Oat protein structure-function properties and value-added application

by

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Abstract

The global protein market is growing with focus on plant-based proteins. Oat protein is a good source of plant protein, and is regarded as a by-product of the β-glucan extraction process; therefore oat protein is waiting research to develop its full potential. Globular proteins in oat closely resemble the proteins in soy, which have demonstrated good gelling properties. Thus, this research aims to develop a new oat protein based gelling ingredient for food and non-food applications. For this, it was necessary to complete a systematic study of the thermal gelation of oat protein under different environmental conditions. Mechanical and rheological properties of oat protein gels were determined and their microstructures were observed. We attempted to better understand gelling mechanisms of oat protein based systems by correlating protein structure changes during heating to gel microstructures and bulk properties. Special emphasis was placed on enzymatic hydrolysis and protein-polysaccharide blending, as two important strategies to improve oat protein gelling properties due to their mild reaction conditions and in consequence higher consumer acceptability.

In the first part of this work, the effect of enzymatic hydrolysis on the structure and gelling properties of oat proteins was investigated. Flavourzyme and trypsin hydrolysates could form gels with similar mechanical strength and water-holding capacity comparable to animal protein. The acidic polypeptide of the 12S fraction exerted great influence over the gelling ability of oat protein. Partial hydrolysis with the appropriate enzyme altered the charges on the protein molecular chains, allowing a balance between attractive and repulsive forces at pH 8 and 9 to establish strong three-dimensional gel networks when heated at 110 and 120˚C. Additionally it was suggested that the partial hydrolysis also led to increased exposure of

hydrophobic groups that remained in the peptide chains, which allowed development of gel with improved strength via hydrophobic interactions. Both oat protein and its hydrolysates gels exhibited excellent water holding capacity at neutral or mildly alkaline conditions.

In the second part of this work, the impact of polysaccharide addition on oat protein gelling properties was investigated. Four different types of polysaccharide were tested including inulin, dextrin, carrageenan and chitosan. A synergistic effect was observed when gels were prepared under conditions that favored segregative phase separation. In contrast an antagonistic effect was observed when gels were prepared under conditions that promoted attractive interactions. This was especially evident for oat protein gels prepared with carrageenan at neutral pH, as a two-fold increase in mechanical strength was observed. Moreover, it has been suggested that strong repulsive forces caused by carrageenan addition resulted in a highly order network structure which permitted the development of hydrogen and hydrophobic interactions to further strengthen the protein networks.

The findings from this work may encourage oat producers to promote the utilization of oat protein as an ingredient for human consumption, which could add economic value to their operations, and also motivate food producers to apply oat protein as a gelling agent in response to the increasing demand for plant-based proteins, which could increase the profit generated by producers and processors.

Preface

Chapter 2 of this thesis has been published as Vanessa Nieto-Nieto, Silvia Amaya-Llano and Lech Ozimek (2012). Continuous Membrane Bioreactor (CMBR) to Produce Nanoparticles from Milk Components, Trends in Vital Food and Control Engineering, Prof. Ayman Amer Eissa (Ed.). In this chapter I was responsible for collecting information for this literature review as well as writing down the manuscript. Dr. Silvia Amaya Llano and Dr. Lech Ozimek were supervisory authors and were responsible for manuscript edits and submission.

Chapter 3 of this thesis has been published as Talina Vanessa Nieto-Nieto, Yi Xiang Wang, Lech Ozimek, Lingyun Chen, Effects of partial hydrolysis on structure and gelling properties of oat globular proteins, Food Research International, Volume 55, January 2014, Pages 418-425. In this chapter my responsibility was to collect and analyze all the data as well as to write down the manuscript. Assistance was received from Dr. Yi Xiang Wang in obtaining the SEM micrographs. Dr. Lingyun Chen was the supervisory author and was involved in concept formation. Dr. Chen was also responsible for manuscript edits and submission.

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was received from Dr. Yi Xiang Wang in obtaining the SEM micrographs. Dr. Lingyun Chen was the supervisory author and was involved in concept formation. Dr. Chen was also responsible for manuscript edits and submission.

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List of Abbreviations

DSC differential scanning calorimetry

β-LG beta lactoglobulin

2-ME 2-mercaptoethanol

Asx Asp+Asn

CMBR continuous membrane bioreactor

CP concentration polarization

CSLM confocal laser scanning microscopy

D denatured

DH degree of hydrolysis

DMSO dimethyl sulfoxide

EMR enzymatic membrane reactor

FAO Food and Agriculture Organization

FITC fluorescein isothiocyanate

FTIR Fourier transform infrared spectroscopy

G' storage modulus

G" loss modulus

Glx Glu+Gln

GOS galactooligosaccharide

GRAS generally recognised as safe

h hydrolysis equivalents

Ho surface hydrophobicity

h_{tot} total amount of peptide bonds in the protein substrate

I intermediate

MF microfiltration

Mw Molecular weight

N native

NF nanofiltration

NLC nanostructured Lipid Carriers

NP nanoparticle

NSPS non-starch polysaccharides

OP oat protein

OP-CA oat protein- carrageenan

OP-CH oat protein-chitosan

OP-DE oat protein-dextrin

OPA o-phthaldialdehyde

OPI oat protein isolate

OPI-A alcalase oat protein hydrolysate

OPI-F flavourzyme oat protein hydrolysate

OPI-I oat protein isolate-inulin

OPI-P pepsin oat protein hydrolysate

OPI-T trypsin oat protein hydrolysate

OS oligosaccharide

SDS sodium dodecyl sulfate

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel

SE-HPLC size exclusion high performance liquid chromatography

SEM scanning electron microscopy

SLN solid lipid nanoparticle

SN-TCA soluble nitrogen after trichloroacetic acid precipitation

T_d denaturation temperature

TG transglutaminase

TNBS 2,4,6-trinitrobenzenesulfonic acid

TPA textural profile analysis

UF ultrafiltration

WHC water holding capacity

WHO World Health Organization

ΔG Gibbs free energy

ΔH_d enthalpy of denaturation

ΔH enthalpy of mixing

ΔS entropy of mixing

ζ zeta potential

Chapter 1

1. Introduction

1.1. Oat

Avena sativa (common oat) is the most important among the cultivated oat varieties. Oat is an annual grass from Asiatic origin. During early growth, the oat plant consists of leaves and a shortened stem, giving a rosette type plant. The tiller grow into additional "branch plants" or tillers and under favorable conditions, the plant can form up to 30 tillers. The main stem and tillers can reach up to 2 or more feet, depending on variety and growing conditions. These stems terminate in a large panicle that bears flowers and seeds or kernels. Each main and lateral stem as well as branch stem terminates in a spikelet that is removed during threshing. Generally two kernels, but occasionally one, are produced per spikelet. The oat kernel, also termed caryopsis or groat, is the part remaining after the removal of palea and lemma. It is elongated spindle shaped, up to about 0.5 inches length and 0.125 inches or less in width. It is generally covered with fine, silky hairs and includes the seed coat layers of cells, starchy endosperm and embryo. Oat is an important food grain in temperate regions of the world. Modern oat probably originated from the Asian wild red oat, which grew as a weed in other grain crops [1,2].

Oat contains high amounts of valuable nutrients such as soluble fibers, proteins, unsaturated fatty acids, vitamins, minerals, and antioxidants. The main constituent of oat is carbohydrate, with starch comprising the majority of this carbohydrate reserve. Very few sugars and

oligosaccharides are also present and account for less than 1 g carbohydrate/100 g oats. Relatively high amounts of dietary fiber at 9 g/100 g oats and proteins, at 15-20 g per 100 g oats, make up a significant proportion of the grain's macronutrient content. Protein accounts for 15–20% of the oat groat weight [3]. Oats are also notable for their lipid content with considerable amount of linoleic acid [4]. Vitamins and minerals comprise the minor organic and inorganic (ash) components of the grain, respectively [3]. Naked oat is a genetic variety of this grain with improved chemical composition. A larger amount of total protein and crude fat and lower content of crude fiber characterize this particular variety. The characteristic feature of the protein is its good amino acid composition, resulting in higher nutritive protein value than other cereal grains although lysine is still the limiting amino acid [5].

Since 2005-2006, the world production of oats has averaged around 24.6 million tonnes (Mt). The European Union is the world's largest oat producing region followed by Russia, Canada, United States and Australia. Global oat imports continue to be dominated by the Unites States followed by Germany, Mexico and Japan. The United States alone accounts for about 80% of all world oat imports. Canada is the world's largest oat exporter and was expected to account for 45-50% of world oat exports in 2009-2010. The European Union (mainly Finland and Sweden) and Australia are the next largest export regions. Although Russia is the world's largest single oat producer, it consumes its oats domestically and does not generally enter the export market [6].

According to the latest market outlook report by Agriculture and Agri-Food Canada (2010), the four major commercial markets for oats are:

- **Milling/Industrial market**, which requires oats that meet stringent purity requirements; have good groat yield, uniformity and color (not stained).
- **Performance feed market**, mainly the southern US horse market, demands the highest quality white oats.
- **General feed market**, mainly for beef cattle and horses is small relative to the market for barley and corn.
- **Specialty market**, which includes organic, birdseed, and health food markets.

Despite that only a minor amount of the total oat production is destined to human consumption, various oat-derived products are included in the everyday diet of many households. In recent years, the demand of oat for human consumption has increased due to the dietary benefits of β-glucan. Daily consumption of 3 g of soluble fiber derived from oat in combination with a diet low in saturated fat and cholesterol may reduce the risk of heart disease. Thus the FDA [7] has allowed this health claim to be made on the labels of oat-containing foods, which must provide at least 0.75 grams of soluble fiber per serving [8] and more recently a similar health claim has been also established in Canada [9]. The incorporation of β-glucan into food products has motivated the industry to develop various methods for its concentration and extraction [10]. In many of these processes the residue is comprised of a protein rich fraction [10], thus a natural by-product of this practice is the oat protein, which could be exploited as value added ingredient.

Many different types of products can be produced from the oat grain depending on the processing steps and conditions. For example oat flakes, steel cut groats, oat bran, oat flour, extruded oat products, oat bread and oat pasta and noodles. In general, the processing of oats

includes cleaning, heat treatment, de-hulling, cutting and flaking (milling). Since, oat contains high levels of lipids, which can be hydrolyzed to fatty acids by lipolytic enzymes found in the oat grain and later on cause rancidity due to further oxidation of the final oat product. The first step after the grain has been clean is heat treatment in order to inactivate oxidative enzymes and also the characteristic oat aroma is developed. During the flaking of oats, steam is added to increase the moisture content in order to soften the groats so that they can form flakes with minimum breakage. The flaking of intact oat groats produces rolled oats that are the thickest of the standard oat flake products, which are about 0.5 to 0.9 mm thick. The thicker flakes require longer cooking periods and maintain flake integrity for extended periods [11,12]. By cutting the groats into pieces, thinner flakers can be produced; usually 0.25 to 0.6 mm thick, and these thinner flakes require less cooking time. Thinner flakes are often used for instant oatmeal. Oat flour can be produced by milling oat flakes or groats using a special hammer mill where exhaust air is drawn through the system to prevent the relatively high fat flour from sticking to the sieves of the mill. Oat flour is used as an ingredient in a wide variety of food products. Oat bran is produced by grinding clean oat groats or rolled oats. Sieving, bolting and/or other suitable means separate the resulting oat flour. Oat bran is not more than 50% of the starting material and has a total beta-glucan content of at least 5.5% (dry weight basis) and a total dietary fiber content of at least 16% (dry weight basis). To produce oats for instant baby foods, rolled oats are milled and mixed with water, drum dried, and the thin film formed in the extract is milled and put in the products. Oatmeal is consumed on a worldwide basis, but North America, the United Kingdom and northern Europe represent the principal markets. Cold plate cereals or ready to eat products represent the second major use for oat products. Oat products are also used as ingredients in a wide

variety of bread and other baked products. These ingredients provide unique flavor and moisture retention characteristics, as well as enhance the nutritional benefits of the products. Oatmeal and oat flour are major components in infant foods. In many instances, this is the baby's first introduction to solid foods. Oat flour is a major constituent in granola bars and baking mixes. It is used as a thickening agent in soups and sauces and as a meat extender and fat replacer [12].

1.2. Oat protein

Oat contains high amounts of protein, averaging 11–15% in an oat kernel with a hull. In groats, with the high cellulose, low protein hull removed, oat protein can be as high as 12.4–24.5%, making it the highest amount of protein among commonly consumed cereal grains [3]. Additionally, the nutritive quality of oat protein is superior to other cereal proteins due to its higher lysine content [3]. Glutelins in oat are found in very low proportion. Albumins represent at most 10% of the oat storage proteins. Likewise, oat prolamins form approximately 10-15%. The albumin and prolamin fractions together account for 20 to 25% of the total protein. Therefore the globulin proportion in oat is as high as 70 to 80% [13]. The major albumin is reported to have a molecular weight of 15 kDa and a minor component of 6 kDa was also found. And the isoelectric point of this fraction composed of various components was found between 4-7.5 [14]. Unlike most cereals, oat displays a much-reduced level of alcohol soluble proteins or prolamins, which are most efficiently extracted from the endosperm with ethanol solution. Prolamins are deficient in lysine and responsible for the lower nutritive profile of the high prolamin cereals in comparison to oat [13]. Oat prolamins have two major components with molecular weight of 20 and 43 kDa, respectively and a minor of 36 kDa [14]. The major

oat globulin fraction has a sedimentation coefficient of 12S [15]. The 12S globulin of oat is similar to the 11S or legumin-type storage proteins of legumes. Other minor fractions of the oat globular proteins are the 3S and the 7S, which are believed to be vicilin-like proteins [16]. The 12S fraction is composed of disulfide-stabilized dimers, each consisting of a small basic subunit (20-24 kDa) and a larger acidic subunit (27-37 kDa) [17]. Since each subunit contains only a single conserved cysteine residue [18], the basic and acidic units are linked by a disulfide bond to yield a 53-58 kDa dimer [17]. The dimers formed by acidic-basic units further associate into a hexamer of approximately 322 kDa [15].

Isoelectric focusing analysis showed that the acidic subunit of oat globulin 12S contains about 20 major bands with isoelectric point between 5 and 7 and the basic subunit has 4 or 5 major bands with isoelectric point between 8 and 9. The two groups of subunits also differed in their amino acid composition. The content of basic amino acids such as histidine, arginine and lysine is higher in the basic subunit than in the acidic. The acidic subunit showed to be rich in glutamate + glutamine and the basic subunits in aspartate + asparagine. Both subunits contained cysteine, but methionine was not detected in the acidic subunits and less than one residue per mole was detected in the basic subunits [17]. The amino acid composition of the oat globulin is similar to that of soybean glycinin, except that the oat globulin is higher in tyrosine and phenylalanine and lower in aspartate + asparagine, proline and lysine [15].

The 3S fraction was found to consist of at least two major components with molecular weight of 15 and 21 kDa. The 7S globulin of oats has some structural resemblance to the second major storage protein type of legumes, the 7S vicilin. Many vicilins have major subunits with molecular weight between 50 kDa and 60 kDa. The 7S globulin of oat does not appear to be associated by disulfide bonds. The 7S globulin of oat contains considerably more glycine

than any reported vicilin [17]. In general the 3S and 7S fractions, both contained high glycine, but the 7S components contained more glutamate + glutamine and arginine [17].

The amino acid content analysis in previous research, indicated that seven of the eight essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine), made up approximately 32.3% and 31.2% of total amino acids for oat protein isolate (OPI) and oat flour respectively [19]. The percentage of some essential amino acids or amino acid pairs of the OPI fulfilled or exceeded their respective percentages stated in the ideal protein of World Health Organization (WHO). However, the overall quality of OPI was slightly compromised by the low level of lysine and methionine, which accounted for only 72.7% and 51.2% of the lysine and methionine required for children as recommended by Food and Agriculture Organization of the United Nations (FAO)/WHO [19].

1.3. Protein gels

Functional properties directly impact the behavior of proteins in food systems during processing, storage, preparation and consumption, which will determine the usefulness of the protein as a food ingredient [20]. Functional properties can be classified as organoleptic, which have a direct impact on the color, flavor, texture of food; as hydration related such as solubility and viscosity; surface related such as emulsifying and foaming; or textural such as gelling properties and dough formation [21]. It is common that food proteins often show various functional properties. The different functionalities that proteins can exert may depend on various molecular features such as the type and availability of amino acids, molecular size, shape, conformation, net charge and protein/protein interactions [20]. These molecular features are considered as inherent factors of the protein, nonetheless there are also external

factors that may also have a direct impact on the functional properties exerted by the protein. These external factors primarily relate to the environmental conditions (pH, ionic strength, temperature) under which the protein is used. Thus it is common to find that proteins extracted by different methods or that have been processed by different treatments, often have different functionalities. It must be considered that the external factors have direct effect on the protein conformation and thus over the intrinsic factors [20,21].

Gelation is one of the most important functional properties of protein as it provides texture and support in foods. Gels have been defined as three-dimensional networks that entrap large quantities of water while remaining insoluble in aqueous solution due to different interactions [22], or as an intermediate state between a solid and a liquid, possessing both elastic (solid) and flow (liquid) characteristics [23]. Proteins can form gels and provide a structural matrix for holding water, flavor, sugar and food ingredients in various food applications but also to provide texture and mechanical support in foods. Protein gelation is useful not only for the formation of solid viscoelastic gels but also for improving other functional properties related to the way protein interacts with water molecules producing increase of thickness or increased water absorption of the food matrix.

A Protein solution can be changed into a gel by a range of processes that increase intermolecular interactions. Once the degree of intermolecular linking reaches a point where a continuous network is formed, the macroscopic property of elasticity is developed and the system is considered a gel [24]. This is reflected as a viscoelastic system that shows a storage modulus (G') larger than the loss modulus (G'') [23].

The formation of a gel can be classified broadly as physical induced (heat, pressure) and chemically induced (acid, ionic, enzymatic) gelation reactions [25]. Thermal gelation is the

most common method to obtain protein gels and in this process, heat induces unfolding of protein, which leads to a non-native conformational state and hence altered functional behavior. Generally, heating temperatures above the minimum denaturation temperature of the proteins is required for gel formation and specifically, the heating conditions used to form a gel greatly influence its rheological properties [26]. A simple model for protein denaturation is:

$$N \rightleftarrows I \rightarrow D$$

Where a native (N) structure is reversibly converted to an intermediate (I) state where tertiary structure is changed but much of the secondary structure remains, and further unfolding produces a denatured (D) state [24]. There are various molecular properties associated with each state that have an impact on functional properties. Molecular weight and primary structure will not be changed during the denaturation, nevertheless the isoelectric point can vary due to intermediate and denatured (unfolded) structures exposing charged amino acids to new local environments. The main changes are in secondary and tertiary structure that can alter the surface exposure of amino acids. This cumulates in an increase in interaction potential, favoring aggregation [24]. A subsequent stage after protein unfolding is the association and aggregation of unfolded molecules with newly exposed amino acids to form complexes of higher molecular weight [23]. The association and aggregation stage can only result into a cross-linked network if the protein concentration is high enough to allow interconnection of the complexes formed [27]. If the protein concentration is low, the association and aggregation stage will cause precipitation. In contrast at the critical gel concentration, aggregates will react or crosslink with other aggregates and after such

intermolecular aggregation stage, the number of crosslinks has increased as well as the molecular weight of the final aggregate [28].

1.4. Network structure of protein gels

In thermal gelation, more factors other than the heating temperature will have an effect on the unfolding and aggregation process. For example, the pH can change the net charge of the molecules and alter the attractive or repulsive forces between molecules as well as the interactions between molecules and solvent. In a similar way the salt content can increase the ionic strength of the gel, which causes the electrostatic forces between molecules to be reduced or neutralized. Thus these factors will define the form of association (type of interactions) that will participate into the gel network formation. Depending on the initial pH and ionic strength it is possible to obtain either transparent or turbid gels due to very well studied structural differences [29].

At low ionic strength, turbidity increases around the isoelectric point of the protein, where the net charge is zero. Likewise at pH values far from the isoelectric point, increasing the ionic strength reduces the electrostatic interactions and enhances turbidity [30]. These conditions, promote the formation of particulates gels, which are characterized by random association into large and seemingly spherical aggregates linked together to form the gel network [27].

Under conditions that promote strong repulsive interactions between polypeptide chains transparent gels are formed, these so-called fine stranded gels are composed of less flexible linear strands and are generally prepared at pH values far from the isoelectric point of the protein and under low ionic strength. In the case of fine stranded gels the rate of aggregation

is slow as the attractive forces between denatured polypeptide chains are small, since repulsion forces, dominate the molecules present [23,25,27]. Gels with fine stranded structures have high water retention capacity whereas gels having nonhomogeneous or particulate structure present a high degree of syneresis [31]

1.5. Gelation mechanism

In general, the gel formation process is considered a multi-step process in which as previously mention the first pre-requisite is to achieve a sufficient degree of unfolding in the protein molecules in order to attain a new reorganization of the protein matrix.

Previously, the gel formation mechanism, for fine stranded and particulate gels prepared with β-lactoglobulin, was studied [27] and it was proposed that the structural differences were related to the different course of the gel formation process. In the case of fine stranded gels dimers were dissociated into monomers prior to aggregation and under conditions promoting the formation of fine stranded gels, the protein undergoes significant structural changes before the aggregation step takes place. In contrasts, in the case of particulate gels the protein experiences a slight unfolding and remains in the dimeric form until aggregation. Since monomers may aggregate in a different way than dimers, different structures may be produced. Consequently as the protein is more unfolded in the case of fine stranded gels, a closer parallel alignment of the polypeptide chains takes place, forming linear strands. This favors intermolecular interactions and hence, more and stronger interactions are developed in fine stranded gels. On the other hand, near the isoelectric point the protein structure is less disturbed and remains in the dimeric form and the gelation process commences with dimers

as the building block, which may hinder the formation of strong bonds as aggregation takes place [27].

Similar mechanisms of protein gelation have been proposed. For example, Clark (2001) proposed a kinetic model for the formation of uniform (pH far from isoelectric point, and low salt content) thermally induced protein gels, which considered three steps for network formation. The first step consisted of the protein unfolding. The second step involved the aggregation of linear filament via nucleation and growth and the third step entailed the random cross-linking of the filaments [32]. For the nucleation and growth process, it was proposed that a number of unfolded monomers had to form a stable nucleus before other unfolded species could add on, one by one, in a linear growth mechanism and the growth of the linear aggregates was terminated by reaction with other chains or with other reactive species [32]. Various models have been proposed to explain the gel formation mechanism, however the common consideration is that basic units (reactive molecules) work as building blocks. The way the building block link will be defined by some inherent characteristics such as size and reactivity (exposure of reactive amino acids). This will ultimately influence the degree of order under which the protein matrix is reorganized and will affect the mechanical characteristics of the gel [25]. The fractal model has been very useful for describing aggregation and gelation of proteins. According to this model, protein gels are formed by primary particles that aggregate to form clusters with a fractal structure, which may ultimately lead to a gel assembled from fractal clusters. The fractal model also considers that the gel formation process is also affected by other relevant factors such as the; 1) the size of the building blocks of the fractal clusters, 2) the amount of protein incorporated in the fractal cluster at the moment the gel is formed and, 3) the way in which the fractal clusters are linked together [33].

The percolation model associated to gelation envisions a lattice where monomers are placed and then randomly introduce a certain proportion of bonds connecting these monomers. This is done by estimating the probability of the site occupation, or of the bond formation in the lattice. As the bonding advances, clusters of monomers (aggregates) are formed, and at a critical threshold of bonding, the gel point is observed in the form of a molecule that now covers the entire percolation lattice [34]. The physical properties (molecular weight, viscosity, elastic modulus) of a system change as the critical gel point is reached [34].

1.6. Interactions involved in gel structure

The gel properties are developed due to the crosslinking of the basic units or building blocks leading to the assembly of a three-dimensional network. This assembly is done by the balance between repulsive and attractive interactions. The repulsive interactions are always nonspecific and with momentary duration. They result from excluded volume effect and/ or electrostatic interactions and tend to be weak, except at very close range or very low ionic strength [35]. Van der Waals, hydrophobic and hydrogen bonding and even ionic interactions under the right pH conditions work as attractive interactions [35]. Thus the simultaneous occurrence of all these interactions, both repulsive and attractive will determine the arrangement of the three-dimensional network. Next, these interactions are discussed in detail, as they are important for gel formation and property development.

1.6.1. Electrostatic interactions

Electrostatic interactions take place between electrically charged molecules. Oppositely charged molecules (counter-ions) are preferentially attracted, whereas molecules of the same charge (co-ions) are repelled. In the case of proteins, balanced electrostatic interactions are

critical in determining the gel network structure and influencing other gel properties. The net charge on protein molecules greatly affects electrostatic attractive or repulsive forces and thus interactions among protein molecules and with the solvent [26]. The sign, magnitude and distribution of the charge on protein molecules are affected by changes in the ionic strength and pH. When the protein is solubilized and the pH is adjusted to values below the isoelectric point, the protein molecules will be positively charged. If the pH is adjusted above the isoelectric point, then the protein molecules will be negatively charged. At the isoelectric point the net charge is zero, because the number of positive and negative charges on the protein are equal [36,37]. Similarly the strength and range of these interactions decrease with increasing ionic strength [38]. A strong electrostatic repulsion force extensively opposes protein-protein interactions preventing gel formation. Intramolecular electrostatic interactions, both attractive and repulsive, control the level of folding/unfolding and expansion of macromolecules, as well as their molecular flexibility [39].

1.6.2. Excluded volume effect

These are repulsive interactions of short range arising from the highly unfavorable overlap of full electron clouds. These interactions are also known as "steric repulsion" as they restrict the relative spatial arrangements of macromolecules. The excluded volume effect is due to the fact that the volume occupied by one biopolymer molecule in solution is not available to other biopolymer molecules. Thus the size and shape of the biopolymer molecule/particle (as determined by the macromolecular conformation/flexibility or aggregate architecture) is of prime importance in relation to steric interactions. The excluded volume effect is associated with a reduction in the mixing entropy of the system. The resulting steric interactions

contribute predominantly to phase separation that is commonly observed in mixed polymer systems [39].

1.6.3. Hydrogen bonds

Hydrogen bonds are short-range attractive interactions with ionic character [39] as this type of bond occurs when a hydrogen atom attach to an electronegative atom such as nitrogen, fluorine, oxygen or sulfur [35]. Hydrogen bonds can be so strong that they cause appreciable alignment of the participating molecules [36] as in the case of α-helix and β-sheet structures of proteins. This indicates the important contribution of hydrogen bonds to the secondary structure of proteins and also the important involvement during folding/unfolding [39]. Hydrogen bonds are commonly observed between charged and neutral biopolymers or between two neutral biopolymers, forming junction zones between different biopolymer molecules [40]. These bonds are enhanced by cooling [26] and become weaker with increasing temperature [39], which contributes to the loss in native biopolymer conformation (denaturation) [41].

1.6.4. Hydrophobic interactions

Hydrophobic interactions occur between non-polar groups separated by water. In an aqueous media these attractive interactions make a strong contribution to biopolymer self-association and complex formation [39]. Hydrophobic interactions between non-polar segments of adjacent polypeptides are enhanced after heating as the protein molecule is expected to unfold and expose buried non-polar groups prior to network formation [26]. Hydrophobic interactions are mainly promoted by conformational and structural changes [35]. As these non-polar groups are exposed in water, water molecules need to reorganize around the hydrophobic exposed group; this changes the interaction energy and entropy of the system. As these changes are

thermodynamically unfavorable, whenever there is another non-polar group exposed in the near surroundings, the formation of an intermolecular hydrophobic bond will be favored in order to minimize the contact between water and non-polar groups [36,42].

1.6.5. Van der Waals

Van der Waals interactions have little influence in the conformation of biopolymers in solution, as not many changes are observer for this particular type of bond during the folded/unfolded transition. Nonetheless, strong van der Waals interactions could work as attractive forces favoring aggregation whenever a biopolymer molecule is large enough to act as colloidal particle [36]. This type of interaction occurs between permanent dipoles and/or induced (temporary and fluctuating) dipoles, and thus depends on the polarizabilities and ionization energies of the two [37]. Van der Waals attraction is a fairly long-range interaction of electrostatic origin, however at very short proximity, such attractive interaction is transformed into strong repulsion [39].

1.6.6. Disulfide bonds

Disulfide bonds are covalent bonds, which are very strong chemical linkages found in globular proteins. Upon unfolding, proteins may expose internal disulfide bonds or sulfhydryl groups, which can undergo disulfide/sulfhydryl exchange reactions with other unfolded polypeptides chains, yielding covalent intermolecular disulfide bonds [42]. This reaction usually occurs under alkaline conditions. Proteins are capable of forming both intramolecular and intermolecular disulfide bonds under the appropriate conditions [36]. The role of intermolecular covalent disulfide bonds in protein gelation may be related to their ability to increase the length of the polypeptide chain, rather than as an initial network primer. Having a longer polypeptide chain may increase molecular entanglements within the

gel structure, thereby restricting the relative thermal motions of the polypeptide [26]. The formation of disulfide bonds requires a close proximity of the reactants and also enough time since disulfide bond formation proceeds slowly as a result of the high activation energy [42].

The interactions mentioned above play an important role to determine protein gel microstructures and consequently the gel behavior under specific environmental conditions. Understanding when and how these interactions occur will allow design of desirable structures targeting different applications.

1.7. Protein modification to improve gelling properties

Proteins may have limited functionality that could constraint their utilization in food systems, consequently, in many cases the modification of proteins is necessary in order to improved or perhaps correct deficiencies in the functional properties exerted by a specific protein. As it was mention earlier, changing some environmental conditions can easily alter protein structures, for example; pH, salt content or temperature among many others conditions. It is expected that as a result of an altered structure the functional properties of the protein can be modified as well. The modifications produced in the functional properties of the protein may be desirable or might be detrimental; nonetheless the application of various methods to intentionally modify the functional properties of proteins has been studied by various researchers with the underlining purpose of improving specific functional properties of proteins [43-47]. Basically, chemical and enzymatic modification are two approaches used widely to modify protein functional properties. In addition to these techniques, the inclusion of polysaccharide to modify protein properties has also been widely studied [48-51]. A brief

review of the most common techniques is presented in the following text with emphasis on their capacity to improve protein gelling properties.

1.7.1. Chemical modification

Chemical modification uses different derivatizing reagents that can modify the charge, hydrophobicity, molecular size and conformation of protein molecules to yield desirable functional properties. Additionally, acid or alkali treatments are used for the purpose of deamidation or peptide bond cleavage to produce hydrolysates [52]. The primary effect on chemical modification procedures is to increase protein solubility in general, and other functional properties in consequence [53]

1.7.1.1. Acylation

Acylation is known to alter protein conformation by promoting unfolding and increasing dissociation of subunits from quaternary structure, as well as shifting the isoelectric point to lower values [47]. This is done in a chemical reaction under which the positively charged lysine Ɛ-amino groups are replaced with negatively charged carboxyl groups [54,55]. Therefore, the electrostatic attraction between charge groups is reduced and hence critical protein-protein interactions [53]. Acylation of protein using succinic anhydride is called succinylation [53]. The change in conformation of succinylated proteins results from their high net charge and replacement of short-range attractive forces in the native molecule with short-range repulsive forces [47]. Succinylation enhances hydration and solubility, improves thermal stability, and generally results in partial to complete unfolding of the protein molecule as the extent of the reaction is allowed to proceed [53]. The gel hardness of oat protein gels was greatly increased by both acetylation and Succinylation, particularly at lower pH [56]. Higher G' and G'' values were observed in canola protein, after the protein structure was modified

through acetylation or succinylation. This improvement was associated to increased surface hydrophobicity caused by the acylation process [57].

1.7.1.2. Deamidation

Deamidation refers to the conversion of asparagine or glutamine amide groups to carboxylic groups [58]. Thus glutamine and asparagine change into glutamic acid and aspartic acid respectively. Proteins can be deamidated in acidic or basic environment, nonetheless the harsh conditions used (extreme pH and high temperature) can lead to protein denaturation and peptide bond hydrolysis which either produces a beneficial or detrimental effect on the functional properties depending on the modification degree [58]. Hence, moderate conditions are recommended to improve functional properties. Deamidation can alter functional properties of proteins by increasing the number of their negative charges thus the enhanced electrostatic repulsion [59] among protein molecules resulting into partial unfolding and exposure of hydrophobic amino acid residues [60]. The isoelectric point of the protein is also lowered as a consequence of the deamidation reaction. Deamidated proteins showed improved emulsifying properties [60] and foaming properties [59]. Deamidation of glutelin from *Akebia trifoliata* with edible organic acids such as citric and malic acid caused a significant improvement of the gel network strength. It was proposed that deamidation induced unfolding of glutelin, producing exposure of more sulfhydryl groups and hydrophobic sites, causing extensive intermolecular protein-protein interactions [61]. Oat protein was deamidated by mild acid hydrolysis. The pH for heat-induced gelation shifted from 9.7 to 7.5 and a weak gel with good water retention ability was formed, Sample with 40.9% deamidation degree had better gel-forming ability. This was attributed to increase in net negative charge and surface hydrophobicity and a decrease in molecular size [62].

1.7.1.3. Phosphorylation

During phosphorylation, phosphate groups can be covalently attached to free ε-amino groups on proteins using various reagents such as phosphorus oxychloride, phosphoric acid or phosphorus pentoxide to effectively increase the net negative charge of the protein [53,63,64]. Some of the targeted groups are the –OH group of seryl, threonyl, and tyrosyl residues and the N of the ε-amino and imidazole group of lysyl and histidyl residues of proteins, and under some conditions the guanidino groups of arginine may be phosphorylated (above pH 11) [65]. Phosphorylation has proven to be a useful method for improving the functional properties of protein as it generally improves solubility, by increasing net hydration of protein chemical groups due to the hydrophilicity of the covalently attached phosphate group [64]. The changes in these properties are a direct result of increased surface electronegativity, thus the electrostatic repulsive forces are increased [53]. Nonetheless, some phosphorylation reactions have led to protein crosslinking, which has shown to have an adverse effect on solubility but it appears to increase viscosity and improve the gelling properties [64]. Increased surface hydrophobicity has been reported after dry-heating phosphorylation, possibly the buried hydrophobic groups become exposed due to strong repulsive forces produced by the negative charges introduced by phosphate groups [66]. Gelling properties have been improved by phosphorylation of a conjugated whey protein in terms of hardness, resiliency, and water holding capacity values. In addition a transparent gel was formed, this was not possible with whey protein before modification [67]. Similarly, a firmer and transparent heat-induced gel of phosphorylated egg white protein was obtained with improved water holding capacity. Increased repulsion of negative charges caused by the

introduction of phosphate groups, makes the phosphorylated protein more unfolded, exposing buried hydrophobic patches. This creates a controlled balance of hydrophobic attractive interactions and electrostatic repulsion for the improvement of gelling properties [66]. Nevertheless, phosphorylated food proteins may not be well accepted in the market, as normally this type of reaction requires harsh reaction conditions and it is difficult to remove unreacted reagents from the final product [64,68].

1.7.1.4. Glycation

Glycation refers to the attachment of sugar residues to proteins through the Maillard reaction [69]. The Maillard reaction, or non-enzymatic browning was first described by the French biochemist Louis Maillard at the beginning of the 20th century. It consists of a condensation of the carbonyl group of a reducing sugar with the ε-amino group of lysyl residues of proteins and results in the so-called Amadori product via the formation of a Schiff base and water release [70]. Heating is often used to accelerate this spontaneous reaction. Since lysine is the limiting essential amino acid in many food proteins, its destruction can reduce the nutritional value of proteins [71]. In most foods it is the $\boldsymbol{\varepsilon}$-amino group of the lysine residues in proteins that are the primary source of reactive amino groups. The conjugation reaction could be limited if the treated protein is a protein with reduced lysine content. Nonetheless, other amino groups can participate in the reaction such as the imidazole group of histidine, the indole group of tryptophan and the guanidine group of arginine but to a lesser extent [69]. The Maillard reaction can be performed under wet or dry conditions. Normally the reaction carried out under dry conditions is preferred as it provides higher stability, faster rate of reaction and easier storage and handling than the aqueous dissolved wet method [72]. Several reaction factors, such as temperature, time, pH, water activity, characteristics of the protein,

properties of the reducing sugar (mono- or polysaccharide) and the amino group:reducing sugar ratio have an impact on the yield and type of Maillard reaction product [73]. Maillard reaction has been used to improve the functional properties, such as solubility, heat stability, emulsifying properties of proteins as covalent bonds are formed between the amino group of the protein and the reducing sugar [74]. After glycation, the amount of lysine residues which carry a positive charge are blocked, thus a change in the isoelectric point of the protein is produced as the net charge of the protein becomes less positive [73,75]. The change in the net charge of the glycated protein also results in the change of surface hydrophilicity/hydrophobicity balance, which greatly affects the functional properties of the protein [73]. Increase of the molecular weight is an obvious result of the glycation reaction, as the attachment of the reducing sugar to the protein forms as larger molecule. Additionally, changes in the functional properties may be attributed to repulsive steric interactions provided by the glycated saccharides, as the steric interactions are a function of the number and effective chain length of saccharides attached on the protein molecules [75]. One advantage of the Maillard reaction over other chemical modification techniques is the utilization of relatively milder and safer conditions. Nonetheless there is some controversy on whether the Maillard reaction products exhibit some toxicity [76]. The Maillard reaction significantly modified the mechanical properties of whey protein-dextran gels and even prevented fracture when conjugate gels were subjected to 80% deformation in uniaxial compression test [77]. Similarly, glycated ovalbumin gels showed increase breaking stress and strain and the appearance of these gels was transparent [78]. Soy protein-xylose Maillard gels showed less syneresis, higher breaking force and were more elastic. The improvement on these gelling properties was attributed to formation of additional covalent crosslinks [79].

1.7.2. Enzymatic modification

Enzymatic modification is generally carried out under mild pH and temperature conditions, more specific and less prone to yielding undesired side reactions than chemical modification procedures. Furthermore, enzymes can be inactivated after the desired functional property has been reached, leading to better control of the final product quality [52]. The utilization of enzymes holds the advantage that the toxicity is reduced and a wide variety of enzymes are commercially available from animal, plant and microbial sources. The choice of enzyme depends on the protein source and end user requirements [80]. The rate of reaction is influenced by the pH and temperature. Only at a certain pH the enzyme is catalytically active as the amino acid residues at the active site possess a particular charge allowing contact with the substrate [81]. Increasing temperature can accelerate the rate of reaction, since the atoms in the enzyme molecule have grater energy and a greater tendency to move. However, as the temperature rises to values close to the denaturation temperature, the activity of the enzyme is terminated. This is due to the unfolding of the protein chain after the breakage of weak bonds; hence the overall reaction velocity drops [82]. Thus, by controlling pH and temperature of the process, the extent of reaction and in consequence the final product can be carefully custom-made.

1.7.2.1. Transglutaminase (TG)

Various enzymes such as transglutaminase, peroxidase and polyphenol oxidase are suitable for cross-linking of protein. Especially TG has found numerous applications within the food industry [83]. TG catalyzes the acyl-transfer reaction between γ-carboxyamide groups of glutamine residues and the ε-amino group of lysine in proteins, leading to inter or intramolecular cross-linking [84]. Three reactions can be catalyzed by TG [85].

- Cross-linking of proteins, resulting in the formation of high molecular weight polymers.
- In the presence of primary amines, TG can cross-link the amines to the glutamines of a protein (acyl-transfer reaction).
- In the absence of lysine residues or other primary amines, water will react as a nucleophile, resulting in deamidation of glutamines.

Changing the functionality of food protein is the main purpose of TG treatment and each type of reaction will produce different changes in the protein structure and therefore in the functional properties. As a result of the crosslink reaction, larger polymers are formed as it can be observed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [86]. In the case of the acyl-transfer reaction, changes in the charge distribution on the protein surface, as well as change in the hydrophobicity can be produced as the reactive lysine may be substituted by several compounds containing primary amino groups, resulting into different derivatives [87]. Similarly to the chemical deamidation, in the case of the enzymatic deamidation reaction, the functional properties such as solubility, emulsification foaming and gelation can be improved. This reaction is suitable for substrates with low lysine and high glutamine content, such as in the case of gluten [85]. The crosslinking reaction allows formation of a well-developed viscoelastic gel network with increased G' and G'' values. TG-induced gelation is characterized for the development of covalent bonds that constitute crosslinking points in the gel network [88]. In the case of soy glycinin-rich gels, it has been proposed that in addition to covalent bonds other interactions such as hydrophobic, hydrogen bonds and disulfide bonds were involved in the formation of the gel network [89]. TG treatment of pea

protein reduced the minimum protein concentration to form heat-induced gels from 5.5% (w/v) to 3% (w/v). Additional cross-linking among protein molecules increases the protein gelation ability, thus less protein is required for gel formation [90].

1.7.2.2. Enzymatic Hydrolysis

The enzymatic hydrolysis reaction consists of the cleavage of the peptide bonds that constitute the protein chains by the specific catalytic action of an enzyme. During the hydrolysis, a water molecule is added per every peptide bond broken [91]. This process is also called proteolysis for the particular case of protein as a substrate and a protease as the catalyst. Enzymatic hydrolysis of proteins is an important bioprocess to improve the physical, chemical, functional and nutritional properties of proteins [92]. The hydrolysis of peptide bonds can increase the number of charged groups and hydrophobicity, decrease molecular weight and modify molecular configuration. Changes in functional properties of protein hydrolysates are greatly dependent on the type of enzyme used, process variables, such as temperature and pH, extent of the hydrolysis, which define the amino acid sequence within the peptide structure [93].

The degree of hydrolysis (DH) is used to measure the extent of the hydrolytic degradation, and the number of peptide bonds cleaved during the reaction is the entity, which most closely reflects the catalytic action of proteases [94]. Several methods of monitoring DH during protein hydrolysis have been proposed. These include the pH-stat, osmometry, soluble nitrogen after trichloroacetic acid precipitation (SN-TCA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), o-phthaldialdehyde (OPA), amino acid nitrogen and formol titration methods [95].

With regards to the application, protein hydrolysated may be broadly classified in two categories depending on the DH.

- **Limited hydrolysis:** DH lower than 10% is used for modifying the functional properties such as solubility, emulsifying, gelling or foaming properties as well as water and oil adsorption [91,96].
- **Extensive hydrolysis:** DH higher than 10% is used as an excellent source of nitrogen in hypoallergenic formulae and enteral diets and to activate biological activity of peptides [91,96].

Recent research has reported the effect of enzymatic hydrolysis over the gelling properties of different proteins. In the case of peanut protein treated with alcalasae, it was proposed that limited enzymatic hydrolysis produced a more unfolded conformation with exposed sulfhydryl and disulfide bonds inside the molecules, which greatly contributed to the formation of the gel network [43]. Another example is the limited enzymatic hydrolysis of canola protein as a pretreatment for crosslinking with TG. It was suggested that enzymatic hydrolysis opened up the protein structure, enhancing the effectiveness of TG and improving gelation properties [97]. The effect of enzymatic hydrolysis on various substrates [98-100] have been studied and results indicate that the improvement of the gelling properties depends on the enzyme-substrate combination as well as the hydrolysis conditions. With increasing DH, decreasing protein-protein interactions [43] are expected and the gelling properties may be impaired. Hence, controlling hydrolysis conditions and the extent of the reaction by monitoring the DH, is of the upmost importance in order to produce ingredients with new functionalities.

1.7.2.2.1. Enzymatic membrane reactors

In the manufacturing of protein hydrolysates, the enzymatic bioconversion processes are of increasing use in the production and transformation of protein raw materials. Predominantly, batch hydrolysis of proteins in jacketed stirred tank reactor is the traditional procedure to obtain hydrolysates with intended use as food ingredients. From the operational point of view, batch type protein hydrolysis is simple and easy to control [91]. However hydrolysis in the batch process involves high cost due to the large quantity of enzyme that is requires, high energy, labor and capital cost. The typical batch reaction involves mixing the enzyme and substrate in a large vat and holding temperature and pH constant for several hours. Upon reaching the required degree of hydrolysis the reaction is terminated by changing the pH and/or increasing the temperature to inactivate the enzyme, thus the enzyme is used only once [101]. As alternatives, enzyme immobilization onto supports and membrane filtration have been proposed. In both cases the aim of the process is; to hydrolyze the protein in order to adjust its functional properties, remove the enzyme from the final product and to recover and reuse the enzyme in further reaction if possible [91]. The enzymatic membrane reactor (EMR) combines a membrane separation process with an enzymatic reaction. Thus, the utilization of EMR ensures two processes (catalysis and separation) working simultaneously and continuously [102]. The main objective of EMR is to ensure the complete rejection of the enzyme in order to maintain the full activity inside the reaction volume. Depending on the case, the enzyme could circulate freely on the retentate or could be immobilized onto the membrane surface or inside its porous structure [103]. The use of soluble enzymes in biotransformation presents significant advantages over immobilized enzymes in terms of productivity, selectivity and economics. Immobilization procedures often lead to reduced

activity due to covalent or physical attachment to a carrier and additional mass transfer resistance within the particles. Furthermore, the immobilization step generates extra catalyst manufacturing cost. The introduction of EMR made it possible to retain the enzyme in the reactor, which enables enzyme lifetime of several batches and continuous process operation [104]. The filtration process also facilitates the recovery of the final hydrolysates without need of further steps to remove undesired components. Filtration is the separation of two or more components from a fluid stream. The filtration membrane works as a selective barrier, allowing the flow of certain molecular and ionic components and retaining other components present in a liquid or a vapor mixture. In membrane separation, particles are separated on the basis of their molecular size and shape with the use of pressure and specially designed semipermeable membranes [105]. Membrane filtration process is the most feasible downstream strategy for industrial manufacture of enzymatically-modified proteins. Membrane technology has a number of advantages as compared to chromatographic purification techniques. These advantages include low energy requirements, hence sustainable processing, and easy modification of the critical operational variables such as pressure, temperatures, feed flow rate, agitation etc., and relatively easy scale up. In addition, product inhibition of the enzyme catalyzed reactions can be diminished if the setup is integrated with continuous or semi-continuous product removal [102]. Ultrafiltration (UF) membranes are routinely employed to enrich protein hydrolysates [106], in which, the applied pressure ranges from about 3 to 7 bars. The separation principle is based on the diameters of molecules and partially on their charge and affinity for the membrane [107]. UF membranes are capable of retaining species in the range of 300-500,000 Da of molecular weight, with pore size ranging from 0.001 to 0.1 μm [108]. An obstacle towards wider acceptance of membrane technology are

the problem of concentration polarization and membrane fouling, thus membrane cleaning is a very important issue in membrane technology. During the separation process, the withdrawal of permeated solutes at the permeate side of the membrane causes a build-up of retained solutes at the surface of the membrane. This creates a difference in concentration of retained solutes in the bulk phase and at the adjacent side of the membrane surface. Such phenomenon is called concentration polarization (CP) [107]. Moreover, membrane fouling is a process in which solutes or particles of the feed stream deposit on the membrane surface, which is so-called external fouling, or into the membrane pores, which is called internal fouling, resulting in a decrease in membrane performance. Due to membrane fouling, the flux declines dramatically and the selectivity of the membrane is modified. Both internal and surface fouling materials act as additional layers of resistance to the transmission of solutes throughout the membrane. CP is part of reversible membrane fouling, which can be removed by physical methods such as high shear force or back flushing. Severe irreversible membrane fouling may require intense chemical cleaning or even replacement of the membrane, which limits the economic efficiency of the application of membrane process [107].

The application of EMR has been especially useful in the preparation and production of innumerable value added ingredients from various food components including lipids, proteins and carbohydrates. In the case of protein derived ingredients, the production of bioactive peptides [109-112] has been the main focus of development and fine-tuning of reaction conditions. In contrast the limited amount of literature reports available indicate that the preparation of protein hydrolysates with improved functional properties has been less explored [113]. Nonetheless the application of EMR for production of novel food ingredients such as protein derived gelling agents should be studied, taking into account that, through

this technology with the usage of the appropriate membrane and by reaching the appropriate DH, is possible to control the molecular weight distribution of the hydrolysates and that the molecular weight of the hydrolysates has strong influence on the functional properties of the hydrolysates [94].

In view of the many valuable food ingredients produced by membrane technologies, some of the strategies implemented for the concentration, purification or separation of these values added ingredient could be adopted in the preparation of protein hydrolysates with techno-functional applications, such as the implementation of integrated membrane processes that include sequential filtration process [114] or electro-membrane filtration [115].

1.8. Protein-polysaccharide interactions

Mixtures of proteins and polysaccharides are often used in various products with the purpose of improving or obtaining different properties in the food. These mixtures of protein and polysaccharide are done in the expectation that the polysaccharide can modify the functional properties of the protein through the different interactions established between the two components [35]. The use of protein–polysaccharide mixtures to change food properties with especial emphasis in gelation have been widely studied [48-51].

Polysaccharides are polymers of one or more sugars that are the building blocks. The most common building blocks of naturally occurring polysaccharides are glucose, fructose and galactose [116]. Thus the type of sugar and the nature of the linkages determine the structure and function of the polymer [117]. Polysaccharides are grossly divided into two groups, starch and non-starch polysaccharides (NSPS). The NSPS group includes polysaccharides of the plant cell wall such as cellulose, hemicellulose and pectin as well as plant gums, mucilages

and hydrocolloids [116]. The length of the polymer chain, the degree of branching, the molecular weight and volume and also the fact that many polysaccharides have other chemical groups which conferee an electric charge to the molecule are relevant features that may impact the way the polysaccharide interact with proteins [118].

1.8.1. Associative and segregative interactions

Basically protein-polysaccharide interactions can be classified as segregative or associative. These types of interactions arise from the fact that proteins and polysaccharides are unlike polymers. When interactions between protein-polysaccharide are less favorable than protein-protein and polysaccharide-polysaccharide, there will be a tendency of the system to segregate into regions where one of the components is surrounded by others of the same type, whereas favorable protein-polysaccharide interactions will promote association between the two polymers [119]. These two types of inter-polymer interactions are responsible for the immiscibility and the complexing of biopolymers [120], and depend on the thermodynamic characteristics of the individual polymers [121]. In the case of segregative interactions, protein and polysaccharide repel each other and are denoted as incompatible. In contrast, in the case of associative interactions, protein and polysaccharide attract each other [122]. A system containing two separate phases can be form by mixing protein and polysaccharides with the same electric charge. In this case, both protein and polysaccharide separate due to electrostatic repulsion which makes the system unstable and parts into two phases, one rich in protein and the other rich in polysaccharide [123] (Figure 1 a). A system can separate into two phases not only due to electrostatic repulsion but also due to high steric exclusion. At a sufficiently low concentration, the protein and polysaccharide are well mixed and form a

one-phase solution, but once the concentration exceeds a certain level, phase separation occurs and a two-phase solution is formed [38].

Once two non-interacting polymers are mixed together a single phase is formed, where the two polymers are evenly distributed throughout the medium (Figure 1 b). In this case the polymers are considered "co-soluble". Miscibility or co-solubility commonly occurs at low biopolymer concentrations [123].

Formation of protein-polysaccharide complexes may occur between a positively charged protein and a negatively charge polysaccharide. In this case the pH of the solution should be below the isoelectric point of the protein. The same could occur at a pH above the isoelectric point, in this case the negatively charged protein can interact with a positively charged polysaccharide. The chance to form a complex is reduced as the pH gets close to the isoelectric point of the protein, since at this pH the net charge of the protein is zero and no electrostatic attractive interactions can be established between the protein and the polysaccharide [35,124]. In solution, complex formation could result into the formation of a soluble or insoluble complex (Figure 1 c and d, respectively). In the case of a soluble complex only one phase where the complexes are uniformly distributed is observed. Insoluble complex formation leads to the formation of two distinct phases, where one phase is rich in both protein and polysaccharide and the other phase is depleted in both [123]. Insoluble complex formation also known as associative phase separation [125] is attributed to charge neutralization [35]. Even though the pH and ionic strength of the solution are important parameters to control the type of interactions formed between protein and polysaccharide, other characteristic such as the type of protein and polysaccharide, molecular weight, charge density and hydrophobicity are also relevant to the extent of complexation [126]. While

electrostatic attraction is the main driving force in the complexation process, other interactions such as hydrogen bonds, hydrophobic interaction may provide a minor contribution to the stability of the protein-polysaccharide system [38]. Complex formation can also take place between similarly charged protein and polysaccharide, since the protein might have patches on the protein surface that carry the opposite charge to that of the polysaccharide and associative interactions are developed [127].

The mixing process is a spontaneous process when change in the Gibbs free energy ($\Delta G = \Delta H - T \times \Delta S$) is negative. In contrast thermodynamic incompatibility of polymers takes place when the Gibbs free energy of mixing is positive. Since the mixing entropy is a function of the number of individual particles being mixed, the value of the entropy of mixing (ΔS) of polymers is much smaller than that corresponding to monomers. A homogenous mixture of unlike polymers could be prepared if the enthalpy of mixing (ΔH) is negative. This means that the attractive forces between unlike macromolecules are equal to or larger than those between like macromolecules. Indicating that polymers are compatible and able to form soluble inter-polymer complexes [120]. The phenomenon of incompatibility relates to the occupation of the solution volume by the macromolecules and the repulsion by unlike molecules. The differences in excluded volume effects between incompatible polymers and their competition for space determines the critical conditions of a system phase separation [120]. Segregation or phase separation leads to a reduction of the polymer concentration near the other particle. This reduction of the polymer concentration is due to loss of conformational entropy of polymers near an interphase. A reduction of polymer concentration near the interphase is called depletion [122], and is observed when the solvent-

polymer interactions are favored to the detriment of protein-polysaccharide and solvent-solvent interactions [125].

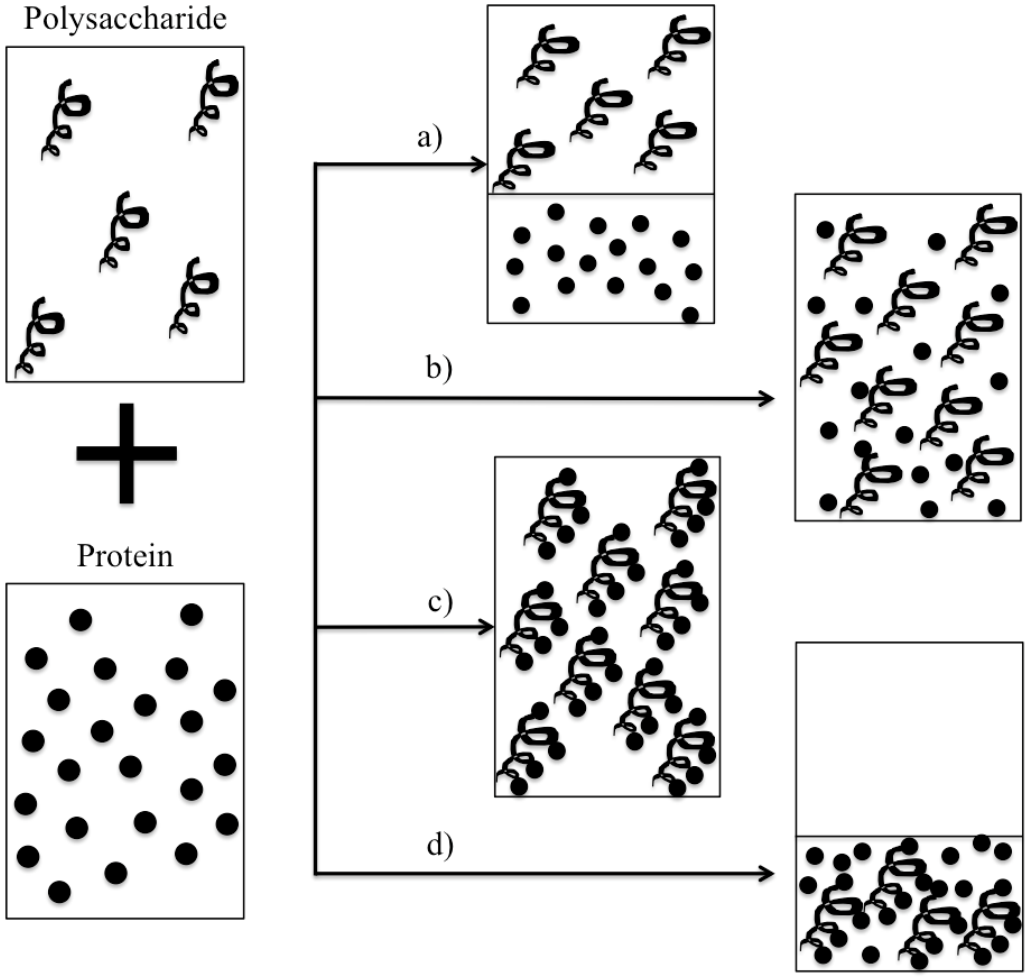

Figure 1 Behavior of protein-polysaccharide mixtures due to associative or segregative interactions. a) Incompatible and two phase system, b) Compatible and one phase system, c) Soluble complexes and one phase system and d) Insoluble complexes and two phase system.

Phase separation is very common and it occurs naturally in any mixture of polymers in which inter-polymer interactions are not favorable, thus, polymer molecules tend to be surrounded by others of the same type, which is known as thermodynamic incompatibility [119]. Thermodynamic incompatibility is encouraged by high molecular weights, by low tendency for the protein and polysaccharide to interact attractively and by the size of the difference between their individual interactions with the solvent [128].

Knowing the possible ways in which protein and polysaccharides are expected to interact in solution, during gelation the associative or segregative interactions established between protein and polysaccharide will take place at the same time as the gel network is formed. Thus protein-polysaccharide gels can be classified into three types; interpenetrating, coupled and phase separated [129]. A similar classification but with less common nomenclature for this types of gels was proposed by Zasypkin et al. (1997) and this classification includes mixed, complex and filled gels [130]. Each type of gel structure has very distinct microstructure and therefore very distinct gelling properties that may be applied for diverse applications.

1.8.1.1. Interpenetrating network

Interpenetrating networks or mixed gels are formed when two components gel separately and form independent networks. Both networks are continuous throughout the sample, and interactions between them are minimal or "topological" [30,129]. In this type of gel the two networks are seen passing through one another at the individual strand, or pore level and the structure is essentially uniform [128].

1.8.1.2. Coupled network

Couple or complex networks are formed in the presence of favorable or associative interactions between proteins and polysaccharides. Thus, intermolecular binding between the

two polymers contributes to the formation of this type of network [30]. The association of polysaccharides and proteins is mostly due to electrostatic attraction between a negatively charged polysaccharide and a protein below its isoelectric point for example, and the strength of the attraction depends on the type and density of the charges present on the molecules [119,131]. Thus, attractive electrostatic interactions are the main interaction responsible for the stability of the gel network [132]; nonetheless other weak energy interactions, especially hydrogen bonding but also hydrophobic interactions can have a significant contribution [127]. In this type of gel both protein and polysaccharide are expected to be closely bonded since they are linking at a molecular level to form the gel network [128].

1.8.1.3. Phase separated network

Phase separated gels are formed due to segregative interactions. In this case is expected that protein and polysaccharide repel each other and/or that both proteins and polysaccharide show a different affinity for the solvent [121]. Phase separated network gels or filled gels, are the most common type of multicomponent gels. The microstructure formed in these gels is the result of the competition between the phase separation process and the gel formation process. Gel formation arrests the phase separation process before it reaches equilibrium by forming a two-layer system [118]. In the case of partial phase separation the gel will appear with inclusions or particles of one phase filling the matrix of the second phase [30]. In a phase-separated gel, usually the faster gelling constituent forms the continuous phase that supports the discontinuous inclusions of the second and slower gelling component [133]. When a protein-polysaccharide mixture is heated, both processes (aggregation and phase separation) occur simultaneously, with very different kinetics [134]. Phase separation in solution can be detected by the formation of 'water in water emulsion' in which one phase exists as a

continuous matrix with the other dispersed through it as small liquid droplets, this emulsion structure can be trapped by network formation, giving a biphasic co-gel with one phase continuous and the other dispersed [119]. The minimum concentration for gelation usually decreases when another incompatible biopolymer is added. This is due to an excluded volume effect. In a phase-separated system, the same effect may be due to water redistribution between phases during gelation [130]. Often phase-separated gels seem to be homogenous at the macroscopic level, nonetheless at the microscopic level these gels show heterogeneity [125]. Phase separation is expected to begin either by spinodal composition or by nucleation and growth and either to progress to two phases in equilibrium or being trapped at some stage by gelling [128].

The utilization of protein and polysaccharide mixtures allows modification of the functional properties of proteins. Similarly enzymatic hydrolysis permits protein modification. Both of these techniques may be advantageous as these may show higher consumer acceptability compared to chemical modification techniques.

1.9. Research hypothesis and objectives

The global protein market is growing and is projected to reach $24.5 billion by 2015 with a focus on plant-based proteins. Rising cost, sustainability concerns and the growing consumer desire to eat clean as well as the beneficial health effects are the reasons behind the market requirements of plant proteins [135]. Soy protein is the most widely consumed plant protein and it leads the non-animal protein market [136]. Soy protein has proven to be a good source of protein and has been widely utilized in the food industry due to its many good functional properties. Of particular interest is the use of soy in tofu and as a gelling agent in the food

industry [137]. Soy protein is currently incorporated in comminuted meats and dairy foods where thickening and gelling properties are of major significance [138]. Despite the fact that soy protein is so widely demanded, there are hurdles for its commercialization mainly in the European Union market but also in the rest of the world due to its genetically modified character and the pending regulatory approval. Gluten is another plant protein with increased use as a food ingredient to provide a range of functional properties at a more modest price than competitors such as milk and soy proteins [139]. Nonetheless, other sources of plant protein need to be considered as the plant protein sector is thriving and growing.

Oat is a good source of plant protein and recently the human food market for oat has grown mainly due to the growing public awareness of the health benefits of β-glucan. This soluble dietary fiber component of oat is known to reduce blood cholesterol [140], and regulate blood glucose levels [141]. Several techniques have been developed to isolate β-glucan from oat grain as a healthy ingredient in food products. The remaining components such as protein and starch are awaiting research to develop their full value [142]. Hence, the utilization of oat protein as a natural by-product of the β-glucan extraction process increases the economic benefit of its utilization.

Oat has the highest protein level (12–20%) [143] among cereals with a superior amino acid profile due to higher amounts of limiting amino acids lysine and threonine [16]. Globular proteins in oat closely resemble proteins in soy. There are strong similarities between the 12S fraction of oat and the 11S fraction of soy (glycinin), which has demonstrated good gelling properties; consequently oat protein has potential to act as a gelling agent. The recognition and development of such significant functionality from oat protein can considerably stimulate its utilization in the food market. Ma et al. have notably contributed to this area of

research [144,145]. However, such efforts are still limited compared to those on dairy and soy proteins. Two previous publications demonstrated that oat protein could form strong gels under alkaline conditions (pH 9-10), but at acidic and neutral pH, very weak gels with poor water holding capacity were obtained [144,145].

Enzymatic hydrolysis is a preferable tool to alter functional properties of proteins because of milder processing conditions required, easier control of reaction and minimal formation of by-products [146]. The final composition and thus the use of the hydrolysates will depend on the protein source, the type of enzyme used and the hydrolysis conditions [96]. Recent research has reported the effect of enzymatic hydrolysis on the gelling properties of proteins including soy protein [98], rice bran protein [99], sunflower protein [100], and canola protein [97]. Results from these studies indicate that improvement of the gelling capacity is highly enzyme specific. To date, knowledge of the effects of enzymatic hydrolysis on the gelling capacity of oat protein is very limited. The application of EMR for production of novel food ingredients such as protein derived gelling agents should be consider as viable alternative as the molecular weight of the hydrolysates can be carefully controlled through this technology. Also the molecular weight has a considerable effect on the gelling properties of the hydrolysates.

On the other hand, the gelling properties can be affected by interaction with polysaccharides. Protein and polysaccharide are often mixed to develop food products with novel textural properties. The interactions develop among protein and polysaccharide will define the microstructure of food products and thus the resulting texture or mechanical properties. In spite of good potential, research effort to develop oat protein-polysaccharide gel is lacking.

In the light of this, this research tested the following hypotheses:

I. Gelling properties of oat protein can be improved by partial hydrolysis.
II. Gelling properties of oat protein can be improved by regulating protein-polysaccharide interactions to achieve a synergistic effect.

The overall objective of this research was to improve the gelling properties of oat protein by enzymatic modification and by formation of protein-polysaccharide blends with the following specific objectives:

I. To review literature reports on the application of enzymatic membrane reactors to produce added-value ingredients from food components.
II. To study the impact of partial hydrolysis on oat protein thermal gelling properties under different processing conditions.
III. To investigate the effect of oat protein and polysaccharide interactions on oat protein thermal gelling properties under different environmental conditions.
IV. To understand the oat gel formation mechanism.

The properties of oat protein gels under different environmental conditions with emphasis on the gel mechanical strengths and water-holding capacity will be investigated, as these properties are the most important gel characteristics for food applications. If the defined gel physical properties would be in the range of similar properties of animal protein derived gels,

value-added opportunities would exist for oat protein to be used as a new gelling ingredient in food formulations such as meat binder and fat replacer to create food with improved quality and nutritive value, or used in meat analogues for vegetarian foods. In this way additional revenue return could be generated to oat producers and processors to enhance their sustainability.

Chapter 2

2. Continuous Membrane Bioreactor (CMBR) to Produce Nanoparticles from Milk Components

2.1. Introduction

Membrane technology has been used in the dairy industry as alternative to some unit operations since the early 1970s. The new applications of membranes were often due to the development of membrane science. The commercial availability of Nanofiltration membranes allowed the demonstration of some biological activities in milk and whey peptide sequences [147]. Nanotechnology is used in different stages of the membrane development and has gain great interest among the agro-food sector and it involves the manufacture, processing and applications of structure devices and systems controlling the shape and size of particles at the nanometer scale [148]. The size range that holds so much interest is typically from 100 nm down to the atomic level; approximately 0.2 nm, because in this range material can have different and enhanced properties compared with the same material at a large size. As the particle size decreases, a greater proportion of atoms are found at the surface compared to inside. For example, a particle size of 30 nm has a 5% of its atoms on the surface, at 10 nm 20%, and at 3 nm 50% of the atoms are in surface. Thus a nanoparticle (NP) has a much greater surface area per unit mass compared with larger particles, leading to grater reactivity. In addition to the surface area effect, quantum effect can also govern the properties of matter as size is reduced to the nanoscale, affecting the optical, electrical and magnetic behavior of materials [149].

The application of nanotechnology in the food industry covers many aspects, such as food safety, packaging material, disease treatment, delivery systems, bioavailability, and new tools for molecular and cellular biology and new material for pathogen detection. However the four major areas in food industry to benefit from nanotechnology are the development of new functional materials, micro and nanoscale processing, new product development and the design of nanotracers and nanosensors for food safety and biosecurity [150].

It is the interest of this chapter to discuss the production of bioactive NPs from food commodities and to explain how nanotechnology in a continuous membrane bioreactor (CMBR) and in particular nanofiltration processes can be employed to alter food products. For this purpose, the term "bioactive food component" refers to nonessential biomolecules that are present in foods and exhibit the capacity to modulate one or more metabolic processes, which results in the promotion of better health [151].

A major strategy for the delivery of these components into food is through encapsulation, which consists in coating one or several components (core) within a secondary material (encapsulant). This is used to mask the color and taste of nutrients, and to protect sensitive nutrients during processing, storage, and transportation [152]. Bioactive compounds are added into food, looking to provide health benefits and protection as antioxidants and anti-aging agents, reducing the risk of cardiovascular diseases or with the intention to boost the nutritional content of the product. The application of nanotechnology in the food industry is still in a developing stage, however in recent years; research has been carried out in order to formulate food-grade encapsulants to enable the delivery of desirable bioactive compounds through the food supply. Due to their sub-cellular size, NPs offer promising means of improving the bioavailability of bioactive compounds, especially poorly soluble substances

such as functional lipids (e.g. carotenoids, phytosterols, ω-3 fatty acids), natural antioxidants, and other compounds that are widely used as active ingredients in various food products. NPs can dramatically prolong compound residence time in the gastrointestinal tract by decreasing the influence of intestinal clearance mechanisms and increasing the surface available to interact with the biological support. They can also penetrate deeply into tissues through fine capillaries, cross the epithelial lining fenestration and are generally taken up efficiently by cells, thus allowing efficient delivery of active compounds to target sites in the body [153].

2.2. Nanomaterials

NP can be produced from a variety of different materials, from metals like gold and silver, organic solvents or some food derived ingredients. The use of food-grade ingredients is generally accepted by the regulatory agencies and has shown to exert good results as nanomaterials and would contribute to dissipate the fear among consumers given the fact that these polymers are part of the human diet. Food-grade proteins and polysaccharides, such as whey protein, casein, gelatin, soy protein, zein, starch, cellulose, and various other hydrocolloids are used. In addition, they may contain other components, such as water, lipids, minerals and sugars [154].

2.2.1. Lipid-based nanoparticles

Lipid-based nanoparticles can encapsulate compounds with different solubilities, but in particular these particles are useful carriers of poorly water-soluble compounds. The term "lipid" is used in a broad sense to include phospholipids, triacylglycerides, fatty acids, steroids and waxes [155].

Different structures have been proposed using lipids as the main encapsulant constituent, some of the most commonly discussed structures are: liposomes and solid lipid nanoparticles (SLN).

Liposomes are spherical, self-closed structures formed by one or more concentric lipid bilayers with an encapsulated aqueous phase in the center and between the bi-layers [156]. They can accommodate hydrophilic, lipophilic and amphiphilic compounds in their aqueous and or lipid compartment. These structures are used as carrier systems for the protection of bioactive compounds by improving their solubility and bioavailability and preventing their unwanted interaction with other molecules [157]. The preparation of SLN is carried out at high temperatures, generally above the melting point of the lipid component used and further a cooling stage is required to solidify the lipid [158]. Some disadvantages of SLN are the limited loading capacity of the bioactive compound and expulsion of the bioactive compound during storage. In order to overcome these drawbacks Nanostructured Lipid Carriers (NLC) were developed [159]. NLC are composed of oily droplets embedded in a solid lipid matrix, this provides more stability with a controlled nanostructure that improves the bioactive compound loading and firmly incorporates the bioactive compound during storage [160]. Lipid-based nanoparticles, have been used to contain different bioactive compounds such as vitamins (α-tocopherol) [161], β-carotene [162], and □-3-fatty acids [163].

2.2.2. Protein-based nanoparticles

Proteins have very good gelling and emulsifying properties and for this reason they are widely used as encapsulating material. Thanks to the consistent primary structure of proteins a wide variety of nutrients can be incorporated allowing them to form complexes with polysaccharides, lipids or other biopolymers. In order to improve bioavailability, the food

industry is currently attempting to increase the circulation time of conventional nanocarriers in the gastrointestinal tract, by surface coating with protein [153]. Food proteins can undergo denaturation due to exposure to high temperatures or pressure. The resulting denaturated product can then re-assemble, building a new structure [164]. Or well if proteins are combined with polysaccharides, these materials can form biopolymeric nanostructured particles by complexation [165]. When a globular protein becomes denaturated, its physical and chemical interaction change appreciably through exposure of nonpolar and sulphur-containing groups that were originally present within the compact interior of the globular protein. Consequently, denaturated proteins have a greater tendency to aggregate irreversibly, with each other through hydrophobic bonding and disulfide bond formation [154]. Nanoemulsions are dispersions of nanoscale droplets produced after mixing two immiscible phases, made by the application of high shear. The rupture of droplets may be achieved by ultra-sonication or microfluidisation. The amount of surfactant required to stabilize nanoemulsion is greater [166]. To prevent the droplet from recombining into larger droplets a thin encapsulating layer is introduced to help stabilize the system; this layer is traditionally made up of proteins or phospholipids which act as surfactants [167].

A particular case of protein based NP are nanotubes, derived from partially hydrolyzed α-lactoalbumin. This nanostructure is very stable and strong and has a 8.7 nm cavity that can be used to contain bioactive compounds [168]. Milk proteins such as casein, α-lactoalbumin and β-lactoglobulin, lactoferrin, bovine serum albumin, have been widely used in the development of nanostructures with very different applications. However their physicochemical properties facilitate their functionality as vehicle of bioactive compounds [169]. Nevertheless, the use of

protein in the building of nanostructures is not limited to milk proteins, vegetable proteins have been used as it is the case of pea protein [170] and zein [171].

2.2.3. Polysaccharide-based nanoparticles

Polysaccharides are used to improve texture in foods or as stabilizer agents in emulsions, and some of the most commonly used are: starch, xanthan gum, pectin, alginate and chitosan. In combination with proteins and lipids, nanostructured particles can be formed. According to their structural characteristics, these polysaccharide-based nanoparticles are prepared mainly by four mechanisms: covalent crosslinking, ionic crosslinking, polyelectrolyte complexation, and self-assembly of hydrophobically modified polysaccharides, Liu et al. (2008) [172] reviewed these four mechanisms in detail. Of these methods polyelectrolyte complexation is commonly used in the preparation of nanoparticles containing food derived bioactive compounds. When proteins and polysaccharides carry an opposite charge, complex formation is driven by attractive electrostatic interactions between the two biopolymers [173]. Attractive interactions and complexation begin at values slightly above the protein's isoelectric point; further pH reduction induces greater complexation [174]. It is important to establish the electrical characteristics of the polysaccharide molecules used, since electrostatic interactions may be used to assemble specific biopolymer structures. The electrical charge on polysaccharide depends on the nature of the ionic groups along the backbone chain, as well as solution conditions. Some polysaccharides are neutral (starch, cellulose) some anionic (alginate, carrageenan, xanthan, gum Arabic) and some cationic (chitosan) [175]. Sugar beet pectin was used to produce a nanostructured system for stabilization, protection and delivery of hydrophobic bioactive compounds. For this, vitamin D was bounded to β-lactoglobulin,

later a complex between the carrier and the polysaccharide of the opposite charge was formed. This system has been proposed for enrichment of clear and non-fat beverage [165].

2.3. Nanotechnology and nanofiltration

The application of membrane technology in the nanoscale range has increased as a result of the continuous application of nanotechnology in the food sector. In the same way that nanoparticles are produced as an encapsulated system based on food ingredients and designed to have a broad range of applications, nanofiltration can be utilized as a nanoscale process to produce food ingredients with added value. In the membrane based separation process, the driving force for material transport through selective membranes is a pressure difference, thus these processes are called pressure-driven membrane process, and such is the case of microfiltration (MF) and ultrafiltration (UF). In the case of nanofiltration (NF) a pressure difference is not the only driving force; selective removal of ions based on charge is another feature of this technology [176]. Thus the selective separation process results in nanometer scale products. Nanofiltration membranes have apparent pore diameter between 0.5 and 5 nm and are capable of removing from water, both organic matter and ions of mineral salts [177]. In such way nanofiltration can separate components according to their molecular size without a change phase of the solvent; this avoids thermal damage and requires a lower consumption of energy [178]. Nanofiltration is currently used to supply high quality drinking water [177], desalting milk, whey and other dairy fluids [147], additionally a wide range of value added components can be obtained after a nanofiltration treatment.

2.4. Membrane bioreactors in the nanoscale process

Biocatalytic membrane reactors combine selective mass transport with chemical reactions, and the selective removal of products from the reaction site increases the conversion of product-inhibited or thermodynamically unfavorable reactions. Membrane reactors using biological catalysts can be used in production, processing and treatment operations [179].

In a bioreactor, the conversion of raw materials into value added products is carried out by enzymatic hydrolysis; further, in combination with a NF membrane the separation of substrate and product will occur; as the membrane will only allow the passage of certain components known as "permeate" (the product) and retains/rejects other components known as "retentate" [176].

The use of biocatalysts for large-scale production is an important application because it enables biotransformations to be integrated into productive reaction cycles. Biocatalysts (e.g. enzymes, microorganisms and antibodies) can be used: (1) suspended in solution and compartmentalized by a membrane in a reaction vessel or (2) immobilized within the membrane matrix itself. In the first method, the system might consist of a traditional stirred tank reactor combined with a NF membrane-separation unit. In the second method, the NF membrane acts as a support for the catalyst and as a separation unit [179].

Some of the advantages of the use of membrane bioreactor as a nanoscale process are that the catalytic/separation process [103,179]:

- Does not require additives
- Is able to function at moderate temperature and pressure
- Reduces the formation of by-products

- Catalytic enzymes are extremely efficient and selective compared with chemical catalysts.
- The enzyme is retained and reused
- Substrate/product inhibition is reduced
- The end product is free of enzyme
- One single step operation (reaction/separation).

However, the use of NF membranes is not limited to the enzymatic membrane reactors. NF has been commonly used as a pre-concentration step in the milk processing operations in order to reduce the energy consumption in heat based treatments. The whey that is obtained during cheese making with a very high mineral content, could be used for the manufacture of other valuable food ingredients such as whey protein concentrate and whey protein isolate, however the mineral content should be reduce to provide a higher quality. With NF the demineralization of whey can be achieve, since NF membranes are often used for separation of charged solutes [180].

Thus, membrane technology at this scale can be used for [181]:

- Separation of high molecular weight component from low molecular weight components.
- Concentration of mixture or selected fractions
- Purification

Table 1 presents an overview of the NF processes that have been proposed in the food sector. NF has been used for concentration or purification of compounds with biological activity such as antioxidants, prebiotics and bioactive peptides. Alternatively NF has also been proposed for separation of valuable components such as lactose or undesirable components such as salts, biogenic amines, toxins or heavy metals.

2.4.1. Recovery of compounds with antioxidant activity

Concentration of bioactive compounds can be achieved by NF treatment, because this process does not require high temperatures in comparison to other concentration treatments such as evaporation. Thus, by using mild temperatures the functional properties of these temperature sensitive compounds are preserved [182].

Due to their beneficial effects on human health, isoflavones were concentrated from soybean using a NF membrane. However, in order to ensure a good yield and to avoid the fouling of the membrane due to the accumulation of fat globules in the membrane, defatted soy flour was used as raw material and the final results of this study showed that the nanofiltration process for the concentration of isoflavones is viable [182].

In order to separate low molecular weight and/or hydrophobic components, the nanofiltration membrane needs to meet certain characteristics that will be defined by the compound that is being recovered. Darnoko and Cheryan (2006) [178] selected a hydrophobic membrane to separate β-carotene from the palm methyl esters, increasing the concentration in from 0.45 g/L to 1.88 g/L (34).

Table 1 Nanofiltration processes proposed for concentration, purification or separation of relevant food ingredients.

Separated component	Component separated from	Membrane material	NF module	MWCO (Da)	T (˚C)	Pressure (kPa)	Reference
Phenolic compounds	Aqueos mate *(Ilex paraguariensis)* extract	--------	Spiral wound	150-300	25	690	[183]
Aroma and protein	Tuna cooking juice	Polyamide coated with polyesthersulfone	Tubular	400	40	3500	[184]
Anthocyanins	Açai juice	Semi-aromatic polyamide layer on top of a polysulphone microporous support	Plate and frame	--------	35	1000 1500 2000 3000	[185]
		Aromatic polyamide		~1000			
		--------		--------			
		Thin film		~1000			
		Polyesthersulfone					
Fructooligosaccharides	Sucrose from sugar cane	--------	Spiral wound	400	95	4000	[186]
Alcohol	Wine	Polyamide	--------	300	45	500 1500	[187]
				400		1000 2000	
Biogenic amines	Model solution	--------	Flat	1000	70	3500	[188]
Mineral salts	Whey	Aromatic polyamide	Spiral wound	300	--------	500-2400	[189]
Cyanobacterial toxins	Drinking water	Polyamide composite with a microporous supporting layer	Flat sheet	200	--------	8000	[190]

Flavonoids and polyphenolic compounds from *Sideritis scardica* were concentrated up to 3-4 times. The concentrated extracts preserve their high antioxidant activity and could be used as a source of concentrated biologically active material. Also the separation of flavonoids form low molecular polyphenols was possible in base of their molecular weight difference [191]. Conidi, et al. (2011) [114] proposed an integrated membrane process based on the preliminary UF of enzymatically depectinised juice with a 100 kDa membrane to remove suspended solids followed by a NF step with a 450 Da membrane. The separation and concentration of polyphenols of the bergamot juice was possible as the retentate contained the phenolic compounds, which showed a high total antioxidant activity.

Crude rice bran is a by-product of rice milling, rich in phytochemicals with high nutritional value such as γ-oryzanol. A two-step nanofiltration system was set up for enrichment of this phytochemical in rice oil. The first membrane stage, produced the separation of glycerides and γ-oryzanol, promoting the oil enrichment in this phytochemical. In the second membrane stage the oil was refined to acceptable consumption levels of free fatty acids and its γ-oryzanol content was further enhanced. The antioxidant activity of the resulting product oil possessed a significantly higher antioxidant capacity than the feed oil (45.9, 19.3 μmol Trolox/g respectively). This results show that the product generated through NF membrane process has a superior nutritional value [192].

2.4.2. Oligosaccharides

Non-digestible oligosaccharides (OS) are able to stimulate the growth of bacterial flora, hence they are used as prebiotics [193]. The resulting commercial OS products contain plenty of side resultants of low molecular weight sugars such as glucose, fructose, sucrose, galactose

and lactose, lowering the performance of the end product. Therefore, high purity of OS products is required [194]. Thus NF seems to be a viable alternative for industrial scale purification of OS mixtures. Many recent reports of the application of membrane technology to produce and concentrate OS have been published using different sources such as whey protein concentrate [195], caprine milk [196], rice husk [197] and soybean waste water [198].

In a recent study, apple pomace samples were subjected to simultaneous saccharification and fermentation resulting in a mixture of lactic acid and OS. Lactic acid was further removed by ion exchange and the mixture was processed with two sequential steps using NF membranes in order to refine and concentrate the following OS: glucooligosaccharides, galactooligosaccharides, xylooligosaccharides and arabinooligosaccharides. More than 90% of low molecular weight compounds (residual lactic acid, arabinose and NaCl) were removed from the solution. On the other hand, just a limited part of the OS was lost in the permeate. As a result of the treatments, the mass fraction of the OS in the final product increased from 0.360 up to 0.677 kg/kg of non-volatile compounds. These results confirm that coupling two NF stages (discontinuous diafiltration followed by concentration) is a suitable alternative for obtaining OS concentrates. The refined product obtained in this work showed degrees of purity in the range reported for commercial OS and was assayed for its prebiotic potential and the results showed the promotion of beneficial bacterial growth [199].

Eucalyptus wood-derived xylooligosaccharides were obtained using UF and NF membranes (1-50 kDa) for separation and concentration. The UF unit was used to separate OS from higher molecular weight products or to fractionate OS of different degree of polymerization. In addition a NF unit was used for concentrating liquors and/or for removing undesired low

molecular weight compounds, such as monomeric sugars or phenolic compounds, increasing the xylooligosaccharide concentration 3.8 times [200].

In other cases the separation process can be primarily intended to recover the low molecular weight sugars, such is the case of lactulose, which is very useful in the treatment of chronic constipation and is normally produced by isomerization of lactose using hydroxide/boric acid forming a complex. This complex can be split by changing conditions from basic to acidic and the borate is converted into NaCl and H_3BO_3. NF was used as an effective method for the desalination of lactulose syrup, thus the disaccharides (lactulose and lactose) were retained by the membrane in the presence of high concentrations of NaCl and H_3BO_3 in the syrup. In this way NF provides a commercial alternative to chromatography for concentration and purification [201]. NF has been used in the winemaking for sugar control; by reducing the sugar concentration in must, before fermentation with the purpose to reduce the alcohol content in wine. For this García-Martín et al. (2010) [202] proposed a two successive NF steps for sugar removal. The musts obtained were mixed with untreated must or with the retentate of the first NF stage, in order to reduce the alcoholic content by 2°. With this treatment other compound such as polyphenols, anthocyanins, catechins and tannins were partially removed and slight changes in color and aroma were observed.

A goats´ milk product enriched in oligosaccharides (>80%), lactose and free of salts was obtained following the two-stage tangential filtration process proposed by Martinez-Ferez et al. (2006) [196]. Tubular ceramic membranes with molecular mass cut-offs of 50 (ultrafiltration) and 1 kDa (nanofiltration), respectively, were employed and 15 new oligosaccharide structures (4 neutral and 11 acidic), were identified. Goat´s milk is a suitable source of oligosaccharides for applications in human nutrition due to their prebiotic and anti-

infective properties. Nanofiltration membranes can be used for purifying galactooligosaccharides (GOS) products from the monosaccharide hydrolysis products such as glucose and galactose. According to Gosling et al., (2010), [203] up to 88% of the di- and oligosaccharides were recovered from a commercial GOS mixture using a nanofiltration membrane with only 19% of the monosaccharides remaining in the retentate stream [204] Catarino et al., (2008) [194,205] investigated the fractionation of saccharide mixtures with calcium using ultrafiltration and nanofiltration processes and reported that saccharide fractionation was enhanced in the presence of calcium. Feng et al., (2009) [194] reported the separation of sugar solutions in total recycle mode operations using nanofiltration membranes and the process resulted in oligosaccharides yield of 70% and purity of 54%. These results show nanofiltration as an alternative process to industrial chromatography.

2.4.3. Bioactive peptides

Controlled enzymatic hydrolysis of proteins, produces smaller peptide fractions, which can exert a positive effect upon health. These fractions have been defined as bioactive peptides [206] and NF processes are particularly useful for separation of peptides due to the suitable molecular weight cut-off and because of the electrochemical effects, which play an important role in the case of charged molecules [207]. Fish protein hydrolysates were produced and then submitted to a two-stage treatment (UF/NF) to obtain fractions with increased activity. In this study 4 major fractions were obtained. Size chromatography was used to characterize the peptide composition in each fraction. Within some fractions it was possible to find a wide size distribution and not only the expected size [208]. Other similar reports using fish protein as a substrate in a UF/NF fractionation system have been reported [209,210]. Milk proteins have been widely studied as a source of bioactive peptides with a broad range of activities, for this

reason Ting et al. (2007) [211] proposed a NF treatment for the fractionation of peptides derived from whey protein. In this work, peptides were selectively separated, based on their charge and size. NF separation of peptides and amino acids is very dependent on physicochemical parameters such as pH and ionic strength. At pH 9, acidic peptides carry a net negative charge. Since both the peptides and the membrane are negatively charged at this pH, it is likely that electrostatic repulsion between the membrane and the acidic peptides prevented the peptides from permeating the membrane, meanwhile the basic peptides which were positively charged at this pH were present in the permeate. Fractionation of a β-lactoglobulin (β-LG) peptide mixture by nanofiltration (NF) membranes was investigated by Lapointe et al., (2003) [212]. Peptide mixture was prepared by tryptic hydrolysis of commercial β-LG followed by ultrafiltration (UF) for enzyme removal. In this paper, important change in NF selectivity as affected by hydrodynamic conditions and recirculation time via possible peptide–peptide interactions occurring in the so-called weakly attached layer were reported. Roufik et al., (2007) [213] showed that the hypotensive peptide β-lactoglobulin (β-Lg) f142-148, known as lactokinin, bind to bovine β-lactoglobulin variant A (β-Lg A) and this complex could delay the hydrolysis of this peptide by digestive enzymes.

The separation of peptides contained in rapeseed protein hydrolysate using UF/NF membranes was studied by Tessier et al. (2006) [115]. This process was also based on selective separation depending on the peptide and membrane charge. As in the previous example peptides with the same charge as the membrane (co-ions) were concentrated in the retentate whereas peptides with the opposite charge (counter-ions) were able to get through the membrane in the permeate. A pre-treatment step by acid precipitation to remove high molar mass substances was followed by the UF step (3kDa), in which the concentration of small

peptides was allowed. Later the mixture of small peptides was desalted using a NF unit to assess the influence of ionic strength on the fractionation selectivity. The results showed that changes in pH and/or ionic strength modified the nature and the intensity of electrostatic interactions between co-ions, counter-ions and membrane. Thus the control of pH and ionic strength should be key parameter in the selective separation process using NF membranes.

Butylina et al., (2006) [214] described the fractionation and further isolation and characterization of peptides and proteins present in sweet whey by means of ultrafiltration using a regenerated cellulose membrane with a nominal molar mass cut-off value of 10 kg/mol and nanofiltration through sulphonated polyether sulphone membrane with a cut-off of 1kg/mol. The concentration of whey proteins was done below the critical flux. The sieving coefficients for the whey components (proteins, lactose and salts) were estimated. Whey proteins were completely rejected by the ultrafiltration membrane. Nanofiltration of whey permeates obtained after ultrafiltration was conducted at two pH values (9.5 and 3.0) that corresponded to the different charged states of the membrane and of the peptides. The transmission of peptides, amino acids and lactose was found to be mainly affected by the permeability of the fouling layer. The selectivity of the nanofiltration membranes toward peptides compared to lactose was calculated as 0.82 and 6.81 at pH 9.5 and 3.0, respectively.

2.4.4. Other applications

2.4.4.1. Fusel alcohols

Although fusel alcohols provide flavor in rice spirits, a high concentration of these compounds, can lead to off-flavors, cloudy appearance and cause headaches and dizziness. Based on this, NF was proposed to remove fusel alcohols from rice spirits. The best removal results were observed with a 150-300 Da cut-off and an operating pressure of 488.95 kPa.

For these conditions the remaining fusel alcohols content decreased to 2.54 g/L. The alcohol content, soluble solids content and pH (organic acids) varied slightly in the rice spirits processed with NF, however the sensory evaluation results showed that the NF treatment, improved the taste and clarity of rice spirits. Thus NF treatment effectively improved the wine quality [215].

2.4.4.2. Alternative sweeteners

A new alternative non-caloric sweetener can be produce from *Stevia rebaudiana*. The production of this sweetener could involve the use of organic solvents and methanol and ethanol. For this reason a membrane based process has been proposed to manufacture this sweetener. It is possible to use a NF membrane process as a post-treatment or polishing stage after applying a previous concentration treatment. This is the case of a recent study, in which an integrated process with a microfiltration, ultrafiltration and nanofiltration membrane stage was used for the purification of this sweetener [216].

2.4.4.3. Soy sauce

Soy sauce with a reduced salt content was obtained after a NF treatment (150 Da) for removal of NaCl and recovery of nutritional components such as amino acid and fragrance from raw soy sauce. The process consisted in a dilution step followed by concentration and then diafiltration. This mode of operation showed the least processing time and least water consumption, along with a high salt removal and high nutrient retention. The permeate produced in soy sauce desalination could be either reused as processing water or as feed to produce light color soy sauce, all the materials including NaCl, amino acids and water could be fully utilized. Consequently, this process was proposed as an alternative to produce soy sauce with low sodium content [217].

2.5. Conclusion

In consideration of the growing interest in promoting health through food, there is a continuous development of food-grade delivery systems to encapsulate, protect and deliver bioactive compounds (i.e. antioxidants, vitamins, bioactive peptides, antimicrobials). Thus, there is now the possibility to produce food for a healthier population and improve its health and wellness through nanoscale technology; because of this, the "nano-food" market is expected to grow very positively within the next few years. Materials such as proteins, polysaccharides and lipids have shown to exert good results as nanomaterials and would contribute to create less fear among consumers given the fact that these polymers are part of the human diet.

As part of the nanotechnology involved in the food processing, operations such as nanofiltration represent a useful alternative for the separation, concentration, fractionation and purification of food components with added value since many of them can exert a beneficial effect upon health. Nanofiltration in combination with controlled hydrolysis also provides an interesting alternative for production of bioactive compounds derived from food ingredients with some advantages such as the use of mild temperatures, the reuse of the enzymes, the final product free of enzyme or other compounds, making this operation economically attractive and highly likely to be used at an industrial scale.

Chapter 3

3. Effect of partial hydrolysis on structure and gelling properties of oat globular proteins

3.1. Introduction

Gelation is one of the most important functional properties of proteins as it provides texture and support in foods. Generally, thermal gelation of globular proteins involves unfolding of the protein molecules by heating, which leads to exposure of reactive amino acid residues. Later, unfolded molecules re-arrange and aggregate irreversibly via disulfide bridges, hydrogen bonds, hydrophobic and/or van der Waals interactions. Finally, aggregation carries on with association of protein particles and if the protein concentration is sufficiently high, a three-dimensional network is created [27]. This process only takes place in the presence of adequate environmental conditions, such as pH, temperature and ionic strength [25].

Plant proteins are normally considered inferior to animal proteins in terms of gelling properties. Gelatin, egg white and whey proteins are widely used as gelling agents in the food industry, particularly in meat and dairy based systems. In recent years, proteins derived from plant sources are becoming one of the food industry's fastest-growing and most-innovative ingredient segments owing to health (no Bovine Spongiforme Encephalopathy concern), religious and cost reasons. For a long time, soy protein has been the major plant protein gelling ingredient in the market. Yet there is an opportunity for other novel gelling ingredients of plant origin to meet the increasing market requirement for different functionalities and sensory attributes. Canada is the third largest producer of oat in the world,

with an annual production of approximately 2.7 million tons [218]. Canadian oat is commonly used as an animal feed and only a small percentage of the grain is currently used for human consumption. Recently the human food market for oat has been gaining momentum mainly due to the growing public awareness of the health benefits of β-glucan. This soluble dietary fiber component of oat is known to reduce blood cholesterol [140], and regulate blood glucose levels [141]. Several techniques have been developed to isolate β-glucan from oat grain as a health ingredient in food products. The remaining components such as protein and starch are awaiting research to develop their full value [142].

Oat has the highest protein level (12–20%) [143]among cereals with a superior amino acid profile due to higher amounts of limiting amino acids lysine and threonine [16]. This is related to the fact that in most cereals the major storage proteins are alcohol-soluble prolamines whereas in oat, globulins represent 70–80% of the total protein fraction [13]. The major fraction in oat protein is the 12S globulin, which consists of two major subunits with molecular weight of about 32 and 22 kDa called the A- and B-subunits, where the A-subunit is an acidic polypeptide and the B-subunit is a basic polypeptide. The A- and B-subunits are disulfide bonded in the native globulin, forming a dimer with a molecular weight of 54 kDa, which further associates into a hexamer through non-covalent forces [17]. The 7S and 3S are the minor fractions. 7S globulins are polypeptides with molecular weight of 55 kDa, and some minor components with a molecular weight of 65 kDa are also present. The 3S fraction entails at least two major components with molecular weight of about 15 and 21 kDa [16].

Oat 12S globulin resembles the structure of 11S globulin of soy (glycinin), which has demonstrated good gelling properties; hence oat protein has the potential to act as a gelling agent. Identification and development of such key functionality from oat protein can

significantly promote its utilization in the food market. Ma et al. have considerably contributed to this area of research. However such efforts are still limited compared to those on dairy and soy proteins. Two previous publications demonstrated that oat protein could form gels [144,145]. But at acidic and neutral pH, very weak gels with poor water holding capacity were obtained. The gel properties improved after pH 8, but strong gels could only be prepared at pHs 9 - 10. The gel hardness was greatly increased by both acetylation and succinylation [56,219]. The authors suggested that the changes in the functional properties of oat protein after modification resulted from altered conformation and increase in net charge [56,219-221]. This was later confirmed with the study of the thermal aggregation of oat globulin by Raman spectroscopy [222]. In this work, changes in protein interactions and conformation were induced by the addition of protein structure modifying agents such as chaotropic salts, sodium dodecyl sulfate or dithiothreitol, which can either enhance or inhibit thermal gelation of oat globulin.

Enzymatic hydrolysis is a preferable tool to alter functional properties of proteins because of milder processing conditions required, easier control of reaction and minimal formation of by-products [146]. Recent research has reported the effect of enzymatic hydrolysis over the gelling properties of proteins including soy protein [98], rice bran protein [99], sunflower protein [100], and canola protein [97]. Results from these studies indicate that improvement of the gelling capacity is highly enzyme specific. The gelling properties of oat protein treated with trypsin were studied in previous work [56,219], however, weak gel structure was obtained due to the short size of the protein molecules, which may no longer be able to associate to form a strong gel matrix. Since the final composition and thus the use of the hydrolysates will depend on the type of enzyme used and the hydrolysis conditions [96]. A systematic investigation of the

effect of various proteases over the gelling capacity of oat protein is required. Till now, such information is not available, however important for the development of new modification strategy to improve oat protein gelling properties.

Modification of protein conformation can also be achieved through limited hydrolysis, as changes in the secondary and tertiary structure can be produced. This can alter the surface exposure of reactive amino acids, leading to an increase in interactions favoring aggregation [24] and three-dimensional network formation. Our preliminary trials have demonstrated that partial enzymatic hydrolysis can improve oat protein gelling properties under specific conditions. Thus it is hypothesized that oat protein and its hydrolysates could form gels of plant origin with similar properties as those from animal proteins such as egg white. Therefore, this work aims to complete a systematic study of the thermal gelation of oat protein and its hydrolysates under different environmental conditions with an emphasis on the gel mechanical strength and water-holding capacity which are the most important gel characteristics for food applications. If the defined gel physical properties would be in the range of similar properties of animal protein derived gels, value-added opportunities would exist for oat protein to be used as a new gelling ingredient in food formulations such as meat binder and fat replacer to create food with improved quality and nutritive value, or used in meat analogues for vegetarian foods. In this way, additional revenue return could be generated to oat producers and processors to enhance their sustainability.

3.2. Materials and methods

3.2.1. Materials

Naked oat grains (*Avena nuda*) were purchased from Wedge Farms Ltd., Manitoba, Canada. The protein content was 17.2%. Flavourzyme (≥ 500 U/g), alcalase (2.4 U/g), pepsin (≥ 250 U/mg), trypsin (1462 U/mg), sodium dodecyl sulfate (SDS) and trinitro benzene sulfonic acid (TNBS) were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). E-Z run pre-stained protein ladder/marker was purchased from Fisher Scientific (Whitby, ON, Canada).

3.2.2. Protein extraction

Oat grains were ground to flour using a mill (Ultra Centrifugal ZM 200 Retsch, PA) equipped with a 0.5 mm screen. The flour was then defatted with hexane at room temperature. Globular protein was extracted from the defatted oat flour according to the method reported by Wu et al., (1977)[223] with some modifications. Briefly, defatted oat flour was dispersed in an alkali solution adjusted to pH 9.2 using sodium hydroxide at a flour-to-solvent ratio of 1:6 and mixed for 1 h at room temperature. The slurry then passed through a 300 μm wire mesh and the permeated mixture was centrifuged at 7000 ×g for 15 min. Then, the supernatant was collected and pH was adjusted to 5 with 1 M HCl, followed by centrifugation at 7000 ×g for 15 min. The pellet corresponding to the precipitated protein was washed with distilled water and freeze-dried for later use. Protein content of the extracted oat protein was determined using the Leco nitrogen analyzer (FP-428, Leco Corporation, St Joseph, MI) and a nitrogen to crude protein conversion factor of 6.25 was used.

3.2.3. Enzymatic hydrolysis of oat protein

A 2% (w/v) protein suspension was prepared with distilled water. The pH and temperature of the suspension were adjusted to the optimum condition for each enzyme. Hydrolysis with flavourzyme was carried out at pH 7 and 50 °C, alcalase at pH 8 and 50 °C, pepsin at pH 2 and 37 °C and trypsin at pH 8 and 37°C. The enzyme/substrate ratio was set at 1/10 for all treatments. Over the hydrolysis period (30 min) the pH was kept constant with 1 M HCl or 1 M NaOH. At the end of the hydrolysis, the solution was heated at 90 °C for 10 min to inactivate the enzyme. Hydrolysate samples were dialyzed with a 10 kDa dialysis bag against distilled water. Samples were collected, freeze-dried and stored for further experiments. The protein content of the dried hydrolysates was also determined using the Leco nitrogen analyzer (FP-428, Leco Corporation, St. Joseph, MI).

3.2.4. Characterization of oat protein and its hydrolysates

Degree of hydrolysis (DH) was determined by the TNBS assay (Adler-Nissen, 1979). Total number of amino groups was determined in a sample completely hydrolyzed with 6 N HCl at 110 °C for 24 h. The DH was calculated with the following equation.

$$DH = \frac{h}{h_{tot}} \, x \, 100$$

Where h (hydrolysis equivalents) is the amount of peptide bonds cleaved during hydrolysis, which is expressed as millimole equivalents per gram of protein (mmol/g of protein) and h_{tot} is the total amount of peptide bonds in the protein substrate. l-Leucine (0–1.5 mM) was used to generate a standard curve ($R^2 = 0.99$).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to study the molecular weight of the oat protein subunits. Protein samples were mixed with sample buffer (0.125 M

Tris‐HCl pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.5% 2-mercaptoethanol and 1% bromophenol blue w/v) and heated at 100 ° C for 5 min, then cooled to room temperature. After cooling, 30 µL sample (1 mg/mL) was loaded on 4% stacking gel and 12% separating gel and subjected to electrophoresis at a constant voltage of 160 V. After electrophoresis the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in water‐methanol‐acetic acid (4:5:1, v:v:v).

The molecular weight distribution of the samples was determined using a size exclusion high performance liquid chromatography (SE-HPLC) system (Agilent 1200 series) equipped with a Biosuite™ 125/5 μm HR-SEC column (7.8 × 300 mm, Water Corp. MA, USA). The eluent used was 0.2 M phosphate buffer with 0.2 M NaCl (pH 7) at a flow rate of 0.5 mL/min and room temperature. Samples (50 μL) were injected into the system and elution was monitored at 220 nm. Standard molecular markers were used to calculate M_w of the oat protein samples. A calibration curve was made from the log M_w of the markers and their respective elution times ($R^2 = 0.97$).

Amino acid composition analysis of the samples was performed using the Waters AccQ-Tag ™ precolumn method. Dried samples were hydrolyzed under vacuum and after derivatization were loaded on a reversed phased column. The AccQ reagent, 6-aminoquinolyl-N-hydrozysuccinimidyl carbamate, is an N-hydroxysuccinimide-activated heterocyclic carbamate, which converts both primary and secondary amino acids to stable fluorescent derivatives.

The denaturation temperature of OPI and its hydrolysates was determined using a differential scanning calorimeter Q1000 (TA Instruments, New Castle, DE, USA). Approximately 10 μL of a 15% protein (w/v) suspension was weighed on a pre-weighed aluminum pan and

hermetically sealed. An empty hermetic pan was used as reference. The sample was heated at a 10 °C/min, over a temperature range of 30-160 °C. The protein suspensions (15% protein) were prepared at pHs 5, 7 and 9 to study the effect of pH on the protein denaturation temperature. The denaturation temperature (T_d) was computed from the thermograms as the peak value detected by using computer software.

3.2.5. Gel preparation

Gels were prepared by heating the protein sample suspension (15%, w/v) at pHs 5, 7 and 9. The pH of the suspension was adjusted using 1 N NaOH or 1 N HCl. Vacuum was applied to remove air bubbles. Test tubes containing the suspension were tightly closed and placed in an oil bath at 110 °C and 120 °C for 15 min. Once heat treatment was completed, the tubes were cooled in an ice bath and stored in the refrigerator overnight.

3.2.6. Textural profile analysis (TPA)

The mechanical properties of the gels prepared above were evaluated using an Instron 5967 universal testing machine (Instron Corp., Norwood, MA, USA). Gels were released from test tubes and cut into cylindrical pieces (~ 10 mm height, ~ 14 mm diameter). A two cycle compression test using a 50 N load cell was performed at room temperature at a rate of 1 mm/min to evaluate their mechanical properties. Each sample was compressed to 50%, since deformation levels between 20 and 50% have been commonly applied in several works on gel food systems. At this level the sample does not break, but it is still possible to obtain valuable information on important parameters [224]. The textural profile parameters including, hardness, springiness and cohesiveness were calculated. These parameters were determined form the typical Instron force-time curve in which hardness is calculated as the peak

compression force in the 1st bite cycle, and cohesiveness is the ratio of the area under the first and second compression peaks. Springiness is the distance calculated from the area under the second compression peak.

3.2.7. Scanning electron microscopy (SEM)

The morphology observation of the gels was carried out with a Phillips XL-30 scanning electron microscope (FEI Company, Oregon, USA). The samples were frozen in liquid nitrogen and freeze-dried before observation. Dry samples were coated with gold and platinum and a scanning electron microscope was used to observe the microstructure of the gels.

3.2.8. Water holding capacity (WHC)

A gel sample (0.9–1.2 g) was placed into a Vivaspin 20 centrifugal filter unit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and centrifuged at 290 ×g for 5 min at 15 ° C. The weight of the gel was recorded before (W_i) and after (W_f) centrifugation to the nearest 0.0001 mg and the percentage of water loss after centrifugation was expressed as:

$$WHC = 100 - \left(\left(\frac{W_i - W_f}{W_i} \right) x100 \right)$$

3.2.9. Statistical analysis

The results were evaluated by analysis of variance (ANOVA) and significant differences, with minimum significance test set at the 5% level ($p < 0.05$) with Tukey's test to compare all means, using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). All experiments were performed at least in three independent trials and the results were reported as mean ± standard deviation.

3.3. Results and discussion

The protein content of the final oat protein isolate (OPI) was 91.2% ± 2.4. Oat protein was partially hydrolyzed using flavourzyme, alcalase, pepsin and trypsin, which are common in industrial applications [96]. After 30 min of enzymatic treatment, limited hydrolysis was achieved with the final DH% value reaching 7.1% ± 0.54, 5.8% ± 0.44, 5.5% ± 0.41 and 5.3% ± 0.40 for flavourzyme, alcalase, pepsin and trypsin hydrolysates and the samples were labeled as OPI-F, OPI-A, OPI-P and OPI-T, respectively

3.3.1. Characterization of oat protein and its hydrolysates

3.3.1.1. SDS-PAGE

The SDS-PAGE pattern in Figure 2 demonstrates that the predominating protein fraction in the extracted oat protein is 12S globulin (Lane 2). The acidic (12S-A) and basic (12S-B) polypeptides are easily identified. The bands between 43 and 72 kDa and below 17 kDa correspond to the 7S and 3S fractions, respectively.

Hydrolysis with flavourzyme partially affected the acidic polypeptide as the 12S-A fraction bands shifted to a lower molecular weight region (26–34 kDa) (Lane 3). In contrast, alcalase had a much stronger effect upon the 12S-A fraction as it disappeared in OPI-A sample (Lane 4). This 12S-A was less susceptible to trypsin as most of the original bands remained, although the low end of the band shifted to a lower molecular weight range (Lane 6). Flavourzyme is a protease composed of a mixture of endoprotease and exopeptidase, which has been used to prepare short chain peptides and free amino acids [225]. Alcalase is an endoprotease composed of a mixture of different proteases, each with different specificities [226], thus it has a broad specificity toward peptide bonds.

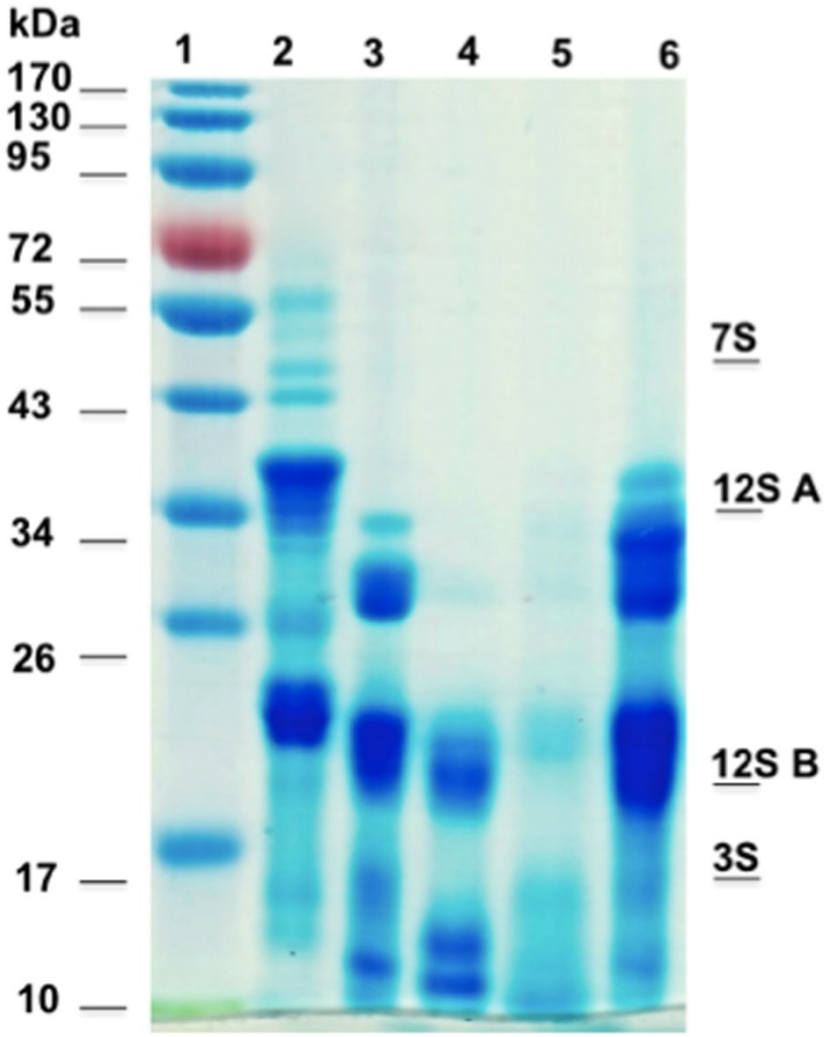

Figure 2 SDS polyacrylamide gel electrophoresis of oat protein and its hydrolysates. Lanes: 1. Standard protein markers, 2. OPI, 3. OPI-F, 4. OPI-A, 5. OPI-P, and 6. OPI-T.

The extensive degradation of the 12S-A subunit can be explained by this broad specificity. In addition, Glu is an abundant amino acid in oat protein and especially in the acidic subunit of oat globulin [17,227]. A Glu-specific endopeptidase has been isolated from a commercial preparation of alcalase [228], therefore the presence of Glu in the 12S-A fraction favors its alcalase degradation. From the enzymes selected in this study, trypsin is probably the most

specific toward its substrate. Furthermore, it cleaves peptides on the C terminal side of Lys and Arg [229], thus the acidic polypeptides 12S-A was less susceptible to trypsin digestion. On the other hand, the 12S-B was fairly resistant to all enzymes tested and only a minor shift in the molecular weight was noticed. The resistance of the basic polypeptide to enzymatic hydrolysis could be explained by the fact that this subunit is buried at the interior of the structure, thus is not as readily accessible as acidic subunit [230,231]. A similar result has been reported for peanut protein isolate in which the acidic subunit of arachin was more susceptible to hydrolysis, whereas the basic subunit was maintained [43].

The case of pepsin is an exception as only faint bands were observed after hydrolysis (Lane 5), indicating that both 12S-A and 12S-B were digested by pepsin. This might be related to the low pH required for pepsin treatment, under which oat protein could be partially unfolded and thus both acidic and basic polypeptides are accessible and susceptible to proteolysis [17,227]. Therefore both units are rapidly digested during 30 min of pepsin treatment.

3.3.1.2. Size exclusion high performance liquid chromatography (SE-HPLC)

SE-HPLC chromatograms of OPI and its hydrolysate samples are shown in Figure 3, divided into three regions, comprising region I (656–22.4 kDa), region II (21.4–0.4 kDa) and region III (< 0.4 kDa). Oat protein isolate shows a dominant peak in region I with molecular weight (M_w) of approximately 190 kDa. This peak almost disappeared in hydrolysates by alcalase and pepsin, meanwhile hydrolysates by flavourzyme and trypsin show a peak dramatically reduced in height, indicating that the oat protein was hydrolyzed by pepsin and alcalase, but only partially by flavourzyme and trypsin. In region II, OPI shows a group of small peaks with molecular weights ranging from 2.4 to 0.7 kDa, whereas this group of peaks dominated in all the oat protein hydrolysate samples, confirming hydrolysis of the oat protein.

Hydrolysates by trypsin, pepsin and alcalase showed a major peak at ~ 0.7 kDa and a shoulder with larger molecular weight. In contrast, the flavourzyme treated sample showed a different contour including a sharp peak with molecular weight of 0.9 kDa and a dramatically reduced shoulder peak, indicating that flavourzyme was more effective at producing low molecular weight polypeptides.

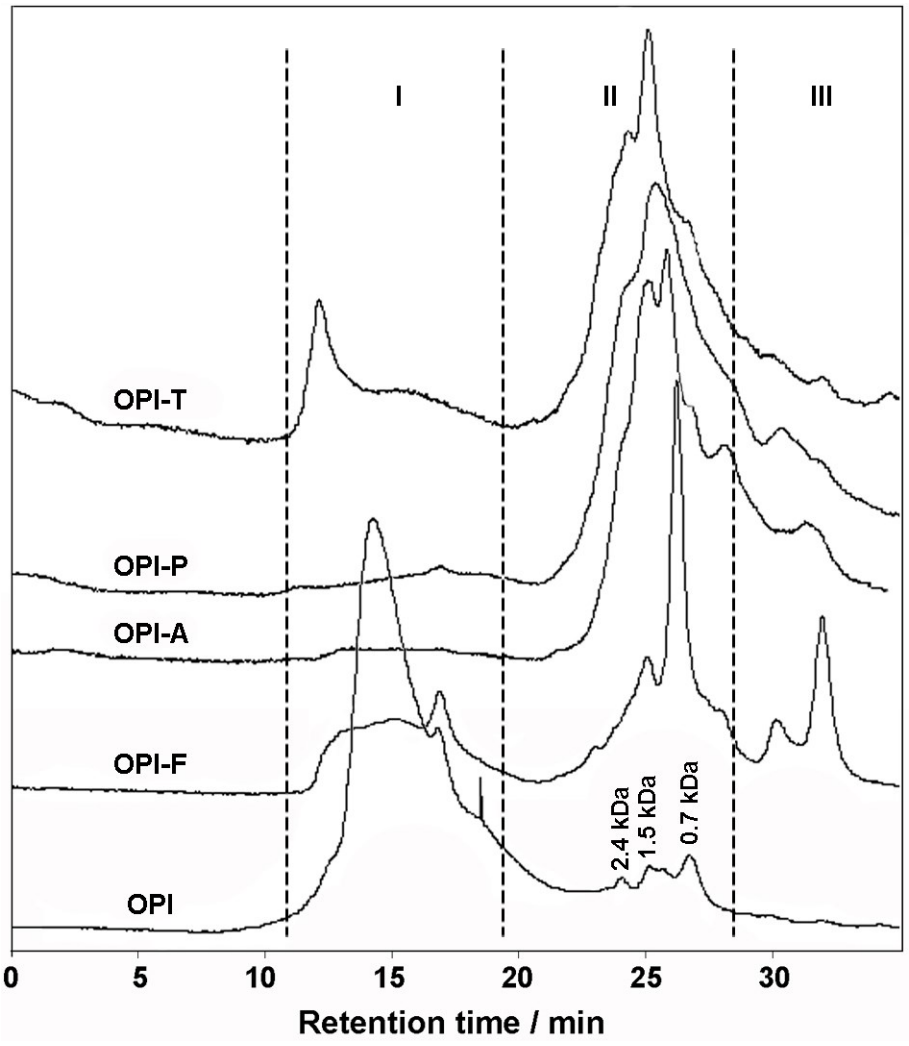

Figure 3 Size exclusion chromatograms of oat protein and its hydrolysates

Oat protein isolates did not show any peaks in region III. On the other hand, all hydrolysates contained small peptides, particularly those treated with flavourzyme as a sharp peak of

approximately 70 Da was observed at the end of the chromatogram. This confirms the ability of flavourzyme to produce small peptides and even free amino acids. Peptides from this region are not likely to participate in the gel formation process due to their small molecular weight, thus the fractions of main interest are contained in regions I and II. Both SDS-PAGE and SE-HPLC observations confirmed that flavourzyme and trypsin hydrolysates maintained greater integrity of the original structure of oat protein, when compared to alcalase and pepsin hydrolysates.

3.3.1.3. Amino acid analysis

As shown in Table 2, oat protein and its hydrolysates contain high levels of Glx (Glu + Gln), since glutamic acid is the most abundant amino acid in oat protein [17,19,227]. Other amino acids at high levels are Gly, Leu and Val. The amount of Glu + Gln was noticeably reduced in the hydrolysate samples. This is in agreement with the enzymatic susceptibility of the acidic polypeptide of oat globulin, considering the acidic polypeptide is rich in Glu. The amount of Asx (Asp + Asn) in the alcalase hydrolysate is significantly higher compared to the other hydrolysates. This confirms that the remaining unit is the basic polypeptide, considering that this polypeptide is rich in Asp [17]. If most of the Glx is considered as Glu, such amino acid composition modification could alter the charge of the polypeptide chains, and thus their functionalities in different pH environments. Reduced Pro residue was also observed in oat protein hydrolysates which could potentially impact the protein properties as this amino acid is believed to play an important role in the stabilization of protein structure due to hydrogen with hydroxyl groups [232].

Table 2 Amino acid composition of oat protein isolate (OPI) and its hydrolysates.

Residue	% Mol

	OPI	OPI-F	OPI-A	OPI-P	OPI-T
Asx[a]	5.5	6.5	10.0	7.1	6.4
Serine	4.2	4.9	4.4	4.8	4.9
Glx[b]	22.4	16.4	14.9	15.5	20.3
Glycine	7.7	8.4	8.7	9.4	9.2
Histidine	n.d.	3.2	n.d.	n.d.	n.d.
Arginine	6.0	8.2	8.6	8.5	7.9
Threonine	3.5	3.7	3.5	4.3	3.5
Alanine	6.7	6.6	6.8	7.0	6.9
Proline	7.3	5.5	5.5	5.9	5.7
Cysteine	2.4	1.6	1.3	2.5	1.6
Tyrosine	2.9	2.9	3.2	2.7	3.1
Valine	7.7	7.3	7.7	7.4	6.8
Methionine	2.1	1.4	1.3	2.0	1.3
Lysine	3.3	4.3	3.7	4.8	3.9
Isoleucine	4.6	5.6	6.1	5.2	5.5
Leucine	8.6	8.2	8.5	8.1	7.9

[a] Asx(Asp+Asn) [b] Glx (Glu+Gln)

n.d. means not detectable

3.3.1.4. Differential scanning calorimetry (DSC)

As shown in Table 3 the extracted oat protein isolate had a denaturation temperature (T_d) value of 112.4 ° C, which is in agreement with previous reports [144,145]. The highest T_d values were observed at pH 7. Its T_d and the enthalpy of denaturation (ΔH) value decreased slightly at pHs 5 and 9, possibly due to partial denaturation of oat protein under acidic or alkali conditions [233]. It is interesting to notice that the oat protein hydrolysates showed significantly increased ΔH values although their T_d values remained almost unchanged. It is possible to speculate that some oat protein fractions, such as 12S have a configuration composed of loosely arranged segments and tightly packed segments.

Table 3 Effect of pH on the denaturation temperature and enthalpy change of OPI and its hydrolysates.

	pH 5		pH 7		pH 9	
	T_d (˚C)	ΔH (J/g)	T_d (˚C)	ΔH (J/g)	T_d (˚C)	ΔH (J/g)
OPI	109.4	0.7494	112.4	0.9826	107.94	0.5763
OPI-F	109.93	1.972	112.52	3.170	110.91	1.810
OPI-T	110.47	3.018	110.61	3.307	107.8	1.262

Normally, these tightly packed segments are highly hydrophobic and are located at the interior of the structure, whereas the loose segments are at the exterior, being more accessible to hydrolysis. After enzymatic cleavage of the exterior loose part, most of the hydrophobic

core structure remained in the hydrolysate samples, which could be much more stable against heat treatment. Thus higher energy is required to disrupt intramolecular bonds to achieve complete denaturation. This type of reaction is called a zipper reaction [94] and it is not unusual to observe the formation of resistant polypeptides even after prolonged hydrolysis due to their compact structure.

3.3.2. Thermal gelation of OPI and derived hydrolysates

In this work, the initial thermal gelation test was conducted at two temperatures near or above oat denaturation temperature (T_d) (110 and 120 °C) at three different pHs (5, 7, 9). The purpose was to screen samples and conditions that allow gel network formation, which is defined for this work as the establishment of a self-supporting structure showing no flow upon inversion after thermal treatment and cooling. As shown in Table 4, the oat protein formed gels under all tested conditions. Oat protein hydrolysated by flavourzyme and trypsin formed gels under almost all conditions, except at pHs 5 and 7 at 110 ° C. Possibly, this temperature was not sufficient to unfold the compact structure of these hydrolysates extensively enough to expose reactive groups that could participate in crosslinking and form a self-supporting structure; conversely at a higher temperature the gelation took place. At pH 9, the protein structure could be more readily opened to expose hydrophobic patches, due to disruption of hydrogen bonds and dissociation of hydrogen from carbonyl and sulfate groups at alkaline conditions, thus a lower energy input was required to favor protein interactions, allowing gel formation at 110 °C.

Oat protein hydrolysates by alcalase were able to form gels only at pH 9 while those by pepsin did not form gels under any conditions. It is possible that the 12S-A fraction of oat globulin exerts great influence over the gelling ability of oat protein, especially under acidic

and neutral pH, as samples with well-preserved 12S-A subunits demonstrated good gelling properties in such pH ranges. This might be partially related to the larger molecular weight of the acidic fraction compared to the basic polypeptide, which allows exposure of more reactive sites on a single polypeptide chain for intermolecular interaction development.

Table 4 Oat protein derived gels formed under different pH and temperature conditions

	pH 5		pH 7		pH 8	
	110˚C	120˚C	110˚C	120˚C	110˚C	120˚C
OPI	✓	✓	✓	✓	✓	✓
OPI-F	✗	✓	✗	✓	✓	✓
OPI-A	✗	✗	✗	✗	✓	✓
OPI-P	✗	✗	✗	✗	✗	✗
OPI-T	✗	✓	✗	✓	✓	✓

✗ No gel

✓ gel formation

Whereas smaller fractions expose little reactive sites limiting the aggregation step, essential for the gel network formation [234]. The formation of gel for alcalase hydrolysate at pH 9 might be attributed to the low surface charge of the basic polypeptide under alkaline condition, which has an isoelectric point of 8–9. Thus the limited net charge could favor network

formation via hydrophobic interactions due to reduced repulsive forces compared to those at pHs 5 and 7 [25]. The hydrolysate prepared with pepsin could not form gels due to loss of both acidic and basic subunits. Gels prepared from oat protein and its hydrolysates by flavourzyme and trypsin were selected for the following experiments, as these samples were able to form gels under a broad range of conditions.

3.3.3. Textural profile analysis (TPA)

The mechanical properties of the gels prepared with oat protein isolate and its hydrolysates at 120 °C were then studied, including hardness (force required to attain a given deformation), cohesiveness (work required to overcome the internal bonding of the material) and springiness (rate at which a deformed material recovers to its original condition after removal of deforming force) [235]. Results of TPA are shown in Figure. 4.a.

Oat protein isolate formed strong gels at pHs 5 (1.90 N) and 7 (1.92 N) at 120 °C. In contrast, softer gels were observed at pH 8 (0.80 N) and very weak gels were obtained at pH 9 (0.07 N). The network structure of a heat-denatured globular protein gel depends greatly on the balance of attractive (hydrogen and hydrophobic interactions) and repulsive (electrostatic) forces among the protein molecules, as determined by pH and ionic strength [144,236]. Thus the right balance between the electrostatically repulsive force and the hydrophobic attractive force should explain the strong gels obtained at pH 5 and pH 7. However, beyond the optimal pH, disproportionate repulsive forces may have led to fewer protein interactions, since very weak gels were formed at pH 9 and intermediate hardness values were observed at pH 8. Similar behavior was observed in β-lactoglobulin gels prepared at pH 8. In this case, excessive repulsive forces created a high energy barrier preventing denatured protein molecules from associating and forming a strong self-

supporting structure [237]. It is interesting to note that flavourzyme hydrolysates formed stronger gels than oat protein isolate under comparable conditions. This improvement was especially substantial at pHs 7–9 as the gel hardness increased from 2.80 N to 4.80 N. Significant ($p<0.05$) increases in gel hardness were not detected at pHs 5–7 for trypsin hydrolysates but hardness values increased ($p<0.05$) to 3.03 N at pH 8, then dramatically improved to 8.80 N at pH 9. The fact that oat protein hydrolysates produced very strong gels at pHs 8–9 indicates that the balance between the electrostatically repulsive force and the hydrophobic attractive force changed as a result of enzymatic hydrolysis. Since enzymatic hydrolysis reduces the amount of Glu (acidic amino acid), the net charge of the hydrolysates at pHs 8–9 could be lower than that of the oat protein isolate, leading to decreased repulsive forces among polypeptide chains. In addition, the augmented hydrophobicity of the peptide chains after partial hydrolysis could contribute to the increased gel strength. A similar observation was reported by Ma (1985) [221,238], in which both surface and exposed hydrophobicity of oat protein increased after trypsin hydrolysis. As mentioned earlier, the acidic polypeptide with hydrophilic character covers the basic polypeptide which has a more hydrophobic character, thus as hydrolysis progresses the acidic polypeptide is broken down and the overall hydrophobicity of the remaining fraction is increased [239]. Thus, the reduced electrostatically repulsive forces and the increased hydrophobicity attractive force are equilibrated at this pH producing the right balance to develop a gel with enhanced hardness. It has been reported that soy protein gels had hardness values of around 2.1 N–2.6 N at neutral pH [240].

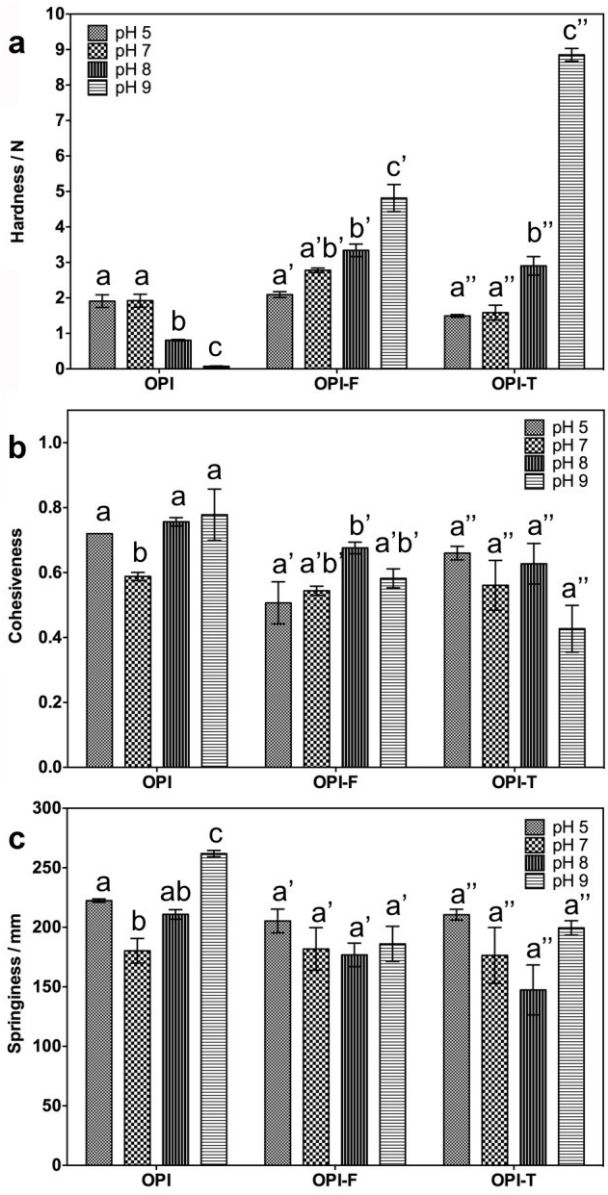

Figure 4 Mechanical properties of oat protein derived gels prepared at 120 °C. a. Hardness (N), b. cohesiveness and c. springiness (mm). Statistical analysis was performed separately for OPI, OPI-F and OPI-T, which was indicated by the use of primed letters (a, a' and a'') Values within the same sample not sharing a common superscript differ ($p<0.05$).

Gels prepared with oat protein isolate, showed a slightly lower value, but those gels prepared with flavourzyme and trypsin hydrolysates were comparable or higher than soy protein gels at the same pH. Gels prepared with flavourzyme and trypsin hydrolysates at pH 9 showed

enhanced hardness, and the results corresponding to trypsin hydrolysate gels are even comparable to egg white protein gels (8.70 N) under the same pH [241]. It is understood that gels prepared at pH 9 could have a limited application as most food products have a pH value between 3 and 8. Nevertheless, egg white has a pH of 7.6–9.7 depending on the storage time and temperature [242], and yet it is commonly used in different applications. In addition, strong oat protein gels were also obtained at pHs 7 and 8 after limited flavourzyme hydrolysis, and at pH 8 after limited trypsin hydrolysis. These gels can be more widely used in different food products.

The effect of trypsin hydrolysis upon the gelling capacity of oat protein was previously studied by Ma and Wood (1986, 1987) [56,219]. The result indicated that trypsin treatment leads to a weak gel structure, probably due to reduction in the size of the protein molecules, which may no longer be able to associate to form a strong gel matrix. Whereas in this work, gels with significantly improved hardness were obtained at pHs 8 and 9 after limited trypsin hydrolysis due to maintenance of appropriate level of peptide size, allowing formation of good three-dimensional networks. The detrimental effect of trypsin hydrolysis was also observed as part of the preliminary experiments for this work (data not shown), in which those hydrolysates produced after long periods of enzymatic treatment would not form a gel at all. Gelling conditions also significantly affect the properties of the resulting gels. The protein concentration and temperature selected for this study were higher than those applied in the work reported by Ma and Wood (1986, 1987) [56,219].

Oat protein isolate gels displayed good cohesiveness (Figure 4.b.) with values of 0.6–0.8 at pHs 5–9. Similar values have been reported for soy protein isolate gels [243]. Gels prepared with flavourzyme and trypsin hydrolysates also presented good cohesiveness, although

slightly lower values were observed ranging from 0.7 to 0.4. The cohesiveness values reported in this study indicate that the gels maintained the integrity of their internal bonds when compressive forces were applied. A low cohesiveness value indicates damage to the internal bonds and thus a tendency to fracture under stress. Most of gels prepared in this study showed good resistance to disintegration due to compression. Both oat protein isolate and its hydrolysate gels showed good springiness (Figure. 4.c.) under the conditions tested. The impact of pH on gel springiness did not show an obvious trend.

3.3.4. Gel morphology

The gel morphology observed using SEM shows the effect of pH on the gel microstructure (Figure 5). It was expected that two types of structures would be observed, either fine-stranded or particulate gels, however the SEM micrographs showed a polymer gel structure for oat protein at pH 7 and for its hydrolysates at pH 9. Typical particulate gels were formed for both oat protein and its hydrolysate gels at pH 5 (SEM micrograph not shown) and for hydrolysate gels at pH 7. Only some specific protein gels, such as gelatin, can be considered polymer gels. Oat protein may have relatively flexible molecular chains, which allow formation of bridges between the interaction points when the balance between electrostatic repulsive forces and hydrophobic forces among polypeptide chains is achieved.

This explains the strong mechanical property of oat protein gel at neutral pH and the significantly enhanced hardness of gels made from hydrolysates in mild alkaline pH. The gel morphology also clearly shows the effect of partial enzymatic hydrolysis on the gel microstructure. Since enzymatic hydrolysis reduces the amount of Glu (acidic amino acid), the net charge of the hydrolysates at pH 7 could be lower than that of the oat protein isolate, leading to decreased repulsive forces among polypeptide chains. Therefore these polypeptide

chains could aggregate rapidly via hydrophobic interactions during heating treatment. Later, these aggregates associated to form particulate networks. Whereas at pH 9, the increased charge on the polypeptide chains led to strong repulsive force to prevent rapid protein aggregation, thus allowed formation of bridges between the interaction points on the polypeptide chains to create polymer gel.

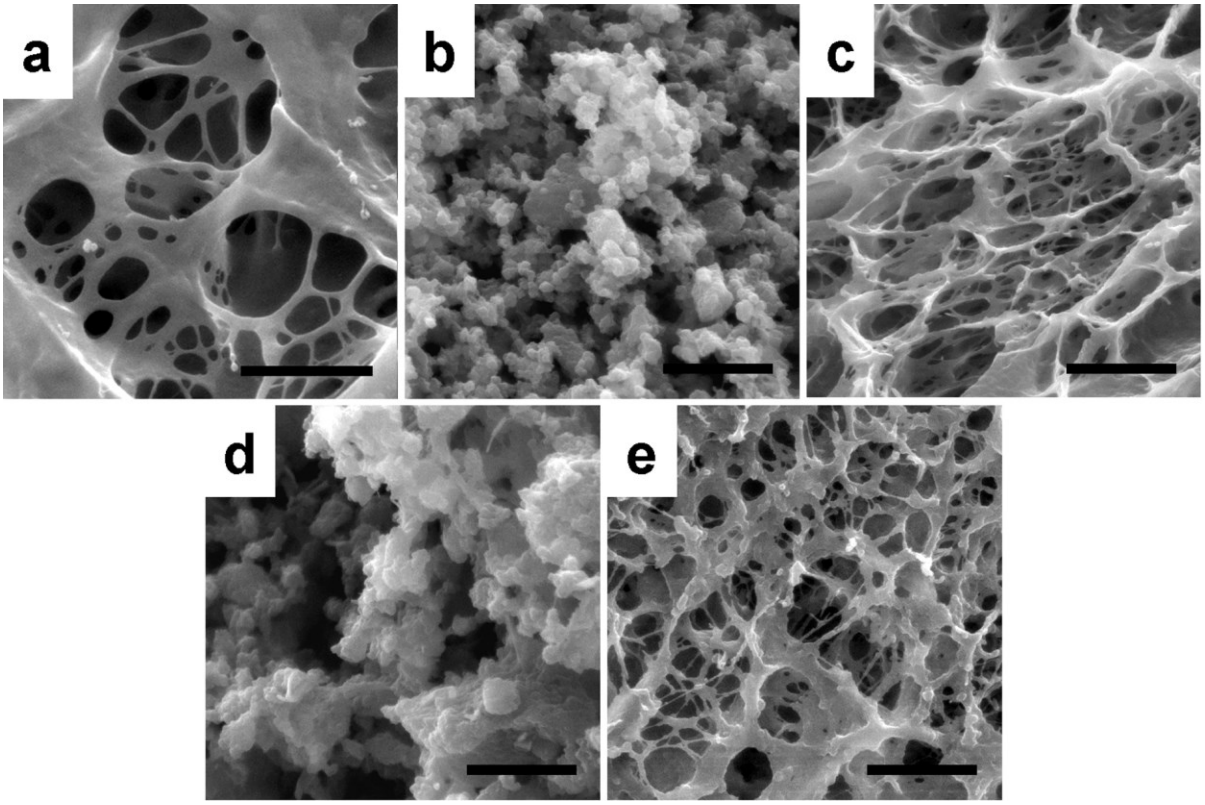

Figure 5 SEM images of the cross section of oat protein derived gels prepared at 120 °C. Scale bar represents 5 µm. a. OPI (pH 7), b. OPI-F (pH 7), c. OPI-F (pH 9), d. OPI-T (pH 7), and e. OPI-T (pH 9).

3.3.5. Water holding capacity (WHC)

Water holding capacity is another important property of food gels and the separation of liquid from the gel network can produce physical modifications such as shrinking or alterations in the palatability of the product due to reduced moisture [244]. These changes can reduce the

quality and acceptability of the product and for this reason a high WHC is required in gels destined for food applications. All gels demonstrated excellent WHC (82.8–95.5%) at pHs 7–9 as shown in Figure 6. Significantly reduced WHC values (61.5–65.2%) were observed at pH 5. According to previous literature, particulate gels formed at pH near the isoelectric point are characterized by an increased pore size that leads to a decrease in capillary forces and therefore a higher water loss [245]. The WHC of gels prepared with oat protein isolate at pH 9 could not be determined as they were very weak. The WHC values reported in this work are comparable to soy protein (82.2%) [246] and whey protein (~ 88%) [247].

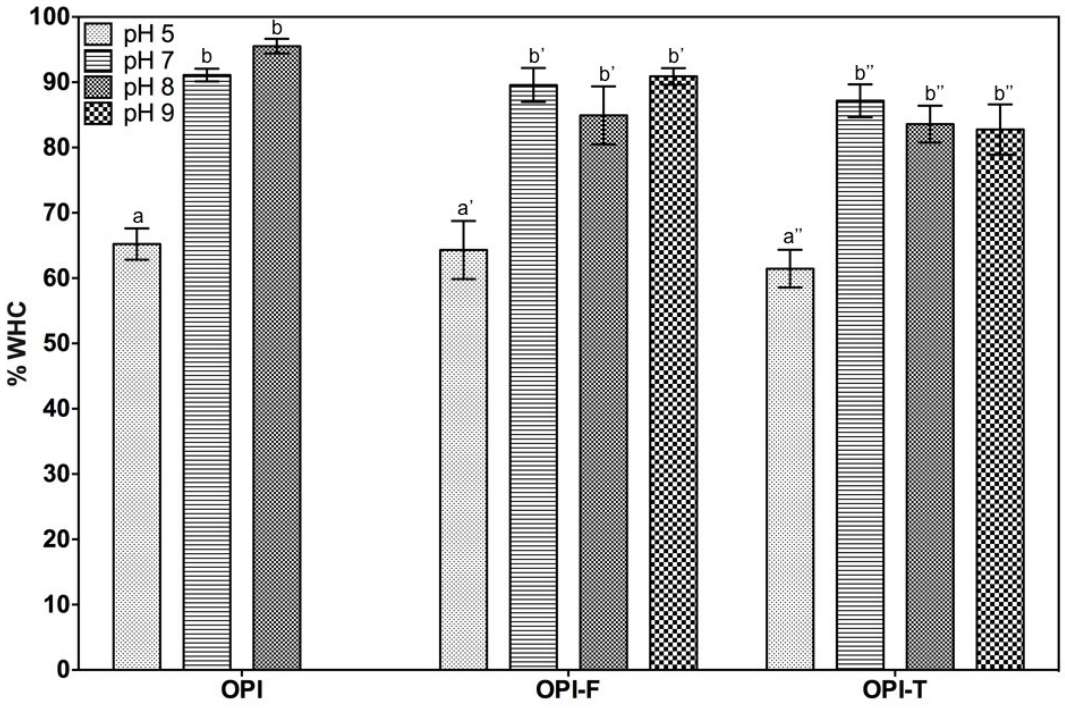

Figure 6 Water holding capacity of oat protein derived gels prepared at 120˚C. Statistical analysis was performed separately for OPI, OPI-F and OPI-T, which was indicated by the use of primed letters (a, a' and a'') Values within the same sample not sharing a common superscript differ ($p<0.05$).

3.4. Conclusion

This study has demonstrated that partially hydrolyzed oat protein could form gels with similar mechanical strength and water-holding capacity as those from animal proteins such as egg white. Thus we can conclude that oat protein gels have potential of replacing those derived from animal proteins to provide texture and structure in food products. This will provide value-added opportunities for oat producers and processors, and at the same time, manufacturers could have access to a new and cost-effective gelling ingredient of plant origin in diversified food formulations.

Chapter 4

4. Inulin at low concentrations significantly improves the gelling properties of oat protein – a molecular mechanisms study

4.1. Introduction

Oats are an important crop worldwide, with an annual production of approximately 21 million tonnes. Canada is a major supplier of oats, making up the majority of world oat trade [248]. However, Canadian oats are mainly used as animal feed and only a portion of the grain is used for human consumption. Oat has recently attracted research and commercial attention mainly due to the growing public awareness of the health benefits of β-glucan, which is known to reduce blood cholesterol and glucose levels. Several techniques have been developed to isolate β-glucan from oat grain as a health ingredient in food products. Protein

is the second largest component (12-20%) in oats after starch. Oat protein have a superior amino acid profile due to a higher content of lysine because globulins represent 70-80% of the total protein in oats, whereas alcohol-soluble prolamines are the major storage proteins in other cereals [13]. The 12S globulin is the major oat protein fraction, which resembles the structure of 11S globulin of soy (glycinin), thus oat protein possess gelling potential [56,144,249]. Plant proteins are normally considered inferior to animal proteins (e.g. gelatin, egg white and whey protein) in terms of gelling properties. In our recent work, trypsin treated oat protein could form gels with comparable mechanical strength to egg white protein at pH 9 [249]. This has provided opportunity for oat protein to be used a new gelling ingredient from plant resources in food formulations such as meat binder and fat replacer, or used in meat analogues for vegetarian foods. However, strong gels could be only obtained at alkali pH when heated to 110-120^{o}C. The gels were weak when formed under acidic and neutral pH at 100^{o}C. This has significantly limited applications of oat protein in food systems that normally have pH values in the range of 2.5 to 7 and heating temperature at 100^{o}C or lower. Therefore novel approaches to enable formation of stronger oat protein gels within a more appropriate pH and temperature for food processing are necessary to promote the utilization of oat protein as a gelling agent.

The gelling properties of proteins can be affected by interaction with other components, such as polysaccharides. Protein and polysaccharide are often mixed to develop food products with novel textural properties. The interactions developed among protein and polysaccharide will define the microstructure of food products and thus the resulting texture or mechanical properties. Interactions between protein and polysaccharides can be either associative or segregative depending on the molecular characteristics of the contributing polymers and the

medium conditions such as pH and ionic strength [122,124]. Coacervate networks are one continuous network and they are formed by favorable attractive interactions between protein and polysaccharide [250]. When no strong interactions exist between protein and polysaccharide, interpenetrating networks are formed, where each polymer is in its own continuous network [129]. Phase-separated networks are formed when interactions between them are repulsive, resulting in a bi-continuous phase or a continuous supporting phase containing inclusions of the other phase [49]. Previous reports show that protein-polysaccharide interactions can be modulated to reinforce the gel forming properties of meat [251], whey [49,252,253], and soy protein [254].

Inulin is a non-digestible polysaccharide naturally occurring in several edible fruits and vegetables. It is formed by fructose molecules linked by β-(2-1) glycosidic bonds, generally with a terminal glucose unit connected to the last fructose by an α-(1-2) bond [255]. Due to the unique nature of inulin bonds, digestive enzymes in the human gut cannot hydrolyze this polysaccharide. Inulin reaches the colon undigested and produces a prebiotic effect since it is fermented by lactic acid bacteria [256]. Additionally, inulin has other interesting biological properties such as enhancing mineral absorption, and reducing both blood lipid levels and the risk of colon cancer [255].The utilization of inulin in the food industry is not limited to its biological properties; it is also incorporated in food formulations as a fat replacer or bulking agent, such as in baked goods, sauces and yogurt [256]. Such a wide range of applications is related to its capacity to form microcrystals that interact with each other forming small aggregates. These aggregates immobilize a great amount of water, thus create a fine creamy texture that provides a mouth sensation similar to that of fat [257]. Previous reports have investigated the influence of inulin addition in milk [258], soy protein gels [254], yogurt [259] and

cheese [260], finding that the protein-inulin system had improved gelling properties. Nonetheless the effect of inulin addition on the gelation properties of oat protein has never been reported. Thus it is hypothesized that inulin addition can produce a synergistic effect which will enable the formation of strengthened oat protein gels.

The aim of this work is to investigate the effect of oat protein and inulin interactions on the gelling properties of oat protein isolate. Mechanical and rheological properties of oat protein gels were determined and their microstructures were observed. We attempt to better understand gelling mechanisms of oat protein-inulin system by correlating protein structure changes during heating to gel microstructures and bulk properties. Improvement of the gelling properties of oat protein at acidic and/or neutral pH may create broad applications of this plant-sourced gelling ingredient in foods. These value-added opportunities may represent very significant sources of revenue to oats producers and processors to enhance their sustainability.

4.2. Material and Methods

4.2.1. Materials

Oat protein isolate (OPI) was extracted according to our previous work [249] and the protein content was determined to be 90.4 % ± 0.6 using the Leco nitrogen analyzer (FP-428, Leco Corporation, St Joseph, MI). Inulin was extracted from chicory root with an average polymerization degree of 25 according to product specifications; 2-mercaptoethanol, urea, sodium dodecyl sulfate, fluorescein isothiocyanate (FITC), Rhodamine B and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada).

4.2.2. Gel preparation

Gels were prepared by heating the protein-inulin suspensions at pH 2.5, 5 and 7 adjusted with 0.1 N NaOH or HCl. The concentration of OPI in the mixtures was kept constant at 15% (w/v), which was revealed to be the optimized oat protein concentration for gel formation [249]. The concentration of inulin varied from 0 to 0.5% (w/v) in the mixture, these values were selected based in preliminary trials. Samples were labeled as OPI, OPI-I 0.1%, OPI-I 0.25% and OPI-I 0.5%, representing inulin content of 0%, 0.1%, 0.25% and 0.5% respectively. Test tubes containing the suspension were tightly closed and placed in an oil bath at 100˚C for 30 min. Once the heat treatment was completed, the tubes were cooled in an ice bath and stored in the refrigerator overnight.

4.2.3. Textural profile analysis (TPA)

The mechanical properties of the gels were evaluated using an Instron 5967 universal testing machine (Instron Corp., Norwood, MA, USA). Gels were dismounted from test tubes and cut into cylindrical pieces (~10 mm height, ~14 mm diameter). A two cycle compression test using a 50 N load cell was performed at room temperature at a rate of 1 mm/min and 50% compression to evaluate their mechanical properties. The textural profile parameters were determined from the typical Instron force-time curve. Compressive stress was calculated as the peak compression force in the 1st bite cycle divided by the initial cross-section area of the gel sample. Springiness is the distance calculated from the area under the second compression peak and gumminess is the product of peak compression force in the 1st bite cycle multiplied by cohesiveness.

4.2.4. Water Holding Capacity (WHC)

A gel sample (~ 1.0 g) was placed into a Vivaspin 20 centrifugal filter unit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and centrifuged at 290 × g for 5 min at 15˚C [249]. The weight of the gel was recorded before (W_i) and after (W_f) centrifugation to the nearest 0.0001 mg and the percentage of water loss after centrifugation was expressed as:

$$\%WHC = 100 - \left(\left(\frac{W_i - W_f}{W_i} \right) x100 \right)$$

4.2.5. Scanning electron microscopy (SEM)

The morphology observation of the gels was carried out with a Phillips XL-30 scanning electron microscope (FEI Company, Oregon, USA) at an acceleration voltage of 6 kV. The samples were frozen in liquid nitrogen and freeze-dried before observations. The cross-section and surfaces of the dry gels were sputtered with gold and platinum, observed and photographed.

4.2.6. Rheological measurements

The rheological measurements were done with a TA Discovery HR-3 rheometer (TA instruments, New Castle, DE, USA). Approximately 1 mL sample was loaded in the bottom plate of the parallel plate geometry; the upper plate was lower to the appropriate geometry gap. To avoid evaporation during heating a solvent trap was used and a thin layer of silicone oil was applied. The temperature of the bottom plate was controlled with a Peltier system. To study the changes in viscoelastic properties as a function of temperature, OPI and OPI-inulin suspensions were subject to a temperature ramp from 25 to 95˚C, then cooled down to 25˚C at a rate of 1.5˚C/min. Sample conditioning took place before and after each temperature ramp for a period of 3 min. The temperature ramp was not run up to the gelling temperature

used in other experiments described in this paper (100˚C) as preliminary experiments reaching 100˚C produced unstable readings due to water boiling, thus the maximum temperature used was 95˚C. All rheological measurements were done within a predetermined linear viscoelastic region at the strain value of 0.05%. To study the molecular interactions involved in the formation of OPI and OPI-inulin gels, a frequency sweep analysis was conducted on gels compressed to 80% of its original height. Gels were prepared as previously described in the gel preparation section at pH 2.5, 5 and 7 and cut into approximately 1 cm (height) sections. The resulting gel disk were submerged for 48 h in solutions of 2-mercapthoethanol (2-ME) (0.2 M), urea (6 M) or sodium dodecyl sulfate (SDS) (1% w/v), which could disrupt disulfide bonds, hydrogen bonds and hydrophobic interactions, respectively.

4.2.7. Confocal laser scanning microscopy (CSLM)

CSLM was used to observe the distribution of inulin within the protein network. A laser scanning confocal microscope Zeiss LSM710 (Carl Zeiss Microscopy, Jena, Germany) was used with a 63× oil immersion objective. Inulin was labeled covalently with FITC. Specifically, 0.5 g of inulin and 10mL of DMSO were stirred overnight, and then 7mg of FITC were added to the inulin-DMSO mixture. The reaction mixture was protected from the light, heated at 90˚C for 2 hours and dialyzed extensively against distilled water in the dark and freeze-dried. Rhodamine B was used for non-covalent labeling of oat protein. A 15% protein suspension was first prepared, and then 40µL of Rhodamine B (5mg/mL) were added to 1 mL of protein suspension. The mixture was stirred for 2 h at room temperature, dialyzed against distilled water in the dark and freeze-dried. Once protein and inulin were labeled, OPI-inulin suspensions were prepared as described previously in the gel preparation section.

Samples were place into a concave microscope glass slide, covered with a lamella, sealed with nail polish and then heated for 15 min at 40, 60, 80, and 100˚C. After heating, samples were cooled in an ice bath and stored in the refrigerator overnight. Unheated sample measured at 25˚C was used as control. The fluorescent images were obtained simultaneously at wavelengths of 488 nm and 516 nm, and then analyzed with ZEN 2009 LE software (Carl Zeiss AG, Oberkochen, Germany).

4.2.8. Particle size measurements

A Zetasizer Nano ZS ZEN1600 system (Malvern Instruments, U.K.) was used to study the evolution of particle size distribution as a function of increasing temperature. For particle size measurements the OPI and OPI-inulin suspensions were heated at 40, 60, 80 and 100˚C for 30 min. Unheated sample measured at 25˚C was used as control. After heating, samples were immediately cooled in an ice bath to room temperature. Samples were then diluted to a total concentration of 0.1% (w/v) and passed through a 0.45 μm pore size filter prior to measurement. Number-based particle size distribution was measured to identify the total number of particles of a given size [261].

4.2.9. Fourier transform infrared (FTIR) spectroscopy

In order to observed changes in protein conformation during heating, the infrared spectra of OPI and OPI-inulin suspensions was recorded using a Nicolet 6700 spectrometer (Thermo Fisher Scientific Inc., MA, USA). OPI and OPI-inulin suspensions (5%, v/w) were dissolved in D_2O. To ensure complete H/D exchange, samples were prepared 48 h before infrared measurements. Suspensions were placed between two CaF_2 windows separated by a 25 μM polyethylene terephthalate spacer in a temperature controlled infrared transmission cell. Temperature was regulated by a Peltier controller (Thermo Fisher Scientific Inc., MA, USA).

Samples were heated from 20 to 80˚C, and the sample was equilibrated and the spectra were automatically recorded every 10˚C. Since the Peltier controller was not able to reach 100˚C, samples heated at 100˚C were prepared by the KBr-disk method. For such, the gels were prepared as previously described and freeze-dried. The dried gel was crush into powder, vacuum–dried at 40˚C overnight and mixed with KBr powder (1:100 w/w). Then the mixtures was compressed to 13 mm discs and used for spectroscopy measurements. To study the amide I region of the protein (1700-1600 cm^{-1}), Fourier self-deconvolutions were performed using the software provided with the spectrometer (Omnic 8.1.210 software). Each spectrum was the result of 128 scans; band narrowing was achieved with a full width at half maximum of 20-25 cm^{-1} and with resolution enhancement factor of 2.0-2.5 cm^{-1}. During measurements Nitrogen was continuously run through the spectrometer.

4.2.10. Statistical analysis

The results were evaluated by an analysis of variance (ANOVA) and significant differences, with minimum significance test set at the 5% level ($p < 0.05$) with Tukey's test to compare all means, using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). All experiments were performed at least in three independent trials and the results were reported as mean ± standard deviation.

4.3. Results and Discussion

4.3.1. Gel texture

The mechanical properties of the gels including compressive stress, springiness and gumminess are summarized in Table 5. Compressive stress indicates the capacity of the material to withstand a given deformation. For OPI alone, the compressive stress values of

the gels were 10.19 and 11.29 kPa at pH 5 and 7, respectively. Previous research suggested that a balance between electrostatic repulsive forces and hydrophobic attractive forces could be achieved at pH 5 and 7, thus allowed formation of gel networks [249]. Hydrogen and disulfide bonds also contributed to the protein network formation by balancing the electrostatic repulsive forces. Whereas, at pH 2.5 strong electrostatic repulsive forces among positively charge amino acid residues prevented attractive interaction development, leading to low compressive stress value (1.53 kPa). Disulfide bonds were unlikely to take place under acidic conditions [262] due to low reactivity of cysteine, which may partially explain formation of very weak networks.

At pH 7, addition of a small amount of inulin (0.25-0.5%) greatly increased the compressive stress of the gels, which equal to 1.67 - 3.33 % dry weight of protein. This effect is especially strong at the highest level of inulin addition (OPI-I 0.5%) as compressive stress values increased from 11.29 to 22.98 kPa. Since inulin is incapable of forming a gel on its own, under any of the concentration utilized in this study, the great improvement of the mechanical strength resulted from a synergistic effect of inulin and OPI. At pH 5 the addition of inulin produced a slight increment of the compressive stress values, however this change was not a significant improvement ($p<0.05$). Gels prepared at pH 5 with and without inulin were prone to syneresis because water release was observed both after heating treatment and mechanical property test. The compressive stress values reported for gels at pH 5 could be overestimated, since the exudation of water resulted in a higher solid content in the actual gel network. At pH 2.5, the addition of inulin did not produce a significant improvement ($p<0.05$) of the compressive stress value either, which ranged from 1.53 to 2.19 kPa. Earlier research showed that heating of inulin under acidic conditions caused intensive hydrolysis,

but little change occurred under neutral or alkaline conditions [263]. Thus the synergistic effect was not observed due to instability of inulin at acidic pH.

The gels prepared at pH 2.5 had the lowest springiness values among all samples, indicating these gels were more affected by the compressive force and took longer to recover. Gels prepared at pH 5 and 7 showed higher springiness values (193-228 mm). Gels prepared at pH 7 had the higher gumminess values than those obtained at pH 2.5 and 5, suggesting that these gels require more energy to be disintegrated. The addition of inulin led to significant increase in springiness and gumminess for gels formed at pH 7 (p<0.05) and the gumminess value in the presence of 0.5% inulin was 2.3 fold of the value observed for OPI gel alone. Thus, the addition of a small amount of inulin also provides the opportunity to tailor other properties of oat protein gels such as springiness and gumminess to meet different sensory requirements.

It is worthy of notice that heat induced OPI-inulin gels at inulin addition level of 0.5% and 100˚C exhibited comparable mechanical strength (22.98 kPa) to those of egg white (22-32 kPa) [264]. Future applications in collaboration with industry may promote the utilization of oat protein as a plant derived gelling ingredient in a wide range of food applications.

Table 5 Mechanical properties of OPI and OPI-inulin gels prepared at 100˚C

	pH 2.5		pH 5		pH 7	
Compressive stress (kPa)						
OPI	1.53 [a]	±0.27	10.19 [a]	±1.32	11.29 [a]	±3.49
OPI-I 0.1%	1.47 [a]	±0.11	9.63 [a]	±1.03	13.93 [a]	±1.95
OPI-I	2.07 [a]	±0.31	10.92 [a]	±2.12	14.41 [a]	±1.39
OPI-I 0.5%	2.19 [a]	±0.38	14.16 [a]	±2.85	22.98 [b]	±1.12
Cohesiveness						
OPI	0.41 [a]	±0.03	0.67 [a]	±0.01	0.55 [a]	±0.04
OPI-I 0.1%	0.47 [a]	±0.06	0.63 [a]	±0.07	0.56 [a]	±0.05
OPI-I	0.39 [a]	±0.04	0.70 [a]	±0.02	0.55 [a]	±0.06

OPI-I 0.5%	0.26 [b]	±0.03	0.68 [a]	±0.05	0.55 [a]	±0.08
Springiness (mm)						
OPI	130.20	±5.37	211.13	±7.74	163.05	±15.42
OPI-I 0.1%	159.83	±21.43	193.13	±6.14	228.21	±20.47
OPI-I	143.73	±21.12	208.16	±4.76	220.73	±9.85
OPI-I 0.5%	197.82	±17.65	208.88	±12.73	217.81	±17.46
Gumminess (N)						
OPI	0.08 [a]	±0.01	0.90 [ab]	±0.09	0.73 [a]	±0.12
OPI-I 0.1%	0.09 [a]	±0.02	0.80 [a]	±0.10	0.90 [a]	±0.14
OPI-I	0.10 [a]	±0.02	0.94 [ab]	±0.15	1.03 [a]	±0.10
OPI-I 0.5%	0.08 [a]	±0.01	1.16 [b]	±0.13	1.68 [b]	±0.18

Values are means ± standard deviation

4.3.2. Water-holding capacity (WHC)

WHC is a key property of gels and low values often result in dry products and low texture stability. As shown in Figure 7, WHC values ranging from 85.09 to 93.29% were recorded for all gels except for those prepared at pH 5 with values around 60%. This is in agreement with the syneresis and observation of water released after the compressive tests at pH 5. With increase of inulin, a slight increasing trend was observed for WHC value at pH 5. At pH 2.5 and 7 the addition of inulin did not significantly impact (p<0.05) the gel WHC.

The OPI and OPI-inulin gels exhibited higher or comparable WHC values to those of gels prepared with soy protein/loctus bean gum (>60%) [265], whey protein/cassava starch (>85%) [266] and egg white protein/konjac glucomannan (90.2%) [48].

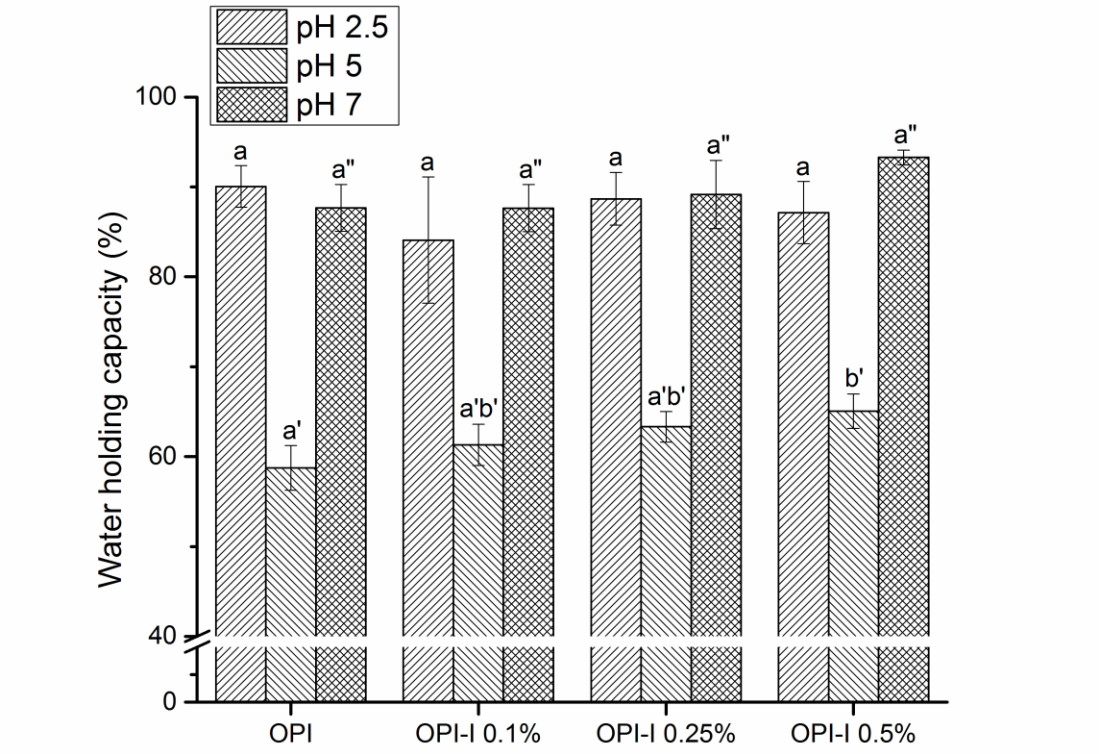

Figure 7 Water holding capacity of OPI and OPI-inulin gels prepared at 100˚C. Statistical analysis was performed separately for each pH, which was indicated by the use of primed letters (a, a' and a"). Values within the same pH not sharing a common superscript differ ($p<0.05$).

4.3.3. Scanning electron microscopy (SEM)

Globular proteins generally form either particulate or fine-stranded gels depending on the environmental pH and ionic strength [262]. At pH 5 (Figure 8) bundles of large spheroid aggregates are randomly distributed along the oat protein networks, forming a characteristic particulate gel. The low net charge of the protein produced minimal repulsion, thus protein molecules unsystematically aggregate to form particulate microstructure. This also favored the development of protein-protein interactions, whereas protein-water interactions were limited, leading to gels with low water holding capacity [267]. It is interesting to notice that oat protein gels (Figure 8) show percolating networks at pH 2.5 and 7, similar to that formed by

gelatin [268]. At pH 2.5 protein aggregates array in such fashion that hollow cells were formed between thin vertical walls. These cells were almost tubular in shape. The cell walls at this pH (Figure 9) seem to be thin and flaky and the addition of inulin did not apparently alter the structure. At pH 7, a similar structure to that formed at pH 2.5 was observed, but the cell walls were thicker and much more interconnected. The addition of 0.5% inulin at pH 7 resulted in highly cross-linked network with significantly increased junction zones. Such microstructure may explain strong oat protein-inulin gel mechanical properties comparable to those of animal proteins.

We realized this unique gel structure might have specific gelling applications in a variety of foods. This triggered our interest to further investigate the molecular mechanism by which oat protein forms such structures. Also, we wanted to know better how small amounts of inulin could greatly improve the gel properties at neutral pH, an environmental condition highly convenient for food applications. So we further investigated the gels with experiments using rheological measurements, Fourier transformed infrared spectroscopy and laser light scattering to study the molecular events taking place during the gel formation.

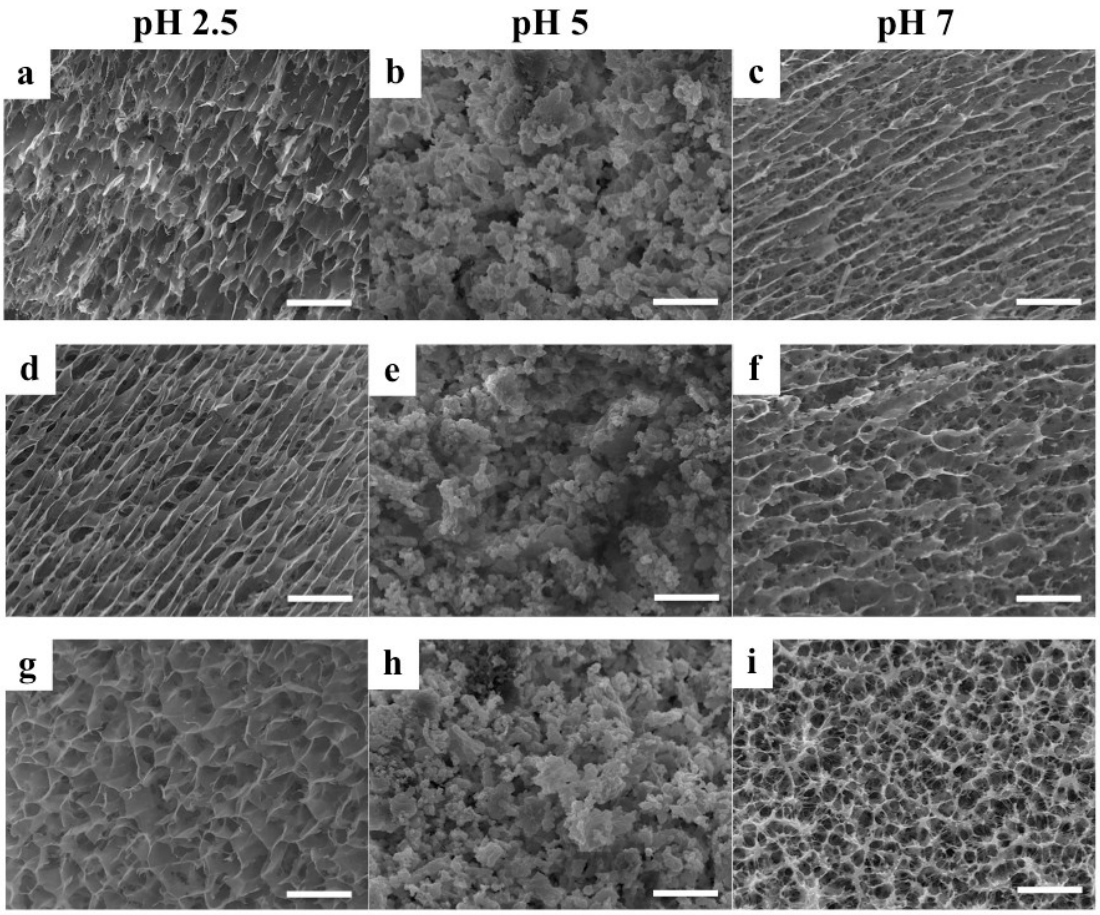

Figure 8 SEM images of the cross section of gels prepared with OPI (a,b and c), OPI-I 0.1% (d, e and f) and OPI-I 0.5% (g, h, and i) at different pH. Scale bar represents 20μm

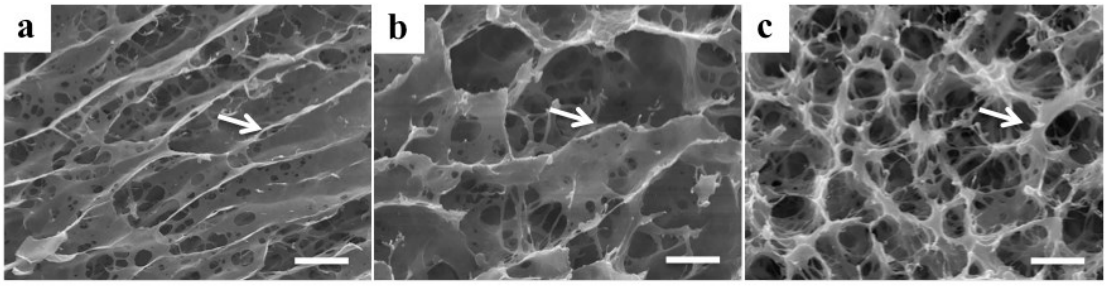

Figure 9 SEM image of the cross section of gels prepared with OPI (a) OPI-I 0.1% (b) and OPI-I 0.5% (c) at pH 7.Scale bar represents 5 μm. Arrows indicate cell walls

4.3.4. Rheological measurements

To study the development of the gel network as a function of heating, a temperature ramp was run for OPI and OPI-inulin suspensions with addition of 0.1 and 0.5 % inulin. Both G' and G" were examined upon heating and cooling, however only G' values are shown, as G" were always lower than G', even as a solution. This means that the elastic behavior dominated the system. A similar phenomenon was observed for egg white, which showed gel-like properties (G'>G'') over the entire temperature range probed and even at low temperature the native protein already forms a weak network that can propagate stress [269]. At pH 7 (Figure 10a) the G' value for OPI decreased at around 50˚C and a plateau was formed until the temperature reached ~75˚C. This decrement could be attributed to weakened hydrogen bonding [270] during heating. The sharp increase of G' from ~70 to 95˚C indicates that the formation of a rigid gel network occurred, as heat prompts protein unfolding, exposing reactive groups of the molecule that enable the molecular interactions such as hydrophobic interactions and disulfide bonds to reinforce the gel network [271]. It is clear, that part of the development of the gel network also took place during the cooling stage because G' further increased during the cooling stage. Addition of small amount of inulin resulted in great increase in G', further confirming the synergistic effect between oat protein and inulin. The addition of inulin also produced two phases in the development of the gel network. According to previous reports [251,270] the first stage (~50˚C) is related to the development of inulin network by molecular entanglement through hydrogen bonds and van der Waals forces [272], whereas the second stage (~70˚C) is related to the protein network development. This result suggests phase separation during gel formation to form two networks, one rich in

inulin and the other rich in protein. In addition, the development of the protein gel network started at a lower temperature (~70˚C) in the presence of inulin compared to OPI alone (~75˚C).

Much lower G' were observed at pH 2.5 (Figure 11). Addition of inulin resulted in significant increase of G' during heating, but the final G' after cooling only slightly enhanced. This is in accordance with the results obtained from gel texture measurements. A decrease in G' value was also observed for OPI at 45-60˚C, but the plateau was much narrower. This suggests that less hydrogen bonds were present in oat protein at pH 2.5 probably caused by partial protein unfolding and/or dissociation under acidic environment. The two-phase network development was not well defined as at pH 7. As discussed earlier, at this acidic pH the stability of inulin is low. The heat treatment could further break down inulin into shorter chains at such pH [263]. Thus the strengthening effect of inulin was limited. At pH 5 (Figure 12), a slight increase in G' was observed during the heating and cooling stages. The inclusion of inulin did not produce a significant improvement in the final G' values (113.13 - 200.57 Pa). These values were much lower than the corresponding values of gels prepared at pH 2.5 and 7. Thus it is confirmed that the high compressive stress values reported for gels at pH 5 were overestimated as the exudation of water produced a higher solid content in the actual gel network.

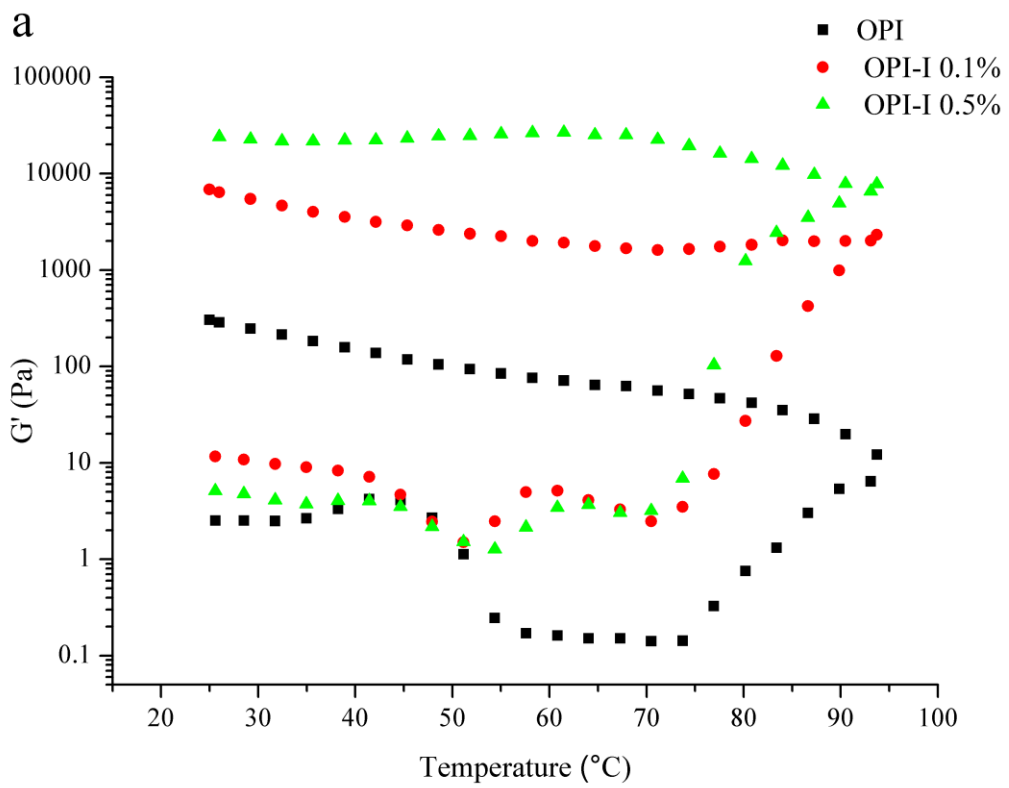

Figure 10 Storage modulus (G') of OPI and OPI-inulin mixtures (0.1 and 0.5%), during heat-induced gelation at pH 7

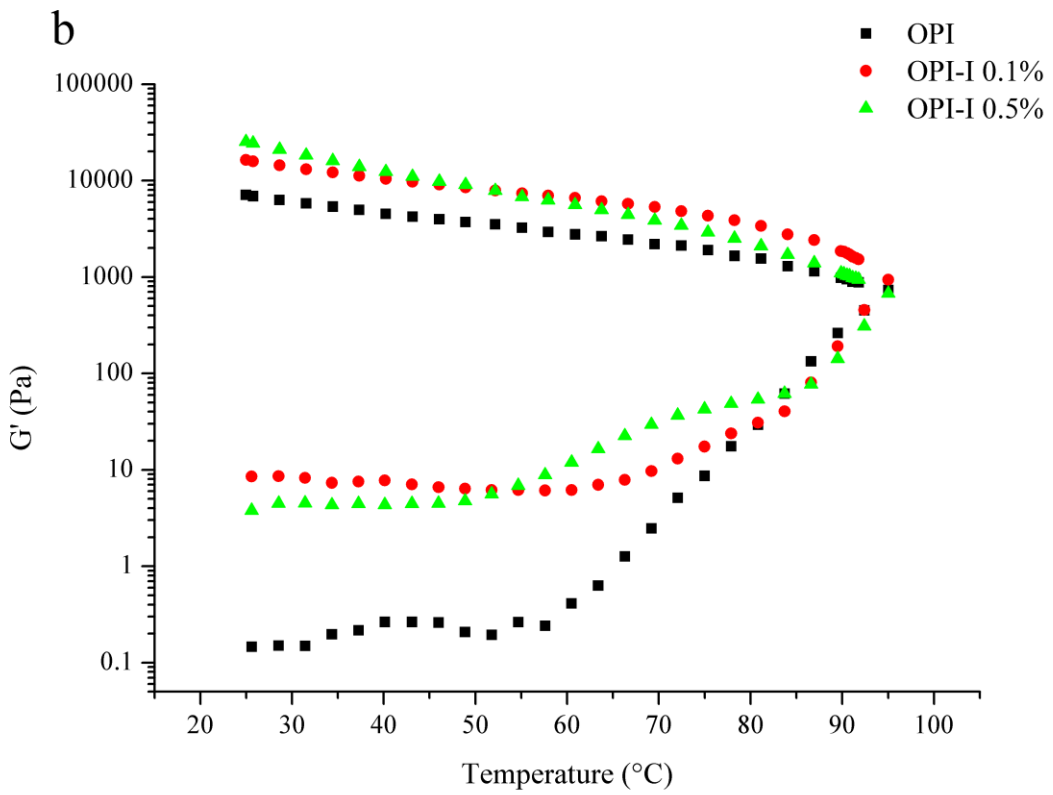

Figure 11 Storage modulus (G') of OPI and OPI-inulin mixtures (o.1 and 0.5%), during heat-induced gelation at pH 2.5

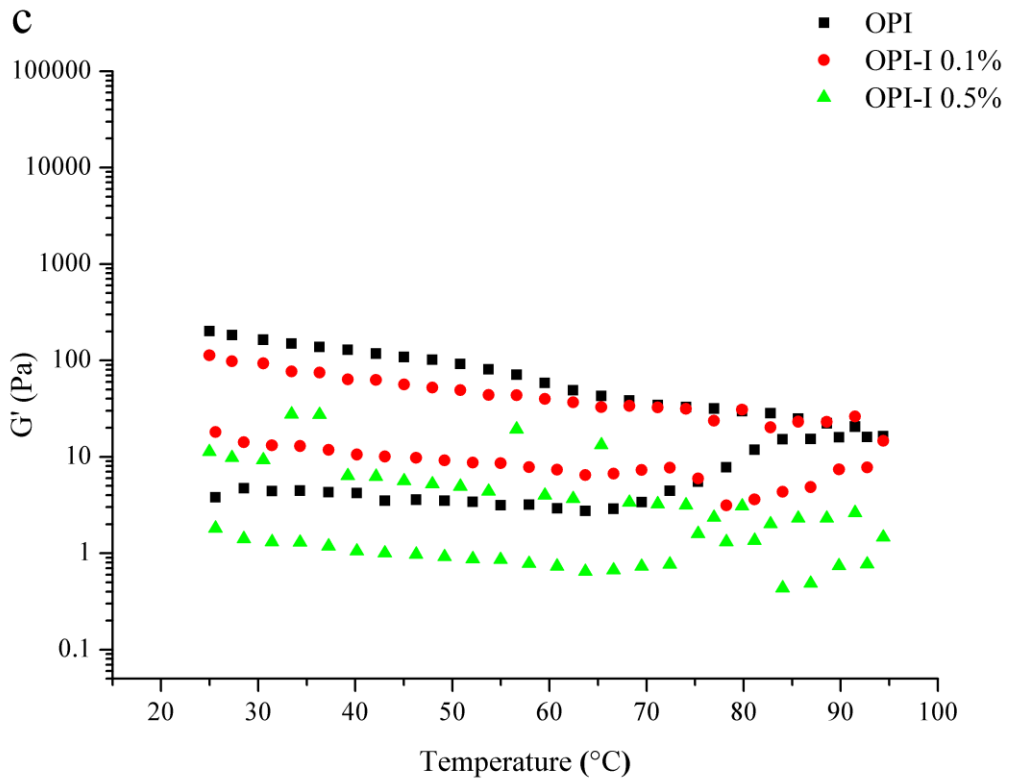

Figure 12 Storage modulus (G') of OPI and OPI-inulin mixtures (0.1 and 0.5%), during heat-induced gelation at pH 5

To determine the type of interactions involved in the gel network formation, a frequency sweep test was conducted on gels treated with different dissociating reagents including urea to interrupt hydrogen bonding, 2-ME to dissociate disulfide bonds and SDS to destroy

hydrophobic interactions. Figure 13 and 14 shows how G' increased with increasing frequency. Physical gels or gels with non-covalent linkages have been described as gels with some frequency dependence [273]. A chemical gel, composed purely of covalent linkages, would show a flat line indicating that neither low nor high frequency produces any alteration in the bonds of the structure [274]. The behavior shown by OPI-Inulin gels indicates that G' is only affected at higher frequency, suggesting that the structure goes through a relaxation process loosening the structure [275].

The test on gels prepared at pH 2.5 was not performed as these gels swelled severely in the presence of dissociating reagents, then ruptured, which did not permit an appropriate measurement. This observation also confirms that the strength of the interactions formed at pH 2.5 was inferior in comparison to those present in gels formed at pH 5 and 7. OPI gels prepared at pH 7 were strongly affected by 2-ME, as the integrity of the gels was lost. This indicates that disulfide bonds performed an essential role for gel formation. Gels in contact with urea and SDS, showed similar response and in both cases G' values were reduced significantly, suggesting that hydrogen bonds and hydrophobic interactions also contributed to the development of three-dimensional networks. In the case of OPI-I 0.5%, gels were affected by 2-ME in the same way as the gels prepared with OPI alone. Thus, disulfide bonds played an important role in the oat protein-inulin gel formation as well. In the presence of urea and SDS, the gel structure was affected to a greater extent as a significant drop of G' was observed. This indicates that addition of inulin strengthened hydrogen bonding and hydrophobic interactions to further improve the gel network structure and mechanical properties. At pH 5 there was not a significant contribution of disulfide bonds in gel formation since the gel submerged in 2-ME showed similar trend compared to the control

sample. Samples submerged in urea and SDS had lower G' values. This suggests that hydrogen bonds and hydrophobic interactions played major role in the maintenance of the gel structure. In the presence of inulin at pH 5, the gels in SDS also exhibited lower G' value, but in urea the gel was less affected as the G' value reduced to a lower extent. Therefore, both hydrogen bonds and hydrophobic interactions are the ruling forces in the establishment of the OPI-inulin gel structure at pH 5. The disulfide bonds were not developed due to the compact structure of the protein near its isoelectric point, where the sulfur hydroxyl groups were hidden inside the protein structure. The main linkages or types of bonds involved in the OPI-Inulin gel formation are hydrogen bonds and hydrophobic forces, which are non-covalent bonds. Even though the involvement of disulfide bonds at pH 7 had an important contribution the behavior shown corresponds to that of a physical gel.

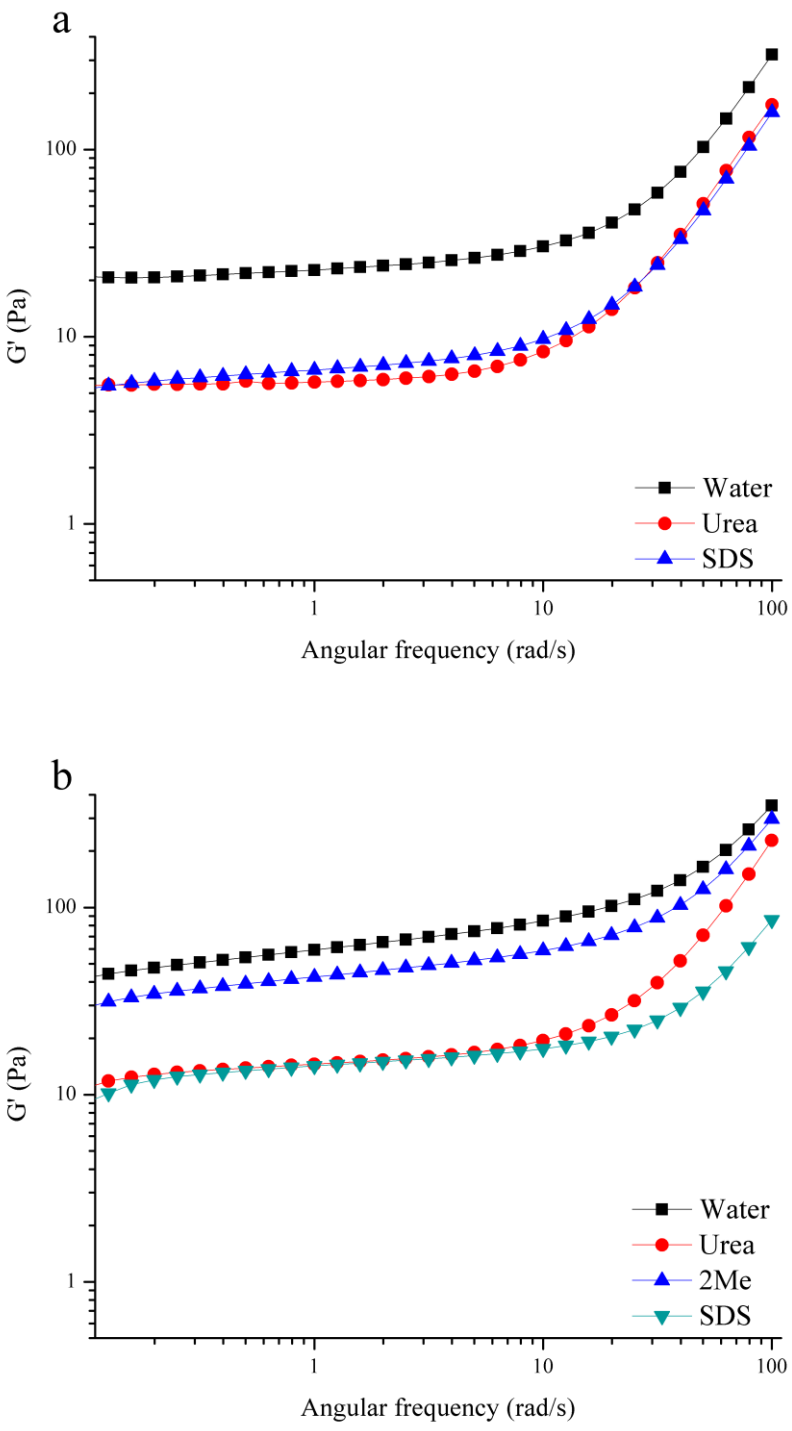

Figure 13 Storage modulus (G') of OPI at pH 7 (a), pH 5 (b) as a function of angular frequency

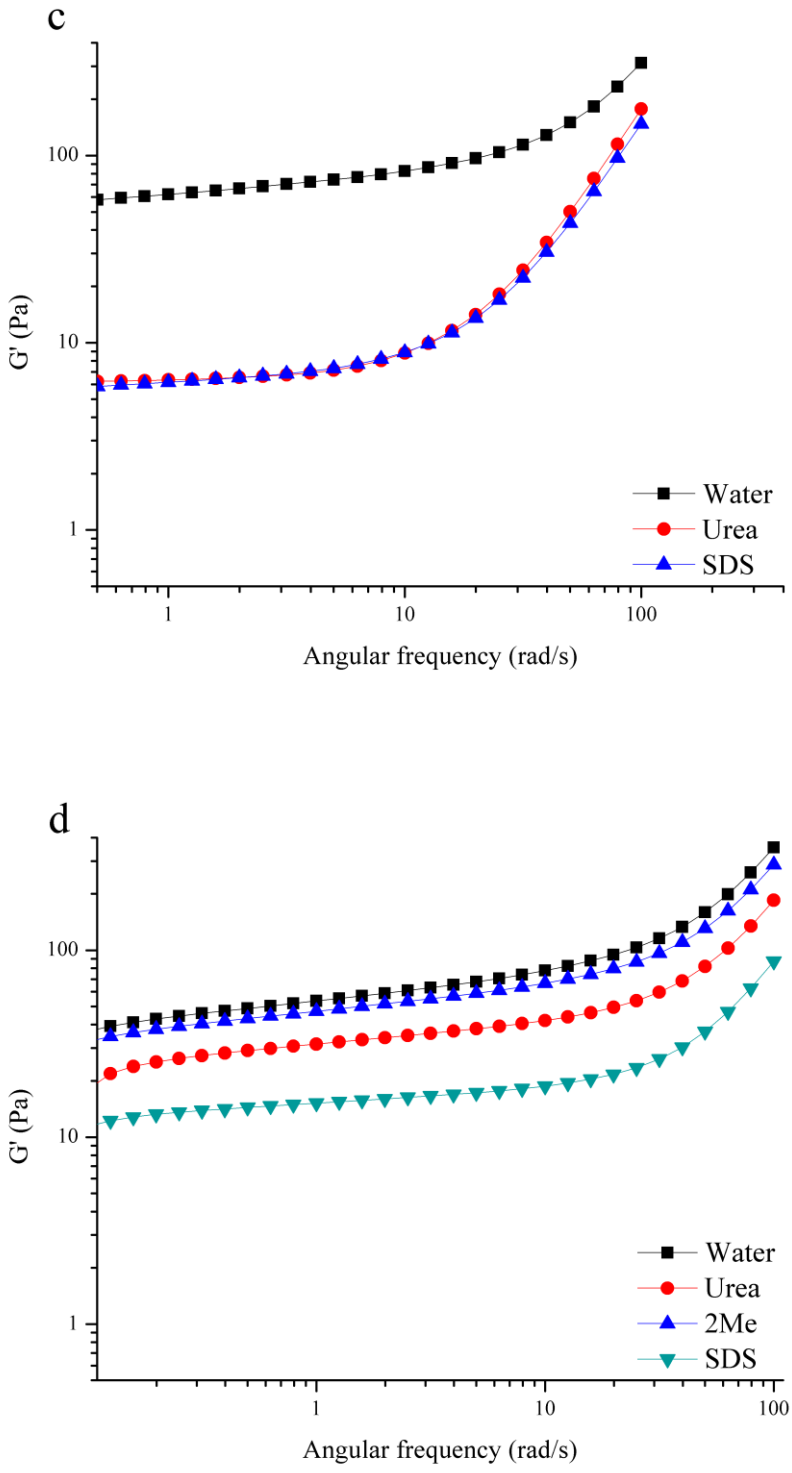

Figure 14 Storage modulus (G') of OPI-I 0.5% at pH 7 (c) and pH 5 (d) as a function of angular frequency

4.3.5. Confocal laser scanning microscopy (CSLM)

The microstructure of OPI and OPI-inulin gels was also observed with CSLM. Red color in the photos corresponds to protein, whereas bright green regions indicate inulin. As shown in Figure 15, OPI-inulin dispersions were homogeneous at pH 7 and room temperature. As temperature increased the protein aggregates grew gradually, and around 60˚C phase separation began. This suggests increased thermodynamic incompatibility of the oat protein and inulin, probably due to the excluded volume effect when the protein started to unfold at an elevated temperature [124]. From this temperature onward it was possible to see two phases, in which the protein network formed a continuous phase with entrapped solid inulin particles ranging in size from several hundreds nanometers to ~1μm. At higher inulin amount (0.5%), early signs of phase separation were noted. The development of the protein network in the presence of inulin was similar to that of protein alone, indicating that inulin did not interfere with the development of the protein network; nonetheless it did reinforce the structure.

At pH 2.5 (Figure 16), much less inulin particles were observed through the heating stage as well as in the final gels due to poor stability of inulin during heating under acidic conditions [263]. Only a few reinforcement sections or junction zones were formed, explaining the limited improvement observed in the reported compressive stress values. In the case of the gels prepared at pH 5, larger aggregates with larger void spaces were formed (Figure 17). No phase separation was seen, not even with increasing temperature. Apparently a protein network composed of compact aggregates was formed and inulin was covered inside those aggregates. Guggisberg et al. (2009) [259] evaluated the effect of inulin addition as a fat replacer in yogurt and their CLSM images suggested that an inulin system could be built in the protein network, as inulin was not visible by CSLM. The larger void spaces indicates

large pores, which led to the release of water from the network, a phenomenon consistent with the low water holding capacity of the gels prepared at this pH.

Gels prepared at pH 7 showed promising application potential due to their excellent mechanical strength and very unique structure. Samples prepared at this pH were selected to further investigate the effect of inulin addition in the gel formation mechanism.

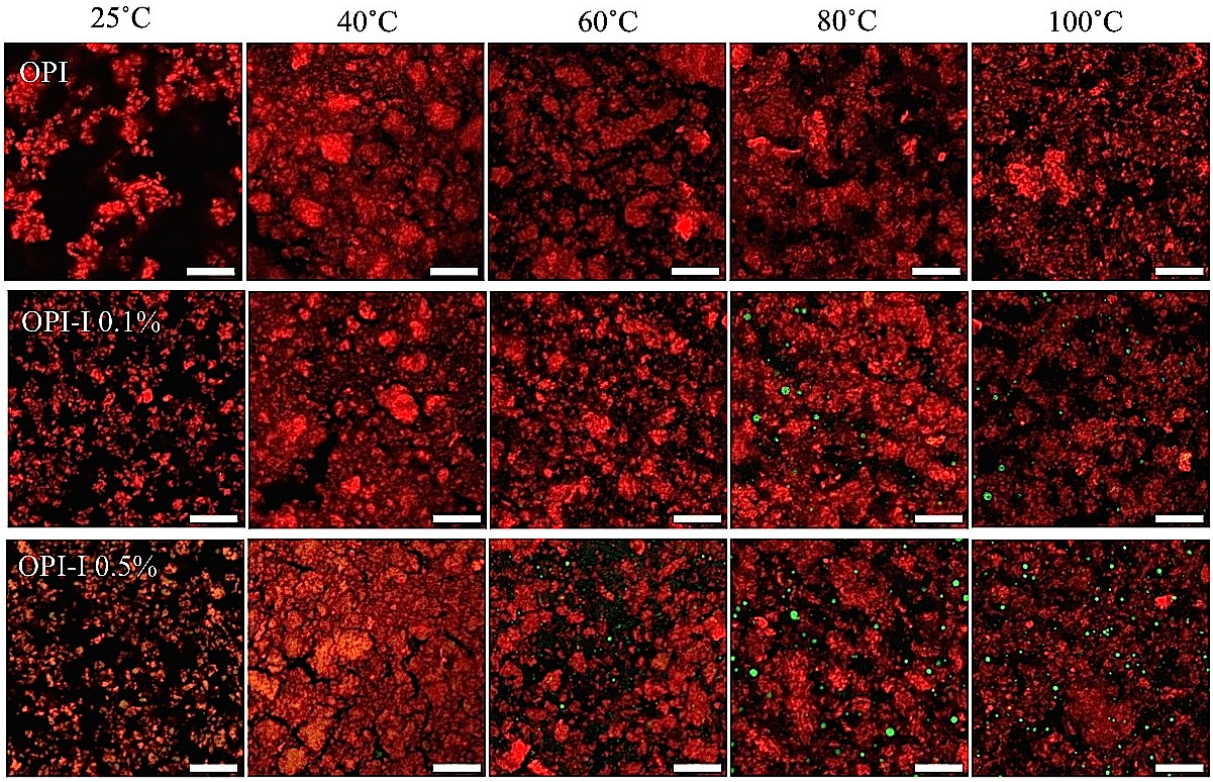

Figure 15 Confocal microscopic photographs of OPI and OPI-inulin (0.1 and 0.5%) at pH 7 as a function of temperature. Scale bar represents 10 μm

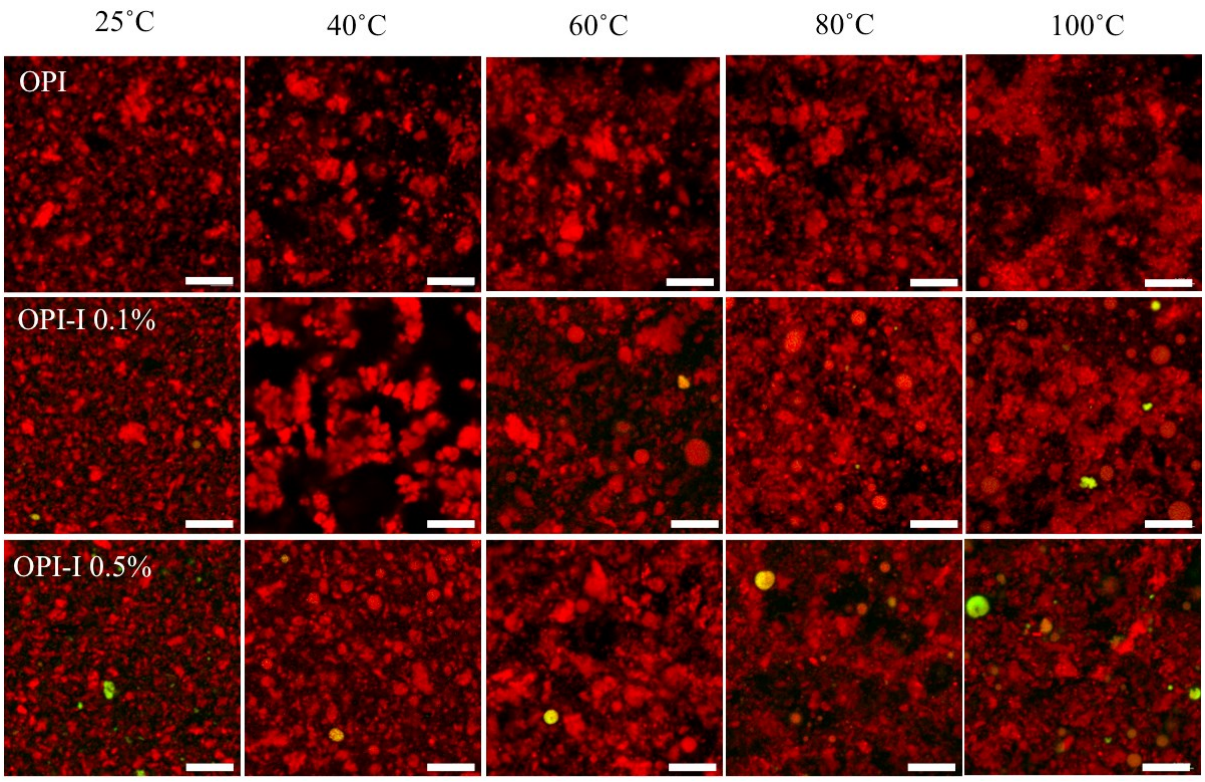

Figure 16 Confocal microscopic photographs of OPI and OPI-inulin (0.1 and 0.5%) at pH 2.5 as a function of temperature. Scale bar represents 10 μm

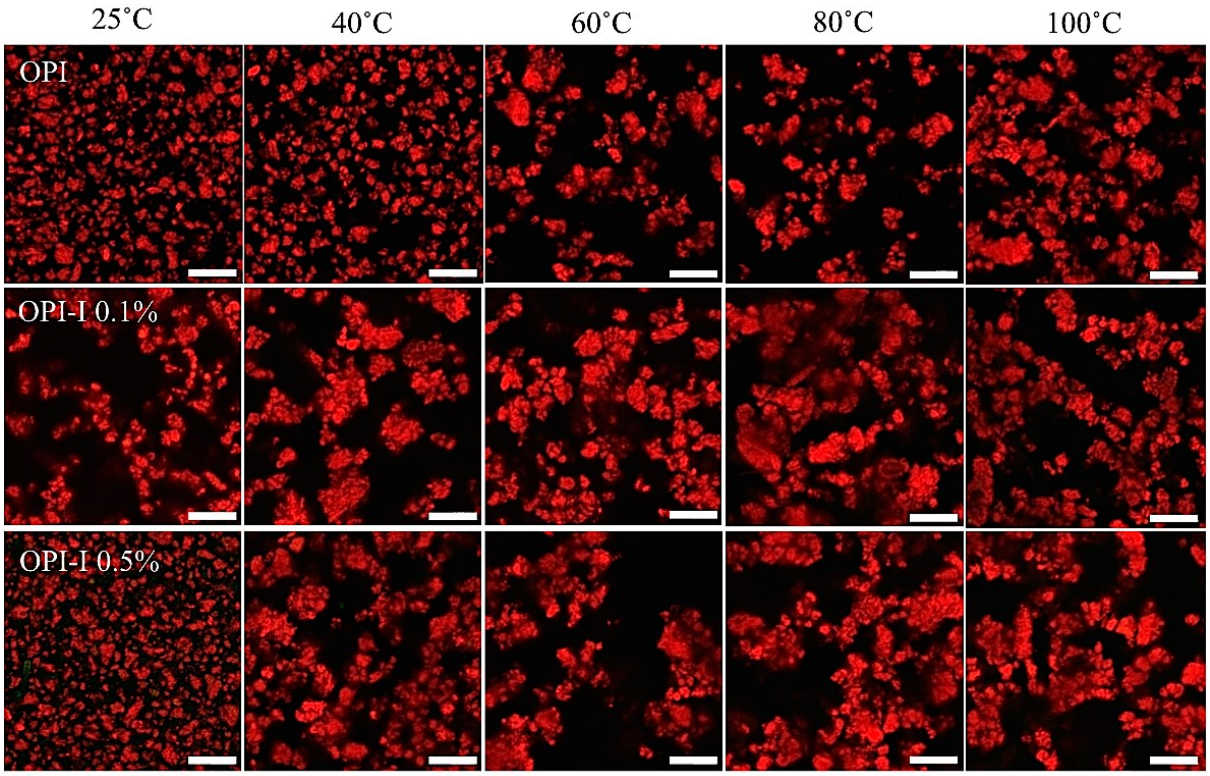

Figure 17 Confocal microscopic photographs of OPI and OPI-inulin (0.1 and 0.5%) at pH 5 as a function of temperature. Scale bar represents 10 μm

4.3.6. Protein size distribution

The change in the size distribution of protein molecules was studied as a function of heating temperature. As shown in Figure 18 for OPI, Figure 19 for OPI-I 0.1% and Figure 20 for OPI-I 0.5%, progressive reduction of the particle size occurred during heating with the same trend for all samples, regardless of the inulin level added. At 100˚C the peak value (diameter) detected for OPI was 10.1 nm, and 8.72 nm and 11.7 nm for OPI-I 0.1% and OPI-I 0.5% respectively. According to previous literature, a diameter value of 11.8 nm was estimated for oat globulin monomers with an extended conformation [276]. Thus the recorded peak values in the current study suggest dissociation of oat protein hexamers down to monomers. The thermal aggregation of oat globulin in previously study suggested oat globulin hexamers dissociated first into trimmers and then into monomers that are highly reactive. These then rapidly associate to larger and more stable molecules [276]. It is likely that the monomers formed after the heating treatment, serve as building blocks in the establishment of the three-dimensional network.

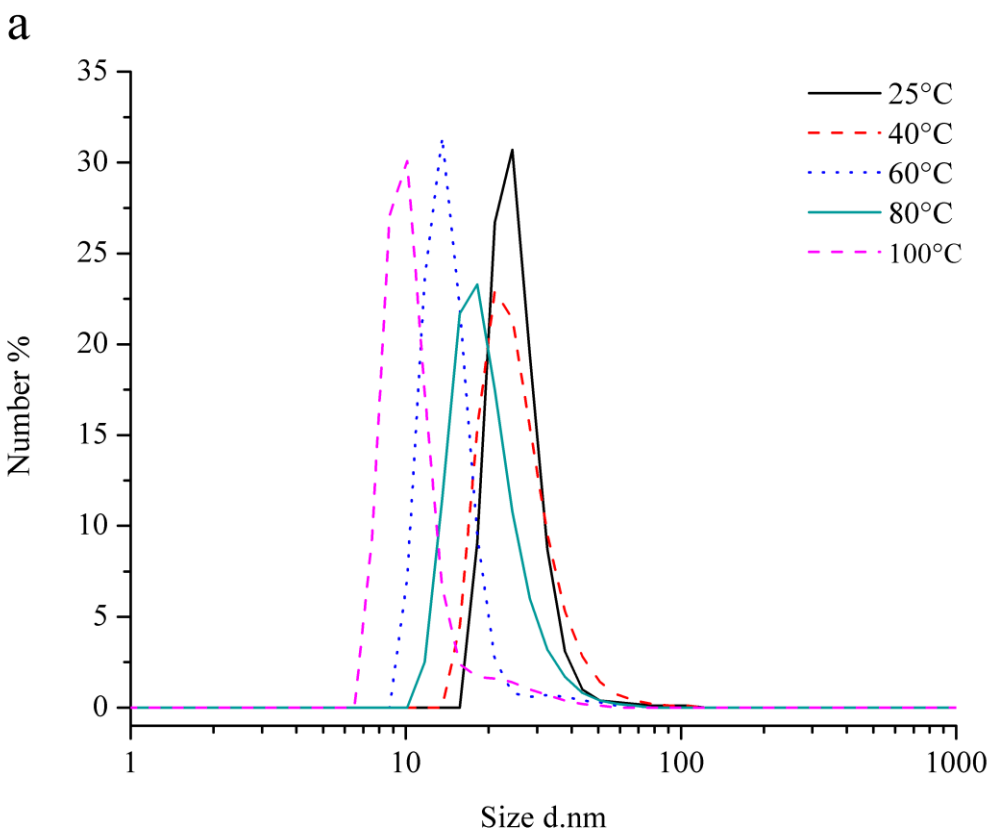

Figure 18 Number based particle size distribution of (a) OPI as a function of heating temperature at pH 7

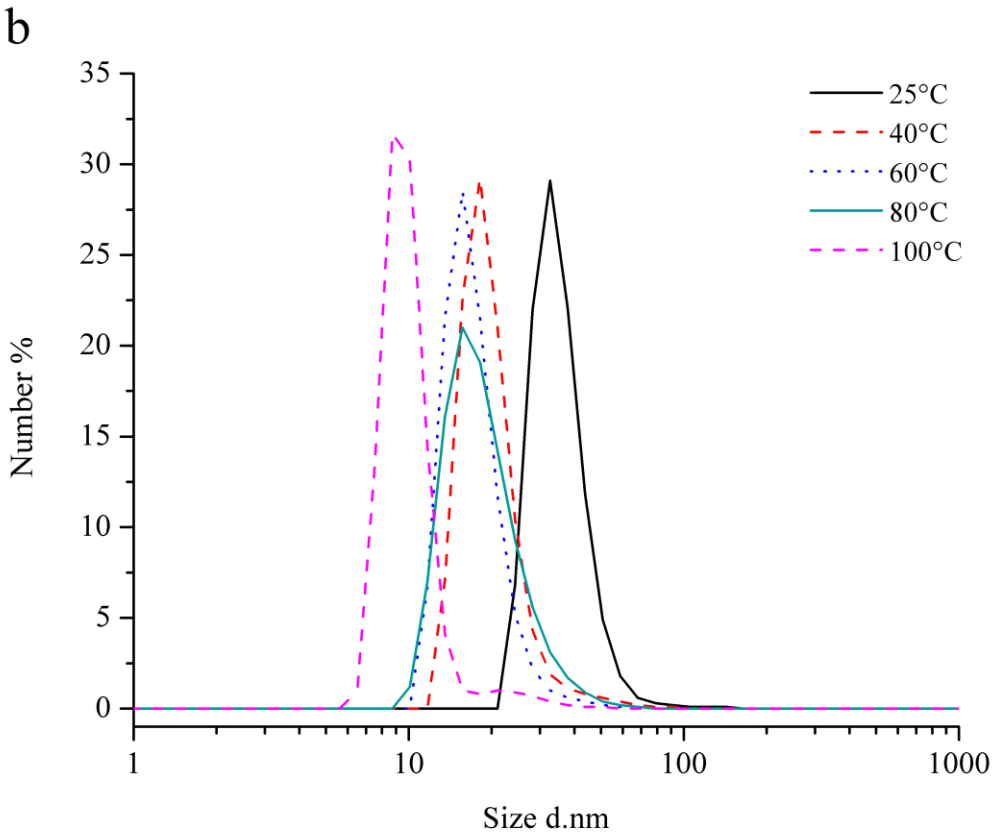

Figure 19 Number based particle size distribution of (b) OPI-I 0.1% as a function of heating temperature at pH 7

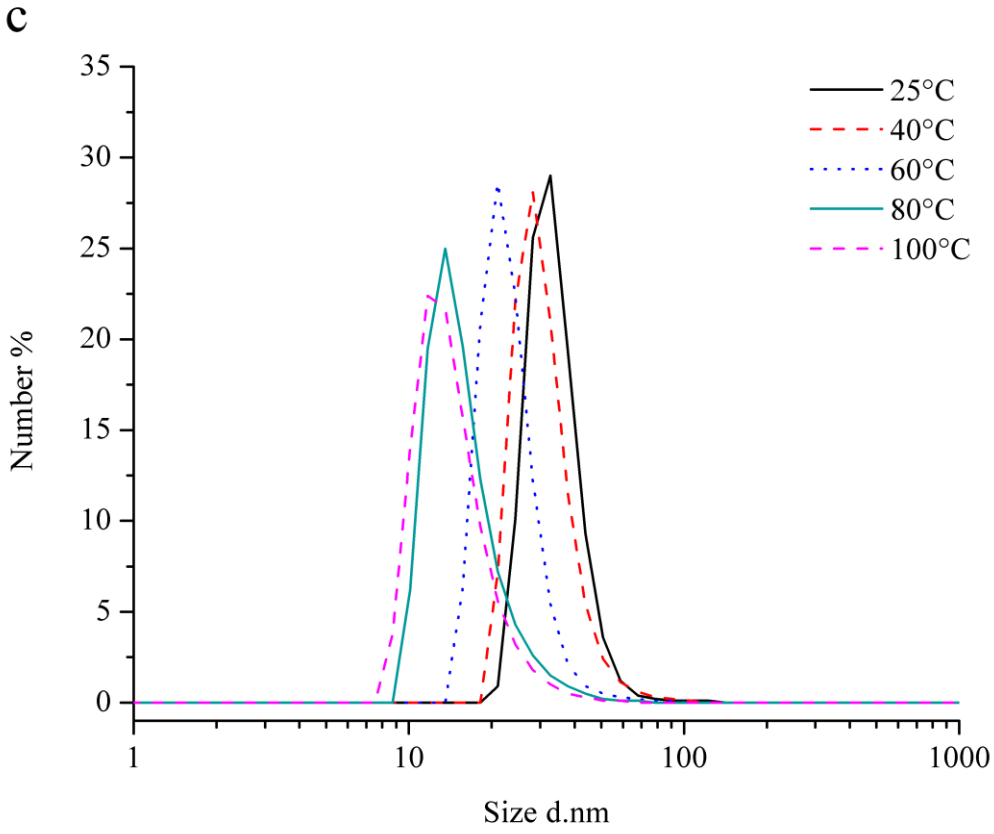

Figure 20 Number based particle size distribution of (c) OPI-I 0.5% as a function of heating temperature at pH 7

4.3.7. Fourier transform infrared (FTIR) spectroscopy

Figures 21, 22 and 23 show the de-convoluted spectra of OPI and OPI-I 0.1% and OPI-I 0.5% respectively, at different temperatures when pD was set at 7. For OPI, the amide I band at 25˚C showed five clear components, including β-turn (1670 and 1658 cm^{-1}), α-helix (1649 cm^{-1}), random coil (1640 cm^{-1}), β-sheet (1630 cm^{-1}) and vibration of amino acid residues (1609 cm^{-1}) [27,277]. This is in agreement with a previous report that α-helix and random coils are the major secondary structures in oat globulin, followed by β-sheet and β-turn [277]. A small peak was also observed at 1618 cm^{-1}, which was assigned to intermolecular β-sheet and is believed to be related to protein aggregation via the exposed reactive groups. As temperature increased from 25 to 80˚C, no major changes in the secondary structure were detected; this could be related to the high heat stability of oat protein with a denaturation temperature of 112.4˚C as revealed by differential scanning calorimetry in our previous work [249]. The absorption at 1690 (β-sheet), 1660 (β -turns) and 1619 cm^{-1} (intermolecular β-sheet) increased gradually with temperature, suggesting more aggregates were formed via exposed reactive sites. The amide I band was significantly altered when the OPI was heated to 100˚C. In order to test whether the aggregation caused by the dry process impacted the protein secondary structure, the dried gel powder formed at temperatures below 100˚C were tested. The results indicated that the dried gels formed at these conditions had similar peaks with the heated suspensions. Thus the protein secondary changes can be attributed to heating at 100˚C. The absorption at 1619 cm^{-1} disappeared, whereas a peak appeared at 1627 cm^{-1}. A similar transition has been related to the dissociation of the dimeric form of β–lactoglobulin into monomers [271]. This transition agrees with previous particle size results in which a progressive reduction of the mean particle size was observed as function of temperature,

confirming the dissociation of oligomers down to the monomeric form. Increased absorption intensity was observed at 1694, 1683, 1671 and 1659 cm^{-1} and two peaks appeared at 1649 and 1638 cm^{-1}. Such changes suggest partial protein unfolding during heating at 100˚C followed by re-organization of protein secondary structure during gel formation [278]. Heating of OPI-I 0.1% and 0.5% from 80 to 100˚C showed similar spectra as OPI alone. This further supports the conclusion that addition of inulin did not significantly interfere or alter the protein network construction.

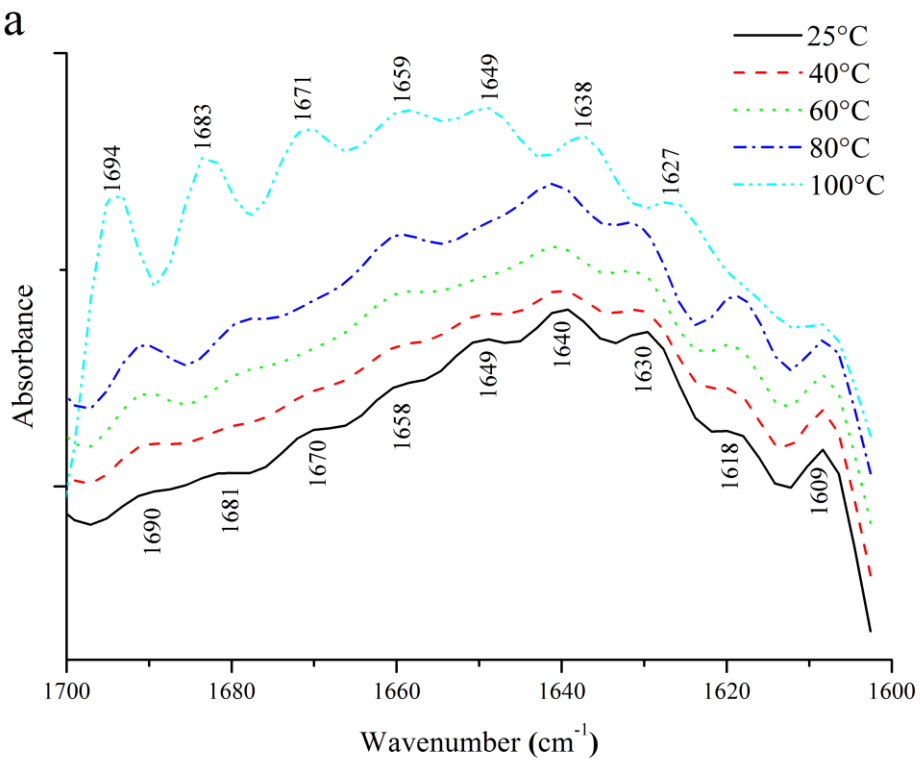

Figure 21 Deconvoluted spectra of (a) OPI at pD 7, as a function of temperature

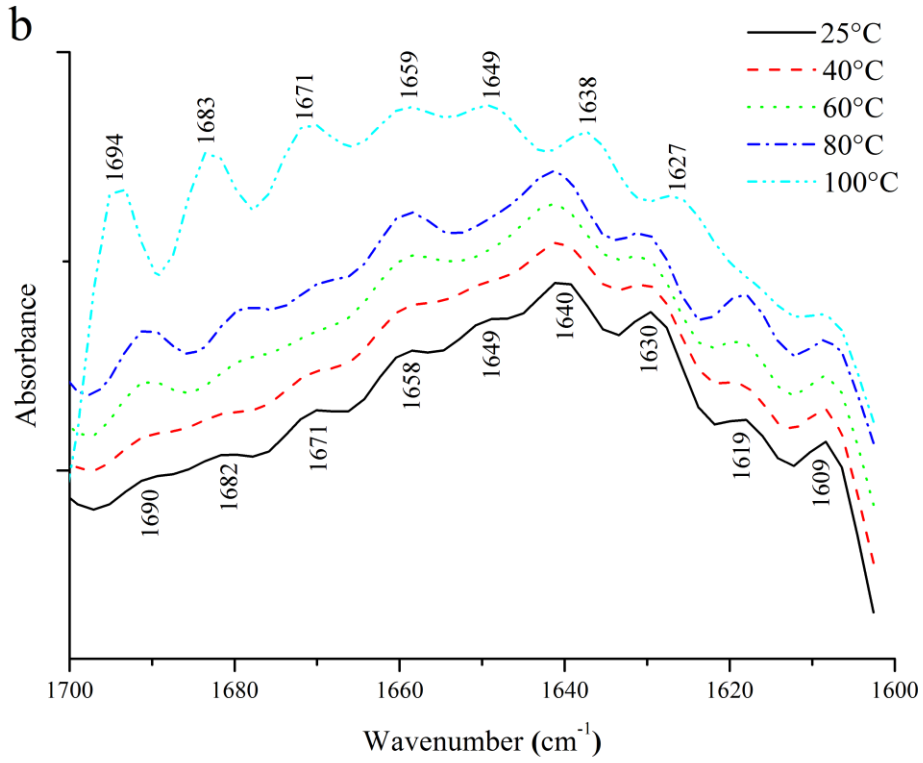

Figure 22 Deconvoluted spectra of (b) OPI-I 0.1% at pD 7, as a function of temperature

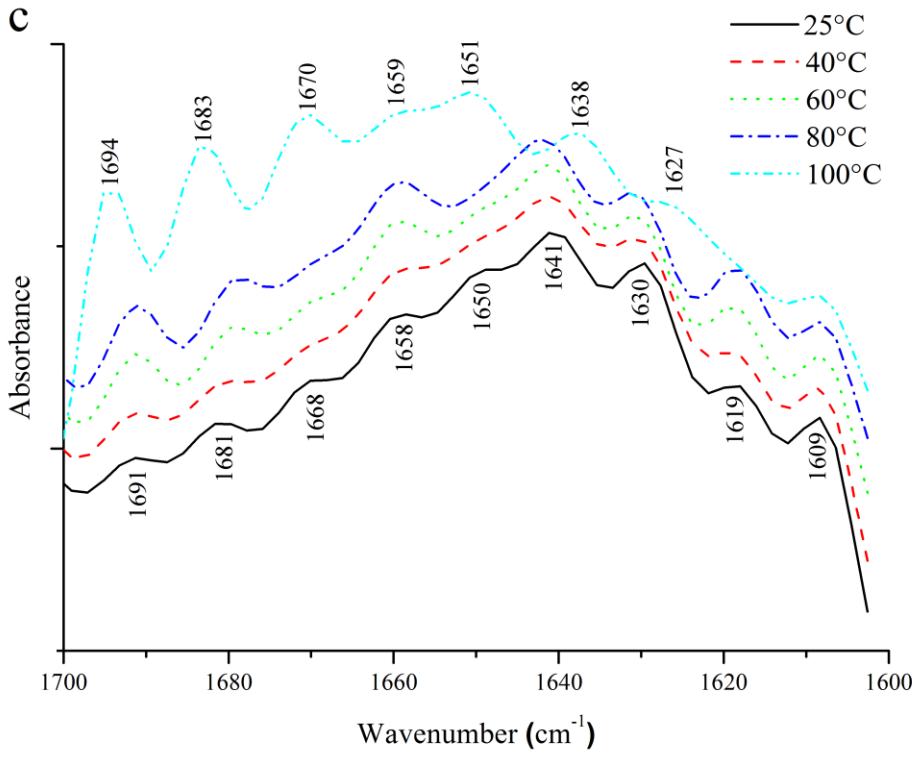

Figure 23 Deconvoluted spectra of (c) OPI-I 0.5% at pD 7, as a function of temperature.

4.3.8. Proposed formation mechanism of OPI-inulin gels at neutral pH

Based on fractal theory, protein particles form a fractal structure, ultimately leading to a gel network built of fractal clusters. Three factors have been found relevant to the type of structure formed: 1) the effective size of the building block of the fractal structure, 2) the amount of protein incorporated in the fractal clusters at the moment of the gel is formed, and 3) the way in which the fractal clusters are linked together [33]. For globular proteins in general (e.g. whey and soy protein), the formation of heat-induced gels involves unfolding of the protein molecules by heating, leading to the exposure of active amino acid residues. This is then followed by protein aggregation and dissociation of these aggregates to form either filament or particulate gels depending on pH and ionic strength [27]. The unique percolating network structure formed by oat protein gels at neutral pH is likely to be associated with monomers as the predominant basic building blocks of the gel network. These smaller units are highly reactive, which would allow the development of the protein gel network at a near molecular level, resulting in development of strong interactions in oat protein gels with high mechanical strength [276]. In addition, at pH 7, heat caused the dissociation of oat globulin hexamers by breaking disulfide bonds linking the acidic and basic subunits. This allowed the re-formation of disulfide bonds during the heating process. This disulfide bonds played major role in gel formation, strengthening gel networks and their mechanical strength.

Addition of inulin led to formation of phase separated gels during heating processing. This was expected, as it is highly unlikely for the OPI-inulin dispersions to establish electrostatic interactions; the protein has a negative charge at pH 7, whereas inulin has no charge. In this case protein-protein or inulin-inulin interactions require less energy than protein-inulin interactions, thus the system separated into a protein-rich and an inulin-rich phase. In fact,

each polymer is excluded from a volume occupied by the other polymer; hence the effective concentration of both polymers is increased [120]. In such way, higher intermolecular interactions were developed in the oat protein network. Furthermore, the greatly improved gel properties attributed to the addition of inulin in small amount can be explained by the strengthening effect of inulin nanoparticles homogeneously distributed inside the protein network. According to the rheological test, addition of inulin increased hydrogen bonding and hydrophobic interactions to further improve the protein gels. Hydrophobic interactions between inulin and other proteins such as casein [279] and β–lactoglobulin [280] have been previously reported because inulin is able to form α-helix in solution [256], which contains a hydrophobic center. Also inulin is rich in hydroxyl groups that are able to take part in supra-molecular interactions, in particular hydrogen bonding [281]. Thus, additional hydrogen bonds and hydrophobic interactions can develop in the border between the continuous network and discontinuous phase that work as a junction zones and provide extra support to the structure.

4.4. Conclusion

This work is the first to reveal unique percolating network structure for heat-induced oat protein gels. Unlike many other globular proteins, which aggregate during heating, oat protein dissociated from hexamers down to monomers. These small units are highly reactive and allowed protein gel network development at a near molecular level. Addition of small amount of inulin dramatically improved gel strength. In addition to the excluded volume effect due to phase separation in the system, the inulin nano-particles contributed to the formation of junction zones in which hydrogen bonds and hydrophobic forces were built at the phase borders, leading to a highly crosslink gel network with a reinforced structure.

Protein and polysaccharides are often used in combination in the development of food products. This research is critically important for future progress in the development of food products including OPI-inulin mixtures. Novel products are now designed based on the understanding of a growing consumer preference for natural and healthy foods, and oat protein-derived ingredients show excellent potential to be well adopted by consumers. The acceptance and utilization of oat and oat-inulin mixtures as value-added ingredients to produce food products of different textures, could potentially contribute to the growth of the food and agricultural industry. Also this research demonstrates the potential of oat protein as an appropriate food gelling agent since the strongest gels were formed at neutral conditions; in previous reports this was only achieved at alkaline conditions.

Chapter 5

5. Effect of different polysaccharides on phase separation during thermal gelation of oat protein.

5.1. Introduction

Plant-based proteins are gaining importance as more consumers are changing their dietary habits by incorporating more plant-based protein in their diets and in some cases, even full replacement of animal protein sources by plant based sources is preferred. Currently soy and wheat proteins dominate the plant protein market. Hence, as the plant-protein ingredient sector grows, there is opportunity for novel protein sources to meet the increasing market requirement for different functionalities and sensory attributes, such as oat. Our previous work revealed that oat protein could form polymer like gels with percolating networks, which may have specific gelling application in a variety of foods. Blends of proteins and polysaccharides are applied to control or improve sensorial attributes in food products by providing texture and water retention. Previous studies have demonstrated that gels with different microstructure can be produced when proteins are mixed with polysaccharides. Coacervate networks are one continuous network formed by favorable attractive interactions between proteins and polysaccharides. Phase separated networks are formed when interactions between protein and polysaccharide are repulsive resulting in a bi-continuous phase. Interpenetrating networks are formed when no interactions are established between polymers and each polymer forms an independent network [129]. The charge density of the polysaccharide and environmental conditions such as pH and ionic strength have direct

influence on the gel structure and in consequence in the gelling properties [282]. By modulating protein-polysaccharide interactions, a synergistic effect can be achieved in the binary gels with improved properties. To date, most of the research has focus on the improvement of gelling properties of dairy proteins in mixture with polysaccharides [134,283,284] and the research efforts on plant protein based binary gels have included soy [264,285], pea [286] and canola [287] protein. Yet the knowledge acquire is still limited.

The objective of this work is to systematically study how the presence of polysaccharide and salt may impact the gelling properties and the microstructures of oat protein gels. Three polysaccharides with different charges were selected including dextrin, carrageenan and chitosan. Dextrin is a low molecular weight carbohydrate (~3 kDa) produced by partial acid hydrolysis of starch, thus exhibiting the α-(1→4)-Glc structure of amylose and the α-(1→4)- and α-(1→4,6)-Glc branched structure of amylopectin, but with lower polymerization [288,289]. Carrageenan is a gel-forming polysaccharide derived from a number of seaweeds, it is a sulfated polygalactan with 15 to 40% ester-sulfate content, which makes it and anionic polysaccharide. It has an average relative molecular weight well above 100 kDa. It is formed by alternate units of D-galactose and 3,6-anhydro-galactose (3,6-AG) joined by α-1,3 and β-1,4-glycosidic linkage [290]. Chitosan is a linear-cationic polysaccharide derived from partial N-deacetylation of chitin and is normally obtained from crustacean shells with the structure α(1→4)-linked 2-amino-2deoxy-β-D- glucan [291].

The impact of polysaccharide addition on gel microstructures, bulk properties including mechanical strength and water holding capacity was evaluated at different pH and salt concentrations. The oat protein gel formation mechanism in the presence of polysaccharide was discussed with especial emphasis on phase-separated networks.

5.2. Materials and methods

5.2.1. Materials

Naked oat grains (*Avena nuda*) were purchased from Wedge Farms Ltd., Manitoba, Canada. The protein content was 16.6% ± 0.64 as determined by Leco nitrogen analyzer (FP-428, Leco Corporation, St Joseph, MI) using a protein calculation factor of 6.25. Oat protein isolate (OP) was extracted according to our previous work [249] and the protein content was determined to be 89.57 % ± 0.73 using the same Leco nitrogen analyzer. Urea, 2-mercaptoethanol, sodium dodecyl sulfate, Rhodamine B, carrageenan and chitosan from shrimp shells were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). Food grade dextrin Capsul 2730 was obtained from Ingredion Canada Inc. (Mississagua, ON, Canada)

5.2.2. Gel preparation

Dextrin, chitosan and carrageenan were selected in this study, representing a neutral, cationic and anionic polysaccharide respectively. Gels were prepared by heating the protein-polysaccharide suspensions at pH 3, 4 and 7 adjusted with 0.1 N NaOH or HCl, in the presence of 0, 50, 150 and 300 mM NaCl. The concentration of OP in the mixtures was kept constant at 15% (w/v) and the concentration of polysaccharide was 0.5% (w/v). These values were determined based on preliminary trials, which allowed gel formation. Samples were labeled as OP, OP-DE, OP-CH and OP-CA, representing gels containing oat protein, oat protein-dextrin, oat protein-chitosan and oat protein–carrageenan respectively. Test tubes containing the suspension were tightly closed and placed in an oil bath at 110˚C for 30 min.

Once the heat treatment was completed, the tubes were cooled in an ice bath and stored in the refrigerator overnight.

5.2.3. Gel properties

The mechanical properties of the gels were evaluated using an Instron 5967 universal testing machine (Instron Corp., Norwood, MA, USA). Gels were dismounted from test tubes and cut into cylindrical pieces (~10 mm height and ~14 mm diameter). A two cycle compression test using a 50 N load cell was performed at room temperature at a rate of 1 mm/min and 50% compression to evaluate their mechanical properties. The textural profile parameters were determined from the typical Instron force-time curve in which compressive stress was calculated as the peak compression force in the 1st bite cycle, divided by the initial cross-section area of the gel sample, and cohesiveness is the ratio of the area under the first and second compression peaks.

Additionally the water holding capacity (WHC) of the gels prepared was evaluated. For this a gel sample (0.9 – 1.2 g) was placed into a Vivaspin 20 centrifugal filter unit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and centrifuged at 453 × g for 10 min at room temperature. The weight of the gel was recorded before (W_i) and after (W_f) centrifugation to the nearest 0.0001 mg and the percentage of water loss after centrifugation was expressed as:

$$\%WHC = 100 - \left(\left(\frac{W_i - W_f}{W_i}\right) x100\right)$$

5.2.4. Scanning electron microscopy (SEM)

The morphology observation of the gels was carried out with a Phillips XL-30 scanning electron microscope (FEI Company, Oregon, USA) at an acceleration voltage of 20 kV. The samples were frozen in liquid nitrogen and freeze-dried before observations. The cross-

section and surfaces of the dry gels were sputtered with gold and platinum, observed and photographed.

5.2.5. Rheological measurements

The rheological measurements were done with a TA Discovery HR-3 rheometer (TA instruments, New Castle, DE, USA). To evaluate the molecular interactions involved in the formation of OP and OP-polysaccharide gels, a frequency sweep analysis was conducted. Gels were prepared as previously described in the gel preparation section at pH 3 and 7 and cut into approximately 1 cm (height) sections. The resulting gel disk were submerged for 48 h in solutions of 2-mercapthoethanol (2-ME) (0.2 M), urea (6 M) and sodium dodecyl sulfate (SDS) (1% w/v), which could disrupt disulfide bonds, hydrogen bonds and hydrophobic interactions respectively. A frequency sweep test was done to evaluate the dependence of G' to frequency (0.1 – 100 rad/s) on gels compressed to 80% of its original height. To study the changes in viscoelastic properties as a function of temperature, OP and OP-polysaccharide suspensions were subject to a temperature ramp from 25 to 95˚C, hold at 95˚C for 5 min, and then cooled down to 25˚C at a rate of 1.5˚C/min. Sample conditioning took place before and after each temperature ramp for a period of 3 min. For these measurements, approximately 1 mL sample was loaded in the bottom plate of the parallel plate geometry; the upper plate was lower to the appropriate geometry gap. To avoid evaporation during heating a solvent trap was used and a thin layer of silicone oil was applied. The temperature of the bottom plate was controlled with a Peltier system. All rheological measurements were done within a predetermined linear viscoelastic region, which was determined in preliminary experiments, setting the strain value at 0.05%.

5.2.6. Turbidity and Particle size measurements

Protein aggregation was followed by measuring the turbidity of samples before and after heating (110˚C, 30 min). Changes in turbidity at 600 nm were recorded at a protein concentration of 0.1% at pH 3 and 7 with 0 and 50 mM NaCl on a Jasco V-530 UV/VIS spectrophotometer (Jasco Corporation, CA, USA). The absorbance was measured using quartz cuvettes with a 10 mm path length.

A Zetasizer Nano ZS ZEN1600 system (Malvern Instruments, U.K.) was used to study the change of particle size distribution caused by heating treatment (110˚C for 30 min). Samples were immediately cooled in an ice bath to room temperature, after completing the heating period. Samples were then diluted to a total concentration of 0.1% (w/v) and passed through a 0.45 μm pore size filter prior to measurement. Number-based particle size distribution was measured to identify the total number of particles of a given size [261].

5.2.7. Surface Hydrophobicity (H_o)

Protein surface Hydrophobicity was determined using the apolar fluorescent dye, ANS [292]. Protein and protein-polysaccharide suspensions (1% w/v) were prepared and heated for 30 min at 110˚C followed by cooling. Samples were diluted in phosphate buffer to obtain five final concentrations ranging from 0.002 to 0.020% (w/v). 20 μL of ANS solution (8.0 mM in 0.1 M phosphate buffer, pH 7) were added to 4 mL sample. The relative fluorescence intensity (RFI) was determined using a Fluorescence spectrophotometer Sprectramax M3 (Molecular devices, CA, USA) set at 390 and 470 as excitation and emission wavelengths, respectively, with a constant excitation and emission slit of 5 nm. The initial slope of the RFI versus protein concentration plot was calculated by linear regression analysis and used as an index of H_o.

5.2.8. Fourier transform infrared (FTIR) spectroscopy

In order to detect changes in the protein conformation caused by addition of polysaccharide. Gel samples were prepared as previously described and freeze-dried. The dried gel was crush into powder, vacuum–dried at 40˚C overnight and mixed with KBr powder (1:100 w/w). The mixtures were compressed to 13 mm discs and used for spectroscopy measurements. To study the amide I region of the protein (1700-1600 cm^{-1}), Fourier self-deconvolutions were performed using the software provided with the spectrometer (Omnic 8.1.210 software). Each spectrum was the result of 128 scans; band narrowing was achieved with a full width at half maximum of 20-25 cm^{-1} and with resolution enhancement factor of 2.0-2.5 cm^{-1}. During measurements Nitrogen was continuously run through the spectrometer.

5.2.9. Confocal laser scanning microscopy (CSLM)

CSLM was used to observe the distribution of the polysaccharide within the protein network. A laser scanning confocal microscope Zeiss LSM710 (Carl Zeiss Microscopy, Jena, Germany) was used with a 10 × objective. Dextrin and carrageenan were labeled covalently with FITC. Specifically, 0.5 g of the polysaccharide and 10mL of DMSO were stirred overnight, and then 7mg of FITC were added to the polysaccharide-DMSO mixture. The reaction mixture was protected from the light, heated at 90˚C for 2 hours and dialyzed extensively against distilled water in the dark and freeze-dried. Rhodamine B was used for non-covalent labeling of oat protein. A 15% protein suspension was prepared, and 40μL of Rhodamine B (5mg/mL) were added to 1 mL of protein suspension. The mixture was stirred for 2 h at room temperature, dialyzed against distilled water in the dark and freeze-dried. Once protein and polysaccharide were labeled, OP-polysaccharide suspensions were prepared as described previously in the gel preparation section. A thin slice of gel was cut

with scalpel and place in a slide. The fluorescent images were analyzed at wavelength of 488 nm and 516 nm. Images were processed with ZEN 2009 LE software (Carl Zeiss AG, Oberkochen, Germany).

5.2.10. Statistical analysis

The results were evaluated by analysis of variance (ANOVA) and significant differences, with minimum significance test set at the 5% level ($p < 0.05$) with Tukey's test to compare all means, using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). All experiments were performed at least in three independent trials and the results were reported as mean ± standard deviation.

5.3. Results and discussion

5.3.1. Effect of polysaccharide

Figure 24 shows the microstructure of oat protein gels in the presence of polysaccharides at pH 3 and 7. Normally, globular proteins form fine stranded gels at pH values that diverge from the isoelectric point (pI) and with low ionic strength. Nevertheless, SEM images of oat protein derived gels showed a percolating network structure similar to that observed in polymer gels. It has been proposed that as a result of heating, oat globulin hexamers dissociated first into trimmers and then into monomers that are highly reactive [276]. The monomers were also unfolded during heating, thus a nearer side by side array of the polypeptide chains took place, which promotes intermolecular interactions and consequently strong percolating networks were formed [27]. At pH 3, the oat protein gels prepared with dextrin and chitosan (OP-DE and OP-CH) (Figure 24 A and E) show networks that consisted of hollow cells formed between vertical walls. The cells appeared to be interconnected in

OP-DE gels, whereas long walls forming large and empty compartments were observed in OP-CH gels. In contrast gels prepared with carrageenan (OP-CA) (Figure 24 C) show a condensed and compact structure with flaky appearance. At pH 7, gels with thick walls and highly interconnected percolating networks were formed by OP-DE and OP-CA (Figure 24 B and D). Whereas, a particulate structure was observed for OP-CH (Figure 24 F) with spherical aggregates distributed among the gel network. To explain the effect that the type of polysaccharide has upon the gelling properties of oat protein, it is necessary to consider the type of interactions involved in the system. Using protein and anionic polysaccharide as an example, at pH > pI, repulsion exists between protein and polysaccharide; at pH < pI, electrostatic attraction exists between protein and polysaccharide, resulting in the formation of coacervates. Coacervate networks are one continuous network formed by favorable attractive interactions between proteins and polysaccharides. Phase-separated networks are formed when interactions between protein and polysaccharide are repulsive, resulting in a bi-continuous phase. Oat protein has a pI of ~5 [223,293], thus OP-CH at pH 3 and OP-CA at pH 7 were likely to form phase separated networks due to repulsive interactions between protein and polysaccharide. However, no clear phase separation could be defined from the SEM photos, probably due to the fact that only small amount of polysaccharide was added in the system. Thus the observed changes in microstructure could be related to the effect that the polysaccharide has on protein gel network formation. The balance between attractive and repulsive forces is useful to explain why gel networks prepared at pH 3 seemed to be less interconnected than those prepared at pH 7. Previous research suggested that the optimum balance in the case of oat protein is reached at pH 5-7, whereas at a higher or lower pH, disproportionate electrostatic repulsive forces may work as an obstacle warding off protein

molecules from crosslinking and forming a strong structure [249]. At pH 7 it is expected that the repulsive forces involved in the system are counterbalanced by other type of attractive interactions, for instance hydrophobic forces that facilitate the intermolecular crosslinking, which produces a more interconnected structure and also provides higher mechanical strength to the gels. The strong repulsion caused by an increased charge, has direct influence on the number as well as the type of interactions taking place.

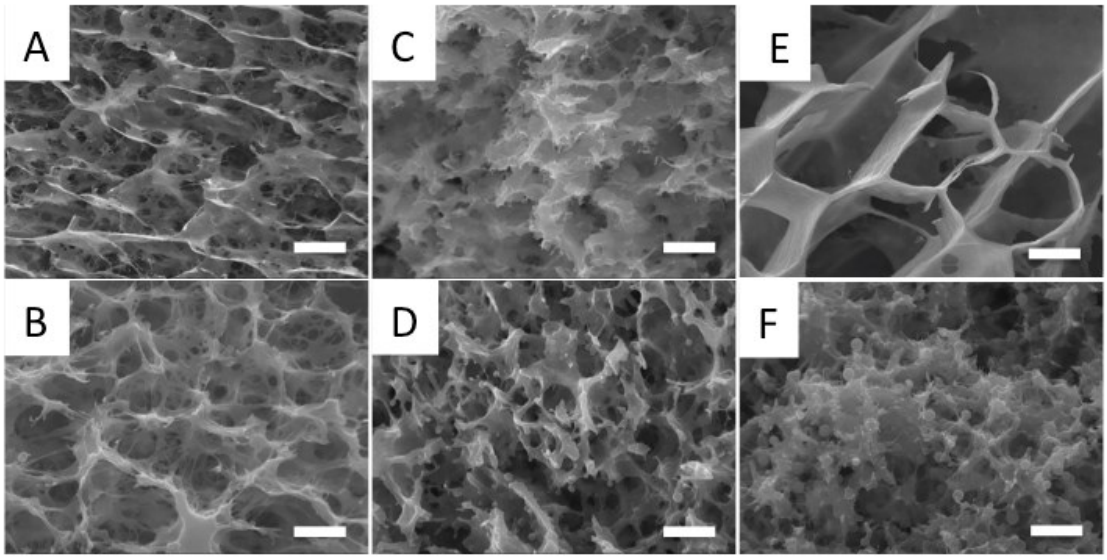

Figure 24 SEM images of the cross section of oat protein gels prepared at 110˚C without salt. Scale bar represents 5 µm. A and B correspond to OP-DE gels prepared at pH 3 and 7 respectively. C and D corespond to OP-CA gels prepared at pH 3 and 7 respectively. E and F correspond to OP-CH gels prepared at pH 3 and 7 respectively.

Under conditions that promote associative interactions, such as in the case of OP-CA system at pH 3 and OP-CH system at pH 7, electrostatic attractive forces are expected. These

attractive forces could restrict protein unfolding and promoted protein random aggregation, leading to the formation of particulate networks with flaky appearance. The gels prepared with dextrin (OP-DE) showed similar microstructure as that made of oat protein alone at both pH 3 and 7. Dextrin being a neutral polysaccharide is not expected to produce any significant change in the surface charge of protein molecular chains thus could be present as a co-solute. Nevertheless, a phase-separated network could still be formed due to the thermodynamic incompatibility between dextrin and oat protein during heating. In our previous work, oat protein-inulin gels with micro phase separated structure were formed [294]. It was speculated that during heating, the protein started to unfold, increasing the thermodynamic incompatibility between oat protein and inulin due to the excluded volume effect [124], thus the system separated into a protein-rich and a inulin-rich phase [124]. At pH 4, particulate gels were observed for oat protein alone and in combination with polysaccharides (SEM images not shown). When pH was near to the pI, the low net charge on protein molecular chains promoted random association of protein aggregates into large and spherical aggregates linked together to form the gel network.

Figure 25 shows the mechanical properties and water holding capacity (WHC) of oat protein gels in the presence of different polysaccharides. Compressive stress indicates the capacity of the material to withstand a given deformation. At pH 3, oat protein had a compressive stress value of 2.99 kPa. This value increased to 4.07 and 3.61 kPa in the presence of dextrin and carrageenan respectively. The compressive stress decreased to 2.66 kPa with addition of chitosan. At pH 7, addition of carrageenan significantly increased ($p<0.05$) the compressive stress value of oat protein gels from 13.21 to 25.95 kPa. The gel strength was also improved (16.87 kPa) with addition of dextrin but decreased in the presence of chitosan (11.61 kPa).

The improvement of the gel strength is associated with the expected phase-separation process produced by the thermodynamic incompatibility between protein and polysaccharides. In this process, polysaccharide molecules were preferentially excluded from the protein surface. As the phase-separation process advanced, protein rich regions and polysaccharide rich regions were formed. The water content is always higher in the phase of a more hydrophilic component with higher excluded volume effect, hence a less concentrated phase rich in polysaccharide and a more concentrated phase rich in protein was formed [295]. The increase in the apparent protein concentration could explain the improved gel strength, as the rigidity of a protein gel is determined by the concentration of protein incorporated into the gel network [295]. In contrast, gels prepared under conditions favoring associative interactions showed reduced gel strength values, since the charge neutralization produced by the polysaccharide, restricted protein unfolding and increased protein aggregation, leading to particulate networks. The restricted protein unfolding associated with weak electrostatic repulsion, prevented the exposure of reactive amino acid residues, which are normally buried at the interior of the protein molecules. Thus the number of possible interactions is reduced. This has direct repercussion in the mechanical strength of the gel.

The WHC is a key property of gels and low values often results in dry products and low texture stability. Oat protein based gels showed good WHC values at pH 3 (86.24-87.84%) and pH 7 (71.28-79.17%). WHC values were maintained or improved with carrageenan at pH 7, chitosan at pH 3 and dextrin at both pH 3 and 7. In phase separated gels, polysaccharides tend to separate into the serum phase and increase the viscosity of the serum phase rendering it less prone to syneresis [296]. Conversely, dramatic decrease of WHC was observed with addition of carrageenan at pH 3 and chitosan at pH 7. Specifically, these samples showed

particulate microstructures in previous SEM observation. In particulate gels, protein aggregation was promoted and protein-water interactions were restricted, leading to reduced gel WHC [267].

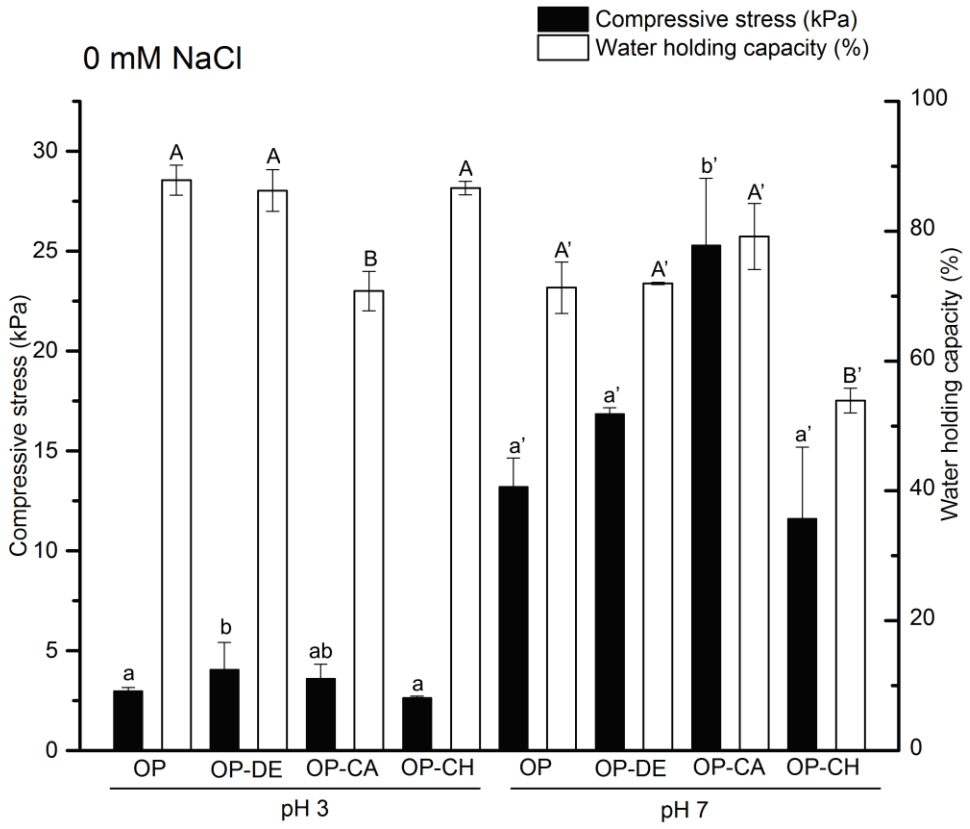

Figure 25 Gel properties of OP and OP-polysaccharide gels prepared at pH 3 and 7 and without salt. Statistical analysis was performed separately for each pH, which was indicated by the use of primed letters (a and a' for compressive stress) (A and A' for water holding capacity). Values within the same pH not sharing a common superscript differ ($p<0.05$).

5.3.2. Effect of salt

Salt is an important food ingredient and salt concentration is a process parameter that is often adjusted in the development of new food products. For this reason, this investigation will provide information on the effect of salt on the gelling properties of the protein-polysaccharide blends. It is known that the presence of salt can suppress electrostatic interactions [88], as salt has a shielding effect on the surface charges, which reduces the repulsive forces between protein molecules and between protein and polysaccharide. Addition of salt can promote the random association of protein aggregates. Nevertheless, at pH 3, such change was not evident for OP-DE and OP-CH gels (Figure 26 A and E) as SEM images show similar microstructure to those formed without salt. These two systems have a high positive surface charge, especially in the presence of chitosan, thus addition of salt at a low concentration (50 mM NaCl) was not enough to suppress electrostatic repulsive forces. In contrast, the gel structure formed at pH 3 with carrageenan in the presence of 50 mM NaCl resulted in particulate gels (Figure 3 C). Since carrageenan neutralized the charge on oat protein molecular chains, a small amount of salt could suppress the remaining electrostatic repulsive forces, inducing formation of spherical aggregates. As it was previously mentioned, the balance between attractive and repulsive forces could be reached at pH 7 [249] to form strong percolating networks in oat protein gels, however the shielding effect of salt disturbed such balance, therefore the addition of salt induced formation of particulate gels in the presence of dextrin and chitosan at this pH (Figure 26 B and F). The network formed by OP-CA (Figure 26 D) was less affected by salt at pH 7 as the morphological differences between the OP-CA images with and without salt are negligible due to high charge in the system. Increasing the salt content from 50 mM to 150 mM intensified the appearance of spherical

aggregates and increased the size of particulate clusters in the gel networks in the presence of all the polysaccharides (SEM image not show).

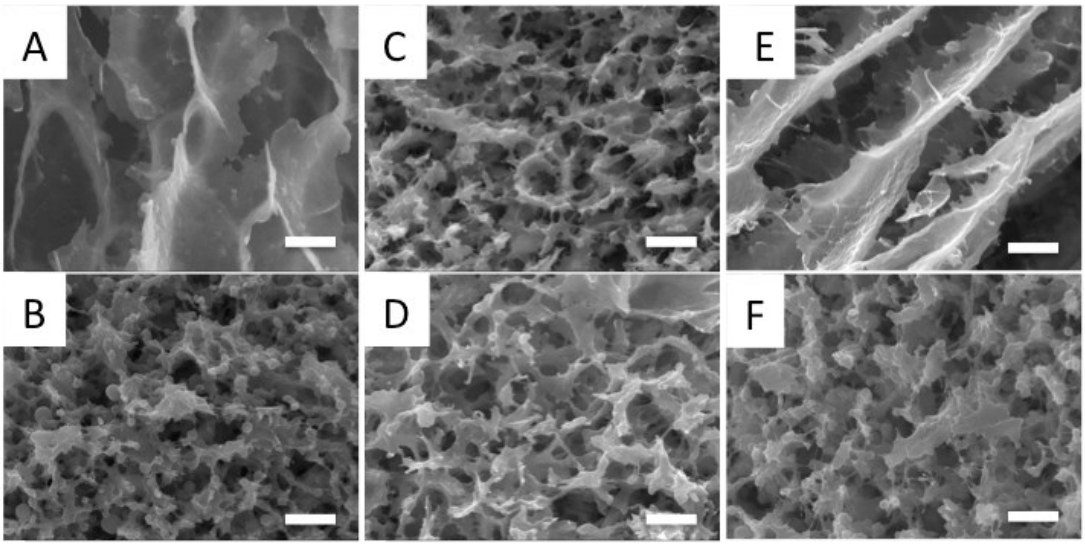

Figure 26 SEM images of the cross section of oat protein gels prepared at 110˚C with 50 mM NaCl. Scale bar represents 5 µm. A and B correspond to OP-DE gels prepared at pH 3 and 7 respectively. C and D correspond to OP-CA gels prepared at pH 3 and 7 respectively. E and F correspond to OP-CH gels prepared at pH 3 and 7 respectively.

At pH 3 compressive stress values significantly increased ($p<0.05$) as the salt content increased up to 150 mM (Figure 27 and 28). Further increase of salt content from 150 to 300 mM reduced the compressive stress values (data not shown). At 150 mM NaCl, the highest compressive stress value (14.02 kPa) was obtained with OP-DE followed by OP (12.16 kPa). Lower values were obtained for OP-CA (11.15 kPa) and OP-CH (9.84 kPa). These values are comparable to oat protein gels formed at pH 7. This feature could be exploited in the development of food gel products under acidic conditions, which has been one of the major limitations of oat protein as a gelling agent. At pH 3, the addition of salt at a low concentration (50 – 150 mM) has a favorable effect, as it reduced the extreme repulsive

forces produced by the large positive surface charge of the system. Thus, by slightly reducing the surface charge a better balance between attractive and repulsive forces was achieved. Increasing the salt concentration favored this balance until the point where the predominant interactions were attractive forces at higher salt concentration such as 300 mM. At pH 7 addition of salt reduced the compressive stress value to certain extend, even at 50 mM NaCl.

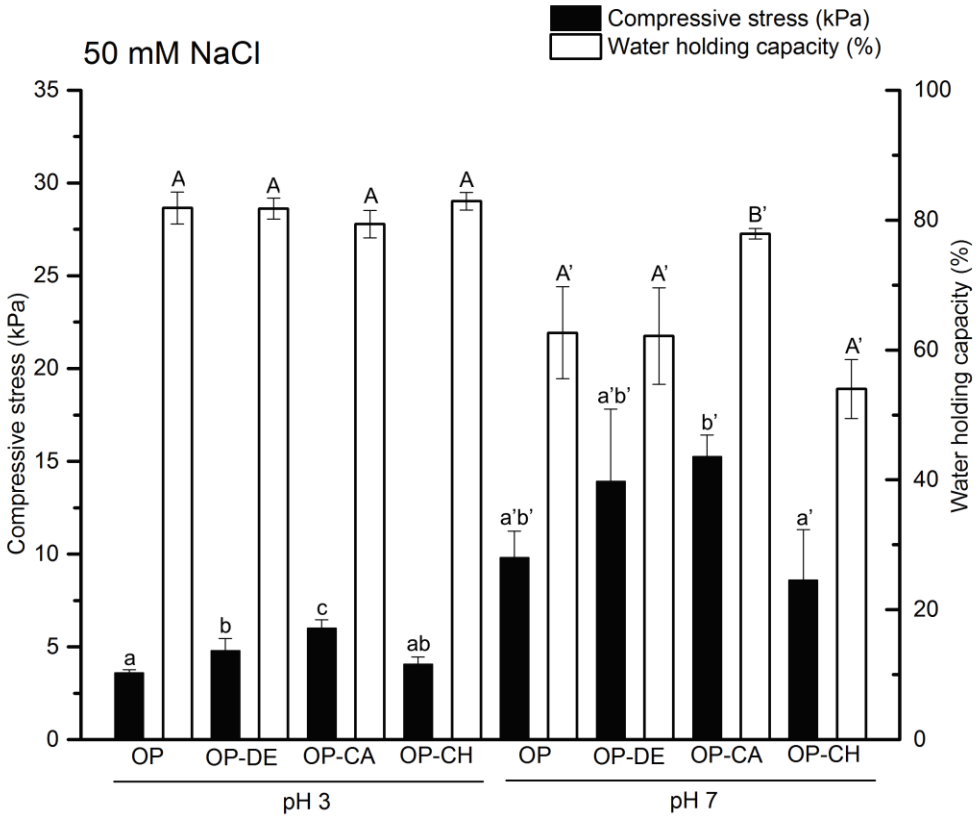

Figure 27 Gel properties of OP and OP-polysaccharide gels prepared at pH 3 and 7 and 50 mM NaCl. Statistical analysis was performed separately for each pH, which was indicated by the use of primed letters (a and a' for compressive stress) (A and A' for water holding capacity). Values within the same pH not sharing a common superscript differ ($p<0.05$).

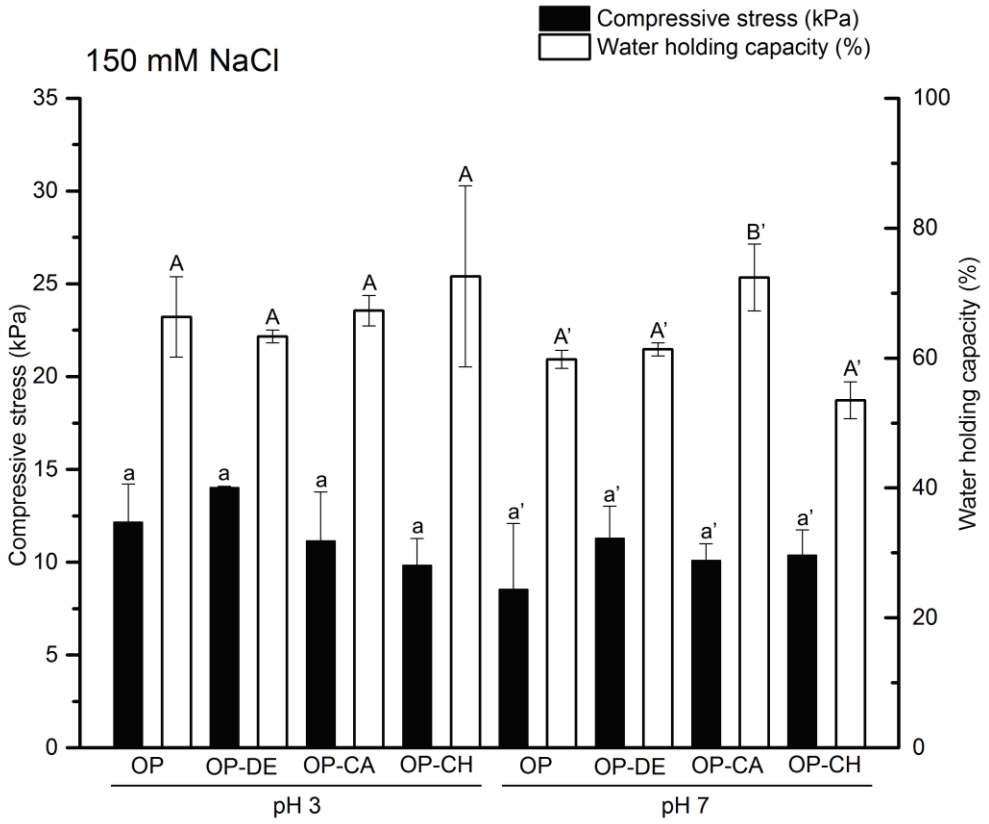

Figure 28 Mechanical properties of OP and OP-polysaccharide gels prepared at pH 3 and 7 and 150 mM NaCl. Statistical analysis was performed separately for each pH, which was indicated by the use of primed letters (a and a' for compressive stress) (A and A' for water holding capacity). Values within the same pH not sharing a common superscript differ ($p<0.05$).

As previously mentioned, the balance between attractive and repulsive forces was reached at pH 7 in oat protein gels. Addition of a small amount of salt could disrupt such balance, leading to reduce compressive stress values. Evidently the threshold for salt addition is different for each pH under which gels were prepared. A higher threshold was observed at pH 3 where presumably electrostatic repulsive forces are stronger allowing adequate balance between repulsive and attractive interactions to form gels with higher compressive stress.

This threshold was lower at pH 7 as a small amount of salt led to significant reduction of the gel strength.

Increasing salt content also caused the WHC to decrease. At pH 3 the WHC diminished to values ranging from 61.22 to 58.43% when the salt concentration was as high as 300 mM NaCl (data not shown). Increasing salt concentration caused a progressive transition into a particulate gel network [267]. Interestingly, WHC values of OP-CH gels were less impacted by salt, showing values of 82.90 and 72.59% as salt content increased from with 50 to 150 mM (Figure 27 and 28). OP-DE gels, also maintained a high WHC (81.78%) at 50 mM. This is in accordance with the previous observation that the OP-CH and OP-DE gels did not undergo significant changes due to the fact that salt at a low concentration (50 mM NaCl) was not enough to suppress the electrostatic repulsive forces in oat protein networks. At pH 7, even low salt concentrations (50 mM) had a detrimental effect on the WHC of the gels. Nevertheless, gels prepared with OP-CA did not show any significant change ($p<0.05$) as the salt content increased. This is also in agreement with the inspection of the gel morphology at pH 7 with 50 mM NaCl, where the formation of spherical aggregates was less evident on OP-CA compared to OP-DE and OP-CH.

The improvement of the gelling properties by blending with polysaccharides is highly related to the type of interactions that are promoted in the system. Thus gelling properties were strengthened without compromising the WHC, when gels where prepared under conditions that favored segregative interactions. In contrast, gels formed under associative interactions, showed reduced WHC values and reduced gel strength. A similar observation was done in a whey protein-xanthan gum system [50]. In this case, xanthan gum had a synergistic effect when gels were prepared under conditions that favored segregative phase separation and an

antagonistic effect when gels were prepare under conditions that promoted attractive interactions, resulting in the protein-polysaccharide complex formation [50].

The effect of salt could also be synergistic or antagonistic depending on prevalent conditions in the system. In some instances and even though salt is known to induce the formation of particulate gels, addition of salt can be advantageous in the interest of regulating the balance between repulsive and attractive interaction. Whenever salt addition takes the system out of this balance, salt exerts a deteriorating effect on the strength and in the WHC of the gels.

Carrageenan had a strong synergistic effect on oat protein gelling properties at pH 7, as the compressive stress was almost doubled. Additionally OP-CA gels at neutral pH showed high WHC even in the presence of salt. Addition of dextrin slightly improved the gel strength at both acidic and neutral conditions. Thus OP-DE, OP-CA were selected for further investigation to understand the influence that the type of polysaccharide has on the gel formation mechanism.

It is worthy of notice that all gels prepared showed a continuous protein surface regardless of the pH or salt content used in its preparation with the exception of gels prepared with OP-CA at pH7, which showed a continuous protein surface with large insertions of carrageenan particles with approximate size of 1.5 mm.

5.3.3. Protein interactions

While electrostatic attractions are the main driving force in protein-polysaccharide complexation, other interactions such as hydrogen bonds and hydrophobic interactions may contribute to the stability of the protein-polysaccharide system [38]. To elucidate the type of interactions involved in the development of the oat protein-polysaccharide gels, gel rheological properties were studied by a frequency test in the presence of dissociating

reagents including urea, 2-ME and SDS; that are known to interrupt hydrogen bonds, disulfide bonds and hydrophobic interactions respectively. The gels prepared at pH 3 and treated with SDS shrank and hardened. This was due to strong interaction develop between positively charged protein and negatively charged SDS, which resulted in highly condensed networks with reduced protein-water interactions. Thus, readings obtained for these samples were considered not reliable and are not included. The participation of disulfide bonds on gels prepared at pH 3 was not expected, as it is known that the ability of disulfide bond formation is reduced at pH values lower than 7 where other interactions become more relevant in the aggregation process [297]. This is associated to the fact that disulfide bonds are unlikely to take place under acidic conditions due to low reactivity of cysteine [262]. Therefore at pH 3, only the involvement of hydrogen bonding was evaluated and all samples treated with urea showed greatly reduced G' values and the reduction levels in OP-DE and OP-CA were higher than gels with OP alone (Data not shown). This could indicate that the addition of the polysaccharide increased hydrogen bonds in the gels. Hydrogen bonds may take place between the C=O and N-H groups of the protein and hydroxyl groups of the polysaccharide [289] and hydrogen bonds are also believed to participate in the secondary stabilization of complexes formed by electrostatic attractive forces [298] such as the one formed by OP-CA at this pH. Hydrophobic bonds may also contribute to gel formation as urea can also disrupt hydrophobic forces according to a previous report [299]. Figure 29 shows the response of G' to the variation on frequency of oat protein derived gels prepared at pH 7 with and without salt. At this pH and in the absence of salt, all samples treated with SDS, urea and 2-ME showed greatly reduced G' values of OP gels. This indicates that hydrogen bonds, disulfide bonds and hydrophobic forces all play essential role to stabilize gel networks.

In the case of OP-DE a similar effect was observed, however the G' was slightly less affected in the presence of 2-ME. In contrast, gels prepared with OP-CA were not affected by 2-ME but were disturbed by SDS and urea. Thus hydrogen bonds and hydrophobic forces constituted the main support of the OP-DE and OP-CA gel networks at neutral pH. Heat is expected to cause the dissociation of oat globulin hexamers by breaking disulfide bonds linking the acidic and basic subunits. This allows the re-formation of disulfide bonds during the heating process and explains the involvement of disulfide bonds in OP gel formation. In the case of gels containing carrageenan the development of new disulfide bonds may be hindered. Probably due to the fact that strong repulsion hampered the required proximity for the SH/S-S- interchange. This situation was not expected in the case of dextrin, since the addition of this polysaccharide has slight effect on the electrostatic repulsion of the system. Previous studies demonstrated that the presence of anionic polysaccharides appeared to protect BSA against extensive aggregation associated to formation of intermolecular disulfide bridges, during or after high-pressure treatment [300]. In spite of depleted disulfide bonds for OP-CA gels, these gels showed superior mechanical strength than that of OP and OP-DE. In the presence of salt, OP gels at pH 3 treated with urea also show reduced G' values. Thus, hydrogen bonds and possibly hydrophobic forces are main contributors in gel network building. In the case of OP-DE, G' values were strongly reduced by treatment with urea, as the development of hydrogen bonds is expected due to the hydroxyl content of dextrin [289]. OP-CA with 50 mM NaCl showed a similar profile to OP-CA without salt (Data not shown).

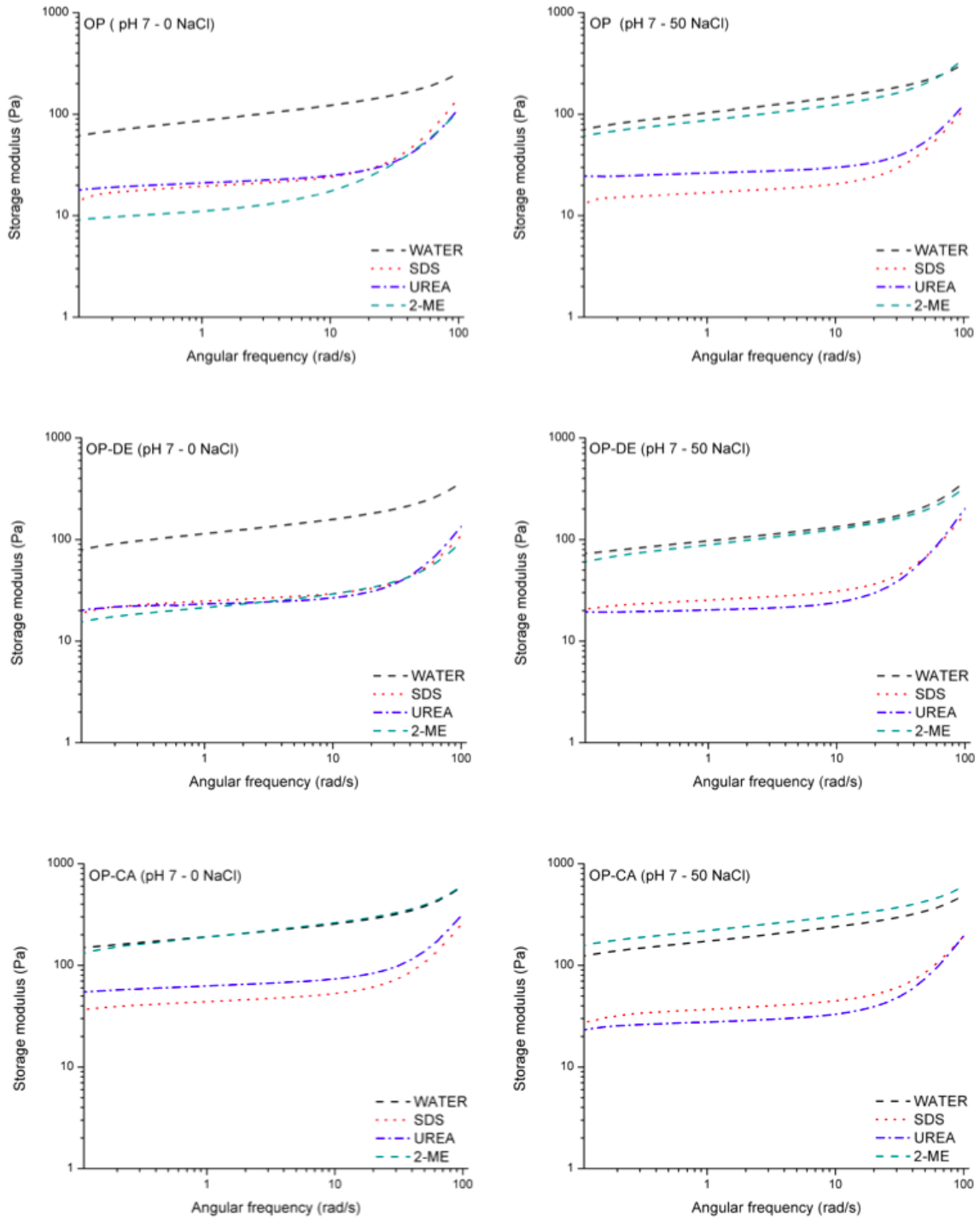

Figure 29 Storage modulus (G') of oat protein derived gels prepared at pH 7 without salt and 50 mM NaCl as a function of angular frequency

This is understandable as hydrogen bonds and hydrophobic forces are not affected by salt and these interactions are expected to provide additional support to the complexes formed. At pH 7 and with addition of 50 mM NaCl the G' values of all gels were mainly affected by urea and SDS, and no effect of 2-ME was observed (Figure 29). As a result of salt addition the contribution of disulfide bonds seems to be less important. This may be related to the shielding effect that salt has in the surface charges, which reduces the repulsive forces between protein molecules and between protein and polysaccharide. This promoted rapid random association of protein aggregates and by doing so, disulfide bonds were not developed due to the compact structure of the protein, where sulfhydryl groups could be hidden inside. Thus, the main forces supporting the gel network in the presence of salt were hydrogen and hydrophobic forces.

5.3.4. Turbidity and particle size

The changes in turbidity were recorded before and after heating OP and OP-polysaccharide suspensions with the purpose of studying how the progression of the heat-induced aggregation is affected by the presence of the polysaccharides. Before heating the turbidity of OP at pH 3, showed the lowest value among samples (Figure 30). OP-DE and OP-CA showed higher turbidity. This may be associated to hydrogen bonds taking place between the protein and hydroxyl groups of dextrin [289] as it was suggested earlier. For OP-CA the increased turbidity is primarily due to electrostatic attraction between the negatively charge carrageenan and the positively charged protein, this produced charge neutralization and bridging effects, leading to an increase in particle size and light scattering efficiency of the protein-polysaccharide complexes [301]. Moreover, hydrogen bonding and hydrophobic interactions are expected to participate in the secondary support of OP-CA complexes. Salt

addition significantly ($p<0.05$) increased the turbidity of OP suspension and the same effect was observed for OP-DE and OP-CA. Salt addition reduced the electrostatic repulsion between proteins, which promoted aggregation. To corroborate the changes in electrostatic forces due to salt addition the zeta potential (ζ) of the OP and OP-polysaccharide suspensions was also determined. In the case of OP the ζ changed from 26±0.4 l to 14±0.2 with addition of 50 mM NaCl at pH 3, for OP-DE from 27±0.2 to 14±0.6 and for OP-CA from 22±0.4 to 10±0.1. The difference in the ζ in the case of OP-CA compared to OP and OP-DE is due to the negative charge of carrageenan, which confirms that charge neutralization and bridging effects are taking place. After heating the sample turbidity increased due to formation of more aggregates, which impaired the light transmission due to the light scattering of particles.

At pH 7, OP-DE also showed higher turbidity than OP. Remarkably the turbidity for OP-CA was lower than OP and OP-DE. Strong repulsive forces between the negatively charged carrageenan and the negatively charged protein prevented aggregation hence large aggregates could not form. Addition of salt resulted in dramatic increase of the turbidity in OP suspension, suggesting the electrostatic repulsion between proteins was greatly affected by salt and aggregation was promoted. Considering that at pH 3, the repulsive forces are larger than at pH 7 and thus, at neutral pH, the balance between repulsive and attractive forces is reached in oat protein suspensions. Therefore, salt addition can easily disrupt this balance and in turn, attractive forces may become the dominant type of interaction resulting in larger aggregates. In contrast at pH 3 the changes in turbidity are limited as the weight of the repulsive forces is higher and thus a higher salt concentration would be needed to produce a similar effect as the one observed at pH 7. Turbidity of OP-DE also increased after salt

addition. Increasing ionic strength has no effect upon hydrogen bonding, thus the higher turbidity of OP-DE was produced due to salt suppression of electrostatic repulsive forces.

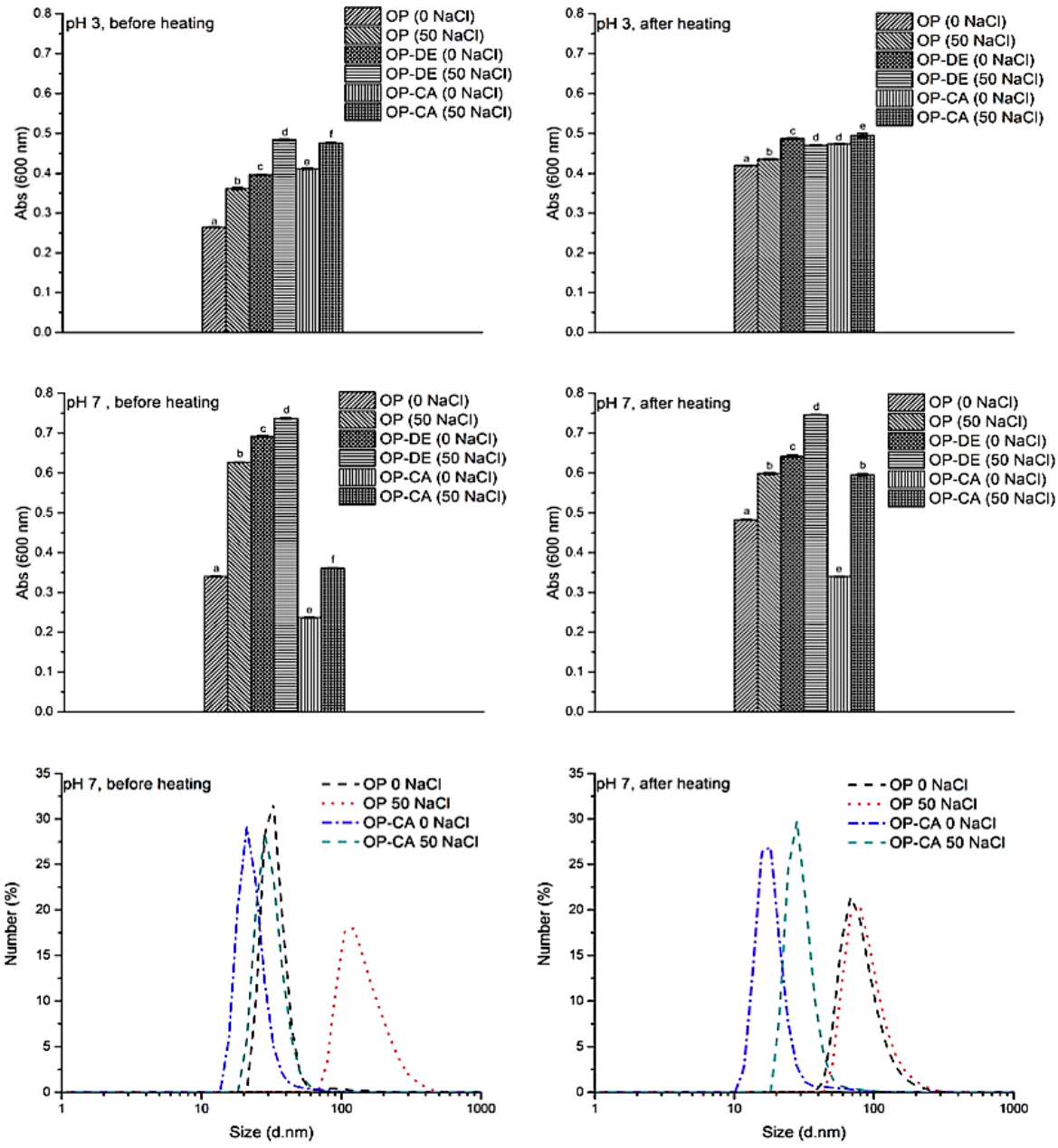

Figure 30 Changes in turbidity and in the particle size distribution (number based) of unheated and heated OP and OP-polysaccharide suspensions without salt and 50 mM NaCl. Values not sharing a common superscript differ ($p<0.05$)

OP-CA also show much lower turbidity than OP and OP-DE, indicating that salt addition had a lesser effect as the total net charge of OP-CA suspension was higher. Accordingly at pH 7, the ζ of OP changed from -29±0.7 to -13±0.3 with addition of 50 mM NaCl, for OP-DE from -28±0.7 to -12±0.2 and for OP-CA from -33±0.5 to -20±0.2. It is interesting to notice that OP-CA still exhibited low turbidity even after heating at pH 7. Both carrageenan and protein carried relatively high negative charge, which produced strong electrostatic repulsion to prevent formation of large aggregate formation [302].

The particle size distribution of the protein aggregates formed in the presence of carrageenan during heating was also monitored at pH 7 and compared to oat protein alone, these results are shown in Figure 30. OP had a peak value of 33 nm in diameter and salt addition resulted in significant increase of the average diameter to 122 nm. This is consistent with turbidity results as salt reduced electrostatic repulsion and therefore facilitated aggregation. OP-CA had a peak diameter value of 21 nm and this increased slightly to 28 nm with addition of salt. This further confirms that strong repulsion existed between carrageenan and the protein, preventing aggregation. After heating the main peak observed for OP was 68 nm, and 79 nm without and with 50 NaCl mM respectively. However, the peak value of OP-CA remained almost the same (16 and 28 nm), as heating and salt addition had little impact on the size. OP-DE showed a similar behavior to oat protein alone (results not shown). These results corroborate that in the presence of carrageenan, strong repulsive forces could limit the size of the aggregates formed and that rapid random aggregation was impaired. The overall reaction rate of heat-induced gelation can be determined by both the unfolding and the aggregation reaction [23]. Under high repulsion conditions, protein molecules will easily unfold, and will aggregate at a lower rate, since the participation of attractive forces is limited [25]. The

combination of high unfolding rate with low aggregation rate allows formation of structures with higher degree of order [25]. Therefore addition of carrageenan could facilitate formation of a highly organized oat protein network. A highly order structure implies that the ensemble of reactive units took place in an intimate lateral sequence, which later on allowed the contribution of other intermolecular interaction as secondary support of the structure culminating in stronger gels [27] which according to the previous rheological tests are hydrogen bonds and hydrophobic forces. This may also explain the two-fold increase in compressive stress with incorporation of small amount of carrageenan at pH 7, in addition to the apparent increase in protein concentration due to phase separation [295].

5.3.5. Surface hydrophobicity

Hydrophobic forces play an important role to stabilize oat protein based gels, therefore changes in surface hydrophobicity after heating as induced by addition of different type of polysaccharide or salt content were also investigated, and the results are demonstrated in Figure 31. Normally, globular proteins are composed of charged side groups at their surface and hydrophobic groups buried at the interior of the structure. After heating, proteins can unfold exposing buried hydrophobic groups; these groups could alter the surface characteristics by providing additional sites for crosslinking. Heating in the presence of dextrin had no significant impact on the surface hydrophobicity as no strong electrostatic interactions could be developed between oat protein and dextrin. In contrasts heating in the presence of carrageenan increased the surface hydrophobicity especially with salt. This suggests exposure of hydrophobic residues [287,303] due to strong repulsive forces in the OP-CA suspension.

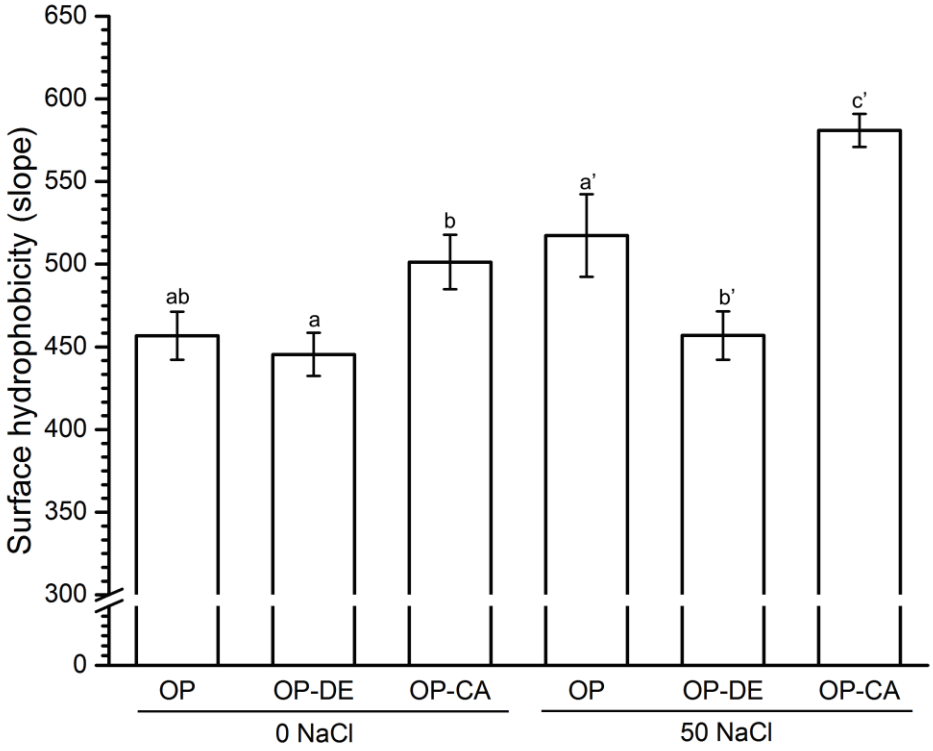

Figure 31 Surface hydrophobicity of OP and OP-polysaccharide suspensions without salt and 50 mM NaCl. Statistical analysis was performed separately for each salt content, which was indicated by the use of primed letters (a and a'). Values within the same pH not sharing a common superscript differ ($p<0.05$).

Considering that ANS is anionic hydrophobic probe [287], the higher surface hydrophobicity observed in the presence of salt may also be related to the shielding effect of salt, which allowed the hydrophobic probe ANS to better approach and attach non-polar binding sites in the oat protein. These exposed hydrophobic groups can function as prospective crosslinking points and promote protein intermolecular interactions as the aggregation process advances. Thus the resulting hydrophobic interactions are part of the secondary support of the gel

structure. The high repulsive forces in the OP-CA system could control the rate of aggregation and expose hydrophobic groups during heating that in turn produced a highly ordered structure [36] with higher level of crosslinking, leading to greatly improved mechanical strength.

5.3.6. Fourier transformed infrared (FTIR) spectroscopy

In order to understand the impact of phase separation on the protein network formation, the protein secondary structure in the gel was studied by FTIR. OP-CA system was addressed due to the interesting observations of the changes on protein aggregation in the presence of carrageenan. Figure 32 shows the deconvoluted spectra of gels prepared with OP without salt and OP-CA with 0 and 50 mM NaCl. The amide I band between 1700 and 1600 cm^{-1} is used to observe changes in the secondary structure of proteins. The spectrum of OP showed seven components [277]: 1693 cm^{-1} (β-sheet), 1680 cm^{-1} (β-sheet), 1670 cm^{-1} and 1659 cm^{-1} (β-turn), 1643 cm^{-1} (random coil), 1625 cm^{-1} (β-sheet) and 1609 cm^{-1} (side chain vibrations). The addition of carrageenan caused remarkable increase in absorption intensity, suggesting that the protein molecules rearranged into a more organized structure [304] in the gel networks both with and without salt.

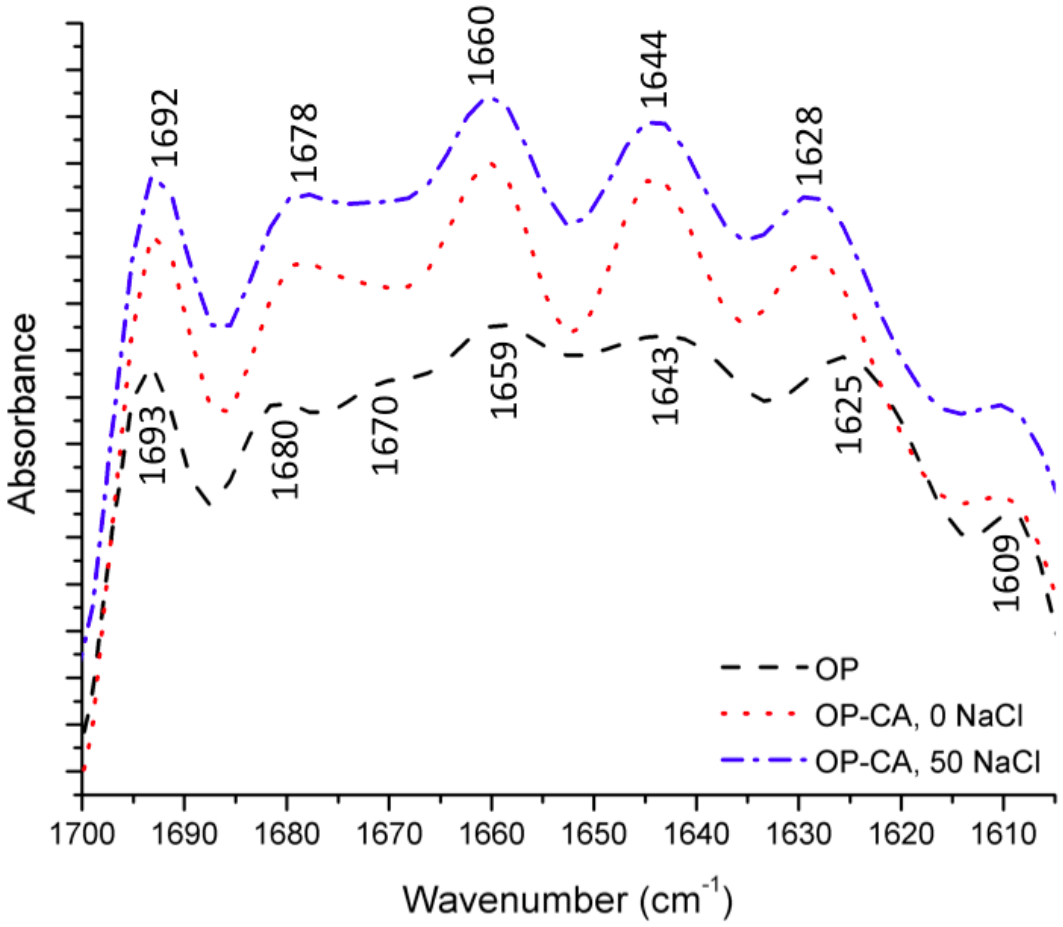

Figure 32 Deconvoluted spectra of OP and OP-CA gels without salt and 50 mM NaCl at pH 7

5.3.7. Confocal laser scanning microscopy (CSLM)

Confocal images of OP showed a continuous protein network, OP-DE and OP-CA exhibited a similar structure to the one formed by OP alone at neutral pH (Figure 33). Nonetheless, OP-CA showed some localized inclusions of transparent gel (carrageenan) randomly distributed among the protein continuous network, confirming phase separation in the gel system.

Confocal images confirm that dextrin and carrageenan were present in the void spaces of the protein network and that dextrin insertions were more and had a smaller size (~20 μm), whereas carrageenan insertions were much larger in size but fewer. These carrageenan inclusions varied in size and could be as large as 1.5 mm. This indicates that a different degree of phase separation occurred in these two systems. A higher degree of phase separation refers to a phase-separated microstructure in which the protein phase contains a small number of large polysaccharide insertions, whereas a lower degree of phase separation is related to a large number of small polysaccharide insertions.

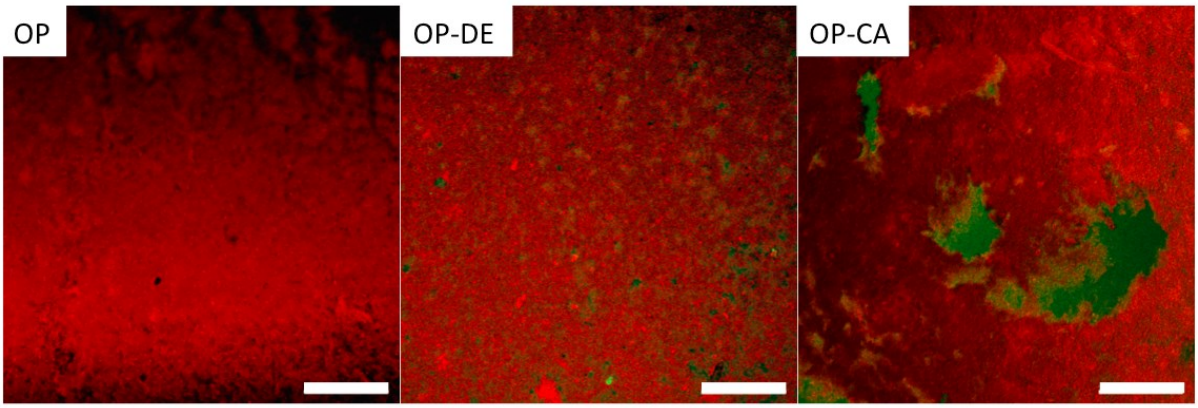

Figure 33 Confocal microscopic photographs of OP and OP-polysaccharide gels formed at pH 7. Scale bar represents 100 μm

5.3.8. Rheological measurements

Phase separation is promoted by heating [124] as the protein denaturation and aggregation increase the system incompatibility by increasing molecular volume or size [120]. During heating of the protein-polysaccharide system, the aggregation and phase separation events occurs simultaneously, with different kinetics [134]. For this reason, the rheological changes during the whole gel formation process were also studied. As shown in Figure 34, the OP-DE system has similar behavior to that of oat protein alone. During the heating stage, both G' values initially decreased with increasing temperature, then increased sharply at 70˚C. During the cooling stage G' still increased until 88˚C. From this temperature and until the end of the cooling stage (25˚C), G' slightly decreased. The final G' values were around 16 kPa for OP and 18 kPa for OP-DE. The development of G' with increasing temperature was different in the presence of carrageenan. A higher initial G' value was observed for OP-CA at 25˚C. A sharp increase occurred at around 55˚C until around 80˚C. Then G' continued to increase from 80-95˚C and during the whole cooling stage, but with a significantly reduced increment rate. The final G' value reached 22 kPa. These results indicate that aggregation started at an earlier stage for OP and OP-DE as the sharp increase of G' took place at a lower temperature (~63˚C) than that of OP-CA (~93˚C). The gel formation process of carrageenan alone (0.05 %w/v) was also monitored (data not shown). The initial G' value was 4.3 Pa, which is significantly lower than OP-CA, and no major peaks were observed during heating. During the cooling stage from 30˚C to 25˚C, the G' value sharply increased from ~4 to 339 Pa which was significantly inferior to OP-CA. This further support previous observation that carrageenan produced a synergistic effect during oat protein gel formation. Strong electrostatic repulsive forces between negatively charged protein molecules and negatively

charged carrageenan led to thermodynamic incompatibility. This means that protein–protein interactions and carrageenan-carrageenan interactions were more thermodynamically favorable than protein-carrageenan interactions, thus the system managed to separate into two phases, each with higher concentration of one of the components and depleted of the other [120].

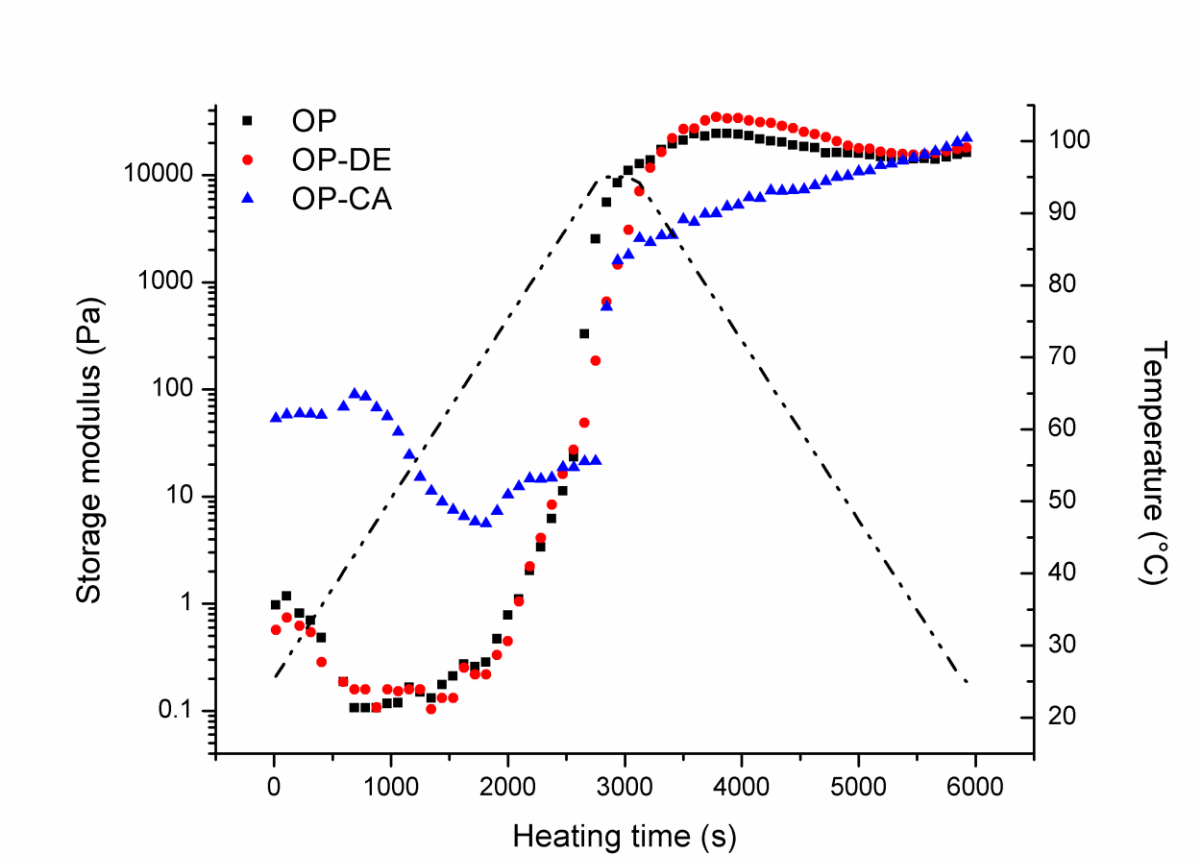

Figure 34 Storage modulus (G') of OP, OP-DE and OP-CA without salt during heat-induced gelation at pH 7. Doted line represents the heating temperature profile. Samples were heated from 25 to 95˚C, hold at 95˚C for 5 min, and then cooled down to 25˚C at a rate of 1.5˚C/min

As a nonionic polysaccharide dextrin could also show thermodynamic incompatibility with the negatively charged oat protein at neutral pH during heating. Phase separation between oat protein and inulin, another neutral polysaccharide, resulted in formation of inulin nanoparticles homogeneously distributed inside the protein network [294]. Thermodynamic incompatibility does not only arise from electrostatic repulsion, it can also be related to differences in the size and shape of the molecules. Before heating, the entropy of mixing allows a homogenous system, but with increasing temperature the size of the aggregates increases, and the entropy of mixing is reduced and therefore the stability of the system [35] leading to phase separation phenomenon. In the interest of better understanding the formation of phase-separated gel networks it is necessary to consider that two events are taking place simultaneously. One is the phase separation process and as it was previously mentioned, this process is stimulated with increasing temperature. The second event is the gelation process. The establishment of a rigid gel network is related to the process of unfolding and the exposure of reactive groups that enable the molecular interactions such as hydrophobic interactions to reinforce the gel network [271]. During heating, as phase-separation proceeds, polysaccharide molecules are preferentially excluded from the protein surface. The polysaccharide rich phase, which is more hydrophilic and has a higher excluded volume effect, will have higher water content and hence a more concentrated phase, rich in protein will be formed [120]. A higher apparent concentration in the protein phase explains the improvement seen in the gelling properties, as more intermolecular interactions were developed in the oat protein network. Other point of consideration is the fact that in the OP-CA gel formation, the process was greatly controlled by strong repulsive forces and in consequence the aggregation process was better organized. This was supported by the fact

that G' values of OP-CA increased at a later time and a higher temperature than OP and OP-DE during the heating process. The extent of the phase separation and thus the heterogeneity of the gels depend on the rate of phase separation compared to the rate of aggregation [305]. During cooling the protein network was further reinforced probably by hydrogen bonds as part of the secondary support of the gel network as it was suggested earlier in section *5.3.3*. Thermodynamic incompatibility of protein and polysaccharides arises from differences on their molecular properties such as shape, size or charge [282]. Hence, the different characteristics produced by OP-DE and OP-CA gels may be related to the difference in the extent of phase separation. In the case of OP-DE the rate of phase separation is slower than the rate at which OP forms gel, as no obvious change in the G' development profile was observed during heating. Thus is speculated that dextrin is uniformly distributed within the OP network. On the other hand, OP-CA showed a higher rate of phase separation, as small carrageenan insertions were visible in the protein continuous networks, possibly due to the slower aggregation process, which allowed the formation of carrageenan rich domains embed in the protein network.

The charge density played an important role to determine the extent of phase separation, as it directly impacts the strength of the electrostatic repulsive forces between protein and polysaccharide as well as the degree of protein unfolding. The difference in molecular weight between dextrin and carrageenan may also contribute to such different degree of phase separation by affecting the volume occupied by the polysaccharide, which in consequence impacted the apparent protein concentration in the protein rich phase for gel formation. The excluded volume effect is significantly greater for molecules with a larger size and non-

spherical macromolecules such as linear polysaccharide [306]. Since dextrin had a much lower molecular weight than carrageenan, thus the space occupied by carrageenan was much higher

5.4. Conclusion

The gel strength improvement by addition of dextrin or carrageenan at neutral pH was studied and results suggested that, as polysaccharides have a more hydrophilic character than proteins, the polysaccharide rich domains hold a large amount of water and consequently the protein rich domain has an increased concentration. The improved gel strength observed in OP-DE and OP-CA gels is then associated to the apparent increase in protein concentration as the firmness of a protein gel is determined by the concentration of particles incorporated into the gel network [295]. It was also revealed that different levels of phase separation occurred in the OP-DE and OP-CA gels and the heterogeneity depends on the rate of phase separation compared to the rate of protein aggregation. OP-CA gels showed a higher level of phase separation, as small carrageenan insertions were visible in the protein continuous network due to the slower aggregation process, which allowed the formation of carrageenan rich domains embed in the protein network. Whereas in the case of OP-DE, the rate of phase separation was slower than the rate at which OP forms gel, thus dextrin particles were uniformly distributed within the protein network. Additionally the polysaccharide rich phase exerts a filling effect by occupying the void spaces of the protein network. This is more evident in the case of carrageenan as gels with higher heterogeneity were formed. The different degree of phase separation produced gels with different strength. Moreover, it has been suggested that strong repulsive forces caused by carrageenan addition resulted in a highly order network structure, thus the ensemble of the reactive protein molecules may take

place in an intimate lateral sequence, which permitted the development of hydrogen and hydrophobic interactions to further strengthen the protein networks.

Chapter 6

6. Final remarks

6.1. Summary and conclusions

The endeavor of this research required the improvement of the gelling properties as the previous work of Ma et al. [144,145] has shown that oat protein can form strong gels under alkaline conditions, but at acidic and neutral pH, very weak gels with poor water holding capacity were obtained. This makes the incorporation of oat protein in food products very challenging. For this, different strategies to improved gelling properties of oat protein were investigated in the precedent chapters yielding favorable results. In the following paragraphs the highlights of each chapter are included in the interest of validating the potential of oat protein as an appropriate food gelling agent.

Chapter 1 includes a literature review, which tried to underline the many unique features that make oat a prospective source of plant protein. This review also covered the thermal gelation of proteins and the description of the different gel network structures that can be produce. Also various proposed gelation mechanisms were addressed. The technological alternatives available for further improvement of gelling properties were included as well. Special attention was given to two different strategies for improvement of gelling properties. These strategies included, enzymatic hydrolysis and protein-polysaccharide interactions. Unlike the other alternatives available, these strategies have not been previously used for improvement of the gelling properties of oat protein. Additionally, these two strategies may be regarded by

consumers as more “natural” and thus better accepted. In the last section of Chapter 1 the motivation for this research and the objectives were detailed.

Enzymatic hydrolysis is a desirable tool for the improvement of functional properties of proteins. For this reason in Chapter 2 the utilization of EMR was presented as an interesting alternative for production of protein hydrolysates derived from food ingredients. The combination of enzymatic hydrolysis and membrane technology presents some practical and economical advantages that make this technology suitable for preparation of value added ingredients at industrial scale. Functional properties such as gelation are strongly influenced by the molecular weight of the hydrolysates. Thus, through the application of EMR, the molecular weight of the end product can be carefully controlled. In this chapter the preparation of various value added ingredients from different food components including lipids, proteins and carbohydrates was also discuss. Some examples of the design implemented for the concentration, purification or separation of these values added ingredient such as the implementation of integrated membrane processes which includes sequential filtration process or electro-membrane filtration were also discussed.

In Chapter 3, the effect of partial hydrolysis on structure and gelling properties of oat globular proteins was investigated. A systematic study of the thermal gelation of oat protein and its hydrolysates under different environmental conditions was performed with the underlining objective of improving the gelling properties. The improvement of the gelling properties is enzyme specific. Alcalase and pepsin hydrolysated showed reduced gelling capacity. In contrast, flavourzyme and trypsin hydrolysates could form gels with similar mechanical strength and water-holding capacity comparable to egg white. The acidic polypeptide of the 12S fraction exerted great influence over the gelling ability of oat protein,

as only the hydrolysates with well-preserved acidic polypeptide demonstrated good gelling properties, possibly due to the larger molecular weight of this fraction. Partial hydrolysis with the appropriate enzyme altered the charges on the protein molecular chains, allowing a balance between attractive and repulsive forces at pH 8 and 9 to establish strong three-dimensional gel networks when heated at 110 and 120˚C. Additionally it was suggested that the partial hydrolysis also led to increased exposure of hydrophobic groups that remained in the peptide chains, which allowed development of gel with improved strength via hydrophobic interactions. Both oat protein and the hydrolysate gels exhibited excellent water holding capacity at neutral or mildly alkaline conditions. The results of this study indicate that oat protein have promising potential to be used as a new cost-effective gelling ingredient of plant origin to provide texture and structure in food products.

In Chapter 4 the second strategy for the improvement of gelling properties was addressed. For this the development of protein-polysaccharide interactions, for the enhancement of the gelling properties of oat protein was investigated. Oat protein was mixed with small amounts of inulin and the gelling mechanism of such system was studied by correlating protein structure changes during heating to gel microstructures and bulk properties. Unlike many other globular proteins that aggregate during heating, oat protein dissociated from hexamers down to monomers. In turn, monomers worked as building blocks, which are highly reactive and allowed protein gel network development at a near molecular level, resulting in the development of strong interactions in gel networks with high mechanical strength. Heat treatment also disrupted the disulfide bond that links the acidic and basic polypeptides in the 12S fraction of oat. As a consequence of this, new disulfide bonds were produced during the gel formation process, which further strengthened the gel networks. A strong synergistic

effect was achieved by adding a small amount of inulin into the oat protein gel system. Inulin and oat protein showed thermodynamic incompatibility during heating, causing the system to separate into two different phases, one enriched in protein and the other one enriched in polysaccharide. Consequently, a higher apparent concentration in the protein phase explains the improvement seen in the gelling capacity of oat protein, since a higher number of interactions were developed in the oat protein network. Moreover, inulin contributed to the formation of junction zones in which hydrogen bonds and hydrophobic forces were built at the phase borders, leading to a vastly cross-linked gel network with a reinforced structure. Since mixtures of protein and polysaccharides are often used in the development of food products. It should be noted that strong oat protein-inulin gels were formed at neutral pH when heated at 100˚C. This has greatly broadened the application of oat protein based gels for food and non-food applications as the denaturation temperature of oat protein has been determined to be 120˚C and strong gels were only produced at pH 9 or higher.

Chapter 5, attempted to broaden the scope of application of protein-polysaccharides interactions for improvement of oat protein gelling properties based on the positive results obtained in in Chapter 4. This triggered our interested to systematically investigate the impact of different polysaccharide on oat protein gelling properties. Three different types of polysaccharides with different electric charge were used in order to promote different types of interactions. In this work it was proposed that the thermodynamic incompatibility between oat protein and dextrin or carrageenan arises from differences in the charge density and size of the molecules. Accordingly, gels with different mechanical properties and characteristics were formed with lower or higher degree of phase separation. Rheological measurements of OP-DE during heating showed G' development similar to that obtained by OP, thus it was

speculated that the rate of phase separation is slower than the rate at which OP forms the gel, hence dextrin is uniformly distributed within the OP network. In contrast, a higher rate of phase separation was expected with OP-CA as the aggregation process was greatly controlled by electrostatic repulsion allowing the system to phase separate in a larger extent. This was confirmed as G' values of OP-CA increased at a later time and a higher temperature than OP and OP-DE during the heating process. As phase separation and aggregation processes are simultaneous, the rate at which each process takes place will determine the extent of phase separation reached by a protein-polysaccharide system. Since the addition of carrageenan slowed down the aggregation process due to strong repulsive forces a highly organized structure was formed which also contributed to enhancement of the gel strength. Additionally, phase separation also produced a higher apparent concentration in the protein phase, which in turn increased the number of interactions develop in the oat protein network.

This research confirmed the initial hypotheses, that oat protein gelling properties can be improved by enzymatic modification or by mixing with polysaccharides. In both cases, modification ultimately promotes interaction development in protein gel networks. The protein gel microstructure, mechanical properties, water holding capacity and other properties are closely related to the type of interactions produced after modification. Proper control and modulation of processing conditions, that favor those interactions associated with a strengthening effect, is imperative for the design of novel applications. Basically, the investigation of the gel formation mechanism at a molecular level provided the knowledge necessary to do a rational design. Taking advantage of this, optimized properties for particular applications can be produced.

6.2. Significance of this work

The plant-based protein market is growing rapidly due to the increasing demand for nutritious and healthy food products. Additionally, consumers have a favorable image of a protein-based diet. For many years plant based gelling agents have been incorporated into foods to reduce cost, to improve processing yield, to decrease caloric content or cholesterol and to confer texture and support in foods. This research demonstrates that oat protein gels can be produced under more food appropriate conditions, considering that the precedent work of Ma [144,145] exhibited limited applications. Significant improvement of the oat protein gelling properties was achieved and the scope of application was also expanded to food and non-food applications. To our knowledge, this work is the first to address the improvement of gelling properties through enzymatic hydrolysis or protein-polysaccharide interactions. Furthermore the systematic study of the molecular mechanism of oat protein gelation and the correlation of protein structure changes during heating to physical properties of the gels, allowed the generation of new knowledge associated to the molecular events taking place during the gel structure formation, which is key for the rational design of different textures with specific mechanical, rheological or microstructural characteristics.

The findings from this work may encourage oat producers to promote the utilization of oat as an ingredient for human consumption, which could add economic value to their operations, and also motivate food producers to apply, oat protein as a gelling agent in response to the increasing demand for plant-based proteins, which could increase the profit generated by producers and processors. Ultimately, the stimulated utilization of this crop may also benefit consumers by having more choices, furthermore delivering the inherent health benefits of this crop.

6.3. Perspectives

The initial hurdle for the utilization of oat protein as a food ingredient has to be the extraction process at industrial level. It is necessary to develop sustainable and economical method for isolation of oat protein. Only if derived from a cost-conscious process, oat protein may be considered a cost-effective gelling agent.

Secondly the introduction of oat protein into a multicomponent food system such as comminuted meat products will allow assessment of the multiple interactions with other ingredients as well as the evaluation of the impact that the other ingredients such as fat, starch or meat proteins may have on oat protein gelation in the full system. Consequently sensory evaluation should be conducted in the interest of assessing consumers' acceptance.

Additionally, biomedical applications generate plenty of interest and give great opportunity for oat protein as a biopolymer. Taking advantage of its GRAS character, biocompatibility and biodegradability, multiple uses related to tissue engineering as well as the design of novel drug delivery systems may represent an enormous field of application.

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