

SPECIFICATION

Purified proteins & process therefor

Purification of proteins has long been a problem in peptide chemistry. Techniques which have been employed include precipitation, gel filtration, ion exchange chromatography, gel electrophoresis, affinity chromatography, and many others too numerous to mention.

Schemes to isolate naturally occurring, high molecular weight proteins which are present in biological samples in extremely low concentrations have involved multi-step procedures utilizing assays of the aforementioned techniques. In a great number of such cases tremendous quantities of crude starting material must be accumulated and processed at high cost and usually with expenditure of great effort due to large losses of product in the later steps of the purification procedure.

A good case in point is the history of the numerous attempts to isolate and characterize interferon. 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 two decades to be isolated as a homogeneous peptide in amounts sufficient to allow characterization and identification of its specific biological and chemical properties.

In U. S. Patent 3,699,222, which is directed to Isaacs and Lindenmann's original research with interferon, the purification of the active material is limited to ammonium sulfate precipitation followed by dialysis. Such procedures are relatively non-specific and thus the product obtained thereby is still in an extremely crude state.

A multi-step procedure for purifying interferon is disclosed in U. S. Patent 3,414,651 utilizing selective adsorption on an amorphous, aluminosilicate, elution with iodine or thiocyanate solution, further precipitation of unwanted protein with aqueous HCl and then aqueous NaOH, precipitation of interferon from the basic solution with water-miscible solvents such as methanol, ethanol or acetone and finally chromatography of the redissolved interferon on an anion exchange resin such as 2-diethylaminoethyl-cellulose to produce an interferon whose specific activity is indicated to have been enhanced 6,000 fold by the entire process. Specific interferons exemplified were chick and monkey interferon.

A further purification variation is taught in U. S. Patent 3,975,344 where a crude human fibroblast interferon solution derived from the incubation medium of the cell culture was purified by zonal density gradient ultra-centrifugation. This technique was indicated to give higher yields and purification than obtained with the conventional procedure of column chromatography on Sephadex G-100 (Trade Mark).

Recent scientific papers directed to the purification and attempted characterization of interferons can be summarized as follows:

Knight, E., *Proc. Natl. Acad. Sci. USA* 73, 520-3 (1976);
 Törmä, E. T. et al., *J. Biol. Chem.* 251, 4810-6 (1976);
 Bridgen, P. J. et al. *J. Biol. Chem.* 252, 6585-7 (1977);
 DeMaeyer-Guignard, J. et al., *Nature* 271, 622-5 (1978);
 Kawakita, M. et al., *J. Biol. Chem.* 253, 598-602 (1978);
 Berthold, W. et al., *J. Biol. Chem.* 253, 5206-11 (1978);
 Jankowski, W. J. et al., *J. Virology* 16, 1124-30 (1975);
 Davey M. W. et al., *J. Biol. Chem.* 251, 7620-5 (1976);
 Chadha, K. C. et al., *Biochemistry* 17, 196-200 (1978).

While several of the above papers contain claims 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 (HPLC) for purification of proteins is generally known in the art. These references specifically described ion exchange and size exclusion type columns in protein purification. See for example Regnier, F. E. et al., *J. Chromatog. Sci.* 14, 316-20 (1976) and Chang, S.-H. et al., *Anal. Chem.* 48, 1839-45 (1976).

The use of Lichrosorb RP-18 (Trade Mark- octadecyl bound silica microparticle column) in reverse phase partition chromatography was successfully employed to purify peptides such as β -endorphin [see e.g. Rubinstein, M. et al., *Proc. Natl. Acad. Sci., USA* 74, 4969-72 (1977)].

Finally, the partial characterization of three species of interferons from mouse Ehrlich ascites tumor cells (MW = 33,000, 26,000 and 20,000) is described by Cabrer, B. et al., *J. Biol. Chem.* 254, 3681-4 (1979).

In its widest sense the present invention relates to an improved process for purifying proteins having molecular weights greater than about 12,000 with high resolution and high yield on a preparative scale. The process comprises passing an aqueous solution of said protein in an impure state through a buffer equilibrated column of a porous silica matrix to which cyanopropyl, cyclohexyl, phenyl, octyl, octadecyl or glyceryl groups are bonded under HPLC conditions so as to absorb said protein onto said column and thereafter eluting said protein with an increasing or decreasing gradient of an aqueous water miscible solvent and obtaining said protein in selected fractions of said eluate in a state of enhanced purity. These columns, which can be used sequentially and under varying conditions of pH and organic solvent gradient, offer the capability to purify to homogeneity protein materials that are present in extremely small quantities in cell extracts or growth media.

In a preferred specific embodiment of the present invention, the purification process is employed to purify interferon to homogeneity in amounts sufficient to allow chemical characterization of this medicinally valuable substance for the first time. The ability to chemically characterize interferon represents a significant advance in the development of this substance since it will allow the compound to be prepared either by conventional peptide synthesis procedures or alternatively by synthesizing the DNA corresponding to the interferon amino acid sequence and introducing such DNA into a suitable organism, preferably a bacterium, by DNA-recombinant techniques. The resulting organism will then have the capability of producing interferon which can be scaled up by application of fermentation techniques to provide a convenient source of this heretofore scarce compound on a commercial level.

The specific process for producing interferon as a homogeneous protein comprises the following steps in combination:

A) passing an aqueous solution of interferon in an impure state through a buffer equilibrated octyl bonded silica matrix column under high performance liquid chromatography conditions so as to absorb said interferon onto said column and thereafter eluting said interferon from said column with an increasing gradient of an aqueous, buffered, water miscible solvent and obtaining said interferon in selected fractions of said eluate in a state of enhanced purity;

B) passing the selected interferon fractions obtained in Step A through a buffer equilibrated glyceryl bonded silica matrix column to absorb said interferon onto said column and thereafter eluting said interferon from said column with a decreasing gradient of an aqueous buffered water miscible solvent and obtaining interferon as distinct major peaks in selected fractions of said eluate in a state of enhanced purity;

C) passing the selected fractions of interferon corresponding to one of said distinct major peaks obtained in Step B in buffered solution through a buffer equilibrated octyl bonded silica matrix column under HPLC conditions so as to absorb said interferon onto said column and thereafter eluting said interferon from said column with an aqueous, buffered, water miscible solvent and obtaining interferon as a single distinct peak in selected fractions of said eluate in the state of a homogeneous protein and, if desired, repeating the procedure of step C to achieve ultimate homogeneity.

The present invention also relates to the pure and homogeneous interferon species obtained by the improved process.

Octyl or glyceryl modified porous silica microparticle columns (particle size = 10 μ ; average pore size = 100 Å), used in the practice of the present invention, are articles of commerce, available from EM Laboratories of Elmsford, N. Y., USA under the Trade Marks Lichrosorb RP-8 and Lichrosorb diol. Equivalent octyl modified porous silica columns (identified as Chromegabond C-8) are available from E. S. Industries, Marlton, N. J., USA.

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

In performing the instant process the solution of the impure high molecular weight peptide, preferably in aqueous buffer at a pH compatible with the properties of the protein in question, is passed through the silica matrix column. Usually the procedure is carried out under pressure, preferably in the range of from 50 to 5,000 psi (3.4-340 atm). The protein is absorbed into the column and is then subsequently eluted in selective fashion using a gradient of an aqueous water miscible solvent. Suitable water miscible solvents for this purpose include alkanols such as n-propanol, 2-propanol, ethanol, methanol, tert-butanol, or cyclic ethers such as dioxane. Fractionation of the eluate is accomplished by utilizing fraction collectors 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). It is also desirable to monitor the presence of the target protein by a suitable bioassay.

The decision as to whether to employ both column types (columns for "normal" partition chromatography and columns for reverse phase chromatography) and if so what order to select depends in large part on the nature of the protein to be purified. It has been found, for example, that in the specific case of human leukocyte interferon best results are achieved by initially resolving the impure interferon solution through the octyl bonded silica matrix column (reverse phase chromatography) using a buffer pH of about 7.5, preferably a 1 M sodium acetate-acetic acid system, and eluting with a gradient of increasing n-propanol concentration (from 0 to 40% v/v), then passing the collected active fractions through the glyceryl bonded silica matrix column in 0.1 M sodium acetate and eluting with a gradient of decreasing n-propanol (from 72.5 to 50% v/v) and finally passing the separated interferon components through the octyl bonded silica matrix column using a buffer pH of about 4.0, preferably 1 M pyridine-2M formic acid system, and eluting using an increasing n-propanol gradient (from 20 to 40% v/v). In this manner, each of the three separate forms of human leukocyte interferon (α , β and γ) can be resolved into separate, sharp peaks representing the homogeneous proteins. The overall purification starting with the incubation medium to the second octyl bonded silica matrix chromatography step was 60,000 to 80,000 fold while the cumulative yield through the glycerol bonded silica matrix chromatography step ranged from 30 to 50%.

In a specific embodiment of the process of purifying human leukocyte interferon the selected peak fractions from Step B are pooled, n-propanol is removed by extraction with n-hexane and the traces of n-hexane are removed from the aqueous phase prior to proceeding to Step C.

The homogeneous human leukocyte interferon species obtained by the practice of the process of the invention each exhibited a sharp peak on the aforesaid HPLC column and a single narrow band on sodium

dodecyl sulfate (NaDodSO₄) polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. Extraction of the gel gave a single sharp peak of antiviral activity coinciding with the protein band. The specific activity of the pure interferon species was found to be in the range of about $2.6 - 4.0 \times 10^8$ with MDBK bovine cells and in the range of about $1.5 - 4 \times 10^8$ with the Ag 1732 human cell line. The molecular weights ranged from 16,000 to 21,000 as seen in Table 4 (page 22). The results of the amino acid analyses are summarized in Table 5 (page 23).

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 interferons which are an aspect of the invention can be utilized in the same manner as the previously employed crude preparations with adjustments in the dosage to provide the desired level of interferon units. The individual species can be utilized per se or alternatively mixtures of two or more of such species can be employed. Such mixtures can be obtained by mixing the isolated species as desired or stopping the purification procedure where several species of interferon are present but no non-interferon active proteins are present so that the composition is a mixture of homogeneous interferon proteins. Thus, the present invention also includes a pharmaceutical preparation containing a homogeneous interferon and a non-toxic, inert, therapeutically compatible solid or liquid carrier.

The aforesaid purification process, while exemplified by the case of human leukocyte interferon, can also be employed to purify other interferons including human fibroblast interferon and interferons from animal sources as well as other proteins having molecular weights greater than about 12,000. Thus, for example, pro-opiocortin (MW ca. 30'000) has been purified to homogeneity using the procedure of the present invention [see Kimura et al., Proc. Natl. Acad. Sci. USA 76, 1756-9 (1979)]. It is within the skill of the art to select the proper specific conditions to provide the optimum resolution for each different type of protein to be purified.

The induction of interferon production, the initial concentration and fractionation of interferon including gel filtration can be achieved using methods well known in the art. These procedures which yield an aqueous solution of interferon in an impure state are not part of the present invention.

The process and product aspects of this invention are further illustrated by reference to the following Examples.

EXAMPLE 1

Homogeneous Human Leukocyte Interferon from Normal Donors

A. Production of Interferon

Interferon was produced by incubation of human leukocytes from the blood of normal donors (10^7 cells/ml) with Newcastle disease virus (15 hemagglutination units/ml) in a serum-free minimal essential medium containing casein (10 mg/ml) for 16 hours. An average interferon titer of 5000 units/ml was obtained. The procedures used were performed as reported by Mogensen, K. E. et al., Pharmacology and Therapeutics A, 1977, 369-381; Wheelock, E.F., J. Bacteriol. 92, 1415-1421 (1966) and Cantell, K. et al., Appl. Microbiol. 22, 625-628 (1971) with some minor modifications. Interferon titers were determined by a cytopathic effect-inhibition assay which could be performed within 16 hours. All interferon titers are expressed in terms of reference units/ml, calibrated against the reference standard for human leukocyte interferon provided by the National Institute of Health (USA).

B. Concentration and Initial Fractionation of Interferon

Unless otherwise specified, these procedures were performed at 0-4°C. At the end of the incubation, cells and debris were removed by low-speed centrifugation (15 min., 500 × g). Casein was precipitated by acidification with HCl to pH 4.0. After 2 hours, the mixture was centrifuged (10 min., 12,000 × g) and the pellet was discarded. The supernatant (10 liters) was adjusted to 1.5% (w/v) trichloroacetic acid. After 1 hour, the precipitate was collected by centrifugation (10 min., 12,000 × g) and redissolved in 50 ml of 0.1 M NaHCO₃. Triton X-100 (Trade Mark) (0.5 g) was added and then acetic acid (1.5 ml) was added dropwise with stirring. The mixture was stored at 0°C for 1 hour and then for 16 hours at -20°C. It was then thawed and centrifuged (10 min., 17,000 × g). The pellet was discarded and the supernatant was adjusted to 4% trichloroacetic acid. After 1 hour, the mixture was centrifuged (10 min., 12,000 × g). The precipitate was collected and redissolved in 5 ml of 0.5 M NaHCO₃.

C. Gel Filtration

Urea (1.5g) was added to the interferon concentrate and the solution was applied to a column of Sephadex G-100 fine (2.6 × 90 cm) pre-equilibrated with 4 M urea/0.1 M sodium acetate buffer. The column was eluted with 4 M urea/0.1 M sodium acetate, pH 7.5, at room temperature at a flow rate of 0.5 ml/min. Fractions of 12.5 ml were collected. Interferon activity was eluted in fractions 19-23.

D. High Performance Liquid Chromatography

Fractions 19-23 of the Sephadex G-100 run were combined and applied directly through the pump to a Lichrosorb RP-8 column (10 μ, 4.6 × 250 mm). The column was pre-equilibrated with a 1 M sodium acetate buffer (pH 7.5) containing 0.01% (v/v) thioglycol and then eluted with a linear gradient of n-propanol in the

same buffer [1 hour, 0-20%; 3 hours, 20-40% (v/v)] at a flow rate of 0.25 ml/min. Fractions of 0.75 ml. were collected. Interferon was eluted in fractions 23 to 40 [25-30% (v/v) n-propanol].

Fractions 27-33, containing most of the interferon activity were combined, n-propanol was added to a final concentration of 80% (v/v), and the solution was applied through the pump to a lichrosorb diol column (10 μ , 4.6 $\times$ 250 mm) previously equilibrated with a solution of 0.1 M sodium acetate containing 80%, (v/v) n-propanol. The column was then eluted with a 4 hours linear gradient of 72-50% (v/v) n-propanol in 0.1 M sodium acetate at a flow rate of 0.25 ml/min. Fractions of 0.75 ml were collected. Interferon activity was eluted as three distinct major peaks that varied quantitatively from one preparation to another. The peaks were labelled α , β and γ according to their order of elution. The α -fraction was eluted at a concentration of 68% n-propanol, the β -fraction at 66.5% n-propanol, the γ -fraction at 65.5 % n-propanol. The total recovery of interferon activity was better than 80%.

The fractions comprising each peak were pooled separately and purified individually through subsequent steps. Because peak γ was present in high abundance and appeared to be better resolved from other components, it was selected for further purification. Fractions 54-56, comprising peak γ from the diol column, were pooled and 1-propanol was removed by two extractions with an equal volume of hexane. Traces of hexane were removed under a stream of nitrogen. Pyridine and formic acid were added to final concentrations of 1 M and 2 M, respectively, and the solution was applied to a Lichrosorb RP-8 column (10 μ m; 4.6 $\times$ 250 mm) previously equilibrated with 1 M pyridine and 2 M formic acid (pH 4.0). The column was eluted with a linear 20-40% 1-propanol gradient in the 1 M pyridine/formate buffer in 3 hours at a flow rate of 0.2 ml/min. Fractions of 0.6 ml were collected. The major peak of activity coincided with a protein peak. Fractions 45 and 46 (32%, v/v, propanol) comprising this peak were combined and rechromatographed under similar conditions. Interferon was eluted in fraction 31 (32%, v/v, propanol). The specific activity of this fraction was calculated to be 4×10^8 units/mg in relation to bovine serum albumin. This material was used for further characterization. The fluorescence profiles of the high-performance steps were so remarkably reproducible that they provided a continual fingerprint of the entire procedure.

The results of the purification are summarized in Table 1. The overall purification starting with the incubation medium to the second RP-8 column was 60,000-to 80,000-fold. The cumulative yield from step 1 through the diol step ranged from 30 to 50%. Beyond this step, each of the three peaks of interferon was purified separately.

TABLE 1.

Purification of human leukocyte interferon

Step	Units recovered $\times 10^{-6}$	Protein recovered [mg]	Relative specific activity, [units/mg]	Degree of purification	Recovery range per step [%]
1. Incubation medium	50	10,000	5×10^3	1	-
2. pH 4 supernatant	50	2,000	2.5×10^4	5	100
3. 1.5% trichloroacetic acid/precipitate	40	1,000	4×10^4	8	80-100
4. Triton X-100/acetic acid supernatant	40	250	1.6×10^5	32	70-100
5. 4% trichloroacetic acid precipitate	35	175	2×10^5	40	80-90
6. Sephadex G-100	32	57	5.6×10^5	112	70-90
7. Lichrosorb RP-8 (pH 7.5)	28	11	2.5×10^6	500	80-100
8. Lichrosorb diol					
Peak α	11	1.1	1×10^7	5,000	70-90
Peak β	2.5	ND	ND	ND	
Peak γ	12.5	0.21	6×10^7	12,000	
9. Lichrosorb RP-8 (pH 4)	1.6	0.0064	3×10^8	60,000	40-60
10. Lichrosorb RP-8 (pH 4)	8.2	0.021	4×10^8	80,000	40-60

For determination of protein recovered in each fraction, bovine serum albumin was used as a standard. The absolute specific activity determined by amino acid analysis of the homogeneous peak of step 10 was found to be $2-4 \times 10^8$ units/mg (see text). Step 9 was performed on peak γ of Step 8. Step 10 was performed on pooled material from Step 9 of several preparations. ND = not determined.

E. Polyacrylamide Gel Electrophoresis

Samples of interferon (1.5×10^5 units) were incubated in NaDodSO₄ and 2-mercaptoethanol and then applied to a slab gel. After electrophoresis, a single sharp band was obtained upon staining with Coomassie blue. The apparent molecular weight was estimated to be 17,500 in comparison with standard proteins. The gel was then cut into 1-mm slices. Each slice was homogenized in 0.4 ml of 0.5 M NaHCO₃/0.1% NaDodSO₄ and assayed for interferon activity. A single peak of antiviral activity was obtained coinciding with the single protein band. No other peak of activity was observed.

5

F. Amino Acid Analysis

Amino acid analysis of homogeneous human leukocyte interferon (peak γ) was performed with the fluorescamine amino acid analyzer on 0.5 to 1- μ g samples of native and S-carboxymethylated interferon. For measurement of the cysteine/cystine ratio, native interferon was carboxymethylated and then hydrolyzed in 6 M HCl under reducing conditions (0.1% thioglycolic acid). Under these conditions, cysteine is measured as S-carboxymethylcysteine whereas cystine is measured as free cysteine. Amino acid analyses are summarized in Table 2. The specific activity based on amino acid content was found to be $2-4 \times 10^8$ units/mg.

10

15

TABLE 2

Amino acid composition of human leukocyte interferon

5	Amino acid	Residues	5
	Asx	15.2±1.2	
	Thr*	7.5±0.5	
10	Ser*	8.0±0.5	10
	Glx	24.0±0.6	
15	Pro	6.3±0.3	15
	Gly	5.5±0.5	
	Ala	8.2±0.2	
20	Cyc (total)	3.3±0.7	20
	1/2 Cystine ⁺	1.8±0.2	
25	Cysteine ⁺	1.5±0.5	25
	Val	7.8±0.2	
	Met	3.9±0.2	
30	Ile	8.9±0.4	30
	Leu	21.8±1.3	
35	Tyr	5.1±0.2	35
	Phe	9.1±0.3	
	His	3.3±0.4	
40	Lys	11.6±0.5	40
	Arg	7.3±0.5	
45	Trp ⁺⁺	0.7±0.1	45
*	Corrected to time 0.		
50	Measured after carboxymethylation of native interferon.		50
++	Measured after hydrolysis in 6 M HCl/4% thioglycolic acid.		

EXAMPLE 2***Homogeneous Human Leukocyte Interferon from Leukocytes of Leukemic Patients***

Interferon was produced by incubation of human leukocytes, isolated from the blood of leukemic patients (chronic myelogenous leukemia, CML) by leukopheresis, with Newcastle disease virus in a serum-free medium containing casein. Interferon titers of 5,000-40,000 units/ml were routinely obtained.

The purification procedure was the same as the one described for interferon from normal blood (Example 1) and included selective precipitation by 0.5 M acetic acid in the presence of Triton X-100, gel filtration on Sephadex G-100 in 4 M urea, HPLC at pH 7.5 on Lichrosorb RP-8, HPLC on Lichrosorb diol and HPLC on Lichrosorb RP-8 at pH 4.0.

The fractions comprising the α -, β - and γ -peak from the Lichrosorb diol column were pooled separately and purified individually through subsequent steps.

Fractions 43-46 (α -peak) were pooled and n-propanol was removed by two extractions with an equal volume of n-hexane. Traces of hexane were removed under a stream of nitrogen. Pyridine and formic acid were added to a final concentration of 1 M and 2 M respectively, and the solution was applied to a Lichrosorb RP-8 column (10 μ , 4.6 $\times$ 250 mm) previously equilibrated with 1 M pyridine/2 M formic acid (pH 4.0). the column was eluted with a linear 20-40% (v/v) n-propanol gradient in the 1 M pyridine formate buffer in 3 hours at room temperature at a flow rate of 0.2 ml/min. Fractions of 0.6 ml were collected. Interferon activity was eluted as broad peaks in the range of 31 to 35% (v/v) n-propanol. These fractions were combined and rechromatographed under identical conditions. Interferon activity was eluted in two main peaks (α_1 and α_2) at 31 and 32% (v/v) n-propanol. Minor components were eluted by 34% (v/v) n-propanol.

Fractions 47-50 (peak β) from the Lichrosorb diol column were pooled and processed and chromatographed on Lichrosorb RP-8 as described for peak α above. Interferon activity was eluted in two main peaks: β_2 at 32% (v/v) n-propanol and β_3 at 34% (v/v) n-propanol. Rechromatography was not necessary in this case. In some preparations a peak designated β_1 eluting at 31% (v/v) n-propanol was observed.

Fractions 52-54 (peak γ) from the Lichrosorb diol column were pooled and processed and chromatographed on Lichrosorb RP-8 as described for peak α above. Interferon activity was eluted in five major peaks, namely: γ_1 (at 31% n-propanol, v/v), γ_2 (at 32% n-propanol, v/v), γ_3 (at 34% n-propanol, v/v), γ_4 (at 35% n-propanol, v/v) and γ_5 (at 35.5% n-propanol, v/v). Rechromatography was not necessary in this case.

TABLE 3.

Purification of leukocyte interferon from CML cells

Step	Units Recovered $\times 10^{-6}$	Protein Recovered [mg]	Specific** Activity [units/mg]	Degree of Purifica- tion	Recovery [%]
1. Incubation medium	800	20×10^3	4×10^4	1	100
2. pH 4 supernatant	800	4×10^3	2×10^5	5	100
3. 1.5% trichloroacetic acid precipitate	780	2×10^3	3.9×10^5	9.8	97
4. Triton X-100/acetic acid supernatant	760	510	1.5×10^6	37.5	95
5. 4% Trichloroacetic acid precipitate	810	350	2.3×10^6	57.5	100
6. Sephadex G-100	660	130	5.1×10^6	128	82
7. Lichrosorb RP-8 (pH 7.5) (two batches)	510	26	2×10^7	500	64
8. Lichrosorb Diol					
Peak α	149	5	3×10^7	750	
Peak β	148	3	5×10^7	1250	
Peak γ	139	1.7	8×10^7	2000	
Total	436				54

TABLE 3. (continued)

Step	Units Recovered $\times 10^{-6}$	Protein Recovered [mg]	Specific** Activity [units/mg]	Degree of Purification	Recovery [%]
9. Lichrosorb RP-8 (pH 4.0)					
Peak α_1^*	9	35×10^{-3}	2.6×10^8	6,500	
Peak α_2^*	26	65×10^{-3}	4.0×10^8	10,000	
Peak β_2	30	75×10^{-3}	4.0×10^8	10,000	
Peak β_3	13	32×10^{-3}	4.0×10^8	10,000	
Peak γ_1	15	58×10^{-3}	2.6×10^8	6,500	
Peak γ_2	31	77×10^{-3}	4×10^8	10,000	
Peak γ_3	26	74×10^{-3}	3.5×10^8	8,750	
Peak γ_4	3.5	10×10^{-3}	3.5×10^8	8,750	
Peak γ_5	4.5	50×10^{-3}	0.9×10^8	2,250	
Total	158				20

*after rechromatography

**activity determined on MDBK (bovine) cells; protein measured with bovine serum albumine as standard

The results of purification to produce the individual species of interferon are summarized in Table 3.

Species α_2 and β_2 are further distinguishable by their elution characteristics on the Lichrosorb diol column: α_2 is eluted with 68% (v/v) n-propanol (72.5 - 50% gradient) and β_2 with 66.5 % (v/v) n-propanol (72.5 - 50% gradient) at room temperature and a flow rate of 0.25 ml/min.

5 Samples of the interferon species (1.5×10^5 units) were incubated in NaDodSO₄ and 2-mercaptoethanol and then applied to a slab gel. After electrophoresis peak α_1 , α_2 , β_2 , γ_1 , γ_2 and γ_4 gave single bands while peaks β_3 , γ_3 and γ_5 gave two bands. The apparent molecular weights were all in the range of 16,000 to 18,000 except β_3 which gave a 16,500 and a 21,000 band and γ_4 which gave a 21,000 band (see Table 4). 5

Amino acid analysis of the purified human leukocyte interferon fractions were performed with the 10 fluorescamine amino acid analyzer on 0.5-1 μ g samples of interferon. Hydrolysis was performed in 6N HCl under reducing conditions (0.1% thioglycolic acid). The results of the analyses are summarized in Table 5. 10

TABLE 4

15		MW, ± 1000	Spec. Act. on MDBK (Bovine cells, units/mg) $\pm 25\%$	Spec. Act. on AG 1732 (Human cells, units/mg) $\pm 50\%$	Growth-inhibiting Activity**	15
20	α_1	16,500	2.6×10^8	2.6×10^8	+	20
	α_2	16,200	4×10^8	3×10^8	+	
	β_2	16,500	4×10^8	2×10^8	+	
25	β_3^*	21,000	4×10^8	3×10^8	+	25
	γ_1	17,700	2.6×10^8	2×10^8	+	
30	γ_2	17,700	4×10^8	1.5×10^8	+	30
	γ_3^*	17,200	3.5×10^8	1.5×10^7	+	
	γ_4	21,000	3.5×10^8	4×10^8	+	
35	γ_5^*	16,500	0.9×10^8	2×10^6	+	35

* 2 bands on NaDodSO₄ polyacrylamide gel electrophoresis; MW of major band given

40 ** Procedure of Steward et al., Nature 262, 300 (1976) 40

TABLE 5.

Amino acid analyses of human leukocyte interferons

Species	α_1	α_2	β_2	β_3^*	γ_1	γ_2	γ_3^*	γ_4	γ_6^*
MW,	16,500	16,200	16,500	21,000	17,700	17,700	17,200	21,000	16,500
Residues	142	139	142	181	153	153	148	180	142
Asx	13.8	11.7	11.5	18.2	13.1	13.3	15.0	17.9	13.8
Thr	7.7	8.3	9.0	9.9	8.4	9.6	8.6	7.3	7.6
Ser	9.2	10.0	10.0	14.5	10.2	7.8	10.1	13.5	9.0
Glx	20.3	20.5	21.9	28.5	23.9	25.0	22.8	27.0	20.8
Pro	6.1	4.9	5.0	5.9	4.5	4.8	4.8	6.5	4.2
Gly	5.1	4.5	5.0	3.6	5.7	5.0	3.2	4.8	4.0
Ala	8.4	8.3	7.9	11.5	8.7	8.1	9.3	10.4	9.3
Val	7.4	6.2	6.7	7.5	7.3	7.0	5.5	7.9	5.1
Met	3.7	3.8	4.4	6.0	4.3	3.9	5.6	4.9	4.7
Ileu	7.4	7.0	7.4	9.5	7.9	8.0	6.8	9.7	6.6
Leu	18.0	18.0	18.9	24.4	20.3	20.1	20.5	24.1	19.6
Tyr	4.0	4.4	4.6	5.0	4.8	4.8	3.7	5.0	3.6
Phe	6.9	8.0	8.7	9.9	8.6	9.0	7.2	9.1	6.4
His	3.0	2.8	3.0	3.8	3.7	3.3	2.9	3.8	2.8
Lys	10.5	9.0	8.5	9.3	10.1	10.0	7.6	12.3	12.0
Arg	6.2	7.1	8.0	10.8	8.0	8.5	10.1	8.5	9.0
Cys	3.9	4.0	1.8	2.3	3.3	2.9	3.1	4.1	2.3

Accuracy ± 1.5 residues

*Composition of mixture of both bands

The various species of purified human leukocyte interferon (300 pmol, 6 μ g) were dissolved in aqueous sodium bicarbonate (50 mM, pH 8.5, 50 μ l). Trypsin (0.1 μ g in 2 μ l of HCl, pH 3) was added and the mixture was incubated 14 hours at 37°C. Acetic acid (5 μ l) was added and the mixture was applied to a Licrosorb RP-8 column (10 μ particle size, 4.6 $\times$ 250 mm). The column was eluted at 0.5 ml/min using a 1 hour linear gradient from 0 to 40% (v/v) n-propanol in 0.1M formic acid/0.03M pyridine buffer (pH 3). The peptides were detected by a fluorescamine monitoring system. The results are set forth in Table 6 and are expressed in % n-propanol and in relative size of the peaks (S = small, M = medium, L = large).

TABLE 6

Tryptic peptides of human leukocyte interferons

Species	Elution Positions (% n-propanol)
α_1	3L, 4L, 4.2M, 11.5M, 12.5S, 14.5M, 16S, 18M, 20S, 21S, 22.5S, 29M
α_2	3L, 4L, 4.2m, 11.5M, 12.5S, 14.5M, 16S, 18M, 27S, 29M
β_2	3L, 4L, 4.2M, 11.5M, 12.5S, 14.5M, 16S, 17.5S, 18M, 29M
β_3	3L, 4L, 4.2M, 4.5S, 10M, 12.5S, 14S, 14.5M, 16S, 18L, 19.5M, 27M, 32M
γ_1	3L, 4L, 4.2M, 4.5S, 5S, 6.5S, 11.5S, 12.5S, 14.5M, 16S, 17.5M, 18M, 29M
γ_2	3L, 4L, 4.2M, 4.5S, 5S, 11.5S, 12.5S, 14.5S, 16S, 18L, 29M
γ_3	3M, 4M, 4.2M, 11.5M, 12.5S, 13.5S, 14.5M, 16S, 18L, 20S, 32M
γ_5	3L, 4L, 4.2M, 4.5M, 7S, 7.5S, 10S, 11.5L, 12.5S, 14S, 14.5M, 16S, 18L, 24.5S, 25.5S, 32S

The purified human-leukocyte interferon species were subjected to amino sugar analysis capable of indentifying amino sugars at the 50-100 pmol level. In all instances glucosamine and galactose/mannose amine was below 1 residue per molecule. In most cases a number of small peptides which eluted near the amino-sugars interfered with the analysis. It is thus possible that even the peaks assigned as amino sugars are at least in part of a peptide nature.

Finally, an attempt to sequence 1 nmol of pure γ_2 interferon by a manual Edman procedure which includes back hydrolysis and amino acid analysis using fluorescamine yielded no amino acids on cycles 1 and 2. Treatment of 100 pmol of pure γ_2 human leukocyte interferon with leucine aminopeptidase and aminopeptidase M for 20 hours at 37°C did not affect the biological activity and no amino acids were detected in the incubation medium. When a supernatant of an induction medium (leukocytes and Newcastle diseases virus in minimal essential medium) was treated with aminopeptidases no loss of interferon activity was observed. This indicates that the interferon molecule has a blocked NH₂-terminus even before the purification procedure. As a control crude interferon as well as pure interferon and the β -chain of insulin were incubated together with aminopeptidase M. While the insulin was partially digested as seen by release of amino acids, no loss of interferon activity was observed.

EXAMPLE 3

In analogy of the procedures of Example 1, pooled bovine leukocytes are prepared, induced to produce bovine interferon and the resulting bovine interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

EXAMPLE 4

In analogy to the procedures of Example 1, pooled porcine leukocytes are prepared, induced to produce porcine interferon and the resulting porcine interferon is purified to homogeneity in the form of distinct species of homogeneous proteins

EXAMPLE 5

In analogy of the procedures of Example 1, pooled ovine leukocytes are prepared, induced to produce ovine interferon and the resulting ovine interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

EXAMPLE 6

In analogy to the procedures of Example 1, pooled equine leukocytes are prepared, induced to produce equine interferon and the resulting equine interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

EXAMPLE 7

In analogy to the procedures of Example 1, pooled canine leukocytes are prepared, induced to produce canine interferon and the resulting canine interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

EXAMPLE 8

In analogy to the procedures of Example 1, pooled feline leukocytes are prepared, induced to produce feline interferon and the resulting feline interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

EXAMPLE 9

In analogy of the procedures of Example 1, pooled primate leukocytes are prepared, induced to produce primate interferon and the resulting primate interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

EXAMPLE 10

(a) A total of 3 mg of homogeneous human leukocyte interferon having a specific activity of 2×10^8 units/mg was dissolved in 25 ml of 5% normal human serum albumin. The solution was passed through a bacteriological filter and the filtered solution aseptically subdivided into 100 vials. Each vial contained 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.

(b) An aqueous solution containing 1.5mg of pooled homogenous interferon species α_1 , α_2 , β_2 , γ_1 and γ_2 each present proportionally to their approximate natural abundance, the pooled mixture having a specific activity of about 2×10^8 units/mg, and 100 mg of normal human serum albumin is passed through a bacteriological filter and the filtered solution aseptically subdivided into 100 vials. Each vial will contain about 3×10^6 units of the pure pooled interferons and 1 mg of human serum albumin. The vials containing interferon suitable for parenteral administration are preferably stored in the cold (-20°C) prior to use.

CLAIMS

1. Interferon, in pure and homogeneous form, and exhibiting a specific activity of $0.9 - 4.0 \times 10^8$ units/mg of protein on MDBK bovine cells and of $2 \times 10^6 - 4.0 \times 10^8$ units/mg of protein on the AG 1732 human cell line.

2. The homogeneous interferon of claim 1 which is human interferon.

3. The homogeneous human interferon of claim 2 which is leukocyte interferon.

4. A species of human leukocyte interferon designated α_1 being a homogeneous protein which is (a) eluted as a single peak at a concentration of 31% (v/v) n-propanol (20-40% gradient) in a 1 M pyridine/2 M formic acid aqueous buffer from an octyl bonded silica matrix HPLC $4.6 \times 250\text{mm}$ column at room temperature and a flow rate of 0.2 ml/min;

(b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M;

(c) providing peptide fragments upon treatment with trypsin, said fragments being eluted as peaks at 3, 4, 4.2, 11.5, 12.5, 14.5, 16, 18, 20, 21, 22.5 and 29% n-propanol when the reaction media is passed through an octyl bonded silica matrix HPLC $4.6 \times 250\text{ mm}$ column ($10\text{ }\mu$ particle size) at room temperature using a 0.03 M pyridine/0.1 M formic acid aqueous buffer (pH 3) at a flow rate of 0.5 ml/min. with a 0 to 40% n-propanol gradient;

and which is further characterized by

(d) a blocked amino terminus;

(e) a specific activity on MDBK (bovine cells) of about 2.6×10^8 units/mg;

(f) a specific activity on Ag 1732 (human line) cells of about 2.6×10^8 units/mg;

(g) a molecular weight of about $16,500 \pm 1000$ by polyacrylamide gel electrophoresis;

(h) an amino sugar content of less than 1 residue per molecule;

(i) a positive growth inhibition activity; and

(j) the following amino acid composition ($\pm 15\%$, based on a molecular weight of 16,500):

**EXAMPLE 5**

In analogy of the procedures of Example 1, pooled ovine leukocytes are prepared, induced to produce ovine interferon and the resulting ovine interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

5

EXAMPLE 6

In analogy to the procedures of Example 1, pooled equine leukocytes are prepared, induced to produce equine interferon and the resulting equine interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

5

10

EXAMPLE 7

In analogy to the procedures of Example 1, pooled canine leukocytes are prepared, induced to produce canine interferon and the resulting canine interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

10

15

EXAMPLE 8

In analogy to the procedures of Example 1, pooled feline leukocytes are prepared, induced to produce feline interferon and the resulting feline interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

15

20

EXAMPLE 9

In analogy of the procedures of Example 1, pooled primate leukocytes are prepared, induced to produce primate interferon and the resulting primate interferon is purified to homogeneity in the form of distinct species of homogeneous proteins.

20

25

EXAMPLE 10

(a) A total of 3 mg of homogeneous human leukocyte interferon having a specific activity of 2×10^8 units/mg was dissolved in 25 ml of 5% normal human serum albumin. The solution was passed through a bacteriological filter and the filtered solution aseptically subdivided into 100 vials. Each vial contained 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.

25

30

(b) An aqueous solution containing 1.5mg of pooled homogenous interferon species α_1 , α_2 , β_2 , γ_1 and γ_2 each present proportionally to their approximate natural abundance, the pooled mixture having a specific activity of about 2×10^8 units/mg, and 100 mg of normal human serum albumin is passed through a bacteriological filter and the filtered solution aseptically subdivided into 100 vials. Each vial will contain about 3×10^6 units of the pure pooled interferons and 1 mg of human serum albumin. The vials containing interferon suitable for parenteral administration are preferably stored in the cold (-20°C) prior to use.

30

35

35

CLAIMS

40

1. Interferon, in pure and homogeneous form, and exhibiting a specific activity of $0.9 - 4.0 \times 10^8$ units/mg of protein on MDBK bovine cells and of $2 \times 10^6 - 4.0 \times 10^8$ units/mg of protein on the AG 1732 human cell line.

40

2. The homogeneous interferon of claim 1 which is human interferon.

45

3. The homogeneous human interferon of claim 2 which is leukocyte interferon.

45

4. A species of human leukocyte interferon designated α_1 being a homogeneous protein which is (a) eluted as a single peak at a concentration of 31% (v/v) n-propanol (20-40% gradient) in a 1 M pyridine/2 M formic acid aqueous buffer from an octyl bonded silica matrix HPLC $4.6 \times 250\text{mm}$ column at room temperature and a flow rate of 0.2 ml/min;

50

(b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M;

50

(c) providing peptide fragments upon treatment with trypsin, said fragments being eluted as peaks at 3, 4, 4.2, 11.5, 12.5, 14.5, 16, 18, 20, 21, 22.5 and 29% n-propanol when the reaction media is passed through an octyl bonded silica matrix HPLC $4.6 \times 250\text{ mm}$ column ($10\text{ }\mu$ particle size) at room temperature using a 0.03 M pyridine/0.1 M formic acid aqueous buffer (pH 3) at a flow rate of 0.5 ml/min. with a 0 to 40% n-propanol gradient;

55

and which is further characterized by

55

(d) a blocked amino terminus;

(e) a specific activity on MDBK (bovine cells) of about 2.6×10^8 units/mg;

(f) a specific activity on Ag 1732 (human line) cells of about 2.6×10^8 units/mg;

60

(g) a molecular weight of about $16,500 \pm 1000$ by polyacrylamide gel electrophoresis;

60

(h) an amino sugar content of less than 1 residue per molecule;

(i) a positive growth inhibition activity; and

(j) the following amino acid composition ($\pm 15\%$, based on a molecular weight of 16,500):



Asx	13.8	Ala	8.4	Phe	6.9	
Thr	7.7	Val	7.4	His	3.0	
5 Ser	9.2	Met	3.7	Lys	10.5	5
Glx	20.3	Ile	7.4	Arg	6.2	
Pro	6.1	Leu	18.0	Cys	3.9	
10 Gly	5.1	Tyr	4.0			10

5. A species of human leukocyte interferon designated α_2 being a homogeneous protein which is
- 15 (a) eluted as a single peak at a concentration of 32% (v/v) n-propanol (20-40% gradient) in a 1 M pyridine/2 M formic acid aqueous buffer from an octyl bonded silica matrix HPLC 4.6 $\times$ 250 mm column at room temperature and a flow rate of 0.2 ml/min; 15
- (b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M;
- (c) providing peptide fragments upon treatment with trypsin, said fragments being eluted as peaks at 3, 4, 20 4.2, 11.5, 12.5, 14.5, 16, 18, 27 and 29% n-propanol when the reaction media is passed through an octyl bonded silica matrix HPLC 4.6 $\times$ 250 mm column (10 μ particle size) at room temperature using a 0.03 M pyridine/0.1 M formic acid aqueous buffer (pH 3) at a flow rate of 0.5 ml/min. with a 0 to 40% n-propanol gradient; 20
- (d) eluted as a single peak at a concentration of 68% (v/v) n-propanol (72.5-50% gradient) in 0.1 M sodium acetate aqueous buffer from a glycerol bonded silica matrix HPLC 4.6 $\times$ 250 mm column at room temperature and a flow rate of 0.25 ml/min; 25
- and which is further characterized by
- (e) a blocked amino terminus;
- (f) a specific activity on MDBK (bovine cells) of about 4.0×10^8 units/mg;
- 30 (g) a specific activity Ag 1732 (human line) cells of about 3×10^8 units/mg; 30
- (h) a molecular weight of about $16,200 \pm 1000$ by polyacrylamide gel electrophoresis;
- (i) an amino sugar content of less than 1 residue per molecule;
- (j) a positive growth inhibition activity; and
- (k) the following amino acid composition ($\pm 15\%$, based on a molecular weight of 16,200): 35
- | | | | | | | |
|--------|------|-----|------|-----|-----|----|
| 35 Asx | 11.7 | Ala | 8.3 | Phe | 8.0 | |
| Thr | 8.3 | Val | 6.2 | His | 2.8 | |
| 40 Ser | 10.0 | Met | 3.8 | Lys | 9.0 | 40 |
| Glx | 20.5 | Ile | 7.0 | Arg | 7.1 | |
| Pro | 4.9 | Leu | 18.0 | Cys | 4.0 | |
| 45 Gly | 4.5 | Tyr | 4.4 | | | 45 |

6. A species of human leukocyte interferon designated β_2 being a homogeneous protein which is
- 50 (a) eluted as a single peak at a concentration of 32% (v/v) n-propanol (20-40% gradient) in a 1 M pyridine/2 M formic acid aqueous buffer from an octyl bonded silica matrix HPLC 4.6 $\times$ 250 mm column at room temperature and a flow rate of 0.2 ml/min; 50
- (b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M;
- (c) providing peptide fragments upon treatment with trypsin, said fragments being eluted as peaks at 3, 4, 55 4.2, 11.5, 12.5, 14.5, 16, 17.5, 18 and 29% n-propanol when the reaction media is passed through an octyl bonded silica matrix HPLC 4.6 $\times$ 260 mm column (10 μ particle size) at room temperature using a 0.03 M pyridine/0.1 M formic acid aqueous buffer (pH 3) at a flow rate of 0.5 ml/min. with a 0 to 40% n-propanol gradient; 55
- (d) eluted as a single peak at a concentration of 66.5% (v/v) n-propanol (72.5-50% gradient) in 0.1 M sodium acetate aqueous buffer from a glycerol bonded silica matrix HPLC 4.6 $\times$ 250 mm column at room temperature and a flow rate of 0.25 ml/min; 60
- and which is further characterized by
- (e) a blocked amino terminus;
- (f) a specific activity on MDBK (bovine cells) of about 4.0×10^8 units/mg;
- 65 (g) a specific activity on Ag 1732 (human line) cells of about 2×10^8 units/mg; 65



- (h) a molecular weight of about $16,500 \pm 1000$ by polyacrylamide gel electrophoresis;
(i) an amino sugar content of less than 1 residue per molecule;
(j) a positive growth inhibition activity; and
(k) the following amino acid composition ($\pm 15\%$, based on a molecular weight of 16,000):

5	Asx	11.5	Ala	7.9	Phe	8.7	5
	Thr	9.0	Val	6.7	His	3.0	
10	Ser	10.0	Met	4.4	Lys	8.5	10
	Glx	21.9	Ile	7.4	Arg	8.0	
	Pro	5.0	Leu	18.9	Cys	1.8	
15	Gly	5.0	Tyr	4.6			15

7. A species of human leukocyte interferon designated β_3 being a protein which is
- 20 (a) eluted as a single peak at a concentration of 32% (v/v) n-propanol (20-40% gradient) in a 1 M pyridine/2 M formic acid aqueous buffer from an octyl bonded silica matrix HPLC 4.6×250 mm column at room temperature and a flow rate of 0.2 ml/min; 20
- (b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M;
- (c) providing peptide fragments upon treatment with trypsin, said fragments being eluted as peaks at 3, 4, 25 4.2, 4.5, 10, 12.5, 14, 14.5, 16, 18, 19.5, 27 and 32% n-propanol when the reaction media is passed through an octyl bonded silica matrix HPLC 4.6×250 mm column (10 μ particle size) at room temperature using a 0.03 M pyridine/0.1 M formic acid aqueous buffer (pH 3) at a flow rate of 0.5 ml/min. with a 0 to 40% n-propanol gradient; 25
- and which is further characterized by
- 30 (d) a blocked amino terminus; 30
- (e) a specific activity on MDBK (bovine cells) of about 4.0×10^8 units/mg;
- (f) a specific activity on Ag 1732 (human line) cells of about 3×10^8 units/mg;
- (g) a major molecular weight band of about $21,000 \pm 1000$ by polyacrylamide gel electrophoresis;
- (h) an amino sugar content of less than 1 residue per molecule;
- 35 (i) positive growth inhibition activity; and 35
- (j) the following amino acid composition

	Asx	18.2	Ala	11.5	Phe	9.9	
40	Thr	9.9	Val	7.5	His	3.8	40
	Ser	14.5	Met	6.0	Lys	9.3	
	Glx	28.5	Ile	9.5	Arg	10.8	
45	Pro	5.9	Leu	24.4	Cys	2.3	45
	Gly	3.6	Tyr	5.0			

- 50 8. A species of human leukocyte interferon designated γ_1 being a homogeneous protein which is 50
- (a) eluted as a single peak at a concentration of 31% (v/v) n-propanol (20-40% gradient) in a 1 M pyridine/2M formic acid aqueous buffer from an octyl bonded silica matrix HPLC 4.6×250 mm column at room temperature and a flow rate of 0.2 ml/min;
- 55 (b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M; 55
- (c) providing peptide fragments upon treatment with trypsin, said fragments being eluted as peaks at 3, 4, 4.2, 4.5, 5, 6.5, 11.5, 12.5, 14.5, 16, 17.5 18 and 29% n-propanol when the reaction media is passed through an octyl bonded silica matrix HPLC 4.6×250 mm column (10 μ particle size) at room temperature using a 0.03 M pyridine/0.1 M formic acid aqueous buffer (pH 3) at a flow rate of 0.5 ml/min. with a 0 to 40% n-propanol gradient; and which is further characterized by 60
- 60 (d) a blocked amino terminus; 60
- (e) a specific activity on MDBK (bovine cells) of about 2.6×10^8 units/mg;
- (f) a specific activity of Ag 1732 (human line) cells of about 2×10^8 units/mg;
- (g) a molecular weight of about $17,700 \pm 1000$ by polyacrylamide gel electrophoresis;
- 65 (h) an amino sugar content of less than 1 residue per molecule; 65



- (ii) a positive growth inhibition activity; and
 (j) the following amino acid composition ($\pm 15\%$, based on a molecular weight of 17,700):

Asx	13.1	Ala	8.7	Phe	8.6	
Thr	8.4	Val	7.3	His	3.7	5
Ser	10.2	Met	4.3	Lys	10.1	
10 Glx	23.9	Ile	7.9	Arg	8.0	10
Pro	4.5	Leu	20.3	Cys	3.3	
Gly	5.7	Tyr	4.8			15

9. A species of human leukocyte interferon designated γ_2 being a homogeneous protein which is
 (a) eluted as a single peak at a concentration of 32% (v/v) n-propanol (20-40% gradient) in a 1 M pyridine/2 M formic acid aqueous buffer from an octyle bonded silica matrix HPLC 4.6 $\times$ 250 mm column at room temperature and a flow rate of 0.2 ml/min;
 (b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M;
 (c) providing peptide fragments upon treatment with trypsin, said fragments being eluted as peaks at 3,4, 4.2, 4.5, 5, 11.5, 12.5, 14.5, 16, 18 and 29% n-propanol when the reaction media is passed through an octyl bonded silica matrix HPLC 4.6 $\times$ 250 mm column (10 μ particle size) at room temperature using a 0.03 M pyridine/0.1 M formic acid aqueous buffer (pH 3) at a flow rate of 0.5 ml/min. with a 0 to 40% n-propanol gradient;
 and which is further characterized by
 (d) a blocked amino terminus;
 (e) a specific activity on MDBK (bovine cells) of about 4.0×10^8 units/mg;
 (f) a specific activity on Ag 1732 (human line) cells of about 1.5×10^8 units/mg;
 (g) a molecular weight of about 17,700 $\pm$ 1000 on polyacrylamide gel electrophoresis;
 (h) an amino sugar content of less than 1 residue per molecule;
 (i) a positive growth inhibition activity; and
 (j) the following amino acid composition ($\pm 15\%$, based on a molecular weight of 17,700):

Asx	13.3	Ala	8.1	Phe	9.0	
Thr	9.6	Val	7.0	His	3.3	
40 Ser	7.8	Met	3.9	Lys	10.0	40
Glx	25.0	Ile	8.0	Arg	8.5	
Pro	4.8	Leu	20.1	Cys	2.9	
45 Gly	5.0	Tyr	4.8			45

10. A species of human leukocyte interferon designated γ_3 being a protein which is
 (a) eluted as a single peak at a concentration of 34% (v/v) n-propanol (20-40% gradient) in a 1 M pyridine/2 M formic acid aqueous buffer from an octyl bonded silica matrix HPLC 4.6 $\times$ 250 mm column at room temperature and a flow rate of 0.2 ml/min;
 (b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M;
 (c) providing peptide fragments upon treatment with trypsin, said fragments being eluted as peaks at 3, 4, 4.2, 11.5, 12.5, 13.5, 14.5, 16, 18, 20 and 32% n-propanol when the reaction media is passed through an octyl bonded silica matrix HPLC 4.6 $\times$ 250 mm column (10 μ particle size) at room temperature using a 0.03 M pyridine/0.1 M formic acid aqueous buffer (pH 3) at a flow rate of 0.5 ml/min. with a 0 to 40% n-propanol gradient;
 and which is further characterized by
 (d) a blocked amino terminus;
 (e) a specific activity on MDBK (bovine cells) of about 3.5×10^8 units/mg;
 (f) a specific activity on Ag 1732 (human line) cells of about 1.5×10^7 units/mg;
 (g) a major molecular weight band of about 17, 200 $\pm$ 1000 on polyacrylamide gel electrophoresis;
 (h) an amino sugar content of less than 1 residue per molecule;
 (i) a positive growth inhibition activity; and



(j) the following amino acid composition:

Asx	15.0	Ala	9.3	Phe	7.2	
5 Thr	8.6	Val	5.5	His	2.9	5
Ser	10.1	Met	5.6	Lys	7.6	
Glx	22.8	Ile	6.8	Arg	10.1	
10 Pro	4.8	Leu	20.5	Cys	3.1	10
Gly	3.2	Tyr	3.7			

- 15 11. A species of human leukocyte interferon designated γ_4 being a homogeneous protein which is
- (a) eluted as a single peak at a concentration of 35% (v/v) n-propanol (20-40% gradient) in a 1 M pyridine/2 M formic acid aqueous buffer from an octyl bonded silica matrix HPLC 4.6 $\times$ 250 mm column at room temperature and a flow rate of 0.2 ml/min;
- 20 (b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M; and which is further characterized by
- (c) a blocked amino terminus;
- (d) a specific activity on MDBK (bovine cells) about 3.5×10^8 units/mg;
- (e) a specific activity on Ag 1732 (human line) cells of about 4.0×10^8 units/mg;
- 25 (f) a molecular weight of about 21,000 $\pm$ 1000 on polyacrylamide gel electrophoresis;
- (g) an amino sugar content of less than 1 residue per molecule;
- (h) a positive growth inhibition activity; and
- (i) the following amino acid composition ($\pm$ 15%, based on a molecular weight of 21,000):

30 Asx	17.9	Ala	10.4	Phe	9.1	30
Thr	7.3	Val	7.9	His	3.8	
35 Ser	13.5	Met	4.9	Lys	12.2	35
Glx	27.0	Ile	9.7	Arg	8.5	
Pro	6.5	Leu	24.1	Cys	4.1	
40 Gly	4.8	Tyr	5.0			40

12. A species of human leukocyte interferon designated γ_5 being a protein which is
- 45 (a) eluted as a single peak at a concentration of 35.5% (v/v) n-propanol (20.40% gradient) in a 1 M pyridine/2 M formic acid aqueous buffer from an octyl bonded silica matrix HPLC 4.5 $\times$ 250 mm column at room temperature and a flow rate of 0.2 ml/min;
- (b) resistant to deactivation by treatment with leucine aminopeptidase and aminopeptidase M;
- (c) providing peptide fragments upon treatment with trypsin, said fragments being eluted as peaks at 3, 4, 50 4.2, 4.5, 7, 7.5, 10, 11.5, 12.5, 14, 14.5, 16, 18, 24.5, 25.5 and 32% n-propanol when the reaction media is passed through an octyl bonded silica matrix HPLC 4.6 $\times$ 250 mm column (10 μ particle size) at room temperature using a 0.03 M pyridine/0.1 M formic acid aqueous buffer (pH 3) at a flow rate of 0.5 ml/min. with a 0 to 40% n-propanol gradient; and which is further characterized by
- 55 (d) a blocked amino terminus;
- (e) a specific activity on MDBK (bovine cells) of about 0.9×10^8 units/mg;
- (f) a specific activity on Ag 1732 (human line) cells of about 2×10^8 units/mg;
- (g) having a major molecular weight band of about 16,500 $\pm$ 1000 on polyacrylamide gel electrophoresis;
- (h) an amino sugar content of less than 1 residue per molecular;
- 60 (i) a positive growth inhibition activity; and
- (j) the following amino acid composition:



Asx	13.8	Ala	9.3	Phe	6.4	
Thr	7.6	Val	5.1	His	2.8	
5 Ser	9.0	Met	4.7	Lys	12.0	5
Glx	20.8	Ile	6.6	Arg	9.0	
Pro	4.2	Leu	19.6	Cys	2.3	
10 Gly	4.0	Tyr	3.6			10

13. A homogeneous interferon as claimed in any one of claims 1 to 12 for use as a pharmaceutically active compound. 15
14. A homogeneous interferon as claimed in any one of claims 1 to 12 for use as an antiviral agent.
15. A homogeneous interferon as claimed in any one of claims 1 to 12 for use as an antitumor agent.
16. A homogeneous interferon as claimed in any one of claims 1 to 12 for use as a growth inhibiting agent.
- 20 17. A homogeneous interferon as claimed in any one of claims 1 to 12 for use as an immunosuppressive agent. 20
18. A process for purifying proteins having molecular weights greater than about 12,000 which process comprises passing an aqueous solution of said protein in an impure state through a buffer equilibrated column of a porous silica matrix to which cyanopropyl, cyclohexyl, phenyl, octyl, octadecyl or glyceryl groups are bonded under high performance liquid chromatography (HPLC) conditions so as to absorb said protein onto said column and thereafter eluting said protein with an increasing or decreasing gradient of an aqueous water miscible solvent and obtaining said protein in selected fractions of said eluate in a state of enhanced purity. 25
19. A process for purifying proteins having molecular weights greater than about 12,000 which process comprises passing an aqueous solution of said protein in an impure state through a buffer equilibrated column of a porous silica matrix to which octyl or glyceryl groups are bonded under HPLC conditions so as to absorb said protein onto said column and thereafter eluting said protein from said column with an increasing or decreasing gradient of an aqueous water miscible solvent and obtaining said protein in selected fractions of said eluate in a state of enhanced purity. 30
20. A process for producing interferon as a homogeneous protein which process comprises the following steps in combination: 35
- A) passing an aqueous solution of interferon in an impure state through a buffer equilibrated octyl bonded silica matrix column under high performance liquid chromatography conditions so as to absorb said interferon onto said column and thereafter eluting said interferon from said column with an increasing gradient of an aqueous, buffered, water miscible solvent and obtaining said interferon in selected fractions of said eluate in a state of enhanced purity; 40
- B) passing the selected interferon fractions obtained in Step A through a buffer equilibrated glyceryl bonded silica matrix column to absorb said interferon onto said column and thereafter eluting said interferon from said column with a decreasing gradient of an aqueous buffered water miscible solvent and obtaining interferon as distinct major peaks in selected fractions of said eluate in a state of enhanced purity; 45
- C) passing the selected fractions of interferon corresponding to one of said distinct major peaks obtained in Step B in buffered solution through a buffer equilibrated octyl bonded silica matrix column under high performance liquid chromatography conditions so as to absorb said interferon onto said column and thereafter eluting said interferon from said column with an aqueous, buffered, water miscible solvent and obtaining interferon as a single distinct peak in selected fractions of said eluate in the state of a homogeneous protein and, if desired, repeating the procedure of Step C to achieve ultimate homogeneity. 50
21. The process of claim 20 wherein said interferon is a human interferon.
22. The process of claim 21 wherein said human interferon is a human leukocyte interferon.
23. The process of claim 22 wherein said water miscible solvent is selected from an alkanol or a cyclic ether. 55
24. The process of claim 23 wherein said water miscible solvent is n-propanol, said buffer in Step A provides a pH of about 7.5, and said buffer in Step C provides a pH of about 4.0.
25. The process of claim 24 wherein said buffer in Step A is 1 M sodium acetate/acetic acid and said n-propanol gradient is increased from 0 to 40% (v/v), said buffer in Step B is 0.1 M sodium acetate and said n-propanol gradient is decreased from 72.5 to 50% (v/v), said buffer in Step C is 1 M pyridine 2 M formic acid and said n-propanol gradient is increased from 20 to 40% (v/v). 60
26. The process of claim 25 wherein the selected peak fractions from Step B are pooled, the n-propanol is removed by extraction with n-hexane and the traces of n-hexane are removed from the aqueous phase prior to proceeding to Step C.
- 65 27. A pharmaceutical preparation containing a homogeneous interferon as claimed in any one of claims 65



- 1 to 12 and a non-toxic, inert, therapeutically compatible solid or liquid carrier.
28. An antiviral preparation containing a homogeneous interferon as claimed in any one of claims 1 to 12 and a non-toxic, inert, therapeutically compatible solid or liquid carrier.
29. An antitumor preparation containing a homogeneous interferon as claimed in any one of claims 1 to 12 and a non-toxic, inert, therapeutically compatible solid or liquid carrier.
- 5 30. A growth inhibiting preparation containing a homogeneous interferon as claimed in any one of claims 1 to 12 and a non-toxic, inert, therapeutically compatible solid or liquid carrier.
- 5 31. An immunosuppressive preparation containing a homogeneous interferon as claimed in any one of claims 1 to 12 and a non-toxic, inert, therapeutically compatible solid or liquid carrier.