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Development and monitoring of purification process for nerve growth factor fusion antibody ☆

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Abstract

A purification process and an on-line process monitoring procedure for a human nerve growth factor (NGF) fusion antibody (NAK) was developed. The NAK protein was produced with a recombinant technique by an adherent Chinese hamster ovary cell line in a tissue culture system supplemented with 5–10% fetal bovine serum, then purified from tissue culture supernatant. The overall purity goal for the process was 90%. A capture step was developed and optimized on POROS 50A which served as a purification and concentration step for the NAK product. A second purification step to remove contaminating bovine IgGs was developed and optimized first on a POROS HS/M cation-exchange column using 20-μm particles. The process was then scaled to a POROS 50 HS (50-μm particle) column. Once an optimized process was developed the capture step on POROS 50A was scaled from an 8-ml column to a 0.3-l column on PerSeptive Biosystems AutoPilot system.

1. Introduction

Antibodies are particularly susceptible to denaturation; hence rapid capture and short elution times critically impact product recovery. POROS 50 perfusive chromatographic media, developed by PerSeptive Biosystems [1–4] can concentrate very dilute samples, such as antibodies from cell culture supernatants, with very high throughput [greater than 40 column volumes (cv)/h] thus eliminating an ultrafiltration step at scale-up. A further ramification of this technology is the ability to perform fast analytical assays, both HPLC and immunoassays [5,6,8] to provide immediate feedback to guide the process scientist in the development of a process from the bioreactor to final product stages.

We herein describe the development of a purification, production, scale-up and monitoring scheme for the recombinant fusion protein NAK, consisting of human nerve growth factor [7] fused to the heavy chain terminus of a humanized IgG antibody. The use of Immuno-Detection/RPM to facilitate monitoring of NAK in the purification process will also be presented along with the scale up of the final purification process to pilot scale on the AutoPilot chromatography system [9,10].

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2. Materials and methods

2.1. Development of a protein A purification step

POROS 50A (PerSeptive Biosystems, Cambridge, MA, USA) was packed under constant pressure (5 bar) in a 100 × 10 mm I.D. (8 ml) glass column (Kontes Vineland, NJ, USA). All development runs were performed in 50 m*M* Tris-HCl pH 8.5, 0.5 *M* NaCl at a flow-rate of 500 cm/h (6.5 ml/min). The column was washed with 15 column volumes (cv) of 50 m*M* Tris-HCl pH 8.5 following application of sample to the column. Elution conditions were typically 100 m*M* glycine-HCl, pH 3.0 over 10 cv.

Scale-up work on POROS 50A was performed on a 100 × 60 mm I.D. (300 ml) Vantage column (Amicon, Bedford, MA, USA), packed under constant pressure of 5 bar. The column was operated on a PerSeptive Biosystems AutoPilot skid-mounted system. Runs were performed at linear velocities of 500 cm/h (300 ml/min).

2.2. Development and optimization of a cation-exchange purification step

POROS HS/M, 20-μm cation-exchange column and POROS 50HS media were packed in 100 × 4.6 mm I.D. (1.7 ml) column format for use in this study (PerSeptive Biosystems). pH mapping studies at pH 4.5, 5.5 and 7.5 were automated on a BioCad workstation. This system blended stock solutions of 33 m*M* Hepes, 33 m*M* Mes, 33 m*M* sodium acetate pH 4.5 and pH 7.5, H_2O and 3 *M* NaCl, to obtain the desired pH and ionic strength. Step elution studies on POROS 50HS were performed using 20 m*M* Tris-HCl pH 7.5 with varying concentrations of NaCl (50 m*M*, 100 *mM*, and 150 m*M*). NAK product was subsequently eluted in 1 *M* NaCl steps (10 cv). POROS HS/M and 50HS were operated at linear flow-rates of 3000 cm/h (8 ml/min) and 1000 cm/h (3 ml/min), respectively.

Scale-up runs on POROS 50HS were performed on PerSeptive Biosystems BioCad 250 system at a flow-rate of 1000 cm/h. For the scale up runs, POROS 50HS was packed under constant pressure (5 bar) into 100 × 10 mm I.D. (8 ml) Kontes glass columns.

2.3. SDS-PAGE (reducing)

This analysis was performed using 10–20% Daiichi gels stained with Commassie Blue dye.

2.4. Preparation of an immunodetection sensor cartridge for RPM (real time process monitoring) assays

Anti-NAK antibody was covalently coupled to a 30 × 2.1 mm I.D. CO (epoxy) immunodetection cartridge (PerSeptive Biosystems). The protocol used to perform the coupling was similar to that described in the CO immunodetection cartridge product insert provided with the cartridge. Briefly, a CO cartridge was washed with 100 cv of 1.5 *M* Na_2SO_4, 0.1 *M* Na_3PO_4, pH 9.0 at 1 ml/min (loading buffer). The cartridge was then loaded with forty 100-μl injections of 2 mg/ml purified antibody in loading buffer at 0.2 ml/min. Each injection was separated by 0.5 ml loading buffer. A gradient was formed between the loading buffer and 0.1 *M* Na_3PO_4, pH 9.0 (coupling buffer) until the first sign of breakthrough was noted. At this point, the cartridge was removed from the system, capped and stored at room temperature overnight. After incubation, the cartridge was washed with 10 ml loading buffer at 3 ml/min. Cartridge characterization revealed that the capacity for NAK was approximately 350 μg/ml.

Protein G RPM assays were performed on a 30 × 2.1 mm I.D. protein G cartridge, at a flow-rate of 5200 cm/h. The assays were done in a subtractive mode with an assay time of 25 s. The detection limit for this assay was 1 μg/ml.

Product specific assays were performed on a 30 × 2.1 mm I.D. CO cartridge derivatized with anti-NGF monoclonal antibody. Assays were run at 5200 cm/h in the subtractive mode with a detection limit of 1 μg/ml. Assay time was 25 s.

Real time process monitoring (RPM)

For chromatographic purification monitoring, the RPM system (Fig. 5) utilized subtractive and

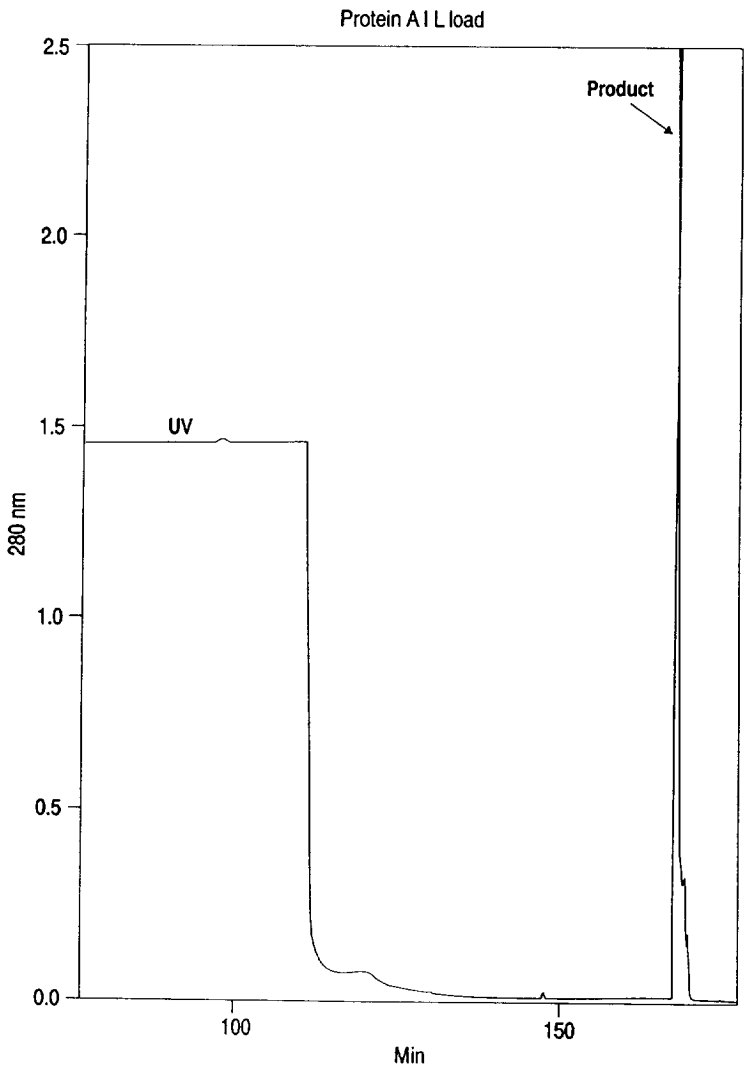

Fig. 1. POROS 50A (protein A) purification of NAK. One liter load on 8-ml column run at a linear velocity of 500 cm/h. The column was washed with 15 column volumes of 50 m*M* Tris-HCl pH 8.5, 500 m*M* NaCl and then eluted with 100 m*M* glycine-HCl pH 3.0, the elution volume of the IgG peak was 2 column volumes.

capture/elution assays. A subtractive assay is performed by passing a small volume of the process stream through an ID cartridge derivatized with a ligand capable of capturing the target analyte. The resulting peak area (devoid of target analyte) is compared with the corresponding peak area of a sample that had not passed through the ID cartridge. The difference in peak areas is proportional to the target analyte's concentration and purity. The RPM software keeps track of accumulated bound analyte and initiates an elution/regeneration cycle when necessary. Subtractive assays can be used for samples with purity levels in the range 5–95% and can be performed at 15 to 30 s intervals.

When high contaminant absorbance or very high or very low sample purities preclude the use of a subtractive assay, a capture/elution assay should be used. During capture/elution assays, a small sample of the process stream is injected

through an ID cartridge derivatized with ligand to capture the target analyte. The captured analyte is then eluted. The breakthrough and eluted peak areas are used to determine purity and concentration. Capture/elution assays can be performed at 1-min intervals, including the time required for cartridge regeneration after each elution cycle.

3. Results and discussion

3.1. Development of protein A purification step

A POROS protein A/50 column (100 × 10 mm I.D.) was used to capture immunoglobulins (NAK and contaminating IgGs) from the culture supernatant. Following equilibration in 50 m*M*

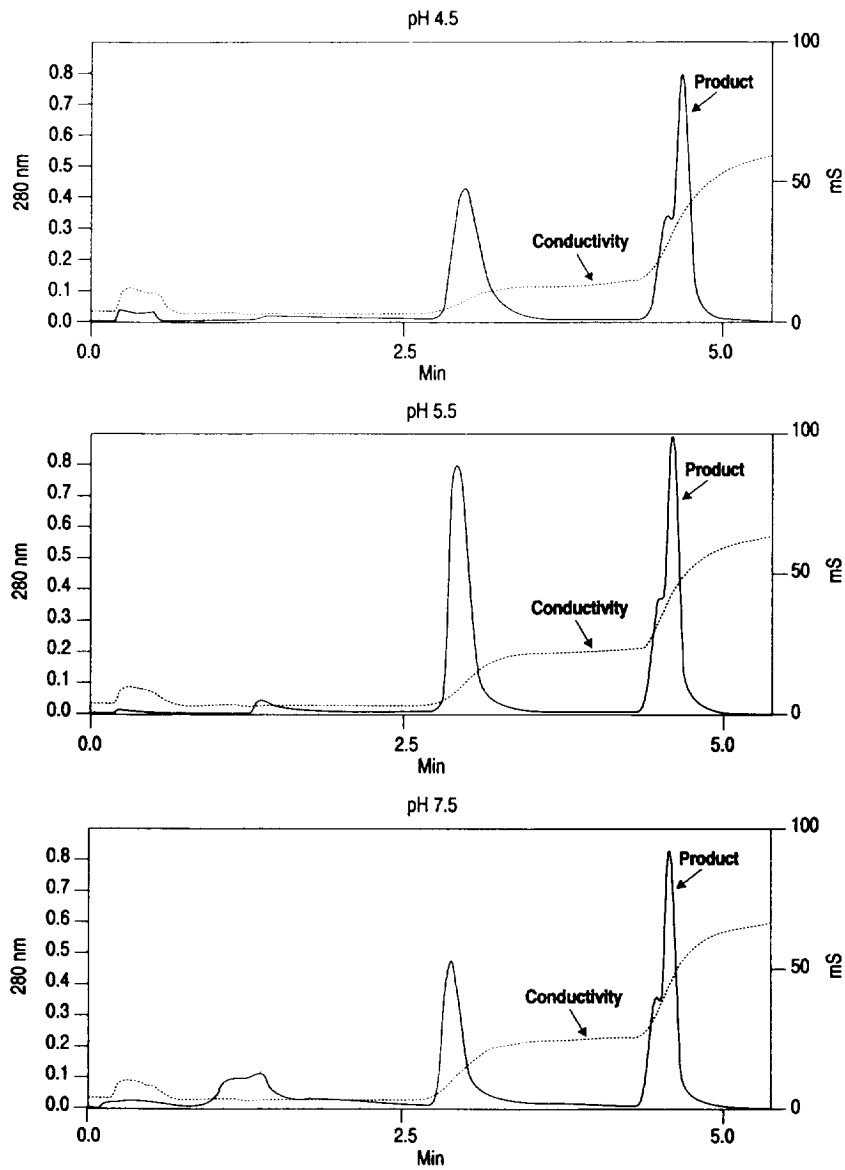

Fig. 2. pH mapping study on POROS HS/M 20-μm strong cation-exchanger. The column was loaded with 3 ml of the protein A pool, and then washed with 10 column volumes of buffer. Elution steps of 250 m*M* NaCl and 500 m*M* NaCl were done to separate the NAK from contaminating bovine IgGs. This column was run at 3000 cm/h with a total run time of 5.5 min.

Tris, pH 8.5, and 0.5 *M* NaCl, 1 l of supernatant was loaded onto the column at 500 cm/h (Fig. 1). The column was washed with 15 column volumes of equilibration buffer and eluted with 100 m*M* Glycine-HCl, pH 3.0. The NAK eluted in less than two column volumes at 0.56 mg/ml – a 33 fold increase in concentration from the initial cell culture concentration. At 500 cm/h, 1 l of supernatant is processed in 3 h, compared to 18 h required when using a conventional protein A soft gel medium operated at 100 cm/h.

This process step was accomplished without the need for concentration of the feed conditioned medium. This becomes especially im-

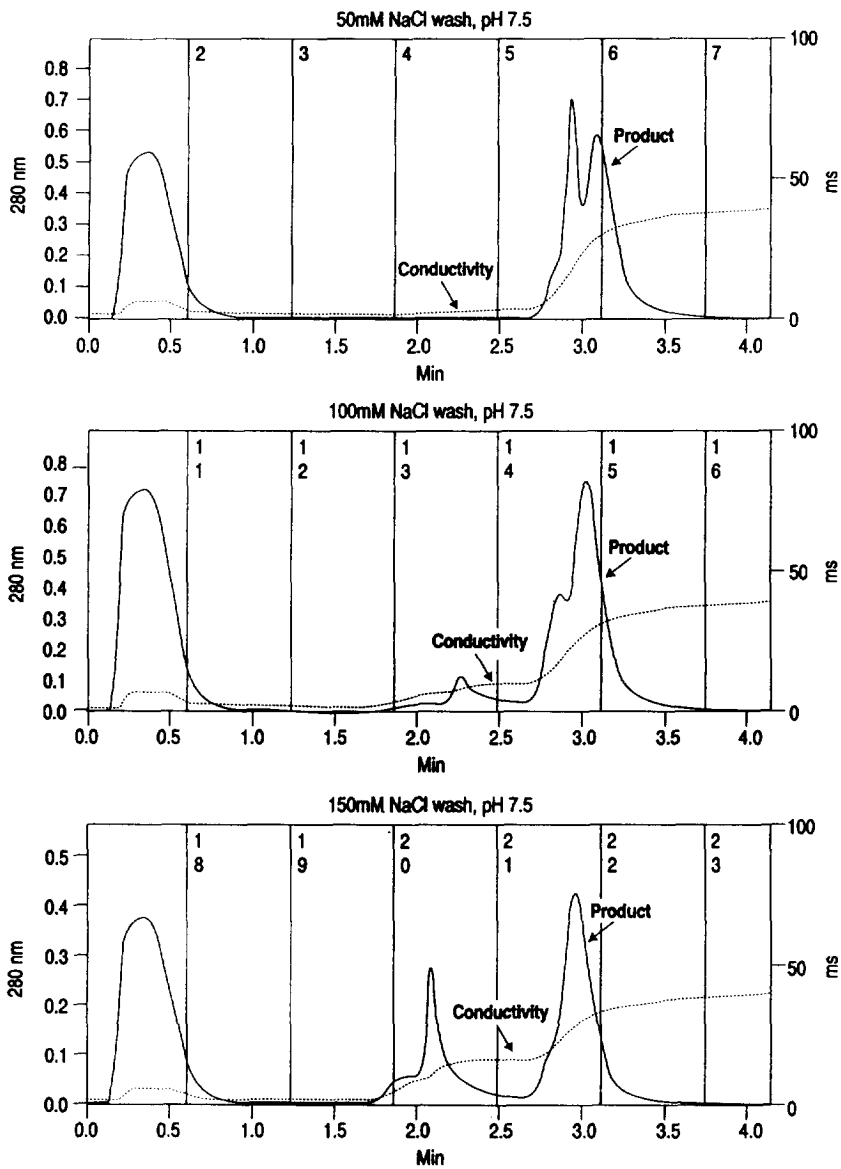

Fig. 3. Optimization of the separation of NAK from bovine IgG on POROS 50HS. The column was loaded with 3 ml of protein A pool and then washed with 10 column volumes of 50 m*M* Tris-HCl pH 7.5. Salt washes of 50 m*M*, 100 m*M* and 150 m*M* were tested to optimize the separation of contaminating bovine IgGs from NAK. These runs were completed at 1000 cm/h with a total run time of 5 min.

portant upon scale-up where speed not only translates into higher throughput but can enhance recovery of active product.

3.2. Development and optimization of a cation-exchange purification step on POROS HS/M and POROS 50HS

Utilizing a POROS HS/M (20-μm particles) cation-exchange column, optimization studies were conducted evaluating pH, column wash and elution conditions. The 20-μm medium is optimally suited for rapid screening experiments where the typical experiments take 5–10 min. Fig. 2 shows an example of a pH study on HS/M at pH 4.5, 5.5, and 7.5. Here it can be seen that substantially more UV absorbing material is found in the non-binding fraction at the higher pH. SDS PAGE (data not shown) analysis demonstrated that the unbound material consisted of non-product IgG and other contaminants.

Based upon the results on the 20-μm POROS HS/M, experiments were conducted on POROS 50HS (50-μm particles) to optimize a process step suitable for scale up. Fig. 3 depicts a salt wash study at pH 7.5. The optimum conditions achieved included a wash at 50 m*M* Tris-HCl/150 m*M* NaCl, pH 7.5, followed by a step elution at 250 m*M* NaCl. Fig. 4 depicts the results of SDS PAGE analysis of the 50HS optimized separation. Here it can be seen that the flowthrough and 150 m*M* NaCl wash (fractions No. 17 and 20) contain non-product IgG whereas the final 500 m*M* NaCl wash (Fraction No. 21) contains product essentially devoid of contaminating IgG.

3.3. On-line monitoring of the protein A scale-up

A 300 m POROS 50A column was loaded with 30 l of cell culture supernatant at 500 cm/h. The run was monitored using RPM for both total IgG breakthrough (protein G cartridge) and NAK breakthrough (anti-NAK cartridge). Total IgG breakthrough was observed early in the loading

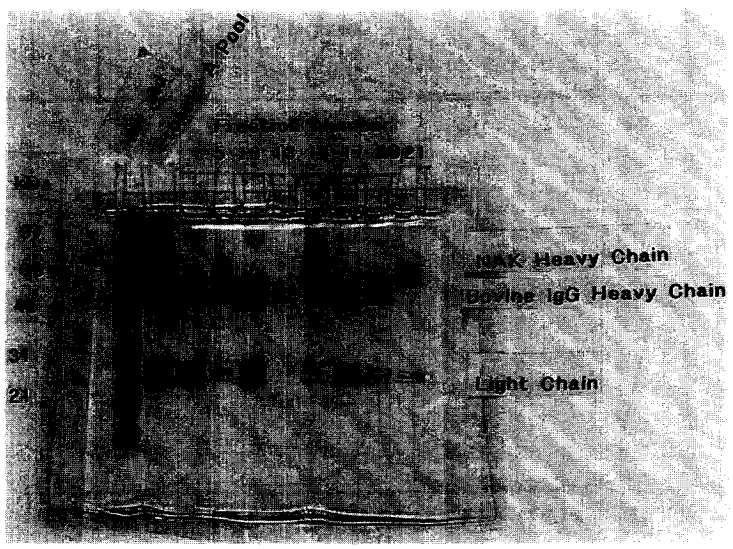

Fig. 4. SDS PAGE (8–20% gradient) analysis of 50HS, cation exchange, optimization steps for purification of NAK. Lane 1: molecular mass markers; lane 2; protein A pool; lanes 3–10: fractions collected during the optimization of the cation exchange separation (Fig. 3).

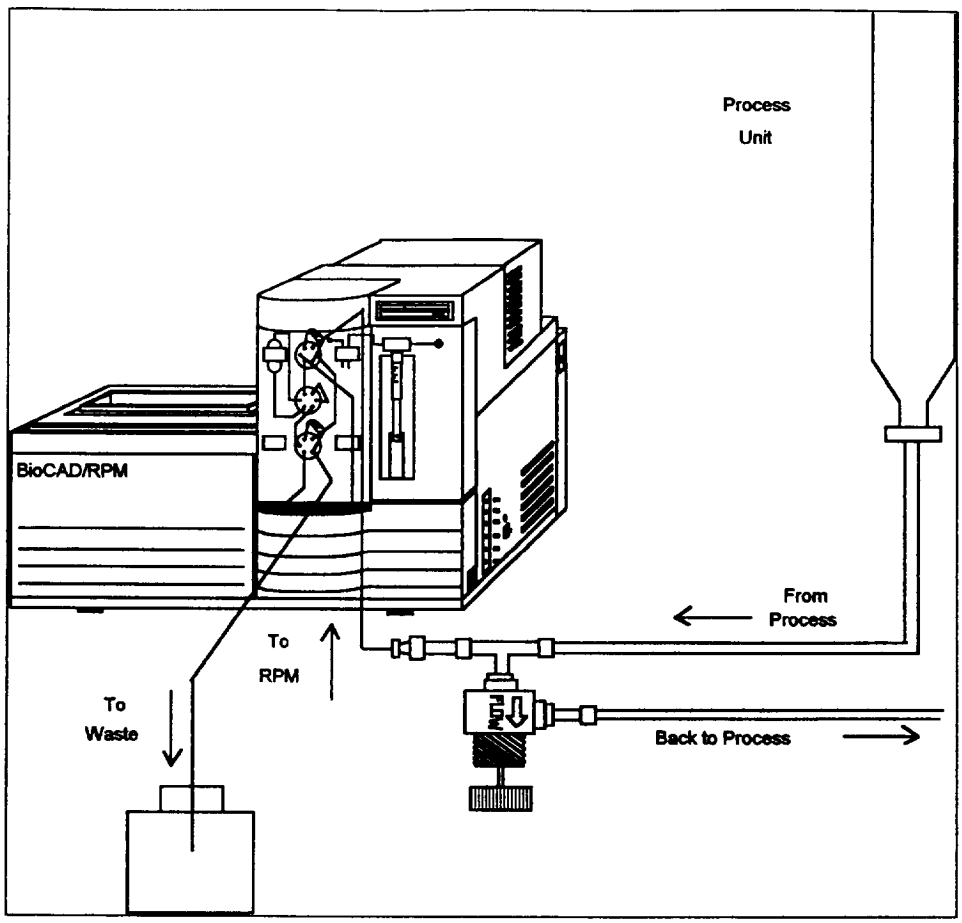

Fig. 5. RPM System. The RPM system is a specialized combination of PerSeptive Biosystems' BioCAD Workstation, POROS chromatography media, and perfusion immunoassay technology. The instrument includes specialized hardware and software for repetitive assay performance, data computation, and automated, accurate sample introduction to the analytical unit. Perfusion immunoassay is a solid-phase immunoassay technique employing a flow-through particle (POROS) that enables biospecific detection with high precision and high speed.

cycle, representing the bovine IgG fraction of the feed. However no product breakthrough was observed during loading (Fig. 6). Product eluted in less than 2 column volumes. On-line monitoring of the protein A column revealed information not attainable from the UV trace alone. Monitoring for both total IgG and NAK breakthrough during the feed allowed us to determine the optimal column load. Also, monitoring revealed competitive binding information, in real time, which would not have to be readily observable with conventional systems.

3.4. RPM of HS50 purification using PerSeptive Biosystems BioCAD 250

A 1-l volume of the POROS 50A pool was loaded onto an 8-ml POROS 50HS column at 1000 cm/h on a BioCad 250 system. RPM was used to monitor the separation of bovine IgG from intact NAK fusion antibody (Fig. 7). The resulting purity was assessed at approximately 90% by the RPM subtractive assay. These assays provide not only immediate in-process data but also provide highly quantitative data about prod-

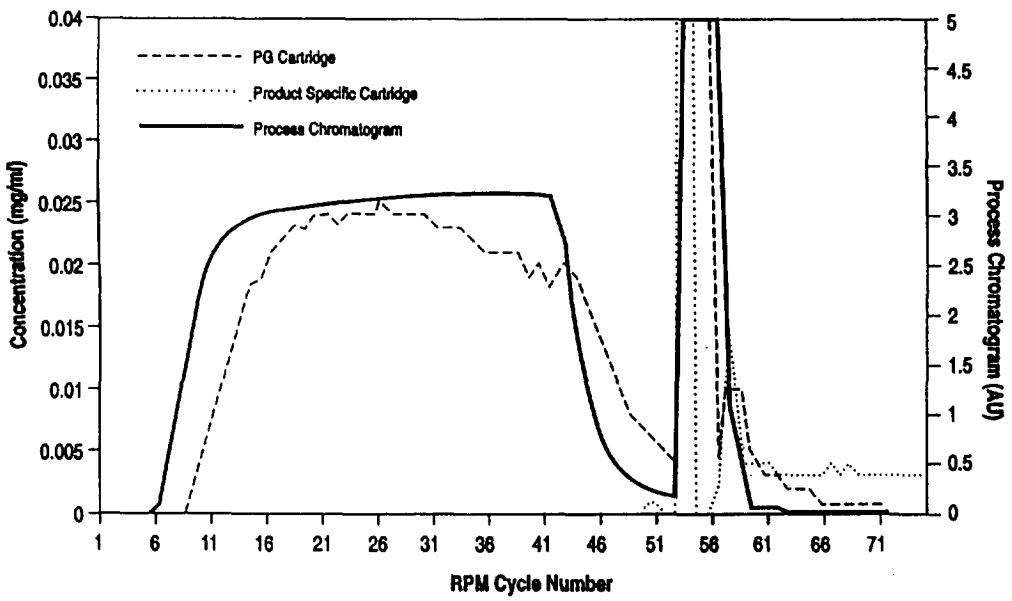

Fig. 6. RPM traces from POROS protein A dilute-feed capture of NAK. Two RPM units operated simultaneously to monitor total IgG and NAK. Capture/elution assays were used with a sample load of 1.5 ml and an assay time of 2 min. The solid trace represents process UV detector signal; the dashed line traces the total IgG and the dotted line the concentration of NAK throughout the process run. Contaminating IgG broke through very early while the NAK did not show breakthrough under these loading conditions.

uct purity (see Fig. 8 for PAGE results). The purified NAK was assessed for activity in a standard neurite outgrowth assay.

3.5. *Activity assay*

A standard neurite outgrowth assay, was performed, on the final product to assess the effect of the purification on the NGF portion of the fusion antibody [11]. The (6–24 cell) neurite outgrowth assay utilizes cultured endothelial cells that form long protrusions (neurites) when exposed to NGF. The cells are cultured in a 96-well plate for one day prior to the addition of NGF. After the addition of NGF the cells are allowed to grow for two more days and then the neurites are counted. All samples are run in parallel with a standard (mouse) NGF. The curve is generated based on the concentration of NGF in the fusion antibody. This assay shows that POROS purified NAK demonstrates the same activity as the standard NGF (Fig. 9).

4. Conclusions

Combining rapid methods development, immunodetection and RPM technologies enabled the rapid development and scale-up of a process for production of the NAK recombinant fusion antibody. It was shown that with this method the NAK fusion protein could be produced with a high degree of purity while retaining activity comparable to standard mouse nerve growth factor.

POROS media enabled a very rapid optimization of process conditions through a systematic methods development approach. In addition, due to the permeable nature of POROS particles, scale up was straight forward with minimal loss in resolution. The increased throughput minimized the number of upstream processing steps such as concentration and diafiltration.

RPM in combination with immunodetection provided immediate feedback to quantitate product in addition to improving the efficiency of the process optimization by offering high precision,

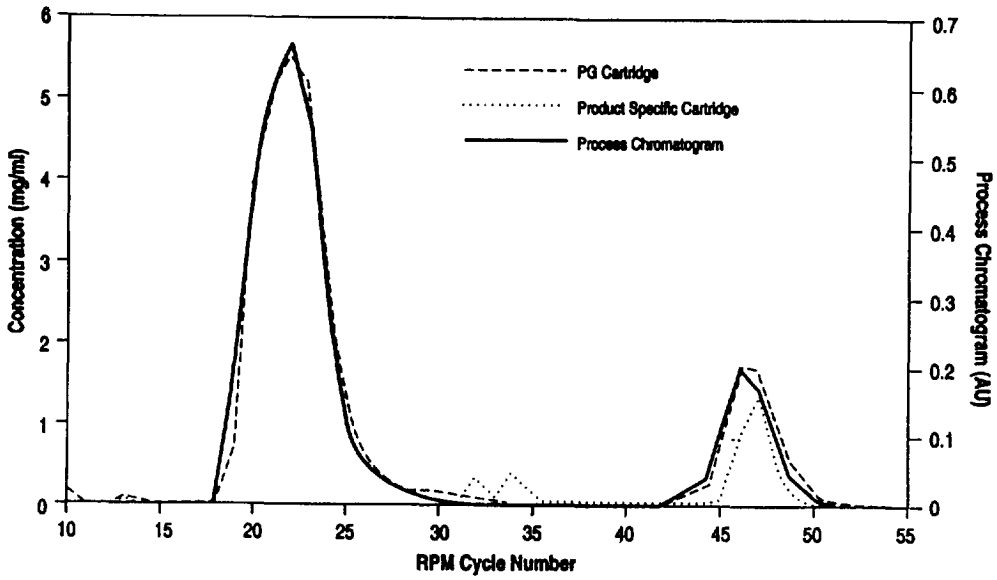

Fig. 7. RPM traces from POROS 50HS separation of NAK. To RPM units operated simultaneously to monitor total IgG and NAK. Subtractive assays were used with a sample load of 20 μl and an assay time of approximately 30 s. The solid trace represents the process UV detector signal; the dashed line traces the total IgG, and the dotted line, the concentration of NAK. The trace of IgG closely matches the process UV signal since the sample had previously been purified over a POROS protein A column. Early portions of the peak contain contaminating IgG while later portions are nearly 100% NAK. Very little NAK was observed as breakthrough under these loading conditions.

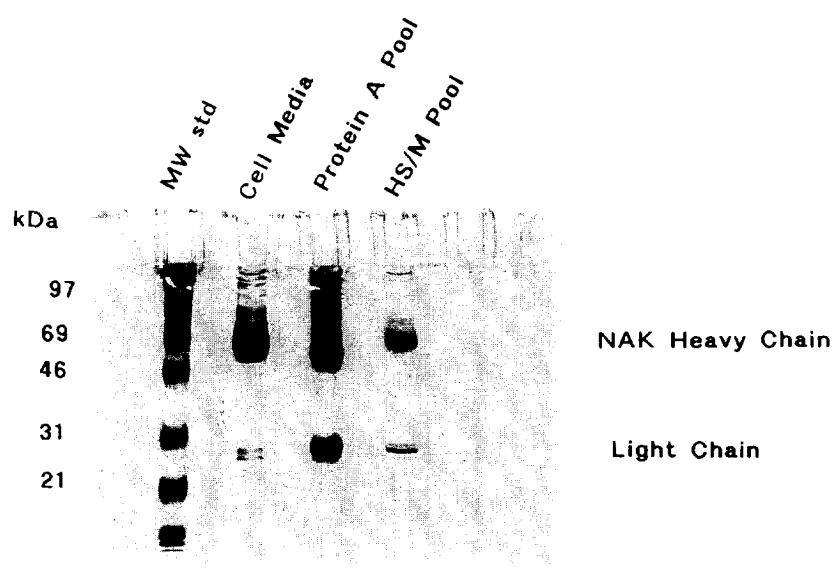

Fig. 8. SDS PAGE (8–20% gradient gels) analysis of final NAK purification process. Lane 1: molecular mass markers; lane 2: cell culture media containing NAK; lane 3: protein A pool; lane 4: final pool of purified NAK.

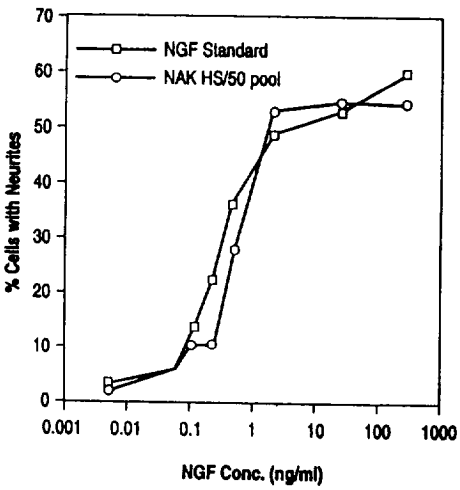

Fig. 9. Analysis of purified NAK by neurite outgrowth assay. PC12 cells were cultured in the presence of NGF or NAK for 24 h and then grown for an additional two days. Percentage of cells containing neurites was calculated.

automated repetitive assays with turnaround times of 15 to 180 s. Normally, a severe bottleneck in process optimization is assay turn around. Through the use of RPM, analytical in-process results were available immediately.

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