RESEARCH ARTICLE

The interaction mechanism and functional properties of active protein and plant active molecule complexes in dairy products

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Received: February 22, 2023; **accepted:** April 22, 2023.

Dairy products are nutrient-rich and popular products, but their counterparts in the dairy industry still suffer from a single structure and serious homogenization. Plant active molecules have an important role in maintaining human health and preventing diseases, but there are few technologies to apply them in food production. To improve this situation, this study attempted to combine lactoferrin (LF), an active protein in dairy products, with two different ginsenoside components, Rb1 and Re, to investigate the interaction mechanisms and functional properties of the resulting non-covalent complexes. The fluorescence spectral results, the type of complex interaction quenching, emulsification, foaming, and foam stability were examined, respectively. The results showed that lactoferrin could interact with two ginsenosides and the generated LF-Rb1 and LF-Re complexes had good foaming properties, which provided a new idea for the application of highly functional molecules in the food field.

Keywords: dairy products; lactoferrin; interaction; complex; ginsenoside.

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Introduction

The development of food science and technology has led to a great change in the current dietary structure of human being. The concept of healthy diet is gradually gaining popularity [1]. Dairy products are loved by many consumers because of their rich protein and other nutrients. Some studies have shown that lactoferrin (LF) in dairy products has different anti-tumor activities against different tumor cells, and in addition to that, it can regulate the original immune system by inducing some activities in the immune system [2]. Ginsenosides, as one of the active plant molecules, have been widely used in pharmaceutical research in recent years. Ginsenosides are composed of numerous components with different functions. The common ginsenoside, Rg3, has been shown to have antitumor and hypoglycemic effects to increase immunity [3]. Lactoferrin, as a milk protein, has a large number of food-derived active molecules on its surface, and therefore, has significant foaming and emulsifying abilities. Ginsenosides, as unique bioactive factors in ginseng, have not only important pharmacological effects, but also being used in food additives in recent years.

Current research on dairy products is relatively homogeneous, and most studies are limited to the aspects such as dairy product preparation

and quality testing [4]. Various constituents of ginsenosides have been shown to have preventive and therapeutic effects in medical studies. However, there are fewer studies related to their use as food additives. Lactoferrin has a high content in dairy products and is preferred by consumers because of its biological activities such as antitumor, antioxidant activity, and antibacterial activity [5]. With the in-depth study of lactoferrin, the various biological functions of lactoferrin are becoming known. Conventional cold-set emulsion gels are composed of a mixture of proteins and oil droplets with lactoferrin and whey proteins making up a relatively large portion of them. Yan, *et al*. examined and analyzed the composition of cold-set emulsion gels in order to investigate its properties. By determining the influence of protein structure, pH, and other influencing factors on the gel strength, it was found that at pH 6.5, 0.5% of whey protein and 1.5% of lactoferrin composition could generate the best gel in the solidified state [6]. To further reveal the biological activity of lactoferrin at different cation-saturated concentrations, Odatsu, *et al*. investigated the synergistic effect of combining different concentrations of lactoferrin and zinc ion to treat human gingival fibroblasts and found that lactoferrin combined with zinc ion was effective in healing wounds in human gums [7]. It has been confirmed that lactoferrin has significant biocidal activity function. El-Fakharany attempted to fuse lactoferrin with nanoparticles to generate dynamic nanoprotein complexes and found that nanopreparations incorporating lactoferrin could serve as good drug delivery tools for disease diagnosis and targeted therapy [8]. Zhang, *et al*. investigated the potential neuroprotective mechanism of ginsenoside Rg1 in attenuating human spinal cord injury and found that ginsenoside Rg1 was effective in reducing neuronal edema and hemorrhage caused by spinal cord injury in humans. In addition, Rg1 was able to inhibit the rate of inflammatory cell infiltration and prevent cell necrosis to further repair the damaged spinal cord [9]. Rc, as one of the important components of ginsenosides, was investigated by Xue, *et al*. for its protective effect on acute cold exposure-induced myocardial injury in rats. The results demonstrated that Rc significantly improved cardiac function, reduced some cardiac enzyme activities, adjusted abnormal blood rheology, and reduced the number of apoptotic cells [10]. Since red and white ginseng have different chemical compositions, their active functions are somewhat different. Yang, *et al*. measured the ginsenoside contents in red and white ginseng and determined their main constituents. The results showed that the average contents of Rb1, Rb2, and Rc in red ginseng were significantly higher than that in white ginseng [11]. Ginseng oxoquinoxaline cyclase plays an important role in the synthesis of ginsenosides. Li, *et al*. studied relevant properties and functions of the ginseng oxoquinoxaline cyclase gene family through gene regulation, gene expression, and gene mutations to provide the basic and necessary genetic knowledge for the biosynthesis and practical application of ginsenosides [12].

Currently, there are many studies either focusing on lactoferrin or ginsenoside, especially on the medical value of ginsenoside for its adjuvant therapeutic effects in medicine [13, 14]. However, there are fewer studies that combine lactoferrin with ginsenosides to analyze the active substances and functional properties of the resulting complexes. This study used two different synthesized lactoferrin-ginsenoside non-covalent complexes to explore their interaction mechanisms, the physicochemical properties, and functional characteristics. The results of this study may establish a perfect dairy industry system and develop more abundant and diverse dairy products.

Materials and methods

Preparation of lactoferrin and ginsenosides

16 mg of lactoferrin (LF) (Hilmar Corporation, Los Angeles, CA, USA) was dissolved in 20 mL of 10 mM Phosphate buffer solution (PBS) at PH 7.0. Ginsenoside Rb1 and Re powders were obtained from Jilin Ginseng Research Institute, Jilin City,

Jilin province, China. Both Rb1 and Re powders were dissolved in anhydrous ethanol to prepare the 10 mM Rb1 and Re solutions, respectively, and then, stored under refrigeration and protected from light.

The effects of ginsenoside Rb1 and Re on lactoferrin fluorescence spectra

The effects of two ginsenosides on the fluorescence spectra of lactoferrin were determined separately by using F-7100 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Briefly, 1 mL of LF was mixed with two ginsenosides solutions respectively at the concentrations of 0, 5, 10, 15, and 20 μM, and then, the fluorescence spectra of samples were measured at 290 K, 300 K, and 310 K, respectively. The excitation wavelength of the spectra was set at 280 nm with a width of 5 nm, and the emission spectra were collected in the range of 300-500 nm. In the final measurement solution, it was necessary to control the concentration of anhydrous ethanol within 2%. The Stern-Volmer fluorescence quenching equation was applied to determine the fluorescence quenching mechanism as below.

$$\frac{F_0}{F} = 1 + K_q \tau_0 [\text{Ginsenoside}] \quad (1)$$

where F was the endogenous fluorescence of lactoferrin after binding to ginsenoside. F_0 was the initial endogenous fluorescence before unbinding. K_q was the rate of quenching. τ_0 was the fluorescence lifetime of lactoferrin. By let the value of lactoferrin be 10^{-8}s, the equation (2) was then obtained.

$$1 + K_q \tau_0 [\text{Ginsenoside}] = 1 + K_{SV} [\text{Ginsenoside}] \quad (2)$$

where K_{SV} was the quenching constant. From equation (2), the fluorescence quenching mechanism of ginsenosides was determined as:

$$\log \frac{F_0 - F}{F} = \log K_a + n \log [\text{Ginsenoside}] \quad (3)$$

where K_a was the binding constant of ginsenoside to lactoferrin. n was the number of binding sites of both. The double logarithmic equation could be used to determine the number of binding sites and constants of both.

Van't Hoff equation below was mainly used to describe the distribution of ions in a solution, thus establishing the relationship between ions and solution temperature.

$$\ln K_a = \frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

where ΔH was the enthalpy change. ΔS was the entropy change. R was the gas constant. T was the temperature.

The thermodynamic equation below enabled the calculation of the Gibbs free energy of the solution.

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

where ΔG was the Gibbs free energy. By calculating the fluorescence data at different temperatures and combining Equation (4) and Equation (5), the thermodynamic parameters between the two, including the enthalpy change, entropy change, and Gibbs free energy change, could be calculated to determine the quenching mechanism between ginsenosides and lactoferrin, and then, the degree of temperature influence on the binding process could also be inferred.

Emulsifiability determination

2 mL of edible corn oil was added to 8 mL of each lactoferrin, LF-Rb1 mixture, and LF-Re mixture, respectively, and dispersed for 3 mins at room temperature with a high-speed disperser (IKAC, Staufen, Germany) to make a crude emulsion. After 0 min and 10 mins of dispersion, 0.01 mL of the crude emulsion sample was added to 5 mL of 0.1% sodium dodecyl sulfate (SDS) and shaken for 15 s before the absorbance was measured at 500

nm. The emulsification activity index was calculated by using the following equation.

$$EAI\left(m^2/g\right)=\frac{2.2303\cdot A_0\cdot N}{c\cdot\varphi\cdot 10000} \quad (6)$$

where A_0 was the absorbance value of the crude emulsion after dilution. N was the dilution multiple. φ was the volume share of corn oil in the solution. c was the protein solution concentration. The emulsion stability index of the complex was calculated by using the following equation.

$$ESI\left(\min\right)=\frac{A_0\cdot t}{\Delta A} \quad (7)$$

where ΔA was the difference between the absorbance value of the crude emulsion diluted by standing for 10 mins and the value at the beginning. t was the time interval.

Foaming and stability determination

LF solution was mixed with Rb1 and Re solutions separately at room temperature in three different molar ratios of 1:0, 1:10, and 1:20, respectively. 10 mL of mixture solution was placed in an Ultra-Turrax T25 high-speed disperser (IKAC, Staufen, Germany) and sheared at 12,000 rpm for 90 s to make the solution generating sufficient bubbles. The foaming performance of the solution was subsequently determined according to the change of the bubbles and the foaming properties of the complex was determined by using the following equation.

$$Foamability=100\times\frac{V_0}{V_T} \quad (8)$$

where V_T was the volume of the sample solution before shearing. V_0 was the volume of the sample solution just after shearing. The foam stability in the complex was determined by using the following equation.

$$\text{Foam stability}=100\times\frac{V_{30}}{V_0} \quad (9)$$

where V_{30} was the volume of the sample solution 30 mins after the end of shear.

Statistical analysis

Each experiment was repeated three times. SPSS 23.0 software (IBM, Armonk, New York, USA) was employed for statistical analysis. The t-test was used for independent sample analysis, and $P < 0.05$ indicated that the statistical results were significantly different.

Results

Analysis of fluorescence spectra

The properties of LF-Rb1 and LF-Re non-covalent complexes were examined by fluorescence spectroscopy. The effects of ginsenosides Rb1 and Re on lactoferrin at two different temperatures were shown in Figure 1. The results demonstrated that, at the temperature 300 K, the fluorescence spectra of lactoferrin showed a strong emission peak at 330 nm as the concentration of Rb1 and Re increased from 0 μM to 200 μM. However, the endogenous fluorescence intensity of lactoferrin showed a regular decreasing trend with the increasing concentrations (Figure 1a and 1b). When the temperature increased to 310 K, with the increases of Rb1 and Re concentrations, the position of the strong emission peaks of the fluorescence spectra of lactoferrin did not change greatly. However, its endogenous fluorescence intensity still showed a regular decreasing trend (Figure 1c and 1d), which indicated that the addition of ginsenosides Rb1 and Re changed the fluorescence properties of lactoferrin, and high concentrations of Rb1 and Re could quench the endogenous fluorescence intensity of lactoferrin, allowing the two to undergo fusion to form a new non-covalent complex.

Interaction quenching type judgment

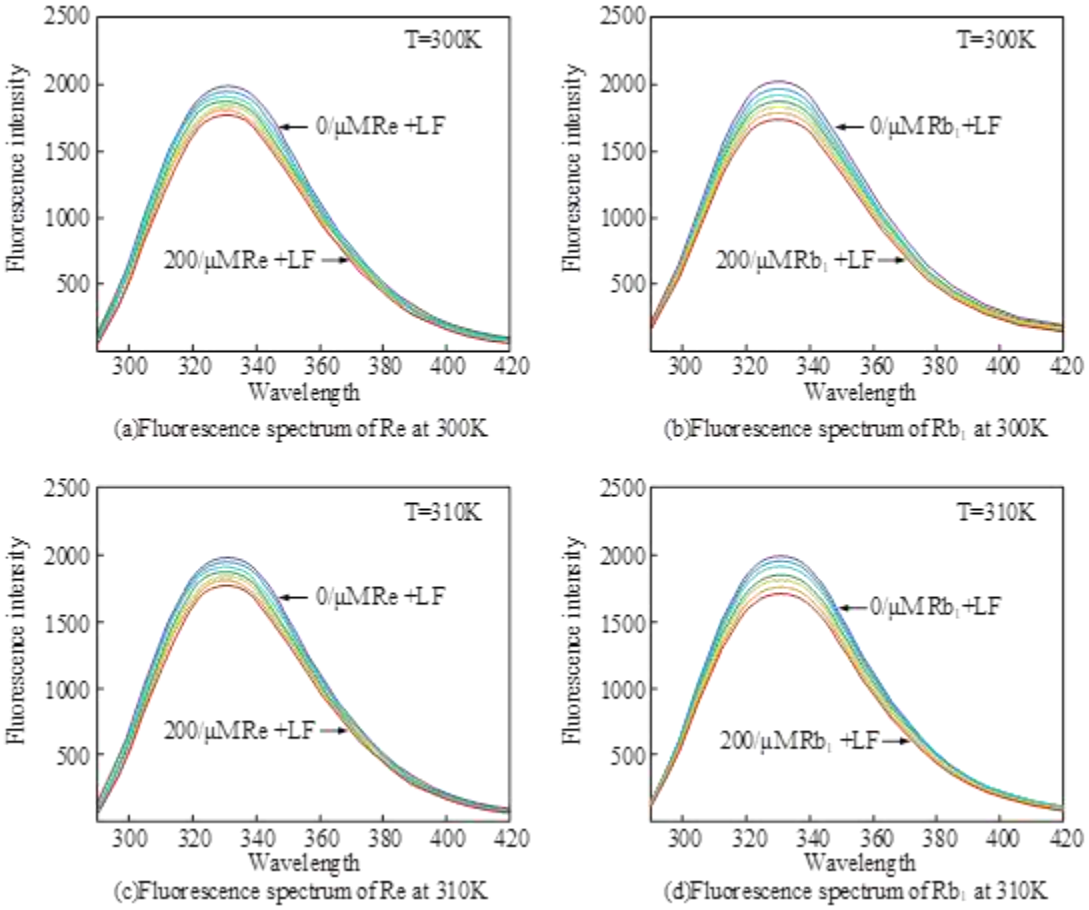

Figure 1. The effects of Rb1 and Re on the fluorescence spectra of LF at different temperatures and different concentrations.

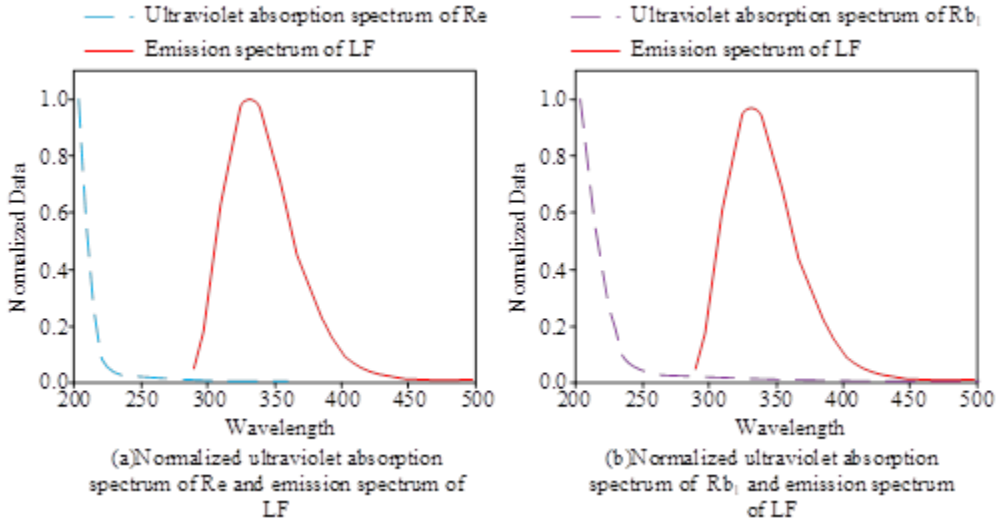

Figure 2. Emission spectrum of LF and UV absorption spectrum of Rb1/Re.

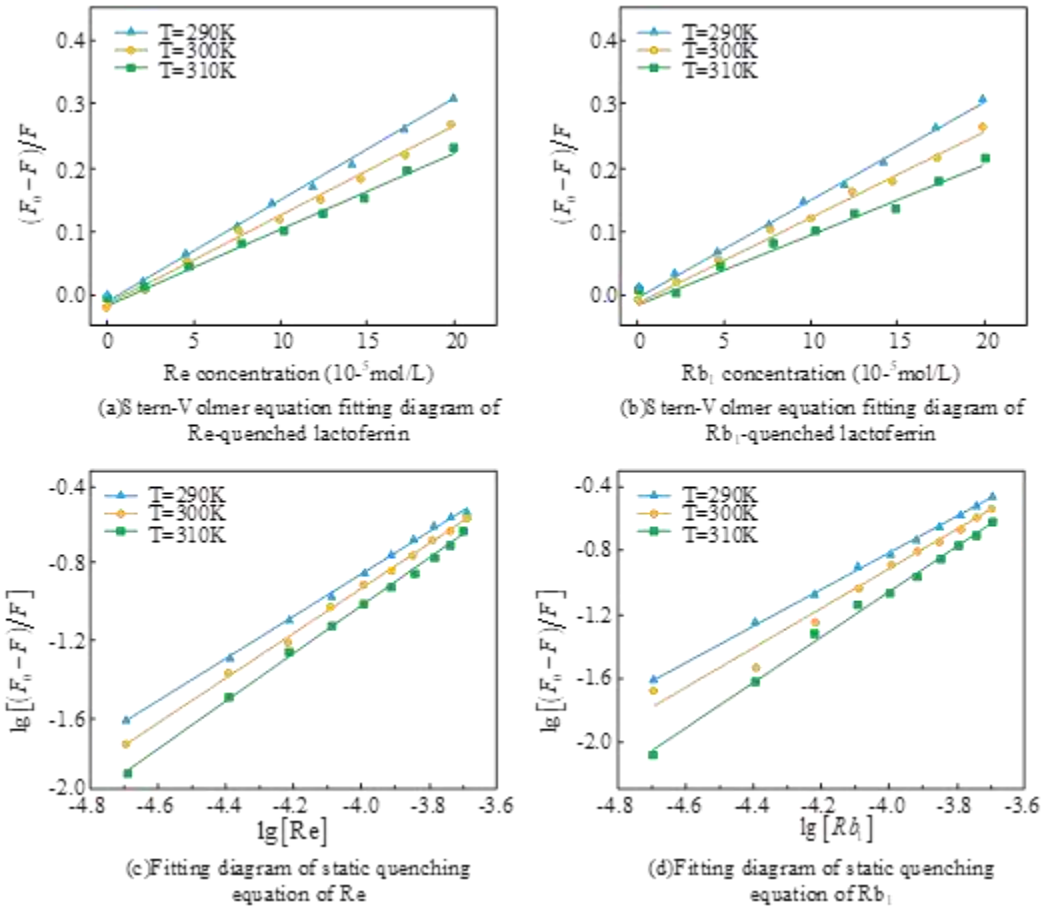

Figure 3. Fitting of Stern-Volmer equation with static quenching equation for Rb1 and Re at different temperatures.

To determine the type of fluorescence quenching of lactoferrin by Rb1 and Re, the emission spectra of lactoferrin with the UV absorption spectra of Rb1/Re were measured (Figure 2). The UV absorption spectra of Rb1 and Re did not overlap with the emission spectra of lactoferrin, so that the internal filtration effect in fluorescence quenching could be excluded. To determine the specific quenching mechanism, the interaction mechanisms of Rb1 and Re with lactoferrin at different temperatures were further investigated in combination with the fluorometric equations described above.

The fitted Stern-Volmer equations for Re and Rb1 at different temperatures with the increase of Re and Rb1 concentrations were shown in Figure 3a and 3b. The Stern-Volmer equations showed a good linear correlation at the temperatures of 290 K, 300 K, and 310 K. The quenching constants of Re on lactoferrin at different temperatures were obtained from the equations described above. The minimum quenching rate constant was 1.30×10^{12}/mol·s. Since the minimum quenching rate constants were all greater than the constants in the dynamic quenching state, it could be determined that the quenching mode of Re on lactoferrin was static quenching. Similarly, the minimum quenching rate constant of Rb1 was also much larger than the maximum dynamic quenching constant. So, it could be determined that the quenching mode of Rb1 on lactoferrin was also static quenching. The static quenching equations of Re and Rb1 at different temperatures were shown in Figure 3c and 3d, respectively. By comparing the static quenching equations of the two, it could be found that Rb1 and lactoferrin were more easily affected by

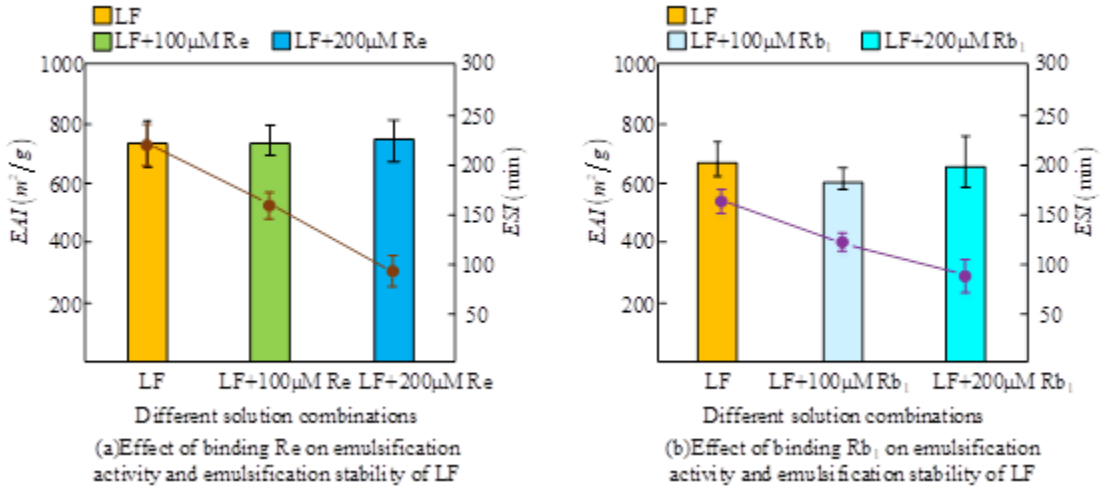

Figure 4. Effects of Rb1-Re complexes on LF emulsification activity and emulsion stability.

temperature during the binding process, while Re and lactoferrin could maintain a more stable performance during the binding process.

Emulsifiability

The effects of combining different concentrations of Rb1 and Re on the emulsification activity and emulsion stability of lactoferrin were shown in Figure 4. The emulsifying activity of lactoferrin did not change significantly when the concentrations of Re and Rb1 were varied between 0 and 200 μM. The results showed that the emulsifying activity index of LF-Re solution was 772 m^2/g at the Re concentration of 200 μM comparing to 756 m^2/g when Re concentration was 0 μM. On the other hand, the emulsifying activity index of LF-Rb1 solution was 635m^2/g when Rb1 concentration was 200 μM comparing to 681 m^2/g when the concentration of Rb1 was 0 μM. Combining different concentrations of Re and Rb1 did not have a large effect on the emulsification activity of lactoferrin ($P > 0.05$), which was due to the facts that there was no excessive hydrophobic interaction force between the two during the binding process, and increasing the hydrophobicity of the protein surface was only beneficial to improve the emulsification activity of the protein. However, the emulsification stability of lactoferrin decreased significantly after combining different concentrations of Rb1 and Re ($P < 0.05$). The emulsion stability of lactoferrin was the best when the concentrations of Re and Rb1 were 0 μM, respectively, with the emulsification stability indexes of LF-Re solution as 235 min and LF-Rb1 solution as 178 min. With the increase of ginsenoside concentration, the emulsification stability of lactoferrin gradually decreased.

Foaming and foam stability

The foaming properties and foam stability of proteins have a key influence on the quality of many foaming food products. The foaming properties and foam stability of the prepared LF-Rb1 and LF-Re solutions were demonstrated in Figure 5. The results showed the foaming properties and foam stability of LF-Re solutions at the different molar ratios with the minimum foamability of 146 at the ratio of 1:0 and the maximum foamability of 203 at the ratio of 1:20., while the maximum foam stability of 91 at the ratio of 1:0 and the minimum foam stability of 69 at the ratio of 1:20. On the other hand, the foamabilities of LF-Rb1 solution were the minimum of 149 at the ratio of 1:0 and the maximum of 186 at the ration of 1:20, while the foam stabilities were the maximum of 83 at the ratio of 1:0 and the minimum of 78 at the ratio of 1:20. With the increase of the solution molar

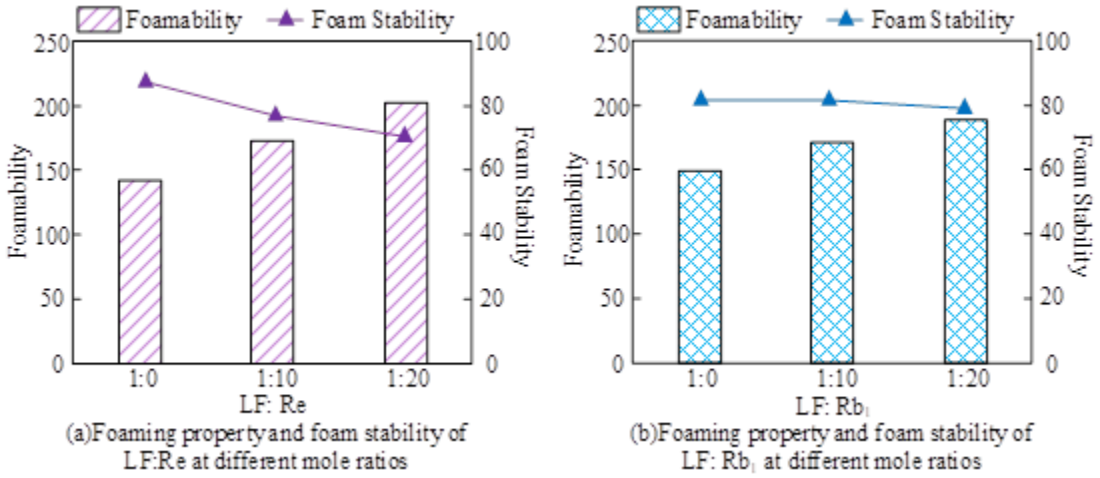

Figure 5. Foaming and stability of prepared LF-Rb1 and LF-Re solutions at different molar ratios.

ratio, the foaming properties of LF-Re and LF-Rb1 solutions were increased. However, the foam stability of LF-Re solution decreased with the increase of molar ratio, while the foam stability of LF-Rb1 solution was basically unchanged.

Discussion

In order to further explore the mechanism and functional characteristics of the interaction between active proteins in dairy products and plant active molecules, this study used lactoferrin combining with two kinds of ginsenosides, Rb1 and Re to investigate the physical and chemical properties of the resulted non-covalent compounds. The results showed that the temperature change had little effects on the non-covalent complexes of LF-Rb1 and LF-Re. However, with the increase of Rb1 and Re concentrations, the fluorescence spectrum of lactoferrin demonstrated a strong emission peak at 330 nm, and the endogenous fluorescence intensity of lactoferrin displayed a downward trend. Through the analysis of various spectrum diagrams and Stern-Volmer equation fitting diagrams, it could be determined that the quenching modes of two ginsenosides for lactoferrin were all static quenching. The Rb1 and lactoferrin were more susceptible to the influence of temperature during the binding process. Re and Rb1 both showed no significant effects on the emulsifying activity of lactoferrin, but reduced lactoferrin emulsifying stability. With the increase of the molar ratio of LF-Rb1 and LF-Re, the foaming ability of both compounds were increased. Through this study, the two non-covalent complexes formed by the combination of ginsenosides Rb1 and Re to LF, respectively, demonstrated good physical and chemical properties. Among them, the influence of temperature on the binding process of LF-Re was less significant than that of LF-Rb1, so that the stability of LF-Re was better. In addition, combining different concentrations of Rb1 and Re could effectively improve the emulsification stability of LF. The two non-covalent compounds generated through this study showed good foaming properties and foam stabilities. Therefore, they both could be considered for the research and development of foaming dairy products in the future. There are still some deficiencies in this study, such as the applications of non-covalent compounds in oil or in water lotion were not investigated. The whole research was only at the experimental level, and additional *in vivo* experiments are needed in follow-up research to fully reveal the physical and chemical properties and mechanism of the complexes. In summary, lactoferrin and ginsenosides Rb1 and Re could combine with each other, and the non-covalent compound

produced could provide new ideas for the research and development of dairy industry.

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