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A NOVEL LOW-DENSITY LIPOPROTEIN WITH LARGE AMOUNTS OF PHOSPHOLIPID FOUND IN THE EGG YOLK OF CRUSTACEA SAND CRAYFISH IBACUS CILIATUS: ITS FUNCTION AS VITELLOGENIN-DEGRADING PROTEINASE

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SUMMARY: Low-density lipoprotein (LDL) with large amounts of phospholipid but not triacylglycerol was isolated from the egg yolk of crustacea sand crayfish *Ibacus ciliatus* as well as lipovitellin. LDL possessed vitellogenin-degrading proteinase activity. Hemolymph vitellogenin was degraded by incubating with LDL at pH 4.5 for 72 hr at 35°C and apolipoprotein profiles of vitellogenin degraded by LDL were very similar to those of lipovitellin in the egg.

The major source of such nutrients as protein and lipid for the developing embryo of all oviparous animals including crustacea is the egg yolk which constituents the non-organellar content of the oocyte. The crustacean egg yolk protein called lipovitellin is derived from hemolymph precursor, vitellogenin (1). Vitellogenin is a lipoprotein and functions as carriers of lipid and protein to the egg yolk (2).

We recently characterized the lipoprotein features of crustacean hemolymph and egg (3). Two different lipoproteins, high-density (HDL) and very-high-density lipoproteins (VHDL), were isolated from the male hemolymphs of freshwater prawn, mitten crab, and striped stone crab, while vitellogenin as well as HDL and VHDL were further found in the female hemolymphs. Low-density lipoprotein (LDL) was present in the egg yolk of only sand crayfish, although lipovitellin was a major egg yolk protein from

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Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; VHDL, very-high-density lipoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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some crustacea including sand crayfish. LDL found in the egg yolk of sand crayfish was of interest because it possessed large amounts of phospholipid but not triacylglycerol as a major lipid component. During the course of our comparative study on crustacean lipoproteins, we noticed the marked degradation of vitellogenin from sand crayfish. We report here LDL isolated from the egg yolk of sand crayfish possesses a vitellogenin-degrading proteinase activity.

MATERIALS AND METHODS

Animals: Live female sand crayfish *Ibacus ciliatus* (average body weight 198g, average egg weight 13.8g) were purchased from a local fish market.

Isolation of egg yolk and hemolymph lipoproteins: To isolate the egg yolk protein, the eggs were homogenized in 5 volumes of 0.15 M NaCl-10 mM phosphate buffer (pH 7.4) containing 3 mM EDTA and 2 mM diisopropyl fluorophosphate. The homogenate was centrifuged at 8,500 rpm for 15 min at 4°C. After centrifugation, the egg yolk protein was separated from a pellet and submitted to the isolation of lipoproteins (4). Potassium bromide was added to the egg yolk protein to a final concentration of 8.9 g KBr/20 ml solution (44.5%). The solution was placed in a centrifuge tube and overlaid with 20 ml of 0.75% NaCl. The tube was centrifuged at 35,000 rpm for 17 hr at 15°C in a 410 rotor using an International B/60 model ultracentrifuge (DAMON/IBC). The floating lipovitellin fraction (density=1.24 g/ml) was collected, adjusted to a density of 1.30 g/ml by addition of solid KBr in a final volume of 20 ml. The solution was then placed in a centrifuge tube, overlaid with 20 ml of 22% KBr solution, and respun at 35,000 rpm for 17 hr at 15°C. The floating LDL fraction (density=1.05 g/ml) was brought to a final KBr concentration of 22% and overlaid in a centrifuge tube with 20 ml of 0.75% NaCl.

Hemolymph was withdrawn by syringe from sinuses at the bases of walking legs. Hemolymph was placed in 0.15 M NaCl-10 mM phosphate buffer (pH 7.4) containing 3 mM EDTA, 3 mM dithiothreitol, 1 mM leupeptin, 2 mM chymostatin, 3 mM pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM diisopropyl fluorophosphate. Following centrifugation (8,000 rpm at 4°C for 15 min) to remove hemocytes, the hemolymph was brought to a density of 1.30 g/ml with solid KBr (20 ml final volume), overlaid with 20 ml of 0.75% NaCl and centrifuged at 35,000 rpm for 17 h at 15°C. The floating vitellogenin fraction (density=1.19 g/ml) was collected, adjusted to a density of 1.25 g/ml by addition of solid KBr in a final volume of 20 ml. The solution was then placed in a centrifuged tube, overlaid with 20 ml of 11% KBr solution, and respun at 35,000 rpm for 17 h at 15°C. VHDL fraction (density=1.27 g/ml) was brought to a final KBr concentration of 44.5% and overlaid in a centrifuge tube with 20 ml of 33% KBr solution. After 17 h at 35,000 rpm, VHDL was separated from hemocyanin. The isolated egg yolk and hemolymph lipoproteins were dialyzed against 0.15 M NaCl (pH 7.4).

Proteolytic degradation of vitellogenin: Vitellogenin (0.4 mg/ml) isolated from the hemolymph was incubated for 24, 48, and 72 hr at 35°C in either 10 mM Tris (pH 7.5) or 10 mM acetate (pH 4.5) with egg yolk LDL (0.4 mg/ml) at a concentration of 1:30 (LDL:vitellogenin). Vitellogenin was also incubated with lipovitellin (0.4 mg/ml) at a concentration of 1:30 (lipovitellin:vitellogenin). The reaction was stopped by addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample treatment buffer (pH 6.8) containing 20 mM Tris, 2% SDS, 2% 2-mercaptoethanol, and 40% glycerol, and boiling at 95°C for 5 min.

Other analyses: Method for SDS-PAGE has been reported elsewhere (3). The protein content was determined spectrophotometrically using Bio-Rad Protein Assay. Phospholipid, triacylglycerol, and cholesterol were determined using enzyme-based assay kits purchased from Kyowa Medex Co., Ltd, Tokyo.

RESULTS AND DISCUSSION

We isolated lipoprotein fractions from both hemolymph and egg yolk of female sand crayfish by a differential density gradient ultracentrifugation, and compared the compositions (Table 1) and apolipoprotein components (Fig. 1). Two lipoproteins with densities of 1.19 and 1.27 g/ml were evident in the hemolymph. The former lipoprotein possessed an identical apolipoprotein with lipovitellin, and was termed vitellogenin. The latter was termed VHDL because of its high density. VHDL was the most abundant lipoproteins in the hemolymph. Both vitellogenin and VHDL contained phospholipid as a predominant lipid component.

Two lipoproteins with densities of 1.25 and 1.05 g/ml were found in the egg yolk from sand crayfish (Table 1). The former lipoprotein was a major component in the egg yolk, and was termed lipovitellin. Lipovitellin possessed high levels of protein because of its high density. The latter possessed much more lipid mass than protein one because of its low density, and was termed LDL. LDL was of interest because it contained phospholipid but not triacylglycerol as a major lipid component.

Apolipoprotein features of lipoproteins isolated from the hemolymph and egg yolk were analyzed by SDS-PAGE (Fig. 1). Vitellogenin had three major

Table 1. Lipoprotein constituents in hemolymph and egg yolk from sand crayfish

	Concentration (mg/ml hemolymph)		Concentration (g/100 g egg)	
	Vitellogenin	VHDL	LDL	Lipovitellin
Protein	0.385 (61.40)	4.988 (93.51)	0.645 (22.20)	4.830 (73.34)
Triacylglycerol	0.038 (6.06)	0.066 (1.24)	0.350 (12.05)	0.385 (5.84)
Phospholipid	0.194 (30.94)	0.270 (5.06)	1.844 (63.48)	1.350 (20.50)
Free cholesterol	0.007 (1.12)	0.006 (0.11)	0.052 (1.79)	0.016 (0.24)
Cholesteryl ester	0.003 (0.48)	0.004 (0.07)	0.014 (0.48)	0.005 (0.08)
Density (g/ml)	1.19	1.27	1.05	1.25

Each lipoprotein fraction was isolated from hemolymph and egg yolk by a differential density gradient ultracentrifugation. The density of each lipoprotein was determined by refractometry. The protein and lipid concentrations of each lipoprotein were determined using Bio-Rad protein and enzyme-based assay kits, respectively. Values in parentheses represent percentages, taking each lipoprotein as 100%.

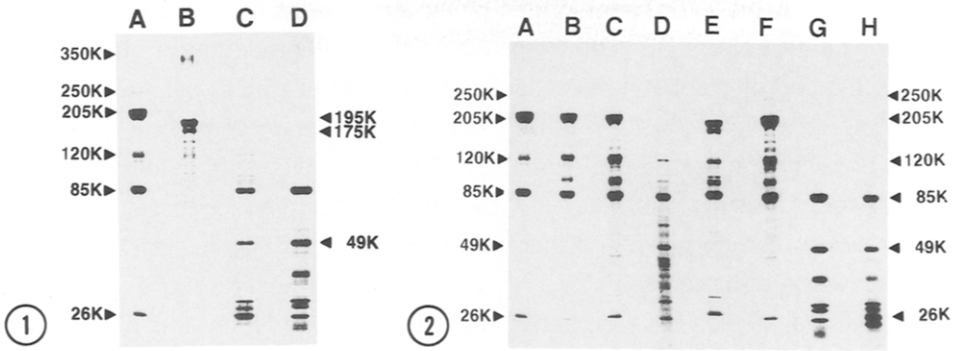

Fig. 1. SDS-PAGE of hemolymph (A,B) and egg yolk (C,D) lipoproteins. A 4-18 percent polyacrylamide gradient slab was electrophoresed at 30 mA for 3.5 hr. Lane A, hemolymph vitellogenin; lane B, hemolymph VHDL; lane C, egg yolk LDL; lane D, egg yolk lipovitellin.

Fig. 2. Degradation of hemolymph vitellogenin by egg yolk LDL. Aliquots (200 μg protein) of vitellogenin were incubated with 6.7 μg of LDL at pH 4.5 (B,C,D) or pH 7.5 (E) for 24 (B), 48 (C), and 72 (D,E) hr at 35°C. Vitellogenin (F) or LDL (G) was incubated separately at pH 4.5 for 72 hr at 35°C. Lane A, vitellogenin; lane H, lipovitellin.

apolipoproteins of molecular weights (Mr) 205,000, 120,000, and 85,000, while two major apolipoproteins of Mr 195,000 and 175,000 were present in VHDL. Lipovitellin consisted of several apolipoproteins, and apolipoproteins of Mr 85,000 and 49,000 were major components. The similar apolipoprotein profiles were observed between LDL and lipovitellin, but LDL possessed apolipoproteins with high molecular weights. Apolipoprotein features of lipovitellin were also similar to those of vitellogenin, although some apolipoproteins found in vitellogenin disappeared in lipovitellin. Considering that vitellogenin is a precursor of lipovitellin, the proteolytic processing of vitellogenin was suggested to occur in the egg yolk. How does LDL function in the egg yolk?

We investigated the degradation of vitellogenin by LDL (Fig. 2). No detectable changes of apolipoproteins in vitellogenin found by incubating with LDL at pH 4.5 for 48 hr at 35°C. However, apolipoproteins of Mr 205,000 and 49,000 disappeared and newly formed, respectively, by incubating vitellogenin with LDL at pH 4.5 for 72 hr at 35°C. Apolipoprotein of Mr 85,000 in vitellogenin was resistant during the incubation with LDL. When vitellogenin and LDL were incubated separately under the experimental conditions, these lipoproteins were stable and no marked changes of apolipoprotein features were found on SDS-PAGE. Apolipoprotein features of vitellogenin incubated with LDL for 72 hr were very similar to those of lipovitellin. The degradation of vitellogenin was also found by incubating with LDL at pH 7.5 for 72 hr at 35°C, but the effect of LDL on the degradation of vitellogenin at pH 7.5 was much

less than at pH 4.5. The degradation of vitellogenin was blocked by incubating it with heat-treated LDL (100°C for 20 min). No degradation of vitellogenin was detected by incubating with lipovitellin instead of LDL at pH 4.5 for 72 hr at 35°C, while vitellogenin was degraded by incubating with both lipovitellin and phospholipid extracted from LDL. These results suggested that LDL possesses a proteinase activity by which vitellogenin is degraded to lipovitellin in the egg yolk and that the phospholipid of LDL is responsible for the activation of proteinase.

Several studies have indicated that the proteolytic processing of egg yolk lipoproteins is induced by the proteinases such as cathepsins D and L, which have optimal pHs of 3-5, isolated from the egg yolk (5-8). LDL with vitellogenin-degrading proteinase activity isolated from the egg yolk of sand crayfish apparently differs from cathepsins D and L. Since LDL isolated by a differential density gradient ultracentrifugation is free from non-lipid-containing proteins, the action of LDL as proteinase does not result from some contaminants in LDL. This paper describes for the first time presence of LDL with vitellogenin-degrading proteinase activity. LDL is a macromolecular complex with large amounts of phospholipid, and the effects of phospholipid and apolipoproteins on the proteinase activity of LDL remain to be explored. Furthermore, it is not clear why LDL requires the long incubation time to degrade vitellogenin. It has recently reported that fibronectin possesses various latent proteinase activities and the long incubation time (48 hr) is required to autolyze fibronectin (9-11). Some similarities of proteinase action may be found between LDL as vitellogenin-degrading proteinase and fibronectin.

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