Structural and Functional Changes of Hen's Egg Yolk Low-Density Lipoproteins with Phospholipase A_2

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Structural and functional changes of hen's egg yolk low-density lipoproteins (LDL) as a result of modifying its phospholipids (PLs) using phospholipase A_2 were examined. The ^{31}P NMR spectrum and enzyme hydrolysis profiles revealed that the PLs are more susceptible in LDL complex than in small unilamellar vesicles (SUV) or PLs emulsions, suggesting higher membrane fluidity of LDL and that the interactions of proteins with PLs are not strong. Although the modification of PLs in LDL did not affect the secondary structure of proteins or immunological property of LDL, the emulsions stabilized with the modified LDL showed considerable heat stability. The differences in thermal behavior of modified LDL by DSC analysis suggested the formation of a structurally heat-stable complex of LysoPLs/polypeptides in LDL during heat treatment. The enhancement of PLs−protein interactions in LDL by phospholipase A_2 could be responsible for the heat stability of emulsions made from the modified LDL.

Keywords: *Egg yolk; low-density lipoproteins; phospholipids−protein interaction; emulsion; heat stability; phospholipase A_2; structural stability*

INTRODUCTION

The major portion of hen's egg yolk is the plasma (~78% of the total liquid yolk). Plasma is composed of livetins and low-density lipoproteins (LDL) (McCully et al., 1962). Hen's egg yolk LDL contains ~12.5% protein and ~80% lipids (Tarner and Cook, 1958). The lipid in LDL consists of 74% neutral lipid (including ~4% free cholesterol and 0.2% of the ester) and 26% phospholipids (PLs) [71−76% phosphatidylcholine (PC), 16−20% phosphatidylethanolamine (PE), and 8−9% sphingomyelin and lysophospholipids (LysoPLs)] (Martin et al., 1963). The diameters of LDL micelles range from ~17 to 60 nm with an average of ~30 nm (Kamat et al., 1972). The structure of hen's egg yolk LDL was incorporated in general theories on the structure of human blood LDL at an early date (Cook and Martin, 1962; Schneider et al., 1973). According to human LDL, triacylglycerols and cholesteryl esters occupy the center of globular lipoproteins particles and the surface consists of PLs, cholesterol, and proteins. Kamat et al. (1972) found that the polar headgroups of PLs in LDL were hindered in molecular motion because of an interaction presumably with LDL polypeptides. It has been suggested that the proteins and PLs of LDL are grouped together in small particles, which are adsorbed to the surface of the triglycerides core (Chang et al., 1977). The surface layer of LDL is probably heterogeneous, and the lipid−protein components do not form a stable association of the kind demonstrated in a number of biological membranes (Holdsworth and Finean, 1972). The lipid core model (Cook and Martin, 1962; Schneider et al., 1973) may be essentially correct, but the different structures of blood and hen's egg yolk lipoproteins is suggested (Burley et al., 1988).

On the other hand, egg yolk is well-known as an effective emulsifying agent for food manufacture. LDL is considered to be the most important contributor to the emulsifying properties of egg yolk (Vincent et al., 1966; Mizutani and Nakamura, 1984). However, structural−functional properties of egg yolk LDL are not well-known. The emulsifying properties and heat stability of protein emulsions were improved substantially through the formation of a complex between the phospholipase-hydrolyzed lecithin (lysolecithin) and free fatty acids (Mine et al., 1992a,b, 1993). Emulsifying capacity and heat stability of egg yolk were improved by fermentation with pancreatic phopholipase (Dutilh and Groger, 1981). This indicates that the emulsifying properties of LDL might be closely related to the structure of PLs−protein complexes in LDL and their interactions at an oil-in-water interface. The main PL in LDL is PC, and it is converted to lysophosphatidylcholine (LPC) by phospholipase A_2. LPC exists as a minor component in PLs but has higher water solubility and emulsifying properties (Ziegeliz, 1995). In this study, the structural and functional changes of egg yolk LDL as related to its emulsifying property were investigated by modifying PC in LDL with phospholipase A_2. The PLs−protein interaction in LDL is also discussed.

MATERIALS AND METHODS

Materials. Fresh shell eggs (White Leghorn) were obtained from Arkel Research Center, University of Guelph. LDL was prepared from fresh egg yolk according to the modified method of Raju and Mahahevan (1974). Triolein (65% practical grade) was obtained from Sigma Chemical Co. (St. Louis, MO) and was purified using silica gel column chromatography (hexane/diethyl ether, 97:3). Egg yolk PC, PE, and LPC were kindly provided from Q. P. Corp., Tokyo, Japan. Other chemicals were purchased from Fisher Scientific (Ottawa, Canada).

Preparation of PLs Vesicles. The lipid mixture containing 90 mg of egg PC, 26 mg of PE, and 20 mg of cholesterol in chloroform/methanol solvent (2:1, v/v) was introduced into a 250 mL round-bottom flask and evaporated using a rotary evaporator under nitrogen gas with the temperature set at 25 °C. After 10 mL of 50 mM imidazole buffer, pH 7.2, was added, the solution was sonicated for 5 min at 60 °C using a Sonifier 250 (Branson, German) at an output of 40 W. After sonication had finished, the liposomes were placed in a centrifuge at 100000*g* for 30 min at 20 °C to sediment a trace of titanium particle which may be liberated from the probe and large

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vesicles. The size of sonicated vesicles was measured by photon correlation spectroscopy (PCS) using a Malvern 4700 optical system attached to a 7032 correlator (Malvern Instruments Inc., Southboro, MA). Measurements were all made at a scattering angle of 90°.

Hydrolysis of LDL, PLs Emulsion, and Vesicle. Oil-in-water emulsions (20 wt % triolein) stabilized with LDL (40 mg/mL) or small unilamellar vesicle (SUV)-PLs (23.2 mg/mL) in 50 mM imidazole buffer, pH 7.2, were prepared using a Microfluidizer (Model 110s, Microfluidics Co., Newton, MA) at an input pressure of 0.2 MPa. Each sample was circulated through the homogenizer for 10 strokes of the pump. The particle size of emulsion was measured by a Mastersizer X. Each 1.0 mL of emulsion (20% oil), containing about 7.2 mg of PC or 32 mg of LDL, and 0.8 mL of SUV-PLs (containing about 7.2 mg of PC) in imidazole buffer, pH 7.2, containing 5 mM $CaCl_2$ was incubated with 0.2 mL of phospholipase A_2 (100 IU/mL, Novo Nordisk, Denmark) at 40 °C for a given time. After incubation, each solution was mixed with 1 mL of 1 M HCl to stop the enzyme reaction and 5 mL of methanol and dried by rotary evaporator. The dried materials were dissolved in 1 mL of chloroform/methanol (2:1, v/v). The liberated LPC was isolated by silica column chromatography (chloroform/methanol/water = 65:25:4, v/v). The production of LPC as the interaction of phospholipase A_2 was quantified using an HPLC (Lichrospher Si-60, 10 mm, Shodex RI SE−50, Toyo Soda CCPM pump) with acetonitrile/methanol/water (75:21:18, v/v) as a mobile phase.

Heat Stability of LDL Emulsions. Oil-in-water emulsions (20% triolein) with 40 mg/mL LDL or modified LDL with phospholipase A_2 in 50 mM imidazole buffer, pH 7.2, were prepared using a homogenizer (Polytron PT 2000, Kinematica, AG, Switzerland) at 22 000 rpm for 1 min. One milliliter of each emulsion was heated at 90 °C for 10 min and cooled to room temperature. The heat stability of the emulsions was evaluated by analyzing the change in particle size of emulsions using a Mastersizer X.

Circular Dichroism Analysis. Far-UV circular dichroism (CD) spectra (260−200 nm) were measured using a JASCO J-720 spectropolarimeter with a 10 mm cell at 25 °C, and data were recorded on-line using an IBM PC. Typically, solutions contained 4 mg/mL LDL, and phospholipase A_2 hydrolyzed LDL in 20 mM phosphate buffer, pH 7.2, containing 0.17 M NaCl was used. To monitor changes in the conformation of LDL during heating, the changes in the CD spectrum at 220 nm were measured. LDL solution (40 mg/mL in phosphate buffer) in a stoppered cuvette (10 mm) was placed in a thermostatically controlled cell holder, and the temperature was varied at a linear rate of 50 °C per hour over the range 50−90 °C. The data were represented in terms of mean residual negative ellipticity (deg·cm^2·dmol^{-1}).

DSC Analysis. Thermal characteristics of LDL were determined using a differential scanning calorimetric (DSC) analyzer (Shimadzu, DSC-50, Kyoto, Japan). A total of 20 μL of LDL or modified LDL solution (50 mg of protein/mL in 20 mM phosphate buffer saline, pH 7.2) was sealed in a preweighed hermetic silver pan. Deionized water was used as a reference. The pans were heated in the calorimeter at a linear rate 2 °C/min over the range 25−110 °C. The peak temperature (T_d) and apparent enthalpy change (ΔH) were analyzed by an IBM-PC computer.

Immunological Measurement. The Ouchterlony method (Ouchterlony and Nilsson, 1986) was carried out to confirm the immunological identity of egg yolk LDL using anti-LDL serum from rabbit. The two rabbits (5 months old, female) were immunized intramuscularly with 5 mg of LDL in saline using an equal volume of Freunds' complete adjuvant (Sigma). Booster injections were repeated three times at 2 week intervals (Harlow and Lane, 1988). The rabbit antiserum was kept at −80 °C until use.

Measurements of ^{31}P NMR Spectra. The LDL, SUV−PLs vesicle, and PLs emulsion prepared according to the method described above were subjected to NMR analysis in the presence of 5 mM $PrCl_3$, which was added after preparation of each sample. The phosphorus signal of NMR is split by the addition of $PrCl_3$ in a bilayer vesicle under the influence of paramagnetic Pr^{3+} in rapid exchange between the phosphate sites on the headgroup in PLs. ^{31}P NMR measurements were performed at 20 °C on a Varian VXR-4000s spectrometer at 161.0 MHz, fitted with the probe (10 mm, 45−165 MHz frequency) using a 45° pulse (25 ms) with 32K data points and a 40 000 Hz spectral window. A rotation spinning rate of 20 rotations/s and decoupler modulation frequency were also used. For NMR, 3.1 mL of samples was placed in a 10 mm precision tube. Imidazole buffer, 50 mM and pH 7.2 containing 0.17 M NaCl and 10 mM EDTA, was used for NMR measurement. The line widths were measured from the resonance at half-height.

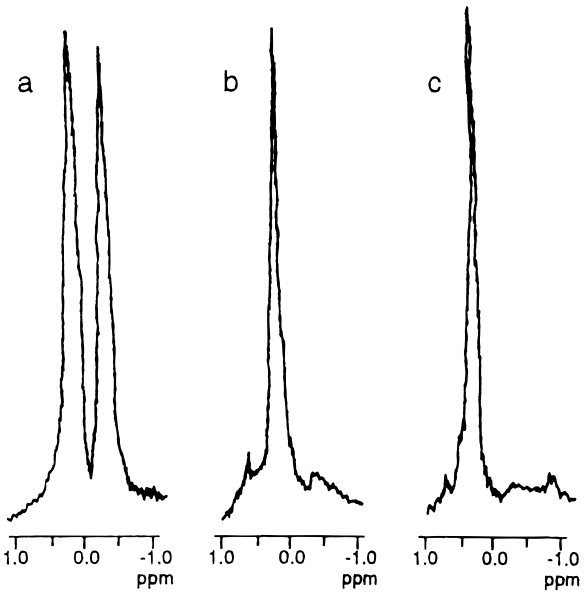

Figure 1. ^{31}P NMR spectra of SUV-PLs, LDL, and PLs emulsion: (a) SUV-PLs; (b) LDL; (c) PLs emulsion. ^{31}P NMR spectra were measured in the presence of $PrCl_3$, which was added to be 5 mM in each sample.

RESULTS AND DISCUSSION

The mean particle sizes of LDL micelles, PLs emulsions, and SUV-PLs vesicles were 47 ± 6, 247 ± 22, and 120 ± 17 nm (n = 5), respectively. The phosphorus signals of SUV-PLs, LDL, and PLs emulsions in the presence of Pr^{3+} showed different patterns (Figure 1). In SUV-PLs vesicle, ∼50% of the phosphorus signal showed a downfield shift (Figure 1a). This indicates that external PLs in a bilayer vesicle showed pseudo contact shift under the influence of paramagnetic Pr^{3+} in rapid exchange between the phosphate sites on the headgroups (Chiba and Tada, 1990). On the other hand, the signals of LDL and PLs emulsions were completely shifted to a downfield shift in the presence of Pr^{3+} (Figure 1b,c). These results show that the PLs of SUV-PLs vesicles were reconstituted on the surface of emulsion droplets after emulsification with triolein. PLs in LDL micelles were also located on the surface of LDL particles as reported previously (Cook and Martin, 1962; Martin et al., 1963; Kamat et al., 1972). In an emulsion state composed of triolein, water, and lipids mixture (PC, PE, and cholesterol), the line widths of ^{31}P NMR spectra of PLs changed to 74 Hz from 104 Hz in SUV-PLs vesicle (Table 1). The phosphorus signals of ^{31}P NMR were influenced by the motional properties of phosphate moieties in molecules (Wu et al., 1984). This result indicates that the headgroup of PLs has motional freedom by reconstituting at the interface. On the other hand, the line width of LDL micelles was broader in comparison with SUV-PLs and PLs emulsion. This may

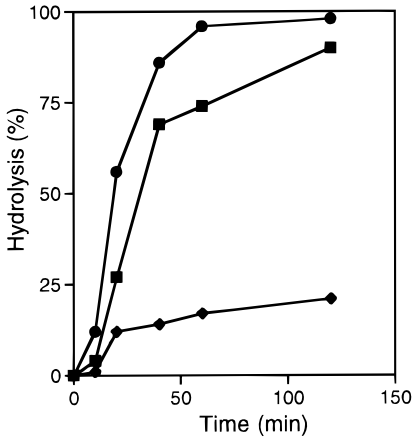

Figure 2. Time courses of phospholipase A_2 hydrolysis of LDL, PLs emulsion, and SUV-PLs: (●) LDL; (■) PLs emulsion; (◆) SUV-PLs.

Table 1. Phosphorus Line Widths of SUV-PLs, LDL, and PLs Emulsion[a]

sample	Hz
SUV-PLs	104 ± 8
LDL	186 ± 13
PLs emulsion	74 ± 6

[a] $n = 4$.

be due to the binding of both PC and PE with lipoproteins. The headgroup of these PLs can be immobilized by interacting with polypeptides in LDL.

The hydrolysis of PLs in SUV-PLs vesicles, PLs emulsion, and LDL micelles by phospholipase A_2 is shown in Figure 2. The hydrolysis reaction profiles and rates were found to depend on a dynamic state of PC in the vesicles or emulsions. The reaction of SUV-PLs vesicles with phospholipase A_2 was slower than that of emulsions. PC in LDL micelles was converted to LPC rapidly, and 96% of PC in LDL was hydrolyzed in 60 min of enzyme treatment. Phospholipase A_2 catalyzes the hydrolytic removal of the acyl moiety at the *sn*-2 position of glyceride molecules. These result indicate the membrane rigidity of SUV-PLs vesicles compared to the emulsion state. Once PLs in the vesicle were reconstituted on the interface, the rigidity of PLs in emulsion became higher and the sensitivity against phospholipase A_2 may be increased. It was reported that lipid and proteins in egg yolk LDL micelles cover areas of the surface and do not form a stable association of the kind demonstrated in a number of biological membranes (Holdsworth and Finean, 1972). It was also suggested that apovitellenin I stabilizes the yolk LDL structure at the surface with PLs arranged strictly parallel with a tightly packed surface (Burley, 1975). The present data support the suggestion that hen LDL has a lipid core/surface layer structure and PLs surround the layer phase. However, the interaction of protein and PLs in the LDL micelles may not be as strong as proposed by Burley (1975).

Figure 3 shows the change of particle size distribution of emulsions stabilized with LDL and modified LDL by phospholipase A_2 after heat treatment. The LDL solution with 92% of PC converted to LPC was used for preparing the emulsion. The mean volume surface diameter ($d_{3,2}$) values of emulsion droplet made from LDL and modified LDL (40 mg/mL of LDL and 20% triolein) were similar (Figure 3). The stability of emulsions composed of LDL was affected by heat treatment and led to oil exudation by heating at 90 °C for 10 min.

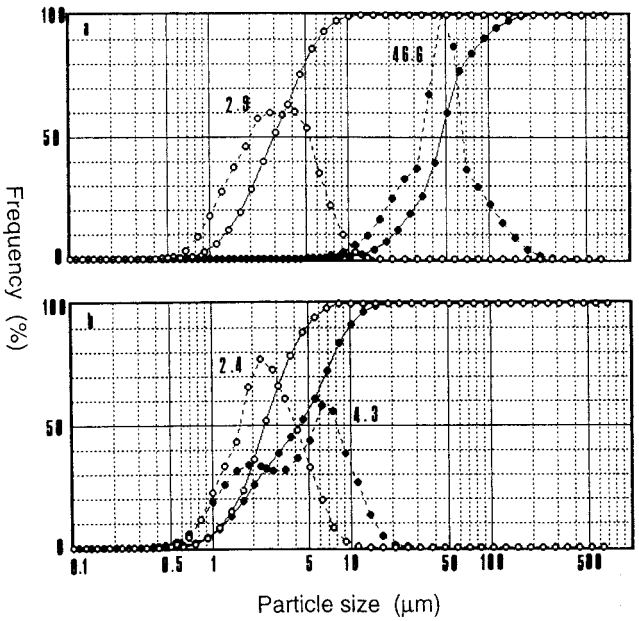

Figure 3. Size distribution of emulsion droplets: (a) emulsion made with LDL; (b) emulsion made with modified LDL; (○) data obtained from fresh emulsions; (●) data from emulsions after heat treatment at 90 °C for 10 min; (− −) frequency of emulsion droplets; (—) accumulation. Parameter was the mean droplet diameter.

The mean droplet size of emulsion made from modified LDL showed heat stability in comparison with unheated one. The particle size distribution of the emulsion shifted toward a little larger size ($d_{3,2}$ = 4.3 μm); however, it remained a stable emulsion after heat treatment. In general, the heat sensitivity of emulsions made with proteins is closely correlated to the conformational change they undergo with heat treatment. It was reported that the mayonnaise made from fermented egg yolk with pancreatic phospholipase A_2 was stable at 100 °C for 30 min without the emulsion breaking (Dutilh and Groger, 1981). It has been suggested that the emulsifying properties of LDL depend on the characteristic structure formed by PL−protein interactions at the interface (Mizutani and Nakamura, 1984). However, the nature of the PLs−protein interactions and the role of PLs in egg yolk lipoproteins as an excellent emulsifier have not been elucidated. Ovalbumin formed a complex with LPC and linoleic acid by sonicating, and the emulsions composed of this complex showed heat stability at 90 °C for 20 min (Mine et al., 1993). This suggests that PLs in LDL may affect the structural changes of LDL with phospholipase A_2. The CD spectra of native and modified LDLs at pH 7.2 are shown in Figure 4. In the far-UV region, no change occurred in the CD spectrum, indicating that no important change in secondary structure of LDL occurred by the modification of PC with phospholipase A_2. A maximum negative ellipticity occurred at 222 nm with shoulders at 205 and 209 nm. Using infrared spectroscopy, Kamat et al. (1972) proposed that the protein in egg yolk LDL appears to be predominantly in disordered and antiparalled β-conformation. The secondary structure of human LDL has α-helix (25%), β-sheet (35%), and random coil (45%), based on the best fit analysis of CD spectrum (Morrisett et al., 1987). It was estimated that the α-helix content in egg yolk LDL was 34.8% according to the modified CD curve-fitting method (Yang et al., 1986). This value is higher than that of human LDL. Thus, it is assumed that the structure of egg yolk

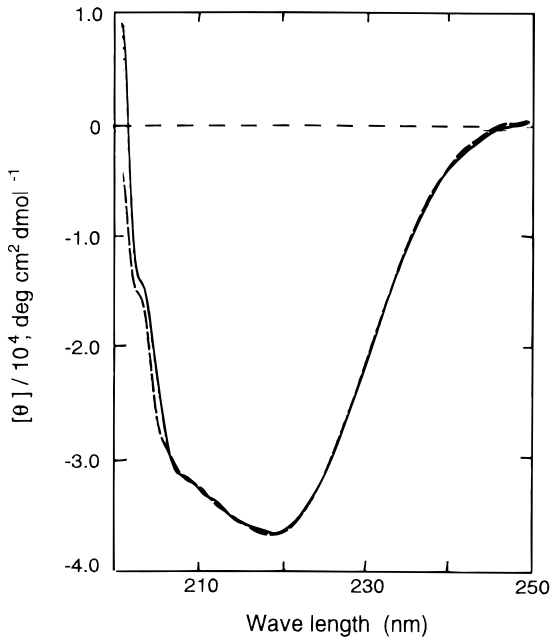

Figure 4. Circular dichroism spectra of LDL and modified LDL: (—) native LDL; (– –) modified LDL.

LDL may be different from human plasma LDLs, which are proposed as amphipathic helical model (Pownall et al., 1987). Recently, one reason to doubt the previously assumed close relationship between the structure of blood and yolk LDL was reported on the basis of both theoretical and experimental facts (Burley et al., 1988). It was also suggested that the association between PLs and proteins in egg yolk LDL is not strong compared to that of human LDL (Holdsworth and Finean, 1972). It is an interesting problem to examine the effect of PLs on the antigenecity of lipoproteins. The LDL modifed with phospholipase D formed a completely fused precipitin line with native LDL against anti-native LDL antibody. However, phospholipase C hydrolyzed LDL did not form any precipitin line with the antibody (Tsutsui, 1985). The changes of the antigenicity of human LDL by delipidation and surfactant and enzymatic digestion were investigated (Sato and Hara, 1969). The apoprotein of human LDL showed a new antigenic property, but the surfactant-treated LDL showed the same antigenic profile as native human LDL. These findings suggest that the modification of PLs in hen's LDL with phospholipase A_2 may affect the immunochemical properties of LDL. The immunochemical properties of the modified LDL were also examined (Figure 5). The precipitin lines between LDL and modified LDL were completely fused, indicating that modification of PC in LDL does not change the antigenicity of egg yolk LDL. Figure 6 shows the changes of α-helix in LDL and modified LDL, which is represented at 222 nm during heating. The two LDL derivatives showed similar profiles up to 80 °C; however, their conformational changes were drastically different at heating temperatures >80 °C. The α-helical content of LDL decreased rapidly at high temperature, while the modified LDL denatured more slowly with the maintenance of considerable secondary structures. Heating at 90 °C for 10 min brought about 40% loss of α-helical content in LDL, but it was only 21% loss in the modified LDL. However, these denaturation profiles of LDL and modified LDL did not agree with the heat sensitivity of the emulsions composed of these LDLs. The emulsions stabilized with LDL were briken down, and almost all

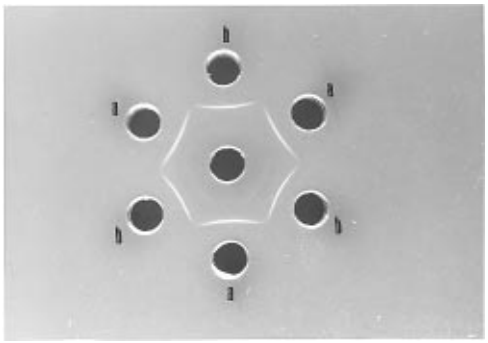

Figure 5. Immunodiffusion pattern of native and modified LDLs: (central well) rabbit antiserum to native LDL (20 μL); (peripheral wells) (a) native LDL; (b) modified LDL (about 30 μg of proteins).

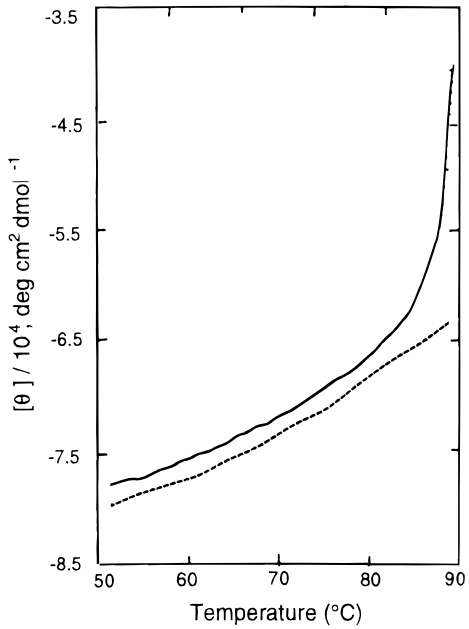

Figure 6. Changes in ellipticity at 222 nm of native and modified LDLs as a function of heating temperature: (—) LDL; (– –) modified LDL.

LDL formed coagulations beyond 75 °C, but not in modified LDL emulsions around 90 °C (data not shown). It may be due to the difference in protein concentration for emulsions (0.5% protein) and the sample used for the CD analysis (0.05% protein). A more important factor could be the difference of PLs–protein interactions between LDL and modified LDL during heating.

The interaction between proteins and surfactant may affect the thermal behavior of proteins, depending on the molar ratio of the bound surfactant. For example, it was shown that binding of SDS anionic surfactant to β-lactoglobulin dimer may increase the thermal stabilization after binding of two molecules. Further binding of SDS led to a decreased thermal stability (Magdassi et al., 1996). The DSC technique is a powerful tool to monitor the thermal behavior of proteins with the interaction of low molecular surfactants. Typical DSC curves obtained with LDL and modified LDL are presented in Figure 7. The corresponding apparent enthalpy (ΔH) changed from 1.25 to 2.82 J/g as a result of modifying PC to LPC in LDL. The peak temperature (T_d) of modified LDL was slightly shifted toward lower temperature (~0.72 °C); however, it was negligible. Previously, it has been shown that the heat stability of ovalbumin is enhanced by coupling with LPC and linoleic acid. LPC bound to the protein through hydro-

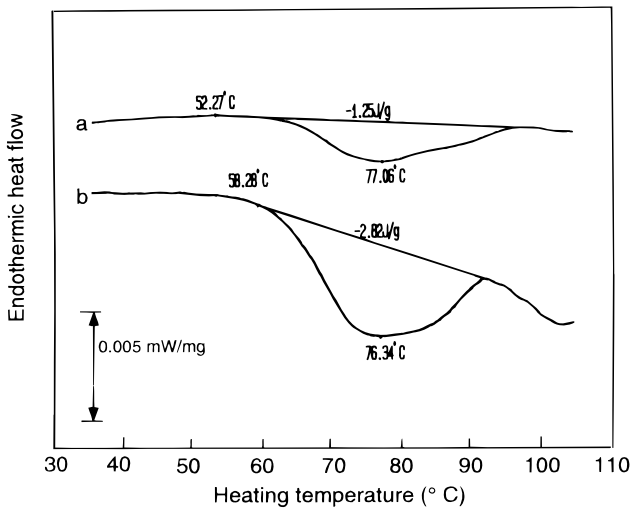

Figure 7. DSC thermograms of native and modified LDLs: (a) native LDL; (b) modified LDL.

phobic interaction and led to conformational change of the protein structure (Mine et al., 1993). The increase in apparent enthalpy (ΔH) on DSC curves as a result of interaction with surfactant is characteristic of hydrophobic, electrostatic, and hydrogen bonds, which are accompanied by stabilization of native protein structure (Tanford, 1970). Modification of PC in LDL did not affect the secondary structure or immunological property of LDL as demonstrated in Figures 4 and 5. The increase in enthalpy in modified LDL is probably due to the increase of interaction with protein and PLs during heating. In general, the surfactants bind to the surface of native proteins. For example, for anionic surfactant the initial interaction will involve cationic amino acid residues (Jones and Brass, 1990), while for nonionic surfactants the binding sites will be in the hydrophobic domain on the protein surface. The hydrophobic core within the β-barrel structure of protein is supposed to be accessible to the apolar molecules of surfactant (Papiz et al., 1986). The difference of the thermal behavior of modified LDL is assumed to be related to the difference of PLs−protein interaction during heating. Heating may facilitate the interaction of protein with LPC or free fatty acid. One explanation of this difference could be that the hydrophobic core within lipoproteins in egg yolk would be exposed to the surface by heat treatment and form the complex with LPC and free fatty acid in LDL by hydrophobic interactions. This firm complex formation could lead to increased thermal stability of lipoproteins and also heat stability of emulsions stabilized with the modified LDL. However, the role of free fatty acids that are released from PC by enzymatic modification is not clear. Further studies on PLs−protein interactions in LDL as a result of modifying phospholipase A_2 related to its structural and functional properties would be desired for better understanding the structure of egg yolk LDL and its functionality.

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