DIONEX

Application Update 173

Enrichment and Identification of Chicken Ovalbumin Phosphopeptides by Immobilized Metal Affinity and Reversed-Phase Chromatographies

INTRODUCTION

Protein phosphorylation is a widely used means of signal transduction for regulating various cellular processes in many organisms.[1] A change in the balance between phosphorylated and dephosphorylated proteins may lead to cellular damage and/or degeneration and has been implicated in certain types of cancer.[2] Characterizing the phosphoproteome is key to understanding the mechanism of cellular processes and identifying targets for therapeutics. Phosphorylation is a very dynamic event with many proteins being phosphorylated at different sites at different times, which makes analysis of phosphoproteins very challenging.[3] Some of the technologies for characterizing protein phosphorylation include matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (MS) and liquid chromatography (LC)-nano electospray tandem MS. Most MS-based protein characterizations are done at the peptide level after protein cleavage with site-specific proteases. Phosphorylated peptides, however, do not ionize very well in the positive-ion mode and are also subject to ion suppression in the presence of an excess of nonphosphorylated peptides. In order to effectively characterize phosphoproteins, specific and efficient enrichment strategies for separating the phosphorylated species from the unphosphorylated ones are required. A variety of enrichment methods, including antibody-based immunoaffinity precipitation, chemical derivatization, immobilized metal affinity chromatography (IMAC), and enrichment on metal oxide surfaces, have been used. Comparative studies between these approaches have shown that the overlap between the methods is poor, suggesting that each method isolates a different subset of the phosphoproteome.[4,5]

Here the authors describe a method using IMAC in the ferric form to selectively isolate and enrich phosphopeptides from a peptide digest. The method exploits the affinity of the phosphopeptide phosphate group to the ferric ions immobilized on the solid support of the ProPac® IMAC-10 stationary phase. The isolated phosphopeptides are eluted from the IMAC column and determined by reversed-phase chromatography using the Acclaim® 300 C18 column and UV detection as described in TN 705.[6] In this update, the authors have optimized the IMAC elution conditions for the ovalbumin phosphopeptides.

The ProPac IMAC-10 column consists of a substrate of nonporous polymeric beads that are 10 µm in size covered by a hydrophilic layer that shields the substrate. Finally, the hydrophilic layer has iminodiacetate (IDA) groups grafted to it. These groups are then charged with ferric iron to bind phosphoproteins. The structure of this resin makes for excellent mass-transfer kinetics when compared to traditional gel-based resins, and results in rapid and complete binding and release of the analyte. The Acclaim 300 C18 columns are designed specifically for peptide-mapping applications and analysis of small proteins. The high efficiency of the 3 µm particle size in a 4.6 × 150 mm format allows for faster separations. Ovalbumin, a chicken egg-white protein that has two serine phosphorylation sites (S69 and S345), was used to model the analysis of phosphopeptides.

AN 99 describes a method to reduce, alkylate, and enzymatically digest ovalbumin into peptides using trypsin.[7] Ovalbumin phosphopeptides were tentatively identified in AN 99 using peptide mapping before and after alkaline phosphatase treatment and absorbance intensity at 214 and 260 nm. This document describes the use of IMAC followed by reversed-phase chromatography to confirm the ovalbumin phosphorylated peptide identification of AN 99.

The Dionex UltiMate® 3000 HPLC system, equipped with a ProPac IMAC-10, an Acclaim 300 C18 column, and a UV detector, is a reliable and easy-to-use automated solution for routine phosphopeptide-enrichment applications.

EQUIPMENT

Dionex UltiMate 3000 HPLC system including:
SRD-3600 Solvent rack with 6 degasser channels (P/N 5035.9230) and Eluent organizer, including pressure regulator and 2 L glass bottles for each pump. Eluents were maintained under helium or nitrogen head space (5 to 8 psi).

DGP 3600M Pump (P/N 5035.0050)

WPS-3000T Well plate sampler (P/N 5823.0020)

FLM-3100 NANO Flow manager (P/N 5720.0010) or

UltiMate 3000 Column compartment (recommended module when using 2 and 3 mm i.d. columns, P/N 5722.0025)

PDA-3000 Photodiode array detector (P/N 5080.9920)

Biocompatible analytical flow cell for PDA (P/N 6080.0220)

REAGENTS AND STANDARDS

Deionized water 18.2 (MΩ-cm)

Hydrochloric acid, ULTREX® II Ultrapure Reagent, 36.5 to 38.0% (J.T.Baker, 9530-33)

Tris (Base), ACS Reagent (tris[hydroxymethyl] aminomethane), (J.T. Baker, X171-7)

Alkaline phosphatase, bovine intestinal mucosa, lyophilized, 35% protein, ≥ 2,000 DEA units/mg protein. One DEA unit will hydrolyze 1 µmol of 4-nitrophenyl phosphate per minute at pH 9.8 at 37 °C. (Sigma-Aldrich, P/N P6772-2KU)

Ovalbumin (Albumin from chicken egg, Sigma-Aldrich, P/N A5503, Grade V, ≥ 98% pure by agarose gel electrophoresis, lyophilized powder)

Iodoacetamide (MP Biomedicals, P/N 100351)

DL-Dithiothreitol (Fluka, P/N 43815)

Trypsin, sequence grade, modified, lyophilized, 20 µg/vial (5 vials/kit), specific activity – 17,000 U/mg (One unit is the amount of trypsin required to produce a change in absorbance [253 nm] of 0.001 per min at 30 °C with the substrate N-α-benzoyl-L-arginine ethyl ester [BAEE]). The substrate is dissolved in 50 mM Tris-HCl, 1 mM $CaCl_2$ (pH 7.6), and the enzyme is diluted in 50 mM acetic acid. Kit includes 100 mM ammonium bicarbonate or trypsin resuspension buffer, 1 mL vial (Promega, P/N V5111)

Ammonium bicarbonate, ReagentPlus®, ≥ 99.0% (Sigma-Aldrich, P/N A6141)

Trifluoroacetic acid (TFA), sequanal grade for making 0.1% v/v TFA solutions (Thermo Scientific, P/N 28904)

Acetonitrile, HPLC grade (Honeywell, P/N 015-4)

Cytochrome C digest, 1.6 nmol/vial, lyophilized (Dionex, P/N 161089)

Tetrasodium pyrophosphate decahydrate (TSPP), $Na_4P_2O_7 \cdot 10H_2O$, (Sigma Chemical, P/N S6422)

Formic acid, 98% (Fluka, P/N 06440)

Acetic acid, glacial (J.T. Baker, P/N 9524)

Ammonium hydroxide, ACS Reagent (Fisher Scientific, P/N A669, 14 N)

Iron (III) chloride hexahydrate, $FeCl_3 \cdot 6H_2O$, (Aldrich Chemical, P/N 207926)

Bovine ß-Casein, monophosphopeptide (Anaspec, P/N 61146)

Bovine ß-Casein, tetraphosphopeptide (Anaspec, P/N 61147)

Tributylphosphine solution, 0.2 M in N-methylpyrollidine (Sigma Chemical, P/N T7567)

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phase A for IMAC

20 mM Formic acid

Add 760 µL of formic acid to approximately 500 mL DI water in a 1000 mL volumetric flask and bring to volume using DI water.

Mobile Phase B for IMAC

20 mM Formic acid titrated to pH 9.0 using ammonium hydroxide

Add 760 µL of formic acid to approximately 500 mL DI water in a 1000 mL volumetric flask, titrate to pH 9.0 using ammonium hydroxide, and bring to volume using DI water.

Mobile Phase A for RP-HPLC

Add 39.1 g of acetonitrile to 950 g of water, followed by the addition of 0.44 g of TSPP and 0.1 mL of TFA.

Note: TSPP is used in the mobile phase for RP-HPLC because it is an effective chelating agent at low pH and is also non-UV absorbent. Because the IMAC column uses iron for binding, the pyrophosphate keeps the RP column from getting contaminated with iron.

Mobile Phase B for RP-HPLC

Add 546 g of acetonitrile to 300 g of water, followed by the addition of 0.11 mL of TFA.

For best performance:

a. Use fresh TFA that is supplied in sealed ampules.
b. Prepare TFA-containing eluents daily.
c. Store the eluents blanketed with inert gas during use on the system (helium or nitrogen).
d. Protect eluents from photoreactions by covering the eluent bottles with aluminum foil.

Monophosphopeptide Standard

Add 1.0 mL of water to the contents of one vial of the mono phosphopeptide standard to make a 100 µg/mL stock solution. Dilute the stock to the working concentration with 100 mM acetic acid.

Tetraphosphopeptide Standard

Add 500 µL of water to the contents of one vial of the tetraphosphopeptide standard to make a 100 µg/mL stock solution. Dilute the stock to the working concentration with 100 mM acetic acid.

100 mM Acetic Acid

Add 575 µL of acetic acid to approximately 75 mL DI water in a 1000 mL volumetric flask and bring to volume using DI water.

Iron Solution for Charging the IMAC Column

Dissolve 0.14 g of iron (III) chloride hexahydrate in 120 µL of glacial acetic acid and then dilute to 20 mL using DI water. Filter the iron solution through a 0.2 µm membrane filter just prior to using the solution for charging the IMAC column.

Charging the IMAC Column

Using a 1 mL disposable syringe, load 0.2 mL filtered iron solution through the column and then rinse the column with 0.2 mL of DI water. Reconnect the column back to the pump and flush with mobile phase A for at least one hour. Connect the column to the detector and monitor the baseline until it is stable. Once charged, the column can be used for three months without recharging. If the column performance is compromised, however, the column should be recharged.

CONDITIONS

Columns: ProPac IMAC-10, 2 × 50 mm (Dionex, P/N 063272)
Acclaim 300 C18, 3 µm Analytical, 4.6 × 150 mm (Dionex, P/N 060266)
Inj. Volume: 30 µL
Temperature: 30 °C
Detection: UV 214 nm, 5 nm bandwidth
Flow Rate: 0.2 mL/min for ProPac IMAC-10
1.0 mL/min for Acclaim 300 C18
Tray Temp: 4 °C

Instrument programs should be performed in the following order and with the following gradient conditions.

SAMPLE PREPARATION

See AN 99 for a detailed description of system qualification, reduction and alkylation, tryptic digestion, and alkaline phosphatase treatment of ovalbumin.[7]

Program 1: IMAC Loading Step

Time	IMAC (Left Pump)			RP (Right Pump)			Event
	% A	% B	Flow (µL/min)	% A	% B	Flow (µL/min)	
-9.0	100	0	200	93	7	1000	Valve Position 1_2
0.0	100	0	200	93	7	1000	Inject; acquire data
2.6	100	0	OFF	93	7	1000	

Program 2: HPLC Analysis of the Unbound Fraction

Time	IMAC (Left Pump)			RP (Right Pump)			Event
	% A	% B	Flow (µL/min)	% A	% B	Flow (µL/min)	
0.0				93	7	1000	Valve Position 10_1
0.25				93	7	1000	Auto-zero detector
1.0				93	7	1000	Acquire data
15.0				31	69	1000	
15.1	100	0	OFF	31	69	1000	
16.0	100	0	OFF	0	100	1000	
20.0	100	0	200	0	100	1000	

Program 3: IMAC Elution Step							
Time	IMAC (Left Pump)			RP (Right Pump)			Event
	% A	% B	Flow (µL/min)	% A	% B	Flow (µL/min)	
0.0	100	0	200	93	7	1000	Valve Position 1_2 Inject; acquire data
1.0	0	100	200	93	7	1000	
10.0	0	100	200	93	7	1000	
13.6	0	100	200	93	7	1000	
13.61	0	100	OFF	93	7	1000	

Program 4: HPLC Analysis of the Bound Fraction							
Time	IMAC (Left Pump)			RP (Right Pump)			Event
	% A	% B	Flow (µL/min)	% A	% B	Flow (µL/min)	
0.0				93	7	1000	Valve Position 10_1
0.25				93	7	1000	Auto-zero detector
1.0			200	93	7	1000	Acquire data
15.0			OFF	31	69	1000	
15.1	100	0	200	31	69	1000	
16.0	100	0	200	0	100	1000	
20.0	100	0	200	0	100	1000	

Note: When configuring the sequence to run the above four programs, only Program 1 gets a sample injection of 30 µL. Programs 2, 3, and 4 should be configured as blank runs with no injection performed.

SYSTEM SETUP

Install the UltiMate 3000 system with the WPS-3000T at the bottom, followed by the FLM-3100, the PDA-3000, and the DGP-3600M pump on top. As shown in Figure 1, connect the left pump of the DGP-3600M to the inlet of the WPS-3000T. Using 0.12 mm i.d. (0.005 in.) tubing, connect the outlet of the WPS-3000T to the IMAC column, the column outlet to port 3 of the 10-port valve, and port 4 to the PDA-3000. Using 0.25 mm i.d. (0.010 in.) tubing, connect the outlet of the PDA-3000 to port 7. Using 0.25 mm i.d. (0.010 in.) tubing, connect the reversed-phase column inlet to port 9 and the outlet to port 5. Using 0.25 mm i.d. (0.010 in.) tubing, connect the right pump of the DGP-3600 to port 10. Make a sample-collection loop from 130 cm of 0.5 mm i.d. (0.020 in.) tubing and connect it to ports 1 and 8. Connect ports 2 and 6 to waste.

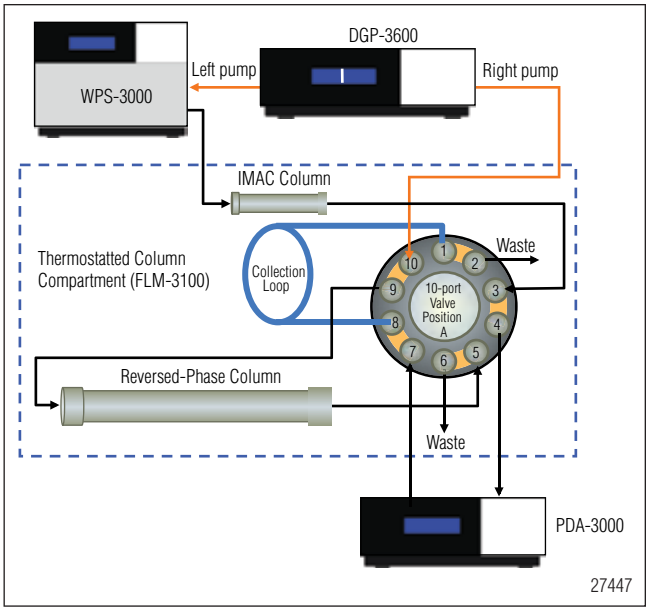

Figure 1. Plumbing diagram of the phosphopeptide enrichment system.

When the 10-port valve is in position 10_1, a sample is injected onto the IMAC column, and the unbound fraction passes through the column to the detector and is trapped in the collection loop when the flow of the left pump is turned off. In valve position 1_2, the contents of the loop are injected onto the C18 column, and the detector monitors the reversed-phase separation of the C18 column. After the above sequence of events is complete, repeat the events with the 10-port valve switched back to the 10_1 position. The IMAC eluent B elutes the bound fraction from the IMAC column to the detector and is trapped in the collection loop when the pump flow is turned off. Switching to valve position 1_2 moves the contents of the loop onto the C18 column, followed by the reversed-phase separation and detection of peptides in the bound fraction.

RESULTS AND DISCUSSION

The system was qualified using a cytochrome C tryptic digest solution, as discussed in AN 99. A retention time RSD of ≤ 0.3% and a peak area RSD ≤ 1.2% using a wavelength of 214 nm were confirmed. The presence of 21 ± 4 peaks and the absence of any significant artifacts were also confirmed. To demonstrate IMAC selectivity, the authors digested ß-casein with trypsin and followed the general procedure described in AN 99, yielding two phosphopeptides: one monophosphorylated FQSpEEQQQTEDELQDK and one tetraphosphorylated RELEELNVPGEIVESpLSpSpSpEESITR.

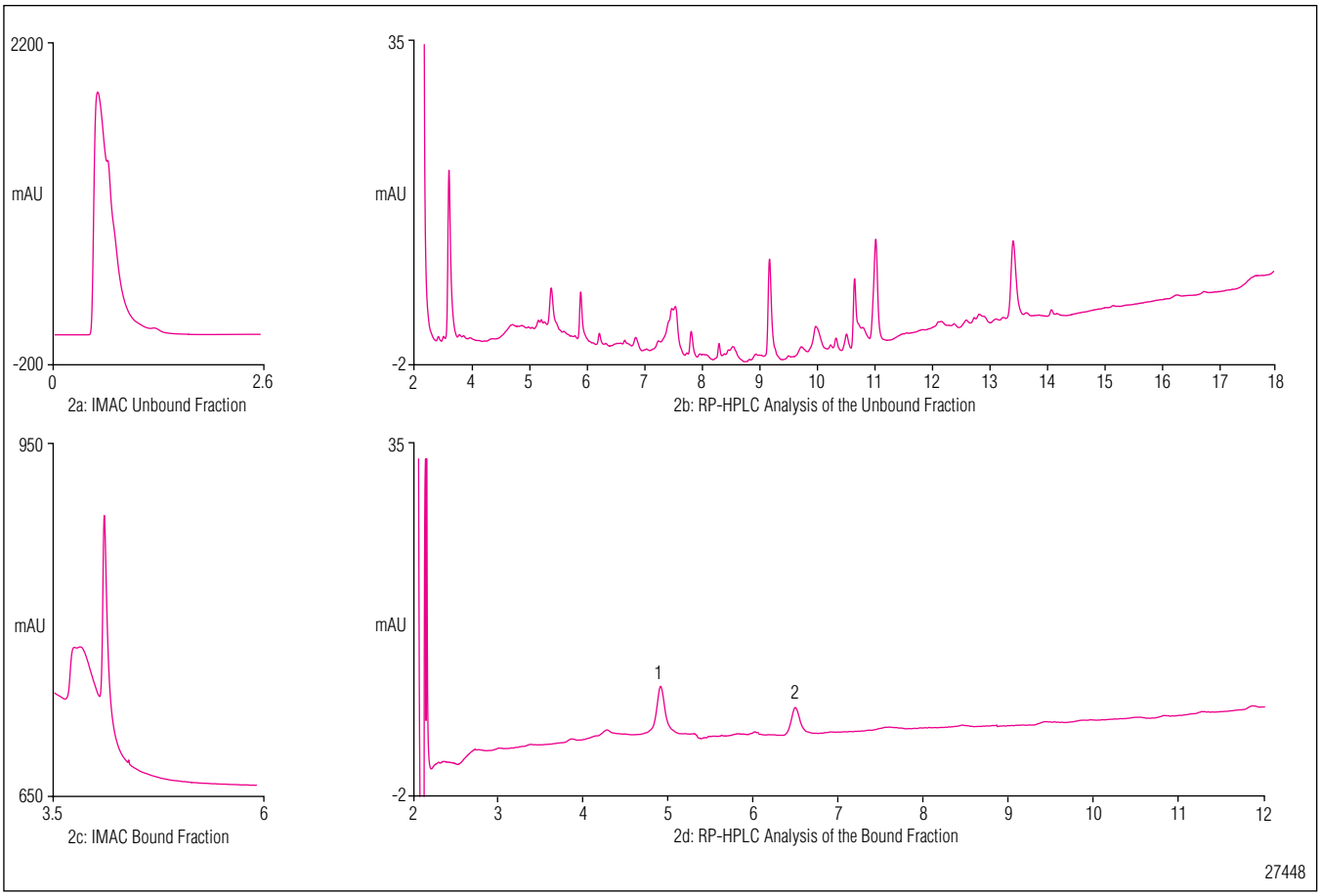

Figure 2. Bovine ß-casein tryptic digest spiked with mono and tetraphosphopeptide standards.

Figure 2 shows the chromatography of automated IMAC enrichment and RP-HPLC analysis when using a tryptic digest of bovine ß-casein spiked with the monophosphopeptide and tetraphosphopeptide standards (using Programs 1 to 4). Figures 2a and 2c show the IMAC unbound and bound fractions, respectively, while Figures 2b and 2d show RP-HPLC of the unbound and bound IMAC fractions, respectively. In Figure 2d, the monophosphopeptide elutes at 4.94 min and the tetraphosphopeptide at 6.52 min. Although the programs were optimized for binding and elution of ovalbumin, the data in Table 1 show reproducible recovery of the mono and tetraphosphopeptide standards spiked into the ß-casein tryptic digest. The retention time RSDs for both peaks were less than 0.055, which indicates reproducible gradient delivery. Peak area RSDs for both peaks were less than 2.1, indicating repeatable sample injections. To calculate recovery, phosphopeptide levels in the ß-casein tryptic digest were first calculated with and without passing through the IMAC column.

A standard addition of 0.15 µg/mL of bovine ß-casein mono and tetraphosphopeptide standards was made to the ß-casein tryptic digest, and recoveries were calculated with and without passing through the IMAC column. The recoveries of mono- and tetraphosphopeptide standards spiked into the ß-casein tryptic digest with the IMAC column were 91.2% and 48.7%, respectively. The lower recovery for the multiply phosphorylated peptide may be due to the fact that this peptide binds more strongly to the IMAC resin and is harder to elute.[8]

Table 1. Reproducibility and Recovery Data from Three Replicate Injections of ß-Casein Tryptic Digest Spiked with Mono- and Tetraphosphopeptide Standards

	Retention Time		Peak Area		
Peak #	Mean (Min)	RSD	Mean (mAU*min)	RSD	Recovery %
1	4.94	0.05	0.3486	1.15	91.2
2	6.89	0.04	0.1605	2.04	48.7

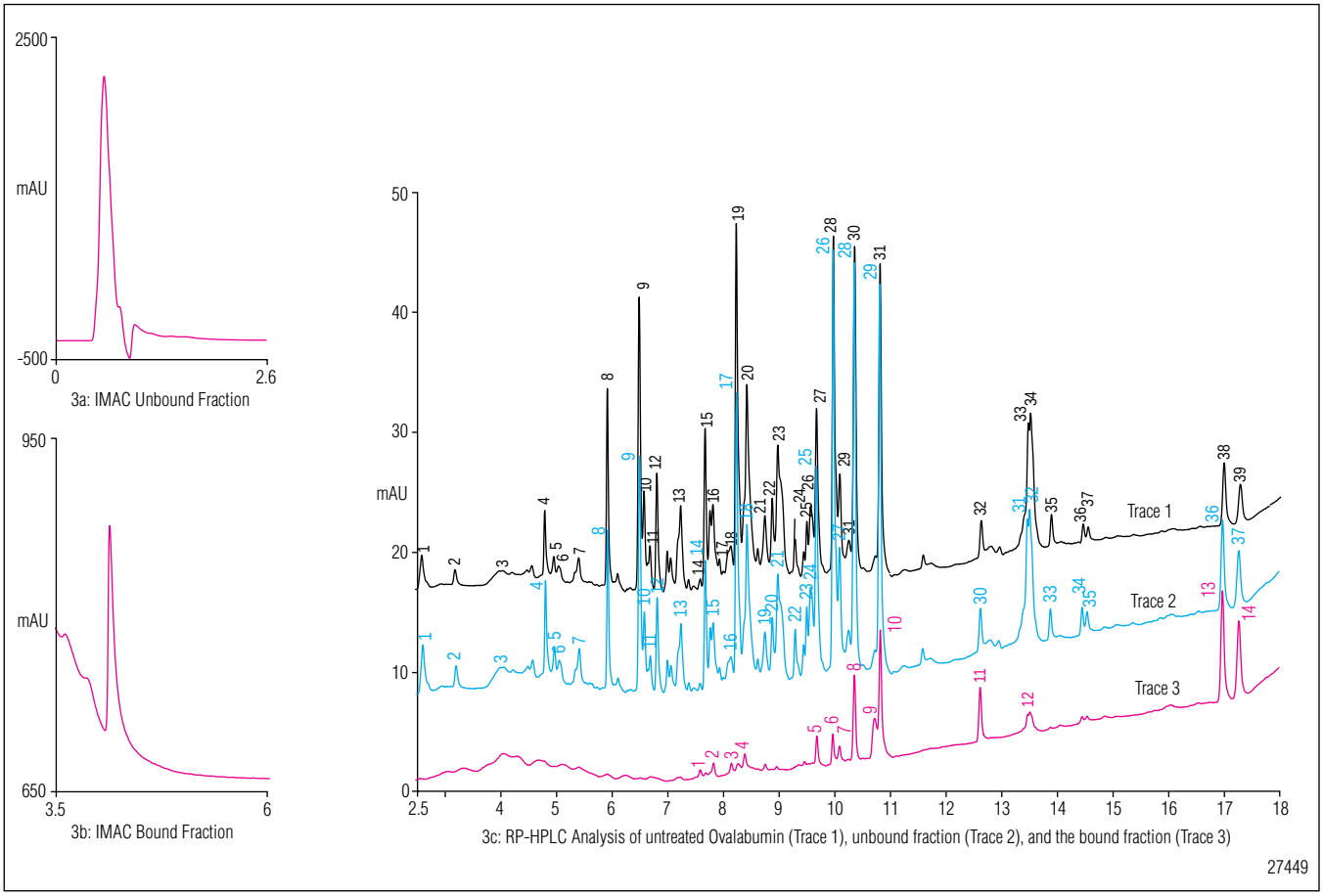

Figure 3. RP-HPLC analysis of the IMAC bound and unbound fractions of the ovalbumin tryptic digest.

Programs 1 and 3 described in the Conditions section have been optimized to effectively capture and elute ovalbumin phosphopeptides from the IMAC column. Figure 3 shows chromatography of automated IMAC enrichment and RP-HPLC analysis of an ovalbumin tryptic digest. Figures 3a and 3b show unbound and bound IMAC fractions of the ovalbumin tryptic digest, respectively. Figure 3c shows an overlay of three reversed-phase separations: (Trace 1) ovalbumin tryptic digest not passed through the IMAC column, (Trace 2) the unbound fraction, and (Trace 3) the bound fraction. The 37 peaks found in the unbound fraction (Trace 2, Figure 3c) exceed the theoretical number of 34 peaks, but this can be attributed to miscleaved peptides, the glycoforms of each glycosylated peptide (multiple peaks for the same peptide sequence), and partial phosphorylation of a given phosphorylation site. Although ovalbumin has only two phosphorylated tryptic peptides—EVVGS*AEAGV DAASVSEFFR and LPGFGDS*IEAQCGTSVNV HSSLR—14 peaks were observed in the bound fraction (Trace 3, Figure 3c). This may be a result of nonspecific binding due to the affinity of some nonphosphorylated peptides to the IMAC stationary phase. Nonphosphorylated peptides containing multiple acidic residues also have a high affinity for the metal ions on an IMAC column and copurify with the phosphopeptides.[9]

To better understand which of the 14 peptides were phosphorylated, the tryptic digest was dephosphorylated using alkaline phosphatase. Figure 4 shows the automated IMAC enrichment and RP-HPLC analysis of an alkaline phosphatase-treated ovalbumin tryptic digest. Figures 4a and 4b show the IMAC unbound and bound fractions of the alkaline phosphatase-treated ovalbumin, respectively. Figure 4c shows an overlay of three reversed-phase separations: (Trace 1) ovalbumin tryptic digest not passed through the IMAC column, (Trace 2) the unbound fraction, and (Trace 3) the bound fraction. The peptide map of ovalbumin that was not passed through the IMAC column shows increased peak

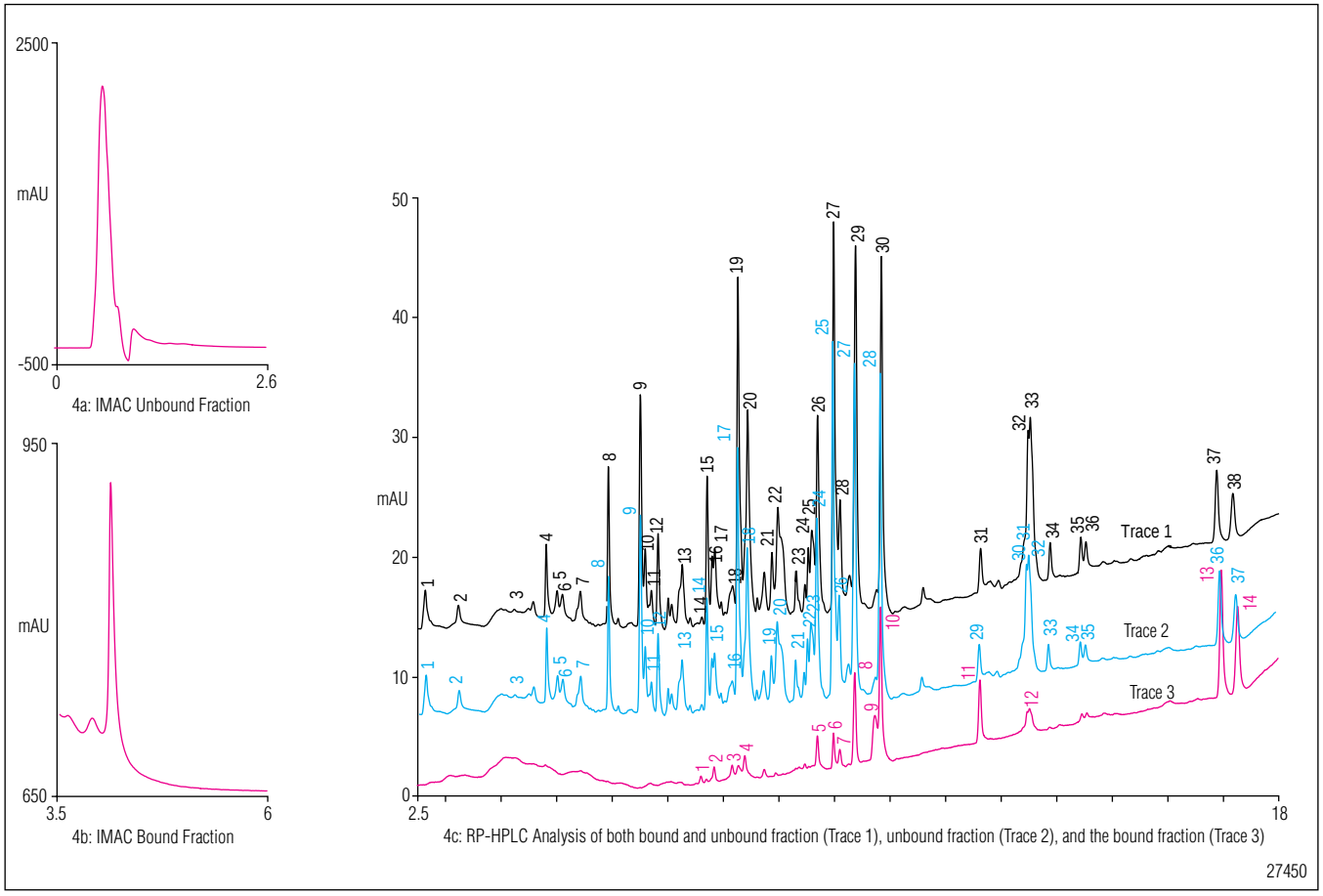

Figure 4. RP-HPLC analysis of the IMAC bound and unbound fractions of the alkaline phosphatase-treated ovalbumin tryptic digest.

areas for several peaks between 7.5 to 11.0 min when compared to the peptide map of the unbound fraction of the phosphorylated ovalbumin. Figure 4c, Trace 3, shows 14 peaks in the bound fraction of the dephosphorylated ovalbumin tryptic digest. When compared to Figure 3c, Trace 3, most of the changes are between 7 and 9 min. Figure 5 shows a closer look at this region of the chromatogram in an overlay of the RP-HPLC analysis of the IMAC unbound and bound fraction of the ovalbumin tryptic digest, with and without alkaline phosphatase digestion. The authors tentatively identify the ovalbumin phosphorylated peptides as the peaks labeled 1 and 2. The chromatography also suggests that neither peptide is completely bound by the IMAC. After dephosphorylation, peak 1 is not found in the bound fraction. In an ideal system, the dephosphorylated ovalbumin tryptic peptides should not bind to the IMAC resin, but they may bind nonspecifically. Each of the phosphorylated peptides—EVVGS*AEAGV DAASVSEFFR and LPGFGDS*IEAQCGTSVNV HSSLR (where the * denotes the phosphorylated amino acid)—has glutamic acid residues (three and one, respectively); each also has one aspartic acid residue. The acidic residues of both of these peptides could allow them to bind nonspecifically to the IMAC column when dephosphorylated. If they do bind, the net loss in negative charge should result in the dephoshorylated peptide peaks eluting later under reversed-phase chromatography conditions due to exposure of the previously phosphorylated amino acid. In addition to the disappearance of peak 1 in the bound fraction, less peak area is seen in the unbound fraction, suggesting that it is not completely bound before alkaline phosphatase treatment. In Figure 5, the authors also observe a new, later-eluting peak in both the bound and unbound fractions of the alkaline phosphatase-treated ovalbumin tryptic digest. This new peak is labeled 1* and tentatively identified as desphosphorylated phosphopeptide 1. After dephosphorylation, only a small amount of peak 2 remains in both bound and unbound fractions. The authors interpret this as a nonspecifically

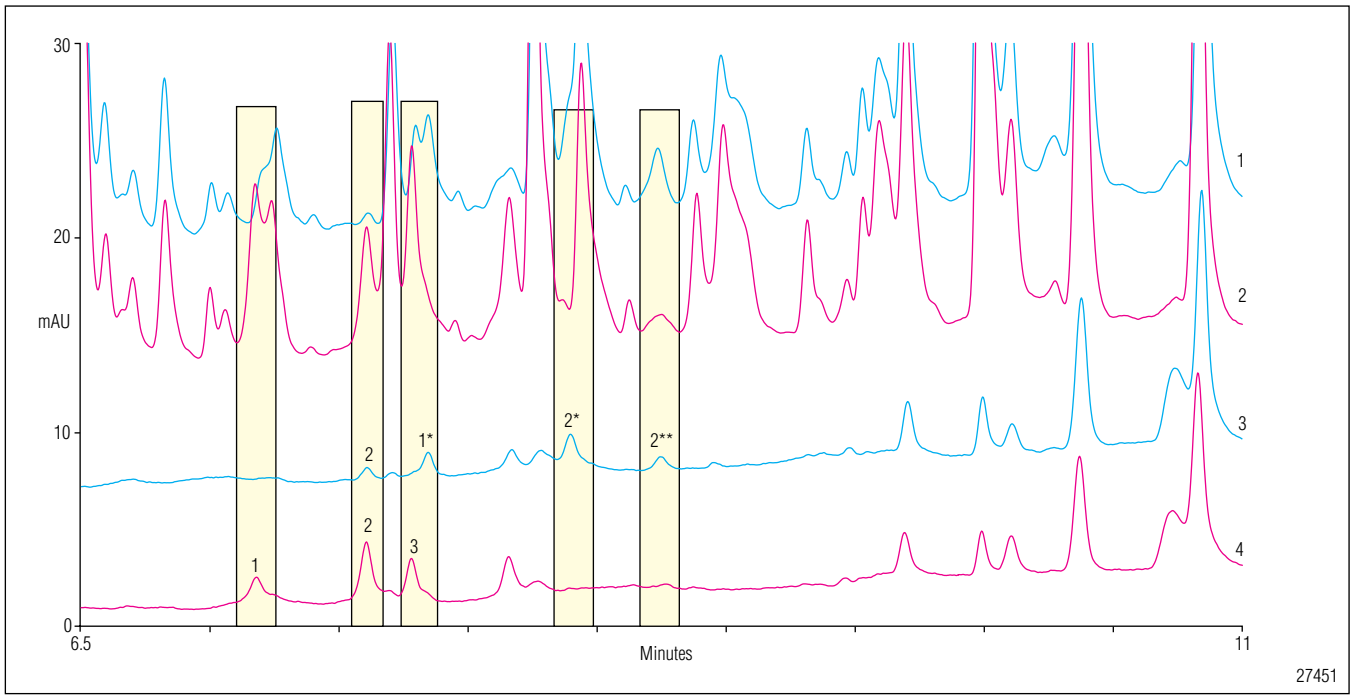

Figure 5. RP-HPLC analysis of the IMAC unbound and bound fractions of the ovalbumin tryptic digest, with and without alkaline phosphatase digestion, measured at 214 nm.

bound peptide that coeluted with the phosphopeptide. It is also possible that the peptide was not completely desphosphorylated. In Figure 5, two new peaks appear, or appear to increase in peak area, in the bound and unbound fractions. These peaks are labeled 2^{*} and 2^{**}. One of these peaks may be the nonspecifically bound dephosphorylated peptide. An increase in peak area of a peak in the native peptide map might also be expected because ovalbumin is known to be partially phosphorylated. It is also possible that peak 3 in the peptide map of the bound fraction of the ovalbumin tryptic peptides was phosphorylated and became 2^{*} or 2^{**} after dephosphorylation. Ovalbumin is known to demonstrate sequence microheterogenity, so perhaps this occurs within a phosphopeptide sequence and results in an additional phosphopeptide population.

AN 99 presents a method for peptide mapping of ovalbumin using the Acclaim 300 C18 reversed-phase column and a combination of alkaline phosphatase digestion and absorbance ratios to tentatively identify phosphopeptides. This method uses the IMAC to enrich phosphopeptides from an ovalbumin tryptic digest before and after alkaline phosphatase treatment, and to then automatically run the reversed-phase peptide map of each fraction. This method may also be used in further experiments to characterize a phosphoprotein.

PRECAUTIONS

Avoid contact and inhalation of any of the materials used. Material safety data sheets (MSDS) for these materials should be reviewed prior to handling, use, and disposal.

CONCLUSION

This method uses the ProPac IMAC-10 stationary phase in the ferric form to isolate phosphopeptides from a peptide digest. Peptide mapping with the Acclaim 300 C18 column in combination with the UltiMate 3000 HPLC system was used to evaluate the IMAC-bound and unbound peptides to help identify the ovalbumin phosphopeptides.

REFERENCES

1. Cantin, G.T.; Shock, T.R.; Park, S.K.; Madhani, H.D.; and Yates, J.R. Optimizing TiO_2-Based Phosphopeptide Enrichment for Automated Multidimensional Liquid Chromatography Coupled to Tandem Mass Spectrometry. *Anal. Chem.,* **2007***, June*, 4666–4673.
2. Schmelzle, K.; and White, F.M. Phosphoproteomic Approaches to Elucidate Cellular Signaling Networks. *Biotechnol.*, **2006**, *17*, 406–414.
3. Feng, S.; Ye, M.L.; Zhou, H.; Jiang, X.; Zou, H.; and Gong, B. Immobilized Zirconium Ion Affinity Chromatography for Specify Enrichment of Phosphopeptides in Phosphoproteome Analysis. *Molecular & Cellular Proteomics*, **2007**, *6*, 1656–1665.
4. Wehr, T. Enriching the Phosphoproteome. *LCGC North America*, **2008**, *June*, 550–559.
5. McLachlin, D.T.; and Chait, B.T. Improved β-Elimination-Based Affinity Purification Strategy for Enrichment of Phosphopeptides. *Anal. Chem.*, **2003**, *75*, 6826–6836.
6. Dionex Corporation, *Automated Enrichment and Determination of Phosphopeptides Using Immobilized Metal Affinity and Reversed-Phase Chromatography with Column Switching*. Technical Note 705, LPN 1763, 2010, Sunnyvale, CA.
7. Dionex Corporation, *Peptide Mapping of Ovalbumin using Reversed-phase High Performance Liquid Chromatography with Phosphorylation Site Prediction*. Application Note 99, LPN 2273, 2009, Sunnyvale, CA.
8. Kange, R.; Selditz, U.; Granberg, M.; Lindberg, U.; Ekstrand, G.; Ek, B.; and Gustafsson, M. Comparison of Different IMAC Techniques Used for Enrichment of Phosphorylated Peptides. *Journal of Biomolecular Techniques*, **2005**, *June*, 91–103.
9. Thingholm, T.E.; Jorgensen, T.J.; Jensen, O.N.; and Larsen, M.R. Highly Selective Enrichment of Phosphorylated Peptides Using Titanium Dioxide. *Nature Protocols*, **2006**, *1*, 1929–1936.
10. Dionex Corporation, *Separation of Protein Phosphoisoforms Using Strong Anion-Exchange Chromatography*. Application Note 214, LPN 2134, 2009, Sunnyvale, CA.

SUPPLIERS

Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103, U.S.A. Tel: 800-521-8956. www.sigmaaldrich.com

Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, U.S.A. Tel: 608-274-4330. www.promega.com

Thermo Fisher Scientific (Pierce Biotechnology, Nalgene, Mallinckrodt Baker, J.T. Baker, and Savant Instruments), 308 Ridgefield Court, Asheville, North Carolina 28806-2210, U.S.A. Tel: 866- 984-3766. www.thermo.com

Sarstedt Inc., 1025, St. James Church Road, P.O. Box 468, Newton NC 28658-0468, U.S.A. Tel.: +1 828 465 4000. www.sarstedt.com

Honeywell International Inc., 101 Columbia Road, Morristown, NJ 07962, U.S.A. Tel: 973- 455-2000. www51.honeywell.com

MP Biomedicals LLC, 29525 Fountain Pkwy, Solon, Ohio 44139, U.S.A. Tel: 800-854-0530. www.mpbio.com

Praxair Specialty Gases and Equipment, 39 Old Ridge bury Road, Dansbury, CT 06810-5113, U.S.A. Tel: 877-772-9247. www.praxair.com

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S./Canada (847) 295-7500

South America
Brazil (55) 11 3731 5140

Europe
Austria (43) 1 616 51 25 Benelux (31) 20 683 9768 (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Sweden (46) 8 473 3380
Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190
Taiwan (886) 2 8751 6655

www.dionex.com

ISO 9001 REGISTERED

LPN 2537 PDF 06/10