Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography A, 1047 (2004) 221–227
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Optimization of diagonal chromatography for recognizing post-translational modifications

Peiran Liu, Christa L. Feasley, Fred E. Regnier*

Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA

Received 5 January 2004; received in revised form 26 April 2004; accepted 22 June 2004

Abstract

Post-translational modifications modulate the activity of most eukaryote proteins. Analysis of these modifications presents a formidable analytical challenge. This paper examines the potential of diagonal chromatography for recognizing post-translational modifications. Diagonal chromatography is the process of using the same chromatographic separation in two dimensions. Between the two dimensions, a chemical modification is applied to all fractions. Substances that have been modified are revealed by the change in their chromatographic properties between the two dimensions. When the modification is specific to a particular type of post-translational modification, peptides that carry the modification have the potential to be revealed. Changes in the retention time of modified peptides have to be large enough to be different from unmodified peptides. Tyrosine, serine, and threonine phosphorylation were identified with diagonal chromatography. Heptafluorobutyric acid was used as an ion-pairing agent to improve the selectivity between serine and threonine phosphorylated peptides and parent peptides after dephosphorylation. The diagonal chromatography method was also examined in the recognition of glycopeptides. However, changes of retention time after deglycosylation were considered to be too small to make this an unequivocal method for the study of glycosylation.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Diagonal chromatography; Post-translational modifications; Retention times; Ion-pairing reagents; Phosphorylation; Phosphopeptides; Peptides

1. Introduction

Proteomics was conceived as a technique that would examine all the proteins in a proteome in a single, integrated set of analytical operations. The problem is that higher animal and plant cells probably express 10 000 or more proteins that are further modified in a large number of ways. Even the most advanced liquid chromatography and two-dimensional (2D) gel electrophoresis methods cannot resolve mixtures of this complexity [1]. A solution to this problem would be to find a way to target the analysis to a particular portion of the proteome. It has recently been proposed that diagonal chromatography can be a powerful tool for targeting methionine-containing peptides from tryptic digests of a proteome [2].

The observation was made decades ago that when a sample was paper chromatographed in one direction and dried, then rotated 90° and chromatographed in a second direction with the same mobile phase that all the analytes appear on the chromatogram in a diagonal line. Analytes that were chemically modified in some way between these two chromatographic dimensions had a different partition coefficient in the second dimension and no longer fell on this line. This technique for recognizing chemical modifications came to be known as *diagonal chromatography* [3,4]. In more recent work it has been noted that this approach can be used in a high-performance liquid chromatography (HPLC) mode as well [2,5]. Fractions collected from an HPLC column are simply treated and then rechromatographed under identical conditions. Chemically modified analytes will potentially have a different retention time when rechromatographed. It has been shown that oxidation of fractions from a tryptic digests after the first dimension of reversed-phase liquid chromatography

* Corresponding author. Tel.: +1 765 4943878; fax: +1 765 4940359.
E-mail address: fregnier@purdue.edu (F.E. Regnier).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.06.126

(RPLC) converts the methionine in methionine-containing peptides to methionine oxide [2]. Generally the retention time of methionine-containing peptides was reduced 1–7 min upon rechromatography. More than 800 proteins from *Escherichia coli* have been identified by this diagonal approach [2].

Analysis of post-translational modifications is a challenging issue in proteomics. This problem is often approached through the use of either an affinity selector that targets a specific post-translational modification (PTM) or by derivatization of a functional group in the PTM with an affinity tag such as biotin [6–9]. The objective of the work described here was to examine the potential of diagonal chromatography for the recognition and characterization of PTMs.

2. Experimental

2.1. Materials

Human transferrin, *N*-glycosidase F (PNGase F), ammonium hydrogencarbonate, heptafluorobutyric acid (HFBA), iodoacetic acid (IAA), cysteine, dithiothreitol (DTT), *N*-tosyl-L-lysyl chloromethyl ketone (TLCK), and all the reagents for trypsin digestion were purchased from Sigma (St. Louis, MO, USA). Sequence grade trypsin was purchased from Promega (Madison, WI, USA). All peptides were obtained from Bachem (Torrance, CA, USA) or Anaspec (San Jose, CA, USA). Trifluoroacetic acid (TFA), triethylamine, tributylamine and tetrabutylammonium phosphate were purchased from Pierce (Rockford, IL, USA). Rat liver extract were kindly provided by Dr. Stephen Hooser from Purdue University School of Veterinary Medicine.

2.2. Proteolysis of protein

Protein was reduced and alkylated in 0.1 M ammonium hydrogencarbonate buffer (pH 8.2) containing 6.2 M urea and 10 mM DTT. After 2 h incubation at 37 °C, iodoacetic acid was added to a final concentration of 20 mM and incubated in darkness for two more hours. Cysteine was then added to the reaction mixture to a final concentration of 40 mM and the reaction allowed to proceed at room temperature for 30 min. After dilution with 0.1 M ammonium hydrogencarbonate buffer to a final urea concentration of 0.8 M, sequence grade trypsin (2% (w/w) enzyme to that of protein) was added and incubated for 24 h at 37 °C. Adding TLCK in slight molar excess stopped digestion.

2.3. RPLC of peptides

Peptide mixtures were separated by gradient elution from a Vydac C_{18} column (250 mm × 4.6 mm, 218TP54) on an Integral Micro-Analytical Workstation (Applied Biosystems, Framingham, MA, USA). The C_{18} column was equilibrated using 100% mobile phase A [1% acetonitrile (ACN)/0.1% TFA in ddl water] at a flow rate of 1 mL/min for two column volumes (CVs). Peptide mixtures were injected and eluted at a flow rate of 1 mL/min in a linear gradient ranging over 60 min from 100% mobile phase A to 70% mobile phase B (95% ACN/0.01% TFA in ddl water). At the end of this period, a second linear gradient was applied in 10 min from 70% B to 100% B at the same flow rate. The gradient was then held at 100% mobile phase B for an additional 10 min. Throughout the analysis, an on-line UV detector set at 214 nm was used to monitor separation of the peptide mixtures.

2.4. Matrix-assisted laser desorption ionization time-of-flight mass spectrometric (MALDI-TOF-MS) analysis

MALDI-TOF-MS was performed using a Voyger DE-RP BioSpectrometry Workstation (PE Biosystems, Framingham, MA, USA). Peptides were prepared by mixing a 1 μl aliquot of a fraction with 1 μl of matrix solution. The matrix solution was a 10 mg/mL solution of α-cyano-4-hydroxycinnamic acid in 50% water–50% ACN with 0.1% TFA. The mixture was spotted onto a well of the MALDI sample plate and allowed to air-dry before being placed in the mass spectrometer. All peptides were analyzed in the reflective, positive ion mode by delayed extraction.

3. Results and discussion

3.1. Diagonal chromatography of complex peptide mixture

It became apparent early in this work that diagonal paper chromatography is not completely equivalent to diagonal chromatography in the HPLC mode. The difference is that fractions must be collected in the HPLC mode to transfer analytes between the dimensions. The nature of this problem is easily seen in the examination of a rat liver extract containing 7–8 mg proteins that had been trypsin digested and chromatographed on an octadecyl silane column eluted with a linear gradient ranging from 0.1% TFA to 60% ACN/0.1% TFA at rate of 1% ACN/min. Obviously, rat liver contains a huge number of peptides. One min fractions were collected and rechromatographed under identical conditions, without any modification of peptides between the two dimensions of chromatography. In all cases, multiple components were seen in each fraction. Moreover, the peaks from fractions were spread across a time window two to three times wider than the original collection window (Fig. 1). Peaks not falling within the collected time window are a type of false positive, i.e., they give the appearance of having been chemically modified when they were not. Although it is quite easy to explain the origin of this phenomenon, as seen in Fig. 2, it means that diagonal chromatography based on fraction collection and rechromatography has a fundamental problem. When applied to very complex mixtures there will always be peptides that fall within a few min beyond the collection

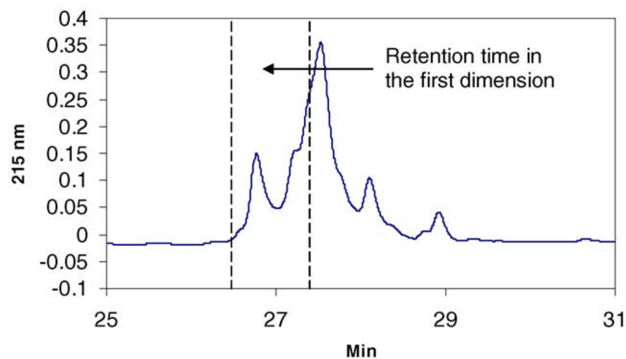

Fig. 1. Reversed-phase chromatogram of a fraction collected during 1 min in the first dimension.

window, depending on peak symmetry. This problem is further illustrated in Fig. 3 where peak 1 in the first dimension is chemically modified and becomes peak 1′ in the second dimension. It is still within the elution window of un-modified peptides even through it has changed in retention time.

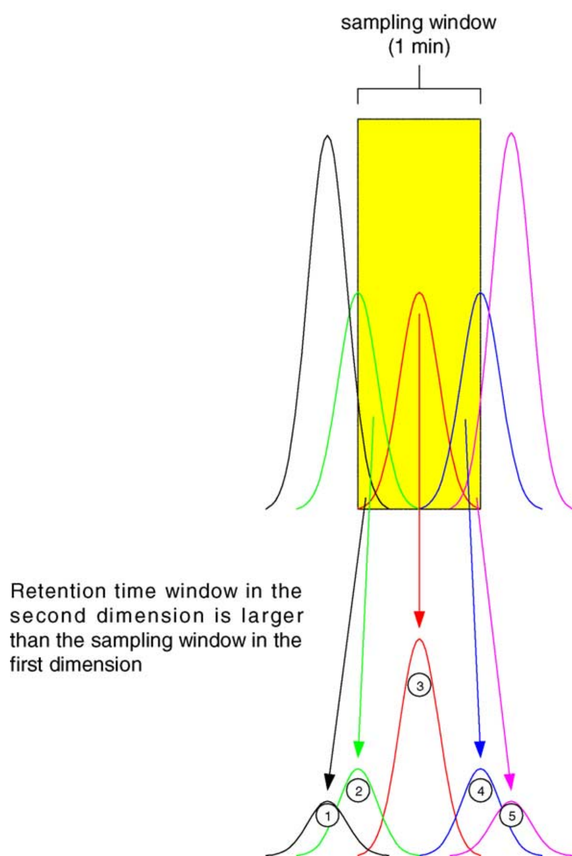

Fig. 2. A graphical illustration of how a fraction from the first dimension of diagonal chromatography will be much wider and show multiple analytes in the second dimension of identical chromatography. The illustration shows that when a peak is collected from the first dimension it contains the leading and trailing edges of peaks of both shorter and longer retention times. When this fraction is rechromatographed these leading and trailing analytes appear as separate peaks.

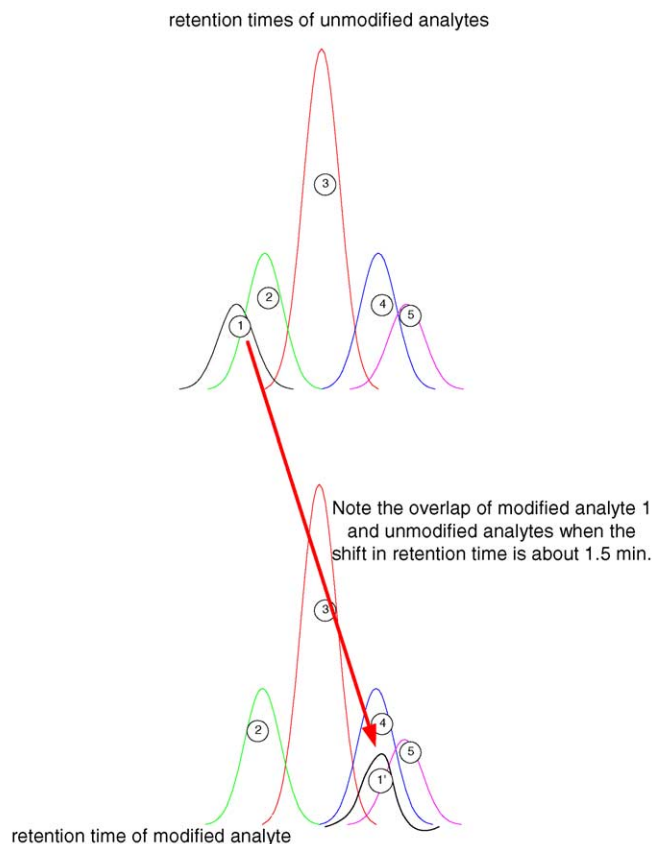

Fig. 3. The appearance of modified and unmodified peaks in the second dimension.

This does not preclude the use of diagonal chromatography in the HPLC mode for recognizing chemically modified species. It means however, that changes in retention resulting from a chemical modification must be at least 2–3 min larger than center of the collected fraction time window.

3.2. Analysis of phosphorylation

With the caveat in mind that retention time changes should be 2–3 min in HPLC-based diagonal chromatography, an analysis of the potential of the technique for recognizing phosphorylation was initiated. The phosphate group in phosphopeptides is very polar. Removing it should cause peptide retention to increase in RPLC. The most common phosphorylation sites in peptides are at serine (S), threonine (T), and tyrosine (Y) residues [10].

The impact of dephosphorylation on the retention time of synthetic phosphopeptides was examined (Fig. 4). The general structure of the phosphopeptides used was L-M-pX-V-R, where X is S, T, or Y. These peptides were combined in an equal molar ratio and split into two fractions. One was analyzed directly by RPLC whereas the other was treated first with alkaline phosphatase, and then chromatographed. Fractions from each separation were collected and analyzed by MALDI-MS to confirm the structure of eluted peptides.

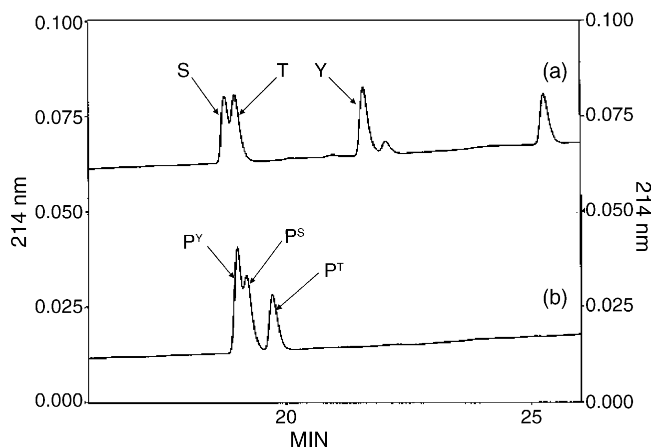

Fig. 4. Reversed-phase chromatograms of a three peptide mixture before and after dephosphorylation. (a) Peptide mixture of LM**S**VR, LM**T**VR and LM**Y**VR. (b) Phosphopeptide mixture of LM**pS**VR, LM**pT**VR and LM**pY**VR.

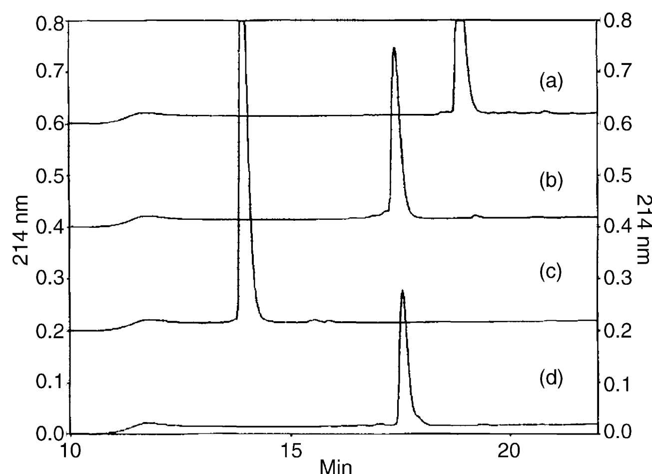

Fig. 5. Reversed-phase chromatograms of peptides with a different number of phosphate groups. (a) Peptide TRDIYETDYYRK. (b) Peptide TRDIYETDYpYRK with one phosphate group. (c) Peptide TRDIpYETDpYpYRK with three phosphate groups. (d) Peptide TRDIYETDpYYRK with one phosphate group.

Dephosphorylation of tyrosine caused a larger shift in retention time (Table 1) than in the cases of serine or threonine. S, T, and Y phosphorylated peptides showed 0.39, 0.80, and 2.57 min shift in retention time, respectively (Fig. 4). This is thought to be due to the fact that removal of the phosphate ester allows the hydrophobic aromatic ring of tyrosine to come in contact with the hydrophobic stationary phase [11].

Many proteins, e.g., casein [12], statherin [13] and neurofilaments contain multiple phosphorylation sites within short peptide sequences. Peptides with multiple phosphate groups were compared to determine the effect of the number of phosphate groups on the retention time. The monophosphorylated peptide isomers, T-R-D-I-Y-E-T-D-Y-**pY**-R-K and T-R-D-I-Y-E-T-D-**pY**-Y-R-K coelute from a reversed phase column (Fig. 5) and produce the same peptide on dephosphorylation. In this case, diagonal chromatography can not distinguish between these two species. The retention time of triphosphorylated peptide T-R-D-I-**pY**-E-T-D-**pY**-**pY**-R-K is decreased by 3.5 min compared to the monophosphorylated analogs. This means the technique is particularly useful for recognizing peptides with multiple phosphorylation sites.

The efficacy of diagonal reversed phase chromatography was examined by mapping a mixture of 10 synthetic peptides: 5 were phosphorylated on tyrosine and 5 were non-phosphorylated. Fractions were collected from the first dimension, treated with alkaline phosphatase, and then rechromatographed. The identity of all peptides was confirmed by MALDI-MS. The retention time of non-phosphorylated peptides was unchanged, and could be mapped in the diagonal of the 2D map (Fig. 6). The retention time of all the tyrosine phosphorylated peptides shifted from 1.3 to 5 min after dephosphorylation. All of the tyrosine phosphorylated peptides, were shifted off the expanded diagonal of the 2D map (Fig. 6) and could be identified as having been modified.

The efficacy of diagonal chromatography is much poorer with serine and threonine phosphorylated peptides. Four S or T phosphorylated peptides showed retention time shifts from 0.4 to 1.4 min after dephosphorylation (Table 1). As shown in Fig. 2, the expanded diagonal line with reversed phase chro-

Table 1
The retention time change of phosphopeptide after dephosphorylation.

Phosphopeptide	Retention time shift (min)
I-**pY**-G-E-F	4.8
T-S-T-G-P-**pY**-Q-G-N-L	3.2
L-M-**pY**-V-R	2.6
T-R-D-I-**pY**-E-T-D-**pY**-**pY**-R-K	5.0
T-R-D-I-Y-E-T-D-Y-**pY**-R-K	1.5
T-R-D-I-Y-E-T-D-**pY**-Y-R-K	1.4
L-M-**pS**-V-R	0.4
L-R-R-A-**pS**-L-G	1.4
K-R-**pT**-I-R-R	0.6
L-M-**pT**-V-R	0.8

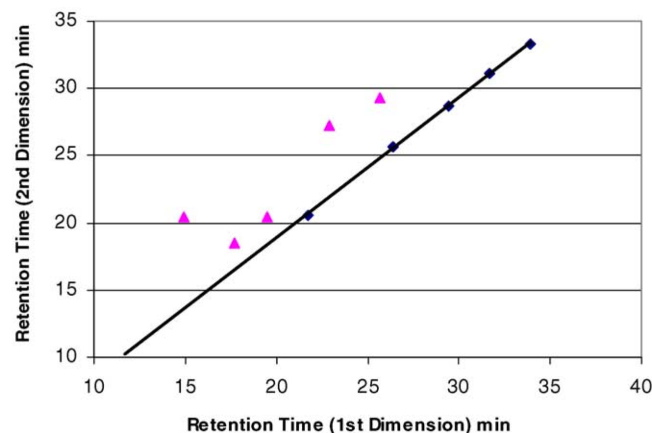

Fig. 6. Two-dimensional mapping of a mixture of tyrosine phosphorylated peptides (triangles) and non-phosphorylated peptides (squares).

matography is two to three times the collection window from the first dimension. Clearly the dephosphorylated peptides fall within this expanded window and cannot be identified as having been phosphorylated.

3.3. Ion pairing agents

The possibility that ion-pairing agents could increase the difference in retention time between phosphorylated and non-phosphorylated peptides was examined. It is well-known that differences in the polarity of peptides can be exploited in their separation by judicious use of ion-paring agents [14–16]. Cationic ion-paring agents pair with acidic residues such as phosphate groups, glutamic acid, aspartic acid, and the C-terminal carboxyl group on peptides. Hydrophobic pairing agents are expected to increase the retention time of peptides carry these groups whereas hydrophilic pairing agents are more likely to decrease their retention time.

Three cationic ion-pairing agents were tested; triethylamine, tributylamine and tetrabutylamine (TBA). Triethylamine and tributylamine caused a switch in elution order, i.e., the phosphopeptides eluted earlier than dephosphorylated peptides. Moreover, the resolution of dephosphorylated and phosphorylated peptides with these ion-pairing agents was no better than that with TFA. TBA was judged not to be useful on the basis of the difficulty of getting good MALDI-MS spectra in the presence of TBA [16].

Anionic ion-pairing agents were examined next. TFA is the most common ion-pairing agent used for peptide separations. Because TFA is weakly hydrophobic and ion-pairs with amines, increases in retention time with this pairing agent are related to the number of amines in the peptide. Surprisingly, heptafluorobutyric acid (HFBA) separated the monophosphorylated peptides from the dephosphorylated analogs better than any other ion-pairing agent, including TFA (Fig. 7).

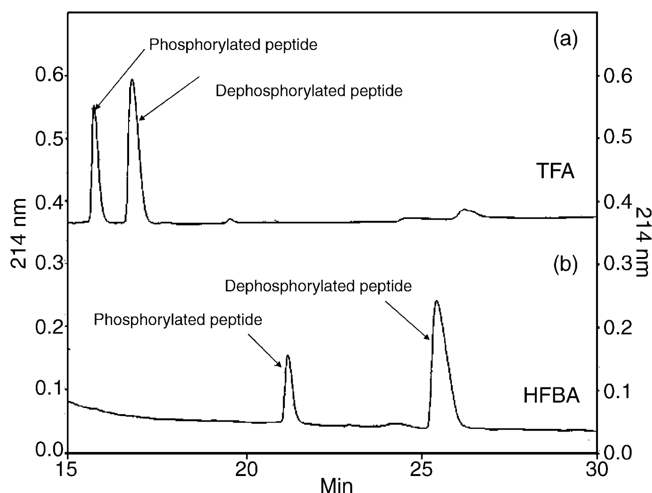

Fig. 7. Reversed-phase chromatograms of the monophosphorylated peptide LPRApSLG and its unphosphorylated parent analogs. (a) Using TFA as the ion-pairing agent. (b) Using HFBA as the ion paring agent.

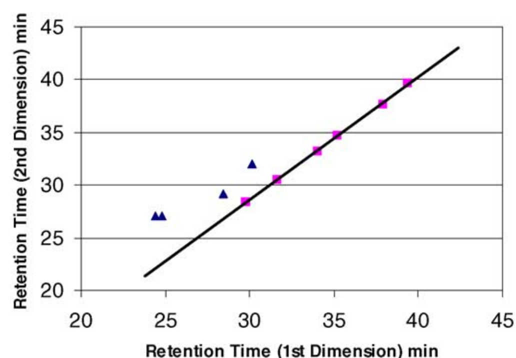

Fig. 8. Two-dimensional mapping of a mixture serine or threonine phosphorylated peptides (triangles) and non-phosphorylated peptides (squares).

With TFA, the phosphopeptide eluted 1 min earlier than the non-phosphorylated parent peptide. HFBA makes both peptides more hydrophobic and causes them to elute later, but the phosphopeptide eluted 4.2 min earlier than the dephosphorylated peptide. The elution order did not change. Clearly, the very polar phosphate group moderates the impact of HFBA on peptide retention. HFBA increased the selectivity over TFA by 3.2 min.

HFBA was used to map a mixture of 10 synthetic peptides: 2 with serine phosphorylation, 2 with threonine phosphorylation, and 6 non-phosphorylated peptides. As expected non-phosphorylated peptides did not shift in retention time and fell on the diagonal of the 2D map. All of the phosphorylated shifted off the expanded diagonal of the 2D map (Fig. 8). S/T phosphorylated peptides can be directly identified by diagonal chromatography using HFBA.

3.4. Analysis of tyrosine phosphorylation through proteolysis

Although chymotrypsin cleaves proteins at aromatic amino acids, it will not cleave peptides at sites containing a phosphorylated tyrosine residue. This fact was exploited in a diagonal chromatography approach to the analysis of peptides phosphorylated on tyrosine. Fractions collected from the first dimension of reversed phase chromatography were dephosphorylated with alkaline phosphatase and then treated with chymotrypsin. Tyrosine phosphorylation sites were subject to chymotrypsin cleavage subsequent to dephosphorylation. The interesting feature of this approach is that a new peptide is formed in which the initial tyrosine phosphorylation site becomes a tyrosine residue at the C-terminus of a new peptide.

3.5. Analysis of glycosylation

Human transferrin has two N-linked glycosylation sites. After transferrin proteolysis, 0.5 mg of the digest was fractionated by reversed phase chromatography. All of the fractions were analyzed by MALDI-MS and then

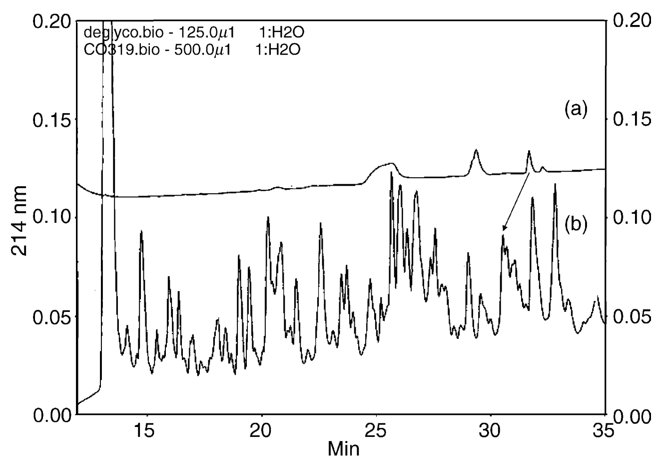

Fig. 9. Reversed-phase chromatograms of deglycosylated peptide from transferrin (a) and tryptic digested transferrin (b).

treated with PNGase F to cleave oligosaccharides from glycopeptides. The fractions were run again on the same reversed phase column under identical conditions. The deglycosylated peptide QQQHLFGS**NVT**DCSGNFCLFR exhibited a shift in retention time of 1.9 min (Fig. 9). The glycopeptide had a mass of approximately 4300 u and some microheterogeneity in the glycan portion of the molecule as judged by mass spectrometry (data not shown). It is common for glycoforms of a glycopeptide to co-chromatograph in reversed phase chromatography.

3.6. Discussion

The great advantage of diagonal chromatography for the analysis of very complex peptide mixtures is that a small number of analytes with unique properties can be moved beyond large numbers of other substances in the second dimension of chromatography, and recognized. The success of this method depends on two critical factors. One is the specificity with which unique features of peptides can be targeted for chemical modification. In the cases of phosphorylated and glycosylated proteins and peptides, there are a variety of very specific techniques for cleaving the PTM from the polypeptide backbone. The second critical variable is that removal of the PTM will cause a sufficiently large change in retention time that it can be recognized. This appears to be the "Achilles heel" of diagonal chromatography in the HPLC mode. When the fraction collected from the first dimension is equivalent to the retention time of a single peak, the time window of the rechromatographed peaks will often be two to three times larger than that of a single peak. Collecting fractions that are much smaller than the average retention width of an analyte could reduce this problem. However, the number of peaks that have to be rechromatographed in the dimension second is proportional to the ratio of the peak width divided by the fraction width. This is not a very good solution.

A better solution is to find chromatography conditions that maximize the difference between the modified and unmodified forms of peptides. The best way to do this in reversed phase chromatography of peptides is with ion-pairing agents. A well-known ion-pairing agent, HFBA, provided the solution in the case of phosphorylated peptides. The situation is likely to be more complicated with glycopeptides.

Aside from the fact that the retention time shift with glycopeptides will be relatively small, *N*-glycosylated peptides are relatively easy to recognize after deglycosylation. Removal of the oligosaccharide residue converts the asparagine residue at the attachment site to aspartic acid. When done in the presence of $H_2{}^{18}O$, it is possible to incorporate 2 mol of ^{18}O into the newly formed carboxyl group on aspartate [17]. Moreover, this aspartate residue will be within the *N*-glycosylation sequence. This compensates for the fact that the relatively small change in retention time upon deglycosylation contributes to uncertainty in recognizing a peptide as having come from a glycoprotein.

A weakness of the method is that identifications are based on peptides from which the PTM has been removed. The site of modification is frequently lost in removal of the PTM. For example, when a phosphopeptide contains multiple S or T residues it may not be possible to know the phosphorylation site from the structure of the parent peptide. The same would be true with *O*-glycosylation. This is a far smaller problem with N-linked glycopeptides, as noted above, and tyrosine phosphorylation. When chymotrypsin is used for proteolysis instead of trypsin and fractions from the first dimension are treated with alkaline phosphatase and rechromatographed, tyrosine phosphorylation sites will be recognized by the presence of a single tyrosine residue at any point in the peptide except at the C-terminus. Multiple tyrosine residues in the interior of a peptide would indicate the presence of multiple phosphorylation sites. The exception would be in the case of a missed chymotrypsin cleavage.

4. Conclusion

It is concluded that diagonal chromatography in the HPLC mode will be of greatest utility when three conditions are met. One is when the retention time of a peptide changes 2–3 min after modification. This is necessary because non-chemically modified peptides frequently elute slightly beyond the time window of the peak collected from the first dimension in the HPLC mode of diagonal chromatography.

The efficacy of diagonal chromatography will also be increased when the difference in selectivity between modified and un-modified version of peptide has been optimized. In the case of RPLC of peptide, this will most likely involve the search for the best ion-pairing agent.

Finally the value of diagonal chromatography will be maximized when it is possible to recognize sites of

post-translational modifications after modification. Based on the work presented here, this is most likely to be the case with tyrosine phosphorylation and N-linked glycosylation.

Acknowledgement

The authors gratefully acknowledge support from NIH grant number GM 59996.

References

[1] H.J. Issaq, Electrophoresis (2001) 22.
[2] K. Gevaert, J. Van Damme, M. Goethals, G.R. Thomas, B. Hoorelbeke, H. Demol, L. Martens, M. Puype, A. Staes, J. Vandekerckhove, Mol. Cell. Proteom. 1 (2002) 896.
[3] O. Mikes, V. Holeysovsky, Chem. Listy 51 (1957) 1497.
[4] W.H. Cruickshank, B.L. Malchy, H. Kapln, Can. J. Biochem. 52 (1974) 1013–1017.
[5] K. Gevaert, M. Goethals, L. Martens, J. Van Damme, A. Staes, G.R. Thomas, J. Vandekerckhove Nat. Biotechnol. 21 (2003) 566.
[6] F.E. Regnier, L. Riggis, R. Zhang, L. Xiong, P. Liu, A. Chakraborthy, E. Seeley, C. Sioma, J. Mass Spectrom. 37 (2002) 133.
[7] L. Xiong, D. Andrews, F. Regnier, J. Proteome Res. 2 (2003) 618.
[8] M.B. Goshe, T.P. Conrads, E.A. Panisko, N.H. Angell, T.D. Veenstra, R.D. Smith, Anal. Chem. 73 (2001) 2578.
[9] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, Nat. Biotechnol. 17 (1999) 994.
[10] J.X. Yan, N.H. Packer, A.A. Gooley, K.L. Williams, J. Chromatogr. A 808 (1998) 23.
[11] J.C. Giddings, J. Chromatogr. A 703 (1995) 3.
[12] M. Adamczyk, J.C. Gebler, J. Wu, Rapid Commun. Mass Spectrom. 15 (2001) 1481.
[13] J.H. Waite, X. Qin, Biochemistry 40 (2001) 2887.
[14] W.S. Hancock, C.A. Bishop, J.E. Battersby, D.R.K. Harding, M.T.W. Hearn, J. Chromatogr. 254 (1979) 339.
[15] W.M.M. Schaaper, D. Voskamp, C. Olieman, J. Chromatogr. 194 (1980) 421.
[16] A. Amini, S.J. Dormady, L. Riggs, F.E. Regnier, J. Chromatogr. A 894 (2000) 345.
[17] L. Xiong, F.E. Regnier, J. Chromatogr. B 782 (2002) 405.