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Proteomic Analysis with Integrated Multiple Dimensional Liquid Chromatography/Mass Spectrometry Based on Elution of Ion Exchange Column Using pH Steps

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A novel integrated multidimensional liquid chromatography (IMDL) method is demonstrated for the separation of peptide mixtures by two-dimensional HPLC coupled with ion trap mass spectrometry. The method uses an integrated column, containing both strong cation exchange and reversed-phase sections for two-dimensional liquid chromatography. The peptide mixture was fractionated by a pH step using a series of pH buffers, followed by reversed-phase chromatography. Since no salt was used during separation, the integrated multidimensional liquid chromatography can be directly connected to mass spectrometry for peptide analysis. The pH buffers were injected from an autosampler, and the entire process can be carried out on a one-dimensional liquid chromatography system. In a single analysis, the IMDL system, coupled with linear ion trap mass spectrometry, identified more than 2000 proteins in mouse liver. The peptides were eluted according to their pI distribution. The resolution of the pH fractionation is ~0.5 pH unit. The method has low overlapping across pH fractions, good resolution of peptide mixture, and good correlation of peptide pIs with pH steps. This method provides a technique for large-scale protein identification using existing one-dimensional HPLC systems.

Protein identification based on mass spectrometry has played an important role in proteomic research. One of the strategies employs MALDI-TOF-MS to obtain peptide fingerprints from tryptic-digested proteins. The proteins/peptides were identified by matching the experimental spectra with the theoretical spectra.[1−2] Another approach is to separate peptides by reversed-phase HPLC and follow by tandem mass spectrometric analysis.[3−5]

The MALDI-TOF-MS method has very low separation capacity and is usually coupled with 2D-gel electrophoresis or other offline separation methods before mass spectrometry. On the other hand, the LC/MS/MS method can separate and identify thousands of proteins in just one analysis. However, for global protein identification, it requires a system able to analyze millions of peptides/proteins quickly. Therefore, a lot of effort has been spent on the optimization of LC conditions to increase the capacity, sensitivity, and resolution.[6−8]

In recent years, multidimensional HPLC separation of peptide mixtures before mass analysis was introduced to meet the demands of a large-scale proteomic analysis by LC/MS/MS.[10−12] To date most multidimensional HPLC separations have been carried out on a strong cation exchange and reversed-phase HPLC. The protein/peptide mixture was fractionated by a strong cation exchange column and followed by reversed-phase separation.[13−14] However, the cation exchange separation requires a salt gradient and as a result is not compatible with mass spectrometry. Therefore, offline cation exchange separation or extensive column wash after sample loading is required before mass analysis. Our new method addresses the problem by injecting or loading a small amount of different pH buffers to elute the protein/peptide mixture

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to the reversed-phase column, eliminating the requirement of having to add salt to the buffers. This allows direct connection of the HPLC system to the mass spectrometer.

A previous paper reported using the pH gradient to measure the pK_a of substances. This indicates that the pH gradient elution can achieve very accurate fractionation according to the pI distribution.[15] We used an integrated column, which combines a strong cation exchange column and a reversed-phase column to separate peptides by their pIs and hydrophobicity. The strong cation exchange column is directly attached to the reversed-phase column and has minimal dead volume to maintain the separation efficiency. The pH buffers are mass compatible, which allows the direct coupling of HPLC to the mass spectrometry. Common acids applied are formic acid, acetic acid, and citric acid; ammonia or substituted amines were used as the base.

Only utilizing a single HPLC pump, an autosampler, and an integrated column, this method can easily achieve two-dimensional HPLC separations. The results are very comparable to any commercial two-dimensional HPLC systems. The method was used to identify more than 2000 proteins from mouse liver. The peptide pI distribution, the buffer system, and the resolution of pIs also are discussed.

EXPERIMENTAL SECTION

Chemicals. All the water used in the experiment was prepared using a Milli-Q system (Millipore, Bedford, MA). Urea, dithiothreitol (DTT), ammonium bicarbonate, and iodoacetamide were all purchased from Bio-Rad (Hercules, CA). Guanidine hydrochloride and citric acid were obtained from Sigma (St. Louis, MO). Trypsin was purchased from Promega (Madison, WI). Formic acid and trifluoroacetic acid were obtained from Aldrich (Milwaukee, WI). Acetonitrile (HPLC gradient grade) was obtained from Merck (Darmstadt,Germany). Trimethylamine was purchased from Applied Biosystems (Foster City, CA). NH_4OH was obtained from Shanghai Chemical (Shanghai, China). All the chemicals were of analytical grade.

Sample Preparation. Mouse liver tissue was cut into small pieces, washed by phosphate-buffered saline. Afterward it was then frozen using liquid nitrogen and rubbed to powder. The powder was suspended in lysis buffer (40 mM Tris, 8 M urea, 4% CHAPS, 65 mM DTT). The suspension was sonicated for 30 s followed by centrifugation at 25000*g* for 1 h. The supernatant contains the total liver proteins. Protein concentrations were estimated with a Bradford protein assay using bovine serum albumin as a standard. The proteins were precipitated overnight with five volumes of ethanol, acetone, and acetic acid (50/50/0.1, v/v/v). The supernatant were carefully removed after centrifugation at 25000*g* for 1 h at 4 °C. The pellet was collected and stored at −20 °C.

Trypsin Digestion. The pellet from 600 μg of proteins before precipitation was redissolved in reducing solution containing 6 M guanidine hydrochloride and 100 mM ammonium bicarbonate at pH 8.3. A 200-μL aliquot of reducing solution was then mixed with 4 μL of 1 M dithiothreitol. The mixture was incubated at 56 °C for 1 h. A 30-μL aliquot of 1 M iodoacetamide was then added, and the mixture was incubated for an additional 40 min at room temperature in darkness. The protein mixtures were spun and exchanged into 100 mM ammonium bicarbonate buffer and then incubated with trypsin (25:1) at 37 °C for 20 h.

pH Buffer Solution. In this study, a series of buffers with different pH values (pH 3, pH 3.5, pH 4, pH 4.5, pH 5, pH 5.5, pH 6, pH ~7, pH 8) were used for the pH step elution. Two kinds of bases were used to adjust the pH of the buffers. The first buffer system contained 10 mM citric acid; trimethylamine was used to adjust the buffer pH from 3 to 7. The pH 8.0 buffer used was aqueous ammonia. The second pH step buffer was 10 mM citric acid, and the pH was adjusted from 3 to 8 with ammonium hydroxide.

2D-LC/MS/MS Shotgun Analysis. The 2D-LC/MS/MS experiments were performed using a Surveyor high-performance liquid chromatography system coupled with a LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA). The Surveyor liquid chromatography system (Thermo) was equipped with an autosampler, one high-pressure mixing pump, and a Bi-Phase integrated column for the 2D-LC separation. The Bi-Phase column included two sections: the front section was a strong cation exchange column (320-μm i.d.; 50-mm length, Column Technology Inc., Fremont, CA), and the rear section was a reversed-phase C18 column (150-μm i.d.; 100-mm length, Column Technology Inc.). The HPLC solvents used were 0.1% formic acid (v/v) aqueous (A) and 0.1% formic acid (v/v) acetonitrile (B). The tryptic-digested peptide mixtures were injected by the Surveyor autosampler using the no-waste injection function. The first dimension of separation elutes the peptides by a stepwise gradient of varied pH buffers. Different pH buffers were applied into the integrated column by autosampler using a full loop injection function. After 100 μL of buffer was applied to the integrated column, the column was washed with 100% mobile phase A. The total injection and wash time of each pH step is ~80 min at a flow rate of 300 μL/min before a (100:1) split ratio. The reversed-phase gradient was from 5 to 65% mobile phase B in 115 min at 200 μL/min flow rate before the split and 2 μL/min after the split.

The mass spectrometer used for this study is a linear ion trap LTQ equipped with a metal needle electrospray interface (ThermoFinnigan, San Jose, CA). The temperature of the ion-transfer capillary was set to 160 °C. A voltage of 3.0 kV was applied to the ESI needle, and the normalized collision energy was at 35.0%. An automated gain control function was used to manage the number of ions injected into the ion trap. One microscan was set for each MS and MS/MS scan. All MS and MS/MS spectra were acquired in the data-dependent mode. The mass spectrometer was set so that 1 full MS scan was followed by 10 MS/MS scans on the 10 most intense ions. The dynamic exclusion function was set as follows; repeat count 3, repeat duration 0.5 min, and exclusion duration 1.5 min. System control and data collection were done by Xcalibur software version 1.4 (Thermo).

Protein Identification. The acquired MS/MS spectra were compared against the MOUSE International Protein Index protein sequence database (version 3.00.1, www.ebi.ac.uk/IPI) using the TurboSEQUEST program in the BioWorks 3.1 software suite (Thermo). All the.out files were filtered by Buildsummary software. An accepted SEQUEST result had to have a ΔCn score of at least 0.1 (regardless of charge state). Peptides with a +1 charge state were accepted if they were fully tryptic digested and had a cross correlation (Xcorr) of at least 1.9. Peptides with a +2

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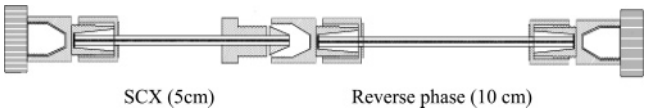

Figure 1. Bi-Phase integrated column used in IMDL strategy.

charge state were accepted if they had an Xcorr >=2.2. Peptides with a +3 charge state were accepted if they had an Xcorr >=3.75.

RESULTS AND DISCUSSION

Bi-Phase column and IMDL Design. Previous 2D-LC/MS methods used two HPLC pumps with one strong cation exchange and one or two reversed-phase columns for the separation.[16] The system usually is fully automated but is expensive and quite complicated. The IMDL technique is a simple, easy method for the 2D-LC/MS/MS separation of protein/peptide mixture that does not require any modification of the existing 1D-LC/MS/MS system. Figure 1 shows the picture of the Bi-Phase integrated column used for this new method. The integrated column includes two sections. The front section utilizes a 5-cm strong cation exchange column and the rear is a 10-cm reversed-phase column. The trypsin-digested peptides were first loaded on the SCX section and then eluted to the reversed-phase section by pH gradient. At low pH, the peptide is positively charged and binds to the strong cation exchange column by ionic interaction. When the pH of buffer increases above the pI of the peptides, the peptides will become negatively charged, and therefore lose their binding to the SCX column, and become extracted to the reversed-phase section. The peptides are then separated by reversed-phase HPLC gradient and followed by tandem mass spectrometry.

Traditional ion exchange separation required a salt gradient to elute peptides; however, the salt contaminant can reduce the efficiency of a reversed-phase column and generate strong background noise in mass spectrometry. Therefore, all the 2D-LC/MS/MS analysis requires is either offline collection or extensive washing after the salt step. The new method uses a pH gradient, and only 10 mM buffer is needed to elute peptides. There was no need for extensive washing after the ion exchange step, and the peptides were eluted according to their pIs. Figure 2 shows the chromatograms of LC/MS separation of the mouse liver proteome determined by the integrated column method. Since the columns are directly attached to each other, there was no dead volume between the ion exchange and reversed-phase sections. This results in high-efficiency separation. Good resolution was observed on all reversed-phase gradients.

The pH buffers were injected by autosampler. There is no need to use a 2D-LC system, and no switching valve or additional controlled software is required. Using the integrated column method, 2D-LC/MS/MS can be easily achieved by any conventional 1D-LC/MS/MS system.

Buffer System of Integrated Multidimensional Liquid Chromatography (IMDL). Various pH buffer systems were tested for the IMDL method to achieve the best resolution for the separation. The peptides have a theoretical pI from 1 to 10 with focus on 3−6. Therefore, a good buffer system should have a buffer capacity from 3 to 7 and should be compatible with mass spectrometry. In this paper, we tried acetic acid, formic acid, and citric acid as buffers for the pH gradient. Citric acid was chosen for the study due to its good buffer range and its compatibility with mass spectrometry. Different concentrations of citric acid were tested: 5, 10, and 30 mM. The 10 mM concentration was selected due to its suitable ion capacity and its ease in equilibrating with reversed-phase mobile phase A.

Trimethylamine and ammonium hydroxide were used to adjust the pH of the citric acid buffers. Both buffers gave good HPLC separation and identified peptides according to their pI. However, the citric acid−ammonium hydroxide buffer identified more peptides at pI <3 and pI >8 than the citric acid−trimethylamine buffer, as shown in Table 1. One hypothesis is that the citric acid−ammonium hydroxide buffer has more buffer capacity then the citric acid−trimethylamine buffer in both pH levels and thus elutes more peptides. Because of this, it was decided that citric acid−amonium hydroxide was the optimal buffer for this study.

Figure 2 shows the chromatogram of LC/MS of IMDL using the citric acid−ammonium hydroxide buffer system, which displays an excellent signal-to-noise pattern and has good distribution of peptides among fractions.

Protein/Peptide Identification by the IMDL Method. The peptides eluted from the integrated column were analyzed by linear ion trap mass spectrometry. The sequence of peptides from LC/MS analysis was identified by comparing MS/MS data with theoretical spectra. The higher number of MS/MS spectra can increase the number of peptides identified by the method. The linear ion trap from Finnigan was an improvement from the previous 3D trap and has a faster scan rate. To obtain a higher number of peptides, we set up a 10 MS/MS analysis following each MS scan. The total scan time for every set of scans was ~1.5 s. The SEQUEST software was used to identify peptide sequence. $\Delta Cn \geq 0.1$ and Xcorr ≥ 1.9 with charge state 1+, Xcorr ≥ 2.2 with charge state 2+, and Xcorr ≥ 3.75 with charge state 3+ were used as cutoff for peptide identification.

Table 1 shows the numbers of peptides identified by citric acid−trimethylamine and citric acid−ammonium hydroxide buffers. A total of 5779 unique peptides were identified by the citric acid−ammonium hydroxide buffer and 5034 peptides were identified by citric acid−TMA buffer. The proteins were identified according to a database search using an in-house BuildSummary software (manuscript submitted to *Proteomics*). Proteins sharing the same identified peptides were assigned to a protein group. A total of 2067 protein groups were identified by citric acid−ammonium hydroxide buffer in one analysis with 600 μg of total proteins before digestion. Within 2067 protein groups, 970 of the protein groups (46.93%) were identified by at least two peptides; the confidence level of the proteins is above average. The citric acid−ammonium hydroxide method identified more proteins then the citric acid−trimethylamine buffer. To define the accuracy of the protein identification, we further used the reverse database to obtain the positive rate as reported.[17] The false positive rates are only 3.77, 1.67, and 0% for 1+, 2+, and 3+ charged peptides, respectively.The very low false positive rate indicates the high confidence of the protein identification based on current filters.

pI Distribution of Identified Peptides. Panels A and B of Figure 3 show the numbers of overlapped peptides across pH

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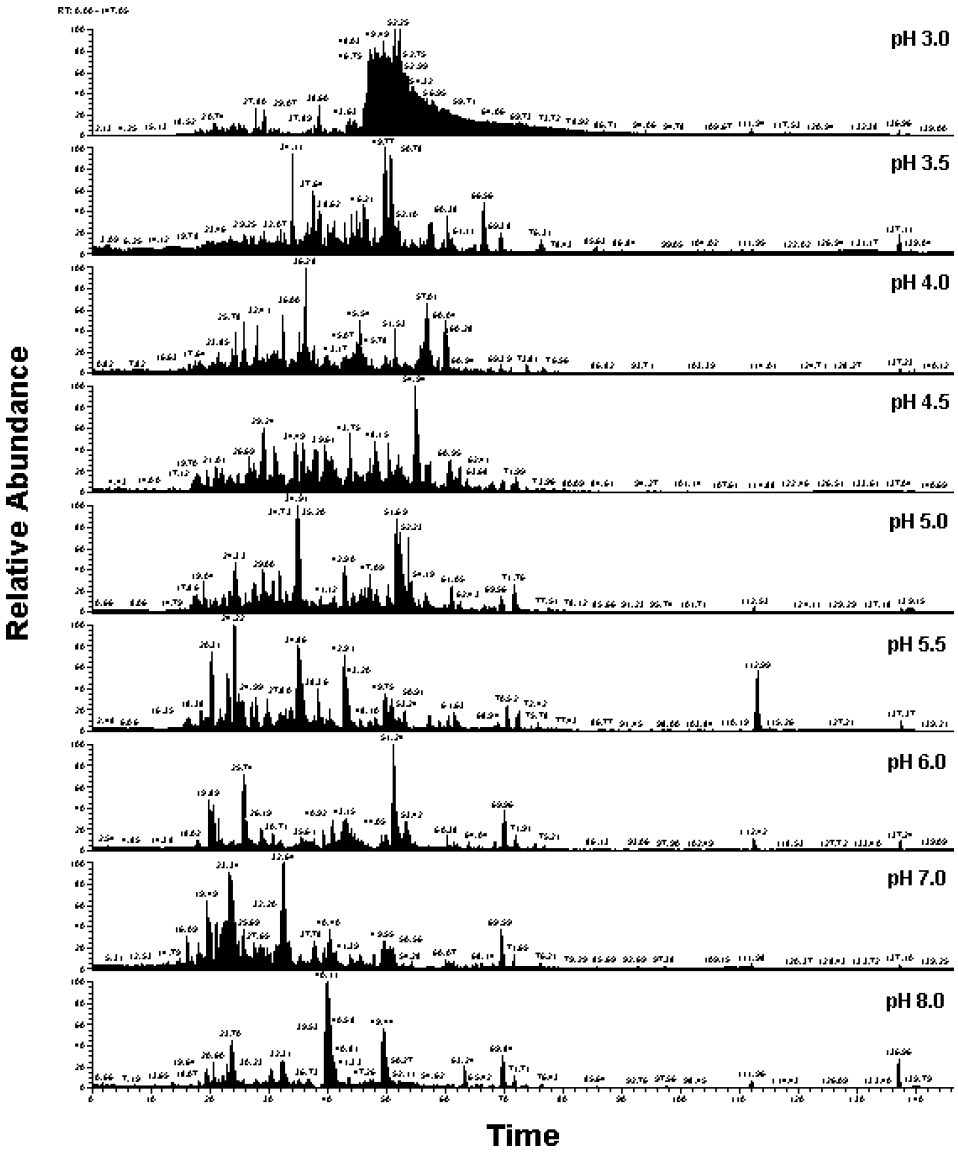

Figure 2. Chromatogram of two-dimensional HPLC-MS/MS of tryptic-digested mouse liver proteins using an integrated column. The first-dimensional SCX-LC uses nine buffers for pH step gradient as shown in Table 1. The mobile phase used in RP-LC, (A) 0.1% formic acid (v/v) in water; (B) 0.1% formic acid (v/v) in acetonitrile; gradient, 5−65% B in 115 min; flow rate, 2 μL/min after splitting.

Table 1. Comparison of Unique Peptides, Peptide Hits, and Protein Group Numbers by 2D-HPLC/MS/MS on an Integrated Column Using CA−TMA and CA−NH_4OH) Buffers[a]

		pH 3.0	pH 3.5	pH 4.0	pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 7.0	pH 8.0	Total
peptide hits	CA−TMA	438	2313	2717	3302	3366	2522	2118	2092	457	19325
	CA−NH_4OH	2985	2904	3001	3447	3707	3055	2085	2112	1488	24784
unique peptides	CA−TMA	208	993	1198	1175	1230	939	746	702	124	5034
	CA−NH_4OH	977	1281	1333	1256	1245	1042	685	656	476	5779
protein groups	CA−TMA										1790
	CA−NH_4OH										2067

[a] Sequest parameters: Xcorr ≥ 1.9 with charge state 1+, Xcorr ≥ 2.2 with charge state 2+, and Xcorr ≥ 3.75 with charge state 3+; ΔCn ≥ 0.1.

steps with ammonium hydroxide or trimethylamine as the counter base, respectively. Both systems display a very low incidence of overlapping peptides across pH steps. The peptides mainly appeared in specific pH steps, which indicates that the pH elution of peptides from the SCX column can achieve a peptide focusing effect. Most peptides accumulate in the region of pH 4−6, while the CA−TMA system seemed to lose some peptides at pH 3 and 8. The problem of peptide overlapping would decrease the number of proteins identified. This is usually observed in the presence of a salt gradient or when poor columns are used (either SCX or RP, data not shown).

In the peptide separation by ion exchange chromatography, the peptides were eluted from the SCX column by an increase of the salt concentration. In general, acidic peptides were eluted at

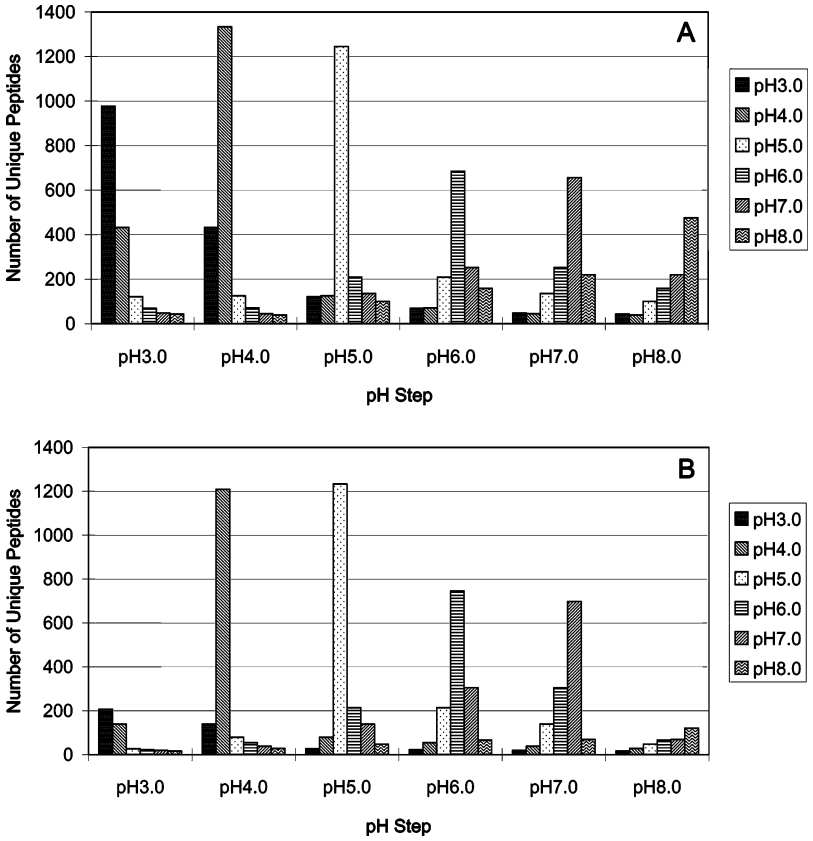

Figure 3. Overlap of unique peptides across pH steps in citric acid−NH_4OH buffers (A) and citric acid−TMA buffers (B).

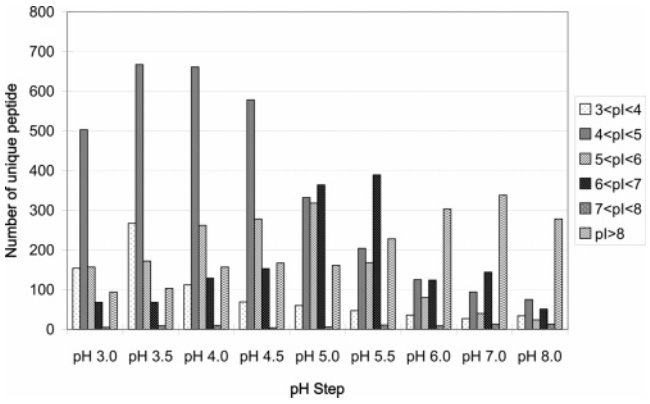

Figure 4. Distribution of unique peptides with their calculated pIs and pH fractions using citric acid−NH_4OH buffer.

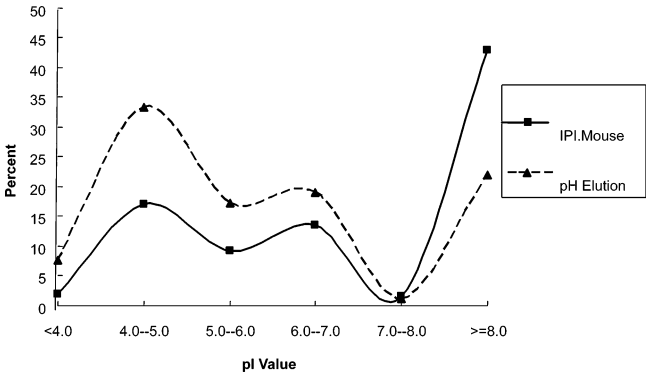

Figure 5. pI profile of unique peptides form IMDL experiment and from database which was calculated by theoretical trypsin digestion.

low salt concentrations and basic peptides by higher salt concentration; the actual peptides did not distribute in salt step according to their pIs.[17] In contrast, in pH elution, the peptides can be washed according to their pI distribution.

Figure 4 shows the correlation of pH steps and pI distribution of peptides identified using a citric acid−ammonium hydroxide buffer system. There was very good correlation of the calculated pI of the peptides and the eluted pH value. The peptides not only appeared in the pH step corresponding to the calculated pI, but the peptides distributed around the expected pH step and shifts were generally within 1 pH unit. Figure 5 shows the distribution of the calculated pI values of tryptic peptides in the database and the pIs of identified peptides. The two curves are very consistent except the database has more peptides with pI >8. Washing the column with 2 M salt after the pH 8 step resulted in only a slight increase in the number of peptides identified. One possibility is that, in the database, most basic peptides were caused by incomplete digestion in which peptides with one or two missed digested sites account for 37.3 and 38.21%, respectively. Since tryptsin cleaves at either the K or R terminal, peptides with a higher number of K and R would have higher pI value. However, in the experimental data, peptides containing one or two missed cleavage sites only reached 6.89 and 2.99%, respectively, and therefore identified less basic peptides. The other possibility is that the basic peptide is less soluble in the buffer and may be lost during the digestion step.

Resolution of Peptides by pH Elution. To achieve high resolution of peptide separation in the ion exchange step, the peptides need to be eluted in a very narrow pH range. In this study, we found that some peptides only appeared in expected pH fractions while others appeared in multiple fractions. We have

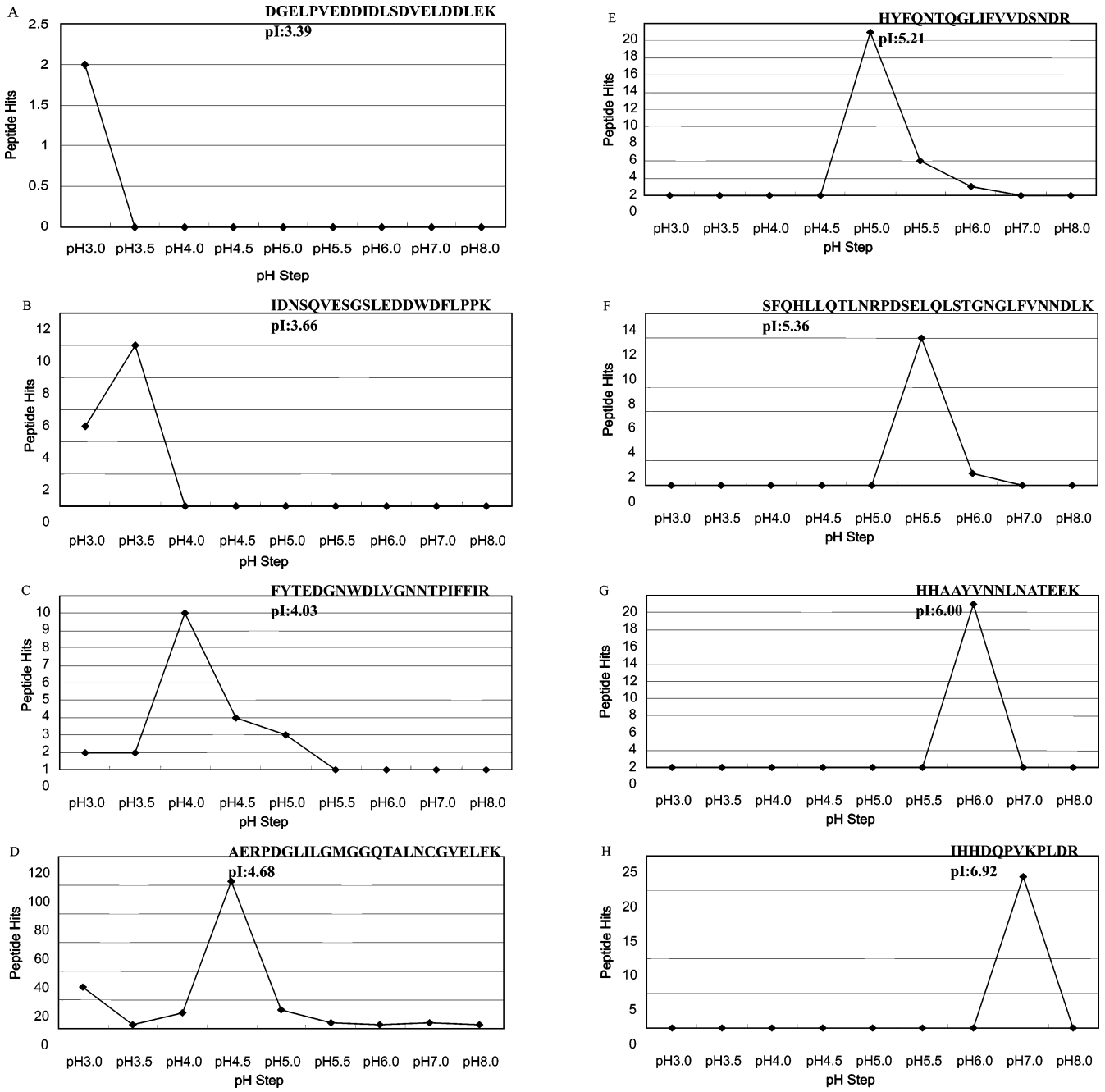

Figure 6. Some specific peptides with different theoretical pI eluted in corresponding pH fractions using citric acid−NH_4OH buffer.

chosen several peptides to calculate the resolution of the IMDL method as shown in Figure 6. A very acidic peptide, DGELPVEDDIDLSDVELDDLEK, with the calculated pI of 3.39, only appeared in pH 3 (Figure 6A) and with only two hits detected. This low-abundant peptide was extracted from the high-abundant peptides ranging from pHs 4 to 6, subsequently making the sensitive detection of these kinds of peptides. Two peptides, HHAAYVNNLNATEEK (calculated pI 6.00) and IHHDQPVKPLDR (calculated pI 6.92), were only observed in the pH 6 (Figure 6G) and pH 7.0 steps (Figure 6H). There were ~20 hits detected, indicating that they are high abundant with excellent focus without any diffusion across pH steps. On the other hand, in the peptide-rich region pH 4−6, FYTEDGNWDLVGNNTPIFFIR (calculated pI 4.03), AERPDGLILGMGGQTALNCGVELFK (calculated pI 4.68), HYFQNTQGLIFVVDSNDR (calculated pI 5.21), and SFQHLLQTLNRPDSELQLSTGNGLFVNNDLK (calculated pI 5.36), were mainly eluted in pH 4.0, 4.5, 5.0, and 5.5, respectively. The peptide AERPDLILGMGGQTALNCGVELFK (calculated pI 4.68), was recorded for over 100 hits, with most of the hits focusing in the pH 4.5 fraction. The 0.5 pH unit resolution gives the pH elution promising potential to detect low-abundant peptides and even to resolve the peptide isoforms caused by posttranslational modification or site mutation.

CONCLUSIONS

We reported an innovative method for the separation of a peptide mixture by integrated multiple dimensional liquid chromatography coupled to mass spectrometry. This method uses an

integrated column, which contains both strong cation exchange and reversed-phase sections. The ion exchange separation was achieved by using a pH step gradient with mass-compatible buffers. The peptides were fractionated according to their pIs and followed by reversed-phase separation. This method demonstrates very low peptide overlapping between different pH fractions, has high resolution of peptides with close pIs, and identified a large number of proteins from the mouse liver proteome. In addition, the system is easy to use, easy to maintain, with no need for an additional HPLC system. This method can be used to separate complicated protein/peptide mixture by two-dimensional HPLC/MS/MS on any one-dimensional HPLC system.

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