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Apolipoprotein of High Density Lipoprotein of Eel Serum

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Abstract

High density lipoprotein (HDL) with density between 1.063 and 1.21 g/m*l* was isolated from eel serum by sequential floatation ultracentrifugation. Apolipoprotein was purified from delipidated HDL by gel filtration on a Toyopearl HW-55F column in the presence of urea. Two main apolipoproteins, designated as apo-AⅠ and apo-AⅡ, were obtained. Apo-AⅠ was purified as a single protein with a molecular weight of 28,000. Apo-AⅡ had a molecular weight of 14,000 and its purity was over 90%.

Synthesis and secretion of apolipoprotein of HDL by using ^{14}C-leucine was studied with cultured eel-hepatocytes. It was demonstrated that cultured eel-hepatocytes synthesized and secreted both apo-AⅠ and apo-AⅡ. We found that cells cultured for 9 days secreted more proteins among cells cultured for 12 days.

Studies on eel serum protein has revealed that lipoprotein, especially high density lipoprotein (HDL), is main component[1]. HDL has two main apolipoproteins designated as apo-AⅠ and apo-AⅡ as described by Shore and Shore[2,3]. Many studies on HDL and its apolipoproteins of mammalian serum have been performed. However, there are only a few reports on those of fish serum. A series of studies on carp plasma proteins have been carried out by Nakagawa *et al.*[4-7], but they did not report on HDL in carp plasma. Though HDL in serum of pink salmon have been studied by Nelson and Shore[8], they did not report on the molecular character of the apolipoprotein. This paper describes HDL of eel serum with particular reference to the purification of its two main apolipoproteins and the synthesis and secretion of these proteins in cultured eel-hepatocytes.

Materials and Methods

Materials

Toyopearl HW-55F was purchased from Toyosoda Manufacturing Co., LTD. Bovine serum albumin as standard protein for protein assay was obtained from Boehringer Mannheim GmbH. Calibration kit for the determination of molecular weight of protein by SDS-PAGE was from Pharmacia LTD. L-(U-^{14}C)-leucine(348mCi/mmol) was purch-

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ased from Radiochemical Center Amersham. Other chemical reagents were obtained from Wako Chemical Co, INC. and Nakarai Chemicals, LTD.

Animal

Eels(*Anguilla japonica*) weighing about 250g were purchased from Sueyoshi Co. in Kagoshima city.

Preparation of eel serum

Eel was cooled with ice water and the abdomen was opened through a middle incision from the anus. The needle with polyethylene tubing connected to maliot flask was inserted into the hepatic portal vein at 15 to 20mm below liver and the liver was perfused with phosphate buffered saline(PBS, pH 7.5). The superior aorta was cut and blood was collected into centrifuging tubes with pipette. Eel serum was obtained by centrifuging the blood at 1,058 $\times g$ for 20min(Kubota K-80). Chloramphenical, NaN_3 and the phenylmethylsulfonyl fluoride(PMSF) were added to serum with a final concentration of 80mg/l, 0.1 g/l, and 1 mM, respectively.

The protein content was determined by the method of Bradford[9].

Isolation of HDL

HDL was isolated according to the method of Brewer *et al.*[10] Density of eel serum was adjusted to 1.063g/ml with solid KBr and the serum was centrifuged with a Kubota International B/60 ultracentrifuge at 193,000$\times g$ for 30h at 15℃. The upper layer($d<1.063$) was removed. The infranate was collected, the density was adjusted to 1.21 g/ml and the solution was centrifuged at 193,000$\times g$ for 44 h at 15℃. The upper layer of which density was adjusted to 1.238 g/ml was centrifuged again at 193,000$\times g$ for 35 h at 15℃ to wash HDL. The upper layer containing HDL fraction was dialyzed against 0.1M NaCl-0.01%EDTA.

After dialysis, HDL fraction in 0.5M saccharose was applied on a Toyopearl HW-55F column to separate HDL from LDL and VLDL contained as minor components. HDL was pooled, dialyzed against the buffer containing 0.01% EDTA-0.01% NaN_3(pH 7.4) and lyophilized.

Purification of apo-A I and apo-A II

HDL was delipidated according to the method of Osborne[11]. Delipidated apolipoproteins were separated to apo-A I and apo-A II by gel filtration on a Toyopearl HW-55F column in the presence of urea.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli[12].

Incorporation of ^{14}C-leucine into intra- and extracellular protein of cultured eel-hepatocytes

Eel liver cells were isolated and cultured according to the method of Hayashi and Ooshiro[13,14]. Isolated liver cells were added to a culture dish(ϕ 10cm, 4.02×10^6 cells/dish) and cultured in 7ml of completely defined medium, which consisted of Williams' medium E, insulin, glucagon, prolactin, growth hormone, epidermal growth factor and H_2SeO_3 as described previously[14].

Protein synthesis was investigated as follows. Cultured cells were washed once with the medium, the medium of 7ml was added and then ^{14}C-leucine was added to the medium(3.5 μ

Ci/dish, 0.57mM of leucine). After incubation at 30℃ in CO_2 incubator, the medium was taken and leupeptin and pepstatin were added to the medium with a final concentration of 0.1mM. Then the medium was applied on a Sephadex G-25 column(ϕ 5×7.5cm), protein fraction was pooled and lyophilized. Lyophilized proteins were dissolved in a small amount of 0.05M Tris-Cl(pH 6.8)-1.67% SDS-0.5M 2-mercaptoethanol and used for the determination of radioactivity as extracellular proteins and for fluorography. On the other hand, to the cells on a dish, trichloroacetic acid(TCA) was added and TCA insoluble fraction was precipitated by centrifugation. The precipitate was dissolved by 0.01 N NaOH and the radioactivity was measured as intracellular proteins.

Fluorography

After electrophoresis, SDS-polyacrylamide gel was stained and destained. The gel was dipped into Amplify(Amersham) for 15min and dried. Dried gel was attached to Fuji X-ray film RX, sealed with aluminum sheet and X-ray film was exposed to ^{14}C-labelled proteins in the gel at −70℃. After several days, X-ray film was developed.

Results and Discussion

Purification of apo-A I *and apo-A* II

HDL fraction with density between 1.063 and 1.21 was obtained by ultracentrifugation. HDL was purified by gel filtration on a Toyopearl HW-55F column as shown in Fig. 1. The

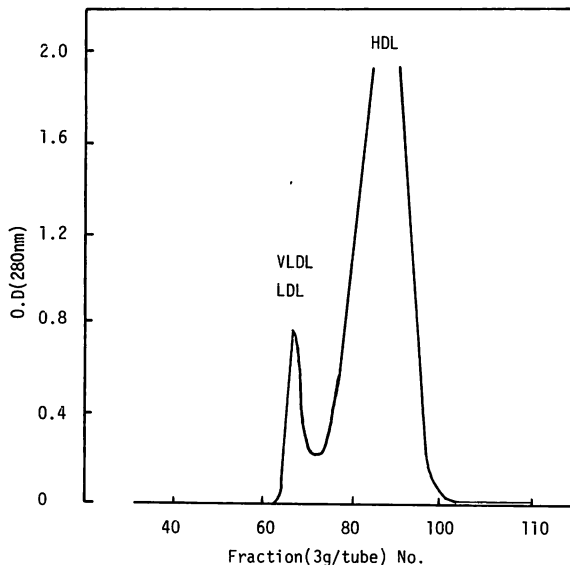

Fig. 1. Gel filtration of lipoprotein(1.063<d<1.21) on a Toyopearl HW-55F column(ϕ 2.6×90cm) Buffer: 0.25M phosphate buffer(pH7.4)-0.1M NaCl-0.01% EDTA

first peak contained VLDL and LDL as reported by Nishi[1] and the second contained mainly HDL.

To purify apolipoprotein of HDL, HDL was delipidated and delipidated HDL in 6M urea was applied on Toyopearl HW-55F column in the presence of 6M urea. As shown in Fig. 2 three components were separated, F-Ⅰ, F-Ⅱ, and F-Ⅲ. F-Ⅰ consisted of undissociated HDL. Main component of F-Ⅱ and F-Ⅲ were apo-AⅠ and apo-AⅡ, respectively. Apo-AⅠ and apo-AⅡ were further purified by rechromatography on a Toyopearl HW-55F column in the presence of 8M urea(Fig. 3). Table 1 showed the recoveries of apo-AⅠ and apol-AⅡ through the purification.

As shown in Fig. 4, HDL consisted of two main apolipoproteins. One had a molecular weight of 28,000 and the other 14,000. The former was designated as apo-AⅠ and the latter was designated as apo-AⅡ. Apo-AⅠ was purified as a single protein and the purity of apo-AⅡ was over 90% from the results of SDS-PAGE.

Purified HDL consisted of 35% of protein and 60.1% of lipid, of which composition was 42.1% of phospholipid and 18% of sterylesters as reported by Nishi[1]. Nelson and Shore[8] reported on serum HDL of pink salmon. Its chemical composition was 60.2% of lipid and 37.4% of protein, and 2.4% of carbohydrate. These compositions were almost the same as those in eel HDL. Among serum HDL of other aquatic animals, serum HDL of prawn was isolated by Teshima *et al.*[15,16] and consisted of 29% of protein and 64% of lipid. Protein content in prawn HDL seems lower than that in eel and salmon HDL.

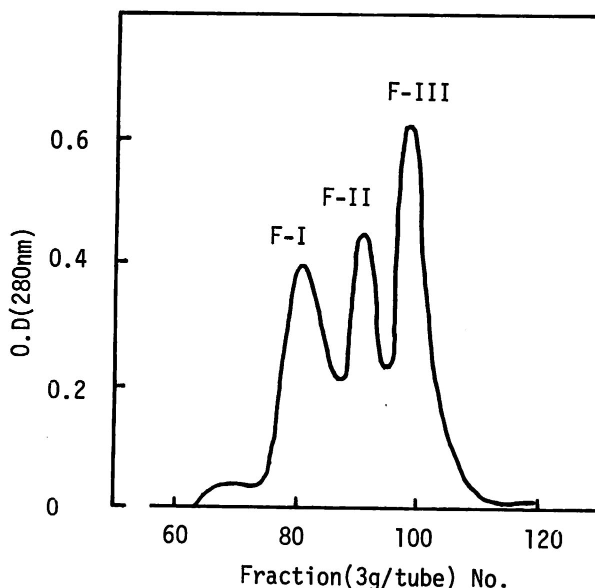

Fig. 2. Gel filtration of delipidated HDL on a Toyopearl HW-55F column(φ 2.6×96cm) in the presence of urea
Buffer: 6M urea-50mM Glycine buffer(pH8.8)-0.5M NaCl

HDL in mammals is well known to consist of two main apolipoproteins, apo-A I and apo-A II. Apo-A I has a molecular weight of 25,000 to 29,000 and apo-A II 8,500 to 9,900[17]. The molecular weight of apo-A I of eel HDL was coincident with that in mammals, but that of eel serum apo-A II, 14,000, was much larger than that in mammals. Apo-A II of human HDL has a molecular weight of 17,414, but consist of dimer[17]. This leaves the possibility that apo-A II of eel HDL is a dimer.

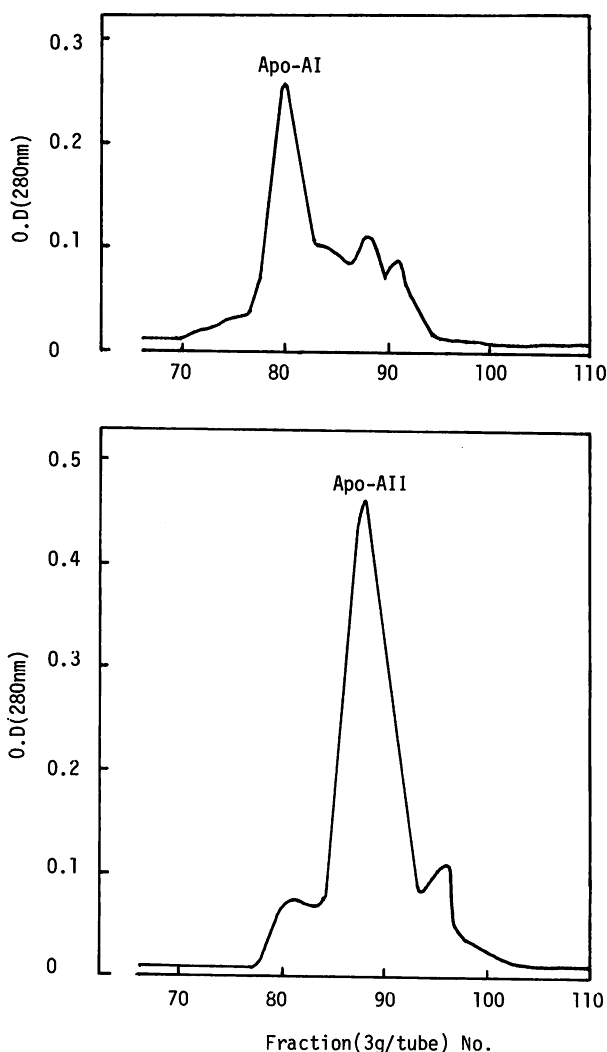

Fig. 3. Rechromatogram of F-II and F-III on a Toyopearl HW-55F column(ϕ 2.6 × 90cm) in the presence of urea
Buffer: 8M urea-10mM Tris-Cl buffer (pH7.5)-1mM EDTA

As reported previously[18,19], the incorporation of ^{14}C-leucine into intracellular proteins was higher that into extracellular proteins during 32h in incubation. Furthermore as molecular weight of apolipoproteins, apo-A I and apo-A II, of HDL were revealed, it was found from the fluorogram of extracellular proteins that HDL were secreted into medium after 8h incubation by cultured hepatocytes.

Effect of cultured days of hepatocytes on the incorporation of ^{14}C-leucine into proteins was investigated. As shown in Table 2, cells cultured for 9 days had greater ability to synthesize protein than cells cultured for 3, 6, and 12 days had. Fluorogram of extracellular proteins revealed that cells cultured for 9 days secreted much more proteins including apolipoprotein of HDL than other cells did(Fig. 5). These results suggest that functions of cultured hepatocytes changed with cultured days without morphological change. Therefore,

Table 1. Recovery of protein through the purification of Apo–A I and apo–A II

Protein	protein content (mg)	Recovery (%) to Total Protein
Eel serum	549.05	100
Lipoprotein (1.063 < d <1.21)	63	11.47
HDL	57.5	10.47
Apo–A I	3.96	0.72
Apo–A II	6.56	1.19

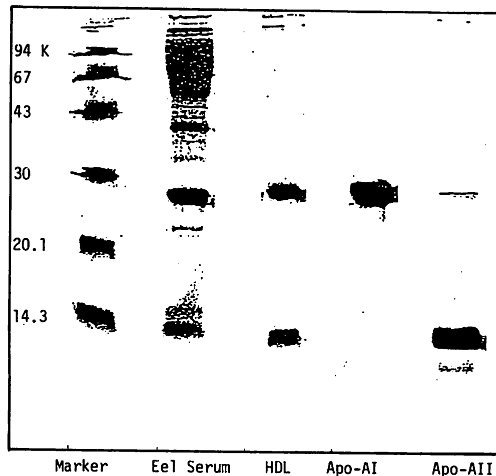

Fig. 4. SDS-polyacrylamide gel electrophoresis of eel serum, HDL, Apo-AI and apo-AII

Table 2. Effect of cultured days of hepatocytes on the incorporation of ^{14}C–leucine into extra–and intracellular protein

Cluture Days	extracellular Protein ($\times 10^{-4}$cpm/mg)	Intracellular protein ($\times 10^{-4}$cpm/mg)
3	4.26	9.15
6	5.59	12.06
9	8.49	12.38
12	2.59	8.48

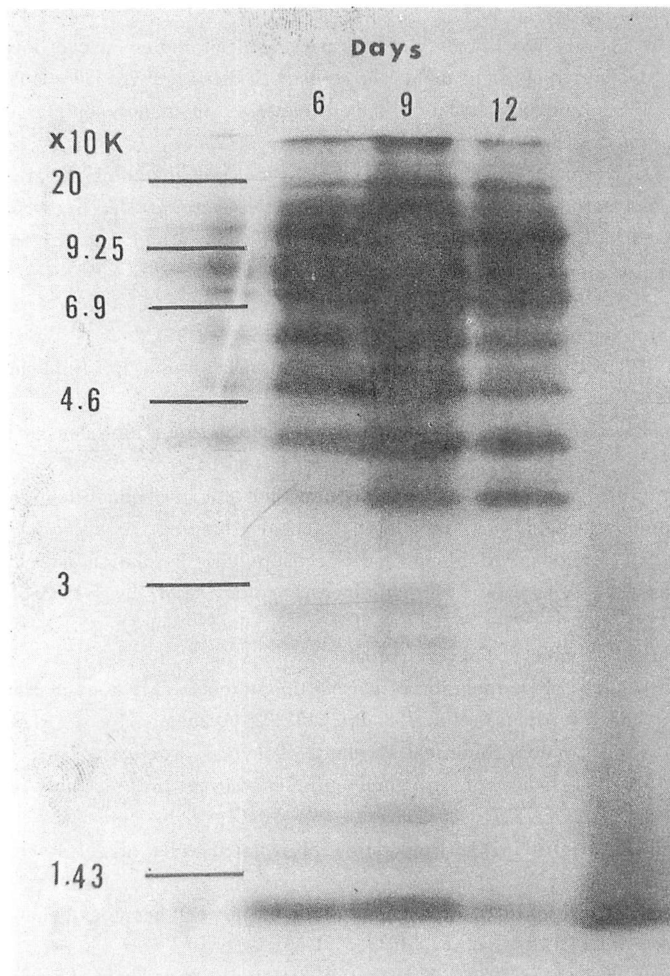

Fig. 5. Fluorogram of extracellular 14 C-proteins secreted by cultured eel-hepatocytes

the rate of apolipoprotein synthesis seems to be a good marker against functions of cultured eel-hepatocytes.

Iijima *et al.*[20] reported changes of lipoproteins in carp plasma after feeding with soybean oil. Triglyceride in VLDL and LDL increased from 3 to 20h after feeding, but that in HDL did not change during 20h. These results suggest that VLDL and LDL are synthesized in intestine after feeding promptly, and that little HDL is synthesized in carp intestine. In fish, HDL seems to be synthesized mainly in liver.

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