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Purification of recombinant human interferon-β by immobilized antisense peptides

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ABSTRACT

Synthetic antisense peptides encoded in the antisense strands of DNA corresponding to the 1–14, 42–54 and 103–115 fragments of the human interferon-β sequence were applied in the purification of recombinant human interferon-β from a mammalian cell culture. The protein fragments were selected on the basis of their computer-predicted exposure on the surface of the protein. The antisense peptides were synthetized by the solid-phase method directly on the resin used as the stationary phase in affinity chromatography. All the tested antisense peptides showed a selective affinity for human interferon-β, permitting a ten-fold purification of the protein.

INTRODUCTION

Several studies have been already reported on the affinity between peptides encoded by the antisense strands of DNA, named antisense (AS) peptides, and those coded by the corresponding sense strands. These include peptides complementary to adrenocorticotropic hormone [1,2], ribonuclease S peptide [3], γ-endorphin [1], substance P [4], lutein izing hormone-releasing hormone (LH–RH) [5], anglotensin II [6], interleukin-2 [7], fibronectin [8], insulin [9] and glycoprotein GPIIa [10]. As the interaction between peptides and the corresponding AS-peptides is selective, their use in affinity technology has been proposed. AS-peptides immobilized on chromatographic supports have been employed to isolate native polypeptides, such as the Arg^8-vasopressin–bovine neurophisin II biosynthetic precursor [11], recombinant c-raf protein [12] and the Arg^8-vasopressin–receptor complex [13].

Human interferon-β (hIFN-β) is a glycoprotein of 166 amino acid residues, secreted by human primary fibroblasts after induction with virus or double-stranded RNA. Alternatively, hIFN-β can be obtained by recombinant bacterial or mammalian cell cultures [14–16]. The interest in this protein is connected with its therapeutic value in viral diseases and against certain types of tumours such as brain tumours and malignant melanomas [17,18]. The purification of hIFN-β from various sources has been attempted by a variety of methods over many years and some procedures have been proposed that yield a homogeneous protein [19–23].

To evaluate the potential of AS peptides in affinity chromatography, we applied this approach in the purification of hIFN-β from a recombinant Chinese hamster overay cell line (CHO-rhIFN-β). Fragments 1–14, 42–54 and 103–115 of the hIFN-β sequence were chosen on the basis of their computer-predicted exposure on the surface of the protein [24]. More precisely, the sequences 42–54 and 103–115 were selected for their highly hydrophilic character deduced from the hydropathic profile [25]. Sequence 1–14 was selected because in the Chou–Fasman-predicted secondary structure [26] it contains a β-turn between two β-sheet strands, which is frequently found in exposed regions of proteins [24].

The sequences of the AS-IFN peptides, AS-IFN 1–14, AS-IFN 42–54 and AS-IFN 103–115, shown

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hIFN-β 1-14

H-Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser-Asn-OH

AS-IFN 1-14

H-Ile-Ala-Ala-Ser-Leu-Gln-Glu-Ser-Lys-Gln-Val-Val-Ala-His-OH

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hIFN-β 42-54

H-Glu-Glu-Ile-Lys-Gln-Leu-Gln-Gln-Phe-Gln-Lys-Glu-Asp-OH

AS-IFN 42-54

H-Val-Leu-Leu-Leu-Glu-Leu-Leu-Gln-Leu-Leu-Asp-Leu-Leu-OH

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hIFN-β 103-115

H-Glu-Glu-Lys-Leu-Glu-Lys-Glu-Asp-Phe-Tyr-Arg-Gly-Lys-OH

AS-IFN 103-115

H-Phe-Ser-Pro-Gly-Glu-Ile-Phe-Phe-Leu-Gln-Phe-Phe-Phe-OH

Fig. 1. Amino acid sequences of the hIFNβ fragments 1–14, 42–54 and 103–115 and of the corresponding AS-IFN peptides.

in Fig. 1 were specified by DNA complementary to the DNA coding the corresponding hIFN-β fragments. The nucleotide sequences of the gene encoding hIFN-β was obtained from the λEMBL4 library of the partial Sau3A fragment of the human lymphoblastoid cell line GM 1416.

The AS-IFN peptides were synthesized by the solid-phase method on a resin, which was used directly as the stationary phase without detaching the peptides, thus avoiding expensive and time-consuming procedures for peptide isolation and immobilization [11–13].

The results achieved in purification with the AS-IFN peptides were evaluated and compared with those obtained with a monoclonal antibody anti-hIFN-β column. An attempt was made to demonstrate the selectivity of the interaction between AS-IFN peptides and CHO-rhIFN-β.

EXPERIMENTAL

Materials

CHO-rhIFN-β produced in recombinant CHO cell culture at a concentration of 0.5 μg/ml with a specific activity of $1.2 \cdot 10^5$ I.U./mg [27] was purified 50-fold by controlled-pore glass (CPG) chromatography [28] and extensively dialysed against 0.125 *M* NaCl–0.1 *M* sodium phosphate buffer (pH 7) [29].

The final solution contained CHO-rhIFN-β with a specific activity of $6 \cdot 10^6$ I.U./mg with a concentration of $3 \cdot 10^6$ I.U./ml, corresponding to 10 μg/ml of IFN-β.

The chromatographic supports containing the immobilized AS-peptides AS-IFN 1–14, AS-IFN 42–54 and AS-IFN 103–115, called R-AS 1–14, R-AS 42–54 and R-AS 103–115, respectively, were prepared on a Biolynx 4175 peptide manual synthesizer, using the standard continuous-flow 9-fluorenylmethoxycarbonyl–polyamide strategy [30] and commercial Ultrosyn resin with 0.1 mequiv./g of sarcosine (Pharmacia–LKB). The resin was activated with 1,6-diaminohexane (Aldrich) prior to use. Details of the synthesis have been described elsewhere [31].

The AS-IFN peptide resins were swollen in dimethylformamide (DMF), then equilibrated with solutions of DMF–water (80:20, 50:50 and 20:80, v/v) and finally conditioned with 0.125 *M* NaCl–0.1 *M* sodium phosphate buffer (pH 7).

Monoclonal antibodies against hIFN-β (mAb-IFN) were purchased from CellTech and purified on Protein A–Sepharose 4 FF (Pharmacia).

Citric acid, diammonium sulphate, disodium hydrogenphosphate, sodium chloride, DMF and triethylamine (TEA) were obtained from Carlo Erba. Acetonitrile (ACN, HPLC grade) and trifluoroacetic acid (TFA) were supplied by Merck. Water was purified with a Milli-Q system (Millipore).

Aqueous solutions were filtered through a 0.45-μm cellulose acetate filter and organic solutions through a 0.5-μm PTFE filter. All the eluents were degassed by purging with helium prior to use.

Apparatus

All the chromatographic experiments were carried out on a Pharmacia apparatus consisting of a Model P-500 pump, a Model MV-7 injection valve equipped with a Super Loop (10 ml), a Model UV-1 monitor, a Model Rec-1 recorder and a Model Frac-100 fraction collector. The eluents were connected to the pump through a Model MV-8 eight-way valve. Samples were introduced into the Super Loop by a Model P-1 peristaltic pump. All the components were controlled by a Model LCC-500 controller.

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a BakerBond Wide-Pore octadecyl column (100 × 4.5 mm I.D., 5-μm particle size) (Baker) and using an HPLC system assembled from two Model 114M pumps, a Model 420 System Organizer, a Model 163 variable-wavelength detector (Beckman) and a Model 7700 Professional computer for data acquisition and reporting (Perkin-Elmer).

Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on Phast System apparatus on precast gels (Phast Gel gradient 10–15, Pharmacia). The electrophoretic gels were evaluated on a Model Ultro-Scan XL laser densitometer (LKB).

Amino acid analyses were performed on an Alpha-Plus amino acid analyser (LKB).

Methods

The IFN-β antiviral activity was measured by the method of cytophathic effect inibition [32]. Protein mass was determined by the Lowry method [33]. mAb-IFN were immobilized on cyanogen bromide-activated Sepharose 4B, following the procedure described by Pharmacia.

The stationary phases R-AS 1–14, R-AS 42–54, R-AS 103–115, Ultrobsyn, DEAE-Sepharose FF and mAb-IFN Sepharose 4B were packed in 100 × 5 mm I.D. columns and conditioned with 0.125 *M* sodium chloride–0.1 *M* sodium phosphate buffer (pH 7.0). All the columns were run under similar chromatographic conditions. In a typical experiment, after having loaded 30 ml of the CHO-rhIFN-β solution, the columns were washed with ten column volumes of 0.125 *M* sodium chloride–0.1 *M* sodium phosphate buffer (pH 7.0), then the adsorbed products were eluted with ten column volumes of 0.1 *M* citric acid (pH 2.0). In the run with human serum albumin (HSA), 3 ml of a 0.1 mg/ml solution were loaded.

In the cation-exchange experiment a prepacked Mono-Q 50 × 5 mm I.D. column (Pharmacia) was used under the above conditions. In all the chromatographic runs the linear velocity was 0.6 cm/min.

Preparative purification of CHO-rhIFN-β by RP-HPLC was carried out at a flow-rate of 1 ml/min on the above RP column. A 30-ml volume of CHO-rhIFN-β solution was loaded on the column, previously conditioned with 30 m*M* TEA–TFA (pH 7.0), then the column was washed with 30 ml of 30

m*M* TEA–TFA in ACN–water (52:48) at pH 7.0 and finally it was eluted with 0.1% TFA in ACN–water (52:48).

RESULTS

The RP-HPLC and SDS-PAGE profiles of the crude solution of CHO-rhIFN-β are shown in Fig. 2. The proportion of CHO-rhIFN-β in the total protein content was about 8%, calculated from the SDS-PAGE profile.

The amino acid analysis of the AS-peptide resins R-AS 1–14, R-AS 42–54 and R-AS 103–115, reported in Table I, gave a purity of the synthesized peptide of *ca.* 90%.

As it has been reported that hydropathic complementary might provide the driving forces in the recognition between sense and antisense peptides [12, 34–36], in Fig. 3 the hydrophatic profiles of AS-IFN peptides are compared with the native hIFN-β sequences.

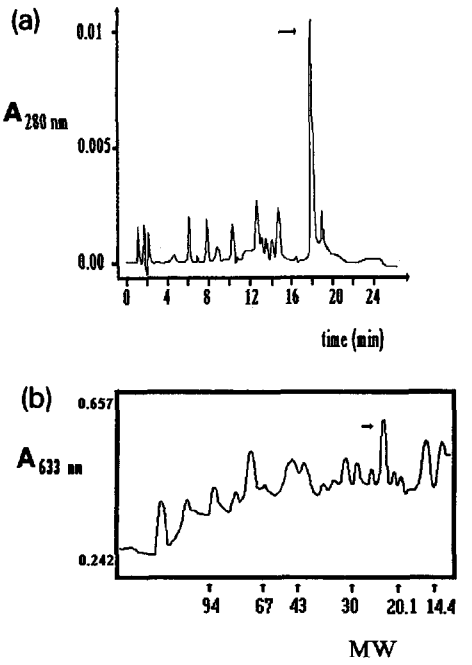

Fig. 2. Chromatographic profiles of crude CHO-rhIFN-β solution. (a) RP-HPLC analysis. Column, 100 × 4.6 mm I.D. Baker-Bond Wide-Pore ODS. Eluents: (A) 0.1% TFA in ACN–water (5:95); (B) 0.1% TFA in ACN–water (95:5). Linear gradient of B from 20% to 55% in 15 min and then 55% for 3 min. Flow-rate, 1 ml/min. Sample, 20 μml of the ten-fold concentrated solution. (b) SDS-PAGE gel scan. Sample, 1 μl of the ten-fold concentrated solution. CHO-rhIFN-β peaks indicated by arrows; MW = molecular weight in kilodalton.

TABLE I

AMINO ACID ANALYSIS OF THE AS-IFN STATIONARY PHASES

Hydrolysis was carried out with 6 *M* HCl at 110°C for 18 h. Theoretical values in parentheses.

R-AS 1–14	R-AS 42–54	R-AS 103–115
His 0.94 (1)	Leu 8.30 (9)	Phe 5.70 (6)
Ala 3.00 (3)	Asp 1.00 (1)	Gix 2.13 (2)
Val 0.86 (1)	Glx 2.21 (2)	Leu 1.00 (1)
Glx 3.35 (3)	Val 0.90 (1)	Ile 0.97 (1)
Lys 1.11 (1)		Gly 9.90 (1)
Ser 1.83 (2)		Pro 0.91 (1)
Leu 0.95 (1)		Ser 0.94 (1)
Ile 0.90 (1)		

The AS-IFN stationary phases gave similar chromatographic behaviours and an elution profile of a representative experiment for the purification of CHO-rhIFN-β on the AS-IFN columns is reported in Fig. 4. As can be seen, almost all the protein material was eluted from the column during the loading of the sample. The amount of CHO-rhIFN-β loaded was chosen to exceed the saturation level of the AS-IFN stationary phases, thus making possible the calculation of the binding capacities. The adsorbed CHO-rhIFN-β was eluted from the column with 0.1 *M* citric acid and no further material was eluted with 1 *M* citric acid.

The chromatographic yields and the CHO-rhIFN-β purity obtained in these runs are summarized in Table II, and in Fig. 5 the scans of the SDS-PAGE gels of the peaks collected at pH 2 are reported. With the stationary phase R-AS 42–54, an increase in the sodium chloride concentration in the wash step to 0.5 *M* slightly reduced the binding capacity to $1.1 \cdot 10^7$ I.U./ml with a specific activity of $6.7 \cdot 10^7$ I.U./mg.

When the AS-IFN columns were replaced with a DEAE-Sepharose FF or Mono-Q column, and tested under the same chromatographic conditions, no adsorption of CHO-rhIFN-β was detected. The same result was obtained when the AS-IFN columns were replaced with the unfunctionalized Ultrosyn column. In addition, no adsorption was

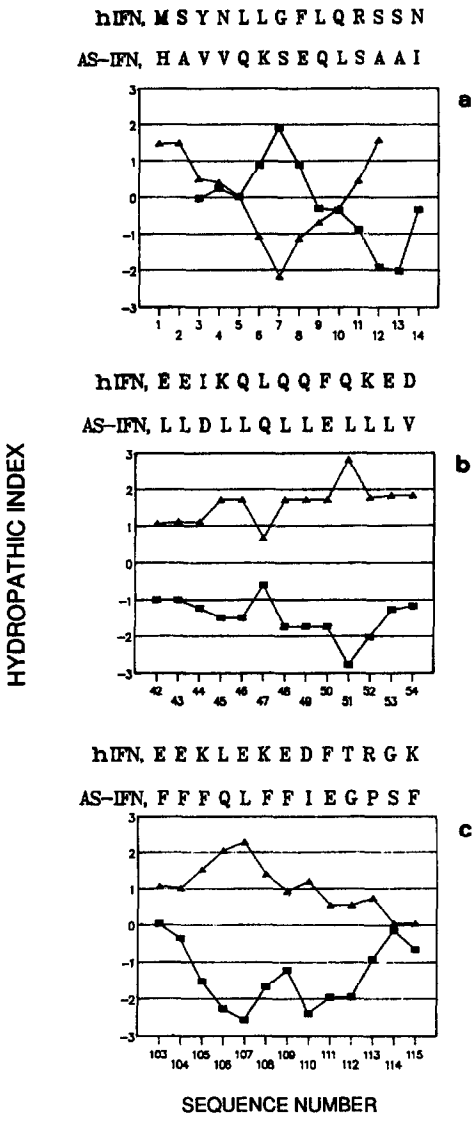

Fig. 3. Comparison of the hydrophatic profiles of (■) hIFN fragments with those of the (▲) AS-IFN peptides. (a) hIFN-β 1–14 and AS-IFN 1–14. (b) hIFN-β 42–54 and AS-IFN 42–54. (c) hIFN-β 103–115 and AS-IFN 103–115. The best complementarity is obtained with the AS-IFN peptides laid down in opposite alignment to the hIFN-β sequences. The hydrophatic indices used are those proposed by Kyte and Doolittle [37].

found when HSA was loaded on the R-AS 42–54 column.

In RP-HPLC, CHO-rhIFN-β was completely retained at pH 7.0 with 30 m*M* TEA–TFA in any ACN gradient, whereas it was eluted with 0.1% TFA at pH 2 in an ACN gradient (see Fig. 2).

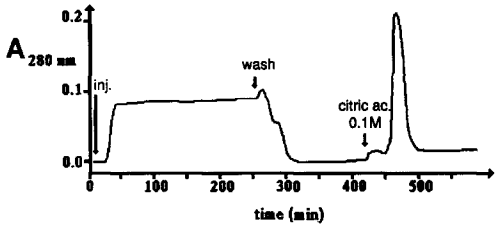

Fig. 4. Elution profile of the preparative purification of CHO-rhIFN-β obtained with R-AS 42–54. Similar elution profiles were obtained with the other AS-IFN stationary phases.

Fig. 6 shows the profile of the preparative purification of CHO-rhIFN-β on the RP column. In the inset the gel scan of the collected material at pH 2 is shown.

An outline of the purification of CHO-rhIFN-β obtained with the mAb-IFN column is reported in Fig. 7; the inset shows the gel scan of the peak collected at pH 2. Purified CHO-rhIFN-β was collected with a specific activity of $1 \cdot 10^8$ I.U./mg and a purity of 82%. The binding capacity was $3.4 \cdot 10^7$ I.U./ml.

A comparison of the RP-HPLC analysis of the purified material obtained with the R-AS 42–54 stationary phase and mAb-IFN Sepharose 4B stationary phase is reported in Fig. 8.

DISCUSSION

The tenfold purification of biologically active CHO-rhIFN-β achieved with each of the AS-IFN stationary phases, R-AS 1–14, R-AS 42–54 and R-AS 103–115, confirms the selective recognition of sense peptides by AS peptides and their useful application in affinity chromatography.

In an attempt to recognize which mechanism is mainly responsible for the interaction of AS-IFN

TABLE II

AS-IFN CHROMATOGRAPHY

Stationary phase	Binding capacity (I.U./ml)	Specific activity (I.U./ml)	IFN-β purity (%)
R-AS 1–14	$9.2 \cdot 10^6$	$6.6 \cdot 10^7$	84.3
R-AS 42–54	$1.4 \cdot 10^7$	$6.4 \cdot 10^7$	86.8
R-AS 103–115	$5.2 \cdot 10^6$	$4.5 \cdot 10^7$	83.2

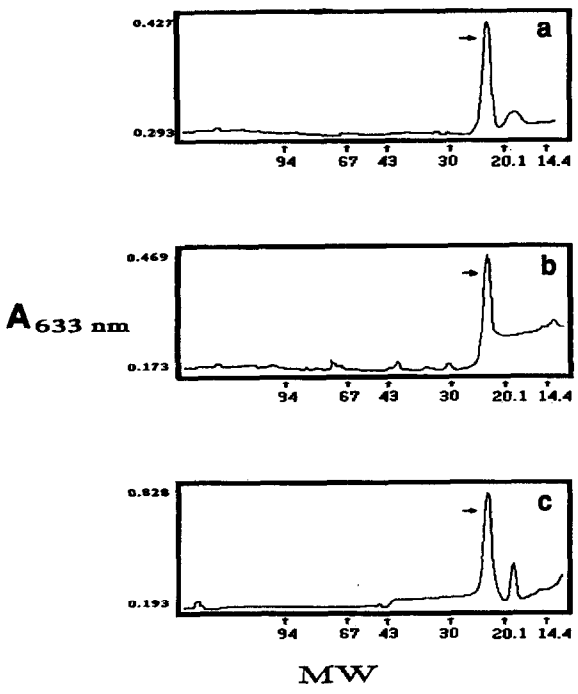

Fig. 5. Gel scans of the peaks collected at pH 2 in AS-IFN chromatography. (a) AS-IFN 1–14; (b) AS-IFN 42–54; (c) AS-IFN 103–115. CHO-rhIFN-β peaks indicated by arrows; MW = molecular weight in kilodalton.

peptides with CHO-rhIFN-β, coulombic attraction, dipolar and/or hydrogen bonding interactions and hydrophobic interaction were evaluated.

The chromatographic behaviour of CHO-rhIFN-β on the AS-IFN columns cannot be explained by a predominant ion-exchange interaction,

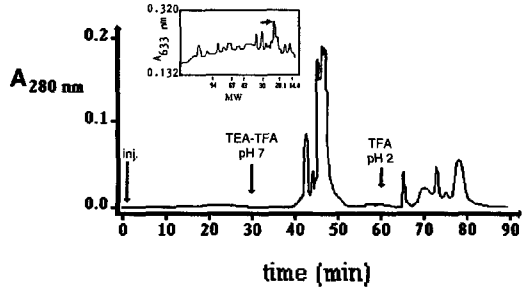

Fig. 6. RP-HPLC profile of the crude CHO-rhIFN-β purification. Inset: gel scan of the peak collected at pH 2 (CHO-rhIFN-β peak indicated by arrow; MW = molecular weight in kilodalton).

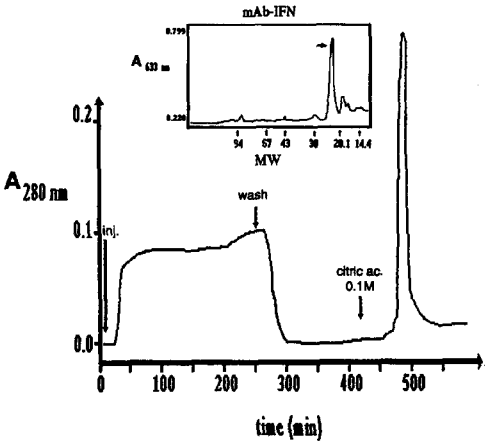

Fig. 7. Profile of the crude CHO-rhIFN-β purification on mAb-IFN column. Inset: gel scan of the peak collected at pH 2 (CHO-rhIFN-β peak indicated by arrow; MW = molecular weight in kilodalton).

as an increase in the ionic strength of the eluents only slightly reduced the binding capacity without affecting the purity of the collected material, and on replacement of the AS-IFN columns with cation- or anion-exchange columns not detectable adsorption

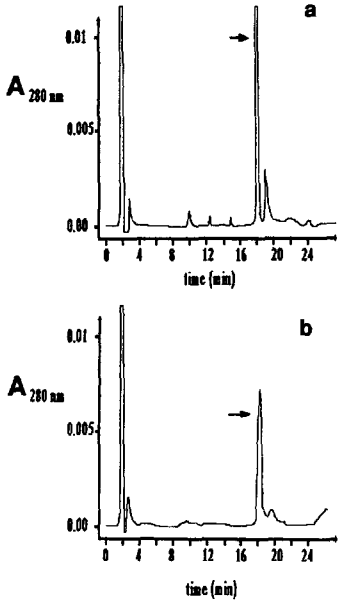

Fig. 8. RP-HPLC analysis of purified CHO-rhIFN-β (peaks indicated by arrows) obtained with (a) R-AS 42–54 and (b) mAb-IFN Sepharose 4B. Conditions as in Fig. 2.

of CHO-rhIFN-β was observed. The purification of CHO-rhIFN-β cannot be related to any non-specific peptide–protein interaction because, for example, HSA was not adsorbed when loaded on R-AS 42–54 stationary phase. Dipolar and/or hydrogen bonding interactions can also be excluded as the main mechanism accounting for the selective adsorption of CHO-rhIFN-β on the AS-IFN stationary phases. Indeed, all the proteins present in the CHO-rhIFN-β solution came from a CPG purification, where they were eluted at pH 2, after being retained at neutral pH. In this kind of chromatographic technique the dipolar and hydrogen bonding interactions have been recognized as making the main contribution to rhIFN-β adsorption [38]. Therefore, the same type of interaction that produced a CHO-rhIFN-β with a purity of 8% in the CPG chromatography cannot justify alone the selectivity obtained in the AS-IFN chromatography.

A hydrophobic interaction could be hypothesized considering the CHO-rhIFN-β behaviour in RP-HPLC at pH 7.0 and 2.0. However, the roughly similar chromatographic performances obtained with the AS-IFN peptides in contrast to their different hydrophobic characters, whose values obtained by summing the hydrophatic contributions of each amino acid are AS-IFN 1–14 +2.9, AS-IFN 42–54 +27.6 and AS-IFN 103–115 + 15.3 and the poor selectivity obtained with the RP purification, also precludes any predominant role of this kind of interaction.

Finally, considering that non-specific adsorptions with the solid-phase synthesis matrix are also absent, we can conclude that only a multi-modal and sequence-dependent binding mechanism is responsible for the interaction of CHO-rhIFN-β with the AS-IFN peptides. This conclusion is supported by the similar chromatographic behaviours of CHO-rhIFN-β with the AS-IFN peptides and mAb-IFN, even though the latter instance a higher binding capacity was achieved.

It is worth noting that the main impurities, still present after the As-IFN chromatography, have molecular weights lower than that of CHO-rhIFN-β and similar products are also present after the mAb-IFN column, thus suggesting possible IFN-β fragments and confirming the specificity of the interaction of the AS-IFN peptides with hIFN-β sequences.

The good hydropathic complementary of the AS-IFN peptides and the corresponding native hIFN-β fragments does not exclude that this could be the reason of the interaction, leaving the possibility of improving the purification performance by optimizing their hydrophatic complementary.

Further, the results demonstrate that it is possible to localize portions of proteins that could interact with properly designed AS-peptides, following the computer-assisted approach previously applied in the search for immunodominant sequences. We believe that the possibility of using as a stationary phase the peptides attached to the solid support used in the synthesis makes the AS-peptides approach more feasible, as it permits rapid screenings of many sequences. Nevertheless, once proper peptides and sequences have been recognized, it would be advisable to use less expensive and more suitable chromatographic supports especially when large-scale purification is needed.

Finally, it could be significant for the explanation of the specific interaction that the best chromatographic performance was obtained with AS-IFN 42–54, *i.e.*, a peptide that should interact with the hIFN-β region where the active site is assumed to be located [39].

CONCLUSIONS

Immunoaffinity chromatography is a very versatile method in protein purification. However, in industrial applications the use of this technique is hampered by the cost of antibodies and by the need to validate the process, where the problems come from the biological sources of antibodies and by their leakage into purified products. Therefore, affinity techniques with synthetic ligands which exhibit selectivity and a wide range of applications similar to immunoaffinity chromatography are desirable.

In this work, it has been demonstrated that AS peptides can be usefully applied as ligands in the purification of a recombinant protein with performances similar to those obtained with a monoclonal antibody column. The use of AS-peptide columns in tandem could improve the purification performance, whereas the proteic sequences of the impurities contain only one biding site among those which chould interact with the chosen AS-peptide station-

ary phases. The possibility of deducing the correct AS peptide sequences only on the basis of the sequence of proteins to be purified makes this approach general and permits a wide application to a number of known proteins.

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