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

Hemant Kumar, Craig R Bunt, Christina Buchanan, John Shaw, Sanjay Garg, Jingyuan Wen. Development of a validated RP-HPLC method for the determination of lactoferrin in β-glucan microparticles formulation. Journal of Pharmaceutical and Biomedical Analysis. Submitted.

Refereed Full Conference Papers

Kumar, H., Bunt, C.R., Chen, E., Shaw, J., and Wen, J., "Formulation and characterisation of poly (lactic acid) nanoparticles for oral delivery of lactoferrin", *Proceedings of the International Symposium on Controlled Release of Bioactive Materials*, 37 (2010), Portland, USA.

Kumar, H., Wen., J, Shaw., J, Yang., M, and Bunt, C., "In-vitro release of lactoferrin from β-glucan films and micro particles", *Proceedings of the International Symposium on Controlled Release of Bioactive Materials,* 36 (2009), Copenhagen, Denmark.

Kumar, H., Wen J., Garg S., Shaw J., and Bunt C., "Determination of molecular mass of a biopolymer, Glucagel using Size exclusion chromatography with multi angle laser scattering technique and Stress studies", *Proceedings of the International Symposium on Controlled Release of Bioactive Materials*, 35 (2008), New York, USA.

Refereed Conference Abstracts

Kumar, H., Wen J., Shaw J., Cornish J and Bunt C., "Stability assessment of a novel particulate formulation of lactoferrin" *Indian Pharmaceutical Congress Association*, (2010), Manipal, India, (Submitted).

Kumar, **H**., Wen, J., Shaw, J., Cornish, J., Nieuwoudt, M., and Bunt, C., "Investigation of structure and thermal stability of microparticles of β-glucan encapsulating Lactoferrin by fourier transform infrared spectroscopy and differential scanning calorimetry" *Australasian Pharmaceutical Sciences Association*, (2009), Hobart, Australia.

Kumar, H., Wen, J, Garg, S, Shaw, J, Dillimore, C, and Bunt, C, "Production of micro particles of β-glucan containing lactoferrin by freezer mill, influence of experimental parameters on particle size distribution and formulation stability", *Australasian Pharmaceutical Sciences Association*, (2008), Canberra, Australia.

Kumar, H., Wen., J, Shi., D, Li., Q, Garg., S, Shaw., J. and Bunt, C., "Oral formulation of lactoferricin, a bioactive peptide from lactoferrin, a whey protein and its in-vitro release studies", *Australasian Pharmaceutical Sciences Association*, (2008), Canberra, Australia.

Kumar, H., Chand, S., Wen, J., Shaw, J., and Bunt, C., **"**Purification of lactoferricin, a bioactive peptide from lactoferrin using micro porous ion exchangers", *Australasian Pharmaceutical Sciences Association*, (2007), Sydney, Australia.

Kumar, H., Garg., S, and Bunt. C., "Rheological properties of β-glucan, a novel biodegradable polymer" *Australasian Pharmaceutical Sciences Association*, (2006), Adelaide, Australia.

Conference Abstracts

Wen, J. Y., **Kumar, H**., and Bunt, C., "Oral delivery of a whey protein, lactoferrin by particular delivery systems for treatment of osteoporosis", *12th Conference on Formulation and Delivery of Bioactives*, (2010), Dunedin, New Zealand

Kumar, H., Garg, S., Wen, J., Bunt, C., "Development of gel permeation chromatography (GPC) method for molecular mass characterization of β-glucan; a novel biodegradable polymer" *NZBIO* Conference (2007), Auckland.

Poster

Kumar, H., Wen, J., Shaw, J., and Bunt, C., "Development and validation of an HPLC method for lactoferrin analysis", Heathex 2009, University of Auckland.

Patents

Kumar, H., Wen, J., and Bunt, C., “Novel formulations, methods of making same and uses therefore” NZ578480, (2009).

Contents

List of Figures

List of Tables

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