High-Performance Ion-Exchange Chromatography for Determination of Protein Microheterogeneity

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ABSTRACT

Protein microheterogeneity is either the result of genetic variants or posttranslational modifications. The separation of individual variants is difficult and requires high-resolution separations. While reversed-phase HPLC is an effective technique for chromatographic separation of many types of protein variants, those types of variants characterized with even a slight change in net charge are best resolved using an ion-exchange separation. This paper presents representative examples of high-resolution ion-exchange separations of protein variants using inert biocompatible chromatography systems. We show separations of phosphoprotein variants of ovalbumin, synthetic bovine serum albumin, and beta-casein; deamidation variants of ribonuclease A and monoclonal antibodies; and C-terminal lysine, acidic, and basic variants of monoclonal antibodies, using anion- and cation-exchange chromatography columns designed for protein separations. For most examples, these separations were improved compared to earlier work by optimizing the eluent and/or separation program.

INTRODUCTION

Protein microheterogeneity can impact a protein's activity and stability as a biotherapeutic. Monitoring stability of therapeutic proteins is regarded as essential for demonstrating safety and efficacy of these drugs, and is expected by the US FDA and other regulating agencies.

In this poster, we used ProPac® ion-exchange columns (Dionex), consisting of linear polymer chains grafted onto hydrophilic polymer beads, to separate a variety of protein variants. The high efficiency of the ProPac ion-exchange resins allowed the resolution of different protein isoforms using simple gradients. The physicochemical properties of these supports eliminated secondary (nonionic) interactions between the protein analytes and the stationary phase, affording minimal band broadening and high selectivity. An inert liquid chromatography instrument (either an UltiMate® 3000 Titanium HPLC system or an ICS-3000 IC system) was used to eliminate the possibility of complex formation between charged proteins and leachable transition metals (e.g., iron), often observed using stainless steel-based chromatography systems. The titanium HPLC and PEEK™ IC system flow paths ensured that neither solvent nor sample came into contact with stainless steel, iron, or other transition metals, removing the risk of contaminating the column. This poster shows the separation of hemoglobin variants (the result of differences in both amino acid sequence and glycation),[1] C-terminal lysine variants of monoclonal antibodies,[2] deamidation variants of ribonuclease A and monoclonal antibodies,[3,4] and phosphorylated variants of bovine serum albumin, casein, and ovalbumin.[5]

EXPERIMENTAL

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

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 Thermostatted Column Compartment without Switching Valves (P/N 5722.0000) or TCC-3200B Thermostatted Column Compartment with 2 PEEK ten-port two-position valves (P/N 5723.0025) for added productivity

VWD-3400 Variable Wavelength Detector (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)

ICS-3000 (PEEK) 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)

Software:

Chromeleon® Chromatography Data Management System

ProPac Columns:

ProPac WCX-10 analytical, 4 × 250 mm (P/N 054993)

ProPac SCX-10 analytical, 4 × 250 mm (P/N 054995)

ProPac SAX-10 analytical, 4.0 × 250 mm, (P/N 054997)

and SAX-10G guard, 4.0 × 50 mm, (P/N 054998)

For experimental and chromatographic conditions, refer to the figures and the respective references described in the Results and Discussion section below.

RESULTS AND DISCUSSION

Amino Acid Sequence and Glycation Variants

The ProPac SCX-10 strong cation-exchange column resolves hemoglobin variants, such as HbS, HbC, HbF, HbA_0, HbA_{1a1}, HbA_{1b2}, HbA_{1b1}, HbA_{1b2}, HbA_{1c}, Pre-HbA_{1c}, HbA_{1d1}, HbA_{1d2}, HbA_{1d3}, HbA_{1e}, and HbA_2 resulting from minor variations in amino acid sequence and glycosylation.[1] This application with its experimental conditions is featured in Dionex Application Note 126.[1] Figure 1 presents the separation of variants HbS, HbC, HbF, HbA_{1c}, HbA_0, and HbA_2.

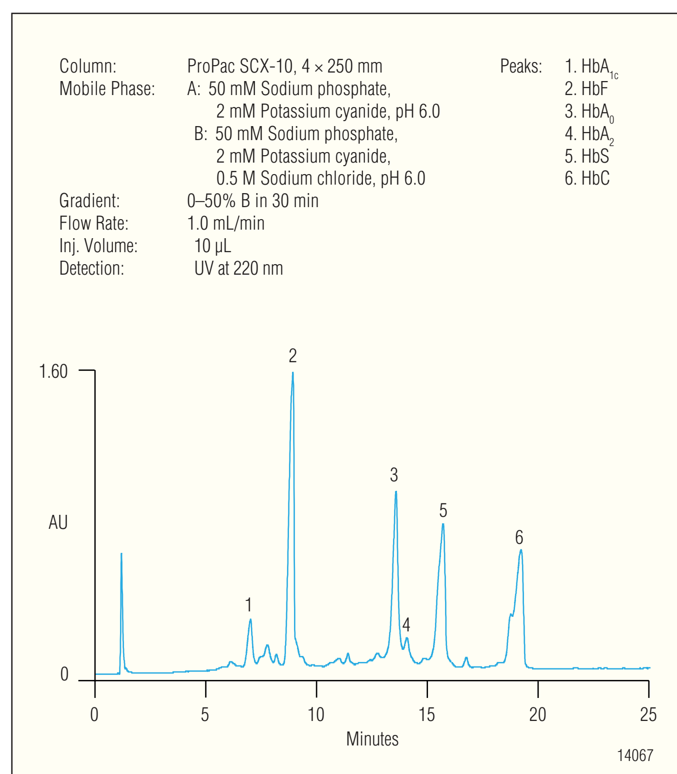

Figure 1. Separation of amino acid sequence and glycosylation variants of hemoglobin, including glycosylated (HbA_{1c}), fetal (HbF), sickle cell (HbS), normal (HbA), and C-type (HbC; causing mild chronic hemolytic anemia).

C-Terminal Lysine Variants

The variation in the number of C-terminal lysine residues on the heavy chain of monoclonal antibodies (MAb) is a common structural variation. C-terminal lysine or arginine residues are often absent in proteins isolated from mammalian cell cultures, even though their presence may be expected on the basis of the gene sequence. This discrepancy, which is common in plasma-derived proteins, results from the activity of one or more basic carboxypeptidases. Incomplete protein processing results in charge heterogeneity which is readily identified by cation-exchange chromatography on the ProPac WCX-10 column. Dionex Application Note 127[2] describes a method for analyzing a humanized IgG_1 MAb for C-terminal lysine variants. Figure 2 shows that these variants are baseline resolved from the native antibody, along with other acidic and basic variants with heavy chain C-terminal lysine heterogeneity, and treatment with carboxypeptide B reduces the number of variants to a single type, without C-terminal lysine.

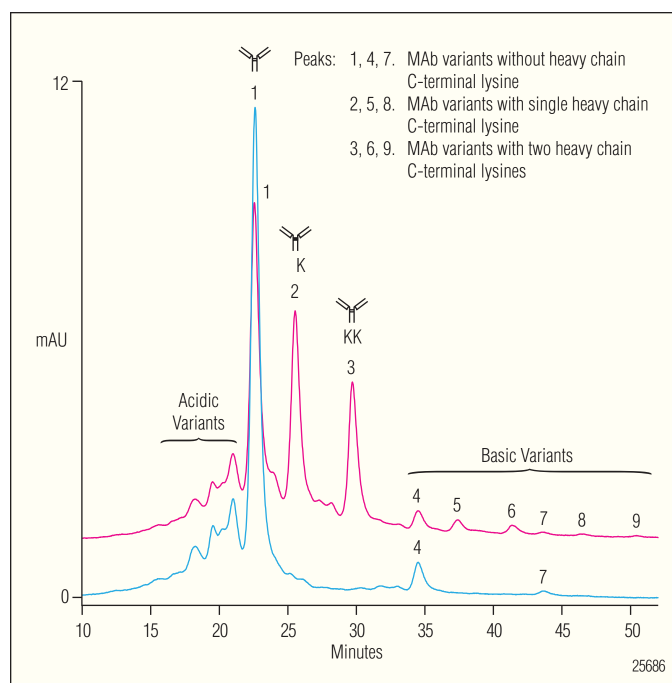

Figure 2. Separation of acidic and basic terminal lysine variants of an IgG_1 monoclonal antibody using the ProPac WCX-10 column and MES-based buffers on the UltiMate 3000 Titanium system. Analysis of IgG_1 monoclonal antibody before (upper trace) and after (lower trace) treatment with carboxy-peptidase B for 2 h at 37 °C.

Deamidation

A common structural modification of recombinant proteins is the deamidation of asparagine (Asn) residues. Proteins and peptides containing Asn adjacent to glycine are particularly susceptible to Asn deamidation, converting Asn to aspartic acid or isoaspartic acid. This modification occurs in a variety of protein-based pharmaceuticals, including human growth hormone, tissue plasminogen activator, and monoclonal

antibodies. Monitoring the extent of deamidation is of interest to quality control and process development chemists concerned with product quality and stability. Dionex Application Notes 125[3] and 128[4] feature applications for determination of protein deamidation, and provides relevant experimental conditions. Figure 3 presents the separation of a humanized monoclonal antibody (without C-terminal lysine variants) through a forced deamidation time-course, using the ProPac WCX-10 column. Figures 4 and 5 present the separation of a ribonuclease A through a forced deamidation time-course, also using this column.

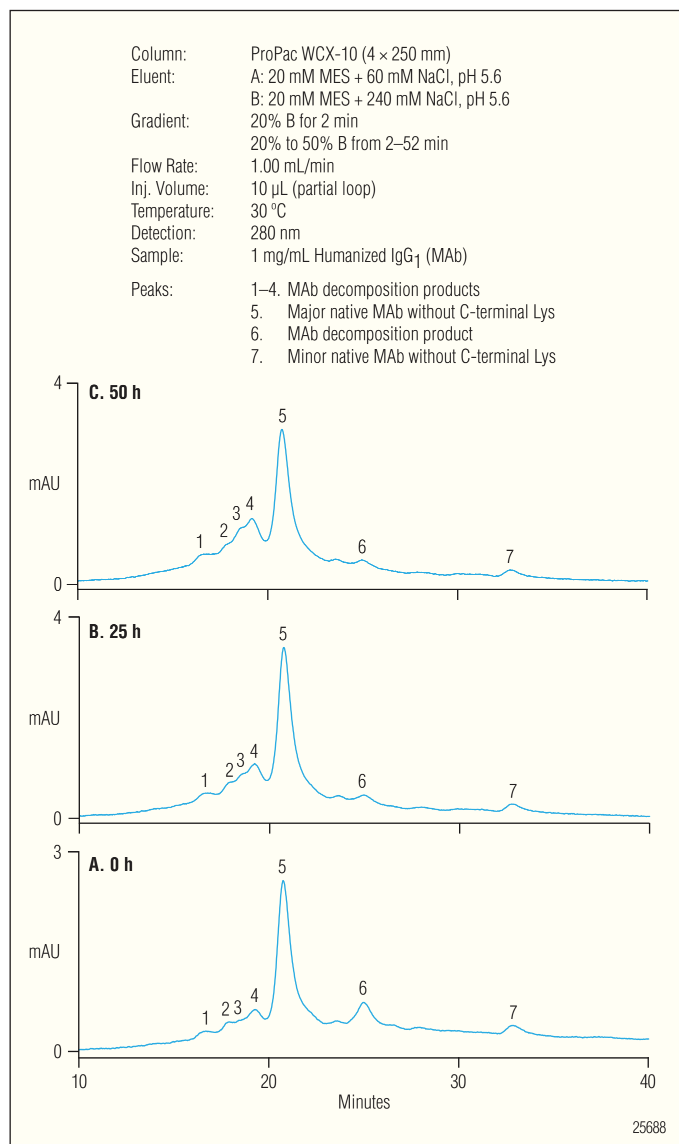

Figure 3. Forced deamidation of carboxypeptidase B-treated monoclonal antibody (1 mg/mL) incubated at 37 °C with 1% ammonium carbonate, 0.05% sodium azide, and protease inhibitor cocktail for 0 h (panel A), 25 h (panel B), and 50 h (panel C).

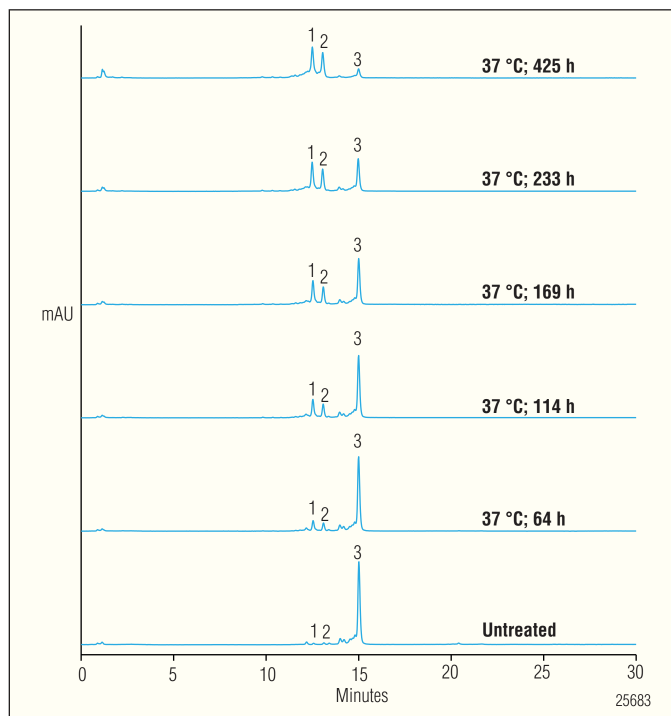

Figure 4. Forced deamidation of ribonuclease A (1 mg/mL) incubated at 37 °C with 1% ammonium carbonate, 0.05% sodium azide, and protease inhibitor cocktail for 0 h, 64 h, 114 h, 169 h, 233 h, and 425 h.

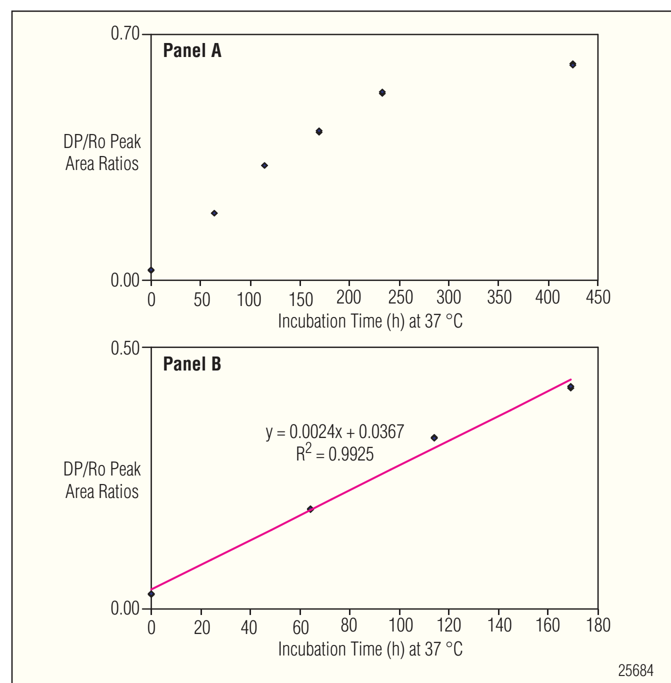

Figure 5. Kinetic plots relating the formation of deamidation peaks, and loss of native ribonuclease A peak, to incubation time under forced deamidation conditions.

Phosphorylation

Approximately 30% of all proteins in a cell are phosphorylated at any given time. Changes in levels of phosphorylated isoforms often signal developmental or pathological disorders. The ProPac SAX-10 resolves several phosphorylated isoforms using a simple gradient. Comparisons of chromatograms for selected model proteins with and without alkaline phosphatase treatment showed that peaks were resolved based on the extent of phosphorylation. In Figures 6–9, we present simple and quick methods using ion exchange chromatography to analyze phosphorylated isoforms of different phosphoproteins: ovalbumin, bovine serum albumin, and casein. These experimental conditions and results are featured in Dionex Application Note 214.[5] Table 1 relates the amount of phosphate determined in five different lots of ovalbumin presented in Figure 7, and Table 2 relates the amount of phosphate measured in the collected fractions for peaks 2, 4, and 7, of Figure 6. The correlation of measured phosphate to peak retention times and the protein content of these peaks confirm a relationship of increasing retention time to increasing ovalbumin phosphorylation.

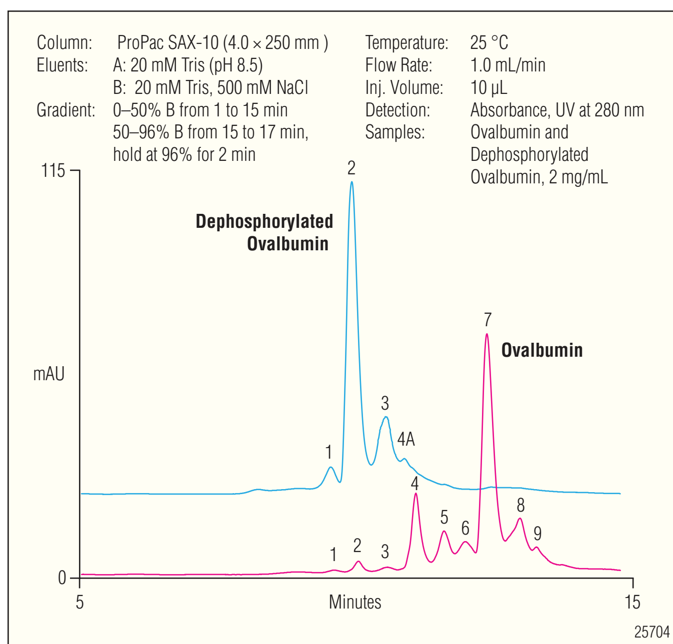

Figure 6. Separation of ovalbumin and its dephosphorylated form using the ProPac SAX-10 column.

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.

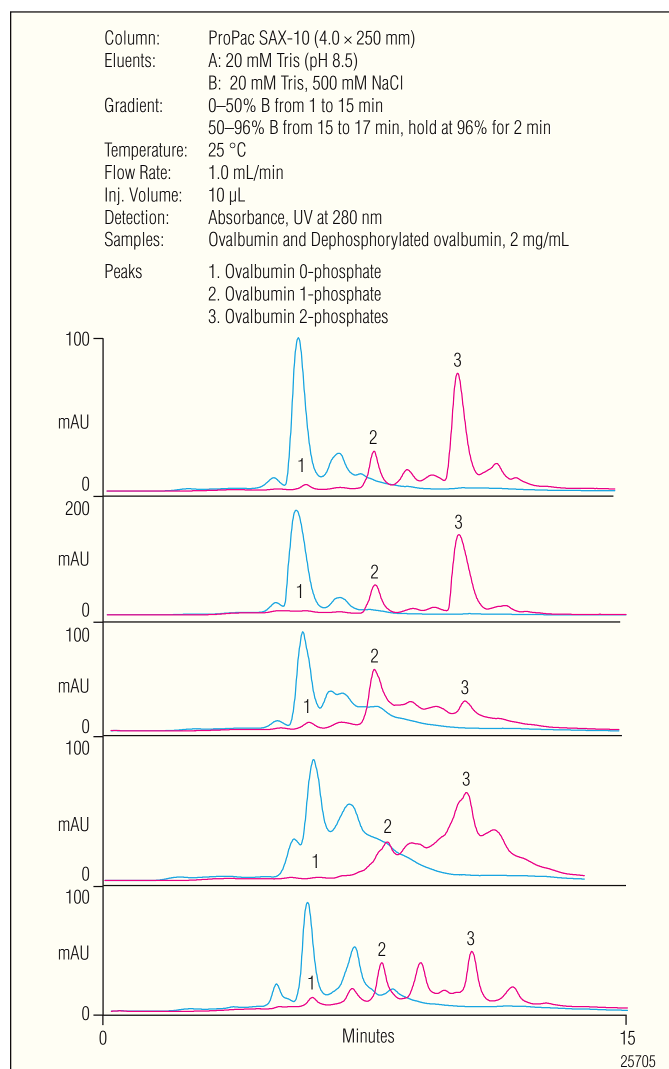

Figure 7. Separations of five lots of ovalbumin and their dephosphorylated forms using the ProPac SAX-10 column.

Sample	Moles of Phosphate/Mole of Protein*
Fraction 1	0.00
Fraction 2	0.93
Fraction 3	1.97

Table 2. Phosphate Content of Ovalbumin Fractions Prepared by ProPac SAX-10 Separation

*Phosphate determined using the method described in Dionex Application Note 210.[6]

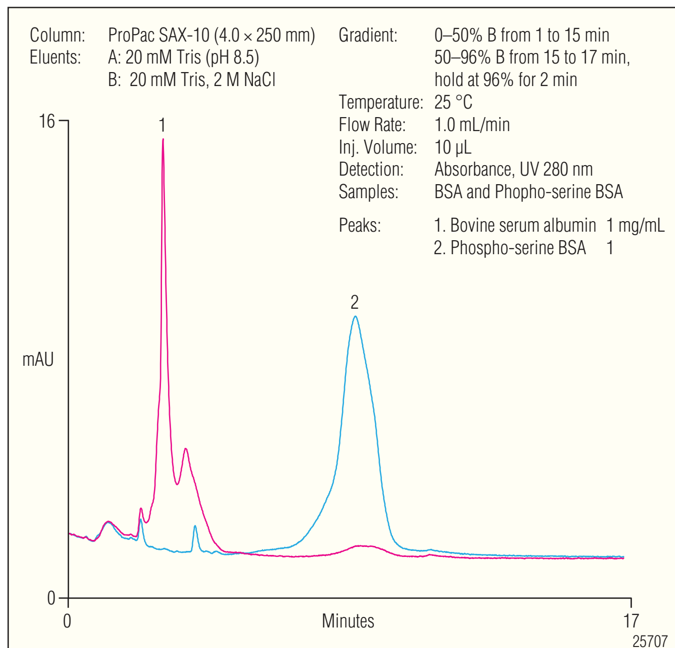

Figure 8. Separations of bovine serum albumin and its synthetically phosphorylated form using the ProPac SAX-10 column.

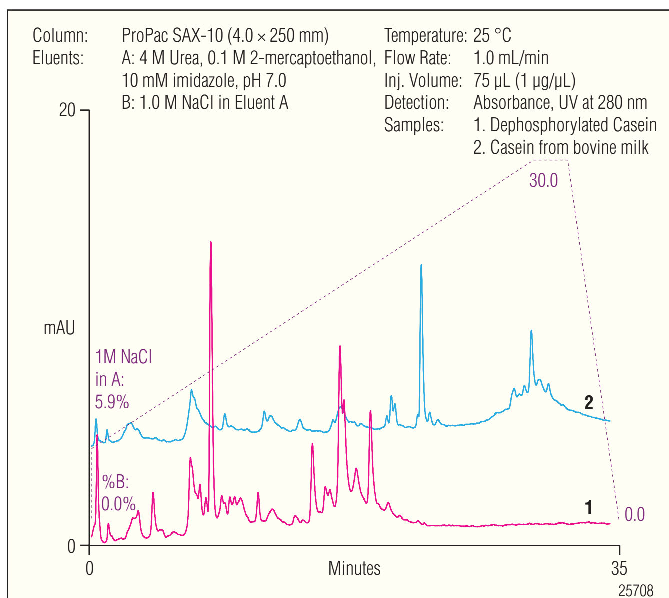

Figure 9. Separations of casein from bovine milk and commercially available dephosphorylated casein using the ProPac SAX-10 column.

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6. Dionex Corporation. *Determination of the Phosphate Content of Phosphorylated Proteins;* Application Note 210, LPN 2097: Sunnyvale, CA, In Production.

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