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Affinity–reversed-phase liquid chromatography assay to quantitate recombinant antibodies and antibody fragments in fermentation broth

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Abstract

An automated dual-column liquid chromatography assay comprised of affinity and reversed-phase separations that quantifies the majority of antibody-related protein species found in crude cell extracts of recombinant origin is described. Although potentially applicable to any antibody preparation, we here use samples of anti-CD18 (Fab′2LZ) and a full-length antibody, anti-tissue factor (anti-TF), from various stages throughout a biopharmaceutical production process to describe the assay details. The targeted proteins were captured on an affinity column containing an anti-light-chain (κ) Fab antibody (AME5) immobilized on controlled pore glass. The affinity column was placed in-line with a reversed-phase column and the captured components were transferred by elution with dilute acid and subsequently resolved by eluting the reversed-phase column with a shallow acetonitrile gradient. Characterization of the resolved components showed that most antibody fragment preparations contained a light-chain fragment, free light chain, light-chain dimer and multiple forms of Fab′. Analysis of full-length antibody preparations also resolved these fragments as well as a completely assembled form. Co-eluting with the full-length antibody were high-molecular-mass variants that were missing one or both light chains. Resolved components were quantified by comparison with peak areas of similarly treated standards. By comparing the two-dimensional polyacrylamide gel electrophoresis patterns of an *Escherichia coli* blank run, a production run and the material affinity captured (AME5) from a production run, it was determined that the AME5 antibody captured isoforms of light chain, light chain covalently attached to heavy chain, and truncated light chain isoforms. These forms comprise the bulk of the soluble product-related fragments found in *E. coli* cell extracts of recombinantly produced antibody fragments.

Keywords: Affinity chromatography; Pharmaceutical analysis; Reversed-phase analysis; Mixed-mode chromatography; Antibodies

1. Introduction

In order to develop productive fermentation processes for biopharmaceutical manufacture it is neces-

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sary to quantitatively monitor product titre. It is also important to monitor product stability since the product may be degraded by post translational modifications, in which case product levels may not be sufficient for recovery efforts. It is equally important to determine the levels of intermediates that give rise to products when generating antibodies and antibody fragments that require assembly of co-expressed light and heavy chains [1–4]. Currently no single product assay can measure intermediates, product, and product variants. Instead, a variety of qualitative and quantitative assays are used to assess the productivity of the fermentation.

Recombinant antibodies and antibody fragments are becoming increasingly common in the treatment of human disease. For example, two recombinant humanized antibodies have been approved for the treatment of metastatic breast cancer and relapsed low-grade non-Hodgkin's lymphoma [5–8]. Both of these full-length antibodies are produced by Chinese hamster ovary (CHO) cell lines and are secreted into the culture medium. When antibody fragments are expressed in *E. coli*, the light and heavy chains are co-expressed and secreted into the periplasm where disulfide bond formation is favored and chain assembly occurs to give rise to a product, such as a Fab′2 [3]. Methods to enhance this process have been developed such that the final form can be recovered directly from fermentation cell pastes [9]. Extraction of the product requires cell disruption and results in a complex mixture of *E. coli* cell proteins (ECPs) with the product. Thus, to monitor product titre efficiently it is necessary to measure light chain, heavy chain, light and heavy chain covalently bound (or associated) (i.e., Fab′, as well as the dimerized product Fab′2) (Fig. 1) and variants of these, in a complex mixture of ECPs.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is commonly used to monitor recombinant protein production in biopharmaceutical fermentations. Although this technique can discriminate or separate purified antibody fragments, e.g., light chain, heavy chain, Fab′, Fab′2 and full-length, host cell proteins (HCPs) of similar electrophoretic mobility often co-migrate with the protein species of interest. While this interference

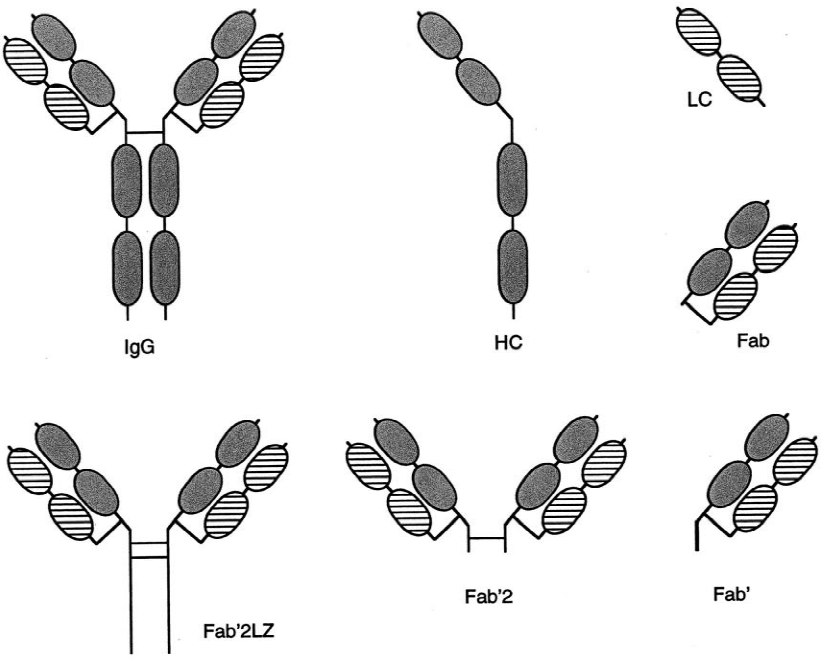

Fig. 1. Structures of a full-length antibody and antibody fragments. Light chain domains are indicated by cross-hatching and heavy chain domains are indicated by shading. The full-length antibody is a homo dimer (disulfide linked) of a light chain (LC) disulfide linked to a heavy chain (HC).

from HCPs can be overcome to some extent by using immunoaffinity detection, neither staining (Coomassie brilliant blue or silver) nor immunoblotting is highly quantitative. When the product related species are present at much greater concentrations than, or are well resolved from host cell proteins, then Coomassie blue-stained SDS–PAGE can be used to estimate product titres. Thus gel electrophoresis is usually better suited for fermentation monitoring of CHO cell-derived products than for *E. coli*-derived products.

Since the product-related proteins share amino acid sequences, they can be quantified using affinity-based techniques. Immunoassays are product-specific assays that can accurately quantitate the antigen (product) from complex mixtures, such as culture media. While accurate quantitation can be achieved when the mixture contains only one chemically distinct antigen, the results can be misleading when there are multiple product variants and interfering host cell proteins.

Thus current technology requires multiple assays to monitor the levels of recombinant protein product and their variants during fermentation development. The aim of our work was to develop a single assay that could quantitate product as well as the bulk of the product-related variants found in *E. coli* cell extracts [and harvested cell culture fluid (HCCF)] during the production of antibodies and antibody fragments. To achieve this, we have used the specificity of an immunoassay to target a common sequence that occurs in most of the variants and subsequently resolve and quantitate these molecules. We here describe the development of that dual column assay.

2. Experimental

2.1. Instrumentation and equipment

Samples were analyzed using an Integral Workstation (Applied Biosytems, Cambridge, MA, USA) configured in the dual column gradient configuration [10]. A minor modification was made to include a second UV detector positioned after the reversed-phase column. This instrument in most regards is similar to any modern dual pump HPLC instrument, but additionally can select from six solvent reservoirs and direct solvent flow via 3×10-port switching valves. These additional capabilities allow multiple solvent pathways and multiple column use. Detection was performed using a built-in dual wavelength UV–Vis detector. Reversed-phase column temperature was controlled using a control unit obtained from Cera (Baldwin Park, CA, USA).

2.2. Samples and reagents

Phosphate-buffered saline, pH 7.2 (PBS), containing 9.4 m*M* sodium phosphate, 136.9 m*M* sodium chloride and 2.7 m*M* potassium chloride is referred to as loading buffer throughout this paper. Assay diluent is sodium chloride (0.15 *M*), sodium phosphate (0.1 *M*), fish gelatin (0.1%), polysorbate 20 (0.05%), Proclin 300 (0.05%). Alternatively, samples can be diluted with 10 mg/ml bovine serum albumin (Sigma). All samples were filtered using Acrodisc PF 0.8-μm/0.2-μm syringe filters (Gelman Sciences, Ann Arbor, MI, USA).

Aqueous 0.1% trifluoroacetic acid (TFA) is the elution buffer as well as the aqueous buffer for the reversed-phase separation. Alternatively, HCl, pH 2.0, can be used as the affinity eluting agent. TFA, HPLC/spectro grade, was obtained from Pierce (Rockford, IL, USA). Acetonitrile, HPLC grade, was purchased from Burdick and Jackson (Muskegon, MI, USA). Sodium cyanoborohydride was purchased from Aldrich (Milwaukee, WI, USA). Purified (Milli-Q) water was produced by a Millipore water purification system. Monoclonal antibodies were obtained from Genentech's hybridoma group. Activated aldehyde immunoaffinity resin (AL-20), Poros R220 reversed-phase resin, empty polyether ether ketone (PEEK) columns (30×2.1 mm) and column packing devices were obtained from Applied Biosystems (Cambridge, MA, USA). Controlled-poreglass (CPG) was obtained from CPG (Linconpark, NJ, USA).

Two-dimensional gel electrophoresis (2DE) equipment and pH 3–10 nonlinear immobilized pH gradient gel strips were purchased from Amersham–Pharmacia Biotech (Piscataway, NJ, USA).

2.3. Immobilization of AME5 anti-human κ Fab to activated glyceryl-coated CPG

The purified Fab was immobilized onto periodate-activated glyceryl-coated controlled pore glass (CPG) to generate the affinity resin. AME5 Fab antibody was immobilized onto activated glyceryl-coated CPG using a modification of the method of Roy et al. [11].

Dry CPG (0.25 g) was washed with purified water and the wetted beads (0.7 ml) were packed into a chromatography column. The beads were activated for 30 min by recirculating 1% sodium metaperiodate (3 ml) (Sigma) through the column. The activated resin was then washed with 20 m*M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.2 (coupling buffer) (35 ml).

AME5 Fab antibody (1 ml) at a concentration of approximately 5 mg/ml in coupling buffer, containing 1 mg/ml of the reducing agent sodium cyanoborohydride (Sigma) was recirculated through the activated resin bed. The coupling of the antibody to the resin was monitored by the decrease in absorption at 280 nm. When there was no further decrease in absorption, any remaining antibody was washed out with coupling buffer and recovered. The coupling density was determined by the difference between the starting amount and the amount recovered after the reaction was completed.

Any remaining active sites on the resin were then reacted by recirculating 1 *M* ethanolamine, pH 8.0 (5 ml) (ICN) in the presence of 1 mg/ml sodium cyanoborohydride for 2 h. The resin was then washed into coupling buffer containing 0.01% thimerosal (GDL International) for storage. The resin was precycled three times between equilibration and elution buffers to be used before any protein was applied.

2.4. Affinity chromatography parameters

Procedures to determine the affinity column capacity, load, loading flow-rate, elution buffer, and stability to elution conditions were performed as described by Battersby et al. [12].

2.5. Sample preparation

E. coli cells and whole broth from small-scale (10 l) fermentations were typically suspended in 100–200 m*M* Tris, pH 8.0, 2 m*M* EDTA, 200 μg/ml lysozyme, then sonicated to lyse cells, and clarified via centrifugation. *E. coli* cells or whole broth from large-scale fermentations (1000 l) were typically extracted mechanically (e.g., Gaulin or microfluidizer) then clarified by flocculation (e.g., low pH or polyethyleneimine treatment) followed by centrifugation. This is the first step in the recovery process and produces what is known as the soluble fraction. In either case, supernatants (from centrifugation) were filtered and diluted 1:1 (v/v) with assay diluent in preparation for loading onto the affinity column. Purified anti-CD18 and anti-tissue factor (anti-TF) standards were diluted to 0.1 mg/ml in assay diluent.

2.6. Reversed-phase chromatography development

Reversed-phase development proceeded as it is usually performed by one skilled in the art. Purified samples consisting of essentially a single component were used to simplify the development process. The organic solvent was 0.09% aqueous TFA–acetonitrile (20:80). Initially the column temperature was maintained at 35°C and the flow-rate was 1 ml/min. Later, temperature (40–75°C) and flow-rate (0.5–3 ml/min) were investigated for their effect on peak shape and retention time. When appropriate conditions had been developed for the highly purified sample, more complex mixtures containing expected fragments and possible impurities were analyzed. Adjustments to the various gradients were then made to optimize the resolution between as many components as possible while keeping the total run time to less than 40 min. This process was then repeated using different reversed-phase columns (Poros R220, Vydac C_4, Poros phenyl, and TSK–phenyl 5PWRP).

2.7. Recovery and quantitation of anti-CD18 Fab′2LZ from E. coli cell extract

The Integral Workstation was configured in the dual column mode [9]. The solvent reservoirs were: Solvent 1A, affinity loading buffer, phosphate-buffered saline (see Section 2.2); Solvent 1B, reversed-phase aqueous buffer and affinity elution buffer, 0.1% TFA in water; Solvent 1C, alternate affinity elution buffer, HCl, pH 2.0; Solvent 2A, water.

Solvent 2B, reversed-phase organic elution buffer, 0.09% aqueous TFA–acetonitrile (20:80). Solvent 2C, non-specific wash solution, 5 *M* NaCl–PBS (20:80). The first column was the affinity column (30×2.1 mm) containing the anti-light-chain (κ) Fab antibody (AME5) immobilized on controlled pore glass. All procedures involving the affinity column were performed at ambient temperature. The second column was the reversed-phase column containing the polymer-based Poros R220 packing material (30×2.1 mm). The reversed-phase column temperature was maintained at 60°C.

The immunoaffinity column was equilibrated in 30% loading buffer (5 ml) and a 50 μl sample was loaded at a flow-rate of 0.1 ml/min. The flow-through was directed to waste. After the sample was loaded the affinity column was washed with 30% loading buffer (2 ml), followed by 100% loading buffer (5 ml) to reduce non-specifically bound components. To further reduce non-specifically bound components, additional salt washes were used at this step in some experiments (up to 1 *M* NaCl) and directed to waste. A final wash with water (3 ml) prepared the affinity column for elution. The affinity column was then connected to the reversed-phase column (by valve switching) and the bound material eluted with elution buffer (2 ml) at a flow-rate of 2 ml/min to transfer the affinity-captured components to the reversed-phase column. During this transfer step the Integral UV detector is located after the affinity column and before the reversed-phase column and hence monitors the material eluted from the affinity column, which becomes the load to the reversed-phase column. In addition to this detector, a second detector was added after the reversed-phase column to monitor its flow-through to confirm that all the components eluted from the affinity column had been captured by the reversed-phase column. Re-equilibration of the affinity column was subsequently performed with loading buffer (4 ml) after removing its connection to the reversed-phase column.

The loaded reversed-phase column was washed with aqueous 0.1% TFA (2 ml). The flow-rate was set to 1 ml/min and a rapid gradient (1 min) was run to 35% solvent 2B (0.09% aqueous TFA–acetonitrile, 20:80) followed by a shallow gradient to 50% solvent 2B over 14 min. Elution is completed by a gradient to 90% solvent 2B over 4 min. The reversed-phase column was then returned to initial conditions over 1 min and re-equilibrated for 3 min at 2 ml/min. Fractions (0.25 ml) were collected across the gradient profile, pooled as appropriate, and lyophilized. Peak fractions were partially characterized using N-terminal sequence analysis, SDS–PAGE and immunoblotting. They were also analyzed by liquid chromatography–mass spectrometry (LC–MS)(see next section). The column eluate was monitored at 280 and 214 nm. Quantitation was performed by comparison of the integrated peak areas with those of standards of known concentrations.

2.8. LC–MS

The lyophilized reversed-phase fractions were analyzed by LC–MS using the following procedure. The fractions were reconstituted in 40% acetonitrile to assure resolubilization of the heavy chain fragments, then diluted to 10% acetonitrile and loaded onto a Poros R220 reversed-phase column (30×2.1 mm). The column was washed with 0.1% TFA then eluted with an acetonitrile gradient as described in the previous section. In some experiments a simple linear gradient was run from 0 to 80% solvent B. The flow-rate was 0.5 ml/min and the column temperature was 60°C. The eluate was monitored for absorbance at 214 nm, split approximately 1:100 and directed into a Sciex API III triple quadrupole mass spectrometer. The data were obtained by scanning from M_r 10 000 to 160 000. The MacBioSpec software package (PE-Sciex) was used to calculate theoretical masses of fragments.

2.9. Two-dimensional gel electrophoresis

Samples to be analyzed by 2DE were solubilized in a solution comprised of 9.0 *M* urea, 4% (v/v) tergitol nonylphenoxy polyethoxy ethanol (NP)-40, 1% (w/v) dithiothreitol (DTT), 4% (w/v) Biolyte 8-10, pH 9.5. 2DE was performed essentially as described by Champion et al. [13]. Briefly, for first dimensional isoelectric focusing, 18-cm pH 3–10 nonlinear immobilized pH gradient gel strips were rehydrated overnight with the samples, then isoelectric focusing took place for a total of 50 000 V h. Second-dimensional SDS–PAGE was performed using the DALT system with large format gels

(200×250×1.5 mm) as indicated by Anderson and Anderson [14]. Upon completion of the second-dimension separation, fixation and silver-staining took place by a modification of the method described [15].

3. Results and discussion

3.1. Reversed-phase analysis development

The assay is comprised of two steps, an affinity step and a reversed-phase step. Both steps were developed separately then combined to produce the single automated procedure. The following section applies to the reversed-phase development.

Reversed-phase analysis of antibodies is generally considered to be a poor choice of analytical methods due to the very poor peak shape and poor recoveries typically attained. However, we are now having considerable success using this technique. Our first published experience with reversed-phase analysis of an antibody-like molecule was with tumor necrosis factor receptor immunoglobulin G, which is a chimeric immunoadhesin molecule comprised of the extracellular portion of human type 1 tumor necrosis factor receptor and the constant domains of human immunoglobulin G (hIgG) heavy chain [12]. The mature molecule is a dimer and is somewhat similar to a hIgG antibody without its two light chains. In contrast to the immunoadhesin, here we present the reversed-phase analysis of anti-CD18, as well as a full-length antibody (anti-TF). AntiCD18 is a Fab′2LZ. It is similar to a full-length antibody with the Fc portion of the heavy chain replaced by a leucine zipper (Fig. 1). The light and heavy chains contain 214 and 277 amino acids, respectively. The last 36 C-terminal residues of the heavy chain comprise the leucine zipper (LZ) designed to facilitate dimerization of the expressed Fab′ fragments. It has a molecular mass of approximately 107 000.

Shown in Fig. 2a–c are chromatograms representative of the reversed-phase development for the analysis of anti-CD18. The reversed-phase column (30×2.1 mm) was a polymer-based Poros R220. The dotted lines in Fig. 2a,b indicate the acetonitrile gradient used. The flow-rate was 1 ml/min and temperature was ambient. This sample is a purified

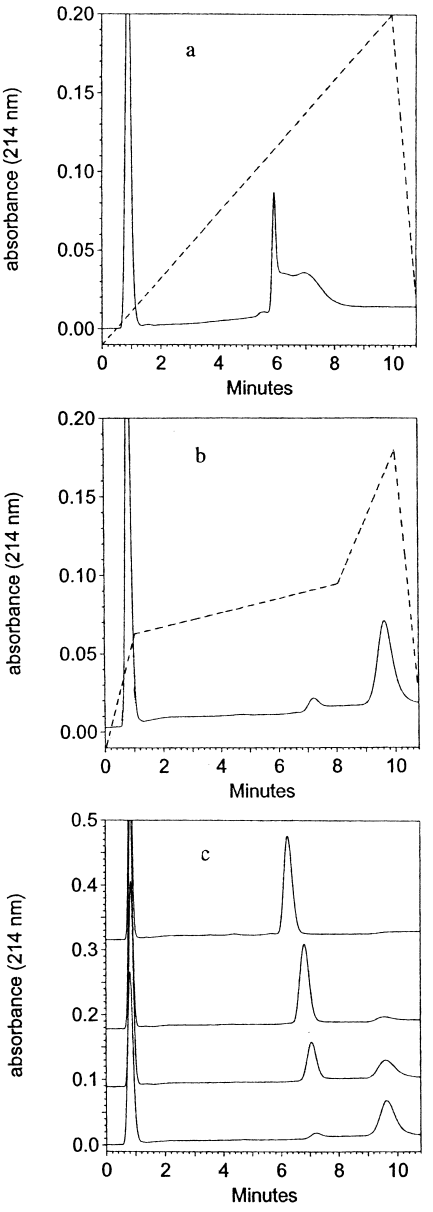

Fig. 2. Chromatograms produced by reversed-phase analysis of anti-CD18 Fab′2 (solid lines). The acetonitrile gradient is shown by the broken line. The same acetonitrile gradient gradients were used for (b) and (c). Column temperature was ambient for (a) and (b). Temperatures for (c) (bottom to top) were ambient, 35, 60 and 70°C. Separation details are described in the Section 2.

standard and is essentially a single component as judged by multiple characterization techniques (data not shown). The chromatogram shown in Fig. 2a was

produced by running a simple linear gradient from 0 to 100% solvent B in 10 min. Although this gradient had a run time of 10 min, it is a 100 column volume gradient due to the small column used (100 μl column volume). The resulting elution profile has a sharp peak (5.8 min) followed by a partially resolved and very broad eluting peak (6–8.5 min). This is the typical peak shape that we have observed for many Fab′2s and full-length antibodies using these conditions.

Shown in Fig. 2b is a chromatogram produced by the next step in the reversed-phase development. It was produced by running a steep gradient from 0 to 35% solvent B over 1 min (0–1 min) i.e., to a percentage acetonitrile just below that required to achieve elution, followed by a relatively shallow gradient to 50% solvent B over 7 min (1–8 min) until the first peak is eluted. This peak (7.3 min) is the same component as in Fig. 2a (5.8 min), but now has a lower peak height and is broader due to the shallower gradient. Note that this peak is tailing badly and the baseline has not returned to the same height as before elution. This tailing is equivalent to the broad eluting component (6–6.5 min) in Fig. 2a, but as with the first peak it now has a very much lower peak height and is much broader, again due to the shallower gradient. Additional analysis indicated that if the gradient is left to run, the balance of the sample is slowly eluted in this long tailing peak (data not shown). However, soon after elution of the first peak the second gradient begins (8 min) causing rapid elution of the tailing material and the creation of the second peak (9.5 min). This second gradient changes the solvents from 50 to 90% solvent B over 2 min. Even though the starting sample is a chemically singular component, we now have the appearance of two peaks. We assumed that this was purely the result of the chromatographic conditions, but there was the possibility that one of these two peaks had been generated by the denaturing reversed-phase conditions. Therefore, these two peaks were analyzed by mass spectrometry. Fifteen separate reversed-phase analyses were run and the mass of each peak was determined and compared (data not shown). The masses were found to be the same within experimental error, indicating that no gross chemical changes had occurred. However it is possible that the tailing material is due to conformational changes and/or dissociable aggregates that the mass spectrometer cannot differentiate.

The profiles in Fig. 2c were produced using the same gradient conditions as in 2b but at various increased temperatures. From bottom to top the temperatures were 25, 35, 50 and 70°C, respectively. As can be seen in these profiles, higher temperature resulted in more material eluting in the first peak. This all-important increase in temperature ensures that above 60°C the sample elutes as a single peak (Fig. 2c, upper profile, 6.3 min). At 60°C and above the peak is symmetrical, with the baseline before and after elution being the same. These final conditions produce a very acceptable reversed-phase analysis. As a cautionary note, we have found that sample diluent can affect the distribution of these components resulting in the two-peak profile, even though the reversed-phase column was at 60°C. Almost all of the reversed-phase chromatograms of anti-CD18 Fab′2 presented in this manuscript were produced by operating the reversed-phase column at 60°C and although we never observed the second peak to be more than 2% of the total peak area at these temperatures, we have since increased the temperature to 70°C. This ensures elution produces only a single peak, regardless of diluent. Additionally, in extending the assay to cover full-length antibodies it was found that this higher temperature was required to elute them in the single peak mode.

We have explained in some detail the reversed-phase development procedure to highlight the fact that under some circumstances a single component antibody (or fragment) can appear to elute in two positions. It is the later eluting position of the second component that causes concern, since this is also where non-specifically captured *E. coli* proteins elute (see Section 3.6). Thus, whenever the second component is observed there is uncertainty as to its composition.

This reversed-phase development process was repeated using several different reversed-phase columns with the results showing the same general trends as seen above (data not shown). The Poros R220 material was selected for routine use in the assay because of its high flow-rate characteristics even though it had higher carryover than the other packing materials. Thus loading, washing and equilibration steps could be performed at 4 ml/min,

reducing run time and increasing sample throughput.

3.2. Assay characteristics

The assay was first developed for the analysis of *E. coli* cell extract of anti-CD18 Fab′2LZ and later extended to full-length antibodies. The assay is comprised of two steps, an affinity step and a reversed-phase step. These are combined to produce the automated procedure. The recovery at each step was determined by comparing the eluted peak area with the peak area obtained when the same amount of material was passed through the detector alone. The affinity step typically had recoveries of 90–95% and the reversed-phase step had recoveries of 98–100%.

Shown in Fig. 3 (upper profile) is the complete chromatogram for the dual column analysis of anti-CD18LZ cell extract (0.05 ml). When this was compared to a similar analysis using commercially available Protein L-Agarose (Sigma) substituted for AME5 Fab CPG, similar reversed-phase chromatograms were obtained (data not shown). It is therefore probable that commercially available Protein L (anti-κ light chain) could be immobilized on hard supports to produce affinity columns similar to those used in this manuscript. Significant events in the analysis are shown labeled in Fig. 3 (upper profile). These are, load affinity, affinity wash (i.e., non-specific elution), affinity elution (with simultaneous capture on reversed-phase) and reversed-phase gradient elution. Seven peaks are observed in the reversed-phase section (see Fig. 4 for enlargement of this area). The peak at 37 min was identified as the desired, intact product peak and from its integrated peak area and using a standard curve generated from the chromatograms shown in Fig. 5A the concentration of Fab′2LZ in the extract was calculated at 0.08 mg/ml. A small amount of carryover (2–3%) is observed as multiple small peaks in the water blank following the analysis of this sample (Fig. 3, lower profile, 30–45 min). Additional washes of the reversed-phase column between analysis can prevent this, but results in a longer run time. To maximize the affinity capture of product and product variants it was necessary to load the affinity column at a flow-rate of 0.1 ml/min while keeping the total load of bound variants to less than 15% of the column capacity (column capacity had been determined to be approximately 200 μg). This slow loading flow-rate accounts for approximately 10% of the assay run time. It was found that we could reduce loading time by increasing the loading flow-rate to 0.5 ml/min when sample loads were near 1% of column capacity while still maintaining a capture efficiency of 95%. How-

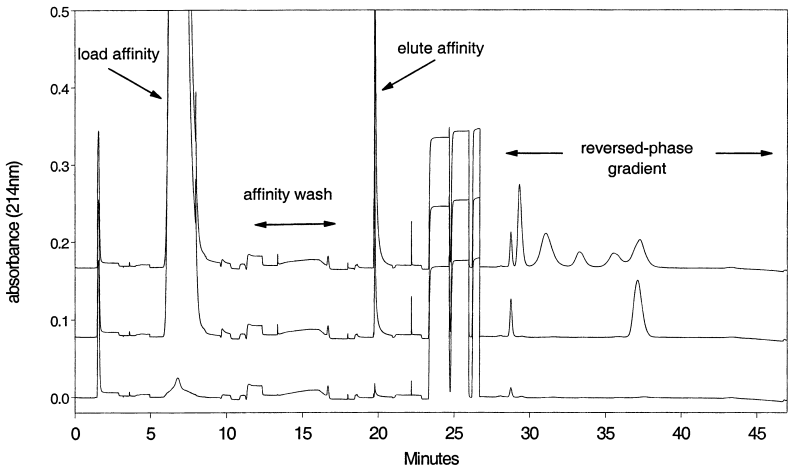

Fig. 3. Chromatograms of the complete dual column assay. Significant events in the procedure are labeled. Samples were anti-CD18 Fab′2LZ *E. coli* cell extract (top), purified anti-CD18 Fab′2 diluted in assay diluent (middle) and a water blank (bottom). Affinity chromatographic conditions are: loading buffer, 30% phosphate-buffered saline (see Section 2.2); elution buffer, HCl, pH 2.0. Remaining chromatographic conditions are described in Section 2.7.

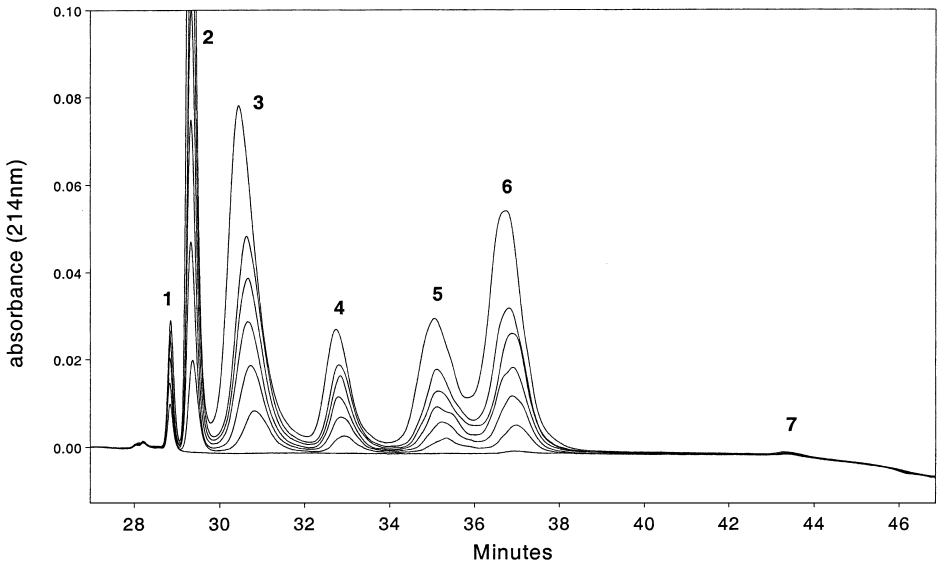

Fig. 4. Chromatograms (overlaid) of reversed-phase section of the dual column assay for various loads of anti-CD18 Fab′2LZ *E. coli* cell extract. The reversed-phase column was a Poros R220 (30×2.1 mm) which was eluted with an acetonitrile gradient. Column temperature was 70°C. Affinity chromatographic conditions as in Fig. 3.

ever, sample concentration and hence load, could only be determined after running the assay, therefore we routinely load the affinity column at 0.1 ml/min. The large peak observed at 5–9 min in the upper two profiles (Fig. 3) is due to the flow-through of non-binding cell extract components and cell culture components. It is essentially absent in the water blank (lower profile) and in Fab′2 standards diluted with buffer (not shown). Fig. 3, middle profile, is the chromatogram for the analysis of anti-CD18 Fab′2 standard (5 μg) diluted with assay diluent, and again we see the large flow-through peak but this time it is due to the 1% fish gelatine included in the diluent as carrier proteins. These carrier proteins increase recoveries in very low protein concentration samples. In the reversed-phase elution step a single symmetrical peak is observed at 37.5 min. Similar analyses were performed with various loads to construct a standard curve from which the concentrations of unknowns were calculated.

The affinity column is stable to the acid elution conditions and repeated exposure to cell extracts. We have observed only minor deterioration in column performance after analyzing hundreds of cell extracts samples over a 6-month period. Over this interval there was an average decrease in peak areas of 3%, with the single largest decrease of 10% being observed for the light chain dimer peak (P4).

3.3. Analysis of anti-CD18 Fab′2 and anti-TF standards

Chromatograms (reversed-phase section) for seven loads (1–9 μg in 10–90 μl, respectively) of anti-CD18 Fab′2 standard, spiked into assay diluent (0.1 mg/ml) are shown overlaid in Fig. 5A. The main peak is symmetrical with no tailing. As loads increased above 10 μg, peak fronting occurred (data not shown). A standard curve (regression coefficient 0.9988) was constructed by plotting the integrated main peak area versus sample load. These data indicate that the assay can accurately quantitate anti-CD18 Fab′2 at this concentration and over this range. Additional studies, using load volumes of 0.05–0.5 ml verified that this assay could be used to quantify anti-CD18 at concentrations of 0.01 mg/ml (data not shown).

The reproducibility of the assay was determined using a 5 μg/50 μl load. Multiple analyses ($n=7$) produced a coefficient of variation for the area of the main reversed-phase peak of 4% (data not shown).

In a similar manner, the assay was used to analyze

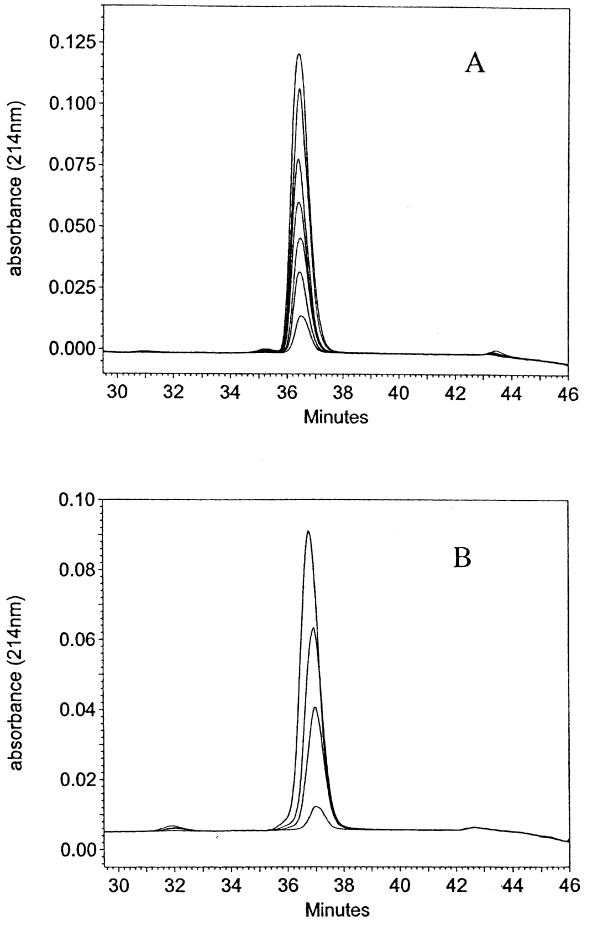

Fig. 5. Chromatograms (overlaid) of reversed-phase section of the dual column assay for loads (1–9 μg) of purified anti-CD18 Fab′2 (A) and anti-TF full-length antibody (B). Integrated peak areas were used to produce standard curves. Chromatographic conditions are described in Section 2.7.

a full-length antibody. Accordingly, purified anti-TF was spiked into assay diluent at 0.1 mg/ml and sample loads of 1–9 μg were analyzed. The chromatograms (reversed-phase section) for these loads are shown overlaid in Fig. 5B. Peak shapes similar to, but slightly broader than, those obtained for the Fab′2 fragments were seen i.e., symmetrical peaks with no tailing. The elution volume was only slightly greater than for anti-CD18 Fab′2. Integrated peak areas were plotted versus sample load and a regression coefficient of 0.9991 was calculated. These data indicate that the assay can accurately quantitate a full-length antibody at this concentration and over this range.

In addition to anti-TF, other full-length antibodies were analyzed using the dual column method. These were anti-Her2, anti-IgE (E25), anti-IgE (E26) and anti-VEGF (vascular endothelial growth factor) (chromatograms not shown). These all produced chromatographic profiles similar to that of anti-TF, i.e., a symmetrical peak with no tailing.

3.4. Analysis of E. coli cell extract (anti-CD18LZ Fab′2)

Using crude *E. coli* cell extract from an anti-CD18LZ production fermentation as the sample, the recoveries of the seven components resolved in the reversed-phase section (see Section 3.5 for identity of these) were determined at various loads. Load volumes of 10, 20, 30, 40, 50 and 90 μl were used and the chromatograms (reversed-phase section of assay) are shown overlaid in Fig. 4. The first peak is non-proteinaceous. Peaks 2–6 comprised 98% of the total peak area and their components all contained, at a minimum, the light chain epitope (see Section 3.5). The last eluting peak, No. 7 (2% of the total peak area) was not recovered in sufficient amounts for detailed characterization but had a retention time equal to both that of non-specifically adsorbed *E. coli* proteins as well as that of the second-peak-form of the antibody. Peaks 2–6 were integrated and peak areas were plotted versus load volume. Regression coefficients were all greater than 0.998, indicating that all components were recovered proportionally to their contribution to the load. These results were indicative of an assay suitable to quantitate changes in concentration of product and product-related variants in the presence of host cell proteins.

Product-related proteins captured by the AME5 affinity column were determined using 2DE. Comparison of 2DE gels of producing and non-producing *E. coli* fermentations showed that several isoforms of the light chain component of anti-CD18 were generated by *E. coli* (to be described in detail elsewhere [16]). All product-related proteins are shown circled in Fig. 6A and were determined by comparative 2DE followed by peptide mass fingerprinting and N-terminal sequencing of the excised spots. Component a was a p*I* (isoelectric point) isoform of light chain, b

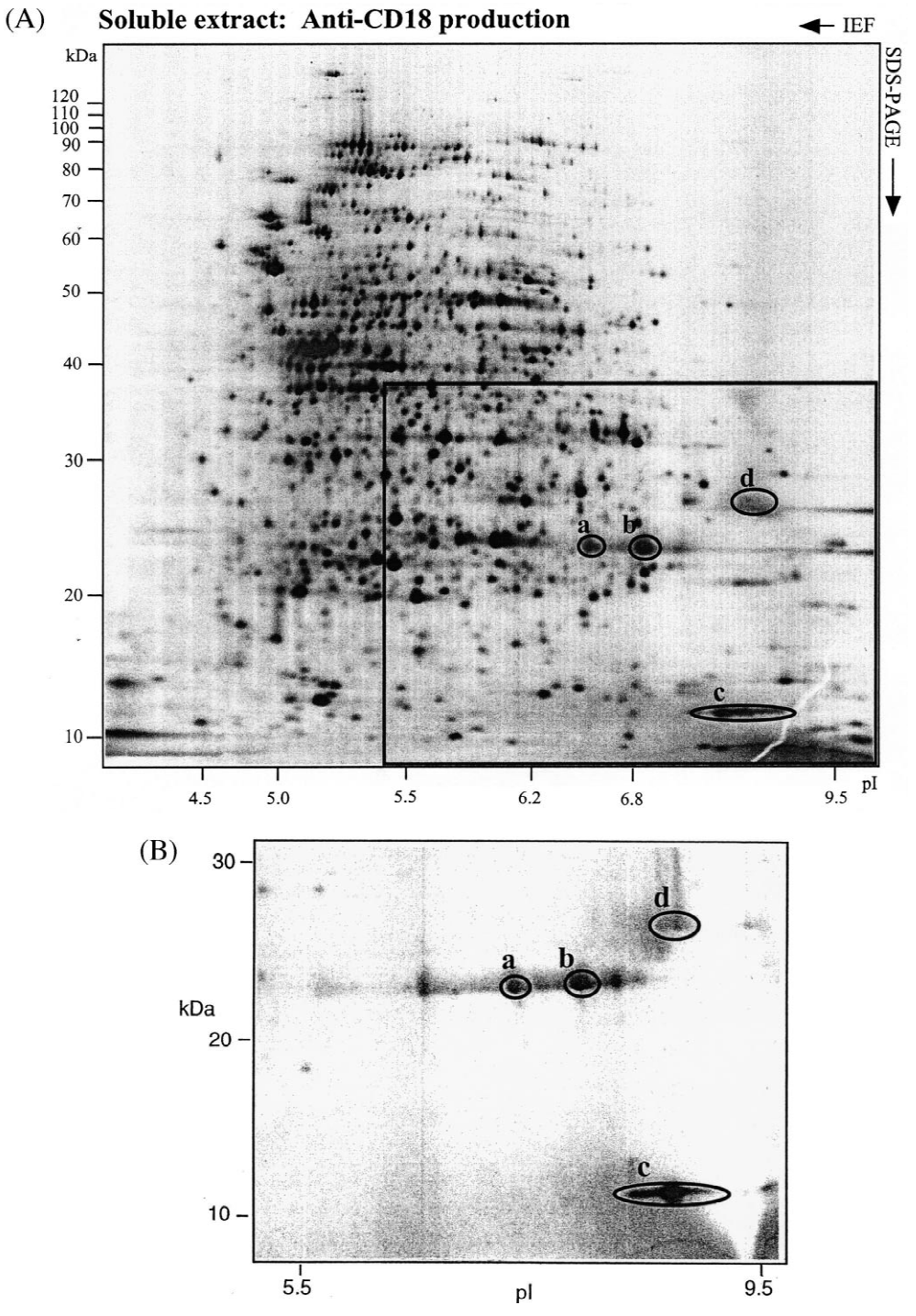

Fig. 6. (A) 2DE analysis (silver stained) of the soluble proteins from anti-CD18LZ produced in *E. coli*. Circled are the product-related proteins. The gel is orientated with the high-molecular-mass proteins toward the top and the acidic proteins to the left. (B) 2DE analysis of the material captured by the AME5 affinity column. Circled are the product-related proteins. Inset to lower right hand corner of 2DE gel as shown in (A). IEF, isoelectric focusing; kDa, kilodaltons.

was intact light chain, c was truncated light chain, and d was heavy chain, which is partially soluble. The reducing, denaturing conditions that the gels are run under dissociate the light and heavy chains. Material recovered by the immunoaffinity step from the *E. coli* production run was also analyzed by 2DE (inset Fig. 6A). Comparison of the 2DE profiles for the production run cell extract (Fig. 6A) and material captured by the AME5 affinity column (Fig. 6B) showed that the AME5 affinity column captures the bulk of the soluble, product-related variants expressed in *E. coli*. Since the AME5 affinity column captures the bulk of the soluble, product-related variants (that are then resolved by reversed-phase analysis) this assay format provides a simple means for determining the capture efficiencies of other affinity columns. This is simply achieved by changing the affinity column (to Protein G, Protein A or any other ligand) and comparing the reversed-phase profiles.

3.5. Product characterization from E. coli cell extracts

The reversed-phase section of the dual column analysis of crude anti-CD18LZ cell extract is shown in Fig. 4. Seven peaks are observed (labeled 1–7). These are baseline resolved except for the partial resolution of peaks 5 and 6. All peaks can be baselined resolved if a longer, shallow gradient is run (data not shown). Peak 1 was observed in the blank, had very little 280 nm absorbance, and was presumed to be non-proteinaceous material. Peaks 2–6 were partially characterized using N-terminal sequence analysis, mass spectrometry, SDS–PAGE analysis and immunoblotting.

Sequence analysis of the material from peaks 2, 3 and 4 indicated a single N-terminal sequence the same as that expected from the light-chain of anti-CD18, i.e., DIQMTQ. These three peaks all showed a positive immune reaction with anti-light chain antibody but not with anti-heavy chain antibody in immunoblots.

Peak 2: LC–MS data showed three significant molecular masses of 12 487.1, 12 388.2 and 12 061.5, with the first in much greater abundance than the other 2. These three masses are in good agreement for light chain amino acid (aa) sequences 1–115 (expected mass=12 488.9), aa 1–114 (expected mass=12 389.8), and aa 1–110 (expected mass=12 063.5), respectively. Considering the combined data, peak 2 was assigned to be truncated light chain.

Peak 3: LC–MS data contained four significant molecular masses of 23 729.9, 23 753.9, 23 570.5 and 23 484.0, all in similar abundances. SDS–PAGE analysis of Peak 3 material resulted in a single band with apparent molecular mass of 27 000. This immuno-blotted with anti-light chain antibody but not anti-heavy chain. The M_r 23 484.0 component is probably the sodium adduct of glutathione, linked through a disulfide bond to the C-terminal cysteine of the light-chain. The theoretical mass of this variant is 23 482. The causes of the other small differences in mass were not identified. Considering the combined data, peak 3 was assigned as light chain and its variants.

Peak 4 had the expected light chain N-terminal sequence, immuno-blotted with anti-light chain but not anti-heavy chain and had a mass of 46 906. After reduction using dithiothreitol, only a single mass of 23 454 was found. This is the expected mass of the light-chain. Thus peak 4 was assigned as light-chain dimer.

Peak 5 contained both light and heavy chain N-terminal sequences in equal molar concentrations. This material showed positive immune reaction when immuno-blotted for both heavy and light chain. Three molecular masses (49 480.1, 51 364.8 and 52 832.4) in near equal amounts were detected by LC–MS. These masses agree within experimental error for variants produced by truncating the C-terminus of the heavy chain of 33, 18 and six residues, respectively. Peak 5 was assigned as Fab′LZ with C-terminal truncation.

Peaks 6 also contained both light and heavy chain N-terminal sequences in equal molar concentrations as well as immuno-blotting for both heavy and light chains. It was a single component by LC–MS with a mass within experimental error of the expected mass of the product, i.e., anti-CD18 Fab′2LZ, mass 107 028.

Peak 7 was recovered in insufficient amounts for characterization.

The analysis and characterization of an *E. coli* cell extract from anti-TF Fab′2, production fermentation

was carried out in a similar manner as described above for anti-CD18 Fab′2LZ. Variants similar to those found in anti-CD18 Fab′2LZ were identified, i.e., a light-chain fragment, free light chain, light-chain dimer, Fab′ and Fab′2. The elution order of these variants were the same as for anti-CD18 Fab′2LZ but retention times were slightly longer for the light-chain fragment, free light chain, and light-chain dimer variants of anti-TF Fab′2 (Fig. 7A). The differences in retention times are a result of differences in amino acid sequences between the two molecules. There is 93 and 85% sequence homology between the light chains and heavy chains, respectively.

Similarly, we analyzed and characterized multiple *E. coli* cell extracts from fermentations in which antibody fragments were expressed. The same class of variants as found in anti-CD18 Fab′2LZ and anti-TF Fab′2 were again found i.e., a light-chain fragment, free light chain, light-chain dimer (as well as the fully assembled form, either Fab, Fab′2). Fig. 7B illustrates this using the analysis of anti-VEGF Fab. Again, these variants were near base line resolved and all had the same general elution order (but different retention times) as those observed in the analyses of anti-CD18 Fab′2LZ and anti-TF Fab′2. To date, all *E. coli* cell extracts from fermentations expressing antibody fragments have contained these variants and based on gel electrophoresis results these variants comprise the majority of the soluble product-related variants produced during these fermentations.

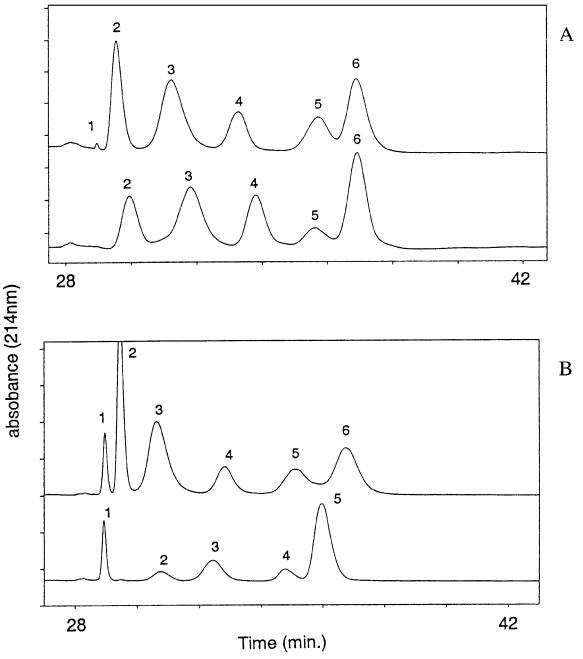

Fig. 7. (A) Chromatograms (reversed-phase section of the dual column assay) comparing the analysis of anti-CD18 Fab′2LZ (upper profile) and anti-TF Fab′2LZ *E. coli* cell extracts. Reversed-phase column temperature was 60°C. Similar numbers represent similar classes of fragments. (B) As (A) but comparing the analysis of anti-CD18 Fab′2LZ (upper profile) and anti-VEGF Fab *E. coli* cell extracts. Reversed-phase column temperature was 70°C.

3.6. Quantitation of full-length antibody from production media

In Sections 3.3 and 3.4 we demonstrated that the assay could quantify antibody fragments from diluent, and *E. coli* cell extact. Additionally, we quantified purified full-length antibodies that had been spiked into diluent (Section 3.3). In this section we report the analysis of a full-length antibody from its production media.

Anti-TF (full-length antibody) is produced in CHO cells and is secreted directly into the cell culture medium. No cellular disruption is required to recover the product. A typical chromatogram (reversed-phase section) for the analysis of anti-TF HCCF is shown in Fig. 8, lower profile. Four peaks are observed. Characterization (data not shown) indicated that the first peak (29 min) was non-proteinaceous. The peaks at 31.6 and 33.8 min were anti-TF fragments. The full-length antibody elutes at 37 min. Co-eluting with the full-length antibody are variants lacking either one or both light chains. As such, the assay can only quantify full-length antibodies when these light chain-deficient variants are absent or present in low amounts. We are currently investigating reversed-phase conditions to resolve these variants.

We did not have samples of full-length antibodies expressed in *E. coli,* since at this time full-length antibodies were produced in CHO cells. Recovery of product expressed in *E. coli* requires cellular disruption to release the product. Disruption also releases cellular proteins and makes analysis of these prod-

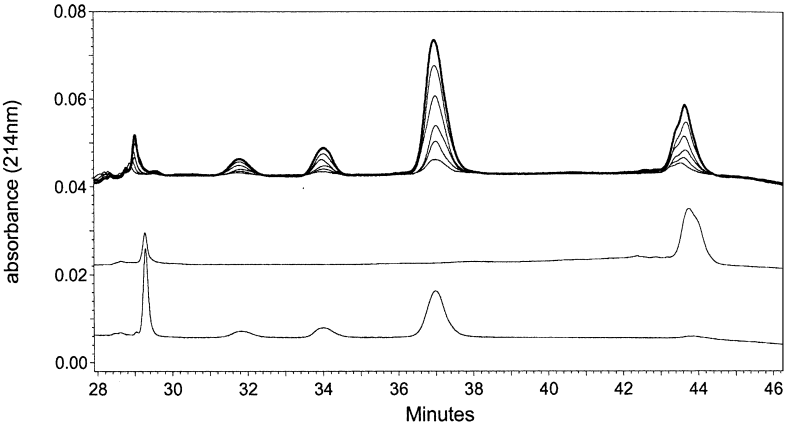

Fig. 8. Analyses of anti-TF (full-length antibody) HCCF (lower), *E. coli* blank run extract (middle) and co-mixes of anti-TF HCCF and *E. coli* blankrun (overlaid, top). Chromatograms show the reversed-phase section of assay.

ucts much more complicated. Anticipating the expression of full-length antibodies in *E. coli* we generated a sample to simulate this so we could test the assay under these conditions. To this end, we mixed anti-TF (full-length antibody) HCCF with *E. coli* blank material (1:1, v/v). (A blank run sample is one in which the cells carry a plasmid that lacks the product gene but where all other fermentation conditions are kept the same as in a production run. Such a sample is a representation of all the ECPs produced by the cells). These mixtures (HCCF and *E. coli* blank) were analyzed at various loads (10–90 μl) and the chromatograms (reversed-phase section) are shown overlaid in Fig. 8, upper profiles. For comparison purposes, the chromatograms of the *E. coli* blank and HCCF are shown (lower two profiles, respectively). The co-mix samples contained all the components expected and in the expected amounts. The three peaks (retention times 31.6, 33.8 and 36.8 min) were integrated and plotted versus load volume and all had regression coefficients greater than 0.99. These results indicated that the assay could quantitate these variants in a typical *E. coli* cell extract mixture. We are thus optimistic that the assay will be suitable to quantify the smaller fragments derived from a full-length antibody produced in *E. coli*. As noted earlier, the full-length antibody and the higher-molecular-mass variants (i.e., light chain deficient) coelute in this assay.

The analysis of the *E. coli* blank (Fig. 8, middle profile) resulted in a broad peak eluting between 43 and 44 min. This was identified as ECPs by 2DE. This peak was also seen in the co-mix. Previous analyses of anti-CD18LZ *E. coli* extracts (Fig. 4) resulted in 2% of this late eluting peak (No. 7), thus such a large peak was unexpected. Furthermore, early in the development process the analysis of a similar blank run sample indicated that essentially no ECPs were being carried through to the reversed-phase column. Hence, to observe increased amounts of ECPs in the assay with this sample was very surprising and clearly indicated that nonspecific adsorbtion had increased. These results suggested that the two blank run ECP pools were different. This difference was eventually traced to different extraction procedures, especially the use of lysozyme (see Section 2.5 for differences in sample preparation methods). The inclusion of lysozyme in the extraction procedure reduced the amounts of ECPs that were binding non-specifically in the assay. Additional analyses of HCCF–*E. coli* blank mixtures with the inclusion of 1 mg/ml lysozyme resulted in the ECP peak being reduced from 40 to less than 4% of the total area (chromatograms not shown).

4. Conclusion

Despite the fact that SDS–PAGE is usually complicated by the presence of host cell proteins of similar molecular mass to the product of interest, this method is commonly used to monitor fermentation

processes for biopharmaceutical production. Since product titres are usually low it is necessary to use silver staining to increase sensitivity. When analyzing these electropherograms there is the natural tendency to correlate darker bands with greater amounts of material even though it is well known that silver staining is not quantitative. Many times this general correlation has been true which tends to only validate the practice, but in others it has been very misleading. Now through the use of the dual column assay we can obtain quantitative data on the product and related variants produced in the fermentation.

We observed the complex mixtures of host cell proteins using the high resolving capabilities of 2DE in large format gels. This allowed us to clearly observe intact light chain, a p*I* isoform of intact light chain, a truncated form of light chain and the partially soluble heavy chain. Using 2D electrophoresis we showed that the dual column assay captures the bulk of the product and soluble product-related variants produced in the fermentation. All *E. coli* cell extracts of antibody fragment preparations contained a light-chain fragment, free light chain, and light-chain dimer as well as the fully assembled molecule. The total capacity of the affinity column is 200 μg, but to ensure complete capture the target load should not exceed 30 μg. We have developed reversed-phase separation conditions that produce symmetrical peak shapes with excellent recoveries for Fab, Fab′2 and intact antibodies while operating with shallow (resolving) gradients. For these reversed-phase analyses we have demonstrated the dramatic effect of temperature on peak shape and resolution. Using these conditions we can base line resolve all the major fragments expected in the cell extract of recombinantly produced antibody fragments. These same fragments can be resolved from a full-length antibody. At this stage, high-molecular-mass variants that are lacking either one or both light chains cannot be baseline resolved from a full-length antibody. In order to maintain resolution on the reversed-phase column, no individual component should exceed 10 μg. Detection was performed by measurement of absorbance at 214 nm. The limit of detection was 0.5 μg, in volumes up to 0.5 ml. Quantitation was reproducible, with a relative standard deviation of 3%.

Before the introduction of this dual-column assay, multiple techniques were required to monitor product titers in fermentations. With this assay we remove all of the interfering host cell proteins while retaining and quantitating the product, product variants and precurser molecules and in doing so we can accurately assess the fermentation process and hence aid its development. In addition to quantitative data, this assay has provided a readily scaleable method for the recovery of these components for characterization. This is especially useful for preparing samples for micro-heterogeneity analysis as these techniques are often not suitable for use with crude cell extract samples.

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