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PURIFICATION OF MOUSE INTERFERON BY SEQUENTIAL CHROMATOGRAPHY

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SUMMARY

Two schemes for the purification of mouse interferon are described, based on the concerted application of various physicochemical and affinity/adsorption column chromatographic techniques. Mouse interferon was purified to a final specific activity of 1.0–8.0 $\times$ 10^8 units/mg when first precipitated with ammonium sulphate and further processed by hydrophobic chromatography and adsorption chromatography on AFFI-Gel 202 and Controlled Pore Glass. It was purified to a final specific activity of 2.5–3.7 $\times$ 10^8 units/mg when first precipitated with ammonium sulphate and further processed by gel filtration with Ultrogel AcA 54, ion-exchange chromatography with Carboxymethyl Bio-Gel Agarose, hydrophobic chromatography with AFFI-Gel 202 and adsorption chromatography with Controlled Pore Glass.

INTRODUCTION

Interferons are notoriously difficult to purify, for three reasons. First, interferons are glycoproteins with an extremely high biological activity; with a predicted specific activity of greater than 1.0 $\times$ 10^9 units/mg protein[1], one unit of activity would constitute less than 1 pg of protein. Secondly, inactivation readily occurs when the protein concentration is reduced to less than 10–25 μg/ml[2,3]. Thirdly, interferon's non-specific stickiness to the experimental apparatus usually means low recoveries[4].

Recent progress in protein purification has led to interferon being separated from its neighbour molecules by a variety of techniques. For example, selecting for differences in physicochemical characteristics, interferons have been purified by size, with gel filtration chromatography[2,3,5–9]; by charge, with either ion-exchange chromatography or isoelectric focusing[3,5,6,8,10]; by solubility in acid or salt[2,3,6,7,10]; and by electrophoretic mobility, with polyacrylamide gel electrophoresis (PAGE)[6]. Alternatively, antibodies, directed against interferon or its contaminants and raised

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in an assortment of animals, have been immobilized on a solid support and employed as a successful tool for purifying interferons[11–16]. This application offers the advantage of having both a large and specific loading capacity.

In recent years, the impetus in interferon purification has been towards affinity and/or adsorption chromatography. Similarly to immunoadsorbents, columns of immobilized ligands usually possess a high specificity and capacity, and thus achieve a high degree of purification. Interferons have thus been purified by virtue of their partiality for certain hydrophobic ligands[17–24], polynucleotides[25], metals[26,27], thiols[28], organomercurials[29] and glass beads[29,30].

Mouse interferon[2,6] and human diploid fibroblast interferon[3] were purified by sequential physicochemical means to a specific activity of greater than 1.0×10^8 units/mg. More recently, a variety of physicochemical, affinity and immunoadsorbent techniques have been used to purify mouse interferon, in a sequential manner, to a specific activity of at least 1.0×10^9 units/mg[7,15,16]. Some of the disadvantages of these approaches were a requirement for dialysis and/or concentration of material between several purification steps[15,16] and the shortage of some reagents[7,16].

Two schemes for the purification of mouse interferon (MIF) are described here, based on the concerted application of various physicochemical and affinity/adsorption column chromatographic techniques. By coordinating the eluting and loading buffers employed, both schemes were designed to reduce the amount of time and manipulation (*e.g.*, dialysis and concentration) required and thereby reduce some of the inherent problems in purifying interferons.

The first scheme, which yielded a 59% recovery, and where the peak material had a specific activity of 8.0×10^8 units/mg or a 116-fold purification, consisted of hydrophobic chromatography with AFFI-Gel 202 and adsorption chromatography with Controlled Pore Glass (CPG). The second, which yielded only 6.7% recovery and where the specific acitivity of the peak material was 3.7×10^8 units/mg protein or 41-fold purification, employed, prior to the two steps described above, gel filtration with Ultrogel AcA 54 and ion-exchange chromatography with Carboxymethyl Bio-Gel Agarose (CM-BGA).

EXPERIMENTAL

Chemicals

Capsid proteins from purified tobacco mosaic virus (TMV) were the generous gift of Professor Jeener (Molecular Biology Dept., Free University of Brussels, Belgium), ovalbumin and DNP-1-α-alanine were obtained from Serva Feinbiochemicals (Heidelberg, G.F.R.), insulin from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), asparaginase (Crasnitine) from Bayer (Leverkusen, G.F.R.), lysozyme (crystallized from hen egg white) and bovine albumin (fraction V) from Armour Pharmaceutical (Eastbourne, Great Britain), AFFI-Gel 202 and CM-BGA from Bio-Rad (Richmond, Calif., U.S.A.), Ultrogel AcA 54 from LKB (Bromma, Sweden) and CPG from Electro-Nucleonics (Fairfield, N.J., U.S.A.).

Cells and viruses

L-929 cells and human diploid skin fibroblasts (VGS strain) were grown in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal calf serum

(FCS). The Kumarov strain of Newcastle disease virus (NDV) was used for interferon induction. The stock virus was prepared in the allantoic fluid of 10-day-old chick embryos. The challenge virus used for all assays was the Indiana strain of vesicular stomatitis virus (VSV). The stock virus was propagated in BSC-1 cells.

Interferons

Crude MIF was produced by a modification of the procedure described by Knight[2]. L-929 cells, grown to near confluency in 0.5 gallon roller bottles, were primed for 2 h at 37° with 100 units/ml of MIF in EMEM containing 10% FCS. The primer was decanted and replaced with 1.4×10^5 plaque-forming units/ml of NDV in EMEM. After incubation for 1 h at 37°, the NDV was discarded and the cells washed in phosphate-buffered saline (PBS), containing magnesium and calcium. The bottles were then replenished with EMEM and incubated at 37° for 18 h.

Interferon assays

MIF was assayed by the inhibition of VSV-induced cytopathogenicity (CPE) in L-929 cells. All assays included an internal standard calibrated against the National Institute of Health mouse reference standard G 002-904-511.

Ammonium sulphate precipitation

The procedure was essentially the same as described by Knight[2]. The pH of crude mouse L-929 interferon was adjusted to 2.0 with 12 *M* HCl. Solid ammonium sulphate, $(NH_4)_2SO_4$, was added to make a 40% saturated salt solution and, under stirring, allowed to incubate at 23° for 15 min. The pH was maintained at 2.0 with 2 *M* HCl. The material was then spun at 1400 *g* at 4° for 30 min and the salt saturation level of the supernatant increased to 65% with solid $(NH_4)_2SO_4$. This mixture was incubated for an additional 15 min at 23° and recentrifuged. The supernatant was decanted and the pellet resuspended in 10 ml of 50 m*M* sodium acetate (NaAc) buffer, pH 5.0. The salt was removed by dialysing the material against the same buffer. The average increase in purification was from *ca.* 10-fold to 50-fold with specific activities ranging from 1.0 to 90.0×10^5 units/mg. This preparation is referred to as 1°-MIF throughout the text.

Gel filtration chromatography with Ultrogel AcA 54

The procedure was modelled after the technique described by Knight[2]. A 1.6 × 63 cm column was packed with Ultrogel AcA 54 and equilibrated in 50 m*M* potassium dihydrogen phosphate buffer (K-PB), pH 5.9. 1°-MIF was dialysed against the equilibration buffer and concentrated with 80% saturated $(NH_4)_2SO_4$ as follows: solid $(NH_4)_2SO_4$ was added to 1°-MIF and, under stirring, allowed to incubate at 23° for 30 min. The mixture was then pelleted at 1400 *g* for 30 min at 4° and the precipitate was resuspended in 2 ml of equilibration buffer. 5 μl of DNP-1-α-alanine were added as a marker and the sample was loaded on to the column. The flow-rate was 10 ml/h and 80 2-ml fractions were collected. The extinction at 280 nm (OD_{280}) of the column effluent was measured with an LKB Uvicord III UV absorption monitor (LKB Produkter, Bromma, Sweden), using a 3-mm optical path length continuous flow cell. In the multiple chromatography experiments interferon purified by this procedure is referred to in the text as 2°-MIF (see Scheme 2).

Ion-exchange chromatography with CM-BGA

This procedure is also a variation of the technique previously described by Knight[2]. A 1.1 × 1.5 cm column of CM-BGA was equilibrated in 50 m*M* K-PB, pH 5.9, and the flow-rate was adjusted to 10 ml/h. The peak fractions from 2°-MIF (see Scheme 2) were loaded directly on to CM-BGA and the column was washed with equilibration buffer. The bulk of the activity was then eluted with 100 m*M* K-PB, pH 8.0, and 1-ml fractions were collected. Absorption at 280 nm was detected as described above. In the multiple chromatography experiments, interferon, purified by this procedure, is referred to in the text as 3°-MIF (see Scheme 2).

Hydrophobic chromatography with AFFI-Gel 202

This procedure has been previously described by Davey *et al.*[19]. The column sizes employed varied according to their use and are further described in the text. Depending on the experiment or scheme, columns, having been regenerated with phosphate-buffered saline (PBS) containing 10 *M* ethylene glycol and 1 *M* NaCl, were equilibrated either with 50 m*M* K-PB or NaAc, pH 5.0.

After the starting material had been loaded, columns were washed with the appropriate equilibration buffer, followed by 20 m*M* sodium phosphate buffer (Na-PB), pH 7.2. The bulk of the activity was then eluted with PBS containing 350 m*M* NaCl (PBS + 350 m*M* NaCl). Absorption at 280 nm was detected as described above. In the multiple chromatography experiments, interferon, purified by this procedure, is referred to in the text as either 2°-MIF or 4°-MIF, in Schemes 1 and 2 respectively.

CPG-adsorption chromatography

CPG beads were regenerated by washing in 65% nitric acid until the yellow suspension turned clear. This was followed by extensively washing the suspension in double-distilled water until the pH reached 5.0. The beads were then equilibrated in PBS.

Depending on their use, columns of various sizes were employed which are further described in the text. Absorption at 280 nm was detected as described above. In the multiple chromatography experiments, interferon, purified by this procedure, is referred to in the text as either 3°-MIF or 5°-MIF, in Schemes 1 and 2 respectively.

Multiple chromatography

Scheme 1. Crude MIF was concentrated and purified by $(NH_4)_2SO_4$ precipitation (1°-MIF) and dialysed against 50 m*M* NaAc, pH 5.0. This was then loaded (flow-rate 10–18 ml/h) directly on to a 0.9 × 12 cm column of AFFI-Gel 202, equilibrated in 50 m*M* NaAc, pH 5.0. After column washes with equilibration buffer (30–60 ml) and 20 m*M* Na-PB, pH 7.2 (30–60 ml), the material (2°-MIF) was eluted with 72 ml of PBS containing 350 m*M* NaCl, pH 7.2. During this last step, the output from the column was pumped directly on to the input portion of the CPG column (0.7 × 5.3 cm). On completion of this step, the first column was disconnected. The CPG column was then washed with 30–60 ml of PBS, followed by 30–60 ml of 400 m*M* glycine–HCl (gly–HCl), pH 3.5. Finally, the column was washed with 400 m*M* gly–HCl (pH 2.0), and 1-ml fractions were collected. All fractions having a pH less than 3.5 were assayed for antiviral activity (3°-MIF).

Scheme 2. Crude MIF was concentrated and purified by $(NH_4)_2SO_4$ precipita-

tion (1°-MIF) and dialysed against 50 m*M* K-PB, pH 5.9. This was then concentrated with 80% saturated $(NH_4)_2SO_4$ and loaded (flow-rate 10 ml/h) on to a 1.6 × 63 cm column of Ultrogel AcA 54, as described above. The peak activity fractions (2°-MIF) were then directly loaded (flow-rate 10 ml/h) on to a 1.0 × 1.5 cm column of CM-BGA, equilibrated in 50 m*M* K-PB, pH 5.9. As described above, the column was then washed in equilibration buffer (10 ml) and the activity (3°-MIF) eluted with 10 ml of 100 m*M* K-PB, pH 8.0. The peak protein-containing samples were pooled, diluted two-fold in double-distilled water, then pH-adjusted with 1 *M* HCl to 5.0 and applied to a 0.7 × 5.3 cm column of AFFI-Gel 202, equilibrated in 50 m*M* K-PB, pH 5.0. After the column had been washed with equilibration buffer and 20 m*M* Na-PB, pH 7.2 (10 ml each), the material was eluted and applied directly on to the CPG column, exactly as described in Scheme 1.

Protein determination

The protein content of samples was determined by the method of Lowry *et al.*[31].

SDS-polyacrylamide gel electrophoresis (PAGE)

Interferon preparations or markers were added to the sample buffer so that the final concentrations were as follows: sucrose, 10%; SDS, 1%; urea, 1.7%; 400 m*M* glycine and 50 m*M* Tris–HCl, pH 6.8. Samples were then boiled for 1 min and loaded (130 μl for preparative gels and 20 μl for analytical) on to a vertical slab gel. The lower gel [containing 15% acrylamide and 0.08% N,N'-methylenebisacrylamide (BIS)] was 13 cm in height and either 3 mm thick for preparative gels or 1.5 mm for analytical gels. The upper gel (containing 3% acrylamide and 0.8% BIS) was 3 cm in height and either 3 mm or 1.5 mm thick for preparative and analytical gels, respectively. The running buffer was as previously described by Laemmli[32].

Samples were electrophoresed at 50 V at room temperature for 20 h. The preparative gel was sliced into 0.5-cm sections and placed into glass tubes containing 0.5 ml of running buffer and 0.4% BSA. The eluted material was then assayed for antiviral activity.

RESULTS

Adsorption chromatography with CPG

The elution scheme previously reported[30] for MIF on CPG was modified. The eluents 10 m*M* gly–HCl, pH 2.0, and 100 m*M* KCl–HCl, pH 2.0, were substituted with 100 m*M* gly–HCl, pH 3.5, and 100 m*M* gly–HCl, pH 2.0. As shown in Fig. 1, 4.5×10^6 units of 1°-MIF (2.5×10^5 units/ml), containing 5.76 mg protein (0.32 mg/ml), or a specific activity of 7.8×10^5 units/mg, were loaded on to a 0.5 × 5.0 cm column of CPG, equilibrated in PBS. After the column had been washed with PBS and 100 m*M* gly–HCl, pH 3.5, the interferon, 2.5×10^6 units (or 55% of the starting material), was eluted with 100 m*M* gly–HCl, pH 2.0. The peak fraction (number 43) contained 5.0×10^5 units of activity and 0.01 mg of protein, which corresponds to a specific activity of 5.0×10^7 units/mg, and represents a 64-fold purification. The bulk of interferon activity eluted in the pH range 2.6–3.2.

Slightly higher recoveries were obtained if the concentration of gly–HCl was increased to 400 m*M* (data not shown). Consequently, in the multiple chromato-

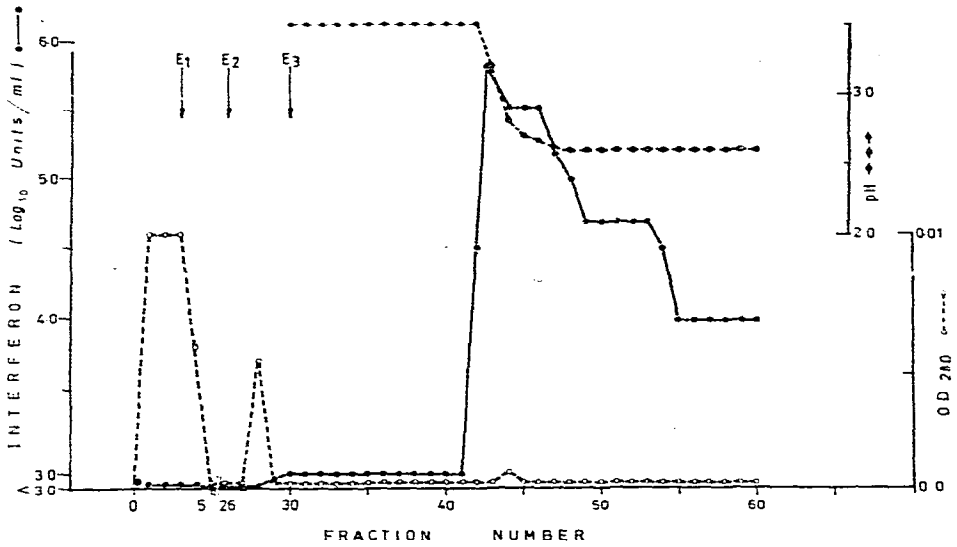

Fig. 1. CPG-adsorption chromatography of 1°-MIF, acid pH elution profile. 18 ml of 1°-MIF were loaded on to a 0.5 × 5.0 cm column of CPG, equilibrated in PBS. The flow-rate was 10 ml/h. Fractions 1–29 contained 6.7 ml/fraction, and fractions 30–60 contained 1 ml/fraction. The eluent PBS is denoted as E_1, 100 m*M* gly–HCl (pH 3.5) as E_2, and 100 m*M* gly–HCl (pH 2.0) as E_3. The effluent was continuously monitored at 280 nm (○– – –○); the fractions were assayed for interferon (●—●), and pH (-●-●-●-).

graphy schemes, CPG columns were washed with 400 m*M* gly–HCl, pH 3.5, followed by 400 m*M* gly–HCl, pH 2.0.

Hydrophobic chromatography of MIF with AFFI-Gel 202

The purification of crude MIF on AFFI-Gel 202 has been previously described[19]. This method has now been applied in the purification of 1°-MIF and the results are depicted in Fig. 2 and summarized in Table I.

3.5×10^6 units of 1°-MIF (1.6×10^5 units/ml), containing 12.2 mg protein (0.55 mg/ml), or a specific activity of 2.9×10^5 units/mg, were loaded on to a 0.9 × 12 cm column of AFFI-Gel 202, equilibrated in 50 m*M* NaAc, pH 5.0. No activity eluted during this buffer wash, whereas 2.5×10^5 units (or 7% of the starting material) eluted with 20 m*M* Na-PB, pH 7.2. The remainder of the recoverable activity, 1.28×10^6 units (or 37%) eluted with PBS containing 350 m*M* NaCl, pH 7.2. With this eluent, one peak fraction (number 55) contained 3.2×10^5 units, and 0.1 mg of protein, which corresponds to a specific activity of 3.2×10^6 units/mg, and represents a 11-fold purification.

Gel filtration chromatography of MIF with Ultrogel AcA 54

Gel filtration with Ultrogel AcA 54 was also tested as a means of further purifying MIF. As indicated in Fig. 3 and summarized in Table I, 2.6×10^7 units of 1°-MIF (2.0×10^6 units/ml), containing 26 mg of protein (2 mg/ml), or a specific activity of 1×10^6 units/mg, were loaded on to a 1.6 × 63 cm column of Ultrogel AcA 54, equilibrated in 50 m*M* K-PB, pH 5.9. Although two peaks of protein were observed, antiviral activity was detected only in the second. The total activity recovered

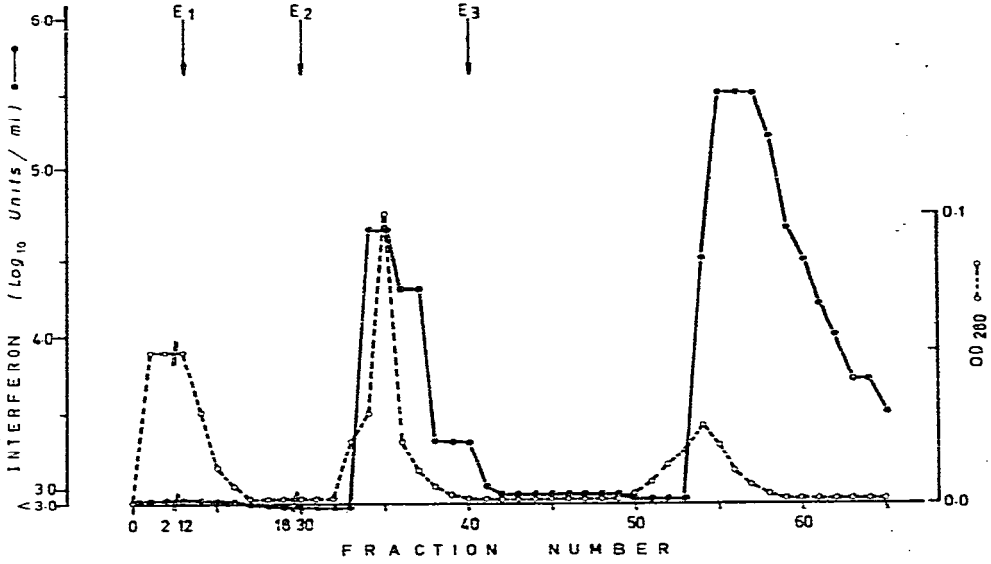

Fig. 2. Hydrophobic chromatography of 1°-MIF with AFFI-Gel 202. 22 ml of 1°-MIF were loaded on to a 0.9 × 12 cm column of AFFI-Gel 202, equilibrated in 50 m*M* NaAc, pH 5.0. The flow-rate was 10 ml/h. Fractions 1–39 contained 2 ml/fraction, and fractions 40–70 contained 1 ml/fraction. The eluent 50 m*M* NaAc (pH 5.0) is denoted as E_1, 20 m*M* Na-Pb (pH 7.2) as E_2, and PBS containing 350 m*M* NaCl (pH 7.2) as E_3. The effluent was continuously monitored at 280 nm (○– – –○) and the fractions were assayed for interferon (●—●).

TABLE I

MULTIPLE CHROMATOGRAPHIC PURIFICATION OF MOUSE L-929 INTERFERON

*Procedure**	*Input*			*Output*					
	Total units applied	*Total protein applied (mg)*	*Specific activity (units/mg)*	*Total units recovered*	*Recovery (%)*	*Units in peak fraction*	*Protein in peak fraction (mg)*	*Specific activity (units/mg)*	*Purification factor*
Scheme 1									
1° → 2°**	3.5×10^6	12.2	2.9×10^5	1.28×10^6	37	3.2×10^5	0.1	3.2×10^6	11
1° → 3°	3.7×10^7	5.3	7.0×10^6	2.2×10^7	59	1.6×10^7	0.02	8.0×10^8	114
Scheme 2									
1° → 2°***	2.6×10^7	26.0	1×10^6	3.2×10^6	12	6.3×10^5	0.021	3.0×10^7	30
1° → 3°†	7.5×10^6	29.0	2.6×10^5	4.8×10^6	64	2.0×10^6	0.032	6.2×10^7	238
1° → 5°	5.3×10^7	5.8	9.1×10^6	3.9×10^6	7.4	2.5×10^6	0.0067	3.7×10^8	41

* The steps described are as follows. Scheme 1: 1°, ammonium sulphate precipitation; 2°, hydrophobic chromatography with AFFI-Gel 202; 3°, CPG-adsorption chromatography. Scheme 2: 1°, ammonium sulphate precipitation; 2°, gel filtration with Ultrogel AcA 54; 3°, ion-exchange chromatography with CM-BGA; 4°, hydrophobic chromatography with AFFI-Gel 202; 5°, CPG-adsorption chromatography.

** See section on results of hydrophobic chromatography of 1°-MIF with AFFI-Gel 202.

*** See section on results of gel filtration of 1°-MIF with Ultrogel AcA 54.

† See section on results of ion-exchange chromatography of 2°-MIF with CM-BGA.

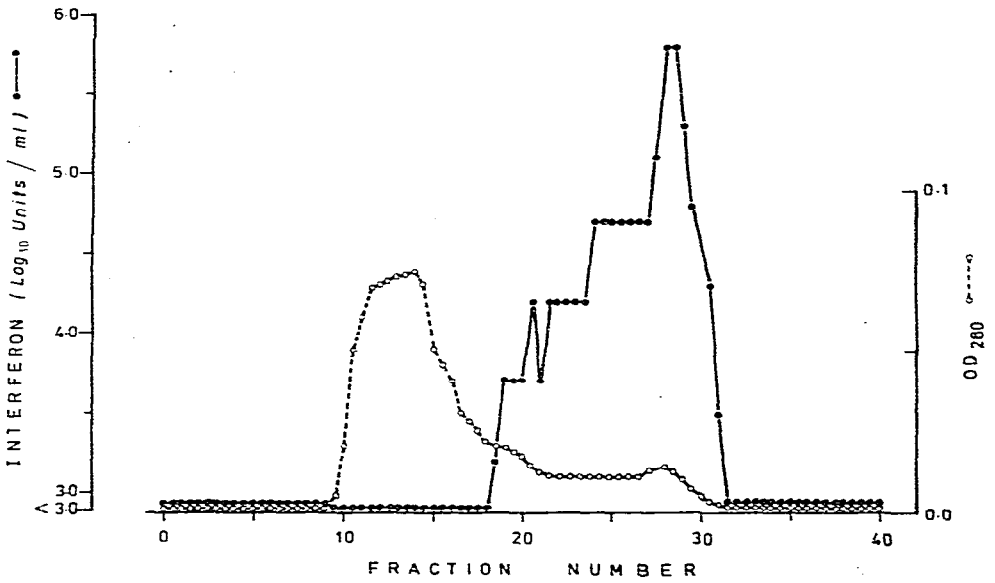

Fig. 3. Gel filtration chromatography of 1°-MIF with Ultrogel AcA 54. 13 ml of 1°-MIF were loaded after concentrating with $(NH_4)_2SO_4$ on to a 1.6 × 63 cm column of Ultrogel AcA 54, equilibrated in 50 m*M* K-PB, pH 5.9. The flow-rate was 10 ml/h and 2 ml/fraction were collected. The effluent was continuously monitored at 280 nm (○– – –○) and the fractions were assayed for interferon (●—●).

under the curve was 3.2×10^6 units, or 12% of the starting material. The peak fraction (number 56) contained 6.3×10^5 units of activity and 0.021 mg of protein, which corresponds to a specific activity of 3.0×10^7 units/mg, and represents a 30-fold purification..

Ion-exchange chromatography of MIF with CM-BGA

MIF was further purified by directly loading the material obtained from Ultrogel AcA 54 on to CM-BGA. As shown in Fig. 4 and summarized in Table I, 9.2×10^6 units of 2°-MIF were applied to a 1.0 × 1.5 cm column of CM-BGA, equilibrated in 50 m*M* K-PB, pH 5.9. After the column had been washed with equilibration buffers, the interferon, 4.8×10^6 units (or 52% of the loaded material), was eluted with 100 m*M* K-PB, pH 8.0. One peak fraction (number 6) contained 2.0×10^6 units of activity and 0.032 mg of protein, which corresponds to a specific activity of 6.2×10^7 units/mg, and, with respect to 1°-MIF employed as the starting material, represents a 238-fold purification.

Multiple chromatography

Two strategies were investigated to purify MIF by the techniques described above. Since, at low protein concentrations, MIF is inactivated as a function of time[2] and/or non-specifically binds to the apparatus employed[4], both schemes were designed to require the minimum amount of time and manipulation (*e.g.*, dialysis and concentrations). To this end, loading and elution buffers were coordinated so that preparations could be directly transferred from one column to the next.

The results from the two procedures are shown in Table I. Because of the

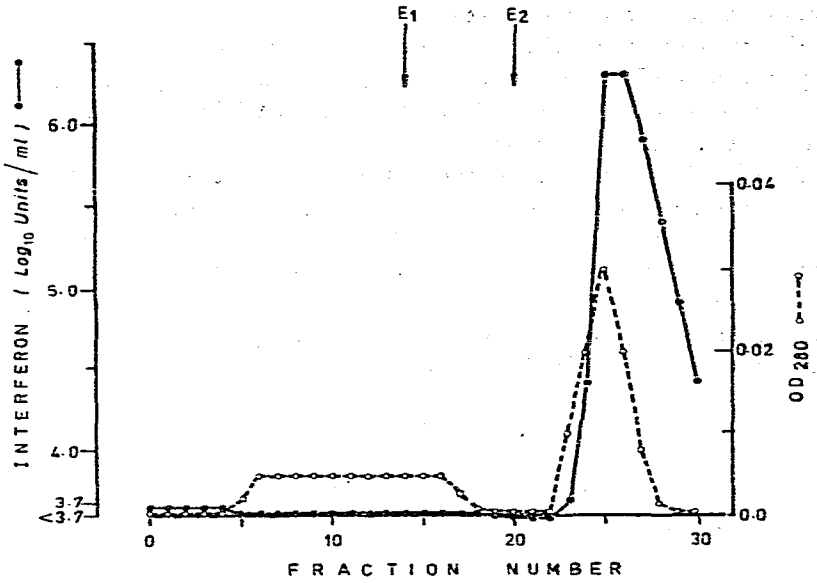

Fig. 4. Ion-exchange chromatography of 2°-MIF with CM-BGA. 10 ml of 2°-MIF were loaded on to a 0.7 × 1.5 cm column of CM-BGA, equilibrated in 50 m*M* K-PB, pH 5.9. The flow-rate was 10 ml/h and 1 ml/fractions were collected. The eluent 50 m*M* K-PB (pH 5.9) is denoted as E_1, and 100 m*M* K-PB (pH 8.0) as E_2. The effluent was continuously monitored at 280 nm (○– – –○) and the fractions were assayed for interferon (●—●).

limited material available, samples were not removed after each step and the data shown are composites of several experiments. The first approach (Scheme 1) consisted of three techniques: $(NH_4)_2SO_4$ precipitation (1°), hydrophobic chromatography with AFFI-Gel 202 (2°), and CPG-adsorption chromatography (3°). The data obtained from the first two steps (1° and 2°) are described above (see hydrophobic chromatography of MIF with AFFI-Gel 202). With the complete procedure, the starting material had 3.7×10^7 units of 1°-MIF (3.2×10^6 units/ml), containing 5.3 mg of protein (0.46 mg/ml), or a specific activity of 7.0×10^6 units/mg, and the final product (3°-MIF) had a total of 2.2×10^7 units (or a 59% recovery). The peak material contained 1.6×10^7 units of activity and 0.02 mg of protein, which corresponds to a specific activity of 8.0×10^8 units/mg, and represents a 114-fold purification.

Scheme 2 consisted of five techniques: $(NH_4)_2SO_4$ precipitation (1°), gel filtration with Ultrogel AcA 54 (2°), ion-exchange chromatography with CM-BGA (3°), hydrophobic chromatography with AFFI-Gel 202 (4°) and CPG-adsorption chromatography (5°). The data obtained from the first three steps (1°, 2° and 1°–3°) are described above under the headings gel filtration and ion-exchange chromatography, respectively.

Of the initial 5.3×10^7 units of 1°-MIF (3.2×10^6 units/ml), containing 5.8 mg of protein (0.36 mg/ml) (or a specific activity of 9.1×10^6 units/mg), a total of 3.9×10^6 units (7.4%) was recovered following application of Scheme 2. The peak material contained 2.5×10^6 units of activity and 0.0067 mg of protein, which corresponds to a specific activity of 3.7×10^8 units/mg and represents a 41-fold purification.

SDS-polyacrylamide gel electrophoresis (PAGE)

The final materials prepared from Schemes 1 and 2 were analysed for interferon activity (Fig. 5) on preparative SDS-PAGE gels. Of the 3.2×10^4 units of Scheme 1 applied, a total of 2.6×10^3 units or 8% of the loaded material was recovered. Two peaks of activity were detected. The first, representing 48% (1.25×10^3 units) of the recovered activity, had a molecular weight of 39,000–44,000 daltons. The second peak, representing 30% (8.0×10^2 units) of the recovered activity, had a molecular weight of 19,000–23,000 daltons.

When Scheme 2 material (1.0×10^4 units of activity) was applied to preparative gels, 17.0% or 1.7×10^3 units of activity were recovered. Only one peak of activity was detected. This represented 74% (1.25×10^3 units of activity) of the recovered material and had a molecular weight of 39,000–44,000 daltons.

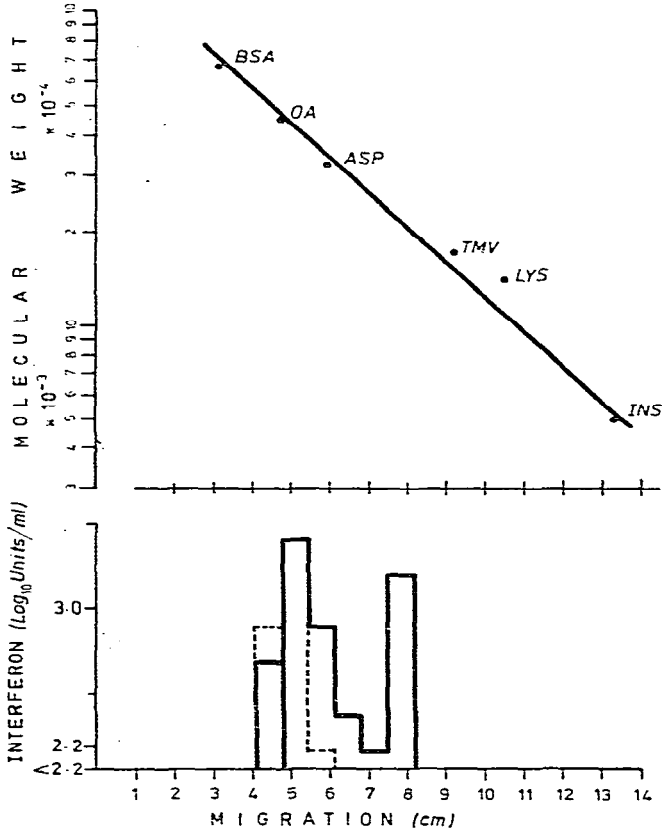

Fig. 5. Preparative SDS-PAGE of MIF from Schemes 1 (———) and 2 (– – –) for the determination of molecular weight. The molecular weight standards were bovine serum albumin (BSA) MW 67,000, ovalbumin (OA) MW 45,000, asparaginase (ASP) MW 33,000, tobacco mosaic virus (TMV) MW 17,000, lysozyme (LYS) MW 14,000 and insulin (INS) MW 5,600.

DISCUSSION

These studies show that MIF can be purified by an assortment of chromatographic techniques. Furthermore, when these techniques were applied in concert, highly purified products were obtained.

The purification of MIF by CPG-adsorption chromatography has been shown previously[29,30]. As demonstrated here (Fig. 1), products with a specific activity of 5.0×10^7 units/mg were obtained when the starting material was 1°-MIF and when solutions of 100 m*M* gly–HCl, pH 3.5 and 2.0, were used as the eluents.

MIF has also been shown to be purified by hydrophobic chromatography with AFFI-Gel 202[19]. In our experiments, however, where the purity of the starting material was higher, the final specific activity (3.2×10^6 units/mg) of the interferon product was lower than that reported earlier. Nevertheless, the technique did offer some additional purification and proved quite useful when employed with other techniques (see below).

Many groups have previously reported the use of gel filtration as a means of purifying MIF[2,6,7], but Ultrogel AcA 54 has not been used before. The gel, a mixture of polyacrylamide and agarose, offered both good recovery (12–123%) and purification (30-fold), yielding a final specific activity of 3.0×10^7 units/mg.

Although we used the buffer system previously described by Knight[2], neither glycerol, which contains impurities that have a tendency to cross-link proteins[33], nor salt appeared to be necessary, despite the low protein content of the applied material. The elimination of the salt allowed direct loading of the material eluted from Ultrogel AcA 54 on to CM-BGA, without an intermediary dialysis step[2].

Ion-exchange chromatography has also been used by many groups[2,3,5,6,8]. The procedure outlined in this study utilized for the first time the ion exchanger CM-BGA, which yielded high recoveries (64%), and, if loaded with material first purified by gel filtration (see above), resulted in a product with a specific activity of 6.2×10^7 units/mg or a 238-fold purification (with respect to 1°-MIF).

The major obstacles in purifying interferons are, at low protein concentrations, its tendency to become inactive[2,3], and/or to bind non-specifically to the apparatus employed[4]. These difficulties might be overcome in two ways: first, by starting with a sufficiently large amount of material so that the protein content of the product continues to sustain activity[7,16]; secondly, by designing a procedure that requires the minimum amount of time, steps and/or manipulation.

Our attempts have been directed at designing a more versatile method of interferon purification. The overall strategy was to select efficient purification techniques where the effluent and influent buffers could be coordinated, and thereby reduce both the number of manipulations and the time required. The first procedure (Scheme 1) involved two affinity/adsorption techniques, hydrophobic chromatography and adsorption chromatography with AFFI-Gel 202 and CPG, respectively. The only manipulation required was dialysis following $(NH_4)_2SO_4$ precipitation. The final product had a specific activity ranging from 1.0 to 8.0×10^8 units/mg and, with respect to 1°-MIF material, it represented a purification of from 16- to 116-fold and a recovery of 59% of activity. When started with the dialysed material, the entire purification procedure usually took no more than one day.

As an extension of the above technique, purification Scheme 2 was attempted.

MIF was first purified by physicochemical means (*i.e.* gel filtration with Ultrogel AcA 54 followed by ion-exchange chromatography with CM-BGA). Except for the need to dialyse the 1°-MIF, the only manipulation required was the dilution and pH readjustment of 3°-MIF. Despite the low recovery (7.4%), the final product had a specific activity of 2.5–3.7 $\times$ 10^8 units/mg and, with respect to 1°-MIF, it represented a purification of from 19- to 44-fold. When started with 2°-MIF, the entire purification procedure usually required no more than a day. The molecular weight(s) obtained for the products of both schemes are similar to values reported previously[2,7,15,16,34].

Considering the low recoveries obtained from Scheme 2 and the inability to detect any protein by SDS-PAGE (data not shown), it is difficult to determine whether Scheme 2 offers any additional purification compared with Scheme 1. If the amount of starting material were increased, the protein concentration of the final product might be within the limits required to sustain activity[2], which might then yield samples with a higher specific activity.

Although neither of these two schemes offers a degree of purification as great as reported by some other groups[7,15,16], they do have certain advantages. All the reagents are commercially available, so there is no need to raising and purify antibodies against MIF[15,16]. In addition, compared to other multistep procedures[7,16] the time and the number of manipulations are kept to a minimum. Lastly, by virtue of the strategy employed, the amount of interferon invested for purification can be reduced.

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