Anal. Chem. **2010,** *82,* 53–56

Letters to *Analytical Chemistry*

Reversed-Phase-Reversed-Phase Liquid Chromatography Approach with High Orthogonality for Multidimensional Separation of Phosphopeptides

Chunxia Song,[†,‡] Mingliang Ye,[*,†] Guanghui Han,[†,‡] Xinning Jiang,[†,‡] Fangjun Wang,[†,‡] Zhiyuan Yu,[†,‡] Rui Chen,[†,‡] and Hanfa Zou[*,†]

CAS Key Laboratory of Separation Sciences for Analytical Chemistry, National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China, and Graduate School of Chinese Academy of Science, Beijing 100049, China

Protein phosphorylation regulates a series of important biological processes in eukaryotes. However, the phosphorylation sites found up to now are far below than that actually exists in proteins due to the extreme complexity of the proteome sample. Here a new reversed-phase-reversed-phase liquid chromatography (RP-RPLC) approach was developed for multidimensional separation of phosphopeptides. In this approach, a large number of fractions were collected from the first dimensional RPLC separation at high pH. And then these fractions were pooled every two fractions with equal time interval, one from the early eluted section and another one from the later eluted section. The pooled fractions were finally submitted to RPLC−tandem mass spectrometry (MS/MS) analysis at low pH. It was found the resulting 2D separation was highly orthogonal and yielded more than 30% phosphopeptide identifications over the conventional RP-RPLC approach. This study provides a powerful approach for efficient separation of phosphopeptides and global phosphorylation analysis, where the orthogonality of 2D separation is greatly improved and the first dimensional separation is of high resolution.

Protein phosphorylation is one of the most important post-translational modifications in eukaryotes, which regulates essential functions such as cell division, signal transduction, and so forth. It is estimated that one-third of all proteins in a cell are phosphorylated at any given time.[1] However, compared with a large number of phosphorylated proteins in the entire eukaryotic cells, the actual number of phosphorylation sites found so far is too insignificant.[2] The greatest challenge in the phosphorylation analysis results from its highly dynamic nature and low abundance. Shot-gun proteomics has been successfully applied to map the phosphoproteome. In this approach, proteins are first digested by trypsin and then the phosphopeptides in the resulting digest are enriched and analyzed by reversed-phase liquid chromatography−tandem mass spectrometry (RPLC−MS/MS). Although many enrichment strategies, such as immobilized metal ion affinity chromatography (IMAC),[3] titanium[4] and zirconium oxides,[5] have proven successful in enriching the phosphopeptides, the mixture of phosphopeptides enriched from proteome samples are still very complex. Therefore, multidimensional separation of phosphopeptides is required to further reduce the sample complexity and increase the phosphoproteome analysis coverage.

Fractionation of peptides by strong cation exchange (SCX) chromatography followed by IMAC enrichment and RPLC−MS/MS analysis of phosphopeptides has been developed for large-scale analysis of the phosphoproteome by Gygi et al.,[6,7] and more than 5 500 and over 13 000 phosphorylation sites were identified in mouse liver and fly embryos, respectively. However, fractionation of phosphopeptides by SCX will lead to loss of some phosphopeptides due to their weak interaction with SCX resins. The salt-containing buffers applied to elute phosphopeptides from the SCX column are also not well compatible with the next dimensional RPLC−MS/MS analysis. Another important disadvantage of SCX is its relatively poor resolution. To overcome these limitations, HILIC was recently applied to fractionate phospho-

* To whom correspondence should be addressed. Prof. Hanfa Zou: phone, +86-411-84379610; fax, +86-411-84379620; e-mail, hanfazou@dicp.ac.cn. Prof. Mingliang Ye: phone, +86-411-84379620; fax, +86-411-84379620; e-mail, mingliang@dicp.ac.cn.

† CAS Key Laboratory and National Chromatographic R&A Center.

‡ Graduate School of Chinese Academy of Sciences.

(1) Witze, E. S.; Old, W. M.; Resing, K. A.; Ahn, N. G. *Nat. Methods* **2007**, *4*, 798–806.

(2) Steen, H.; Jebanathirajah, J. A.; Rush, J.; Morrice, N.; Kirschner, M. W. *Mol. Cell. Proteomics* **2006**, *5*, 172–181.

(3) Andersson, L.; Porath, J. *Anal. Biochem.* **1986**, *154*, 250–254.

(4) Pinkse, M. W. H.; Uitto, P. M.; Hilhorst, M. J.; Ooms, B.; Heck, A. J. R. *Anal. Chem.* **2004**, *76*, 3935–3943.

(5) Zhou, H. J.; Tian, R. J.; Ye, M. L.; Xu, S. Y.; Feng, S.; Pan, C. S.; Jiang, X. G.; Li, X.; Zou, H. F. *Electrophoresis* **2007**, *28*, 2201–2215.

(6) Villen, J.; Beausoleil, S. A.; Gerber, S. A.; Gygi, S. P. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 1488–1493.

(7) Zhai, B.; Villen, J.; Beausoleil, S. A.; Mintseris, J.; Gygi, S. P. *J. Proteome Res.* **2008**, *7*, 1675–1682.

10.1021/ac9023044
Published on Web 12/01/2009

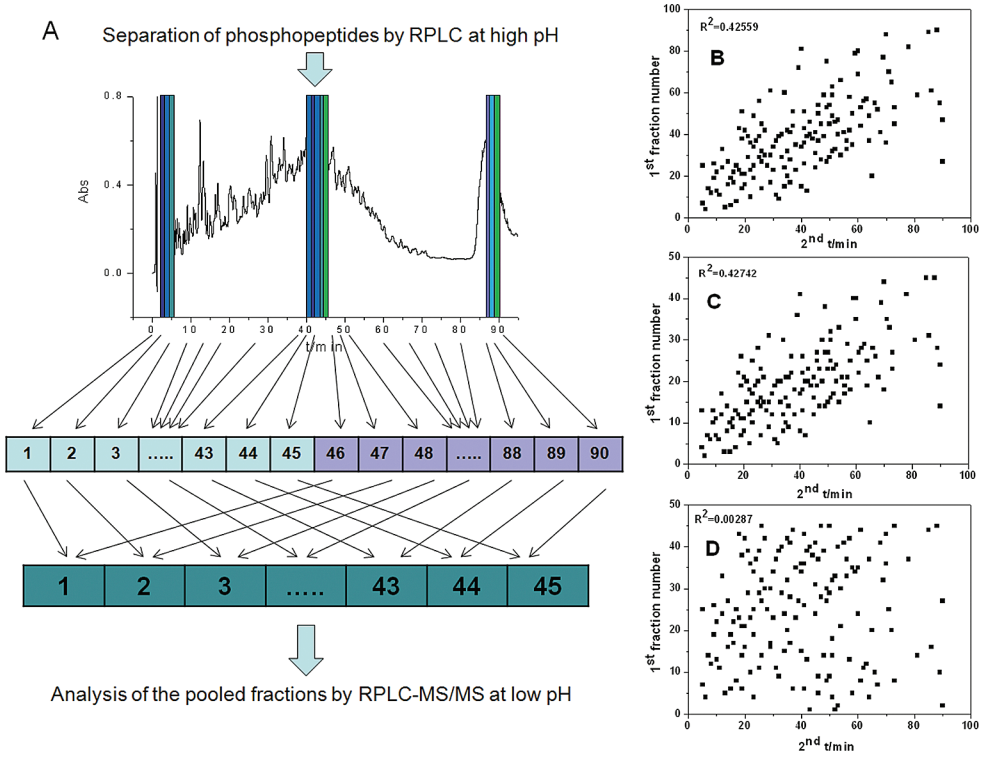

Figure 1. The new RP-RPLC approach with high orthogonality for separation of phosphopeptides. (A) The scheme for fractionation of phosphopeptides in the new approach; (B) 2D retention plots for a hypothetical 2D separation of peptides, with 90 fractions; (C) reducing fraction number by pooling adjacent fractions; and (D) reducing fraction number by pooling equal interval fractions.

peptides and a higher coverage of the phosphoproteome compared with SCX fractionation was obtained.[8,9]

Among all common liquid chromatography modes, RPLC has the highest resolution and could provide the greatest peak capacity.[10] The orthogonality of the RP-RPLC system using mobile phases with different pH values for separation of nonphosphopeptides was evaluated by Gilar et al.,[10,11] and the good performance of this approach for shotgun proteome analysis was also demonstrated by several laboratories.[12,13] However, the nature of phosphopeptides is different from nonphosphopeptides, and the potential of this system for global phosphorylation analysis has not been explored so far. In this work, we evaluated the orthogonality of RP-RPLC separation under different pH for phosphopeptides analysis. It was found the selectivity for RPLC separation of phosphopeptides was significantly changed by changing the pH value. However, the orthogonality of the RP-RPLC separation was not perfect. In order to further improve the orthogonality of the 2D separation, a new RP-RPLC approach was presented as the scheme shown in Figure 1A. It was demonstrated that this new RP-RPLC strategy had superior performance for in-depth phosphoproteome analysis.

EXPERIMENTAL SECTION

Immobilized Metal-Affinity Chromatography (IMAC) and First-Dimensional RPLC Separation. Adult female C57 mice were purchased from Dalian Medical University (Dalian, China). The procedures for preparation of mouse liver lysate and tryptic digestion were performed as previously reported by Zhou et al.[5] The phosphopeptides were enriched from the digest of mouse liver lysate by Ti^{4+}-IMAC microspheres following the protocol described by Yu et al.[14] The first dimensional separation was carried out on a Waters Acquity UPLC system with a photodiode array detector (Waters, Milford, MA) using a 250 mm × 2.1 mm Hypersil GOLD aQ 5 μm C18 column (Thermo, CA). Phosphopeptides enriched from the digest of 8 mg of mouse liver proteins were redissolved in 40 μL of mobile phase A (25 mM ammonium formate (NH_4FA) aqueous buffer, pH 7.5). Mobile phase B was 25 mM NH_4FA in water/acetonitrile (1:9). The gradient elution was performed by 0−10% B (0−80 min) and 10−35% B (80−90 min), and fractions were collected every 1 min. The fractions were pooled as required.

Nanoflow RPLC−MS/MS Analysis and Database Search. The RPLC−MS/MS system consisted of a quaternary surveyor MS pump (Thermo Finnigan, CA) and a LTQ linear IT mass spectrometer (Thermo, CA) with a nanospray source. The capillary separation column was in-house packed with C18 aQ beads (5 μm, 120 Å) from Michrom BioResources (Auburn, CA) to 120 mm length. The lyophilized fractions from the first dimension were resuspended in 10 μL of 0.1% FA solution, and 2 μL of the

(8) Albuquerque, C. P.; Smolka, M. B.; Payne, S. H.; Bafna, V.; Eng, J.; Zhou, H. L. *Mol. Cell. Proteomics* **2008**, *7*, 1389–1396.
(9) McNulty, D. E.; Annan, R. S. *Mol. Cell. Proteomics* **2008**, *7*, 971–980.
(10) Gilar, M.; Olivova, P.; Daly, A. E.; Gebler, J. C. *J. Sep. Sci.* **2005**, *28*, 1694–1703.
(11) Gilar, M.; Olivova, P.; Daly, A. E.; Gebler, J. C. *Anal. Chem.* **2005**, *77*, 6426–6434.
(12) Delmotte, N.; Lasaosa, M.; Tholey, A.; Heinzle, E.; Huber, C. G. *J. Proteome Res.* **2007**, *6*, 4363–4373.
(13) Dowell, J. A.; Frost, D. C.; Zhang, J.; Li, L. J. *Anal. Chem.* **2008**, *80*, 6715–6723.
(14) Yu, Z. Y.; Han, G. H.; Sun, S. T.; Jiang, X. N.; Chen, R.; Wang, F. J.; Wu, R. A.; Ye, M. L.; Zou, H. F. *Anal. Chim. Acta* **2009**, *636*, 34–41.

sample were then manually loaded onto the column. The mobile phase A was 0.1% FA in water and B was 0.1% FA in acetonitrile; gradient elution was performed by 3−25% B in 60 min with flow rate ~200 nL/min. The mass spectrometer was set that one full MS scan was followed by three MS^2 scans and three neutral loss MS^3 scans; the more detailed protocol was the same as that described by Han et al.[15]

The MS^2 and MS^3 spectra were searched using SEQUEST (version 0.27) against a database containing the mouse IPI protein database (version 3.21) and its reversed complement. For phosphopeptides identification by matching the assigned sequences derived from MS^2 and MS^3 data, a homemade software named APIVASE (automatic phosphopeptide identification validating algorithm for Sequest) was applied to validate the identifications as reported by Jiang et al.[16] In this study, to achieve false discovery rate (FDR) < 1%, cutoff filters such as Rank'm, ΔCn'm, and Xcorr's were used to filter the data.

RESULTS AND DISCUSSION

Orthogonality of Conventional RP-RPLC System for Separation of Phosphopeptides. The performance of phosphopeptides separation in the RP-RPLC system using different pH was investigated. Mobile phase containing 25 mM NH_4FA (pH 7.5) was adopted in the first dimensional separation, and mobile phase containing 0.1% FA (pH 2.0) was adopted in the second dimensional separation which was coupled directly with mass spectrometer. Phosphopeptides enriched from the tryptic digest of mouse liver lysate were used as the test sample. The enriched phosphopeptides were separated by a C18 column (250 mm × 2.1 mm) at pH 7.5 over a 90 min gradient elution. The fractions were then collected every 1 min interval for capillary RPLC−MS/MS analysis with mobile phase at a low pH of 2.0. Typical base peak chromatograms by RPLC−MS/MS analysis of early eluted and later eluted phosphopeptide fractions (fractions 38 and 68) from the first dimensional separation are given in Figures S1A and S1B in the Supporting Information. The elution windows of the 1 min fractions from the first dimension were increased to 30−40 min in the second dimension during 60 min LC−MS/MS analysis, which indicated the two RPLC separations under different pH values had good orthogonality.

However, when the chromatograms are looked at more closely, it can be found that the distributions of phosphopeptide peaks are not even across the entire separation window of RPLC−MS/MS analysis. There are fewer peaks in the later region of the separation window for RPLC−MS/MS analysis of the early eluted fraction, while there are fewer peaks in the early region for the later eluted fraction. The correlation of retention time for RP-RPLC separation of nonphosphopeptides was also observed by Gilar et al.[10] This means the 2D separation scheme is not fully orthogonal, and there is space to be further improved.

New RP-RPLC Approach with High Orthogonality for Separation of Phosphopeptides. The frequency of fraction collection is an important issue for 2D separation. To maintain the resolution of the first dimensional separation at a maximum extent, collection of two or three fractions per first dimensional peak is recommended.[17] The peak capacity over 100 could be easily obtained for RPLC because of its high resolution, which means several hundreds of fractions should be collected for the second dimensional separation. However, in a typical experiment for proteome/phosphoproteome analysis, the number of collected fractions is often limited due to the limitation of the overall analysis time. Less than 40 fractions were often collected, and the collected fractions were then directly submitted to the second dimensional separation. In a chromatographic system with highly efficient separation performance, a less frequent fraction collection will certainly undermine the original resolution of this separation method.[11] As the fraction number required for phosphoproteome analysis is much fewer than the first dimensional RPLC could provide, reducing the number of fractions by selective pooling of short-time-interval fractions may provide a solution to improve the orthogonality of the RP-RPLC system.

It can be seen from Figures S1A and S1B in the Supporting Information that the phosphopeptide peaks cluster to different regions of the separation window for the early and later eluted fractions. However, when these two 1 min fractions were pooled into one fraction, it was found the phosphopeptides almost randomly appeared across the entire separation window as the base peak chromatogram shown in Figure S1C in the Supporting Information. On the basis of the above observations, a new approach to increase the orthogonality of the RP-RPLC system was proposed as the schematics shown in Figure 1A. In this approach, a large number of fractions, for example, 90 fractions, were collected in the first dimensional separation. These fractions were divided into two groups: the early eluted group (fractions 1−45) and the later eluted group (fractions 46−90). Then every two fractions from each group with equal time interval (fractions 1 and 46, 2 and 47, and so on) were mixed for the second dimensional separation. Therefore, only half the number of fractions (45) was submitted to LC−MS/MS analysis at last. To demonstrate the effectiveness of this approach to improve the orthogonality of the 2D separation, the 2D maps for the hypothetical RP-RPLC separation of peptides are given in Figure 1B−D. We assume the separations in both dimensions are of high resolution. The distribution of hypothetic peptides in Figure 1B was based on a real study for RP-RPLC separation of peptides.[11] The correlation of the retention time for the two dimensions is obvious, and the orthogonality is not perfect. In the case of Figure 1B, there are too many fractions in the first dimension for proteome analysis. Two ways could be used to reduce the fraction number. The conventional way is to use a wider collection window which in fact is to pool adjacent fractions together, and the new way we proposed in this work is to pick one fraction from the early eluted group and later eluted group, respectively, and then pool them together as shown in Figure 1A. When every two fractions in Figure 1B were pooled by the above two methods, the corresponding theoretical 2D separation profiles were given in parts C and D of Figure 1. It is obvious that the new way can lead to a separation of peptides with much higher orthogonality

(15) Han, G. H.; Ye, M. L.; Jiang, X. N.; Chen, R.; Ren, J.; Xue, Y.; Wang, F. J.; Song, C. X.; Yao, X. B.; Zou, H. F. *Anal. Chem.* **2009**, *81*, 5794–5805.
(16) Jiang, X. N.; Han, G. H.; Feng, S.; Jiang, X. G.; Ye, M. L.; Yao, X. B.; Zou, H. F. *J. Proteome Res.* **2008**, *7*, 1640–1649.
(17) Murphy, R. E.; Schure, M. R.; Foley, J. P. *Anal. Chem.* **1998**, *70*, 1585–1594.

as more separation space is filled and the distribution of peptides is more random.

A key question is whether this new RP-RPLC approach is superior to the conventional method for phosphoproteome analysis. To address this question, we examined the different performance of the conventional and the new RP-RPLC approaches. Six 1 min fractions were collected from the early eluted section (from 9 to 14 min), and another six 1 min fractions were collected from the later eluted section (from 54 to 59 min) in the first dimension. For all the twelve fractions, half of each fraction was mixed by pooling two adjacent fractions (fractions 9 with 10, 11 with 12, and so on) together, which resulted in six new fractions. These fractions in fact were collected by a 2 min window and could be considered as obtained by the conventional method. Another six new fractions were generated by the new approach as following. The left half of the twelve collected fractions were mixed by pooling one fraction from the early eluted section and another fraction from the later eluted section with equal time interval (fractions 9 with 54, 10 with 55, and so on). All the new fractions obtained by the above two approaches were submitted to the RPLC−MS/MS analysis under identical conditions. It was found that 33% more (487 versus 365) unique phosphopeptides were identified from the fractions obtained by the new approach compared with that obtained by the conventional approach (Table S1 in the Supporting Information). Though a lower overlap of phosphopeptides between neighboring fractions (Figure S2 in the Supporting Information) was achieved for the conventional approach because of a wider collection window, the number of phosphopeptides identified was still much less than that of the new approach. The significant increase in the number of identified phosphopeptides is due to the improved orthogonality of the 2D separation.

The ion-suppression effect is an important factor affecting the sensitivity of MS detection. The chance for coelution of phosphopeptides is much lower in the new RP-RPLC approach because the distribution of phosphopeptides is more random across the chromatogram during RPLC−MS/MS analysis. Therefore, the ion-suppression effect is less serious and higher detection sensitivity could be achieved with the new approach. By comparison of the mass spectra for the phosphopeptides identified in both approaches, the mass spectra acquired with the new approach were often of higher quality. For example, the higher quality of the MS/MS spectrum of the triply phosphorylated peptide RpSpSSELpSPEVVEK was acquired with the new approach, which resulted in the identification of this phosphopeptide with higher score (Figure S3 in the Supporting Information). The poor quality of mass spectra acquired with the conventional approach probably resulted from the interference of coeluted phosphopeptides. Above observations clearly demonstrated the super performance of the new RP-RPLC approach for large-scale phosphoproteome analysis.

CONCLUSIONS

In this study, a new RP-RPLC approach was proposed for multidimensional separation of phosphopeptides. By combination, the high resolution of RPLC with a new fractionation format, the orthogonality of the two-dimensional separations was greatly improved in this approach. The suitability of this new approach for large-scale phosphorylation analysis was demonstrated. Recently, we are applying this approach to analyze human liver phosphoproteome which has identified about 8000 phosphorylation sites up to now (controlling FDR <1% on phosphopeptide identification). Though the experiments are not finished yet, it has already become the largest data set for human liver phosphoproteome.

A conventional notion for multidimensional separation is that highly efficient separation can only be obtained when the separations in each dimension are of high orthogonality. However, this notion is not always true. This study provides a solution to increase the orthogonality of 2D separation where the two separations are only partially orthogonal and the first dimensional separation is of high resolution. The proof-of-principle example shown in this study only pooled two fractions with different ways. If the peak capacity of the first dimensional separation is very high and only a few fractions are needed for second dimensional separation, more fractions could be pooled to further increase the orthogonality of the 2D separation.

ACKNOWLEDGMENT

Financial support is gratefully acknowledged from the National Natural Sciences Foundation of China (Grants 20675081 and 20735004), the China State Key Basic Research Program (Grants 2005CB522701 and 2007CB914102), the China High Technology Research Program (Grants 2006AA02A309 and 2008ZX10002-017), the Knowledge Innovation program of CAS (Grants KJCX2.YW.HO9 and KSCX2-YW-R-079), and the Knowledge Innovation program of DICP to H. Zou and the China High Technology Research Program (Grants 2008ZX1002-020) and the National Natural Sciences Foundation of China (Grants 20605022 and 90713017) to M. Ye.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review October 13, 2009. Accepted November 15, 2009.

AC9023044