Characterisation of Biopolymer/Co-Solute Composites for the 
Design of High-Solid Biomaterials with Improved 
Structural Properties

A thesis submitted in fulfilment of the requirements for 
the degree of Master of Applied Science

Divya Sharma
Masters in Food & Nutrition

School of Applied Sciences, SET Portfolio
RMIT University, Melbourne
August 2010
Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Signature

Divya Sharma

Date:
Acknowledgements

It is my proud privilege to express my deepest sense of gratitude and heartful thanks to my expedient guide Prof. Stefan Kasapis, for his inspiring, consistently keep going approach and deep involvement in the study right from its inception to the final completion. His professional excellence and immaculate meticulousness had a direct imprint on the execution of the research and its manuscript. The present study is a testimony to the ardent personal interest taken by him in nurturing this research.

I extend my sincere thanks to RMIT, for the requisite facilities provided and I am truly grateful to Michael Kakoullis, Lilian and Mary for their unstinted support and untiring help in the Food Science Laboratory.

I am thankful to Dr. Bee May, my second supervisor for her valuable help and guidance. It's my pleasure to thank my colleagues, Paul George and Philip Button for all the help and assistance.

To my parents, I owe more than I can express. My achievements in life are a reflection of the values and virtues instilled in me since childhood. It was the affection, love and blessings showered upon me by Rishi Vidhyadhar Ji, which sustained me during the entire course of my study.

Words again fail me when it comes to thank Prof. Suresh Bhargava and Prof. Ann Lawrie for their optimistic support and for standing by me in the time of need.

It is virtually impossible for me to express it in words what it means to have a ‘Guru’ in life. I am blessed to have one as Sri Sri Ravi Shankar. It is because of His Grace and Blessings, I survived this tumultuous period. The belief that you are taken care of by divine kept me going. Guruji you continue to be the APOSTLE OF MY STRENGTH.

Divya Sharma
# Contents and Pagination

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>i</td>
</tr>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Contents and Pagination</td>
<td>vi</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Tables</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xii</td>
</tr>
<tr>
<td>Publication</td>
<td>xv</td>
</tr>
<tr>
<td>Summary</td>
<td>xvi</td>
</tr>
</tbody>
</table>
# Table of Contents

## Chapter 1: INTRODUCTION

1.1 GELATIN.................................................................................................................. 2
1.2 AGAROSE.................................................................................................................. 4
1.3 WHEY PROTEINS..................................................................................................... 7
1.4 GLUCOSE SYRUP..................................................................................................... 15
1.5 WATER...................................................................................................................... 16
1.6 BIOPOLYMER MIXTURES/COMPOSITE GELS......................................................... 16
1.7 PHASE SEPARATION IN BIOPOLYMER MIXTURES................................................... 18
1.8 GLASS TRANSITION IN MIXED SYSTEMS................................................................. 20
1.9 POLYMER BLENDING LAWS OF TAKAYANAGI..................................................... 22

## Chapter 2: METHODOLOGY

2.1 RHEOLOGY............................................................................................................... 24
2.2 DIFFERENTIAL SCANNING CALORIMETRY............................................................... 34
2.3 CONFOCAL MICROSCOPY....................................................................................... 38
2.4 MATERIALS AND EQUIPMENTS USED................................................................. 40
   2.4.1 Gelatin............................................................................................................... 40
   2.4.2 Agarose............................................................................................................. 41
   2.4.3 Whey Protein Isolate........................................................................................ 42
   2.4.4 Glucose Syrup................................................................................................ 43
   2.4.5 Calcium Chloride............................................................................................ 44
   2.4.6 AR-G2 Advanced Rheology Generation 2 Rheometer..................................... 45
   2.4.7 Q2000 Differential Scanning Calorimeter....................................................... 46
   2.4.8 Leica DM6000 CFS Confocal Fixed Stage Microscope.................................. 47
2.5 GEL PREPARATION................................................................................................. 48
Chapter 3: THERMOMECHANICAL STUDY ON THE PHASE BEHAVIOUR OF AGAROSE/GELATIN MIXTURES IN THE PRESENCE OF GLUCOSE SYRUP AS THE CO-
SOLUTE

ABSTRACT........................................................................................................................................53

INTRODUCTION................................................................................................................................54

EXPERIMENTAL SECTION................................................................................................................56

3.3.1 Materials................................................................................................................................56

3.3.2 Methods.................................................................................................................................56

RESULTS AND DISCUSSION..........................................................................................................58

3.4.1 Co-solute Induced Structural Transformation in Agarose and Gelatin Networks..........58

3.4.2 Structural Properties of Agarose/Gelatin Mixtures at Low and High Levels of Glucose Syrup.................................................................63

3.4.3 Utilization of the Free Volume Approach to Identify the Glass Transition Temperature of Agarose/Gelatin/Glucose Syrup Mixture........................................................................68

CONCLUSIONS................................................................................................................................75

Chapter 4: STRUCTURAL PROPERTIES OF WHEY PROTEIN/GLUCOSE SYRUP MIXTURES AS A FUNCTION OF A VARIANCE IN THE LEVEL OF SOLIDS

ABSTRACT........................................................................................................................................76

INTRODUCTION................................................................................................................................77

EXPERIMENTAL SECTION............................................................................................................80

4.3.1 Materials.............................................................................................................................80

4.3.2 Methods.............................................................................................................................81

RESULTS AND DISCUSSION......................................................................................................83
4.4.1 Rheology...........................................................................................................83
4.4.2 Differential Scanning Calorimetric Studies......................................................90
4.4.3 Confocal Laser Scanning Microscopy..............................................................94
4.5 CONCLUSIONS....................................................................................................97

Chapter 5: EPILOGUE AND FUTURE RESEARCH.................................................99
REFERENCES.........................................................................................................102
List of Tables

Table 1.1 – Composition of Different Whey Proteins (%w/w)
Table 1.2 – Uses of Whey Proteins in Human Foods
Table 1.3 – Physiochemical Characteristics of Major Whey Proteins

Table 2.1 – Standard Rheological Parameters
Table 2.2 – Certificate of Analysis of Gelatin
Table 2.3 – Certificate of Analysis of Agarose
Table 2.4 – Certificate of Analysis of Whey Protein Isolate
Table 2.5 – Certificate of Analysis of Glucose Syrup
Table 2.6 – Certificate of Analysis of Calcium Chloride
List of Figures

Figure 1.1 A typical gelatin amino acid composition
Figure 1.2 Fundamental Unit of Agarose
Figure 1.3 Schematic representations of network types: a) Ideal rubber b) Gelatin and c) Agarose
Figure 1.4 Composition of Cheese Whey
Figure 1.5 a) Primary structure of $\beta$ – lactoglobulin b) Primary structure of $\alpha$ lactalbumin
Figure 1.6 a) Tertiary structure of $\beta$ - lactoglobulin b) Tertiary structure of apo-$\alpha$-lactalbumin
Figure 1.7 Network structures in biopolymer mixed gels
Figure 1.8 Main Trends in the behaviour of protein/polysaccharide mixtures
Figure 1.9 Variation of $G'$, $G''$ and tan $\delta$ as a function of temperature, frequency, molecular weight and concentration for amorphous polymers

Figure 2.1a) Creep Strain(%) Curves
Figure 2.1b) Creep Compliance Curves
Figure 2.2a) $G^*$ and $\eta^*$ versus Shear Stress
Figure 2.2b) $G^*$ and $\eta^*$ versus Shear Strain
Figure 2.3a) Temperature as a function of time for a typical DSC and MDSC experiment
Figure 2.3b) Heating rate as a function of time for a typical DSC and MDSC experiment
Figure 2.4 Marvin Minsky’s original confocal designs in transmitted light
Figure 2.5 Advanced Rheology Generation 2 Rheometer
Figure 2.6 Differential Scanning Calorimeter
Figure 2.7 Leica TCS SP5 with integrated fixed stage microscope Leica DM6000 CFS
Figure 3.1a) Cooling profiles of storage modulus for 1.5% agarose with 0, 20, 40, 60 and 70% glucose syrup

Figure 3.1b) Heating profiles of storage modulus for 1.5% agarose with 0, 20, 40, 60 and 70% glucose syrup

Figure 3.2a) Cooling profiles of storage modulus for 7% gelatin with 0, 20, 40 and 70% glucose syrup

Figure 3.2b) Heating profiles of storage modulus for 7% gelatin with 0, 20, 40 and 70% glucose syrup

Figure 3.3a) DSC exotherms for the mixture of 3% agarose plus 15% gelatin with 0, 20, 40 and 60% glucose syrup run at temperatures above zero

Figure 3.3b) DSC exotherms for the mixture of 3% agarose plus 15% gelatin with 57 (--) and 62% (-) glucose syrup run at temperatures above zero

Figure 3.4 Heating profiles of storage modulus for the mixture of 1.5% agarose plus 7% gelatin with 0, 20, 40 and 60% glucose syrup

Figure 3.5 Cooling profiles of storage and loss modulus for 1.5% agarose, 7% gelatin and 66.5% glucose syrup (75% total solids)

Figure 3.6a) Frequency variation of G’ for 1.5% agarose, 7% gelatin and 66.5% glucose syrup

Figure 3.6b) Frequency variation of G” for 1.5% agarose, 7% gelatin and 66.5% glucose syrup

Figure 3.7 Master curve of reduced shear moduli (G’_p and G”_p) as a function of reduced frequency of oscillation (∆a_T)

Figure 3.8 Temperature variation of the factor a_T within the glass transition region and the glassy state of the material

Figure 4.1 Heating profiles of storage modulus for 15% whey protein with 0, 10, 20, 30, 35 and 40% glucose syrup in the presence of 10 mM CaCl_2

Figure 4.2 Frequency Sweep of 15% whey protein in the presence of 10 mM CaCl_2 at 5°C

Figure 4.3 Frequency Sweep of 15% whey protein with 10% glucose syrup in the presence of 10 mM CaCl_2 at 5°C

Figure 4.4 Frequency Sweep of 15% whey protein with 30% glucose syrup in the presence of 10 mM CaCl_2 at 5°C
Figure 4.5  Frequency Sweep of 15% whey protein with 40% glucose syrup in the presence of 10 mM CaCl$_2$ at 5ºC

Figure 4.6  DSC endotherms for the mixture of 15% whey protein isolate with 5, 15, 25, 35, 45, 55 and 65% glucose syrup in the presence of 10 mM CaCl$_2$

Figure 4.7  Variation in the denaturation temperature of 15% whey protein isolate with increasing concentration of glucose syrup

Figure 4.8  Aggregates of 15% whey protein isolate at 80ºC in the presence of 10 mM CaCl$_2$ using rhodamine dye

Figure 4.9  Aggregates of 15% whey protein isolate at 80ºC with 10% glucose syrup in the presence of 10 mM CaCl$_2$ using rhodamine dye

Figure 4.10 Aggregates of 15% whey protein isolate at 80ºC with 20% glucose syrup in the presence of 10 mM CaCl$_2$ using rhodamine dye

Figure 4.11  Aggregates of 15% whey protein isolate at 80ºC with 65% glucose syrup in the presence of 10 mM CaCl$_2$ using rhodamine dye
List of Abbreviations

~ Approximately
α Alpha
β Beta
δ Delta
D Dextro
DE Dextro Equivalent
L Levo
kDa Kilodalton
kPa Kilopascal
pI Isoelectric Point
GME Gelatin Manufacturers of Europe
BSA Bovine Serum Albumin
BSE Bovine Spongiform Encephalopathy
KGM Konjac Glucomanan
LBG Locust Bean Gum
Ig Immunoglobulins
Tg Glass Transition Temperature
T_{max} Maximum Heat Flow Temperature
G' Storage/Elastic Modulus
G'' Loss/Viscous Modulus
G_c Shear Modulus
G^* Complex Modulus
J_c Shear Compliance
tanδ Phase angle
η* Complex Viscosity
mM Millimolar
°C Degree Celsius
kg Kilogram
g Gram
mg Milligram
mm Millimeter
nm Nanometer
μm Micrometer
rad Radian
sec Second
min Minute
Pa Pascal
Hz Hertz
% Percent
w/w By Weight
CaCl₂ Calcium Chloride
KCl Potassium Chloride
EDTA Ethylene Diamine Tetra Acetic Acid
WPC Whey Protein Concentrate
WPI Whey Protein Isolate
WPH Whey Protein Hydrolysate
MTDSC Modulated Temperature Differential Scanning Calorimetry
MDSC Modulated Differential Scanning Calorimetry
DSC Differential Scanning Calorimetry
AR-G2 Advanced Rheology Generation 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFS</td>
<td>Confocal Fixed Stage</td>
</tr>
<tr>
<td>TCS</td>
<td>True Confocal Scanner</td>
</tr>
<tr>
<td>SP5</td>
<td>5 SpectroPhotometer</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>3D</td>
<td>Three Dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>Two Dimensional</td>
</tr>
<tr>
<td>LVR</td>
<td>Linear Viscoelastic Region</td>
</tr>
<tr>
<td>WLF</td>
<td>Williams, Landel and Ferry</td>
</tr>
<tr>
<td>PPW</td>
<td>Protein Polysaccharide Water</td>
</tr>
<tr>
<td>TTS</td>
<td>Time Temperature Superposition</td>
</tr>
<tr>
<td>RMIT</td>
<td>Royal Melbourne Institute of Technology</td>
</tr>
</tbody>
</table>
The publication resulting from this work of study has been submitted to *Food Chemistry*.

Sharma, D., George, P., Button, P., May, B. & Kasapis, S. Thermomechanical study on the phase behaviour of agarose/gelatin mixtures in the presence of glucose syrup as the co-solute.
SUMMARY

The field of structure/function relationships in protein and polysaccharide systems of low solids has been extensively researched. In contrast, there is scant information in the literature on the subject of structure/function relationships in high-solid biopolymer mixtures with immediate applications in the confectionery industry. The overall aim of this research study is to draw attention to this scarcity of data by examining the structure-function relationships of model systems incorporating materials of industrial interest and viability, like agarose, gelatin, whey protein isolate (WPI) and glucose syrup. The Chapter 1 of this study covers, a comprehensive literature review on the subject of the properties of these ingredients in solutions and gels in relation to a widespread range of applications in the dairy and other food industries. Chapter 2 talks about the extended methodology of the techniques utilised in this work to assess the functional properties of our preparations. These include small-deformation dynamic oscillation on shear, modulated differential scanning calorimetry and confocal laser scanning microscopy. Theoretical principles in terms of constitutive equations governing the operational procedures of the instruments, and equations covering the thermodynamics and kinetics aspect of analysis are discussed. Chapter 3 deals with the binary composites of agarose and gelatin in the presence of increasing amounts of glucose syrup, the sugar phase which acts as the co-solute for the polymeric materials.

Agarose/gelatin mixtures in an aqueous low-solid environment form non-interactive bicontinuous networks. Fundamental contributions to the underlying physical chemistry of the structural relationship in high-solid mixtures is made by reporting for the first time: i) addition of glucose syrup to the polymeric blend prevents the formation of stable double helices in the agarose network, which is increasingly “dissolved” in the high-solvent environment, and ii) gelatin, on the other hand, withstands better the co-solute induced change in solvent quality. In addition, estimates of the mechanical glass transition temperature are distinct from the DSC
counterparts in the agarose/gelatin/co-solute system and interpret this result in relation to the distinct property and distance scale being probed by the two techniques of rheology and calorimetry. The single value of $T_g$ estimated by this working protocol argues in accordance with experimental observations for the predominance of the gelatin network in the high-solid mixture. Chapter 4 is a treatise of the structural properties of WPI, which were made by heat denaturing the protein. Furthermore, solvent quality was manipulated by increasing the glucose syrup concentration in the presence of 10 mM calcium chloride. Mechanical analysis shows considerable changes in the structural properties of the gels formed by varying the level of co-solute, which relates to distinct interactive patterns in the mixtures. The nature and extent of these interactive patterns or molecular forces were further examined calorimetrically. Tangible evidence of the aggregation patterns of whey protein molecules is recorded using laser microscopy. This part of the work clearly demonstrates that the protein molecules are able to cluster in a high sugar environment by utilising a limited layer of hydration as compared to aqueous systems. Chapter 5 is a way forward giving a brief account of a range of related topics that can be analysed in future in order to further advance the understanding of the subject of high-solid biomaterials.
Chapter 1: INTRODUCTION

Biopolymers like proteins and polysaccharides known to exhibit gel formation have been of interest and a subject of observation to both scientists and academicians in the past few years. The use of gelling biopolymers to structurally manipulate commercial products in the food, beverage and pharmaceutical industry is common, with an objective of creating variance in the textural profiles. In an increasingly competitive market, the industrialist is faced with the challenge of innovation in terms of ingredient cost, product – added value and expectations of a healthy lifestyle to name a few (Kasapis, 2008). In the last two to three decades, the research in the area of biopolymer mixtures has shown a rapid growth (Tolstoguzov, 2003), outcome of which is the gaining popularity of polysaccharides and proteins usage as stabilizers, thickeners or gelling agents in manufacture of low fat spreads, an alternative to butter. As a healthy preference to urban lifestyle, low fat spreads reduces the risk of coronary heart disease, hypertension and other related ailments. But at the same time offers the consumer, spreadibility and texture similar to butter. Polysaccharides like ‘Starch Hydrolysis Products’ mimic the organoleptic properties of fat to a large extent, however, they impart a not so desirable ‘starchy mouthfeel’.

Gelatin is known for its characteristic ‘melt in mouth’ property and has been used in the food industry since time immemorial. It not only helps release flavour in the food systems but also improves the mouthfeel (Chronakis & Kasapis, 1995). Similarly, the incorporation of a sugar to such a protein – polysaccharide mixture could be an area of keen interest per se to observe its contribution to the organoleptic properties of the end products in the ever prominent confectionery market.

Various mixed systems of polysaccharides and proteins have been studied for their rheological properties and applications in the food industry. Gelatin-agarose composite system
was the one to go through pioneer research and formed the basis to further investigations using other biopolymers. The works on protein polysaccharide composite gels did not indulge into studying systems containing sugars and the loopholes in gelatin-agarose system also need to be investigated, hence, this research aims at studying the effect on the stability, gelling behaviour and mechanical profile on addition of a sugar phase to a three phase system comprising gelatin, agarose and glucose syrup.

1.1 GELATIN

Among various commercial hydrocolloids used in the food industry, gelatin is the most unique, as it not only serves multiple functions but has a wide range of industrial applications too. Gelatin has long been used as a gelling and foaming agent in the food industry, in preparation of pharmaceutical products (like soft and hard capsule, microspheres), in biomedical field (wound dressing and three dimensional tissue regeneration) and in various other non-food applications e.g., photography (Karim & Bhat, 2008). It is obtained by partial hydrolysis of collagen derived from animal skin, white connective tissue and bones (Morrison, Clark, Chen, Talashek, & Sworn, 1998). It is derived from collagen via controlled acid or alkaline hydrolysis. Collagen may come from hide, bone, or other collagenous material (Belitz & Grosch, 1999). The main sources for commercial gelatin are limited to pig (porcine) or cow (bovine) due to the relatively low cost of the final gelatin product.

Composition and structure of gelatin

A typical gelatin is formed of 14% moisture, 84% protein and 2% ash (Rix, 1990). The protein portion consists of several different amino acids, major ones are the glycine, proline and hydroxyproline (Cuppo, Venuti, & Cesro, 2001; Johnston-Banks & Harris, 1990). These
amino acids are arranged in gelatin gels to form long molecular chains, similar to the collagen source from where they came. These chains form structures which eventually results in the entire gel network. Gelatin molecules contain repeating sequences of glycine-X-Y triplets, where X and Y are frequently proline and hydroxyproline. Amino acid glycine is said to be responsible for chain flexibility (Burchard & Ross-Murphy, 1990). Such sequences lead to a triple helical structure in gelatin and its ability to form gels.

![Figure 1.1 A typical gelatin amino acid composition (Burey, Bhandari, Rutgers, Halley, & Torley, 2009).](image)

“The issue of gelatin replacement has existed for many years for the vegetarian, halal and kosher markets but has gained increased interest in the last decade, particularly within Europe with the emergence of bovine spongiform encephalopathy (“mad cow disease”) in the 1980’s” (Morrison, et al., 1998). Ever, since there has been a concern regarding using gelatin derived from possibly infected animal parts. The worldwide production of gelatin in 2007 was about 326000 tons, of which 46% were from pigskin, 29.4% from bovine hides, 23.1% from bones, and 1.5% from other parts (GME, 2008a, 2008b). Therefore, academia and industry, have been looking for various possible alternatives that possess most of the unique functional
properties of mammalian gelatin. Gelatin displays multiple roles in food processing and formulations. The functional properties of gelatin can be divided into two groups (Schrieber & Gareis, 2007). The gelling properties are the ones associated with gel strength, gelling time, setting and melting temperatures, viscosity, thickening, texturizing and water binding. The second group relates to the surface behaviour of the gelatin, for instance, emulsion formation and stabilization, protective colloid function, foam formation and stabilization (as in marshmallow), film formation and adhesion/cohesion (Schrieber & Gareis, 2007).

An ideal gelatin alternative should, therefore, possess all or at least some of these properties exhibited by mammalian gelatin. The “Melt-in-mouth” property of gelatin gives it a special and unique status amongst all other food hydrocolloids. Gelatin melts slightly below physiological temperature of humans and contributes to intensive flavour and aroma release when used in various confectionery and other food products. This characteristic is hard to find in other biopolymer systems and so far, scientists have not been able to find a gelling protein or polysaccharide that can replace gelatin universally (Haug, Draget, & Smidsrød, 2004). Hence, the need to conduct this study with an objective to discover properties similar to gelatin in other gelling agents like alginate, pectin, starch, agar, carrageenan and other polysaccharides from plant sources.

1.2 AGAROSE

Agarose is one of the biopolymers keenly considered by most of the food product and ingredient manufacturers for the development of high sugar/low biopolymer formulations, which is a core to many confectionery products. Agarose, the gelling component of agar, is a neutral polysaccharide obtained from a family of red seaweeds (Rhodophyceae). It is a linear polysaccharide which consists of β-1,3 linked D- galactose and α- 1,4 linked 3,6- anhydro- α L- galactose residues. Sometimes, traces of sulphate groups can be present on the agarose
chain, and may modify the gel properties (Normand, Lootens, Amici, Plucknett, & Aymard, 2000).

Figure 1.2 Fundamental Unit of Agarose, $M = 306 \text{ g. mol}^{-1}$ (Normand, et al., 2000).

Due to its gelling properties the gelation of agarose has been studied extensively (Arnott & Selsing, 1974; Watase, Nishinari, & Hatakeyama, 1988). Agarose gels are formed by aggregation of helical molecules forming junction zones that are stabilised by water molecules. These gels are typically rigid and prone to the phenomenon of “weeping” or spontaneous loss of water on standing that is known as syneresis. They are very turbid and show considerable hysteresis in melting and setting temperatures at low levels of solids. Regarded as physical gels, their gelation mechanism is governed by hydrogen bonding (Braudo, 1992). “Agarose gels are composed of thick bundles of agarose chains, large pores of water and exhibit high turbidity and strong elasticity” (Aymard, et al., 2001). Gel network formation is because of hydrogen bonding between agarose molecules. Agarose gels are formed when random coils in a heated sol become ordered as the sol cools. As cooling progresses, helices are formed which aggregate to form a gel network (Arnott & Fulmer, 1974; Dea, McKinnon, & Rees, 1972; San Biagio, Bulone, Emanuele, Palma-Vittorelli, & Palma, 1996). Agar is one of the most familiar gel materials from which wide range of aids can be made for the separation of biological molecules and particles by techniques such as gel filtration, affinity chromatography and ion-exchange chromatography. It is commonly used in
physical chemistry for preparation of salt bridges, bacteriology (e.g. plating out microorganisms) and virology (viral overlay technique).

As mentioned above, the source of agar is a seaweed of which *Gracilaria* and *Gelidium* are commercially important genera. Most products in the food industry contain a variety of molecular interactions which contributes to an overall structure. As compared to most solid foods, biopolymer gels have a relatively low level of complexity, hence, more useful for rheological investigations. The networks formed by agarose gels are least complex as they are formed solely through hydrogen bonds, making them a useful model to investigate the structure-function relationships (Barrangou, Drake, Daubert, & Foegeding, 2006).

In their work in 1985, McEvoy and his research group showed how networks of Gelatin and Agarose differed from that of ideal rubber as seen in Fig 1.3

![Figure 1.3](image_url)

**Figure 1.3** Schematic representations of network types: a) Ideal rubber. Circles are covalent crosslinks. b) Gelatin and c) Agarose. Heavy lines indicate helical junction zones (McEvoy, Ross-Murphy, & Clark, 1985).
1.3 WHEY PROTEINS

Proteins are highly complex polymers and their functional diversity mainly arises from their chemical make-up. While other biopolymers, such as polysaccharides and nucleic acids, are made up of one or few monomers, proteins and polypeptides are made up of combinations of 20 different amino acids. Whey from the cheese industry is increasingly being used either as animal feed or as a source of protein for human nutrition. There are two types of whey that can be differentiated, firstly, soft whey which comes from rennet coagulation of milk at pH 6.6 (e.g., in Emmental or Cheddar manufacturing) and secondly, the acid whey which is a result of both caseinate manufacture as well as fresh soft cheese production (e.g., Cream Cheese, Camembert, or Petit Suisse), after acid coagulation of milk and in case of cottage cheese, following heating of curd. Whey in the market comes in liquid as well as powder forms, with different compositions (Table 1.1).

<table>
<thead>
<tr>
<th>Whey concentrates</th>
<th>Water</th>
<th>Lactose</th>
<th>Lipids</th>
<th>Minerals</th>
<th>Nitrogen</th>
<th>β-Lactoglobulin</th>
<th>α-Lactalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>93.5</td>
<td>4.5</td>
<td>0.3</td>
<td>0.6-0.8</td>
<td>1.0</td>
<td>0.45</td>
<td>0.2</td>
</tr>
<tr>
<td>Powder</td>
<td>4.0</td>
<td>70</td>
<td>-</td>
<td>9-12.0</td>
<td>13.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Curd</td>
<td>42.0</td>
<td>36.0</td>
<td>-</td>
<td>8.0</td>
<td>9.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Concentrate</td>
<td>4.0</td>
<td>9.5</td>
<td>6.0</td>
<td>3.0</td>
<td>75.0</td>
<td>32.0</td>
<td>15</td>
</tr>
<tr>
<td>UF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Damodaran & Paraf, 1997)
Whey protein is a mixture of proteins (Fig 1.4) with several functional properties and many potential uses.

![Diagram of cheese whey composition](image)

**Figure 1.4** Composition of Cheese Whey. Numbers indicate concentration in g/litre. NPN = products composed of non-protein nitrogen (Damodaran & Paraf, 1997).

The main proteins are β- lactoglobulin and α- lactalbumin. These represent approximately 70% of the total whey proteins and are responsible for the hydration, gelling and surface-active properties (emulsifying and foaming properties) of the whey protein ingredients (Damodaran, 1994). Industrial applications and uses of whey products are listed in Table 1.2. These protein ingredients are also used for therapeutic and nutritional properties in low- calorie diets and in intensive care enteral nutrition especially for the burn victims.
<table>
<thead>
<tr>
<th>Industrial Applications</th>
<th>Functional Properties Expected</th>
<th>Proteins Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bread making</td>
<td>Water holding</td>
<td>WPC or WPC + caesinates</td>
</tr>
<tr>
<td>Biscuit manufacturing</td>
<td>Fat dispersibility</td>
<td>WPI</td>
</tr>
<tr>
<td>Breakfast cereals</td>
<td>Emulsion stabilization</td>
<td>WPI, coprecipitates</td>
</tr>
<tr>
<td></td>
<td>Overrun of foam</td>
<td>Whey</td>
</tr>
<tr>
<td></td>
<td>Gelling properties</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Browning</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aroma enhancement</td>
<td></td>
</tr>
<tr>
<td>Pasta</td>
<td>Binding and texturing effect</td>
<td>Coprecipitates</td>
</tr>
<tr>
<td></td>
<td>Browning</td>
<td></td>
</tr>
<tr>
<td>Confectionery</td>
<td>Emulsion manufacturing</td>
<td>WPC + hydrolysed caseinates</td>
</tr>
<tr>
<td>Chocolate confectionery</td>
<td>Overrun of Foam</td>
<td>WPC</td>
</tr>
<tr>
<td></td>
<td>Browning, aroma</td>
<td>Whey</td>
</tr>
<tr>
<td></td>
<td>Antioxidizing effect</td>
<td>Coprecipitates</td>
</tr>
<tr>
<td>Ice cream</td>
<td>Emulsion stability</td>
<td>WPC + caesinates and total milk proteins</td>
</tr>
<tr>
<td></td>
<td>Overrun of foams</td>
<td></td>
</tr>
<tr>
<td>Category</td>
<td>Use</td>
<td>Ingredients</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Meat products</td>
<td>Gelling properties</td>
<td>WPC, WPI alone or in mixture with caesinate</td>
</tr>
<tr>
<td>Delicatessen Meat</td>
<td>Emulsion making</td>
<td>WPC, WPI alone or in mixture with caesinate</td>
</tr>
<tr>
<td></td>
<td>Water holding (creamy and smooth texture)</td>
<td>WPC, WPI alone or in mixture with caesinate</td>
</tr>
<tr>
<td></td>
<td>Adhesive or binding properties</td>
<td>WPC, WPI alone or in mixture with caesinate</td>
</tr>
<tr>
<td>Sauces</td>
<td>Emulsion stability</td>
<td>WPC + caesinates + egg yolk</td>
</tr>
<tr>
<td>Soups</td>
<td>Water holding</td>
<td>WPC + caesinates + whole egg</td>
</tr>
<tr>
<td>Ready-to-eat food</td>
<td>Gelling properties</td>
<td>WPC + caesinates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WPI</td>
</tr>
<tr>
<td>Milk products</td>
<td>Emulsion stability</td>
<td>Caesinates</td>
</tr>
<tr>
<td>(cheese, yoghurts, light butter)</td>
<td>Water holding</td>
<td>WPC + caesinates</td>
</tr>
<tr>
<td></td>
<td>Gelling properties</td>
<td>WPI</td>
</tr>
<tr>
<td>Alcoholic beverages</td>
<td>Cream stabilization</td>
<td>WPC + caesinates</td>
</tr>
<tr>
<td></td>
<td>Cloudy aspect</td>
<td>WPC or WPI</td>
</tr>
<tr>
<td>Nutritional uses</td>
<td>Protein intake</td>
<td>Whey, WPC, or WPI</td>
</tr>
<tr>
<td></td>
<td>Enteral nutrition</td>
<td>WPC hydrolysates</td>
</tr>
<tr>
<td>Cosmetics</td>
<td>Skin protection</td>
<td>Lactoferrin, WPC hydrolysates</td>
</tr>
<tr>
<td></td>
<td>Antimicrobial properties</td>
<td>Lactoferrin, lactoperoxidase</td>
</tr>
</tbody>
</table>

Source: Centre Interprofessionnel de Documentation et d’information Laitieres, Paris.

(Damodaran & Paraf, 1997)
Composition and structure of whey proteins

β-Lactoglobulin (2-4g/litre in whey) has a molecular mass of 18.3 kDa. It contains 162 amino acid residues with one thiol group and two disulfide bonds (Table 1.3). Out of the seven genetic variants, the A and B variants are abundant. They differ from each other by two amino acid residues (residue 64 is Asp for A and Gly for B; residue 118 is Val for A and Ala for B). Variant A is the most negatively charged at the pH of the milk (6.6) and can be separated from the B variant by electrophoresis or amino acid exchange by anion exchange chromatography. The secondary and tertiary structures show a high degree of organisation with a great proportion of β sheets (43-50% of the residues) and only 10-15% of α helix and 15-20% of β turns (Fig 1.6 a) (Papiz, et al., 1986). The monomer form of β-lactoglobulin resembles that of a cone or a calyx with a hydrophobic pocket capable of binding Vitamin A and fatty acids. The structure of β-lactoglobulin is akin to that of the retinol-binding protein from blood plasma.

**Table 1.3 – Physiochemical Characteristics of Major Whey Proteins**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>β-Lactoglobulin</th>
<th>α-Lactalbumin</th>
<th>Bovine serum albumin</th>
<th>Immunoglobulins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar mass (g/mol)</td>
<td>18,362</td>
<td>14,174</td>
<td>69</td>
<td>150,000-1,000,000</td>
</tr>
<tr>
<td>Cysteinyl residue/mol</td>
<td>5</td>
<td>8</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>Amino acid residues/mol</td>
<td>162</td>
<td>123</td>
<td>582</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>Disulphide bonds/mol</td>
<td>2</td>
<td>4</td>
<td>17</td>
<td>4.x</td>
</tr>
<tr>
<td>----------------------</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td>Thiol function/mol</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Lysyl residues/mol</td>
<td>15</td>
<td>12</td>
<td>59</td>
<td>-</td>
</tr>
<tr>
<td>Arginyl residues/mol</td>
<td>3</td>
<td>1</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Histidyl residues/mol</td>
<td>2</td>
<td>3</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Glutamyl residues/mol</td>
<td>16</td>
<td>8</td>
<td>59</td>
<td>-</td>
</tr>
<tr>
<td>Aspartyl residues/mol</td>
<td>10</td>
<td>9</td>
<td>39</td>
<td>-</td>
</tr>
<tr>
<td>pI (isoelectric point)</td>
<td>52</td>
<td>4.5 – 4.8</td>
<td>4.7 – 4.9</td>
<td>5.5 – 8.3</td>
</tr>
<tr>
<td>Average hydrophobicity</td>
<td>508</td>
<td>468</td>
<td>468</td>
<td>458</td>
</tr>
</tbody>
</table>

(Damodaran & Paraf, 1997)

*α*-Lactalbumin is made up of 123 amino acid residues and four disulphide bridges (Table 1.3 and Figure 1.5 b). It has a molecular mass of 14.2 kDa. The *α* – lactalbumin content of whey is about 1.5 g/litre. The two genetic variants A and B differ from each other by one residue; the residue at position 10 is Gln for A and Arg for B. This protein has a very low content of organised secondary structure: 30% *α* helix and 9% *β* – sheets (Alexandrescu, Evans, Pitkeathly, Baum, & Dobson, 1993). Hence, it has great flexibility. However, the presence of one bound Ca$^{2+}$ ion and four disulfide bridges help it maintain a compact ellipsoidal structure (Figure 1.6 b).

Two other major whey proteins are the bovine serum albumin (BSA) and immunoglobulins (Ig). BSA is a large protein with 582 amino acid residues (Table 1.3) (Brown, 1977). It functions as a carrier protein for transport of non-polar molecules in biological fluids. The protein is compact on the C-terminal side and can be reversibly
denatured by heat or by adding acid or base at 40-50°C. The immunoglobulins form a heterogeneous family of glycoproteins of 148-1000 kDa with antibody properties (Table 1.3) (Morr & Ha, 1993). Though they show denaturation temperatures higher than those of β-lactoglobulin and of α-lactalbumin, they are very heat sensitive in presence of BSA, probably due to interaction with the free thiol group.

The lactoferrin is a metalloprotein with a molecular mass of 80-92 kDa; it contains 703 amino acid residues and 16 disulfide bridges (Anderson, Baker, Norris, Rice, & Baker, 1989; Pierce, Colavizza, & Benaissa, 2005) and one mole of bound Fe³⁺ cation (Nagasako, Saito, Tamura, Shimamura, & Tomita, 1993). A few minor proteins also play an important role in the functional properties of whey proteins. The proteose peptone fraction, which consists of proteolytic fragments of β-casein, contains surface-active peptides. Many enzymes like lactoperoxidase, alkaline phosphatise, catalase, sulfhydryl oxidase and plasmin, are also found in whey.
**Figure 1.5**  
**a)** Primary structure of \( \beta \)-lactoglobulin B (\( * \) = variant A)  
**b)** Primary structure of \( \alpha \)-lactalbumin B (\( ^o \) = variant A) (Damodaran & Paraf, 1997).

**Figure 1.6**  
**a)** Tertiary structure of \( \beta \)-lactoglobulin  
**b)** Tertiary structure apo-\( \alpha \)-lactalbumin  
(Damodaran & Paraf, 1997).
1.4 GLUCOSE SYRUP

Sucrose in the food industry is often used in conjunction with glucose syrup, as it enhances its solubility and retards crystallisation in food products (Belitz, Grosch, & Schieberle, 1999; Edwards, 2000). It not only fortifies the texture but also the sensory properties of the gels and can be used to impart body or mouthfeel, by increasing the product bulk or weight.

Glucose syrup refers to products with a dextrose equivalent (DE) of between 20 and 80 (where 100 indicates pure glucose and 0 indicates no glucose). If the DE of the product is less than 20 then they are termed as maltodextrins and in case of DE more than 80, are called hydrolysates or hydrols. The carbohydrate composition, dextrose equivalent and sulphur dioxide content specify the glucose syrup. Traditionally, 42DE syrup is employed in the confectionery market to obviate sugar crystallisation in the consumer goods during storage (Jackson, 1999). Glucose syrup is essentially shelf-stable, therefore, no preservatives are required in order to avert microbial growth. As confectionery products have a considerably, longer transit periods before they reach the shelves of the supermarkets; it is very important that they do not experience any kind of microbial growth or fermentation or mould spoilage, it is here where the glucose syrup comes to the rescue of manufacturers as it facilitates a lower water activity in the confectionery gels (Jackson, 1999). Its principal role is that of a sweetener, although it also contributes to stabilizing other ingredients like sucrose or gelatin, in addition to the texture, mouthfeel and microbial stability (Belitz & Grosch, 1999; Gillies, 1979). The gelling agents in confectionery gels usually make up for about 10% of the food gel, remaining is glucose syrup or sucrose.
1.5 WATER

Water is a major constituent in many foods, acting as a reactant in hydrolytic processes and supporting chemical reactions (Slade, Levine, & Reid, 1991). Hence, removing water or binding it by increasing the concentration of common salt or sugar in foodstuffs inhibits many reactions and retards microbial growth, thus, facilitating shelf-life of the product. “The physical interaction of water with proteins, lipids, polysaccharides and salts can contribute significantly to food texture, as foods tend to become plastic when their hydrophilic components are hydrated. The glass transition temperature of a food i.e. the temperature where the material shows glassy to rubbery behaviour, is affected by the water content” (Belitz & Grosch, 1999).

In confectionery gels, water often acts as a plasticizer to aid gel formation. Studies reveal that highest affinity for water is exhibited by gelatin when combined with a polysaccharide or sugar system. The physical state of metastable food products depends upon composition, temperature and storage time (Burey, et al., 2009). Water can affect their properties, causing them to be glassy, rubbery or viscous by altering the glass transition temperature. The variations in moisture content result in changes such as premature crystallisation, stickiness, accelerated rancidity, lack of body, difference of chew, hardness, poor handling on cutting machines and product flaws in surface texture.

1.6 BIOPOLYMER MIXTURES / COMPOSITE GELS

A biopolymer mixture is composed of at least two different biopolymers in addition to an aqueous component which forms the largest in terms of volume. The nature of the biopolymer will decide the type of resultant mixture. Possible outcomes can be a fluid-fluid system, a solid dispersed in a fluid or a mixed gel system wherein the gelation of both biopolymers has
taken place. Depending upon the nature of the network developed, a mixed gel system can be further classified as:

i. **Interpenetrating networks** are those where each of the biopolymer, gels separately forming independent networks that interpenetrate into one another.

ii. **Coupled networks** are the ones wherein the biopolymers associate directly to form a single network by covalent linkages, ionic interactions or co-operative junction zones.

iii. **Phase separated networks** are seen more often in mixed biopolymer mixtures. Due to thermodynamic incompatibility solutions of two different biopolymers usually phase separate above a critical concentration which results in a less favourable interactions amongst different polymer segments. This results each biopolymer to exclude the other from its polymeric domain. In a protein-polysaccharide-water system, phase separation occurs when the total polymer concentration exceeds 4%.

![Network structures in biopolymer mixed gels](image)

**Figure 1.7** Network structures in biopolymer mixed gels, modified from (Morris, 1998).

Both single-phase and phase separated solutions are capable of forming binary gels, leading to associative or segregative systems (Morris, Harding, Hill, & Mitchell, 1995).
Associative interactions exist in the ordered junctions found mainly in synergistic gels. Only a few examples are known, alginate of high polyguluronate content with high-methoxy pectin at low pH and konjac glucomannan (KGM) or galactomannans of low galactose content (e.g. LBG) with aggregated double helices of agar/carrageenan polysaccharides or with xanthan. Secondly, gelation in this case occurs through electrostatic complexes typically between negatively-charged polysaccharides and positively-charged proteins (i.e. below their isoelectric point). The segregative interactions are even more common and occur in virtually all biopolymer mixtures where there is no over-riding drive to heterotypic association. Often referred to as “thermodynamic incompatibility”. The segregative interactions are far more common and with gelatin/agarose mixture the tendency of individual molecules to be surrounded by others of the same type leads to composite gels where phase inversion, occurs at a specific mixture composition (Clark, Richardson, Ross-Murphy, & Stubbs, 1983).

1.7 PHASE SEPARATION IN BIOPOLYMER MIXTURES

Most of the food products as gels in the market today are in fact composite gels, containing two or more gelling agents. Nearly, most of the components contribute to structure and physical properties of food, but the two main structural materials are the polysaccharides and proteins (Tolstoguzov, 2000). The biopolymer mixtures are used not only in the food industry but many other industries, to impart textures, appearances, specific flow behaviours and tactile and mouthfeel properties to various products (De Kruif & Tuinier, 2001; Norton & Frith, 2001). Due to their wide use and applications, biopolymer mixtures in the food have been greatly researched. But only recently, the biopolymer phase separation and its effect on the properties of composite gels have been under study (Butler, 2002; De Kruif & Tuinier, 2001;
DeMars & Ziegler, 2001; Norton & Frith, 2001). “Most food components have limited miscibility on a molecular level and tend to form multicomponent heterophase and non-equilibrium dispersed systems” (Tolstoguzov, 2000).

Polymers dissimilar in shape and structure undergo segregation leading to a loss in polymer concentration near the other type of polymer particle. When a critical polymer concentration is exceeded, phase separation takes place (De Kruif & Tuinier, 2001; Norton & Frith, 2001). Interactions of polysaccharides and proteins vary from complete segregation to complexation as shown in the figure below.

![Figure 1.8 Main trends in the behaviour of protein/polysaccharide mixtures (Burey, et al., 2009; De Kruif & Tuinier, 2001).](image-url)
The mechanical properties of a gel are greatly affected by these interactions. Linear polysaccharides are more incompatible with proteins as compared to the branched ones. This is due to the rigidity and presence of less independently available macromolecular segments and lower mixing entropy than in flexible branched polymers (Tolstoguzov, 2000). Polymer concentration and the biopolymer compatibility decides the level of phase separation and shape and appearance of phase components in these systems (Norton et al., 2001; De Kruif et al., 2001). The thermodynamic incompatibility of the gel components is the reason for phase separation in composite gels (Zasypkin, 1997). According to Norton and Frith, phase separation is entropically unfavourable, but enthalpically beneficial as molecules prefer like molecules. Temperature, pH and shear changes are also liable to affect phase separation in biopolymers (Cuppo, et al., 2001; Lorén & Hermansson, 2000; Norton & Frith, 2001; Wolf, Sciocco, Frith, & Norton, 2000; Zasypkin, 1997). Interfacial tension between gel phases is also important in determining the biopolymer composite behaviour. Synthetic polymer blending laws have been applied to predict and quantify mechanical behaviour of such composites (Abdulmola, Hember, Richardson, & Morris, 1996).

1.8 GLASS TRANSITION IN MIXED SYSTEMS

Glass transition of an amorphous polymer system is the change in the system behaviour from rubbery to hard and relatively brittle state of existence. This transition has gained wider appreciation and use because of its application in understanding and controlling the quality of such systems (Burey, et al., 2009). Confectionery gels are a typical example of amorphous polymer systems; therefore, the rubber to glass transition is a significant factor in analysis of these gels. Occurrence of glass transition in a system can be studied by determining its glass transition temperature ($T_g$), but techniques like dynamic mechanical analysis can also be used to observe the changes from rubbery to glassy state. Figure 1.9 shows the master curve
detailing the four regions of mechanical behaviour for amorphous polymer systems (Kasapis, Mitchell, Abeysekera, & MacNaughtan, 2004; Ong, Whitehouse, Abeysekera, Al-Ruqaie, & Kasapis, 1998).

**Figure 1.9** Variation of $G'$, $G''$ and $\tan \delta$ as a function of temperature, frequency, molecular weight and concentration for amorphous polymers (Kasapis, Al-Marhoobi, Deszczynski, Mitchell, & Abeysekera, 2003).

First section of the curve, displays the value of $G''$ (---) or the viscous modulus to be more or greater than the value of $G'$ (-) or the storage modulus. This is because of the molecular flow in protein or polysaccharide solutions is predominant as well as the concentrated preparations of glucose syrup. In the second section, the materials form rubbery or elastic networks of stable physical associations where the $G'>G''$, as seen at room temperature in jelly bears or gummy candies (Loren, Altskar, & Hermansson, 2001). The glass transition region is clearly depicted in the section III, where the viscous response again becomes
dominant i.e. $G'' > G'$, due to cooling of the rubbery food materials and absence of crystallisation. Finally, in section IV the moduli crossover for a third time and the system enters the glassy state at which the rates of chemical, enzymatic and microbial processes decelerate dramatically (Kasapis, et al., 2004). The master curve throws some light upon the molecular movements within the material during changes in frequency or temperature. In glass transition region (section III) backbone adjustments of polymer chains i.e. elastic contribution is limited, although molecular movements capable of dissipating energy are visible. Upon entering the glassy zone (section IV), extremely high frequencies avert configurational rearrangement of polymeric chains; only bending and stretching of bonds are allowed resulting in an elastic, rather than a viscous character (Ong, et al., 1998). The appearance of this curve, the temperatures and the frequencies at which the boundaries of each section, in the curve occur, are pivotal for understanding and analysing the structural behaviour of confectionery gels.

1.9 POLYMER BLENDING LAWS OF TAKAYANAGI

A combination of two simple viscoelastic models (parallel and series) in proportion to their phase volumes helps in prediction of overall viscoelastic properties. In his work, Takayanagi has verified such a model. Dynamic extensional measurements were performed on samples composed of two layers of different synthetic polymers. The strain was imposed either parallelly or perpendicularly to their sides (Richardson & Kasapis, 1998).

This analysis is based on binary composites of pure, mutually insoluble, synthetic polymers whose individual rheological properties are independent of the macroscopic amounts present, and hence is an approximation. Thus, in a binary composite, if X and Y are the two components having shear moduli $G_x$ and $G_y$ respectively, mechanical properties of the
composite may be derived from such individual systems present at phase volume fractions $\Phi_x$ and $\Phi_y$, where $\Phi_x + \Phi_y = 1$. In extreme cases of strain and stress distribution, two equations are derived providing upper and lower bound limits for the value of shear modulus $G_c$ of a composite formed from X and Y.

If a weak material X is dispersed as discrete particles within a continuous matrix of a stronger material Y, the overall shear modulus of the composite is related to the corresponding moduli of the component phases by (Kasapis, Morris, Norton, & Clark, 1993).

$$G_c = \Phi_x G_x + \Phi_y G_y$$  \hspace{1cm} \text{.... (1.1)}$$

Equation (1.1) applies to isotrain conditions wherein the strain is approximately uniform throughout the material. It provides upper bound limits as the overall strength of the composite is higher when the stronger component forms the continuous network. The deformation of the weak filler is dictated by the response of the surrounding stronger matrix. Hence, both components are deformed to the same extent.

Conversely, if a strong material is dispersed discontinuously within a continuous matrix of a weaker material, the overall shear compliance of the composite is obtained as the corresponding weighted average of the individual compliances (Morris, 1992).

$$J_c = J_x \Phi_x + J_y \Phi_y$$  \hspace{1cm} \text{.... (1.2)}$$

i.e. $1/G_c = \Phi_x/G_x + \Phi_y/G_y$  \hspace{1cm} \text{.... (1.3)}$$

Equation (1.3) refers to isostress conditions where the stress may be regarded as constant in both phases, describing a lower limit appropriate to a weaker continuous phase, there by providing lower bound limits. The strong filler is deformed less than the surrounding matrix, and the stress acting upon it is limited to the resisitance of the matrix to the imposed deformation.
Chapter 2: METHODOLOGY

2.1 RHEOLOGY

Rheology is the science of the deformation and flow of matter or in other words, the study of the manner in which materials respond to applied stress and strain. The structure of matter can be physically characterized using this technique (Malkin, Malkin, & Isayev, 2006). It was initially developed by scientists studying printing inks, plastics, rubber but can be applied to a wide range of products (Bourne, 2002). All materials have rheological properties and the area is relevant in many fields of study like geology and mining (Cristescu, 1989), concrete technology (Dealy, 1993), soil mechanics (Tattersall & Banfill, 1983), plastics processing (Vyalov, 1986) and tribology (study of lubrication, friction and wear). The focus of this work is food where understanding rheology is critical in optimising product development efforts, processing methodology and final product quality.

Specifically, there are numerous reasons rheological data are needed in the food industry:

a. Process engineering calculations involving a wide range of equipment such as pipelines, pumps, extruders, mixers, coaters, heat exchangers, homogenizers, calenders and on-line viscometers.

b. Determining ingredient functionality in product development.

c. Intermediate or final product quality control.

d. Shelf life testing.

e. Evaluation of food texture by correlation to sensory data.

f. Analysis of rheological constitutive equation
The macroscopic properties of biopolymers, particularly, molecular shape, aggregation and intermolecular network formation can be conveniently obtained using rheological measurements.

These fall in two broad categories:
(i) Small deformation measurements, in which the structure is retained
(ii) Large deformation studies, which measures the breakdown of structures

While both approaches are extremely useful and characterise different aspects of rheological response, the former has the advantage of being non-destructive; hence ideal for monitoring systems where numerous measurements must be carried out on the sample under different conditions, for instance, when melting a gel at different temperatures. The fundamental operation in all rheological testing is to apply a force to the sample under investigation and measure its deformation or, equivalently, apply a deformation and measure the resistance.

Table 2.1 summarises, the parameters used in rheological measurements. There are two idealised extremes of response – first being the one for a perfectly elastic solid fully obeying Hooke’s law, where stress is directly proportional to strain (with the ratio of stress/strain giving a measure of the rigidity of the material). And the second is for a perfectly viscous (Newtonian) liquid, where the stress is directly proportional to the rate of strain but independent of the strain itself. Here, the ratio of stress/rate of strain is the viscosity of the liquid. Most of the biopolymer systems exhibit both liquid-like and solid-like behaviour and are thus said to be viscoelastic.
Table 2.1 Standard Rheological Parameters

<table>
<thead>
<tr>
<th>PARAMETER (units)</th>
<th>LONGITUDNAL DEFORMATION</th>
<th>LATERAL DEFORMATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRESS (Pa ; 1 Pa = 10 dyne cm$^{-2}$)</td>
<td>$\sigma$</td>
<td>$\tau$</td>
</tr>
<tr>
<td>STRAIN (dimensionless)</td>
<td>$\varepsilon$</td>
<td>$\gamma$</td>
</tr>
<tr>
<td>STRESS / STRAIN (Pa)</td>
<td>$E$</td>
<td>$G$</td>
</tr>
<tr>
<td>RATE OF STRAIN ($s^{-1}$)</td>
<td>$\dot{\varepsilon}$</td>
<td>$\dot{\gamma}$</td>
</tr>
<tr>
<td>STRESS / RATE OF STRAIN (Pa s; 1 Pa s = 10 poise; 1 mPa s = 1 cP)</td>
<td>$\lambda$</td>
<td>$\eta$</td>
</tr>
</tbody>
</table>

(From Morris, 1984)

Small deformation studies using dynamic oscillation experiments on a Rheometer is one of the most popular techniques and is used extensively to study the viscoelastic behaviour and gelation properties of materials. The rheological behaviour of foods can be tested using a rheometer. A rheometer can quantify the food substance by steady shear oscillation or
dynamic oscillation. This technique can be used to measure the storage modulus, viscous modulus, complex viscosity and various other parameters.

The storage and viscous moduli are prime parameters that help in characterization of the viscoelastic systems like gels. A structured system gains energy from the oscillatory motion as long as the motion does not disrupt the structure. This energy in the sample is stored as elastic modulus (G’). It is also referred to as ‘storage modulus’ because it describes the storage of energy in the structure. In other words, it defines the solid-like behaviour of the material and its magnitude depends upon the number of interactions between ingredients in the sample. The higher the number of such interactions and stronger the interaction, higher is the value of G’.

On the other hand, the loss modulus (G’’) describes that part of the energy which is lost as viscous dissipation.

A structured sample in an oscillatory shear creates motion between the ingredients leading to friction. This friction is responsible for the energy to be lost as viscous heating. It is related only to the number of interactions and virtually independent of their strength. Therefore, greater the number of interactions in which friction can be created larger is the value of G’’. The loss modulus is also called as the ‘viscous modulus’ and describes the liquid-like behaviour of a material.

Other useful parameters are the phase angle tan δ and complex viscosity η*. Phase angle tan δ is associated with the degree of viscoelasticity of the sample. A low value of tan δ (or δ) indicates a higher degree of viscoelasticity (i.e. more solid-like behaviour) and vice versa. The phase angle tan δ signifies the ratio of the elastic modulus to the viscous modulus (G’/G’’) and can be used to describe the properties of a sample.

Complex viscosity, η*, describes the flow resistance of the sample in the structured state, originating as viscous or elastic flow resistance to the oscillating movement. Higher the value of complex viscosity, greater is the resistance to flow in the structured state and vice versa (Schramm, 1994).
Concept of Linear Viscoelastic Region (LVR)

The region where the viscoelastic properties of the materials are independent of the imposed stress and strain is called as the linear viscoelastic region. It is a domain where the magnitudes of stress and strain are linearly related. This domain is known as the linear viscoelasticity. For instance, if the deformation is small, or applied sufficiently slowly, the molecular arrangements are never far from (dynamic) equilibrium. In such a case, the mechanical response is then just a reflection of dynamic processes at the molecular level which go on constantly, even for a system at equilibrium. It is important to make sure that all the experiments are performed within the range of the LVR or the linear viscoelastic region.

Controlled stress rheology provides two techniques for determining a polymer’s linear viscoelastic region. These are creep and dynamic oscillation. In creep, a constant stress is applied to the material and the resultant strain is monitored with time. By sequentially increasing the stress, a series of creep curves is obtained (Figure 2.1a). Replotting these curves as compliance, a viscoelastic function defined as strain divided by stress, yields the results as shown in Figure 2.1(b).

In the polymer’s linear viscoelastic region, the compliance curves should overlap. In case the curve produced does not overlap, it clearly signifies that the LVR has been exceeded. In the dynamic oscillation approach, increasing cyclic levels of stress and strain are applied at a constant frequency. The point at which a dynamic viscoelastic function (either $G^*$ or $\eta^*$) deviates by more than 10% from a constant (plateau) value indicates departure from linear viscoelastic behaviour. Figure 2.2(a) and (b) illustrates the oscillatory stress and strain results respectively. These figures display results for polystyrene as an example (TA Instruments, 2010; internet: http://www.tainst.com).
Figure 2.1 a) Creep Strain(%) Curves; Source: TA Instruments; Internet: http://www.tainst.com

Figure 2.1 b) Creep Compliance Curves; Source: TA Instruments; Internet: http://www.tainst.com.
Figure 2.2 a) $G^*$ and $\eta^*$ versus Shear Stress; Source: TA Instruments; Internet: http://www.tainst.com

Figure 2.2 b) $G^*$ and $\eta^*$ versus Shear Strain; Source: TA Instruments; Internet: http://www.tainst.com
Constitutive Relations

The fundamental relations between the stresses (forces) and deformation (shear strains and shear rates) in the materials are referred to as constitutive relations. These are obtained with the help of five different types of sweeps, namely:

*Dynamic Strain or Stress Sweep*

It is the materials response to increasing deformation amplitude (strain or stress). This is monitored at constant frequency and temperature and is useful in determining the LVR.

*Dynamic Time Sweep*

It is the materials response monitored at constant frequency, amplitude and temperature. This parameter is of quite significance for time dependent thixotropic food substances like yoghurt.

*Frequency Sweep*

It is the materials response to increasing frequency (rate of deformation) and is monitored at constant amplitude (strain or stress) and temperature.

*Dynamic Temperature Ramp*

It is the materials response to linear heating at constant frequency and constant amplitude of deformation. The data can be collected at user defined intervals.

Rheological characterization is typically carried out by measuring the storage ($G'$) and loss ($G''$) moduli as a function of temperature, time, frequency and strain using parallel plate geometry. Accordingly, experiments are termed as Temperature Ramps, Time Sweeps, Frequency Sweeps and Strain Sweeps.
Temperature Ramp

In cold setting gels, a cross over of $G'$ and $G''$ takes place at a particular temperature when the polymer solution is cooled at a predefined rate. At this critical point, $G'$ overtakes $G''$ marking the onset of sol-gel transition, commonly known as gelation. The ramp rate greatly influences the gelation profiles obtained through temperature sweep experiment. Such an experiment is carried out at a fixed frequency and amplitude of oscillation.

Time Sweep

A temperature ramp is followed by a time sweep wherein the values of both moduli increase as a function of time at a constant temperature for a certain period until equilibrium is more or less achieved. The extent of increase in the values of $G'$ is much higher than that of $G''$ which is negligible. In case of gels, a curing time is usually given during which the elastic or storage modulus keeps developing until a state of equilibrium is achieved. The time taken to reach this state varies from gel to gel. For instance, in agarose gels, the coil to helix transformation occurs very fast resembling a true first order phase transition. So, an extremely short curing time is sufficient for these gels. But in case of gelatin, an initial phase lasts for several hours followed by a much slower process that continues for a very long time. Therefore, depending on the concentration gelatin gels are subjected to much longer curing times before a pseudo-equilibrium state is achieved, as an absolute equilibrium state is difficult to achieve due to its dynamic nature.

The importance of a time sweep is thus displayed in the fact that it helps in determining if the properties of a system are changing during the time of testing. Such a time sweep is carried out by measuring material response at a fixed temperature, frequency and amplitude of oscillation.
**Frequency Sweep**

A frequency sweep monitors the material response to increasing frequency (rate of deformation) at constant amplitude (strain or stress) and temperature. Frequency is the time required to complete one oscillation. The frequency sweep data is of great help in determining to a large extent, the category under which a given sample can be classified viz. a dilute solution, an entangled solution, a weak gel or a strong gel. Derived parameters such as complex viscosity ($\eta^*$) and $\tan\delta$ provide useful information about the nature of the system being tested. In addition, data from frequency sweeps is also used in time-temperature superpositioning in order to gauge long term properties or extremely high / low frequencies beyond the scope of the instrument or reasonable experimental time.

**Strain Sweep**

A strain sweep helps determine the extent to which a sample undergoes deformation. For dynamic oscillation test measurements, it is vital that all the tests are carried out at amplitude within the linear viscoelastic region of sample. This is to verify that the results are ‘real’ and not merely artifacts. The principle behind this is that if the deformation is small or applied sufficiently slowly, the molecular arrangements are never far from equilibrium. The mechanical response is then just a reflection of dynamic processes at the molecular level which are ongoing even for a system at equilibrium. Within this domain of linear viscoelasticity, the magnitudes of stress and strain are related linearly, and the behaviour for any liquid is completely described by a single function of time. Thus, the material response to increasing amplitude at a constant frequency and temperature is monitored during a strain sweep. This is performed to determine the LVR as all tests are to be carried out at amplitude found within the linear viscoelastic region. Samples are assumed to be stable before carrying out a strain sweep. An unstable sample is subjected to a time sweep prior to the strain sweep,
to determine stability. It must be noted however, that the linear region changes as a function of frequency and temperature.

2.2 DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) is a thermal analysis technique which has already been used for several decades. It is applicable to a variety of materials including polymers, pharmaceuticals, foods and inorganics. DSC measurements provide qualitative and quantitative information as a function of time and temperature regarding transitions in materials that involve endothermic or exothermic processes, or changes in heat capacity (Gill, Sauerbrunn, & Reading, 1993). The ease of sample preparation, the applicability to both solids and liquids, fast analysis time and wide temperature range are some of the advantages contributing to the widespread usage of DSC. It is used to efficiently study the gel-sol transition, the denaturation of proteins, the gelatinization of starch and the state of water in gels (Nishinari, 1997). But there are some limitations associated with it, for instance, it is often not so easy to interpret the heat flow from a DSC experiment if multiple processes are involved over the same temperature range. If the material component is single, different types of transitions can overlap such as melting and recrystallisation in a semi-crystalline material and changes in heat capacity during the exothermic cure reaction of a thermoset (Coleman & Craig, 1996). In a multicomponent material, transitions of the different compounds can overlap. Also, it is not always easy to identify the nature of transition: an enthalpic relaxation peak superimposed on the heat capacity variation at the glass transition temperature can be so large that the transition is confused with a melting transition.

To increase the sensitivity (i.e. signal to noise) for the detection of a weak transition either the sample mass or the scan rate can be increased. For obtaining a better resolution in separating transitions occurring at close temperatures, either the smaller samples are used or
the scan rate is lowered. So increased sensitivity is always at the expense of the resolution an
vice versa (Verdonck, Schaap, & Thomas, 1999). In addition, the detection of weak transitions
is also strongly influenced by the baseline curvature and stability. It is much more difficult to
determine a small jump in the heat capacity when it is superimposed on a curved baseline as
compared to a straight baseline. Mostly, the baseline is not straight as a result of moisture
evaporation from the sample, variations in thermal contact between the sample and the DSC
pans during the scan and the overall baseline characteristics of the specific DSC cell.

There are some quantities such as the absolute value of a material’s heat capacity and its
thermal conductivity which require multiple experiments and cannot be determined
straightforwardly with DSC. The heat capacity cannot be determined in an isothermal
experiment with DSC. Modulated Temperature DSC (MTDSC) overcomes these limitations
and helps in providing new insight into the material’s properties. In this study, we focussed on
the operation and use of the TA instruments model, Q2000, modulated DSC.

Operating principle of MDSC

In DSC the difference in heat flow between a sample and an inert reference is measured as
a function of time and temperature as both the sample and reference are subjected to a
controlled environment (pressure, purge gas). The imposed temperature program is either
isothermal, or the temperature is varied linearly as a function of time. In MDSC, a sinusoidal
modulation is overlaid on the linear ramp as shown in Figure 2.3(a). This example is for a
heating experiment but is analogous for cooling and isothermal experiments. The heating rate
varies in a periodic or modulated fashion, as a result of the temperature modulation (Figure
2.3 b). The modulated heating rate varies between a minimum and a maximum value; these
are determined by the value of the underlying heating rate, the period (or frequency) and the
amplitude of the superimposed temperature wave. Depending on the combination of these
three parameters, the minimum modulated heating rate is positive (heat-only), zero (heat-
isothermal), or negative (heat-cool). The resultant heat flow between the sample and reference
in a DSC or MDSC experiment is described by the general equation:

\[ \frac{dQ}{dt} = C_P b + f(T,t) \]  \hspace{1cm} (2.1)

where \( \frac{dQ}{dt} \) is the resultant heat flow, \( C_P \) is the heat capacity of the sample, \( b \) is the rate of
temperature change (\( \frac{dT}{dt} \)) and \( f(T,t) \) is the heat flow from kinetic processes. For an MDSC
experiment, the resultant heat flow is periodically varying and termed the modulated heat
flow.

Figure 2.3 a) Temperature as a function of time for a typical DSC and MDSC experiment
(Verdonck, et al., 1999).
From the general equation, it follows that the resultant heat flow is composed of two components: one component is a function of the sample’s heat and rate of temperature change, and the other is a function of absolute temperature and time. In conventional DSC only the sum of the two components is determined and is called the total heat flow. In MDSC the total heat flow and the two individual components can be distinguished as the heat capacity component ($C_p b$) or reversing heat flow and the kinetic component ($f(T, t)$) or non-reversing heat flow.

All these signals are calculated from three measured signals: time, modulated temperature, and modulated heat flow. The total heat flow in MDSC is calculated from the average of the modulated heat flow; this average corresponds to the total heat flow in a conventional DSC experiment at the same underlying heating rate. In conventional DSC, $C_p$
can be calculated from the difference in heat flow between two runs on an identical sample at two different heating rates. In MDSC, it is possible to determine $C_P$ in a single experiment because of the periodical variation in the heat rate: the heat capacity is calculated from the ratio of the modulated heat flow amplitude and the modulated heating rate amplitude by discrete Fourier transformation. The reversing heat flow is then calculated by multiplication of $C_P$ with the negative heating rate $b$. The kinetic component or non-reversing heat flow is the arithmetic difference between the total heat flow and the reversing heat flow.

2.3 CONFOCAL MICROSCOPY

Confocal microscopy is an optical imaging technique used to increase optical resolution and contrast of a micrograph by using point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. It enables the reconstruction of three-dimensional structures from the obtained images. This technique has gained popularity in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and material science.

According to Clarke, “Confocal microscopy is a non-destructive sectioning technique, which enables the 3D reconstruction of material surfaces (surface topological studies) or the internal structure of semi-transparent specimens (sub-surface bulk studies). These spatial reconstructions by confocal microscopy entail either high spatial resolution (± 0.2 µm) studies of regions within 5µm to 200µm of the specimen’s surface (dependent on the opacity and heterogeneity of the sample) or, using air objective lenses, correspondingly lower resolution studies of regions within accessible depths of many millimetres. These upper limits to performance are determined essentially by the working depth of the high numerical aperture (NA) objective lens selected for the particular research study” (Clarke, 1980).
In 1957, Marvin Minsky, while working at Harvard University, filed his patent for a ‘double-focussing stage scanning microscope’. In doing so, he set the scene for the development of the confocal microscopes capable of 3D object reconstructions by optical sectioning, which are available today. Figure 2.4 displays Minsky’s original ‘double-focussing’ design where light, emitted by a lamp, passes through a pinhole and is focussed onto the sample by a condenser lens. Light transmitted through the sample is focussed by an objective lens, through a second pinhole, onto a detector. The objective and condenser are confocally (having the same point of focus) aligned, hence the origin of the name ‘confocal microscopy’. By simple ray tracing, it can be seen that light from any region, other than the focal point of both lenses within the sample, is rejected. A confocal microscope based on this design enables the formation of an image from a prescribed section of a thick translucent object, without the presence of out-of-focus information from the surrounding planes. Therefore, confocal images are frequently described as optical sections, enabling rapid 3D reconstruction without the need to physically section the sample.

Figure 2.4 Marvin Minsky’s original confocal designs in transmitted light (Clarke, 1980).

where
A – first pinhole
C – condenser lens
S – sample
O – objective lens
B – second pinhole
Although, Minsky referred to a bright white light source in his original patent, the laser is now the universal light source employed within these confocal systems. The latest version of confocal fixed stage system introduced by Leica Microsystems has been discussed ahead (in the equipments section).

### 2.4 MATERIALS AND EQUIPMENTS USED

**Materials**

All the materials used for this project along with their Certificate of Analysis and the product specifications are clearly mentioned.

**2.4.1 Gelatin**

The gelatin powder was supplied by Sigma Aldrich (G2500). It forms a strong gel at 7% concentration with bloom strength ~ 300 Bloom. The protein concentration of the sample is 81% and is faintly yellow in color.

Table 2.2 Certificate of Analysis of Gelatin:

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Gelatin from porcine skin, Type A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Number</td>
<td>G2500</td>
</tr>
<tr>
<td>Product Brand</td>
<td>SIGMA</td>
</tr>
<tr>
<td>CAS Number</td>
<td>9000 - 70 - 8</td>
</tr>
<tr>
<td>TEST</td>
<td>LOT 036K0144</td>
</tr>
<tr>
<td>Appearance (Color)</td>
<td>Off - White</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Appearance (Form)</td>
<td>Powder</td>
</tr>
<tr>
<td>Solubility (Color)</td>
<td>Faint Yellow</td>
</tr>
<tr>
<td>Solubility (Turbidity)</td>
<td>Hazy</td>
</tr>
<tr>
<td>% Protein (Biuret)</td>
<td>81</td>
</tr>
<tr>
<td>Gel Strength (Bloom No.)</td>
<td>306</td>
</tr>
<tr>
<td>Date of QC Release</td>
<td>April 2006</td>
</tr>
<tr>
<td>Recommended Retest Date</td>
<td>April 2011</td>
</tr>
</tbody>
</table>

### 2.4.2 Agarose

The agarose powder was supplied by Sigma Aldrich (A0576). It forms a strong gel at 1.5%. The content of water, ash and sulphate according to the supplier was 2.94, 0.10 and 0.06%, respectively.

#### Table 2.3 Certificate of Analysis of Agarose:

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Agarose, Type I-B, Low EEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Number</td>
<td>A0576</td>
</tr>
<tr>
<td>Product Brand</td>
<td>SIAL</td>
</tr>
<tr>
<td>CAS Number</td>
<td>9012 - 36 - 6</td>
</tr>
<tr>
<td>TEST</td>
<td>LOT 112K1630</td>
</tr>
<tr>
<td>Appearance (Color)</td>
<td>Off-White Powder</td>
</tr>
<tr>
<td>Appearance (Form)</td>
<td>Powder</td>
</tr>
<tr>
<td>Solubility (Turbidity)</td>
<td>Solution at 1.5 GM Plus 100 ML of Water</td>
</tr>
<tr>
<td>Moisture Content</td>
<td>2.94%</td>
</tr>
<tr>
<td>pH Test (1.5% Gel)</td>
<td>6.78</td>
</tr>
<tr>
<td>Residue on Ignition</td>
<td>0.10%</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Sulfate</td>
<td>0.06%</td>
</tr>
<tr>
<td>Gel Strength (1.5% Gel)</td>
<td>3500 G/CM2</td>
</tr>
<tr>
<td>Gel Point (1.5% Gel)</td>
<td>36.5 DEG C</td>
</tr>
<tr>
<td>Melting Temperature</td>
<td>89.3 DEG C</td>
</tr>
<tr>
<td>Turbidity (1.5% Gel)</td>
<td>19 NP Coleman</td>
</tr>
<tr>
<td>Electroendosmosis</td>
<td>0.11</td>
</tr>
</tbody>
</table>

### 2.4.3 Whey Protein Isolate

WPI was supplied by Fonterra Australia Pty. Ltd. It contains 88.71% proteins, 0.93% fat and has a moisture content of 4.83%. The ash content and pH according to the supplier was 3.3% and 6.9 respectively.

**Table 2.4 Certificate of Analysis of WPI:**

<table>
<thead>
<tr>
<th>Product Description</th>
<th>Whey Protein Isolate, Insanitized 894</th>
</tr>
</thead>
<tbody>
<tr>
<td>Customer</td>
<td>Fonterra Australia Pty. Ltd</td>
</tr>
<tr>
<td>COA ID</td>
<td>8944-500-02</td>
</tr>
<tr>
<td>COA Number</td>
<td>81571201-2</td>
</tr>
<tr>
<td>Batch/Cypher</td>
<td>GT20</td>
</tr>
<tr>
<td>Manufacture Date</td>
<td>20 February, 2009</td>
</tr>
<tr>
<td>Protein (6.38xN) as is</td>
<td>88.71 % m/m</td>
</tr>
<tr>
<td>Fat</td>
<td>0.93 % m/m</td>
</tr>
<tr>
<td>Moisture</td>
<td>4.83 % m/m</td>
</tr>
<tr>
<td>Ash</td>
<td>3.3% m/m</td>
</tr>
<tr>
<td>pH</td>
<td>6.9</td>
</tr>
<tr>
<td>Product</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Lactose Monohydrate</td>
<td>0.8% m/m</td>
</tr>
<tr>
<td>Bulk Density</td>
<td>0.33 g/ml</td>
</tr>
<tr>
<td>Flavour DFC</td>
<td>Typical</td>
</tr>
<tr>
<td>Aerobic Plate Count</td>
<td>1200 cfu/g</td>
</tr>
<tr>
<td>Coliforms</td>
<td>&lt;1 cfu/g</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Not detected</td>
</tr>
<tr>
<td>Yeasts and Moulds</td>
<td>&lt;1 cfu/g</td>
</tr>
<tr>
<td>Coag Positive Staphylococci</td>
<td>Not detected</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

2.4.4 Glucose Syrup

Glucose syrup was supplied by Manildra Group (Batch No. 27/07/08). It contains 81.2 g total solids per 100 grams and has a Dextrose Equivalent of 43.4. Its sulphur dioxide content is 102 mg/kg. The supplier clearly mentions that the product does not contain any Genetically Modified ingredients and is gluten free though it is wheat derivative.

Table 2.5 Certificate of Analysis of Glucose Syrup:

<table>
<thead>
<tr>
<th>Product Name</th>
<th>43BE Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Number</td>
<td>N27/07/08</td>
</tr>
<tr>
<td>Product Brand</td>
<td>MANILDRA GROUP</td>
</tr>
<tr>
<td>TEST</td>
<td>T21438</td>
</tr>
<tr>
<td>Appearance (Colour)</td>
<td>Transparent</td>
</tr>
<tr>
<td>Appearance (Form)</td>
<td>Syrup</td>
</tr>
<tr>
<td>Total Solids g/100g</td>
<td>81.2</td>
</tr>
<tr>
<td>D.E</td>
<td>43.4</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>pH (10% Dist. Water)</td>
<td>5.2</td>
</tr>
<tr>
<td>Sulphur dioxide mg/kg</td>
<td>102</td>
</tr>
<tr>
<td>Certificate of Cleanliness</td>
<td>58031</td>
</tr>
</tbody>
</table>

2.4.5 Calcium Chloride

Calcium Chloride was supplied by Sigma Aldrich (C1016). It is available as white granules with a molecular weight of 110.98 g/mole. It forms a clear to hazy colourless solution at 100mg/ml in water, which becomes clear to very slightly hazy after addition of 1 drop of Hydrochloric Acid.

**Table 2.6 Certificate of Analysis of Calcium Chloride:**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Calcium Chloride, &gt;93.0%, anhydrous, granular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Number</td>
<td>C1016</td>
</tr>
<tr>
<td>Product Brand</td>
<td>SIAL</td>
</tr>
<tr>
<td>CAS Number</td>
<td>10043-52-4</td>
</tr>
<tr>
<td>Molecular Formula</td>
<td>CaCl$_2$</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>110.98</td>
</tr>
<tr>
<td>TEST</td>
<td>LOT 121K0128</td>
</tr>
<tr>
<td>Appearance (Colour)</td>
<td>White</td>
</tr>
<tr>
<td>Appearance (Form)</td>
<td>Granules</td>
</tr>
<tr>
<td>Solubility (Colour)</td>
<td>Clear to Hazy Colourless Solution at 100mg/ml in Water</td>
</tr>
<tr>
<td>Purity By EDTA Titration</td>
<td>95.6% (supplier information)</td>
</tr>
</tbody>
</table>
Equipments

2.4.6 AR-G2 Advanced Rheology Generation 2 Rheometer

AR-G2 is the latest rheometer and the first of its kinds to have a patent pending magnetic thrust bearing technology for ultra-low nano-torque control. It is the most advanced controlled stress, direct strain and controlled rate rheometer available. The instrument at RMIT is also fitted with the environmental test chamber which is designed to use controlled convection/radiant heating concept. The best for polymer science and is fitted with parallel peltier plates (TA Instruments, 2009).

Figure 2.5 Advanced Rheology Generation 2 Rheometer.

Source: TA Instruments (www.tainstruments.com).
Samples prepared freshly were loaded on the pre-heated plate of the rheometer, the exposed edges of the sample, after the geometry is in contact with the sample, were covered with dimethyl polysiloxane oil to avoid moisture loss.

2.4.7 Q2000 Differential Scanning Calorimeter

The Q2000 Differential Scanning Calorimeter is TA instrument’s top-of-the-line, research graded DSC, with unmatched performance in baseline stability, sensitivity and resolution. It has the most powerful DSC technology commercially available called the Advanced Tzero technology. The reasons for the modulated DSC to lead the industry are the 50-position intelligent autosampler and digital mass flow controllers. This instrument has a patented technology known as the Tzero technology only available from the TA instruments (TA instruments, 2009).

Figure 2.6 Differential Scanning Calorimeter.

Source: TA Instruments (www.tainstruments.com).
Samples of 7-10 mg were weighed approximately and sealed in hermetic aluminium pans. Hermetic sealing is important to avoid the leakage of water or other volatile ingredients, thermal analysis was made between 0 – 90°C at a scanning rate of 2°C/min.

2.4.8 Leica DM6000 CFS Confocal Fixed Stage Microscope

Leica Microsystems sets a new standard with the integration of the Leica DM6000 CFS fixed stage microscope into the Leica TCS SP5 confocal platform. There are various beneficial features to its credit. To illustrate, searching for the perfect spot in the sample requires a large field of view, whilst the precise positioning of the micropipettes requires high magnification. Furthermore, high quality confocal image scanning requires a large numerical aperture for best performance. To fulfill these experimental pre-requisites, a camera with an adjustable magnification and specially adapted objectives are needed.

Any direct manipulation of the system potentially disturbs the delicate positioning of the micropipettes within the sample. This emphasizes the importance of a remote control for the imaging set up, providing convenient access to all relevant functions. The Leica TCS SP5 with the integrated fixed stage microscope Leica DM6000 CFS is fully equipped with the above mentioned features and gives excellent experimental results under all conditions.
Confocal Laser Scanning Microscopy was used to observe the whey protein aggregates. Native whey protein isolate was dyed using rhodamine dye and heated to a temperature of 80°C on a peltier platform to induce aggregation. An emission band width of 553-619 nm was used which is appropriate for rhodamine dye. Objective lens of dimension N PLAN L 20.0 X 0.40 DRY was employed. Scanning frequency of 400 Hz was applied to capture the images.

2.5 GEL PREPARATION

Pure agarose and gelatin, were dissolved in distilled water at 90°C and 65°C respectively till clear solutions were obtained. Solutions of varying concentrations were made to obtain
individual calibration curves. Individual gel of agarose was made at 1.5% which was brittle and prone to synersis. The gelatin gel displayed its typical elastic character at a concentration of 7%. Both were combined to form a binary mixture by maintaining agarose at 1.5% and gelatin at 7%. To achieve this, agarose was first dissolved at 90°C and then the solution was cooled down to about 65°C before adding gelatin. After the added gelatin dissolved completely, the required final composition was achieved by adjusting the proportion of water. The speed of magnetic stirrer was maintained at approximately 300 rpm.

Tertiary mixtures were made by maintaining the concentration of agarose at 1.5%, gelatin at 7% and varying the sugar concentration from 10-70%, for rheology experiments. But for calorimetric experiments the concentration of both agarose and gelatin was doubled to 3% and 15% respectively. Glucose syrup concentration again varied from 10-70%. Binary mixtures were prepared as discussed above. However, before adding the sugar phase, the temperature of the binary mixture was further brought down to 60°C and maintained thereafter. Appropriate amount of the sugar was added into the mixture at this temperature. The stirring speed of the magnetic stirrer was carefully monitored in order to obtained a homogenised tertiary mixture. The freshly prepared sample was loaded onto the rheometer for mechanical analysis. For DSC experiments, the prepared sample was kept overnight at 4°C, following which thin and tiny slices of gel were meticulously cut and hermetically sealed for calorimetric analysis.

In case of whey proteins, the gels formed are flimsy in nature as compared to the agarose-gelatin gels which are brittle and elastic. Gels were made at near-neutral pH, the whey protein isolate or WPI concentration was kept constant at 15% and calcium chloride concentration of 10 mM was used. However, the sugar phase was systematically varied using concentrations from10-70% of glucose syrup for the binary mixture. The single system comprising of 15% WPI and 10mM CaCl2 was prepared at 25°C. Preparation of binary mixtures was done by adding 10mM CaCl2 solution to the given concentration of glucose syrup, followed by 15%
whey protein isolate. The mixing of the whole system was carried out at normal room temperature of 25°C for nearly 4 hours. The speed of the magnetic stirrer was kept from low to medium. Once proper mixing with gradual stirring was achieved, the given sample was used for analysis on both AR-G2, the rheometer and Q2000, the differential scanning calorimeter.

2.6 MEASUREMENT TECHNIQUES

Small and large deformation measurements were carried out on an advanced rheology generation two (AR G2, TA instruments) using parallel plate geometry (40 mm diameter; 1000 microns gap). Biopolymer solutions of desired compositions were prepared, loaded at 70°C and allowed to gel in situ on the rheometer. Measurements of storage (G’) and loss (G’’) moduli as a function of temperature, time, frequency and strain were made. Initial gelation was allowed to occur by cooling the solutions at a ramp rate of 1°C/min and frequency of 1 rad/sec to a final temperature of 0°C in order to observe effects of the soft or hard filler respectively. The level of strain maintained was 0.1% for both agarose and gelatin. The development of moduli was subsequently, monitored as a function of time. At equilibrium, i.e. when the experimental moduli had not increased more than 0.5% in a 15 min time interval; the samples were subjected to an increasing frequency of oscillation (0.1-100 rad/sec) at a fixed amplitude (0.1%). Alternatively, in order to determine the linear viscoelastic region (LVR) of the samples, strain sweeps were conducted by increasing the amplitude of oscillation at a fixed frequency (1 rad/sec). It was ensured that all measurements were carried out at levels of strain well within the LVR. Time Sweep of 60 minutes was conducted for all samples, in order to maintain uniformity. This led to frequency sweep at 0°C being followed by a second temperature ramp from 0°C to 90°C at 1°C/min, again at a fixed amplitude and frequency.
For Differential Scanning Calorimetry (DSC), gels were prepared as described earlier and gelation was witnessed by allowing the solutions to cool at room temperature. Later, they were stored at 4°C for a period of 12 hours. Thin gel pieces were then used in the following protocols.

DSC measurements were performed on MDSC (Q2000, TA instruments). The instrument used a refrigerated cooling system to achieve temperatures down to -90°C and a nitrogen DSC purge gas flow at 25ml/min. Samples were loaded and sealed in hermetic aluminium pans. The DSC heat flow was calibrated using a traceable indium standard and the heat capacity response using a sapphire standard. Size of samples ranged between 7-10mg. Samples of agarose, gelatin or mixtures of the same along with glucose syrup were equilibrated at 90°C to eliminate erroneous phenomena due to thermal history during sample preparation and loading. The pans were then cooled to 0°C and reheated to 97°C at a scan rate of 2°C/min. Samples were subjected to modulate at +0.530°C temperature at every 40 seconds. The reference was an empty hermetically sealed aluminium DSC pan.

Whey Protein single systems and binary mixtures with glucose syrup of required concentrations were prepared and loaded on the rheometer at a temperature of 40°C. Heating at a ramp rate of 1°C/min was carried out till 80°C, followed by a time sweep of 20 min duration at the same temperature. After this step, cooling was performed at a ramp rate of 1°C/min and a frequency of 1rad/sec till a final temperature of 5°C was reached. A strain of 0.1% was maintained throughout the analysis. Frequency sweep (0.1-100 rad/sec) was executed at 5°C in order to determine the timescale dependence of the sample to control deformation of 0.1% strain.

For DSC analysis the whey protein samples were loaded after overnight refrigeration at 4°C. These samples were equilibrated at 40°C and then heated to 90°C at a scan rate of 2°C/min. Following which the pans containing samples were cooled to 0°C at a scan rate of 2°C/min. As mentioned previously, the samples were subjected to modulate at +0.530°C
temperature at every 40 seconds. The reference being an empty hermetically sealed aluminium DSC pan.

Confocal microscopy has emerged out to be a powerful technique for material scientists being a non destructive sectioning technique enabling a 3D reconstruction of surfaces or the internal structure of semitransparent surfaces. Elimination of the out of focus light in specimens by point illumination and spatial pinhole results in better optical resolution and contrast. This modern version of the fluorescence microscopy facilitates to characterise the surface topology of hydrogels and polymeric structures within a range of 5 µm to 200µm which is relevant for our investigation.

Leica TCS SP5 was used to conduct studies on the whey protein isolate system. 3D structural characterisation of the whey protein aggregates in different concentrations of the glucose syrup was made possible by the XYZ – Z stack operational mode of the microscope. Rhodamine 6G dye was used to stain the sample. Rhodamine C_{27}H_{29}ClN_{2}O_{3} is normally used because of its high photostability and quantum yield. The lasing range of rhodamine is between 555 - 585nm and the compromised range in consideration of the laser beam for this research work falls within the range of 553 – 619nm. An independent peltier base was attached replacing the conventional specimen and slide holder which has a temperature range of -90 °C to 600 °C. Sample for analysis with the rhodamine dye was placed on the cavity slides under glass cover slips with waxed edges to minimize the moisture loss at high temperature. The peltier base was then heated to 80°C to denature the protein molecule resulting in its aggregation.
Chapter 3: THERMOMECHANICAL STUDY ON THE PHASE
BEHAVIOUR OF AGAROSE/GELATIN MIXTURES IN THE
PRESENCE OF GLUCOSE SYRUP AS THE CO-SOLUTE

3.1 ABSTRACT

In an effort to draw attention to the subject of structure/function relationships in high-solid biopolymer mixtures, this investigation produces binary composites of agarose with gelatin and increases systematically the amount of glucose syrup, which is the co-solute in this system. Experimental work was carried out using small-deformation dynamic oscillation on shear and modulated differential scanning calorimetry. Agarose/gelatin mixtures in an aqueous low-solid environment form non-interactive bicontinuous networks. Addition of glucose syrup to the polymeric blend prevents the formation of stable double helices in the agarose network, which is increasingly “dissolved” in the high-solvent environment. Gelatin, on the other hand, withstands better the co-solute induced change in solvent quality. At subzero temperatures, materials go through the rubber-to-glass transition whose DSC glass transition temperature \( T_g \) is governed by the total level of solids in the system. Estimation of the mechanical \( T_g \) acquires physical significance by utilising the theory of free volume as modelled by the Williams, Landel and Ferry (WLF) equation. The single value of \( T_g \) estimated by this approach argues in accordance with experimental observations for the predominance of the gelatin network in the high-solid mixture.

Keywords: agarose, gelatin, glucose syrup, rheology, calorimetry, microscopy
3.2 INTRODUCTION

Creative use of functional ingredients such as proteins and polysaccharides in processed foods requires an understanding of structure-function relationships of individual components and molecular interactions in their mixtures (van de Velde, Weinbreck, Edelman, van der Linden, & Tromp, 2003). Such an undertaking leads to the development of single-component gels followed by the formulation of complex binary blends and ternary systems. For example, calcium-polymer complexes impact dramatically on the viscoelasticity of high-guluronate alginate or low-methoxy pectin gels and their interactions in mixture with other polyelectrolytes (Fang, et al., 2008). Broadly speaking, three distinct topologies of coupled, interpenetrating and phase separated networks are formed upon mixing two biopolymers to form a binary system (de Jong & van de Velde, 2007). We have found that the phenomenon of phase separation can be manipulated to create acceptable consistency in products with spreadable texture (Kasapis, 2000).

Good understanding of the thermodynamic principles that govern phase separation phenomena in low-solid aqueous systems, e.g. at 5 or 10% total solids has been achieved. Thus, when small molecules such as water and ethanol are mixed together, the natural tendency is to attain a high degree of disorder. This is physically possible since the molecules are small and can move freely. Hence the entropy of mixing is the important thermodynamic factor. In the case of biopolymer mixtures, however, the chains are obviously more restricted and cannot move so freely. Hence ‘disorder’ or entropy of mixing become much less important. The interaction between different biopolymer chains, i.e. the enthalpic contribution, thus becomes the dominant factor, which determines the free energy of mixing (Antoniou, Buitrago, Tsianou, & Alexandridis, 2010).

When interactions between the two polymers are thermodynamically unfavourable, the biopolymers are said to be ‘thermodynamically incompatible’. These types of interactions are
referred to as ‘segregative’ (Harrington, Foegeding, Mulvihill, & Morris, 2009). At low concentrations this can result in a single gelled phase, accompanied by an increase in the rate of gelation. However, if the concentration of the individual polymers is high enough (as is, for example, in low fat products), eventually two different phases may separate, where each phase is enriched in one polymer and depleted in the other. In the case of protein-polysaccharide-water systems (PPW), phase separation generally occurs when the total concentration is greater than 4%, although variations can be found from system to system (Fitzsimons, Mulvihill, & Morris, 2008).

Despite the extensive research in low-solid PPW systems, there is scant information in the literature on the structural properties of high-solid counterparts (> 60%) where part of the water is replaced with small-molecule co-solutes. Work on single polysaccharide/co-solute preparations with industrial relevance, i.e., up to 2% macromolecule plus up to 90% sugar solution, demonstrated the formation of materials with rubbery consistency, which upon cooling transform into a clear glass (Tsoga, Kasapis, & Richardson, 1999). Such transformation is observed both with small-deformation mechanical measurements and modulated differential scanning calorimetry at a range of cooling rates. Thermomechanical profiles allow pinpointing of the glass transition temperature ($T_g$) either as an empirical indicator of convenience or as the outcome of theoretical modeling. The value of $T_g$ can then be utilised as an index of quality control in state diagrams of confections, dried fruit leathers, various commodity products and in conjunction with predictions of the second quality-control concept of water activity (Kasapis, 2004). This communication attempts to advance the current state of affairs by preparing binary mixtures of agarose/gelatin and adding increasing amounts of glucose syrup thus observing deviations from the structural properties of the low-solid systems.
3.3 EXPERIMENTAL SECTION

3.3.1 Materials

The agarose sample was supplied by Sigma. It is a material of high gel-strength, which achieves storage modulus values of $2.8 \times 10^4$ Pa at $5^\circ$C (1.5% gel in Figure 3.1a). According to the supplier, water, ash and sulfate contents were of less than 7, 0.25 and 0.12%, respectively. Gelatin was a high-quality first extract from pigskin with a mass average molecular weight of 162,400 (as provided by Sigma) and a Bloom value of 305 g. Storage modulus values at $5^\circ$C for the 7% gel was $0.9 \times 10^4$ Pa in Figure 3.2a. The glucose syrup used was a Cerestar product. The dextrose equivalent of the sample is $\approx 42$. The total level of solids is 82% and glucose syrup compositions in this work refer to dry solids. Gel permeation chromatography established the polydisperse nature of glucose syrup, which upon cooling transforms from a viscous solution to a clear glass (Shrinivas & Kasapis, 2010).

3.3.2 Methods

**Sample Preparation:** The polysaccharide solution was prepared by dissolving at 90°C with stirring for 20 min and then dropping the temperature to 70°C for mixing with gelatin and various concentrations of glucose syrup. The hydration temperature of the protein did not exceed 70°C and low-solid solutions were readily prepared with gentle stirring within 15 min. Besides the single systems, binary mixtures of agarose with gelatin were also made in the presence of glucose syrup at the required level of solids ready for analysis.

**Rheology:** Low amplitude oscillatory measurements of the real ($G'$; storage modulus) and imaginary ($G''$; loss modulus) parts of the complex shear modulus ($G^* = G' + iG''$), and tan δ...
($G''/G'$) were performed with the AR-G2, which is a controlled strain rheometer with a magnetic thrust bearing technology (TA Instruments, New Castle, DE). Low-solid samples were loaded onto the pre-heated plate of the rheometer (70°C), their exposed edges covered with a silicone fluid obtained from BDH chemicals (50 cS) to minimize water loss and cooled to 5°C at a rate of 1°C/min. This was followed by a 60-min isothermal run, frequency sweep from 0.1 to 100 rad/s at 5°C and a heating scan at 1°C/min to 90°C.

In high-solid samples, the experimental temperature range was extended from 60 to −50°C thus accessing molecular motions that cover the glassy state, the softening dispersion (glass transition region), ‘rubbery plateau’ and the flow region. Scan rate for cooling runs was 1°C/min, the frequency of oscillation for each isothermal profile was between 0.1 and 100 rad/s, the applied strain was 0.01% and the plate size for the parallel-plate measuring geometry was either 40 or 20 mm.

**Differential Scanning Calorimetry:** Measurements were performed on MDSC Q2000 with autosampler (TA Instruments Ltd). The instrument used a refrigerated cooling system to achieve temperatures down to -90°C and a nitrogen DSC cell purge at 25 ml/min. Hermetic aluminium pans were used. The DSC heat flow was calibrated using a traceable indium standard ($\Delta H_f = 28.3 \text{ J g}^{-1}$) and the heat capacity response using a sapphire standard. At the beginning of each experiment, gelatin was heated to 70°C and agarose to 90°C to eliminate erroneous phenomena due to thermal history during sample preparation and loading. Then samples were cooled to 0°C (low solids) or -90°C (high solids) and heated to 90°C at 2°C/min to enhance resolution of molecular thermal events. Samples of 7 to 10 mg were analysed at ±0.53°C temperature amplitude of modulation and 40 s period of modulation. The reference was an empty hermetically sealed aluminium DSC pan. Three runs were recorded and the average of essentially overlapping traces is reported.
3.4 RESULTS AND DISCUSSION

3.4.1 Co-solute Induced Structural Transformation in Agarose and Gelatin Networks.

Understanding the nature of agarose and gelatin networks in an aqueous environment is a prerequisite to advancing a study on the effect of increasing the concentration of co-solute in these systems. Agarose is a linear copolymer of alternating (1→3)-β-D galactopyranose and (1→4)-3,6 anhydro-α-L-galactopyranose repeat units whose hydration unleashes fluctuating, disordered coils (L. Barrangou, Daubert, & Allen Foegeding, 2006). Figure 3.1 (a – b) reproduces the cooling and heating profiles of the storage modulus obtained at a controlled scan rate (1°C/min) for 1.5% agarose preparations under changing conditions of solvent quality. In the presence of water molecules, agarose chains undergo a conformational transition at about 35°C and create a rigid gel at the end of the cooling routine (2.8 x 10⁴ Pa at 5°C; Figure 3.1a). This was followed by an isothermal run for 60 min and a frequency sweep between 0.1 and 100 rad/s at the same temperature (5°C; data not shown here).

Mechanical spectra at the end of the isothermal run generate a flat frequency response for both storage and loss modulus with a low tan δ value \( (G''/G') \sim 0.025 \), which is typical of hydrogels (Foegeding, 2007). Subsequent heating of the aqueous agarose gel in Figure 3.1b unveils substantial thermal hysteresis with the network melting at a temperature just below the boiling point (Figure 3.1b). The mechanical evidence from this study is congruent with the formation of an enthalpic network where the structural knots are highly aggregated intermolecular associations. On the basis of X-ray fibre diffraction and optical rotation evidence, it has further been postulated that an agarose aggregate is composed of a plethora of co-axial double helices (Matsuo, Tanaka, & Ma, 2002).
Addition of glucose syrup to the agarose gels produces a systematic variation in the viscoelasticity of the cooling profiles (Figure 3.1a). As the amount of co-solute increases to 20 and 40% in preparations, the values of storage modulus also increase with the levelled $G'$ traces approaching a constant value. However, the values of $G'$ traces reach a maximum point and start declining at 60 and 70% co-solute. At 70% addition, for example, the sharp gelling process is no longer evident and instead traces are displaced to lower temperatures. Furthermore, traces maintain considerable temperature dependence towards the end of the cooling routine and finish off well below the values of $G'$ recorded for the aqueous system.
Within the time constraints of this experimental protocol, storage modulus values of the 1.5% agarose plus 70% glucose syrup gel do not recover sufficiently and remain low during heating until all data converge at near melting temperatures (Figure 3.1b).

![Figure 3.1 b) Heating profiles of storage modulus for 1.5% agarose with 0 (▲), 20 (■), 40 (●), 60 (◇) and 70% (□) glucose syrup (scan rate: 1°C/min; frequency: 1 rad/s; strain: 0.1%).](image)

The rheological approach was extended to the second polymeric component of this investigation, and Figure 3.2 (a – b) illustrates thermal profiles of the protein in water and water/co-solute environments. As for agarose, there is an extensive literature on the cold setting properties of aqueous gelatin preparations whose gelation involves the formation of a
triple helix, consisting of three left-handed helices (α-chains) wound around each other into a right-handed super-helix (Hawkins, Lawrence, Williams, & Williams, 2008). In the 7% gelatin preparation, a sharp sol-gel transition commences at about 25°C (for a scan rate of 1°C/min), which levels off towards the end of the cooling run (Figure 3.2a). In the presence of 20 and 40% glucose syrup, the temperature at the onset of the gelation process shifts to 27 and 30°C, respectively, and this is accompanied by higher $G'$ values than the aqueous gelatin network.

Figure 3.2 a) Cooling profiles of storage modulus for 7% gelatin with 0 (●), 20 (■), 40 (▲) and 70% (○) glucose syrup (scan rate: 1°C/min; frequency: 1 rad/s; strain: 0.1%).

In contrast with the observations at low and intermediate levels of co-solute in the preceding paragraph, the nature of modulus development and its thermal stability is entirely
different at high levels of solids. Thus there is a jump in thermal stability to about 50°C for the onset of gelation in samples with 7% gelatin and 70% glucose syrup, which is accompanied by a gradual structure development upon cooling. Heating of the high-solid material in Figure 3.2(b) follows back the trace of its cooling counterpart thus producing minimal thermal hysteresis. By comparison, thermal hysteresis is more pronounced in samples with lower levels of solids and in the case of 20% added glucose syrup it is about seven degrees centigrade.

Figure 3.2 b) Heating profiles of storage modulus for 7% gelatin with 0 (●), 20 (■), 40 (▲) and 70% (○) glucose syrup (scan rate: 1°C/min; frequency: 1 rad/s; strain: 0.1%).
3.4.2 Structural Properties of Agarose/Gelatin Mixtures at Low and High Levels of Glucose Syrup.

Work of the preceding section on single protein and polysaccharide preparations serves as a reference for characterisation of the phase behaviour in mixtures of the two polymers. Standard or modulated differential scanning calorimetry can be used to accurately measure the temperature and heat flow of molecular events thus providing complementary information to that obtained by rheological means (Rahman, Al-Saidi, & Guizani, 2008). Mixtures with a wide range of polymer, co-solute and total solids were prepared for thermal analysis, and Figure 3.3 (a – b) depicts thermograms of typical examples scanned at 2°C/min.

Cooling exotherms of gelatin and agarose are particularly helpful to determine the nature of physical interactions in their mixture, as shown in Figure 3.3(a). This reveals well-defined exothermic events, but with considerable variation in size, shape and temperature range as a function of increasing level of glucose syrup (up to 60%). Relatively sharp peaks with maximum heat flow temperature ($T_{max}$) of 37 and 21°C denote the co-operative conformational transition of agarose and gelatin segments, respectively. Results are congruent with mechanical evidence from the cooling profiles in Figures 3.1(a) and 3.2(a), which also indicate an early onset of structure development in polysaccharide networks. It can thus be argued that networks are formed independently in the absence of direct and heterotypic interactions between the two polymeric components.
Inclusion of 20% glucose syrup in the binary mixture maintains the two thermal events whose $T_{max}$, however, shift to higher temperatures hence arguing for increased thermal stability of networks in the presence of co-solute. The area under the peak of the DSC scan provides an estimate of the enthalpy change of the gelation process (or coil-to-helix transition to be more precise) in biopolymers. Examination of the four thermograms in Figure 3.3(a) argues that gradual addition of co-solute reduces dramatically and severely disrupts aggregation of individual agarose helices. Consequently, the exothermic peak of the polysaccharide diminishes and becomes undetectable within the constraints of the current
experimental routine at 60% co-solute in the mixture. The gelatin peak withstands the extensive change in solvent quality but it becomes quite broad and forms a tail that should be attributed to molecular polydispersity and a range of relaxation processes induced at the high-solid regime.

Addition of high levels of co-solute prevents ice formation at subzero temperatures thus allowing monitoring of the glass transition temperature in supersaturated materials (Han & Tan, 2009). Direct measurements of heat capacity were performed on the gelatin/agarose/glucose syrup mixture at a total-solid content of 75 and 80%. As shown in Figure 3.3(b), the heat capacity increases (absolute numbers) during heating driven by the generation of excess volume due to thermal motion and molecular mobility.

![Figure 3.3(b)](image)

**Figure 3.3 b)** DSC exotherms for the mixture of 3.0% agarose plus 15% gelatin with 57 (- -) and 62% (-) glucose syrup run at subzero temperatures (scan rate: 2°C/min).
Conventionally, the onset and end of the heat capacity change are considered to demarcate the glass transition region thus affording pinpointing of the calorimetrically derived glass transition temperature at the middle of the transition (Seo, et al., 2006). This is recorded to be about -56.3 and -45.6°C for preparations with 75 and 80% total level of solids, an outcome which documents that the thermal spectrum of proteins or polysaccharides in the presence of a high-sugar environment is dominated primarily by the vitrification of the latter.

**Figure 3.4** Heating profiles of storage modulus for the mixture of 1.5% agarose plus 7% gelatin with 0 (●), 20 (●), 40 (▲) and 60% (■) glucose syrup (scan rate: 1°C/min; frequency: 1 rad/s; strain: 0.1%).
Further explorations of phase behaviour in our blends were carried out using rheological means and comparing with the corresponding patterns recorded for single preparations. Figure 3.4, reproduces such profiles for binary systems with increasing addition of co-solute under controlled heating at 1°C/min. Heating of the 1.5% agarose plus 7% gelatin gel exhibits a thermally labile transition at low temperatures (< 40°C) followed by a second wave of structure melting at higher temperatures (> 85°C). From Figures 3.1(b) and 3.2(b), it is clear that these correspond to the melting profiles of gelatin and agarose networks, respectively. Calorimetry in Figure 3.3(a) argues convincingly for the non-interactive nature of the two polymers and since the composite gel remains partially intact with melting of the gelatin component, the agarose network should form a continuous phase. Furthermore, the dramatic drop in the values of storage modulus (from 34.6 kPa at 5°C to 1.6 kPa at 35°C presently) argues strongly that gelatin also forms a continuous phase. This type of phase topology (i.e., a bicontinuous network) has been proposed using blending-law analysis, since filler composites with a single continuous phase cannot weaken more than 50% following melting of the “infinitely hard” discontinuous filler (Morris, 1992; Picout, Richardson, & Morris, 2000).

Incorporation of 20 and 40% glucose syrup in the polymeric mixture reproduces the bimodal profile with comparable gelatin traces at the low end of the experimental temperature range (log \( G' \geq 4.2 \) below 20°C in Figure 3.4). However, melting of the agarose component is accompanied by a reduction in the log modulus values at the onset of the second step, which at 35°C are about 3.2 and 2.5 with 0 and 20% co-solute, respectively. The corresponding value of log modulus at 40% addition is 1.5 at 46°C. It appears that the increasing shortage of water molecules deprives gradually the polysaccharide associations of a hydration layer, which is required for the thermodynamic stability of intermolecular helices and the subsequent building of a three dimensional network (Trombetta, Bona, & Grazi, 2005). The result is in agreement with the diminishing and eventual disappearance of the agarose exotherm in the thermograms of Figure 3.3(a). Following the melting of the gelatin helices in gels of 20 and
40% glucose syrup, the values of storage modulus increase and reach a plateau before weakening once more. This observation is reproducible and may be attributable to the progressive migration of non gelling concentrations of agarose from the gelatin phase to join the existing network of the polysaccharide that acts as a nucleus toward the partial reactivation of the process of network formation. Finally, preparations with 60% glucose syrup (i.e., 68.5% total solids) approach glassy consistency (log $G' = 5.0$ at 5°C) whose structural properties at subzero temperatures are the main subject of interest in the following section.

3.4.3 Utilisation of the Free Volume Approach to Identify the Glass Transition Temperature of Agarose/Gelatin/Glucose Syrup Mixture.

Having discussed the phase behaviour of the agarose/gelatin mixture at low and intermediate levels of co-solute, we prepared samples at 75 and 80% total solids and ventured to subzero temperatures in order to record mechanical properties. Figure 3.5 shows an extensive variation in shear modulus (five orders of magnitude) over hundred-and-twenty degrees centigrade for a mixture of 1.5% agarose, 7% gelatin and 66.5% glucose syrup cooled at 1°C/min.

At this solids content, the system is a viscous solution at temperatures above 50°C but controlled cooling allows the solid-like component of the network to dominate leading to a rubbery region with a relatively high ‘sol fraction’ ($\tan \delta \approx 0.6$ at 40°C). Qualitatively, the temperature effect on our mixture follows the changes that characterise amorphous synthetic polymers in their passage from the melt to the rubbery and the glass transition zone (Liu, Wang, Wang, & Wang, 2007). Thus in the absence of crystallinity or ice formation in the present system (also confirmed from the thermograms in Figure 3.3b) a spectacular development of viscoelasticity is observed in the form of a glass transition region. Eventually, the glassy state is reached where the values of storage modulus exceed $10^9$ Pa.
Figure 3.5 Cooling profiles of storage and loss modulus for the mixture of 1.5% agarose, 7% gelatin and 66.5% glucose syrup (75% total solids) scanned at 1°C/min (frequency: 1 rad/s, strain: 0.01%).

There is considerable uncertainty in the empirical derivation of the glass transition temperature from thermal profiles like the one in Figure 3.5. Such derivations have been taken in the past as the initial drop in the values of storage modulus upon heating or the maxima in loss modulus and $\tan \delta$ traces within the realm of the rubber-to-glass transition. As it has been pointed out, however, in the absence of a distinct molecular process associated with these selections, the approach denotes merely an empirical index of convenience (Kasapis, 2005).
In this work, we consider that a ‘true’ glass transition relates to the conformational rearrangements of the material in question, which is a process that should be followed equally by changing the temperature or frequency (time) of measurement. Thus, one is able to perform a time-temperature superposition (TTS) on the basis that the effect of a change in temperature is primarily to shift the frequency scale (Ronan, Alshuth, Jerrams, & Murphy, 2007). To explore the applicability of TTS to the high-solid agarose/gelatin system, we recorded a series of mechanical spectra around the experimentally accessible rubber-to-glass transformation and the outcome is reproduced logarithmically in Figure 3.6 (a – b).

**Figure 3.6 a)** Frequency variation of $G'$ for 1.5% agarose, 7.0% gelatin and 66.5% glucose syrup. Bottom curve is taken at -20°C (■); other curves successively upwards – 24°C (□), - 28°C (●), - 32°C (◇), - 36°C (▲), - 40°C (△), - 44°C (●), - 48°C (○), – 52°C (X) and -56°C (+).
Figure 3.6 b) Frequency variation of $G''$ for 1.5% agarose, 7.0% gelatin and 66.5% glucose syrup. Bottom curve is taken at -20°C (■); other curves successively upwards – 24°C (□), - 28°C (◆), - 32°C (◇), - 36°C (▲), - 40°C (△), - 44°C (●), - 48°C (○), - 52°C (X) and -56°C (+).

The plotted data for $G'$ and $G''$ cover the temperature range of -20 to -56°C in order to demonstrate the utility of the “synthetic polymer approach” in pinpointing $T_g$. Modulus data at the upper range of temperature exhibit a rapid reinforcement of viscoelasticity through the glass transition region. Further cooling brings the system within the glassy consistency and experimental traces become relatively flat. Horizontal superposition of the mechanical spectra around an arbitrary chosen reference temperature ($T_o = -20°C$) generates the master curve of viscoelasticity shown in Figure 3.7. This transcends through the glass transition region and the
glassy state over a combined frequency (i.e. time) range of observation of about twelve orders of magnitude.

Figure 3.7 Master curve of reduced shear moduli ($G'_p$ and $G''_p$) as a function of reduced frequency of oscillation ($\omega a_T$) based on the frequency sweeps of the preparation in Figure 6 (reference temperature = -20°C).

Superposition of the mechanical spectra during vitrification of the agarose/gelatin/glucose syrup system at 75% total solids generates a series of shift factors ($a_T$), which are plotted in logarithmic format as a function of temperature in Figure 3.8. Following the synthetic polymer approach, we fitted factors $a_T$ based on the concept of free volume via the mathematical form
of the Williams, Landel and Ferry (WLF) equation (van der Put, 2010):

$$\log a_T = - C_1^o (T - T_g)/(C_2^o + T - T_g)$$ \quad \ldots \ldots (3.1)$$

For dynamic mechanical data on shear, $a_T = G'(T)/G'(T_o)$ and $C_1^o$ and $C_2^o$ are the so-called WLF constants (Ferry, 1991).

A straightforward calculation recasts the WLF equation as a linear dependence of the experimental temperature steps on the derived shift factors. The intercept and gradient of the linear fit or the software available with the rheometer (Rheology Advantage Data Analysis – V5.7.0 for AR-G2) allow estimation of parameters in equation (3.1), which then yields the glass transition temperature for this system. As shown in Figure 3.8, the minimisation routine fails to resolve the differences between the calculated and the observed values of shift factors at the lower end of the temperature range. This is the realm of the glassy state where our data indicate that the WLF framework becomes inappropriate. Progress in viscoelasticity in the glassy state can be followed by the modified Arrhenius equation in the following form (Binder, Baschnagel, & Paul, 2003; Gunning, Parker, & Ring, 2000):

$$\log a_T = \frac{E_a}{2.303R} \left( \frac{1}{T} - \frac{1}{T_o} \right)$$ \quad \ldots \ldots (3.2)$$

Equation (3.2) implies that the dependence of log relaxation times on temperature is linear thus yielding a constant energy of activation ($E_a = 129.8$ kJ/mol). The point of deviation from the WLF fit of the glass transition region, which introduces the exponential Arrhenius prediction in the glassy state, can be considered as the mechanical glass transition temperature with physical significance ($T_g = -38^\circ$C). It appears, therefore, that the glass transition temperature is a true turning point where large configurational vibrations requiring free volume cease to be of overriding importance. Instead, the emerging constant energy of activation reflects the need to overcome an energetic barrier for the occurrence of local rearrangements from one state to the other.
Figure 3.8 Temperature variation of the factor $a_T$ within the glass transition region (■) and the glassy state (●) of the material in Figure 3.7, with the solid lines reflecting the WLF and modified Arrhenius fits of the shift factors throughout the vitrification regime (dashed line pinpoint the $T_g$ prediction).

Similar analysis was performed on the mechanical data of the mixture at 80% solids (data not shown here) yielding a $T_g$ value of about $-25^\circ$C. In both cases, estimates of the mechanical glass transition temperature are distinct from their DSC counterparts discussed earlier. Therefore, results support an underlying process that becomes increasingly apparent in the literature. This relates to the observation that besides a thorough description of the material in terms of its composition or preparation history, the glass transition temperature depends on the analytical method and protocol employed (Kasapis, Sablani, Rahman, Al-Marhoobi, & Al-Amri, 2007; Schmidt, 2004). Thus the discrepancies observed in the values of the mechanical
and DSC $T_g$ in Figures 3.3(b) and 3.8 should not be seen as an experimental artifact but, rather, as a reflection of the distinct property and distance scale being probed by the two techniques of rheology and calorimetry.

### 3.5 CONCLUSIONS

This work dealt with high-solid materials of agarose/gelatin/glucose syrup that undergo a rubber-to-glass transformation as a function of temperature or time (frequency) of observation. The two polymers form a bicontinuous network in the mixture, with the addition of glucose syrup as the co-solute having a distinct influence on these components. The gelatin network appears to remain intact and exhibits increasing thermal stability in the changing solvent quality of the high-solid environment. There is evidence in the literature that the interaction between the protein and small polyhydroxy compounds is general and non specific, which minimizes the interfacial area between the two phases (Oakenfull & Scott, 1986). This leads to a reduction in the chemical potential of gelatin to a thermodynamically favourable state evidenced experimentally in this work. Agarose, on the other hand, appears to dissolve in the high co-solute environment being unable to sustain an extensive aggregated structure, as for its aqueous counterparts. In a separate investigation of the structural properties of $\kappa$-carrageenan (with added KCl) in the high solid environment of glucose syrup, the sulfated galactan maintains a positive trend in the development of helical structures at higher co-solute content than agarose (Kasapis, et al., 2003). Therefore, network formation withstands the high co-solute environment with increasing charge density, and addition of suitable interlinking counterions, in the polygalactan sequences.
Chapter 4: STRUCTURAL PROPERTIES OF WHEY PROTEIN/GLUCOSE SYRUP MIXTURES AS A FUNCTION OF A VARIANCE IN THE LEVEL OF SOLIDS

4.1 ABSTRACT

To achieve fundamental understanding of globular protein – sugar interaction with considerable industrial relevance on whey proteins, demands a detailed thermo-mechanical investigation towards its thermodynamic compatibility mechanism. The analysis was carried out the heat denatured whey protein isolate by changing the concentration of glucose syrup in the presence of 10mM calcium chloride. Experiments employed small deformation dynamic oscillation on shear and modulated differential scanning calorimetry. The mechanical analysis depicted the characteristic property of the gel on varying the sugar concentration which can be related to its interactive pattern. The thermodynamic analysis and delay in the denaturation of the whey protein isolate with increasing concentration of glucose syrup was analysed using differential scanning calorimetry. Confocal Laser Scanning Microscopy was utilized for observing the aggregation pattern at varying concentration of glucose syrup on a temperature controlled platform. Stabilisation of protein molecules and their ability to cluster in a high sugar environment with less water of hydration, as compared to the low solids aqueous counterparts, is evident from the research work.

Keywords: whey protein isolate, glucose syrup, rheology, calorimetry, microscopy
4.2 INTRODUCTION

Globular proteins are utilized in food, pharmaceutical, and health-care products because of their unique functional attributes, for example, enzyme catalysis, ligand binding and transport, surface activity and self-association. The expression of these functional attributes in a particular product depends on the molecular structure, chemical environment and thermal-mechanical history of the proteins. The optimization of the design and operation of processing technologies used to manufacture protein-containing materials depends on a thorough understanding of the influence of processing conditions and material composition on protein properties (McClements, 2002).

Polymeric aggregation of protein in food systems, in addition to their nutritional value, can be utilised as a thickening and stabilising agent which in turn reflects textural properties. Whey protein is one such agent, which is a potential textural modifier, nutraceutical agent capable of microencapsulation and a strong replacer of egg whites, soy protein or gelatin in food. Variation in the protein concentration ingredients, and processing classifies whey proteins as whey protein concentrate (WPC), whey protein isolate (WPI), and whey protein hydrolysate (WPH). Alpha lactalbumin and beta lactoglobulin are the major protein fractions where beta lactoglobulin accounts mainly for the polymeric property of the whey protein (Fox & McSweeney, 1998).

The denatured protein molecules show adequate interaction and aggregate to form a network which is dynamically stable. This property of induced association becomes a phenomena of vital importance in food systems. There are two phases in the globular protein aggregation: firstly, where the protein initially enhances the interaction within the molecules by changing the structural configuration which is secondly, followed by the aggregation of the molecules resulting in gelation (Fitzsimons, Mulvihill, & Morris, 2007). When heated above
the denaturation temperature i.e. about 70°C, the tertiary structure is unfolded, particularly the alpha-lactalbumin and beta–lactoglobulin by which the hydrophobic sites are exposed and sulfhydryl/disulfide reactions trigger the aggregation of the molecules. The gelation of whey proteins can be induced as a function of temperature, pressure, pH, salt concentration or even mechanical shearing.

The concentration of the protein and co-solute is in direct relation to the formation of soluble whey protein aggregates (Puyol, Perez, & Horne, 2001). The structure of protein aggregate is determined by the electrostatic interaction between the molecules. Attractive and repulsive forces between the particles depends upon the ionic strength, pH and denaturing pattern, which can be altered suggesting that structural configuration can be manipulated depending on functionality (Boye, Alli, Ismail, Gibbs, & Konishi, 1995). All the studies so far have been carried out primarily by altering the salt concentration, pH and other denaturing parameters like pressure, mechanical shearing etc. The study on the replacement of hydration environment has never been investigated on whey protein systems.

Therefore, a polymer science approach on the aggregation of whey protein systems in a sugar environment can be useful in determining its structural properties with changing “solvent quality”. To start with, rheological studies can give an idea on the structural matrix of aggregated protein molecules and its interactive pattern. Observations within the linear visco-elastic region (LVR) using dynamic oscillatory experiments will provide us with the information on the matrix development in relation to temperature and its responsiveness to various time scales using frequency sweeps. Thermodynamic observation can be made by differential scanning calorimetry (DSC), where the enthalpic change and the denaturation temperature can be observed with changing concentration of the co-solute (Fitzsimons, et al., 2007). The microscopic observation gives tangible evidence on the pattern of aggregation and interaction of the protein molecules. It also gives vital information on the particle size of the native whey protein in the sugar environment. In particular, the technique of confocal
microscopy can be very helpful in visualising distinctly the protein – polysaccharide phase based on the respective dye used (Turgeon & Beaulieu, 2001).

Studies on the glass transition temperature of the whey protein/sugar system are critical in designing ideal processing and storage conditions. To illustrate, whey proteins are regarded a potential replacer of many foods like processed meat and dairy products which in most instances are stored frozen. Therefore, the response or behaviour of whey protein gel at subzero temperatures is imperative for successful product development. Using DSC, the glass transition temperature ($T_g$) can be estimated. This is regarded as an index of quality control below which all biological, chemical and physical reactions are considerably retarded. In addition, the mechanical glass transition temperature can be estimated by reproducing the master curve of linear viscoelasticity. This is achieved by cooling the sample to subzero temperatures and incorporating the time-temperature superposition principle (TTS) using the Arrhenius and WLF derived mathematical modelling.

There is effectively no information on the mannerism of whey protein isolate in a high sugar environment. So, this lead into exploring the mechanical, thermal and microscopic observations of the aggregation pattern of whey proteins in high sugar surroundings. On the other hand, the effect of sugar on the gelation pattern of whey protein isolate has been investigated but only at low levels of solids. Sugars have two different effects on the protein gelation, the first being the stabilization of protein against heat denaturation where a delayed denaturation can be observed for the proteins on heating. This is of prime importance and the second effect being the Maillard reaction (Kulmyrzaev, Cancelliere, & McClements, 2000). The cause for the stabilisation of the protein against the heat is not well understood. The stabilisation of protein by sugars can be due to the preferential sugar hydration due to the positive effect sugars have on the surface tension of water (Arakawa, Bhat, & Timasheff, 1990).
It has been stated in the literature that the concentration of sugar can play an essential role in the gelation kinetics of whey proteins. Thus at low concentrations of sugar in the system (10 - 30%), a decrease in the gelation time can be observed which implies that the involved mechanism strengthens the protein-protein interaction. As we compromise the solvent quality with increasing levels of solids, the protein molecules have a tendency to aggregate and stay closer in order to attain a better state of thermodynamic stability (Kulmyrzaev, Cancelliere, et al., 2000). These investigations were taken further in this study by achieving concentrated systems of up to 65% sugar, and monitoring the behaviour of whey protein both mechanically and thermally. The dispersion of whey protein particles in the high sugar environment is of importance and its stability with time was also examined. Such studies of relevance navigate towards the textural quality of food materials as a function of water content and storage conditions (Guo & Narsimhan, 1991).

4.3 EXPERIMENTAL SECTION

4.3.1 Materials

Whey protein isolate sample was supplied by Fonterra Australia PTY LTD with a protein concentration of 88.71%, as analysed by Kjeldal method. According to the supplier, fat, moisture and ash were in the concentration of 0.93%, 4.83% 3.3%, respectively. Native pH of the sample was reported as 6.9. General microbiological testing was performed and was reported as ‘not detected’ by Fonterra Australia. Glucose syrup used was from Manildra Group with total solids of 81.21% and the dextrose equivalent of the sample is 43.4. Calcium chloride was provided by Sigma Aldrich Co. Sydney Australia with its purity by EDTA titration reported to be 95.6%.
4.3.2 Methods

**Sample preparation:** Samples prepared had whey protein isolate in constant concentration of (15% w/w) and varying the concentration of glucose syrup (0 - 65%) in 10 mM CaCl₂. The required amount of glucose syrup was dissolved in 10 mM CaCl₂ with a magnetic stirrer at room temperature. Whey protein isolate powder was then added in batches at regular intervals and stirred for 2 hours until well dispersed. The solution was kept in the refrigerator at a temperature of 4°C, overnight (for hydration) before subjecting it to thermal analysis.

**Rheology:** Mechanical analysis of the denatured whey protein isolate gel was accomplished using the small deformation dynamic oscillation on shear. Unlike, the old age shearing of the material to determine properties, dynamic oscillation was used to understand both the viscous and elastic property of the material simultaneously with changing conditions. Measurements of the storage modulus (G’) and loss modulus (G’”) from the complex modulus (G*) were obtained from AR-G2 (TA instruments, New Castle, DE) under various experimental conditions and parameters.

AR-G2 possesses an air bearing mounting an iron trust plate on the shaft which is held by the electromagnetic actuators placed above and below the measuring plate, facilitating reduced friction and allowing accurate mapping of the slight fluctuations in friction as the air bearing rotates. All values were recorded within the range of linear viscoelastic region (LVR). Strain sweep was performed prior to all the experiments to determine the LVR range and a fresh sample was used for analysis with the controlled deformation value picked from LVR. Time scale observation of the sample was under controlled deformation and was observed with different time frames known as frequency sweeps.
Native whey protein isolate with glucose syrup was loaded at 40 °C and heated to 80 °C to denature the protein molecule. To attain complete denaturation, the samples were subjected to isothermal at 80 °C for 20 minutes and then cooled to 5°C. Temperature ramping was performed at 1°C/min throughout the experiment. The set gel at 5°C was then subjected to a frequency sweep, where the frequency or time scale of observation was varied from high to low for a constant strain of 0.1%.

**Differential Scanning Calorimetry:** Calorimetric studies were performed on DSC Q2000 with autosampler (TA Instrument Ltd). This was attached to a refrigerated cooling system to attain temperatures down to – 90°C and a nitrogen DSC cell purge at 25 ml/min. Hermetic aluminium pans were used for the analysis. Samples of 8 to 12 mg were weighed into the aluminium pans and sealed with a hermetic lid. Modulated heat flow at ±0.53°C was recorded every 40 sec. An empty hermetically sealed aluminium DSC pan was used as a reference. Samples were loaded at room temperature and heated to 90 °C and then cooled back to 0 °C at a ramp rate of 2 °C/min to achieve full denaturation of the protein molecule. For high solid systems, the samples were cooled down to – 90 °C to attain the glassy state. Signals were then split into non reversible and reversible components for the observation of the first order and second order phase transitions, respectively.

**Confocal Laser Scanning Microscopy:** Leica TCS SP5 confocal laser scanning microscope was used to observe the whey protein aggregates. Rhodamine dye was added to the native whey protein isolate and heated to 80°C on a peltier platform to induce aggregation. Objective lens of dimension N PLAN L 20.0 X 0.40 DRY was used throughout the experiment. An emission bandwidth of 553 – 619 nm was used which is appropriate in relation to the dye used for the experiment.
The three dimensional view of the protein aggregated network was observed using the xyz – Z stack and Z- wide operational mode. The images were captured at a scanning frequency of 400 Hz with both line and frame averaging for better resolution.

4.4 RESULTS AND DISCUSSIONS

4.4.1 Rheology

Rheology has always been the mainstay in different fields, including food science, to analyse the materials for their different properties like flow, viscosity, stability etc. This has been developed into a more sophisticated and dynamic area where the strength of different matrices, their viscoelasticity and responsiveness to different parameters could be evaluated (Lowe, Allen Foegeding, & Daubert, 2003).

The aggregation of whey protein after denaturation can be well analysed by the pattern in the development of storage and loss modulus and changes in the accompanying phase angle (Dierckx & Huygebaert, 2002). Varied concentration of glucose syrup and a steady concentration of whey protein isolate (15% w/w) was appropriately observed by mechanical mode of analysis.
Figure 4.1. Heating profiles of storage modulus for 15% whey protein with 0 (■), 10 (□), 20(▲), 30(Δ), 35(●) and 40(○) % glucose syrup in the presence of 10 mM CaCl$_2$ (scan rate: 1°C/min; frequency: 1 rad/s; strain: 0.1%).

Figure 4.1 compares different concentrations of glucose syrup in relation to the pattern of development of storage modulus. All results obtained were within the linear viscoelastic region, which was analysed by performing a strain sweep. The time scale of observation for the dynamic oscillation has been set at 1 rad/s. Whey protein in 10 mM CaCl$_2$ with no sugar has a tendency for early denaturation and aggregation. Thus, we could observe that the sample with no sugar while approaching 66 °C starts to aggregate which is evident from the development of storage modulus. When the aqueous solvent system is replaced by 10% glucose syrup, a delayed aggregation was seen at a higher temperature of about 74 °C. This trend was expected
to continue until the intermediate levels of sugar around 30%. It was observed as the concentration of the glucose syrup was increased, the viscosity of the continuous aqueous phase also increases which in turn inhibits the mobility of the denatured protein molecules to form an aggregated structure.

It is quite evident from Figure 4.1 that protein denaturation has been delayed to higher temperatures on further increase in the concentration of glucose syrup, which supports the assertion that sugar can stabilise the protein molecules in a high solid environment (Dierckx & Huyghebaert, 2002). At high levels of solids, the stability of the protein molecules plays a vital role in structure development and a rapid aggregation can be observed for the respective systems. From the literature, it is expected that at higher concentration of polysaccharide (above 30%), the protein molecules attain thermodynamic stability by undergoing rapid aggregation. This could be attributed to the mechanism that prevents uniform dispersion and aggregation of the protein molecules due to the reduction of solvent quality and the preferred association of sugars with water molecules. As a “footnote”, variation in such a pattern can be further manipulated by altering the concentration of the whey protein or by varying the ionic strength by increasing the amount of calcium chloride.

This Thesis also presents work on the partial and full denaturation of whey protein. The protein molecules denature partially when the heat energy is insufficient and the system ends with both denatured and native molecules being present simultaneously in the form of a composite morphology. This affects the strength of the gel formation and the overall textural consistency. In this work, denaturation and aggregation of the whey protein to various extents was achieved and then cooled to 5 °C so as to bring the system to a lower energy state where a good gel with a reinforced texture could be achieved. Time scale observation on set gel from 0.1 rad/sec to 100 rad/sec is helpful in the explanation of the gel characteristics with a frequency sweep. Figure 4.2 shows a characteristic set gel having a considerable high strength
where the values of $G'$ and $G''$ are well separated from each other and remain linear throughout the experimental observation.

**Figure 4.2.** Frequency sweep of 15% whey protein in the presence of 10 mM CaCl$_2$ at 5 °C ($G'$, $G''$, $|\eta^*|$); strain: 0.1%.
Figure 4.3. Frequency sweep of 15% whey protein with 10% glucose syrup in the presence of 10 mM CaCl$_2$ at 5 °C ($G'(\blacksquare)$, $G''(\square)$, $|\eta^*|$ (▲)); strain: 0.1%.
Figure 4.4 Frequency sweep of 15% whey protein with 30% glucose syrup in the presence of 10 mM CaCl\(_2\) at 5 °C (G'(■), G''(□), |η*| (▲)); strain: 0.1%.
Figure 4.5. Frequency sweep of 15% whey protein with 40% glucose syrup in the presence of 10 mM CaCl$_2$ at 5 °C ($G'(\blacksquare)$, $G''(\square)$, $|\eta^*|$ (▲)); strain: 0.1%.
Figure 4.3 corresponds to the system with a 10% sugar addition where a considerable decrease in the gel strength could be observed. This outcome could be partially due to a decrease in the capacity of the system to readily hydrate. The storage and loss modulus in the mechanical spectrum has a tendency for a linear progression as approaching high frequencies. Increase of the total solids in the system by the addition of sugar is shown in Figure 4.4 (30% sugar) and Figure 4.5 (40% sugar). These two mechanical spectra reproduce a dramatic change in the nature of the sample as observed in the conventional frequency range. There is an increased frequency dependence of the modulus traces which start merging at the top end of the experimentally accessible frequency range. This type of behaviour can be considered as part of the master curve of viscoelasticity for a sample heading towards its glass transition from the current rubbery consistency as a function of the time scale of observation. For all the samples analysed presently, the complex viscosity decreases with increasing frequency, which is a clear indication of a shear thinning behaviour.

4.4.2 Differential Scanning Calorimetric Studies

Calorimetric analysis of the whey protein system is important in order to understand the thermodynamic state of the sample and to observe the endothermic and exothermic reactions accompanying such thermodynamic transformation. The major endothermic reaction for the whey protein system is its denaturation at high temperature where the unfolding of the protein molecule happens exposing the hydrophobic groups and facilitating the sulfhydryl/disulfide reactions. These play a critical role in the aggregation and subsequent gelation of the protein molecules. Change in the state of the network can be analysed from the heat flow (W/g) of the thermograms. According to the conventional experimental settings, all the exothermic reactions have an upward peak and endothermic reactions have a downward peak.
Previous studies conducted on whey proteins in the presence of carbohydrates have been cited in the literature with focus on the state of hydration of proteins in the presence of co-solutes (Arakawa, et al., 1990; Jou & Harper; Kulmyrzaev, Bryant, & McClements, 2000).Replacement of water with sucrose or sorbitol and its effect on the thermal stability of protein has been a topic of interest so far only to low and intermediate levels of solids, which makes this research study distinguishable from the previous works. With changing concentration of glucose syrup in the samples, whey protein is expected to have a gradually different denaturation profile. Increasing concentrations of sugar in the system, results in a stable protein network as recorded from the raw DSC data in Figure 4.6. To obtain these results, samples were loaded at room temperature and heated to 85 °C at a scan rate of 2°C/min. A shift in the endothermic peak of denaturation to higher temperatures with increasing concentration of sugars in the preparation was clearly observed.

By calculating the area under the endothermic peak, one can estimate the change in enthalpy during the process of denaturation. Previous studies suggested that the change in enthalpy for the globular proteins is independent of co-solute concentration (Baier & McClements, 2005). It was reported (Kilara & Harwalkar, 1996) that this parameter changes slightly without a specific pattern, with both types of observation being close to the results obtained in this study. Figure 4.7 reproduces the peak denaturation temperature at various concentration of glucose syrup with 15% whey protein. It is evident from the result that the denaturation temperature has been shifted to higher degrees with increasing concentration of glucose syrup in a consistent manner. For the system with 15% whey protein and 5% glucose syrup, the denaturation temperature picked at 71.3 °C and this progressed to higher degrees with increasing concentration of the co-solute. In the presence of 65% glucose syrup and the remaining whey protein which makes the total level of solids in the sample to 80%, the denaturation temperature was 91.3 °C.
Figure 4.6. DSC endotherms for the mixture of 15% whey proteins isolate with 5(■), 15(○), 25(▲), 35(●), 45(x), 55(–) and 65%(∆) glucose syrup in presence of 10 mM CaCl$_2$ at a scan rate of 2 °C.
Figure 4.7. Variation in the denaturation temperature of 15% whey protein isolate with increasing concentration of glucose syrup from the samples of Figure 4.6. (--) linear fit
4.4.3 Confocal Laser Scanning Microscopy

Heat set whey protein gels have been visualised for their three dimensional microstructure using high magnification microscopy (Chen, Moschakis, & Pugnaloni, 2006). In this study, confocal laser microscopy was employed to observe the aggregation of the whey protein isolate at 80°C with different concentrations of glucose syrup. This gave way in performing a comparative analysis on the aggregate size observed.

Figure 4.8. Aggregates of 15% whey protein isolate at 80°C in the presence of 10 mM CaCl₂ using rhodamine dye and an emission band width between 553 – 619 nm at a scan speed of 400 Hz.
The above picture illustrates the aggregates of whey protein as red clumps with an average aggregate size of 10 μm using rhodamine dye. The sample on glass slide was placed on peltier base and heated up to 80°C to facilitate observation of the aggregation pattern. From the preliminary observation of the sample in the absence of glucose syrup, the protein aggregate sizes are considerably large and discontinuous from this 2D view.

Figure 4.9. Aggregates of 15% whey protein isolate at 80°C with 10% glucose syrup in the presence of 10 mM CaCl₂ using rhodamine dye with an emission band width between 553 – 619 nm at a scan speed of 400 Hz.
Figure 4.10. Aggregates of 15% whey protein isolate at 80°C with 20% glucose syrup in the presence of 10 mM CaCl\(_2\) using rhodamine dye with an emission band width between 553 – 619 nm at a scan speed of 400 Hz.

Figure 4.11. Aggregates of 15% whey protein isolate at 80°C with 65% glucose syrup in the presence of 10 mM CaCl\(_2\) using rhodamine dye with an emission band width between 553 – 619 nm at a scan speed of 400 Hz.
Comparing Figures 4.8 to 4.11, the aggregate size in whey protein isolates with different concentrations of glucose syrup in the mixture can be noticed clearly. Approaching an intermediate concentration of 20% glucose syrup in the system, the network of the whey protein aggregates arranges itself into an ordered fashion where the continuous network is clearly visible. However, the aggregate size of the whey protein isolate has been considerably reduced to less than 5 μm on an average scale. At even higher concentrations of glucose syrup (65%), the aggregate particles are even smaller but possess a trend of forming a continuous cohesive network. Further magnification into the network was not possible with N PLAN L 20.0 X 0.40 DRY lens. The thermodynamic incompatibility of protein in a carbohydrate environment is quite noticeable from this microscopic observation, as compared to the compatible gelling polysaccharide/sugar systems that are very homogeneous. In other words, glucose syrup has great influential property on the structural formation of whey proteins.

4.5 CONCLUSIONS

These observations and discussion about the high-solid systems was made in correlation with the existing understanding of the structural properties of protein/sugar samples at low levels of solids and the gelling carbohydrate/sugar mixtures. Thermodynamic incompatibility between the protein molecules and sugars stands out to be the driving factor for the recorded results in this Thesis. Rheology and DSC studies suggest that the protein molecules stabilise themselves in the high-sugar environment with delayed processes of denaturation and aggregation. The comparative aggregate size determination using microscopy suggests that whey protein isolate in the absence of sugar tends to form large and non-uniform aggregates whereas at intermediate levels of sugar the aggregate size is reduced but possesses a better
arrangement pattern with a uniform particle size. The phase behaviour of the system analysed by the confocal laser scanning microscopy proposes that even at high concentration of glucose syrup in a highly viscous environment, the protein molecules after denaturation are effective for structure development with a comparatively small aggregate size. The present study into the high-solid systems of whey protein isolate and glucose syrup as a co-solute was carried out with a view to secure fundamental understanding on the behaviour of a globular protein at an environment of reduced hydration and increased solids in relation to aggregation patterns of structure formation.
Chapter 5: EPILOGUE AND FUTURE RESEARCH

The overall aim of this research study is to initiate a debate on the formulation of a scientific basis for an optimal choice of constituents and composition in high-solid materials comprising biopolymers, their mixtures and sugars (i.e. glucose syrup). High-solid biomaterials with industrial applications increasingly include a number of non-starchy polysaccharides and proteins to deliver a range of properties such as structure, storage stability, processability, etc. Thus the ingredients of this investigation, i.e. agarose, gelatin, whey protein isolate and glucose syrup are found in a wide range of high-solid food products. The market value of these products is significant. In the case of processed foods, for example, the global confectionery market was worth an estimated AUD 174 billion in 2005, having risen by almost 19% in value terms compared with levels in 2001. Because high-solid materials have low-water content, they have a long shelf life and are relatively inexpensive to handle and transport. Foods that fit this category include confectionery products like gummy bears, jelly babies, jellybeans and wine gums. The unfrozen phase of ice cream can also be considered as a highly dissolved solid matrix of sugars and mixed biopolymers. Other preparations that can be considered in this category are dried fruit products (fruit leathers, roll-ups, etc.), high-protein snack bars and new product ideas like the yogurt roll-up.

Work of this Thesis has tried to address a common observation among artisans in the field that by and large, manufacturing of high-solid materials is regarded as being craft based. Thus, there have been no systematic studies on the kinetics of structure development of products containing sugars or their substitutes and various biopolymers. The lack of understanding and control of these properties is hampering the ability of the industry to match the kinetics of structure formation to the timescale of manufacturing processes and
storage in order to improve the consumer acceptance of many high-solid products. For instance, the difficulty of studying the distribution of relaxation mechanisms in phase-separated mixtures in relation to the glass transition region and the specific interactions between chemical moieties in the glassy solid is acknowledged in the literature.

To a good extent, “functional solutions” to contemporary needs, spanning the full range of relevant time, length and concentration scales have been carried out in protein-polysaccharide gelling systems at low levels of solids (< 40% in the formulation). However, findings of the work in this Thesis argue that the behaviour of high-solid systems (> 70%) cannot be predicted by combining the current extensive understanding of the behaviour of biopolymers at high-water contents and the properties of sugars at low-water contents. In high-solid systems, synergistic interactions or phase separation of macromolecules in the presence of amorphous sugars transforms polymeric networks from being enthalpic aggregated to lightly cross-linked (i.e. entropic). It is clearly the case displayed by the agarose/glucose syrup mixture at high levels of solids in the current study. This process may play a significant role in determining the meso- and macroscopic properties of high-solid materials and end products.

The present work has also been carried out in order to provide preliminary insights, which in future research may lead to gelatin replacement. The protein, which for almost a century has been produced on an industrial scale, often used as structuring agent in high-solid foods and pharmaceuticals, but is now increasingly falling ‘out-of-fashion’ with consumers and producers alike. This change in the social attitudes can be reasoned out to finding a way to diet and health problems or perceptions such as the Bovine Spongiform Encephalopathy (BSE) scare, vegetarianism or the go-green mantra and religious dietary laws for Hindus and Muslims alike. Furthermore, high co-solute/gelatin products tend to become sticky during handling, or when stored in warehouses at relatively high ambient temperatures resulting in partial structural collapse, welding and crystallization (caking) of
the products. For these reasons, work of this research study focused on aspects of partial gelatin replacement (gelatin/agarose mixtures) and that of total protein replacement as in the case of WPI/co-solute mixtures.

The work of this study also aims to briefly allude additional aspects of interest in this area. Firstly, the science of structure development in relation to functional properties from the point of view of the polymer matrix and co-solute, builds the basis of addressing the issues of physics and chemistry of a “replacement composition for sugar” in a glassy material, which are poorly understood. In the 1970’s it was the rapid introduction of high-fructose corn syrup into the food supply that has been recognized as an important factor contributing to the obesity epidemic that swept the world in the last 30 years. Acquiring comprehensive knowledge of functional properties in basic preparations and systems containing sugars should provide the fundamental information in terms of a baseline of behaviour (i.e. mouthfeel) that must be imitated for the development on a sound technological basis of products that satisfy the special dietary need of not causing a surge in the blood sugar. Secondly, understanding the molecular dynamics of biopolymer/co-solute systems in the glass transition region (as achieved here in this study for the agarose/gelatin/glucose syrup system) ensures the stability and control of the kinetics of diffusional mobility of bioactive compounds thus designing high-solid materials with nutraceutical properties. Development of tablet and capsule delivery systems, which are stable and effective, requires understanding of the molecular packing and interactions of the biopolymer matrix with co-solute and how this affects the encapsulated bioactive compound. Fundamental understanding of the physicochemical interactions of active components and polymeric matrices and sugars in the glassy state will thus enhance product functionality and increase bioavailability.
REFERENCES


