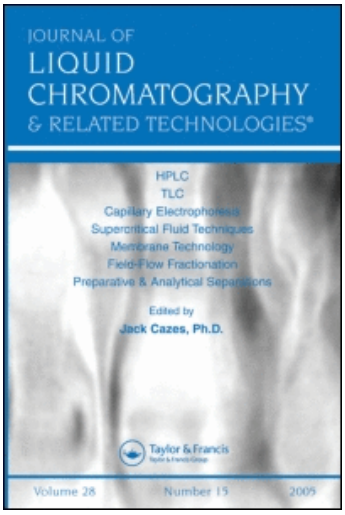

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High-Performance Gel-Permeation Chromatography of Bovine Skim Milk Proteins

G. P. Dimenna[ab]; H. J. Segall[a]
[a] Department of Physiological Sciences School of Veterinary, Medicine University of California Davis, California [b] Division of Drug Metabolism, A. H. Robins Company Richmond, Virginia

To cite this Article Dimenna, G. P. and Segall, H. J.(1981) 'High-Performance Gel-Permeation Chromatography of Bovine Skim Milk Proteins', Journal of Liquid Chromatography & Related Technologies, 4: 4, 639 — 649
To link to this Article: DOI: 10.1080/01483918108059961
URL: http://dx.doi.org/10.1080/01483918108059961

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(4), 639-649 (1981)

HIGH-PERFORMANCE GEL-PERMEATION CHROMATOGRAPHY OF BOVINE SKIM MILK PROTEINS

G. P. Dimenna[1] and H. J. Segall

Department of Physiological Sciences
School of Veterinary Medicine
University of California
Davis, California 95616

ABSTRACT

The separation of bovine skim milk proteins by gel-permeation high performance liquid chromatography was examined. Toya-Soda TSK-GEL (Type SW) columns were used with an eluent of .05 M phosphate buffer (pH 6.80) containing .1 M sodium sulfate at .5 ml/min. Bovine whole milk was centrifuged to remove lipids, and the resultant skim milk directly injected. A 2000SW column yielded three protein peaks: 1 = casein, IgG and BSA; 2 = β-lactoglobulins and BSA; and 3 = α-lactalbumin and BSA. A 3000SW plus 2000SW column system with a 30 μl injection volume yielded four protein peaks: 1 = minor amounts of α_{s1} - and β-casein; 2 = casein, BSA and IgG; 3 = β-lactoglobulins; and 4 = α-lactalbumin. A 3000SW plus 2000SW column system with a 10 μl injection volume yielded five protein peaks: 1 = casein; 2 = IgG; 3 = BSA; 4 = β-lactoglobulins; and 5 = α-lactalbumin. Both the single column and dual column applications yielded three nonprotein peaks, which were dialyzed from solution. Thus, a high speed analytical separation of milk proteins was achieved according to molecular size, but this application is highly dependent on sample size.

INTRODUCTION

Gel-permeation chromatography (GPC) on Sephadex is used to separate proteins according to their molecular size. Sephadex is not mechanically

Send Correspondence to: Dr. H. J. Segall
Department of Physiological Sciences
School of Veterinary Medicine
University of California
Davis, California 95616

[1]New address: Division of Drug Metabolism, A. H. Robins Company
Richmond, Virginia, 23220.

stable under pressure resulting in relatively low flow rates and lengthy analysis time.

A number of GPC column packings have recently been developed that exhibit good resolution and can be operated under moderate pressure (1-4). Data on the applicability of these GPC columns for biological analysis is rapidly expanding, but many applications require proteins to be denatured with SDS to achieve good separation (5,6). New GPC column packings such as TSK-GEL (Type PW), consisting of microspheres of a hydrophilic polymer, have recently offered improved resolution (7-9).

This study was undertaken to establish a rapid method to separate and isolate milk proteins. Environmental toxicants such as pyrrolizidine alkaloids or aflatoxins are known contaminants in milk (10-12). These toxicants and their metabolites may be covalently bound to milk proteins, yet no direct high-speed analytical method has been developed to rapidly separate and isolate milk proteins. The application of high performance liquid chromatography (HPLC) utilizing Toya-Soda TSK-GEL columns for the separation of bovine skim milk proteins is discussed.

EXPERIMENTAL

Sample Preparation

Bovine whole milk samples were obtained from the bulk tank at the University dairy facilities. Milk was centrifuged twice at 200 x g for 10 min and 10,000 x g for 20 min to remove lipids. Total protein concentration was determined in an aliquot of the skim milk (13). The concentration of protein in the skim milk samples ranged from 2.7 to 3.5%. Whey was obtained by acid-precipitating casein from skim milk (14).

Chromatography

The chromatography system consisted of a Waters Associates 6000A pump and U6K injector, Schoeffel 770 variable-wavelength UV detector (280 nm), and Varian A-25 recorder. The following 7.5 x 300 mm columns were used: TSK-125

(Toya-Soda 2000SW, 10 μm particle size, 25 nm pore size, Bio-Rad, Richmond, CA) and MicroPak TSK 3000SW (10 μm particle size, 150 nm pore size, Varian, Sunnyvale, CA). Protein exclusion limits are approximately 100,000 daltons for the TSK-125 column and >350,000 daltons for the TSK 3000SW column (15). The eluent was 0.05 M phosphate buffer (pH 6.80) containing 0.1 M sodium sulfate. The flow rate was 0.5 ml/min with a backpressure of 100 psi per column. Ten or 30 μl aliquots of the skim milk were injected, and individual peaks collected. The peaks from individual runs were pooled, dialyzed against deionized water (1000 dalton cut-off membrane, Spectraphor, Los Angeles, CA), then lyophilized.

Electrophoresis

The proteins in each peak were characterized against milk protein standards by discontinuous polyacrylamide gel electrophoresis (16). The gel concentrations in the stacking and running gels were 3.5 and 7.5% with 7 M urea, or 5 and 9% without urea. The gels were stained with 0.05% Coomassie Blue in glacial acetic acid:methanol:water (10:25:65), and destained in glacial acetic acid:methanol:water (10:25:65).

Protein Standards

Protein standards, α-lactalbumin, cytochrome-C, pepsin, β-lactoglobulins A and B, ovalbumin, bovine serum albumin (BSA), and immunoglobulin G (IgG) were purchased from Sigma Chemical Company (St. Louis, MO.).

RESULTS AND DISCUSSION

Figure 1 shows the elution curves of various commercial milk protein standards on the 2000SW column. IgG (161,000 daltons), BSA (69,000 daltons), β-lactoglobulins A and B (dimers, 36,000 daltons), α-lactalbumin (14,150 daltons) elute according to their molecular size, and exhibited retention times of 12.1, 12.6, 14.6, 14.9 and 16.1 min. The void volume (V_o) was 5.7 ml using blue dextran (average MW = 2 x 10^6) as a marker. The total column volume (V_t) was 10.5 ml using phenylalanine (MW = 165.2) as a marker. The

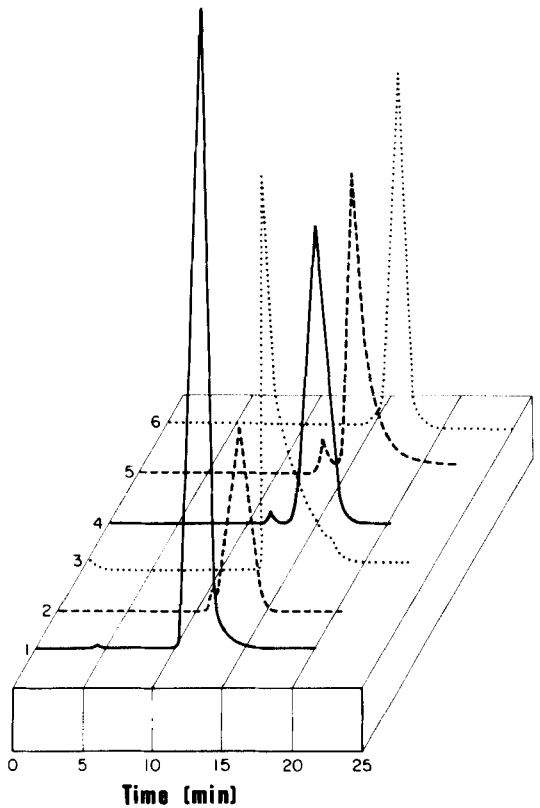

Figure 1. Elution curves of commercial milk proteins and blue dextran. Column 7.5 mm x 300 mm Toya-Soda 2000SW; Schoeffel model 770 variable wavelength uv detector (280 nm); eluent, .05 M PO_4 buffer (pH 6.80) containing .1 M Na_2SO_4; flow rate of .5 ml/min. Peaks and retention times: 1 = blue dextran (11.4 min); 2 = BSA (12.6 min); 3 = IgG (12.1 min); 4 = β-lactoglobulin A (14.6 min); 5 = β-lactoglobulin B (14.9 min); and 6 = α-lactalbumin (16.1 min).

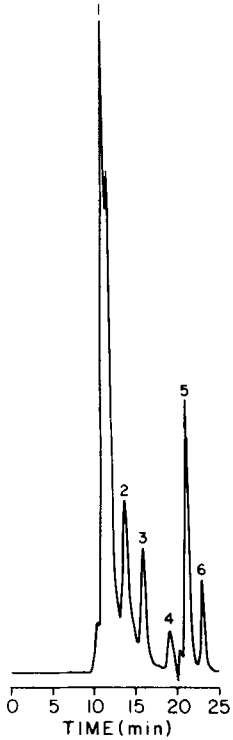

Figure 2. Elution curve of skim milk. Conditions as in Figure 1, 30 µl injected.

elution volumes (V_e) of BSA and IgG were similar, though their molecular weights are markedly different. Jones et al. (17), using a 10 nm pore C_8 reversed-phase support, found that BSA did not chromatograph as efficiently as ribonuclease (13,700 daltons), which was attributed to BSA having a lower diffusion rate and equilibrating more slowly with the stationary phase. However, this argument may not be valid with an aqueous gel-permeation system.

Figure 2 illustrates the elution curve of skim milk obtained with the 2000SW column. A total of six peaks were obtained, and the percent distributions of protein in each peak were 70.7, 18.0, 7.0, 0.7, 3.2 and 0.3%. The total recovery of protein was 91.0%. The V_t was 12.5 ml as compared to 10.5 ml for

phenylalanine. Thus, some milk component(s) had absorbed onto the column. Others have found similar absorption problems with low molecular weight compounds on the Toya-Soda (Type SW) and Waters I-125 gel-permeation columns (17). The proteins in each peak were characterized electrophoretically against milk protein standards in the presence or absence of 7 M urea in the gels. Peak 1 contained the casein proteins, IgG, and BSA; peak 2 contained β-lactoglobulins; peak 3 contained α-lactalbumin; peaks 2 and 3 were contaminated with BSA; and peak 3 was contaminated with β-lactoglobubin. No protein bands were obtained with peaks 4-6, and these samples were not further analyzed. Although the molecular weights of individual casein proteins are on the order of 20,000 daltons (18), they eluted with the V_0. The milk caseins are in a micellar form, which is spherical in shape and may be on the order of 40-300 nm in diameter with particle weights of 10^6 to 3 x 10^9 daltons (14). Therefore, the casein micelle is probably remaining intact during the chromatographic run. In agreement, Hill and Hansen (19) and Morr et al. (20) separated skim milk proteins on Sephadex G-100, and casein proteins eluted exclusively in the first fraction.

Figure 3 shows the elution curve of whey with the 2000SW column. The elution profile of whey is directly comparable to that of skim milk, except peak 1 is a minor component and peaks 2 and 3 are the major components of the elution profile; providing further evidence that the casein micelle elutes with the V_0. Peaks 4-6 could be dialyzed (1000 dalton cut-off membrane) out of solution.

Since casein, IgG and BSA eluted in the V_0, a dual column system (3000SW plus 2000SW), was used to increase the exclusion limit. Figure 4 shows the comparison between V_e and protein molecular weight with the 3000SW and 2000SW columns. Addition of the 3000SW effectively increased the molecular weight exclusion limit and resolution over the 2000SW column alone. Also, molecular weight of proteins could be effectively estimated with these gel-permeation columns.

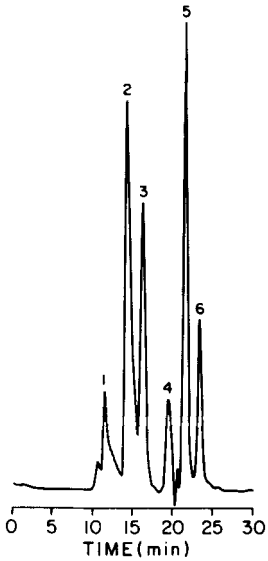

Figure 3. Elution curve of the whey fraction of skim milk. Conditions as in Figure 1, 25 µl injected.

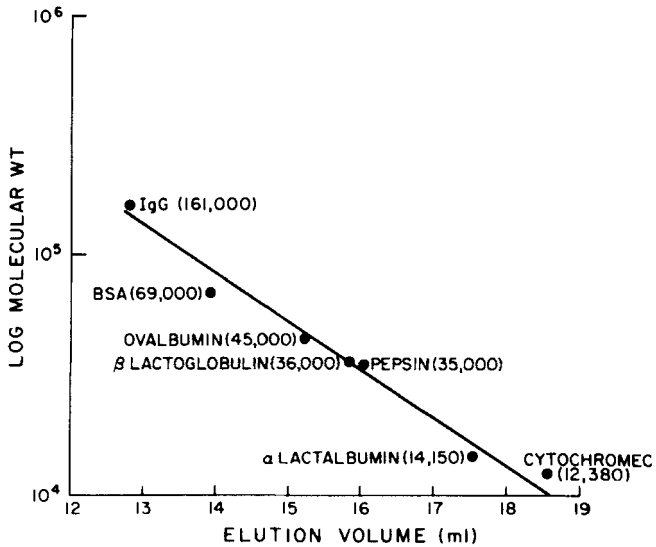

Figure 4. Protein molecular weight versus elution volume. Columns 7.5 mm x 300 mm Toya-Soda 3000SW plus 2000SW; other conditions as in Figure 1.

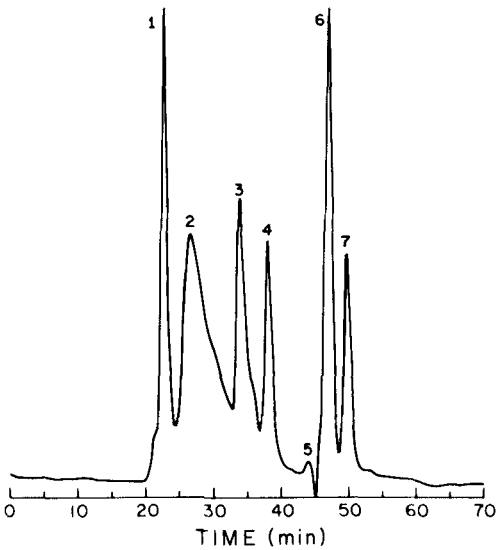

Figure 5. Elution curve of skim milk. Conditions as in Figure 4, 30 µl injected.

Figure 5 shows the elution profile of skim milk (30 µl injected) on the 3000SW plus 2000SW columns. A total of seven peaks were obtained, and the percent distributions of protein in each peak were 21.3, 32.8, 22.8, 15.4, 2.5, 4.7 and 0.5%. The total recovery of protein was 86.0%. Peak 1 contained a minor amount of α_{S_1}-and β-casein; peak 2 contained the remainder of caseins, BSA and IgG; peak 3 contained β-lactoglobulins with contamination from α_{S_1}-and β-casein; peak 4 contained only α-lactalbumin. The presence of α_{S_1}-and β-casein in peak 3 suggests that the casein micelle is not remaining intact during the chromatographic run. No proteins were present in peaks 5-7. The 3000SW plus 2000SW column system with a 30 µl injection volume did not enhance milk protein separation, since BSA and IgG eluted with casein (peak 2).

When 10 µl of skim milk was injected onto the 3000SW plus 2000SW columns, eight peaks were obtained as shown in Figure 6. The percent distributions of protein in each peak were 26.7, 3.0, 6.7, 27.4, 18.6, 3.3, 14.3 and 0%.

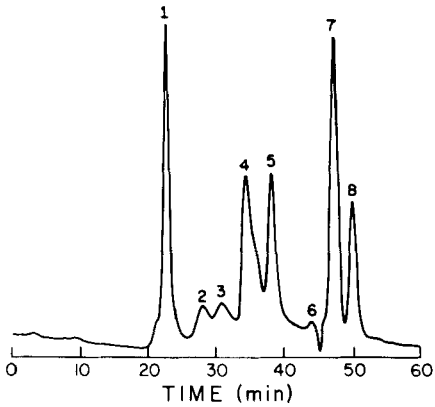

Figure 6. Elution curve of skim milk. Conditions as in Figure 4, 10 µl injected.

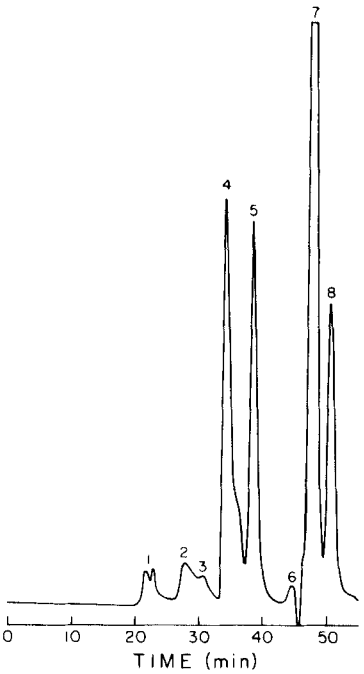

Figure 7. Elution curve of the whey fraction of skim milk. Conditions as in Figure 4, 30 µl injected.

However, total recovery of protein was $\leq$ 75%. The low protein recovery may be a result of assay error, since only .3 mg protein was injected. The proteins from this chromatogram were characterized by comparing the retention times with standards: peak 1 = casein, 2 = IgG, 3 = BSA, 4 = β-lactoglobulin, 5 = α-lactalbumin, and 6-8 = nonprotein. When casein was precipitated from the skim milk sample and an aliquot of whey injected, peak 1 was a minor component of the chromatogram (Figure 7), providing further evidence that the casein proteins eluted with peak 1 (Figure 6).

The TSK-GEL (Type SW) columns were effective in separating proteins according to molecular size in a nonpractical (Figure 4) and a practical application (Figure 6). However, separation of bovine skim milk proteins was highly dependent on sample size, as a 10 µl versus a 30 µl injection volume resulted in a better separation of casein proteins from whey proteins and IgG from BSA.

ACKNOWLEDGEMENTS

This work was funded by Grant PFR 78-06924 from the National Science Foundation.

REFERENCES

1. Becker, N. and Unger, K. K., Separation studies of amino acids, proteins and enzymes on bonded 1,2-dihydroxy-1,2-hydroxyl amino- and amino silica packings, Chromatographia, 12, 539, 1979.

2. Randau, D., Bayer, H. and Schnell, W., Chromatographische anwendung des polyathylenglykol-dimethacrylat-gels Merckogel® PGM 2000 unter normal-und hochdruck, J. Chromatogr., 57, 77, 1971.

3. Regnier, F. E. and Noel, R., Glycerolpropylsilane bonded phases in the steric exclusion chromatography of biological macromolecules, J. Chromatogr. Sci., 14, 316, 1976.

4. Vondruska, M., Sudrich, M. and Mladek, M., Determination of some gel permeation chromatographic parameters of the gel Spheron P-1000, J. Chromatog. 116, 457, 1976.

5. Barford, R. A., Sliwinski, B.J. and Rothbart, H. L., Observations on the rapid size-exclusion chromatography of proteins, Chromatographia, 12, 285, 1979.

6. Vivilecchia, R. V., Lightbody, B. G., Thimot, N. Z and Quinn, H. M., The use of microparticulates in gel permeation chromatography, J. Chromatogr. Sci., 15, 424, 1977.

7. Fukano, K., Komiya, K., Sasaki, H. and Hashimoto, T., Evaluation of new supports for high-pressure aqueous gel permeation chromatography: TSK-GEL SW Type columns, J. Chromatogr., 166, 47, 1978.

8. Hashimoto, T., Sasaki, H., Aiura, M. and Kato, Y., High-speed aqueous gel-permeation chromatography of proteins, J. Chromatogr. 160, 301, 1978.

9. Kato, Y., Sasaki, H., Aiura, M. and Hashimoto, T., High-performance aqueous gel-permeation chromatography of oligomers, J. Chromatogr. 153, 546, 1978.

10. Dickenson, J. O., Cooke, M. P., King, R. R. and Mohamed, P. A., Milk transfer of pyrrolizidine alkaloids in cattle, J. Am. Vet. Med. Assoc., 169, 1192, 1976.

11. Johnson, A. E., Changes in calves and rats consuming milk from cows fed chronic lethal doses of Senecio jacobaea (Tansy Ragwort), Am. J. Vet. Res. 37, 107, 1976.

12. Winterlin, W., Hall, G. and Hsieh, D. P. H., On-column chromatographic extraction of aflatoxin M_1 from milk and determination by reversed phase high performance liquid chromatography, Anal. Chem., 51, 1873, 1979.

13. Lowry, O. H., Rosebrough, N. J., Farr, A. C. and Randall, R. J., Protein measurement with the folin phenol reagent, J. Biol., Chem., 193, 265, 1951.

14. McKenzie, H. A., Milk proteins. Chemistry and molecular biology, Academic Press, New York, 2, 1971.

15. Wehr, C. T., High speed exclusion chromatography of proteins, Varian Technical Bulletin LC 100, 1980.

16. Melachouris, N., Discontinuous gel electrophoresis of whey proteins, casein, and clotting enzymes, J. Dairy Sci., 52, 456, 1969.

17. Jones, B. N., Lewis, R. V., Paabo, S., Kojima, K., Kimura, S. and Stein, Effects of flow rate and eluent composition on the high performance liquid chromatography of proteins. J. Liq. Chromatogr. 3, 1373, 1980.

18. Welinder, B. S., Gel permeation chromatography of insulin, J. Liq. Chromatogr., 3, 1399, 1980.

19. Whitney, R. M., Brunner, J. R., Ebner, K., E., Garrell, Jr., H. M., Josephson, R. V., Morr, C. V and Swaisgood, H. E. Nomenclature of the proteins of cow's milk: Fourth revision. J. Dairy Sci., 59, 785, 1976.

20. Hill, R. D. and Hansen, R. R., The separation of milk protein on dextran gel, J. Dairy Res. 31, 291, 1964.

21. Morr, C. V., Kenkare, D. B. and I. A. Gould, Fractionation of skimmilk proteins by Sephadex gel filtration, J. Dairy Sci., 47, 621, 1964.