.22 µm) were used so that no particles came out with the sample. While replacing the buffer, the same cartridge was used so that any particle that might have reached the lower end of the filter cartridge would go back into the cell. All the withdrawn samples were filtered using a 0.22 µm filter cartridge, centrifuged and the supernatant was analysed by a HPLC method.

4.3.7 Stability of formulation

4.3.7.1 Physical appearance

The physical appearance of samples stored at different storage conditions were observed visually for any colour change and agglomeration each month and pictures were taken using a Panasonic DMC-FZ5 camera (Panasonic, Japan). The physical appearance, particularly colour, of bLF was also evaluated before and after milling.
4.3.7.2 Molecular weight of β-glucan

The long term stability of β-glucan2 bLF milled particles was evaluated by storing the formulation in triplicate at different storage conditions (25°C/60% RH and 40°C/70% RH). β-glucan2 bLF milled particles (500 mg) samples were placed in glass bottles and stored in stability chambers (Binder, USA) maintained at specific storage conditions. Control samples of β-glucan2 particles and bLF (500 mg each) were also stored under the same conditions. Samples were removed periodically, at 1, 2, 3, 4, 5 and 6 months, and visually examined for change in colour and physical appearance of the particles. For average molecular weight determination of β-glucan2, a 5 mg/ml dispersion was prepared in 0.1 M NaNO₃ solution and SEC-MALLS was carried out as described in Section 2.4. All the stability samples were prepared in triplicate. Statistical difference between the groups was evaluated by SPSS and one-way analysis of variance (ANOVA) and p values < 0.05 were considered statistically significant.

4.3.7.3 Stability of bLF

Short term and long term stability of bLF in the formulation were studied. Short term stability of bLF was investigated immediately after milling with HPLC, FTIR spectroscopy, DSC and LC-MS/MS. Long term stability of bLF was investigated by FTIR spectroscopy, mass spectrometry, electrophoresis and LC-MS/MS.

4.3.7.4 FTIR spectroscopy

To assess the stability of the formulation, IR spectra were recorded immediately after milling at optimised milling conditions. IR spectra were recorded on a Bruker Tensor 37 (Bruker Optik, GmbH, Germany) spectrometer using a Miracle Micro ATR attachment with diamond crystal. A small amount of sample with or without bLF, both before and after milling, was placed on the diamond crystal and compressed gently using the pressure clamp. The crystal was cleaned carefully to avoid any contamination from the previous sample. Scanning was carried out in the 4000-400 cm⁻¹ region with a resolution of 4 cm⁻¹, averaging 64 scans. The spectra were recorded and the data were analysed using OPUS software (Bruker Optik, Germany) version 6.5.
4.3.7.5 Raman spectroscopy

The spectra of the samples (unmilled β-glucan2, milled bLF and particles encapsulating bLF) were recorded on a Reninshaw System 1000 Raman Imaging Microscope (Reninshaw, UK), consisting of a single grating spectrograph, a Leitz microscope and an air cooled CCD array detector. The laser used as an excitation source was a Reninshaw solid-state diode laser emitting a line in the near-infrared at 785 nm and 26 mW with a grating of 1200 lines/mm. The data were processed using a Grams 32 spectroscopic software (Thermo Fisher Scientific Inc., USA).

4.3.7.6 Modulated temperature differential scanning calorimetry (MTDSC)

For thermal analysis, unmilled and milled samples (5.0 ± 0.2 mg) were heated at the rate of 10°C/min from -50 to 300°C for bLF, and -50 to 500°C for both β-glucan2 and β-glucan2 bLF milled particles. The samples were then cooled down at 5°C/min. The modulation parameters used were 0.5°C every 20 sec on a Q1000 Tzero™ module DSC instrument (TA instrument, USA). The instrument was duly calibrated for enthalpy with Indium. Fresh samples were dried in a vacuum drying oven at 30°C for 24 hours and analysed using the same parameters as mentioned above. Data were analysed using Universal Analysis software (TA Instruments, USA) version 4.1D. All the samples were analysed in duplicate and the data reported as mean ± SD.

4.3.7.7 Thermo gravimetric analysis (TGA)

TGA was carried out to evaluate any weight loss or gain of the formulation as a function of temperature. This technique is best used to evaluate decomposition of a substance as a function of temperature. A DSC peak related to solid-solid phase transformation would not show a weight loss in TGA. However, removal of water or solvent as a function of temperature should show a weight loss peak in TGA over the same temperature range. Samples (5 mg) were accurately weighed along with an aluminum pan and heated at 10°C/min to 500°C (unmilled and milled β-glucan2 and β-glucan2 bLF milled particles) or 300°C (unmilled and milled bLF) using a STA 1500 instrument. Data were recorded and analysed with Microsoft Excel.

4.3.7.8 X-ray diffraction studies

Wide angle x-ray diffraction patterns were recorded using a Rigaku Miniflex II desktop X-ray diffractometer with Cu Kα radiation at a wavelength of 1.54Å in the angular
range 5-70° (2θ) at a scan rate of 1.0 min⁻¹ with operating voltage of 30 kV and a current of 15 mA. The XRD studies were carried out on individual samples of bLF, β-glucan2, the physical mixture of β-glucan2 and bLF (90% and 10% w/w respectively) and β-glucan2 bLF milled particles.

4.3.7.9 HPLC-UV and LC-MS/MS studies of bLF and bLF extract from β-glucan2 bLF milled particles

Solutions of unmilled bLF and bLF milled (1mg/ml) were prepared in MQ water. These solutions were then diluted with buffer (0.1%TFA in water) and injected into HPLC. β-glucan2 bLF milled particles that were stored at RT for 6 months were weighed (20 mg) and dispersed in 20 ml of MQ water. The dispersion was then heated to 40°C for one hour and centrifuged at 16000 rpm for 10 min and the sample was diluted further with buffer (0.1%TFA in water) and 20 µl was injected into HPLC. From all three samples, bLF fractions were collected.

HPLC fractions were concentrated in a vacuum centrifuge to approximately 100 µl. A 50 µl aliquot was taken for enzymatic digestion. To this sample, 0.5 µl of 1 M DTT was added and the sample heated at 56°C for 1 hour to reduce disulphide bonds. To bring the pH up to 8, a 10 µl aliquot of 1 M ammonium bicarbonate was added, followed by 2.5 µl of 1 M iodoacetamide (IAM). Samples were incubated in the dark at RT for 1 hour to alkylate cysteines, followed by quenching of excess IAM with a further 1.25 µl of DTT. To each sample, 4 µl of 12.5 ng/µl sequencing grade modified porcine trypsin (Madison, Wisconsin, USA) was added and the samples were incubated at 37°C overnight to digest. Samples were then acidified with 5 µl of 10% formic acid and diluted to 0.5 ml with 0.1% formic acid. Solid phase extraction was performed on 10 mg Oasis HLB SPE cartridges (Waters, Milford, MA, USA) using the protocol described in the manual.

Samples were then concentrated in a vacuum centrifuge to approximately 15 µl. A 10 µl injection was made of each digest onto a 0.3 x 5 mm Pepmap C₁₈ trap column (LC Packings, USA) which was then switched in-line with a 0.3 x 100 mm Zorbax 300SB C₁₈ 3.5 µm column after 3 min for subsequent gradient elution over 45 min. The solvent gradient was formed at 6 µl/min using 0.1% formic acid in water as buffer A and 0.1% formic acid in acetonitrile as buffer B as follows: 0-3min 10% B;33 min 35% B;36 min 95%;39 min 95%; 40.5 min 10% B; 45 min 10% B. The column eluent was plumbed into the electrospray ionisation source of a QSTAR XL hybrid
Quadrupole Time-of-Flight mass spectrometer (Applied Biosystems, Foster City, CA, USA). Each cycle consisted of a TOF-MS scan from 300-1600 m/z from which the top three most abundant multiply-charged species were then selected for MS/MS. The resulting data were then searched using Mascot 2.0.05 (Matrix Science, UK) against a small in-house database containing the bLF protein sequence. The data from bLF extracted from particles was also searched on the internet using Mascot against NCBI's protein sequence database to confirm that bLF was the major component of the observed HPLC peak.

4.3.7.10 Circular dichroism spectroscopy

Circular dichroism (CD) analysis was done to evaluate the secondary structure of the protein in samples of unmilled and milled bLF and bLF extracted from the particles by the extraction method described in Section 4.3.5. The concentration of bLF was calculated by measuring the absorbance at 280 nm. The sample solutions were then all diluted with MQ water to give a final absorbance below 1.0. The spectrum was recorded in the far UV mode region (200-320 nm) by using a Applied Photophysics Pistar, CD instrument (UK) with a 1 mm quartz cylindrical cell. Before the spectra were recorded, the system was purged with nitrogen at 3 l/ min for 10 min. A baseline spectrum was recorded with the MQ water and subtracted from the spectra of the samples. All the data recorded were the mean of three determinations. The data is presented after smoothing to the same level for all the samples.

4.3.8 Investigation of the interaction between β-glucan2 and bLF

The full amount of bLF could not be recovered from the microparticles. This was a prompt to undertake interaction studies using a surface plasmon resonance (SPR) machine from Biacore 2000 (Pharmacia, USA). A running buffer, 20 mM HEPES Buffered Saline-EDTA and P20 (HBS-EP), was prepared by dissolving 1.19 g HEPES, 4.39 g NaCl and 0.56 g EDTA in 450 ml distilled water. 25 µl surfactant P20 (Biacore) was added and the pH was adjusted to 7.4 with 2 M NaOH. The volume was made up to 500 ml with distilled water. All the solutions were prepared in HBS-EP. Immobilisation buffer at pH 6.5 (10 mM sodium acetate) was prepared. Stock solutions of bLF (1 mg/ml) and β-glucan2 (1-10 mg/ml) were prepared in HBS-EP. CM5 carboxymethylated dextran chip (Pharmacia Biotech, Sweden) was prepared by priming the surface in HBS-EP. Immobilisation was carried out at 5 µl/min. Flow cell 1 (FC) was activated and
deactivated in EDC (1-ethyl-(3-dimethylaminopropyl)-carbonimide / NHS (N-hydroxysuccinimide) and ethanolamine respectively. FC2 was activated, immobilised and deactivated with bLF to a level of ~2500 Ru. A β-glucan2 solution of 50 µl (1 mg/ml) was passed over the immobilized bLF and response was recorded. Response from blank control was subtracted. To regenerate the surface various salts were used, including 1 M NaCl, low pH HCl and 2 M Guanidine HCl, which were each run at a flow rate of 20 µl/min. Another concentration of β-glucan2 (10 mg/ml) was also passed over immobilised bLF to investigate the effect of concentration on binding. Response was recorded and data were analysed using Bia Evaluation software V 4.1.1 (Biacore, USA). SDS-PAGE (10%) was run to further investigate the results that were observed in the SPR technique. The following samples were run on the gel: bLF standard, β-glucan2 solution (1mg/ml), lichenase treated β-glucan2 solution, lichenase treated bLF (1 mg/ml), lichenase only and the supernatant and pellet from β-glucan2 bLF milled particles treated with lichenase. The gel was stained with staining solution for 30 min and the composition is of the staining solution is described in Section 3.3.3, then observed for the position of different bands.

4.4 Results and discussion

4.4.1 Analytical method

4.4.1.1 Chromatographic conditions

Different analytical columns and combinations of mobile phase consisting of organic and aqueous phase components were investigated at different flow rates and temperatures. With the C₈ column, there was significant carryover of about 3-4% of bLF even with low flow rates. With C₁₈, C₄ and Mono S, the sensitivity was not adequate, with insufficient improvement using different concentrations of TFA (0.08-0.1% v/v) to optimise peak shape or different gradient conditions in an attempt to provide optimal resolution of the bLF peak.

The mobile phases A and B were optimised for different concentrations of TFA and ACN. The best performance was achieved using a C₅ column with wide pore diameter size, proving optimal for a large molecule like bLF to bind and elute easily. A flow rate of 0.05 ml/min over 19 min provided sufficient time to bind to the stationary phase. Increasing the flow rate to 0.2 ml/min resulted in fast elution of bLF, giving a sharp and symmetric peak at 24.55 min, well separated from the solvent front and other small impurities in the sample.
4.4.1.2 Method validation

A calibration curve was constructed by plotting concentration versus peak area. The linear equation obtained was \( y = 82619x - 48068 \), and the correlation coefficient \( (r^2) \) was 0.999. The calibration curve showed good linearity over the concentration range of 6.25-100 µg/ml and three calibration curves were generated on three consecutive days. The LOD and LOQ were 3.0 µg/ml and 6.25 µg/ml respectively. The inter-day and intra-day precision and accuracy results are summarised in Table 4.3. The mean percentage RSD for the inter-day and intra-day precision was 1.9 ± 0.25 and 2.9 ± 0.25 respectively. The mean inter-day and intra-day accuracy results were 99.9 ± 1.7 and 99.1 ± 1.2, which showed a good agreement between the measured and true values. Figure 4.2 shows a representative chromatogram of a standard solution of bLF (50 µg/ml). The mean recovery of bLF from spiked samples was 97.91% with a RSD of 1.8% (n=9). The HPLC method developed was found to be reliable, linear, precise, accurate and sensitive.

Table 4.3 Inter- and intra-day accuracy and precision results for the determination of bLF by the HPLC method. Replicate samples (n=3) of each concentration of bLF were assayed.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Accuracy (%)</th>
<th>Precision (%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inter-day</td>
<td>Intra-day</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>98.2</td>
<td>97.9</td>
</tr>
<tr>
<td>60</td>
<td>100.2</td>
<td>99.2</td>
</tr>
<tr>
<td>100</td>
<td>101.5</td>
<td>100.3</td>
</tr>
<tr>
<td>Mean</td>
<td>99.9</td>
<td>99.1</td>
</tr>
<tr>
<td>SD</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Figure 4.2 A representative HPLC chromatogram of bLF (50 µg/ml) at 214 nm. The elution time was 24.55 min.
4.4.2 Casting of films

4.4.2.1 Visual and SEM evaluation of films

The SEM micrograph of the β-glucan2 film (Figure 4.3) looked much smoother compared to the β-glucan2 bLF film (Figure 4.4), where some depressions were observed. There was no apparent difference observed in the cross section of the two films.

4.4.2.2 Yield and moisture content of films and microparticles

The average yield of the β-glucan2 bLF milled films after drying were found to be 94.5% ± 2.5%, n=3. The average yield of the β-glucan2 bLF milled particles was found to be 92.2% ± 3.2%. Moisture content of the films was found to be 8.4% ± SD 3.5% and moisture content of the β-glucan2 bLF milled particles was 7.2% ± SD 2.8%.
Figure 4.3 SEM micrograph of β-glucan2 film (top) and a cross section of β-glucan2 film (bottom).

**Figure 4.3** SEM micrograph of β-glucan2 film (top) and a cross section of β-glucan2 film (bottom).
Figure 4.4 SEM micrograph of β-glucan2 bLF film (top) and a cross section of β-glucan2 bLF film (bottom).
4.4.2.3 **Staining of the film**

Only a few blue spots were noticed on the surface of the film after staining the film with Prussian Blue, which indicates that most of the bLF was entrapped within the film as shown in Figure 4.5 (bottom).

![Figure 4.5](image)

**Figure 4.5** Light microscope pictures of β-glucan2 film (top) and β-glucan2 bLF film after staining with Prussian Blue. Circled areas indicate regions stained blue (bottom).
4.4.3 Optimisation of milling Process

4.4.3.1 Milling parameters

The major advantage of cryo-milling was to safeguard the integrity of bLF and β-glucan2 as the milling takes place at very low temperatures (−150°C). Figure 4.6 shows the SEM micrograph of β-glucan2 particles at lower and higher magnification and Figure 4.7 shows the SEM micrograph of β-glucan2 milled particles and β-glucan2 bLF milled particles. Unmilled β-glucan2 particles appeared agglomerated with rough surface. Milled unmilled β-glucan2 has slightly smooth surfaces and more ordered.

Gaisford et al (2010) observed a sharp reduction in particle size after short milling times and size increased subsequently after milling for a longer time using the ball mill at normal temperature. It has been reported that size reduction was attributed to the mechanical force that was applied to the crystal, while the increase in size after extended milling time might be due to humidity or excessive heat that might have led to recrystallization of some of the amorphous material and particle-particle agglomeration (203). Moreover, with the size reduction and formation of new surfaces the particle gets electrostatically charged, which might result in agglomeration of particles. These previous studies suggest that there is a limit to the extent to which particle reduction takes place and continued milling might result in changes in surface chemistry and the material becoming disordered. In one of the studies, cryo-milled crystalline material had undergone major changes at the surface, changing from a crystalline to a amorphous form after 1 hour of cryomilling. However the DSC profile still showed a crystalline peak, which indicated that the bulk of the material still had the same crystalline structure. This suggest that the major effects of milling take place at the surface (204) and by milling for such a long time at cryogenic temperatures, it is still possible to keep the majority of the structure intact. Milling is a high energy process and it is expected that some changes in the surface chemistry are unavoidable during milling (203). However, it is quite feasible to attain the reduction in particle size without any major change in structure by milling under cryogenic conditions. Overall, milling processes have the advantages of low cost and the ability to be scaled up easily. The objective is to optimise the process to attain the desired particle size without any significant changes in the structure of the drug or excipients.
Figure 4.8 shows the average particle size obtained at different milling conditions. On visual examination there were no apparent changes in the shape or colour of the particles. bLF loading and number of cycles did not significantly influence particle size. With increasing milling time the particle size significantly (p=0.093) increased with both loading concentrations. The initial studies had shown that milling for up to an hour using cycles of 2 min followed by 2 min of cooling resulted in an average particle size range of 5-9 µm. As previously reported, cryomilling for a longer duration resulted in structural changes at the surface, so longer milling times were not used. Moreover, the desired average particle size (below 10 µm) was attained with milling conditions for shorter time periods. Therefore milling conditions of (2, 2, 10), which used a higher loading concentration of bLF and resulted in low average particle size were selected for further studies. Unmilled bLF and unmilled β-glucan2 individually were also milled under the same milling conditions for all further studies.
Figure 4.6 SEM micrograph of unmilled β-glucan2 (top) and with higher magnification (bottom).
Figure 4.7 SEM micrograph of milled β-glucan2 (top) and β-glucan2 bLF milled particles (bottom).
Figure 4.8 Effect of various milling conditions upon average particle size. Key: #, #, # milling time in min, cycle in numbers, bLF loading in %. Error bars are ± SEM, n=3.
4.4.3.2 Zeta potential of different formulations

Zeta potential is the difference in electrical potential between a tightly bound layer of ions on the surfaces of particles and the liquid in which the particles are suspended. It is considered an important and useful indicator of the net surface charge on the particles and yields valuable information about the colloidal stability of a colloidal dispersion (205). As a cationic protein, bLF carries a net positive charge, therefore it is important to investigate the net charge on the particles encapsulating bLF at different conditions. β-glucan2 alone at pH 1.2 and 7.4 exhibited a zeta potential of +2.6 ± 1.3 mV and -1.37 ± 1.8 mV respectively (Table 4.4), indicating a charge reversal at pH 7.4. However, β-glucan2 with entrapped bLF had a zeta potential of +8.87 ± 2.1 mV, which might indicate some adsorption of bLF on the surface of particles. When β-glucan2 was dispersed in simulated gastric and simulated intestinal solutions, zeta potential recorded was +1.46 ± 1.2 mV and -3.13 ± 2.1 mV respectively. The zeta potential with PEG 2000 was +0.85 ± 0.5 mV, which might be due to coating of PEG2000 over bLF that was adsorbed. Likewise the zeta potential of the formulation with CAP and Kollicoat was quite similar at pH 1.2 and when measured in simulated intestinal fluid the net charge on the particles was negative in all the formulations, with the maximum in formulations with Kollicoat (-15.8 ± 2.4 mV). It has been reported that β-glucan2 at lower pH carries a positive charge and it carries a negative charge at higher pH (206). Since in our formulations the major component is β-glucan2 (88-90% w/w), it was expected that all our formulations would have a positive charge at lower pH. The bLF that was adsorbed on the surface of the particles might have hydrolysed to peptides and those peptides, which were also cationic in nature, thus contributed to the increase in marginal zeta potential in simulated gastric fluid. The zeta potential of all the formulations was found to be a net negative charge, which is similar to previous reports. The zeta potential of the formulation with Kollicoat was substantially higher than other formulations in simulated intestinal fluid and this might be because of the anionic character of Kollicoat.
Table 4.4 Effect of pH or simulated biological fluids upon zeta potential of β-glucan2 only or β-glucan2 bLF milled particles with or without various excipients. Replicate (n=3) zeta potential (mV) along with standard deviations.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>β-glucan2 bLF milled particle</th>
<th>β-glucan2 bLF milled particle with PEG2000</th>
<th>β-glucan2 bLF milled particle with CAP</th>
<th>β-glucan2 bLF milled particle with Kollicoat</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 1.2</td>
<td>+2.61 ± 1.3</td>
<td>+8.87 ± 2.1</td>
<td>+0.85 ± 0.5</td>
<td>+1.25 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>-1.37 ± 1.8</td>
<td>-1.68 ± 1.6</td>
<td>-0.66 ± 2.9</td>
<td>-1.90 ± 1.1</td>
</tr>
<tr>
<td>Simulated gastric fluid</td>
<td>+1.46 ± 1.2</td>
<td>+3.01 ± 1.7</td>
<td>+0.72 ± 1.8</td>
<td>+2.33 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>-3.13 ± 2.1</td>
<td>-7.30 ± 1.2</td>
<td>-6.53 ± 1.8</td>
<td>-15.8 ± 2.4</td>
</tr>
<tr>
<td>Simulated intestinal fluid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.4.4 Drug entrapment efficiency

The drug entrapment efficiency of various films and particles is summarised in Table 4.5. The entrapment efficiency of the β-glucan2 bLF film with hydrophobic excipients (Kollicoat) was found to be higher compared to the β-glucan2 bLF films without any excipients. The films with Kollicoat had the highest entrapment efficiency, at around 91%. After milling the films, the entrapment efficiency was around 80% in β-glucan2 bLF milled particles with PEG2000 and around 67% in β-glucan2 bLF particles without any excipients. Kollicoat is a partially neutralised methacrylic acid-ethyl acrylate copolymer (1:1) and contains stabilisers such as sodium lauryl sulphate and polysorbate 80. It forms an aqueous dispersion or solution in organic solvents.

<table>
<thead>
<tr>
<th>Excipients</th>
<th>Composition (β-glucan2:excipients:bLF)</th>
<th>Formulation</th>
<th>Entrapment Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>90:0:10</td>
<td>Film</td>
<td>67.3 ± 3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Particles</td>
<td>71.8 ± 0.6</td>
</tr>
<tr>
<td>Kollicoat</td>
<td>88:2:10</td>
<td>Film</td>
<td>91.5 ± 2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Particles</td>
<td>63.2 ± 4.5</td>
</tr>
<tr>
<td>PEG 2000</td>
<td>88:2:10</td>
<td>Film</td>
<td>70.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Particles</td>
<td>80.9 ± 2.0</td>
</tr>
<tr>
<td>CAP</td>
<td>88:2:10</td>
<td>Film</td>
<td>82.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Particles</td>
<td>69.7 ± 2.3</td>
</tr>
</tbody>
</table>

The values are shown as mean ± SEM, n=3
4.4.5 Extraction efficiency validation

4.4.5.1 Effect of duration on extraction of bLF from β-glucan2 bLF films and β-glucan2 bLF milled particles

The effect of extraction duration was evaluated for 3, 6, 12 and 24 hours. The results of extraction are presented in Table 4.6

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Duration (hours)</th>
<th>Average bLF recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-glucan2 bLF film</td>
<td>3</td>
<td>52.65 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>60.10 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>62.82 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>62.02 ± 2.9</td>
</tr>
<tr>
<td>β-glucan2 bLF milled particles</td>
<td>3</td>
<td>24.36 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>30.54 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>32.35 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>38.11 ± 2.6</td>
</tr>
</tbody>
</table>

The values are shown as ± SEM, n=3
4.4.5.2 Effect of pH and volume on the extraction of bLF from β-glucan2 bLF films and β-glucan2 bLF milled particles

The mean recovery of bLF from β-glucan2 bLF films and particles at pH 4.0 and 5.5 is summarised in Table 4.7. It is apparent from the data presented that the pH did not have any major effect on the recovery of bLF from films and particles.

Similarly, there was no apparent difference in recovery of bLF when 10 mg of films and 10 mg of particles were dispersed in 10 or 20 ml of phosphate buffer (pH 7.4). The data is presented in Table 4.7.

The extraction of bLF was found to be lower from particles than from films, pointing towards interactions, possibly hydrogen bonding between protein and cellotriosyl residues of β-glucan2 after milling which might have resulted in loss of osteoblast proliferation effect as discussed in sections 4.4.8. Other proteins and peptides like insulin and glutathione was investigated with β-glucan2 using the same methodology and similar results were observed indicating interactions (data not reported here).
Table 4.7 Average (n=3) bLF recovery from β-glucan2 bLF films or β-glucan2 bLF milled particles at pH 4.0, 5.5 and volume 10 and 20 ml for 3 hours in phosphate buffer (pH 7.4).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Variable</th>
<th>Average bLF recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-glucan2 bLF film</td>
<td>pH of media</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>53.82 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>52.02 ± 2.4</td>
</tr>
<tr>
<td>β-glucan2 bLF film</td>
<td>Volume of media (ml)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>52.14 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>54.12 ± 2.6</td>
</tr>
<tr>
<td>β-glucan2 bLF milled particle</td>
<td>pH of media</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>25.62 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>24.12 ± 2.5</td>
</tr>
<tr>
<td>β-glucan2 bLF milled particle</td>
<td>Volume of media (ml)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>23.51 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>25.85 ± 1.5</td>
</tr>
</tbody>
</table>

The values are shown as ± SEM, n=3
4.4.5.3 Recovery of bLF from β-glucan2 bLF milled particles after enzyme treatment

Lichenase (endo-1, 3 (4)-β-glucanase) is a specific enzyme that specifically breaks β-(1-4) linkages adjacent to (1-3)-linkages in the β-glucan molecule (127). Enzymatic hydrolysis was chosen as this method is more specific and gentle and would not affect encapsulated bLF. The total average recovery of bLF from the particles was 57.87% ± SD 5.3 (n=3). The average recovery of bLF from the supernatant after 12 hours incubation with PBS buffer was 31.1%. Recovery of bLF from the pellet was around 26.8%. The recovery of bLF from the pellet suggests that bLF could be bound to insoluble portion of β-glucan2. After the treatment with enzyme there was still some insoluble material observed.

4.4.5.4 Evaluation of interactions between bLF and β-glucan2 or sucrose

The recovery of bLF from the β-glucan2 bLF milled particles was found to be 29.89%, whereas the physical mixture of β-glucan2 and bLF showed 52.3% recovery and mixing bLF with sucrose showed 105% recovery (Table 4.8). The recovery of bLF from sucrose was close to 100%, which strongly suggests that interactions might take place between bLF and β-glucan2. Sucrose is a non-reducing sugar similar to β-glucan wherein oxygen is attached to two carbons at β-position where the ring cannot open and is expected to behave similarly.

Table 4.8 Average recovery (n=3) of bLF from different combinations of carbohydrates and bLF.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Condition</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>β-glucan2 bLF milled particle</td>
<td>29.89 ± 3.1</td>
</tr>
<tr>
<td>2</td>
<td>β-glucan2 blank with bLF (10% w/w)</td>
<td>52.34 ± 2.5</td>
</tr>
<tr>
<td>3</td>
<td>bLF heated to 80°C</td>
<td>98.54 ± 2.3</td>
</tr>
<tr>
<td>4</td>
<td>Sucrose with bLF (10% w/w)</td>
<td>105.15 ± 0.8</td>
</tr>
</tbody>
</table>

The values are shown as ± SD, n=3
4.4.6  *In vitro* release of bLF

The *in vitro* release profiles of bLF from different formulations are shown in Figure 4.9. β-glucan2 bLF film without excipients showed a burst release of 60% within the first hour and released very little bLF after this. The addition of PEG2000 reduced the burst effect to approximately 30% and release was sustained for approximately 3 hours until approximately 70% of bLF was released, after which very little additional bLF was released. Including Kollicoat increased the burst effect dramatically to 80-90% with nearly 100% release by the end of the first hour.

β-glucan2 bLF particles produced from the film showed reduced bLF release. With PEG2000 or without excipients, release of bLF showed a burst of approximately 25-30% within the first 30 min and very little after this time. β-glucan2 bLF particles produced from films with the addition of Kollicoat showed reduced burst effect and sustained bLF release over 8 hours.

Generally the release of drug from a particulate system involves three different mechanisms. These are diffusion of the drug from the surface, diffusion from the swollen rubbery matrix and release due to polymer erosion. On contact with an aqueous medium, the hydrophilic polymer matrix starts hydrating at the surface and water soluble drugs diffuse out in the early stages, causing a burst effect (100). Apart from diffusion of water soluble drugs, the polymer undergoes a relaxation process that results in slow direct erosion of the hydrated polymer in order to inhibit high initial burst effect.

In previous studies, it was found that when the polymer concentration in a physical mixture was above 85% w/w, the effect of particle size in release studies was negligible. With a low polymer concentration, drug release rate increased with increase in particle size (207). It was expected that addition of hydrophobic excipients such as Kollicoat would help to reduce the burst effect and improve sustained release of bLF from the particles. It was assumed that during the slow drying process of the films, some of the bLF moved to the surface and resulted in the initial burst effect. At the same time, addition of hydrophobic excipients decreased the interaction between β-glucan2 and bLF and resulted in release of almost all entrapped bLF from the film and the particle. With the addition of PEG2000, which was supposed to have formed a layer around the bLF, the initial burst effect was reduced to 30%. Milling produces major effects on the surface of the particles and might have altered the surface.
characteristics, resulting in reduced initial burst effect. One of the reasons for incomplete release of bLF might be binding to β-glucan2.

Figure 4.9 Average release of bLF from β-glucan2 bLF milled particles (△) or films (▲) with PEG 2000 particles (○), with PEG 2000 films (●), with Kollicoat particles (□) or with Kollicoat films (■). Release assessment media PBS (pH 7.4), Error bars are ± SEM, n=3.
4.4.7 Stability of bLF and β-glucan2 bLF milled particles

4.4.7.1 Stability of bLF

Protein aggregation is considered to be the most common protein instability encountered in protein drug development and there are various techniques in use to monitor aggregation in protein formulations, as explained in Section 1.2.7. IR spectroscopy and modulated DSC techniques were used to monitor aggregation after milling (208). bLF was milled alone and monitored for any aggregation. Both these techniques along with mass spectrometry were employed for long term stability evaluation.

Although glycoproteins such as bLF are generally considered more stable than other proteins (209), they are still prone to various instabilities.

Stability results are presented in Figure 4.10. Short term storage of samples at 4°C, 25°C and 37°C for 24 hours indicated that bLF was stable under experimental conditions and showed no degradation. The stability was evaluated as samples were stored at 4°C and the films were dried at 25°C and the temperature of the column was maintained at 37°C. Statistical analysis was carried out using one way ANOVA, which found no significant difference (p > 0.05)

![Figure 4.10](image.png)

**Figure 4.10** Average initial (white) stability of bLF at different temperatures (4, 25 and 37°C) and after 24 hours (dark). Error bars are ± SEM, n=3.
4.4.7.2 Long term stability of formulation

The β-glucan2 bLF milled particles stored at 25°C/60% RH did not show any change in colour and agglomeration over the entire storage period of 6 months. The off white colour was maintained throughout the storage period. Samples stored at 40°C/70% RH showed a change in colour to yellow in the second month of storage. The intensity of the yellow colour increased with storage time until month four. The β-glucan2 bLF particles stored at 40°C/70 RH also showed agglomeration (Figure 4.11).

The average molecular mass of the β-glucan2 bLF milled particle samples stored at different storage conditions is shown in Figure 4.12. There was no significant difference over six months of storage in average molecular mass of samples stored at 25°C/60% RH (p>0.05) and there was a significant decrease in average molecular mass in the samples stored at 40°C/70% RH (p<0.05). The studies showed that the transition temperature started at around 35-40°C and was greatly influenced by the presence of moisture. After drying the β-glucan2 powder, the transition temperature increased. β-glucan2 constitutes 90% w/w of the β-glucan2 bLF film and particles, and at high temperatures and high relative humidity it appeared to degrade because of the high mobility of the molecules. It was suggested in previous studies that thermal stability of different β-glucans depends on the degree of branching and the number of glucose side chains of β-glucan. However β-glucans with β-(1→3) and β-(1→4) linkages are unbranched and linear. It is highly probable that internal molecular mobility is responsible for the degradation in molecular weight.
Figure 4.11 Unmilled β-glucan2 (top left), fresh β-glucan2 bLF milled particles (top right), β-glucan2 bLF milled particles after storage for 6 months at 25°C/60% RH (bottom left) and 40°C/70% RH (bottom right).
Figure 4.12 Average β-glucan2 molecular weight over 6 months at 25°C/60% RH (open) and 40°C/75% RH (closed). Error bars are ± SEM, n=3.
4.4.7.3 Spectroscopic analysis of fresh unmilled and milled samples

IR spectroscopy is considered to be a suitable technique for surface characterisation as the IR beam transmitted through the crystal penetrates only a few µm or tenths of µm into the sample (210). During milling, most of the effect takes place on the surface of the particles, as explained in Section 4.4.3.1, so this technique is considered valuable in studying the surface of the particles. The other advantage of this technique is its simple sample preparation and non-intrusive measurement.

The FTIR spectra of unmilled and milled bLF are shown in Figure 4.13 and 4.14. The spectra showed the presence of strong bands in the 1500-1700 cm\(^{-1}\) region, which is the Amide I and Amide II band area and a conformationally sensitive region. The spectra of unmilled and milled bLF did not show any change in bands. There was a slight increase in the relative intensities in the region of 1600 cm\(^{-1}\).

FTIR spectra of unmilled and milled \(\beta\)-glucan2 are shown in Figure 4.15 and 4.16. The spectra showed strong bands at 1074 cm\(^{-1}\), corresponding to C-C and C-O stretch modes and represent the presence of glucopyranose. A strong band at 1156-1165 cm\(^{-1}\) represents the linear structure of \(\beta\)-D-glucan linked through (1\(\rightarrow\)3) linkage. A band at 896 cm\(^{-1}\) corresponds to \(\beta\)-linked glycosidic bonds (211). The bands at 1650 and 3415 cm\(^{-1}\) correspond to stretching and bending modes of the hydroxyl group of water. Generally carbohydrates are characterised by bands around 1040 cm\(^{-1}\) (C-O bond from alcohol group), 2940 cm\(^{-1}\) (C-H stretch) and 3400 cm\(^{-1}\) (O-H stretch). Small changes in the relative intensities around 1040 cm\(^{-1}\) were evident in Figure 4.16, but they are considered to be very minor changes and would not be due to any major changes in structure.

The spectra of unmilled and milled \(\beta\)-glucan2 did not show any change in peak shapes. However, when the spectrum of \(\beta\)-glucan2 bLF milled particles was compared with unmilled bLF and \(\beta\)-glucan2, it showed a shift towards higher frequency bands (Figure 4.17 and 4.18). The peaks at 1526 cm\(^{-1}\) and 1640 cm\(^{-1}\) were shifted to 1533 and 1643 cm\(^{-1}\) respectively. These shifts fall in the Amide I (1600-1700 cm\(^{-1}\)) and Amide II (1510-1580 cm\(^{-1}\)) band regions which are conformationally-sensitive regions in the protein. The Amide I band corresponds with C=O stretching vibration, weakly coupled with C-N stretching and in plane N-H bending (212). The shift of spectra towards higher wavenumbers after milling indicates interactions.
between bLF and β-glucan2. This interaction might have changed the secondary structure of bLF, which might have an effect on the biological activity of bLF.

Figure 4.13 IR spectra of unmilled bLF (blue) and milled bLF (red).

Figure 4.14 IR spectra (expanded region) of unmilled (red) and milled bLF (blue).
Figure 4.15 IR spectra of unmilled (blue) and milled β-glucan2 (red).

Figure 4.16 IR spectra (expanded region) of unmilled (blue) and milled β-glucan2 (red).
Figure 4.17 IR spectra of β-glucan2 bLF milled particles (red) and addition of 90% β-glucan2 and 10% bLF result (blue), the circled regions show changes observed in the spectra following cryomilling.
Figure 4.18 IR spectra (expanded) of \(\beta\)-glucan2 bLF milled particles (red) and addition result of 90% \(\beta\)-glucan2 and 10% bLF (blue).
Raman spectroscopy is a supplementary technique to IR spectroscopy in characterisation of proteins, both in solid and liquid form. Raman spectra of unmilled β-glucan2, unmilled bLF and β-glucan2 bLF particles are presented in Figure 4.19. There appear to be some minor changes in the region of 1100-1150 cm\(^{-1}\) and 1300-1350 cm\(^{-1}\) in the relative intensities. 1300-1340 cm\(^{-1}\) is the Amide III (N-H and C-H bend) band area region.

4.4.7.4 Raman spectroscopy

Raman and IR spectra showed minor blue shifts in the vibrational modes in the regions of the Amide I and Amide II bands. This was due to strain on these modes, probably induced by the milling. Small changes in the relative intensities were also observed around 1074 cm\(^{-1}\) in the C=O stretch mode of milled β-glucan2, however, these changes are considered minor and would not be due any major change in structure.

![Figure 4.19 Raman spectra of unmilled β-glucan2 (red), unmilled bLF (blue) and β-glucan2 bLF milled particles (green).](image)
4.4.7.5 DSC studies

Proteins have specific secondary and higher levels of structure that are maintained by weak non-covalent interactions. Various formulation stresses have the potential to disrupt these weak interactions. For a stable protein formulation, it is important to keep these interactions intact for the desired biological activity of proteins (213). MTDSC is a suitable technique to detect changes in transition temperature and enthalpy with higher sensitivity and resolution (214).

The DSC thermograms for unmilled bLF (as received), milled bLF, without vacuum drying are presented in Figure 4.20. The corresponding data of bLF is presented in Table 4.9. Thermograms of the above samples after vacuum drying at 30°C for 24 hours are presented in Figures 4.23 and Table 4.10. The thermograms in Figure 4.20 showed two endotherms. The first was a broad endotherm, which is characteristic for proteins and is due to the large internal heterogeneity of macromolecules or dehydration or enthalpy relaxation in the solid (215, 216). The second endotherm was observed at 221.7°C. The peak maximum reflects the apparent denaturation temperature or transition temperature ($T_m$). It has been reported that proteins also show a second weak endothermic peak that represents denaturation and decomposition. bLF has been reported to have two transitions (217). The $T_m$ of diferric human LF is reported as around 90°C (218). In this study the second $T_m$ was observed at 221.7°C.

bLF, either unmilled or milled for 4 min, did not show any major change in transition temperature or enthalpy change. There was no difference in the second transition peak and the difference in enthalpy of the first peak, which is about 4-5%, can be accounted for by the uncertainty of determination. This indicates that no change had taken place on milling the bLF alone using the cryomill.

The TGA traces presented in Figure 4.25 showed the presence of moisture in the range where the endothermic peak was observed by DSC. After vacuum drying, the transition temperature of unmilled bLF was shifted to 111°C (Table 4. 10) with a lower enthalpy. Elkordy et al (2008) reported that a decrease in the enthalpy change in the second scan might be due to denaturation of the protein, as partially unfolded proteins require less heat energy to denature completely (215). This indicates that the presence of water influences the transition temperature and this might have led to
partial unfolding of bLF. Milled bLF did not show any significant variation in transition temperature.

The endotherm with unmilled and milled β-glucan2 was recorded at a peak temperature of 91.2 and 99.4°C respectively with broadening of peak, and an exothermic peak at 314°C, which represents the decomposition of β-glucan2 (Figure 4.21, and Table 4.11). The peaks at 91.2 and 99.4°C appeared due to water loss. When β-glucan2 and β-glucan2 bLF milled particles were heated to a temperature below the decomposition temperature, then cooled and reheated, the endotherm disappeared (Figure 4.22). These results are well correlated with TGA results, in which about 10% loss of water was recorded in the same temperature range (Figure 4.26). The increase in endotherm peak temperature for milled particles is believed to be due to loss of water during drying of the films at 25°C overnight. Further vacuum oven drying of β-glucan2 bLF milled particles at 30°C for 24 hours resulted in a higher endotherm peak temperature due to further loss of water (Figure 4.24). This indicates that water present in the sample had an influence on the endotherm peak temperature. There was some water which was strongly bound to β-glucan2 molecules and still present after vacuum drying requires higher temperature to give endotherm peak (Table 4.11 and 4.12). The endotherm peak of the β-glucan2 bLF milled particles shows a lower peak temperature of 70.8°C and higher enthalpy of 297.1 J/g compared to the unmilled and milled β-glucan2. The intensity of the decomposition peak was lower, indicating that addition of bLF might have made β-glucan2 more stable. Sarmento et al (2006) reported that thermograms of insulin-loaded nanoparticles shifted to a lower melting transition when compared with unloaded nanoparticles due to interactions (219). Similar results were observed in our studies, indicating that there could be an interaction between bLF and β-glucan2. The increase in enthalpy indicated a highly ordered structure of the mixture. To further investigate interactions between bLF and β-glucan2, surface plasmon resonance studies were conducted. These are discussed in detail later in the chapter.
Table 4.9 Thermodynamic parameters for unmilled bLF and milled bLF derived from MTDSC measurements without vacuum drying.

<table>
<thead>
<tr>
<th>Samples</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>∆H&lt;sub&gt;m&lt;/sub&gt; (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmilled bLF</td>
<td>89.4 ± 0.7</td>
<td>221.7 ± 0.9</td>
</tr>
<tr>
<td>Milled bLF</td>
<td>91.2 ± 0.8</td>
<td>221.3 ± 1.0</td>
</tr>
</tbody>
</table>

Table 4.10 Thermodynamic parameters for bLF and milled bLF derived from MTDSC measurements with vacuum drying at 30°C for 24 hours.

<table>
<thead>
<tr>
<th>Samples</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>∆H&lt;sub&gt;m&lt;/sub&gt; (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmilled bLF</td>
<td>111.6 ± 1.7</td>
<td>218.4 ± 0.6</td>
</tr>
<tr>
<td>Milled bLF</td>
<td>93.6 ± 0.5</td>
<td>219.9 ± 1.0</td>
</tr>
</tbody>
</table>
Table 4.11 Thermodynamic parameters for unmilled β-glucan2, milled β-glucan2 and β-glucan2 bLF milled particles derived from MTDSC measurements without vacuum drying.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Endotherm peak temp (°C)</th>
<th>ΔH_m (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmilled β-glucan2</td>
<td>91.9 ± 0.8</td>
<td>273.7 ± 1.0</td>
</tr>
<tr>
<td>Milled β-glucan2</td>
<td>99.4 ± 0.5</td>
<td>277.2 ± 0.8</td>
</tr>
<tr>
<td>β-glucan2 bLF milled particles</td>
<td>70.8 ± 1.1</td>
<td>297.1 ± 1.3</td>
</tr>
</tbody>
</table>

Table 4.12 Thermodynamic parameters for unmilled β-glucan2, milled β-glucan2 and β-glucan2 bLF milled particles derived from MTDSC measurements vacuum drying at 30°C for 24 hours.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Endotherm peak temp (°C)</th>
<th>ΔH_m (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmilled β-glucan2</td>
<td>135.5 ± 1.2</td>
<td>241.6 ± 1.0</td>
</tr>
<tr>
<td>Milled β-glucan2</td>
<td>131.7 ± 0.9</td>
<td>233.6 ± 0.3</td>
</tr>
<tr>
<td>β-glucan2 bLF milled particles</td>
<td>134.7 ± 1.2</td>
<td>181.4 ± 1.1</td>
</tr>
</tbody>
</table>
**Figure 4.20** DSC thermograms of bLF unmilled (a, solid line) and milled (b, dash line).
Figure 4.21 DSC thermograms of (a) unmilled $\beta$-glucan2, (b) milled $\beta$-glucan2 and (c) $\beta$-glucan2 bLF milled particles.
Figure 4.22 DSC thermograms of unmilled β-glucan2 (top) and β-glucan2 bLF milled particles (bottom) heated to 150°C at 10°C/min, cooled at 5°C and re-heated to 300 and 350°C respectively.
Figure 4.23 DSC thermograms of (a) unmilled bLF and (b) milled bLF after vacuum drying at 30°C for 24 hours.

Figure 4.24 DSC thermograms of (a) unmilled β-glucan2, (b) milled β-glucan2 and (c) β-glucan2 bLF milled particles after vacuum drying at 30°C for 24 hours.
Figure 4.25 TGA traces of bLF unmilled (black) and milled (red) showing water loss with temperature increase.

Figure 4.26 TGA traces of unmilled β-glucan2 (green), milled β-glucan2 (red) and β-glucan2 bLF milled particles (black).
4.4.7.6 XRD studies

The XRD pattern presented in Figure 4.27 shows that β-glucan2 and bLF are both amorphous and β-glucan2 has some crystallinity (c) compared to bLF (a), which showed a broader pattern compared to β-glucan2. Mixing of bLF and β-glucan2 made the structure more amorphous because of the physical presence of bLF. There was no apparent difference in the pattern or shift in the peaks of unmilled bLF and milled bLF except the intensity. The pattern of β-glucan2 bLF milled particles, where most of the bLF was encapsulated, showed a similar pattern to the physical mixture. This indicates that there is no change in structure of bLF, β-glucan2 and The XRD pattern of unmilled and milled bLF is also presented separately to have a wider view (Figure 4.28)

![XRD patterns](image)

Figure 4.27 XRD patterns of: (a) unmilled bLF, (b) milled bLF, (c) unmilled β-glucan2, (d) β-glucan2 and bLF physical mixture without milling and (e) β-glucan2 bLF milled particles.
Figure 4.28 XRD pattern of bLF (a) unmilled and (b) milled.
4.4.7.7 MALDI TOF analysis of fresh samples and stability samples

Mass spectrometry results presented in Figure 4.29 show the molecular mass of fresh samples of unmilled bLF, milled bLF and bLF extracted from β-glucan2 bLF milled particles. The molecular mass of the storage samples after 4 months at 25°C/60% RH are presented in Figure 4.30. The molecular mass of fresh samples and stability samples stored at 25°C/60% RH were found to be within the 0.3-0.5% limit of instrumental error for this mass range. However, stability samples stored at 40°C/70% RH for 4 and 6 months showed higher molecular mass. At higher temperature and humidity β-glucan2 had degraded after storage as described in Section 4.4.7.2. The increase in mass of bLF extracted from β-glucan2 bLF milled particles after storage at higher temperature and humidity might be due to an association or interaction of a degraded part of β-glucan2 with bLF. The control sample without β-glucan2 showed molecular mass within instrumental error limits when stored at both the conditions for 4 and 6 months. The molecular mass of bLF from particles was found to have increased after 6 months of storage at 25°C/60% RH and after 4 and 6 months of storage at 40°C/70% RH. All the results of mass spectrometry are summarised in Table 4.12. There was no major difference found in the mass of bLF when stored at 25°C/60% RH for 4 months.

Table 4.12 Molecular mass of bLF and bLF extracted from β-glucan2 bLF milled particles stored at different storage conditions for 4 and 6 months using MALDI-TOF.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Storage time (months)</th>
<th>Molecular Mass (Da)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>bLF (UM)</td>
<td>bLF (M)</td>
<td>bLF from particles</td>
</tr>
<tr>
<td>Fresh</td>
<td>0</td>
<td>82,950</td>
<td>82,817</td>
<td>82,940</td>
</tr>
<tr>
<td>25°C/60% RH</td>
<td>4</td>
<td>82,732</td>
<td>82,564</td>
<td>82,684</td>
</tr>
<tr>
<td>40°C/70% RH</td>
<td>4</td>
<td>82,960</td>
<td>82,632</td>
<td>85,650</td>
</tr>
<tr>
<td>25°C/60% RH</td>
<td>6</td>
<td>82,750</td>
<td>82,389</td>
<td>85,649</td>
</tr>
<tr>
<td>40°C/70% RH</td>
<td>6</td>
<td>82,825</td>
<td>82,580</td>
<td>85,662</td>
</tr>
</tbody>
</table>
Figure 4.29 Mass spectra of fresh bLF samples: unmilled (A), milled (B) and extracted from β-glucan2 bLF milled particles (C).
Figure 4.30 Mass spectra of bLF samples after storage at 25°C/60 RH for 4 months: unmilled (a) milled (b) and extracted from β-glucan2 bLF milled particles (c)
4.4.7.8 FTIR studies of stability samples of bLF and β-glucan2 bLF milled particles

IR spectra of unmilled bLF stored at 25°C/60% RH and 40°C/70% RH are shown in Figure 4.31 (top). Unmilled bLF did not show any change in the overall spectra recorded after storage at 25°C/60% RH or 40°C/70% RH for 6 months. However, unmilled β-glucan2 showed changes in the 1000-1100 cm⁻¹ and 1200-1500 cm⁻¹ regions at both 25°C/60% RH and 40°C/70% RH for 6 months (Figure 4.31 bottom).

After storage for 6 months at 25°C/60% RH, β-glucan2 bLF milled particles showed significant changes in the entire spectra, especially in the 3000-3500 cm⁻¹ region. Changes were also observed at 1050 cm⁻¹ and around 1600-1700 cm⁻¹. The changes in the 1600 cm⁻¹ and 3400 cm⁻¹ regions were probably due to water absorption by β-glucan2 (Figure 4.32 top). Figure 4.32 shows the IR spectrum of β-glucan2 bLF milled particles recorded after storage at 40°C/70% RH for 6 months. Significant changes were observed across the entire spectrum in the regions of 1050 cm⁻¹, 1300-1500 cm⁻¹ and 3000-3500 cm⁻¹, indicating major changes in the structure. The major changes in the IR spectra can be correlated with the change in colour to yellow of β-glucan2 bLF milled particles after storage at 40°C/70% RH.
Figure 4.31 IR spectra of unmilled bLF after storage at 25°C/60% RH (blue) and 40°C/70% RH (red) [top] and unmilled β-glucan2) after storage at 25°C/60% RH (blue) and 40°C/70% RH (red) [bottom] for 6 months.
Figure 4.32 IR spectra of β-glucan2 bLF milled particles after storage at 25°C/60% RH (top) and unmilled β-glucan2 after storage at 40°C/70% RH (bottom) for 6 months.
4.4.7.9 Stability of bLF and bLF from particles by HPLC-UV and LC-MS/MS

HPLC fractions of bLF, bLF extracted at 40°C for 1 hour from the particles stored at RT for 6 months and bLF extracted from the same stored particles at RT for 3 hours were collected and analysed by LC-MS/MS. The glycopeptides formed by this technique would not be expected to be matched automatically by the database searching software (Mascot) due to poor fragmentation data, and this was found to be the case.

Data from bLF standard fraction digest were searched against all NCBIun protein sequences (11673899 as at 2 September 2010) and only bLF and trypsin matches were found, indicating that the HPLC fraction was pure. Peptides were matched to regions of the protein from residue 27-708 in all three samples, with an overall sequence coverage ranging from 51-62%. This strongly suggests that the bLF protein backbone was still intact after milling and storage. The data for all three collected fractions were searched against the in-house database containing the bLF protein sequence allowing for the expected modifications due to protein degradation. There was no evidence observed for the oxidation of methionine, histidine or tryptophan residues in any sample, except for one peptide match in the fraction from the RT extract. There were 3-5 deamidated peptide matches found in each sample including the standard, suggesting negligible differences between the three samples. The typically low number of modified peptide matches may well be formed artefactually after the HPLC step, as is commonly observed with this technique (Appendix 3).
4.4.7.10 **Circular dichroism (CD) studies**

The CD spectra of unmilled and milled bLF appeared similar but bLF from the β-glucan2 bLF milled particles had shifted to the left (Figure 4.33), indicating a change in the secondary structure of bLF after milling with β-glucan2. When milled alone, bLF maintained its intact secondary structure after milling. It was clear from previous results that a soluble part of β-glucan2 was present in the extract from β-glucan2 bLF milled particles, in a much higher concentration than bLF itself. The change in the secondary structure of bLF observed on the CD spectra could be entirely due to the presence of a soluble portion of β-glucan2, which might have masked or changed the conformation of bLF.

![Figure 4.33 CD spectra of fresh samples of unmilled bLF (solid line), milled bLF (broken line) and bLF extracted from β-glucan2 bLF milled particles (dash line)](image-url)
4.4.8 Interaction between β-glucan and bLF

In previous studies, it has been reported that bLF binds with a number of other molecules and that this process is central to its biological role (220, 221). Patel (2000) reported that when a dye, methylene blue, was incubated with β-glucan gel, only 33% of the dye absorbed could be recovered even after several days, indicating strong interactions between β-glucan and dye (222).

A surface plasmon resonance (SPR) biosensor was used to investigate and characterise the interactions between bLF and β-glucan2. SPR technology enables monitoring of specific biomolecular interactions between a ligand (bLF) and an analyte (β-glucan2), which is measured as an arbitrary response unit which is a measure of change in refractive index (223). The major advantage of SPR is real time direct determination of binding activity in crude, complex mixtures without the need to label any components. When a solution of β-glucan2 (1 mg/ml) was injected across immobilised bLF, the initial response was fast, followed by a steady interaction during injection (Figure 4.34). This indicates that there was probably something in the β-glucan2 that was interacting with bLF. After the injection it was expected that the curve would come back to baseline due to natural dissociation of the molecule, but it did not touch baseline, indicating that natural dissociation of β-glucan2 is slow. Following a number of regeneration steps when a solution of β-glucan2 (1 mg/ml) was passed over immobilised bLF for 2.5 min at 20 µl/min, a lower response was observed. The shape of the curve changed (Figure 4.33), which indicates damage or changes to the bLF conformation on the surface following regeneration attempts. An interaction was still observed and the proportion of β-glucan2 remaining bound and the rate of natural dissociation was similar to that observed in the first attempt (Figure 4.35). When a high concentration of β-glucan2 solution (10 mg/ml) was passed over immobilised bLF for 2.5 min at a flow rate 20 µl/min, a larger response was observed. Some natural dissociation was observed after binding. A similar effect was observed when a solution of β-glucan2 (10 mg/ml) was passed again over immobilised bLF, indicating that the response was reproducible.

It is apparent from this brief study that β-glucan2 strongly interacts with bLF, dissociation was very slow and many steps of regeneration with various regenerating agents were necessary for the baseline to be achieved. However, this regeneration process resulted in either denaturation of the surface of the ligand (bLF) or a conformational change that resulted in decreased binding of the analyte when passed
over the bLF surface. Denaturation was the most likely reason as indicated by further injections. The response was low after regeneration but the dissociation was still slow, which indicates that there might be strong interactions between the two entities.

To investigate further, SDS-PAGE (10%) was run to investigate which portion of β-glucan2 was interacting with bLF (Figure 4.36). The insoluble portion of β-glucan2 after treated with the lichenase enzyme showed a thick intensity band around the bLF band, whereas there was no other band except that of bLF in lane 8, in which the supernatant also contained soluble β-glucan2. Lane 12 also showed only bLF when a physical mixture of β-glucan2 and bLF in solution was loaded onto the gel. Supernatant treated with Lichenase enzyme shows some binding shown in lane 9. It is evident from this that either the non protein component of β-glucan2 or a protein component not observed on the gel was binding with bLF.
Figure 4.34 SPR sensorgram of β-glucan2 (1 mg/ml) interacting with bLF. Stage 1: baseline signal of HBS buffer followed by injection of β-glucan2; stage 2: steady state of interaction; stage 3: A high proportion of the molecule interacting with bLF remains bound following the end of the injection.

Figure 4.35 SPR sensorgram of β-glucan2 (1mg/ml) interacting with bLF after a number of regeneration steps.
**Figure 4.36** SDS-PAGE (10%) scan of samples. Lanes 1, 3 and 5: bLF with Lichenase enzyme; Lanes 2, 4 and 6: Lichenase enzyme; lane 7: bLF milled; Lane 8: supernatant after bLF extraction; Lane 9: supernatant after lichenase treatment; Lane 10: Lichenase treated insoluble pellet of β-glucan2 bLF particles; Lane 11: β-glucan2 only; Lane 12: bLF and β-glucan2 physical mixture. Std LF represents bLF.
4.5 Conclusion

A suitable HPLC method was developed for the quantification of bLF and analysis of short term bLF stability. Throughout the project, HPLC peak shape and retention time were monitored. HPLC fractions of unmilled bLF, milled bLF and bLF extract from the particles were collected and digested with trypsin enzyme and peptides sequence was matched from the data base to check the integrity of the peak eluting.

The formulation was prepared by casting films of β-glucan2 with bLF and particles were produced with an optimised cryomilling method. Formulations were characterised for morphology, particle size and the formulation with highest loading and lowest particle size was selected for further study. The release study profile showed that release of bLF into the media from the particle was very low. Hydrophilic (PEG2000) and hydrophobic (Kollicoat) excipients were added to alter the release profile. The Kollicoat film showed a burst effect but particles with Kollicoat had a reduced burst effect and a sustained release was observed. β-glucan2 with bLF particles showed release of only 30% of the loaded bLF.

Long term stability (6 months) of the β-glucan2 in the formulation was evaluated and was found to be stable at 25°C/65% RH but not at 40°C/75% RH. Spectroscopic analysis of the unmilled and milled bLF and unmilled and milled β-glucan2 showed no major changes in the spectra but β-glucan2 bLF milled particles did show some changes in the Amide I and II area. A change in Raman spectra can only be observed if the concentration of the material of interest is more than 1% in the sample. However, IR spectroscopy can detect changes even if the concentration is low. The lack of changes observed on Raman spectra suggests that the changes occurred on a very small proportion of the bLF, which may have been the bLF on the surface of the particles. DSC studies also suggested an interaction phenomenon after milling. bLF extracted from the β-glucan2 bLF milled particles had a similar molecular mass when extracted from both samples stored at 25°C/65% RH for 4 months and fresh samples. However, the molecular mass of bLF was higher when extracted from samples stored at 40°C/75% RH.

Interaction studies by SPR strongly suggest that there was strong binding between bLF and β-glucan2. Further SDS-PAGE suggested that binding was prominent with the insoluble portion of β-glucan2. The LC-MS/MS analysis of bLF extracted from the
β-glucan2 bLF milled particles indicated that the bLF was intact after storage for 6 months at RT.
CHAPTER 5
Evaluation of Formulation using Cell Culture and Animal Model
5.1 Introduction

Orally administered drugs are absorbed from the GIT across the intestinal mucosal membrane by many mechanisms, namely passive transcellular, passive paracellular, carrier mediated and receptor mediated mechanisms. It is essential to evaluate the absorption of oral formulations using in vitro and appropriate in vivo models. Before carrying out in vitro absorption and in vivo studies, it was envisaged to evaluate the osteogenic activity of bLF extracted from the β-glucan2 bLF milled particles in order to assess the biological activity of entrapped bLF. To perform its desired actions, bLF was expected to pass in therapeutic doses through the potential barrier of the GIT. To study the absorption of bLF and bLF from β-glucan2 bLF milled particles, a Caco-2 cell monolayer was used. In vivo studies were carried out to investigate the bioavailability of bLF and its effect on bone using a specific animal model, ovariectomised mice. The in vivo studies were conducted with the assistance of Dr Anne Blais, UMR-914 INRA-Agro Paristech PNCA, Paris, France.

The specific aims of this chapter are:

1. To evaluate in vitro osteoblast activity of the bLF extracted from the β-glucan2 bLF milled particles.

2. To evaluate the uptake of bLF and bLF from β-glucan2 bLF milled particles by Caco-2 cells.

3. To evaluate delivery efficiency of bLF and bone mineral density improvement in a mouse model.

5.2 Materials

A bovine LF ELISA kit with accessory starter kit (E101) was purchased from Bethyl Laboratories, Inc (USA). Caco-2 cell lines were obtained from American Type Culture Collection (ATCC, USA). Dulbecco’s modified Eagle medium (DMEM), fetal calf serum (FCS), non-essential amino acids, L-glutamine, trypsin with EDTA (0.25%), penicillin-streptomycin-glutamine, Hanks balanced salt solution (HBSS), HEPES (pH 7.4), PBS (pH 7.4) and MEM non-essential amino acid solution were purchased from Invitrogen Ltd (USA). All other chemicals used in the study were at least reagent
grade. Water used in the preparation of buffers was obtained by reverse osmosis (Milli Q unit, Millipore, USA).

5.3 Methods

5.3.1 Osteogenic activity of bLF

Biological activity of the bLF and bLF extracted from β-glucan2 bLF milled particles was investigated using an osteoblast proliferative assay method as described in Section 3.3.6.6. β-glucan2 bLF milled particles (20 mg) were dispersed in 20 ml MQ water and stirred slowly for 3 hours at room temperature, then centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was carefully taken off and freeze dried using a freeze dryer (Labconco Corporation, USA). After freeze drying, the samples were analysed using a validated HPLC method. It was important to assess the osteogenic activity of the encapsulating polymer since the soluble portion of β-glucan2 was extracted along with bLF. β-glucan2 milled particles were solubilised by heating at 80°C for 30 min and filtered through a 0.45 µm cartridge filter (Millipore, USA) to get the soluble portion and its osteogenic activity was determined. The data were analysed using ANOVA with post hoc Dunnett’s tests. A 5% significance level was used throughout. Data are presented as ± SEM, n=6.

5.3.2 Reproducibility of ELISA method

Validation of the ELISA assay was undertaken for its reproducibility over a range of known bLF concentrations and compared with a Bethly kit LF standard curve.

A stock solution of bLF (1 mg/ml) was prepared in PBS (pH 7.4). From this stock solution, samples were diluted with sample diluent (included in the kit) in the detection range 7.8-500 ng/ml. A sample solution was also prepared from this stock with diluent to give a concentration of 100 ng/ml. A 96 well plate was also provided with the kit and was used for the assay. The assay was carried out as per the manufacturer’s protocol (224). In brief, the process was as follows:

1. A coating plate was prepared using capture antibody (1:100) in coating buffer and 100 µl was added into each well and incubated for 60 min at room temperature.
2. The antibody was tipped off after 60 min and the wells were washed with 300 µl wash buffer three times.

3. Post coat solution (200 µl) was added to each well and incubated for 30 min.

4. The plate was washed 3 times with washing buffer.

5. The standards of bLF (calibrator) were prepared within the detection range of the kit (7.8-500 ng/ml). The samples were diluted to a final concentration with sample diluent. Standards (100 µl) were added into each well in duplicate and sample (100 µl) was added into each well in 7 replicates. The plate was incubated for 60 min at room temperature.

6. The plate was washed five times with 300 µl wash buffer.

7. Goat anti-Bovine LF-HRP conjugate detection antibody was diluted (1:100,000) in sample diluents and 100 µl was added into each well and incubated for 60 min.

8. HRP detection antibody was tipped off and the plate was washed with 300 µl wash buffer.

9. Equal parts of substrate reagents A and B (TMB peroxide) were mixed and 100 µl was added to each well and incubated for 15 min. The plate was covered with tinfoil during the incubation.

10. The reaction was stopped by adding 100 µl of 2 M H₂SO₄ and absorbance was measured at 450 nm using a multi-detection microplate reader, BioTek® Instruments, Inc. (USA) within 20 min of addition of acid. Data was analysed using Gen 5™ software (USA).

5.3.3 Evaluation of uptake of bLF from β-glucan2 bLF milled particles using Caco-2 cell monolayer

Caco-2 cells are extensively employed as an in vitro model for drug absorption and uptake studies as they have features similar to the absorptive intestinal cells, such as microvilli, carrier mediated transport systems and paracellular transport through tight junctions (225).
Caco-2 cell lines were established as follows. The Caco-2 cell medium was prepared by adding 50 ml FBS, 5 ml Penicillin-streptomycin-glutamine and 5 ml 1% non-essential amino acids to 440 ml DMEM buffer. The medium was filtered through 0.22 µm filter paper. This medium was stored at 4°C. The freezing vial containing Caco-2 cells from the liquid nitrogen cylinder was collected and the lower part of the vial was immersed in a water bath maintained at 37°C. The cell suspension was transferred into 15 ml sterile centrifuge tubes and 5 ml of DMEM medium was added drop by drop with slight agitation. The cell suspension was centrifuged at 1500 rpm for 10 min, then the supernatant was discarded to remove DMSO and the cells were resuspended in 15 ml DMEM. These cells were transferred to a 75 cm² flask and incubated at 37°C, 5% CO₂, 95% air, in a saturated humidity incubator. The medium was changed in the first 48 hours and then every 2-3 days. The cells were further sub-cultured when confluence reached 80-90%. The cells were washed with 5 ml PBS at 37°C, then PBS was discarded and 2.5 ml 0.25% trypsin-EDTA solution was added to detach the cells from the surface of the flask. The flask was kept in the incubator for 5 min and then cells were observed under a converse microscope for change in shape to round. Trypsin solution was discarded, then DMEM medium was added and the cell suspension was split into further fresh flasks with DMEM medium and incubated at 37°C.

For uptake studies, cells were washed with PBS (pH 7.4) and trypsinised. Viable cell counting was carried out by mixing tryphan blue (1:1) with the cell suspension and counting using a haemocytometer. The number of viable cells was determined with the aid of a light microscope at 100x magnification. Only live cells (unstained) were counted in the four large corner squares of the haemocytometer and the mean of four counts was taken for calculation. Viable cell numbers were calculated by multiplying the total mean number of live cells counted x 10,000 x dilution factor. The cells were cultured in sterile tissue culture dishes (60 x 15 mm) (Becton Dickinson, France). The cell density of counted viable cells was adjusted to 5 x 10⁵ and seeded in 5 ml DMEM medium in each of the petri dishes. The culture dishes were incubated in an incubator at 37°C. Cells were cultured for 7-8 days until they reached 80-90% confluence.

All the petri dishes were observed under the microscope for confluency to the same level. Just before the uptake study, the medium was removed and the cells were washed twice with 5 ml HBSS with HEPES (pH 7.4). HBSS was discarded and a further 2 ml HBSS was added. The cells were then incubated in an incubator for 10
min. bLF solution was prepared in HBSS with concentrations of 25, 50 and 100 µg/ml. A stock solution (100 µg/ml) was prepared and diluted to 50 and 25 µg/ml with HBSS.

In the same way, a dispersion of particles was prepared of the same concentration calculated on the basis of theoretical loading of 10%. 15, 7.5 and 3.5 mg of particles were suspended in 15 ml each of HBSS, to get 100, 50 and 25 µg/ml respectively. The particle dispersion was prepared immediately before incubating the particles with Caco-2 cell lines.

To each culture dish, 2 ml of bLF solution was added in duplicate and incubated for a predetermined time (15, 30, 45, 60, 90, 120, 900 and 2700 sec and 8 and 24 hours). For particles, the incubation times were 15, 30, 45, 60 and 2700 sec and 8 and 24 hours. Uptake was stopped by aspirating the media off. The culture dishes were quickly rinsed five times with 5 ml ice cold HBSS (pH 7.4). The last washing was collected for monitoring the possible residual bLF. Cell lysis buffer (400 µl) was added to each culture dish to break down the cell membrane. The composition of cell lysis buffer is listed in Table 5.1. Cells were scraped off with a cell scraper (BD Biosciences Labware, USA) and transferred into Eppendorf tubes with lysis buffer and stored at -20°C until analysis. After all the time points were finished, all the samples were thawed and centrifuged at 14000 rpm for 15 min at 4°C. Supernatant was taken off gently and analysed using an ELISA kit, as described in Section 5.3.1. The pellet was dissolved in 1N NaOH solution (400 µl) and analysed for total protein using a Pierce® BCA protein assay kit (Thermo Scientific, USA).

Table 5.1 Composition of cell lysis buffer.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantities (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS (1 M, pH 7.4)</td>
<td>0.5</td>
</tr>
<tr>
<td>EDTA (0.5 M, pH 8.0)</td>
<td>0.2</td>
</tr>
<tr>
<td>NaCl (5 M)</td>
<td>1.5</td>
</tr>
<tr>
<td>Brij (10% w/v)</td>
<td>4.38</td>
</tr>
<tr>
<td>Tween 20 (10% v/v)</td>
<td>0.625</td>
</tr>
<tr>
<td>Distilled Water to</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>
5.3.4 *In vivo evaluation of the formulation*

Among the several potential biological functions of LF discussed in Section 1.2.1.3, its role in osteoporosis is considered to be of immense importance. Osteoporosis affects the older population and postmenopausal women in particular, in terms of lifestyle and life expectancy. Osteoporosis is characterised by an increased risk of fracture, disruption of bone micro-architecture and reduced bone mineral density (BMD). Bone loss originates from an imbalance between bone formation and bone resorption, especially after menopause, which induces increase of bone turnover by excess osteoclast activity. It has been reported by a number of research groups that LF from different sources has an anabolic effect on bone, it stimulates mitogenesis, and is responsible for differentiation and survival of osteoblasts. LF not only stimulates proliferation and survival of osteoblasts but also inhibits osteoclastogenesis (226).

To evaluate the effect of the β-glucan2 bLF milled particles on bones, a small animal model of postmenopausal osteoporosis was used. This model is considered to be a validated model to represent clinical features of the postmenopausal condition induced by estrogen deficiency in adult humans. The model is called the ovariectomised (Ovx) mouse model. C3H ovariectomised (Ovx) or sham-operated (Sham) mice were used as previously described by Blais *et al.* (2009) (227). In brief, 70 female C3H/HeN strain mice were housed in a temperature controlled room (22 ± 1°C) and fed a standard pellet diet prior to the study. The diet composition is summarised in Table 5.2. Fifty 12-week old female C3H mice were ovariectomised and twenty were sham operated (Sham). The surgery was performed under anaesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg). Morphine (2.5 mg/kg) was given to the animal to avoid any pain. Just after surgery, five groups of ten Ovx mice and two groups of ten sham mice were formed and fed for 16 weeks with either the control diet including 140 g of total protein/kg of diet (Ovx C) or with a diet in which bLF (5 and 10 g/kg of the diet) and the β-glucan2 bLF milled particles encapsulating bLF (1 and 10 g of the β-glucan2 bLF milled particles encapsulating 100 mg and 1 g of bLF respectively) was mixed thoroughly with the diet and the total milk protein content was adjusted as shown in Table 5.2. At week 9 after surgery, the blood of each mouse was collected from the orbital sinus to evaluate immunoreactive bLF concentration. At week 17 after the surgery the animals were euthanised and whole blood was collected by cardiac puncture. The body composition was determined and the skeleton and muscles were weighed. BMD of the entire body,
lumbar spine and right femoral bone of each animal was measured under anaesthesia at 1, 5, 9, 13 and 17 weeks after ovariectomy. Radiographic dual-energy X ray absorptiometry analysis was performed using a Lunar Piximus densitometer (GE Medical System, software version 1.4x Lunar) to determine the femoral BMD. Immunoreactive bLF concentrations in mouse plasma were determined by ELISA as previously described (228). The ELISA method has less than 0.1% cross-reactivity with murine LF, which was not considered significant (229). The immunoreactive bLF was measured after 2 and 4 months of feeding a diet supplemented with bLF or β-glucan2 bLF milled particles. The further method of analysis was followed as described by Blais et al (2009) (227). Statistical difference between the groups was evaluated by Student’s t-test and p values <0.05 were considered statistically significant.

Table 5.2 Composition of diet fed to mice with bLF and β-glucan2 bLF milled particles.

<table>
<thead>
<tr>
<th>Ingredients, g/kg diet</th>
<th>Control</th>
<th>Ovx 5</th>
<th>Ovx 10</th>
<th>Ovx F 1</th>
<th>Ovx F 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cow's milk protein</td>
<td>140</td>
<td>135</td>
<td>130</td>
<td>139</td>
<td>130</td>
</tr>
<tr>
<td>bLF</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>1*</td>
<td>10*</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>622.4</td>
<td>622.4</td>
<td>622.4</td>
<td>622.4</td>
<td>622.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.30</td>
<td>100.30</td>
<td>100.30</td>
<td>100.30</td>
<td>100.30</td>
</tr>
<tr>
<td>Soyabean oil</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Alpha-cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>AIN 93 mineral mixture</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>AIN 93 M vitamin mixture</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

1* and 10* represent the 1 and 10 g particles with theoretical loading of 100 mg and 1 g of bLF respectively. Ovx 5 and Ovx 10 represent 5 g and 10 g of bLF which were not encapsulated.
5.4 Results and discussion

5.4.1 ELISA standard curve

ELISA is the most commonly used method for detection and quantitative determination of LF. However, LF exists from a wide variety of different sources, which may hamper the characterisation and determination of exact LF concentrations in a sample. Therefore it is important that the LF sample has same binding determinants as the LF of the kit. The antibodies in the kit used bind specifically to bLF. The standard curve was generated using four logistic (4-PL) curve fit. The curves generated using bLF from the kit and bLF from Fonterra (Figure 5.1) were found to be of good fit, which indicates that both bLF samples had the same determinants. The average concentration of the sample (100 ng/ml) was found to be 98.42 ng/ml with ± SD of 7.2 (n=7). The kit was used to analyse the bLF uptake by Caco-2 cells.

![Figure 5.1 Standard curve of bLF from the kit (▲) and bLF from Fonterra (♦).](image-url)
5.4.2 Investigation of biological activity of formulation

It was observed that there was a strong interaction between β-glucan2 and bLF in the investigations discussed in Chapter 4. The biological activity of β-glucan2 bLF milled particles was investigated and compared with unmilled and milled bLF. Similarly, the biological activity of the encapsulating polymer, β-glucan2, was evaluated under similar conditions. Unmilled and milled bLF showed significant osteogenic activity at 10 and 100 µg/ml compared to control, indicating that milling did not denature the bLF when milled alone (Figure 5.2). bLF extracted from the β-glucan2 bLF milled particles showed significant activity at 100 µg/ml (p<0.0001) and did not show any significant activity at 1 and 10 µg/ml (p=0.4170). However, activity of extracted bLF compared to standard bLF and milled bLF (Figure 5.2) had reduced significantly. This might be due to interference of soluble β-glucan2, which was present along with extracted bLF, or due to a conformational change in the bLF resulting from the interaction between β-glucan2 and bLF. In the investigation, it was observed that IR spectra and transition temperature in the DSC studies had changed after the milling of β-glucan2 and bLF. The interactions were found to be strong between β-glucan2 and bLF, which might have had an effect on the biological activity.

β-glucan2 at 100 µg/ml showed significant osteogenic activity compared to control, whereas it did not show any significant effect at 1 and 10 µg/ml (Figure 5.3 top). bLF at 10 and 100 µg/ml showed significant proliferation of osteoblasts as shown in Figure 5.2 and Figure 5.3 bottom.

With both β-glucan2 and bLF having osteoblast proliferation activity at 100 µg/ml, a synergistic effect was expected when the two were given together.
Figure 5.2 Osteoblast mitogenic assay of bLF: From left to right: control; bLF unmilled; bLF milled for 4 min using cryomill; bLF extracted from β-glucan2 milled particles for 3 hours and freeze dried. Error bars represent ± SEM, n=6, *p<0.05.
Figure 5.3 Osteoblast mitogenic assay of β-glucan2 at different concentrations (top) and bLF at different concentrations (bottom). From left to right (top): control; β-glucan2 at 1, 10 and 100 µg/ml; control; β-glucan2 at 0.01, 0.1 and 1 µg/ml. Bottom: control; bLF std; bLF extract at 10 µg/ml; control; bLF std and bLF extract at 100 µg/ml. Error bars represent ± SEM, n=6, *p<0.05.
5.4.3 Uptake studies evaluation

Caco-2 cells spontaneously differentiate and form tight junctions. This property is also a characteristic of the cells lining the intestine. The uptake of bLF at different concentrations is shown in Figure 5.4. The uptake of bLF appeared to increase linearly at all tested concentrations (25, 50 and 100 µg/ml) and there was a significant increase in the uptake of bLF at 50 and 100 µg/ml compared to 25 µg/ml. Uptake of bLF from the β-glucan2 bLF milled particles also appeared to be dose dependent and there was a significant increase in the bLF uptake at 50 and 100 µg/ml compared to 25 µg/ml (Figure 5.5). Interestingly, the uptake of bLF at 100 µg/ml was found to be lower than at 50 µg/ml.

At 25 µg/ml, uptake of bLF from the particles significantly increased after incubation for 24 hours with Caco-2 cells (Figure 5.6), but this trend was reversed for the 50 and 100 µg/ml concentration. The uptake increased significantly until 8 hours on incubation at 50 and 100 µg/ml, then gradually reduced.

LF absorption from intestinal cells and other organs takes place through receptor mediated endocytosis or transcytosis (168, 230). Fisher et al (2007) reported that the mechanism of transport of LF from the intestinal cells could be two fold, with a degradative pathway leading to transcellular transport and a minor pathway of the passage of LF by transcytosis through M cells present on Peyer's patches. (229). It has been reported that LF is not absorbed into the circulation, but produces its biological function through a network of mucosal immunity and systemic immunity (231). Brock et al (1992) reported that LF binds to intestinal mucosal cells and while LF itself did not get across the cell monolayer, some LF fragments did cross through (232). However, this argument was not supported by a number of researchers who found immunoreactive LF in the circulation and various organs in mice (227, 229). At 50 and 100 µg/ml concentrations of bLF from β-glucan2 bLF milled particles, uptake or binding increased until 8 hours followed by a decline in uptake when incubated for 24 hours (Figure 5.6). This might be because of inhibition of an internalisation process due to the presence of excess bLF at higher concentration as reported by Ashida et al (2004) (233). However, this phenomenon was not observed with 25 µg/ml. At 900 seconds, equilibrium was reached with 50 and 100 µg/ml and no further increase in uptake was observed when bLF was incubated with Caco-2 cells.
Figure 5.4 Average bLF uptake by Caco-2 cells at different concentrations: 25 µg/ml (▲), 50 µg/ml (♦), 100 µg/ml (■). Error bars are the range between samples, n=2.

Figure 5.5 Average bLF uptake from β-glucan2 bLF milled particles by Caco-2 cells at different concentrations: 25 µg/ml (▲), 50 µg/ml (♦), 100 µg/ml (■). Error bars are the range between samples, n=2.
5.4.4 In vivo studies of β-glucan2 bLF milled particles

The BMD and bLF concentration in mouse plasma following oral administration are summarised in Figure 5.7 and 5.8 respectively. The Ovx procedure was done on 12-week old mice when the BMD of the mice was still increasing. The procedure reduced BMD gain (227) and consequently after 2 months the average total BMD of Sham mice was significantly higher than that of Ovx mice. After 17 weeks all the bLF supplemented diets were able to restore total BMD of Ovx mice to the level of Sham mice. When fed with a diet supplemented with β-glucan2 bLF milled particles (Ovx F1 and Ovx F 10), Ovx mice showed a significant improvement in BMD compared to control (Ovx C) (p< 0.05). At week 17 in the Ovx F 1 fed group, which was the group receiving the lowest concentration of bLF (100 mg of entrapped bLF), BMD was significantly higher compared to the control group (Ovx C), but a further increase in the quantity of β-glucan2 bLF milled particles did not increase the BMD compared to Ovx F 1. The higher dose of bLF did not further increase BMD, probably because of immune exclusion as reported in earlier studies (229).
The immunoreactive bLF concentration (Figure 5.8) in individual mouse peripheral blood was found to increase significantly after ingestion of the bLF diet for 17 weeks. The immunoreactive bLF concentration in the plasma is dependent not only on the bLF concentration in the diet but also on the bLF formulation. The immunoreactive bLF concentration measured in mice receiving the β-glucan2 bLF milled particles (Ovx F10) was significantly higher compared with mice receiving Ovx5 and Ovx10. Ovx F1 and Ovx F10 contained only 100 mg and 1 g of bLF respectively loaded into the β-glucan2 bLF milled particles. The higher bLF absorption from the mouse GIT could be related to bLF being encapsulated and protected by the β-glucan2 against proteolytic digestion, leaving more bLF accessible for absorption through LF receptors in the mouse intestine. This suggests that encapsulated bLF is more bioavailable than the unencapsulated bLF mixed in the diet. β-glucans from cereals are partially digested in the GIT by microflora, so it is hypothesised that bLF that was bound by β-glucan would be released and available for absorption at the site of absorption. The increase in concentration of bLF in peripheral blood can be correlated with the increase in BMD at week 17.

![Figure 5.7](image-url)  
**Figure 5.7** Average BMD gain of mice fed with diet supplemented with different concentrations of bLF. The Ovx or sham mice were fed for 4 months with either control diet for sham and Ovx control or a diet including Sham 10 (Sham 10), 5 (Ovx 5) and 10g/kg (Ovx 10) bLF. F1 (Ovx F1) and F10 (Ovx F10) are the formulations containing 1 g and 10 g of particles, encapsulating 100 mg and 1 g of bLF respectively. Error bars are ± SEM, n=10.
Figure 5.8 Average immunoreactive bLF in the plasma of mice fed with diet supplemented with different concentration of bLF. The Ovx or sham mice were fed for 4 months with either control diet for Sham and Ovx control (Ovx C) or a diet including Sham 10 (Sham 10), 5 (Ovx 5) and 10g/kg (Ovx 10) bLF. F1 (Ovx F1) and F10 (Ovx F10) are the formulations containing 1 g and 10 g of particles, encapsulating 100 mg and 1 g of bLF respectively. Error bars are ± SEM, n=10.

5.5 Conclusion

The *in vitro* results presented suggest that bLF shows linear uptake at all concentrations. At higher concentrations, bLF showed an increase in uptake until 8 hours, then reduced uptake on further incubation with Caco-2 cells. However, the uptake increased after 8 hours at 25 µg/ml. This can be correlated to *in vivo* results where mice fed with diet supplemented with bLF or β-glucan2 bLF milled particles had a significant increase in plasma concentrations of immunoreactive bLF after 2 months but there was no significant increase after 4 months of feeding mice with the same diet. This suggests there was saturation of LF receptors and that equilibration was reached, after which there is no more uptake of bLF.

The toxicity studies of β-glucan2 and bLF were not carried out as bLF and β-glucan2 are from natural sources and are ingested as a part of a normal diet. In previous toxicity studies of β-glucan2, rats were fed β-glucan2 for 28 days and no toxicity was found (234).

After four months of feeding mice a diet supplemented with 1 g/kg or 10 g/kg of β-glucan2 bLF milled particles, the plasma concentration of immunoreactive bLF was significantly higher compared to controls. The total BMD of the body also increased.
significantly after feeding the Ovx mice with a diet supplemented with β-glucan2 bLF milled particles. It was observed that β-glucan2 at 100 µg/ml also had significant osteoblast proliferation activity, it was not used as a control in in vivo studies. The effect on BMD might be the synergistic effect of bLF supplemented diet and β-glucan2. However, higher concentrations of immunoreactive bLF in plasma strongly suggest that the bioavailability of bLF increased when diet supplemented with β-glucan2 bLF milled particles was fed to the mice.
CHAPTER 6
General Discussion and Future Directions
Effective delivery of protein therapeutics has been a major focus of research recently, because of the availability of more recombinant proteins with therapeutic applications. The success of these therapeutic applications largely depends upon efficient drug delivery systems. Most of the protein products are delivered by parenteral routes whilst oral delivery is still the most favoured delivery system. The physiochemical properties of most proteins, including molecular size, hydrophilicity and susceptibility to enzymatic degradation, pose a challenge for their effective absorption. It is imperative that each protein and peptide be treated in its own way for successful delivery to achieve a specific pharmacological and therapeutic requirement. Among the various DDS formulation strategies, particulate delivery systems such as polymeric microparticles have been investigated extensively. While a wide range of polymeric systems have been studied, the use of natural biodegradable polymers to deliver proteins continues to be an area of active research.

This study investigated the feasibility of the use of a naturally occurring biopolymer, β-glucan, as an encapsulating polymer carrier for bLF and production of microparticles with a novel technique called cryomilling. The selected formulation was evaluated for stability and in vitro and in vivo studies were carried out to assess its uptake, osteogenic activity on bone cells, and improvement in bioavailability using a small animal model.

Bovine LF, a whey protein, possesses multifunctional biological activities, including antimicrobial, immunomodulatory, anti-inflammatory and anti-carcinogenic activity. It has been recently reported to have potent osteoblast anabolic activity. It is a strong cationic protein and susceptible to hydrolytic degradation when ingested orally. β-glucan is a polysaccharide extracted from barley by a non-organic solvent method employing only water, heat and enzymes naturally present in the barley grain. It forms thermo-reversible translucent gels depending upon the molecular weight and source. Though bLF has a high molecular weight (80 kDa), it is absorbed from intestinal epithelial cells by two pathways, specific receptor mediated transcytosis and non-specific transcytosis. It is then shifted to the lymphatic system and transferred to the thoracic duct lymph, then finally enters the systemic circulation (235). It has been reported that bLF is less immunogenic when taken orally.

In Chapter 2, two grades of β-glucan, named β-glucan1 and β-glucan2, were characterised for their physicochemical properties, including molecular weight, rheological behaviour, mechanical strength and density. β-glucan2 was selected for.
the formulation because of its high molecular mass which could form a robust matrix for protein encapsulation, and is less dispersed compared to β-glucan1. Preformulation studies were undertaken to select the concentration of β-glucan1 and 2 in solution to entrap bLF. After investigating the rheological and mechanical properties of β-glucan, the concentration of 2% w/w was selected because β-glucan does not form a gel at this concentration at room temperature or 37°C. The stress studies were undertaken on β-glucan2 to investigate its behaviour. The results suggest that no degradation takes place under normal gastric pH and temperature. β-glucan from barley (in general) is only partially digested by microflora in the GIT.

Chapter 3 reports a method of conversion of bLF to the bioactive fraction bLFcin by controlled hydrolysis using pepsin. The other objective of this study was to purify a low molecular weight peptide from the N-terminal and investigate its activity on bone. It was expected that lower molecular weight fractions would be more bioavailable than those of high molecular weight (192). There were several reports of purification of the peptide (17-41) and its antibacterial activity. The activity of the synthetic peptides with a lower molecular weight than bLFcin from the N-terminal of bLF have been reported in one study.

Various techniques were employed to purify bLFcin. Adsorptive membrane chromatography technique was selected to purify bLFcin from bLF hydrolysate as this technique was easy to use, short processing time and higher purity. The osteoblast activity assay of the purified peptide (17-41) at 0.1 µg/ml was not significant compared to the control and only showed activity at 1 µg/ml. At higher concentrations (10 µg/ml), bLF showed a much higher magnitude of response compared to purified bLFcin. It was reported that the C lobe has higher osteogenic activity than the N lobe and the full length bLF molecule has higher activity (192). Therefore bLF was selected for the final formulation.

In Chapter 4 a two step process was developed using film casting followed by milling to produce particles of β-glucan2 containing bLF. Formulation studies and development of analytical methods were carried out to further characterise the β-glucan bLF milled particles. A reliable HPLC quantification method for bLF was developed and validated. The validation work showed that the method was accurate, precise and presented a good linearity over 6.25-100 µg/ml of bLF.
To produce microparticles, films were cast by selecting low (1% w/w) and high (10% w/w) concentrations of bLF in β-glucan2 for loading. The films were milled using a cryomill containing liquid nitrogen and different parameters of milling were optimised to achieve the lowest particle size with highest loading. The formulation with the lowest particle size and highest loading was selected. Hydrophobic (Kollidac), hydrophilic (PEG2000) and CAP excipients were added to the formulation (2% w/w of β-glucan) to evaluate their effect on entrapment and release of bLF from films and particles. The results presented showed that films with Kollidac showed higher entrapment efficiency than films with PEG2000. It was expected that hydrophobic excipients would reduce burst effect but, on the contrary, they showed high burst effect. The addition of PEG2000 reduced burst release of bLF, but very little bLF was released overall when PEG2000 was added. Many methods were tried to extract bLF from particles but the recovery was found to be low. An enzymatic hydrolysis was carried out with lichenase enzyme to recover maximum bLF from particles. The results presented show that approximately 57% of bLF could be recovered.

Further studies were undertaken to investigate the interaction between β-glucan2 and bLF by using surface plasmon resonance. Strong binding was observed between bLF and β-glucan2 (1 and 10 mg/ml concentration). An SDS-PAGE of samples treated with lichenase enzyme was run. The result indicated that the binding of bLF was mainly with the insoluble part of β-glucan2. The insoluble part of the β-glucan2 is mainly made up of long blocks of cellobiosyl residues which have β-(1-4) linkages. When extracted at higher temperatures (65°C), β-glucans have a higher content of β-(1-4) linkages and a tendency to aggregate through strong hydrogen bonding along the cellulose-like regions (108, 236). Therefore cellobiosyl residues might have interacted with bLF as discussed in section 4.4.8.

Therapeutic proteins undergo various stresses during formulation development, so thorough characterisation was required after each step. Characterisation was carried out using various techniques such as IR spectroscopy, Raman spectroscopy, gel electrophoresis, DSC, LC-MS/MS and mass spectrometry. The stability of bLF and the formulation was assessed by all these techniques and the results suggest that when the bLF and β-glucan2 were each milled alone, no significant difference was observed in IR spectra and DSC. However when bLF and β-glucan2 were milled together, the IR spectra shifted to a higher wave number and Tm, endotherm peak (β-glucans2) and enthalpy changes were evident. bLF extracted from the particles showed similar molecular weight to standard bLF on SDS-PAGE gel. To assess the
integrity of the bLF protein backbone after milling, bLF was extracted from the β-glucan2 bLF milled particles for 3 hours, centrifuged and injected into HPLC to collect the fraction associated with the peak. The fraction associated with the peak from standard bLF was also collected and peptide identification was done using LC-MS/MS and compared with the database. The results presented in Chapter 4 strongly suggest that the protein was intact. The change in IR spectra and DSC profile of milled particles suggests loss of activity of bLF, which was evaluated further by an in vitro osteoblast proliferation assay. The osteoblast mitogenic assay indicated loss of significant osteogenic activity after milling compared to standard bLF. The possibility of degradation of bLF at the surface of the particle might be one of the reasons for the change in IR spectra. However changes in the IR spectra were not significant to destroy the structure of bLF. The low osteogenic activity could be due to interference of soluble portion of β-glucan2 which was present along with bLF. DSC studies pointed towards interactions that might have resulted in loss of bLF activity.

The stability studies of the β-glucan2 bLF milled particles when stored at different storage conditions (25°C/60% RH and 40°C/75% RH) were carried out over six months. In this time, bLF stability was assessed in 4 and 6 months and β-glucan2 stability was assessed monthly. The molecular weight of the β-glucan2 was analysed after storage at 25°C/60% RH and no significant change was observed. However, there was a significant change in molecular weight observed after storage at 40°C/70% RH. The IR spectra of the unmilled bLF control stability sample did not show any changes after 6 months of storage at 25°C/70% RH and 40°C/70% RH. However unmilled β-glucan2 showed changes after storage at 25°C/70% RH and 40°C/70% RH. The β-glucan2 bLF milled particles showed significant changes in the entire spectra after storage at 25°C/70% RH for 6 months. The major changes in the spectrum of β-glucan2 bLF milled particles were observed after storage at higher temperature and humidity conditions for 6 months. These changes were probably due to water absorption by β-glucan2. The bLF extract showed changes in molecular mass after storage at 40°C/70% RH for 4 and 6 months. Control samples of unmilled and milled bLF did not show any changes at both the storage conditions after 3 and 6 months. The results suggest that the stability of both β-glucan2 and bLF was found to be influenced by moisture and temperature. This can be correlated to with the change in colour of β-glucan2 bLF milled particles stored at 40°C/70% RH.

Chapter 5 reports the in vitro and in vivo activity of bLF delivered by the β-glucan bLF milled particles.
The \textit{in vitro} uptake of bLF in solutions and bLF from the particles was investigated using Caco-2 cell monolayers and the data presented indicate that at all tested concentrations (25, 50 and 100 µg/ml), the cellular uptake showed dose-dependent increase. Significant increase in the uptake of bLF at 50 and 100 µg/ml compared to 25 µg/ml. The uptake of bLF from the particles was also found to be dose dependent. bLF from the particles at 25 µg/ml showed a trend of continuously increasing uptake when incubated for 24 hours. However, at concentrations of 50 or 100 µg/ml, the uptake saturated at 8 hours of incubation followed by a drop in uptake. This phenomenon might be due to the presence of excess bLF at the surface of Caco-2 monolayers as observed by Ashida \textit{et al.} (2004) (233). The uptake of particles by Caco-2 cells could be studied further by confocal microscopy to ascertain their exact location in the Caco-2 cells.

The \textit{in vivo} results showed an enhanced delivery of bLF into the plasma when the ovariectomised mice were fed with a diet supplemented with $\beta$-glucan2 bLF milled particles compared with a diet supplemented with only bLF. The increase in bLF concentration in plasma could be linked to an increase in BMD. There was no significant improvement in bioavailability and BMD after certain time periods even after feeding the mice for longer durations. These \textit{in vivo} findings support the results that were observed in Caco-2 cell studies and also reported by many research groups, as discussed in Section 5.4.4 of Chapter 5.

The results presented in this thesis suggest that cryomilling did not have any harmful impact on the structure and activity of the protein when milled alone, but changes were observed in the structure when it was milled with $\beta$-glucan2, a natural polysaccharide. This might have led to loss of some of the biological activity. The \textit{in vivo} results presented show the usefulness of this technique and suggest that the change in the structure or loss of activity might only be with bLF that was present on the surface. Therefore cryomilling can be a useful technique for entrapment of therapeutic proteins and can be explored for particle production. The results demonstrated that $\beta$-glucan2 has the potential to protect proteins and can be explored further for oral delivery of therapeutic proteins.

This study was carried out to investigate the feasibility and potential of a natural biopolymer for use in a protein delivery system. During the project, the following limitations were encountered. The \textit{in vitro} release of bLF from the films and particles did not achieve complete release. Other excipients or physicochemical properties of
the β-glucan2 could be modified to achieve an optimum release profile. The *in vitro* biological activity of the recovered bLF showed that activity had reduced significantly. Formulation parameters need to be further optimised to reduce the degradation of bLF, especially at the surface of the particles. Although bLF has shown to be more bioavailable from the β-glucan2 bLF milled particles, further studies are required to investigations the role of β-glucan2 on BMD. bLF is a strong cationic protein and has shown strong interactions with β-glucan2. Our studies suggest that an insoluble portion of β-glucan2 interacts with bLF and this needs to be further investigated in future studies.
Appendix 1

The overlaid chromatogram of stress samples for β-glucan2: β-glucan2 (green), β-glucan2 treated with 0.2 M HCl (dark blue), β-glucan2 treated with 2 M HCl (pink), β-glucan2 treated with 6 M HCl (red), β-glucan2 treated with 0.1 M HCl at 120°C (light blue).
# Appendix 2

## Amino acid three letter and one letter codes

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Three letter code</th>
<th>One letter code</th>
</tr>
</thead>
<tbody>
<tr>
<td>alanine</td>
<td>ala</td>
<td>A</td>
</tr>
<tr>
<td>arginine</td>
<td>arg</td>
<td>R</td>
</tr>
<tr>
<td>asparagine</td>
<td>asn</td>
<td>N</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>asp</td>
<td>D</td>
</tr>
<tr>
<td>asparagine or aspartic acid</td>
<td>asx</td>
<td>B</td>
</tr>
<tr>
<td>cysteine</td>
<td>cys</td>
<td>C</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>glu</td>
<td>E</td>
</tr>
<tr>
<td>glutamine</td>
<td>gln</td>
<td>Q</td>
</tr>
<tr>
<td>glutamine or glutamic acid</td>
<td>glx</td>
<td>Z</td>
</tr>
<tr>
<td>glycine</td>
<td>gly</td>
<td>G</td>
</tr>
<tr>
<td>histidine</td>
<td>his</td>
<td>H</td>
</tr>
<tr>
<td>isoleucine</td>
<td>ile</td>
<td>I</td>
</tr>
<tr>
<td>leucine</td>
<td>leu</td>
<td>L</td>
</tr>
<tr>
<td>lysine</td>
<td>lys</td>
<td>K</td>
</tr>
<tr>
<td>methionine</td>
<td>met</td>
<td>M</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>phe</td>
<td>F</td>
</tr>
<tr>
<td>proline</td>
<td>pro</td>
<td>P</td>
</tr>
<tr>
<td>serine</td>
<td>ser</td>
<td>S</td>
</tr>
<tr>
<td>threonine</td>
<td>thr</td>
<td>T</td>
</tr>
<tr>
<td>trpophan</td>
<td>try</td>
<td>W</td>
</tr>
<tr>
<td>tyrosine</td>
<td>tyr</td>
<td>Y</td>
</tr>
<tr>
<td>valine</td>
<td>val</td>
<td>V</td>
</tr>
</tbody>
</table>
Appendix 3

Peptide identification of tryptic digest using LC-MS/MS of bLF extracted from β-glucan2 bLF milled particles. Total sequence coverage: 51%

Matched peptides shown in **Bold Red**

1 MKLFVPALLS LGALGLCLAA PRKNVR\textit{WCTI SQPEWFKCRR WQWRMKK} KLGA
51 PSITCVRRA ALECIR AIAE K\textit{KADAVTLDG GMVFEAGRDP YKLRPVA} AE!
101 YGTKEPQTH YYAVAVVKKG SNFQLDQLQG RK\textit{SCHTGLGR SAGWI} PMGI
151 LRPYLSWTES LEPLQGAVA\textit{K} K\textit{FASCVPCI DRQAYPNLCQ LCKGE} GENQC
201 ACSSREPYFG YSGAFKCLQD GAGDVAFVKE TTVENLPEK ADRDQYELLC
251 LNNSRAPVDA FKECHLAQVP SHA\textit{VARSVD GKE} DLWKL SKAQEKF\textit{G} KKN
301 KSR\textit{SFQLFGS PPGQ} RDLFLK DS\textit{ALGFLRIP SKVDSAL} YLG SRYLTTLKLN
351 RETAEEVKAR YTRVWCA\textit{V} PEEQ\textit{KK}CQQW SQ\textit{QSG}NVTC A\textit{TAS}TTDDCI
401 VLVLKGE\textit{AD}A LNLDGG\textit{YI}YT AGKCGLV\textit{P} VL AEN\textit{RKSSKH S} LDC\textit{VL}RPT E
451 GYLAVAVKK ANE\textit{GLT} WNSL KD\textit{K}CSCHTAV D\textit{RTAGW} NIM \textit{GLIVNQ} TGSC
501 AFDEFFSQSC A\textit{PGADPKSRL CALCAGD} DQG LD\textit{KCV} NSK E\textit{KYGYTGAFR}
551 CLA\textit{EDVG} DA F\textit{V}K\textit{N} DT\textit{V} WE N\textit{T} GE\textit{SADW}A K\textit{N} N\textit{REDFR} L \textit{CLDGT} RKP V
601 TEAQ\textit{SCHLAV AP} NHAV\textit{V} SRS \textit{D}RA\textit{AHVK} VL L\textit{HHQAL} F\textit{GK} N\textit{KNCP} DKFCL
651 FK\textit{SETKL} LF \textit{D}N\textit{TECLAKL GGRPYEEYL GTEY} VAT\textit{A} A\textit{N} LKC\textit{STSP} LL
701 E\textit{ACAFLTR}
References


55. Ahsan F, Arnold JJ, Yang T, Meezan E, Schwiebert EM, Pillion DJ. Effects of the permeability enhancers, tetradecylmaltoside and dimethyl-β-cyclodextrin, on


175. Miyazawa K, Mantel C, Lu L, Morrison DC, Broxmeyer HE. Lactoferrin-lipopolysaccharide interactions: effect on lactoferrin binding to...


