Simple pH Treatment as an Effective Tool to Improve the Functional Properties of Ovomucin

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Abstract: Ovomucin has been considered to contribute a lot to the excellent functional properties of egg white. This work focused on investigating the effects of pH and protein concentration on foaming and emulsifying properties of ovomucin to evaluate the proper use of this egg protein as a functional food ingredient, and to clarify its contribution to the functional properties of egg white under different pH conditions. Protein solubility and surface hydrophobicity were measured through the pH ranged from 2.3 to 11.0. Alkali treatment gave ovomucin improved emulsification properties, which were correlated well with the surface hydrophobicity ($r \geq 0.89$, $P < 0.01$). Although ovomucin showed lower foaming capacity in acid and neutral solution, enhanced foaming stability was observed with weak acid-treated ovomucin (pH 5 to 6) compared to native ovomucin. These results demonstrated that acid and alkali treatment, which leads to partial unfolding of ovomucin can improve functional properties of ovomucin, with the greatest improvement for emulsification properties being from the alkali treatment and for foaming stability being from weak acid treatment. These results are helpful to produce unfolding ovomucin suitable for wide range of applications in food industry, and to provide useful information on the proper use of egg white in different food systems.

Keywords: egg white, emulsify, foaming, ovomucin, pH

Practical Application: Ovomucin plays a critical functional role in egg white products. However, it is typically insoluble in distilled water or common salt solutions, which has thus limited its commercial applications. Alkaline treatment resulted in gradual increase in solubility, which markedly enhanced the emulsifying properties, on the other hand foaming stability of ovomucin can be promoted by weak acid treatment. The results of this work help to produce unfolding ovomucin suitable for wide range of applications in food industry, and to provide useful information on the proper use of egg white in different food systems.

Introduction

Egg albumen is widely used in food industry due to its various functional properties such as gelling, foaming, and emulsification (Wang and Wang 2009; Mleko and others 2010). As the main viscous micro-glycoprotein in egg albumen, ovomucin plays a critical functional role in egg products, as it is instrumental in forming strong flexible films at the air–water interface, and producing viscoelastic gel matrixes, being responsible for the gel structure of thick albumen (Rabouille and others 1989). Generally, half of the albumen of a freshly laid egg consists of a gel, that is, the thick albumen which is interposed between two liquid fractions, the outer thin and inner thin albumen. Previous studies have shown that the foam stability of thick egg albumen is superior to that of the thin egg albumen (Hammershøj and Qvist 2001). However, protein compositions in the thin egg albumen were almost the same as those in the thick albumen. And the difference only existed in the ovomucin content. The ovomucin content in thick albumen is twice as that in thin egg albumen (Hiidenhovi and others 2002). In addition, other researches also showed that the foam stability of egg albumen was greatly increased by the addition of ovomucin (Mine 1995). Thus, ovomucin has been considered to contribute a lot to the foaming properties of egg albumen. Although the foaming and emulsion property of ovomucin have been extensively investigated, little attention has been paid to the influences of pH or other environmental parameters on emulsifying and foaming properties of ovomucin. Moreover, investigation on the functional characteristics of some specific proteins and their variation in egg albumen has important significance for explaining the mechanism of the functional properties of egg albumen, one of which is ovomucin.

Ovomucin is typically insoluble in distilled water or common salt solutions. However, the insoluble form can be converted to soluble ovomucin with the increase of albumin pH during the storage of fresh egg (Sato and others 1976). Therefore, it can be inferred that the desirable functional attributes of ovomucin can be achieved via adjusting pH to a certain value. Large differences in the functional properties of proteins such as emulsification and foaming properties as affected by pH or other environmental factors, such as temperature, ionic strength have been observed among different food proteins (Kilara and others 1986; Valuev and others 2006; Zhang and others 2008). According to these reports, both the flexibility and hydrophobicity of the protein seemed to be the important structural factors of these functional properties (Dàvila and others 2007; Tömösközi and others 2008). In addition, the viscous nature and association caused by protein–protein

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doi: 10.1111/j.1750-3841.2012.02761.x

Journal of Food Science A Publication of the Institute of Food Technologists

interaction may also be important for the functional properties of proteins (Martinez and others 2007). The flexibility, hydrophobicity, and viscous nature could be highly sensitive to pH (Jahaniaval and others 2000). Thus, to investigate the functional properties of protein as a function of pH shift is of great interest. As a viscous macromolecular glycoprotein, ovomucin is one of the main proteins contribute a lot to the functionality of egg white. But up to now, the role of acid or alkali unfolding on functionalities of ovomucin is still unknown.

Therefore, it is quite necessary to investigate the influences of some pretreatments, such as adjusting pH, on the quality of ovomucin. The current study mainly investigated the influences of pH shift on the solubility, foaming, and emulsifying properties of ovomucin in order to predict the relationship between the functional properties and structural factors of ovomucin molecular, and to clarify the contribution of ovomucin to the functional properties of egg albumen in different food systems with various pH.

Materials and Methods

Materials

Fresh eggs laid within 2 d from a strain of *Hy-Line Brown* available in China were purchased from local poultry farm. Upon arrival to the laboratory, the undamaged eggs were used for ovomucin preparation without delay. The electrophoresis reagents were all purchased from Biosharp. Standard proteins (ovalbumin, ovotransferrin, ovomucoid, and lysozyme from egg) were purchased from Sigma Inc. (St. Louis, Mo., U.S.A.). The other reagents were of reagent grade unless otherwise stated.

Preparation and purity determination of ovomucin

Ovomucin was prepared according to reported method (Omana and Wu 2009) with a minor modification. After separated with the yolk, egg white was diluted with 5 volumes of 100 mM NaCl solution and stirred gently for 30 min on a magnetic stirrer (Model 85–2, Sile Corporation, Shanghai, China). Then the pH of the intermixture was adjusted to approximately 6 using 1 M HCl. The dispersion was kept overnight at 4 °C and separated by centrifugation at 10000 *g* for 15 min at 4 °C. The precipitate was resuspended in 500 mM NaCl solution and kept for 6 h at 4 °C. After centrifugation at 10000 *g* for 10 min at 4 °C. The precipitate was then washed repeatedly with 10 mM NaCl solution until the supernatant became protein-free. The sample was then lyophilized after dialysis against distilled water. Ovomucin purity was checked using sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE), gel filtration chromatography (GFC, SRT SEC-300, Sepax Technologies, Inc., Newark, Del., U.S.A.), and RP-HPLC (C4) coupled with high-performance liquid chromatography. The purity determination process was closely referred to the description in previous report (Omana and Wu 2009). The prepared samples were stored at 4 °C and used within 3 d after preparation. The storage period did not change its electrophoretic pattern.

Protein pH-solubility

Ovomucin was dispersed in distilled water (1% ovomucin, w/v) with different pH by mixing it with a vortex. And then the ovomucin solutions were magnetically stirred for 12 h and centrifuged at 2000 *g* for 15 min at 4 °C. The stirring process did not significantly influence the functional characteristics and spectral pattern of ovomucin (data were not shown here). Nitrogen content of the supernatants was determined by micro-Kjeldahl technique. Total protein content of each sample was calculated by multiplying the nitrogen content by a factor of 6.25. Ovomucin content was calculated as the following formula:

$$\text{Solubility of ovomucin (\%)} = \frac{\text{protein content (in the supernatant)} \times \text{purity \% (in the supernatant)}}{\text{protein content (ovomucin powder)} \times \text{purity \% (ovomucin powder)}} \times 100$$

The purity of ovomucin was determined as described above.

Surface hydrophobicity

The protein surface hydrophobicity (S_0) of the supernatant was analyzed by fluorescence spectrometry using the 1-anilinonaphthalene-8-sulphonate (ANS) as fluorescence probe (Cardamone and Puri 1992). Ovomucin treated with different pH were serially diluted with its own buffering solution to a final volume of 2 mL with protein concentration ranging from 0.05 to 0.25 mg/mL. After stabilizing at 20 °C, 10 μL ANS were added to each sample. The S_0 was determined in duplicates on fluorescence Spectrometer RF-5301 (Shimadzu Corp., Kyoto, Japan). The mixtures were excited at 374 nm and the relative fluorescence intensity was measured at 485 nm. The initial slope (S_0) of the fluorescence intensity versus protein concentration (mg/mL), calculated by linear regression analysis, was used as an index of protein surface hydrophobicity.

Emulsifying activity and emulsion stability

Ovomucin solutions different pH with concentration of 0.5, 1.5, and 2 mg/mL, respectively, were prepared as described above before forming emulsion with corn oil. Emulsifying activity index (EAI) of ovomucin was determined according to the method of Pearce and Kinsella (1978). To prepare emulsions, 10 mL of corn oil and 40 mL of ovomucin solution in 1/20 mM buffer were homogenized in an Ultra Turrax (Hansen Co., Staufen, Germany) at 12000 rpm for 1 min at 25 °C. 0.1 mL of the emulsion was taken from the bottom of the container immediately after homogenization, and diluted with 1 mg/mL SDS solution. The turbidity of the diluted emulsion was then measured at 500 nm using a spectrophotometer. The emulsion stability index (ESI) of ovomucin was estimated by measuring the turbidity of the emulsion at 500 nm first immediately after emulsion formation and then after keeping at 22 °C for 30 min. EAI (m^2/g) and ESI (min) values were calculated by the following equations:

$$\text{EAI}(m^2/g) = \frac{2 \times 2.303 \times \text{DF} \times A_0}{c \times \text{Ø} \times 10000}$$

$$\text{ESI}(\text{min}) = \frac{A_0}{A_0 - A_{30}} \times 30$$

where DF is the dilution factor of the emulsion (20); Ø is the optical path (1 cm); θ is the oil volume fraction in the emulsion (0.25); A_0 and A_{30} are the absorbance of the diluted emulsions at 0 and 30 min, respectively; and c is the protein concentration in unit volume (g/mL) of protein in the aqueous phase.

Foaming capacity and foam stability

The foaming capacity of proteins was determined by measuring the electric conductivity of foams when air was introduced into

5 mL of ovomucin solution in a glass filter (G-4) at a constant flow rate of 90 cm^3/min (Kato and others 1983). The conductivity reading was recorded automatically using recorder software connected with conductivity meter. Foaming capability (FC) was defined as the conductivity of foams immediately produced after air had been introduced into protein solution for 15 s, that is, initial conductivity (C_i). Foam stability was represented as the foam stability index, $C_0 \times \Delta t/\Delta C$, where ΔC is the change in conductivity, occurring during the time interval (Δt), and C_0 is the conductivity at 0 s. That is, foam stability index indicates the time of disappearance of foams (Kato and others 1983).

Statistical analysis

Data were analyzed by analysis of variance using the general linear model (Version 8.0; SAS Institute Inc., Cary, N.C., U.S.A.). Duncan's multiple range tests was used to determine the differences among samples. $P < 0.05$ or less was considered significant. All of the determinations were carried out in duplicate.

Results and Discussion

Purification of ovomucin

SDS–PAGE of the prepared ovomucin (Figure 1B, line 3) revealed a strong band above 250 kDa and two bands between 250 kDa and 130 kDa, which correspond to ovomucin subunits (Shan and others 2012). Only weak bands for ovalbumen (45 kDa) and lysozyme (14.3 kDa) were seen in the SDS–PAGE, respectively. The SDS–PAGE pattern of ovomucin was similar to that reported in previous studies (Omana and Wu 2009). The final purity of ovomucin used in this study was calculated to be 96.7% as further assessed by GFC (Figure 2A), and confirmed by RP-HPLC (Figure 1C). There was only one major peak corresponding for ovomucin in the elution profile from GFC. The peaks from the gel filtration curve showed the presence of a major peak corresponding to ovomucin and two minor peaks corresponding to ovalbumin and lysozyme, which were identified via standard proteins.

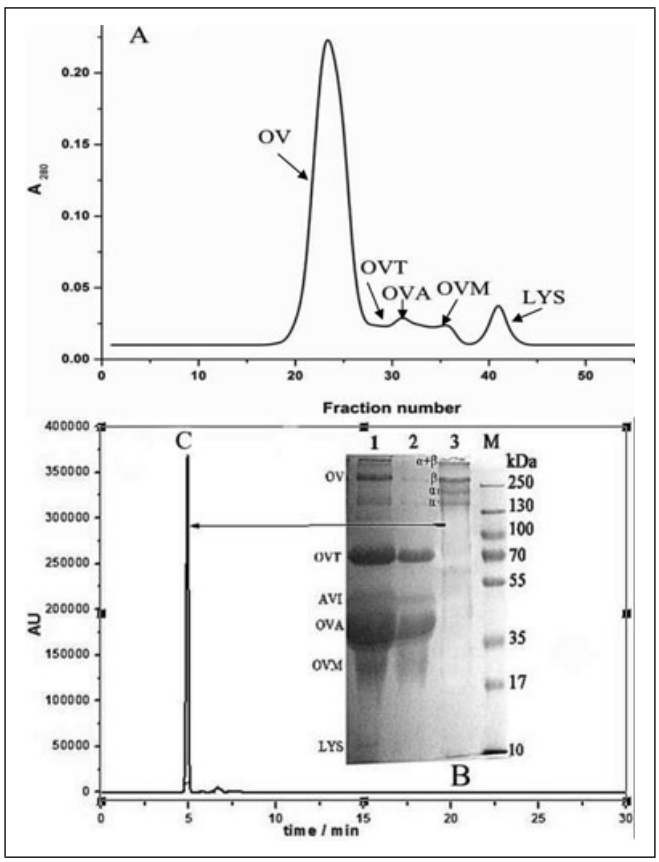

Figure 1–(A) GFC of ovomucin on SRT SEC-300; (B) SDS–PAGE of ovomucin (lane 3) extracted from egg albumen. M (Lane 4) designates molecular weight markers. As a reference to monitor ovomucin, intact egg white (lane 1) and egg white derived from ovomucin (line 2) were denatured and applied to the gel, 10 μL sample (0.5 mg/mL) per lane was loaded on the gel; C = RP-HPLC of ovomucin on C4. OV = ovomucin; OVT = ovotranderrin; OVA = ovalbumin; OVM = ovomucoid; LYS = lysozyme.

Solubility and surface hydrophobicity of native and pH-treated ovomucin

Solubility is the most important functional property of a protein because it can usually influence some other functional properties, such as gelation, emulsification, and foam formation. It is important to note in Figure 2A that the solubility of ovomucin was in fact significantly ($P < 0.05$) affected by pH in neutral and alkaline solutions (pH 7 to 11). And in acid solutions (pH 2 to 6), it was highly insoluble. The minimum ovomucin solubility of 0.15% was observed at pH 5, which is around the isoelectric point of ovomucin (Campbell and others 2003), while maximum solubility of 64.05% emerged at pH 11 or higher. The solubility of ovomucin in alkaline solution (pH 9) was 252 times higher than that of ovomucin in distilled water. Therefore, the solubility of ovomucin in alkaline solution is higher than that in acid aqua, even at low ionic strength. However, pH higher than 11 is not desirable because of undesirable changes, such as protein hydrolysis which could affect the functionality and sensory quality of ovomucin.

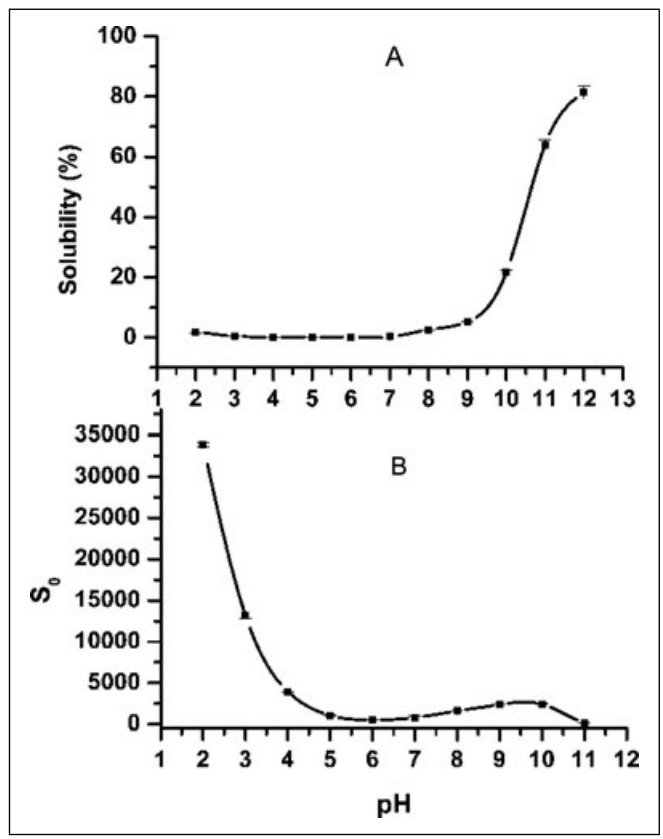

Figure 2–Solubility (A) and surface hydrophobicity (B) of ovomucin as a function of pH. Error bars show standard deviations ($n = 3$).

It was reported that the hydrophobic interactions play an important role in mediating the protein conformation, protein–protein interactions, and thus influence the solubility (Roach and others 2005) and interfacial properties of proteins (Schein 1990). To obtain a complete picture on the nature of the enhanced solubility of the refolded ovomucin, the surface hydrophobicity of ovomucin was assayed using the hydrophobic fluorescent probe ANS. It was observed in Figure 2B that pH treatment resulted in a sharply decrease ($P < 0.05$) in S_0 when the pH of the solutions was increased from 2 to 5. With the continuous increase in pH, it was verified a tendency of S_0 increase initially and then decrease slightly with increasing pH to 11. In our present work, alkaline hydrolysis of ovomucin was accompanied by a decrease of surface hydrophobicity. The possible reason is that peptides released from the native structure of ovomucin reveal great flexibility, which helped them to adopt a conformation with hydrophilic groups more exposed outward. Since ANS is not sensitive to the pH range from 2 to 8 (Gibrat and Grignon 1982), changes in surface hydrophobicity as determined by ANS probe was mainly attributed to the changes in protein–protein interaction.

Emulsifying properties of native and pH-treated ovomucin

The emulsification properties of native and refolded ovomucin were tested by its ability to emulsify and stabilize corn oil in water. The effect of pH on emulsion capacity of ovomucin with different concentrations is shown in Figure 3. At each of the tested conditions, both EAI and ESI tended to increase as protein content increase. Alkaline treatment significantly improved the emulsification ability of ovomucin ($P < 0.05$). Emulsification ability increased by a factor of 1.71 and 1.40 for the pH 10.0 treated ovomucin at content of 0.25% and 0.15%, respectively, compared to native ovomucin ($P < 0.05$) (Figure 3A and Table 1). It can also be seen from Figure 3B that the alkali treatment also resulted in significant increase in the ESI of ovomucin concentrations ($P < 0.05$), with the pH 10.0 treatment yielding the highest activity index.

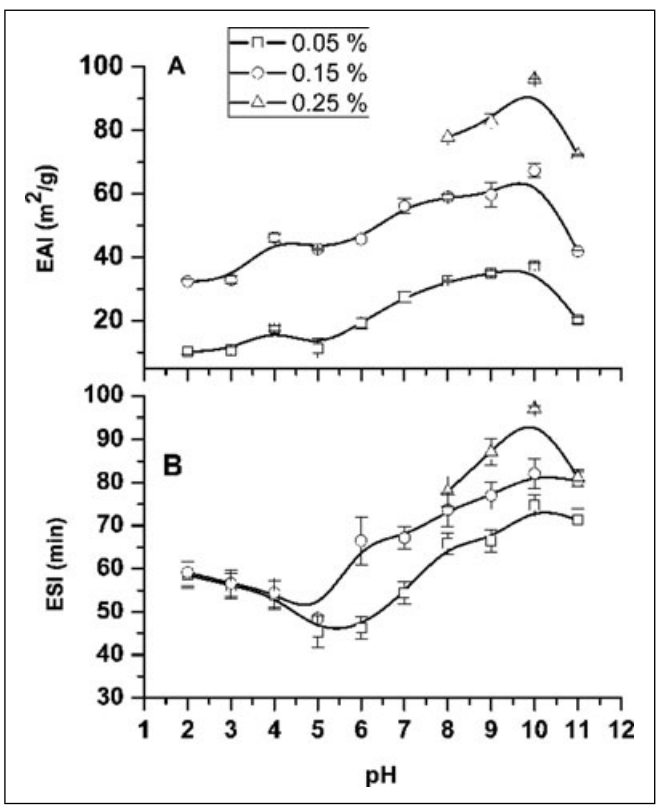

Figure 3–Effect of pH treatment (2 to 11) on emulsifying capacity (A) and stability (B) of ovomucin. Error bars show standard deviations ($n = 3$).

Results from Figure 3A and B revealed that EAI was pH dependent and alkaline pH improved the emulsion capacity more than the acidic pH did. Only slight fluctuations in EAI values were observed for ovomucin dispersed at pH 3 and 4. The dependence of EAI on pH was consistent with what has been expected as it coincides with a general regular pattern. It is known that emulsion capacity of a total protein depends upon the hydrophilic–lipophilic balance, which is affected by pH (Sathe and others 1982). Differences in the structure of the biopolymer interface could also influence emulsion stability. Moderate hydrolyzation of ovomucin in alkaline conditions may increase the protein solubility and expose more hydrophobic groups to water and oil interface, consequently resulting in EC increase and stable emulsion. Nevertheless, if protein hydrolyses excessively into smaller molecular at strong alkaline conditions, which could reduce the film-forming ability, the emulsion will decrease significantly. This is mainly because that the film forming ability of protein is the necessary precondition for emulsification. Like the results of EAI, ESI was also pH dependent. It was suggested that various factors, including pH, droplet size, net charge, interfacial tension, viscosity, and protein conformation, could affect the values of ESI (Hung and Zayas 1991). Therefore, ovomucin could demonstrate variability under different conditions which may accelerate the polysaccharide part to be dissolved in water, and increase the solution viscosity, thereby improve emulsion stability. These findings could lead the author to a conjecture that there is no positive correlation between solubility and emulsifying properties while the emulsifying properties of ovomucin were related to surface hydrophobicity.

Foaming properties of native and pH-treated ovomucin

Foam formation is governed by three factors, transportation, penetration, and reorganization of the molecule at the air–water interface. Therefore, to exhibit good foaming, a protein must be capable of migrating at the air–water interface, unfolding and rearranging at the interface (Phillips and others 1987). Data in Figure 4A suggested that the foaming power of soluble ovomucin markedly increased with the pH (pH range from 5 to 11), which corresponded to the increase of solubility. The FC of ovomucin at 0.05%, 0.15%, and 0.25% (w/v) concentrations reached a maximum of 315.75, 520.69, and 1157.52 $\mu\Omega$/cm, respectively, at pH 10, and then decreased when pH > 10 due to alkaline hydrolysis. The profile of foaming ability against pH for the protein isolate was more or less similar to that of its solubility against pH. However, the opposite was observed in FS of ovomucin, where the

Table 1–Increase in emulsification activity index (EAI) and emulsification stability index (ESI) of alkali treated ovomucin compared to native ovomucin[a].

	Increase in EAI			Increase in ESI		
pH	0.05%[b]	0.15%[b]	0.25%[b]	0.05%[b]	0.15%[b]	0.25%[b]
7 (control)	1.00	1.00	1.00	1.00	1.00	1.00
8	1.18	1.22	1.39	1.21	1.29	1.30
9	1.27	1.24	1.48	1.22	1.35	1.45
10	1.36	1.40	1.71	1.37	1.44	1.61
11	0.74	0.87	1.29	1.31	1.41	1.35

[a]The values represent the EAI and ESI for the pH treated ovomucin divided by the values for the native ovomucin at pH 7.0.[b]Concentration of ovomucin (w/v).

maximum attained at pH 5. A decrease of FS ($P < 0.05$) with increasing pH was observed when pH > 5 (Figure 4).

Hydrophobic interactions among the protein molecules are reported to be reduced at pH far from the isoelectric point of protein, which could facilitate the flexibility of protein molecules, hence enabling protein to spread more rapidly to the air–water interface (Adebowale and Lawal 2003). Therefore, appropriate alkaline conditions might be attributed to the increase in the flexibility of the protein molecules which diffuse more rapidly to the air–water interface to encapsulate air particles, leading to enhanced foaming. However, a foam stability reduction occurs since more microscopic molecules produced by alkaline hydrolysis do not have the strength needed to maintain the stable foam. While proper acidic conditions might be propitious to maintain the foam stability of ovomucin. Solubility of ovomucin makes an important contribution to the foaming behavior of protein. The pH of the dispersing solution dramatically influences foaming properties, especially foam stability.

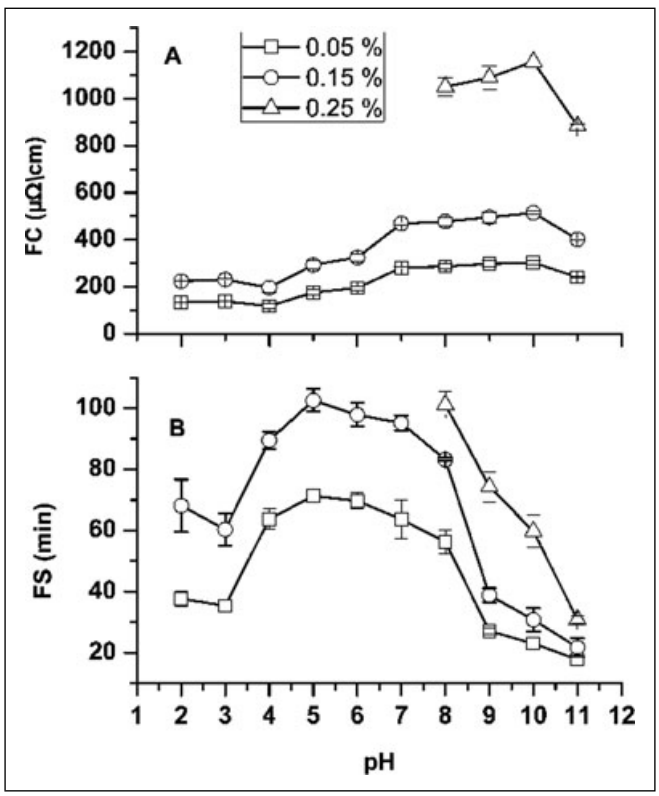

Figure 4–Effect of pH treatment (2 to 11) on foaming capacity (A) and stability (B) of ovomucin. Error bars show standard deviations ($n = 3$).

Table 2–Correlations analysis structural factor and functional properties of ovomucin.

Correlation coefficient (r)	pH	Solubility (%)	Hydrophobicity (S_0)
pH	1		
Solubility (%)	0.82*	1	
Hydrophobicity (S_0)	−0.67*	−0.34	1
EAI (mg/m^2)	0.71*	−0.41	0.89**
ESI (min)	0.46	−0.61	0.92**
Foaming ability ($\mu\Omega$/cm)	0.86**	0.83*	0.80*
Foaming stability (min)	−0.96**	−0.75*	−0.34

*Correlations are significant at $P < 0.05$. **Correlations are significant at $P < 0.01$. The values for foaming and emulsifying properties are determined at pH from 6 to 11 as those at 0.15% protein concentration.

Correlation analysis

That there is some correlation between the solubility, S_0 and functional properties is not very surprising considering the results obtained above. To verify this correlation, specific correlation analysis was carried out using bivariate correlation analysis. Since soluble protein is needed for good functionality, particularly emulsions and foams (Liu and Damodaran 1999), we only used the values determined at pH > 6.

As shown in Table 2, there were negative significantly correlations between pH and S_0, pH and FS with correlation coefficient of −0.67($P < 0.01$) and 0.96 ($P < 0.05$), respectively. This indicated that these parameters decreased as pH increased. On the other hand, positive correlation ($P < 0.05$) between solubility and FA, and a negative correlation ($P < 0.05$) between solubility and FS were obtained (Table 2), which indicated that FA increased as solubility increased, while FS decrease. However, the correlation between S_0 and FA was not significant. Thus, we can suggest that solubility was more important to influence the foaming stability of ovomucin than the S_0. It was also observed that there was a positive and highly significant correlation between the surface hydrophobicity and the emulsifying activity of ovomucin at different pH (correlation coefficient r were both greater than 0.80, $P < 0.01$)), but not for solubility and the emulsifying activity. This indicated that the loss of S_0 of ovomucin induced the decrease of EAI and ESI. Therefore, it is confirmed that the surface hydrophobicity may play a more important role in protein emulsifying property than the solubility do.

A higher surface hydrophobicity of ovomucin may contribute to its higher ESI because an extensive protein–protein interaction, caused by hydrophobic interaction on the surface of the protein, would form a strong oil–water interface, resulting in a stable emulsion (Wasswa and others 2008). Similar to the oil–water interface, increased surface hydrophobicity of the formed complexes (due to polysaccharide induced conformational changes of the protein upon binding) could have resulted in greater absorption at the air–water interface. Moreover, the improved stability and capacity of protein using glycosylation have been reported (Kato and others 1993; Martinez and others 2007), because that the use of polysaccharides control protein absorption to the air–water interface (Ganzevles and others 2006). Since ovomucin is a highly glycosylated protein, the functional properties of ovomucin may also be due to the carbohydrate side chains in addition to the surface properties. However, further investigations are needed to determine the contribution of carbohydrate chain to functional properties of ovomucin.

Conclusion

Results in the current study showed that the emulsifying properties (emulsifying activity and emulsion stability) of ovomucin concentrate were markedly enhanced under moderate alkaline conditions which were correlated well with the surface hydrophobicity, while enhanced foaming stability was observed with weak acid-treated ovomucin (pH 5 to 6) compared to native ovomucin. Therefore, promotion for emulsification properties can be achieved by alkali treatment, while the greatest improvement for foaming stability was observed from the alkali treatment.

Conflict of Interest: The authors have declared no conflict of interest.

Acknowledgments

The authors gratefully acknowledge the financial support provided by modern Agro-industry Technology Research system of

China (Nrs. CARS-41-K23) and the Natl. Natural Science Foundation of China (Grant Nr. 31101320). We are very grateful to Liu Yanxiu, professor from College of Foreign Languages, Huazhong Agricultural Univ. for her language support.

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