



Short communication

Insight into the effect of glycerol on stability of globular proteins in high protein model system

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ABSTRACT

A model system was established to investigate the effect of glycerol on stability of globular proteins. To study whether protective effect of glycerol is dependent on the nature of protein, four kinds of globular protein were used including α -lactalbumin, β -lactoglobulin, native lactoferrin and lysozyme. Differential scanning calorimetry (DSC) was used to study the thermal stability of the proteins with glycerol aqueous (0–80% v/v). The results showed that the stability of β -lactoglobulin and lysozyme (hydrophobicity-dominated proteins) increased with glycerol concentration. However, that of α -lactalbumin and lactoferrin (metalloproteins), regardless of the ion-binding state, initially increased and then decreased. Different protective effects were observed beyond 50% (v/v) glycerol concentrations in liquid phase. The secondary structure and tryptophan exposure remained unchanged. In conclusion, the protection of glycerol on protein stability was highly dependent on the nature of proteins.

1. Introduction

High protein snack bars are becoming popular with consumers demanding special diet, particularly those engaged in bodybuilding. The textural hardening of high protein snack bars during storage is the main deterioration effect upon the sensory quality and acceptability. Dairy proteins are major protein resources used in high protein snack bars (Lu et al., 2016). Protein type predominates the hardness intrinsically. In a protein/solvent binary model system, the hardness of the model bars was dependent on the species of protein, i.e. sodium caseinate > α -lactalbumin $\approx$ whey protein isolate > β -lactalbumin (Hogan, Chaurin, O'Kennedy, & Kelly, 2012). The texture hardening of high protein bar is significantly attributed to the aggregation of protein (Zhou, Liu, & Labuza, 2008a, Zhou, Liu, & Labuza, 2008b). The protein aggregation involves the intermolecular interactions, which initiates from the destabilization of protein, in particular, of globular protein.

The stability of protein relies on its structural variety to a great extent. The primary structure of the protein naturally determines its native three-dimensional structure (Pechkova, Sivozhelezov, & Nicolini, 2007). Protein stability refers to its ability to resist adverse influences on tertiary and quaternary structures. The steric structure of protein greatly depends on the non-covalent intramolecular interactions among

side-chains of amino acid residues, including hydrogen bonds, hydrophobic interactions, electrostatic interactions and van der Waals' forces (Fágán, 1995; Pucci & Rooman, 2017). Compactness is used to estimate the protein stability, which is subjected to the density of the hydrophobic cores, the side-chains distribution at the hydrophilic exterior and overall electrostatic interactions (Argos et al., 1979; Pechkova et al., 2007; Liu & Bolen, 1995). The ability to maintain the compactness is equivalent to the energy threshold for conformational changes, which varies from protein to protein, resulting in the differences in protein stability (Chiti et al., 1998).

Polyols, such as glycerol and sorbitol, are commonly used to alleviate the hardening because they are not only excellent humectants but also can enhance the stability of protein (Liu, Peng, Tran, & Labuza, 2009). In the presence of polyols, the fluctuation of protein molecules decreases (Pazhang, Mehrnejad, Pazhang, Falahati, & Chaparzadeh, 2016). The protective mechanism of polyols on protein still remains debatable. Preferential hydration has been the dominated mechanism to elucidate this protecting effect. Specifically, the exclusion of glycerol from protein surface leads to the favorable interaction between the water and protein molecules. (Timasheff, 1993; Timasheff, 2002). Liu, Ji, Zhang, Dong, and Sun (2010) reported that glycerol showed little hydrogen bonding with protein using molecular dynamic simulation

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which is in favor of the preferential hydration which emphasizes a non-contact protective effect of polyol. However, Kamiyama, Sadahide, Nogusa, and Gekko (1999) reported that direct interaction between glycerol and protein helped stabilize the proteins in a molten globe state due to preferential solvent interactions including hydrophobic and electrostatic interaction. Vagenende et al. (2009a,b) using molecular dynamics reported that the significant electrostatic and hydrophobic interactions of specific side chains on the surface of protein with glycerol led to the formation of an amphiphilic surface. The amphiphilic surface prevented the protein from undergoing conformational changes and intermolecular aggregations. Moreover, electrodynamic force was proposed to be responsible for the changes in protein stability due to cosolvents including glycerol (Damodaran, 2012; Damodaran, 2013).

All above mentioned studies focused on the alterations of the solvent environment suggesting a non-specific protective effect (Davis-Searles, Saunders, Erie, Winzor, & Pielak, 2001). The purpose of this study is to provide the further understanding of the effects of glycerol on the stability of globular proteins in high protein model system. Globular protein with different native structures were used including α -lactalbumin, β -lactoglobulin, lactoferrin and lysozyme. Lactoferrin and α -lactalbumin are ion-binding proteins but with distinct ion binding mechanism. Lysozyme and β -lactoglobulin are non-ion-binding proteins with former in a less compact folded state. Furthermore, α -lactalbumin and lysozyme have highly similar secondary and tertiary structures other than the ion-binding property. The most direct technique to study the thermodynamic behavior of proteins is differential scanning calorimetry (DSC). Thus, thermal denaturation detected by DSC was used to monitor the protein stability. In addition, confocal Raman spectroscopy was used to detect the structure of protein in the presence of glycerol.

2. Materials and methods

2.1. Materials

Alpha-lactalbumin (JE-022-6-414) and beta-lactoglobulin (Bio Pure, JE-003-6-922) powders were kindly provided by Davisco Foods International, Inc. (Minnesota, MN, USA). Lactoferrin powder (Tatura-Bio Lactoferrin Powder 500201) was obtained from Tatura Milk Industries (Tatura, Victoria, Australia). Lysozyme (62971) was purchased from Sigma-Aldrich (St. Louis, USA). Glycerol (PubChem CID: 753) was purchased from Alfa Aesar (Thermo Fisher Scientific Co., Shanghai, China).

2.2. Sample preparation

Glycerol was mixed with double distilled water at concentrations ranging from 0 to 80% (v/v). The weight of both glycerol and water were recorded to calculate the molar fraction. The density of glycerol and water used were 1.26 and 1.00 g·m⁻³ at room temperature (20 °C, David, 2004), respectively. All the experiments were done at room temperature. Glycerol solution and protein powder with a mass ratio of 1:1 were mixed and equilibrated at room temperature for 12 h. The appearance of samples were recorded by digital camera (DS100, Nikon, Tokyo, Japan). Each sample was prepared in duplicates.

2.3. Confocal Raman spectroscopy

Raman spectra were recorded using a confocal Raman microscopy (LabRAM HR Evolution, HORIBA Jobin Yvon SAS, Lille, France) equipped with a nitrogen-cooled detector. The samples were observed at 50 times magnification under a reflective mode and the regions of spectra collection were snapshoted. The spectra were obtained under excitation wavelength of 532 nm, within the range of 400–2000 cm⁻¹. The spectral data were baseline-corrected and normalized using LabSpec 6 (HORIBA Jobin Yvon SAS, Lille, France). Additionally, the

Raman intensity was normalized against the phenylalanine band at (1003 cm⁻¹). Quantitative estimation of secondary structure was performed based on amide I (1645–1700 cm⁻¹) using Peakfit 4.12 (Seasolve Software, Framingham, MA, USA).

2.4. Differential scanning calorimetry

DSC (Polyma, Netzsch, Selb, Germany and DSC1, Mettler Toledo, Columbus, OH, USA) was used to monitor the thermal stability of protein in the 0–80% glycerol solutions. The temperature and heat flow were calibrated with indium. Samples were heated from 25 to 95 °C at 5 °C/min under a nitrogen atmosphere. The heat flow of samples were normalized by the weight of protein and the peak integrated using Proteus Analysis (Netzsch) and STARE Thermal Analysis Software (Mettler Toledo) to calculate the enthalpy changes during denaturation. Duplicate samples were measured.

2.5. Statistical analysis

The significance analysis was done using SPSS (PASW Statistics 18, IBM Co., NY, USA) using one way ANOVA to determine significant differences between means ($p < 0.05$).

3. Results and discussion

High protein model system with 50% protein content was prepared using four kinds of globular protein. DSC was used to measure the thermal denaturation of them in the presence of glycerol. Generally, glycerol is regarded as a protein protectant, the protective effect depending on glycerol concentration. As shown in Fig. 1, glycerol was able to increase the denaturation temperature (T_d) of the proteins. However, the degree of increase was not identical among four kinds of proteins.

The β -lactoglobulin and α -lactalbumin are high abundant globular proteins present in whey, which are commonly used in high protein containing foods. The T_d enhancement of β -lactoglobulin (Fig. 1A) followed a linear trend with glycerol concentration, in agreement with general expectation. Specifically, at 80% (v/v) glycerol, the T_d of β -lactoglobulin was 10 °C higher than control. Interestingly, with the increase of glycerol concentration, the T_d of α -lactalbumin (Fig. 1B) was initially enhanced and then reached a maximum at around 50% (v/v) glycerol, 3 °C higher in denature temperature than control. At 80% (v/v) glycerol, it was even lower than the control.

Lactoferrin is the minor protein in whey but is important bioactive globular protein. The ferric-free and ferric-saturated lactoferrin are defined as apo- and holo-lactoferrin, found in native bovine lactoferrin (Hu et al., 2008; Shimazaki, Kawano, & Yoo, 1991). Holo-lactoferrin, with two ferric ions, denatures at a higher temperature than apo-lactoferrin (Hu et al., 2008). We observed two separate peaks in the DSC thermogram of native lactoferrin at 64.3 and 87.5 °C (Fig. S1C). The effect of glycerol on the lactoferrin under different ion-binding state was investigated using native lactoferrin. As shown in Fig. 1C and 1D, the T_d of apo- and holo-lactoferrin was increased by 5 and 3 °C in 0 to 50% glycerol, respectively. Similarly, the T_d was not further improved by increasing glycerol concentration. Additionally, the stability changes of both freshly prepared apo- and holo-lactoferrin (preparation as described in Supplementary information) in the presence of glycerol showed no difference from those in native lactoferrin (data not shown). Lactoferrin under different ion-binding state showed a similar tendency in stability with the increasing glycerol concentration, suggesting that effect of glycerol is independent of the ion-binding state. On the other hand, it should be noted that at 80% glycerol, the T_d of apo-lactoferrin was 1 °C higher than control while that of holo-lactoferrin was 1 °C lower than control. Despite this, the T_d of holo-lactoferrin in 80% glycerol was still higher than that of apo-lactoferrin, suggesting that the high glycerol concentration did not alter the ion-binding state.

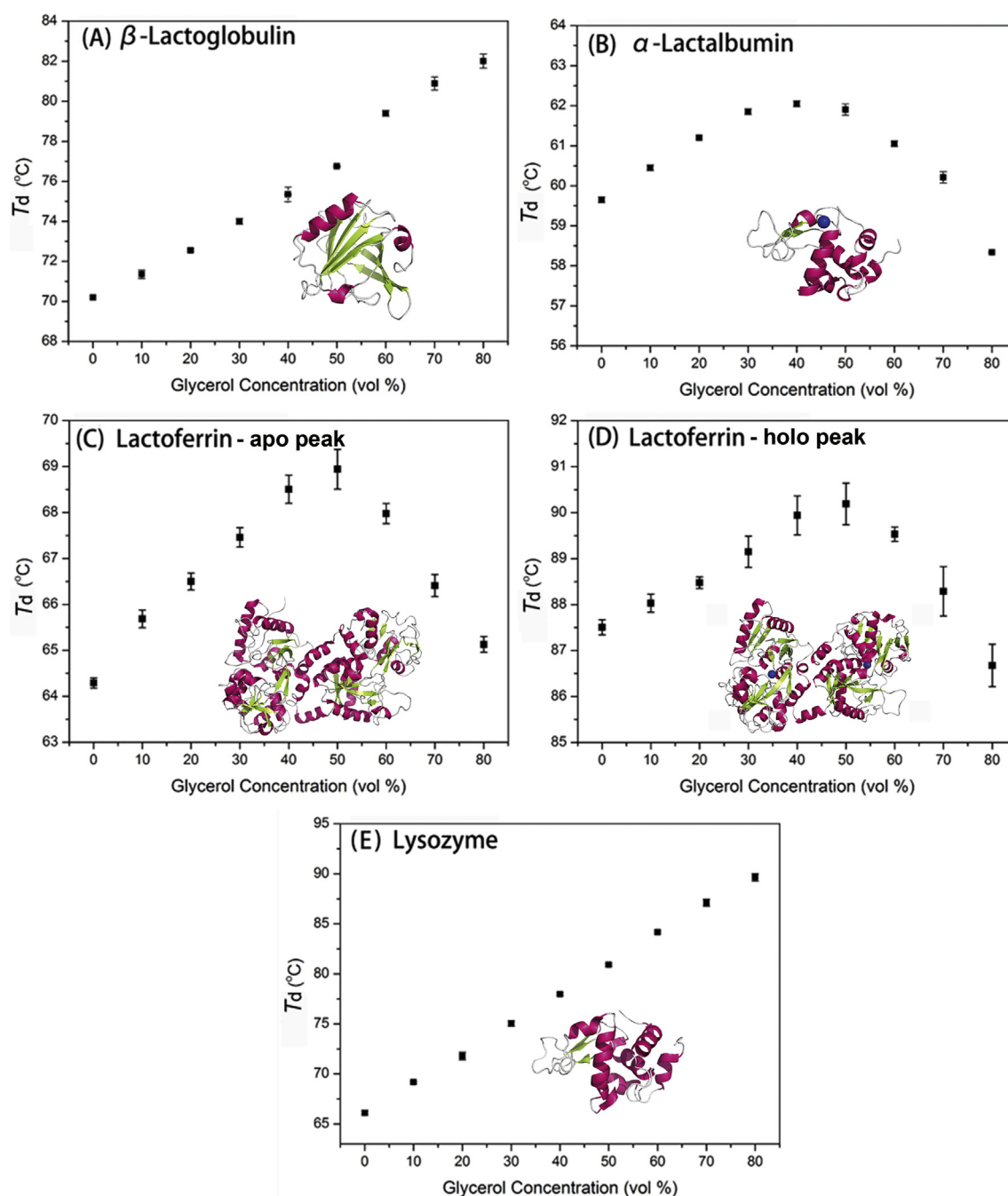


Fig. 1. Effect of glycerol on the denaturation temperature of β -lactoglobulin (A), α -lactalbumin (B), apo-lactoferrin (C), holo-lactalbumin (D) and lysozyme (E). The crystal structure of β -lactoglobulin, α -lactalbumin, apo-lactoferrin, holo-lactalbumin and lysozyme is achieved from protein data bank, whose ID are 3blg (Qin et al., 1998), 1f6s (Chrysina, Brew, & Acharya, 2000), 1i6b (Kumar, Khan, Yadav, & Singh, 2002), 1blf (Moore, Anderson, Groom, Haridas, & Baker, 1997) and 1lse (Kurinov & Harrison, 2010). The crystal structure of each protein is attached, in which the α -helix, β -sheet, random coil and ions are in hot pink (—), limon (—), grey (—) and blue (●), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In short, in the ternary model system, two distinctive ways of protein stability changes were observed with the increasing proportion of glycerol. The α -lactalbumin and lactoferrin, classified as metalloprotein, were similar but different from β -lactoglobulin. Noteworthy, the β -lactoglobulin belongs to lipocalin family with a hydrophobic cavity made from 8 anti-parallel β -sheet and in neutral condition it is prone to exist in a dimer form. The difference presented prominently when glycerol beyond 50% in liquid phase, which might contributed to the penetration of protein hydration layer by glycerol (Hirai et al., 2018). The question is, does the effect of glycerol on the stability of protein depend on the native structure of protein?

To verify the hypothesis, lysozyme was involved because of its structural similarity to α -lactalbumin (Polverino, Frare, Gottardo, Van, & Fontana, 2002) and structure-stabilizing mechanical resemblance with β -lactoglobulin. As shown in Fig. 1E, the T_d enhancement followed a linear trend with glycerol concentration, 25 °C higher than control at 80% (v/v) glycerol.

Lysozyme and α -lactalbumin, consisting of 129 and 123 amino residues respectively, have bilobal structure, with a cleft in between where calcium presents for α -lactalbumin but lysozyme. One lobe of theirs is formed by α -helices and the other by small β -sheet and irregular structures (McSweeney & Fox, 2013). The stabilizing effect of

glycerol on lysozyme increased with concentration while that on α -lactalbumin reached a maximum at 50% glycerol. On the other hand, the increment of T_d lysozyme was much higher than α -lactalbumin because of the glycerol, which is in agreement with the previous reports (Romero, Albis, Lozano, & Sancho, 2009). These suggests that glycerol has a stronger protective effect on lysozyme than α -lactalbumin, despite the similar steric structure of these two proteins. It is noteworthy that lysozyme is not an ion-binding protein whose stability is not dominated by the binding ions compared with α -lactalbumin.

For both lysozyme and β -lactoglobulin, hydrophobic interaction plays an extremely important role in stabilizing steric structure. The active site cleft of lysozyme has been reported being surrounded by a number of hydrophobic residues (McSweeney & Fox, 2013). Interferences on these hydrophobic interaction by residue mutation result in dramatic alteration on its thermal stability (Shih, Holland, & Kirsch, 1995). In terms of the above non-ion-binding protein, they displayed a similar increasing tendency with the glycerol concentration. As initially proposed, the structure and dynamic of water change because of the presence of polyols, resulting in an enhancement of the hydrophobic interaction between pairs and subsequently stabilizing the protein native structure (Back, Oakenfull, & Smith, 1979).

The enthalpy changes determined by DSC are shown in Table 1. Denaturation enthalpy of α -LA and β -LG and lactoferrin decreased with increasing glycerol concentration. However, the case is different in lysozyme, whose denaturation enthalpy fluctuates with glycerol concentration. The specificity of lysozyme might be related to the distinct micro-structure of model mixture system, in line with the particularity of micro-texture shown in Fig. S2. The microscopic images of the mixture of α -lactalbumin, β -lactoglobulin and lactoferrin with glycerol aqueous suggested their texture was fine and homogeneous expect the presence of some air pockets. In the case of lysozyme, the texture is rough in model system but becomes finer with the increase of glycerol concentration. The homogeneity of α -lactalbumin, β -lactoglobulin and lactoferrin is similar but differ from lysozyme model system. The appearance in micro-scale is dependent on the solvation of protein during preparation (Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2010). With the increase of glycerol and decrease of water in the liquid phase, the solvation state of different source of protein could be altered changing its conformational flexibility, might resulting in the enthalpy differences during the thermal denature.

Moreover, the protein structure in model system was studied using confocal Raman spectroscopy which provides the backbone and side-chain information of protein, free from the interference of water compared with infrared spectroscopy. The Raman intensity was normalized against the intensity of band 1003 cm^{-1} , the breathing vibration of phenylalanine which is insensitive to the conformational and environmental changes (Barrett, Peticolas, & Robson, 1978). Although the high proportion of glycerol leads to some Raman peaks, the unique Raman

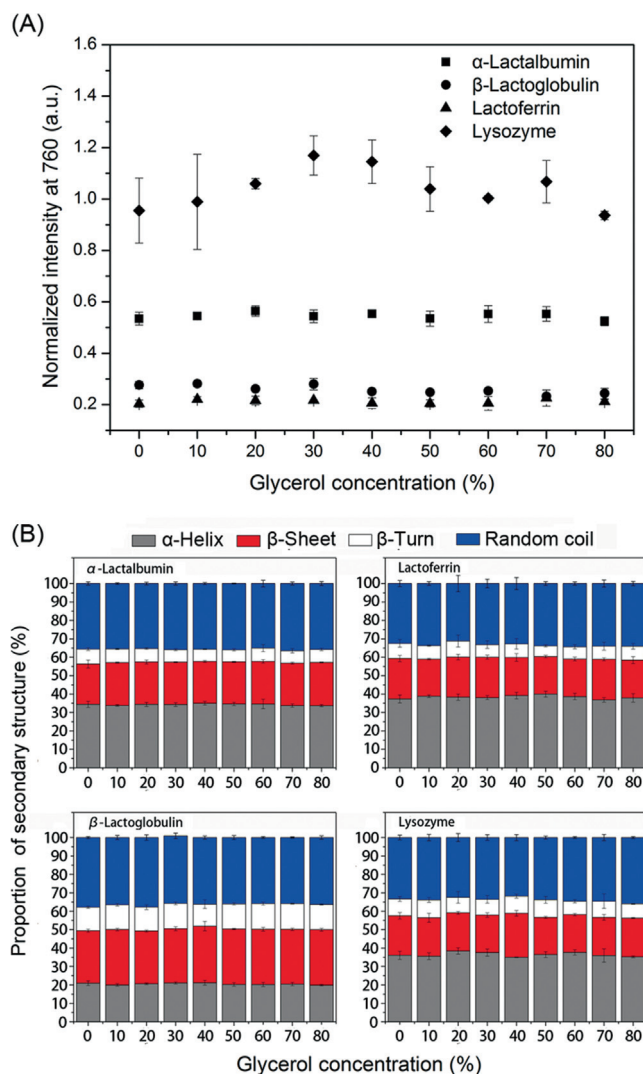


Fig. 2. Tryptophan exposure (A) and secondary structure (B) of protein in high protein model system at various glycerol concentration.

peaks generated by protein including the indole-ring vibrations at 760 cm^{-1} and Amide I at $1645\text{--}1690\text{ cm}^{-1}$ were used to evaluate the environmental and conformational structure in the presence of glycerol.

The changes on the tertiary structure level can be predicted using the Raman peak intensity at 760 cm^{-1} (I_{760}) which decreases with the exposure of buried tryptophan residues (Lord & Yu, 1970). As shown in

Table 1
Enthalpy changes of proteins with glycerol concentration.

Glycerol % (v/v)	ΔH (J/g of protein)				
	β -Lactoglobulin	α -Lactalbumin	Lactoferrin (holo peak)	Lactoferrin (apo peak)	Lysozyme
0	11.6 ± 0.1^{ab}	14.1 ± 0.1^a	2.3 ± 0.1^a	12.0 ± 1.0^a	25.0 ± 2^a
10	11.4 ± 1.0^{ab}	12.2 ± 0.2^b	2.3 ± 0.3^a	14.0 ± 1.0^a	27.2 ± 0.2^{ab}
20	11.8 ± 0.1^a	11.6 ± 0.3^b	2.3 ± 0.2^a	12.4 ± 0.3^a	26.4 ± 1^{ab}
30	11.5 ± 0.3^{ab}	10.2 ± 1.1^c	2.2 ± 0.0^a	12.0 ± 0.2^a	28.0 ± 2^{abc}
40	10.6 ± 1.0^b	9.9 ± 1.0^c	2.1 ± 0.3^a	11.0 ± 1.0^a	28.9 ± 1.4^{bc}
50	8.8 ± 1.0^c	9.4 ± 0.6^c	1.9 ± 0.2^a	11.0 ± 1.0^a	30.9 ± 0.4^c
60	7.3 ± 1.0^d	9.2 ± 0.2^c	1.6 ± 0.0^a	8.5 ± 0.1^b	31.1 ± 1.5^c
70	7.3 ± 0.3^d	8.9 ± 0.3^c	1.7 ± 0.1^a	8.0 ± 1.0^b	27.1 ± 1.1^{ab}
80	6.8 ± 0.0^d	7.4 ± 1.0^d	0.7 ± 0.0^b	7.1 ± 0.3^c	27.1 ± 2.5^{ab}

¹Results are displayed as mean $\pm$ SD.

²The significant differences ($p < 0.05$) in each column are represented by different letters.

Fig. 2A, the I_{760} of α -lactalbumin, β -lactoglobulin and lactoferrin showed no significant changes with the increasing glycerol concentration while that of lysozyme fluctuated dramatically. The exposure of tryptophan residues reflects the solvation of protein. The I_{760} suggests that the solvation state of α -lactalbumin, β -lactoglobulin and lactoferrin were not altered because of glycerol in such high protein model system. Although I_{760} of lysozyme model system is not as even as the other three systems, it does not decrease. The I_{760} fluctuation of lysozyme model system, particularly at low glycerol concentration, might be attributed to the homogeneity, in line with the micro-structure and appearance. The above results indicate that the increasing glycerol do not lead to obvious changes of solvation state of tryptophan, which implies that the distribution of hydrophobic sidechains are free from the alteration by glycerol.

The quantity of secondary structure of protein in model system was estimated from Amide I vibration, with Raman shift between 1645–1700 cm^{-1} (Fig. 2B). The predominant secondary structure of α -lactalbumin, lactoferrin and lysozyme is α -helix while β -lactoglobulin is predominated by β -sheet, in agreement with the previous studies. With the increasing glycerol concentration, the proportion of secondary structure showed little changes in model system made from all four protein resources. This indicates that the presence of glycerol does not influence the secondary structure. Furthermore, it also proves that intramolecular hydrogen bonds are seldom interfered by glycerol regardless its concentration.

Above all, two proteins with a common characteristic of hydrophobic regions were stabilized by glycerol in a linear trend with increasing glycerol. Specifically, hydrophobic core and band were dominating factors to stabilize β -lactoglobulin and lysozyme, respectively. For another two metalloproteins including α -lactalbumin and lactoferrin, the maximal stability were observed at 50% glycerol. Although lysozyme and α -lactalbumin process similarity in secondary and tertiary structure, their stability changed differently with glycerol. Hence, the mechanism of glycerol on the stability of proteins deserves further study regarding the protein nature.

4. Conclusion

The protein stability in the high protein model system was studied in the presence of glycerol. Two distinct ways of change in protein stability with glycerol concentration have been observed. The stability of hydrophobicity-dominated protein increases with glycerol concentration while metalloproteins reaches a maximum stability subsequently decreases at high glycerol concentrations. Nevertheless, the secondary structure and solvation of tryptophan shows little changes because of the glycerol. Overall, the protective effect of glycerol on protein in high protein model system presents different outcomes upon protein with different structural characteristics. Accordingly, the protein recourse and its nature should be taken into consideration in formulating the high protein foods, such as reducing the use of glycerol in a high α -lactalbumin or lactoferrin formulation.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2018.11.117>.

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Supplementary Information

Figure S1

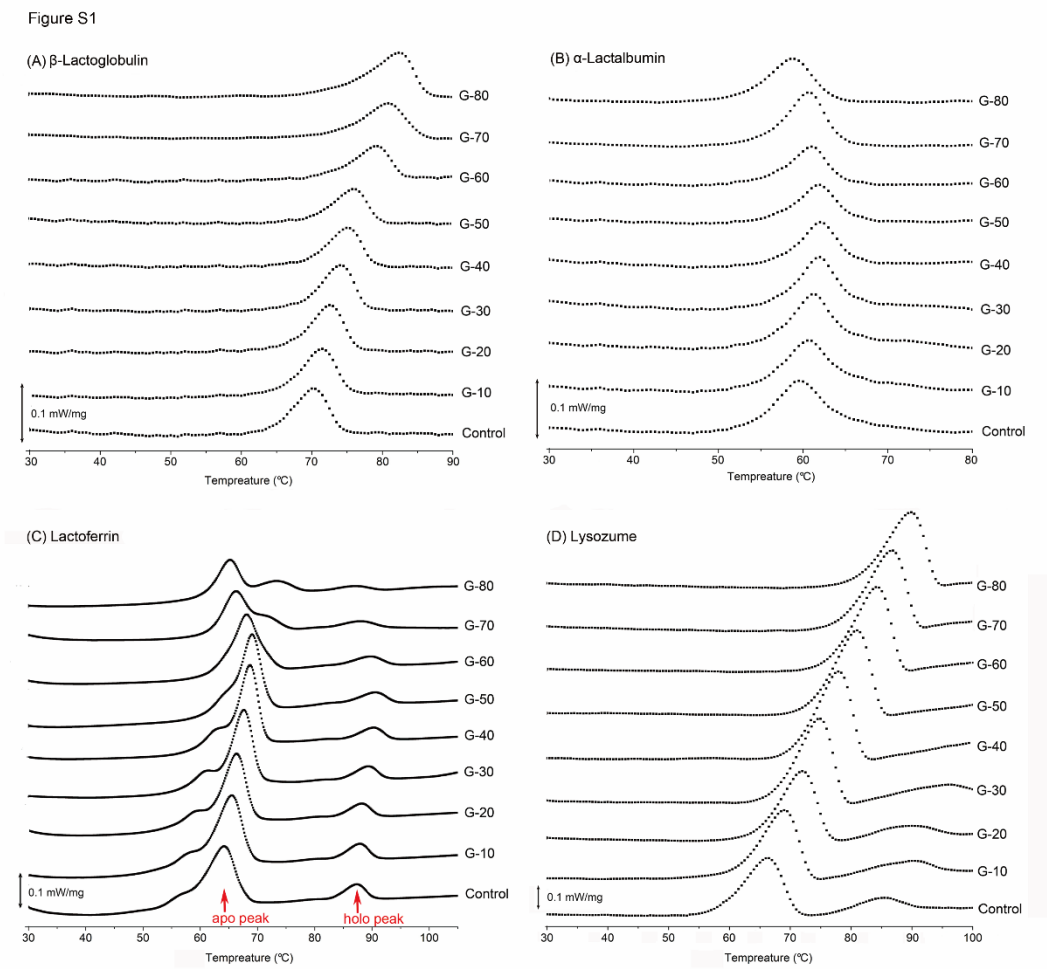


Figure S1. DSC therograms of β -lactoglobulin (A), α -lactalbumin (B) native lactoferrin(C) and lysozyme (E)

Figure S2

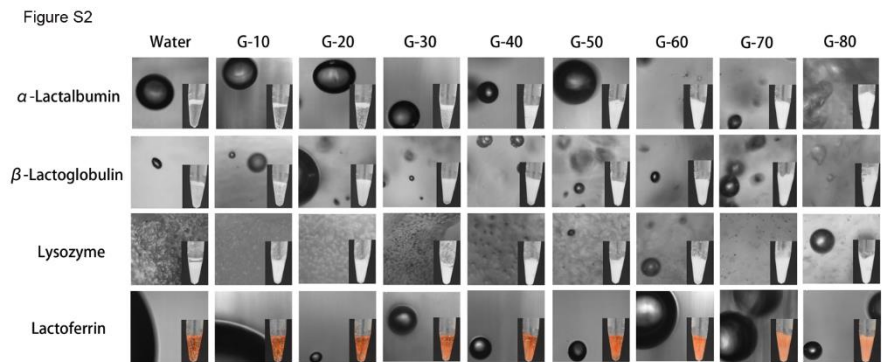


Figure S2. Micro and macro appearance of of high protein model system characterized by confocal microscopic and digital images

Preparation of apo and holo lactoferrin

Apo lactoferrin was obtained according to the method of Mazurier and Spik (1980) with some modification. The native lactoferrin was dissolved in 1.05 M acetic acid/sodium acetate buffer (pH 4.0) at protein concentration of 1%, 0.2 M sodium phosphate. Ion depletion was carried on at 4°C overnight. Subsequently the solution was dialyzed against Milli-Q water for 48 h with three changes of water at 4°C. The pH of depleted lactoferrin was adjusted to 5, 7 and 9 using 1 M NaOH and dialyzed against Milli-Q water for 24 h. Freeze-drying for 48 h was carried on finally.

Holo-lactoferrin was prepared according to the method described by Bokkhim *et al.* (2014). Native lactoferrin solution (1%) of was prepared in 10 mM Tris-Cl buffer containing 75 mM NaCl (pH 7.2, adjusted by 0.1M HCl). Fresh ferric nitrilotriacetic acid (FeNTA) solution constituted of 9.9 mM ferric nitrate and 8.5 mM nitrilotriacetic acid (pH 7.0 adjusted using solid sodium bicarbonate). FeNTA was added to the lactoferrin solution reaching a lactoferrin:iron molar ratio of 1:2. The mixed solution was incubated at ambient temperature for two hours and subsequently dialyzed against Milli-Q water for 48 h with three changes of water. The pH of ion-saturated lactoferrin was adjusted to 5, 7 and 9 using 1 M HCl or 1 M NaOH and dialyzed against Milli-Q water for 24 h. Freeze-drying for 48 h was carried on finally.

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