# Emulsifying and Structural Properties of Ovalbumin

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The emulsifying properties of ovalbumin were investigated, and the relationships between the emulsifying properties and protein structure were also discussed. The emulsifying properties of ovalbumin were dependent on pH, concentration of protein dispersion, oil-phase volume, and presence of salts. The pH was the most important variable in the emulisfying activity. The emulsifying activity of ovalbumin was relatively high in the acidic pH region. The surface hydrophobicity of ovalbumin was greater at acidic pHs than at neutral pHs. No significant difference was found between the secondary structure of ovalbumin at pH 3 and that at pH 7, but microenvironmental changes were shown around the aromatic amino acid residues in acid solution. The line widths of $^{31}P$ NMR spectra in ovalbumin at pH 3 or 8 suggested that the conformation of ovalbumin was more flexible at acidic pHs than at neutral pHs. Thus, the high emulsifying activity of ovalbumin at acidic pHs was assumed to correlate with the greater surface hydrophobicity and flexibility of the molecule.

## INTRODUCTION

Proteins have the ability to stabilize emulsion because they behave in a similar manner to surface-active agents. Many proteins including casein, soybean protein, muscle protein, and egg protein have been used in various emulsified food products.

The mechanism of the adsorption of protein on fat–water interfaces is of great interest. Various factors that affect the emulsifying properties of proteins, including adsorption on the interface, were proposed by many investigators (Kitchener and Mussellwhite, 1968; Birdi, 1973; Friberg, 1976; Tornberg, 1978; Phillips, 1981; Yamauchi et al., 1980; Matsudomi et al., 1985). However, general rules on the relationships between the protein structure and the adsorptivity at fat–water interfaces during the emulsification step are unknown.

The proteins of egg white constitute a mixture of materials of great importance to the food industry. Egg white proteins present three or four major characteristics critical to the preparation of a wide variety of foods and food products. Ovalbumin is the major constituent of egg white, and important food ingredient with high functionality including emulsifying properties and foam stability (Powrie and Nakai, 1987; Horn, 1980).

For this paper, the emulsifying and physical properties of ovalbumin have been investigated at various pHs. Relationships between the emulsifying properties and protein structure are discussed.

## MATERIALS AND METHODS

**Preparation of Ovalbumin.** Ovalbumin was prepared from fresh egg white by the crystallization method in sodium sulfate and recrystallized five times (Kekwick et al., 1936).

**Measurement of Emulsifying Properties.** The emulsifying properties were determined by the method of Pearce and Kinsella (1978). To prepare emulsion, ovalbumin solution (0.5–5.0% protein in distilled water adjusted to pH 3–9 by HCl or NaOH) was mixed with various amounts of soybean oil. The mixture (4 mL) was homogenized with a Physcotron instrument (Nition Rikaki Corp.) equipped with generator shaft NS-10 at 20 000 rpm for 1 min at 30 °C. A 25 μL emulsion sample was taken from the bottom of the container on the passage of times and was diluted with 5 mL of a 0.1% sodium dodecyl sulfate solution. The absorbance of the diluted emulsion was measured at 500 nm. The emulsifying activity was determined by the absorbance measured immediately after emulsion formation, and the emulsion stability was estimated by measuring the half-life (seconds) of the turbidity of the emulsion.

**Measurement of the Surface Hydrophobicity.** The surface hydrophobicity was determined by the method of Kato and Nakai (1980). Ten microliters of an ethanolic solution of *cis*-parinaric acid ($3.6 \times 10^{-3}$ M) was added to 2 mL of a 0.1% protein solution, adjusted to pH 3–9 by HCl or NaOH. The mixture was excited at 325 nm, and the relative fluorescence intensity was measured at 420 nm by using a fluorescence spectrophotometer (Hitachi Model 203). The relative fluorescence intensity reading was adjusted to 1.0 when 10 μL of *cis*-parinaric acid solution was added to 2 mL of distilled water adjusted to pH 3–9 in the absence of protein. The fluorescence intensity was plotted against protein concentration, and the initial slope was calculated.

**Determination of Adsorbed Protein.** The amount of protein adsorbed on the oil globule surface during emulsification was determined as follows. After emulsification, the emulsion was immediately centrifuged at 5000*g* for 20 min, and the aqueous portion was removed by aspiration. To the floated cream portion was added threefold distilled water (pH adjusted to 3.0–9.0). The diluted cream was gently mixed and centrifuged as before. Washed cream was obtained by repeating this step five times. The protein adsorbed on the fat globule surface was extracted with sodium dodecyl sulfate (SDS) and 2-mercaptoethanol as follows: To 1 mL of the washed cream was added 1 mL of 4% SDS–1% 2-mercaptoethanol, and the mixture was heated in a boiling water bath for 5 min. The content of protein was determined by the standard Kjeldahl procedure (Kj.N × 6.52).

**CD and Fluorescence Spectral Measurement.** Circular dichroism (CD) was measured on a Jasco J-500A spectropolarimeter at wavelengths from 190 to 350 nm. Ovalbumin (1 mg/mL) in 10 mM sodium phosphate buffer (pH 7.0) or 20 mM HCl–potassium phosphate buffer (pH 3.0) was measured with a 1-mm cell in the far-UV region. Ovalbumin (2 mg/mL) in the same buffer solution was measured with a 1-cm cell in the near-UV region. Fluorescence spectra were recorded with a Hitachi 203 fluorescence spectrophotometer. The excitation wavelength was 295 nm, and emission was from 300 to 400 nm.

**Fat Globule Size Distribution.** The fat globule size distribution in the emulsion was measured by using a particle size distribution analyzer and principles of centrifugal sedimentation (Horiba, CAPA-700, Tokyo).

**Measurement of $^{31}P$ NMR Spectra.** $^{31}P$ NMR spectra were recorded on a VXR-400S instrument at 22 °C, using a 45° pulse, with 32K data points, a 4000-Hz spectral window, and a 1.5-s pulse delay. Proton was decoupled. The buffer was prepared 20 mM Tris-HCl, 1 mM EDTA, and 25% $D_2O$ at pH 8.0 or 25 mM sodium acetate, 1 mM EDTA, and 25% $D_2O$ at pH 3.0. Concentration of protein was 5.0%.

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**Table I. Effect of $CaCl_2$ and NaCl on Emulsifying Activity and Emulsion Stability of Ovalbumin at Different pHs[a,b]**

| | pH | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 | | 5 | | 7 | | 9 | |
| | EA[c] | ES[d] | EA | ES | EA | ES | EA | ES |
| OVA | 0.796 ± 0.03 | 324 ± 23 | 0.526 ± 0.04 | 522 ± 34 | 0.483 ± 0.05 | 480 ± 31 | 0.439 ± 0.04 | 456 ± 28 |
| OVA + 10 mM $CaCl_2$ | 0.683 ± 0.04 | 360 ± 18 | 0.609 ± 0.07 | 150 ± 16 | 0.593 ± 0.06 | 126 ± 22 | 0.555 ± 0.09 | 150 ± 27 |
| OVA + 0.2 M NaCl | 0.648 ± 0.05 | 330 ± 27 | 0.496 ± 0.06 | 72 ± 11 | 0.427 ± 0.07 | 30 ± 14 | 0.428 ± 0.08 | 60 ± 20 |

[a] 25% soybean oil (w/w) in 1% protein (w/v) dispersion was emulsified. [b] Each value is an average of three replications ± standard deviation. [c] EA, emulsion activity. [d] ES, emulsion stability.

**Table II. Effect of Protein Concentration on Emulsion Activity and Emulsion Stability of Ovalbumin[a,b]**

| | oil concn, % | | | | | |
|---|---|---|---|---|---|---|
| | 12.5 | | 25.0 | | 50.0 | |
| protein concn, % | EA[c] | ES[d] | EA | ES | EA | ES |
| 0.1 | 0.076 ± 0.02 | <15 | 0.142 ± 0.02 | <15 | 0.131 ± 0.02 | <15 |
| 0.5 | 0.335 ± 0.04 | 276 ± 22 | 0.311 ± 0.04 | 156 ± 11 | 0.269 ± 0.04 | <30 |
| 1.0 | 0.474 ± 0.04 | 485 ± 31 | 0.520 ± 0.06 | 480 ± 28 | 0.422 ± 0.03 | 198 ± 22 |
| 2.0 | 0.643 ± 0.03 | 600 ± 26 | 0.823 ± 0.05 | 576 ± 32 | 0.719 ± 0.04 | 1680 ± 54 |
| 3.0 | 0.672 ± 0.05 | 204 ± 18 | 0.939 ± 0.07 | 648 ± 29 | 0.871 ± 0.09 | 2580 ± 190 |
| 4.0 | 0.779 ± 0.04 | 144 ± 16 | 1.026 ± 0.11 | 660 ± 22 | 0.963 ± 0.08 | 8800 ± 277 |
| 5.0 | 0.795 ± 0.06 | 132 ± 15 | 1.111 ± 0.14 | 750 ± 31 | 0.929 ± 0.07 | 9470 ± 344 |

[a] Emulsification was performed at pH 7.0. [b] Each value is an average of three replications ± standard deviation. [c] EA, emulsion activity. [d] ES, emulsion stability.

**Table III. Fat Globule Size Distribution in the Emulsion and Adsorption of the Ovalbumin on the Fat Surface Prepared at Different pHs[a,b]**

| | pH | | | |
|---|---|---|---|---|
| | 3 | 5 | 7 | 9 |
| diameter,[c] μm | 12.03 ± 0.4 | 15.10 ± 0.3 | 15.70 ± 0.5 | 17.06 ± 0.6 |
| adsorbed protein,[d] mg/g of oil | 11.34 ± 1.2 | 8.68 ± 0.9 | 5.88 ± 0.5 | 4.92 ± 0.4 |

[a] 25% soybean oil (w/v) in 1% protein (w/w) dispersion was emulsified. [b] Fat globule size was measured by using a particle size distribution analyzer. [c] Mean volume diameter. [d] Each value is an average of three replications ± standard deviation.

## RESULTS

The emulsifying property of ovalbumin and the effect of salts (0.2 M NaCl and 10 mM $CaCl_2$) at pH 3–9 were measured. As shown in Table I, the emulsifying activity was high at pH 3 and decreased with an increase in pH. Addition of salts (0.2 M NaCl or 10 mM $CaCl_2$) in 1% ovalbumin dispersion affected the stability of the emulsion prepared at pH 5–9. On the other hand, the emulsifying activity was increased by addition of $CaCl_2$ but decreased by addition of NaCl. The effect of protein concentration and oil-phase volume on the emulsifying activity and emulsion stability is shown in Table II. The emulsifying activity and emulsion stability depended on the protein concentration and the oil-phase volume. The emulsifying activity and emulsion stability were very low when the protein concentration was below 0.5%. The stable emulsion was formed by using high protein concentration and high percentage of oil-phase volume.

The fat globule size of the emulsion and the amount of protein adsorbed onto the fat globule surface prepared at pH 3–9 are shown in Table III. The fat globule size of the emulsion was dependent on pH at the emulsification. The globule size was minimal at pH 3 and increased with increasing pH. The adsorption of ovalbumin was dependent on pH of the protein dispersion at the emulsification. The amount of adsorbed protein was maximal at pH 3 and decreased with increase of pH. The amount of ovalbumin adsorbed on the fat globule surface at pH 7 increased with the increase of protein concentration as shown in Table IV.

Since the adsorption of proteins onto oil surface by emulsification occurs through hydrophobic interaction, the surface hydrophobicity of protein molecule must be important for emulsifying properties (Kato and Nakai, 1980). Surface hydrophobicity of ovalbumin at pH 3–9 was measured by a fluorescent probe method using *cis*-parinaric acid. The results are shown in Table V. The surface hydrophobicity of ovalbumin was greatest at pH 3.

**Table IV. Effect of Protein Concentration on the Amount of Protein Adsorbed onto Fat Globule Surface[a,b]**

| protein concn, % | adsorbed protein, mg/g of fat | protein concn, % | adsorbed protein, mg/g of fat |
|---|---|---|---|
| 0.5 | 3.91 ± 0.3 | 3.0 | 32.10 ± 1.8 |
| 1.0 | 6.23 ± 0.7 | 4.0 | 47.87 ± 1.9 |
| 2.0 | 18.03 ± 1.2 | 5.0 | 45.89 ± 2.0 |

[a] Emulsification was performed at pH 7.0. The fat content of the emulsion was 25% (w/w). [b] Each value is an average of three replications ± standard deviation.

**Table V. Surface Hydrophobicity of Ovalbumin at Different pHs[a]**

| | pH | | | |
|---|---|---|---|---|
| | 3 | 5 | 7 | 9 |
| OVA | 338 | 135 | 100 | 100 |

[a] Expressed as relative values.

We examined the conformational change of ovalbumin at different pHs by CD spectral measurement (Figure 1). The far-UV CD spectra of ovalbumin at pH 7.0 and 3.0 gave almost the same curve (Figure 1A). These results show that the secondary structure of ovalbumin at pH 3.0 and at neutral pH was about the same. However, the near-UV CD spectrum of ovalbumin at pH 3.0 was different from the spectrum at pH 7.0 (Figure 1B). The decrease in ellipticity around 250–290 nm in the acid solution

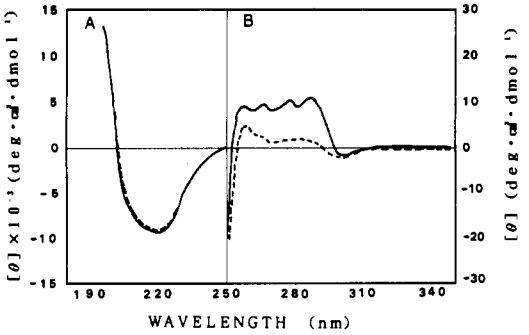


**Figure 1.** CD spectra of ovalbumin at pH 3.0 (- - -) and 7.0 (—). (A) Ovalbumin (1 mg/mL) in 10 mM sodium phosphate buffer (pH 7.0) or in 20 mM HCl–potassium phosphate buffer (pH 3.0) was measured with a 1-mm cell in the far-UV region. (B) Ovalbumin (2 mg/mL) in the same buffer solution was measured with a 1-cm cell in the near-UV region.

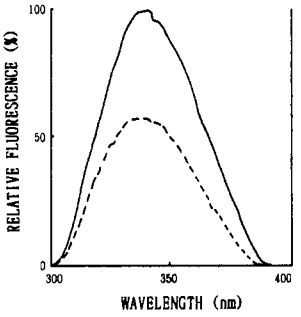


**Figure 2.** Fluorescence spectra of ovalbumin at pH 3.0 (- - -) and 7.0 (—). The excitation wavelength was 295 nm, and emission was from 300 to 400 nm.

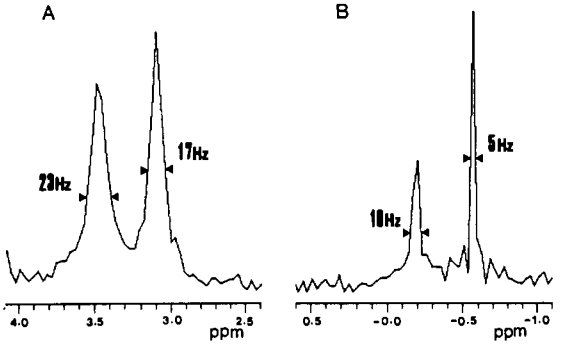


**Figure 3.** $^{31}P$ NMR spectra of ovalbumin at different pHs. Concentration of ovalbumin was 5.0% (w/v) in (A) 20 mM Tris-HCl and 1 mM EDTA, pH 8.0, or in (B) 25 mM sodium acetate and 1 mM EDTA, pH 3.0; 10 000 acquisitions at 22 °C.

indicated that the aromatic amino acid residues were transferred to a less ordered structure than in neutral solution.

Microenvironmental changes around tryptophan residues were examined by the intrinsic fluorescence of ovalbumin. A maximum of fluorescence emission was found at 338 nm for both pH 7.0 and pH 3.0 (Figure 2). The fluorescence intensity was reduced by acidification.

Ovalbumin is a monomer comprising 385 residues with a molecular weight of 45 000 and contains two phosphoryl residues at serine 68 and 344 residues (Taborsky 1974; Nisbet et al., 1981). The two phosphoserine residues were well resolved in a $^{31}P$ NMR spectra, and that was ready assigned (Vogel and Bridger, 1982). Figure 3 shows a proton-decoupled $^{31}P$ NMR spectra of a 5% ovalbumin solution at pH 8.0 or 3.0. The line widths of $^{31}P$ NMR spectra were affected by pH. $^{31}P$ NMR spectra of both SerP-68 and SerP-344 at pH 3.0 were sharper than those at pH 8.0. The line widths of $H_3PO_4$ in $^{31}P$ NMR spectra were not affected by pH using buffer.

## DISCUSSION

Many studies on the chemical and enzymatic modification of proteins have attempted to improve protein functionality. Emulsification is one of the most important functionalities of food proteins. The emulsifying properties of ovalbumin were improved by coupling ovalbumin to dextran (Kato et al., 1990) or freeze-drying and spray-drying (Kitabatake et al., 1989), but various factors that affect the emulsifying properties of ovalbumin are not sufficiently elucidated.

The emulsifying activity and emulsion stability of ovalbumin were dependent on pH at emulsification, concentration of protein dispersion, oil-phase volume, and presence of salts. The pH was the most important variable in the emulsifying activity. The emulsifying activity of ovalbumin was high at pH 3. These results, as well as the pH-dependent adsorption behavior of ovalbumin at the emulsified oil surface, suggest that some structural changes of ovalbumin molecules occur at acidic pHs and affect the emulsifying properties.

Formation of a stable emulsion was not established below 0.5% protein concentration under the conditions used herein. Oil-phase volume was an extremely important factor in the formation of a stable emulsion. As the concentration of oil was increased, a higher stability rating of the emulsion was observed, especially at higher protein concentration, which seemed to be due to the remarkable increase of the viscosity of the emulsion. The amount of protein adsorbed on the fat globule surface was also correlated to the concentration of the ovalbumin dispersion. Studies on the emulsions prepared from meat protein and corn oil (Acton and Saffle, 1970, 1971) have shown that the increase in the protein concentration facilitates the adsorption of the protein and the increase in the adsorbed protein may cause a reduction of the interfacial tension which increases stability of the emulsion.

Though the surface hydrophobicity of ovalbumin significantly changed when the pH was lowered, no significant difference was found between the secondary structure of ovalbumin at pH 3 and that at pH 7 as observed by far-UV CD measurement, but the near-UV CD spectrum in pH 3 was different from that in pH 7. The decrease in ellipticity in the positive peaks around 255–295 nm indicates small conformational changes of the ovalbumin molecule around its tyrosine and tryptophan residues. The fluorescence spectrum of ovalbumin at pH 3.0 was also different from that at pH 7.0. These changes have occurred because of a change in the ionic state of carboxyl ions that are close to tryptophan residues.

The line width of phosphorus is much influenced by the motional properties of phosphoprotein (Vogel, 1989). The line width of $^{31}P$ NMR spectra on ovalbumin was sharper at pH 3.0 than at pH 8.0. These results suggest that the conformation of ovalbumin was more flexible at acidic pHs than at neutral pHs.

Recently, the existence of a new type of structure state, the "molten globule state", has been reported in acidic conditions for certain globular proteins (Ohgushi et al., 1983; Ptitsyn, 1987; Arakawa et al., 1987; Baum et al., 1989). In this state, the molecule is the compact globule form just as in the native state, but the tertiary structure can slowly fluctuate. Koseki et al. (1988) have reported that the molten globule state was observed with ovalbumin at lower pH. From these findings, it is suggested that the globular conformation and secondary structure of oval-

bumin may be almost the same at acidic and neutral pHs. However, the side chains in the molecule are more flexible at lower pHs than at neutral pHs, and they are very susceptible to denaturation.

It is concluded that the emulsifying activity of ovalbumin was higher at lower pHs than at neutral pHs, because of the greater surface hydrophobicity and flexibility of the molecule at acidic conditions.

Detailed analyses on the protein structure and adsorption behavior of ovalbumin are necessary to understand the mechanism of the adsorption of protein on fat–water interfaces.

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