Journal of Chromatography, 259 (1983) 169–175
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,577

Note

Mouse interferon: rapid purification by adsorption on controlled pore glass and high-performance liquid chromatography

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(Received November 2nd, 1982; revised manuscript received November 30th, 1982)

A major problem in studying the mechanism of action of interferon, and indeed, in the clinical evaluation of interferon, lies in the difficulty with which interferon is purified. Although both mouse[1] and human[2,3] interferon have been purified to homogeneity, the methods used have been time consuming, involving reagents or procedures which can denature the protein being purified. Both the use of harsh purification conditions and the use of multiple step procedures increase the likelihood of inactivating interferon. Thus, we decided to investigate the utility of high-performance liquid chromatography (HPLC) using mild elution conditions in the purification of mouse L cell interferon in order to increase the speed and cumulative recovery of interferon compared to traditional processes.

Controlled pore glass (CPG) chromatography of proteins was introduced by Bock *et al.*[4] and was subsequently extended to the purification of crude human interferon[5–7] and mouse L929 cell interferon[5]. In view of its simplicity and high capacity, CPG is a useful technique for the concentration of crude interferon. The possible utility of HPLC in the purification of interferon was suggested by Rubinstein *et al.*[8], who employed reversed-phase (RP) and normal-phase (NP) partition HPLC in the purification of human leukocyte interferon. The reported recovery during their HPLC steps ranged from 40–90% of the antiviral activity. We have attempted to take advantage of the rapidity of HPLC while avoiding the possible deleterious effects of the organic modifiers needed in RPLC and NPLC on the stability of interferon by employing ion exchange and size-exclusion HPLC in the purification of mouse L cell IFN. The peak specific activity of the purified mouse interferon we obtain is 438-fold higher than the crude mouse interferon. The overall recovery of interferon activity is 141%. These data indicate that use of CPG-HPLC can be a rapid and efficient method of purification of mouse L929 cell interferon, for the purpose of utilization in pharmacological studies in our laboratory.

0021-9673/83/0000–0000/$03.00

EXPERIMENTAL

Production and assay

Crude mouse interferon was produced by mouse L929 cell cultures which were acid treated to inactivate endogenous virus prior to infection with Newcastle Disease virus[9]. Crude preparations contained $1.6 \cdot 10^4$ units/ml and $1.3 \cdot 10^4$ units/mg of protein. Column eluents were monitored for antiviral activity with a microtiter plaque reduction assay on mouse L cells using vesicular stomatitis virus as the challenge virus[10]. Assays were calibrated with an NIH standard; titers were expressed as NIH reference units per ml.

High-performance liquid chromatography

Column used were an anion-exchange column (SynChropak AX 300, 6.5 μm, 250 × 4.1 mm I.D., SynChrom, Linden, IN, U.S.A.), cation exchange column (SynChropak CM 300, 6.5 μm, 250 × 4.1 mm I.D., SynChrom) and size exclusion column (Spherogel-TSK 3000 SW, d_p 10 μm, 300 × 7.5 mm I.D., Beckman Instruments, Berkeley, CA, U.S.A.). A Beckman Model 421 system controller and a Model 210 injector were used. Column eluents were monitored on a Hitachi Model 100-4018 ultraviolet–visible variable detector and results were plotted on a Hewlett-Packard Model 3390A integrator.

Concentration and purification of crude interferon

Controlled-pore glass beads (200–400 mesh, Electro-Nucleonics, Fairfield, NJ, U.S.A.) were added to 730 ml crude mouse L929 cell interferon at a concentration of 10 mg/ml. Absorption was carried out at 4°C for 3 h, with constant stirring. CPG was collected on nylon mesh and washed extensively with cold, sterile phosphate-buffered saline (PBS) to remove unbound protein[11]. The washed IFN containing CPG was then loaded into a 20 × 1.5 cm column and flushed with PBS at 4°C. The column effluent was monitored by UV absorbance at 280 nm. When a stable baseline was achieved, indicating removal of unbound proteins, the interferon was eluted with 0.5 *M* $(CH_3)_4N^+Cl^-$ in PBS[12,13]. The column was monitored for interferon activity; the interferon containing peak was pooled (56 ml) and was concentrated 10-fold (6 ml) using a 43-mm PM-10 Amicon Diaflo membrane at 4°C (2 h). Concentrated sample (1 ml) was applied on a semi-preparative size-exclusion column (HPLC) and eluted isocratically with 0.02 *M* sodium phosphate and 0.15 *M* NaCl, pH 7.25 at a flow-rate of 0.5 ml/min. Eluents were monitored by UV absorbance at 280 nm. A typical size-exclusion HPLC run is completed in 40 min.

Protein determination

Protein concentrations were determined by the method of Lowry *et al.*[14], with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Although both mouse and human interferons have been substantially purified from natural sources[1–3], the methods used have typically been time consuming, involving multiple concentration, absorption, chromatographic and electrophoretic

steps. Partially as a result of the time needed for a multi-step purification, and partially as a result of the sometimes harsh conditions used, the recovery of highly purified interferon has typically been low. In this study it has been our aim to enhance the recovery of mouse L cell interferon by employing rapid purification techniques while avoiding the use of destabilizing conditions. Chromatography on CPG offers several advantages as an initial step in the purification of mouse L cell interferon, including a high binding capacity and sufficient specificity to effect a substantial initial purification (10–50 fold) while serving as a means to concentrate the interferon present in crude preparations[5,15]. With the introduction of tetraalkylammonium salts as an elution agent for chromatography[7,12] the recovery and stability of interferon produced by this technique has been greatly enhanced[13,16]. Using tetramethylammonium chloride as the eluting agent, we typically obtained a purification factor of 21-fold with a 91% recovery of interferon activity (table I).

TABLE I

PURIFICATION OF MOUSE INTERFERON

	Total activity (units)	*Spec. act. (units/mg)*	*Purification factor*	*Recovery (%)*
Crude mouse interferon	$1.16 \cdot 10^7$	$1.3 \cdot 10^4$	–	100
Controlled-pore glass chromatography	$1.06 \cdot 10^7$	$2.7 \cdot 10^5$	21	91
Ultrafiltration ($\geq$ 10,000 daltons)	$1.02 \cdot 10^7$	–	–	88
Size-exclusion HPLC				
Σ fractions 1 5	$1.83 \cdot 10^6$	$9.8 \cdot 10^4$*	8	16
Σ fractions 6 20	$1.45 \cdot 10^7$	$2.1 \cdot 10^6$*	162	125
(fraction 9)	$3.0 \cdot 10^6$	$3.4 \cdot 10^6$	262	(26)
(fraction 19)	$2.4 \cdot 10^5$	$5.7 \cdot 10^6$	438	(2)

* Average value.

In attempting to utilize the speed of HPLC in the purification of mouse L cell interferon we first investigated ion-exchange HPLC as a possible purification protocol. Figs. 1 and 2 show typical results obtained with cation-exchange (Fig. 1) and anion-exchange (Fig. 2) HPLC of crude and CPG concentrated mouse L cell interferon. In all cases, interferon activity coeluted with the major, unstained protein peak. Attempt to improve the separation of interferon activity from the major protein contaminate by altering pH and/or salt concentration of the elution buffer were unsuccessful (data not shown). Thus, although cation exchange HPLC is clearly of considerable utility in monitoring the efficiency of the purification of mouse L cell interferon (Fig. 1), it is of itself incapable of separating the interferon activity from the major contaminating protein species. Consequently, we have used the high capacity, batch procedures of CPG adsorption and ultrafiltration as the initial steps of our purification.

The CPG and ultrafiltration-concentrated mouse L cell interferon was also fractionated on a semipreparative size exclusion HPLC column (TSK-3000 SW, Beckman Instruments). Interferon activity eluted from this column as a broad peak,

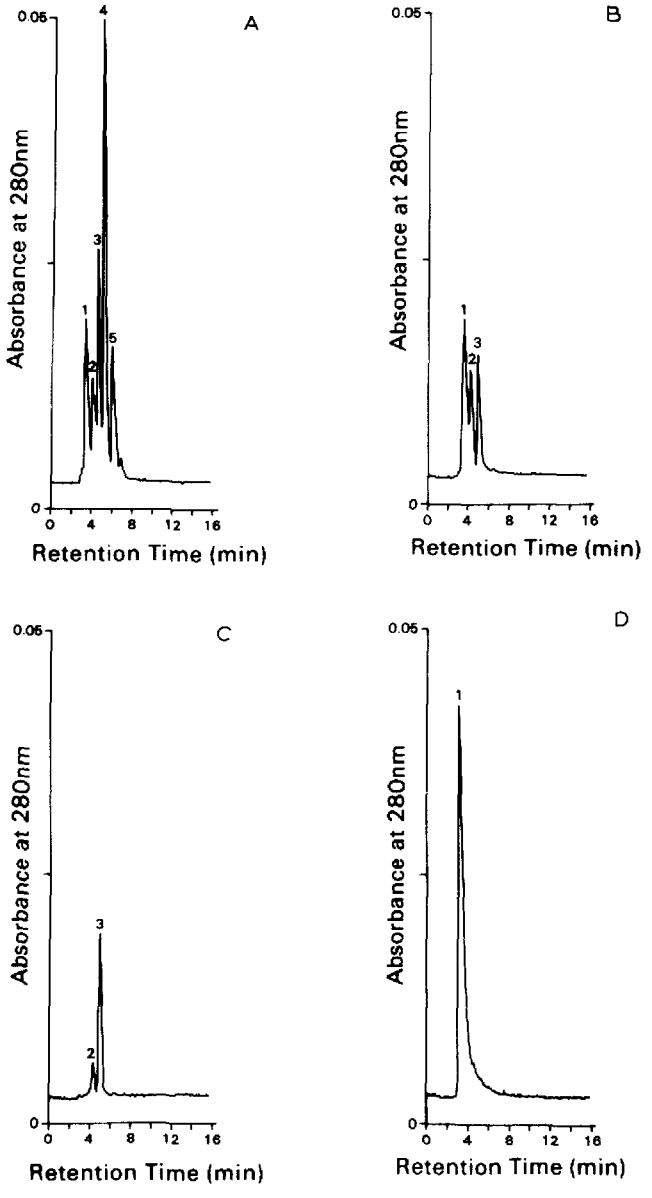

Fig. 1. Cation-exchange HPLC of CPG and ultrafiltration fractions of mouse L cell interferon. A SynChropak CM 300 column was eluted isocratically with 0.02 *M* sodium phosphate and 0.15 *M* NaCl, pH 7.25 at a flow-rate of 0.5 ml/min. (A) 10 μl crude mouse interferon, (B) 40 μl CPG purified mouse interferon, (C) 50 μl of CPG purified mouse IFN ultrafiltrate (MW $<10{,}000$ daltons), and (D) 10 μl of CPG purified mouse IFN ultrafiltrate retentate (MW $\geq 10{,}000$ daltons). Interferon activity is found in the first eluting protein peak in all cases.

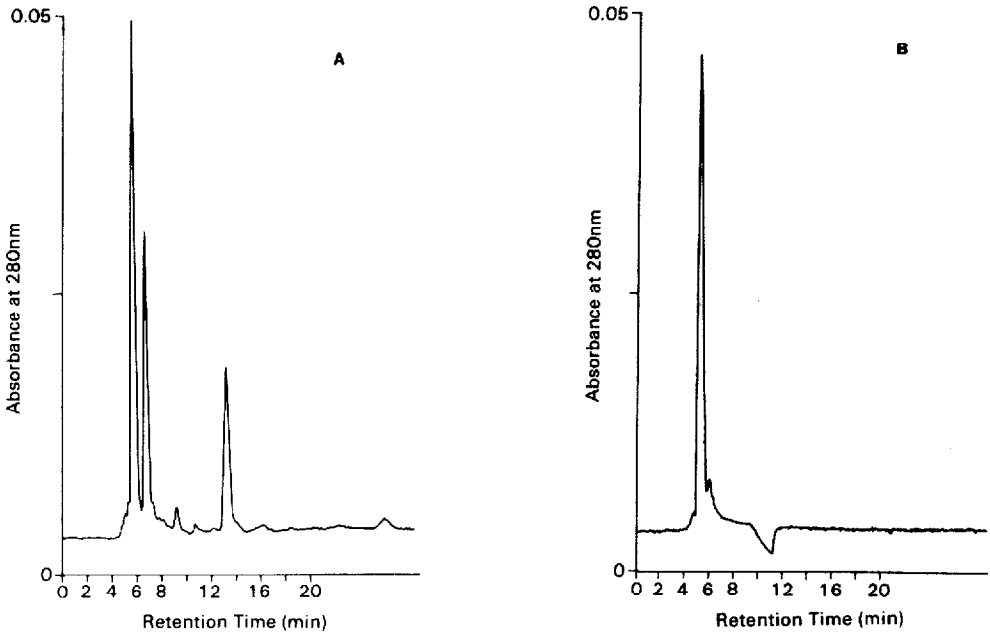

Fig. 2. Anion exchange HPLC of CPG and ultrafiltration fractions of mouse L cell IFN. A SynChropak AX 300 column was used. Column conditions as Fig. 1. (A) Crude mouse interferon, and (B) CPG purified and concentrated (MW $\geqslant$ 10,000 daltons) mouse interferon. Interferon activity is found associated with peak 1.

slightly after the major protein component (Fig. 3). The major protein contaminate of the concentrated CPG-preparation coeluted with a bovine serum albumin marker on the TSK-3000 SW column. We thus tentatively identified this peak as serum albumin present in the cell culture media, an identification which is supported by the failure of the component to bind on the AX300 anion-exchange column (Fig. 2). A typical purification scheme for mouse IFN utilizing adsorption chromatography on CPG and size exclusion HPLC is presented in Table I. The major interferon containing peak, which was partly resolved from the 68,000 daltons contaminant contained 125% of the original interferon activity, with an average specific activity of $2.1 \cdot 10^6$ units/mg. The most highly purified fraction of this peak, containing 26% of the original interferon activity, had a specific activity of $3.4 \cdot 10^6$ units/mg, and represented a 262-fold purification. The trailing peak of interferon activity contained only 5% of the total original interferon activity, but was highly purified, with the peak fraction representing a 438-fold purification and a specific activity of $5.7 \cdot 10^6$ units/mg.

The data reported here show that it is possible to obtain a significant purification mouse L cell interferon in only two days using adsorption on CPG, ultrafiltration and size exclusion HPLC. The interferon obtained by this procedure is stable for over two months at -20°C. Overall recovery of activity from the purification was greater than 140%. We attribute the high recovery partly to the speed which the use of HPLC allows and partly to our use of nondenaturing conditions throughout the purification. The use of tetramethyl ammonium chloride as the elution agent in CPG

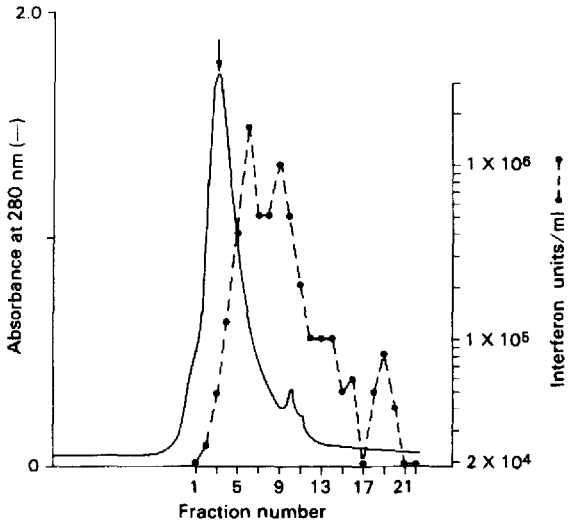

Fig. 3. Semi-preparative scale elution profile of purification of mouse interferon on TSK 3000 SW size-exclusion column. ———, absorbance at 280 nm; ●------●, interferon activity. The elution volume of a 68,000-daltons molecular weight (bovine serum albumin) marker is shown by an arrow (↓). The column was eluted isocratically with 0.02 *M* sodium phosphate and 0.15 *M* NaCl, pH 7.25 at a flow-rate of 0.5 ml/min.

chromatography[12,13] allows the elimination of elution agents such as ethylene glycol. The substitution has been shown to enhance the recovery and stability of the resulting interferon[13,16]. The greater than quantitative recovery of antiviral activity is not unusual in interferon purification[11], and may be due to a combination of variation of the assay procedures and to the removal of interferon inhibiting and/or viral enhancing components present in the crude preparations.

The presence of a bimodal area of interferon activity in the size exclusion HPLC column profile (Fig. 3) is suggestive of a partial separation between the alpha (MW = 22,000 daltons) and beta (MW = 40,000 daltons) species typically present in mouse L cell interferon preparations. The identity of the small, trailing, nominally low-molecular-weight peak of interferon activity (fractions 18–20, Fig. 3) is problematical. This peak, which was not found in all size exclusion experiments performed, could conceivably result from the interaction of a small portion of the interferon with the column matrix. In contrast, the two higher molecular weight, major peaks of interferon were consistently present in all experiments. Re-chromatography of the peak areas or the use of addition size exclusion column connected in series should increase the resolution of the alpha and beta mouse interferon, without significantly increased the experimental time. Similarly, the purity of the mouse interferon produced by this procedure should be significantly enhanced without sacrificing the speed of the protocol by increasing the length or number of size exclusion columns used. These experiments and further studies using the highly purified interferon samples in pharmacological studies are currently in progress in our laboratory.

CONCLUSION

The method described here is a rapid and reproducible technique suitable for analytical and preparative scale purification of mouse interferon. The IFN obtained by these procedures was stable; the specific activity of the peak fraction was $5.7 \cdot 10^6$ units/mg protein, a 438-fold higher than crude mouse interferon. Using this protocol mouse L cell interferon can be purified to a specific activity of over $2 \cdot 10^6$ units/mg in two days. Thus, our data indicate that CPG and HPLC using nondenaturing elution conditions can be useful for the rapid production of mouse interferon, allowing its utilization as a model system for pharmacological testing and disease control.

ACKNOWLEDGEMENTS

We thank Joyce Sabados for her help in preparation of crude mouse interferon, and Robert B. Lewis for his technical assistance.

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