Journal of Chromatography, 326 (1985) 225–233
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 551

PREPARATION OF SHEEP AND CATTLE IMMUNOGLOBULINS WITH ANTIBODY ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

MARY JO F. SCHMERR*, KATHRYN R. GOODWIN, HOWARD D. LEHMKUHL and RANDALL C. CUTLIP
National Animal Disease Center, P.O. Box 70, Ames, IA 50010 (U.S.A.)

SUMMARY

High-performance anion-exchange chromatography (HPIEC) and high-performance size-exclusion chromatography (HPSEC) were used to purify serum IgM and IgG from sheep and cattle. Pooled serum from normal cattle and sheep and serum from sheep, infected with two different viruses, were prepared for HPIEC by chromatography on CM-Affi-Gel Blue. After HPIEC, fractions containing IgG and IgM were pooled and concentrated and further purified by HPSEC. The purity of fractions from HPIEC and HPSEC were evaluated by immunoelectrophoresis, protein-A-Sepharose affinity chromatography, and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Monoclonal antibody specific for bovine IgG2 was used to assay for IgG2 and IgG2 contamination of other fractions. The antibody activity to ovine adenovirus 5 and to ovine progressive pneumonia virus was assayed by neutralization and immunodiffusion. Antibody activity was retained against both viruses in the fractions containing IgG1 and IgM. This high-performance liquid chromatography procedure was a rapid preparative method to produce specific immunoglobulins and could be used to evaluate the purity of immuno-reagents.

INTRODUCTION

The development of size-exclusion gels[1–3] and ion-exchange resins[4], suitable for high-performance liquid chromatography (HPLC) of proteins has reduced the time required for separation and has enhanced resolution. Improvement over conventional chromatographic methods used to purify ruminant immunoglobulins was needed. After long elutions from size-exclusion columns and many cycles on ion-exchange resins, poor yields, cross contamination, and dimerization of IgG1 were persistent problems[5].

To improve upon these methods, we used two preparative high-performance columns (a DEAE ion-exchange column and a size-exclusion column) to purify both bovine and ovine immunoglobulins. By combining these two techniques, we were able to resolve IgM preparatively and to obtain the subclasses of IgG in good yields.

0021-9673/85/$03.30

MATERIALS AND METHODS

Sera

Sera from the blood of ten or more sheep or cattle were collected and pooled and stored at −20°C. Serum was also prepared from a sheep persistently infected with ovine progressive pneumonia virus (OPPV) and a sheep infected with *Mastadenovirus ovi* 5 (OA-5).

Affinity chromatography

CM-Affi-Gel Blue (Biorad, Richmond, CA, U.S.A.) chromatography was performed according to recommendations of the manufacturer[6]. A 30-ml syringe, containing approximately 20 ml of gel, was used as the column. A volume of 5 ml of serum was placed on the column.

Protein-A-Sepharose chromatography (PAS) was performed by using a descending stepwise pH gradient. Buffers were made by adjusting the pH of 0.5 *M* disodium hydrogen phosphate with 0.1 *M* citric acid. Two gradients were formed: one by descending one half pH unit steps and one by four buffers with pH values of 8.0, 6.5, 4.5 and 2.5.

Fractions were collected and the absorbance and pH were determined.

Sample preparation

After CM-Affi-Gel Blue chromatography, the unbound fractions were pooled and precipitated with 50% saturated ammonium sulfate. The precipitate was collected by centrifugation and resuspended in 50 m*M* sodium phosphate buffer, pH 7.0, and dialyzed against this buffer. After application to the (HPIEC) column, the three major peaks were collected and pooled. After precipitation with 50% saturated ammonium sulfate, the precipitates of the pooled peaks were collected and dialyzed against 0.1 *M* sodium phosphate, pH 7.0, 0.2 *M* sodium chloride for high-performance size-exclusion chromatography (HPSEC).

HPIEC and HPSEC

Chromatography was performed with a system consisting of a Beckman 100A pump, an Altex 210 injector, a LKB UVICORD UV detector, equipped with a HPLC flow cell, and a Waters 730 M data module.

HPSEC was performed on a 600 × 21.5 mm I.D. column, TSK 3000 SW (Beckman, Palo Alto, CA, U.S.A.), by isocratic elution with 0.1 *M* sodium phosphate, pH 7.0, containing 0.2 *M* sodium chloride (buffer A). Absorbance was monitored at 254 nm. HPLC markers (U.S. Biochemicals, Cleveland, OH, U.S.A.) were used to establish a molecular-weight curve. DEAE-HPIEC was carried out on a 21.5 × 150 mm I.D. DEAE 545 TSK IEC column. The column was equilibrated with 50 m*M* sodium phosphate buffer pH 7.0. After sample application, the column was washed with 30 ml of buffer. The bound fractions were eluted with a one-step gradient, using 90 ml of the above buffer, containing 0.5 *M* sodium chloride. Absorbance was monitored at 254 nm.

Electrophoresis

Fractions from column eluates were analyzed by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE)[7], using 3–27% acrylamide gradient gels (Separation Sciences, Attleboro, MA, U.S.A.). Samples were solubilized in 0.01 *M* Tris, pH 8.0 containing 1% SDS, 0.001 *M* EDTA, 6 *M* urea and 5% β-mercaptoethanol. SDS-PAGE markers (Bio-Rad, Richmond, CA, U.S.A.) were also tested. The gels were stained for 16 h in an aqueous solution, containing 0.125% Coomassie Blue R250 (Eastman Kodak, Rochester, NY, U.S.A.), 10% acetic acid, and 50% methanol.

Immunoelectrophoresis was performed on pooled column fractions, using the method of Grabar and Williams[8]. Two percent special Noble agar (Difco Lab., Detroit, MI, U.S.A.) in 0.15 *M* borate buffer pH 8.6 was used to form the gel, and either rabbit anti-normal bovine serum or goat anti-sheep immunoglobulins was used in the troughs.

Virus assays

Immunodiffusion for antibodies to OPPV was performed according to the method of Cutlip *et al.*[9] and virus neutralization of OA-5 was carried out by a microtiter test[10].

Radioimmunoassay

Solid-phase radioimmunoassays were performed according to the method of Srikumaran *et al.*[11]. HPLC column fractions of 100 μl were coated on polyvinyl plates and tested with monoclonal antibodies, produced to bovine immunoglobulins[12].

RESULTS AND DISCUSSION

Chromatography on CM-Affi-Gel Blue removed most of the albumin and protease activity from the serum[6]. The immunoglobulins and other serum proteins did not bind to this column. This initial purification step eliminated contamination of the immunoglobulin fractions with albumin.

The profile of HPIEC of bovine immunoglobulins is shown on Fig. 1. As expected from conventional DEAE ion-exchange chromatography, IgG2 (peak 1) did not bind[13]. This fraction was IgG2, as observed by HPSEC and PAS (Fig. 2a), and immunoelectrophoresis (Fig. 3, lane 2).

A peak (Fig. 1. peak 2) that was often eluted after IgG2 in older samples may be due to dimerization, which often occurs in bovine samples[5]. This peak consisted of both IgG1 and IgG2, as analyzed by immunoelectrophoresis (data not shown).

Peaks 3 and 4 contained bovine IgG1 and IgM, but no IgG2. This was readily observed on PAS (Fig. 2b) and by the reaction of monoclonal antibody specific for IgG2 (Table I). Rechromatography of peak 4 (Fig. 1, inset) enriched IgM, but IgG1 was a major contaminant. IgG1 and IgM were readily separated by HPSEC, as analyzed by immunoelectrophoresis. In addition, as observed on SDS-PAGE (Fig. 4), heavy chains from IgM did not contaminate the IgG1 fractions and IgG1 heavy chains did not contaminate the IgM fractions. If dimerization of IgG1 occurred, it could be readily discerned on HPSEC by observation of a peak with a molecular weight of 320000 daltons. The percent of recovery for immunoglobulins on both columns was 75% or greater. Pooled ovine serum gave results similar to bovine serum.

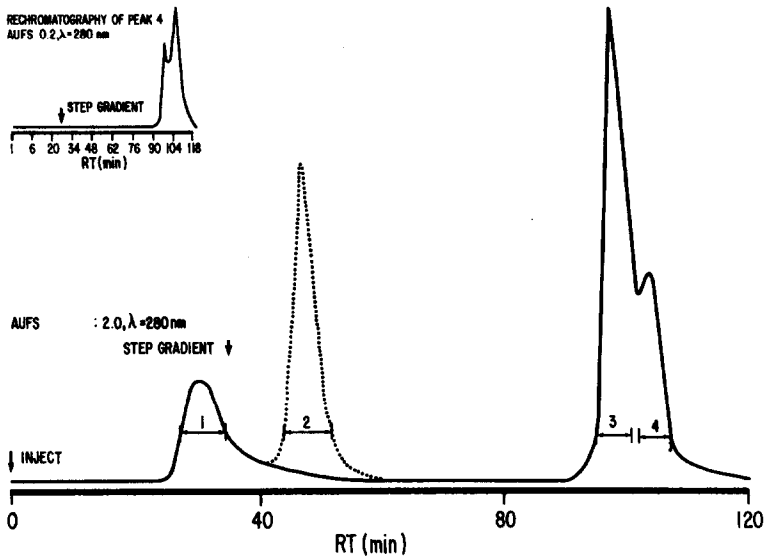

Fig. 1. DEAE-IEC of bovine immunoglobulins. (——) Peak fractions that were pooled. (------) Peak that occurs some of the time. Chromatographic conditions: sample, 2 ml of pooled bovine serum; column, DEAE-IEC 15 × 2.1 cm I.D.; mobile phase, 50 m*M* sodium phosphate, pH 7.0; step gradient, 0–0.5 *M* sodium chloride; flow-rate, 1 ml/min; run time, 120 min.

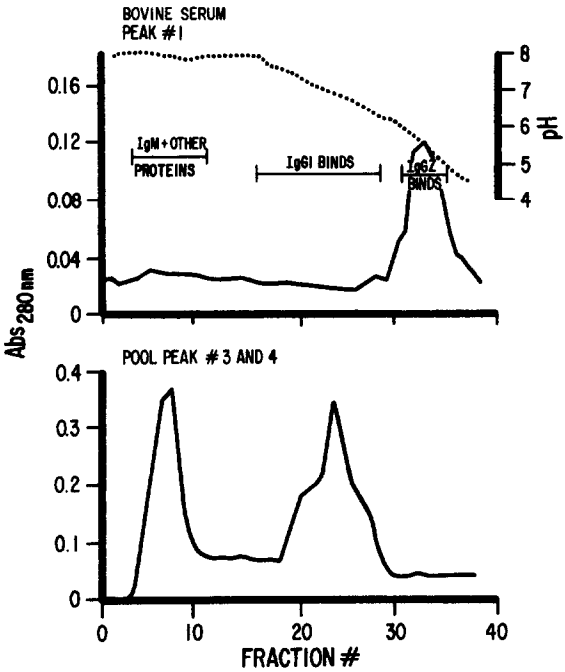

Fig. 2. (a) PAS of HPIEC peak No. 1. (b) PAS of HPIEC Nos. 3 and 4.

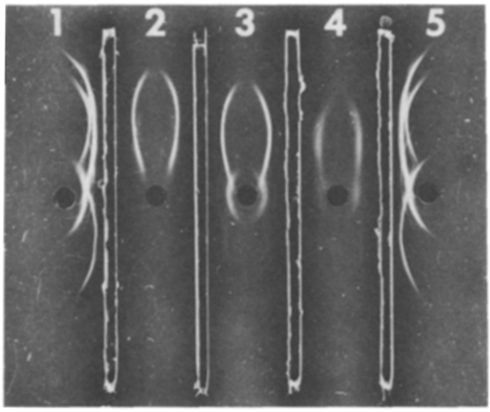

Fig. 3. Immunoelectrophoresis pattern of bovine immunoglobulins. Lanes 1 and 5, CM-Affi-Gel Blue sample; lane 2, HPIEC peak 1; lane 3, HPIEC peak 3; lane 4, HPIEC, peak 4. Rabbit anti-normal bovine serum was used in the troughs.

TABLE I

BINDING OF MONOCLONAL ANTIBODY TO HPIEC COLUMN FRACTIONS OF BOVINE IMMUNOGLOBULINS

	*Starting material (cpm)**	*cpm Peak 1*	*cpm Peak 3*	*cpm Peak 4*
4A11**	870	1050	70	105
1H7***	1935	1170	1270	1230

* Sample cpm − background cpm.
** Monoclonal antibody, specific for bovine IgG2.
*** Monoclonal antibody, crossreactive to bovine IgG and IgM.

Antibody activity was studied in sera from two individual sheep, one of which was infected with OA-5 and another with OPPV. These antisera, processed on CM-Affi-Gel Blue, were applied to HPIEC (Fig. 5a and b). The pooled peaks were tested for antibody activity for the respective viruses (Fig. 5a and 6). The first HPIEC peak (IgG2) gave a low titer to OA-5, but peaks 2 and 3 (IgG1 and IgM) gave high titers to the virus (Fig. 5a). The total recovery of neutralization activity to this virus was *ca.* 80%. Antibody activity by the immunodiffusion test for OPPV was negative for the IgG2 peak (Fig. 6, well No. 1). This was probably due to the much lower levels of OPPV antibody present in the IgG2 fraction and the inability of the immunodiffusion test to detect low levels of antibody activity. Pool Nos. 2 and 3 both gave precipitin lines for OPPV (Fig. 6, well Nos. 3 and 7). The HPIEC pools were further processed on HPSEC. Peak 1 from HPIEC gave only one peak on HPSEC (Fig. 7a) and no precipitin line (Fig. 6, well No. 2). Peak 2 from HPIEC gave two peaks on HPSEC (Fig. 7b). HPSEC peak 1 had no immunodiffusion activity, but the second HPSEC gave a strong precipitin line (Fig. 6, well No. 5). After PAS of this peak (Fig. 8), only one peak was observed in the IgG1 binding region. This PAS peak also gave a strong precipitin line (Fig. 6, well No. 6). The profile of peak 3 from

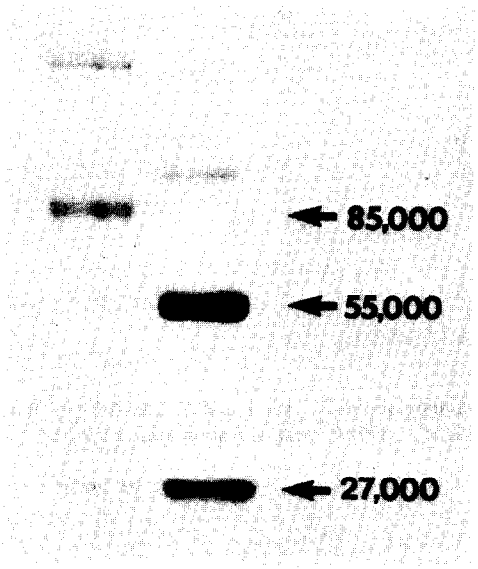

Fig. 4. SDS-PAGE of fractions from HPSEC of peak 3 from HPIEC of bovine immunoglobulins. Lane A: Peak fraction which eluted first in HPSEC, *ca.* 2 μg of protein were applied to the gel. Lane B: Peak fraction which eluted second in HPSEC, *ca.* 5 μg of protein were applied to the gel. Arrows indicate estimated molecular weights as determined from a standard curve. 85 kdaltons represents the MW of the heavy chain of IgM; 55 kdaltons that of IgG; 27 kdaltons is the MW of light chains.

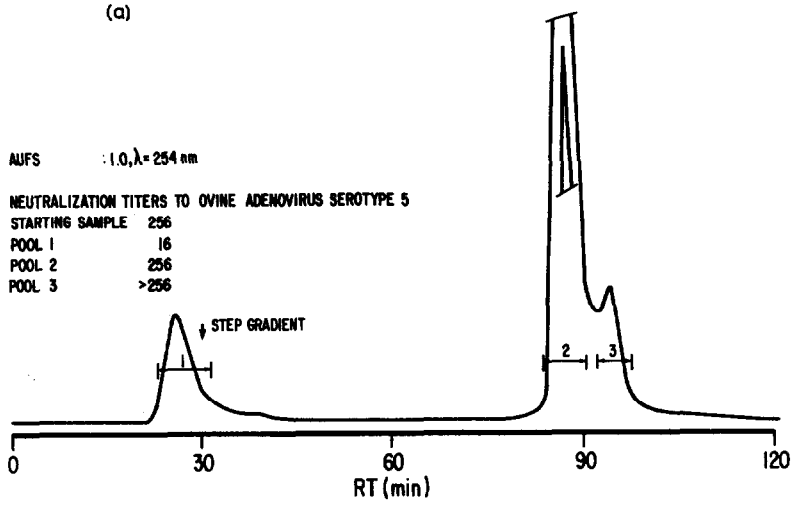

Fig. 5.

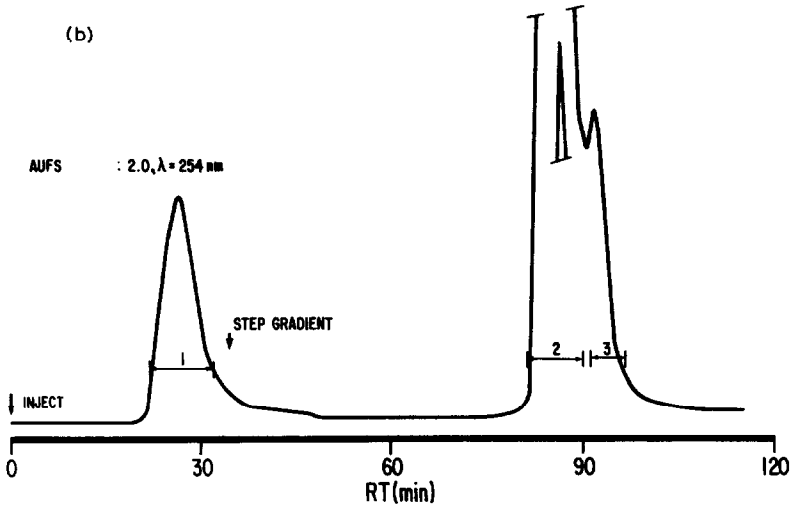

Fig. 5. DEAE-IEC of ovine sera. (a) Serum 2473 (infected with OA-5). Chromatographic conditions: sample, 2.5 ml sheep serum No. 2473; column DEAE-IEC 15 × 2.1 cm I.D.; mobile phase, 50 m*M* sodium phosphate, pH 7.0; step gradient, 0–0.5 *M* sodium chloride; flow-rate, 1 ml/min; run time, 110 min. Virus neutralization titers of the pooled fractions are given. (b) Serum 1242 (infected with OPPV). Chromatographic conditions as in a, except sample volume was 3.0 ml sheep serum 1242.

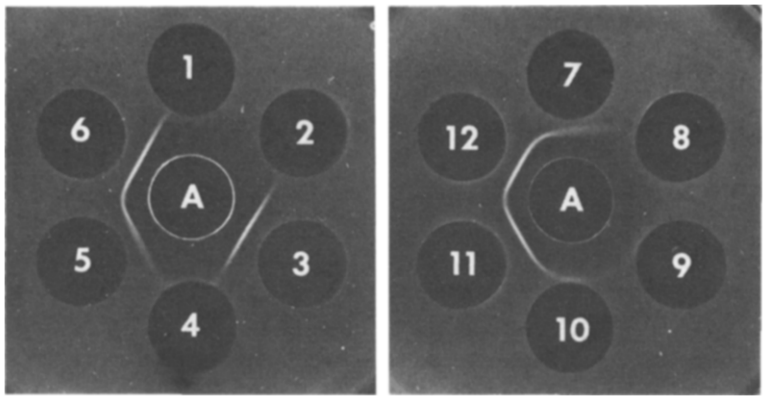

Fig. 6. Immunodiffusion activity of purified fractions from serum 1242. A, Glycoprotein from OPPV. Well Nos: (1) HPIEC peak (pk) 1; HPSEC pk 1; (3) HPIEC pk 2, (4) HPSEC pk 1 of HPIEC pk 2; (5) HPSEC pk 2 of HPIEC pk 2; (6) PAS of pk 2; (7) HPIEC pk 3; (8) HPSEC pk 1 of HPIEC pk 3; (9) HPSEC pk 2 of HPIEC pk 3; (10) HPSEC pk 3 of HPIEC pk 3; (11) CM-Affi-Gel Blue sample; (12) serum 1242.

HPIEC on HPSEC was slightly different with three peaks: an excluded peak, an IgG peak, and a peak at a molecular weight of *ca.* 100 kdaltons. Of these three peaks, the two peaks with smaller molecular weights gave weak immunodiffusion lines.

Immunoelectrophoresis patterns of peaks from HPIEC and HPSEC are shown in Fig. 9. Peak 1 from HPIEC is IgG2. Peak 2 from HPIEC contained IgG1, IgM and two other components that were not identified. After HPSEC of the HPIEC

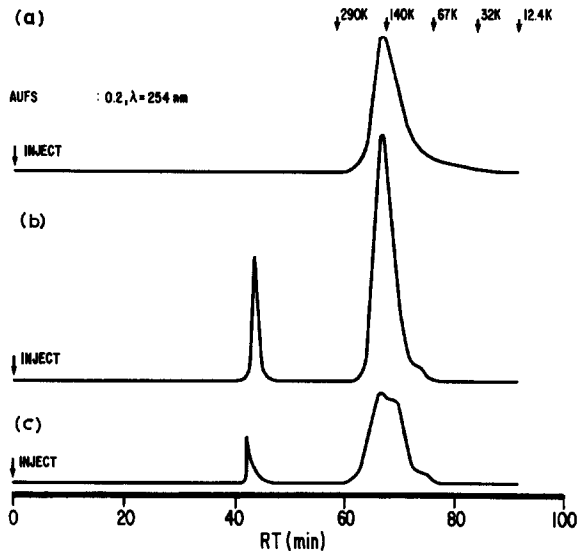

Fig. 7. HPSEC of pooled HPIEC peaks of serum 1242. Arrows indicate retention times of molecular weight standards. Chromatographic conditions: column, TSK 3000 SW 60 × 2.1 cm I.D.; mobile phase 0.1 *M* potassium phosphate, pH 7.0, containing 0.2 *M* sodium chloride; flow-rate, 2 ml/min. Samples: (a) HPIEC peak 1 of serum 1242; (b) HPIEC peak 2 of serum 1242; (c) HPIEC peak 3 of serum 1242.

peaks, IgG1 was clearly separated from IgM and the other components. IgM was still contaminated. Perhaps by adjusting the gradient and the pH in HPIEC, the components, one of which is probably transferrin, could be separated from the IgM. These contaminating components were not found in the IgM fraction of the immu-

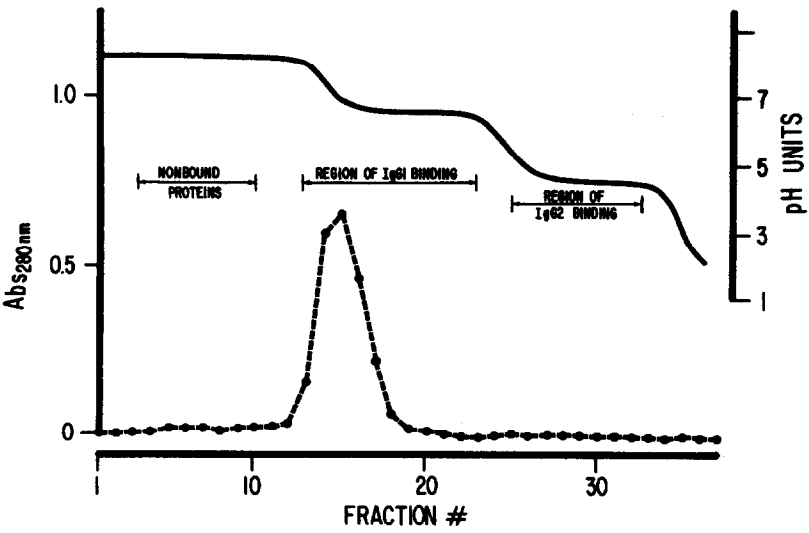

Fig. 8. Protein A-Sepharose chromatography of HPSEC peak 2 from HPIEC peak 2.

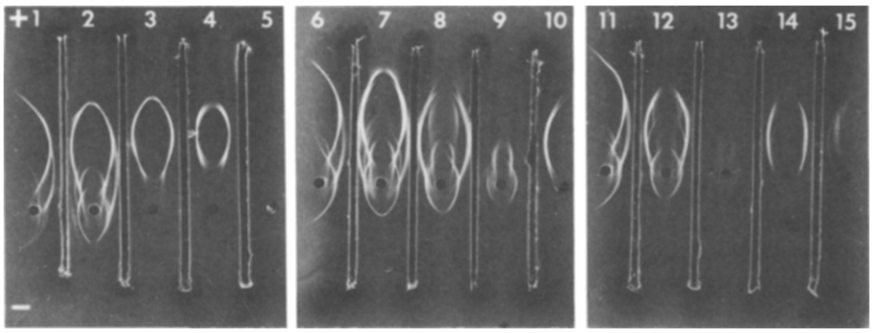

Fig. 9. Immunoelectrophoresis pattern of purified serum 1242 immunoglobulins. Lanes 1 and 6, 1242 serum; lanes 2, 7 and 11, CM-Affi-Gel Blue sample; lane 3, HPIEC pk 1; lane 4, HPSEC pk 1 of HPIEC pk 1; lane 8, HPIEC pk 2; lane 9, HPSEC pk 1 of HPIEC pk 2; lane 10, HPSEC pk 2 of HPIEC pk 2; lane 12, HPIEC pk 3; lane 13, HPSEC pk 1 of HPIEC pk 3; lane 14, HPSEC pk 2 of HPIEC pk 3; lane 15, HPSEC pk 3 of HPIEC pk 3 (Goat anti-sheep immunoglobulins was used in the troughs.)

noelectrophoresis plate after peak 3 of HPIEC was chromatographed by HPSEC (Fig. 8, line 11).

In summary, use of HPIEC and HPSEC gave excellent results for the purification of ruminant immunoglobulins. Recovery rates of protein and antibody activity were very high, compared to conventional procedures. In addition, these methods were rapid. Since a one-step gradient was used for the HPIEC, only one HPLC pump was necessary. These purified immunoglobulins will aid in monoclonal antibody production and testing and will provide good reagents for studying the immune response in sheep and cattle.

ACKNOWLEDGEMENTS

The authors appreciate the excellent technical assistance of Mary Sue Brown. We also thank Carolyn Thomas for manuscript preparation and Gene Hedberg for his excellent technical drawings.

REFERENCES

1 C. T. Wehr, and S. R. Abbott, *J. Chromatogr.*, 185 (1979) 453.
2 Y. Kato, K. Komiya, Y. Sawada, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 190 (1980) 305.
3 M. Okazaki, K. Shiraishi, Y. Ohno and I. Hara, *J. Chromatogr.*, 223 (1981) 285.
4 G. Vanecek and F. E. Regnier, *Anal. Biochem.*, 109 (1980) 345.
5 J. E. Butler, *Veterinary Immunology and Immunopathology*, 4 (1983) 43.
6 Bio-rad Lab., Product Information 1061 E, *CM Affi-Gel Blue*, April 1978.
7 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
8 P. Grabar and G. A. Williams, *Biochim. Biophys. Acta*, 10 (1953) 193.
9 R. C. Cutlip, T. A. Jackson and G. A. Laird, *Amer. J. Vet. Res.*, 38 (1977) 1081.
10 H. D. Lehmkuhl and P. M. Gough, *Amer. J. Vet. Res.*, 38 (1977) 1717.
11 S. Srikumaran, A. J. Guidry and R. A. Goldsby, *Amer. J. Vet. Res.*, 43 (1982) 21.
12 R. A. Van Deusen, in N. Stern and H. R. Gamble (Editors), *Hybridoma Technology in Agricultural and Veterinary Research*, Rowman and Allenheld, Totowa, NJ, 1984, p. 15.
13 J. P. Mach and J. J. Pahud, *J. Immunol.*, 106 (1971) 552.