Fundamental studies of the effect of glass transition temperature on enzymatic activity in high-solid biomaterials

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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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; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Vinita Chaudhary
14/2/2013
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Publications and presentations

Most of the work reported in this thesis has previously been presented in the following papers:

**Journal publications**

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**Fully refereed conference proceedings papers**


continued
Publications

Other conference presentations

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Abstract

This PhD research utilizes our understanding of the molecular dynamics of carbohydrates at the vicinity of the glass transition temperature (\(T_g\)) with the aim to enhance the stability and quality of processed foods where enzymatic processes are critical considerations. In this context, enzymatic activity in relation to storage temperature addresses the issue of diffusion-controlled substrate/enzyme interactions. Attempts have been made in the literature to follow the rates of enzymatic reactant consumption but were unable to follow convincingly the kinetics of molecular processes within the glass transition region. Our work indicates that the so-called “universal” \(C_1\) and \(C_2\) values of the free volume theory should not be employed in fundamental research that probes molecular property, since they cannot describe adequately the structural complexity or the specific free volume of a given bio-macromolecule. Problems encountered with this type of approach include negative estimates or physically unrealistic high values of free volume.

To advance fundamental understanding in this field, we developed carbohydrate or protein matrices based on distinct patterns of glycosidic or peptide linkages found in deacylated gellan, gelatinised starch and whey protein isolate (WPI). Characterisation techniques included small-deformation mechanical spectroscopy, micro and modulated differential scanning calorimetry, light and scanning electron microscopy, Fourier transform infrared spectroscopy and wide angle x-ray diffraction. Experimental results of the vitrification properties in the various polymeric matrices, obtained using these physicochemical techniques, were modeled on the basis of a combined theoretical framework of the time-temperature superposition principle, mechanical shift factor, reaction rate theory and free volume theory. This framework of analysis was able to produce fundamental values of the glass transition temperature, as the threshold of two distinct molecular processes, and pinpoint specific \(C_1\) and \(C_2\) values of the free volume theory for polymeric matrix.

Once the physics of the polymeric material within the glass transition region were understood, we used directly the glassy matrix as the substrate in gelatinised starch/\(\alpha\)-amylase preparations or designed non-metabolisable glassy matrices to incorporate the reactive system of \(p\)NPG/\(\alpha\)-glucosidase in deacylated gellan and
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\( pNPG/\alpha\text{-glucosidase} \) in whey protein isolate. A UV-visible spectrophotometric procedure was adapted to analyse the hydrolytic activity of \( \alpha\text{-glucosidase} \) on \( pNPG \) within the condensed gellan or WPI systems, whereas the activity of \( \alpha\text{-amylase} \) on starch was monitored using reducing sugar analysis with a dinitrosalicylic acid assay. In a further addition to this field, the work introduced to the literature the concept of spectroscopic shift factor as a means of estimating the energy of activation associated with enzymatic hydrolysis of the substrate in vitrified systems. Results strongly argue for a pronounced effect of the gelling bio-macromolecule on enzymatic activity near the mechanical \( T_g \) of the matrix, and that the mechanical \( T_g \) should be considered and utilised as an effective tool in the quality control and development of novel formulations with desirable structural property and bio-functionality.
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1 (a) Cooling profiles of storage modulus for 1% gellan with 0% (♦), 10% (■), 20% (▲), 30% (●), 40% (◇), 50% (□), 60% (△), 70% (○), 79% (−), and 83% (+) polydextrose (scan rate: 1°C/min; frequency: 10 rad/s; strain: 0.01%) and (b) variation of normalized storage modulus as a function of sugar concentration for 1% gellan with varying concentration (w/w) of polydextrose (♦).

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2 Cooling profiles of storage and loss modulus for 2% gellan with 83% polydextrose (●,♦), and 85% polydextrose (○,◊) scanned at 1°C/min (frequency: 1rad/s; strain: 0.01%).

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7. Plot of the spectroscopic shift factor in the form of the ratio of rate constants as a function of experimental temperature.
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<td>$\phi$</td>
<td>Binding energy</td>
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<tr>
<td>$\nu$</td>
<td>Frequency</td>
</tr>
<tr>
<td>$\nu_0$</td>
<td>Initial velocity</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Shear rate</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength</td>
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<tr>
<td>$\Delta H$</td>
<td>Change in enthalpy</td>
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<tr>
<td>$A$</td>
<td>Absorbance</td>
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<tr>
<td>$\AA$</td>
<td>Angstrom</td>
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<tr>
<td>ANZ</td>
<td>Australia New Zealand</td>
</tr>
<tr>
<td>ARG-2</td>
<td>Advanced generation rheometer-2</td>
</tr>
<tr>
<td>$a_T$</td>
<td>Shift factors</td>
</tr>
<tr>
<td>$b$</td>
<td>Radiation</td>
</tr>
<tr>
<td>$B$</td>
<td>Constant taken as 1 in Williams, Landel and Ferry equation</td>
</tr>
<tr>
<td>$B.$</td>
<td>Bacillus</td>
</tr>
<tr>
<td>BSE</td>
<td>Back scattered electrons</td>
</tr>
<tr>
<td>$c$</td>
<td>Velocity of light in vacuum</td>
</tr>
<tr>
<td>$c$</td>
<td>Concentration</td>
</tr>
<tr>
<td>$C_1$</td>
<td>Parameter of free volume theory</td>
</tr>
<tr>
<td>$C_2$</td>
<td>Parameter of free volume theory</td>
</tr>
<tr>
<td>CA</td>
<td>California</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>Co.</td>
<td>Company</td>
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<tr>
<td>$C_p, C_s$</td>
<td>Heat capacity</td>
</tr>
<tr>
<td>$C_r$</td>
<td>Heat capacity of the reference</td>
</tr>
<tr>
<td>cS</td>
<td>centiStoke</td>
</tr>
<tr>
<td>CTE</td>
<td>Coefficient of thermal expansion</td>
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<tr>
<td>$d$</td>
<td>Delta</td>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>DMA</td>
<td>Dynamic mechanical analysis</td>
</tr>
<tr>
<td>DMTA</td>
<td>Dynamic Mechanical Thermal Analysis</td>
</tr>
<tr>
<td>DNS</td>
<td>Dinitro salicylic acid</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>E</td>
<td>Energy</td>
</tr>
<tr>
<td>$E_a$</td>
<td>Activation energy</td>
</tr>
<tr>
<td>EMR</td>
<td>Electromagnetic radiation</td>
</tr>
<tr>
<td>ESEM</td>
<td>Environmental scanning electron microscope</td>
</tr>
<tr>
<td>ETC</td>
<td>Environment test chamber</td>
</tr>
<tr>
<td>f</td>
<td>Heat flow</td>
</tr>
<tr>
<td>$f_o$</td>
<td>Fractional free volume</td>
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<tr>
<td>FTIR</td>
<td>Fourier transformation infra-red</td>
</tr>
<tr>
<td>$G'$</td>
<td>Shear storage modulus</td>
</tr>
<tr>
<td>$G''$</td>
<td>Viscous modulus</td>
</tr>
<tr>
<td>$G', E'$</td>
<td>Storage modulus</td>
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<td>Complex modulus</td>
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<tr>
<td>$G''$</td>
<td>Shear loss modulus</td>
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<td>$H,h$</td>
<td>Heat flux</td>
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<tr>
<td>$H_f$</td>
<td>Heat flow</td>
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<tr>
<td>I</td>
<td>Intensity</td>
</tr>
<tr>
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<td>International</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-red</td>
</tr>
<tr>
<td>IUBMB</td>
<td>International Union of Biochemistry and Molecular Biology</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>k</td>
<td>Rate constant</td>
</tr>
<tr>
<td>$k_0$</td>
<td>Rate constant at reference temperature</td>
</tr>
<tr>
<td>Ltd.</td>
<td>Limited</td>
</tr>
<tr>
<td>LVR</td>
<td>Linear viscoelastic region</td>
</tr>
<tr>
<td>max</td>
<td>Maximum</td>
</tr>
<tr>
<td>mbar</td>
<td>Millibar</td>
</tr>
<tr>
<td>MDSC</td>
<td>Modulated differential scanning calorimetry</td>
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### Abbreviations

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<td>Missouri</td>
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<tr>
<td>Mol</td>
<td>Mole</td>
</tr>
<tr>
<td>NaCO₃</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>PDX</td>
<td>Polydextrose</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>pNPG</td>
<td>$p$-nitrophenyl $\alpha$-D-glucopyranoside</td>
</tr>
<tr>
<td>Pty.</td>
<td>Private</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>R</td>
<td>Thermal resistance</td>
</tr>
<tr>
<td>R</td>
<td>Gas constant</td>
</tr>
<tr>
<td>$R^2$</td>
<td>Coefficient of determination for a regression curve or line</td>
</tr>
<tr>
<td>rad</td>
<td>Radian</td>
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<tr>
<td>RMIT</td>
<td>Royal Melbourne Institute of Technology</td>
</tr>
<tr>
<td>RMMF</td>
<td>RMIT Microscopy and Microanalysis Facility</td>
</tr>
<tr>
<td>SA</td>
<td>South Australia</td>
</tr>
<tr>
<td>sd</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Beta</td>
</tr>
<tr>
<td>St.</td>
<td>Saint</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
</tr>
<tr>
<td>T</td>
<td>Transmittance</td>
</tr>
<tr>
<td>tan $\delta$</td>
<td>Phase angle</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>TMA</td>
<td>Thermo-mechanical analysis</td>
</tr>
<tr>
<td>TN</td>
<td>Total nitrogen</td>
</tr>
<tr>
<td>$T_r$</td>
<td>Temperature of reference</td>
</tr>
<tr>
<td>TTS</td>
<td>Time Temperature Superposition</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
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Abbreviations

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<td>uv</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VIC</td>
<td>Victoria</td>
</tr>
<tr>
<td>vis</td>
<td>Visible</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight by weight</td>
</tr>
<tr>
<td>WAXD</td>
<td>Wide angle x-ray diffraction</td>
</tr>
<tr>
<td>WLF</td>
<td>Williams, Landel and Ferry</td>
</tr>
<tr>
<td>WPI</td>
<td>Whey protein isolate</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>αₜ</td>
<td>Thermal expansion coefficient</td>
</tr>
<tr>
<td>γ</td>
<td>Shear strain</td>
</tr>
<tr>
<td>δ</td>
<td>Delta</td>
</tr>
<tr>
<td>ε</td>
<td>Molar absorptivity</td>
</tr>
<tr>
<td>η</td>
<td>Viscosity</td>
</tr>
<tr>
<td>η'</td>
<td>Dynamic viscosity</td>
</tr>
<tr>
<td>η*</td>
<td>Complex viscosity</td>
</tr>
<tr>
<td>σ</td>
<td>Shear stress</td>
</tr>
<tr>
<td>θ</td>
<td>Theta</td>
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Explanatory notes

The purpose of these notes is to briefly describe the approaches adopted during the preparation of this thesis. They relate to the calculation and expression of enzyme activity data, spelling, units of measurement, as well as the referencing of literature sources:

1. Where alternative spellings are in common use then the British rather than the American approach has been adopted in the text. Examples include words ending with -ise (rather than -ize) and some technical terms.

2. For the presentation of experimental results, SI units have been used throughout this thesis. For enzyme activity, the primary unit in the SI system is the katal (corresponding to one mole per sec) and so data in this thesis is expressed in terms of nanokatals.

3. The background and derivation for the calculation of enzymatic activity are provided in the Appendix to this thesis.

4. In the citation and listing of references and information sources, the current recommendations to authors for Food Chemistry (published by Elsevier) have been applied throughout.
Chapter 1

Introduction

The purpose of this chapter is to provide a brief overview of the research program described in this thesis on the kinetics of enzyme activity in glassy carbohydrate and protein matrices at temperatures in the vicinity of the glass transition. This has involved preparation and thermo-mechanical and physic-chemical characterization of matrices at different levels of solid content. In addition, the use of a series of specific matrices for encapsulation of enzymes and formation of enzyme-substrate complexes has provided the basis for the evaluation of enzyme activity in the vicinity of the glass transition temperature ($T_g$). This project has been developed on the basis of the following issues:

- The concept of $T_g$ has become an important tool in understanding the time-dependant structural and mechanical properties that relate to storage stability and texture of amorphous food products and pharmaceutics. Briefly, the glass transition is a kinetic and relaxation process associated with the primary, $\alpha$-relaxation of the material where there is a prominent difference in the molecular mobility between the glassy and rubbery states.

- At temperatures well below $T_g$, molecular mobility can be considered to be negligible over the lifetime of a given industrial product, which is expected to remain stable. Above $T_g$, however, long-range cooperative motions are possible, with the difference between $T_g$ and the processing/storage temperature ($T - T_g$) controlling the rate of physical, chemical and biological changes.

- There are many areas of food processing where enzymes may impact upon the quality of products and this effect may be beneficial or deteriorative. Enzyme assays are useful as indicators of the sufficiency of various treatments including those involving heat. Applications of enzymes as reagents in the analysis of various food molecules often utilise the specificity and hydrolytic abilities of these fascinating catalysts.

- There is a range of gums/hydrocolloids/biopolymers including guar and locust bean gums, xanthan, carboxy methyl cellulose, alginate, deacylated gellan, starch, gelatin
as well as whey protein isolates, and have been increasingly utilized in various food products. This primarily reflects their functional properties of water binding, gelation, emulsion stabilization, encapsulation, fat replacement, viscosity building, suspension, foam stabilization, and also of providing rubbery/glassy consistency essential in certain food groups, particularly confectioneries.

- Up until now, very few studies are available on the interactions between polymeric matrices (e.g., starch, dietary fibre, proteins with co-solute) in relation to their $T_g$ and chemical, structural or enzymatic functionality.

- Furthermore, attempts in the literature to follow the rates of reactant consumption with predictions of the reaction rate or free volume theories have been unsuccessful with neither theoretical approach being able to follow convincingly the kinetics of molecular processes in the region of the glass transition.

Therefore, this research project was designed with the following objectives:

- To follow the phase transitions of conformationally distinct biopolymers as a function of temperature and, in particular, in the vicinity of the rubber-to-glass transition by adapting the advanced synthetic polymer approach; and

- To identify suitable enzymes for inclusion in a biopolymer matrix with a view to developing and evaluating model systems that control the kinetics of enzymatic activity within the glassy state.

Accordingly, this study is based on the hypothesis that the $T_g$ of different matrices would have an effect on the activity of different enzymes and would provide a much needed theoretical understanding of the enzyme stability and kinetics in glassy matrices. This would then serving as a basis for subsequent work within the food and pharmaceutical industries and the development of novel product systems. Therefore, this project seeks to introduce selected enzymes into hydrocolloid matrices, study the effects at high levels of solid concentration spanning the rubber to glass regime and develop an experimental protocol that will be able to quantify, in a reproducible manner, the enzymatic activity thus yielding physically realistic parameters of the free volume for matrix/enzyme and substrate/enzyme interactions.
Chapter 2

Background and literature review

The purpose of this chapter is to provide background and review the relevant scientific literature on glass transition, enzymes and their interactions in matrices prepared from hydrocolloids. The areas covered are the theory behind glass transition, enzymes, their significance, interactions and the particular enzymes used in the current study. In addition the various hydrocolloids used for the development of matrices are also briefly reviewed.

2.1 The amorphous state and state transitions

In order to predict the state and the behavior of food during processing, distribution and storage it is very important to understand the phenomenon of glass transition and its relationship with physicochemical changes. The glass transition is shown by amorphous solids and in these materials the molecules are randomly disposed rather than being arranged in definite crystalline structures. In the glassy state, amorphous materials have relatively high viscosity with values in the order of $10^{12}$ Pa and become very brittle (Ferry 1980 and Noel, Ring and Whittam, 1990) depicting almost negligible mobility and diffusion (Karel, 1985 and Drapon, 1985). These latter constraints have been regarded as a factor that may affect rates of deteriorative changes including enzymatic reactions in food systems (Slade and Levine, 1991 and Roos, 1998).

Those solid materials having glassy amorphous structures melt upon heating and when subsequently quenched, they convert to an amorphous solid form, like that of glass, and this contrasts with the behavior of many liquids in which crystallization occurs (Ediger, Angell and Nagel, 1996). In general, when a molten liquid is cooled at a slow rate, crystal formation begins as the kinetic energy of molecules is reduced sufficiently so that it no longer surpasses the binding energy of neighbouring molecules. If an ordered crystalline system is to form, the molecules must move from their current location to an energetically preferred point, and thus, it is a slow process. With a
Background and literature review

decrease in temperature the molecular motion slows and, if the cooling rate is sufficiently fast, the molecules can never reach their preferred point. Thus, disordered a glass is formed as substances enter dynamic arrest and this occurs at a temperature known as the glass transition temperature ($T_g$). Once this transition has taken place, the molecules and atoms are subjected to only vibrational and not translational/rotational motion. Vitrification is the termed used for the conversion of a crystalline to a glassy solid and this depends on the cooling rate, cleanliness of liquid, viscosity at melting temperature and similarity of liquid packing.

With increases in temperatures through and above the glass transition, glassy materials enter into a supercooled liquid state, soften and become rubbery/leathery so that they may behave as a syrup with decreased viscosity and increased translational mobility. This increase in molecular mobility can be correlated with increased rates of physical, chemical and biological changes, since the temperature difference between $T_g$ and the storage temperature ($T-T_g$) has been found to control the rates of physical, chemical and biological changes (Champion Meste and Simatos, 2000).

2.1.1 Factors affecting $T_g$ within matrices

A range of factors have been shown to influence the temperature at which the transition is observed to occur in matrix systems and these are now reviewed briefly.

2.1.1.1 Molecular weight

- An increase in molecular weight decreases chain end concentration in straight chain polymers. It has been observed that the molecular weight dependence of $T_g$ can be changed by changing the end groups. Example: effect of molecular weight of polyvinylpyrrolidone (PVP) on $T_g$ (Roth, Pound, Kamp, Murray and Dutcher, 2006).

2.1.1.2 Molecular structure (Champion, Meste, Simatos, Roudaut and Contreras-Lopez, 2004)

- The presence of bulky, inflexible side groups or chains increases $T_g$ as these reduce mobility.
As length of side groups increases the polymer chains tend to be further apart which increases the free volume thus resulting in a lower $T_g$.

Bond rotation is decreased in those polymers with double bonds in their backbone structure and as a result these have lower $T_g$.

Cross linking of chains decreases mobility or the free volume and thus increases $T_g$.

The presence of a plasticiser increases the free volume within a matrix by increasing the space between the polymer chains so that these can slide past each other more readily, even at low temperature, resulting in a decrease in $T_g$ (Ljungberg, Andersson and Wesslen, 2003).

Water or moisture content acts as a plasticiser due to the formation of hydrogen bonds with polymeric chains, thereby increasing the distance between polymeric chains and decreasing the $T_g$. A well-documented example is wheat starch (Ohkuma, Kawai, Viriyarattanasak, Mahawanich, Tantratian and Takai, (2008) and Goula, Karapantsios, Achilias and Adamopoulos, 2008).

2.1.1.3 The cooling rate is directly proportional to $T_g$, with higher cooling rates resulting in higher values for $T_g$. An example of this is seen in studies on rice starch gels (Hsu, Heldman, Taylor and Kramer, 2003).

2.1.1.4 Effect of entropy and enthalpy: the value of $T_g$ is high if that of the entropy is high as the entropy of an amorphous material is higher than that of a crystalline material (Debenedetti, and Stillinger, 2001).

2.1.1.5 Pressure decreases the free volume, ultimately resulting in a high $T_g$.

2.1.1.6 Polymer film thickness affects $T_g$ by affecting the mobility of molecules i.e. the thicker the film the higher is its $T_g$.

2.1.1.7 Flexibility of the polymer chain also affects the $T_g$ as it is related to the mobility; rigid chains will have higher $T_g$ than those that are flexible. Overall flexibility itself is affected by backbone flexibility, presence of pendant groups, branching, bond
Background and literature review

interactions, functionality, alkyl chain length and the presence of polar groups (He, Li and Ren, 2003).

2.2 The importance of glass transition temperature

Since it was first developed, the concept of $T_g$ has proven useful as it contributes to our understanding of the behavior of many different food materials, particularly as these influence processing, storage and sensory attributes of food products.

- Improved processing and handling qualities: For confectionery products it is desirable that these are not sticky; this can be achieved by increasing the $T_g$ of the raw materials used. The resultant product will also be harder and is more readily processed. The glassy state makes the substance tougher, with enhanced strength which facilitates packaging as well.

- Improved dissolution and bioavailability: amorphous materials which are randomly arranged require less energy to dissolve as compared to highly ordered crystalline materials. At room and body temperatures, low $T_g$ materials are in a rubbery state and problems are encountered during dissolution whereas, high $T_g$ materials are in glassy state and dissolve quite readily. This can be utilised for the enhancement of drug delivery systems (Gothoskar, 2005).

- Improved physical stability: glass solutions can be formed when drugs, bioactive compounds and polymer/biopolymers are entirely miscible in the molten state and remain as an amorphous one-phase system when cooled, this produces a product with better physical stability as there are fewer sites available for bonding with water molecules. Glassy materials demonstrate improved storage ability and physical stability (Jans and Mielck, 1996).

The glass transition temperature can also be utilised to elaborate the relationship between the kinetics of vitrification and metastability of systems in order to develop innovative product formulations and methods of processing.
2.3 The synthetic polymer approach

In the context of food systems, in order to evaluate the mechanical glass transition temperature the time temperature superposition principle (TTS) is applied. This adopts the procedures applied in the study of synthetic polymers and assumes that by changing the temperature, the complete relaxation spectrum is affected by the same factor. In oscillation experiments, temperature is held constant and frequency or time is varied. The data obtained spans over a two to four decade range in terms of frequency/time. By repeating such tests over a number of temperatures, one can obtain a set of isothermal dependencies of storage and loss modulus in graphs of shear versus frequencies. On shifting these linear viscoelastic parameters along the x-axis such that they are superimposed on one another, a master curve is generated at a particular temperature, representing the time response of a material over a wide range of frequencies at a chosen reference temperature.

To develop a mechanistic understanding of the rubber-to-glass transition, polymer scientists make extensive use of the concept of molecular free-volume that forms basis for deriving the Williams, Landel and Ferry (WLF) equation. This was first presented by Ferry (1980) who described free volume ($u_f$) as the spaces between the packing irregularities of long chain segments or the space required for their string-like movements. It can be mathematically expressed as:

$$\log a_f = -\frac{C_1^0 (T - T_0)}{C_2^0 + T - T_0}$$

(2)

where, $C_1^0$ and $C_2^0$ are the WLF constants at $T_0$ and relate to the free volume theory as follows:

$$C_1^0 = \frac{B}{2.303 f_0}$$ and $$C_2^0 = \frac{f_0}{\alpha_f}$$

(3)

and, 

$f_0$ is the fractional free volume (the ratio of free to total volume per gram of material)

$\alpha_f$ is the thermal expansion coefficient; and

$B$ is usually taken as one for simplicity.
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According to Cavaille, Jordan, Perez, Monnerie and Johari, (1987) the assumption of a rapid and linear development of the fractional free volume at temperatures above the glass transition can be considered in terms of the expansion coefficient $\alpha_f$. By plotting $1/\log a_T$ against $1/ (T-T_0)$, fitting of the shift factors to the WLF framework can be achieved. In addition, the two parameters $C_1^0$ and $C_2^0$ can be obtained from the slope and intercept of the linear fit, respectively.

The shifting of the frequency scale with temperature depends on the progress of factor $a_T$ (Matveev, Grinberg and Tolstoguzov, 2000). The master curve also covers the glassy state, the shift factors from WLF equation do not fit in the glassy state, instead can be better described by the Andrade mathematical expression. In this, the reaction rate is proportional to exp ($E_a/RT$), where $E_a$ is the activation energy of the molecular process and is expressed for a set of two different temperatures, as follows:

$$
\log a_T = \frac{E_a}{2.303R} \left( \frac{1}{T} - \frac{1}{T_0} \right)
$$

From this approach, the concept of activation energy ($E_a$) evolves for an elementary flow process in the glassy state, which is independent of temperature. As within the glassy state, the factor $a_T$ is an exponential function of the reciprocal absolute temperature, so the logarithmic form with a constant energy can be used for calculating numerical values.

It has been found that, in polymer melts the proportion of free volume is usually 30% of the total volume and at glass transition it collapses to approximately 3% (Ferry 1980). Also, at this point, the thermal expansion coefficient of free volume ($\alpha_f$) shows a discontinuity which is reflected as a change in slope in the graph of the linear dependence of total volume with temperature. Mechanistically, this is the point of transformation from effects related to the free volume in the glass transition region to the process of an energy barrier to rotation in the solid-like environment of the glassy state. Thus, this point of discontinuity can be considered as a valid reference for the $T_g$ and its application has been explored in detail by Kasapis (2006) who investigated model confections as well as dehydrated foodstuffs using state diagrams and adsorption isotherms.
For a period of decades, manufacturing of high-solids products was regarded as being craft-based, until the developments in understanding inspired by the advanced sophisticated polymer approach described above. This facilitated the formulation of a physicochemical method for the interpretation of vitrification phenomena (Ferry, 1991 and Levine and Slade, 1986). From these advances, the physical properties of carbohydrate-water systems in terms of their temperature- and water-dependent phase transitions have resulted in wide use of phase and state diagrams to predict both behaviour during processing and the storage stability of carbohydrate-based foods and pharmaceuticals (Roos, 1995). State diagrams represent the pattern of change in the physical state of a material as a function of increasing levels of solids (Kokini, Cocero, Madeka and de Graaf, 1994) and their use has facilitated the transfer of technology from the material science of aqueous solid solutions to the functional attributes of ingredients used in the food-processing industry (Karel, Buera and Roos, 1993). The combination of equilibrium (crystallization) and metastable (vitrification) processes as a function of time, pressure and temperature of processing or preservation has extended the basic understanding of physical properties of foodstuffs (Goff, Caldwell, Stanley and Maurice, 1993).

Thermo-mechanical analysis has been utilized to study the important preparations of the confectionery industry particularly that of sugar in the presence of gelatin and/or gelling polysaccharides. Further insight on their structural properties is fundamental for future innovation in the development of novel products. In addition to the area of confectionery, these materials can also be used in a variety of other increasingly important applications including encapsulation of flavors and the preservation of bioactive molecules within glassy carbohydrate matrices (Kasapis, Mitchell, Abeysekera and MacNaughtan, 2004).

More recently, the analysis of glass transition was further refined by the stress relaxation dynamics of “the coupling model” in conjunction with the WLF equation which resulted in the fundamental definition of network or mechanical $T_g$. This has provided a novel concept, quite distinct from the commonly reported calorimetric $T_g$ of sugars and polyols at high levels of solids (70-90 %), thus, opening new vistas into the structure and mechanical properties of this class of materials (Kasapis, 2004 and Kasapis, 2006).
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2.4 Enzymes: a brief introduction

Enzymes are proteins that enhance the rate of chemical reactions, that is, they catalyze chemical reactions by lowering the activation energy for a reaction. Dixon and Webb (1979) defined enzymes as “proteins with catalytic properties due to their power of specific activation”. The prime reason for the importance of enzymes as a group of molecules for the food industry is their capability to specifically manipulate all the major biological macromolecules, proteins, carbohydrates, lipids and nucleic acids, as well as smaller molecules including amino acids, sugars and vitamins. Enzyme activity is affected by various parameters particularly enzyme concentration; substrate concentration; environmental conditions of pH, temperature, ionic strength and moisture; presence or absence of inhibitors; activators or co-factors. Kinetics provides a systematic approach for the analysis and quantification of the effect of these parameters on enzyme activity.

2.4.1 Enzyme classification and hydrolytic enzymes

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) is responsible for classifying the enzymes. This Enzyme Commission (EC) assigns each enzyme with a recommended name and a four part distinguishing number. EC numbers divide enzymes into six primary groups according to the type of chemical reaction catalysed:

1. Oxidoreductases;
2. Transferases;
3. Hydrolases;
4. Lyases;
5. Isomerases; and
6. Ligases

Enzymes in which water participates in breakage of covalent bonds of the substrate, with concurrent addition of the element of water to the principles of those bonds are known as “hydrolases or hydrolytic enzymes” (Whitaker, 1996). Hydrolases are a large group of enzymes having in common the involvement of water as a co-
substrate of the primary substrate in formation of products (Whitaker, 1972). Hydrolytic reactions yield two products resulting from the cleavage of the parent substrate molecule. Whitaker (1972) has proposed more detailed information on the mechanism of hydrolysis and the classification of hydrolytic enzymes.

2.5 Factors influencing enzyme activity and their significance in low-moisture food systems

2.5.1 Effects of water

Water influences enzymes in several different ways within food systems. Whitaker (1995) and Drapon (1985) have explained in detail the role of water as a determinant of enzyme activity and stated that it plays four important functions in all enzyme catalyzed reactions by:

- Influencing the folding of the protein
- Acting as transport medium for both the substrate and enzyme
- Hydrating the protein
- Ionizing prototropic groups at the active site of the enzyme

2.5.2 Effects of substrate

An enzymatic reaction involves two steps: a binding step in which the substrate is attracted to and “captured” by the enzyme, followed by the actual reaction step that results in the formation of the products. The effects of substrate concentration can be better observed by keeping all other parameters constant, while the initial concentration of the substrate is varied. Enzymes operate most efficiently (by increasing the rate of the reaction) when an excess of substrate is available. If the substrate concentration is relatively low compared to that of the enzyme, successful collisions are few. When the substrate concentration is very high compared to the enzyme concentration, successful collisions occur very frequently. This ensures that most of the enzyme molecules present are bound in the form of an enzyme–substrate (ES) complex and product(s) formation occurs at a maximum rate (Mathewson, 1998). Once all enzyme molecules have combined with substrate(s), further increases in substrate concentration will not
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increase the rate of reaction (Whitaker, 1972; Mathewson, 1998). The velocity at this point is referred to as the maximum velocity, \( V_m \).

Andersen, Thiesen and Broe (1969) postulated that sucrose hydrolysis was a two-step transfer in which the first step is the formation of an active enzyme-fructose complex and the next step a liberation of glucose and a simultaneous transfer of the fructosyl group to water or sucrose. This observation was later confirmed by Bowski, Saini, Ryu and Vieth (1971). The ability of the amino acids at the active sites of an enzyme to interact with the substrate depends on their electrostatic state (Kang, Marangoni and Yada, 1994) that is, whether they are charged or uncharged as well as their spatial orientation. If the pH is not appropriate, the charge on one or all of the required amino acids is such that the substrate can neither bind nor react to produce a product (Mathewson, 1998). Most enzymes are active in a rather narrow pH range and the range and optimal pH values vary for different enzymes. For many the range is 6.0-8.0, although many enzymes have been characterised as having different pH properties. Silver and Haskel (1990) observed a 10-fold increase in the activity of \( \alpha \)-chymotrypsin in a membrane system, when the pH of the medium was lowered from 8.5 to 4. However, it has been reported that the dependence of reaction rate on pH is often related to water activity \( (a_w) \) (Schwimmer, 1980; Bell and Labuza, 1992, 1994).

2.5.3 Effect of temperature

Just as has been observed for pH, temperature affects the three-dimensional structure of an enzyme (Whitaker, 1972; Mathewson, 1998). Supplying the system with more energy in the form of heat helps to overcome the energy barriers, including that necessary for ionization of groups in the active site of the enzyme-substrate complex. This occurs because the warmer the system, the faster the molecules move and thus, the reactants collide more often and with greater energy.

The effect of temperature on enzymes in amorphous food systems has been extensively studied (Schebor, Buera and Chirife, 1996; Cardona, Schebor, Buera, Karel and Chirife, 1997; Mazzobre, buera and Chirife, 1997a and b; Burin, Buera, Hough and Chirife, 2002). However, these studies focused on the thermal resistance or stability of the enzyme as related to \( T_g \), rather than on enzyme activity. Drapon (1985) reported that the effect of temperature on enzyme activity was related to moisture content by demonstrating that at a higher moisture content, inactivation and denaturation of a lipase
occurred at a lower temperature, while at low moisture content, a higher temperature was required for the same processes. The effect of temperature on rate of enzyme-catalyzed reactions has also been studied extensively. Whereas most studies have investigated temperatures reflecting either ambient or higher (elevated) conditions, Whitaker (1995) postulated that decreasing the temperature of a system to below 0 °C decreases the rate of enzyme-catalyzed reactions. Lund, Fennema and Powrie (1969) attributed such observations to the conversion of part of the bulk water in the system to ice, causing reduction in mobility, and an increased substrate inhibition as a result of the resultant increase in substrate concentration.

2.5.4 Effect of glass transition

Slade and Levine (1994) suggested that the glassy state of an amorphous matrix could be responsible for the rate control of diffusion-limited rates of chemical reactions that take place at low moisture contents. According to Buera, Chirife and Karel (1995), the limitation in diffusion disappears and a drastic increase in the reaction rates as a result of increased water content and plasticizer effects may be noticeable as the glass-rubbery transition occurs. However, experimental studies presented to demonstrate this, were conducted on aqueous solutions and matrices made up of low solid content. Furthermore, a comprehensive review of the scientific literature has shown us that no experimental data has yet related this to the behavior of enzymes in matrices at temperatures in the vicinity of the transition.

2.5.5 Effect of polysaccharides

The presence of polysaccharides in a viscoelastic liquid has been shown to result in the entanglement of the chains and restrict diffusion of water molecules (Goff, Caldwell and Stanley, 1993). It was observed that dextrans underwent a conformational change from random coil to a more compact coil when the concentration was raised from 4.7 to 19 % (McCurdy, Goff, Stanley and Stone, 1994). These authors speculated that the conformational change could modify the diffusion rate of small molecules. Contreras-Lopez, Champion, Hervet, Blond and LeMeste, (2000) demonstrated by using fluorescein, that pullulan at a concentration of 10 % induced a significant decrease in measured translational mobility. This effect of the polysaccharide was attributed to its ability to form physical entanglements. Similar behavior was observed for gelatin gels by Gillies, Sutcliffe, Wu and Belton, (1996) and Contreras-Lopez et al., (2000) reported
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that translational motion was virtually stopped when the concentration of gelatin reached 55%.

Roozen, Hemminga and Walstra, (1991) also investigated the influence of polysaccharides on molecular motions and suggested that small molecules can freely rotate in cavities, "holes", created within a polysaccharide matrix. They postulated that the structure and composition of such a system could affect the motions of molecules, affecting the probability that substrate(s) and enzyme molecules might collide. Molecular motions can subsequently influence the reaction rates and in water-restricted systems, it could be assumed that mobility would be limited. The activity of the enzyme would also be dependent on its closeness to the substrate and poor miscibility could also lead to reduced reaction rates since it may reduce interactions between molecules. Therefore it is important that the enzyme should be distributed in such a way that it is available in the vicinity of the substrate.

2.6 Enzymes used in the present study

For the purposes of the current study two particular enzymes were selected for investigation in model matrices. Those selected were α-amylase and α-glucosidase, both relevant in food systems and the purpose here is to briefly provide background on these.

2.6.1 α-Amylase

Based on the type of reaction catalyzed and the substrate specificity, IUBMB has adopted the systematic name of 4-α-D-glucan glucanohydrolase for the enzyme popularly known as α-amylase and the number applied is EC 3.2.1.1. It is an endoglucanase that catalyzes the breakdown of internal glucosidic bonds in starch and similar polysaccharides to yield dextrins and oligosaccharides with the anomeric C1-OH in the α-configuration. α–Amylase plays an important role in carbohydrate metabolism of microorganisms, plants and animals. For industrial purposes, several bacterial species belonging to the genus *Bacillus* (including *B. licheniformis*, *B. stearothermophilus* and *B. subtilis*) are used in the production of α-amylase through controlled fermentation with the resultant preparations being in the form of off-white to brown, amorphous powders or liquids.
In comparison with the enzyme from other sources, these bacterial amylases are generally stable at temperatures in excess of 90 °C, relatively less dependent on Ca$^{2+}$ ions and are active over a wide pH range. These properties enable them to be used for a variety of commercial applications including preparation of starch syrup, dextrose, alcohol, beer and bakery products. They are also used for the liquefaction of starch to produce dextrins. These can be further saccharified by another enzyme, glucoamylase, to produce glucose feed stock for corn syrup, fuel ethanol or beverage alcohol production. The addition of α-amylase into bakery formulations ensures a continuous supply of fermentable sugar for yeast growth and gas production. In the textile industry α-amylase aids in providing a finish to the product by desizing the starch that has been used as a coating to the threads during mechanical weaving. In addition, as this enzyme acts upon starch, it is incorporated into detergents in order to remove starchy stains and also to increase efficiency and reducing requirements for harsh chemical ingredients in the laundry powder (Wong and Robertson, 2002).

In the current study dinitrosalicylic acid reagent (DNS) (Fig. 2.1 a) was used to determine the reducing sugar produced by α-amylase after hydrolysing starch matrix by adopting the procedure given by Miller (1959). The colored product 3-amino,5-nitrosalicylic acid (Fig. 2.1 b) formed was read spectrophotometrically in the Absorbance (A) mode.

![Fig. 2.1 Representative structures of (a) 3,5-dinitrosalicylic acid and (b) 3-amino, 5-nitrosalicylic acid](image-url)
2.6.2 \( \alpha \)-Glucosidase

This enzyme has the systematic name of \( \alpha \)-D-glucoside glucohydrolase with IUBMB enzyme number of EC 3.2.1.20, and is also known by a variety of common names including maltase, glucoinvertase and glucosidosucrase. It catalyses the hydrolysis of terminal non-reducing, (1\( \rightarrow \) 4) – linked \( \alpha \)-D-glucose residues of a number of substrates, for example disaccharides, oligosaccharides and other aryl-alkyl- \( \alpha \)-glucopyranosides and releases \( \alpha \)-D-glucose. It is found to hydrolyse oligosaccharides rapidly in comparison to polysaccharides on which it acts relatively slowly or not at all. For convenience, in the assay of activity of the enzyme the artificial substrate \( p \)-nitrophenyl \( \alpha \)-D-glucoside (Fig. 2.2, \( p \)NPG, also known as 4-nitrophenyl \( \alpha \)-D-glucoside) is commonly used. Typically, \( \alpha \)-glucosidases from a variety of sources exhibit activity within a pH range of 5.0-6.0 (Kelly and Fogarty, 1988).

![Fig. 2.2 The structure of \( p \)NPG, used as the substrate for assay of \( \alpha \)-glucosidase](image)

It has been reported that many organisms producing extracellular amylolytic enzymes also produce an intracellular \( \alpha \)-glucosidase, which is the final enzyme involved in the pathway of metabolism from starch to glucose (Costantino, Brown and Kelly, 1990). The enzyme has been isolated from various bacteria including some moderate thermophiles. Extracellular \( \alpha \)-glucosidase was purified by Suzuki, Yuki, Kishigami and Abe, (1976) from \( B. \) thermoglucosidus who characterised the enzymes as having a temperature optimum of 75 °C but it was inactivated rapidly at higher temperatures. In other reports, \( B. \) caldovelox DSM 411 (Giblin, Kelly and Fogarty, 1987) and \( B. \) stearothermophilus ATCC 12016 (Suzuki, Shinji and Eto, 1984) have also been used to isolate the enzyme and from these sources the enzymes exhibited optimal activity at 60 and 70 °C, respectively. Costantino, Brown and Kelly, (1990) have purified and
characterized α-glucosidase from a hyperthermophilic archaebacterium, *Pyrococcus furiosus* and this exhibited a temperature optimum in the range of 105 to 115 °C.

In addition to the evolutionary significance of α-glucosidase, these enzymes provide the opportunity to elucidate the structure and function of proteins at relatively high temperatures. There is biotechnological potential for the bacterial enzymes in advantageously replacing enzymes from existing sources, as well as identifying opportunities in different and new applications.

### 2.7 Relationship between $T_g$, reaction pathways and enzymatic processes

In low moisture foods, enzymatic reactions account for various deleterious changes and the rates of these changes may be related to changes in the physical states including the glass transition. This has been recognized as a possible factor affecting kinetics of enzymatic changes in high solid and frozen foods (Lim and Reid, 1991; Slade and Levine, 1991; Roos and Karel, 1991 and Roos 1998). As a system approaches the glassy state the translational and rotational motions of the molecules become negligible. It has therefore been hypothesized that chemical and biological reactions would have reduced rates in the glassy state (Le Meste, 1995 and Cardona et al., 1997). According to Roos (1995), there is likely to be a dramatic rise in rates within and above the $T_g$ range in amorphous materials that may be attributed to the decrease in viscosity. Above $T_g$ various changes with more significant rates can occur and are influenced by the temperature difference between $T_g$ and storage temperature ($T$) generally presented as ($T-T_g$). Physical stability can be assumed to be maintained over a practical time frame within the glassy state (Levine and Slade, 1986; Slade and Levine, 1991 and Jouppila, Kansikas and Roos, 1998).

Molecular mobility facilitates the arrangement of molecules and possibly enhances enzymatic reactions as a result of plasticizing effect of water. The amount of available water has been found to affect the rates of enzymatic reactions in amorphous food systems (Schwimmer, 1980; Silver and Karel 1981 and Chen, Aull and Bell, 1999). Water plays a major role in plasticizing the materials and this is evident from the effect it has in the depression of $T_g$ (Roos and Karel 1991; Slade and Levine, 1991 and Biliaderis, Lazaridou and Arvanitoyannis, 1999). The composition and structure of
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Amorphous systems may also affect the mobility of the components. It has been shown that polymers are able to form entanglements in which small molecules can be entrapped (Sperling, 1992 and Contreras-Lopez et al 2000). These polymeric materials can be used to improve physical and structural properties of food systems and also to modify the reactivity (Kasapis, Al Marhoobi and Giannouli, 1999) and kinetics of deteriorative changes occurring in foods (Contreras-Lopez et al 2000). At this time no experimental studies have extended to those involving enzyme systems in amorphous matrices.

Various chemical reaction pathways and enzymatic processes are major considerations for improving the stability and quality of processed foods. To control these it is important to understand the molecular dynamics of carbohydrate and proteins at the vicinity of $T_g$. Mostly enzyme activity relates to the diffusion-controlled substrate/enzyme interaction and chemical studies focusses in the prevention of flavour and colour degradation and control of the oxidative reactions such as fat rancidity (Burin, Jouppila, Roos, Kansikas and del Pilar Buera, 2000 and Thomsen, Lauridsen, Skibsted and Risbo, 2005).

The interactions of reducing sugars (fructose, glucose and xylose) with lysine in several matrices made of trehalose, maltodextrin, or polyvinylpyrrolidone (PVP) have been monitored through studies on non-enzymatic browning (Bell, 1996; Lievonen, Laaksonen and Roos, 1998; Craig, Parker, Rigby, Cairns and Ring, 2001 and Lievonen, Laaksonen and Roos, 2002) few other examples include the degradation of aspartame, the acid-catalyzed hydrolysis of sucrose, the deterioration of saffron quality, the methyl esterase-catalysed de-esterification of pectin and the alkaline phosphatase-catalysed hydrolysis of disodium $p$-nitrophenylphosphatase (Bell and Hageman, 1994; del Pilar Buera, Chirife and Karel, 1995; Tsimidou and Biliaderis, 1997; Chen, Aull and Bell, 1999; Terefe and Hendrickx, 2002; Terefe, Mokwena, Loey and Hendrickx, 2002; Terefe, Arimi, Loey and Hendrickx, 2004; Terefe, Nhan, Vallejo, Loey and Hendrickx, 2004 and Terefe, Delele, Loey and Hendrickx, 2005).
2.8 Hydrocolloids as food ingredients

2.8.1 Introduction

Hydrophilic colloids or hydrocolloids, commonly referred to as “gums”, are long chain, high molecular weight polymers that disperse in water yielding a thickening and sometimes gelling effect. These molecules have often been classified into three categories of natural (derived from various plant and animal sources), modified natural (including cellulose derivatives) and synthetic (PVP, carboxyvinyl polymers). Some of the most commonly used hydrocolloids are listed in Table 2.1 along with the functions for which they are being used in various applications (Glicksman, 1979). The specific hydrocolloids selected and utilized in this study are further briefly described.

Table 2.1 Common food hydrocolloids and their functions

<table>
<thead>
<tr>
<th>Gums/hydrocolloids/biopolymers</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guar locust bean</td>
<td>Water binding/gelation</td>
</tr>
<tr>
<td>Deacylated gellan</td>
<td>Viscosity building</td>
</tr>
<tr>
<td>Xanthan</td>
<td>Emulsion stabilisation</td>
</tr>
<tr>
<td>Starch</td>
<td>Suspension stabilisation</td>
</tr>
<tr>
<td>Carboxy methyl cellulose</td>
<td>Encapsulation</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Foam stabilisation</td>
</tr>
<tr>
<td>Alginate</td>
<td>Fat replacement</td>
</tr>
<tr>
<td>Whey protein isolate</td>
<td>Rubbery/glassy consistency</td>
</tr>
</tbody>
</table>

2.8.2 Gellan

Gellan is a linear, anionic, extracellular heteropolysaccharide produced by Pseudomonas elodea. It has a molecular weight of approximately 0.5 x 10^6 Daltons. As shown in Fig. 2.3, it is composed of tetrasaccharide repeating units of 1,3-β-D-glucose, 1,4-β-D-glucuronic acid, 1,4-β-D-glucose and 1,4-α-L-rhamnose (Gibson 1992). It also
contains approximately 1.5 acyl substituents per tetrasaccharide repeating unit. Kuo, Mort and Dell (1986) identified these as an L-glyceric ester on L-2 of the 3 linked D-glucose and an acetic ester on L-6 of the same glucose residue. Their presence, particularly the glycerate groups, hinders chain association and is responsible for the change in gel structure brought about by de-esterification.

De-esterification yields a polymer with a well-defined un-substituted, tetrasaccharide repeating unit (Fig.2.4). This form of gellan is commercially available and is proprietary to Kelco (Kang, Colgrove and Veeder, 1980; 1982 and 1983).

During gelation, gellan gum undergoes disorder-order transitions as the hot solution cools. This transition has been attributed to the coil coil-helix transition. The textural properties of gellan are dependent on its acyl content (Morris, 2006) and divalent cations, including calcium and magnesium, co-ordinate to the carboxylate
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group to stabilize the gel formed. X-ray diffraction analysis indicates that the molecule forms a parallel half-staggered double helix in which each polysaccharide chain is a helix, specifically described as left handed 3–fold (Baird, Talashek and Chang, 1992). Gellan gum has the ability to produce thermo-reversible gels, as a result of changes of either increasing ionic strength or decreasing temperature. The latter has a very strong influence upon the conformation of gellan and a double stranded helix is formed at low temperature whereas polymer is single stranded at higher temperatures. The transition between the two takes place at approximately 35 °C, and below this, molecules form a very stiff structure in an ordered state. Gels are prepared by adding the gelling gum to water under conditions of shear, followed by heating to 75 °C, then adding ions and cooling to set. It is particularly noteworthy that, for gelation to occur, the concentration of gellan gum can be as low as 0.05 %.

Gellan gum is compatible with many other gelling agents including agar and κ-carrageenan and is highly efficient in gel formation providing a variety of textures. A further advantage is that it can replace other existing alternatives and be readily incorporated into many current processes, thereby promising a broad potential for application in the food industry. It has been claimed that its versatility provides food processors with a unique hydrocolloid, which, used by itself or in combination with other food hydrocolloids, provides functionality in gelling, texturizing, stabilizing, suspension, film forming and adhesion. The potential to vary the gel properties, in a systematic and controlled manner, should lead to enhanced functionality and wider application in food and industrial applications. Some examples of the claimed applications are listed in Table 2.2 (Gibson, 1992). It is noted that although gellan has been investigated over some time, regulatory approval for its use and the release of the commercial food ingredient have occurred relatively recently in comparison with the other food hydrocolloids. Accordingly it is not yet widely utilised and there is considerable potential for the application of this ingredient in the development of novel products.
Table 2.2 Potential food applications for gellan

<table>
<thead>
<tr>
<th>Area of application</th>
<th>Typical products</th>
<th>Typical use level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water based jellies</td>
<td>Dessert jellies, aspics</td>
<td>0.15-0.2</td>
</tr>
<tr>
<td>Jams and jellies</td>
<td>Low calorie spreads, imitation jams, bakery fillings</td>
<td>0.12-0.3</td>
</tr>
<tr>
<td>Confectionery</td>
<td>Pastille type confectionery, marshmallows</td>
<td>0.8-1.0</td>
</tr>
<tr>
<td>Fabricated foods</td>
<td>Fabricated fruit, vegetables and meats</td>
<td>0.2-0.3</td>
</tr>
<tr>
<td>Icings</td>
<td>Bakery icings, frostings</td>
<td>0.05-0.12</td>
</tr>
<tr>
<td>Dairy products</td>
<td>Milk desserts</td>
<td>0.1-0.2</td>
</tr>
<tr>
<td>Pie fillings</td>
<td>Fruit pie fillings</td>
<td>0.25-0.35</td>
</tr>
</tbody>
</table>

2.8.3 Starch

Amongst polysaccharides, starch is one of the most widely produced biopolymers in the world. Because of biocompatibility and degradability, starch has been of interest in pharmaceuticals, thermoplastics, paper, textile and various other applications. These polymers have similar thickening and gelling properties to those that are observed for other gums. Starches are carbohydrate polymers of glucose composed of two distinct fractions: amylose, a linear, straight-chain, $\alpha$-1,4-glucopyranose polymer, and amylopectin, consisting of sequences of $\alpha$-1,4-glucopyranose units with periodic branching of side chains through 1,6 linkages (Fig. 2.5). Amylose is the non-ramified fraction typically having about 4,000 glucose units, and amylopectin is the branched fraction a degree of polymerization of approximately 100,000 glucose units.
The amylose component contributes to the gelling properties of the starches by forming hydrogen bonds with neighbouring molecules thereby building up a three-dimensional network. On the other hand, as amylopectin is highly branched, close physical association between molecules is prevented, thereby inhibiting or preventing gelation. Typically, starch gelation occurs after a dispersion of granular starch in water has been firstly heating the above its gelatinization temperature, followed by cooling the resultant liquid. This process of using starch as a food ingredient can be speeded up by pre-gelatinizing the starch using heating or cooking in excess water and then drying the slurries. The resulting pre-gelatinized starch would then be more readily dissolved in cold-water and gelling at low temperatures although some of the gel quality and strength would be compromised as a result of this approach to the process. With advances in science and technology many chemically modified starches are now available for specific applications reflecting their changed properties and giving important advantages when compared with native starches (Glicksman, 1979).

Starch is naturally hydrolysed by several amylolytic enzymes and their action will influence the textural implications of starches and the processing of foods containing this polymer. \( \alpha \)-Amylase is an endo- enzyme specific to \( \alpha \)-(1,4)-D-
glucopyranosidic bonds located within polyglucose chains. The degradation products of amylolysis are dextrins and oligosaccharides including maltose (Dumoulin, Cartilier, and Mateescu, 1999).

### 2.8.4 Whey protein isolate (WPI)

Whey protein is a natural by-product of cheese and casein manufacture and it possesses unique nutritional and physiological functionality compared to other dietary proteins. It is an excellent source of functional proteins as well as peptides, lipids, vitamins, minerals and lactose. The functional properties, especially of the proteins and peptides have helped to transform whey from being regarded as a waste material to a valuable dairy stream containing nutritional components available to be utilised in the agri-food, biotechnology, medical and related markets (Smithers, 2008). Whey protein can be defined as the soluble proteins remaining in milk serum after coagulation of caseins at pH 4.6 and approximately 20 °C. It is primarily a mixture of β-lactoglobulin, α-lactalbumin, bovine serum and immunoglobulins (Considine, Noisuwan, Hemar, Wilkinson, Bronlund and Kasapis, 2011).

Whey proteins are widely utilized due to their high nutritional value and also due to their ability to form gels, emulsions and foams. Structural functionality of whey proteins depends on amino acid composition and sequence, net charge, the ratio of hydrophobicity to hydrophilicity as well as molecular flexibility. Further modifications can be achieved by the external factors of protein concentration, pH, temperature and ionic environment to mention but a few (Eissa, Puhl, Kadla and Khan, 2006). β-Lactoglobulin is the principal gelling agent of whey protein. Gels prepared from these proteins have been used as pH-sensitive hydrogels for the controlled delivery of biologically active substances (Gunasekaran, Xiao and Ould Eleya, 2006). Particular advantages in using whey proteins as potential devices for controlled release in pharmaceutical area, food and bio-processing applications, are that no chemical cross linking agents are required in their preparation and these are entirely biodegradable.
2.8.5 Polydextrose

Polydextrose was developed in the 1970s by scientists at Pfizer. It is a highly branched, low-molecular-weight, randomly-bonded polysaccharide of glucose with an average degree of polymerization of approximately 12 glucose units. It resists digestion in the upper gastrointestinal tract and is partially fermented in the colon, yielding an energy value of 4.18 kJ/g (equivalent to 1 kcal/g). Since regulatory approval was given, it has gradually found application as a low calorie bulking agent which can replace all or part of sugar and some fats in the foods while maintaining the pleasant texture and mouth feel (Stowell, 2009). Polydextrose is prepared commercially by vacuum bulk poly-condensation of a molten mixture of food-grade starting materials which are glucose, sorbitol, citric acid or phosphoric acid. A representative structure for polydextrose is depicted in Fig. 2.6.

![Representative structure for polydextrose](image)

\[R = \text{HYDROGEN} / \text{POLYDEXTROSE} / \text{GLUCOSE} / \text{SORBITOL} / \text{CITRIC ACID}\]

**Fig. 2.6** Representative structure for polydextrose (Craig, Troup, Auerbach and Frier, 1998)

Polydextrose is being used in a range of food applications, from beverages through to confectionery products, for both its physiological and technological benefits. Well-documented physiological effects of polydextrose include oral health (it has been shown to be non-cariogenic), dietary fiber properties, reducing glycemic impact as well
as prebiotic properties. Technologically, polydextrose provides enhancement of sweetness (example in flavoured milk drinks with 4 % w/v fructose, sucrose sweetness increases with increasing levels of polydextrose addition), it can be used to balance and reduce the sweetness level of food products and is also suitable for savoury applications (Stowell, 2009).

Polydextrose has been reported to act both as a humectant and as a crisping agent in foods. The amount of water in a food system greatly influences polydextrose functionality and its subsequent effect on the glass transition temperature of the composite food. Polydextrose powder is an amorphous glass with an anhydrous glass transition temperature of 110 °C, which is significantly higher than that of most other carbohydrates and is partly a function of its relatively low molecular weight. This high $T_g$ of polydextrose can be helpful in raising the composite $T_g$ of foods. Even at very low temperatures products may undergo undesirable changes in texture (ice and solute crystallization, starch retrogradation), structure (collapse/shrinkage) and chemical composition (oxidation, colour degradation). All of these can be prevented by use of polydextrose and/or starch retrogradation by providing structure and/or raising the composite $T_g$. According to Slade and Levine (1991) as different sugars have lower $T_g$ value than that of polydextrose, (Table 2.3) the composite $T_g$ of a food can be raised by replacing sugar with polydextrose.

Polydextrose is able to retain moisture and therefore, can function as a humectant in foods and slows undesirable changes in the moisture content. These attributes positively affect texture and shelf life in a range of applications, including confectionery, baked goods and reformed meat products (Chetana, Srinivasa and Reddy, 2005).

<table>
<thead>
<tr>
<th>Component</th>
<th>Value of $T_g$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>-28</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-32</td>
</tr>
<tr>
<td>Fructose</td>
<td>-42</td>
</tr>
<tr>
<td>Glucose</td>
<td>-43</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>-43.5</td>
</tr>
<tr>
<td>Polydextrose</td>
<td>-24</td>
</tr>
</tbody>
</table>

Table 2.3 Values of $T_g$ for selected carbohydrate ingredients (Slade and Levine, 1991)
2.9 Summary of current knowledge

On the basis of an extensive review of the scientific literature it is evident that the phenomenon of the glass transition is relevant to a variety of amorphous food systems. In addition, it has been recognised that reduced mobility in the glassy state is important for a range of bioactive components. Although it has been hypothesized that enzymes and their interaction with their substrates may be influenced within the glassy state, there has been no rigorous, systematic investigation of this important issue. Accordingly two enzymes and three different matrix systems have been selected to include a series of polysaccharides including traditional (starch) as well as novel (gellan and polydextrose) ingredients along with a protein based hydrocolloid for fundamental studies of the behavior of enzymes under a range of conditions that will encompass the transition from the rubbery to glassy states.

It is hoped that this will then form the basis of an enhanced understanding of enzymes and hydrocolloids in food formulations, and the potential of their interactions. Fundamental research in enhancing the concept of mechanical $T_g$ to analyse the behavior of enzymes in high solid matrices might lead us to innovative developments and also provide sound predictive capability and knowledge to modify and formulate existing and new food products and pharmaceutics.
Background and literature review

References


Background and literature review


Background and literature review


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Background and literature review


Background and literature review


Background and literature review


Chapter 3

Materials and methods

The purpose of this chapter is to describe the chemicals, reagents, equipment and instrumentation as well as methods used during this study. In addition, the theory behind analytical methods has been introduced. This includes the measurement of glass transition temperature by various approaches to differential scanning calorimetry (DSC), including modulated (MDSC) and micro DSC, small deformation on shear, dynamic mechanical analysis (DMA). Descriptions are also provided on procedures used to study physic-chemical behaviour by Fourier transform infrared spectrometry (FTIR), wide angle X-ray (WAXD), scanning electron microscopy (SEM) and spectrophotometry.

3.1 Basic principles for methods utilised
3.1.1 Thermal analysis
3.1.1.1 MDSC

Conventional DSC is an analytical technique in which the difference in heat flow between a sample and an inert reference is measured as a function of time and temperature: both the sample and reference are subjected to a controlled environment of time, temperature, atmosphere and pressure. It can differentiate between two types of thermal processes namely, exothermic by measuring loss of heat energy and endothermic by measuring gain in heat energy. The area under the exo- or endothermic peaks is proportional to the heat energy released or absorbed by the sample during a particular process (Rahman, Machado-Velasco, Sosa-Morales, and Velez-Ruiz, 2009). In conventional DSC, the temperature regime seen by the sample and reference is linear and the heating or cooling rates can be as fast as 200 °C/minute or as slow as 0 °C/minute (isothermal). Equation 3.1 can be used to describe either DSC or MDSC (Thomas, 2005) and Fig. 3.1 depicts the schematic diagram of a heat flux type DSC.
\[ \frac{dH}{dt} = C_p \frac{dT}{dt} + f(T,t) \]  

(3.1)

where
\[ \frac{dH}{dt} = \text{total heat flow rate}; \]
\[ C_p = \text{heat capacity}; \]
\[ \frac{dT}{dt} = \text{heating rate}; \]
\[ f(T,t) = \text{heat flow (a function of temperature and time)}; \]
Total heat flow = the sum of all heat flow;
Reversing heat flow = heat capacity component, \( C_p \times \frac{dT}{dt}; \) and
Non reversing heat flow = kinetic component, \( f(T,t) \)

Fig. 3.1 Schematic diagram of a heat flux type DSC showing the heat fluxes between DSC components and the corresponding DSC thermal model (Rady, 2009)

Although, DSC is an invaluable analytical tool, the technique has some limitations with respect to its ability to delineate complex transitions into individual contributing components. Many thermograms contain complex transitions with overlapping events, which may potentially be resolved by decreasing the heating rate and sample size at the expense of sensitivity. These limitations can lead to difficulties in the understanding and interpretation of thermal events. Alternatively, MDSC offers a solution to overcome some of the analytical limitations of DSC, by subjecting the sample to a more complex heating program with sinusoidal temperature modulation along with an underlying linear heating ramp (Rabel, Jona and Maurin, 1999).
Chapter 3

In the current study MDSC was utilized to investigate the thermal properties of research samples, gelatinization and glass transition in particular. Thermal transitions can be both reversing type, for example glass transition and melting, or non-reversing including evaporation, cold crystallization and decomposition. Most materials exhibit either of these, however, several material transitions, including polymer melting can show both reversing and non-reversing phenomena, as evident from the thermogram of bilayer film containing polycarbonate (PC) and amorphous polyethylene terephthalate (PET) (Fig. 3.2). The interpretation of transitions between 130 and 150 °C, exhibited by the DSC curve (solid line) is very difficult due to overlapping of transitions.

![MDSC thermograph of PET/PC bilayer film](image)

Fig. 3.2 MDSC thermograph of PET/PC bilayer film

MDSC results clearly show that PC $T_g$ is a reversing transition and PET recrystallization is non-reversing (Gill, Sauerbrunn & Reading 1993). Fourier transformation analysis is utilised to calculate total heat flow in MDSC and this is the average value of the raw modulated heat flow signal and the difference between the total heat flow and heat capacity (reversing) component gives the kinetic (non-reversing) component of total heat flow. To conclude, MDSC is advantageous in separating the glass transition from enthalpic relaxation phenomena and therefore, has added significant value in internal characterization and enhanced interpretation of thermal events arising from multiple origins.
3.1.1.2 Micro DSC

During food processing heat is involved at different steps. The food undergoes different types of transformations, including melting, crystallization, gelation, gelatinization, denaturation and oxidation during heating, cooling or freezing. All these transformations occur in a certain range of temperature and are associated with heat variations. Thermal analysis techniques, particularly DSC, are used as a primary approach for investigating these properties of foods. However, food processing involves mixtures of food constituents and not just simple systems; these may be mixed or diluted with a liquid (water, milk) or with a powder (sugar, salt, yeast). For simulation of such transformations and interactions, the limited volume and the lack of in situ mixing, constitute the major drawbacks of techniques involving DSC.

Micro-calorimetry provides an ideal solution for such investigations because it has the capacity to work on bulk materials and diluted solutions with a very high sensitivity. Principally, it uses the heat flux calorimetric principle for food characterization and the calorimeter consists of a measurement chamber surrounded by a detector (thermocouples, resistance wires, thermisters, and thermopiles) to integrate the heat flux exchanged by the sample contained in an adapted vessel. The chamber is insulated in a surrounding heat sink made of a material having high thermal conductivity. The heat flux for a given sample at a temperature $T_s$ can be calculated by the following equation:

$$\frac{dq_s}{dt} = -\frac{dh}{dt} + C_s T_s \frac{dT_s}{dt}$$  \hspace{1cm} (3.2)

where, $dh/dt = \text{heat flux produced by the transformation of the sample or reaction}$; $C_s = \text{heat capacity of the sample including that of container}$. The heat flux $dq_s/dt$ is exchanged with the thermostatic block at a temperature $T_p$ through a thermal resistance, $R$, which can be described as follows:

$$\frac{dq_s}{dt} = \frac{T_p - T_s}{R}$$  \hspace{1cm} (3.3)

Using equation 3.2 we know that the thermal contribution due to the heat capacity of the sample and the container is very large and will provide a major disturbance at the introduction of the container into the calorimeter. From equation 3.3, it is evident that any temperature perturbation of the thermostatic block will affect the calorimetric measurement. A calorimeter designed to be symmetrical can resolve these issues, incorporating two identical calorimetric chambers; one housing a container with the
sample and an identical reference container placed in the thermostatic block at the same
temperature, $T_p$. The heat flux difference is measured between the two chambers:

$$\frac{dq}{dt} = \frac{dqs}{dt} - \frac{dqr}{dt} = -\frac{dh}{dt} + \frac{CsTdTs}{dt} - \frac{CrdTr}{dt}$$

(3.4)

here, $C_r$ is heat capacity of the reference, including the container, and $T_r$ is temperature
of the reference. Equation 3.3 becomes:

$$\frac{dq}{dt} = Tr - Ts \div R$$

(3.5)

or by derivation

$$Rd2q/dt2 = dTr/dt - dTs/dt$$

(3.6)

By combining equations 3.5 and 3.6, the characteristic equation for the
calorimetric measurement can be obtained as:

$$\frac{dh}{dt} = -\frac{dq}{dt} + (Cs - Cr)dTp/dt - RCs2q/dt2$$

(3.7)

If $dh/dt$ corresponds to an endothermic transformation or reaction, the $dh/dt$ value is
positive and it is negative if $dh/dt$ corresponds to an exothermic transformation or
reaction. In an isothermal run, the parameter $dT_p/dt$ is null. In a small perturbation of the
temperature, $T_p$, of the thermostatic block, the corresponding thermal effect will be
minimized if the $Cs$ and $C_r$ heat capacities are similar. The last term $R Cs2q/dt2$, also
known as thermal lag, depends primarily on the thermal resistance or the time of
response of the calorimeter and the heat capacity of the sample and the container. For a
long period ($t >> RCs$) it will be negligible. Table 3.1 provides an overview of some
endothermic or exothermic effects occurring in various types of food (Parlouër, and
Benoist (2009).
Table 3.1  Endothermic and exothermic effects for different food types

<table>
<thead>
<tr>
<th>Food component</th>
<th>Endothermic effect</th>
<th>Exothermic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat, oil</td>
<td>Melting, lipidic transition</td>
<td>Crystallization, oxidation</td>
</tr>
<tr>
<td>Protein</td>
<td>Denaturation</td>
<td>Aggregation, crystallization</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Denaturation</td>
<td>Aggregation, enzymatic reactions</td>
</tr>
<tr>
<td>Starch</td>
<td>Gelatinization, glass transition</td>
<td>Retrogradation, oxidation</td>
</tr>
<tr>
<td>Milk</td>
<td>Melting</td>
<td>Crystallization, oxidation</td>
</tr>
<tr>
<td>Hydrocolloid,</td>
<td>Melting</td>
<td>Gelation</td>
</tr>
<tr>
<td>gelatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Melting, glass transition</td>
<td>Crystallization, decomposition</td>
</tr>
<tr>
<td>Yeast</td>
<td></td>
<td>Fermentation</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td>Growth, metabolism, fermentation</td>
</tr>
</tbody>
</table>

3.1.2 Rheology

The term rheology was coined in 1920s, and was inspired by a Greek quotation, “panta rei”, meaning “everything flows”, and it can be defined as the study of the flow of matter: particularly liquids but also soft solids or solids under conditions in which they flow rather than deform elastically. It applies to substances which have a complex structure, including, sludges, suspensions, polymers, many foods, bodily fluids, and other biological materials; it focuses on the flow and deformation behaviour of materials which are in the transient between solids and flow region (Barbosa-Canovas, Kokini, Ma and Ibarz, 1996). It can also be used to define the relationship between the applied force/ stress and the deformation of materials.

As it provides a enhanced understanding in regards to the physics of amorphous solids, cross-linked rubbers and polymeric glasses (Mcakenna 2012) it plays an important role in the field of food manufacturing offering useful information about raw materials, during processing and final production (Dorbraszczyk and Morgenstern 2003 and Steff, 1996). In practice, rheology is principally concerned with extending the “classical” disciplines of elasticity and (Newtonian) fluid mechanics to materials whose mechanical behaviour cannot be described with the classical theories.
3.1.2.1 Small deformation on shear

Uni-directional shear is applied on the samples in a large deformation rheological test or flow test and, in this, the geometry turns continuously in one direction. As a consequence the sample can be destroyed and thus it is now commonly used to analyse fluids (Rao, 2007). One of the most common techniques widely used to study the flow behaviour and gelation properties of materials involves small deformation studies using dynamic oscillation experiments on a rheometer. Bi-directional movement is provided by the geometry while rheological measurements are being taken. Complex modulus \((G^*)\) in Pascal units is the total resistance of the materials to the oscillatory shear. It consists of two components storage modulus \((G')\), viscous modulus \((G'')\) and these are related by the following equation:

\[
G^* = (G'^2 + G''^2)^{1/2}
\]

(3.8)

The storage and viscous moduli are of prime importance to characterize viscoelastic systems including gels. As the name suggests, storage energy in a structure is described by the storage modulus or it defines how “solid-like” a material is. Its magnitude depends on the number of interactions between different constituents in a sample under study. The value of the storage modulus is directly proportional to the number of interactions and their strength.

On the other hand, the viscous or loss modulus, describes the part of energy lost as viscous dissipation. It is related only to the number of interactions and is independent of their strength. Furthermore, the degree of viscoelasticity of a sample can be evaluated by phase angle or \(\tan \delta\) which is the ratio of loss modulus to storage modulus \((G'' / G')\). Hence, a sample is more viscous or liquid-like if it has a high value of \(\tan \delta\) while the sample with low \(\tan \delta\) would be more elastic or solid-like. Table 3.2 describes the standard rheological parameters.
Table 3.2  Standard rheological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Symbol</th>
<th>Units (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear stress</td>
<td>Force per unit area</td>
<td>$\sigma$</td>
<td>Pa</td>
</tr>
<tr>
<td>Shear strain</td>
<td>Relative deformation in shear</td>
<td>$\gamma$</td>
<td>-</td>
</tr>
<tr>
<td>Shear rate</td>
<td>Change of shear strain per unit time</td>
<td>$\dot{\gamma}$</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Resistance to flow</td>
<td>$\eta$</td>
<td>Pa.s</td>
</tr>
<tr>
<td>Shear storage modulus</td>
<td>Measure of elasticity of material</td>
<td>$G'$</td>
<td>Pa</td>
</tr>
<tr>
<td>Shear loss modulus</td>
<td>the ability of the material to dissipate energy</td>
<td>$G''$</td>
<td>Pa</td>
</tr>
<tr>
<td>Complex viscosity</td>
<td>Resistance to flow of the sample in the structured state, originating as viscous or elastic flow resistance to the oscillating movement</td>
<td>$\eta^*$</td>
<td>Pa.s</td>
</tr>
<tr>
<td>Dynamic viscosity</td>
<td>Internal friction of liquid</td>
<td>$\eta'$</td>
<td>Pa.s</td>
</tr>
<tr>
<td>Phase angle</td>
<td>Degree of viscoelasticity</td>
<td>$\tan \delta$</td>
<td>-</td>
</tr>
</tbody>
</table>

The greater the number of interactions in which friction can be created, the larger is the value of loss modulus. For rheological characterization, the storage and loss moduli can be measured as a function of:

1. Temperature, in order to gain useful information about gel formation;
2. Time, also known as a gel-cure experiment. It is important to determine if the properties of a system change over fixed time along with stabilising the sample for further explorations;
3. Frequency: this helps in classification of samples as being, for example, a weak or strong gel, depending on the kind of spectra obtained and this approach is also utilized in theoretical modelling of reduced variables;
4. Strain. This is primarily used to determine the linear viscoelastic region (LVR) of a system.

Accordingly, experimental measurements are termed as temperature ramps, time, frequency and strain sweeps and these are conducted at some fixed variables of strain, frequency or temperature (Rao, 2007).
3.1.2.2 DMA

This is a technique in which a small deformation is applied to a sample in a cyclic manner; this allows the material to respond to stress, temperature, frequency and other variables under investigation. The term is also used to refer to the analyzer that performs the test. DMA is also called DMTA (Dynamic Mechanical Thermal Analysis) and it differs from thermo-mechanical analysis (TMA) in applying an oscillatory force at a set frequency to the sample and reports changes in stiffness and damping whereas, TMA applies a constant static force to a material and observes the material changes as temperature or time varies. DMA data is used to obtain modulus information while TMA gives a coefficient of thermal expansion (CTE). Whist both techniques detect transitions, of the two, DMA is much more sensitive.

DMA works by applying a sinusoidal deformation to a sample of known geometry. The sample can be subjected to a controlled stress or a controlled strain, for a known stress, the sample will then deform a certain amount. In DMA this is done sinusoidally and deformation is related to the stiffness of the sample. DMA measures stiffness and damping, which are reported as modulus and tan δ. On application of a sinusoidal force, the modulus can be expressed as an in-phase component, the storage modulus, and an out of phase component, the loss modulus. The storage modulus, either E’ or G’, is the measure of elastic behaviour of the sample and the ratio of the loss to the storage is the tan δ (often called damping). It is a measure of the energy dissipation of a material. Modulus values change with temperature and transitions in materials can be seen as changes in the E’ or tan δ curves. This includes not only the glass transition and the melt, but also other transitions that occur in the glassy or rubbery plateau. Tg is seen as a large drop (a decade or more) in the storage modulus when viewed on a log scale against a linear temperature scale. A concurrent peak in the tan delta δ also seen. The values most commonly used to report Tg are onset of the E’ drop, the peak of the tan δ, and the peak of the E’ curve (Menard, 2008).

3.1.3 Chemical analysis: FTIR

One of the preferred methods of infrared spectroscopy is FTIR and in this IR radiation is passed through a sample. Some of the radiation is absorbed by the sample while the remainder is transmitted and the resulting spectrum represents absorption and transmission at a molecular level. This creates a molecular fingerprint of the sample, with absorption peaks that correspond to the frequencies of vibration between the bonds
of the atoms making up the sample. As each sample is a unique combination of atoms, no two compounds produce infrared spectra that are exactly the same. Therefore, infrared spectroscopy results in a positive identification (qualitative analysis) of each different kind of materials and also the size of the peaks in the spectrum provides a direct indication of the amount of the material present. According to Rees (2010) FTIR can be used for inorganic or organic compounds and also provides the following advantages over other dispersive or filter methods of infrared spectral analysis:

- It is a non-destructive technique;
- It provides a precise measurement method without the need of external calibration;
- The speed of collecting a scan can be increased to every second;
- One second scans can be co-added together to effectively remove random noise, thereby increasing sensitivity; and
- It has greater throughput and is mechanically simple with only one moving part.

The general components of FTIR are depicted in Fig. 3.3 and can be described as follows (Thermo-Nicolet, 2001):

**The energy source:** glowing black body produces infrared energy beam that passes through a small aperture which is capable of controlling the amount of the energy impinging on the sample, and eventually transferred to the detector.

**The interferometer:** The sample absorbs specific frequencies of the energy from the passing energy beam, which characterises the sample. The beam then passes through the interferometer where the spectral encoding takes place and then the signal exits the interferometer.

**The detector:** Finally the beam passes to the detectors which are designed to detect and measure the signal as an interferogram.

**The computer:** Software in a computer is designed to perform the Fourier transformation on the signals received from the detector and producing the final infrared spectrum.
3.1.4 WAXD

The basic principle of all X-ray diffraction phenomena is scattering of X-ray electrons (Liebhafsky, Pfeiffer, Winslow and Zemany, 1966) and is used to provide information regarding the structure of materials at atomic resolution (I’Ason, Bacon, Lambart, Miles, Morris, Wright and Nave, 1987). WAXD scattering is used to measure the degree of crystallinity and also to assess the structure at the unit cell level in comparison to the small angle scattering, which provides information at the lamellar level (Mano, 2007).

Readings of the intensity on the detector are shown as an angular position of the crystal monochromator when, a tungsten target is bombarded by constant rate of electrons. These positions are related to known wavelengths and are specified in terms of $2\theta$, the angle between incident and reflected beam and corresponding to twice the angle. X-ray diffraction is usually taken within certain angles of $2\theta$ at a constant rate per time unit (min) and the relative crystallinity is calculated by dividing the area of a particular peak by the total area of all of the peaks observed (Rindav-Westling, Standing, Hermansson and Gatenholm, 1998).

The solid matter can then be described as amorphous or crystalline: as being in the crystalline state when the atoms aggregate systematically and an amorphous state when the arrangement is random. When an X-ray beam bombards an atom the electrons in the orbits around the atom start to oscillate in all the directions with the same frequency as the incoming beam. This produces destructive interference, that is the combining waves are out of phase and there is no resultant energy leaving the solid sample. On the other hand, constructive interference occurs when the atoms in a crystal are aggregated in a regular pattern and in limited directions. As a result, the waves will be in phase with
well-defined X-ray beams radiating from the sample in different directions, the angle \( \theta \) can then be estimated according to the distance between planes (Jenkins & Snyder, 1996). The signals are also affected by the bonds existing within the materials, for example, if the energy of the particles is greater than the binding energy of the atomic electron, there is a possibility that the atomic electron could be ejected from its atomic position with a kinetic energy \( (E - \phi) \) equal to the difference between the energy \( (E) \) of the original particle and the binding energy \( (\phi) \) of the atomic electron (Arnaud, Smith and Semiginowska, 2011 and Jenkins, 2000).

### 3.1.5 Structural analysis using SEM

SEM produces an image of a sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals containing information about the characteristics of the sample, particularly surface topography, composition as well as electrical conductivity. Secondary electrons, back scattered electrons (BSE), characteristic X-rays, light (cathodoluminescence), specimen current and transmitted electrons are the different types of signals produced by an SEM.

In the most common or standard detection mode, secondary electron imaging, the SEM can produce very high-resolution images of a sample surface revealing details having a size of approximately 1 to 5 nm in size. SEM micrographs yield a characteristic three-dimensional appearance making them very useful for understanding the surface structure of a sample. A wide range of magnifications is also possible from about \( \times 25 \) (equivalent to that of a powerful hand-lens) to \( \times 250,000 \), corresponding to 250 times the magnification limit of the best light microscopes. The components of an SEM are shown in Figure 3.4 and generally consist of a lens system, electron gun, and detector as well as visual and photo recording cathode ray tubes. The beam from the electron gun is passed through electron lenses in order to decrease spot size, thus producing a clear image. Subsequently, the signals from the beam-specimen from different locations are collected by the electron detector. This then converts the collected signals to point–by–point intensity differences on the monitor providing an image of the sample (FEI, 2008 and Kimseng & Meissel, 2001).
3.1.6 Spectrophotometry

Spectroscopy is based on the fact that electromagnetic radiation (EMR) interacts with atoms and molecules in discrete ways to produce absorption or emission profiles specific to individual elements. Our eye acts as detector to perceive color and the range of wavelengths in the EMR detected by human eyes is known as the visible range. A sine wave is used to represent EMR and the wavelength, $\lambda$, is the distance between two successive peaks (Fig. 3.5). This can also be expressed as a function of the frequency, $\nu$, the number of peaks passing a given point per second and the two terms are related through the equation $c = \nu \lambda$ where, $c$ a constant corresponding to the velocity of light in a vacuum (Burgess, 2007).

Fig. 3.5 The wavelength ($\lambda$) of electromagnetic radiation

The full electromagnetic radiation spectrum is continuous and each region merges slowly with the next. For spectrophotometric purposes, light in the ultraviolet and
visible regions are selected in terms of wavelength values expressed in nanometers. Other units encountered in the literature are the Angstrom (Å) and the millimicron (m μ),

\[ \text{Im} = 1 \text{m} \mu = 10 \text{Å} = 10^{-9} \text{meters} \]  \hspace{1cm} (3.9)

The wavelengths for various spectral regions as set by the Joint Committee on Nomenclature in Applied Spectroscopy are these are listed in Table 3.3.

### Table 3.3 Wavelengths for different spectral regions of EMR

<table>
<thead>
<tr>
<th>Region</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Far ultraviolet</td>
<td>10-200</td>
</tr>
<tr>
<td>Near ultraviolet</td>
<td>200-380</td>
</tr>
<tr>
<td>Visible</td>
<td>380-780</td>
</tr>
<tr>
<td>Near infrared</td>
<td>780-3000</td>
</tr>
<tr>
<td>Middle infrared</td>
<td>3000-30,000</td>
</tr>
<tr>
<td>Far infrared</td>
<td>30,000-300,000</td>
</tr>
<tr>
<td>Microwave</td>
<td>300,000-1,000,000,000</td>
</tr>
</tbody>
</table>

During passage of monochromatic electromagnetic radiation with the intensity \( I_0 \) through a solution of an analyte, some part of it is absorbed whereas the remainder is transmitted. When the intensity of the transmitted monochromatic radiation (measured on the side of the solution where the detector is located) is \( I \), the absorbance of radiation (A) can be defined as:

\[ A = \log_{10} \left( \frac{1}{T} \right) = \log_{10} \left( \frac{I_0}{I} \right) \]  \hspace{1cm} (3.10)

where, \( T \) is the transmittance (\( I/I_0 \)).

The measurement of absorption of radiation by a solution of molecules is governed by Beer’s law, according to which, the absorbance (A) is proportional to the path length through which the radiation (b) and the concentration of the substance in the solution (c):

\[ A = \varepsilon bc \]  \hspace{1cm} (3.11)
When b is expressed in centimeters and c in moles per litre (M), \( \varepsilon \) is known as the molar absorptivity and it is constant for a particular substance. According to Beer’s law the absorbance is proportional to the molar absorptivity, the path length and the analyte concentration in the solution. When \( \varepsilon \) and b are known, the unknown molar concentration can be determined from the reading of absorbance. The path length is specified for the equipment used and measurements are normally conducted at a path length of 1 cm. the absorbance is dependent on the wavelength, solvent, pH and to some extent on the temperature. So it is important that these parameters are controlled for each solution including blanks, standards and sample solutions, if accurate results are to be obtained.

Ultraviolet and visible spectrophotometers are regarded as one of the most important analytical instruments in the modern day laboratory as they are simple to use and versatile in operation leading to high speed, accuracy and cost effectiveness (Hansen, Pedersen-Bjergaard and Rasmussen, 2012 and Thermo Spectronic 2012).

3.1.7 Spray drying

Spray drying is a relatively well-established technique with reports of the first spray dryer having been constructed as early as 1878. In spray drying, a liquid feed is transformed from a fluid state (solution, dispersion or paste) into a dried particulate material by spraying the prepared fluid into a hot drying medium. Spray drying technique involves rapid evaporation of the solvent from the droplets and therefore is widely utilized for drying materials which are relatively heat sensitive including various foods, pharmaceuticals along with other active ingredients (flavors, enzymes) (Re, 1998).

The advantages of spray drying have ensured its continued dominance in many of its original applications. These advantages include availability of equipment, high production capacities (up to 4,000 kg/h), low process costs (20 and 30% of that of freeze drying and vacuum drying, respectively), a wide choice of carrier solids, excellent retention of volatiles, low effective process temperatures as well as high stability of finished ingredients. In the past decade, spray drying has increasingly been applied to microencapsulation of a variety of food and pharmaceutical components. The resultant capsules produced by spray drying often have irregular geometry and may be aggregates of a small number of particles, each having small droplets of core materials dispersed throughout the structure (Thies, 1996).
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Spray drying is a continuous processing operation; it involves a number of steps, which are atomisation, mixing of spray and air, evaporation and product separation. The initial step consists of selecting a suitable carrier of wall material most commonly from one of the following: hydrolysed starches, emulsifying starches, gums and proteins (gelatin, casein, soya protein and whey protein). Each of these has its own advantages and limitations and selection depends on the characteristics of the active ingredient being encapsulated. Feed preparation is the preliminary stage in spray drying and the concentration/viscosity require adjustment to facilitate adequately atomization and drying. Also involved is emulsification, dispersion or dissolution of the core materials in a relatively concentrated solution of shell material. The resultant liquid is then introduced as droplets into the heated chamber of the spray drier, in which the atomized particles are mixed with hot air flowing in either a concurrent or counter-current direction. Rapid dehydration through evaporation of water takes place with its contact within the heated chamber and the final shape of particles is set once they are sufficiently dry with typical particle diameters of between 10 and 300 µm. The powdered product can then be collected from the outlet of the spray drier (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007; Madene, Jacquot, Scher, & Desobry, 2006; Re, 1998 and Thies, 1996).

Many different types of spray dryer are available, differing in their size, shape, air flow pattern and type of atomization. The latter can be accomplished by the use of single-fluid-high-pressure spray nozzle, a two-fluid nozzle or a centrifugal wheel (Reineccius, 2001). In the current study a spray drier fitted with a double fluid nozzle was utilized. In this arrangement, the inner nozzle develops the liquid jet and the outer nozzle develops the compressed air jet which functions to atomise the liquid as it emerges from the jet to form the required fine spray. For cleaning purposes, the jet and outer nozzle can be unscrewed from the main assembly (Fig. 3.6).
3.2 Materials

All chemicals including gellan, polydextrose, starch, whey protein isolate and enzymes used in analytical procedures were of analytical grade or of the highest purity available, unless otherwise specified. Details of both the supplier and selected product codes for the chemicals used in the current study are presented in Table 3.4.

3.2.1 Polysaccharides

3.2.1.1 Gellan

Deacylated gellan gum, Kelcogel; batch no. 77109A, was provided by Nutrasweet Kelco Company (Santiago, CA). It is the extracellular polysaccharide produced by the microorganism Sphingomonas elodea during aerobic fermentation.
Table 3.4  Details of chemicals and suppliers

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Chemical(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrasweet Kelco (Santiago, CA)</td>
<td>Deacylated Gellan</td>
</tr>
<tr>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
<td>3,5-dinitrosalicylic acid α-Glucosidase, from (<em>Bacillus stearothermophilus</em>), Calcium chloride, L-Glutathione, p-Nitrophenyl α-D-Glucoside (pNPG), Potassium phosphate, monobasic, anhydrous, Silicone oil, Starch from wheat, unmodified,</td>
</tr>
<tr>
<td>MG Nutritionals, Murray Goulburn Co-operative Co. Ltd, VIC, Australia</td>
<td>Whey Protein Isolate</td>
</tr>
<tr>
<td>Tate &amp; Lyle (South Melbourne, VIC, Australia)</td>
<td>Polydextrose</td>
</tr>
<tr>
<td>Megazyme Int. (Wicklow, Ireland)</td>
<td>α-amylase (<em>B. licheniformis</em>)</td>
</tr>
<tr>
<td>BDH, Port Fairy, VIC Australia</td>
<td>Potassium acetate, Sodium carbonate anhydrous</td>
</tr>
<tr>
<td>Merck KgaA, Frankfurter StraBe 250, Germany</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>EMD Chemicals, Inc. (San Diego, CA)</td>
<td>D(+) Glucose, anhydrous</td>
</tr>
<tr>
<td>Chem-supply (GILLMAN, SA)</td>
<td>Sodium potassium tartrate</td>
</tr>
<tr>
<td>Millipore Australia Pty. Ltd.</td>
<td>Millipore Type 2 water</td>
</tr>
</tbody>
</table>

3.2.1.2 Starch

Starch from wheat, unmodified (C₆H₁₀O₅)n, is a high polymeric carbohydrate. It was purchased from Sigma-Aldrich (St. Louis, MO, USA) in the form of white powder with moisture content of 9.6 per cent.

3.2.2 Protein: Whey protein isolate

In this study standard whey protein isolate (WPI) from MG Nutritionals, Murray Goulburn Co-operative Ltd, VIC, Australia was used. The composition of the WPI as reported by the supplier was 91.3% protein (TN × 6.38), 0.7% fat, 3.5% moisture, 3.8% ash and 0.44% lactose. pH of 10% (w/w) solution was 6.3, bulk density was 0.45 g/mL and a standard plate count produced 9,900 cfu/g.
3.2.3 Co-solute: polydextrose

Polydextrose used was Sta-Lite III powder, supplied by Tate & Lyle ANZ, Pvt. Limited (Decatur, IL) (Illinois – USA). The specifications supplied by the manufacturer were that the powder was of 90% purity with 4% moisture and had passed microbiological testing under the food grade standards. It was described as a bulk amorphous polymer with an average degree of polymerization of twelve glucose residues. With the 1,6-glycosidic linkage predominating, polydextrose is described as a randomly bonded condensation polymer of D-glucose, sorbitol and citric acid.

3.2.4 Enzymes

3.2.4.1 $\alpha$-Amylase: $\alpha$-Amylase ($B.\ licheniformis$) solution was purchased from Megazyme, and used without dilution.

3.2.4.2 $\alpha$-Glucosidase from $B.\ stereothermophilus$ was from Sigma-Aldrich, it came as a lyophilized powder which was dissolved in phosphate buffer prior to use.

3.3 Apparatus and auxiliary equipment

The items of equipment used, together with the details of manufacturers and model numbers are presented in Table 3.5.
Table 3.5 Description of instrumentation and equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer/supplier</th>
<th>Model no</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDSC</td>
<td>TA Instruments</td>
<td>DSC Q 2000</td>
</tr>
<tr>
<td>Micro DSC</td>
<td>SETARAM, Caluire, France</td>
<td>micro-DSC-III calorimeter</td>
</tr>
<tr>
<td>Rheometer</td>
<td>TA Instruments, New Castle, USA</td>
<td>ARG-2</td>
</tr>
<tr>
<td>DMA</td>
<td>Perkin Elmer, MA, USA</td>
<td>DMA 8000</td>
</tr>
<tr>
<td>FTIR</td>
<td>Perkin Elmer, MA, USA</td>
<td>Spectrum 100</td>
</tr>
<tr>
<td>SEM</td>
<td>FEI</td>
<td>FEI Quanta 200 ESEM</td>
</tr>
<tr>
<td>WAXD</td>
<td>Germany</td>
<td>Bruker Endeavour D4</td>
</tr>
<tr>
<td>UV-VIS Spectrophotometer</td>
<td>Perkin Elmer, Singapore</td>
<td>Lambda 35</td>
</tr>
<tr>
<td>Spray dryer</td>
<td>Keison Products, England</td>
<td>LabPlantSD Basic FT30MKIII</td>
</tr>
<tr>
<td>Freeze dryer</td>
<td>Operon Co. Ltd, South Korea</td>
<td>Operon Freeze dryer -55 °C</td>
</tr>
<tr>
<td>Fridge freezer</td>
<td>ARB, Australia</td>
<td></td>
</tr>
</tbody>
</table>

3.3.1 DSC systems

3.3.1.1 DSC-Q2000

This unit was equipped with Advanced Tzero™ and Modulated DSC™ technologies, in addition to Platinum™ software and a 50-position auto sampler facilitating scheduling of tests automatically and during off–work periods (TA Instruments, n.d.-b). The MDSC Q2000 instrument used in this study is shown in the Fig. 3.7 and this was found to provide high stability, sensitivity and resolution.
3.3.1.2 Micro DSC (SETARAM)

For the study of thermal profiles of low to intermediate solids systems, a micro DSC (Fig. 3.8) was utilized as it was found to be more sensitive in revealing thermal events.

3.3.2 Rheometers

3.3.2.1 Advanced Rheometer Generation 2 (ARG-2)

The unit utilised is described by the manufacturer as a state-of-the-art instrument with patented magnetic thrust bearing technology which equips it for ultralow, nano-torque control benefitting characterization of sensitive structures, requiring smaller sample volumes and better resolution at the low frequencies. It is a cutting-edge
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rheometer due to its superior capabilities of controlled stress, direct strain and controlled rate.

The instrument also features Smart Swap™ Geometries that detects and stores geometry information automatically. The ARG-2 (Fig. 3.9) is also operational with a peltier plate that can hold temperature between -20 °C and 200 °C or with an environment test chamber (ETC) which is designed on the controlled convection/radiant heating concept. It is primarily used for polymer/biopolymer applications and can be used with parallel plate, cone and plate, disposable plate and rectangular torsion clamps for solids. The ETC has a temperature range of -160 °C to 600 °C with heating/cooling rates of up to 60 °C/min (TA Instruments, n.d.-a).

Fig. 3.9 Advanced Rheometer Generation 2 (ARG-2)
3.3.2.2 DMA 8000

This instrument is shown in Fig 3.10 and is described by the manufacturer as having an innovative design, being light weight and having a small footprint. It has the unique feature of rotating analysis head, which can be oriented through a full 180° for optimal analysis head configuration for virtually any test type and sample geometry. It has a wide temperature range (-190 °C to 600 °C) along with wide frequency range (0.001 to 600 Hz). It can measure within +10 to -10 N of force and comes with optional accessories of humidity generator and controller which delivers the capability to apply and accurately control relative humidity to the sample environment.

Fig. 3.10 DMA 8000

Any sample can be loaded and analysed with ease by using material pockets that are specifically designed to work with the DMA 8000 These innovative pockets allow powdered or samples which cannot support their own weight including drugs, gels, natural products like tea, coffee, herbs. and low viscosity materials to be investigated by DMA. There are minimal connections from motor to sample minimising any compliance issues; it has no air bearing in its design thus reducing maintenance and no thermal drift and/or no issues from environmental studies.

3.3.3 Spectrum 100

Spectrum 100 (Fig. 3.11) is a highly sensitive instrument with advanced digital signal processing that reduces signal artefacts and improves response linearity. Exclusive optics maximize the source output, increasing the signal-to-noise ratio
without sacrificing source lifetime and high-quality. Low angle, off axis optics minimize aberrations providing the highest optical efficiency. Kinematically mounted optics ensures perfect optical alignment, delivering consistent results and accessory vapour seal prevents dust or solvent contamination thereby, reducing measurement variations. Spectrum software provides user friendly interface that minimizes training and ensures consistent operation.

![Spectrum 100](image)

**Fig. 3.11 Spectrum 100**

### 3.3.4 Bruker D4 Endeavour Wide Angle Diffractometer

This instrument (Fig. 3.12) features a high precision 2 circle goniometer, modern X-ray optics and detectors which yield excellent analytical results with unrivalled speed. It can handle a wide variety of samples of different dimensions and consistency which can be loaded at same time and can be analysed differently. Its software DIFFRAC plus allow fully automatic process of measurements, analyses and sample changing. Data exchange between the equipment and the operator software uses TCP/IP based client /server principle meaning one can start and monitor measurements from any computer in the network.

This work utilised the D4 Endeavour unit available within the Integrated Victorian X-ray Structural Determination and Materials Characterization Facility located at RMIT University. It was equipped with a 66 position auto-sampler and innovative 1-dimensional X-ray detector- Lynxeye which enormously reduces measurement time per sample.
3.3.5 SEM

The SEM used in this study is located the RMIT Microscopy and Microanalysis Facility (RMMF) and features three imaging modes – high-vacuum, low-vacuum and ESEM™ hence accommodating the widest range of samples of any SEM system. It minimizes the amount of sample preparation as low and ESEM vacuum capabilities enable charge free imaging and analysis of non-conductive specimens and/or hydrated specimens. Table 3.6 lists some of the specifications of the unit which is depicted in and Fig. 3.13.

Table 3.6 Specifications of the FEI Quanta 200 ESEM

<table>
<thead>
<tr>
<th>Resolution</th>
<th>High vacuum</th>
<th>Low vacuum</th>
<th>ESEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.0 nm at 30 kV (SE)</td>
<td>3.0 nm at 30 kV (SE)</td>
<td>3.0 nm at 30 kV (SE)</td>
</tr>
<tr>
<td></td>
<td>4.0 nm at 30 kV (BSE)</td>
<td>- 4.0 nm at 30 kV (BSE)</td>
<td>- &lt; 12 nm at 3 kV (SE)</td>
</tr>
<tr>
<td></td>
<td>10 nm at 3 kV (SE)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chamber vacuum</td>
<td>High-vacuum: &lt; 6e-4 Pa</td>
<td>Low-vacuum: 10 to 130 Pa</td>
<td>ESEM-vacuum: 10 to 2600 Pa</td>
</tr>
</tbody>
</table>
3.3.6 UV-VIS (Lambda 35)

The spectrophotometer (Fig. 3.14) is described as being suitable for conducting measurements on liquids, solids, pastes and powder samples. It features true double beam operation with high throughput, low stray-light optics and pre-aligned deuterium and tungsten-halogen lamps. It has the advantage of giving very high stability, accuracy and reproducibility. Its wavelength range is 190 to 1100 nm; bandwidth of 0.5 to 4 nm and can be used for following modes of operation: Scanning, wavelength program, time drive, rate, quant or scanning quant.
3.3.7 Spray Dryer

SD-Basic spray dryer, FT30MK III (Fig. 3.15), is a bench mounted laboratory scale spray dryer for processing aqueous emulsions, solutions, suspensions and colloids. A self-priming peristaltic pump delivers the liquid sample through a small diameter jet into the main chamber and at the same time compressed air enters the outer tube of the jet which causes the liquid to emerge as a fine atomized spray into the drying chamber. Jet nozzle assembly (0.5 mm standard, 1.0 and 1.5 mm jets are optional) comes with manually operated de-blocking needle for effective cleaning between runs. Other characteristics are: Product flow rate: 0-1500 mL/h; air inlet temperature: 200 °C max; heater capacity: 3 kW and drying air through flow: 70 m³/hr (fixed) (SD basic spray dryer instruction manual, 2012).

Fig. 3.15 SD-basic spray dryer (FT30MK III)
3.3.8 Operon Freeze dryer

This bench-top freeze dryer was used to prepare samples for gold plating carried out in preparation for examining them by SEM. It has maximum cold trap temperature range of -70 to -90 °C and requires a 200 or 400 L per minute vacuum pump.

Fig. 3.16 Operon freeze dryer

3.3.9 Fridge freezer

The ARB fridge freezer (Fig. 3.18) was used in the current study to provide temperature controlled conditions in the range of -18 to 10 °C.

Fig. 3.17 ARB fridge freezer
References


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SD Basic spray dryer instruction manual retrieved 1st October 2012 http://www.keison.co.uk/labplant_sdbasic.shtml
Chapter 4

Results and discussion

Structural studies on matrices of deacylated gellan with polydextrose

Journal article published in *Food Chemistry* presented as Chapter 4
1. Introduction

Decayed gellan gum, a microbial polysaccharide, is now commercially available and has good prospects as a food ingredient. It has the potential for partial or total replacement of existing gelling agents, and thus, is an important tool for the development of new food products (Gibson, 1992). Gellan is produced with aerobic fermentation by the micro organism Pseudomonas elodea. It is an excellent gelling agent, having functional properties similar to alginate, carrageenan, pectin, gelatin and starch (Chandrasekaran, Millane, Arnott, & Atkins, 1988; Sanderson & Clark, 1983), and is a biodegradable polymer. Gellan gum is a linear anionic heteropolysaccharide having a tetrasaccharide repeating unit consisting of rhamnose, \( \alpha \)-glucose and \( \alpha \)-glucoronic acid in the ratio of 1:2:1. As it forms a relatively transparent gel, which is particularly resistant to heat and acid when compared to those formed by other polysaccharides, gellan is increasingly used in the food industry and biotechnology (Bajaj & Singhal, 2007; Evageliou, Galanaki, Gardeli, & Komaitis, 2011).

The precise gelation properties depend crucially on the presence of cations. It is now accepted that gellan gum may undergo a thermally reversible helix-coil transition, with the junction zones of gellan gum gels being formed by aggregation of double helical regions of the polymeric network (Champman, Chilvers, Miles, & Morris, 1990; Crescenzi, Dentini, Coviello, & Rizzo, 1986; Dentini, Coviello, Burchard, & Crescenzi, 1988; Grasdalen & Simidsrod, 1987; Milas, Shi, & Rinaudo, 1990). The disorder-to-order transformation upon cooling is analogous to that occurring during the gelation of other polysaccharides. There is a considerable body of literature regarding gellan gelation in terms of varying ionic conditions; examples include: Grasdalen and Simidsrod (1987), Miyoshi, Takaya, and Nishinari (1996), Nickerson and Paulson (2005), Tang, Tung, and Zeng (1997a, 1997b). Compression strength and deformation of gels formed with mono- and di-valent cations have been reported by Tang, Tung, and Zeng (1996). Texture, clarity and the influence of temperature with fructose and sucrose have been investigated by Tang, Mao, Tung, and Swanson (2001).

Sworn and Kasapis (1998) studied the viscoelastic properties of gellan gums as affected by conformation and molecular weight of co-solute. Formation of composite structures in the gellan polysaccharide/sugar systems have been engineered and studied by Whittaker, Al-Ruqaie, Kasapis, and Richardson (1997). In addition, tangible evidence of the morphology of the gellan network in a high sugar environment has been elaborated by the use of transmission electron microscopy (Kasapis, 2006; Kasapis, Abeysekera, Atkin, Deszczyynski, & Mitchell, 2002). However, there is a deficiency of systematic studies on the kinetics of structure development of materials containing sugars, proteins, dietary fibre components and/or other polysaccharides. This is hampering the ability of the industry to match the kinetics of formation to the timescale of manufacturing processes in order to enhance consumer acceptance of many high-solid formulations (Al-Marhoobi & Kasapis, 2005).

Presently, we have focused on characterising the thermomechanical properties of deacylated gellan gum with varying...
concentrations of polydextrose, which is a low-calorie bulking agent that can replace sugar and fat in foods while maintaining texture and mouthfeel (Mitchell, 1996). Polydextrose is prepared commercially by vacuum polycondensation of a molten mixture of food-grade starting materials, i.e. glucose, sorbitol, and either citric or phosphoric acid. It is not sweet and is used to replace the physical functionality of high-calorie ingredients including sugar, not their sweetness. It yields only 4.18 kJ/g, hence being advantageous as a dual purpose ingredient that can reduce calories from carbohydrates as well as fats (Lindsay, 2008).

Gellan and polydextrose in mixture have been used to produce low-calorie, sugar or gelatin-free jelly sweets, where the final total solids content exceeds 80%. In comparison to other gums, deacetylated gellan does not appreciably add to the viscosity of the depositing mix, hence is functional for confectionary work (Craig, Troup, Auerbach, & Frier, 1998; Stowell, 2009). In this study, we utilised several physicochemical techniques and theoretical modelling to discuss the structural properties of the gellan/polydextrose binary mixture in some detail. We believe that the ability to vary the solid-like properties, in a systematic and controlled manner, has the potential to lead to enhanced functionality and wider applications in the food and pharmaceutical industry for these preparations.

2. Materials and methods

2.1. Materials

Decayed gellan powder, Kelcogel trade name, was provided by Nutrasweet Kelco Company (Santiago, CA, USA). Polydextrose used was Sta-Lite III powder, supplied by Tate & Lyle, ANZ, Pvt. Limited (Decatur, IL, USA). The polydextrose powder was of 90% purity with 4% moisture and had passed the microbiological testing under the food grade standards. It was a bulk amorphous polymer with an average degree of polymerisation of twelve glucose residues. Calcium chloride was from Sigma Aldrich and Milli-Q water of equal weight (±30 g). Both pans were then placed into the calorimeter chamber and equilibrated for ten minutes to eliminate the effect of thermal history, prior to heating to 90 °C at 0.5 °C/min followed by cooling at the same rate to 5 °C.

For high levels of solids, a modulated differential scanning calorimeter (DSC Q2000) from TA instruments (New Castle, DE, USA) with nitrogen purge gas (flow rate of 50 ml/min) was used to perform thermal measurements. The instrument was fitted with a refrigerated cooling system to achieve temperatures down to −90 °C. Calibration of the heat flow signals using a traceable indium standard (ΔHf = 28.3 J/g) and the heat capacity response using a sapphire standard enabled accurate measurements. Tzero pans with lids and sample size of 8 mg were used. Samples were heated to 97 °C, cooled to −90 °C and again heated to 95 °C at modulation amplitude of 0.53 °C for each period of 40 s. The glass transition was recorded from the second heating scan using a scan rate of 1 °C/min and an empty Tzero pan was taken as the reference. In both rheological and calorimetric studies, essentially overlapping triplicates of mechanical spectra for rheology and thermograms for calorimetry are reported.

2.3.2. Calorimetric measurements

For low and intermediate solids, DSC measurements were carried out with a Setaram micro DSC-III calorimeter, (Caluire, France). Approximately 900 mg of sample was filled into a DSC pan and sealed hermetically; a reference pan was also taken with Milli-Q water of equal weight (±30 μg). Both pans were then placed into the calorimeter chamber and equilibrated for ten minutes to eliminate the effect of thermal history, prior to heating to 90 °C at 0.5 °C/min followed by cooling at the same rate to 5 °C.

2.3.3. Fourier transform infrared spectroscopy

Perkin Elmer Spectrum 100 spectrometer, equipped with MIRacle™ ZnSe single reflection ATR plate (Perkin-Elmer, Norwalk, CT, USA), was used to obtain the FTIR spectra for gellan and polydextrose preparations in an effort to identify the nature of interactions between the two constituents. Absorbance mode was utilised to obtain the spectra for the wavelength range of 600–4000 cm⁻¹ with a resolution of 4 cm⁻¹. This was corrected against the background spectrum of the solvent at ambient temperature before plotting.

2.3.4. Wide angle X-ray diffraction

Measurements were performed using Bruker D8 Endeavour (Karlsruhe, Germany). An accelerating voltage and current of 40 kV and 40 mA, respectively, were employed to produce the diffractograms of gellan and polydextrose alone and their mixtures. Samples were freeze-dried and powdered prior to placing on sample magazine. These were then continuously scanned to obtain the raw data in a 2θ range between 5 ° and 90 ° in measuring intervals of 0.1 °, and subsequently analysed using the Bruker Advanced X-ray Solutions software, DIFFRAC™ESD Evaluation (Eva), version 10.0 revision 1.

2.3.5. Environmental scanning electron microscopy

FEI Quanta 200 ESEM (Hillsboro, OR, USA) was used to obtain micrographs of gellan and polydextrose matrices in order to provide tangible evidence of changes in network morphology and phase topology of samples under investigation. This was targeted as a function of thermal treatment and polymer/co-solute composition. Freeze dried and gold plated samples were imaged under a...
high-vacuum mode at an accelerating voltage of 30 kV, spot size 5 and working distance between 9.2 and 11.2 mm.

3. Results and discussion

3.1. Structural properties of polysaccharide in the presence of increasing levels of co-solute

The cooling profiles of storage modulus for 1% gellan with varying concentrations of polydextrose are depicted in Fig. 1a at a scan rate of 1 °C/min, 0.01% strain and frequency of 10 rad/s. It was observed that at low levels of polydextrose (up to 30%, w/w) stronger and more thermally stable structures were formed. Nishinari, Watase, Williams, and Phillips (1990) and Nishinari, Watase, Miyoshi, Takaya, and Oakenfull (1995) emphasised the role of hydrogen bonding in the proliferation of the stress supporting polymer–polymer interactions in the presence of sugars, with hydrogen bond disruptors (e.g. urea or guanidine hydrochloride) reducing the network strength.

The nature of modulus development changes entirely at intermediate levels of the co-solute (40–60%, w/w) in Fig. 1a for which there is an abrupt drop in the values. This reduction in polysaccharide network strength is accompanied by a decline in the enthalpy of the coil-to-helix transition obtained from micro DSC with controlled cooling (scan rate of 0.5 °C/min) in Fig. 2a. Sworn and Kasapis (1999) have suggested that this drop in mechanical strength of polysaccharide at intermediate levels of co-solute (glucose syrup and 1:1 mixtures of glucose syrup with sucrose) can be attributed to the transformation from a highly enthalpic and aggregated structure to a network having reduced cross-linking.

Furthermore, high levels of solids (from 70–83%, w/w) in Fig. 1a exhibit a progressive four decade enhancement of shear modulus. This ranges from just below $10^4$–$10^8$ Pa at 0 °C as the sample increasingly goes through a glass transition. It has been argued that this dramatic change in viscoelasticity is the result of enhanced flexibility of chain segments between intermolecular associations.

![Fig. 1](image1.png)

**Fig. 1.** (a) Cooling profiles of storage modulus for 1% gellan with 0 (●), 10% (■), 20% (▲), 30% (●), 40% (○), 50% (□), 60% (△), 70% (◇), 79% (●) and 83% (+) polydextrose (scan rate: 1 °C/min; frequency: 10 rad/s; strain: 0.01%) and (b) variation of normalised storage modulus as a function of sugar concentration for 1% gellan with varying concentration (w/w) of polydextrose (●).

![Fig. 2](image2.png)

**Fig. 2.** (a) Micro DSC cooling profiles for 1% gellan with 0%, 20%, 40%, 60%, and 79% polydextrose successively upwards (scan rate: 0.5 °C/min), (b) trend of the change in enthalpy for 1% gellan with varying concentrations (w/w) of polydextrose and (c) MDSC cooling (—) and heating (×××) profile for 1% gellan with 79% polydextrose, and 80% polydextrose to subzero temperature (scan rate: 1 °C/min).
that render important entropic contributions to the elasticity of the network at the upper range of co-solute concentrations (Groot & Agterof, 1995). Changes in mechanical properties are also demonstrated through the variation of normalised storage modulus of gellan and polydextrose matrices shown in Fig. 1b, which clearly shows three distinct regions of network strength with polydextrose content. The rheological behaviour of polydextrose is similar to that of other co-solutes described in the literature, i.e. the addition of sucrose, fructose, glucose or glucose syrup at concentrations of up to 40% (0.5–2.0 mol/L) creates stronger and more thermally stable structures for the industrially important polysaccharides of κ-carrageenan, agarose and deacetylated gellan gum (Watase, Nishinari, Williams, & Phillips, 1990). The present work, however, extends observations to higher levels of co-solute solids thus unveiling additional molecular processes in the condensed state.

3.2. Effect of temperature on low and high solid gellan and polysaccharide matrices

The protocol of first heating and then cooling samples in micro DSC at a controlled scan rate of 0.5 °C/min was successful in revealing well defined exothermic events. The average of essentially three overlapping traces for each preparation is reported here and the area delimited by the peaks in Fig. 2a has been used to obtain values of change in enthalpy (ΔH) in Fig. 2b. These were found to increase up to 40% solids and then decrease dramatically to show almost flat thermal events. Results are in accordance to mechanical observations in the preceding section, hence confirming the reduced ability of gellan gum to form macromolecular associations at higher levels of co-solute.

The cooling and heating profiles of gellan with polydextrose, and polydextrose alone samples (total solid concentration in both cases of 80%, w/w) exhibit glass transition phenomena obtained from MDSC at the controlled scan rate of 1 °C/min (Fig. 2c). Due to the reduced thermal motions and molecular mobility during cooling, the heat capacity decreases along with the volume of the gel. Traditionally, the onset and endset of the heat capacity change are considered as the empirical bounds of the glass transition region, thus providing the calorimetrically derived glass transition temperature (Tg) at the central point of the transition. This is observed at approximately −45 °C for both 1% gellan with 79% polydextrose, and the sample of 80% polydextrose alone. It has been suggested by Goff, Caldwell, and Stanley (1993) and Roos (1993) that the thermal spectrum of proteins or polysaccharides in the presence of a high sugar environment is dominated by the vitrification of the latter. Thus, the calorimetric Tg for polysaccharide-sugar samples can be predicted by the total level of solids in the system, with the low polysaccharide concentration acting as a “cross contaminant” in the mixture.

3.3. The viscoelastic master curve in high solid matrices of gellan with polydextrose

Progress in viscoelasticity upon cooling both polysaccharide with co-solute, and co-solute alone samples at 80% solids are shown in Fig. 3. Extensive temperature variation at a controlled scan rate from 70 to −50 °C results in three distinct regions, i.e. the rubbery plateau, glass transition and glassy state. The storage modulus develops six orders of magnitude from 10⁶ Pa in the rubbery plateau at 70 °C to almost 10¹⁰ Pa at −50 °C in the glassy state, where the values of loss modulus tends to drop. The gellan/polydextrose mixture enters the glassy state at about −28 °C, as compared to the polydextrose alone sample at −30 °C. Results are consistent with those previously reported for deacetylated gellan with sucrose or glucose syrup as the co-solute recording values close to −26 °C for the onset of the glassy state (Kasapis, 2006).

According to Ferry’s free volume theory and Ngai’s coupling model, a fundamental definition of the rheological glass transition temperature can be advanced as the conjunction of the glass transition region and the glassy state (Ferry, 1980; Ngai & Plazek, 1995; Plazek & Ngai, 1991). It is evident from Fig. 2c (calorimetry data) and Fig. 3 (rheological data) that the mechanical Tg is recorded at a higher temperature (−28 °C) than the calorimetric counterpart (−45 °C). Therefore, the latter is not altered by the presence of low levels of polysaccharide indicating that the micro-mobility of the sugar is not affected by the presence of the gelling macromolecule.

3.4. Theoretical modeling of viscoelastic data in high solid gellan/polydextrose mixtures

Further evaluation of the mechanical glass transition temperature requires theoretical modeling via the time temperature superposition principle (TTS). The method assumes that, by changing the temperature, the complete relaxation spectrum is affected by the same factor. In oscillation experiments, temperature is held constant and frequency or time is varied. The experimental data spans over a two to four decade range in frequency/time. By repeating such tests over a number of temperatures, we obtain a set of iso-thermal dependencies of storage and loss modulus in shear versus accessible frequency of oscillation (Figs. 4a and b for the gellan/polydextrose mixture). On shifting these linear viscoelastic parameters along the x-axis such that they are superposed on one another, a viscoelastic master curve is generated, which represents the time response of a material over a wide range of frequencies at a particular reference temperature. The master curve for deacetylated gellan gum with polydextrose, and a single polydextrose matrix at 80% levels of solid is depicted in Fig. 5a.

To develop a mechanistic understanding of the rubber-to-glass transition, synthetic polymer scientists have made extensive use of the concept of molecular free volume that generates the WLF equation (Ferry, 1980). This can be mathematically expressed as:

\[
\log a_T = \log\left[\frac{G(T)/G(T_0)}{G_0}/G(T_0)\right] = \frac{-B(2.303f_0)(T - T_0)}{(f_0/a_T) + T - T_0}
\]

where, \(f_0\) is the fractional free volume (the ratio of free to total volume per gram of material at an arbitrary reference temperature, \(T_0\),
\( \alpha \) is the thermal expansion coefficient, and \( B \) is usually taken as one for simplicity.

Cavaille, Jordan, Perez, Monnerie, and Johari (1987) proposed that the assumption of a rapid and linear development of the fractional free volume at temperatures above the glass transition temperature can be considered in terms of the expansion coefficient \( \alpha \).

The terms \( B/2.303 \phi_0 \) and \( f_0/\alpha \) are known as the WLF parameters, \( C_1^0 \) and \( C_2^0 \), respectively. Shifting of the frequency scale with temperature depends on the progress of factor \( \alpha T \) obtained from Eq. (1) (Matveev, Grinberg, & Tolstoguzov, 2000). The glass transition region of the deacylated gellan and polydextrose mixture extends from about \(-28 \) to \(10 \) °C in Fig. 3, and viscoelastic data within this region provide a good fit of the empirically derived shift factors from the WLF equation shown for the upper range of temperatures in Fig. 5b.

The master curve in Fig. 5a also covers the glassy state of the gellan/polydextrose mixture, where the values of \( G' \) approach a maximum and those of \( G'' \) level off below. Shift factors within the glassy state, i.e. at the low end of the experimentally accessible temperature range in Fig. 5b, describe the mechanics of the glassy state. This region does not follow the WLF equation, but can be better described by the Andrade mathematical expression (Peleg, 1992), as follows:

\[
\log \alpha_T = \frac{E_a}{2.303R} \left( \frac{1}{T} - \frac{1}{T_g} \right)
\]  

The treatment produces the concept of activation energy \( (E_a) \) for an elementary flow process in the glassy state, which is independent of temperature. Within the glassy state, the factor \( \alpha_T \) is an exponential function of the reciprocal absolute temperature, so the logarithmic form of Eq. (2) with a constant energy can be used for calculating numerical values (Gunning, Parker, & Ring, 2000).

The analysis described above for the binary mixture was also utilised for single polydextrose preparations and corresponding mechanical data with shift factor fits are illustrated in Figs. 5a and b. In the glassy state, this approach yielded values of \( E_a \), which were found to be 15.02 J/mol for the mixture and 9.87 J/mol for the co-solute alone. In addition, the two parameters \( C_1^0 \) and \( C_2^0 \) of the WLF equation can be obtained, which were 14.91° and 52°, and 11.07° and 53° for the binary mixture and polydextrose alone, respectively. These correlate well with the corresponding parameters obtained for vitrification of amorphous synthetic polymers and diluted systems (Dannhauser, Child, & Ferry, 1958; Ferry, 1980; Slade & Levine, 1993).

The conjunction of the glass transition region with the glassy state defined by the progression of the fundamental indicator, \( \alpha_T \),
in Fig. 5b pinpoints the mechanical glass transition temperature for the deacylated gellan and polydextrose as −28, and −30 °C for 80% polydextrose alone. Mechanistically, this is the point of transformation from free volume derived effects in the glass transition region to the process of an energy barrier of rotation in the solid-like environment of the glassy state. Thus, the mechanical $T_g$ can be considered as the threshold of two distinct molecular processes that can be utilised objectively to assess the temperature dependence of molecular processes during vitrification.

3.5. Tangible evidence of phase morphology in gellan/polydextrose matrices

In the present investigation, environmental scanning electron microscopy was used to obtain micrographs for the phase morphology in gellan gels with varying polydextrose concentrations. As illustrated in Fig. 6a, 1% gellan in an aqueous media forms uniformly spread aggregates of a helical configuration that can be readily visualised. A serial dilution is observed, with the addition of the co-solute from low to high levels of solids, in the density of the helical strands of the polysaccharide (Fig. 6b–f). The visually distinct aggregates start dispersing and are no more identifiable, an outcome which is accompanied by the evolution of a distinct amorphous structure. Similar effects of dilution have also been reported by Kasapis (2006) and Kasapis et al. (2002) who employed transmission electron microscopy to study the morphology of gellan in the high co-solute environment of glucose syrup. The amorphous nature of high solid gellan/polydextrose preparations further supports the case of theoretical modeling using concepts of glass transition phenomena in the preceding sections.

![Micrographs for 1% gellan with various polydextrose concentrations](image-url)
3.6. Micromolecular aspects of gellan and polydextrose interactions

Fourier transform infrared spectroscopy (FTIR) and wide angle X-ray diffraction (WAXD) were used to study possible molecular interactions between the two constituents of gellan and polydextrose in preparations. Infrared spectra of single gellan or polydextrose systems and their binary mixtures are depicted in Fig. 7a. For the polydextrose chain, a variety of molecular events corresponding to specific chemical linkages are shown: O–H stretching (3500 cm⁻¹), C–H stretching (2900 cm⁻¹), C–O stretching of aldehyde (1627 cm⁻¹), and stretching vibration of the COC glycosidic linkage (1180–930 cm⁻¹) (Mickova, Kopikova, & Synytsya, 2007). Gellan shows a significant absorption peak at wave numbers around 3463 cm⁻¹, in a similar manner to those recorded for the polysaccharide in the presence of polyvinyl alcohol (PVA) in films, being attributed to a major OH group signal (Sudhamani, Prasad, & Sankar, 2003). In conclusion, the profile of these absorptions in the gellan/polydextrose mixture matches the expected spectra for the individual components, hence arguing against the presence of chemical (covalent) interactions between the two constituents in our low or high-solid mixtures.

Finally, Fig. 7b presents the diffractograms from wide angle X-ray scattering for single or mixed systems of gellan and polydextrose. WAXD was utilised to analyse for amorphous character and the presence of traces of crystallinity in different mixtures. Gellan exhibits a broad peak at 22° characteristic of a non-crystalline network. According to Payne, McCormick, and Francis (1999), a broad peak recorded at 22° with shouldering until 50° for the co-solute and macromolecule/co-solute samples (as observed currently) is the fingerprint of amorphous materials, with their characteristics dense morphology being shaped up from the processing conditions of freeze drying used in the preparations of these samples. Overall, the absence of sharp peaks in the diffractogram supports the amorphocity of the constituents and mixtures of this work; it also supports the experimental observations of rheology, calorimetry and the working framework of glass transition theory employed in this investigation.

4. Conclusions

The present work was an effort to provide a comprehensive picture of the structural behaviour of deacylated gellan in the presence of high levels of polydextrose. Deacylated gellan in mixture with polydextrose depicted similar thermomechanical profiles as other gelling polysaccharide matrices in the presence of sugars like sucrose, fructose or glucose syrup, cited in the literature review of the manuscript. This is an encouraging outcome suggesting that the binary mixture could be used for novel confectionery formulations. The preceding statement is further reinforced by the observation that condensed gellan/polydextrose samples exhibit substantial amorphicity without unwanted chemical bond formation during preparation at high temperature followed by cooling to ambient conditions. Rubbery consistency of the binary mixture can be further manipulated at subzero temperatures, where glassy behaviour is recorded experimentally and modelled theoretically. Thus, a link between experimental observation and molecular understanding has been substantially accomplished and should facilitate explorations of these high solids materials for industrial applications.

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References


Chapter 5

Results and discussion

Effect of a glassy gellan/polydextrose matrix on the activity of $\alpha$-glucosidase

Manuscript accepted for publication in *Carbohydrate Polymers* published by Elsevier, presented as Chapter 5

Note that further supplementary details regarding the calculation of enzymatic data is presented in the Appendix to this thesis
EFFECT OF A GLASSY GELLAN/POLYDEXTROSE MATRIX ON THE
ACTIVITY OF $\alpha$-GLUCOSIDASE

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An investigation of the ability of the enzyme $\alpha$-glucosidase to act on the substrate 4-nitrophenyl $\alpha$-D-glucopyranoside ($p$NPG) while embedded in glassy carbohydrate matrices (deacylated gellan with polydextrose and polydextrose alone) is presented. Physicochemical characterisation of the matrices was achieved using the techniques of modulated differential scanning calorimetry, small deformation dynamic oscillation on shear, Fourier transform infra-red spectroscopy, wide angle x-ray diffraction and scanning electron microscopy. A UV-visible spectrophotometric procedure was adapted for the analysis of the activity of $\alpha$-glucosidase in hydrolysing $p$NPG in the condensed carbohydrate systems. In order to derive a relationship between the structural properties of the matrix and the enzymatic activity, mechanical spectra were recorded using the combined framework of the Williams, Landel and Ferry equation with the time-temperature superposition principle. Theoretical modeling and experimental observations strongly argue for a pronounced effect of the gelling polysaccharide/co-solute mixture on enzymatic activity near the mechanical $T_g$ of the matrix.

Keywords: deacylated gellan, polydextrose, glass transition temperature, $\alpha$-glucosidase
INTRODUCTION

In high solid systems, numerous changes can take place depending on physical state, properties of food materials and the physicochemical environment. During the various processing steps, distribution and storage of food materials, enzymatic reactions may contribute to a significant extent to these transformations, which may be desirable or deleterious. Therefore, it is important to understand these reactions for technological improvement, quality preservation and extension of the shelf life of foods (Drapon, 1985).

As one of the primary components of food, water is involved and affects a number of interactions occurring during the various stages of food production (Roos 1995). Amorphous food systems exhibit a unique phenomenon which is temperature, time (or frequency) and composition dependent. For example, these are material-specific changes occurring upon heating a “glassy” mechanical solid to a “rubbery” viscous fluid commonly referred to as the “glass transition” (Sperling, 2006). This transition has been discussed as a possible factor affecting the kinetics of enzymatic changes in low-moisture processed foods (Slade and Levine, 1991; Roos and Karel, 1991; Roos, 1998).

A reduction in the translational and rotational motions of component molecules occurs during transition to the glassy state, thus supporting the hypothesis that chemical and biological reactions have reduced rates in glassy systems (Le Meste, 1995; Cardona, Schebor, Buera, Karel and Chirife, 1997). Extensive literature is available on the effect of temperature on enzymes in amorphous systems including studies by Schebor, Buera and Chirife, 1996; Mazzobre, Buera and Chirife, 1997a; Mazzobre, Buera and Chirife, 1997b; Burin, Buera, Hough and Chirife, 2002. However, the focus of previous research has been on the thermal resistance or stability of the enzymes as related to the glass transition temperature ($T_g$) rather than on enzymatic activity. In addition, the interactions between polymer matrices (e.g. proteins or polysaccharides with co-solute at high levels of solids) and the recorded calorimetric or mechanical $T_g$ in relation to enzymatic activity remains to be elucidated.

Microbial exo-polysaccharides have been invaluable ingredients in a variety of applications over a long period. One of them is deacylated gellan, which due to the novel property of forming thermo-reversible gels is gaining importance being utilised in diverse fields encompassing food, pharmaceutical and other industries (Giavasis,
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Harvey and McNeil, 2000). It is produced through a fermentation process by a pure culture of *Pseudomonas elodea* yielding an anionic, high molecular weight, polysaccharide gum with a tetrasaccharide repeating unit of one α-L-rhamnose, one β-D-glucuronic acid and two β-D-glucose residues. The supplier describes it as having good flavour release and gel strength, requiring low usage levels (below 0.5% w/w) for most applications (Kelco, 2004; Rozier, Mazuel, Grove, and Plazonnet, 1989; Carlfors, Edsman, Petersson and Jörnving, 1998). On the other hand, polydextrose is utilized in various products including beverages through to confectionery for both its physiological and technological benefits, e.g. oral health benefit, dietary fiber properties, reducing glycemic impact, prebiotic behaviour, and an ability to act both as humectant and crisping agent in foods (Stowell, 2009).

The present study takes advantage of the distinct structural properties of the aforementioned materials to characterise a high solid mixture (85%) with the aim to develop a model system for entrapping the enzyme-substrate complex of α-glucosidase and 4-nitrophenylα-D-glucopyranoside (pNPG). This allows examination of enzymatic behaviour in the vicinity of the glass transition temperature as recorded calorimetrically and rheologically for the binary mixture and a single polydextrose matrix.
2. MATERIALS AND METHODS

2.1 Materials

Deacylated gellan powder, Kelcogel trade name, was from Nutrasweet Kelco Company (Santiago, CA). The primary structure of gellan gum is composed of a linear tetrasaccharide repeating unit $\rightarrow [3]-\beta-D-\text{Glc}-p-(1 \rightarrow 4)-\beta-L-\text{Glc}A-(1 \rightarrow 4)-\beta-D-\text{Glc}-p-(1 \rightarrow 4)-\alpha-L-\text{Rhap}-(1 \rightarrow)_n$. Polydextrose (Sta-Lite III powder) also known as PDX is a D-glucose polymer reaction product with citric acid or phosphoric acid and sorbitol with predominating $\beta (1 \rightarrow 6)$ bond. It was supplied by Tate & Lyle, ANZ, Pvt. Limited (Decatur, IL), as per manufacturer’s certificate of analysis; it was of 90% purity with 4% moisture and had passed microbiological testing under the food grade standards. The enzyme used ($\alpha$-D-glucoside glucohydrolase (EC 3.2.1.20, product number G3651) from Bacillus stearothermophilus, Sigma-Aldrich (St. Louis, MO, USA) commonly known as maltase), was as a lyophilized powder containing potassium phosphate buffer salt. Substrate ($\rho$NPG), potassium phosphate, L-glutathione and calcium chloride were also purchased from Sigma-Aldrich and sodium carbonate was from BDH, Port Fairy, VIC Australia. Milli-Q water from Millipore was used in all experiments.

2.2 Sample preparation

For physico-chemical studies: A clear solution of the polysaccharide was obtained by slowly dispersing weighed amounts of deacylated gellan powder in hot (90°C) Milli-Q water and stirring constantly on a hot plate for 20 min. Calcium chloride (14 mM) was added to the hot solution to prepare a dispersion containing divalent cations. A weighed amount of polydextrose was separately dispersed in cold Milli-Q water and placed in a preheated water bath at 75°C until fully dispersed. The temperature of the gellan sample was reduced to 75°C and the polydextrose solution was carefully added so that no bubbles formed. Small amounts of excess water were removed by evaporation at temperatures close to 75°C for all the samples. A solution containing only polydextrose was also prepared as described.

For enzymatic studies: Weighed amounts of gellan powder were slowly dispersed in hot (90°C) Milli-Q water with constant stirring on a hot plate for 20 min to form a clear solution of the polysaccharide before adding calcium chloride (14 mM). A
weighed amount of polydextrose was separately dispersed in potassium buffer (pH 6.8) with stirring for 20 minutes without heat and then placed in a preheated water bath at 50°C for 2 hours. After a clear solution was obtained, the temperature of the gellan sample was reduced to 50°C and polydextrose solution carefully added so that no bubbles formed. Following preliminary optimization and adaptation from the enzyme assay procedure described by Sigma, volumes of substrate (1.2 mL), enzyme (0.1 mL) along with glutathione (0.2 mL) were added to gellan/polydextrose mixtures and small amounts of water was evaporated to bring the final concentration to 85% solids.

2.3 Methods

**Small deformation dynamic oscillation:** To characterize the development of linear/non-linear viscoelastic parameters as a function of temperature, the technique of small amplitude oscillation on shear was chosen. The data for the elastic ($G'$) and viscous ($G''$) components of the network can be derived by the technique without destroying the structure of the matrix and for this a controlled strain rheometer (ARG-2) with magnetic thrust bearing technology (TA Instruments, New Castle, DE) was utilized. An environmental test chamber (ETC) and liquid nitrogen were used to study the samples (85% w/w) which were loaded onto the preheated 5 mm measuring geometry of the rheometer at either 75°C (deacylated gellan and polydextrose) or 25°C (polydextrose) and covered with silicone oil (50 cS from BDH) to prevent moisture loss.

A temperature ramp was then implemented to sub-zero temperatures of -34 and -42°C, respectively, for the mixture and co-solute alone at a scan rate of 1°C/min, frequency of 1 rad/sec and 0.01% strain, which was within the linear viscoelastic region. At the completion of cooling runs, frequency sweeps were recorded in the range of 0.1 to 100 rad/s with 4°C temperature intervals to obtain a series of data for theoretical modelling. Overall, the experimental temperature range was able to cover molecular motions covering the glassy state, glass transition region and the flow region. For each experimental preparation, two replicates were analysed with the flow-to-glass transition region being readily reproducible as a function of temperature or time scale of measurement.

**Calorimetric measurements:** Samples consisting of gellan with polydextrose or polydextrose alone were hermetically sealed in $T_{zero}$ pans and subjected to modulated differential scanning calorimetry (MDSC) measurements (DSC Q2000, TA instruments,
New Castle, DE). A refrigerated cooling system attached to the calorimeter enabled achievement of temperatures down to -90°C. The heat flow signals were calibrated by the use of a traceable indium standard ($\Delta H_f = 28.3 \text{ J/g}$) and the heat capacity response using a sapphire standard. Samples (8 mg) were heated to 97°C, cooled to -90°C and reheated to 95°C, at a modulation amplitude of 0.53°C for each period of 40 s. An empty $T_{\text{zero}}$ pan was taken as the reference, with nitrogen purge gas at a flow rate of 50 mL/min and systems were scanned at 1°C/min. Results reported are of individual traces selected as representative of three replicates, which were effectively superposing traces.

**Fourier transform infrared spectroscopy (FTIR):** In order to identify the nature of interactions between the two constituents, FTIR spectra of gellan and polydextrose preparations were obtained using Absorbance mode on a Perkin Elmer Spectrum 100 spectrometer, equipped with MIRacle™ ZnSe single reflection ATR plate (Perkin-Elmer, Norwalk, CT). The wavelength range of 600 – 4000 cm$^{-1}$ with a resolution of 4 cm$^{-1}$ was investigated and this was corrected against the background spectrum of the solvent at ambient temperature before plotting.

**Wide angle X-ray diffraction (WAXD):** The diffractograms of gellan and polydextrose alone and their mixtures were obtained using a Bruker D$_4$ Endeavour (Karlsruhe, Germany). Freeze dried samples were placed on the sample magazine and scanned under accelerating voltage and current of 40 kV and 40 mA, respectively, to obtain the raw data in a 2$\theta$ range between 5 and 90° in measuring intervals of 0.1°. The Bruker Advanced X-ray Solutions software, DIFFRAC plus Evaluation (Eva), version 10.0 revision 1 was used to analyse the data and then plot the diffractograms.

**Scanning electron microscopy (SEM):** Micrographs of gellan and polydextrose matrices were obtained using FEI Quanta 200 ESEM (Hillsboro, OR, USA) in order to study the network morphology and phase topology of the samples under investigation. A high-vacuum mode at an accelerating voltage of 30 kV, pressure of 0.54 mbar, spot size 5 and working distance between 9.2 and 11.2 mm was utilised to image freeze-dried and gold-plated preparations.

**Estimation of enzyme activity using UV-visible Spectroscopy:** Polysaccharide and co-solute matrices containing the enzyme-substrate complex were immediately cooled to the required temperature in an ARB fridge-freezer (temperature range of -18 to 10°C). Temperature was authenticated by the use of a thermocouple. Samples were individually packed using aliquots of 0.1 g in small containers. These were then equilibrated for an hour before inactivating enzymes by addition of 8 ml of 100 mM
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sodium carbonate solution and vortexing vigorously for one minute. Absorbance was recorded at 400 nm on a Lambda 35 UV-vis spectrophotometer (Perkin Elmer, Singapore). Measurements of enzyme activity at each experimental temperature were carried out in triplicate and average values are reported in terms of nanokatals per gram of the matrix.
3. RESULTS AND DISCUSSION

3.1 Estimation of the glass transition temperature using MDSC

The molecular mechanisms involved in different relaxations including glass transition are commonly investigated by the use of MDSC as it is a versatile and sensitive technique in providing the total heat flow along with the reversible (kinetic) and non-reversible (heat capacity) components (Lopez, Champion, Blond and Le Meste, 2005; Rabel, Jona and Maurin, 1999). In the present study, the technique allowed us to observe the phenomenon of glass transition, when the condensed systems of gellan with polydextrose, and polydextrose alone (total solid concentration in both cases of 85%, w/w) were subjected to heating and cooling cycles at the controlled scan rate of 1°C/min (Fig. 1).

During cooling, the heat capacity and volume of the gel decreases, as a result of reduced thermal motion and molecular mobility, so that the calorimetric $T_g$ can be derived as the central point of the onset and endset of the heat capacity change. This was observed at approximately -38°C for both polydextrose and its mixture with gellan.
The similar values of $T_g$ for both samples confirm the suggestion of Goff, Caldwell and Stanley (1993) and Roos (1993) that the thermal spectrum of proteins or polysaccharides in the presence of a high sugar environment is dominated by the vitrification of the latter. It appears, therefore, that in mixtures of low polysaccharide and high sugar, the former merely acts as “cross contamination” and the calorimetric $T_g$ can be predicted by the total level of solids in these systems.

3.2 Characterisation of viscoelastic behaviour in single and mixed systems of polydextrose and deacetylated gellan

Calorimetry in the preceding paragraph elucidated micromolecular aspects of vitrification phenomena. The purpose in this section is to investigate the macromolecular effects of addition of 2% deacetylated gellan (14 mM CaCl$_2$) to a concentrated (83%, w/w) polydextrose preparation. This approach has been adapted from the “synthetic polymer approach”, which commonly follows the viscoelastic properties of three dimensional structures by performing small deformation dynamic oscillation or stress relaxation experiments. Fig. 2 depicts the variation of storage ($G'$) and loss ($G''$) modulus of thermally reversible mechanical profiles for condensed single polydextrose samples and polydextrose/gellan mixtures at a total solids level of 85% (w/w).

There is a strong thermal effect on the behaviour of both systems over the temperature range of 75 to -40°C resulting in three distinct regions, i.e. the rubbery plateau for the binary mixture, followed by the glass transition and glassy state for both systems. The storage modulus develops five orders of magnitude from $10^5$ Pa in the rubbery plateau at the beginning of the cooling run to about $10^{9.5}$ Pa at the end of the cooling run for the mixture. Mechanical traces are displaced to higher temperatures, an outcome which argues that the process of vitrification is rapid, as compared to single polydextrose preparations. The latter exhibit a crossover of the two modulus traces at about -20°C, being indicative of the onset of the glassy state, which is considered as an empirical index of the mechanical glass transition temperature.
Fig. 2 Cooling profiles of storage and loss modulus for 2% gellan with 83% polydextrose (●,♦), and 85% polydextrose (○,◊) scanned at 1°C/min (frequency: 1rad/s; strain: 0.01%).

It appears that the values of mechanical $T_g$ are distinct from those recorded in Fig. 1 from calorimetry observations. The latter are consistent with recorded estimates for small molecule polyhydroxyl compounds in condensed matrices (Kalichevsky, Jaroskiewicz, Ablett, Blanshard and Lillford, 1992; Biliaderis, Lazaridou and Arvanitoyannis, 1999; Slade and Levine, 1991). Differences in the predictions between the two techniques could be due to several factors including those induced by the analysis of experimental data (e.g. definition of $T_g$ in the DSC curve), choice of parameters in mechanical tests and, significantly, coupling of distinct structural units with particular relaxation times in the two modes of experimentation.

3.3. Quantitative exploration of the structural properties of gellan at high levels of polydextrose

To provide a means of identification of the molecular dynamics involved in secondary transitions, we evolved mechanistic explanations via the time-temperature superposition principle (TTS). Glass formation is such transformation taking place from a solid- to a liquid-like consistency, where there is a change in ‘state’ but not in “phase”.
In the present work, TTS was utilized in Figures 3(a-d) by recording a series of mechanical spectra within the accessible frequency range of 0.1-100 rad/s for both gellan with polydextrose and polydextrose alone. These were taken at a constant temperature interval of 4°C upon heating the binary gel from -34 to 6°C and from -40 to 4°C for polydextrose. As observed from the data at low temperatures, mechanical spectra remain relatively flat, but transform into a steep drop in modulus with increasing frequency at high temperatures (e.g. -36 and 0°C, respectively in Fig. 3d).

Next, an arbitrary reference temperature was selected within the glass transition region ($T_o = -10$ and -16°C, respectively for binary and single preparations) and remaining mechanical spectra were shifted horizontally along the log frequency axis. This provides a relationship between viscoelasticity and reference temperature of the heating or cooling run as long as the frequency of the former is multiplied by a shift factor, $a_T$. For TTS to be applicable, it is critical that the mechanical spectra of $G'$ and $G''$ generate the same factors $a_T$ and superimpose thoroughly, otherwise modelling should be rejected.

### 3.4 Theoretical modelling to interpret the mechanical data

Mechanical spectra recorded for both materials could be readily superposed in Fig. 4a. Superposition yields composite (or master) curves of viscoelasticity spanning sixteen decades of frequency from $10^{-4}$ to $10^{11}$ rad/s in Fig. 4a. This is the analogue of the temperature profile discussed in Figure 2 by reproducing the transition zone and glassy state. Generated shift factors were first modeled with the Arrhenius rate law that describes the temperature dependence of molecular parameters in various chemical and physical reactions (Hrma, 2008). The reaction rate is proportional to $\exp \left( \frac{E_a}{RT} \right)$, where $E_a$ is the activation energy of the molecular process and is expressed for a set of two different temperatures, as follows:

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

This type of analysis generates a straight line, with the gradient reflecting the activation energy of relaxation processes. As illustrated in Fig. 4b, good linear fits are obtained for the low temperature ranges, which according to Fig. 2 cover the glassy state.
Fig. 3 Frequency variation of $G'$ (a) and $G''$ (b) for 2% gellan with 83% polydextrose, the lowest curve is taken at 6°C (-), other curves successively upwards 2°C ($\times$), -2°C ($\ast$), -6°C ($\circ$), -10°C (●), -14°C (□), -18°C (■), -22°C (Δ), -26°C (▲), -30°C (◊), -34°C (+), and frequency variation of $G'$ (c) and $G''$ (d) for 85% polydextrose, the lowest curve is taken at 4°C (▲), other curves successively upwards 0°C (*), -4°C(●), -8°C (◇), -12°C (■), -16°C (□), -20°C (●), -24°C (◇), -28°C (◇), -32°C ($\times$), -36 °C (Δ), -40 °C (+).
Fig. 4a Master curve of gellan/polydextrose, at the reference temperature of -10°C, for the reduced storage and loss modulus (●,◆) as a function of reduced frequency of oscillation along with the master curve of 85% polydextrose (○,◊) at the reference temperature of -16°C.
Fig. 4b Temperature variation of the factor $a_T$ within the glass transition region (●) and the glassy state (■) for gellan/polydextrose and the single polydextrose preparation (○,□), with the solid lines reflecting the WLF and modified Arrhenius fits of the shift factors throughout the vitrification regime and dashed lines pinpointing the $T_g$ predictions (all data from the frequency sweeps of the preparations in Figure 3).

Deviation from Arrhenius predictions is noted at higher temperatures, where rationalisation of molecular events was attempted using the concept of molecular free volume. This approach assumes that the space available to a group of molecules is the total volume actually taken by the molecules (sum of volume due to the molecular oscillations and van der Waals radii) plus large scale vibrations (Meinders and van Vliet, 2009). The mathematical expression of free volume theory is given by Williams, Landel and Ferry (WLF equation) in the following (Ferry1980):
\[ \log a_T = -\frac{C_1^0(T - T_o)}{C_2^0 + T - T_o} \]  

(2)

where, \( C_1^0 \) and \( C_2^0 \) are the WLF constants at \( T_o \) and relate to the free volume theory as follows:

\[ C_1^0 = \frac{B}{2.303 f_o} \quad \text{and} \quad C_2^0 = \frac{f_o}{\alpha_f} \]  

(3)

where, \( f_o \) is the fractional free volume (the ratio of free to total volume per gram of material), \( \alpha_f \) is the thermal expansion coefficient and \( B \) is usually taken as one for simplicity.

Utilisation of the combined WLF/free volume framework yields good fits for the temperature dependence of shift factors at the upper range of temperatures, which from Figure 2 correspond to the glass transition region of our samples. This threshold of behaviour where large configurational adjustments contributing to changes in free volume are superseded by a barrier to rotation with a constant activation energy can be considered as the mechanical \( T_g \) (~20 and -14°C for polydextrose and its mixture with gellan, respectively).

Equations (2,3) permit calculation of the fractional free volume and thermal expansion coefficient at \( T_g \) being 0.021 and \( 3.88 \times 10^{-4} \) deg\(^{-1} \) for the mixture, and 0.039 and \( 7.22 \times 10^{-4} \) deg\(^{-1} \) for polydextrose. Values are within the ranges reported earlier for amorphous synthetic polymers and diluted systems (Tsui, Paraskos, Torun, Swager and Thomas, 2006). Experimental observations in Figure 2 are consistent with theoretical modelling in Figure 4 arguing that mechanical profiles in the rubber-to-glass transition are strongly influenced by the network forming polysaccharide. The magnitude of the gellan contribution to rheological properties is thus represented by the concept of mechanical (or “network”) \( T_g \) being distinct from the corresponding \( T_g \) values for polydextrose obtained rheologically and by calorimetry.
3.5 Phase morphology and interactions in gellan/polydextrose mixtures

Scanning electron microscopy (SEM) was used to obtain images in Figures 5a and 5b. Gellan in aqueous media forms uniformly spread aggregates of a helical configuration that can be readily visualised as thick aggregates protruding from the featureless background. These structures are not readily identifiable upon addition of 83% polydextrose, an outcome which is accompanied by the evolution of a distinct amorphous structure. Similar phase morphology has been reported in micrographs of gellan with sugars obtained using transmission electron microscopy (Kasapis, 2006; Kasapis, Abeysekera, Atkin, Deszczynski and Mitchell, 2002). Tangible evidence from microscopy supports the case of amorphicity in gellan/polydextrose samples put forward via thermomechanical analysis and theoretical modeling in the preceding sections.

![Fig. 5 Micrographs for (a) 2% gellan and (b) 2% gellan with 83% polydextrose (1000x magnification).]

To look for possible interactions between the two constituents of our mixture, Fourier transform infrared spectroscopy (FTIR) was employed. Infrared spectra obtained are depicted in Figure 6a. Results are consistent with those reported by Mickova, Copikova, and Synytsya, (2007), and Sudhamani, Prasad, and Sankar, (2003) for polydextrose and gellan, reporting a variety of molecular events that correspond to
specific chemical linkages within each material: O—H stretching (3500 cm\(^{-1}\)), C—H stretching (2900 cm\(^{-1}\)), C—O stretching of aldehyde (1627 cm\(^{-1}\)) and stretching vibration of the COC glycosidic linkage (1180-930 cm\(^{-1}\)) for polydextrose. A significant absorption peak at wave numbers of approximately 3463 cm\(^{-1}\) for gellan is attributed to a major OH group signal. In conclusion, these profiles do not provide evidence of chemical interactions between the two components of the mixture under the present experimental conditions.

![FTIR spectra](image)

**Fig. 6 (a)** FTIR spectra the lowest curve is for 2% gellan and others successively upwards are for 2% gellan with 83% polydextrose, and 85% polydextrose alone.

Wide angle X-ray diffraction (WAXD) was also employed and diffractograms obtained are presented in Figure 6b. According to Payne, McCormick, and Francis, (1999), a broad peak recorded at 22° with shouldering until 50° for the polydextrose and gellan/polydextrose samples (as observed currently) is the fingerprint of amorphous materials, with their characteristic dense morphology resulting from the processing conditions of freeze drying used in the preparation of these samples. Therefore, the experimental observations of rheology, calorimetry and the working framework of glass transition theory employed in this investigation are further supported by the absence of sharp peaks in the diffractogram confirming that the constituents and mixture of this investigation being amorphous.
3.6 The activity of enzyme embedded within the glassy carbohydrate matrices

In order to facilitate the utilization of the techno- and biofunctional benefits of gellan and polydextrose, as reviewed, a novel series of experiments was designed to evaluate the possibility of using these as entrapping agents for enzymes while seeking to broaden our understanding of the behaviour of an enzyme within the matrix in the vicinity of the glass transition temperature. Following the characterization of the gellan/polydextrose matrix, the enzyme α-glucosidase was chosen and its activity upon the substrate pNPG using a UV-vis spectrophotometer was monitored in order to allow measurements within the matrix held at various temperatures.

For this, a series of preliminary experiments was used to modify and adapt a “spectrophotometric stop rate determination method” described by Sigma to suit our project aims and conditions. The critical conditions of temperature (≤ 50°C), time of preparation (approximately 45 minutes) and pH (6.8) were rigorously maintained to assist in the assay of α-glucosidase. A clear trend was observed for the release of the hydrolysis product recorded after 60 minutes of equilibration, which was sufficient to
control the conditions of low moisture and low temperatures to which the matrix and enzyme were subjected (Table 1).

Table 1 Absorbance recorded for the polydextrose matrix and in combination with gellan

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°C)</th>
<th>85% polydextrose</th>
<th>2% gellan with 83% polydextrose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-8</td>
<td>-10</td>
<td>-12</td>
</tr>
<tr>
<td>60</td>
<td>0.476</td>
<td>0.441</td>
<td>0.453</td>
</tr>
<tr>
<td>60</td>
<td>0.415</td>
<td>0.438</td>
<td>0.440</td>
</tr>
<tr>
<td>60</td>
<td>0.462</td>
<td>0.456</td>
<td>0.441</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>0.451</td>
<td>0.445</td>
<td>0.444</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>0.031</td>
<td>0.009</td>
<td>0.007</td>
</tr>
</tbody>
</table>

The enzymatic activity expressed in terms of nanokatals/gram of the matrices was calculated from the average values after sixty minutes of observation from data in Table 1 using equation (4) which is based upon that described by Sigma:

$$\text{Activity (nanokatals/gram)} = \frac{(\Delta A_{400})(8)(10^6)}{(18.3)(10^3)(3600)(0.1)}$$

where, $\Delta A_{400}$ is the change in absorbance during incubation at the particular temperature, 8 is the volume of NaCO₃ used (mL), $10^6$ converts milli to nanomoles, 18.3 is the millimolar extinction coefficient of $p$-nitrophenol at 400 nm, $10^3$ expresses volumes in units of litre, 3600 is the time of observation (s), and 0.1 is the amount of the matrix taken for analysis (g).
Results presented in Figure 7 depict the effect of the gellan polysaccharide on the ability of α-glucosidase to act on its substrate (pNPG) in the vicinity of the $T_g$ value established in earlier phases of this study (-14°C). Little effect of temperature on activity was observed below $T_g$, contrasting with the trend at increasing temperatures above this index. In the case of the matrix consisting of only polydextrose, the enzyme activity curve remains relatively flat throughout the range of temperatures trialled. It is noted that the $T_g$ of the polydextrose system (-20°C) is well below the experimentally accessible temperatures for the enzymatic assay investigated here. This indicates that the gellan polysaccharide by accelerating the vitrification process of the matrix considerably affects enzymatic phenomena in these systems.

![Figure 7](image)

**Fig. 7** The influence of temperature on the activity of α-glucosidase in a matrix of 2% gellan with 83% polydextrose (♦) in comparison to 85% polydextrose (◊). Enzyme activity is expressed in terms of nanokatals per gram of the matrix.

Recent reports on the effect of gelling polysaccharides on bioactive components and their mobility have described the diffusion of caffeine in high solid matrices of glucose syrup and glucose syrup/κ-carrageenan (Kasapis and Shrinivas, 2010; Jiang and Kasapis, 2011). The current findings confirm the previous observations emphasising the role of network glass transition temperature in diffusional processes of bioactive compounds and proteins. Whilst it has long been known that enzymes have limited
activity within low moisture systems, this is a systematic study of the effects of
temperature on the activity of an enzyme in the vicinity of the glass transition
temperature that has been estimated using theoretical modeling. Therefore the
mechanical glass transition temperature has the potential to be applied in the control not
only of the mobility of bioactive compounds but also of enzymatic activities that might
influence product attributes. There are direct implications for quality control from this
work in the processing of biomaterials in addition to traditional considerations based on
$T_g$ values established using calorimetric techniques.

CONCLUSIONS

Physicochemical characterisation in this study demonstrates that a dilution of the
gellan helix, visible in the aqueous state, occurs when high amounts of polydextrose
are added to preparations. There are no chemical interactions between the two
constituents in the mixture, which is amorphous in nature and converts upon cooling
from the rubbery to the glassy consistency. Further examination of the binary system
argues that the calorimetric glass transition temperature depends on the high
concentration of the co-solute, on the other hand, the presence of gellan influences the
mechanical $T_g$. This particular polysaccharide/co-solute arrangement has a pronounced
effect on substrate mobility and the hydrolytic effect of the enzyme, $\alpha$-glucosidase.
Results indicate that the mechanical $T_g$ should be considered and utilised as an
effective tool in the quality control and development of novel formulations with
desirable structural properties and biofunctionality.

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REFERENCES


Chapter 5


Chapter 6

Results and discussion

Optimization of alpha-amylase activity in the glassy starch matrix

Manuscript prepared for review and publication with the Journal of Agricultural and Food Chemistry published by the American Chemical Society

Note that further supplementary details regarding the calculation of enzymatic data is presented in the Appendix to this thesis
Chapter 6

OPTIMIZATION OF ALPHA-AMYLASE ACTIVITY IN A GLASSY STARCH MATRIX

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Chapter 6

ABSTRACT

A novel study designed to optimize the protocol for estimation of α-amylase activity in a glassy starch matrix is presented. The experimental temperature range was from -14 to 10°C, and the techniques of modulated differential scanning calorimetry, small deformation dynamic mechanical analysis and UV-vis spectroscopy were employed. The value of glass transition temperature ($T_g$) was obtained using both calorimetry and mechanical analysis. The vitrification profile of the starchy matrix was used to assess the enzymatic activity assayed as the rate of release of reducing sugar, which was measured using a dinitrosalicylic acid procedure. Thus the condensed starchy matrix served the dual purpose of acting as a substrate as well of producing a pronounced effect on the ability of enzyme to hydrolyse the carbohydrate. Activation energies were estimated throughout the glass transition region of condensed starch preparations based on the recently introduced concept of the spectroscopic shift factor. Results were further used to demonstrate a pronounced effect of $T_g$ on the reaction rate of starch hydrolysis by α-amylase.

Keywords: starch, α-amylase, glass transition temperature, spectroscopic shift factor
1. INTRODUCTION

Amongst biopolymers, starch is one of the most abundant polysaccharides, and because of its biocompatibility and degradability, it has been of interest in pharmaceuticals, thermoplastics, paper, textile and a variety of other applications. Starch from most plants is composed of two distinct fractions: amylose, which is the non-ramified fraction containing approximately 4,000 glucose units and amylopectin, the branched fraction with a typical degree of polymerisation of around 100,000. Starch is naturally hydrolysed by several amylolytic enzymes and α-amylase (4-α-D-glucan glucanohydrolase) is an endo-enzyme specific to α-(1, 4)-D-glucopyranosidic bonds located within glucan chains. The degradation products of amylolysis are primarily mainly composed of dextrins and oligosaccharides including maltose (Dumoulin, Cartilier and Mateescu, 1999).

It is documented that native and modified starches, as well as non-starchy polysaccharides and proteins, exhibit vitrification processes in condensed regimes. Such consistency has been utilised to enhance texture, processability, storage stability and delivery control of a diverse range of foods (Liu, Bhandari and Zhou, 2006; Rahman, 2006). In addition, preparations of these biomaterials at low water contents have extended shelf life properties making them relatively inexpensive to handle and transport around the world. Accordingly, these ingredients are successfully incorporated into commercial products in the food and pharmaceutical industries, including confections, the unfrozen phase of ice cream, dried fruit leathers and the hard gelatine capsule as a pharmacotherapeutic (Wang, Zhang and Chen, 2008).

Enzyme kinetics provide a systematic approach to the analysis and quantification of the effects of the various parameters that influence the activity of an enzyme. Among those commonly considered are enzyme and substrate concentrations, environmental conditions of pH, temperature, ionic strength and moisture, as well as the presence of inhibitors, activators or co-factors (Whitehurst and Oort, 2010). In the current study, the aim has been to utilize the concept of glass transition temperature ($T_g$) from the materials science as a means to control the kinetics of enzymatic activity in carbohydrate matrices. $T_g$ can be described as the point, or narrow region, on the temperature scale where a discontinuity is observed in the value of the thermal
expansion coefficient \( (\alpha_f) \), where below \( T_g \) the configurational rearrangements of polymer chain backbones occur relatively slowly. Amorphous polymers and any liquid that can be cooled to a sufficiently low temperature without crystal formation exhibit this phenomenon (Ferry, 1980).

To date, very few studies are available on the physicochemical behaviour of polymer matrices in relation to \( T_g \) and their chemical or enzymatic functionality. We believe that investigating the molecular dynamics of biopolymers, at the vicinity of \( T_g \), in relation to chemical or enzymatic processes would lead to enhanced stability and quality of processed foods. Most of the chemical studies reported so far focussed on the prevention of flavour and colour degradation or control of the oxidative reactions particularly those leading to rancidity. In the context of enzymatic activity in foods, the interaction of the enzyme and substrate(s) are important and this is a diffusion controlled process (Burin, Jouppila, Roos, Kansikas and Buera, 2000; Thomsen, Lauridsen, Skibsted and Risbo, 2005).

Interactions of simple sugars including fructose, glucose and xylose with lysine in matrices prepared from trehalose, maltodextrin or polyvinylpyrrolidone have been extensively reported in the field of non-enzymatic browning (Lievonen, Laaksonen and Roos, 2002; Craig, Parker, Rigby, Cairns and Ring, 2001; Miao and Roos, 2004). The rate of reactant consumption was evaluated and attempts were made to evaluate these based on the amplitude of the difference between the glass transition and the experimental temperature \( (T-T_g) \). The question still remains as to the fundamental mechanism governing various physicochemical and enzymatic processes in the glass transition region. In the current research, a novel two-step approach has been chosen in order to develop a protocol that monitors the thermomechanical properties of a glassy starch matrix and its effect on the hydrolytic activity of \( \alpha \)-amylase. The work aims to provide a much needed theoretical understanding of enzyme stability and mobility in glassy matrices, hence to serve as a basis for innovation in related industrial applications.
2. MATERIALS AND METHODS

2.1 Materials

Starch from wheat, unmodified \((C_6H_{10}O_5)_n\), a highly polymeric carbohydrate, was purchased from Sigma-Aldrich (St. Louis, MO, USA) in the form of white powder with moisture content of 9.6% (w/w). Moisture was estimated (AACC 1983, Method 44-15A) before preparing each sample for DSC work. \(\alpha\)-Amylase \((Bacillus licheniformis)\) enzyme solution from Megazyme Int. (Wicklow, Ireland) was used as purchased.

2.2 Sample preparation

In the first phase of the project, unmodified wheat starch was taken to prepare high solid samples for thermal and mechanical evaluations. In the second stage, samples were prepared for enzymatic assay with a solids content of 77.5% (w/w), which was selected on the basis of the thermomechanical studies.

Preparation of starch for thermomechanical studies: Unmodified wheat starch samples were prepared with a range of solids content from approximately 50 to 87.5% (w/w) using the direct pan procedure described by Zeleznak and Hoseney (1987). Samples were also prepared using the glass vial method described by Yu and Christie (2001).

Sample preparation for enzymatic assay: Unmodified wheat starch was gelatinised by heating a 10% (w/w) starch solution at 90°C for 10 min and the resultant material was freeze dried. Individual samples weighing 0.1 g each were prepared and stored in desiccators until investigated.

2.3 Methods

Calorimetric measurements: A modulated differential scanning calorimeter (DSC Q2000) from TA instruments (New Castle, DE, USA) with nitrogen purge gas (flow rate 50 mL/min) was used to perform thermal measurements. The instrument was fitted with a refrigerated cooling system to achieve subzero temperatures (as low as -90 °C in this study). The heat signals were calibrated by a traceable indium standard \((\Delta H_f = 28.3 \text{ J/g})\) and the heat capacity response by a sapphire standard to ensure accuracy of measurements. \(T_{zero}\) pans and lids with 8 mg of samples were used, and an empty \(T_{zero}\) pan was the reference. Preliminary studies were done using scan rates of 1, 2, 5 and
10°C/min in order to compare the clarity of peaks and to optimize the rate for subsequent analyses. A ramp rate of 10°C/min was selected to provide high quality results. All pans were scanned at the heating rate of 10°C/min, from 30 to 95°C, followed by cooling to -90°C and reheating to 95°C. Glass transition temperatures were recorded from the second heating scan.

Rheological measurements: A dynamic mechanical analyser (DMA 8000) from Perkin Elmer (Waltham, MA, USA) was used to investigate the vitrification properties of our starch sample. Compression (aluminium pocket encapsulated) mode was used to scan the samples at 2°C/min through a temperature range of -65 to 110°C with liquid nitrogen as the coolant and a frequency of 1 rad/s.

Spectrophotometry: A Lambda 35 UV-vis spectrophotometer from Perkin Elmer (Waltham, MA, USA) was used to record the absorbance in glucose equivalents at 540 nm following enzymatic hydrolysis of starch.

Enzymatic study: A dinitrosalicylic acid (DNS) method described by Miller (1959) was chosen and adapted to the assay of reducing sugars as it was found in preliminary trials to be more sensitive than the procedure given by Somogyi (1952) at the high level of solids in this investigation. An ARB fridge-freezer was used to control temperature within the range of -18 to 10°C. The actual temperatures were authenticated using a thermocouple before analysis commenced.
3. RESULTS AND DISCUSSION

3.1 Effect of temperature on starch matrices at varying moisture levels

The DSC curves presented in Fig. 1 give the average of triplicate values/scans for starch concentrations in the range of 50 to 87.5% (w/w). Gelatinization was clearly observed when a temperature ramp of 10°C was applied, since the sensitivity of thermal events increases with heating rate (Katayama, Carpenter, Manning, Randolph, Setlow and Menard, 2008). In these first heating scans, gelatinization of starch is observed to occur at temperatures in the range of 55 to 70°C for the experimental solids content. Copeland, Blazek, Salman and Tang (2009) reported that the gelatinization temperatures of most starches are observed to occur between 60 and 80°C, which is consistent with the results of our study. They also stated that gelatinization occurs when native starch is heated in the presence of sufficient moisture; the granules absorb water, swell and the crystalline organization is irreversibly disrupted. Our data demonstrate that sufficient moisture must be available for the process of gelatinisation to occur, since at the top end of solids content endothermic events are not clearly discernible.

Fig. 1 Gelatinization of wheat starch samples as a function of concentration obtained using MDSC at a heating rate of 10°C/min. The lowest curve is 50% (w/w) solids; other curves upwards are 60, 70, 72.5, 75, 77.5, 80 and 87.5%, respectively.
In subsequent cooling scans, high intensity exothermic peaks were observed for moisture content of around 25% (w/w; data not shown here). These were attributed to crystallization of free (available) water, but were not visible as the moisture content was reduced to about 13% (w/w) in preparations. MDSC mode was “activated” during the second heating of these materials to increase the sensitivity and resolution of analysis as the resultant scans could be resolved into “reversing” and “non-reversing” heat flow (Rabel, Jona and Maurin, 1999). Fig. 2 reproduces this deconvolution of the total heat flow for a starch sample of 77.5% (w/w) solids, where the onset of devitrification is observed in the reversing signal at temperatures above -10°C.

Fig. 2 Second heating profile of 77.5% (w/w) wheat starch sample obtained using MDSC at a heating rate of 10°C/min. Presenting total heat flow (Δ), reversing heat flow (○) and non-reversing heat flow (□) as a function of temperature.

In the current study, the $T_g$ value of -10°C for the starch matrix at 77.5% moisture was found to be highly repeatable and is compared with values available from the literature. Table 1 lists $T_g$ data for starches from various sources that range from 5 to as high as 100°C. Results reflect the characteristics of particular sources and experimental protocol of analysis but, also, the effect of moisture content, which is pronounced on the glass transition temperature of starch in the high solids end (70 to 95%, w/w) of the state diagram.
### Table 1 Compilation of literature data on $T_g$ values of starch systems

<table>
<thead>
<tr>
<th>Authors</th>
<th>$T_g$ value</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeleznak and Hoseney (1987)</td>
<td>30-90°C for wheat starches (13-18.9% moisture content)</td>
<td>$T_g$ would be below ambient temperatures if water content was above 20%</td>
</tr>
<tr>
<td>Stepto and Tomka (1987)</td>
<td>25°C for extruded potato starch (15-20% moisture content)</td>
<td></td>
</tr>
<tr>
<td>Van Soest, Benes and De Wit (1996)</td>
<td>5°C for extruded potato starch with 14% moisture content</td>
<td>$T_g$ could not be measured for moisture content above 14%</td>
</tr>
<tr>
<td>Shogren (1992)</td>
<td>No $T_g$ for corn starch on initial scan. On second scan, $T_g$ detected at 20-60°C (25-50% moisture content)</td>
<td>Polysaccharide was found to be highly ordered</td>
</tr>
<tr>
<td>Lourdin, Coignard, Bizot and Colona (1997)</td>
<td>90-100°C for potato starch (13-15% moisture content)</td>
<td></td>
</tr>
<tr>
<td>Rindlava, Hullemen and Gatenthaloma (1997)</td>
<td>75-95°C for potato starch (13-15% moisture content)</td>
<td>$T_g$ decreased linearly as the moisture content increased</td>
</tr>
<tr>
<td>Liu, Yu, Liu, Chen and Li (2009)</td>
<td>59.2, 61.4 and 67.3°C for corn starch with 13.3, 11.6 and 8.7% moisture.</td>
<td>Used high speed DSC with heating rate up to 250°C/min</td>
</tr>
<tr>
<td>Various researchers (Biliaderis 1992; Chinachoti 1996; Maurice, Slade, Sirett and Page 1985)</td>
<td>Considered that DSC may not be suitable to study the $T_g$ of starch because the heat capacity of the transition is too weak and is frequently masked by the gelatinization endotherm</td>
<td></td>
</tr>
</tbody>
</table>
3.2 Mechanical glass transition properties

To further investigate the vitrification properties of the starch matrix, mechanical spectroscopy was employed. When a parallel plate rheometer was trialled, it was observed that the sample was very brittle, breaking as soon as the geometry was lowered onto the surface so that it was not possible to perform the analysis. Accordingly, DMA was chosen where the sample in a powder form could be analysed, and results in Fig. 3 provide further information on the starch matrix. The midpoint of devitrification upon heating in the $E'$ trace or the peak of the tan $\delta$ trace can be considered as an expedient indicator of the mechanical glass transition temperature.

![Graph showing storage $(E')$, loss $(E''$, tan $\delta$ variation as a function of temperature for 77.5% (w/w) wheat starch sample measured at a rate of 2°C/min and a frequency of 1 rad/s.]

This was found to be around -3°C for the starch matrix at 77.5% (w/w) solids, which is somewhat higher than the prediction using calorimetry ($T_g$ about -10°C). The empirical nature of these measurements and the distinct relaxation processes of starch molecules measured by the two techniques should be considered in this context. Results from the thermomechanical analyses, discussed in Figures 1 to 3, produce a solid grounding of the glassy behaviour in our system as a function of temperature, which can be utilised as a basis for subsequent enzymatic work.
### 3.3 Optimization of the enzymatic assay on starch

**Amount of sample and enzyme:** Prior to enzymatic studies, unmodified wheat starch was heat treated and freeze dried so that a matrix could be produced at high solids content equivalent to that used in thermomechanical studies. Based upon an estimation of the amount of starch taken, the amount of enzyme to be added was calculated from the data of the enzymatic activity for the α-amylase provided by the manufacturer. The amount of enzyme corresponded to the release of 30 µmoles per minute per gram of the sample, and the flow chart of optimising the enzymatic assay is reproduced in Fig. 4.

**Equilibration time:** Initially the time of the reaction, that is, the period after introduction of the enzyme into the sample matrix was taken as 24 h to allow sufficient time for it to act. Based on preliminary results, the equilibration time was reduced to 1 h, 30 and 15 min, so that the relationship between absorbance values and time of incubation were within linear range of the enzymatic action. It was found that periods of 15 min gave reproducible results in comparison to other time periods and the former was routinely applied.

**Solvent requirements for the enzymatic assay:** A solution of 0.1 M NaOH was used to inactivate α-amylase in the starch matrix. Possible interference due to colour development was evaluated by estimating the response of standard glucose solutions, with the colorimetric reagent (DNS), in direct comparison between samples with NaOH and MilliQ water. As the resultant readings of absorbance were identical, NaOH was selected as the reagent for inactivation of the enzyme. Initial experiments were conducted with 25, 50 and 100 mL of the solution and the absorbance was noted. It was found that 25 and 100 mL of solution developed very high and low absorbance values, respectively. On this basis, 50 mL of 0.1 M NaOH was selected for subsequent use.

**Timeframe of enzymatic activity observation:** Recording absorbance was initially 12 h, but was reduced to 60, 30, 15 and 5 min based on the preliminary results of this investigation. Five min time interval was selected in the end for the experimental work. Thus, several aliquots of starch and enzyme mixtures were prepared and incubated at each experimental temperature for 15 min and then, in a sequence of 5 min intervals, were subjected to the routine in Fig. 4 leading to measurements of absorbance that reflect enzymatic activity.
Temperature range of interest: Based on the findings of the rheological study in the preceding section, experimental temperatures at intervals of four degree centigrade above and below the prediction of $T_g$ (about $-3^\circ C$) was considered for the enzymatic part of this work.

Wheat starch gelatinised (10% starch solution heated at 90°C for 10 min and freeze dried)

Sample weighed in small containers (0.1 g each) (stored overnight in refrigerator/freezer at the temperature of interest)

$\alpha$-Amylase added (0.029 mL) with thorough mixing (to achieve 77.5% solids) and equilibrated for 15 min at the temperature of interest

NaOH solution (50 mL, 0.1 M) added to each sample and vortexed vigorously

Aliquot of sample taken (1.5 mL), added to DNS reagent (2.0 mL) and mixed

Heated in water bath at 100°C for 15 min

Cooled under running tap water and held for 15 min

Absorbance read at 540 nm against blank

Fig. 4 Flow chart of an optimized assay procedure for $\alpha$-amylase action on starch.
3.4 Molecular dynamics of the enzyme in a carbohydrate matrix

The concept of initial velocity ($v_0$) described by Whitaker (2003) was used to estimate the rate of reaction for the various temperatures under consideration. Initial velocity was determined as near time zero in the time course of the reaction as possible. No more than 5% of the original substrate should be converted to product to determine $v_0$. This approach largely eliminates problems caused by changes in the degree of saturation of the enzyme with substrate during reaction time while also minimising the influence of the reverse reaction occurring when product accumulates. As it is based on a continuous recording, not on one point assay, the substrate concentration at time zero can be used. A typical standard curve obtained with glucose using the DNS method is presented in Fig. 5; it had an $R^2$ value of 0.998 and was used to calculate the amount of reducing sugar released enzymatically during starch hydrolysis.

![Graph](image)

Fig. 5 Standard graph for glucose release as a function of concentration (mM) measured using DNS at 540 nm.

During standardization of the protocol, the time interval for observations was reduced to 5 min from 1 h so that readings were taken within the linear region of the initial slope in the time course of enzymatic activity. The focus here is that of assessing the significance of the starch matrix characteristics upon the ability of α-amylase to act...
and produce reducing sugars. The outcomes are presented in Figures 6 to 8. It is evident that the amount of glucose released increases as a function of time at a particular temperature, and does so as a function of temperature (Fig. 6).

Fig. 6 Absorbance as a function of time at -14 (◇), -10 (○), -6 (●), -2 (○), 2(▲), 6 (Δ) and 10 (■) °C with an observation interval of 5 min.

The initial slope values derived from the time course data presented in Fig. 6 were plotted as a function of temperature in Fig. 7 for each experimental temperature. There is a clear change in the pattern of enzymatic activity within the starch matrix as a function of temperature, and this discontinuity is observed at a temperature corresponding to the mechanical $T_g$. 

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Fig. 7 Rate of reaction as a function of temperature for hydrolysis of wheat starch by α-amylase at the vicinity of the mechanical $T_g$. The units of reaction rate are picomoles per second per gram of starch substrate (i.e., picokatals of enzymatic activity per gram) following adjustment to 77.5% (w/w) solids for starch and including the addition of an enzyme solution at 0.029 mL.

The discontinuity in the relationship and the much greater influence of temperature on enzymatic activity above the $T_g$, as compared to subzero temperatures, indicates reduced molecular mobility. This limits the interaction of the enzyme with its substrate corresponding to reduced kinetics of amylolysis below $T_g$. For starch, it has previously been reported that granule characteristics influence susceptibility to attack by α-amylase, including crystallinity, granule size and available specific surface, amylose to amylpectin ratio, porosity, structural inhomogeneities and the degree of integrity (Copeland et al., 2009). As far as we are aware, this is the first report of an observation that emphasizes the importance of glassy starch matrix on the functionality of the enzyme-substrate complex.
Further reduction in temperature below $T_g$ results in a negligible rate of enzymatic activity, e.g. at -14°C in Fig. 7. The glassy state of the starch matrix hinders the diffusional mobility of the enzyme and this process was examined at a molecular level with the predictions of the reaction rate theory applied via the modified Arrhenius equation for a set of two experimental temperatures (Peleg, Engel, Gonzalez-Martinez and Corradini, 2002). In conventional food science, the standard approach, i.e. with a single temperature term, is used to evaluate the effect of temperature on viscosity ($\mu$) and microbial inactivation during thermal treatment. In our case it takes the form:

$$\log\left(\frac{k_0}{k}\right) = \frac{E}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)$$  

(1)

where, $k$ is the reaction rate, $k_0$ is the rate constant at a reference temperature $T_0$, $E$ is the “energy of activation” in J/mol, and $R$ is the gas constant. The most obvious advantage of the modified Arrhenius model is that, in applicable systems, knowing the values of $k$ (or $\mu$) at any two temperatures is sufficient to calculate $E/R$. Once calculated, this ratio can be used to estimate the magnitude of $k$ (or $\mu$) at any other temperature within the pertinent range.

The Arrhenius model in equation (1) entails that the plot of $\log (k_0/k)$ versus $1/T$ is a straight line with a slope ($E/R$) from which the “energy of activation” is extracted. To make headway on this, and also add to our understanding the kinetic patterns of enzymatic activity, the recently proposed concept of spectroscopic shift factor was used (Kasapis and Shrinivas, 2010). In doing so, we considered a very acceptable linear relationship observed for the 25 min absorbance-time data in Fig. 6. This section of the spectrum can be treated as a zero-order reaction with the gradient being the rate constant at $k = \frac{dx}{dt}$. For each experimental temperature, a spectroscopic shift factor, $\log (k_0/k)$, was developed, where $k_0$ is the rate constant at the reference temperature of -2°C. Outcome is plotted in Fig. 8 covering the temperature range of -10 to 10°C.
Fig. 8 Plot of spectroscopic shift factor in the form of the ratio of rate constants as a function of experimental temperature.

Application of the modified Arrhenius model to the data of Fig. 8 in the current study produces activation energy values of 6.28 and 0.42 kJ/mol for the temperatures below and above $T_g$, respectively. Direct comparison of molecular dynamics between carbohydrate matrices with glassy consistency and enzymatic activity can now be made, which demonstrates the increasing difficulty to enzymatic diffusional mobility for hydrolytic action to occur at temperatures below the mechanical $T_g$. 
4. CONCLUSIONS

This investigation utilised a novel approach in combining thermomechanical analysis and UV-vis spectroscopy in order to identify the kinetics of enzymatic activity on a polysaccharide matrix in the glassy state. Physicochemical measurements, i.e. rheology and calorimetry, were complementary in recording the relaxation dynamics in the form of the glass transition temperature of condensed matrices of unmodified wheat starch. The spectroscopic protocol added to our understanding of the molecular nature of the starch matrix by unveiling the hydrolytic activity of $\alpha$-amylase on the said matrix. Enzymatic activity changes appear to respond to the vitrification phenomena in the starch network as monitored by rheology allowing estimation of activation energies associated with molecular processes within the temperature range of industrial interest. The pronounced effect of the mechanical glass transition temperature on enzymatic activity emphasizes the importance of molecular interactions in a dense polysaccharide matrix for the quality control of material properties and the ways these are measured.

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REFERENCES


Chapter 7

Results and discussion

Enzymatic catalysis in a whey protein matrix at temperatures in the vicinity of the glass transition

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Note that further supplementary details regarding the calculation of enzymatic data is presented in the Appendix to this thesis
ENZYMATIC CATALYSIS IN A WHEY PROTEIN MATRIX AT TEMPERATURES IN THE VICINITY OF THE GLASS TRANSITION

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ABSTRACT

The current study deals with the effect of temperature on the activity of the enzyme α-glucosidase incorporated into a whey protein matrix through spray drying. Thermomechanical characterisation of the matrix was achieved using the techniques of modulated differential scanning calorimetry and small-deformation dynamic mechanical analysis. In solutions of the protein, as the concentration was raised from 75 to 94% (w/w), denaturation occurred at increasing temperatures. In contrast, after spray drying, denaturation was not observed in calorimetric scans. The glass transition temperature ($T_g$) measured in the dried particles using dynamic mechanical analysis was about 40°C. An optimised procedure was developed whereby α-glucosidase and its substrate p-nitrophenyl α-D-glucopyranoside were incorporated into the whey matrix. The effect of temperature on enzymatic catalysis was investigated and, below 40°C, activity was low and relatively independent of temperature. In contrast, the rates of product formation markedly accelerated as temperatures were increased beyond $T_g$. These novel observations strongly emphasise the pronounced effect of mechanical $T_g$ of the protein matrix on enzymatic activity.

Keywords: whey protein isolate, glass transition temperature, α-glucosidase, spray drying
1. INTRODUCTION

Over recent decades, the transition between glassy and rubbery states in amorphous solid materials has attracted particular attention reflecting its significance as a determinant of the stability characteristics in food and pharmaceutical systems (Slade and Levine, 1995). The glass transition temperature ($T_g$) is an important characteristic of such materials and is strongly influenced by moisture content. Structural stability of individual components within an amorphous material is high in the glassy state, and these matrices can retain their physical state for periods of years or even longer. This reflects the inhibition of large-scale molecular rearrangements and macroscopic flow at temperatures below $T_g$.

Increased chemical stability is observed in glassy systems, as the rates of reaction will be very low, with an early report describing the Maillard reaction between carbohydrates and proteins (Eichner and Karel, 1972). However, several dynamic processes can still proceed in the glassy state at measurable rates. Examples are the diffusion of water (Tromp, Parker, and Ring, 1997) as well as other small molecules including gases (Schoonman, Ubbink, Bisperink, Le Meste and Karel, 2002) along with local rearrangements of the carbohydrate molecules resulting in glassy-state aging of the matrix (Noel, Parker, Brownsey, Farhat, MacNaughtan and Ring, 2005).

Enzymes are important in many food systems, as they may be naturally occurring components of ingredients, produced during fermentation or spoilage and also following intentional incorporation into the formulation. In the context of the varied roles of enzymes, both as deteriorative agents and also in enhancing product quality and ease of processing, relatively few researchers have addressed the influence of the glass transition upon the rates of enzymatically catalysed reactions within amorphous matrices. Recently, we have reported upon the activity of $\alpha$-glucosidase/$\beta$NPG complex as affected by a glassy polysaccharide matrix (Chaudhary, Small and Kasapis, 2013).

Whilst carbohydrate systems are widely employed as amorphous matrices in foods and pharmaceuticals, a variety of protein ingredients also have potential for the formulation of commercial products. Proteins provide a range of useful functional properties (Shahidi and Han, 1993) and these have been described for gelatin (Draye, Delaey, Van de Voorde, Van Den Bulcke, Reu and Schacht, 1998) and wheat gliadin...
In addition, whey protein gels have been used as pH-sensitive hydrogels for the controlled delivery of biologically active substances (Gunasekaran, Xiao and Ould Eleya, 2006) and their aggregates have been shown to participate in the formation of hydrogels, gel beads and submicron particles for protecting and controlling the release of bioactive ingredients (Nicolai, Britten and Schmitt, 2011). Advantages in using whey proteins in controlled-release applications is their association without the need for chemical cross-linking agents and their biodegradability: two of the major requirements for use in pharmaceutical, food and bioprocessing areas (Gunasekaran, Ko and Xiao, 2007).

Whey powders are also increasingly used in the development of nutritional preparations where enzyme stabilization is required in the dry state. Several authors (Stellwagen, Cronlund and Barnes, 1973; Yang, Marchio and Yen, 1994; Schebor, Burin, Buera, Aguilera and Chirife, 1997) have reported and attributed the increased stability of β-galactosidase in liquid or solid dairy systems to lactose (substrate) binding protection. The low molecular mobility of various components in the glassy state appears to govern changes that affect the enzymatic stability in dry amorphous dairy systems including mobility of enzyme side chains for folding processes, reactant (amino-carbonyl) mobility for the Maillard reaction and lactose mobility for the crystallization process of the matrix. Therefore, the physical properties of the matrices, which are used to encapsulate enzymes often exert control by affecting enzyme activity and stability (Burin, Joupilla, Roos, Kansikas and Buera, 2004).

The increasing interest in the properties of whey proteins as encapsulating agents for the food industry reflects the need for effective and selective delivery of bioactive agents to the site of action. As natural polymers, biomacromolecules provide advantages over synthetic polymers due to their great availability, low cost, low toxicity and ease of modification (Chasin and Langer, 1990). The objective of the present study has been to extend our previous findings on enzyme activity in the deacylated gellan matrix by investigating the relationship between $T_g$ and behaviour of α-glucosidase encapsulated in a model system prepared by spray drying of whey protein isolate.
2. MATERIALS AND METHODS

2.1 Materials

In the current study, the standard whey protein isolate (WPI) used was from MG Nutritionals, Murray Goulburn Co-operative Ltd, VIC, Australia. According to the supplier, the composition of the WPI was 91.3% protein (TN × 6.38), 0.7% fat, 3.5% moisture, 3.8% ash and 0.44% lactose. The pH of a 10% (w/w) solution was 6.3, bulk density of the powder was 0.45 g/mL and a standard plate count produced 9,900 cfu/g.

The enzyme used (α-D-glucoside glucohydrolase (EC 3.2.1.20), product number G3651) from Bacillus stearothermophilus was from Sigma-Aldrich (St. Louis, MO, USA). The lyophilized powder (3.2 mg, containing potassium phosphate buffer salt) was dissolved in potassium phosphate buffer (10 mL, 67 mM, pH 6.8 at ambient temperature) prior to use. Substrate (p-nitrophenyl α-D-glucopyranoside; pNPG), potassium phosphate and L-glutathione (acting as stabiliser) were also purchased from Sigma-Aldrich and sodium carbonate was from BDH, Port Fairy, VIC, Australia. Milli-Q water from Millipore was used in all experiments.

2.2 Sample preparation

Preparation of whey protein for thermal studies: WPI dispersions of 30% (w/w) solids were prepared by mixing the powder in Milli-Q water at ambient temperature. Dispersions were stirred for ~2 hrs using a magnetic stirrer to ensure maximum dissolution. In order to achieve thorough hydration and to remove air bubbles, dispersions were stored overnight at 4°C. On the following day, a rotary evaporator was used at 40 ± 1°C to remove water and prepare a series of solutions at concentrations of 75, 77.5, 80, 82.5 and 85% (w/w). A spray dried sample with 94% solids content was also subjected to thermal analysis.

Sample preparation for dynamic mechanical analysis, scanning electron microscopy and enzymatic studies: WPI dispersions of 10% (w/w) solids were prepared by mixing the powder in Milli-Q water and holding at 4°C overnight to ensure thorough hydration. Following optimization of the spray drying procedure, the dispersion was treated using a Lab Plant SD Basic FT30MKIII spray drier (Keison products, Chelmsford, Essex, UK) to produce microcapsules for dynamic mechanical analysis and scanning electron microscopy.
Microcapsules for the enzymatic assay were also produced by following the same procedure after mixing the WPI dispersion (50 g in 500 mL MilliQ water) with potassium phosphate buffer (25 mL, 67 mM), glutathione (3.32 mL, 3 mM), enzyme solution (1.67 mL equivalent to 42 units) and \( p \)-NPG (20 mL, 20 mM), with the mixture being constantly stirred while spray drying. The microcapsules were produced in triplicate and stored at -30°C for subsequent analysis.

2.3 Methods

**Calorimetric measurements:** Samples consisting of WPI (75 to 94% solids, w/w) were hermetically sealed in \( T_{\text{zero}} \) pans and subjected to modulated differential scanning calorimetry (MDSC) measurements (DSC Q2000, TA instruments, New Castle, DE). To calibrate the heat flow signals, a traceable indium standard (\( \Delta H_f = 28.3 \text{ J/g} \)) and a sapphire standard were utilised. Samples of 8 mg were heated to 90°C, cooled to -90°C and reheated to 90°C, at a modulation amplitude of 0.53°C for each period of 40 s. Temperatures down to -90°C were achieved by using a refrigerated cooling system attached to the calorimeter. An empty \( T_{\text{zero}} \) pan was taken as the reference, nitrogen purge gas was at a flow rate of 50 mL/min, and controlled scanning was carried out at 2°C/min.

**Rheological measurements:** Estimates of the glass transition temperature of spray dried WPI (94% solids) was obtained using a dynamic mechanical analyser (DMA 8000) from Perkin Elmer (Waltham, MA, USA) with liquid nitrogen as the coolant. The sample was subjected to deformation mode of compression within a temperature ramp of -100 to 90°C, scan rate of 2°C/min and frequency of 1 rad/s. Results reported for thermal and rheological experiments are of individual traces selected as representative of three replicates, which were effectively superposing traces.

**Scanning electron microscopy (SEM):** Images of WPI particles produced by spray drying were obtained using an FEI Quanta 200 ESEM instrument (Hillsboro, OR, USA). Samples were gold-coated and imaged using high-vacuum mode at an accelerating voltage of 30 kV, pressure of 0.54 mbar and spot size 5. Regarding the moisture content of our materials, these were analysed gravimetrically by oven-drying at 100°C for 24 hrs.

**Spectrophotometry and enzymatic assay:** In 10% (w/w) WPI solution (500 mL) we added 1.67 mL enzyme equivalent to 42 units and 20 mL of \( p \)-NPG (20 mM
concentration). This solution was spray dried, and to analyse the enzymatic activity in spray dried WPI (94% solids), containing the above enzyme-substrate complex, a spectrophotometric approach from Sigma was adapted and utilised. For this, samples were individually packed in small containers (0.1 g each) and stored at -30°C to prevent enzymolysis until experimentation commenced. In doing so, the spray dried powders were equilibrated at various temperatures (25, 30, 35, 40, 45, 50 or 55°C) selected to extend the range of observations above and below the mechanical $T_g$ value of 40°C established using DMA. Five containers with 0.1 g sample each were stored at the aforementioned temperatures for an hour before commencing the “incubation” and taking readings at 0, 5, 10, 15 and 20 minutes.

At each time, the enzyme was inactivated by addition of sodium carbonate solution (8.0 mL, 100 mM) and vortexing vigorously for one minute. Absorbance of the resultant solution was recorded at 400 nm on a Lambda 35 UV-vis spectrophotometer (Perkin Elmer, Singapore) for each time interval of 5 min. Measurements of enzyme activity at each experimental temperature were carried out in triplicate and average values are reported.
3. RESULTS AND DISCUSSION

3.1 Effect of temperature on high-solid WPI matrices

High-solids preparations of WPI, which for the purposes of this investigation are considered as those with solids level above 70%, were subjected to MDSC. The technique measures the difference in heat flow between sample and inert reference as a function of time and temperature, with both sample and reference being subjected to a controlled environment of time, temperature, atmosphere and pressure (Rahman, Machado-Velasco, Sosa-Morales, and Velez-Ruiz, 2009). It facilitates assessment of disturbances in the conformational arrangement of proteins in solutions and gels during heating (Paulsson and Dejmek, 1990). Our samples were scanned relatively slowly (at 2°C/min), and Fig. 1 depicts thermograms of the samples (75-94% solids, w/w) during heating from 30 to 90°C.

![Thermograms of samples during heating](image)

**Fig. 1** Denaturation of whey protein samples as a function of concentration varying from 75 to 94% obtained using MDSC (scan rate: 2°C/min).

Well-defined troughs with some variation in size and range of temperature bands reflect the main characteristics of the endothermic event observed for each preparation. The denaturation event ranged from approximately 72 to 81°C (midpoint transition...
temperatures), and these shifted towards higher temperatures as the polymer concentration increased, an outcome indicating increased stability with higher material density. Results confirm those reported by Fitzsimons, Mulvihill and Morris (2007) in whey protein preparations of lower concentrations. In the context of utilising spray drying for encapsulation of the enzyme-substrate complex, spray dried powder was also subjected to calorimetry. In contrast to aqueous WPI pastes at 75 to 85% (w/w) solids, the WPI powder did not show evidence of a thermal event reflecting its very high solids content (94%) and the denaturation effect of the spray drying process.

3.2 Optimisation of the spray drying procedure

Regarding the spray drying of heat sensitive materials, settings commonly used involve spraying at an inlet temperature of about 100°C with low flow rates. In order to prepare samples of WPI, which are suitable for investigation of glass transition phenomena, temperatures around 70°C were trialled here with relatively high flow rates. It has been reported that α-glucosidase from *Bacillus stearothermophilus* remains stable at temperatures above 70°C (Suzuki, Shinji and Eto, 1984), nevertheless, the above inlet temperature was chosen due to the general sensitivity of enzymes with increasing thermal treatment. We succeeded in drying WPI at 70°C inlet temperature with a speed setting of 5, corresponding to a flow rate of 6.68 mL/min, at an air pressure of 4 kgf/cm². The resultant outlet temperature was in the range of 42 to 44°C, and the average moisture content of the powder was found to be 6 percent in the end product. Once the spray drying conditions were optimised, a mixture of WPI, buffer, glutathione, enzyme and substrate was spray dried to produce a material where the enzymatic activity was analysed by the procedure described in the Materials and Methods section and depicted as a flow chart in Fig. 2.
WPI spray dried with potassium phosphate buffer, glutathione, α-glucosidase and pNPG

↓

Sample weighed in small containers (0.1 g each)

↓

Equilibrated for 1 hr in incubator at the temperature of interest

↓

Reaction terminated on a five minute interval by adding NaCO₃ solution (8 mL, 100 mM) with vortexing

↓

Absorbance read at 400 nm against a blank

Fig. 2 Flow chart of an optimised assay procedure for α-glucosidase/pNPG complex spray dried in a whey protein matrix.

3.3 Estimation of the mechanical $T_g$

Aluminium-pocket encapsulated sample of WPI following spray drying was subjected to small-deformation mechanical spectroscopy (DMA) in an effort to record glass related phenomena. The peak of the tan $\delta$ trace is a common indicator used to report the glass transition temperature of biomaterials (Menard, 2008). In the present study, the $T_g$ value is found to be about 40°C, which has been derived from the peak of the tan $\delta$ trace as the ratio of loss and storage modulus values upon devitrification of the WPI matrix during controlled heating (Fig. 3).
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Fig. 3 Storage ($E'$; ◆) and loss ($E''$; ◊) modulus, and $\tan\delta$ (▲) variation as a function of temperature for 94% spray dried whey protein sample (scan rate: 2°C/min; frequency: 1 rad/s).

3.4 Microstructural features

The outer topography of the particles of spray-dried powders revealed by the use of scanning electron microscopy indicates that the microcapsules were of relatively uniform size and spherical in shape with very low levels of breakage (Fig. 4). Spherical microcapsules with diameters in the range of 3 to 40 μm have been reported earlier for spray dried WPI powders by Rosenberg and Sheu (1996) and Rosenberg and Young, (1993).
Fig. 4 Microcapsules of spray dried whey protein isolate with 94% solids at different magnifications shown on the microscopy images.
The importance of whey protein in influencing the drying and rheological characteristics, at the very high concentration of the material in the wall system, of the microcapsule has been reported by Rosenberg and Young, 1993 and Young, Sarda and Rosenberg 1993. They reported that limited surface indentations were observed with increasing proportions of WPI in spray dried preparations. As the microstructural features of the capsules affect the retention of volatile components during storage (Chang, Scire and Jacobs, 1988), production of intact spray dried capsules in the current study is an indicator of high protection of the encapsulated enzyme-substrate complex through the use of whey protein isolate as the wall ingredient.

3.5 The behaviour of the enzyme-substrate complex encapsulated in the glassy whey protein matrix

In order to facilitate utilization of the techno- and biofunctional benefits of spray dried whey protein, as reviewed, a novel series of experiments was designed to evaluate the possibility of using the material as an encapsulating agent for enzymes. A further objective has been to deepen our understanding of the behaviour of an enzyme-substrate complex, within the protein matrix at temperatures in the vicinity of $T_g$. Following estimation of the glass transition temperature of spray dried WPI, the activity of the enzyme $\alpha$-glucosidase upon its $p$NPG substrate was monitored using a UV-vis spectrophotometer in order to allow measurements within the matrix held at the temperature range of interest.

To suit our project aims and conditions, a series of preliminary experiments was used to modify and adapt a “spectrophotometric stop rate determination method” described by Sigma. A clear trend was observed for the release of the hydrolysis product recorded after 60 minutes of equilibration at specific temperatures around $T_g$. Thus, it was sufficient to control the conditions of low moisture (6% in spray dried whey protein) and ambient to high temperatures (25-55°C) to which the encapsulated enzyme-substrate complex was subjected (Fig. 5).
The enzymatic activity expressed in terms of nanokatals/gram of the matrices was calculated from the slope values of absorbance/time of observation trends reproduced in Figure 5 using the following equation:

\[
\text{Activity (nanokatals/gram)} = \frac{\text{Slope} \times (8 \times 10^6)}{(18.3 \times 300)}
\]  
where, 8 is the volume of NaCO₃ used (mL), 10 converts 0.1 to 1 of the matrix weight taken for analysis (g), \(10^6\) converts milli to nanomoles, 18.3 is the millimolar extinction coefficient of \(p\)-nitrophenol at 400 nm, and 300 is the time of observation (s).

The outcome of this approach is presented in Figure 6, which depicts the effect of the WPI matrix on the ability of \(\alpha\)-glucosidase to act on its substrate (\(p\)NPG) in the

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**Figure 5** Values of absorbance as a function of time at 25 (■), 30 (□), 35 (▲), 40 (△), 45(●), 50 (◊) and 55 (●) °C with an observation interval of 5 min.
vicinity of the $T_g$ value established in earlier phases of this study using dynamic mechanical analysis (about 40°C).

**Fig. 6** Enzymatic activity as a function of temperature for the hydrolysis of pNPG by $\alpha$-glucosidase at the vicinity of the mechanical $T_g$, expressed in terms of nanokatals per gram of the whey protein matrix.

The activity was found to be considerably affected below $T_g$ (low and relatively constant nanokatal values) in comparison to the highly accelerating values observed at temperatures above this index of quality control. To expand our understanding of the molecular nature of the protein matrix in influencing the mobility of the enzyme-substrate complex, experimental data were further subjected to a modified Arrhenius equation utilizing the recently introduced spectroscopic protocol by Kasapis and Shrinivas, 2010:

$$\log \left( \frac{k}{k_0} \right) = \frac{E}{R} \left( \frac{1}{T} - \frac{1}{T_0} \right)$$

where, $k$ is the reaction rate, $k_0$ is the rate constant at a reference temperature $T_0$, $E$ is the “energy of activation” in J/mol, and $R$ is the gas constant.

For each experimental temperature in Figure 5, we considered a very acceptable linear relationship observed in the 20 min absorbance-time data. This can be treated as a zero-order reaction with the gradient being the rate constant at $k = \frac{dx}{dt}$. A
spectroscopic shift factor, \( \log \left( \frac{k_o}{k} \right) \), is thus developed, where \( k_o \) is the rate constant at the reference temperature of 40°C in this study. Calculated spectroscopic factors are plotted in Figure 7 covering the temperature range of 25 to 55°C. The hydrolytic ability of the enzyme appears to respond to the vitrification phenomenon in the WPI network, as monitored by rheology, and allowed estimation of activation energies associated with molecular processes within the experimental temperature range. Activation energy was found to be 0.99 and 2.59 kJ/mol above and below \( T_g \), respectively, indicating the direct effect of mechanical \( T_g \) on the ability of the enzyme to act on its substrate.

![Graph of Log k_o/k vs Temperature](image)

**Fig. 7** Plot of the spectroscopic shift factor in the form of the ratio of rate constants as a function of experimental temperature.

In recent reports, the pronounced effect of the mechanical \( T_g \) on the molecular mobility of micronutrients has been demonstrated. Thus the diffusion of caffeine in high-solid matrices involving glucose syrup and glucose syrup/\( \kappa \)-carageenan systems at the glass transition region has been studied (Kasapis and Shrinivas, 2010; Jiang and Kasapis, 2011). The effect of deacylated gellan/polydextrose matrices on the ability of \( \alpha \)-glucosidase to hydrolyse a substrate (pNPG) in the vicinity of \( T_g \) has been reported in this Thesis. To our knowledge, this PhD Thesis is the first to systematically investigate...
353 the effect of glassy matrices (WPI, unmodified wheat starch and deacylated gellan) on
354 enzymatic activity with molecular aspects of $T_g$ as the reference point.
355
4. CONCLUSIONS
356
358 The current study is successful in utilising and optimising the technique of spray
359 drying to produce and evaluate high-solid microcapsules with whey protein isolate as
360 their wall material where an enzyme-substrate complex is incorporated. Well defined
361 denaturation troughs were visible at elevated temperatures in condensed WPI solutions
362 using calorimetry, although it was not possible to record a thermal glass transition
363 region at subzero temperatures. On the other hand, we were able to identify a
364 mechanical $T_g$, which, in combination with UV-vis spectroscopy, allowed use of a
365 spectroscopic shift factor in modeling enzymatic activity in the glassy state of the
366 polymeric matrix. It was found that the mechanical $T_g$ plays a major role in determining
367 enzymatic activity, an outcome which emphasises the potential of this approach in the
368 control of molecular processes that influence quality attributes of food materials.
369
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Chapter 7


Chapter 8

General discussion and conclusions

The purpose of this chapter is to briefly summarise the results obtained during the current study, draw final conclusions and make recommendations for further research.

8.1 Introduction

In the development of this project, a survey of the literature indicated that, since it was first introduced approximately twenty (1990’s) years ago, the concept of the transition from the glassy to the rubbery states has been investigated in a variety of food-related systems, amongst others. It is generally accepted that, within the glassy state, mobility is reduced and structural and molecular stability are higher. Although it is also expected that enzymes will have relatively low activities within low moisture systems, there has been virtually no reports of fundamental research designed to elucidate the influence of temperature on catalytic activity in food systems under conditions where the transition in the amorphous states might take place. It is likely that this, at least partially, reflects the challenges of effectively and reliably measuring enzymatic activity under the relatively low moisture conditions involved. In addition, the incorporation of enzyme along with substrate into model matrix systems is difficult and the usual approaches to measurement of activity in aqueous solutions require considerable modification if they are to be applied for the purposes of the current investigation.

The results described in this thesis fall into four broad areas. These are:

1. Selection, development and characterization of suitable matrix systems;
2. The establishment of the levels of co-solute polydextrose for use in gellan-based matrices for this study;
3. The selection of particular enzymes and the development of assay procedures appropriate to the low-moisture matrices developed in the earlier phases of the work;
4. The measurement of enzymatic activities within the model matrix systems and evaluation of the effect of temperature.

The results for each of these are now reviewed as a basis for presenting the primary conclusions of this project and a discussion of areas recommended for further research.

8.2 Selection, development and characterization of suitable matrix systems

To investigate the effect of $T_g$ on enzymatic activity we require a suitable matrix system. For this, we selected suitable hydrocolloids as these are able to develop novel products and modify the existing ones by their wide range of functionality. From a long list of possible hydrocolloids that could be used, we selected starch and gellan, the former being the most abundant polysaccharide in nature and the latter relatively new for food products. We also included whey protein to expand our research from being concentrated to polysaccharides to include protein as well. All three were characterized in terms of their thermomechanical and physicochemical properties where applicable. Characterization of these materials on a macro and micro level assisted us in selecting the matrix most appropriate for further investigation involving enzymes. In particular, suitable matrices of gellan with co-solute, starch and whey protein isolate have been characterized by different thermomechanical and physicochemical methods. The polysaccharides employed depict structural profiles, which are under control and have been utilised to advance our research in terms of enzymatic effects on $T_g$.

8.3 Evaluation of polydextrose as co-solute in a gellan matrix

Hydrocolloids like $\kappa$-carrageenan, alginate, gellan have been studied and reported in detail with different sugar as their co-solutes. As these hydrocolloids can form gels at very low levels of solids (as low as 0.05 %), co-solutes are added to give body to the product and also to modify the functional properties of these hydrocolloids. We selected polydextrose as a co-solute to gellan as it is gaining popularity as dietary fibre and also there have been few publications on polydextrose particularly relating to rheological characterization and applications in food products. Current study revealed that deacylated gellan with polydextrose depict similar thermomechanical profiles like sucrose, fructose or glucose syrup, which is an encouraging outcome suggesting that the binary mixture could be used for novel confectionery formulations. The preceding
statement is further reinforced by the observation that condensed gellan/polydextrose samples exhibit substantial amorphicity without unwanted chemical bond formation during preparation at high temperature followed by cooling to ambient conditions.

8.4 Development of enzymatic assays for the low-moisture matrices

For each matrix under study, a series of preliminary experiments were conducted to modify and adapt existing enzymatic assay to suit the condition of low-moisture dictated by our objective. In the process, the assays were tested and optimized along with derivation of equations to calculate the enzymatic activity. Different approaches to estimate enzyme activity, from literature were considered, before the final set of observations were recorded and subsequently analysed. Procedure for sample preparation and enzymatic assay has been optimized by studying matrices as a substrate or as an encapsulant for the enzyme-substrate complex.

8.5 Enzymatic activity within model matrix systems and the effect of temperature

Whilst it has long been known that enzymes have limited activity within low moisture systems and at low temperatures, all the cited reports have investigated enzymatic activity at a single temperature. In contrast, this is a systematic study where we have studied the enzymatic activity at a range of temperatures in the vicinity of the glass transition temperature. The results indicate that enzymatic activity is relatively independent of temperature below $T_g$ but temperatures above $T_g$ have a strong influence on enzymatic activity. This result was observed in all three matrices of the current study including the polysaccharides and the proteins.

8.6 Major conclusions

The final conclusions of this study are summarized here:

1. Conformationally distinct biopolymers, gellan, starch and WPI, have been characterized by following their phase transitions in the vicinity of the rubber-to-glass transition.
2. The synthetic polymer approach has been utilized to predict the mechanical $T_g$ and specific C1 and C2 values of the free volume theory have been estimated for the polymeric matrix.
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3. Model systems controlling the kinetics of enzymatic activity within the glassy state have been successfully developed and evaluated.

4. Mechanical $T_g$, in combination with UV-vis spectroscopy, allowed use of a new concept, the spectroscopic shift factor, in modeling enzymatic activity in the glassy state of the polymeric matrix.

5. The current findings provide compelling evidence on the role of network glass transition temperature in diffusional processes of bioactive compounds and proteins.

6. To our knowledge, this PhD Thesis is the first to systematically investigate the effect of glassy matrices (deacylated gellan, unmodified wheat starch and WPI) on enzymatic activity with molecular aspects of $T_g$ as the reference point.

8.7 Possible areas for future research

This study has concentrated on the effect of a high-solid matrix on the enzymatic activity while serving as the substrate to the enzyme (starch/α-amylase system) or encapsulating the enzyme (gellan/polydextrose/α-glucosidase or WPI/α-glucosidase) in the vicinity of its $T_g$. It would be of value to extend this work to other matrix-enzyme systems as food is a complex material, and there are many other beneficial enzymes that can also be utilised. Accordingly, studies of different matrices may provide further insights on the effect of $T_g$.

Another issue highlighted by the studies reported here is the paucity of information on the effect of $T_g$ on the enzymatic activity or on bioavailability of bioactive compounds like vitamins. It is reinforced by the findings of the current study that characterization is an important consideration. The procedures validated and adopted here would form a useful basis for further studies in this area. The findings of this study provide a strong basis of knowledge for further investigations aimed at the utilization of the concept of glass transition affecting enzymes/bioactive compounds. As different factors are important in the case of a particular enzyme and related to a specific matrix, it is likely that a variety of different strategies will need to be considered.

In conclusion, there have been rapid developments in our knowledge over recent years in the areas of glass transition and the factors that can be utilised for our benefit. The research reported here is the first systematic investigation to study the effects of
temperature on the activity of an enzyme in the vicinity of the glass transition temperature that has also been estimated using theoretical modeling. It is hoped that this work might form the basis of further studies as the mechanical glass transition temperature has the potential to be applied in the control not only of the mobility of bioactive compounds, reported earlier in the literature, e.g. caffeine, but also of enzymatic activities that might influence product attributes.

There are direct implications for quality control from this work in the processing of biomaterials in addition to traditional considerations based on $T_g$ values established using calorimetric techniques. It appears that much remains to be done to ensure adequate development of a mechanical $T_g$ concept as a means for quality control for further novel production in food and allied industries.
Appendix

**Description of the derivation of equations used in the assay of enzymes embedded within amorphous matrices**

The purpose of this appendix is to briefly describe the derivation of the calculations used in the assay of the enzymes. These relate to \( \alpha \)-glucosidase, firstly in the polysaccharide matrix and secondly within the protein matrix, as described in Chapters 5, and 7, respectively. In addition the approach used for \( \alpha \)-amylase is presented to supplement the material in Chapter 6.

**Assay of \( \alpha \)-glucosidase in the gellan-polydextrose matrix**

A series of preliminary experiments was used to modify and adapt a “spectrophotometric stop rate determination method” described by Sigma for \( \alpha \)-glucosidase, to suit our project aims and conditions. The critical conditions of temperature (\( \leq 50^\circ \text{C} \)), time of preparation (approximately 45 minutes) and pH (6.8) were rigorously maintained to assist in the assay of \( \alpha \)-glucosidase. The optimized process is depicted in Figure A1. The enzymatic activity expressed in terms of nanokatals/gram of the matrices was calculated from the average values after sixty minutes of observation using equation (1) which is based upon that described by Sigma:

\[
\text{Activity (nanokatals/gram)} = \frac{\Delta A_{400}}{(18.3)(10^3)(3600)(0.1)} \quad (1)
\]

Where

\( \Delta A_{400} \) is the change in absorbance during incubation at the particular temperature,
8 is the volume of NaCO\(_3\) used (mL),
\( 10^6 \) converts milli to nanomoles,
18.3 is the millimolar extinction coefficient of \( p \)-nitrophenol at 400 nm,
\( 10^3 \) expresses volumes in units of litre,
3600 is the time of observation (s); and
0.1 is the amount of the matrix taken for analysis (g).
Matrix prepared with 2% gellan and 83% polydextrose using buffer solution incorporating substrate (1.2 mL) and enzyme (0.1 mL) and 0.2 mL glutathione (10 g in total with 85% solids)

↓

Sub-samples (0.1 g) individually packed and stored in refrigerator or freezer at temperature of interest for 60 minutes

↓

Enzyme inactivated by adding Na$_2$CO$_3$ (8 mL, 100 mM)

↓

Vortexed vigorously for one minute

↓

Absorbance measured at 400 nm

Fig. A1 Flow diagram for enzymatic assay of α-glucosidase for the gellan-polydextrose matrix
Appendix

Derivation of equation for calculation of α-glucosidase activity in gellan-polydextrose matrix

Absorbance values were adjusted:

$$\Delta A_{400} = A_{400} \text{ (sample)} - \Delta A_{400} \text{ (blank)}$$

The concentration of $p$-nitrophenol in the solution

$$= \Delta A_{400}/18.3 \text{ millimoles per litre}$$

The amount of $p$-nitrophenol in 1 mL of solution

$$= \Delta A_{400}/(18.3 \times 10^3) \text{ millimoles}$$

The amount of $p$-nitrophenol in 8 mL of solution (the total volume of the assay)

$$= \Delta A_{400} \times 8/(18.3 \times 10^3) \text{ millimoles}$$

However, this is the amount of nitrophenol released by the enzyme present in 0.1 g of matrix.

Therefore, the amount of $p$-nitrophenol for 1 g matrix

$$= \Delta A_{400} \times 8/(18.3 \times 10^3 \times 0.1) \text{ millimoles}$$

This was released in 60 minutes, therefore to express in terms of seconds

The amount of $p$-nitrophenol per second per 1 g matrix

$$= \Delta A_{400} \times 8/(18.3 \times 10^3 \times 0.1 \times 3600) \text{ millimoles}$$

Based upon the activity of the enzyme, the activity is best expressed in units of nanomoles per second which corresponds to the SI unit of nanokatals

The amount of $p$-nitrophenol per second per 1 g matrix

$$= (\Delta A_{400} \times 8 \times 10^6)/(18.3 \times 10^3 \times 0.1 \times 3600) \text{ nanomoles}$$

Therefore the activity of the enzyme in the matrix:

$$\text{Activity (nanokatals/gram)} = \frac{(\Delta A_{400})(8)(10^6)}{(18.3)(10^3)(3600)(0.1)} \quad (1)$$
Appendix

Assay of α-amylase in the starch matrix

A dinitrosalicylic acid (DNS) method described by Miller (1959) was chosen and adapted to the assay of reducing sugar after starch hydrolysis by α-amylase. The optimised process is depicted in Fig A2. Here the matrix served the dual purpose of being a matrix and a substrate to the enzyme. A standard glucose curve was established and was utilised to estimate the rate of reaction from the linear part of the slope.

Fig. A2 Flow chart of an optimized assay procedure for α-amylase action on starch.
**Assay of α-glucosidase in the whey protein matrix**

The “spectrophotometric stop rate determination method” described by Sigma was also adapted for the protein matrix. A clear trend was observed for the release of the hydrolysis product recorded after 60 minutes of equilibration, which was sufficient to control the conditions of low moisture and ambient to high temperatures to which the encapsulated enzyme/substrate complex was subjected. The optimised process is depicted in Fig A3.

The enzymatic activity expressed in terms of nanokatal/gram of the matrices was calculated from the average values after sixty minutes of observation from the slope values using equation (2):

\[
\text{Activity (nanokatal/gram)} = \frac{\text{Slope}(8)(10)(10^6)}{(18.3)(300)}
\]

Where,

8 is the volume of NaCO₃ used (mL), 10 converts 0.1 to 1 of the matrix taken for analysis (g), 10⁶ converts milli to nanomoles, 18.3 is the millimolar extinction coefficient of p-nitrophenol at 400 nm; and 300 is the time of observation (s).

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**Flow chart of an optimised assay procedure for α-glucosidase/pNPG complex spray dried in a whey protein matrix.**

- WPI spray dried with potassium phosphate buffer, glutathione, α-glucosidase and pNPG
  - Sample weighed in small containers (0.1 g each)
  - Equilibrated for 1 hr in incubator at the temperature of interest
  - Reaction terminated on a five minute interval by adding NaCO₃ solution (8 mL, 100 mM) with vortexing
  - Absorbance read at 400 nm against a blank