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Separation of Protein Phosphoisoforms Using Strong Anion-Exchange Chromatography

INTRODUCTION

Posttranslational modifications (PTMs) of proteins are considered one of the major determinants of an organism's complexity. Protein phosphorylation is one of the most studied PTMs because it provides a rapid, reversible means of regulating protein activity and numerous other cellular processes including cell differentiation, proliferation, and migration.¹ It is estimated that approximately thirty percent of all proteins in a cell are phosphorylated at any given time.² Changes in levels of phosphorylated isoforms often signal developmental or pathological disorders.³ To better understand the role of phosphorylation in regulation, and for clinical diagnostics, it is important to differentiate phosphorylated forms of proteins from their dephosphorylated forms.

Conventional antibody-based phosphopeptide mapping methods used to assay for protein phosphorylation are laborious, difficult to control, and require the use of large amounts of ³²P. Additionally, these methods can only be used when the protein of interest is relatively abundant in the tissue homogenate. Mass spectrometric analysis of protein phosphorylation has several inherent problems. Phosphopeptides are often missed during the mass spectrometric analysis due to (1) selective suppression of ionized phosphorylated proteins relative to large amounts of ionized unphosphorylated proteins, and (2) lower detection efficiencies of phosphopeptides compared to their unphosphorylated cognates.⁴

This application note demonstrates the use of the ProPac[®] SAX-10 column for separation of phosphorylated protein variants. This novel anion-exchange column consists of linear polymer chains grafted onto polymer beads coated with a hydrophilic surface. An inert liquid chromatography instrument is used to eliminate the possibility of complex formation between phosphorylated proteins and leachable transition metals (e.g., iron) often observed using stainless steel-based chromatography systems. The high efficiency of the strong anion-exchange resin allows the resolution of several phosphorylated isoforms using a simple gradient. Comparisons of chromatograms for selected model phosphoproteins with and without alkaline phosphatase treatment, which cleaves phosphate from the amino acid side chains, shows that peaks are resolved based on the extent of phosphorylation.

The phosphate content of the phosphorylated proteins is determined using ion chromatography with suppressed conductivity detection, as described in Application Note 210. Here, we present a simple and quick method combining anion-exchange chromatography and UV detection to analyze phosphorylated isoforms of different phosphoproteins: ovalbumin, synthetically phosphorylated BSA, and casein.

EQUIPMENT

ICS-3000 liquid chromatography system consisting of:

- ICS-3000 SP single pump (P/N 061707) or DP dual pump (P/N 061713)
- ICS-3000 VWD variable wavelength detector (P/N 064653) with PEEK™ flow cell, 10 mm, 11 µL (P/N 066346)
- ICS-3000 TC (P/N 064444) or DC (P/N 061767)
- AS Autosampler (P/N 056859)

Or

UltiMate® 3000 Titanium system consisting of:

- 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 head space (5–8 psi)
- LPG 3400AB Quaternary Analytical Pump (P/N 5037.0015) or DGP-3600AB Dual Ternary Analytical Pump (P/N 5037.0014) for dual gradient capability
- WPS-3000TBPL Biocompatible Analytical Autosampler (P/N 5823.0020)
- TCC-3000 Column Compartment without switching valves (P/N 5722.0000) or TCC-3200B Column Compartment with 2 PEEK 10-port, 2-position valves (P/N 5723.0025) for added capability
- VWD-3400 Variable Wavelength Detector without flow cell (P/N 5074.0010) or PDA-3000 Photodiode Array Detector (P/N 5080.0020)
- Biocompatible Analytical Flow Cell for VWD (P/N 6074.0200) or Biocompatible Analytical Flow Cell for PDA (P/N 6080.0220)

Chromleon® 6.8 Chromatography Management Software

Other Equipment

SpeedVac™ Evaporator System (ThermoQuest Savant E/C Division) consisting of :

- SpeedVac, Model SVC100
- Refrigerator Vapor Trap, Model RVT400
- Vacuum Gauge, Model VG-5
- Welch Duo-Seal Vacuum Pump, Model 1402 capable of pulling 0.2 Torr (200 µm Hg) vacuum

Microcentrifuge, Model 5415C (Eppendorf)

Sterile assembled micro-centrifuge tubes with 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

Biodialyser™ microdialysis system with 500 dalton molecular weight cutoff (MWCO) cellulose acetate membranes (AmiKa, Inc., The Nest Group, P/N SSM0500)

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

Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair Specialty Gases)

REAGENTS AND SAMPLES

Deionized water 18.2 (MΩ-cm)

Hydrochloric Acid, 36.5-38.0% (J.T.Baker, 9530-33)

Tris (Base), ACS Reagent (J.T. Baker, X171-7)

Sodium Chloride, ≥99.5% (Sigma-Aldrich, S7653)

Alkaline phosphatase, bovine intestinal mucosa, lyophilized, 35% protein (Sigma-Aldrich, P6772-2KU)

Ovalbumin from chicken egg white, Grade V, VI, >98%, lyophilized powder (Sigma-Aldrich, A5503, and A2512, 45 kDa), five lots

Bovine serum albumin (BSA), ≥96%, (Sigma-Aldrich, A6003, 69 kDa)

Micro BCA™ Protein Assay kit (Pierce Biotechnology, 23250)

Phospho-serine-BSA, 2 mg/mL in 10 mM phosphate buffer (pH 7.4), (Sigma-Aldrich, P3717, 45 kDa)

2-Mercaptoethanol, Molecular biology grade (Sigma-Aldrich, M6250)

Imidazole, Molecular biology grade (Sigma-Aldrich, I5513)

Urea, Molecular biology grade (Sigma-Aldrich, U5378)

Casein from bovine milk (Sigma-Aldrich, C8654)

Dephosphorylated casein from bovine milk (Sigma-Aldrich, C4032)

CONDITIONS

Chromatography Conditions for the Separation of Ovalbumin and BSA Isoforms

Columns: ProPac SAX-10 column,
4.0 × 250 mm, (P/N 054997)
ProPac SAX-10G column,
4.0 × 50 mm, (P/N 054998)
Flow Rate: 1.0 mL/min
Temperature: 25 °C
Inj. Volume: 10 µL
Detection: UV, 280 nm

Eluents and Gradient Program for Ovalbumin:

A: 20 mM Tris (pH 8.5)
B: 20 mM Tris,
500 mM Sodium chloride (pH 8.5)

Time (min)	A%	B%
0.0	100.0	0.0
15.0	87.5	12.5
17.0	76.0	24.0
19.0	76.0	24.0
19.1	100.0	0.0
25.0	100.0	0.0

Eluents and Gradient Program for BSA and Synthetically Phosphorylated BSA:

Eluents: A: 20 mM Tris (pH 8.5)
B: 20 mM Tris,
2 M Sodium chloride (pH 8.5)

Time (min)	A%	B%
0.0	100.0	0.0
2.0	100.0	0.0
30.0	10.0	90.0
39.0	10.0	90.0
39.1	100.0	0.0

Chromatography Conditions for Separation of Casein Isoforms

Columns: ProPac SAX-10 column,
4.0 × 250 mm, (P/N 054997)
ProPac SAX-10G column,
4.0 × 50 mm, (P/N 054998)
Flow Rate: 1.0 mL/min
Temperature: 25 °C
Inj. Volume: 75 µL
Detection: UV, 280 nm
Eluents: A: 4 M Urea, 0.1 M 2-mercaptoethanol,
10 mM imidazole, pH 7.0
B: 1 M Sodium chloride in 4 M urea,
0.1 M 2-mercaptoethanol,
10 mM imidazole, pH 7.0

Gradient Program:

Time (min)	A%	B%
-10.0	100.0	0.0
0.0	100.0	0.0
3.0	95.0	5.0
30.0	70.0	30.0
32.0	70.0	30.0
35.0	100.0	0.0

Phosphate Determination

Phosphate determinations were performed using an IonPac® Fast Anion IIIA analytical column (3 × 250 mm, P/N 062964) using 25 mM potassium hydroxide. Method details are described Application Note 210.⁷

PREPARATION OF SOLUTIONS AND REAGENTS

20 mM Tris (pH 8.5)

Dissolve 2.42 g of Tris in approximately 1700 mL of 18.2 MΩ-cm DI water. Adjust the pH to 8.5 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 2 L with DI water and filter through a 0.2 µm filter.

20 mM Tris, 200 mM Sodium Chloride (pH 8.5)

Dissolve 2.42 g of Tris and 23.38 g of sodium chloride in approximately 1700 mL of 18.2 MΩ-cm DI water. Adjust the pH to 8.5 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 2 L with DI water and filter through a 0.2 µm filter.

20 mM Tris, 2 M Sodium Chloride (pH 8.5)

Dissolve 2.42 g of Tris and 233.76 g of sodium chloride in approximately 1700 mL of 18.2 MΩ-cm DI water. Adjust the pH to 8.5 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 2 L with DI water and filter through a 0.2 μm filter.

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate (Na_2HPO_4) in water to a final solution volume of 1.0 L.

4 M Urea, 0.1 M 2-mercaptoethanol, 10 mM Imidazole, pH 7.0

Dissolve 240.24 g of urea in approximately 700 mL of water. Dissolve 0.6808 g imidazole in the urea solution. Add 90 μL of 2-mercaptoethanol and adjust the pH to 7.0 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 1 L with DI water and filter through a 0.2 μm filter.

1 M Sodium Chloride in 4 M Urea, 0.1 M 2-Mercaptoethanol, 10 mM Imidazole, pH 7.0

Dissolve 58.44 g sodium chloride in approximately 700 mL of water. Dissolve 240.24 g in the urea solution, and then dissolve 0.6808 g of imidazole to the urea solution. Add 90 μL of 2-mercaptoethanol and adjust the pH to 7.0 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 1 L with DI water and filter through a 0.2 μm filter.

SAMPLE PREPARATION

5 mg/mL Ovalbumin Standard

Dissolve 20 mg ovalbumin into 4 mL DI water in a 20 mL scintillation vial. Gently swirl the solution until thoroughly mixed. Dispense 200 μL aliquots of the 5 mg/mL ovalbumin into 1.5 mL sterile micro-centrifuge tubes. Store the solutions at –20 °C or less.

5 mg/mL Bovine Serum Albumin (BSA) Standard

Dissolve 20 mg BSA into 4 mL DI water in a 20 mL scintillation vial. Gently swirl the solution until thoroughly mixed. Dispense 200 μL aliquots of the 5 mg/mL BSA into 1.5 mL sterile micro-centrifuge tubes. Store the solutions at –20 °C or less.

1 mg/mL Casein Standard

Dissolve 20 mg casein into 20 mL eluent A. Gently swirl the solution to mix. Dispense 200 μL aliquots of the 1 mg/mL casein into 1.5 mL sterile micro-centrifuge tubes. Store the solutions at –20 °C or less.

1 mg/mL Dephosphorylated Casein Standard

Dissolve 20 mg dephosphorylated casein into 20 mL eluent A. Gently swirl the solution to mix. Dispense 200 μL aliquots of the 1 mg/mL casein into 1.5 mL sterile micro-centrifuge tubes. Store the solutions at –20 °C or less.

Dephosphorylation of Protein with Alkaline Phosphatase

Digest 96 μg of ovalbumin using 19 units of alkaline phosphatase and 120 μL of 50 mM Tris buffer (pH 9). Bring the total volume to 300 μL using DI water. Incubate the mixture on a heat block at 37 °C for 5 h. Centrifuge the digested protein at 14,000 rpm for 5 min, and pipette the supernatant into 1.5 mL sample vials. Prepare, heat, and centrifuge control samples of Tris buffer, alkaline phosphatase, and proteins in buffer in the same manner as the protein samples. See Application Note 210 for preparation of reagents and a detailed discussion of the assay. Determine total protein in all proteins according to the instructions on the Micro-BCA Protein Assay kit.

Microdialyze ovalbumin fractions overnight in water in order to remove salt, then dry in the SpeedVac. Because the fractions are eluted in high concentrations of salt, it is important to remove the salt to allow quantitation of the phosphate that is present at much lower concentrations. Resuspend the dried fractions in 50 mM Tris buffer prior to alkaline phosphatase digestion.

RESULTS AND DISCUSSION

The ProPac SAX-10 column was selected for the separations discussed in this application note because it is a high resolution column well suited for the separation of closely related anionic protein variants. The column has a nonporous core particle and a hydrophilic layer over the core to prevent unwanted secondary interactions. The strong anion-exchange moieties grafted onto the hydrophilic layer provide increased capacity, while still maintaining high resolution.

Ovalbumin is a 43 kDa glycoprotein present in avian egg white with two potential serine phosphorylation sites at positions 68 and 344.^{5,6} Reported phosphate values for ovalbumin preparations vary from 1.75 to 1.89, indicating that both sites are phosphorylated. Figure 1 shows ovalbumin (magenta trace) and its dephosphorylated form after alkaline phosphatase treatment (blue trace). The figure shows

that treatment with alkaline phosphatase results in earlier elution of peaks, which is consistent with a loss of negative charge. The early eluting peaks are unaffected by alkaline phosphatase treatment. Ovalbumin has been reported to have four isoforms with varying phosphate content, one with zero, two with one and one with two phosphates.⁶ The figure shows that treatment with alkaline phosphatase reduced the number of peaks from nine to one large and three small peaks. The first three peaks in the dephosphorylated sample correspond to the peaks in the native sample, while peak 4A corresponds to the initial shoulder of peak 4 in the native sample. This confirms that the separation of the ovalbumin sample is in part due to differences in the phosphorylation state of the protein backbone. Peak 2 appears to represent ovalbumin with no attached phosphate. Peaks 1 and 3 in the dephosphorylated sample may represent the known sequence microheterogeneity of ovalbumin. Peaks 2, 4, and 7 in the native preparation seem to represent varying phosphate contents, and all the minor peaks (1, 3, 5, and 6) represent different phosphorylation states of ovalbumin due to sequence microheterogeneity.

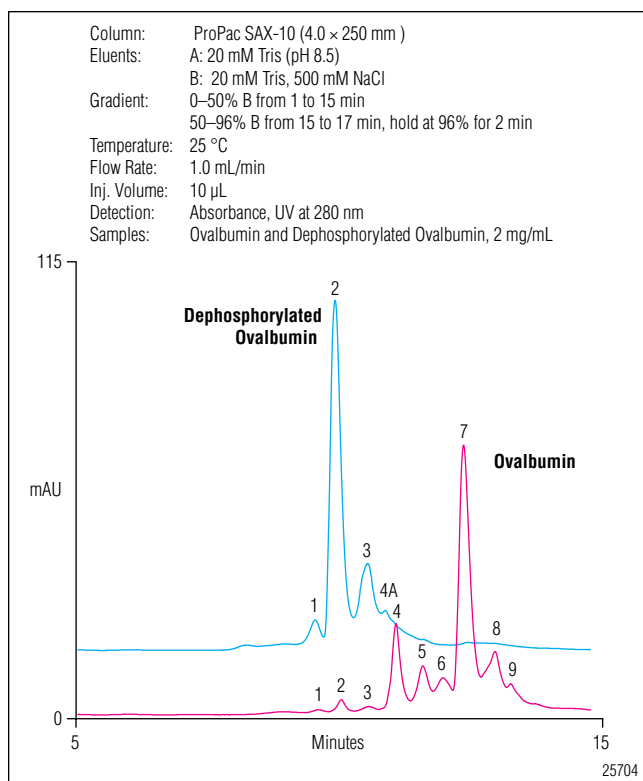


Figure 1. Separation of ovalbumin before and after alkaline phosphatase treatment (dephosphorylation) using the ProPac SAX-10 column

Figure 2 shows the separation of five lots of ovalbumin before and after dephosphorylation with alkaline phosphatase. The magenta trace shows the separation of native ovalbumin and the blue trace shows the separation of dephosphorylated ovalbumin. All the traces show a reduction of peaks after alkaline phosphatase treatment. Based on the chromatography in Figure 1 and reports of varying phosphate content, peak 1 was identified as an isoform with no phosphate, peak 2 as an isoform with one phosphate, and peak 3 as an isoform with two phosphates. Following alkaline phosphatase treatment, all the traces show the presence of only peak 1, an ovalbumin isoform with zero phosphates.

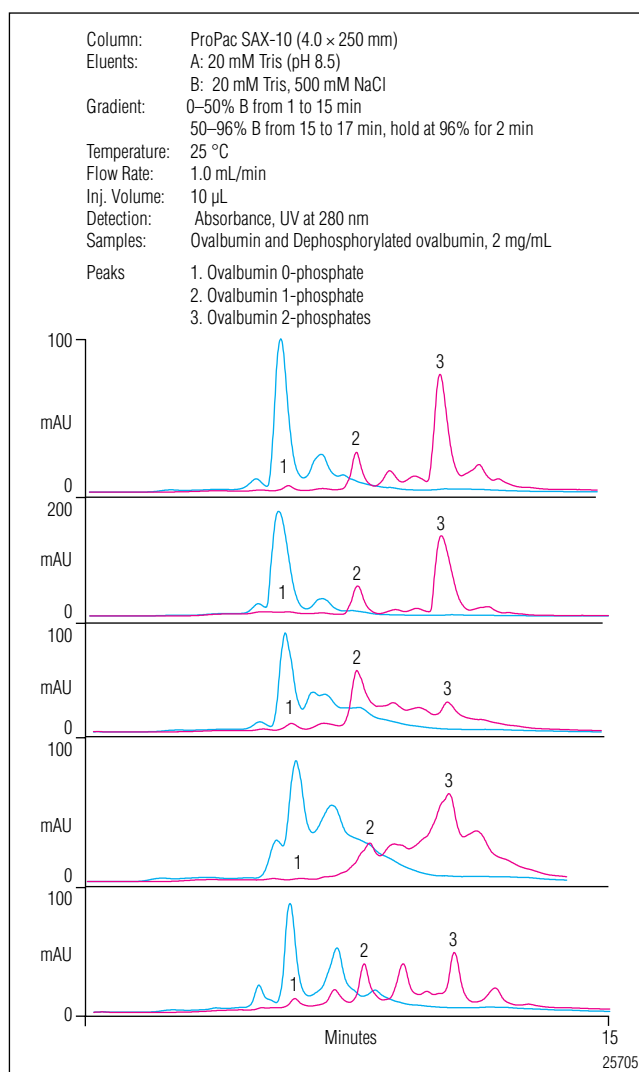


Figure 2. Separations of 5 lots of ovalbumin and their dephosphorylated forms using the ProPac SAX-10 column

Table 1 shows the ratios of the peak areas of peaks 2 and 3 to the total peak area prior to alkaline phosphatase treatment and the amount of phosphate removed after treatment, as measured by ion chromatography.⁷ The amount of phosphate in each ovalbumin lot was predicted based on the sum of weighted peak area ratios of peaks 2 and 3. The predicted values for phosphate content were consistent with the IC determinations, suggesting that the assignment of peaks 2 and 3 as single and doubly phosphorylated isoforms of ovalbumin is correct.

Table 1. Phosphorylation Analysis of Five Commercial Ovalbumin Samples				
Protein Grade and Lot	Ratio of peak 2 area to total peak area mAU*min	Ratio of peak 3 area to total peak area mAU*min	*Predicted Mole Ratio (Phosphate/Protein)	Found Mole Ratio (Phosphate/Protein)
Ovalbumin, grade V, lot 1	0.27	0.61	1.49	1.64
Ovalbumin, grade VI, lot 5	0.24	0.63	1.50	1.22
Ovalbumin, Grade V, lot 2	0.57	0.20	0.97	1.14
Ovalbumin, grade VI, lot 4	0.22	0.39	1.00	0.78
Ovalbumin, grade VI, lot 3	0.30	0.26	0.82	0.37

* Predicted mole ratio of phosphate to protein calculated based on the following formula: Predicted mole ratio of phosphate to protein = 2(ratio of peak 3) + ratio of peak 2

Fractions shown in Figure 3 were collected from one lot of ovalbumin, and the phosphate content of each was assayed as described in Application Note 210.⁷ After dialysis the three collected fractions were treated with alkaline phosphatase and then analyzed for free phosphate. Table 2 presents the moles of phosphate measured per mole of protein. This data confirms the conclusion that peaks 2 and 3 are singly and doubly phosphorylated isoforms of ovalbumin.

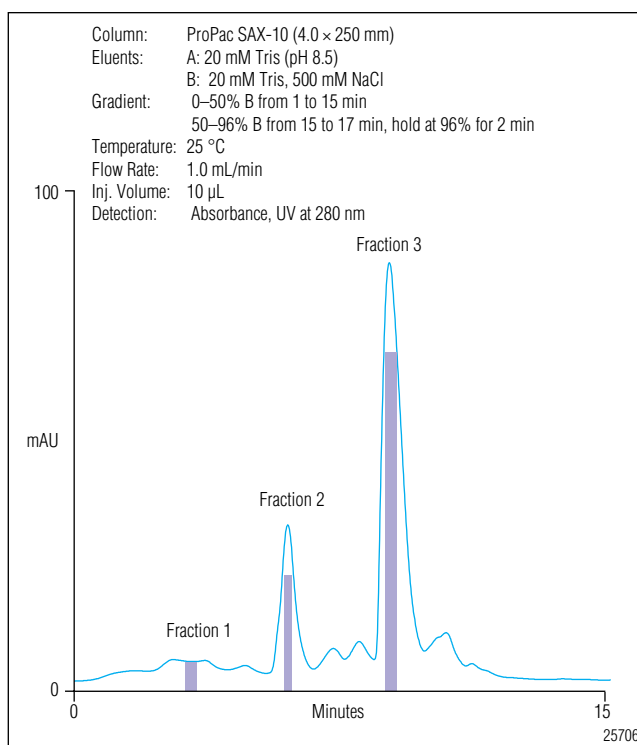


Figure 3. UV chromatogram showing the collection of fractions from a lot of ovalbumin separated on a ProPac SAX-10 column.

Table 2: Phosphate Content Of Ovalbumin Fractions Prepared By ProPac SAX-10 Separation	
Sample	Moles of Phosphate/Mole of Protein
Fraction 1	0.00
Fraction 2	0.93
Fraction 3	1.97

Bovine serum albumin (BSA) is a 66 kDa single polypeptide chain consisting of 583 amino acid residues.⁸ The pI of BSA is 5.1 with a charge of -17 at pH 7.4.⁹ O-phospho-L-serine BSA is a commercially available synthetically phosphorylated form of bovine serum albumin. The ProPac SAX-10 column was selected for this separation based on pI of the protein. Figure 4 shows the separations of BSA and a commercially available synthetically phosphorylated form on a ProPac SAX-10 column. Phosphorylated BSA is retained longer on the column than BSA as would be predicted based on its increased negative charge.

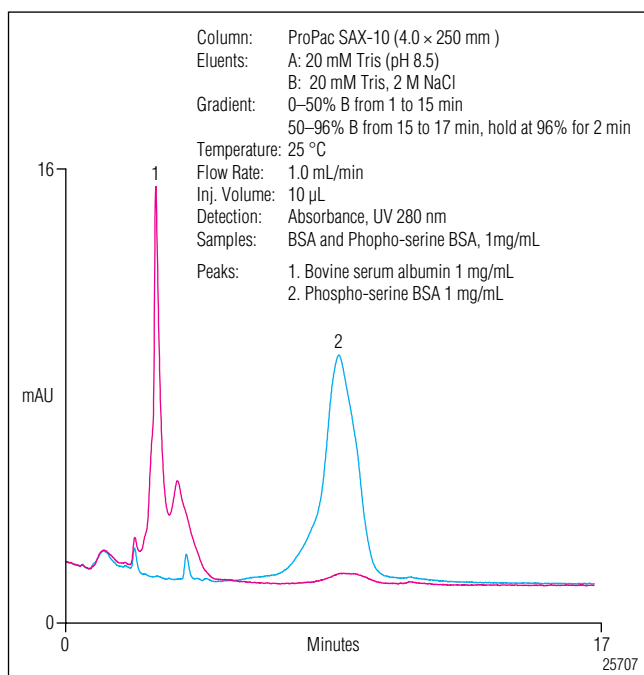


Figure 4. Separations of bovine serum albumin and its synthetically phosphorylated form using the ProPac SAX-10 column

Caseins are a unique group of acid insoluble phosphoproteins that represent approximately eighty percent of the total protein in bovine milk.¹⁰ Caseins can be differentiated according to the homology of their primary structures and based on their relative electrophoretic mobility into the following families: α 1, α 2, β , and κ -caseins. The α 1 and α 2 subunits comprise 40% and 10% of casein in bovine milk and contain 8 and 11 phosphoserine residues respectively. The β and κ -subunits constitute 45% and 5% of casein in bovine milk and contain 5 and 1 phosphoserine residues respectively.¹¹ Figure 5 shows the separations of casein and a commercially available dephosphorylated form of casein on a ProPac SAX-10 column. This figure reveals that casein is a complex sample with considerable heterogeneity and that some of its retention is due to phosphorylation. The majority of the peaks in the commercially available dephosphorylated form of casein show less retention than the majority of the peaks in the untreated casein preparation due to a net loss of negative charge.

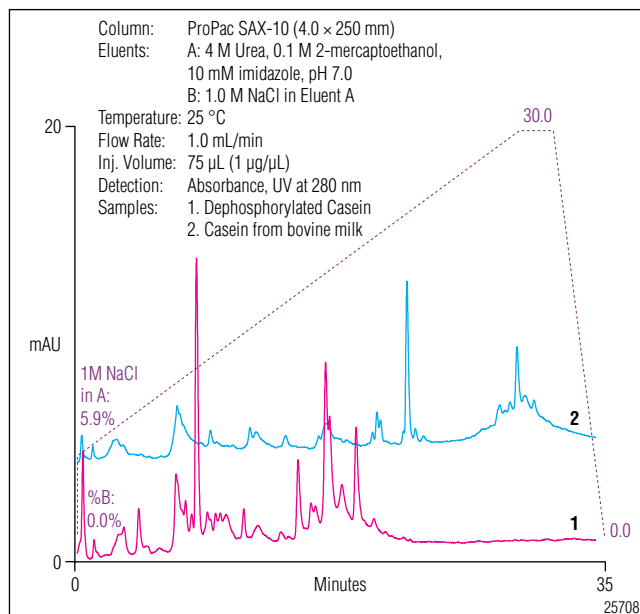


Figure 5. Separations of casein from bovine milk and commercially available dephosphorylated casein using the ProPac SAX-10 column

SUMMARY

Phosphorylation of proteins confers variability in the protein's charge, resulting in charged isoforms. Grafted pellicular anion and cation exchangers are useful for resolution of charged protein isoforms.¹² In this application note we demonstrate a quick and simple method to separate phosphorylated isoforms from their dephosphorylated forms using the ProPac SAX-10, a high efficiency strong anion-exchange column. This column can separate proteins with differences as small as one or more phosphates on a single amino acid residue. Phosphoprotein analysis on an inert PEEK or titanium chromatography system ensures that there are no protein-transition metal complexes that reduce protein recovery, or transition metal fouling of the ProPac column.

PRECAUTIONS

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

SUPPLIERS

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

Mallinckrodt Baker, Inc., 222 Red School Lane, Phillipsburg NJ 08865, Tel: 908-859-2151, www.solvitcenter.com.

Nalge Nunc International, 75 Panorama Creek Drive, Rochester, NY 14625. U.S.A., Tel: 800-625-4327, www.nalgenunc.com.

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

Pierce Biotechnology, Inc. P.O. Box 117, Rockford, IL 61105, Tel: 800-874-3723, www.piercenet.com.

Savant Instruments/E-C Apparatus (a division of Thermoquest), 100 Colin Drive, Holbrook, NY 11741-4306 USA, Tel: 800-634-8886, www.thermoquest.com.

The Nest Group, Inc., 45 Valley Road, Southborough, MA 01772-1323. Tel: 800-347-6378 www.nestgrp.com

Eppendorf, One Cantiague Road, PO Box 1019, Westbury, NY 11590, Tel: 800-645-3050, www.eppendorf.com

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

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