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Physico-Chemical Characterisation and Functionality of the Polysaccharide Extracted from the New Zealand Black Tree Fern, *Cyathea medullaris* (Mamaku)

A thesis presented in partial fulfilment of the requirements for the degree of

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in

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May Wee Sui Mei

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Preface

What do the New Zealand black tree fern and the Russian Matroyshka doll have in common?

The Matroyshka doll is a doll in a doll in a doll. And the tree fern? It is but a fern in a fern in a fern. The fronds make up the fern tree, which is made up of smaller frond representations called the primary pinna, which is made of up the secondary pinna, which is, (you guessed it) made of up the tertiary pinna. Looking at the tertiary pinna is no different from looking at the secondary, or the primary pinna. In other words, the fern is constructed of self-similar shapes. The figure below is an actual tertiary pinna of the black tree fern scanned as an image. It looks just like an entire fern leaf blade doesn't it?

This self-similarity or macrocosmic characteristic of nature is seemingly random and sometimes oblivious to the human mind trained by classical geometry. To Benoit Mandelbrot, the recursive nature of the tree fern is what he came to describe as ***fractals*** *in 1977. Fractals are a family of shapes, characterised by irregularities and self-similarity which are still statistical in nature. Triangles, squares and circles are shapes, as much as flowers, snowflakes, clouds, waves and even Brownian motion can be.*

Keeping in mind that fractals are the underlying fundamentals of nature can prepare us to recognise the order within the disorder, and not be bound by current knowledge. For every researcher dealing with nature, there is always one thing to remember: God might not play dice, but it will most certainly play hide-and-seek. It is now for us to embark on a journey to seek out what wonders might lie within the polysaccharide extracted from the New Zealand black tree fern in this thesis.

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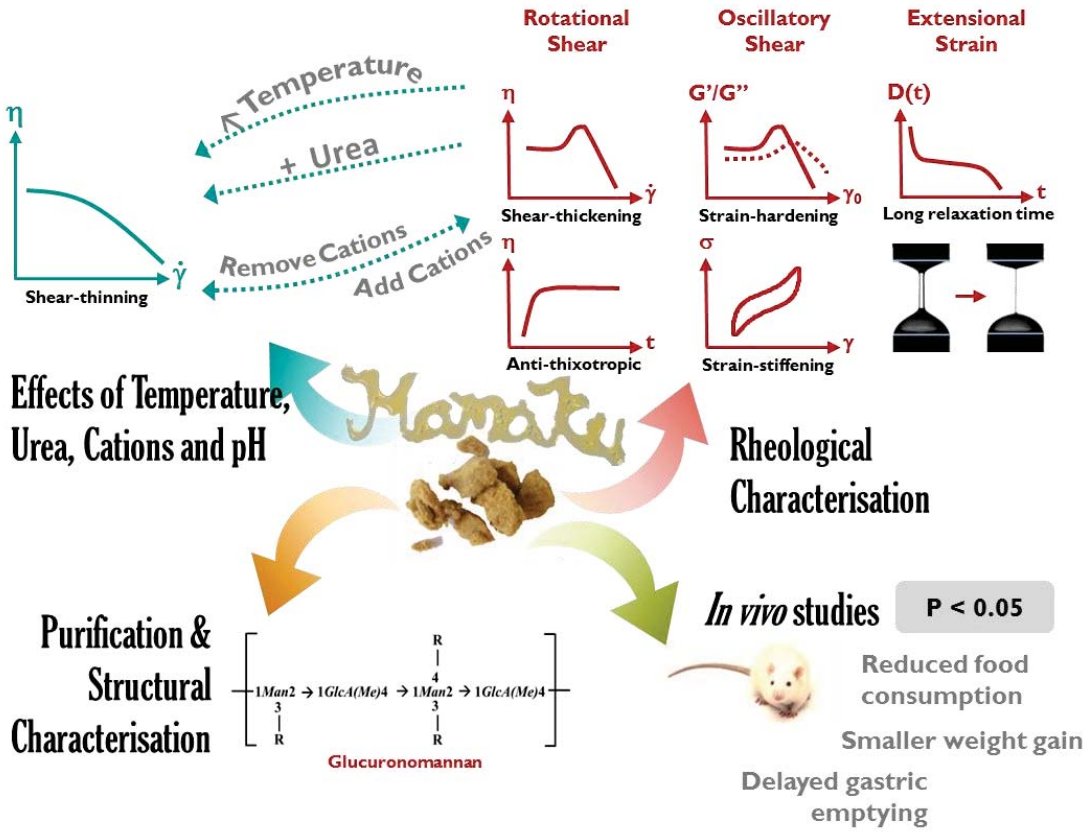
“I don’t know what I’m looking for.”

“Why not?”

“Because … because … I think it might be because if I knew
I wouldn’t be able to look for them.”

- Douglas Adams, Hitchhiker’s Guide to the Galaxy

Abstract



The aim of this thesis was to characterise the polysaccharide extracted from the New Zealand black tree fern, *Cyathea medullaris*, or mamaku in Māori using a combination of rheological, structural and *in vivo* research techniques. Polysaccharides are biopolymers with diverse functionalities that have found their way into many applications in the food, cosmetic or pharmaceutical industries. Novel sources of polysaccharides may have promising functional properties for new or existing applications, therefore it is essential to have fundamental knowledge of their properties. The native and endemic New Zealand black tree fern produces mucilage (containing the polysaccharide) which is extracted from the thick fleshy stem pith of the frond.

Rheological properties of the polysaccharide were characterised using rotational shear, oscillatory shear, and extensional rheology. The combination of these techniques provided information on how the polysaccharide deformed under shear, strain and extension. Rotational shear was further classified into tests for shear-dependent viscosity/normal stresses, time-dependent viscosity, and shear-history dependent viscosity. The polysaccharide (5% w/w) exhibited shear-thickening (4-10s^{-1}), positive first normal stress differences coinciding with shear-thickening, anti-thixotropy (under constant shear with time at shear rates between 4-10s^{-1}), and thixotropy (at 1s^{-1}, pre-sheared at 10s^{-1}) or rheopexy (at 10s^{-1}, pre-sheared at 1000s^{-1}) depending on shear-history. Oscillatory shear was classified into linear and nonlinear rheology, *i.e.* small amplitude (SAOS) and large amplitude oscillatory shear (LAOS) respectively. Under linear strain deformation, the polysaccharide displayed viscoelasticity and a power-law dependence on concentration for relaxation time ($\lambda_s \sim c^{3.6}$). Complex viscosity did not superimpose on shear viscosity at higher shear rates/angular frequency (nonlinear region),

therefore not complying with the Cox-Merz rule. The LAOS response in the nonlinear region was characterised by new large-strain and minimum-strain moduli parameters (G'_L and G'_M), as well as the traditional first-harmonic storage modulus G'. The polysaccharide (10% w/w mamaku) was found to exhibit first a linear viscoelastic region (0.1-20% γ_0), followed by strain-softening (20-800% γ_0), then strain hardening (800-2000% γ_0) and finally a second strain-softening region due to viscous flow (>2000% γ_0) for all three elastic moduli measurements. Closer examination of Lissajous plots in the intercycle strain-hardening region revealed deviation from ellipsoidality *i.e.* sigmoidal shapes, which were representative of intracycle strain-stiffening. Finally, the evolution of filament diameter with time and extensional relaxation time were characterised using a capillary breakup extensional rheometer (CaBER). The polysaccharide exhibited long extensional relaxation times (4.6s), high extensional viscosities (~10^4) and large Trouton ratios (~10^4).

Factors *i.e.* temperature, urea concentration, cations (ionic strength) and pH were tested to investigate how changes in the environment would affect the rheological properties of the polysaccharide. These factors are also intrinsically related to intermolecular interactions which may be present in the polysaccharide e.g. hydrogen bonding, hydrophobic interaction and electrostatic attractions. Thus the molecular origin of its rheological behaviour could also be elucidated through these effects. Shear-thickening was lost at higher temperatures (≥50°C) but enhanced at low temperatures. The peak viscosity during shear-thickening exhibited an Arrhenius' Law dependency with an activation energy of flow of ~90 kJ/mol (5% w/w). Hydrogen bonds are sensitive to temperature and inversely proportion to temperature in the order of kT, which indicated that hydrogen bonds are likely to be involved in shear-thickening of the polysaccharide. The addition of urea, a hydrogen-bond disruptor (chaotropic agent) suppressed shear-thickening completely in 5% w/w mamaku solution at a concentration of 5M. Urea molecules compete for hydrogen bonding sites with the polysaccharide and lower the lifetime of polymer-polymer associations. Removal of salts from the native mamaku solution via dialysis resulted in loss of shear-thickening as well. However, shear-thickening was reinstated upon addition of salts (NaCl, KCl, $N(CH_3)_4Cl$, $CaCl_2$, $MgCl_2$, $LaCl_3{\cdot}7H_2O$, $AlCl_3{\cdot}6H_2O$) back. Mono-, di- and trivalent cations screen the electrostatic charges on the polysaccharide thus lowering the viscosity as the polysaccharide adopts a more compact configuration. In addition, trivalent cations also cause chain collapse (precipitation) and re-dissolution of the polysaccharide, a phenomenon known as re-entrant condensation in polyelectrolytes. Lastly, shear-thickening was also recovered in the dialysed extract at pH 2-4. Similarly, the protons (H^+) screen the electrostatic charges which lowered the viscosity of the polysaccharide. Screening of electrostatic repulsion appeared to promote shear-thickening rather than ionic cross-linking, since monovalent cations and protons were able to recover shear-thickening.

Chemical structure is an important identity for any polysaccharide. In addition, the chemical structure can provide insight as to how the polysaccharide may have participated in shear-thickening. The native mamaku extract was further purified prior to structural characterisation via ultracentrifugation, starch hydrolysis, de-proteinisation and ethanol (80% w/v) precipitation. This method of purification yielded approximately 15% of purified material, removing most of the starch, minerals and simple sugars from the native extract. The purified fraction retained its shear-thickening character and had a molecular weight of 1.94 x 10^6 Da. Structural characterisation determining monosaccharide composition and glycosyl linkages were carried out using methylation, HPLC/GC, GC-MS and NMR techniques. The structure of the mamaku polysaccharide was

suggested to be a glucuronomannan backbone (methylesterified 4-Glc*p*A (27.9 mol%) with 2,3- (9.2 mol%) and 2,3,4-linked Man*p* (10.9 mol%)) with branched sugar side-chains of galactose, arabinose, xylose, non-methylesterified glucuronic acid (8.2 mol%) and other simple sugars at the O-3 and O-4 of the mannose residues.

Piecing the information obtained from the various characterisation techniques together helped to elucidate the molecular origin of shear-thickening, anti-thixotropy, strain-hardening and extensional-hardening. They were postulated to be of the same event, *i.e.* intra- to intermolecular association between polysaccharide chains during shear, strain or extension via hydrogen bonding. Stretching the polysaccharide exposed associative groups within the long chain, which interacted in a cooperative zip-like manner. The hydrogen bonds were suggested to take place via the hydroxyl (-OH) groups of mannose or carbonyl/carboxyl groups (-C=O/-COOH) of the glucuronic acids.

Finally, the satiety effects of the mamaku gum were tested *in vivo* in rats. The functional ability of the polysaccharide to confer satiety was postulated to arise from its high viscosity as well as its shear-thickening behaviour, which alters gastric antrocorporal contractions and delays gastric emptying. Oral gavage of the rats with mamaku gum (15% w/w) showed a significant reduction in short term food consumption ($p<0.05$), smaller weight gains ($p<0.05$), as well as prolonged gastric emptying ($p<0.05$) as compared to rats gavaged with water. Therefore the polysaccharide could potentially be used as a satiety aid in food products.

Biopolymers which exhibit such complex rheological properties that can be easily controlled by manipulating environmental factors are rarely or never before encountered. Clearly, the mamaku polysaccharide would find its way into novel applications, starting with satiety enhancers.

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