Structural Aspects of β-Lactoglobulin during Self-assembly into Amyloid-like Fibrils

A thesis presented in partial fulfilment of the

requirements for the degree of

Doctor of Philosophy

in

Food Technology

at
Riddet Institute, Massey University,
Palmerston North
New Zealand.

Anant Chandrakant Dave

2014

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Riddet Institute
FOOD | INNOVATION | HEALTH

Abstract

This study explores the structural characteristics of *β*-lactoglobulin (*β*-Lg) during its self-assembly into long amyloid-like fibrils on heating at low pH and low ionic strength. *β*-Lg (1%, w/v) was heated at 80°C, pH 2 and low ionic strength and the different processes occurring during self-assembly were characterized using a variety of techniques including circular dichroism spectroscopy, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and mass spectrometry.

The results of this study indicate that fibril formation from *β*-Lg self-assembly consists of four processes: 1) protein unfolding; 2) heat- and acid- induced protein hydrolysis 3) nuclei formation and; 4) growth of nuclei into mature fibrils by peptide self-assembly. It was found that the heat-induced unfolding of *β*-Lg and its acid hydrolysis promoted self-assembly by removing structural constraints and generating assembly-capable peptides. The peptides from the N-terminal region (1-53) of *β*-Lg were found to play an important role during nucleation and may form the core of the fibrils. The characterization of fibril composition strongly indicated the presence of disulfide bonding in fibrils and the native disulfide bond Cys66-Cys160 in *β*-Lg appeared be conserved during fibril formation.

The substitution of amino acid residues in *β*-Lg variants A, B and C did not significantly affect the kinetics of different self-assembly processes. The fibrils from *β*-Lg A, B and C had similar morphology, but were slightly different in their peptide compositions. The latter may be explained on the basis of sites of genetic substitutions, in particular, the Asp64 of *β*-Lg A that is Gly in variants B and C. In comparison, glucosylation and lactosylation of *β*-Lg strongly inhibited fibril formation primarily by inhibition of peptide self-assembly due to the steric conformational

restrictions. The inhibitory effect of glycation varied with the type of sugar and the degree of glycation. Lactosylation produced a stronger effect than glucosylation, but glycation with either sugar did not appear to have any effect on the morphology of fibrils.

The modification of the aqueous phase composition by glycerol and sorbitol (0-50 % w/v) greatly decreased the rate of *β*-Lg self-assembly and the effect of glycerol and sorbitol on *β*-Lg self-assembly was concentration-dependent. Sorbitol inhibited the self-assembly by stabilizing *β*-Lg against unfolding and acid-hydrolysis, resulting in fewer fibril-forming peptides, whereas glycerol inhibited peptide self-assembly without affecting unfolding and acid-hydrolysis. Although, both polyols increased the viscosity of the solutions, viscosity did not affect the self-assembly of peptides, indicating that, under these conditions, the self-assembly was not diffusion-limited.

The effects of *β*-casein on *β*-Lg self-assembly were investigated by heating *β*-Lg-*β*-casein mixtures (molar ratios 1:0.0625 to 1:1). Heating under fibril-forming conditions resulted in acid hydrolysis of both proteins at approximately equal rate. *β*-Casein produced a small but consistent effect in inhibiting *β*-Lg self-assembly in heated *β*-Lg-*β*-casein mixtures. The transmission electron microscopy images of solutions showed irregular, coiled and ribbon-like structures co-existing with the *β*-Lg fibrils. These aggregates were absent in respective heated control samples of either protein indicating that *β*-Lg assembly-competent peptides have alternate competing pathway during self-assembly. The limited effect of *β*-casein on *β*-Lg self-assembly may be explained by the aggregation of *β*-casein peptides by a separate alternate pathway which competed with their interaction with *β*-Lg peptides.

Overall, the findings of this study have advanced our understanding of the mechanisms of self-assembly of globular proteins and provided insights into the ways to decouple self-assembly processes. This may help to design protocols for the control of globular protein self-assembly and extend the functionality and applications of protein fibrils.

Acknowledgements

The journey of my Ph.D. studies has been extremely special for me since pursuing research was a long-held dream that I shared with many people. This work has come to be from the contribution of time, resources and expertise of a number of people who have placed their faith in me and allowed me to pursue my dream. I express my deepest sense of gratitude to everyone who has contributed to the successful completion of this work.

It is with great pleasure that I express my immense gratitude to Distinguished Professor Harjinder Singh for supervising my research. His constant guidance and encouragement have been a huge source of motivation and inspired me to work harder. I appreciate the opportunity to study at the Riddet Institute, and the freedom to explore my own ideas.

I thank Dr. Simon Loveday, my co-supervisor, for introducing me to research, in helping me take my first steps and inspiring me all through this journey. His patience, especially during my writing phase has been a learning that I will be carrying with me wherever I go.

I also wish to extend my deepest and sincerest thanks to my co-supervisor, Dr. Skelte Anema (Fonterra Research and Development Centre, Palmerston North). His critical evaluation, advice and suggestions for my work during the planning, execution and writing phases of my research have been instrumental in the success of my research. His continued support and encouragement have been one of the main driving forces for maintaining the standard of my work.

Acknowledgements

I thank Professor Geoffrey Jameson, Institute of Fundamental Sciences (IFS), Massey University for his useful advice and guidance for protein unfolding studies. His valuable critical suggestions for my studies and assistance in interpreting and summarising the findings of the studies in the publications have been invaluable. I acknowledge his earnest interest in my wellbeing and in helping me to evolve as a researcher. I also thank Dr. Gillian Norris (IFS) for her valuable guidance in the peptide sequencing studies. Working at her laboratory has personally helped me immensely and added a completely different dimension to my work. I am grateful to Mr. Trevor Loo for the stimulating discussions on proteomics research and his useful advice regarding the protein unfolding and mass spectrometry studies. Working with him has helped me immensely in developing and sharpening my general laboratory skills.

I also wish to acknowledge the following specific contributions from:

- Nutrition laboratory Institute of Food, Nutrition and Human Health, Massey University for proximate analysis of the protein used in the study.
- Mr. Mallesh Peram for introducing and familiarizing me with the electrophoresis techniques.
- Ms. Leiza Turnbull for lyophilisation of all my samples.
- Dr. Simon Loveday for writing the SigmaPlot software codes for curve fiting.
- Dr. Skelte Anema for providing the purified genetic variants of the protein *β*-lactoglobulin.
- Dr. Geoff Jones (IFS) for his valuable guidance with statistical analysis.
- Mr. Doug Hopcroft and Ms. Jordan Taylor for their assistance with transmission electron microscopy of samples.

- Mr. Trevor Loo (IFS) and Ms. Diana Carne (Centre for Protein Research, University of Otago) for running the samples on the mass spectrometer and analysis of the mass spectrometry data.
- Mr. Manu Singh for his skillful assistance in standardization of protocols for circular dichroism spectroscopy and mass spectrometry.
- The referees of my peer-reviewed publications for providing critical feedback and appreciating my work in the submitted manuscripts.
- Dr. Gareth Rowlands (Institute of Fundamental Sciences, Massey University, Palmersto North, New Zealand) and the staff of The Graduate Research School, Massey University, Palmerston North, New Zealand, for facilitating my thesis examination process.
- Prof. Edward Allen Foegeding (North Carolina State University, USA), Dr. Nigel Larsen (Plant & Food Research, Lincoln, New Zealand) and Dr. Alistair Carr (Institute of Food, Nutrition and Human Health, Massey University, Palmersto North, New Zealand) for reviewing the work in this thesis and being my PhD thesis examiners.

I wish to acknowledge the financial support provided by the Riddet Institute, in the form of Riddet Institute Scholarship and travel grant for attending international conference in Australia. I also thank Fonterra Cooperative Ltd. and the New Zealand Foundation for Research Science and Technology (contract number DRIX0701) for funding my research.

I am grateful to Distinguished Professor Paul Moughan for his concern for my wellbeing and constant encouragement throughout my research. I gratefully acknowledge the contribution of the Riddet Institute administration and information

technology team who have worked tirelessly to facilitate my research. A big thank you to Ms. Ansley Te Hiwi, Ms. Terri Palmer, Ms. Felicia Stibbards, Ms. Paula McCool, Mr. John Henley-King and Mr. Matt Levin. I also wish to thank Ms. Willi Stevenson-Wright and Dr. Shantanu Das, who were with the Riddet Institute for a major duration of this work, for their constant encouragement and motivation.

I thank the Riddet Institute researchers Dr. Shane Rutherford, Dr. Jaspreet Singh, Dr. Carlos Montaya, Dr. Aiqien Ye, Dr. Ashling Ellis, Dr. Lovedeep Kaur, Dr Sharon Henare and Dr. Xiang-Qian Zhu for promoting a stimulating atmosphere that has greatly facilitated the flow of ideas during my research work. The 'epicentre' of the experimental work in this research was the Riddet Institute laboratory. I wish to extend my heartfelt thanks to Ms. Janiene Gilliland and Mr. Chris Hall for maintaining the 'epicentre'. I acknowledge the constant support from the Riddet Institute staff including, Mr. Russell Richardson, Ms. Chanapha Sawatdeenaruenat, Ms. Namrata Taneja, Mr. Amit Taneja and Mr Jian Cui. Their support and encouragement helped me to maintain a positive attitude when the experiments did not work as planned.

I am equally thankful to all my past and present lab-mates with whom I have spent several hours here. My heartfelt thanks to Mr. Mallesh Peram, Mr. Qing Guo, Mr. Vikas Mittal, Ms. Lakshmi Dave, Ms. Natascha Stroebinger, Mr. Shakti Singh and Mr. Devastotra Poddar for creating a conducive, friendly atmosphere that made working in the lab all the more enjoyable.

I acknowledge the emotional support of close friends who have contributed to this work by facilitating my stay in Palmerston North. Thanks to all my friends including Mr. Sebastian Riedle, Mr. Rajesh Deshpande, Mr. Pankaj Sharma, Ms. Nancy Taneja, Ms. Jinita Das, Ms. Wibha Desai and the members of the Indian Gujarati commmunity

in Palmerston North, for making my stay in Palmerston North comfortable and for being there when the pangs of going back home struck! I wish to acknowledge the constant support and encouragement from Dr. Anwesha Sarkar (University of Leeds, Leeds, UK). Over the the last decade she has been a source of constant motivation and supported me like an elder sister. I thank her for all the valuable advice she has offered and, in particular, for inspiring me to embark on this journey.

This endeavour could only be successful because of the unconditional love and constant support from my family. I acknowledge the endless support from my father Dr. Chandrakant J. Dave, my mother Mrs. Hemlata Dave, my sister Ms. Priyanka Dave and my parents-in-law Dr. A.V.R.L Narasimhacharya and Mrs. Vani Acharya who have shared my dream, stood like strong pillars provinding a solid emotional foundation and inspired me to complete my research. I thank them for their blessings and am extremely grateful for their sacrifices that they have made in order to allow me to complete this work.

Finally, I wish to acknowledge the contribution of my best friend, who has stood besides me unconditionally for the last 10 years. A special friend who has been extremely patient, understading and caring throughout this journey as I realized this dream. A friend who has celebrated all the successes and milestones of this project with me, and supported me firmly when I found this journey challenging. A big thank you to my wife Lakshmi Dave for being there always, from taking care of home while I was busy with experiments or thesis writing to being the first to correct the drafts of the manuscripts or thesis chapters.

Contents

List of Figures

List of Tables

List of Publications

Dave, A. C., Loveday, S. M., Anema, S. G., Loo, T. S., Norris, G. E., Jameson, G. B., & Singh, H. (2013). *β*-Lactoglobulin self-assembly: Structural changes in early stages and disulfide bonding in fibrils. *Journal of Agricultural and Food Chemistry,61*(32), 7817-7828.

Dave, A. C., Loveday, S. M., Anema, S. G., Jameson, G. B., & Singh, H. (2014). Modulating *β*-lactoglobulin self-assemby into nanofibrils at pH 2 using polyols. *Biomacromolecules,15*(1), 95-103.

Dave, A. C., Loveday, S. M., Anema, S. G., Jameson, G. B., & Singh, H. (2014). Glycation as a tool to probe the mechanism of *β*-lactoglobulin self-assembly. *Journal of Agricultural and Food Chemistry*. *62*(14), 3269-3278.

Dave, A. C., Loveday, S. M., Anema, S. G., Jameson, G. B., & Singh, H. ***In press*** Formation of nano-fibrils form the A, B and C variants of *β*-lactoglobulin. *International Dairy Journal.*

.