

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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



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 to 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, glycosylation and lactosylation of β -Lg strongly inhibited fibril formation primarily by inhibition of peptide self-assembly due to the steric conformational

Abstract

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 fitting.
- 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.

Acknowledgements

- 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

Acknowledgements

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 community

Acknowledgements

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. Anwasha 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 providing 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

Abstract	I
Acknowledgements	V
List of Figures	XVII
List of Tables	XXXI
List of Publications	XXXIII
1. Introduction	1
2. Review of literature	7
2.1. Amyloid fibrils	8
2.1.1. Defining characteristics of amyloid fibrils	11
2.1.1.1. Characteristic cross β -structure	11
2.1.1.2. Nucleation-dependent polymerization	12
2.1.2. Structural basis for self-assembly.....	13
2.1.2.1. Role of amyloidogenic sequences	13
2.1.2.2. Role of protein structure.....	16
2.1.3. Factors affecting the self-assembly of proteins	18
2.1.3.1. Factors promoting protein self-assembly	18
2.1.3.2. Factors inhibiting protein self-assembly	20
2.2. β -Lactoglobulin	23
2.2.1. Native structure	23
2.2.2. Genetic variants of β -Lg.....	27
2.2.3. Heat-induced unfolding of β -Lg.....	29
2.2.4. Heat-induced aggregation of β -Lg	31
2.2.5. Factors affecting thermal aggregation of β -Lg.....	33
2.2.5.1. Genetic variation	34
2.2.5.2. Effects of polyols	35
2.2.5.3. Glycation	36
2.2.5.4. Chaperone-like properties of β -casein.....	38

Contents

2.3.	Self-assembly of β -Lg into amyloid-like fibrils	42
2.3.1.	Conditions of β -Lg self-assembly.....	42
2.3.2.	Mechanism of β -Lg self-assembly at low pH.....	44
2.3.3.	Heat-induced acid hydrolysis	46
2.3.4.	Characteristics of β -Lg fibrils.....	50
2.3.5.	Sequences present in fibrils	53
2.3.6.	Factors affecting kinetics of self-assembly	54
2.3.6.1.	Temperature.....	54
2.3.6.2.	pH and ionic strength	55
2.3.6.3.	Cations.....	56
2.3.6.4.	Seeding	56
2.3.6.5.	Shear	57
2.3.7.	Stability of β -Lg fibrils	58
2.4.	Objectives	59
2.4.1.	Experimental Approach.....	59
2.4.2.	Experimental techniques.....	60
3.	Materials and Methods.....	61
3.1.	Materials	61
3.1.1.	Water	61
3.1.2.	Whey protein isolate	61
3.1.3.	Trypsin.....	62
3.1.4.	β -Lg variants A, B and C.....	62
3.1.5.	Bovine β -casein	62
3.1.6.	Chemicals	62
3.2.	Instruments	65
3.2.1.	Centrifuges.....	65
3.2.2.	Waterbath.....	66
3.2.3.	Spectrofluorometer	66
3.2.4.	Spectrophotometer.....	66
3.2.5.	pH meter	66
3.2.6.	Circular dichroism spectrometer	67
3.2.7.	High performance liquid chromatography system	67

3.2.8. Mass spectrometers	67
3.2.9. Transmission electron microscope	67
3.2.10. Other instruments	67
3.2.11. General consumables.....	68
3.3. Methods.....	68
3.3.1. Isolation of β -lactoglobulin	68
3.3.2. Preparation of fibrils.....	69
3.3.3. Thioflavin T assay for detection of fibrils.....	69
3.3.4. Separation of fibrils using ultracentrifugation.....	74
3.3.5. Polyacrylamide gel electrophoresis (PAGE).....	76
3.3.5.1. Native non-denaturing PAGE	78
3.3.5.2. Glycine SDS-PAGE	79
3.3.5.3. Tricine SDS-PAGE	80
3.3.5.4. Running, staining and imaging of gels.....	81
3.3.5.5. 2-Dimensional nonreducing-reducing (2D NR-R) SDS-PAGE	82
3.3.5.6. Densitometry	82
3.3.6. CD spectroscopy.....	83
3.3.6.1. Introduction	83
3.3.6.2. Sample preparation.....	85
3.3.6.3. Recording scans.....	86
3.3.7. Mass Spectrometry	88
3.3.7.1. Introduction	88
3.3.7.2. Determination of molecular weights.....	91
3.3.7.3. Sequence characterization by MALDI-TOF MS/MS	92
3.3.7.4. Characterization of sequences in fibrils by ESI- MS/MS	93
3.3.8. Transmission electron microscopy.....	95
3.4. Software packages.....	96

4. Characterization of β-Lactoglobulin self-assembly: Structural changes in early stages and disulfide bonding in fibrils	97
4.1. Abstract	97
4.2. Introduction	99
4.3. Materials and Methods	101
4.4. Results	102
4.4.1. Characterization of β -Lg isolated from WPI	102
4.4.2. Self-assembly of β -Lg	105
4.4.3. Hydrolysis of β -Lg in heated samples	106
4.4.4. Structural transitions in the lag phase	108
4.4.4.1. Hydrolysis of β -Lg	108
4.4.4.2. Unfolding of β -Lg during heating	110
4.4.5. Characterization of fibril composition	115
4.4.5.1. Composition of fibrils after different heating times ...	115
4.4.5.2. Disulfide bonding in fibrils	120
4.4.5.3. Characterization of sequences of fibril peptides	122
4.5. Discussion	138
5. Self-assembly from β-Lg A, B and C	147
5.1. Abstract	147
5.2. Introduction	149
5.3. Materials and Methods	150
5.4. Results	151
5.4.1. Characterization of β -Lg variants	151
5.4.2. Unfolding of β -Lg	153
5.4.3. SDS-PAGE of heated samples	156
5.4.4. Self-assembly of β -Lg A, B and C	159
5.4.5. Morphology of fibrils	160
5.4.6. Composition of fibrils	162
5.5. Discussion	163

6. Glycation as a tool to probe the mechanism of β-lactoglobulin self-assembly	171
6.1. Abstract	171
6.2. Introduction	173
6.3. Materials and methods	175
6.3.1. Preparation of glycated β -Lg	176
6.4. Results	177
6.4.1. Preliminary experiments	177
6.4.2. Characterization of glycated β -Lg for self-assembly studies	180
6.4.3. Unfolding behavior of glycated β -Lg	183
6.4.4. Heat-induced acid hydrolysis	187
6.4.5. Self-assembly from glycated β -Lg	191
6.4.6. Morphology of fibrils	196
6.5. Discussion	199
7. Modulating β-Lg self-assembly using polyols glycerol and sorbitol	203
7.1. Abstract	203
7.2. Introduction	205
7.3. Materials and Methods	207
7.3.1. Preparation of fibrils	208
7.3.2. Effect of polyols on ThT assay	208
7.4. Results	209
7.4.1. Effect of polyols on the rate of β -Lg self-assembly	209
7.4.2. Effects of polyols on pre- self-assembly processes	213
7.4.2.1. Heat-induced acid hydrolysis	213
7.4.2.2. Unfolding behavior of β -Lg in presence of polyols	218
7.4.3. Morphology of fibrils in presence of glycerol and sorbitol	224
7.5. Discussion	227

Contents

8. Interactions of β-Lactoglobulin and β-casein during self-assembly	233
8.1. Abstract	233
8.2. Introduction	235
8.3. Materials and Methods	237
8.3.1. Preparation of β -casein	237
8.3.2. Preparation of samples for self-assembly	238
8.4. Results	240
8.4.1. Self-assembly from β -Lg in the presence of β -casein	240
8.4.2. Heat-induced acid hydrolysis of β -Lg and β -casein	241
8.4.3. Composition of aggregates in the heated solutions	250
8.4.4. Morphology of aggregates	251
8.5. Discussion	253
9. Overall discussion and avenues for future work.....	263
9.1. Summary	263
9.1.1. Unfolding of β -Lg.....	264
9.1.2. Heat-induced acid hydrolysis	264
9.1.3. Self-assembly.....	265
9.1.4. Fibril composition and amyloidogenic sequences.....	267
9.1.5. Morphology of fibrils	268
9.2. Overall discussion	271
9.3. Applications.....	281
9.4. Avenues for Further Work	283
10. References	287
11. Annexures	322
12. DRC 16 forms: Statement of contribution to doctoral thesis	
containing publications.....	349

List of Figures

Figure 1.1 Overview of following thesis chapters.	6
Figure 2.1 Schematic representation of characteristic X-ray fibre diffraction arising from the cross- β structure of fibrils. The distance between two β -strands is approximately 4.8 Å, while between the anti-parallel arrangements of β -strands is 9.6 Å. The distance between two β -sheets is 10-11 Å. Adapted from Serpell (2000).....	12
Figure 2.2 Free energy landscape of protein aggregation. The area in the pink region represents different transitional states during aggregation. Adapted from Hartl et al. (2009).....	17
Figure 2.3 Aggregation pathways for misfolded proteins. N, I and U represent native, intermediate and unfolded polypeptide conformation states of the protein. Adapted from Dobson (2003a).	18
Figure 2.4 Sequence of β -Lg A (Variant B shows presence of glycine instead of aspartic acid at residue 64 and alanine instead of valine at residue 118) adapted from the Protein Data Bank [©] (PDB ID 1BSO) (Qin et al., 1998a). Dotted lines indicate disulfide bonding. Solid arrows indicate β -strands; waveforms indicate helices and arches indicate loops.	24
Figure 2.5 pH-dependent reversible association behavior of β -Lg. Adapted from Cheison et al. (2011).	27
Figure 2.6 Proposed mechanism of acid hydrolysis of proteins. Adapted from Blackburn et al. (1954).....	47

List of Figures

- Figure 2.7** Characteristics of β -Lg fibrils (A) wide angle X-ray diffraction pattern of β -Lg fibrils, adapted from Bromley et al. (2005); (B) TEM and (C) AFM image of β -Lg fibrils, adapted from Adamcik et al. (2010); (D) Distribution of proto-filaments in β -Lg fibrils, numbers indicate number of protofilaments, adapted from Lara et al. (2011)..... 52
- Figure 2.8** Comparison of sequences found in β -Lg fibrils reported by (i) Akkermans et al. (2008b) and (ii) Hettiarachchi et al. (2012). The numbers on the top show the locations of aspartic acid residues in the sequence, based on the sequence of β -Lg A. (A) and (B) show the peptides from β -Lg A and B..... 54
- Figure 3.1** (A) Structure of Thioflavin T molecule and (B) orientation of ThT molecules bound to fibrils. The ThT molecules align themselves in the channels formed by the ordered arrangement of side-chain residues in cross- β sheets of fibrils. Adapted from Krebs et al. (2005). 71
- Figure 3.2** Residual Thioflavin T intensities of supernatants obtained by ultracentrifugation at different speeds for 30 and 60 minutes (20 °C). β -Lg 1% (w/v) was heated at 80 °C at pH 2 for 12 h. Sample intensities were corrected by subtracting the intensity of the blank containing ThT solution without any sample. 75
- Figure 3.3** Components of circularly polarized light (A) without absorption and (B) after absorption by chiral component. Adapted from Kelly et al. (2005). 84
- Figure 3.4** Schematic representation of mass spectrometer. 89

Figure 4.1 Native PAGE pattern of β -Lg isolated from WPI by salt precipitation.....	103
Figure 4.2 SDS-PAGE (with or without reducing agent) patterns of β -Lg isolated from WPI by salt precipitation method. I represents interface of stacking and resolving gel. For description of bands 1 to 4, see text.....	104
Figure 4.3 Deconvoluted MS spectrum of the isolated β -Lg.....	105
Figure 4.4 Thioflavin T (ThT) fluorescence intensity (empty circles) at 486 nm for 1% β -Lg at pH 2 heated at 80 °C. Error bars show standard deviations for triplicate measurements for three separate samples and the solid line show fit of Equation 3.1. Filled circles represent normalized intensity of the SDS-PAGE band corresponding to intact monomer. Error bars show standard deviations from three separate samples while the solid line shows fit of Equation 3.8.	106
Figure 4.5 Reduced SDS-PAGE of β -Lg heated at 80 °C and pH 2 for different times. M_0 , Molecular mass marker in kDa; 0, unheated sample, numbers above the lanes indicate the heating times in hours.	107
Figure 4.6 Reduced SDS-PAGE of β -Lg (1% w/v) heated at 80 °C and pH 2 during the lag phase. M_0 , Molecular mass marker (kDa); 0, unheated sample; Numbers above the lanes indicate the heating time in minutes. For description of bands A-E, M and N, see text.	110
Figure 4.7 CD spectra of β -Lg at pH 2 and 80 °C. (A) Near-UV scans, (10 mg/mL). (B) Far-UV spectra (0.01 mg/mL). Conditions for data collection were: path length 10 mm, temperature 80 °C and scans were averaged at 2-minute intervals each.	113

List of Figures

- Figure 4.8** Relative change in ellipticity at (A) 293 nm representing the loss of tertiary structure, and (B) 208 nm representing change in secondary structure, calculated from data shown in Figure 4.7. Solid lines are for visual reference. 114
- Figure 4.9** High-tension (HT) curves of circular dichroism scans shown in Figure 4.7 (B) showing high absorption by the sample between 180 and 200 nm. 115
- Figure 4.10** Thioflavin T fluorescence intensities at 486 nm in heated β -Lg (1%) samples (filled circles) and supernatants in (empty circles) obtained after centrifugation at 2.4×10^5 g for 60 minutes. The pellets were used for SDS-PAGE analysis shown in Figure 4.12. Solid line shows the fit of Equation 3.1. 116
- Figure 4.11** Comparison of reduced SDS-PAGE profiles of centrifuged samples at different heating times. M_0 , Molecular mass marker, weights in kDa; 0, unheated sample; U, heated and uncentrifuged; S, supernatant; P, pellet. Numbers above the lanes indicate heating times in hours. Uncentrifuged sample and supernatants were diluted 1:10 with the PAGE sample buffer. Surface-washed pellets were suspended in the loading buffer without dilution. For description of bands M, N and A to E see text. 117
- Figure 4.12** SDS-PAGE profiles of pellets obtained after centrifugation of heated samples (1% β -Lg) at different stages of self-assembly. Pellets were obtained by ultracentrifugation and were complete-washed (see Chapter 3 for details). The washed pellets were suspended in PAGE loading buffer with 4% SDS and 100 mM DTT

-
- and allowed to dissolve for 7 days 20 °C. Samples were run on tricine SDS-PAGE gels prepared in house. M₀: Molecular weight marker (weights in kDa); U: unheated; numbers above the lanes indicate heating times in h. For description of bands A to E, M and N see text..... 119
- Figure 4.13** SDS-PAGE comparison of peptide bands in fibrils under reducing and non-reducing conditions. Complete-washed pellets obtained after ultracentrifugation for 12 h. M₀, Molecular mass marker (weights in kDa); 0, unheated sample; U, heated and uncentrifuged sample; P_R, complete-washed pellet suspended in reducing PAGE buffer; P_{NR}, complete-washed pellet suspended in non-reducing PAGE buffer. For description of bands C, D and E, see text..... 121
- Figure 4.14** 2D SDS-PAGE of the pellet obtained from a sample heated for 12 h. Complete-washed pellet suspended in buffer containing 4% SDS and run under non-reducing conditions in 1D. The target gel lane was excised, reduced and run in the 2nd dimension. P_R: reduced sample obtained from the same sample..... 122
- Figure 4.15** Typical MS/MS spectra of showing ions with assigned masses for peptide SLAMAASDISLLDAQSAPLR (with oxidation of methionine) from band C in Table 4.2 with a cut-off ion score of 100 (expect value 6.3×10^{-7})..... 130
- Figure 4.16** Schematic representation of regions found in peptides from in-gel digestion ESI-MS/MS and MALDI-TOF MS/MS. The dark blocks in the sequence show location of aspartic acid residues indicating potential sites of acid hydrolysis of β -Lg. Sequence of β -Lg variant
-

List of Figures

- A is shown. Variant B has glycine instead of aspartic acid at residue 64 and alanine instead of valine at residue 118. 137
- Figure 4.17** Schematic illustration of how disulfide-bonded peptides come to be present in fibrils. Red colored regions indicate the N- terminal region (1-53) of the β -Lg sequence. 141
- Figure 5.1** Native non-denaturing PAGE of β -Lg A, B and C. 151
- Figure 5.2** De-convoluted MS spectra of β -Lg variants. (A) β -Lg A, (B) β -Lg B and (C) β -Lg C. 152
- Figure 5.3** Spectra of native β -Lg A, B and C at pH 2. (A) NUV spectra (1 mg/mL) and (B) FUV spectra (0.01 mg/mL). 154
- Figure 5.4** Unfolding of the β -Lg A, B and C upon heating at 80 °C. The NUV scans in (A), (C) and (D) show effect of heating on the tertiary structure, while FUV scans in (B), (D) and (E) show the effect of heating on the secondary structure of β -Lg A, B and C respectively. The concentration of β -Lg was 1 mg/mL for the NUV scans, and 0.01 mg/mL for the FUV scans. 155
- Figure 5.5** Relative losses of ellipticities at (A) 293 nm and (B) 208 nm calculated from Equations 3.11 and 3.12. Solid lines are for visual reference. 156
- Figure 5.6** Reducing tricine SDS-PAGE profiles of control and heated samples after heating for 12 and 24 h at 80 °C. M_0 Molecular weight marker; molecular weights are in kDa. For description of band Q, see text. 157
- Figure 5.7** Residual intensities of β -Lg band at different heating times. Each value represents an average of two separate experiments. Solid lines

indicate fit of Equation 3.8. Thick solid lines represent β -Lg A; dots, β -Lg B; and dashes, β -Lg C.	158
Figure 5.8 ThT fluorescence intensities at 486 nm in heated samples of β -Lg A, B and C (1% w/v) at 80 °C and pH 2. Error bars represents standard error of measurements from two experiments with two replicates in each. Solid lines indicate fit to Equation 3.1. Thick solid lines represent β -Lg A; dots, β -Lg B; and dashes, β -Lg C.	159
Figure 5.9 TEM images of fibrils from β -Lg A, B and C after 24 h at 80 °C.....	161
Figure 5.10 Reducing tricine SDS-PAGE of fibrils formed after heating at 80 °C for 12 h and separated by ultracentrifugation. M ₀ , Molecular weight marker; S, supernatant; P pellet containing fibrils. Molecular weights in kDa; for descriptions of (i), bands A to E and R, see text. ...	162
Figure 6.1 De-convoluted MS spectra of glucosylated β -Lg (A) 8 h, (B) 16 h and (C) 36 h. Numbers in the figure represent the number of glucose residues attached on the β -Lg variant A.....	178
Figure 6.2 De-convoluted MS spectra of lactosylated β -Lg (A) 3 days, (B) 5 days and (C) 7 days. Numbers in the figure represent the number of lactose residues attached on the β -Lg variant A.....	179
Figure 6.3 De-convoluted MS spectra of glucosylated (A) LowGlu β -Lg and (B) HighGlu β -Lg. Peak labels show the number of attached glucose residues.....	181
Figure 6.4 De-convoluted MS spectra of lactosylated (A) LowLac β -Lg and (B) HighLac β -Lg. Peak labels show the number of attached lactose residues.....	182

List of Figures

- Figure 6.5** Comparison of CD spectra of β -Lg glycosylated with glucose and lactose (A) NUV (10 mg/mL β -Lg) and (B) FUV (0.01 mg/mL β -Lg) region..... 184
- Figure 6.6** CD spectra of LowGlu β -Lg in the (A) NUV region (10 mg/mL β -Lg) and (B) in the FUV region (0.01 mg/mL β -Lg). The numbers in the figures indicate heating time in minutes..... 185
- Figure 6.7** Relative change in ellipticity at (A) 293 nm in the NUV region and (B) 208 nm in the FUV region. Solid lines are for visual reference. For details of x and y-axes see Section 3.3.6.3, Chapter 3..... 186
- Figure 6.8** Tricine SDS-PAGE profiles of control and glycosylated samples after heating for 12 h at 80 °C, at pH 2. M_0 , Molecular weight markers in kDa; C, heated control..... 187
- Figure 6.9** The normalized band intensities of the un-hydrolyzed β -Lg band at different times in glycosylated samples. Each value is an average of quantifications from two sets of independent experiments. The solid lines represent fits to the first-order exponential decay model of Equation 3.8..... 188
- Figure 6.10** The normalized band intensities of the un-hydrolyzed β -Lg band at different times in the controls with no sugar added. Each value is an average of quantifications from two sets of independent experiments. The solid lines represent fits the first-order exponential decay model in Equation 3.8. 190
- Figure 6.11** (A) ThT fluorescence intensity of control and glycosylated β -Lg (1% w/v) during heating at pH 2 and 80 °C. Each data point is an average of triplicates from two independent experiments, and error bars are

standard deviations. Solid lines indicate the fit to Equation 3.1. (B) Normalized parameters of self-assembly calculated from Equations 3.2 and 3.4. The kinetic parameters of individual samples were normalized by those of control sample.	192
Figure 6.12 Self-assembly from β -Lg (1% w/v) without sugars used as control during glycation and treated similarly to the glycated samples. Error bars represent standard error of triplicate measurements in samples. Each value represents an average of triplicate values. Solid lines indicate fits to Equation 3.1.....	194
Figure 6.13 Tricine SDS-PAGE profiles of centrifugally separated fibrils made by heating glycated β -Lg at 80 °C for 24 h. M_0 , Molecular weight markers in kDa; C, control fibrils made from the same heat treatment.....	195
Figure 6.14 Residual ThT intensities in supernatants obtained for upon ultra centrifugation. Heated β -Lg solutions obtained after heating at 80°C for 24 h were ultracentrifuged. The pellets obtained after ultracentrifugation were used for SDS-PAGE (Figure 6.13).....	196
Figure 6.15 TEM images of fibrils from glycated β -Lg after heating for 24 h at 80 °C. (A) LowGlu β -Lg; (B) HighGlu β -Lg.....	197
Figure 6.16 TEM images of fibrils from glycated β -Lg after heating for 24 h at 80 °C. (A) LowLac β -Lg; (B) HighLac β -Lg.....	198
Figure 6.17 Proposed mechanism of inhibition of self-assembly of glycated β -Lg by the steric effect of the sugar adduct.	201
Figure 7.1 Thioflavin T (ThT) fluorescence intensity at 486 nm for β -Lg (1%, w/v) heated at 80 °C and pH 2 in presence of different levels (%,	

List of Figures

- w/v) of (A) glycerol and (B) sorbitol. Solid lines indicate the fit using Equation 3.1. Error bars represent the standard errors of measurements from two separate experiments with three replicates in each..... 210
- Figure 7.2** Normalized ThT fluorescence intensities β -Lg sample containing fibrils diluted using different levels of polyols (0-50% w/v, used for self-assembly studies). The ThT fluorescence intensity in the undiluted sample was 920 fluorescence units. The intensity of the sample diluted using water (pH 2) was used for normalization. A value close to 1 indicates that the fluorescence intensity in samples diluted with water or polyols were similar. 213
- Figure 7.3** Reduced SDS-PAGE of samples heated at 80 °C for 12 h in the presence of different concentrations of (A) glycerol or (B) sorbitol. (M_0) Molecular weight marker in kDa; (U) unheated sample. Numbers above the lanes indicate polyol concentrations (% w/v)..... 214
- Figure 7.4** Normalized β -Lg band intensities from SDS-PAGE gels of samples containing different concentrations of (A) glycerol and (B) sorbitol. Values are the average of two independent experiments. Solid lines indicate fit to Equation 3.8. 215
- Figure 7.5** Effect of glycerol (A) and sorbitol (B) on the normalized kinetic parameters of hydrolysis and self-assembly..... 217
- Figure 7.6** CD spectra of β -Lg at 20 °C in presence of different levels of (A) and (C) glycerol and (B) and (D) sorbitol. For the NUV scans (A) and (B), the concentration of β -Lg was 1 mg/mL, while that for FUV

-
-
- spectra (C) and (D) was 0.01mg/mL. The sample scans were corrected by subtracting the baseline scans and smoothed. 220
- Figure 7.7** NUV (A, B) or FUV (C,D) circular dichroism spectra of β -Lg during heating at 80 °C and pH 2 with 30% glycerol (A,C) or 30% sorbitol (B,D). Numbers indicate the heating time in minutes. 221
- Figure 7.8** Relative loss of ellipticity at 293 nm in β -Lg solutions during heating at 80 °C with 0-50% glycerol (A) or sorbitol (B) calculated from Equations 3.13 and 3.14. For details of the notations of Y axis see Section 3.3.6.3 in Chapter 3. The same symbols representing glycerol concentrations in (A) apply for sorbitol concentrations in (B). Solid lines are a visual guide only. 222
- Figure 7.9** Normalized ellipticities in the samples at 293 nm at the end of 10 minutes at 80 °C. Absolute ellipticities in the samples with different levels of polyols (θ_{polyol}) were normalized by the ellipticity of the control sample without any polyols (θ_{control}). Values >1 indicates a higher residual signal in the sample and hence the stabilized state of β -Lg. Solids lines are a visual guide. 223
- Figure 7.10** High tension curves for FUV scans of β -Lg in presence of 30% w/v of (A) glycerol and (B) sorbitol during heating at 80 °C. The data in A and B represent the high tension (HT) values for the same scans shown in Figure 7.7 C and D. Solid lines are a guide and are not fitted with any mathematical model. 223
- Figure 7.11** Transmission electron microscopy images of β -Lg fibrils separated from heated solutions. (A) Without any polyol; (B) With 20%

List of Figures

- glycerol; and (C) With 20% sorbitol. Samples heated at 80 °C, pH 2 and 24 h. 225
- Figure 7.12** Transmission electron microscopy images of β -Lg fibrils separated from β -Lg samples heated at 80 °C, pH 2 and 24 h in the presence of (A) 50% (w/v) glycerol and (B) 50% (w/v) sorbitol at 80 °C, pH 2 and 24 h. 226
- Figure 7.13** Schematic illustration of how β -Lg fibril assembly is affected by sorbitol (slowing unfolding) and glycerol (slowing assembly of peptides). 228
- Figure 7.14** Viscosity of polyol solutions at 80 °C at a shear rate of 1 s⁻¹. Polyol solutions were made in Milli Q water without pH adjustment. Measurements were carried out on an AR-G2 rheometer (TA Instruments, New Castle, DE) using a double gap measuring system equilibrated at 80 °C for two minutes prior to measurements. 230
- Figure 8.1** ThT fluorescence intensities at 486 nm in samples of heated β -Lg- β -casein mixtures. The control sample contained β -Lg alone. Error bars show the standard errors of measurements from two separate experiments with two replicates for each data point. Solid lines indicate fit of Equation 3.1. 240
- Figure 8.2** Reduced SDS-PAGE of β -Lg and β -casein mixtures heated at 80 °C for (A) 12 h and (B) 24 h. M₀ molecular weight marker (kDa); C, control. Lanes marked 1 to 5 represent β -Lg and β -casein mixtures at different molar ratios. Lane marked 1 represents a molar ratio 1:0.0625; 2, 1:0.125; 3, 1:0.250; 4, 1:0.50; 5, 1:1. 242

-
-
- Figure 8.3** Reduced SDS-PAGE of β -casein controls heated at 80 °C for (A) 12 h and (B) 24 h. M_0 molecular weight marker (kDa); Lanes marked 1 to 5 represent the same β -casein concentrations as used in β -Lg- β -casein mixtures of Figure 8.4. 243
- Figure 8.4** Peptide compositions of heated solutions after 12 h at 80 °C. M_0 molecular weight marker (kDa); C, control β -Lg (without β -casein); 1, β -Lg and β -casein mixture (molar ratio 1:1) and 2, control β -casein (without β -Lg) at same concentration as in 1. For description of band F to J, see text. All samples analyzed under reducing conditions. 245
- Figure 8.5** Residual SDS-PAGE monomeric band intensities of (A) β -Lg and (B) β -casein in heated β -Lg- β -casein mixtures. Symbols in (B) represent the same β -casein concentrations as used in (A). Values are an average of two separate estimations. Solid lines indicate fit of Equation 3.8. 248
- Figure 8.6** Composition of aggregates formed from the heated and ultracentrifuged samples containing β -Lg (1%, w/v) with (0.65% w/v, molar ratio 1:0.5) or without β -casein. Samples were heated at 80 °C for 12 h. M_0 represents molecular weight markers (kDa); H, heated solution; S, supernatant and P, Pellet. Supernatants were diluted 1:10 in reducing PAGE sample buffer, while the pellets were suspended in 1.2 ml PAGE sample buffer without further dilution..... 250
- Figure 8.7** TEM images of β -Lg samples heated (A) without, and (B to D) with β -casein at molar ratios of 1:0.5 (B and C) or 1:1 (D and E). Samples

List of Figures

heated at 80 °C for 24 h selected for TEM analysis. For description of white arrows see text.....	252
Figure 8.8 TEM images of β -casein samples heated at 80 °C for 24 h without β -Lg. The β -casein concentration was equivalent to that in mixed solutions at molar ratio of 1:0.5.....	253
Figure 8.9 Charge distribution on β -casein polypeptide at pH (A) 6.7 and (B) 2.6. Adapted with permission from Moitzi et al. (2008), Copyright 2008 American Chemical Society.....	254
Figure 8.10 Overview of the hydrophobicity (pH 7) of β -casein peptides G to J of Figure 8.4 calculated using the method of Kyte et al. (1982). At pH 2, the hydrophobicity of the peptides is likely to be higher due to protonation of carboxylic groups of Asp and Glu (Kuhn et al., 1995). Bands G (A), I (D) and J (E) contain phosphate groups bound to serine residues.	257
Figure 8.11 Proposed mechanism for the effect of β -casein on β -Lg self-assembly. (i), (ii) and (iii) represent aggregation pathways.....	259
Figure 9.1 Mechanism of β -Lg self-assembly at 80 °C, pH 2 at low concentrations (<3% w/v).	266
Figure 9.2 Strategies to decouple acid hydrolysis from self-assembly by modifying (A) glycation of β -Lg or (B) composition of aqueous solution by glycerol.	277

List of Tables

Table 2.1 Human diseases associated with amyloid fibrils.....	9
Table 2.2 Sites of differences in β -Lg A, B and C.	28
Table 2.3 Non-native conditions promoting fibril formation from β -Lg.	43
Table 2.4 Overview of experimental techniques used in this study.	60
Table 3.1 List of chemicals and reagents used and their vendors.	63
Table 3.2 Composition of native and SDS-PAGE gels.....	79
Table 3.3 Composition of resolving and stacking gels for tricine SDS-PAGE analysis.	81
Table 4.1 Proximate composition of extracted β -Lg.....	103
Table 4.2 Peptides from β -Lg hydrolysis present in fibrils analyzed by MALDI- TOF MS/MS.....	123
Table 4.3 Peptide sequences of peptide bands A to E (Figure 4.12) in the pellet (12 h) analyzed by ESI-MS/MS after extraction following in-gel digestion with trypsin.	125
Table 4.4 MS/MS data from Mascot search showing masses of the assigned ion peaks shown in Figure 4.16.....	131
Table 4.5 Peptides obtained by in-gel digestion of unheated β -Lg followed by ESI-MS/MS. β -Lg loaded on to SDS-PAGE gel (resolving gel concentration 20%). After running, the β -Lg band was excised and processed along with the peptide bands A-E which have been characterized in Table 4.3.	133
Table 4.6 Possible sequences of peptides present in SDS-PAGE bands of fibrils..	144
Table 5.1 Rate constants of monomer hydrolysis calculated from Equation 3.8....	158

List of Figures

Table 5.2 Kinetic parameters of self-assembly calculated from Equations 3.2 to 3.4.....	160
Table 5.3 Possible sequences present in the peptide bands of fibrils of β -Lg A, B and C, shown in Figure 5.10.....	165
Table 6.1 Rate constants of acid hydrolysis of glycated β -Lg calculated from data in Figure 6.9 and Equation 3.8.....	189
Table 6.2 Rate constants of acid hydrolysis of β -Lg used as glycation control calculated from the data in Figure 6.10 and Equation 3.8.....	190
Table 6.3 Kinetic parameters describing β -Lg self-assembly calculated using Equations 3.2 to 3.4.....	193
Table 6.4 Kinetic parameters describing self-assembly calculated from Equations 3.1-3.4 for β -Lg samples used as controls during glycation experiments. The ThT fluorescence intensities are shown in Figure 6.12 above.	194
Table 7.1 Kinetic parameters from Equation (3.2-3.4) showing effect of glycerol and sorbitol on β -Lg self-assembly.	211
Table 7.2 Fit parameters from Equation 3.8 describing the kinetics of acid hydrolysis of β -Lg during heating under fibril forming conditions.	216
Table 8.1 Composition of β -Lg- β -casein mixtures.....	239
Table 8.2 Kinetic parameters of self-assembly calculated from Equations 3.2-3.4.....	241
Table 8.3 Predicted sequences of β -casein peptides shown in Figure 8.6.....	246
Table 8.4 Rate constants of acid hydrolysis of β -Lg and β -casein in heated β -Lg- β -casein mixtures during heating at 80 °C.....	249
Table 9.1 Summary of the results from Chapters 5-8.....	269

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-assembly 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 from the A, B and C variants of β -lactoglobulin. *International Dairy Journal*.