DIONEX

Application Note 99

Peptide Mapping of Ovalbumin Using Reversed-Phase High-Performance Liquid Chromatography and Prediction of Phosphopeptide Elution

INTRODUCTION

Protein variants arise from post-translational modifications, such as glycosylation, oxidation, and phosphorylation. Variants of proteins produced for medicinal purposes can occur during manufacturing, handling, and storage, and can impact the activity and stability of the biotherapeutic.[1] Protein phosphorylations at serine, threonine, and tyrosine residues are the most-studied and best-characterized of all post-translational modifications (PTMs).[2] Peptide mapping is used routinely to study PTMs and is capable of identifying a single PTM difference in a protein. Peptide mapping is commonly used in the biopharmaceutical industry to establish product identity by confirming the primary structure of a product.[3] The chicken egg white protein ovalbumin has a molecular weight of 44,281 Da and is 386 amino acid residues long. Ovalbumin has two serine phosphorylation sites (S69 and S345) thus making it a good model for the analysis of protein phosphorylation.[4]

This application note (AN) describes a method to reduce, alkylate, and enzymatically digest ovalbumin into peptides using trypsin. These peptides are separated by a reversed-phase HPLC method using an Acclaim® 300, C18 column. The Acclaim 300 column with its 300 Å pore size and 3 µm silica particle size is designed for the rapid analysis of peptide mixtures or proteins. The stable bonding of the Acclaim 300, C18 columns results in predictable reversed-phase separations with minimal secondary interactions and makes them compatible with LC/MS applications. Treating the ovalbumin tryptic digest with alkaline phosphatase removes phosphate from the phosphopeptides. Comparing tryptic maps with and without alkaline phosphatase treatment enables a tentative identification of the phosphopeptides. The Dionex UltiMate® 3000 HPLC system equipped with an Acclaim 300, C18 column is an excellent solution for routine peptide mapping applications.

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EQUIPMENT:

Dionex UltiMate 3000 system consisting of:

- SRD-3600 Solvent rack with six degasser channels (P/N 5035.9230)
- Eluent organizer, including pressure regulator (eluents were maintained under helium or nitrogen head space 5–8 psi)
- 2 L glass bottles for each pump
- DGP 3600M UltiMate 3000 quaternary low-pressure proportioning analytical pump system (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)

SpeedVac® Evaporator System (Thermo Scientific Savant or equivalent) consisting of:

- SpeedVac Concentrator, Model SVC100
- Refrigerator vapor trap, Model RVT400
- Vacuum gauge, Model VG-5
- Welch DUOSEAL™ vacuum pump, Model 1402, capable of pulling 0.2 Torr (200 µm Hg) vacuum

Microcentrifuge, Model 5415C or equivalent

Microcentrifuge tubes, sterile with assembled screw cap, 1.5 mL (Sarstedt 72.692.005)

Filter unit, 0.2 µm nylon (Nalgene® Media-Plus with 90 mm filter, Nalgene Nunc International, P/N 164-0020) or equivalent nylon filter

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent)

0.3 mL Polypropylene (Vial Kit, P/N 055428) injection vials with caps

Reacti-Therm™ III heating module with Reacti-Block™ (Pierce Chemical Co., P/N 18940ZZ or equivalent)

Nitrogen 4.8 grade, 99.998%, <2 ppm oxygen (Praxair Specialty Gases)

SnakeSkin™ pleated dialysis tubing (Pierce, P/N 68100)

SnakeSkin dialysis tubing clips (Pierce, P/N 68011)

REAGENTS AND STANDARDS

Deionized water 18.2 (MΩ-cm)

Hydrochloric acid, ULTREX® II Ultrapure Reagent, 36.5–38.0% (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.* (Sigma-Aldrich, P6772-2KU)

Ovalbumin (albumin from chicken egg, Sigma-Aldrich, 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. Kit includes 100 mM ammonium bicarbonate or trypsin resuspension buffer, 1 mL vial (Promega, P/N V5111)**

Ammonium bicarbonate (Sigma, A6141)

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

Fetuin, fetal calf serum, lyophilized powder (Sigma-Aldrich, F2379, 250 mg, 48.4 KDa)

Micro BCA protein assay kit (Thermo Scientific, P/N 23235)

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

Sodium hydroxide, >98%, pellets, anhydrous (Sigma-Aldrich, P/NS8045)

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

*One DEA unit will hydrolyze 1 µmole of 4-nitrophenyl phosphate per min at pH 9.8 and 37 °C.

**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, 1mM $CaCl_2$ (pH 7.6), and the enzyme is diluted in 50 mM acetic acid.

CONDITIONS

Method

Columns	Acclaim 300, C18, 3 µm, Analytical, 2.1 × 150 mm, (P/N 060264) Acclaim 300, C18, 3 µm, Guard, 2 × 10 mm, (P/N 060395) Guard Kit (holder and coupler), (P/N 059526)
Eluents:	(A) 5% Acetonitrile, 0.1% TFA (B) 95% Acetonitrile, 0.1% TFA
Flow Rate:	0.2 mL/min
Temperature:	50 °C
Inj. Volume:	10 µL
Detection:	UV, 214 nm and 260 nm Minimum peak height setting for peak counts: 3.0 mAU (for ovalbumin and fetuin) and 1.0 mAU for cytochrome C

Gradient Method for Ovalbumin, Cytochrome C, and Fetuin

Time (min)	A%	B%
–33.0	95	5
0.0	95	5
45.0	20	80
60.0	20	80
60.1	0	100
65.0	0	100

PREPARATION OF SOLUTIONS AND REAGENTS

Eluents

5% Acetonitrile, 0.1% TFA

Add the contents of 1 ampule (1 mL) TFA to approximately 800 mL of 18.2 MΩ-cm DI water. Add 50 mL acetonitrile to the mix and make up the volume to 1000 mL with DI water.

95% Acetonitrile, 0.1% TFA

Add the contents of 1 ampule (1 mL) TFA to approximately 800 mL acetonitrile. Add 50 mL DI water to the mix and make up the volume to 1000 mL with acetonitrile.

As a precautionary note, TFA oxidizes over time and this may result in interfering chromatographic peaks. For best performance:

a. Use fresh TFA that is supplied in sealed ampules.
b. Prepare fresh 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 them with aluminum foil.
e. Though not used for this work, 0.08% TFA in mobile phase B will partially compensate for the rise in baseline observed when executing the gradient.

Sample Preparation Reagents

100 mM Ammonium Bicarbonate (pH 8.0–8.5)

Dissolve 1.58 g of ammonium bicarbonate in approximately 180 mL of 18.2 MΩ-cm DI water, bring the volume to 200 mL with DI water.

7 M Guanidine Hydrochloride Solution

Dissolve 3.34 g guanidine hydrochloride in 5 mL of 100 mM ammonium bicarbonate solution.

0.10 M HCl

Add 8.3 mL of concentrated HCl (11.65 M) to a volumetric flask containing 500 mL of DI water. Bring the volume to 1 L with DI water.

1 M Dithiothreitol (DTT) Solution

Dissolve 0.077 g DTT to 5 mL in 100 mM ammonium bicarbonate solution. This solution should be prepared just prior to use.

1 M Sodium Hydroxide Solution

Dissolve 0.200 g sodium hydroxide in 250 µL of 18.2 MΩ-cm DI water.

1 M Iodoacetic Acid

Dissolve 0.0465 g iodoacetic acid in 250 µL of 1 M sodium hydroxide solution. Warning: photosensitive, shield from light, and prepare fresh each day.

50 mM Ammonium Bicarbonate

Dissolve 7.91 g ammonium bicarbonate in approximately 1800 mL DI water and bring the volume to 2000 mL with DI water. Store at room temperature for up to 2 weeks.

Preparation of 50 mM Tris buffer (pH 9)

Dissolve 0.606 g of tris in 18.2 MΩ-cm DI water. Adjust to pH 9.0 (± 0.05) with 0.1 M HCl, if needed. Store at 4 ºC for up to 4 weeks.

Alkaline Phosphatase Working Solutions

To prepare 700 units/mL (0.7 units/µL) working solution of alkaline phosphatase, dissolve the entire contents of the 35% alkaline phosphatase, bovine intestinal mucosa bottle in 3 mL of 50 mM tris buffer. Swirl gently to dissolve. Dispense 200 µL aliquots into separate 1.5 mL micro-centrifuge tubes. Store the aliquots at –20 to –40 ºC for up to 6 months.

Preparation of Cytochrome C for System Qualification

Reconstitute the lyophilized cytochrome C (1.6 nmol/vial) in 200 µL of 5% acetonitrile, 0.1% TFA (eluent A) to obtain a concentration of 8 pmol/µL. Reconstitute the peptides by vortexing and wait for at least 10 min before use. Store all unused material at –20 °C for up to 2 weeks.

Procedure

Part I: System Qualification

1. Perform four replicate 10 µL injections of the cytochrome C tryptic digest solution for system qualification.
2. Confirm retention time RSD of ≤ 0.3% and peak area RSD ≤ 1.2% using a wavelength of 214 nm.
3. Confirm the presence of 21 ± 4 peaks, and the absence of any significant artifacts.

Part II: Reduction and Alkylation (Fetuin and Ovalbumin)

1. Weigh 5 mg of each protein (ovalbumin, and fetuin as a control, in triplicate) in a microcentrifuge tube and add 1 mL of the 7 M guanidine chloride solution. Gently mix to dissolve.
   a. Fetuin controls are used to demonstrate the reproducibility of the trypsin digestion method.
   b. A reagent control (without protein) is also prepared by adding 1 mL of 7 M guanidine chloride to a vial. The guanidine unfolds the protein by destabilizing both intra- and interchain electrostatic attractions.
2. Add 10 µL of 1 M DTT to each vial, followed by gentle mixing. Incubate for 60 min at 60 °C. Mix gently every 5 min. DTT reduces the protein's disulfide bonds.
3. Cool to room temperature.
4. Add 20 µL of 1 M iodoacetamide to the mixture. Gently mix and incubate in the dark for 30 min at room temperature. Iodoacetamide alkylates the cysteine and histidine residues and prevents proteins from assuming their native conformation, facilitating proteolytic digestion.
5. Add another 40 µL of 1 M DTT to each solution. Gently mix and incubate for 30 min at room temperature.
6. Dialyze (10 KD MW cutoff) each sample for 24 h at room temperature in 2 L of 50 mM ammonium bicarbonate while stirring with a magnetic stirrer. The dialysis exchanges the reduction, and alkylation buffer with ammonium bicarbonate is used for trypsin digestion in part III below.
7. After dialysis, a precipitate may form. Carefully measure the entire volume of the preparation, (including the precipitate) and transfer to a clean vial. Repeat the process for the reagent control. Calculate protein concentration based on the dialysis volume. If the protein concentration is unknown or questionable due to precipitation, determine the concentration using a Pierce micro BCA protein assay kit.[5]

Part III: Trypsin digest

Unmodified trypsin autoproteolyzes, generating fragments that can interfere with peptide mapping. The sequencing grade-modified trypsin, recommended for this method is altered by reductive methylation, making it resistant to proteolytic digestion.

1. The trypsin digest requires the following: reduced and alkylated protein, two vials of trypsin (20 µg/vial) and 100 mM ammonium bicarbonate (provided with the trypsin kit).
2. Prepare the trypsin by adding 25 µL of 100 mM ammonium bicarbonate to a lyophilized trypsin vial. Pipette 0.5–2 mg of each reduced and alkylated protein to a trypsin vial. Gently mix and transfer the contents to the second vial of trypsin. Digest fetuin in triplicate to evaluate reproducibility of the trypsin digestion method.

3. Based on the volume of protein solution added to the vial of trypsin, pipette an equivalent volume of the reagent control (from the reduction and alkylation) into a vial of trypsin. Transfer the contents of the first tube into a second vial of trypsin to prepare the reagent control.
4. Incubate the protein mixtures and the reagent control in a 37 °C water bath for 20 h. Freeze to stop the reaction.
5. Remove ammonium bicarbonate from the sample by placing frozen samples into a SpeedVac evaporator system. Add 100 µL of water to dissolve the dried solid.

Part IV: Evaluating the tryptic digestion using the fetuin tryptic digest control samples

1. Analyze three 10 µL injections of each fetuin tryptic digest control and the reagent control to evaluate the tryptic digestion.
2. Confirm the presence of a consistent peak count for all the three fetuin digests with no significant artifacts. Artifacts include extra peaks due to nonalkylated or nondigested protein or incompletely digested protein, nonspecific restriction peaks, and interfering contaminants from the digestion components.
3. Confirm a retention time RSD of ≤ 0.3% and a peak area RSD of ≤ 4.0% for replicate digests.

Part V: Alkaline phosphatase treatment

1. Alkaline phosphatase-treated tryptic digest: Pipette 96 µg of trypsin-digested ovalbumin or other peptide mixture to a vial containing 120 µL of 50 mM tris buffer (pH 9) and 107.6 µL of alkaline phosphatase (19 units). Add DI water to a total volume of 300 µL.
2. Untreated tryptic digest: Pipette 96 µg of trypsin-digested protein to a vial containing 227.6 µL of 50 mM tris buffer (pH 9). Add DI water to a total volume of 300 µL.
3. Reagent control with alkaline phosphatase: Pipette a volume of reagent control (equal to that used for the tryptic digest in steps 1 and 2) into a vial containing 120 µL of 50 mM tris buffer (pH 9.0) and 107.6 µL of alkaline phosphatase (19 units). Add DI water to a volume of 300 µL.
4. Reagent control without alkaline phosphatase: Pipette the same volume of reagent control used in step 3 into a vial containing 227.6 µL of 50 mM tris buffer (pH 9.0). Add DI water to make a volume of 300 µL.
5. Incubate the mixtures at 37 °C for 5 h. Freeze to stop the reaction. Store the mixtures at –20 °C for up to 6 months.

RESULTS AND DISCUSSION

Trypsin is an endopeptidase that specifically cleaves peptide bonds on the carboxyl side of lysine and arginine residues in proteins.[4] Peptides derived from the trypsin digestion of ovalbumin were separated by reversed-phase HPLC. High retention time repeatability is a requirement for peptide mapping. The predigested cytochrome C from Dionex was used to qualify the HPLC system. Figure 1 shows an overlay of four injections of the cytochrome C tryptic digest. Based on published amino acid sequence information, the tryptic digest of cytochrome C should result in 21 peptides that range in size from 1–37 amino acid residues. This is however, the theoretical peak count assuming no miscleavages

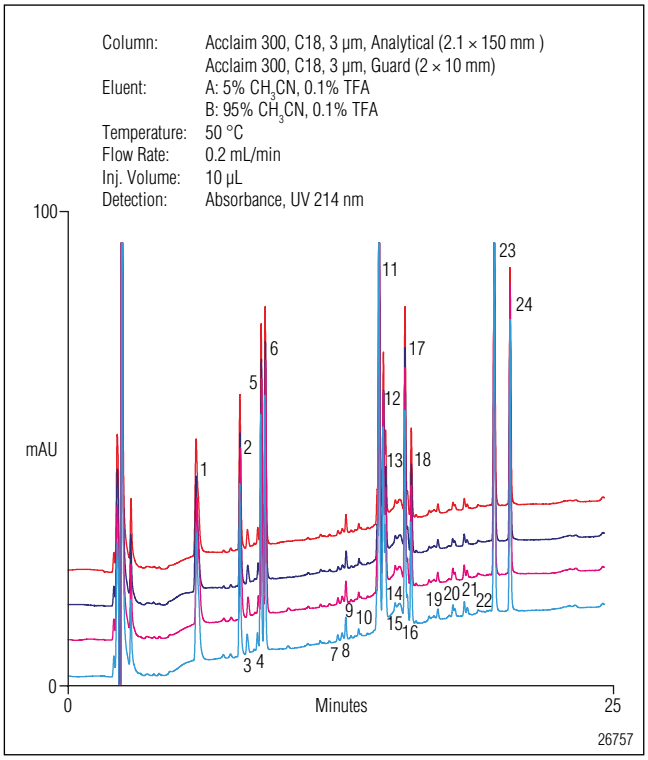

Figure 1. Overlay of four injections of the cytochrome C tryptic digest.

have occured, and that all the shortest peptides retain on the C18 column. The tryptic digest of this protein using our method contained 24 peaks. The peak count for the predigested cytochrome C surpassed the theoretical number of tryptic peptides. This discrepancy in the peak counts can result from peak threshold values set too low during peak integration. The presence of small amounts of contaminating proteins in the protein preparation, miscleavages, and contaminants in test-tubes and other labware can also increase peak counts. Commercially available trypsin can have small amounts of contaminating proteases that cause nonspecific digestion of proteins resulting in extra peaks. Post-translational modifications can also increase peak counts. Table 1 shows the average retention times and RSDs for eight representative early and late eluting peaks

Table 1. Retention Time and Peak Area Reproducibilities of Eight Arbitrarily Chosen Peaks for Four Replicate Injections of the Cytochrome C Tryptic Digest Measured at 214 nm

	Retention Time		Peak Area	
Peak #	Mean (min)	RSD	Mean (mAU/min)	RSD
1	5.99	0.30	5.750	0.22
5	8.36	0.28	0.430	0.6
11	12.90	0.02	0.350	0.45
14	14.70	0.04	2.250	1.1
18	15.70	0.01	3.160	1.08
21	17.20	0.04	0.170	0.69
25	19.90	0.04	7.290	1.09
26	20.60	0.03	5.460	0.55

*See Figure 1 for peaks analyzed

from the cytochrome C peptide map. The retention time RSDs for these peaks were less than 0.3, which indicates reproducible gradient delivery. Peak area RSDs were less than 1.2, indicating repeatable sample injections.

The trypsin digestion method was qualified based on results for three separate tryptic digests of fetuin and determining the peak count reproducibility. Figure 2 shows an overlay of three fetuin digests and shows a consistent peak count of 36 peaks for each of the samples. The peak area RSDs for the three digests were less than 3.5, indicating the digestion procedure was reproducible. Based on published amino acid sequence information, the tryptic digest of fetuin should result in 25 peptides. The peak count for fetuin surpasses the theoretical number of tryptic peptides and is attributed to miscleaved peptides and to the fact that fetuin is a glycosylated protein. A glycosylated peptide has a different retention than a nonglycosylated peptide with the same amino acid sequence. The number of sugar residues also affects retention of the peptide, and this results in extra peaks in a tryptic map. A comparison of peptide maps before and after treatment with enzymes such as PNGase F can indicate which peaks are glycopeptides. Reference 7 describes the isolation and enzymatic carbohydrate removal of at least nine different glycopeptides peaks present in fetuin. The 36 peaks that we observed in the

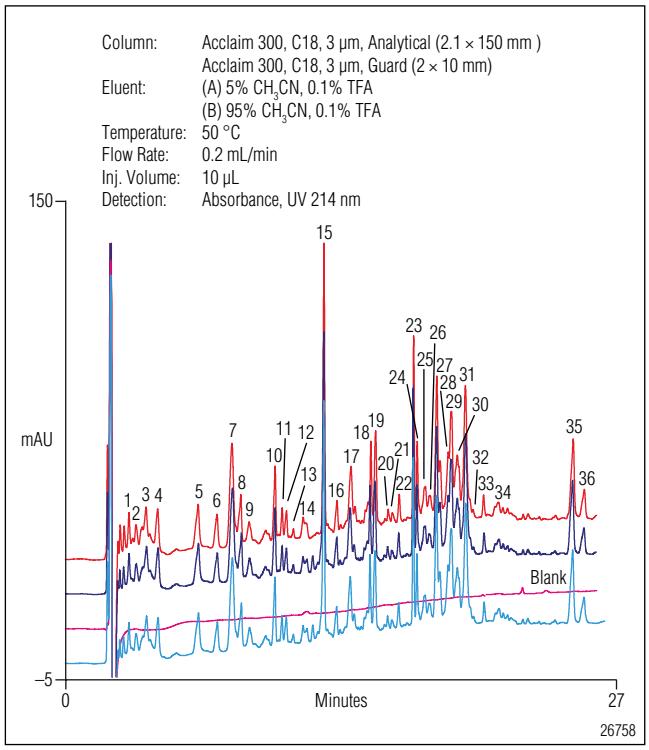

Figure 2. Overlay of three fetuin tryptic digests showing digestion and separation reproducibility.

fetuin peptide map are therefore, well within the expected peak count range for this glycosylated protein.

Table 2 shows the expected 34 peptides and their amino acid sequences for an ovalbumin tryptic digest. These peptides range in length from 2–33 amino acids. Figure 3 shows separation of a tryptic digest of ovalbumin. The 35 peaks found surpass the theoretical number of 34 peaks, but within the range of 34–36 peaks possible when both phosphorylated and dephosphorylated peptide forms are considered.

Table 2. The Expected Tryptic Peptides of Ovalbumin

Amino-terminal…….	
MGSIGAASME FCFDVFK	DEDTQAMPFR
ELK	VTEQESKPVQ MMYQIGLFR
VHHANENIFY CPIAIMSALA MVYLGAK	V ASMASEK
DST R	MK
TQINK	I LELPFASGTM SMLVLLPDEV SGLEQLESII NF
VVR	LTEWTS SNVMEER
F DK	K**
LPGFGD S*I EAQCGTSVNV HSSLR	IK**
DILNQ ITKPNDVYSF SLASR	VYLPR
LYAEE R	MK
YPILPEYLQ CVK	MEE K
ELYR	YNLTSVLMA MGITDVFSSS ANLSGISSAE SLK
GGL EPINFQTAAD QAR	ISQAVHA AHAEINEAGR
ELINSWV ESQTNGIIR	EVVG S*AEAGV DAASVSEFFR
N VLQPSSVDSQ TAMVLVNAIV FK	ADHPFLFCIK
GLWEK	HIATNAVLFF GR
AFK	CVSP
	……Carboxy-terminal

S* Phosphorylated Serine
** This is an unlikely cleavage as trypsin is a poor exopeptidase

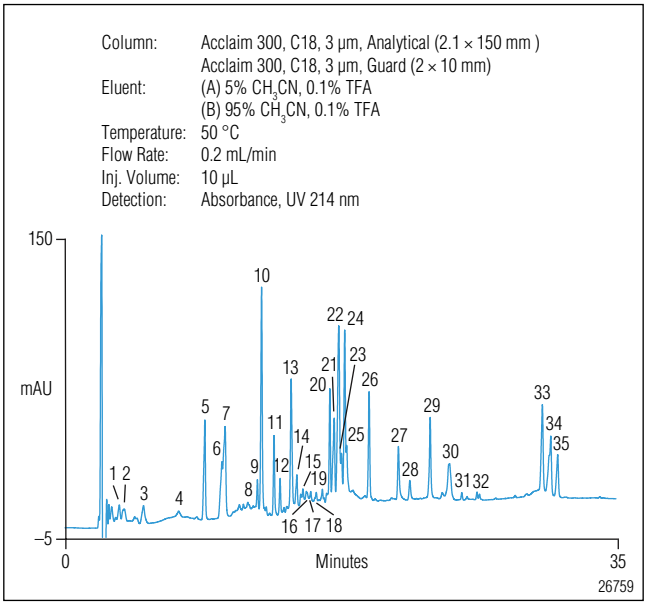

Figure 3. Tryptic map of ovalbumin.

Figure 4 shows a comparison of an ovalbumin peptide map with and without alkaline phosphatase treatment at 214 nm. The highlighted peaks 11 and 15 are believed to be the phosphorylated peptides EVVGS*AEAGV DAASVSEFFR and LPGFGDS*I EAQCGTSVNV HSSLR. When treated with alkaline phosphatase, the phosphates are removed from peptide peaks 11 and 15 resulting in a net loss of negative charge, therefore eluting later as peaks 11* and 15*. To evaluate the phosphopeptide identification and to suggest the elution order of the two phosphorylated peptides, the two separations were plotted at 260 nm, because both phosphopeptides contain phenylalanine. The phosphopeptide EVVGS*AEAGV DAASVSEFFR has two phenylalanine residues and should have a stronger absorbance at 260 nm compared to

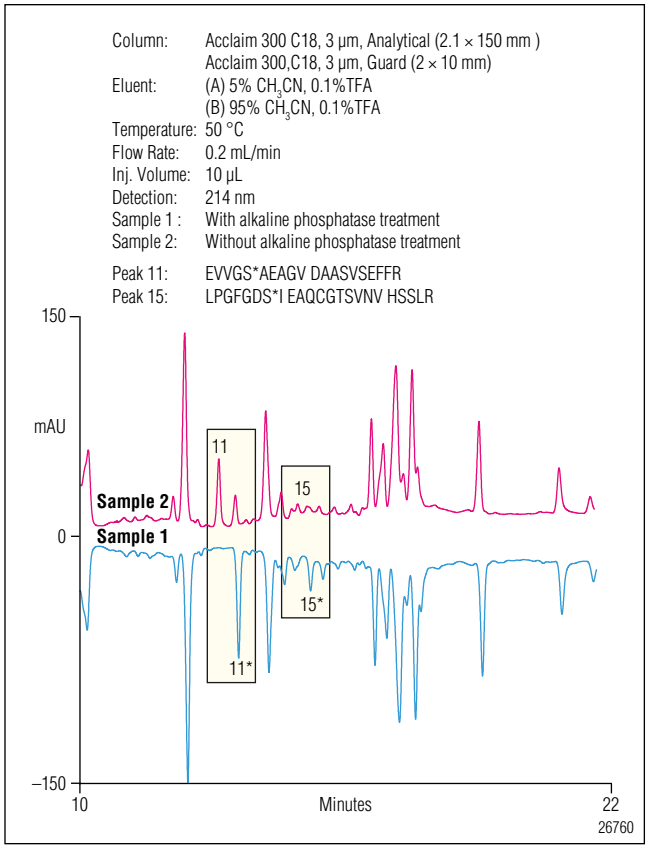

Figure 4. Ovalbumin peptide map with and without alkaline phosphatase digestion measured at 214 nm.

LPGFGDS*I EAQCGTSVNV HSSLR, that has only one phenylalanine. Figure 5 shows the data to be consistent with our hypothesis that peaks 11 and 15 are phosphorylated, and the absorbance intensities suggest they are EVVGS*AEAGV DAASVSEFFR and LPGFGDS*I EAQCGTSVNV HSSLR, respectively. The calculated average hydrophobicity (cal/mol)[6] for EVVGS*AEAGV DAASVSEFFR was 643 and for LPGFGDS*I EAQCGTSVNV HSSLR was 772 (ignoring the phosphate contribution in both peptides).

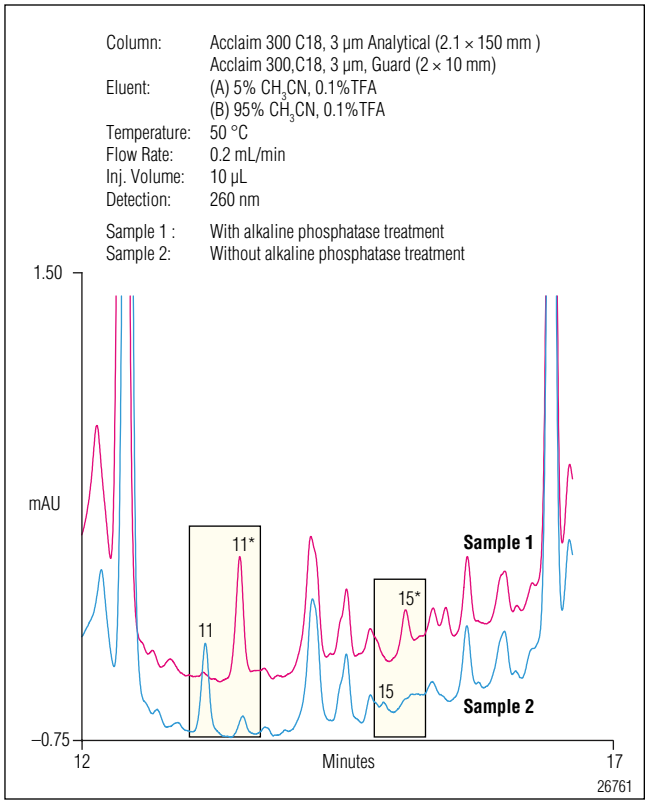

Figure 5. Ovalbumin peptide map with and without alkaline phosphatase digestion measured at 260 nm. The identification of the two phosphopeptide peaks is possible by comparing the relative peak heights at 214 and 260 nm wavelengths.

The calculated hydrophobicity of these two peptides was consistent with their elution order, supporting their identification in the peptide map.

CONCLUSION

This application note demonstrates a quick and simple method to perform peptide mapping using the Acclaim 300 C18 reversed-phase column designed for high-resolution separation of proteins and peptides. This method also demonstrates the capability of the Acclaim 300 column in combination with the UltiMate 3000 chromatography system to separate and identify phosphopeptides in a peptide map.

PRECAUTIONS

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

LIST OF SUPPLIERS

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

Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, 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, Tel: 866-984-3766, www.thermo.com

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

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

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

Praxair Specialty Gases and Equipment, 39 Old Ridgebury Road, Dansbury, CT 06810-5113, Tel: 877-772-9247, www.praxair.com.

REFERENCES

1. Kauffman, J.S. Analytical Testing to Support Biopharmaceutical Products. *Biopharm. International,* **2007,** *April,* 20–25.
2. Dionex Corporation. *Phosphopeptide Enrichment Using a TiO_2 Nano Precolumn,* Application Note 531, LPN 1898, Sunnyvale, CA.
3. Knight, Z.A.; Schilling, B.; Row, R.H.; Kenski, D. M.; Gibson, B.W.; Shokat, K.M. Phosphospecific Proteolysis for Mapping Sites of Protein Phosphorylation. *Nat. Biotechnol.* **2003,** *21,* 1047–1054.
4. Boerner, R.; Jeyarajah, S., Cook, S.; Acharya, P.S.; Henderson, I.; Schrimsher, J.L.; Shepard, S.R. Identifying and Modulating Disulfide Formation in the Biopharmaceutical Production of a Recombinant Protein Vaccine Candidate. *J. Biotechnol.* **2003,** *103,* 257–271.
5. Pierce Biotechnology. *Instructions for BCA Protein Assay Kit,* Doc. No. 1296.
6. Bigelow, C. C.; Channon, M. Hydrophobicity of Amino Acids and Proteins. Handbook of Biochemistry and Molecular Biology. 3rd Ed. Proteins. Volume 1. Fasman, G.D. (ed). CRC Press, Cleveland, OH, 1976, 209–243.
7. Rohrer, J.S.; Cooper, G.A.; Townsend, R.R. Identification, Quantification, and Characterization of Glycopeptides in Reversed-Phase HPLC Separations of Glycoprotein Proteolytic Digests. *Anal. Biochem.* **1993,** *212,* 7–16.

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LPN 0581-01 PDF 12/09

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