인체 LDL에서 분리한 Apo B-100의 표면 및 중심부위에서 지질과 결합하는 peptide에 관한 연구

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Lipid Binding Peptides Region of Surface and Core Fraction from Apo B-100 of Human LDL

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요 약

Objective: 인체내 LDL의 증가는 동맥경화 발병과 비례한다고 알려져 있으며, 최근, LDL의 변형된 형태인 산화 LDL(oxidized LDL) 및 여러 변형된 LDL(modifided-LDL)은 혈관내에서 macrophage와 작용하여 동맥경화의 초기단계를 형성한다고 알려 있다. 이러한 LDL은 지질과 단백질(apo B-100)로 구성되어 있다. 동맥경화 발병과 직결된 modified-LDL의 지질 및 단백질이, 어떠한 구조적인 형태로 변화되는지 확실히 알려져 있지 않으므로, 우선 native-LDL의 형태적 특성을 조사하기 위하여, 인체 LDL에서 분리한 Apo B-100의 표면 및 중심부위에서 지질과 결합하는 peptide를 알아보고자 하였다.

Methods: 본 연구에서는 apo B-100의 표면 및 중심부위의 peptide가 지질과 결합하는 부위를 알기 위해, LDL의 단백질 성분인 apo B-100의, 지질에 둘러싸인 중심(core) 부위와 표면(surface)을 각각 분리하여, 지질을 제거한 중심(core) 부위와, 표면(surface)부위를 trypsin과 pronase의 두가지 효소를 사용하여 peptide로 만든후, DMPC(dimyristoyl phophatidylcholine) vesicle에 결합시켜 지질과 결합하는 부위를 연구하였다.

Results: 본 연구의 결과, LDL의 지방산 조성은 불포화 지방산으로는 linoleic acid와 oleic acid가 주요 성분이었으며, 포화지방산은 palmitic acid와 stearic acid가 주요 성분이었다. Apo B-100의 중심(core)과 표면(surface)을 trypsin과 pronase의 두가지의 효소를 사용하여 peptide를 만든후 DMPC와 재결합 시킨후, 지질과 결합하는 peptide를 HPLC의 peptide mapping 방법으로 조사하였다. 실험의 결과, LDL의 주요 단백질인 apo B-100에서 지질과 결합이 가능한 8개의 peptide부위 (lipid-associating peptide)를 알수 있었다.

Conclusion: 이러한 결과로서, 지질과 결합되어 있는 LDL의 주요 단백질인 apo B-100는, 중심(core) 및 표면(surface)의 여러 부위에서 광범위하게 지질과 결합되어 형성되어 있음을 알수 있었으며, 동맥경화와 관련된 변형된 LDL(modified-LDL)에서는 이와는 다른 형태의 지질 및 단백질의 결합형태를 조성되어 있을것으로 예측된다.

중심단어: LDL(Low Density Lipoprotein), apo B-100
중심 및 표면 부위의 부분(Core and surface fraction), 지질결합부위(lipid-associating peptide)

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Introduction

Apo B-100 is an important component in the system of plasma low density lipoproteins (LDL). It has functions as the ligand for the LDL receptor in peripheral cells. In humans, much of the apo B-100 of VLDL is transferred to IDL and subsequently to LDL. Apo B-100 is synthesized by liver and is an obligatory constituent of VLDL, IDL, and LDL[1,2,3].

The LDLs are removed from the circulation by both high-affinity receptor-mediated and receptor-independant pathways, the liver being the major organ responsible for LDL clearance. LDLs are removed less efficiently by the hepatic receptors through binding to apo B-100, gradually gain access to extravascular compartments of various organs and tissues which contain LDL receptors, so that an appreciable fraction is taken up in extrahepatic tissues as well[2,3]. The distribution of LDL to various tissues depends on mainly the rate of transcapillary transport and the activity of LDL receptors on cell surfaces.

These negatively charged regions presumably constitute the ligand-binding sites of the receptor which interact electrostatically with the positively charged region of the apolipoprotein ligands.

LDL are heterogeous in their lipid contents, size and density and small LDL subspecies may increase risk of atherosclerosis possibly due to differences in the conformation of apo B in the particle[1,2,3]. Elavated LDL cholesterol and Apo B-100 concentrations are associated with an incresed risk of atherosclerosis.

Small, dense LDL particles are more atherogenic than larger, buoyant LDL particles. Small, dense LDL particles contain less phospholipids and unesterified cholesterol in their surface monolayer than large, buoyant LDL particles. This difference in lipid content appears to induce changes in the conformation of apo B-100, leading to more exposure of proteoglycan-binding regions[4]. Although the primary structure of apo B-100 has been determined, its changes in structure and conformation on LDL are still poorly understood.

In the present study, a number of apo B-100 core and surface peptides by enzyme digestion have been characterized. A comparison of the behavior of the core and surface should provide information about the regions of apo B-100 involved in LDL metabolism and also about the structural features concerning on the formation of atherosclerosis. We have studied the characterization of surface and core fraction in order to search for physical features of inside and outside of apo B-100. The distribution of lipid-associating regions of the surface and core peptide fraction of apo B-100 by recombinding proteolytic peptides with DMPC vesicle was also charaterized.

Materials and Methods

1. Materials

Trypsin and pronase were purchased from Sigma chemical Co. (St. Louis.USA). Acetonitrile, methanol, HPLC grade water, and other solvents for HPLC were obtained from E. Merck (Darmstadt, Germany) and J.T.Baker (Phillipsburg, USA). DMPC (dimyristoyl phosphatidylcholine) was obtained from Sigma chemical Co. (St. Louis, USA).

2. Isolation of Low Density Lipoproteins

The blood from healthy donors was used, and aprotinin (0.055 Units/ml), 0.05% EDTA and 0.05% NaN_3 were added to the plasma. The LDL, 1.025 <

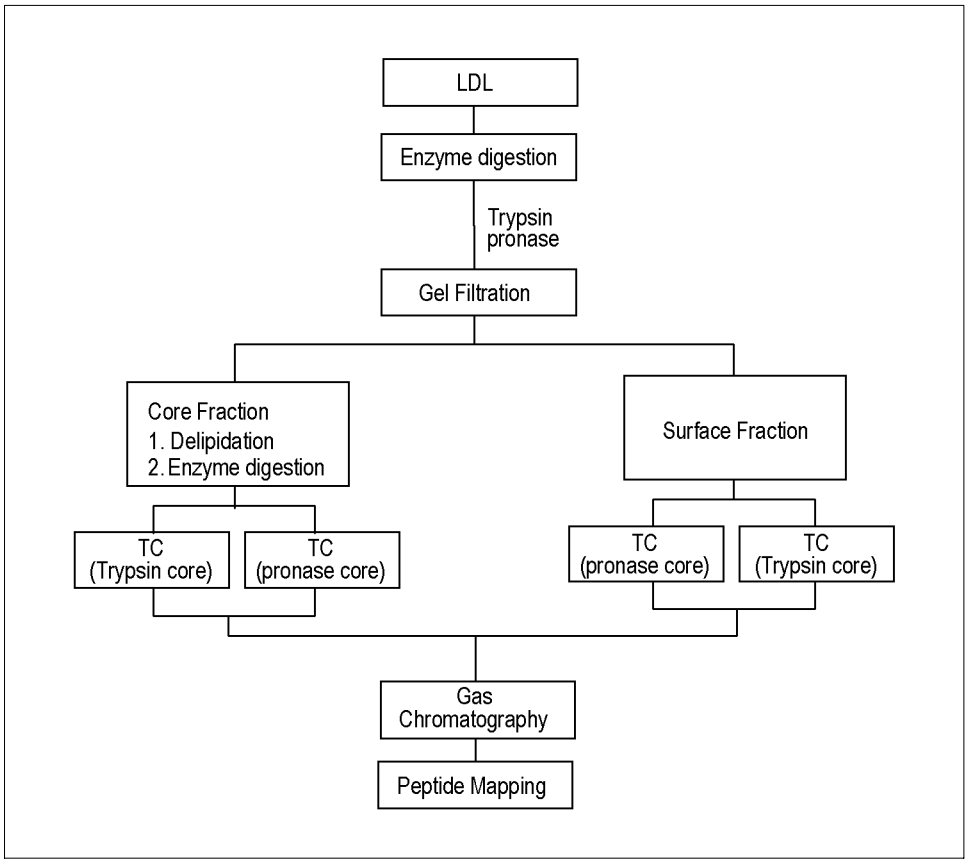

Fig. 1. Separation of surface and core peptide fraction from apo B-100 of human LDL.

d < 1.055 g/mL, were isolated by sequential ultracentrifugation using a KBr gradient at 40,000 rpm for 15 hrs at 4℃, followed by one more runs for 24 hrs under the same conditions[5,6]. The protein concentration of the LDL was determined by the method of modified Lowry et al[7]. Purified LDL was dialyzed against 0.1 M ammonium bicarbonate (NH_4HCO_3), pH 8.0 buffer with a No.2 dialysis bag (Spectra/por). The purification of LDL was identified by 5～14% SDS-PAGE and the result was identified if there was only one protein band at the position of apo B-100.

3. Enzyme Digestion

Enzymatic digestion of LDL was performed with trypsin or pronase (Fig. 1). The enzyme/substrate ratio was 1:150 (w/w). The mixture was stirred at room temperature for 8 hrs. The enzyme hydrolysate was applied to a Sephadex G-50 (2.6×40 cm) column at a flow rate of 4 ml/10 min. Each 4 mL fraction was monitored by ultraviolet absorbance at 220 nm[8,9] Surface fractions of apo B-100 generated by digestion of LDL with trypsin or pronase and the remaining core fragments were pooled and lyophilyzed. The lyophilyzed core fragments were delipidated by extracting three times with 50 ml of ether/ethanol (3:1, v/v). Each extraction was carried out by adding the solvent to the samples by vortexing and placing the sample in a freezer (-20℃) for 1 hr, and then centrifuged at 2,000 rpm and

removed the solvent by aspiration. The core fragments were redigested with the trypsin or pronase (enzyme/substrate ratio was also 1:150). 50 μL of surface and core fractions were used for the idendification with 5～14% gradient SDS-PAGE.

4. Gas Chromatography

Gas chromatography analysis was performed with a Hitachi model 163 equipped with F.I.detector [10,11,12,13]. A 2 m×3 mm (ID) glass column containing GP 3% SP-2310/2% SP-2300 on 100/120 chromosorb was used for the GLC analysis. The temperature was as follows; injector, 220℃: column, 130℃ for 1 min, and detector, 220℃. The flow rates were : nitrogen, 39 ml/min : hydrogen, 36 ml/min : and air, 500 ml/min. The sensitivity and sample size were 8×10^2 afs and 1 μL, respectively. Chart speed was 10 mm/min.[15,17]

5. Isolation of Lipid Associated Peptides from Apo B-100

DMPC (dimyristoyl phosphatidylcholine) vesicles were prepared by sonication. 300 mg of DMPC in chloroform were placed in pyrex tube and the organic solvent was removed with a stream of nitrogen. The tube was placed in a lyophilizing flask and place under a vacumn for 10 min to remove the last traces of solvent. 15 mL of Tris buffered saline (10 mM Tris, 1 mM EDTA, 1 mM NaN_3, 100 mM NaCl) solution was put into the tube and placed on ice and sonicated until clarified. The sample was centrifuged for 20 min at 10,000 rpm in a J-20 rotor to sediment the titanium particles. The supernatant is now more than 95% single bilayer vesicles. The digested apo B-100 (100 mg in 5 mL) was mixed with sonicated single bilayer vesicles of DMPC (300 mg in 15 mL) at 2 4℃, the transition temperature of the lipid. Lipid-protein complex was monitored using UV-spectrophotometer at 220 nm. The density of the sample was adjusted to 1.15 mg/mL by the addition of solid KBr and subjected to ultracentrifugation at 4℃ 30,000 rpm in a Beckman 70 Ti rotor for 18 hrs to separate the peptides that bind to DMPC from the remainder of the peptides. The floated lipid-protein complexes were removed with a pasteur pipette and dissolved in 10% sodium cholate in standard buffer before applying the sample to a 2.8×40 cm column of Sephadex G-50 in 0.1 M ammonium bicarbonate for desalting. The fractions that eluted in the void volume were lyophilized and subjected to additonal purification by HPLC.

6. Peptide mapping of lipid binding peptides from Apo B-100

Core and surface fragments of apo B-100 were fractionated on a Waters HPLC system equipped with a variable wavelength detector. The column temperature was set at 50℃. For fractionation, a Vydac C18 reverse phase column (1.0×25 cm) was used with a trifluoroacetic acid (TFA) buffer system at a flow rate 1.5 ml/min, buffer A: 0.1% TFA in water (v/v), buffer B: 0.08% TFA on 95% acetonitrile and 5% water (v/v/v). A linear gradient of buffer B at 1% B per minute was applied. The eluted fractions were monitored at 220 nm.

Results and Discussion

1. Enzyme digestion of LDL

To remove surface fraction from lipid-containing core fraction, LDL was digested with trypsin or

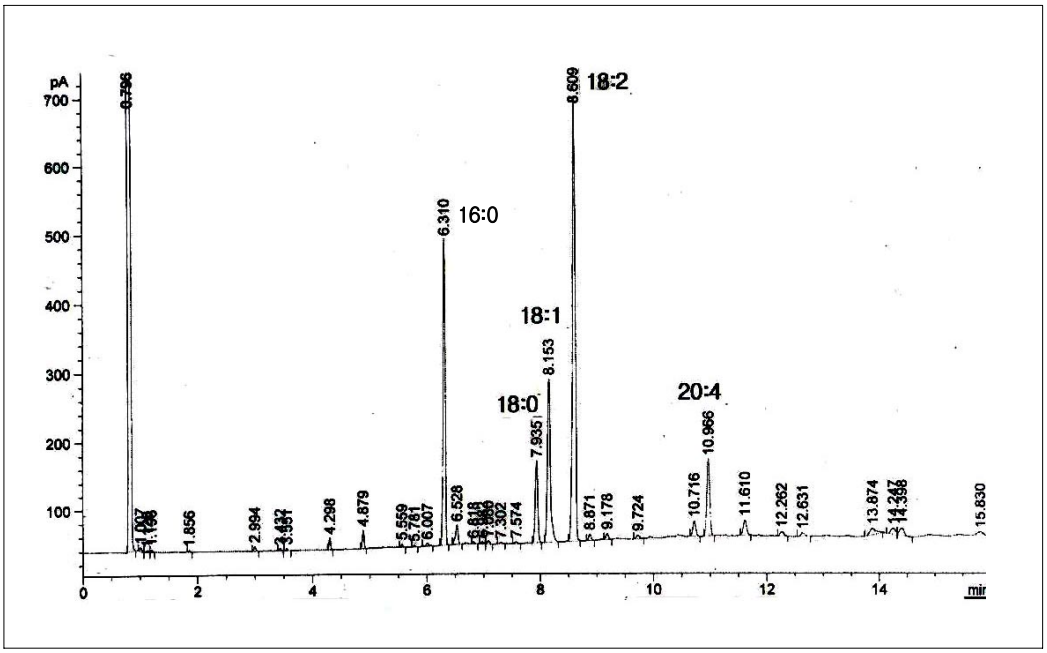

Fig. 2. Fatty acid pattern of LDL.
LDL was extracted with ether and ethanol (3:1,v/v). For the characterization of fatty acid composition, the extracted lipid-moiety was separated into two subclasses, first extraction and second extraction.

pronase (Fig. 1). A unspecific cleavage enzymes, pronase was chosen to compare the effect of trypsin, a specific-cleavage enzyme. Trypsin is a pancreatic serine protease with substrate specificity based upon positively charged lysine and arginine side chains. But, pronase acts a unspecific endogenous and exogenous cleavage of apo B-100 polypeptide. Proteolytic hydrolysis of apo B-100 by either trypsin or pronase generated peptides with mass from approximately 20～70 kDa[14,15,17]. Therefore, pronase-digested peptides showed much smaller size than trypsin-digested peptides. The first group surface of apo B-100 of peptides was labelled as trypsin-surface (TS) or pronase-surface (PS), and the second group core of apo B-100 as trypsin-core (TC) or pronase-core (PC). From the previous results[7], pronase digestion was more extensive to remove exposed surface protein fraction from LDL compared to trypsin digestion. However, elastase digested peptide did not show any significant peaks on HPLC peptide mapping[7].

2. Fatty acid composition of LDL

Fig. 2 shows a fatty acid composition of LDL. The major fatty acids were palmitic (16:0), oleic (18:1) and linoleic (18:2) acid, approximately 60% of the total fatty acid content. Palmitoleic (16:1) and stearic (18:0) were constituted of the remaining 10%. From the above result, the major fatty acids of LDL were palmitic and linoleic acid.

3. Lipid-binding of core and surface peptides of apo B-100

Core and surface peptides of apo B-100 by

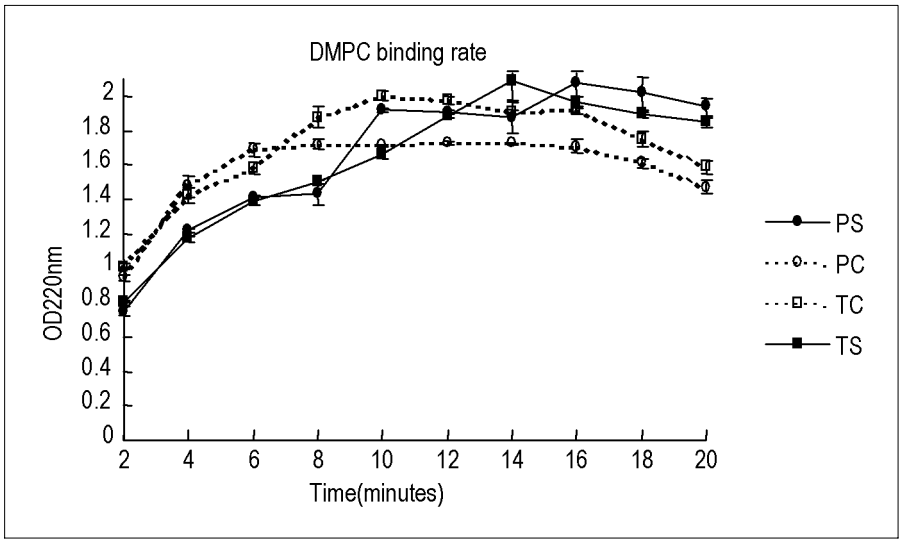

Fig. 3. Lipid binding of core and surface peptides to DMPC single bilayer vesicles.
● : Pronase-surface, ■ : Trypsine-surface
○ : Pronase-core, □ : Trypsine-core

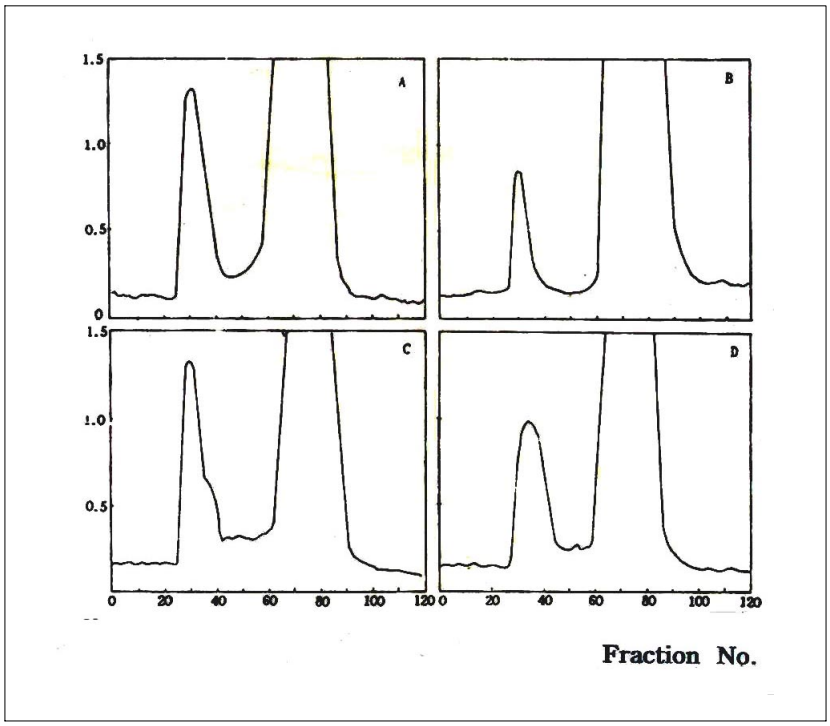

Fig. 4. Gel Permeation chromatography of core and surface peptides by enzyme digestion with Sephadex G-50
A: Trypsin-core, B: Trypsin-surface,
C: Pronase-Core, D: Pronase-surface

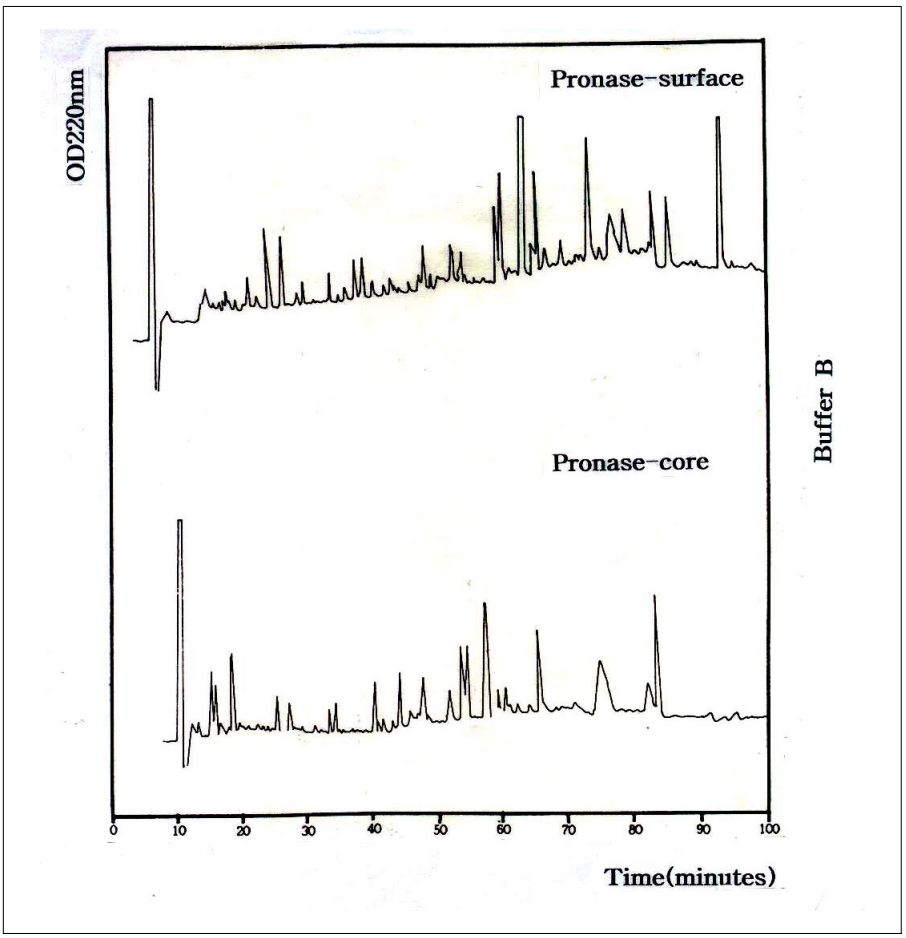

Fig. 5. HPLC chromatogram of the lipid-associating core and surface peptide by pronase.

enzymatic digestion were associated with DMPC single bilayer vesicle at the transition temperature (24℃) and then, the lipid-bound peptides from apo B-100 were stabilized by using the cholate removal technique. The formation of small discoidal particles between DMPC vesicles and the apo B-100 fragments (TC, TS, PC and PS) was monitored by measuring the absorbance at 220 nm as a function of time (Fig. 3). DMPC binding rate of all groups showed a progressive increase up to 10 min. And, lipid-binding reached a maximum plateau between 10 and 16 min and decreased slowly whereas PC showed a decrease in binding rate after 10 min. The spontaneous association of apo B-100 peptides with DMPC, a saturated phospholipid, was considered by the insertion of the apo B-100 into pre-existing lattice vacancies in the bilayer[2].

4. Separation of lipid-associated peptides by HPLC

Lipid-associated core and surface peptides were isolated by gel permeation chromatography on a sephadex G-50 (2.8×40 cm column) for chracterization of lipid-binding peptides as illustrated in Fig. 4. Lipid-bound peptide mixtures were collected in void volumes (first peak) and lipid-unbound free

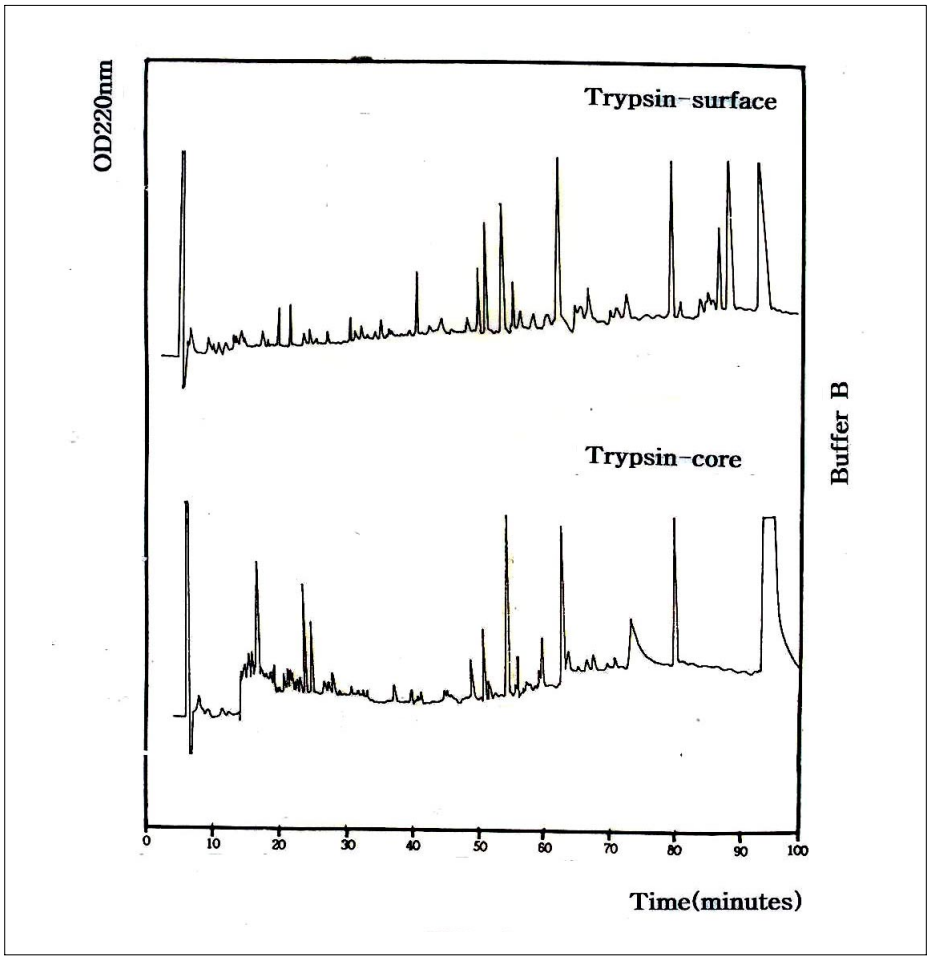

Fig. 6. HPLC chromatogram of the lipid-associating core and surface peptide by trypsin.

peptides (later peak) was discarded. Lipid-peptide mixture (void volume) showed much smaller amount than lipid-inaccessible peptides. The lipid-associated surface and core peptides of apo B-100 were separated and identified by HPLC. To compare the lipid-associating regions of surface and core reptides of apo B-100 with those of other peptides that are known to associate with lipids spontaneously, we have performed HPLC mapping using Vydac C18 column and TFA buffer system. The chromatograms obtained by HPLC were shown in Fig. 5 and Fig. 6. Lipid-associated surface and core peptide (either pronase or trypsin digestion) showed different HPLC chromatogram. It indicated that surface and core peptides have different lipid-binding sites. Lipid-bound peptides were appeared between 50 and 90 min from HPLC chromatogram, and phospholipid peaks were shown from 10 to 50 min. Phospholipids are much polar than lipid-associated peptides, therefore lipid-associated peptide sample elute later than phospholipid components.

Table 1 summarizes the retention times of lipid-associated peptides by trypsin or pronase digestion from HPLC chromatogram. Four major peaks for

Table 1. Retention times of Lipid Binding Peptides Cleavaged by Pronase or Trypsin from HPLC Chromatogram

unit; minutes

Peak No.	Trypsin Cleavaged Peptides		Pronase Cleavaged Peptides	
	Core	Surface	Core	Surface
1	98.15	96.89	-	-
2	-	91.91	-	-
3	-	90.47	-	-
4	82.70	82.57	82.13	81.58
5	75.39	75.24	72.83	74.22
6	-	-	-	72.26
7	39.00	69.21	68.96	68.15
8	-	-	-	66.36
9	63.83	63.99	63.66	63.13
10	60.59	-	-	60.50
11	-	58.01	-	-
12	56.62	56.89	-	55.83
13	54.88	54.97	54.61	53.85
14	-	52.04	51.13	50.60
15	51.18	51.48	51.13	50.60
16	48.95	-	-	-
17	-	41.08	40.93	-

all groups (TC, TS, PC and PS) showed the identical retention times 51, 55, 64, 69, and 83 minutes. And, other possible lipid-associating peptides are 52 min (TS, PC, PC), 56 min (TC, TS. PS) and 75min (TC, TS, PS). Therefore, eight possible lipid-associating peptides were found from the HPLC chromatogram. Most of possible lipid-associating peptide peaks were collected manually for the identification.

The lipid-binding peptides were derived from a mixture of small peptides from apo B-100, and were not representative of the full apo B-100 polypeptide. Human LDL contains 80% lipid and 20% protein by weight and possess a very high affinity for lipids[8]. This high affinity for lipids suggests a structural role for apo B-100 in the formation and maintenance of lipoprotein particles. And Yang[1] reported that lipid-binding regions of apo B-100 are widely distributed from apo B-100 can associate in vitro with lipids to from LDL-like particles. These results are well accord with our findings. Reduction of the phospholipid content in the surface monolayer LDL by treatment with secretory phospholipase A_2 ($sPLA_2$) forms small,

dense LDL with an enhanced tendency to interact with ptoteoglycans[19]. For the characterization of the surface and core peptides of apo B-100 from human low density lipoprotein, LDL was digested with either trypsin or pronase.

In conclusion, apo B-100 is an important protein component in the system of plasma lipoprotein. It has functions as the ligand for the LDL-receptor in peripheral cells. And LDL is the major carrier of cholesterol in plasma, and increased concentration is correlated with the development of atherosclerosis[23]. Changes of physical and structural properties of apo B-100 has been known to be related to the development of atherosclesosis, and further studies should be continued.

Abstract

Apo B-100 is a major physical ligand for the LDL receptor. LDLs are the major cholesterol carrier in circulation. LDL subspecies increase risk of atherosclerosis due to differences in the conformation of apo B in the particle. The difference in lipid content appears to induce changes in the conformation of apo B-100, leading to more athrogenic. In the present study, the distribution of lipid-associating region of surface and core peptides of apo B-100 has been investigated by recombining peptides with DMPC vesicles. Fragments of apo B-100 were generated by digestion of LDL with trypsine or pronase. Proteolytic fragments and DMPC (dimyristoyl phosphatidylcholine) were reassembled at the transition temperature of the lipid and stabilized by cholate. Eight lipid-associating regions of apo B-100 were investigated by peptide mapping, and peptides were collected as possible lipid-binding peptides from HPLC seperation. Our results indicate that lipid-associating regions of apo B-100 are widely distributed within apo B-100 polypeptide.

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