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Analysis of recombinant human interleukin-11 fusion protein derived from *Escherichia coli* lysate by combined size-exclusion and reversed-phase liquid chromatography

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Abstract

A two-dimensional size-exclusion–reversed-phase high-performance liquid chromatographic assay has been developed for the quantitation of recombinant human interleukin-11 fusion protein (rhIL-11 FP) expressed in *E. coli* cells. The sample preparation procedure included the optimization of lysis buffer components to achieve maximum rhIL-11 FP recovery through the disruption of associations between rhIL-11 FP and *E. coli* components. The *E. coli* cells were dialyzed into lysis buffer and lysed by a French Press prior to two-dimensional chromatographic analysis. A size-exclusion column was used first to remove high- and low-molecular-mass *E. coli* components. Then reversed-phase chromatography was used to separate and quantify the rhIL-11 FP. The assay was linear over the range of 0.0294 to 0.235 mg/ml. The limit of quantitation, 0.0294 mg/ml, was based on % normalized residuals and precision criteria not exceeding 10%. The reproducibility of the assay for lysate samples was good on a daily (% R.S.D.=1.0; n=5) and a day-to-day (% R.S.D.=3.2; n=10) basis. The assay was also monitored by an external control, in which day-to-day reproducibility was good (% R.S.D.=2.2; n=9). Selectivity and chromatographic peak identification were based upon gel electrophoresis and N-terminal sequencing of the rhIL-11 FP peak collected from the reversed-phase column.

Keywords: *Escherichia coli*; Proteins; Interleukins

1. Introduction

High-performance liquid chromatographic (HPLC) methods have been shown to play an important role in biotechnology process development, especially with regard to process and product monitoring [1–3]. The importance of optimizing processes can not be overemphasized in the expression and purification of potential biopharmaceuticals. Various analytical methods such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), enzyme-linked immunosorbent assay (ELISA) and HPLC have been used to analyze proteins. SDS-PAGE suffers from a lack of precision due to the non-selective staining procedure of not only the sample but of the standard as well, and may not be sensitive enough to detect subtle protein concentration changes due to process changes. ELISA methods are difficult to automate and require antibodies to be selective in a variety of process buffers, including crude lysate mixtures.

Reversed-phase (RP) HPLC has been used to purify several *E. coli* derived proteins, such as insulin-like growth factor II and Colicin V [4,5].

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However, in these examples, the *E. coli* proteins were purified for further structural and biological characterization and not used for quantitative analysis in the traditional sense. Additionally, RP-HPLC has been used to monitor processing steps for the determination of product purity, while other complementary methods have been used for product concentration [6].

Quantitative analysis of *E. coli* expressed proteins in upstream samples has been performed by reversed-phase chromatography. Upstream samples, including crude cell suspensions and lysates, represent separation challenges due to the complexity of the sample. *E. coli* lysates are composed of not only the analyte of interest but also endogenous proteins, nucleic acids, cell fragments and small molecules. Crude cell suspensions and cell lysates, after some type of sample pretreatment, have been injected onto reversed-phase columns and the expressed protein quantified [7].

However, simple off-line sample preparation may not be adequate for the particular sample at hand especially if the other components affect analyte recovery, cause chromatographic supports to foul and/or coelute with the analyte. The combination of some or all these influences may further complicate the chromatographic profile, resulting in poor selectivity or difficulty in integration and analyte band identification. Therefore the reduction or removal of non-analyte components would be advantageous, providing a more rugged, robust, and reproducible assay. There are numerous sample preparation methods which may be conducted off-line or on-line [8,9]. Off-line methods may be simpler to perform, but require multiple steps which increase the likelihood of analyte loss. On-line methods allow automation and less sample handling, but require more complex instrumentation and programming.

Multidimensional chromatography has been reviewed and described by a number of definitions [10]. One definition is the coupling of chromatographic columns with different modes of retention. This coupling of columns has been demonstrated for the separation of rhProinsulin fusion protein in *E. coli* [11]. This paper provides another example of this type of multidimensional chromatography as applied to quantify rhIL-11 FP.

During cell lysis, the target protein produced in *E. coli* is usually contained in inclusion bodies. Processing involves the release and resolubilization, in a detergent solution, of the target protein to its native state. This process is typically associated with a limited recovery. An alternative to this process is expression of the target protein with another protein as a fusion protein which remains soluble during lysis. The two proteins are later separated by enzymatic or chemical cleavage. As an example of this type of system, rhIL-11 is expressed as a thioredoxin fusion protein (FP) in *E. coli* [12]. rhIL-11 is a cytokine which stimulates blood platelet growth, and is being studied as an agent to treat severe thrombocytopenia, among other indications.

Generation of soluble fusion proteins is a new technology and has required new analytical methodology capable of recovering, separating and quantifying a target protein from crude cell lysates rich in proteins, DNA and cell wall fragments, etc. Generally, an ELISA is used to quantify components from such a complex mixture. However, a number of random, non-specific interactions of rhIL-11 FP occur with other proteins which may sterically interfere with antibody binding. This results in irreproducible methodology with an ELISA. Therefore, another method was needed which is not based on antibody interactions. In this paper, an analytical method is reported which is based upon two-dimensional chromatographic separation and quantification of rhIL-11 FP from crude *E. coli* lysate. Using this method the concentration of rhIL-11 FP was measured and used to assess expression levels, leading to data for mass balances and yield determinations during process development.

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, USA). Sequencer-grade trifluoroacetic acid was obtained from ABI (Foster City, CA, USA). Distilled water was further purified with a Barnstead Nanopure System (Syborn Barnstead, Boston, MA, USA). Bis-Tris propane and sodium chloride were obtained from Fluka (Ronkonkoma, NY, USA). Ethanol was obtained from Aaper Alcohol and Chemical (Shelbyville, KY, USA).

2.2. Procedure

Lysis buffer

The lysis buffer consisted of 15 m*M* Bis-Tris propane–750 m*M* sodium chloride–5% ethanol, pH titrated with HCl (pH 6.95 to 7.05).

Preparation of rhIL-11 FP standards and external control

The rhIL-11 FP standard was formulated and lyophilized for storage. One milliliter of the lysis buffer was added to a vial of lyophilized rhIL-FP, resulting in a concentration of 0.47 mg/ml. The 0.47 mg/ml solution was serially diluted to the following concentrations: 0.235, 0.176, 0.117, 0.0588, 0.0294, 0.0147, and 0.0074 mg/ml. The external control, which was used to monitor assay performance, was a partially purified lysate sample. The external control, kept at −80°C, was thawed at room temperature and diluted 1:19 (50 μl external control +950 μl lysis buffer).

Preparation of E. coli rhIL-11 FP cell lysates

Several 10-ml aliquots of T0 and post-cooling *E. coli* cells were obtained directly from a production campaign and stored at −80°C. T0 cells were collected before induction of rhIL-11 FP synthesis, and post-cooling cells were collected after cell density reached an optimum value and the culture cooled to 28°C. Cells were thawed in a Precision water bath (Chicago, IL, USA), at 70° to 80°C. The sample volume was measured and centrifuged in a Beckman J-6B centrifuge (Palo Alto, CA, USA) at 1400 *g* for 45 min (0° to 4°C). The supernatant was discarded and replaced with 10 ml of 15 m*M* Bis-Tris propane–750 m*M* NaCl–5% ethanol pH 7, then the cells were resuspended and centrifuged again. This sequence was repeated a total of three times. On the third time, the supernatant was not discarded.

Cells were vortex-mixed to suspend the cells, placed in a cold room (4°C) and the tubes were rotated overnight. After the overnight rotation the cells were lysed by a SLM Instruments French Press (Urbana, IL, USA) fitted with a mini cell pressurized to 18 000 p.s.i. (ca. 124 MPa) and maintained at ambient temperature. The cells were lysed by the French Press in two passes. Lysed cells were centrifuged at 1400 *g* for 30 min (0° to 4°C). The supernatant was removed and normalized to 14 ml with the lysis buffer. The 14-ml lysate was filtered with Millipore Millex SLSV 5-μm filters (Bedford, MA, USA). Aliquots of the filtrate were diluted 1:5 with the lysis buffer (50 μl of filtrate+250 μl of lysis buffer), frozen in liquid nitrogen and stored at −80°C until analysis.

T0 lysates were used to demonstrate that no rhIL-11 FP was being produced prior to initiation of induction. Samples analyzed to quantify rhIL-11 FP were the post-cooling sample lysates.

Preparation of E. coli cell pellet

The pellet obtained after centrifugation of the post-cooling lysate was analyzed for rhIL-11 FP recovery determination. The pellet was obtained from *E. coli* cells lysed in the optimal lysis buffer. The stored pellet was thawed, solubilized with SDS-PAGE loading buffer (Laemmli buffer) and sonicated. The mixture was centrifuged (15 min at 1400 *g*) and supernatant removed and filtered for analysis.

Experimental system

The chromatographic system consisted of a BioCAD HPLC (PerSeptive BioSystems, Cambridge, MA, USA) with a Model 717 autosampler (Waters, Milford, MA, USA) controlled at 8°C to 12°C. Size-exclusion chromatography (SEC) was the first dimensional separation method, utilizing a BioSil SEC-250 column (30×0.78 cm; BioRad, Richmond, CA, USA). The mobile-phase composition was 15 m*M* Bis-Tris propane–200 m*M* NaCl–5% ethanol, pH 6.95 to 7.05. In the second dimension, a Poros R1/H reversed-phase column (10×0.46 cm; PerSeptive BioSystems) was used. The reversed-phase solvents were composed of (A) 0.1% TFA in water (v/v) and (B) 0.1% TFA in 8:2 (v/v) acetonitrile and water.

The chromatographic system was programmed to inject 100 μl of sample onto the SEC column. The elution program was the following: for 6.60 min the system was run in isocratic mode with the SEC column in-line: then at 6.60 min the RP column was placed in line and the eluate from the SEC column was loaded onto the RP column for 4.70 min. After the 4.70 min load, the SEC column was placed off-line and the reversed-phase elution gradient began. The gradient was from 34 to 72% B in 50 min. After RP reequilibration (seven column volumes) at 34% B the column was set off-line and the

SEC column was placed in-line. The low-molecular-mass components remaining on the SEC column were eluted within one column volume with the SEC mobile phase. The separation was monitored at 214 nm and total assay time was 105 min. All flow-rates were 1 ml/min, except during line flushing and column equilibration (5 ml/min and 2 ml/min, respectively). The RP column temperature was maintained at 40°C, while the SEC column remained at ambient temperature.

Data analysis of assay performance

To determine linearity, response curves were generated with rhIL-11 FP standards. Accuracy, based upon % normalized rhIL-11 FP residual values, and precision based upon % R.S.D. values for each rhIL-11 FP standard, were used to determine the rhIL-11 FP lower working range. For this assay, the limits of accuracy and precision were set at an upper limit of 10%. Assay reproducibility and sample stability were measured by the rhIL-11 FP standards, the external control and post-cooling lysate samples. Recovery was based on spikes of rhIL-11 FP standard into the lysate and analysis of the pellet. Peak identification and selectivity were determined by N-terminal sequencing and gel electrophoresis, respectively. N-terminal sequencing was performed with an ABI 477A Protein Sequencer and 120A Analyzer (Foster City, CA, USA). For gel electrophoresis, ISS 10% to 20% Standard TC SepraGels (Integrated Separation Systems, Natick, MA, USA) were used. Gels were stained either with silver stain II (Daiichi Pure Chemicals, Tokyo, Japan) or Coomassie Brilliant Blue R-250 (BioRad, Richmond, CA, USA). Coomassie-stained gels were used in the pellet analysis and scanned with a BioImage Scanner (Millipore, Bedford, MA, USA).

3. Results and discussion

3.1. Optimization of NaCl concentration in lysis buffer

The lysis buffer composition was optimized to enhance the disruptions of rhIL-11 FP associations, whether electrostatic and/or hydrophobic, with *E. coli* derived components. The disruption of these interactions would free rhIL-11 FP and maximize the recovery. Based on preliminary studies, which showed that these interactions could be reduced by the presence of NaCl [13], the concentration of NaCl in the lysis buffer was investigated. Sodium chloride concentrations were varied (0, 200, 500, 750 and 1000 m*M*) in the lysis buffer, while Bis-Tris propane and ethanol (EtOH) remained at 15 m*M* and 5%, respectively. To monitor the effect of the NaCl concentration in the lysis buffer, *E. coli* cells were dialyzed and lysed in each lysis buffer, then the rhIL-11 FP peak area was measured by the two-dimensional assay, vide infra. A plot of the rhIL-11 FP peak area with respect to NaCl concentration is shown in Fig. 1.

As the NaCl concentration increased, the rhIL-11 FP peak area reached a maximum at 750 m*M* NaCl. Therefore, 750 m*M* was selected as an optimum NaCl concentration for the lysis buffer.

3.2. Selection of lysate sample treatment

Off-line methods

Preliminary sample treatment was required for the lysate sample, since the harsh denaturing reversed-phase chromatographic conditions alone were not effective in disrupting rhIL-11 FP associations or providing the required selectivity and reproducibility. Other sample treatment methods were investigated, such as precipitation, enzymatic treatment, heat treatment, and chromatography (ion-exchange and size-exclusion). The precipitation, enzymatic, and heat treatments demonstrated a lack of specificity and recovery. The ion-exchange column resin became fouled with the many components of the lysate. The size-exclusion column provided a reproducible profile with the selectivity to remove high- and low-molecular-mass *E. coli* cell components (such as lipids), and improved the rhIL-11 FP recovery by promoting disruptions. SEC is a non-surface mediated chromatographic technique; therefore, its stationary-phase coating was not noticeably altered by a dynamic layer of lipids or other lysate components. Removal of lipid components by SEC made it possible to obtain a reproducible reversed-phase chromatographic method for analysis with a long column lifetime. Therefore, SEC was chosen for

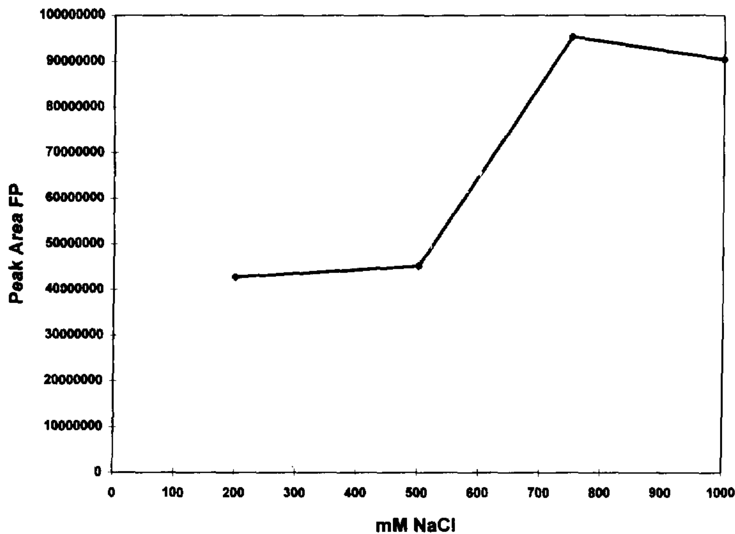


Fig. 1. rhIL-11 FP peak area, determined by SEC–RP-HPLC, as a function of NaCl concentration in the lysis buffer. The lysis buffer consisted of 15 m*M* Bis-Tris propane–5% ethanol (pH 7) with varying NaCl concentrations: 0, 200, 500, 750, and 1000 m*M*. Chromatographic conditions are described in the text.

sample treatment prior to reversed-phase chromatography.

On-line methods

SEC followed by reversed-phase chromatography are facile to couple for multidimensional separations; however, solvent compatibility between the mobile phases is required [14]. Each chromatographic mode was individually optimized and then coupled.

3.3. SEC optimization

Mobile-phase composition of the SEC was optimized for NaCl and EtOH concentration. Increases in either NaCl and/or organics have been shown to disrupt hydrophobic and electrostatic associations [13], thereby increasing recovery and resolution. The concentrations of NaCl (100 to 1000 m*M*) and EtOH (5 to 39%) were varied individually. The chromatographic profile was evaluated with respect to its three major bands: high-molecular-mass band (HMW), middle band (MB), and low-molecular-mass band (LMW) (Fig. 2). The HMW band consisted primarily of rhIL-11 FP aggregates, cell membrane and DNA fragments. The MB was a complex heterogeneous mixture of *E. coli* proteins including rhIL-11 FP and rhIL-11 FP–*E. coli* protein aggregates. The LMW band consisted of lysate buffer species, monomeric forms of *E. coli* cell components and small molecules.

Increasing the NaCl concentration resulted in a decrease in the peak area of the HMW band with a corresponding increase in the peak area of the LMW band, and the total peak area of the MB decreased slightly (Fig. 3A). The decrease in peak area, especially at NaCl concentrations greater than 200 m*M*, of the MB was due to the decline in peak shape, resolution and UV response.

Increasing the EtOH concentration resulted in a similar trend. Both the HMW and MB peak areas decreased, while that of the LMW increased (Fig. 3B). Decreased rhIL-11 FP peak height and resolution were observed at and above 9% EtOH. Apparently the EtOH had a similar effect on the MB as the NaCl.

It is important to point out that the FP standard's peak area remained quasi constant throughout all mobile-phase composition studies (data not shown). However, peak width of the FP standard increased with an increase in NaCl or EtOH concentration in the mobile phase.

At concentrations above or equal to 400 m*M* NaCl and 9% EtOH, the chromatographic profile was adversely affected with respect to UV response, and

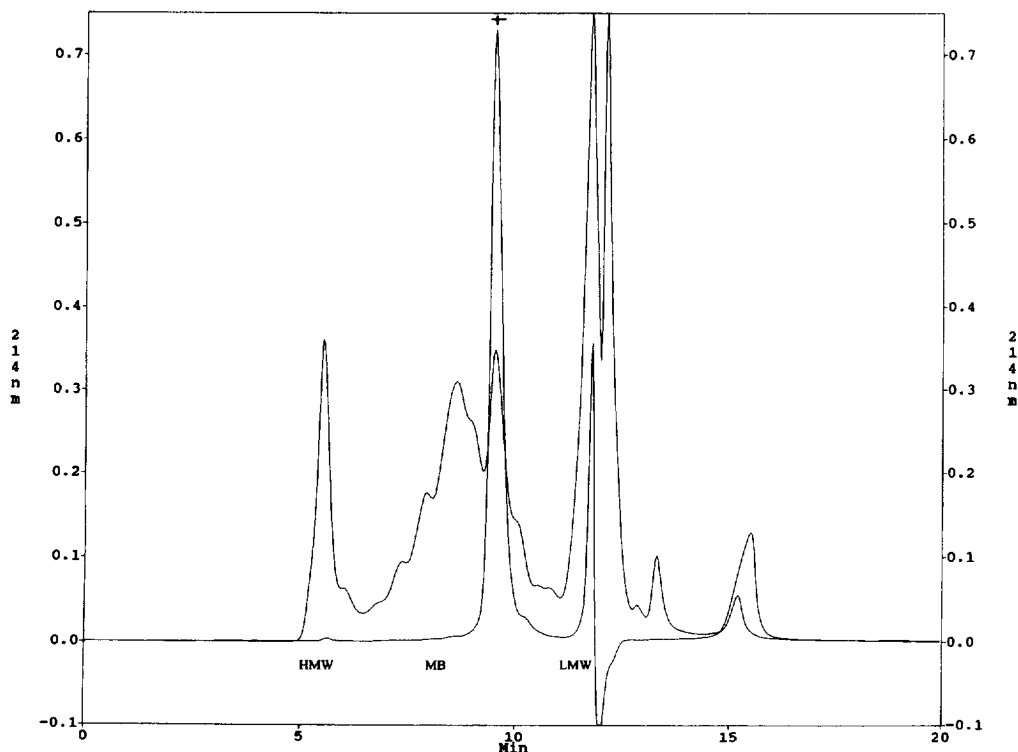


Fig. 2. Overlaid SEC chromatograms of rhIL-11 FP standard (23.5 μg) and post-cooling lysate (1:5), obtained with optimum SEC mobile phase: 15 m*M* Bis-Tris propane–200 m*M* NaCl–5% ethanol (pH 7). Three bands are identified: high-molecular-mass band (HMW), middle band (MB) containing the rhIL-11 FP and low-molecular-mass band (LMW). + Denotes the rhIL-11 FP band.

resolution of the MB with the LMW. Based upon our observations, the selected SEC mobile phase had the following composition: 200 m*M* NaCl, 5% EtOH in 15 m*M* Bis-Tris propane at pH 7.0.

3.4. Optimization of reversed-phase chromatography for SEC–RP-HPLC

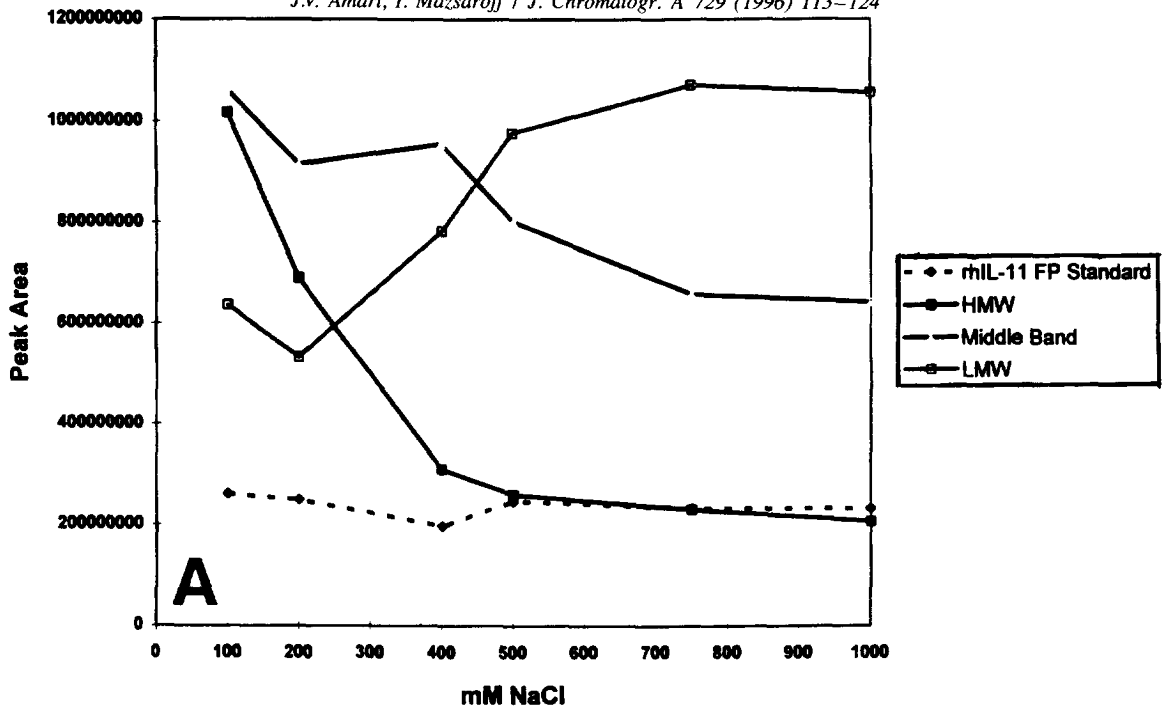
RP-HPLC was used as the analytical method after SEC sample treatment. The SEC and RP-HPLC were compatible with respect to mobile phases, and good recovery of the rhIL-11 FP was obtained on each column. The RP-HPLC gradient was optimized to improve the resolution (R_s) and thus integration of the rhIL-11 FP band in the post-cooling lysate. In preliminary studies, the gradient was 25 to 72% B in 25 min. To measure R_s, the component eluting directly in front of rhIL-11 FP was used in the R_s equation. The compositions of the A and B buffers were not varied; instead, the effect of varying the gradient slope was studied. All flow-rates were 1 ml/min. The following gradients were investigated and resulted in the calculated slope (% B/min) and measured R_s:

1. 25 to 72% B in 25 min (slope=1.88), R_s=0.58
2. 34 to 72% B in 25 min (slope=1.50), R_s=0.60
3. 25 to 72% B in 50 min (slope=0.94), R_s=0.83
4. 34 to 72% B in 50 min (slope=0.76), R_s=0.86
5. 25 to 72% B in 75 min (slope=0.62), R_s=0.84

The highest R_s was obtained with gradient 4. Therefore, the reversed-phase portion of the method was modified to incorporate gradient 4 for subsequent use.

3.5. Optimization of SEC fractionation time

The SEC lysate profile indicated the presence of HMW and LMW solutes with the rhIL-11 FP



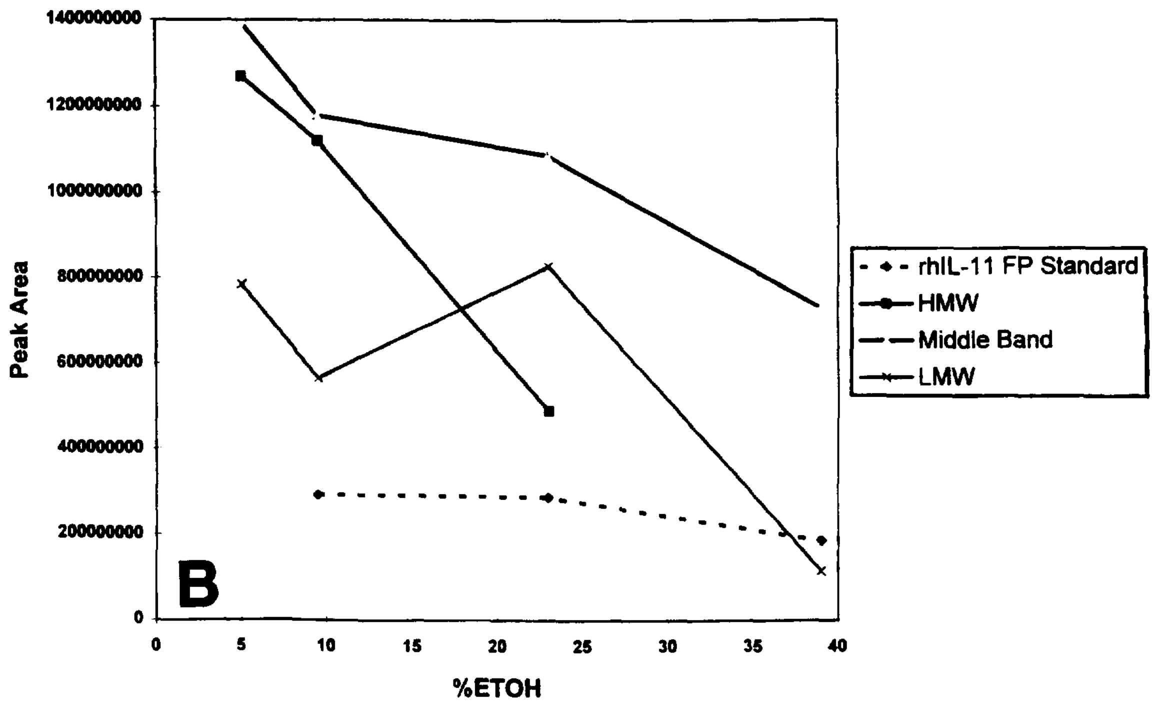


Fig. 3. (A) NaCl concentration effects on peak areas of rhIL-11 FP standard, HMW, MB and LMW, based on SEC. Conditions listed in text. (B) Ethanol concentration effects on peak areas of rhIL-11 FP standard, HMW, MB and LMW, based on SEC. Conditions listed in text.

contained in the MB. The rhIL-11 FP was not detected in either the HMW or LMW bands, under the optimized sample treatments, based on gel electrophoresis. However, a host of *E. coli* proteins, ranging from M_r 6500 to 200 000, in addition to the rhIL-11 FP was contained in the MB. Therefore, the HMW and LMW bands were not loaded onto the RP-HPLC column; only the MB encompassing the SEC elution window of the rhIL-11 FP was loaded onto the RP-HPLC column.

A fractionation time study was conducted to determine the load volume and time window of the MB onto the RP-HPLC column. Reversed-phase chromatography was used to monitor the effect of fractionation time on the rhIL-11 FP peak area. Multiple start fractionation times were investigated (6.6, 8.0, 8.5, and 9.0 min) while maintaining a fixed end time (11.5 min). The 6.6 to 11.5 min fractionation time encompassed the entire MB (valley of the HMW to valley of the LMW), while the other subsequent start times went deeper into MB. It should be mentioned that the BioCAD's extensive valving configuration enabled the easier coupling of the two columns (SEC–RP-HPLC) on a single system.

Reversed-phase results indicated that the rhIL-11 FP peak area remained constant for the first two start fractionation times (6.6 and 8.0 min) and then decreased by greater than 14 and 32%, as fractionation went deeper (8.5 and 9.0 min, respectively) into the MB, Fig. 4. The loss of rhIL-11 FP in the post-cooling lysate samples with respect to the later start times may have originated from rhIL-11 FP associations with *E. coli* proteins. The lysis buffer and SEC step may not be able to disrupt all rhIL-11 FP–*E. coli* protein associations. However, the harsh denaturing RP-HPLC conditions were able to disrupt all the remaining rhIL-11 FP–*E. coli* protein associations. Therefore, the entire MB was loaded onto the RP-HPLC for analysis. The rhIL-11 FP standard

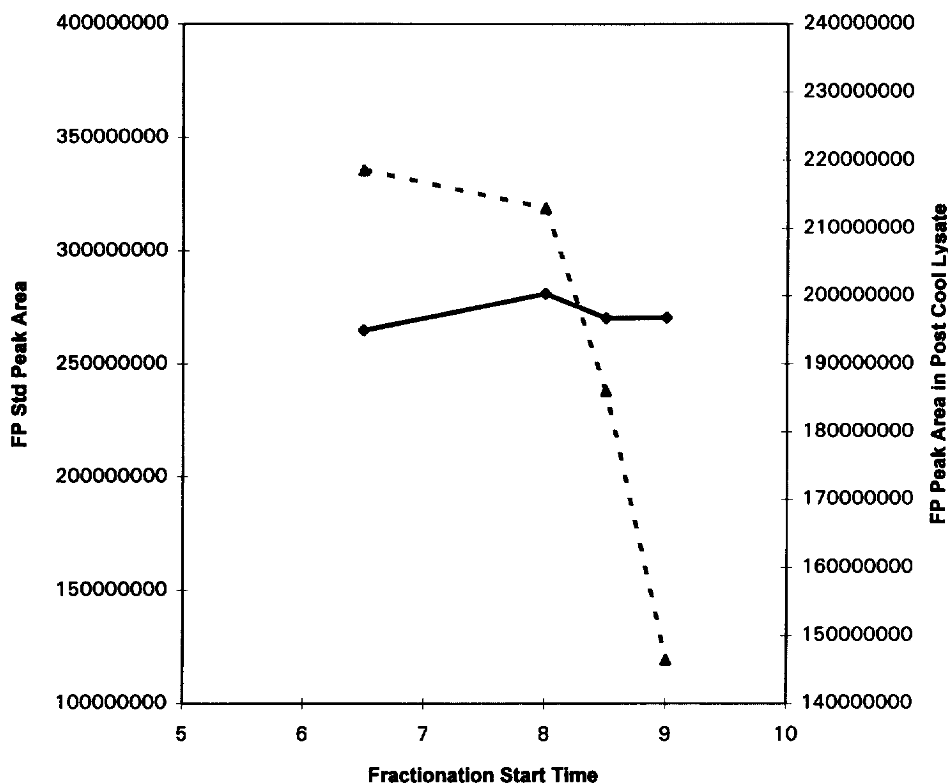


Fig. 4. Effect of fractionation time on peak area of rhIL-11 FP standard (solid line) and rhIL-11 FP in post cooling lysate (dashed line), determined by SEC–RP-HPLC. Reversed-phase gradient 25 to 72% B in 25 min.

exhibited no significant decrease in peak area; the observed decrease was less than 2.5% over the entire fractionation time study.

3.6. Evaluation of assay performance

Assay performance was based upon a number of criteria. To assess assay performance the rhIL-11 FP working range was determined by generation of nine response curves. The upper working range was set at 23.5 μg rhIL-11 FP (0.235 mg/ml). The nine curves were combined to produce a master calibration curve with the linear best-fit equation, $y = 10\,545\,079x - 17\,263\,724$, and an R^2 of 0.999. The % R.S.D. of the nine slopes was 4.2.

Due to the complexity of the sample and analytical method, % normalized residuals and % R.S.D. for precision were set at a limit of 10. To determine the lower working range, based upon the nine response curves, both % normalized residuals and % R.S.D. for precision had to be less than or equal to 10 for the same rhIL-11 FP load. At a load of 2.94 μg rhIL-11 FP (0.0294 mg/ml), both % normalized residuals and % R.S.D. for precision were less than 10. The lower working range of 2.94 μg rhIL-11 FP also defined the limit of quantitation for the assay. Therefore, the rhIL-11 FP working range was from 2.94 to 23.5 μg rhIL-11 FP with mean % normalized residuals ranging from 2.2 to 6.5 and % R.S.D. for precision ranging from 3.0 to 8.4.

Assay performance, based upon precision of the chromatographic system, was monitored with an external control. The external control was a partially purified rhIL-11 FP in-process sample. The % R.S.D. ($n=9$) for the rhIL-11 FP peak area in the external control was 2.6. For each external control injected, the amount of rhIL-11 FP was calculated from the master response curve. The mean mass of rhIL-11 FP obtained was 10.8 μg with a % R.S.D. of 2.2. Therefore, throughout the evaluation of assay performance consistent and reproducible results were obtained for the assay. A representative external control chromatogram overlaid with the rhIL-11 FP standard is illustrated in Fig. 5.

Experiments were conducted to measure the reproducibility of the SEC–RP-HPLC assay. The rhIL-11 FP peak, in the post-cooling lysate, was detected and identified based on retention time of the standard (Fig. 6) and rhIL-11 FP spikes. All post-cooling lysate injections ($n=10$) made throughout the assay performance studies exhibited identical profiles, with a % R.S.D. of 4.1 and 3.2 for rhIL-11 FP peak area and measured amount, respectively. The post-cooling lysate used throughout assay performance experiments was obtained from a single 10-ml aliquot, which was prepared on a single day (day 1). To examine the reproducibility of the lysis, the SEC step, and the RP-HPLC separation, additional aliquots of post-cooling *E. coli* cells from the same production batch were lysed and analyzed on two separate days (days 2 and 3). rhIL-11 FP Peak areas for day 2 and day 3 lysate samples exhibited % R.S.D.s of 10 and 3.3, respectively, while measured rhIL-11 FP amounts exhibited % R.S.D.s of 8.5 and 2.9. There was very good agreement between the rhIL-11 FP peak areas and calculated mass of rhIL-11 FP in the post-cooling lysate for different lysis days, indicating reproducible lysis, sample preparation steps and assay performance.

The stability of the post-cooling lysates (five injections) within the cooled autosampler was evaluated within a single assay performance study. The elapsed time between the first and last injection of a post-cooling lysate was 45 h. The five profiles were chromatographically identical, indicating no adverse impact upon the sample. Peak areas and mass of rhIL-11 FP exhibited a % R.S.D. of 1.4 and 1.0, respectively.

Two recovery studies were conducted to determine the effect of the lysate matrix and the amount of extractable rhIL-11 FP in the *E. coli* cell pellet. For the matrix effect study, two levels of rhIL-11 FP were spiked into the post-cooling lysate and analyzed. The two spike levels of rhIL-11 FP were 5.88 and 11.75 μg, which were representative amounts of rhIL-11 FP in post-cooling lysate. Recovery of the rhIL-11 FP spike ranged from 98 to 103%. Therefore, the matrix had no significant influence on the recovery of rhIL-11 FP.

The determination of extractable rhIL-11 FP present in the pellet was not possible by either SEC or SEC–RP-HPLC due to incomplete resolution and inability to confidently confirm the presence of rhIL-11 FP. Therefore fractions were collected, which encompassed the elution window of rhIL-11 FP, off the SEC–RP-HPLC columns and analyzed by gel

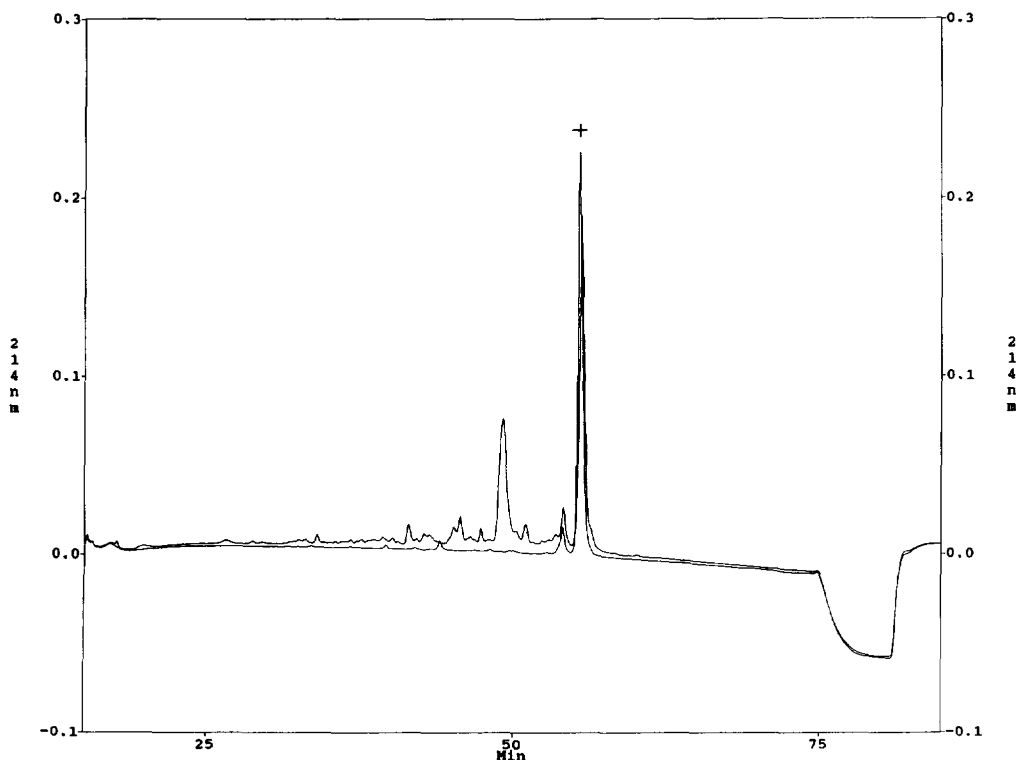


Fig. 5. Overlaid SEC–RP-HPLC chromatograms of rhIL-11 FP standard (11.75 μg) and external control (1:19). + Denotes the rhIL-11 band. Conditions listed in text.

electrophoresis. Additionally, rhIL-11 FP standards, diluted in the SDS-PAGE buffer, were also collected in the same manner as the pellet fractions, and used for calibration of the BioImage scanner. The BioImage scanner was used to quantify the amount of extractable rhIL-11 FP in the pellet, based upon % integrated optical density (IOD). Two gels were developed with two staining methods, the first used Western development to confirm that extractable rhIL-11 FP was contained in the pellet fractions, and the second was a Coomassie stain for BioImage measurement. The Western confirmed the presence of extractable rhIL-11 FP in a single fraction (data not shown). A regression line of IOD for the Coomassie-stained gel was plotted against μg of rhIL-11 FP standard. The determined concentration of extractable rhIL-11 FP in the pellet was 0.0473 mg/ml, accounting for all dilution and concentration steps. For the post-cooling lysate, the determined rhIL-11 FP concentration was 0.707 mg/ml. Therefore, the recovery of rhIL-11 FP in the post cool lysate was 93.7%.

The identification of the rhIL-11 FP was based not only upon retention time and spiked lysates, but also on gel electrophoresis and N-terminal sequencing of the collected rhIL-11 FP peak. Multiple post-cooling lysate samples were injected and the rhIL-11 FP peak was collected, combined, and concentrated. The concentrated rhIL-11 FP band was split into two aliquots, one for gel electrophoresis and the other for N-terminal sequencing. Gel electrophoresis with silver staining indicated a band which migrated to the same location as the rhIL-11 FP standard. No other bands were detected in the lane with silver staining. The gel is illustrated in Fig. 7. For N-terminal sequencing, the first 15 amino acids were sequenced (data not shown). The obtained sequence of the peak matched the sequence of the standard, thus confirming the peak which was quantified to be rhIL-11 FP.

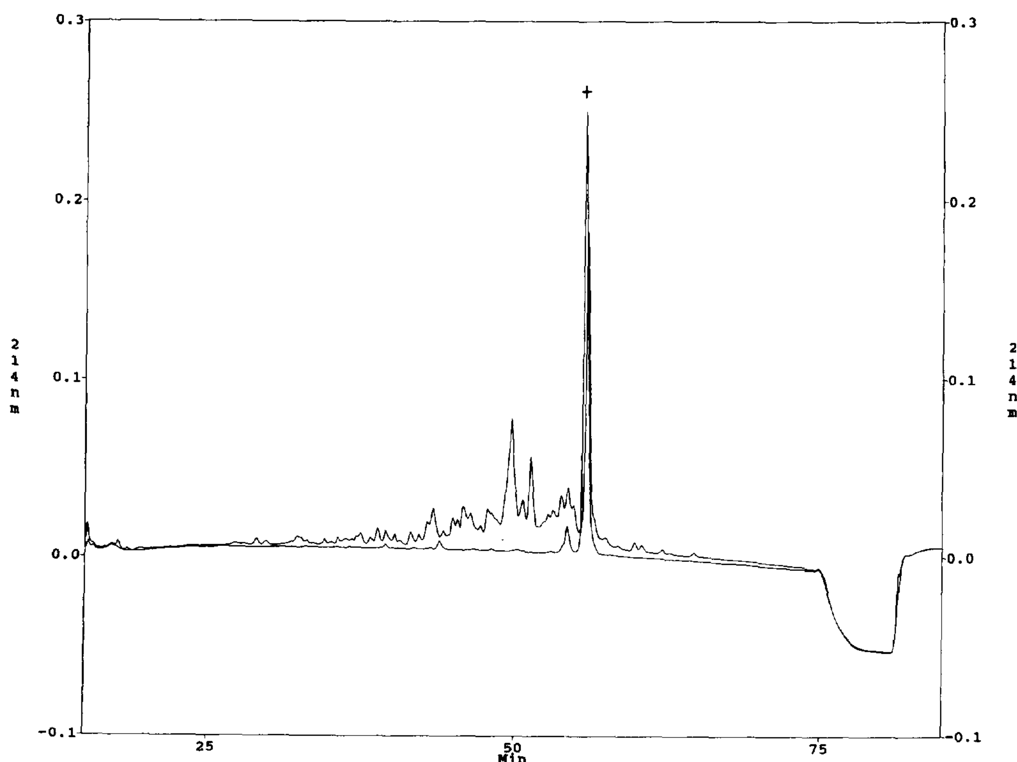


Fig. 6. Overlaid SEC–RP-HPLC chromatograms of rhIL-11 FP standard (11.75 μg) and post-cooling lysate. + Denotes the rhIL-11 band. Conditions listed in text.

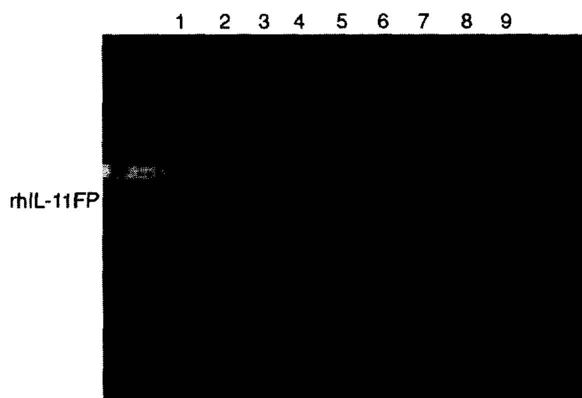


Fig. 7. Gel electrophoresis with silver stain of collected rhIL-11 FP band from SEC–RP-HPLC. Lane 1: molecular mass markers (M_r 6500 to 200 000). Lane 2: 0.24 μg rhIL-11 FP standard. Lane 3: post-cooling lysate (1:200). Lanes 4, 6, 7, and 8 are blanks. Lane 5: 0.24 μg rhIL-11 FP standard diluted in reversed-phase buffers. Lane 9: collected post-cooling lysate rhIL-11 FP band.

4. Conclusions

E. coli cells when lysed with a French Press produced a very complex mixture. Lysis buffer composition was optimized to maximize rhIL-11 FP recovery, through the disruption of rhIL-11 FP associations with *E. coli* cell components. A two-dimensional chromatographic method was utilized to separate and quantify rhIL-11 FP. In the first dimension, SEC was used for sample treatment, which eliminated high- and low-molecular-mass *E. coli* components. In the second dimension, RP-HPLC was used to dissociate the remaining rhIL-11 FP–*E. coli* aggregates, and then to separate and quantify rhIL-11 FP. Percent normalized residual and precision values were below 10%, meeting the assay criteria. rhIL-11 FP recoveries based upon the spiked matrix and with respect to the extractable rhIL-11 FP in the pellet, were greater than 98% and 93%, respectively. The selectivity of the method was very

good as indicated by gel electrophoresis and N-terminal sequencing. A single band was detected from the gel and the sequence matched that of the rhIL-11 FP standard. The method, including sample preparation, has shown a high degree of reliability and reproducibility.

Data obtained by this method has been used to assess fermentation productivity, and to calculate a mass balance for process steps until the first chromatographic unit operation. In addition, this method may be applicable for other downstream samples.

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References

[1] R.L. Garnick, N.J. Solli and P.A. Papa, Anal. Chem., 60 (1988) 2546.
[2] W.S. Hancock, in W.S. Hancock (Editor), High Performance Liquid Chromatography in Biotechnology, Wiley, New York, 1990, Ch. 1, p. 1.
[3] V. Anicetti and W.S. Hancock, in R.G. Harrison (Editor), Protein Purification Process Engineering, Marcel Dekker, New York, 1993, Ch. 2, p. 11.
[4] T.C. Furman, J. Epp, H.M. Hsiung, J. Hoskins, G.L. Long, L.G. Mendelson, B. Schoner, D.P. Smith and M.C. Smith, Bio/Technology, 5 (1987) 1047.
[5] M.J. Fath, L.H. Zhang, J. Rush and R. Kolter, Biochemistry, 33 (1994) 6911.
[6] C. Cachia, P.-J. Flamion and J.-P. Schreiber, J. Chromatogr., 539 (1991) 343.
[7] P. DePhillips, B. Buckland, K. Gbewoyo, S. Yamazaki and R. Sitrin, J. Chromatogr. A, 663 (1994) 43.
[8] J.G. Dorsey, J.P. Foley, W.T. Cooper, R.A. Barford and H.G. Barth, Anal. Chem., 64 (1992) 353R.
[9] J.G. Dorsey, J.P. Foley, W.T. Cooper, J.F. Wheeler and H.G. Barth, Anal. Chem., 66 (1994) 500R.
[10] S.A. Berkowitz, in J. Calvin Giddings, E. Grushka and P.R. Brown (Editors), Advances in Chromatography, Dekker, New York, 1989, Ch. 4, p. 175.
[11] J.S. Patrick and A.L. Lagu, Anal. Chem., 64 (1992) 507.
[12] E.R. LaVallie, E.A. DiBlasio, S. Kovacic, K.L. Grant, P.F. Schendel and J.M. McCoy, Bio/Technology, 11 (1993) 187.
[13] Personal communication.
[14] W. Kopaciewicz and F.E. Regnier, Anal. Biochem., 129 (1983) 472.