EFFECT OF $CACL_2$ ON VISCOELASTIC PROPERTIES AND MICROSTRUCTURE OF CALCIUM-INDUCED COLD-SET WHEY PROTEIN ISOLATE GELS

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ABSTRACT

The role of $CaCl_2$ concentration on viscoelastic properties and microstructure of Ca^{2+}-induced cold-set whey protein isolate gels (containing 10% protein w/v, pH 7) was investigated. An increase in [$CaCl_2$] from 10 to 120 mM increased shear stress at failure and decreased shear strain. Asymptotic residual modulus in stress relaxation test suggested that gels containing high $CaCl_2$ behave like solid more than gels containing lower $CaCl_2$. Scanning and transmission electron micrographs revealed that gels containing higher [$CaCl_2$] had larger aggregate size and more porous than gels containing lower $CaCl_2$.

INTRODUCTION

Whey proteins are defined as the proteins remaining in milk serum after the removal of caseins.[1] Whey protein concentrate (WPC) and whey protein isolate (WPI) can be used as food additives or converted into highly functional and nutritional ingredients for medical and pharmaceutical uses due to their excellent nutritional value and a wide range of functionalities.[2,3] The major functional properties of whey proteins include: a) solubility throughout the entire pH range, b) gelation when subjected to heating under proper protein and ionic conditions, c) emulsification, and d) foam stabilization.[3]

The ability to form heat-induced gels at high temperature is a functional property important in many food systems, such as processed meat, bakery and dairy products.[4] Overall, gelation of globular proteins (such as whey protein) is a two-step process involving denaturation and aggregation of unfolded proteins.[5] The mechanisms of whey protein gelation in the presence of salts have been studied through heat-induced processes in which protein denaturation, association of unfolded proteins, strand formation of aggregates and association of strands into a network[6] occur almost simultaneously during heating. As a result, the role of salt on the mechanisms of gelation can not always be distinguished from those of the heating conditions (*e.g.*, temperature, heating rate and duration of heating). Unlike heat-induced gelation, salt-induced cold gelation takes place in two distinctly separate stages. The first is unfolding and association of globular proteins into high-molecular weight-soluble aggregates during heat treatment[7] This is followed by cooling and subsequent salt addition (such as $CaCl_2$). The latter results in network formation via Ca^{2+}-mediated interactions of the soluble aggregates. Thus, cold gelation provides an opportunity to study the effect of [$CaCl_2$] independently of the initial protein denaturation.

Gels with different appearances (*i.e.*, clear to opaque), texture (*i.e.*, soft, hard, elastic or

brittle) and water holding capacity (*i.e.*, excellent or poor) can be made by manipulating processing conditions such as protein concentration, pH, ionic strength, temperature, etc.[8] This is because these extrinsic factors result in the conformational alteration of proteins (*e.g.*, size, shape, net charge, charge distribution, hydrophobicity/hydrophilicity ratio) and consequently gel network geometry.

The extent of gelation/aggregation of unfolded whey proteins to form a gel network was mainly determined by the $CaCl_2$ concentration, where maximum aggregation was obtained at 30 mM.[9] Viscoelastic property, measured as Young's modulus, indicated that gels were most rigid at 30 mM $CaCl_2$, at which point the extent of aggregation was the highest. Therefore, the objective of this study was to further evaluate the effect of [$CaCl_2$] on fracture properties and stress relaxation behaviour of Ca^{2+}-induced cold-set WPI gels and their relation to the gel microstructure.

MATERIALS AND METHODS

Gel preparation

Whey protein isolate [BiPro, Davisco International, Inc., Le Sueur, MN, 92.5 % protein determined by Macro-Kjeldahl[10] using N-factor 6.38] suspensions (10% protein w/v) were prepared in double distilled water at pH 7 adjusted by adding 0.1 M HCl or NaOH. The isolate contained 1.51 % ash (manufacturer data sheet). The suspensions were heated in polycarbonate tube (19 mm diameter) at 80°C for 30 min, cooled to room temperature (24°C) for 2 h and dialyzed against $CaCl_2$ solutions (0-150 mM) through a 20.4 or 25.5 mm diameter membrane (Spectra/Por #1 membrane, MW cut off 6,000-8,000; Spectropor, Los Angeles) using method described by Barbut and Foegeding.[11] The volume ratio of the protein suspension and dialyzing solution was 1:19. The experiment was replicated in three separate trials. Gels obtained after dialysis for 16 h at room temperature were characterized as follows:

Calcium content

Samples were dried, digested by heat and acid using H_2O_2 as a catalyst at the Analytical Service Laboratory, Department of Land Resource Science, University of Guelph. After digestion, the samples were diluted with deionized water and total calcium was determined by an Atomic Absorption Spectrophotometer Varian Spectr AA 300 (Varian Inc., Mulgrave, Victoria).

Fracture properties

Cylindrical gel sections (20.4 mm diameter x 10 mm high; n=3/treatment/trial) were compressed between lubricated stationary bottom plate and a moving upper plate using a TA-XT2 Texture Analyzer (Stable Microsystems, Haslemere, UK) at a rate of 0.8 mm/s until fractured.

Assuming that a specimen is practically incompressible, shear stress (a) can be calculated as[12]:

$$\sigma = \frac{F(L-\Delta L)}{\pi r^2 L} \quad (1)$$

where F is the compressive force at the moment of failure, L is the original sample length, ΔL is the corresponding deformation at failure, and r is the original radius. Shear strain was calculated as Hencky strain (ε):

$$\varepsilon = -\ln\left[1-\frac{\Delta L}{L}\right] \quad (2)$$

Stress relaxation behaviour

Gels were cut into 20 mm long cylinders and compressed at a rate of 0.8 mm/s. The specimens were compressed to 95% of their original height (*i.e.*, 5% deformation) using a stress relaxation test (n=3/treatment/trial). Force values were collected at 0.16-second intervals over a period of 600 s. F is the compressive force at a given time and ΔL is the difference in the deformation.

Each relaxation curve was fitted by the normalized and simplified equations[13]:

$$\frac{\sigma_0-\sigma_t}{\sigma_0} = \frac{abt}{1+bt} \quad (3)$$

and

$$\frac{\sigma_0 t}{\sigma_0-\sigma_t} = \frac{1}{ab}+\frac{t}{a} \quad (4)$$

where σ_0 is initial stress at time zero, σ_t is stress at time t, a and b are constants. The constant a was used to calculate an asymptotic residual modulus (E_A) in the following equation[14]:

$$E_A = \frac{\sigma_0(1-a)}{\varepsilon} \quad (5)$$

Colour

Internal colour of gel sample (5.7 mm path length; n=4/treatment/trial) was measured using the standard CIELAB colour system and reported as reflectance spectrum using the Colormet Fiber-Optic Spectrophotometer (Instrumar Engineering Ltd., St John's, NF). The Colormet was standardized with a white Teflon tip provided by the manufacturer.

Scanning and transmission electron microscopy (SEM and TEM)

Gel samples (with 10 and 120 mM $CaCl_2$) were cut (approximately 1 x 1 x 2 mm), fixed in 2% glutaraldehyde + 1% paraformaldehyde in 0.1 M PIPES buffer at pH 7 for 6 h, rinsed, post fixed in 1% OsO_4 overnight and dried in a graded series of ethanol.[15] For SEM, the samples were critical point dried, sputter coated with 30 nm of gold/palladium and viewed at 15 kV. For TEM, the samples were embedded in Epon, thinly sectioned, stained with uranyl acetate and lead acetate and viewed at 80 kV.

Statistical analysis

Data from the three separated trials were analyzed by the Statistical Analysis System[16] using the General Linear Model procedure (GLM). Differences among treatments were determined by Least Significance Difference procedure using $p \leq 0.05$.

RESULTS AND DISCUSSION

Statistical analysis showed no significant trial effect ($p>0.05$) on Ca content in cold-set WPI gels, fracture properties, stress relaxation behaviour and reflectance spectra. Overall, $CaCl_2$ concentration had significant effect on both viscoelastic properties ($p\leq0.05$) and internal colour ($p\leq0.0001$).

Viscoelastic properties

Raising [$CaCl_2$] from 10 to 30 mM significantly ($p\leq0.05$) increased gel strength determined as shear stress at failure (σ) and decreased shear strain (ε) (Table 1). However, further increase to 120 mM $CaCl_2$ did not significantly affect those values although the Ca content was

increased (Table 2). Stress and strain values of gels formed at ≥30 mM $CaCl_2$ indicate that these gels were harder and less cohesive than gels formed at 10 mM $CaCl_2$. Stress relaxation behaviour (Table 3) showed that [$CaCl_2$] had no effect on slope ($1/a$), which was related to the level of stress decay during relaxation.[13] Note that the a value represents a hypothetical asymptotic level of stress not relaxed for a long time (Fig.1), However, Table 3 shows that the magnitude of a was affected by the gel diameter, particularly at high [$CaCl_2$]. This, in part, appears to be due to the small linear viscoelastic range of the gels formed at high [$CaCl_2$], which had larger aggregate size than those formed at 10 mM $CaCl_2$ (Figs.2,3). Another explanation is that the degree of gelation of the 25.5 mm-diameter gels was somewhat less than that of the 20.4 mm-diameter gels. This might have been due to the dialysis method of $CaCl_2$ into the dialysis tube to form gel, which could have resulted in a slightly lower degree of gelation in gels with a larger diameter (after 16 h). The slight reduction in initial stress of the 25.5 mm diameter gels can also be used to support this explanation.

The intercepts ($1/ab$) were affected by [$CaCl_2$] but not the diameter of the gel. The intercept is related to the rate at which stress relaxes.[13] By calculation, b values were not significantly affected by [$CaCl_2$]; although there was a tendency for them to increase as [$CaCl_2$] increased. In general, the higher b values suggests that the gels relax more quickly than those possess smaller b values.[13] However, the highest asymptotic residual modulus or gel solidity (E_A) was obtained at 30 mM $CaCl_2$. This trend was also observed in the Young's modulus results previously reported.[9] An asymptotic residual modulus has been shown to be related to the number of effective covalent crosslinks in particle gels such as bovine serum albumin, egg white and soybean protein gels[17,18,19] in equilibrium deformation test since the weak non-covalent interactions are in relaxed state.

Table 1 Effect of [$CaCl_2$] on shear stress and strain at failure of Ca^{2+}-induced cold- set WPI gels formed by dialyzing against different $CaCl_2$ solutions at 24°C after 16 h dialysis.

[CaCl] (mM)	True failure stress (kPa)	True failure strain
10	18.0^b	1.228^a
30	20.7^a	1.072^b
120	20.0^a	1.119^{ab}

Means (n=9) followed by a different superscript are significantly different ($p \leq 0.05$)

Table 2 Means ±standard deviation (n=2/treatment) of calcium content in WPI suspensions/ gels (dialyzed for 16 h at 24°C)

[$CaCl_2$] in dialyzing solution (mM)	Calcium content (% dried weight basis)
0*	0.19±0.00
10	0.87±0.08
30	1.42±0.30
120	3.43±0.54

*no gel

Table 3 Stress relaxation parameters of Ca^{2+}-induced cold-set WPI gels (10% protein w/v) formed by dialyzing against different $CaCl_2$ solutions at 24°C for 16 h.

[CaCl] (mM)	Gel diameter (mm)	Slope (1/*a*)	Intercept (1/*ab*)	Initial stress (kPa)	Asymptotic residual modulus (E_A) (kPa)
10	20	1.620^{ab}	95.170^{a}	42.5^{c}	56.1^{c}
	25	1.592^{ab}	92.753^{ab}	38.3^{c}	49.5^{c}
30	20	1.540^{b}	89.173^{b}	79.6^{a}	97.1^{a}
	25	1.654^{a}	91.703^{ab}	67.7^{b}	92.8^{a}
120	20	1.538^{b}	89.333^{b}	63.6^{b}	77.3^{b}
	25	1.688^{a}	91.747^{b}	61.3^{b}	86.7^{ab}

Means (n=9) followed by a different superscript are significantly different ($p \leq 0.05$).

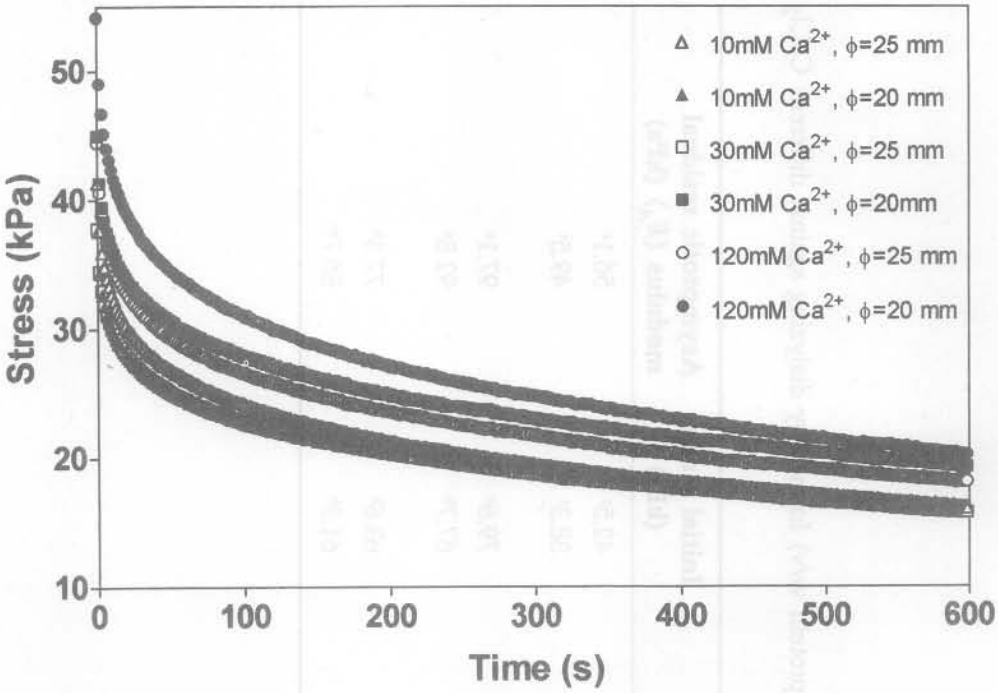

Fig.1 Stress relaxation curve.

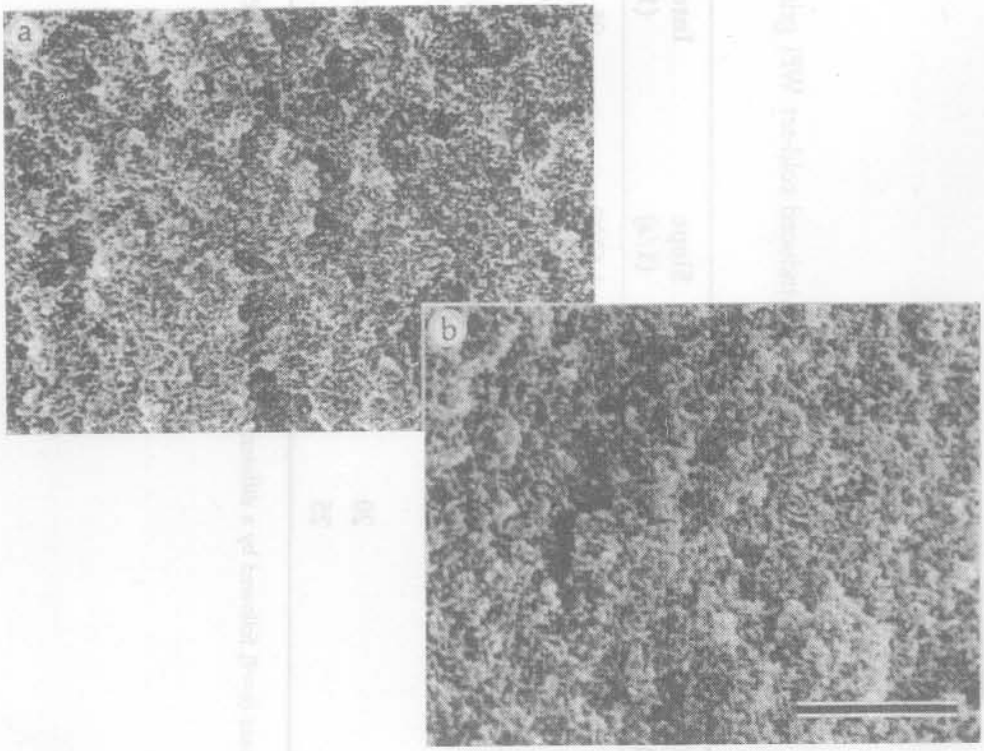

Fig.2 Scanning electron micrographs of cold-set WPI gels prepared by dialysis against 10 mM (a) and 120 mM (b) $CaCl_2$. Bar = 5 μm.

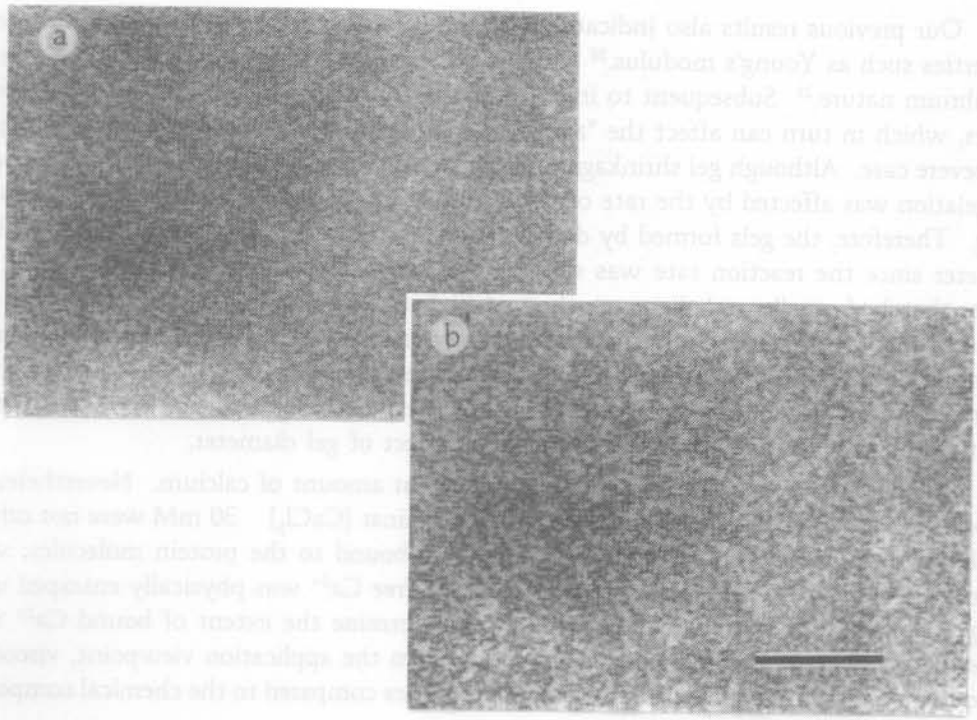

Fig.3 Transmission electron micrographs of cold-set WPI gels prepared by dialysis against 10 mM (a) and 120 mM (b) $CaCl_2$. Bar = 2 μm.

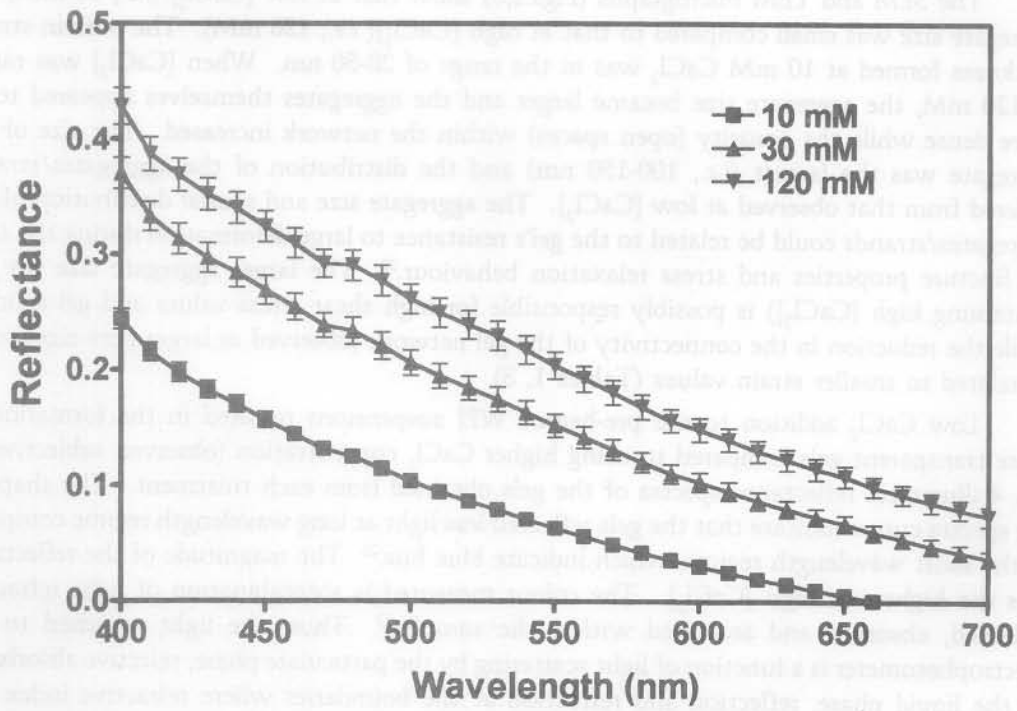

Fig.4 Effect of $CaCl_2$ concentration on internal reflectance spectra of cold-set WPI gels. Protein suspensions (pH 7) were pre-heated at 80°C for 30 min and cooled before $CaCl_2$ addition at 24°C. Bars represent standard deviation.

Our previous results also indicated that the gelation time of WPI affected viscoelastic properties such as Young's modulus.[20] This is because gelation is considered to be of a non-equilibrium nature.[21] Subsequent to initial gelation, a time-dependent crystallization process occurs, which in turn can affect the "aging" phenomenon or causing shrinkage and syneresis in a severe case. Although gel shrinkage was not noticed in this study, it should be noted that the gelation was affected by the rate of $CaCl_2$ diffusion, the rate of aggregation, gelation and aging. Therefore, the gels formed by dialyzing against 10 mM $CaCl_2$ was not affected by gel diameter since the reaction rate was slower than the ones containing higher [$CaCl_2$]. The latters that had smaller gel diameter showed higher level of stress relaxed than the larger diameter ones (Fig.1). It is likely that the aging process in the smaller gels started earlier than the larger ones and can be detected by the parameter $1/a$. The parameters $1/ab$ and E_A are governed by the distribution and connectivity of the protein aggregates. Thus, they are less sensitive than the 1/a values in determining the effect of gel diameter.

Table 2 shows that the gels contained different amount of calcium. Nevertheless, the viscoelastic properties of gels formed by dialyzing against [$CaCl_2$] 30 mM were not different (Tables 1, 3). This is because not all of Ca^{2+} was bound to the protein molecules; which, consequently affected viscoelastic properties. Some free Ca^{2+} was physically entraped within the gel network. To date, it is still difficult to determine the extent of bound Ca^{2+} in the concentrated system like food gels. Considering from the application viewpoint, viscoelastic properties are more relavent to the functional properties compared to the chemical composition of food ingredients.

Microstructure

The SEM and TEM micrographs (Figs.2,3) show that at low [$CaCl_2$] (*i.e.*, 10 mM) the aggregate size was small compared to that at high [$CaCl_2$](*i.e.*, 120 mM). The protein strand thickness formed at 10 mM $CaCl_2$ was in the range of 20-50 nm. When [$CaCl_2$] was raised to 120 mM, the aggregate size became larger and the aggregates themselves appeared to be more dense while the porosity (open spaces) within the network increased. The size of the aggregate was the largest (*i.e.*, 100-150 nm) and the distribution of the aggregates/strands differed from that observed at low [$CaCl_2$]. The aggregate size and spatial distribution of the aggregates/strands could be related to the gel's resistance to large deformation during the tests for fracture properties and stress relaxation behaviour.[22] The larger aggregate size (in gels containing high [$CaCl_2$]) is possibly responsible for high shear stress values and gel solidity, while the reduction in the connectivity of the gel network (observed as larger pore size) could be related to smaller strain values (Tables 1, 3).

Low $CaCl_2$ addition to the pre-heated WPI suspensions resulted in the formation of more transparent gels compared to using higher $CaCl_2$ concentration (observed subjectively). Fig. 4 illustrates reflectance spectra of the gels obtained from each treatment. The shape of the spectra curves indicate that the gels reflected less light at long wavelength regime compared to the short wavelength regime, which indicate blue hue.[23] The magnitude of the reflectance was the highest at high [$CaCl_2$]. The colour measured is a combination of light refracted, reflected, absorbed and scattered within the sample.[23] Thus, the light returned to the spectrophotometer is a function of light scattering by the particulate phase, selective absorbance by the liquid phase, reflection and refraction at the boundaries where refractive index and wavelength change.[24] The larger aggregates can scatter more light than the smaller aggregates. Although the spectra curves showed significant differences ($p \leq 0.0001$) among each other (determined by paired t-tests), the means of difference at [$CaCl_2$] between 30-120 mM were

smaller than those between 10-30 mM. This qualitatively suggests a transition in the microstructure from a translucent to an opaque gel at around 30 mM Ca^{2+}. Overall, the magnitude of reflectance was in accordance with microstructure observed under SEM and TEM.

The fact that gels formed at 10 mM $CaCl_2$ was clearer than gels formed at higher [$CaCl_2$] indicates that a finer protein strand network was formed at low $CaCl_2$ concentration. The relationship between gel clarity and protein aggregate/strand size has been described that the fine-strand gel network (*i.e.*, more transparent gel) is usually the result of less aggregation and more order in the gel structure.[15] The effects of [$CaCl_2$] on gel transparency can be partly explained by the DLVO theory. At low [$CaCl_2$] (*i.e.*, 10 mM $CaCl_2$), in which gels are transparent and have a smaller aggregate size, the presence of a repulsive electrostatic energy barrier might slow down the aggregation of the pre-heated protein molecules. This is in contrast to gels obtained at high [$CaCl_2$], in which aggregation rates were much faster, mainly due to changes in the distance distribution of repulsive forces.

In summary, WPI gels with different viscoelastic properties can be prepared by changing [$CaCl_2$]. An increase in $CaCl_2$ resulted in gels with larger aggregate size, harder and less cohesive than gels containing lower [$CaCl_2$]. However, the high value of asymptotic resudual modulus (which is related to the number of effective chemical crosslinks) of gels containing high [$CaCl_2$] suggested that there are other chemical forces (such as covalent) apart from electrostatic forces (as described by DLVO theory) involved in stabilizing the network of cold-set WPI gels.

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