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Process monitoring of the production of γ-interferon in recombinant Chinese hamster ovary cells

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ABSTRACT

The production of recombinant γ-interferon was monitored using high-performance liquid chromatographic methods. These methods were able to distinguish between glycosylated and non-glycosylated forms of γ-interferon by complexing the carbohydrate with borate. Sufficient quantities of standard glycosylated γ-interferon were not available for peak identification so immunological techniques were used to identify γ-interferon variants. These techniques were validated with the non-glycosylated form. The non-glycosylated form was then shown to be retained only on a cation-exchange column, while the glycosylated form, complexed with borate, was retained only on an anion-exchange column. Samples were drawn at 2-h intervals over a 60-h production cycle and analyzed by both anion- and cation-exchange chromatography. Results indicated that the production of each form was coincidental and that the glycosylated form of γ-interferon is produced in greater abundance than non-glycosylated.

INTRODUCTION

The production of recombinant proteins in mammalian cell culture is a process of growing importance. Unlike prokaryotic hosts (*e.g. E. coli*), eukaryotic production systems are more adept at producing recombinant proteins with the proper post translational modifications [1,2]. However, these modifications add complexity to the production system because the product recombinant protein may consist of additional classes of post translational variants. When analyzing unmodified proteins, only primary structural variants and folding variants were important. Post translational variants add another dimension of complexity which multiplies previous complications.

Previously, various researchers have demonstrated various methods for analyzing γ-interferon (IFN-γ) [3–5]. It was the aim of this research to develop rapid, automated, analytical methods for the purification of both glycosylated and non-glycosylated IFN-γ. Further, to test the ruggedness of the analytical methods, a 60-h experiment was performed to demonstrate the reliability of the techniques and to monitor the actual production process for recombinant IFN-γ.

EXPERIMENTAL

Instrumentation

The HPLC system used was a BioCAD Perfusion Chromatography Workstation (PerSeptive Biosystems, Cambridge, MA, USA) with a built-in variable wavelength UV–Vis detector, pH meter and conductivity probe. The HPLC columns used were a POROS S/H cation-exchange

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column (100 × 4.6 mm), a POROS Q/M anion-exchange column (100 × 4.6 mm), a POROS A/M protein A affinity column (30 × 2.1 mm), and a POROS G/M protein G affinity column (30 × 2.1 mm) (all columns from PerSeptive Biosystems, Cambridge, MA, USA).

Reagents

Tris(hydroxymethyl)aminomethane, boric acid, hydrochloric acid and sodium chloride were used for HPLC (Mallinckrodt, Paris, KY, USA). Schiff's reagent (Mallinckrodt), sodium periodate (Sigma, St. Louis, MO, USA), and sodium metabisulfite (Allied Chemical, Morristown, NJ, USA) were used for Fuchsin–Sulfite staining of glycoproteins. Anti-human γ-interferon polyclonal antibody was used for immuno-subtractive chromatography (Endogen, Boston, MA, USA) as was anti-human γ-interferon monoclonal antibody (ICN Biomedicals, Costa Mesa, CA, USA). The monoclonal antibody was directed against the C-terminal region of γ-interferon. Therefore, it is expected to cross-react with a variety of glycoforms of γ-interferon since the glycosylated region is located more closely to the N-terminal domain. Non-glycosylated γ-interferon standard was used in HPLC calibrations (a generous donation from Genentech, San Francisco, CA, USA). An ELISA kit (Endogen, Boston, MA, USA) was used to verify peak identities. A small amount of glycosylated γ-interferon standard was obtained (Sigma) which was used to show that the glycosylated form of γ-interferon was not retained on either the cation- or anion-exchange sorbent but could be retained on an anion-exchange sorbent in the presence of borate.

Cell culture

Recombinant Chinese hamster ovary cells (γ-CHO) engineered to produce γ-interferon were used in these studies. A 1:1 mixture of Dulbecco's Modified Eagle's (DME) medium and Ham's F12 medium (JRH Biosciences, Lenexa, KS, USA) was used to cultivate the cells. The medium was supplemented with 10% fetal bovine serum (FBS, Sigma), 4 m*M* L-glutamine, $2.4 \cdot 10^{-7}$ *M* methotrexate (Sigma), 10 units/l of penicillin and 10 mg/l of streptomycin. Cells were cultivated on Plastispex microcarriers (JHR Biosciences, 90–150 μm diameter) in 250-ml spinner flasks (Bellco). Microcarriers were washed 3 times with phosphate-buffered saline in siliconized spinner flasks (Sigmacote, Sigma) and sterilized by autoclaving for 30 min. After a final wash with culture medium, the microcarriers were seeded with cells at $2 \cdot 10^5$ cells/ml. The spinner flasks were then placed in an incubator (37°C, 10% CO_2, humidified) on a spinner plate (Bellco). The agitation was kept constant throughout the culture period at 50 rpm. Cell confluency on microcarriers was evaluated microscopically by taking culture samples daily. Upon confluency, the growth medium was replaced with medium without FBS. Samples from this serum-free cell culture were collected to monitor the production of IFN-γ.

RESULTS AND DISCUSSION

The aim of the research was to develop rapid automatable analytical methods that could be performed in real-time. This means that the methods can take no longer than 30 min if samples are taken hourly and the two forms of IFN-γ are to be analyzed separately. We chose perfusion chromatography because of its speed and reliability. Proteins may be separated in as little as 15 s with this technology [5]. Polystyrene-based columns were chosen for their durability and tolerance at extremes of pH. The advantage of the chromatographic based system is the ability to automate sample collection, product analysis, data management and feed back control.

The BioCAD workstation has recently been used to process monitor the production of monoclonal antibodies in hybridoma cultures [6]. However, the focus of the hybridoma study was automated sample handling of cell culture media and the integration of sampling, analysis, and data management. Unlike the hybridoma monitoring study, this set of experiments is complicated by the fact that more than one product needs to be determined and more than one method is required for these determinations.

The first objective was to develop HPLC methods for the determination of dilute quantities of IFN-γ in conditioned media. To enhance the response of the HPLC detector to poly-

peptides, the wavelength was set at 225 nm. The extinction coefficient of IFN-γ is higher at this wavelength than either 254 or 280 nm. Although IFN-γ absorbs even more at wavelengths below 225 nm, the mobile phase also begins to absorb significantly in this region. Therefore, 225 nm was chosen as an optimum for these conditions. Detection was also enhanced by using larger injection volumes; 1000-μl injections of conditioned IFN-γ media were used when dealing with dilute samples.

Sufficient quantities of glycosylated IFN-γ standard were not available for HPLC calibration. Antibodies to IFN-γ were obtained and immobilized on a protein G affinity column. The sample was then passed through this column, to remove any IFN-γ, before subsequent ion-exchange analysis. These chromatograms were then compared to chromatograms in which antibody was not used (Fig. 1). This subtractive technique was first described by Riggin *et al.* [7] for the analysis of human growth hormone. The technique was validated for non-glycosylated IFN-γ for which the standard was available. The missing peak in the conditioned media sample was then further confirmed by spiking the sample with non-glycosylated IFN-γ (Fig. 2). Once the subtractive technique was validated, it was applied to the glycosylated form of IFN-γ, for which standard was not available. To confirm the identity of the putative IFN-γ peaks, they were collected and shown to contain IFN-γ by enzyme-linked immunosorbent assays (ELISA) [data not shown]. They were also analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and stained with Fuchsin–Sulfite reagent to show that they were glycosylated, and also exhibited an apparent molecular mass shift to higher molecular mass.

The results of mobile phase optimization show that the non-glycosylated form of IFN-γ will adsorb to a cation exchanger at pH 7.0 in 20 m*M* Tris. It may then be eluted with a 10-min gradient to 1.0 *M* NaCl in 20 m*M* Tris pH 7.0 at 4.0 ml/min. Most of the other proteins in the conditioned media have lower isoelectric points than IFN-γ and are not retained by the column. They elute in the void volume.

The glycosylated form of IFN-γ did not adsorb to the cation exchanger at pH 7.0. There may be shielding of the amino acid side chains by the carbohydrate portion of the molecule. It is not retained by the anion-exchange column either,

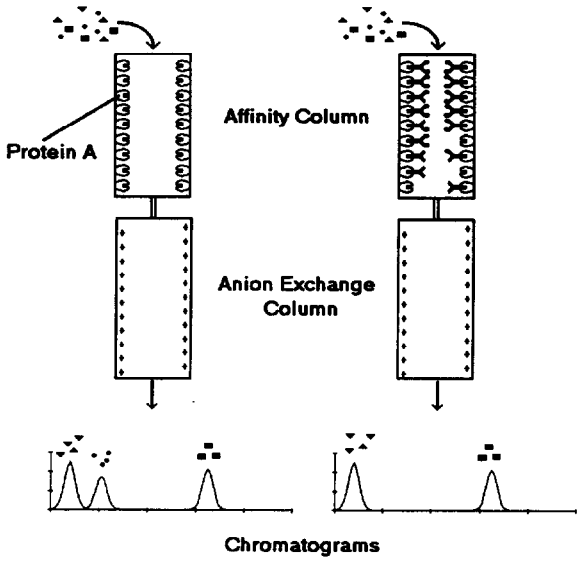

Fig. 1. Subtractive chromatography can be used to identify peaks. The sample is passed through a protein A column loaded with an antibody which will remove the protein of interest. A chromatogram of this sample is compared with a chromatogram of the same sample without antibody to determine the retention time of the protein.

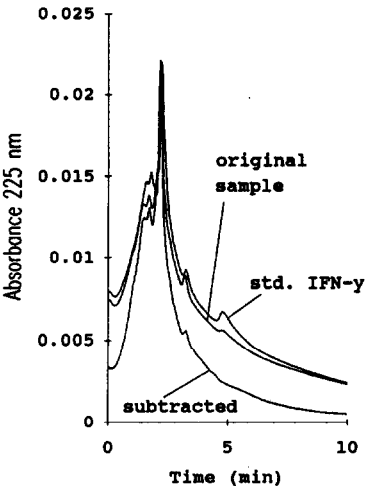

Fig. 2. Subtractive chromatography for γ-interferon. Conditioned γ-CHO medium was analyzed by cation-exchange chromatography (original sample). The subtracted sample was also analyzed, and the last small peak disappeared (subtracted). To further confirm the location, pure IFN-γ was added to the original sample (std. IFN-γ).

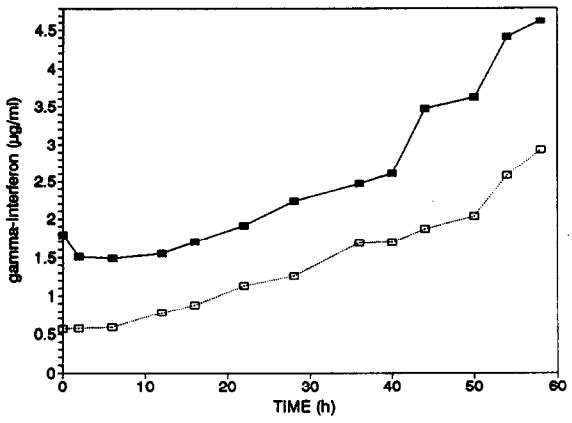

Fig. 3. Production of IFN-γ. □ = non-glycosylated and ■ = glycosylated IFN-γ.

unless the buffer contains borate (borate complexes with *cis* diols in the carbohydrate portion of the molecule, imparting negative charge [8,9]). This charge then causes retention of the glycosylated form of IFN-γ on the anion-exchange column. The pH of the mobile phase in this case was above 9. It is this alkaline pH which dictates the use of pH-stable packing material such as polystyrene. Silica-based media will dissolve at this alkaline pH.

Unfortunately, the antibiotics and methotrexate in the media co-eluted with the glycosylated IFN-γ. It is ironic that they also co-eluted with IFN-γ in capillary electrophoresis as well [10]. Since the antibiotics and methotrexate are small molecules, they were removed from the samples by size-exclusion cartridges (PD-10) prior to analysis. This precluded totally automated sample handling, but only added 5 min to the length of the assay. However, the added benefit of removing many other contaminating species, such as phenol red, was also acquired, making the chromatograms somewhat simpler to interpret.

The second objective was to demonstrate the ruggedness of the method and test its suitability for process monitoring. The production of both forms of IFN-γ were monitored with these HPLC methods over the course of a 60-h fermentation. Samples were removed from the bioreactor and analyzed immediately by the HPLC methods previously mentioned. Production curves show that both forms of IFN-γ are produced at roughly the same rate, although there is more glycosylated IFN-γ than non-glycosylated (Fig. 3).

CONCLUSIONS

Rapid HPLC methods for the determination of glycosylated and non-glycosylated IFN-γ were developed. These methods were then used to monitor IFN-γ production over a 60-h fermentation. These experiments demonstrate the efficacy of rapid, automated, chromatographic analyses for monitoring the production of recombinant proteins. Future work should be directed toward developing a direct interface between the HPLC and the bioreactor, to complete the on-line monitoring system.

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REFERENCES

1 R.R. Burgess, in D.L. Oxender and C.F. Fox (Editors), *Protein Engineering*, Alan R. Liss, New York, 1987, p. 81.
2 C.F. Goochee, M.J. Gramer, D.C. Andersen, J.B. Bahr and J.R. Rasmussen, *Bio/Technology*, 9 (1991) 1347.
3 A.L. Smiley, W.-S. Wu and D.I.C. Wang, *Biotechnol. Bioeng.*, 33(9) (1989) 1182.
4 J. Notani, Y. Saitoh, M. Terada, H. Yamada, Y. Ishii, S. Satoh, M. Tomoi and M. Niwa, *Biotechnol. Prog.*, 5(1) (1989) 40.
5 N.B. Afeyan, S.P. Fulton and F.E. Regnier, *LC/GC*, 12(9) (1991) 824.
6 S.K. Paliwal, T.K. Nadler, D.I.C. Wang and F.E. Regnier, *Anal. Chem.*, in press.
7 A. Riggin, J.R. Sportsman and F.E. Regnier, *J. Chromatogr.*, 632 (1993) 37.
8 S. Chapelle and J.-F. Verchere, *Tetrahedron*, 44 (1988) 4469.
9 S. Hoffstetter-Huhn, A. Paulus, E. Gassmann and H.M. Widmer, *Anal. Chem.*, 63 (1991) 1541.
10 C. Lee, personal communication.