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Real-time isoform analysis by two-dimensional chromatography of a monoclonal antibody during bioreactor fermentations

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Abstract

A humanized monoclonal antibody specific for L-selectin exhibits two distinct isoforms that are distinguishable by a charged group modification on one of the antibody light chains. The added charge allows baseline separation of the isoforms by anion-exchange chromatography. Since this modification most likely results from specific enzymatic activity within the Golgi complex, it is possible that fermentation conditions may affect the relative amounts of the isoforms produced. Herein is described a two-dimensional chromatographic method for quantifying the relative amounts of the isoforms from fermentation broths, in real time.

Keywords: Monoclonal antibodies; Proteins

1. Introduction

HuDREG-55 is a humanized IgG4 monoclonal antibody that binds to human L-selectin, an adhesion molecule associated with neutrophil interactions along activated endothelium at inflamed sites [1,2]. It has been hypothesized that treating patients with HuDREG-55 could potentially block neutrophil adhesion to activated endothelium, and prevent further tissue damage caused by neutrophil transmigration into tissues. During purification of HuDREG-55, it was observed that affinity purified antibody could be separated into at least two major isoforms using anion-exchange chromatography. Extensive characterization of the isoforms [3] suggested that the difference could be attributed to a tyrosine sulfation of one light chain on one of the antibody isoforms. HuDREG-55 preparations, therefore, consist of one isoform with unmodified light chains, and a second isoform with one modified and one unmodified light chain. Tyrosine sulfation is a common post-translational modification that has been shown to occur in the trans-Golgi and is catalyzed by the enzyme tyrosylprotein sulfotransferase [4]. Cell culture conditions have been found to affect post-translational modifications, such as glycosylation, in various proteins. For instance, *N*-linked glycosylation of Chinese hamster ovary (CHO) cell-derived recombinant interferon gamma was found to deteriorate over time during batch culture, affecting the macroheterogeneity [5] and microheterogeneity [6] of the glycan structures. Additionally, nutrient composition in both batch and fed-batch culture fermentation has been reported to affect sialic acid composition of CHO-derived recombinant γ-interferon, with some tissue extracts having a potentially adverse effect on product sialylation [7]. In other experiments, monitoring the product quality of CHO-derived γ-interferon during fermentation showed more stable glycosylation efficiency and product quality during fed-batch compared with batch cultures [8]. Monoclonal antibodies have also demonstrated varying

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glycosylation patterns when derived from differing culture conditions. A murine monoclonal IgG exhibited varying sialic acid content depending on ascites production, or cell culture with serum-containing or serum-free medium [9]. Chimeric human/mouse antibodies of IgG3b and IgG2b isotypes showed increased galactosylation when prepared in still culture compared with continuous flow, hollow-fibre culture or mouse ascites production [10]. More sophisticated methods are being applied to carbohydrate analysis of therapeutic proteins, to discern bioprocess effects on protein glycosylation. High pH anion-exchange chromatography with pulsed amperometric detection was used to demonstrate that a human IgM exhibited oligosaccharide differences in type and distribution depending on whether production was via mouse ascites or cell culture [11]. Also, micellar electrokinetic capillary chromatography has been described as a useful method for monitoring bioprocess effects on protein glycosylation in real-time [12]. The fermentation conditions used to produce HuDREG-55 could affect the ratio of isoforms generated during drug manufacture. The following report details the development and implementation of a two-dimensional chromatography method to analyze cell culture supernatants during a fermentation run. Sequential protein A affinity chromatography and anion-exchange chromatography allow the analysis of cell culture samples in less than 22 min. This allows for real-time process monitoring of relative amounts of the HuDREG-55 isoforms, and makes possible the optimization of cell culture conditions and harvest times, accordingly.

2. Experimental

2.1. General conditions

An Integral™ microanalytical workstation from Perseptive Biosystems (Framingham, MA, USA) was used for the experiments outlined in this report. Table 1 shows the optimized conditions used for two-dimensional analyses, described after methods development activities were complete. The column used in the number 1 position on the Integral was a Poros PA ImmunoDetection cartridge (3 cm×2.1 mm) packed with protein A-immobilized 20 μm particles from Perseptive Biosystems. The column used in the number 2 position on the Integral was a Poros HQ/H (3 cm×2.1 mm) cartridge packed with QAE-derivatized 10 μm particles, also from Perseptive Biosystems. The Integral was configured for dual-column mode, allowing the monitoring of column 1 when in-line, and column 2 when in-line, and between columns when both were in-line. An injection volume of 500 μl was delivered through a PEEK (polyether ether ketone) sample loop for all samples. Mobile phase solutions, shown in Table 1, were prepared from analytical reagent grade chemi-

Table 1
Optimized chromatographic conditions employed during two-dimensional analyses of cell culture supernatants

Parameter	Affinity step	Ion-exchange step
Column	Protein A	Anion-Exchange
Packing	Poros PA immunodetection	Poros HQ/H
Size	3 cm×2.1 mm	3 cm×2.1 mm
Mobile phase solvent	1A, 25 m*M* potassium phosphate, 75 m*M* potassium chloride, 2.5% isopropanol, pH 7.3	1B, 50 m*M* Tris, pH 8.5
	2A, 25 m*M* potassium phosphate, 75 m*M* potassium chloride, 2.5% isopropanol, pH 1.7	2B, 50 m*M* Tris, pH 8.5, 1 *M* NaCl
Gradient	Step	Step
UV detection	280 nm	280 nm
Flow-rate	1.5 ml/min	1.5 ml/min

Table 2
Optimized program steps and gradient settings employed during two-dimensional analyses of cell culture supernatants

Step	Action	Condition
1	Column 1 equilibration	30 CV of 60% 1A–40% 2A
2	Sample loading	500 μl sample injection
3	Column 1 wash	30 CV of 60% 1A–40% 2A
4	Column 1 elution to column 2	8 CV of 50% 1A–50% 2A (both columns in line)
5	Column 2 binding step	10 CV of 100% 1B
6	Column 2, 1st step-gradient	50 CV of 70% 1B–30% 2B
7	Column 2, 2nd step-gradient	20 CV of 62% 1B–38% 2B
8	Column 2, cleaning	20 CV of 100% 2B
9	Column 2, re-equilibration	10 CV of 100% 1B
10	Column 1, cleaning	10 CV of 50% 1A–50% 2A
11	Column 1, re-equilibration	10 CV of 60% 1A–40% 2A

CV, column volume.

cals that were obtained from Mallinckrodt (St. Louis, MO, USA). Table 2 shows the optimized program steps used to control valve, pump, and gradient conditions during the analysis. Separations were performed at ambient temperature. Buffer 2B, Table 1, was used at full strength to clean the anion-exchange column between sample injections. Purified preparations of the two HuDREG-55 isoforms were kindly provided by Dr. Marie-Luise Hagmann, of Boehringer Mannheim Therapeutics (Penzberg, Germany).

2.2. Method development

Initial method development was accomplished using affinity purified HuDREG-55 containing both modified and unmodified isoforms. A quantitative Protein A method had previously been used to quantify antibody in cell culture media [13]. Because the elution solvent described in the procedure was at low pH and high ionic strength, solvent compatibility between the two separation modes was investigated. An 8-μl volume ''pancake'' mixer was introduced between columns 1 and 2, to help dilute and neutralize the pH of the eluent from protein A. The elution time from protein A was also evaluated. Buffer systems for protein A separation were modified to evaluate the effects of lower ionic strength on HuDREG-55 binding to the anion exchanger. Solvents for loading the anion-exchange column were varied to evaluate buffer ion concentration, to accommodate the low pH eluent from the protein A column.

2.3. Method performance

The performance of the optimized method was established using spike and recovery experiments in media samples. Unmodified and modified HuDREG-55 were mixed at ratios of 4:0, 3:1, 2:2, 1:3 and 0:4 in culture media, at a final concentration of 1 mg/ml. Sample injections were performed in triplicate, using a 400-μl injection volume in full-loop mode. Accuracy was determined by correlating the area counts obtained for each isoform with the spiked amount. In order to determine the sensitivity of this two-dimensional assay, modified HuDREG-55 was spiked into culture medium at concentrations ranging from 0.625 to 20 μg. In this case, 500 μl injection volumes were used in full-loop mode.

Samples were analyzed in triplicate. Sensitivity was determined by correlating the area counts obtained versus the mass of modified HuDREG-55 spiked. The limit of detection was defined as the lowest mass of modified HuDREG-55 that yielded a detectable peak with calculated area counts. Precision of the assay was estimated by expressing the mean coefficient of variation obtained from triplicate determinations of the spike and recovery experiments.

2.4. Fermentation samples

The optimized two-dimensional chromatographic analysis was carried out to quantify the relative amount of the two major isoforms of HuDREG-55 in cell culture supernatants. Supernatants taken at vari-

ous time points throughout bioreactor fermentations were analyzed. Samples from two, 10 l pilot scale fermentations and from two 750 l manufacturing scale fermentations were collected and analyzed. The average fermentation time for pilot scale and manufacturing scale fermentation runs were eleven and seven days, respectively. Cell culture samples were collected at 12 h intervals and filtered through a 0.2-μm syringe filter to remove cells. Samples were stored at −80°C until analysis, although this rapid two-dimensional method could be used in real-time, if needed.

3. Results

3.1. Method development

One of the most challenging aspects of combining two established separation methods in a two-dimensional analysis system is solvent compatibility. Fig. 1 shows the initial analysis of an affinity purified HuDREG-55 sample in dual-column mode. Note that most of the protein eluted from the protein A column does not bind to the anion-exchange column under these conditions. Fig. 2 shows the same separation using the pancake mixer and changing the 1 min linear gradient elution to a 0.5-min step gradient elution. In this case, more HuDREG-55 bound to the anion-exchange column. Originally, the optimized conditions for the anion-exchange separation of the two isoforms included a starting NaCl concentration of 0.2 *M*. This condition was established to minimize the binding of other materials in the sample to the anion-exchange column. Adjustments to the starting NaCl concentration were controlled by the percentage of buffer 2B in the solvent mix. This resulted in more efficient binding of HuDREG-55, as demonstrated in the chromatogram shown in Fig. 3. However, not all of HuDREG-55 that was eluted from protein A bound to the anion-exchanger under these conditions. To better control the pH of the material loading on the anion-exchanger, the Tris buffer concentration was increased from 10 to 50 m*M* in solvents 2A and 2B. In addition, the binding solvent mix for the anion-exchange column was set for zero NaCl (100% 2A). These changes resulted in the chromatogram shown in Fig. 4. The peak at approximately 2 min in Fig. 4 shows that some HuDREG-55 has not bound to the anion-exchange column under these conditions. Also, the pH profile, compared to that in Fig. 3, shows an improvement in reducing pH

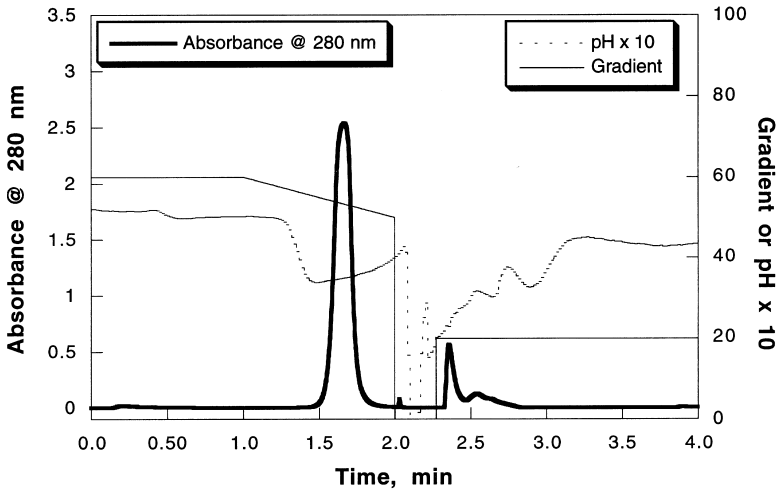

Fig. 1. Initial dual-column separation obtained for an affinity purified mixture of HuDREG-55 isoforms. Solvents 1A and 2A were 150 m*M* potassium chloride, 50 m*M* potassium phosphate and 5% isopropanol with pH values of 7.3 and 1.7, respectively. The elution step for column 1 was 60% 1A–40% 2A to 50% 1A–50% 2A for 1 min. Solvents for column 2 were 1B containing 10 m*M* Tris, pH 8.5, and 2B containing 10 m*M* Tris, 1 *M* NaCl, pH 8.5. The right hand *y*-axis shows the gradient (in %) and the pH ×10.

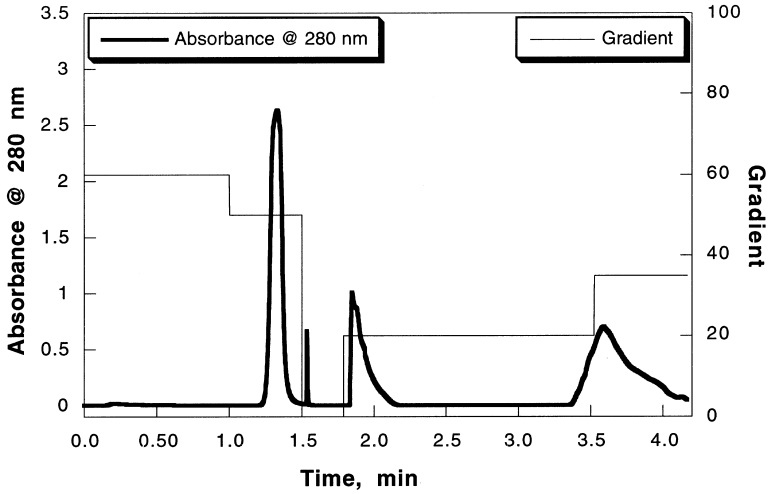

Fig. 2. Dual-column separation obtained for a HuDREG-55 mixture of two isoforms using an in-line ''pancake'' mixer and step-wise pH gradient elution from protein A. Protein A elution was done with 50% 1A–50% 2A for 0.5 min. The solvents for binding on the anion exchanger were 80% 1B–20% 2B (See Fig. 1 for solvent composition).

variations during the elution/loading step. Standard solvent systems used for analytical protein A separations contain relatively high ionic strength, with NaCl at 150 m*M*. By reducing this ionic strength twofold, the chromatogram shown in Fig. 5 was obtained. Using these conditions, Fig. 5 demonstrates the complete binding and elution of HuDREG-55 on the anion-exchange column. The step gradient ap-

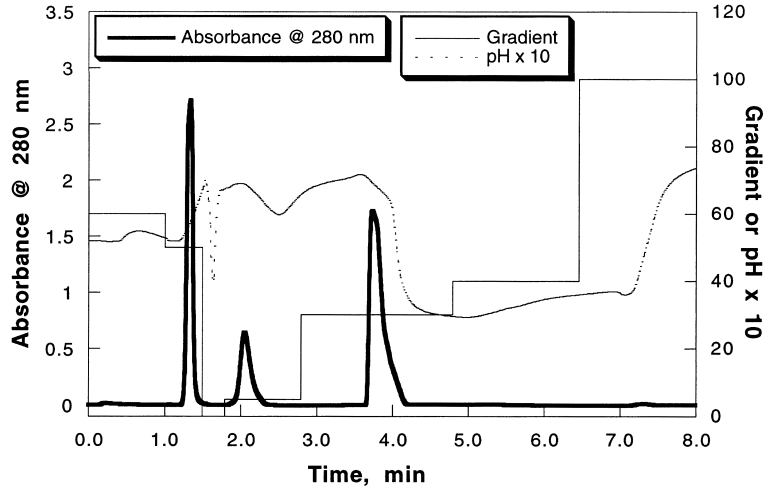

Fig. 3. Dual-column separation obtained for a HuDREG-55 mixture of two isoforms after adjusting the NaCl concentration of the loading buffer used on the anion-exchange column. The amount of salt was decreased from 20 to 5% during the binding step for the ion-exchanger. The right hand *y*-axis shows the gradient (in %) and the pH ×10.

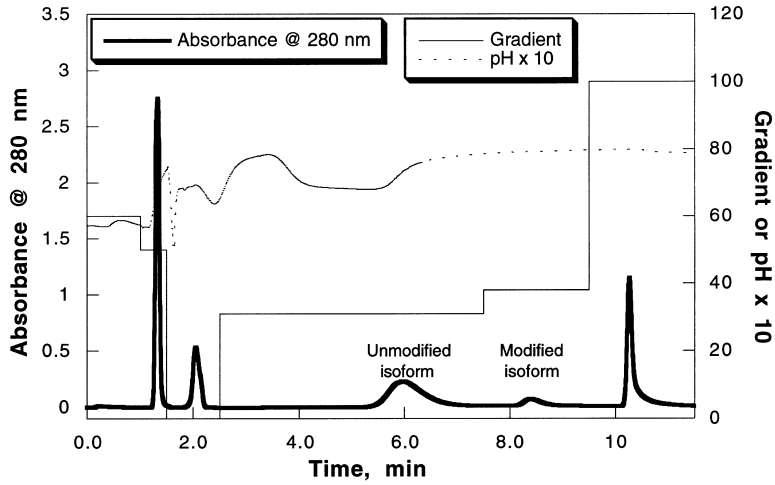

Fig. 4. Dual-column separation obtained for a HuDREG-55 mixture of two isoforms after adjusting the Tris concentration of the solvents used on the anion-exchange column. Solvents for column 2 were changed from 10 to 50 m*M* Tris. Also, NaCl was eliminated during binding to column 2. The right hand *y*-axis shows the gradient (in %) and the pH ×10.

plied to the anion-exchange column allows for the separation and quantitation of the two isoforms of HuDREG-55.

3.2. Method performance

The accuracy of the optimized method was evaluated using a series of spike and recovery experiments. Varying ratios of HuDREG-55 isoforms, in the range of 100 to 400 μg total load, were prepared in cell culture medium and analyzed. Linear regressions were performed to assess the correlation of area counts obtained versus mass of the isoforms injected. The equation $y=-4.44\cdot10^{6}+4.54\cdot10^{5}x$ was

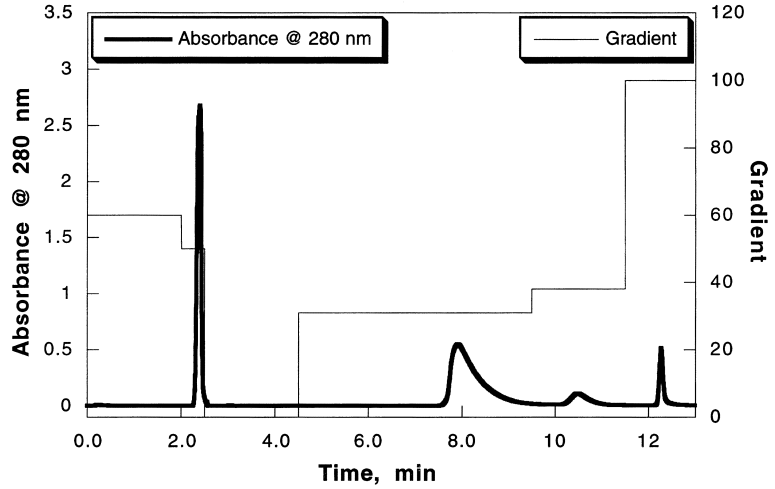

Fig. 5. Dual-column separation obtained for a HuDREG-55 mixture of two isoforms after reducing the ionic strength of the solvents used for the protein A column. The solvent mixture used for elution of column 1 was 50% 1A–50% 2A (with 1A and 2A being 75 m*M* potassium chloride, 25 m*M* potassium phosphate and 2.5% isopropanol, pH values of 7.3 and 1.7, respectively), for 0.5 min. The solvent mixture used for the binding step of column 2 was 100% 1B, for 2 min. Solvents 1B and 2B were 50 m*M* Tris, pH 8.5, and 50 m*M* Tris, 1 *M* NaCl, chloride, pH 8.5, respectively.

obtained from the regression analysis of unmodified HuDREG-55, yielding a R^2 value of 0.996. The equation $y=-3.00\cdot10^7+4.56\cdot10^5x$ was obtained from the regression analysis of modified antibody, yielding a R^2 value of 0.955. The relatively high degree of correlation between the amount of unmodified and modified isoforms spiked, and that recovered (area counts) for each of the peaks, demonstrates proportional recovery for both isoforms of HuDREG-55 from medium. In spike recovery studies, evaluating modified HuDREG-55 only in the lower range (0.625 to 20 μg total load), the method was sensitive to 0.625 μg, or 1.25 μg/ml in culture medium. Regression analysis of the area counts obtained versus the mass of modified isoform spiked yielded the equation $y=-1.57\cdot10^5+3.19\cdot10^5x$ with $R^2=0.996$. The average coefficient of variation for the spike and recovery experiments was 6.8% throughout the concentration range evaluated.

3.3. Fermentation samples

With the confidence that this dual-column method is quantitative and sensitive, the culture supernatants from bioreactor fermentations were analyzed. Due to the limited volume of samples, single injections were

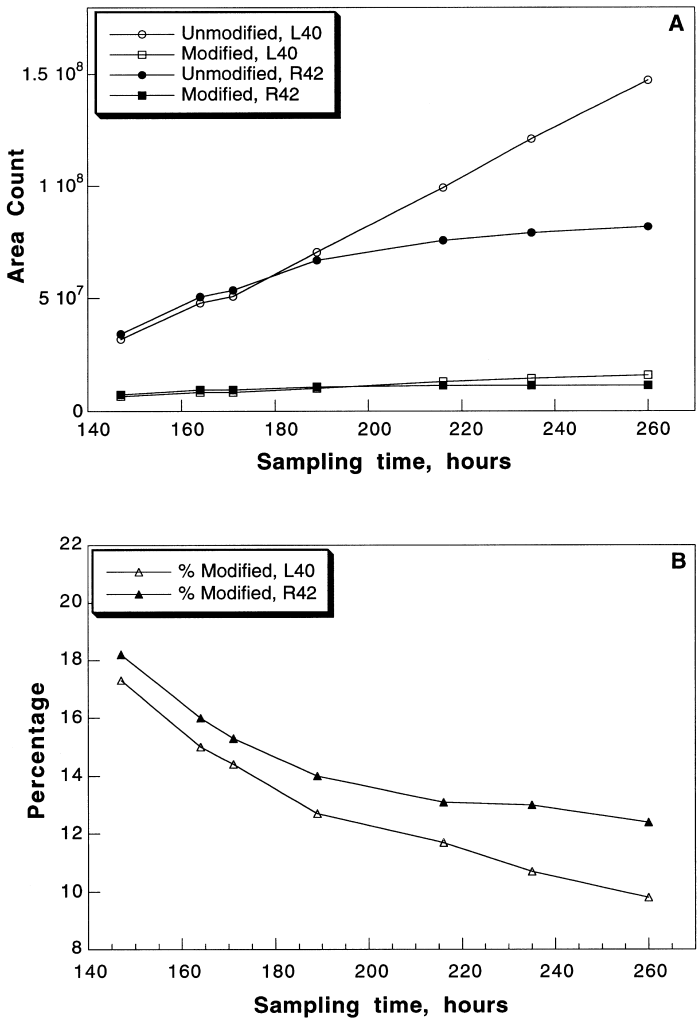

Fig. 6. Antibody titers by area counts (A) and the percentage of modified isoform (B) obtained using dual-column separation of samples collected during 10 l pilot scale fermentations.

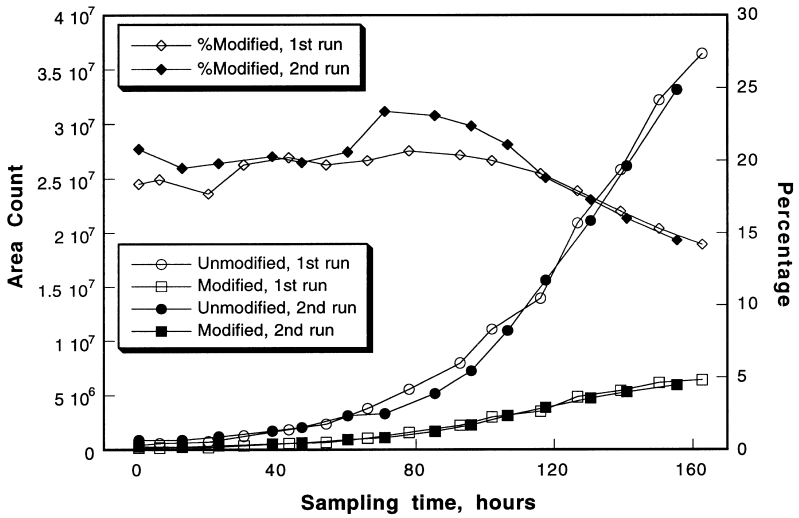

Fig. 7. Antibody titers by area counts and the percentage of modified isoform obtained using the dual-column separation of samples collected during 750 l manufacturing scale fermentations.

performed for all of the samples. Area counts of unmodified and modified isoforms of HuDREG-55 were used to generate the antibody titer curves shown in Fig. 6. As can be seen from these curves, product titers increased during the six days of sampling, as indicated by the increasing total area counts. The percentage of modified HuDREG-55 decreased with time. Although the variation between the antibody titers and the percentage of modified HuDREG-55 between the two, 10 l bioreactors has not yet been explained, the tendency towards increasing antibody titers and decreasing percentages of modified HuDREG-55 was consistent. Fig. 7 shows data for manufacturing fermentations at the 750 l scale. As can be seen, similar results were found to those obtained at the pilot scale. Antibody titers of both isoforms increased during the seven days of fermentation, but not to the same degree. Hence, the percentage of modified isoforms, as indicated by the right hand *y*-axis in the figure, dropped from approximately 20% in the earlier half of the cultures to less than 15% at the end of the cultures.

4. Discussion

A humanized anti-L-selectin antibody exhibits two isoforms, distinguishable by a charged group modification on Tyr-31 on one of the light chains. The presence or degree to which this modification exists may be attributable to the influence of bioreactor fermentation conditions on the intracellular activity of tyrosylprotein sulfotransferase. Others have previously reported two-dimensional chromatographic methods where size-exclusion was performed in the first dimension followed by protein A affinity chromatography in the second dimension [14]. Here, we described the development of a dual-column method based on combining protein A affinity chromatography with anion-exchange chromatography to analyze cell culture supernatants and evaluate the post-translational modification event on HuDREG-55 during the fermentation process. The most challenging aspect in adapting individual methods to a multi-dimensional mode is solvent compatibility. Several configuration and solvent changes were made to overcome solvent incompatibility problems from protein A to anion-exchange chromatography in this system. Lowering the elution volume from a low pH step prior to loading on the ion-exchange matrix was an advantage. Similarly, diluting the ionic strength of the elution buffer in the first column step, as well as adding buffering capability by increasing the buffer content of the ion-exchange equilibration solvent, added value to the system. Finally, the addition of

the pancake mixer just upstream from the ion-exchange column helped reduce the impact of ionic strength and pH from the elution solvent. As experience is gained with the Integral instrument, more configuration and solvent variations will be examined and implemented. The Integral is compatible with the use of analytical scale columns and, in conjunction with the instruments low delay volumes, allows the derivation of very fast methods. This dual-column method proved to be quantitative, sensitive and fast. Once optimized, it takes less than 22 min to analyze a sample using this method, allowing real-time monitoring of cell cultures. Analysis of HuDREG-55 culture supernatants by this two-dimensional method has demonstrated that the proportion of the unmodified light chain isoform increased with fermentation time and that the highest proportions of unmodified HuDREG-55 will be achieved with longer fermentation runs.

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