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Effects of Salt and Heparin on the Activation of Lipoprotein Lipase (LPL)

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Abstract

Lipoprotein lipase (LPL) is an acylglycerol hydrolase and is the extrahepatic enzyme responsible for the hydrolysis of triglyceride-rich plasma lipoproteins. LPL has been isolated from bovine milk by affinity chromatography on heparin-sepharose in 2M NaCl, 5mM barbital buffer, pH 7.4. Para-nitrophenyl butyrate (PNPB) was used as a substrate for the determination of LPL activity. Molecular weight of LPL was 55KD on 10% SDS-PAGE. When the effects of heparin on LPL activation were compared, LPL activity of heparin added group increased approximately 5 times higher than that of heparin non-added groups. These results indicated that heparin involved in the stabilization of LPL structure that led to increase enzyme activity. Furthermore, LPL activity increased about 4 times compared to the absence of heparin at various pH. LPL was stabilized when heparin was added either low or high salt concentrations. With the presence of heparin, NaCl concentration did not affect LPL activity at pH range 6~9.

Key words : lipoprotein lipase (LPL), purification, salt, heparin, bovine milk

INTRODUCTION

The enzyme, LPL, is localized on the surface of the capillary endothelium and is widely distributed in extrahepatic tissue, including heart, skeletal muscle, and adipose tissue. Although the LPL isolated from these tissues is capable of breaking down plasma triglyceride (TG) of TG-rich lipoproteins, including chylomicrons and very low density lipoproteins (VLDL), it has only limited activity toward artificial acylglycerol emulsions unless serum is added as a source of activator (1-3).

This enzyme is synthesized in adipose, muscle, and several other tissues and is transported to the luminal surface of capillary endothelial cells (3,4). The function of LPL is to direct the influx of plasma TG in the form of fatty acids into the peripheral tissues for storage and to provide fuel for energy requirement. For this reason, the control of LPL is an important regulatory step for directing traffic of TG-fatty acids to fulfill the energy requirements of peripheral tissues in a tissue specific manner (5-7).

In addition to lipoprotein lipids, LPL also catalyzes the hydrolysis of water-soluble short chain fatty acyl ester substrates such as p-nitrophenyl acetate and p-nitrophenyl butyrate (8,9). For maximal hydrolysis of long chain fatty acyl esters, the enzyme requires apo C-II (10), a protein constituent of triglyceride-rich lipoproteins and high density lipoprotein (11) ; apo C-II does not enhance the hydrolysis of water-soluble substrate or of short chain triacylglycerols such as tributyrylglycerol. The deficiency or dysfunction of LPL was found to be associated with the pathogenesis of hypertriglyceridemia (12).

Human LPL has 87~94% identy with other mammalian LPLs (1). Therefore, bovine milk LPL was used instead of human LPL for the test. The purposes of this study were to purify the LPL from bovine milk and to investigate the effects of salt and heparin on the changes of activation and physical properties of LPL.

MATERIALS AND METHODS

Materials

Bovine fresh milk was obtained from Dairy Farm (Kangwon National University). Heparin agarose, he-

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parin, p-nitrophenyl butyrate (PNPB) were purchased from Sigma (St. Louis, USA). Acetonitrile, methanol, and HPLC grade water were obtained from J.T Baker (USA). Triglyceride GII Kit was purchased from Wako chemicals (Japan). Other chemicals used were the highest reagent grade.

Isolation of lipoprotein lipase from bovine milk

Unpasteurized raw skimmed milk was prepared from fresh whole milk by centrifugation. In the cold room, the skimmed milk was stirred with heparin agarose (200ml/4L of milk). After overnight, filtration was begun using 500ml coarse sintered glass funnel. The heparin agarose where the lipase was bound washed as described by Yang *et al.* (13) ; 5mM barbital, and 0.2% triton X-100, pH 7.4 were used for washing. The lipase bound resin was suspended in the mixed solution of 0.75M NaCl, 25% glycerol, 5mM barbital-(pH 7.4) and packed into a column, 2.8×20cm. After washing with the same buffer, lipase was collected overnight by step-wise elution with a solution of 2.0M NaCl, 25% glycerol, and 5mM barbital, pH 7.4, at a flow rate of 16ml/hr. Each fraction was measured at 280nm, and purity was analyzed by 10% SDS-PAGE.

SDS electrophoresis analysis

SDS-PAGE was performed on 10% gels using an electrophoresis buffer consisting of 25mM Tris, 192 mM glycine and 0.1% SDS (pH 8.3). Samples were diluted in a sample buffer consisting of 10mM Tris (pH 8.8), 1mM EDTA, 50g/L SDS. Electrophoresis was performed at a constant 30mA and gels were fixed for 20min in 30% methanol, 9% acetic acid, 61% water and stained for 1hr with 1% Coomassie Blue R-250 in 10% acetic acid. Destaining was performed in 63 % water, 7% acetic acid, and 30% methanol.

Separation of LPL using HPLC

A Waters Associates HPLC system was used for the separation and identification of LPL. Separation was carried out with a Vydac C_{18} column at a flow rate of 1.5ml/min. A trifluoroacetic acid (TFA) buffer system (A : 0.1% TFA in water, V/V ; B : 0.08% TFA in 95 % acetonitrile and 5% water, V/V/V) was used for the separation. A gradient of buffer B was linearly increased at a rate of 1%/min. Protein concentration was measured at 280nm.

Measurement of lipase activity

Lipase activity was assayed in a reaction mixture containing PNPB (50μl), LPL (24μg) and heparin (2μg) with a final volume of 1.0ml of 0.1M sodium phosphate, pH 7.2, containing 0.9% NaCl. The hydrolysis of PNPB was determined by monitoring the increase in absorbance at 400nm continuously using a no-enzyme incubation mixture as a blank or by measuring the absorbance after extracting p-nitrophenyl butyrate from the reaction mixture (9,14).

Effect of salt and pH on the activity

To determine the effects of salt on LPL activity, each LPL fraction (295μM/min/mg) was dialyzed against several NaCl concentrations (18hr, 40° C). The salt concentrations used were 0.15, 0.75, 1, 2, and 3M NaCl. The effects of pH on LPL activity were examined under the condition where the salt concentration was varied from 0.15 (the physiological salt concentration) to 3M. 0.1M of HCl or NaOH was used to adjust the pH. The MWCO (molecular weight cut-off) of dialysis tube was 12,000~14,000.

RESULTS AND DISCUSSION

Purification of LPL

The yield of LPL from bovine milk was shown in Table 1. From total 4L of milk, the purified LPL was 3.36mg/L by heparin-agarose chromatography. Specific activities of the whole milk and the purified LPL were 4.30 and 295.13μM/min/mg, respectively.

In Fig. 1, purified LPL using heparin agarose chromatography was shown in one peak from 40 to 50 fraction tubes.

Table 1. The yields of lipoprotein lipase

	Volume (ml)	Total protein concentration	Specific activity (μM/min/mg)	Yield (%)
Whole milk	4,000	46.56g	4.30	100
Skim milk	3,800	40.28g	*	*
Purified LPL	28	13.44mg	295.13	1.98

* Not detected

Identification of LPL by 10% SDS-PAGE

Purified LPL was identified on 10 % SDS-Polyacrylamide Gel Electrophoresis (Fig. 2). LPL, resolved in buffer containing 2M NaCl, was dialyzed against the various concentrations of NaCl solution, 0.15, 0.75, 1, 2 and 3M, respectively, to compare the effect of NaCl on LPL activity. Two bands, 55KD and 100KD, were found on SDS at 0.15M NaCl dialyzed (lane 7) and H_2O-dialyzed LPL (lane 8), but only one band, 55KD, was observed when LPL was dialyzed against NaCl concentration varied from 0.75 to 3M (lanes 3~6).

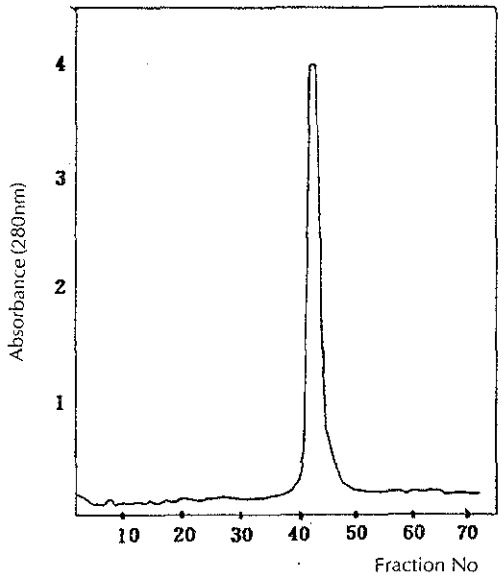

Fig. 1. The purification of lipoprotein lipase using heparin agarose chromatogram.

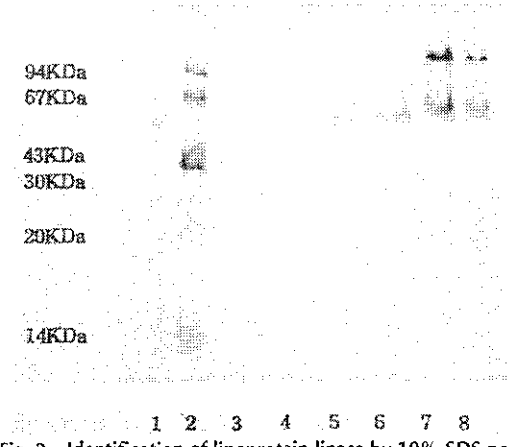

Fig. 2. Identification of lipoprotein lipase by 10% SDS polyacrylamide electrophoresis.
(1) No dialyzed LPL (2) Standard
(3) 3M NaCl dialyzed LPL (4) 2M NaCl dialyzed LPL
(5) 1M NaCl dialyzed LPL (6) 0.75M NaCl dialyzed LPL
(7) 0.15M NaCl dialyzed LPL (8) H_2O dialyzed LPL

Wang *et al.* (15) reported that LPL had a mature protein of 498 amino acids with a calculated molecular weight of 54,700. Therefore, it could be concluded that 55KD band was LPL. Lverius and Ostlund (16) reported that LPL was a monomer, but showed several different MW by the methods. Sedimentation equilibrium ultracentrifugation using 6.6M guanidine HCl, under the reducing condition, showed the MW of LPL as 48,300 but 50,800 MW was calculated from analytical gel chromatography method. Also he reported analyses of the sedimentation coefficient ($S^\circ_{20,w}$=5.40S) and the diffusion coefficient ($D^\circ_{20,w}$=48.8μm^2/S) in a buffer of physiological pH and ionic strength yielded a molecular weight of 96,000. He also determined that the native enzyme appeared to be a dimer of presumably identical subunits in solution.

As evident from the results described above, 100 KD band (Fig. 2) from 0.15M NaCl-dialyzed LPL might be a dimer of two identical monomer (55KD).

Separation of LPL by HPLC

HPLC chromatogram of LPL was shown in Fig. 3. The chromatogram of purified LPL (at 2M NaCl) showed three peaks, retention times of 43.3, 46.3, and 49.3min. Two peaks; at the retention times of 43.3 and 49.3min, were shown on the chromatogram of blank. From the comparison of chromatograms of blank and LPL, it can be concluded that the peak of the purified LPL appeared at the retention time of 46.30min. Also, Sigma LPL (commercial LPL enzyme) was used as standard for comparison. The differences of peak area between 2M NaCl containing LPL (not dialyzed) and dialyzed LPL (0.15M NaCl) might have originated from the dialysis because dialysis caused the dilution of total concentration of LPL. The eluted two peaks at blank might be due to acetonitrile.

Effects of NaCl on LPL with or without heparin

Fig. 4 illustrates the effects of salt on LPL activity in the absence and the presence of heparin (2μg/ml in 0.1M Sodium Phosphate buffer). LPL was dialyzed against 0.15, 0.75, 1, 2, and 3M NaCl. The effects of

various salt concentrations on LPL activity were studied.

First, the presence of heparin increased LPL activity approximately six times higher than the absence of heparin. With heparin, the highest activity of LPL was shown at 0.15M NaCl concentration (physiological salt concentration of human body), and LPL activity decreased as the NaCl concentration increased. It was elucidated that LPL bound with heparin at endothelial cell at physiological salt concentration (0.15 M NaCl) became more stable, and induced the maximal LPL activity as active sites of LPL were stabilized. It might be the reason why heparin led to the stabilization of LPL and also led to increase enzyme activity.

Second, in the absence of heparin, the highest activity of LPL appeared at 3M NaCl concentration, and LPL activity increased with increasing the NaCl concentration.

However, in the presence of heparin, 0.15M NaCl (physiological condition) showed the substantial increased activity. Comparing LPL activities of heparin added and heparin non-added groups, LPL activity was much more improved approximately 5 times in the heparin added group.

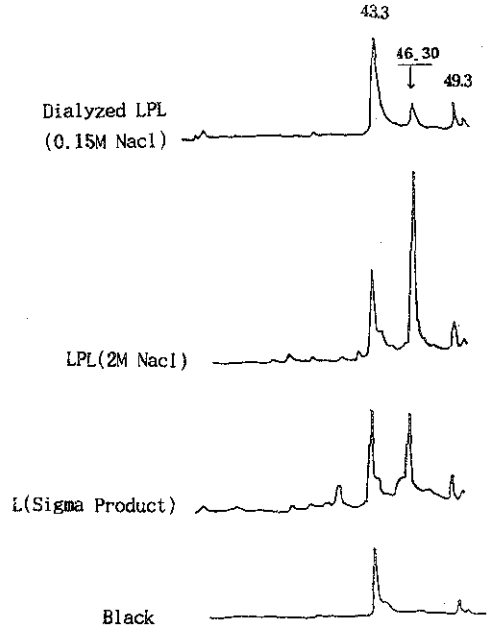

Fig. 3. HPLC chromatogram of LPL at 280nm.

In the absence of heparin, the lowest activity of LPL was obtained at 1M NaCl, and LPL activity was progressively increased up to 3M NaCl. It can be concluded that LPL (without heparin) becomes a form of free enzyme, which is delocalized from the endothelium, and changed to inactive lipase.

In the presence of heparin, the activity decreased up to 2M NaCl, but at 3M NaCl, activity increased slowly. LPL (without heparin) showed the highest activity at 3M NaCl concentration. It might be due to the effect of NaCl, and ionized salt enhanced the stability of LPL instead of heparin. However, it should be poin-

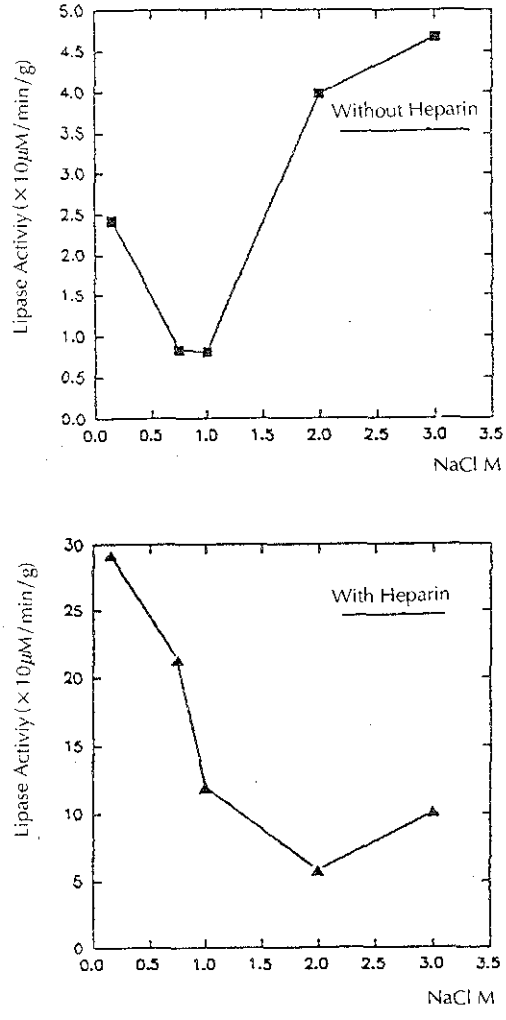

Fig. 4. Activity of LPL as a function of salt concentration in the presence or absence of heparin.

ted out that inhibition of LPL activity by NaCl is only *in vitro* phenomena with considerable variation. LPL binds the endothelial surface through interaction with heparin sulfate (18,19). Thus, the present results indicate that the interaction between LPL and heparin leads to the stabilization of LPL.

Effects of pH on LPL with or without heparin

The lipase activity at all pH values were tested in the presence or the absence of heparin (Fig. 5 and 6). At low or high pH, the loss of activity became more rapid. In the presence of heparin, lipase became stable at pH 6~9. In the absence of heparin, at low NaCl concentration (0.15M), optimum pH was 6 while optimum pH was 8 at high salt concentration (3M). As increasing salt concentration, optimum pH was shifting from acidic to basic conditions. However changes of LPL activity were not found as varying the NaCl concentration.

However, when heparin was added (Fig. 6), LPL activity increased about 3~7times compared to the absence of heparin. Again, we can conclude that LPL activity is improved with heparin, and heparin has a role to lead the stabilization of LPL. But, regardless of NaCl concentration, optimum pH ranges (6~9) were

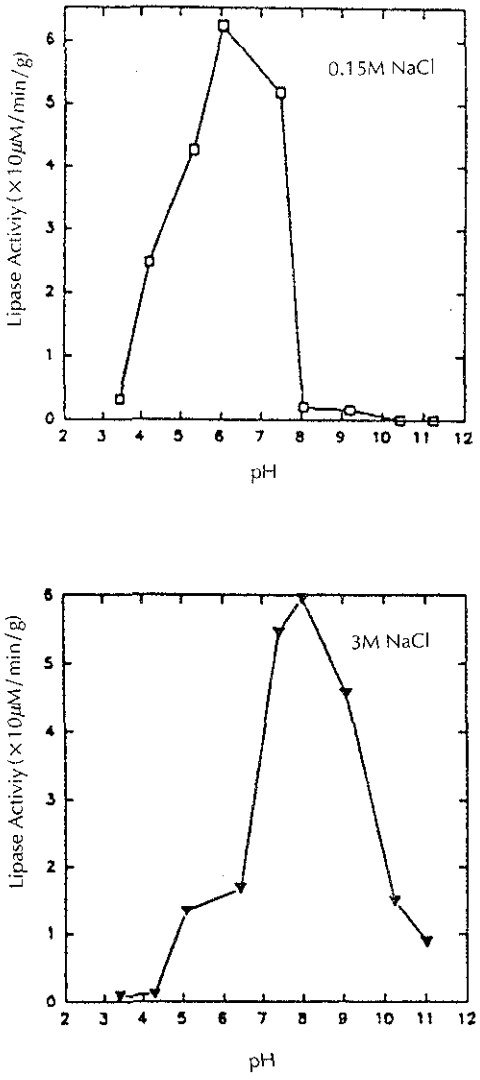

Fig. 5. Activity of LPL as a function of pH in the absence of heparin.

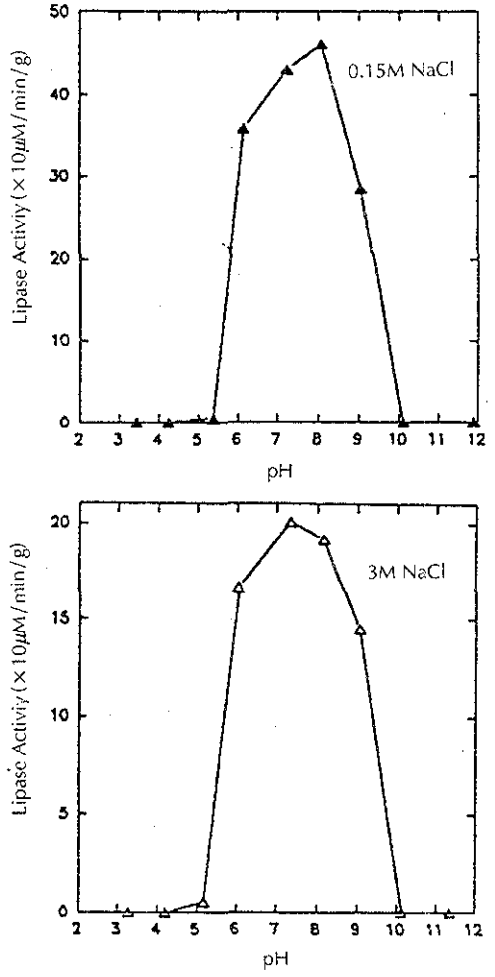

Fig. 6. Activity of LPL as a function of pH in the presence of heparin.

not changed. But, LPL activity of 0.15M NaCl showed two times higher than that of 3M NaCl concentration. This result indicates that the physiological conditions (0.15M NaCl, pH 7.4, 37° C) stabilize and increase the LPL activities.

In conclusion, the function of LPL is to direct the influx of plasma TG in the form of fatty acids into the peripheral tissues for storage and to provide fuel for energy requirement. For this reason, the control of LPL is an important regulatory step for directing traffic of TG-fatty acids to fulfill the energy requirement of peripheral tissues in a tissue specific manner. A deficiency or dysfunction of LPL has been found that it is associated with the pathogenesis of hypertriglyceridemia (1,4,6).

Heparin-releasable LPL binds the endothelial surface through interaction with heparin sulfate. The use of heparin affinity chromatography provided the direct evidence that there is a direct interaction between LPL and heparin. In addition, the release of LPL into circulation by intravenous administration of heparin implies that this treatment causes detachment of LPL from the endothelium due to competition of heparin with cell-surface glycosaminoglycans (11).

Our results showed that the interaction of LPL and heparin leads to the stabilization of LPL. This stabilizing effect may be explained by the role of heparin as activator of LPL. In addition to stabilizing of LPL, the interaction of heparin with LPL has also been shown to retard the hepatic clearance of the enzyme in circulation (2,4,18). Also, this enzyme is bound to the vascular endothelium by affinity for heparin sulfate (18), and only low levels of LPL are found in the circulation. The binding of LPL to the endothelium is thought to be weakened by local fatty acid accumulation that may result from lipolysis of plasma triglycerides (19).

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Lipoprotein Lipase(LPL)의 활성에 미치는 NaCl 및 Heparin의 영향 연구

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요 약

Lipoprotein lipase (LPL)는 acylglycerol 가수분해효소 (hydrolase)이며 중성지질 (triglycerol)이 풍부한 plasma lipoprotein을 가수분해하는 extrahepatic에 존재하는 효소이다. LPL은 capillary endothelium의 표면에 위치하며 심장, 골격근과 지방조직 등의 조직에 넓게 분포되어 있으며 지질대사에 중요한 작용을 한다. LPL의 기능은 plasma TG를 지방산의 형태로 바꾸며, 저장을 위하여 말초조직으로 유입하며, 체내에 에너지를 연료로서 공급하게 된다. 체내의 endothelium에서 분리된 LPL이 chylomicron과 VLDL 등의 TG-rich lipoprotein의 TG를 분해시킬 수 있다해도, serum이 activator의 source로서 더해지지 않는다면 제한적인 활성을 가지며, 또한 salt와 heparin에 많은 영향을 받는 것으로 여겨지고 있다. LPL은, 우유로 부터 2M NaCl, 5mM barbital buffer, pH 7.4에서 heparin affinity chromatography로 분리하였다. p-Nitrophenylbutyrate (PNPB)는 LPL activity의 결정을 위해서 기질로서 사용하였다. LPL의 분자량은 10% SDS-PAGE에서 55KDa이었다. LPL의 활성은 heparin을 첨가하였을 경우, 대조군에 비해 대략 5배 정도 활성이 증가 되었다. 이것은 heparin이 LPL을 안정하게 하므로서, 효소활성을 증가시킨다는 사실을 증명하였다. 또한 LPL의 활성은 여러 pH 범위에서, 대조군과 비교하였을 때, heparin의 첨가시 약 4배 정도가 증가하는 것을 관찰할 수 있었다. NaCl의 존재에도 heparin이 공존하는 경우 최적 pH (6~9)가 변화되지 않았으며, 이것은 heparin이 높거나 혹은 낮은 salt 농도 하에서도 LPL을 안정화 시키기 때문이다. 결론으로, 지질대사에 주요한 LPL의 활성은 NaCl과 heparin에 의해 많은 영향을 받는 것으로 나타났으며, 이것은 NaCl과 heparin에 의해, LPL의 구조적인 안정성을 증가시킴으로써 LPL의 활성을 높이는 것으로 사료된다.