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Novel Application of Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) in Shotgun Proteomics: Comprehensive Profiling of Rat Kidney Proteome

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In shotgun proteomics, multidimensional liquid chromatography (MDLC) is commonly used to reduce sample complexity and increase dynamic range of protein identification. Since reversed-phase chromatography is mostly used as the second-dimensional separation before mass spectrometric analysis, the improvement of MDLC primarily depends on the first dimension of separation. Here, we present a novel whole proteome analysis method that separates peptides based on ERLIC. Tryptic peptides were retained on a weak anion exchange column through ERLIC with a high organic mobile phase. They were then distributed into multiple fractions based on both p*I* and polarity through the simultaneous effect of electrostatic repulsion and hydrophilic interaction when eluted using a salt-free pH gradient of increasing water content. Applying this to rat kidney tissue, we identified 4821 proteins and 30 659 unique peptides with high confidence from two replicates using LTQ-FT. This was 36.2% and 64.3% higher, respectively, than was obtained with the widely used SCX separation mode. Notably, the identification of both highly hydrophobic and basic peptides increased over 120% using the ERLIC method. The results indicate that ERLIC is a promising alternative to SCX as the first dimension of MDLC. In total, 5499 proteins and 35 847 unique peptides of rat kidney tissue are characterized.

Keywords: ERLIC • SCX • WAX • HILIC • mixed-mode chromatography

Introduction

For some years shotgun proteomics has been the method of choice for analysis of complex samples because of its ease of automation, high throughput, and sensitivity. In part this reflects the recent development of mass spectrometers with high resolving power, mass accuracy, and high scan speed.[1,2] Undersampling and ion suppression effects from coeluting peptides of high abundance are the bottlenecks that prevent successful identification of numerous peptides of low abundance.[3−5] Reducing the complexity of the sample facilitates analysis of the whole proteome and discovery of biomarkers in cell, tissue, and plasma samples. Thus, multidimensional liquid chromatography (MDLC) is generally used to reduce sample complexity and increase dynamic range and sensitivity of peptide identification by minimizing the undersampling and ion suppression problems.[6]

Currently, the most commonly used MDLC is the online or offline combination of strong cation exchange (SCX) with reverse-phase (RP) chromatography that separate peptides orthogonally based on charge and hydrophobicity, respectively.[7−10] Online MDLC has many advantages, such as ease of automatization and minimization of sample loss, and it is especially useful when sample amount is limited. However, offline MDLC is more flexible than online separations with the following advantages: (a) buffers that afford good chromatograpic separations but are not compatible with MS analysis can be used; (b) larger amounts of sample can be processed facilitating identification of peptides of low abundance; (c) each dimension of separation can be optimized separately, which usually results in better resolution of very complex samples.[3,11] To date, RP is used as the last dimension of separation before MS analysis in most laboratories because it desalts and separates peptides in one step, and its buffers are also fully compatible with ESI MS. Therefore, improvement of the performance of MDLC depends primarily on the first dimension of separation.[12]

In recent years, many efforts have been made to develop first-dimensional separations with good orthogonality to RP. Several chromatographic methods, such as size exclusion chromatography (SEC), RP with high-pH elution, and hydrophilic interaction liquid chromatography (HILIC), have been investigated by many researchers, and detailed comparison with SCX-RP has also been done.[13−17] Moreover, OFFGEL isoelectric focusing with immobilized p*I* strips have become very popular as the first-dimensional separation, with many researchers making direct comparisons with SCX-RP.[18−21] The above-mentioned methods may have certain advantages in analyzing different proteomes/subproteomes, such as better sensitivity or repeatability, but none of them have significantly better overall performance than SCX-RP. Similarly, they also separate peptides based on only one property of peptides, such as size, hydrophobicity, and p*I*.[4,19,20,22] Thus, SCX-RP is still the most widely used MDLC in shotgun proteomics.

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Electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) was recently introduced by Alpert for separation of biomolecules and phosphopeptide enrichment.[23] Its ability to enrich phosphopeptides from cell extracts has been validated.[24] It has been extended to the fractionation of N-linked glycopeptides from complex samples.[25] In addition, simultaneous enrichment of glyco- and phospho-proteomes from the same sample has been demonstrated.[26] In an ERLIC separation, at high concentration of organic mobile phase, analytes can still be retained in the aqueous layer around the stationary phase through hydrophilic interaction even if they have the same charge as the stationary phase. The opposing interactions allow the isocratic resolution of complex peptide mixtures.[23] Because both electrostatic interaction and hydrophilic interaction are used in ERLIC separations, both of which show good orthogonality to RP,[3,17] we hypothesize that it has potential to be the first dimension of separation in MDLC. At high concentrations of organic mobile phase, nearly all peptides will be retained on a hydrophilic anion-exchange column, acidic peptides through electrostatic attraction and basic peptides (with the same charge as the stationary phase), and neutral peptides through hydrophilic interaction alone. Thus, we hypothesize that, when the retained peptides are eluted with a shallow gradient of increasing water content and decreasing pH, ionic interaction and hydrophilic interaction will effect a mixed-mode resolution of peptide mixtures based on more than one physical property in a single column. This paper describes our testing of this hypothesis with the proteomic analysis of trypsin-digested rat kidney proteins using ERLIC-RP method. The results were compared with those obtained by the widely used SCX-RP method. The presented method was found to have a significantly better performance than SCX-RP in identifying proteins and peptides from rat kidney tissue, especially highly hydrophobic and basic peptides.

Experimental Section

Sample Preparation and Digestion. Rat kidneys were obtained from adult Sprague−Dawley rats, snap-frozen in liquid nitrogen, and kept at −80 °C until use. The tissue was cut into small pieces, washed with phosphate-buffered saline, and ground into fine powders in liquid nitrogen with a pestle. The powders were then suspended in lysis buffer (8 M urea, 50 mM Tris-HCl, pH 8.0) with protease inhibitor cocktail (P8340, Sigma) and 10 mM PMSF added in the ratio of 1:50 and 1:20 (v/v), respectively. The suspension was sonicated for 10 s thrice on ice and centrifuged at 20 000*g* at 4 °C for 30 min. The protein concentration of the supernatant was then determined by the bicinchoninic acid (BCA) assay. About 20 mg of sample lysate was reduced with 20 mM DTT at 37 °C for 3−4 h and alkylated with 80 mM iodoacetamide for 45 min in the dark. After the concentration of urea was diluted to 1 M with 50 mM NH_4HCO_3, trypsin was added at a ratio of 1:100 (trypsin/sample). It was then incubated at 37 °C for 4 h. For complete digestion, incubation was continued at 37 °C for about 12 h after a second addition of the same amount of trypsin. The obtained tryptic peptides were desalted using a Sep-Pak C18 cartridge (Waters, Milford, MA) and dried in a SpeedVac (Thermo Electron, Waltham, MA).

SCX Separation. Peptides from 2 mg of protein were fractionated using a PolySULFOETHYL A column (4.6 × 200 mm, 5 μm, 200 Å, PolyLC, Columbia, MD) on a Shimadzu Prominence UFLC system in replicates. Forty-six fractions were collected during a 50 min gradient of 100% buffer A (10 mM KH_2PO_4 in 25% acetonitrile (ACN, pH 3.0) for 5 min, 0−5% buffer B (Buffer A with 500 mM KCl, pH 3.0) for 2 min, 5−20% buffer B for 18 min, 20−40% buffer B for 10 min, 40−100% buffer B for 5 min, and 100−0% buffer B for 5 min followed by 5 min at 100% A at a flow rate of 1 mL/min. The fractions were then dried in vacuum, desalted with Sep-Pak C18 cartridges, and redissolved in 0.1% formic acid (FA) for LC-MS/MS analysis.

ERLIC Separation. Peptides from 2 mg of protein were fractionated using a PolyWAX LP anion-exchange column (4.6 × 200 mm, 5 μm, 300 Å, PolyLC, Columbia, MD) on a Shimadzu Prominence UFLC system in replicates. Forty-six fractions were collected with a 140 min gradient of 100% buffer A (90% ACN/0.1% acetic acid, pH 3.6) for 10 min, 0−8% buffer B (30% ACN/0.1% FA, pH 3.0) for 20 min, 8−27% buffer B for 30 min, 27−45% buffer B for 10 min, 45−81% buffer B for 20 min, and 81−100% buffer B for 20 min followed by 30 min at 100% buffer B at a flow rate of 0.5 mL/min. The fractions were then dried in vacuum and redissolved in 0.1% FA for LC-MS/MS analysis.

LC-MS/MS. The fractionated peptides were separated and analyzed on a Shimadzu UFLC system coupled to a LTQ-FT Ultra (Thermo Electron, Bremen, Germany). One-third of the peptides in each fraction were injected into a Zorbax peptide trap column (Agilent, CA) via the autosampler of the Shimadzu UFLC so that they were concentrated and desalted simultaneously. The peptides were separated in a capillary column (200 μm × 10 cm) packed with C18 AQ (5 μm, 300 Å, Michrom BioResources, Auburn, CA). The flow rate was maintained at 500 nL/min. Mobile phase A (0.1% FA in H_2O) and mobile phase B (0.1% formic acid in ACN) were used to establish the 60 min gradient compose of 45 min of 8−35% B, 8 min of 35−50% B and 2 min of 80% B followed by re-equilibrating at 5% B for 5 min. The peptides were then analyzed on LTQ-FT with an ADVANCE CaptiveSpray Source (Michrom BioResources) at an electrospray potential of 1.5 kV. A gas flow of 2, ion transfer tube temperature of 180 °C and collision gas pressure of 0.85 mTorr were used. The LTQ-FT was set to perform data acquisition in the positive ion mode as previously described[24] except that a m/z range of 350−1600 was used in the full MS scan.

Data Analysis. The raw data were first converted into the dta format using the extract_msn (version 4.0) in Bioworks Browser (version 3.3, Thermo Fisher Scientific, Inc.), and then the dta files were converted into Mascot generic file format using an in-house program as described.[27] Intensity values and fragment ion m/z ratios were not manipulated. The IPI rat protein database (version 3.40, 40 381 sequences) and its reversed complement were combined and used for the searches. The database search was performed using an in-house Mascot server (version 2.2.04, Matrix Science, Boston, MA) with precursor mass tolerance of 10 ppm and MS/MS tolerance of 0.8 Da. Two missed cleavage sites of trypsin were allowed. Carbamidomethylation (C) was set as a fixed modification, and oxidation (M), phosphorylation (S, T and Y) and deamidation (N) were set as variable modifications. The obtained peptide/protein list for each fraction was either exported to Microsoft Excel or processed using an in-house script for further analysis. The dta files of peptides of which the Mascot scores were over 20 in each fraction were combined and converted into Mascot generic file format using an in-house program. It was then searched again using Mascot to generate the peptide/protein list for false discovery rates (FDR = 2.0 × decoy_hits/total_hits)

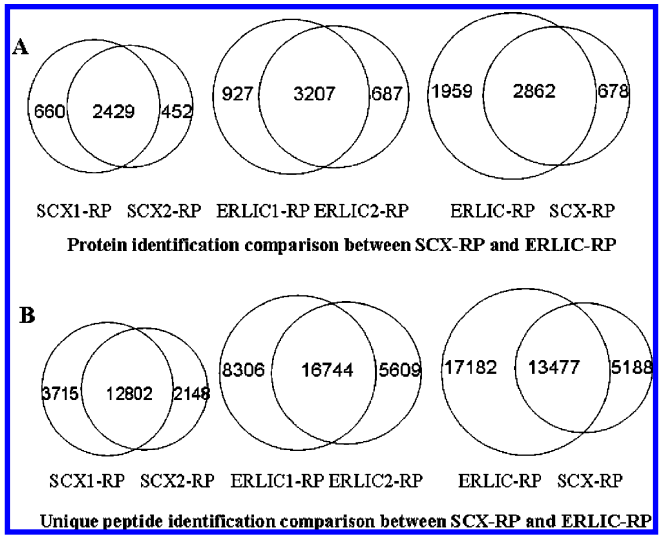

Figure 1. Comparison of protein identification in SCX-RP and ERLIC-RP (A); comparison of peptide identification in SCX-RP and ERLIC-RP (B). SCX1-RP and SCX2-RP and ERLIC1-RP and ERLIC2-RP were replicate runs. The ERLIC-RP and SCX-RP figures are the total number of proteins/peptides identified from both replicate runs.

evaluation. FDRs were evaluated using an in-house script according to the target-decoy strategy as previously described.[28]

The theoretical p*I* values of peptides were calculated based on the algorithm from ENBOSS;[29] peptide GRAVY values were calculated in the same way with the ProtParam tool from Swiss-Prot.[30] The average p*I* and GRAVY values of identified peptides with scores above homologue or identification cutoff scores in each fraction were calculated using an in-house program.

Protein Classification and Functional Annotation. Proteins identified in this study were categorized according to their respective subcellular locations using online Gene Ontology tools.[31]

Results and Discussion

To determine the performance of ERLIC-RP in shotgun proteomics analysis, we comprehensively compared its capability with the widely used SCX-RP method in profiling complex trypsin-digested rat kidney tissue. The protein extract from a higher eukaryote organ is an appropriate sample for testing the separation performance of our fractionation method due to its high complexity and dynamic range. Tryptic peptides were fractionated into multiple fractions in order of decreasing p*I* and polarity when the retained peptides in WAX (weak anion exchange) column are eluted with a shallow gradient of increasing water content and decreasing pH, as indicated by the p*I* and GRAVY value range of peptides identified from ERLIC-RP. ERLIC-RP identified more proteins and peptides than SCX-RP. In addition, many highly basic and hydrophobic peptides were only identified by the ERLIC-RP sequence. Another significant advantage of our method is that desalting after first-dimension fraction is unnecessary because no salts are used in the mobile phases. Desalting is not only labor-intensive, but also results in a certain degree of sample loss.

Protein and Peptide Identifications. Both SCX and ERLIC separations were done in duplicate. The results of protein and peptide identifications are shown in Figure 1 and Supplementary Data 1−4. For SCX-RP, 3540 proteins and 18 665 unique peptides were identified from two replicate analyses, with 2429 proteins and 12 802 unique peptides in common in both analyses. This indicates good repeatability at both protein and peptide levels. The two replicates of ERLIC-RP also showed similarly good repeatability at both levels, as revealed by the LC (first dimension) and LC-MS/MS base peak (second dimension) chromatograms of the 46 fractions of ERLIC and SCX separations (Supplementary Data 5 and 6). The numbers of proteins and unique peptides identified from two replicates were 36.2% (4821 versus 3540) and 64.3% (30 659 versus 18 665) higher, respectively, than with SCX-RP. The results from Mascot MS/MS ion search of both ERLIC-RP and SCX-RP experiments are shown in Supplementary Data 3−4, respectively. To our knowledge, the number of proteins identified from the two ERLIC-RP replicate runs is the largest one reported for rat kidney tissue, which indicates the power of ERLIC separation as the first dimension of MDLC. The good repeatability between replicate analyses could facilitate future comparative analysis of complex samples under different conditions. In a comparison of ERLIC-RP and SCX-RP identifications, there was an overlap of 2862 proteins (52.0%) and 13 477 unique peptides (37.6%). In total, 5499 proteins and 35 847 unique peptides of rat kidney tissue are characterized by a combination of ERLIC-RP and SCX-RP. This indicates that although ERLIC-RP has much better performance, the two fractionation methods complement each other to some extent. Presumably this is because they separate peptides based on different properties.

Separation Efficiency of SCX and ERLIC. The separation efficiency of peptides by SCX and ERLIC were assessed by measuring the overlap of unique peptides across the 46 fractions. The number of unique peptides identified in each fraction and its overlap with other fractions was calculated using an in-house program and used to generate a 3-D column chart with Microsoft Excel (Supplementary Data 7). For SCX separation, a shallow gradient was used in order to minimize the clustering of similarly charged peptides since it separates peptides based mainly on their charges.[17] The unique peptides identified in each fraction had little overlap with the adjacent several fractions as shown in Supplementary Data 7, indicating that the separation was efficient. However, most peptides were identified in fractions 13−37, while less peptides were identified in other fractions. As most tryptic peptides carried two, three or four positive charges, and SCX fractioned peptides based on the cationic interaction with the stationary phase, then tryptic peptides tend to elute in SCX in clusters. For ERLIC, mixed-mode separation of peptides was achieved due to the combination of ion-exchange chromatography (IEX) and HILIC. Consequently, it significantly reduced the clustering of peptides with any single property in common, and peptides were distributed more evenly among the fractions than was the case with SCX. As shown in Supplementary Data 7, the unique peptides identified in each fraction of ERLIC also had only slight overlap with adjacent fractions, indicating an efficient separation.

The p*I* and GRAVY Value Distribution of Peptides. The average number of unique peptides identified in each fraction is illustrated in Figure 2A for SCX and ERLIC. Compared with SCX, more peptides were identified in most fractions of ERLIC. This probably reflects the ability of ERLIC to separate peptides more uniformly into different fractions, reducing sample complexity. The average p*I* and GRAVY values of all peptides in each fraction are illustrated for SCX and ERLIC in Figure 2B and 2C, respectively. For ERLIC, the ranges of average p*I* and GRAVY values are significantly wider than those for SCX, with both of them declining gradually as the fractions progress.

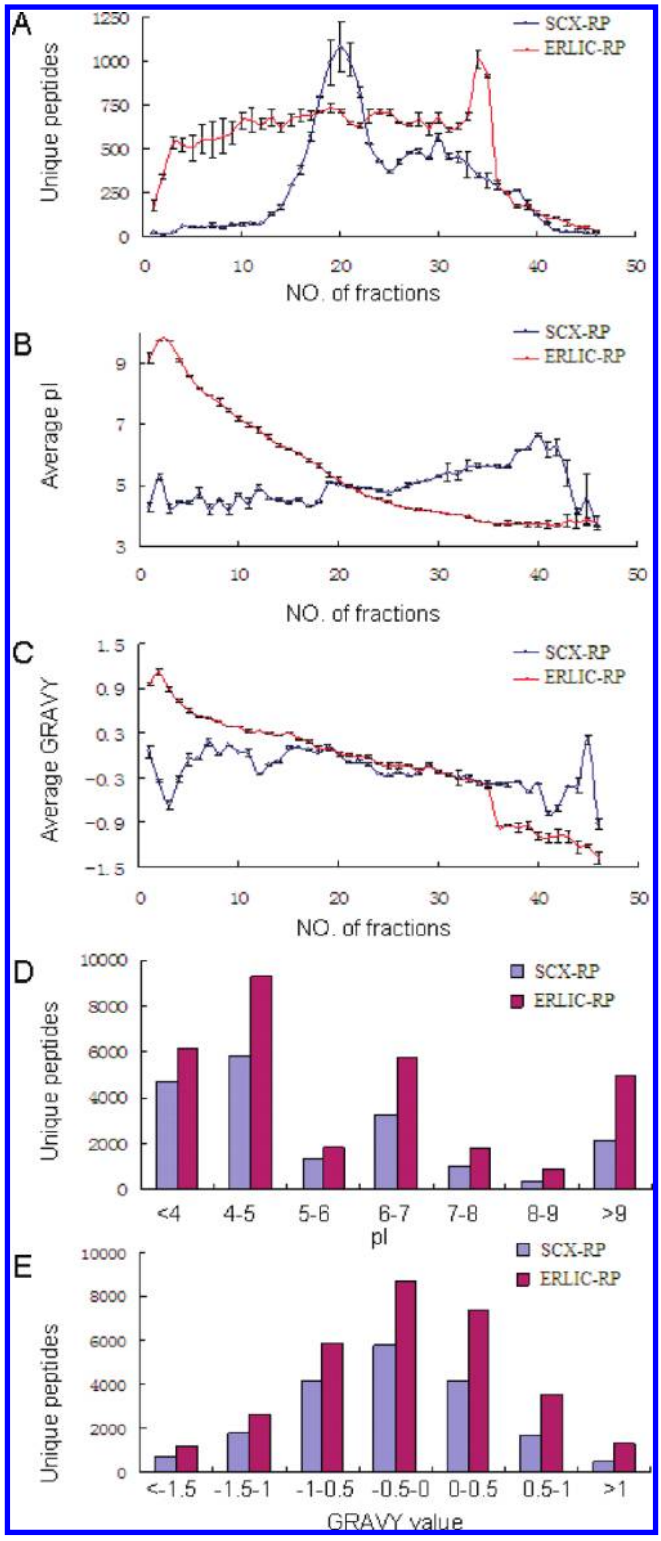

Figure 2. Peptide analysis. Number of unique peptides identified in each fraction of SCX-RP and ERLIC-RP (A); the average p*I* (B) and GRAVY value (C) of peptides identified in each fraction of SCX-RP and ERLIC-RP; distribution of unique peptides according to their p*I* (D) and GRAVY value (E). The labeled standard deviation bars span differences between replicate runs.

There is no such tendency for SCX regarding the GRAVY value and only limited gradual increase in average p*I* value. IEX separates peptides based on their charge. In ERLIC, both the charge and polarity contribute simultaneously to the peptide

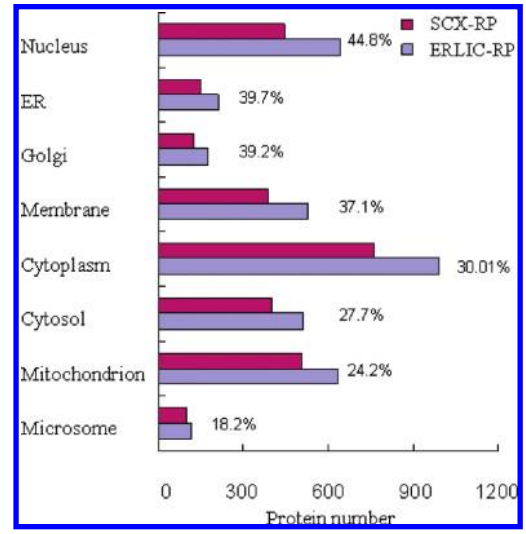

Figure 3. Comparison of Gene ontology annotations of identified proteins in SCX-RP and ERLIC-RP according to cellular compartment. The percentage indicates the increase of proteins identified in ERLIC-RP over SCX-RP in each subcellular location.

retention and overall separation. In this study, our optimized gradient and the proper choice of loading and elution solvents are the key to the separation of peptides based on the dual properties of charge and polarity. Acetic acid was chosen as the electrolyte in the starting mobile phase because it is a weak displacing agent (being only ~5% dissociated in water) and because its solution has a pH high enough for the carboxyl groups in peptides to have some degree of negative charge. Both factors promote retention of tryptic peptides on an anion-exchange material. The formic acid used in the second mobile phase is sufficiently acidic to uncharge most carboxyl groups, promoting elution. Because the pH of our gradient for ERLIC starts at a pH high enough for carboxyl groups to have some degree of ionization and is decreased slowly thereafter, peptides are separated into each fraction according to their p*I*. By contrast, performance of SCX at pH 3 or less leads to separation of peptides largely on the basis of the absolute number of their basic residues rather than the overall content of ionizable residues. That said, elution using pH steps has also been used with SCX columns to achieve the separation of peptides according to their p*I*.[32]

To investigate the differences in the properties of identified peptides from SCX-RP and ERLIC-RP, their distribution in specific ranges of p*I* and GRAVY values was statistically analyzed. As shown in Figure 2D and 2E, ERLIC-RP identified more peptides than SCX-RP in every p*I* and GRAVY range. The disparity was greatest with peptides that were quite basic or hydrophobic; appreciably more of these were identified in ERLIC-RP. For example, 4940 unique peptides with p*I* over 9 were identified in ERLIC-RP, which was 128.3% higher than with SCX-RP. Their relative representation in ERLIC-RP was also 39.0% higher than with SCX-RP (16.1% versus 11.6%). Similarly, the number of unique peptides with GRAVY value over 0.5 in ERLIC-RP was also 127.0% higher than with SCX-RP, and their relative representation in ERLIC-RP was also 38.2% higher than with SCX-RP (15.9% versus 11.5%). This suggests that ERLIC-RP can be used efficiently in the analysis of membrane proteins with high hydrophobicity, basic p*I*, and low abundance.[33] It is plausible that the high concentration of organic solvent in the loading buffer is helpful in the solvation

of hydrophobic peptides. It should be noted that Liu et al. have demonstrated the presence of a small number of peptides in complex tryptic digests that are not eluted from an SCX column with a salt gradient but are eluted by a gradient to 100% propanol.[34] Presumably this subset of peptides elutes with all the others in ERLIC. However, loading samples at high concentration of organic solvents also has some obvious disadvantages. For example, some highly hydrophilic peptides may become insoluble in high concentrations of organic solvents,[35] and then they will not be identified by ERLIC. In fact, in our studies, we did observe some white precipitate when peptide samples were dissolved in the loading solvent. Although this may have resulted in a failure to identify some highly hydrophilic peptides, ERLIC-RP still identified many more proteins and unique peptides than did SCX-RP. Of course, the set of precipitated peptides is an extra fraction that can be analyzed together with the ERLIC fractions. Despite the fact that both *N*-glycopeptides and phosphopeptides can be enriched simultaneously with ERLIC chromatography with 70%ACN, 2%FA sample loading buffer, surprisingly, we did not identify many *N*-glycopeptides and phosphopeptides with this fractionation method with 90% ACN and 0.1% acetic acid as sample loading buffer. We hypothesize that these hydrophilic modified peptides may precipitate at 90% of ACN. It should be noted that our objective was to assess the performance of ERLIC with tryptic peptides in general. Our greatest concern was to ensure that all peptides in a digest were retained. Accordingly, we started with a very high level of ACN (90%). Other studies of HILIC of tryptic digests did not report solubility problems when dissolving the peptides in 70% ACN[35] [but running % ACN not reported] or running at 80% ACN.[16] Further experimentation should identify a level of organic solvent that suffices for retention of all tryptic peptides in ERLIC without precipitating some of them.

Protein Classification and Functional Annotation. To investigate whether the proteins identified in ERLIC-RP differ significantly in subcellullar location from those identified in SCX-RP, they were categorized independently. As shown in Figure 3, ERLIC-RP identified more proteins than SCX-RP in each listed cell component, with the greatest disparity involving locations rich in membrane proteins: nucleus, ER, Golgi, and membrane (44.8%, 39.7%, 39.2%, and 37.1% higher, respectively, than with SCX-RP).

Mechanism of Peptide Separations in ERLIC. The gradient shape and solvents were optimized to afford adequate retention of most tryptic peptides. Their distribution among the fractions was unusually uniform. The gradients to lower pH and higher water content both serve to promote elution, through increasing electrostatic repulsion and decreasing hydrophilic interaction. Peptides are separated on the basis of both p*I* and polarity. This decreases the chances that peptides similar in any one of those properties will coelute. That may be an argument favoring more widespread applications of mixed-mode separations like this one; a frequent complaint about fractionation of tryptic digests by SCX is the clustering of peptides with the same charge. If either electrostatic interaction or hydrophilic interaction is too strong with a particular gradient, then the selectivity for one property or the other will be perturbed. Presumably, the combined selectivity for both properties explains why ERLIC-RP identifies many more proteins and unique peptides than SCX-RP. In addition, both the IEX and HILIC modes operating in ERLIC have good orthogonality to RP,[3,17] so the overall orthogonality should be excellent. This is reflected in the identification of many unique peptides in each fraction of ERLIC.

Gilar et al. noted that elution of tryptic peptides in HILIC is generally in order of least to most basic.[35] The trend resembled that of SCX but with the superimposed hydrophilic interaction increasing the overlap between sets of peptides of different charge. Now, that study involved an uncoated silica column at pH 4.5. At that pH, there would be significant negative charge from the silanol groups, which could account in part for the increase in retention with basicity of the peptides. However, the same trend was noted by Boersema et al. using a ZIC-HILIC column at pH 3.[16] Basic residues are the most hydrophilic ones and ordinarily are the most important in promoting retention in HILIC whatever the column used.[36] ERLIC is a special case; the electrostatic component selectively antagonizes the retention due to the basic residues. This tunes down their contribution to retention overall. Whether the net result is retention or repulsion is determined by the balance of the two forces, as described in ref 23. At 70% ACN or less, the electrostatic repulsion is more significant than the hydrophilic interaction, and peptides with a net positive charge (which includes most tryptic peptides at pH 3.6 or less) will be repelled by the PolyWAX LP stationary phase. This was exploited previously to separate nonphosphopeptides from phosphopeptides, which retain negative charge at low pH. Since the objective here was the retention of all peptides in a tryptic digest, a concentration of ACN was used that was high enough (90%) to promote hydrophilic interaction sufficient for retention of basic peptides despite the electrostatic repulsion, a typical combination of forces in ERLIC. The electrostatic repulsion still influences selectivity under these conditions, as is evident from the fact that the most basic peptides eluted earliest (Figure 2B), the opposite of the trend noted above with regular HILIC.

Comparison of ERLIC-RP with Previously Reported Mixed-Mode Chromatography. In an excellent study by Ning et al.,[37] intact proteins were separated using a combination of SCX and RP columns, and 1933 proteins were identified over a wide dynamic range because of good resolution with a simultaneous gradient of increasing salt concentration and pH in the SCX step. Separating solutes in a single run based on two different properties of the solutes is generally termed mixed-mode chromatography (MMC). Because the final objects of analysis in LC-MS/MS are peptides, then separation at the peptide level is critical.[12] Recently, SCX/RP MMC was used in fractionating mixtures of standard peptides, phosphopeptides, and sialylated glycopeptides.[38] HILIC/CEX (cation exchange chromatography) has also been used in separation of cyclic peptides and modified products of synthetic peptides.[39,40] However, MMC procedures have not been widely utilized in proteomic research yet because of the following factors: (1) until now, none of them have been validated with real complex samples; (2) convenient methods for manipulation of two modes simultaneously have not always accompanied the reports; (3) some of them involve the use of very complex gradients using three or more buffers for elution, which prevents them from being employed by other laboratories without advanced HPLC equipment; (4) the desalting of the eluted peptides is time-consuming and arduous and may also result in some degree of sample loss. In contrast, in this study of the application of ERLIC for analysis of a whole proteome, only two solvents without salts were used along with a commercially available column, that is, PolyWAX LP. The

avoidance of sample loss from desalting may also be a reason why more proteins and unique peptides are identified in ERLIC-RP than with SCX-RP. All of the above-mentioned features suggest that ERLIC is superior to previously reported MMC combinations for proteomics applications.

Conclusions

We have presented here a peptide fractionation method based on ERLIC. The ERLIC conditions have been modified to permit the retention and fractionation of nearly all the peptides in the tryptic digest of a whole organ. For the first time, a gradient without salts was introduced to implement the separation of peptides by ion-exchange and hydrophilic interaction in a mixed-mode fashion with one column. Peptides were then distributed into multiple fractions based on both p*I* and polarity. Compared with the widely used SCX-RP sequence, the ERLIC-RP sequence identified significantly higher numbers of proteins and unique peptides from rat kidney tissue. Interestingly, many more basic and hydrophobic peptides were identified in this method than in SCX-RP, which is encouraging for potential use in the analysis of membrane proteins. As previously reported MMCs have mostly been used in the separation of peptides with only slight compositional differences and for pharmaceutical compounds because of their outstanding separation power, the newly introduced ERLIC might also be extended to similar applications.

Abbreviations: MDLC, multidimensional liquid chromatography; ERLIC, electrostatic repulsion-hydrophilic interaction chromatography; SCX, strong cation exchange; RP, reverse-phase; ACN, acetonitrile; WAX, weak anion exchange; HILIC, hydrophilic interaction liquid chromatography; FA, formic acid; IEX, ion-exchange chromatography; MMC, mixed-mode chromatography.

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Supporting Information Available: The complete list of proteins and peptides identified in ERLIC-RP and SCX-RP (Supplementary Data 1 Proteins Identified in ERLIC-RP with FDR.xls, Supplementary Data 2 Peptides Identified in ERLIC-RP with FDR.xlsx, Supplementary Data 3 Proteins Identified in SCX-RP with FDR.xls, Supplementary Data 4 Peptides Identified in SCX-RP with FDR.xlsx). The ERLIC and LC-MS/MS base peak chromatograms of 46 fractions of the two ERLIC-RP replicates (Supplementary Data 5 ERLIC 2 Replicates (ERLIC and LC-MS Chromatograms).pdf). The SCX and LC-MS base peak chromatograms of 46 fractions of the two SCX-RP replicates (Supplementary Data 6 SCX 2 Replicates (SCX and LC-MS Chromatograms).pdf). The data and figures showing the ERLIC and SCX separation efficiency by measuring overlap of unique peptides across the 46 fractions (Supplementary Data 7 Overlap of Unique Peptide Across All Fractions.xls). This material is available free of charge via the Internet at http://pubs.acs.org.

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