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PROFILING OF NORMAL HUMAN LEG LYMPH PROTEINS USING THE 2-D ELECTROPHORESIS AND SELDI-TOF MASS SPECTROPHOTOMETRY APPROACH

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ABSTRACT

The parenchymatous cells are supplied by nutrients transported in fluid from blood across the capillary wall. This fluid, called tissue fluid (TF), contains proteins originating from plasma as well as those synthesized and secreted by tissue cells. The protein composition of TF remains largely unknown. The TF which has entered lymphatics is called lymph (L). Harvesting L and measuring its proteins concentrations and identifying them provide an insight into biochemical processes in the TF. Here we describe our initial evaluation of the normal human prenodal L protein profile of m.w. 2.5 to 12.5kDa using the ProteinChip SELDI MS system and compare it with that of plasma (P) protein. This is the first study in the literature providing evidence for the presence of the so far non-identified proteins in L as well as proteins identified in L but absent from P and conversely present in P but not in L. Evident differences between paired L and P samples have been found, along with similarities. Thirteen proteins were detected in P and seven in L in the region of 2.5 to 12.5 kDa. Five identical proteins, although of different relative intensity, were found in L and P. The proteins specific for L but not P had 7070 and 8619 ion values. P proteins absent from L were of 3890, 3969, 4078, 6863, 7676, 7778, 7847 and 7937 ion values. In addition to detecting some so far unknown proteins in L, these preliminary findings throw a new light on our understanding of the mechanism of transcapillary transport of low m.w. proteins. They challenge the commonly accepted notion of unlimited free diffusion of peptides across the capillary membrane.

The tissue parenchymal cells are surrounded by a layer of tissue fluid (TF). The free TF remains in thermodynamic equilibrium with the interstitial gel. Under physiological conditions, there is a continuous movement of the free fraction of TF toward initial lymphatics (previously known as lymphatic capillaries). The TF which has entered lymphatics is called lymph (L). There is a large body of evidence that the two fluids, TF and L, are identical (1). Thus, measurement of L proteins and their identification provide an insight into biochemical processes in the tissue cell environment (2-6). Peripheral L contains all the protein fractions of plasma (P), but at lower levels (1). Most of the protein in TF and L is derived from blood P by diffusion, filtration and vesicular transport. In addition, a small fraction is derived from protein synthesized by the tissue's cells. Due to the molecular sieving

that occurs during transport of macromolecules across the blood capillary wall, the percentage of the total protein that is contributed by the small-molecular-weight (m.w.) proteins tends to be greater than that in serum (7). The contribution of the floating L cells to the L protein pool seems to be negligible. The activity of enzymes and their inhibitors may be different in L draining from tissue compared to P (8).

The identification and validation of proteins of the TF and L in normal conditions and associated with a particular disease state has so far been carried out only sporadically, although it is indispensable for obtaining knowledge on the in vivo biochemical events in the cell environment as well as establishing the etiology of edema of tissues caused by various pathological factors. For example, a long-lasting edema of the skin and subcutaneous tissue of limbs may be caused by inflammation, trauma, destruction of lymphatics and nodes by infection, lack of TF drainage because of malfunction of initial lymphatics in idiopathic lymphedema and inborn malformations of lymphatics. The protein spectrum will be different in each of these conditions. So far we have identified several L proteins of m.w. above 12 kDa (1) using classic biochemical methods. However, knowledge of the TF and L proteins of m.w. below 12kDa remains rudimentary. Within the very low m.w. range a large number of regulatory proteins and protein break-down products is contained.

The reasons for scarcity of studies on TF proteins have been difficulties in sampling of TF and lymph and, until recently, lack of sensitive and specific methods for protein profiling from complex biological materials. In recent years, two-dimensional (2D) gel electrophoresis has become the principal tool for separation and analysis of multiple proteins (9). In the course of time this method has been modified, however, as it is labor intensive, requires good quality of material, lacks inter-laboratory reproducibility and is not very useful for clinical application. The comparisons of 2D gel-protein maps are difficult and the need for protein staining and the subsequent sample handling limits the sensitivity. Surface enhanced laser desorption/ionization time-of-flight mass spectrophotometry (SELDI-TOF MS) is a novel approach used for protein profiling in biological materials (10).

The ProteinChip Biology System uses SELDI-TOF MS to retain proteins on a solid-phase chromatographic surface that are subsequently ionized and detected by TOF MS. This system is most effective at profiling low molecular weight proteins (<20kDa), providing a complementary visualization technique to 2D-PAGE. It is very sensitive and its versatility allows exploiting an unlimited type of samples. The studies on animal L proteins using the 2D-electrophoresis and SELDI-TOF system have recently been initiated by Leak (11).

Here we describe our initial evaluation using the ProteinChip SELDI MS system to assess the normal human prenodal L protein profile of m.w. 2.5 to 12.5kDa and compare it with P protein profile. This first study of its type in the literature has provided evidence for the presence of hundreds of so far not identified proteins in L as well as proteins identified in L but absent from P and conversely present in P but not L.

MATERIAL AND METHOD

Study Participants

Five healthy male volunteers, age 20-26 years, had one of their leg lymphatics cannulated, and L samples were collected over 24 hours. All signed an informed consent. The study was approved by the Medical Research Center, Polish Academy of Sciences ethical committee.

Lymph and Serum Collection

In patients with patent lymphatics, a superficial lymph vessel draining the skin of

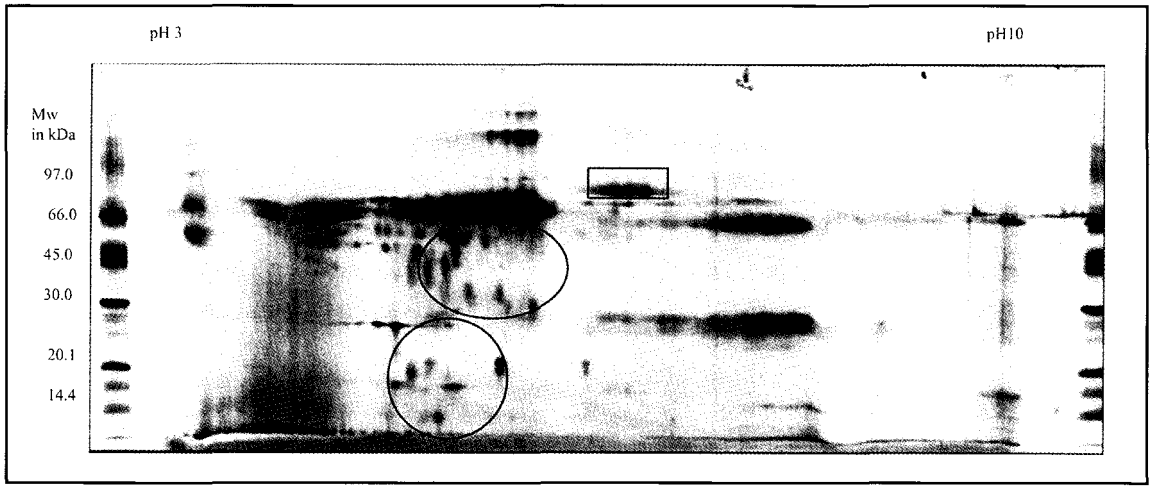

Fig. 1. The 2-D electrophoresis of a normal prenodal human leg lymph (L) sample. The whole protein spectrum. The gel for L shows minor protein spots for fibrinogen (rectangle), and a more differentiated region between 30 and 60 kD and 5 –7 pI (elipse). The region analyzed by SELDI is in the circle.

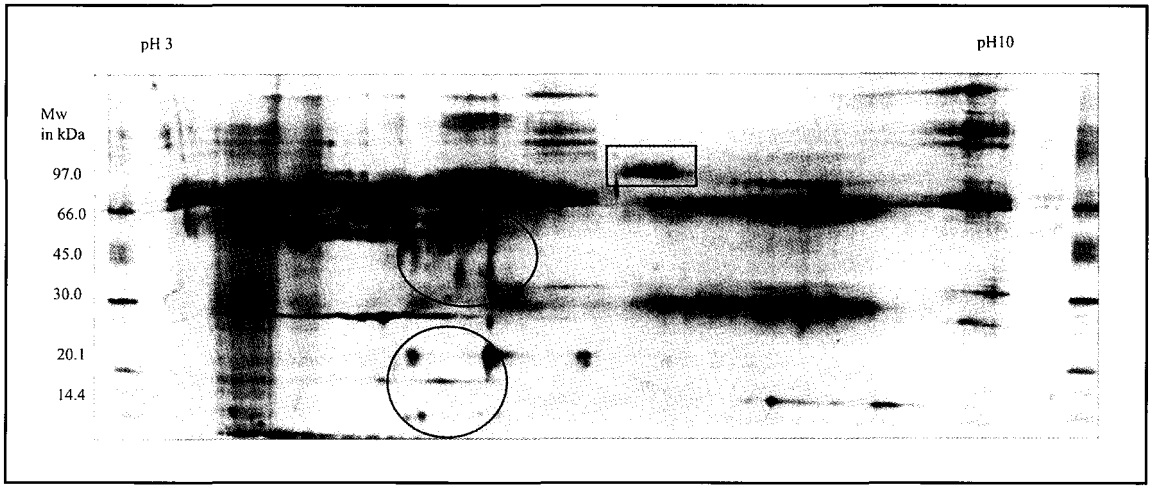

Fig. 2. The 2-D electrophoresis of a normal human plasma (P) sample. The whole protein spectrum. The gel for P shows major protein spots for fibrinogen (rectangle) and a less differentiated region between 30 and 60 kD and 5 –7 pI (elipse). The region analyzed by SELDI is in the circle.

the foot and lower part of calf was cannulated with a siliconized polyethylene cannula (1.6-mm outer diameter, 1.1-mm inner diameter) (Adams Clay, Raritan, NJ) (12). The cannula was inserted into the lymphatic in a retrograde fashion and L was collected in sterile test-tubes containing 0.1 ml of heparin (Novo, Copenhagen, Denmark) without preservative (12). The collection period lasted for 6-24 hr. Two to five ml aliquotes were spun down at 280g, the supernatants devoid of cells were kept frozen at -70°C until further use. Blood samples were taken from the cubital vein into 10 ml syringes with EDTA. P was separated and 2 ml aliquots were processed as for L. For

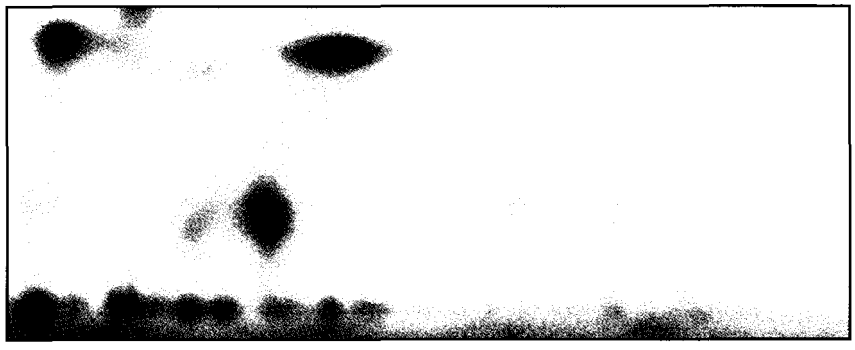

Fig. 3. The 2-D electrophoresis of a normal prenodal human leg lymph sample. The protein spectrum of m.w. from 2.0 to 15.0 kDa analyzed using ProteinChip SELDI-TOF analysis system.

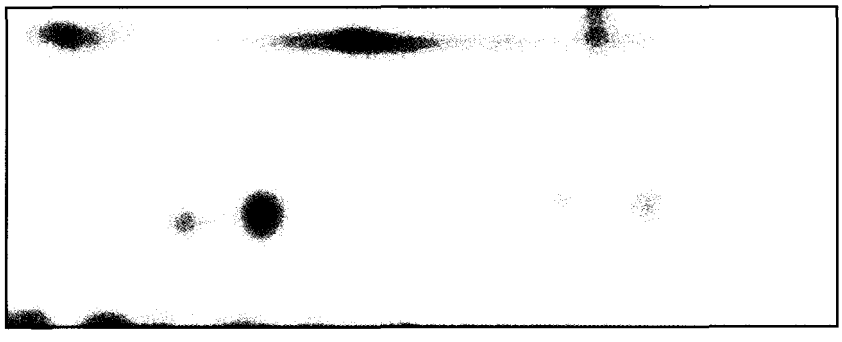

Fig. 4. 2-D electrophoresis of a normal human plasma sample. The protein spectrum of m.w. from 2.0 to 15.0 kDa analyzed using ProteinChip SELDI-TOF analysis system.

these studies, the L and P protein concentration was adjusted to 100µg/ml.

Two-Dimensional (2D) Electrophoresis of L and P

All reagents and equipment were obtained from Amersham Biosciences. The 18 cm 3-10 NL IPG strips were rehydrated overnight to their original thickness (0.5mm) with 350µl of solution containing 1µl of sample, 8M urea, 2% w/v CHAPS, 2% IPG Buffer 3-10, 10mM DTT, protease/ phosphatase inhibitors cocktail and tracking dye bromophenol blue. The first dimension (IEF) was performed at 15°C with the use of Immobiline Dry Strip Kit, Multiphor II, Multi Temp III and EPS 3501XL. Voltage was linearly increased from 300 to 3500V for total 45,000Vh. After the IEF separation, the strips were either stored at -80°C until further use or equilibrated and run (second dimension) on ExelGel 2-D homogeneous 12.5 with SDS buffer strips. Gels were silver stained, scanned and analyzed with Image Master 2-D software.

Protein Chip SELDI Analysis of L and P

The L and P samples were applied to a weak cation exchange (WCX2) protein chip analyzed using a Ciphergen SELDI TOF mass spectrometer. A Biomek 2000 robotic liquid handler was used to prepare the surface of the WCX2 protein chip, apply the samples, wash the protein chips and apply a matrix solution.

The analysis was done by comparison of

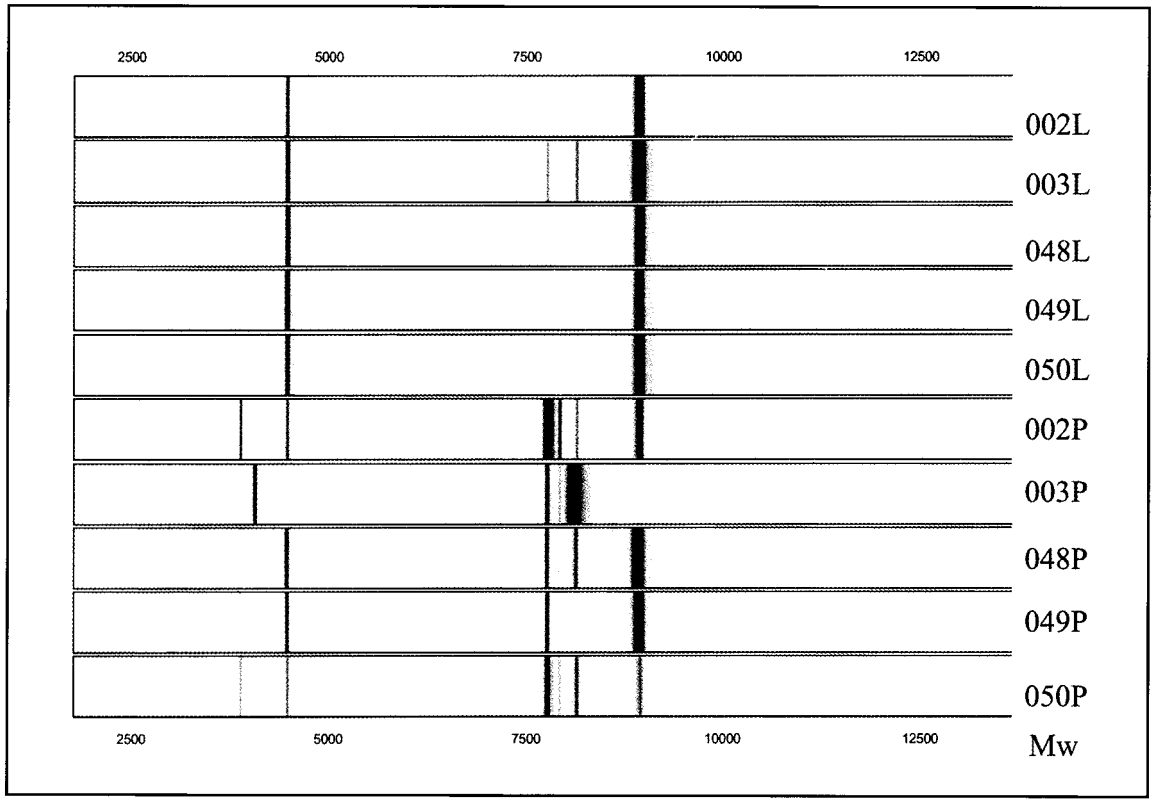

Fig. 5. The gel view of L and P protein SELDI-TOF system analysis of 5 healthy volunteers. Upper panel – 5 L samples, lower panel – 5 P samples. Evident differences between L and P band localization are seen in each individual.

two spectra through all the samples. A peak map was generated and compared to the next sample keeping only the peaks that are found in both samples. This iterative process was performed on all samples, and the final peak maps were compared. Results were presented as protein gel views and mass spectra. The mass spectra of individual subjects L and P were compared. This final comparison showed that all the peaks identified in L were also identified in P except for ion value 8549.7. As the data illustrate, there are more ions present in P than in L. *(Fig. 7)*. This finding is consistent with 2D-PAGE analysis.

RESULTS

The gel views of 2D L and P electrophoresis are shown on *Figs.1* and *2*. The gel view depicts the whole spectrum of resolved peptides. A detailed description has been presented in the figure legends. Proteins of 2.5 to 12.5kDa m.w. comprising the region seen on *Figs. 3* and *4* have been analyzed using SELDI-TOF system.

The gel view and mass spectra of SELDI analysis of normal L and P are shown in *Figs. 5* and *6*. The protein profile patterns of L were similar in all 5 samples with some additional peaks in subject #3. Only minor differences were seen between the P samples. Interestingly, evident differences between paired L and P samples have been found *(Fig. 7)*. Fourteen proteins were detected in P and seven in L. Five identical proteins, although of different relative intensity, were found in L and P. The proteins specific for L but not P had 7070 and 8619 ion values.

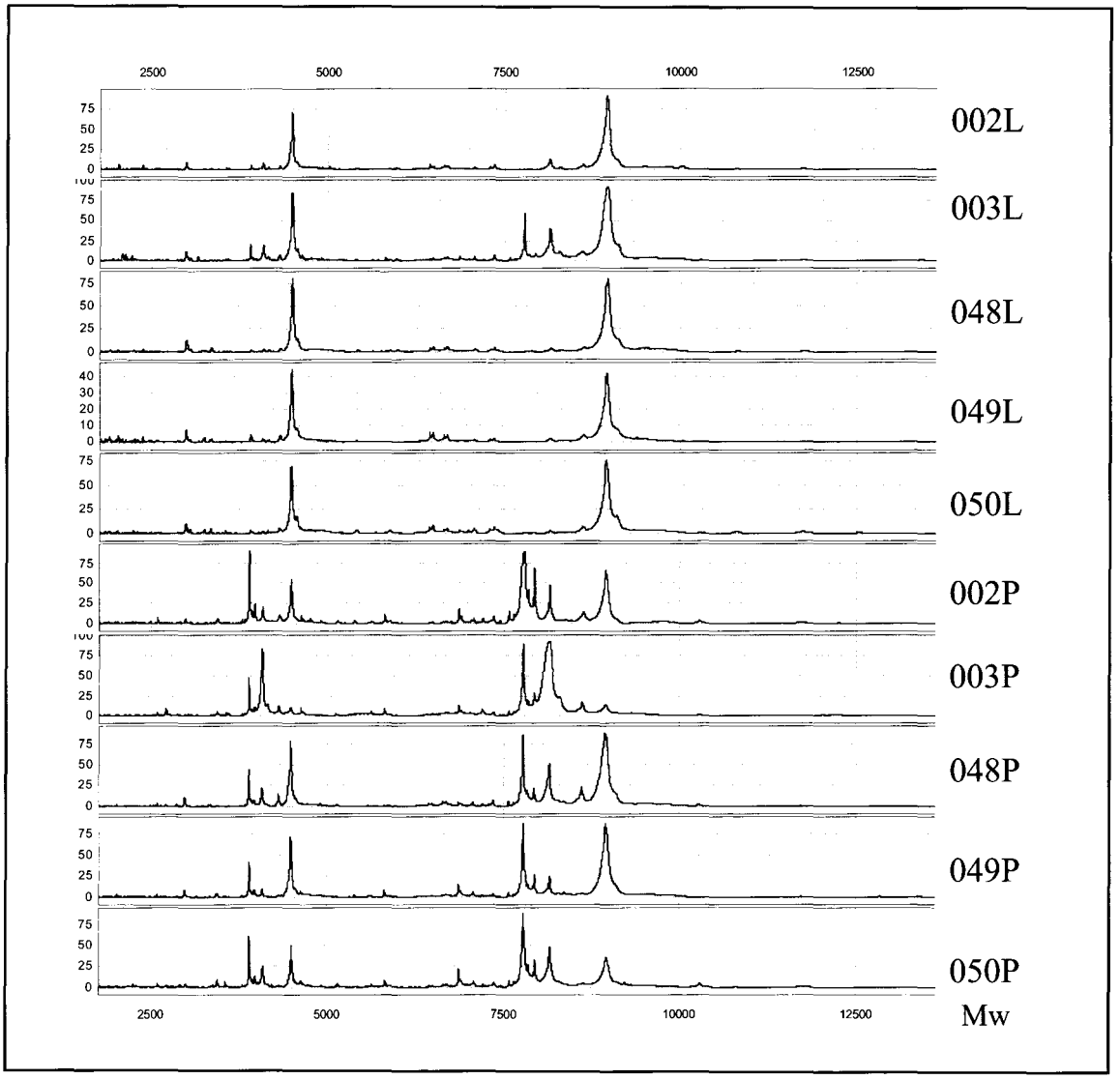

Fig. 6. The spectra view of samples shown in Fig. 3 and 4. Upper panel – 5 L samples, lower panel – 5 P samples. Major differences in peaks between L and P. The relative intensity is displayed along the y-axis, and the mass is presented on the x-axis as the molecular weight to charge ratio.

P proteins absent from L were of 3890, 3969, 4078, 6863, 7676, 7778, 7847 and 7937 ion values.

DISCUSSION

This preliminary study using the SELDI-TOF analysis system has shown that human prenodal leg L contains proteins absent from P, and conversely P contains proteins not detectable in L. Proteins detected in L could be the products of parenchymal cells in areas drained by the cannulated lymphatic. Such proteins are physiologically transported with the L stream to the blood circulation. Due to their low concentration, they may be diluted in P and not detectable there. The blood proteins are filtered across the capillary

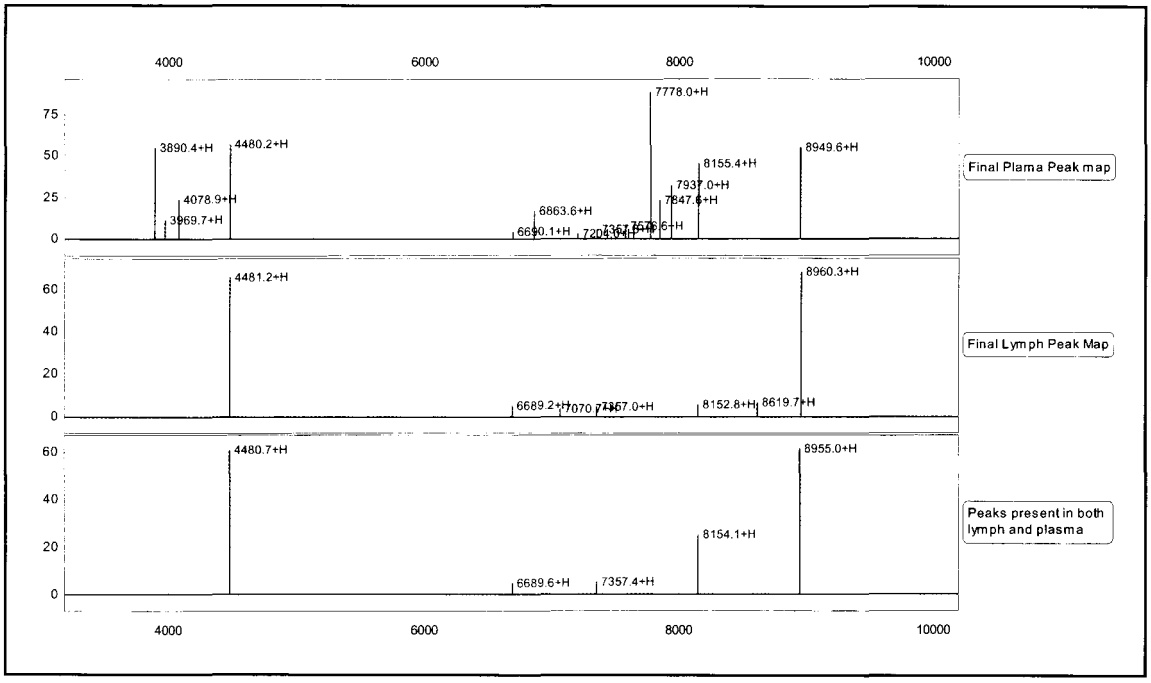

Fig. 7. The map view of P (top) and L (middle) protein peaks. Lower panel – peaks present in L and P. Note that there are peaks present in P but absent from L and conversely present only in L (8619,7⁺H ion value in circle).

membrane in a fashion inversely proportional to the molecular weight and radius (7). This applies mostly to proteins of m.w. above 70 kDa whereas proteins of low m.w. diffuse freely from P to the TF. Detection of proteins (peptides) of m.w. between 4.0 to 8.0 kDa in P not diffusing to the TF suggests either their binding to other P proteins or changes in configuration preventing passage through the capillary pores to the interstitial space. The identity of proteins detected in L but absent from P has not been established. These might be peptides cleaved from proteins secreted in the tissue (neurokinins, defensins, fragments of extracellular matrix, etc.).

In addition to detecting some so far unknown proteins in L, these preliminary findings throw a new light on our views on the mechanism of transcapillary transport of low m.w. proteins. They challenge the commonly accepted notion of free diffusion of peptides across the capillary membrane. Proteins retained in plasma and those detected solely in L have so far not been characterized. Their amino acid sequences are being analyzed.

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