Journal of Chromatography, 131 (1977) 383–390

CHROM. 9388

RAPID PURIFICATION OF AN RNA TUMOR VIRUS AND PROTEINS BY HIGH-PERFORMANCE STERIC EXCLUSION CHROMATOGRAPHY ON POROUS GLASS BEAD COLUMNS

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(Received June 4th, 1976)

SUMMARY

High-performance steric exclusion chromatography on a 1250-Å pore size polyethylene glycol-treated glass bead column was used to purify avian myeloblastosis virus and hamster melanoma virus from plasma protein and tissue culture media. The purified hamster melanoma virus was still infectious and the avian myeloblastosis virus-associated RNA-directed DNA polymerase showed a 1100-fold purification of the virus from one column treatment. Electron microscopy of the purified virus showed intact particles, with surface projections evident. The time required for column purification of the virus was 5 min.

INTRODUCTION

In the course of our studies on the ability of various nucleosides to cause the release of RNA tumor viruses from cultured tumor cells, it became necessary to process rapidly many samples of cell culture fluid in order to determine the amount of virus released into the medium at various times after the chemical treatment, and to obtain pure virus for reverse transcriptase and protein studies. It has been shown by others[1,2] that sucrose gradient centrifugation results in both damaged virus particles and samples that contain cellular debris. Thus the availability of high-performance liquid chromatography (HPLC) and the success of others[3,4] in purifying other types of animal viruses by exclusion chromatography on porous glass beads led us to this technique.

We used avian myeloblastosis virus (AMV) and hamster melanoma virus (HaMV) as a model to determine whether an RNA tumor virus could be recovered intact from glass bead columns under conditions of rapid elution. In addition, since we are interested in the characteristics of the polymerases ("reverse transcriptase") of the recovered virus, we attempted to determine whether the solubilized polymerase,

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known to be very labile, could be recovered using the same type of chromatographic system.

Owing to the large size of RNA tumor viruses (800–1200 Å in diameter) it seemed reasonable to believe that they could be eluted in the void volume of a column packed with large pore glass beads, in which most macromolecules, even large proteins, would be retained. It has been shown, however, that, although the glass beads can be treated in order to lessen adsorption, precautions effective for one virus or protein do not necessarily protect another from adsorption[5]. In addition to their large size, their lipid-containing outer coat makes RNA tumor viruses quite different from other bacterial or animal viruses purified by porous glass bead chromatography[3,4].

In this study we used HPLC to separate plasma proteins from RNA tumor viruses by steric exclusion chromatography on glass beads of a defined pore size. In addition, a preliminary purification of RNA-directed DNA polymerase from disrupted virions was achieved.

METHODS

A Varian 4100 liquid chromatograph, equipped with a UV detector (254/280 nm) was used in all of the following experiments. Columns used were stainless-steel tubing, 1/4 in. O.D., 4.5 mm I.D., and 1, 2, or 3 m long. Columns were fitted for 1/8 in. O.D., 2 mm I.D., 15 cm long pre- and post-columns for connection to injector and detector. Extrusion of the packing was prevented by 2-μm frits mounted in a Swagelok fitting placed between the post column and the detector. Separations were done at ambient temperature for AMV and at 0° for HaMV. All columns were coiled, with a coil diameter of 30 cm.

Controlled pore glass (CPG) beads of mesh sizes 80–120 or 120–200 were used. CPG was purchased from Analabs (North Haven, Conn., U.S.A.) or from Electro-Nucleonics (Fairfield, N.J., U.S.A.); the manufacturer provided bead size and pore size distribution, which varied from ± 5 to $\pm 10\%$. CPG adsorbs some compounds, especially proteins with isoelectric points above pH 7. The adsorption was greatly reduced by a modification of the method of Hawk *et al.*[5], in which the beads were treated with a de-aerated 3% aqueous solution of polyethylene glycol (PEG).

100 g of CPG beads were added to 500 ml of a degassed solution of 3% 20 M PEG in distilled water. A vacuum was pumped on the solution to insure that air held in bead pores was removed to allow PEG to enter the pores. The solution was shaken occasionally to free any air bubbles adsorbed to beads. After 30 min the solution was put on a line vacuum overnight. The excess PEG was removed from the beads by repeated distilled water washes until no foam was noticed. The beads were air dried under a vacuum. We did not attempt to oven dry the PEG-treated beads for several oven dried preparations showed adsorption of proteins; also the interaction of PEG with silica gels changes drastically with heating to produce a hydrophobic surface[6].

The beads were dry packed into the column, which was tapped vigorously after the addition of each 1.0-ml aliquot. The column was then connected to the pump and equilibrated with degassed buffer flowing at 200 ml/h. The equilibration removes air in the bead pores, and further compresses the column packing. Usually 1–4 cm^3 of CPG were added after the packing had settled under pressure. We routinely emptied columns for cleaning after 80 chromatographic runs. The glass beads may be

cleaned by soaking in concentrated nitric acid, rinsing with water, and again treating with PEG.

Ortec (Oak Ridge, Tenn., U.S.A.) Gradipore polyacrylamide gradient gels were used with an Ortec 4100 pulsed power supply for electrophoresis studies.

All proteins were obtained from Sigma (St. Louis, Mo., U.S.A.), except for alkaline phosphatase (*Escherichia coli*, 35 units/mg) purchased from Worthington (Freehold, N.J., U.S.A.). Protein samples for chromatography were 1 mg/ml in elution buffer unless otherwise noted. J. Beard kindly supplied AMV in plasma collected from infected chickens. RNA-directed DNA-polymerase assay was described by Reid and Albert[7]. Alkaline phosphatase was assayed by the method of Garen and Leventhal[8]. Catalase was detected by its reaction with hydrogen peroxide. *p*-Nitrophenylphosphate was assayed using alkaline phosphatase, and sodium chloride by silver precipitation.

Samples were injected via an injection port, or a loop injector equipped with either a 0.2- or 1.0-ml loop (the 1-ml loop was used routinely for the virus injections).

Elution volumes (V_e) were measured from an injection artifact to the UV peak. For molecular weight determination, elution positions are expressed as $K_{av} = (V_e - V_0)/(V_t - V_0)$, in which V_0 is void volume, and V_t is total included volume[9].

Protein molecular weights used were those listed by Andrews[9] except that used for once lypholized catalase[10]. Protein concentrations were determined by the method of Lowry *et al.*[11].

RESULTS

AMV can be separated routinely from plasma proteins and smaller molecules by high-speed liquid chromatography on glass beads having a pore diameter of 1250 Å (CPG-10-1250, 80–120 mesh, pore size ±10%). The virus-containing plasma is centrifuged at 5000 *g* for 5–10 min to remove fibrinogen. The supernatant is then injected into the chromatographic column. Eluting virions are detected: (1) by assaying for detergent-requiring polymerase activity *versus* protein concentration; (2) by a difference in UV absorption (eluent absorption at 254 nm minus absorption at 280 nm); (3) by electron microscopy. Routinely, only the difference spectrum is checked, since the position of the virus peak, once determined, does not vary more than 0.1 ml, which is within experimental error. Good separation is achieved on a 2-m column, from which virions elute in 5 min if a flow-rate of 120 ml/h is maintained. Under these conditions the virions injected in a 1-ml sample of plasma elute in a volume of about 3.5 ml. Table I shows that on the basis of RNA-directed DNA-polymerase activity, which is known to be associated with the virus particle, essentially 100% recovery is achieved. The slight increase in activity (more than 100% recovery) could be due to the removal of inhibitors. In addition, it can be seen that a 1,100-fold purification of the virus-associated enzyme was achieved.

Similar studies using HaMV showed that, after correcting for dilution, a 12-fold increase in total activity for the viral reverse transcriptase was obtained. Of more importance is the result that the column-purified virus could produce transformation of hamster embryo cells in tissue culture. It was felt that the increase in activity resulting from column treatment was due to the removal of polymerase inhibitors. It was therefore difficult to determine the amount of purification achieved with the virus.

TABLE I

RNA-DIRECTED DNA-POLYMERASE ACTIVITY BEFORE AND AFTER CHROMATOGRAPHY OF THE AMV VIRUS

Sample	*[^{3}H]TMP incorporated (pmoles·ml^{-1}·h^{-1})*	*Protein concentration (mg/ml)*	*[^{3}H]TMP incorporated (pmoles per hour per mg of protein)*
AMV in crude plasma	80 ± 3	1.4	57
AMV after CPG chromatography	23 ± 1*	0.0013	63,200

* Dilution of 3.5 on the column.

However, it is felt that it is of the same order of magnitude as that found for AMV, based on the fact that the same number of infectious virus particles were found by focus-forming assays before and after column purification.

Fig. 1 shows polyacrylamide gel electrophoresis patterns of both chromatographically purified AMV and AMV in plasma. The purified virions just enter the 4% gel with no detectable plasma protein present. By comparison, the plasma is seen to contain many proteins in large quantity, which run behind catalase.

Another check on the nature of the virus preparation was achieved by electron

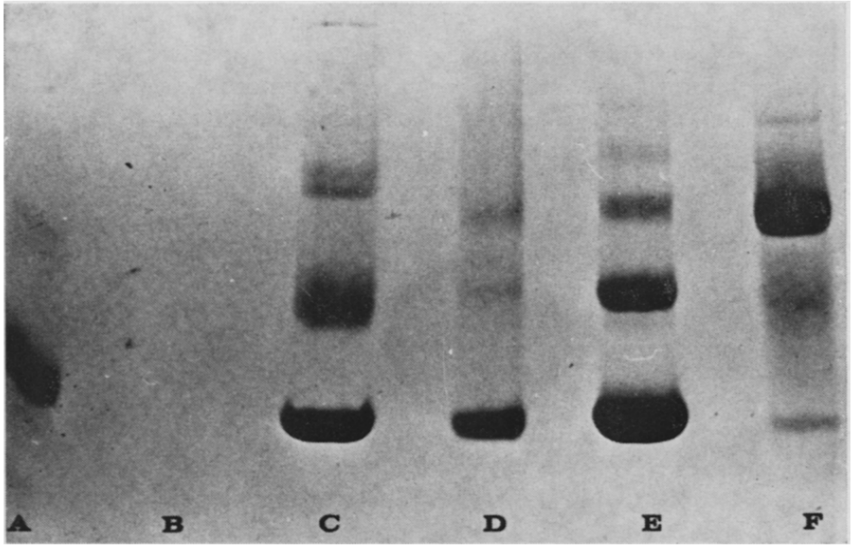
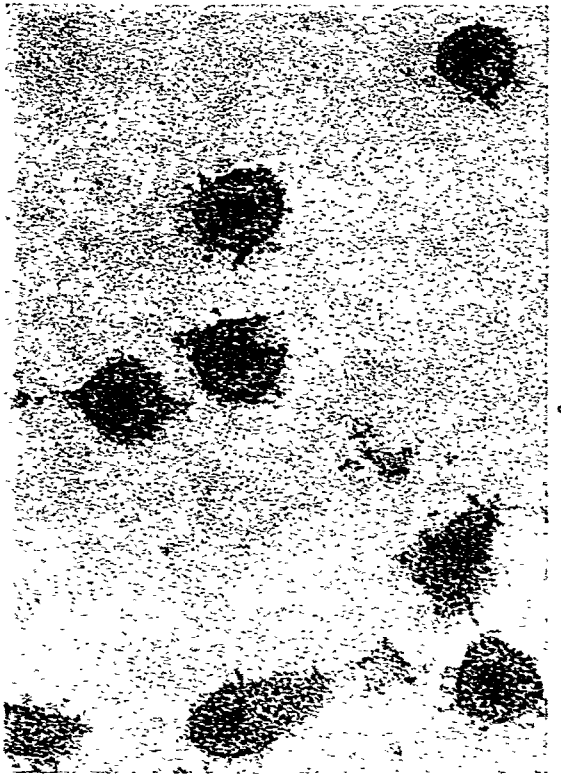


Fig. 1. Polyacrylamide gel electrophoresis of chromatographically purified AMV. A, D, E, and F contain 50 μg of hexokinase, arginase, bovine serum albumin, and catalase, respectively. C contained 7 μl of AMV plasma. B contained 30 μl of purified virus. Protein, plasma, and virus preparation samples were all diluted with equal volumes of 50% sucrose–borate buffer (pH 9.6) before being added to the gels. Electrophoresis was on a 4 to 46% gradient Gradipore gel, with pH 9.6 borate buffer. A 4100-pulse power supply was used, set at 25 mA, 225 V, 75 pulses/sec for the first 10 min, 100 pulses/sec for the next 15 min, and 150 pulses/sec for the last 5 min after which the tracking dye had eluted. Gels were stained with Schwartz amido black overnight.



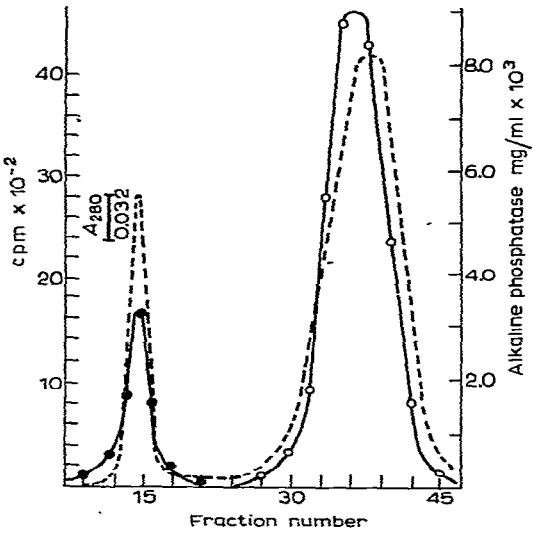


Fig. 2. Chromatographically purified AMV (× 200,000).

Fig. 3. Separation of alkaline phosphatase (*E. coli*) from AMV. A solution of *E. coli*, alkaline phosphatase- and AMV-containing plasma was injected into a 2 m × 1/4 in. CPG-10-1250 glass bead column using a 200-μl loop injector. The sample contained 0.035 mg of alkaline phosphatase. Elution was with Tris buffer (0.01 *M*, pH 8.3) at a flow-rate of 30 ml/h, with a back pressure of 100 p.s.i. Fractions of 0.5 ml were collected. The polymerase and phosphatase activities were assayed as described under Methods. ●, Detergent-requiring polymerase activity; ○, alkaline phosphatase activity; — — —, absorbance at 280 nm.

microscopy of a virus preparation that was pelleted after chromatography; Fig. 2 shows an electron micrograph of such a preparation which was pelleted under conditions such that material of *s* value of 88S or more would have pelleted with virus. One preparation of virus chromatographed on beads of 363 Å pore size contained debris when visualized by electron microscopy. Thus, a larger pore size separates virus from plasma material that was not separated by the smaller pore beads. The virions shown in Fig. 2 are of the size expected for AMV, that is, 800–1200 Å in diameter[12]. The fine structure of the C-type virus particles has not been damaged. The inner dense core, as well as surface projections are clearly visible[12]. Preservation of virus morphology requires low salt eluent buffers. Elution with 0.2 *M* phosphate buffer, instead of our usual 0.01 *M* Tris, appears to dehydrate the virions so that they appear elongated and disrupted.

Fig. 3 shows the results of an experiment in which AMV was separated from a sample of *E. coli* alkaline phosphatase which had been added to virions in plasma.

Plasma contains a small amount of activity that assays as alkaline phosphatase. When this background activity was substracted from the recovered activity it was found that the recovery of the *E. coli* alkaline phosphatase was quantitative.

We have modified the method in order to purify virus from larger volumes of plasma. After the preliminary low-speed centrifugation, AMV in plasma or HaMV in tissue culture fluid is pelleted by centrifugation at 100,000 *g* for 60 min. Supernatant plasma is removed; virus is resuspended in a small amount of buffer (usually 1.2 ml). This resuspended material is chromatographed; UV-absorbing material is well separated from the virus peak on a 2-m column. The elution volume for virus on this column was 3.5 ml.

Similarly, columns of 363 Å pore size beads (CPG-10-370, mesh size 80–120, pore size ±5.4%), pretreated with PEG, can be used to separate proteins of molecular weight in the 10,000–400,000 dalton range. V_0 was determined as the volume at which AMV eluted. V_t was determined as the volume at which sodium chloride, *p*-nitrophenylphosphate, or phenol eluted. Fig. 4 lists the proteins that were chromatographed and their K_{av} values *versus* logarithm of molecular weight. The first peak eluted was assumed to be the protein. Often a second peak eluted, generally with a V_e expected for the protein subunit. We note that the K_{av} values generally agree well with those obtained by Hawk *et al.*[5]. The graph of log MW versus K_{av} is quite linear between V_0 and V_t. The proteins used in this study possess isoelectric points, of pH 7.5 or less, *e.g.*, lysozyme. Proteins with high isoelectric points tend to adsorb to the CPG beads in our system; others have noted similar adsorptive effects[3,13]. The adsorption may be due to sites not sensitive to the PEG treatment.

The RNA-directed DNA-polymerase of AMV can be separated from other viral components using chromatographic columns similar to those described above. Purified virus is pelleted by ultracentrifugation at 100,000 *g*. The pellet is suspended in 1.2 ml of solubilization buffer containing 1% NP-40 (a non-ionic detergent), 0.1 *M* NaCl, and 0.01 *M* Tris, pH 8.3. The solution is incubated for 1 h at room temperature. Chromatography on CPG-10-700 of the detergent-disrupted virus gave a peak of polymerase activity only slightly displaced from V_0 (see Fig. 5). In order to stabilize

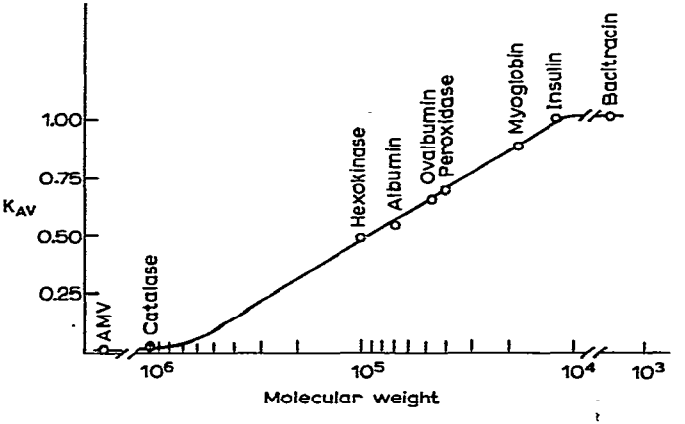


Fig. 4. Separations of proteins by molecular weight on a CPG column. Chromatography on a coiled, PEG pre-treated, CPG-10-370 (80–120 mesh) column, 3 m × 1/4 in., of various proteins using 0.1 *M* sodium phosphate, pH 7.5.

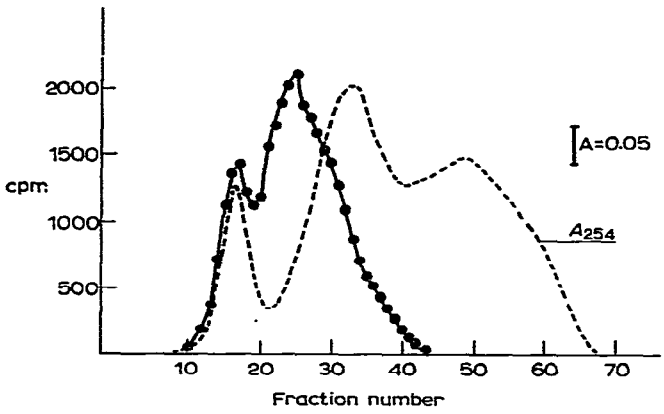


Fig. 5. Chromatography of solubilized viral proteins. Chromatography on a coiled, PEG pre-treated, CPG-10-700 column 2 m × 1/4 in. of NP-40-solubilized viral proteins. The results of polymerase activity shows two peaks, one associated with viral RNA and the other slightly ahead of the bulk of the viral proteins. ●, Polymerase activity; — — —, absorbance at 254 nm. The viral RNA was identified by its relative absorbance at 260 nm and 280 nm.

the polymerase it is desirable to make the solution 50 m*M* in dithiothreitol. However, since dithiothreitol has a strong UV absorption, it was not added for the run monitored by the UV detector. Preliminary experiments indicate that the polymerase may be well separated from proteins and RNA by coupled columns which contain CPG-10-370 and CPG-10-700, respectively.

DISCUSSION

Our results indicate that AMV and HaMV assayed by viral polymerase, can be quantitatively recovered after separation from plasma proteins on a CPG column. Some viral polymerase activity occurs in protein peaks eluting after the virus peak; however, this activity does not require detergent, and can be attributed to solubilized polymerase, perhaps from virus that was disrupted during the thawing of the plasma solution.

The results of chromatography of globular proteins agree well with the observations made from chromatography on Sephadex and similar packings, that K_{av} plotted against the log of molecular weight gives a straight line[9]. The relationship is a consequence of the variation of Stokes' radius with molecular weight. The determination of molecular weights by this technique would entail assuming that the protein is globular and without any great symmetry. Because of the adsorptive properties of CPG, one can underestimate the molecular weight of a substance whose progress throughout the column was retarded by adsorption. However, retardation seems to occur only with proteins whose isoelectric points are greater than 7.5. Detergent-liberated reverse transcriptase can be separated from the main bulk of low-molecular-weight protein in the virus, in good yield, in spite of the extremely labile nature of this enzyme. The good recovery of activity is probably due to the short time with which a column run may be performed on the glass bead column.

Rapid separation is often useful for experimentation with labile viruses and

proteins. The technique described above is rapid, and lends itself to careful control of chromatographic conditions. The columns can be packed and stored until needed. Finding the proper conditions of separation is limited only by ease of performing the required assay.

Two cautions are warranted. First, if one desires to retain the infectivity of viruses, one should avoid radiation damage, which might be caused by the UV detector. However, once the elution volume of a virus is determined, detectors need not be used. Secondly, the glass bead adsorptive characteristics are not well understood. PEG treatment seems to diminish the charge–charge adsorptive phenomena, but adsorption may still occur. AMV appears to be completely excluded from the pores of CPG-10-1250, so there is little possibility of virus adsorption within the pores. The strategy of avoiding potential adsorption by excluding the macromolecule from the bead pores is similar to the approach taken by Haller *et al.*[14] in the purification of immunoglobulin M on porous glass beads. Thus, for a particular separation the PEG treatment may not be necessary. If it is, one should be wary of accidentally removing the PEG by solutions containing sugars or some detergents.

Separations of proteins are improved as the adsorptive characteristics of the glass beads are decreased, and through the use of smaller beads and longer columns. The speed and precision of high-performance liquid chromatography may lend themselves to the purification of other labile viruses and proteins.

ACKNOWLEDGEMENTS

We thank Dr. Joseph Beard for the avian myeloblastosis virus and Dr. Gerald Hawk for helpful discussions. Supported by PHS grant from the National Eye Institute, NIH training grant for T.D. and RPB Research Professorship for T.W.R.

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