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SPECIFICATION

Homogeneous fibroblast interferon and method for manufacture thereof

5 The present invention deals with human fibroblast interferon as a homogeneous protein and a method for the manufacture thereof by combining affinity chromatography and high pressure liquid chromatography (HPLC). 5

Since its first discovery by Isaacs and Lindenmann in 1957, interferon whether of the leucocyte or fibroblast form has resisted the attempts of researchers at institutions throughout the world spanning over 10 two decades to be isolated as a homogeneous peptide in amounts sufficient to allow characterization and 10 identification of its specific biological and chemical properties. While several authors claim to have purified mouse or human interferons to homogeneity none of the classical proofs of homogeneity of protein materials were given nor were any properties of the allegedly pure compounds described.

The use of high performance liquid chromatography for purification of proteins is generally known in the 15 art. References specifically describe ion exchange and size exclusion type columns in protein purification (e.g. Regnier and Noel, *J. Chromatog. Sci.* 14, 316 [1976] and Chang et al., *Anal. Biochem.* 48, 1839 [1976]) and the use of Lichrosorb RP-18 (octadecyl bound silica microparticle column) in reverse phase partition chromatography was successfully employed to purify peptides such as β -endorphin (Rubinstein et al., *Proc. Natl. Acad. Sci. USA*, 74, 4969 [1977]). 15

20 It has been known in the art to employ affinity chromatography as a procedural step in the purification of human fibroblast interferon. Thus Davey et al., *J. Biol. Chem.* 251, 7620 (1976), describe the use of Concanavalin A-Sepharose 4B affinity chromatography to produce purified human fibroblast interferon. Jankowski et al., *Biochemistry* 15, 5182 (1976), utilize Blue Dextran-Sepharose for the same purpose. Finally, according to U.S.P. 4,172,071 (de Maeyer et al.) purification of various crude solutions of leukocyte and 25 fibroblast interferon is accomplished by affinity chromatography over a Blue-Dextran-Sepharose column yielding preparations which have specific activity of between 1 and 5×10^6 International Units of interferon. No additional purification steps are disclosed and the interferon preparations obtained are indicated to contain a high content of products with interferon type activity freed to a major extent from contaminating proteins. 25

30 The present invention provides human fibroblast interferon as a homogeneous protein and also provides a method for its manufacture. 30

The improved process of the present invention involves a combination of affinity chromatography and high pressure liquid chromatography steps to achieve efficient purification of human fibroblast interferon.

More specifically, the process of the present invention comprises the following steps in combination:

35 A. passing an aqueous solution of human fibroblast interferon in an impure state through an affinity chromatography column so as to absorb said interferon onto said column and obtaining said interferon in selected fractions of said eluate in a state of enhanced purity; and 35

B. passing the selected said interferon fractions obtained in Step A through one or more buffer equilibrated hydrocarbon bonded silica matrix column(s) under high pressure liquid chromatography 40 conditions wherein said hydrocarbon is cyclohexyl, phenyl, diphenyl, n-octyl, n-octadecyl or 3-cyanopropyl, to absorb said interferon onto said column and thereafter eluting said interferon from said column with a gradient of an acidic aqueous buffered mixture of water miscible solvents and obtaining interferon as a single distinct peak in selected fractions of said eluate in the state of a homogeneous protein. 40

The present invention also includes a pharmaceutical preparation comprising human fibroblast interferon 45 as a homogeneous protein. 45

In the affinity chromatography process of the present invention Concanavalin A-Sepharose 4B or Blue Dextran-Sepharose can be used, the use of the latter being preferred in view of the fact that such procedure provided substantially higher yields than the Concanavalin A-Sepharose 4B procedure and also provides a more stable product.

50 For the Concanavalin A-Sepharose 4B procedure the following protocol can be employed: 50

(a) 20-30 column volumes of crude human fibroblast interferon (specific activity about 10^4 units/mg) in 5% (v/v) fetal calf serum containing Eagle's minimal essential medium are pumped onto a Concanavalin A-Sepharose 4B column at a flow rate of from 30-60 cm/hr;

(b) wash with phosphate buffered saline (PBS) pH 7.2;

55 (c) wash with PBS-0.1M α -methyl mannoside (α -MM); and 55

(d) elute with PBS-0.1M α -MM containing 50% (v/v) ethylene glycol.

The resulting purified interferon obtained from step (d) exhibits a specific activity of about 10^7 units/mg with a recovery of 10-30%.

A suitable protocol for utilizing Blue Dextran-Sepharose in the affinity chromatography step is as follows:

60 (a) 10-40 column volumes of culture supernatant ($1-3 \times 10^4$ units/ml, 0.1-1 mg protein/ml) are adjusted to 1.25 M NaCl by addition of saturated NaCl solution and then pumped on the column at a linear flow rate of 20-40 cm/hr; 60

(b) the column is washed with 5-15 column volumes of 1M NaCl/0.02-0.05M phosphate and then with 5-15 column volumes of 1M NaCl/0.02-0.05M phosphate containing 15% (v/v) ethylene glycol (pH 7.2); and

65 (c) interferon is eluted with 1M NaCl/0.02-0.05M phosphate containing 50% (v/v) ethylene glycol (pH 7.2). 65

The resulting interferon has a specific activity of approximately 3×10^8 units/mg and can be obtained from this step in a recovery of about 80%.

The Blue Dextran-Sepharose employed in the above affinity chromatography step can be conveniently prepared by coupling Blue Dextran [Cibacron Blue F3GA coupled to Dextran] to cyanogen bromide-activated Sepharose 4B at pH 9.5.

The aforesaid coupling procedure, as well as the washing and ageing of the resin, the design of column loading and elution is important for obtaining maximum yield and highest degree of purity of the product human fibroblast interferon.

In cases where the interferon sample is harvested from a serum free medium purification to homogeneity is accomplished by one or two passages through a Blue Sepharose-4B column eluting with a 30-50% (v/v) ethylene glycol/buffered aqueous solution (pH 7.2).

In order to achieve further purification of the interferon produced in the affinity chromatography step the interferon is processed through one or more high pressure liquid chromatography steps with high resolution and high yield on a preparative scale. The liquid chromatography procedure utilizes columns containing a porous silica matrix to which cyclohexyl, n-octyl, n-octadecyl, phenyl diphenyl or 3-cyanopropyl groups are bonded. These columns, which can be used sequentially and under varying conditions of pH and organic solvent gradient, can purify human fibroblast interferon to homogeneity as determined by obtaining a single band on sodium dodecylsulfate (NaDodSO₄) polyacrylamide gel electrophoresis, a constant specific activity, and a single peak on HPLC with activity and protein levels superimposable.

The irregularly shaped, totally porous silica microparticle columns (particle size about 10 microns and a pore size of about 100 Å) having cyclohexyl, n-octyl, n-octadecyl, phenyl or 3-cyanopropyl groups bound thereto used in the practice of the invention are articles of commerce.

A convenient high pressure liquid chromatography system for utilizing the aforesaid columns is described in U.S. Patent No. 4,116,046 (Stanley Stein).

In performing the instant process, the solution of the human fibroblast interferon purified by affinity chromatography in aqueous buffer of pH 4-8 is passed through the silica matrix column. A preferred buffer for purposes of the present invention is pyridine/formic acid/water (8:8:84, v/v). Usually the procedure is carried out under pressure, preferably in the range of from 3.4 to 340 atm. The interferon is absorbed to the column and is then subsequently eluted in selective fashion using a gradient of an aqueous buffer plus varying amounts of a water miscible solvent. Suitable water miscible solvents for this purpose include alkanols such as n-propanol, iso-propanol, n-butanol, tert.-butanol, ethanol, and methanol. Particularly preferred for the elution of human fibroblast interferon is a mixture of 2-propanol and n-butanol.

Fractionation of the eluate is accomplished by utilizing fraction collectors in a manner known per se with concomitant monitoring of the protein content in each fraction by peptide monitors operating at high sensitivity. A suitable system for this purpose is disclosed by Bohlen et al., *Anal. Biochem.* 67, 438 (1975). Note also U.S. Patent No. 3,876,881.

The selection of the specific resin types to be employed in the high pressure liquid chromatography steps depends on the source and purity of the human fibroblast interferon starting material for this step. Thus material produced by affinity chromatography on a Concanavalin A-Sepharose column is conveniently purified to homogeneity by utilizing high pressure liquid chromatography on a cyclohexyl bonded silica column followed by an n-octyl bonded silica column. On the other hand when a Blue Dextran column is utilized in the affinity chromatography step, purification to homogeneity can be accomplished by one or more passages through an n-octyl bonded silica column or alternatively by passage through the n-octyl bonded silica column followed by passage through a 3-cyanopropyl bonded silica column and then, if required, through a diphenyl bonded silica column. When a Blue Sepharose-4B column and a serum free preparation is employed, a single passage through an n-octyl bonded silica column will achieve a homogeneous preparation.

Due to the sensitivity of human fibroblast interferon activity in the presence of organic solvents, e.g. the propanols, n-butanol or 2-methoxyethanol at neutral pH, chromatography was performed at acidic pH. Since human fibroblast interferon has a more hydrophobic nature than leucocyte interferon and since there are other more hydrophobic proteins present in the partially purified fibroblast interferon preparations, a mixture of 2-propanol and n-butanol was found to provide the preferred solvent for elution of reverse phase columns rather than n-propanol. Use of such mixture serves to limit the organic solvent concentration necessary for complete elution of all absorbed proteins and thus reduces artifacts occurring at high loading due to lower solubility of proteins.

The homogeneous human-fibroblast interferon obtained in accordance with the present invention was derived as a single peak in the last stage high performance liquid chromatography column and provided a single narrow band on NaDodSO₄ polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. Extraction of the gel gave a single peak of antiviral activity coinciding with the protein band. Any conventional antiviral assay for human fibroblast interferon activity can be used for this purpose.

The molecular weight of the homogeneous human fibroblast interferon as determined by the polyacrylamide gel procedure was approximately 20,500. The specific activity of this purified material was approximately 4×10^8 units/mg. The N-terminal partial sequence obtained on a sample of a homogeneous human fibroblast interferon was H-Met¹-Ser-Tyr-Asn-Leu⁵-Leu-Gly-Phe-Leu-Gln¹⁰-Arg-Ser-Ser-Asn-Phe¹⁵-Gln-...-Gln-Lys¹⁹....

Interferons have exhibited antiviral, antitumor, growth inhibition and immunosuppression activity. These activities have been obtained even at the clinical level using $1-10 \times 10^6$ units daily with relatively crude preparations, less than 1% of which was human interferon. The purified, homogeneous human fibroblast interferon of the present invention can be utilized in the same manner as the previously employed crude preparations with adjustments in the dosage to provide the equivalent desired level of interferon units.

In another aspect, therefore, the present invention provides human fibroblast interferon as a homogeneous protein, or a pharmaceutical preparation containing it, for use in the treatment of viral and neoplastic disease states.

The process and product aspects of this invention are further illustrated by reference to the following Examples. All interferon titers are expressed in terms of reference units/ml or units/mg against a reference standard for human leucocyte interferon (G023-901-527) provided by the U.S. National Institutes of Health.

EXAMPLE 1

Concanavalin A Affinity Chromatography

50 ml of Concanavalin A-Sepharose 4B which is equilibrated with PBS pH 7.2 containing 0.1M α -MM was packed in a 50 ml polypropylene column equipped with a polyethylene disk frit. It was loaded at 180 ml/hr (35.5 cm/hr) flow rate with 1.25 liters of crude interferon (2×10^7 units) having a specific activity of 2×10^4 units/mg. The charged column was washed with 150 ml of PBS buffer and 600 ml of PBS buffer containing 0.1M of α -MM. Interferon was finally eluted with the above buffer containing 50% (v/v) ethylene glycol. Yield of the procedure 9% (1.8×10^6 units) with a specific activity of about 1×10^7 units/mg. A broader cut including additional fractions yielded 15% (3×10^6 units) with a specific activity of $2-4 \times 10^6$ units/mg.

HPLC

A total of 120 ml of eluent from the Concanavalin A-Sepharose 4B column (2.5×10^6 units; approx. $2-4 \times 10^6$ units/mg) was applied directly to a Chromegabond 10 μ cyclohexyl column (4.6×300 mm) which had been equilibrated with pyridine/formic acid/isopropanol/water (8:8:20:64; v/v; buffer A) at flow rates in the range of 0.4-0.8 ml/min., keeping the maximal backpressure below 306 atm. The system used for the HPLC procedures was essentially that described by Bohlen et al. (Anal. Biochem. 67, 438 [1975]).

The following gradient from equilibrating buffer A to final buffer B (pyridine/formic acid/isopropanol/n-butanol/water, 8:8:25:20:39, v/v) was run at 0.4 ml/min.: 0-25% B, 32 min.; 25-60% B, 225 min.; 60-100% B, 63 min. (all percentages being by volume). Fractions of 2.0 ml were collected. A pool comprising fractions 26-29 (2×10^6 units) in the center of the activity peak was taken and diluted with 4 ml of pyridine/formic acid. This solution was applied through the pump to a Chromegabond 10 μ n-octyl column (4.6×300 mm). The elution conditions were the same as for the cyclohexyl column above. The specific activity in the center of the activity peak was approximately 4×10^8 units/mg with a total yield of purified human fibroblast interferon of 10^6 units.

It should be noted that the elution position of the interferon on both the cyclohexyl and n-octyl columns is very close to the main central peak when interferon purified on Concanavalin A is subjected to HPLC on either column. If the first HPLC step is carried out on n-octyl column the interferon elutes right before the main central peak, while it elutes immediately after that peak under the same chromatography conditions on the cyclohexyl column.

Samples of HPLC purified human fibroblast interferon were run on 12.5 or 15% (w/v) NaDodSO₄ polyacrylamide gels in Tris/glycine buffer with 1 μ g of protein for the run. After electrophoresis, a single band was obtained upon staining with Coomassie blue. The same sample material was run in a parallel gel and the gel sliced for antiviral assay. The maximum of the antiviral activity coincided with the center of the stained band. A molecular weight of about 20,500 was determined using bovine serum albumin, chymotrypsin, cytochrome C and ribonuclease as standards. The relative mobility of the human fibroblast interferon band was the same whether 2-mercaptoethanol was in the sample or not.

EXAMPLE 2

Preparation of the Blue Dextran-Sepharose 4B resin

Fifty grams of packed Sepharose 4B were washed with 1 liter of water and resuspended in 50 ml of distilled water. Fifteen grams of finely divided cyanogen bromide were added to the slowly stirred solution and the pH was immediately adjusted to and maintained at 11 ± 0.2 by dropwise addition of 10N NaOH. Temperature was maintained at $20 \pm 5^\circ\text{C}$ by small additions of crushed ice. When the reaction had subsided (15-20 min.) about 1 volume of ice-water was added, the suspension transferred into a Buchner funnel and further washed with 5 volumes of 0.01N HCl.

The activated resin was immediately suspended in 50 ml of 0.4M sodium carbonate buffer (pH 9.5) containing 1.00 g of dissolved Blue Dextran and rotated overnight at 4°C in a round bottom flask. The resin was then washed with 10 volumes of 1M NaCl, 2 volumes of 50% (v/v) ethylene glycol containing 1M NaCl and 0.05M sodium phosphate (pH 7.2), left overnight in the 50% (v/v) ethylene glycol containing 1M NaCl and 0.05M sodium phosphate (pH 7.2), left overnight in the 50% (v/v) ethylene glycol washed with 1 volume 80% (v/v) ethylene glycol and 5 volumes of Gibco No. 11 minimum essential medium containing 5% (v/v) fetal calf serum (MEM) and left in this medium overnight at 50°C . After further washings with 3 volumes of 80% (v/v) aqueous ethylene glycol containing 1M NaCl and then with 2 volumes of MEM the resin is then slurried in

MEM and packed in a column for use.

Affinity Chromatography

1.5 liter of crude fibroblast interferon (2×10^4 units/ml; 1 mg protein/ml; specific activity 2×10^4 units/mg protein) were mixed with 0.25 volumes of saturated NaCl-solution (ca. 6.3M). This solution was pumped through Blue Dextran-Sepharose 4B (35 ml) packed in a 50 ml polypropylene column equipped with a porous polyethylene disk at the bottom at a flow rate of 100 ml/hr (20 cm/hr). The charged resin was washed in sequence with 200 ml of 1M NaCl containing 0.05M sodium phosphate buffer (pH 7.2); 500 ml of 1M NaCl containing 0.05M sodium phosphate (pH 7.2) and 75 ml of ethylene glycol and finally eluted with 500 ml of 1M NaCl containing 0.05M sodium phosphate (pH 7.2) and 250 ml ethylene glycol. About 90% of the interferon came down in a peak with the front of the 50% (v/v) ethylene glycol. The specific activity in the peak maximum, where more than 50% of the total interferon eluted, was 1×10^7 units/mg.

HPLC

(A) A total of 125 ml of pooled interferon eluted from the Blue Dextran-Sepharose 4B column (3×10^7 units; 1×10^7 units/mg) were pumped on a 4.6×300 mm Chromagabond 10 μ octyl column which had been pre-equilibrated with 1M NaCl containing 50% (v/v) ethylene glycol. Immediately after sample application buffer A (pyridine/formic acid/isopropanol/n-butanol/water, 8:8:25:20:39, v/v) with the following program was run at a flow rate of 0.45 ml/min.: 0-25% B, 30 min.; 25-55% B, 190 min.; 55-100% B, 20 min.; 100% B, 60 min. (all percentages being by volume). Interferon activity was found to coincide essentially with a single protein peak found in fractions 18-23 (each fraction contained 1.5 ml of eluent).

(B) When approximately one-fourth the load in run (A) above was applied to the same column and the elution conditions used for the cyclohexyl column in Example 1 were employed interferon activity was found to coincide with a protein peak found in fractions 24-26.

(C) Using the same loading, gradient program and linear flow rate as in (A) and the A and B buffers used in (B) with a 9.6×500 mm Chromagabond n-octyl column which thus provided about 1/9th the loading per column volume, and resulted in better separation quality.

(D) The material exhibiting the highest specific activity (4×10^8 units/mg) from run (A) above was rechromatographed on a cyanopropyl column using pyridine/formic acid/water (8:8:84, v/v) and a 1 hour 0-40% (v/v) n-propanol gradient at 0.3 ml/minute to provide a symmetrical main peak.

NaDodSO₄ Polyacrylamide Gel Electrophoresis

Electrophoresis as described in Example 1 of samples from procedures (A), (B) and (C) above showed as major band the 20,500 MW band, which when gels were sliced and assayed coincided with the center of the activity peak. The second strongest band (10,500 MW/20-50% of total material as estimated from the staining intensity) varied from sample to sample and had no antiviral activity. If samples were not treated with 2-mercaptoethanol, a 40,000 MW band could be observed, which had borderline activity. Amino acid analysis of the 20,000 MW and 40,000 MW bands were indistinguishable while the analysis of the 10,000 MW band was very significantly different.

Electrophoresis of a sample from procedure (D) above provided a single band which had a molecular weight of about 20,500 and a specific activity of 4×10^8 units/mg. An amino acid analysis of this homogeneous peptide carried out on a 24 hour hydrolysate in 6N HCl gave the following values relative to leucine taken arbitrarily as 25.0:

Asx	15.4 ± 0.2	Ala	11.8 ± 0.4	Tyr	9.6 ± 0.2
Thr*	7.8 ± 0.3	Cys	n.d.	Phe	7.3 ± 0.1
Ser*	7.7 ± 0.2	Val	6.9 ± 0.1	His	4.5 ± 0.1
Glx	24.1 ± 0.4	Met	2.8 ± 0.4	Lys	11.6 ± 0.4
Pro	2.8 ± 0.4	Ile	8.7 ± 0.1	Arg	8.8 ± 0.4
Gly	4.9 ± 0.3	Leu	25.0	Trp	n.d.

* Uncorrected value
n.d. not determined

EXAMPLE 3

Blue Dextran-Sepharose Affinity Chromatography

(a) Five liters of culture supernatant containing 19400 units/ml of fibroblast interferon were filtered through a 0.3μ filter and then applied to a column containing a bed volume of 275 ml of Blue Dextran-Sepharose. The supernatant was adjusted to 1.25M NaCl by the addition of 1350 ml of a saturated solution of NaCl (6.3M) and pumped onto the column at a flow rate of 360 ml/hr. About 4-6% of the interferon

passed through the column in the flow-through fraction that was not retained by the column.

(b) The column was then washed with 1300 ml of an aqueous solution containing 15% (v/v) ethylene glycol, 1M NaCl, and 0.02M sodium phosphate (pH 7.2) at a flow rate of 420 ml/hr. About 1% of the interferon applied to the column was eluted.

(c) Interferon was then eluted with an aqueous solution containing 50% (v/v) ethylene glycol, 1M NaCl, and 0.02M sodium phosphate (pH 7.2) at a flow rate of 420 ml/hr. Little interferon was eluted with the first 200 ml of the eluent. Most of the interferon applied to the column was eluted with the next 300 ml of eluent as shown in the following table:

	Fraction No.	Fraction Volume [ml]	Protein [μ g/ml]	Interferon Titer [units/ml]	Specific Activity [units/mg]	
10						10
15	1	200	not determ.	122	-	15
	2	50	43	3.8×10^5	9.02×10^6	
	3	50	82	11.64×10^5	1.42×10^7	
20	4	50	63	7.72×10^5	1.23×10^7	20
	5	50	62	1.94×10^5	3.13×10^6	
25	6	50	49	9.7×10^4	1.97×10^6	25
	7	50	41	3.64×10^4	8.87×10^5	

The total yield of interferon recovered was calculated as 114% (due to assay variability).

HPLC

Fractions 2-6 from the Blue Dextran-Sepharose affinity column (250 ml; 13×10^7 units; specific activity 8.12×10^6 units/mg) were pumped on a 9.5×500 mm n-octyl bonded silica matrix column at a flow rate of 4 ml/min. Buffer A (pyridine/formic acid/2-propanol/n-butanol/water, 8/8/20/2.5/61.5, v/v) was pumped through the column then at a flow rate of 2 ml/min. for 12 minutes. Subsequently the following gradient was run to buffer B (pyridine/formic acid/2-propanol/n-butanol/water, 8/8/25/25/34, v/v) at a flow rate of 1.25 ml/min: 0-20% B, 18 minutes; 20-29% B, 57 minutes; 29% B isocratic for 27 minutes; 29-30% B, 3 minutes; 30-100% B, 36 minutes; 100% B, 54 minutes (all percentages being by volume).

The interferon activity emerged in the isocratic region of the gradient and coeluted with a peak detected with a fluorescamine monitoring system. 45×10^6 units of interferon were recovered (yield 35%) and a specific activity of 2×10^8 units/mg was obtained for the pool (50 ml). After 2 months of storage in stoppered polypropylene vials at -20°C the activity had dropped to 10% of the value after the HPLC step. No protein was lost.

The 50 ml pool from the previous HPLC column (specific activity ca. 0.2×10^8 units/mg) was mixed with 1 ml thiodiglycol and 20 ml n-propanol. A total of 4.26×10^6 units was found in the assay for this mix. To this were added 100 ml of 0.1% Triton X-100/0.3% (v/v) thiodiglycol. This mix assayed to 5.1×10^6 units total and was pumped on a $5 \mu\text{m}/80\text{\AA}$ 4.6×250 mm Spherisorb CN column which had previously been equilibrated with a mixture of pyridine/formic acid/water (8:8:84, v/v, buffer A), at a flow rate of 1.5 ml/hr for 30 minutes. The following gradient was run to buffer B, pyridine/formic acid/n-propanol/water (8:8:50:34, v/v), at a flow rate of 0.5 ml/min: 0-25% B, 30 min; 25-50% B, 210 min. (both percentages being by volume). The interferon activity emerged together with the only peak observed. 4.2×10^6 units were recovered (82%) in three 1 ml fractions.

The above pool representing 69% of the total protein applied onto the first cyano column was mixed with 2 ml of 0.1% (v/v) Triton X 100 and pumped onto the same cyano column used above. The same gradient as above with half the total gradient time was run. All activity (11.75×10^6 units; 280% recovery) eluted together with the only peak.

The interferon activity pool from the second cyano column (4 ml) was pumped onto a 4.6×250 mm diphenyl bonded silica matrix column, previously precycled with a linear 2 hr gradient from buffer A to B (same buffers as for the cyano column) and equilibrated with buffer A, at a rate of 1.5 ml/min.

The following gradient was run at 0.5 ml/min: 0-25% B, 22 min; 25-50%, 98 min. (both percentages being by volume). The second peak which eluted while the gradient instrument indicated 33% (v/v) buffer B, coincided with the interferon activity plot. A total of 2.6×10^6 units (22%) and 40 μ g of protein eluted with this peak.

The diphenyl bonded silica matrix column was prepared as follows:

(16)

Silica support (10 μ m, 10 nm pore size) was soaked in 6N HCl (10:1, v/w) for 24 hours with occasional shaking. The support was washed on a sintered glass filter with water until the wash was neutral and then washed with acetone and methanol to remove the water. After drying in vacuum overnight, the support (10 g) was refluxed in dry toluene (100 ml) containing 10 ml of dichlorodiphenylsilane for 6 hours. The bonded support was removed from the toluene solution with a sintered glass filter and washed with 200 ml of toluene. Following this, the bonded support was treated in a Soxhlet extractor sequentially with 250 ml of toluene, acetone and methanol for 8 hours each and was then treated with trimethylchlorosilane by the same procedures as described above.

The bonded support was packed in 4.6 $\times$ 250 mm stainless steel columns at 340 atm. using a mixture of chloroform (10.7 ml) and n-butanol (2.3 ml) to suspend 3 g of support. The columns were then washed with ethanol (75 ml).

A sample of the center fraction of the resulting interferon peak was analyzed for amino acid composition under varying hydrolysis conditions. The results are summarized below:

All hydrolyses in 5.7N HCl in acid washed evacuated glass tubes. Sample buffer and HCl blanks are subtracted.

	Asx	15.4	17.6	17.95	15.2
	Thr*	7.8	8.6	8.4	7.3
	Ser*	7.7	8.3	9.1	9.9
20	Glx	24.1	21.1	21.4	n.d.
	Pro	2.8	n.d.	n.d.	3.36
	Gly	4.9	6.9	6.6	7.4
	Ala	11.8	10.7	10.7	9.9
	Cys	n.d.	3.96	3.4	n.d.
25	Val	6.9	7.84	8.2	6.9
	Met	2.8	4.5	4.5	3.7
	Ile	8.7	8.6	8.9	7.8
	Leu	25.00**	25**	25**	25**
	Tyr	9.6	10.3	9.0	8.1
30	Phe	7.3	9.5	9.8	9.0
	His	4.5	7.5	6.9	6.1
	Lys	11.6	12.3	11.8	11.25
	Arg	8.8	11.25	10.6	8.8
	Trp	n.d.	n.d.	n.d.	2.93
35		24 hrs. TGA	24 hrs. TGA	48 hrs. TGA	24 hrs. TGA

40 TGA thioglycolic acid

* Uncorrected values

** Arbitrary value

n.d. not determined

45 Hexosamine analysis after hydrolysis of interferon with 5.7N HCl containing 0.02% (v/v) thioglycolic acid at 110°C for 6, 14 and 24 hours, using the eluent from the center peak fraction of the diphenyl column, indicated that fibroblast interferon contains about 3 residues of glucosamine. No galactosamine or mannosamine has been found.

Endgroup determination was carried out with a 90 pmol sample of the center fraction of the diphenyl column fibroblast interferon peak and resulted in detection of Met as N-terminus.

150 pmol samples each of fibroblast and leukocyte interferon were submitted to tryptic digestion. The digests were chromatographed and no peptide fragment common to both interferons was found.

Sequencing of an amount of 1.25 nmol of fibroblast interferon confirmed Met as N-terminus and also confirmed the sequence 3-10 reported by Hunkapillar and Hood, Biochemistry 17, 2124 (1978), which is Tyr³-Asn-Leu-Leu-Gly-Phe-Leu-Gln¹⁰. The results of a further sequence analysis of 5.9 nmoles of fibroblast interferon indicate the following NH₂-terminal sequence:

H-Met¹-Ser-Tyr-Asn-Leu⁵-Leu-Gly-Phe-Leu-Gln¹⁰-Arg-Ser-Ser-Asn-Phe¹⁵-Gln-.....-Gln-Lys¹⁹-....

EXAMPLE 4

60 Crude interferon was prepared from human fibroblasts (cell line GM 2504A). The crude material was made 1M in sodium chloride by the addition of saturated sodium chloride. This material was passed through a column of Blue Sepharose-4B (bed volume 20-25 ml, diameter 2.5 cm, 2.5 ml/min.). The crude material was kept on ice while loading. After loading, the column was washed with 250 ml of the following solution (2.5 ml/min.):

65 30% (v/v) ethylene glycol, 1M NaCl, 50mM Na₂HPO₄ (pH 7.2).

Finally, the interferon was eluted at a flow rate of 2.5 ml/min. with 250 ml of the following solution:
50% (v/v) ethylene glycol, 1M NaCl, 50mM Na₂HPO₄ (pH 7.2).

5 Typical assay results: 5% activity in flow thru
10% activity in 30% (v/v) wash
85% activity in 50% (v/v) wash 5

Peak fractions from the first Blue Sepharose column were pooled and diluted to 10% (v/v) ethylene glycol with the following solution:

10 2M NaCl, 50mM Na₂HPO₄ (pH 7.2). 10

This material was loaded on a Blue Sepharose column (20-25 ml bed volume, 2.5 cm diameter, flow rate 2.5 ml/min). It was washed with 250 ml of a solution containing 2M NaCl, 50mM Na₂HPO₄ (pH 7.2) and 30% (v/v) ethylene glycol and was eluted with a solution containing 2M NaCl, 50mM Na₂HPO₄ (pH 7.2) and 50% (v/v) ethylene glycol

15 15

Typical assay results: 10% activity in flow thru
30% activity in 30% (v/v) wash
60% activity in 50% (v/v) wash

20 HPLC 20

Sample: The first sample used was the 50% (v/v) ethylene glycol cut from Blue Sepharose. Later samples were ones that had been run through Blue Sepharose twice, in which case interferon in either the 30% or 50% (v/v) ethylene glycol cut could be used.

25 A 25 x 0.46 cm Lichrosorb RP-8 column was used. The column was first washed with water and then the Blue-Sepharose eluate was pumped on. A 5 ml mixing chamber was used before the pump. The HPLC column was run at 22 ml/hr with the following step-wise gradient of n-propanol, at a constant pH of 4.2, maintained with 1M formic acid/0.8M pyridine. 25

	n-propanol (%) (v/v)	time (min.)	
30	1. 0	10	30
	2. 30	40	
	3. 32	40	

35 The column was then washed with 60% (v/v) n-propanol and re-equilibrated with 0% (v/v) for next run. 35

Interferon eluted at the end of the 32% (v/v) step (between 80 and 90 min). This material was homogeneous as determined by NaDodSO₄ acrylamide gel electrophoresis staining with Coomassie Blue or prelabeling the sample with Fluram.

40 Amino acid analyses (24 hours hydrolysis, 0.2% (v/v) TGA, 5.7N HCl) were carried out on nine separate samples comprising four separate preparations. The average amino acid composition is set forth below with values relative to leucine taken arbitrarily as 22.0. The results were as follows: 40

	Residues	Residues	
45	Asx 15.1 ± 0.7	Met 4.5 ± 0.7	45
	Thr* 7.0 ± 0.5	Ile 9.4 ± 0.1	
	Ser* 7.5 ± 0.5	Leu 22.0	
	Glx 22.2 ± 0.5	Tyr 8.8 ± 0.2	
	Pro not determined	Phe 8.0 ± 0.3	
50	Cys 3.0 ± 0.3	His 4.5 ± 0.1	50
	Gly 6.9 ± 0.7	Lys 11.0 ± 0.6	
	Ala 7.4 ± 1.0	Arg 11.9 ± 0.7	
	Val 5.7 ± 0.5	Trp not determined	

55 * uncorrected value 55

Based on the several samples prepared by different procedures and analyzed at different times as set forth above in this and prior Examples, a representative amino acid analysis (± 15%) for homogeneous fibroblast interferon utilizing a 24 hour hydrolysate in 6N HCl with 0.2% (v/v) thioglycolic acid is as follows:

	Asx	15.1	Gly	6.9	Tyr	8.8
	Thr*	7.0	Ala	7.4	Phe	8.0
	Ser*	7.5	Val	5.7	His	4.5
	Glx	22.5	Met	4.5	Lys	11.0
5	Pro	3.0	Ile	9.4	Arg	11.0
	Cys	3.0	Leu**	22.0	Trp	3.0

* uncorrected value

10 * arbitrary value

EXAMPLE 5

Parenteral Dosage Form with Homogeneous Human Fibroblast Interferon

A total of 1.5 mg of homogeneous human fibroblast interferon having a specific activity of 4×10^8 units/mg is dissolved in 25 ml of 5% (v/v) normal human serum albumin. The solution is passed through a bacteriological filter and is then aseptically subdivided into 100 vials. Each vial contains 6×10^6 units of the pure interferon suitable for parenteral administration. The vials are preferably stored in the cold (-20°C) prior to use.

The words "Sephacrose", "Vibacron", "Coomassie", "Triton", "Spherisorb" and "Lichrosorb" are all registered Trade Marks.

CLAIMS

1. Human fibroblast interferon as a homogeneous protein.
- 25 2. Human fibroblast interferon as a homogeneous protein according to claim 1 characterised by
 - (a) a specific activity of about 4×10^8 units/mg;
 - (b) an apparent molecular weight of about 20500 by sodium dodecyl sulfate polyacrylamide gel electrophoresis;
 - (c) the following amino acid composition ($\pm 15\%$) based on 24 hours hydrolysis in 6.0N HCl containing 0.2% (v/v) thioglycolic acid:

	Asx	15.1	Gly	6.9	Tyr	8.8
	Thr*	7.0	Ala	7.4	Phe	8.0
	Ser*	7.5	Val	5.7	His	4.5
35	Glx	22.2	Met	4.5	Lys	11.0
	Pro	3.0	Ile	9.4	Arg	11.9
	Cys	3.0	Leu**	22.0	Trp	3.0

40 * uncorrected value

** arbitrary value

(d) the partial amino acid sequence

H-Met¹-Ser-Tyr-Asn-Leu⁵-Leu-Gly-Phe-Leu-Gln¹⁰-Arg-Ser-Ser-Asn-Phe¹⁵-Gln-.....Gln-Lys¹⁹.....and

(e) a maximum of 3 residues of glucosamine and no residue of galactosamine or mannosamine per molecule.

3. Human fibroblast interferon as a homogeneous protein, substantially as hereinbefore described.

4. Human fibroblast interferon as defined in any one of claims 1 to 3 for the treatment of viral and neoplastic disease states.

5. A process for producing human fibroblast interferon as a homogeneous protein which process comprises the following steps in combination:

A. passing an aqueous solution of human fibroblast interferon in an impure state through an affinity chromatography column so as to absorb said interferon onto said column and thereafter eluting said interferon from said column and obtaining said interferon in selected fractions of said eluate in a state of enhanced purity; and

B. passing the selected said interferon fractions obtained in Step A through one or more buffer equilibrated hydrocarbon bonded silica matrix column(s) under high pressure liquid chromatography conditions wherein said hydrocarbon is cyclohexyl, phenyl, diphenyl, n-octyl, n-octadecyl or 3-cyano-propyl, to absorb said interferon from said column with a gradient of an acidic aqueous buffered mixture of water miscible solvents and obtaining interferon as a single distinct peak in selected fractions of said eluate in the state of a homogeneous protein.

6. The process of claim 5 wherein said affinity chromatography column is a Concanavalin A-Sepharose column; said high pressure liquid chromatography is carried out on a cyclohexyl bonded silica matrix column using as eluent a pyridine/formic acid/isopropanol/n-butanol/water solution with increasing concentrations of n-butanol followed by passing the pooled active fractions through an n-octyl bonded silica matrix column using the same elution conditions as for the cyclohexyl column.



Asx	15.1	Gly	6.9	Tyr	8.8
Thr*	7.0	Ala	7.4	Phe	8.0
Ser*	7.5	Val	5.7	His	4.5
Glx	22.5	Met	4.5	Lys	11.0
Pro	3.0	Ile	9.4	Arg	11.0
Cys	3.0	Leu**	22.0	Trp	3.0

* uncorrected value

10 * arbitrary value

EXAMPLE 5

Parenteral Dosage Form with Homogeneous Human Fibroblast Interferon

A total of 1.5 mg of homogeneous human fibroblast interferon having a specific activity of 4×10^6 units/mg is dissolved in 25 ml of 5% (v/v) normal human serum albumin. The solution is passed through a bacteriological filter and is then aseptically subdivided into 100 vials. Each vial contains 6×10^6 units of the pure interferon suitable for parenteral administration. The vials are preferably stored in the cold (-20°C) prior to use.

The words "Sephacrose", "Vibacron", "Coomassie", "Triton", "Spherisorb" and "Lichrosorb" are all registered Trade Marks.

CLAIMS

1. Human fibroblast interferon as a homogeneous protein.
2. Human fibroblast interferon as a homogeneous protein according to claim 1 characterised by
 - (a) a specific activity of about 4×10^6 units/mg;
 - (b) an apparent molecular weight of about 20500 by sodium dodecyl sulfate polyacrylamide gel electrophoresis;
 - (c) the following amino acid composition ($\pm 15\%$) based on 24 hours hydrolysis in 6.0N HCl containing 0.2% (v/v) thioglycolic acid:

Asx	15.1	Gly	6.9	Tyr	8.8
Thr*	7.0	Ala	7.4	Phe	8.0
Ser*	7.5	Val	5.7	His	4.5
Glx	22.2	Met	4.5	Lys	11.0
Pro	3.0	Ile	9.4	Arg	11.9
Cys	3.0	Leu**	22.0	Trp	3.0

* uncorrected value

** arbitrary value

(d) the partial amino acid sequence

H-Met¹-Ser-Tyr-Asn-Leu⁵-Leu-Gly-Phe-Leu-Gln¹⁰-Arg-Ser-Ser-Asn-Phe¹⁵-Gln-.....Gln-Lys¹⁹-....and

(e) a maximum of 3 residues of glucosamine and no residue of galactosamine or mannosamine per molecule.

3. Human fibroblast interferon as a homogeneous protein, substantially as hereinbefore described.
4. Human fibroblast interferon as defined in any one of claims 1 to 3 for the treatment of viral and neoplastic disease states.

5. A process for producing human fibroblast interferon as a homogeneous protein which process comprises the following steps in combination:

A. passing an aqueous solution of human fibroblast interferon in an impure state through an affinity chromatography column so as to absorb said interferon onto said column and thereafter eluting said interferon from said column and obtaining said interferon in selected fractions of said eluate in a state of enhanced purity; and

B. passing the selected said interferon fractions obtained in Step A through one or more buffer equilibrated hydrocarbon bonded silica matrix column(s) under high pressure liquid chromatography conditions wherein said hydrocarbon is cyclohexyl, phenyl, diphenyl, n-octyl, n-octadecyl or 3-cyano-propyl, to absorb said interferon from said column with a gradient of an acidic aqueous buffered mixture of water miscible solvents and obtaining interferon as a single distinct peak in selected fractions of said eluate in the state of a homogeneous protein.

6. The process of claim 5 wherein said affinity chromatography column is a Concanavalin A-Sepharose column; said high pressure liquid chromatography is carried out on a cyclohexyl bonded silica matrix column using as eluent a pyridine/formic acid/isopropanol/n-butanol/water solution with increasing concentrations of n-butanol followed by passing the pooled active fractions through an n-octyl bonded silica matrix column using the same elution conditions as for the cyclohexyl column.

7. The process of claim 6 wherein said eluent has an initial composition in said high pressure liquid chromatography system of pyridine/formic acid/isopropanol/water (8:8:20:64, v/v) and a final eluent composition of pyridine/formic acid/isopropanol/n-butanol/water (8:8:25:20:39, v/v).
8. The process of claim 5 wherein said affinity chromatography column is a Blue Dextran-Sephadex column; said high pressure liquid chromatography is carried out on an n-octyl bonded silica matrix column using as eluent a pyridine/formic acid/isopropanol/n-butanol/water solution with increasing concentrations of n-butanol. 5
9. The process of claim 8 wherein said eluent has an initial composition in said high pressure liquid chromatography system of pyridine/formic acid/iso-propanol/n-butanol/water (8:8:20:3:3:60.6, v/v) and a final composition of pyridine/formic acid/iso-propanol/n-butanol/water (8:8:25:20:39, v/v). 10
10. The process of claim 8 or claim 9 wherein the pooled active fractions eluted from said n-octyl bonded silica matrix column is rechromatographed on a 3-cyanopropyl bonded silica matrix column using as eluent a pyridine/formic acid/n-propanol/water solution with increasing concentrations of n-propanol.
11. The process of claim 10 wherein said eluent has an initial composition of pyridine/formic acid/water (8:8:84, v/v) and a final composition of pyridine/formic acid/n-propanol/water (8:8:40:44, v/v). 15
12. The process of claim 8 wherein said eluent has an initial composition in said high pressure liquid chromatography step of pyridine/formic acid/isopropanol/water (8:8:20:64, v/v) and a final composition of pyridine/formic acid/isopropanol/water (8:8:25:20:29, v/v).
13. The process of claim 10 wherein the pooled activity fractions from said 3-cyanopropyl bonded silica matrix column are passed through said column a second time and the pooled active fractions from said second passage are passed through a diphenyl bonded silica matrix column. 20
14. The process of claim 5 wherein said human fibroblast interferon is harvested from a serum free preparation, said affinity chromatography column is a Blue Sepharose 4B column and said high pressure liquid chromatography is carried out on an n-octyl bonded silica matrix using as eluent a step gradient of n-propanol, in a constant mixture of pyridine/formic acid/water. 25
15. The process of claim 14 wherein said step gradient of n-propanol consists of 0% for ten minutes, 30% for 40 minutes and 32% for 40 minutes and wherein said interferon elutes from 80 to 90 minutes, all percentages being by volume.
16. A process for producing human fibroblast interferon as a homogeneous protein, substantially as hereinbefore described. 30
17. A pharmaceutical preparation suitable for parenteral administration for treatment of viral and neoplastic disease states said preparation comprising an effective amount of human fibroblast interferon as a homogeneous protein and a conventional pharmaceutical parenteral carrier material.
18. A pharmaceutical preparation comprising an effective amount of human fibroblast interferon as a homogeneous protein, substantially as hereinbefore described. 35
19. A pharmaceutical preparation according to claim 16 or claim 17, for use in the treatment of viral and neoplastic disease states.