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Reversed-phase high-performance liquid chromatography assay for recombinant acidic fibroblast growth factor in *E. coli* cell suspensions and lysate samples

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ABSTRACT

A reversed-phase HPLC assay for acidic Fibroblast Growth Factor (aFGF) expressed in *E. coli* is described. The assay was developed on a Polymer Labs. PLRP-S macroporous poly(styrene-benzene) column. A sample preparation procedure was developed to permit quantitation of aFGF in crude samples, such as cell suspensions and cell lysates. The assay was linear over the range of 25–140 μg/ml with an accuracy of 5%. By using a POROS column containing a stationary phase with "through-pores" and a superficial mobile phase velocity of 1440 cm/h, the analysis could be performed in 3 min. The polymeric supports used for this assay were durable; periodic washing with sodium hydroxide maintained column performance for extended periods.

INTRODUCTION

In order to develop and subsequently validate a large-scale recombinant protein purification process, a careful and detailed evaluation of the process performance is required. Typically, during the development phase of a multi-step purification process, dozens of in-process samples may be generated for analysis of purity and recovery. Early on, when many possible steps are being simultaneously evaluated for possible inclusion, the length of time required for this analysis can be considerable, and can dictate to a significant extent the overall development time. Developing fast assays, or assays that require minimal sample preparation but are nonetheless applicable throughout the process, is therefore an important aspect of any process development effort.

In the case of *E. coli* derived proteins, determining host cell expression levels, monitoring fermentations, and optimizing a capture step requires that the concentration of a target protein be accurately determined in cell suspensions or cell lysates. Analysis of samples such as these, in complex matrices with high levels of endogenous protein, lipids, DNA and other cellular contaminants, can be problematic. Enzyme or biological assays may require substantial sample purification prior to assay and can give misleading information when truncated or chemically modified products are present. Immunoassays, such as ELISA [1], and particle concentration fluorescence assays [2] have been used to quantitate protein concentrations in crude samples. In most cases however these may require specialized reagents and equipment, such as antibodies, and are difficult to automate. SDS-PAGE is slow, difficult to quantitate and offers limited sample throughput.

Reversed-phase HPLC offers very high res-

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olution, sensitivity and precision and has often been applied to difficult protein separation problems. It is a valuable method for in-process monitoring, and for determination of chemical modification [3], protein conformation, or enzymatic degradation. In addition, HPLC systems are widely available and completely automated. For the crude samples described above however, silica-based reversed-phase columns may lose efficiency and resolution very rapidly due to non-specific adsorption of contaminants. They are also difficult to clean once degraded. Recently, polymer-based reversed-phase columns have been developed and utilized [4] for protein separations. Polymeric columns have exhibited higher recoveries [5], and increased column stability at both low and high pHs [6,7]. As we will demonstrate, polymeric columns appear much less prone to degradation with crude samples, and can be regenerated easily and repeatedly. By using these supports, the benefits of RP-HPLC, namely high speed, high resolution, and high recovery, along with complete automation, can be applied to samples generated through a protein purification process, including very crude early stage samples (cell suspensions and lysates).

We describe herein the assay of acidic fibroblast growth factor, (aFGF) [8,9] in both cell suspensions and cell lysates, as well as a rapid sample preparation procedure applicable for both cell lysate samples and cell suspensions. The method was evaluated for linearity, accuracy, precision and specificity. With some modification, the method was adapted for high-speed analysis.

EXPERIMENTAL

HPLC grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid was obtained from Pierce (Rockford, IL, USA). Water was obtained from a Millipore Milli-Q system (Bedford, MA, USA). Ultrapure grade guanidine hydrochloride was obtained from Bethesda Research Laboratories (Gaithersburg, MD, USA).

A polymeric PLRP-S column 50 mm × 4.6 mm I.D. with a particle diameter of 8 μm and nominal pore size of 300 Å was obtained from Polymer Laboratories (Amherst, MA, USA). A POROS RP/H column 50 mm × 4.6 mm I.D. was obtained from PerSeptive Biosystems (Cambridge, MA, USA).

The gradient HPLC system consisted of two Rainin Rabbit pumps, a Macintosh SE computer with Rainin Dynamax HPLC Method Manager software (version 1.1), a Gilson Model 811 gradient mixer, and a Gilson Model 231 autosampler. The autosampler rack was chilled by a Lauda circulating chiller to a temperature of 6°C. Columns were heated to 60°C by an Eldex lCH-150 column heater unless indicated otherwise. A Gilson Model 116 UV detector set at 280 nm was used for detection.

Cell disruptions were carried out with a Manton Gaulin Laboratory Homogenizer (Model 15M-8TA).

Preparation of aFGF stock solutions in PBS

Purified aFGF in phosphate buffered saline (PBS) from a trial purification process was concentrated using an Amicon Centriprep 10 (Amicon, Beverly, MA, USA) membrane to a final concentration of 14.0 mg/ml. The concentrated stock solution was diluted in PBS to obtain stock solutions of 2.5, 5.0, 7.0 and 10.0 mg/ml.

Preparation of aFGF standards and control samples

Preparation of blank cell lysate. E. coli cells of the same host strain used for aFGF expression but without the aFGF plasmid were suspended in PBS (1:4, v/v). The suspended cells were lysed by two passes through a Manton Gaulin Homogenizer operated at a pressure of 9000 p.s.i. (1 p.s.i. = 6894.76 Pa). The resultant cell lysate was aliquoted and stored at − 70°C until use.

Preparation of aFGF standards in blank cell lysate. The aFGF stock solutions described above were diluted 1 in 100 with blank cell lysate to obtain standards with final aFGF concentra-

tions of 25, 50, 70, 100, and 140 μg/ml cell lysate.

Preparation of aFGF control samples in blank cell lysate. Stock solutions of 2.5, 7.0, and 14.0 mg/ml were diluted 1 in 100 with blank cell lysate to obtain 3 control standards with aFGF concentrations of 25 (Low control), 70 (Mid control), and 140 (High control) μg aFGF/ml blank cell lysate. Five samples were prepared for each control concentration.

Sample preparation procedures

Cell lysate samples. In-process aFGF lysate samples were prepared by passing cell suspensions two times through a Manton Gaulin Laboratory Homogenizer at a pressure of 9000 p.s.i. To prepare cell lysate samples for HPLC assay, an aliquot of lysate (100 μl) is placed in a 1.5-ml microcentrifuge tube. 7 *M* guanidine hydrochloride (200 μl) with 5 m*M* DTT (dithiothreitol) is added and vortexed. After mixing, 500 μl of acetonitrile–7 *M* guanidine–TFA (20:80:0.2, v/v/v) is added to the same tube and vortexed, then centrifuged for 5 minutes (10 000 *g*). The supernatant is removed and placed in an autosampler vial.

Chemical cell disruption. Frozen cell paste samples were thawed and suspended in 4 volumes of PBS buffer then vortexed for 30 s. A 500-μl aliquot of each of the samples of suspended cells was added to 4.0 ml of 7 *M* guanidine hydrochloride containing 5 m*M* DTT and rotated for 10 min. After rotation, an aliquot (300 μl) was placed in a 1.5-ml microcentrifuge tube. The sample is then vortexed with 500 μl of acetonitrile–7 *M* guanidine–TFA (20:80:0.2) and centrifuged for 5 min (10 000 *g*). The supernatant is removed and placed in an autosampler vial.

Whole fermentation broth samples were centrifuged for 5 min (10 000 *g*). The resulting cell pellets were resuspended in 2 to 4 volumes of PBS and vortexed for 30 s. The resulting cell suspension was prepared as noted above for resuspended frozen cell paste samples.

HPLC methods

For the Polymer Labs PLRP-S column the HPLC assay was performed using a two-stage linear gradient. Following injection the gradient increased linearly from 8% acetonitrile–0.1% TFA to 28% acetonitrile–0.1% TFA over 1 min then linearly to 42% acetonitrile–0.1% TFA over 14 min at a flow rate of 2 ml/min throughout. The injection volume was 100 μl. Column temperature was maintained at 60°C.

For the POROS RP/H column the gradient increases linearly from 8% acetonitrile–0.1%TFA to 28% acetonitrile–0.1% TFA over 20 s, then linearly to 41% acetonitrile–0.1% TFA over 2 min 25 s, at a flow rate of 4 ml/min throughout. The injection volume was 20 μl. Column temperature was maintained at 60°C.

Assessment of linearity, precision, specificity, viability and column durability

The aFGF standards prepared in blank cell lysate were assayed to determine linearity. The five replicates of the control samples were assayed to assess variability in the method. Specificity was determined by comparison of a blank cell lysate sample to the spiked standards. Column durability was determined by making 40 injections of a cell lysate sample and comparing retention times and peak areas for these injections.

RESULTS AND DISCUSSION

Sample preparation procedure

To develop and optimize a capture step for aFGF, it was necessary to quantitate the amount of aFGF present in lysate samples obtained from pilot scale cell breakages. To determine the mass of aFGF present in these samples with a high degree of accuracy (±5%) presented problems because no enzymatic assay existed and the mitogenic bioassay [8] could not be used because of interference of *E. coli* lipopolysaccharide in the sample. It was therefore desirable to develop a sample preparation procedure that would allow for quantitation by HPLC.

aFGF has limited solubility at the low pH typically used for reversed-phase chromatography and, at concentrations encountered in cell

lysates, can precipitate when the pH is rapidly lowered. The addition of 200 μl 7 *M* guanidine to 100 μl of cell lysate produces a final guanidine concentration of about 4.6 *M*. At this guanidine concentration, aFGF unfolds rapidly; this unfolded aFGF is soluble at low pH and does not precipitate upon subsequent acidification. This high guanidine concentration should also assist in disassociation of any aFGF in electrostatic aggregation with cell constituents as well as solubilization of inclusion bodies if present. This unfolding/disaggregation step proved critical to the entire sample preparation scheme; if omitted, the recovery of aFGF was drastically lowered for these samples. When 6 *M* urea was substituted for guanidine, the yield of aFGF in lysate samples was higher than obtained with omission of the unfolding/disaggregation step, but not as high as obtained with guanidine. High concentration of urea was less effective in dissociating electrostatic interactions between aFGF and cell constituents, resulting in loss of aggregated aFGF. After unfolding, the solution is acidified by addition of acetonitrile–7 *M* guanidine–TFA (20:80:0.2), and centrifuged at high speed to remove insoluble material.

A chromatogram of a typical cell lysate sample is shown in Fig. 1A. The aFGF peak is well resolved from all the *E. coli* contaminants, which are confined to the early portion of the gradient and the portion of the gradient used to clean off the column between injections, thereby producing a clean chromatographic profile amenable to accurate quantitation.

The method is specific for aFGF. When a blank *E. coli* cell paste that does not express aFGF is prepared, no interfering peaks are detected in the region of aFGF elution, as shown in Fig. 1B.

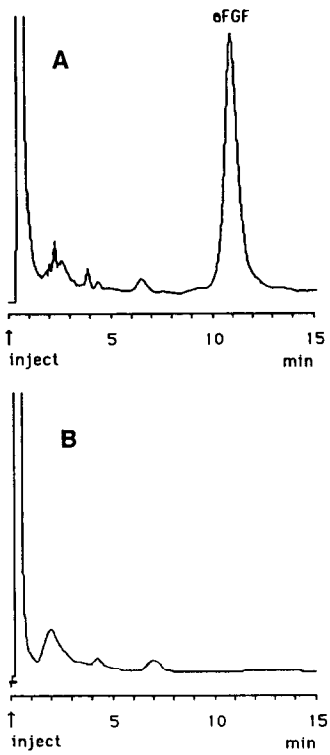

Fig. 1. Chromatographic profiles of an aFGF cell lysate sample (A) and a blank cell lysate sample (B) containing no aFGF. No interference peaks were detected in the aFGF region of the blank cell lysate. Preparation of samples is described under Sample preparation procedures. Chromatographic conditions: Polymer Labs PLRP-S column (50 mm × 4.6 mm I.D.); eluent A, 0.1% TFA in water, eluent B 0.1% TFA in 80:20 acetonitrile–water; two-stage linear gradient program, 1 min linear gradient from 10 to 35% B; followed by a 14-min linear gradient from 35 to 52% B at a flow rate of 2 ml/min throughout. Injection volume 100 μl for each, detection wavelength 280 nm.

Linearity, accuracy, precision and specificity

A standard curve prepared from the spiked cell lysate standards described in Experimental is shown in Fig. 2. The regression coefficient of 0.999 for the least-squares fit to the data is quite good considering the crude state of the samples and demonstrates linearity for the concentration range of 25–140 μg aFGF/ml.

Data from the spiked control samples are shown in Table I. Five replicate samples were prepared for each concentration level. The low control standard, with a nominal concentration of 25.0 μg/ml shows a mean measured concentration of 26.2 μg/ml, with an R.S.D. of 5.4%. The mid control standard with a nominal concentration of 70.0 μg/ml shows a mean measured concentration of 70.2 with an R.S.D. of 2.0%. The high control standard, with a nominal concentration of 140.0 μg/ml shows a mean measured concentration of 136.0 μg/ml, with an R.S.D. of 3.0%. The percentage difference be-

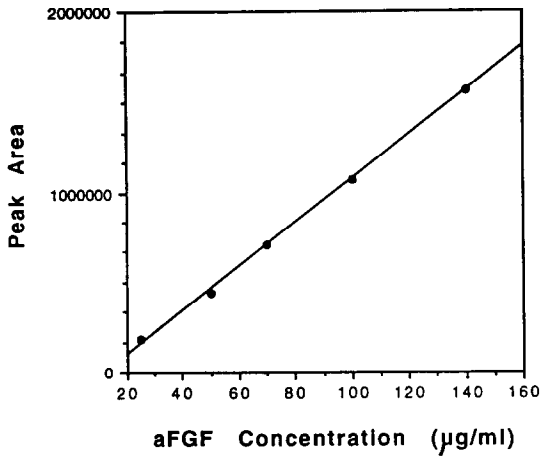

Fig. 2. A five-level calibration curve prepared from aFGF standards. Preparation of aFGF standards is described under Preparation of aFGF standards and control samples. Chromatographic conditions as in Fig. 1.

tween the nominal and measured concentration was under 5.5% for all concentration levels, which was within the desired accuracy range.

Repeatability and column degradation

The ability to process multiple samples from crude matrices can be difficult due to column degradation. Initial attempts to develop this assay on silica based reversed-phase columns were unsuccessful: these columns were significantly and irreversibly degraded after fewer than 10 injections of lysate samples prepared as described above (data not shown).

Polymeric supports have been shown to give higher protein recoveries and significantly longer lifetimes [5–7]. In addition, these supports can be regenerated with sodium hydroxide to restore performance. The durability of the PLRP-S column was tested by making 40 consecutive injections of a prepared lysate sample. Both the retention times and peak areas obtained for these injections were very stable. The average peak area was 2 169 209 uV ± 27 076 uV, the R.S.D. value is therefore less 1.3%, indicating that prepared lysate samples do not appreciably degrade the column over at least 40 consecutive injection cycles. Fig. 3 shows an overlay of the chromatograms of the first and fortieth injection of prepared lysate; peak shape is virtually identical, and more importantly, the peak area of the first and fortieth injection were within 1.5%. Following these injection sequences, blank gradients showed no evidence of "ghosting". For this assay application the polymeric column proved to be more resistant to degradation than silica-based columns, in practice this allowed for heavy use. Periodic (bimonthly) washing with 60

TABLE I

DATA FROM SPIKED CONTROL SAMPLES

Five samples were prepared for each control concentration.

Sample	Measured concentrations (μg/ml)		
	Low control (25 μg/ml)	Mid control (70 μg/ml)	High control (140 μg/ml)
1	26.3	69.9	138.4
2	25.9	69.3	131.6
3	28.7	70.8	129.6
4	24.4	68.7	137.0
5	25.7	72.3	138.3
Mean	26.18	70.20	135.99
S.D. (μg)	1.4	1.4	4.1
R.S.D. (%)	5.3	2.0	3.0
Diff. (%)	4.7	0.2	2.9

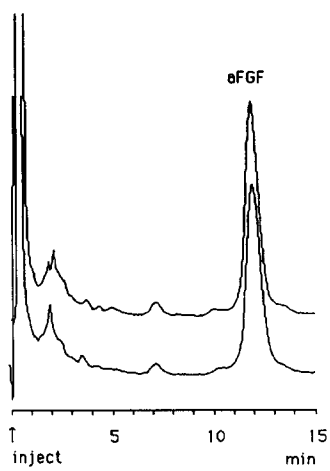

Fig. 3. Comparison of the 1st (lower trace) and 40th (upper trace) chromatograms obtained from consecutive injections of a cell lysate sample. Peak areas and retention times for aFGF remained stable through the injection series. Chromatographic conditions as in Fig. 1.

ml of methanol–0.2 *M* NaOH (1:1, v/v) prevented deterioration in peak height and separation quality.

Comparison of mechanical and chemical disruption of cells

In order to rapidly prepare cell paste and cell suspensions for HPLC assay, a guanidine disruption procedure was developed as described in Experimental. Chaotropic agents such as guanidine are commonly and effectively used to solubilize protein samples prior to assay [7]. Recently, guanidine has also been shown to be capable of releasing protein from intact *E. coli* by solubilizing the inner membrane and altering the outer wall to allow protein release without causing cell fragmentation [10]. Since preserving native conformation through the sample pretreatment is neither required nor desirable for this aFGF assay, very high guanidine concentrations were examined for aFGF release. The release and solubilization of aFGF from chemically disrupted cells was compared to that obtained by mechanical disruption. Frozen cells were resuspended in PBS buffer (1:4, v:v). A sample of this suspension was retained and the remainder lysed by two passes through a Manton Gaulin at 9000 p.s.i. The retained cells were chemically disrupted by adding 500 μl of cell suspension to 4 ml of 7 *M* guanidine and rotating at room temperature for 20 min. Samples were prepared in triplicate for both the chemically and mechanically disrupted cell suspensions. The assay results were normalized to account for the difference in total dilution between the two disruption methods. The mean concentration for chemically disrupted cells was 97% of the value obtained for mechanically disrupted cells (S.D. = 3.4%), indicating that the chemical disruption is as effective as homogenization in causing release of aFGF from the cells.

Fig. 4 shows a typical chromatogram of a chemically disrupted cell suspension. The chromatographic profile of this sample is virtually identical to the profile obtained by mechanical disruption (Fig. 1A), with no contaminants eluting in the region of aFGF. The addition of 500 μl of cell suspension to 4 ml of 7 *M* guanidine results in a final guanidine concentration of about 6 *M*. As with the sample preparation of cell lysates described earlier, it is envisioned that disruption of cells in the presence of a high concentration of a charged chaotrope will enhance both the solubilization of inclusion bodies and the disaggregation of aFGF from cellular constituents. Since the chemical disruption procedure is so simple, multiple samples can be rapidly processed.

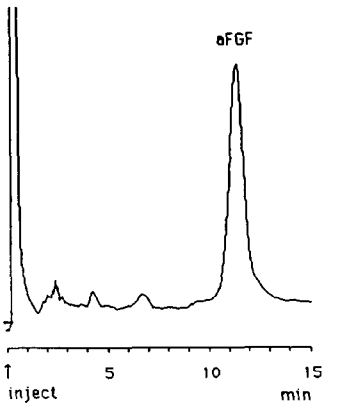

Fig. 4. The chromatogram of a cell suspension sample. The sample was chemically disrupted by guanidine HCi as described under Chemical cell disruption. Chromatographic conditions as in Fig. 1.

Monitoring fermentations and determining aFGF expression levels

In the development of a bacterial fermentation process both the yield and quality of a target protein need to be determined for different expression systems and under differing fermentation conditions to optimize product expression. A rapid, accurate assay for the target protein that can differentiate small changes in product concentration can greatly enhance this development process. The chemical disruption of cell suspensions followed by the sample pretreatment method described above have been applied to this fermentation effort for aFGF.

By removing small samples from active fermentations at different time points, the aFGF production in fermentors can be followed. In a typical analysis, whole broth samples are centrifuged to obtain a cell pellet. Equivalent cell mass samples are generated for each time point.

A comparison of aFGF concentration per cell mass can then be made. The production of aFGF in a fermentor monitored at 12, 14, and 16 h after induction is shown in Fig. 5. The aFGF titers could be accurately quantitated through the entire fermentation; because the chemical lysis and sample preparation procedures are simple and can be performed quickly, this information can be obtained concurrently with the fermentation. The ability to accurately determine aFGF levels in cell suspensions allowed for fermentation conditions to be more readily optimized. As media or fermentation conditions are varied, relatively small differences in the resulting aFGF expression levels can be determined. This is exemplified by the chromatograms shown in Fig. 6. In this comparison, the maximum aFGF titers from two different trial fermentations differed by less than 15%. Yet this difference could be easily discerned; the ability to accurately discriminate these smaller percentage improvements is significant since optimization usually consists of making several such stepwise improvements.

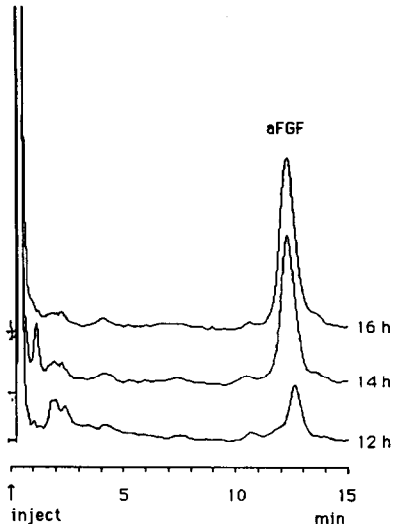

Fig. 5. The production of aFGF in a fermentation monitored at 12, 14, and 16 h after induction. Whole broth samples were removed at each time point and centrifuged to produce a cell pellet. The cell pellet is suspended in buffer and chemically disrupted by guanidine HCl as described under Chemical cell disruption. Chromatographic conditions as in Fig. 1.

High-speed analysis

For fermentation and process development projects, where large numbers of samples must be analyzed, obtaining a high sample throughput is important. The recent development of "perfusion chromatography" has resulted in significant reduction in analysis times. Chromatography on POROS supports permits very high superficial velocities to be achieved (>1000 cm/h) while maintaining efficiency and resolution [11–13]. To reduce analysis time, the aFGF assay was adapted to a POROS R/H column. By increasing the flow rate to 4 ml/min (superficial velocity = 1445 cm/h) and modifying gradient conditions a decrease in run time from 15 min to 3 min was achieved while maintaining the quality of the separation. A chromatogram of an aFGF cell lysate sample assayed on the POROS R/H column is shown in Fig. 7. The aFGF is well resolved from the sample constituents at the higher superficial mobile phase velocity used for POROS supports. Duplicate sample sets analyzed on both columns and similar validation experiments showed equivalent accuracy and reproducibility for the POROS column; R.S.D. values of 5% or less for 3 control samples

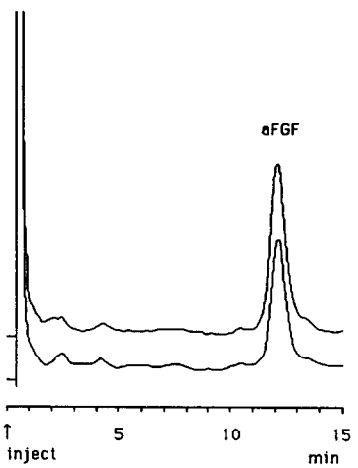

Fig. 6. A comparison of aFGF titers from two different trial fermentations. Lower trace, fermentation 1 (145 units/ml); upper trace, fermentation 2 (178 units/ml). Sample preparation and chromatographic conditions as in Fig. 5.

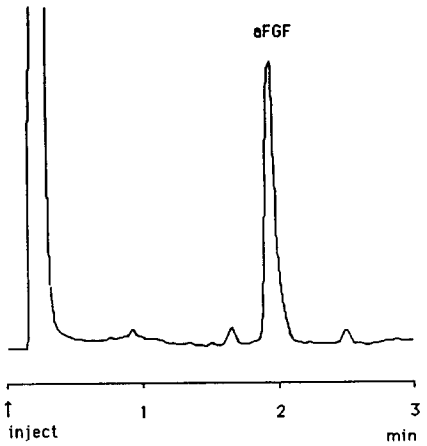

Fig. 7. High-speed analysis of an aFGF cell lysate sample. Chromatographic conditions: PerSeptive Biosystems POROS R/H column (50 mm × 4.6 mm I.D.); eluent A, 0.1% TFA in water, eluent B 0.1% TFA in 80:20 acetonitrile–water; two-stage linear gradient program, 20 s linear gradient from 10 to 35% B; followed by a 2:25-min linear gradient from 35 to 51% B at a flow rate of 4 ml/min throughout. Injection volume 20 μl, detection wavelength 280 nm.

analyzed in quadruplicate over the range of 50–250 μg/ml.

The quantitation of aFGF recovery through the entire process scheme, starting with the whole cell paste can be done very rapidly using one HPLC method for all sample streams. A purification procedure for aFGF consisted of cell lysis, centrifugation to remove cell debris, capture chromatography on an ion-exchange column to concentrate and purify the aFGF, followed by an affinity chromatography polishing step. The HPLC analysis of the suspended cells prior to lysis, the lysed cells and the chromatography column eluates are shown in Fig. 8. The cell suspension and lysate samples require the sample preparation methods previously described, samples from the capture column and other chromatographic products are sufficiently clean that simple dilution in 0.1% TFA is all that is required prior to analysis. Because the aFGF concentration could be determined for any sample type in 3 min, a complete process recovery analysis, (including side-fractions, column flow through and waste streams) comprising 60–80 samples, can be completed in about four h.

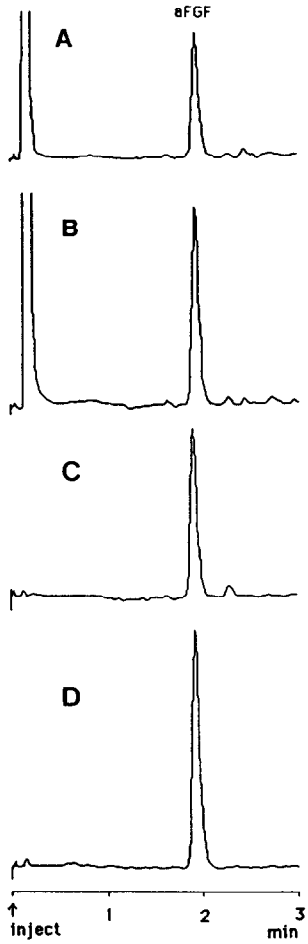

Fig. 8. Chromatograms from a pilot scale purification of aFGF. The cell suspension sample (A) was chemically disrupted by guanidine HCl as described under Chemical cell disruption, then prepared as described under Sample preparation procedures. The cell lysate sample (B) was prepared as described under Sample preparation procedures. The capture column product (C) and the affinity column product (D) were diluted with PBS prior to chromatography. Chromatographic conditions as in Fig. 7.

CONCLUSIONS

The use of polymeric reversed-phase supports in tandem with a simple sample preparation procedure has enabled the development of an assay for aFGF in cell suspensions and cell lysates with mean precision and accuracy values of 5%. The polymeric columns described here have been in use for over six months, periodic (biweekly) washing with 60 ml of 50%

methanol–0.2 *M* NaOH have prevented any noticeable deterioration in separation quality. Samples generated from either fermentation or any processing step can be assayed with the same HPLC method, eliminating the need for immunoassays to quantitate aFGF in very crude matrices. Enhanced throughput can be obtained using Perfusion Chromatography columns operated at high superficial velocity. The resulting chromatography is fast enough to obtain results concurrent with either fermentation or processing operations, with the potential for real time on-line analysis.

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