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Short Communication

Measurement of Heat and Pressure Induced Denaturation of Whey Protein Isolate Using Reversed-Phase HPLC and FTIR-Spectroscopy

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HIGHLIGHTS

- Heat (IHT) and pressure (HPT) stresses caused 88% and 58% WPI to denature, respectively.
- Reversed phase-HPLC determined the denatured protein through aggregation.
- FTIR together with HPLC is required for better characterization of denatured protein.

ABSTRACT

The protein molecules experience various external stresses leading to denaturation of protein during the process of transforming original whey to the concentrated whey proteins or while the concentrated proteins are used in the protein-enriched food formulation. This study was designed for a comparative assessment of the denaturation of whey protein isolate (WPI) under an important thermal stress, isothermal heat treatment (IHT), and high hydrostatic pressure treatment (HPT). The type and extent of denaturation were determined using reversed-phase HPLC and FTIR spectroscopy. The HPLC results demonstrated that the isothermal heat treatment caused higher denaturation of protein due to IHT at 80oC for 600s (88.38%) than that of HPT (58.5%). However, the infra-red spectroscopic analyses suggested that the HPT caused severe destruction of the structural conformation of WPI. The state of protein has a great impact on food formation; hence, the findings of this study would alert the concentrate protein producers and protein-enriched food manufacturers to prepare more active functional foods.

Introduction

There has been a substantial increase in the demand of concentrated whey protein for direct consumption or for being used as an ingredient in food formulation. This protein is extracted from the whey; a liquid remaining after milk has been curdled and strained. As a whole whey protein is a mixture of numerous proteins where α-lactalbumin, β-lactoglobulin, immunoglobulin, and bovine serum albumin are the major factions constituting more than 95% of the whole mass (Ng-Kwai-Hang, 2011; Haque et al., 2014). These proteins are quite commonly used as nutritional supplements or as additives

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in planned foods because of its ability to supply a high proportion of essential amino acids (Godfrey et al., 1996; Hoffman and Falvo, 2004). The folded 3-dimensional (3-D) structure of whey proteins is an extra advantage in giving functional attributes such as foam and gel formation properties during food processing. Moreover, the 3-D globular whey protein has been found as a fantastic wall material for oils and food flavours. This protein owns superior encapsulation efficiency (up to 89.6%) over other proteins such as soy protein (up to 75.9%) (Kim et al., 1996; Jimenez et al., 2004). However, for better functionality, maintaining the native structure of the protein is essential.

Based on the desired parameters of the final product, the original whey needs to be processed through a single or combined treatment such as drying, pressure, and heat treatments. The previous studies reported that the external stresses such as heat stress and spray drying stress can cause considerable denaturation (up to 90%) of whey protein (Anandharamakrishnan et al., 2007; Haque et al., 2013). However, in the majority of the previous reports, the denaturation of protein was quantified by measuring the loss of protein through aggregation, which is the ultimate fate of denatured proteins. But the denaturation of protein may start far before than aggregation with the conformational perturbation under stresses. Therefore, assessing both the conformational changes and aggregation losses is required to present the complete state of denaturation of protein. By principle, the reversed-phase HPLC quantify the loss of protein through denaturation and successive aggregation (Ferreira et al., 2001; Anandharamakrishnan et al., 2008). On the other hand, Fourier Transform Infra-Red (FTIR) spectroscopy determines the denaturation of protein through assessing the conformational alteration or secondary structural alteration of protein (Fu et al., 1994; van de Weert et al., 2001). Hence, both the instruments were used in this study for assessing the denaturation of protein under the subjected stresses. The pre-information from laboratory experiments, such as the current study, would alert the manufacturers to consider the necessary measures for controlling the denaturation of protein during food processing. The current study aims at presenting a comparative depiction of heat and pressure stresses induced denaturation of WPI using HPLC and IR spectroscopy.

Materials and Methods

Materials

Whey protein isolate (WPI 895) was donated by Fonterra Cooperative, New Zealand. Other proteins such as α-Lactalbumin (α-Lac), β-lactoglobulin (β-Lg A & B), and bovine serum albumin (BSA) having electrophoretic purity ≥ 95% were purchased from Sigma-Aldrich (New South Wales, Australia) and were used as standards in the reversed-phase HPLC tests. HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich and Fisher Scientific, respectively. Deuterium oxide (D2O) was purchased from Sigma Aldrich (New South Wales, Australia). All proteins and chemicals were used as received without further purification.

Isothermal heat treatment (IHT)

A sample volume of 0.5 mL (10% WPI solution) was taken in 4 mL glass vials (3 replications) and heated at 65°C and 80°C for 600 s. The experiment was carried out in a gently stirred water bath to keep the temperature uniform and the temperature was observed by inserting a micro-thermocouple into an additional vial containing the same amount of solution. These two temperatures (65oC and 80oC) were selected for this study because this set of temperatures is a widely used practice in preheating and concentrating stages during powder milk production (Oldfield et al., 2005). This temperature range is also recommended as outlet temperature of the spray drying step of powder milk production considering the performance of dryer and the induced stress by drying process on the biomolecules including protein in milk (Haque et al., 2015).

High hydrostatic pressure treatment (HPT)

The 600 MPa hydrostatic pressure was applied on protein solution as described in our previous article (Haque et al., 2013). Briefly, the samples of 10% (w/v) aqueous WPI solutions were subjected to high pressure using Quintus Press-QFP 35L, Avure Technologies, Kent, WA, U.S.A. for 600 s. The samples (sample size of 10 mL) were vacuum sealed in adequately pressure resistant flexible polyethylene bags before compression. The pressure medium was demineralised water; the rate of pressure builds up was 100 MPa per 20 sec and required only 2 min to reach the set pressure value. When the compression was completed the pressure release was achieved within 5 sec. The recorded temperature was 22±0.3°C in the system throughout the holding time.

Determination of denaturation of WPI using reversed-phase HPLC

The details of the procedure for determining quantity of the denaturation by the Reversed-phase HPLC were

described in Haque et al. (2013) and Parris and Baginski (1991). Shortly, at first, the treated samples and a control (without subjecting any treatment) sample were collected in microtubes and diluted HCL was added to reach the pH at 4.6. After 30 min the tubes were centrifuged at 13,000 rpm (11,500 g) for 10 min. The supernatant was collected and injected onto the reversed-phase HPLC. The column Aeris WIDEPORE 3.6 XB-C18, 150×2.1mm, Phenomenex Pty. Ltd., was used to separate the denatured and undenatured protein fractions. In these tests a 0.1% (v/v) aqueous solution of trifluoroacetic acid (TFA) was used as solvent A and 0.1% TFA in 80% aqueous acetonitrile (v/v) was used as solvent B. Linear increment of solvent B, from 36% to 56% over 20 min, and 56 to 60% over 10 min, was maintained and the process was completed by lowering the solvent B from 60% to 36% in 5 min. Finally, the column was re-equilibrated for 3 min before the next run started. The temperature of the column and the flow rate of the solvent were maintained at 40°C and 0.5 mL/min, respectively. The injection volume of the sample was 40 µL. A UV-detector at wavelength of 215 nm was used in these tests. The undenatured or intact protein was calculated by Equation (1) using the sum of areas of the peaks of individual whey protein fractions (BSA, α-Lac, β-LgA and β-LgB) present in treated and control WPI.

$$\text{Undenatured (residual) protein (\%)} = \Sigma A_{treat} / \Sigma A_{control} \times 100 \quad (1)$$

where ΣA_{treat} is the sum of areas under peaks of the protein fractions of the treated sample and $\Sigma A_{control}$ is the sum of areas under peaks of the protein fractions of the control sample

Determination of denaturation of WPI using FTIR

The procedure for estimation of secondary structural alteration of WPI is described in our previous article (Haque et al., 2014). FTIR was run using Perkin Elmer Frontier FTIR instrument operated by CPU32M software. The treated and control samples were diluted in deionized water at 1% (w/v) concentration. The samples were scanned within 650 to 4000 cm^{-1} wave range using triglycine sulphate (TGS) detector. A total of 8 scans at 4 cm^{-1} resolution were accumulated at 0.2 cm/s scanning speed. The protein spectra were analysed by using Perkin Elmer's proprietary software (Version 10.05.03) and peak fit software Peak Fit version 4.12 (Sea Solve Software Inc. Framingham, USA). A 9 point second derivative analysis using the Perkin Elmer software was used to locate the peak position in the spectra. The Peak Fit Software was used for quantitative analysis of the secondary structure of the protein. The original spectra (of amide region-I, 1600-1700 cm^{-1}) without any smoothing were fitted with Gaussian shape and were analysed by local least square (LLS) algorithm. Percentage of secondary structures (α-helix, β-sheets, β-turns and random coils) was estimated using Equation 2 (Ngarize et al., 2004; Yazdanpanah & Langrish, 2013).

$$\text{Secondary structure (\%)} = A_{ind} / A_{total} \times 100 \quad (2)$$

where Aind is the sum of area of individual secondary structure in amide I band and Atotal is the sum of area of total secondary structure in amide I band.

The position or location of the bands for each secondary structure (β-sheets, α-helix and βturns) of the tested proteins was determined as suggested in the literature (Kong & Yu, 2007; Byler & Susi, 1986). The bands from 1620 to 1640 cm^{-1} and 1674 cm^{-1} to 1680 cm^{-1} were assigned to β-sheets. The bands from 1641 to 1647 cm^{-1} was assigned to random coil. The bands within 1648 to 1660 cm^{-1} were assigned to α-helix. Similarly, the bands appearing at and in the vicinity of 1663 cm^{-1}, 1671 cm^{-1}, 1683 cm^{-1}, 1688 cm^{-1}, and 1694 cm^{-1} were assigned to β turns. The peaks between 1600 cm^{-1}-1619 cm^{-1} were considered as aromatic side-chains.

Results and Discussion

Denaturation measured by reversed-phase HPLC

Denaturation loss of WPI measured by HPLC is presented in Fig. 1.

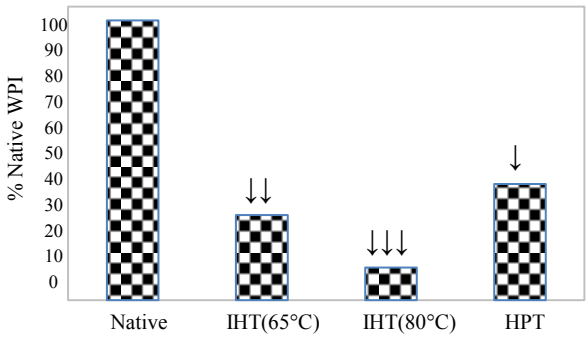

Figure 1. Denaturation of WPI during isothermal heat treatment (IHT) and high pressure treatment (HPT). Number of downward arrows are expressing the extend of denaturation of WPI under stresses. The increased number of arrows indicate the increased amount of protein is denatured.

It was found that the denaturation of WPI under IHT at 65oC and 80oC were 69.55% and 88.38%, respectively, whereas this value was 58.5% under HPT stress. It is expected that the heating at higher temperature caused more protein to denature. But, a vast amount of protein was also denatured under pressure stress, where the

temperature was constant within 22±0.3°C. The quantity of the denaturation under HPT, in the current study, confirms the previous findings of Felipe et al., (1997), where they found about 52% goat milk whey protein to denature under similar stress condition. Of course, the extent of the denaturation may vary depending on the type of protein, their inter and intra molecular linkages, and mode of pressurization (Navarro et al., 2015; Grossi et al., 2016). However, the nature of the denaturation under HPT is not well explained. The results and instrumental explanations of the reversed-phase HPLC suggest that both the treatments (IHT, HPT) for a prolonged time have resulted in irreversibly denatured protein through dissociation of the polypeptides and their further coagulation to insoluble aggregates. The denaturation product of WPI from these treatments has modified size and weight, hence, it could be separated through centrifugation and reversed-phase column. It is well known for the case of IHT that the polypeptides unfold or uncoil due to heat stress and gradually the unfolded polypeptides stick together to form aggregates. In the case of HPT, although the protein molecules do not face severe heat stress, the molecules experience exotic compressive pressure which results in breakdown of the secondary and tertiary bonds such as hydrogen bond, hydrophobic bond, and disulphide bond. The reversed phase-HPLC assay used in this study quantified the denatured protein based on the separation of aggregated proteins, however, could not assess the conformational alteration due to the perturbation of tertiary and secondary structural bonds of the polypeptides. Therefore, assessing structural conformation of the protein by FTIR spectroscopy is able to give a better explanation of HPT denaturation of WPI.

Denaturation measured by FTIR

The absorption spectra of experimental protein solutions are presented in Fig. 2.

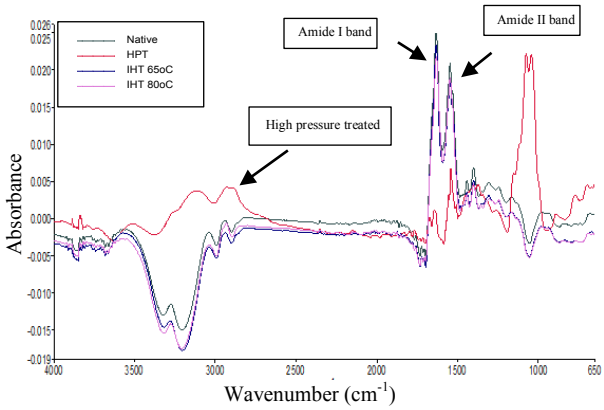

Figure 2. FTIR absorbance spectra of WPI before (Native) and after treatments.

The IHT stress caused visible alteration of the IR peaks (Fig. 3).

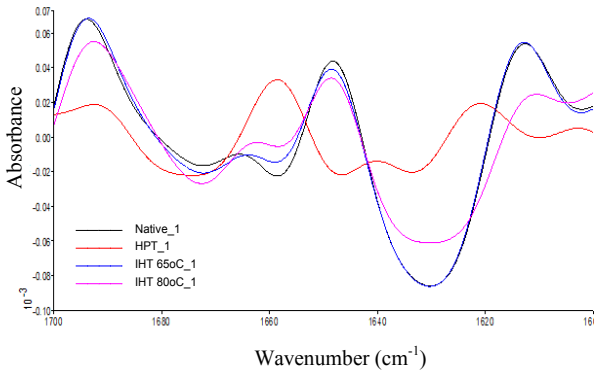

Figure 3. Nine (9) point second derivative spectra of native and treated WPI (amide region-I).

However, an exciting change was found in high-pressure treated WPI. The pressure-induced stress markedly decreased the prominent band regions for protein (Amide I, II, III; 1300-1700 cm^{-1}) and similarly increased the band region 900-1000 cm^{-1} instead. The spectral region above 2900 cm^{-1} was also visibly affected. It determines that high hydrostatic pressure on protein solution for a long time (10 min in this experiment) changed both the fingerprint region (450-1500 cm^{-1}) and functional groups of the protein.

With protein molecules, the characteristics bands were mainly produced through stretching vibration of C=O and C-N bonds and bending vibration of N-H bonds (Byler and Susi, 1986; Hastings et al., 2018). In this study, the native and IHT protein samples produced the prominent IR peaks within the band region of 1400-1700 cm^{-1} (Mosharaf, et al., 2018). However, the pressure-treated protein produced the immensely decreased bands within this region. This finding is supported by Vadim et al. (1996), where they observed that the vibration band from C=O bond (band region 1620-1680 cm^{-1}) disappeared due to high hydrostatic pressure. The mystery of the changes in pressure-treated spectrum is still not well explained. The large peak around the band region of 1000 cm^{-1} in the pressure-treated spectrum senses that a massive exchange has happened due to the relaxation of the intermolecular or intra-molecular hydrogen bonds because of the denaturation of the proteins. The prolonged shear stress might induce laxative effect and cause alteration amongst the bonds. This effect increased with increasing time of high pressure application (Fig. 4). The appearance of the enormous peaks might be attributed to the formation of a

single bonded C-O group whose characteristic band region is 1000-1100 cm^{-1} (Silverstein et al., 1981; Siwatt, 2014).

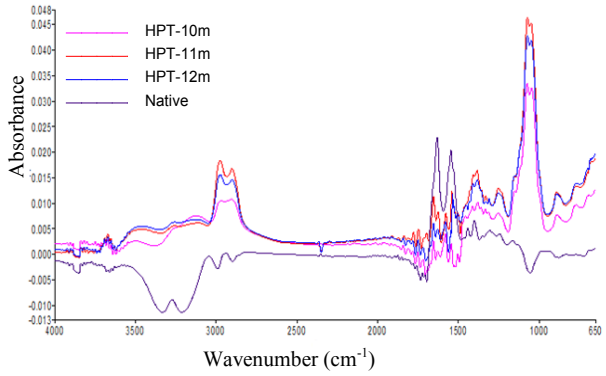

Figure 4. Changes of FTIR spectra of WPI with increased time of hydrostatic pressure treatment.

Secondary structural alteration of WPI

The secondary structural alteration was not visible from the intrinsic spectra presented in Fig. 2. Hence, the band narrowing mathematical tool, 9 points second derivative, was used to locate the changes of the peaks (Fig. 3). The peak fit software quantified the located peaks (Fig. 5). Both the native and IHT whey proteins were found to produce four quantitative peaks. However, most interestingly it was found that no definite peak erupted in the pressure treated spectrum (spectrum is not inserted). This observation suggested that the protein molecules might have completely lost the associated hydrogen bonds because of prolonged high-pressure treatment. In secondary structural estimation, the α-helix, β-sheet, and β-turn contents were decreased during IHT which were compensated by the appearance of random coil and increment of side-chains (Table 1). The IHT induced stress on WPI decreased α-helix, β-sheet, and β-turn contents by about 12.00%, 8.00%, and 4.00%, respectively which were compensated by newly appeared unordered coil 13.35% and increment of side-chain by 11.00%.

Table 1. Percentage of secondary structural features (α-helix, β-sheet etc.) of WPI before and after treatments.

Sample	α-helix	β-sheet	β-turn	random coil	side-chain
Native	21.58	46.22	21.86	--	10.34
IHT (65°C)	17.72	43.84	20.61	--	17.82
IHT (80°C)	10.00	38.29	17.30	13.35	21.06

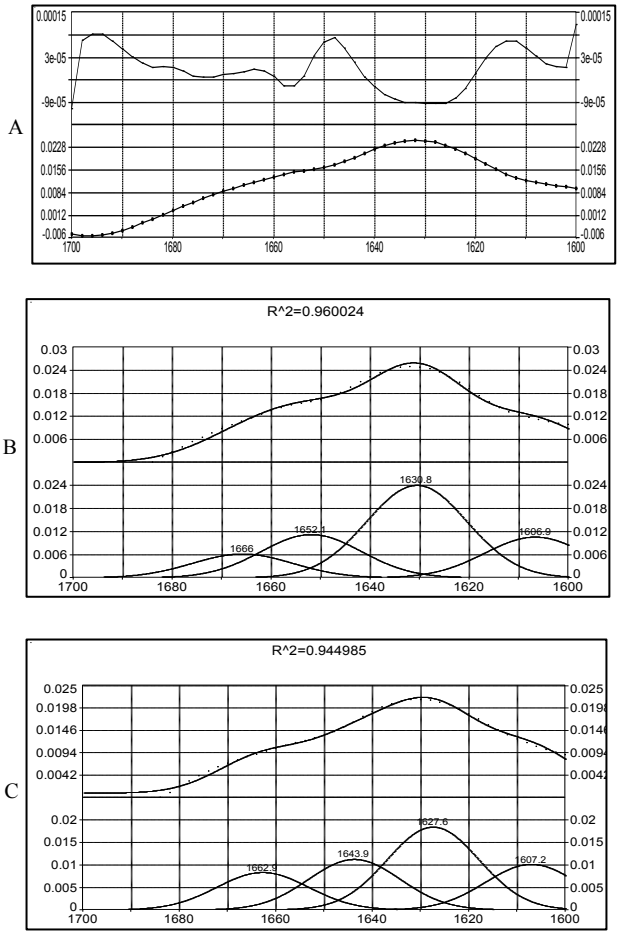

Figure 5. A) Absorbance & second derivative spectra of native WPI and fitted spectra of B) native and C) IHT (80°C) WPI.

Conclusion

The comparative assessment of WPI denaturation under IHT and HPT stresses measured by reversed-phase HPLC and FTIR which showed variation in results. As expected, the higher heating temperature resulted in the higher denaturation loss through aggregation. In HPLC quantification, it was found that about 89% WPI was lost by denaturation at 80oC whereas the HPT caused about 58% loss of native protein through the aggregation of the denatured protein. Significant alterations of the secondary structural properties were observed in the IHT stressed protein. A massive exchange among the intermolecular or intra-molecular hydrogen bonds of the protein samples was assumed due to prolonged hydrostatic pressure.

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Competing Interests

The authors declared that there is no conflict of interest.

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