Characterization of β-Lactoglobulin Fibrillar Assemblies

Thesis presented for the award of Doctor of Philosophy (Ph.D.)

By

Daniela Oboroceanu, B.Sc.

Under the supervision of Prof. Edmond Magner Dr. Mark A.E. Auty

Submitted to the Faculty of Science and Engineering, University of Limerick

October 2011
Declaration:

The work presented in this thesis was carried out at the University of Limerick and Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, unless otherwise stated. It has not previously been submitted to this or any other university.

____________________

Daniela Oboroceanu
Abstract

The overall goal of this research was to study the mechanism of heat-induced whey proteins fibrils and to characterise their properties for possible food applications.

Experimental parameters such as protein concentration, pH, ionic strength, time and temperature of heating were varied to optimize the process of fibril formation of β-lactoglobulin (β-lg). Fibrillar structures could only be formed at acidic pH; the optimal conditions were pH 2, low ionic strength and 2% (w/w) protein concentration.

A two-stage mechanism of β-lg fibril formation was proposed: 1) denaturation, partial unfolding and increase in β-sheets content, with hydrolysis of monomers, followed by 2) the linear aggregation of polypeptide fragments into fibrils via non-covalent interactions, accompanied by hydrolysis and a decrease in β-sheets content and overall secondary structure. In this study, β-lg and whey protein isolate (WPI) – derived fibrils were observed by atomic force microscopy (AFM). Their height was ca. 2 - 3 nm, while their length and periodicity was up to 15 µm and ~ 30 nm, respectively.

Various process treatments such as high pressure, heating, shearing and acidification can induce changes in the physicochemical properties of whey proteins and their fibrillar aggregates. The studies on pH stability of β-lg fibrillar structures morphology revealed that the longer fibrils break up in the isoelectric range (pH 4.6 - 6) but were stable starting with pH 7 to 12.

High dynamic pressure treatment (microfluidization) produced changes in the general physical dimensions of whey protein fibrils and in the secondary structures of their constituent units. Long fibrils (15 µm) were fractured under high-pressure treatment, the end result being shorter length fibrils (< 350 nm) with the same thickness of 2-3 nm.

The study on the foaming properties of whey protein fibrils revealed that foam capacity and stability were related to the protein concentration, pH, whipping time, thermal and/or high pressure treatment of the protein and their assemblies. Results indicated the fibrillization of whey proteins increased foam capacity and stability compared to non-fibrillar whey proteins.
Publications:

Peer reviewed journals:


Oral and Poster presentations:


“Atomic, force microscopy of biological materials using AC mode in liquid and
dual AC mode imaging in air.” – 31st Annual MSI Meeting, University of
Limerick, University of Limerick, 22-24th of August 2007; (Poster).

“Characterization of dynamic nano-fibrillar aggregates from β-lactoglobulin”,
AFM Forum at UCD – Workshop, UCD, Dublin, November 20-21, 2008;
(Poster).

fibrillar aggregates due to self-assembly processes of β-lactoglobulin whey
proteins.” Self-assembly and Self-organisation at Surfaces and Interfaces,
Cambridge, UK December 10 – 12, 2008; (Poster).

(Poster).

of beta-lactoglobulin nano-fibrillar assembly.” – The 14th International Food
Science & Technology, Shanghai, China; 2008; (Oral presentation).

assembly of beta-lactoglobulin at low pH.” International, 5th Symposium on
Food Rheology and Structure, Zurich, Switzerland, 15-18 June 2009; (Poster).

Microfluidization on the Formation of β-lactoglobulin Fibrils.” International
Dairy Federation, Berlin, Germany, 20-24 September 2009; (Oral presentation).

IV
Acknowledgements

I would like to express my sincere appreciation and acknowledgments to Prof. Edmond Magner and Dr. Mark Auty for giving me the opportunity to explore this interesting research area under their supervisions, for their patience, guidance, encouragement and support throughout my Ph. D. studies.

Also I would like to thanks to Dr. Lizhe Wang for her support and help in the lab during the project and for her friendship. My acknowledgments are going to Dr. Andre Brodkorb for his scientific support and useful discussions, for the encouragements given over the years.

I would like to express my acknowledgements to Prof. Paul Venema from Food Physics Group, Wageningen University, Netherlands for the shear birefringence measurements and the useful scientific discussions.

I would like to thanks to Vivian Gee for her wonderful friendship, for the great time we spent together inside and outside the lab, and for her help in all the matters; to Aniket Abhyankar, Ian Ciron and Alexa Raluca for their friendship and great support in Moorepark over the years.

My acknowledgments are also for Dr. David Corcoran for facilitating me the participation at “PATTERNS” Marie Curie network meetings. I would like to thanks to Catherine Lenihan for her friendship, all her advices on Irish life and help.

Special thanks to Dr. Marina Serantoni for her patience in teaching me AFM and her support in the first months of my arrival in Ireland. Also, I wish to thanks to all the scientific and technical personnel from Moorepark and MSSI that helped me over the years.

To Joan O’Riordan and Brid O’Brien-May in MSSI and to Eileen Lehane in Moorepark, thanks for helping me in dealing with all the administrative matters.
I would like to dedicate this thesis to my husband George and to thank him for his great help, his full support and love throughout my studies.

The research presented in this thesis was mainly conducted at Teagasc Food Research Center in Moorepark, Fermoy, Co.Cork, with the most of the laboratory works carried out at the National Food Imaging Centre, Food and Chemistry Department. The research was supported by the Teagasc Walsh Fellowship Scheme and funded by the Irish Department of Agriculture, Fisher and Food under the Food Institutional Research Measure (06RDT-MFRC-432 – Project Title: Nano-fibrillar milk protein assemblies as precursors to novel food structures) as part of National Development Plan.
# Table of Contents

Chapter 1. Introduction and literature review

1.1. Introduction 1

1.2. Whey proteins and products 2

1.2.1. Whey protein products 2

1.2.2. β-Lactoglobulin 2

1.2.3. α-Lactalbumin 4

1.2.4. Bovine serum albumin 5

1.3. β-Lactoglobulin aggregates 5

1.3.1. Denaturation of β-lactoglobulin 5

1.3.2. Aggregation of β-lactoglobulin 7

1.3.3. β-Lactoglobulin fibrils 8

1.4. Protein functional properties. Foams. 13

1.5. Methods for characterizing the assembly of whey protein aggregates 15

1.5.1. Atomic force microscopy 15

1.5.2. Attenuated total reflectance - Fourier transform infrared spectroscopy 22

1.5.3. Fluorescence spectroscopy 27

1.5.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis 29

1.6. Outline of thesis 31

1.7. References 33

Chapter 2. Characterization of β-lactoglobulin assemblies as a function of pH and heating time

2.1. Introduction 47

2.2. Materials and methods 48

2.2.1. Sample preparation 48
Table of Contents

2. 2. 2. Protein preparation 49

2. 2. 3. AC-mode atomic force microscopy 49

2. 2. 4. Attenuated total reflectance-Fourier transform infrared spectroscopy 50

2.3. Results and discussion

2.3.1 Heat denatured whey proteins aggregates investigated by atomic force microscopy 50

2.3.2. Attenuated total reflectance-Fourier transform infrared spectroscopy 54

2.4. Conclusions 58

2. 5. References 60

Chapter 3. Characterization of the assembly of β-lactoglobulin fibrils

3. 1. Introduction 65

3. 2. Material and methods 67

3. 2. 1. Sample preparation 67

3. 2. 2. Protein fibrillar dispersion 67

3. 2. 3. AC-mode atomic force microscopy 67

3. 2. 4. Scanning transmission electron microscopy 68

3. 2. 5. Attenuated total reflectance-Fourier transform infrared spectroscopy 68

3. 2. 6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis 69

3. 3. Results and discussion 70

3. 3. 1. Atomic force microscopy of β-lactoglobulin fibrils 70

3. 3. 2. Scanning transmission electron microscopy of β-lactoglobulin fibrils 75
Table of Contents

3. 3. 3. Attenuated total reflectance-Fourier transform infrared spectroscopy of β-lg fibrils formation 76

3. 3. 4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis 79

3. 4. Conclusions 81

3. 5. References 82

Chapter 4. The effect of pH on the structure of β-lactoglobulin fibrils

4. 1. Introduction 87

4.2. Materials and methods 88

4.2.1. Materials 88

4.2.2. Preparation of β-lactoglobulin fibrils 88

4.2.3. AC-mode atomic force microscopy 88

4.2.4. ATR-FTIR spectroscopy and STEM 89

4.3. Results and discussion 89

4.3.1. Atomic force microscopy 89

4.3.2. Scanning electron microscopy 92

4.3.3. Attenuated total reflectance-Fourier transform infrared spectroscopy 94

4.4. Conclusions 96

4.5. References 97

Chapter 5. The effect of high pressure microfluidization on the structure and length distribution of whey protein fibrils

5. 1. Introduction 99

5.2. Materials and methods 100

5.2.1. Preparation of β-lg and WPI fibrils 100
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.2. Microfluidization treatment</td>
<td>101</td>
</tr>
<tr>
<td>5.2.2.1. Microfluidization of native β-lg and WPI prior to fibrillization</td>
<td>101</td>
</tr>
<tr>
<td>5.2.2.2. Microfluidization of fibrils</td>
<td>102</td>
</tr>
<tr>
<td>5.2.3. AC-mode atomic force microscopy</td>
<td>102</td>
</tr>
<tr>
<td>5.2.4. Flow-induced birefringence</td>
<td>103</td>
</tr>
<tr>
<td>5.2.5. Reversed phase-high performance liquid chromatography</td>
<td>103</td>
</tr>
<tr>
<td>5.2.6. Attenuated total reflectance-Fourier transform</td>
<td>104</td>
</tr>
<tr>
<td>5.2.7. Thioflavin T fluorescence</td>
<td>105</td>
</tr>
<tr>
<td>5.3. Results and discussion</td>
<td>105</td>
</tr>
<tr>
<td>5.3.1. Atomic force microscopy</td>
<td>105</td>
</tr>
<tr>
<td>5.3.1.1. AFM observations and length distribution of fibrils formed</td>
<td>105</td>
</tr>
<tr>
<td>5.3.1.2. Atomic force microscopy of microfluidized fibrils</td>
<td>107</td>
</tr>
<tr>
<td>5.3.2. Shear birefringence</td>
<td>109</td>
</tr>
<tr>
<td>5.3.3. The effect of microfluidization on the secondary protein structure of β-lg and fibrils</td>
<td>110</td>
</tr>
<tr>
<td>5.3.3.1. Effect of microfluidization on protein secondary structure</td>
<td>111</td>
</tr>
<tr>
<td>5.3.3.2. ATR-FTIR measurement of microfluidized fibrils</td>
<td>113</td>
</tr>
<tr>
<td>5.3.4. Thioflavin T fluorescence</td>
<td>114</td>
</tr>
<tr>
<td>5.3.4.1. ThT fluorescence of microfluidized protein prior fibril formation</td>
<td>114</td>
</tr>
<tr>
<td>5.3.4.2. ThT fluorescence measurement of microfluidized fibrils</td>
<td>114</td>
</tr>
<tr>
<td>5.4. Conclusions</td>
<td>116</td>
</tr>
</tbody>
</table>
Chapter 6. Foam stability of whey protein fibrils

6.1. Introduction 121

6.2. Materials and methods 123

6.2.1. Materials 123

6.2.2. WPI fibrillar dispersions 123

6.2.3. Microfluidization treatment 124

6.2.4. Shear mixing by Ultra Turrax 124

6.2.5. Whipping treatment 124

6.2.6. Foam properties 124

6.2.7. Microscopic analysis of the bubble size 126

6.2.8. AC-mode atomic force microscopy 126

6.3. Results and discussion 127

6.3.1. Effect of whey protein concentration, pH value, whipping time and thermal treatment on WPI foam properties 127

6.3.2. Effect of dynamic high pressure via microfluidizer or high shear Ultra Turrax treatment on WPI foam properties 135

6.3.2.1. The effect of microfluidization 135

6.3.2.2. The effect of shear mixing Ultra-Turrax 138

6.4. Conclusions 145

6.5. References 146

Chapter 7. General discussions, conclusions and future work

7.1. Conclusions 151

7.2. Recommendations for future work 153
# Table of Contents

7.3. References 156

## Appendices

Appendix I: Aggregation of $\alpha$-lactalbumin and bovine serum albumin investigated by atomic force microscopy 157

Appendix II: Recommendations for future work 161

Appendix III: Publication 1 177

Appendix IV: Publication 2 185
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>attenuated total reflectance - Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>α-la</td>
<td>α-lactalbumin</td>
</tr>
<tr>
<td>β-lg</td>
<td>β-lactoglobulin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>EWP</td>
<td>egg white protein</td>
</tr>
<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MF</td>
<td>microfluidization</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reversed phase high pressure liquid chromatography</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>STEM</td>
<td>scanning transmission electron microscopy</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH</td>
<td>sulphydryl group</td>
</tr>
<tr>
<td>S-S</td>
<td>disulphide bonds</td>
</tr>
<tr>
<td>WPI</td>
<td>whey protein isolate</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1. Amino acid sequence of β-lactoglobulin B from Protein Data Bank (accession number P02754). 3

Figure 1.2. The three-dimensional structure of bovine β-lactoglobulin (PDB-ID entry - 2 BLG) generated using Pymol package. 4

Figure 1.3. Schematic representation of an atomic force microscope. 16

Figure 1.4. Diagram illustrating the force regimes under which each of the three most basic AFM imaging modes operate. 19

Figure 1.5. Principle of the attenuated total reflection method. 24

Figure 1.6. Typical vibrations in the IR spectrum of bovine serum albumin. 27

Figure 1.7. Overview of thesis. 31

Figure 2.1. AFM images of (a) native 2% (w/w) β-lactoglobulin solution at pH 7 and (b) aggregates formed after heating at 80 °C for 30 min. 52

Figure 2.2. AFM images of aggregates present in a 2% (w/w) β-lactoglobulin solution at pH 7 after heating for 20 h at 80 °C. b) High resolution image of 500 nm x 500 nm area showing β-lg structures. 52

Figure 2.3. AFM images of 2 % (w/w) β-lactoglobulin solution at pH 12 heated at 80 °C for a) 0 min; b) 20 h. Inset: Higher resolution image of 500 nm x 500 nm area showing native or aggregates of β-lg. 54

Figure 2.4. (a) Amide I of ATR-FTIR spectra of 2% (w/w) β-lactoglobulin at pH 7 heated at different time (up to 24 h); (b) Curve fitting for amide I of ATR-FTIR spectra for native 2% (w/w) β-lg at pH 7; (c) Amide I spectra of the heated samples after native β-lg at pH 7 spectrum subtraction varying from 5 min to 24 h. 55

Figure 2.5. (a) Amide I of ATR-FTIR spectra of 2% (w/w) β-lactoglobulin at pH 12 heated at different times (up to 24 h); (b) Curve fitting for amide I of
ATR-FTIR spectra for 2% (w/w) β-lg at pH 12; (c) Amide I spectra of the heated samples after native β-lg at pH 12 spectrum subtraction varying from 5 min to 24 h.

Figure 3.1. AFM height images of β-lg at pH 2 and 80 °C as a function of the heating time at 0, 15, 35, 45, 65 and 85 min. Images were obtained in air-dried mode. Scan size = 600 nm.

Figure 3.2. AFM height images of β-lg at pH 2 and 80 °C as a function of the heating time at 100, 200, 300 min, and 20 h. Images were obtained in air-dried mode. Scan size = 600 nm. The arrows in the image at 100 min show the early stages of fibril formation.

Figure 3.3. AFM (a) height and (b) phase images of β-lg at pH 2 and 80 °C heated for 200 min. β-lg fibrils had a (c) height of ~2-3 nm and a (d) periodicity of ~30 nm. Images were collected in air-dried mode. Scan size = 600 nm. The arrows in the height image show the precursors of mature fibrils.

Figure 3.4. (a) 10 µm scan of AFM height images of β-lg at pH 2 and 80 °C heated for 20 h. Images were collected in air-dried mode. The arrows indicate denatured protein not incorporated into fibrils. (b) Magnified (2 µm x 2 µm) area of panel a, confirming the presence of non-incorporated proteins as well as fibrils.

Figure 3.5. (a) AFM height image of β-lg at pH 2 and 80 °C heated for 20 h and imaged in water (pH 2.6). Fibril height values were 3 nm. (Inset) Higher resolution height image of 1 µm x 1 µm area, confirming periodicity of fibrils. (b) AFM height image of WPI fibrils at pH 2 and 80 °C heated for 20 h and imaged in water. (Inset) Cross-section height profile along the fibril axis highlighting periodicity.

Figure 3.6. STEM negatively stained image of 2% (w/w) β-lg at pH 2 and 80 °C heated for 20 h. Scale bar = (a) 100 nm and (b) 20 nm.
Figure 3.7. Amide I ATR-FTIR spectra of 2% (w/w) β-lg heated at 80 °C varying
(a) from 1 to 109 min and (b) from 134 to 1560 min (26 h). Amide I spectra
of the heated samples after native β-lg spectrum subtraction varying
(c) from 5 to 109 min and (d) from 134 min to 26 h.

Figure 3.8. SDS-PAGE of 2% (w/w) β-lg at pH 2, heated at 80 °C for various times.
Lane 8, polypeptide molecular-weight marker (~14.4 kDa for α-lactalbumin,
~18.4 kDa for β-lg, and ~67 kDa for bovine serum albumin).
Lanes 1-7 represent β-lg heated for 0, 45, 85, 100, 200, 300 min, and 20 h,
respectively.

Figure 4.1. (a) AFM height image of β-lg fibrils at pH 2. (b) Magnified (500 nm x 500
nm) area with a cross-section along the fibril axis, highlighting the periodicity of
fibrils of 40 nm (c) and height (~5 nm) (d).

Figure 4.2. AFM height image of β-lg fibrils at (a) pH 2, (b) pH 4, (c) pH 5, (d) pH 7,
(e) pH 8 and (f) pH 12.

Figure 4.3. SEM image of β-lg fibrils at pH 4.6. Sample was prepared on carbon film
on copper grid. Scale bar: (a) 1 µm, (b) 200 nm (magnified imaged of
Figure 4.3.a).

Figure 4.4. STEM image of β-lg fibrils at pH 7. Scale bar: (a) 1 µm, (b) 200 nm
(magnified image of Figure 4.4.a).

Figure 4.5. ATR-FTIR spectra of β-lg fibrils at different pH varying from 2 to 6 (a)
and from pH 7 to pH 12 (b). The insets emphasize the changes of the absorbance
peak (full squares) and its corresponding wavenumber (down pointing full
triangles) for different pH.

Figure 5.1. Atomic force microscopy height images (5 µm × 5 µm and 500 nm × 500
nm scans) of fibrils, together with cross-section profiles of native (0 MPa) β-lg
(a and b) and microfluidized β-lactoglobulin at 170 MPa (e and f). The profiles
shows typical heights of ~ 3 nm for 0 MPa (c) and ~ 5 nm for 170 MPa (g) and fibril periodicity of 30 nm (d and h). Cross section profiles were acquired from 500 nm × 500 nm images, the fibril height and periodicity being measured from cross-sections both perpendicular and along fibril axis, respectively. Images were obtained using AC mode in air.

Figure 5.2. Atomic force microscopy height images (2 µm × 2 µm and 500 nm × 500 nm scans) of β-lg fibrils microfluidized at 50 MPa (a and b) and 170 MPa (c and d), showing shortened fibrils. Images were obtained in AC air-dried mode.

Figure 5.3. The effect of microfluidization on fibril frequency length distributions of β-lactoglobulin fibrils at pH 7 obtained after microfluidization pressure up to 170 MPa (◊, 0 MPa; □, 50 MPa; ∆, 100 MPa; ×, 150 MPa; ○, 170 MPa). Fibril length was measured using Image J software and a total of 90 fibrils were measured from 4 separated images.

Figure 5.4. The effect of microfluidization on fibril formation. Fibril length distribution was obtained from flow birefringence measurements at different applied pressures (—, 0 MPa; ——, 50 MPa; ~~~, 75 MPa; ◊, 100 MPa; □, 150 MPa; ●, 170 MPa) during the microfluidization process. Data are derived from the decay curves of shear birefringence analysis. The arrow indicated that the signal decreases as he pressure increases.

Figure 5.5. ATR-FTIR spectra of microfluidized a) β-lg and c) WPI fibrillar structures. Amide I band of the treated samples after native protein subtraction is also shown (b and d).

Figure 5.6. Normalized Thioflavin T fluorescence intensity of WPI solutions microfluidized up to 170 MPa (□, 0 MPa; ▲, 50 MPa; ×, 75 MPa; ∆, 100 MPa; ○, 150 MPa; ♦, 170 MPa) before (a) and after (b) fibril formation.

Figure 6.1. AFM images showing the presence of WPI aggregates after 20 h of heating.
at 80 °C of 2% (w/w) WPI dispersions at pH 7 (a) and pH 2 (b) before whipping. Their presence after whipping treatment can be observed in (c) and (d), respectively.

Figure 6.2. Foam expansion vs WPI concentration before and after thermal treatment for different pH of WPI dispersions whipped for 15 min. Error bars denote standard deviation (± SD).

Figure 6.3. Foam expansion vs whipping time of 2% (w/w) native or heated WPI dispersions and EWP. Error bars denote ± SD.

Figure 6.4. Foam drainage time evolution for a) 1% (w/w), b) 2% (w/w) and c) 3% (w/w) concentrations of unheated, heated WPI dispersions and EWP.

Figure 6.5. Liquid volume fraction vs WPI concentration before and after thermal treatment for different pH of WPI dispersions whipped for 15 min. Error bars denote ± SD.

Figure 6.6. Optical microscopy images of the foams obtained for 2% (w/w) WPI fibrillar dispersions at pH 2 (a) and 7 (b), heated WPI dispersions at pH 7 (c) and EWP (d) after 15 min of whipping. The histograms inserted in optical images show the bubbles diameter distribution. The Sauter diameter and liquid volume fraction values were also included. The black scales represent 200 µm for all optical images.

Figure 6.7. AFM images showing the change in fibrils length after treatment with a) dynamic high pressure using microfluidization at 100 MPa and b) shear mixing using Ultra-Turrax at maximum speed (24,000 rpm).

Figure 6.8. Foam expansion of native and fibrillar WPI dispersions, 2% (w/w) at pH 7, for different applied pressures during microfluidization treatment. Error bars denote ± SD.

Figure 6.9. Foam drainage time of 2% (w/w) native WPI dispersions at pH 7 for
different applied pressure (up to 170 MPa) during microfluidization treatment.

Figure 6.10. Foam drainage time of 2% (w/w) fibril WPI dispersions at pH 7 for different applied pressure (up to 170 MPa) during microfluidization treatment.

Figure 6.11. Foam expansion of 2% (w/w) native WPI and fibrillar dispersions at pH 2 and 7 after shear mixing by Ultra Turrax. Error bars denote ± SD.

Figure 6.12. Foam drainage time of 2% (w/w) WPI native and fibrillar dispersions at pH 2 and 7 after shear mixing treatment by Ultra Turrax. The first, second and third symbol columns correspond to 0, 11200 and 24000 rpm, respectively.

Figure 6.13. Liquid volume fraction vs (a) microfluidization and (b) Ultra Turrax mixing of unheated or heated WPI for different pH of WPI dispersions whipped for 15 min. Error bars denote ± SD.

Figure A.I.1. AFM images of aggregates present in a 2% (w/w) protein dispersion at pH 2 for 20 h heating at 80 °C: a) α-lactalbumin b) bovine serum albumin (in addition of 0.03 NaCl). Inset: Higher resolution height image of 600 x 600 nm area showing α-la and BSA aggregates, respectively.

Figure A.II.1. AFM height image of reconstituted (a) freeze dried β-lg fibrils at pH 2.4 ± 0.5 at room temperature; (b) spray dried β-lg at pH 2.35 ± 0.3 for 20 h at 80 °C. Insets represent magnified regions (500 nm x 500 nm) of Figure A.II.1. a and b, respectively.

Figure A.II.2. a) AFM height image of digested β-lg fibrils at pH 7; b) GPC elution profile of the freeze dried β-lg fibrils after enzyme action during simulated gastrointestinal test. Inset: the corresponding molecular weight at the elution time.
Figure A. II. 3. (a) AFM height image of β-lg fibrils at pH 2 after 50 mM NaCl addition and 2 h at 4 °C. Magnified (500 nm x 500 nm) area of Figure A.II.3.a, highlighting the periodicity of fibrils. (b) AFM height image of β-lg fibrils at pH 2 after 50 mM NaCl addition and reheated for 2 h at 80 °C. Images were obtained using AC mode in air.

Figure A. II.4. (a) AFM image showing cross-linking of β-lg fibrils with transglutaminase at pH 8 and (b) sodium alginate-fibrils composite gel.

Figure A.II.5. Visual aspect of 2% (w/w) WPI at pH 7 heated for 20 h (a) and 2% (w/w) WPI fibrils at pH 7 (b) and 20% (w/w) sunflower oil before (left) and after (right) microfluidization at 50 MPa.

Figure A.II.6. LUMiFuge results showing the transmission profiles of emulsions containing 20% (w/w) sunflower oil for (a) 2% (w/w) WPI at pH 7 heated for 20 h and (b) 2% (w/w) WPI fibrils at pH 7 before (left) and after (right) microfluidization treatment at 50 MPa.

List of Tables

Table 5.1. Summary of experimental samples and procedures.

Table 5.2. Effect of high pressure microfluidization on native β-lactoglobulin as determined by reversed phase-HPLC.

Table A.II.1. Characterization of WPI gels with different salt content and storage at 4 °C for 72 h.
Chapter 1

Introduction and Literature Review
1.1. Introduction

Increasing preferences of consumers for more tasty, healthy, convenient, and natural food products have provided the dairy industry with an opportunity to develop and supply milk protein ingredients to improve the functional properties of food. Whey proteins are widely used as ingredients in food because of their functional properties that include emulsification, gelation, thickening, foaming and water-binding capacity [1, 2]. Protein assemblies are abundant in food products and the exploration of their morphology, physical and chemical characteristics can lead to a better understanding of the engineering of the food materials. The assembly of proteins into fibrils has received considerable attention in recent years. This attention arises from the potential utility in modifying the material properties of food products to create high protein foods, meat replacement and/or low calorie products. Whey proteins, like many food proteins (e.g. soy and egg-white) can form fibrils under denaturing conditions such as low pH, ionic strength and long heat treatment. Acidification, heating, shearing, and/or high-pressure treatment can induce changes in the physicochemical properties of whey proteins and their assemblies.

Techniques for characterization of β-lactoglobulin (β-lg) fibrils include: atomic force microscopy (AFM), attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy, scanning transmission electron microscopy (STEM), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gel permeation chromatography and thioflavin T fluorescence spectroscopy.
Chapter One

1.2. Whey proteins and products

1.2.1. Whey protein products

Bovine milk contains ~ 3.5% protein of which ~ 80% is casein and ~ 20% in whey [3, 4]. These fractions can be separated from each other after casein precipitation at pH 4.6 or by adding an enzyme called “rennet” to form a soft curd in the first step in cheese manufacturing [3, 4]. The whey contains lactose, proteins, water - soluble vitamins and minerals, and it is used by the dairy industry to produce a variety of value-added functional ingredients including whey protein concentrate (which contains lactose and ~ 30 - 80% proteins), whey protein isolate (without lactose and > 90% protein) and whey protein hydrolysate (hydrolyzed whey proteins are more easily absorbed).

Whey protein concentrate (WPC) or isolate (WPI) are usually sold in a powdered form. Industrially, a membrane separation process is applied in the manufacturing of ordinary whey powder and whey protein concentrates, while ion-exchange chromatography technique is employed in the manufacture of whey protein isolate.

The whey fraction contains a great variety of different individual proteins, the majority being β-lactoglobulin (~ 55%), α-lactalbumin (~ 20%), bovine serum albumin (~ 5 %), immunoglobulins (Ig) and other minor proteins including lactoferrin (Lf).

1.2.2. β-Lactoglobulin

Bovine β-lactoglobulin (β-lg) represents ~ 55% of bovine whey proteins. Being relatively easy to isolate, β-lg has become a standard protein for biochemical and physical studies of denaturation and aggregation mechanisms [3-9]. β-Lactoglobulin is a globular protein that contains 162 amino acids residues, has a molecular weight (MW) of 18,362 Da and an isoelectric point (pI) of 4.6 - 5.2 [3-7]. β-Lactoglobulin contains
eight genetic variants in bovine milk: A, B, C, D, E, F, G and H; the most widely studied are A and B. The difference between these two variants consists in amino acids placed in positions 64 (Asp-Gly) and 118 (Val-Ala) \cite{3, 5, 6}. Thus, variant A has an aspartic acid residue and a valine residue in these two positions, respectively. Conversely, variant B has glycine and alanine in the above mentioned positions.

Native β-lg at neutral pH exists as a mixture of monomers and non-covalent dimers, comprising two homologous monomers that dissociate into native monomers at acidic pH <3.5 or basic pH >7.5 \cite{5, 6, 10-12}. The β-lg monomer structure is strongly stabilized by two disulfide bonds (Cys66-Cys160 and Cys106-Cys119) and a free sulfydryl group (Cys121) buried in the core (β-barrel) of the protein structure \cite{5, 6}. The complete amino acid sequence of β-lg monomer obtained from the Protein Data Bank (Accession number P02754) is shown in Figure 1.1. The sulfhydryl residues play an important role in the protein aggregation at neutral or basic pH \cite{13}.

LIVTQTMKL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP EGDLEILLQK WENGECAQKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK KYLLFCMENS AEPEQSLACQ CLVRTPEVDD EALEKFDKAL KALPMHIRLS FNPTQLEEQC HI

**Figure.1.1.** Amino acid sequence of β-lactoglobulin B from Protein Data Bank (accession number P02754).

The secondary structure of the β-lg monomer is composed of β-sheets (40-50%) formed by nine strands (labelled from A to I), a single α-helix (10-20%) on the outer surface of the β-barrel, turns (8-10%), and random coils (30-40%) \cite{5, 3}. The β-barrel is formed from the first eight β-strands (A to H) arranged to create a central hydrophobic pocket (Figure 1.2).
Figure 1.2. The three-dimensional structure of bovine β-lactoglobulin (PDB-ID entry - 2 BLG) generated using Pymol package.

Conformational changes of the protein structure were observed under physical-chemical modification such as heat, pressure, changing the pH, protein concentration, ionic strength, addition of enzymes. All these factors govern the mechanism of denaturation and aggregation of β-lg \cite{14, 15} and are discussed in detail in the next chapters.

1.2.3. α-Lactalbumin

α-Lactalbumin (α-la) is a globular protein with MW of 14,178 Da, with a primary structure consisting of 123 amino acids residues. α-Lactalbumin has four intrachain disulfide bonds and no free thiol group unlike β-lg. α-Lactalbumin has a pI of 4.2 – 4.8 and exists as a monomer \cite{3, 4}. Native α-la consists of a large α-helical domain and a small β-sheet domain, which are connected by a calcium binding loop given an ellipsoidal shape with a hydrophobic pocket \cite{3, 16}.
1.2.4. Bovine serum albumin

Bovine serum albumin (BSA) is a large globular protein with a MW of 66,776 Da and represents ~ 5 % of whey protein. It consist of 582 amino acids residues, with 17 disulphide bridges and one free sulphydryl group. The BSA monomer has an ellipsoidal shape and a pI between 4.7 and 5 \[3, 4, 17\]. Heat induced aggregation of BSA involves denaturation of protein monomer and exposure of hydrophobic pockets to the aqueous solvent \[17, 18\].

1.3. β-Lactoglobulin aggregates

Previous literature indicates that β-lg is the only whey protein that can form fibrils upon prolonged heating at 80 °C, pH 2.0, at low concentration and low ionic strength. No fibrils form upon heating pure α-la or pure BSA at pH 2, but fibrils are formed in pure β-lg and WPI solutions \[15, 19, 20\]. The conversion of β-lg monomers into aggregates was studied over recent years by controlling the heating treatment, whey protein concentration, pH, salt type \[1\]. Recent findings \[21\] indicated that a different path of fibril formation occurs in comparison to that presented in the general accepted mechanism \[14\]. Prior to aggregation and fibril formation, the globular monomer suffers conformational changes after denaturation, but this aspect will be discussed in detail in chapter 3.

1.3.1. Denaturation of β-lactoglobulin

Heating globular proteins in aqueous solution leads to partial loss of the native structure and increased mobility of the chain segments, but generally not to complete unfolding of the chain. The process that involves a change from the native protein conformation to a more unfolded one is called denaturation \[22\]. The reversible or
irreversible denaturation of β-lg depends on pH \[^{[23]}\], ionic strength, \[^{[24]}\] concentration and purity of the protein \[^{[25]}\], temperature \[^{[26]}\] and genetic variants \[^{[27, 28]}\].

Like many other proteins, native β-lg is sensitive to heating in the 65 - 100 °C range. Under ambient conditions and neutral pH β-lg exists as a mixture of monomers and non-covalently linked dimers. By increasing the temperature the equilibrium between the monomers and dimers is shifted toward the monomeric form. Near the isoelectric point (pH 3.5 to 5.2), the dimers associate to form octomers \[^{[10-12]}\]. Under acidic (pH < 3.4) or basic (pH > 7.5) conditions, they dissociate and become monomers \[^{[29]}\].

Reversible denaturation of proteins can occur at 58 - 60 °C, and affects mainly the α-helix structure; these structural changes become irreversible at temperatures above 80 °C. The thermal denaturation at 80 °C of β-lg at low protein concentration involved dissociation of the native monomers/dimers into native or denatured monomers by breaking of intramolecular disulphide bonds (S-S), accompanied by exposure of free sulphhydril group (-SH) to the solvent \[^{[22, 26, 30, 31]}\]. After denaturation, new intermolecular covalent bonds (S-S) formed by SH interchange/oxidation or non-covalent interactions (hydrophobic, hydrogen bonding, electrostatic, van der Waals) can cross link protein molecules leading to the formation of irreversible β-lg aggregates \[^{[3, 15, 26, 30-34]}\]. In the native state, the interactions between the globular protein molecules are repulsive (mainly electrostatic, hydration and entropic). These interactions are sufficiently strong to overcome the attractive interactions (mainly van der Waals, hydrophobic) and, therefore, the proteins exist as individual entities or as small aggregates (e.g. dimers, octomers etc.) \[^{[31]}\].
1.3.2. Aggregation of β-lactoglobulin

Intensive research on the kinetics of the mechanism of whey protein aggregation has been performed in order to understand the forces that drive protein-protein association.

During heat-induced denaturation, β-lactoglobulin unfolds and forms aggregates with different sizes through a series of steps that involve physical and/or chemical bonds between non-native monomers \([21-23, 27, 30, 31, 35-37]\). The aggregation process is governed by the equilibrium between attractive and repulsive electrostatic interactions \([22, 31, 38]\).

Roefs and Kruif \([26]\) proposed a kinetic aggregation model for the heat-induced denaturation and aggregation of β-lg at 65 °C, neutral pH and low protein concentration. The model is based on -SH/S-S exchange reactions between monomers leading to the formation of polydisperse, disulfide-linked aggregates. The kinetic model assumes that the protein molecules aggregate in a similar manner to the polymerization mechanism following three major steps: an initiation, propagation, and a termination step.

The initiation step consists of a number of reversible reactions, when the dimer splits into monomers, followed by an irreversible reaction, when the free -SH group of native β-lg (B) is exposed to the solvent and the protein monomer becomes reactive (B*) and available to react with other protein molecules.

\[
\begin{align*}
B_2 & \rightleftharpoons 2 B \\
& \rightarrow B^* \text{ (activation)}
\end{align*}
\]  

(1)

The reactive -SH group of B* reacts with one of the two intramolecular S-S bonds of other native β-lg monomer (B) to form a dimer \(B_2^*\); a new intermolecular disulfide bond is formed and a new reactive -SH group is now available on the second molecule, thus leaving a reactive dimer. The reactive dimer in turn can react in the same way with a native monomer and this step (propagation reaction) can be repeated many times:
In the propagation reaction, only one of the two intramolecular disulphide bonds and only one sulphydryl group/monomer are reactive. Consequently, linear aggregates will be formed. The polymerization process stops when two reactive intermediate multimers $B_i^* \text{ and } B_j^*$ react with each other (termination reaction) forming a polymer without a reactive -SH group:

$$B_i^* + B_j^* \rightarrow B_{i+j}, \ i, j \geq 1 \ (\text{termination})$$  \hspace{1cm} (3)

Clark et al. \cite{39} had developed a model for $\beta$-lg gelation which is similar to the polymerization model proposed by Roefs and Kruif \cite{26}. The model recognizes three main stages in the process of heat-gelation of globular protein: an initial protein unfolding step or dimerization, linear fibrillar aggregation via nucleation and growth, and random association of the fibrils. The growth rate of the linear aggregates was determined by the rate of reaction with other chains, or activated single species. Apart from the -SH/S-S interchange, non-covalent interactions become important at high temperature and/or low pH \cite{35, 40-42}.

### 1.3.3. $\beta$-Lactoglobulin fibrils

The aggregation of $\beta$-lg upon prolonged heating at 80 °C into fibrils was widely investigated by many research groups \cite{9, 16, 43-45}. A generally accepted nucleation and growth model for fibrils \cite{46} was based on a polymerization mechanism first proposed by Roefs and Kruif \cite{26}. This model considered that the fibrils were formed from denatured monomers. However, recently it was suggested that fibrilization takes place through assembly of hydrolyzed protein due to non-covalent interactions \cite{21}. Therefore, a
second model based on the fact that the constituents units of the fibrils were polypeptides was developed. This second model for fibril formation mechanism is developed in chapter 3 of this thesis.

Monomeric model

The first model proposed involves a nucleation step and a simple addition reaction for the growth of the fibrils. The partially or total heat denaturation of β-lg at 80 °C (activation step) was followed by a nucleation step, when an unfolded molecule is either attached to another unfolded or completely denatured molecule. Obviously, denaturation must be a first-order reaction with a rate constant which may or may not depend on the ionic strength. The model predicts a critical protein concentration and ionic strength that influence the kinetic rate constants for β-lg to aggregate into fibrils, the rest remains monomeric.

Starting from the nucleation-growth model Arnaudov et al. [14], Bolder et al. [47] developed a mechanism for β-lg fibril formation consisting from the following steps: activation of β-lg monomer (partial denaturation), nucleation (assembly of active monomers), reversible aggregates formed by growth up from the nuclei by addition of monomers to both ends of the intermediated aggregate. These aggregates conclude the polymerization process and can consolidate in mature and irreversible fibrils. The aggregation reversibility was present in the earlier stages of the β-lg fibril formation process. The aggregation irreversibility appears upon increasing the heating time and formation of intermolecular β-sheets between monomers that determine fibrils formation. The fibrils will no longer have active ends and the polymerization process is closed. At longer heating times (24 h at 80 °C) a significant fraction of β-lg (“dead-end” species) coexists with the mature fibrils. The irreversible denatured/hydrolyzed monomers and/or oligomers of low molecular weight formed during the consolidation
of the mature fibrils cannot aggregate any further. Arnaudov et al. [14] and Bolder et al. [47] found that above a critical protein concentration [2.5% (w/w)] the conversion rate of β-lg monomer into long fibrils was higher than that into low molecular weight named “dead-ends”. Arnaudov et al. [14] defined the critical concentration as being the concentration where the conversion of protein into fibrils was too low to be determined with nuclear magnetic resonance spectroscopy. Previous reports showed that the fibrils were not formed at critical protein concentration and all ionic strength value [14, 47]. In the literature, the reported values for the critical concentration of fibril formation of β-lg at pH 2 varied from protein concentrations as low as 0.33% (w/w) up to 2.5% (w/w). It was observed that the critical concentration decreases with increasing ionic strength, mainly “dead-end” species that cannot aggregate any further being formed.

Qualitative information on protein aggregation at 80 °C were monitored by “tapping mode” AFM (see section 1.4.1) for different protein concentrations and heating time from 1 h to 24 h. Semi-flexible fibrils which are polydisperse in length and monodisperse in cross-section can be obtained on heating β-lg [0.5 - 2% (w/w)] at 80 °C for 4 - 17 h [14, 48, 49]. Occasionally, these fibrils were split towards the ends. A cross section along the fibril structure presents a fluctuation in height (4 nm), periodicity (~25 nm) and length (longer than 1 µm) [14].

The effect of salt

A detailed investigation carried out using AFM was performed on the effect of ionic strength upon the morphology of the fibrils using β-lg at pH 2.0 under heating at 80 °C [44, 49]. The results of those studies showed that the length of the fibrils formed at different ionic strength present a non-monotonic behavior [15, 32, 45, 50-53]. At low NaCl or CaCl concentration (up to 50 mM) the β-lg fibrils were long and straight [45, 50], while in 100 mM NaCl or CaCl, short and curved “worm-like” fibrils were present. It was also
noted that the fibrils had many bends. The fibril length distribution changed non-
monotonically with increasing ionic strength. An increase in ionic strength (using NaCl
or CaCl$_2$) and a low protein concentration can screen electrostatic repulsion between the
monomers leading to faster rates of aggregation (fibril nucleation) and a smaller amount
of “dead-ends” [15, 32, 50-53]. In these conditions of higher ionic strength, fibril nucleation
is faster, more fibrils are formed, and as a result the mean length of the fibrils is shorter.
Conversely at all ionic strengths studied, the fibrils had similar thicknesses of about one
or two monomers (2 - 4 nm) and a periodicity of 25 - 28 nm, which suggests that the
molecular mechanism of fibril formation is most likely independent of the ionic strength
and specific for this protein.

In the presence of 0.1 M NaCl, fine-stranded aggregates of β-lg appearing as
strings of partially unfolded monomers were observed [15, 54]. These strings were shorter
and more flexible than the fibrils detected by Arnaudov et al. [45]. Different periodicities
of the fibril structure was observed by Gosal et al. [51], where the AFM images showed
β-lg fibrils after protein monomer heating at 80 °C with lengths of between 0.1 and 2
µm and heights of 3.6 ± 0.5 nm, that are in agreement with other observations [45]. An
increase in the ionic strength and protein concentration induces an increase in the
amount of β-lg dimers at pH 2, but the amount decreases with the temperature [32].

The effect of pH

Long semi-flexible unbranched β-lg fibrils with similar morphology were
observed from pH 1.6 to pH 2.4 [50]. At pH 2 the fibrils had diameters of 5 - 10 nm and a
length up to 10 µm. At pH 2.2, the fibrils exhibit a ‘wavy’ appearance while at pH 1.6 a
small number of tightly-curled fibrils were present [50]. Using transmission electron
microscopy, long fibrils at pH 2 in the absence of salt were found, but at high pH (up to
9) and 0.05 M NaCl, no fibrils were formed [43]. At constant heating temperature and
protein concentration, the aggregation kinetic was slower at lower ionic strength \[32, 55\].

After a heating time of about 10 h, the conversion of monomers into fibrils reached a constant value \[32\]. This is due to the fact that the protein is more resistant to thermal denaturation at low pH than in alkaline conditions. Thus, it was observed that, only between 40% and 70% of the protein in the solution is converted into fibrils. Besides a strong dependence on ionic strength, the rate of conversion increased significantly with increasing β-lg concentration \[9, 49\].

**Polypeptide model**

A more recent study suggests that β-lg fibrils formed at pH 2 were composed of peptides and that intact β-lg monomers was not present in the fibrils \[21\]. Only peptides with a low charge, hydrophobicity and high capacity to form β-sheets were present in the fibrils \[21\]. The rest of the sequence that has a high charge and low capacity to form β-sheets were completely absent from the fibrils. The increased length of the fibrils at low ionic strength cannot be attributed only to electrostatic interactions along the chain. This could be due to specific binding between the proteins, such as the formation of intermolecular β-sheet \[43, 55, 56\]. Changes of β-lg secondary structure upon heating appear during the formation of the aggregates at all pH (acidic to alkaline) and were monitored using FTIR \[55-58\]. During thermal treatment the hydrogen bonds stabilizing the native structure of β-lg are disrupted, causing loss of the α-helix and β-sheets structures, and creating new β-sheets arrangements. Protein aggregation is frequently characterized by the increase of a new intermolecular β-sheets band, involved in cross-linking of unfolded proteins \[59, 60\]. Strong bands in the region 1620-1630 cm\(^{-1}\) and 1685-1695 cm\(^{-1}\) were seen in the amide I region of aggregated protein spectra. It appears, therefore, that only the amide I can be used to distinguish between intra - and intermolecular β-sheet formation, because it is a complex composite, consisting of a
number of overlapping component bands representing helices, β-structures, turns and random structures \(^{[43]}\).

Over the years, studies performed by a number of research groups showed using SDS-PAGE analysis, that under reducing or non-reducing conditions (added mercaptoethanol), disulphide-linked aggregates were unfolded, and could be broken up and separated by size. All intra- and intermolecular disulphide bonds were cleaved, resulting in a linear polypeptide chain \(^{[61, 62]}\).

The effect of pressure

Dynamic or static high pressure treatments affect native β-lg structures and may increase the rate of protein aggregate formation \(^{[63-71]}\). Studying the effect of pressure treatment can give information on the mechanical properties of the aggregates. Generally, high pressure treatments using a homogenizer are used in the dairy industry to mix proteins and oil, disrupting the fat particles and producing fine and stable emulsions \(^{[64, 72, 73]}\). Recent studies suggest that dynamic pressure affects the native protein structure and can induce aggregation, significantly differences when compared to heat induced-aggregates being observed \(^{[72]}\). On applying dynamic high pressure to the protein particles, intra and intermolecular bonds in the network may break, leading to fragmentation of protein structure \(^{[74]}\). The effect of pressure on β-lg fibrils has not been studied to date.

1.4. Protein functional properties. Foams.

Food quality attributes, as identified by human senses, are related to the characteristics of the constituent ingredients and the interaction between them or with the surrounding environment \(^{[75]}\). Food proteins are used as highly functional structuring agents in food systems. Functional properties of globular food proteins such as whey
proteins include swelling, viscosity, water binding, gelation, emulsification, foaming, and flavour binding [76-79].

Food foams include breads, cakes, ice creams, mousses, meringues etc., and are formed by dispersing large amounts of gas into relatively small amounts of liquid by severe mechanical stresses such as beating or whipping [79-84]. Their stability is affected by numerous factors such as the protein adsorption from solution at the liquid/gas interface, the surface rheological properties, diffusion of the gas out and into foam cells, size distributions of the cells, liquid surface tension, external pressure and temperature [85].

Foams are destabilized by drainage that causes thinning of the interstitial liquid film and by its rupture [86, 87]. The drainage of the liquid takes place throughout the liquid film and leads to formation of polyhedral cellular structures surrounded by channels called lamellae, the vertices of the polyhedra being called “Plateau borders”. Due to the curvature of the liquid film in this region the liquid pressure is lower than in the lamellae leading to liquid flow. If the resulting forces overcome the surface tension that supports the liquid channels the film will rupture and the foam will collapse [86, 87].

Protein foam formation requires the ability of proteins present in the liquid to rapidly adsorb at a large interfacial area (air/liquid) and to stabilize it against internal and external forces. The foaming capacity of proteins shows a good correlation with the overall free energy of the system which is higher than before air dispersion [87, 88]. After dispersion of air into the continuous liquid phase, the overall free energy of the system increases leading to a large interface and a minimized of interfacial tension [87, 88]. If the system tends to equilibrium, the overall free energy of the system tends to a minimum value and a phase separation will be obtained by decreasing the interfacial surface and an increase of the interfacial tension [87, 88]. The drainage of the liquid will depend on the physical properties of the liquid, particularly viscosity. As the liquid drains from the
foam, the bubbles will coalesce. The coalescence can be stabilized by the presence of the proteins at the liquid/air interface that can modify the surface tension of the liquid. As the surface tension is lowered, the bubbles become more deformable, being easier to disperse \cite{87, 89, 90}. The presence of the proteins at two interfaces of lamellae can lead to the appearance of long-range repulsive forces such as electrostatic or steric repulsion. These forces can prevent the close approach of the two interfaces \cite{89, 90}. During adsorption at the interface, the proteins may unfold and denature, exposing previously buried hydrophobic residues of the protein to migrate to the gas phase, with the hydrophilic segment exposed to water \cite{77, 83, 88}. Surface hydrophobic patches facilitate initial anchoring of the protein and the overall behavior of protein molecules at the interface will depend on their primary and secondary structure and degree of aggregation \cite{87, 88}.

Whipped food proteins such as egg white thus form a stable, cohesive air-water interfacial film, characterized by reduced interfacial tension due to the rapidly spreading of the liquid around the air bubbles during whipping \cite{77, 80-82, 88}. It is known that whey proteins adsorb to air interfaces as partially denatured molecules and/or aggregates. However, the foaming properties of whey protein fibrils have not yet been studied. Therefore, the effect of heat treatment and fibrillization on the foaming properties of whey proteins at pH 2 and pH 7, will be presented in chapter 6. The results will be compared with those of egg white, a standard foaming agent used in the food industry.

1.5. Methods for characterizing the assembly of whey protein aggregates

1.5.1. Atomic force microscopy

Atomic force microscopy is a very high-resolution type of scanning probe microscope and it became over the years a main tool for imaging, measuring and
manipulating matter at the nano-scale \cite{91, 92}. Unlike classical electron microscopy techniques, AFM can image almost any type of surface (polymers, ceramics, composites, glass, and biological); the sample preparation is minimal and the investigation of the sample can be made in air or in liquid such as biological buffers. Another advantage of the AFM is the ability of the probe tip to be scanned across a surface, probing the repulsive and attractive forces existing between the probe tip and the sample surface, and, thus, to produce a high resolution three-dimensional topographic images of the surface. The resolution in the $x$-$y$ plane ranges from 0.1 to 1.0 nm while in the $z$ direction (normal to the sample surface) is 0.01 nm \cite{91, 92}.

Most common AFM instruments are based on an optical lever detection method that monitors the changes in the deflection of a flexible cantilever as it responds to mechanical interactions of the tip with a surface. A laser beam deflection system is used, where a laser is reflected from the back of the reflective AFM lever and onto a position-sensitive detector.

The basic elements and operation principle of the AFM used in this thesis are presented in Figure 1.3.

\textit{Figure 1.3.} Schematic representation of an atomic force microscope.
The AFM instrument consists of a piezoelectric scanner system employed to move the sample relative to the tip when a voltage is applied (Figure 1.3). In order to make a line-by-line image, the sample surface and the tip are brought close together or are in contact.

The scanning operation causes a deflection of the cantilever because of the change in surface topography or in tip-sample interaction forces. This deflection of the AFM cantilever is detected using a laser light source that shines onto the back of the cantilever. A lens focuses the incident light beam from the light source to the lever. The light reflects off the cantilever and up to a recollimation lens and mirror to the position sensitive detector, represented by a segmented photodiode with four quadrants. The position of the reflected beam on the photodiode is determined by the angle of the deflected cantilever. The cantilever bends vertically upwards or downwards because of attractive and repulsive forces and the reflected beam is directed to the photodiode. The photodiode is segmented into four quadrants, the deflection signal being calculated by the difference in signal detected between the top two and the bottom two quadrants. Thus, any deflection of the cantilever will produce a change in the position of the laser spot on the photo-detector.

A summary of the scanning mode technique is described here. In the first stage, the tip is at a height of few hundred nanometers from the sample surface, to ensure no deflection of the cantilever.

As the separation distance decreases, the forces between the cantilever tip and the sample rapidly increase and the cantilever starts to bend under the influence of attractive van der Waals interactions (second stage). The second stage occurs at a short distance before contact with the surface, where the tip of the cantilever jumps to contact. At this point the tip lands on the surface bending the cantilever which attempts to pull the tip back off the sample (stage 3). When the tip is pushed back, the cantilever
straightens out again (stage 4). When the cantilever is approximately straight the force exerted by it on the sample is nearly zero (stage 5).

*Scanning modes*

The AFM has three primary scanning modes, contact, non-contact and “tapping” mode, which are differentiated by the nature of the interaction forces involved in each case when the scanning probe is or is not in contact with the sample surface [91-93].

*Contact mode*

In *contact mode* operation, the AFM tip scans in direct physical contact with the sample surface across it, like a phonograph stylus playing a record, its advantage being the precise control of the applied forces to the sample. As the tip scans in contact mode, the tip-sample interactions occurs in the repulsive force regime as illustrated in Figure 1.4, and the topographic changes present on sample surface cause the deflection of the tip and the cantilever.

The relationship between the cantilever’s motion, d, and the force required to generate the motion, F, is given by Hooke’s law:

\[ F = -k \cdot d \]

where \( k \) is the force constant. It is possible to fabricate a cantilever with a force constant, \( k \), of 1 N/m or less. Since motion of less than one angstrom can be measured, forces of less than 0.1 nanoNewton are detectable.

Two working modes are available in contact mode: constant and variable force applied to the sample surface.

In constant force imaging mode, a feedback mechanism is used to maintain the deflection, and hence the force between the tip and surface sample at a constant value. The scanning of the sample surface is achieved by sending drive signals to the x, y scanner such that the sample moves along one line in the x direction, after which the
sample is moved in the \( y \) direction and the process repeated. When the tip encounters an object on the surface of the sample, the cantilever begins to bend and the feedback loop adjusts the \( z \) piezoelectric scanner to move the tip in the appropriate direction to return the cantilever to its original deflection or set-point. The feedback loop adjusts the height of the sample (to maintain constant deflection) by varying the voltage applied to the \( z \) scanner (by the extending and retracting piezoelectric scanner).

The topographical image of the sample surface and height information are generated and created by plotting the \( z \) correction signal from the feedback loop against \( x \) and \( y \).

![Diagram illustrating the force regimes under which each of the three most basic AFM imaging modes operate.](image)

**Figure 1.4.** Diagram illustrating the force regimes under which each of the three most basic AFM imaging modes operate.

In variable force imaging mode, the feedback mechanisms are switched off so that \( z \)-height remains constant and the freely deflection of the tip in contact with the sample is monitored to produce a topographic image. The height data in the images is directly measured from the AFM tip and not from the feedback loop as in the constant force mode.

Contact mode is used primarily on hard samples which are relatively smooth with low lying surface features. For soft surfaces or fragile samples (biological
specimens or polymers), cantilever movement across the sample in contact mode may damage the tip or the sample surface, reducing image resolution. Therefore, for correct imaging, the high forces between the tip and the sample have to be monitored and adjusted continuously. Another disadvantage of this imaging mode is represented by the existence of capillary forces between the tip and surface of the sample. At the contact point of the small cantilever radius and sample, a nucleation star of a water vapor condensation can appear due to humidity. Imaging in ambient conditions, the tip will be pulled down towards the sample by the capillary forces associated with the meniscus of the liquid. These forces in contact mode and ambient environment can be large enough to destroy the sample. These disadvantages can be eliminated by using the non-contact or the “tapping” mode \[91-93\], that monitor the tip-sample interactions in the attractive regime (Figure 1.4).

Non-contact mode

In the non-contact imaging mode, the cantilever that oscillates at its resonant frequency is brought close to the surface. The changes in relatively long-range force (van der Waals and electrostatic) between the atoms of the tip and the sample are detected by monitoring the changes in the tip vibrations. These attractive forces are low and induce a damping effect on the sinusoidal oscillating cantilever. As the probe gets closer to the sample surface, these attractive forces induce changes during the scanning in the oscillation amplitude, frequency and phase of the vibrating cantilever. These changes can be detected by the feedback-control loop and used to control the tracking of the tip over the surface.

Amplitude detection in the non-contact method is usually used for high-amplitude cantilever vibration. When the cantilever vibration amplitude is relatively small and/or
higher sensitivity is needed for stable feedback, the phase detection method is used \cite{91-93}.

**"Tapping" mode**

With *intermittent contact* (alternatively named "tapping" or AC mode), the tip oscillates at its resonant frequency, close to the sample surface where it repeatedly comes into and out of contact with it. In this way the trapping of AFM tip by the capillary forces caused by the water molecules surrounding the sample in air is prevented. By using "tapping" mode the capillary forces and the lateral forces on the samples are reduced due to the shorter time spent of the cantilever on the sample surface, the distortion or damaging of the sample being avoided.

During the scan, the tip experiences attractive and repulsive forces that depend on the chemical and mechanical properties of the sample. Conventional AFM tips are limited in their ability to resolve these time varying forces \cite{45}. When biological samples are investigated it is important to bind the sample on the substrate in order to avoid dragging by the tip, but, at the same time, such binding should not cause structural alterations to the sample. Also, to avoid sample dragging, it is preferable to use non-contact or "tapping" imaging modes when biological samples are examined. The ideal solid substrate for biological samples to be deposited and investigated has to be smooth at an atomic level and it should have a high affinity for the studied sample. Atomic level smoothness is required in order to avoid incorrect interpretation of the sample topography. The affinity should allow its adsorption but should keep the sample at least partially functional. The adsorption is due to electrostatic, polar or van der Waals interactions that can be adjusted by changes in pH, ionic strength or solution composition. Glass and muscovite mica \cite{94} are commonly used as substrates. Muscovite mica consists of layers of (Si,Al)$_2$O$_5$ ionically bound to a central layer of Al$_2$(OH)$_2$, the negative charge being compensated by potassium ions. The glass surfaces have the
disadvantage that they exhibit irregularities on the nanometer scale. Samples have to be placed on clean substrates. Depending on the substrates, different cleaning procedures have to be applied. For example, for glass substrates, cleaning has to be done in such a manner that the surface remains hydrophilic. Due to its cleaving properties, mica is easily cleaned with sticky tape that when pulled off, produces a hydrophilic and smooth surface at atomic level. In aqueous samples, both substrates can be used, as they present negative surface charges that allows an easy adsorption of positive surface charge molecules. Another substrate used is extremely smooth gold surface deposited on mica. The substrate has to be used immediately after preparation otherwise it will be quickly covered with hydrocarbons due to air substrate-interactions. The contamination problem appears when AFM tips absorb these hydrocarbons. In order to clean the organic contamination, the substrate or the tip can be exposed to an UV radiation. Great care has to be taken with all aqueous solutions that are used in the measurements or cleaning of the equipment. It is recommended to use ultra-pure water, as it contains lower levels of hydrocarbons and other contaminants than the conventional bi-distilled water. Due to their ease of handling and preparation, mica substrates were the main substrates used in this thesis.

1.5.2. Attenuated total reflectance - Fourier transform infrared spectroscopy

One of the analytical protein techniques available today is infrared spectroscopy, its great advantage being that, virtually any sample in any state can be studied. Thus, with an appropriate choice of sampling technique liquids, solutions, pastes, powders, films, gases and surfaces can all be examined \textsuperscript{[95]}. Infrared (IR) radiation refers broadly to the part of the electromagnetic spectrum between the visible and microwave regions and IR spectroscopy begun in 1800 when the astronomer Friedrich Wilhelm Herschel analyzed the spectrum of sunlight. In order to divide the sunlight into its colors Herschel...
Chapter One

directed the sunlight through a glass prism and measured the heating ability of each color using thermometers with blackened bulbs. Beyond the red part of the spectrum he observed the highest heating ability of all. He concluded that beyond the red part of the spectrum should be a kind of radiation invisible to the human eye. This radiation became known as “infrared” (below red) light. Herschel placed a container filled with water between the prism and thermometers and observed that the temperature measured was lower than the one measured without water. As a consequence he concluded that the water must partially absorb this radiation [96]. The infrared spectrum is between 0.7 µm and 350 µm, but for organic chemistry and related areas such as biology and food science, the region of interest is the mid-infrared region, namely between 5 µm and 40 µm.

Infrared spectrometers became commercially available in the 1940s and used prisms as dispersive elements. In the mid 1950’s these were replaced by diffraction gratings. The introduction of Fourier-transform spectrometers was one of the most significant advances in IR spectroscopy. This type of instrument employs an interferometer and exploits the well established mathematical process of Fourier-transformation. The use of Fourier-transform infrared (FTIR) spectroscopy has dramatically improved the quality of IR spectra and minimized the time required to obtain data. Fourier transform instruments use a Michelson interferometer that has a fixed and a moving mirror, their relative positions being accurately measured. The light from a laser (usually a helium - neon laser) with a defined wavelength is split in two. One half is sent to the fixed mirror and the other half to the moving one. The beams are recombined afterwards. The position of the moving mirror determines if constructive and destructive interference is obtained. The intensity of the recombined beam is a sine wave as function of position, and the nodes in the sine wave act as a ruler. The IR light beam that contains all the wavelengths of experimental interest is split, sent to the
mirrors, and recombined. Different IR waves will interfere at different mirror positions. The total intensity is collected by the instrument as function of mirror position. The recorded function of intensity versus mirror position is Fourier transformed into intensity as a function of frequency - that is, the normal infrared absorption spectrum. All IR frequencies from the Michelson interferometer are recorded and transformed simultaneously by a computer. Thus the spectrum is usually collected within seconds without requiring scanning.[97]

Many measurement methods are available for sample analysis using IR spectroscopy. The most used techniques are transmission and attenuated total reflection (ATR) methods. In transmission analysis, the sample has to be optically thin since variations in concentration or chemical compositions within the sample can lead to erroneous results and the sample has to be as homogenous as possible. The ATR method requires little to no preparation of the sample and is widely used in biological/food science.

Attenuated total reflectance spectroscopy utilizes the total internal reflection phenomenon (Figure 1.5).

![Attenuated total reflectance spectroscopy](Image)

Figure 1.5. Principle of the attenuated total reflection method.
A beam of IR radiation is sent through a crystal onto the sample. The beam experiences total internal reflection when the angle of incidence at the interface between the sample and crystal is greater than the critical angle. The beam penetrates a fraction of a wavelength beyond the reflecting surface and when a material that selectively absorbs radiation is in close contact with the reflecting surface, the beam loses energy at the wavelength where the material absorbs. The resultant attenuated radiation is measured and plotted as a function of wavelength by the spectrometer and gives rise to the absorption characteristics of the sample. Experimentally, modern spectrometers display the spectroscopic data as function of wavenumbers, which are reciprocal wavelengths in units of cm\(^{-1}\).

The electromagnetic wave that penetrates into the sample is called an evanescent wave. The infrared light beam penetrates the sample and the depth of penetration \(d_p\) can be quantitatively described by Harrick’s approximation:

\[
d_p = \frac{\lambda}{2\pi \cdot n_1 \cdot (\sin^2 \theta - (\frac{n_2}{n_1})^2)^{\frac{1}{2}}}
\]

where \(\lambda\) is the wavelength, \(n_1\) – the refraction index of the crystal, \(n_2\) – the refraction index of the sample, \(\theta\) - the incidence angle.

The depth of penetration \(d_p\) is defined as the distance between the surface of the sample and the position where the intensity of the penetrating evanescent wave dies off to \((1/e)^2\) or 13.5%, or its amplitude has decayed to \(1/e\).

In an absorption spectrum the intensity of a peak is directly proportional to the concentration of the sample substance. This is defined by Lambert - Beer law:

\[
A = \varepsilon \cdot L \cdot c
\]

where
A = log(I/I_0) - the absorption maximum at a given wavelength measured in arbitrary units (a.u.), I - the intensity of the incident beam, I_0 - intensity of the light beam leaving the sample.

$\varepsilon$ - molar absorption coefficient defined as absorption probability at a given wavelength measured in L mol^{-1} cm^{-1}

L - path length for samples in the cell or sample thickness for pressed pellets for films (cm)

c - sample concentration (mol L^{-1})

Infrared spectroscopy is based on the vibrations of the atoms within a molecule. An infrared spectrum is commonly obtained by passing IR radiation through a sample and determining what fraction of the incident radiation is absorbed at a particular energy. The energy at which any peak in an absorption spectrum appears corresponds to the frequency of a vibration of a part of a sample molecule. The interactions of IR radiation with matter may be understood in terms of changes in molecular dipoles associated with vibrations and rotations.

For proteins, the main contributions to infrared spectra are from the amide I and amide II bands (Figure 1.6). The amide I contribution is observed in the 1700 - 1600 cm^{-1} interval and is due, almost entirely, to the C=O stretch vibrations (~ 80%) coupled with in-plane NH bending (< 20%). The frequencies of the amide I components are related to the secondary structural elements of the proteins \cite{95, 98}. Due to the unique molecular geometry and hydrogen bonding pattern each secondary structural element will have a different well defined C=O stretching frequency. On the other hand, the contribution of the amide II to the spectrum occurs in the 1600 - 1500 cm^{-1} interval and derives mainly from in-plane NH bending (40 – 60%) and from CN stretching vibrations (18 - 40%). Therefore, the amide II group is less sensitive than amide I group in reflecting the conformational changes of the proteins \cite{95}. 
The amide I band for native β-lg contains region correspond to β-sheets, α-helix, turns, and random coils. Thus, the bands at 1630 cm$^{-1}$ and 1675 cm$^{-1}$ correspond to β-sheets structures, 1686 cm$^{-1}$ and 1645 cm$^{-1}$ to turns and random coils, while that at 1652 cm$^{-1}$ represent α-helix. The side chain residues are reflected by 1605 cm$^{-1}$ line. The bands do not correspond only to the secondary structures of the individual molecules, but also depend to a certain extent on the interaction between the molecules.

**Figure 1.6.** Typical vibrations in the IR spectrum of bovine serum albumin.

### 1.5.3. Fluorescence spectroscopy

Fluorescence spectroscopy is an important investigation tool used extensively in many areas of analytical sciences (e.g. biochemistry, biophysics, medical and food science) due to its extremely high sensitivity$^{[99, 100]}$ that allow detailed, real time observation of the structure and dynamics of the analyte. It can be used either for qualitative (analysis of the emission spectrum of the analyte) or quantitative (to measure the fluorescence of the analyte solution at one wavelength) measurements$^{[100]}$. 
In fluorescence spectroscopy the emitted electromagnetic radiation from the analyte as it relaxes from an excited electronic energy level to its corresponding ground state is measured, where the analyte was initially activated to a higher energy level by the absorption of radiation in the UV or visible range. During a fluorescence measurement the processes of activation (photon absorption) and deactivation (fluorescence emission) occur simultaneously. For each unique molecular system, there will be an optimum radiation wavelength for sample excitation and another, of longer wavelength, for monitoring fluorescence emission. Even though the excitation and emission wavelengths depend on the system studied, the fluorescence spectrum will be located at higher wavelengths (lower energy) than the absorption spectrum because of the energy loss in the excited state due to vibrational relaxation. As with absorption assays, under optimal conditions, there will be a direct linear relationship between the concentration of the analyte in the unknown solution and the fluorescence intensity.

In general, an instrument used for fluorescence spectroscopy contains the radiation source, the wavelength selector, the sample holding cell, the radiation detector and a readout device. It uses a light beam tailored for the type of fluorescence detection needed. Usually, the ultraviolet part of the electromagnetic spectrum is used to excite the electrons in the molecules causing them to emit light of lower energy that can be within the visible and non-visible part of the spectrum.

**Thioflavin T fluorescence**

To confirm the presence of fibrillar or “amyloid” structures, hydrophobic fluorescent dyes such as thioflavin T (ThT), Congo red or 8-anilinonaphthalene sulfonate (ANS) can be used. ThT undergoes characteristic spectral changes on binding to the β-sheets of proteins. Therefore, by monitoring the changes in the fluorescence signal, a better understanding of fibril stability can be obtained. When ThT
was used with native β-lactoglobulin solutions, a low fluorescence signal was reported in the literature due to the relatively low number of binding sites \[^{101, 103}\]. Investigating solutions of β-lactoglobulin fibrillar spherulites viewed under cross polars using confocal microscopy, Krebs et al. \[^{103}\] suggested that ThT binds with its long axis parallel to that of the fibrillar structures present in spherulites. The binding sites of ThT molecules were found to be the channels formed by the β-sheet residues, the intensity of the fluorescence signal being proportional with the number of available run channels \[^{103}\]. Therefore, in the present study, ThT fluorescence was used to monitor fibril formation and stability as the number of available ThT binding sites should vary depending on the applied treatment.

### 1.5.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Electrophoresis describes the migration of charged molecules in medium under the influence of an electric field \[^{61}\]. Their rate of migration depends on the strength of the field, on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique. Many important biological molecules such as amino acids, peptides, proteins, nucleotides, and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-). Depending on the nature of the net charge, the charged particles will migrate either to the cathode or to the anode \[^{104, 105}\].

There are two basic types of materials used to make gels: agarose and polyacrylamide. The SDS-PAGE technique was introduced by Raymond and Weintraub in 1959 and since then it is a widely use technique. SDS-PAGE is an abbreviation for
sodium dodecyl sulphate -polyacrylamide gel electrophoresis \[^{[104, 105]}\]. In spite of the many physical arrangements for the apparatus, and regardless of the medium through which molecules are allowed to migrate, all electrophoretic separations depend upon the charge distribution of the molecules being separated. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide. SDS break up the 3D structure of the protein leaving only the primary structure conferring a negative charge to the polypeptide in proportion to its length – i.e.: the denatured polypeptides become "rods" of negative charge with equal charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the rod configuration necessary for separation by size: this is done with \(\beta\)-mercaptoethanol. During denaturation, SDS-PAGE migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

Proteins are commonly analyzed by SDS-PAGE using a modified method by Laemmli in 1970 \[^{[105]}\], in which the separating gel contained of 15 % acrylamide and a 4 % stacking gel, both containing 0.1% SDS. The samples are loaded in wells of the polyacrylamide gel. The proteins move into the gel when an electric field is applied. The gel minimizes convection currents caused by small temperature gradients, and it minimizes protein movements other than those induced by the electric field.

Proteins can be visualized after electrophoresis by treating the gel with a stain such as Coomassie blue, which binds to the proteins but not to the gel itself. Each band on the gel represents a different protein (or protein subunit); low molecular weight proteins are found near the bottom of the gel.
1.6. Outline of thesis

The overall aim of this study is to investigate the mechanism of assembly of whey proteins into fibrils and to characterise their properties for possible use in food applications.

The specific objectives are to:

1) optimize and understand the mechanism of heat-induced fibrillar aggregation of bovine β-lactoglobulin;

2) investigate the β-lg fibril stability at different pH and the effect of high dynamic pressure using microfluidization (MF);

3) determine the functional properties of whey protein fibrils, particularly foam, and their potential use as food ingredients.

Figure 1.7. shows a schematic overview of the thesis content.

**Figure 1.7. Overview of thesis.**

The thesis is divided into seven chapters, beginning with an introductory chapter containing a literature review about whey proteins and aggregation processes followed
Chapter One

by an introduction on the investigations techniques used throughout this study (chapter 1).

The first objective of the thesis is to obtain an improved understanding of the mechanism of formation of β-lg fibrils. Therefore, chapter 2 and chapter 3 were dedicated to the study of heat induced aggregation of whey proteins, specifically of β-lg. Experimental parameters such as protein concentration, pH, ionic strength, time and temperature of heating were varied to optimize the process of fibril formation.

The second objective of the thesis was to study the effect of acidification, heating, shearing, and/or high - pressure treatment on β-lg and their aggregates (chapter 4 and 5). The pH stability of β-lg fibrils length was studied in chapter 4. The effect of high dynamic pressure on β-lg before and after fibril formation was investigated using AFM, STEM, ATR-FTIR and fluorescence spectroscopy, while the length distribution of the fibrils was determined using flow-induced birefringence.

The final experimental part of the thesis was focused on the functional properties of whey protein fibrils (chapter 6), in particular on their foam capacity and stability. The foaming properties were related to the protein concentration, pH, whipping time, thermal and/or high pressure treatment of the protein and their assemblies.

The general conclusions of the thesis are detailed in chapter 7 together with a discussion on the possible applications of the fibrils. The results of a series of trials performed in order to observe the behavior of protein fibrils in the presence of other ingredients are described. These results provide an indication of the properties of whey protein fibrils that might be exploited and developed for possible applications.


29. S. Guinand and J. Tonnelat, *Etude thermodynamique de la dissociation réversible de la β-lactoglobuline B pour des Ph supérieurs à 5.5*. Biochimica et Biophysica Acta. 1962, **59**: p. 737-739.


Chapter 2.

Characterization of β-lactoglobulin assemblies as a function of pH and heating time
2.1. Introduction

Fundamental research of whey protein is required because of their extensive use as food ingredients (e.g. thickening or gelling agents, etc.). Whey proteins can adopt different conformations under certain conditions such as different protein concentration, pH, ionic strength, temperature or solvent composition. They have the ability to form long fibrillar structures at acidic pH and large aggregates at higher pH. Whey proteins often undergo heat treatment during food processes such as spray drying and this can lead to uncontrolled denaturation, aggregation and poor quality products. The major component of whey protein is β-lactoglobulin (β-lg) and it has become a standard protein in describing the physical-chemical properties of protein structure [1-3].

Native β-lg at neutral pH was found to exist as a mixture of monomer and a non-covalent dimer that dissociates into native monomers at acidic (< 3.5) or basic pH (> 7.5) [4-6]. Irreversible denaturation of β-lg occurs on heating above 65 °C at neutral pH or on storage in alkaline solution at 25° C (slowly in the pH range 8 - 9.5, more rapidly in solutions of higher pH) [4, 7].

Thermal treatment of low concentrations of β-lg at 80 °C and pH 7 for 20 h involved dissociation of native monomers/dimers into native or denatured monomers. This was accompanied by sulphydryl group (-SH) exposure to the solvent and followed by protein cross linking via intermolecular disulphide (S-S) and non-covalent bonds, which lead to irreversible aggregate formation [3, 8-19]. As denaturation progresses, the free SH group was exposed to the solvent and the intramolecular disulphide bridges break up [8, 14, 15]. In the native state, the interactions between the globular protein molecules are repulsive (mainly electrostatic, hydration and entropic). These interactions are sufficiently strong to overcome the attractive interactions (mainly van der Waals, hydrophobic) and, therefore, the proteins exist as individual entities or as small aggregates (e.g. dimers, octomers etc.) [15, 16]. After protein denaturation,
intermolecular S-S bonds formed by SH - S-S interchange or SH oxidation led to β-lg aggregates formation \[8, 14-16\]. In order to study the influence of pH on the aggregation process and the possibility for fibrillar structure formation at different pH, experiments at pH 7 and 12, were performed. β-Lactoglobulin solutions with a protein concentration of 2% (w/w) were heated at 80 °C, the aggregation process and the changes in the secondary structure as function of heating time being monitored using ATR-FTIR. The resultant aggregates were investigated using oscillatory or “AC” mode of AFM. In biopolymer studies, AFM has become a standard investigation technique for visualizing the structures at the molecular scale \[20-22\]. Changes in the protein secondary structure caused by heat denaturation and aggregation were monitored in the amide I region (1600 - 1700 cm\(^{-1}\)). They were observed as alterations in the shape of the infrared absorption spectra. The observed amide I band is a complex composite, consisting of a number of overlapping component bands representing α-helix (1654 cm\(^{-1}\)), low and high-frequency components of β-structures (1630 - 1675 cm\(^{-1}\)), turns (1686 cm\(^{-1}\)) and random structures (1645 cm\(^{-1}\)) corresponds to the disordered parts of polypeptide backbones \[23-25\].

2.2. Materials and methods

2.2.1. Sample preparation

Bovine β-lactoglobulin [approximately 90% pure (PAGE), 3 x crystallized and lyophilized; mixture of genetic variants A and B, reference L0103, Lot 095K7006], HCl and NaOH were obtained from Sigma Aldrich (Ireland). Whey protein isolate BiPro\(^{TM}\) [~98% (w/w) protein on dry basis: 65% β-lactoglobulin, 25% α-lactalbumin, 8% bovine serum albumin] was obtained from Davisco Foods International Inc. (Le Sueur, MN,
USA). Purified water [Milli-Q® water, resistivity (18.2 MΩ cm, at 25 ºC), Millipak® Express] was used throughout. All other reagents were analytical-grade.

2.2.2. Protein preparation

A total of 2 g β-lactoglobulin or whey protein isolate (WPI) was dissolved in 98 g Milli-Q® water, stirred overnight to ensure full hydration of the protein and the pH was adjusted to 12.0 using 6 M NaOH. Dispersions were centrifuged at 45,000 × g for 3 h at 25 ºC. The supernatant was filtered through 0.45 µm filters (Minisart, Sartorius, Germany), and subsequently heated at 80 ºC in a water bath (Grant, JPAT, Ltd.).

2.2.3. AC-mode atomic force microscopy

β-Lactoglobulin/WPI dispersions were investigated using a MFP-3D-BIO™-atomic force microscope (Asylum Research UK, Ltd., Oxford, U.K.). Samples of β-lg/WPI heated at 80 ºC for 0 min, 30 min, 100 min and 20 h were investigated using AC mode AFM. Prior to imaging, the dispersions were diluted in Milli-Q® water to a final concentration of 0.02% (w/w) (pH 7.0 ± 0.5 and 12.0 ± 0.5) to facilitate imaging of single protein aggregate. Selected samples were also diluted in 6M NaOH (pH 12.0) for AFM imaging to confirm whether dilution affected the aggregates appearance. No differences were observed between images obtained after Milli-Q® water or NaOH dilution, and, therefore, only images obtained from water-diluted solutions are presented. The imaging was performed in air-dried mode, 10 µL of β-lg or WPI diluted with Milli-Q® water being deposited onto a freshly cleaved mica surface and subsequently dried in a desiccator. An aluminum reflex coating cantilever with a tetrahedral tip (AC 240; spring constant, 1.8 N/m; resonant frequency, 70 - 90 kHz; Olympus Optical Co., Ltd., Japan) and scan rate at 1 Hz was used for air-dried samples. The tetrahedral shape is ideal for obtaining precise morphology of soft samples because
of its high lateral resolution. The radius of curvature of the tetrahedral tip was less than 10 nm. Mica was used as the substrate for all samples because of its flatness and overall negative charge when cleaved.

2.2.4. Attenuated total reflectance-Fourier transform infrared spectroscopy

Attenuated total reflectance-Fourier transform infrared spectroscopy measurements of freshly prepared 2% (w/w) β-lg in water at pH 7 or 12 were performed using a Bruker Tensor 27 spectrometer (Bruker Optik, GmBH, Ettlingen, Germany) fitted with a thermally controlled BioATR Cell II at 25°C that was specifically designed for proteins in aqueous solution. Spectra were acquired and averaged over 128 scans at a resolution of 4 cm\(^{-1}\) using Bruker Opus 5.5 software. After atmospheric compensation for absorbance of CO\(_2\) and H\(_2\)O as vapour and solute, respectively, the amide I band (1600 - 1700 cm\(^{-1}\)) was vector-normalized and the spectrum of the unheated protein sample was subtracted from the spectra of heated samples. Spectra were obtained for native and heated β-lg. The heated β-lg at 80 °C for 5 min, 10 min, 100 min, 300 min and 24 h corresponded to the stages of aggregate formation. The individual components of spectral bands were obtained using Lorentzian functions. The number of peaks and their relative positions were determined from the second order derivative of the experimental spectrum.

2.3. Results and discussion

2.3.1 Heat denatured whey proteins aggregates investigated by atomic force microscopy

Atomic force microscopy images were obtained for β-lg dispersions [2% (w/w), pH 7 and 12] that were heated at 80 °C for 0 min, 30 min, 100 min, and 20 h,
respectively. Changes of compact, globular β-lg into aggregates were monitored by AFM (Figure 2.1, Figure 2.2 and Figure 2.3). At pH 7, native β-lg solutions contained a mixture of globular structures. AFM cross section profiles reveal that heights of native β-lg molecules are ca. 2.0 nm ± 0.35 nm (Figure 2.1.a) being smaller than the hydrodynamic diameter values of β-lg monomer presented in the literature (4 - 6 nm)\textsuperscript{16} and can be explained by β-lg packing when deposited on the substrate \textsuperscript{26}. The AFM image shows that the protein molecules are tight packed and, therefore, only the upper region of the protein molecule will be detected by AFM. Thus, the measured height of 2 nm is closer to the radius value of the β-lg than the diameter. The presence of β-lg molecules in dispersions as monomers and dimers form can be explained based on strong repulsive interactions between them that prevent the native proteins from aggregating \textsuperscript{15, 16}.

Upon prolonged heating (up to 20 h) the monomers were denatured (the hydrophobic part being exposed), the hydrophobic interactions between them increased and large aggregates were formed. Denatured monomers and/or dimers formed globular structures with heights of 2.5 nm ± 0.5 nm (Figure 2.1.b). The conformation adopted by the native/denatured protein depends on the balance between attractive interactions that favour the native state and repulsive interactions (configurational entropy) that favour the unfolded protein \textsuperscript{15}. The unfolded proteins can either aggregate or remain as individual molecules function of the balance between attractive and repulsive interactions between them.
Figure 2.1. AFM images of (a) native 2% (w/w) β-lactoglobulin solution at pH 7 and (b) aggregates formed after heating at 80 °C for 30 min.

Figure 2.2. AFM images of aggregates present in a 2% (w/w) β-lactoglobulin solution at pH 7 after heating for 20 h at 80 °C. b) High resolution image of 500 nm x 500 nm area showing β-lg structures.

The size and shape of the aggregates did not significantly change even after the samples were heated over a longer time period (20 h) (Figure 2.2.a and b). Only small aggregates and their assembly in clusters of height 5.5 nm ± 0.5 nm were observed.

Recently, Ikeda et al. proposed two stages in the β-lg aggregation process [3]. After heating 2% (w/w) β-lg at pH 7 at 80 °C for 60 min large aggregates (18 ± 4 nm)
composed of polydisperse granular aggregates were obtained starting from the monomer of < 2 nm height \(^3\). No fibrils were formed at pH 7 as at pH 2. This can be explained by the fact that the main contribution in the aggregation is due to the intermolecular covalent bonds (-SH/ S-S interchange, -SH oxidation reaction). While the initial aggregation of the protein was mainly due to hydrophobic interactions, disulfide bonds have an important role in strengthening (the second stage) of the aggregates through intra- and intermolecular S-S bonding.

On occasion, when the sample of β-lg aggregates at pH 7 was deposited on the mica substrate prior to AFM analysis, fractal structures were obtained (images not showed). The patterns observed are due to the fingering effect that usually appears when a solution containing only spherical nanoparticles dries on a surface.

At pH 12, globular β-lg structure with heights of 1.5 nm ± 0.5 nm were observed (Figure 2.3.a). Under prolonged heating (up 20 h), denaturation of monomers was followed by protein aggregation. A mixture of non-aggregates and aggregates of β-lg were observed in the aqueous dispersion, the height of the protein aggregates increasing to 2 - 3 nm (Figure 2.3.b). As expected for 2% (w/w) β-lg dispersion at pH 12, the granular aggregates were mainly formed by intermolecular S-S bonds \(^3\) and non-covalent interactions \(^{15, 16}\). After protein denaturation, attractive interactions (hydrophobic, disulfide) are very strong, but there are short range interactions and lead to small aggregates formation even after 20 h heat. As at pH 7, fractal structures as previously described can be formed when the solution is placed on the substrate.

Similar experiments as for β-lg solutions were carried out using WPI. The AFM results are similar to those obtained for β-lg at high pH and 80 °C and can be explained by the dominance of β-lg in WPI composition.
**Figure 2.3.** AFM images of 2 % (w/w) β-lactoglobulin solution at pH 12 heated at 80 °C for a) 0 min; b) 20 h. Inset: Higher resolution image of 500 nm x 500 nm area showing native or aggregates of β-lg.

### 2.3.2. Attenuated total reflectance-Fourier transform infrared spectroscopy

Attenuated total reflectance-Fourier transform infrared spectroscopy measurements allowed observation of changes in the secondary structure during the denaturation and self-assembly processes of β-lg at 80 °C \[12, 23, 24, 27\]. The spectra of β-lg, native and heated at pH 7 (Figure 2.4.a) illustrate that heat-treatment at pH 7 had a pronounced effect on the secondary structure of the protein.

Curve fitting of the amide I band of native β-lg at pH 7 that had been heated at 80 °C for different time intervals (up to 24 h) revealed absorption bands at 1618, 1626, 1633, 1641, 1652, 1663, 1680 cm\(^{-1}\) that are in good agreement with those previously presented in the literature \[12, 23, 24, 28\] (Figure 2.4.b).
Increasing the heating time induced disruption of the native structure of β-lg\textsuperscript{[15]}, causing changes in the 1620 - 1635 cm\textsuperscript{-1} and 1630-1680 cm\textsuperscript{-1} region attributed to the intra and intermolecular bonds (Figure 2.4.a). The mixture of monomers and dimers of β-lg dispersions at pH 7 was suggested by the presence of two components in this region at 1626 and 1633 cm\textsuperscript{-1} as illustrated in Figure 2.4.b\textsuperscript{[12, 24, 27, 28]}.

The ATR-FTIR results suggest that the process of formation of β-lg aggregates at pH 7 takes place in two stages. In the first stage (up to 10 min heating time), dissociation of the dimers into monomers followed by denaturation is characterized by a
decrease in band intensity and a shift of the intramolecular β-sheets band (1626 and 1633 cm\(^{-1}\)) and a slightly decrease of the α-helix content (1652 cm\(^{-1}\)). The heated β-lg spectra are similar to that of unheated solution, though minor changes suggest a partial denaturation of β-lg at this stage\(^{[23]}\).

The second stage (100 min - 24 h) was characterized by the progressive decrease or elimination of the intramolecular β-sheets content (1626 and 1633 cm\(^{-1}\)). A new intermolecular β-sheet band appeared at 1622 cm\(^{-1}\) which can be attributed to the formation of extensive β-sheets region involved in intermolecular cross-linking of the unfolded protein. Irreversible denaturation and aggregation processes induced a decrease in the secondary protein structure. This was accompanied by the decrease in α-helix (1652 cm\(^{-1}\)), unordered structures (1641 cm\(^{-1}\)), turns and β-sheets (1663 and 1680 cm\(^{-1}\)) content. A small increase of the side-chain residue shoulder (1618 cm\(^{-1}\)) was also observed (Figure 2.4.a). All these changes facilitate the formation of large aggregates of β-lg at pH 7 after prolonged heating\(^{[24, 27, 28]}\).

Subtraction of the native β-lg spectrum from the heated samples spectra confirmed the conformational changes of the secondary structures during the heating. This was reflected by the significant increase of the intermolecular β-sheets band in the 1623 - 1600 cm\(^{-1}\) range (Figure 2.4.c). The large increase of the 1614 cm\(^{-1}\) band (10 min - 24 h) and the decrease of the 1694 cm\(^{-1}\) band (specific to the rearrangement of β-sheets after S-S bonds cleavage) observed in Figure 2.4.c can be ascribed to denatured monomers present in the aggregates structure\(^{[24, 27]}\). Stable and irreversible aggregates were formed by non-native S-S bonds or non-covalent bonds (hydrogen bonds, hydrophobic and/or van der Waals interactions) after prolong heating of β-lg at pH 7\(^{[14, 27]}\).

The effect of heat treatment on the internal structure of the native β-lg at pH 12 can be observed in Figure 2.5.a. The β-lg monomer-dimer ratio changed as the
monomers number increased with the pH changing from 7 to 12. High monomer concentration was reflected by the main monomer band present in the 1620 and 1635 cm\(^{-1}\) range as illustrated in Figure 2.5.a. Curve fitting of the amide I band of native β-lg at pH 12 that had been heated at 80 °C for different time intervals (up to 24 h) revealed absorption bands at 1624, 1634, 1644, 1654, 1665, 1678 cm\(^{-1}\) which are in good agreement with those in the literature\(^{[23, 24]}\) (Figure 2.5.b). Irreversible denaturation and aggregation of the protein was characterized by the IR spectra after subtraction of the native β-lg from the heated samples and showed a decrease in the content of intramolecular bonds and an overall increase in the amount of intermolecular β-sheets (1628 - 1630 cm\(^{-1}\)) formed during aggregation (Figure 2.5.c). The denaturation and aggregation of β-lg at pH 12 induced a loss in the secondary structure characterized by a decrease in the α-helix, intramolecular β-sheets, turns and random coils.
Chapter Two

Figure 2.5. (a) Amide I of ATR-FTIR spectra of 2% (w/w) β-lactoglobulin at pH 12 heated at different times (up to 24 h); (b) Curve fitting for amide I of ATR-FTIR spectra for 2% (w/w) β-lg at pH 12; (c) Amide I spectra of the heated samples after native β-lg at pH 12 spectrum subtraction varying from 5 min to 24 h.

2.4. Conclusions

Above the denaturation temperature (80 °C), β-lactoglobulin aggregation depends on pH. Thus, in neutral and alkaline dispersions protein aggregation follows the general model of Roefs and Kruif [5]. After thermal denaturation, globular β-lg (~2 nm height) aggregate via covalent and non covalent bonds [13, 15, 16] into larger clusters ~6 nm height. The aggregation process can be summarized in three steps: 1) reversible or irreversible denaturation of the protein followed by 2) aggregation of denatured proteins
into primary aggregates accompanied by the denaturation and 3) further aggregation of
the primary aggregates into clusters of larger aggregates after 20 h heating.

The aggregation mechanism of the whey protein at neutral and alkaline pH
seems to take place more rapidly than the fibril model presented in chapter 1. The fibril
aggregation model implies longer heating times for mature and consolidated structures
to be obtained. After heating at pH 7 and 12, β-lg aggregates form within minutes into
spherical and/or short length aggregates. Moreover, these aggregate structures can
assemble into large clusters via covalent and non covalent interactions [15].

The heat induced loss in the secondary structure of the native β-lg was due to
denaturation and aggregation processes. ATR-FTIR has been used to compare the
structure of 2% (w/w) β-lg at pH 7 or 12. The amide I region of the infrared spectra
illustrated the conformational changes on the β-lg structure induced by thermal
treatment at different pH. During the thermal treatment, reversible denaturation of β-lg
at pH 7 shifts the monomer-dimer equilibrium (in the first 10 min of heating). After
prolonged heating times, denaturation was followed by an aggregation step in the same
time with a continuous irreversible protein denaturation. The pronounced effect of the
thermal treatment on protein leads to the diminishing and/or the disappearance of
intramolecular bonds (1626 and 1633 cm⁻¹) and the formation of intermolecular bonding
β-sheets structures at 1622 cm⁻¹. The thermal treatment on β-lg at pH 12 leads to the
appearance of new intermolecular β-sheets (1628-1630 cm⁻¹) emphasizing the
aggregation of the denatured protein.
2.5. References


Chapter 3.

Characterization of the assembly of

β-lactoglobulin fibrils

3.1. Introduction

Whey proteins in bovine milk contain a mixture of β-lactoglobulin, α-lactalbumin, and bovine serum albumin, all of which may form fibrillar aggregates under specific conditions \(1, 2\). β-Lactoglobulin is the major whey protein in cow’s milk, representing ~ 50% of the total whey protein content. β-Lactoglobulin is a globular milk protein, whose secondary structure contains approximately 50% β-sheet, 9 - 12% α-helix, 8 - 10% turn, and 30 - 35% random coil \(3\) and forms fibrillar aggregates 2 - 4 nm in thickness upon prolonged heating at low pH and low ionic strength \(4, 5\). No fibrils are formed upon heating pure α-la or BSA at pH 2, but fibrils are formed in pure β-lg and WPI solutions \(1, 2, 6, 7\). Various studies have shown that β-lg structures have the potential to be used as functional ingredients in food products because of their unique functional properties, for example, emulsification, gelation, thickening, foaming, and water-binding capacity \(8-10\). These protein fibrils could be used as thickening ingredients in low-calorie products, for example, as replacements for polysaccharide based ingredients or meat replacement products and high protein content foods. The addition of small amounts of protein fibrils may increase the viscosity and shear thinning behaviour of food products. Arnaudov et al. \(11\) established the critical protein concentration for fibril formation as 2.5% (w/w) using nuclear magnetic resonance (NMR) spectroscopy and static light scattering, while AFM data suggested that the fibrils had an irregular helical structure, with a thickness of one or two protein monomers and a periodicity of approximately 28 nm. The effect of increasing the ionic strength (using NaCl or CaCl\(_2\)) on the morphology of the fibrillar aggregates formed from β-lg at pH 2.0 upon heating at 80 °C lead to an increase in the rate of fibril formation but a decrease in length, most likely because of screening of electrostatic
repulsions\textsuperscript{[4, 6, 11, 12]}. At low ionic strength, the $\beta$-lg fibrils are long and straight, whereas at high ionic strength ($\geq 0.08$), they are short and curved. The fibrils obtained at all ionic strengths exhibit similar thicknesses and periodic morphology. Similar results showed that fine-stranded aggregates of $\beta$-lg appeared as strings of monomers\textsuperscript{[13]}.

Nevertheless, a more recent study suggests that $\beta$-lg fibrils formed at pH 2 were composed of peptides and that intact $\beta$-lg monomers were not present in the fibrils\textsuperscript{[14]}. In this study, oscillatory or “AC” mode of AFM was used to investigate the fibrillar assembly process of $\beta$-lg heated at 80 °C. A difficulty with previously reported AFM data is that images were obtained in an air, which could affect the appearance of the fibrils\textsuperscript{[15]}. To overcome this drawback, AFM imaging of $\beta$-lg and WPI in liquid and air environments was performed and the results were compared. It has been argued that electrostatic or hydrophobic interactions with the mica surface affect the morphology and elongation pattern of the fibrils as determined by AFM\textsuperscript{[16]}. Therefore, to confirm that fibrillar structures were formed, STEM was also performed on selected samples. Previous research showed that the $\beta$-sheets display a spectral band at $\sim 1630$ cm$^{-1}$, while the $\alpha$-helix display at 1654 cm$^{-1}$\textsuperscript{[17, 18]}. To clarify the process of fibril formation in a $\beta$-lg dispersion heated at 80 °C and pH 2, changes in the secondary protein structure were followed in real time using ATR-FTIR at 80 °C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis under reducing conditions was also performed to investigate time-dependent changes in the molecular weight of $\beta$-lg fractions, specifically to determine whether fibrils were composed of $\beta$-lg monomers or polypeptide fragments. The fibril formation processes of $\beta$-lg and WPI are similar.
3. 2. Materials and methods

3. 2. 1. Sample preparation

β-Lactoglobulin or whey protein isolate were used as in chapter 2 of this thesis. HCl was purchased from Sigma Aldrich (Ireland). Phosphotungstic acid was purchased from Agar Scientific (Stansted, Essex, UK). Molecular weight standards were purchased from GE Healthcare (Chalfont St. Giles, UK). All other reagents were analytical-grade.

3. 2. 2. Protein fibrillar dispersion

2% (w/w) β-lactoglobulin or whey protein isolate fibrillar dispersion at pH 2 was prepared following the same procedure as in chapter 2.

3. 2. 3. AC-mode atomic force microscopy

β-Lactoglobulin dispersions were investigated using a MFP-3D-BIO™ atomic force microscope (Asylum Research UK, Ltd., Oxford, U.K.) as previously described in chapter 2. Samples of β-lg heated at 80 °C for 0, 15, 35, 45, 65, 85, 100, 200, 300 min, and 20 h, respectively, were used for AFM analysis. Prior to imaging, the dispersions were diluted in Milli-Q® water to a final concentration of 0.02% (w/w) (pH 2.6 ± 0.3) to facilitate imaging of single fibrils. Selected samples were also diluted in HCl (pH 2.0) for AFM imaging to confirm whether dilution affected the fibril appearance. No differences were observed between images obtained after water or HCl dilution, and, only images obtained from water-diluted solutions are presented. 10 µL of β-lg diluted with Milli-Q® water was deposited onto a freshly cleaved mica surface and subsequently dried in a desiccator. For AFM imaging in water environment 100 µL of diluted β-
lg/WPI was deposited on the mica surface. After a period of 10 min to allow for protein adsorption, AFM images were obtained using sharp and tall V-shaped “Biolever 150” silicon nitride tips (spring constant, 0.03 N/m; Olympus Optical Co., Ltd., Japan). The radius of curvature of the tip was 40 ± 3 nm, with a tip height of 5 µm, which helped to prevent the cantilever from touching the specimen. Oscillatory frequencies in water were in the range of 5 - 7.5 kHz. The scan rate was set at 1 Hz. Mica was used as the substrate for all samples because of its flatness and overall negative charge when cleaved.

3. 2. 4. Scanning transmission electron microscopy

Samples of fibrillar dispersions were prepared for STEM using negative staining. A droplet of the β-lg fibrils sample, which had been diluted 1:10 in water, was deposited onto carbon support film on a 400 mesh copper grid (Agar Scientific Ltd., Stansted, Essex, U.K.). Excess liquid was removed after 1 min, using filter paper. A drop of staining solution [0.1% (w/w) phosphotungstic acid] was added, and excess solution was removed after 30 s with filter paper. Electron micrographs were taken using a Zeiss Supra 40 VP field emission scanning electron microscope (Carl Zeiss, Cambridge, U.K.) fitted with a Gemini Multi-mode® STEM detector. Bright field STEM images were acquired at 20 kV.

3. 2. 5. Attenuated total reflectance-Fourier transform infrared spectroscopy

Attenuated total reflectance Fourier transform infrared spectroscopy measurements of freshly prepared 2% (w/w) β-lg in water at pH 2 were performed using a Bruker Tensor 27 spectrometer as described in chapter 2. Spectra were obtained for
native and heated β-lg. The samples were heated directly on the crystal of the BioATR Cell at 80 °C and measurements were performed at 1, 5, 10, 21, 29, 39, 48, 59, 73, 88, and 109 min corresponding to the early stages of fibril formation. Subsequent measurements were performed on samples heated in a water bath for 109, 134, 135, 140 min, 21 h, 22 h, and 26 h, transferred immediately onto the ATR microcell, sealed, and equilibrated at 80 °C for at least 5 min before measurement. This procedure was chosen to eliminate the effect of sample evaporation when samples were heated for long time periods (>150 min) on the crystal.

3.2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to the method of Laemmli [19] under reducing conditions. Sigma grade β-lg was used for SDS-PAGE analysis. Native β-lg samples were placed in glass vials and heated at 80 °C in a water bath. The vials were removed from the bath at defined time intervals (5, 10, 20, 30, 40, 100 min and 20 h) and immediately cooled on ice. Heated and unheated β-lg samples were mixed with reducing buffer (62.2 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, and 5% β-mercaptoethanol) at a sample/buffer ratio of 1:14 and heated for 5 min at 90 °C. SDS-PAGE was performed using 12% polyacrylamide gels, at a constant voltage of 155 V in a Mini Protean II system (BioRad, Alpha Technologies, Dublin, Ireland). The gels were stained with 0.5% Coomassie Brilliant Blue R-250 (Sigma, Aldrich, St. Louis, MO). Molecular weight standards were run on the gel to allow the estimation of the molecular weight of the samples.
3.3. Results and discussion

3.3.1. Atomic force microscopy of β-lactoglobulin fibrils

To investigate the formation of β-lg fibrils, AFM height images were performed on β-lg dispersions [2% (w/w), pH 2] that were heated at 80 °C for 0 min, 15 min, 35 min, 45 min, 65 min, 85 min, 100 min, 200 min, 300 min and 20 h, respectively. The formation of fibrils from β-lg monomers was monitored by AFM measurements in air environment (Figure 3.1 and 3.2). At pH 2, native β-lg appeared as a globular monomer with a height of approximately 2 nm (0 min in Figure 3.1). During heating for 1 - 45 min, the monomers swelled and deformed, forming aggregates (Figure 3.1). After 65 min of heating, the monomers appeared to reduce in size. Upon prolonged heating (> 85 min), short and long paired fibrils with split ends were observed (Figure 3.2). The short fibrils were assumed to be precursors of the longer fibrils. After 100 min heating, samples consisted of a mixture of small aggregates and fibrils (Figure 3.2). Fibrillar subunits were observed after 200 min of heating (arrows in Figure 3.3.a and b). More and longer fibrils were formed in samples heated from 300 min to 20 h (Figure 3.4). Single β-lg fibrils had a linear morphology and periodic structure with a periodicity of ~30 - 40 nm (Figure 3.3.d), heights of ~2 - 3 nm (Figure 3.3.c) and lengths of up to 10 µm or more (Figure 3.4.a).

The thickness of overlapping fibrils was nearly 2 times higher (~ 6 nm) than single fibrils (Figure 3.3.a and b), with results being consistent with previous reports[20]. AFM phase image contrast provides high spatial resolution of surfaces, highlighting heterogeneities and relative surface property differences, allowing for a clear distinction between fibrils subunits (Figure 3.3.b). Thus, it can be seen that subunits of well-
developed fibrils exhibit similar mechanical properties (adhesion to the tip and hardness) because they induce the same changes in the phase image.

**Figure 3.1.** AFM height images of β-lg at pH 2 and 80 °C as a function of the heating time at 0, 15, 35, 45, 65 and 85 min. Images were obtained in air-dried mode. Scan size = 600 nm.
Figure 3.2. AFM height images of β-lg at pH 2 and 80 °C as a function of the heating time at 100, 200, 300 min, and 20 h. Images were obtained in air-dried mode. Scan size = 600 nm. The arrows in the image at 100 min show the early stages of fibril formation.

Average fibril heights were calculated by measuring the tip height at various points \( n = 10 \) for 10 different fibrils for each set of experiments. The cross-sectional height profiles, as measured using the MFP-3D software, demonstrate the periodicity of the fibrils and are shown in Figure 3.3.
Figure 3.3. AFM (a) height and (b) phase images of β-lg at pH 2 and 80 °C heated for 200 min. β-lg fibrils had a (c) height of ~2 - 3 nm and a (d) periodicity of ~30 nm. Images were collected in air-dried mode. Scan size = 600 nm. The arrows in the height image show the precursors of mature fibrils.

The results indicate that some denatured proteins are not incorporated into fibrils but form unordered aggregates, confirming the suggestion by Bolder et al.\cite{5} that not all denatured and hydrolyzed β-lg is incorporated into fibrils even on prolonged heat treatment (they appear as white dots shown by arrows in panels a and b of Figure 3.4).

The height of the fibrils was smaller than those previously reported by Ikeda et al.\cite{6}, who studied β-lg dispersions at pH 2 in the presence of 0.1 M NaCl after heating at 80 °C.

They observed fibrils of 4 nm in height, and concluded that the fibrils consisted of strings of partially unfolded β-lg monomers\cite{6,20}.
Figure 3.4. (a) 10 µm scan of AFM height images of β-lg at pH 2 and 80 °C heated for 20 h. Images were collected in air-dried mode. The arrows indicate denatured protein not incorporated into fibrils. (b) Magnified (2 µm x 2 µm) area of panel a, confirming the presence of non-incorporated proteins as well as fibrils.

Atomic force microscopy images of samples in water clearly show the β-lg fibrils and confirm their periodic nature (periodicity of 34 nm) (Figure 3.5.a). Similar AFM experiments performed using WPI also showed the presence of fibrils. AFM height measurements in water show that WPI fibrils have similar characteristics to β-lg fibrils and occasionally showed paired fibrils in the form of a double helix with a periodicity of ~36 nm and height of ~7 nm (Figure 3.5.b). The height of single fibril imaged in water ranged between 2.9 and 4.3 nm. Fibrils with periodic structures were seen in both the air and the water (height) images, demonstrating that the periodicity and dimensions are real and not an artefact of air drying.
Figure 3.5. (a) AFM height image of β-lg at pH 2 and 80 °C heated for 20 h and imaged in water (pH 2.6). Fibril height values were 3 nm. (Inset) Higher resolution height image of 1 µm x 1 µm area, confirming periodicity of fibrils. (b) AFM height image of WPI fibrils at pH 2 and 80 °C heated for 20 h and imaged in water. (Inset) Cross-section height profile along the fibril axis highlighting periodicity.

However, small differences in heights measured by the two modes can be observed; these are inevitable because in air the fibril volume will decrease upon drying. Measurements in air using AFM indicated a loss in height of ~25% compared to the values obtained for measurements in liquid. These observations were in agreement with Moreno-Herrero et al. [21] who studied Alzheimer paired helical filaments imaged under physiological conditions and in air environments using AFM.

3. 3. 2. Scanning transmission electron microscopy of β-lactoglobulin fibrils

Electron microscopy confirmed the presence of fibrils, indicating that these features were not caused by the charged mica surface, as assumed previously [16] (Figure
3.6. The diameters of fibrils, measured from STEM images using the microscope software, were approximately 3 nm, which is comparable to the heights measured by AFM.

![Figure 3.6. STEM negatively stained image of 2% (w/w) β-lg at pH 2 and 80 °C heated for 20 h. Scale bar = (a) 100 nm and (b) 20 nm.](image)

**Figure 3.6.** STEM negatively stained image of 2% (w/w) β-lg at pH 2 and 80 °C heated for 20 h. Scale bar = (a) 100 nm and (b) 20 nm.

### 3. 3. 3. Attenuated total reflectance-Fourier transform infrared spectroscopy of β-lg fibrils formation

Attenuated total reflectance-Fourier transform infrared spectroscopy experiments allowed *in-situ* observation of changes in the secondary structure during the denaturation and self-assembly processes of β-lg at 80 °C. As previously described in chapter 2, the amide I band is very sensitive to changes in the secondary structure of β-lg during the denaturation and aggregation processes \(^{22, 23}\). The spectra of native and heated β-lg (Figure 3.7) illustrate that heat-treatment at pH 2 had a pronounced effect on the secondary structure of the protein.
Curve fitting of the amide I band of β-lg incubated at pH 2 and heated for 1 min at 80 °C revealed absorption bands at 1614, 1624, 1631, 1640, 1650, 1660, 1670, and 1680 cm⁻¹, which are in good agreement with previous reports [18, 22, 23].

Figure 3.7. Amide I ATR-FTIR spectra of 2% (w/w) β-lg heated at 80 °C varying (a) from 1 to 109 min and (b) from 134 to 1560 min (26 h). Amide I spectra of the heated samples after native β-lg spectrum subtraction varying (c) from 5 to 109 min and (d) from 134 min to 26 h.

The observed amide I band is a complex composite, consisting of a number of overlapping component bands representing α-helices (1654 cm⁻¹), low and high-frequency components of β-structures (1630 and 1670 cm⁻¹), turns (1681 cm⁻¹) and
random coils (1645 cm\(^{-1}\)) corresponding to the disordered parts of polypeptide backbones \(^{[24]}\). During heating, hydrogen bonds stabilising the native structure of β-lg are disrupted, causing loss of α-helix and β-sheet structure, while new intermolecular β-sheets can be created (Figure 3.7.a and b). The increase in intensity of a specific band at 1624 cm\(^{-1}\) was attributed to the formation of extensive regions of β-sheets involved in intermolecular cross-linking between unfolded proteins and characterized protein aggregation \(^{[15, 25]}\). Subtraction of the native spectra from those of the heated sample showed that there was a significant increase in the absorbance at 1624 cm\(^{-1}\) (Figure 3.7.c and d). The bands at 1646, 1630, and 1624 cm\(^{-1}\) broaden, suggesting a general loss in secondary structure.

The ATR-FTIR data suggest that the fibril formation process takes place in two stages. In the first stage (up to approximately 100 min heating time), a decrease in the α-helix content and an increase in intermolecular β-sheets content were observed, similar to those associated with disordered aggregates \(^{[18]}\). The second stage (between 100 min to 26 h) is accompanied by a decrease in β-sheets content and a slight increase in α-helix content, corresponding to the formation long fibrils after prolonged heating. Changes in the secondary structures are due to protein degradation, fibril assembly, and protein reorganization in the fibril.

When the AFM results are taken into account, in the first stage, the β-lg proteins denature, become partially unfolded, and gradually degrade. All these phenomena lead to an increase in the β-sheets content. In the second stage, the decreased β-sheets content is associated with the formation of long fibrils.
3.3.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Reducing SDS-PAGE results showed that native β-lg monomers have a broad band around 18.4 kDa (0 min, lane 1 in Figure 3.8). No significant changes were observed for heating times shorter than 45 min. Above a heating time of 45 min, protein fragments (peptides) smaller than the β-lg monomers were detected (lanes 3 - 7 in Figure 3.8). It can be seen from the AFM results (Figure 3.2) that β-lg dispersions heated for 100 min contain a mixture of aggregates and short fibrillar structures. Thus, the low molecular weigh protein fragments observed during SDS-PAGE experiments indicate that these assemblies could be broken down by the reducing sample buffer. It has recently been shown that β-lg at pH 2, heated at 80 ºC (from 25 min to 24 h) is hydrolyzed into peptides\(^{[14]}\).

AFM and STEM results show that only the dispersions heated for > 300 min contain very long fibrils (>10 µm), together with some non-fibrillar protein particles, which were not incorporated into fibrils. In lane 7, only peptides were observed, suggesting that β-lg fibrils were composed of peptide fragments. Similar results were obtained when non-reducing SDS-PAGE was used (data not shown). These observations appear to contradict previous studies which indicate that protein fibrils are not completely broken down by SDS-reducing buffers\(^{[5]}\). This may be attributed to the lower protein concentrations and higher buffer concentrations used in the present study when heating the sample in reducing sample buffer.

It should also be noted that no stained proteinaceous material was seen either at the top of the stacking gel or within the wells. Furthermore, no fibrillar material was detected by AFM or SEM analysis of fibrillar dispersions prepared in SDS - reducing
buffer under these experimental conditions (results not shown), suggesting that fibrils had been dissociated.

These observations indicate that the effect of chaotropic agents on protein fibrils is complex and warrants further systematic study. It can therefore be tentatively concluded that β-lg components within the nano-fibrils were associated via non-covalent interactions \cite{5}. This fibril formation mechanism contrasts with that of fibrils formed at higher ionic strength, which is characterised by shorter fibrils formed from partially intact β-lg monomers or dimers \cite{20}.

**Figure 3.8.** SDS-PAGE of 2% (w/w) β-lg at pH 2, heated at 80 °C for various times. Lane 8, polypeptide molecular-weight marker (~14.4 kDa for α-lactalbumin, ~18.4 kDa for β-lg, and ~67 kDa for bovine serum albumin). Lanes 1-7 represent β-lg heated for 0, 45, 85, 100, 200, 300 min, and 20 h, respectively.
3.4. Conclusions

Studies using AFM, STEM, real-time ATR-FTIR and SDS-PAGE were performed to investigate the fibrillar aggregation process of β-lg. This study demonstrates that AFM imaging combined with more established analytical techniques gives unique insight into the transformation of native proteins into fibrillar structures. The sequence of AFM images of β-lg dispersions clearly demonstrates the progress of fibril aggregation as a function of heating time. STEM results confirm AFM observations of β-lg fibrils in air and in water.

This study shows, for the first time, AFM imaging of β-lg and WPI-derived fibrils in water under ambient conditions and the presence of double helical structures in situ. SDS-PAGE analysis suggests that β-lg fibrils consist of polypeptide fragments linked via non-covalent intermolecular bonds, and are not formed from intact monomers. The real-time monitoring of the assembly processes in-situ using ATR-FTIR correlated with AFM and SDS-PAGE results, suggesting a two-stage mechanism of fibril formation: (1) denaturation, partial unfolding and increase in β-sheets content with hydrolysis of monomers followed by (2) linear aggregation of polypeptide fragments into fibrils via non-covalent interactions, continued hydrolysis and an accompanying decrease in β-sheets content and overall secondary structure.
3.5. References


Chapter 4.

The effect of pH on the structure of β-lactoglobulin fibrils
4.1. Introduction

β-Lactoglobulin can form fibrillar structures with lengths of over 10 µm and thicknesses of 2 - 4 nm when heated at 80 °C, low pH and low protein concentrations. β-Lactoglobulin fibrils consist of polypeptide fragments linked via non-covalent intermolecular bonds. Changes in the physico-chemical and mechanical properties of β-lg fibril were observed when the pH was changed from acidic to alkaline values. During heating, changes in the secondary structure of β-lg were attributed to the rearrangement of intra-and intermolecular bonds as monitored by ATR-FTIR spectroscopy. An increase in intermolecular β-sheets content and a decrease in α-helix content characterized the fibrils formation process. Veerman et al. showed that the length of the β-lg fibril was stable when the pH was increased from 2 to 8. Akermans et al. found that at low pH (up to 3) the fibril length (1-10 µm) was constant but when the pH was increased further the fibrils become shorter and formed loose clusters at pH 4 and 8 or dense clusters at pH 5 and 7. Aggregation of WPI fibrils at high pH conditions with storage were observed by Bolder et al. leading to large aggregates, clusters at pH 7 and gels at pH 10. The formation of disulphide bonds between non-fibrillar protein or fibrils led to an increase in the viscosity of the protein solution and the formation of clusters or gels. These disulphide bonds were not formed at pH 2. Depletion interactions between the long fibrils induced by monomers (or small aggregates) might also lead to gel formation. A gel network appeared when disulphide bonds were formed between fibrils. Gel electrophoresis showed that disulphide bonds were formed in dispersions of WPI fibrillar structures at pH 7 and 10 but not at pH 2.

The aim of present study is to investigate the stability of β-lg fibril over the pH range 2 to 12. The structure of β-lg fibril over this pH range was monitored using AFM,
scanning electron microscopy (SEM), scanning transmission electron microscopy (STEM) and ATR-FTIR spectroscopy.

4.2. Materials and methods

4.2.1. Materials

Bovine β-lactoglobulin, HCl, NaOH, phosphotungstic acid were used as described in previous chapters 2 and 3 of this thesis.

4.2.2. Preparation of β-lactoglobulin fibrils

β-Lactoglobulin fibrils were prepared by heating a 2% (w/w) β-lg at pH 2 for 20 h as previously described in chapter 3. The initial fibril dispersion was split in 11 batches and the pH of each individual batch was adjusted to different pH values up to 12 by the addition of 1 M NaOH. To avoid fibril precipitation occurring in the isoelectric range (4.6 – 6), larger amounts of acid were added.

4.2.3. AC-mode atomic force microscopy

β-Lactoglobulin dispersions were investigated using a MFP-3D-BIO™-AFM instrument (Asylum Research UK Ltd., Oxford, UK) as described in chapter 3. Prior to imaging, the fibrillar dispersions were diluted in Milli-Q® water to a final concentration of 0.02% (w/w). Protein dispersions (10 µL) were deposited onto freshly cleaved mica and subsequently dried in a desiccator. The height profile of the β-lg fibril was measured directly using the MFP-3D software. Fibril height was established as the average value after the height of the fibril was measured at various points for 10 different fibrils for each set of experiments.
4.2.4. ATR-FTIR spectroscopy and STEM

ATR-FTIR measurements of freshly prepared 2% (w/w) β-lg fibril at different pH (pH 2-12) were performed using a Bruker Tensor 27 spectrometer as described in chapter 2. Fibrillar dispersions at different pH were analyzed using STEM as described in chapter 3.

4.3. Results and discussion

4.3.1. Atomic force microscopy

The height of β-lg fibrils was not affected when the pH was increased from 2 to 12. However the length of the fibrils changed, especially at pH close to the isoelectric range. AFM images revealed that long fibrils were formed after 20 h at 80 °C of 2% (w/w) β-lg at pH 2 (Figure 4.1.a, b and Figure 4.2.a), in agreement with previous reports [1-3]. The β-lg fibrils displayed a linear morphology and lengths of up to 15 µm. Cross-sections along the axes of the fibril showed height of 2 – 4 nm and a periodicity of ~30 - 40 nm (Figure 4.1.c, d). Occasionally, when single fibrils overlapped, the thickness was ca. 7 nm with a periodicity of ~36 nm emphasizing their helical structure [1].

When the pH of the fibril dispersion was increased from 2 towards to the isoelectric range (pH 4.6 – 6), the protein dispersions became more turbid and the fibrils exhibited variable lengths and formed clusters (Figure 4.2.b, c). At pH close to the isoelectric range, fibril lengths appear to decrease to less than 2 µm while height was constant at 4 nm. From pH 7 to pH 12, the fibrillar dispersions become clearer and a mixture of aggregates, short and long fibrils was observed by AFM (Figure 4.2.d, e and f).
Figure 4.1. (a) AFM height image of β-lg fibrils at pH 2. (b) Magnified (500 nm x 500 nm) area with a cross-section along the fibril axis, highlighting the periodicity of fibrils of 40 nm (c) and height (~5 nm) (d).
Figure 4.2. AFM height image of β-lg fibrils at (a) pH 2, (b) pH 4, (c) pH 5, (d) pH 7, (e) pH 8 and (f) pH 12.

On changing the pH of the fibrillar dispersions from acidic to alkaline pH at ambient temperature, a weak gel network was formed, especially in the isoelectric range. Gel formation most likely occurred via fibril aggregation by intermolecular
disulfide bonds or non-covalent interactions\textsuperscript{[7, 9]}. This aggregation may be explained by the amphoteric nature of the β-lg fibrils, the charge of the fibrils being non-uniformly distributed and dependent on the residues of the amino acids that form their constituent units\textsuperscript{[3]}.

Above the isoelectric range, the fibrils are negatively charged and the electrostatic and hydrophobic interactions among the fibrils can induce the connections between different fibrils. This contribution of the electrostatic and hydrophobic interactions induced an increase in the number and the thickness of the contact points between fibrils leading to aggregation\textsuperscript{[8, 9]}. At acidic pH the electrostatic repulsions are strong keeping the fibrillar dispersions clear.

\section*{4.3.2. Scanning electron microscopy}

To observe fibril aggregation, WPI fibrillar structures at pH 4.6 were examined by SEM. Scanning electron microscopy confirmed that the diameters of the β-lg fibrils were comparable to the fibrils height obtained by AFM for all pH. The lengths (~ 15 µm) and the diameters (~ 4 nm) of β-lg fibrils at pH 2 were also comparable to those measured by AFM. No differences were observed in fibril morphology when the fibrils were prepared at pH far from the isoelectric range. Clusters of small aggregates, short and long fibrils were formed in the isoelectric range (Figure 4.3.a, b).
Figure 4.3. SEM image of β-lg fibrils at pH 4.6. Sample was prepared on carbon film on copper grid. Scale bar: (a) 1 µm, (b) 200 nm (magnified image of Figure 4.3.a).

Scanning transmission electron micrographs of negatively stained fibrils showed that at neutral pH, β-lg fibrillar dispersions contained a mixture of small aggregates, short and long fibrils (Figure 4.4.a, b). The smallest fibril length was found to be ~ 0.5 µm. This differs from the previous reported findings where only short fibrils formed by long fibrils fracture were observed \(^{[4, 7]}\).

Figure 4.4. STEM image of β-lg fibrils at pH 7. Scale bar: (a) 1 µm, (b) 200 nm (magnified image of Figure 4.4.a).
4.3.3. Attenuated total reflectance-Fourier transform infrared spectroscopy

Changes in the secondary structures of β-lg fibrils at different pH (2-12 range) were observed using ATR-FTIR spectroscopy (Figure 4.5.a, b). The amide I band of β-lg fibrils at pH 2 revealed absorption bands at 1617, 1643, 1651, 1660, 1680 and 1695 cm\(^{-1}\) which are in good agreement with previous findings\(^{[1]}\). For native β-lg the amide I band is a complex composite, consisting of a number of overlapping component bands representing α-helices (1654 cm\(^{-1}\)), low and high-frequency components of β-structures (1630, 1670 cm\(^{-1}\)), turns (1681 cm\(^{-1}\)) and random coils (1645 cm\(^{-1}\)) corresponding to the disordered parts of polypeptide backbones\(^{[11, 12]}\). Changes in the IR spectrum corresponded not only to the secondary structure of the individual protein molecules but also to the interaction between them\(^{[11, 12]}\). ATR-FTIR spectra of β-lg fibrils exhibited an overall increase from pH 2 to 10, followed by a decrease below its initial absorbance at pH 2 for the pH 11 and 12 dispersions. A shift in the absorbance maxima from 1644 to 1630 cm\(^{-1}\) was observed when the pH was in the isoelectric range (pH 4 - 6 in Figure 4.5.a). A further increase in the pH value reversed the above shift back towards its initial position (1641 cm\(^{-1}\) for pH 12). At pH 10 the recorded spectrum had its maximum intensity value at 1643 cm\(^{-1}\) (Figure 4.5.b).

On increasing the pH, spectra of the β-lg fibrils showed an overall decrease of the α-helix and random coil content (1651-1660 cm\(^{-1}\)) especially at pH 2, 3, 11 and 12 (Figure 4.5). The decrease in the intensity at 1680 cm\(^{-1}\) (pH 5-12) and 1693 cm\(^{-1}\) (pH 2-12) could be assigned to a decrease in β-sheets and turns content. The shift of the maximum in the isoelectric range may be attributed to intramolecular β-sheets formation, the solutions exhibiting a mixture of small aggregates and fibrils.
Figure 4.5. ATR-FTIR spectra of β-lg fibrils at different pH varying from 2 to 6 (a) and from pH 7 to pH 12 (b). The insets emphasize the changes of the absorbance peak (full squares) and its corresponding wavenumber (down pointing full triangles) for different pH.

The dissociation of β-lg fibrils with pH induced a small loss in the secondary structure, especially at pH 11 and 12, where the dispersions are characterized by
diminished content of $\alpha$-helix (1651 cm$^{-1}$), intermolecular $\beta$-sheets and random coils (1659, 1680 and 1691 cm$^{-1}$) and by an increased content of turns and unordered structures (1643 and 1666 cm$^{-1}$). ATR-FTIR showed an overall increase in the $\beta$-sheets content up to pH 10, characterizing the partial or complete cleavage of the fibrils as the pH was varied.

4.4. Conclusions

Long $\beta$-lg fibril with similar lengths (up to 15 µm), periodicity (30 – 40 nm) and height (2 – 4 nm) were found to be predominant above and below the isoelectric range as shown by AFM and STEM. Aggregates and shorter fibrillar structures start to appear as the pH was varied from 4 to 6, suggesting a break up of longer fibrils closer to the isoelectric range. The presence of the fibrillar structures in protein dispersions at isoelectric pH and their secondary structure changes was showed by ATR-FTIR spectroscopy.
4.5. References


Chapter 5.

The effect of high pressure microfluidization on the structure and length distribution of whey protein fibrils

5.1. Introduction

High pressure technology is increasingly used by the food industry and conformational changes in whey proteins after hydrostatic high pressure treatment have been studied \[^{1, 2}\]. Microfluidization is a dynamic high pressure homogenization process used by the pharmaceutical and food industries to produce fine and stable emulsions \[^{3-5}\]. The effect of high pressure (> 50 MPa) microfluidization on whey protein fibrils has not been studied.

During microfluidization, the liquid sample is split into two streams that are reunited under high pressure and velocity in an interaction chamber \[^{6}\]. During the high dynamic pressure induced flow that subsequently occurs, intensive disruptive forces are generated as the two streams collide with each other, exhibiting a higher energy density compared with hydrostatic high pressure treatments or conventional homogenization techniques. During microfluidization, the protein particles are disrupted by the intense turbulence and shear flow effects. When protein fibrils are placed in such a flow, the elongational component of the flow may lead to fibril breakdown if the energy applied to the fibril is larger than the binding energy between fibril units. It has already been shown that strain rates of 8 - 10^7 s\(^{-1}\) were able to fracture WPI fibrils after a single pass, the average length of the fibrils then remaining relatively constant after five consecutive passes \[^{7}\]. However, the microfluidization process generates highly turbulent flow at very high shear forces, making it difficult to measure the precise flow components which influence protein denaturation and protein fibril structures.

Studies suggest that microfluidization affects the native protein structure and may produce significantly different structures when compared with heat induced denaturation and aggregation \[^{6}\]; intra - and intermolecular bonds can break, leading to protein fragmentation \[^{8}\]. There are few reports on the effect of high pressure microfluidization on protein secondary structure.
Quantitative measurement of fibril lengths at the nanoscale is challenging but recently, the flow-induced shear birefringence technique was developed to measure the length distribution of fibrils in suspension \cite{9-11}. Performing flow-induced birefringence measurements on β-lg fibrils in the semi-dilute concentration regime, Rogers et al. \cite{11} showed that the fibrils did not break up during shear treatment up to shear rates of 200 s$^{-1}$.

In this study, the effect of a single pass dynamic high pressure microfluidization on β-lg and WPI dispersions before and after fibril formation were investigated. Fibril length distribution of β-lg and WPI before and after microfluidization was measured by shear birefringence and AFM. The effect of single pass microfluidization on the degree of β-lg denaturation was measured using reversed phase high pressure liquid chromatography (RP-HPLC). Protein secondary structure was monitored by ATR-FTIR and fluorescence spectroscopy. For investigation methods requiring large quantities of fibrillar dispersions, WPI was used as this is a standard food ingredient.

5.2. Materials and methods

5.2.1. Preparation of β-lg and WPI fibrils

The bovine β-lactoglobulin, WPI and Thioflavin T were used as in previous chapters of this thesis. Fibrillar dispersions were prepared as previously described \cite{12}. The pH of the β-lg or WPI dispersions was adjusted to 4.6 using 6 M HCl and centrifuged (3700 x g for 30 min at 25 ºC) to remove the denatured protein. The pH of fibrillar dispersions was adjusted to 7.0 with 6 M NaOH for subsequent microfluidization treatment and analysis.
A summary of the samples and analyses is presented in Table 5.1. Unless otherwise specified, analyses were performed in triplicates.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Microfluidization treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native protein (before fibril formation)</td>
</tr>
<tr>
<td></td>
<td>β-lg</td>
</tr>
<tr>
<td>Atomic force microscopy</td>
<td>+</td>
</tr>
<tr>
<td>Shear birefringence</td>
<td>(+)</td>
</tr>
<tr>
<td>Reversed phase HPLC</td>
<td>+</td>
</tr>
<tr>
<td>ATR-FTIR spectroscopy</td>
<td>+</td>
</tr>
<tr>
<td>Thioflavin T fluorescence</td>
<td>NT</td>
</tr>
</tbody>
</table>

\* Microfluidization pressure from 0 to 170 MPa. NT, not tested; (+), data not shown.

Table 5.1. Summary of experimental samples and procedures.

5.2.2. Microfluidization treatment

5.2.2.1. Microfluidization of native β-lg and WPI prior to fibrillization

An amount of 300 mL of β-lg or WPI dispersions [2% (w/w) protein concentration at pH 4.6] were microfluidized (M110-EH-30 Microfluidizer® Processor, Microfluidics, Newton, MA, USA) using a single pass, at 50 MPa, 75 MPa, 100 MPa, 150 MPa and 170 MPa pressure. After microfluidization, the pH of the β-lg or WPI
dispersions was adjusted to 2.0 using 6 M HCl and heated at 80 °C for 20 h in a water bath (Grant Instruments Ltd., Cambridge, UK) to form fibrils \[^{12}\].

### 5.2.2.2. Microfluidization of fibrils

To study the effect of dynamic high pressure on fibril structure, a series of fibrillar dispersions of \(\beta\)-lg or WPI were prepared as described above. Fibrillar dispersions were microfluidized at 50 MPa, 75 MPa, 100 MPa, 150 MPa, and 170 MPa, with the inlet dispersions being at room temperature.

To prevent possible heat-induced protein denaturation that might occur during the microfluidization process, the interaction chamber was cooled with ice. The chamber was made from an aluminium oxide type ceramic, which showed no abrasion throughout the experiments. Flow rates were calculated for WPI and fibrillar dispersions based on the volume of liquid collected at the outlet in 1 min. Flow rates increased with pressure from 6 to \(8 \times 10^{-6} \text{ m}^3 \text{s}^{-1}\) for 50 and 170 MPa, respectively.

### 5.2.3. AC-mode atomic force microscopy

\(\beta\)-Lactoglobulin or WPI dispersions (microfluidized before or after fibrils formation) were investigated using a MFP-3D-BIO™-AFM (Asylum Research UK Ltd. Oxford, UK) as described previously \[^{12}\]. Prior to imaging, the dispersions were diluted in Milli-Q® water to a final concentration of 0.02% (w/w). Protein dispersions (10 µL) were deposited onto freshly cleaved mica and subsequently dried in a desiccator. Fibril heights were established as the average value after the height of the fibril was measured in various points for 10 different fibrils for each set of experiments. Fibril height profiles were obtained directly using the MFP-3D software. Fibril length frequency distributions were measured from AFM images using Image J v1.43 image analysis.
software. A total of 90 fibrils were measured from 4 separate AFM images and fibril length frequencies calculated as a dimensionless number \( L' \) (Eqn. 1):

\[
L' = \frac{L_p}{L_{(\text{mean } 0)}}
\]  

(1)

where \( L_p \) = Length of fibrils at different microfluidization pressures and \( L_{(\text{mean } 0)} \) = mean fibril length at 0 pressure.

### 5.2.4. Flow-induced birefringence

Length distributions of \( \beta \)-lg or WPI fibrils (before or after microfluidization) were performed in triplicate using a strain-controlled ARES rheometer (Rheometric Scientific Ltd., Piscataway, New Jersey, USA) with Couette geometry (rotating cup with a diameter of 33.8 mm and a static bob with a diameter of 30.0 mm). A laser beam of wavelength 670 nm passed vertically through the gap between the cup and bob, and the birefringence was measured with a modified optical analysis module \(^{[13]}\). A shear rate of 5 s\(^{-1}\) was applied for 60 s and from the decay curve of the flow-induced birefringence after the cessation of flow the length distribution of the fibrils was determined \(^{[11]}\). The analysis was performed in a temperature-controlled room at 20 °C. The protein samples were diluted with HCl at pH 2 to a final protein concentration 0.25% (w/w).

### 5.2.5. Reversed phase-high performance liquid chromatography

The effect of microfluidization on native \( \beta \)-lg was investigated using RP-HPLC. The chromatographic system consisted of an Agilent 1200 series binary pump with standard auto sampler, a thermostatic column department and a 1200 series diode array detector (DAD) (Agilent Technologies, Santa Clara, CA, USA). Separation of the \( \beta \)-lg fractions was achieved using an Agilent 300 SB-C18 RP Poroshell column. The injection volume of the sample was 5 µL [1% (w/w) protein], the flow rate being 0.5
mL min\(^{-1}\) and the column temperature was set at 35 °C. Solvent A consisted of a 900:100:1 (by vol) mixture of water, acetonitrile and trifluoroacetic (TFA), while solvent B was a 900:89.1:0.9 (by vol) mixture of acetonitrile, water and TFA. A solvent gradient was generated after injection, starting at 26% B and increasing to 37% B after 10 min\(^{-1}\), the proportion of B then increased to 45% after 13 min\(^{-1}\) 10 sec \(^{-1}\) and subsequently to 100% B after 3 min\(^{-1}\) 40 sec \(^{-1}\). This was held for 3 min\(^{-1}\), before returning to starting conditions. After the microfluidization treatment, \(\beta\)-lg dispersions were centrifuged (3,700 x g for 30 min at 25 ºC) to remove the denatured protein and the supernatant was filtered and used for HPLC analysis. The \(\beta\)-lg samples were prepared by dilution (v/v) using a disassociating buffer containing 7 M urea, 0.02 M bis-Tris-propane and 0.5% mercaptoethanol \([14]\). The samples were left at room temperature for at least one hour in the disassociating buffer before being passed through a 0.22 \(\mu\)m filter to remove any insoluble material. Data were collected at 214 and 280 nm and quantification was based on integration of the data at 214 nm. Commercial \(\beta\)-lg and WPI were used as standards. The experiments were run with \(\beta\)-lg since a pure protein is required for the analysis. The results can be extrapolated for WPI samples since previous studies indicate that only \(\beta\)-lg forms fibrils at low pH and ionic strength\([12, 15, 16]\).

5.2.6. Attenuated total reflectance-Fourier transform infrared spectroscopy

ATR-FTIR measurements of freshly prepared 2% (w/w) \(\beta\)-lg or WPI heated before or after microfluidization at 80 ºC for 20 h were performed using a Bruker Tensor 27 spectrometer as described in chapter 2.
5.2.7. Thioflavin T fluorescence

Thioflavin T (ThT) solution (3 mM) was prepared by dissolving ThT powder (4.79 mg) in Milli-Q® water (4.52 mL), the pH adjusted to pH 7 with 6 M NaOH and filtered (Minisart, Sartorius, 0.2 µm) to remove undissolved powder. ThT solution (20 µL) was added to 2.98 mL of the 0.02% (w/w) fibrillar dispersions and the fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer (JVA Analytical Ltd., Dublin, Ireland).

Excitation and emission wavelengths of 420 and 485 nm, respectively, were used (slit widths 5 nm). The emission spectra of the protein dispersions were normalized \cite{17}.

5.3. Results and discussion

5.3.1. Atomic force microscopy

5.3.1.1. AFM observations and length distribution of fibrils formed from microfluidized β-lg or WPI

AFM height images were performed using AC mode in air to monitor the morphology changes of fibrils prepared with microfluidized protein. The length of the fibrils was not affected by the pressure applied on the native protein up to 170 MPa, as indicated by AFM and shear birefringence results. At 0 MPa (i.e., non-microfluidized), fibrils exhibited a linear morphology (Figure 5.1.a, b), a height of 2 - 3 nm (Figure 5.1.c) and periodicity of 30 - 40 nm (Figure 5.1.d), and lengths of up to 15 µm. The thickness of overlapping fibrils was nearly twice (~ 6 nm) that of single strand fibrils, consistent with previous reports \cite{12, 18}. AFM height images of WPI showed similar fibrillar structures (results not shown). No significant difference in fibril length was observed for samples that had been microfluidized at 50 MPa, 75 MPa, 100 MPa, 150 MPa, or 170
MPa. AFM images of fibrils formed from β-lg microfluidized at 170 MPa are shown in Figure 5.1.e, f.

**Figure 5.1.** Atomic force microscopy height images (5 μm × 5 μm and 500 nm × 500 nm scans) of fibrils, together with cross-section profiles of native (0 MPa) β-lg (a and b) and microfluidized β-lactoglobulin at 170 MPa (e and f). The profiles shows typical heights of ~ 3 nm for 0 MPa (c) and ~ 5 nm for 170 MPa (g) and fibril periodicity of 30 nm (d and h).
Cross section profiles were acquired from 500 nm × 500 nm images, the fibril height and periodicity being measured from cross-sections both perpendicular and along fibril axis, respectively. Images were obtained using AC mode in air.

A slight increase in fibril height ranging from 4 nm to 5.75 nm was observed in samples that had been microfluidized (Figure 5.1.g). This increase may be due to pressure-dependent transfer of water into the protein interior \[^{[19]}\]. Unlike the heat denatured proteins that transfer their non-polar groups into water, the pressure-denatured proteins retain a compact structure, with water molecules penetrating their core \[^{[19]}\]. AFM showed slight changes in fibril periodicity between native (Figure 5.1.a, b) and microfluidized β-lg (Figure 5.1.e, f). These changes were characterized by the partial loss of globular shape of fibril constituent units following microfluidization (Figure 5.1.e, f).

### 5.3.1.2. Atomic force microscopy of microfluidized fibrils

AFM height images of microfluidized β-lg fibrils are shown in Figure 5.2. Results indicate that high pressure microfluidization of 50 MPa (Figure 5.2.a, b) or above (Figure 5.2.c, d) broke up fibrillar structures without affecting fibril height. No fibrils longer than 300 nm were observed for 170 MPa applied pressure (Figure 5.2.c, d).

It can be clearly seen that the fibril length distributions changed with applied pressure (Figure 5.3). At 0 MPa, fibril lengths ranged from 1 to 15 µm. The length generally decreased with increasing microfluidization pressure and ranged from approximately 25 nm to 425 nm at 50 MPa and from 12 nm to 275 nm at the highest pressure applied (Figure 5.3). Small differences in fibril length distributions can be seen when the pressure was raised from 50 to 100 MPa or from 150 to 170 MPa.
Figure 5.2. Atomic force microscopy height images (2 µm × 2 µm and 500 nm ×500 nm scans) of β-lg fibrils microfluidized at 50 MPa (a and b) and 170 MPa (c and d), showing shortened fibrils. Images were obtained in AC air-dried mode.

Results further indicate that fibril lengths exhibited a narrower size range when microfluidized compared with the initial conditions, suggesting that microfluidization could be used to control fibril length.
Figure 5.3. The effect of microfluidization on fibril frequency length distributions of β-lactoglobulin fibrils at pH 7 obtained after microfluidization pressure up to 170 MPa (◊, 0 MPa; □, 50 MPa; △, 100 MPa; ×, 150 MPa; ○, 170 MPa). Fibril length was measured using Image J software and a total of 90 fibrils were measured from 4 separated images.

5.3.2. Shear birefringence

Shear birefringence measurements were performed to measure the length distribution of fibrils formed from microfluidized protein (Figure 5.4). Results indicate that the length distributions of fibrils prepared from microfluidized WPI were similar for the various pressure treatments, although a slight decrease (as shown by the arrow in Figure 5.4) of the signal from 50 to 170 MPa was observed. Most fibrils were 1.5 - 7 µm in length, the results being comparable with the measurements made from AFM images (Figure 5.1). Shear birefringence results for β-lg were similar to those of WPI (results not shown). The length distribution of the microfluidized shortened fibrils could
not be measured using the shear birefringence technique, since fibrils smaller than 1µm could not be aligned by the flow and a birefringence value could not be obtained.

**Figure 5.4.** The effect of microfluidization on fibril formation. Fibril length distribution was obtained from flow birefringence measurements at different applied pressures (—, 0 MPa; – – –, 50 MPa; ~~~, 75 MPa; ◊, 100 MPa; □, 150 MPa; ●, 170 MPa) during the microfluidization process. Data are derived from the decay curves of shear birefringence analysis. The arrow indicated that the signal decreases as the pressure increases.

**5.3.3. The effect of microfluidization on the secondary protein structure of β-lg and fibrils**

The content of native β-lg after microfluidization was quantified by RP-HPLC (Table 5.2). The native monomeric β-lg concentration decreased by approximately 30% when microfluidized over the 50-170 MPa range.
<table>
<thead>
<tr>
<th>Microfluidization pressure (MPa)</th>
<th>Concentration native β-lactoglobulin (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.84</td>
</tr>
<tr>
<td>50</td>
<td>0.59</td>
</tr>
<tr>
<td>75</td>
<td>0.47</td>
</tr>
<tr>
<td>100</td>
<td>0.65</td>
</tr>
<tr>
<td>150</td>
<td>0.53</td>
</tr>
<tr>
<td>170</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Table 5.2. Effect of high pressure microfluidization on native β-lactoglobulin as determined by reversed phase-HPLC.

5.3.3.1. Effect of microfluidization on protein secondary structure prior to fibril formation

ATR-FTIR spectrum of microfluidized β-lg dispersions were recorded for different pressures up to 170 MPa (Figure 5.5.a, b). In the ATR-FTIR spectrum of β-lg (no pressure) bands were observed at 1630 and 1681 cm⁻¹ suggesting that the monomeric structure of β-lg was mainly comprised of β-sheets even though α-helix bands at 1652 cm⁻¹ and turns at 1690 cm⁻¹ were detected (Figure 5.5.a, b). These results are in agreement with literature reports that indicate globular β-lg in its monomeric form at pH 4.4 is mainly composed of intramolecular β-sheets (1630 cm⁻¹), α-helix (1654 cm⁻¹), turns and unordered structures [20-22]. Changes in these bands occurred on microfluidization of the protein, especially in the 1620 cm⁻¹ range. As the pressure increased from 50-170 MPa, the absorbance band corresponding to α-helix (1652 cm⁻¹) increased and the contribution to the absorbance spectra from the β-sheets decreased (1629 cm⁻¹) suggesting a loss of secondary structure due to protein unfolding (Figure 5.5.a, b).
Figure 5.5. ATR-FTIR spectra of microfluidized a) β-lg and c) WPI fibrillar structures. Amide I band of the treated samples after native protein subtraction is also shown (b and d).

Different experimental findings were presented by Subirade et al. [23] wherein the hydrostatic pressures up to 140 MPa did not affect the β-sheet content of the protein structure. This discrepancy might be due to the fact that during microfluidization, intensive disruptive forces are generated when the fluids collide with each other or with a solid surface. Thus, after subtraction of the native β-lg spectrum from the microfluidized samples spectra, the experimental results showed that overall the α-helix (1650 cm⁻¹) content increased while intra and intermolecular β-sheets content (1628 and 1618 cm⁻¹) decreased with pressure (Figure 5.5.b).

Starting with 50 MPa, the spectra subtraction (Figure 5.5.b) revealed an increase with pressure of the absorption value at 1680 and 1695 cm⁻¹ assigned to β-sheets and β-turns structures. The new arrangement of the intra and intermolecular bonds by
 formation of new intermolecular β-sheets (1680 cm\(^{-1}\)) may have been caused by denaturation/aggregation of β-lg during the microfluidization process. The disappearance of the 1628 cm\(^{-1}\) band might be explained by the conversion of intramolecular β-sheets into intermolecular β-sheets, β-turns and non-native α-helix by the high pressure treatment\(^{[19]}\). Pressure induced changes in secondary structure may be related to changes in the globularity of the subunits shape and fibril height as observed by AFM (Figure 5.1.b, d, f, h).

5.3.3.2. ATR-FTIR measurement of microfluidized fibrils

ATR-FTIR spectra were recorded for WPI fibrils at microfluidized at pressures from 50 to 170 MPa (Figure 5.5.c, d). For the control (0 MPa), bands were observed at 1617, 1647, 1654, 1663, 1670, 1676, 1685 and 1696 cm\(^{-1}\) (Figure 5.5.c). ATR-FTIR spectra of microfluidized fibrils showed a sharp increase in 1600-1630 cm\(^{-1}\) range, with a maximum increase at 1618 cm\(^{-1}\) and 1620 cm\(^{-1}\) (at 50 - 75 MPa), indicating the presence of intermolecular β-sheets\(^{[12]}\). A corresponding decrease in the secondary structure signal was observed after microfluidization of the fibrils. After subtraction of the 0 MPa spectrum from the microfluidized samples spectra, the experimental results show that overall the α-helix (1654 cm\(^{-1}\)) content increased while intermolecular β-sheets component (1618 cm\(^{-1}\)) decreased in WPI fibrils with pressure (100 - 170 MPa) (Figure 5.5.d). The decrease in 1618 cm\(^{-1}\) band can be attributed to the breaking of fibril into short fibrillar structures under the action of the high pressure. The increase observed in the other bands can be attributed to the conversion of intermolecular β-sheets into β-turns and non-native α-helix. This increase was not as obvious as for microfluidized β-lg presented in Figure 5.5.b. A possible reason for this difference is that the constituent units of the fibrils formed under thermal treatment are polypeptides rather than native proteins\(^{[12]}\). Thus, for non-fibrillar β-lg, high pressure acts only to
denature the native protein, whilst for microfluidized fibrils the pressure acts on the constituent units and also to disrupt the intermolecular bonds between the polypeptides.

**5.3.4. Thioflavin T fluorescence**

**5.3.4.1. ThT fluorescence of microfluidized protein prior fibril formation**

ThioflavinT fluorescence of microfluidized WPI dispersions at pH 7 was recorded with a maximum at 485 nm (Figure 5.6.a). Unheated WPI had a small ThT fluorescent intensity caused by the β-sheets originally present in the native state. A decrease in the fluorescence intensity signal with pressure can be observed and it was related to the decrease of the β-sheet content under the action of the applied pressure observed in the ATR-FTIR measurements.

**5.3.4.2. ThT fluorescence measurement of microfluidized fibrils**

The microfluidized WPI fibrils had a higher ThT fluorescent intensity than the unheated WPI indicating higher β-sheet content present at 0 MPa (Figure 5.6.b). Thus, in the microfluidized fibril (WPI) dispersion, the ThT can bind to both inter- and intramolecular β-sheets associated with the polypeptides, while in the unheated microfluidized WPI the ThT fluorescence intensity is mainly derived from intramolecular β-sheets present in the native state [24]. The fluorescence peak appeared at 485 nm and an overall increase was observed in fluorescence intensity with the pressure, showing that the β-sheet content increased with the pressure.
Figure 5.6. Normalized Thioflavin T fluorescence intensity of WPI solutions microfluidized up to 170 MPa (□, 0 MPa; ▲, 50 MPa; ×, 75 MPa; Δ, 100 MPa; ○, 150 MPa; ♦, 170 MPa) before (a) and after (b) fibril formation.

The maximum of this increase was observed at 50 MPa pressure. For larger pressures, a decrease in ThT intensity of the protein aggregates was recorded, but it remained larger than the initial value measured at 0 MPa.
The overall increase in the intensity can be explained by the fact that the pressure broke the fibrils, creating a larger number of small fibrillar aggregates and thus a larger number of binding sites to which ThT could attach.

5.4. Conclusions

AFM images show that fibrils lengths formed from β-lg or WPI microfluidized up to 170 MPa were not influenced by the applied pressure during microfluidization treatment of the native protein, although a slight loss of periodicity and increase in fibril height was observed. The maximum length of protein fibrils was 15 µm and their height was 5-6 nm. Shear birefringence measurements of fibrillar suspensions prepared from microfluidized β-lg or WPI confirmed that the fibril length was not affected by high pressure microfluidization. The ATR-FTIR and ThT fluorescence data indicated that following high dynamic pressure treatment the secondary structure of the protein changes, most likely by rearrangement of the intramolecular β-sheets into intermolecular β-sheets. This study revealed that high pressure microfluidization breaks up fibrillar structure into more regular sized fragments <350 nm in length. Shortened fibrils could withstand pressures up to 170 MPa without breaking up further.

The results indicate that the dynamic high pressure microfluidization not only affects the general physical dimensions of the long β-lg or WPI fibrils, but also alters the secondary structures of their constituent units as shown by the ATR-FTIR and ThT fluorescence spectroscopy measurements.
5.5. References


properties as determined by Fourier transform infrared spectroscopy.


Chapter 6.

Foam stability of whey protein fibrils
6.1. Introduction

Whey protein isolate (WPI) and egg white protein (EWP) are often used as foaming agents in the food industry in the manufacture of meringues, cake, whipped toppings and leavened bakery products [1-9]. Previous studies have shown that WPI or EWP can improve and maintain the quality (texture, volume) of “foamed” food, in particular foaming capacity and stability [3, 9-11]. The foaming properties of WPI are influenced by protein concentration, pH, high pressure, thermal treatment, foam procedure, whipping time, by their nature and behaviour at interfaces (denaturation, protein-protein interactions) and by their interactions with other food ingredients [1-18].

Partial denaturation of 5% (w/w) WPI after 1 min heating at 70 °C improved foam capacity when compared with unheated whey protein [16], but excessive heating times led to formation of aggregates that can alter the foam properties [4-6,11, 12, 16,19-21]. An optimal mixture of unheated and heat-induced aggregates of WPI can enhance foam stability [12, 16]. The foaming properties of WPI were improved by the presence of an optimal aggregate quantity (10 % w/w) at the interface. However, increasing the percentage of aggregates to >50% (w/w) led to large high molecular weight clusters that promoted foam collapse [5]. Bals et al. [21] showed that heat denaturation of WPI in the range 60 – 90 °C leads to aggregate formation that can reduce foam capacity and rigidity although other studies demonstrated that foam stability can be improved [16]. WPI at pH 5 heated for 10 min at 80 °C improved foam stability by 65 % but this is slightly decreased when heated at pH 4 and 7 [19]. Salt (NaCl) addition to the protein solution increased aggregates formed during thermal treatment, but these aggregates exhibited reduced mobility and lower surface hydrophobicity, the foam-liquid stability being increased [5, 17]. Nicorescu et al. [5] showed that heat induced aggregates at 2% (w/v) WPI and 50 mM NaCl, pH 7 at 70 – 100°C have a reduced mobility and affinity for air-liquid interface due to their higher surface energy. The optimal foam stability
was obtained when the protein was denatured at 80 °C, above this temperature the foam stability decreased \cite{5, 21}.

Zhu and Damodaran \cite{16} showed that native WPI contributed to foam formation due to rapid adsorption at the interface, while WPI aggregates contribute to foam stability. Other studies reported that high dynamic pressure \cite{13} or hydrostatic \cite{14} treatment enhanced the foaming ability and stability of WPI. Bouaouina et al. \cite{13} showed that high pressure treatment using a homogenizer increased the whey protein surface hydrophobicity leading to improved foaming properties. The increase in hydrophobicity was attributed to the increased exposure of hydrophobic sites resulting from the disruption of protein aggregates \cite{13}.

Cayot et al. \cite{19} found that the foaming capacity of whey proteins varies with the preparation method, the most common procedures to insert a gaseous phase in protein aqueous solution being either whipping or bubbling \cite{19, 22}. In the whipping method the atmospheric gas was incorporated in the liquid phase by cutting the liquid surface coarse foam being initially formed. As the whipping continues the foam becomes dispersed, smaller bubbles being formed. In the bubbling techniques the gas was inserted into the liquid phase through small orifices, forming bubbles that rose in the liquid and increased their volume \cite{22}.

As described in chapter 3, whey proteins, particularly β-lg, can form extended fibrils when heated at 80 °C at pH 2 and low ionic strength. The potential of fibrils as functional ingredients has not been fully investigated. Although some studies indicate that the fibrillar whey proteins can form low salt gels \cite{23}, there have been no published studies investigating the foaming properties of whey protein fibrils. In the present study the influence of pH, protein concentration, whipping time and high dynamic pressure on whipped WPI foams is described.
The aims of this study are:

a) To compare the foaming capacity and stability of whipped dispersions of fibrillar with non-fibrillar whey protein. Dispersions of native or heated WPI (80 ºC, 20 h) of three concentrations [1, 2 or 3 % (w/w)] and two pH values (2 and 7) were whipped for three different intervals (5, 10 or 15 min). The pH was raised from 2 to 7 for a part of the fibrillar dispersions and their foam properties were also investigated.

b) To study the effect of high dynamic pressure by microfluidization (b1) or shear mixing by Ultra Turrax (b2) on the foaming properties of whey protein fibrils.

The resulting foams were evaluated by calculating foam expansion, drainage time, drainage weight and bubble size characterised by optical microscopy. The WPI dispersions were investigated by AFM before and after heat and/or pressure/shear treatments to monitor changes in the protein structure. The foaming properties of the WPI dispersions were also compared with those of EWP, traditionally a standard food ingredient for producing good quality foams.

6.2. Materials and methods

6.2.1. Materials

Whey protein isolate BiPro™ [~98% (w/w) protein on dry basis: 65% β-lactoglobulin, 25% α-lactalbumin, 8% bovine serum albumin] was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). Fresh eggs were purchased from a local grocery store. An amount of 225 mL egg white (protein content ~ 12%) was manually separated from the yolk.

6.2.2. WPI fibrillar dispersions

Whey protein isolate fibrillar dispersions at concentration of 1, 2 and 3 % (w/w) were prepared as previously described in chapter 3.
6.2.3. Microfluidization treatment

Native or fibrillar WPI dispersions [2 % (w/w) protein at pH 7] were used to study the pressure effect on WPI foam properties. The pH of WPI fibrils was adjusted from 2 to 7 using 6 M NaOH to prevent corrosion of the microfluidization equipment. The WPI dispersions were microfluidized (M110-EH-30 Microfluidizer® Processor, Microfluidics, Newton, USA) at pressure range 0-170 MPa (50 MPa, 75 MPa, 100 MPa, 150 MPa, 170 MPa) as previously described in chapter 5.

6.2.4. Shear mixing by Ultra Turrax

Native and heated WPI [2% (w/w) protein, pH 2 or 7] and WPI fibrillar dispersions [2% (w/w) WPI concentration, pH 7] were used to study the effect of shear mixing on WPI foam properties. WPI dispersions were prepared using a high shear speed disperser Ultra Turrax (IKA®T18 basic, Ultra-Turrax®, IPAT Ltd., IKA Werke GmbH, Germany) with a S18N-19G stainless steel dispersing tool and set to rotational speeds of 11,200 or 24,000 rpm.

6.2.5. Whipping treatment

Whey protein samples were whipped using a heavy duty blender (Heavy Duty, Kitchen Aid Inc., St. Joseph, Michigan, USA) at room temperature. Approximately 225 mL WPI dispersions were placed in a steel bowl [7 quarts (6.7 L) capacity] and mixed using a wire whip beater at maximum and constant speed setting 10 for 5, 10 or 15 min.

6.2.6. Foam properties

Foam capacity was defined as the quantity of foam formed per unit of solution and represented the maximum foam volume measured after expansion. Foam stability represents the capacity of the foam to retain its structure over time and can be evaluated
either by measuring the volume of the foam or weighting the volume of the separated liquid (drained) over time \([2, 12]\). Foam expansion \([24, 25]\) was determined by level-filling a 225 mL plastic weighting boat with foam and then weighing. Its value was calculated using the expression:

\[
\text{Foam expansion (\%) = } \frac{Unwhipped\ solution\ wt(g) - foam\ wt(g)}{Unwhipped\ solution\ wt(g)} \times 100
\]

Foam stability was determined by evaluating foam drainage. 225 mL of maximum expansion foam was transferred into a pyrex filter funnel of 9.9 cm inner top diameter, 0.7 cm inner stem diameter and 8.5 cm stem length. A small plug of glass wool was placed in the top of the funnel stem to retain the foam but allowing the liquid drainage. The time required (in min) to drain the entire foam was determined \([26]\). All foaming experiments and measurements were replicated three times for statistical purposes, the results presented in this study being the mean values. The weight of the liquid drained from foam was measured every 5 min for up to 120 min. Foam drainage was calculated as:

\[
\text{Foam drainage (\%) = } \frac{Solution\ drained\ from\ foam\ wt(g)\ }{Unwhipped\ solution\ wt(g)} \times 100
\]

The packing degree of the bubbles \([27, 28]\) was characterized by estimating the liquid volume fraction defined as \(\varepsilon = \frac{\rho_{\text{foam}}}{\rho_{\text{liquid}}}\) and was calculated as \(\varepsilon = \text{wt foam}/\text{wt liquid}\) for constant volume.

The Sauter mean bubble diameter \(d_{32}\) \([5]\) was estimated using the following relationship:
\[ d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \]

in which \( n_i \) is the bubbles number that belong to the \( i \)th size class of average diameter \( d_i \).

6.2.7. Microscopic analysis of the bubble size

The shape and the size of the resulting air bubbles were investigated using bright field optical microscopy. Approximately 200 \( \mu \)L of the foam was deposited on a glass slide without a cover slip. Images were acquired using an optical microscope (Olympus BX-51, Olympus Corporation, Tokyo, Japan) with 4x objective and equipped with a CCD camera (Jenoptik C14 Imagic). The recorded images were then transferred and analyzed using image analysis software [Image Access Premium8 software (Imagic Bildverarbeitung AG, Glattbrugg, UK)]. The bubbles size distribution was determined using Image J v1.43 image analysis software, the image processing program developed at the National Institutes of Health, USA.

6.2.8. AC-mode atomic force microscopy

WPI dispersions were investigated using a MFP-3D-BIO™-AFM (Asylum Research UK Ltd. Oxford, UK) as previously described in chapter 2. Prior to imaging, the WPI dispersions were diluted in Milli-Q\textsuperscript{®} water to a final concentration of 0.01 - 0.03 % (w/w) (pH 2 - 7 ± 0.5). Thus, 10 \( \mu \)L of WPI diluted with Milli-Q\textsuperscript{®} water was deposited onto freshly cleaved mica and subsequently dried in a desiccator.
6.3. Results and discussion

6.3.1. Effect of whey protein concentration, pH value, whipping time and thermal treatment on WPI foam properties

Whey protein isolate concentration, pH, whipping time and thermal treatment can affect the foam properties of WPI. Hence, unheated (native) or heated WPI dispersions of three different concentrations [1, 2 or 3% (w/w)] at pH 2 and 7 were used to study the changes that appear in foam properties. Heating the WPI dispersions at 80°C for 20 h leads to the formation of aggregates (Figure 6.1.a) in the pH 7 dispersions, while in the pH 2 dispersions leads to long fibrils of 2-3 nm height and up to 15 µm length (Figure 6.1.b).

Figure 6.1. AFM images showing the presence of WPI aggregates after 20 h of heating at 80 °C of 2% (w/w) WPI dispersions at pH 7 (a) and pH 2 (b) before whipping. Their presence after whipping treatment can be observed in (c) and (d), respectively.
On changing the pH of the WPI fibrillar dispersion from 2 to 7, a mixture of short, long fibrils and small aggregates was present as seen in chapter 4. Unheated and heated WPI dispersions were whipped for different time intervals and the properties of the resulting foams were investigated. After whipping, no significant changes were observed on the size of the aggregates (Figure 6.1.c) but the height of the fibrils was changed to 0.5 - 1.2 nm (Figure 6.1.d), as shown by AFM measurements.

The effects of whey protein concentration, pH and heat treatment on foam expansion are presented in Figure 6.2. Maximum foam expansion was obtained for all concentrations of WPI fibrillar dispersions at pH 7, while the minimum value was obtained by the 1% (w/w) native WPI solution at pH 7.

![Figure 6.2](image)

**Figure 6.2.** Foam expansion vs WPI concentration before and after thermal treatment for different pH of WPI dispersions whipped for 15 min. Error bars denote standard deviation (± SD).

The foam expansion results (Figure 6.2) suggested that the thermal treatment improved the WPI foam properties since the values for the heated protein dispersions...
were larger than for unheated dispersions at all concentrations and pH. Decreasing the pH at constant WPI concentration enhanced the foam capacity. Thus, larger foam expansion values were observed at pH 2 than at pH 7 for the unheated or heated dispersions. An exception can be observed for 3% (w/w) WPI concentration in unheated state where the foam expansion for pH 7 is larger than for pH 2 solution. For WPI fibrillar dispersions the pH increase from 2 to 7 lead to improved foam expansion values.

In general, an increase in WPI concentration leads to an increase in the foam expansion value, an exception being the 3% (w/w) WPI unheated solution at pH 2 (Figure 6.2). When compared with EWP dispersions, the foam expansion results (Figure 6.2) show that from all dispersions only the WPI fibrillar dispersions at pH 2 and 7 display similar or larger foam capacity with EWP.

The effect of whipping time on foam expansion values of the WPI dispersions can be observed in Figure 6.3 where the foam expansion values for unheated or heated 2% (w/w) WPI dispersions at different pH and for EWP are presented. These results suggest that for all WPI solutions the largest foam expansion can be achieved after 15 min of whipping.
Figure 6.3. Foam expansion vs whipping time of 2% (w/w) native or heated WPI dispersions and EWP. Error bars denote ± SD.

Even though EWP is considered the strongest foam agent, WPI fibrillar dispersions have similar or larger foam expansion values after whipping for 15 min (Figure 6.2 and 6.3). Foam stability can be quantitatively estimated by weighing the drained liquid over time. To observe the changes in the WPI foam stability, a graph of WPI dispersions foam drainage (%) as function of drainage time (Figure 6.4) for different WPI concentrations, pH and thermal treatment was plotted.
For all WPI concentrations the most stable foams were formed by dispersions of WPI fibrils at pH 7 since they exhibit the longest drainage time [up to 120 min for 3% (w/w) protein]. The shortest drainage time (on 40 min) were observed for unheated WPI dispersions at pH 2. The foams obtained for 1% (w/w) WPI fibrillar dispersions at pH 7 are less stable than EWP foam since EWP displays longer drainage time, although as the WPI concentration is increased this situation is reversed.

For all concentrations, thermal treatment of the WPI dispersions at pH 2 improves foam stability, longer drainage times being measured for the heated dispersions than for the unheated ones. A similar effect was obtained for 1% (w/w) WPI dispersion at pH 7, while for 2% (w/w) at pH 7 the thermal treatment has no significant effect on the drainage time. However, for the 3% (w/w) WPI dispersions at pH 7, thermal treatment has the opposite effect, the drainage time being much longer (30 min).
for the unheated WPI dispersions. For unheated WPI dispersion at pH 7 the drainage time remains relatively constant as the concentration increased, but the amount of drained liquid decreased.

In general, it can be concluded that thermal treatment, decreased pH and/or increase in WPI concentration can all improve the WPI foam properties. Considering the foam expansion and the foam stability results, the best foam were achieved for samples containing fibrils [2% (w/w) WPI fibrils at pH 2 and 7] using a whipping time of 15 min. Consequently, this sample will be used for the second part of the study.

**Bubble size distribution and liquid volume fraction**

The foam is a large volume of gas mixed with a much smaller amount of a fluid, but in time these two components tend to separate, the fluid drains and the foam becomes dryer \(^{[28]}\). The bubbles compress together, their packing degree being characterized by the liquid volume fraction \(\varepsilon\) (Figure 6.5) \(^{[27, 28]}\). Micrographs and image analysis data for foam bubble size are shown in Figure 6.6. In foams with small liquid fractions \((\varepsilon \leq 0.05)\) the bubbles are more tightly packed exhibiting a polyhedral shape with slightly curved faces and well defined edges \(^{[28]}\). An increase in a liquid fraction decreased the bubbles packing and starting with the critical value \(\varepsilon_c = 0.36\) the bubbles were no longer deformed, this critical value representing the random close packing of solid spheres \(^{[28]}\). Thus, the thin liquid film properties will influence the foam stability, the presence of the protein and/or protein aggregates in the liquid film determining the lamellae structure and morphology. The pH, protein concentration and heat treatment determined changes in the bubble packing.

In this study, the value of \(\varepsilon\) varied from 0.077 for 2% (w/w) WPI fibrillar dispersions at pH 7 to 0.24 for 1% (w/w) native WPI dispersions at pH 7 (Figure 6.5). The liquid volume fraction for EWP foams after whipping was 0.107. Hence, the WPI
foams obtained showed polydisperse bubble size distributions (Figure 6.6). Foams containing the ideal bubble polyhedral shape were not seen in any sample.

![Figure 6.5](image)

**Figure 6.5.** Liquid volume fraction vs WPI concentration before and after thermal treatment for different pH of WPI dispersions whipped for 15 min. Error bars denote ± SD.

The WPI fibrillar dispersions foams at pH 2 and 7 displayed bubbles with different diameter distributions (Figure 6.6). At pH 2, the WPI fibrillar dispersions formed foams containing bubbles with diameters mostly distributed between 100 and 400 µm (d$_{32}$ = 257.6 µm), but some bubbles with low diameter values (≤ 100 µm) were also present. For WPI fibrillar dispersions at pH 7 the foam bubbles were smaller with a maximum diameter of 120 µm (d$_{32}$ = 66 µm).

The heated WPI dispersions at pH 7 exhibit only bubbles with large diameters (100 - 400 µm), no small bubbles being present (< 100 µm). The bubble diameter distribution for EWP dispersions was < 120 µm, similar to that of WPI fibrillar structures dispersions at pH 7, but with fewer bubbles with diameters > 80 µm. The bubble size distribution of EWP foams was skewed to lower values diameters while for
WPI fibrillar dispersions at pH 7 foams, the bubble size were more evenly dispersed over the entire range.

**Figure 6.6.** Optical microscopy images of the foams obtained for 2% (w/w) WPI fibrillar dispersions at pH 2 (a) and 7 (b), heated WPI dispersions at pH 7 (c) and EWP (d) after 15 min of whipping. The histograms inserted in optical images show the bubbles diameter distribution. The Sauter diameter and liquid volume fraction values were also included. The black scales represent 200 µm for all optical images.
6.3.2. Effect of dynamic high pressure via microfluidizer or high shear Ultra Turrax treatment on WPI foam properties

6.3.2.1. The effect of microfluidization

2% (w/w) native and fibrillar WPI at pH 7 were microfluidized at different high dynamic pressures (up to 170 MPa) and the resulting dispersions were whipped for 15 min as described previously. AFM measurements were performed on WPI dispersions before and after the microfluidization process. The microfluidization treatment of the WPI fibrillar dispersions caused fracture of the fibrils into small segments (height of 2 - 3 nm and length of 15 – 400 nm, Figure 6.7.a). The entire process leading to fibril fracture as a function of the applied pressure has been previously described in chapter 5. The foam capacity and stability of unheated and WPI fibrillar dispersions at pH 7 before and after microfluidization treatment were characterized, as previously, by calculating the foam expansion and the drainage as function of time, respectively.

Figure 6.7. AFM images showing the change in fibrils length after treatment with a) dynamic high pressure using microfluidization at 100 MPa and b) shear mixing using Ultra-Turrax at maximum speed (24,000 rpm).
The foaming capacity for the native and fibrillar WPI was not significantly influenced by the microfluidization treatment (Figure 6.8).

**Figure 6.8.** Foam expansion of native and fibrillar WPI dispersions, 2% (w/w) at pH 7, for different applied pressures during microfluidization treatment. Error bars denote ± SD.

The foam stability of native 2% (w/w) WPI dispersions at pH 7 (Figure 6.9) was improved when low pressures (up to 75 MPa) were applied during the microfluidization process and dwindled at high values (over 100 MPa). This variation in the stability is reflected by the drainage time. The drainage time of all foams formed from microfluidized WPI was always smaller than for EWP foam (Figure 6.9).
Figure 6.9. Foam drainage time of 2% (w/w) native WPI dispersions at pH 7 for different applied pressure (up to 170 MPa) during microfluidization treatment.

The foam drainage time evolution for WPI fibrillar dispersions at pH 7 after microfluidization treatment can be observed in Figure 6.10. Pressure treatments of up to 170 MPa have no significant influence on the longer time (100 min) stability of the WPI fibrils. A lag in drainage onset (up to 25 min) was observed, indicating short-term stability of the foams.
Figure 6.10. Foam drainage time of 2% (w/w) fibril WPI dispersions at pH 7 for different applied pressure (up to 170 MPa) during microfluidization treatment.

6.3.2.2. The effect of shear mixing Ultra-Turrax

The effect of shear mixing using Ultra-Turrax at two rotational speeds (11,200 and 24,000 rpm) on the foam properties of 2% (w/w) native or fibrillar WPI dispersions at pH 2 and 7 were examined. As seen in the microfluidization process, the shear mixing Ultra-Turrax treatment resulted in long WPI fibrils being broken into shorter length fibrils (150 - 400 nm) (Figure 6.7.b). There appears to be little difference in foam expansion between 11,200 and 24,000 rotor speeds (Figure 6.11).
The changes in drainage over time (Figure 6.12) show that the foam stability is decreased by the Ultra-Turrax shear mixing treatment since the drainage time is larger for the untreated dispersions than for the treated ones. The WPI fibrillar dispersions at pH 7 had the most stable foams among the treated or untreated dispersions.

Figure 6.11. Foam expansion of 2% (w/w) native WPI and fibrillar dispersions at pH 2 and 7 after shear mixing by Ultra Turrax. Error bars denote ± SD.
Figure 6.12. Foam drainage time of 2% (w/w) WPI native and fibrillar dispersions at pH 2 and 7 after shear mixing treatment by Ultra Turrax. The first, second and third symbol columns correspond to 0, 11200 and 24000 rpm, respectively.

The value of $\varepsilon$ varied from 0.075 for 2% (w/w) WPI fibrillar dispersions at pH 7 microfluidized at high pressure (over 100 MPa) to 0.15 for 2% (w/w) WPI dispersions at pH 7 microfluidized (Figure 6.13.a and b). The liquid volume fraction for EWP foams after whipping was 0.107.

Ultra-Turrax mixing at the maximum speed produced an increase in the liquid volume fraction for fibrillar dispersions and a decrease for the other samples.
Figure 6.13. Liquid volume fraction vs (a) microfluidization and (b) Ultra Turrax mixing of unheated or heated WPI for different pH of WPI dispersions whipped for 15 min. Error bars denote ± SD.
General discussion

On whipping of the WPI dispersions, energy was supplied into protein liquid dispersions to incorporate air \[2, 3, 11\]. Thus, a cohesive air-water interfacial film was formed, the spreading of the liquid around the air bubbles would be influenced by the protein and/or protein aggregates adsorbing to the interface \[3, 4, 7-10, 12\]. Protein adsorption at the interface suggests unfolding and denaturation by exposing hydrophobic residues of the protein to the air phase and its hydrophilic segment to aqueous phase \[4, 7-10, 12\], the hydrophobic segments facilitating the initial anchoring of the native/denatured protein.

Native WPI at neutral and acidic pH leads to a faster adsorption at air-liquid interface due their high diffusivity and forms a thick interfacial film \[16\]. After long thermal treatment (20 h at 80 °C) at pH 2 and 7, WPI associates into fibrils and aggregates, respectively (Figure 6.1.a and b). These larger size assemblies should diffuse more slowly to the air - liquid interface. Consequently, they should have a lower capacity to stabilize the interface and foam properties. Thus, one should expect that heated dispersions will not have better foaming properties. However, when fibrils and/or aggregates were present, the foam capacity of the WPI dispersions was increased (Figure 6.2., 6.3., 6.6. and 6.11.). The presence of very long fibrils (up to 15 µm) further increased the foam capacity of the WPI dispersions, the best foam being achieved for WPI fibrillar dispersions at pH 7 containing a mixture of small aggregates, short and long fibrils. When the fibrils were fractured by applying high dynamic pressure using microfluidization (at over 100 MPa) and Ultra-Turrax treatments the foam capacity decreased but still remained larger than that of non-treated WPI and EWP dispersions.

Foam stability is generally affected by the drainage of the liquid film and bubble coalescence. In order to produce a high volume stable protein foam, drainage of liquid from between the bubbles must be minimized \[2, 3, 10, 12\]. It was already shown that
mixtures containing various amounts of protein aggregates with different sizes can stabilize the foam via two mechanisms \[^{29, 30}\]. If the aggregates adsorb on to the liquid interface, they will increase its viscoelasticity and the foam becomes stable. If the aggregates remain in the liquid phase, they can undergo a percolation process due to confinement, leading to a gel-like network formation \[^{29, 31}\]. In WPI fibrillar dispersions at pH 7 clusters of long and short fibrils were observed (see chapter 4). Considering the thickness of the liquid films separating the bubbles (up to 10 µm), it can be concluded that the fibrils were constrained, possibly forming the gel like network described above. For heated WPI dispersions at pH 7 (Figure 6.1.a and c), the aggregates that have high surface energy will have a reduced mobility and affinity to the liquid-air interface. These results suggested that the foams obtained from thermal treated WPI dispersions at all pH might be stabilized via both proposed mechanisms.

Foam stability is also related to the rheological properties of the interfacial film and can be enhanced increasing film viscosity, achieved either by raising the protein concentration or by adjusting the pH away from isoelectric point. The presence of the fibrils increased the viscosity of the WPI dispersions, higher viscosity being displayed at pH 7 than at pH 2 \[^{31}\]. A higher viscosity should produce a more stable interfacial film leading to a reduction of the drainage and a more stable foam. The different foam stabilities observed in Figure 6.4., 6.9., 6.10., and 6.12. can be related with changes in the WPI solution viscosity, with dispersions with the largest viscosity [1 - 3% (w/w) WPI fibrils at pH 7] producing the most stable foams.

After microfluidization treatment, all WPI fibrillar dispersions exhibited a short-term stability (estimated by the time of the first drop), followed by a long term stability (emphasized by the drainage curves). Thus, the time of the first drop drained was increased by up to 20 min for the WPI dispersions containing fibrils or aggregates while
for untreated WPI dispersions this time period was up to 10 min. A positive effect on long-term stability was displayed by the WPI fibrillar dispersions at pH 7.

The second factor contributing to foam stability is bubble coalescence which is determined by the balance between capillary and disjoining pressures \[12\]. The capillary pressure occurs due to the pressure difference between the air and liquid phase, its value being given by the Laplace equation \[12\], while the disjoining pressure arises from forces between the two interfaces of the liquid lamella. When no proteins are present the disjoining pressure is negligible and the film collapses due to Laplace capillary pressure. When proteins are adsorbed at the two film interfaces, the interaction between them tends to increase the thickness of the liquid film and to prevent its thinning \[12\]. If adsorbed at surfaces the polymeric chains tend to arrange in tail, loop and/or train configurations \[12\]. When two such surfaces are brought near each other, steric repulsion interactions appear, thickening the film. In the liquid film of the WPI fibrils foam such polymeric structures (long and/or short fibrils) are present and can lead to an increase in the steric repulsion stabilizing the film against coalescence. Thus, when long fibrils were present, the foam stability was larger than for the short fibrillar dispersions obtained after dynamic high pressure treatment or aggregates dispersions.

The WPI aggregates dispersions at pH 7 exhibited a low foam capacity and this might be explained by the hydrophobic interactions that are present and can inhibit the aggregates to form a cohesive interfacial film. Conversely the heat treatment increased the hydrophobicity of the WPI at pH 7 dispersion leading to more stable foams.

The improvement of foam properties of heated WPI dispersions may be correlated to changes in the secondary structure of whey protein (an increase of β-sheets content). The increase of molecular flexibility of whey protein by cleavage of disulfide bonds, the re-oxidation of SH groups in the protein film at the air-water interface might also improve the foaming capacity \[12, 19, 20\]. A stable structured and cross-linked whey
protein unfolds and adsorbs more slowly than a flexible, non-cross-linked one with lower rigidity which effectively stabilize the newly formed interface \[^{[4, 9, 12, 32]}\]. The lack of flexibility of the protein aggregates leads to the need for higher protein concentrations at the interfaces to adequately decrease interfacial tension. Thus, the foam capacity increased with WPI concentration for all heated WPI dispersions.

The degree of bubble packing and the amount of liquid can also affect the foam stability, the higher the initial liquid content (or the denser the foam), the faster the foam being can be destabilised \[^{[6]}\]. Foams with the smallest liquid volume fraction \(\varepsilon\) (and as consequence the less amount of liquid) were the most stable ones, this being a possible explanation for the larger stability exhibited by the WPI fibrillar dispersions at pH 7 \((\varepsilon = 0.077)\).

6.4. Conclusions

The results demonstrate that prolonged thermal treatment of WPI dispersions at acidic or neutral pH improved their foaming properties. Fibrillization of whey protein significantly improved both foaming capacity and stability when compared to the non-fibrillar heat denatured protein. Furthermore, whipped foams prepared with fibrillar whey proteins compared favourably with those produced with egg white protein. Dynamic high pressure and Ultra-turrax treatments had little effect the WPI foam properties. Further work is needed to clarify the physico-chemical basis for the observed improved foaming properties of fibrillar whey proteins.
6.5. References


Chapter 7.

General discussions, conclusions and future work
7.1. Conclusions

The overall goal of this thesis was to investigate the mechanism of β-lactoglobulin fibrils formation and to study their behaviour in different processing environments that can lead towards possible applications in food.

The assembly of fibrils from β-lactoglobulin (β-lg) was examined as a function of protein concentration, pH, ionic strength, time and temperature of heating (chapter 2 and 3). At neutral and alkaline pH, the β-lg molecule undergoes conformational changes under thermal treatment and is aggregated via non-native disulfide bonds and/or non-covalent bonds into larger aggregates. No fibrils were obtained even after prolonged heating at neutral and alkaline pH (chapter 2). A decrease of native secondary structure, suggested by the disappearance of intramolecular bonds (1626 and 1633 cm\(^{-1}\)) and appearance of newly formed intermolecular bonds (1622 cm\(^{-1}\)) caused by the thermal denaturation and aggregation of protein was demonstrated using ATR-FTIR.

A detailed study of the aggregation of whey protein in acidic conditions showed that long fibrils can be formed using β-lg at low protein concentration and heating at 80 °C for 20 h (chapter 3). Results obtained from monitoring the assembly processes \textit{in-situ} and in real time using ATR-FTIR spectroscopy correlated with AFM, STEM and SDS-PAGE results suggested that a two-stage mechanism of β-lg fibril formation occurred: 1) denaturation, partial unfolding and increase in β-sheets with hydrolysis of monomers followed by 2) linear aggregation of polypeptide into fibrils via non-covalent interactions, accompanied hydrolysis and decrease in β-sheets and overall secondary structure (chapter 3). “Tapping” mode AFM images of samples either in air or in water clearly show the structures of β-lg and whey protein isolate fibrils, having a height of 2-3 nm, length up to 15 μm and periodicity of ~ 30 nm. The two other major proteins of whey protein, α-lactalbumin (α-la) and bovine serum albumin (BSA) did not form
fibrils even after 20 h of heating at 80 °C, but only formed globular protein aggregates when observed under the same conditions as β-lg (Appendix I, Figure A.I.1.a and b).

After the mechanism of fibril formation was optimized, the behaviour of the fibrils in different environments was monitored. Acidification, heating, shearing, and/or high-pressure treatments induced changes in physico-chemical properties of whey proteins and of the fibrils (chapter 4 and 5). The pH stability of fibrils was monitored and the pH adjustment led to changes in the length of the whey protein fibrils. The length of the fibrils decreased when the pH of the protein dispersion was raised from 2 to isoelectric range (4.6-6) but were longer at pH 7-12. Short and long fibrils were present over the entire pH range as observed by AC mode-AFM in chapter 4.

Another factor that induced changes in the physical dimensions of the whey protein fibrils and their secondary structure was high dynamic pressure treatment using a microfluidizer (chapter 5). Dynamic high pressure treatment of the native β-lg induced 40% protein denaturation, as shown by RP-HPLC. This was accompanied by slight changes in secondary structure revealed by ATR-FTIR and ThT fluorescence spectroscopy. Fibrils formed from high pressure treated β-lg/WPI were slightly thicker but similar in length to the fibrils formed from non-pressure treated proteins (AFM, STEM, shear birefringence). Dynamic high pressure treatment of β-lg/WPI fibrils fractured the fibrils into regular short size fibrillar structures but did not completely dissociate them.

Whey protein foaming capacity and stability were found to be related to protein concentration, pH, time of whipping, pressure and heating treatments. Fibril based foam properties were compared with those of native or denatured whey protein and also with egg white protein (chapter 6). Foams produced from fibrils showed significant improvements in foaming capacity and stability when compared with non-fibrillar whey protein. High dynamic pressure had no significant effect on whey protein fibrils foam
properties. Furthermore, foams prepared with fibrillar whey protein (≤ 3% protein) were comparable with egg white protein (> 10%) foams, the traditional foam ingredient in food industry. Results suggested that fibrillized protein is an effective foaming agent even at relatively low concentration of protein [1-3% (w/w)].

7.2. Recommendations for future work

Preliminary trials were performed to investigate further properties of the whey protein fibrils for their potential incorporation in food products. The initial results of these studies and recommendations for future investigations are summarised below, the experimental conditions and the tests results being further described in the Appendix II.

Dehydration of fibrils by freeze and spray-drying treatments

In general, whey proteins are used in industry as powdered ingredients as they can be easily stored, transported and/or incorporated in food products. The potential of fibrils as dried food ingredients should thus be explored. Preliminary results when fibrils where freeze or spray dried are presented in the Appendix II. 1. The reconstitution of dried fibrils in Milli-Q® water was also tested. Reconstitution of freeze and spray dried β-lg fibrils in Milli-Q® water at room temperature showed that the fibrils were still present but were significantly shorter (Figure A. II. 1. a). Further investigations are therefore required to determine the optimum conditions for whey protein powder production and fibril reconstitution.

Fibril digestion

When considering the incorporation of fibrils into food products, their digestibility should be investigated by incubation in simulated gastrointestinal fluid. Preliminary digesting experiments using the AFM and gel permeation chromatography
(GPC) results are presented in Appendix II. Results suggest that fibrils may be only partially digested in such an environment. These results seem to contradict recent findings in the literature that reported a complete destruction of the fibrillar structures by pepsin followed by the self-assembly of the resulting peptides into thinner fibrils with storage after enzyme activity ceases \cite{1}. The presence of fibrillar structures in our preliminary trials may be due to differences in the simulated gastrointestinal fluid used or from the shorter exposure time of the fibrils to the enzymes. Another factor to be considered is that the mature fibrillar structures [2% (w/w) protein formed after 20 h at 80 °C] could be more stable to enzymatic action than fibrils formed with 1% (w/w) protein and will therefore require longer digestion periods.

Whey protein fibrillar gels

As observed in chapter 3, the fibrils have a large aspect ratio. Therefore, they can increase the viscosity, shear thinning behavior and the gelation properties of the solutions and can act as thickening agents when introduced in food products \cite{2,3}.

During the trial experiments (Appendix II.3.) hot or cold induced β-lg fibrillar gels were investigated as a function of the concentration of CaCl$_2$, NaCl and glucono-δ-lactone (GDL) for possible application in food products. Whey protein fibrils were found susceptible to cross-linking by transglutaminase. A firmer gel was obtained by mixing fibrillar dispersion with 1% (w/w) sodium alginate. To understand and optimize the gelling properties, further investigations are required to determine the influence of the fibril length, pH, salt concentration, etc.

Emulsion properties of whey protein fibrils

Proteins are commonly used in food products as stabilizers for food emulsions and provide nutritional benefits to the products \cite{4-6}. Preliminary experimental tests on
the emulsifying properties of the native and heated whey protein isolate and their fibrils have been performed using different methods of mixing (microfluidizer, Ultra-Turax, Silverson) (Appendix II.4). A small improvement of the emulsions using fibrils was observed. The fibrils and/or oil concentration, pH, pressure treatment was observed to have influence the fibrils emulsifying properties, further investigations being recommended.

Meat replacement and edible/biodegradable film

Meat analog products often have inferior sensory quality as they do not have the same heterogeneous texture as meat. Fibrils are highly anisotropic and, if they can be aligned in different spatial conformations (e.g. in a flow field [7]), they have the potential to be used as fillers to obtain a fibrous texture. Meat analogues using 4% (w/w) WPI fibrils were produced but exhibited a tough and brittle texture.

Fibrils might be also incorporated or laminated into biodegradable films as a nano-filler in order to prevent moisture transfer. The results of the preliminary film testing were encouraging, suggesting that multi-laminar films containing fibrils may be formed. These films appeared to have hydrophobic surfaces, based on higher contact angles seen when water droplets were deposited on the film surface.
7.3. References


Appendices
Appendix I: Aggregation of α-lactalbumin and bovine serum albumin investigated by atomic force microscopy

Materials and methods

Sample preparation

Bovine serum albumin (≥ 97% purity, by agarose gel electrophoresis; Ref. A 4378, Lot 021k7606), HCl and NaOH were obtained from Sigma Aldrich (Ireland). α-Lactalbumin (95% purity) were obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). Purified water [Milli-Q® water, resistivity (18.2 MΩ cm, at 25 ºC), Millipak® Express] was used throughout. All other reagents were analytical-grade.

Protein dispersions

Both bovine serum albumin and α-lactalbumin dispersions were prepared using the same procedure as for β-lactoglobulin or whey protein isolate described in chapter 3. To obtain two different protein concentration 2 and 6 g were dispersed in 98 g and 94 g, respectively, of Milli-Q® water and stirred overnight to ensure full hydration of the protein. The pH was adjusted to 2.0 using 6 M HCl. After centrifugation and filtration, the protein dispersions were heated at 80 ºC for 0, 30, 100 min and 20 h, respectively. NaCl and CaCl₂ (0.03 M) were added after heating and sample was heated for another 2 h at 80 ºC.

AC-Mode Atomic Force Microscopy

Bovine serum albumin and α-lactalbumin dispersions were investigated using a MFP-3D atomic force microscope (Asylum Research UK, Ltd., Oxford, U.K.). Samples of heated protein were investigated using AFM analysis using the same procedure as for β-lactoglobulin or whey protein isolate described in chapter 3. Prior to imaging,
dispersions were diluted in Milli-Q® water to a final concentration of 0.02 or 0.06% (w/w), at pH 2.0, to facilitate imaging of single protein aggregate. Thus, for imaging in air-dried mode, 10 µL of BSA and α-la diluted with Milli-Q® water was deposited onto a freshly cleaved mica surface and subsequently dried in a desiccator.

Results and discussion

Aggregation of α-lactalbumin and bovine serum albumin investigated by atomic force microscopy

The other two major proteins of whey protein, α-lactalbumin (α-la) and bovine serum albumin (BSA) don’t form using the same procedure as for β-lactoglobulin or whey protein isolate described in chapter 3 fibrils even after 20 h of heating at 80 °C or on the addition of NaCl. Only protein aggregates were formed (Figure A.I.1.a and b). The cross-sectional height profiles, as measured using the MFP-3D software showed large aggregates of α-la after long heating times (up to 20 h) with heights of up to 8 nm (Figure A.I.1.a). Denatured α-la molecules formed small (1-5 nm) and large aggregates (~8 nm). On heating a solution of bovine serum albumin for 20 h at 80 °C in the presence of NaCl a mixture of non-aggregated protein (~1 nm), small aggregates (~2-4 nm) and large aggregates (~8-9 nm) was formed (Figure A.I.1.b). Even when the protein concentration was raised to 6% (w/w) in the absence or presence of salt, no fibrils were obtained. Therefore, no further investigations were pursued.
Figure A.I.1. AFM images of aggregates present in a 2% (w/w) protein dispersion at pH 2 for 20 h heating at 80 °C: a) α-lactalbumin b) bovine serum albumin (in addition of 0.03 NaCl). Inset: Higher resolution height image of 600 nm x 600 nm area showing α-la and BSA aggregates, respectively.
Appendix II. 1. Dehydration of fibrils by freeze and spray-drying treatments

Materials and methods

Materials

Bovine $\beta$-lactoglobulin, HCl, NaOH were used as in previous chapters 2 and 3 of this thesis.

Preparation of $\beta$-lactoglobulin fibrils

$\beta$-Lactoglobulin fibrils were prepared following the procedure previously described in chapter 3.

AC-Mode atomic force microscopy

$\beta$-Lactoglobulin dispersions were investigated using a MFP-3D-BIO™-AFM instrument (Asylum Research UK Ltd., Oxford, UK) as described in chapter 3.

Freeze and spray drying

Fibrillar dispersion (500 mL) of 2% (w/w) $\beta$-lg at pH 2 was transferred to a 1 L flask, shell frozen in methanol bath (Heto Lab Equipment A/S, Denmark) at – 45 °C for 20 min and dried at - 45 °C under vacuum for 72 h using a Labconco freeze drier and Centri Vap Mobile System (Labconco Corporation). 1L of the $\beta$-lg fibrillar dispersions [2% (w/w) at pH 2] was inserted in a spray dryer (Buchi Mini B-191, Flawil, Switzerland) for 45 min and powdered samples were obtained. The inlet temperature was maintained at 180 °C throughout and outlet air temperature at 93 °C ± 3 °C. The freeze and spray dried samples were stored in a refrigerator at 4 °C for analysis.

Reconstituted $\beta$-lg fibrils were prepared by dispersing freeze/spray dried $\beta$-lg fibrils powder in Milli-Q® water (2 h at room temperature) in a final protein concentration of 2% (w/w) and pH of 2.4 ± 0.5. Alternatively, freeze or spray dried $\beta$-lg fibrils powder
were dispersed in water and heated for 20 h at 80 °C to reconstitute the long fibrillar structures.

**Results and discussion of dehydration of fibrils**

Freeze and spray drying of β-lg fibrils lead to a mixture of short fibrillar structure, aggregates and non aggregated proteins as imaged by AFM (Figure A.II.1).

**Figure A.II.1.** AFM height image of reconstituted (a) freeze dried β-lg fibrils at pH 2.4 ± 0.5 at room temperature; (b) spray dried β-lg at pH 2.35 ± 0.3 for 20 h at 80 °C. Insets represent magnified regions (500 nm x 500 nm) of Figure A.II.1. a and b, respectively.

The cross section profile along the fibrils revealed that short fibrils have the same periodicity of 30 – 40 nm (inset magnitude 500 nm x 500 nm of A.II.1.a) as for the untreated long fibrils (chapter 3). The height of the short fibrils after reconstitution of freeze dried fibrils powder dissolved in Milli-Q® water at room temperature exhibited an increase to ca. 4 – 7.5 nm with lengths shorter than 1 µm. The AFM measurements showed that these shorter fibrils maintained their length and height even when the pH of the protein dispersion was raised to neutral pH (data not shown). Following the reconstitution of the freeze or spray dried fibrillar dispersions by reheating for 20 h at
80 °C, long fibrils (up to 3 µm length and 2 – 4 nm height) and small aggregates (< 0.5 µm length) were obtained (Figure A.II.1.b).

Appendix II. 2. Fibrils digestion

Materials

The materials and the procedure used for β-lactoglobulin fibrils preparation were the same as in Appendix II.1.

Pepsin from porcine gastric mucosa (lyophilized powder, 2,530 units/mg solid; 2,890 units/mg protein; P7012), α-chymotrypsin-agarose from bovine pancreas (lyophilized powder stabilized with lactose, 116.3 mg solid; 430 units/g solid; 2580 units/g agarose; C9134), Trypsin from porcine pancreas (1473 units/mg, 93615) were purchased from Sigma-Aldrich (Ireland).

Separation of fibrils and non-aggregated protein

In order to study the effect of enzyme on fibril structure and their digestion, β-lg fibrillar dispersions were diluted and separated using centrifugal membrane filters (MWCO 100 kDa, Vivaspin, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The β-lg dispersions containing a mixture of fibrils, small aggregates and non aggregated proteins, were diluted to different concentrations [0.04, 0.08 and 0.1 % (w/w)] and centrifuged at 2,800 x g for 30 min at 20 °C to remove small aggregates and the non aggregated proteins. This procedure was repeated three times to assure the removal of the non-fibrillar material from dispersion. After each centrifugation step, the recovered fibrillar dispersions were washed with HCl solution (pH 2). After the third centrifugation step, only fibrillar structures remained in dispersions. Prior to digestion tests, the fibrils were freeze dried and analysed with AFM and GPC as previously described.
Simulated gastrointestinal environment

Simulated gastric juice was prepared using 0.2% (w/w) NaCl, 0.06% (w/w) KH$_2$PO$_4$, 0.011% (w/w) CaCl$_2$ and 0.037% (w/w) KCl, adjusted to pH 2.0 using 1 M HCl and autoclaved at 120 °C \cite{21}. Pepsin (2.45 g) was then added to 35 mL of gastric juice. Simulated ileal juice at pH 7 was prepared using 0.61% (w/w) NaCl, 0.07% (w/w) KH$_2$PO$_4$, 0.03% (w/w) NaH$_2$PO$_4$, 0.1% (w/w) NaHCO$_3$ \cite{21}. Trypsin (300 mg) and chymotrypsin (20 mg) were added to 10 mL of ileal juice.

Freeze dried β-lg fibrils at pH 2 [35 mg of 0.1% (w/w)] were incubated in 35 mL of gastric juice (containing pepsin) at 37 °C for 1 h with constant stirring. Subsequently, the pH of the new dispersion was raised to 7 using 6 N NaOH; therefore, 5 mL of gastric juice at pH 7 (containing pepsin and freeze dried fibrils) was incubated with 5 mL of ileal juice (containing trypsin and chymotrypsin) at 37 °C for 2 h with constant stirring. The samples were diluted with MilliQ® water to a final protein concentration of 0.025% (w/w), filtered and analyzed by gel permeation chromatography.

Gel permeation chromatography

The chromatographic system consisted of a Waters™ Dual λ Absorbance Detector 2484, Waters™ 600S Controller and Waters 717 Plus Autosampler (Waters Corporation, Milford, MA, USA). Separation of β-lg fibrils fractions was achieved using TSK G 3000SW XL (7.8 mm ID x 30 cm, 5µm) and TSK G 2000SW XL (7.8 mm ID x 30 cm L, 5 µm, TosoHaas, Montgomeryville, PA, USA) columns in series. Samples were filtered through 0.2 µm filters (Minisart, Sartorius, Germany) prior to injection into the column. The protein fractions were eluted in isocratic mode at a flow rate of 1 mL min$^{-1}$ using 30% acetonitrile containing 0.1% (v/v) trifluoroacetic. Chromatographic data were detected and collected at wavelength detector at 214 nm.
Pure β-lg and WPI were purchased from Sigma-Aldrich (Ireland) were used as standards.

**Results and discussion on digestibility of β-lg fibrils**

Prior to performing digestibility tests, β-lg fibrils were separated from the non-aggregated material. After β-lg fibril separation using Vivaspin centrifugal filters, AFM measurements revealed an absence of non-aggregated proteins and the same characteristics (length and height) of the fibrils as in chapter 3. The length of β-lg fibrils were affected by freeze drying treatment and also in simulated gastrointestinal environment.

**Digestibility of β-lg fibril**

β-Lactoglobulin fibrils were not fully digested when sequentially exposed to gastric and ileal juice. The AFM measurements showed that short (50 – 200 nm) and thin (1.5 ± 0.5 nm) fibrils were still present after digestion (Figure A.II.2.a). GPC chromatograms of the fibrils before and after digestion (Figure A.II.2.b) show that the samples contained high and low molecular weight structures. Fragments of β-lg fibrils were still present after the enzyme action during digestive treatment.
Figure A.II.2. a) AFM height image of digested β-lg fibrils at pH 7; b) GPC elution profile of the freeze dried β-lg fibrils after enzyme action during simulated gastrointestinal test. Inset: the corresponding molecular weight at the elution time.

As expected, the large aggregates eluted from the column earlier than the peptides segments. In the absence of enzyme, aggregates larger than 20 kDa eluted after 11.94 min from the column (Figure A.II.2.b). After gastric juice treatment, the amount of eluted large aggregates (≥10 kDa) decreased, while peptides of low molecular weight
Appendix II

(< 5kDa) appeared with an elution time of 15.70 min indicating that enzymatic cleavage had occurred.

Treatment with ileum juice increased the low molecular weight peptide content (< 0.5 kDa). However, even after this treatment the presence of aggregates with molecular weight larger than 10 kDa can be still observed indicating that the fibrils are not fully digested as seen by AFM. The increase in the amount of low molecular weight structures (< 0.5 kDa) is indicative of the cleavage of the original peptides by enzyme during the digestion process.

Conclusions

1. Dehydration of fibrils

Freeze or spray drying treatment did not completely destroy the β-lg fibrils, small fibrillar structures were always present. Rehydration of the dried fibrils revealed larger heights (4-7.5 nm) and smaller lengths (1 µm) of fibrils.

2. Digestion of fibrils

Sequential digestion with pepsin, trypsin and chymotrypsin reduced the length of rehydrated fibrils, but did not appear to digest the fibrils completely.

Appendix II. 3. Whey protein fibrillar gels

Materials

Bovine β-lactoglobulin, HCl, NaOH were used as in previous chapters 2 and 3 of this thesis. Glucono-δ lactone (GDL) was purchased from Roquette Italia S.p.A. (AL, Italy).
Appendix II

Preparation of β-lactoglobulin fibrils

β-Lactoglobulin fibrils were prepared following the procedure previously described in chapter 3.

AC-mode atomic force microscopy

β-Lactoglobulin dispersions were investigated using a MFP-3D-BIOTM-AFM instrument (Asylum Research UK Ltd., Oxford, UK) as described in chapter 3.

Whey protein fibrils gel preparation

The pH of a series of fibrillar dispersions was adjusted to 7.0 with 6M NaOH for subsequent GDL treatment and analysis. In order to study the capacity of fibrils to form gel network, 50 mM, 100 mM and 3 M NaCl or CaCl₂ and 0.5% (w/w) GDL were used. After addition of salt in fibrillar dispersion, a series of samples was heated for 2 - 68 h while another series was refrigerated for 72 h at 4 °C.

Results and discussion on whey protein fibrils gel

Fibrils gel in presence of NaCl, CaCl₂ and GDL

As shown in chapter 3 all the results for β-lg can be generalized to the WPI. The WPI gelation properties depend on protein concentration, pH value, ionic strength and thermal treatment conditions. AFM height images were performed in air to monitor the morphology changes of whey protein isolate and β-lactoglobulin fibrils after addition of NaCl or CaCl₂. The AFM images showed long fibrils formed after 20 h heating at 80 °C of 2% (w/w) β-lg at pH 2 (see chapter 3). After NaCl or CaCl₂ addition and short refrigeration time (2 h) the length of the fibrils was not changed (Figure A. II. 3. a). The periodicity of fibrillar structures was 40 - 60 nm and their height 2 - 6 nm.
Figure A. II. 3. (a) AFM height image of β-lg fibrils at pH 2 after 50 mM NaCl addition and 2 h at 4 °C. Magnified (500 nm x 500 nm) area of Figure A.II.3.a, highlighting the periodicity of fibrils. (b) AFM height image of β-lg fibrils at pH 2 after 50 mM NaCl addition and reheated for 2 h at 80 °C. Images were obtained using AC mode in air.

Addition of NaCl, CaCl$_2$ and GDL in 2% (w/w) WPI at pH 2 and 7, formed a weak cold gel after storage at 4 °C for a 72 h (Table A. II. 1). At pH 2 or 7, the fibril dispersion without salt appeared as a viscous solution rather than a gel.

When the fibrils and NaCl or CaCl$_2$ dispersions were heated for 2 h at 80 °C a cross-linked network was observed by AFM (Figure A. II. 3. b). The fibrils network determines an increase in the viscosity that leads to the weak gelation. If the protein dispersion from the Table A.II.1 were reheated at 80 °C for 2 – 68 h no strong gel were formed. Visually, slight increase in the capacity of the protein aggregates to form gel network as increasing the salt concentration or heating time was observed. Gel strength increased with increasing the concentration of NaCl, aggregates via covalent or noncovalent bonds occurred in the fibrillar gel when NaCl concentration reached 3 M.
### Table A.II.1. Characterization of WPI gels with different salt content and storage at 4 °C for 72 h.

**Fibrils gels with transglutaminase or sodium alginate**

β-Lactoglobulin fibril was found to be susceptible to cross-linking by transglutaminase or sodium alginate. Transglutaminase was added in fibrillar dispersions to a final enzyme concentration of ~1% (w/w) and the pH was adjusted to 6 and 8. Subsequently, the fibrillar dispersions were stirred for 5 h at 50 °C and 1 h at 80 °C. No changes were observed at pH 6. At pH 8 a network of fibrils was observed by AFM (Figure A.II.4.a), most probably due to enzymatic cross-linking with transglutaminase. The fibrils were cross-linked at random directions, a network of aggregates and fibrils being obtained (Figure A.II.4.a).
Figure A.II.4. (a) AFM image showing cross-linking of β-lg fibrils with transglutaminase at pH 8 and (b) sodium alginate-fibrils composite gel.

A preliminary test on fibrils/polysaccharide composite hydrogels was performed using WPI fibrils in a matrix of 1% (w/w) sodium alginate solution resulting in a gel structure. The visual appearance of the sodium alginate-fibrils composite gel can be seen in Figure A.II.4.b.

Conclusions

A weak gel network of whey protein fibrils at pH 2 or 7 was observed as function of different concentration of NaCl, CaCl\textsubscript{2} and GDL after heating or refrigeration. A cross-linking of the fibrils was obtained using transglutaminase and a stable gel was obtained after fibrils addition in a sodium alginate matrix. These results might constitute the starting point for a study on whey protein fibril gelation.
Appendix II. 4. Emulsion properties of whey proteins fibrils

Materials

Whey protein isolate, HCl and NaOH were used as described in chapters 2 and 3 of this thesis. Commercial sunflower oil was used.

Preparation of WPI fibrils

WPI fibrils were prepared following the procedure previously described in chapter 3. The pH was adjusted to 7 to prevent the corrosion of the microfluidization equipment (see chapter 5).

Preparation of WPI emulsions

To produce emulsions, the protein dispersions and oil were mixed by applying high shear Ultra Turrax, Silverson and dynamic high pressure by microfluidizer treatments. As the results were similar for all the methods used, only the results obtained after microfluidization are presented in this thesis. Dispersion of 2% (w/w) WPI fibrils at pH 7 and 2% (w/w) WPI heated at pH 7 for 20 h with 20% (w/w) sunflower oil were microfluidized at 50 MPa.

LUMiFuge

A LUMiFuge® 116 particle separation analyser was used to investigate the sedimentation kinetics of 2% (w/w) WPI fibrils and heated 2% (w/w) WPI at pH 7/oil mixtures during centrifugation. This work focuses on the sedimentation stability of dispersions using a recently introduced separation automated–the LUMiFuge® 116 (L.U.M.GmbH, UK) system. The system measures near infrared (NIR) transmission profiles continuously during centrifugation. Separation kinetics can thus be studied under accelerated conditions and two samples run in parallel using volumes 0.5mL. The
0.5 mL sample volume (in a 1.12 cm diameter tube, Cell Type 110-131xy, polycarbonate) forms a rather shallow suspension sample compared to the distance from the axis of rotation to the base of the tube (11.2 cm), thereby minimising the lateral particle movement that could invalidate gravitational settling. The 3000 rpm rotor speed is equivalent to 1200 g acceleration with reference to the base of the tube. NIR transmission profiles were recorded continuously every 20 s during the 1 h 24 min (non-microfluidized emulsions) and 12 h (microfluidized emulsions) separation process. The LUMiFuge software – SEPView 4.1(L.U.M.Ltd.) – calculates the integral of every transmission curve over the chosen sample radius. Integral transmission increases linearly as a function of time in the initial sedimentation phase.

**Results and discussion on emulsion properties of whey proteins fibrils**

The changes in the visual appearance of the dispersion of 2% (w/w) WPI fibrils at pH 7 and 2 % (w/w) WPI heated at pH 7 for 20 h with 20% (w/w) sunflower oil before and after microfluidization at 50 MPa were recorded. Visually, these two components (protein dispersions and oil) seem to be well homogenised after microfluidization treatment at 50 MPa (Figure A.II.5.).

LUMiFuge results (Figure A.II.6) show that a phase separation of the oil - protein components was present. The oil signature is visible in the left hand side while protein dispersion can be observed in the right hand side.
Figure A.II.5. Visual aspect of 2% (w/w) WPI at pH 7 heated for 20 h (a) and 2% (w/w) WPI fibrils at pH 7 (b) and 20% (w/w) sunflower oil before (left) and after (right) microfluidization at 50 MPa.

Figure A.II.6. LUMiFuge results showing the transmission profiles of emulsions containing 20% (w/w) sunflower oil for (a) 2% (w/w) WPI at pH 7 heated for 20 h and (b) 2% (w/w) WPI fibrils at pH 7 before (left) and after (right) microfluidization treatment at 50 MPa.
Conclusions

A small improvement of the emulsions using fibrils was observed after microfluidization at 50 MPa, but further studies on fibrils and/or oil concentration, pH, pressure treatment, are recommended as these factors will influence fibril emulsifying properties.
Characterization of β-Lactoglobulin Fibrillar Assembly Using Atomic Force Microscopy, Polyacrylamide Gel Electrophoresis, and in Situ Fourier Transform Infrared Spectroscopy

DANIELA CHEROCCIO1,*, LIZA WANG1, ANDRÉ BRECKMEIR1, EDMOND MAGNÉ1,2, and MARK A. E. AUSTY*1

1Mooresville Food Research Centre, Teagasc, Mooresville, Fennery, County Wicklow, Ireland, and Materials and Surface Science Institute and Department of Chemical and Environmental Sciences, University of Limerick, Limerick, Ireland

The aggregation process of β-lactoglobulin (β-lg) from 0 min to 20 h was studied using atomic force microscopy (AFM), scanning transmission electron microscopy (STEM), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and in situ attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). Fibril assembly was monitored in real time using AFM up to 20 h. From 0 to 85 min, β-lg monomers deformed, expanded, and aggregated to a common aggregate. After 65 min, fibrillar structures were formed, exceeding 10 μm in length. Fibrillar structures were confirmed by STEM. Secondary structural changes occurring during fibril formation were monitored by ATR-FTIR at 80 °C and indicated a decrease in α-helix content and an increase in β-sheet content. SDS-PAGE indicated that fibrils were composed of polyglycine and not intact monomers. In this study, β-lg and whey protein isolate (WPI)-derived fibrils, including some double helices, were observed by AFM under ambient conditions and in their native aqueous environment.

KEYWORDS: β-Lactoglobulin, whey protein isolate; proteins; peptides; self-assembly; fibrils; AFM; ATR-FTIR; SDS-PAGE; STEM

INTRODUCTION

Whey protein isolate (WPI) is extensively used as a functional ingredient in the food industry. Whey proteins in bovine milk contain a mixture of β-lactoglobulin (β-lg), α-lactalbumin, and bovine serum albumin, all of which may form fibrillar aggregates under specific conditions (1, 2). β-lg is the major whey protein in cow's milk, representing ~50% of the total whey protein content. β-lg is a globular milk protein, whose secondary structure contains approximately 50% β-sheet, 9~12% α-helix, 8~10% turn, and 30~35% random coil (3). β-lg and forms fibrillar aggregates 2-4 nm in thickness upon prolonged heating at low pH and low ionic strength (4, 5). No fibrils are formed upon heating pure α-lactalbumin or bovine serum albumin at pH 2, but fibrils are formed in pure β-lg and WPI solutions (6, 7). Various studies have shown that β-lg structures have the potential to be used as functional ingredients in food product foods because of their unique functional properties, for example, emulsification, gelation, thickening, foaming, and water-binding capacity (8-10). These proteins fibrils could be used as thickening ingredients in low-calorie products, for example, as replacements for polyvinylalcohol-based ingredients or as fat replacement products and high protein content foods. The addition of small amounts of protein fibrils may increase the viscosity and shear-thinning behavior of

*To whom correspondence should be addressed. Telephone: +353-25-624420. Fax: +353-25-62440. E-mail: mark.austy@teagasc.ie

© 2010 American Chemical Society

Published online 20/02/2010
pubs.acs.org/JAFC

177
Appendix III

Obercours et al.

Mili-Q water as described above. A total of 180 μL of diluted β-lg was deposited on the mica surface. After a period of 30 minutes allowing adsorption, AFM images were obtained using sharp and talc-Y-tipped “ultracur” 150° silicon nitride tips (spring constant, 0.08 N/m; Olympus Optical Co., Ltd., Japan). The radius of curvature of the tips was 40 ± 1 nm, with a tip height of 5 μm, which helped to prevent the entanglement of the mica surface with the mica surface. The results were compared. It has been argued that electrostatic or hydrophobic interactions with the mica surface affect the morphology and orientation of the fibrils as determined by AFM [59]. Therefore, to confirm that fibril structures were formed, scanning transmission electron microscopy (STEM) was also performed on selected samples in this study. Previous research showed that β-sheet content presents a spectral band at 1650 cm⁻¹, while α-sheet content presents a spectral band at 1654 cm⁻¹ (50, 71). To clarify the process of fibril formation in a β-sheet disturbance at 90°C and pH 2, we followed changes in secondary protein structure in real time using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and energy-dispersive X-ray spectroscopy (EDS-PAGE) analyses under reducing conditions. These changes were also performed to investigate time-dependent changes in the molecular weights of β-lg fractions, specifically to determine whether fibrils were composed of β-lg monomers or polymeric fragments. The fibril formation process of β-lg and WPI are similar. For investigation methods requiring high amounts of fibril dispersion, WPI is a standard ingredient used by the food industry. β-lg (Sigma grade) was used for EDS-PAGE analyses.

MATERIALS AND METHODS

Sample Preparation. Bovine β-lg (approximately 90% pure [PAGE], 3x crystallized and hydrophobic mixture of genetic variants A and B, reference LID1, Lot 40973B) and HCl were obtained from Sigma-Aldrich (Milwaukee, WI). WPI in 10% (w/v) protein on a dry basis; 45% β-lg, 25% α-lactalbumin, and 15% (w/v) α-lactalbumin was acquired from Danisco Food Quality International, Inc. (Le Creusot, FR). Phosphotungstic acid was purchased from Agar Scientific (Stansted, Essex, UK). Molecular weight standards were purchased from GE Healthcare (Chalfont, St. Giles, UK). Purified water (Milli-Q water, resistivity 18.2 MΩ cm, at 25°C, Millipak Express) was used throughout. All other reagents were analytical grade.

Dispersion Conditions. All samples of β-lg or WPI was dispersed in 0.01 M Mili-Q water and stirred overnight to allow full hydration of the protein, and the pH was adjusted to 2 using 5 M HCl. Dispersions were centrifuged at 4000 rpm for 1 h at 25°C. The supernatant was filtered through a 0.45 μm filter (Millipore, Munktell, Miki, Nissei, Japan) and subsequently, heated at 80°C in a water bath (Grant, FPAT, Ltd.).

AC Mode AFM β-lg dispersions were investigated using a tappingMode AFM (AFM-3D atomic force microscope (Avantes Research UK, Ltd., Oxford, UK)). Samples of β-lg heated at 88°C for 2 min, 12 min, 15 min, 45 min, 65 min, 85 min, 100 min, 200 min, 300 min, and 400 min, respectively, were used for AFM analysis. Prior to imaging, the dispersions were diluted in Mili-Q water to a final concentration of 20 g L⁻¹ (pH 2.6 ± 0.3) to facilitate imaging of single fibrils. Selected samples were also diluted in HCl (pH 2.0) for AC Mode AFM imaging to confirm whether dilution affected the film appearance. No differences were observed between images obtained after water or HCl dilution, and to simplify the paper, only images obtained from water-diluted samples are presented. Thus, 10 μL of diluted sample with Milli-Q water was deposited onto a freshly cleaved mica surface and subsequently dried in a desiccator. An aluminum reflector coating containing with a totalized tip (AC 240, spring constant, 1.8 N/m, resonant frequency, 78 kHz; Olympus Optical Co., Ltd., Japan) and scan rate at 1 Hz was used for air-dried samples. The terrace shape is ideal for analyzing precise morphology of soft samples because of its high lateral resolution. The radius of curvature of the terahedral tip was less than 10 nm. For imaging in water, β-lg or WPI dispersions were diluted in Mili-Q water as described above. A total of 180 μL of diluted β-lg was deposited on the mica surface. After a period of 30 minutes allowing adsorption, AFM images were obtained using sharp and talc-Y-tipped “ultracur” 150° silicon nitride tips (spring constant, 0.08 N/m; Olympus Optical Co., Ltd., Japan). The radius of curvature of the tips was 40 ± 1 nm, with a tip height of 5 μm, which helped to prevent the entanglement of the mica surface. The results were compared. It has been argued that electrostatic or hydrophobic interactions with the mica surface affect the morphology and orientation of the fibrils as determined by AFM [59]. Therefore, to confirm that fibril structures were formed, scanning transmission electron microscopy (STEM) was also performed on selected samples in this study. Previous research showed that β-sheet content presents a spectral band at 1650 cm⁻¹, while α-sheet content presents a spectral band at 1654 cm⁻¹ (50, 71). To clarify the process of fibril formation in a β-sheet disturbance at 90°C and pH 2, we followed changes in secondary protein structure in real time using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and energy-dispersive X-ray spectroscopy (EDS-PAGE) analyses under reducing conditions. These changes were also performed to investigate time-dependent changes in the molecular weights of β-lg fractions, specifically to determine whether fibrils were composed of β-lg monomers or polymeric fragments. The fibril formation process of β-lg and WPI are similar. For investigation methods requiring high amounts of fibril dispersion, WPI is a standard ingredient used by the food industry. β-lg (Sigma grade) was used for EDS-PAGE analyses.

RESULTS AND DISCUSSION

AFM of β-lg Fibrils. To investigate the formation of β-lg fibrils, AFM height images were performed on β-lg dispersions (2% w/v, pH 2) that were heated at 80°C for 4 min (5 min, 15 min, 45 min, 65 min, 85 min, 100 min, 200 min, 300 min, and 400 min, respectively). Changes of β-lg monomers into fibrils were monitored by AFM measurements in air (Figure 1). At pH 2, native β-lg appeared as a globular monomers with a height of approximately 2 nm (shown in Figure 1). During heating for 45 min, the monomers swelled and deformed, forming aggregates (Figure 1). After 65 min of heating, monomers appeared to reduce in size. Upon prolonged heating (>85 min), short and long fibrils with split ends were observed. The short fibrils were assumed to be precursors of the longer fibrils. At 100 min of heating, samples consisted of a mixture of small particles/aggregates and fibrils.
Fibrillar subunits were observed after 200 min of heating (arrows in Figure 2a). More and longer fibrils were formed in samples heated from 300 min to 20 h (Figure 3). Single β-lg fibrils had a linear morphology and periodic structure, with a periodicity of ~30–40 nm (Figure 2b), heights of ~2–3 nm (Figure 2c), and lengths of up to 10 μm or more (Figure 3a). The thickness of overlapping fibrils was nearly 2 times higher (~6 nm) than single fibrils (panels a and b of Figure 2), with results being consistent with previous reports (27). The AFM phase image contrast provides high spatial resolution of surface topography, highlighting heterogeneities, and relative surface property differences, allowing for a clear distinction between fibril subunits (Figure 2b). Thus, it can be seen that subunits of well-developed fibrils exhibit similar mechanical properties (adhesion to the tip and hardness) because they induce the same changes in the phase image. Average fibril heights were calculated by measuring the tip height at various...
Appendix III

180

Figure 2. AFM (a) height and (b) phase images of β-lg at pH 2 and 36 °C heated for 20 min. β-lg fibrils had a (c) height of ~2–3 nm and a (d) periodicity of ~30 nm. Images were collected in air. Scan size = 500 nm. The arrows in the height image show the presursors of mature fibrils.

Figure 3. (a) 10 µm scan of AFM height images of β-lg at pH 2 and 80 °C heated for 20 h. Images were collected in air. The arrows indicate denatured protein not incorporated into fibrils. (b) Magnified (2 µm scan) area of panel a, confirming the presence of nonincorporated protein as well as fibrils.

points (n = 10) for 19 different fibrils for each set of experiments. The cross-sectional height profiles, as measured using the MFP-3D software, demonstrate the periodicity of the fibrils and are shown in Figure 2. The results indicate that some denatured proteins are not incorporated into fibrils but form unordered aggregates, confirming the suggestion by Bolder et al. [5] that not all denatured and hydrolyzed β-lg is incorporated into fibrils even with prolonged heat treatment (they appear as white dots shown by the arrows in panels a and b of Figure 3). In this study, the height of the fibrils was smaller than those previously reported by Ikeda et al. [6], who studied β-lg dispersions at pH 2 in the presence of 0.1 M NaCl after heating at 80 °C. Ikeda and colleagues observed fibrils 4 nm in height, and they concluded that the fibrils consist of strings of partially unfolded β-lg monomers [6, 23]. AFM images of samples in water clearly show the β-lg fibrils and confirm their periodic nature (periodicity of ~34 nm) (Figure 3a). Similar AFM experiments performed using WPI also showed the presence of fibrils. AFM height measurements in water show that WPI fibrils have similar characteristics to β-lg fibrils and occasionally showed paired fibrils in the form of a double helix with a periodicity of ~36 nm and height of ~7 nm (Figure 3b). The heights of single fibrils imaged in water ranged between 2.9 and 4.3 nm. Fibrils with periodic structures were seen in both the air and the water (height) images, demonstrating that the periodicities and dimensions are real and not an artifact because of air drying. However, small differences between height values measured in the two modes can be observed; these are inevitable because in air the fibril volume will decrease upon drying. Measurement in air using AFM indicated a loss in height of ~25% compared to the values obtained for measurements in liquid. These observations were in agreement with Morena-Herrera et al. [14], who studied Alzheimer paired helical filaments imaged under physiological conditions and in air environments using AFM.

STEM of β-lg Fibrils. Electron microscopy confirmed the presence of fibrils, indicating that these features were not caused by the charged mica surface, as assumed previously (19) (Figure 5). The diameters of fibrils, measured from STEM images using the microscope software, were approximately 3 nm, which is comparable to the heights measured by AFM.

ATR–FTIR of β-lg Fibrils Formation. ATR–FTIR experiments allowed the observation of changes in the secondary structure during the denaturation and self-assembly processes of β-lg at 80 °C. The amide I band (1600–1700 cm⁻¹) of ATR–FTIR spectra is very sensitive to changes in the secondary structure of β-lg during the denaturation and aggregation processes (21, 26). The spectra of native and heated β-lg (Figure 6) illustrate that heat treatment at pH 2 had a pronounced effect on the secondary structure of the protein. Curve fitting of the amide I band of β-lg incubated at pH 2.0 and heated for 1 min at 80 °C revealed absorption bands at 1614, 1634, 1631, 1640, 1650, 1670, and 1686 cm⁻¹, which are in agreement with those in the literature (21). The observed amide I band is a complex composite, consisting of a number of overlapping components representing α helices (1634 cm⁻¹), low- and high-frequency components of β structures (1650 and 1670 cm⁻¹), turn (1611 cm⁻¹), and random coils (1645 cm⁻¹), corresponding to the disordered parts of polypeptide backbones (27). During heating, hydrogen bonds stabilizing the native structure of β-lg are disrupted, causing the loss of α helices and β sheets, and new intermolecular parallel β sheets can be created (peaks a and b of Figure 6).
Figure 4. (a) AFM height image of β-lg at pH 2 and 80 °C heated for 20 h and imaged in water (pH 2.6). Fibril height values were 3 nm. (Inset) Higher resolution height image of 1:1 mixture, confirming periodicity of fibrils. (b) AFM height image of WPI/β-lg at pH 12 and 80 °C heated for 20 h and imaged in water. (Inset) Cross-section height profile along the fibril axis highlighting periodicity.

Figure 5. STEM negatively stained image of 2.0% (w/w) β-lg at pH 2 and 80 °C heated for 26 h. Scale bar = (a) 100 nm and (b) 20 nm.

Figure 6. Amide I FTIR spectra of 2% (w/w) β-lg heated at 80 °C varying (a) from 1 to 139 min and (b) from 134 min to 26 h. Amide I spectra of the heated samples after native β-lg spectrum subtraction varying (c) from 0 to 139 min and (d) from 134 min to 26 h.

The increase in intensity of a specific band (1624 cm⁻¹) was attributed to the formation of extensive regions of β-sheets involved in intermolecular cross-linking between unfolded proteins and characterized protein aggregation (60, 28–32). Subtraction of the native spectra from those of the heated sample showed that there was a significant increase in the absorbance at
Appendix III

This may be attributed to lower protein concentrations and higher buffer concentration used when heating the sample in reducing sample buffer. It should also be noted that no stained proteinaceous material was seen either at the top of the stacking gel or within the wells. Furthermore, no fibrillar material was detected by AFM or SEM analysis of fibril dispersions prepared in SDS-reducing buffer under our experimental conditions (results not shown), suggesting that fibrils had not been dissociated. These observations indicate that the effect of chaotropic agents on protein fibrils is complex and warrants further systematic study. It can therefore be reasonably concluded that β-g components within the nanofibrils were associated within the fibril aggregate (37). This fibril formation mechanism contrasts with that of fibrils formed at higher ionic strengths, which is characterized by shorter fibrils formed from partially intact β-g monomers or dimers (27).

CONCLUSIONS

Studies using AFM, STEM, real-time ATR-FTIR, and SDS–PAGE were performed to investigate the fibrillar aggregation process of β-g. This study demonstrates that AFM imaging combined with more established analytical techniques gives unique insight into the transformation of native proteins into fibrillar structures. The sequence of AFM images of β-g dispersions clearly demonstrates the progress of fibril aggregation as a function of the heating time. STEM results confirmed AFM observations of β-g fibrils in air and in water. This study shows, for the first time, AFM imaging of β-g and WPI-derived fibril in water under ambient conditions and the presence of double-helical structures in situ. SDS–PAGE analysis indicates that β-g fibrils consist of polymeric fragments linked via non-covalent interactions in fibrils and are not formed from intact monomers. The real-time monitoring of the assembly processes in situ using ATR–FTIR correlated with AFM and SDS–PAGE results, suggesting a two-stage mechanism of fibril formation: (1) denaturation, partial unfolding, and increase in β-sheet content with hydrolysis of monomers followed by (2) near-regular aggregation of polymeric fragments into fibril via non-covalent interactions, continued hydrolysis, and an accompanying decrease in β-sheet content and overall secondary structure.

ACKNOWLEDGMENT

The authors thank Amir Mosleh (Applications Scientist, Asylum Research, U.K.) for his assistance with AFM imaging in fibrils.

LITERATURE CITED

Appendix III

Received for review August 11, 2009. Revised manuscript received February 15, 2010. Accepted February 15, 2010. This work was funded by the Irish Department of Agriculture and Food through the FIRM Project 09/RDF-MFRC-62.
Appendix IV

The effect of high pressure microfluidization on the structure and length distribution of whey protein fibrils

Daniela Oborocanu, Lihe Wang, Arty Knes-Nijboer, André Brodkorb, Paul Venema, Edmond Magner, Mark A.E. Avers, 

Agricultural Research Center, AgriPark, County Galway, Ireland
Molecular and Cellular Science Institute and Department of Chemical and Environmental Sciences, University of Limerick, Limerick, Ireland
Centre for Sustainable Biofuels-Global Impacts, Environment and Agriculture Technologies, Australian Greenhouse Office, Luxembourg
Physical and Chemical Chemistry of Foods, Department of Agrotechnology and Food Sciences, Wageningen University, The Netherlands

ABSTRACT

The effect of high pressure microfluidization on native β-lactoglobulin (β-lg) or whey protein isolate (WPI), both before and after heat-induced protein fibril formation at pH 2.0, was investigated using atomic force microscopy (AFM), shear birefringence, reversed phase-high pressure liquid chromatography (RP-HPLC), and flow-induced birefringence, respectively. High pressure (>50 MPa) microfluidation treatment of β-lg induced a WPI protein denaturation, accompanied by changes in secondary structure fibril formation from high-pressure treated β-lg or WPI similar to length and stiffness from non-pressure treated proteins. High pressure (>50 MPa) microfluidization of fibrils formed from β-lg or WPI resulted in their breakup into more uniformly sized and much shorter fibrils. Microfluidization pressures of up to 170 MPa resulted in slightly shorter fibrils but did not completely disintegrate them.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Native β-lactoglobulin (β-lg) is a major component of whey protein isolate (WPI), which is used by the food industry as a functional ingredient (Aggelis & Johnston, 2006; Sánchez & Page, 2002). Native β-lg is a globular protein but forms micellar assemblies over 10 nm in length with a thickness of 2–3 nm at high temperatures, low pH, and low ionic strength (Abdessamad et al., 2008; Kodlan & Matheson, 2002). Recent research has shown that heat-induced β-lg fibrils comprise polymeric fragments linked via non-covalent intermolecular interactions (Abdessamad et al., 2008; Oborocanu et al., 2009). High pressure technology is increasingly used by the food industry and conformational changes in whey proteins after hydrostatic high pressure treatment have been studied (Barney, Kitchener, & Chell, 1994; Meersman & De Koster, 2006). Microfluidization is a dynamic high-pressure homogenization process used by the pharmaceutical and food industries to produce fine and visible emulsions (Christiansen, Vangervliet, Langeland, Rønberg, & Egelrud, 2004; McElvany, 2005; Golda, 1999; Sánchez & Page, 2002). The effect of high pressure (>50 MPa) microfluidization on whey protein fibrils has not been studied.

During microfluidization, the liquid sample is split into two streams that are sandwiched under high pressure and velocity in an interaction chamber (McElvany, 2005). During the high dynamic pressure induced flow that subsequently occurs, intensive disruptive forces are generated as the two streams collide with each other, exhibiting a larger energy density compared with hydrostatic high pressure treatment or conventional homogenization techniques. During microfluidization, the protein particles are disrupted by the intense turbulence and shear flow effects. When protein fibrils are placed in such a flow, the entanglement component of the flow may lead to fibril breakup if the energy applied to the fibril is large than the binding energy between fibril units. Thus, it was already shown that strain rates of 8–107 s−1 were able to fracture the WPI fibrils after a single pass, the average length of the fibrils being afterwards relatively constant even for five consecutive passes (Krenk-Nijboer, Venema, Baptist, & van der Linden, 2010). However, the microfluidization process generates highly turbulent flow at very high shear forces (Richard Xie, Microfluidics Corp, personal communication).
Appendix IV

communication, making it difficult to measure the precise flow components which influence protein denaturation and protein fibril structures.

Studies suggest that microfluidization affects the native protein structure and may produce significantly different structures when compared with heat-induced denaturation and aggregation (McKenna, 2005). Intra- and inter-molecular bonds can break, leading to protein fragmentation (Ramasamy & Vasiljevic, 2009).

There are few reports on the effect of high pressure microfluidization on protein secondary structure. Infrared absorption spectroscopy of the amide I region (1600–1700 cm⁻¹) of β-lactoglobulin had been the subject of numerous studies (Alain, Pajoum, & Subarica, 1996; Leefers, Dijkstra, & Verschoor, 1998; Gorbunova et al., 2010). The observed amide I band is a complex composite consisting of a summation of overlapping components bands representing α-helix (1654 cm⁻¹), low and high-frequency components of β-structures (1643–1673 cm⁻¹), turns (1656 cm⁻¹) and random structures (1645 cm⁻¹) (Kavanagh, Clark, & Brown-Murphy, 2000; Chornomos et al., 2010).

Changes in the secondary structure can also be monitored using ThT fluorescence, a known marker that becomes strongly fluorescent upon binding to native structures such as those present in whey protein aggregates and amyloid fibrils (Alscherma et al., 2008b; Carnotta, Baxi, Waninger, & Kochel, 2000; Kreis, Brownley, & Donald, 2005; Kreis et al., 2005). It has been observed that ThT binds to microfluidized β-lactoglobulin with its long axis parallel to the fibrils and conclude that the reaction channel on the β-sheet of the fibrils act as a binding site of the dye, but their model cannot fully explain the specific binding of ThT to native protein. Quantitative measurement of fibril length at the nanoscale is challenging but recently, the flow-induced shear birefringence technique was developed to measure the length distribution of fibrils in suspension (Akselrod, 2008; Stoddard, van der Goot, Verona, van der Leij, & Boom, 2004; Roger, Verona, & Van der Leij, & Boom, 2009). Performing flow-induced birefringence measurements on β-lactoglobulin in the semi-dilute concentration regime, Rogers et al. (2005) showed that the fibrils did not breakup during shear treatment up to shear rates of 200 s⁻¹.

In this study, the effect of a single pass dynamic high pressure microfluidization on β-lactoglobulin and WPI dispersions before and after fibril formation was investigated. In bio-polymer studies, atomic force microscopy (AFM) has become a powerful technique to visualize structures at the molecular scale (Hendr, 2002; Morris et al., 2001; Ortiz, Zhang, Rhee, & Beito, 2007). Therefore, in order to study the effect of dynamic high pressure on fibrillar structure, a series of fibrillar dispersions of β-lactoglobulin and WPI were prepared as described above. Fibril samples were microfluidized at 50 MPa, 75 MPa, 100 MPa, 150 MPa and 170 MPa before and after microfluidization by shear birefringence. The effect of the single pass microfluidization on the degree of β-lactoglobulin denaturation was measured using a 180°-polarized-light optical microscopy and fluorescence microscopy. Further, the ThT fluorescence microscopy was monitored by atomic force microscopy (AFM) and ThT fluorescence spectroscopy. For investigation methods requiring large quantities of fibril dispersion, WPI was used as this is a standard food ingredient.

2. Materials and methods

2.1. Materials

Bovine β-lactoglobulin (approximately 99% pure by polyaclaramide gel electrophoresis, κ-carrageenan and gellan gum) mixture of genetic variants A and B (Ref. L0100, Lot 05/068/006) was from Sigma Aldrich Ltd (Tadley, Berks, UK). Whey protein isolate (WPI) was a commercial product, consisting of 85% β-lactoglobulin, 8% bovine serum albumin, obtained from Danisco Foods International Inc, (Le Suar, MN, USA). Thioflavin T (Ref. T350-SG, Lot 052755) and HCl were purchased from Sigma Aldrich Ltd. Purified water (Milli-Q® water resistivity 18.2 MΩ cm at 25°C, Millipak® E2 0025, UK) was used throughout.

2.2. Preparation of β-lactoglobulin and WPI fibrils

Fibrillar dispersions were prepared as described by Gorbunova et al. (2010). The pH of the β-lactoglobulin dispersions was adjusted to 4.6 using 6 M HCl and centrifuged (3000 g at 30 min at 25°C) to remove the denatured protein. The pH of fibrillar dispersions was adjusted to 7.0 with 6 M NaOH for subsequent microfluidization treatment and analysis.

A summary of the samples and analyses is presented in Table 1. Unless otherwise specified, analyses were performed in triplicate.

Table 1: Summary of experimental samples and procedures.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Microfluidization treatment</th>
<th>Native protein (before fibril formation)</th>
<th>Denatured protein (after fibril formation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>β-Lactoglobulin</td>
<td>Whey protein isolate</td>
</tr>
<tr>
<td>A.Atomic force microscopy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Shear birefringence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Scanned phase-DIC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. ThT-FRL fluorescence</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Microfluidization pressure from 0 to 300 MPa for all experiments. Data not shown.

To study the effect of dynamic high pressure on fibrillar structure, a series of fibrillar dispersions of β-lactoglobulin and WPI were prepared as described above. Fibril samples were microfluidized at 50 MPa, 75 MPa, 100 MPa, 150 MPa and 170 MPa before and after microfluidization by shear birefringence. The effect of the single pass microfluidization on the degree of β-lactoglobulin denaturation was measured using a 180°-polarized-light optical microscopy and fluorescence microscopy. Further, the ThT fluorescence microscopy was monitored by atomic force microscopy (AFM) and ThT fluorescence spectroscopy. For investigation methods requiring large quantities of fibril dispersion, WPI was used as this is a standard food ingredient.
Flow rates were calculated for WFI and fibril dispersions based on the volume of liquid collected at the outlet in 1 min. Flow rates increased with pressure from 6 to 8 × 10^{-6} m^3 s^{-1} for 50 and 120 MPa, respectively.

2.4. AC-mode atomic force microscopy

β-lactoglobulin or WFI dispersions (microfluidized before or after fibril formation) were investigated using an MFP-3D-BOY® AFM (Asylum Research Inc. Ltd, Oxford, UK) as described previously (Oboromeou et al., 2010). Prior to imaging, the dispersions were diluted in Milli-Q® water to a final concentration of 0.20 g L^{-1}. Protein dispersions (50 µl) were deposited onto freshly cleaved mica and subsequently dried in a desiccator. An attenuation reference cantilever with a rectangular tip (AC 400® spring constant, 5.8 N m^{-1}; resonance frequency, 79.07 Hz; Olympus Optical Co., Ltd, Tokyo, Japan) was used for imaging air-dried samples. Fibril height was established as the average value after the height of the fibril was measured in various pores for 36 different fibrils for each set of experiments. Fibril length profiles were obtained directly using the MFP-3D software. Fibril length and frequency distributions were measured from AFM images using Image J (NIH) image analysis software. A total of 180 fibrils were measured from four separate AFM images and fibril length frequencies calculated as a dimensionless number, L (Eqn. (1)),

\[ L' = L / L_{max} \]

where \( L \) = length of fibril at each different microfluidization pressures and \( L_{max} \) = mean fibril length at 0 pressure.

2.5. Flow-induced birefringence

Length distributions of β-lg or WFI fibrils (before or after microfluidization) were performed in triplicate using a strain-controlled ARES rheometer (Rheometric Scientific Inc., Piscataway, New Jersey, USA) with Couette geometry (rotating cup with a diameter of 33.8 mm and a static bob with a diameter of 30.8 mm). A laser beam of wavelength 670 nm passed vertically through the gap between the cup and bob. Birefringence was measured with a modified optical analysis module (Klein et al., 2007). A shear rate of 5 s^{-1} was applied for 90 s and from the decay curve of the flow-induced birefringence after the cessation of flow, the length distribution of the fibrils was determined (Klein et al., 2005). The analysis was performed in a temperature-controlled room at 20 °C. The protein samples were diluted with HCl at pH 2 to a final protein concentration of 25 g L^{-1}.

2.6. Resolved phase-high performance liquid chromatography

The effect of microfluidization on native β-lg was investigated using reversed-phase high-performance liquid chromatography (RP-HPLC). The chromatographic system consisted of an Agilent 1200 series binary pump with standard auto sample, a thermostat column compartment and a 1200 series diode array detector (Agilent Technologies, Santa Clara, CA, USA). Separation of the β-lg fractions was achieved using an Agilent 350 5C-18 RP PBonded column. The injection volume of the sample was 5 µl of 0.1 g L^{-1} protein concentration. The flow rate being 0.5 ml min^{-1} and the column temperature was set at 25 °C. Solvent A consisted of 50:50 H2O:ACN (by vol) mixture of water, acetic acid and trifluoroacetic acid (TFA), while solvent B was a 900:100:1.0 (by vol) mixture of acetonitrile-water-TFA. A solvent gradient was generated after injection, starting at 20% B and increasing to 50% B after 10 min; and subsequently to 100% B after 3 min -10^{-4} 40 s^{-1}. This was held for 3 min -10^{-4}, before returning to starting conditions. After the microfluidization treatment, the protein was centrifuged (15000 g for 30 min at 25 °C) to remove the centrifuged protein and the supernatant was filtered and used for HPLC analysis. The β-lg samples were prepared by dilution (v/v) using a di-sodium citrate buffer containing 7% ura, 0.02 M Tris-HCl, and 0.5% mercaptoethanol (Visser, Stienen, & Rellermans, 1991). The samples were left at room temperature for at least 1 h in the di-sodium citrate buffer before being passed through a 0.22 µm filter to remove any insoluble material. Data were collected at 214 and 280 nm and quantification was based on integration of the data at 214 nm. Commercial β-lg (Sigma Aldrich) and WFI (Rifco) were used as standards.

The experiments were run with β-lg since a pure protein is required for the analysis. The results can be extrapolated for WFI samples using the WFI procedures. The ATR-FTIR measurements of freshly prepared 20 g L^{-1} β-lg or WFI heated before or after microfluidization at 80 °C for 20 h were performed using a Bruker Tensor 27 spectrometer (Bruker Optics GmbH, Ettlingen, Germany) fitted with a thermally controlled Bio ATR Ge® II, which was specifically designed for measuring proteins in aqueous solution. The spectra were acquired at 25 °C and averaged over 128 scans at a resolution of 4 cm^{-1} using Bruker Optics GPI software. After atmospheric compensation of CO2 and H2O as vapour and solute, respectively, the amide I band (1650–1700 cm^{-1}) was vector normalized and the spectrum of the control sample was subtracted from the spectra of the treated samples.

2.7. Thioflavin T fluorescence

Thiorflavin T (ThT) solution (3 mM) was prepared by dissolving ThT powder (4.70 mg) in Milli-Q® water (4.52 ml), the pH adjusted to pH 7 with 5 M NaOH and 0.2 M HCl (NaOH, Salter, 0.2 ml) to remove undissolved powder. ThT solution (20 ml) was added to 298 ml of the 0.2 g L^{-1} fibril dispersion and the fluorescence was measured using a Victor 1400 fluorescence spectrophotometer (PerkinElmer, Norwalk, USA). Excitation and emission wavelengths of 420 and 483 nm, respectively were used (slit widths 5 nm). The emission spectra of the protein dispersions were normalized (Grabiel et al., 2009).

3. Results and discussion

3.1. Atomic force microscopy

3.1.1. AFM observations and length distribution of fibrils formed from microfluidized β-lg or WFI

AFM height images were obtained in air to monitor the morphology changes of fibrils prepared with microfluidized proteins. Fibril length was not affected by the pressure applied on the native protein up to 170 MPa, as indicated by AFM and shear birefringence results.

At 0 MPa (i.e., non-microfluidized), fibrils exhibited a linear morphology (Fig. 1a and b), a height of 2–3 nm (Fig. 1c) and periodicity of 30–40 nm (Fig. 1d), and lengths of up to 15 μm. The thickness of overlapping fibrils was nearly twice (66 nm) that of single strands (38 nm), consistent with previous reports (Amouras, de Vries, Ippers, & van Mierlo, 2003; Oboromeou et al., 2010). AFM
height images of WPJ showed similar fibrillar structures (results not shown). No significant difference in fibril length was observed for samples that had been microfluidized at 50 MPA, 75 MPA, 150 MPA, or 170 MPA. AFM images of fibrils formed from β-lg microfluidized at 170 MPA are shown in Fig. 1e and f. A slight increase in fibril height ranging from 4 nm to 5.75 nm was observed in samples that had been microfluidized (Fig. 1g). This increase may be due to pressure-induced transfer of water into the protein interior (Considine, Patel, Aueraa, Singh, & Cerny, 2007). Unlike the basic denatured proteins that transfer their non-polar groups into water, the pressure-denatured proteins retain a compact structure, with water molecules penetrating their core (Considine et al., 2007). Atomic force microscopy showed slight changes in fibril periodicity between native (Fig. 1a and b) and microfluidized β-lg (Fig. 1e and f). These changes were characterized by the partial loss of globular shape of fibril constituent units following microfluidization (Fig. 1e and f).

3.1.2. Atomic force microscopy of microfluidized fibrils

AFM height images of microfluidized β-lg fibrils are shown in Fig. 2. Results indicate that high pressure microfluidization of 50 MPA (Fig. 2a and b) or above (Fig. 2c and d) incompletely formed fibril structures without affecting fibril height. No fibrils larger than 300 nm were observed for 170 MPA applied pressure (Fig. 2c and d).
Appendix IV

It can be clearly seen that the fibril length distributions changed with the applied pressure (Fig. 3). At 0 MPa, fibril lengths ranged from 1 to 15 μm. Fibril length generally decreased with increasing microfluidization pressure and ranged from approximately 25 nm to 425 nm at 50 MPa and from 32 nm to 375 nm at the highest pressure applied (Fig. 3). Small differences in fibril length distributions can be seen when the pressure was varied from 50 to 100 MPa or from 150 to 170 MPa. Results further indicate that fibril lengths exhibited a narrower size range when microfluidized compared with the initial conditions, suggesting that microfluidization could be used to control fibril length.

3.2 Shear birefringence

Shear birefringence measurements were performed to measure the length distribution of fibrils formed from microfluidized protein (Fig. 4). Results indicate that the length distributions of fibrils formed from microfluidized protein had a bimodal distribution, with peaks at around 300 nm and 2 μm. The length distribution of fibrils formed under different pressures had a similar pattern, with a slight increase in the percentage of fibrils in the 300 nm range and a decrease in the percentage of fibrils in the 2 μm range as the pressure increased.
Appendix IV

Table 2: Effect of high pressure microfluidization on native β-lactoglobulin determined by reversed-phase HPLC.

<table>
<thead>
<tr>
<th>Microfluidization pressure (MPa)</th>
<th>Concentration of native β-lactoglobulin (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.84</td>
</tr>
<tr>
<td>36</td>
<td>0.59</td>
</tr>
<tr>
<td>75</td>
<td>0.47</td>
</tr>
<tr>
<td>100</td>
<td>0.65</td>
</tr>
<tr>
<td>150</td>
<td>0.53</td>
</tr>
<tr>
<td>170</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Fibrils prepared from microfluidized WPI were similar for the various pressure treatments, although a slight decrease in length as shown by the arrow in Fig. 4 of the signal from 50 to 170 MPa was observed. Most fibrils were 15–7 μm in length. Shear birefringence results for β-lg were similar to those of WPI (results not shown). The length distribution of the microfluidized shortened fibrils could not be measured using the shear birefringence technique, since fibrils smaller than 1 μm could not be aligned by the flow and a birefringence value could not be obtained.

3.3. The effect of microfluidization on the secondary protein structure of WPI and fibrils

The content of native β-lg after microfluidization was quantified by RP-HPLC (Table 2). The native monomeric β-lg concentration decreased by approximately 50% when microfluidized over the 50–170 MPa range.

3.3.1. Effect of microfluidization on protein secondary structure prior to fibril formation

ATR-FTIR spectrum of microfluidized β-lg dispersions were recorded for different pressures up to 170 MPa (Fig. 5a and b). The ATR-FTIR spectrum of β-lg (no pressure) bands was observed at 1630 and 1681 cm⁻¹ suggesting that the monomeric structure of β-lg was mainly composed of β-sheets content even though α-helix at 1025 cm⁻¹ and turn at 1099 cm⁻¹ were detected (Fig. 5a and b). These results are in agreement with the literature that indicates that at pH 4.4 globular β-lg in the monomeric form is mainly composed of intra-molecular β-sheets (1630 cm⁻¹) and turn content (1099 cm⁻¹). Changes in these bands occurred on microfluidization of protein, especially in 1620–1631 cm⁻¹ range. As the pressure increased from 50 to 170 MPa, the absorbance band corresponding to α-helix (1026 cm⁻¹) increased and the contribution to the absorbance spectra from the β-sheets decreased (1620 cm⁻¹) suggesting a loss of secondary structure due to protein unfolding (Fig. 5a and b). Similar experimental findings were presented by Subrad, Leopiz, Alain, and Paquin (1996) wherein the hydrostatic pressures up to 140 MPa did not affect the β-sheet content of the protein structure. This discrepancy might be due to the fact that during microfluidization, intense disruptive forces are generated when the fluids collide with each other or with a solid surface. Thus, native β-lg spectrum subtraction from the microfluidized samples spectra, the experimental results showed that overall the α-helical (1650 cm⁻¹) content increased while intra- and inter-molecular β-sheets component (1628 and 1096 cm⁻¹) decreased with pressure (Fig. 5b).

Starting with 50 MPa, the spectra subtraction (Fig. 5b) revealed an increase with pressure of the absorption value at 1680 and 1099 cm⁻¹ assigned to β-sheets and α-helical bands. The new arrangement of the intra- and inter-molecular β-sheets by formation of new inter-molecular β-sheets (1680 cm⁻¹) might have been caused by denaturation/aggregation of β-lg during the microfluidization process. The disappearance of the 1026 cm⁻¹ band might be explained by the conversion of intra-molecular β-sheets into inter-molecular β-sheets, α-helical and non-native α-helix by
the high pressure treatment (Considère et al., 2007). Pressure-induced changes in secondary structure may be related to changes in periodicity and fibril height as observed by AFM (Fig. 3b, d, f, h).

3.3.2. ATR–FTIR measurement of microfluidized fibrils

ATR–FTIR spectra were recorded for WPI fibrils at microfluidized at pressures from 50 to 170 MPa (Fig. 5c and d). No control (0 MPa) bands were observed at 1617, 1547, 1654, 1563, 1670, 1675, 1675 and 1694 cm⁻¹ (Fig. 5c). ATR–FTIR spectra of microfluidized fibrils showed a sharp increase in 1600–1630 cm⁻¹ range, with a maximum increase at 1618 cm⁻¹ and 1620 cm⁻¹ (at 50–75 MPa), indicating the presence of inter-molecular β-sheets (Oberhauser et al. 2010). A corresponding decrease in the secondary structure signal was observed after microfluidization of the fibrils. After subtraction of the 0 MPa spectrum from the microfluidized samples spectra, the experimental results show that overall the α-helix (1654 cm⁻¹) content increased while inter-molecular β-sheets component (1618 cm⁻¹) decreased in WPI fibrils with pressure (100–170 MPa) (Fig. 5d). The decrease in 1618 cm⁻¹ band can be attributed to the breakdown of fibrils into sheet-like fibril structures under the action of the high pressure. The increase observed in the other bands can be attributed to the conversion of inter-molecular β-sheets into β-pleated and non-native α-helices. This increase was not as obvious as for microfluidized β-sheets presented in Fig. 5a. A possible reason for this difference is that the constituent units of the fibrils formed under thermal treatment are polypeptides rather than native proteins (Oberhauser et al. 2003). Thus, for non-native β-sheets, high pressure acts to disrupt the native protein, while for microfluidized fibrils the pressure acts on the constituent units and also disrupts the inter-molecular bonds between the polypeptides.

3.4. Thioflavin T fluorescence

3.4.1. ThT fluorescence of microfluidized protein prior fibril formation

The early ThT fluorescence of microfluidized WPI dispersions at pH 7 was recorded with a maximum at 485 nm (Fig. 6a). Unfluided WPI proteins had a small ThT fluorescence intensity caused by the β-sheets originally present in the native protein. A decrease with the pressure in the fluorescence intensity signal can be observed and it was related to the increase of the β-sheet content under the action of the applied pressure observed in the ATR–FTIR measurements.

3.4.2. ThT fluorescence measurement of microfluidized fibrils

The microfluidized WPI fibrils had a higher ThT fluorescent intensity than the unfluided WPI protein indicating higher β-sheet content present at 0 MPa (Fig. 6b). Thus, in the microfluidized fibril WPI dispersion, the ThT can bind to both intra- and inter-molecular β-sheets associated with the polypeptides, while in the unfluided microfluidized WPI the ThT fluorescence intensity is mainly derived from intra-molecular β-sheets present in the native protein (Krebs et al. 2005). The fluorescence peak appeared at 485 nm and an overall increase was observed in fluorescence intensity with the pressure, showing that the β-sheet content increased with the pressure. The maximum of this increase was observed at 50 MPa pressure. For larger pressures, a decrease in ThT intensity of the protein aggregates was recorded, but it remained larger than the initial value measured at 0 MPa. The overall increase in the intensity can be explained by the fact that the pressure broke the fibrils, creating a larger number of small fibril-like aggregates and thus a larger number of binding sites to which ThT could attach.

4. Conclusions

AFM images show that fibril lengths formed from β-sheet or WPI microfluidized up to 170 MPa were not influenced by the applied pressure during microfluidization treatment of the native protein, although a slight loss of periodicity and increase in fibril height was observed. The maximum length of protein fibrils was 15 μm and their height was 2–3 μm. Shear birefringence measurements of fibres prepared from microfluidized β-sheet or WPI confirmed that the fibril length was not affected by high pressure microfluidization. The ATR–FTIR and ThT fluorescence data indicated that following high dynamic pressure treatment the secondary structure of the protein changes, most likely by rearrangement of the intra-molecular β-sheets into inter-molecular β-sheets. This study revealed that high-pressure microfluidization breaks up fibrillar proteins into more regularized fragments <250 μm in length. Shortened fibrils could withstand pressures up to 170 MPa without breaking up further. The results indicate that the dynamic high pressure microfluidization not only affects the general physical dimensions of the long β-sheet or WPI fibrils, but also alters the secondary structures of their constituent units as shown by the ATR–FTIR and ThT fluorescence measurements.

Acknowledgements

The authors acknowledge the support of the Department of Agriculture and Food, Food Industry Research Measure Project (O8615/MTRC-832) for funding this work and would like to thank Anthony Keat for assistance with microfluidization of samples.