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Virtual 2-D Gel Electrophoresis of High Density Lipoproteins

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Abbreviations: CHAPS (3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate); DTT dithiothreitol; FAPH 50% formic acid/25% acetonitrile/15% isopropanol/10% water v/v/v/v; HDL high density lipoproteins; IEF isoelectric focusing; IPG immobilized *pH* gradient; MALDI-MS matrix-assisted laser desorption ionization-mass spectrometry; PAGE polyacrylamide gel electrophoresis; TOF time-of-flight

Abstract

High-density lipoproteins (HDLs) isolated by immunoaffinity chromatography and separated by immobilized *pH* gradient-isoelectric focusing (IPG-IEF) were examined by mass spectrometry directly, applying a new proteomics technology, *virtual 2-D gel electrophoresis.* A preliminary examination of HDL particles has revealed at least 42 unique masses for protein species with isoelectric points between *pH* 5.47-5.04, some of which have not been observed previously. By delivering masses of intact proteins from complex cellular mixtures in a format that correlates *directly* to classical 2-D gel analyses, virtual 2-D gel electrophoresis constitutes a general discovery tool to expose and monitor protein isoforms and post-translational modifications. Furthermore, its general ability to deliver ions from sub-picomole level proteins enmeshed in complex cellular mixtures potentially fulfills the need of top-down proteomics to obtain intact protein ions from *microscale* samples. Additional comparison of such data to 2-D gel analyses and their identified proteins may elucidate the functions of the individual apolipoprotein components and the cardioprotective effects of HDL.

INTRODUCTION

Apolipoproteins, providing the scaffold on which lipoprotein particles are assembled, participate in lipid metabolism mechanically and enzymatically. Their relationship to cardiovascular disease, *e.g.*, high density lipoprotein levels in plasma are inversely correlated to premature coronary heart disease, deems them of continual interest [1]. Despite that interest, however, the mechanisms by which high density lipoproteins (HDL) protect against atherosclerosis are not fully understood. Is reverse cholesterol transport, protection against oxidation, or some other process key to their effects? Clearly much remains to be learned about the structure and function of apolipoproteins arising from mutation, alternate splicing, processing, truncation, and deamidation [2-4].

Today's proteomic technologies, focused on rapidly listing and quantifying intracellular and extracellular gene products, are leading a large-scale incursion into the study of cellular networks of proteins [5-9]. These approaches best address puzzles for which a missing piece is a novel or unexpected protein, or for which the amounts of various protein pieces change in response to a stimulus. However, interactions are also affected by proteins' appearances: the extent of their glycosylated plumage, whether they are adorned with acyl-, sulfo-, or phospho-groups, if their propeptides are trimmed, and whether they have been alternatively spliced. These latter effects challenge current proteomic technologies.

Virtual 2-D gel electrophoresis [10-15], based on combining a first dimension isoelectric focusing (IEF) separation on polyacrylamide gels with matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) surface scanning of the dried gel (Fig. 1), was developed to address the challenges of post-translational modifications. With this process, a virtual 2-D gel can be created, generating an image like that of 2-D polyacrylamide gel

electrophoresis (PAGE), but in which mass spectrometry is substituted for the second dimension SDS-PAGE (size-based) separation. The mass spectrometry dimension provides improved mass accuracy and resolution in a format directly linked to classical 2-D PAGE analyses, with the latters' applications to immunoblotting, in-gel chemistries, Edman degradation, mass spectrometric peptide mapping, and image analysis/processing. That direct link is achieved by the virtual 2-D gel method's use of the *same*, highly reproducible, immobilized pH gradient gels employed in classical 2-D PAGE, hence identical *pI* scales are delivered by the two methods, facilitating comparison. Its synergy with other technologies, both analytical- and preparative-scale, should offer many discovery opportunities for this infant technology.

These studies extend virtual 2-D gel methods to human proteins, with the overall goal of employing them on a system with many modifications. HDL particles provide an excellent test system, because glycosylation, phosphorylation, truncation, signal peptide cleavages, pro-peptide cleavages, sulfation, fatty acylation, and alternative splicing are all established for apolipoproteins [16-20]. Acylation by palmitate or palmitoleate [18-23] should, in principle, be detectable on these gels, based on our previous virtual 2-D gel observations of *E. coli* proteolipids *mulI* and *osmE* (SwissProt accession numbers P02937 and P23933, respectively) [13].

Previously, James *et al*. [24] examined HDL particles by 2-D PAGE under denaturing, reducing conditions with carrier ampholyte-based tube gels and SDS-PAGE separations. Farwig *et al*. [25] focused delipidated HDL on immobilized *pH* gradient (IPG) gels under denaturing, non-reducing conditions, and employed passive elution [12, 26-31] to extract proteins for subsequent MALDI-MS. Relying on IPG gels, they [25] were able to sidestep carrier ampholytes [27], essential to non-IPG slab gel isoelectric focusing, but which also bind strongly

to proteins. Their extraction with formic acid [25], while among the most effective MS-compatible means to recover proteins from polyacrylamide gels [32], has two possible drawbacks: formylation of serine and threonine residues and cleavage of acid-labile bonds (especially aspartyl-proline). Heterogeneous formyl esters have provided an impetus to alter classic biochemistry protocols; *e.g.*, substitution of 70% trifluoroacetic acid for 70% formic acid in CNBr cleavage cocktails [33, 34]. Formylation of proteins incubated in 50% formic acid/25% acetonitrile/15% isopropanol/10% water v/v/v/v (FAPH) has been demonstrated [29]. Nevertheless, formic acid is a superior solvent, and remains particularly valuable in mass analysis of membrane proteins and in extracting proteins from polyacrylamide gels.

EXPERIMENTAL

HDL Sample Preparation

Isolation of HDL from human sera was accomplished by selected-affinity immunosorption of apoA-I, as described by McVicar *et al* [35]. Selected-affinity immunosorption utilizes a subpopulation of monospecific apoA-I antibodies preselected to dissociate from apoA-I under gentle elution conditions maintaining the HDL complex.. First, goat anti-apoA-I antibodies eluted from apoA-I Sepharose at *pH* 3.0, were neutralized and sent to us by Dr. John Kane at the University of California, San Francisco. This preselected antibody (40 mg) was coupled to 8 g of cyanogen bromide-activated Sepharose 4B (Amersham-Pharmacia) at *pH* 7.0, with gentle rocking for 4 hr. Then the gel was neutralized for 6 hours with 1 M sodium carbonate (*pH* 8.2) at room temperature. The wet gel was then packed into a column (1 x 20 cm), after which it was washed with 10 column volumes of 0.15 M NaCl/0.015 M tris, *pH* 7.4.

In a typical run, 0.25 mL (later increased to 0.50 mL) of human plasma was run through the column using the same running buffer, and 50 fractions of 0.7 mL were taken (35 mL) until the optical density decreased to 0.005 or less. Then, a 1 M acetic acid solution (*pH* 3.0) was put through the column, and fractions of 0.7 mL were collected. A peak emerged in fractions 59-62, and as the fractions emerged, they were immediately diluted with an equal volume of 2 M tris base, to give a *pH* of 8.2, and read in the spectrophotometer. Typical optical densities were 0.079, 0.208, 0.184, 0.071 for the peak fractions, which often came off in four tubes. The one or two central tubes with the highest absorbance were dialyzed overnight against two 100 mL volumes of 0.15 M NaCl/0.015 M tris, *pH* 7.4.

Isoelectric Focusing

In preparation for isoelectric focusing, HDL particles isolated above were dialyzed against 7 M urea. To 110 μL of dialyzed HDL were added: 9.7 mg dithiothreitol (DTT), 230 μL 9 M urea, 9 μL Triton X-100, 2.5 μL of 3-10 carrier ampholytes, and a crystal of bromophenol blue, yielding a final concentration of 8.3 M urea, 45 mM DTT, 2.55% Triton X-100 (v/v), and 1% 3-10 ampholytes (v/v). Alternatively, the detergent combination Triton X-100 (2.5% v/v) and CHAPS (2% w/v) was employed with the chaotropes 7 M urea/2 M thiourea. Detergent concentrations in excess of 2% were maintained to dissociate lipid from the proteins, but the freed lipid was not removed. The HDL extract was loaded onto 18-cm, *pH* 4-7 IPG gels by in-gel rehydration. Following overnight rehydration at room temperature, isoelectric focusing was carried out at 20^{o}C on a Multiphor II flatbed electrophoresis apparatus (Amersham Biosciences) for 61000 Volt-hours. Voltages were applied as follows: 150 V for 0.5 hr, 300 V for 0.5 hr., 300-500 V ramp for 1 hr, 500-3500 V ramp for 2 hr., and 3500 V for 16 hr. Following

isoelectric focusing, the polyacrylamide gels were stored at -80^{o}C, until processed for mass spectrometry.

Urea, Pharmalyte™ 3-10 ampholytes, Triton X-100, and bromophenol blue were purchased from Amersham Biosciences (Piscataway, NJ), while DTT was obtained from Bio-Rad (Hercules, CA). CHAPS (3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate) was acquired from Pierce (Rockford, IL), while glycerol, and puriss-grade thiourea were provided by Sigma/Aldrich/Fluka (Milwaukee, WI). Immobilized *pH* gradient gels (*pH* 4-7, 18-cm length) were purchased from Amersham Biosciences. Positions of bands visualized by MALDI-MS or by silver staining were converted to isoelectric points by assuming a linear *pH* 4-6.6 gradient across the gel's full length (approximately 180 mm), based on an Amersham-supplied plot of *pH* versus distance for *pH* 4-7 Immobiline DryStrip™ gels at 20^{o}C and 8 M urea.

Silver-Staining Immobilized pH Gradient Gels

IPG gels to be stained were incubated in 20% (w/v) trichloroacetic acid for 20 minutes followed by incubation in 40% ethanol/10% acetic acid/50% H_2O (v/v/v) for an additional 20 minutes. After these two fixation steps, the gels were sensitized in 30% ethanol/7.5% glutaraldehyde/62.5% H_2O (v/v/v) containing 72 g/L sodium acetate and 2.5 g/L potassium tetrathionate. Following sensitization, the gels were washed four times (10 minutes each wash) in H_2O. The gels were incubated in aqueous silver nitrate solution (0.2 g/L $AgNO_3$ in 0.25 mL/L formaldehyde) for at least 45 minutes, after which they were washed for 1 minute in H_2O. The washed gels were incubated in developer (30 g/L K_2CO_3, 7.5 mg/L $Na_2S_2O_5$) until bands appeared, at which point the gels were placed in aqueous stop solution (20 mL/L acetic acid,

50 g/L tris base) for 10 minutes. Finally, stained gels were soaked in 5% glycerol/10% acetic acid/85% H2O (v/v/v) for 10 minutes and imaged. Based on the low sensitivity by silver staining and long development times required, the amount of protein per band appears to be under 1 pmol. Losses during dialysis may have been significant.

Acetic acid was obtained from EM Science (Darmstadt, Germany). Trichloroacetic acid, formaldehyde, glutaraldehyde, sodium acetate, potassium tetrathionate, tris base, $AgNO_3$, K_2CO_3, $Na_2S_2O_5$, and glycerol were obtained from Sigma/Aldrich/Fluka (Milwaukee, WI).

Processing Gels for Mass Spectrometry

Gels were washed for 10 minutes in 1:1 acetonitrile:0.2% TFA followed by a 10 minute soak in saturated sinapinic acid/1:1 acetonitrile:0.2% TFA. (Blotting gels with water-moistened filter paper prior to the sinapinic acid soak was often beneficial to remove oil remaining from the isoelectric focusing. Glycerol (1.5% v/v) was added to the sinapinic acid solution above to stabilize the gel's attachment to the polyester backing under vacuum and during laser-irradiation. *This step is important to prevent dried gels from lifting away from the sample stage and short-circuiting the high voltage connection. A secondary concern is that "lifting" gels deliver inferior and inconsistent mass accuracies, consequences of their uneven desorption surface.* After drying at room temperature overnight, the 3 mm wide gel strips (plastic backing still attached) were cut to 3.5-4 cm lengths and mounted onto the mass spectrometer's modified sample stage with double stick adhesive tape [10-13, 36, 37].

For consistency with general methods, carrier ampholytes were included in the IEF rehydration buffer. Washing IPG strips prior to incubating them in matrix, as described above, reduces carrier ampholyte concentrations sufficiently for successful MALDI analyses.

Ampholine™ and Pharmalyte™ carrier ampholytes, available from Amersham, have been successfully tested in this application. (Ampholines™ are polyaminocarboxylic acids derived from reacting polyamines such as pentaethylene hexamine with alpha, beta-unsaturated acids such as acrylate or ethyl acrylate, while Pharmalytes™, made by copolymerizing amines, amino acids, and dipeptides with epichlorohydrin, contain peptide bonds [38].) However, the detergent CHAPS can yield a cluster ion background below 5000 *m/z* with a characteristic 615 Da spacing. It can be reduced by washing the IPG gel more extensively and/or by lowering the CHAPS concentration of the IEF rehydration buffer. We have found cluster ion backgrounds to be most noticeable at CHAPS concentrations of 2-4% (w/v). Alternatively, the lower *m/z* range can be examined in reflector mode, *i.e.*, instrumental conditions to which the clusters are less stable.

MALDI Mass Spectrometry

Prior to mass analysis, 0.2-0.3 μL of cytochrome *c*/sinapinic acid solution was dispensed along one edge of the dried IPG gel at multiple *pH* positions for external and internal mass calibration.

MALDI-mass spectra were acquired on an Applied Biosystems Voyager DE-STR time-of-flight (TOF) mass spectrometer (Framingham, MA) operated with 337 nm irradiation, delayed extraction, and positive ion detection. The instrument was operated primarily in linear mode, but reflector mode was used to improve the mass accuracy of ions below 10 kDa, especially in regions where calibrant ions were not visible.

As mentioned above, a portion of the sample stage was modified (recessed) to provide additional clearance between the gel surface and the instrument's extraction grid. The modification is intended to lessen the chance of damaging the Voyager DE-STR extraction grid.

This consideration is important for mass spectrometers designed to position the sample plate close (*i.e.*, a few millimeters) to a voltage-biased element.

MALDI matrix was prepared as follows: sinapinic acid (saturated) in 33% CH_3CN/67% H_2O (v/v) optionally acidified to 0.1% with trifluoroacetic acid (TFA). Sinapinic acid was purchased from Sigma/Aldrich/Fluka, while TFA (sequencing grade) was obtained from Pierce. HPLC-grade water and acetonitrile were purchased from EM Science (Darmstadt, Germany).

In our previous studies mass analyzing proteins directly from dried immobilized *pH* gradient gels [11, 13], we searched databases assuming a mass accuracy of ± 0.1-0.2% for proteins below 50 kDa in size. Generally, 0.1% accuracy or better was achievable for reasonably abundant proteins below 20 kDa in size. Our previous IEF-MALDI studies have also established that proteins separated on *pH* 4-7 IPG strips are not alkylated by acrylamide, despite the reducing conditions employed during electrophoresis [12, 13]. The lower *pH* range spanned by these gels is not conducive to acrylamide addition onto cysteine, in contrast to the conditions encountered in classic SDS-PAGE separations with tris-glycine gels.

RESULTS AND DISCUSSION

The isoelectric focusing conditions described here differ from those of Farwig *et al*. [25] by employing reducing conditions and carrier ampholytes. Also, lipid was not removed from the solution prior to isoelectric focusing (*e.g.*, by solid phase extraction) so that potential proteolipids might be recovered, and for consistency with previous 2-D electrophoresis studies [24]. MacFarlane's laboratory removed lipid from the sample by rapid solid phase extraction from C-18 media [39]. Both laboratories employed urea during IEF.

Isoelectric points are likely to differ somewhat for reducing *vs.* non-reducing conditions, and thus between these HDL studies. Differences arise from: (1) ionization of free sulfhydryls, (2) differing conformations of proteins containing *intra*molecular disulfide-linkages and of reduced proteins alter the local environments of charge sites, (3) new species arising from *inter*molecular disulfide linkages. Theoretical calculations of isoelectric point assume reducing and denaturing conditions [40-42]. We do not expect the presence or absence of carrier ampholytes to affect protein isoelectric points.

A silver-stained IEF gel, of loading equal to that employed for mass spectrometry, revealed 9 major bands at *pH* 6.52, 5.46, 5.37, 5.27, 5.16, 5.08, 5.00, 4.94, and 4.25. (Fig. 1.) Additional, faint bands were observed at *pH* 5.76, 5.68, 5.69, 5.42, and 5.34.

ApoA-I is the major protein comprising HDL [1]. Mature apoA-I (M_r 28079 Da) is predicted to focus at *pH* 5.27, while its precursor, proapoA-I, (M_r 28962 Da) should focus at *pH* 5.45. ProapoA-I is converted to apoA-I by proteolytic excision of six N-terminal amino acids. Multiple isoforms of both mature apoA-I and pro-apoA-I have been observed in HDL, reflecting deamidations and palmitate acylation [18, 22, 43]. Phosphorylated isoforms have also been reported [44].

The discussion to follow describes analysis over the pH 5.89-4.91 segment of the IEF gel, primarily within the *pH* 5.49-5.03 region. Analysis and interpretation of the remaining segments is not complete to date. Our analysis revealed masses consistent with mature apoA-I (*i.e.*, 28 kDa) from *pI* 5.47-5.04, a region spanning the predicted *pI*. The isoelectric point heterogeneity reflected by this broad range of apoA-I migration is consistent with that observed in pure apoA-I, as reflected in the densitometry trace presented by Farwig *et al.* [25]. Their trace from isoelectrically focused commercial apoA-I displayed as many as 12 isoforms between

pI 4.52 and 5.33. Previous studies suggest that up to 6 discrete bands could arise from undeamidated, singly deamidated and doubly deamidated forms of both the pro- and mature protein. Other isoforms could reflect truncations [3], fatty acid acylation [18, 22], phosphorylation [44], or uncharacterized modifications. The Swiss-2DPAGE repository (http://us.expasy.org) includes 2-D gel images of human plasma samples. Their immunoblotting results attributed five 23 kDa spots that lay between *pI* 5.0-5.5 to apoA-I isoforms, as well as four additional spots migrating from 7-9.5 kDa and focusing at *pH* 4.79, 4.86, 6.97, and 7.26, respectively.

Recently, a mass of 28102 Da was reported for a commercial apoA-I standard [25]. After the standard was isoelectrically focused, masses of 28236, 28179, 28133, and 28115 were reported for components centered about *pH* 4.87, 4.94, 5.02, and 5.10, respectively, with the *caveat* that mass accuracy had not been optimized. Chemical modification of the apolipoproteins by the FAPH solvent system was also suggested as possible [25].

Performing mass measurements on the apoA-I components of HDL from the matrix-embedded IPG strips proved challenging, despite multiple measurements performed with internal and external calibration. We obtained an average mass of 28076 (± 4) Da from four measurements performed over *pH* 5.20-5.17, a major apoA-I band on our gel, in good agreement with 28078.6 Da, predicted from the primary sequence. Mass spectra from this region are illustrated in Fig. 2. Spectra obtained at *pI*'s above or below this range, however, revealed broad tailing to higher *m/z* and an increase in the apex mass for the 28 kDa species, characteristic of modifications, retained impurities, and/or counterion adducts. As a result, the average from mass measurements performed over *pH* 5.47-5.04 yielded 28104 Da. At *pH* 5.17, secondary peaks rising over the broad tail were observed at 28214 and 28265 Da. Palmitoyl-ApoA-I (M_r 28315)

[18, 22], if present, could be obscured by the high *m/z* tailing. ApoA-I is not annotated as glycosylated in the SwissProt database, but non-enzymatic glycation is possible. Glucosylated apoA-I, A-II, B, C-I, and E have been observed in diabetic plasma [45]. Moreover, it has been reported that glucosylated protein comprises triglyceride-rich lipoproteins in hyperglycemic subjects, but high-density lipoproteins in euglycemic subjects [45]. Identifying altered populations of sugars linked to apoA-I and apoA-II has been cited as potentially helpful in elucidating mechanisms of diabetes and cardiovascular disease [46, 47]. Other potential contributors to heterogeneity include oxidation or counterion adduction. Previous MALDI-MS analyses by Bondarenko *et al*. [3] have reported 98 Da adducts to apoA-I (measured M_r 28075.0), thought to arise from phosphoric acid.

In an earlier IPG-IEF study of HDL [25], proteins were extracted from 3 gel segments spanning 4.90-5.05 *pH* units, 5.05-5.15 *pH* units, and 5.15-5.28 *pH* units. Their analysis returned apoA-I masses of 28174, 28065, and 28153, respectively, provided again with the *caveat* that mass accuracy had not been optimized. Those spectra, too, revealed significant differences in the width of the apoA-I mass distribution, with the sharpest peak corresponding to the *pH* 5.10 segment.

Except for variants such as A-I_{MILANO}, apoA-I lacks cysteine residues [1]. Consequently, apoA-I isoelectric points determined under reducing and non-reducing conditions should be comparable. We believe that the apoA-I band ascribed to *pH* 5.20 in our study corresponds to the 5.10 band examined by MacFarlane's laboratory [25].

The preceding IPG-IEF study also revealed numerous HDL components spanning *pH* 4-6.5 from stained gels [25]. Eight regions were analyzed by MALDI-MS, returning masses

for the 9 proteins: apoA-I, apoA-II, apoC-I, proapoC-II, apoC-III_0, apoC-III_1, apoC-III_2, SAA_4, and serum albumin.

Mass spectra acquired over 0.45 *pH* units (*pH* 5.49-5.03) have been analyzed in detail, revealing 42 masses (See Figs. 2-4). In addition to the measurements for mature apoA-I, described above, a smaller protein (M_r 27972 based on an average of replicate measurements) was also observed at *pH* 5.20 (Fig. 2B). Within our mass accuracy, this mass is consistent with apoA-I', a recently reported apoA-I isoform lacking C-terminal glutamine, (calc. M_r 27950.4) [3]. Somewhat higher in *pH* at 5.44, we observed a 28983 Da protein (Fig. 4). ProapoA-I, estimated to migrate 0.18 *pH* units higher in *pH* than its processed product (apoA-I) has a sequence-predicted mass of 28961.6, and may be responsible for this feature. ProapoA-I constitutes less than 4% of the total mass of A-I isoforms in humans [43].

Fig. 3 illustrates a spectrum acquired at *pH* 5.07. In addition to ions corresponding to singly and doubly charged apoA-I, several smaller ions are observed. These proteins, with masses of 10032, 10177, 11050, and 11746, were not detected at *pH* 5.20 (Fig. 2). Just below the cytochrome *c* calibrant ion (M_r 12361.1) in Fig. 3, a 12105 *m/z* singly charged ion is also observed. We note that a pair of slightly larger ions had been observed in Fig. 2, at 12160 and 12175 Da, with the latter ion possibly reflecting an oxidation. At *pI* 6.02, unique species with masses 9910, 12865, and 13797Da are apparent (Fig. 5). Identities for these assorted ions below 14 kDa in size are not obvious. Characterized apoC proteins (C-I, C-II, C-III_0, C-III_0*) have predicted isoelectric points of 7.93, 4.66, 4.72, and 4.72, respectively, excluding them from the *pH* range examined. ApoC-III isoforms differ in number of sialic acid residues (0, 1, or 2) and in whether or not they possess any sugar backbone [4, 48]. Accounting for the added sialic acids of apoC-III_1 and apoC-III_2, providing overall isoelectric points below 4.72, it is predicted that they

will also be excluded. A *pI* of 9.15 is calculated for mature apoC-IV, although sialic acid glycosylated-forms have been observed to focus around *pH* 8. Although apoA-II monomer is predicted to focus at *pH* 5.05, within the range examined, its calculated mass of 8690.9 Da is too low to account for the ions observed. The Swiss 2-DPAGE plasma gel attributes 4 spots focusing within the *pH* 4.85-5.0 region of the IEF dimension and migrating between 9.1-11.1 kDa by SDS-PAGE to truncated forms of apoA-IV. At present, these apoA-IV spots best explain the low molecular weight ions we observe, but further 2-D gel analysis employing peptide mapping or immunoblotting would be enlightening.

Broad features from 22.6-25 kDa are present over the *pH* ranges 5.47-5.30 and *pH* 5.20-5.04, but are absent or very weak from *pH* 5.29-5.21. The 22000-27000 *m/z* region varies as one compares Figs. 2-5; *e.g.*, note the heterogeneous peaks between 22 and 25 kDa in Fig. 3, revealing at least four different species, and the appearance of a 26182 Da ion in Fig. 4. A new 21874 Da ion appears at *pI* 6.02 in Fig. 5. Clearly the 22000-27000 *m/z* region is complex.

Protein ions within this 22-27 kDa range do not match the sequence-calculated masses of known HDL proteins. Nevertheless, attribution of these ions to common HDL proteins remains likely, especially because glycosylation, truncation, and other modifications are prevalent. Proteins focusing in a *pH* range comparable to that of apoA-I, yet migrating as though smaller in size, have been revealed by silver-stained two-dimensional gels [24]. When probed with antibodies to apoA-I, apoA-II, apoA-IV, apoC's, apoD or apoE, these spots did not react [24]. A protein candidate consistent with the observation regarding antibody activity is apolipoprotein M (apoM). It is present in HDL and known to retain its signal peptide [49]. ApoM has a predicted mass of 21253 Da and calculated isoelectric point of 5.66 for an unglycosylated form. That the protein is known to be glycosylated on Asn-135 [49], suggests a higher molecular weight and

perhaps an altered isoelectric point. Apolipoprotein M is potentially responsible for masses 21.25 kDa or larger. Alternatively, immunoblotting of human plasma gels, (SWISS 2-D PAGE) ascribed a spot estimated to lie at pI 4.88 and 27 kDa to apoD, along with 11 spots slightly lower in isoelectric point. Hochstrasser's laboratory [24] observed apoD focusing between *pI* 4.3-5.1 and migrating as though sized between 27000-32000 Da. Glycosylated apolipoprotein D from plasma and axillary extracts has been characterized [17, 50]. Based on the mass-analyzed peptides encompassing N-linked glycosylated residues Asn^{45} and Asn^{78} [17], it was possible to calculate minimum, major, and maximum masses for plasma apoD as 23340, 24287, and 25966, respectively. Swiss 2-DPAGE annotates a *pI* 5.11, 22 kDa spot as arising from a truncated form of apoA-IV.

Larger proteins are also observed in MALDI from IPG strips; *i.e.*, 36, 50, 57, 61, and 72 kDa in Fig. 2, 36, 43, 50, 57, and 61 kDa in Fig. 3, and 37, 48, 50, and 67 kDa from Fig. 4. The SWISS 2DPAGE plasma gel includes three 43 kDa apoA-IV spots between pH 5.2 and 5.0 and three apoE spots observed at 35.4 kDa/*pI* 5.23, 35.0 kDa/*pI* 5.35, and 34.3 kDa/*pI* 5.49. The apoE primary sequence predicts a mass of 34236.68 and a *pI* of 5.52. Although apoE is known to be glycosylated, there is also evidence for a form without the carbohydrate [48]. The apoA-IV primary sequence predicts a 43375 Da protein focusing at *pI* 5.18. Other potential proteins include apoL-1, for which a truncated form of M_r 38461, *pI* 5.98 and a mature form of 41082 Da, *pI* 5.71 are predicted based on primary sequence. Glycosylation is also expected in apoL-1.

CONCLUSIONS

In mass analyzing a *pH* 5.89-4.91 segment of the IPG gel, masses consistent with mature apoA-I were observed from *pI* 5.47-5.04, a region spanning the predicted *pI* and consistent with

known heterogeneity. An average mass of 28076 Da was obtained from four measurements performed over *pH* 5.20-5.17, in good agreement with 28078.6 Da, predicted from the sequence. Broad tailing to higher *m/z* and an increased apex mass were observed in spectra from *pI*'s above or below this range.

Mass spectra acquired over 0.45 *pH* units revealed 42 masses. In addition to mature apoA-I described above, a M_r 27972 protein was observed at *pH* 5.20, consistent with apoA-I', a recently reported apoA-I isoform lacking C-terminal glutamine (calc. M_r 27950.4) [3]. A 28990 Da protein, observed at *pH* 5.44, may correspond to proapoA-I (predicted M_r 28961.6), estimated to migrate 0.18 *pH* units higher in *pH* than mature apoA-I. ApoD, apoIV, and/or apoM may give rise to masses observed between 22-27 kDa.

This study, extending virtual 2-D gel electrophoresis technology to human samples, demonstrates that synergies between virtual 2-D gels and classical 2-D gels are strong and useful. Newly acquired intact masses can be compared to gel images and data reported more than 15 years ago to provide new insights. This MS method's ability to deliver masses from polyacrylamide gels better allows estimation of the relevant mass heterogeneity in different molecular species, a particular help when exploring systems with many protein modifications.

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FIGURE CAPTIONS

Figure 1. Silver-stained IPG gel (*pH* 4-7) of human high density lipoproteins

Figure 2. Mass spectra obtained at *pH* 5.20 on a *pH* 4-7 IEF gel of human HDL. a) IPG-IEF spectra reveal a range of species, including apoA-I, a major protein constituent of HDL. b) Expanding about the 2+ apoA-I ion reveals a truncated isoform, attributed to apoA-I'. c) Proteins up to 71 kDa in size are detected at this isoelectric point.

Figure 3. Mass spectrum acquired directly from an IEF gel at *pH* 5.07. (Ions from the cytochrome *c* standard (*****) are off-scale.) An expanded view of the 20-27 kDa region is included in the inset.

Figure 4. Differences in peak widths reflect varying amounts of heterogeneity for proteins isoelectric at *pH* 5.44. Cytochrome *c* dimer ion indicated by ***** (non-specific cluster from the internal standard) appears at 24721 *m/z*. ProapoA-I is observed at 28983 *m/z*.

Figure 5. Mass spectrum acquired at *pH* 6.02 from an immobilized pH gradient gel. ***** marks cytochrome *c* calibrant ion.

Figure 1

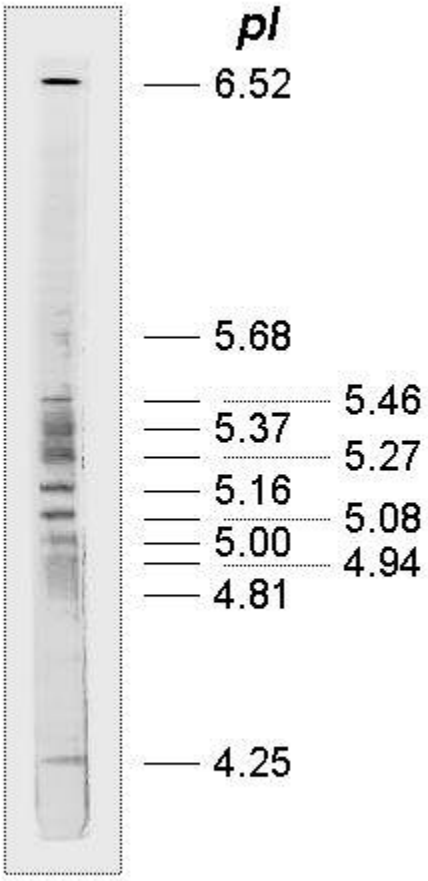

Figure 2

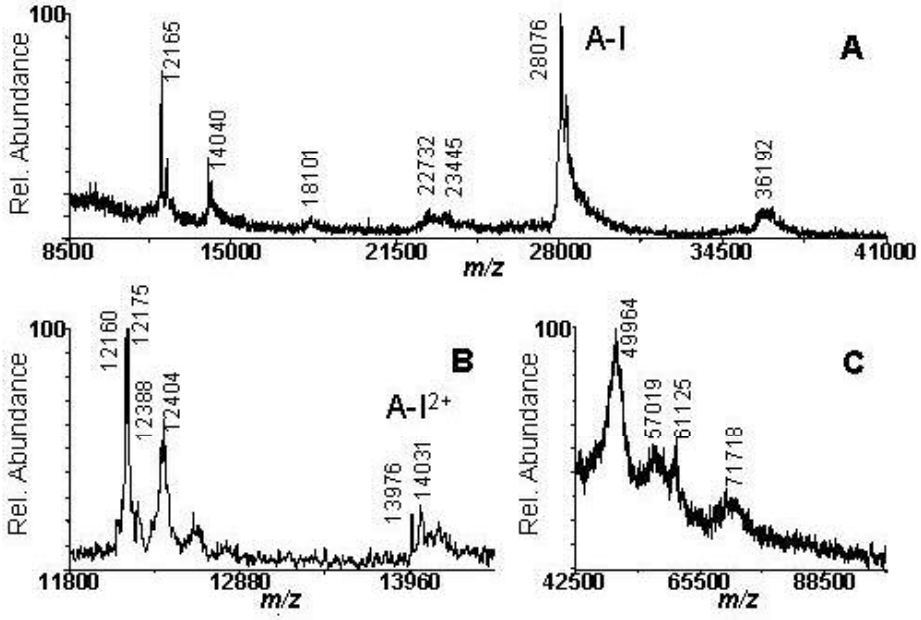

Figure 3

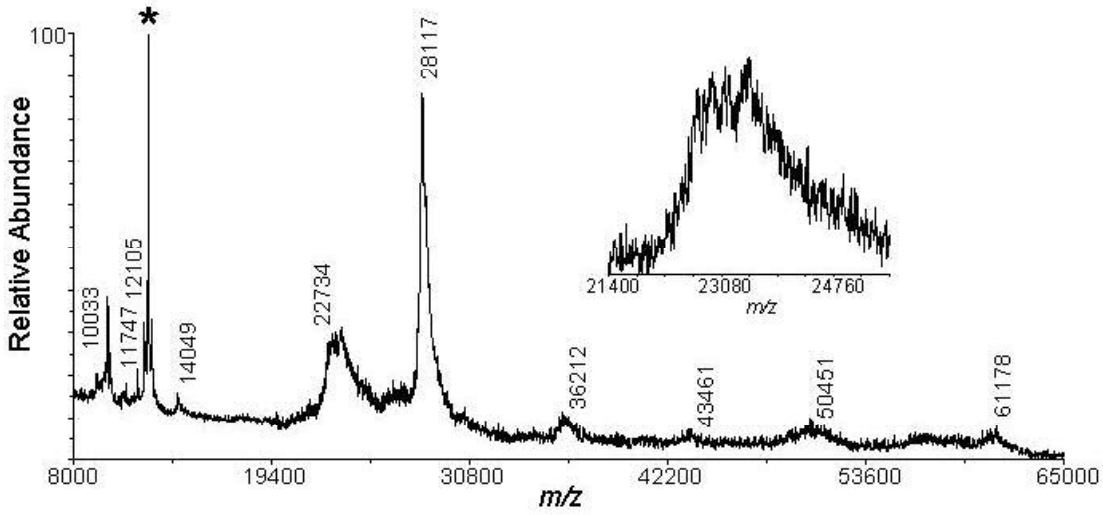

Figure 4

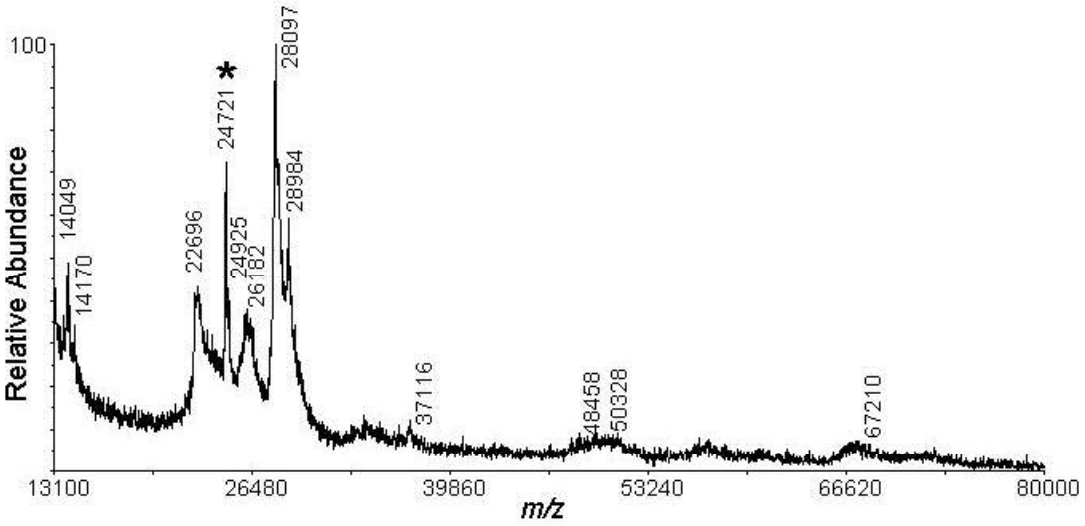

Figure 5