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**DEVELOPMENT AND EVALUATION OF A
β-GLUCAN BIOPOLYMER FORMULATION
OF LACTOFERRIN PRODUCED USING A
NOVEL CRYOMILLING TECHNIQUE**

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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.

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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

2.6	Stress studies of β-glucan.....	41
2.6.1	Methods	41
2.6.1.1	Sample preparation.....	41
2.6.1.2	Molecular weight measurement	42
2.7	Results and Discussion.....	42
2.7.1	Visual and microscopic examination of β-glucan.....	42
2.7.2	Rheological properties of β-glucan1 and β-glucan2	44
2.7.2.1	Effect of pH on different concentrations of β-glucan1 and 2	44
2.7.2.2	Effect of temperature on viscosity of different concentrations of β-glucan1 and 2	49
2.7.3	Mechanical characterisation of β-glucan1 and 2	56
2.7.4	Molecular weight determination of β-glucan1 and 2	58
2.7.4.1	Density measurement of β-glucan1 and 2	60
2.7.4.2	Particle size determination of β-glucan1 and 2.....	60
2.7.5	Stress studies of β-glucan2.....	62
2.8	Conclusion	65
CHAPTER 3	Lactoferrin Characterisation, Derivitisation and Purification of Lactoferricin	66
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

3.5.3.1	Ultrafiltration.....	95
3.5.3.2	Derivitisation of bLF and process optimisation	96
3.5.3.3	Gel filtration and preparatory ion exchange chromatography	99
3.5.3.4	Fast protein liquid chromatography	101
3.5.3.5	Ion exchange using microporous membrane.....	102
3.5.3.6	Characterisation of purified peptide.....	104
3.5.3.7	Biological activity of bLF and bLFCin	106
3.6	Conclusion	108
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
4.3.1.1	Preparation of standard solution	112
4.3.1.2	Linearity	112
4.3.1.3	Method validation.....	113
4.3.1.4	Specificity.....	113
4.3.1.5	Sensitivity.....	113
4.3.1.6	Repeatability	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	<i>In vitro</i> 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	<i>In vitro</i> 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	<i>In vivo</i> 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	<i>In vivo</i> 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 \rightarrow 3) and (1 \rightarrow 4) linkages and Cellotriosyl and Cellotetraosyl residues are shown as G4G4G or G4G4G4G. B: Linear unbranched β -(1 \rightarrow 3) and (1 \rightarrow 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 μ m) and back lit β -glucan2 (bottom, bar=100 μ m).....	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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 bar are \pm 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 \pm 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 bar are \pm 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 \pm 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 \pm 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 \pm SEM, n=100.....	61
Figure 2.24	Representative chromatograms of 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 \pm 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 μ g); lane 3, bLF (10 μ g); lane 4, bLF (15 μ g); lane 5, b LF (20 μ g); lane 6, bLF (25 μ g).	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, lane1, 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, lane1, 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, lane1, low molecular mass markers; lane 2, fraction 1 with 2 M NH ₄ 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 \pm 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 \pm 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 μ g/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 \pm SEM, n=3.....	137
Figure 4.9	Average release of bLF from β -glucan2 bLF milled particles (\triangle) or films (\blacktriangle) with PEG 2000 particles (\circ), with PEG 2000 films (\bullet), with Kollicoat particles (\square) or with Kollicoat films (\blacksquare). Release assessment media PBS (pH 7.4), Error bars are \pm 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 \pm 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 \pm 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 (\blacktriangle) and bLF from Fonterra (\blacklozenge).....	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 \pm 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 μ g/ml; control; β -glucan2 at 0.01,0.1 and 1 μ g/ml. Bottom: control; bLF std; bLF extract at 10 μ g/ml; control; bLF std and bLF extract at 100 μ g/ml. Error bars represent \pm SEM, n=6, *p<0.05.	193
Figure 5.4	Average bLF uptake by Caco-2 cells at different concentrations: 25 μ g/ml (\blacktriangle), 50 μ g/ml (\blacklozenge), 100 μ g/ml (\blacksquare). 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 μ g/ml (\blacktriangle), 50 μ g/ml (\blacklozenge), 100 μ g/ml (\blacksquare). 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 μ g/ml (\blacktriangle), 50 μ g/ml (\blacklozenge), 100 μ g/ml (\blacksquare). 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 \pm 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 \pm SEM, n=10.....	198

List of Tables

Table 1.1	Recent industrial technologies for oral protein/peptide delivery. Adapted from (79) with permission.....	13
Table 1.2	Common mechanisms and causes of protein instability. Adapted from (29) with permission.	16
Table 2.1	Biologically active glucans and their sources. Modified from (113) with permission.	34
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.	50
Table 2.3	Area and force maxima of β -glucan1 and β -glucan2 gels at different concentrations.	56
Table 2.4	Average molecular weight and polydispersity index of β -glucan1 and β -glucan2.	59
Table 2.5	Average molecular weight of β -glucan2 at given stress conditions.....	62
Table 3.1	Typical composition of bovine milk and whey from (140) with permission.	68
Table 3.2	Composition and physiological functions of major milk and whey proteins from (137) with permission.	70
Table 3.3	Protein profile of whey. Adapted from (151) with permission.	73
Table 3.4	Composition of separating gel (10 ml) and stacking gel (5 ml).	82
Table 3.5	Composition of resolving gel, spacer and stacking gel for Tricine- SDS-PAGE.	86
Table 3.6	Peptides identified and amino acid sequencing in tryptic digests of bLF by LC-MS/MS	94
Table 4.1	HPLC gradient conditions for bLF elution.....	112
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 β - glucan2 milled particles.	118
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.	127
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.....	139
Table 4.5	Average (n=3) entrapment efficiency of different β -glucan2 bLF films or β -glucan2 bLF milled particles.	140
Table 4.6	Average (n=3) bLF recovery from β -glucan2 bLF film or β -glucan2 bLF milled particle.....	141
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).....	143
Table 4.8	Average recovery (n=3) of bLF from different combinations of carbohydrates and bLF.....	144
Table 4.9	Thermodynamic parameters for unmilled bLF and milled bLF derived from MTDSC measurements without vacuum drying.....	159

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

ACE	Angiotensin 1 converting enzyme
AA2P	L-ascorbic acid-2-phosphate (AA2P)
ANOVA	Analysis of variance
AR	Analytical reagent
ATR	Attenuated total reflection
AUC	Area under the curve
APS	Ammonium persulphate
AB	Acrylamide bisacrylamide
ATCC	American type cell culture
BSA	Bovine serum albumin
Beta Glucan	β -glucan
bLF	Bovine Lactoferrin
bLFCin	Bovine Lactoferricin
BMD	Bone mineral density
CAGR	Cumulative annual growth rate
CPPs	Cell penetrating peptides
Caco-2	Colon carcinoma cancer cells
CE	Capillary electrophoresis
CD	Circular dichroism
C*	Critical concentration
CAP	Cellulose acetate pthalate
DDS	Drug delivery systems
DNA	Deoxyribose nucleic acid
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
DEE	Drug encapsulation efficiency
DMSO	Dimethyl sulphoxide
dn/dc	Differential refractive index
Da	Dalton
DMEM	Dulbecco's modified Eagle medium
ED	Electrodialysis
EDTA	Ethylene diamine tetra acetic acid
ESI	Electro spray ionisation
ELISA	Enzyme linked immune sorbent assay
FPLC	Fast protein liquid chromatography
FBS	Fetal bovine serum
FCS	Fetal calf serum

FTIR	Fourier transform infrared spectroscopy
GIT	Gastrointestinal tract
GALT	Gut associated lymphoid system
GPC	Gel permeation chromatography
GRAS	Generally regarded as safe
HEPES	(4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid)
HBSS	Hank's balanced salt solution
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
IAA	Iodo acetic acid
IAM	Iodoacetamide
ICH	International Conference on Harmonization
IR	Infrared spectroscopy
J/g	Joules/gram
kDa	Kilo Dalton
LF	Lactoferrin
LFcin	Lactoferricin
LOQ	Limit of quantification
LOD	Limit of detection
MF	Microfiltration
MALDI-TOF	Matrix assisted laser desorption ionisation- time of flight
MTDSC	Modulated temperature differential scanning calorimetry
ME	Microemulsion
MS	Mass spectroscopy
Mw	Molecular weight
MQ	Milli Q
MEM	Minimum essential medium
M/Z	Mass/charge
mV	Milli volt
NF	Nanofiltration
NK	Natural killer cells
O/W	Oil in water
Ovx	Ovariectomised mice
PLA	Polylactic acid
PLGA	Polylactic glycolic acid
PBS	Phosphate buffer saline
PACA	Polyalkylycyanoacrylate
PTH	Parathyroid hormone
PVA	Polyvinyl alcohol

PEG2000	Polyethylene glycol 2000
PMMA	Polymethacrylate
RI	Refractive index
RH	Relative humidity
RSD	Relative standard deviation
Rpm	Revolutions per minute
RT	Room temperature
Sham	Sham operated mice
SEM	Standard error of the mean
SEM	Scanning electron microscope
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC-MALLS	Size exclusion chromatography connected to multi angular laser light scattering
SD	Standard deviation
TEMED	Tetramethyl ethylenediamine
Tm	Melting transition/ melting tempertaure
TFA	Trifluro acetic acid
TGA	Thermo gravimetric analysis
TMB	Tetramethyl benzidine
Tricine	N-(Tri(hydroxymethyl)methyl)glycine
UK	United Kingdom
USFDA	United States Food and Drug Administration
UV	Ultra violet
UF	Ultrafiltration
W/O	Water in oil
WPI	Whey protein isolate
WPC	Whey protein concentrate
XRD	X- ray diffraction
η_0	Zero shear viscosity