Cryogelation of Egg White Protein

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Abstract

Gelation of egg white protein (EWP) solutions can be induced as a consequence of freezing and thawing in the presence of a denaturant (urea). This mechanism of cryogelation can be affected by the relative concentrations of protein and urea, as well as freezing conditions including freezing temperature and freezing duration.

The compression peak force (CPF) of the obtained cryogels was measured using large deformation texture analysis to indicate the gel strength of samples. In the range of added urea concentration from 1M to 6.6M the CPF of samples was seen to decrease, whilst increasing EWP concentration from 5% (w/v) to 15% (w/v) resulted in a strengthening of gel structure with increasing CPF. Lower freezing temperatures (e.g. -30^{o}C) caused a decrease in CPF of samples compared to -18^{o}C and samples stored for 68 hours had higher CPF than those stored for 20 hours (at 1M added urea).

Water holding capacity (WHC) of cryogel was determined by measuring the amount of released water from samples gravimetrically. With increasing concentration of urea the WHC of samples was seen to initially decreased, followed by a progressive increase as the concentration of urea was raised. This trend was observed for most protein concentration with the exception of 5% EWP which showed a significant increase at 4M urea, followed by a drop at higher urea concentrations. Increasing EWP concentrations tended to result in higher WHC in general, although samples with 10% and 12% EWP had relatively similar WHC values. WHC of samples frozen at -30^{o}C was higher than those frozen at -18^{o}C at high urea concentrations. However, increasing the frozen storage time did not appear to affect the WHC.

The microstructure of EWP cryogel was observed using scanning electric microscopy (SEM) and transmission electric microscopy (TEM). The SEM data showed a porous structure for all samples. The increase in the concentration of added urea and the decrease in freezing temperature seemed to reduce the porosity and connectivity of the structure especially at low EWP concentrations. There was also some observed difference of the gel wall thickness between some different samples. The TEM data provided a clear distribution of protein and pores within the gel phase.

The effect of urea on the thermal stability of protein molecules was studied using nano differential scanning colorimetry (nano DSC). Results showed that the addition of urea progressively denatured the protein with increasing urea concentration. Proteins appeared to be further denatured as a consequence of the freezing-thawing process.

The effect of urea addition on freezing point depression and ice content was calculated, allowing the protein content in the unfrozen phase to be determined. The relative concentrations of protein, urea, frozen and unfrozen water in the frozen state provided some indications as to how the extent of denaturation coupled with freeze concentration in the unfrozen phase contributed to the cryogels structures being formed. A number of correlations were determined that assisted in developing a mechanistic understanding of the cryogelation effect. Findings demonstrate a potential means of creating food gel structures with novel structural and material characteristics.

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1 Introduction

Gelation is one of the most important processes present in food, enabling the manipulation of the structure, material properties, physical stability and sensory attributes of many different food systems. Gelation can be defined as the transition from sol to gel material states, in which particles or macromolecules of polymers aggregate, resulting in the assembly of a percolating three-dimensional network in which the solvent (water in most case) is effectively entrapped. Jelly, jam, confectionery products, desserts, etc. are common examples of food products in the market for which the structure comprises the gel state (Banerjee & Bhattacharya, 2012). A range of permitted food hydrocolloids are frequently used in such products and are commonly termed as gelling agents. These are typically based on polysaccharide materials (such as agar, alginates or pectins) or a variety of proteinaceous materials such a gelatine, soy proteins and the proteins comprising milk (Banerjee & Bhattacharya, 2012). In the case of protein gelation, gels derived from assembly of globular proteins play an important part in the food industry because of their nutritional value and the ability to provide particular textural attributes to food derived from their specific network structures (Sun & Arntfield, 2010; Vankleef, 1986).

Egg white provides an example of a composite globular protein system widely used as a food ingredient for its excellent functional and nutritional properties. It has good ability to improve the texture and the consistency of food through gelation (in addition to stabilising other colloidal structural states such as foaming), which can be a medium to deliver flavours and nutrients (Su et al., 2015). Approximately 90% of egg white dry matter is protein (predominantly globular proteins), and which account for the gelation properties of egg white (Lechevalier, Croguennec, Anton, & Nau, 2011). A summary of the process of gelation of egg white involves firstly hydrophobic interactions before sulfhydryl-disulfide reactions and finally, the rapid formation of hydrogen bond, leading to the formation of spherical aggregates and a corresponding large increase in elasticity (Yoshinori Mine, 1995).

There are a number of physical and/or chemical pathways for inducing gelation. Physical gelation usually includes the application of heat or pressure, while the application of acid, ions, enzymes and denaturants like urea, can be considered as chemical gelation (Balny & Masson, 1993; Barbut & Foegeding, 1993; Britten & Giroux, 2001; Ju, Otte, Zakora, & Qvist,

1997; Puyol, Perez, & Horne, 2001; Xiong & Kinsella, 1990b). Likewise, a number of different structural mechanisms are involved in the formation of gels, including hydrophobic interactions, electrovalent cross-linking, covalent cross-linking through formation of disulphide bonds and molecular unfolding.

However, a relatively new form of gel---namely the cryotropic gel, or cryogel, has come under increasing study in the past few decades (Konstantinova & Lozinsky, 1997; Lozinsky et al., 1986; Rodionov, Grinberg, Burova, Grinberg, & Lozinsky, 2015; Vainerman, Lozinsky, & Rogozhin, 1981). Basically, the formation of cryogel is achieved through a freezing-thawing process. In frozen system, there are frozen phase consist of ice crystals of the pure solvent, and unfrozen phase, where solutes are highly concentrated leading to interaction and aggregation, and retention of the gel state on thawing (Carvalho et al., 2014; Lozinsky & Okay, 2014). Hence, it allows gels to be obtained from very dilute solutions thanks to the significantly increased concentration of solutes in the unfrozen phase. Cryogels can have unique combinations of properties such as macroporous structure, chemical and mechanical robustness (Carvalho et al., 2014). Therefore, they have the potential to provide a very different texture and structure from ordinary gels formed in more traditional ways. This phenomenon reveals the opportunities of obtaining structured new food products (Konstantinova & Lozinsky, 1997; Vainerman et al., 1981). Other than that, efforts have been made to use cryogel in encapsulation and removal of bitter taste of citrus juices (Lopez-Fouz et al., 2007; Sowasod, Nakagawa, Charinpanitkul, & Tanthapanichakoon, 2013).

There are mainly four stages of cryotropic gelation processes: ① Preparation of the feed system, ② freezing of the feed, ③ incubation of the gelation system in the frozen state and ④thawing of the frozen system. The conditions of each stage more or less affect significantly the formation of cryogel and thus affect the properties and the structure of resulting materials (Lozinsky & Okay, 2014). Hence, it is necessary to fully understand what specific effects of those conditions have on the cryogelation system in order to control and obtain the desired properties of the formed cryogel.

In order to form cryogels from solutions comprising globular proteins, denaturation of protein molecules is required. Hence, heat and denaturants such as urea have been applied to achieve protein molecular denaturation (Konstantinova & Lozinsky, 1997; Rodionov et al.,

2015; Shimoyamada, Tomatsu, & Watanabe, 1999). In these studies, a number of different proteins, including ovalbumin, bovine serum albumin (BSA) and soybean protein, were used as precursors of cryogelation. The effects of various conditions (e.g. different concentrations of protein and urea, different freezing temperatures and storage durations) have also been investigated. Properties of gel samples such as gel yield fraction, and micro-morphology were measured. A recent study concerning cryogelation of BSA (Rodionov et al., 2015) has highlighted how the concentration of precursor and process parameters affect cryogelation and the relevant gel structure, while research undertaken by Konstantinova & Lozinsky, (1997) has revealed the relationship between initial concentration of urea, initial concentration of ovalbumin and the portion of liquid phase in the frozen samples.

However, because of the combination effects of many factors as mentioned above, the relationship between these affecting factors and properties of cryogels still remains not fully understood. Therefore, the overall aim of this research is to explore the relationship between the properties of resulting cryotropic gel and the conditions of cryogelation processes for the potential application in food by employing cryotropic gel. The specific objectives of this study were as follow:

- To investigate the effects of concentration of urea on the properties of cryogel
- To investigate the effects of concentration of protein on the properties of cryogel
- To investigate the effects of freezing temperature on the properties of cryogel
- To investigate the effects of freezing time on the properties of cryogel
- To understand how the properties of cryogel are affected by the combination of various factors
- To understand the behaviour of protein, urea and water at each stage of cyogelation process

2 Literature Review

2.1 Food gels

A gel in an intermediate material state between liquid and solid which has both flow (liquid) and elastic (solid) characteristics (Banerjee & Bhattacharya, 2012). Typical food products in the form of gel include jellies, jam, confectionery products, yogurt, and desserts (Banerjee & Bhattacharya, 2012). Structurally, a food gel can be considered as a high moisture three dimensional network that is able to resist flow under external forces and more or less, retain mechanical rigidity (Banerjee & Bhattacharya, 2012). The formation of gel requires the presence of a gelling agent which is normally a biopolymeric material (e.g. polysaccharides and proteins), an appropriate solvent in which gelling agents are dissolved and certain conditions (such as pH, heat, pressure, enzyme) to promote molecular interactions leading to gelation.

2.2 Gelation of globular proteins

The nutritional properties and the contributions to food texture arising through the specific gelation of globular proteins play important parts in particular manufactured foods (Sun & Arntfield, 2010; Vankleef, 1986). Gelation is considered as one of the most useful functional properties as it is able to modify structure, physical and material properties (Ikeda & Nishinari, 2001a).

A protein gel is defined as a coherent, percolating protein network formed through physical-chemical interactions such as van der Waals, electrostatic or hydrophobic interactions, as well as formation of hydrogen or covalent bonds. These diverse interactions allow formation of intermolecular bonds that are able to entrap the liquid component, generally water or its solutes providing rigidity and elasticity on the whole system (Baier & McClement, 2005). Protein gels can be highly hydrated, containing up to 98% water (Bhattacharya & Jena, 2007). The specific case of globular protein gelation requires the unfolding of native protein structure by a driving force, before aggregation providing three-dimensional network of aggregates or strands of molecules cross-linked by usually non-covalent bonds or less frequently covalent bonds (Totosaus, Montejano, Salazar, & Guerrero, 2002). The nature of aggregation of proteins depends on conditions of their environment such as pH, temperature, mineral composition, mechanical agitation and so on. With sufficient protein

concentration, aggregation of proteins results in the formation of a three-dimensional network throughout the entire system that leads to the formation of gels (Baier & McClements, 2005).

Interactions in protein gel formation were classified into intrinsic factors and extrinsic factors (Table **2**.**1**) (Phillips et al., 1994, as cited in (Totosaus et al., 2002)).

Table 2.1 Classification of interactions in protein gel formation

Intrinsic factors	Extrinsic factors
Hydrophobicity	Protein concentration
Electrostatic Interactions	pH
Disulphide bonds	Temperature
Molecular weight	Ionic strength and type of Ion
Amino acid composition	Pressure

2.2.1 Formation of protein gelation

The formation of protein gelation can be induced through various mechanisms. The major mechanisms of gelation of proteins are generally categorised into physical and chemical ones (

Table **2**.**2**) and further information regarding these mechanisms are provided in more details in the latter sections.

Table 2.2 Physical and chemical means to induce protein gelation

Physical	Heat	Network formation resulting from unfolded native protein due to heat. Ordered matrix is also formed from the aggregated molecules.
	High pressure	High pressure (200–500 MPa) induces disulphide bonds and hydrophobic interactions between protein molecules, resulting in a rearrangement of gel structure.
Chemical	Ion	Electrostatic repulsion or charges are reduced after initial heating and salt addition, which lead to a gel formation. A hydrophobic effect is induced by disruption of secondary structure.
	Urea	Urea promotes denaturation by decreasing hydrophobic interactions as well being able to induce intermolecular thiol-disulphide oxidation of thiol groups, which forms a network.
	Acid	Slow acidity allows denaturation of proteins to form aggregates and cluster.
	Enzymatic	Enzyme catalyses cross-linking between protein glutamine residues to form a gel structure.

Adapted from Totosars et al. (2002)

2.2.1.1 Heat-induced gelation

Denaturation is required by most globular proteins to form gels, the most common method of which is to introduce heat in protein solution to increase their thermal motion leading to breakdown of intramolecular and intermolecular bonds that contributes to the stability of native structure (Baier & McClements, 2005). Since heat-induced gelation accounts for the structure present in a lot of everyday heat-set foods, it tends to be one of the most commonly studied mechanism relating to foods (Totosaus et al., 2002). The introduction of heat to a globular protein solution initially contributes to the conformational changes of protein molecules, exposing hydrophobic groups to the solvent, which triggers the subsequent aggregation of denatured proteins by means of hydrophobic interactions and/or covalent bonds (Chodankar, Aswal, Kohlbrecher, Vavrin, & Wagh, 2009; Xiong & Kinsella, 1990b). Ferry (1948), as cited in Totosause et al.,(2002), considered that heat-induced gelation followed this two-step mechanism; however, Baier and McClements (2005) considered the formation of heat-induced gel comprised 3 sequential steps which are denaturation, aggregation and cross-linking. Given that the unfolding of protein molecules may be only partial, their particle size is not considerably different from that of native protein molecules, while sulphydryl groups and non-polar regions that are believed to take part in the intermolecular cross-linking flip to the surface of the molecules (as cited in Ikeda (Ikeda & Nishinari, 2001b)).

When it comes to whey protein, heat-induced gels are formed by means of this aggregation process. Notably, the manipulation of the strength of interactions in solutions (e.g. through addition of salt) are able to strongly influence the structure and properties of the resulting gel, with aggregation and network formation resulting from the exposure of initially embedded hydrophobic amino acids and sulfhydryl groups induced by thermal denaturation (Bryant & McClements, 1998; Puyol et al., 2001).

2.2.1.2 Pressure-induced gelation

High pressure causes conformational changes in proteins molecules, also leading to denaturation or aggregation, depending on the conditions and the protein system in which high pressure is applied (Balny & Masson, 1993; Dumay, Kalichevsky, & Cheftel, 1998; Smith, Galazka, Wellner, & Sumner, 2000). It was believed that structures of protein gels formed by means of high pressure involved mainly hydrogen bonds rather than disulphide bonds

(although these may also be present under certain high pressure conditions), resulting in very different gels to those produced by heat (Angsupanich, Edde, & Ledward, 1999). Other researchers suggested that the disruption of hydrophobic groups and ionisation of charged groups caused by an increase in electrostriction and which has been considered the major contribution to gelation upon pressurisation (Briscoe, Luckham, & Staeritz, 2002). This hypothesis also considers a lesser disruption of hydrogen bonds (Briscoe et al., 2002). High pressure-induced gels offer an additional mean for modification of food texture as they tend to be softer but resistant to breaking compared to heat set equivalents (Hoover, 1993).

Taking a specific example, the gelation time of 13w/v% milk protein concentrates was reduced significantly (from 53300s to 7500s) when pressures of up to 1000 bar were applied (Briscoe et al., 2002). Also, it was firstly shown that concentrated milk with a solid content >25%(w/w) can form gel by the application of high pressure of >250 MPa (Kumeno, Nakahama, Honma, Makino, & Watanabe, 1993).

2.2.1.3 Acid-induced gelation

Whey protein at neutral pH is highly soluble forming solutions with low viscosity due to strong electrostatic repulsions between molecules. Acidification towards the isoelectric point of the protein does not itself result in gelation, but the screening of repulsive charges does allow greater approach between molecules (Bryant & McClements, 1998). Gelation of protein induced by acid usually still requires a mechanism to promote molecular bonding, e.g. heating. Acidification after heat treatment also lowers the negative charges leading to weakened electrostatic repulsion that can likewise allow aggregation and gelation (Alting, de Jongh, Visschers, & Simons, 2002).

2.2.1.4 Ion-induced gelation

Repulsive forces are diminished with the addition of ion to protein solution, generating protein-protein associations such that self-supporting gels can be formed more readily in combination with other mechanisms (Totosaus et al., 2002). For example, the addition of salts was observed to enhance the formation of thermocoagulum of egg albumin and the effects followed the lyotropic series (Shimada & Matsushita, 1981). For bovine serum albumin, the formation of coagulum was enhanced by salts of higher order on lyotropic series but inhibited by those of the lower order when the concentrations of salt were

increased. For soybean protein coagulum, the turbidity was increased at alkaline pH, with an inhibited thermocoagulation (Shimada & Matsushita, 1981). The study also reported that salts affected the coagulum formation of egg albumin in the order of SO_4^{2-}> $C1^-$> Br^- > I^- > $SCN^-$and Ca^{2+}>>Li^+>Na^+≈Cs^+, while the order followed as SO_4^{2-}> $C1^-$> Br^- and Ca^{2+}>>Li^+>Na^+ ≈Cs^+ when it comes to bovine serum albumin. On the other hand, NaCl was showed to stabilize pea protein molecules against thermal denaturation as NaCl contained samples had higher T_d and △H values (T_d = 94.28 ^oC, △H =17.84J/g protein) than NaCl extracted samples (T_d = 86.21 ^oC, △H =15.81J/g protein) (Sun & Arntfield, 2010).

The concentration of ion content also affects the gelation properties. The compressive strength of β-lactoglobulin gel has been revealed to be maximum at 200mM NaCl, while the maximum gel strength was obtained at 10mM when $CaCl_2$ was added instead, which is much more effective in increasing gel strength than NaCl (Mulvihill & Kinsella, 1988). Moreover, 3% whey protein isolate (WPI) diluted from 4-9% WPI preheated at 80 ^oC for 30 mins was shown to form gel with the presence of 20mM $CaCl_2$ (Ju & Kilara, 1998).

2.2.1.5 Urea-induced gelation

Urea as a protein denaturant has been considered as a possible alternative approach to induce protein gelation by changing the conformation of protein molecules other than heat treatment (Xiong & Kinsella, 1990b). Although urea destabilizes hydrogen bonds and hydrophobic interactions in proteins, other forces like SH=SS (sulphydril/disulphide) interactions and sulphydryl oxidation account for the mechanism of urea-induced gelation (Xiong & Kinsella, 1990a). It was proposed by Huggins et al. (1951)as cited in (Totosaus et al., 2002), that a chain reaction between protein sulphydryl and disulphide groups allows a reticulum with intermolecular protein disulphide bonds to be knitted to the protein within the framework, leading to gel formation.

Studies have shown that egg ovalbumin and bovine serum ovalbumin (BSA) are able to form gels with concentrated urea such as 8M/L at temperatures as low as 30-40^oC (Frensdorff, Watson, & Kauzmann, 1953; Huggins, Tapley, & Jensen, 1951). Whey protein isolate (WPI) at a concentration of 11% has been shown to spontaneously form a gel in the presence of 6M/L urea (Xiong & Kinsella, 1990b).

2.2.1.6 Enzyme-induced gelation

Enzyme-induced gelation refers to either gelation arising from hydrolysis (e.g. protease enzymes) or through cross-linking between protein chains that leads to formation of intermolecular stranded networks (e.g. through use of transglutaminase) (Totosaus et al., 2002).

One example of proteolytic gelation is for 0.5% pre-heated WPI or 2% untreated WPI, which is able to form gels as induced by 1% protease from Bacillus licheniformis (BLP) upon incubation at pH=7 and 50^{o}C without addition of salts (Otte, Schumacher, Ipsen, Ju, & Qvist, 1999). The enzyme-induced gelation process was enhanced significantly as temperature, protein concentration and enzyme concentration increased, while the presence of up to 100mM NaCl and 15mM $CaCl_2$ decreased the gelation time (Otte et al., 1999).

2.3 Factors affecting gel properties

2.3.1 pH

Altering pH value affects the efficiency of gelation. The effects of pH on gelation of whey protein concentrate were investigated at pH values of 5.2, 5.9 and 7.0 and at 71^{o}C. Gelation time, as the function of protein concentration, decreased more at higher pH values (Taylor, Gladden, & Fryer, 1994). For heat-denatured WPI with the presence of protease, as pH increased from 6.2 to 8.0, gel time increased gradually from about 10 mins to 30 mins, while rate of gelation increased from 0.4 min^{-1}at pH 6.2 to 0.6 min^{-1} at pH 6.8, before dropping back to 0.4 min^{-1} at pH 8.0. In addition, gel strength increased from around 1000 Pa at pH 6.2 to 2600 Pa at pH 7.8 (Otte et al., 1999).

The turbidity of formed gels is also affected by pH. For example, as pH is increased from 2.5 to 6.0, the turbidity of some gels, as determined by absorbance measurements made at 500nm, dropped dramatically from 2.5 at pH 4.0 to near 0 at pH 4.5-5.0. As for other gels, the turbidity increased from 0 to around 2, before decreasing to 0 again (Alting et al., 2002).The absorbance of 0.045% w/v native whey protein solution and pectin solution at 600nm was 0 at pH 1-4, which indicated that there was no aggregation at pH below 4. The absorbance of solutions of heated whey protein and pectin with different ratios all

decreased with various degrees from about 1.5-2.2 to near 0 as pH decreased from 4 to 1 (Li & Zhong, 2016).

2.3.2 Temperature

Gel properties, and in particular their mechanical behaviour, can be greatly influenced by the thermal conditions applied during the gelation process. For example, the material properties of three kinds of gels [WPC, M (WPC/λ-carrageenan (λ - C) mixtures), DM (WPC/ λ -C spray dried mixtures) were investigated at two heating temperatures (74^{o}C and 80^{o}C) (Spahn, Baeza, Santiago, & Pilosof, 2008). The hardness of the three gels at 80^{o}C was obviously higher than at 74^{o}C. The springiness of WPC and M gels at 80^{o}C was slightly higher than their counterparts at 74^{o}C (about 0.10 for WPC and 0.05 for M), while that of DM gel at 74^{o}C (0.87) was slightly higher than that at higher temperature (0.80). The adhesiveness of DM and M gels increased from around 4.8 and 5.0 g.s at 74^{o}C to 6.5 and 7.5 g.s respectively, while that of WPC gels slightly decreased from 6.8 to 6.5. The three gels all have stronger cohesiveness at 80^{o}C varying from 0.62 to 0.75.

In the case of milk concentrates at ambient pressure, as temperature of milk concentrates (16% w/w) increased from about 313 k to 349 k, gelation time gradually decreased from over 50000s to 5000s (Briscoe et al., 2002).

Water holding capacity (WHC) has also been shown impacted by temperature. As the temperature increased from 70^{o}C to 90^{o}C during the heat treatment process of gelation of native amaranth protein isolate, WHC barely changed (all at slightly more than 80% level) (M. V. Avanza, M. C. Puppo, & M. C. Anon, 2005). However, when temperature increased to 95^{o}C, WHC considerably decreased to about 72%.

2.3.3 Protein concentration

Protein concentration is widely reported as having a major contribution to gel strength. An example is a study of gel properties prepared using pea protein isolates (PPI) for which the protein concentration was increased from 4 to 18%, and for which elastic modulus was shown to scale with protein concentration, resulting in stronger gels (Sun & Arntfield, 2010). This is because higher protein concentrations provide increased opportunities for cross-

linking of protein molecules (Sun & Arntfield, 2010). Similarly, the increase in the concentration of both pre-heated and untreated WPI (from 2% to 9%) was found to improve gel strength (with storage modulus G' increased from 0 to 400 Pa and 0 to 3600 Pa respectively), as well as to decrease gel time and reduce the rate of gelation (Otte et al., 1999). In a third example, the gel strength of amaranth protein gels also increased dramatically as protein concentration increased from 5% (G'=0 Pa) to 15% (G'=700Pa with proteins after heating at 90^{o}C and G'=4000Pa with proteins after heating at 90^{o}C and after cooling at 25^{o}C) (M. Avanza, M. C. Puppo, & M. C. Anon, 2005). In addition, higher concentration of native amaranth protein isolate (in the range of 5-20 w/v%) has been found to provide better water holding capacity, which increased from about 60% to 90% at both 90^{o}C and 95^{o}C (M. V. Avanza et al., 2005).

2.4 Characterization of gels

2.4.1 Rheological characterization

Gelation represents a change in material state from sol to gel and this transition can be readily characterised in terms of measurement of mechanical properties through the use of various rheological techniques, which can in turn be related to microstructural characteristics (Banerjee & Bhattacharya, 2012). It is important to note that instrumental food rheology measurement systems can be broadly categorised into empirical, fundamental and imitative tests (

Table **2.3**).

Mechanical properties can be measured for both small and large deformation (Spahn et al., 2008). Small deformation properties are measured with non-disruptive methods, which determine the intrinsic mechanical behaviour of the gel system, while large deformation properties are tested by tension or compression (Stading & Hermansson, 1991) and are more commonly used to study the change in mechanical properties as a consequence of structural failure and the breaking of bonds under an applied force.

Table 2.3 Rheological and textural measurements of gels

Nature of test	Type of measurement	Instrument used	Measurement parameters	Applications	References
Fundamental tests	Compression	Texture measuring system	Modulus of elasticity, Poission's ratio	Surimi gel	(Kim, Decker, & McClements, 2006)
	Stress relaxation	Texture measuring system	Residual stress, relaxation time	Gellan gels	
	Creep	Controlled stress rheometer	Shear modulus, creep compliance	Soy and gelatin gels	(Kamata & Kinsella, 1989)
	Oscillation	Controlled stress rheometer	Storage modulus (G), loss modulus (G),phase angle, complex modulus and viscosity	Viscoelastic characterization of rice, soy gels, mixed gels	(Bhattacharya & Jena, 2007; Jena & Bhattacharya, 2003; Keogh, Laine, & Oconnor, 1996; Kim et al., 2006)
Empirical	Puncture force	Texture measuring system	Puncture Characteristics	Characterization of rice gel	(Jena & Bhattacharya, 2003; Kim et al., 2006)
	Compression	Texture measuring system	Peak force, firmness, compression energy	Measurement of gel quality and gel strength	(Kim et al., 2006)
Imitative	Texture profile analysis (TPA)	Texture measuring system	Parameters of texture profile analysis like hardness, brittleness, adhesiveness, springiness, cohesiveness	Food gels	(Pons & Fiszman, 1996)

organized by Banerjee & Bhattacharya (2012)

2.4.2 Structural characterization

Structural characterization plays an important role in gel characterization as the structure of samples determines the gelation properties. Relevant characterizations include heat flow measurements, microscopic characterizations and molecular characterizations (

Table **2**.**4**)

Table 2.4 Structural measurements of gel

Type of measurement	Instrument used	Measurement parameters	Applications	References
Structural Characterization	Differential scanning calorimeter (DSC)	Heat flow	Gellan and polyvinyl alcohol blend film	(Sudhamani, Prasad, & Sankar, 2003)
	X-ray diffraction and SAXS	Particle size analysis	Nano delivery system in food	(Luykx, Peters, van Ruth, & Bouwmeester, 2008)
	Colorimeter	Calorie measurement	Gellan edible film	(Leon, Lamanna, Gerschenson, & Rojas, 2008)
Microscopic characterization	Light microscopy (LM)	Area of the granules	Tapioca starch gel	(Vittadini, Carini, & Barbanti, 2006)
	Scanning electron microscope (SEM)	Structural arrangement of components	Macro- and microstructure of cryogels	(Konstantinova & Lozinsky, 1997)
	Transmission electron microscopy (TEM)	Structural distribution of constituents	The protein network of concentrated skimmed milk and milk-sugar mixtures in pressure-induced gelation	(Keenan, Young, Tier, Jones, & Underdown, 2001)

	Atomic force microscope	Structure of the molecules	Structural characteristics of nanoparticles	(Luykx et al., 2008)
	Confocal laser scanning microscopy (CLSM)	microstructure of gels	microstructure of gels of protein and pectin mixtures	(Li & Zhong, 2016)
Molecular Characterization	NMR	Conformation changes on gelation	Structural features of the constituents	
	FTIR	Molecular structure	Infrared spectra of the components	(Sudhamani et al., 2003)
	FT-RAMAN; Near infrared resonance	Molecular Characterization	Functional characteristics of pectins	

modified from the form organized by Banerjee & Bhattacharya (2012)

2.4.3 Other properties and their characterization

2.4.3.1 Water-holding Capacity (WHC)

Microcentrifuge-based water-holding tests can be used to determine WHC of gels as demonstrated by the characterisation of whey gels by (Kocher & Foegeding, 1993). Figure **2.1** shows the schematic of this method. Held water curve was recorded as the function of g force at constant time of 10 minutes.

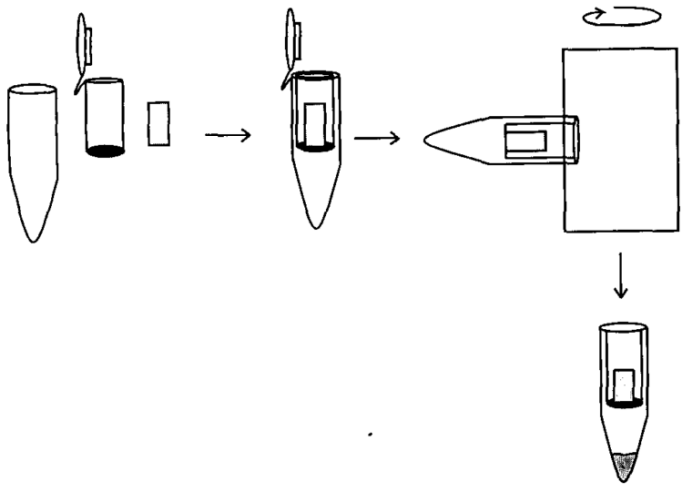


Figure 2.1 Schematic of Micocentrifuge-based water-holding method

Also, WHC was determined by means of NMR (Hinrichs, Gotz, & Weisser, 2003). A low-resolution NMR spec-trometer system, MINISPEC mq20 (Bruker AnalytikGmbH, Rheinstetten, Germany) was used for T_2 experiments at 20 MHz as resonance frequency of 1H, while a magnet SWB 200 (BrukerAnalytik GmbH, Rheinstetten, Germany) was used to

perform diffusion experiments. In this technique, it is believed that the larger the relaxation times, T_2, the larger the mobility. T_2 is also analogous to the voluminosity of solutions.

In the study of heat-treated collagen, approximate 5 g of gel samples were centrifuged at 10,000g at 25^{o}C for 10 minutes. The WHC of gels were calculated using equation [2.1] (de Moraes & Cunha, 2013):

$$\mathrm{WHC} = \frac{W_{sample} - W_{releasedwater}}{W_{sample}} \times 100 \quad [2.1]$$

where $w_{releasedwater}$ is the mass of water released after centrifugationand w_{sample} is the initial mass of sample before centrifugation.

2.4.3.2 Aggregation properties

Aggregation properties of gels have been studied spectrophotometrically by measuring the absorbance using a UV/Vis spectrophotometer (Li & Zhong, 2016) to provide an indication of change in turbidity. An example of this is the absorbance measurements made at 600 nm of a 0.45% w/v native whey protein solution, which were shown to decrease from 1.5 to about 0.2 as pH decreased from 4 to 1.

The differences in molecular weight of β-Lg and WPI aggregates with different treatment were determined by using SDS-agarose continuous gelelectrophoresis (0.4% (w/w) agarose) (Alting et al., 2002). In the study of pH-Induced cold gelation of whey proteins, turbidity of gels as the absorbency at 500 nm was performed on a Cary 1E UV-Visspectrophotometer (Varian) equipped with a temperature controller at 20^{o}C and at 1.5% protein concentration (Alting et al., 2002).

Minimum gelling concentration has also been studied as one of the aggregation properties of protein gels. It is considered to take takes one protein molecule to denature, two or more to aggregate and many more to form a gel matrix (Clark & Ross-Murphy, 1987). Therefore, it requires concentrations an order of magnitude higher than from gelatin or gel forming carbohydrates to obtain gel from globular proteins (as cited in Sun & Arntfield, 2010). In Sun and Arntfield's study, proteins samples at a specific range of concentration were used to undergo a gelation process, after which the tubes containing gelled samples were inverted

and the one with lowest concentration that did not flow was considered as the minimum gelling concentration

2.4.3.3 Sensory properties

Another important characteristic of gels is their sensory properties, as different gels may result in different touch feelings. Several specific sensory properties of gels have been tested. For example, as the method suggested by Drake and Gerard (1999), sample was placed on the first two fingers of the hand and pressed between the two fingers and the thumb. Stickiness was determined using the degree of samples sticking to the fingers by pressing the samples in the hand for 3 to 6 times. In another study, hardness evaluation was determined by pressing the samples between thumb and forefinger to find whether hard or soft, while elasticity was evaluated by manually twisting samples to find whether elastic or inelastic (Bhattacharya & Jena, 2007).

2.5 Egg white gelation

The excellent functional properties of egg white, especially its ability to improve the texture and consistency of food through a formed gel, which also provides a medium to deliver flavours and nutrients, makes it an important ingredient in food (Su et al., 2015). Egg white thus plays an important part in food industry such as surimi products and some other meat products (Badii & Howell, 2006; Dawson, Sheldon, & Ball, 1990). Other than proteins, egg white is also an important source of iron, unsaturated fatty acids, phosphorus, trace minerals and vitamin A, B, E and K (Y. Mine, 2007).

2.5.1 Compositions of egg white

More than 90% of dried egg white matter is comprised of a mixture of predominantly globular proteins, with carbohydrates accounting for only 0.5-0.6% (Lechevalier et al., 2011; Yoshinori Mine, 1995). About 55% of the total carbohydrate is located within the protein structures (Strixner & Kulozik, 2011). The amount of lipid in egg white powder is 0.01%, which is negligible compared with egg yolk (Strixner & Kulozik, 2011). Table 2.5 and Table 2.6 shows the major constitutes of egg white protein and their physicochemical and functional properties (Strixner & Kulozik, 2011), while Table 2.6 indicates their thermal denaturation properties.

Table 2.5 Composition and physicochemical properties of the major egg white proteins (according to Tilgner, 2009)

Protein fraction	Rel. amount [%]	SH/SS [–]	Molecular weight [kDa]	IEP [–]	Glycosylated [–]
Ovalbumin	54	4/2	45	4.6–4.8	✓
Ovotransferrin	12–13	0/15	76–77.7	6.1–6.6	✓
Ovomucoid	11	0/9	28	4.1	✓
G2 Globulin	4.0	–	40–49	5.5	✓
G3 Globulin	4.0	–	49–58	4.8–5.8	✓
Lysozyme	3.4–3.5	0/4	14.3	10.7	–
Ovomucin	1.5–3.5	–	230–8300	4.5–5.0	✓

Table 2.6 Denaturation temperature TD [°C] for the major egg white proteins in the native and isolated status measured by differential scanning calorimetry (Tilgner, 2009)

	Denaturation temperature TD (^{o}C)			
	In the native form (egg white)		In the isolated form	
Protein fraction	pH 7	pH 9	pH 7	pH 9
Ovalbumin	84.5	84.0	84.0	84.0
Ovotransferrin	65.0	69.5	61.0	62.0
Al^{3+} Ovotransferrin	76.5	–	73.5	72.5
Ovomucoid	–	–	79.0	79.0
Lysozym	74.0	–	75.0	72.5
Globuline	–	–	92.5	–

2.5.2 Gelling properties of egg white powder

Proteins are responsible for gelling properties in egg white. The success of many baked or cooked food products depends on the coagulation of proteins, particularly the irreversible heat coagulation of egg white proteins (Strixner & Kulozik, 2011). The modification of

stability of protein in solution - for example, the disruption of the equilibrium between repulsive forces (electrostatic, steric) and attractive (van der Waals), causes gelation (Lechevalier et al., 2011). Heat induced gelation of egg (white) is the most widely encountered process for forming gelled structured within food preparation and manufacturing. The interactions involved in heat induced gels of egg proteins are predominantly electrostatic and some observable highly energetic interactions (Lechevalier et al., 2011).

All egg white proteins coagulate when heated, except for ovomucoid and ovomucin (Johnson & Zabik, 1981). The contribution to egg white thermal gelation is mainly from ovalbumin (the predominant protein fraction) and ovotransferrin (Lechevalier et al., 2011).

2.5.2.1 Effects of ions

There are effects of ions on gelling properties of egg white. As indicated in Section 2.2.1.4 for conditions of high ion strength during heating process, the combination of electrostatic charge screening coupled with increased hydrophobic interactions can increase molecular resistance to denaturation, but can promote intermolecular interactions between molecules (Doi, 1993). Conversely, low ionic strength conditions amplifies electrostatic repulsions, favouring denaturation, but decreasing propensity towards aggregation through intermolecular interaction (Raikos, Campbell, & Euston, 2007) (Lechevalier et al., 2011). As a result, the high ion strengths tend to lead to random aggregation of partially denatured proteins (opaque gels), while linear polymeric aggregation tends to occur in low ion strength conditions (transparent gel) (Figure **2**.**2**).

Gelling properties of egg white proteins are also affected by the type of salts. For example, it was shown that the addition of low concentration of $CaCl_2$ decreased the denaturation temperature of ovalbumin (Hegg, 1979). Also, Ca^{2+} decreased the homogeneousity of egg white gels by decreasing the string of beads structure (Croguennec, 2002). Mg^{2+} has similar action on egg white and ovalbumin gels to Ca^{2+} (Mine, 1995). For the addition of Fe^{3+}, it was shown to provide a uniform, fine-structure gel with decreased particles as well as to increase the denaturation temperature of ovotransferrin (Croguennec, 2002).

Figure 2.2 Effect of protein charge on thermal gelation of egg white (Doi, 1993)

2.5.2.2 Effects of protein concentration

Like gels of other proteins mentioned at Section 2.3.3, gel properties of thermally treated egg white are strongly dependent on concentration. For example, egg white gel was found to have higher rigidity ratio values (1.4) at low concentration and shift to lower value (around 1) at high concentration, which indicates the gel is more elastic as egg white protein concentration increases (Foegeding, Li, Pernell, & Mleko, 2000). It is also suggested that over a critical gelation concentration, gel firmness increases as protein concentration increases (Lechevalier et al., 2011).

2.5.2.3 Effects of pH

The influences of pH on heat-induced egg white gels have been studied by some researchers. One interesting effect of pH is on the microstructure of heat-induced egg white gels. For example, microstructure of gel samples at various pH was also revealed using SEM and TEM in the same study (Figure **2.3**), indicating the effects of pH on the aggregation of egg white protein molecules (Handa, 1998). Similar SEM micrographs were obtained (Croguennec, Nau, & Brule, 2002). Both studies have shown that the gel microstructure is coarser at pH 5 and 7(Figure **2.3** and **Figure 2.4**). An earlier research also agrees with this finding, where the three dimensional network of heat-induced egg white gel at pH 5 examined with SEM was found to be coarse and random with large irregular shape voids (Woodward, 1985). Handa's study also included the effects of pH on other properties such as textural characteristics, where

hardness, chewiness, elasticity, etc. were maximum at pH 11, and water holding capacity that was observed higher at alkaline pH.

Figure 2.3 Scanning electron micrographs (left) and transmission electron micrographs (right) of egg white gels at vari-ous pH: A. pH 3; B. pH 5; C. pH 7; D. pH 9; E. pH 11; F. pH 7 with 10% SDS. Bar=100 nm (Handa, 1998).

Figure 2.4 Effect of pH on egg white gels microstructure at μ 120 mM ionic strength observed by transmission electronic scanning microscopy (×5000): a) pH=5; b) pH=7; c) pH=9 (Croguennec et al., 2002)

2.6 Cryotropic gelation

The term “cryotropic gelation” is derived from the Greek kryos (frost) and tropos (cause), which refers to the gel formation caused by cryogenic treatment (freezing-frozen storage-thawing) of the precursor system (Lozinsky & Okay, 2014). In cryotropic gelation, gels are assembled at temperatures below the freezing/melting point of the initial liquid system. Early research on cryotropic gelation demonstrated that the freezing of myofibrillar proteins

resulted in not only a lower solubility of these proteins post-thawing, but under certain conditions also resulted in the formation of thermally stable gels with anisotropic or isotropic structure providing mechanical rigidity and a retained predetermined shape (Rogozin, Slonimskij, Rogovina, Vajnerman, & Pivovarov, 1981). This phenomenon indicates the potential of obtaining gelled protein structures using alternative processing pathways. This study of proteins assembles into coherent structured under freezing conditions and the factors contributing to this effect has been termed “cryostructurization” (Vainerman et al., 1981).

2.6.1 The mechanism of cryogelation

The cryogel system is formed heterogeneously under freezing conditions, and consists of a solid phase (frozen crystal solvent which is usually solute free) and unfrozen liquid microphase (Carvalho et al., 2014). The unfrozen content usually contributes the amount of 0.1-10% of the total sample (Gusev, Lozinsky, Vainerman, & Bakhmutov, 1990; Konstantinova & Lozinsky, 1997; Lozinsky, Golovina, & Gusev, 2000) and contains any added solutes. When the initial solution or sol-gel forming precursor is frozen, “cryotropic gelatinization,” cryo-gelling or cryo-structuration occurs (Carvalho et al., 2014). Crystallization of the solvent is the dominant factor that differentiates between cooling-induced gelling and cryogelatinization (Carvalho et al., 2014). As with other forms of gelation, cryogelation requires the presence of a gelling component in the initial solution (typically a biopolymeric species, noting that not all biopolymers will have a propensity to form cryogels). For biopolymer solutions capable of forming cryogels, the formation of the gel structure follows the pathway described in Figure **2.5** (Carvalho et al., 2014). For a frozen system, there should be a retained fraction of non-frozen solvent, where precursor and reagent are concentrated and formation of gel takes place. The concentrating of the component in the unfrozen phase is the reason to explain the formation of cryogel from very dilute solution of polymers (Konstantinova & Lozinsky, 1997). This so-called unfrozen liquid microphase (UFLMP) is formed, while the solvent starts to crystallize (Carvalho et al., 2014). Gelation occurs within this domain, and the gel structures are subsequently retained on thawing of the sample.

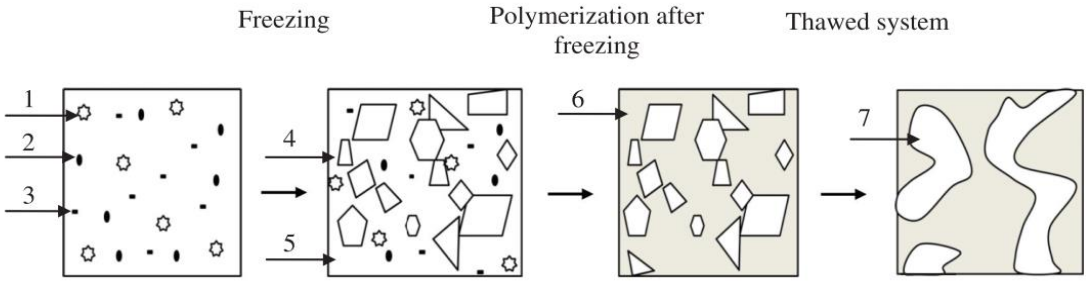


Figure 2.5 Main scheme for the formation of polymeric cryogels: 1, precursor; 2, solvent; 3, reagent; 4, polycrystals of frozen solvent; 5, unfrozen liquid microphase (ULMP); 6, cross-linked polyme

The formation of cryotropic gels from globular proteins typically requires pre-denaturation or *in situ* denaturation of precursors. The ways of denaturing include the addition of denaturant, for example, urea in ovalbumin solutions (Konstantinova & Lozinsky, 1997) and bovine serum albumin (Rodionov et al., 2015), as well as heat denaturation. e.g. as applied in soymilk and soybean protein (Hashizume, 1978; Shimoyamada, Koseki, Yamauchi, & Watanabe, 2002). In the case of cryogelation of ovalbumin solutions, it was suggested that under frozen condition, partial denaturation of protein globules is sufficient for the formation of aggregation and cryogelation of ovalbumin molecules (Konstantinova & Lozinsky, 1997).

Cryotropic gelation has been studied in detail for synthetic polymers, where it was suggested that the formation of cryotropic gel of thiol-containing polymers results from SS bonds cross-linking the polymer chains (Vainerman et al., 1981). Structures and functional properties of macromolecules of proteins are considered to be much more complicated than those of synthetic polymers (Konstantinova & Lozinsky, 1997).

2.6.2 Characterization of cryogels

2.6.2.1 Gel fraction and Gel fraction yield

In the case of cryogels, which can be structurally inhomogeneous, it can be challenging to measure experimentally the real content of gel phase, which refers to the separate system containing the crosslinking polymer and immobilized solvent within the whole sample volume (Lozinsky & Okay, 2014). That being said, some methods have been developed to determine what is called “gel fraction yield” of cryogels.

The gel-fraction yield (Y) was calculated using a formula by Rodionov and the others (2015) using equation [2.1]:

$$Y = (m_d : m_{th}) \times 100\% \quad [2.1]$$

Where m_d is the weight of the dried sample and m_{th} is the 'theoretical' weight of the sample calculated by assuming that all the protein containing in the feed solution was incorporated into the 3D network of the resultant BSA-cryogel (Rodionov et al., 2015).

In Konstantinova and Lozinsky's study (1997), The yield of gel fraction is the ratio $((C_0 - C_s)/C_0) \times 100\%$, where C_0 is the concentration of the protein in the solution before freezing and C_s, is the concentration of the soluble protein in the solution after removal of the insoluble cryogel by filtration through a glass filter. The concentrations Co and Cs were measured using a biuret method at a wavelength of 540 nm with a Specol 221 spectrophotometer.

2.6.2.2 Liquid phase measurement

The study on the liquid phase of cryogel is of importance as it indicates the structural properties of the samples. A measure of cryotropic gel water holding capacity can be calculated by determining the weight of water released from the thawed gels (Shimoyamada et al., 2002).

Non-frozen water in frozen samples has also been measured using ^{1}H NMR, which showed that the concentration of urea instead of protein affects the liquid phase content in frozen samples (Konstantinova & Lozinsky, 1997). In this particular study the volume of unfrozen water was found to increase as the urea concentration increased due to the freezing point depression influence of the added urea.

2.6.2.3 Structural characterization

Electronic microscopies are commonly used to intuitively observe the microstructure of cryogel, while other techniques have been applied to study the denaturation of protein molecules as the precursors of cryogel.

The structures of cryotropic gels can be viewed using scanning electron microscopy (SEM) (Aguilera & Rojas, 1996; Carvalho et al., 2014; Konstantinova & Lozinsky, 1997), enabling

variations in the structure and distribution of the porous matrix to be visualised. Some examples of SEM and TEM images of cryogels are shown in Figure **2**.**6** and Figure **2**.**7**.

Figure 2.6 Scanning electron micrographs of cryogels. The cryogels were obtained by freezing-thawing of 5% ovalbumin solutions containing urea at concentrations of I mol/dm3 (A and C) and

2.5 mol/dm3 (B and D). The time of storage in the frozen state at 262 K was 24h (Konstantinova & Lozinsky, 1997)

Figure 2.7 Electron micrographs (TEM) of cryoPVAG obtained from 10 % solution of 16/1 mark PVA at -10oC for 1 day: a) preparative technique of freezing-drying; b) preparative technique of contrasting with phosphorus-tungsten acid (Lozinsky et al., 1986).

2.6.2.4 Circular dichroism (CD) and ultraviolet (UV) spectra

CD and UV spectra have been used to determine the extent of denaturation of ovalbumin macromolecules in ovalbumin solutions containing urea after frozen-storage-thawed process (Konstantinova & Lozinsky, 1997), which indicated the degree of denaturation of studied molecules by showing the comparison between initial solutions and frozen-thawed solutions in the spectra graphs.

2.6.3 Factors affecting gelation properties of cyogels

2.6.3.1 Concentration of components in initial solutions

Pores of gels formed from solution containing 5% ovalbumin and 2.5mol/L urea were observed to be bigger than those from solution containing the same concentration of ovalbumin and lower concentration of urea (1mol/L) (Konstantinova & Lozinsky, 1997). However, it has been shown in the same study that urea concentration does not affect the yield of gel fraction as it grows sharply first with the increase of urea concentration in the low range yet remains unchanged at 92-95% when urea concentration reaches a critical point.

With a different method of measurement from the one indicated in Section 2.6.2.1, the increase in urea concentration from 0.5 to 2.5mol/L led to a decrease in yield of gel fraction from 91.0% to 78.7%, while there was a maximum in the swelling degree through this series (Rodionov et al., 2015).

In the case of bovine serum ovalbumin (BSA) solution containing 1.0mol/L urea and 0.01mol/L Cys after frozen at -15^{o}C, the swelling extent of the gel phase decreased from 1.92±0.07 to 1.79±0.07 as the concentration of BSA in the initial solutions increased from 3.0 to 5.0 g/dL, coming with stronger gels, while the yield of gels fraction did not significantly dependent on BSA concentration (Rodionov et al., 2015).

2.6.3.2 Freezing temperature

Yield of gel fraction increased gradually from 20% to about 30% in the range of -2~-8^{o}C, while a sharp increase was observed around -8^{o}C, followed by a stable amount as the temperature was lower than -8^{o}C (Konstantinova & Lozinsky, 1997).

There is evidence showing that when freezing temperature was decreased in the range of -15~-25^{o}C at constant concentrations of urea and bovine serum albumin (BSA), there was weaker formed gel, as well as smaller gel fraction yield and bigger swelling extent of gel phase (Rodionov et al., 2015). In addition, the decrease in size of macrospores in samples was observed when temperature was lowered from -15^{o}C to -20^{o}C (Rodionov et al., 2015). As indicated by the author, the reduced size of macrospores is due to the smaller porogen particles at lower cryostructuring temperatures. However, the pore size was the biggest at relatively medium frozen temperature of -15^{o}C in the range of -10^{o}C to -30^{o}C for chitosan cyogels, because of the interference of supercooling phenomenon (Lozinsky, 2014).

2.6.3.3 Storage time

Yield of gel fraction of cryogels from 5% ovalbumin solution containing 1mol/L urea at various temperatures increased from 0 to 80-98% as storage time was increased to about 100 minutes, yet remained stable as storage time was longer (Konstantinova & Lozinsky, 1997). This observation reflects the time it takes for the component in the unfrozen phase to fully interact under specific conditions

2.6.3.4 pH of initial solutions

For 2.5% ovalbumin solutions, the yield of gel fraction is sensitive to pH in the presence of urea at low concentration (0.125mol/L), which reaches maximum when pH<4 and is minimal at 6<pH<9, while it does not dependent on pH with urea>0.5mol/L (Konstantinova & Lozinsky, 1997). According to the author, different from the effects of pH on heat-induced egg white gels at positive temperatures mentioned at Section 2.5.2.3, the reason of its effects on egg white cryogels is assumed to be the lack of sufficient denaturation of all the proteins when it comes to low concentration of urea, which leads to the sensitivity of the system to acid denaturation.

2.6.3.5 Relevant expressions

As shown below, some key expressions indicating the relationships between parameters of cyogelation have been provided in some literature. Such mathematic models provide not only a better understanding of the effects of parameters on cryogelation, but also good references for the interpretation of results of the experiments.

2.6.3.5.1 Concentrating extent

The concentrating extent β, which is the ratio of the total concentration of solutes within the unfrozen phase to that in the initial system at a negative temperature T can be estimated using the following formula (Sergeev, Batyuk, Stepanov, & Sergeev, 1973):

——— [2.2]

Where T_0 is the crystallization point of the solvent, Δ is cryotropic constant, C_i is the initial concentration of the solvent i.

2.6.3.5.2 Volume of unfrozen phase

The relationship between the volume of unfrozen phase V_f and initial concentration of urea C_0^u and has been obtained from some cryostropic correlations (Konstantinova & Lozinsky, 1997):

$$\frac{}{\Delta(T \quad T)} \qquad [2.3]$$

Where Δ is the cryotropic constant, T_0 is the temperature of experiment and T is the freezing temperature.

2.6.3.5.3 Protein concentration in unfrozen phase

Based on the same cryotropic correlations, the concentration of protein in the unfrozen C_f^p phase is expressed as follow (Konstantinova & Lozinsky, 1997):

$$\frac{\Delta(T \quad T)}{} \quad [2.4]$$

Where C_0^p is the initial concentration of protein.

2.7 Conclusions

The formation and characterization of conventional protein gels have been fairly fully studied. Rheological properties are considered to be the most important properties of a gel as its viscoelasticity changes during sol-gel transition. As for cryotropic gels, other than rheological properties, porous properties are equally important since they are what mainly make cyotropic gels different from conventional gels.

When the precursor is protein, the main factors affecting cyotropic gelation are the initial concentration of denaturant and precursors, the freezing temperature and the freezing duration. The initial concentration of precursors affects the concentration of them in the unfrozen phase in a frozen sample and therefore affects the formation of the gel. The initial concentration of denaturant affects the freezing point of the solvent as well as the volume of unfrozen phase. The affected freezing point of the solvent determines the minimum freezing temperature to form a cryotropic gel, while the affected volume of unfrozen phase alters the concentration of precursors --- more specifically, the increased concentration of denaturant increases the volume of unfrozen phase and therefore decreases the concentration of precursors. Also, a sufficient concentration of denaturant is required in order to achieve fully denaturation. However, the initial concentration of denaturant is less important for protein denaturant as this is highly concentrated in the unfrozen phase which becomes sufficient to denature protein.

In a certain range, freezing temperature affects the yield of gel fraction and the pore size within the gel. Freezing duration provides sufficient time for solute to interact with each

other, therefore yield of gel fraction usually increases as freezing duration is increased, but only before it reaches the point where the interaction has been completed. After this point, yield of gel fraction remains unchanged.

Compared to conventional gelation, cryotropic gelation is more complicated for the multiple factors affecting the gelation properties stating above. These factors also affect each other and therefore cause some complex changes. For example, the increase of initial concentration of urea increases the volume of unfrozen phase, resulting in the decrease of protein concentration in the unfrozen phase, which affects the gel strength of the thawed product. The relationship between this factors and cryogelation properties has been roughly understood. However, it is still very challenging to define the precise relationship in order to obtain the gel product with desired properties by controlling the parameters in the system. Hence, more efforts still need to be put into further investigating these relationships based on previous studies.

3 Materials and methods

3.1 Materials

Egg white powder (EPW) was provided by LM Wright & Co, New Zealand. The CoA of the product is present in Table **3.1**. Urea was obtained from Thermo Fisher Scientific Ltd (New Zealand) with ≥99% purity.

Table 3.1 COA of the egg white powder

pH	Moisture %	Cbt	Coli	E.Coli	Enter.	Staph.	Mould/ Yeast	Salm./ 375gr.
6.80	6.05	<100	<10	<10	<10	ABS	<10	ABS

3.2 Sample preparation

3.2.1 Preparation of initial solutions

Egg white powder was dissolved in R.O water on stirrers with magnetic bars in each solution at 20^{o}C for 30 minutes at the range of concentrations of 5-15 %w/v. Urea at the

concentration of 1-6.6M was added to the dispersed solution and well dissolved. Foam formed during stirring was maximally removed from the solutions.

3.2.2 Preparation of cryotropic gels

Prepared solutions were transferred to containers and stored at desired negative temperature (depending on the experiment). After storage of 20h/68h, frozen samples were taken out of the freezer and spontaneously thawed at 20^{o}C.

3.3 Characterization of samples

3.3.1 Gel Strength

Gels strength of samples was determined by a compression test, by using a texture analyser (Texture analyser TAXT plus) at 5kg load cell, with test speed 1mm/s, post-test speed 2mm/s, deformation distance 6mm. Samples were compressed by a 10mm round probe. Results were obtained from the graph generated by the software. The peak force of each test was used to indicate the gel strength of the samples. The test was conducted in triplicate.

3.3.2 Water holding capacity (WHC)

WHC of the frozen samples was determined gravimetrically by leaving them on grids for 4 hours at ambient temperature (20^{o}C) in order to measure the amount of free water released from the gels. The degree of WHC was calculated using equation [3.1]:

$$\text{WHC\%}=\frac{Ws-Ww}{} \times 100 \qquad [3.1]$$

Where w_s is the weight of the frozen sample, w_w represents the weight of released water.

3.3.3 Sample processing for scanning electron microscope (SEM)

SEM imagines were obtained in the assistance of Manawatu Microscope Image Center (MMIC) at Massey University.

10x10x10mm cubes of sample were placed in primary fixative (Modified Karnovsky's fixative (3% gluteraldehyde 2% formaldehyde in 0.1M phosphate buffer, pH 7.2)) for at least 8 hours at room temperature. They were then washed three times (10-15 minutes each) in phosphate buffer (0.1M, pH 7.2) followed by dehydration in graded ethanol series (25%, 50%, 75%, 95%, 100%) for 10-15 minutes each with a final 100% ethanol wash for 1 hour. Samples were critical point dried using liquid CO_2 as the CP fluid and 100% ethanol as the

intermediary (Polaron E3000 series II critical point drying apparatus). Samples were mounted on to aluminium stubs using double sided tape and sputter coated with approximately 200nm of gold (Baltec SCD 050 sputter coater) and viewed in the FEI Quanta 200 Environmental Scanning Electron Microscope at an accelerating voltage of 25kV (method provided by MMIC).

3.3.4 Sample processing for transmission electron microscope (TEM)

TEM imagines were obtained in the assistance of Manawatu Microscope Image Center (MMIC) at Massey University.

Samples were trimmed to the correct size and shape and were fixed in Modified Karnovsky's Fixative (3% Gluteraldehyde (Merck) (v/v) 2% Formaldehyde (w/v) in 0.1M Phosphate Buffer (pH7.2)) for at least 2 hours. Buffer washed in 0.1M phosphate buffer (pH7.2) 3x for 10min each. Post fixed in 1% Osmium Tetroxide in 0.1M phosphate buffer for one hour maximum. Buffer washed as above 3x for 10min each. Dehydrated through a graded acetone series (25%, 50%, 75%, 95%, 100%) for 10-15min each followed by 2x changes of 100% for one hour each. Samples were then put into 50:50 resin: acetone and placed on the stirrer overnight. This was replaced by fresh 100% resin (Procure 812, ProSciTech Australia) for 8 hours on the stirrer. This step was repeated twice more (overnight in 100% resin, 8 hours in 100% resin). Samples were embedded in moulds with fresh resin and cured in a 60^{o}C oven for 48hrs.

Light microscope sections were cut at 1 micron using a glass knife on the ultramicrotome (Leica EM UC7, Germany) and heat fixed onto glass slides. These were stained with 0.05% Toluidine Blue for approximately 12 seconds and viewed under the light microscope. The block was then trimmed down to the selected area and cut using a Diamond Knife (Diatome, Switzerland) at 100nm (light gold sections). These were stretched with chloroform vapour and mounted on a grid using a Quick Coat G pen (Daido Sangyo, Japan). Grids were stained in Saturated Uranyl Acetate in 50% Ethanol for 4 min, washed with 50% ethanol and MilliQ water and then stained in Lead Citrate (Venable and Coggeshall, 1965) for a further four minutes. This was followed by a wash in MilliQ water. Samples were viewed using a Philips CM10 Transmission Electron Microscope (Eindhoven, The Netherlands) before March 2014

and viewed with an FEI Tecnai G^2 Spirit BioTWIN (Czech Republic) after March 2014 (method provided by MMIC).

3.3.5 Nano Deferential scanning colorimetry (Nano DSC)

1% (w/v) EWP and corresponding 0-6.6M urea were dissolved in buffer solution (10mM Na-phosphate, pH 7). Cryogel suspensions were prepared by freezing solutions containing 1% (w/v) EWP and 1-6.6M urea respectively. Solutions were frozen at -18^{o}C for 20 hours and thawed at ambient temperature. The obtained cryogel suspensions were mixed with phosphate buffer at the ratio of 1:1. Calorimetric measurements were carried out using a nano differential scanning calorimeter (602000, TA Instrument, USA) at the heating rate of 2^{o}C min^{-1} within the temperature range of 40-130^{o}C and at the excess pressure of 3ATM. Operation control and data acquisition were performed using DSCRun software. Thermodynamic analysis of experimental data was conducted in NanoAnalyze software.

4 Cryotropic gelation of different proteins

To investigate the influence of cryotropic treatment on globular proteins, three different protein solutions --- soy protein isolate (SPI), egg white powder (EPW) and whey protein isolate (WPI) (WPI solutions were preheated at 40-50^{o}C for 30 minutes) were selected to undergo freeze-thaw process in the presence of urea. The freezing temperature was -18^{o}C and the storage duration was 20 hours. The concentration of urea was 1, 3, 5 and 8 M depending on specific protein solution. Initial visual inspection on thawing was used to determine a phase diagram which indicates the sol-gel state of products formed at different concentrations of urea and protein (Figure **4**.**1**).

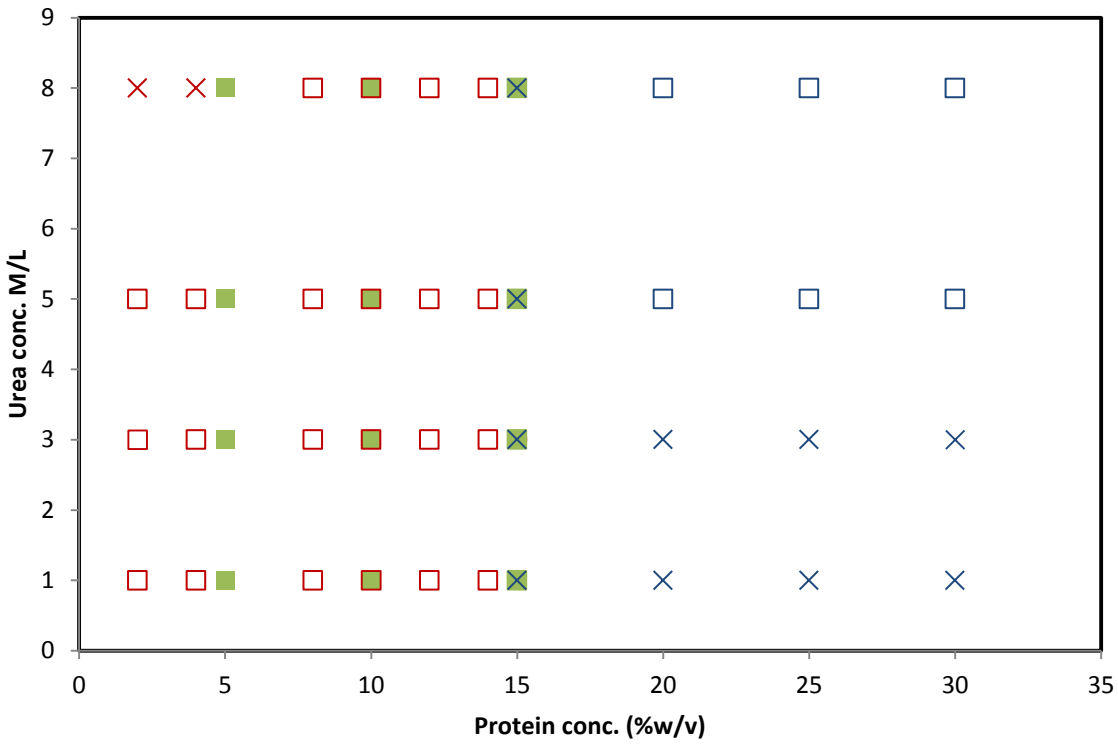


Figure 4.1 Phase diagram of samples obtained from solutions containing urea and different proteins at different concentrations. Samples were frozen at -18°C for 20 hours. ☐ Gel samples obtained from SPI solution; × Sol samples obtained from SPI solution; ■ Gel samples obtained from EWP solution; ☐ Gel samples obtained from WPI solution; × Sol samples obtained from WPI solution; ■ Gel samples obtained from SPI and EWP solutions; ■ Gel samples obtained from EWP solution and sol samples obtained from WPI solution.

For SPI solutions of >8%, gels were formed at all concentrations of urea studied. The 2% and 4% SPI solutions failed to form gel at 8M urea. In contrast, EWP solution gelled at all experimental concentrations of protein and urea (noting that cryogels were not formed in the absence of urea). WPI gel was only formed at high concentrations of protein and urea. However, because of the high concentration of both protein and urea in the initial WPI solution, the formation of a gel had already occurred before freezing of the system. In addition, the gels formed before the samples were frozen were observed to be not much different from their counterparts after freezing-thawing process (regarding the appearance of the samples and the gel strength sensed by fingers). Therefore, it is believed that there is very little influence of cryotropic condition on the gelation of WPI.

Figure **4.1** provides an indication of the visual appearance of gels from different proteins. The EWP gel displayed a fibrous macrostructure, markedly different from typical EWP gels

formed by heat treatment. The SPI gel was also fibrous, but more loosely structured, and consequently was found to be very weak and breakable. The WPI gel was transparent and no fibrous structure was observed.

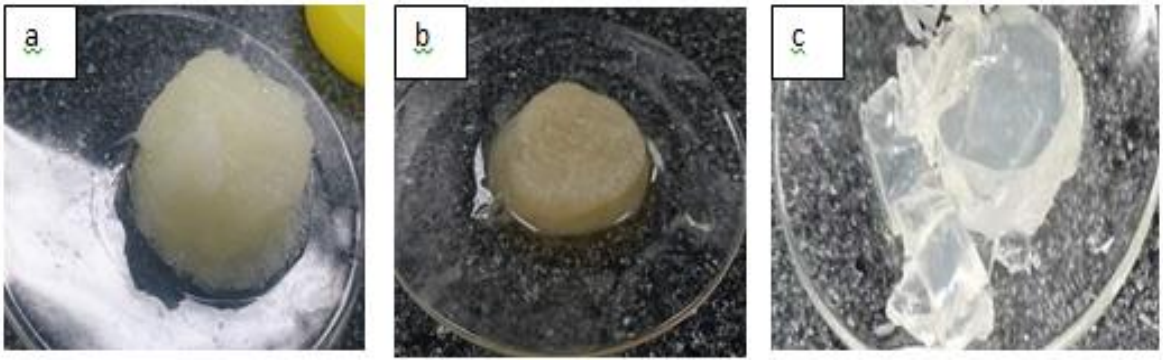


Figure 4.2 Cryotropic gels of different proteins (a. 10% EWP × 5mol/L urea; b. 10% SPI × 5mol/L urea; c. 25% WPI × 5mol/L urea)

4.1 Discussion

The hypothesis for protein cryogelation is that it arises from initial urea induced protein unfolding that exposes intermolecular bridging sites that are able to form disulphide crosslinks as a consequence of freeze concentration and compaction between ice crystals (which provides a rationale for the observed fibrous structures). Egg protein, soy protein and whey protein are common globular proteins, and were considered appropriate systems to test this hypothesis. Among these proteins, whey protein has the least amount of SS and SH groups (Edwards, Creamer, & Jameson, 2008; Fukushima, 2011; Strixner & Kulozik, 2011), noting that although BSA in whey protein has 17 disulphide bonds, it only accounts for 3% of the total whey protein (Edwards et al., 2008). Moreover, the treatment of urea addition alone was shown not to work on isolated BSA (Rodionov et al., 2015). It is possible that the globular conformational changes induced by urea alone in freezing conditions are also not sufficient to enable subsequent cryogelation.

This may also apply to the SPI, noting that although soy protein fractions contains a number of sulphur bearing amino acids, it is possible that not all of these may be available for interacting and crosslinking. In addition, the higher viscosity of soy protein solutions may reduce the volume of frozen phase in the frozen samples, leading to reduced compaction of protein within the ice network and thus producing a weak, poorly structured gel.

Overall, given the observed properties of gels of different proteins, EPW was selected for further investigation of this research, on the basis that it formed more robust cryogel structures as a consequence of freezing protein solutions in the presence of urea.

5 Effects of different gelling conditions on material properties of EWP cryotropic gels

5.1 Effects of urea

5.1.1 Effects of urea on gel strength

To determine the influence of protein and urea concentration on cryogel properties, material testing using large deformation texture analysis was used to investigate gel strength. This was challenging due to the fibrous, inhomogeneous nature of the cryogels formed. Compression testing was found to provide a reasonable empirical measure of gels material properties, with compression peak force (CPF) providing an indication of gel strength. Usually the bigger the CPF value, the stronger the gel is in relative terms. The CPF of cryotropic gels formed from EPW solutions in the presence of urea is shown in Figure **5**.**1**. The value of peak force is the highest at 15% EWP×3M urea and the lowest at 5% EWP×6.6M urea. The findings shows a general trend of peak force decreasing as urea concentration is increased and increasing when protein concentration increased, with the exception being a slightly lower anticipated value at 10% protein in comparison with 7% protein (at 1M urea) and a significant decrease in CPF as protein concentration is increased from 12% to 15% EWP concentration (also at 1M urea). Moreover, samples comprising 15% EWP did not follow the general trend of decreasing CPF with increasing urea concentration in line with the other protein concentrations, with mean peak force value increasing from 1.302N at 1M urea to 1.797N at 3M urea, before decreasing as urea concentration was increased above 3M.

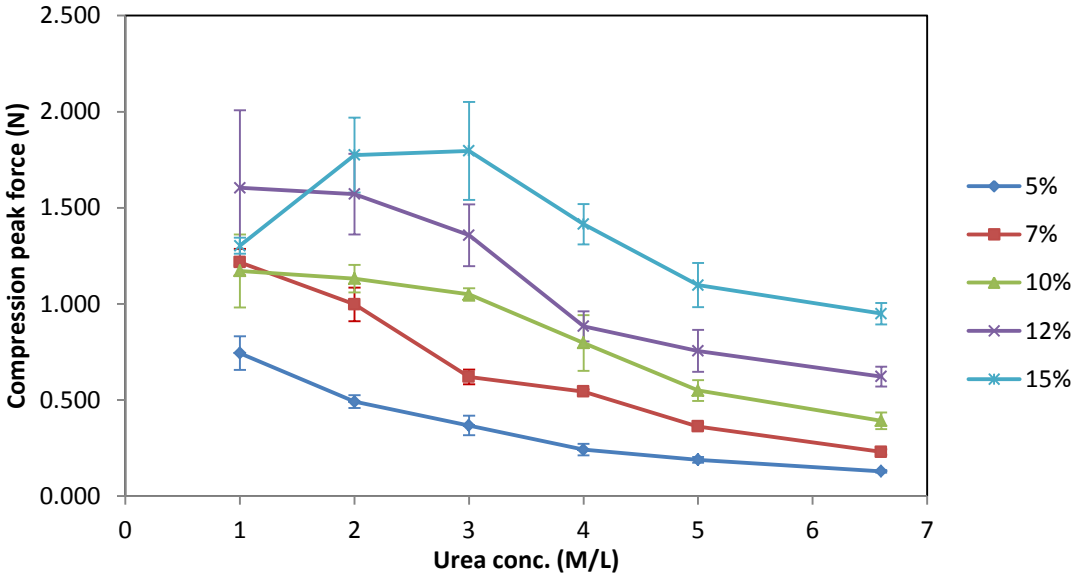


Figure 5.1 Compression peak force of cryotropic gels in the function of urea concentration at different protein concentrations. Samples were storage for 20h at -18°C.

5.1.2 Effects of urea on water holding capacity

Water holding capacity (WHC) of cryotropic gels provides an indication of gel structures ability to bind water within the protein networks indicating both gel strength and homogeneity. WHC of cryogels formed at different concentrations of protein and urea is shown in Figure **5.2**. At higher concentrations of EWP (10%, 12% and 15%), WHC decreases slightly as urea concentration is increased in the range 1 – 4M. However, as the concentration of urea is increased above 4M, the WHC of the samples is seen to increase. Samples comprising 10% and 12% EWP show similar WHC values across all concentrations of urea. For 7% EWP, WHC increases with increased urea concentration (with the exception of 2M urea, where a dip in WHC is observed). It is worth noting that the WHC of the sample containing 5% protein reaches a peak at 4M urea, which is remarkably higher than that at any other urea concentrations and other EWP concentrations at the same amount of urea.

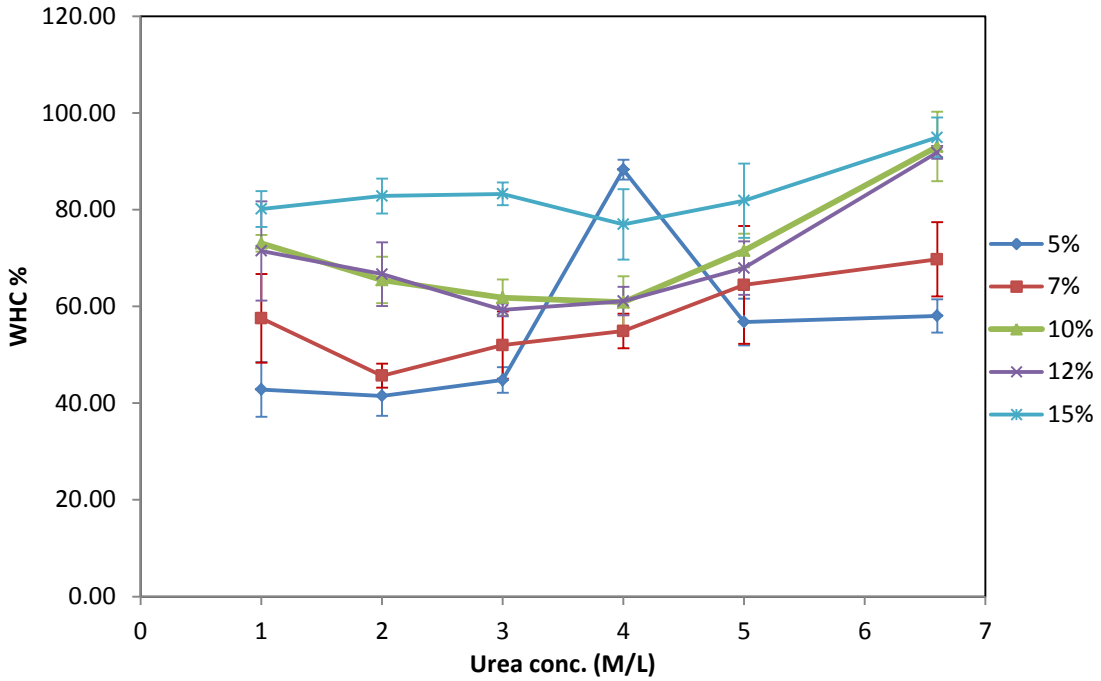


Figure 5.2 Water holding capacity of cryotropic gels in the function of urea concentration at different protein concentrations. Samples were storage for 20h at -18°C.

5.2 Effects of freezing temperature

Samples at EWP concentration of 5%, 10% and 15%, and urea concentration of 1M, 3M and 5M, were also frozen at -10°C and -30°C for 20 hours respectively to determine whether ice content had any influence on cryogel properties. Samples stored at -10°C were predominantly still in the sol state, except those at 10% EWP×5M urea and 15% EWP×5M urea which formed weak gels. At freezing temperature of -30°C, most samples were observed to undergo gelation. Thus, -30°C was chosen as an additional freezing temperature for further study of the properties of cryotropic gels.

Figure **5.3** shows the compression peak force of cyotropic gels formed at -30°C at different concentrations of EWP and urea. For 5% EWP, gels formed at urea concentration ≥ 2M were too weak to undergo compression test. This was the same for 7% EWP gel formed at urea concentration ≥ 4M. Therefore, CPF data is not shown at these points. Samples containing higher EWP concentration had higher CPF at equivalent concentrations of urea. At 7% EWP, CPF decreased as urea concentration increased. For 10% and 12% EWP CPF initially increased as urea concentration was increased from 1M to 2M and thereafter decreasing as urea concentration was increased above 2M. For 15% EWP initial increase in CPF was also observed at low to intermediate urea concentrations, before decreasing at urea levels, with maximum force being measured at 3M urea concentration.

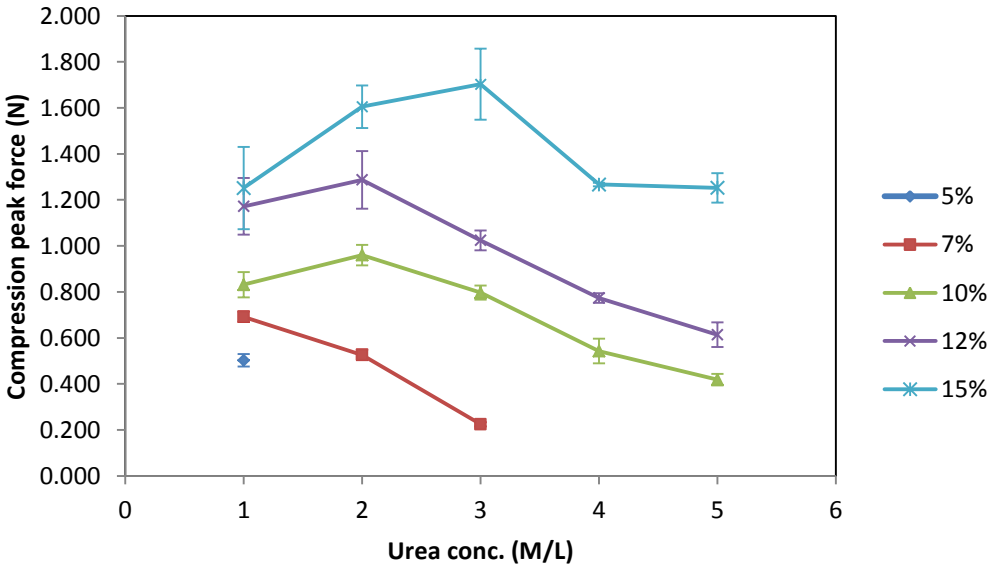
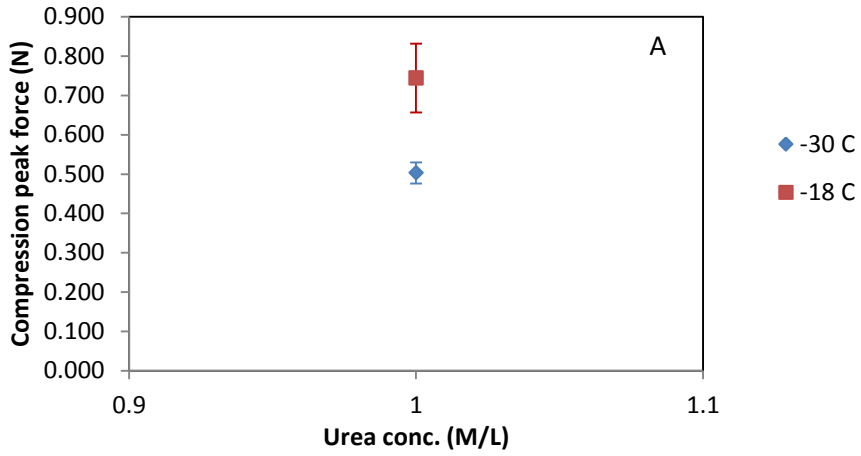
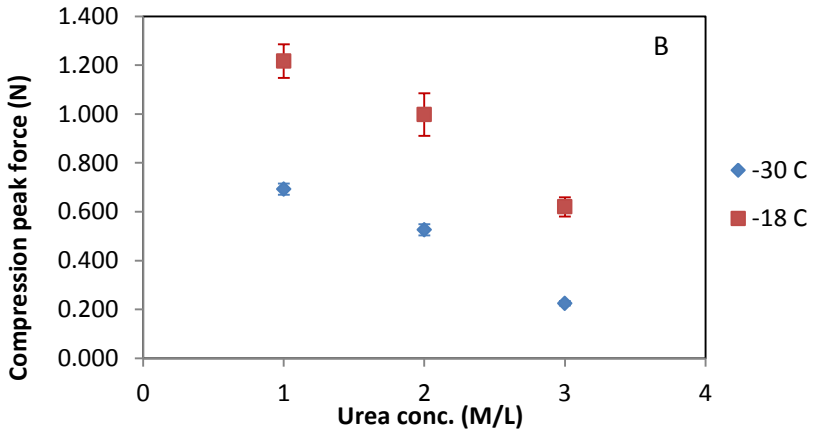


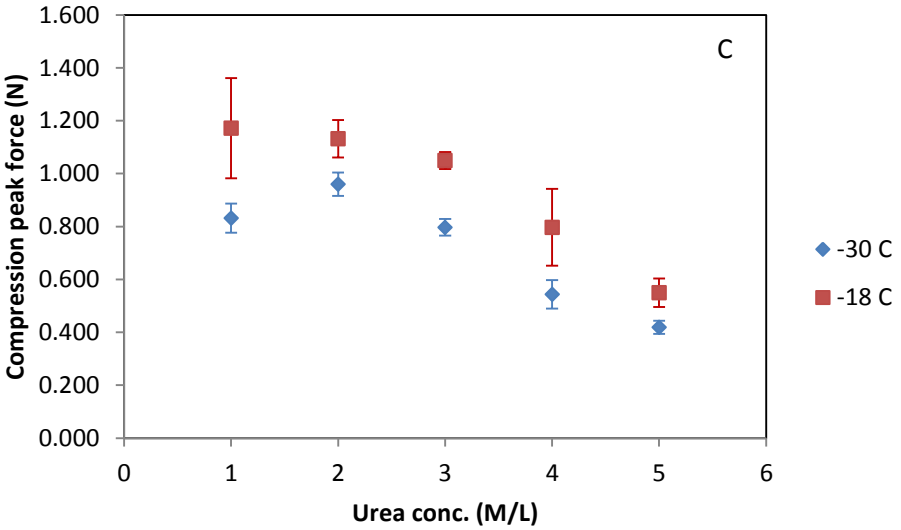
Figure 5.3 Compression peak force of cryotropic gels in the function of urea concentration at different protein concentrations. Samples were storage for 20h at -30°C.

5.2.1 Comparison of gel strength of samples formed at different temperatures

Gel strength of samples formed at -30°C and -18°C respectively is compared in Figure **5.4**. Essentially, both freezing temperatures have the same general trend for CPF as a consequence of urea addition. However, gels formed at -18°C were seen to be relatively stronger than their counterparts at -30°C, particularly at lower protein concentrations (5% and 7%), while the difference is marginal at 15% EWP. At 10% and 12%, the difference in CPF between two temperatures is more obvious at lower urea concentrations (e.g. CPF at 10% protein×1M urea at -30°C is 0.4N lower than that at -18°C) while results are more similar at higher urea concentrations (e.g. points at 10% protein×5M urea, 12% protein×4M urea and 12% protein×5M urea).


A
Compression peak force (N)
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0.9
1
1.1
Urea conc. (M/L)
-30 C
-18 C


B
Compression peak force (N)
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
1
2
3
4
Urea conc. (M/L)
-30 C
-18 C


C
Compression peak force (N)
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
1
2
3
4
5
6
Urea conc. (M/L)
-30 C
-18 C

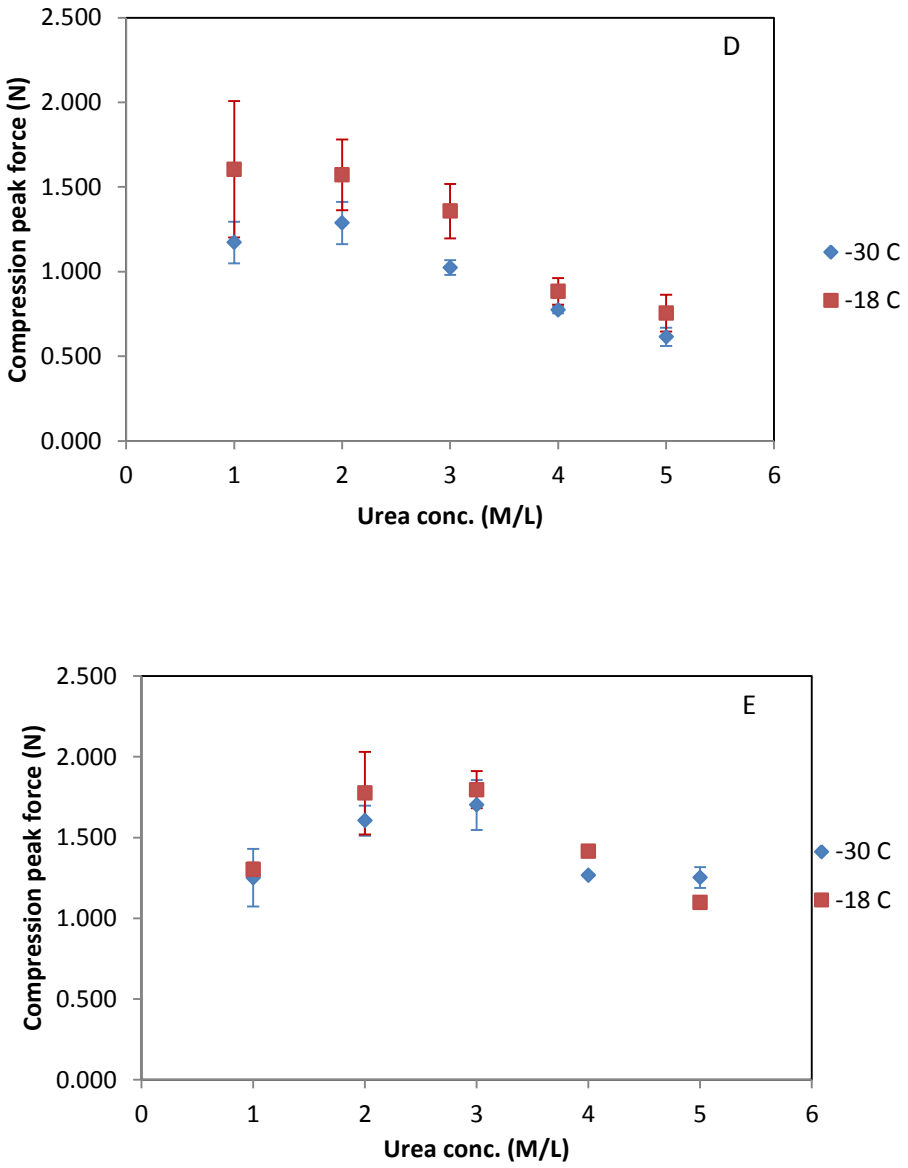


Figure 5.4 Comparison of compression peak force of samples frozen at -30°C and -18°C. Freezing duration was 20h. (A) 5% EWP; (B) 7% EWP; (C) 10% EWP; (D) 12% EWP; (E) 15% EWP.

5.2.2 Effects of freezing temperature on water holding capacity

Figure **5.5** shows urea concentration dependent water holding capacity (WHC) of thawed samples frozen at -30°C at different EWP concentrations. For samples containing 5% EWP and urea ≥ 3M, and those containing 7% EWP and urea ≥ 4M, gel samples failed to maintain on the grid after thawed due to the lack of rigidity and the released water and gel phase were not properly separated. Thus, data at these points is not shown. Basically, WHC of samples tended to decrease first and then increase as urea concentration increased, with the exception being that WHC increased from 44.65% at 1M urea to 58.50% at 2M urea at 5% EWP. For samples containing 10%, 12% and 15% EWP respectively, WHC reaches to 100% at

5M urea, which indicates there is no released water at this point. At the same time, these gels are very weak without being able to stay in shapes on the grid, as well as showing little fibrous structure.

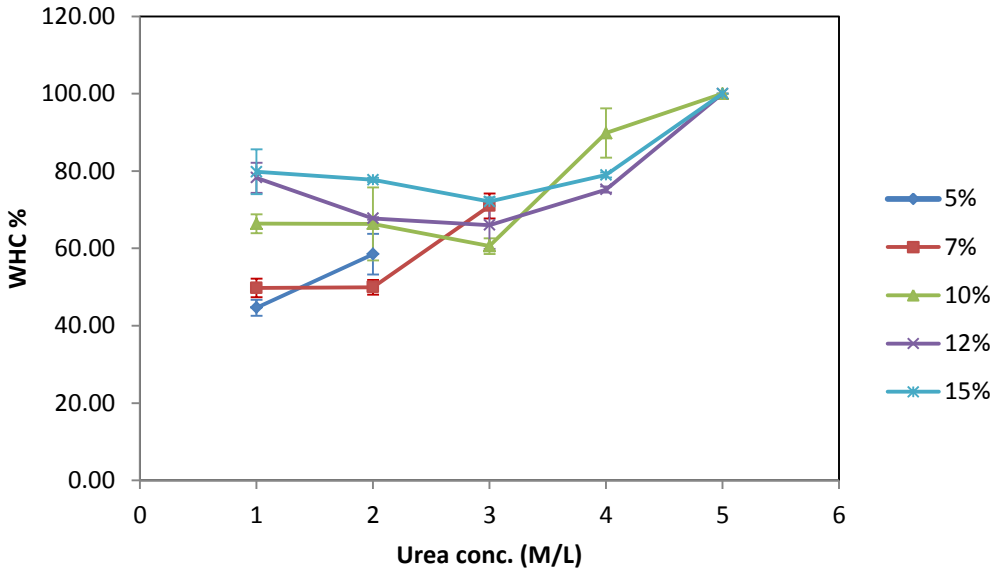
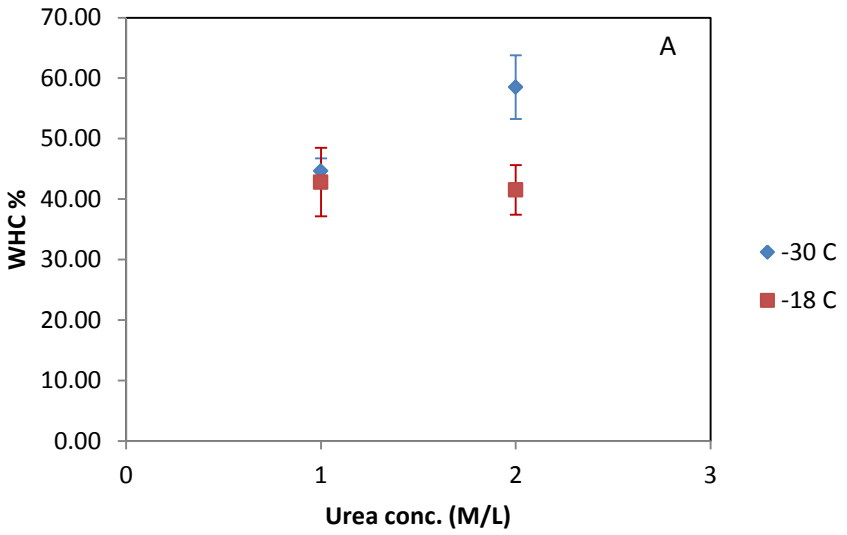
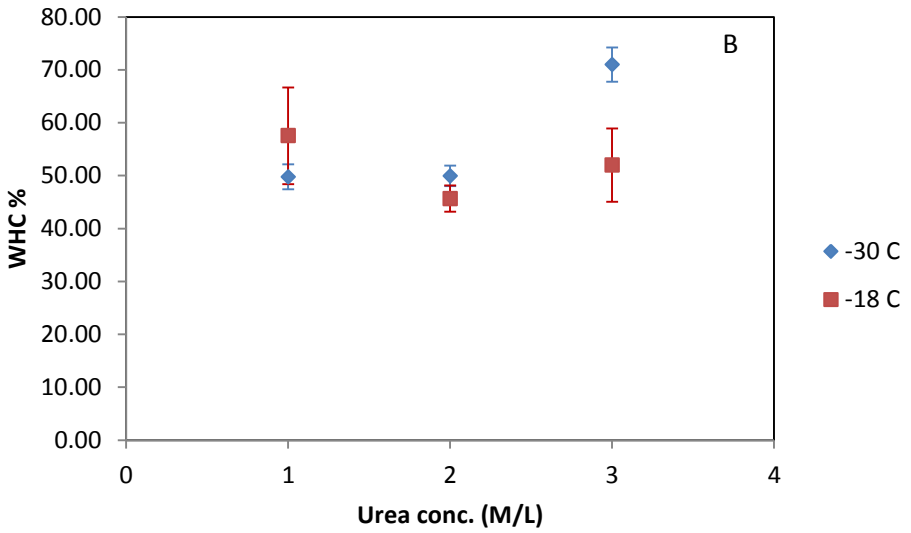


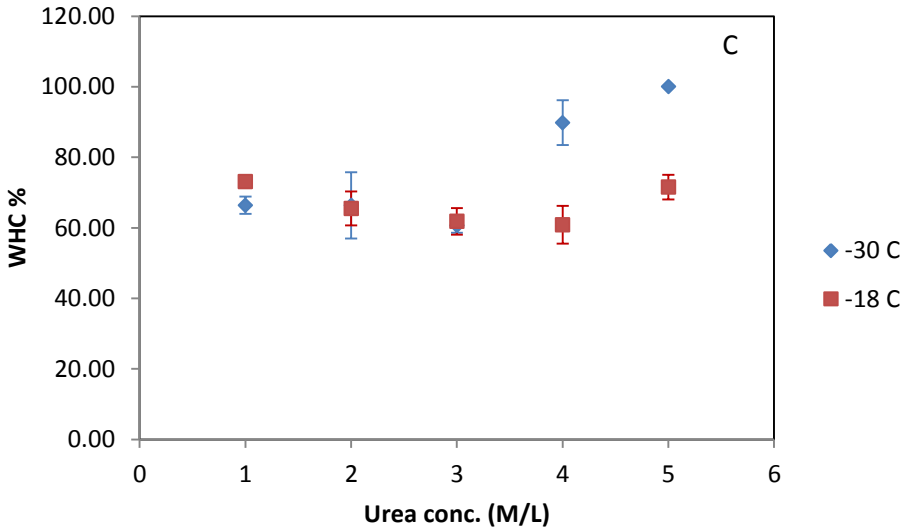
Figure 5.5 Water holding capacity of frozen-thawed samples at different concentrations of EWP and urea. Samples were frozen at -30°C for 20h.

5.2.3 Comparison of water holding capacity of samples formed at different temperatures

WHC of thawed samples frozen at -18°C and -30°C is compared in Figure **5.6**. Water holding capacity of samples from both batches showed similar values at lower urea concentration for each EWP concentration series. However, at higher concentrations of urea, WHC of samples frozen at -30°C was significantly higher than those frozen at -18°C. In the urea concentration range of 1M to 5M, and for samples containing 5% EWP, the maximum WHC is at 2M urea, while for samples containing 7% EWP this occurs at 3M urea. WHC of samples frozen at -30°C tended to be higher than that at -18°C for urea concentrations ≥4M and when the concentration of EWP was 10% and 12%. At 15% EWP, this trend started at 5M urea.


A
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
WHC %
0
1
2
3
Urea conc. (M/L)
-30 C
-18 C


B
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
WHC %
0
1
2
3
4
Urea conc. (M/L)
-30 C
-18 C


C
120.00
100.00
80.00
60.00
40.00
20.00
0.00
WHC %
0
1
2
3
4
5
6
Urea conc. (M/L)
-30 C
-18 C

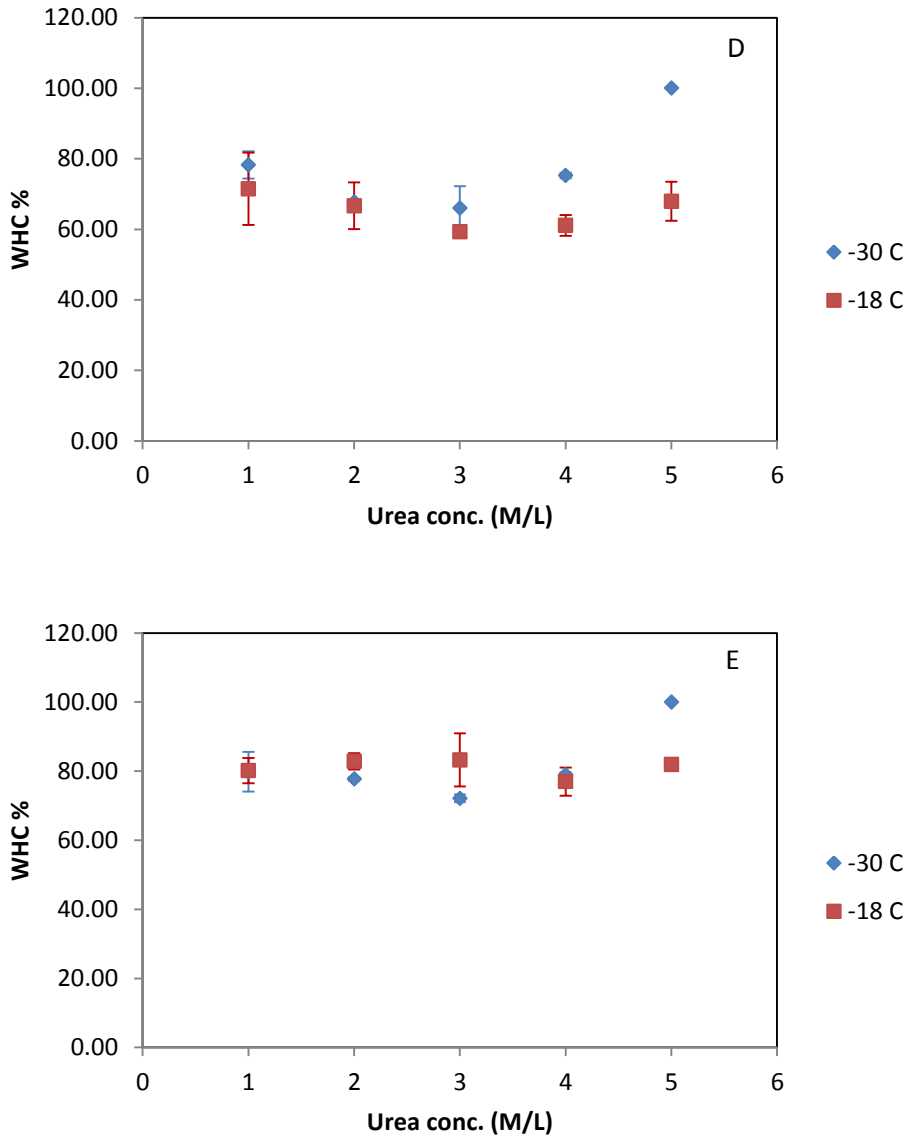


Figure 5.6 Comparison of water holding capacity of samples frozen at -30°C and -18°C. Freezing duration was 20h. (A) 5% protein; (B) 7% protein; (C) 10% protein; (D) 12% protein; (E) 15% protein.

5.3 Effects of freezing duration

EWP samples were additionally frozen for 68 h to determine whether freezing time had any influence on either material or water holding properties. CPF data for samples stored at -18 °C for 68 hours and then thawed is shown in Figure **5.7**. For thawed samples containing 5% or 10% EWP, CPF decreased as the concentration of urea was increased. For those containing 15% EWP, CPF initially increased from around 1.5N at 1M urea to 1.75N at 3M urea, before decreasing to around 1.2N at 5M urea. With the exception of the sample comprising 15% EWP and 1M urea (which displayed a lower CPF than 10% EWP sample with equivalent urea concentration), the gel strength of samples increased with increasing protein concentration at equivalent urea concentrations.

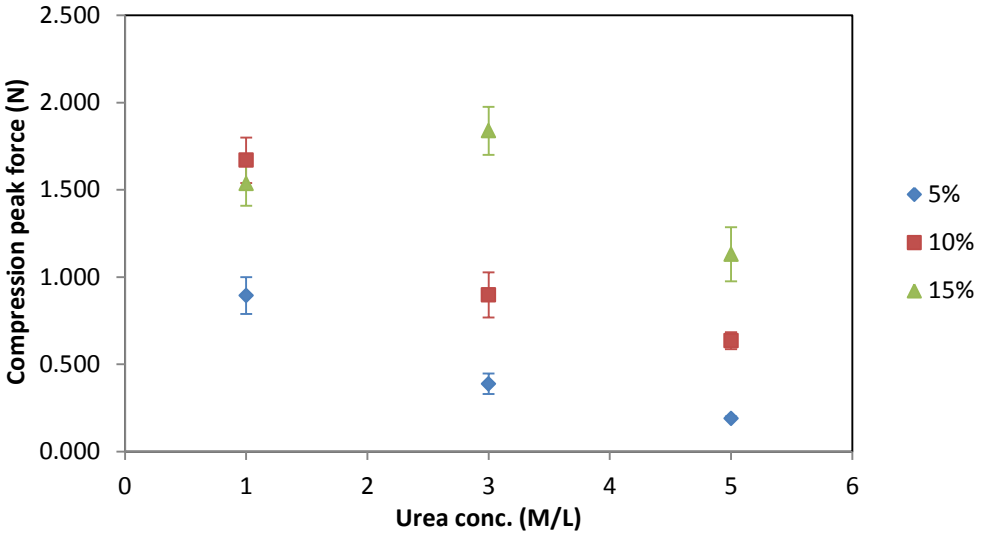
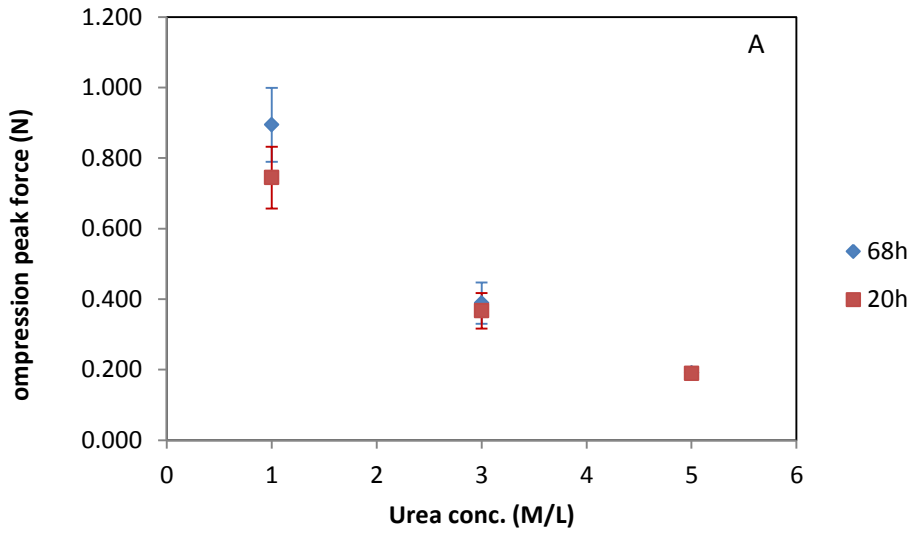


Figure 5.7 Compression peak force of cryotropic gels in the function of urea concentration at different protein concentrations. Samples were storage for 68h at -18°C.

5.3.1 Comparison of gel strength of samples formed at different storage durations

Figure **5.8** compares the CPF of thawed samples frozen for 20 and 68 hours respectively. At higher urea concentrations (3M and 5M), there appears to be little difference between two batches of samples across all sets of EWP concentrations. However, at lower concentrations of urea (1M), it could be seen that the CPF of samples stored for 68 hours was higher than those stored for only 20 hours.



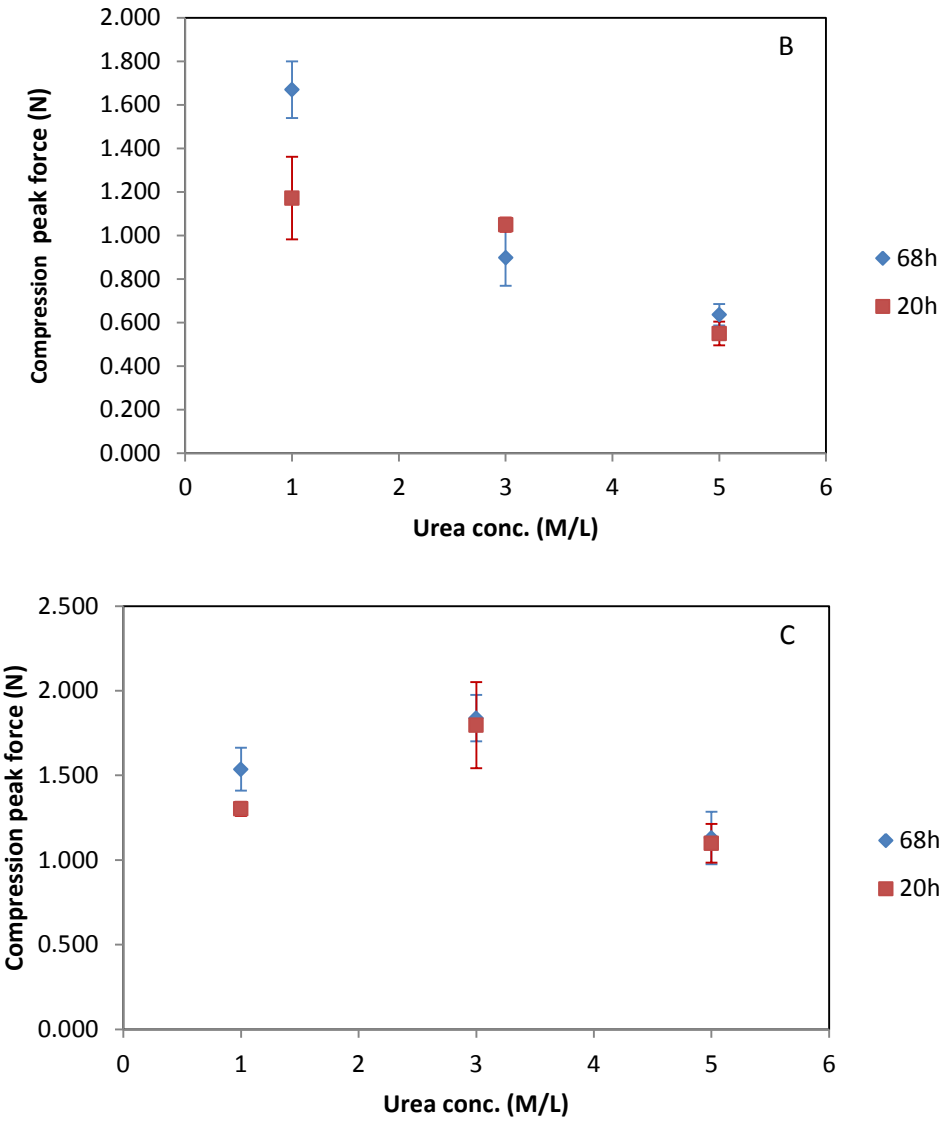


Figure 5.8 Comparison of compression peak force of samples stored for 20h and 68h. Freezing temperature was -18^{o}C. (A) 5% EWP; (B) 10% EWP; (C) 15% EWP.

5.3.2 Effects of freezing duration of water holding capacity

As shown in Figure **5.9** the WHC of thawed samples at 5% and 10% EWP increased marginally as the concentration of urea was increased. WHC was only less than 8% higher when urea concentration was increased from 1M to 5M at 5% EWP, and is only around 5% higher at 5M urea than at 1M urea with 10% EWP. For samples containing 15% EWP WHC slightly decreased at 3M urea and increased at 5M urea.

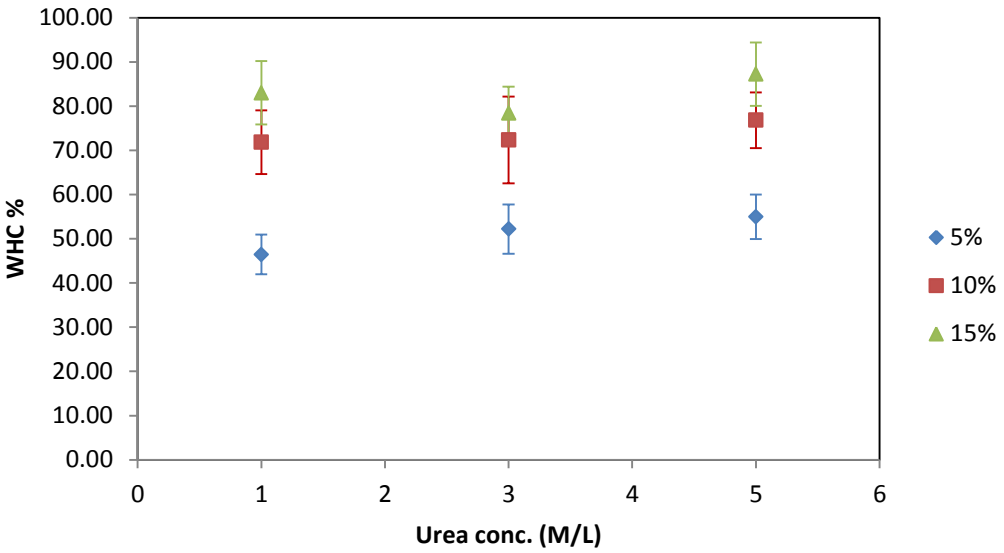


Figure 5.9 Water holding capacity of frozen-thawed samples at different concentrations of EWP and urea. Samples were frozen at -18°C for 68h.

5.3.3 Comparison of gel strength of samples formed at different storage durations

At 3M urea, WHC of samples stored for 68 hours was about 8% higher than those stored for 20 hours at 5% EWP, and about 10% higher at 10% EWP. Except for that, WHC of samples stored for different duration was similar (Figure **5.10**).

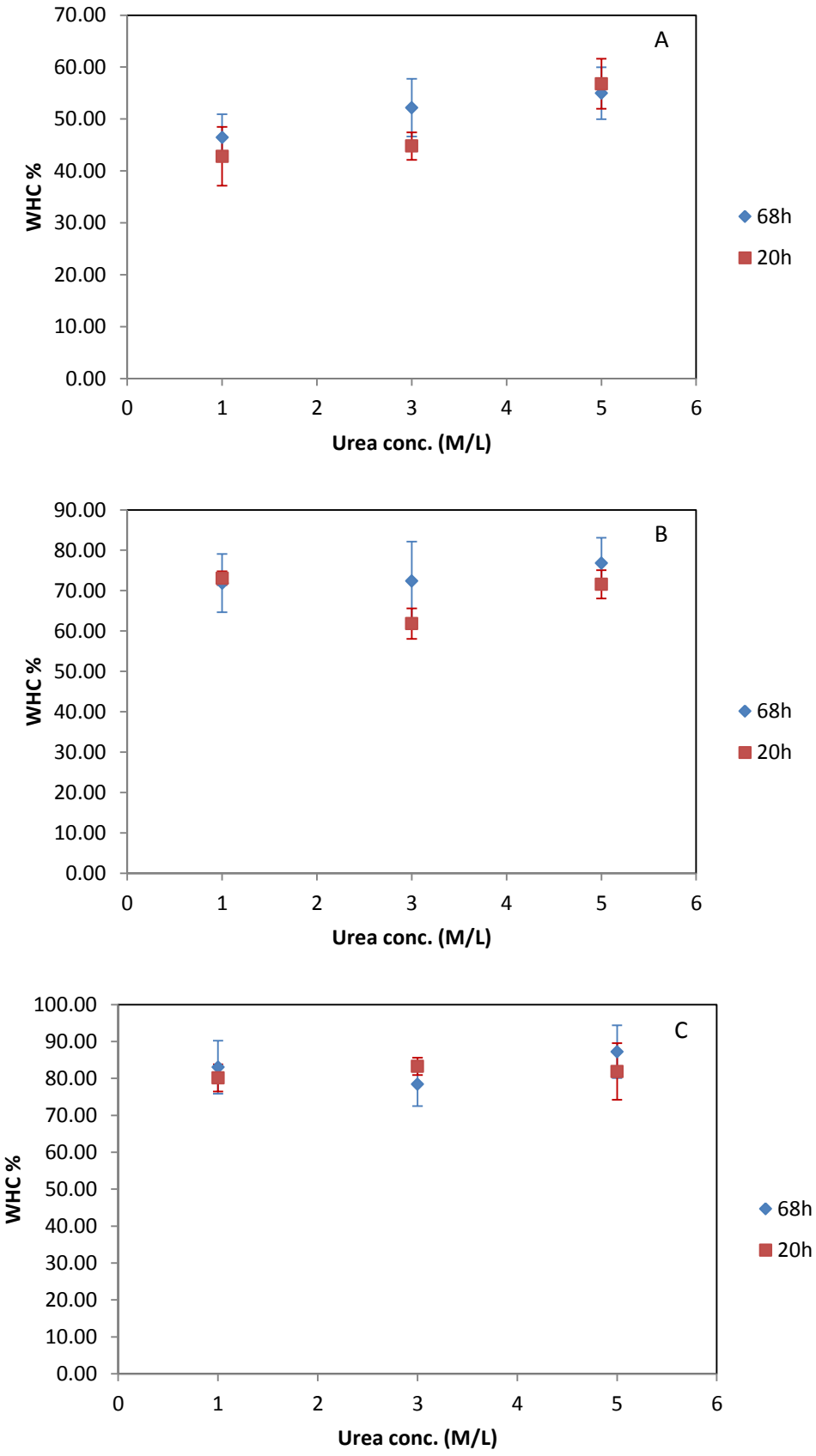


Figure 5.10 Comparison of water holding capacity of samples stored for 20h and 68h. Freezing temperature was -18^{o}C. (A) 5% EWP; (B) 10% EWP; (C) 15% EWP.

5.4 Discussion

5.4.1 Selection of experiment conditions

Preliminary studies showed that initial concentration of EWP lower than 5% resulted in essentially small final gel-fraction yield, which made the formed gel too weak for mechanical characterization. At an initial EWP concentration higher than 15%, the initial solution and the resultant cryogels were too dense, which was considered unnecessary for the experiment. Thus, the range of 5-15% EWP concentration was chosen for the experiment. For urea, an initial concentration higher than 7M would denature high concentrated protein solutions in a short time and could cause pre-gelation in the solution before freezing the samples. To make proper interval, 1-5M urea concentrations were chosen for the experiment. Considering that 6.6M is supposed to be the concentration of urea that achieves fully denaturation of protein, this concentration was also added to the design of the experiment. It has been shown that 200 minutes of freezing is sufficient for fully interaction between solutes in the unfrozen phase, as there was no more gel formed after this duration (Konstantinova & Lozinsky, 1997). Considering the different experiment temperature from Konstantinova and Lozinsky's study, to make sure the contents would completely interact, 20 hours of freezing was selected in the present experiment. To investigate the influence of long freezing period on the resultant cryogels, 68 hours of storage in the freezer was also investigated. Freezing temperatures of -10^{o}C, -18^{o}C and -30^{o}C were based on the existing equipment in the laboratory.

5.4.2 Effects of different conditions on cryotropic gels

5.4.2.1 Effects of initial concentrations of components in the initial solution

Generally speaking, at either freezing temperature or freezing duration, the higher the initial EWP was, the stronger the resultant cryotropic gels were (Figure **5.1**, Figure **5.3** and Figure **5.7**). Similar trend was obtained for cryogels formed from bovine serum albumin (BSA)-urea-Cys solutions which were observed stronger at higher initial BSA concentration (Rodionov et al., 2015). It was also shown in Rodionov and his co-workers' study that the swelling degree of the polymeric network within the pore walls of resultant cryogels decreased as the initial BSA concentration increased, which indicates the larger amount of interchain crosslinks formed in the gel phase at more concentrated BSA. As the initial urea concentration

increased, gel strength decreased at most constant initial EWP concentrations (Figure **5**.**1**). However, at high EWP concentration (15%) the resultant cryogels started at relatively low gel strength before reaching a maximum and afterwards decreasing. This trend at 15% EWP agrees with the observation in Rodionov and his co-workers' study, where samples went through soft spongy→elastic spongy→spongy→weak spongy as the initial concentration of urea was increased from 0.5M to 2.5M. The possible explanation for the difference at 15% EWP series is that the mechanical property of cryogels is the result of a balance between initial EWP concentration and urea concentration. There is an optimum combination of these two concentrations where the greatest gel strength can be obtained. This optimum combination is not necessarily higher concentration of EWP or lower concentration of urea, as the concentration of urea also affects the porous structure of the cryogels (Section 6.2.1), which as well influences their mechanical properties. It also needs to be mentioned that since after thawing the shape of cryogels will change (usually shrink) when the water in released from melted crystals, to assure uniform shape and size of the cryogels for accurate results, the samples were in open containers through freezing and measurement to prevent water release. For this operation, the free water contained in the samples for compression test is believed to somehow affect the result of the test.

The resultant cryogels also showed better water holding capacity at higher initial concentration of EWP (Figure **5**.**2**). The trends of WHC at 10% and 12% EWP series almost overlap. It is probably because that the 2% difference between 10% and 12% EWP is not sufficient to make noticeable changes on the WHC, compared to the same difference between two low EWP concentrations (5% and 7%) and greater difference (3%) between any other adjacent EWP concentrations. The maximum WHC unexpectedly occurred at 5% EWP×4M urea. For other EWP concentrations, WHC reached a minimum before increasing as the urea concentration increased. It should be mentioned that the released water is from melted crystals in the thawed samples instead of from the gel phase, and is the result of the combination of the formed structure of the cryogels and gravity.

5.4.2.2 Effects of freezing conditions

The freezing process is the critical step for both the formation and the properties of the resultant cryogels, as it leads to the transformation of the feed liquid into a frost-bound

solid, which strongly alters the concentration of the components and other characteristics such as viscosity and thermal mobility in the unfrozen phase (Lozinsky & Okay, 2014). Freezing conditions also influence the size and shapes of the solvent growing polycrystals and thus influence the morphology of the resultant cryogels (Lozinsky & Okay, 2014). In this research cryogels formed at lower temperature (-30^{o}C) were weaker than those formed at higher temperature (-18^{o}C) (Figure **5.4**). However, the difference of gel strength between cryogels comprising 15% EWP formed at these two temperatures seems to be little (Figure **5.4**E), which indicates that the gel strength of cryogels containing high protein concentration is less sensitive to freezing temperature in a certain range. Despite that, weaker cryogel strength at lower freezing temperature was also observed with BSA containing cryogels (Rodionov et al., 2015). Cryogels formed at different freezing temperatures showed similar WHC at low urea concentrations but had better WHC at higher urea concentrations (Figure **5.6**). Presumably the initial solution could be more sensitive to higher urea concentrations at lower temperature and thus the interaction between components in the unfrozen phase and the structure have been altered which would affect the WHC.

When it comes to longer freezing duration (68h), only cryogels at low urea concentration (1M) were observed to be stronger relative to samples with the shorter freezing times, while at higher urea concentrations there was little difference. Furthermore, there was seen to be no significant difference of WHC between cryogels at 68h and 20h freezing duration (taking in to account the standard deviations). Hypothetically there should be little changes when the freezing duration is lengthened, based on the assumption that the protein molecules have already undergone interaction at an earlier stage in the freezing process. That said, in this case long freezing storage does seem to affect the gel strength, at least at low urea concentration.

6 Effects of different conditions on structural properties of cryogel

To investigate how different conditions affect the mechanical and water holding capable properties of EWP cryogel, the structure of samples was also studied.

6.1.1 Macrostructure of samples

Figure 6.1 - Figure **6.4** show the appearance of cryotropic gels at various concentrations of urea and EWP frozen at -18^{o}C and -30^{o}C respectively. For thawed samples frozen at -18^{o}C, fibrous features occur at low concentrations of urea (1M). Gel fibrosity at this urea concentration is seen to become more extensive as protein concentration is increased. In contrast, fibrous structures become increasingly less apparent as urea concentration is increased (3M, 5M) and when protein concentration is low (5% and 10% EWP). At higher concentrations of EWP (i.e. 15%), the fibrous nature is re-established, particularly at low urea concentrations, and with decreasing fibrous properties as the concentration of urea is increased.

Gels formed through -30^{o}C show weaker and less fibrous structure compared to their counter parts formed through -18^{o}C storage. Particularly, weak gels with little structure were formed at 5% EWP and 3M urea or 5M urea. Little fibrous structure could be found at high concentrations of EWP and urea (Figure **6.4**).

It is worth noting that solutions containing high concentrations of EWP and urea transited from sol to gel after being left at ambient temperature for 20 hours (**Figure 6.5**). The formed gels are weaker than those formed at freezing conditions with the same concentrations of EWP and urea, as detected by sensory measurements (compressing with fingers). Also, they had no fibrous features.

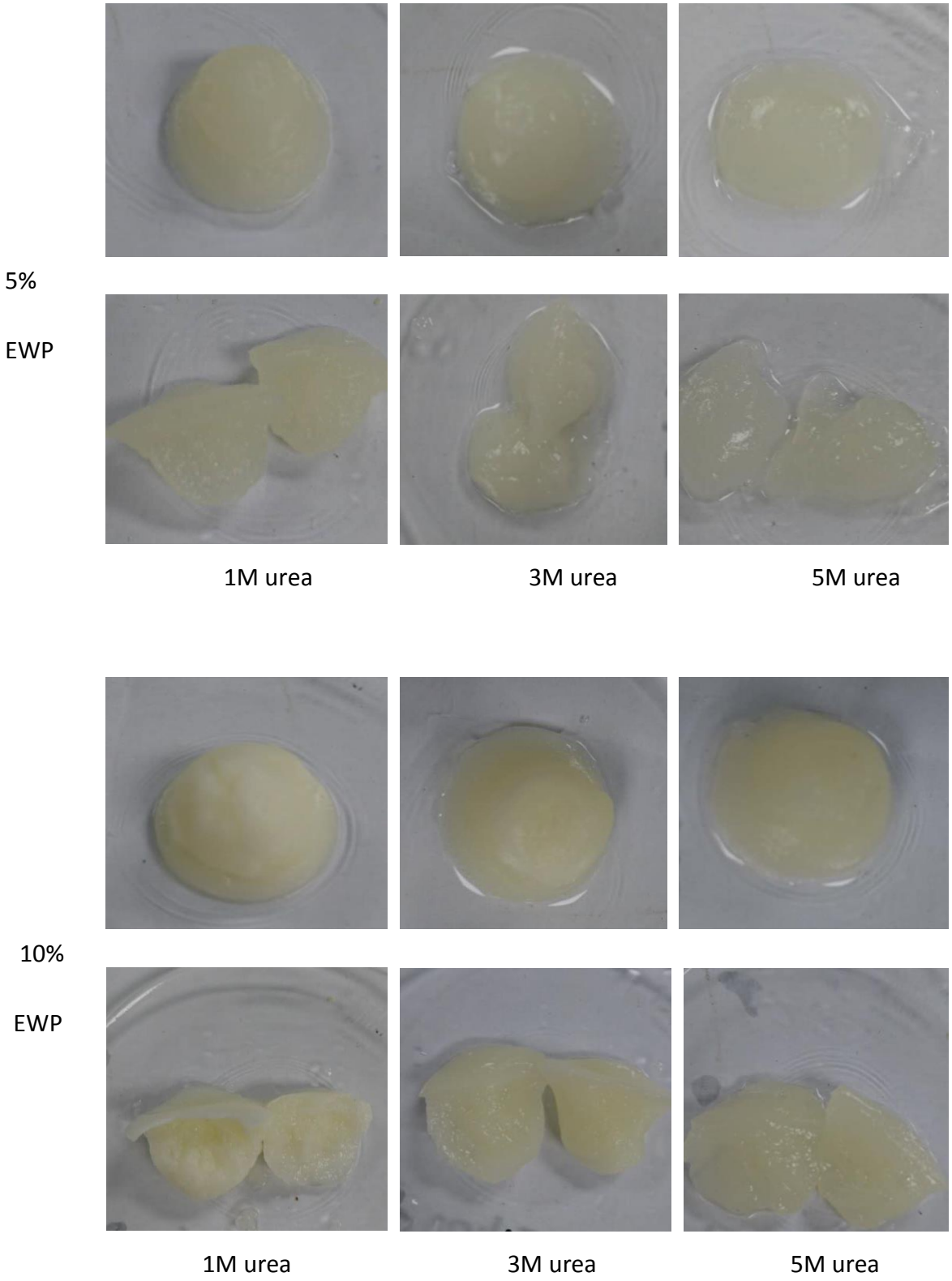


Figure 6.1 Appearance of the surface and the cross section cryotropic gels frozen at -18^{o}C. Samples were stored for 20 h.

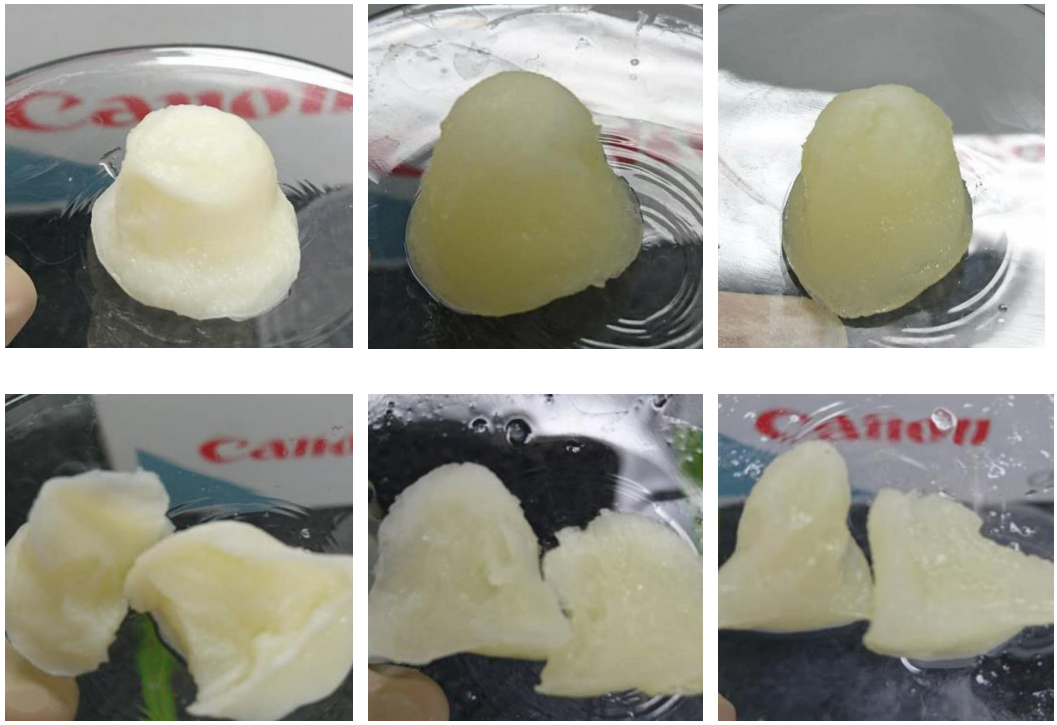


Figure 6.2 Appearance of the surface and the cross section cryotropic gels frozen at -18^{o}C. Samples were stored for 20 h.

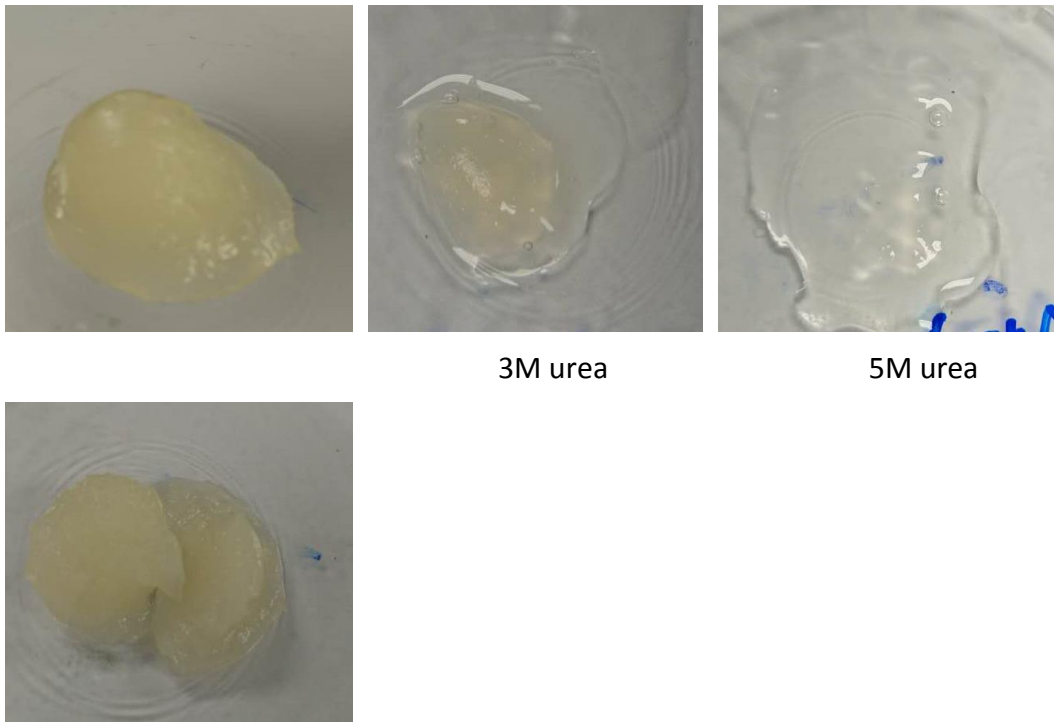


Figure 6.3 Appearance of the surface and the cross section cryotropic gels frozen at -30^{o}C. Samples were stored for 20 h (images of cross sections of samples at 5%EWP and 3M, 5M urea are missing due to the lack of rigidity of the samples).

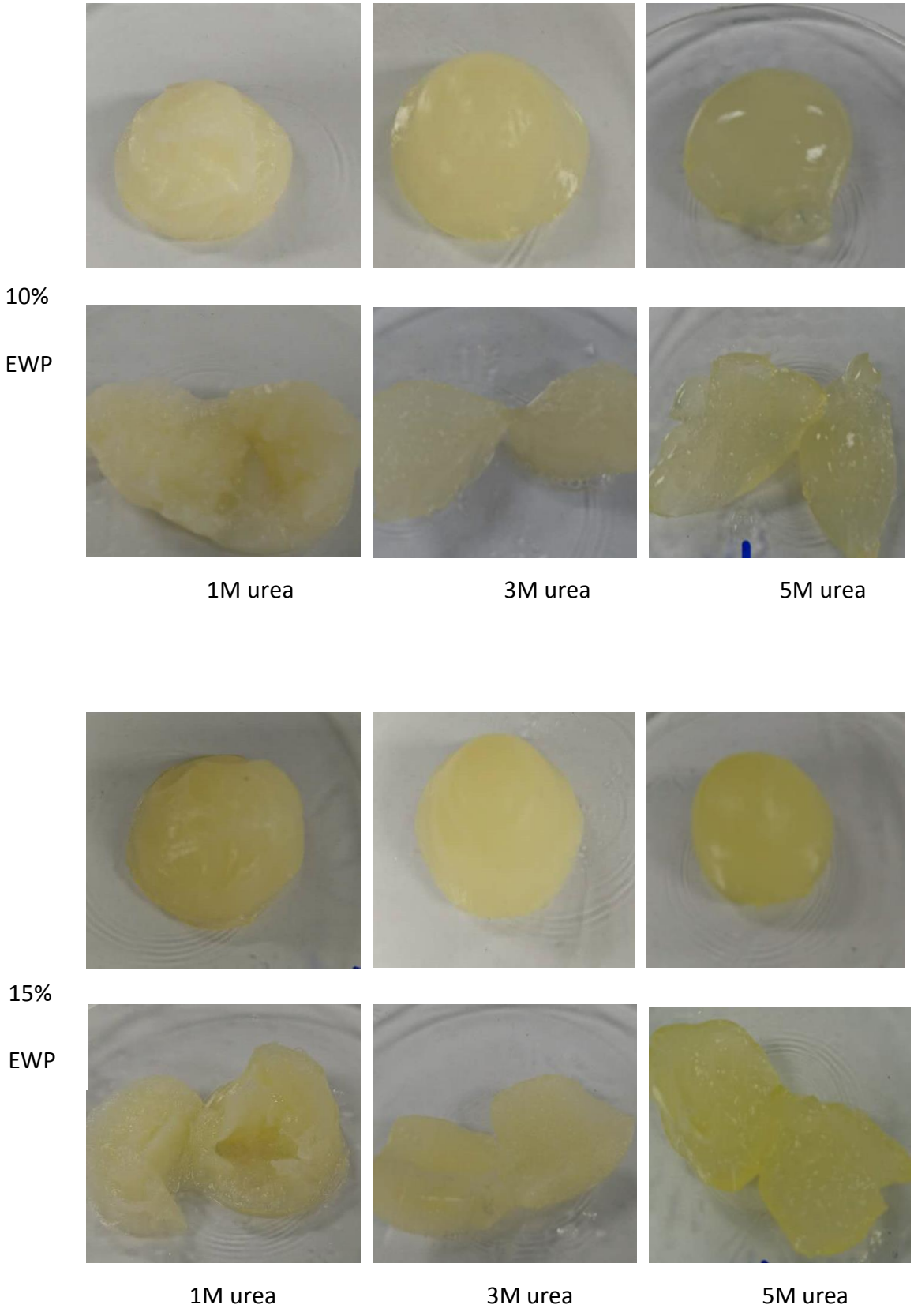


Figure 6.4 Appearance of the surface and the cross section cryotropic gels frozen at -30^{o}C. Samples were stored for 20 h.

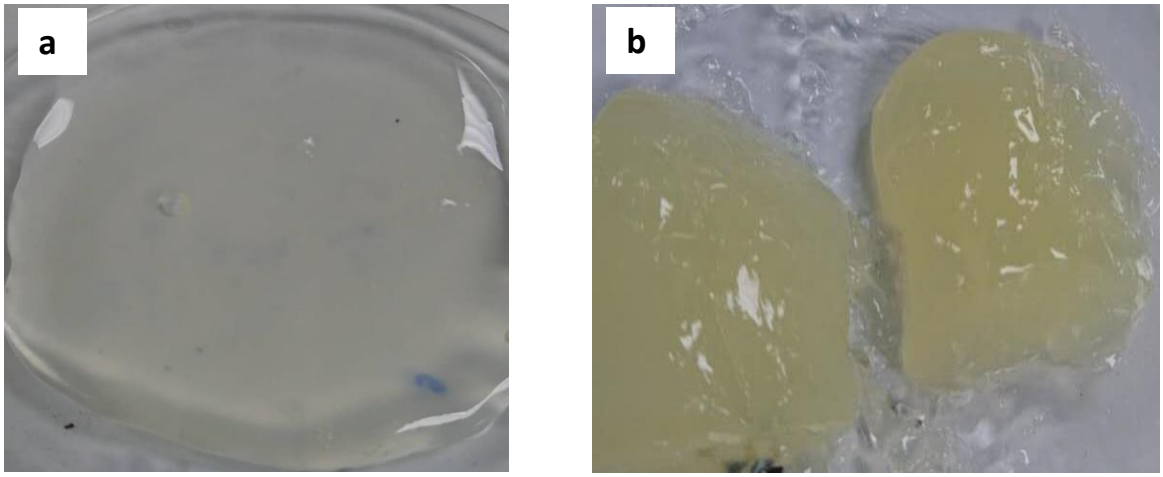


Figure 6.5 Gels formed at ambient temperature (20°C). (a) 10% EWP×5M urea; (b) 15% EWP×5M urea

6.1.2 Microstructure of samples observed with SEM

SEM microscopy of cryogels at various EWP and urea concentrations formed at -18°C and -30°C is shown in **Figure 6.6** and **Figure 6.7** respectively. All samples show extensive inhomogeneity of gel structuring, which may be expected due to assembly of the gel networks as a consequence of entrapment and compaction within the ice crystal structures during freezing. However, it is possible to make some observations relating particular structural characteristics to solution properties.

As shown in the images, for the same amount of EWP, the apparent density of cryogel structure decreases with increasing urea concentration. This tendency is especially obvious with lower concentration of EWP. A clear porous structure can be observed in the microscopy of cryogel with 5% EWP and 1M urea frozen at -18°C (Figure **6**.**6**a), where the "gel strands" are thin and dense and the macro-pores are more open. Whilst, when the concentration of urea was increased to 3M (Figure **6**.**6**b), the structure was less regular where the gel phase had been "swollen" and the pores are deformed. The regularity of the structure also decreased as urea concentration increased with 10% EWP. The change was greater at higher urea concentration (Figure **6**.**6**c). With high concentration of EWP (15%), this change as urea concentration increased was hardly observed (Figure **6**.**6**f-i).

Compared to cryogels frozen at -18°C, those frozen at -30°C seem to have a less pronounced structure especially at high urea concentrations. For samples with 5% EWP and 3M or 5M urea (Figure **6**.**7**b and c), the gel strands were considerably thick and the pores were much

smaller. Even for samples with 15% EWP there was a noticeable increase of the thickness of the gel phase at 3M and 5M urea (Figure **6**.**7**h and i).

Figure 6.8 shows an example of longitudinal sections of cryogel, which indicates that the gel walls actually lay on one and another in pieces.

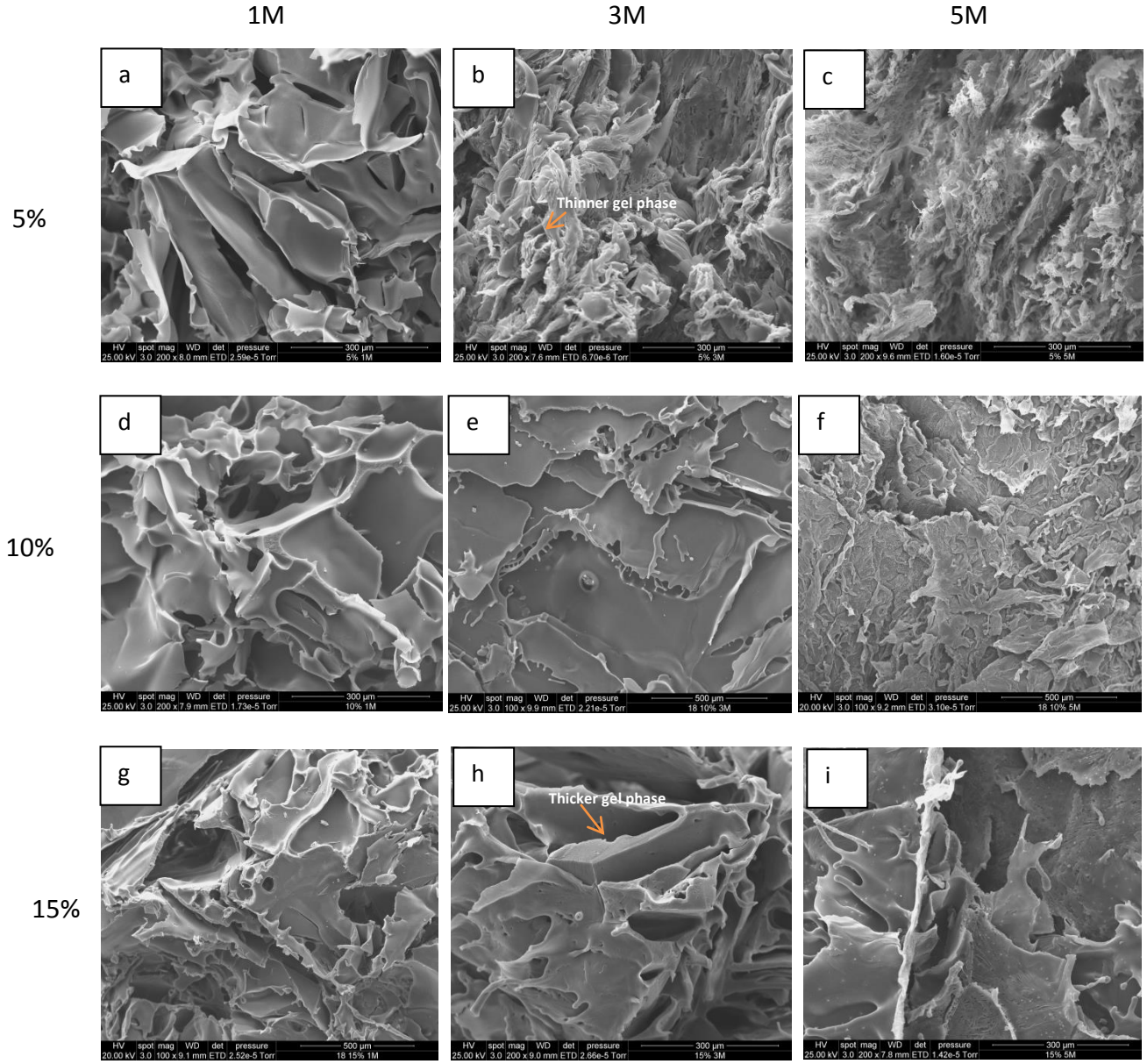


Figure 6.6 Scanning electric microscope images of cross sections of the porous structure of frozen-thawed samples at different EWP concentrations and urea concentrations. Samples were stored at -18^{o}C for 20h.

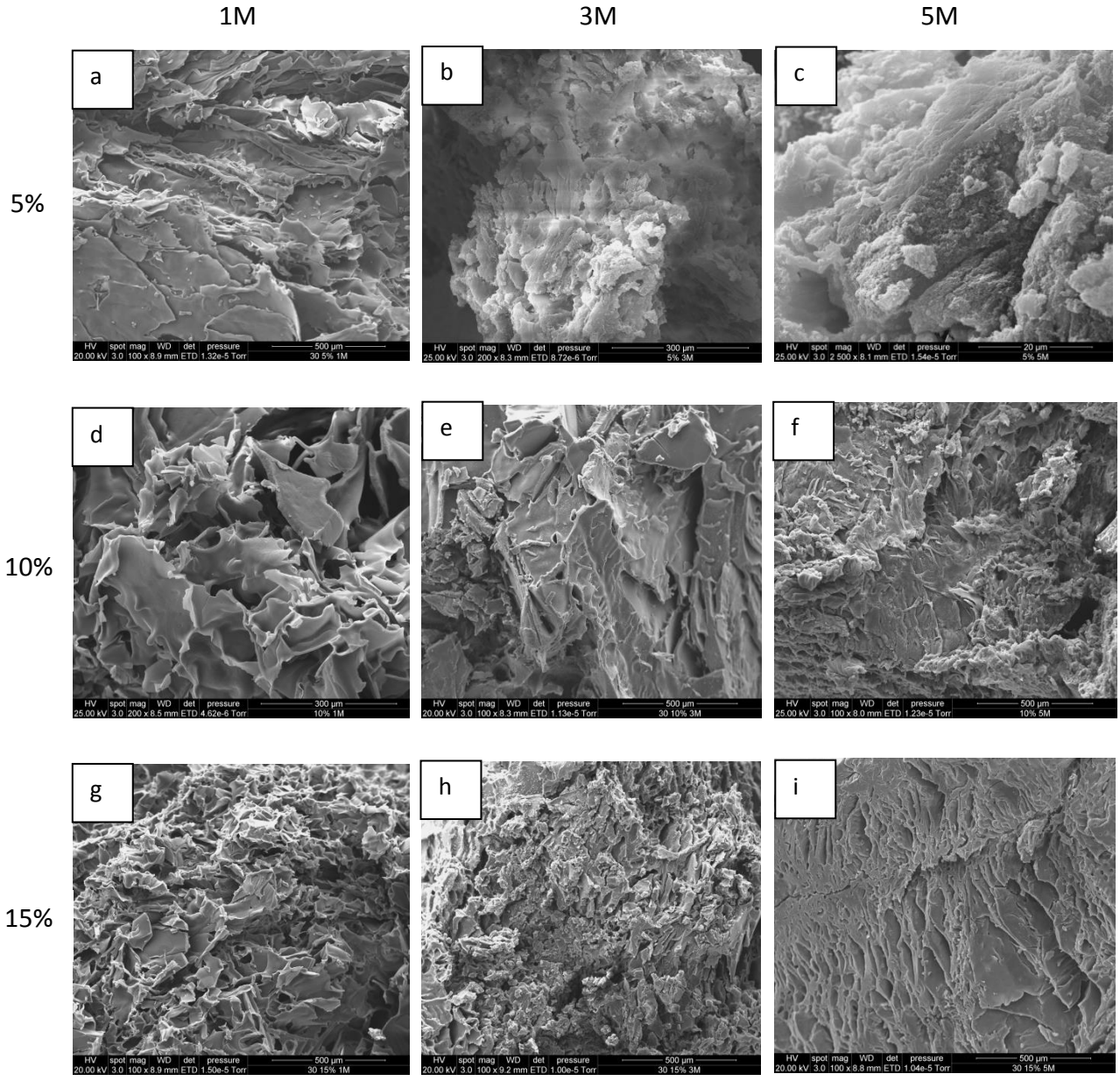


Figure 6.7 Scanning electric microscope images of cross sections of the porous structure of frozen-thawed samples at different EWP concentrations and urea concentrations. Samples were stored at -30^{o}C for 20h.

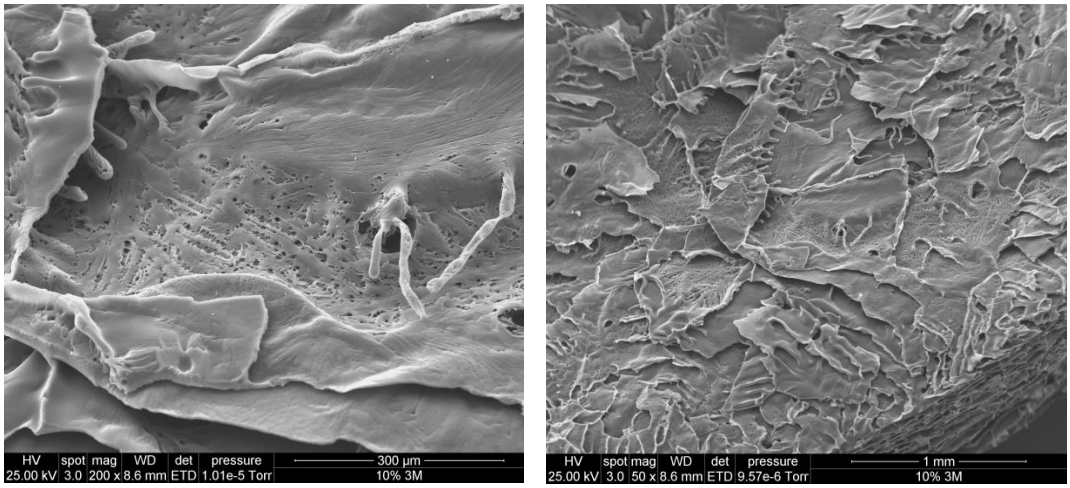


Figure 6.8 Scanning electric microscope images of longitudinal sections of frozen-thawed samples at 10% EWP and 3M urea. Samples were stored at -18^{o}C for 20h.

6.1.3 Micro-structure of cross sections of gel phase of samples observed with SEM and TEM

The SEM images of the cross sections of the gel phase of samples are shown in **Figure 6.9**. Samples with initial 5% EWP and 1M urea show a neat cross section with sparsely distributed small pores. The density of protein with the cross section increased when the initial concentration of EWP was increased to 10% and 15% with the same concentration of urea. At higher urea concentrations (3M and 5M), denser and bigger pores appeared in the gel phase at all EWP concentrations.

The TEM images (**Figure 6.10**) indicate the relative distribution of protein and pores in the gel phase. Pores tend to be more dense and smaller as the concentration of urea is increased. At 1M urea, pores are in clear, regular rounded shapes at 5% EWP (Figure **6**.**10**a). When the concentration of EWP is increased to 10% (Figure **6**.**10**e), the shape of pores becomes irregular with less clear boundaries. EWP concentration continuously increases (15%) (Figure **6**.**10**h), these irregular pores and protein are evenly distributed in the gel. The individual pore area is narrower and denser. For both EWP concentrations, pores are back to dots-like distribution (Figure **6**.**10**f, g, i, j) at higher urea concentration as they are at lower EWP concentration (Figure **6**.**10**a-c).

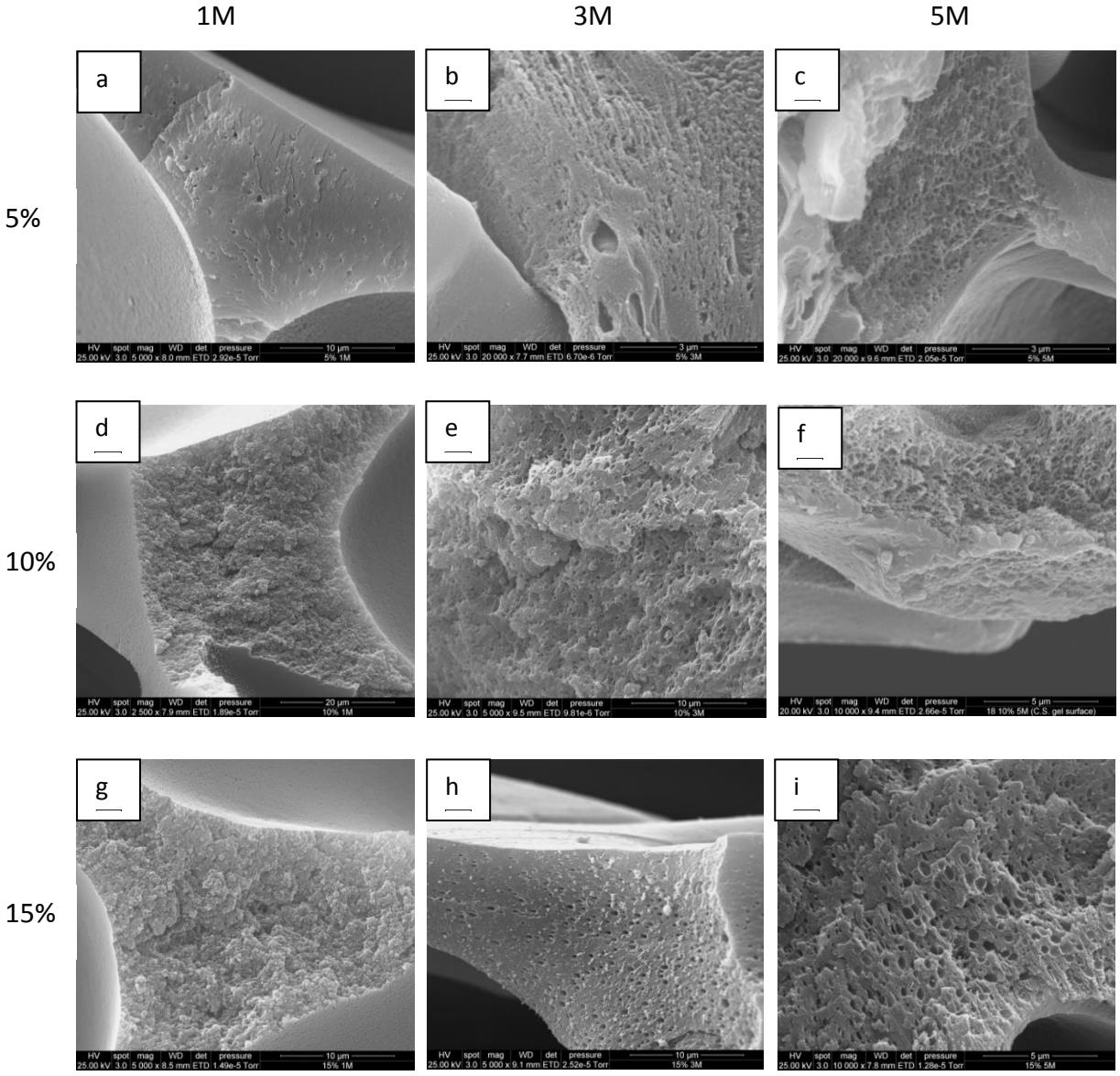


Figure 6.9 Scanning electric microscope images of cross sections of the gel phase of frozen-thawed samples. Samples were stored at -18^{o}C for 20h.

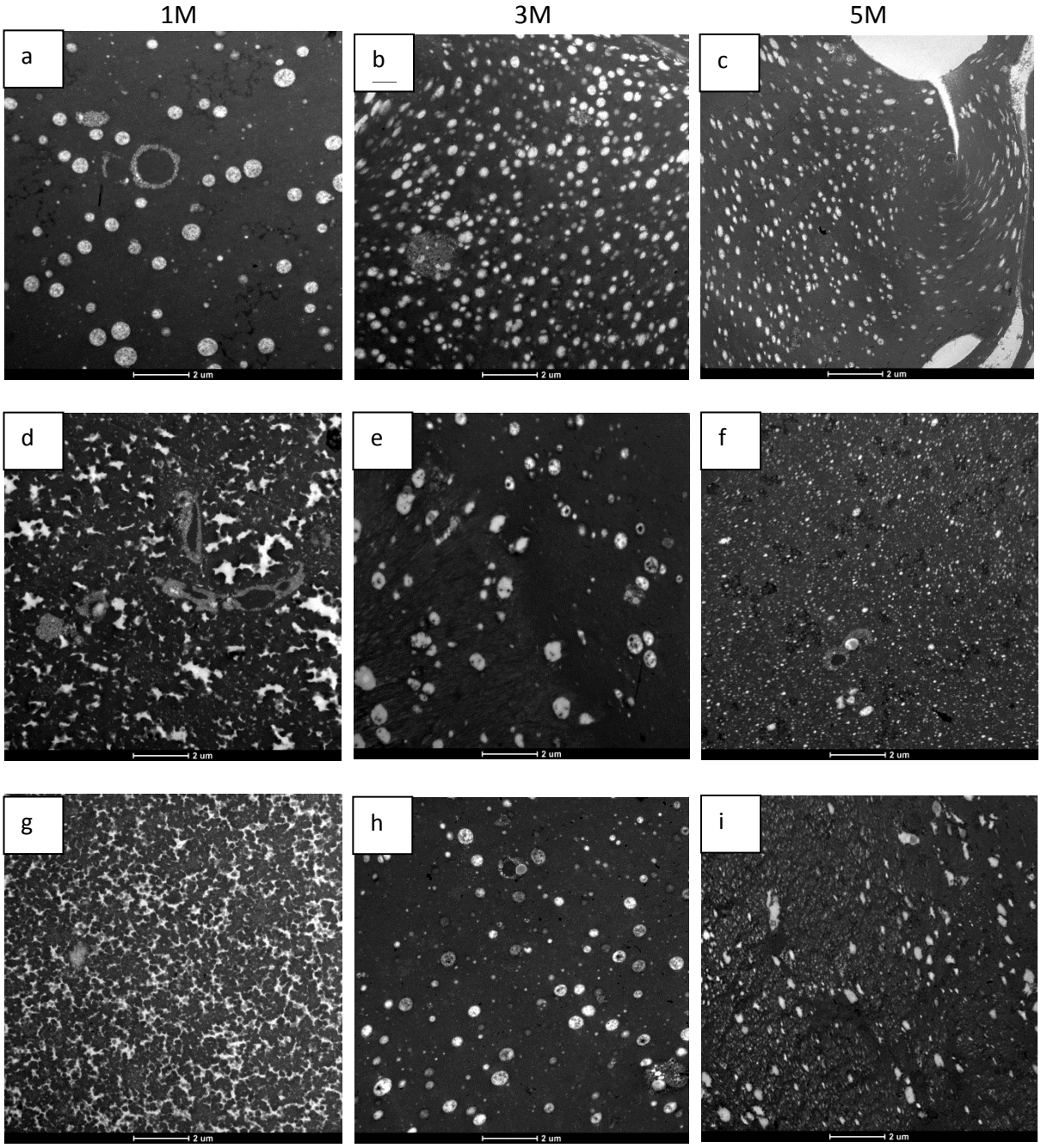


Figure 6.10 Transmission electric microscope images of cross sections of frozen-thawed samples. Samples were stored at -18^{o}C for 20h. (a) 5% EWP×1M urea; (b) 5% EWP×3M urea; (c) 5% EWP×5M urea; (d) 10% EWP×1M urea; (e) 10% EWP×3M urea; (f) 10% EWP×5M urea; (g) 15% EWP×1M urea; (h) 15% EWP×3M urea; (i) 15% EWP×5M urea.

6.1.4 Nano differential scanning calorimetric (nano DSC) measurements

DSC measurements were conducted to investigate the influence of urea concentration on the denaturation of EWP. **Figure 6.11** shows the thermal denaturation of EWP solutions containing urea at concentrations from 1M to 6.6M. For native EWP (a), an endothermic denaturation peak was observed at ~78^{o}C. With the addition of 1M urea (b), denaturation

peak occurred at lower temperature (around 75°C) but the enthalpy of denaturation showed little difference. However, as the concentration of urea contained in the solution increased further, both protein denaturation temperature and the enthalpy of denaturation decreased. At 6.6M urea the denaturation temperature had dropped to ~60°C, markedly lower than that (78°C) without the presence of urea, and with a corresponding reduction in enthalpy.

Figure 6.12 shows the DSC thermograms of thawed EWP cryogels over the same heating range used to observe denaturation in the EWP solutions containing urea. It was interesting to note the absence of any endothermic transitions across the applied temperature range. The implication of the data is that cryogelation facilitates complete protein denaturation across all urea concentrations studied (noting that denaturation does not occur as a consequence of freezing in the absence of urea), most likely due to freeze concentration effects. It also appears that denaturation is a critical step in the gelation mechanism of EWP cryogels.

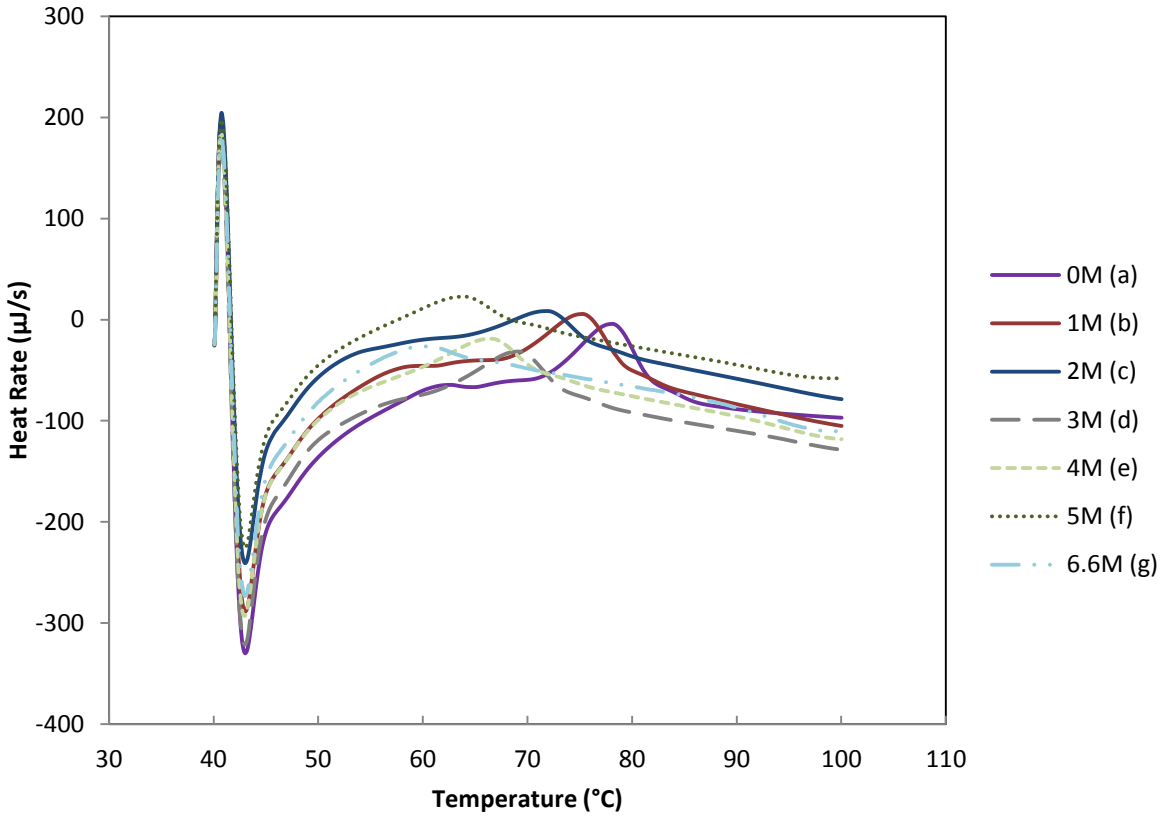


Figure 6.11 Thermograms of EWP solutions containing urea at various concentrations. ↓ Exothermal

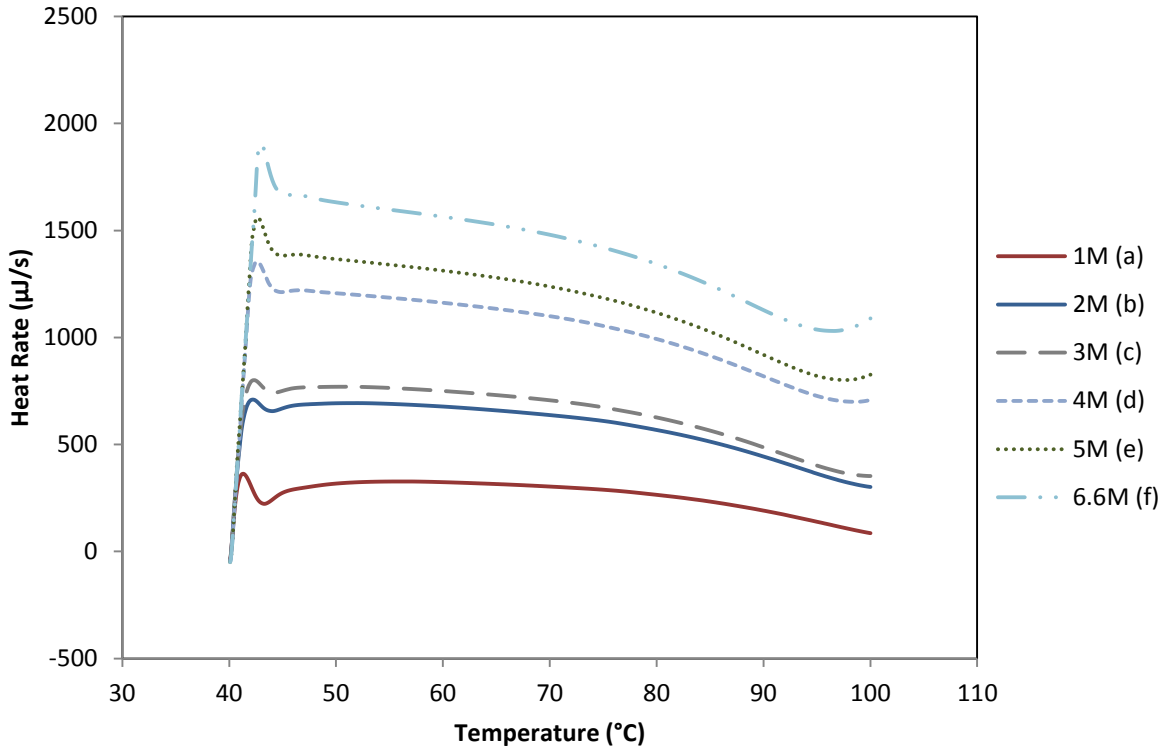


Figure 6.12 Thermograms of EWP cryogel suspensions containing urea at various concentrations. ↓ Exothermal

6.2 Discussion

6.2.1 Macro- and micro-structure of samples

The investigation of cryogel microstructure can provide a number of insights relating to the macroscopic properties of cryogels as impacted by protein and urea concentration. Samples with clearly observed porous microstructures tended to have more sponge-like appearance. This was seen to be the case for samples containing 5% EWP and 1M urea, 10% EWP and low concentrations of urea and samples containing 15% EWP at all urea concentrations. For samples containing initial 5% EWP and 3M, 5M urea respectively frozen at -18^{o}C, their gel walls in micro-images were more swollen with smaller pores and it was harder to visually recognize the sponge-like fibrous feature of the resultant cryogels. Their counterparts frozen at -30^{o}C had even less porous structure with lower amount of pores, resulting in small rigidity of the resultant crygelsPrevious studies have shown the effect of initial urea concentration and freezing temperature on the structure of resultant cryogel.

Konstantinova and Lozinsky (1997) observed a reduced pore size and increased thickness of the wall of the pores in cryogel with initial 2.5M urea and 5% ovalbumin, compared to that with initial 1M urea and the same amount of ovalbumin. At a close concentration in this study (5% EWP and 1M, 3M urea respectively), samples with 1M urea showed similar thickness of the wall of pores and pore size. Samples with 3M urea did not have as regular porous structures as the one with 2.5M urea in Konstantinova and Lozinsky study, but did show the increase in the thickness of the gel wall phase and the decrease in pore size (pore deformation) to some extent. The size of macro-pores of BSA cryogels was found either diminish at freezing temperature of -15^{o}C or pass through a slight maximum for samples formed at -20^{o}C with increasing feed urea content (Rodionov et al., 2015). The study also showed a decrease in the size of macropores of the samples as the freezing temperature was decreased from -15^{o}C to -20^{o}C. The smaller porogen particles at lower freezing temperature may help to explain this tendency (Rodionov et al., 2015). Both findings agree with the observed trend in this study, where the size of macro-pores of samples decreased at higher urea concentration and lower freezing temperature. Furthermore, it has been pointed out that when there is no super cooling, the crystal size of the solvent is smaller at lower freezing temperature (Lozinsky & Okay, 2014). The smaller size of crystals in the solvent should be responsible for the deformation and diminishing of macropores of samples frozen at -30^{o}C. A high initial concentration of EWP seems to guarantee a proper porous structure at high amount of feed urea content (**Figure 6.6** g-i). It gives the sufficient protein concentration in the unfrozen phase and thus provides a decent cohesiveness of the gel phase to maintain a porous structure.

It should be pointed out that the porous structure of cryogel is not as uniform as that of a comparable EWP heat set gel, perhaps not surprising given that gel fibres are formed in the unfrozen interstices between ice crystals, and thus the gel structure will be a consequence of the fractal properties of the ice crystal network. Furthermore, the formation of the frozen phase may be inhibitory to the formation of a percolating network, thereby accounting for the more inhomogeneous gel structure of the cryogel. In this regard, it should be noted that the microstructure images only show a very small part of the sample that may differ from the other. The variation of the microstructure of cryogel contributes to the difficulty when it

comes to identifying the accurate properties. However, it still provides a general idea about the microstructure of cryogel under different conditions.

6.2.2 Differential scanning calorimetric measurements

Duplicate experiments showed a good reproducibility of denaturation temperature (±0.5^{o}C). A previous DSC study on fresh egg white has shown four denaturation peaks on the thermogram (Ferreira, Hofer, & Raemy, 1997). There is only one denaturation peak on the thermogram of native EWP in this study (**Figure 6.11**a), which is believed to indicate the denaturation of ovalbumin fraction, as the denaturation temperature (78^{o}C) also coincides with that shown in the DSC study on ovalbumin fraction alone (Ferreira et al., 1997). The absence of other peaks may result from either the lack of sensitivity of the equipment to other protein fractions in egg white or their deactivation during the process of egg white dehydration.

The decreasing denaturation temperature and diminished denaturation areas on the thermograms for samples with addition of urea compared to that without urea indicate a reduction in the strength of interactions maintaining the protein in a folded state. The degree of denaturation of EWP is higher at higher urea concentration. EWP does not seem to be fully denatured at such a high concentration of urea as 6.6M, although the small denaturation area indicates it has been mostly denatured. Urea is commonly used for protein denaturation. Urea weakens hydrogen bonds and hydrophobic interactions in proteins as well as exposing sulphydryl and disulphide groups in protein molecules to induce forces like SH=SH interaction and sulphydryl oxidation interaction (Kinsella, 1982; Xiong & Kinsella, 1990a). Also, urea has been considered to denature proteins by interfacing between natively buried parts of protein and water (Colombo, Ribotta, & Leon, 2010; Stumpe & Grubmuller, 2007). Moreover, the increase in “permittivity” of water for the apolar residues caused by urea is another reason for the loss of protein structure(Li-Chan & Ma, 2002).

The frozen-thawed samples are believed to be fully denatured after the freezing-thawing process regardless of the concentration of urea added to the initial solution based on the disappearance of denaturation peaks on the thermograms (**Figure 6.12**). The tendency of the generation of a curve at high temperature (100^{o}C) on the thermogram with high urea

concentration (**Figure 6.12**e and f) may result from the high amount of urea in the solutions. Similarly, diminshed denaturation peaks for BSA cryogel suspensions have been shown in Rodionov and his team's study (2015), which indicated a strong denaturation of the protein compared to native BSA. The study also eliminated the possibility of irreversible cold denaturation during freezing that may cause protein denaturation and confirmed that the presence of denaturant/reductant mixtures is the cause of conformational changes of BSA in cryogels. However, Konstantinova and Lonzinky (1997) evidenced that the degree of denaturation of ovalbumin solutions containing 0.25M and 2M urea respectively after freezing-thawing is small based on the slight distinction of UV and CD spectra between samples with and without undergoing freezing-thawing process. The denaturation peak of EWP in the presence of 2M urea had been significantly diminished (**Figure 6.11**c) compared to native EWP (**Figure 6.11**a), even a slight further denaturation may cause the disappearance of denaturation peak on the thermogram. Either way, all studies have evidenced the further denaturation of protein after freezing-thawing process. This further denaturation should therefore be due to the concentrated urea in the unfrozen phase of the frozen sample.

7 Further interpretation of the mechanism of cryotropic gelation using modelling correlations

7.1 Correlations between urea concentration and the system

7.1.1 Correlation between initial urea concentration and unfrozen liquid phase in the frozen samples

Some equations ([7.1]-[7.5]) describing correlations between initial concentrations of component in the solution and the volume of unfrozen phase of frozen samples are suggested (Konstantinova & Lozinsky, 1997).

$$/V_0 = C_0^u / C \qquad [7.1]$$

[7.2]

$$\Delta(T \quad T) \quad [7.3]$$

$$/\Delta(T \quad T) \quad [7.4]$$

$$\Delta(T \quad T)/C \quad [7.5]$$

Where are concentrations of protein and urea respectively in the initial solution before freezing, and C_f^u are the concentrations in the unfrozen phase in the frozen sample, is the volume of solution before freezing, V_f is the volume of unfrozen microphase in the frozen sample, T_0 is the temperature of freezing of the pure solvent, T is the freeze temperature of the experiment, Δ is cryoscopic constant of water.

To confirm the influence of urea on the phase state of the frozen samples, Konstantinova and Lozinsky (1997) conducted the ^{1}H experiment. Figure **7.1** shows how the volume of liquid phase of frozen samples from different solutions changes at various urea concentrations. Results calculated from equation [7.1] show that the volume of liquid phase increases as the concentration of urea increases. Also the results from ^{1}H experiment show the same trend with or without ovalbumin in the initial solution. Therefore it has been concluded in the article that urea influences the volume of liquid phase of frozen samples while there is no influence of protein in the solution. Also, it has been proved that equation [7.1] describes the correlation between urea concentration and the volume of liquid phase properly.

It can be concluded from equation [7.3] that when the difference between freezing temperature of the pure solvent and the temperature of the experiment is constant, the concentration of urea remains constant in the unfrozen phase in the frozen sample. Figure **7.1** shows that the volume of liquid phase increases as the concentration of urea in the initial solution increases in a certain range, following a positive linear relationship. It means although the initial concentration of urea is increased, the volume of the unfrozen phase also increases proportionately, which explains the constant concentration of urea in the unfrozen phase.

Figure 7.1 Relative content of the liquid phase in frozen samples at various urea concentrations. (•) [water-urea solutions (data calculated from the diagram using the equation (1)]; (Δ) water-urea solutions (data calculated from 1H NMR experiments); (I) 5% ovalbumin water-urea solutions (data calculated from ^{1}H NMR experiments). T=262K. (Konstantinova & Lozinsky, 1997)

7.1.2 Correlation between initial urea concentration and freezing point depression of the solvent in the system

According to equation [7.6], Figure **7.2** that describes the influence of urea at the freezing point of water has been obtained. The freezing point decreases with increased urea molality in a linear manner.

[7.6]

Where ΔT_f is the change of freezing point, i is the number of particles urea splits into as when dissolved as solute, K_f is freezing point depression constant of water and m is the effective molality of urea.

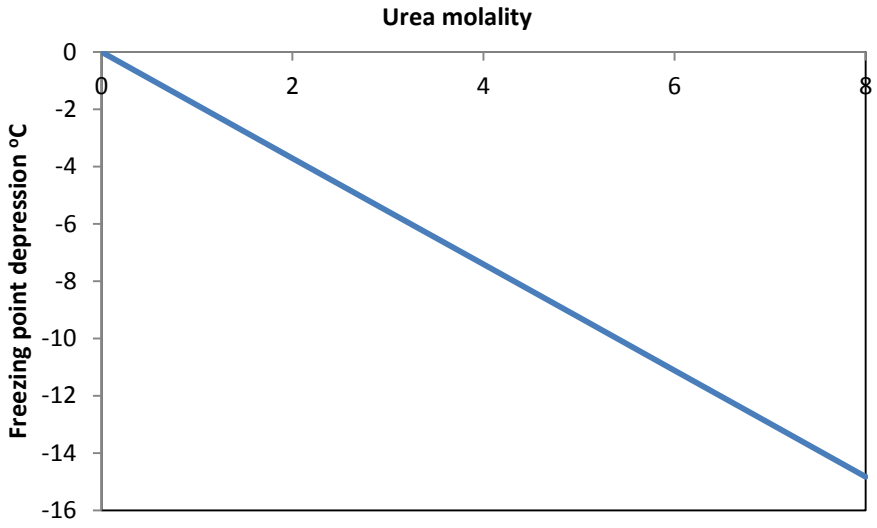


Figure 7.2 freezing depression of water at various urea molalities

7.2 Correlation between freezing temperature and unfrozen phase in the frozen samples

According to equation [7.4] and [7.6], the relationship between the volume of unfrozen phase and freezing temperature regarding different initial concentrations of urea can be described theoretically using Figure **7.3**. Data is presented at points where temperature under freezing point of water at certain urea concentration. It reveals that the lower the temperature under freezing point, the smaller the volume of unfrozen phase. In addition, the lower the urea concentration the smaller the volume of unfrozen phase.

Based on the condition of this research, relationship between the volume of unfrozen phase and the initial concentration of urea at -18^{o}C and -30^{o}C as freeze temperature is shown in Figure **7.4**. At -18^{o}C the volume of unfrozen phase increases more rapidly than that at -30^{o}C and also the values are higher at all urea concentrations.

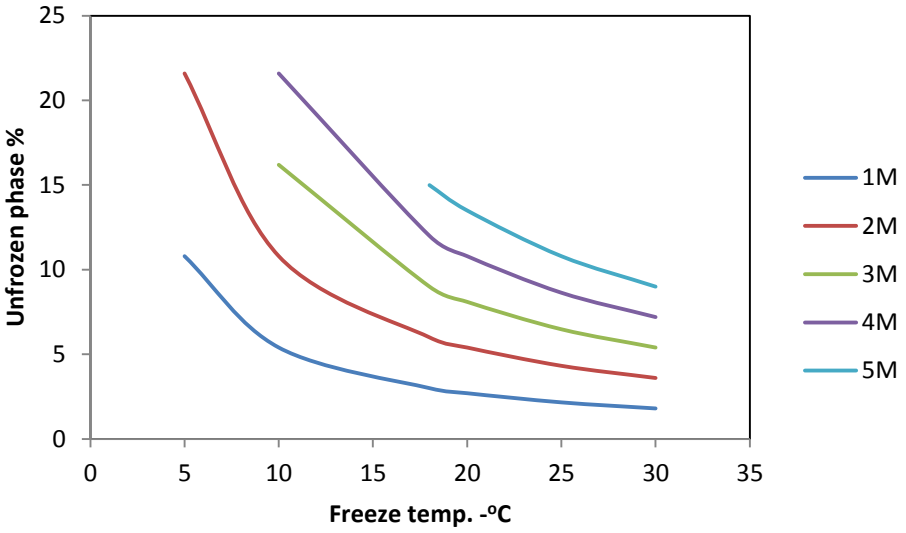


Figure 7.3 Correlation between freezing temperature and the volume of unfrozen phase in the frozen sample.

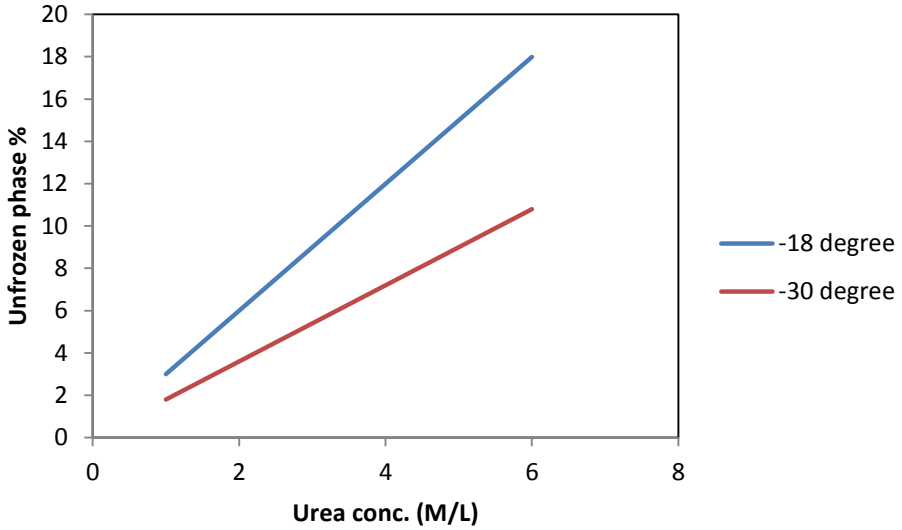


Figure 7.4 Correlation between initial urea concentration and the volume of unfrozen phase at freeze temperature of -18°C and -30°C.

7.3 Relative protein concentration in the unfrozen phase

7.3.1 Correlation between initial urea concentration and relative protein concentration in the unfrozen phase in the frozen sample

Relative concentrations of protein (C_p^f) in the unfrozen phase of frozen samples were shown in Table **7.1** and Figure **7.5**, calculated using equation [7.5]. C_p^f increases as the initial concentration of protein increases and the initial concentration of urea decreases. However,

based on equation [7.5] C_p^f at high urea concentration (e.g. 6.6M) is supposed to be even lower than the initial concentration before freezing, which is impossible. Therefore, it is assumed that this equation is only valid in a certain range of initial urea concentration (e.g. $C_u^0 \leq 5.5M$), as C_u^0 gets higher and higher, the value of C_p^f should be infinitely approaching the initial concentration C_p^0. Based on this, a corrected correlation between initial urea concentration and relative protein concentration has been drawn (**Figure 7.6**).

Table 7.1 Relative concentration of protein in the unfrozen phase of samples frozen at -18°C - 30°C and at various initial concentrations of urea and EWP

	Relative protein conc.									
urea	5%		7%		10%		12%		15%	
conc. (M)	-18°C	-30°C	-18°C	-30°C	-18°C	-30°C	-18°C	-30°C	-18°C	-30°C
1	27.767	46.279	38.874	64.790	55.534	92.557	66.641	111.069	83.302	138.836
2	13.884		19.437	32.395	27.767	46.279	33.321	55.534	41.651	69.418
3	9.256		12.958	21.597	18.511	30.852	22.214	37.023	27.767	46.279
4	6.942		9.719		13.884	23.139	16.660	27.767	20.825	34.709
5	5.553		7.775		11.107	18.511	13.328	22.214	16.660	27.767
6.6	4.207		5.890		8.414		10.097		12.621	

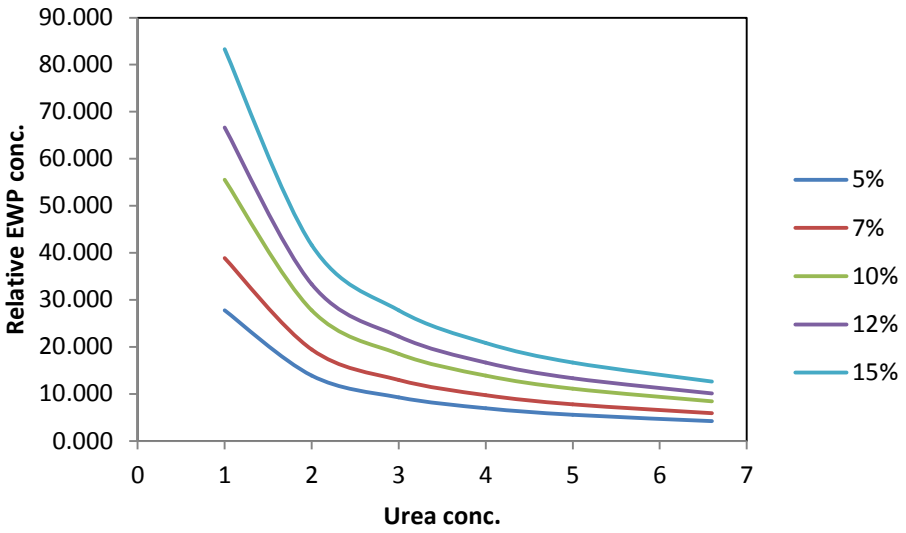


Figure 7.5 Relative concentration of EWP in the unfrozen phase of samples frozen at -18°C at various initial concentrations of urea and EWP.

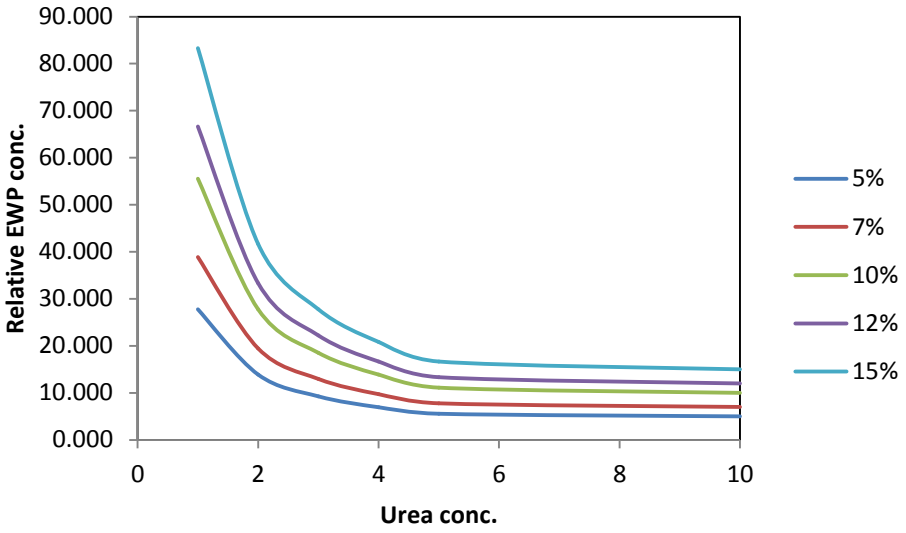


Figure 7.6 Corrected correlation between initial urea concentration and relative protein concentration at the freezing temperature of -18°C.

7.3.2 Relationship between relative EWP concentration and properties of cryotropic gels

Compression peak force and water holding capacity of frozen-thawed samples regarding the relative concentration of EWP are shown in Figure **7.7** and Figure **7.8**. CPF increased gradually when initial EWP concentration was at 5% and 7%. At 10% and 12% initial EWP, CPF increased as relative EWP concentration was increased from lower value to higher value. When the relative concentration kept increasing, CPF tended to be stable and only

increased slightly. At 15% initial EWP, CPF firstly increased from about 0.9N at 12% relative EWP concentration to the maximum (1.8 N) at 27% relative EWP concentration, before decreased from the maximum point as the relative EWP concentration continuously increased. For WHC, as relative EWP concentration increased, WHC of samples with 5% initial EWP dramatically increased about 30% (from around 58% to 88%), followed by a drop and getting relatively stable. For samples containing 7% initial EWP, WHC starting at 70% at the lowest relative EWP concentration reached a minimum value (45%) at 20% relative EWP and bounced to 57% when relative EWP concentration was doubled to about 40%. WHC of samples containing 10% and 12% initial EWP respectively also decreased remarkably before a gradual increase with close values between the two series. WHC of samples at 15% initial EWP decreased slightly after a decrease-increase trend as the relative EWP concentration went higher.

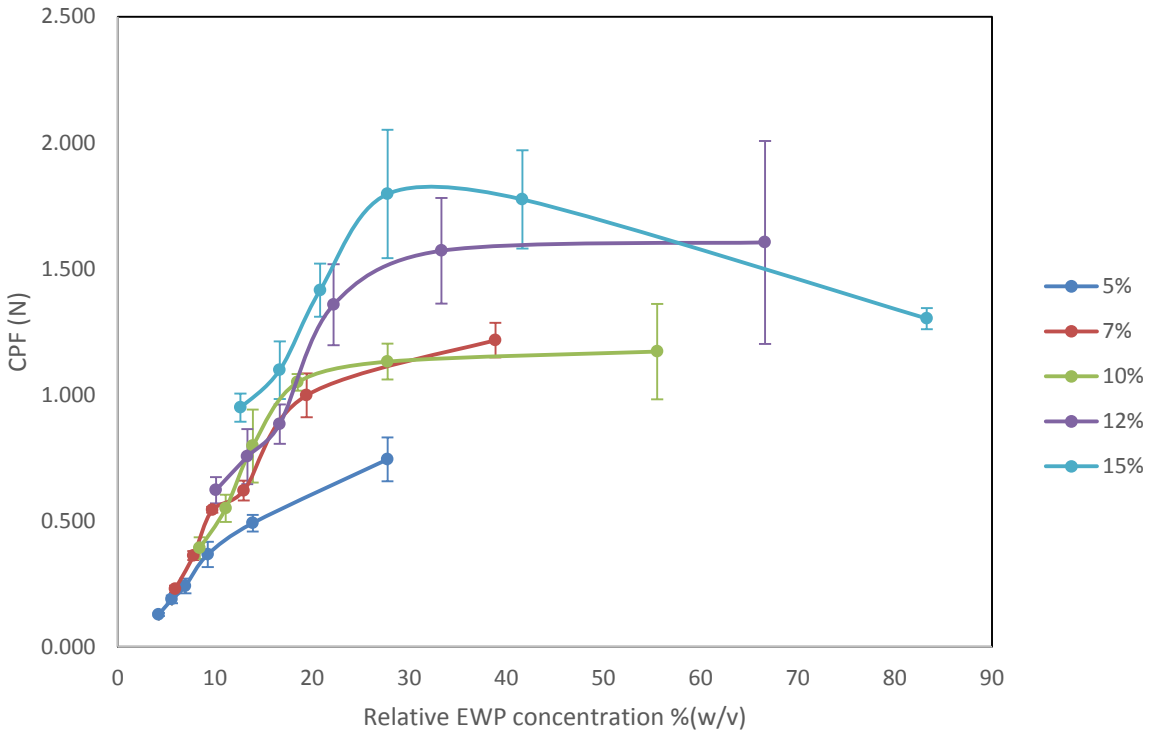


Figure 7.7 Compression peak force of frozen-thawed samples regarding relative concentration of EWP at various initial EWP concentrations. Samples were stored at -18^{o}C for 20h.

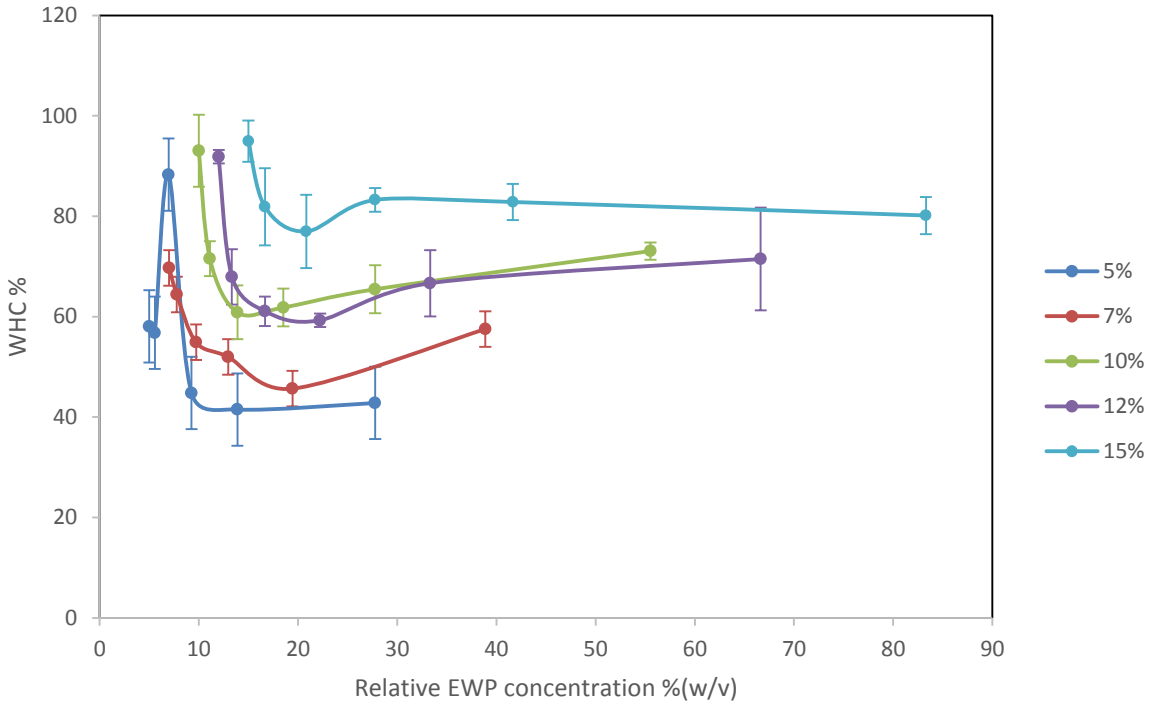


Figure 7.8 Water holding capacity of frozen-thawed samples regarding relative concentration of EWP at various initial EWP concentrations. Samples were stored at -18°C for 20h.

7.4 Discussion

Now that the correlations between sample preparation conditions and material and properties have been presented, the data and observations obtained in this study can be further discussed based on these correlations.

7.4.1 Effects of initial concentration of urea in the system

Urea has essential effects on the whole process of the formation of cryogels with EWP. Firstly, it denatures protein molecules so the unfolded protein molecules are able to interact and form a three-dimensional network in the frozen sample (Konstantinova & Lozinsky, 1997; Totosaus et al., 2002; Xiong & Kinsella, 1990b) (Section 6.2.2). Secondly, it determines the freezing point of the solvent (water in this case) with cryoscopic effect (Section 7.1.2). Thirdly, it affects the volume of liquid phase of the frozen sample and thus alters the actual concentrations of protein and urea in the liquid phase (Section 7.1.1). The last two effects are also believed to influence the porous structure of the resultant cryogels (Section 6.1.2).

It is believed that the freezing point depression caused by the addition of urea should be responsible for the feed did not freeze at the temperature of -10°C (Section 5.2). However,

according to Figure **7.2** , when urea molality is no more than 5M the freezing point of water as the solvent is above -10^{o}C, which indicates that the feed should be expected to freeze at the freezing temperature of -10^{o}C. The opposite result is possibly due to the increasing urea concentration in the solvent during the pre-formation of crystals. More specifically, as crystals are partially formed at the early stage, the concentration of urea is increased because of the reduced liquid phase of the solvent and thus increases the freezing point compression. The increase of freezing point depression along with insufficiently grown crystals make a negative circle during the whole duration of freezing and consequently cause the failure of freezing the feed. Another possible explanation is supercooling phenomenon (Lozinsky & Okay, 2014).

In most cases, the increase in initial concentration of urea causes a decrease in gel strength of the resultant cryogels (Section 5.1.1, Section **Error! Reference source not found.**, Section **Error! Reference source not found.**). Given the fact that the volume of the unfrozen phase decreases as the initial concentration of urea increases (Section 7.1.1), it is not hard to conclude that the relative protein concentration also decreases as the protein phase is less compressed in the greater unfrozen phase (Section 7.3). A lot of studies have shown that higher protein concentration results in stronger gels (M. V. Avanza et al., 2005; Otte et al., 1999; Sun & Arntfield, 2010). In other words, lower protein concentration decreases gel strength of resultant gels. Therefore, as the initial concentration of urea increases, a less rigid gel is formed in the gel phase, which contributes to lower gels strength of the cryogels.

However, not all samples followed the consistent decrease tendency in gel strength as the initial urea concentration increased, especially for samples at high initial EWP concentrations such as 15% (Section 5.1.1, Section **Error! Reference source not found.**, Section **Error! Reference source not found.**). That is most likely because of the change in porous structure caused by the greater decrease in initial concentration of urea, which alters the mechanical properties of the sample. Section 6.1.2 (SEM) shows the influence of initial urea concentration on the morphology of macropores. It is possible that the increase in freezing point depression caused by increasing initial concentration of urea results in the difference in crystal growing and thus affects the formation of macropores when crystals melt. Furthermore, as mentioned in Section 5.4.2, the free water contained in the sample has effects as well, and this is also due to the urea concentration.

Hypothetically, there should be less released water (equals to greater WHC) as the initial concentration of urea increases as at higher urea concentration the crystal phase is higher, which converts to free water when being thawed. Different from this expectation, most samples were observed with a decrease in WHC before an increase (Section 5.1.2, Section 5.2.2, Section 5.3.2). The measurement of the WHC in this study was a gravimetrical measurement, which indicates not only the volume of free water in the samples but the mechanical characteristics of samples themselves affect the results. Also, the mechanical properties of samples are determined by the resultant structure (pore size and shape, gel phase) affected by the amount of urea in the initial system. Therefore, urea affects the WHC basically through affecting the volume of unfrozen phase and the structure of the samples.

7.4.2 Effects of the concentration of EWP in the system

As the precursor in the system EWP plays an important role in the formation of cryogels. The main effect of the initial concentration of EWP is to alter its relative concentration in the unfrozen phase (Figure **7.5**). At a constant initial urea concentration, the relative EWP concentration increases as its initial concentration increases (Table **7.1**). The relationships between relative EWP concentration and properties of resultant cryogels have been shown in Figure **7.7** and Figure **7.8**. These figures show that the same relative EWP concentration can have multiple corresponding values of the certain property. It further proves that relative protein concentration in the unfrozen phase is not the only factor that affects the properties of cryogels. At low relative EWP concentrations, the corresponding values of CPF are closer, which possibly indicates that at these low concentrations the CPF of samples does relay more on protein concentrations than other factors.

Generally, WHC of thawed samples decreases with increasing relative EWP concentration at low concentrations, followed by a more slightly increase or decrease (series 15% in Figure **7.8**). Apart from the size and quantity of the crystals in frozen samples, the gel phase of cryogel is another important factor affecting the WHC for its mechanical effect that can squeeze the water out of the cryogel working with gravity. It can be said from the microstructure of samples (Section 6.1.2) that the morphology varies at different initial concentrations of EWP and urea and at different freezing temperature (which leads to different relative EWP concentration). As a result, the change of relative EWP may alter the mechanical properties of the gel phase that can either help squeeze out the free water of

hold it inside the cryogel structure. Again, the multiple corresponding values at the same relative EWP concentration more or less indicate that the relative concentration of EWP is not the dominant factor affecting WHC of thawed samples.

7.4.3 Effects of freezing temperature

Below freezing point of the solvent, the volume of unfrozen phase should decrease with decreasing temperature as indicated in Section 7.2. With decreasing unfrozen phase, the walls between pores in samples will be expected to be thinner and the relative protein concentration in the unfrozen phase should additionally be higher at lower freezing temperature (Table **7.1**). However, there is not sufficient evidence to show thinner gel walls at lower freezing temperature based on SEM images in this study. On the contrary, noticeably thicker walls can be observed at specific concentrations of EWP and urea at -30^{o}C compared to -18^{o}C. The model of the relationships between freezing temperature and the volume of unfrozen phase may need to be further verified due to the inconsistency between the model and the result of the experiment.

Results show that the cryogels obtained at lower temperature (-30^{o}C) were weaker (Section **Error! Reference source not found.**). Meanwhile, WHC at lower freezing temperature was found higher only at high urea concentrations (Section 5.2.3). The decrease in the strength of porous microstructure and the increase in thickness of wall between macropores of samples may account for this phenomenon.

8 Overall Discussions

Apparently, the structural characteristics of cryogel determine their macroscopic behaviour. Urea has remarkable contribution to the structure of cryogel. This is evidenced by the change of porous structure and the thermal denaturation of native EWP and EWP in the presence of urea (Section 6.1.2). The effect of the variation of urea concentration on protein denaturation seems to be little as proteins were fully denatured regardless of the initial urea concentration. The freezing temperature also influences the microstructure. One of the influences is the smaller porogen particle at lower temperature as mentioned earlier (Rodionov et al., 2015). The other one is the smaller crystal size at lower temperature (Lozinsky & Okay, 2014). In fact, urea and the freezing temperature are believed to work together on the porous structure by affecting crystal generation morphologically and

volumetrically. The influence of EWP (or protein) is more within the gel phase to provide the foundation and strengthen the structure with high enough concentration. The effect of freezing duration seems to be minor except the increase in freezing duration increases the gel strength of the samples at low urea concentration. However, the reason of the increased gel strength at 1M urea at long freezing duration remains not properly explained.

Essentially, the properties of cryogelation are affected by the combination of the properties of gel wall between pores (e.g. thickness, intensity of protein, hardness) and the morphological properties of macropores (mainly the size of pores and the number of pores in a certain area).

9 Conclusions and recommendation

9.1 Conclusions

This research focuses on the effects of the concentration of the components (urea and EWP in this case) and the conditions on the behaviour and properties of cryogel. Based on the comparison of SPI, EWP and WPI undergoing the freezing-thawing process with the presence of urea, EPW showed the best cryotropic gelation characteristics among these three different globular proteins. Overall, the gel strength of EWP cryogel decreased as the concentration of urea in the feed system increased in the range of 1-6.6M, and increased as the concentration of EWP in the feed system increased from 5% to 15% (w/v). The gel strength was generally lower at lower freezing temperature (-30^{o}C) compared to that at -18^{o}C. The 68 hours of freezing increased the gel strength of samples at low urea concentration compared to the 20 hours freezing storage. The water holding capacity of EWP cryogels went through a minimum before an increase as the concentration of urea was increased for samples containing 7-15% (w/v) EWP frozen at -18^{o}C. Opposite to this, samples containing 5% EWP reached a remarkable maximum at the urea concentration of 4M. Lower freezing temperature (-30^{o}C) increased the WHC high urea concentrations. The lengthened freezing duration did not show effects on WHC.

The structural characteristics of EWP cryogel is affected by the concentration of urea and EWP in the initial system and freezing temperature. The increase in urea concentration tends to reduce the size of macropores in cryogel and the porous structure at low EWP

concentration. High EWP concentration can prevent the loss of porous structure caused by the increase in urea concentration. Additionally, lower freezing temperature contributes to smaller pore size and weakens the structure in cryogel. Urea destabilises the protein structure of EWP. The degree of denaturation of EWP was higher at higher urea concentration. Also, a higher degree of denaturation occurred in cryogel after freezing-thawing process.

9.2 Recommendations for future work

Gel strength measurement could be conducted after removing the water content from melted crystals in cryogel. A way to obtain cryogels in uniform shape and size should be developed. A more accurate method of WHC measurement for cryogel should be improved from the existing one. Quantification of the size of macropores would help better understand the porous structure of cryogel.

The alternative ways to predenatured the protein molecules are of interest, especially the use food grade agents that have the similar functions as urea on cryogelation. Other than that, the effects of other factors such as ions and pH in the initial feed system should be investigated. Also, the study of alternative precursors other than egg white protein (other globular proteins or polymers) for cryogelation has the potential of research.

The application of cryogel in food products such as meat products or cakes is always expected. How cryogel works with an actual food product and the new texture and other characteristics it provides are of high interest.

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