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Fast quantitation of recombinant plasminogen activator inhibitor type 1 in bacterial lysates by micropellicular reversed-phase liquid chromatography

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Abstract

A rapid reversed-phase HPLC assay is described for quantitating recombinant plasminogen activator inhibitor type 1 (PAI-1) in cultures of *Escherichia coli*. The assay utilized a short nonporous micropellicular C_{18} column with an acetonitrile gradient in 0.1% trifluoroacetic acid. The selective resolution of recombinant PAI-1 from major host proteins occurred within 1.3 min. Regeneration of initial chromatography conditions was fast and allowed successive injections every 3 min. The assay's limit of detection for recombinant PAI-1 was 0.5 μg in 5-μl injections of bacterial lysates and the assay was linear to 20 μg. The assay's apparent recovery was between 94 and 108% throughout the quantitative range. In a direct comparison to gel electrophoresis the reversed-phase assay proved superior in monitoring recombinant PAI-1 titers in cultures of *E. coli*.
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1. Introduction

Tissue-type plasminogen activator (t-PA) is a serine protease that catalyzes the conversion of plasminogen to the enzymatically active protein plasmin. Plasmin is responsible for the lysis of fibrin clots, but its involvement in other processes such as angiogenesis, hemostasis, metastasis of tumors, cell migration, and ovulation has been suggested [1,2]. The primary physiological regulator of t-PA activity is plasminogen activator inhibitor type 1 (PAI-1, $M_r \cong 50\,000$) [3]. PAI-1 is a member of a class of serine protease inhibitors called serpins [4]. The interaction between PAI-1 and t-PA leads to the formation of a stable acyl bond between the two molecules [4]. Such action reduces the fibrinolytic activity of circulating t-PA. The consequences of which have suggested an etiology for thrombotic disorders such as coronary artery disease and myocardial infarction [3]. Modulation of PAI-1 activity through therapeutic intervention might ameliorate these conditions or provide a means for regulating other processes involving plasmin.

The concentration of PAI-1 in plasma is approximately 20 ng/ml [5]. This low level is not sufficient for the studies required to develop an effective therapy. Fortunately, several recombinant sources of PAI-1 have been described [6,7]. Notable among

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these are *Escherichia coli* systems that are amenable to large-scale production of recombinant proteins. Although PAI-1 is a glycoprotein, the nonglycosylated version produced in *E. coli* is active in regulating t-PA [7]. This suggests that *E. coli* fermentations can be a viable source of large-scale production of active PAI-1 to further therapeutic studies.

The development of reproducible fermentations requires fast, selective, robust and quantitative assays for monitoring the desired product. When the desired product is a recombinant protein produced in *E. coli* this can be particularly challenging for two reasons. Firstly, the *E. coli* genome predicts over 4200 host proteins and secondly, recombinant proteins produced in *E. coli* are generally not excreted but remain cell associated [8]. Efficient and fast separation of recombinant products, such as PAI-1, from all these other analytes can be a daunting task. Nevertheless, this work shows how a short nonporous micropellicular C_{18} column operated at elevated temperature can facilitate development of a fast reversed-phase liquid chromatographic method for quantitating recombinant PAI-1 in lysed cultures of *E. coli*.

2. Experimental

2.1. Materials

Trifluoroacetic acid (TFA) and acetonitrile were from Burdick and Jackson (Muskegon, MI, USA). Guanidine was from Boehringer Mannheim (Indianapolis, IN, USA). The source of dioxane-free isopropyl β-D-thiogalactopyranoside (IPTG) was Gibco BRL (Gaithersburg, MD, USA). Carbenicillin was from Teknova (Half Moon Bay, CA, USA). Sources of powdered growth media are described elsewhere [9]. All other chemicals were from J.T. Baker (Phillipsburg, NJ, USA).

2.2. PAI-1 expression

Expression of recombinant PAI-1 was from the human gene cloned into plasmid pET11b in *E. coli* strain MM294(DE3). The growth of bacterial cultures was in shake flasks in M101 medium containing 4% yeast extract, 2% glycerol, and 50 μg/ml carbenicillin at 37°C [9]. The addition of IPTG to 1 m*M* induced PAI-1 gene expression. The growth of MM294(DE3) carrying a plasmid lacking the PAI-1 gene was in an identical manner and used as a control to analyze bacterial lysates in the absence of recombinant PAI-1 production. The purification of recombinant PAI-1 followed published procedures [10].

2.3. Chromatography

Protein and bacterial sample preparation included resuspension in 6 *M* guanidine·HCl, followed by brief vortexing, and incubation for a minimum of 20 min at room temperature prior to clarification by microcentrifugation. Sample injections were 5 μl onto a HY-TACH micropellicular (2 μm, nonporous) C_{18} column (30×4.6 mm I.D.) from Glycotech (Hamden, CT, USA). The injection valve, sample loop, and column temperatures were 80°C throughout the run and analyte detection was by UV absorbance at 280 nm. Mobile phase A consisted of water–acetonitrile (80:20) in 0.1% TFA and mobile phase B was water–acetonitrile (20:80) in 0.1% TFA. A linear gradient from 25% to 55% B over 1.5 min with a flow-rate of 3 ml/min resolved PAI-1 from major host proteins. Column re-equilibration was complete within the next 1.5 min. The chromatography system was from Thermo Separation Products (San Jose, CA, USA) and included a P4000 quaternary pump, an AS3000 autosampler and column oven, and a UV6000LP photodiode array detector. An SN4000 system controller interfaced to a personal computer running ChromQuest software controlled the chromatography system.

2.4. Electrophoresis and Western blots

Electrophoresis utilized 10% Bis–Tris NuPAGE gels (Invitrogen, Carlsbad, CA, USA) under reducing conditions with a 3-(*N*-morpholino)propanesulfonic acid running buffer according to the manufacturer's instructions. GelCode Blue colloidal Coomassie stain (Pierce, Rockford, IL, USA) enabled the visualization of electrophoresed proteins. The ChemiImager 4000 (Alpha Innotech, San Leandro, CA, USA) scanned stained gels to determine the staining den-

sity of individual bands. For Western blotting, the electrotransfer of proteins to nitrocellulose paper (Schleicher & Schuell, Keene, NH, USA) proceeded as described elsewhere [11] but with the addition of 0.1% sodium dodecyl sulfate (SDS) to the transfer buffer. Goat anti-human PAI-1 (Biopool, Ventura, CA, USA) was the primary antibody in the western blot followed by rabbit-anti-goat antibody conjugated to alkaline phosphatase (Sigma, St. Louis, MO, USA) as described previously [12–14].

3. Results

In attempts to optimize the production of recombinant PAI-1 in *E. coli* we sought to develop a fast, robust, high-throughput and quantitative process-monitoring assay. We chose a reversed-phase assay utilizing a short nonporous micropellicular C_{18} column operated at 80°C. Utilizing an acetonitrile gradient in 0.1% TFA, recombinant PAI-1 in *E. coli* lysates eluted at 1.3 min with a peak width at half height of 4 s (Fig. 1). A similar bacterial lysate that did not express PAI-1 did not exhibit this peak. The peak at 1.3 min reappeared upon addition of purified recombinant PAI-1 to these cells prior to lysis (Fig. 1). Isolation and SDS–polyacrylamide gel electrophoresis (PAGE) analysis of the eluent at 1.3 min demonstrated that the corresponding peak consisted of one predominant protein species having the same electrophoretic mobility as purified recombinant PAI-1 (Fig. 1, lane A). Additionally, anti-PAI-1 antibodies reacted to the isolated peak in a Western blot (Fig. 1, lane C). These data suggested that the peak eluting at 1.3 min in these bacterial lysates represented recombinant PAI-1 and demonstrated the assay's selectivity.

Separation and column re-equilibration were complete within 3 min after an injection, which allowed rapid analysis of recombinant PAI-1 samples. The short run time suggested the assay had the capability of high throughput if it could reproducibly monitor recombinant PAI-1 in numerous lysate samples. Towards this end, a series of 50 consecutive injections were made of a single bacterial lysate containing recombinant PAI-1 and the results are shown in Fig. 2. Despite the crude nature of the sample, column performance did not appreciably

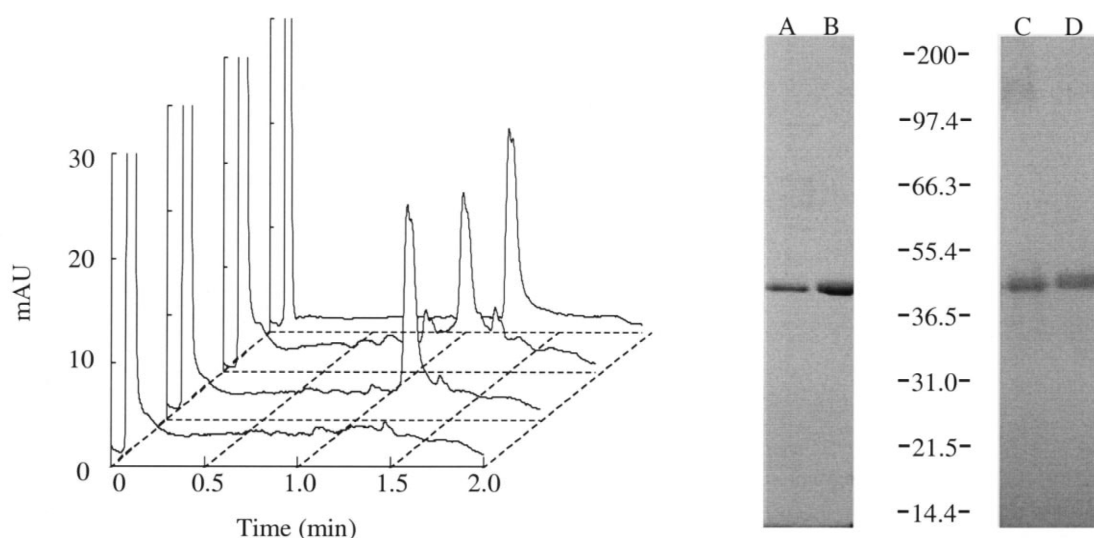

Fig. 1. Reversed-phase chromatograms of recombinant PAI-1 and crude *E. coli* lysates. Left panel: the chromatograms represent from the top to the bottom of the figure: a purified sample of 1.1 μg of recombinant PAI-1, an induced *E. coli* culture expressing PAI-1, an induced *E. coli* culture lacking the PAI-1 gene but spiked with 1.1 μg of purified recombinant PAI-1 prior to solubilization, and an induced *E. coli* culture lacking the PAI-1 gene. Right panel: gel electrophoresis of the isolated peak at 1.3 min from an induced *E. coli* culture expressing PAI-1 (lane A) and purified recombinant PAI-1 (lane B) and western blot analysis with anti-PAI-1 antibodies of the same isolated peak at 1.3 min (lane C) and purified recombinant PAI-1 (lane D). The electrophoretic migration of molecular mass markers is shown in kilodaltons.

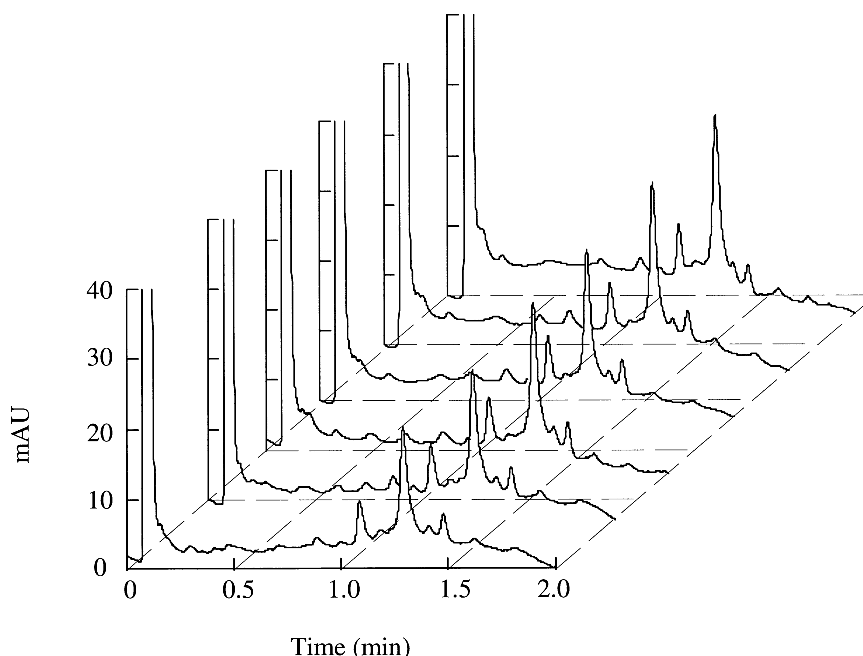

Fig. 2. Reproducibility of chromatograms of crude *E. coli* lysates. An *E. coli* culture expressing PAI-1 was lysed and chromatographed as described under Experimental. Fifty consecutive injections were made. The chromatograms represent injections 1, 10, 20, 30, 40, and 50 from the bottom to the top of the figure, respectively.

decrease during this injection sequence. The retention time of the recombinant PAI-1 peak in the fiftieth injection differed by less than 3 s from that of the first injection. The standard deviation of the PAI-1 peak height over 50 injections was less than 10%. These data demonstrated that the assay was robust and had the capability of high throughput.

The assay demonstrated peak height linearity for purified recombinant PAI-1 from 0.2 to 20 μg ($n=3$, $r^2=0.998$, data not shown). Yet, to define the quantitative range of the assay we determined the recovery of recombinant PAI-1 from *E. coli* lysates throughout the linear range. An *E. coli* culture carrying a plasmid lacking the PAI-1 gene was induced with IPTG and then harvested 2 h later. The cell pellets received a defined amount of recombinant PAI-1 in a minimal volume prior to lysis with 6 *M* guanidine·HCl and reversed-phase (RP) HPLC analysis. Comparing the peak heights of the spiked PAI-1 samples to those of purified recombinant PAI-1 samples treated and analyzed in an identical manner determined the recovery of the assay. The results reported in Table 1 demonstrated acceptable recoveries between 94 and 108% and defined the assay's limit of detection as 0.5 μg and its quantitative range as 0.5 to 20 μg.

Analysis of the time-dependent production of PAI-1 in an induced culture of *E. coli* illustrated the utility of the assay. Strain MM294(DE3) was grown as described under Experimental and induced with

Table 1
Recovery of PAI-1 from *E. coli* lysates[a]

Amount of PAI-1 spike (μg)	Recovery ($n=3$) (%)
0.2	199.6±5.9
0.5	102.4±3.9
1.0	106.4±3.8
2.0	98.0±6.5
5.0	108.0±2.8
10.0	98.4±3.6
20.0	93.5±4.1

[a] Aliquots (1 ml) of an induced *E. coli* culture lacking the PAI-1 gene were harvested by microcentrifugation. Each bacterial pellet then received a defined amount of purified recombinant PAI-1 prior to solubilization and chromatography as described under Experimental.

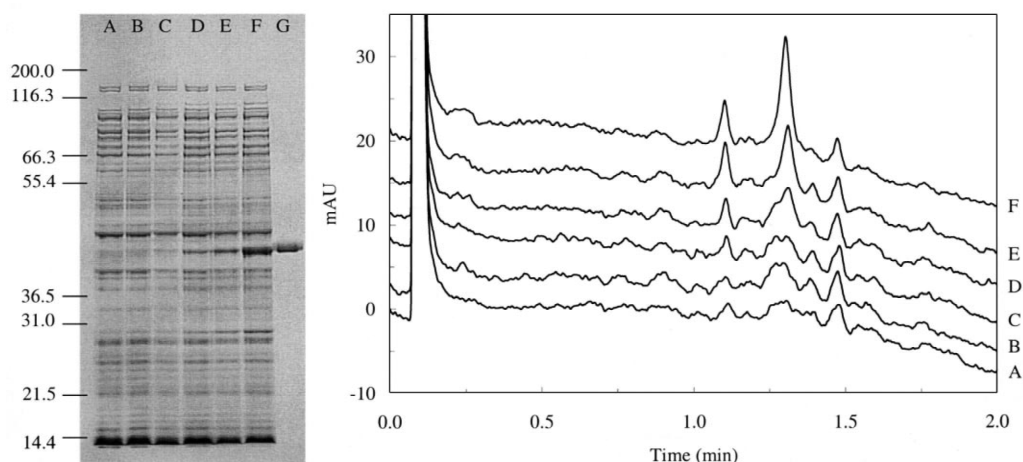

Fig. 3. Time-dependent production of recombinant PAI-1 in *E. coli* after induction. *E. coli* strain MM294(DE3) carrying the gene for PAI-1 was grown and induced with IPTG. At various times thereafter, samples were taken and processed for SDS–PAGE or RP-HPLC as described under Experimental. The gel lanes and chromatograms correspond to: (A) pre-induction, (B) 5 min post-induction, (C) 15 min post-induction, (D) 30 min post-induction, (E) 60 min post-induction, (F) 120 min post-induction, and (G) purified recombinant PAI-1. The electrophoretic migration of molecular mass markers is shown in kilodaltons to the left of the gel. The additional peak in the chromatogram at ~1.1 min and the band in the gel at $M_r \approx 30\,000$ (30 kilodaltons) were both identified as β-lactamase, a protein unrelated to PAI-1.

IPTG to initiate PAI-1 expression. At various times thereafter, SDS–PAGE and RP-HPLC analysis determined the PAI-1 content of 1-ml culture aliquots. In the results shown in Fig. 3, both analytical methods demonstrated a time-dependent increase in recombinant PAI-1 production after induction. Gel electrophoresis did not reliably detect PAI-1 production until 30 min post-induction whereas the RP-HPLC assay quantitated PAI-1 production within 15 min after induction. In another experiment, standard curves of purified recombinant PAI-1 were run simultaneously on both the gels and the HY-TACH column and used to quantitate the time-dependent production of PAI-1. The results shown in Table 2 demonstrated that both methods detected a time-dependent increase in volumetric recombinant PAI-1 titers, but the titers were at least two-fold greater when measured by the RP-HPLC assay.

Table 2
Volumetric production of recombinant PAI-1 in *E. coli* after induction[a]

Time after induction (min)	Volumetric PAI-1 titer (mg/l)	
	SDS–PAGE (n=2)	RP-HPLC (n=3)
Pre-induction	<6.4	0
5	<6.8	1.8±0.5
15	<9.3	15.8±1.4
30	12.8	44.2±4.0
60	30.1	88.2±7.1
120	66.5	169.0±10.1

[a] Samples from an *E. coli* culture expressing PAI-1 were analyzed for PAI-1 titers by either SDS–PAGE or RP-HPLC as described in the legend of Fig. 3 and under Experimental.

4. Discussion

Optimization of recombinant protein production in fermentations requires fast, selective, robust and quantitative analytical assays. On occasion, analysts employ immunoassays for this purpose because of their high selectivity and throughput. However, immunoassays are slow in providing results for real time *E. coli* fermentation optimization and process monitoring. These assays also suffer from the limitation of detecting only antibody-reactive products, which may not be equivalent to the total amount of recombinant protein. Gel electrophoresis lacks sufficient speed, selectivity, and throughput to be a process monitoring technique. Capillary electrophoresis, on the other hand, does show considerable promise for process monitoring [15]. It has the

capability of high throughput, quantitation, and highly efficient separations. Still, as a process-monitoring tool, capillary electrophoresis lacks robustness. It is difficult to reproduce numerous consecutive runs consistently and determining the recovery of an analyte can be difficult. Therefore, we chose a reversed-phase assay utilizing a short nonporous micropellicular C_{18} HY-TACH column operated at elevated temperature to satisfy all assay requirements.

The reversed-phase assay for recombinant PAI-1 was selective and fast. An isolated fraction of the eluent at 1.3 min was devoid of proteins except for recombinant PAI-1, which demonstrated the assay's selectivity. Individual chromatography runs required only 3 min. Consecutive injections of up to fifty crude bacterial lysates did not alter the column's performance significantly. The reproducibility of these injections was high. The peak height of all 50 injections deviated by less than 10% from the average. Also, the retention time of the fiftieth injection was less than 3 s different from that of the first. However, we did note that after additional injections (>60) the retention of the PAI-1 peak started to change by more than 6 s. Also, after several hundred injections of crude bacterial lysates the recovery of PAI-1 did start to change significantly. Cleaning the column with nitric acid injections and a formic acid–isopropanol (50:50) mobile phase at 80°C was effective and could be used at set intervals during long injection sequences to minimize these changes. Nevertheless, considering the crude nature of the lysate samples, the assay was robust for process monitoring and it also had the capability of high-throughput.

The RP-HPLC assay was quantitative. It was linear over a 100-fold range of purified recombinant PAI-1. However, spike and recovery studies revealed that only 0.5 μg to 20 μg of PAI-1 had acceptable recovery and this defined the assay's quantitative range. Within this range, the standard deviation of replicate recovery analyses was <10%, attesting to the reproducibility of the assay. The unusually high recovery at 0.2 μg of PAI-1 was likely due to insufficient resolution from low level endogenous host proteins. Although these host proteins were present in all the lysate samples they only made significant contributions to the PAI-1 peak height when the sample's PAI-1 content was low. The inability to detect 0.2 μg of PAI-1 in a lysate is not a significant drawback of the assay. Recombinant PAI-1 was greatly overexpressed in *E. coli* making process monitoring for fermentation optimization still possible. Nevertheless, potentially both the sensitivity and the selectivity of the assay could be increased. Monitoring for intrinsic protein fluorescence [16] or utilizing fluorescence derivatization [17] increased the sensitivity and selectivity, respectively, of micropellicular HPLC assays described previously for process monitoring.

In a direct comparison to SDS–PAGE for determining recombinant PAI-1 titers, the micropellicular RP-HPLC assay proved superior in a number of ways. The speed of the analysis and the sample throughput of the RP-HPLC assay were clearly greater. Gel electrophoresis was incapable of detecting the lower levels of recombinant PAI-1 produced early after induction. This reflected both the lower sensitivity and the decreased selectivity of the electrophoretic assay. Conversely, the RP-HPLC assay not only detected recombinant PAI-1 within 15 min after induction, but the assay quantitated these levels reproducibly thereafter with a standard deviation of <10%. This ability is due to the increased resolution and selectivity of micropellicular RP-HPLC brought about by highly efficient separations. The volumetric titers determined by both methods differed by approximately two-fold. The increased volumetric titers determined by the RP-HPLC method are attributed to differences in the sample preparation procedures used prior to either assay. At least a portion of the recombinant PAI-1 exists intracellularly in insoluble inclusion bodies. For RP-HPLC analysis, resuspending the bacterial pellet in 6 *M* guanidine lysed the cells and effectively solubilized the inclusion bodies. In contrast, gel electrophoresis employed the ionic detergent SDS for cell lysis. SDS is less effective than guanidine at solubilizing inclusion bodies thus explaining the lower recombinant PAI-1 titers observed by SDS–PAGE analysis.

The use of nonporous micropellicular sorbents for process monitoring is increasing [16–18]. These resins offer several advantages over conventional microporous particles for fermentation monitoring [19]. First, columns packed with nonporous micropellicular sorbents have increased mass transfer of

solutes. The increase is partly due to the small size of the particles (~2 μm) and the presence of a thin-layer stationary phase [19]. Further increases in mass transfer are achieved by utilizing an elevated temperature that lowers the viscosity of the mobile phase and increases the diffusivity of the proteins. The enhanced mass transfer made possible by these properties increased the separation efficiency of the lysates and produced a PAI-1 peak with a retention time of 1.3 min and a peak width at half height of approximately 4 s. Second, the ability to use elevated temperatures with nonporous micropellicular sorbents also contributes to the speed of the analysis by allowing the use of high flow-rates. The high flow-rate, the lack of pores, and a short column length allowed for rapid re-equilibration of the column. For the assay described here, these features produced a 3 min run time for each injection which allowed high throughput analysis of numerous samples. Third, nonporous particles also have the advantage of higher protein recovery because of the lack of protein entrapment within pores. This partly explains the excellent recovery of recombinant PAI-1 from *E. coli* lysates.

In this paper we described the use of a nonporous micropellicular sorbent for monitoring the production of a specific recombinant protein in *E. coli*. It is likely that this application can be expanded and made more general. Often protein investigations can be greatly facilitated by acquiring large amounts of the specific protein under study. *E. coli*, which has a number of powerful promoters for heterologous protein expression [20], is often the host of choice for producing large amounts of recombinant protein. In *E. coli* it is not unusual for recombinant proteins to reach greater than 10% of the total bacterial cell protein [21]. Therefore, by micropellicular chromatography it would not be difficult to identify an over expressed recombinant protein in an induced lysate when compared to a noninduced lysate. This can allow rapid screening of numerous isolates to identify an appropriate clone. We have already achieved success using this procedure on several different recombinant constructs (data not shown). Even in instances where *E. coli* expression is not high, more sensitive modes of detection such as low UV absorbance, intrinsic protein fluorescence, or fluorescence derivatization [22,23] can enable early clone selection. Furthermore, cell lysis procedures can be developed such that the assay identifies clones that produce high levels of soluble recombinant protein. Other heterologous expression systems such as yeast, mammalian and viral systems might also be amenable to this approach. These systems have potentially more endogenous proteins and often have lower levels of recombinant protein expression than many *E. coli* systems. However, the increased separation efficiency afforded by nonporous micropellicular sorbents and the added sensitivity achieved by detection methods discussed previously make these challenges manageable. Finally, electrospray ionization mass spectrometric detection may be coupled to nonporous micropellicular RP-HPLC for unambiguous rapid identification of a recombinant protein in a crude lysate [24].

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