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Rheological and structural characterization of whey protein gelation induced by enzymatic hydrolysis

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ABSTRACT

Whey proteins hydrolyzed by *Bacillus licheniformis* protease (BLP) form soft and turbid aggregate gels with potential food and biotechnological applications. The purpose of the study was to characterize protease-induced whey protein gelation by comparing different protein and enzyme concentrations in terms of gel mechanical and microstructural properties, and conformational changes in the protein secondary structure due to hydrolysis and gelation. Gels formed with whey protein isolate (WPI), at concentrations 5 and 10% (w/v), and BLP concentrations, BLP/WPI (w/w), of 1, 3, and 5% were studied. Regardless of the enzyme concentration, gels with 10% WPI were strong and elastic while those with 5% WPI were weak. Gelation time decreased as the enzyme concentration increased for both protein concentrations. Gel strengths values of 10% WPI samples were independent of BLP concentrations at the end of the incubation period. Creep tests performed on the resulting gels showed that 10% WPI gels with different BLP concentration had similar elasticity, slightly increasing with BLP amount. Remarkable differences were observed in the microstructures of gel prepared with different concentrations of protein and BLP. Changes in the protein secondary structure measured during the gelation were small before gelation. However, sudden changes were observed when the samples gelled, and also after 7 h of incubation at 50 °C (time in which samples reached a plateau in G* as seen by rheology tests). Results revealed that without enzyme, hydrolysis of the protein was not promoted and the protein secondary structure remains the same; only a slight denaturation was observed when the protein was incubated at 50 °C.

1. Introduction

Gelation is one of the most important processes to develop a variety of food and pharmaceutical products and whey proteins are used commonly for these purposes. Various approaches used to obtain whey protein gels are well-documented in the literature. They include heating (Comfort & Howell, 2002; Donovan & Mulvihill, 1987; Morr & Foegeding, 1990; Paulsson, Hegg, & Castberg, 1986; Taylor, Gladden, & Fryer, 1994), chemical action (Katsuta, Hatakeyama, & Hiiraki, 1997; Xiong & Kinsella, 1990) and enzymatic treatments (Doucet, Gauthier, & Foegeding, 2001; Doucet, Gauthier, Otter, & Foegeding, 2003a; Doucet, Otter, Gauthier, & Foegeding, 2003b; Eissa, Bisram, & Khan, 2004; Eissa & Khan, 2005; Ipsen, Otte, Lomholt, & Qvist, 2000; Ju, Otte, Madsen, & Qvist, 1995; Ju, Otte, Zakora, & Qvist, 1997; Otte, Ju, Frergemand, Lomholt, & Qvist, 1996a; Otte, Schumacher, Ipsen, Ju, & Qvist, 1999).

In the case of enzyme-induced gelation, the native globular structure of whey proteins is destabilized by enzymatic hydrolysis which leads to the formation of smaller protein/peptide fragments that tend to aggregate to form gel networks with well-defined mechanical properties and microstructures (Otte et al., 1996a). The physico–chemical properties of enzyme-induced whey protein gels obtained with *Bacillus licheniformis* protease (BLP) have been reported in various studies (Ipsen et al., 2000; Ju et al., 1995, 1997

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&; Otte et al., 1996a, 1999; Otte, Ju, Skriver, & Qvist, 1996b). These studies determined key parameters (e.g. concentration of protein, enzyme concentration, ionic strength, pH and temperature) that affected gelation. By using chromatographic and spectroscopic techniques, major peptides leading to aggregation and gelation were identified. Rheological methods were used to determine gel strength and gelation time. These studies were complemented with electron microscopy analysis to characterize the microstructure of the gels. Doucet and co-workers studied whey protein gelation induced by extensive hydrolysis of the protein using the enzyme Alcalase 2.4L® (Doucet et al., 2003a &, 2003b, 2001). They compared heat- and protease- induced whey protein isolate (WPI) gels in terms of gelation profiles, gel strength and type of gel formed. Along these studies, the degree of hydrolysis, peptide profile after extensive hydrolysis and the interactions involved in the gel network were characterized. Research on the subject has mostly focused on the hydrolysis phenomena and the formation of peptides leading to aggregation and enzyme-induced WPI gelation (Creusot, Gruppen, van Koningsveld, de Kruif, & Voragen, 2006; Doucet et al., 2003b; Ipsen et al., 2000; Ju et al., 1997; Otte et al., 1996b; Spellman, Kenny, O'cuinn, & Fitzgerald, 2005). However, still there is a lack of information presenting a detailed rheological characterization of BLP-induced WPI gelation and resulting gels. Furthermore, conformational changes of the protein secondary structure during enzymatic hydrolysis and gel formation have not been fully investigated (Otte et al., 1997). The knowledge of structural changes in the protein as a function of time during hydrolysis and gelation is essential for a better understanding of BLP-induced WPI gelation. In that respect, the main goal of this study was to analyze BLP-induced gelation of whey proteins by considering key rheological parameters describing the system and the structural changes undergone by the proteins due to hydrolysis and gelation.

Rheological characterization of gels can be studied by a number of methods involving the characterization of viscoelastic materials under shear (more commonly used test), torsion and extensional strains (Beaulieu, Sylvie, Turgeon, & Doublier, 2001; Foegeding, Gonzalez, Hamann, & Case, 1994; Havea, Watkinson, & Kuhn-Sherlock, 2009; McSwiney, Singh, & Campanella, 1994a; McSwiney, Singh, Campanella, & Creamer, 1994b; Tung, Britt, & Tang, 1994; Vardhanabhuti, Foegeding, McGuffey, Daubert, & Swaisgood, 2001). BLP-induced whey protein gels have an aggregate microstructure (Ju et al., 1997) which is promoted mainly by non-covalent interactions (Creusot & Gruppen, 2007). Upon hydrolysis, unfolded proteins with exposed hydrophobic zones, in addition to formed peptides having hydrophobic amino acid residues, are linked by hydrophobic interactions to form aggregates that finally constitute the gel network (Ipsen et al., 2000; Ju et al., 1995; Otte at al., 1996b). Identification of the resultant gel network in terms of strength, structure and stability is of key significance to the use of these gels in applications involving foods and biomaterials.

This work presents a comprehensive study on gelation of whey proteins promoted by enzymatic hydrolysis. Rheological tests along with microscopy to assess the properties of the formed gels, in terms of gel viscoelasticity, type and structure were carried out. A novel approach to describe viscoelasticity of the gels in terms of creep and recovery behavior is also presented. The approach uses the concept of fractional calculus which has been already employed to characterize the viscoelastic properties of macromolecular gels (Koeller, 1984; Orczykowska & Dziubiński, 2012). Additionally, structural changes in the protein secondary structure due hydrolysis and gel formation were investigated using circular Dichroism Spectroscopy (CD).

2. Materials and methods

2.1. Materials

WPI was kindly supplied by Davisco International Foods (Minnesota, USA) and BLP (13.744 AU-A/G, batch no PL 100013) was kindly supplied by Novozymes A/S (Bagsvaerd, Denmark). According to the manufacturer, a typical WPI batch contains 61–70% Beta-lactoglobulin (β-Lg), 23–31% alpha-lactalbumin (α-La), 2–4% Bovine Serum Albumin (BSA) and 1–5% Immunoglobulin (IgG).

DSC pans were purchased from DSC Consumables Inc. (Austin, MN, USA) and chemicals used were purchased from Sigma Chemicals C. (St. Louis, MO, USA).

2.2. Methods

2.2.1. Sample preparation

Solutions of WPI with concentrations 5 and 10% (w/v) were prepared by dissolving the protein in tris–HCl buffer (75 mM, pH 7.5) and maintained overnight at 4 °C. BLP was added to the solutions at concentrations corresponding to an enzyme to substrate ratio of 1, 3, and 5% (dry matter); hereby designed as 1%, 3% and 5% BLP, and well mixed prior to rheology, CD spectroscopy and Differential Scanning Calorimetry (DSC) measurements. Separately, samples were prepared using the same procedure and incubated in a water bath at 50 °C for 10 h. These samples were used for microscopy and modulated DSC (MDSC) analyses performed immediately after incubation.

2.2.2. Small amplitude oscillatory strain (SAOS) tests

Enzyme-induced protein gelation was followed with a controlled stress rheometer (ARG2, from TA Instruments, New Castle, DE, USA) using a parallel plate geometry with a gap of 1 mm. Samples were placed onto a Peltier temperature controlled plate. Once the selected gap was achieved, the exposed edges of the sample were coated with a thin layer of silicon oil to prevent evaporation. Samples were subjected to dynamic oscillation with controlled strain of 1% at 1 Hz frequency. The strain of 1% was previously determined as within the linear viscoelastic range of samples passing through all stages of the gelation process.

Time sweep measurements were performed while heating the sample from 25 to 50 °C at a rate of 1 °C/min, then holding it at 50 °C for 10 h and then cooling to 25 °C at a rate of 1 °C/min. Once the heating/holding/cooling cycle was completed, the samples were subjected to creep (15 min) and recovery (15 min) tests at 25 °C. The applied stress was 1 Pa, which was within the linear viscoelastic behavior of the sample. Recovery strains were calculated by dividing the difference between the measured initial and final strains by the initial strain.

Frequency sweep tests were performed in the range of frequencies 0.1–10 Hz at a strain of 1% and 25 °C. Temperature sweep tests were carried out in the range of temperature 25–95 °C at a strain of 1%, frequency of 1 Hz and with a heating rate of 5 °C/min. Storage modulus (G'), loss modulus (G'') and complex modulus (G^*) values were determined during the measurements. All measurements were conducted by duplicate. Gelation time was determined as the time at which $\tan \delta = 1$ (Ipsen, Otte, & Qvist, 2001).

2.2.3. Creep and recovery analysis

Creep and recovery data were analyzed by using the approach of fractional derivative, which has been extensively used in polymer studies and recently introduced to the characterization of food materials (David & Katayama, 2013; Jaishankar & McKinley, 2014; Schaffter, Corvalan, & Campanella, 2015). One of the advantages of this approach is that both the creep and recovery curves can be

analyzed together and also the gel characterization is done using a smaller number of viscoelastic parameters than those used by current methods, thus facilitating the analysis of the data and its physical interpretation.

Eq. (1) below describes creep and recovery curves in terms of the parameter α which provides an indication of the degree of elasticity of the sample; where lower values of α indicate elastic gels whereas large values indicate more viscous samples. It must be noted that, values of α range between 0 and 1, a value of 0 would be indicating a purely elastic material whereas a value of 1 means a purely viscous one. For viscoelastic materials, like the gels studied in this work, α values range between 0 and 1. Other parameters of relevance in the test are λ_1 and λ_2 which represent the inverse of the gel elastic modulus during creep and recovery, respectively. These parameters are associated with the structure of samples and vary when the stress is applied during the creep experiment which disturbs the sample structure (Schaffter et al., 2015). Conversely, if the structure of the sample is not disturbed these values remain identical.

$$J(t) = \frac{\varepsilon(t)}{\sigma_0} = \frac{1}{\Gamma(\alpha+1)}\left(\lambda_1 t^{\alpha} H(t) - \lambda_2 (t-t_m)^{\alpha} H(t-t_m)\right) \quad (1)$$

In Eq. (1), $J(t)$ is the material compliance with a unit of %/Pa, $\varepsilon(t)$ is the instantaneous strain measured in % strain, $\sigma(t)$ is the applied stress, which to maintain a linear behavior in the sample was selected as 1 Pa for this test, t_m is an experimentally measured time at which the stress is removed to initiate the recovery test, Γ is the gamma function described by Abramowitz and Stegun (1964), and $H(t)$ is the Heaviside or step function defined as:

$$H(t) = \begin{cases} 0 & if \quad t<0 \\ 1 & if \quad t>0 \end{cases} \quad (2)$$

The rheological parameters α, λ_1 and λ_2 were determined by fitting the experimental creep-recovery data of the formed gel to Eq. (1) using the Simplex Nelder-Mead algorithm implemented in Matlab. Further details of the procedure are described by Schaffter et al. (2015).

2.2.4. Microscopy

For confocal electron microscope imaging an inverted Nikon Eclipse Ti-S (Japan) model microscope was used. Samples were non-covalently stained with Rhodamine solution (2 mg/ml). Observations were made at an excitation of 561 nm wavelength laser and emission was recorded between 570 and 620 nm wavelength range. A 60× magnification was applied for all samples with 1.4 numerical aperture and 0.8 au pinhole.

2.2.5. Circular dichroism (CD) spectra analysis

Far-UV CD measurements were performed in a Jasco J-1500 spectrometer (Easton, MD, USA). WPI samples mixed with BLP in the right proportions were diluted 500-fold and placed into quartz cells with a path length of 0.1 cm. A temperature scan was performed first from 25 to 50 °C with a 1 °C/min heating ramp, then, a 10 min intervals were performed at 50 °C for a period of 6 h, followed by a cooling step from 50 to 25 °C with a 1 °C/min ramp. Scan rate was 10 nm/min and band width was 0.2 nm. Results are presented in terms of mean residue ellipticity ([θ]) in units of deg.cm^2/dmol, which were determined according to the following equation: [θ] = [θ] = MWR × [θ]/10 × d × c, where θ corresponds to the observed ellipticity (deg), MRW is the mean residue weight, d is the pathlength (cm), and c is the protein concentration (g/ml). All spectra were obtained by subtracting buffer base-line spectra.

2.2.6. Modulated Differential Scanning Calorimetry analysis (MDSC)

MDSC measurements were performed using a DSC instrument (Q2000 from TA Instruments, New Castle, DE, USA). Samples (~10 mg) were weighed and hermetically sealed in hermetic aluminum pans. They were heated from 25 to 90 °C with a ramp of 5 °C/min. Temperature was modulated as 1 °C every 60 s.

2.2.7. Statistical analysis

All experimental work was conducted by duplicate. New solutions of whey proteins were prepared for all the studies using the same whey protein supply lot (given in section 2.1). Results are presented as means with their corresponding standard error or standard deviation. For the statistical analysis of the data, StatGraphics Centurion XV software was used and the analysis of variance (ANOVA) was done. When statistical differences were found, LSD test ($P < 0.05$) was carried out.

3. Results and discussion

3.1. Rheological characterization of enzyme-induced WPI gels

3.1.1. SAOS test to characterize gelation

Fig. 1 illustrates BLP induced gelation profiles of solutions with 10% (A) and 5% (B) WPI and the different enzyme concentrations used in the study. Samples with 10% WPI concentration formed stronger gels at slightly longer gelation times than samples having

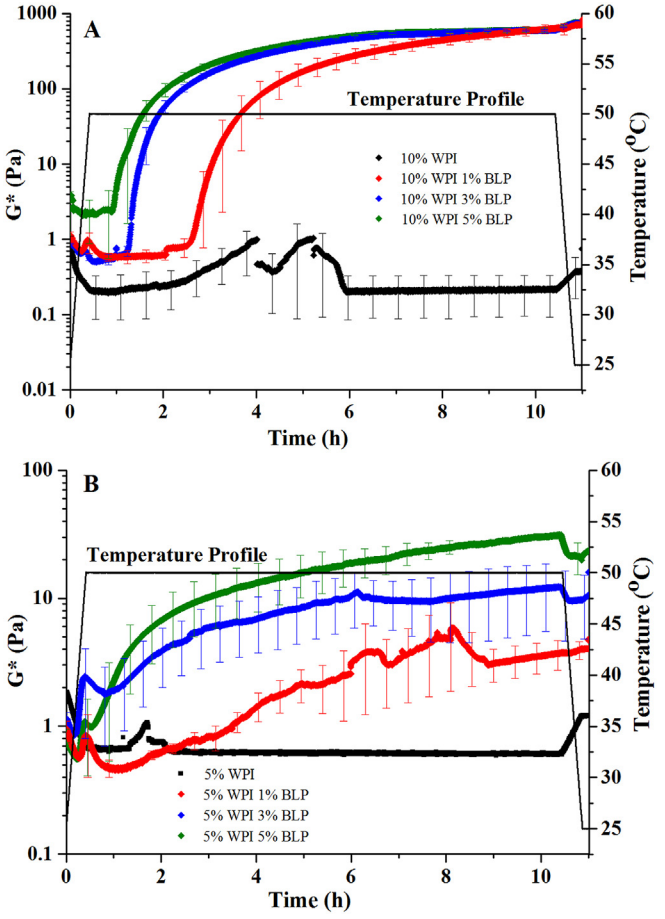

Fig. 1. BLP induced-WPI gelation: 10% WPI (A); 5% WPI (B) with 1, 3 and 5% (w/w) enzyme (BLP) concentrations. Control samples, 10% and 5% WPI, did not contain enzyme.

5% WPI concentration. Gelation times for samples with 10% WPI and BLP concentrations of 1, 3 and 5% were 159, 81 and 60 min, respectively, whereas gelation times for samples with 5% WPI were 142, 63 and 46 min for BLP concentrations 1, 3 and 5%, respectively (Table 1). As enzyme concentration increased the gelation time decreased for both the 10% and 5% WPI samples.

After the incubation time (~11 h), complex modulus values (G*) measured at 25 °C for 10% WPI gels prepared with 1, 3 and 5% BLP were 704, 734 and 752 Pa, whereas for the 5% WPI gels prepared with 1, 3 and 5% BLP were 4.1, 10.5 and 31 Pa, respectively. As can be seen, WPI concentration has a big influence in G* values, meanwhile BLP concentration has not (G* had a slight tendency to increase as the BLP concentration increased, although no statistical differences were found). These results suggest that protein concentration is one of the major contributors to the mechanical properties of the BLP-induced WPI gels.

Otte et al. (1999) studied the effects of different parameters including protein (0.5–9%) and enzyme (0.1–3%) concentrations on BLP-induced gelation of heat-treated and untreated whey proteins and reported that a decrease in WPI concentration resulted in a decrease in the rate of gelation and gel strength as well as an increase of gelation times. Furthermore, they found that decreasing enzyme concentrations resulted in decreasing gelation times following a non-linear relationship, but it did not affect gel strength. Findings in this work mostly agree with results of other researchers except that gelation times were almost independent of the WPI concentration at all concentrations of BLP enzymes. Samples with low WPI and with the highest BLP concentration had lower gelation times most probably due to faster hydrolysis and peptide formation leading to aggregation favored by the high enzyme respect to the low WPI amount.

Control samples, 10% and 5% WPI without BLP enzyme, did not form a gel after heating at 50 °C for 10 h. As it is shown in Fig. 1A and B, for both systems 5% WPI and 10% WPI without the enzyme it is observed a slightly increase in G* at 2 h and between 2 and 6 h. However, in both cases that increase stops at longer times indicating that no gels were formed. This behavior can be explained by a partial unfolding of proteins during incubation at 50 °C. Gelation did not occur without enzyme, hence its presence is essential to form a gel.

3.1.2. Frequency sweep tests

Frequency sweep SAOS tests presented in Fig. 2 provides information about the gel structure (Stading & Hermannson, 1990). Gels formed by covalent linkages truly elastic and frequency independent, while physical gels, mostly formed by non-covalent interactions, exhibit slight frequency dependence (Doucet et al., 2001). Enzyme-induced WPI aggregate gels are formed mostly through non-covalent hydrophobic interactions (Creusot & Gruppen, 2007), hence, a slight dependence of the viscoelastic moduli with frequency would be expected. However, for the 10% WPI concentration gels both the storage and loss moduli were independent of the applied frequency (Fig. 2A). This might be due to formation of well-structured aggregate gels. Conversely, 5% WPI gels were weak gels and exhibited frequency dependence on the storage and loss moduli (Fig. 2B). On the other hand, systems with low protein and enzyme concentrations (5% and 1% respectively) exhibited an extremely weak gel behavior as they are formed by weak aggregate networks, so when high frequencies were applied (Fig. 2B1 and 2B2) artifacts as inertial effects affected the measured viscoelastic moduli as measurements were at the lower limit of the instrument capacity. Increasing enzyme concentration for gels with 5% WPI concentration led to extensive aggregation, thus the behavior of the gels showed less frequency dependence (Fig. 2B3). The artifacts observed while testing the weak gels at high frequencies were not observed for gels formed with high enzyme concentrations (specifically 3% and 5%).

Table 1
Gelation times obtained in WPI-BLP gel systems.

Gel systems	Gelation time (min)
10% WPI–1% BLP	159 ± 4^{c}
10% WPI–3% BLP	81 ± 5^{a,b}
10% WPI–5% BLP	60 ± 5^{a}
5% WPI–1% BLP	142 ± 1 ^{b,c}
5% WPI–3% BLP	63 ± 47^{a}
5% WPI–5% BLP	46 ± 27^{a}

Mean values with different letters were significantly different when LSD test was applied ($P < 0.05$).

3.1.3. Temperature sweep tests

Temperature sweep tests carried out in the range 25–95 °C with a heating rate of 5 °C/min showed clear differences in the viscoelastic behavior when gels with 10% and 5% WPI concentrations were compared (Fig. 3A and B). As the temperature increased, G′ decreased slightly for 10% WPI gels while it increased for 5% WPI gels at all BLP concentrations. The decrease in G' of the 10% WPI concentration gels with increasing temperature can be explained by the reduction of physical interactions (hydrogen bonds) among molecules with increasing in temperature, thus resulting in a decrease in the gel elastic behavior. In 10% WPI gel, the formed peptides could be closer than in the 5% WPI gels, thus hydrogen bonding interactions could be more likely to be involved and they decreased with increasing temperatures. However, for the 5% WPI gels, the storage modulus increased with increasing temperature. For the 5% WPI concentration gels, hydrogen bonding might be less effective as they are short-range interactions, while long-range interactions such as hydrophobic and electrostatic ones, which increase with temperature might become more effective. Also, the effect of dehydration during testing could be higher in these weak systems.

3.1.4. Creep and recovery

Creep and recovery results of BLP-induced gels with 10% and 5% WPI containing 1, 3 and 5% BLP enzyme concentration and using two creep and recovery cycles are shown in Fig. 4. Results for 10% WPI gels show a typical viscoelastic behavior for all enzyme concentrations. The elasticity of the sample increases with the concentration of enzyme (Fig. 4A). Conversely, 5% WPI concentration gels shows the typical behavior of non-elastic liquids (Fig. 4B). These results are in close agreement with those obtained with the SAOS tests which are described in the previous section indicating that stronger gels are formed with higher WPI protein concentrations. In the case of 10% WPI gels, the recovery strain for 10% WPI with 1, 3 and 5% BLP were 84, 84, and 94 for the first creep and 81, 80 and 91% for the second creep, respectively. These results clearly indicate an increase in the gel elasticity with increasing enzyme concentrations.

Eq. (1) assumes that the elasticity modulus of the sample during creep and recovery are different. However, if the applied stress is small enough the viscoelastic behavior of the material would be linear and these parameter values could be fairly close to each other. As discussed the viscoelastic parameter associated with the elasticity of the sample is designed asα. The closer the parameter α is to 1 the more viscous the material is; the value $\alpha = 1$ indicates a non-elastic liquid. Conversely, the closer the parameter α is to 0 the more elastic the material is; $\alpha = 0$ indicates a perfectly elastic material (Schaffter et al., 2015). Since 5% WPI concentration samples exhibited a viscous liquid behavior, the creep curves were not

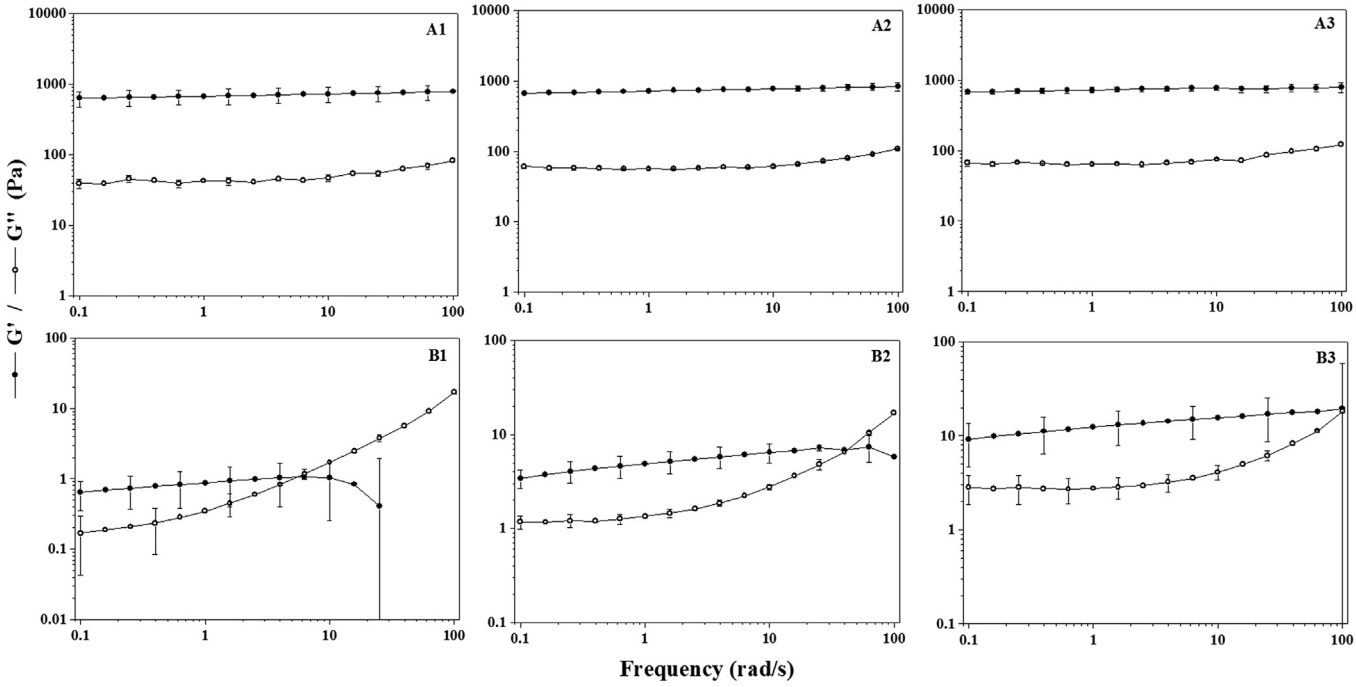

Fig. 2. Frequency sweep curves of BLP induced- 10% WPI gels with 1% (A1), 3% (A2), and 5% (A3) BLP, and 5% WPI gels with 1% (B1), 3% (B2), and 5% (B3) BLP. Strain used was 1%, which is within the linear viscoelastic region.

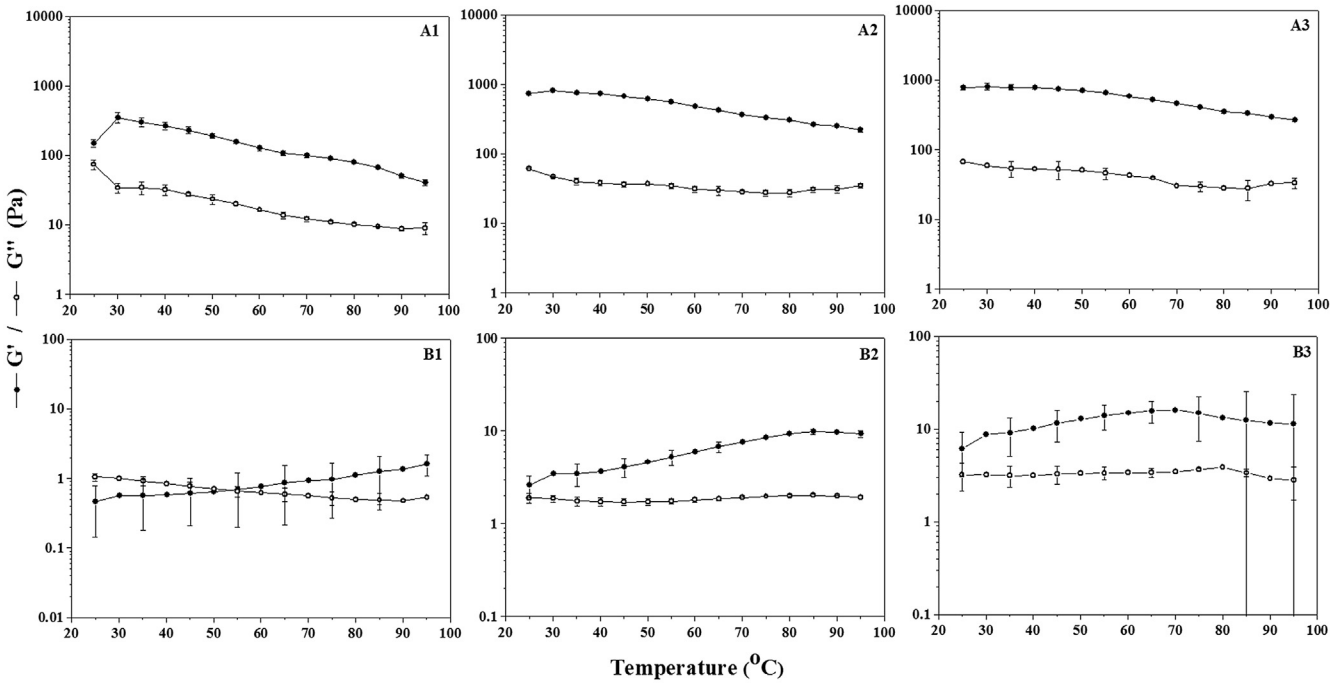

Fig. 3. Temperature sweep curves of BLP induced- 10% WPI gels with 1% (A1), 3%, 5% (A3) BLP, and 5% WPI gels with 1% (B1), 3% (B2), 5% (B3) BLP. Measurements were carried out at a strain rate of 1% with 1 Hz frequency.

further treated. Fig. 5 illustrates the fit of experimental data (for 10% WPI concentration gel) to Eq. (1). Values of the corresponding parameters for the gels are given in Table 2. From these values it appears that all samples had a similar level of elasticity for all BLP concentrations. However, lower values of λ were found for samples with higher BLP concentrations, which indicate an increase in the gel strength or gel elastic modulus. It was also noted that the creep and recovery compliances were not identical resulting in different elasticity moduli for the creep and recovery portions of the test (Table 2). That is a clear indication that the stress used in the test modified the structure of the samples. This conclusion is also reinforced by the observed changes in the second creep test cycle, where, for the three samples tested, there were observable changes in the rheological parameters (Table 2). For samples with different

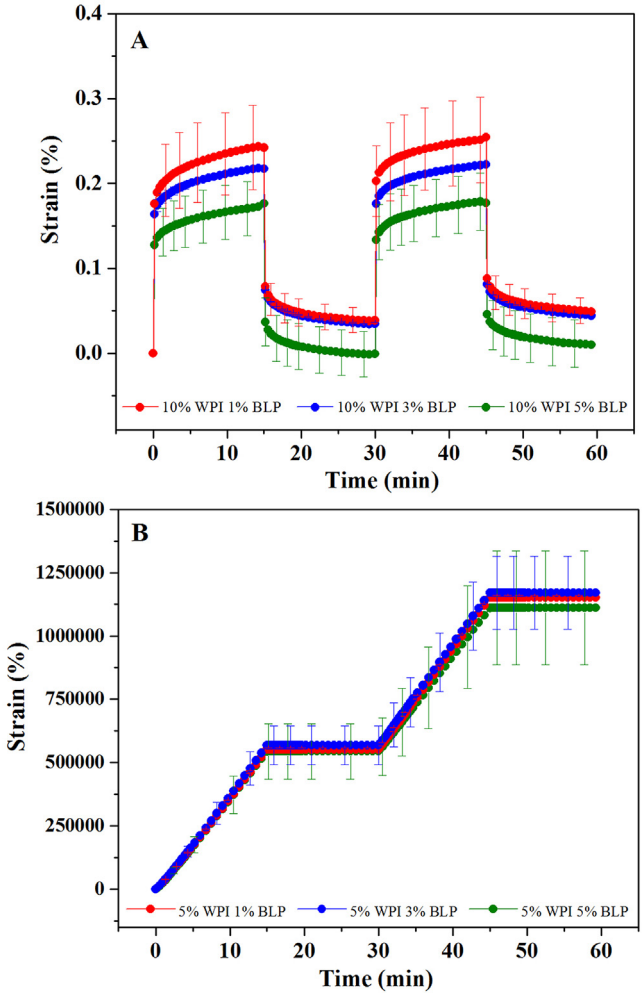

Fig. 4. Creep & Recovery curves of BLP induced- 10% (A) and 5% (B) WPI gels with 1, 3 and 5% BLP concentrations. Two creep and recovery steps were followed for 15 min each. Stress applied was 1 Pa.

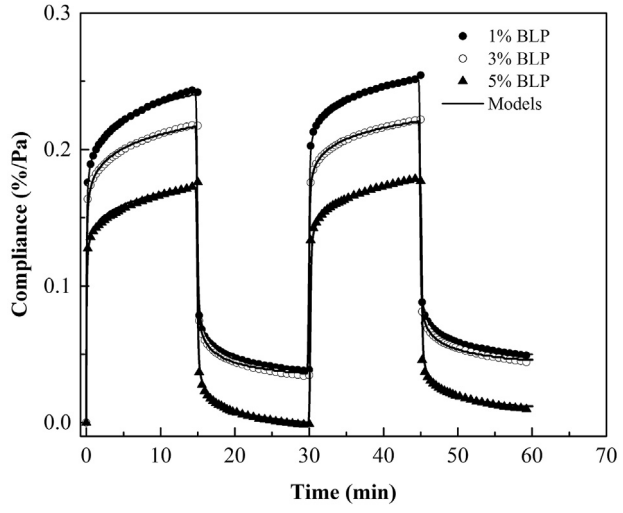

Fig. 5. Successive creep and recovery experiments on enzyme-induced 10% WPI gels. The t_ms for both experiments were 15 min. 10% WPI–1% BLP data are represented by filled circles; 10% WPI- 3% BLP data are represented by empty circles; 10% WPI- 5% BLP data are represented by filled triangles. Black lines represent models.

BLP concentrations it appeared that they became more elastic with larger elasticity modulus (lower values of α andλ) with increasing BLP concentrations probably caused by water syneresis during the second cycle. That trend was however reverted for the 5% BLP enzyme gel in which the modulus of elasticity decreased in the 2nd creep cycle although the elasticity of the sample increased (Table 2).

3.2. Gel microstructure

Gels were also prepared by incubating the WPI-BLP solutions at 50 °C for 11 h in micro centrifuge tubes. Gels obtained at the end of the incubation period are illustrated in Fig. 6Aa and 6Ba. By examining both G* values and the appearance of these gels, it seems that samples with 10% WPI concentration in the presence of the BLP enzyme formed strong gels whereas samples with 5% WPI concentration in the presence of the BLP enzyme formed weak liquid-like gels. Turbidity increased with increasing protein and enzyme concentrations (Fig. 6Aa and 6Ba). This is probably due to an increase in aggregation generated by interactions of larger amount of peptides produced during the hydrolysis. Otte et al. (1996b) reported that turbidity increased due to formation of aggregates of increasing size during BLP-induced gelation. β-Lg is the main whey protein responsible for WPI gelation and its peptides produced during BLP-hydrolysis are prone to form aggregates rapidly, thus increasing turbidity (Doucet & Foegeding, 2005). Phase separation was observed only in the 5% WPI with 5% BLP sample after storage of the gels at +4 °C (data not shown).

3.2.1. Confocal microscopy

All gel samples were labeled with the fluorescent dye Rhodamine B and visualized by a confocal microscope (Fig. 6Ac and 6Bc). The 10% WPI gels with varying BLP concentrations are illustrated in Fig. 6A- row c whereas the 5% gels WPI samples with varying BLP concentrations are illustrated in Fig. 6B- row c. No differences were observed on samples prepared without enzyme (Fig. 6AIc and 6BIc) since gelation did not occur. Aggregates could have been formed increasing the viscosity the samples to provide them with a paste appearance but the size of these aggregates remained below 200 nm which is the resolution limit of the light microscopes, including confocal microscopes (Spotti, Santiago, Rubiolo, & Carrara, 2012).

Distributions of protein aggregates, appearing in red in the images due to the presence of Rhodamine B, are visible in the micrographs. Samples with low protein concentration had a loose and more disorganized structure due to a lower amount of aggregates in the solution when compared with samples formed with higher protein concentrations. The appearance of the 10% WPI concentration gel containing 1% BLP (Fig. 6AIIc) was different than the others showing a unique reticular-like structure instead of a structure where aggregates prevailed as in the other gels formed with the 10% WPI samples.

3.3. CD spectra

Changes in secondary structure of WPI proteins upon hydrolysis and gel formation were investigated by CD spectroscopy. Fig. 7A and 7B represent far-UV CD spectra of samples formed with 10% and 5% WPI concentrations respectively and with different enzyme concentration levels, taken at the beginning (0 h) and at the end of incubation time (7 h). This incubation time was selected based on the rheological data considering the time taken by the sample to reach a plateau for G* value which was about 7 h (Fig. 1). Samples without enzyme show a broad negative band between 208 nm and 222 nm. Assignments of the bands indicating the presence of

Table 2
Fitting parameters for the fractional derivative based model to describe creep and recovery of 10% WPI gels. The adjustment of the experimental data is illustrated in Fig. 5. 1st and 2nd Creep refer to the first and second of two successive creep and recovery tests for each sample, respectively. The stress applied was 1 Pa.

Gel systems	Creep constant ($\lambda_1\sigma_o$)	Recovery constant ($\lambda_2\sigma_o$)	Derivative order (α)
10% WPI–1%–BLP/1st Creep	0.1901 ± 0.0396	0.171 ± 0.030	0.07335 ± 0.00015
10% WPI–1%–BLP/2nd Creep	0.1737 ± 0.0316	0.172 ± 0.029	0.06205 ± 0.00065
10% WPI–3%–BLP/1st Creep	0.1725 ± 0.0058	0.153 ± 0.003	0.07135 ± 0.00725
10% WPI–3%–BLP/2nd Creep	0.1499 ± 0.0001	0.148 ± 0.002	0.06695 ± 0.00005
10% WPI–5%–BLP/1st Creep	0.1353 ± 0.0260	0.143 ± 0.006	0.07280 ± 0.00040
10% WPI–5%–BLP/2nd Creep	0.1429 ± 0.0072	0.140 ± 0.005	0.06870 ± 0.00040

ANOVA could not be applied since variances were not homogeneous according to the Levenés test with $p < 0.05$.

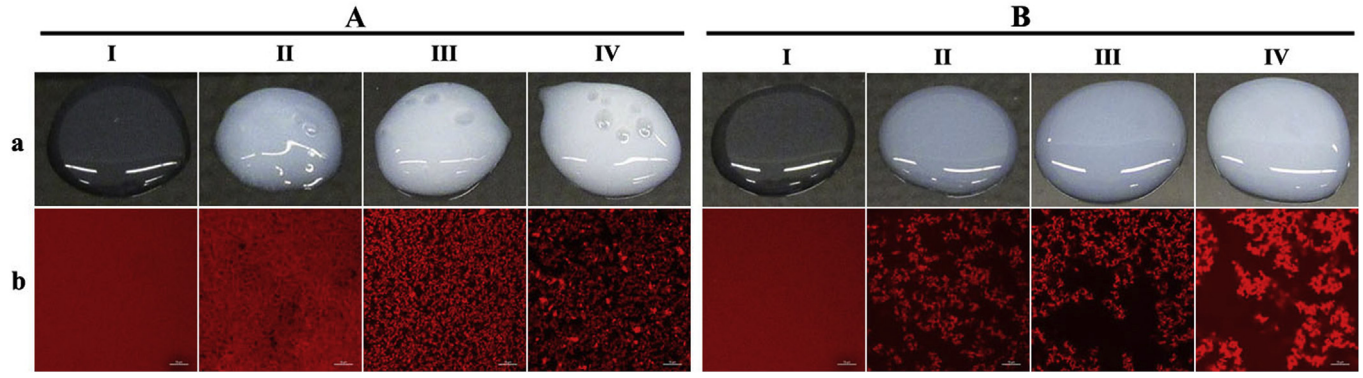

Fig. 6. a. Gel images **b.** Confocal EM images of BLP-induced WPI gels: 10% WPI and (A) and 5% WPI gels (B) without BLP (I); with 1% (II); 3% (III); 5% (IV) BLP. In confocal images, bar represents 10 μm and whey protein aggregates appeared red due to Rhodamine B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

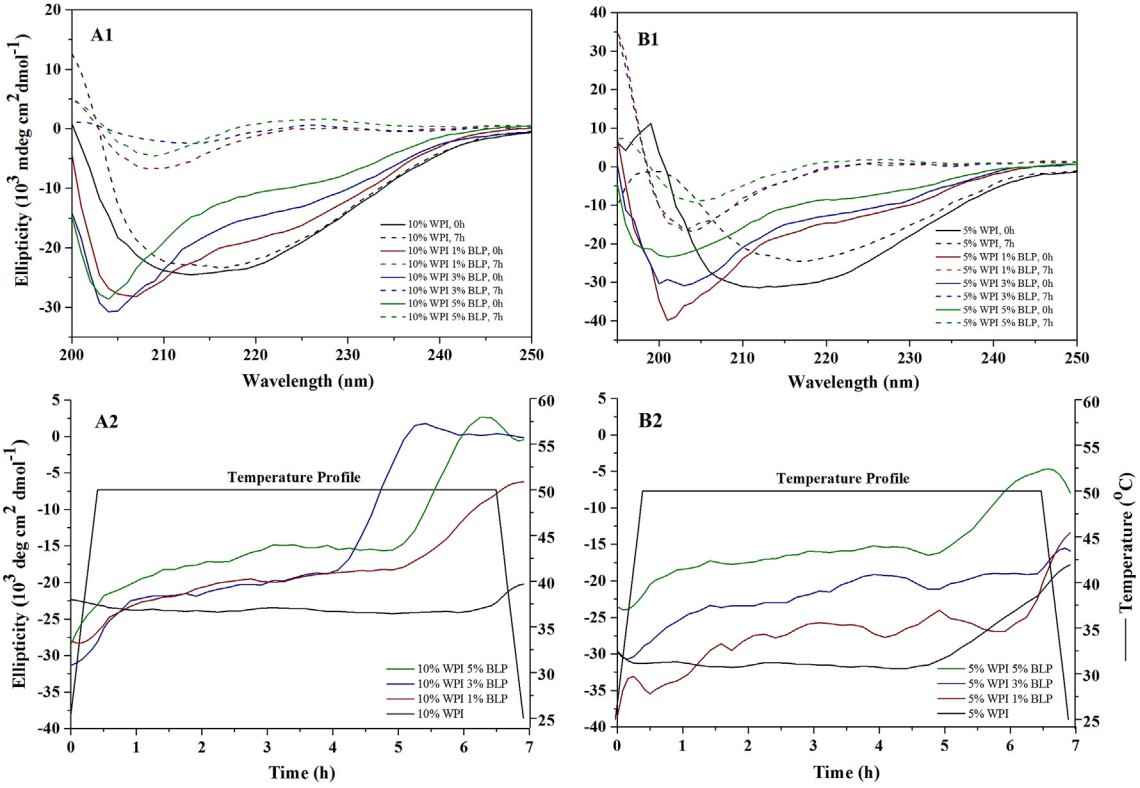

Fig. 7. Far-UV CD spectra of BLP induced-WPI gels: 10% WPI (A1), 5% WPI (B1) gels with 1, 3, and 5% BLP concentrations. Full lines represent data at the beginning of the incubation, whereas, dashed lines represent data at the end of the incubation. Time analysis of BLP induced-WPI gels based on CD data: 10% WPI (A2), and 5% WPI (B2).

helical, β-sheet and random/disordered structures were done as described in the literature (Greenfield, 1996, 2006; Kelly, Jess, & Price, 2005). Negative bands at 208 and 222 nm are attributed to α-helical proteins whereas proteins with β-sheet conformation show a negative broad band at 218 nm. Conversely, disordered protein conformations exhibited low ellipticity around 210 nm (Fig. 7A1 and B1).

Given the nature of the samples and how they were prepared, it is logical to assume that there co-exist different protein conformations within the gel samples, Thus, it might be assumed that both α-helix and β-sheet structures exist in the broad band observed in the samples containing only protein (for both 10% and 5% WPI concentrations). Interestingly, this broad band between at 208–222 nm was shifted to 204–208 nm range for 10% and to 200–204 nm for 5% WPI concentration samples after adding the BLP enzyme at the beginning of the incubation period at room temperature. It was observed for all samples containing enzymes held at room temperature only for few minutes prior to CD measurements. The shifting of the broad band might suggest that the enzyme started to work immediately after mixing with the protein solution, which might lead to detectable conformational changes in the protein structure, probably due to the high enzyme substrate specificity and selectivity of the BLP-WPI system. BLP is a serine endopeptidase, highly specific to Glu and Asp residues, and digests whey proteins specifically to induce aggregation and gelation (Creusot & Gruppen, 2007; Ipsen & Otte, 2007). As reported recently, faster hydrolysis is achieved through selectivity of the enzyme at specific cleavage sites due to different factors. Butre, Sforza, Gruppen, & Wierenga (2014) investigated in detail using WPI and BLP enzyme selectivity for individual cleavage sites affecting hydrolysis rate.

The 7-h incubation period included 30 min for a heating ramp from 25 °C to 50 °C with a heating rate of 1 °C/min, the 6 h-incubation time at 50 °C and 30 min of a cooling ramp from 50 °C to 25 °C with a cooling rate of 1 °C/min. At the end of this incubation period, protein secondary structure was largely retained in the 10% WPI sample without BLP, and the WPI protein was unfolded. In the case of the gel samples containing enzyme, the protein secondary structure was remarkably changed during the 7-h incubation period due to hydrolysis and gelation. The native helical structure was affected and irregular/disordered protein structures appeared which show clearly new bands in Fig. 7A1 and B1 (dashed lines). Similar results have been obtained during hydrolysis of β-Lg (about ~70% of predominant protein in WPI) with 2% BLP that led to a gradual decrease in β-sheet and α-helix structures (17% and 15% decreases, respectively) after 15-h incubation at 40 °C (Otte et al., 1997). It was suggested that secondary structure conformation was largely retained by the presence of peptides released during the hydrolysis. In addition the very stable β-sheet structures closing at Glu sites were not disrupted under relatively mild conditions. Conversely, differences in hydrolysis conditions and the potential presence of α-La (another whey protein present in WPI) may lead to selective cleavage at specific sites thus affecting the hydrolysis and changing the protein secondary structure significantly.

The 7-h incubation period including hydrolysis and gelation were followed in time with CD measurements by taking scans at 10 min intervals. Changes in ellipticity, for a certain wavelength (for 10% WPI without BLP and with 1%, 3%, and 5% BLP were 214 and 207, 204, and 204, respectively; for 5% WPI without BLP and with 1%, 3%, and 5% BLPwere 216 and 201, 203, and 203, respectively), versus time are illustrated in Fig. 7B1 and B2. These plots provide information to analyze the time dependency of the structural conformation in the protein. A strong relationship was obtained between sudden structural changes in the protein and the beginning of the gelation measured by rheological testing. Sudden change in ellipticities were observed in samples containing the BLP enzyme, for the 10% WPI concentration samples at around 4.5 h and for the 5% WPI samples (especially with 5% BLP) after 5 h of incubation, which agreed with the formation of the plateau in the complex modulus G* observed in the rheological temperature sweep experiments. This finding suggests that during the hydrolysis of the WPI protein some losses in the secondary structure occurred but a significant loss in both helical and β-sheet structure occurs when a gel is established.

3.4. Modulated Differential Scanning Calorimetry (MDSC)

Fig. 8 illustrates MDSC thermograms for 10 and 5% WPI concentration samples with different enzyme concentrations (heat flow curves were shifted to facilitate comparison). In both cases, there were slight but observable denaturation endotherms in the protein samples without enzyme after 10-h incubation at 50 °C. As β-lactoglobulin (β-lg) is the major protein present in WPI, a clear thermal denaturation peak attributed to β-lg was observed at around 72 °C in agreement with data previously reported by Boye and Alli (2000). Enthalpy values obtained for 10% and 5% native WPI were 4.2 J/g and 1.7 J/g, whereas those for heat-treated 10% and 5% WPI were 3.8 J/g and 0.95 J/g. Also two other shoulder type transition peaks corresponding to denaturation of α-lactalbumin (α-la) were observed at about 35 and 64 °C in samples that do not contain enzyme. Enzyme-induced WPI gels showed no denaturation endotherms because almost all proteins were already denatured by enzymatic hydrolysis. Only the 10% WPI concentration gels with different BLP enzyme concentrations showed very small denaturation peaks at about 78 °C, probably as consequence of non-hydrolyzed β-Lg residues. MDSC results were in agreement with CD findings clearly showing that protein was mostly denatured during hydrolysis of WPI in the presence of the BLP enzyme. As control, a not incubated sample (NI) showed that incubation did not denature the protein (Fig. 8).

4. Conclusion

BLP-induced WPI gelation was analyzed in terms of rheological characterization, gel microstructure, and structural changes in protein during hydrolysis and gelation. Hydrolysis, aggregation and gelation mechanisms differed and depended on protein and enzyme concentrations. Strong viscoelastic particulate gels were obtained from samples with 10% WPI concentration regardless of the BLP enzyme concentration. Gelation rate was increased with increasing enzyme concentration. Viscous liquids with little or none elasticity were obtained from samples having 5% WPI concentration and the degree of viscoelasticity depended on the enzyme concentration in the samples.

Rheological data was analyzed using standard techniques, and a novel approach to study creep and recovery data. The novel approach was based on the concept of fractional derivative, which not only facilitated data analysis but also provided a much simpler interpretation of the creep-recovery tests.

The time to achieve significant denaturation of protein structure due to hydrolysis and aggregation was closely related to time that take to form stable gels at the same temperature. That feature allows for a good control of the gel structure and its mechanical properties and gives BLP-induced WPI gels a different perspective for potential uses not only in food but also in biomedical applications.

The characterization of these systems is important in other areas of research because it can aid the development of novel applications on food and material science, for example the production of matrices for transport and delivery of active compounds or the

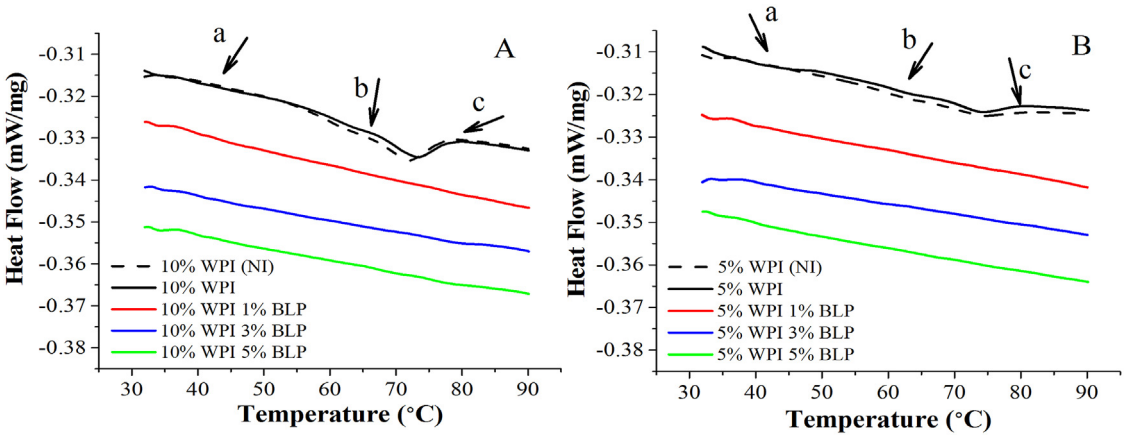

Fig. 8. MDSC thermograms of BLP induced-WPI gels: 10% WPI (A) and 5% WPI (B). NI:Not incubated.

growth of cells, due to the formation of gel microstructures with tunable porosity and biocompatibility.

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