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Characterization of lipoprotein a by capillary zone electrophoresis

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Abstract

Lipoprotein a [Lp(a)] has been recognized as a significant marker for premature coronary heart disease (CHD). In this paper, we present the results of Lp(a) analysis based on capillary zone electrophoresis (CZE). CZE separation of Lp(a) and its reduced species, lipoprotein a^- [Lp(a^-)] and apolipoprotein a [apo(a)], was accomplished using 50 m*M* borate buffer containing 3.5 m*M* sodium dodecyl sulfate (SDS) and 20% (v/v) acetonitrile (ACN). Low density lipoprotein (LDL) and high density lipoprotein (HDL) were separated under the same buffer conditions. The electrophoretic mobilities of both Lp(a) and Lp(a^-) were found to be different from that of LDL. Benzyl alcohol (BA) and methanol (MeOH) were used as electroosmotic flow markers. BA molecules associated with Lp(a^-) and LDL to enhance their UV absorbance, but did not change their effective electrophoretic mobilities. Our results show that CE is a very efficient and effective technique for lipoprotein analysis.

1. Introduction

Lipoproteins (LPs) are a class of large biomolecules which are closely associated with CHD. Among them, lipoprotein a [Lp(a)] has been recognizd as a significant marker for predicting the risk of CHDs due to its genetic trait [1]. As a consequence, detection of elevated Lp(a) levels with rapid, efficient analytical methods could make a significant contribution to solving the problems of assaying Lp(a) in blood. Lp(a) has many of the features of low density lipoprotein (LDL). It consists of an LDL-like particle, lipoprotein a^- [Lp(a^-)], and apolipoprotein a [apo(a)] [2]. Apo(a) is attached to Lp(a^-) through a single disulfide bond which can be cleaved via chemical reduction.

Immunoassay [3,4] is the prevailing technique for measuring Lp(a) concentration in serum or plasma. However, because of the high heterogeneity of Lp(a) molecules detection methods with reliable standards and reference materials have long been sought [5]. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [6] is fundamental for characterization of Lp(a) polymorphism, but its performance is time-consuming.

Capillary electrophoresis (CE) has been used for the analysis of large biomolecules in many situations. Apoliproteins have been separated with capillary zone electrophoresis (CZE) [7–9] and lipoproteins with capillary isotachophoresis (ITP) [10]. In this paper, we present the results

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of a CZE method for the identification of Lp(a) and other lipoprotein species.

2. Experimental

2.1. Instrumentation

All CE data were collected with a Beckman P/ACE System 5510 (Beckman Instruments, Fullerton, CA, USA) equipped with System Gold software and a diode array detector. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D. and 375 μm O.D. were used for the CE analysis. SDS-PAGE gels were casted with a gradient former (Jule, New Haven, CT, USA) and electrophoresis was performed with a vertical slab-gel unit SE 600 (Hoefer Science, San Francisco, CA, USA) and power supply FB 458 (Fisher Scientific, Pittsburg, PA, USA). A Beckman ultracentrifuge L7-65 with a 70Ti rotor was used for Lp(a) separation from plasma. Sample desalting was performed with Slide-A-lyzers (Pierce, Rockford, IL, USA).

2.2. Materials

Double distilled water, deionized with a Milli-Q system from Millipore (Bedford, MA, USA) and filtered with a 0.22-μm filter, was used in the preparation of all CE buffers. Sodium borate, sodium bromide, EDTA, and BA were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Bovine serum albumin (BSA), fibrinogen, low density lipoprotein (LDL), high density lipoprotein (HDL), SDS (70% purity), dithiothreitol (DTT), and β-mecaptomethanol (ME), phenylmethyl-sulfonyl fluoride (PMSF), aprotinin, and coomassie brilliant blue R 250 were purchased from Sigma (St. Louis, MO, USA). Sodium azide was from Aldrich (Milwaukee, WS, USA). Acetonitrile (ACN), acetic acid, and methanol were purchased from EM Science (Gibbstown, NJ, USA). Acrylamide was obtained from Boehringer (Mannheim, Germany), and N′N′-methylenebisacrylamide from Life Technologies (Gaithersburg, MD, USA). Prestained high-molecular-mass standards (M_r 49 000–194 000) for SDS-PAGE were obtained from Bio-Rad (Hercules, CA, USA). Lysine-Sepharose 4B gels were obtained from Pharmacia Biotech (Piscataway, NJ, USA). Lp(a) plasma concentration was determined by enzyme-linked immunosorbant assay (ELISA). Lp(a) ELISA kit was purchased from Strategic Diagnostic Industries (Newark, DE, USA). All chemicals were of analytical-reagent grade or electrophoresis grade and used without further purification.

2.3. Isolation of Lp(a) from blood

Lp(a) was isolated from blood plasma via ultracentrifugation [11] and purified by lysine-Sepharose affinity chromatography [12]. The subject selected had a Lp(a) plasma level of 1.08 g/l as determined by ELISA. The plasma was mixed with 0.15% EDTA, 0.01% sodium azide, 0.0001% PMSF, and 0.4 μM aprotinin. Its density was adjusted to 1.15 g/ml by adding sodium bromide. The density adjusted plasma was spun at 45 000 rpm for 24 h at 5°C. After fractionation, the top floating thick orange band was aspirated with Pasteur pipettes and its Lp(a) concentration was determined to be 4.75 g/l by ELISA. This fraction was dialyzed against phosphate buffered saline (PBS) solution for 20 min at 4°C. The fraction density was then readjusted to 1.05 with sodium bromide and subjected to spinning at 54 000 rpm for 20 h at 5°C. The bottom fraction [Lp(a) = 2.00 g/l by ELISA] was aspirated, dialyzed, and purified by lysine-Sepharose affinity chromatography.

2.4. SDS-PAGE

Gradient polyacrylamide gels of 3–10% were self-casted with a Jule gradient former. SDS (0.1%)–Tris (0.02 M)–glycine (0.2 M) buffer of pH 8.3 was used. Prestained SDS-PAGE standards and Lp(a) (15–20 ng each) were mixed with either nonreducing buffer for 30 min at room temperature or reducing buffer for 7 min at 100°C. The samples then were loaded on the gel wells and the gel was run for 8–10 h. The gel

was stained and destained following the established procedure [13].

2.5. CE methods

The fused-silica capillaries used had a total length of 57 cm and an effective length of 50 cm. Bared fused-silica capillaries were initially rinsed with 1 *M* NaOH and water and conditioned between runs with 0.1 *M* NaOH and water. Using 50 m*M* borate buffer containing 3.5 m*M* SDS and 20% (v/v) ACN, CE data were collected at 214 nm, 17.50 kV, 20°C, and pressure injection between 1–5 s. Lipoprotein samples were stored at 4°C in 0.15 *M* NaCl with 0.01% EDTA at pH 7.4. Each lipoprotein sample was diluted 10 times with run buffer before injection. Reduction of Lp(a) into apo(a) and Lp(a^-) was performed by adding ME (0.09 *M*) to the diluted sample and boiling the sample for 7 min. The amount of lipoproteins injected was between 1–8 ng (0.5 fmol–4 fmol). Diluted BA (5 μl in 100 ml water) or methanol (1:1 in water) was used as the electroosmotic flow marker.

3. Results and discussion

3.1. Lp(a) reduction determined by SDS-PAGE

The ultracentrifugal separated and lysine-Sepharose 4B purified Lp(a) fraction was positively identified with Lp(a) ELISA. The Lp(a) was further analyzed by SDS-PAGE in non-reduced and reduced forms as shown in Fig. 1, where one and two bands were displayed correspondingly [14]. This result indicated that a fairly pure Lp(a) sample was isolated from plasma and Lp(a) reduction was complete.

3.2. Characterization of Lp(a) and its reduction products by CZE

Using 50 m*M* borate buffer of pH 10, Lp(a) produced very narrow peaks after it was premixed with 1% SDS–0.5 *M* Tris buffer (Fig. 2). The plate number of the Lp(a) peak was calculated to be higher than 10^7/m. However, the reduced

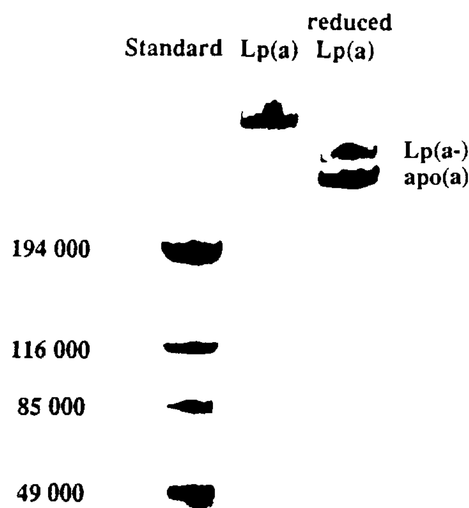

Fig. 1. Lp(a) in non-reduced and reduced forms determined by SDS-PAGE.

Lp(a) products, Lp(a^-) and apo(a), were detected as one peak under the conditions used. The SDS concentration in the sample buffer (35 m*M*) was above the critical micellar concentration (CMC = 8 m*M*) and the running buffer was free of SDS. Therefore, the reduced Lp(a) particles formed micelles which migrated together and were detected as one peak. A similar result was observed for ovalbumin and conalbumin [15]. The loss of resolution and the peak sharpening effect were caused by the mobility gradients as indicated.

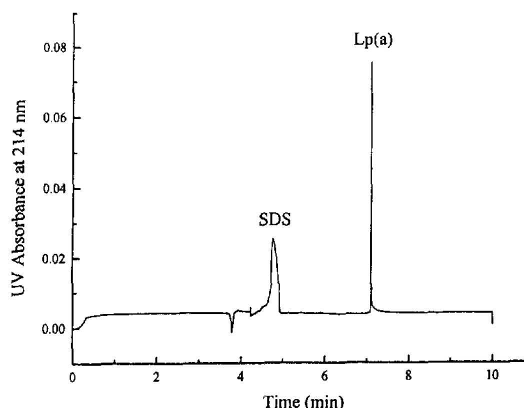

Fig. 2. Lp(a) in 50 m*M* sodium borate buffer at pH 10. The sample was premixed with 1% SDS–0.5 *M* Tris buffer. Running condition: 30 kV, 20°C, pressure injection for 5 s.

Using 20% acetonitrile (ACN), 3.5 m*M* SDS, 50 m*M* sodium borate (pH 9) as buffer and BA as electroosmotic flow marker, Lp(a) in its non-reduced and reduced form was examined as shown in Fig. 3. Comparing Fig. 3a and 3b, Lp(a) could be easily characterized. The LDL peak in Fig. 3a was identified using LDL samples purchased from Sigma. Two LDL samples from Sigma were tested, one isolated from plasma via size-exclusion chromatography and the other being lyophilized powder redissolved in phosphate-buffered saline (0.15 *M* NaCl, pH 7.4). The effective mobilities of these two LDL samples were very close. Unlike Lp(a), LDL remained intact after treatment with reducing buffer. Fig. 3 shows two interesting results. First, both Lp(a) and Lp(a^-) were different from LDL in terms of their effective electrophoretic mobilities (μ_{eff}). Second, LDL still existed in Lp(a) even after Lp(a) was purified by lysine-Sepharose affinity chromatography.

The LDL samples from Sigma were originally used to distinguish Lp(a^-) from apo(a), because Lp(a^-) has been assumed to be an LDL-like particle with regard of its lipid–protein ratio and particle size [16]. Our CE data revealed a significant difference in electrophoretic mobility between Lp(a^-) and LDL. We further examined LDL samples from different sources and observed fairly consistent μ_{eff} values (Table 1). Consistency of μ_{eff} values also applied to HDL samples (Fig. 4). Structurally, LDL consists of a lipid core with its surface partially covered by apo B-100 [2]. Cholesterol ester and triglyceride molecules, which are hydrophobic, are in the center of the lipid core, while the less hydrophobic free cholesterol and phospholipid molecules are on the surface layer. The apolipoproteins are hydrophilic and rest on the surface of the lipoproteins. Lp(a) is structurally different from LDL in having an additional apo(a). Our CE data indicate that both Lp(a) and Lp(a^-) are different from LDL in terms of their μ_{eff}. In CE, the μ_{eff} of a species is determined by its charge-to-size ratio. LDL and Lp(a) have comparable particle sizes [15]. SDS molecules can add nega-

Table 1
The effective electrophoretic mobilities of lipoproteins

Lipoproteins	(sources)	$\mu_{eff} \times 10^{-5}$ (cm^2/V s)
Lp(a)	(subject's plasma)	15.9 (±0.2)
apo(a)	(subject's plasma)	14.5 (±0.2)
Lp(a^-)	(subject's plasma)	19.6 (±0.1)
LDL	(Sigma, liquid)	26.2 (±0.07)
	(Sigma, lyophilized powder)	24.1 (±0.1)
	(subject's plasma)	26.1 (±0.2)
HDL	(Sigma, liquid)	19.6 (±0.1)
	(Sigma, lyophilized powder)	19.5 (±0.1)

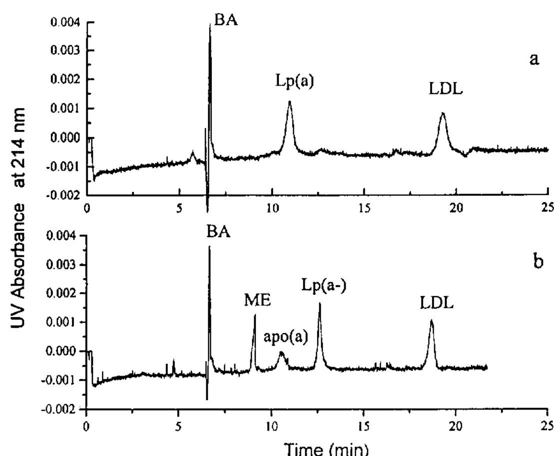

Fig. 3. Lp(a) in non-reduced (a) and reduced (b) forms. Running condition: 17.5 kV, 20°C, pressure injection for 1–2 s.

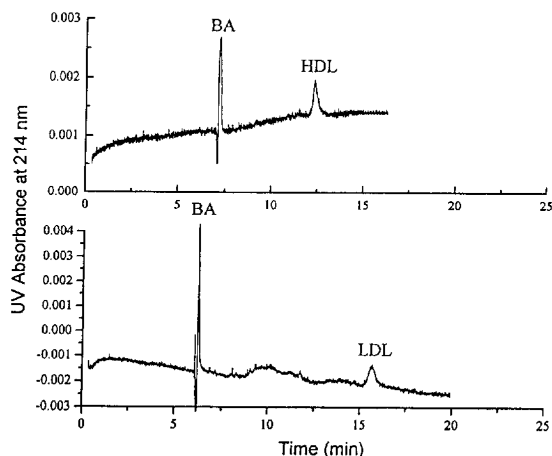

Fig. 4. HDL and LDL separation under the same conditions as in Fig. 3.

tive charges to lipoproteins by attaching to both lipid molecules and protein backbones. The average amount of lipid (w/w) in Lp(a) and LDL is 66 and 77%, respectively [16]. Because the surface of LDL is covered more by lipid than Lp(a), it is likely that more SDS molecules will attach to the LDL surface resulting in an increase of negative charges on the LDL particles. Hence, the charge-to-size ratio of LDL molecules exceeded that of Lp(a) and consequently a larger μ_{eff} was observed for LDL.

The existence of LDL in Lp(a) samples was clearly shown in Fig. 3 but could not be seen on the slab SDS-PAGE (Fig. 1). When Lp(a) was isolated as density fractions using ultracentrifugation, it was likely to be contaminated by LDL and HDL. Apo(a) in Lp(a) consists of many repeats of a unique structural domain, the kringle domain [2], which can specifically bind to lysine. Since kringle domains do not exist in LDL or HDL, it should be possible to purify Lp(a) from LDL and HDL by lysine-Sepharose affinity chromatography. Based on our CE data, HDL did not appear in the Lp(a) electrophoregrams. On the one hand, the detection of LDL in Lp(a) demonstrated the superior sensitivity of CE over slab SDS-PAGE; on the other hand, it implied that Lp(a)–LDL complexes exist in plasma because LDL could not have survived the affinity chromatographic separation unless it is tightly associated with Lp(a) via lipophilic interaction. Evidence for the high affinity of Lp(a) for LDL has been reported [17].

Both BA and methanol were used as neutral markers in our CE studies. Neutral marker was injected following each sample injection. Unexpectedly, the two markers caused considerable differences in the signal intensities of the LDL (Fig. 5) and Lp(a^-) (Fig. 6) peaks. MeOH appeared as a positive peak in Figs. 5 and 6 because the background electrolyte buffer contained 20% ACN which showed a lower UV absorbance than MeOH. When methanol was used as the neutral marker, the LDL peak in the Lp(a) sample was either very small or non-observable. In contrast, the LDL peak was well detected simply by switching to BA as marker. The aqueous solubility of BA is limited due to its

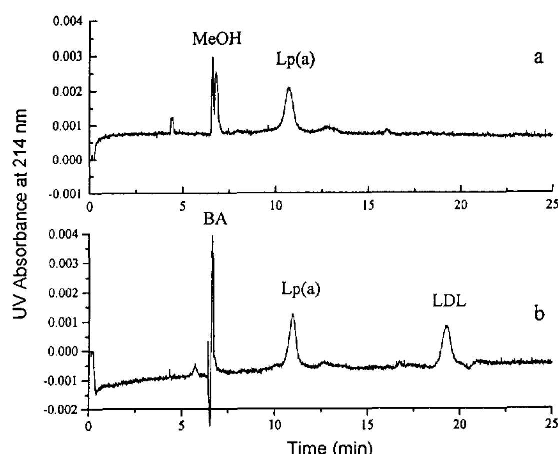

Fig. 5. UV enhancement effect of BA on LDL. The neutral markers used were (a) MeOH and (b) BA. The running conditions were the same as in Fig. 3.

hydrophobic aromatic ring which causes its intense UV absorbance. In LDL, the lipid molecules on the surface layer are hydrophobic and contribute little to the UV absorbance of the LDL particles. When BA was injected following the sample injection, it passed through the sample zone during migration. Presumably, part of the BA molecules became attached to the surface lipid portion to such an extent that the UV absorbance of the LDL molecules was no-

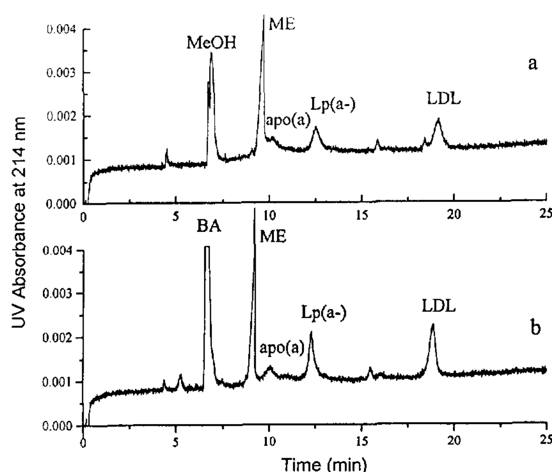

Fig. 6. UV enhancement effect on Lp(a^-) and LDL. The neutral markers used were (a) MeOH and (b) BA. The running conditions were the same as in Fig. 3.

tably enhanced but its overall C/S ratio was not alternated. This UV-enhancement effect observed for LDL and Lp(a$^-$) largely exceeded that of Lp(a) and HDL. It demonstrates that there is more lipid exposed on the surface of LDL and Lp(a$^-$) compared with Lp(a) and HDL. In other words, Lp(a) and HDL are covered more by protein on their surfaces. Based on the literature, little is known about the nature of the interaction between apo(a) and Lp(a$^-$) other than that the two components can be easily separated via chemical reduction. Our CE results indicate that apo(a) molecules have more interactive contacts with the Lp(a$^-$) surface besides the disulfide bond linkage. In LDL particles, apo B-100 molecules are believed to be wrapped around the lipid core [2]. If we consider that the size of apo(a) molecules (300–800 kDa) is comparable to that of apo B-100 (500 kDa), it is conceivable that apo(a) covers a considerable part of the Lp(a$^-$) surface.

The CZE method reported here is advantageous over the widely used ELISA and SDS-PAGE techniques. First, all immunoassays rely on specific antibody recognition of epitopes on the Lp(a) molecules. The nature and variety of the epitopes selected by Lp(a) antibodies is unknown. This feature causes the problems in reliable standardization and comparison [5]. In contrast, CZE detection of Lp(a) is based on the UV absorbance of the whole molecule. Therefore, it avoids the dependency on specific epitopes encountered in immunoassays. Secondly, Lp(a) and LDL show very low mobilities on SDS-PAGE due to their extremely large sizes as well as lipid contents, which makes the determination of molecular masses and isoforms difficult. On the other hand, all lipoproteins studied by us show very different electrophoretic mobilities. (iii) ELISA and SDS-PAGE require 3–10 h to accomplish, while all the measurements of Lp(a) and other lipoproteins presented above are achieved within 20 min. In addition, neither immunoassays nor SDS-PAGE can be used to simultaneously determine the concentration and isoforms of lipoproteins, whereas further development of CE methods will likely be successful in this respect.

4. Conclusions

Lp(a) and its reduction products, Lp(a$^-$) and apo(a), can be separated and identified with sodium borate buffer containing SDS and ACN. LDL and HDL from different sources have consistent effective electrophoretic mobilities under the same conditions. Both Lp(a) and Lp(a$^-$) are significantly different from LDL in their electrophoretic mobilities. Because of the lipophilic properties of Lp(a$^-$) and LDL, they can absorb BA on their surface thereby enhancing their UV absorbance. Based on the different electrophoretic behaviors of Lp(a) and LDL, we speculate that Apo(a) does not simply attach to Lp(a$^-$) via a disulfide bond, but has also other interactions with the Lp(a$^-$) surface. All the CE data can be obtained within 20 min, which is very time efficient compared to immunoassay and SDS-PAGE.

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References

[1] G. Utermann, J.J. Menzel, H.G. Kraft, H.C. Duba, H.G. Kemmler and C. Seitz, J. Clin. Invest., 80 (1987) 458.

[2] R.M. Lawn, Science, 266 (1992) 54.

[3] C. Labor, J. Shepherd and M. Racine, Clin. Chem., 36 (1990) 591.

[4] W.C. Taddei-Peters, B.T. Butman, G.R. Jones, T.M. Venetta, P.F. Macomber and J.H. Ransom, Clin. Chem., 39 (1993) 1382.

[5] J.J. Albers and S.M. Marcovina, Chem. Phys. Lipids, 67/68 (1994) 257.

[6] J.W. Gaubatz, K.I. Ghanem, J. Guevara, Jr., M.L. Nava, W. Patsch and J.D. Morrisett, J. Lipid Chem., 31 (1990) 603.

[7] T. Tadey and W. Purdy, J. Chromatogr., 583 (1992) 111.

[8] T. Tadey and W. Purdy, J. Chromatogr. A, 652 (1993) 131.

[9] A. Goux, A. Athias, L. Persegol, L. Lagrost, P. Gambert and C. Lallemant, Anal. Biochem., 218 (1994) 320.
[10] G. Schmitz and C. Möllers, Electrophoresis, 15 (1994) 31.
[11] J.W. Gaubatz, G.L. Cushing and J.D. Morrisett, Methods Enzymol., 129 (1986) 180.
[12] M.L. Snyder, D. Polacek, A.M. Scanu and G.M. Fless, J. Biol. Chem., 267 (1992) 339.
[13] J.F. Robot and B.J. White, Biochemical Techniques–Theory and Practice, Waveland Press, Prospect Heights, IL, 1990, p. 140.
[14] G.M. Fless, M.E. ZumMallen and A.M. Scanu, J. Lipid Res., 26 (1985) 1224.
[15] E. Kenndler and K. Schmidt-Beiwl, J. Chromatogr., 545 (1991) 397.
[16] G.M. Kostner, in A.M. Scanu (Editor), Lipoprotein a, Academic Press, San Diego, CA, 1990, p. 183.
[17] G.M. Kostner and H.K. Grillhofer, J. Biol. Chem., 266 (1991) 21287.