Effects of a Limited Proteolysis of Ovalbumin on Interfacial Adsorptivity Studied by ^{31}P Nuclear Magnetic Resonance

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Effects of a limited proteolysis of ovalbumin on the emulsifying property were investigated with ^{31}P NMR spectra in correlation with the interfacial adsorptivity of the protein. The interfacial adsorptivity was evaluated by the droplet size distribution. The interfacial adsorptivity of *p*-ovalbumin, which was formed by cleaving the N-terminal residues (1–22) of ovalbumin, were markedly decreased, but those of plakalbumin, which was formed by cleaving the C-terminal residues (346–385) of ovalbumin, were increased. The line widths of the phosphorus signals of plakalbumin were much broadened by emulsification and those of *p*-ovalbumin were scarcely changed by emulsification compared to those of ovalbumin. The dynamic states of two phosphoserine moieties of ovalbumin in emulsions were elucidated by their susceptibility to acid phosphatase digestion. The line widths of ^{31}P NMR spectra were well correlated with the interfacial adsorptivity of ovalbumin.

INTRODUCTION

Proteins are generally surfactive, and many proteins have good emulsifying properties which are beneficial for manufacturing various emulsified foods (Mitchell, 1968). The emulsifying property depends strongly on protein–lipid interactions occurring at an oil/water interface during emulsification. As a result of this widespread importance, there is a long history of studies of interfacial adsorptivity of proteins and stabilized emulsions with proteins (Kitchener and Mussellwhite, 1968; Miller, 1971; MacRitchie, 1978). Various factors that affect the emulsifying properties of proteins including the adsorption on interfaces were proposed by many investigators (Carpenter and Saffle, 1965; Peason et al. 1965; Birdi, 1978; Tornberg, 1978; Friberg, 1976; Yamauchi et al., 1980; Phillips, 1981). However, the mechanism of protein–lipid interaction at an oil/water interface is not clearly understood.

Ovalbumin, a major protein of egg white, is critical to functional properties of egg white such as the gelling, foaming, and emulsifying properties. Previously (Mine et al., 1991), we have studied the emulsifying properties of ovalbumin at various pHs in relation to its molecular structure and found that emulsifying activity of ovalbumin is higher at acidic conditions. The emulsifying properties of ovalbumin were improved by coupling with dextran (Kato et al., 1990). It is necessary to investigate closely the relationship between the protein structure and the emulsifying properties of ovalbumin.

It is an interesting problem to elucidate the dynamic state of a protein molecule on the interface, but there is much difficulty in the evaluation of the dynamic state of protein by conventional physicochemical techniques. NMR methods provide useful information about lipid–protein interactions (Seelig, 1978; Trumbetas et al., 1979; Yeagle, 1982) or characterization of emulsion (Van Den Enden et al., 1990; Lönnqvist et al., 1991). Previously, we investigated the interfacial adsorptivity of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) by ^{31}P NMR and found that the headgroup motion of PC and LPC was correlated with the interfacial adsorptivities and with their emulsion stability (Chiba and Tada, 1989, 1990a,b). The line width of ^{31}P NMR spectra of LPC was well correlated with its interfacial adsorptivity. Ovalbumin contains two phosphoserine residues at serines 68 and 344 that give rise to two well-resolved signals in a ^{31}P NMR spectrum (Vogel and Bridger, 1982). Thus, the ^{31}P NMR technique can be expected to be applied in the evaluation of the interfacial adsorptivity of ovalbumin.

The limited proteolysis of protein is a useful technique to elucidate the relationship between the molecular structures and functional properties of proteins (Shimizu et al., 1987). Ovalbumin is hydrolyzed strictly into *p*-ovalbumin by removing the N-terminal residues (1–22) with pepsin (Kitabatake et al., 1988) and into plakalbumin by removing the C-terminal residues (346–385) with subtilisin (Ottesen, 1958).

In this paper, we discuss the effects of limited proteolysis on emulsifying properties of ovalbumin and the relationship between the interfacial adsorptivity and the line widths of ^{31}P NMR of ovalbumin in emulsions.

MATERIALS AND METHODS

Preparation of Ovalbumin. Ovalbumin was prepared from fresh egg white by crystallizing out of aqueous sodium sulfate and recrystallized from aqueous ammonium sulphate five times (Kekwick et al., 1936).

Preparation of *p*-Ovalbumin and Plakalbumin. *p*-Ovalbumin was prepared by pepsin digestion (Kitabatake et al., 1988). Pepsin crystals (Sigma, from porcine stomach mucosa, 1:60 000) were added to a 1% ovalbumin solution (w/v) in 0.1 M sodium acetate buffer (pH 4.0) to give an enzyme/substrate ratio of 1/25 (w/w), and the mixture was incubated at 25 °C for 20 h. After incubation, the reaction was stopped by adjusting to pH 7.5 with 0.1 N NaOH. This solution was dialyzed against water to be salt free, followed by lyophilization. Plakalbumin was prepared according to the method of Ottesen (1958). Subtilisin (Novo Industry) was added to a 1% ovalbumin solution (w/v) in 0.01 M sodium phosphate buffer (pH 6.3) to give an enzyme/substrate ratio of 1/2000 (w/w), and the mixture was incubated at 30 °C for 1 h. After incubation, the reaction was stopped by adjusting to pH 4.0 with 0.1 N HCl. This solution was dialyzed against water (pH 4.0) and lyophilized. The crude *p*-ovalbumin or plakalbumin was purified by CM-cellulose column chromatography using a 0.1 M ammonium acetate buffer (pH 6.2) or a linear gradient of 0.1 M ammonium acetate buffer (pH 6.2)–0.025 M

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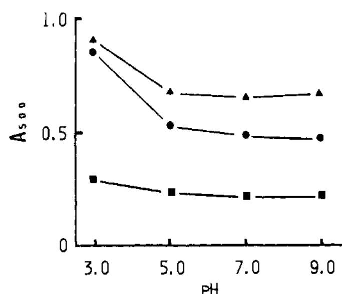

Figure 1. Emulsifying activity of ovalbumin (●), *p*-ovalbumin (■), and plakalbumin (▲) at different pHs. Twenty-five percent of soybean oil (w/w) in 1% protein (w/v) dispersion was emulsified.

sodium carbonate buffer (pH 10) until a single band was given by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Emulsification and Evaluation of Emulsion. The emulsifying properties were determined by the turbidimetric technique (Pearce and Kinsella, 1978). Three milliliters of 1 or 5% protein (w/v) solution in distilled water, adjusted to pH 3.0–9.0 by HCl or NaOH, was mixed with various amounts of soybean oil to prepare emulsions. The mixture was homogenized with a Physcotron instrument (Nition Rikaki Corp., Tokyo) equipped with generator shaft NS-10 at 12 000 rpm for 3 min at 20 °C. A 25-μL emulsion sample was taken from the bottom of the container and diluted with 5 mL of 0.1% SDS solution. The emulsifying activity was determined by the absorbance measured immediately after emulsification. The particle size was measured by centrifugal sedimentation (particle size distribution analyzer, Horiba CAPA-700, Tokyo) or by laser light scattering photometer (submicron particle sizer, Pacific Scientific Nicomp Model 370-HPL).

Measurement of Surface Hydrophobicity. The surface hydrophobicity of the proteins was determined by using *cis*-parinaric acid (Kato and Nakai, 1980).

Measurement of Protein Adsorbed on the Interface. The amount of protein adsorbed on the interface surface in emulsion was measured as follows. Immediately after the emulsification, it was centrifuged at 1000*g* for 20 min, and the aqueous portion was removed by aspiration. Three-fold-distilled water, pH adjusted to 3.0–9.0, was added to the floated cream portion. 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 interface was extracted with SDS and 2-mercaptoethanol (2-ME) as follows. One milliliter of 4% SDS-1% 2-ME was added to 1 mL of the washed cream, and the mixture was heated in a boiling water bath for 5 min. The content of protein was determined according to the standard Kjeldahl procedure (Kj. N. × 6.52).

Measurement of ^{31}P NMR Spectra. ^{31}P NMR measurements were performed at 20 °C on a Varian VXR-4000s spectrometer at 161.0 MHz, fitted with a probe (10 mm, 45–165 MHz frequency), using a 45° pulse, with 32K data points, a 40 000-Hz spectral window, a 20 round/s spinning rate, and a 2.0-s pulse delay. Proton was fully decoupled by 20 power mode and 9900-Hz decoupler modulation frequency. NMR samples were 3.1 mL in 10-mm precision tubes. The line widths were measured from the signals at half-height.

Phosphatase Digestion. Phosphatase digestion was performed by incubating 3 mL of 5% protein in 10 mM sodium acetate buffer, pH 3.0, or emulsion prepared at pH 3.0 containing same protein with 16 units of acid phosphatase (from potato type II, Sigma) for a given time. The degradation percentage of the two phosphoserine residues was measured by ^{31}P NMR spectra after incubation for a given time.

RESULTS AND DISCUSSION

Figure 1 shows the emulsifying activities of ovalbumin, *p*-ovalbumin, and plakalbumin. The emulsifying activity of ovalbumin was high at pH 3.0 and decreased with an increase of pH. The emulsifying activity of plakalbumin was about 1.5 times higher than that of ovalbumin at pH 5.0–9.0. On the other hand, the emulsifying activity of

Table I. Adsorption of Ovalbumin, *p*-Ovalbumin, and Plakalbumin on the Interface Prepared at Different pHs[a,b]

protein adsorbed, mg/g of oil	pH 3	pH 5	pH 7	pH 9
ovalbumin	11.34 ± 0.8	8.68 ± 0.6	5.88 ± 0.4	4.92 ± 0.4
p-ovalbumin	4.12 ± 0.3	3.00 ± 0.2	3.08 ± 0.3	3.33 ± 0.4
plakalbumin	20.72 ± 1.2	17.73 ± 1.3	16.74 ± 0.9	14.88 ± 0.7

[a] 25% of oil (w/w) in 1% ovalbumin (w/v) dispersion was emulsified. [b] Each value is an average of three replications ± standard deviation.

Table II. Mean Droplet Size of Emulsion Composed of Ovalbumin, *p*-Ovalbumin, and Plakalbumin Prepared at Different pHs[a,b]

	pH 3	pH 5	pH 7	pH 9
ovalbumin	12.0 ± 0.4	15.1 ± 0.5	17.0 ± 0.4	15.9 ± 0.6
p-ovalbumin	38.0 ± 1.5	39.0 ± 2.2	43.1 ± 2.3	44.1 ± 1.8
plakalbumin	6.7 ± 0.3	8.1 ± 0.6	10.7 ± 0.9	12.3 ± 0.7

[a] 25% of oil (w/w) in 1% ovalbumin (w/v) dispersion was emulsified. [b] Each value is an average of three replications ± standard deviation.

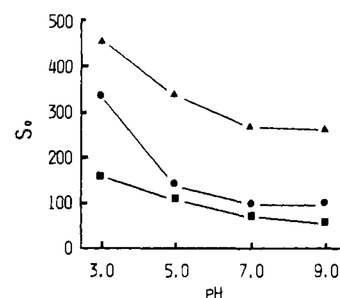

Figure 2. Surface hydrophobicity of ovalbumin (●), *p*-ovalbumin (■), and plakalbumin (▲) at different pHs. S_0 is expressed as relative values.

p-ovalbumin was markedly decreased at all pHs used herein. The emulsion stability of *p*-ovalbumin was very low, and oil was separated immediately after emulsification. These results suggest that the N-terminal residues are important for the emulsifying property of ovalbumin.

Tables I and II show the amount of proteins adsorbed on the interface and the mean droplet size of emulsion prepared at pH 3.0–9.0, respectively. The interfacial adsorption of protein and the mean droplet size of emulsion were dependent on the pH of the dispersion of protein. The amount of the adsorbed protein was decreased, and the droplet size of emulsion became larger with increasing pH. The amount of plakalbumin adsorbed on the interface was about 2–3 times larger and formed smaller droplets than ovalbumin. On the other hand, the amount of *p*-ovalbumin adsorbed on the interface was lower than that of ovalbumin. A significant correlation was observed between the emulsifying capacity and the hydrophobicity of proteins determined fluorometrically (Kato and Nakai, 1980). The N-terminal residues (1–22) of ovalbumin are hydrophobic and are in the core of the protein, while the C-terminal residues (346–385) are normally on the surface of the protein (Nisbet et al., 1981; Wright et al., 1990). Thus, we expected that the surface hydrophobicity of ovalbumin played an important role in its emulsifying property and that it is changed by partial hydrolysis. Figure 2 shows the surface hydrophobicity of ovalbumin, *p*-ovalbumin, and plakalbumin at different pHs. The surface hydrophobicity of these proteins was maximum at pH 3.0 and decreased with increasing pH. The surface hydrophobicity of plakalbumin was higher and that of *p*-ovalbumin was lower in comparison with that of ovalbumin. These results show that the surface hydrophobicity of oval-

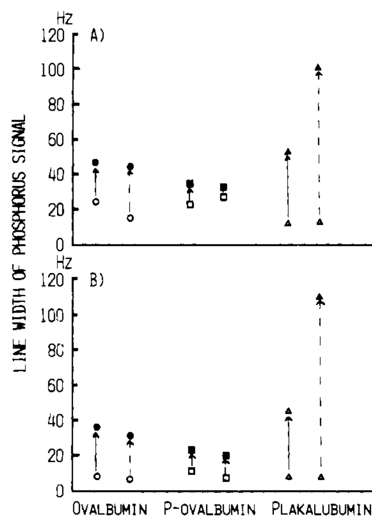

Figure 3. Phosphorus line widths of ovalbumin (●), *p*-ovalbumin (■), and plakalbumin (▲) in aqueous dispersion and emulsion at pH 9.0 (A) and 3.0 (B). (—) SerP-68; (- - -) SerP-344. Twenty-five percent of soybean oil (w/w) in 1% protein (w/v) dispersion was emulsified.

bumin decreased by the removal of the N-terminal residues and increased by the removal of the C-terminal residues. The effect of limited proteolysis on the secondary structure of ovalbumin was studied by circular dichroism analysis (CD), however, no significant difference was observed for ovalbumin, *p*-ovalbumin, and plakalbumin (data not shown). The results indicated that the removal of the N- or C-terminal residues of ovalbumin does not affect its secondary structure but changed their surface hydrophobicity.

From these observations, the difference in the emulsifying properties of ovalbumin and its partially hydrolyzed forms can be predicted to correlate with their dynamic states on the interface. We expected that the differences of phosphorus signals of ^{31}P NMR were observed between the highly adsorptive protein and lesser ones. Thus, the aqueous dispersion and emulsion composed of ovalbumin, *p*-ovalbumin, and plakalbumin prepared at pH 3.0 or 9.0 were subjected to ^{31}P NMR analysis, and the line widths of ^{31}P NMR spectra were measured (Figure 3). The line widths of ^{31}P NMR spectra of ovalbumin and plakalbumin obviously broadened by emulsification, and the broadening ratio of the line widths of ^{31}P NMR spectra were larger at pH 3.0 than those of ones at pH 9.0. The phosphorus line widths of plakalbumin in emulsion were broader than those of ovalbumin. In contrast, the line widths of ^{31}P NMR of *p*-ovalbumin were scarcely changed by emulsification compared to those of ovalbumin and plakalbumin. These data are well correlated to the results of the amount of adsorbed protein on the interface and the mean droplet size of emulsion of ovalbumin and its partially hydrolyzed forms (Tables I and II). The phosphorus signals of ^{31}P NMR spectra are influenced by the motional properties of the phosphate moiety in the molecules, and peak broadening of phosphorus signals of ^{31}P NMR spectra is observed by increasing the magnitude of the negative phosphorus chemical anisotropy (Wu et al., 1984; Gorenstein, 1982). From these results, the peak broadening of ^{31}P NMR spectra was interpreted as indicating that motions of the phosphoserine moiety in ovalbumin and plakalbumin were restricted by adsorbing on the interface. It was also suggested that the extensive peak broadening of ^{31}P NMR spectra of plakalbumin in

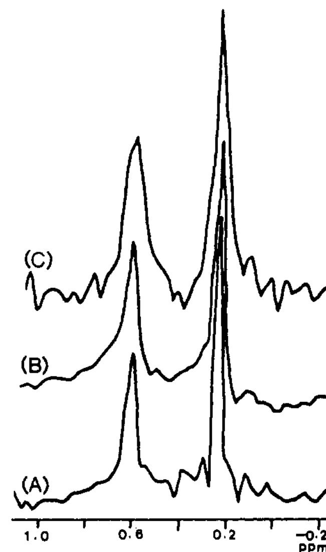

Figure 4. ^{31}P NMR spectra of ovalbumin in an aqueous dispersion and emulsions. (A) Aqueous dispersion of 5% ovalbumin at pH 3.0.; (B, C) emulsions at pH 3.0. The oil/protein (w/w) ratios were 4 and 8, respectively. Chemical shifts were referenced to 85% H_3PO_4 at 0 ppm.

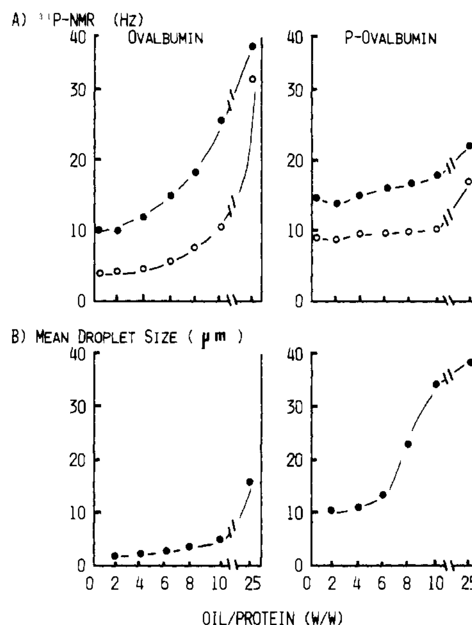

Figure 5. Changes of phosphorus line widths (A) and the mean droplet size of the emulsion (B) of ovalbumin or *p*-ovalbumin at varied oil/protein (w/w) ratios. (A) (●) SerP-68; (○) SerP-344. The emulsions were prepared with 5% ovalbumin dispersion in distilled water at pH 3.0 and with soybean oil. The droplet size was measured by the submicron particle sizer or particle sizer distribution analyzer.

emulsion was correlated with its higher interfacial adsorptivity and the amount of protein adsorbed on the interface.

Consequently, we attempted to evaluate the relationship between the line widths of ^{31}P NMR spectra of ovalbumin and its interfacial adsorptivity at various oil/protein (w/w) ratios. Figure 4 shows typical ^{31}P NMR spectra of ovalbumin in an aqueous dispersion and in emulsion at the various oil/protein (w/w) ratios. In the emulsion of ovalbumin, the phosphorus line widths became broader with the increase of oil/protein ratio. The relationship between the line widths of ^{31}P NMR spectra and the mean droplet size in the emulsion of ovalbumin and *p*-ovalbumin at the various oil/protein ratios is shown in Figure 5.

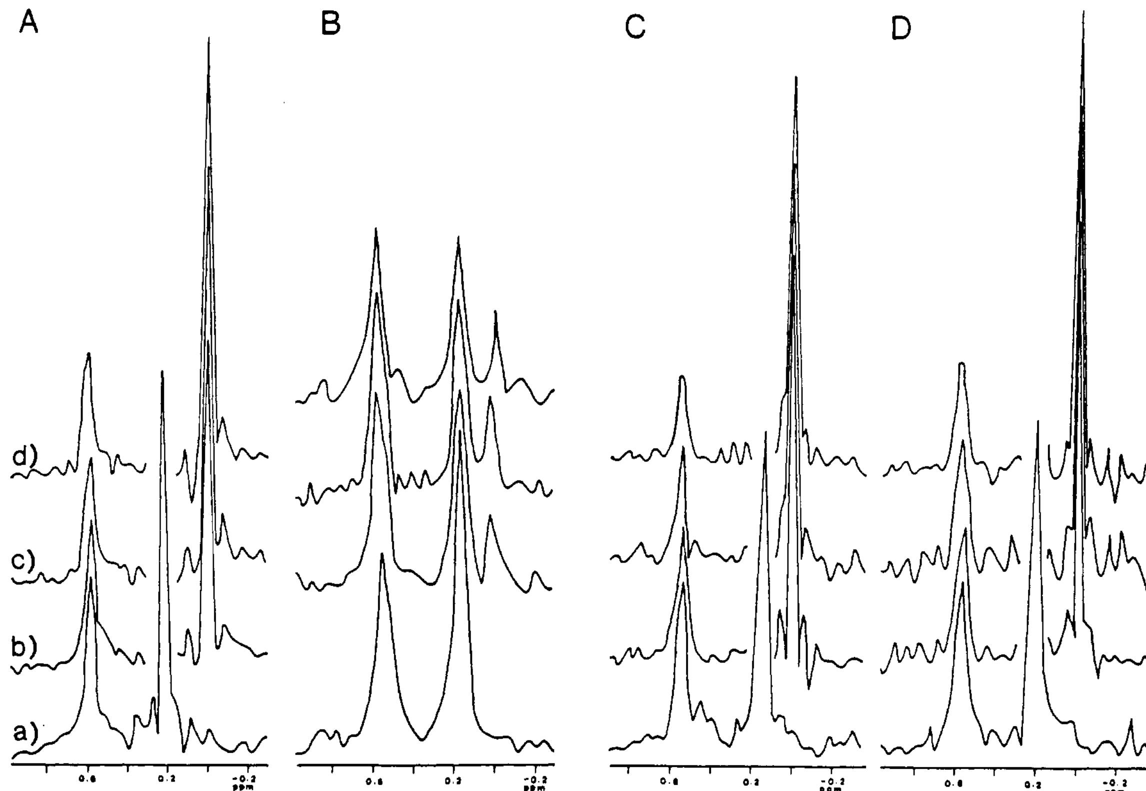

Figure 6. ^{31}P NMR spectra of ovalbumin and *p*-ovalbumin treated with acid phosphatase for 0 (a), 6 (b), 12 (c), and 18 h (d) in aqueous dispersion and emulsion. (A, B) Ovalbumin; (C, D) *p*-ovalbumin; (A, C) aqueous dispersion; (B, D) emulsion. The oil/protein (w/w) ratio was 8. Chemical shifts were referenced to 85% H_3PO_4 at 0 ppm.

When the oil/ovalbumin (w/w) ratio was under 8, the line width of ^{31}P NMR spectra gradually increased by emulsification and the mean droplet size was less than 3.0 μm. The mean droplet size became larger with the increase of the oil/protein (w/w) ratio over 10, and emulsion became unstable. It was suggested that the amount of ovalbumin was in excess when the oil/protein ratio was under 6, and excess ovalbumin existed in an aqueous phase and could be adsorbed on the interface with increasing oil content. The peak broadening of ^{31}P NMR spectra was interpreted as indicating that the motions of the phosphoserine moiety in ovalbumin were restricted by adsorbing on the oil/water interface. On the other hand, the amount of ovalbumin became deficient with increase of the oil/protein (w/w) ratio over 10 and larger droplets were formed. Moreover, the phosphorus line widths of these emulsions were much broadened. The reason for these phenomena was assumed to be that the motions of the phosphoserine moiety in ovalbumin were more restricted due to the changes of curvature of the droplet surface by increasing droplet size. Interestingly, the line widths of ^{31}P NMR spectra of *p*-ovalbumin scarcely changed despite the increasing amount of oil. The mean droplet size was very large (>10 μm), and the emulsion was unstable at any stage employed here. These results suggested that the interfacial adsorptivity of *p*-ovalbumin was markedly lower and that most of the *p*-ovalbumin was not adsorbed on the interface by emulsification despite the increasing amount of oil.

Furthermore, we investigated the susceptibility to acid phosphatase digestion of the emulsion composed of ovalbumin or *p*-ovalbumin when the oil/protein (w/w) ratio was 8 at pH 3.0 to confirm the relationship between the line width of ^{31}P NMR spectra and the interfacial adsorptivity of the proteins. Figure 6 shows typical ^{31}P NMR spectra of ovalbumin and *p*-ovalbumin in aqueous dispersion and emulsion treated with acid phosphatase. The three separated phosphorus signals were assigned to phosphoserine-68, phosphoserine-344 of ovalbumin or *p*-ovalbumin, and inorganic phosphate released from the proteins by acid phosphatase digestion (Vogel and Bridger, 1982). We determined the degradation percentage of the two phosphoserine residues of ovalbumin and *p*-ovalbumin by comparing the ^{31}P NMR spectra after incubation with acid phosphatase for a given time in aqueous dispersion and emulsion (Figure 7). ^{31}P NMR analysis of the time course of digestion by acid phosphatase showed that phosphoserine-344 was always more susceptible than phosphoserine-68 of ovalbumin and *p*-ovalbumin. Vogel and Bridger (1982) have reported that phosphoserine-344 is mobile as it exists on the surface of the protein molecule but that phosphoserine-68 is more restricted as it is in the interior of the protein. The line width of phosphoserine-344 in ovalbumin and plakalbumin was more broadened than that of phosphoserine-68 by emulsification (Figure 3). The susceptibility to acid phosphatase digestion of the two phosphoserines of ovalbumin was decreased by

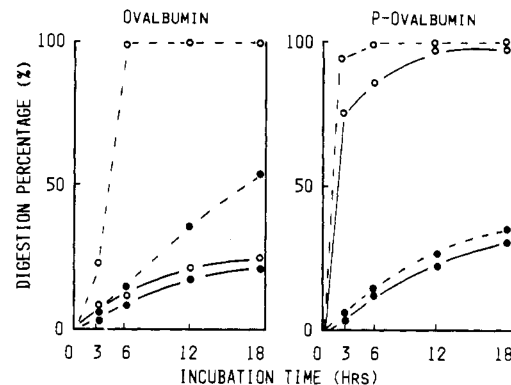

Figure 7. Degradation percentage curves of the two phosphoserine residues of ovalbumin or *p*-ovalbumin in aqueous dispersion and emulsion. (●) SerP-68; (○) SerP-344; (- - -) aqueous dispersion; (—) emulsion.

emulsification; the acid phosphatase digestion of phosphoserine-344 was much decreased by emulsification. The results suggest that the phosphate residue of phosphoserine-344 is restricted by the interaction between the part of the protein molecule around phosphoserine-344 and the oil phase. In contrast, the susceptibility to acid phosphatase digestion of *p*-ovalbumin was scarcely influenced by emulsification, and the motion of two phosphoserines of *p*-ovalbumin was not restricted by emulsification. These results agreed well with the relationship between the line width of ^{31}P NMR spectra and interfacial adsorptivity of ovalbumin or *p*-ovalbumin.

In conclusion, the emulsifying activity of ovalbumin was much influenced by limited proteolysis in correlation with its interfacial adsorptivity. The interfacial adsorptivity of ovalbumin is markedly decreased by removing the N-terminal residues and increased by removing the C-terminal residues. The line width of ^{31}P NMR spectra of ovalbumin and partially hydrolyzed ovalbumin was well correlated with their interfacial adsorptivities. ^{31}P NMR spectra in emulsion of ovalbumin provide an effective index in the evaluation of its interfacial adsorptivity.

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