

Citric Acid as a Facilitator for the Integration of Okara in Soymilk Gels

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contribution

Amaan Fathima was the sole author of all chapters which were written under the supervision of Dr. Christine Moresoli. The question explored associated with the preliminary tests (Chapter 3) and the main research (Chapter 4) were conducted at the University of Waterloo. With the exception of the preparation methodology for reference gels that were adopted from the research of Jing Sy and Minh Xuan Nguyen, Amaan Fathima was responsible for forming the hypothesis, developing the formulation of the gels, conducting experiments, analyzing and interpreting the results, and writing the chapters. The samples for SEM were prepared and analyzed with the assistance of Minh Xuan Nguyen, and the imaging was obtained through the service at the Waterloo Advanced Technology Laboratory. Dr. Christine Moresoli provided guidance with the design of experiments, data interpretation, review and editing of the chapters.

Abstract

This study aims to explore the effects of citric acid (CA) in the development of soymilk okara (MO) gels with improved integration of okara in soymilk protein matrix. A two-step approach was adopted. The first step focused on changes in the preparation of ingredients, soybeans, soymilk (M) and okara (O), and their effects on gel texture. The addition of CA at 0.14%, 1.25% and 2.5% (w/w) to M induced protein coagulation, significantly enhancing the mechanical and viscoelastic properties of thermally prepared soymilk gels. The particle size profile of O was manipulated by drying duration producing uniform MO gels with shorter drying time. The surface morphology of CA-treated okara (OCA), visualized with SEM, indicated that the structure of O loosened in OCA0.14 and degraded in OCA2.5.

The second step focused on the preparation of soymilk okara gels with CA at two concentrations, 0.14% and 2.5% (w/w). The role of CA on the structure of okara and the coagulation of soymilk was examined by comparing three different formulations: (i) CA treatment of okara prior to its addition to soymilk (M+OCA), (ii) CA treatment of soymilk prior to okara addition (M+CA+O), and (iii) CA addition to a mixture of soymilk and okara (MO+CA). The CA soymilk okara gels were obtained by heat treatment and their physicochemical and texture attributes were examined. The concentration and order of CA addition had different effects on the gels. FTIR, TGA and SEM analyses of CA-treated O (OCA) revealed structural modification in OCA2.5, including decomposition and release of pectic substances, which were not detected in OCA0.14.

The CA0.14 soymilk okara gels exhibited similar failure stress, failure strain and Young's modulus, while viscoelasticity was influenced by the sequence of CA addition, in comparison to MO. Elasticity (G_o') of M+OCA and MO+CA increased threefold and viscosity (G_o'') doubled, whereas M+CA+O exhibited 4.5-fold increase in G_o' and 3.5-fold increase in G_o'' . At CA2.5, failure stress of the CA soymilk okara gels was similar to MO but a lower failure strain and higher Young's Modulus which indicated less cohesive, stiffer gels. The G_o' and G_o'' of M+OCA increased threefold and twofold, while nearly 4.5-fold in G_o' and 4-fold in G_o'' increase were observed for M+CA+O and MO+CA. The gel microstructure visualized with CLSM revealed

changes in the protein network from strand-like in MO to particulate in the presence of CA, with CA2.5 soymilk okara gels having a denser protein network than CA0.14 soymilk okara gels. However, CLSM provided limited insights on the effect of the sequence of CA addition on gel microstructure.

In conclusion, the properties of soymilk okara gels explored in this study indicate that the gel texture can be modulated by altering CA concentration or CA sequence of addition. The CA-modification of O was not essential as similar or superior improvement in texture were achieved by pre-aggregating soymilk proteins with CA in the M+CA+O and MO+CA gel formulations.

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List of Abbreviations

Abbreviation	:	Description
AC	:	Analogue Cheese
BSA	:	Bovine Serum Albumin
CA	:	Citric acid
CNF	:	Cellulose nanofibril
EEP	:	Electrical Equivalence Point
fbM	:	Soymilk extracted from soybeans that had been freeze-treated at -20°C for 5 hours
fbMO	:	MO gel prepared from soymilk and dried okara that were extracted from soybeans that had been freeze-treated at -20°C for 5 hours
fbO _w	:	Wet okara extracted from soybeans that had been freeze-treated at -20°C for 5 hours
IEP	:	Isoelectric Point
M	:	Soymilk
MO	:	Gel prepared by combining soymilk and dried okara
MO _{25%}	:	Soy-okara gel containing only 25% of O when compared to regular MO gel
MO _{50%}	:	Soy-okara gel containing only 50% of O when compared to regular MO gel
MO _{75%}	:	Soy-okara gel containing only 75% of O when compared to regular MO gel
MO _{100%}	:	Regular MO gel
MO _{125%}	:	Soy-okara gel containing 25% additional O when compared to regular MO gel
MO _{150%}	:	Soy-okara gel containing only 50% additional O when compared to regular MO gel
MO _{175%}	:	Soy-okara gel containing only 75% additional O when compared to regular MO gel

MO _{200%}	:	Soy-okara gel containing twice the amount of O when compared to regular MO gel
MO _F	:	MO gel prepared from freeze-dried okara (-61°C, 0.940 mbar for 23-24 hours)
M+OCA __	:	Gel prepared by combining soymilk with citric acid-treated (_% w/w concentration) okara
MO+CA __	:	Gel prepared by combining soymilk and dried okara which was subsequently treated with citric acid (_% w/w concentration)
M+CA __ +O	:	Gel prepared by treating soymilk with citric acid (_% w/w concentration) with subsequent addition of dried okara to the mixture
MTGase	:	Microbial transglutaminase
O	:	Oven-dried okara
O _F	:	Freeze-dried okara (-61°C, 0.940 mbar for 23-24 hours)
O _w	:	Wet okara
OCA __	:	Okara treated with _% w/w citric acid concentration with subsequent oven drying
PBAC	:	Plant-based Analogue Cheese
WHC	:	Water holding capacity

List of Symbols

Symbol	:	Description
A_x	:	Absorbance at wavelength x (nm)
A_{595}	:	Absorbance at wavelength 595 nm (for protein solubility)
A_{600}	:	Absorbance at wavelength 600 nm (for turbidity)
C	:	Concentration
G_o'	:	Storage modulus (kPa)
G_o''	:	Loss modulus (kPa)
M_{x50}	:	Subscript x50 indicates dilution of gel mixture with distilled water by a factor of 50
M_{x100}	:	Subscript x100 indicates dilution of gel mixture with distilled water by a factor of 100
M_{x200}	:	Subscript x200 indicates dilution of gel mixture with distilled water by a factor of 200
W_r	:	Weight of water released from gel that was refrigerated overnight and ultracentrifuged for 20 min at 10,000 rpm (for WHC estimation)
W_t	:	Initial weight of gel measured for WHC estimation
ω	:	Angular frequency (rad/s)

Chapter 1: Introduction

1.1. Research Aim

Cheese, a source of protein, fat and micronutrients such as calcium, phosphorous, some types of vitamin B and vitamin A, is a nutrient-rich food product [60]. The main goal in manufacturing cheese is to convert milk, a perishable product, into a stable product with longer shelf-life [1] [2]. However, recent times have seen a shift in consumer interest towards cheese alternative sourced from plants. Analogue cheeses (ACs) or imitation cheeses offer multiple health benefits, while catering to various dietary needs [61]. Lactose intolerance, high saturated fat content and cholesterol, along with concerns of low yield and spoilage during storage [63], are some of the problems associated with traditional dairy cheeses. ACs may address these problems and offer the flexibility of formulating products with enhanced nutrition profile [62] [64] and diverse functionality ranging from the ability to flow, melt or shred [64]. High protein cheese analogues sourced from legumes, specifically from soybeans (which is also referred to as tofu or soy gel), are the products of interest in this research study.

Traditionally, quality tofu is produced from soymilk [168], which utilizes only 53% of the soybean mass [33]. The remaining soybean mass is a fibrous by-product, okara, which is commonly used as animal feed or discarded that negatively impacts the environment [33] [168] [154]. The rich dietary fiber profile of okara, along with its proteins [168], makes okara a potentially good additive that can enhance nutrition profile of food products [151] [168] and benefit health [33] [168]. Moreover, texture is an important consideration when mixing proteins and polysaccharides, such as soymilk and okara, as they possess different structures, properties and functionalities. Mixing proteins and polysaccharides offers the ability to create various textures that can be regulated by modifying the interactions between them and changing the environmental conditions (temperature, pressure, pH, ionic strength) [41]. However, the insoluble okara fibers cause deterioration of the texture and sensory attributes of soy gels [154] [168]. Therefore, recent studies have focused on the use of okara in producing high-fiber tofu, while tackling the concern of quality degradation.

Mechanical processing [129] [154] and non-mechanical methods [34] [154] have been explored to reduce the size of okara particles, modify its surface properties, enhance its compatibility in

protein gel matrix and therefore improve gel homogeneity [154]. Some of the methods explored include pulverization [120 [150], high pressure homogenization [35], microwave treatment [135], chitosan-pectin double layer coating [34], and enzyme crosslinking [154]. However, non-physical approach to okara modification using food grade organic acids and its utilization in producing okara-added soymilk gel, as proposed by Sy 2022 [58] and Nguyen 2023 [59], remains a limited area of research with potential for growth.

Organic poly-carboxylic acids such as citric, malic or tartaric acid have been used as soymilk coagulants in tofu production [83]. Citric acid (CA) has been applied on polysaccharide to hydrolyze [43] [46], modify surface through esterification [44] [45] [112] [175], and to extract soluble polysaccharides such as pectin [49] [50] [149]. Moreover, organic acids have mediated in the formation of protein-polysaccharide complexes for the preparation of emulsions or polysaccharide-modified protein isolates, by modifying the surface charges of protein. However, the polysaccharides used to date are charged, soluble, or have a linear, well-defined structure [58 – 62]. These polysaccharides significantly differ from the complex and heterogeneous structure of okara.

In our study, CA is hypothesized to modify okara structure through hydrolysis, esterification and release of soluble polysaccharides, that can improve its integration in soymilk protein matrix. The excess CA in modified okara can influence soymilk proteins by inducing aggregation and accelerating the subsequent thermal gelation. Therefore, the effects of CA on altering soymilk proteins or okara polysaccharides were explored to assess which process influences the texture and microstructure of soymilk okara gels.

1.2. Objectives

The main objective of this research was to develop and characterize soymilk okara gels in the presence of citric acid (CA). This objective was achieved through the steps illustrated in **Figure 1.1** and detailed below.

The first step (Chapter 3) was to explore the manipulation of the ingredients, soybeans, extracted soymilk and filtered okara, as well as the soymilk okara mixture, to analyze the effects on the texture of the resulting gels. Specifically,

- **Soybean:** (1) Gels were prepared from soymilk and okara extracted from frozen soybeans to determine the effects of cold-denaturation of soy proteins on the gel properties.
- **Okara:** Gel texture was modulated by incorporating okara (2) of different particle size range, (3) dried in oven or freeze-dried, and (4) at various concentrations in soymilk. (5) Additionally, the surface morphology of citric acid-modified okara was visualized with SEM.
- **Mixture:** (6) The influence of citric acid-induced coagulation of soymilk proteins, before thermal gelation, was assessed on soymilk gel texture.
- **Gel:** (7) The changes in the surface morphology of M gel, in the presence of okara in MO gel, were visualized with SEM.

The second step was to assess the effects of different sequences of CA addition in soymilk okara mixtures, specifically (a) to modify okara before its addition to soymilk, (b) to coagulate soymilk protein before incorporating okara, and (c) to a mixture of soymilk and okara. Two CA concentrations were investigated – 0.14 and 2.5% (w/w). Subsequently, the effects of CA sequencing and concentration on okara structure, and the gel homogeneity, texture and chemical interactions involved were examined.

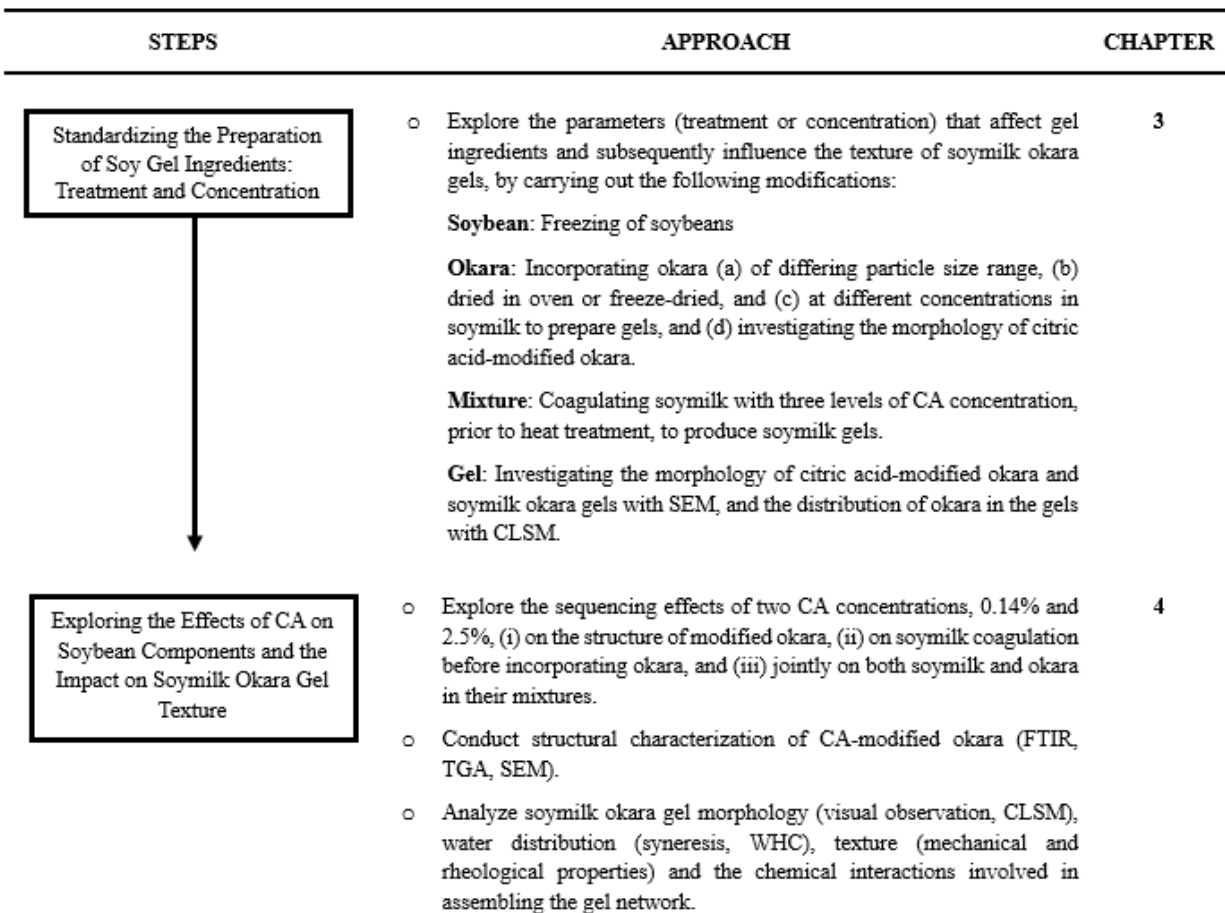


Figure 1.1: Flowchart of the research goals and associated approaches of the Master thesis.

1.3. Thesis Structure

The thesis is organized into five chapters that present the information outlined below:

Chapter 1: Introduction

An overview of the thesis that includes the research aim, objectives, and thesis structure.

Chapter 2: Literature Review

- Section 2.1 and 2.2 provides a brief summary of dairy cheese production, particularly paneer, as it involves the use of organic acid and heat and dairy cheese analogues.
- Section 2.3 to Section 2.5 explore a specific protein cheese analogue - tofu, its components, and literature on the production of high-fiber soy gels.
- Section 2.6 provides details on the effects of citric acid on polysaccharides and proteins

- Section 2.7 is a general review on the associative or segregative interactions between proteins and polysaccharides in mixtures.

Chapter 3: Standardizing the Preparation of Soy Gel Ingredients: Treatment and Concentration

- Section 3.1 and Section 3.2 provide an overview of the initial questions explored in Chapter 3 and methodology, with regards to the manipulation of the ingredients, soybeans, extracted soymilk and filtered okara, as well as the soymilk okara mixture, and analyzing the outcomes on the texture of gels.
- Section 3.3 and Section 3.4 address some factors that influence the texture of soymilk okara gels, including freezing soybeans, and incorporating okara of different particles size range, dried through different methods, or at different concentrations in soymilk gels.
- Section 3.5 assesses the influence of citric acid-induced coagulation of soymilk proteins, before thermal gelation, on soymilk gel texture.
- Section 3.6 explores the methodology of SEM to visualize okara surface morphology and distribution in gels.

Chapter 4: Exploring the Effects of Citric Acid on Soybean Components and the Impact on Soymilk Okara Gel Texture

- Characterizes the effects of citric acid on okara modification, detected using FTIR, TGA and SEM.
- Analyzes and discusses the effects of citric acid concentration and sequencing on the macro- and micro-structures, texture profiles, and chemical interactions in soymilk okara gels, as well the mechanism of gelation.

Chapter 5: Conclusions and Recommendations

- Provides a summary of the key findings of the thesis and recommendations for future, based on the gaps detected in literature and the current research.

Chapter 2: Literature Review

2.1 An Overview of Dairy Cheese

The objective of manufacturing cheese is to convert milk, a perishable product, into a stable product [1] [2]. It is a two-phase composite material or gel wherein a protein matrix is embedded with fat globules that act as filler material [3]. The moisture content of cheese is used to classify cheese from soft to very hard, irrespective of the routes used to produce cheese [2]. Cheese is manufactured either through acidification, referred to as acid-coagulated cheese which are generally soft, or enzymatically with rennet, termed as rennet-coagulated cheese, to produce firm cheese [2]. **Figure 2.1** illustrates the structural components of cheese from macro – to nano-scale that collectively affect cheese properties [3]. Casein, fat globules and water at the microscale assemble into curd particles that aggregate together into a solid mass but with visible cracks at the junctions between the individual curd particles [3].

The following sections will review the composition of dairy milk, particularly the protein components, and provide a general overview of the cheese making process with specific details on the production of heat and acid coagulated cheese such as paneer.

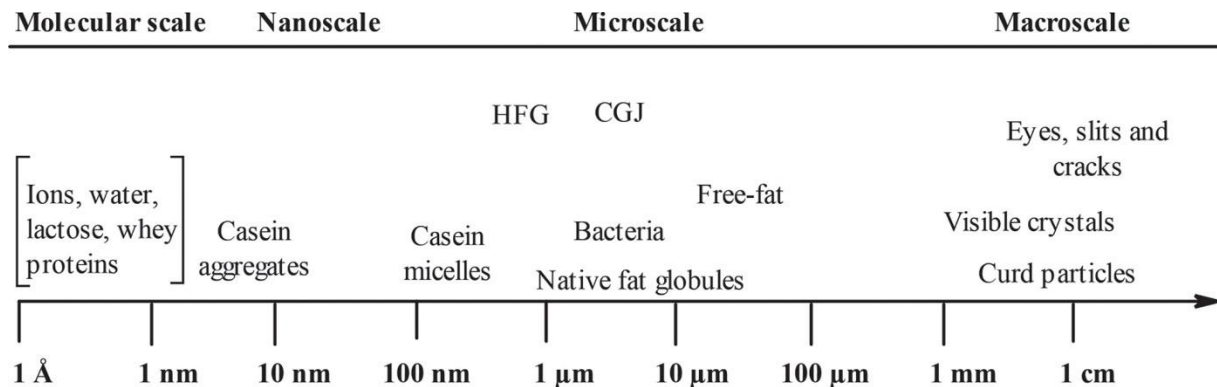


Figure 2.1: Hierarchical structure of cheese spanning from a molecular length scale to macroscale (HFG = homogenized fat globules, CGJ = curd granule junction). Reprinted from P. Lamichhane, A. L. Kelly, J. J Sheehan, “Symposium review: Structure-function relationships in cheese,” *Journal of Dairy Science*, vol. 101, no. 3, pp. 2692–2709, Mar. 2018, with permission.

2.1.1 Milk Protein Composition

Milk is composed of 3.5% protein that are typically classified into two fractions, based on the protein solubility after acidification at pH 4.6 and 30°C [4]. Casein constitutes 80% of the milk proteins that precipitates at 30°C and pH 4.6 whereas the remaining soluble protein fraction at these conditions is comprised of the whey or serum proteins [4].

Casein is a phosphoprotein wherein the phosphate groups allow casein to associate with calcium, as illustrated in **Figure 2.2** [5]. According to a study, the amino acid sequences in the primary structure of proteins, solubility and the behavior in presence of calcium ions, varies across the different fractions of casein proteins [6]. The largest and the least soluble fraction of casein proteins, at low calcium ion concentration, is the class of α -casein, comprised of amphiphilic α_{s1} -casein (38%) and hydrophilic α_{s2} -casein (10%). Different from α -casein, the class of κ -casein (36%) remains soluble in the presence of calcium and stabilizes casein in milk by forming colloidal micelles. The most hydrophobic fraction, β -casein (13%), and trace amount of γ -casein (3%) are also present in milk. Majority of the casein proteins are present in milk as colloidal particles or micelles [4] that are linked to other micelles via colloidal calcium phosphate (CCP), as illustrated in **Figure 2.2**. Calcium phosphate is water insoluble but its dispersion in the bulk liquid indicates that CCP is entrapped in casein micelles [4].

Whey protein, comprised of β -lactoglobulin and α -lactalbumin subunits, is the liquid fraction consisting of the residual soluble proteins left behind after the extraction of casein through isoelectric precipitation (pH 4.6) and removal milk solids [4].

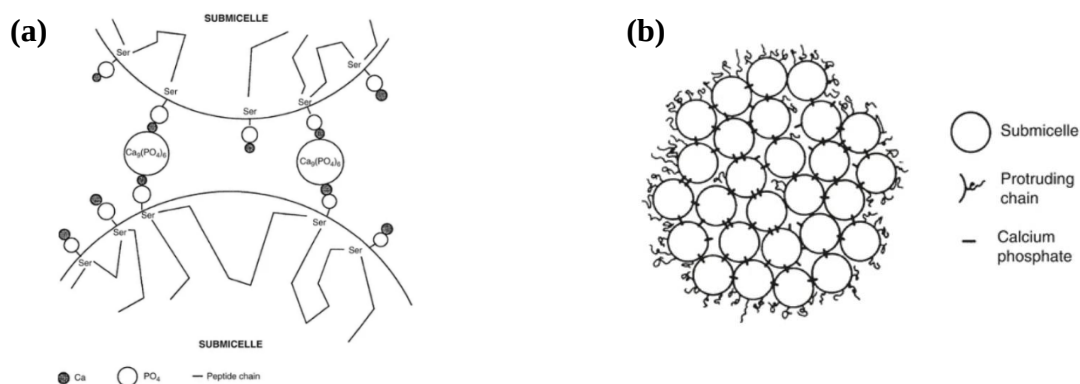


Figure 2.2: (a) Association of colloidal calcium phosphate with the casein protein phosphate groups. Reprinted from P. F. Fox, T. Uniacke-Lowe, Paul L.H. McSweeney, and J. A. O'Mahony, "Salts of Milk," Springer eBooks, pp. 241–270, Jan. 2015, with permission. (b) Submicelle model of casein. Reprinted from P. F. Fox, T. Uniacke-Lowe, P. L. H. McSweeney, and J. A. O'Mahony, "Milk Proteins," Dairy Chemistry and Biochemistry, pp. 145–239, 2015, with permission.

2.1.2 Dairy Cheese Production

Cheesemaking is a milk-dehydrating process that leads to a concentrated mass of milk fats and casein proteins [1]. The objective of cheesemaking is to convert liquid milk into a viscoelastic solid material through sol-gel transition, referred to as curd that is ten times richer in protein content than milk [60]. Since this greatly depends on the composition of milk, a pre-requisite stage of cheesemaking is standardizing the casein to fat content of milk [2]. The protein content is increased by adding milk protein concentrate or caseinate whereas the fat content is adjusted with cream [2]. The extent of dehydration or concentration depends on the cheese type (soft or hard) and is regulated with a series of steps – milk acidification and protein coagulation, dehydration through cooking or pressing to promote the release of the liquid fraction of milk (whey), molding for curd processing, and ripening with salts [1]. The following section discusses a specific acid-coagulated cheese-like product, paneer, the factors that influence its texture and an overview of mechanism for the conversion of milk into paneer.

2.1.2.1 Paneer

Paneer is a non-fermented, unripened and non-meltable soft cheese that contains 22–25% fat and 16–18% protein, and is characterized by a soft, cohesive texture with a mildly acidic flavor with slightly sweet taste [81]. It is prepared by heating milk followed by acidification with an organic acid coagulant [7] [80] [81]. During acidification, the milk curds separate out from whey [7] [80] [81] which is subsequently pressed to obtain a gel that does not melt, flow or disintegrate on being cooked [79]. The milk proteins form a gel network with all other milk components such as lipids and ions that become entrapped in the structure [7], except for soluble whey proteins, lactose and minerals which are not incorporated into paneer [81]. An extensive heat treatment, however, can denature the β -lactoglobulin subunit of whey proteins that causes self-aggregation or initiates

interactions with the κ -casein present on the surface of casein micelles, and therefore reduce protein loss and increase the proteins recovered from milk [79].

2.1.2.1.1 Factors Influencing the Yield and Texture of Paneer

A correlation between the texture of paneer with the milk source such as buffalo or cow milk has been established in previous studies that vary in the amount and size of casein proteins and milk fats, as well as the calcium and phosphorous mineral content [7] [80]. The larger size of casein micelles and fat globules, and higher amount of calcium in buffalo milk, when compared to cow milk, aids in more extensive network between the casein micelles. As a result, paneer from buffalo milk has a firmer, harder texture whereas cow milk paneer is soft and spongy.

The yield of paneer depends on the protein and fat content in milk, coagulating temperature and duration of heat treatment. The type and strength of coagulant utilized affect paneer texture and therefore the moisture expelled or retained during the post-processing stage of pressing the milk curd [7]. The moisture content of paneer decreases with increasing coagulant concentration, regardless of the type of coagulant employed [7]. The use of salt coagulants like calcium chloride and calcium phosphate supplements the calcium content of milk. Calcium ions neutralize protein charges, induce protein aggregation, bridge the polypeptide chains of both casein and whey proteins, increase the amount of proteins participating in the gel network as whey proteins are coagulated that facilitates their interaction with casein micelles, and thus enhance the yield [80].

The acidification of milk proceeds differently according to the type of organic acid used as coagulant such as citric acid, tartaric acid and malic acid, in the preparation of paneer due to differences in the pKa values [7]. The amount and rate of H⁺ ions released affects the extent of milk protein denaturation which was observed to be lowest with malic acid and highest with citric acid. Moreover, the degree of protein denaturation alters protein-water interactions. The high hydration capability of casein proteins prevents effective interactions between proteins which was observed when malic acid was used as a coagulant. As a result, paneer produced with malic acid has a loose protein network with the softest and weakest texture, highest moisture and highest loss of proteins as whey whereas citric acid was the most effective in enhancing the gel texture. Furthermore, the concentration of coagulant is another factor that determines the amount of participating proteins in the paneer network [7] [81]. High acid concentration caused proteins to

rapidly denature to a higher degree, increased the loss of milk proteins as whey and reduced protein-water interactions that produced a dry weak paneer, whereas low coagulant concentration slowed down protein aggregation that occurred uniformly throughout the milk volume producing cohesive, strong gels [7].

Heat treatment has a significant effect on the physicochemical, sensory and microbiological properties, and the yield of paneer [80]. Heating milk is essential to destroy the pathogenic microorganisms and extend shelf life of paneer. Heat treatment denatures and insolubilizes the whey proteins which increases the availability of milk proteins to participate in the network. The denatured whey proteins coprecipitate with casein during acidification, thus increasing the proteins recovered and yield of milk curd [80]. An increase in the heating temperature from 60°C to 86°C decreased the moisture content of paneer while maintaining a high coagulating temperature (more than 70°C), resulted in a dry, hard texture of paneer [81]. In the post-processing pressing stage, the texture of paneer is further enhanced by altering the applied pressure, which affects the water content and texture of paneer [81], and duration of ageing or ripening during which the proteins within the aggregate particles of the gel network rearrange [82].

2.1.2.1.2 Mechanism of Acid-Induced Gelation of Milk Proteins

The insights from the acidification mechanism of skim milk with Glucono-Delta-Lactone (GDL) or HCl across studies are summarized in Fox et al. [82]. At the near neutral pH of milk, the solubility of casein and the steric repulsion due net negative surface charge of proteins stabilizes the casein micelles. Upon acidification, the surface charges are neutralized and isoelectric point is attained that destabilizes the micelles and induces interaction between casein.

The casein molecules in the micellar structure undergo two stages during acidification. The first phase is associated with the dissociation of the micellar structure. The expanded, unfolded structure of casein begins to disintegrate into smaller micellar particles, during the heat treatment, as the pH is lowered [82]. The increasing acidic environment, in the presence of an acid coagulant, solubilizes the colloidal calcium phosphate that causes dissociation of the micellar structure. The decrease in pH from 6.6 until pH 5.5 does not alter the shape or structure of casein where electron microscopy micrographs have exhibited the presence of some amount of small micellar particles. Dissociation of the micellar structure predominates at pH 5.5 which suggests the onset of micelle

disintegration that opens the structure and makes the micelles less compact, compared to the native micelle state. The unfolding of the micellar structure is accompanied with the dissolution of the exposed colloidal calcium phosphate in the acidic environment. A pH decrease to 5.2 destabilizes the proteins as the isoelectric point of β -casein is attained and the dispersed casein begin to interact. As the repulsive forces are reduced near the isoelectric point (IEP), hydrophobic interactions dominate among casein micelles which drives their aggregation and precipitation into insoluble particles. Calcium ions aid precipitation by crosslinking the casein polymer chains as well as the whey proteins [81] [8]. By pH 4.6 - 4.8, discrete casein particles larger than the native micelles are formed, hydrophobic interactions dominate leading to casein micelles coagulation, and the aggregated protein particles rearrange into a particulate network [82].

2.2 Cheese Analogues

2.2.1 Overview

Analogue cheeses and imitation cheeses are cheese-like products wherein the dairy fat or protein is partially or completely substituted with plant-based ingredients [62] [64]. Additives such as preservatives, salt, acidifying and flavoring agents are used in the formulations to create a gel that mimics the texture and form of cheese [62]. The difference between a cheese analogue (or substitute) and imitation cheese lies in the nutrition of the product [62]. Products that resemble cheese in texture and sensory attributes but are inferior in nutrition are classified as imitation cheese while cheese substitutes contain comparable nutrition [62].

Analogue and imitation cheeses have gained interest due to the multiple health benefits they offer, while catering to various dietary needs [61]. Lactose intolerance, high saturated fat content and cholesterol, along with concerns involving low yield and spoilage during storage [63], are some of the problems associated with traditional dairy cheeses. Analogue cheese address these problems and offer the flexibility of formulating products that are lactose-free, contain low to no fat and probiotics [61], have an enhanced nutrition profile through fortification with vitamins, minerals and proteins [62] [64], and possess diverse functionality ranging from the ability to flow, melt or shred [64]. An addition to the recent interest in healthier alternatives to cheese is the development of low-salt analogues. Kamath et al. summarized the multiple uses of salt as a key ingredient that

aids in assembling the three-dimensional gel network in analogue cheese, preventing acidic-bitter flavors or microbial spoilage [62]. Additionally, its role as a ‘melting salt’ in the production of dairy-based cheese loosens the protein structure and solubilizes the proteins to produce a homogeneous gel from subsequent heat treatment. Attempts on low-sodium salt analogues across studies has been reviewed by Bansal et al. [67]. Some changes adopted in cheese analogue production include adding flavor enhancers to counter the acidity or bitterness [62] [67], use of other mineral salts (CaCl_2 , MgCl_2 , KCl) [66] [67], and incorporating probiotics to prevent pathogenic growth during storage while enhancing the health benefits [67] [66].

Analogue cheeses are classified into three groups depending on the key ingredients involved in structuring the product’s matrix. Dairy cheese analogues contain caseinates, whey protein, dried milk powder or milk fats along with additives to enhance the flavor profile or texture properties such as the meltability of dairy cheese. Partial dairy analogues replace a part of the milk protein or fat content with plant-based ingredients such as starch [65]. The starch-containing analogues are significantly softer but more cohesive which reflects the internal structural strength of cheese. Non-dairy or plant-based analogue cheese source both the protein and fat content from plant sources and do not require the ripening stage which otherwise adds to the cheese production costs [62] [64]. However, while traditional cheese is prepared with milk as the primary ingredient, plant-based analogue cheese mimicking its texture, flavor and sensory attribute requires the use of a range of ingredients and processing routes [60]. In the processing route referred to as the fractionation route, the plant material is purified to isolate a particular component, usually one among the protein, polysaccharide and fat constituents [60]. Legumes, zein, rice or potato proteins are the common sources for the protein component. Lipids are extracted from seeds of soybean, sunflower, rapeseed, coconut or palm oil. A range of functional polysaccharides have been utilized including starch, pectin, cellulose, alginate, carrageenan, gum and agar. A mixture of the isolated constituents is processed through thermal changes (heating, freezing), pH modification, enzyme or salt addition to form a cheese-like gel. The other processing route is tissue disruption route where whole plant-based raw material such as legumes or nuts are utilized as the starting material or in some cases filtered to separate the plant-based milk from a fibrous by-product [60]. The extracted milk is exposed to high or low temperatures, enzymes, changing pH or ionic strength conditions to solidify into a gel

While plant-based analogue cheese offer health benefits, mimicking the texture of dairy cheese is a challenge as the use of globular plant proteins generally results in products with low elasticity, hardness and cohesiveness properties [64], with a gritty texture that is sensorily undesirable in cheese-like products [62]. Additionally, high protein products often source the proteins from legumes that are associated with a poor, beany off-flavor [62] [64]. The following section explores studies that focused on producing high protein, bean-based cheese analogues, and addressed some of these challenges.

2.2.2 Gelation of Dairy and Plant Proteins

The structural differences between plant-derived proteins and dairy proteins contribute to the difficulty in mimicking the behavior of casein micelles and therefore the progress of gelation [60]. Casein micelles are flexible and possess an open structure with phosphate groups that function as binding sites for calcium [60]. In contrast, plant proteins that predominately belong to the category of globular proteins, have a tightly packed structure and different surface chemistry [60] [68]. Globular proteins are folded mainly through hydrophobic effect with some contributions from disulfide bonds, hydrogen bonding, electrostatic interactions and van der Waals forces [68]. Moreover, the structural and compositional differences in amino acid sequence causes a difference in properties directly responsible for gelation such as the isoelectric point and denaturation temperatures of the plant protein subunits, therefore imparting their different behavioral response from casein micelles [68].

The heat and cold-induced gelation of plant globular proteins and dairy casein micelles is illustrated in **Figure 2.3** [69]. The heat induced gelation of plant globular proteins involves a series of dissociation and association stages. The native proteins unfold and dissociate into smaller protein particles as the denaturation temperature is reached. The flexible state of unfolded proteins initiates the opportunity to interact with other proteins through hydrogen bonds, formation of disulfide bonds, or via the exposed hydrophobic regions. The association leads to the formation of aggregates that further associate into the gel network. Protein concentration, protein subunit composition, pH and ionic strength influence the gelation process. While globular proteins can form gel structures in the absence of coagulants, casein gelation often involves coupling of heat treatment with an acid or a salt coagulant. Change in pH initiates dissolution of the colloidal

calcium phosphate links between casein micelles and screening of charges that overcomes the electrostatic repulsion and leads to aggregation, with heat facilitating gelation.

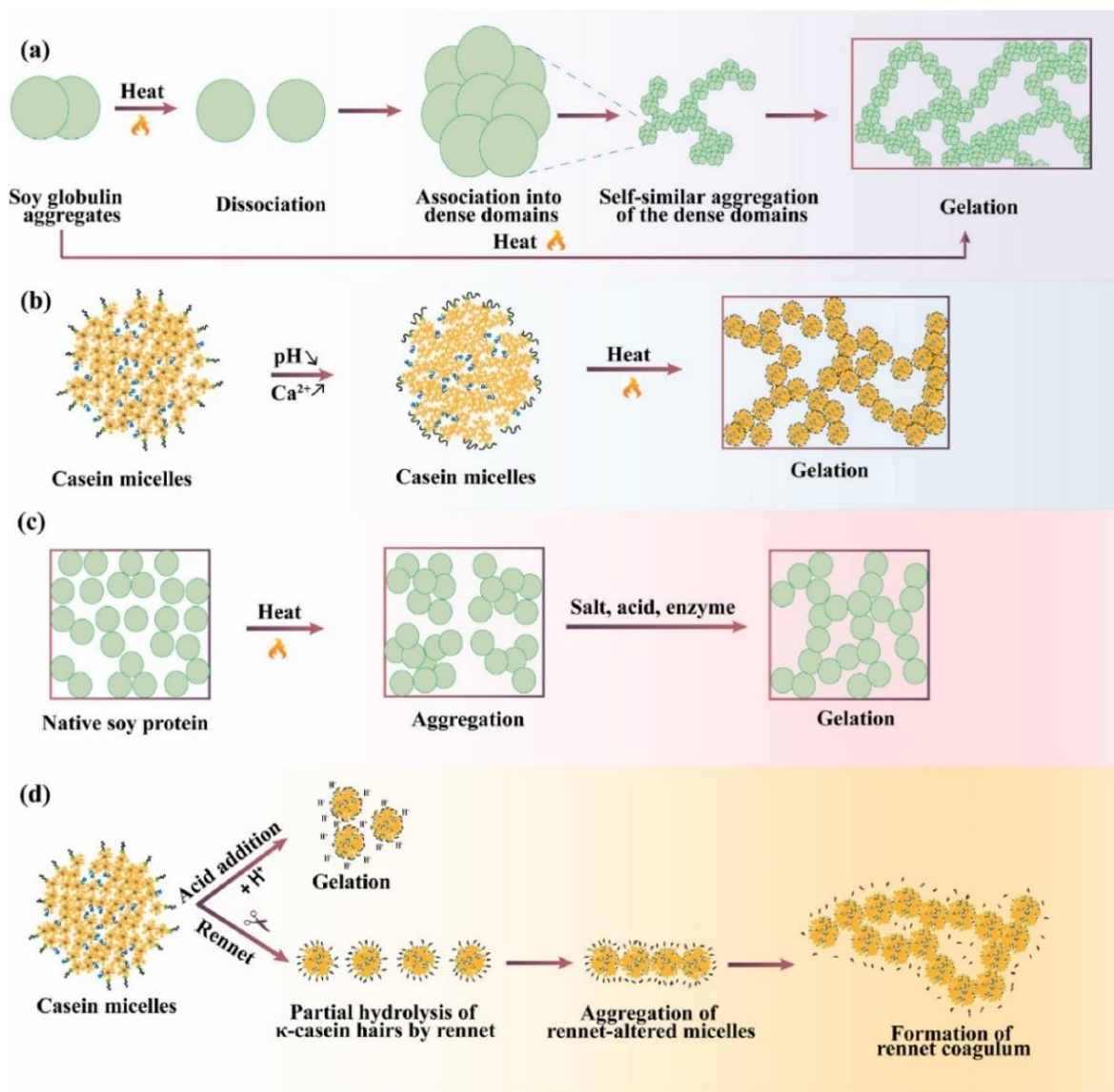


Figure 2.3: Schematic representation for globular protein and casein gelation: heat-induced (a, b) and cold-induced (c, d) gelation [69]. Reprinted from Q. Tang, Y. H. Roos, and S. Miao, “Structure, gelation mechanism of plant proteins versus dairy proteins and evolving modification strategies,” *Trends in Food Science & Technology*, vol. 147, p. 104464, Mar. 2024, with permission.

Cold-induced gelation is a milder process used for the encapsulation of heat sensitive substances. Plant-derived globular proteins are pre-heated above their denaturation temperature and far from

the isoelectric point to obtain an unfolded, flexible state with exposed functional groups. Low protein concentration is used in this process to prevent heat-induced aggregation. Following denaturation, an acid coagulant (GDL), divalent salt (CaSO_4 , MgCl_2) or enzyme (MTGase) aid in forming the gel network. Casein micelles are gelled at cold temperatures in the presence of acid (GDL, lactic acid bacteria) or rennet. An acidic environment disrupts the electrostatic interactions of the micelles, releases the colloidal calcium, and screens the charges causing repulsion that leads to aggregation. On the other hand, rennet causes proteolysis of the micelles which subsequently aggregate at adequate temperature, with the assistance of calcium ions as the crosslinking agents. A few research studies that focused on producing high-protein gels as cheese substitutes are summarized in **Table 2.1**.

Table 2.1: Selected publications on Plant Protein Gels

Plant Protein	Gelling Route	Gel Properties
In-lab protein isolate extraction via alkaline extraction and isoelectric precipitation from twelve legumes - black kidney bean, speckled kidney bean, panda bean, cowpea, mung bean, adzuki bean, rice bean, black soybean, soybean, chickpea, broad bean, and pea. [74]	pH 3.0 or 7.0 using HCl or NaOH Heat treatment	7S-rich (Phaseolin) black kidney/ speckled kidney/ panda beans had the highest elasticity ($G_o' = 6.6 - 7$ kPa) and hardness whereas chickpea, broad bean and pea protein gels were the least elastic ($G_o' = 0.18 - 0.29$ kPa) and amongst the weak gels - Gels formed at pH 3.0, closer to isoelectric point, were opaque with a particulate microstructure whereas pH 7.0 produced comparatively transparent gels with a fine-stranded network containing small pores
In-lab protein isolate extraction via alkaline extraction and isoelectric precipitation from eight legumes - mung bean, black bean, adzuki bean, rice bean, black kidney bean, speckled kidney bean, chickpea, cowpea, soy bean and pea [75]	pH 3.0, 5.0, 7.0 and 9.0 using HCl or NaOH Heat treatment	- Surface charge of all protein isolates and protein solubility were the least at pH 5.0 whereas the surface hydrophobicity was maximum at pH 5.0 - Mung/ black kidney/ speckled kidney bean and cowpea are suitable for producing gels at acidic environments as they exhibit strong gelling capacity at pH 3.0 - Black/speckled kidney bean proteins possess strong gelling ability at pH 7.0 due to the lower critical protein concentration required to induce gelation
Commercial fava bean, pea and mung bean protein isolates [146]	pH 7.0 Homogenization Heat treatment	- Homogenization had a positive effect on enhancing the interactions in the network, delaying gel failure and improving gel stiffness - Mung bean isolate produced brittle gels whereas fava bean and pea isolated produced ductile gels. - Fava bean isolate produced the strongest gel, followed by pea and mung bean isolate, whether or not the protein dispersion was homogenized
In-lab preparation of chickpea isolate [76]	pH 3.0 or 7.0 NaCl or CaCl_2 Heat treatment	- Gels were produced at pH 3.0 in the presence of salt, with the gel strength increasing with increasing ionic strength. Gels with CaCl_2 were stronger than with NaCl. - Viscoelasticity of gels improved with ionic strength at pH 3.0, whereas decreased at pH 7.0

In-lab extraction of kidney bean and pea protein isolate [77]	Heat treatment	- Kidney bean protein gels possess higher elasticity (G') (2.13 – 10.02 kPa) and strength than pea protein gels (0.10 – 0.27 kPa) due to a greater proportion of β-sheets in kidney bean proteins that possess large surface areas than α-helix secondary structure and enables greater extent of intermolecular hydrogen bonding between the proteins. - Kidney bean protein is suitable for applications requiring strong gels whereas the high water and oil absorption ability of pea protein finds application as a suitable adsorbent at fat-water interface.
In-lab protein isolate extraction via alkaline extraction and isoelectric precipitation from panda bean, soy bean and pea [78]	pH 3, 5, 7, 9 Heat treatment	- At pH 7, 8% (w/v) panda bean protein could form a firm gel while SPI (10% w/v) and PPI (12% w/v) had a higher critical protein concentration requirement to form gels. The minimum protein concentration required for gelation decreased further to 4% w/v of panda bean protein isolate at pH 3, attributed to its ability in forming fibrils at acidic pH - Panda bean protein gels had a highest elasticity (G') and strength followed by SPI and PPI, at all pH conditions, due to its high β-sheet content - Hardness of panda bean protein gel was 2.4 – 7.7 times higher than SPI gel and 6.7 – 58.6 times harder than pea protein gel over the pH range.

2.3 Soybean Composition and Soy Products

Soybeans is one of the richest sources of plant proteins among legumes and the concentrated presence of isoflavones, associated with multiple health benefits such as reducing the risks of cancer, cardiovascular diseases and osteoporosis, makes soybeans unique compared to other legumes [192].

A soybean seed is comprised of the hull and the embryo consisting of the embryonal axis attaching two cotyledons [9]. Soybean hull contains high amount of cellulose (28.6–52.3%) and hemicellulose (18.5–33.8%), along with some lignin (2.3–13.1%), pectin (4.2%) and protein (9.4%) [12]. The cotyledons of the embryo make up 90% of the seed weight wherein the smaller oil bodies (0.2–0.5 μm) surround the much larger protein (2–10 μm), present within the individual densely packed subcellular structures [9]. Approximately 40-41% of the cotyledon weight is protein, 8.1 – 24% is lipids, and carbohydrates represent 35% of the seed, on dry weight basis [9]. The cotyledon contains protease inhibitors such as Bowman-Birk trypsin-chymotrypsin inhibitor and the Kunitz trypsin inhibitor, lectin, phytic acid (storage form of 65-80% of soybean phosphorus content) and isoflavones. These are considered as anti-nutritional components for humans that trigger allergic response, indigestion or reduce nutrient absorption, yet are essential for the transfer of nutrients and defense against organisms [9]. The activity of these anti-nutritional substances is reduced considerably after dehulling, washing or exposing soybeans to heat treatment [9] (**Table 2.2**). Lipoxigenase enzyme is another minor component of soybean seed that is responsible for the undesirable beany flavors [9].

Table 2.2: Antinutritional factors present in soybeans, their effects on health and methods of elimination [160] [162]

Category	Effect on consumption	Elimination of anti-nutritional factor
Enzyme / Protease inhibitor (trypsin)	Positive: Anticarcinogenic properties and can be used to control diabetes mellitus (with the aid of amylase inhibitors) Negative: Reduces protein digestibility and sulfur-amino acid quantity	Dehulling has no effect as it is present in cotyledon Heat eliminates up to 80 % of the trypsin inhibitor activity (in products like soymilk, tofu) Heat, roasting beans, microwave, boiling, or fermentation modify the enzyme structure and cause loss of binding capacity

Lectin	Positive: Antioxidative, antimicrobial, insecticidal, and antitumor properties and can be used to modulate blood glycemic level Negative: Cause obesity, chronic inflammation, nausea and irritable bowel syndrome	Boiling or autoclaving causes irreversible denaturation which alters the binding sites. 1 h of boiling soybeans at 95C reduces lectin activity by 93 - 99%. Fermentation for up to 72 hours almost completely eliminates the activity. High pressure (over 500 MPa) treatment of soybeans reduced lectin activity by 36 - 40%
Oxalic acid (or oxalates)	Negative: Binds to minerals in gut causing mineral deficiencies and renal stones	Being water soluble, boiling, steaming and soaking eliminates 30-87%, 42-46% or 40-76% oxalic acid, respectively
Phytic acid	Positive: Antioxidative, antidiabetic, and anticarcinogenic properties Negative: Impairs absorption of Fe, Zn, Ca and Mg causing mineral deficiencies	Cooking soybeans for 1h at 95 °C reduces phytate content by 10-25%. The enzymes during sprouting break down phytates in the seeds and reduce content by over 60%
Saponin	Positive: Reduces the absorption of dietary lipids, cholesterol and bile acids and has antibacterial, antiprotozoal and insecticidal activities Negative: Binds to the small intestine cells which minimizes absorption and utilization of nutrients	Soaking decreases saponin content which can be further effective after hydrothermal processing of soybeans
Tannin	Positive: Antioxidative, antidiabetic, anti-inflammatory, antiallergic, antimicrobial, and anticarcinogenic properties Negative: Indigestible and can have mutagenic and hepatotoxic activities	Being heat resistant, dehulling, soaking, or fermentation are the methods used to reduce tannin content. Soaking for 12–14h eliminates 54.6% of tannin in soybeans, dehulling removes 68 - 99 % of tannin content, sprouting breaks down protein-tannin complex which releases free tannins into water. Other techniques include radio frequency treatment (17% removal), high hydrostatic pressure (26 - 40% removal) and microwave (16-19%) treatment

2.3.1 Soybean Major Constituents

Soybean proteins are classified into enzymes, structural, membrane, and storage proteins based on their functionality in the seed, amongst which storage proteins comprise 65-80% of the protein content [9]. Glycinin belonging to the legumin proteins (11S globulins) and the vicilin (7S globulins) β -conglycinin are the major fraction of storage proteins, constituting about 40% and 25% of soybean seed protein, respectively [9] [10] [11]. The less abundant storage proteins are 2S, 9S and 11S globulins [11].

β -conglycinin (7S) is comprised of three subunits β , α and α' that interact through hydrogen bonds and hydrophobic interactions [9]. Glycinin (11S) is a hexamer consisting of two trimers that are associated through hydrophobic, electrostatic, hydrogen bonds and each trimer contains acidic and basic polypeptide subunits that are covalently linked to each other with disulfide bridges [9] [10] [11]. The 11S contains more sulfur-containing amino acids than 7S [9] [11]. The different composition, structures and molecular weights of 7S and 11S (**Table 2.3**) give them different functionality [9]. 7S denatures at a temperature of 68°C whereas 11S requires a higher denaturation temperature of 86°C [10]. Soy proteins have an isoelectric point (IEP) near pH 4.5 and are considered soluble outside the pH range of 3-6 [10]. However, individually, the surface charge of 7S and 11S is neutralized at pH 4.8 and 6.4, respectively [11].

Table 2.3: Properties of Glycinin and β -conglycinin Soy Proteins [10] [11] [13] [14] [15]

Properties	β -conglycinin (7S)	Glycinin (11S)
Molecular weight	Trimer; 180 – 210 kDa	Hexamer; 320 – 360 kDa
Isoelectric Point	4.5 – 4.9	6.4 – 8
Denaturation	68 – 74°C	83 – 86°C
Interactions	α and α' subunits have one cysteine residue (-SH) and can participate in disulfide bond. β subunit does not contain cysteine residue. Structure is mainly maintained by hydrogen bonds, electrostatic and hydrophobic interactions.	At least one disulfide bond links the acidic and the basic subunits that maintains the structure in addition to hydrogen bonds, electrostatic and hydrophobic interactions.

Soybean storage lipids are mainly present as triacylglycerols consisting of the fatty acids linoleic, oleic, palmitic, linolenic and stearic acids [9] and some phospholipids [10]. Lipids are present in the form of oil bodies in the cotyledon that are encapsulated with small molecular weight proteins called oleosins [10].

In addition to soy proteins, carbohydrates are the next most abundant component of soybean seeds. Approximately 86% of the hull (dry weight) and 35% of cotyledon (dry weight) is composed of

carbohydrates [9]. The polysaccharides of the cotyledon mainly consist of pectin, hemicellulose and cellulose microfibril cross-linked with proteins in the primary cell whereas cellulose and hemicelluloses are the building blocks of the secondary cell wall [10]. The adhesive in the extracellular space that holds the cells together primarily contains pectin and attachment proteins such as hydroxyproline-rich glycoproteins [10]. In addition to the structural polysaccharides, soybean seeds contain non-structural soluble sugars with sucrose, raffinose and stachyose being the most abundant [9]. Some amount of starch is present as storage polysaccharide in the cotyledon, although, it functions as a reserve material that converts into energy source, glucose, as the seed matures [9].

2.3.2 Soymilk

The aqueous extract from soybeans, also known as soymilk, is composed of approximately 3% proteins, 2% carbohydrates and 2% fat [21]. Traditionally, soaked soybeans are cold-ground with water, steamed, and filtered from soybeans fiber (okara) to extract soymilk [21] [22] [23]. While the soymilk yield is high from the conventional process, its rancid-oil taste has encouraged modifications in the extraction process to improve the flavor, shelf-life and proteins recovered during filtration of soymilk. Temperature has a major influence on the flavor profile of soymilk due to the degradation of lipoxygenase (LOX) and trypsin inhibitors. Grinding soybeans at cold temperatures (3°C) or above 80°C as carried out in the hot-grinding method, reduced the presence of flavor-producing components in soymilk [23] [24]. In the hot-grinding method, in addition to grinding the soaked soybeans with hot water, sodium bicarbonate or caustic soda is used as the alkaline environment aids in inactivating LOX and reducing the rancid-oil flavor. However, this is at the expense of poor, chalky mouthfeel due to the temperature of heating stage that exceeds the soy protein denaturation temperature [21]. The rancid-oil flavor is eliminated when the heat treatment is applied to the soaked soybeans by blanching the beans. However, the chalky mouthfeel persists due to denaturation of proteins into insoluble particles [21]. The airless cold-grind method was developed to obtain a pleasant mouthfeel and flavored soymilk by processing the soybeans in the absence of oxygen and limiting heat exposure to less than 50°C before and

during the grinding stage. Table 2.4 compares the various soymilk extraction methods and their properties [21].

Table 2.4: A comparison of basic soymilk manufacturing processes [21]. Adapted from S. K. Giri and S. Mangaraj, “Processing Influences on Composition and Quality Attributes of Soymilk and its Powder,” Food Engineering Reviews, vol. 4, no. 3, pp. 149–164, May 2012, with permission.

	Traditional Method	Hot-Grinding Method	Hot-Blanching Method	(Canadian) Airless Cold-Grinding
Methodology	Soaked whole beans cold ground, filtered and cooked, without use of chemicals	Whole or dehulled beans hot ground and filtered, and involves use of NaHCO_3 / HCl	Whole or dehulled beans hot ground, homogenized at high pressure and filtered, and involves use of NaHCO_3 / HCl	Whole or dehulled beans processed in oxygen-deficient environment, cold ground, filtered and cooked, without use of chemicals
Soymilk	Dissolved solids; 70-90% protein retained	Mostly dissolved solids; 60-80% protein retained	Mostly suspended solids; 98% protein retained	Dissolved solids; 70-90% protein retained
Odor	Rancid	Less rancid	Roasted nut	Cereal
Mouthfeel	Smooth	Chalky	Very chalky	Smooth

2.3.3 Okara

Okara is the soybean residue or dreg produced during soymilk extraction from soybeans, consisting of the ruptured cotyledon cells [25] [26]. Large quantity of okara is produced as a by-product, about 1.2 kg of fresh okara for every kg of soybeans utilized for tofu manufacture, that has the potential of being utilized as a nutrient and fiber-rich additive in food products [26]. Dietary fiber is the major component of dried okara comprising of 42.4 – 58.1% that is largely present as insoluble dietary fibers (50%) with some low-soluble fibers (5%) [25] [26]. Cellulose (19%), hemicellulose (41%) and lignin (38%) are assembled into the fiber structure of okara whereas the monosaccharides and oligosaccharides include glucose, fructose, galactose, arabinose, sucrose, stachyose and raffinose [26]. The pectic polysaccharides comprised of galacturonan and rhamnogalacturonan are distributed at the reducing and nonreducing ends of the okara polysaccharide [25]. Additionally, okara contains protein (15.2 – 33.4%) [26], lipids (8.3–10.9%),

isoflavones, lignans, phytosterols, saponins, and phytates [25]. The composition of okara depends on multiple factors such as the soybean cultivar, soymilk and okara extraction methods [26], and other pre- or post-processing chemical, enzymatic, fermentation, high-pressure or micronizing treatments applied on soybeans that alter the composition of extracted okara [25].

2.3.4 Tofu

Tofu can be viewed as a soy-based cheese analogue [64]. Based on its texture characteristics, tofu is classified into firm, medium or soft, packed or pressed and fermented tofu [193]. The quality of tofu depends on the selection of soybean seeds, grinding of beans, filtering, heating and coagulating stages, operating conditions such as time, temperature, pressure, coagulant type, pH, ionic strength and microbial growth, and whether or not the product is pressed [193]. Additionally, the intrinsic factors of soybean protein amino acid and subunit compositions, and the secondary structure [193] affect the transition from liquid soymilk or soy dispersion to a gel.

Tofu is produced through hot or cold gelation routes, although cold gelation involves a pre-heating stage to denature the soy proteins before inducing aggregation at low temperatures. In either methods, gelation proceeds through four stages [194]. Firstly, heating of soy proteins unfolds the proteins, dissociates the native globulins into smaller units [194] and exposes the buried polar or non-polar groups on the surface. Subsequently, proteins associate mainly through non-covalent interactions and form thermally-irreversible aggregates [194]. Finally, the protein aggregates form a network that gives rise to the gel structure [194]. Each of these stages is affected by protein concentration, temperature, pressure, pH and ionic strength of the system [96].

The use of coagulants to assist the protein coagulation process affects the quality, texture and yield of tofu produced, as coagulants can modulate the rate of association which influences the protein-protein and protein-water interactions in the gel network [193]. The coagulation mechanism differs between acid, salt and enzyme coagulants used in tofu manufacture. Each coagulant type has unique benefits as well as disadvantages. Tofu produced with acid coagulants at slow acidification rate have a soft texture and sour flavor, excess salt coagulants generally give a bitter taste with

gritty texture, whereas the enzyme coagulants produce tofu with low hardness and require prolonged duration for production [193].

Acid coagulants such as GDL or organic acids (citric, malic, tartaric, lactic acids) supply hydrogen ions, lower the pH and neutralize the surface charge of proteins [195]. When the pH of the protein dispersion is close to the IEP, the proteins aggregate, insolubilize and precipitate [96] [193] [195]. Salt coagulants include chlorides and sulphates of magnesium or calcium where the Mg^{2+} or Ca^{2+} ions crosslink proteins and assist in forming the gel network structure. Additionally, salt screens the surface charge of proteins through neutralization and reduces electrostatic repulsion between the polypeptide chains [196]. Through counter ion exchange of the H^+ ions of protein with the salt metal ions, the pH of the system is lowered which along with the shielding effect of salt facilitates protein aggregation [99]. Enzyme coagulant such as microbial transglutaminase (MTGase) crosslink the polypeptide chains and assist in building the gel structure [193].

Research on soy gels often use soy protein isolate (SPI) as the protein source to study the effect of protein concentration [94], coagulants [72] [73] [86], or gelling conditions on the textural properties [71]. A study on twenty different commercial SPI correlated amino acid and protein subunit compositions with the texture of Chiba tofu, a variant of tofu that incorporates starch in the formulation [70]. Significant differences in the secondary structure of proteins, the amino acid composition and hardness of the tofu produced from different SPI sources were observed. The presence of more sulfur-containing amino acids, β -sheets and 11S:7S ratio in SPI was beneficial in producing gels with more uniform texture, and dense structure. This study demonstrated the difficulty in comparing the properties of soy gels produced across studies that utilize commercial SPI from different sources and extraction methods.

2.4 Incorporation of Okara in Soy Products

Okara offers many potential health benefits related to hypoglycemia, diabetes, cardiovascular diseases, obesity, and antioxidant properties [164]. The insoluble dietary fibers (IDF) aid with digestive conditions such constipation or bowel cancer while soluble dietary fibers (SDF)

modulate metabolic functions such as lowering cholesterol level and controlling blood sugar levels [164]. IDF, however, cannot be digested by humans and therefore, attempts have been made at converting it into SDF to enhance the benefits of okara [164]. Moreover, the high IDF content of okara limits its application in food systems. It adversely affects the sensory aspect and functionality of the food products when incorporated in food and therefore requires modification through physical, chemical or enzymatic methods [137]. The following sections explore some physical and non-physical approaches to okara modification, and their subsequent use in producing high-fiber soy gels.

2.4.1 Mechanical Modification of Okara

The effect of steam explosion treatment on the SDF and IDF content of okara was previously studied wherein dried okara was exposed to superheated steam at different pressure conditions (0.5, 1.0, 1.5 and 2.0 MPa) and durations (30, 60 or 120s) [164]. The instantaneous release of pressure after the treatment ruptures the cellular structure of the polysaccharides making the cell wall of okara. As a result, low molecular weight components are released from hydrolysis, thermal and mechanical degradation, and disruption of hydrogen bonds disrupted, thus converting cellulose and hemicellulose into SDF. The treated okara was observed to constitute higher oligosaccharide and SDF content, lower IDF, increased water solubility from exposure of hydrophilic groups but reduced WHC as the porous structures of okara were degraded.

Wet media milling treatment was utilized to modify okara functionality wherein the shearing force of the beads in the milling chamber mechanically reduce IDF particles into nanofibers [137]. The particle size of IDF decreased after up to 6 h of treatment beyond which the fine particles aggregated via van der Waals and electrostatic interaction. The anionic surface of nanofibers became less negatively charged and the water solubility was enhanced from the increased exposure of hydroxyl groups.

Cavitation jet treatment, based on the principle of formation of cavities in a fluid whose expansion and contraction generates localized regions of high temperature and pressure that breaks down large particles into smaller particles, was explored to treat okara [127]. The dry, defatted okara

powder, processed in a cavitation chamber at 22.5°C, 0.01 MPa for 3 to 15 min duration had higher SDF and reduced IDF content. Moreover, okara treated for up to 10 minutes had enhanced water solubility, swelling capacity, water holding capacity (WHC) and oil holding capacity (OHC), which reduced on over-processing for longer durations due to change in surface characteristics of okara.

High pressure homogenization and ultrasound treatment of okara fibers were explored for the preparation of nanocellulose [119]. A range of disruptive forces are involved in high-pressure homogenization (100, 120, or 140 MPa at 10,000 rpm for 5 minutes) such as cavitation, turbulence, and shear effects, whereas cavitation is the predominant disrupting force in ultrasound treatment (400W and 600 W for 15 or 30 minutes). The combination of ultrasonication (400, 500, or 600 W for 30 min) and high-speed shearing (5000, 10000 or 15000 rpm) treatments on okara IDF in another study demonstrated enhanced swelling capacity, WHC and OHC [27]. Ultrasound treatment disintegrated the tightly packed cellulose in the cell wall and destroyed the crosslinking between polysaccharides. Combined with high-speed shearing, the constituent cell layers of the fibers were disintegrated at the cellular level resulting in a decrease in particle size. However, over-processing deteriorated the WHC and OHC properties as the fibers lost their porous structure, and physical interactions such as van der Waals and electrostatic interactions caused aggregation of the okara particles.

Other physical treatments explored across studies include microwave treatment [135], superfine grinding in a micronizer [29] and high pressure homogenization [30] that either successfully converted the IDF component in okara fibers into SDF or modified the properties of the fibers.

2.4.2 Chemical and Enzymatic Modification of Okara

A variety of non-physical methods of okara treatment with the objective to reduce the size okara fibers, increase SDF content or isolate soluble fractions of okara polysaccharides are reported in literature. Microbial fermentation of okara decomposes IDF into SDF [135]. Alkaline peroxide treatment applied (1 – 3% w/v) at a range of temperatures (20 – 50°C) modifies the okara fibers by disrupting the hydrogen bonding involved in the structure [32]. This results in unraveling of the

structure and increases surface roughness, facilitating water penetration into the fiber network. As a result, the hydration property of alkaline modified okara is enhanced, along with improved oil holding capacity, soluble fiber content and soluble okara protein content. Hydrolysis of okara fibers into SDF has also been achieved using a mixture of carbohydrase enzymes [28]. Nanocellulose can be produced from okara through TEMPO oxidation of the fibers followed by sonication or high pressure homogenization treatment in another study [31]. TEMPO oxidation disrupts the amorphous regions of okara cellulose, exposes the crystalline regions, and alters the surface charge of fibers to become more negative as the hydroxyl groups are replaced with carboxyl groups. Subsequent sonication or homogenization disrupts the exposed crystalline regions through cavitation, turbulence, or shearing and significantly reduces the particle size of fibers into nanocellulose with high thermal stability and functional surface.

2.5 Citric Acid Treatment

Citric acid (CA) is an organic, weak acid that possesses three carboxylic acid groups with pKa values of 3.1, 4.7 and 6.4. The functional groups and acidic character of CA equips it to function as a preservative and a metal ion chelator in food applications but most importantly, its role as a pH modifier, a crosslinker and an extracting agent from polymer hydrolysis [47] were of interest in this study that are detailed in the following sections.

2.5.1 CA Hydrolysis

High CA concentration is being used as a replacement for mineral acids for the extraction of cellulose nanofibers (CNF) and nanocrystals (CNC) by hydrolyzing the fibers of materials such as eucalyptus cellulose kraft pulp [42]. In the study, low pK_a of CA (pK_{a1} = 3.15) along with high concentration of CA (65 w/w%) and high operating temperature (120°C for 1.5 – 6 h), lead to hydrolysis of amorphous regions of cellulose that produced CNC/CNF and carboxylation of the cellulose surface via esterification. Lower CA concentration was employed to pre-treat biomass such as the insoluble dietary fiber extracted from citrus peel (CIDF) [43]. The study explored CIDF treatment with acetic acid (10% w/w) or citric acid (5% w/w) followed by heating (50°C for 1h),

as well as with oxalic acid (0.9% w/w) at room temperature. The particle size of the treated fibers decreased indicating rupture of the fiber structure and the conversion of some insoluble fibers into soluble fibers, and FTIR spectra indicated carboxylation of the surface hydroxyl groups. Another study emphasized the requirement of high CA concentration (0.35 - 11.65%) and long reaction times (100°C, 102.4 min) for polysaccharide degradation, particularly the cellulose and hemicellulose fractions in sugarcane bagasse, that was visualized from the ruptured structures of CA-treated fibers under SEM [46].

2.5.2 Esterification and Cross-linking of Polymers

The carboxylic acid groups of CA enable it to esterify macromolecules, and the presence of tri-carboxylic acid groups makes CA capable of crosslinking. However, esterification and crosslinking of macromolecules generally requires elevated temperature, high acid concentration and long reaction duration. The surface of kenaf core fibers was modified with CA induced esterification by soaking the fibers in 11.5% (w/v) CA solution followed by two-stage oven-drying [44]. A similar methodology was adopted for the surface modification of soybean straw [45] with 11.5% (w/v) CA. Citric acid esterification was detected in FTIR, although, FESEM micrographs recorded no significant changes in the highly porous, ordered fibril microstructure of soybean straw after CA treatment. A study on the soaking of ramie cellulose in 80% (w/w) citric, malonic or malic acids overnight, followed by 3 h heating at 140°C, lead to hydrolysis and surface modification via esterification of the fibers and produced CNC [112].

When two or more of the carboxylic acid groups of CA are consumed in esterification reaction, it functions as a crosslinking agent between the involved macromolecules. Crosslinked carboxymethyl cellulose was produced at low freezing temperature (-20°C) by treating the polysaccharides with CA (2% w/w) or fumaric acid (5%) for 36 hours. An ester bond vibration peak observed in FTIR spectra confirmed the crosslinking [173].

The carboxyl groups of CA can induce crosslinking of proteins by esterifying the hydroxyl groups of protein chains. Soy proteins were previously crosslinked with CA (0.6 – 6.7% w/w) by stirring the dispersion at 40°C for 2 minutes followed by 3 h curing at 180°C [175]. However, as high

temperatures can also denature proteins and alter their functionality, crosslinking reaction has also been carried out at low temperatures.

Wheat gliadin protein was crosslinked with CA solution (12.8% w/w) during 4 hours treatment at 50°C, with sodium carbonate acting as the alkali catalyst that facilitated deprotonation of CA by raising the pH. An increase in pH deprotonates the carboxyl group of CA into COO⁻ and the protein amino group into NH₂, resulting in interactions that form an amide bond through nucleophilic substitution. Crosslinking within or between polypeptide chains occurs when multiple carboxyl groups of CA are deprotonated and interact with proteins. Without an alkali catalyzing CA deprotonation by increasing the pH, CA lowers the pH below the isoelectric point at which the amine groups of wheat gliadin become positively charged. While weak electrostatic interactions may be initiated, the reduced nucleophilicity of positively charged amine group (NH₄⁺) prevents formation of amide bond between protein and CA. Another study also highlighted the necessity of an alkali to catalyze the esterification reaction between malic acid or CA with gliadin, soy or zein protein [120].

2.5.3 Pectin Extraction

CA has been used in studies to extract pectin from pectin-rich fruit sources such as apple [49] and citrus [50] peel. Despite the milder conditions of organic acid, compared to the harsh mineral acids that are conventionally used for pectin extraction, pectin extracted with citric, malic or tartaric acids has higher molecular weight and higher apparent viscosity which are desirable qualities for food applications requiring gel forming, thickening or stabilizing agent. Another study extracted soluble polysaccharides from soy hull with 0.6% (w/w) CA combined with microwave treatment and the presence of soy pectin was detected in the composition profile and FTIR spectra [149].

2.6 Protein-Polysaccharide Gels

Protein gels and polysaccharide gels have been explored across multiple studies for different applications. In food applications, texture is an important aspect of consideration and texture is developed when homogeneity is destabilized which then subsequently generates heterogeneity in

the system [37]. Binary gel system consisting of protein and polysaccharide offers this advantage of producing various gel structures and textures that can be regulated by modifying the interactions and gelling conditions [41]. Texture is an inevitable consequence when mixing proteins and polysaccharides as they belong to different classes of biopolymers that possess different chemical structures, properties and functionality. Diverse phase behaviors can be generated, as a result, depending on the long or short-range interactions between the biopolymers, and their affinity to each other and to water. Additionally, combining the individual nutrition and functional properties of proteins and polysaccharides facilitates the production of functional food gels [41].

Multiple studies have reviewed the various interactive or repulsive behaviors between proteins and polysaccharides [40 – 47], and are illustrated in **Figure 2.4**. The interactions depend on the biopolymer concentration, type, and mixing ratio, as well as the operating conditions of the system that include pH, temperature and ionic strength. Therefore, the type of interaction between protein and polysaccharide can be modulated by changing these parameters such that an incompatibility between the polymers is converted to compatibility. The behavior of protein-polysaccharide mixture is classified into either being associative or segregative.

In dilute systems containing low biopolymer concentration, the mixture is stable and the protein and polysaccharide molecules are co-soluble in the solvent. An increase in concentration or when protein and polysaccharides molecules carry similar surface charge, repulsive forces dominate which makes them thermodynamically incompatible. This results in the homogeneous mixture of the two polymers to segregate into two phases wherein one phase is protein-rich and the other is a polysaccharide-rich phase.

Association of protein and polysaccharides is predominantly driven by electrostatic interactions. pH, ionic strength and temperature of the environment, isoelectric point of proteins and pKa of polysaccharide acid groups influence the effective interactions formed between the biopolymers. Heating can unfold the biopolymers and expose the buried polar and non-polar groups that consequently modify the surface charge properties. Modulating the operating pH relative to the isoelectric point of proteins, which is the pH at which the charge on proteins transitions between positive or negative, is the key to induce protein-polysaccharide interactions. Except for a few

polysaccharides such as chitosan that carries a net positive charge, most polysaccharides are negatively charged over a wide range pH range. Therefore, effective electrostatic protein-polysaccharide interactions occur at pH slightly above the IEP of protein at which the protein surface contains patches of positive charge that associate with the negatively charged polysaccharide, despite the overall net negative charge on the protein. As the complexes aggregate with decreasing pH, their size and density increase resulting in possible sedimentation that induces phase separation. Additionally, when the inter-polymer interactions are of short-range, the biopolymers interact closely and form dense aggregates that sediment. Alternatively, when the biopolymers associate via long-range interactions, the less dense aggregates remain soluble and dispersed in the solution.

Proteins with polysaccharides can be associated via hydrophobic interactions. However, modulating hydrophobic interactions often requires heating to alter the protein configuration (tertiary and quaternary structures) which exposes the buried hydrophobic groups, increasing their surface hydrophobicity. Yet, the resulting complexes formed are irreversible and resistant to pH or ionic strength changes. In contrast, electrostatic interactions are easier to tune by modifying the environmental pH or ionic strength. To utilize the advantages of both interactions, design of food matrices often involves aggregation of protein and polysaccharide with electrostatic interaction, and then subsequently stabilizing the formed complexes through heat treatment.

Coacervation is another type of association between proteins and polysaccharides wherein electrostatic interactions between oppositely charged polymers are the dominant driving force [36]. Coacervates are generally comprised of a polysaccharide core surrounded or encapsulated with proteins. A consequent observation that may occur depending on the density of the aggregates formed is phase separation of the coacervates as they sediment from the solvent phase containing small amount of the macromolecules. While offering the advantages of linking protein with polysaccharide and encapsulating materials that are sensitive to moisture, light or oxidation, coacervation occurs in narrow pH range, usually below the isoelectric point of the involved protein. Therefore, an increase in pH can disrupt the integrity of the coacervates formed through electrostatic interactions. Heat treatment can stabilize these coacervates, particularly if the

polymers possess hydrophobic regions since hydrophobic interactions can replace the weak electrostatic interactions in holding the coacervate together.

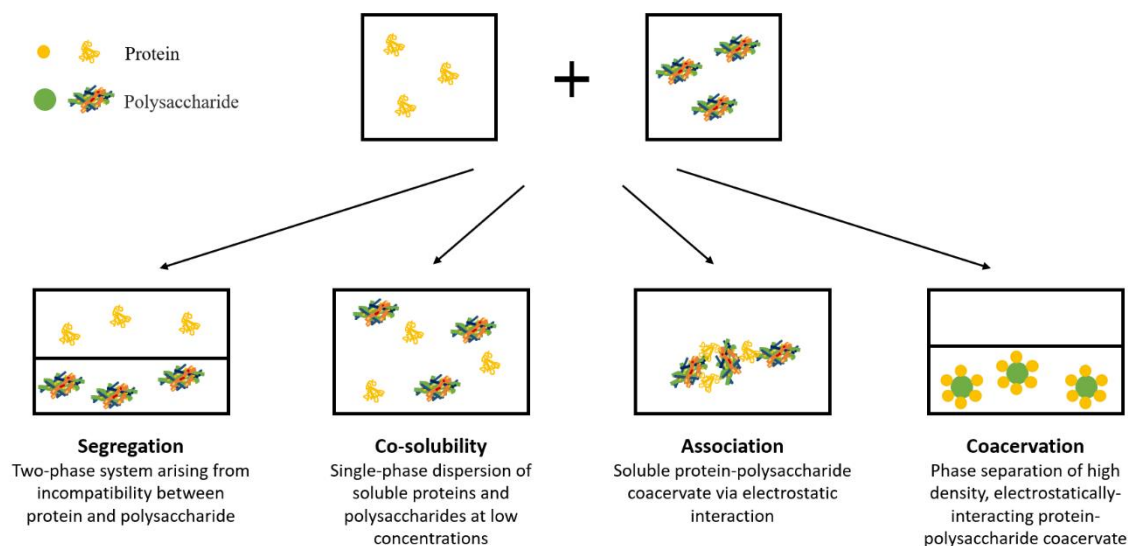


Figure 2.4: Trends in the behavior of protein and polysaccharide mixtures. Adapted from C. G. de Kruif and R. Tuinier, “Polysaccharide protein interactions,” *Food Hydrocolloids*, vol. 15, no. 4–6, pp. 555–563, Jul. 2001, with permission [40]

The association between plant-based proteins and polysaccharides for food applications most commonly occurs via physical interactions [39] through simple physical mixing without the involvement of chemical reactions, although this is a disadvantage of electrostatic complexes as they are susceptible to destabilization on changing the temperature, pH or ionic strength [102]. Charged polysaccharides electrostatically link with the charged groups of proteins [52] whereas neutral polysaccharides associate weakly with proteins through hydrophobic interactions or hydrogen bonding [52]. Studies have explored electrostatic coacervate or complex formation of SPI with linear cationic chitosan [53], anionic gellan gum, neutral guar gum [52], linear anionic carboxymethyl cellulose [53], high methoxy pectin [54], pectin-containing soy soluble polysaccharide [54] [57], carrageenan and alginate [56]. Moreover, various legume proteins including mung bean protein isolate [55] and pea protein isolate [57] have been tested for forming interactions with polysaccharides. Majority of the studies explored complexation of proteins with polysaccharides that have high surface charge density, possess a linear or well defined molecular

structure, respond to pH changes, or are soluble in water. These polysaccharides significantly differ from the complex, heterogeneous structures of cellulose, hemicellulose, lignin and pectin present in okara which pose limitation on its application for preparing protein-polysaccharide complexes.

Alternatively, proteins and polysaccharides can be chemically and permanently associated with each other through conjugation [38]. Chemical interaction through glycosylation is a type of covalent conjugation forming protein-polysaccharide complexes. Maillard reaction is a non-enzymatic spontaneous, glycosylation reaction that involves a sequence of three stages. In the initial stage referred to as glycation of proteins, heat causes the amino groups of proteins to covalently bond with the reducing sugar of carbohydrates through condensation reaction to form Amadori products [16] [17]. The intermediate stage of Maillard reaction involves degradation of the Amadori products on continuing heating which results in the production of highly reactive compounds that further undergo reactions to form advanced glycation end-products (AGEs) in the final stage. For the production of functional proteins, Maillard reaction is limited to the glycation stage and prevented from progressing into producing AGEs or melanoidins that are associated with loss in nutritional value, off-flavors and formation of undesirable, uncontrollable toxic products [16]. Maillard reaction has been observed to occur in okara during drying [18] [19] [20]. The oven-drying of wet okara at increasing temperatures from 50 to 70°C for 10 hours was observed to cause faster moisture loss and browning which was associated with Maillard reaction [18].

2.7 Soymilk and SPI Gel with Modified Okara

Traditionally, tofu is produced from soymilk which utilizes only 53% of the soybean mass (on dry basis) [33], while the by-product okara is often discarded leading to resource wastage and environmental contamination [33] [168] [154]. The rich dietary fiber profile of okara (50-60%), along with the presence of proteins (20-30%), lipids (10-12%), minerals and isoflavones [168], makes okara a good additive that improves resource utilization [151], enhances nutrition profile of food products [168] and offers positive effects in preventing health concerns [33]. The main

challenge to address when incorporating okara in soymilk and SPI gels is the disrupting effect of okara on their quality. The large particle size and high water absorption capacity of okara weakens the protein network and coarsens the gel texture [151] [154] [168]. **Table 2.5** presents a summary of soymilk and SPI gels with okara and the influence okara had on the gel texture.

Table 2.5: Published studies on the soymilk or SPI gels with okara

Okara Treatment	Soy Gel Preparation	Results
Fresh okara was freeze-dried, micronized in pulveriser and passed through sieves to segregated it into four categories based on particle size – (a) 250 – 380 μm, (a) 150 – 180 μm, (a) 120 – 150 μm and (a) less than 75 μm [108]	Treated okara was added to soymilk. Mixture was heated at 75°C for 10 min followed by a second heating stage at 90°C for 10 min. Mixture was cooled down (<25°C) before adding 0.3% (w/w) GDL and then heated at 85°C for 1 h. Control: Gel prepared by similar method, without okara	• Decrease in particle size of O increased SDF content, WHC and OHC. • Soymilk oil droplets in heated mixture decreased in size after O addition • Presence of O increased the WHC of the GDL-induced gel compared to control due to water immobilizing ability of O. Additionally, WHC increased with decreasing size of O due to higher hydration capacity of smaller O particles • Except the gel containing 250 – 380 μm O, presence of O increased gel strength. Gel strength increased with decrease in O size as small fibers acted as filler that could be entrapped in the protein network. • Control had a smooth, homogenous, compact microstructure whereas gels with O had a rough, heterogeneous texture and relatively loose network. Reducing particle size reduced negative effect of O on gel microstructure. Homogeneity was retained in gel with <75 μm O.
No treatment; Okara was ground in high-speed grinder for 2 min [168]	Okara and SPI suspension was treated with high intensity ultrasound probe at 500, 600, 700 and 800W for 20 min, while maintaining <25°C temperature 1% (w/v) GDL was added to the treated suspension and heated at 80°C for 30 min Control: SPI and okara gel without ultrasonic treatment	• Cavitation effect of sonication reduced the average particle size of okara and SPI suspension up to 600W, beyond which particle size did not vary due to aggregation of particles via van der Waals forces and electrostatic attraction, as well as protein denaturation. • Rheological properties significantly increased with increase in treatment up to 600W, when compared to control. Higher sonication power, however, caused a decrease in viscoelasticity. • Ultrasonic treatment significantly increased gel WHC, compared to control as treatment decreased particle size and exposed polar groups. Treated gel had denser, more uniform texture with small pores. Above 600W treatment, WHC decreased as the porous structure of okara fibers was destroyed that decreased its ability to entrap water. • Gel strength increased after treatment and had similar trend as WHC at intense treatment condition (>600W)

Dietary fiber fraction of okara was extracted with chemical treatment, dried and pulverized to obtain MDF (110 μm average). NDF (370 nm average) was prepared by further subjecting MDF to wet media milling for 8 h. [150]	Soymilk and MDF or NDF (DF concentration of 0 – 50% v/v) suspension was heated to 95°C and maintained for 10 min. Water bath was then cooled down to 85°C and coagulant 0.02 mol/L MgCl_2 was added. Mixture was heated at 80°C for 10 min. Tofu curd was pressed for 30 min in mold to drain whey. Control: Soymilk gel prepared by similar method, without DF	• WHC of gels with MDF or NDF was higher than control. The WHC of MDF containing gels increased up to 30% MDF concentration and decreased. Gels containing NDF decreased with increasing NDF concentration. • Gel strength and elasticity decrease with increasing MDF or NDF concentration compared to control, with MDF gels having relatively higher TPA properties than NDF gels. • MDF did not alter the continuous protein phase but at high concentrations, MDF aggregated and increased the number and size of cavities in the continuous phase. • NDF gel possessed large number of cavities at low concentration and fragmented the continuous phase.
No treatment [154]	Soymilk and okara were homogenized at 40 MPa to reduce the okara particle size < 50 μm, heated to 95 °C and maintained for 5 min GDL Tofu: 0.3% w/w GDL (final) was added to cooled mixture, and heated at 85°C for 30 min TGase Tofu: To GDL-gel mixture, 0.15% (w/w) TGase was added, incubated at 50 °C for 240 min and then at 85 °C. Control: GDL-Tofu without okara	• The okara in high fiber GDL-Tofu weakened the gel structure and strength compared to control. Addition of TGase significantly increased the gel strength as TGase assisted crosslinking of soy proteins. • Okara particles disrupted the continuation protein network but TGase promoted formation of protein aggregates that encapsulated the okara particles • WHC increased in the presence of okara, with further increase on adding TGase to the gel, due the ability of dietary fibers to absorb water.
No treatment [33]	Soy flour (ground soybean) suspensions (15% and 20%), either thermally treated at 95 °C for 10 min or treated by ultra-high-pressure homogenization (UHPH) in a homogenizer at 100 and 150 MPa 10% GDL solution was added to treated suspension (final GDL concentration of	• UHPH decreased the size of large insoluble particles of soy flour from 300 μm to less than 30 μm • Tofu containing 15% soy flour concentration and thermally treated (control) was harder than UHPH treated tofu. Among the UHPH gels, an increase in pressure of the treatment increased hardness. This trend was different for 20% soy flour suspension where UHPH treatment significantly increased hardness compared to thermally treated tofu (control).

	0.5%) and heated at 80°C water bath for 30 min	• WHC increased with increase in soy flour concentration and UHPH treatment. • Thermally treated tofu had a honeycomb structure but with large particles inlaid in the network. UHPH tofu had a regular honeycomb protein network without large particles.
Dietary fiber fraction of okara was extracted chemically, dried and pulverized and then subjected to wet media milling for 8 h to obtain NDF (370 nm average) [151]	Soymilk combined with NDF (0 – 40%) was heated up to 95°C and maintained for 10 min. MgCl₂ or GDL (0.015mM and 0.02mM) was added to the heated suspension and incubated at 80°C for 10 min. Curd is pressed for 30 min with 1.5 kg load. Control: Soymilk tofu prepared by same method, without NDF	• Tofu with MgCl₂ as coagulant had a porous, particulate microstructure. Tofu with GDL as coagulant had a strand-like, compact microstructure • GDL tofu was softer with higher moisture content and WHC • Addition of NDF decreased gel strength due to increased moisture and porous structure • NDF inhibited protein unfolding during heating and inhibited exposed functional groups of unfolded protein to interact
Okara IDF coated with a layer of chitosan followed by a layer of pectin (O-IDF/C/P) [34]	Soymilk was heated to 95°C for 10 min and cooled. O-IDF or O-IDF/C/P (10 g/L) was added to soymilk and mixture was homogenized for 30 s. 5% (w/v) GDL (0.35% final concentration) was added to suspension and heated at 85°C for 30 min Control: Soymilk gel prepared by similar method, without O	• Control had a smooth surface and had homogenous, compact structure. O-IDF tofu had a coarse, heterogeneous texture with cracks. Incorporation of O-IDF/C/P smoothened tofu surface, similar to control, and had some fine pores. • Except for flavor, O-IDF/C/P tofu had a favorable appearance, texture and mouthfeel. Layering of chitosan and pectin on okara improved its compatibility and incorporation in tofu.
Suspension of vacuum-dried okara was first ground in high-speed colloid mill and then treated by high pressure homogenization (HPH) at 40 – 100 MPa [35]	SPI and okara (0-60% v/v) suspension was heated at 100°C for 5 min and cooled to 45°C. TGase was added to the suspension and incubated at 45°C for 60 min Control: SPI gel prepared by same method, without okara	• Increasing intensity of HPH treatment decreased okara particle size • Elasticity of gels with okara was higher than control, and elasticity increased with increase in okara content.

Chapter 3: Standardizing the Preparation of Soy Gel Ingredients: Treatment and Concentration

3.1 Overview

Chapter 3 explores the effects of changes to ingredients (soybeans, extracted soymilk and okara) and soymilk okara mixture on the texture properties of the gels. The effect of freezing soybeans, the particle size, concentration, citric acid-modification, and drying of okara, and the citric acid coagulation of soymilk before thermal gelation were examined. Scanning electron microscopy (SEM) was adopted to characterize the morphology of dried okara and visualize okara distribution in soymilk gels. A visual representation of the above objectives is presented in **Figure 3.1**. The experimental methodology for the preparation of soymilk and okara from soybean and their associated gels was adapted from [59].

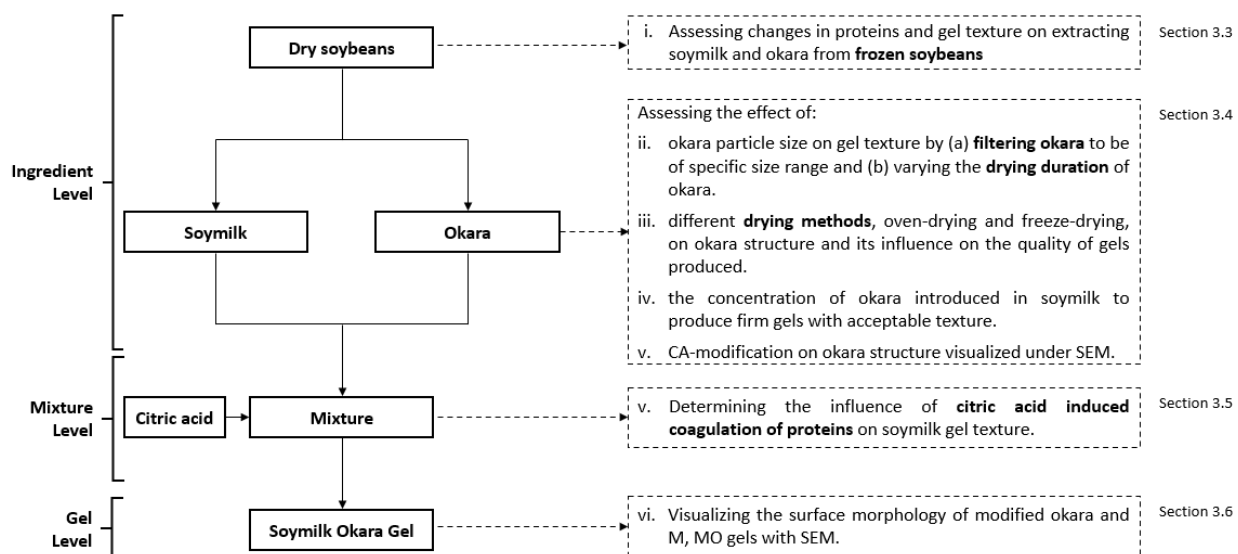


Figure 3.1: Objectives of the preliminary experiments for standardizing the preparation of soymilk okara gels

3.2 Materials and Methodology

3.2.1 Materials

Soybeans (Nupak, Freshco), obtained in bulk from a local grocery store (Waterloo, Ontario, Canada) were stored in food storage bags at room temperature. The chemical reagents were of analytical grade which included sodium phosphate dibasic heptahydrate (Acros Organics, India) and sodium dihydrogen phosphate dihydrate (Acros Organics, India), hydrochloric acid (Fisher Scientific), sodium hydroxide (Sigma Aldrich, India), sodium chloride (Fisher Scientific), urea (VWR, Canada), Bio-rad dye reagent and Bovine Serum Albumin (Bio-rad Laboratories Inc., USA, Batch # 64499500).

3.2.2 Preparation and Characterization of Ingredients

3.2.2.1 Extraction of Soymilk and Wet Okara

Dry soybeans were soaked overnight in DI water at a soybean mass to DI water volume ratio of 1:3 (w/v) for 22-24 hours. The soaked beans were washed under running DI water to remove the released anti-nutritional components, dehulled, and then ground for 2 minutes at high speed in 4 cycles of 30 seconds in a blender (Waring Commercial Grinder E11524 USA). The slurry was filtered through a cheesecloth (Scenglos, Woodridge, IL) to separate soymilk (M) from wet Okara (O_w), and both products were stored in the refrigerator until further use.

The preparation of soymilk and okara from frozen soybeans used for **Section 3.3** was adapted from [107], with some modifications in procedure as outlined in **Figure 3.2**. The dehulled soybeans were frozen in a freezer at for 5 h and then thawed overnight in the refrigerator. The thawed soybeans were equilibrated at room temperature before proceeding with grinding and filtering of slurry with a cheesecloth to obtain soymilk (fbM) and okara (fbO_w) that were stored in a refrigerator, without boiling, until further use.

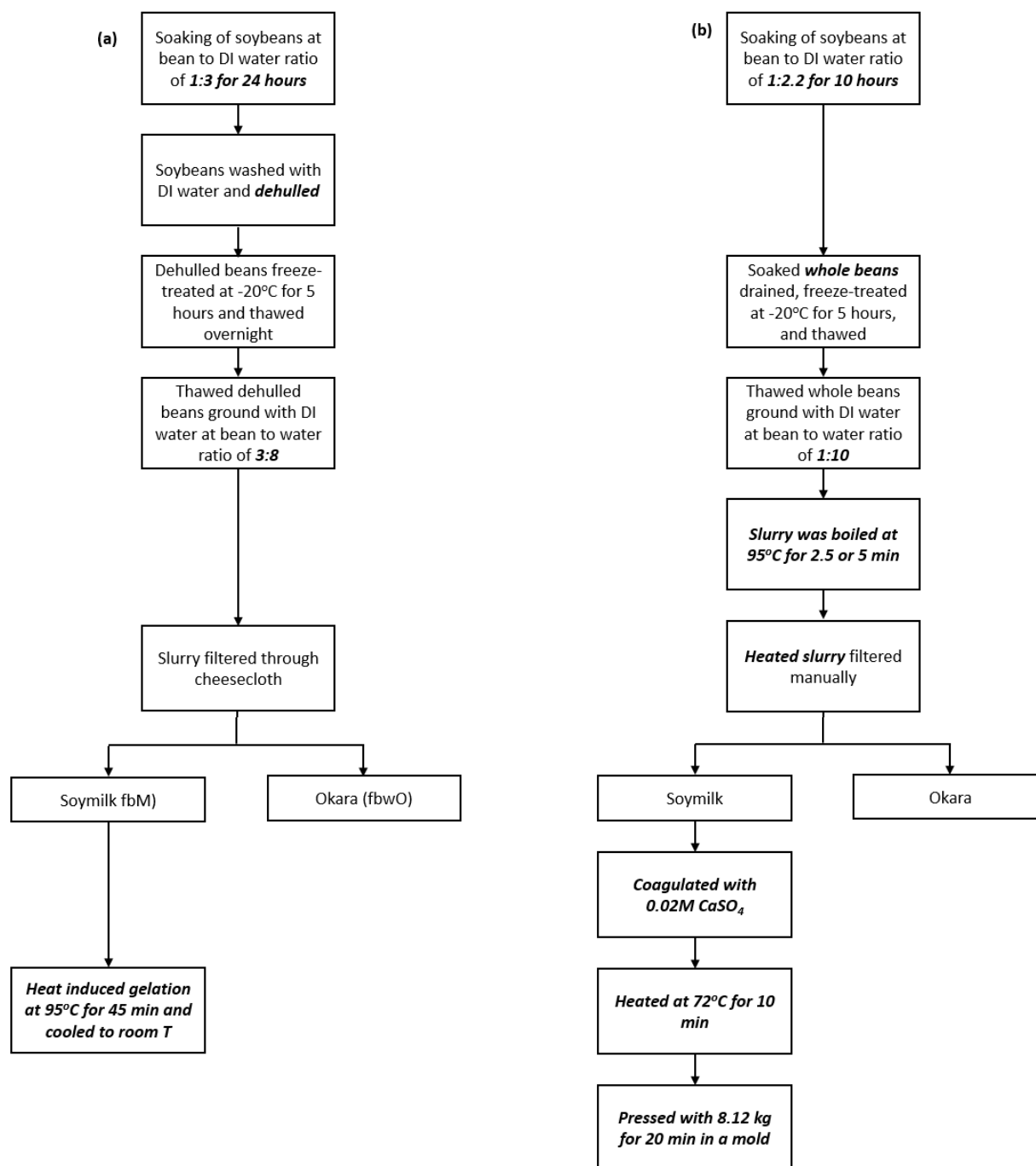


Figure 3.2: Schematic of the preparation of soymilk gel from frozen soybeans: (a) adopted in this study, and (b) procedure of Noh et al. 2005; the italicized text highlights the modifications made to the methodology.

3.2.2.2 Soymilk Solid Content

The quality of soymilk extracted from soybeans was evaluated from its solid content. 1 g of M was measured into a 20 mL beaker and dried in forced air oven at 60°C for 3 hours following which the mass was recorded. The solid content was calculated using **Equation 3.1**:

$$\% \text{ solid content} = \frac{W_{\text{solid}}}{W_{\text{soymilk}}} \times 100 \quad \text{Equation 3.1}$$

3.2.2.3 Preparation of Dried Okara

Approximately 10 g of O_w was dispersed in 50 g of DI water and mixed on a magnetic stirrer (Fischer Isotemp 11-100-49sh, USA) for 10 minutes to ensure complete dispersion. The okara slurry was transferred to a 100 mm diameter petri dish, previously lined with parchment paper. The petri dishes were placed in a pre-heated forced air oven (Fischer Isotemp 737F, USA) maintained at 60°C for 22-24 hours to remove moisture and then pulse-ground in a coffee grinder (Coffee & Spice Grinder CBG110SC, Black & Decker Co and Spectrum Brands Inc., USA) for one minute into powder to obtain O.

Similar drying methodology was carried out on fbO_w to obtain fbO used in **Section 3.3**. For **Section 3.4** assessing the influence of drying duration on the particle size distribution of O, O_w dispersed in DI water was dried at 60°C for 19 (O_{19h}) and 24 hours (O_{24h}). Air-dried okara (O_{Air}) was obtained by exposing a thin layer of O_w to room temperature overnight. Freeze-dried okara (O_{F-I}) was prepared by spreading 10g O_w along the walls of a borosilicate vial, previously cooled in freezer at -20°C overnight. O_{F-II} was prepared by mixing 5g O_w with 25g DI water over magnetic stirrer at 600-700 rpm for 10 minutes to form a slurry. The slurry was layered along the walls of a borosilicate vial, previously cooled in freezer at -20°C overnight. The borosilicate vials containing sample were dipped in liquid N_2 for 5 minutes and subsequently attached to a freeze dryer (Freeze Dryer ALPHA 1-4 LSC, Christ). Freeze-drying was carried out for 22-23 hours (main drying stage) under a vacuum pressure of 0.940 mbar and ice condenser temperature of -61°C to obtain O_F . O_{F-I} was ground for one minute and used for FTIR analysis and gel preparation. O_{F-II} was used for SEM analysis without prior grinding. Both O_{F-I} and O_{F-II} were stored in a scintillation vial sealed with parafilm until analysis.

Citric acid-modified okara OCA0.14, OCA1.25 and OCA2.5 were prepared by mixing 10 g of O_w in 50 g CA solutions prepared by dissolving 0.084 g, 0.75 g and 1.5 g CA in DI water, respectively. The dispersions were stirred at 600 – 800 rpm for one hour at room temperature over a magnetic stirrer, transferred into a petri dish and dried in a forced air oven at 60°C for 19 - 20 hours.

3.2.2.4 Estimating the Particle Size Distribution of Okara

Okara, with initial weight w_i , was passed through a series of sieves with mesh pore sizes of 212, 150, 106, 75, 63 and 45 μm . A rubber spatula was used to aid the fine okara particles to pass through each mesh screen. The mass of okara retained on the sieves, w_{sieve} , was weighed and used to estimate the percentage of okara that is larger than the mesh pore size using **Equation 3.2**,

$$\text{weight \% of okara collected on the sieve} = \frac{w_{\text{sieve}}}{w_i} \times 100 \quad \text{Equation 3.2}$$

The particle size analysis was carried out twice to evaluate the average weight % and the ground okara was subsequently categorized into three size ranges as (i) more than 106 μm , O_{III} , (ii) between 106 - 63 μm , O_{II} , and (iii) less than 63 μm , O_{I} , to prepare soymilk okara gels.

3.2.2.5 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of 32 scans at a resolution of 4 cm^{-1} was collected in the wavenumber range of 500 to 4000 cm^{-1} with the Nicolet 6700 FTIR spectrometer (Thermo Scientific Nicolet 6700, Waltham, MA, USA) for dried M and O samples. The transmittance was baseline corrected and normalized using Omnic software (Thermo Scientific) for comparison of the spectral peak shifts and magnitude changes.

3.2.2.6 Visualization of Soymilk Coagulation with Droplet Test

The approach to assess the influence of CA concentration on the coagulation of M was adapted from the methodology developed for the tracking of MgCl_2 induced coagulation of soymilk [147]. CA solutions of concentrations 0.14, 0.168, 0.376, 0.75, 1.5, 2.5, 3 and 5% (w/w) were prepared by dissolving solid citric acid (0.07, 0.084, 0.188, 0.375, 0.75, 1.25, 1.5, 2.5 g) in DI water to a total mass of 50g. The pH of each solution was recorded with a pH meter (Mettler Toledo, S47 SevenMulti Dual). The CA solutions were heated in a water bath at 95°C for 5 minutes to ensure

dissolution. 30 μ L drop of M was added to 200 μ L drop of warm CA solution and the coagulation process was captured with a digital camera (Samsung S23 FE).

3.2.2.7 Turbidity of Mixture

The potential characterization of CA-coagulated M particles was assessed with change in the turbidity of M+CA mixtures. M+CA0.14 and M+CA2.5 mixtures were prepared by dissolving 0.084 g and 1.5 g CA, respectively, in 46 $\pm$ 2 g M. Approximately 0.5g and 1g of the mixtures were diluted with DI water in 100 mL beakers to a total weight of 50g and stirred at room temperature for 5 minutes to obtain dispersions diluted by a factor of 50x and 100x (by weight), respectively. The turbidity of the mixtures was measured in triplicates as the absorbance at 600 nm (A_{600}) in the UV-Vis Spectrophotometer (Genesys 10S, Thermo Scientific) with a 1 cm light pathlength quartz cuvette and DI water as the blank.

3.2.3 Preparation of Gels and Analysis of Gel Properties

3.2.3.1 Preparation of Gels

M prepared from soybeans was used for the preparation of gels after overnight refrigeration. Approximately 42-45 g of M, depending on the extracted O:M ratio, was transferred to 100 mL beakers (Pyrex) that was previously coated with PAM cooking oil for the preparation of M gel. The MO gel was prepared by initially making a paste of ground O with a portion of M (15-20 ml). The paste was added to the remaining M, mixed over a magnetic stirrer at 500-700 rpm for 15 minutes, and then transferred into a 100 mL beaker coated with PAM oil. The beakers were sealed with aluminum foil and placed in a water bath (Neslab RTE-111, USA) maintained at 95°C for 45 minutes to carry out thermal gelation. The resulting gels were allowed to cool down to room temperature to consolidate the gel network, and stored overnight in the refrigerator until further analysis.

Gels from frozen soybeans using fbM and fbO for **Section 3.3** were prepared similarly. Similar gel preparation methodology was used to prepare soymilk okara gels for **Section 3.4** to assess the effects on gel texture as a result of (i) O drying duration using O_{19h}, O_{24h}, OCA0.14_{19h}, OCA0.14_{24h}, (ii) O particle size using O_I, O_{II} and O_{III}, and (iii) O drying method using O_F. The gels to assess the

effect of O concentration on texture, ranging from 1.4 – 11.2% (by weight) of the gel composition, were prepared by mixing M with 25 to 200% of O used in the standard MO formulation to produce MO_{25%}, MO_{50%}, MO_{75%}, MO_{125%}, MO_{150%}, MO_{175%}, MO_{200%}.

To determine the effects of CA-induced coagulation of M, the gels for **Section 3.5** were prepared by gradually adding 0.084g, 0.75g and 1.5g of citric acid (solid) to 46.6±0.05g M to obtain soymilk citric acid mixtures (M+CA) with 0.14%, 1.25% and 2.5% (w/w) CA concentrations, respectively. The M+CA mixtures were stirred over a magnetic stirrer at room temperature for 15 minutes, transferred to 100 mL PAM-coated beakers, sealed with aluminum foil and heated at 95°C for 45 minutes in water bath. The amounts of CA were chosen such that the M+CA gels contain similar amount of CA as used in M+OCA gels to study the interaction between CA and M. Due to the absence of O that adds to the mass of M+OCA, however, the actual concentration of CA in M+CA mixtures is 0.18%, 1.61% and 3.22% (w/w).

For SEM analysis of M and MO gels carried out in **Section 3.6**, half of the M and MO mixtures were transferred into 100 ml beakers such that the gels produced were 20 cm high. Gel samples 0.8x0.8x0.2 cm gel samples were cut from the cross-section, transferred into a borosilicate vial that was previously chilled overnight in a freezer at -20°C, dipped in liquid N₂ for 5 minutes., and freeze-dried (Freeze Dryer ALPHA 1-4 LSC, Christ) for 22-23 hours (main drying stage) under a vacuum pressure of 0.940 mbar and ice condenser temperature of -61°C.

The above procedure was performed to produce gels for the macrostructure, water holding capacity, gel syneresis and protein-protein interactions analysis. Minor modifications were made in the gel preparation methodology for evaluating the texture properties. For uniaxial compression test, the mixture was divided and distributed into two PAM oil coated 100 mL such that the height of the mixture in the beakers was at 20 mm, and then the beakers were exposed to gelling conditions. Prior to measuring the mechanical properties, the gels from each beaker were cut into 3 cylinders of diameter 20 mm and thickness 10 mm with a corer. The samples were allowed to equilibrate to room temperature for 1 h prior to the analysis. For viscoelasticity measurement, the mixture was distributed into a 100 mm and a 90 mm diameter petri dish. The petri dishes were previously lined with parchment paper, coated with PAM oil and then filled with 23-25 g mixture in the larger petri dish and 20-21 g mixture in the smaller petri dish, such that a 2-3 mm thick gel

was produced. Following gelling conditions in the water bath and subsequent overnight refrigeration, the gels were cut into 4x4 mm squares, extracting at least 2 samples per petri dish for viscoelasticity analysis.

3.2.3.2 Macrostructure Analysis

The gels were cut along the cross section and images of the cross-sections were captured with a digital camera (Samsung S23 FE) taken from a consistent height of approximately 20 cm above the gel. The cross-sectional images were processed with ImageJ software to analyze porosity and pore distribution. A 30 x 30 mm region was converted into 8-bit grayscale image, auto-adjusted for brightness and contrast, and the threshold was set at 100 to analyze the macro-pore distribution, pore area coverage and pore size.

3.2.3.3 Scanning Electron Microscopy Characterization (SEM)

The okara (O, O_{Air}, O_F, OCA0.14, OCA1.25 and OCA2.5) for **Section 3.4** and gel samples (M, MO) for **Section 3.6** were loaded onto a pin stub that was covered with conductive carbon tape. The samples were sputter-coated with gold and then the surface morphology was examined with Zeiss Leo 1530 FESEM for samples O_{Air}, O and OCA2.5 and FEI Quanta FEG-250 ESEM for the all other samples at similar conditions of 20kV voltage and in high vacuum environment, at magnifications of 100x, 500x and 1000x.

3.2.3.4 Estimation of Gel Syneresis

The gels after overnight refrigeration, were observed to discharge some water. The color of liquid exuded from gels was recorded and weighed to estimate gel syneresis using **Equation 3.3**:

$$\% \text{ syneresis} = \frac{W_{\text{liquid released}}}{W_{\text{whole gel}}} \times 100 \quad \text{Equation 3.3}$$

3.2.3.5 Estimation of Water Holding Capacity (WHC)

About 5 ± 1 g of the gels were transferred into 13.2-mL thin walled polypropylene centrifuge tube (Beckman Coulter) and weighed (W_t). The gel samples were centrifuged in an ultracentrifuge (Beckman Coulter, Optima XPN100, swing bucket SW 41 Ti) at room temperature for 20 min at

a centrifugal force of 12,074 x g, corresponding to the rotor speed of 10,000 rpm. Following ultracentrifugation, the water released was obtained from the mass of the supernatant above the compressed gel mass, transferred to a scintillation vial and weighed (W_{r1}) and the remaining water sticking to the surface of the centrifuge tube wall which was absorbed using a Kimwipe and weighed (W_{r2}). The total water released (W_r) was accounted as the sum of W_{r1} and W_{r2} . The water holding capacity was calculated using **Equation 3.4**,

$$\text{WHC (\%)} = \frac{W_t - W_r}{W_t} \times 100 \quad \text{Equation 3.4}$$

3.2.3.6 Protein-Protein Interactions

Protein-protein interactions of the gel were evaluated from the protein solubilized after soaking a gel sample in solution containing reagents targeting specific interactions. Four different soaking solutions were prepared in 0.05M phosphate buffer (pH 7.0) - SA (0.05M NaCl), SB (0.6M NaCl), SC (0.6M NaCl + 1.5M urea), SD (0.6M NaCl + 8M urea). Phosphate buffer (0.05 M, pH 7.0) was prepared by dissolving 7.744 g of sodium phosphate dibasic heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) and 2.9118 g of sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) in 1000 mL DI water. The pH of the buffer was monitored with pH meter (Mettler Toledo, S47 SevenMulti Dual) when adjusting pH of the buffer to 7.0 with 1M HCl and 1M NaOH solutions.

A soy gel sample (0.6 g) was homogenized for a minute in 10mL of the different soaking solutions (SA, SB, SC and SD) with a vortex mixer and soaked overnight for at least 12 hours at room temperature. Subsequently, the soaked gels were ultracentrifuged (Beckman Coulter Ultracentrifuge Optima XPN100, USA) for 50 minutes at room temperature at a speed of 10,000 rpm. The supernatant of gels soaked in SA, SB and SC was diluted twice and diluted 10-fold for SD with phosphate buffer (pH 7.0). 10 mL of the diluted supernatant samples and 200 mL of Biorad dye reagent solution were added to a 96-wells microplate (polysyrene) in triplicates with an Eppendorf micropipette and scanned with microplate reader (Bio-tek) at an absorbance wavelength of 595 nm. The solubilized protein content was evaluated with the Bradford assay methodology adapted from [59]. A protein standard curve of Bovine serum albumin (BSA) was

constructed within a concentration range of 0.05 – 0.5 mg protein/mL solution, following which the interactions were estimated based on **Table 3.1**.

Table 3.1: Soaking solutions and their target intermolecular bond disruptions, adapted from [59]

Soaking solution	Reagents (0.05M phosphate buffer at pH 7.0)	Protein solubility difference	Target Interactions
SA	0.05M NaCl	SB-SA	Electrostatic
SB	0.6M NaCl		
SC	0.6M NaCl + 1.5M urea	SC-SB	Hydrogen
SD	0.6M NaCl + 8M urea	SD-SC	Hydrophobic

3.2.3.7 Mechanical Properties

Six replicates of cylindrical samples (20mm diameter, 10mm height) cut out from gels were subjected to uniaxial compression in a tensile tester (Shimadzu Autograph AGS-X) equipped with a 500-N load cell to gauge the gel strength. The compression was carried out at room temperature and at a rate of 40 mm/min, until the gel failed or the load plates were less than 5 mm apart.

The total stress applied on a gel sample, denoted as true stress ($\sigma(t)$), and the deformation in the dimensions of the gel sample, referred to as true strain (ϵ), were calculated with **Equation 3.5** and **Equation 3.6**.

$$\text{True stress: } \sigma(t) = \frac{F(t)}{A_0} \times \frac{l(t)}{l_0} \quad \text{Equation 3.5}$$

$$\text{True strain: } \epsilon = \ln\left(\frac{l(t)}{l_0}\right) \quad \text{Equation 3.6}$$

where l_0 represents the initial height (m), A_0 the initial gel area (m^2), and $l(t)$ (m) the diminished height of the gels recorded at time t that resulted from the applied compression force $F(t)$ (N). The true stress versus true strain curve indicated a linear region with a peak, followed by a drop in stress. The peak point was established as the failure point of the gel. The slope of the linear region

up to the failure point in the true stress-strain curve, shortened to consider only the region between one-third to two-thirds of the failure point, was used to estimate the Young's modulus of the gels.

3.2.3.8 Viscoelastic Properties

Gel samples of 4x4cm and 2-3cm thickness were equilibrated at room temperature for 1h and then the small amplitude oscillatory shear test was performed using a rheometer (Thermo Scientific, HAAKE MARS III, USA, located in E6 5007) with a parallel plate geometry of 35 mm in diameter and 1 mm measuring gap. The sample was loaded on the sample stage when a 10-mm gap space between the parallel plates was attained slowly by setting the speed of the upper plate at 1.25 mm/min, to minimize any abrupt uniaxial deformation to occur prior to the start of the test. The excess gel jutting out of the plate area was trimmed, after the gel was compressed to a gap space of 1.025 mm [0.025 mm higher than the measuring position of 1 mm]. Once the desired measuring position of 1 mm separation distance between the two parallel plates was reached, the samples were allowed to rest for an additional 3-5 minutes to attain mechanical equilibrium. The frequency sweep test was then conducted in triplicates at an angular frequency range of 1 – 100 rad/s at 0.1% strain. These parameters were chosen based on a preliminary strain sweep test that was used to determine the linear viscoelastic region (LVR) of the gels.

The dynamic dependency of the storage (G') and the loss moduli (G'') on angular frequency (ω) was represented with the power law model, **Equation 3.7** and **Equation 3.8**

$$G' = G'_0 \omega^{n'} \quad \text{Equation 3.7}$$

$$G'' = G''_0 \omega^{n''} \quad \text{Equation 3.8}$$

The parameters G'_0 (Pa) and G''_0 (Pa) are the storage and loss moduli coefficients recorded at angular frequency of 1 rad/s, respectively. G'_0 provides a measure of the rigidity of the gels and the elasticity of the gel structure while G''_0 is associated with the energy dissipated as heat and the liquid behavior of the gel. The exponents n' and n'' (both dimensionless) denote the influence of ω on each modulus and $\tan \delta$, the ratio between the loss modulus (G'') and storage modulus (G'), gives an indication of weak- or strong-gel behavior.

3.2.4 Statistical Analysis

The statistical analysis was carried out with Statistica (version 14.0.0.15, TIBCO software Inc) on WHC (n=2), viscoelasticity properties (n=3) and mechanical properties (n=6). The error bars indicate the standard deviation. The statistical difference was evaluated with one-way analysis of variance (ANOVA) and the Tukey's post-hoc test was utilized at 5% significance level to determine the significant differences between the averages (paired means).

3.3 . Freezing of Soybeans

3.3.1 Objective

The objective of this experiment was to explore the use of frozen soybeans to potentially soy milk gel texture properties. The effects of freezing soybeans was previously studied to examine the changes in heat-induced soy milk gel in the presence of CaSO_4 coagulant [107]. The results indicated faster coagulation and increased hydrophobic interactions during gelation as more proteins participated in the network. SEM depicted a more uniform, orderly microstructure and the compact gel network increased the gel hardness, chewiness and gumminess. Similar results were observed in another study where the hardness of ‘tofu’ prepared from ground whole soybeans, that were frozen for 5 or 24 hours and subsequently dried at 40°C , improved [143]. Temperatures below freezing point of water can mechanically damage soybean components during ice crystal formation when the crystals penetrate and break down the cellular structures [132]. The ice crystals force neighboring proteins closer to each other that induces protein denaturation and aggregation [132]. As a result, the surface hydrophobicity of proteins increases, making them more susceptible to heat-induced denaturation and aggregation via hydrophobic interactions [142]. Moreover, The examination of soybeans before and after freezing under TEM, illustrated in **Figure 3.3**, revealed microstructural changes from the coalescence of discrete storage protein vacuoles in cotyledon cells into large, irregular vacuoles after freezing [120].

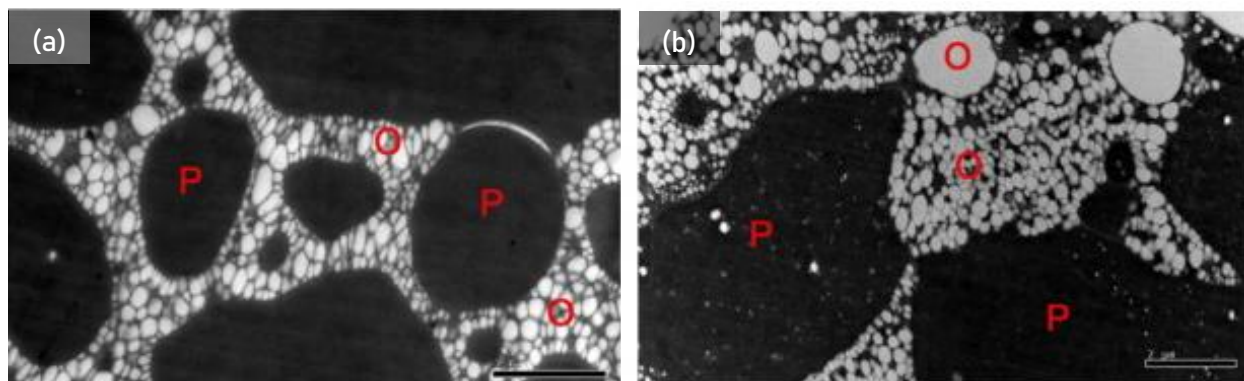


Figure 3.3: Transmission electron microscope images of (a) untreated soybean (control) and (b) soybeans frozen for 1 day where ‘P’ indicates protein storage vacuole and oil bodies are indicated by ‘O’. Reprinted from W. Lili, C. Yeming, and L. Zaigui, “The effects of freezing on soybean microstructure and qualities of soy milk,” *Journal of Food Engineering*, vol. 116, no. 1, pp. 1–6, May 2013 [120], with permission.

3.3.2 Results

3.3.2.1 Qualitative Analysis

The qualitative analysis of the soymilk and okara extracted from untreated and frozen soybeans, estimated by comparing the extracted O:M ratio, weight of dried O and solid content of M, is presented in **Table 3.2**. These estimates were similar suggesting similarity in that the soymilk okara gels prepared from untreated or frozen soybeans and that variation in texture results from difference in the denatured state of soy proteins after freeze-treatment.

Table 3.2: Ratio of soymilk to okara (w/w) extracted (n=3), weight of okara after oven drying at 60°C for 23-24 h (n=3) and the solid content of soymilk calculated with Equation 3.1 (n=2). Data is expressed as mean $\pm$ standard deviation.

	Untreated Soybeans	Frozen Soybeans
O:M ratio (w/w)	0.227 $\pm$ 0.01	0.232 $\pm$ 0.02
Weight of dried okara (g)	2.44 $\pm$ 0.11	2.40 $\pm$ 0.09
Solid content % (w/w)	14.9 $\pm$ 0.9	15.63 $\pm$ 0.5

3.3.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR absorption spectra of M, fbM, O and fbO were divided into four regions, as illustrated in **Figure 3.4**. In region I of soymilk materials, M and fbM, the broad band at 3650 – 3100 cm^{-1} is associated with the overlapped stretching vibrations of hydrogen involved in intermolecular hydrogen bonding, N-H bonds in protein [121] [162], and O-H groups in polysaccharide structures [137] [138]. This band was centered at 3273 cm^{-1} in both M and fbM and the higher intensity in fbM suggests structural changes in protein during freezing of soybeans leading to exposure of more hydroxyl groups. Region II peaks are ascribed to bending and stretching of CH_n in aliphatic chains of proteins [121] and polysaccharides [137] [138]. The symmetric and asymmetric CH_n vibrations are situated at 2923 cm^{-1} and 2854 cm^{-1} and similar in M and fbM. Region III (1800 – 1200 cm^{-1}) contains the structural information of protein and C=O bonds of ester carbonyl groups or carboxylate ions. A change in soy protein structure from freeze-treatment was indicated by the increase amide I (C=O), II (N-H) and III (N-H, C-N) bands in fbM situated at 1632, 1535 and 1236

cm⁻¹ peaks, respectively [121] [172]. While the esterified C=O bond vibration at 1743 cm⁻¹ was similar, asymmetric and symmetric vibrations of carboxylate group (COO⁻) detected at 1632 cm⁻¹ and 1394 -1388 cm⁻¹ increased in intensity in fbM. The increase in the peak intensity of OH, amide I and COO⁻ group vibrations suggests that the denaturation of proteins during soybean freezing led to exposure of these groups that were previously buried in the native protein structure. Region IV (1300 – 800 cm⁻¹) is the fingerprint region of polysaccharides and the protein-rich soymilk had a weaker spectrum in this region. The notable peaks are tensile vibration of C-O in C-O-H bend and C-O in C-O-C stretch in the skeletal structure of polysaccharide centered at 1150 and 1047 - 1038 cm⁻¹ [138] [166]. These peaks were slightly more intense in fbM indicating a possible release of soluble polysaccharides after the disruption of soybean cellular structure during freezing, and therefore higher transfer of soluble polysaccharides into soymilk during extraction.

Looking at the FTIR spectra of okara materials, the single band of hydrogen bond vibration at 3292 cm⁻¹ (region I) and the symmetric-asymmetric stretching modes of CH_n bonds situated at 2922 and 2855 cm⁻¹(region II) are less intense in fbO than O, which is possibly an effect of freezing on the structure of polysaccharide. In region III, the amide I, II and III peaks at 1628, 1537 and 1237 cm⁻¹ are similar in O and fbO which suggests that freezing did not alter okara proteins bound to polysaccharide chains. Additionally, the carboxylate ion peaks at 1628 and 1394 - 1388 cm⁻¹ for okara polysaccharides did not exhibit differences, although fbO had a lower intensity for the ester bond C=O vibration at 1742 cm⁻¹. In region IV, with the exception of the C-O in C-O-C stretch at 1047 – 1038 cm⁻¹, the vibration of C-O in C-O-H bend (1158 - 1150 cm⁻¹), β-glycosidic linkage between the sugar monomers of polysaccharides (890 cm⁻¹) [133] [168], and the other peaks in this region associated with vibrations of C-O stretch [126], out of plane C-H bend [123], ring structure deformation of CH₂ groups [116] [130] and ring compounds of aromatics [116], had a stronger intensity in fbO than O.

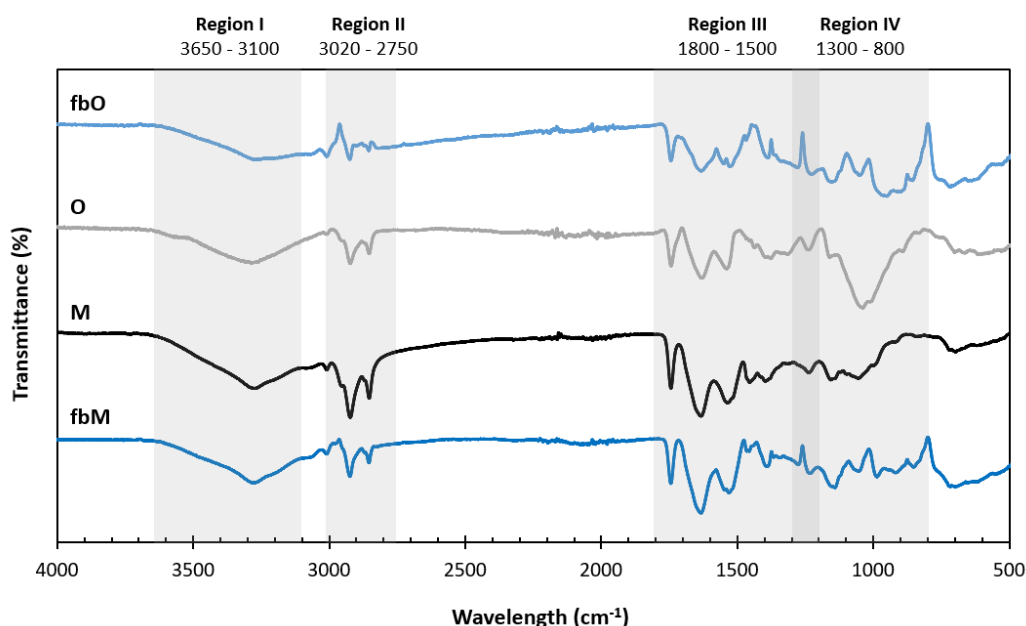


Figure 3.4: FTIR spectra of soymilk and okara from untreated (M, O) and frozen soybeans (fbM, fbO) within the range from 4000 – 500 cm^{-1} .

3.3.2.3 Macrostructure Characterization

The cross-section surface characteristics of gels and porosity detected with ImageJ are illustrated in **Figure 3.5**. M and fbM gels had a smooth surface with a few large cavities, with M gel containing fewer, larger cavities when compared to the smaller, numerous cavities in fbM. Void fraction or gel porosity of 1.01% in M and 1.31% in fbM were similar. The addition of O and fbO to soymilk made the gels heterogeneous and rigid. The pore distribution analyzed suggested an increase in porosity of MO and fbMO gels with an estimated void fraction of 3.4 and 2.8%, respectively. The macrostructure analysis of gels prepared from untreated or frozen soybeans reveals that freezing soybeans did not notably impact the gel porosity or appearance.

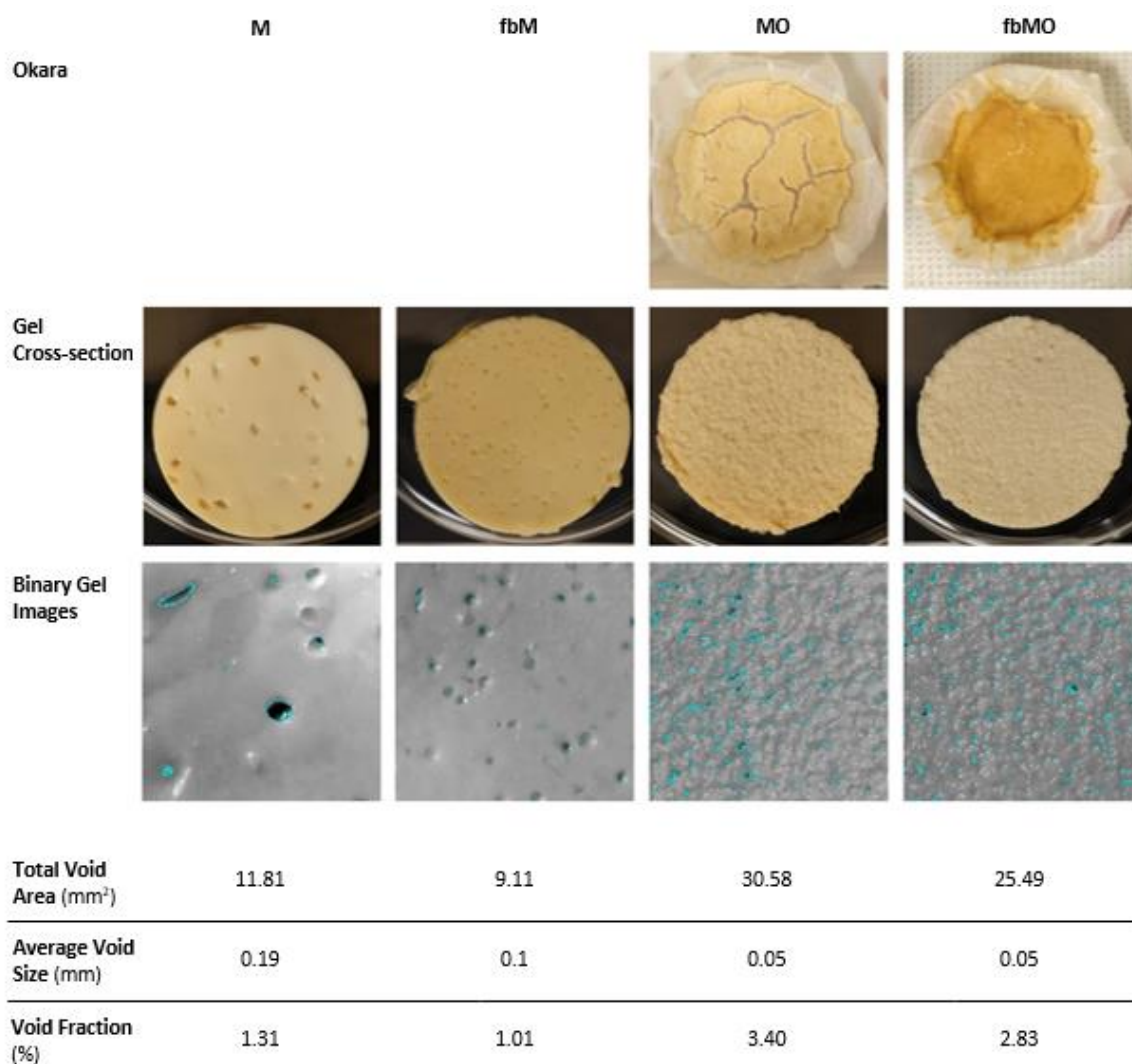


Figure 3.5: Visual observation of dried okara (O, fbO), soymilk okara gel cross section (M, fbM, MO, fbO), binary images of gels along with the void regions (blue lines) detected by ImageJ, and porosity estimates based on the binary images generated with ImageJ.

3.3.2.4 Syneresis and Water Holding Capacity

The discharged liquid after overnight refrigeration of gels represents syneresis or the unbound water contained in the gel network. Water is passively exuded during storage from the increased crosslinking between the protein aggregates of the gel structure [107] [150] [153]. Water holding capacity (WHC) is an indicator of protein-water or polysaccharide-water interaction involved in a

gel and its ability to entrap, bind and immobilize water through capillary action [83] such that water is not released even after centrifugation [140] [152] [155].

Syneresis and WHC of gels M, MO, fbM and fbMO are illustrated in **Figure 3.6**. The homogenous, compact network of the M and fbM gels exuded minimal amount of water during storage with the syneresis evaluated as 0.76% and 0.89%, respectively. The dietary fibers present in MO and fbMO effectively retained water [87] in the gel network and prevented syneresis.

The WHC of M and fbM gels, 71.5 - 78.4%, and MO and fbMO gels, 60.85 - 69.32% was statistically similar ($p > 0.05$). No statistical difference was observed in the WHC of gels, suggesting that the pores and heterogeneity introduced in presence of okara did not deteriorate the gels ability to retain water in the network. Both syneresis and WHC properties indicate that freezing soybeans did not enhance the water retention of soy gels, making the additional freezing step unnecessary from the perspective of water entrapment.

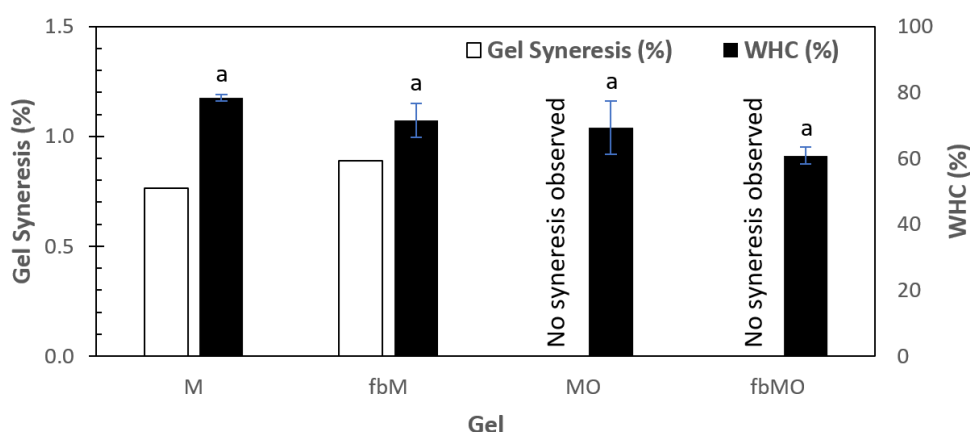


Figure 3.6: Gel syneresis and water holding capacity (WHC) of soymilk okara gels after overnight refrigeration at 4°C. Data of syneresis is a single measurement. Data of WHC is expressed as mean $\pm$ standard deviation ($n = 2$). Statistically different means (ANOVA followed by Tukey's post-hoc: $p < 0.05$) do not share a common letter.

3.3.2.5 Protein-Protein Interactions

The different protein-protein interactions in the network of M, fbM, MO and fbMO gels are illustrated in **Figure 3.7**. Hydrophobic interactions are the main contributor in assembling the soymilk gel structure [83] and is the key force responsible for an orderly, dense protein network

structure [107]. Ionic interactions and hydrogen bonding have a comparatively minor participation in the gel network as these are disrupted during heat treatment [83].

The hydrophobic interactions in the fbM gel were lower than in the M gel whereas fbMO gel had a comparable magnitude as MO gel. The denaturation of the proteins followed by their aggregation during freezing of soybeans [107], may reflect higher resistance to the solubilization of the protein aggregates in the fbM gel. On the other hand, the presence of okara disrupts the protein network in both MO and fbMO gels which hinders protein aggregation and therefore reduces the proteins participating in the network. Additionally, the coarsened texture of gels in presence of fibers has been previously attributed to an increase in protein concentrations at localized spots [140]. Fibers induce localized aggregation of proteins [104] resulting in regions of tight protein network that are difficult to solubilize, therefore reducing the solubilization of proteins.

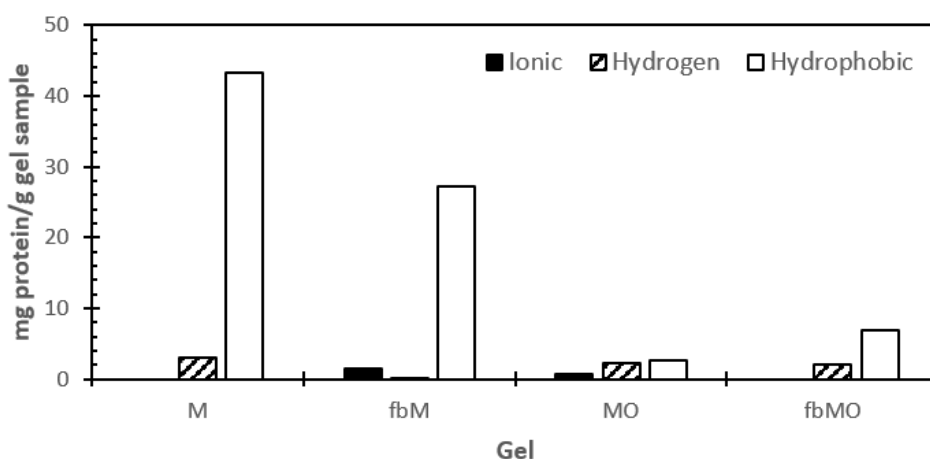


Figure 3.7: Electrostatic interactions, hydrogen bonds and hydrophobic interactions between the proteins in M, MO, fbM, and fbMO gels.

3.3.2.6 Mechanical Properties

The mechanical properties of the gels under compression are illustrated in **Figure 3.8**. The stress-strain curve is divided into three regions. Region I is associated with the linear elasticity of the gels where the applied stress caused a proportional yet recoverable deformation to the gels' height. Freezing soybeans decreased the elastic region in fbM while had no effect in fbMO gel when compared to gels from untreated soybeans. The failure region (region II) displayed a clear peak in

all gels where M and fbM had a shallow, broad peak which is characteristic of ductile materials [146] while the broad yet sharp peak of the MO and fbMO gels is characteristic of brittle materials [146]. The peak of region II was followed by flattening of the curve or constant stress in region III for all gels which is associated with the response of the deformed gel fragments.

The failure properties of the gels in region II are illustrated in **(b - d)**. Gel fbM had statistically similar failure stress and Young's modulus as M whereas the difference in failure strain indicated that fbM was less cohesive. The failure properties of the gels in the presence of okara were affected more significantly. The addition of okara to the gels, prepared from untreated or frozen soybeans, increased the gel strength (high failure stress) and stiffness ((high Young's Modulus), but at the expense of increased brittleness or reduced cohesivity (low failure strain) when compared to soymilk gels [145]. However, fbMO was weaker (lower failure stress) and had lower stiffness (lower Young's modulus) than MO. The mechanical properties indicate that freezing soybeans did not enhance the texture properties of soymilk okara gels.

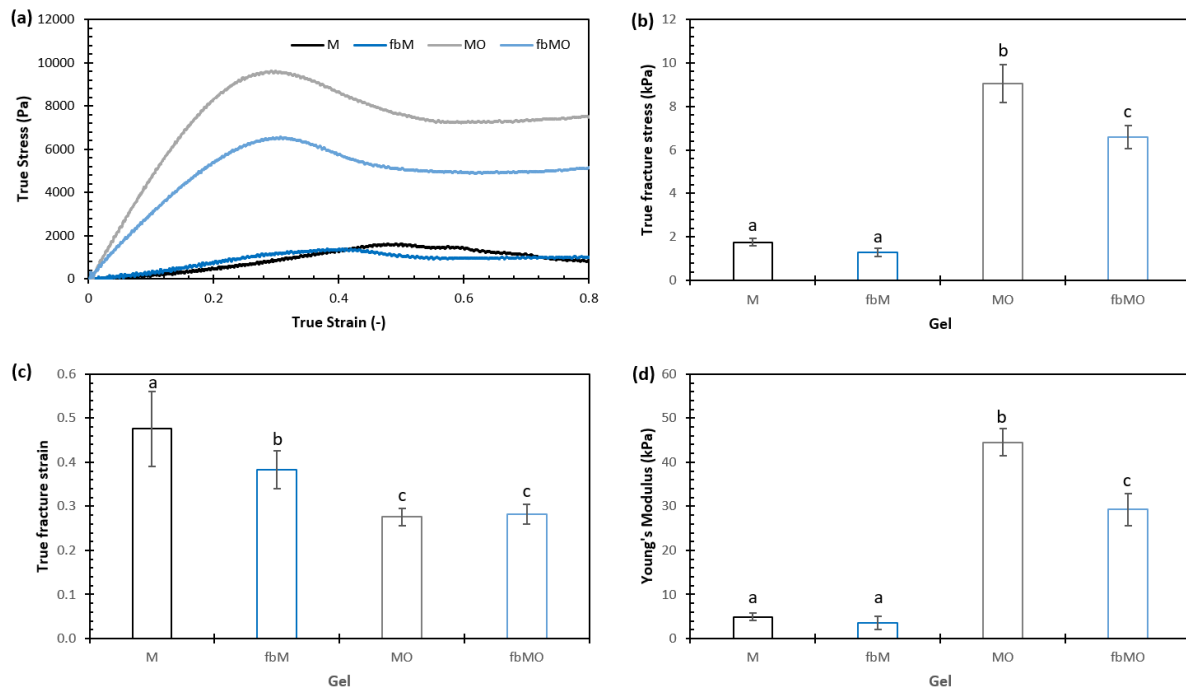


Figure 3.8: Mechanical properties of M, fbM, MO, fbMO gels: (a) Typical stress – strain curves, (b) Failure stress (kPa), (c) Failure strain, and (d) Young's modulus (kPa). Data of (b – d) is expressed as mean $\pm$ standard deviation; means that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$) ($n = 6$).

3.3.2.7 Viscoelastic Properties

The viscoelastic properties of the M, fbM, MO and fbMO gels were measured after overnight cooling of the gels as illustrated in **Figure 3.9**. All gels displayed a consistently higher storage modulus, G' , than loss modulus, G'' , which is associated with an elastic gel behavior wherein the gel disposition is more solid-like than liquid-like [155] [187] [88]. There was no crossover of G' and G'' which is characteristic of a continuous network in the gel structure [189]. The loss tangent, $\tan(\delta)$, which is the ratio of loss modulus to storage modulus, is another measure reflecting the viscoelastic property of gels [153] [180]. A value of $\tan(\delta) < 1$ was observed for all gels (**Figure 3.9 (b)**) and is indicative of solid behavior as elasticity was prominent over the viscous attribute of gels [190]. The progress of G' and G'' over frequency reflects a weak dependence of G' and G'' on angular frequency with estimates of n' within the range of 0.10 – 0.14 and between 0.08 – 0.18 for n'' (**Table 3.3**). An estimate of n' , $n'' > 0$ is a characteristic behavior of weak gels wherein non-covalent bonds are predominantly responsible in assembling the physical gel network [155] [97] [144].

The viscoelastic parameters, storage moduli (G_o') and loss moduli (G_o'') at the frequency of 1 rad/s, are presented in **Table 3.3**. The M and fbM gels had statistically similar G_o' and G_o'' . On the other hand, MO and fbMO gels displayed statistically similar viscous response (G_o'') while the elastic component (G_o') of the MO gel was significantly higher. The presence of okara in the soymilk gels significantly increased the viscoelasticity (G_o' , G_o'') of gels, which was reported previously [191]. However, the viscoelastic properties of the soymilk okara gels indicate that freeze-treatment of the soybeans did not enhance the texture.

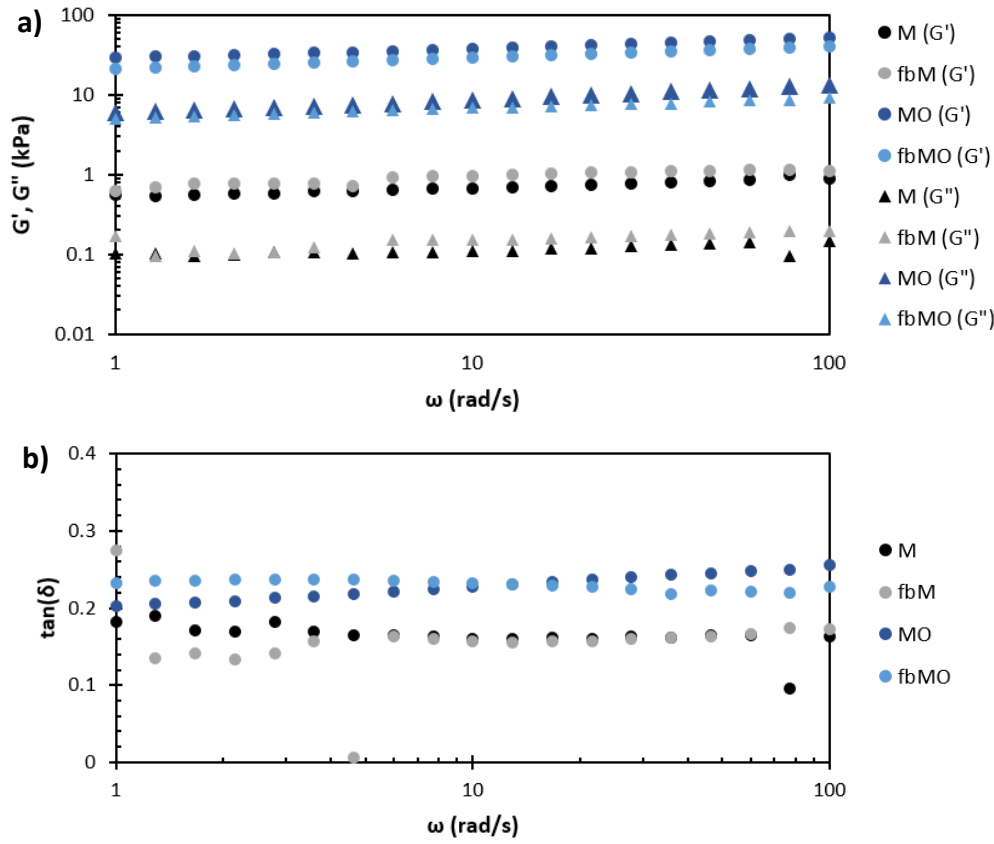


Figure 3.9: Viscoelastic properties of M, fbM, MO, fbMO gels: Typical curves of a) storage modulus G' (circle) and loss modulus G'' (triangle), and b) $\tan \delta$ over angular frequency (ω).

Table 3.3: Power law parameters of the viscoelastic properties of M, fbM, MO, fbMO gels (Equation 3.7 and Equation 3.8). Data expressed as mean $\pm$ standard deviation ($n = 3$); means within a column that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$).

Gel	G'_0 (kPa)	n'	r_1^2	G''_0 (kPa)	n''	r_2^2
M	0.51 ± 0.03^a	0.09 ± 0.04^a	0.60	0.08 ± 0.02^a	0.12 ± 0.08^a	0.44
fbM	0.77 ± 0.16^a	0.12 ± 0.02^a	0.87	0.12 ± 0.04^a	0.13 ± 0.07^a	0.38
MO	32.61 ± 6.95^b	0.13 ± 0.01^a	1.00	6.67 ± 1.52^b	0.18 ± 0.01^a	1.00
fbMO	21.32 ± 2.50^c	0.14 ± 0.01^a	1.00	5.11 ± 0.51^b	0.12 ± 0.01^a	1.00

3.3.3 Conclusion

The objective of this experiment was to examine protein denaturation by freezing soybeans as a potential pathway to enhance gel texture. The qualitative analysis of soymilk (M and fbM) based on solid content did not reflect differences, however, dried okara (O and fbO) had distinct textures where O dried as a crisp solid while fbO had a film-like texture. FTIR absorption spectra of these components (M, O, fbM, fbO) reflected changes in structure as the peak of hydrogen bonding, C-H bonds, carboxylate ion and polysaccharide fingerprint region vibrations varied in intensity. However those changes were not beneficial in enhancing the mechanical or viscoelastic properties of soymilk and soymilk okara gels and therefore, freezing of soybeans was not explored further.

3.4 Influence of Okara Particle Size, Drying Method and Concentration on Soy Gel Texture

3.4.1 Objective

The size of ground okara (O) introduced in soymilk (M) gels has been previously demonstrated to influence gel texture [139]. The incorporation of ground O, ranging from 150–380 μm and less than 75 μm in size, in GDL-induced M gels revealed that large particles (250–380 μm) disrupted the protein network and produced weaker gels. In contrast, smaller size O is effectively entrapped in the protein network. Furthermore, small size O comprised of a higher soluble fiber content increases the viscosity of the mixture, slows down the aggregation process of proteins and the gelation rate, thus facilitating the assembly of a gel network with enhanced WHC and elasticity from the increased participation of proteins [139]. Based on these observations, the objectives of this set of experiments were to analyze the effect of the size distribution of O by fractionation and change in drying duration of O on the porosity and texture of soymilk okara gels, assessed qualitatively. The influence of drying methods, air-drying, oven-drying and freeze-drying, on O was examined by scanning electron microscopy (SEM) and from qualitative observations of the gel. SEM was also adopted to analyze the effect of citric acid (CA) on structural changes O at 0.14%, 1.25% and 2.5% (w/w) CA concentration. Finally, the suitable concentration of O that produced stronger M gels with high dietary fiber content but without notably deteriorating texture was determined qualitatively.

3.4.2 Results and Discussion

3.4.2.1 Effect of Okara Size on Gel Porosity

The size distribution of the different fractions of okara obtained by sieving is illustrated in **Figure 3.10**. A decrease in the size made ground O appear lighter in hue (**Figure S.2**), which was observed in a previous study where color changes were ascribed to the ‘micronization’ of the particles [139]. The two main fractions of okara particles were 75 – 106 μm and <45 μm which represent 25.3% and 18.5% (by weight) of the initial okara, respectively. The other sizes were present in smaller quantities of 10% or lesser. Approximately 11.7% of the initial okara was lost as a result of manual

sieving. The effect of okara size on gel texture was assessed by combining the okara fractions into three groups, comprising of 29 ± 4 % each of the initial okara, to prepare MO_I, MO_{II} and MO_{III} gels.

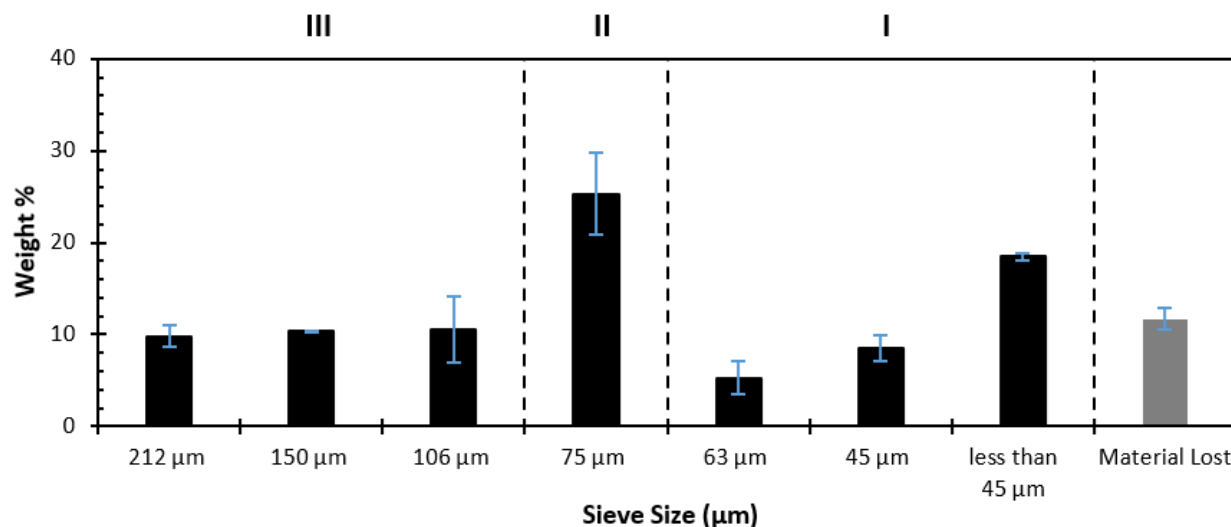


Figure 3.10: Particle size distribution of okara based on weight % collected on sieves with decreasing pore size. The particle size is classified into three categories where category I comprises of particles more than 106 μm, category II of particles between 75 to 106 μm, and category of particles less than 45 μm. Data is presented as mean $\pm$ standard deviation (n=2).

The soymilk okara gels prepared from three different okara fractions are depicted in **Figure 3.11**, with MO gel as the reference for comparison. The gel rigidity and strength, assessed by finger touch, were similar across all gels. Difference in the pore distribution at the gel cross-section was observed. MO_I gel containing okara less than 75 μm appeared visually similar to MO, both smooth with spherical-shaped pores uniformly distributed throughout the gel volume. The gel MO_{II} and MO_{III}, containing okara fractions 75 - 106 μm and >106 μm, respectively, had increasingly rougher internal cross section, higher porosity and grainier appearance when compared to MO which indicated that the bigger size of these fractions in the gel significantly disrupted the protein network. The increases in porosity was supported from the image analysis by ImageJ. The MO and MO_I gels contained similar void fraction of 2.62% and 2.95%, respectively. On the other hand, MO_{II} and MO_{III} gels displayed a higher porosity of 5.87% and 4.54%, respectively. The reference MO gel, containing non-fractionated okara, had uniformly distributed pores and smooth appearance, the additional step of filtering okara to be of a specific size range (<75 μm) was not necessary, and therefore, not considered further.

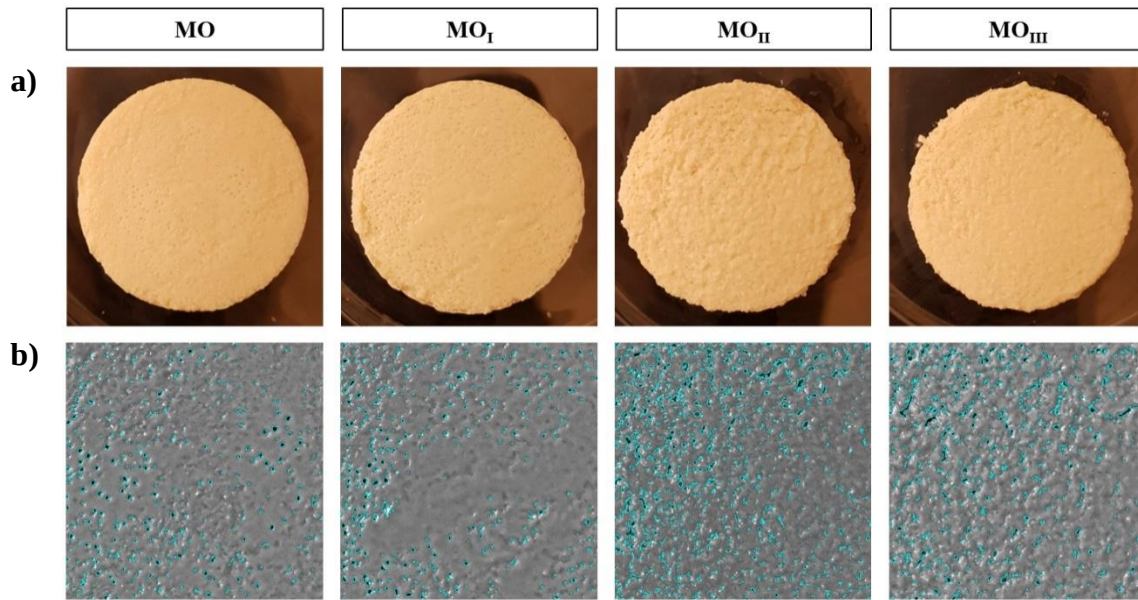


Figure 3.11: (a) Visual observation of soymilk okara gels prepared with O (MO) and okara fraction <75 μm (MO_I), between 75 - 106 μm (MO_{II}) and >106 μm (MO_{III}); (b) Binary images of gels with pores detected (blue lines) generated by ImageJ.

Table 3.4: Porosity estimates of the internal cross-section of soymilk okara gels, containing okara with different size ranges, based on the binary images generated with ImageJ ($n = 1$).

	MO	MO _I	MO _{II}	MO _{III}
Total Void Area (mm²)	23.64	26.65	52.96	40.97
Average Void Size (mm)	0.04	0.04	0.03	0.04
Void Fraction (%)	2.62	2.95	5.87	4.54

3.4.2.2 Effect of Different Okara Drying Durations on Gel Texture

Drying duration influences the hardness of dried O and its subsequent grinding that affects its size. Two different drying durations, 19h and 24h, were considered to assess their influence on the grinding of O, resulting size distribution of O and mechanical properties of soymilk okara gels, as illustrated in **Figure 3.12**. Dried O_{19h} had a uniform yellow surface whereas O_{24h} developed dark, burnt patches from the prolonged drying and dried into a harder material. The ground O_{24h} contained a higher proportion of coarse particles than O_{19h}. The coarse fragments were manually

sifted from the fine powder, and were comparatively larger in size than the coarse particles sifted from O_{19h} . $OCA0.14$ dried for 19h and 24h were observed to be similar in color (**Figure 3.12**) and appeared to have similar proportions of coarse and fine particles.

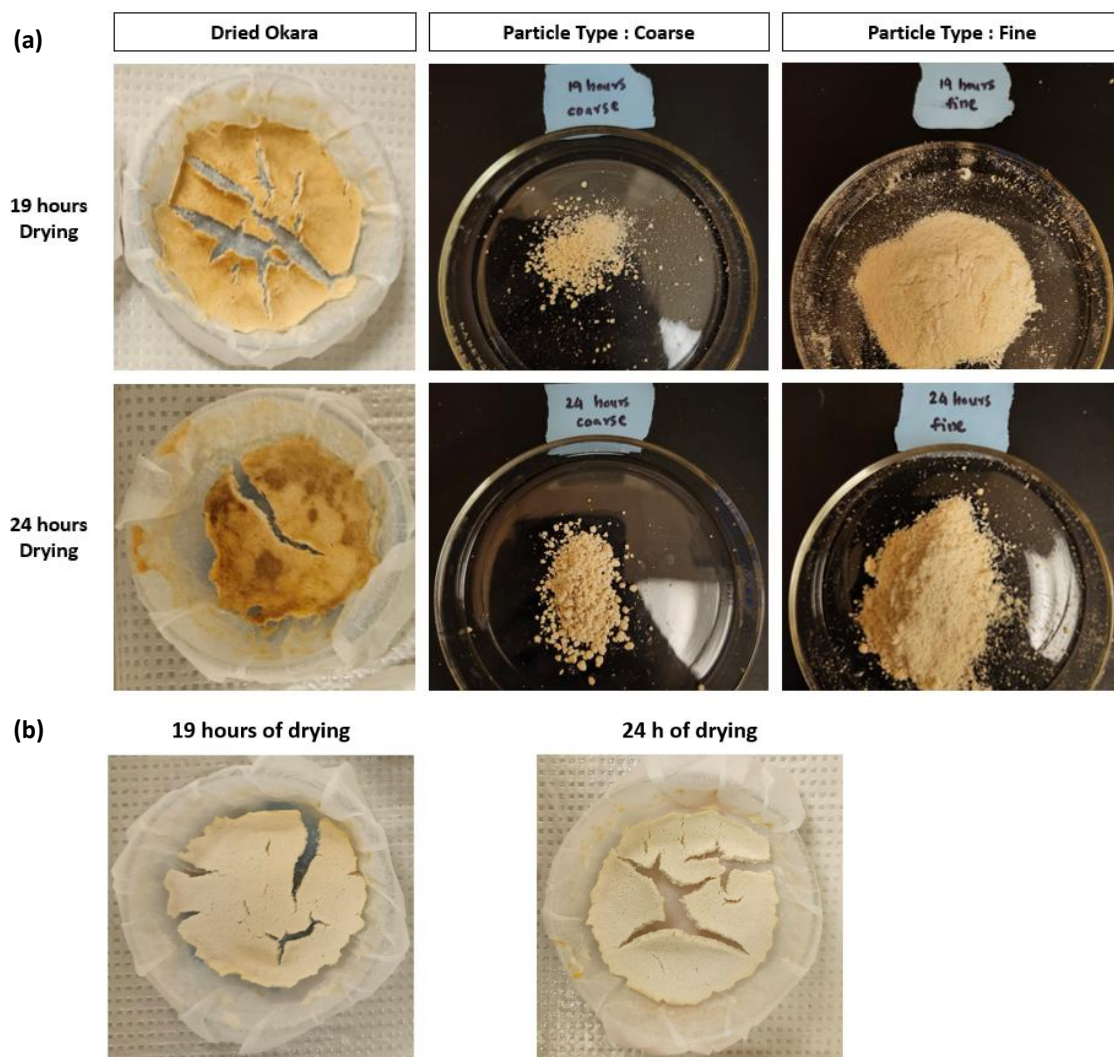
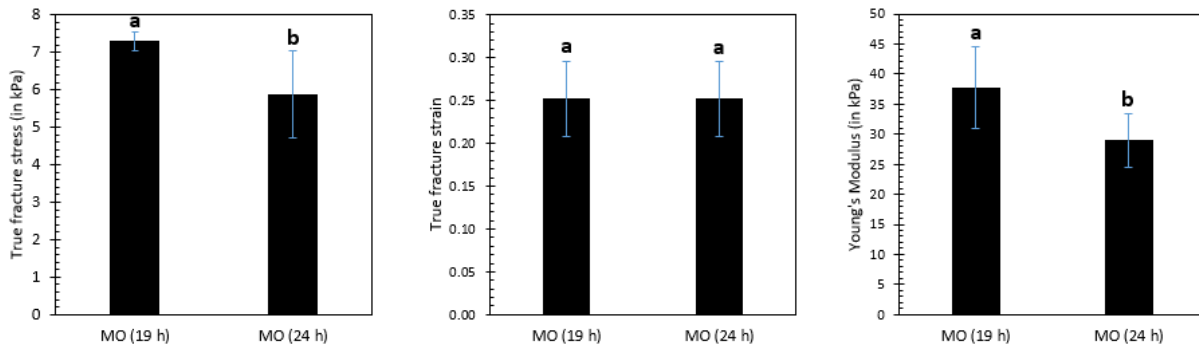


Figure 3.12: Visual observation after 19 h and 24 h drying of (a) O, and coarse and fine fractions of the ground O obtained through manual separation (without sieve); (b) $OCA0.14$

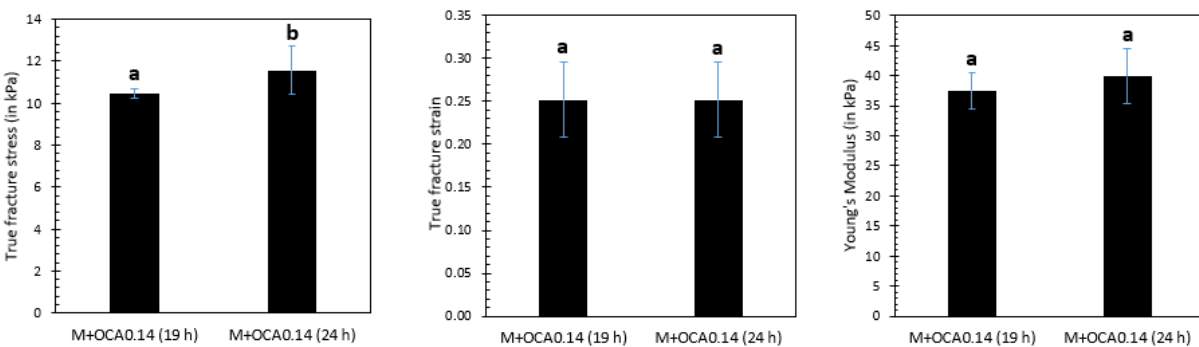
The mechanical properties of the soymilk okara gels produced using okara dried for 19 and 24 h are presented in **Figure 3.13**. The statistically higher failure stress and Young's modulus of the MO_{19h} gel relates to higher gel strength and stiffness than the MO_{24h} gel. The smaller standard deviation and similar profile of the stress-strain curves for MO_{19h} gel suggests it was more

homogenous than MO_{24h} gel. The OCA0.14 produced more homogeneous gels for okara dried for 19h (M+OCA0.14_{19h}) but had statistically lower gel strength ($p < 0.05$) compared to M+OCA0.14_{24h} gel. The cohesiveness (failure strain) and stiffness (Young's modulus) of M+OCA0.14 soymilk okara gels, however, were statistically similar ($p > 0.05$).

a) MO gels with O dried for 19 or 24 h



b) M+OCA0.14 gels with OCA0.14 dried for 19 or 24 h



c) Stress-strain curve of MO and M+OCA0.14 gels

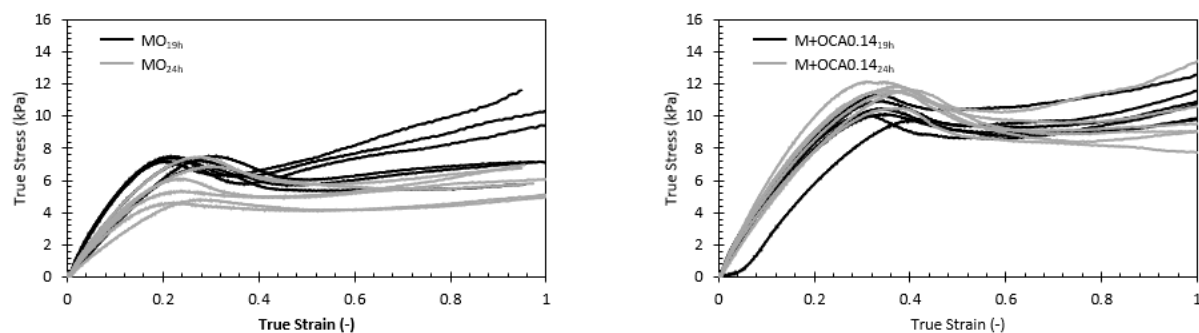


Figure 3.13: Failure stress (kPa), failure strain and Young's modulus of (a) MO and (b) M+OCA0.14 soymilk okara gels with okara dried for 19 or 24 h, and (c) stress-strain curves of gels. Data is presented as mean $\pm$ standard deviation. Means that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$) ($n = 6$).

3.4.2.3 Effect of Different Drying Methods on Okara Morphology

The microstructure of ground okara obtained after air drying (O_{Air}), oven drying (O) and freeze drying (O_F) captured with SEM, is displayed in **Figure 3.14**. O had a wrinkled surface comprised of densely stacked flaky layers (lamellar structure) and deformed honeycomb-like structures of the parenchyma layer present in soybean. Grinding of O disintegrated the soybean cell layers and deformed the structure. O_{Air} had dense, layered structure as O but also contained regions of orderly intact honeycomb-like structure, unlike the deformed structures in O which suggests that heating affects okara. O_F preparation did not involve mechanical processing such as grinding, therefore, much of orderly honeycomb-like structures of the parenchyma layer remained intact indicating that freeze-drying was the least destructive drying method.

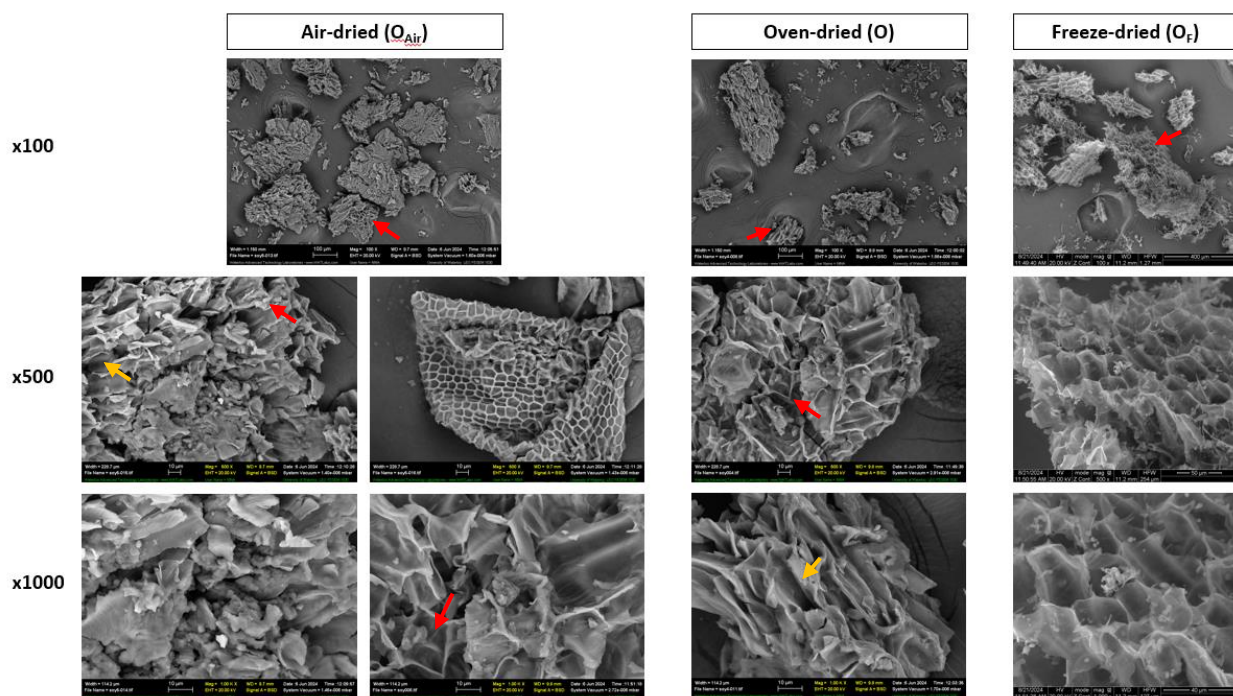


Figure 3.14: SEM micrographs of okara prepared with different drying methodology – air-dried, oven dried at 60°C and freeze-dried, visualized at magnifications of x100, x500 and x1000. The ↗ arrow indicates regions of honeycomb-like structure of soybean seed parenchyma layer while the deformed, stacked layer structure is represented by ↘ arrow.

3.4.2.4 FTIR Characterization of Freeze-Dried Okara

The FTIR absorption spectra of O_F was compared with O as illustrated in **Figure 3.15**. A broad band centered at 3293 cm^{-1} (region I), associated with the overlapped stretching vibrations of

protein N-H groups hydrogen bonding of O-H groups [137] [138], was more intense in O than O_F. A possible reason could be that the structural changes in O caused during heating exposed the buried OH groups. Additionally, oven drying is less effective in removing water than freeze-drying due to which some water could be retained in O, that contributed to the higher OH group detected. The effective removal of water through freeze-drying suggests that the hydroxyl groups of fibers were available for interaction. The peaks in region II of CH_n stretching symmetric vibration at 2922 cm⁻¹ [137] [138] in both O and O_F and the asymmetric vibration that shifted from 2853 cm⁻¹ in O to 2879 cm⁻¹ in O_F were less intense in O_F. The exposure of O to oxygen at high temperature can induce structural changes from non-enzymatic browning or oxidative thermal degradation. A similar observation of darkened color was reported for fermented okara dried in hot air oven (60°C) or microwave vacuum dryer (60°C, 10kPa) when compared to the light color of freeze-dried (-36°C, 40Pa) okara [105]. The ochre O and white O_F hue observed supports this observation. The amide I, II and III peaks centered at 1630, 1535 and 1236 cm⁻¹ [121] [172], respectively, and esterified (C=O) carboxyl group vibration at 1743 cm⁻¹ became less intense in O_F while the peak of carboxylate ion stretching (COO⁻) at 1386 - 1376 cm⁻¹ was similar in the okara materials [138] [171]. Significant differences were observed in the peak intensity of O and O_F in the fingerprint region of polysaccharides (region IV, 1300 – 800 cm⁻¹) associated with the vibrations of C-O in C-O-H bend (1159 - 1153 cm⁻¹), C-O-C stretch (1048 – 1038 cm⁻¹) [138] [166], and β-glycosidic link between the sugar monomers (890 cm⁻¹) [111] [137], suggesting that heat during oven-drying induces structural changes in O.

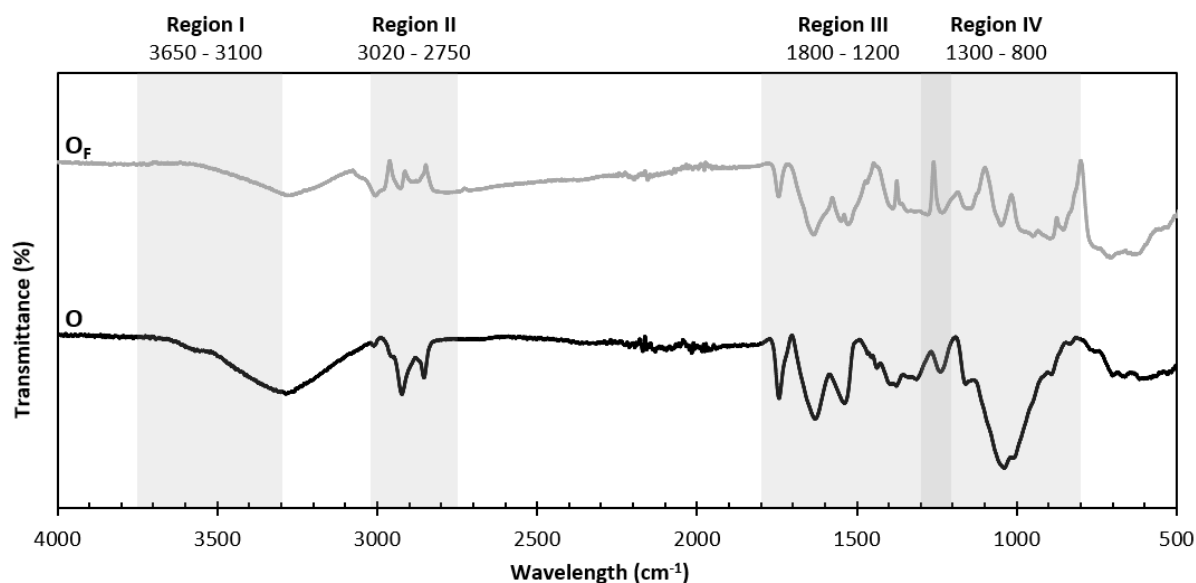


Figure 3.15: FTIR spectra of O and O_F in the wavelength range of 4000 – 500 cm⁻¹.

3.4.2.5 Effect of Okara Drying on Macrostructure of Soymilk Okara Gels

Okara is generally dried to reduce its moisture content and prevent spoilage. The method used can affect the functional properties and quality of okara [109] [110]. Freeze drying produces okara with optimal water holding capacity, oil binding capacity and swelling capacity, when compared to hot air or vacuum drying [109]. These properties are important considerations when incorporating okara in soymilk gels affecting the compatibility of okara with the protein network. Moreover, tofu containing freeze-dried okara was reported to be of superior sensory quality compared to tofu with oven dried or microwave dried okara [108]. Therefore, the purpose of this study was to investigate the influence of oven drying and freeze drying on okara and the soymilk okara gels.

Dried okara produced by oven drying and freeze drying, had different color, as presented in **Figure 3.16**. The oven dried okara (O) had an ochre hue while the freeze-dried okara (O_F) was white and less dense than O as it was perceived to occupy a larger volume despite their similar mass (approximately 2.4g). The color of okara was reflected in the color of soymilk okara gels such that MO_F gel exhibited a lighter color than MO. While the porosity of the gels was similar (**Table 3.5**), MO_F gel was perceived to be slightly firmer than MO gel upon finger compression. While the

lighter color of MO_F gel is attractive, the higher energy and low processing capacity of freeze drying equipment limits its use for tofu products.

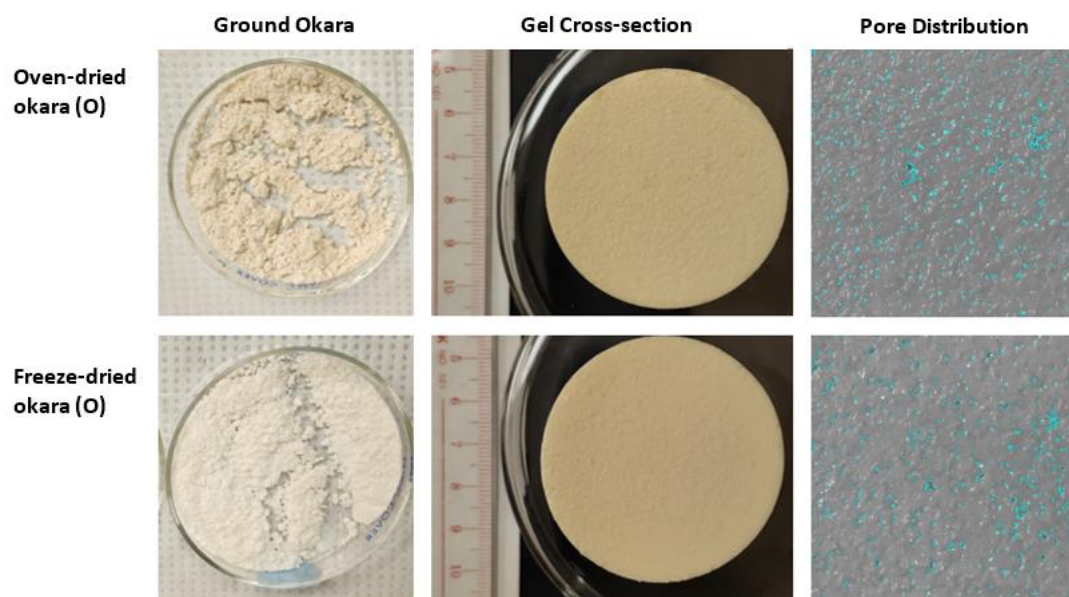


Figure 3.16: Visual observation of okara obtained after (a) oven-drying (O) and (b) freeze-drying (O_F) along with the cross-section of soymilk okara gels (MO, MO_F) and their porosity (blue lines) detected by ImageJ.

Table 3.5: Porosity of MO and MO_F gels based on the binary images generated with ImageJ

Gels	Total Area (mm ²)	Average Pore Size (mm)	Void Fraction (%)
MO	21.731	0.016	2.408
MO _F	21.508	0.017	2.384

3.4.2.6 Citric Acid-Modified Okara Morphology

The surface morphology of dried, ground O, OCA0.14, OCA1.25 and OCA2.5, captured with SEM, are displayed in **Figure 3.17**. The untreated okara, O, had a wrinkled surface comprised of densely stacked flaky layers (lamellar structure) and deformed honeycomb-like structures of the soybean parenchyma layer. Oven drying followed by grinding of okara disintegrated the soybean cell layers and deformed the structure. The CA0.14 treatment of okara did not significantly alter its structure. The irregular honeycomb-like forms of okara persisted but were less densely packed

and more dispersed than in O (magnifications 500x and 1000x) suggesting opening of the structure. Some parenchyma layer structures were detected in OCA1.25 but were destroyed in OCA2.5 suggesting that the flaky layered structure of O collapsed, transforming into a smooth surface of OCA1.25 and OCA2.5, and degraded into small particles that speckled the surface (magnifications 500x, 1000x). Furthermore, the gumminess of OCA2.5 observed visually suggests that the breakdown of okara cellular structure possibly facilitated the release of pectic substances onto the surface. These observations indicate that the CA treatment was different according to concentration. At 0.14% CA, there was little effect on okara with minimal loosening of its structure while at 2.5% CA okara was significantly degraded.

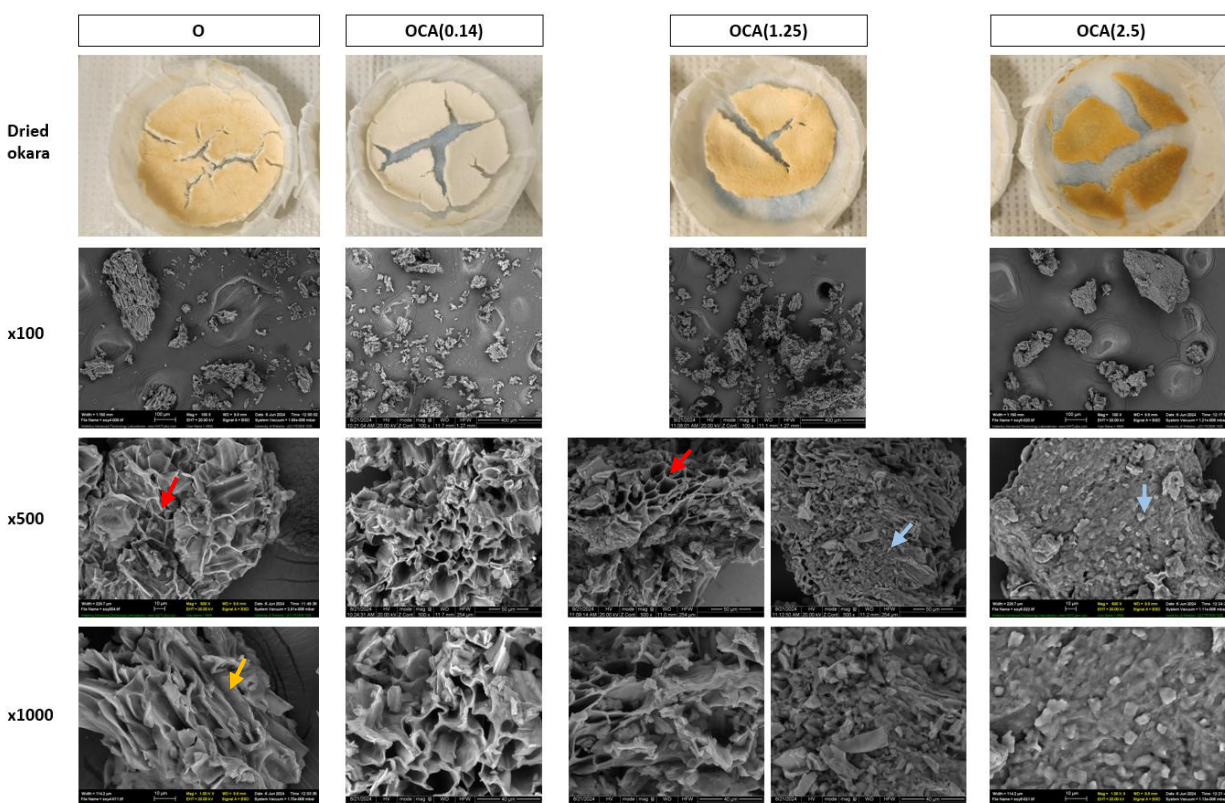


Figure 3.17: SEM micrographs of untreated (O) and citric acid modified okara (OCA0.14, OCA1.25, OCA2.5), obtained after drying in forced air oven at 60°C, visualized at magnifications of x100, x500 and x1000. ↗ indicates regions of honeycomb-like structure of parenchyma layer. The deformed, stacked layer structure is represented by ↗ and ↗ arrow signals smooth surface resulting from the release of pectin.

3.4.2.7 Effect of Okara Concentration on Soymilk Okara Gel

An increase in O concentration in soy gels has been previously reported produce gels with higher solid content and WHC which enhanced the gel yield, hardness and elasticity (G') [85]. Polysaccharides can improve gel texture and WHC, despite their non-gelling character, by increasing viscosity of the system that facilitates gelation [140], and entangle in the protein network that improves the viscoelasticity of gel network [141]. In this study, an increase in okara concentration consistently enhanced the firmness or rigidity of MO gels that was perceived from the resistance experienced while cutting along the gel cross-section, along with increase in yellowness and dryness.

The MO gels with 0 – 11.2% (w/w) O concentration were organized into three groups, as illustrated in **Figure 3.18**, based on their heterogeneous, porous appearance noted qualitatively. The effects of okara concentration on the surface characteristics and the pore distribution at the cross-section of soymilk okara gels are presented in **Table 3.6**. The increase in average pore size and pore area in the gels with increasing okara concentration supports the visual observation of increased porosity and roughness of the gels. Group I gels, M, MO_{25%} and MO_{50%} with the okara content ranging from 0 to 2.8% (w/w) had a homogenous, smooth surface with soft texture, silky appearance and a few small pores that increased in count with increasing okara content. These gels, along with MO_{75%}, discharged some water during overnight storage that was recorded as syneresis (**Figure 3.18-d**). Group II gels, MO_{75%}, MO, MO_{125%} and MO_{150%} contained 4.21 – 7.95% (w/w) O and includes the reference gel MO (5.59% O). These gels were firm with numerous pores, supported by the increased porosity estimated by ImageJ. Moreover, except MO_{75%} gel, these gels did not exude water after storage suggesting that water was effectively absorbed by okara fibers [154] and entrapped in the porous network of the gels. Additionally, the wet surface of the gels in groups I and II reflecting light indicated the presence of free water. Group III gels, MO_{175%} and MO_{200%}, (9.81-11.2% w/w O) had a dry, crumbly texture with a more porous cross-section than MO gel. These gels did not discharge water which is ascribed to the high fiber content and the inherent water-absorbing character of okara fibers [154]. Unlike the group I and II gels, group III gels did not have a shining wet surface.

The results indicate that the concentration of okara can be utilized as a factor to modulate the strength and rigidity of soymilk okara gels. While low O content (less than 4.21%) produces gels of similar texture as M gel and high O content (more than 7.95%) makes gels unpleasantly rough, the MO gels with 4.21 – 7.95% O concentration appear to possess a satisfactory texture. Moreover, this range includes the MO gel prepared according to the standard procedure adopted from [59].

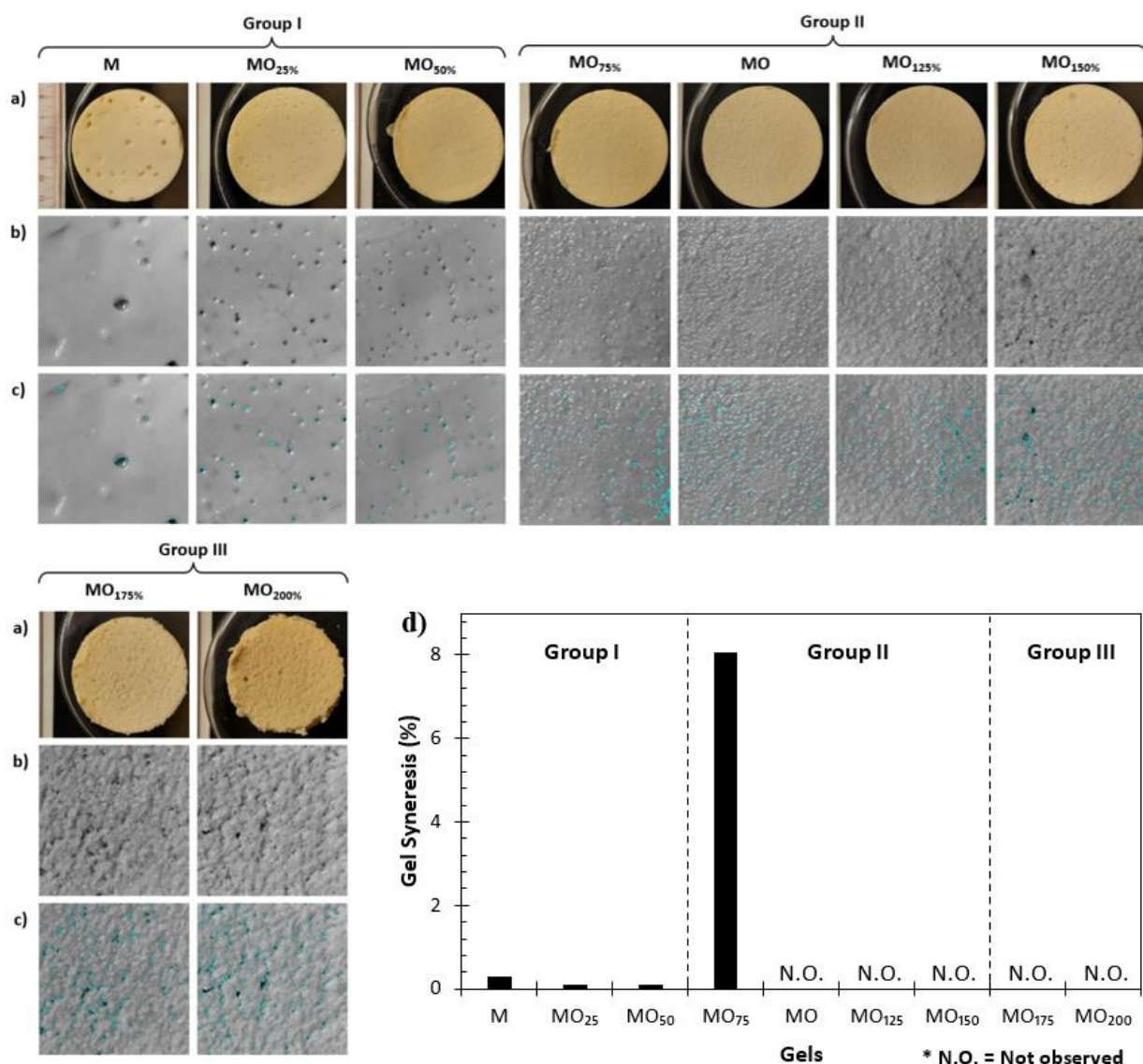


Figure 3.18: (a) Visual observation of soymilk okara gel cross-section categorized into three groups based on qualitative analysis of heterogeneity. Group I (M, MO_{25%}, MO_{50%}) has 0 - 2.8% okara content, Group II (MO_{75%}, MO, MO_{125%}, MO_{150%}) contains 4.21 - 7.95% okara, and Group III (MO_{175%}, MO_{200%}) has 9.81 - 11.2% of gel weight as okara (on weight basis); (b) Binary images generated by ImageJ of 30x30 mm gel section; (c) Porous regions (blue lines) detected by ImageJ; (d) Syneresis of the soymilk okara gels recorded after overnight storage at 4°C.

Table 3.6: Void area estimates of soymilk okara gels, with okara concentration (C_o) ranging from 0 - 11.2% (w/w), based on the binary images generated with ImageJ.

Group	Gel	C_o (w/w %)	Total Void Area (mm ²)	Average Void Size (mm)	Void Fraction (%)
I	M	0.00	5.62	0.28	0.62
	MO _{25%}	1.40	10.05	0.12	1.12
	MO _{50%}	2.80	8.74	0.08	0.97
	Average		9.36 ± 0.92	0.16 ± 0.11	0.90 ± 0.25
II	MO _{75%}	4.21	14.09	0.02	1.57
	MO	5.59	10.95	0.01	1.22
	MO _{125%}	6.99	20.12	0.03	2.23
	MO _{150%}	7.95	24.93	0.04	2.76
	Average		17.52 ± 6.23	0.03 ± 0.01	1.94 ± 0.69
III	MO _{175%}	9.81	36.39	0.08	4.04
	MO _{200%}	11.20	46.01	0.08	5.11
	Average		41.20 ± 6.80	0.08 ± 0.00	4.58 ± 0.76

3.4.3 Conclusion

The size of okara affects its distribution in the gel network and the porosity of gels. Incorporation of large O particles (>75 µm) made MO gel rougher, more porous and heterogeneous while fine particles (<75 µm) did not alter gel appearance when compared to MO gel containing unfractionated O indicating fractionation of okara was not necessary. The drying duration of okara changes its size distribution after grinding and therefore affected the characteristics of MO gels. A drying duration of 19h was found to be favorable for producing a more homogenous MO gel with higher gel strength due to a higher proportion of fine particles. Freeze-dried O was perceived to produce a lighter hued and firmer gel, however, high energy requirements and low processing capacity of freeze-drier limits its use. CA-modification of O from SEM micrographs revealed loosening of the stacked layer structure of okara at CA0.14 which degraded into smooth structures on increasing the concentration to CA1.25 and CA2.5. Finally, an increase in O concentration made MO gels firmer, with 4.21 - 7.95% (w/w) O that includes the reference MO gel formulation perceived to have an appropriate balance of rigidity, porosity and dryness.

3.5 Influence of Citric Acid Concentration on Soymilk Protein and Gel Texture

3.5.1 Objective

The objective of this experiment was to investigate the influence of citric acid on the coagulation of soymilk proteins at three citric acid concentrations, 2.5% (w/w) adapted from [59], 1.25% (w/w), and 0.14% (w/w) as per [83] that demonstrated superior properties of ‘tofu’ prepared within the concentration range 0.12 to 0.18% (w/v). The mechanical and viscoelastic properties of the gels were evaluated, and the effect of CA on the transition of soymilk protein from native to denatured or aggregated state was examined using turbidity.

3.5.2 Results

3.5.2.1 Citric Acid Induced Coagulation of Soymilk

The effect of citric acid on soymilk was assessed with the droplet test, developed in [147], and is illustrated in **Figure 3.19**. The coagulation behavior of soymilk was categorized into three groups according to CA concentration. In category I, 0.14% - 0.168% (w/w) CA, M was dispersed in CA droplet, appearing as a turbid white solution with few specks of aggregates. Category II concentration, 0.376% - 0.75% (w/w) CA, lead to a coagulated mass surrounded by a turbid solution, which suggests that a greater portion of M proteins coagulated while the remainder were dispersed as soluble proteins. In category III, 1.5% - 5% (w/w) CA, a spherical mass was observed to be surrounded by white radial streaks. The behavior of soymilk according to CA concentration indicates differences in the aggregation of M proteins and suggests differences in the assembly of the gel network and therefore, variation in gel texture.

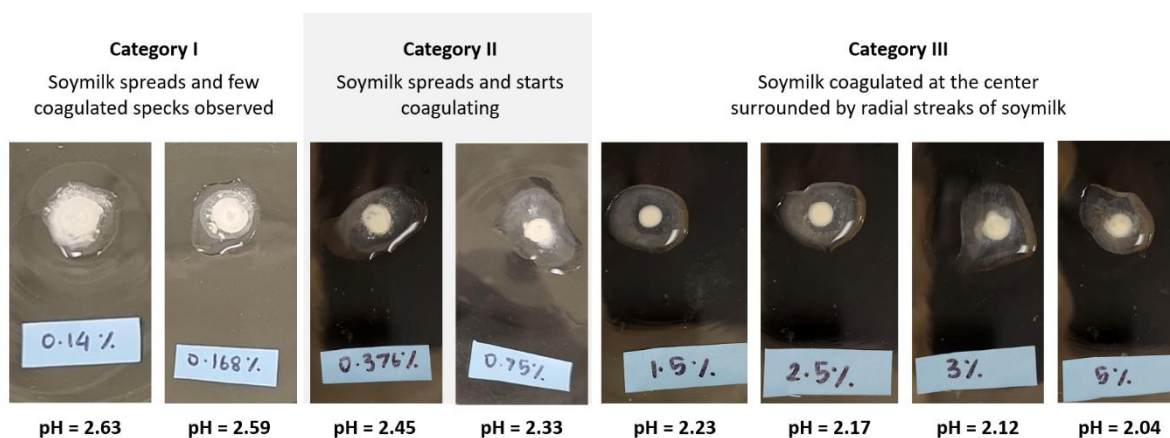


Figure 3.19: The coagulating behavior of a drop of soymilk in a drop of heated citric acid solution, categorized into three groups based on the different behaviors of soymilk behavior at 0.14% - 0.168% (w/w) (Category I), 0.376% - 0.75% (w/w) (Category II), and 1.5% - 5% (w/w) (Category III) citric acid concentration.

3.5.2.2 Soymilk Gels with Citric Acid

The porosity of the soymilk gels prepared at three different CA concentrations, 0.14%, 1.25% and 2.5% (w/w), was examined from the gel cross-section as illustrated in **Figure 3.20** and **Table 3.7**. The M+CA0.14 and M+CA2.5 gels were smooth and cohesive with spherical voids while the M+CA1.25 gel was characterized by a rough and porous surface which is supported by the higher porosity estimates (**Table 3.7**). The pH of the M+CA1.25 mixture, before thermal gelation, was measured to be 3.90 which was closest to the isoelectric point (IEP) pH 4.5 - 4.6 of soy proteins reported previously [83] [96]. The neutralization of protein charges followed by protein aggregation in M+CA1.25 resulted in curdling of M and gave rise to the heterogeneous texture observed.

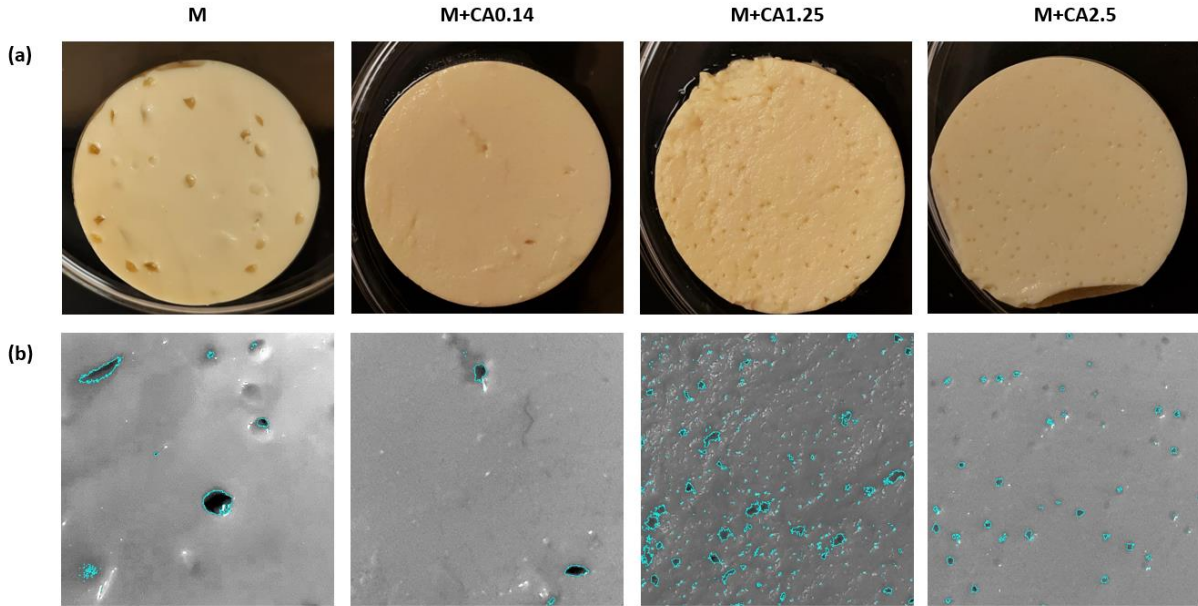


Figure 3.20: Visual observation of the cross-section of M, M+CA0.14, M+CA1.25 and M+CA2.5 gels; (b) Binary images of 30x30mm region of gels generated along with the porous regions or air bubbles (blue lines) detected by ImageJ.

Table 3.7: Porosity estimates of M, M+CA0.14, M+CA1.25 and M+CA2.5 gels based on binary images generated with ImageJ.

Gel	pH	Total Void Area (mm ²)	Average Void Size (mm)	Void Fraction (%)
M	6.37	11.81	0.19	1.31
M+CA0.14	5.54	4.19	0.38	0.46
M+CA1.25	3.90	24.49	0.04	2.71
M+CA2.5	3.33	7.28	0.08	0.81

The mechanical properties of M+CA gels are illustrated in **Figure 3.21**. The behavior of stress-strain curve is divided into three regions. Region I is associated with the linear and non-linear elasticity of the gels where the applied stress causes a recoverable deformation to the gel height. Depending on CA concentration, the elastic region either extended up to a strain beyond (M+CA0.14) or lesser (M+CA2.5) than that of M gel. Moreover, the slope of the elastic region

increased with increasing CA concentration. Region II is where the gel failure occurs and a peak was observed for all gels, except in M+CA1.25. The shallow peak of M is characteristic of ductile materials whereas the comparatively deeper peaks of M+CA0.14 and M+CA2.5 is characteristic of brittle materials [146]. M+CA1.25 gel had a distinct behavior where the elastic region that extended up to a strain of 0.2 was followed by a plastic region wherein the slope of the stress-strain curve increased sharply. No clear failure point could be detected and therefore, the failure properties could not be estimated. The behavior of M+CA1.25 reflects its fragile structure that was qualitatively observed and the tensile equipment was unable to detect the failure of such a soft material. Region III records the response of the failed gels to compression and does not provide relevant information on texture.

The mechanical properties of M+CA0.14 and M+CA2.5 gels compared to M gel indicate that protein coagulation induced by citric acid, prior to thermal gelation, enhanced the gel texture. The gel strength (high failure stress) and stiffness ((high Young's Modulus) increased with CA concentration and were significantly higher than M gel. M+CA0.14 gel displayed a similar failure strain as M gel, whereas the significantly lower failure strain of M+CA2.5 indicated its brittleness or low cohesiveness. Overall, CA induced pre-aggregation at 0.14% and 2.5% concentration was beneficial in improving the gel rigidity.

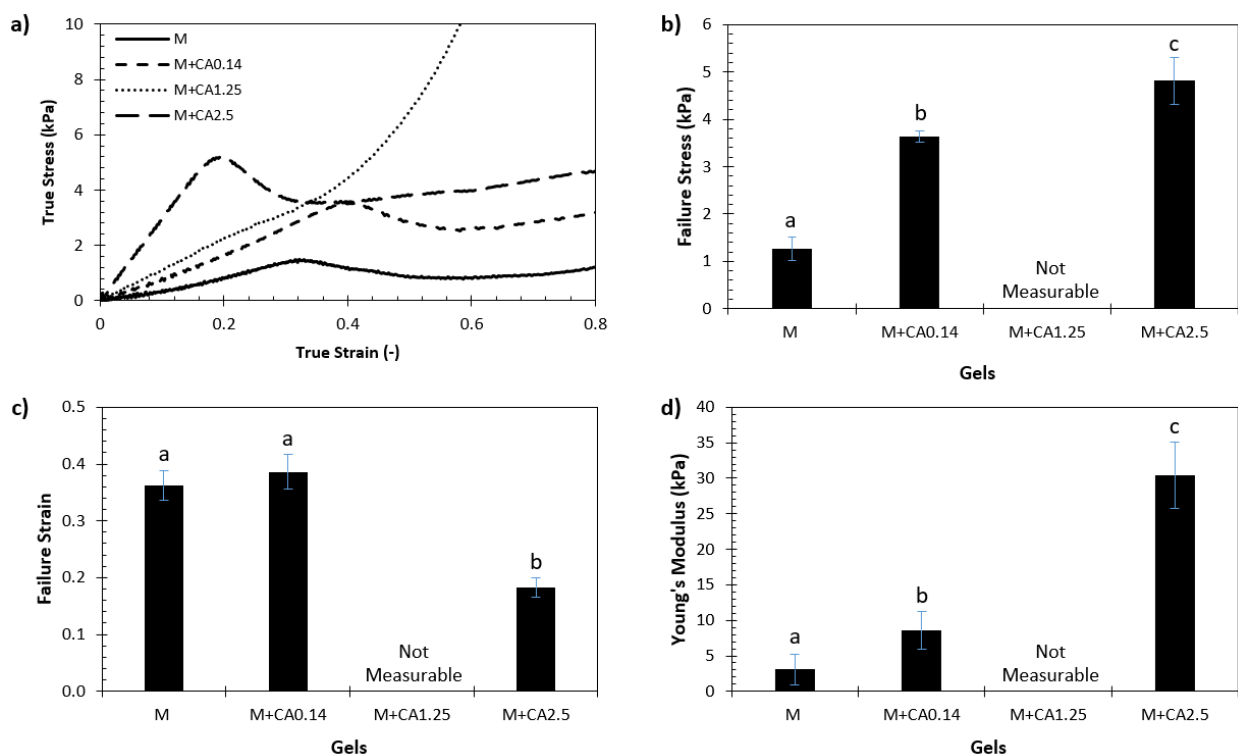


Figure 3.21: Mechanical properties of soy gels M, M+CA0.14, M+CA1.25 and M+CA2.5: (a) Typical failure stress – failure strain curves, (b) True failure stress (kPa), (c) True failure strain, (d) Young's modulus (kPa). Data of (b – d) expressed as mean $\pm$ standard deviation ($n = 6$) where means that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$).

The viscoelastic properties of the M, M+CA0.14, M+CA1.25 and M+CA2.5 gels is illustrated in **Figure 3.22**. All gels displayed a consistently higher storage modulus, G' , than loss modulus, G'' , which indicated an elastic gel behavior wherein the gel disposition was more solid-like than liquid-like [155] [187] [88]. G' and G'' did not crossover which is characteristic of a continuous gel network [189]. The weak dependency of G' and G'' on angular frequency was quantified with the parameters n' and n'' of the power law model, as presented in **Table 3.6**. The values of n' within the range of 0.10 – 0.14 and n'' between 0.08 – 0.14 support the formation of weak physical gels that were assembled predominantly by non-covalent bonds [155] [97] [144].

The storage moduli (G_o') and loss moduli (G_o'') at the frequency of 1 rad/s, are listed in **Table 3.8**. In the presence of CA0.14 and CA2.5, the viscoelasticity (G_o' , G_o'') of gels increased significantly than M gel, with more pronounced effect at higher CA concentration. However,

M+CA1.25 gel displayed the highest viscoelasticity. A similar G' profile according to pH was observed in a previous study that prepared gels with β -conglycinin (15% w/w) and NaCl (2.5%) by heating the dispersion at 80°C over a pH range of 3.3 to 7.3 [148]. The dynamic viscoelastic measurements observed in the study, to track the progress of gelation, recorded the highest G' at the end of gelation period for the gel prepared at pH 3.8, followed by pH 3.3 and then pH 5.5 which recorded the lowest G' . This supports the highest G'_0 and G''_0 observed for M+CA1.25 gel (pH 3.89) followed by M+CA2.5 (pH 3.33) and M+CA0.14 (pH 5.54) gels.

Table 3.8: Power law parameters expressing the viscoelasticity of soymilk gels. Data is expressed as mean $\pm$ standard deviation ($n = 3$). Means within one column that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$).

Gel	G'_0 (kPa)	n'	r_1^2	G''_0 (kPa)	n''	r_2^2
M	0.36 ± 0.07^a	0.12 ± 0.02^a	0.84	0.07 ± 0.01^a	0.08 ± 0.05^a	0.28
M+CA0.14	7.82 ± 0.91^b	0.12 ± 0.01^a	1.00	1.91 ± 0.30^b	$0.12 \pm 0.01^{a,b}$	1.00
M+CA1.25	34.56 ± 0.75^c	0.13 ± 0.01^a	1.00	4.14 ± 0.23^c	0.14 ± 0.01^b	0.99
M+CA2.5	10.55 ± 0.35^d	0.14 ± 0.02^a	1.00	2.84 ± 0.11^d	$0.13 \pm 0.02^{a,b}$	0.99

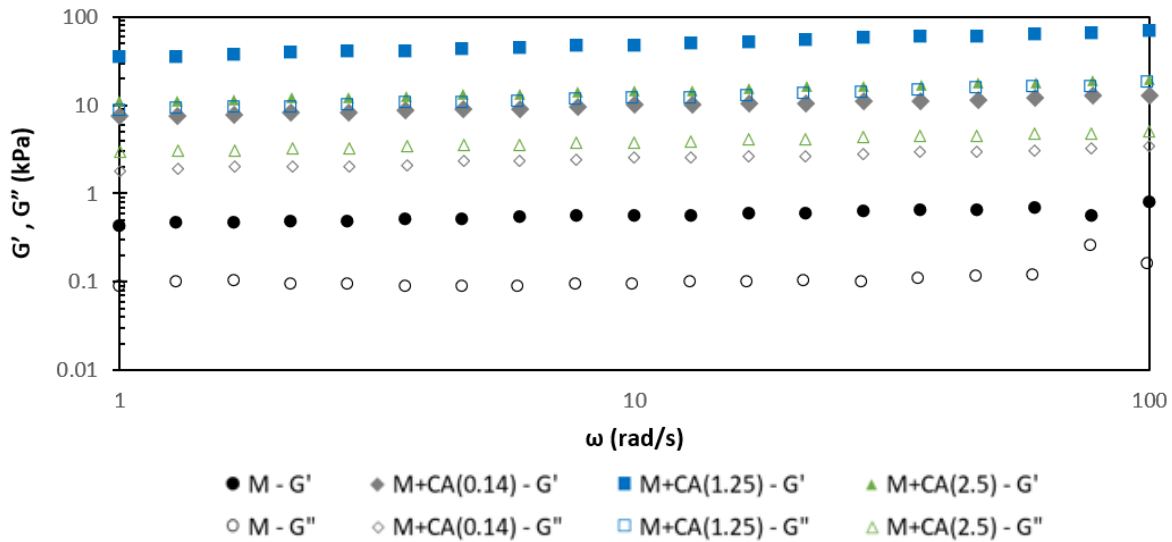


Figure 3.22: Typical curves of storage modulus G' (filled symbols) and loss modulus G'' (empty symbols) of M, M+CA0.14, M+CA1.25 and M+CA2.5 gels.

The differences in gel texture observed from the mechanical or rheological properties may have resulted from differences in the microstructure. The assembly of gel network is affected by the size of protein particles, resulting from the variation in the native, partially unfolded or aggregated states of proteins before gelation. Change in turbidity reveals a change in the size of particles or the number of aggregates and signals modification in the structure of proteins [100] [92]. An alteration in the surface charge of proteins minimizes electrostatic repulsion, causes aggregation of soluble proteins into insoluble particles [103], increases the number of aggregates and thus, increases turbidity. Thus, to analyze the state of protein through changes in the size of protein particles or aggregates, the turbidity of the gel mixtures was measured and presented in **Table 3.9**. An increase in absorbance is caused by increased light scattering due to the presence of larger sized particles or higher particle concentration [100] [92], both reflecting greater turbidity. Although insoluble protein aggregates were detected in M+CA0.14 and M+CA2.5 mixtures, turbidity of the mixtures was statistically similar ($p > 0.05$) to M. Turbidity could not effectively characterization the changes in soymilk proteins due to aggregation.

Table 3.9: Average Turbidity of M, M+CA0.14 and M+CA2.5 mixtures diluted by a factor of 100x and 50x. Data is presented as mean $\pm$ standard deviation (n=3). Means that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$)

Gels	A ₆₀₀ of Mixture (50x dilution)	A ₆₀₀ of Mixture (100x dilution)
M	2.01 $\pm$ 0.00 ^a	1.37 $\pm$ 0.00 ^a
M+CA0.14	2.27 $\pm$ 0.00 ^a	1.74 $\pm$ 0.01 ^a
M+CA2.5	2.03 $\pm$ 0.00 ^a	1.34 $\pm$ 0.00 ^a

3.5.3 Conclusion

The addition of citric acid at concentrations 0.14%, 1.25% and 2.5% (w/w) caused coagulation of soymilk proteins before thermal gelation and was beneficial in enhancing the texture of the soymilk gels. The gel strength and stiffness increased with increasing CA concentration, with an exception at CA1.25 at which the gel failure could not be identified and the mechanical properties could not be evaluated. The viscoelasticity of the gels also echoed enhanced gel material properties in presence of citric acid, with more pronounced effect with increasing CA concentration, and M+CA1.25 gel was an exception as it recorded the highest viscoelastic properties. Based on the

mechanical and rheological properties of the gels, the benefits of adding CA0.14 and CA2.5 was explored further in the preparation of soymilk okara gels wherein CA was used either to treat okara or as a soymilk coagulant.

Turbidity was used as a simple, potential method to characterize the changes in soymilk proteins in soymilk, okara and/or citric acid diluted mixtures, before thermal gelation. However, the actual contribution of the soymilk proteins to the mixture turbidity could not be isolated effectively due to the interference from the large okara particles and the settling of the insoluble aggregated protein particles formed in the presence of citric acid. Therefore, turbidity was an ineffective method for characterizing the changes in soymilk proteins.

3.6 Visualization of Okara Distribution in Gels with Microscopy

3.6.1 Objective

The objective of this test was to visualize changes in the surface morphology of okara modified with citric acid at 0.14%, 1.25% and 2.5% concentrations and oven-dried at 60°C with a scanning electron microscope (SEM). Additionally, the surface microstructures of freeze-dried soymilk okara gels M and MO were captured under SEM to detect the network of protein aggregates and the disruption of the protein network in presence of okara fiber.

3.6.2 Results and Discussion

The surface microstructures of freeze-dried M and MO gels captured with SEM are displayed in **Figure 3.23**. The M gel was comprised of a dense, continuous, strand-like protein network with small pores distributed throughout the gel surface. A similar cellular, porous structure was reported in SPI gels prepared through thermal treatment at 80°C [169]. The presence of okara particles in MO gel disrupted the continuous protein network [154]. The black regions indicating the pores [168] in MO gel were comparatively larger than in M. The honeycomb-like as well as lamellar structures of okara particles [168] were detected at the surface of the gel sample, and the okara structures were surrounded by dense regions of proteins because okara facilitates formation of protein-rich regions [104].

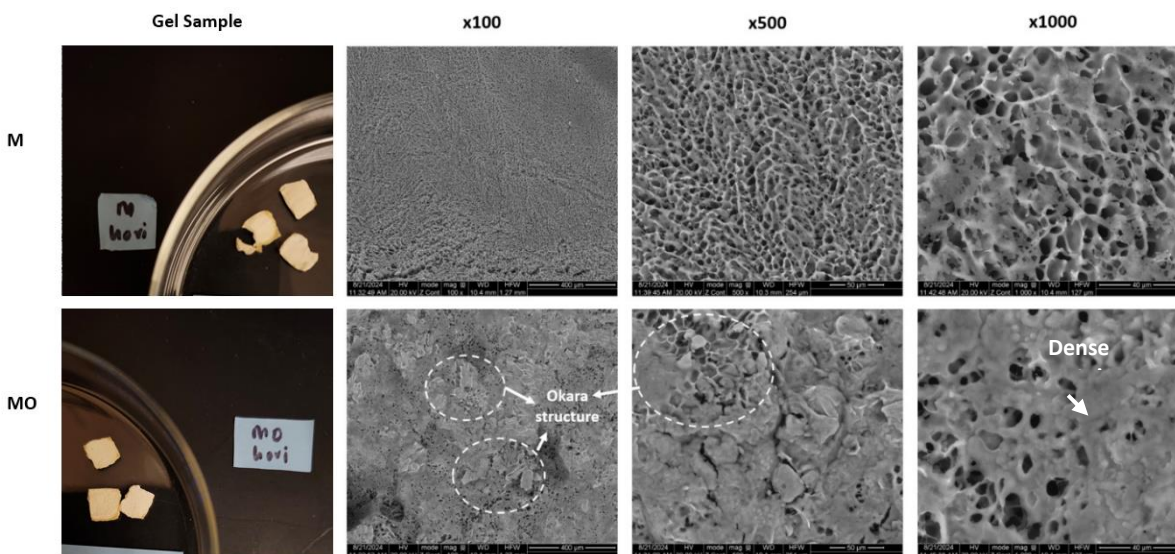


Figure 3.23: SEM micrographs of freeze-dried samples of M and MO gels visualized at magnification of x100, x500 and x1000.

3.6.3 Conclusion

The SEM analysis of the gel samples revealed a continuous, strand-like network of proteins in M gel. The protein network was disrupted by the honeycomb-like and lamellar structures of okara particles in MO gel. These structures of okara were also visualized under CLSM. Calcofluor White (CW) dye at the concentration of 0.1% (w/w) and volume of 150 μ L was determined to be suitable for staining the okara polysaccharides to obtain the CLSM micrographs that visualized the microstructure and distribution of okara in the soymilk okara gels.

Chapter 4: Effects of Citric Acid on Soymilk and Okara Components and Gels

4.1 Abstract

This study aimed to produce high-fiber soymilk gels with enhanced texture by incorporating okara (O). To improve the integration of O in soymilk (M) protein gel matrix, citric acid (CA) at 0.14% and 2.5% (w/w) was utilized for its ability to modify okara and coagulate soymilk proteins. Three citric acid soymilk okara gel formulations were investigated: (i) CA treatment of okara prior to its addition in soymilk (M+OCA), (ii) CA treatment of soymilk prior to okara addition (M+CA+O), and (iii) CA addition to a mixture of soymilk and okara (MO+CA). The FTIR, TGA and SEM analyses of CA-treated O (OCA) revealed structural modification in OCA2.5, including okara decomposition and release of pectic substances, which were absent in OCA0.14.

The presence of CA in the three formulations altered the microstructure of soymilk okara gel from strand-like to a particulate network, thus affecting its texture. In comparison to MO gel, the CA0.14 soymilk okara gels exhibit similar failure stress (3.9-5.2 kPa), failure strain (0.3-0.31) and Young's modulus (16.3-20.9 kPa), while viscoelasticity was influenced by the sequence of CA addition. The elasticity (G_o') of M+OCA0.14 and MO+CA0.14 increased threefold and viscosity (G_o'') doubled, whereas an increase 4.5-fold in G_o' and 3.5-fold in G_o'' was exhibited by M+CA0.14+O. The CA2.5 soymilk okara gels had similar failure stress (2.8-4.8 kPa) to MO, but a lower failure strain (0.16-0.23) and higher Young's Modulus (21.9-53 kPa), indicating lower cohesiveness and higher stiffness. G_o' and G_o'' of M+OCA increased threefold and twofold, while M+CA+O and MO+CA showed a further increase of nearly 4.5-fold in elasticity and 4-fold in viscosity in comparison to MO gel. The texture properties suggest that CA had an incremental effect on gel stiffness and viscoelasticity. The CA-modification of O was not essential as similar or superior improvement in texture were achieved by pre-aggregating M proteins with CA in M+CA+O and MO+CA formulations.

Keywords: Soymilk Gel; Okara; Citric Acid; Isoelectric Point; Chemical Interactions; Gel Texture Properties; Microstructure; SEM

4.2 Introduction

Cheese analogues or imitation cheeses sourced from plant ingredients have garnered interest as they cater to various dietary needs such as free of lactose, saturated fat, and cholesterol [91], and can fortify nutrition profile [62] [64]. Additionally, they can be designed to possess flow, melt or shred properties [64]. Tofu, or soymilk gel, is a well-established product sourced from soybeans that can be regarded as a cheese analogue with high protein content.

Traditionally, tofu is produced from extracted soymilk [168] which utilizes 53% (dry basis) of the soybean mass [33]. Each kg of soybean processed into tofu produces approximately 1.2 kg of fresh okara waste, a fibrous by-product susceptible to microbial degradation. Okara is either used as animal feed, or discarded leading to resource wastage [33] [168] [154]. The rich dietary fiber profile of, along with its protein content [168], makes okara a valuable resource that improves the nutrition profile of food products and aid in health concerns related to hypoglycemia, diabetes, cardiovascular diseases and obesity [164]. The addition of dietary of fibers in soy gel systems is hypothesized to enhance gel texture, as fibers promote the formation of localized protein-rich regions and contribute to stiffer gels, as noted in a review of protein-polysaccharide food systems [37]. Moreover, using proteins and polysaccharides for food applications offers the flexibility of generating various textures that can be manipulated by modifying interactions with a change in the production conditions (temperature, pressure, pH, ionic strength) [87]. However, the incorporation of insoluble okara fibers deteriorates the texture and sensory attributes of soy gels [154] [168]. Therefore, recent studies are making efforts towards utilizing okara for producing high-fiber soy gels while addressing the challenge of quality degradation.

A variety of treatments have been explored to reduce the size of okara particles, modify its surface properties, enhance its compatibility in protein gel matrix and therefore improve gel homogeneity [154]. Some treatments include pulverization [108] [128], high pressure homogenization [35], microwave treatment [123], double layer coating with chitosan and pectin [34], and enzymatic crosslinking [154]. The utilization of non-physical approach to okara modification using food grade organic acids, however, in producing okara-added soymilk gel as proposed in [58] [59] remains a limited area of research. CA has been applied on polysaccharides to hydrolyze [43] [46], modify surface through esterification [44] [45] [104] [138], extract soluble polysaccharides such as

pectin [49] [50] [149], and as a coagulant of soymilk in tofu production [83]. The compatibility between soymilk proteins and okara polysaccharides can be enhanced by facilitating the formation of electrostatically interacting complexes, formed upon manipulating the surface charge of proteins relative to their isoelectric point. Various polysaccharides have been tested to date to complex with soy proteins. These polysaccharides significantly differ from the complex, heterogeneous composition of okara, and are often are charged, soluble, or have a linear, well-defined structure [51-55].

The aim of this study was to explore CA sequencing in soymilk okara gel formulations to isolate its effects on (a) okara modification before incorporating it in soymilk (M+OCA), (b) soymilk coagulation before mixing okara (M+CA+O), and (c) combining coagulation of soymilk and modification of okara (MO+CA) for potentially enhancing protein-polysaccharide compatibility in the soymilk gel network. CA is hypothesized to hydrolyze and esterify okara, and release soluble polysaccharides. These structural modifications can improve okara integration in the soymilk protein gel matrix. Additionally, residual CA in the treated okara was hypothesized to initiate pre-aggregation of soymilk proteins and accelerate gelation.

Two CA concentrations were investigated, 0.14 and 2.5% (w/w), to assess fiber degradation, protein coagulation and aggregate formation in gels. The okara modifications resulting from CA treatment was examined visually and characterized through TGA, FTIR and SEM. The response of soymilk protein aggregation to CA was obtained by visual observations. The impact of CA concentration and addition sequence on the texture of soymilk okara gels was assessed through macro-pore distribution analysis, estimation of expressible and entrapped water with gel syneresis and water holding capacity, chemical forces, failure properties under compression, viscoelastic properties, and microstructure visualization.

4.3 Materials and Methods

4.3.1 Materials

Soybeans (Nupak), obtained in bulk from a local grocery store (Waterloo, Ontario, Canada) were stored in food storage bags at room temperature. The chemical reagents are analytical grade

including citric acid anhydrous (Fischer Scientific, Fair Lawn, NJ, USA), Rhodamine B (Sigma Aldrich, India) and Calcofluor White (Polyscience Inc.), sodium phosphate dibasic heptahydrate (Acros Organics, India), sodium phosphate monobasic monohydrate (Acros Organics, India), hydrochloric acid (Fisher Scientific), sodium hydroxide (Sigma Aldrich, India), sodium chloride (Fisher Scientific), urea (VWR, Canada), Bio-Rad dye reagent and Bovine Serum Albumin (Bio-Rad Laboratories Inc., USA, Batch # 64499500).

4.3.2 Preparation of Ingredients

4.3.2.1 Soymilk and Wet Okara Extraction

Dry soybeans were soaked overnight in DI water at a soybean to DI water ratio of 1:3 (w/v) for 22-25 hours. The soaked beans were washed under running DI water to remove the water soluble anti-nutritional components, dehulled, and then ground for 2 minutes at high speed in 2 cycles of 1 minute each in a blender (Waring Commercial Grinder E11524 USA). The slurry was filtered through a cheesecloth (Scenglos, Woodridge, IL) to separate soymilk (M) from wet Okara (O_w), and both products were stored in the refrigerator until further use. Table 6.1. summarizes the procedure to track consistency in the quality of M and O_w .

The solid content of soymilk was determined in triplicates by drying 1 g of soymilk, contained in a 20 mL beaker, at 60°C for 3 hours in a forced air oven (Fischer Isotemp 737F, USA). The solid content was evaluated using **Equation 4.1**:

$$\% \text{ solid content} = \frac{W_{\text{solid}}}{W_{\text{soymilk}}} \times 100 \quad \text{Equation 4.1}$$

where, W_{solid} was the dried soymilk after 3 hours in oven and W_{soymilk} was the initial weight of soymilk.

Table 4.1: Mass of soybeans after overnight hydration and dehulling, and mass of soymilk and okara extracted. The solid content was calculated using Equation 4.1. Data is expressed as mean $\pm$ standard deviation (n = 3).

Dehulled hydrated soybeans ^a	243.99 $\pm$ 1.60
Okara (O_w) ^a	96.88 $\pm$ 1.79

Soymilk (M) ^a	44.17 ± 1.39
O_w:M Ratio ^b	0.22 ± 0.005
Solid content of soymilk (w/w %) ^c	16.21 ± 0.81

a: Calculated based on 125.43 ± 0.42 g dry soybeans.

b: Calculated by dividing the mass of wet okara to soymilk.

c: Calculated with Equation 4.1.

4.3.2.2 Treatment and Drying of Okara

Approximately 10 grams of O_w was dispersed in 50 grams of DI water on a magnetic stirrer (a. CanLab, b. Fischer Isotemp 11-100-49sh, USA) for approximately 5 minutes to ensure complete dispersion. The okara slurry was transferred to a 100 mm diameter petri dish, previously lined with parchment paper. The petri dishes were placed in a pre-heated forced air oven (Fischer Isotemp 737F, USA) maintained at 60°C for 19 - 20 hours to remove all moisture and then ground (Coffee & Spice Grinder CBG110SC, Black & Decker Co and Spectrum Brands Inc., USA) for one minute into powder to obtain O.

For the treatment of okara with citric acid (CA), two CA concentrations were investigated, 0.14 and 2.5% (w/w). CA solutions were prepared by dissolving 0.084 g CA in 49.916 g DI water and 1.5 g CA in 48.5 g DI water for CA0.14 and CA2.5, respectively. 10 grams of wet okara (O_w) was dispersed in CA solutions and stirred at 600 – 800 rpm for an hour at room temperature with a magnetic stirrer (CanLab or Fischer Isotemp 11-100-49sh, USA). The mixtures were then transferred to a petri dish lined with parchment, dried in a forced air oven at 60°C for 19 - 20 hours and ground for one minute to obtain ground OCA0.14 or OCA2.5.

4.3.3 Characterization of Okara

4.3.3.1 Thermogravimetric Analysis (TGA)

The methodology to assess the stability of dried M, O and CA treated okara (OCA0.14, OCA2.5) by thermogravimetric analysis (TA Instrument, TGA Q500) was adopted from [59]. 13±1 mg of powdered sample was heated in an inert nitrogen environment at the rate of 10 °C/min from room temperature to 600 °C. The weight loss of the sample over the temperature range was recorded.

The Universal Analysis 2000 software (TA Instruments) generated the derivative thermogravimetric (DTG) data representing the rate of mass change upon heating.

4.3.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

Thirty-two scans at a resolution of 4 cm⁻¹ of FTIR spectra of dried M, O and CA treated okara (OCA0.14, OCA2.5) in the wavenumber range of 500 to 4000 cm⁻¹ was collected with the Nicolet 6700 FTIR spectrometer (Thermo Scientific Nicolet 6700, Waltham, MA, USA). The baseline was corrected and the transmittance signal normalized with the Omnic software (Thermo Scientific) was used to for the comparison of the spectral peak shifts and magnitude changes across samples.

4.3.3.3 Environmental Scanning Electron Microscopy (ESEM)

The oven dried, ground soymilk, okara and modified okara were loaded onto a pin stub that was covered with conductive carbon tape. The samples were sputter-coated with gold and then the surface morphology was examined by ESEM (FEI Quanta FEG-250 ESEM) at magnifications of x100, x500, x1000, x3000, high voltage of 20kV and high vacuum environment.

4.3.4 Preparation of Soymilk Okara Gels

4.3.4.1 Preparation of M and MO Formulations

The soymilk in the formulations was used after overnight refrigeration which was kept consistent in all tests and batches. The M mixture was prepared by weighing 44±2g soymilk that was equilibrated at room temperature. The MO mixture was prepared by initially making a paste of dried, ground O with one-third of the soymilk required for the formulation, in a petri dish. The paste was mixed with the remaining M, contained in a 200 mL beaker, with a magnetic stirrer to ensure uniform distribution of okara solids. Mixing over magnetic stirrer was carried out for 15 minutes at low speed of 400 – 600 rpm to prevent air bubbles that can potentially influence the texture and heterogeneity of the gels produced. At the end of the mixing duration, the pH of the mixture was recorded with a pH meter (Mettler Toledo, S47 SevenMulti Dual).

4.3.4.2 Preparation of MO Formulations with CA

Three MO formulations were prepared at two CA concentrations (0.14% and 2.5% w/w) that was added in a different order to examine changes in mixtures and resulting gels.

The first formulation, M+OCA0.14 and M+OCA2.5, was to observe the effect of adding CA modified O (OCA) to M. A paste was first prepared by mixing ground OCA0.14 or OCA2.5 with one-third mass of total M used (44 ± 2 g). The paste was added to the remaining M, contained in a 200 mL beaker, and mixed over a magnetic stirrer at 400-600 rpm for 15 minutes. The pH of the mixtures was recorded with a pH meter during the addition of okara in periods of 30 seconds and after 1, 3, 6, 9, 12, 15 minutes following the addition of OCA.

The second formulation, M+CA0.14+O and M+CA2.5+O, was to use M coagulated with CA in the preparation of MO mixture. The first step was to gradually add 0.084g or 1.5g of solid citric acid to soymilk (44 ± 2 g) under constant stirring and mixed for 15 minutes. The pH of the mixture was recorded after 1, 3, 6, 9, 12, 15 minutes following the addition of CA. The next step was to prepare a paste by mixing about one-third of the soymilk citric acid solution with ground O. The paste was subsequently added to the remaining soymilk solution and stirred for an additional 15 minutes to obtain a homogenous dispersion and its pH was recorded.

The third formulation, MO+CA0.14 and MO+CA2.5, was to assess the effect of CA added to MO mixture. A paste of O with one-third portion of total M required (44 ± 2 g) was first prepared. The paste was added to the remaining M, contained in a 200 mL beaker, and mixed over a magnetic stirrer at 400-600 rpm for 15 minutes. Subsequently, 0.084g or 1.5g of solid citric acid was gradually added to the soymilk okara mixture and stirred for an additional 15 minutes to obtain MO+CA0.14 and MO+CA2.5 mixtures, respectively. The pH of the mixture was recorded after 1, 3, 6, 9, 12, 15 minutes following the addition of CA.

4.3.4.3 Thermal Gelation

All gels were obtained by thermal treatment of the mixtures at 95°C for 45 minutes in a water bath with thermocouple (Neslab RTE-111, USA). The resulting gels were cooled to room temperature naturally for one hour to consolidate the gel network, and subsequently refrigerated overnight at

4°C, for at least 12 h before further analysis. Some minor modifications were made according to the type of testing.

The gel samples for macrostructure analysis, water holding capacity, gel syneresis and protein-protein interactions were prepared by transferring the mixture into a 100 mL beaker coated with PAM cooking oil. The beaker was then sealed with aluminum foil and placed in a hot water bath for gelation. The gel samples for uniaxial compression were prepared by distributing the mixture into two 100 mL beakers that were coated with PAM cooking oil. The beakers were filled with the mixture up to a height of 10 mm, sealed with aluminum foil and then exposed to gelling conditions.

For rheology analysis, the mixture was distributed between a 100 mm and a 90 mm diameter petri dish to prepare the gel samples. The petri dishes were previously lined with parchment paper, coated with PAM cooking oil, and then filled with the gel mixture up to a weight of 20-25 g to obtain a 2-3 mm thick gel product. The petri dishes were sealed with 2 layers of aluminum foil and exposed to gelling conditions.

4.3.5 Analysis of Gel Properties

4.3.5.1 Macrostructure Analysis

Gels were cut along the cross section with a knife and the internal cross-section of the gel was captured with a digital camera (Samsung S8) at a height of approximately 20 cm above the surface of the gel. The cross-sectional images were processed with ImageJ software. A 30 x 30 mm region was converted into 8-bit grayscale image, auto-adjusted for brightness and contrast, and the threshold was set at 100 to examine the pore distribution with the total void area, void fraction and average void size estimated in ImageJ.

4.3.5.2 Microstructure Analysis

Approximately 4g of a given M, O and CA mixture was prepared and transferred to a 20-mL disposable scintillation vial. The vial was covered with aluminum foil to prevent degradation of the dye. A 20µL volume of 0.2% (w:v) Rhodamine B (RB) (Sigma Aldrich) solution and 150µL of 0.1% (w:v) Calcofluor White (CW) (PolyScience Inc.) were added to the mixture. After an hour of stirring at room temperature to ensure sufficient staining, samples were refrigerated overnight.

About 2 drops of RB and CW labelled soy gel mixtures were placed in a concave microscope slide (Fisherbrand, 25x75 – 1mm), gently pressed down with cover slide (Fisherbrand, 22x22 – 1.5mm), sealed with nail polish and wrapped in aluminum foil. The slides were placed in a forced air oven (Fischer Isotemp 737F) at 95°C for 45 min to replicate gelling conditions and stored in the fridge until the microstructure imaging and analysis. The microstructure of the gel samples was recorded using a confocal laser scanning microscope (Zeiss LSM 700 AxioObserver, Germany) at three magnifications, 20X, 63X and 100X, with the image size set at 1080 x 1080 pixels, pinhole size fixed at 1 to 1.5 airy unit and laser power at 2.0%. The detector gain was varied within the range 350-500 for RB and 600-800 for CW depending on the objective lens. The excitation and emission wavelengths were set at 555nm and 560 – 800 nm for RB detection and 405 nm and 410 – 480 nm for Calcofluor White, respectively.

4.3.5.3 Gel Syneresis

The gel samples, after overnight refrigeration, were observed to discharge some water. The color of the liquid exuded from gels was noted and weighed to estimate gel syneresis (%) as the ratio of liquid discharged to the initial weight of the gel, according to **Equation 4.2** and adopted from [59] and [150],

$$\% \text{ syneresis} = \frac{W_{\text{liquid released}}}{W_{\text{whole gel}}} \times 100 \quad \text{Equation 4.2}$$

4.3.5.4 Water Holding Capacity

About 5 ± 1 g of the gels were transferred into 13.2-mL thin walled polypropylene centrifuge tube (Beckman Coulter) and weighed (W_t). The gel samples were centrifuged in an ultracentrifuge (Beckman Coulter, Optima XPN100, swing bucket SW 41 Ti) at room temperature for 20 min at a centrifugal force of $12,074 \times g$, corresponding to the rotor speed of 10,000 rpm. Following ultracentrifugation, the total water released was estimated from the supernatant over the compressed gel mass which was transferred into a scintillation vial and weighed (W_{r1}), and the water stuck at the surface of the centrifuge tube wall which was absorbed using a Kimwipe and weighed (W_{r1}). The total water released (W_r) by the gels was estimated as the sum of W_{r1} and W_{r2} . The water holding capacity (WHC) was calculated using **Equation 4.3**,

$$\text{WHC (\%)} = \frac{W_t - W_r}{W_t} \times 100$$

Equation 4.3

W_r (g) denotes the total weight of the water released after centrifugation and W_t is the initial weight (g) of approximately 5 g.

4.3.5.5 Protein-protein Interactions

Protein-protein interactions in the soymilk gel network was obtained from the solubilized proteins released after soaking the gel in aqueous solutions with different composition. A buffer solution, 1000 mL of 0.05M phosphate buffer (pH 7.0) was prepared in a 1000-ml volumetric flask by dissolving 7.744 g of sodium phosphate dibasic heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) and 2.9118 g of sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) in DI water. The pH was monitored with a pH meter (Mettler Toledo, S47 SevenMulti Dual) when adjusting pH of the buffer to 7.0 with 1M HCl and 1M NaOH solutions. Four different solutions were prepared in 0.05M phosphate buffer (pH 7.0) as the solvent - SA (0.05M NaCl), SB (0.6M NaCl), SC (0.6M NaCl + 1.5M urea), SD (0.6M NaCl + 8M urea). The SA solution with low NaCl concentration does not disrupt specific interactions whereas SB with higher NaCl concentration disrupts the electrostatic interactions in the gel structure. The presence of low urea concentration in SC overcomes the hydrogen bonding in the protein network and an additional disruption of the hydrophobic interactions by the higher urea concentration of SD.

A 0.6g soymilk okara gel sample was homogenized for one minute in 10mL of each of the four solutions (SA, SB, SC and SD) with a vortex mixer and soaked overnight for at least 12 hours at room temperature. Subsequently, the gel was ultracentrifuged (Beckman Coulter Ultracentrifuge Optima XPN100, USA) for 50 minutes at room temperature at a speed of 10,000 rpm. The supernatants from the gels soaked in solutions SA, SB and SC were used undiluted while from the solution SD was diluted 10-fold with phosphate buffer (pH 7.0). The supernatant was analyzed for its protein content using the Bradford assay methodology [94] adapted from [66]. A volume of 10 mL of the diluted supernatant and 200 mL of Biorad dye reagent solution were transferred with an Eppendorf micropipette into microplate (polystyrene) wells in triplicates and analyzed with microplate reader (Bio-tek) at an absorbance wavelength of 595 nm. The solubilized protein content was estimated from a protein standard curve of Bovine serum albumin (BSA), constructed

within a concentration range of 0.05 – 0.5 mg protein/mL solution, following which the interactions were estimated as described in **Table 4.2**.

Table 4.2: Soaking solutions and target interactions disrupted, adapted from [59].

Soaking solution	Reagents (0.05M phosphate buffer at pH 7.0)	Protein solubility difference	Target intermolecular interactions
SA	0.05M NaCl	SB-SA	Electrostatic
SB	0.6M NaCl		
SC	0.6M NaCl + 1.5M urea	SC-SB	Hydrogen
SD	0.6M NaCl + 8M urea	SD-SC	Hydrophobic

4.3.5.6 Mechanical Properties

The gels prepared in each beaker were cut into 3 cylindrical samples of 10 mm diameter and 20 mm thickness with a corer, producing a total of 6 replicates per formulation. The gel samples were allowed to equilibrate to room temperature for 1 h prior to the analysis. The cylindrical gel samples were subjected to uniaxial compression with a tensile tester (Shimadzu Autograph AGS-X) equipped with a 500-N load cell to gauge the mechanical properties. The compression was carried out at room temperature and at a rate of 40 mm/min, until the gel sample failed or the load plates were less than 5 mm apart.

The total stress applied on the gel with a 500-N load cell, denoted as true stress ($\sigma(t)$), and the deformation in the dimensions of the gel, referred to as true strain (ϵ), were calculated using **Equation 4.4** and **Equation 4.5**,

$$\text{True stress: } \sigma(t) = \frac{F(t)}{A_0} \times \frac{l(t)}{l_0} \quad \text{Equation 4.4}$$

$$\text{True strain: } \epsilon = \ln\left(\frac{l(t)}{l_0}\right) \quad \text{Equation 4.5}$$

l_0 represents the initial height [in m], A_0 is the initial gel area [m²], and $I(t)$ [in m] is the height of the gels recorded at time t that resulted from the applied compression force $F(t)$ [in N]. The true stress versus true strain curve displayed a linear region followed by a peak. The peak represented the failure point of the gel. The slope of the linear region up to the failure point in the true stress-strain curve, shortened to consider the region only between one-third to two-thirds of the failure point, was used to estimate the Young's modulus of the gels.

4.3.5.7 Viscoelastic Property

Duplicate gels prepared in petri dish for each formulation were cut into 4x4 mm squares of 2-3mm thickness to obtain at least 2 samples per petri dish. The 4x4cm gels were allowed to equilibrate at room temperature for 1h.

Small amplitude oscillatory shear test was performed using a rheometer (Thermo Scientific, HAAKE MARS III, USA, located in E6 5007) with a parallel plate geometry of 35 mm diameter and 1 mm gap. The sample was loaded on the sample stage when a 10-mm gap space between the parallel plates was attained. The upper plate was lowered slowly at a set speed of 1.25 mm/min, to minimize any abrupt uniaxial deformation of the sample. After the gels were compressed to a gap space of 1.025 mm [0.025 mm higher than the measuring position of 1 mm], the excess gel jutting out of the plate area was trimmed, following which the samples were allowed to rest for 5 minutes to attain mechanical equilibrium. The frequency sweep test was then conducted in triplicates at an angular frequency range of 1 – 100 rad/s at 0.1% strain. These parameters were chosen based on a preliminary strain sweep test to determine the linear viscoelastic region (LVR) of the gels (**Figure S.8**).

The power law models specified in **Equation 4.6** and **Equation 4.7** were used to track the dynamic dependency of storage (G') and loss moduli (G'') on angular frequency (ω),

$$G' = G'_0 \omega^{n'} \quad \text{Equation 4.6}$$

$$G'' = G''_0 \omega^{n''} \quad \text{Equation 4.7}$$

G'_0 (Pa) and G''_0 (Pa) are the storage and loss moduli coefficients recorded at angular frequency of 1 rad/s, respectively. G'_0 provides a measure of gel rigidity or elasticity while G''_0 is associated with

the energy dissipated as heat and the viscousness (or liquid behavior) of the gel sample. The exponents n' and n'' (both dimensionless) denote the influence of ω on each moduli and along with $\tan \delta$, which is the ratio between the loss modulus (G'') and storage modulus (G'), these parameters indicate weak or strong gel behavior.

4.3.6 Statistical Analysis

Statistical analysis was carried out with Statistica (version 14.0.0.15, TIBCO software Inc) on three experimental sets, wherein each set consisted of a single value for porosity, gel syneresis, and protein-protein interactions, duplicates for WHC, triplicates for viscoelasticity, and 6 replicates for the mechanical properties. The statistical significance of difference was evaluated with one-way analysis of variance (ANOVA) and the Tukey's post-hoc test was used at 5% significance level to determine the significant differences between the averages (paired means).

4.4 Results

4.4.1 Characterization of Okara

4.4.1.1 Qualitative Analysis

The pH of CA okara dispersion decreased with increasing CA concentration (**Table 4.3**), and affected the brittleness and color of modified okara (

Figure 4.1). Dried OCA0.14 had a bright white appearance compared to the ochre hue of O, with a crumbly texture that could be ground into fine powder like O. In contrast, dried OCA2.5 was an ochre hued crisp sheet with some gumminess that was ground into a dense, grainy powder. The different characteristics of okara affects their behavior when mixed with soymilk. The fine particles of O and OCA0.14 absorbed soymilk to form a foamy, thick paste and their mixtures with soymilk, MO and M+OCA0.14, contained uniformly distributed okara. On the other hand, OCA2.5 paste had a liquid consistency with clumped particles of gummy OCA2.5. As a result, M+OCA2.5 mixture contained large particles that settled down when left undisturbed.

Table 4.3: Composition and pH of okara mixtures (O, OCA0.14, OCA2.5) prior to drying, and the mass of dried solid. Data is expressed as mean $\pm$ standard deviation (n = 3).

Samples	Components			pH ^a	Dried solid ^b (g)
	Wet O (g)	CA (g)	DI water (g)		
O	10	0	50	6.59 $\pm$ 0.07	2.64 $\pm$ 0.03
OCA0.14	10	0.084	49.916	4.49 $\pm$ 0.04	2.78 $\pm$ 0.22
OCA2.5	10	1.5	48.5	2.58 $\pm$ 0.01	4.14 $\pm$ 0.04

^a pH values of okara and citric acid dispersion after the 15 minutes mixing period and prior to drying of the dispersion

^b Mass of solid after 19-20 h drying in forced air oven maintained at 60 °C

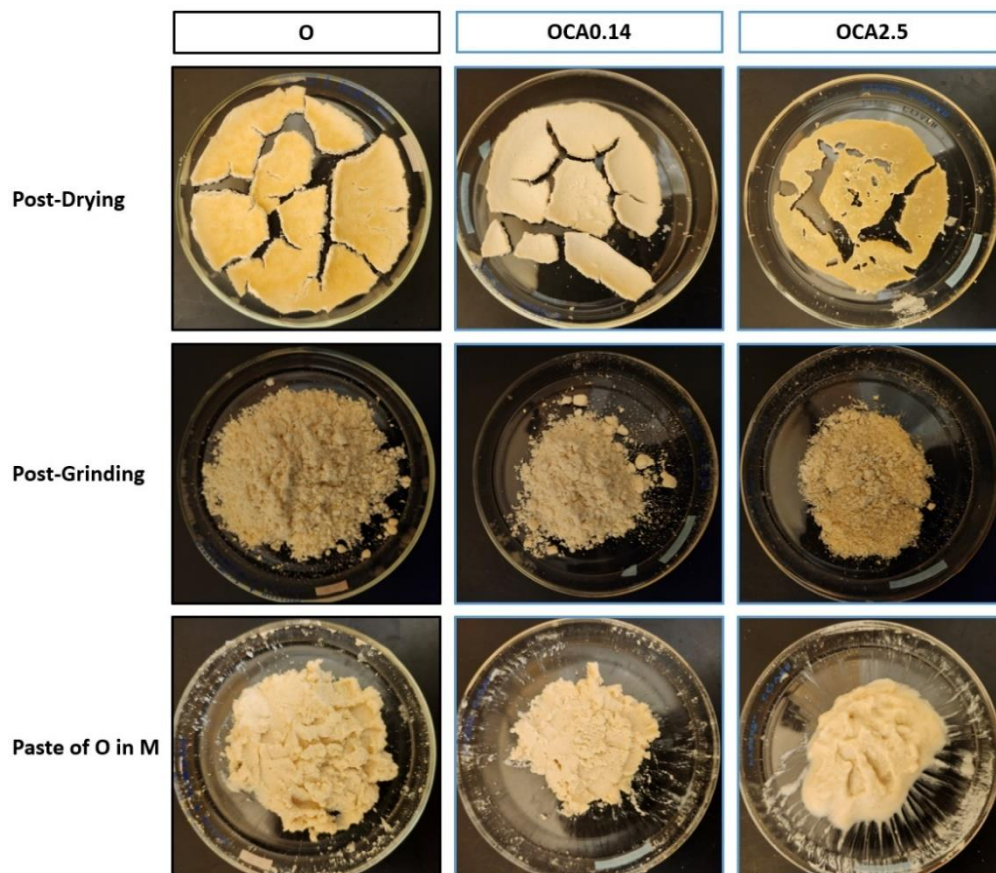


Figure 4.1: Visual observation of O, OCA0.14, OCA2.5 after drying for 19-20 hours at 60°C in oven, after drying and grinding, and as a paste with soymilk.

4.4.1.2 Thermal Stability

The thermal stability of citric acid modified okara materials was analyzed with TGA using dried soymilk, untreated okara and CA as references of comparison. The weight loss (TGA) and rate of change in weight loss (DTG) profiles in **Figure 4.2** illustrate the extent of their thermal degradation over five stages in the temperature range of 25 – 600°C.

The initial weight loss in M and O at 25-150°C occurs from the evaporation of adsorbed water, both free and intramolecular bound water, along with the release of low molecular weight volatile compounds [131] [111]. Their degradation in stage 2, 150-240°C, is attributed to the cleavage of unstable hydrogen bonds in soy protein [160] and unstable side chains of soluble soy polysaccharides such as arabinose and galactose [118]. In stage 3, 220-330°C, a major weight loss observed and is associated with the stage-wise degradation of the peptide chains in M, with DTG peaks situated at 228.8°C and 301.2°C [131] [160]. Soy protein decomposition commences with the disruption of the non-covalent interactions, and subsequently the cleavage of covalent bonds in the primary structure [136]. Similarly, O experienced a significant weight loss with DTG peaks at 255.9°C and 292.8°C ascribed to the initiation of thermal depolymerization of hemicellulose [133] and cellulose [119] [134], respectively. The significant weight loss in stage 3 extended to stage 4, 330 – 430°C, for both M and O. The degradation of soluble fibers comprising galacturonan [118], hemicellulose and cellulose continued at these temperatures, in addition to okara proteins, soy fats [134] and the pyrolysis of lignin with a peak at 356°C [133]. In the last stage, 450 – 600°C, minor weight loss was observed which is associated with the decomposition of residual cellulose, lignin [116] and the carbonaceous materials formed from degraded proteins or fibers at lower temperatures [117].

The CA treatment of okara influenced its thermal stability differently according to CA concentration. OCA0.14 had a generally similar weight loss profile as O with minor shifts in peak temperatures. In contrast, OCA2.5 was more thermally sensitive with an accelerated degradation occurring at a lower temperature extending beyond the first heating stage. The presence of CA in OCA0.14 and OCA2.5 could be related to DTG peaks at 162.7°C in OCA0.14 and 146.3°C in OCA2.5 that represented the melting point of CA present in excess [115]. The subsequent rapid decomposition of OCA2.5 and a DTG peak at 202.5°C aligned with the decomposition of CA

which indicated the presence of a significant excess CA [115]. The higher weight loss in OCA2.5 at this stage may possibly be due to the presence of more water, reflected by its gummy texture, that evaporated during heating. The DTG peaks in the latter heating stages shifted from 255.9°C, 292.8°C and 356.8°C in O to lower temperatures 244.8°C, 331.3°C and 379.4°C in OCA2.5, associated with the decomposition of hemicellulose, cellulose and lignin, respectively. The higher carboxylic acid group content from excess CA or the presence of thermally sensitive ester bonds in OCA2.5 possibly induced these changes, as reported previously in studies on CA-modified paper pulp-starch complex [176] and CNC/CNF extracted from bagasse pulp [177]. However, CA-induced crosslinking of okara hypothesized is less probable as it would otherwise delay decomposition or improve thermal stability, as reported previously for CA-modified carboxymethyl cellulose [175]. At the end of stage 5, the residual char decreased from 22.8% in O to 21.1% in OCA0.14 and 19.5% in OCA2.5. This finding along with the earlier onset degradation and higher weight loss in the previous stages suggest lower thermal stability of OCA2.5, possibly due to structural degradation induced during CA treatment.

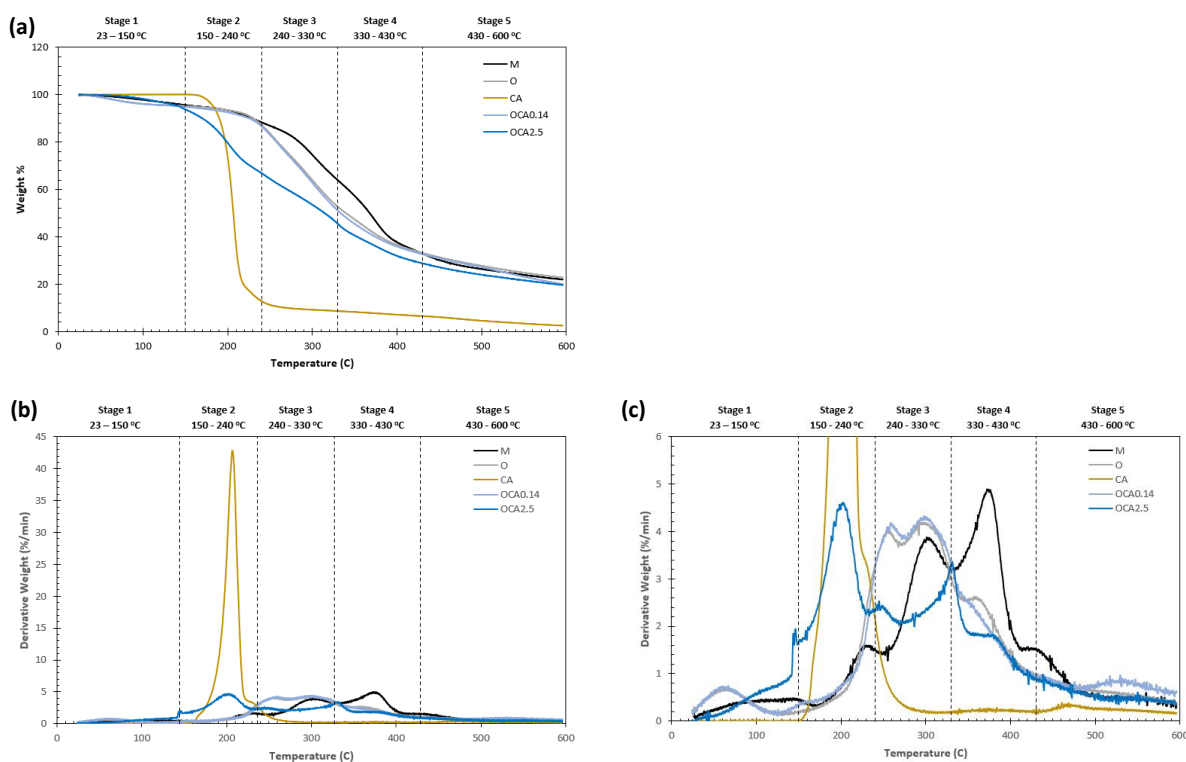


Figure 4.2: (a) Thermogravimetry (TGA) and b) derivative thermogravimetry (DTG) curves of control (M, O, CA) and citric acid modified okara materials (OCA0.14, OCA2.5); c) magnified DTG curve of M, O, OCA0.14, OCA2.5.

4.4.1.3 Fourier Transform Infrared Spectroscopy (FTIR)

The structural changes induced in CA-modified okara was analyzed by comparing the FTIR spectra of OCA0.14 and OCA2.5 with protein-rich M and polysaccharide-rich O. **Figure 4.4** presents FTIR spectra of the materials over four regions. Region I ($3650 - 3100 \text{ cm}^{-1}$) is associated with the absorption band of hydroxyl groups. Region II ($3020 - 2750 \text{ cm}^{-1}$) captures the symmetrical – asymmetrical vibration of CH_n . Regions III ($1800 - 1500 \text{ cm}^{-1}$) and IV ($1300 - 800 \text{ cm}^{-1}$) contain the characteristic peaks of amide groups and polysaccharides, respectively.

The spectra of M and O generally exhibited similar peak profile with differences in the characteristic bands of protein and polysaccharide. The broad band in region I at $3650 - 3100 \text{ cm}^{-1}$ is associated with the overlapped stretching vibrations of hydrogen involved in intermolecular hydrogen bonding, N-H bonds in protein [121] [162], and O-H groups in polysaccharide structures [137] [138]. Region II peaks are ascribed to the stretching of CH_n in aliphatic chains of proteins [121] and polysaccharides [137] [138]. The symmetric and asymmetric CH_n vibrations are situated at 2922 cm^{-1} and 2853 cm^{-1} . A stronger intensity of these peaks in M suggests a relatively higher moisture content and exposure of C-H bonds in proteins compared to O which is comprised of a complex intertwined network of polysaccharides. Region III contains the structural information of protein and carbonyl-carboxyl distribution in polysaccharide. The amide I (C=O), II (N-H) and III (N-H, C-N) bands at 1631 , 1535 and 1235 cm^{-1} peaks, respectively [121] [172], are more intense in protein-rich M than O. The peaks of amide bands overlap with the vibration of esterified carbonyl group (C=O) situated at 1743 cm^{-1} , and asymmetric and symmetric vibrations of carboxylate group (COO^-) detected at $1631 - 1625 \text{ cm}^{-1}$ and 1394 cm^{-1} [138] [171]. Multiple small peaks within $1460 - 1376 \text{ cm}^{-1}$ are associated with the C-H deformation and bending vibration of CH_n in protein side chains [121] and polysaccharides [137] [138]. The fingerprint region of polysaccharides, region IV, contained more prominent peaks in the spectra of O than M. The peak of tensile vibration of C-O bending in C-O-H and stretching in C-O-C between sugar rings of cellulose and hemicellulose are centered at 1038 cm^{-1} and 1155 cm^{-1} [138] [166]. The overlapping

peaks of β -glycosidic linkage between the sugar monomers of okara hemicellulose, cellulose and pectin were detected within the range $920 - 885 \text{ cm}^{-1}$ [111] [137] [171].

The FTIR spectra of CA-treated O materials, OCA0.14 and OCA2.5, will be discussed in comparison to O. CA facilitates esterification, Maillard reaction and acid hydrolysis that modify the structure of okara and influence the functional groups detected, as illustrated in **Figure 4.3**.

The functional groups of O polysaccharides involved in **esterification reaction** with CA are detected in regions I to III. The broad band of O-H stretching band (region I) reduced in intensity in OCA0.14 and flattened in OCA2.5. A decrease in this peak intensity occurs from the disruption of hydrogen bonds [127] [138]. Furthermore, COO^- vibration peak of galacturonic acid present in pectin and hemicellulose (region III) became less intense, whereas the vibration of side group C-H bonds (region II) and ester C=O bonds (region III) increased, with the effects more pronounced in OCA2.5. Changes in these functional groups suggest esterification of the okara polysaccharides with CA. Similar observations on the increase in ester carbonyl bond while decrease in carboxylate ions detected were reported for CA-esterified ramie cellulose [112] and wood [114]. Additionally, the increased intensity of ester carbonyl peak in OCA2.5 could reflect the C=O groups of excess citric acid or C=O of uronic acid detected from the **pectic substances released** during CA-modification [125]. The appearance of a small peak at 1085 cm^{-1} in OCA2.5, possibly associated with the peak of arabinogalactans in pectin that was previously reported at 1078 cm^{-1} [122], and its gummy texture observed further suggest the release of pectic materials.

In addition to the gummy texture, dried OCA2.5 appeared darker in hue possibly due to **Maillard reaction**. An alteration to the conformation of okara protein, resulting from change in pH or formation of protein-polysaccharide interactions, are indicated by the reduced vibration of C=O peptide bond stretching (amide I band) [124]. The amide II and amide III bands decreased in intensity, suggesting conjugation between okara proteins and polysaccharides via Maillard reaction [122]. Furthermore, the peak at 1160 cm^{-1} (C-O-C stretching) intensified suggesting its overlap with the vibration of C-N bonds in Amadori products formed during Maillard reaction. A study on conjugated SPI-SSPS (soy soluble polysaccharides) reported appearance of a new band at $1130-1160 \text{ cm}^{-1}$ associated with the C-N stretching of secondary amine in Amadori products [122].

Structural modifications in OCA0.14 and OCA2.5 resulting from the **acid hydrolysis** of okara polysaccharides induced by CA are indicated by a decrease in peak intensity corresponding to the C-H bending vibrations ($1460\text{--}1376\text{ cm}^{-1}$, region III), C-O in C-O-H bending ($1057\text{--}1038\text{ cm}^{-1}$, region IV), and β -glycosidic linkage ($891\text{--}888\text{ cm}^{-1}$, region IV). These changes were more pronounced in OCA2.5, suggesting partial decomposition of okara fibers into oligosaccharides [137] [138] or a reduction in the polymerization degree of the polysaccharide chains [172].

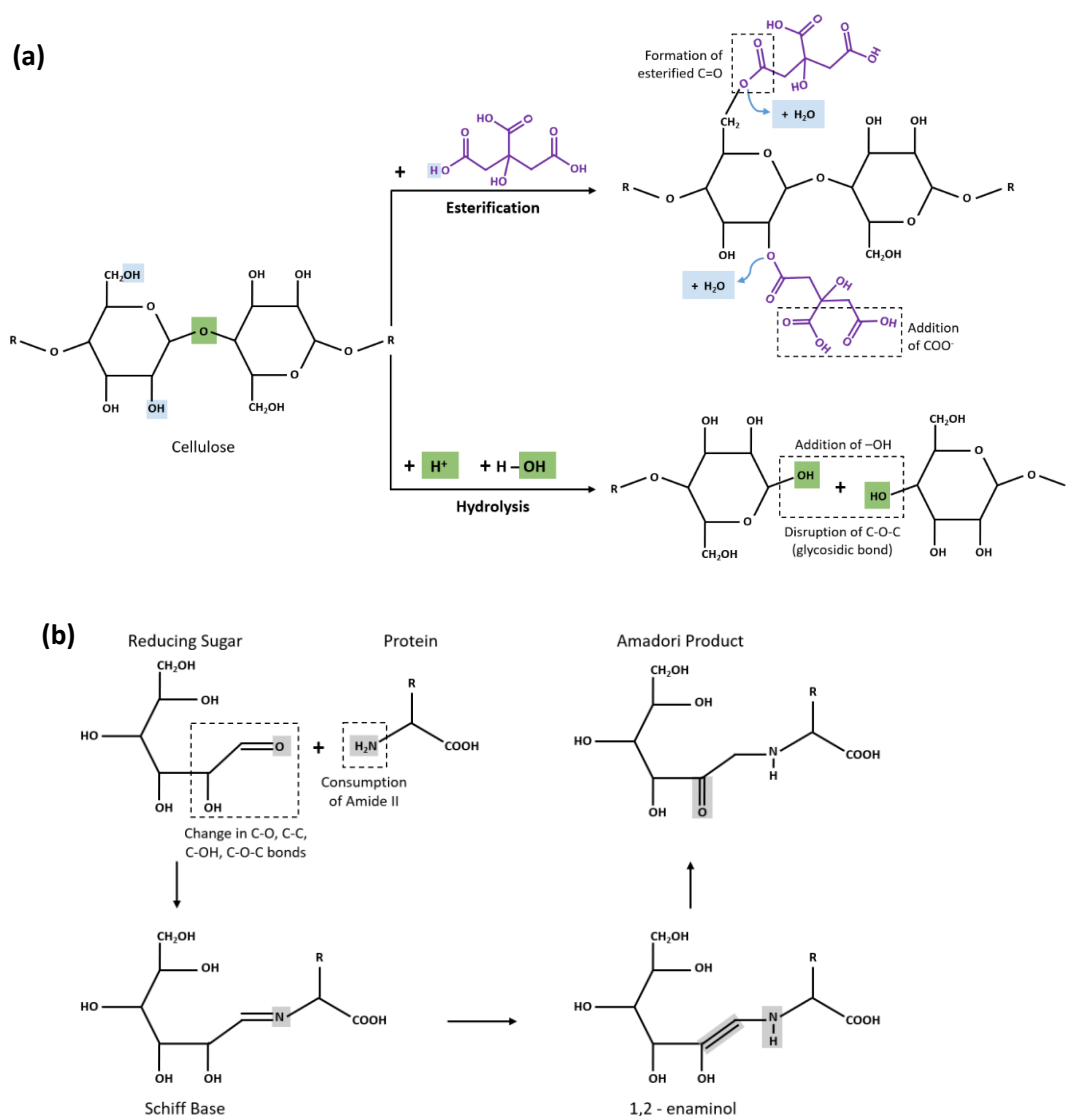


Figure 4.3: (a) Reaction mechanism of citric acid induced esterification [112] [131] [174] or hydrolysis [112] of polysaccharides; (b) Maillard reaction between reducing sugar and protein, adapted from [59] with modifications.

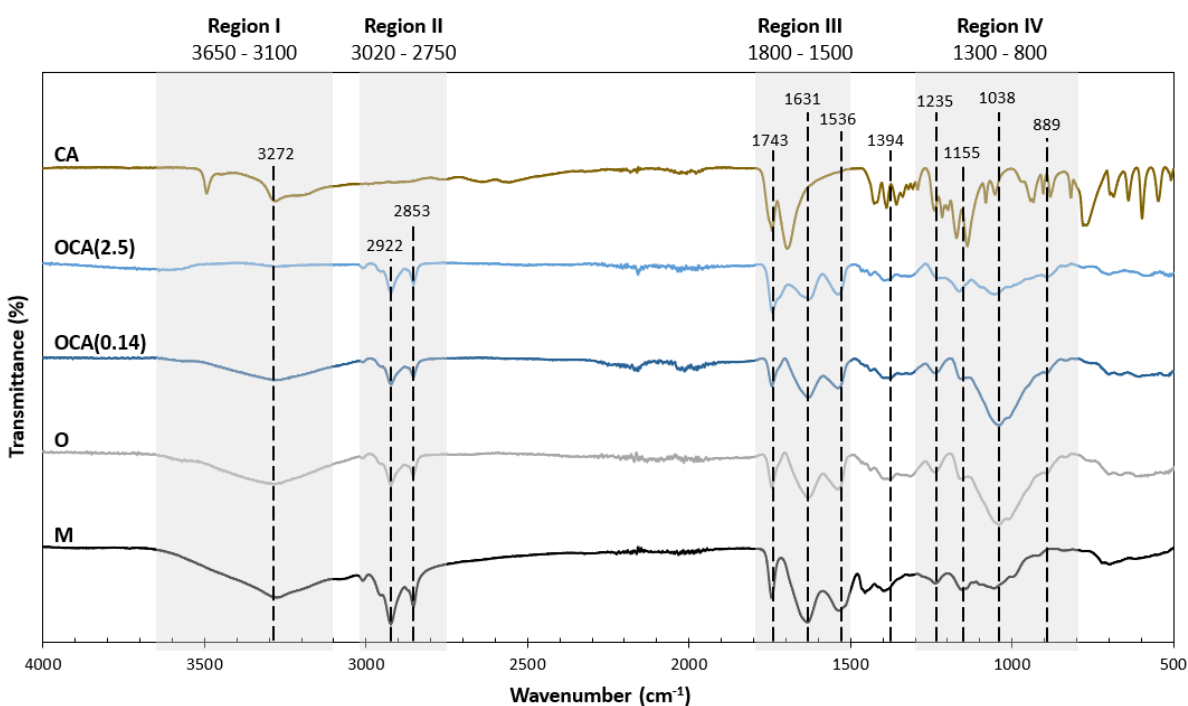


Figure 4.4: FTIR spectra of M, O, OCA0.14 and OCA2.5 in the wavenumber range of 4000 – 500 cm^{-1} .

4.4.1.4 Morphology of Okara

The surface morphology of okara and citric acid modified okara captured with SEM are displayed in **Figure 4.5**. O, had a wrinkled surface comprised of densely stacked flaky layers (lamellar structure) and deformed honeycomb-like structures of the soybean parenchyma layer. Oven drying followed by grinding of O disintegrated the soybean cell layers and deformed its structure. CA0.14 treatment did not significantly alter the structure of okara where the irregular honeycomb-like forms were preserved but appeared less densely packed and more dispersed than in O (magnifications 500x and 1000x). CA2.5 treatment destroyed the parenchyma lamellar layers, collapsed the structure of okara into a smooth surface, and degraded okara into small particles that speckled the surface (magnifications 500x, 1000x). The breakdown of the cellular structure in okara at CA2.5 may be related to the release of pectic substances and explains the gumminess of OCA2.5 observed visually (**Figure 4.1**). Thus, CA treatment could loosen okara structure at 0.14% and cause significant degradation at 2.5%.

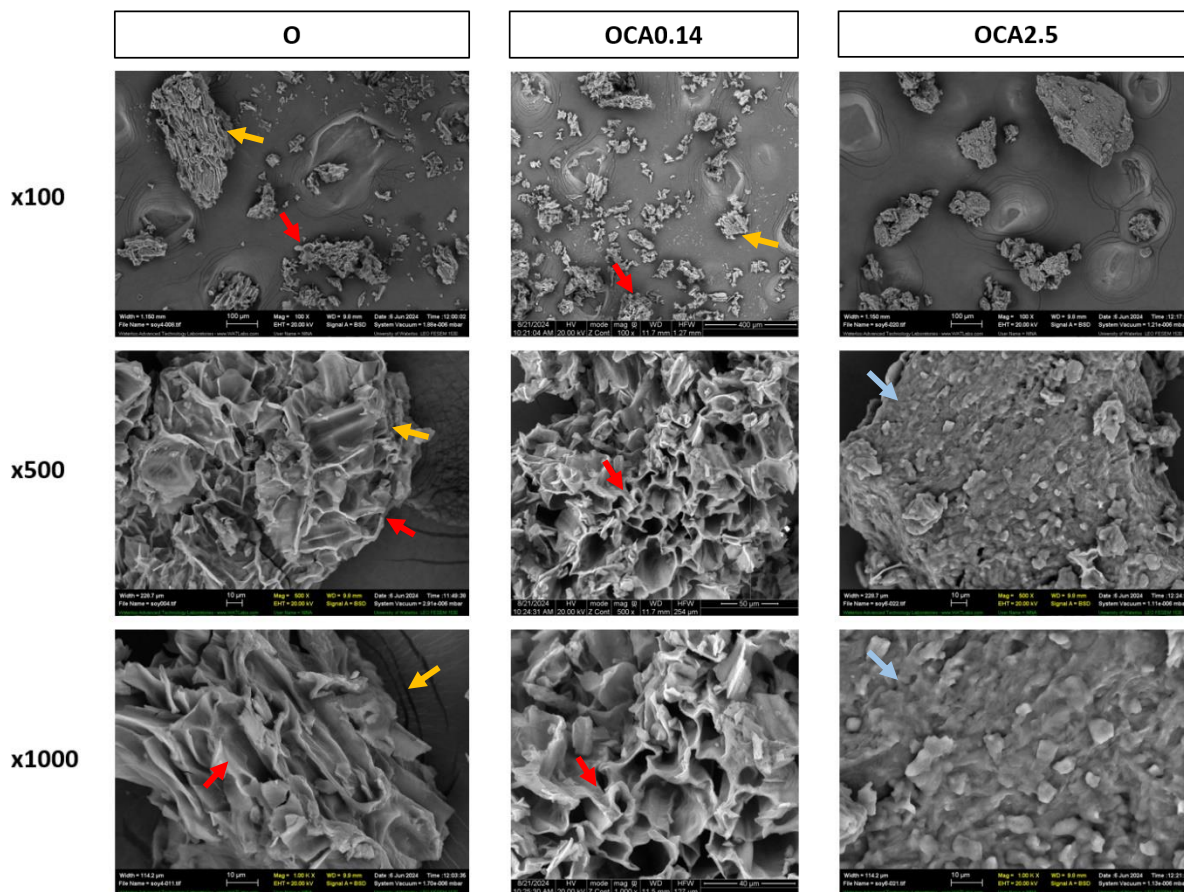


Figure 4.5: SEM micrographs of okara (O) and citric acid modified okara (OCA0.14, OCA2.5), obtained after drying in forced air oven at 60°C, visualized at x100, x500 and x1000 magnifications. The red arrow  indicates regions of honeycomb-like structure of soybean seed parenchyma layer while the deformed, stacked layer structure is represented by the yellow arrow . The blue arrow  signals smooth surface possibly from the release of pectic substances

4.4.1.5 Microstructure Analysis

The microstructure of oven-dried O visualized by CLSM is illustrated in **Figure 4.6** where the polysaccharides labelled with Calcofluor White are depicted in blue and Rhodamine B-stained proteins are represented in red. The irregular honeycomb-like structures and disrupted cell walls indicate the presence of intact cotyledon cells (ICC) [132]. At magnification objectives 20x and 63x, two protein regions are observed. The protein clusters around the honeycomb-like structures are possibly the proteins from soymilk as some soymilk remains stuck to O during extraction. The

other protein region is a thin internal lining within the fibrous structures of O that are possibly the proteins inherently present attached to O polysaccharides.

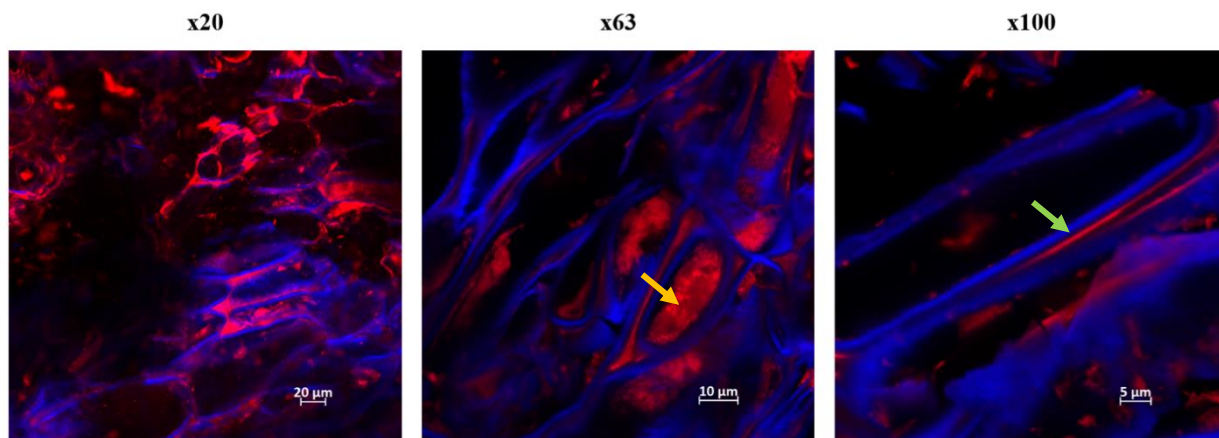


Figure 4.6: CLSM micrographs of dried okara at three magnifications x20, x63 and x100. The honeycomb-like blue regions represents Calcofluor White labelled polysaccharides and the red region represents Rhodamine B labelled proteins of soymilk that was extracted with okara (↗) or proteins within okara structure (↖).

4.4.2 Effect of CA on the pH of Soymilk Okara Mixtures

The pH of M and MO mixtures was 6.40 and 6.46, respectively, and remained unaffected during their preparation. The addition of CA to MO formulations lowered pH. **Figure 4.7** illustrates the pH profile upon introducing CA in the mixtures where a decrease was recorded in the initial 3 minutes following which the pH stabilized at 5.64 – 5.70 in CA0.14 mixtures and 3.51 – 3.62 in CA2.5 mixtures.

The pH change was relatively similar for all CA formulations at a given CA concentration. The initial sharp drop in pH arises from the dissociation of CA releasing H⁺ ions that shift the pH of the aqueous dispersion from near neutral to acidic. Changes in the pH of the system affect the ionization state of the charged amino acid groups of soy proteins, which causes them to either be protonated (pH < pKa) or deprotonated (pH > pKa). At CA0.14, the pH of the mixtures is above the isoelectric point (IEP) of soy proteins while below their IEP at CA2.5. A shift in the ionization state disrupts the electrostatic interactions and hydrogen bonds maintaining the structure of native proteins. An alteration in the protein charges can initiate its unfolding due to localized regions of

repulsion or induce protein aggregation from localized electrostatic attraction, and further influence the pH of mixture. The exposed hydrophobic groups of the unfolded proteins in the aqueous environment will lead to protein aggregation and insolubilization. This further modifies protein structure and its charge, and therefore, alters the pH of the mixtures.

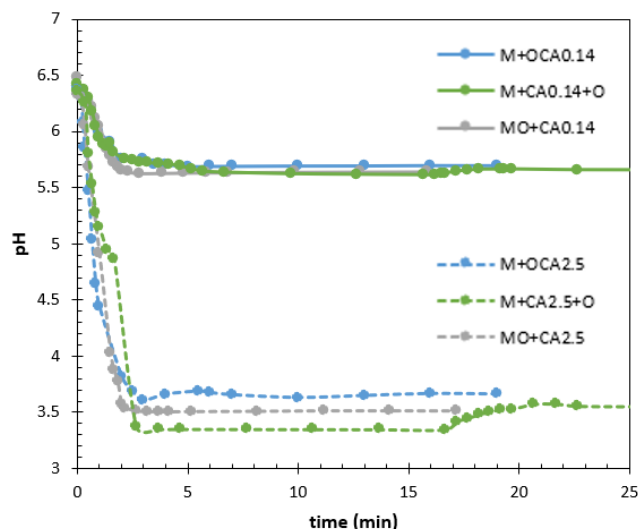


Figure 4.7: Profile of pH over time during the preparation of M+OCA, MO+CA and M+CA+O mixtures at CA0.14 (solid line) and CA2.5 (dashed line).

4.4.3 Analysis of Gel Properties

4.4.3.1 Macrostructure Analysis

The size and distribution of voids within gels reflect the macroscopic porosity and structural defects that can influence gel strength and water distribution. The void distribution detected by ImageJ from the optical images of gel cross-section are presented in **Figure 4.8** and **Table 4.4**.

The M gel displayed a smooth, homogenous cross-section with $0.99 \pm 0.36\%$ void fraction from the traces of air bubbles. The presence of O in MO gel introduced pores and heterogeneity, significantly increasing ($p < 0.05$) the void fraction detected, $2.40 \pm 0.07\%$. O was previously reported to disrupt the homogeneity of tofu and its protein matrix, which could be lowered by reducing the size of O particles [108].

All MO formulations containing CA at a given concentration exhibited similar color and heterogeneous appearance as MO, with comparable ($p>0.05$) average void size. The porosity of the gels was 2.49 – 3.15% at CA0.14 which was statistically similar ($p>0.05$) to MO gel. The porosity of M+CA2.5+O and MO+CA2.5 gels, ranging between 3.3 – 3.5%, was comparable to MO gel whereas M+OCA2.5 gel with a void fraction of 3.8% was statistically more porous ($p<0.05$). As large okara particles cause a more consequential disruption of gel the network, the larger, coarser OCA2.5 particles observed after grinding possibly contribute to the increased pore distribution and area in M+OCA2.5. In summary, CA concentration or addition sequence generally did not alter the macroscopic gel porosity.

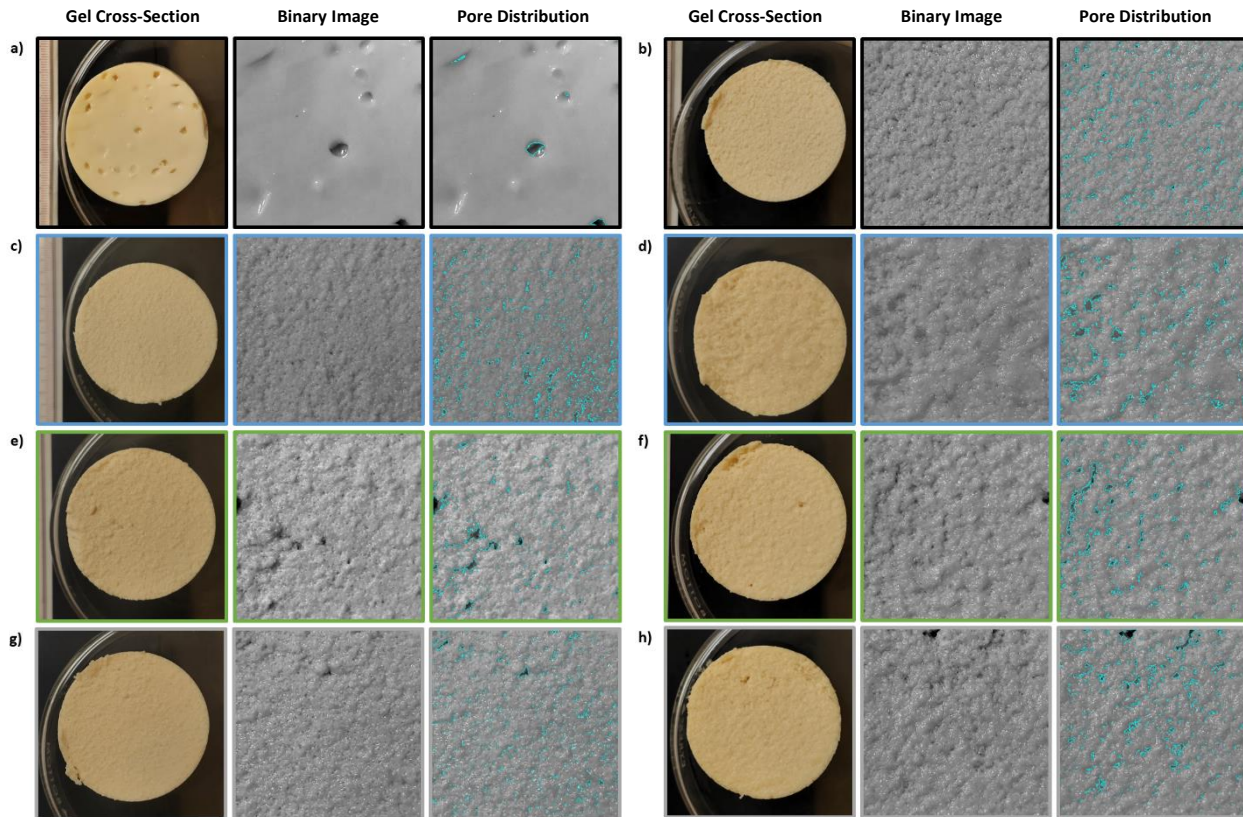


Figure 4.8: Visual observation of the optical image of the internal cross-section of soymilk okara gels with binary images of 30x30mm region generated by ImageJ, and the void regions (blue lines) detected by ImageJ in the soymilk okara: (a) M, (b) MO, (c) M+OCA0.14, (d) M+OCA2.5, (e) M+CA0.14+O, (f) M+CA2.5+O, (g) MO+CA0.14, (h) MO+CA2.5.

Table 4.4: Porosity estimates of the internal cross-section of soymilk okara gels (M, MO, M+CA+O, MO+CA) at citric acid concentrations of 0.14% and 2.5% (w/w) based on the binary images generated with ImageJ. Data is expressed as mean $\pm$ standard deviation (n = 3). Means that do not share a common letter within a column are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$) (n = 3)

Gel	Total Pore Area (mm ²)	Porosity (%)	Average Pore Size (mm)
M	8.95 $\pm$ 3.21 ^a	0.99 $\pm$ 0.36 ^a	0.24 $\pm$ 0.04 ^a
MO	21.62 $\pm$ 0.61 ^b	2.40 $\pm$ 0.07 ^b	0.03 $\pm$ 0.01 ^b
M+OCA0.14	28.82 $\pm$ 7.28 ^{b,c}	3.15 $\pm$ 0.88 ^{b,c}	0.03 $\pm$ 0.01 ^b
M+CA0.14+O	27.99 $\pm$ 2.83 ^{b,c}	3.01 $\pm$ 0.47 ^{b,c}	0.04 $\pm$ 0.00 ^b
MO+CA0.14	24.51 $\pm$ 0.19 ^b	2.49 $\pm$ 0.30 ^b	0.04 $\pm$ 0.02 ^b
M+OCA2.5	35.16 $\pm$ 3.64 ^c	3.82 $\pm$ 0.40 ^c	0.06 $\pm$ 0.03 ^b
M+CA2.5+O	31.61 $\pm$ 1.82 ^{b,c}	3.53 $\pm$ 0.25 ^{b,c}	0.05 $\pm$ 0.00 ^b
MO+CA2.5	29.31 $\pm$ 4.07 ^{b,c}	3.26 $\pm$ 0.43 ^{b,c}	0.05 $\pm$ 0.03 ^b

4.4.3.2 Gel Syneresis

Water in gels is present in unbound and bound form which is influenced by the gel constituents, and impacts its texture properties. The unbound water occupying the macro-pores of gels exuded passively during storage, or syneresis, is a result of the gel network becoming increasingly denser as the protein aggregates in the network continue to associate through electrostatic interaction or hydrogen bonds during refrigerated storage [107] [150]. Syneresis observed from the gels is presented in **Figure 4.9**. The M gel visually has a homogeneous, non-porous structure and a dense, compact protein network that is observed in microstructure imaging (**Figure 4.14**). Lacking pores in the gel network that are otherwise a pathway for release of water, M gel thus released minimal liquid of 0.37% after overnight storage. MO exhibited negligible syneresis, 0.05%, in spite of its porous texture which is likely attributed to the inherent water-absorbing characteristic of O that effectively retained water in the network [150].

The presence of CA in MO gels displayed differences in water released at the two CA concentrations. Syneresis was negligible at CA0.14, 0.01 – 0.04%, and was statistically similar to MO gel ($p > 0.05$). At CA2.5, gels experienced a significantly higher syneresis ($p < 0.05$), 1.25 –

2.9%, than MO gel, with the exception of MO+CA2.5. The rapid assembly of the protein network due to faster acidification rate at CA2.5 (**Figure 4.7**) limits protein-water interactions. This was commented previously in a study on pressed soymilk gels using organic acid coagulant where a limited time for interactions contributed to high expressible water content with increasing acid concentration [83]. The higher syneresis at CA2.5 may have resulted from protein crosslinking during storage due to the presence of excess CA. The crosslinking of polypeptide chains during storage of salt-coagulated soymilk gels in the absence [179] and presence of micro- or nano-okara dietary fiber [150] was associated with increased syneresis. These crosslinking interactions make the protein matrix of CA2.5 gels denser that forces water to diffuse out from the gels. The microstructure of CA2.5 gels relative to CA0.14 soymilk okara gels exhibited a denser protein aggregate network (**Figure 4.14**).

4.4.3.3 Water Holding Capacity (WHC)

WHC represents the water bound to the hydroxyl groups or entrapped in the micro-pores of the gel network via capillary action preventing water release even when the gel is subjected to an external centrifugal force [150]. **Figure 4.9** presents the water retained in the gels.

WHC was observed to be statistically similar ($p>0.05$) between M (73.5%) and MO (77.8%), and indicated that a major portion of the water was effectively entrapped the gel matrix. The presence of O had limited effect on the bound water, despite the disruption it caused to the protein network producing a heterogeneous gel. This is attributed to the inherent ability of dietary fibers in binding water and their rigidity that resist gel compression during centrifugation preventing waster loss [120] [175]. The soymilk okara gels containing CA had a similar ($p>0.05$) WHC as MO gel, except the statistically lower ($p<0.05$) WHC of M+CA0.14+O gel (62.4%), suggesting the accelerated gelation in the particulate network of CA-containing gels had a negligible influence on protein-water interactions.

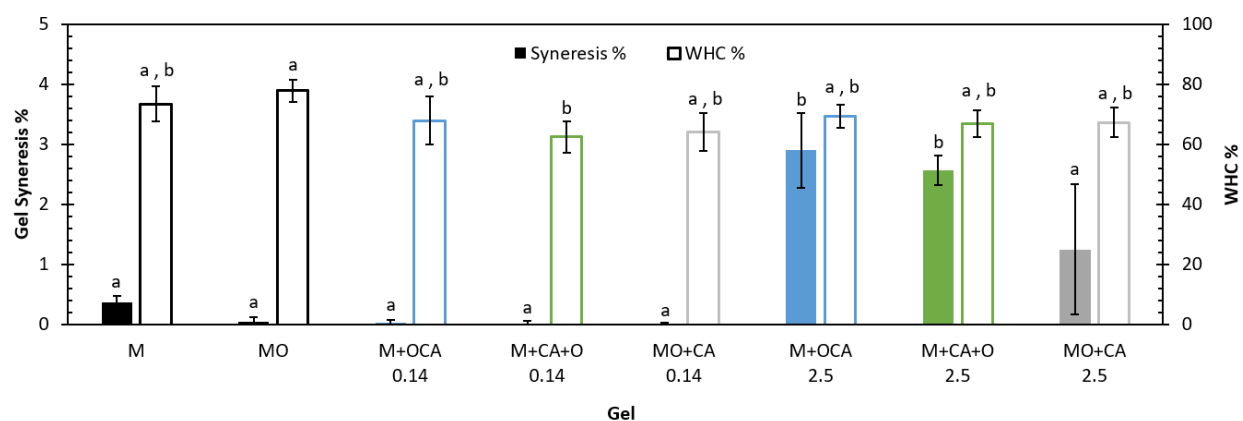


Figure 4.9: Syneresis and water holding capacity (WHC) of M, MO, M+OCA, M+CA+O and MO+CA gels at two CA concentrations, 0.14% and 2.5% (w/w), after overnight refrigeration at 4°C. Data is expressed as mean $\pm$ standard deviation ($n = 3$), with the error bar representing the standard deviation. Statistically different means (ANOVA followed by Tukey's post-hoc: $p < 0.05$) do not share a common letter

4.4.3.4 Protein-Protein Interactions

The chemical forces involved in the protein network of the soymilk okara gels, estimated from the proteins solubilized upon disruption of targeted non-covalent interactions, are illustrated in **Figure 4.10**, with their relative magnitudes presented in **Table 4.5**. Protein gels are primarily assembled through non-covalent hydrogen bonds, electrostatic and hydrophobic interactions. Electrostatic interactions and hydrogen bonds have a relatively minor role in all gels, contributing less than 10% collectively. These interactions are disrupted during thermal gelation as commented for heat-induced gelation of soymilk with and without an organic acid coagulant [83]. Hydrophobic interactions are the main driving force assembling the network of all soymilk gels, which is consistent with previous reports on its key role in forming soy protein gel network [83] [151].

Some differences in the magnitude of hydrophobic interactions, measures as protein solubilized, was recorded according to the gel type. Hydrophobic interactions were statistically different in MO gel in comparison with M gel, decreasing by 40%. The heterogeneous texture of MO gel indicates that O sterically hinders the self-aggregation of proteins into a continuous network, thereby decreasing protein interactions. O can restrict the unfolding of protein native structure to expose buried hydrophobic groups which is a prerequisite to initiate interactions and subsequently

gelation. SPI in the presence of okara dietary fibers was reported previously to have lower surface hydrophobicity and exposed hydrophobic regions which hindered self-aggregation of SPI during thermal treatment [104].

The MO gels with CA0.14 possessed statistically comparable ($p>0.05$) hydrophobic interactions to MO gel suggesting that coagulation of soymilk proteins had an insignificant influence on chemical interactions at this concentration. At CA2.5, the hydrophobic interactions in M+OCA and M+CA+O gels were similar ($p>0.05$) to MO gel and these gels at CA0.14 indicating that CA concentration did not alter chemical interactions. In contrast, MO+CA2.5 gel contained significantly higher ($p<0.05$) hydrophobic interactions than MO and MO+CA0.14 soymilk okara gels, with a large variation. These findings suggest that generally, the sequence of CA addition did not notably affect protein interactions in MO gels at both CA concentration, with MO+CA2.5 gel being an exception.

Table 4.5: Relative magnitudes of ionic interactions, hydrogen bonds and hydrophobic interactions (%) in M, MO, M+OCA, M+CA+O, MO+CA gels at CA0.14 and CA2.5 concentration. Data is presented as mean $\pm$ standard deviation. Means that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$) ($n = 3$)

Gels	Ionic Interaction (%)	Hydrogen Bonds (%)	Hydrophobic Interaction (%)
M	1.51 ± 0.95^a	2.46 ± 0.70^a	96.01 ± 0.99^a
MO	0.85 ± 0.75^a	5.85 ± 2.84^a	93.29 ± 2.15^a
M+OCA0.14	2.22 ± 1.70^a	3.91 ± 2.34^a	93.85 ± 3.57^a
M+CA0.14+O	1.96 ± 0.72^a	2.90 ± 0.72^a	95.13 ± 1.34^a
MO+CA0.14	2.09 ± 1.72^a	7.41 ± 6.78^a	90.49 ± 7.44^a
M+OCA2.5	1.28 ± 2.22^a	3.62 ± 3.42^a	95.09 ± 1.65^a
M+CA2.5+O	0.10 ± 0.17^a	3.65 ± 0.63^a	96.24 ± 0.51^a
MO+CA2.5	0.39 ± 0.36^a	2.00 ± 1.75^a	97.59 ± 1.51^a

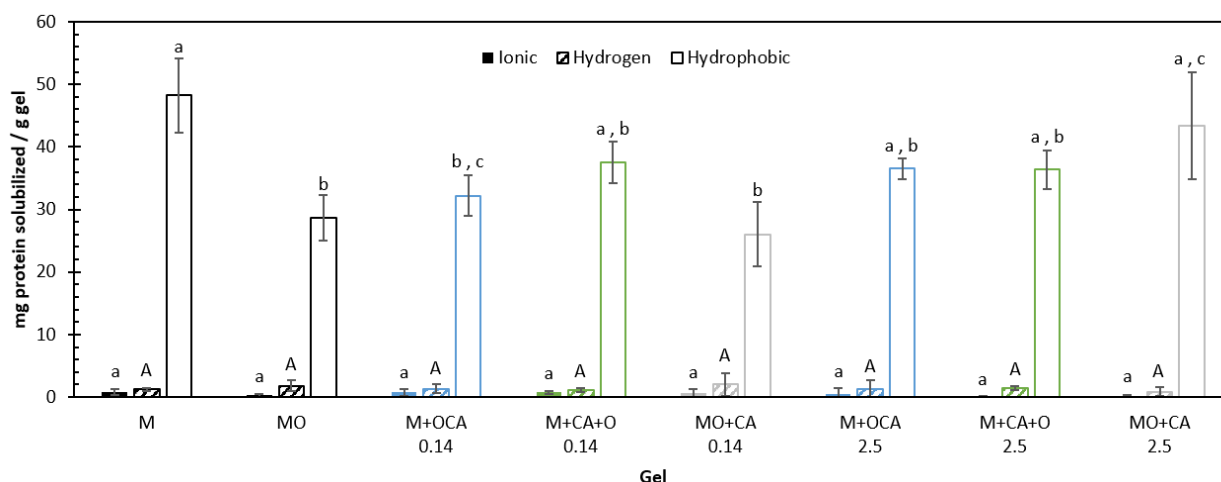


Figure 4.10: Chemical forces of ionic interactions, hydrogen bonds and hydrophobic interactions given as protein solubilized in M, MO, M+OCA, M+CA+O, MO+CA gels at CA0.14 and CA2.5 concentration. Data is presented as mean $\pm$ standard deviation. Means that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$) ($n = 3$).

4.4.3.5 Mechanical Properties

The stress-strain curves upon uniaxial compression of soymilk okara gels depict the progress of gel deformation over three regions of elastic deformation, failure and response of the fragments to after gel failure. **Figure 4.11** summarizes the distinctions observed in the stress-strain curve profiles of the soymilk okara gels depicted in **Figure 4.12 (a)**. The weak physical gels prepared in this study are neither sufficiently brittle to fracture nor ductile to deform flexibly and therefore, the gel failure is described to be more brittle-like or ductile-like in characteristic. These properties will be discussed in regard to the stress-strain curve profile of the gels.

The stress-strain curve in region I, which is associated with linear and non-linear elastic response or the temporary deformation of gels upon the application of a compressive stress, displayed different slopes according to the gel type. M gel had the lowest slope that extended to a higher strain while the presence of O in the other gels increased the slope. The gels retain their original form on removing the load applied and the slope of the curve represent gel stiffness, or Young's modulus. In region II, the load on the gel initiates its permanent deformation and subsequently failure. The permanent deformation region in the stress-strain curve of the soymilk gels, displayed a peak that varied in intensity across the gels. The sharp peaks of M and all CA0.14 soymilk okara

gels suggested brittle-like characteristic of these gels. In contrast, MO and all CA2.5 gels exhibited shallow peaks that is characteristic of a more ductile-like material. A previous study on pea, faba and mung bean protein isolate gels associated the shallow and sharp peaks to the failing of gels that is more ductile- or brittle-like, respectively [146]. The stress-strain curve in region III following gel failure either flattened in M and MO gels or displayed a positive slope of various degree in the soymilk okara gels containing CA. However, relevant information could not be derived from this region as it records the response after the gels had failed (**Figure S.6**).

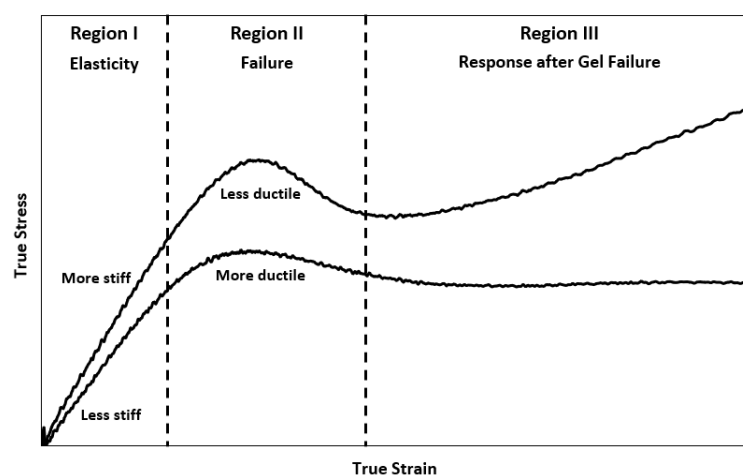


Figure 4.11: Hypothetical representation of the stress-strain curve as a gel is deformed under compression. The stress-strain curve is divided into three regions. The linear region I provides information on the elasticity of the gels, with higher slopes corresponding to higher gel stiffness. Region II captures the gel failure behavior, with the peak intensity reflecting the ductility of the gel and the response of the gels after failure is exhibited in region III.

The mechanical properties of the soymilk okara gels extracted from regions I and II upon subjection to compression force are displayed in **Figure 4.12 (b-d)**. A brief explanation of the texture associated with the gel failure properties is provided in **Table 4.6**. The failure properties of the homogenous M and heterogeneous MO reveal a significant ($p < 0.05$) increase in the failure stress and Young's modulus but a decrease in failure strain upon incorporating O. O transformed the weak, deformable yet cohesive M gel into a stronger, stiffer but less cohesive MO gel.

The effect of CA on the mechanical properties of MO gel proceeded differently according to CA concentration. The CA0.14 soymilk okara gels had statistically ($p > 0.05$) similar failure stress,

failure strain and Young's Modulus as MO gel. Defects from heterogeneity weaken and initiate gel disintegration [156] [186]. The resemblance in heterogeneity and coarseness of MO gels with and without CA0.14 (**Table 4.4**) relates to their comparable mechanical properties. The gels at CA2.5 have statistically similar ($p>0.05$) failure stress as MO, however, failure strain (cohesivity) and Young's Modulus (stiffness) depended on the sequence of CA addition. The M+OCA2.5 gel had similar cohesiveness and stiffness to MO gel, whereas M+CA2.5+O and MO+CA2.5 gels were significantly ($p<0.05$) less cohesive but stiffer than MO gel. In M+OCA gels at both CA concentrations, a significant portion of CA is consumed in treating O independently which reduces CA available to interact with soymilk. In contrast, all added CA in the other two formulations interacts directly with soymilk, which along with the influence of O in imparting rigidity, facilitating localized clustering of proteins and higher protein aggregation level reflect the improved properties at lower pH (3.51 – 3.66).

The above observations suggest that the pre-aggregation of M initiated with CA enhanced gel stiffness, with the effect more pronounced at CA2.5, while gel strength was not altered and gel cohesivity deteriorated. The CA-modification of O is not effective in improving the mechanical properties of soymilk okara gels. The statistically similar gel strength of most gels could be associated with the high load cell utilized (500 N) and the low sensitivity of the equipment to detect cracks in soft materials like soy gels.

Table 4.6: Texture properties of soy gels and their physical interpretation in literature

Property	Physical Interpretation	Characteristic
Failure stress	Relates to the extent to which gel withstands compression force before disintegrating and resists regional plastic deformation [146]. Ductile materials deform significantly, plastically, and absorb the applied force before failing whereas brittle materials deform elastically and fracture soon after yield stress. [106]	Strength or firmness
Failure strain	Relates to the degree of deformation before a gel fails (or fractures), reflecting its resistance to crumble during compression arising from the strength of internal bonds that assemble the gel structure. High failure strain equips a gel with high resistance [156].	Cohesiveness
Young's Modulus	Ratio of failure stress to failure strain which provides a measure of the resistance offered by a gel to disintegrate its surface [146]	Stiffness

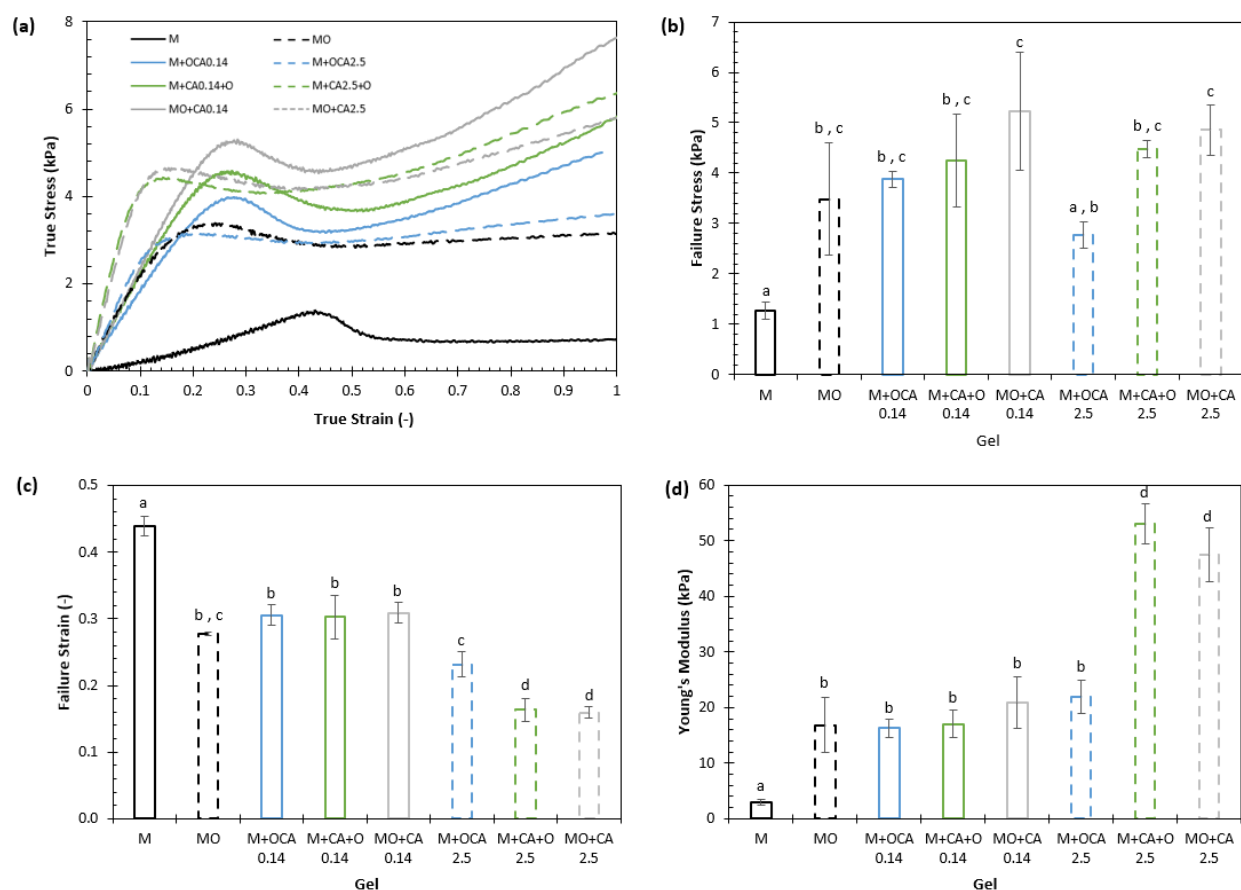


Figure 4.12: Mechanical properties of soymilk okara gels (M, MO, M+OCA, M+CA+O and MO+CA) at two citric acid concentration levels of CA0.14 [solid] and CA2.5 [dashed]: (a) Typical stress – strain curves, (b) True failure stress (kPa), (c) True failure strain, (d) Young's modulus (kPa). Data of (b – d) expressed as mean $\pm$ standard deviation ($n = 3$) where means that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$).

4.4.3.6 Viscoelastic Properties

Elasticity, viscosity and the extent of viscoelasticity of soymilk okara gels, represented as G' , G'' and $\tan(\delta)$, are illustrated in **Figure 4.13** and **Table 4.7**. The consistently higher storage modulus (G') than loss modulus (G'') in all gels, with no crossover of G' and G'' , is characteristic of elastic gels comprised of a continuous network [88] [51]. The ratio between G' and G'' , or loss tangent, $\tan(\delta) < 1$ furthermore reflects the solid-like disposition of gels assembled from sufficient interactions within the protein matrix [190]. The solid-like behavior of all gels is supported by the decrease in complex viscosity (η) with increasing frequency, describing the flow resistance of

viscous components [89]. A more prominent solid-like behavior is associated with an intense decrease in complex viscosity [89]. The frequency dependency of η is more notable in MO than M gel, and intensified further upon adding CA in the gels. The firmness of gels perceived qualitatively along with the improved stiffness and viscoelasticity supports the solid-like character of the gels. The weak frequency dependency of G' and G'' indicated by the power law model parameters n' , $n'' > 0$ suggests that all gels have characteristics of weak physical gels that are predominantly formed by non-covalent bonds [155]. This is supported by the analysis of protein-protein interactions where non-covalent hydrophobic interactions were the main driving force in assembling the gel network (**Figure 4.10**). The viscoelastic properties of the soymilk okara gels were compared based on the power law coefficients, G_o' and G_o'' . In comparison to M gel, the presence of okara in MO significantly ($p < 0.05$) increased G_o' and G_o'' . The rigid O fibers reinforced the structure of MO which is also reflected in its enhanced mechanical properties. The contribution of O to elasticity (G') in soymilk okara gel was previously attributed to O reducing the free water in gel which otherwise facilitates the flow of protein matrix and transferring the applied shear stress from the protein network to O [191].

The presence of CA in MO gels influenced the viscoelasticity differently depending on CA concentration or addition sequence. At CA0.14, M+OCA and MO+CA gels exhibited a significant increase ($p < 0.05$) of threefold in elasticity ($G_o' = 36.1 - 37.9$ kPa) and twofold in viscosity ($G_o'' = 7.7 - 7.8$ kPa). M+CA0.14+O gel exhibited a 4.5-fold increase in elasticity ($G_o' = 55.6$ kPa) and a 3.5-fold increase in viscosity ($G_o'' = 11.5$ kPa). The reduction in pH upon the addition of CA0.14 was visually observed to induce soymilk coagulation prior to thermal gelation. Similarities in porosity and mechanical properties of these gels with MO gel suggests that other factors such as difference in microstructure of CA0.14 soymilk okara gels caused by the coagulation of soymilk prior to thermal gelation contributed to the enhanced viscoelasticity. Protein pre-coagulation benefited the texture of M+CA+O gel while the presence of okara in M+OCA and MO+CA gels had some inhibitory effects on protein aggregation at this CA concentration that caused a variation in the viscoelastic properties. Lowering the pH of SPI in a previous study enhanced protein participation and formed highly elastic, coarse gel assembled from thick network strands of large protein aggregates, that are more resistant to shear deformation than thin protein strands [90].

CA2.5 concentration had a more pronounced impact on the viscoelasticity of MO gel. The M+OCA2.5 gel had a significant increase ($p < 0.05$) of threefold and twofold in G' (32.6 kPa) and G'' (7.2 kPa), respectively. M+CA2.5+O and MO+CA2.5 gels exhibited a further increase of nearly 4.5-fold in elasticity ($G' = 56.7 - 57.4$ kPa) and 4-fold in viscosity ($G'' = 12.6 - 13$ kPa) when compared to MO gel. The higher viscoelasticity of M+CA2.5+O and MO+CA2.5 gels in contrast to M+OCA2.5 may be due to all added CA available that facilitated increased protein aggregation and protein participation in the gel network.

The findings from rheology suggest that the presence of CA in the soymilk okara gels had an incremental effect on gel viscoelasticity. The CA-modification of O was not necessary as the viscoelasticity improved similarly in MO+CA0.14 or superiorly in M+CA0.14+O, M+CA2.5+O, MO+CA2.5 gels by inducing pre-aggregation of M with CA.

Table 4.7: Power law parameters of Equation 4.6 and Equation 4.7 obtained from frequency sweep tests, expressing the viscoelastic properties of M, MO, M+OCA, M+CA+O and MO+CA gels at CA0.14 and CA2.5. Data is expressed as mean $\pm$ standard deviation ($n = 3$). Means within one column that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$).

Gel	G' (kPa)	n'	R_1^2	G'' (kPa)	n''	R_2^2
M	0.54 ± 0.16^a	0.11 ± 0.01^a	0.80	0.09 ± 0.03^a	0.09 ± 0.01^a	0.39
MO	12.45 ± 0.63^b	$0.13 \pm 0.01^{a,b}$	1.00	3.13 ± 0.11^b	0.09 ± 0.00^a	0.99
M+OCA0.14	37.89 ± 6.97^c	$0.13 \pm 0.01^{a,b}$	0.99	7.73 ± 1.57^c	$0.18 \pm 0.00^{b,c}$	1.00
M+CA0.14+O	55.58 ± 3.37^d	0.14 ± 0.00^b	0.99	11.51 ± 0.64^d	$0.17 \pm 0.00^{b,c,d}$	0.99
MO+CA0.14	36.07 ± 1.16^c	0.14 ± 0.01^b	0.99	7.79 ± 0.24^c	$0.18 \pm 0.02^{b,c}$	1.00
M+OCA2.5	32.59 ± 3.49^c	0.14 ± 0.01^b	0.99	7.21 ± 0.79^c	0.19 ± 0.00^b	0.99
M+CA2.5+O	57.39 ± 1.18^d	$0.13 \pm 0.01^{a,b}$	0.99	12.97 ± 0.57^d	0.14 ± 0.01^d	0.99
MO+CA2.5	56.72 ± 2.75^d	$0.13 \pm 0.00^{a,b}$	0.99	12.60 ± 0.57^d	$0.15 \pm 0.01^{c,d}$	0.99

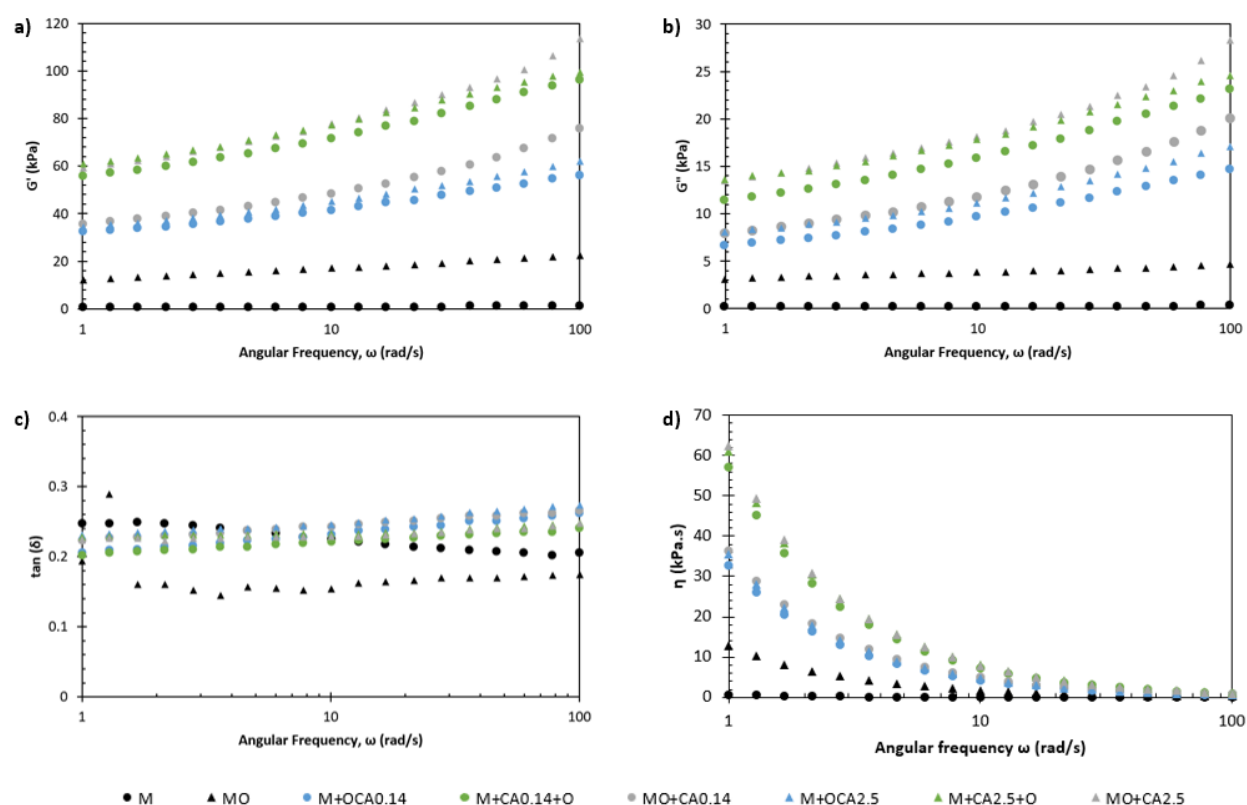


Figure 4.13: Viscoelastic properties of soy gels (M, MO, M+OCA, M+CA+O, MO+CA): Typical curves of a) storage modulus G' , (b) loss modulus G'' , c) $\tan(\delta)$ and (d) complex viscosity over angular frequency (ω).

4.4.3.7 Microstructure Analysis

The microstructures of soymilk okara gels visualized with CLSM at different magnifications are presented in **Figure 4.14**, with a focus on the assembled protein matrix. The distribution and structure of okara in the gels are presented in **Figure S.11**. The soymilk proteins stained with Rhodamine B are exhibited in red whereas the blue structures represent okara stained with Calcofluor White. The black, unstained regions are comprised of micropores, water and oil. The micrographs of CA2.5 gels are limited to protein regions due to the unstable 405 nm laser required for the visualization of Calcofluor White staining.

The M gel shows a compact, dense matrix of proteins arranged in a strand-like network, with the strands visible at 100x magnification. This observation is consistent with previous studies on

thermal aggregation of globular soy proteins [156]. The soluble dietary fibers of soymilk are not detected. The MO gel retained the dense protein network of M gel, but the continuity of the network is disrupted by O distributed.

The microstructure of M+OCA, M+CA+O and MO+CA gels at both CA concentrations have a particulate protein network comprised of spherical protein aggregates. The protein network reflects protein pre-coagulation due to the decrease in pH, which was also visually observed from the insoluble aggregates formed in the mixture. The protein aggregates are dispersed with black regions indicating micropores in the protein network. A similar transition in the microstructure from a homogeneous to a particulate network of spherical aggregates upon lowering pH was observed in a previous study on SPI gel [101]. The change in gel microstructure explains the improvement in gel viscoelastic properties of MO gels with CA (**Table 4.7**).

In comparison to MO gel, the protein matrix of CA0.14 soymilk okara gels became less dense with higher content of smaller black regions. The CA2.5 gels have a denser protein matrix than the CA0.14 soymilk okara gels with fewer black regions. The differences in the size of protein aggregates at CA0.14 (pH 5.64 – 5.67 > IEP) and CA2.5 (pH 3.51 – 3.66 < IEP) which were formed from native and denatured proteins, respectively, relate to their pH differences and their proximity to the IEP of soy proteins [101]. The protein aggregates in M+OCA gels are more widely dispersed than those in the M+OCA and M+CA+O gels at both CA concentrations. As discussed previously, the residual CA when OCA is added to soymilk is low since CA is consumed in modifying okara. In contrast, all CA is available when added to soymilk in M+CA+O and MO+CA gels. As a result, protein participation is higher in the latter gel formulations which is reflected in their denser protein network. Similar okara structures are observed in all three formulations at both CA concentrations (**Figure S.11**). This observation suggests that the degradation of O with CA treatment, observed by FTIR and TGA, with more pronounced effects in OCA2.5 than in OCA0.14, did not improve the distribution of O. Therefore, CA modification of O is not essential to improve its integration in the protein network, which is consistent with the observations of gel mechanical and viscoelastic properties.

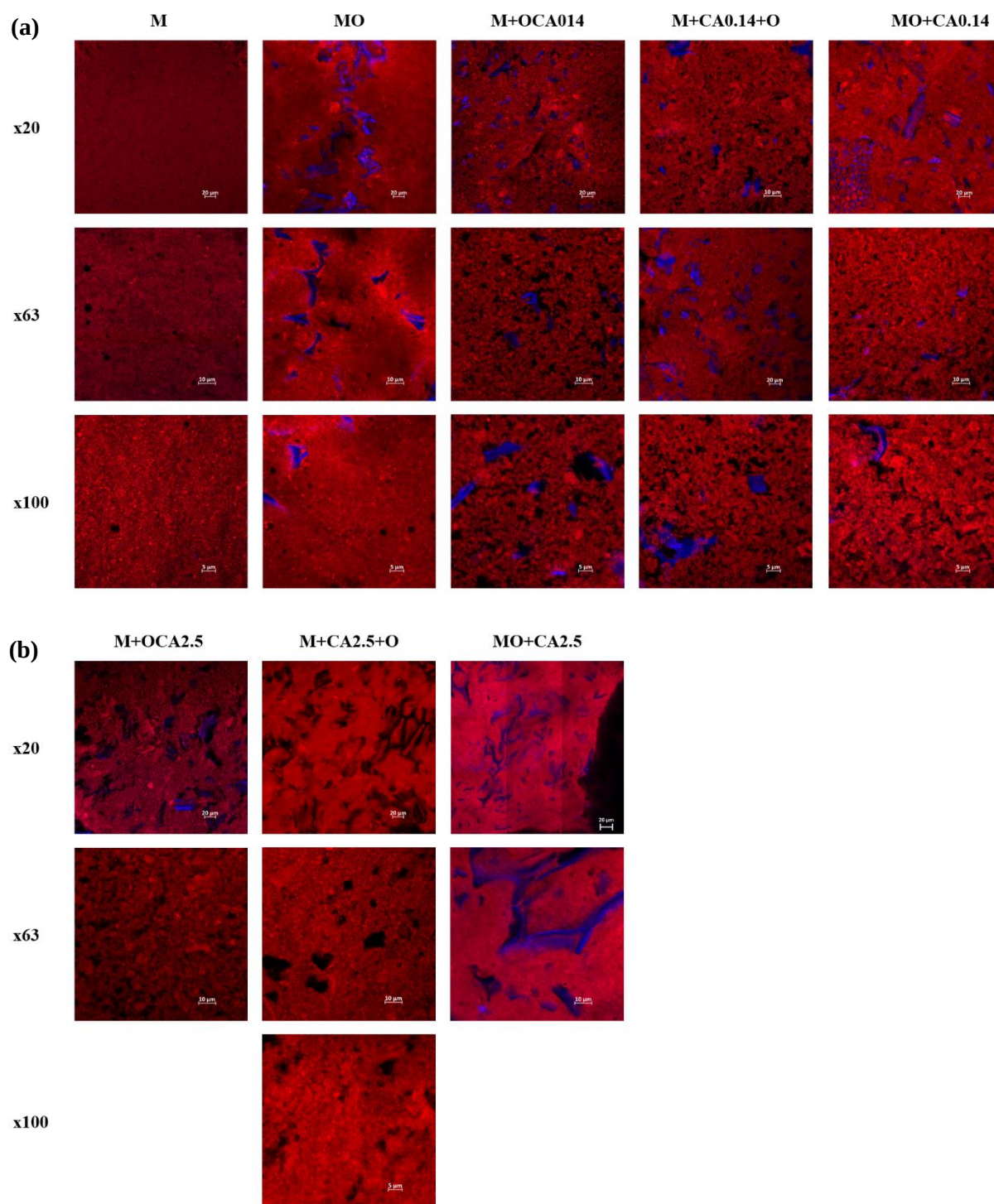


Figure 4.14: CLSM micrographs of soymilk okara gels stained with Rhodamine B labelled proteins (red region) and Calcofluor White stained okara polysaccharide (blue region) at three magnifications x20, x63 and x100. (a) M, MO, M+OCA0.14, M+CA0.14+O, MO+CA0.14 (b) M+OCA2.5, M+CA2.5+O, MO+CA2.5.

4.4.4 Proposed Mechanism of Gelation

Heat induced gelation of soy proteins involves two steps [96] [153] [188]. A pre-requisite of gel formation is denaturation of soy proteins wherein the soy proteins unfold, dissociate as the hydrogen bonds are disrupted, and expose the buried hydrophobic groups. The exposed hydrophobic regions of soy proteins subsequently interact to re-associate into aggregates that are different from the native soy protein structures, which further interact to assemble the three-dimensional gel structure. In this section, potential mechanisms associated with the preparation of MO gels with CA added in different sequences, M+OCA, M+CA+O and MO+CA and the subsequent thermal gelation, will be discussed. The preparation of the gels involves two stages. Stage I is the response of the proteins in soymilk to pH decrease upon CA addition and stage II is the thermal gelation of the mixture. An overview of the mechanisms for each stage is proposed in **Figure 4.15** and **Figure 4.16**, and detailed in the next sections.

4.4.4.1 Citric Acid Modification of Okara

The citric acid induced modification to the structure and functionality of okara was adapted from the mechanism proposed in [59] and is illustrated in **Figure 4.15**. The representation of okara fibers presented in [59] was modified to include the proteins embedded in the okara structure based on the microstructure observations obtained by CLSM and from the amide peaks detected in the FTIR spectra. The representation of pectin with an egg box model described by [59] was not considered in this study because it depicts the extraction and gelation of pectin through chelation with calcium ions [181] and does not capture pectin in its native form. The cell wall models of plants [185] and pea hull [184] represent pectin as strands attached to the other fiber constituents such as cellulose via non-covalent bonding [170] [182].

The qualitative analysis of O and OCA indicated differences in their color and texture. OCA0.14 had the lightest hue followed by O, both possessing a soft, crumbly texture. The darker ochre hue and gumminess of OCA2.5 indicated structural modification of okara from the citric acid induced reactions, including Maillard reaction and release of pectic substances.

The thermal degradation analysis of OCA revealed minimal modification of the constituent polysaccharides at CA0.14 while significant differences were recorded in OCA2.5 when compared

to O. The TGA/DTG curves indicated a disruption of hydrogen bonds as the thermal degradation of OCA2.5 was initiated at a lower temperature. As the weight loss in O over 150 – 220°C is associated with the degradation of soluble and low molecular weight polysaccharides, the accelerated degradation in OCA2.5 suggests the presence of pectic substances and hydrolyzed fibers. However, pure CA degrades at 150 – 240°C and the overlap in the decomposition of excess CA in OCA2.5 makes it difficult to confirm the presence of soluble or low molecular weight polysaccharides. Therefore, the observations of TGA analysis were further explored with FTIR.

CA facilitated structural changes in O, specifically at CA2.5, that were captured from the FTIR peaks associated with polysaccharide functional groups. The **decomposition** of okara into **oligosaccharides** was detected in OCA which increased with increasing CA concentration as the peaks of C-O, C-H and C-OH decreased in intensity. The vibrations associated with the rhamnogalacturonan backbone or sugar side chains of **pectin** were not confirmed as the peaks overlap with the C-O, C-H, C-OH and C-O-C bond vibrations of other polysaccharides. The decreased intensity of COO⁻ vibration in OCA2.5 may have resulted from the esterification of COO⁻ in galacturonic acid groups of released **pectic substances**, although this peak overlaps with the vibration of okara protein and hemicellulose. Citric acid induced **esterification** of okara polysaccharides was detected in OCA2.5 as the ester carbonyl band increased in intensity. Additionally, the **Amadori products of Maillard reaction** was detected at 1160 – 1155 cm⁻¹, although this overlaps with C-O-C vibration suggesting the change in peak intensity was possibly due to polysaccharide conformation changes.

The SEM micrographs of okara confirmed the structural changes in okara after citric acid treatment observed in TGA and FTIR. The compact, layered structure of O was disrupted as it loosened in OCA0.14 and the hexagonal parenchyma structures were not detected in OCA2.5.

4.4.4.2 Citric Acid Addition in Soymilk Okara Mixtures

The mixtures containing CA were not characterized extensively and the mechanism proposed in **Figure 4.15** is based on the visual observations recorded on the formation of insoluble protein aggregates and the increased viscosity of the mixture at both CA0.14 and CA2.5 concentrations. As the pH of the mixture decreased from 6.4 ± 0.05 in M or MO mixture to 5.64 - 5.67 (pH>IEP)

in CA0.14 mixtures and 3.51 - 3.66 (pH<IEP) in the presence of CA2.5, the charge of proteins was altered to net negative or positive, respectively [102]. The surface charge modification induced protein unfolding, exposure of hydrophobic groups and subsequently protein aggregation via non-covalent hydrophobic interactions [90]. A previous study on soy proteins and 3.53% CA concentration reacted for 2 minutes at 40°C followed by drying at 180°C indicated esterification and crosslinking of proteins [173]. The possibility of CA induced esterification of the M proteins in our study requires further analysis due to the different treatment conditions.

In M+OCA mixture, the majority of CA is consumed in modifying O, with some excess CA in OCA that coagulated M. In M+CA+O mixture, the action of CA primarily occurs on the proteins constituting M. CA in MO+CA mixture acts on both M proteins and O polysaccharides simultaneously although the effect on M was more apparent from visual observations. The addition of CA in each mixture caused an immediate coagulation of M proteins in contact with solid CA, with the effect more pronounced at CA2.5 and in M+CA+O and MO+CA formulations. The charge and structure of O polysaccharides in M+CA+O and MO+CA mixtures may potentially be altered by CA, as reported in studies on ramie cellulose [112] and wood [114] modified through CA esterification. However, esterification of O polysaccharides in these mixtures is less probable due to the short mixing and thermal-gelation durations.

4.4.4.3 Soymilk Okara Gels containing Citric Acid

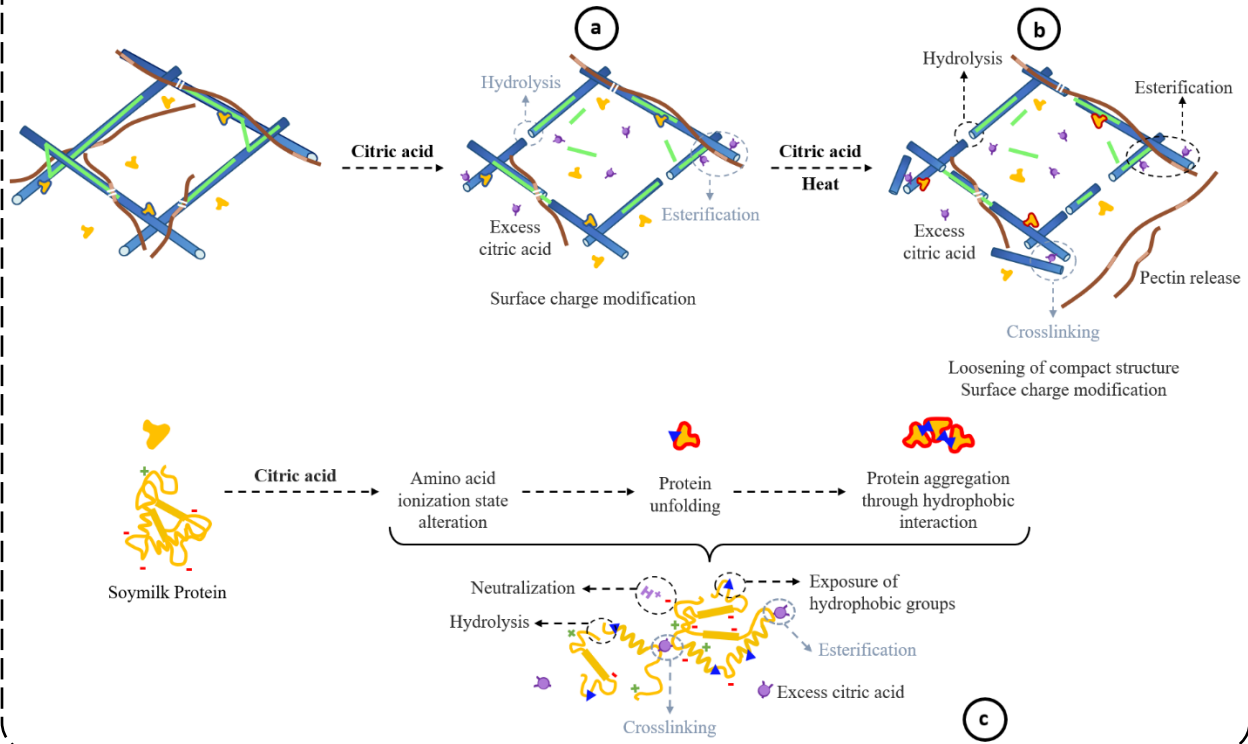
The influence of CA on the mechanism of the thermal gelation of M+OCA, M+CA+O and MO+CA gels is illustrated in **Figure 4.16**, along with a comparison of the properties with the reference MO gel, obtained from statistical analysis. The properties of M+CA0.14 and M+CA2.5 gels with no replicates are also considered in the following discussion to isolate the influence of CA on soymilk proteins (**Figure S.3 and Figure S.4**).

The microstructure of M and MO gels had a compact, dense protein network, with some disruptions in MO gel matrix from the presence of O. The presence of CA in M+OCA, M+CA+O and MO+CA gels resulted in a particulate gel network due to the pre-aggregation of proteins induced by CA in their mixtures before heat treatment. The pre-aggregation of SPI proteins with divalent salts was reported to accelerate heat-induced gelation and cause an increase in the

aggregate size [99], and pH alters the thickness of the soy gel network strands that are assembled from the aggregates [90]. The CA2.5 formulations (lower pH) caused a greater degree of protein aggregation than CA0.14 in all gels, observed from the denser protein matrix in CLSM micrographs. Moreover, the insoluble protein aggregates formed at CA0.14 (pH 5.64 – 5.67 > IEP) were mainly comprised of the native soy protein structures [90] and were different from the protein aggregates formed at CA2.5 (pH 3.51 – 3.66 < IEP) post acid-denaturation. A previous study on the effect of pH on SPI aggregation commented that the aggregates of denatured proteins are larger than native protein aggregates [101].

The protein aggregates in M+OCA gel in CLSM micrographs was less densely packed than in M+CA+O and MO+CA gels at both CA concentrations. These differences could be related to the lower CA content available to act on M in M+OCA whereas the CA content was higher in M+CA+O and MO+CA gels which initiated significant aggregation of proteins. The aggregate size and distribution in the gel network structure influence the gel's resistance to shearing in rheology test. The lower pre-aggregation of proteins, and the smaller, interspersed network aggregates in M+OCA gels contributed to their lower viscoelasticity.

Citric Acid Effects on Okara Polysaccharide and Soymilk Protein



Stage I: Interaction between Protein, Polysaccharide and Citric Acid in Mixtures

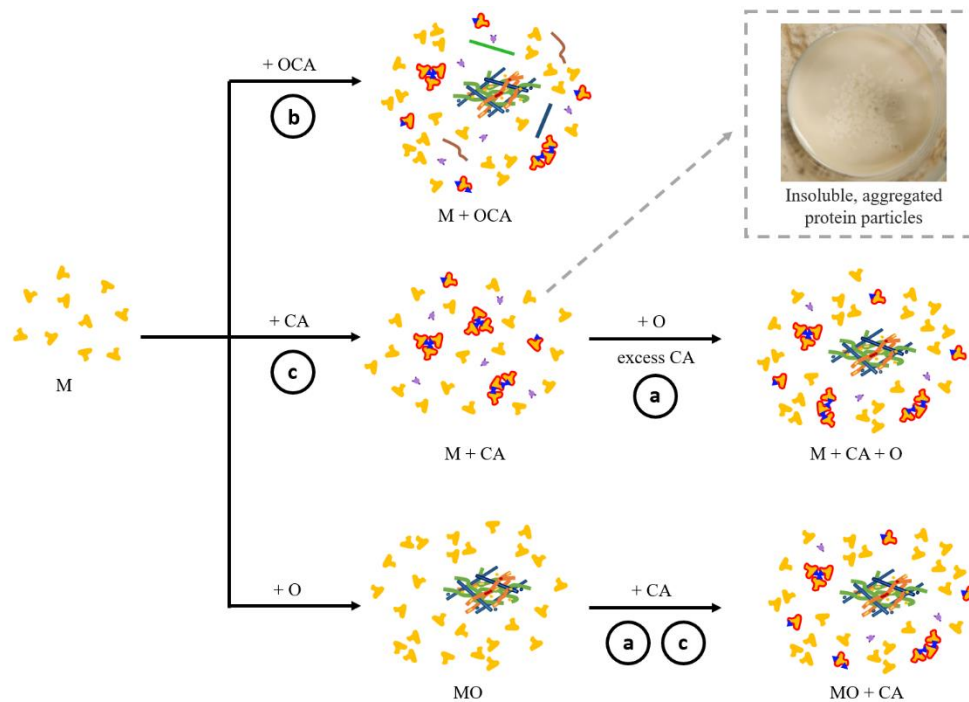


Figure 4.15: Proposed structural modifications caused by CA: (a) O during 15 min mixing at room temperature, (b) OCA after drying at 60°C for 19-20 h, and (c) soymilk proteins. Proposed interactions occurring between soymilk proteins, okara and CA in the M+OCA, M+CA+O and MO+CA mixtures (Stage I).

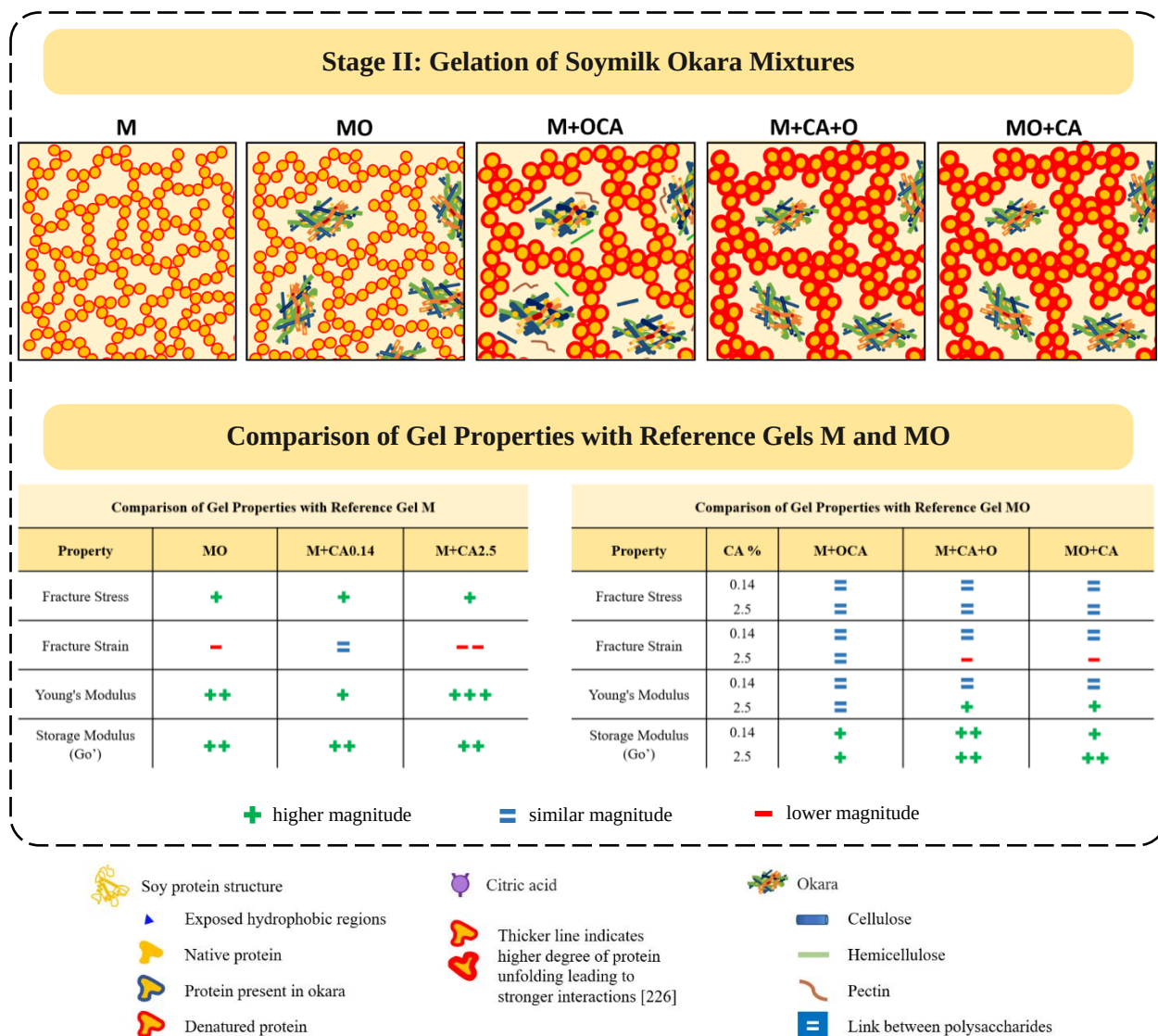


Figure 4.16: Proposed mechanism of the progress of thermal gelation in M, MO, M+OCA, M+CA+O, and MO+CA gels (Stage II).

4.5 Conclusion

In this study, the effects of citric acid (CA), a poly-carboxylic acid, on okara (O) and soymilk (M) in CA soymilk okara gels were evaluated. The goal was to compare and contrast the effect of CA on the structural modification of O and on the coagulation of M by examining three different sequences to produce a CA soymilk okara mixtures and assessing the compatibility between okara and soymilk in the gel matrix at two different CA concentration.

The structure of okara, examined by FTIR, TGA and SEM, was altered at CA2.5. CA induced hydrolysis which modified the okara structure, releasing soluble polysaccharides such as pectic substances observed by the gummy nature of OCA2.5. Esterification of okara was also observed at CA2.5. These observations were not seen in OCA0.14 which had a similar FTIR spectra as O.

The effects of CA concentration and sequencing on the soymilk okara gels were analyzed through estimating the porosity, microstructure, water holding capacity (WHC), failure and viscoelastic properties, and chemical interactions involved in the assembly of the gels. The incorporation of okara disrupted the protein network and introduced heterogeneity which was observed macroscopically and microscopically. The presence of CA0.14 and CA2.5 as okara modifier (M+OCA) and coagulating agent (M+CA+O, MO+CA) did not significantly alter the heterogeneity, hydrophobic interactions, or failure stress properties of these gels compared to MO gel. However, the viscoelasticity of the gels increased by threefold in M+OCA gels and MO+CA0.14, and by 4.5 folds in M+CA+O gels and MO+CA2.5. The increased viscoelasticity of the gels M+CA2.5+O and MO+CA2.5 was accompanied by increased gel stiffness (Young's Modulus) and decreased cohesivity (failure strain).

The benefits of CA on the textural properties of soymilk okara gels were limited to enhancing the viscoelasticity of the gels at CA0.14, while increasing viscoelasticity and stiffness of gels at CA2.5 when compared to MO gels. Further research is needed to study the changes caused in soymilk proteins by CA and their aggregation mechanisms to modulate the gel structure as desired. Additionally, sensory evaluation can provide insights on the textural properties that need further investigation to develop high-fiber, high-protein plant-based cheese analogues.

Chapter 5: Conclusion and Recommendations

5.1 Conclusion

The research presented in this thesis focused on the potential of citric acid (CA) to improve the integration of okara (O) in soymilk (M) gels produced by thermal gelation. The overall goal was to compare and contrast the effect of CA on the structure of O and the coagulation of M by examining three different sequences to produce a CA okara soymilk mixture at two different CA concentration. The study consisted of two specific objectives: (1) standardization of soymilk okara mixtures by adjusting the concentrations and preparation of the ingredients, and (2) analysis of soymilk okara gel characteristics prepared with two CA concentrations.

The outcomes on the standardization of soymilk okara ingredients are summarized in the following four categories:

Soybean:

The freezing of soybeans caused structural changes in soymilk and okara detected in the FTIR spectra but did not affect the texture properties of the soymilk okara gels produced from untreated or frozen soybeans. Therefore, the freezing of soybeans was not explored further.

Okara:

The particle size of okara affected the soymilk okara gel homogeneity and surface roughness. Gels with fine particle okara ($<75\ \mu\text{m}$) and unfractionated okara were similar in homogeneity and porosity, and therefore, fractionation of okara was not considered for further experimentation.

The drying duration of okara affected its grinding where longer drying time altered the particle size distribution and resulted in more heterogeneous MO gels. Therefore, a shorter drying time, 19 h, was adopted for subsequent experimentation.

The visualization of okara by SEM provided information on changes in the microstructure of okara modified with CA where a loosened and more porous okara structure were identified. The higher CA concentration in OCA1.25 and OCA2.5 smoothened the surface morphology that may be related to the release of pectic substances from okara. Therefore, the addition of CA modified okara in soymilk okara gels was considered for subsequent gel texture analyses.

Soymilk:

The addition of CA at 0.14% (w/w) (CA0.14) and 2.5% (w/w) (CA2.5) to soymilk enhanced the strength and viscoelasticity of the soymilk gels that were more pronounced at CA2.5. Therefore, CA0.14 and CA2.5 were utilized in the preparation of CA modified okara and soymilk okara gels.

Gel:

The okara content in the soymilk okara mixtures indicated that increasing okara content enhanced the strength of MO gels, but made the gels heterogeneous, rough and dry. Okara content 4.2 - 8.0% (w/w) produced strong and smooth gels and therefore, 5.6% which is used for the standard MO gel, was adopted in the preparation of soymilk okara gels.

The second objective focused on the effects of CA (0.14 and 2.5% (w/w)) on soymilk okara mixture and the gels produced thereafter with three different formulations: (i) CA treatment of okara prior to its addition to soymilk (M+OCA), (ii) CA treatment of soymilk prior to okara addition (M+CA+O), and (iii) CA addition to a mixture of soymilk and okara (MO+CA). The CA0.14 and CA2.5 soymilk okara gels had similar heterogeneity, protein-protein interactions, and failure stress to MO gel. The microstructure of the gels suggested transition from strand-like network in MO gel to a particulate network in CA soymilk okara gels, with the protein network being denser at CA2.5 than CA0.14. The elasticity of CA0.14 soymilk okara gels increased by 3-fold in M+OCA and MO+CA, and 4.5-fold in M+CA+O gels, while gel stiffness (Young's Modulus) and cohesivity (failure strain) were similar to MO gel. At CA2.5, M+OCA gel has similar stiffness and cohesivity to MO gel but 3-fold increase in elasticity. The M+CA2.5+O and MO+CA2.5 gels had lower cohesiveness, higher stiffness, and an improved elasticity that increased by 4.5-fold in comparison to MO gel. The gel formulations explored in the study indicate that CA facilitated the integration of okara in soymilk gels and modulated their textural properties. CA enhanced the gel viscoelasticity at CA0.14, while gel viscoelasticity and stiffness at CA2.5 was improved when compared to MO gels.

5.2 Recommendations

The following recommendations are formulated based on the results of this study and are presented in regards to ingredient, mixture and gel preparation and characterization:

Ingredient:

1. Oligosaccharides content of okara

Oligosaccharides may be released during the CA treatment of okara. The consumption of oligosaccharides can have a prebiotic or bloating effect on human gut [239] [240]. Oligosaccharide content can be assessed by evaluating the molecular weight or particle size distribution with dynamic light scattering (DLS), or quantified through HPLC [241].

2. Hydrophilicity and hydrophobicity of okara

The water (WHC) and oil (OHC) holding capacities of CA modified okara can provide a measure of the changes in its hydrophilicity and hydrophobicity. A change in WHC of okara can suggest structural modification, whereas OHC will indicate the change in its hydrophobicity and therefore provide insights on potential hydrophobic interactions between okara and soymilk proteins.

3. Partial decoupling and quantifying the effects of CA on okara

The specific effects of CA on soymilk proteins or okara can be assessed by isolating acid hydrolysis, esterification and crosslinking with alternative chemicals. A weak monocarboxylic acid such as formic or acetic acid can reflect the extent of hydrolysis and esterification of okara, whereas EDTA can simulate the crosslinking ability of CA. Additional characterization methods such as NMR, or kinetic studies that manipulate the conditions to favor a specific reaction, can complement the quantification of the various CA-induced reactions.

4. CA induced reactions and structural changes in soy proteins

Analysis of soymilk aggregation by CA could provide insights on the gelation mechanism. The aggregation of soymilk could be assessed with dynamic temperature sweep rheology measurements to track gelation rate and the protein particle size estimated with dynamic light scattering. Structural changes of the proteins resulting from reactions between protein

and CA such as esterification and crosslinking could be investigated through stage-wise conductometric and potentiometric titration [55].

Mixture

5. Controlled aggregation of soymilk proteins

The control of the soymilk protein aggregation could be obtained with short heating cycles that build on the denaturation temperature specific to the 7S and 11 S soy proteins. For instance, further developing a preliminary experiment that was conducted could be considered where soymilk was exposed to a short heating cycle at 60°C (below soy protein denaturation temperature), 75°C (above denaturation of 7S protein), and 95°C (above denaturation of all soy proteins), and subsequently cooled in ice bath. Further investigation may reveal insights on soymilk protein denaturation, aggregation and gelation mechanisms.

Gel

6. Oil phase in CA soymilk okara gels

The presence of CA in the CA soymilk okara gels may affect the oil distribution in the gels. Staining the CA soymilk okara mixtures with Nile Red can aid in visualizing the distribution of oil droplets in the gels and differentiating the oil phase from the micropores or water.

7. Gel texture with okara

The addition of CA-modified okara enhanced the viscoelastic component of the soymilk okara gel texture but not the gel strength. Gel strength, on the other hand, increased with increasing okara content. An increase of CA-modified okara, or combined with native okara may improve gel mechanical and viscoelastic properties.

The interaction between okara and soymilk may be enhanced by functionalization of okara. This may be achieved by coating okara to alter its surface functionality, such as dual coating with chitosan and pectin [38] reported previously, and facilitate interaction with soymilk proteins.

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Appendix

Appendix A: Standardizing the Preparation of Soy Gel Ingredients: Treatment and Concentration

Appendix A1: Effect of Freezing Soybeans on Soymilk Okara Gel Texture

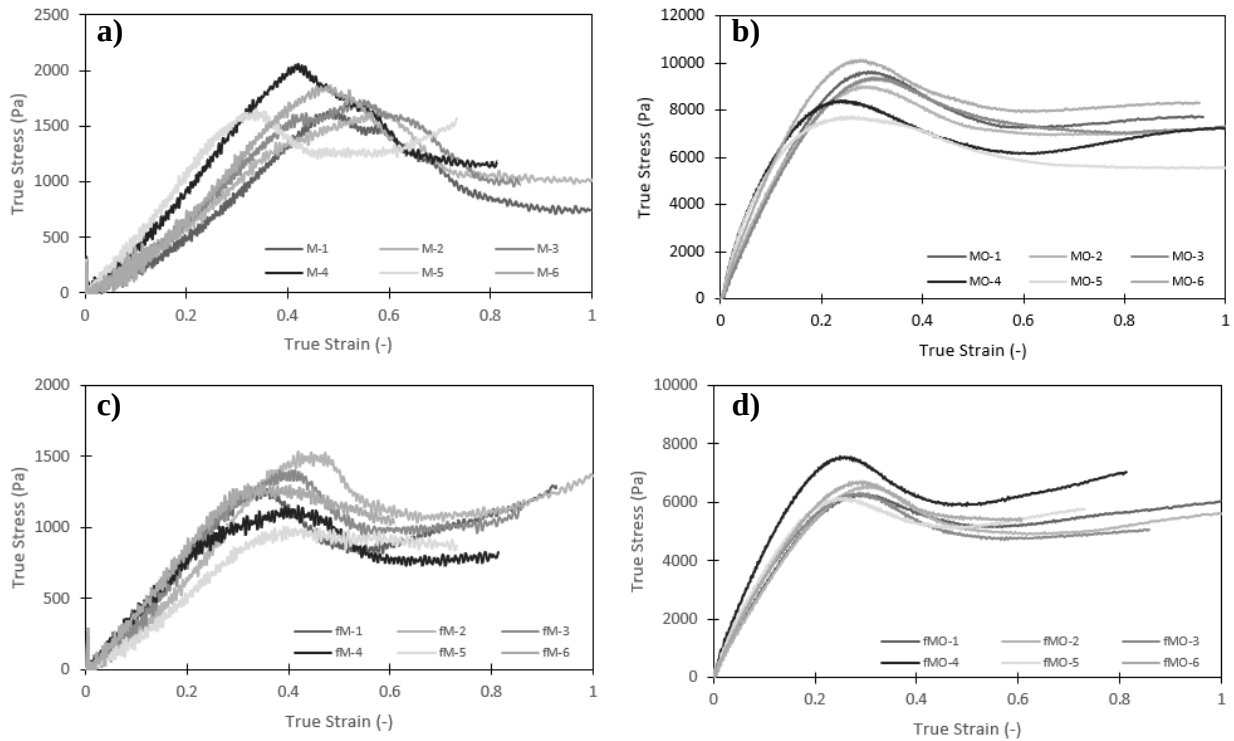


Figure S. 1: Stress-strain curves of soymilk okara gels a) M, (b) MO, (c) fM, (d) fMO.

Appendix A2: Influence of Okara Particle Size, Drying Method and Concentration on Soy Gel Texture



Figure S. 2: Visual observation of the color difference between the various size fractions of dried okara obtained through filtration (a) > 212 μm (b) 212 - 106 μm (c) 106 - 75 μm (d) 75 - 63 μm (e) 63 - 45 μm and (f) < 45 μm; (g) Color difference between okara particles of size >212μm and <45μm.

Appendix A3: Effects of Citric Acid Concentration on Soymilk Gel Texture

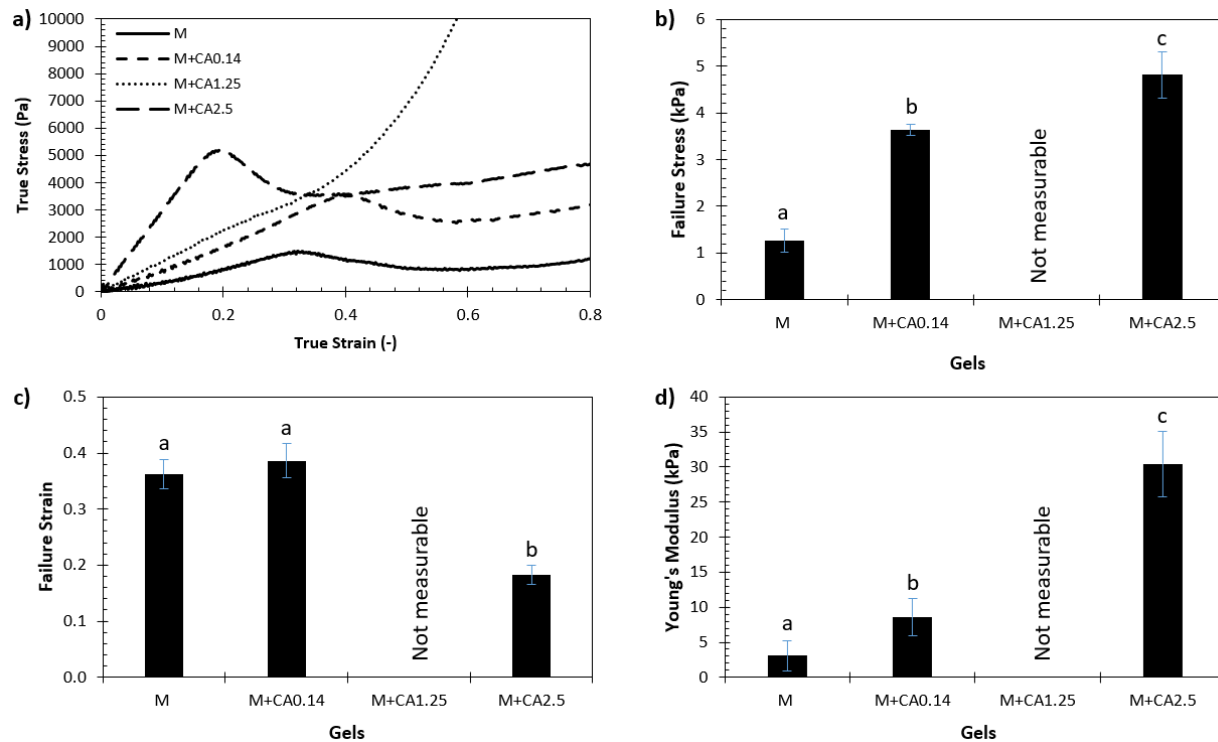


Figure S. 3: Mechanical properties of soy gels M, M+CA0.14, M+CA1.25 and M+CA2.5: (a) Typical failure stress – failure strain curves, (b) True failure stress (kPa), (c) True failure strain, (d) Young's modulus (kPa). Data of (b – d) expressed as mean $\pm$ standard deviation ($n = 6$) where means that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$).

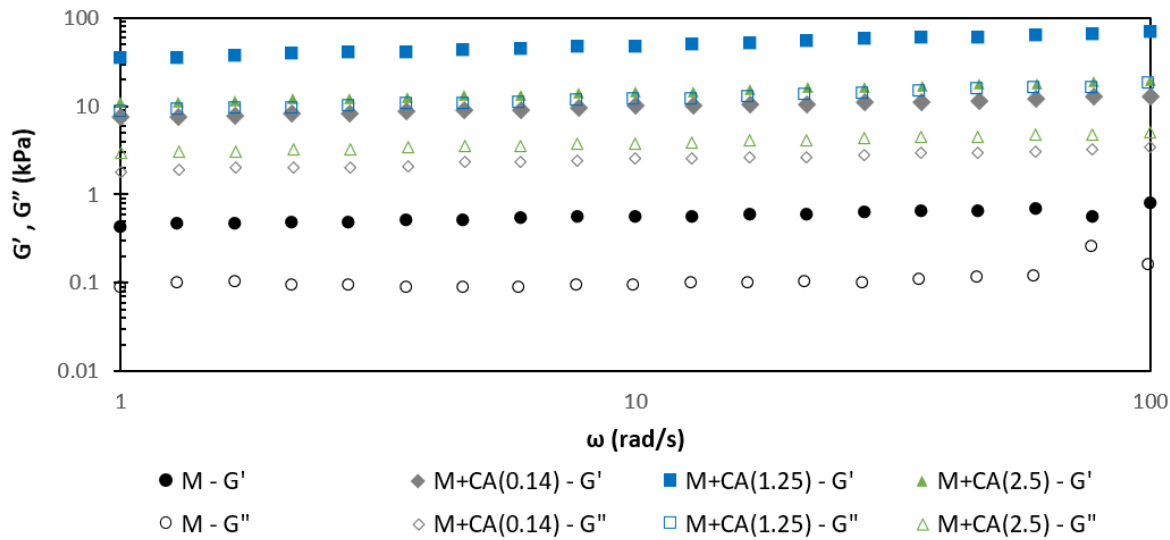


Figure S. 4: Typical curves of storage modulus G' (filled symbols) and loss modulus G'' (empty symbols) of M, M+CA0.14, M+CA1.25 and M+CA2.5 gels.

Table S. 1: Power law parameters Equation 3.7 and Equation 3.8 obtained from frequency sweep tests, expressing the viscoelastic properties of M, M+CA0.14, M+CA1.25 and M+CA2.5 gels. Data expressed as mean $\pm$ standard deviation ($n = 3$). Means within one column that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$).

Gel	G'_0 (kPa)	n'	r_1^2	G''_0 (kPa)	n''	r_2^2
M	0.36 ± 0.07^a	0.12 ± 0.02^a	0.84	0.07 ± 0.01^a	0.08 ± 0.05^a	0.28
M+CA0.14	7.82 ± 0.91^b	0.12 ± 0.01^a	1.00	1.91 ± 0.30^b	$0.12 \pm 0.01^{a,b}$	1.00
M+CA1.25	34.56 ± 0.75^c	0.13 ± 0.01^a	1.00	4.14 ± 0.23^c	0.14 ± 0.01^b	0.99
M+CA2.5	10.55 ± 0.35^d	0.14 ± 0.02^a	1.00	2.84 ± 0.11^d	$0.13 \pm 0.02^{a,b}$	0.99

Appendix A4: Determining Soymilk Aggregation in the Presence of Okara or Citri Acid

Table S. 2: Publications on the methodology of measuring turbidity

Protein Matrix	Treatment	Turbidity Measure Objective	λ (nm)	Solvent	* C_{Protein} (% w/w)	Result
Freeze-dried SPI that was extracted from low-denatured soybean flakes [92]	Succinylation of SPI with solid succinic anhydride	Turbidity of heat induced aggregates of SPI measured as a function of NaCl concentration (0 - 1 mol/L)	540	Phosphate buffer (pH 7.0)	5 mg/mL (0.5% *)	Turbidity increased with the concentration of NaCl up to 0.2 mol/L as the salt ions screened electrostatic repulsion and facilitated protein association via hydrophobic interactions.
Enriched soy protein (SP) extracted from defatted soy flour [98]	SP treated with different salts (MgCl_2 and MgSO_4) over a range of salt concentration and range of heating temperatures	1. Turbidity changes measured as a function of C_{salt} 2. Turbidity changes tracked immediately after addition of salt	600	MOPS buffer	0.1% v/v (- *)	1. Turbidity increased with salt concentration, and was more pronounced in presence of MgCl_2 , above 0.7 mM. 2. Major changes in turbidity occurred within the first 5 min. At 50 C and 70 C, higher turbidity observed indicates either larger or higher number of aggregates formed.
Freeze-dried fractions of 7S and 11S that were isolated from soy flour [93]	High intensity ultrasound (HUS) treatment of proteins	Turbidity measured as a function of HUS treatment time (0, 5, 20, 40 min) applied on protein dispersions	600	Standard buffer (pH 7.0, 0.05M Tris-HCl buffer, 0.01% Sodium Azide)	2% w/v for 7S (2% *) 0.25% w/v for 11S (aggregated in buffer) (0.25 *)	Increase in HUS treatment time decreased turbidity due to: - decrease in particle size of 7S fraction - changes in 3° and 4° structure of "non-aggregated proteins"
Freeze-dried soluble SPI aggregates (SSA) extracted from defatted soybean flakes	GDL induced gelation of SSA at 25°C and 50°C	Change in turbidity of SSA dispersion over time due to GDL acidification was tracked	540	DI water (to avoid phosphate buffer interference on pH changes	40 mg/mL (4% *)	Turbidity increased rapidly in the initial 40 minutes after the addition of GDL. Particles of dispersion with the smallest intermolecular electrostatic repulsion, observed fastest increase in aggregate size (i.e., turbidity)

[94]				caused by GDL)		
SPI extracted from defatted soybean flakes [101]	Acid treatment of SPI with HCl at varying pH between 5.8-6.4	Change in turbidity over time during heating of SPI dispersion at 80°C and at different pH (5.8-6.4)	500	Salt-free Milli-Q water with Sodium Azide (to prevent bacterial growth)	18 g/L (1.8% *)	Either no change (pH 5.8-5.9) or an initial decrease (pH 6.0-6.4) in turbidity was observed up to different time-lengths followed by increase in turbidity
SPI extracted from defatted soybean meal [99]	SPI pre-aggregated with different salts (CaSO ₄ , MgSO ₄ , ZnSO ₄)	Turbidity of pre-aggregated SPI dispersion	600	DI water	2 mg/mL (0.2% *)	Turbidity increased with the concentration of salts up to 15 mM due to electrostatic screening and salt bridge effects inducing protein aggregation. The variation in turbidity across the pre-aggregates formed with different salts was due to variation in their coagulation capacity
SPI extracted from: • commercial soy flour • soy flour prepared in-lab with soybean [100]	-	Turbidity measured as a function of protein concentration	600	Distilled water	10 to 100 mg/mL (1 to 10% *)	Turbidity increased with protein concentration. SPI sourced from soy flour prepared in-lab had higher turbidity, possibly due to protein polymerization caused by lipoxygenase oxidation activity which is reduced in commercial flours due to high temperature treatments involved in their preparation

* C_{Protein} (% w/w) is the concentration of protein reported in the publication in various units which is converted to % w/w

Appendix A4: Visualization of Okara and its Distribution in Gels

Table S. 3: Publications on SEM analysis of untreated and treated okara and extracted pectin

Sample	Sample Analyzed with SEM	SEM Conditions	SEM Observations
Okara obtained from local supplier [111]	Control: Fresh okara dried at 60°C and crushed to pass 0.25 mm mesh sieve Treatment: 1. LAB fermentation 2. Microwave treatment in presence and absence of citric acid. Treated okara was freeze-dried and crushed to pass 0.25 mm mesh sieve	Sample: Samples dried at 105°C for constant weight Coating: Gold Voltage: 10kV Magnification: x1500	Control okara: Aggregated with a wrinkled surface. Fermentation treated okara: Looser structure indicating enzymatic degradation of proteins and cellulose/ hemicellulose that damage the epidermal structure. Microwave treated okara: Microwaves ruptured the cells resulting in okara with porous structure. The loose, porous structure of treated okara improves its adsorption ability.
Okara prepared in-lab. [163]	Control: Okara filtered from heat treating soy flour dispersion at 95°C, followed enzymatic hydrolysis, and then freeze-dried Treatment: Processing soy flour dispersion with high-speed shear treatment before okara extraction	Sample: Freeze-dried sample Coating: Pb Voltage: 10kV Magnification: x1000	Control okara: Contained honeycomb-like structures, typical of parenchyma, along with seed coat components as okara was extracted from whole soybeans. Grinding and thermal treatment disintegrated soybean into its component cell layers but did not cause degradation up to cellular level. High speed shear treatment: Disintegrated cell layers at cellular level. The parenchyma layer was deformed while the other layers (palisade layer and hourglass cells) were fractured.
Okara obtained from local supplier [164]	Control: Spin flash drying of okara Treatment: Steam explosion at pressures of 0.5 - 2 Mpa for 30 - 120 s, followed by drying at 80°C and grinding to pass through 40-80 mesh sieve.	Sample: Okara samples dried at 105°C for constant sample weight Coating: Gold Voltage: 15kV Magnification: x1000	Control: Ordered, loose sheet structure with folds Steam explosion treatment: After weak treatment, lamellar structures were destroyed but inner structure remained intact. Intense treatment destroyed nucleated structure inside okara into small particles.

Bagasse from local supplier [165]	Control: Dried in air oven for 72 h at 70°C Treatment: Fibers soaked in 1% NaOH or acrylic acid solution before drying at 70°C for 72 h	Sample: Okara samples dried at 105°C for constant sample weight Coating: Gold Magnification: x250	Control: Comprised of hexagonal cellular structures, with hollow cavity called lumen, that are bound by lignin and pectin. Chemical Treatment: NaOH and acrylic acid treatments destroyed and compressed the cellular structure (honeycomb-like), decreasing the cavity area.
Okara from local supplier [138]	Control: Okara enzymatically hydrolyzed in trypsin to degrade proteins and freeze-dried Treatment: Ultrasound treatment for 30 min at 20kHz at various powers followed by enzymatic hydrolysis and freeze-drying	Sample: Freeze-dried Coating: Gold Voltage: 5kV Magnification: x1000	Control: Smooth regular surface with compact texture due to no intermolecular interaction, and no fragments observed indicating enzyme hydrolysis removed the surface impurities including proteins Ultrasonic treatment: Okara degraded into flaky, stacked structure. The typical honeycomb structure of okara was retained as parenchyma remained intact, however at high intensity, parenchyma layer was deformed (compact) and hourglass cell layer fractured indicating disruption of crosslinking between the polysaccharide molecules.
Okara prepared in-lab [166]	Control: Freeze-dried Treatment: Fermentation treated in 3 different cultures	Sample: Freeze-dried Coating: Gold Voltage: 100kV Magnification: x500, x1000	Control: Dense, loose, sheet-like and corrugated structure Fermentation treatment: Destroyed fiber structure, wrinkled surface, loose flaky structure resulting from degradation of protein, starch and cellulose
Pectin extraction from sisal fiber [167]	Control: Sisal fiber Treatment: Combination of HCl acid, enzymatic and ultrasonic extraction of pectin	Sample: vacuum dried at 40°C Voltage: 10kV	Control: Compact, smooth, honeycomb-like structures Treatment: Enzymatic treatment resulted in porous structure due to hydrolysis of connection between polysaccharides and partial release of pectin. Ultrasonic treatment collapsed the fiber structure. Combined enzymatic-ultrasonic treatment broke cell walls (mechanical destruction) and resulted in porous irregular structure, aiding high pectin extraction.

Appendix B: Exploring the Effects of Citric Acid on Soybean Components and the Impact on Soymilk Okara Gel Texture

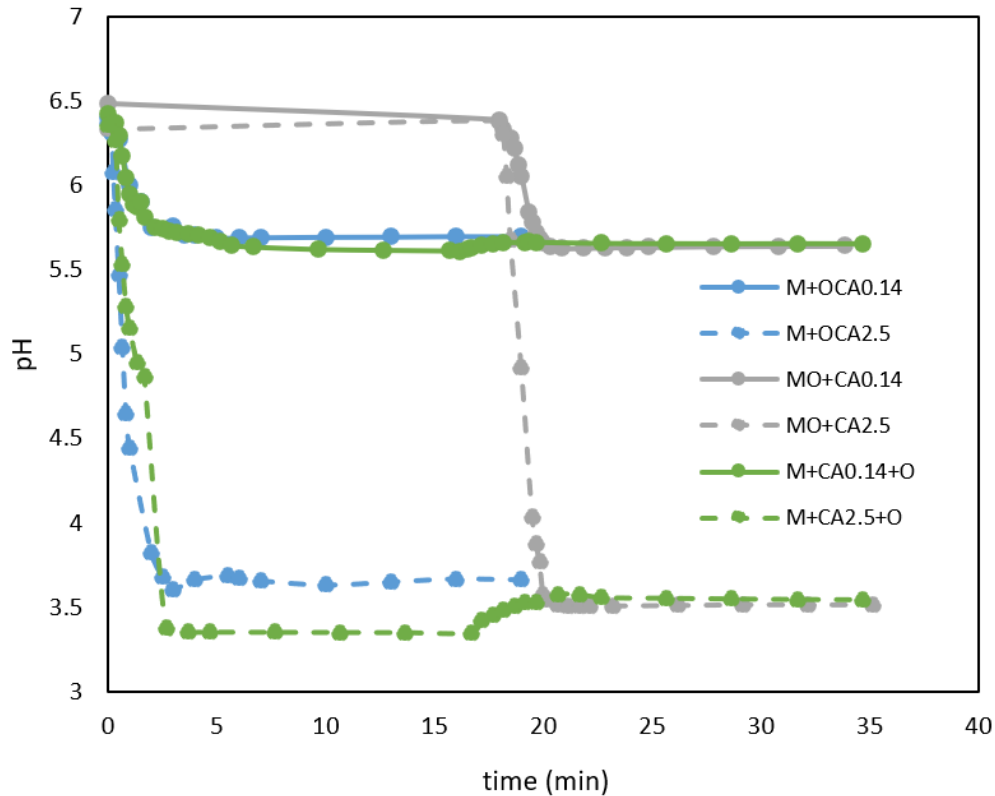


Figure S. 5: pH changes of soymilk okara mixtures during 15 – 30 min mixing duration preparation

Table S. 4: Final pH of soymilk okara mixtures after 15-30 min mixing and before thermal gelation. Data expressed as mean standard deviation (n = 2). Means within one column that do not share a common letter are statistically different (ANOVA followed by Tukey's post-hoc: $p < 0.05$).

M	MO	M+OCA		M+CA+O		MO+CA	
		0.14%	2.50%	0.14%	2.50%	0.14%	2.50%
6.42±0.06	6.35±0.1	5.61±0.04	3.28±0.59	5.62±0.04	3.52±0.06	5.51±0.06	3.45±0.01

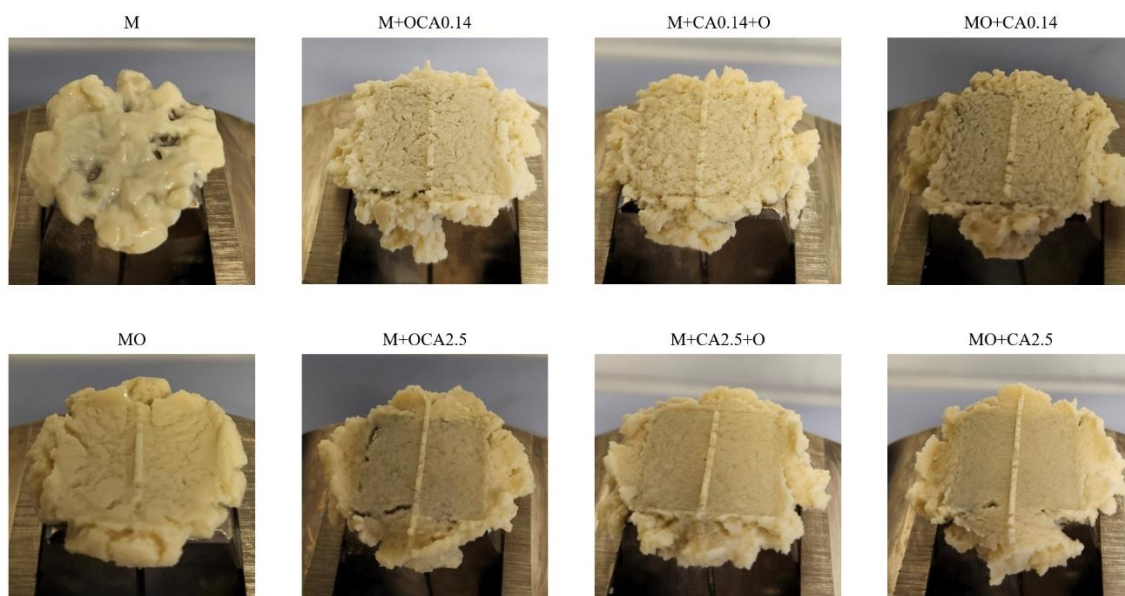


Figure S. 6: Fragments of deformed gels a) M, (b) MO, (c) M+OCA0.14 (d) M+CA0.14+O, (e) MO+CA0.14, (f) M+OCA2.5 (g) M+CA2.5+O, (h) MO+CA2.5, after failing from uniaxial compression under 500 N load.

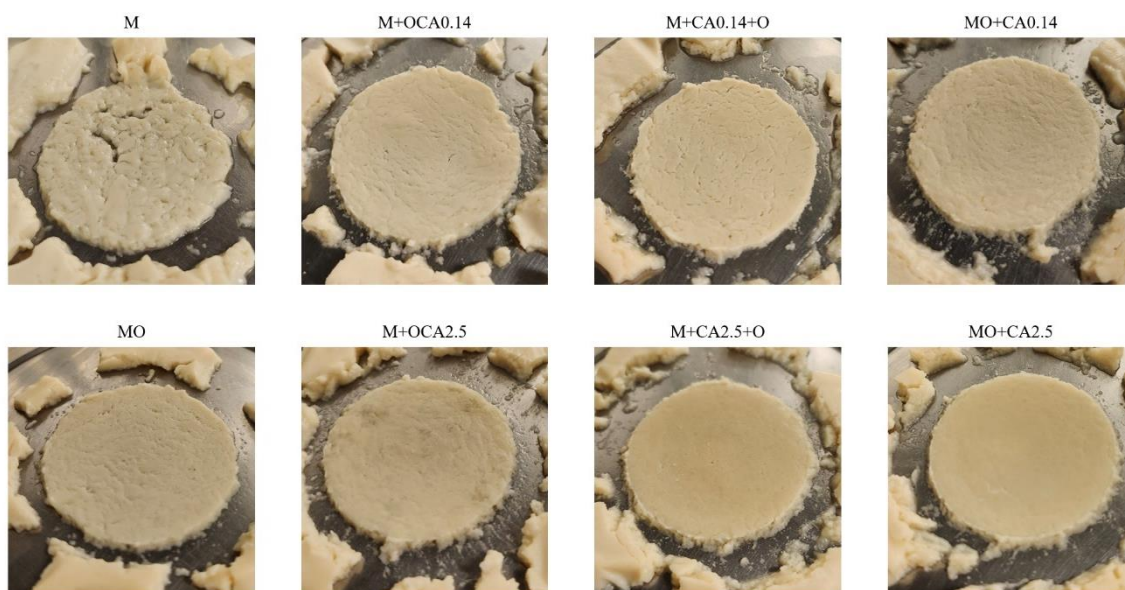
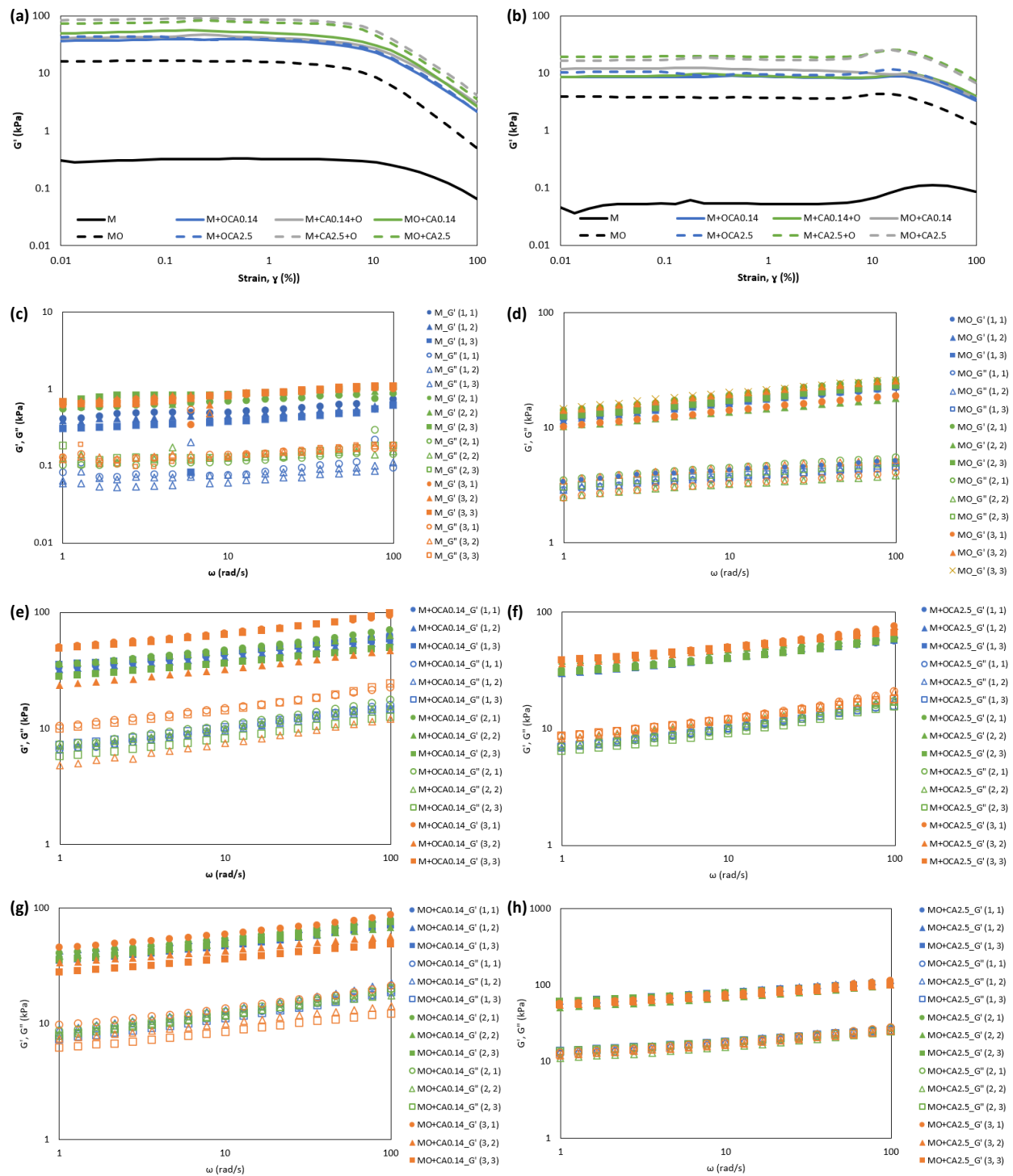


Figure S. 7: Deformed gels a) M, (b) MO, (c) M+OCA0.14 (d) M+CA0.14+O, (e) MO+CA0.14, (f) M+OCA2.5 (g) M+CA2.5+O, (h) MO+CA2.5, after deformation from shear stress in rheometer.



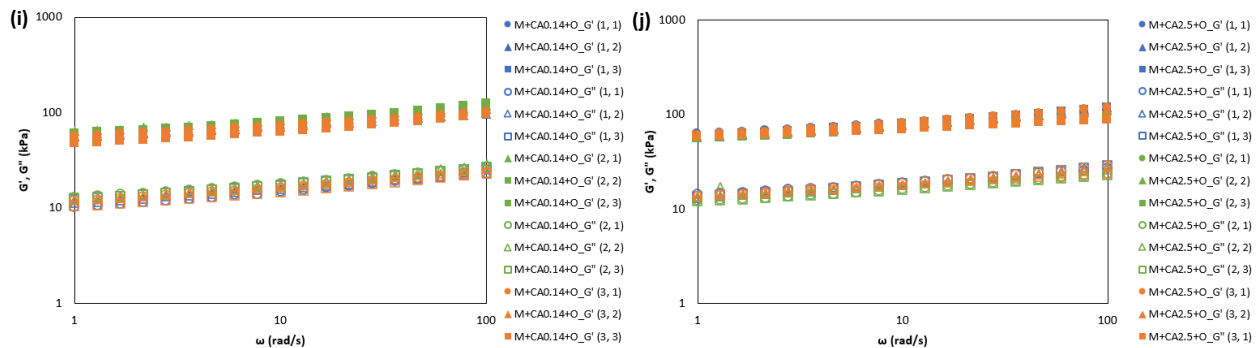
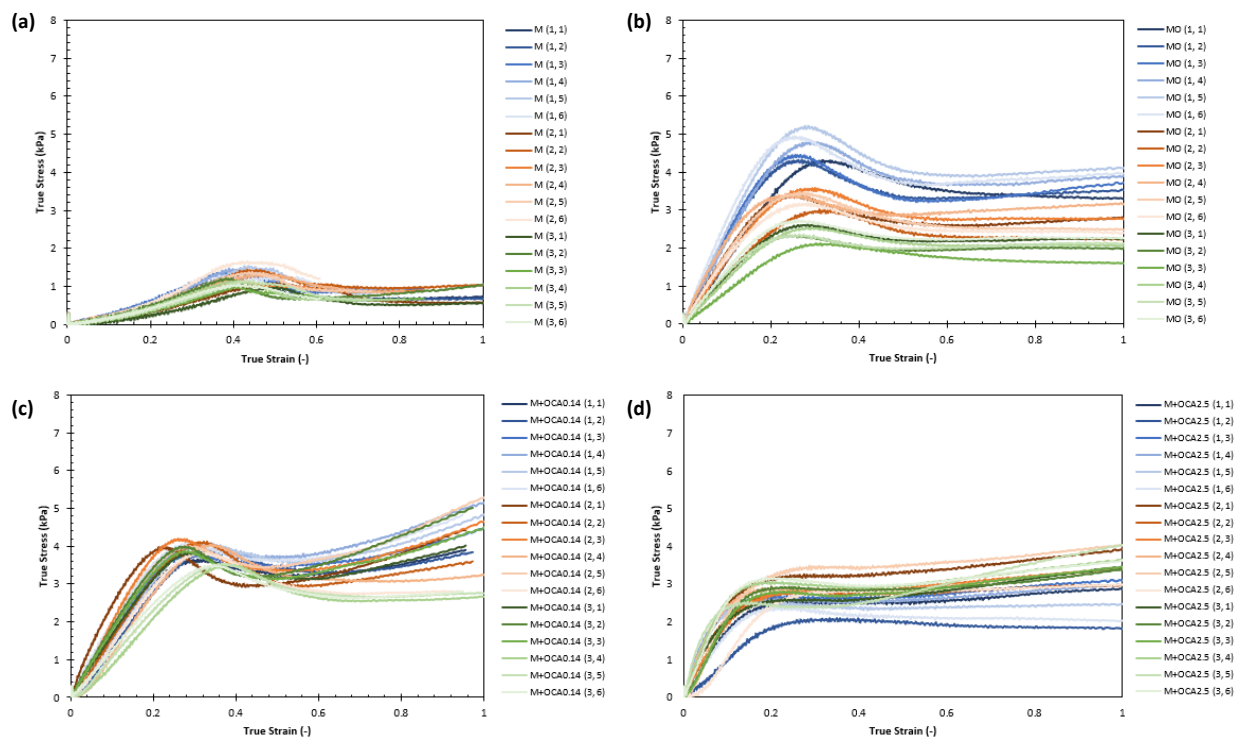


Figure S. 8: Curves of (a) storage modulus, G' and (b) loss modulus, G'' over strain (γ) to determine the linear viscoelastic region; Curves of storage modulus, G' and loss modulus, G'' over angular frequency, ω of gels (c) M, (d) MO, (e) M+OCA0.14, (f) M+OCA2.5, (g) MO+CA0.14, (h) MO+CA2.5, (i) M+CA0.14+O, and (j) M+CA2.5+O.



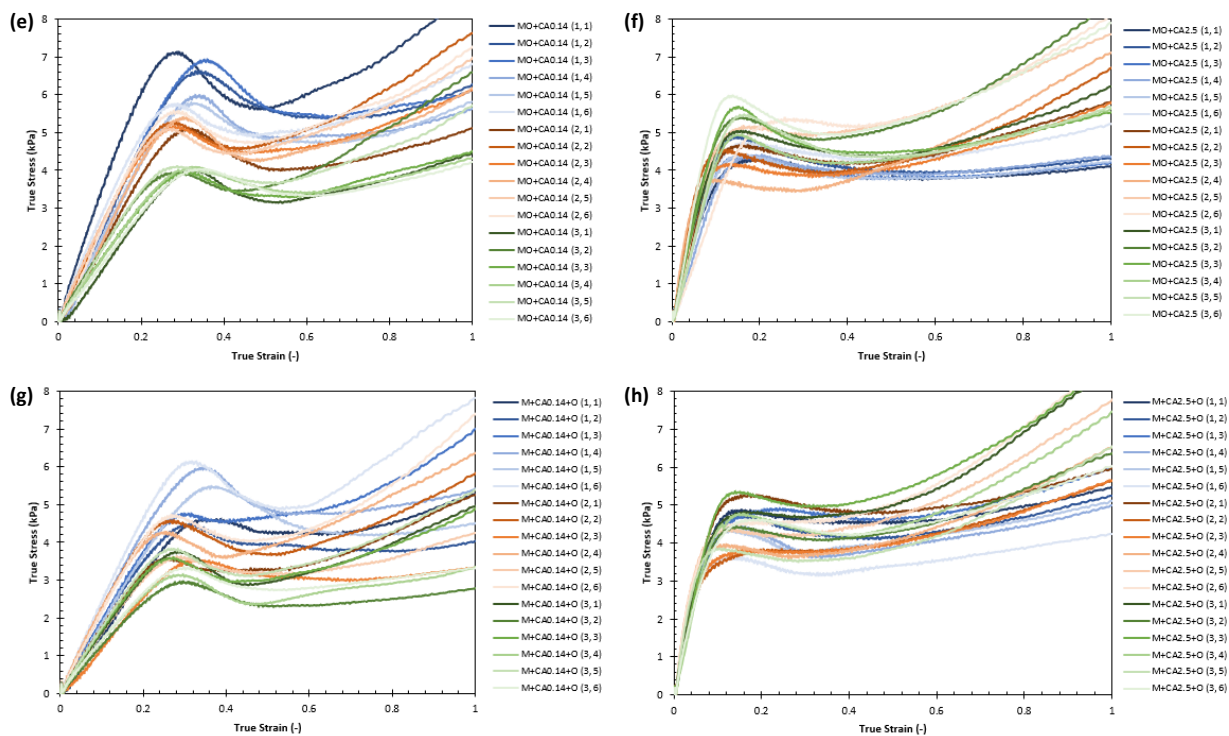
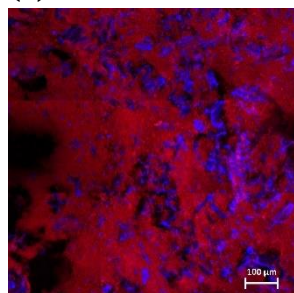
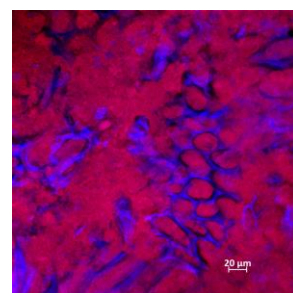
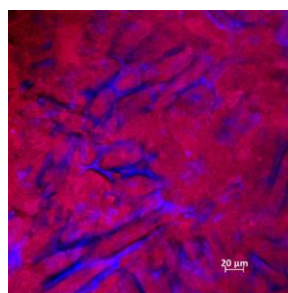


Figure S. 9. Failure stress – failure strain curves of soymilk gels (a) M, (b) MO, (c) M+OCA0.14, (d) M+OCA2.5, (e) MO+CA0.14, (f) MO+CA2.5, (g) M+CA0.14+O, and (h) M+CA2.5+O.

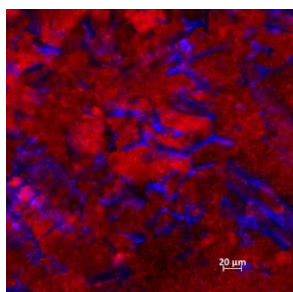
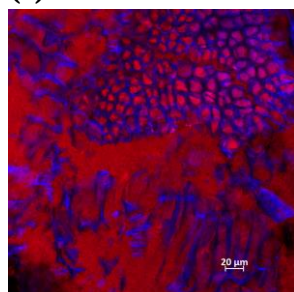
(a) M+OCA0.14



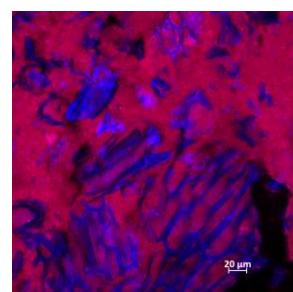
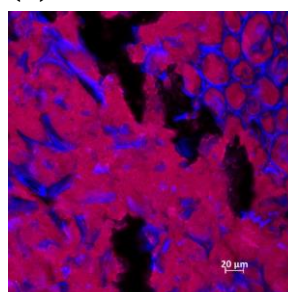
(b) M+OCA2.5



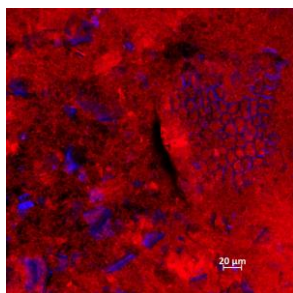
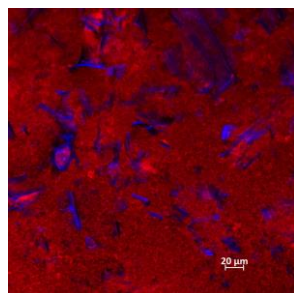
(c) M+CA0.14+O



(d) M+CA2.5+O



(e) MO+CA0.14



(f) MO+CA2.5

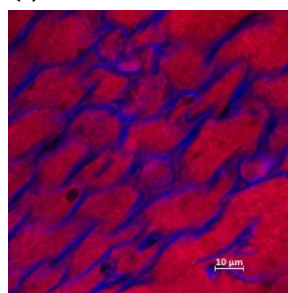


Figure S. 10: Various structures of okara (rod-like, honeycomb-like, fragmented) and its distribution in soymilk okara gels visualized under CLSM

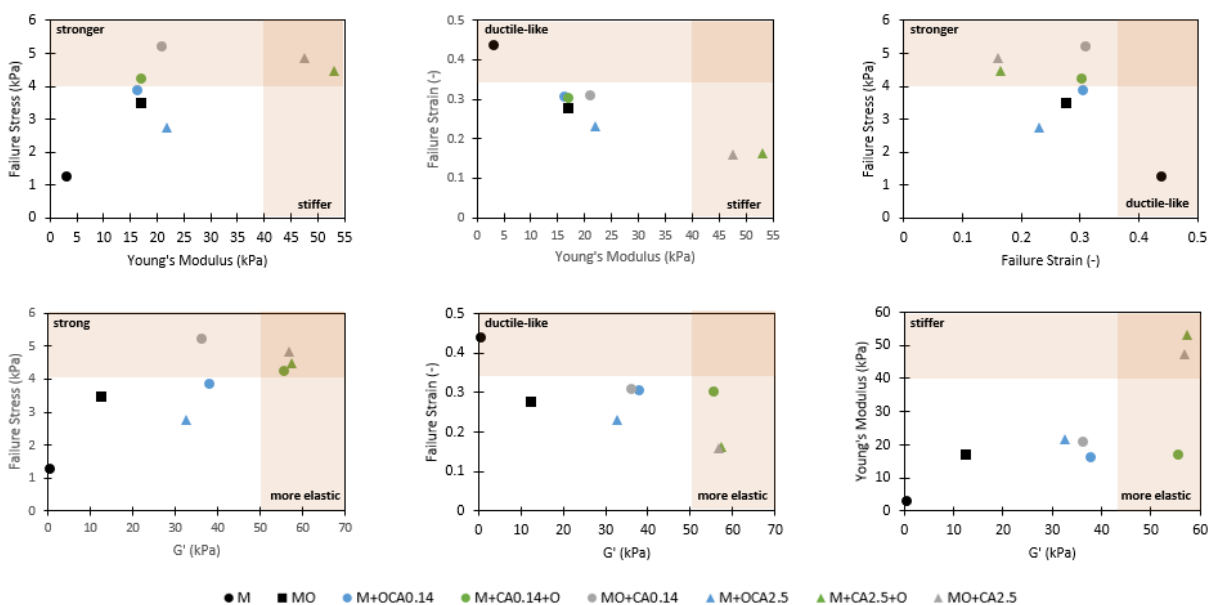


Figure S. 11. Correlation between failure and viscoelastic properties

Table S. 5: Properties of reference gels M and MO, and soymilk okara gels M+OCA, M+CA+O, MO+CA containing citric acid at two concentration levels – 0.14% and 2.5% (w/w)

DATA	M	MO	M+OCA(0.14)	M+CA(0.14)	M+CA(0.14)+O	MO+CA(0.14)
WHC%	73.45	77.80	67.85	50.25	62.41	64.08
Syneresis %	0.37	0.05	0.04	0.79	0.02	0.01
Void Fraction	0.99	2.40	3.15	0.46	3.01	2.49
Fracture Stress (kPa)	1.27	3.48	3.88	3.63	4.25	5.22
Fracture Strain	0.44	0.28	0.31	0.39	0.30	0.31
Young's Modulus (kPa)	2.99	16.88	16.31	8.60	17.04	20.95
Go' (kPa)	0.54	12.45	37.89	7.82	55.58	36.07
Go'' (kPa)	0.09	3.13	7.73	1.91	11.51	7.79
Ionic	0.78	0.27	0.75	0.01	0.76	0.65
Hydrogen	1.21	1.80	1.31	1.09	1.14	2.06
Hydrophobic	48.26	28.59	32.22	34.62	37.50	26.03

DATA	M	MO	M+OCA(2.5)	M+CA(2.5)	M+CA(2.5)+O	MO+CA(2.5)
WHC%	73.45	77.80	69.34	60.83	66.89	67.32
Syneresis %	0.37	0.05	2.90	5.02	2.57	1.25
Void Fraction	0.99	2.40	3.82	0.81	3.53	3.26
Fracture Stress (kPa)	1.27	3.48	2.76	4.82	4.48	4.85
Fracture Strain	0.44	0.28	0.23	0.18	0.16	0.16
Young's Modulus (kPa)	2.99	16.88	21.91	30.42	53.10	47.51
Go' (kPa)	0.54	12.45	32.59	10.55	57.39	56.72
Go" (kPa)	0.09	3.13	7.21	2.84	12.97	12.60
Ionic	0.78	0.27	0.51	0.00	0.04	0.17
Hydrogen	1.21	1.80	1.36	1.19	1.38	0.83
Hydrophobic	48.26	28.59	36.51	38.43	36.38	43.43

Appendix B1: CLSM Visualization Okara Distribution

A preliminary test to determine the suitable CW concentration for staining, at 15 μL , indicated the use of the higher 0.1% (w/v) CW concentration over 0.01% (w/v) CW to effectively stain the okara polysaccharides. At 0.01% CW, the polysaccharides were undetectable whereas with 15 μL of 0.1% CW, okara was detected but at high detection gain or signal amplification (1000) which resulted in significant noise in the micrograph (**Figure S.15-a**). Therefore, 15 μL CW was an insufficient volume for staining the okara fibers and further tests were conducted by staining the samples with 150 μL of 0.1% CW solution.

The micrographs okara dispersions stained with 150 μL of 0.1% CW solution are shown in **Figure S.15 (b, c)**, displaying the various structures of ground okara. Thermally processed insoluble okara fibers, extracted from soybean slurry via centrifugation, that was visualized under in a previous study identified disrupted cell walls as well as regions of continuous irregular hexagonal structures of intact cotyledon cells (ICC) [161]. Similar structures were observed in the okara dispersion in this study.

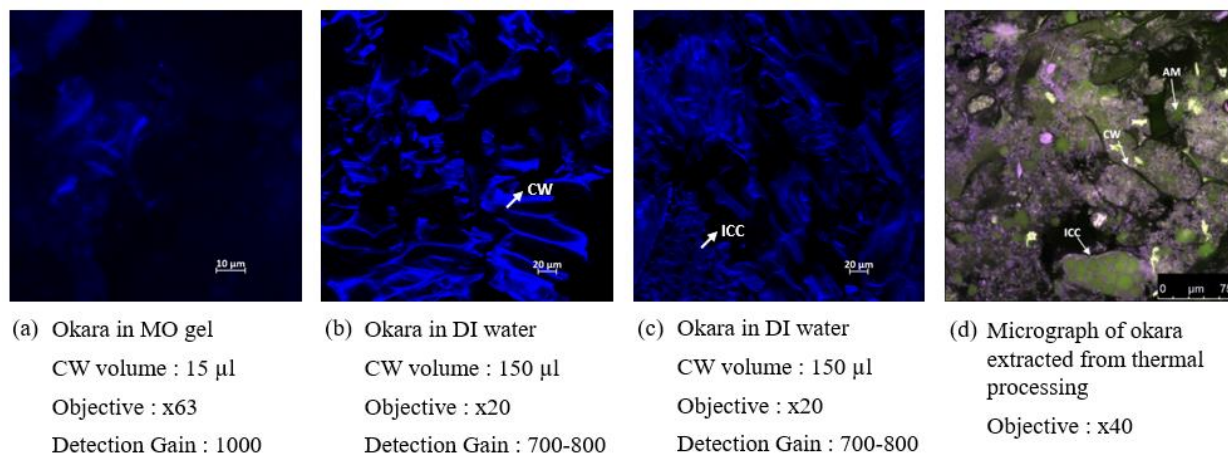


Figure S. 12. Micrographs of (a) okara in MO gel stained with 15 μL Calcofluor White (0.1% w/v), detected with an objective lens of x63 (oil immersion); (b - c) okara dispersed in water stained with 150 μL Calcofluor White (0.1% w/v) and detected with an objective lens of x20; (d) CLSM microstructure image of okara, produced using thermal processing at 80°C, visualized with acridine orange, displaying the intact cotyledon cells (ICC) and cell walls of disrupted cells (CW). Reprinted from K. E. Preece, E. Drost, N. Hooshyar, A. Krijgsman, P. W. Cox, and N. J. Zuidam,

“Confocal imaging to reveal the microstructure of soybean processing materials,” *Journal of Food Engineering*, vol. 147, pp. 8–13, Feb. 2015 (Creative Commons CC-BY license) [161]

The efficacy of 150 μL of 0.1% CW solution was assessed in staining the okara distributed in MO and MO_{x2} gels, in the presence of non-polysaccharide components such as proteins and lipids, as illustrated in **Figure S.16**. In addition to the broken cell wall and intact cotyledon cells previously identified in okara dispersion (**Figure S.15**), rod-like fiber structures were found in the soymilk okara gels. The MO_{x2} gel micrograph was color saturated at the same equipment settings (detection gain of 700-800). Dense okara regions were detected where the okara particles were clustered and stacked together due to a higher okara content and high affinity of okara particles for each other, which hindered the visualization of polysaccharide structures. However, this test confirmed that 150 μL was a suitable volume of 0.1% CW solution to sufficiently stain 0.2-0.4g okara added to soymilk okara gels.

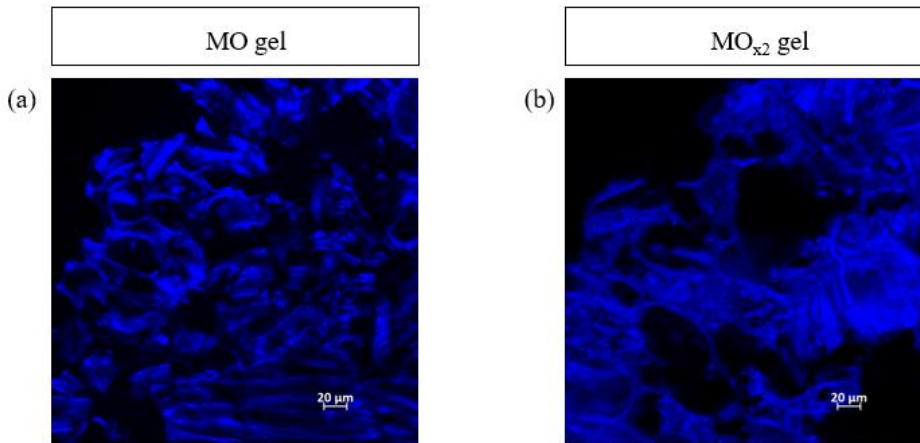


Figure S. 13. CLSM micrographs of gels (a) MO and (b) MO_{x2} .

Table S. 6: Calcofluor White amount and concentrations used across publications, and the equivalent amount of CW required for approximately 0.2g okara in the 4g gel mixture sample prepared for analysis under CLSM in our study

Gel Formulation	Staining for CLSM	Polysaccharide : CW ratio in publications	Equivalent CW Volume Required
Surimi and konjac glucomannan (KGM) (2 wt%) gels in the presence of sodium chloride (3 wt%) [183]	1 g surimi-KGM gel was stained with 40 μ l aliquots of staining solution containing 0.01% (w/v) RB and 0.1% CW	0.02g KGM in 1 g gel was stained with 40 μ l of 0.1% CW	380 μ L of 0.1% CW
Emulsion gels of 0.75 wt% egg white protein (EWP) was mixed with insoluble soybean fiber (ISF) (0.375, 0.75, 1.5 wt%). EWP-ISF suspension was mixed with soybean oil at 4:1 ratio [184]	20 μ L of CW (1 mg/mL) was used to stain 1 mL of EWP-ISF suspension (ISF concentration in emulsion was 0.3, 0.6 and 1.2 wt%)	0.003 - 0.012 g ISF present in 1mL emulsion was stained with 20 μ L of 0.1% CW	300 - 1260 μ L of 0.1% CW
Aqueous suspension of β -glucans of concentrations 0.125 - 1 % (w:v) [185]	20 μ L of the aqueous suspension of β -glucan was stained with 5 μ L of CW (0.01%)	0.025 – 0.2 mg β -glucans in 20 μ L of the aqueous suspension was stained with 5 μ L of CW (0.01%)	4 - 40 μ L of 0.1% CW
Ground soybean cotyledon suspended in water at a 1:10 (w/v) ratio; 20% of soybean cell wall is cellulose (0.2g cellulose in 10 mL water) [186]	30 μ l of dye mixture of 0.002 wt% CW, 0.001% RB, and 0.001 wt% BODIPY was added to 30 μ l of sample (assuming 10 μ l of each dye)	0.0006 g cellulose stained with 10 μ l of 0.002 wt% CW (equivalent to 0.03g cellulose stained with 10 μ l of 0.1 wt% CW)	65 μ L of 0.1% CW

*CW = Calcofluor White

RB = Rhodamine B