ION EXCHANGE CHROMATOGRAPHY FOR THE PURIFICATION OF RECOMBINANT HUMAN ALPHA-2B INTERFERON. COMPARATIVE STUDY OF MEDIUM AND HIGH RESOLUTION GELS

✉ Rebeca Bouyón,[1] José R Hernández,[1] Tania Aleaga,[1] Hector Santana,[1] Miriela Gil,[1] Raudel Sosa,[1] Yaí Cruz,[1] Alberto Agraz[2] and Luis Herrera[3]

[1]Interferons Production Division, [2]Biopharmaceutical Development Division, [3]Production Division. Center for Genetic Engineering and Biotechnology, PO Box 6162, Havana, Cuba. Phone: (53-7) 21 6022; Fax: (53-7) 21 8070; Telex: INGENCU 512330; E-mail: alberto.agraz@cigb.edu.cu

ABSTRACT

A comparative study was made of Mono S HR gel, and medium/high resolution Fractogel TSK SP-650 (S) and SP-Sepharose High Performance (SP-Sepharose HP) cationic exchangers for the purification of recombinant human α 2b interferon (rec hu-α 2b IFN). The protein was eluted through a linear gradient or by stepwise elution with approximately 0.1 - 0.19 M of NaCl. For the 3 gels a 55 % recovery with more than 98 % purity was obtained. The medium/high resolution gels showed higher protein loading capacity (50 mg/mL of gel) than the Mono S column (10 mg/mL of gel), and the productivity was increased. The purification step cost was reduced about 2,500 - 3,000 fold. Fractogel TSK SP-650 and SP-Sepharose HP can substitute high resolution Mono S for the final chromatographic purification of rec hu-α 2b IFN.

Key words: chromatography, recombinant human α 2b interferon, IFN

Biotecnología Aplicada 1997;14:189-192

RESUMEN

Se realizó un estudio comparativo entre el gel de intercambio catiónico de alta resolución Mono S y los geles de media/alta resolución TSK SP-650 (S) y SP-Sepharose HP en la purificación del interferón α 2b humano recombinante (rec hu-α 2b IFN). La proteína fue eluída de los 3 geles evaluados mediante un gradiente lineal o un gradiente escalonado a 0,1 - 0,19 M de NaCl aproximadamente con un 55 % de recobrado y una pureza superior al 98 %. Con los geles de media/alta resolución se obtuvo una mayor capacidad de carga (50 mg/mL de gel) que el obtenido con la columna Mono S (10 mg/mL de gel) así como un aumento en la productividad y la reducción del costo de la etapa en 2 500 - 3 000 veces. Los geles Fractogel TSK SP-650 y SP-Sepharose HP pueden sustituir al gel de alta resolución Mono S en la purificación cromatográfica final del rec hu-α 2b IFN.

Palabras claves: cromatografía, interferón α 2b humano recombinante, IFN

Introduction

Ion exchange chromatography (IEC) is a separation technique used in biotechnological and biochemical industries for the purification of proteins. Using IEC, the proteins can be separated by electrostatic interaction between charged groups present on the protein surface and those of the stationary phase (1, 2). Separation of molecules with only small differences in net charge can be achieved with this technique.

Efforts have been made to develop new IEC gels, from the early cellulose-based gels (3) to the more recent "rigid" gels, which combine high resolution, high flow rates and high loading capacity (4, 5).

Fractogel TSK SP-650 from Merck (Darmsstadt, Germany) and SP Sepharose High Performance from Pharmacia-Biotech (Uppsala, Sweden) are "semi-rigid" gels designed for and extensively used in chromatographic separation of proteins, showing medium to high resolution with reliable chemical and mechanical stability. The Fractogel TSK matrix is a copolymer of oligoethyleneglycol, glycidylmethacrylate and pentaerythrol-dimethacrylate with an M_r fractionation range of 5×10^4 to 5×10^6 for globular proteins and a particle size range of 25-50 μm. On the other hand, SP-Sepharose High Performance (SP-Sepharose HP) is a beaded agarose matrix, highly cross-linked for chemical and physical stability, with an M_r exclusion limit of 4×10^6 for globular proteins and a particle size of about 34 μm.

Cationic IEC has been used to purify recombinant human α 2b interferon (rec hu-α 2b IFN), a protein of 166 aminoacids that shows anti-tumoral, antiviral and immunoregulatory activities (6-13). High Resolution FPLC Mono S column (Pharmacia-Biotech, Sweden) has been used to separate de-

✉ Corresponding author

1. Robert K. Scopes, protein purification. Principles and practice, 2nd. ed, Springer-Verlag, New York 1987.

2. Janson, Ryden L. Protein purification. Principles, high resolution methods and applications. VCH ed, New York 1989.

3. Peterson, Sober HA. Chromatography of proteins. I. Cellulose ion-exchange absorbents. J Am Chem Soc 1956;78: 751-755.

4. Fulton M, Meys J, Protentis NB, Afeyan J, Carlton, Haycock J. Preparative peptide purification by cation exchange and reverse-phase perfusion chromatography. BioTecniques 1992;12: 742.

5. Dion DM, O'Connor K, Phillips D, Vella GJ, Warren W. New family of high-resolution ion exchangers for protein and nucleic acid purifications from laboratory to process scales. J Chromatog 1990;535:127-145.

graded products and contaminant proteins with very good results (14), but at a relativity high cost, mainly due to the high price of the matrix.

The present work shows a comparative study of alternative "rigid" cationic exchangers of medium/high resolution (Fractogel TSK SP-650 (S) and SP-Sepharose HP) for the purification of the rec hu-α 2b IFN.

Materials and Methods

Chromatographic matrixes and chemicals

All reagents used were analytical grade. Buffers were prepared with Super Q quality water (Millipore, USA) and filtered through a 0.2 μm membrane (Sartorius, Germany).

The starting material was rec hu-α 2b IFN that has been refolded and partially purified by immunoaffinity chromatography with anti-interferon monoclonal antibodies (14).

Mono S and SP-Sepharose HP were purchased from Pharmacia-Biotech, Sweden, and Fractogel TSK SP-650 (S) from Merck, Germany.

Chromatographic and analytical methods

Experiments were done on a FPLC system using HR 16/10 columns (Pharmacia-Biotech, Sweden) packed with 20 mL of the gels (the Mono S column was purchased prepacked). Data from the detection system were automatically collected and processed using the BIOCROM software (CIGB, Cuba) (15).

The protein dissolved in the starting buffer (buffer A: sodium acetate 50 mM, pH 4.8) was pumped through the columns at a constant linear flow velocity of 150 cm/h. The adsorbed protein was eluted by increasing the saline concentration through a linear gradient with buffer B (buffer A + 1 M NaCl), in 60 min. Eluted proteins were collected in fractions. The columns were regenerated with 1 M NaCl.

Protein concentration was measured according to Lowry (16) and the purity was determined by SDS polyacrylamide gel electrophoresis in 15 % PA gels (17). Proteins were detected with silver staining (18).

Results and Discussion

Performance of gels

Figure 1 shows the elution profiles obtained in the purification of rec hu-α 2b IFN, with the three gels loaded with 10 mg of protein/mL of gel. There was no difference, in terms of resolution, for the separation of the rec hu-α 2b IFN peak (main peak) and the contaminants. The main contaminants were molecules with higher molecular weights than the rec hu-α 2b IFN and dimers of this protein. Thus,

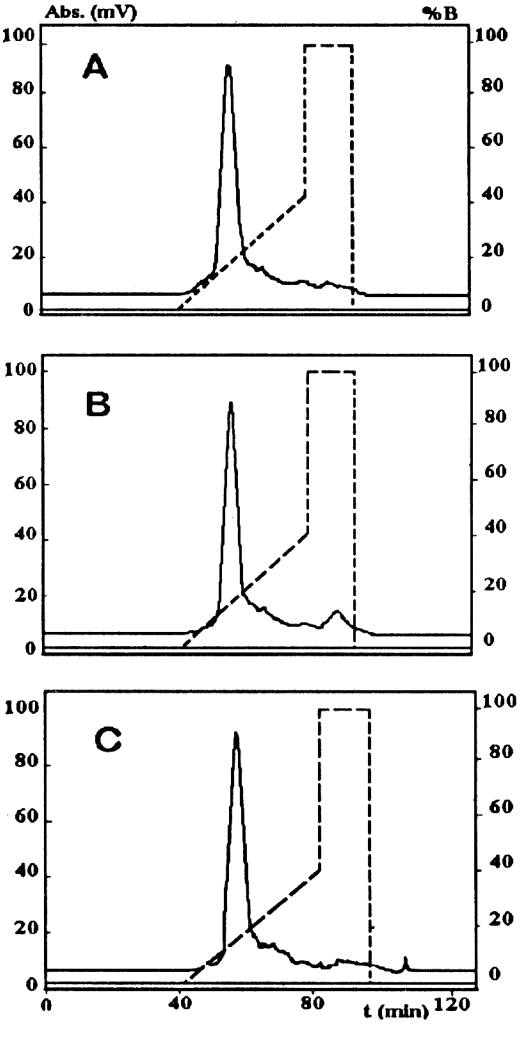

Figure 1. Chromatographic profiles with linear gradient elution of 60 min. A) SP-Sepharose HP, B) Mono S, C) Fractogel TSK SP-650 (S).

cationic exchange separates the monomeric and correctly folded rec hu-α 2b IFN from these undesired products (Figure 2).

Recoveries of monomeric rec hu-α 2b IFN with medium and high resolution gels were approximately the same (about 55 %). There was a portion (approximately 25-30 %) of the protein loaded in the column that could not be recovered, but that was partially removed only after washing with 1 M NaOH. This may be caused by precipitation of the protein inside the column or by a very strong adsorption of the protein to the matrix. These results influenced the lifetime of the columns: for Mono S gel it was 10 runs approximately, and for both the Fractogel TSK SP-650 gel and SP-Sepharose HP gel it was 15 runs.

It is uncommon to find such short lifetimes mainly dealing with IEC. The observed effect could be caused by protein precipitation or the presence of

6. Issacs A, Lindnmann J. Virus Interference. Proc Soc London Ser B 1957;147: 258-267.

7. Pestka S. Cloning of the human interferons. Methods in Enzymology 1981;78B: 599-601.

8. Cantell K, Hirvonen S, Kauppinen HL, Myllyia G. Production of interferon in human leukocytes from normal donors with de use of Sendai virus. Methods in Enzymology 1981;78A:29-38.

9. Cantell K, Hirvonen S, Koistinen V. Partial purification of human leukocyte interferon on a large scale. Methods in Enzymology 1981;78A:499-505.

10. Bodo G. Procedures for large-scale production and partial purification of Hu-IFN from lymphocyte (Namalwa) cultures. Methods in Enzymology 1981; 78A:69.

11. Menge V, Kula MG. Purification techniques for human interferons. Enzyme microb Technol 1984;6:135.

12. Langer JA, Pestka S. Structure of interferons. Pharmac Ther 1985;27:371-401.

13. Silva A, de la Fuente J, Pérez G, Quiñones Y, Novoa L, Herrera L. Altos niveles de expresión del gen del interferón α-2 humano bajo el control del promotor derecho del fago lambda en *E. coli*. Interferón y Biotecnología 1988; 5: 40-47.

14. Quiñones Y *et al.* High purity recombinant human alpha -2 interferon free from oligomeric forms in *E. coli*. Highlights Modern Biochem 1989;2: 1237-1246.

15. Gómez A. BioCROM. Sistema automatizado de cromatografía líquida. Manual de usuario. Versión 2.1, 1992.

16. Lowry OH, Rosenburough NJ, Farry AR, Randall J. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265.

17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680.

18. Wray W, Boulikas T, Wray VP, Hancock R. Silver staining of proteins in polyacrylamide gels. Anal Biochem 1981;118:197-203.

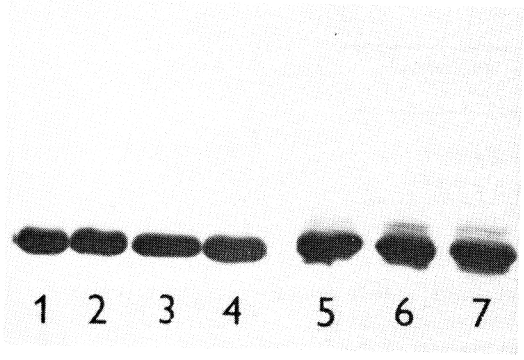

Figure 2. SDS-PAGE of fractions eluted from the IEC columns. 1) standard of highly purified rec hu-α 2b IFN; 2 to 4) main elution peak (monomeric and correctly folded rec hu-α 2b IFN); 5 to 7) contaminant fractions with higher molecular weights than rec hu-α 2b IFN. 2 and 5) Mono S; 3 and 6) SP Sepharose HP; 4 and 7) Fractogel TSK SP-650 (S).

host cell contaminants in the protein sample that remains inside the gel even when using "cleaning in place" procedures. These problems could block the column and reduce its lifetime.

In all matrixes, unfolded rec hu-α 2b IFN molecules eluted in the first fractions (up to 0.05 M NaCl) and molecules with higher molecular weights than the rec hu-α 2b IFN eluted in the fractions collected after reaching 0.15 M NaCl for the Fractogel TSK SP-650 gel and 0.27 M NaCl for the SP-Sepharose HP gel. The main peak (correctly folded rec hu-α 2b IFN) eluted between 0.1 and 0.15 M NaCl.

Elution gradient

Stepwise elution (with increasing concentration of NaCl) was considered a way to increase recovery and reproducibility of the IEC step, and therefore improve performance in the scaling-up of the step.

Steps at 5, 10, 15 and 20 % of B (B = A + 1 M NaCl) were used with Fractogel TSK, and 19, 21, and 23 % of B for the SP-Sepharose HP.

Correctly folded monomeric rec hu-α 2b IFN was eluted from the column at 10 % of buffer B for the Fractogel TSK (Figure 3a) and at 19 % of buffer B for SP-Sepharose HP (Figure 3b). Purity was higher than 98 %, a result comparable to that obtained by linear gradient elution on both gels, and even comparable to that on the Mono S column. Recovery of rec hu-α 2b IFN was about 55 %.

Loading capacity

The loading capacity of the different gels was evaluated in the range from 10 to 50 mg IFN/mL matrix, using de same starting material at a constant linear flow velocity of 150 cm/h.

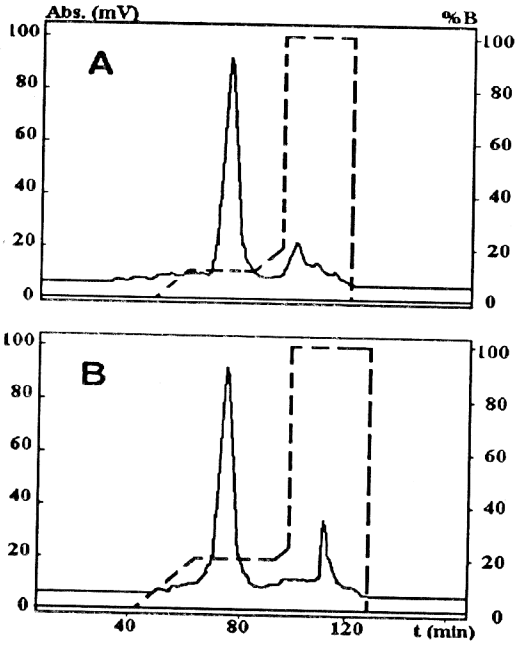

Figure 3. Chromatographic profiles with stepwise gradient elution. A) Fractogel TSK SP-650 (S), B) SP-Sepharose HP.

The resolution as well as the recovery of rec hu-α 2b IFN on the Mono S gel were affected at protein loads higher than 10 mg/mL (Table 1). In the case of Fractogel TSK SP-650 and SP-Sepharose HP gels, loading from 10 to 50 mg/mL of gel showed equivalent recovery without loss of resolution, that resulted in considerably higher productivity while higher loads produced losses of protein in the non-retained fraction.

All the experimental results are the average of 10 runs.

Economic analysis

The results obtained with Fractogel TSK SP-650 and SP-Sepharose HP drastically influenced the cost/benefit ratio of the step, not only because of the improvement in productivity achieved by these gels, but by the lower cost of these gels.

The cost of this step was estimated (Table 2) considering lifetime productivity and cost of the gel. The cost of the buffers used and the cost of the different steps for the isolation of the final product

Table 1. Loading capacity of the matrixes under study.

Loaded protein	Recovery (%)		
(mg/mL gel)	Mono S	SP-Sepharose HP	TSK SP-650 (S)
10	55	55	54
20	50	50	56
30	45	45	55
40	45	51	59
50	39	49	51

Table 2. Productivity and the cost of the step in the different matrixes.

Gels	Recovery (%)	Productivity (g/day x mL gel)	Step cost index*
Mono S	55	0.085	1
SP-Sepharose HP	49	0.656	3.4×10^{-4}
Fractogel TSK SP-650 (S)	51	0.613	4.6×10^{-4}

*Value relative to the cost of the Mono S gel (Step cost = gel cost/gram of IFN purified x mL of gel)

were taken as constants for the 3 gels studied (the quality of the final product obtained after the ion exchange step was the same for the 3 gels).

Cost was reduced to 2 100 fold using Fractogel TSK SP-650 (s) and 2 900 fold with SP-Sepharose HP. This analysis excluded the chromatography equipment which in the case of high performance gels include expensive systems and therefore enhances its importance.

Conclusions

Fractogel TSK SP-650 and SP-Sepharose HP can substitute high resolution Mono S for the final chromatographic purification of rec hu-α 2b IFN. This modification in the final purification procedure improves productivity and decreases the cost of the IEC step. Elution by a stepwise gradient facilitates the scaling-up of the purification process.

These results show that a new generation of "semi-rigid" gels of medium/high resolution can be evaluated as substitutes of HPLC/FPLC high resolution columns in processes designed to produce recombinant proteins. The advantages is not only their lower cost but the simplicity of operation due to the use of low pressure chromatography, which requires less sophisticated equipment and is easier to scale up.

Received in August, 1996. Accepted for publication in February, 1997.