Ö. Tarhan and O.H. Campanella / Hacettepe J. Biol. & Chem., 2020, 48 (3), 301-307

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: www.hjbc.hacettepe.edu.tr

Microstructure and Rheology of Whey Protein Based Hydrogels

Peyniraltısuyu Proteinleri Bazlı Hidrojellerinin Mikroyapısı ve Reolojisi

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ABSTRACT

Protein hydrogels have great potential for food and biomedical applications due to their ability to create three dimensional gel networks. A dairy industry by-product whey contains valuable proteins capable of forming gels with the ability of holding excess amount of water and entrap active ingredients. This enables a wide use of whey proteins for various applications in food formulations. Whey protein gelation is commonly promoted by heat and the gel characteristics can be enhanced by blending whey proteins with suitable carbohydrates. Mechanical properties and microstructure of gel networks determine their availability for target applications such as entrapment of active agents by maintaining their stability, target delivery and texture improvement of food products. The objective of this study was to investigate structural and mechanical features of whey protein -sodium alginate gels using microscopy, spectroscopy and rheometry. Rheological and structural properties of gels obtained by different preparation protocols and composition differed significantly. Porosity in the gel microstructure changed remarkably as the gel composition changed. Also, some conformational changes were tracked by studying the secondary structure of proteins during gel formation. Further research will focus on investigation of these whey protein-based gels for the entrapment ability and release behavior of bioactive components.

Key Words
Whey proteins, gelation, sodium alginate, microstructure, spectroscopy.

ÖZ

Protein hidrojeller üç boyutlu jel ağ yapılarından dolayı gıda ve biyomedikal kullanım için önemli potansiyele sahiptirler. Süt endüstrisinin yan ürünü olan peyniraltı suyu fazla miktarda su ve aktif bileşen tutabilme yeteneğine sahip jeller oluşturabilen değerli proteinler içerir. Bu yetenek peyniraltı suyu proteinlerinin gıda işlemede çeşitli uygulamalarda yaygın olarak kullanımına olanak sağlar. Peyniraltı suyu proteinlerinin jelleşmesi yaygın olarak ısıl işlemle tetiklenir ve peyniraltı suyu proteinlerinin karbonhidratlarla harmanlanmasıyla jel özellikleri istenen şekilde geliştirilir. Jellerin mikroyapısı ve mekanik özellikleri aktif ajanların kararlıklıklarını koruyarak tutma, hedef bölgeye teslim ve gıda ürününün tekstürünü geliştirme gibi hedef uygulamalara elverişliliğini belirler. Bu çalışmanın amacı, peyniraltı suyu protein jellerinin yapısal ve mekanik özelliklerinin mikroskop, spektroskopi ve reometre kullanarak araştırılmasıdır. Farklı işlemlerle elde edilen jellerin reolojik ve yapısal özellikleri dikkate değer şekilde farklılık göstermiştir. Jel mikroyapısının gözenekliliği jel kompozisyonu değiştikçe önemli ölçüde değişmiştir. Ayrıca, jel oluşumu sırasında protein ikincil yapısında konformasyonel değişiklikler izlenmiştir. Sonraki çalışmalar, peyniraltı suyu bazlı jellerin biyoaktif maddeleri hapsetme becerisi ve salınım davranışının araştırılması üzerine odaklanacaktır.

Anahtar Kelimeler
Peyniraltı suyu proteinleri, jelleşme, sodium aljinat, mikroyapı, spektroskopi.

Article History: Received: Jan 11, 2020; Revised: Jun 5, 2020; Accepted: Jun 5, 2020; Available Online: Jul 1, 2020.

DOI: https://doi.org/10.15671/hjbc.669837

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INTRODUCTION

Hydrogels can form a three dimensional network of polymers capable of holding large amount of water. Polymers containing hydrophillic groups such as hydroxyl, carboxyl, amino and sulphonyl are stabilized by hydrogen bonding, electrostatic intreractions and chemical crosslinkings [1, 2]. Natural hydrogels are obtained by using biological polymers such as proteins. Proteins have various applications in food and biomedicine due to their unique functional properties such as gelation. The native structure of proteins can be destabilized by heat, chemical or enzymatic treatments. Peptide fragments exposed due to these treatments aggregate and form gel networks. The resultant gels have good mechanical and barrier properties. Those properties differ according to the type protein source. Animal based collagen, casein, whey and egg proteins can form transparent and opaque gels with good barrier properties [3, 4]. Plant based proteins such as soy, zein, and wheat form transparent hydrogels and have good barrier properties [5, 6, 7]. Protein gelation occurs via unfolding and aggregation of proteins due to heat, chemical crosslinking, enzymatic hydrolysis, and high pressure, Resultant gels can be transparent or turbid depending on gel composition and preparation conditions.

Protein hydrogels are biodegradable, and can have high biocompatibility, elasticity, mechanical stability, good transport properties and tunable porosity. They can be used in transporting and target delivery of bioactive compounds and nutraceuticals to enhance bioavailability, and improve texture of foods as well. Thus, protein gelation affects texture, quality, and sensory attributes of jelly foods. Hydrogel-based edible films and coatings can be used for packaging purposes as well. In the case of biomedicine, these films are used for transport and controlled delivery of drugs. Injectable hydrogel preparations can trigger delivery of cells and drug in the body. Non-food applications of hydrogels are scaffolding in tissue engineering, drug-delivery systems, contact lenses, wound dressings, cosmetics, waste treatment, seperation systems, and biosensors [8, 9]. The present research reports whey based protein gels with some characteristic features to be considered for potential food and biomedical applications.

Whey, the valuable by-product of dairy industry is further processed by membrane filtration and chromatographic techniques to obtain whey protein powders of commercial use. Major protein fractions in whey are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and bovine serum albumin (BSA). Various treatments such as heating, chemical crosslinking and enzymatic hydrolysis induce gel formation. Protein-protein and protein-water interactions determine the nature of gels obtained. Aggregate gels with opaque and turbid appearance are obtained when the pH of the reaction medium is closed to the isoelectric point, pI, of the protein [10]. However, fine-stranded, transparent and stronger gels are formed when pH is away from pI. Gel strength and viscoelasticity are critical parameters to design target application schemes with desirable properties. Gelation characteristics can be measured using several techniques but rheometry has been widely used because of the very significant changes on the rheological properties of the material at the gel point. r. Storage and loss moduli profiles obtained by small amplitude oscillatory strain (SAOS) measurements indicate changes in the viscoelastic properties of the system and thus the sample gelation point and gel stiffness [11, 12]. Mechanical properties of protein hydrogels can be improved by blending the protein with suitable polysaccharides. Polysaccharide-protein gels may show higher stiffness and stability due to the formation of an enhanced polymer network. Dextran, carboxymethylcellulose (CMC) and chitosan incorporated into whey protein gels exhibited considerably high strength and stability [13, 14]. Sodium alginate is one of the natural polymers used to form hydrogels in combination with whey as the carrier of active ingredients [15]. Whey/alginate (WALG) gels are prepared and characterized in in this work given their soft texture and transparent appearance which make these gels suitable for food and non-food applications.

The microstructure of gels, which can be studied by microscopy, is an important property because its porous nature may affect binding and entrapment of bioactive ingredients. Gel composition and processing conditions determine the size of pores formed. Thus, the density and capacity of the gel network may vary due to changing pore size. Cryo-electron microscope is a versatile tool enabling determination of pore size of whey protein gels [12]. Gel samples are frozen in liquid nitrogen slush and the ice is sublimated under high vacuum, then the imaging of fractured gel surfaces coated with platinum can create a specific surface that can be see with promotes high resolution.

Protein unfolding and denaturation achieved by heating or other conditions promote characteristic structural

changes that can be followed by spectroscopic techniques. For example, structural features of native protein changes in relative proportions are based on specific secondary structures detected by these spectroscopic techniques [16]. Circular dichroism (CD) spectroscopy is commonly used to determine secondary structure of proteins in their native and denatured forms. Structural changes due to different treatments and potential new crosslinking can provide a comprehensive evaluation to assess the surface active groups and available bindings to be involved in the gelation of these systems.

By considering various aspects of protein gelation, the purpose of this research is to investigate heat induced whey protein/alginate gels by characterizing their gels microstructure, and rheology and secondary structure changes upon gelation using electron microscopy, small amplitude oscillatory strain tests and CD spectroscopy.

MATERIALS and METHODS

Materials

Whey protein isolate (WPI) (BiPRO) and α-lactalbumin (α-La) (BIPRO) was kindly provided by Davisco Foods International Inc. (Minnesota, USA). According to manufacturer, a typical WPI batch contains 61-70% b-Lg, 23-31% a-La, 2-4% BSA, and 1-5% IGg. On dry basis, the percent composition was 97% (w/w) protein, 0.2% (w/w) fat, 1.9% (w/w) ash, and 4.8% (w/w) moisture. A typical α-La batch has 97.8% (w/w) protein, 0.1% (w/w) fat, 1.9% (w/w) ash, and 5.2% (w/w) moisture on dry basis. ***Bacillus licheninformis*** Protease (BLP) (Activity: 13.744 AU-A/G, batch no PL 100013) was kindly provided by Novozymes A/S (Bagsvaerd, Denmark). Other reagents used were of analytical grade.

Methods

Sample Preparation

WPI and α-La solutions were prepared at a constant protein concentration of 10% (w/w) by dissolving WPI and α-La powders in Tris-HCl buffer (75 mM, pH 7.5) overnight at 4°C. Protein solutions were prepared with (1%, w/v) and without sodium alginate. Gelation was propmoted by heat treatment. Temperature ramps used for rheology and CD measurements are described in sections 2.2.3 and 2.2.4, respectively. Gel samples for microscopy analysis were prepared using the same procedure and incubated in water baths at 65°C and 80°C. Those values were chosen based on thermal denaturation temperatures of beta-lactoglobulin (78°C), major protein fraction in whey and alpha-lactalbumin (64°C) [17].

Cryo-SEM Microscopy

Cryo-SEM experiments were performed using a GATAN Alto 2500 Cryo Units (JEOL Ltd., Tokyo, Japan) attached to a FEI Nova Nano630 SEM (Oregon, USA) according to Spotti et.al., 2017 [12]. Samples of α-La, α-La-NaAl, α-La-WPI, and α-La-WPI-NaAl were incubated at 65°C, whereas WPI and WPI-NaAl were incubated at 80°C, temperatures selected according to gelation profiles of protein and protein/carbohydrate solutions. The images were recorded at 2500, 5000 and 10000X, and the images of 5000X were used for comparison of the gels.

Dynamic Oscillation Measurements

Heat-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 water evaporation. Samples were subjected to dynamic oscillation with controlled strain of 1% at 1 Hz frequency. The strain of 1% was previously determined to be within the linear viscoelastic range of the gels. Temperature/time sweep measurements were performed while heating the sample from 25 to 65°C/80°C at a rate of 1°C/min, then holding it at 65°C / 80°C up to 3 h and then cooling to 25°C at a rate of 1°C/min. Storage modulus (G') and loss modulus (G'') values were determined during the measurements and ploted as a function of time. All measurements were conducted by duplicate. Gelation time was determined as the time at which tan d ≤ 1 [18].

Circular Dichroism (CD) Spectroscopy

Protein solutions were prepared with concentrations of 0.1 mg/mL in 100 mM Tris-HCl buffer (75 mM, pH 7.0) for CD measurements. CD measurements were performed using a Jasco J-1500 Spectropolarimeter (JASCO UK Ltd., Great Dunmow, UK). Temperature ramps for CD measurements during heating and cooling steps were similar to those one used during the gelation tests. Scans were taken during a heating step from 25 °C to 65/80°C with a heating rate of 5°C/min. Then, 10 min-interval scans were taken at 65/80°C for 6 h, and scans were taken during a cooling step rom 65/80°C to 25°C with a rate 5°C/min. Quartz cells with a 0.1 cm path length were used in the measurements and the scan rate used was 10 nm/min with, with a 0.2 nm band width. Recorded spectrum was an average of 2 scans from 200 to 250 nm. Results were expressed in terms of mean residual ellipticity (θ) in units of deg. cm/decimol (dmol), and

were determined according to the following equation:

$$\theta = (MRW)/10.l.c \quad (1)$$

where θ corresponds to the measured ellipticity angle (mdeg), MRW is the mean residue weight (114.63), *l* is the optical path length (cm), and c is the protein concentration (g/ml). All spectra were obtained by subtracting the buffer base-line from the recorded sample spectra.

RESULTS and DISCUSSION

Microstructure of Whey Protein/Alginate Gels

Unfolding of the native whey protein globular structure via heating leads formation of intra- and inter-chain crosslinking, thus fine-stranded and clear hydrogels are formed. Protein composition and the heating scheme strongly affect the nature of gelation process, the gel structure and its appearance. Also, crosslinking with other macromolecules may have additional changes during the gelation process henomena. The α-La-enriched WPI was mixed with sodium alginate and the gelation process was investigated. Cryo-SEM images of WPI/α-La/NaAl gels at different compositions are shown in Figure1). The microstructures of heat-induced individual WPI, α-La, mixture WPI/ α-La and NaAl gels can be observed in the first row of the series of pictures (Figure 1a-1d) whereas gels prepared with NaAl and blends with WPI, α-La and WPI/ α-La are illustrated in the second row (Figure 1e-1g). It is clear gels prepared solely with whey protein without alginate have a regular porous structure with varying pore size. NaAl gels have an irregular microstructure with soft and clearer appearance (Fig 1d). In the case of WPI/NaAl and α-La/NaAl the structure of the gels becomes denser. The network of WPI/α-La/NaAl gel is quite dense, however the gel was softer and more transparent in appearance. It has been previously reported that heat-induced whey protein gels are strong and rigid and have low viscoelasticity. The images clearly show that three components gels have the capacity of enhancing their entrapment ability of active ingredients within their dense network.

Mechanical strength, porous nature and viscoelasticity are significant properties of hydrogels for desirable food and non-food applications. Entrapment of bioactive agents within the pores of the gel structure facilitates transportation and target release of drugs and bioactive compounds via controlled disassembly of the gels. Surface chemistry of these gel structures are also critical to determine and direct binding and release behavior of these components to the gel matrix. New findings regarding microstructure and appearance of whey/alginate hydrogels may help to discover potential applications in controlled delivery and packaging purposes.

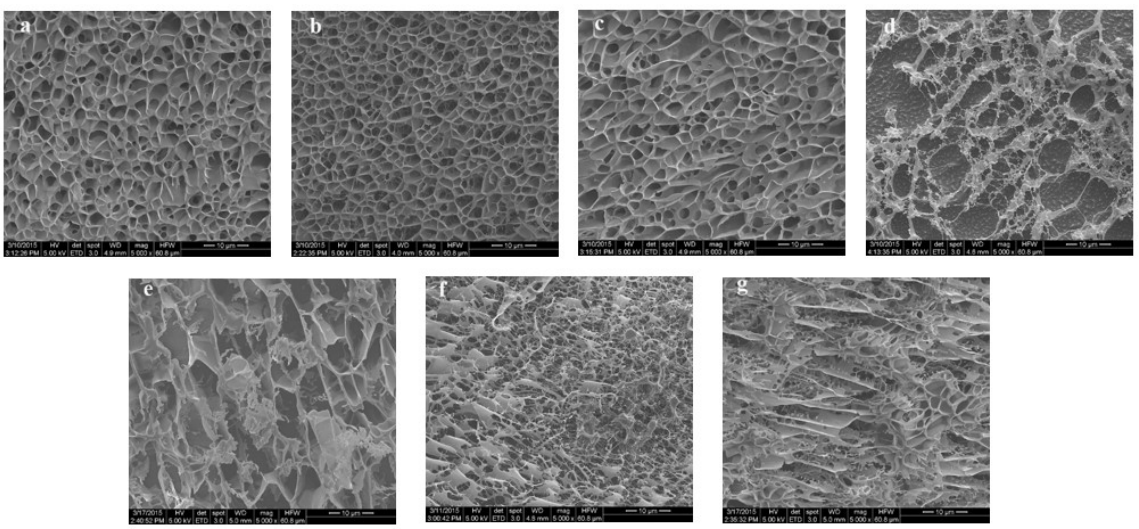

Figure 1. Cryo-SEM images of WPI/α-La/NaAl gels at different compositions. a) α-La, b) WPI, c) WPI/α-La, d) NaAl, e) α-La/NaAl, f) WPI/NaAl, g) WPI/α-La/NaAl.

Rheology of WP/Alginate Gels

Gelation process of whey proteins induced by heat and sodium alginate crosslinking was followed by monitoring changes in the storage modulus (G') and loss modulus (G'') as a function of time and temperature. Gelation profiles of protein/alginate blends are illustrated in Figure 2. Two major protein fractions in whey, β-Lg and α-La have thermal denaturation at 78°C and 64°C, respectively [16]. As protein denaturation promotes aggregation and possible gelation which is achieved at 80°C and 65°C by inducing denaturation of main proteins in whey. According to previous studies, WPI forms rigid gels during heating at around 80-90°C mostly due to denaturation of β-Lg [11, 12]. Due to thermal denaturation, gel formation of α-La protein itself was observed as quite slow (data not shown). In case of WPI/NaAl blend, gel formation started at around 50°C after 10 min of the temperature ramp. The crossover of G' and G'' is considered as the gelation point and considered as the temperature where G', an indication of the sample elasticity, surpasses G'', and indication of the sample viscous thus indicating the increase in elasticity due to network formation [19]. The temperature was maintained at 80°C for 60 min, which was the time until an approximate plateau was seen in the storage modules, and then a temperature ramp was used in a cooling step that triggered an increase in the gel stiffness, as measured by the storage modulus (Figure 2a). A transparent but weak gel was obtained during the heating/cooling process of α-La/NaAl blend (Figure 2b). From the data shown in Figure 2b is clear that heating up to 80°C did not significantly induced gelation of the α-La/NaAl samples after 60 minutes heating at 80°C. However, keeping this sample at 65°C but for a longer time (almost 18 h) resulted in a stronger gel (data not shown). When α-La was added to the WPI sample and subjected to the long heating process at 65°C weak and little opaque gels were obtained after about 75 min (Figure 1c). In the case of α-La added to the WPI with and NaAl mixture, gelation started at lower temperatures and about 60 min (Figure 1d). This indicates that the addition of α-La to WPI did not lead to desirable gel formation within the tested time period without adding NaAl. Heating of WPI samples at 80°C resulted in gelation mainly

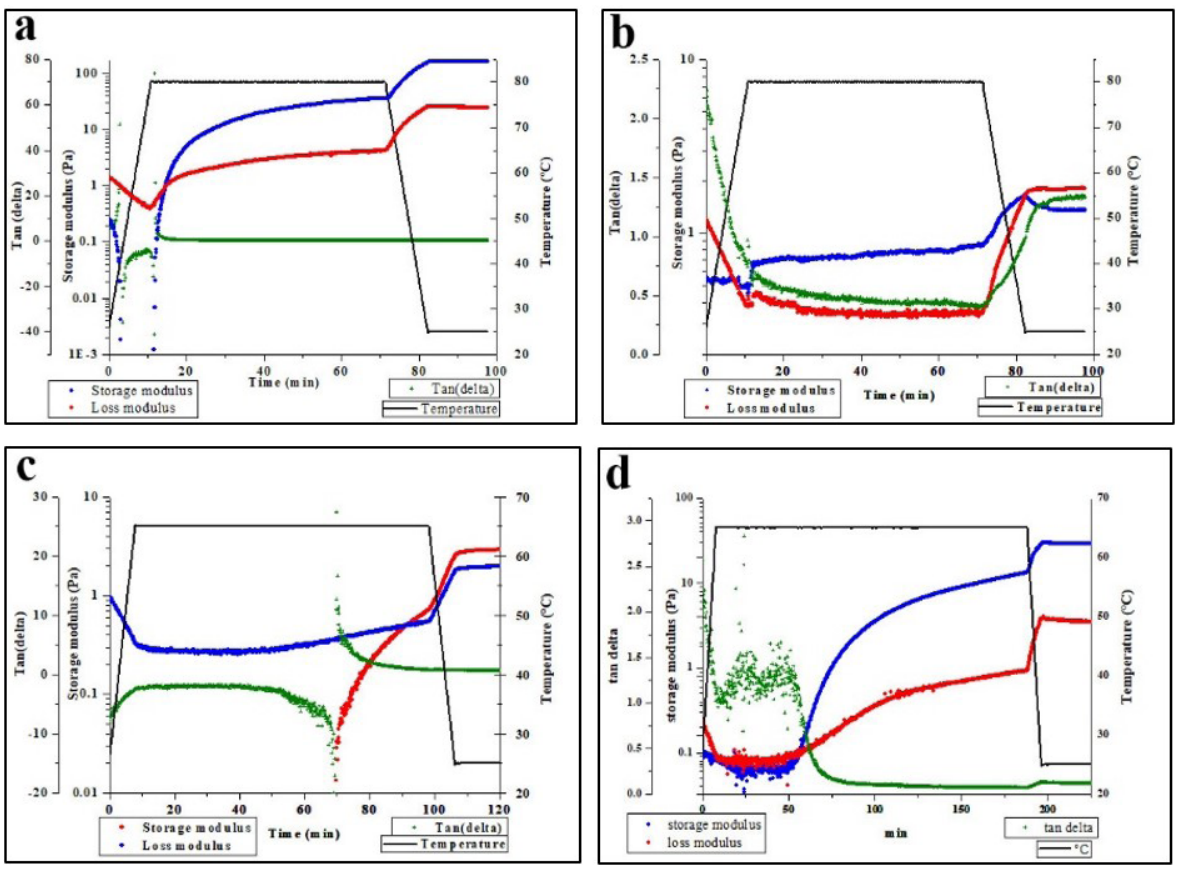

Figure 2. . Gelation profiles of WALG samples at different compositions. a) WPI/NaAl, b) α-La/NaAl c) WPI/α-La, d) WPI/α-La/NaAl. Gelation induced via heating from 25°C to 65/80 °C, maintaining at to 65/80 °C, and cooling to 25°C.

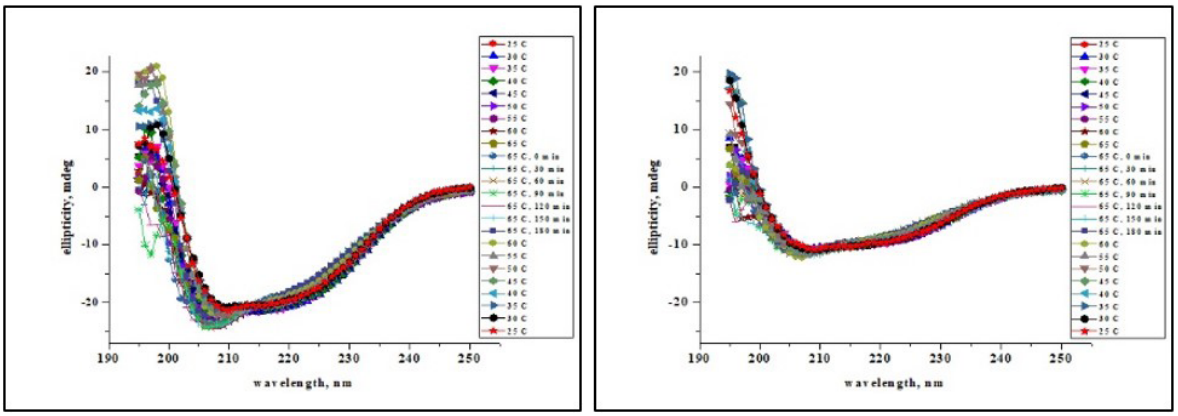

Figure 3. CD spectra of a) WPI/α-La, and b) WPI/α-La/NaAl gel samples.

due to thermal denaturation of β-Lg. Thus, exposure of WPI/α-La/NaAl blends to 65°C allowed us to conclude that the gelation of WPI/α-La blends was enhanced by the incorporation of sodium alginate. Gel rheology differed due to varying protein /alginate composition and the heating process. Key findings indicated that heat induced WP hydrogels obtained with mixing NaAl were clear, soft and viscoelastic.

CD Spectroscopy of WP/Alginate Gels

Secondary structure elements such as α-helices, β-sheets and random coils can help to characterize the molecular conformation of soluble proteins. Conformational changes occurring in the whey protein secondary structure due to heat treatment and chemical crosslinking was followed by CD spectroscopy. The ellipticity of WP/alginate samples during heating from 25°C to 65°C, holding at 65°C for 30 min, and cooling from 65C to 25°C are presented below (Figure 3). This temperature profile was selected based the rheology tests. The blends of WPI/α-La and WPI/α-La/NaAl samples were subjected to CD analysis to monitor these structural changes. In the case of WPI/α-La, ellipticity close to 208 nm and 222, attributed to α-helices [20, 21], decreased as the temperature increased to 65°C (Figure 3-a). A negative broad band at 218 nm indicates the presence of a β-sheet structure whereas disordered protein conformation exhibits low ellipticity at 210 nm. Although some conformational changes in the protein structure were captured, it is apparent that mostly secondary structure was retained. A less decreased ellipticity was observed in samples containing both whey proteins blended with sodium alginate (Figure 3-b).

CONCLUSION

Whey protein/alginate gels were investigated using cryo-electron microscope, rheometry and CD spectroscopy in this study. Gel microstructure was significantly affected by composition. Protein gels had regular porous structure whereas alginate formed an irregular network structure. Whey proteins blended with sodium alginate gels formed denser and irregular networks having a higher like hood of better entrapment capacity. Heating above thermal denaturation temperature induced aggregation and stiffer gel formation. Blending whey proteins with sodium alginate resulted in gel with different physical and rheological properties. More transparent and softer viscoelastic hydrogels were obtained in the presence of sodium alginate. WPI tended to form stronger gels when heated whereas α-La promoted the formation of weak gels. According to CD-spectroscopy findings changes of the secondary structure of α-La enriched WPI proteins were not significant. In the case of sodium alginate incorporation protein gels appear to have low dispersion in CD ellipticity with some alterations in helical and β-sheet structures. Besides, secondary structure of proteins was mostly retained in whey/alginate gels when exposed to heat treatment.

Acknowledgments

The authors would like to thank Chia Ping Huang from Life Science Microscopy Facility, Dr. Patricia Bishop from Chemistry Department in

Purdue University for their technical assistance in cryo-SEM, and CD analyses, respectively. The author Dr. Ozgur Tarhan was financially supported by The Scientific and Technological Research Council of Turkey (TUBITAK) for postdoctoral research in USA.

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