Application note

Investigating heat-induced gelation of whey protein using simultaneous rheology and FTIR spectroscopy

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Bovine milk contains two groups of proteins: casein and whey proteins. Whey is the liquid byproduct remaining after milk has been curdled during the cheese manufacturing process.

Whey protein products such as whey protein concentrate and isolate are mixtures of globular proteins, consisting mainly of β-lactoglobulin and α-lactalbumin. Globular proteins have excellent nutritional benefits and exhibit exceptional physicochemical properties. As a result, globular proteins are used in a wide range of food applications as foaming agents, stabilizers, emulsifiers, and gelling agents (to name a few).

When sufficiently concentrated aqueous whey protein solution is heated, it induces denaturation and aggregation of the protein molecules, and a gel-like network can be formed (Figure 1). The protein gel structure directly influences the sensory response and structure of food products, and thus, gelation is considered one of the most important functional properties of proteins [1]. Whey protein gels are commonly utilized in the food industry for structuring products and controlling the overall food texture.

The thermal gelation process of globular proteins typically involves two main steps: thermal denaturation and unfolding of the proteins, followed by intermolecular aggregation and rearrangement [1] (Figure 1). The physical gelation behavior of whey protein solutions can be studied via rheology, where the bulk viscoelastic properties can be used to examine the transition from a pourable liquid solution to a solid-like, rubbery gel network. Conversely, Fourier-transform infrared (FTIR) spectroscopy can be employed to gain insight into protein structural changes that occur at the molecular level during the gelation process. IR spectroscopy of proteins shows the absorption of specific amide bond vibrations within the polypeptide chain, primarily the Amide I (stretching vibration in the C=O bond) and Amide II (bending vibrations of the N–H bond) regions [2]. The use of FTIR spectroscopy for discerning secondary and tertiary protein structures is relatively fast and inexpensive compared with other common analytical methods.

Overall, the thermal denaturation of whey protein is a complex physicochemical process driven by various inter- and intra-molecular interactions [3–5]. Thus, this study uses rheology coupled with *in situ* FTIR spectroscopy to examine thermally induced gelation of a whey protein solution. By utilizing simultaneous FTIR spectra and rheological data, molecular-level interactions and conformational changes during the protein gelation process can be directly correlated with increases in bulk viscoelastic properties, providing unique insight into the complex denaturation behavior of whey protein.

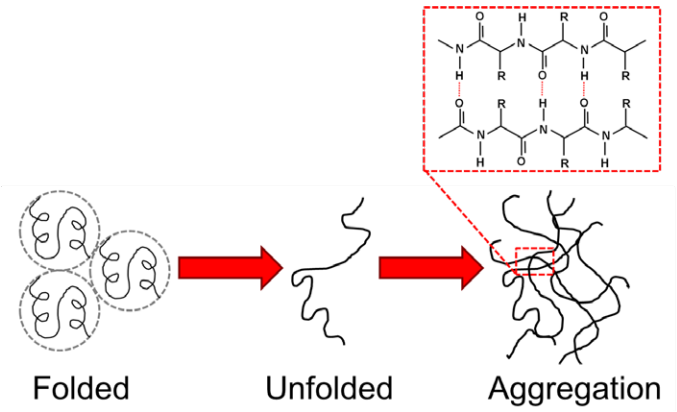

Figure 1. Schematic representation of the whey protein heat-induced gelation process depicting molecular unfolding and denaturation, followed by aggregation via intermolecular β-sheet hydrogen bonding.

Materials and Methods

Materials

The whey protein solution was prepared by dispersing whey protein isolate powder (Now Foods, Bloomingdale, IL, USA) in deionized water at a concentration of 50% (w/w). Prior to testing, the whey protein solution was equilibrated for at least 1 hour at room temperature to ensure complete hydration of the protein particles [6].

Rheology

Rheological measurements were performed using a Thermo Scientific™ HAAKE™ MARS™ 60 Rheometer equipped with a 35 mm diameter parallel-plate geometry at a gap height of 1 mm. All measurements were conducted under small-amplitude oscillatory shear, using a set frequency of 1 Hz and a constant strain of 1%, and data was collected every 10 s. The whey protein samples were loaded onto the rheometer at 20 °C and held constant for 10 min while collecting rheological data. After the initial equilibrium step, the temperature was increased from 20 °C to 90 °C at a rate of 2 °C/min. The temperature was held constant at 90 °C for 10 min and then decreased from 90 °C back to 20 °C at the same 2 °C/min rate as the increasing temperature step. After the decreasing temperature sweep, the temperature was again held constant at 20 °C for 10 min to complete the full temperature cycle. To prevent evaporation, the sample edge was sealed with mineral oil and covered using an insulated sample hood.

Rheonaut

Simultaneous rheology and FTIR spectroscopy measurements were performed using the patented Thermo Scientific Rheonaut Module coupled to the Thermo Scientific Nicolet™ iS™ 10 FTIR Spectrometer. The incident infrared light was directed from the side port of the Nicolet iS10 FTIR Spectrometer and into the Rheonaut Module, which acts as both the temperature control module and the bottom plate of the rheometer. The bottom rheometer plate features a monolithic diamond element that serves as the ATR (attenuated total reflection) sensor, offering a single internal reflection. The ATR diamond is integrated into the center of the motor-driven lower plate, which can be positioned via software control at different radial locations from the center to a maximum distance of 45 mm. The Rheonaut Module was also equipped with a Peltier temperature element for temperature control from 0 to 100 °C (an electrical unit is also available for temperatures from ambient up to 400 °C).

The Rheonaut Module coupled with the iS10 FTIR Spectrometer operates in the mid-infrared spectral range from 400 cm^{-1} to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1}. Each *in situ* FTIR spectrum consists of 8 co-added scans. In total, 180 data sets, each containing rheological and spectral data, were collected. Sequential FTIR spectra (in parallel with the rheological measurements) were collected over a predetermined time window using the SERIES collection function within the Thermo Scientific OMNIC™ Software package.

Results and Discussion

FTIR Spectroscopy: Whey Protein Heat Treatment

Full-range FTIR spectra (3900 – 900 cm^{-1}) of the 50% w/w aqueous whey protein solution at 20 °C before and after heat treatment are shown in Figure 2a. Prominent FTIR features were observed across the complete absorbance spectrum, which can be divided into two distinct regions: X–H (X=C, N, O) stretching region (4,000 – 2,500 cm^{-1}) and the double bond/fingerprint region (1800 – 900 cm^{-1})[7]. Within the X-H stretching region, multiple peaks were observed from 3000 – 2800 cm^{-1}, which are commonly associated with acyl chain C-H stretching and are used to determine lipid content. Conversely, in the region from 3700 – 3000 cm^{-1}, only one broad spectral feature centered around 3300 cm^{-1} was observable, making it difficult to delineate individual spectral markers. For protein solutions, this region is usually impacted by the influence of water, and the observed broadening is most likely caused by hydrogen bonding and/or water-protein binding.

The double-bond and fingerprint region from 1800 – 900 cm^{-1} (Figure 2b) describes a protein's secondary structure, which consists primarily of hydrogen bonding interactions between carbonyl oxygen and amine hydrogen atoms along the backbone peptides. Protein molecules are commonly characterized based on the infrared absorption of their "amide bands." For the whey protein solution studied here, three major amide bands were observed: Amide I (1700 – 1600 cm^{-1}; stretching vibration in the C=O bond), Amide II (1570 – 1510 cm^{-1}; bending vibrations of the N–H bond), and Amide III (1400 – 1200 cm^{-1}; mainly N-H bending and the C-N stretching vibration) [2]. Due to the influence of water, only a single broad peak was observed for both the Amide I and II bands. However, all three amide bands displayed distinct increases in peak intensity and were clearly impacted by the heat-treating process (Figure 2b). In addition to an increase in intensity, the maxima of the Amide I and Amide II bands also shifted to lower wavenumbers after heating, i.e., downshifts from 1629 to 1622 cm^{-1} and 1545 to 1535 cm^{-1}, respectively. The Amide I band is often considered the best indicator of secondary structural changes as the Amide II and III bands are significantly less "pure" since they also incorporate C–N stretching [8]. Therefore, the Amide I band was used here as a spectral marker during the heat-induced gelation process.

Heat-induced gelation of whey protein is a multistep process that includes protein molecule unfolding, aggregation, and the growth and rearrangement of ordered clusters [9]. It is believed that the aggregation of whey protein molecules is caused by intermolecular β-sheet hydrogen bonding (Figure 1), which is indicated by the Amide I band. Intermolecular β-sheet activity is commonly associated with features between 1620 – 1635 cm^{-1} [10, 11] while aggregated proteins show bands due to intermolecular antiparallel β-sheets around 1614 – 1624 cm^{-1} and at 1684 cm^{-1}[12-14].

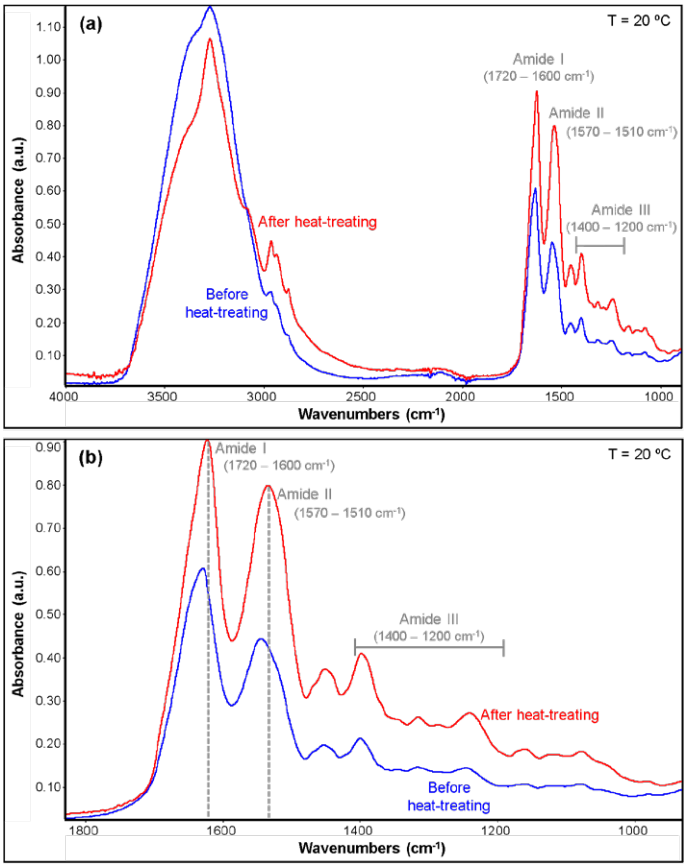

Figure 2. (a) Full FTIR spectra and (b) FTIR spectra of the Amide bands for the 50% w/w whey protein solution at 20 °C before and after heat treatment.

[12-14]. Unfortunately, due to the influence of water, all features associated with the Amide I band merged into a single broad peak centered at ~1629 cm^{-1} before heat treatment. As a result, the overall intensity of the 1629 cm^{-1} peak (I_{1629}) as well as the Amide I band wavenumber (defined as the wavenumber at the maximum peak intensity) were both used as spectral markers to track whey protein aggregation during the *in situ* rheology-FTIR measurements.

Simultaneous Rheology and FTIR Spectroscopy Using the Rheonaut

Tracking Amide I band peak intensity

Prior to heating, the 50% w/w whey protein solution was held at 20 °C for 10 min to get a baseline signature for both rheology and FTIR spectroscopy. As expected, the untreated protein solution behaved as a free-flowing viscous liquid, where G" was significantly greater than G' and both moduli remained steady at constant temperature (the first 10 min of Figure 3a). Similar to G' and G", the 1629 cm^{-1} peak intensity (I_{1629}) also remained relatively stable during the initial temperature hold at 20 °C (primary right y-axis Figure 3a).

To initiate the heat-induced gelation process, the temperature was then increased from 20 °C to 90 °C at a rate of 2 °C/min. As the temperature increased, both G' and G" began to decrease as the overall viscosity of the solution was reduced with increasing temperature. Meanwhile, the 1629 cm^{-1} peak intensity remained constant, displaying no observable change with the initial increase in temperature. However, when the temperature reached 60 °C (about 30 min into the measurement), both G' and G" began to increase exponentially, eventually increasing by three to four orders of magnitude (Figure 3a). The observed rapid increase in moduli was accompanied by a simultaneous increase in the 1629 cm^{-1} peak intensity, suggesting that the observed rheological behavior was directly associated with secondary structural changes of the whey protein molecules. The synchronized increases in both the rheological response and the Amide I band intensity indicate the start of the gelation process.

As the temperature was further ramped to ~74 °C (37 min), the increase in G' and G" began to slow. The moduli eventually reached a maximum of 80 °C (40 min) and remained fairly constant (with a slight decrease in G") until the end of the increasing temperature ramp to 90 °C (45 min). Correspondingly, as the increase in moduli began to subside from 74 to 90 °C (37-45 min), the I_{1629} also exhibited a slower growth, displaying two distinct rates of change during the increasing temperature ramp step: a rapid initial rise followed by a more gradual increase. The two different observed peak intensity growth rates, which directly corresponded with the observed rheological behavior, suggest that two separate molecular-level processes were present during the temperature ramp.

Once the temperature reached 90 °C, G' was now significantly greater than G" (i.e., a crossover was observed), indicating that the whey protein mixture had transformed from a liquid solution into a solid-like network during the increasing temperature ramp. Then during the isothermal hold step at 90 °C (45-55 min), G' and G" began to increase once again, even though temperature remained constant. Increases in G' during gelation have been directly correlated with increases in intermolecular crosslinks and/or entanglements within the network [15, 16]. In parallel, the 1629 cm^{-1} peak intensity continued to increase during the isothermal hold at 90 °C; however, it started to increase at a significantly faster rate (Figure 3a). Previous studies have shown that prolonged heating at 90 °C not only accelerates protein aggregation rates, but also leads to the rapid formation of firmly structured gels and an overall more elastic gel network [15,17]. Therefore, the simultaneous increase in both the Amide I band intensity and the elastic modulus, G' at 90°C, can be attributed to accelerated protein aggregation and increases in crosslink density.

Then as the temperature was decreased back to 20 °C, both G' and G" continued to increase as the whey protein sample further hardened with the reduction in temperature. Ultimately the growth in moduli plateaued, and the whey protein specimen reached its final stabilized gel structure. During the cooling step, G' increased and further deviated from G", indicating further structural development as the sample transitioned into an elastically dominated protein network. The observed physical transformation of the whey protein solution was thermally irreversible, suggesting a permanent molecular-level transformation.

Correspondingly, during the cooling step, the 1629 cm^{-1} peak intensity also continued to increase. However, the rate of increase began to slow at ~60 °C (70 min) and eventually subsided as the system was further cooled. Once the temperature had returned to 20 °C, a significant difference in the starting and ending 1629 cm^{-1} peak intensities was clearly evident, suggesting that permanent heat-induced conformational changes of the protein had occurred (i.e., the observed protein gelation process was irreversible). The irreversible increase in elasticity of the system (denoted by the rise in G') is most likely due to the formation of intermolecular bonds such as β-sheet hydrogen bonding [15, 17], which was directly corroborated by corresponding increases in the Amide I band intensity.

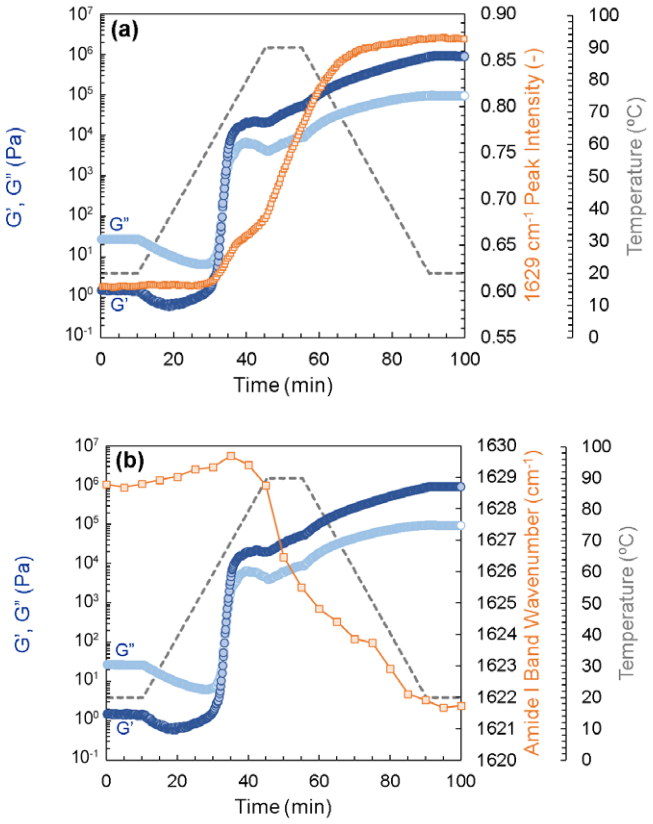

Figure 3. Elastic modulus G' and viscous modulus G" (filled and open circles, respectively; plotted on the left y-axis) overlaid with (a) 1629 cm^{-1} peak intensity and (b) Amide I band wavenumber (squares, primary right y-axis) plotted versus time for the 50% w/w whey protein solution during the temperature rampup, hold, and rampdown procedure. The corresponding temperature is plotted as a dashed line on the secondary right y-axis.

Tracking Amide I band wavenumber

The Amide I band intensity not only increased during the three-step heat-treatment protocol, but a significant decrease in the peak wavenumber was also observed (Figure 2b). Therefore, the wavenumber at the maximum Amide I band intensity was also tracked during heat treatment and overlaid with the rheological response (Figure 3b). Initially, the Amide I band was constant during the first temperature hold step at 20 °C. Then as the temperature ramped up to 90 °C, there was a slight but continual increase in peak wavenumber. The observed increase is relatively minute; however, upshifts of the Amide I band wavenumber have been previously associated with the unfolding of β-sheet structures [18], which would agree with the observed decrease in viscoelasticity and would also fit with the accepted protein gelation model: unfolding followed by aggregation and crosslinking. Nonetheless, as the temperature reached ~70 °C (35 min), the Amide I wavenumber reached a maximum point and then began to decrease slightly with further heating to 90 °C. However, during the isothermal hold step at 90 °C (45-55 min), the Amide I band displayed an abrupt decrease shifting from 1629 cm^{-1} down to 1625 cm^{-1}. Then as the temperature decreased and the whey protein specimen continued to solidify, the wavenumber continued to shift downward until it finally stabilized around 1622 cm^{-1} at 20 °C.

Downshifts of the Amide I band are typically indicative of strongly hydrogen-bonded β-sheets [19], and the 1624 cm^{-1} peak has been specifically attributed to intermolecular β-sheet crosslinking between unfolded proteins and used to characterize protein aggregation [20-22]. Thus, the sudden decrease in wavenumber initiated at 90 °C, which directly coincided with the observed increase in G' and G" (Figure 3b) as well as the rapid growth in the 1629 cm^{-1} peak intensity (Figure 3a), suggests that protein aggregation and crosslinking was induced and further exacerbated once the temperature had reached and was held constant at 90 °C. Again, this agrees with previous studies that extended heating at 90 °C accelerates protein aggregation rates, leading to the formation of more elastic gel structures [15, 17].

Conclusions

Simultaneous rheology and FTIR spectroscopy measurements were used to examine the gelation process of whey protein, both under changing and constant temperature. This multimodal analytical technique allowed the bulk mechanical properties of the whey protein specimen (G' and G") to be directly correlated with conformational changes at the molecular level (Amide I band peak intensity and wavenumber) in real time. The observed increases in viscoelasticity were not only directly associated with increases in the 1629 cm^{-1} peak intensity, but the rates at which G' and G" increased also paralleled the rate of increase in band intensity (i.e., rapid increases in the rheological response correlated with fast growth rates in peak intensity and vice versa), suggesting that the observed rheological behavior was driven by conformational changes and aggregation of the protein molecules. Changes in G' and G" were also clearly associated with changes in the Amide I band wavenumber. Initially, the Amide I band displayed a slight wavenumber increase, coinciding with an overall decrease in viscoelasticity, which is indicative of molecular denaturation or unfolding. Then during the isothermal hold step at 90 °C, the Amide I wavenumber exhibited an abrupt decrease, which was accompanied by further increases in G' and G" as well as rapid growth in band intensity, indicating that the protein aggregation rate had accelerated and the overall crosslink density within the gelled network had increased. The observed physical transformation of the whey protein solution was thermally irreversible, suggesting a permanent molecular-level transformation. This was corroborated by an irreversible increase in Amide I band intensity and an irreversible decrease in band wavenumber.

The simultaneous rheology with *in situ* FTIR spectroscopy allowed for deconvolution of the multi-step whey protein gelation process, including protein molecule unfolding, aggregation, and aggregate growth and rearrangement. The hyphenated technique provides corroborating evidences, from both macroscopic and molecular perspectives, to unravel a complex, multi-step process that cannot be fully revealed by either analytical technique alone. The case study presented in this note demonstrates the unique analytical capability unleashed by the Rheonaut Module. While this work focused on whey protein gelation, the underlying principles applied here should be applicable to a wide range of material processes including polymerization, crosslinking, curing behavior, as well as other shear-induced phenomena.

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