Purification and Characterization of Very Low Density Lipoprotein in Commercial Broiler and Crossbred Village Chickens by Fast Protein Liquid Chromatography

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ABSTRACT : Very low density lipoprotein (VLDL) of commercial broiler (CB) and crossbred village chicken (AK) was purified using Fast Protein Liquid Chromatography (FPLC). The fraction collected was then confirmed as VLDL using 4% polyacrylamide gel electrophoresis and transmission electron microscopy (TEM). The particle size of VLDL is 46.8±8.6 nm. The VLDL fraction was then subfractionated and the apolipoprotein (apo) profile was studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The CB and AK have almost similar types of apo in both subfractions 1 and 2. The AK showed the presence of apoAI, AIV, D and E whereas the CB had apo AIV, D, E and H. The apo AIV and apo E were present in both subfractions of AK and CB. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 12 : 1780-1785)***

Key Words : Very Low Density Lipoprotein, Broiler, Crossbred Village Chicken, Fast Protein Liquid Chromatography, Apolipoprotein

INTRODUCTION

Lipoproteins are heterogeneous lipid-protein macromolecule complexes that circulate in the plasma and lymph. There are several classes of lipoproteins, such as high density lipoprotein (HDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL) and chylomicrons (Smith et al., 1978). Each lipoprotein is classified on the basis of its floatation density (Redgrave et al., 1975) or size (Evans et al., 1977). VLDL density is less than 0.013 g/ml, with a size range of 21.5-71.7 nm, LDL 1.023-1.046 g/ml, 6.0-49.6 nm and HDL 1.052-1.130 g/ml, 6.0-17.9 nm, respectively.

There are various methods to separate lipoproteins. Traditional ultracentrifugation technique, sequential (Havel et al., 1955) or one-step gradient (Redgrave et al., 1975) are the most commonly used procedures for isolating lipoproteins. However, the technique has its drawbacks. It requires relatively large amounts of plasma, usually between 5 and 10 ml. Moreover, samples are exposed to the high centrifugal forces and salt concentrations during the separation process. These conditions lead to considerable alteration of the lipoprotein particles, resulting in loss of several apolipoproteins, primarily apo C and E from the respective fractions (Kunitake and Kane, 1982).

Size exclusion chromatography using agarose columns is an alternative to ultracentrifugation for the isolation of lipoproteins. By using this method, samples are not exposed to high centrifugal forces and salt concentrations. However, this method has its disadvantages. It is time-consuming (36-48 h per sample) and result in large sample dilutions. The eluted fractions are usually pooled, concentrated or extracted to enable for lipoproteins chemical component analysis (Gibson et al., 1983; Rudel et al., 1986).

Some workers (Carroll and Rudel, 1983; Okazaki et al., 1983) reported that high performance liquid chromatography (HPLC) can be used to separate lipoproteins. However, they found that the matrix in the column could interact with the lipoprotein particles and cause poor recoveries as well as clogging of the column.

These difficulties have prompted a development of a new procedure, Fast Protein Liquid Chromatography (FPLC), for the rapid and quantitative analysis of lipoprotein fractions. The FPLC has been used to separate lipoprotein in rat, rabbit and human (Ha and Barter, 1985; März et al., 1993; Innis-Whitehouse et al., 1998). The purpose of this paper was to study the isolation of chicken's VLDL using FPLC and to characterise the apolipoprotein (apo) of the VLDL.

MATERIALS AND METHODS

Two breeds of chicken, Avian (commercial broiler, CB)

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Table 1. Feed compositions of starter and finisher diets for two breeds of chicken

Ingredients	Starter diet	Finisher diet
Corn	506.00	549.00
Soybean meal	293.84	269.00
Wheat pollard	62.00	66.74
Crude palm oil	36.00	32.00
Fish meal	76.00	50.00
L-lysine	2.50	2.50
DL-methionine	2.00	2.00
Monodicalcium phosphate	10.00	14.00
Calcium carbonate	6.80	9.90
Choline chloride	0.60	0.60
Salt	2.50	2.50
Multimineral*	1.00	1.00
Multivitamin**	0.60	0.60
Antioxidant	0.10	0.10
Salinomycin 12%	0.06	0.06
Calculated analyses:		
Crude protein (%)	22.00	18.50
ME (kcal/kg)	3,100	2,950

* The multiminerals provide the following amounts per 2 kg of diet: Manganese 20,000 mg, Iron 80,000 mg, Zn 80,000 mg, Copper 100,000 mg, Cobalt 200 mg, Iodine 300 mg, and Selenium 300 mg.

** The multivitamins provide the following amounts per 100 kg of diet: Vitamin A, 500 IU, Vitamin D_3 625 IU, Vitamin E 7,500 g, Vitamin B_1 750 g, Vitamin B_2 2,500 g, Vitamin B_6 2,500 g, Vitamin B_{12} 0.10 g, Niacin 1,000 g, Folic acid 250 g, Biotin 0.125 g.

and Sasso crossed (crossbred village chicken, AK) were used in this study. The birds were housed in floor pens (3.72 m^2) containing a deep litter of wood shavings. Feed and water were given *ad libitum*. Both breeds of birds were offered same type of feed. Feed compositions for starter and finisher diets are shown in Table 1. They were kept on a 24 h photoperiod.

The birds were fasted for 18 h prior to blood sampling in order to ensure portomicrons were cleared from the circulation. The birds were slaughtered and the blood samples were collected into vacutainer tubes (Beckton Dickinson, UK) containing disodium EDTA as anticoagulant, at a final concentration of 1 mg/ml of blood. The blood sample was mixed by inverting the collection tube gently in order to avoid hemolysis. The blood samples were then pooled in a clean glass test tube and centrifuged at 1,500×g for 30 minutes under 4°C. Plasma was transferred to a clean storage test tube using a Pasteur pipette equipped with a rubber bulb. The plasma was then kept under -20°C for VLDL separation.

The VLDL was separated from the plasma by Fast Protein Liquid Chromatography (Äkta-FPLC) (Amersham Pharmacia Biotech, Sweden). The Äkta-FPLC consisted of a controller UPC-900, one P920 pump, an injection valve (INV-907) to load sample into a sample loop, a mixer (M-925) and an automated fraction collector (Frac-900). Two ml plasma sample was filtered through a 0.22 μm membrane filter (Minisart® Sartorius AG, Goettingen, Germany) and injected into an XK 16/70 (16 mm internal diameter, 700 mm in length) Superose 6 prep grade column (Amersham Pharmacia Biotech, Sweden) equilibrated with 0.15 M NaCl, 10 mM Na_2HPO_4, 5 mM Na_2EDTA, 0.02% NaN_3, pH 7.4. The buffer was filtered with 0.22 μm membrane filter and degassed before use, and the system was operated at 4°C. The eluent was monitored at 280 nm for protein content and collected in 2 ml fractions.

The VLDL fractions collected from FPLC were dialysed with 0.12 M ammonium acetate, 3 mM ammonium carbonate and 1.34 mM Na_2EDTA, pH 7.4. The dialysed VLDL was negatively stained with 2% (w/v) methylamine phosphotungstate. The stained preparation was examined under TEM (Hitachi H-7100). The particle size was determined by Ultrastructure Size Calculator (Slide Guide™).

Fractions corresponding to peaks of absorbance at 280 nm were pooled and their identity confirmed by polyacrylamide gel electrophoresis. The gels were 4% (w/v) (37.5% acrylamide, 1% bis-acrylamide) and made up in 1.5 M Tris-HCl, pH 8.8 for separating gel and in 0.5 M Tris-HCl, pH 6.8 for stacking gel. The VLDL samples (16 μl containing 10 μg protein) were mixed with 4 μl sample buffer (1 ml 0.5 M Tris-HCl, pH 6.8, 0.8 ml glycerol, 0.4 ml 2β-mercaptoethanol, 0.4 ml 1% bromophenol blue, 5.4 ml deionised water). The plasma was diluted 16-fold with the same sample buffer before use. The running buffer was 0.3% (w/v) Tris base and 1.44% (w/v) glycine, pH 8.3. The electrophoresis was carried out at 150 V, 150 mA and 20 W for 45 minutes. Following electrophoresis, the gel was stained with 0.2% (w/v) Sudan Black for 1 h and destained with 50% (v/v) ethanol for 30 minutes.

The VLDL was dialysed with 0.05 M NaCl, 5 mM Tris and 0.02% NaN_3, pH 7.4. $MnCl_2$ was added to not more than 1 mg of VLDL protein to a final concentration of 0.025 M. Subfractionation was carried out, using heparin-Sepharose column (Amersham Pharmacia Biotech, Sweden). The column was equilibrated with three column volumes (CV) of Buffer A (0.05 M NaCl, 5 mM Tris, 25 mM $MnCl_2$ and 0.02% NaN_3, pH 7.4). Three CV of buffer A were passed through the system to wash out the unbound protein followed by five CV of Buffer B (0.2 M NaCl, 5 mM Tris and 0.02% NaN_3, pH 7.4) and four CV of Buffer C (2.8 M NaCl, 5 mM Tris and 0.02% NaN_3, pH 7.4). Then, the system was washed with three CV of Buffer D (0.5 M NaCl, 0.01 M glycine and 0.02% NaN_3, pH 5.5) and Buffer E (0.5 M NaCl, 0.01 M glycine and 0.02% NaN_3, pH 8.5), respectively. Fractions were collected at 0.5 ml/min and absorbance was monitored at 280 nm. The fractions were dialysed against 0.15 M NaCl, 0.01% Na_2EDTA, 0.02% NaN_3, pH 7.4 for 24 h. The collected fractions were freeze-

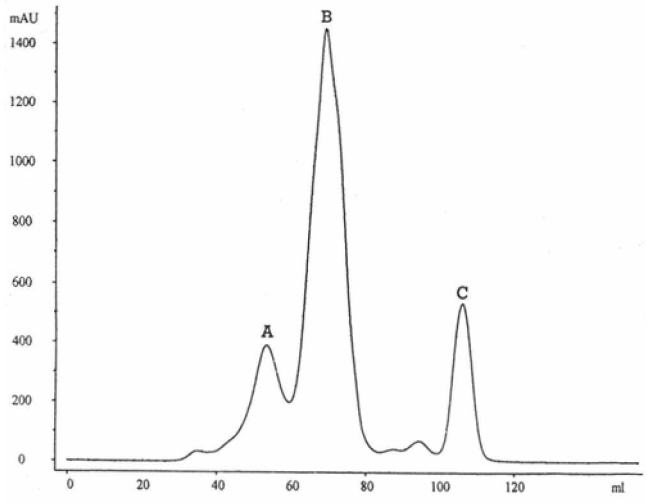

Figure 1. Chromatogram of chicken's plasma isolated by FPLC. Peaks A, VLDL; B, LDL and C, HDL.

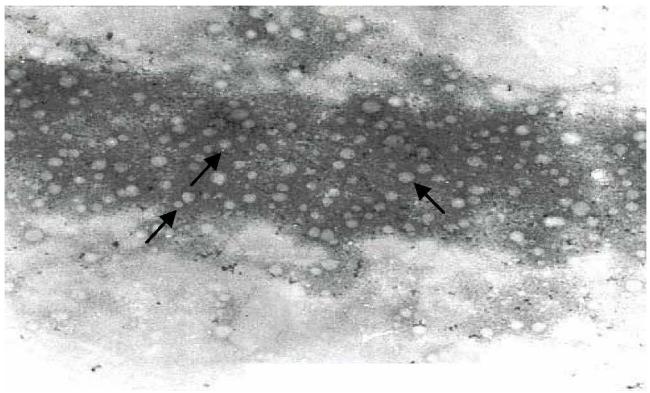

Figure 2. Negative stained transmission electron micrograph of peak A (×30,000). Arrows indicate empty vesicles believed to be VLDL with the size of 46.8±8.6 nm.

dried and studied for apolipoprotein profiles.

Very low density lipoprotein subfractions were delipidated by the method applied of Wright (1993). Five percent and 16% gel were used to determine the apolipoprotein profile of the VLDL subfractions. The 5% gel was for the high molecular weight apolipoprotein and 16% gel was for the low molecular weight apolipoprotein. Samples were dissolved in 20 µl sample buffer (1 ml 0.5 M Tris-HCl, pH 6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2β-mercaptoethanol, 0.4 ml of one percent bromophenol blue, 3.8 ml deionised water) and boiled in hot water for five minutes. The sample was then centrifuged at high speed for 30 seconds. The separating gel buffer was 1.5 M Tris-HCl, pH 8.8 and the stacking gel buffer was 0.5 M Tris-HCl, pH 6.8. The gel was prepared according to Laemmli System. Running buffer was 1.5% Tris base, 7.2% glycine and 0.5% SDS, pH 8.3. The buffer was diluted five times prior to electrophoresis. Two types of molecular weight standard were used, a low range standard (14400-94000 Dalton) and a high range standard (53000-212000 Dalton) that were purchased from Amersham Pharmacia Biotech, Sweden. Electrophoresis was carried out at 150 V, 150 mA and 20 W. Following electrophoresis, the gel was stained, using silver staining (Wray et al., 1981). The apolipoproteins were identified by comparing the sample bands with standard using Alphalmager™ 1220 Documentation and Analysis System with Alphalmager 1.220 Version 5.5 software (Alpha Innotech Corporation, California, USA). The proportion of the apolipoprotein was also determined with the same software.

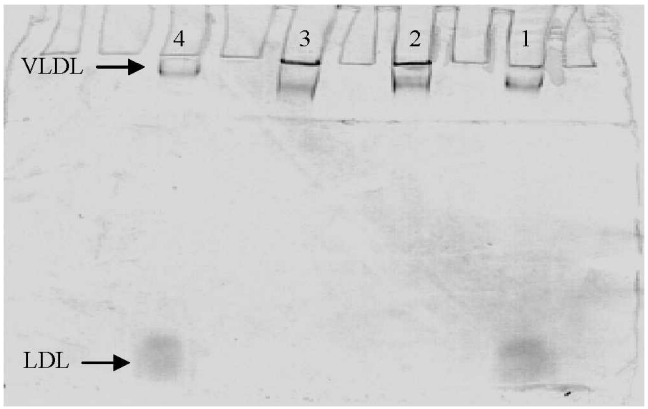

Figure 3. Four percent PAGE gel of AK and CB plasma samples. 1: AK's plasma sample. 2: Peak A fraction of AK's plasma. 3: Peak A fraction of CB's plasma. 4: CB's plasma sample. CB = commercial broiler; AK = village chicken.

Table 2. Types of apolipoprotein present in subfraction 1 and 2 of village chicken (AK) and commercial broiler (CB)

Subfractions	AK	CB
1	AI, AIV, E	AIV, D, E, H
2	AI, AIV, D, E	AIV, E

RESULTS

The elution profile of lipoproteins in the plasma fraction isolated by FPLC is presented in Figure 1. Three peaks were observed from the chromatogram. Peaks A, B and C were identified as VLDL, LDL and HDL, respectively.

Figure 2 shows the negative stained electron micrograph of peak A (×30,000). Particles measured are 46.8±8.6 nm in size. Figure 3 shows 4% polyacrylamide gel. Lane 1 is the plasma sample collected from AK. Lane 2 is peak A fraction of AK plasma. Lane 3 is peak A fraction of CB plasma and lane 4 is plasma sample collected from CB. First and second bands are VLDL and LDL, respectively. HDL had been eluted from the gel as the molecular size is too small to be retained in 4% gel. Peak A fraction collected from AK (Lane 2) and CB (Lane 3) migrated equal distance to VLDL bands of the whole plasma samples (Lanes 1 and 4).

Figures 4-7 show the apolipoprotein profiles of subfractions 1 and 2 for AK and CB. In AK, three bands

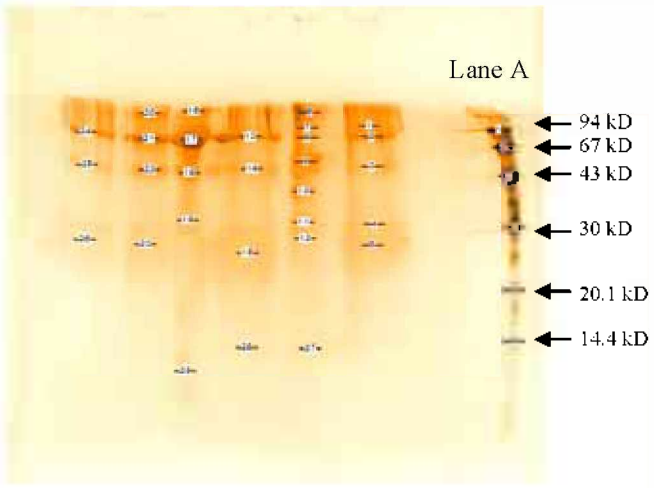

Figure 4. 16% SDS-PAGE gel of AK subfraction 1. Lane A consists of marker proteins: 1. Phosphorylase b, 2. Albumin, 3. Ovalbumin, 4. Carbonic anhydrase, 5. Trypsin inhibitor, 6. α-Lactalbumin. Band 15 is apo AI, band 10 is apo AIV and bands 4, 11 and 19 are apo E. The other bands are other proteins. AK = village chicken.

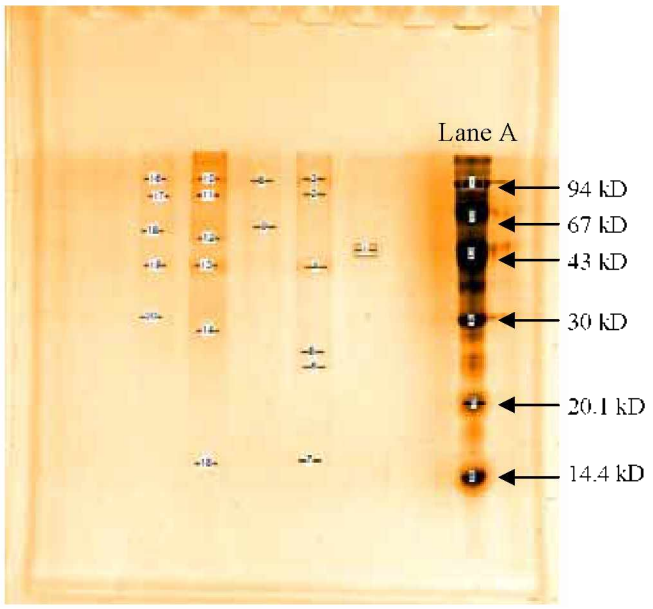

Figure 5. 16% SDS-PAGE gel of AK subfraction 2. Lane A consists of marker proteins: 1. Phosphorylase b, 2. Albumin, 3. Ovalbumin, 4. Carbonic anhydrase, 5. Trypsin inhibitor, 6. α-Lactalbumin. Band 5 is apo AI, band 14 is apo D, band 20 is apo E and bands 4, 13 and 19 are apo AIV. The other bands are other proteins. AK = village chicken.

with molecular mass 27 kD, 35 kD and 46 kD and four bands with molecular mass 28 kD, 32 kD, 35 kD and 47 kD were observed in subfractions 1 and 2, respectively. In CB subfraction 1, four bands with molecular mass of 32 kD, 36 kD, 47 kD and 49 kD were detected. However, in CB subfraction 2, only two bands with molecular mass of 35 kD and 46 kD were detected (Table 2).

DISCUSSION

In gel filtration chromatography, the molecules are eluted with decreasing particle size (Voet and Voet, 1990; Lehninger et al., 1993). Molecules with the largest size will be first eluted out from the column. Three peaks were observed from the chromatogram. Peak A may represent VLDL as the particle size is the largest among the lipoproteins. In order to prove that peak A is VLDL, the peak A fraction collected was subjected to TEM to measure the particle size and polyacrylamide gel electrophoresis was carried out. According to Evans et al. (1977), the particle size of hen VLDL is 21.5-71.7 nm, 6.0-49.6 nm for LDL and 6.0-17.9 nm for HDL. The particle size of peak A measured from the TEM was 46.8±8.6 nm, within the range of VLDL size. The reported molecular mass of VLDL is between 4×10^7 to 5×10^6 Dalton (Van Gent and Van Tol, 1990). In the absence of appropriate molecular weight markers in this range, whole chicken plasma was used for comparative purposes. Peak A fraction only showed one band in the gel and it migrated equal distances to the VLDL bands of the whole plasma samples. This again proved that peak A is purely VLDL.

März et al. (1993) found that when applied VLDL prepared by ultracentrifugation to FPLC, two fractions were obtained. A major one eluting at void volume and a minor one coeluting with LDL. However, by using FPLC a pure fraction can be obtained as is shown in Figure 3.

In ultracentrifugation, lipoproteins are exposed to high centrifugal forces and salt concentrations that result in detachment of apolipoprotein from the surface of lipoprotein particles. In contrast, März et al. (1993) were never able to detect free apolipoproteins in their experiments, using FPLC. Moreover, these researchers reported that abnormal lipoprotein particles are easily recognised as FPLC provides a continuous size distribution of particles, unlike ultracentrifugation that separates lipopproteins at fixed density cutoffs. They concluded that isolation of human lipoprotein by FPLC is precise and reliable.

Furthermore lipoproteins are separated according to their floatation density during using ultracentrifugation. Ha and Barter (1985) found that the density range defined for human plasma lipoprotein classes may not correspond exactly to that of rabbit or rat. The difference in the particle size of human high density lipoprotein (HDL) and that of rabbit or rat indicates that the density range of HDL may vary between these animal species. However, FPLC separates lipoproteins according to particle size. Any species' lipoproteins can be separated although their densities may not be determined.

Ha and Barter (1985) have used FPLC to carry out the separation of human, rabbit and rat plasma on a column of

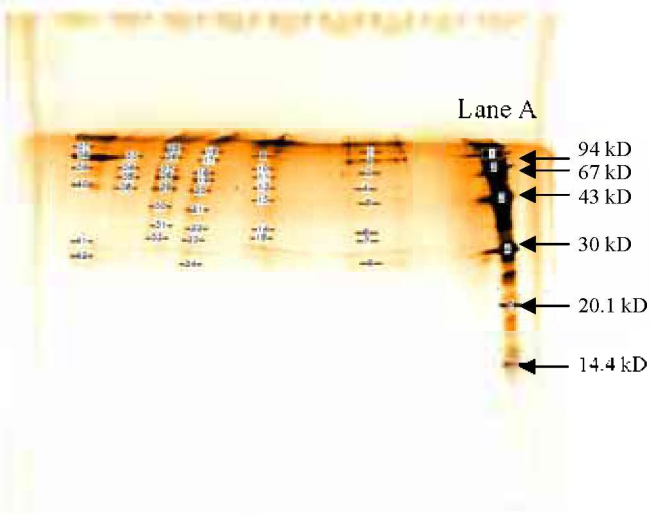

Figure 6. 16% SDS-PAGE gel of CB subfraction 1. Lane A consists of marker proteins: 1. Phosphorylase b, 2. Albumin, 3. Ovalbumin, 4. Carbonic anhydrase, 5. Trypsin inhibitor, 6. α-Lactalbumin. Band 42 is apo D, bands 7, 23 and 41 are apo E, bands 5 and 30 are apo H and band 21 is apo AIV. The other bands are other proteins. CB = commercial broiler.

Superose 6B. They used a flow rate of 0.75 ml/min and a good lipoprotein separation was achieved within 2-3 h. The fractions collected from the column were concentrated enough to be assayed directly for cholesterol and triacylglycerol (TAG) without any requirement for prior concentration of sample. März et al. (1993) also carried out an on-line enzymatic assay by combined enzymatic reagents with the lipoprotein eluate in special heated coils. This procedure solves the difficulties in determining low concentration lipid.

Innis-Whitehouse et al. (1998) validated that the TAG and cholesterol levels in the eluted fraction can be determined from only 200 µl plasma sample. They also demonstrated that the cholesterol contents of LDL and HDL as determined by FPLC was highly correlated to values obtained by the ultracentrifuge method and precipitation with dextran sulfate/$MgCl_2$. Hence, FPLC is a good alternative method for separating lipoproteins if the plasma sample volume is a limiting factor.

The apolipoprotein profiles of AK subfraction 1, three bands with molecular mass 27 kD, 35 kD and 46 kD were observed. These three bands showed similar molecular mass to that of human apo AI, E and AIV. Hence, AK subfraction 1 may consist of apo AI, E and AIV. In AK subfraction 2, four bands with molecular mass 28 kD, 32 kD, 35 kD and 47 kD were found. These molecular mass results indicated them to be apo AI, D, E and AIV, respectively. In CB subfraction 1, four bands with molecular mass of 32kD, 36 kD, 47 kD and 49 kD, suggesting apo D, E, AIV and H may be present in this subfraction. However, only two bands with molecular mass of 35 kD and 46 kD were detected in

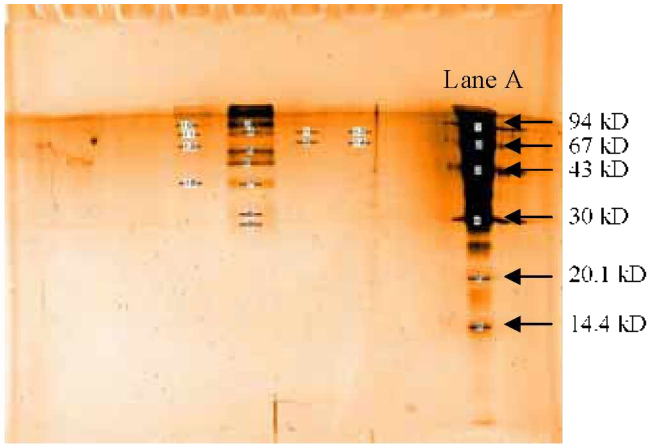

Figure 7. 16% SDS-PAGE gel of CB subfraction 2. Lane A consists of marker proteins: 1. Phosphorylase b, 2. Albumin, 3. Ovalbumin, 4. Carbonic anhydrase, 5. Trypsin inhibitor, 6. α-Lactalbumin. Bands 7 and 15 are apo AIV and band 8 is apo E. The other bands are other proteins. CB = Commercial broiler.

CB subfraction 2. They were probably apo E and AIV on the basis of their molecular mass. These results indicate that AK contained almost similar types of apolipoproteins with CB.

In conclusion, preparation of pure VLDL samples from chicken plasma can be simply achieved using FPLC. The whole purification period is less than 6 h. No further purification step is needed as only one band is obtained from the polyacrylamide gel. This shows that the fractions collected are not contaminated by other lipoproteins. The CB and AK have similar types of apo in both subfractions 1 and 2. The AK showed the presence of apo AI, AIV, D and E whereas the CB had apo AIV, D, E and H. Both subfractions of AK and CB contained apo AIV and E.

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