

Fuoidan improves the structural integrity and the molecular stability of β -lactoglobulin

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Abstract β -lactoglobulin (β -lg) was covalently bonded with fuoidan through Maillard reaction at 60 °C for 96 h under 79% RH condition. The molecular characters of the conjugate were investigated using fourier transform infrared spectroscopy (FT-IR), atomic force microscopy (AFM), and circular dichroism (CD) spectroscopy. And, its thermal properties, surface activity, and zeta-potential were compared with intact β -lg, β -lg-fuoidan mixture, and fuoidan under different pH conditions. AFM indicated that the conjugate was nano-structured, regular spherical-shaped and generally large sized compared to β -lg-fuoidan mixture. CD spectra and FT-IR showed that tertiary structure of β -lg slightly unfolded, but little change in secondary structure occurred. This explained that glycation under Maillard condition resulted in a molten globule state of β -lg. Differential scanning calorimetry (DSC) data exhibited that fuoidan shifted the temperature of phase transition and improved thermal stability of β -lg molecule. In addition, the conjugate prominently decreased the surface tension with pH-dependency.

Keywords Maillard reaction · β -lactoglobulin · Fuoidan · Conjugation · Molten globule state

Introduction

For decades, the glycoconjugates formed through the Maillard reaction between widely various proteins and physiologically active hydrocolloids have been investigated [1]. In this reaction, the aminocarbonyl bonding forms a Schiff base, and then the loss of water from the labile Schiff base releases Amadori product [2]. This molecular modification under a mild condition without introducing major structural changes of biopolymers has to enhance the functional properties physicochemically and physiologically [3].

Fuoidan is the anionically charged and sulfated fucans extracted from brown seaweed. Fuoidan derived from *Undaria pinnatifida* has complex structures, which are differently proportioned with sugar linkages (1,3-linked fucose, and 1,3-, 1,4-, and 1,6-linked galactose) substituted with sulfates such as 2- or 4-positioned fucosyl residues and 3- or 6-positioned galactosyl residues [4]. Based on their biologically unique structures, significant interests in synthesizing glycoconjugates formulated from fuoidan and proteins have been focused for further studies as follows: the enhanced emulsifying properties [5–7] and anti-complementary activity in the classical pathway [8].

β -lactoglobulin (β -lg) is a small globular protein which consists of 162 amino acid residues ($M_w \sim 18,300$ g/mol). This protein has a sensitively but reversibly interchange between monomer and dimer depending on pH conditions, ionic strength, protein concentrations, and temperature [9]. Due to the molecular sensitivity of β -lg, glycation with mono- and disaccharides has been elaborated to increase the molecular stability of β -lg and to improve solubility and emulsifying capacity [10, 11].

However, less studied Maillard reaction using β -lg with high-molecular weight polysaccharide has been focused.

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Accordingly, the objectives of this study are to formulate the conjugate between β -Ig and fucoidan via Maillard reaction under a mild dry condition, and to investigate its molecular characteristics in various ways.

Materials and methods

Materials

β -lactoglobulin (β -Ig) was obtained from McGill University (Montreal, Quebec, Canada) and then used without a further purification after checking SDS-patterns and protein quantification. A dried powder of fucoidan extracted from the cultured Korean *U. pinnatifida sporophylls* (Miyeokkwi) was purchased from Haerim Fucoidan Co., Ltd. (Wando, Korea). All the other chemicals used were of analytical grade.

Conjugation condition

Maillard reaction was carried out according to Kim and Shin [6, 12] without a modification. Briefly, each β -Ig and fucoidan was completely dissolved in distilled water at 1% (w/v) with stirring at room temperature. In sequence, each solution was mixed at weight ratio 1:3 (approximately, 1:2.77 molar ratio) with homogeneous stirring for 1 h and then freeze dried (β -Ig-fucoidan mixture). In order to prepare the conjugate between two polymers, the individually lyophilized samples were placed in a desiccator, where the temperature and the relative humidity was controlled with saturated KBr, and then incubated at 60 °C for 96 h. Heated β -Ig and heated fucoidan were prepared by incubating native β -Ig and native fucoidan individually under the same conditions as β -Ig-fucoidan conjugate.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with 4–10 and 4–12.5% polyacrylamide gradient. The samples heated at 90 °C for 10 min in a 0.125 M Tris-HCl sample buffer (pH 6.8) with 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol. After electrophoresis, a Coomassie brilliant blue G-250 and Schiff staining was performed for protein and carbohydrate, respectively. The protein and carbohydrate bands were identified by using Xpert Prestained Protein Marker (GenDEPOT, Barker, TX, USA).

Atomic force microscopy

The morphology of β -Ig (native and heated), β -Ig-fucoidan mixture and β -Ig-fucoidan conjugate was imaged using a

5500 AFM instrument with PicoView (software ver. 1.6.4) (Agilent Tech., Santa Clara, CA, USA). Each sample was diluted to 10 ppm using distilled and deionized water, and 2 μ L of each sample was placed on a freshly cleaved mica substrate (MTI Korea, Seoul, Korea) and dried in fume hood at least 2 h prior to imaging.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were profiled using a Chirascan plus (Applied Photophysics, Leatherhead, UK) at 25 °C. Samples were prepared to 1 mg/mL of protein concentration, and the distilled water was scanned as a control. CD spectra were collected in the far-UV region (180–260 nm), and the data was expressed as mean residue ellipticity in deg cm²/dmol and analyzed by Neural Networks method using CDNN secondary structure analysis software version 2.1 (Applied Photophysics, Leatherhead, Surrey, UK) to calculate the α -helix, β -sheet, β -turn, and random coil proportions in each sample.

Fourier transform infrared spectroscopy

The IR spectra were obtained using a Nicolet iS50 FT-IR spectrometer equipped with Omnic software (version 9.2) and attenuated total reflectance (ATR) ZnSe crystal (Thermo Fisher Scientific, Madison, WI, USA). Each spectrum is the average of 64 scans at 4 cm⁻¹ resolution. Measurements were recorded between 1750 and 1450 cm⁻¹.

Differential scanning calorimetry

Thermal property was analyzed using a DSC7 instrument (Perkin Elmer Inc., Wellesley, MA, USA). The samples were weighed 10 mg accurately into stainless steel pans and 30 μ L of deionized water was added to each pan. The pans were hermetically sealed and left to stand for 2 h for hydration. After hydration, the sample pans were heated from 30 to 120 °C at a scan rate of 10 °C/min under a constant nitrogen purge. Reference pan was prepared with only 30 μ L of deionized water. The onset (To), peak (Tp), conclusion (Tc) temperature and enthalpy change (ΔH) were calculated from the thermograms using PyrisTM software.

Surface tension

The surface tension on the water surface was measured using a Sigma 703D tensiometer (KSV Instruments Ltd., Helsinki, Finland) with a duNoüy ring (ring radius: 9.545 mm) at ambient temperature. The protein stock solution was prepared in distilled and deionized water and

then diluted to the desire concentration (0–1 mg/mL of protein) at pH 3.0, 5.0, and 7.0 adjusted with phosphate buffer. Prior to the measurement, the apparatus was calibrated with distilled water to get a baseline.

Zeta-potential measurements

The charge distribution of β -lg and fucoidan-conjugated β -lg was measured by electrophoretic light scattering method using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK). Each sample was dissolved in distilled and deionized water, and its charge distribution in different pH conditions was measured in triplicates.

Statistical analysis

The collected data were subjected to one-way ANOVA ($p < 0.05$) using SPSS software version 21 (SPSS Inc. Chicago, IL, USA). Significant differences were determined using Duncan comparison procedure at $p < 0.05$.

Results and discussion

SDS-PAGE of β -lg-fucoidan conjugates

SDS-PAGE patterns can confirm whether proteins were conjugated with polysaccharides through the reactions. Figure 1 showed that the band of native β -lg appeared at approximately 17 and 35 kDa as monomer and dimer, respectively. Meanwhile, only β -lg and fucoidan showed no band in the Schiff stained gel and protein stained gel, respectively. Otherwise, the fucoidan-conjugated β -lg exhibited a weak protein band, but no band was observed in β -lg-fucoidan mixture. In the Schiff stained gel, the mixture showed a weakly stained band at high molecule region, but conjugate showed stain at 43–56 kDa [Fig. 1A]. Figure 1B exhibited conjugate has more smeared and stained at high molecular weight bands than the mixture and fucoidan. The combined presence of the polydispersed protein and carbohydrate band, near the top of the running gels, provided further evidence for covalent bonding between the free amino groups at the position of lysine residues on β -lg and the reducing ends of fucoidan.

Morphology of β -lg-fucoidan conjugates particle

The morphology of the conjugates was observed by atomic force microscopy images as shown in Fig. 2. Native β -lg was mostly observed to small globules of uniform size, and heated β -lg showed that the numbers of particles decrease but the size of particles increased. Meanwhile, particles of β -lg-fucoidan mixture have irregular shapes and sizes, but

the conjugated molecules were regular spherical in shape and the particles were larger than molecular mixture. This result might be because the interaction between β -lg and fucoidan was weaker in mixture than in conjugate, resulting in polydispersed distribution.

Likely, whey protein isolate and maltodextrin glycosylated at high temperature showed increased particle size [13]. Zhang et al. [14] reported that β -conglycinin-dextran conjugate has larger size than heated β -conglycinin and mixture, and Jones et al. [15] reported that β -lg with pectin, after heat treatment, exhibited large particles. Kim and Shin [12] reported an increase in size for mixture and conjugate, compared to native protein, conjugate having the biggest particle size. This indicates that conjugate is a higher molecular weight polymer.

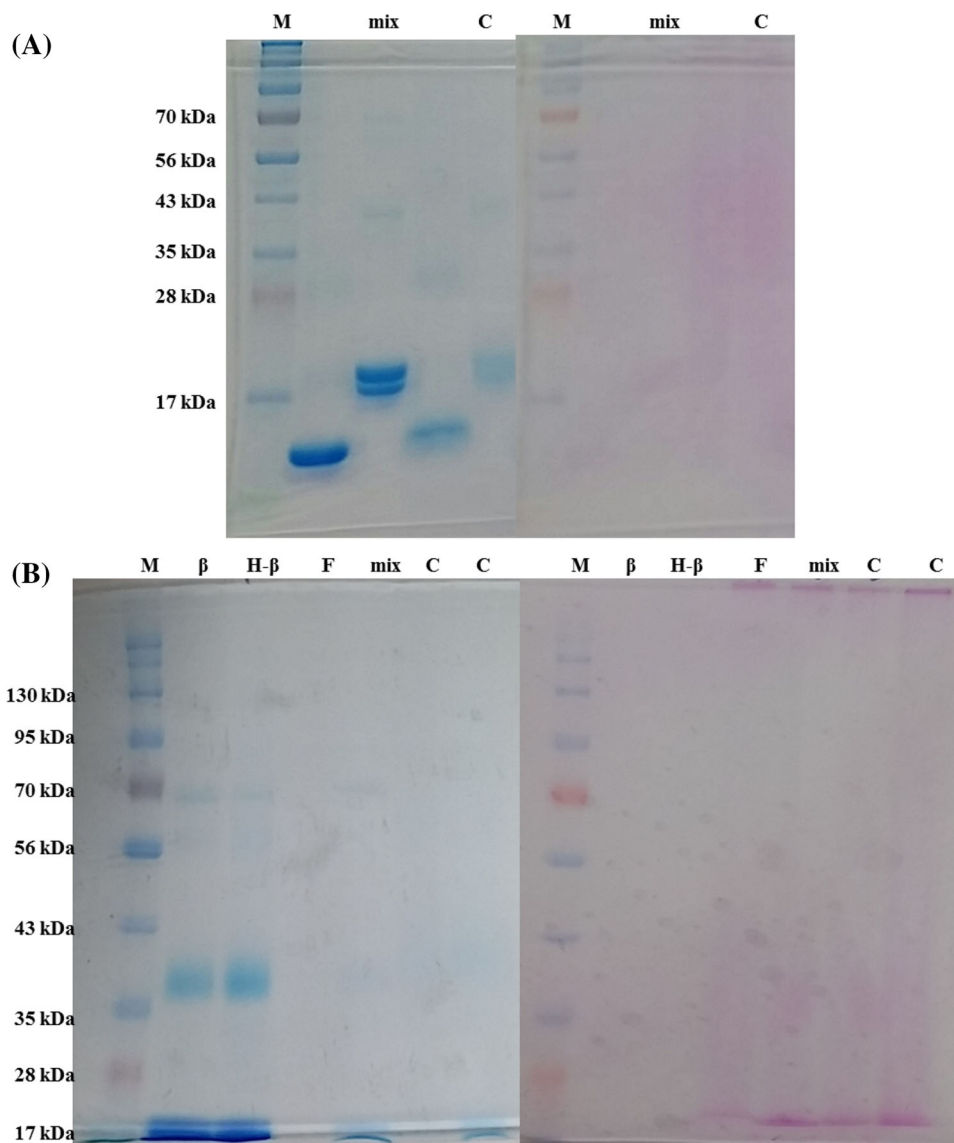
Structural characteristic of β -lg-fucoidan conjugates

Figure 3A showed the CD spectra of secondary structure in the far-UV region (180–260 nm). CD is an excellent method for rapidly evaluating the secondary structure, folding and binding properties of proteins, absorbing unequal light such as left-handed and right-handed circularly polarized light [16]. The far-UV CD signal occurred from absorption of peptide bonds and this indicated the protein had secondary structure, which can be obtained by dividing the concentration (mass terms, mg/mL) by the mean residue weight [17]. Native β -lg had a negative maximum at 216 nm since β -lg was rich in β -sheet. In this study, native β -lg has 13% of α -helix and 38.8% of β -sheet structure, which is similar to α -helix but slightly different from β -sheet reported by Chevalier et al. [18].

Compared with native β -lg, heated β -lg and conjugate exhibited slightly increased β -sheet and slightly decreased random coil, respectively. It could be caused by the exposed internal sites of tertiary protein structure. Heat treatment of β -lg under a mild condition induced its partial unfolding and more β -lg hydrophobic residues could be exposed leading to changing the surface charges and hydrophobicity. Ngarize et al. [19] reported that β -lg heated at 90 °C for 30 min showed increased β -sheet, but our finding was that mild heating of β -lg at 60 °C induced no change in secondary structure.

In sequence, the protein-carbohydrate interaction after Maillard reaction was measured using FT-IR spectroscopy, which has been well-known as a particularly useful technique to detect molecular vibration for the study of protein-carbohydrate systems [20, 21], providing information about the molecular structures and chemical composition [22]. The amide I band depends on protein secondary structure, caused primarily by the stretching of the C=O bonds, and amide II caused by the deformation of N–H bonds and C–N bonds stretching [22]. The amide I and amide II regions

Fig. 1 SDS-PAGE patterns of native β -lg (β), heated β -lg (H- β), fucoidan (F), β -lg-fucoidan mixture (mix) and β -lg-fucoidan conjugate (C) in the weight ratio 1:3 incubated at 60 °C for 96 h. (A) was 4–10% polyacrylamide gradient and (B) was 4–12.5% polyacrylamide gradient



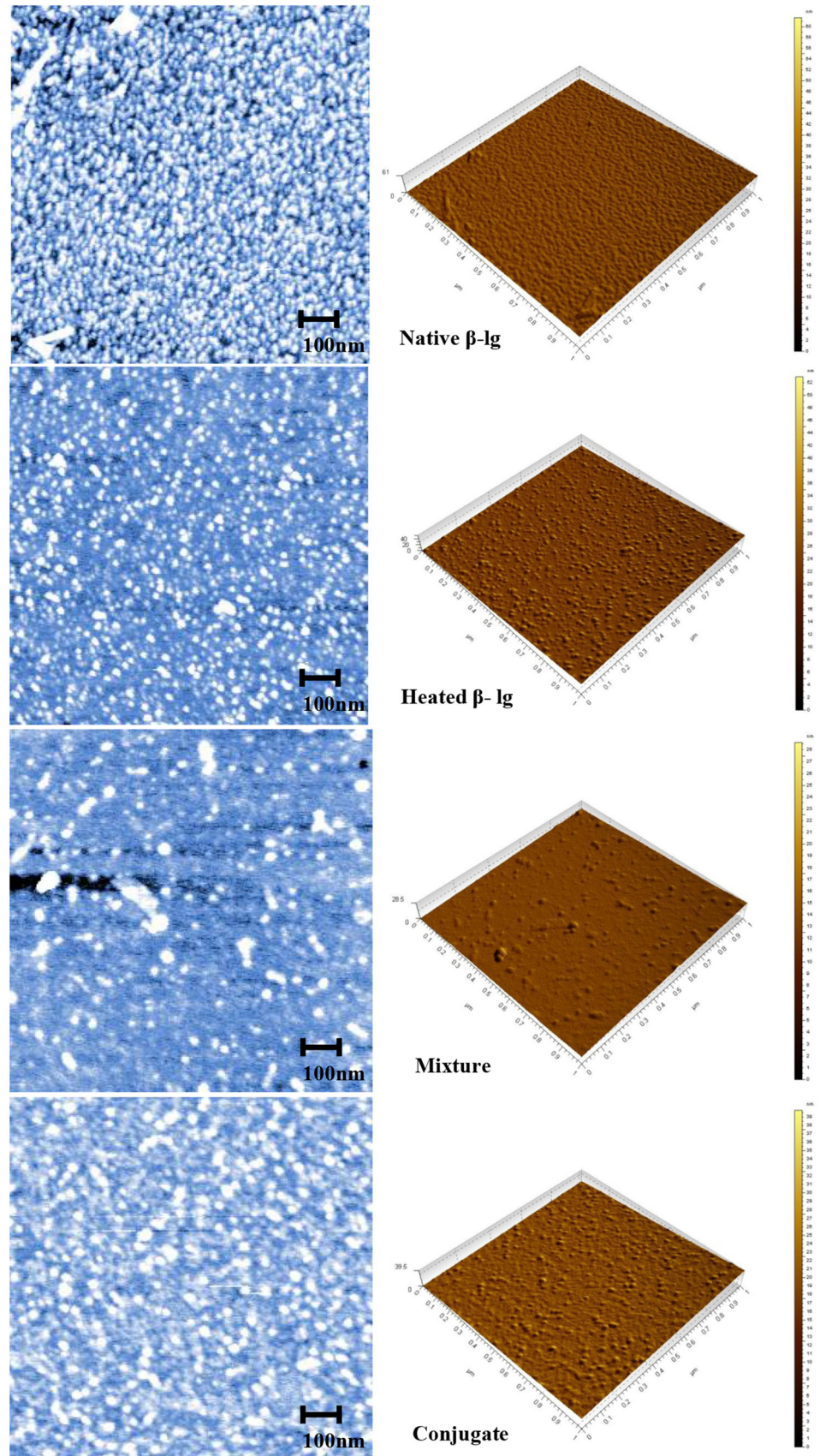
ranged from 1700 to 1600 cm^{-1} and from 1600 to 1500 cm^{-1} , respectively [23]. Figure 3B exhibited FT-IR spectra of the native β -lg, heated β -lg, β -lg-fucoidan mixture, and β -lg-fucoidan conjugate in the infrared amide I and II region (1750–1450 cm^{-1}). After heating, the intensities of the peaks at amide I and II increased in heated β -lg and β -lg-fucoidan conjugate more than native β -lg and β -lg-fucoidan mixture, respectively. And the shift of the peaks from 1629 to 1628 cm^{-1} , and from 1514 to 1519 and 1525 cm^{-1} , were observed in β -lg. In the mixture and conjugate, the amide I peak at 1631 cm^{-1} did not shift, but the amide II peak shifted from 1538 to 1542 cm^{-1} . Liu et al. [21] observed an increase in the peaks of amide I and II during the heating. The changes in FT-IR spectra indicate the exposure of the secondary structure of β -lg after heating.

Compare to CD spectra, no change or increase in the secondary structure was observed, otherwise the tertiary structure of β -lg was slightly unfolded, exposing an internal part of its secondary structure under mild heating condition at 60 °C and 79% relative humidity. Therefore, it was conferred that β -lg was transformed into molten globule state during the Maillard reaction.

Thermal analysis of β -lg-fucoidan conjugates

DSC is widely used to investigate the changes in protein conformation occurring during heat treatment. In DSC thermogram, protein unfolding is an endothermic reaction, whereas aggregation and precipitation indicate an exothermic reaction. The changes in endothermic heat flow and thermodynamic parameter of each polymer (e.g. native β -lg, heated β -lg, two polymer's mixture, two polymer's

Fig. 2 Atomic force microscopy image ($1.0 \times 1.0 \mu\text{m}$) of native β -lg, heated β -lg, β -lg-fucoidan mixture, and β -lg-fucoidan conjugate



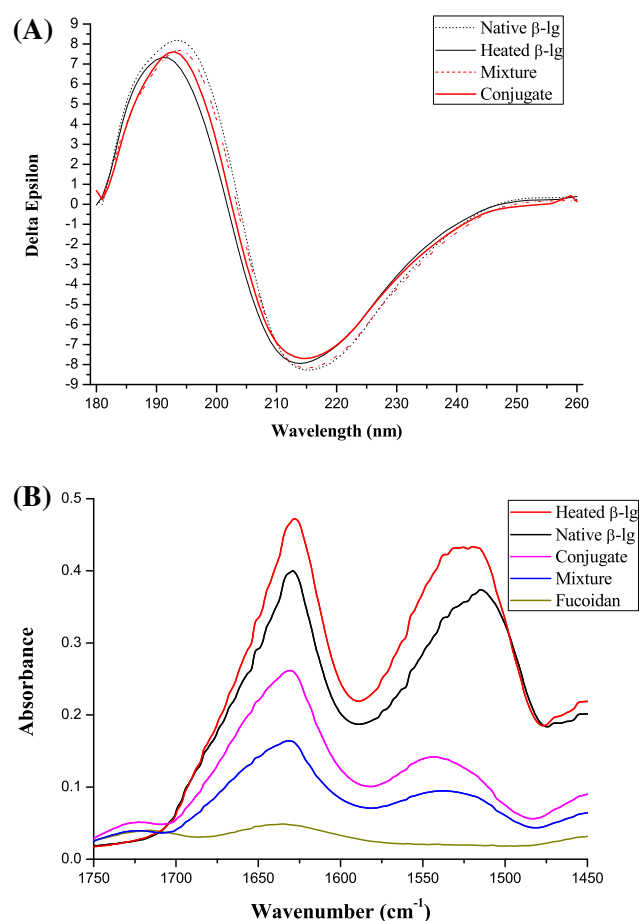


Fig. 3 Circular dichroism spectra (A) and Infrared spectra (B) of native β -Ig, heated β -Ig, β -Ig-fucoidan mixture, and β -Ig-fucoidan conjugate

conjugate, native fucoidan, and heated fucoidan) are presented in Table 1.

Native β -Ig exhibited a single endothermic transition at the peak temperature (T_p) of 75.93 ± 0.58 °C. This is in a good agreement with Boye and Alli [25] reporting a single peak transition of β -Ig. Heated β -Ig absorbed the heat the most at 77.75 ± 0.01 °C which is thermal transition point as endothermic peak (T_p). This means the transition peak

was similar to that of the native β -Ig, indicating no significant difference in the peak temperature, onset temperature (T_o), and conclusion temperature (T_c), whereas the enthalpy value (ΔH) for the heated β -Ig was lower than native β -Ig. Kim et al. [6] reported the thermodynamic parameter of T_p and ΔH directly related to thermal stability of the protein, and consequently the content of the ordered structure of the protein has high thermal transition temperature and the amount of energy absorption. Thus, the changes in molecular integrity of protein have an impact on the state of protein conformations [26].

Taken together, the data on thermal transition suggest that heated β -Ig exists in molten globule state, corresponding to the results of CD and FT-IR analyses. The partial denaturation process has been widely investigated in BSA [27], apomyoglobin [28] and α -lactalbumin [29].

Unlike protein, native fucoidan had the lowest peak temperature (T_p) and ΔH value of 56.68 ± 3.18 °C and 0.16 ± 0.00 J/g, respectively. On the other hand, Kim and Shin [12] reported that fucoidan has a single peak with high temperature at 97.54 °C. After the heating, they were increased to T_p of 89.43 ± 4.33 °C and 0.50 ± 0.39 J/g, respectively. Yoshimura et al. [30] reported that xyloglucan did not have a peak but when mixed with corn starch it showed T_p at 66 – 68 °C. The observed temperatures of onset, peak, and conclusion were shifted to higher temperature for β -Ig-fucoidan mixture or β -Ig-fucoidan conjugate. Generally, a high T_p value is associated with higher thermal stability; therefore, the β -Ig with fucoidan obtains an increased thermal stability and subsequently becomes highly resistant to denaturation. Jones et al. [31] reported the mixture of β -Ig with hydrocolloids shifted the T_p , and this is in good agreement with the thermal profiles of fucoidan-complexed β -Ig in our study. However, interestingly, the conjugate had a lower ΔH value than the mixture. Ibanoglu [32] explained that since the partial denaturation of the protein surrounded or shielded with highly charged hydrocolloids occurred prior to the heat treatment, less energy was required for protein denaturation. Liu [24]

Table 1 Thermal properties of native β -Ig, heated β -Ig, β -Ig-fucoidan mixture, β -Ig-fucoidan conjugate, native fucoidan, and heated fucoidan by DSC

Sample	Temperature (°C)			ΔH (J/g)
	T_o	T_p	T_c	
Native β -Ig	69.81 ± 0.60^c	75.93 ± 0.58^b	83.44 ± 0.79^c	9.42 ± 1.35^a
Heated β -Ig	70.35 ± 0.15^c	77.75 ± 0.01^b	84.22 ± 0.18^c	5.03 ± 0.58^b
β -Ig-fucoidan mixture	85.24 ± 0.17^a	89.59 ± 0.00^a	94.16 ± 0.19^b	2.73 ± 0.10^c
β -Ig-fucoidan conjugate	81.74 ± 2.20^b	90.81 ± 1.92^a	100.57 ± 0.68^a	1.26 ± 0.24^d
Native fucoidan	54.57 ± 1.82^d	56.68 ± 3.18^c	57.99 ± 4.45^d	0.16 ± 0.00^d
Heated fucoidan	86.45 ± 2.97^a	89.43 ± 4.33^a	91.87 ± 1.84^b	0.50 ± 0.39^d

Different superscript letters within rows represent a significant difference ($p < 0.05$) by Duncan comparison procedure ($n = 3$)

suggested that the change in ΔH might be due to the nature of the protein covalently linked with polysaccharides.

Surface tension of β -lg-fucoidan conjugates

The effects of various concentrations (from 0 to 1 mg/mL of protein) of native β -lg, heated β -lg, β -lg-fucoidan mixture, β -lg-fucoidan conjugate, native fucoidan, and heated fucoidan at distilled water and pH 3.0, 5.0, and 7.0 on surface properties are presented in Fig. 4. The results showed that surface tension depended on the protein concentration and pH environment. Native β -lg decreased the surface tension from 69 to 57 mN/m with increasing protein concentration at pH 3. Sakurai et al. [33] reported that most of protein molecules are in their “close” state at pH 3.0. The ability of a protein to reduce surface tension depends on conformational changes that occur as the protein is adsorbed. Heated β -lg was in a partially denatured state, i.e. molten globule-like state, which provides a more flexible conformation that can be easily adsorbed to the surface [34]. On the other hand, surface tension values of β -lg-fucoidan mixture were little higher than those of protein under acidic condition. At pH5, native and heated β -lg did not lower the surface tension (Fig. 4), suggesting low solubility or partial aggregation of protein in near pI solution ($pI = 5.18$). However, since anion-charged fucoidan redeemed the surface charges of β -lg, the mixture and the conjugate could lower the surface tension at pH 5. Interestingly, partially denatured β -lg (heat-treated) lowered the surface tension than the native, suggesting the changes in the surface charge distribution through a partial conformational change. At a neutral pH, both native and heated β -lg decreased the surface tension with increasing protein concentration and β -lg-fucoidan conjugate lowered the surface tension most. This can be explained by the dominant anionic action of fucoidan molecules to the surface charge distribution. However, little change in the surface tension was shown in both the native and heated fucoidan under different pH circumstances, confirmed that the anionic fucoidan contribute the charge effects but no surface-active properties at any concentration and pH. In addition, the protein-polysaccharide conjugate was more surface-active than was the polysaccharide itself.

Zeta-potential of β -lg-fucoidan conjugates

All the sample solutions had negative zeta-potential value (Table 2). Heat treatment caused an increase in the negative zeta-potential of the proteins unfolded and aggregated [35]. Native β -lg had a charge of -19.38 ± 1.71 mV but heating decreased the potential into -29.34 ± 4.22 mV, suggesting that a partial denaturation caused the conformational change and then this

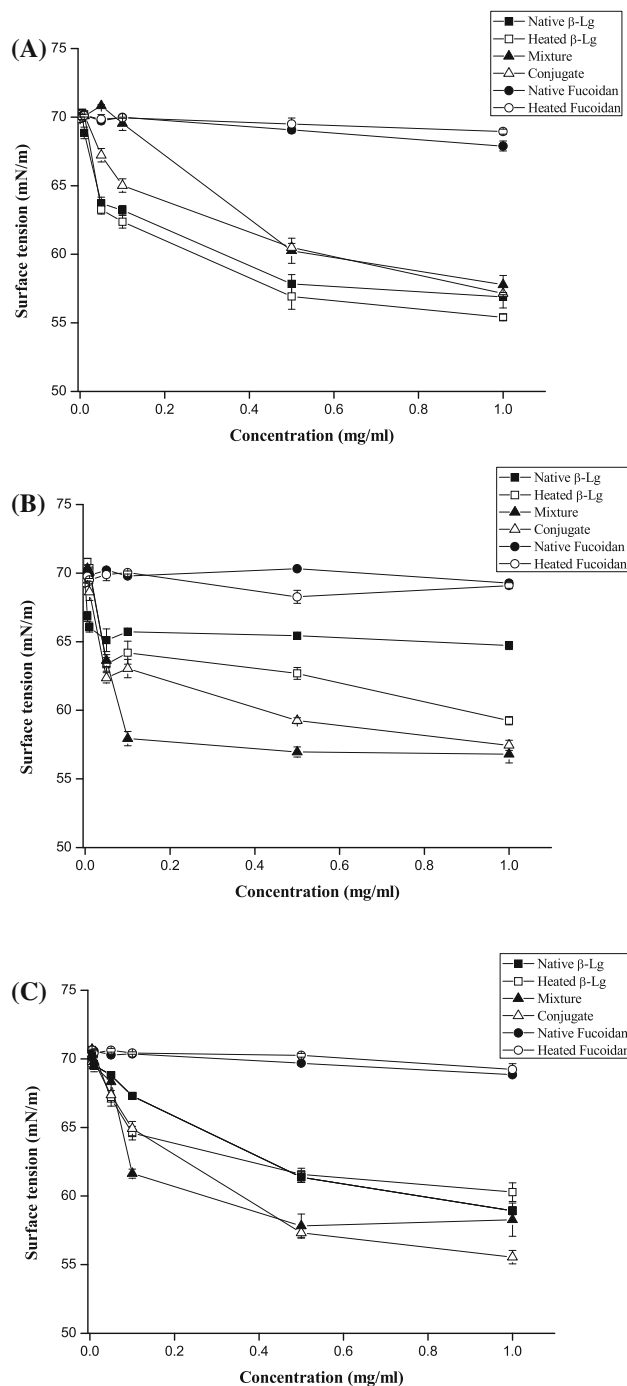


Fig. 4 Changes in surface tension in response to native β -lg (filled square), heated β -lg (open square), β -lg-fucoidan mixture (filled triangle), β -lg-fucoidan conjugate (open triangle), native fucoidan (filled circle), and heated fucoidan (open circle) as a function of protein concentration (0, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 mg/mL) at pH 3 (A), pH 5 (B), and pH 7 (C)

affected the surface charge distribution. The conjugate revealed the lowered zeta-potential of the native β -lg (-19.38 ± 1.71 to -23.17 ± 1.28 mV), but there was no significant difference in the mixture. And, addition of fucoidan into the protein (both the mixture and the

Table 2 Z-potential and pH value of native β -Ig, heated β -Ig, β -Ig-fucoidan mixture, β -Ig-fucoidan conjugate, native fucoidan, and heated fucoidan

Sample	Z-potential (mV)	pH
Native β -Ig	-19.38 ± 1.71^a	6.92 ± 0.01^a
Heated β -Ig	-29.34 ± 4.22^c	6.80 ± 0.01^b
β -Ig-fucoidan mixture	-22.27 ± 0.81^b	5.94 ± 0.02^c
β -Ig-fucoidan conjugate	-23.17 ± 1.28^b	5.82 ± 0.04^d
Native fucoidan	-23.04 ± 0.93^b	4.64 ± 0.00^e
Heated fucoidan	-18.74 ± 1.30^a	4.51 ± 0.01^f

Different superscript letters within rows represent a significant difference ($p < 0.05$) by Duncan comparison procedure (Zeta-potential, $n = 9$; pH, $n = 3$)

conjugate) slightly lowered the solution pH to the acidic environment.

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References

- Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME. Essentials of glycobiology. Cold Spring Harbor Laboratory Press, NY, USA. pp. 115–127 (2009)
- Horvat Š, Jakas A. Peptide and amino acid glycation: new insights into the Maillard reaction. *J. Pept. Sci.* 10: 119–137 (2004)
- Li Y, Lu F, Luo C, Chen Z, Mao J, Shoemaker C, Zhong F. Functional properties of the Maillard reaction products of rice protein with sugar. *Food Chem.* 117: 69–74 (2009)
- Hemmingson JA, Falshaw R, Furneaux R, Thompson K. Structure and Antiviral Activity of the Galactofucan Sulfates Extracted from *UndariaPinnatifida* (Phaeophyta). *J. Appl. Phycol.* 18: 185–193 (2006)
- Kim D-Y, Shin W-S. Roles of fucoidan, an anionic sulfated polysaccharide on BSA-stabilized oil-in-water emulsion. *Macromol Res.* 17: 128–132 (2009)
- Kim D-Y, Shin W-S. Functional improvements in bovine serum albumin–fucoidan conjugate through the Maillard reaction. *Food Chem.* 190: 974–981 (2016)
- Kim D-Y, Shin W-S, Hong W-S. The unique behaviors of biopolymers, BSA and fucoidan, in a model emulsion system under different pH circumstances. *Macromol Res.* 18: 1103–1108 (2010)
- Tissot B, Montdargent B, Chevolut L, Varenne A, Descroix S, Gareil P, Daniel R. Interaction of fucoidan with the proteins of the complement classical pathway. *Biochim. Biophys. Acta, Proteins Proteomics.* 1651: 5–16 (2003)
- Roefs P, de Kruif KG. Association behavior of native β -lactoglobulin. *Biopolymers.* 49: 11–20 (1999)
- Morgan F, Léonil J, Mollé D, Bouhallab S. Modification of bovine β -lactoglobulin by glycation in a powdered state or in an aqueous solution: effect on association behavior and protein conformation. *J. Agric. Food Chem.* 47: 83–91 (1999)
- Rada-Mendoza M, Villamiel M, Molina E, Olano A. Effects of heat treatment and high pressure on the subsequent lactosylation of β -lactoglobulin. *Food Chem.* 99: 651–655 (2006)
- Kim D-Y, Shin W-S. Characterisation of bovine serum albumin–fucoidan conjugates prepared via the Maillard reaction. *Food Chem.* 173: 1–6 (2015)
- Liu G, Zhong Q. Thermal aggregation properties of whey protein glycosylated with various saccharides. *Food Hydrocoll.* 32: 87–96 (2013)
- Zhang X, Qi J-R, Li K-K, Yin S-W, Wang J-M, Zhu J-H, Yang X-Q. Characterization of soy β -conglycinin–dextran conjugate prepared by Maillard reaction in crowded liquid system. *Food Res Int.* 49: 648–654 (2012)
- Jones OG, Lesmes U, Dubin P, McClements DJ. Effect of polysaccharide charge on formation and properties of biopolymer nanoparticles created by heat treatment of β -lactoglobulin–pectin complexes. *Food Hydrocoll.* 24: 374–383 (2010)
- Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc.* 1: 2876–2890 (2006)
- Kelly SM, Price NC. The use of circular dichroism in the investigation of protein structure and function. *Curr Protein Pept Sci.* 1: 349–384 (2000)
- Chevalier F, Chobert J, Dalgalarondo M, Choiset Y, Haertle T. Maillard glycation of β -lactoglobulin induces conformation changes. *Nahrung.* 46: 58–63 (2002)
- Ngarize S, Herman H, Adams A, Howell N. Comparison of changes in the secondary structure of unheated, heated, and high-pressure-treated β -lactoglobulin and ovalbumin proteins using Fourier transform Raman spectroscopy and self-deconvolution. *J. Agric. Food Chem.* 52: 6470–6477 (2004)
- Cooper E, Knutson K. Fourier transform infrared spectroscopy investigations of protein structure. *Pharm Biotechnol.* 7: 101–143 (1995)
- Liu Q, Kong B, Han J, Sun C, Li P. Structure and antioxidant activity of whey protein isolate conjugated with glucose via the Maillard reaction under dry-heating conditions. *Food Struct.* 1: 145–154 (2014)
- van der Ven C, Muresan S, Gruppen H, de Bont DB, Merck KB, Voragen AG. FTIR spectra of whey and casein hydrolysates in relation to their functional properties. *J. Agric. Food Chem.* 50: 6943–6950 (2002)
- Curley DM, Kumosinski TF, Unruh JJ, Farrell HM. Changes in the Secondary Structure of Bovine Casein by Fourier Transform Infrared Spectroscopy: Effects of Calcium and Temperature. *J. Dairy Sci.* 81: 3154–3162 (1998)
- Liu Y, Zhao G, Zhao M, Ren J, Yang B. Improvement of functional properties of peanut protein isolate by conjugation with dextran through Maillard reaction. *Food Chem.* 131: 901–906 (2012)
- Boye J, Alli I. Thermal denaturation of mixtures of α -lactalbumin and β -lactoglobulin: a differential scanning calorimetric study. *Food Res Int.* 33: 673–682 (2000)
- Choi S-M, Mine Y, Ma C-Y. Characterization of heat-induced aggregates of globulin from common buckwheat (*Fagopyrum esculentum* Moench). *Int J Biol Macromol.* 39: 201–209 (2006)
- Shin W-S, Hirose M. Thiol-dependent gelation of the domain I-truncated fragment of bovine serum albumin. *Biosci Biotechnol Biochem.* 59: 817–821 (1995)
- Nishii I, Kataoka M, Tokunaga F, Goto Y. Cold denaturation of the molten globule states of apomyoglobin and a profile for protein folding. *Biochemistry.* 33: 4903–4909 (1994)
- Xie D, Bhakuni V, Freire E. Calorimetric determination of the energetics of the molten globule intermediate in protein folding: apo- α -lactalbumin. *Biochemistry.* 30: 10673–10678 (1991)
- Yoshimura M, Takaya T, Nishinari K. Effects of xyloglucan on the gelatinization and retrogradation of corn starch as studied by

- rheology and differential scanning calorimetry. *Food Hydrocoll.* 13: 101–111 (1999)
31. Jones O, Decker EA, McClements DJ. Thermal analysis of β -lactoglobulin complexes with pectins or carrageenan for production of stable biopolymer particles. *Food Hydrocoll.* 24: 239–248 (2010)
 32. Ibanoglu E. Effect of hydrocolloids on the thermal denaturation of proteins. *Food Chem.* 90: 621–626 (2005)
 33. Sakurai K, Konuma T, Yagi M, Goto Y. Structural dynamics and folding of β -lactoglobulin probed by heteronuclear NMR. *Biochim. Biophys. Acta, Gen. Subj.* 1790: 527–537 (2009)
 34. Mezzenga R, Fischer P. The self-assembly, aggregation and phase transitions of food protein systems in one, two and three dimensions. *Rep. Prog. Phys.* 76: 046601 (2013)
 35. Kehoe J, Foegeding E. The characteristics of heat-induced aggregates formed by mixtures of β -lactoglobulin and β -casein. *Food Hydrocoll.* 39: 264–271 (2014)