Adsorption Behavior of Egg Yolk Low-Density Lipoproteins in Oil-in-Water Emulsions

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Adsorption behavior of egg yolk low-density lipoprotein (LDL) constituents in oil-in-water emulsions (20% triolein) was examined. The mean particle size was decreased with increase in LDL concentrations and reached a plateau at 60 mg/mL of LDL concentrations. The average particle size and concentration of lipoproteins at the interface were greater for emulsions made at pH 3.0 and 5.0 than at pH 7.0 and 9.0, resulting from the formation of lipoprotein dimers at acid pHs. Electrophoretic analysis revealed that the three polypeptides (64, 43, and 19 kDa) in LDL constituents did not adsorb at the interface, independent of the LDL concentration, pHs, and NaCl content. On the other hand, cholesterol in LDL was preferentially adsorbed to the interfaces at the low LDL concentration. The ratio of phosphatidylcholine and phosphatidylethanolamine was increased with increased of LDL concentration. These results suggest that egg yolk LDL micelles breakdown when the micelles come into contact with the interface and rearrangement of lipoproteins, cholesterol, and phospholipids take place following adsorption at an O/W interface.

Keywords: *Egg yolk; low-density lipoproteins; emulsion; adsorption behavior; cholesterol; phospholipids; oil-in-water interface*

INTRODUCTION

Egg yolk is an effective emulsifying agent for food products such as mayonnaise and bakery products. However, emulsifying properties of most individual egg yolk constituents are not well known (Yang and Baldwin, 1995). Low-density lipoprotein (LDL) is a major component of hen's egg yolk and has been considered as a main factor in the emulsifying properties of egg yolk (Vincent *et al*., 1966). LDL contains 86−89% lipids, having 27% phospholipids, 69% triacylglycerol, and 4% cholesterol and cholesterol ester and 12.5% of proteins (Martin *et al*., 1963). LDL is considered to be a large spherical particle with a core of triacyglyceride and a surface layer of both phospholipids and proteins (Evans *et al*., 1968; Schneider *et al*., 1973). The mean droplet size of emulsions made with LDL was much smaller and more stable during storage than that for emulsions made with bovine serum albumin. The emulsifying properties of egg yolk LDL were little affected by pH or salt concentration (Mizutani and Nakamura, 1984; 1985; Carrillo and Kokini, 1988). Vincent *et al*., (1966) reported that livetin and phospholipids of egg yolk contribute to its low surface energy. Sell *et al*. (1935) have suggested that a lipid−protein complex rather than lecithin is the emulsifying agent of yolk. Dutilh and Groger (1981) observed that both emulsifying capacity and heat stability of egg yolk improved by fermentation with pancreatic phospholipase. The emulsifying properties and heat stability of protein emulsions were improved substantially through the complex formed among the phopholipase hydrolyzed lecithin (lysolecithin) and free fatty acid (Mine *et al*., 1992a,b, 1993). These results indicate that the emulsifying properties of LDL might be due to the characteristic structure of its lipid−protein complexes and interactions at oil-in-water interfaces. However, little is known regarding the adsorption behavior of LDL constituents on oil-in-water emulsions. The objective of this work was to investigate the adsorption behavior of each LDL constituents in triacylglycerol-in-water emulsions.

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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 method of Raju and Mahadevan (1974) with a minor modification in ion exchange chromatography using Q-Sepharose (Pharmacia Biotech, Quebec, Canada) column. LDL concentrations were estimated from protein concentration determined by modified Lowry procedure (Markwell *et al*., 1978) based on 12.5% protein concentration in LDL (Martin *et al*., 1963). Triolein (65% practical grade) was obtained from Sigma Chemicals Co. (St. Louis, MO). Crude triolein was purified to remove mono- and diglyceride by silica gel column chromatography using a solvent mixture composed of hexane:diethyl ether (97:3, v/v). The solvent in the elution was evaporated on a rotary evaporator and the residual oil transferred into an amber bottle and saturated with nitrogen gas before storage at 4 °C. Purity of the triolein preparation was >98% after analysis by thin layer chromatography-flame ionization detection (TLC-FID) using a Iatroscan system (Iatroscan MK-5, Iatron Laboratories Inc., Tokyo, Japan). Other chemicals were purchased from Fisher Scientific (Ottawa, Canada).

Preparation of LDL Emulsions. Oil-in-water emulsions (20 wt% triolein) stabilized with LDL in concentrations ranging from 10 to 80 mg LDL/mL in the aqueous phase were prepared using Microfluidizer (model 110S, Microfluidics Co., Newton, MA) at an input pressure of 0.3 MPa, which corresponds to a pressure drop of 42 MPa. The following buffers were used for the preparation of emulsions: 50 mM imidazole buffer, pH 6.5, containing 100−1500 mM NaCl, 50 mM sodium acetate-HCl buffer, pH 3.0 or 5.0, 50 mM imidazole buffer, pH 7.0, and 50 mM borate buffer, pH 9.0, containing 100 mM NaCl, respectively.

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Determination of Emulsifying Properties. The mean droplet size of emulsions (mean volume surface diameter, $d_{3,2}$) and its surface area were measured by light scattering using a Mastersizer X (Malvern Instruments Inc., Malvern, U.K.) with optical parameters defined by the manufacture's presentation code 0303. The surface protein concentration of LDL in emulsions was calculated by the amount of protein adsorbed to the oil droplets and using the estimation of emulsion droplet surface area by Mastersizer X.

The emulsions were centrifuged at 20 °C and 15000*g* for 30 min and the cream was washed twice with 5 mL of appropriate buffer with each washing followed by centrifugation; the subnatants were pooled together and filtered (0.10 mm) for protein determination. The protein contents in the pooled sample were determined according to the modified Lowry method. Protein content of the cream was estimated as a difference between protein concentration of the subnatant solution and the total protein used to make the emulsion. The amount of protein present in the cream phase was divided by the specific surface area (from Mastersizer results) of its emulsion to give the amount of protein bound per squared meter.

Analysis of Adsorbed Polypeptides at the Interface. The washed cream was mixed with 3 vol of chloroform−methanol (2:1, v/v) for dilipidation. The polypeptides fraction was dilipidated again by same solvent, followed by evaporation to dryness under N_2 gas. These polypeptides fraction adsorbed to an oil droplet was subjected to SDS−PAGE to compare its electrophoretic patterns with the original LDL and unadsorbed polypeptides fraction as described above. SDS−PAGE analysis was performed on slab gels (4−15% Tris−glycine gel, Bio-Rad Laboratories, Mississauga, Ontario) according to the method of Laemmli (1970). Apo-LDL, which was obtained from LDL by delipidation with chloroform−methanol (2:1, v/v), was used as a control. The gels were scanned on a Sharp JX-330 scanner (Sharp Electronics, Tokyo, Japan) and the R_f of each band was determined using the Pharmacia ImageMaster 1D software, version 2.0.

Analysis of Phospholipids and Cholesterol at the Interface. The adsorbed lipids fraction to an oil droplet in emulsions was extracted from a given volume of the washed cream of emulsion by adding 3 vol of chloroform−methanol (2:1, v/v). For the first step, triacylglycerol (triolein) derived from emulsion oil was eliminated from the lipids mixture using a prepacked Sep-Pak silica cartridge (Waters Co., Milford, MA) with 5% ethyl acetate−hexane. Next, cholesterol, phosphatidylethanolamine (PE), and phosphatidylcholine (PC) derived from LDL were eluted from the silica column with methanol−water (98:2, v/v) after washing the column with 5% ethyl acetate−hexane and followed to dryness under N_2 gas by rotary evaporator. The column was dehydrated by elution with the following solvent combination: (1) 5 mL of ethyl acetate, (2) 10 mL of 50% (v/v) ethyl acetate in acetone, (3) 5 mL of ethyl acetate and (4) 20 of mL of hexane. This same procedure was used to regenerate the column after each use. The extracted cholesterol and phospholipids were then analyzed using an Iatroscan system. Each sample was dissolved in 0.5 mL of chloroform−methanol (2:1, v/v) before application on Chromarods (Chromarod-S3, Iatron Laboratories, Inc.). The developing conditions used for the separation of samples were as follows: the rods were first developed with a solvent system of chloroform−methanol−water (70:30:3, v/v) for 10 min, dried, and then redeveloped with a solvent system of petroleum ether−diethyl ether−acetic acid (80:30:0.2, v/v) for 30 min. The composition ratio of cholesterol, PE, and PC was determined from each peak area of TLC-FID Iatroscan chromatograms. Egg PC (QP Corporation, Tokyo, Japan), bovine liver PE (Avanti Polar Lipids, Inc., Alabama, CA), and cholesterol (Sigma) were used as standards. Composition ratio of lipids extracted from the emulsion cream phase was expressed as PE/cholesterol and PC/cholesterol ratio. Lipids from LDL particle obtained by the same method as described above were used as a control.

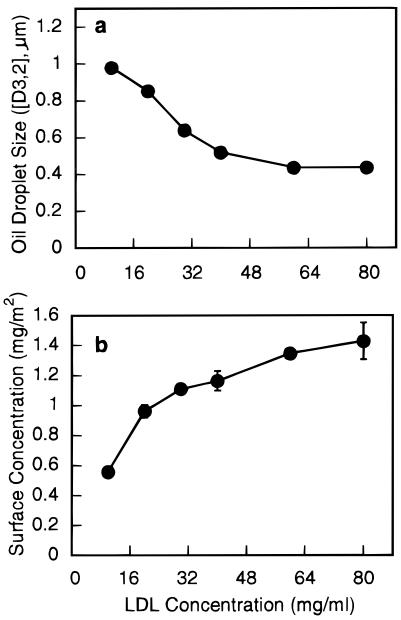

Figure 1. Average particle size (a) and surface protein concentration (b) of oil droplets as a function of LDL concentration in the emulsions. The emulsions were made at pH 6.5. Data are the average of triplicate measurements.

RESULTS AND DISCUSSION

Oil Droplet Size and Surface Concentration of LDL Emulsions. The average diameters of emulsion droplets formed by Microfluidizer with different LDL concentration ranged from 430 to 980 nm depending on the total LDL concentration from 10 to 80 mg/mL. The mean droplet size of emulsions ($d_{3,2}$) measured with Mastersizer and surface coverage as a function of the total LDL concentration is shown in Figure 1. At concentration above 60 mg/mL of LDL, the droplet sizes were almost independent of the LDL concentration. For the lower LDL concentration (<40 mg/mL), the insufficient protein in serum cannot cover all of the freshly created oil surface, so that droplet size increases. On the other hand, the surface coverage per squared meter varied from about 0.56 mg/m^2 to 1.4 mg/m^2, for the lowest to highest LDL concentration (Figure 1b). However, plateau was reached at 60 mg/mL of LDL concentration for 1.35−1.4 mg/m^2. The emulsion droplet distribution showed almost symmetrical distribution curve, in which the particle size was given in a logarithmic scale on the *x* axis, except at low LDL concentration (10 mg/mL) (data are not shown). The ratio of adsorbed protein to the original varied from 60 to 42%, for lowest, to 10 to 80 mg/mL of LDL concentration, estimated from combination the results with Figure 1b data and the equation, $S = 6/d_{3.2}$, where *S* is the average surface area (Walstra, 1983). These values are smaller than those obtained from other proteins [for example, the value of whey protein isolate is 85−87% (Hunt and Dalgleish, 1994)]. This means that some LDL constituents cannot adsorb at the interface, independent of its concentration.

Figure 2 shows the effect of pHs on the droplet size and surface concentration of LDL emulsions containing 60 mg/mL of LDL. Egg yolk LDL formed emulsions with smaller droplet sizes at pH 7.0 and 9.0 than those at pHs 3.0 or 5.0. In addition to particle sizes, the surface protein concentration per unit surface was markedly different between the pHs. The protein

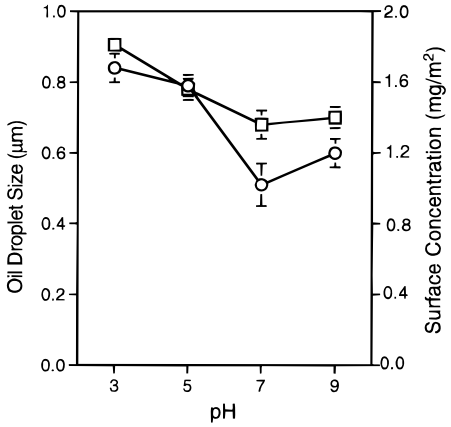

Figure 2. Average particle size and surface protein concentration of LDL emulsions as a function of pHs. (○) Average particle size, (□) surface protein concentration. Each emulsion was prepared with 60 mg/mL of LDL at different pHs. Data are the average of triplicate measurements.

adsorbed per unit surface at pH 3.0 and 5.0 was 1.81 and 1.56 mg, respectively, while it was 1.26 and 1.36 mg at pH 7.0 and 9.0, respectively, for 60 mg/mL LDL concentration. This data suggests that the adsorption behavior of LDL on oil-in-water emulsions is different at different pHs. In addition, there was no noticeable change in particle size for pH 7.0 and 9.0 emulsions at LDL concentrations greater 60 mg/mL. On the contrary, at pH 3.0, the change in droplet size did not reach a minimum, an indication that the droplet size was still dependent on LDL concentration even at the level greater than 80 mg/mL (data are not shown).

The results would indicate that LDL is better a emulsifier at pH 7.0 and 9.0 than at acidic pHs. Since effective reduction of interfacial tension requires unfolding of adsorbed proteins at the oil/water interface so that their apolar segments can come into contact with the oil phase (Parker, 1987), it is possible that the dimerization of lipoproteins at pH below 5.0 reduced their capacity to unfold and interact with the oil phase at pH 3.0, when compared to the lipoprotein molecules present at pH 7.0 and 9.0. (Burley and Cook, 1962). These results suggest that protein−protein interactions at the interface between the LDL is greater at pH 3.0 and 5.0 than at pH 7.0 and 9.0. The higher surface protein concentration at pH 3.0 when compared to pH 7.0 and 9.0 can also be explained on the basis of increased dimerization of lipoproteins at pH values below 5.0. Moreover, the increased charge on the proteins at high pH values decreases binding to the interface since an energy barrier is presented as a result of electrostatic repulsions between charged groups in the adsorbed protein and similarly charged groups in the protein in solution. Another reason that LDL formed emulsions with a larger droplet size and thicker film than those at pH 7.0 and 9.0 may be related to its structural characterization. Kisseoglou and Sherman (1983) studied the interfacial tension-time behavior of egg yolk lipoproteins at the oil-in-water emulsions. At pH 6.2, the initial value of interfacial tension was very low, and it increased substantially when the pH was decreased. Disruption of LDL particle at an oil-in-water interface has been attributed to weakening of protein−protein forces. At low pH, the positive charge may weaken the micelle structure and so promote greater degradation of LDL when contact is made with the oil-in-water interface (Evans *et al.*, 1974). If the pH in serum phase is close to the isoelectroric point (pH 5.5−5.8) of egg yolk lipoproteins, the initial adsorption rate will probably be controlled by the rate at which micelles diffuse to the interface and by the interfacial pressure barrier to adsorption which arise from the previously adsorbed LDL micelles. At any other pHs, the increased potential energy barrier, arising from the electric charge, also inhibits penetration of the interface by LDL micelles and produces a higher initial interfacial tension, resulting emulsions with a larger droplet size. The effects of NaCl concentration on the oil droplet size and the surface concentration were also examined at 60 mg/mL of LDL concentration (Table 1). At different NaCl concentrations ranging from 100 to 1500 mM in the aqueous phase at pH 6.5, there was little difference in the droplet size ($d_{3,2}$) and in the surface protein concentration in LDL emulsions. The presence of NaCl in serum increased aggregation of the emulsions but had little effect on coalescence during aging for 2 days (data are not shown).

Table 1. Effect of NaCl Concentration on the Average Particle Size ($d_{3,2}$) and the Surface Concentration of LDL Emulsions[a]

condition NaCl (mM)	droplet size[b] ([$d_{3,2}$], μm)	surface concentration[b] (mg/m^2)
100	0.503 ± 0.014	0.990 ± 0.016
500	0.520 ± 0.010	1.052 ± 0.015
1000	0.523 ± 0.020	1.077 ± 0.034
1500	0.517 ± 0.026	1.067 ± 0.034

[a] Each emulsion (20% triolein) was prepared by 50 mg/mL of LDL in 50 mM imidazole−HCl buffer, pH 6.5. [b] Mean ± SD (n = 3).

Adsorbed Polypeptides at the Interface. It has been reported that LDL consists of about six major polypeptides that range in molecular mass from about 10 to 180 kDa and several unidentified minor polypeptides. (Raju and Mahadevan, 1976; Yamauchi *et al.*, 1976; Burley and Sleigh, 1980). However, their name and molecular mass of each band from SDS−PAGE patterns are not agreed among each researcher. Here, I used the names of polypeptide with their molecular mass which was estimated from R_f on SDS−PAGE gel to avoid the confusion. The present SDS−PAGE analysis indicated LDL compose 7 major polypeptides ranging 19−225 kDa and some minor polypeptides. From the migration patterns of polypeptides in SDS−PAGE gels (Figure 3), the preferential adsorption among polypeptides in LDL emulsions was observed. Almost all major polypeptides in LDL components adsorbed on the oil droplet, but three major polypeptides remained in serum (Figure 3a). The same results were obtained at LDL concentration ranging from 10 to 80 mg/mL. Even at the lower concentration (10 mg/mL) of LDL, these three polypeptides did not adsorb at an O/W interface. The molecular sizes of these unadsorbed polypeptides were estimated at about 64, 43, and 19 kDa. The effects of pH and NaCl concentration on the adsorption behavior in LDL emulsions was also studied (Figure 3b). Minor differences were observed in the migration pattern of SDS−PAGE at pH 3.5. Higher molecular mass band of 170 kDa corresponding to apovitellenin VI (Burley and Sleigh, 1980) was decreased at the interface, and the 48 kDa polypepetide appeared in serum at pH 3.5. Furthermore, some polypeptides were polymerized at the interface at pH 6.5. There was little difference of adsorbed components in LDL polypeptides at the emulsions containing high NaCl content. Two unadsorbed

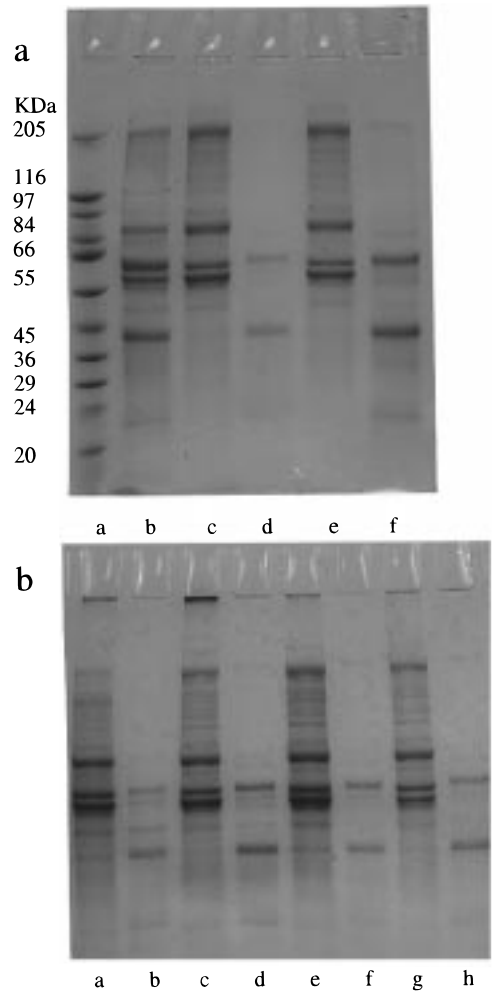

Figure 3. SDS−PAGE profiles of adsorbed polypeptides in the emulsions stabilized by LDL at various conditions. (a) Adsorbed polypeptides at pH 6.5. Lane b, apo-LDL; lanes c and d at 10 mg/mL LDL; lanes e and f at 60 mg/mL of LDL; lane a, molecular mass markers, respectively. Lanes c and e, adsorbed polypeptides, lanes c and e, unadsorbed polypeptides. (b) Emulsions at 60 mg/mL of LDL. Lanes a and b at pH 3.5; lanes c and d at pH 6.5; lanes e and f at pH 8.5 containing 100 mM NaCl, respectively; lanes g and h at pH 6.5 containing 1500 mM NaCl, respectively.

polypeptides described above were also found in the unadsorbed fraction under each condition. Therefore, this preferential adsorption of polypeptides in LDL was observed independent of the conditions of emulsion preparation. Interestingly, two of unadsorbed polypeptides (64 and 43 kDa) were not found in previous papers (Raju and Mahadevan, 1976; Yamauchi *et al.*, 1976; Burley and Sleigh, 1980).

Egg yolk plasma (water soluble fraction) is composed of 85% LDL and 15% of livetin. The livetin fraction consists of α-, *β*-, and *γ*-livetins in egg yolk (Martin *et al.*, 1957). Williams (1962) identified *γ*-livetin as egg yolk immunoglobulin (IgY). The molecular mass of the light and the heavy chains of IgY are 25 and 65 kDa, respectively (Jensenius *et al.*, 1981). The molecular mass of one of unadsorbed polypeptides (64 kDa) is very closed to one of the heavy chains of IgY (65 kDa). Thus, I was concerned whether this 64 kDa polypeptide was from LDL constituents or contamination from livetin fraction under the purification process of LDL. This is an important factor to discuss as the adsorption behavior of LDL constituents at the interface. Both fractions

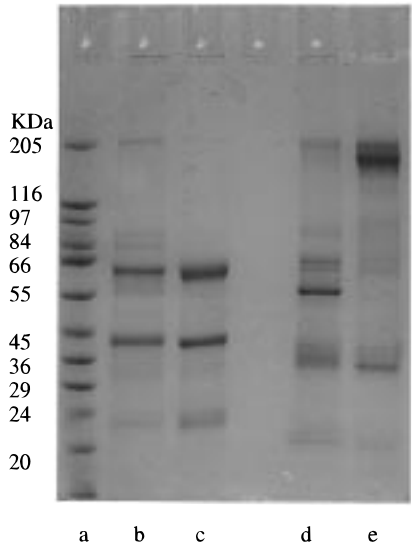

Figure 4. SDS−PAGE patterns of unadsorbed polypeptides (lanes b and c) in LDL emulsion and livetin (lanes d and e) from egg yolk plasma fraction under reducing (lanes b and c) or nonreducing condition (lanes d and e). (a) Molecular marker.

of unadsorbed polypeptides and livetin were compared in SDS−PAGE under reducing and nonreducing conditions (Figure 4), because livetin fraction contains IgY which is composed by heavy and light chains linking by disulfide bond. These two fractions showed very similar migration patterns under reducing conditions (Figure 4, panels b and c); however, quite different patterns were found under nonreducing conditions (Figure 4, panels d and e). Therefore, it was concluded that the unadsorbed polypeptides of 64 and 40 kDa were originated from LDL micelles.

Adsorbed Phospholipids and Cholesterol at the Interface. Egg yolk LDL consists of 70% neutral lipid, 26% phospholipids (71−76% PC, 16−20% PE, and 8−9% sphingomyelin and lysophospholipids), and 4% free cholesterol (Martin *et al.*, 1963). Lipoproteins and phospholipids are major constituents of LDL to act as an emulsifier (Yang and Baldwin, 1995). However, the role of cholesterol or phospholipids−cholesterol−protein interaction in LDL at an oil-in-water emulsion is little understood. The composition of adsorbed cholesterol and phospholipids, which is derived from LDL components on the oil droplet in the emulsion, was compared with that LDL (Figure 5). Changes in the ratios seemed to be dependent on the concentration of LDL (Figure 5a). Both composition ratios (PE/cholesterol and PC/cholesterol) became larger as the total LDL concentration increased. However, even at higher concentration of LDL, these values of lipids obtained from the emulsion were lower than those of LDL as a control. The cholesterol adsorbed preferentially at the interface at low LDL concentration. This indicate that cholesterol has higher affinity to the oil droplets in the emulsion than PC or PE. In contrast to the adsorption behavior of polypeptides, this preferential adsorption of cholesterol was dependent on the concentration of LDL. The first interactions between each components of LDL and the oil droplets in emulsion would be made by hydrophobic effects in aqueous system. At the adsorption, polypeptides, which are hydrophobic or have a hydrophobic region on the surface, and cholesterol in an LDL particle may be attracted to the oil droplets (triolein) with a hydrophobic interaction. When the surface concentration of the polypeptide is low, there may be

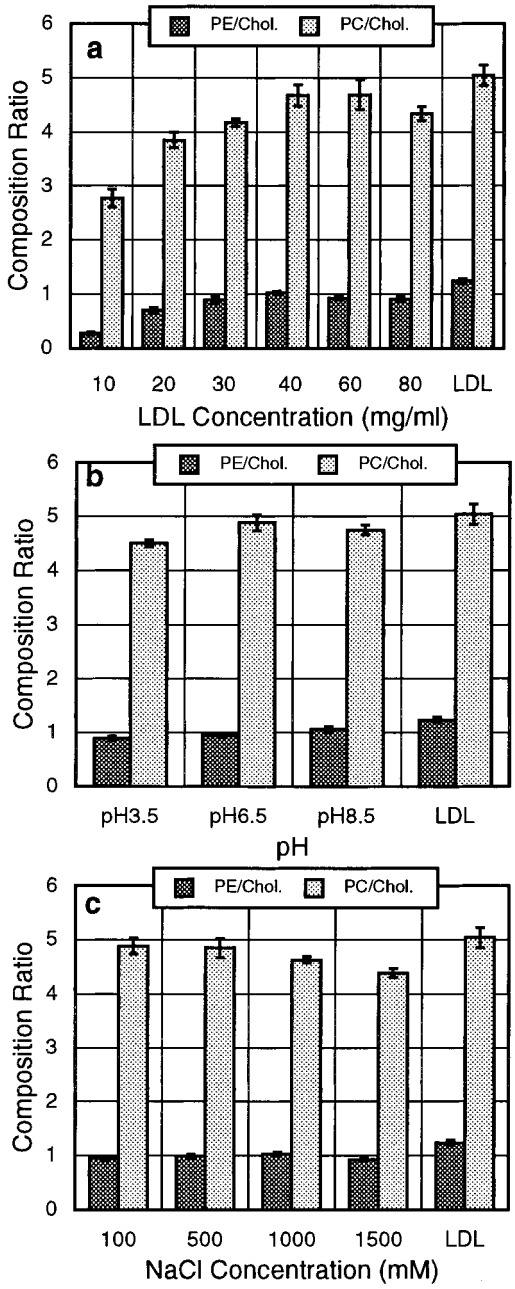

Figure 5. Relative composition ratio of adsorbed lipids from LDL at the oil-in water interface stabilized by LDL at various conditions. (a)The emulsions were prepared at pH 6.5 containing 100 mM NaCl as a function of LDL concentration. (b) The emulsions were made at 60 mg/mL of LDL concentration at various pHs. (c) The emulsions were prepared at 60 mg/mL of LDL concentration at pH 6.5 as a function of NaCl concentration. Data are the average of triplicate measurements.

large gaps, i.e., bare areas of oil surface. Under such conditions, it might be difficult for phospholipids to adsorb tightly on the large gaps compared to cholesterol or polypeptides, because it is more polar lipid. This suggests that polypeptides and cholesterol in LDL would play a role as "wedge" on the surface of oil droplets in emulsion. The ratio of PE/cholesterol and PC/cholesterol at the interface increased with increasing LDL concentration. These data indicate that the phospholipids−protein interaction is stronger than that of cholesterol−protein. The adsorption of phospholipids on to the interface may be affected by LDL concentration under preparation of emulsions. The ratio of PE/cholesterol and PC/cholesterol at pH 3.5 was slightly lower than that at pHs 6.5 and 8.5 (Figure 5b). This indicates that more cholesterol was adsorbed at the interface at low pHs. The ratio of PC/cholesterol slightly decreased with rising concentration of NaCl, but no effect was observed in the PE/cholesterol (Figure 5c).

Lipid−protein complexes in LDL have a spherical structure (Nichols *et al.*, 1969). The surface layer of LDL is probably heterogeneous and consists of charged molecules such as phospholipids and protein which associate not so strongly with each other (Evans *et al.*, 1968; Holdworth and Finean, 1972). It has been reported that the superior emulsifying properties of LDL mainly depend on the characteristic structure of its lipid−protein complex, and emulsifying properties of protein will be enhanced by the complex formation with egg PC (Nakamura *et al.*, 1988) or lyso PC (Mine *et al.*, 1992a,b). The addition of egg-PC enhanced the stability of the emulsion containing low concentration of casein, and protein and egg-PC can coexist on the oil/water interface (Fang and Dalgleish, 1993). Kiosseoglou and Sherman (1983) have suggested a possibility of the LDL micelle breakdown following adsorption from the measurement of interfacial tension decay. However, they did not show any direct evidence to support their hypothesis. The results in this study showed that both components of polypeptides and lipids (cholesterol and phospholipids) in LDL components could interact at oil-in-water interfaces and play an important role for forming a stable emulsion. Furthermore, the analysis of each adsorbed components would emphasize that the rearrangement of the component in LDL could occur during emulsifying process. Further study on lipid−protein interaction of LDL components at oil-in-water interfaces would be required to get better understanding of the emulsifying property of egg yolk lipoproteins.

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