Emulsifying and Structural Properties of β -Lactoglobulin at Different pHs

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The emulsifying properties of β -lactoglobulin (β -Lg) in relation to its molecular structure were investigated at pH 3~9. In the acidic pH region, β -Lg showed relatively low emulsifying and surface activity, while its surface hydrophobicity was greater than at neutral pHs. The conformational stability of β -Lg varied depending on pH, *i.e.* its conformation was more rigid and resistant to denaturation at pH 3 than at pH 7. The low emulsifying and surface activity of β -Lg at acidic pHs was assumed to be due to such low denaturability (flexibility) of the molecule. Cleavage of the intramolecular disulfide bonds increased the denaturability, as well as the emulsifying activity, of β -Lg at pH 3.

Whey proteins have various functional properties as well as excellent nutritional quality. Because of such functionalities as emulsifying, foaming, and gelling properties, whey protein concentrates (commercially available whole whey proteins) are used as valuable ingredients for many processed food products.

Emulsification with whole whey proteins has been studied by many researchers as reviewed by Morr¹⁾ and Cheftel and Lorient.²⁾ Emulsifying and fat-binding properties of individual whey proteins such as β -lactoglobulin (β -Lg) and α -lactalbumin (α -La), however, remain obscure. Tornberg³⁾ has studied emulsifying properties of a whey protein concentrate compared with those of casein and soybean proteins, and found that whey proteins diffused rapidly from the aqueous phase to the oil surface on emulsification, forming a thin adsorbed layer on the oil globule surface. Contribution of individual whey proteins to the formation of such adsorbed layer has been studied by Oortwijn *et al.*⁴⁾ and by Yamauchi *et al.*^{5,6)} Both groups found that the adsorption of individual whey proteins was highly selective. At neutral pHs, the predominant constituent protein of the adsorbed layer was

β -Lg. We found, however, the adsorption of β -Lg was strongly reduced by a decrease in pH. At pH 3, the content of β -Lg in the adsorbed layer was only 12.9%, whereas it was 61.6% at pH 9.⁶⁾ It is suggested that some pH-dependent structural changes of β -Lg affect its adsorption and emulsification properties.

For this paper, the emulsifying and structural properties of the isolated β -Lg have been investigated at various pHs. Relationships between the emulsifying property and protein structure are discussed.

MATERIALS AND METHODS

Preparation of β -Lg. β -Lg (a mixture of variants A and B) was isolated from bovine fresh skim milk by the procedure of Aschaffenberg and Drewry,⁷⁾ and then purified by gel filtration on Cellulofine GC-200m (Chisso Co., Ltd.).

Measurement of emulsifying activity. The emulsifying activity index (EAI) of β -Lg was determined by the turbidimetric procedure of Pearce and Kinsella.⁸⁾ Two milliliters of a β -Lg solution (1%, w/v) and 0.5 ml of soybean oil were mixed and homogenized at 30°C for 3 min by a Polytron PTA-7 (Kinematica, Switzerland) at maximum speed. An aliquot (0.5 ml) of the emulsion was diluted with a 0.1% sodium dodecyl sulfate solution and its turbidity was measured at 500 nm.

Measurement of surface hydrophobicity.

i) *Hydrophobic chromatography.* Butyl-Toyo Pearl 650M (Toyo Soda Co., Ltd.) was packed in a glass column (1 × 20 cm) and equilibrated with an aqueous solution adjusted to pH 3, 5, 7, or 9. A 0.5% β -Lg solution (0.5 ml) adjusted to the same pH was applied to the column. Elution was carried out at room temperature at a flow rate of 3 ml/h with the solution used for equilibration. Protein was measured at 280 nm using a ISCO UA-5 UV monitor. The hydrophobicity of β -Lg at each pH was calculated as follows;

$$\text{hydrophobicity} = \frac{[\text{elution volume (ml)} - \text{gel bed volume (ml)}]}{\text{gel bed volume (ml)}} \times 100.$$

ii) *Fluorescent probe method.* The procedure was basically that of Creamer *et al.*⁹⁾ To an aqueous solution of β -Lg adjusted to pH 3, 7, or 9 was added an 1-anilino-2-naphthalene sulfonate (ANS) solution to give a final concentration of 1.5×10^{-5} M. The ANS- β -Lg conjugates were excited at 370 nm and the relative fluorescence intensity was measured at 470 nm using a Hitachi 650-10S fluorescence spectrophotometer. The fluorescence intensity was plotted against protein concentration and the initial slope was calculated.

Circular dichroism (CD) spectra. CD was measured on a Jasco J-20 spectropolarimeter using a 0.1% β -Lg solution.

Ultracentrifugal analysis. Sedimentation velocity analysis was performed at 60,000 rpm and at 30°C on a Hitachi KCA-1A analytical ultracentrifuge. Protein concentration ranged from 0.5 to 1.0% in water (adjusted to pH 3, 7, or 9). The sedimentation coefficient was calculated and extrapolated to zero protein concentration.

Measurement of surface tension. Surface tension was measured with a Wilfelmie surface tensiometer (Kyowa Kagaku, Type CBVP A-3) using a glass plate (24 mm × 24 mm). A β -Lg solution (0.001%, w/v) adjusted to pH 3 or 7 was used. Measurement was performed at 20°C.

Denaturation of β -Lg with urea or guanidine hydrochloride (GuHCl). β -Lg was dissolved in varied concentrations of urea (0~8 M) or GuHCl (0~6 M). The denaturation of β -Lg caused by these agents was evaluated by fluorescence study. The sample solution was excited at 296 nm and the wavelength at which the emission fluorescence was maximal was measured by using a Hitachi 650-10S fluorescence spectrophotometer. The wavelength for the emission maxima was plotted against the concentration of urea or GuHCl.

Reduction of disulfide bonds in β -Lg. A 2% concentration (w/v) of β -Lg was dissolved in 0.1 M Tris-HCl buffer (pH

8.2) containing 8 M urea and 1% 2-mercaptoethanol. The solution was incubated at 30°C for 6 hr and then dialyzed against 0.1% 2-mercaptoethanol. About 70% of the disulfide bonds were shown to be cleaved by these treatments. The solution was appropriately diluted with 0.1% 2-mercaptoethanol and served for analyses.

RESULTS AND DISCUSSION

Emulsifying activity of β -Lg at different pHs

The emulsifying activity index (EAI) of β -Lg at pH 3~9 was measured. As shown in Fig. 1, EAI of β -Lg was low at pH 3 and increased with an increase in pH. These results, as well as the pH dependent adsorption behavior of β -Lg at emulsified oil surfaces,^{5,6)} suggest that some structural changes of β -Lg molecules occur at acidic pHs and affect their emulsifying properties.

Hydrophobicity of β -Lg at different pHs

Since the adsorption of proteins onto oil surfaces by emulsification occurs through hydrophobic interaction, the hydrophobicity of the protein molecule must be important for its emulsifying properties. Surface hydrophobicity of β -Lg at pH 3~9 was measured by two different methods—a hydrophobic chromatography method and a fluorescent probe method using ANS. The results are shown in Table I. By both methods, the increase in the hydrophobicity at pH 3 was great.

Relationships between protein hydrophobicity and protein functions, such as emulsifying and foaming properties, have been investigated and reviewed by Kinsella¹⁰⁾ and

FIG. 1. Effect of pH on Emulsifying Activity of β -Lactoglobulin.

TABLE I. SURFACE HYDROPHOBICITY OF β -LACTOGLOBULIN AT DIFFERENT PHs

pH	3	5	7	9
Hydrophobic chromatography method	78	6	1	0
Fluorescent probe method ^a	100	— ^b	7	10

^a Expressed as relative values.

^b Not determined because of the low solubility at this pH.

Nakai.¹¹⁾ A positive correlation between the surface hydrophobicity and emulsifying activity was first observed by Kato and Nakai¹²⁾ using various proteins. Such correlation was confirmed by several following studies.^{13~16)} However, the results obtained in this study was outside of the correlation proposed by these authors. Some factors other than hydrophobicity are suggested to be important for the emulsifying properties of β -Lg.

Conformation of β -Lg at different pHs

Though the surface hydrophobicity of β -Lg significantly changed upon lowering the pH, no significant difference in the secondary structure of β -Lg at pH 3 and 7 was observed by CD measurement (Fig. 2) in agreement with the result reported by Teller *et al.*¹⁷⁾

β -Lg is known to form dimers at neutral pH. The monomer-dimer equilibrium is affected by pH and the dissociation to monomers increases as the pH is lowered.¹⁸⁾ Mackenzie¹⁹⁾ observed that the dissociation was appreciable at below pH 3.5 and above pH 7.5. However, the monomer-dimer association phenomenon is very complex, and it is affected by many other factors such as temperature, protein concentration, and ionic strength. The degree of association of β -Lg molecules under the condition used for emulsification (1% protein, 30°C, in water adjusted to pH 3, 7, or 9) was investigated by sedimentation velocity analysis, although this was not ideal for the analysis. The sedimentation coefficients, s_{30}^0 , of β -Lg at pH 3, 7, and 9 were 2.72, 2.65, and 2.04 (s), respectively. The similar S values at pH 3 and 7 suggest that the different EAI values

FIG. 2. Circular Dichroism of β -Lactoglobulin at pH 3 (---) and pH 7 (—).

(Fig. 1) and adsorption behavior of β -Lg at these pHs^{5,6)} have no relationship to the degree of monomer-dimer association of the molecules.

Denaturability of β -Lg at different pHs

On emulsification, proteins are adsorbed on oil globule surfaces. This process is, in most cases, accompanied by some structural changes of the proteins, namely surface denaturation.²⁰⁾ We have found that adsorbed whey proteins including β -Lg on emulsified oil surfaces were more easily digested with proteases than the native whey proteins.⁶⁾ This suggests that whey proteins are denatured on the oil surface. Denaturability of proteins might, therefore, be an important factor affecting their emulsifying properties.

Rate of surface denaturation of β -Lg was estimated by surface tension measurement of β -Lg solutions at pH 3 and 7. As shown in Fig. 3, the surface pressure π of a β -Lg solution increased much faster at pH 7 than at pH 3. The rate of change of π is considered to be a function of protein structure. It probably reflects the ability of the protein molecules to penetrate and rearrange in the surface.²¹⁾ Graham and Phillips have demonstrated that β -casein, a flexible protein, gives a more rapid increase in surface pressure than globular pro-

FIG. 3. Course of the Increase in Surface Pressure of β -Lactoglobulin at pH 3 and pH 7.

FIG. 4. Urea and Guanidine-HCl Denaturation Curves for β -Lactoglobulin at Different pHs.

At pH 3 (●), pH 7 (○), and pH 9 (□).

teins such as lysozyme.²²⁾ They concluded that adsorption kinetics was dependent upon the flexibility of the protein. The faster increase for β -Lg at pH 7 suggests that the conformation of a β -Lg molecule is more rigid at pH 3 and flexible at pH 7.

Effects of urea and GuHCl on denaturation of β -Lg at pH 3~9 were investigated. Figure 4 clearly demonstrated that denaturation of β -Lg occurs more easily at pH 7 and 9 than at pH 3. Five molar urea or 4M GuHCl was necessary to denature β -Lg at pH 3, *i.e.* the structure of β -Lg seemed to be stable at pH 3. De Wit and Klarenbeek²³⁾ reported that the stability of β -Lg to heat denaturation was greater at acidic pHs, in good agreement with our results.

These results strongly suggest that β -Lg has a relatively stable or rigid conformation at pH 3 and resists surface denaturation. Low emul-

FIG. 5. Urea Denaturation Curves for β -Lactoglobulin (●) and Reduced β -Lactoglobulin (○) at pH 3.

TABLE II. RELATIVE EMULSIFYING ACTIVITY AND SURFACE HYDROPHOBICITY OF REDUCED β -LACTOGLOBULIN

	β -Lactoglobulin	Reduced β -lactoglobulin
Emulsifying activity	100	153
Surface hydrophobicity ^a	100	300

^a Measured by fluorescent probe method using ANS.

sifying activity and less adsorption at pH 3 might be at least partly owing to this property. Importance of the conformational instability of the molecule of proteins in their emulsifying properties has been recently suggested by Kamata *et al.*²⁴⁾ using soybean proteins.

Cleavage of intramolecular disulfide bonds

Intramolecular disulfide bonds are considered to stabilize the native structure of protein molecules by lowering the entropy of unfolded forms of the chain. β -Lg has two disulfide bonds in its molecule. Reduction of these bonds is expected to destabilize the conformation of β -Lg, resulting in an increase in its emulsifying activity. The effect of reduction of the disulfide bonds on the emulsifying property and conformational stability of β -Lg was investigated.

For cleavage of disulfide bonds, reduction with 2-mercaptoethanol or dithiothreitol followed by carboxymethylation of free SH groups is often used. Incorporation of the carboxymethyl groups, however, might change

the surface properties of the β -Lg molecule. Therefore, in the present study, the reduced β -Lg was used for analyses without blocking its free SH groups. Instead, the experiments were carried out in the presence of 0.1% 2-mercaptoethanol.

As shown in Fig. 5, the reduced β -Lg became more susceptible to denaturation by urea than native β -Lg. The EAI of the reduced β -Lg is shown in Table II. Reduction caused about a 50% increase of the EAI value. These results, therefore, suggest that increased flexibility of the β -Lg molecule caused by S-S cleavage favorably affected its emulsifying activity, although the increase in EAI of the reduced β -Lg should not be attributed solely to the increased flexibility caused by cleaving the disulfide bonds. Gross changes in molecular shape or conformation of β -Lg probably occurred during the process of reduction, since the reduction was found to increase the surface hydrophobicity (Table II) and changed the CD spectrum (data not shown).

The physicochemical properties of β -Lg are known to be dependent upon the pH. However, there is limited information about the effect of pH on the functional properties of β -Lg. This study revealed that the emulsifying properties of β -Lg changed depending on the pH. Low emulsifying activity of β -Lg was shown at pH 3. Studies on the structural properties of β -Lg molecules suggest that the low EAI at pH 3 is owing to the low denaturability (flexibility) of the molecules at this pH.

The primary structure of β -Lg has already been established.²⁵⁾ Creamer *et al.*²⁶⁾ predicted the secondary structure of β -Lg and suggested that a number of segments may have an α -helical conformation. Brown *et al.*²⁷⁾ have suggested that such helical conformation of β -Lg must be important when β -Lg hydrophobically interacts with phospholipid vesicles. Whether the similar interaction plays some role in oil (triglyceride)- β -Lg interaction on emulsification is still unknown. Detailed analyses on the dynamic structure and adsorption behavior of β -Lg are necessary to understand

its emulsifying property.

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