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Role of high-performance liquid chromatographic protein analysis in developing fermentation processes for recombinant human growth hormone, relaxin, antibody fragments and lymphotoxin

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Abstract

Development of efficient and reliable fermentation processes for protein pharmaceuticals is aided by the availability of accurate quantitative and qualitative product analyses. We have developed a variety of single and dual column chromatographic separations that meet the needs of process development and examples will be provided of how the resulting data has been used to optimize the culture process. For single column methods, reversed-phase chromatography has been the most versatile, permitting the reliable quantitation of many yeast, Chinese hamster ovary (CHO) cell and *Escherichia coli*-expressed products in the matrix of culture broth or cell extract. Analysis of secreted human growth hormone synthesized in *E. coli*, along with clipped and unprocessed forms, will be discussed. Another reversed-phase assay for direct analysis of a peptide product (B-chain relaxin) and its degradation products secreted into *E. coli* fermentation medium has allowed the purification of the responsible protease. Cation-exchange has proven extremely useful for the direct analysis of antibody fragments synthesized in *E. coli*, allowing the separation and quantitation of the desired Fab′ and Fab'_2, as well as the unwanted products of glutathione addition and translational read-through. Assay development is often complicated by the presence of host proteins with chromatographic behavior that is similar to that of the product. Commercial instrumentation now permits the facile development of multidimensional chromatographic assays. We show examples of coupled receptor affinity–reversed-phase assays for a mistranslation product and for covalent multimers of *E. coli*-synthesized lymphotoxin.

Keywords: Fermentation processes; Proteins; Growth hormone; Relaxin; Lymphotoxin; Antibodies

1. Introduction

Liquid chromatography is widely recognized to play a vital role in the quality control analysis of purified, formulated protein pharmaceuticals [1]. By assaying product macro- and micro-heterogeneity, information about process consistency and product stability can be obtained. Protein variants including misfolded, deamidated, aggregated, proteolyzed and oxidized forms can arise at any step in the manufacturing or storage of the pharmaceutical. By determining the source and cause of a particular variant, it is often possible to limit or prevent its formation, thereby maximizing the yield of the desired form.

During development of the fermentation process for a new protein, researchers typically rely on gel

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electrophoresis (with protein staining or immunodetection) and immunoassays for information about product quality and titer in the culture. Electrophoresis can reveal only variants with significant molecular mass differences and then only when they are present at high enough levels. Reliable quantitation is often difficult due to interference from host proteins with similar mobility. Immunoassays can be highly misleading when used to quantitate product in a complex matrix, such as solubilized *E. coli* cells or culture medium from Chinese hamster ovary (CHO) cell culture. Among the problems that can be encountered include overquantitation of variants, such as proteolytic fragments or aggregates, and interference from host proteins or solubilizing agents. In some cases, the expressed protein is not the final product (for which antibodies are available) but is a fusion between the desired product and another polypeptide added to facilitate translation or translocation. For all their shortcomings, electrophoresis and immunoanalysis provide for high throughput, parallel processing of many samples, a desirable feature for assays supporting process development.

Although high-performance liquid chromatography (HPLC) can reveal subtle modifications of molecular structure and provide for accurate quantitation of the product and its variants, it is not routinely used for product quality analysis of fermentation products. There are several reasons for this. The high efficiency packings used are easily fouled by unknown components found in fermentation samples and crude protein extracts, limiting assay robustness. The complexity of these samples, containing numerous host proteins, media nutrients and metabolic byproducts, can result in serious chromatographic interference with the analyte of interest. Finally, unlike more "universal" methods like polyacrylamide gel electrophoresis (PAGE), each HPLC assay needs to be developed individually in order to maximize its performance, utility and reliability. Despite these problems, several examples have appeared in the literature of HPLC assays for recombinant proteins in the matrix of crude *E. coli* extract or broth [2–4].

We have developed numerous chromatographic assays for proteins produced in *E. coli*, yeast, and mammalian cell culture. Depending on the product and expression system, the molecule may reside internally as inclusion bodies or be secreted into the culture medium or cellular periplasm. In this paper, we will show examples of how the choice of column, mobile phase and sample preparation can prolong assay reliability and provide specific process information for those proteins made in *E. coli*. In each case, we will also demonstrate how the availability of this analytical information has allowed improvements in the fermentation process to be made, leading to more consistent production or to a higher quality product entering the recovery process stream.

2. Experimental

2.1. Neutral pH separation of rhGH variants from E. coli extracts

Unless otherwise noted, all separations in this study were carried out on a Waters (Milford, MA, USA) HPLC consisting of two model 510 pumps, a WISP 712 autosampler, with gradient control and data acquisition by a Maxima 820 workstation or with Waters ExpertEase software. A Kratos model 783 UV monitor was used for detection. Unless otherwise noted, all reagents were of analytical grade or the best available. Acetonitrile was of HPLC grade from either J.T. Baker (Phillipsburg, NJ, USA) or Burdick and Jackson (Muskegon, MI, USA). Deionized water was obtained using a Milli-Q system (Millipore, Bedford, MA, USA) and all mobile phases were filtered prior to use with 0.2 μm nylon filters (Millipore).

Periplasmic contents are extracted by osmotic "shock". Frozen *E. coli* paste is suspended in six volumes of 10 m*M* Tris–HCl, pH 7.8, by vortex-mixing at room temperature for 30 min. The extract is clarified by centrifugation in a microcentrifuge at 12 000 *g* and the rhGH variants in the supernatant are resolved on a 250×4.6 mm Polymer Labs (Amherst, MA, USA) PLRP-S column (300 Å pore size, 8 μm particle size). Mobile phase A contains 10% acetonitrile, 35% 30 m*M* ammonium bicarbonate, pH 7.8, and 55% water. Mobile phase B contains 70% acetonitrile, 20% 30 m*M* ammonium bicarbonate, pH 7.8, and 10% water [8]. A gradient containing linear segments of 40–50% B over 5 min followed by 50–75% B over 30 min was used. The

flow-rate was 0.5 ml/min, the column temperature was maintained at 52°C and detection was based on absorbance at 280 nm.

In several of the separations described in this report, elevated temperatures were used to optimize the columns' selectivity and obtain the required resolution of analyte from host proteins [39]. The columns we used appear to be sufficiently durable to withstand these conditions and give acceptable assay lifetimes. In all of the assays described in this report, sample stability was checked by repeated injection of a single prepared sample at widely spaced intervals throughout an assay set (with times of up to 24 h). We have not observed significant losses in analyte. If necessary, a refrigerated autosampler can be used, however, high concentrations of denaturing agents in the sample may precipitate at low temperature.

2.2. Acid pH separation of rhGH variants from E. coli extracts

Total protein is extracted by sonication of cell paste suspended at approximately 25 mg/ml in 2% sodium dodecyl sulfate (SDS; Research Organics, Cleveland OH, USA), 50 m*M* Tris–HCl, pH 7.8. Ultrasonic disruption was carried out with a Heat Systems (Ultrasonics, Plainview, NY, USA) unit equipped with a micro tip, using 10–15 pulses at a 40% duty cycle at the maximum power setting for that tip. Extract is clarified by microcentrifugation at 12 000 *g* and supernatant samples are reduced with 0.1 *M* dithiothreitol (DTT, Boehringer Mannheim, Indianapolis, IN, USA) prior to injection of 50 μl aliquots.

Chromatography is performed on the following columns in series; SDS removal cartridge (10×3 mm, Michrom BioResources, Auburn, CA, USA), Zorbax Stablebond C_3 guard cartridge (300 Å pore size, 5 μm particle, 12.5×4.0 mm) and Zorbax C_3 Stablebond 250×4.6 mm analytical column (300 Å pore, 5 μm particle size, MAC-MOD Analytical, Chadds Ford, PA, USA). Mobile phase A contained 0.12% trifluoroacetic acid (TFA; Sequenal grade, Pierce Chemicals, Rockford, IL, USA) in water; solvent B contained 0.1% TFA in acetonitrile. The flow-rate was 1 ml/min, the column temperature was maintained at 50°C and UV detection was at 214 nm. The multiple segmented gradient system used for reduced samples was 35–53% B over 5 min; 53–56.5% B over 35 min; 56.5–95% B over 2 min; 95% B for 2 min; 95–35% B over 3 min. The next sample could be injected following re-equilibration for 8 min (for a total run time of 55 min). The analytical guard cartridge was replaced each time the assay was run (about every twenty samples) and the SDS removal cartridge was replaced after ~200 injections. During assay development, a blank cell extract (from host cells containing a plasmid without the recombinant gene and grown under identical conditions) was mixed with purified product to determine the effect of the matrix on assay range, linearity and recovery. Standards of the analytes of interest in the blank matrix were run on a daily basis to determine the performance of the column and the assay.

2.3. Separation of B-chain relaxin and its proteolytic degradation products in E. coli culture supernatants

E. coli fermentation supernatants are diluted with an equal volume of 8 *M* guanidine–HCl–50 m*M* Tris–HCl, pH 8.0–10 m*M* EDTA and are then reduced by addition of DTT to a final concentration of 100 m*M*. Recombinant relaxin standards are diluted into an *E. coli* fermentation blank supernatant prior to denaturation and reduction in order to generate an assay standard curve. When the standard was prepared in this way, a linear response was obtained. For a product in fermentation broth, the titer is expressed as mass per unit volume (i.e. g/l).

A 1 *M* triethylammonium acetate (TEAA) stock is made from 14 ml of HPLC-grade triethylamine (gold label, Aldrich, Milwaukee, WI, USA), 5 ml of glacial acetic acid (Mallinkrodt, Paris, KY, USA) and 99 ml of water. The pH is adjusted to 7.5 with acetic acid. Mobile phase A contains 1% TEAA stock in water (v/v); solvent B contains 0.5% TEAA stock–19.5% water–80% acetonitrile (v/v). Chromatography is carried out on a Polymer Labs PLRP-S column (300 Å pore, 8 μm particle size, 150×4.6 mm) equipped with a PLRP-S guard cartridge (5.0×3.0 mm). A multistep gradient is performed as follows; 10–30% B over 1 min, 30% B for 2 min, 30–45% B over 5 min, 45–65% B over 8 min, 60–90% B over 1 min, 90–10% B over 3 min. The flow-rate was 2 ml/min,

the temperature is maintained at 50°C and detection was at 214 nm.

2.4. Cation-exchange separation of E. coli-expressed humanized antibody fragments against CD18

Total soluble protein is released from *E. coli* (typically at about 100 mg paste/ml of extraction buffer) by treatment with 20 μg/ml hen egg lysozyme (Sigma, St. Louis, MO, USA) in 0.2 *M* Tris–HCl, pH 8, in the presence of 2 m*M* EDTA and 2 m*M* iodoacetamide (Sigma) for 15–30 min at room temperature. Samples are sonicated as described above for rhGH (Section 2.2) and then extracts are clarified by microcentrifugation at 12 000 *g*. Samples are diluted with 3 volumes of deionized water and then 200 μl of each is loaded onto a Bakerbond Carboxy
Sulfon column (J.T. Baker, Phillipsburg, NJ, USA; 300 Å pore size, 5 μm particle, 50×4.6) equipped with a 10×4.3 mm Bakerbond CarboxySulfon guard cartridge (MetaChem Technologies, Torrance, CA, USA). Mobile phase A was composed of 1% 0.5 *M* sodium phosphate, pH 7.0, 94% water and 5% 2-propanol (v/v); solvent B consisted of 20% 0.5 *M* sodium phosphate, pH 7.0, 75% water and 5% 2-propanol (alcohol is added to inhibit microbial growth in buffers). A 30-min gradient from 0–50% B followed by a 2 min gradient from 50–100% B is run at 1 ml/min, at ambient temperature, with detection at 280 nm. The total run time is 42 min.

Post-extraction production of glutathione–Fab′ was carried out by lysing *E. coli* cells as described above with lysozyme, EDTA, Tris–HCl (but without iodoacetamide) and sonication. At varying times following cell lysis, iodoacetamide is added to a final concentration of 2 m*M*. Cell debris is removed by centrifugation and the supernatant is analyzed by CarboxySulfon chromatography for Fab′ and GS–Fab′.

2.5. Tryptic mapping of a high mass Fab′ variant

A 0.8-g amount of *E. coli* paste was extracted in 5 ml of 0.2 *M* Tris–HCl, pH 8.0, 2 m*M* EDTA, 20 mg/ml lysozyme, 7 m*M* iodoacetamide (Sigma). Following sonication and centrifugation, the extract was buffer exchanged into mobile phase A using a PD-10 column (Pharmacia, Uppsala, Sweden). Variant Fab′ peaks were collected from multiple 1.75 ml injections of extract on a 250×4.6 mm Bakerbond CarboxySulfon column using a modified gradient: Linear gradient segments of 0–15% B in 2 min followed by 15–20% B over 28 min and then 20–45% B in 15 min. The flow-rate was 1 ml/min at ambient temperature. Peak fractions from several runs were pooled and then reinjected using the standard method to test for peak purity.

A 100-μg aliquot of each pool was transferred to a microcentrifuge tube and dried. A 0.5-ml volume of unfolding buffer (6 *M* guanidine–HCl in 30 m*M* Tris–HCl, pH 7.5) and 10 μl of 1 *M* dithiothreitol (DTT) were added and incubated for 4 h at room temperature. At the end of this time, 10 μl of an iodoacetic acid solution (0.54 g/ml in 1 *M* NaOH) was added, and incubation continued for an additional 30 min. This solution was then dialyzed overnight against 10 m*M* N-(2-acetamido)-2-aminoethanesulfonic acid (ACES), 20 m*M* $CaCl_2$, pH 7.0. The samples were then transferred to microcentrifuge tubes and digested at 37°C with N-tosylphenylalanine chloromethyl ketone (TPCK)–trypsin (Cooper Biomedical) at an initial 2% (w/w) ratio. After 4 h, a second addition of 2% (w/w) enzyme was made. The digestion proceeded for an additional 4 h and was then terminated by freezing. Half of the digested protein, 50 μg, was loaded onto a reversed-phase HPLC column, (Vydac C_{18}, 250×2.1 mm, 300 Å pore size, 5 μm particle size; The Separations Group, Hesperia, CA, USA) on a Hewlett-Packard (Palo Alto, CA, USA) 1090 HPLC system controlled by a Hewlett-Packard Chemstation. The initial mobile phase was aqueous 0.1% TFA. Following a 5-min isocratic hold, there was a linear gradient from 0 to 45% of 0.08% TFA in acetonitrile (Burdick and Jackson) over 115 min (0.39%/min). The flow-rate was 0.25 ml/min, the column temperature was maintained at 45°C, and the absorbance was monitored at both 214 and 295 nm. All peaks were collected, and the amino acid sequence of the new peak in the basic Fab′ variant was determined using standard N-terminal sequencing methods.

2.6. Soluble vs. refractile lymphotoxin by reversed-phase HPLC

Soluble lymphotoxin (LT) is extracted by treatment with 20 μg/ml hen egg lysozyme–2 m*M* EDTA–0.2 *M* Tris–HCl, pH 8, followed by sonication (as described above for Fab′; Section 2.4). After centrifugation, the supernatant is removed to a second tube and the pellet is re-extracted with an equal volume of 8 *M* urea (Sequenal grade, Pierce) in 25 m*M* Tris–HCl, pH 8.0, with sonication. This solution is also clarified by centrifugation and the samples are analyzed on a Bakerbond C_4 column (300 Å pore size, 5 μm particle size, 250×4.6 mm) with a gradient of 40–50% B in 30 min. Mobile phase A contained 0.12% TFA (Sequenal grade, Pierce) in water; solvent B contained 0.1% TFA in acetonitrile (v/v). The flow-rate was 1 ml/min, the column temperature was 40°C and detection was at 214 nm.

2.7. Monomer, dimer and trimer lymphotoxin by affinity/reversed-phase HPLC

Two dimensional HPLC of soluble LT is performed on an Integral workstation (PerSeptive Biosystems, Framingham, MA, USA) using a 75×2 mm column of TNF receptor IgG [36] immobilized onto Protein A controlled pore glass (Bioprocessing, Durham, UK) with dimethylpimelimidate–HCl (Pierce) [40]. Cell extract is loaded and washed with phosphate buffered saline and eluted with 12 m*M* HCl. LT is captured on a Polymer Labs PLRP-S column (4000 Å pore size, 8 μm particle, 50×4.6 mm) and variants are separated with a gradient of 30–50% B in 5 min at a flow-rate of 2 ml/min at ambient temperature. Solvent A contained 0.12% TFA in water, solvent B was 0.1% TFA in acetonitrile (v/v). Detection is at 214 nm. Pre-kill sample is taken at the end of fermentation; post-kill is after tank and contents are heated to 60°C and immediately cooled to ambient temperature.

2.8. High resolution 2-D separation of LT and an Ile-to-Val mistranslation product

Extraction and affinity capture of LT are done as describe above. Prior to acid elution, the affinity column is washed with 2.5 ml of 0.5 *M* tetramethylammonium chloride (Sigma), 0.01% Tween 20, in 25 m*M* Tris–HCl, pH 7.3, with 150 m*M* NaCl to remove any non-specifically bound protein. The second, reversed-phase, dimension is performed on a Polymer Labs PLRP-S column (250×2.1 mm, 300 Å pore size, 8 μm particle). Solvent A contained 0.05% TFA (Pierce Sequenal) in water, solvent B was 0.05% TFA in acetonitrile (v/v). Gradient elution was as follows; 35–38.3% B in 8 min, 38.3–41.3% B in 15 min, 41.3–48.3% B in 7 min, 48.3–80%B in 0.5 min. The flow-rate was 0.4 ml/min, the column temperature was ambient and absorbance detection was at 214 nm.

3. Results

3.1. Human growth hormone

Recombinant human growth hormone (Nutropin, Somatotropin for injection) is expressed in *E. coli* downstream of a signal peptide which directs translocation of the growing polypeptide into the bacterial periplasm [5–7]. Signal peptidase cleaves off the hydrophobic 23 amino acid STII leader to yield the mature sequence with the correct N-terminus. Within the oxidizing periplasmic environment, the protein folds into the correct structure and the disulfide bonds are formed. During development of our fermentation protocol, two prominent variants were observed on SDS–PAGE. Through amino acid sequencing, these were identified as endoproteolytically clipped rhGH ("two-chain") [8] and product still containing the leader peptide ("unprocessed"). The first has a clip between T142 and Y143 with the resulting fragments still bound together by the disulfide bridge between C165 and C53 [8]. The relative proportion of each of these two variants was seen to vary with modifications in fermentation conditions, however, in the absence of a reliable, quantitative assay, it was impossible to determine which process was best and to assess process reproducibility. Immunoassays of total product titer often did not correlate well with final recovered yield, since these variants are removed during purification

and because interferences in *E. coli* extracts can lead to erroneous results.

Several reversed-phase methods were available for purified rhGH [8–10] and two of these were adapted to provide information about the two fermentation variants present in crude *E. coli* extracts. In acidic mobile phases [eg. TFA–acetonitrile (ACN)], there is excellent resolution of unprocessed from mature rhGH, but two-chain is not well resolved from the main peak. At neutral pH (e.g. ammonium bicarbonate–ACN, pH 7 [8]), rhGH is resolved from host proteins and from the two-chain and deamidated forms (Fig. 1A), however, under these conditions, unprocessed does not elute from reversed-phase resins and must be quantitated using a second, acid pH system (data not shown). Fig. 1B shows how the neutral pH reversed-phase assay was used to demonstrate that the periplasmic protease, degP [11,12] is responsible for the production of two-chain rhGH. Additionally, this assay allowed selection of a medium for manufacturing using the wild type host that minimizes product clipping (data not shown).

For routine monitoring of the production process and for further process development, a single sepa-

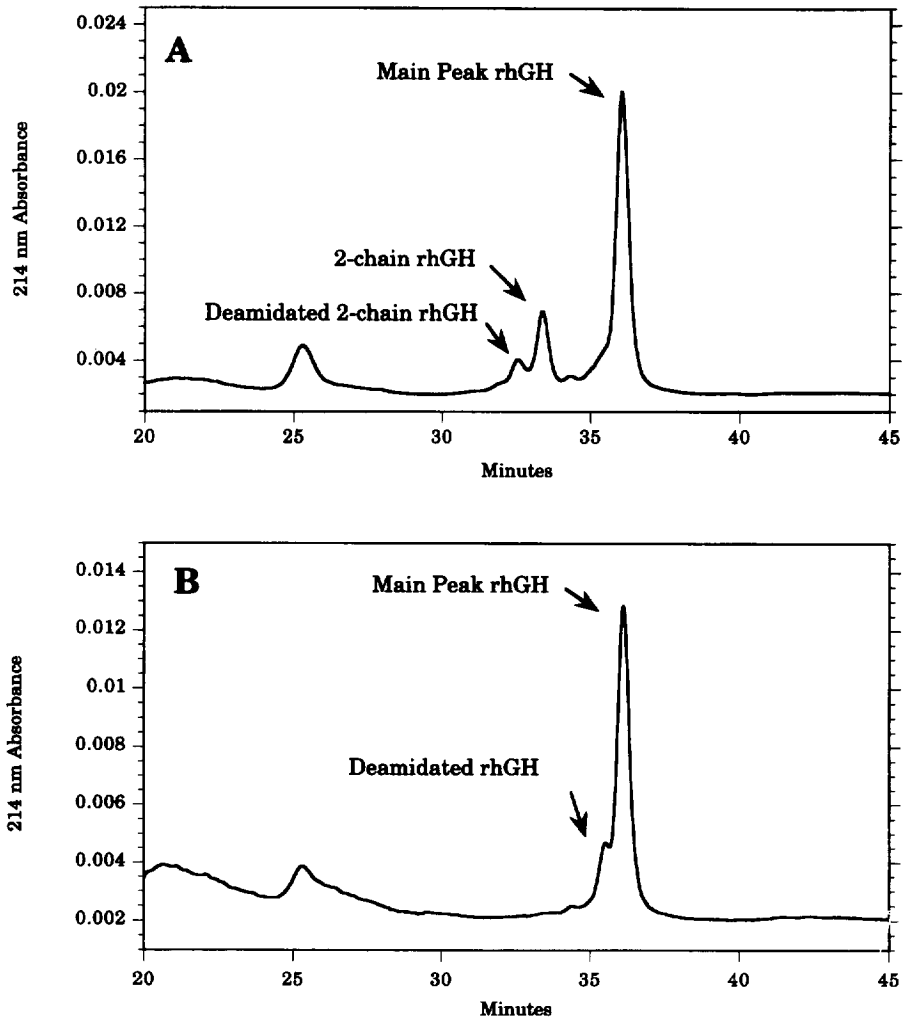

Fig. 1. Neutral pH reversed-phase HPLC of recombinant human growth hormone in *E. coli* cell extract from (A) the wild-type production host and from (B) a strain carrying a mutation in the gene for the degP protease [10]. Strains carry identical plasmids and are grown under the same conditions.

ration for these variants in cell paste was desirable. By optimizing the gradient, column selection, column temperature and sample pretreatment, we developed a method that permits the reliable quantitation of main peak rhGH and the two principal variants (Fig. 2).

In order to ensure the complete extraction of all variants, samples are sonicated in the presence of 2% SDS. This gives a reliable value for the titer of "main peak" rhGH and for unprocessed rhGH, which is not efficiently solubilized by any other chaotrope (data not shown). Although the use of SDS may overestimate the content of soluble monomer (by dissociating aggregates), it directly measures the total expression and indirectly indicates the reproducibility of the process. For these samples (and for the lymphotoxin and antibody fragment assays described below), product titers are calculated as mass of recombinant protein (in g) per mass of cell paste (in kg). In some cases, samples are also dried in a vacuum oven to a constant mass to determine the water content of the cell paste. This allows for

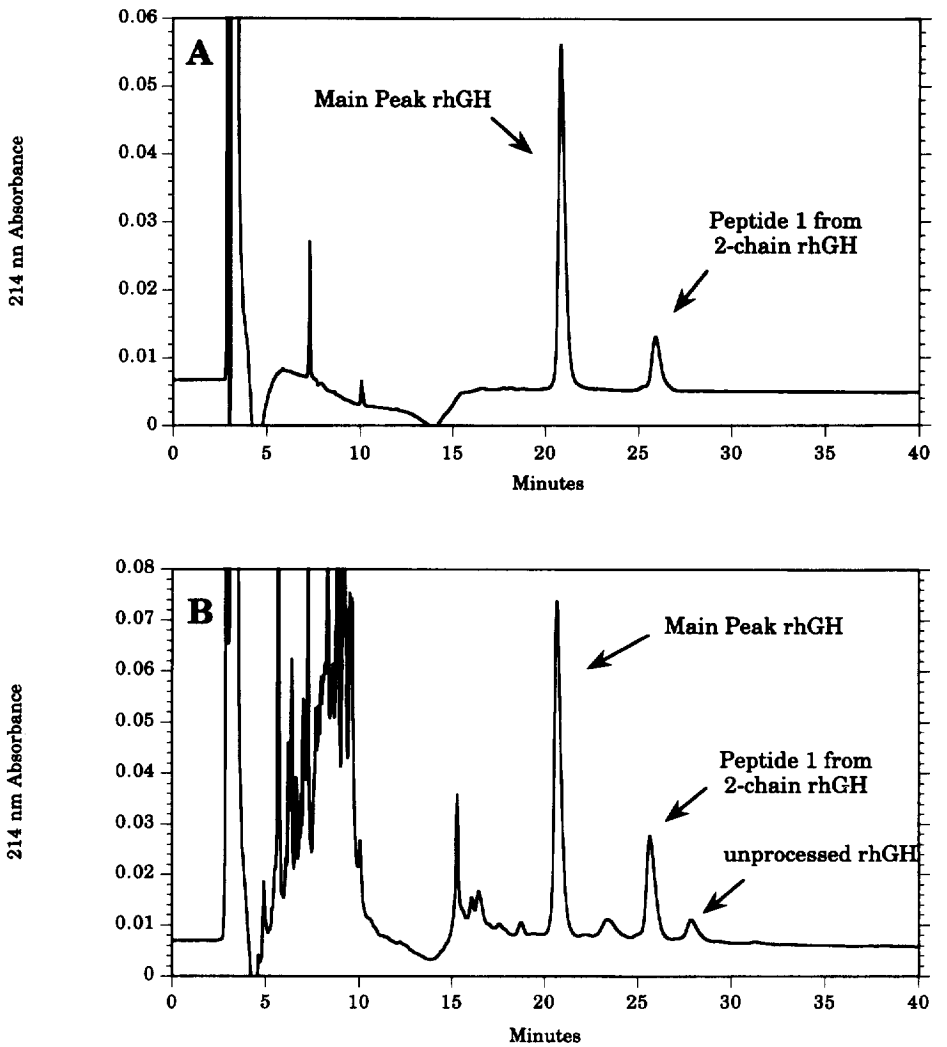

Fig. 2. TFA–acetonitrile reversed-phase HPLC method for rhGH variants. (A) SDS–DTT treated standards of rhGH and the two-chain form and (B) SDS–DTT extracted rhGH cell paste spiked with authentic two-chain rhGH showing resolution of unprocessed, two-chain, and authentic rhGH from *E. coli* host proteins.

expression of product titer as a percentage of the dry mass and eliminates differences in titer due to the operation of the large scale centrifuge. Titers may also be expressed using an equivalent cell mass measurement that is based upon the optical density at 550 nm, due to light scattering by a bacterial suspension.

In the case of rhGH, the inclusion of an in-line SDS removal cartridge before the guard cartridge and analytical column resulted in significantly improved resolution of the analytes [13,14]. This cartridge is regenerated at the end of each gradient by a wash with organic mobile phase and has an effective life of close to 200×50 μl injections. We have seen several peaks of unprocessed rhGH in some samples, likely due to disulfide-mediated aggregation. Reduction of the sample prior to injection converts the unprocessed protein to a single peak, and additionally converts the clipped form into the two resulting peptides. The 142 amino acid N-terminal peptide elutes between unprocessed and main peak growth hormone (Fig. 2B). The resulting method can be used to monitor the effects of process conditions on the accumulation of product variants and to assess process consistency in manufacturing. We find that after about twenty injections, the resolution begins to degrade, but replacement of the guard cartridge restores the separation to its initial state. Given the low cost of the guard cartridges, we do not attempt to regenerate them.

3.2. Relaxin B chain

Relaxin, a hormone involved in cervical ripening during late pregnancy, is made up of disulfide-linked A and B peptides [15]. Initial work on producing recombinant relaxin involved separate fermentation protocols for the two chains followed by in vitro chain recombination [16]. Unlike rhGH, expression of relaxin B chain downstream of a signal peptide leads to accumulation of the product in the culture medium, not in the periplasm. Attempts at producing this 29 amino acid peptide were complicated both by proteolysis of the product and by significant difficulty in quantitating it. Reversed-phase HPLC, using TFA–acetonitrile–water, of purified B29 and its primary N-terminal proteolytic fragments (B16 and B17) gave excellent results, however, five–ten injections of *E. coli* culture supernatant from a production run led to the rapid deterioration of peak shapes and to complete inability to quantitate the peptides. Using assay robustness as our end-point (i.e. number of injections until column "death"), numerous unsuccessful strategies were tried in order to improve assay longevity. These included the use of internal surface reversed-phase resins, two-column methods, sample pretreatments, and alternate resins. The use of in-line guard cartridges, which have proven effective in most other cases of column fouling, did not protect the analytical column from deterioration. Additionally, an assay requiring a change in guard columns every five injections would not be practical.

Ultimately, we found that a change in mobile phase to one containing acetonitrile and triethylammonium acetate at pH 7.5 led to robust and reliable chromatograms (Fig. 3B). Accurate quantitation was achieved by diluting the standard in "blank" culture supernatant from the same *E. coli* host and grown under identical conditions (Fig. 3A), but containing plasmid without product sequence. This leads to more reliable recovery of the standard and to standard curves that are representative of the sample in its matrix.

With this assay, it was possible to undertake the fractionation of culture supernatants and cell extracts and to isolate the periplasmic protease responsible for conversion of B29 to B16. The successful completion of this project led to its identification as the previously described protease, prc [17] or tsp [18], an enzyme responsible for the cleavage of penicillin binding proteins and other proteins with non-polar C-termini (Dorothea Reilly, unpublished results).

3.3. Antibody fragments (Fab′ and Fab'_2) produced in E. coli

CD18 is the common subunit of the LFA-1 family of integrins, including Mac-1, a cell surface adhesion molecule on leukocytes, which plays a central role in inflammation [19]. A murine monoclonal antibody against this protein has been humanized to investigate its potential utility in various inflammatory diseases [20] and it is being expressed in bacteria. Efficient processes for production of antibody frag-

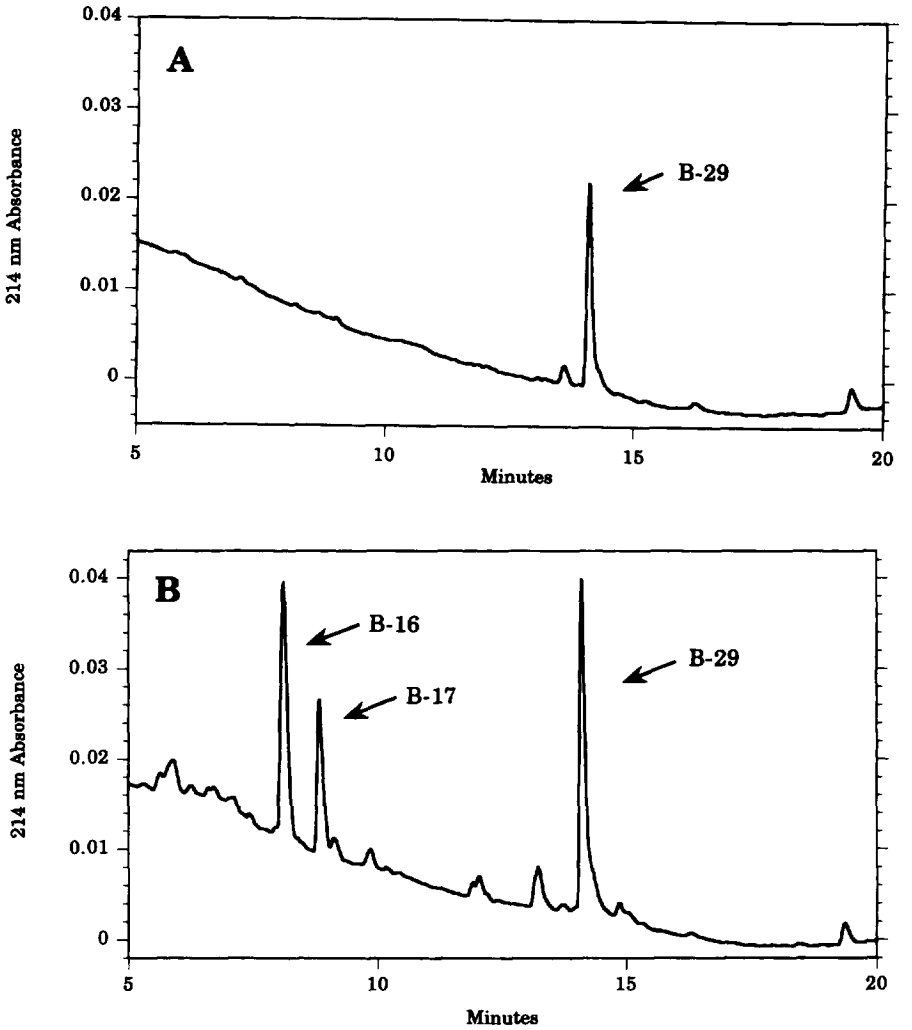

Fig. 3. Neutral pH reversed-phase chromatography of (A) reduced and denatured recombinant relaxin spiked into blank *E. coli* broth and (B) a process fermentation supernatant showing the B-chain product and proteolytic degradation forms.

ments (Fab′ and Fab'_2) in *E. coli* have been developed at Genentech. Co-expression of heavy and light chains of an antibody fragment with signal peptides on each chain results in their secretion to the periplasm, with subsequent folding, chain association and disulfide bond formation [21,22]. If the hinge region sequence of the heavy chain contains one or more cysteines, two Fab′s can potentially associate to yield disulfide bonded Fab'_2. As a human therapeutic, a Fab'_2 may be preferred due to its higher molecular mass and subsequently longer in vivo half-life. It was therefore important to develop a reliable analytical method to quantitate levels of Fab′, Fab'_2, and other possible product variants. A variety of methods existed for the analysis and purification of monoclonal antibodies, including anion-exchange, hydroxyapatite, size-exclusion and hydrophobic interaction [23]. More recently, several "mixed-mode" cation exchangers have been introduced specifically for this application [24–26]. We have found that one of these (Bakerbond Carboxy Sulfon) provides a specific, robust separation of *E.*

coli-expressed antibody fragments and serves as the basis of a reliable method for the quantitation of the desired product and several biosynthetic variants.

Although several alternative mobile phases are recommended in the literature on this support [26], we have found that a simple phosphate gradient (5–50 m*M*) at pH 7.0 gives excellent resolution of Fab′ and Fab'_2. *E. coli* has few abundant proteins with p*I*>6.5 so there is no significant interference from host components in the quantitation of these antibody fragments (this one has a p*I* of 8.3, based on isoelectric focussing).

Fig. 4 provides an example of the type of separation we have obtained from crude extracts of *E. coli* cell paste. In this application, cells are disrupted by sonication after treatment with lysozyme and EDTA in the presence of 0.2 *M* Tris–HCl, pH 8. In this case, iodoacetamide has been added to the lysis buffer (at 2 m*M*) to prevent post-extraction conversion of Fab′ to Fab'_2. The resulting chromatogram is complex, revealing a wide range of product variants. Using a number of techniques, including mass spectrometry, tryptic mapping and PAGE, we have characterized many of these forms.

The major peak seen in these extracts (at 16.3 min) corresponds to authentic Fab′, confirmed by SDS–PAGE, size-exclusion chromatography (SEC) and mass spectrometry. Fab'_2 is only a minor component of these extracts (with a retention time of 22 min) and post-extraction incubations without thiol-blocking reagents do not lead to increases in this species (see below). The two peaks eluting just after Fab′ (at 16.9 and 17.4 min) have identical molecular masses and can both be converted to forms with the same

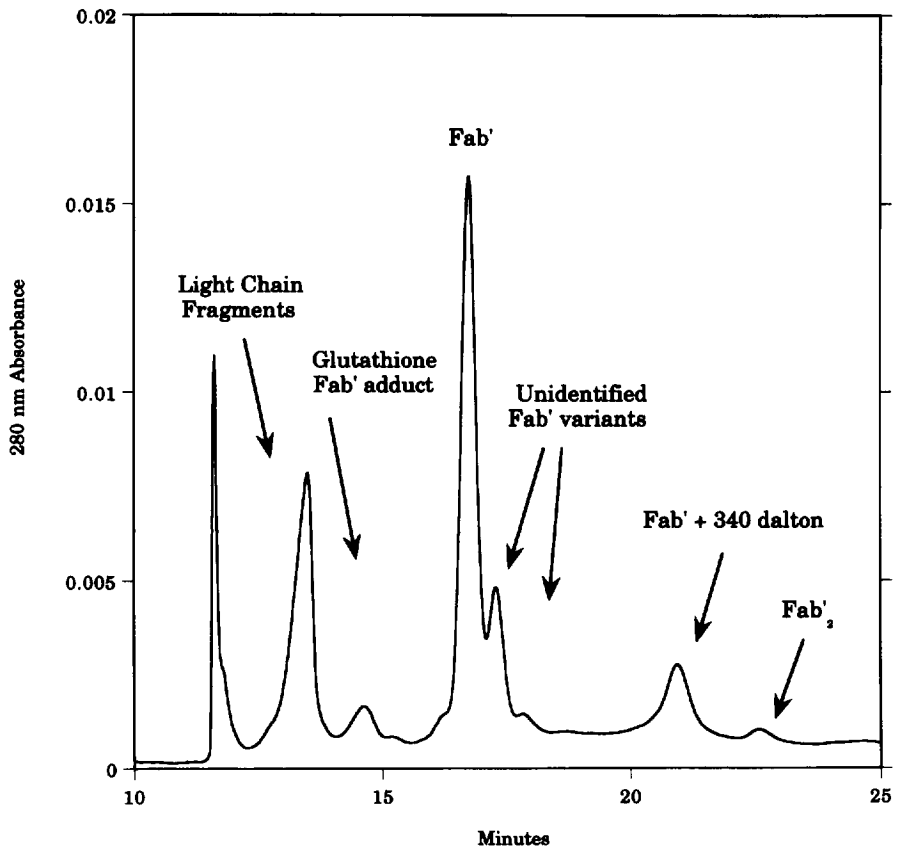

Fig. 4. Cation-exchange chromatography (on Bakerbond CarboxySulfon) of *E. coli* synthesized antibody fragments against CD18 showing principal product variants.

retention as Fab′ by treatment with dilute reducing agent. These may correspond to products containing incorrect disulfide bonds.

By ionspray mass spectrometry and amino acid sequencing, the prominent peak at 13.1 min was identified as two N-terminal fragments of light chain cleaved after S114 or V115 (data not shown). The peak at 14.3 min was originally seen as a significant component of samples taken from the purification process. It is usually a minor component of our fermentation samples and we have identified it as a species containing a mixed disulfide between glutathione and the Fab′ hinge cysteine (GS–Fab′). The initial assignment was made by electrospray mass spectrometry of the purified variant peak (of Fab′+306) and has been confirmed by MS–MS of the glutathione moiety, following reduction [27]. When iodoacetamide is added at varying times following the initial cell lysis, a time-dependent increase in this species is observed, concurrent with the disappearance of Fab′ (Fig. 5A–C), indicating that its formation primarily occurs post-fermentation. Fahey et al.

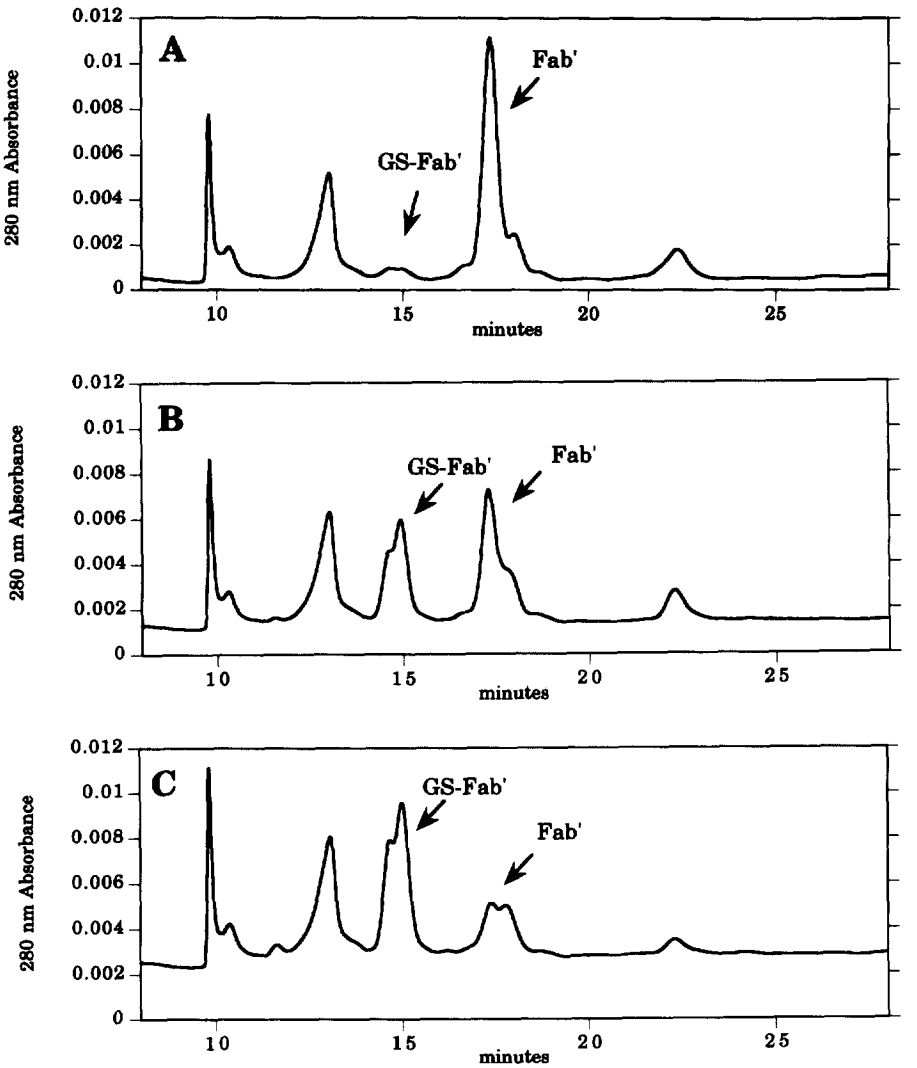

Fig. 5. Bakerbond CarboxySulfon analysis for monitoring the conversion of *E. coli* Fab′ to glutathione-Fab′. Cells are extracted without sulfhydryl protecting agent and iodoacetamide is added at varying times following cell lysis (A, 0 min; B, 30 min and C, 240 min).

[28] have reported glutathione levels in *E. coli* of 25–30 mmol/g dry mass. The observation that GS–Fab′ is formed post-extraction, while the inter- and intra- chain disulfides are formed during the culture, suggests that GSH–GSSG levels are low in the periplasm and that the mixed disulfide can only be formed after the contents of periplasm and cytoplasm are mixed during cell disruption. By using reversible thiol protecting agents during the extraction process, glutathione reaction with the free hinge sulfhydryl can be prevented and the ultimate yield of Fab'_2 from in vitro Fab′ dimerization is maximized.

The peak at 20 min can constitute up to 20% of the Fab′ area and its identity was determined using a variety of techniques. Following collection and desalting, its M_r was determined by electrospray MS to be 340 higher than that of the main Fab′ peak. Mass spectrometry following reduction indicates that the excess mass is still associated with the heavy chain (eliminating the possibility of a disulfide adduct). The correct N-termini were found by sequencing, so aberrant processing of the signal peptide was not responsible. Comparison of a tryptic digest of the purified peak with that of the Fab′ indicated that the C-terminal hexapeptide (THTCPP) was missing (Fig. 6A, lower trace). In its place, a new peptide

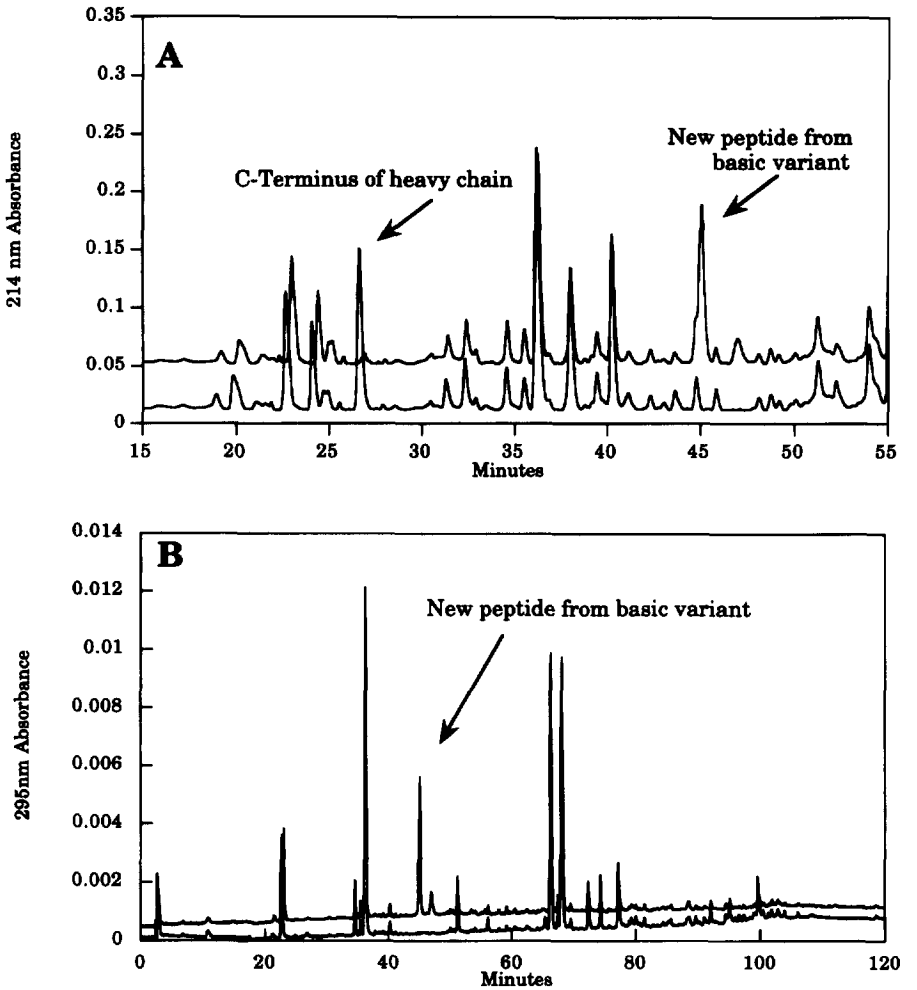

Fig. 6. Tryptic digests of preparatively collected CarboxySulfon fractions of authentic Fab′ (lower trace in each panel) and of a more basic Fab′+340 dalton variant (upper trace in each panel). Panel A shows a portion (from 15 to 55 min) of the two chromatograms monitored at 214 nm. In panel B, the same separations are monitored at 295 nm, indicating the presence of a new tryptophan-containing peptide at 44 min.

was seen (Fig. 6A, upper trace), which had significant absorbance at 295 nm, indicating the presence of a tryptophan (Fig. 6B, upper trace). Amino acid sequencing of the new peptide gave THTCPPWR. We believe that this form of the protein results from read-through translation of the opal stop codon with insertion of tryptophan [29]. In our plasmid, translation would continue six codons to the next stop (amber). This initial read-through product is a substrate for the optT protease present on the exterior of *E. coli* [30–32] and is cleaved to the Fab′+WR product we observe in cell extracts. This peak is not observed in an ompT⁻ host, where a broad peak with an even longer retention time is seen (data not shown). Substitution of an ochre stop codon for the opal eliminates accumulation of this variant.

3.4. Lymphotoxin (tissue necrosis factor-β)

Human lymphotoxin (LT, TNF-β) was purified and cloned into *E. coli* by Genentech researchers [33,34]. Its sequence is 30% homologous to tumor

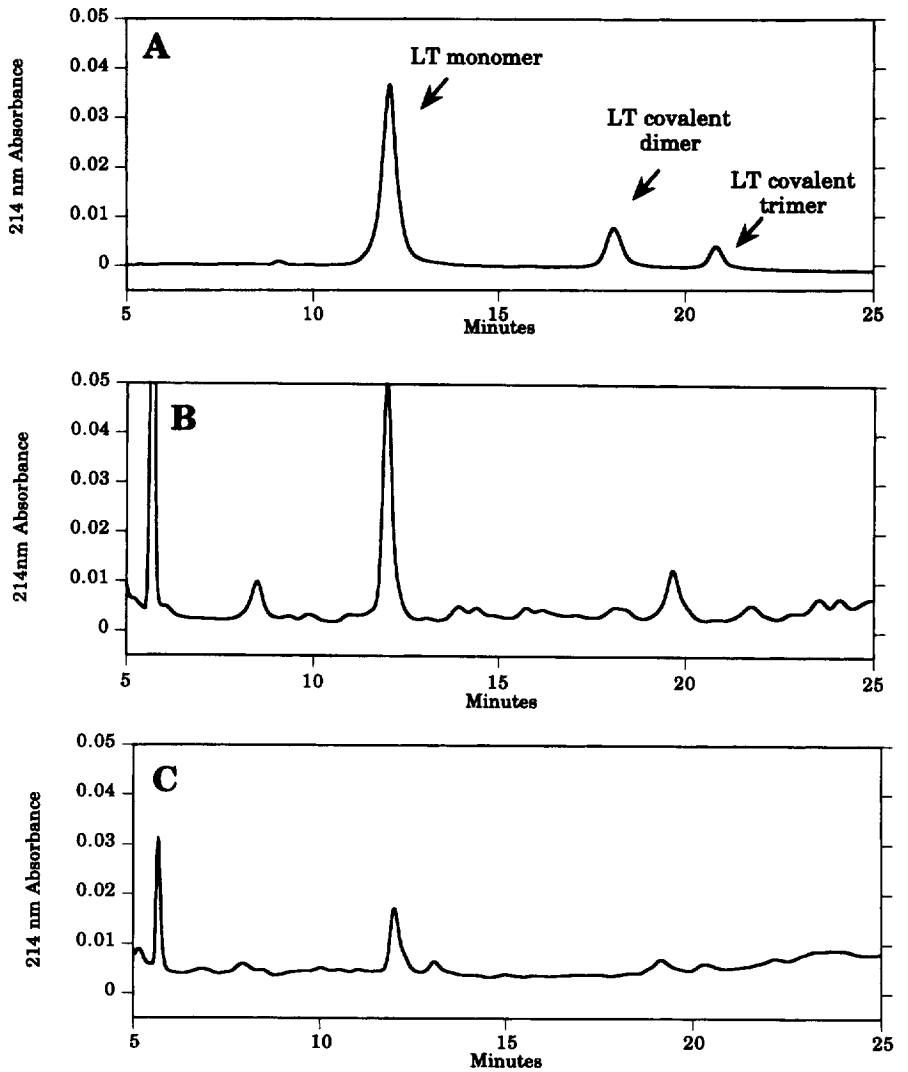

Fig. 7. Reversed-phase HPLC of purified recombinant human lymphotoxin containing high levels of covalent dimer and trimer (A). Soluble (B) and refractile (C) fractions are obtained from two-step extraction of an *E. coli* paste sample. In this case, 78% of the product is soluble and 22% is present as insoluble aggregate.

necrosis factor alpha, and both proteins bind to the same two receptors in vitro [35]. Like TNFα, it is a trimer of identical subunits and in various bioassays they both exhibit potent anti-tumor cytotoxicity. In order to evaluate LT in animal cancer models, we developed an efficient *E. coli* cytoplasmic expression scheme. A variety of chromatographic systems have been developed to determine product quality and quantity of this lymphokine.

A simple reversed-phase gradient system has provided robust, reliable quantitation of LT (Fig. 7). In addition, by modifying the extraction protocol, it has been possible to determine the distribution of the product in soluble, properly folded (Fig. 7B) and refractile, aggregated (Fig. 7C) forms. Since only the folded protein is active and recovered, it is important to evaluate the effects of plasmid construction and fermentation conditions on titer of recoverable (soluble) LT. Table 1 summarizes the results of several fermentation experiments analyzed by reversed-phase HPLC. Plasmids 2 and 3 are capable of much higher overall production (especially when grown at 30°C), however, the improved titer does not translate into recoverable yield since the soluble LT titer is only 50–80% of that seen with the first plasmid.

Unlike rhGH, which is more hydrophobic than the majority of the *E. coli* host proteins, LT elutes at a solvent strength close to that of several host proteins. Although this is not a problem when quantitating the "main peak" of a highly expressed protein like LT, it presents a challenge to the analysis of minor product variants. In order to improve the resolution and reliability of our assays, we have incorporated on-line affinity chromatography coupled to reversed-phase HPLC to remove the bulk of the host protein from the soluble LT. An immobilized immunoadhesin, composed of recombinant TNF receptor coupled to the Fc portion of a human IgG1 [36], provided a high affinity, robust and specific matrix for removing soluble LT from a complex mixture (*E. coli* extract). LT can be eluted from the TNFrIgG with acid and captured onto a reversed-phase resin with high efficiency. By manipulating reversed-phase conditions, we have obtained information about several unique product variants made during the fermentation.

Table 1
Reversed-phase HPLC analysis of fermentation cell paste samples extracted and analyzed for soluble (active) and refractile (aggregated) lymphotoxin as described in Section 2

Plasmid	Temperature	Lymphotoxin titer (mg/l/OD)		% soluble
		soluble	refractile	
Original	37	9.6	0.3	97
	30	11.4	0.2	98
2	37	7.7	0.7	91
	30	5.4	68.9	8
3	37	6.0	15.3	39
	30	6.8	64.5	11

E. coli production host is transformed with each plasmid and grown under identical conditions (except for temperature).

Although LT and TNFα are non-covalent trimers, when purified from *E. coli* they have been shown to contain low levels of covalent, non-reducible dimers and trimers [37,38]. TNFα has also been shown to form these covalent forms upon storage [38]. The molecular basis of this reaction has not been determined but the covalent bonds between the subunits do not seem to affect binding to the TNF receptor column. Using coupled affinity–reversed-phase chromatography, we could demonstrate that these covalent dimers and trimers accumulate during the course of the culture to about 4% dimer and 0.5% trimer (Fig. 8A) and that during heat inactivation of the culture, this can rise to as much as 15% dimer and 3% trimer. By optimizing the heat inactivation time and temperature, it is possible to limit the formation of these variants, yielding a more consistent product (Fig. 8B). Use of continuous heat inactivation can further limit the formation of these variants (not shown).

Reversed-phase analysis of purified LT revealed a low level of a hydrophilic variant containing an amino acid substitution of valine for isoleucine at position 12. Using a modification of the coupled affinity–reversed-phase system to improve resolution in the second dimension, it was possible to determine the effect of fermentation conditions on production of this variant without needing to purify the protein. Fig. 9 demonstrates how the analysis can be used to assess the effects of isoleucine levels in the initial medium on the accumulation of this mistranslation product.

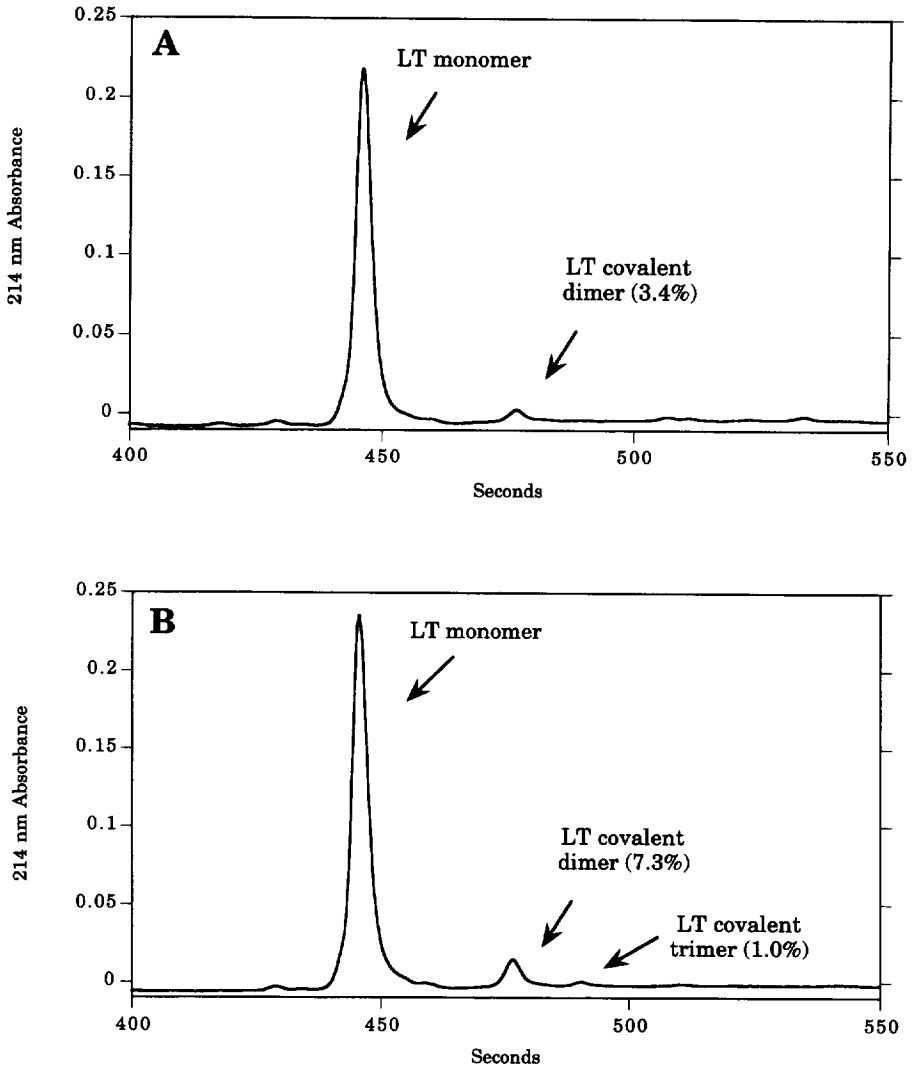

Fig. 8. Two-dimensional affinity–reversed phase analysis of soluble lymphotoxin extract for covalent dimer and trimer content. Panel A shows extract made from live paste (with 3.4% dimer and undetectable trimer) and solid line is from heat-killed cells (with 7.3% dimer and 1% trimer).

4. Conclusions

The use of HPLC for analysis of proteins in the complex matrix of fermentation broth or host cell extract can provide important quantitative information about product titer and quality. There are, however, several barriers to successfully implementing such assays on a routine basis, including column fouling and co-elution of host constituents.

While it may initially seem a daunting task to design a specific HPLC assay for a product and its variants in the presence of the many other constituents present in a crude product matrix, we have frequently found that this can be accomplished. In the case of secreted human growth hormone, it was possible to take advantage of the product's relative hydrophobicity to design specific single column assays. In the case of antibody fragments (and other

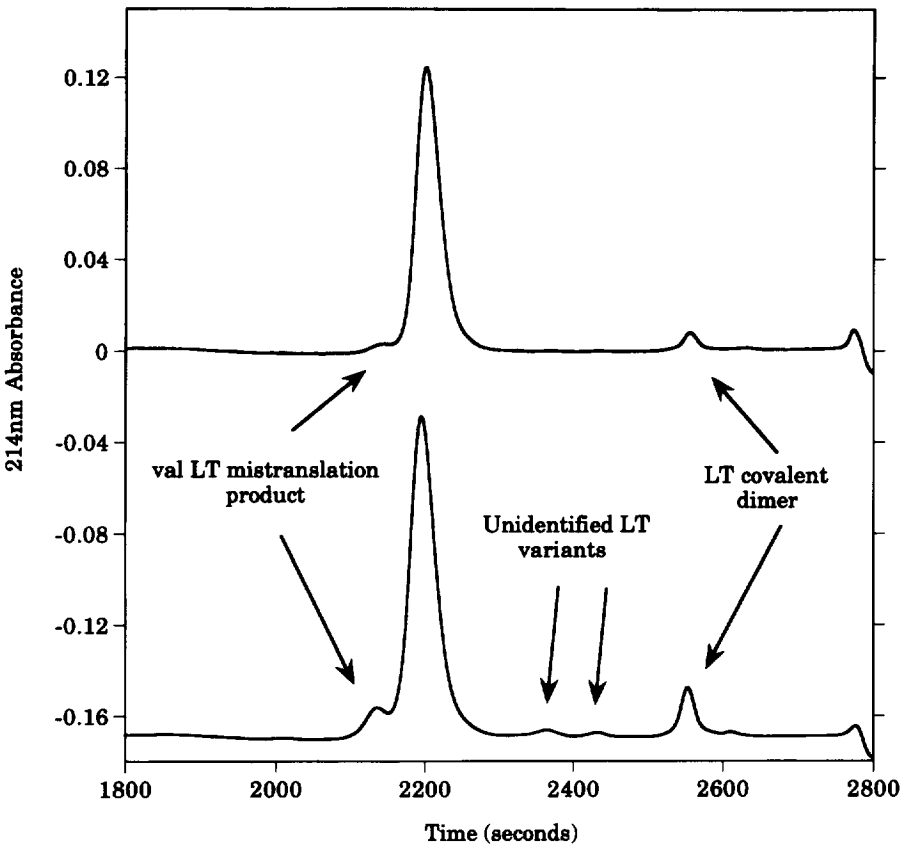

Fig. 9. Two-dimensional affinity–reversed phase analysis of soluble lymphotoxin extract for analysis of isoleucine-to-valine mistranslation product. Lower trace corresponds to extract from the initial process (with low Ile), while the upper trace is from cells grown in medium with optimized initial isoleucine content (resulting in lower mistranslation).

proteins with a basic p*I*), it was possible to make use of relatively specific mixed-mode cation-exchange resins that are sold commercially for antibody separations. In the case of LT, it proved possible to monitor the concentration of the primary product with a single column assay since its titer was very high relative to that of any co-eluting host proteins. In more difficult cases, two-dimensional separations can be used, as described above for the quantitation of minor lymphotoxin variants.

Column fouling usually occurs at some rate when HPLC methods are applied to these crude product matrices. We have been able to achieve acceptable column lifetimes by making use of guard columns and by careful optimization of the assay conditions, as described here for the B-chain relaxin method. For routine assays, we have found that the use of replaceable guard cartridges is preferred over column regeneration schemes for prolonging the life of the analytical column. Two-dimensional separations, where the bulk of the crude extract is shunted to waste prior to column switching, can also dramatically extend the number of assays obtained from the high resolution analytical column.

Since sample preparation precedes analysis, it can be used as a tool in assay design. In the case of lymphotoxin, we have used a two-step extraction to differentiate soluble, folded LT from refractile, aggregated product. In other cases (such as rhGH and relaxin B-chain) we have used a single extraction (or broth treatment) with denaturing agent to give information about total product. Finally, with the antibody fragments, we analyzed only the soluble, folded product after extraction without denaturant.

As mentioned above, the use of coupled chromatography can be a powerful aid in resolving analytes from contaminants, however, it may require that expensive instrumentation be dedicated to a routine assay. Furthermore, it requires that, if affinity is the first dimension, the sample be in a form or solvent compatible with binding to antibody, receptor or ligand. Chaotropes, reducing agents or detergents may interfere with binding as well as destroy or significantly reduce the life of the affinity column. In the case of LT, analysis of the soluble, properly folded trimer was facilitated by the use of affinity to an immobilized receptor, followed by reversed-phase LC. This method permitted reliable quantitation of minor variants without requiring product purification. Furthermore, it permitted rapid method development.

Wherever possible, a single, robust, reliable chromatographic method is preferred. For LT, a gradient reversed-phase method on a C_4 silica column provided reliable quantitation of the "main peak" material in soluble and urea extracted (refractile) pools. In these "one column" methods, throughput is limited by the need to maintain adequate resolution between the product and host peaks. Optimization of the gradient, flow-rate, column temperature, column selection and mobile phase composition can usually be used to obtain the needed separation, however, assay development time can be long.

While these methods require greater effort to develop, the quantitation obtained enables improvement of the production process in ways that would not be possible using only immunoassay or gel electrophoresis techniques. This is certainly justified for a product in the late stages of development or in routine manufacturing.

Acknowledgments

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