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Analysis for residual host cell proteins and DNA in process streams of a recombinant protein product expressed in *Escherichia coli* cells

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Abstract

Analyses of crude samples from biotechnology processes are often required in order to demonstrate that residual host cell impurities are reduced or eliminated during purification. In later stages of development, as the processes are further developed and finalized, there is a tremendous volume of testing required to confirm the absence of residual host cell proteins (HCP) and DNA. Analytical tests for these components are very challenging since (1) they may be present at levels that span a million-fold range, requiring substantial dilutions; (2) are not a single component, often existing as fragments and a variety of structures; (3) require high sensitivity for final steps in process; and (4) are present in very complex matrices including other impurities, the product, buffers, salts and solvents. Due to the complex matrices and the variety of potential analytes, the methods of analysis are not truly quantitative for all species. Although these limitations are well known, the assays are still very much in demand since they are required for approval of new products. Methods for final products, described elsewhere, focus on approaches to achieve regulatory requirements. The study described herein will describe the technical rationale for measuring the clearance of HCP and DNA in the entire bioprocessing to purification from an *Escherichia coli*-derived expression system. Three analytical assays, namely, reversed-phase high-performance liquid chromatography (RP-HPLC), enzyme-linked immunosorbent assay (ELISA), and Threshold Total DNA Assay, were utilized to quantify the protein product, HCP and DNA, respectively. Product quantification is often required for yield estimation and is useful since DNA and HCP results are best expressed as a ratio to product for calculation of relative purification factors. The recombinant *E. coli* were grown to express the protein of interest as insoluble inclusion bodies (IB) within the cells. The IB were isolated by repeated homogenization and centrifugation and the inclusion body slurry (IBS) was solubilized with urea. After refolding the product, the solution was loaded on several commonly used ion exchangers (CM, SP, DEAE, and Q). Product was eluted in a salt gradient mode and fractions were collected and analyzed for product, HCP and DNA. The IBS used for this study contained about 15 mg/ml product, 38 mg/ml HCP and 1.1 mg/ml DNA. Thus, the relative amounts of HCP and DNA in the IBS was excessive, and about 10^3 times greater than typical (because the cells and IB were not processed with the

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normal number of washing steps during isolation). This was of interest since similar samples may be encountered when working with non-inclusion body systems, such as periplasmic expressions, or in cases where the upstream unit operations under-perform in IB cleaning. The study described herein describes the development of three robust methods that provide the essential process data needed. These findings are of general interest to other projects since applications of similar analytical technology may be used as a tool to develop processes, evaluate clearance of impurities, and produce a suitable product.

Keywords: HCP; Host cell impurities; DNA; Chromatography; Protein purification; Impurity clearance; *E. coli* inclusion bodies; Ion exchange resins

1. Introduction

Development of new recombinant proteins has increased greatly over the past several years. In microbial systems, these proteins are generally obtained from the fermentation of microorganisms and the protein of interest is released from the cells. Often the release involves cell-lysis and this leads to a simultaneous release of undesirable species such as bacterial host cell proteins (HCP), nucleic acids (DNA and RNA), endotoxin, and other host cell impurities [1,2]. This requires a purification process to purify the protein from all these impurities, not only as demonstration of good manufacturing procedures (GMPs), but also to reduce the potentially negative impact that the presence of these impurities in biopharmaceutical therapeutics may have on the safety of the product [3,4]. Unless DNA and HCP have been cleared during processing, and reduced to acceptable levels (typically ng/mg for HCP and pg/mg for DNA), the product is unlikely to meet industry and regulatory requirements [10–13] or be used for clinical or commercial purposes [1,4]. Thus, acceptable identification and quantification of these impurities is often a complicated task in itself [1].

Typically, inclusion bodies (IB) in the fermentation product are processed by cell disruption, centrifugation, washing, and removal of supernatant, such that HCP and DNA are greatly reduced in the process stream before purification. It is, therefore, unusual to begin a series of chromatographic purifications with material that has not had its burden of these impurities already reduced. Further, due to the widespread use of fast flow ion exchangers in protein purification [4–9]. Pharmacia Sepharose Fast-Flow SP, CM, Q, and DEAE ion exchangers were used in this study. The purpose of the study described herein was to investigate the performance of the different analytical assays with material that had not been processed up front, and contained about 10^3-fold greater quantities of DNA and HCP than normal (15 mg/ml product, 38 mg/ml HCP and 1.1 mg/ml DNA). The interest in starting with highly contaminated material was 2-fold: (1) to provide material of sufficient level of impurity to calculate clearance factors more accurately, and (2) to better understand performance of chromatography steps when the starting material is less pure than the normal case. This is especially relevant when working with non-inclusion body systems, such as periplasmic expressions, or in the case when the upstream unit operations under-perform in IB cleaning. The current study evaluates the performance of the analytical methods and the impact on the refold efficiency and purification recovery.

The protein of interest in this study is a dual agonist of both the granulocyte colony stimulating factor (G-CSF) and fetal liver tyrosine kinase 3 (flt-3) receptors developed as a component of cancer vaccine protocols to stimulate dendritic cell proliferation.

2. Process

2.1. Materials and instrumentation

Escherichia coli was used as host for the production of the protein. The seed flask was transferred aseptically to a Braun Biostat-E fermentor containing pre-sterilized medium. Aeration, agitation, pH, temperature, dissolved oxygen

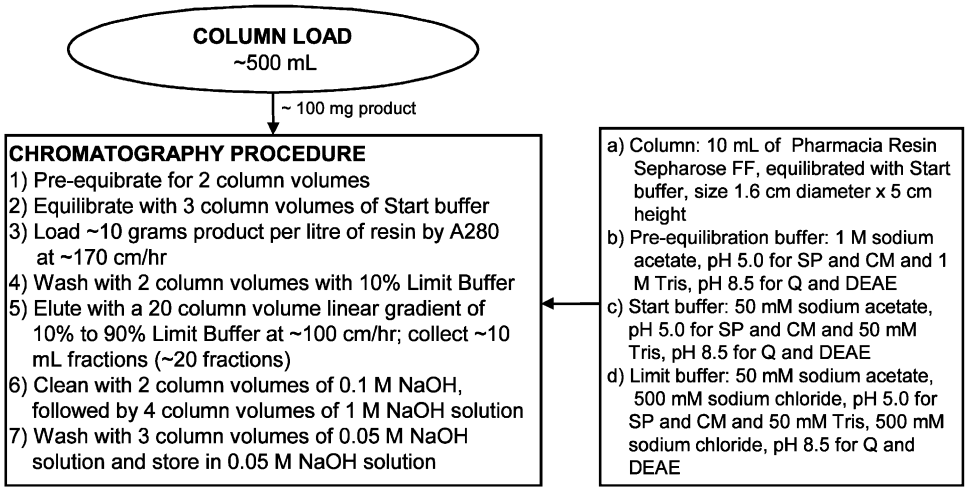

Fig. 1. Procedure for chromatography step.

levels, glucose level, and nitrogen source (ammonium hydroxide) were monitored and maintained within specifications. Fermentation was terminated 6 h after induction and the IB were harvested. The broth was recovered and transferred to a Beckman J2-21 centrifuge. The supernatant was discarded and the cells were separated using a Beckman JCF-Z continuous flow rotor. The cell paste was removed from the rotor and mixed with water using an Ultra Turrax T25 mixer. Cell disruption was accomplished using a Microfluidics microfluidizer and the slurry was then aliquotted and stored at $\leq -20\,^{\circ}C$. Minimal inclusion body (IB) washing was done during the fermentation and isolation processes for the purpose of this study. In normal operations, these washes are used to remove the bulk of the nucleic acids and other host cell impurities.

The inclusion body slurry (IBS) was thawed at 2–10 °C for 48–72 h and then homogenized. The protein concentration in the IBS was measured by reversed-phase high-performance liquid chromatography (RP-HPLC) performed under reducing and denaturing conditions and this was used to estimate the protein charge to the refold. The IB were dissolved in a reducing environment and the protein allowed to refold in the presence of appropriate additives. The refold was terminated after the holding period by addition of 6 M acetic acid to adjust the pH to 5.0 ± 0.3 for SP and CM Loads and to 8.5 ± 0.3 for Q and DEAE Loads. The refold solution was stirred for 1 h at 2–12 °C and filtered through a 0.2 μm Sartorius Sartopure P filter (Catalogue Number 5231307H700B). The filtered solution was directly loaded on the appropriate chromatography column.

The chromatographic procedure is illustrated in the Flowchart shown in Fig. 1. Experiments were performed using 1.6×5 cm (10 ml) columns from Amersham Biosciences. The procedure consisted of seven steps, namely, column pre-equilibration, equilibration, loading, wash, elution, cleaning and storage. The lengths of these steps and the respective buffers used with the different columns are also indicated in Fig. 1. Each column was loaded with 500 ml of refold solution containing about 100 mg of the product. This resulted in a protein loading of 10 mg product/ml resin for the 10 ml column, which was about half the usable capacity of the SP column for this product [9]. Considering that more than twice HCP than product was present in the source of IB (see page 2), the columns were overloaded if total protein is considered. The dynamic capacity given by the manufacturer, Amersham Pharmacia Biotech, for these resins were as follows: SP FF—70 mg

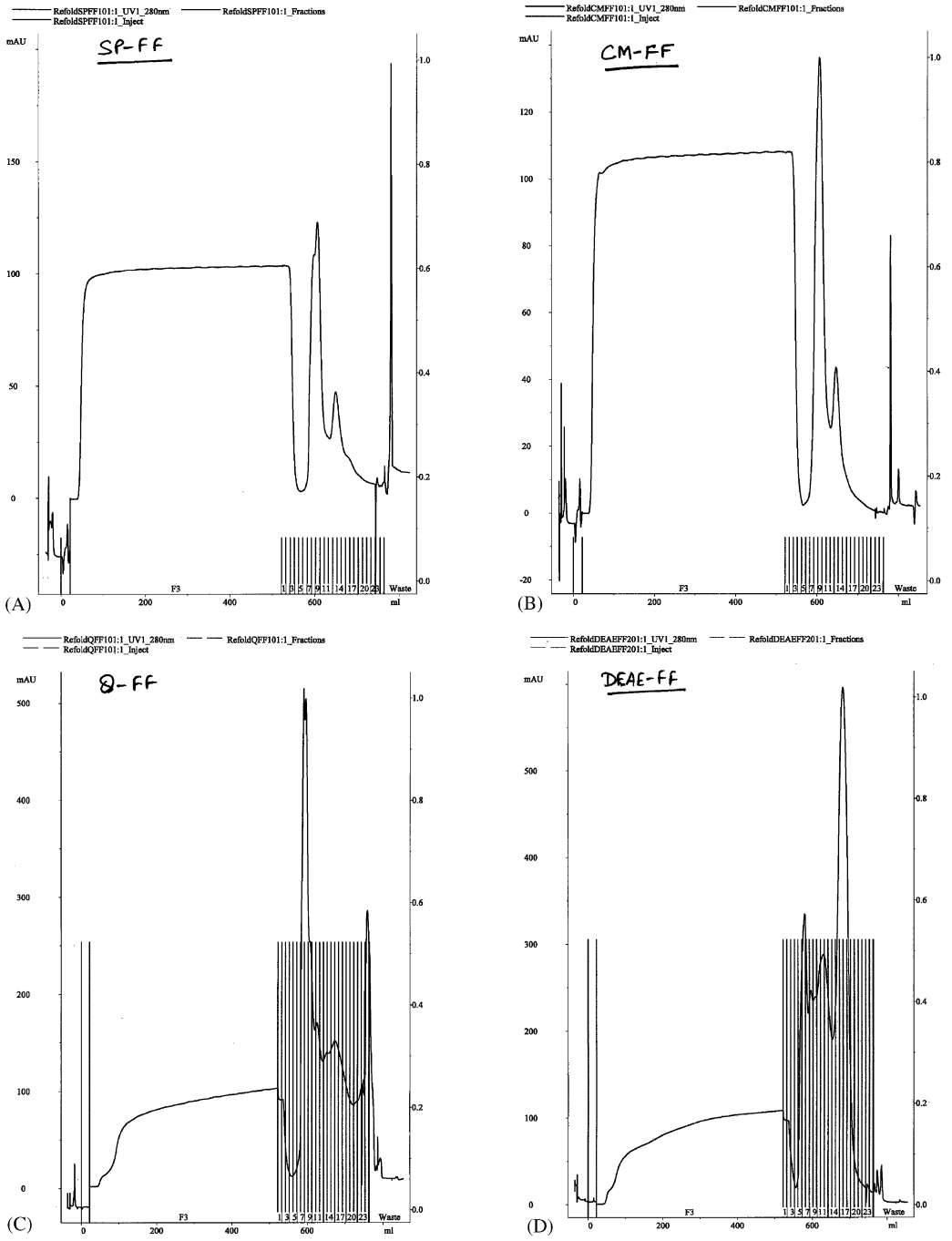

Fig. 2. Performance of different ion-exchange resins.

Table 1
Recovery comparison of different chromatography resins

Resin	Pool #	mg/ml protein by RP-HPLC	ml	mg	Total mg per column	Percent recovery protein[a] (%)
Pharmacia SP FF	1	0.027	50	1.35		
	2	0.061	60	3.66	5.01	5.0
Pharmacia CM FF	1	0.037	50	1.85		
	2	0.051	50	2.55	4.4	4.4
Pharmacia DEAE FF	1	0.66	60	39.6		
	2	0.55	60	33	72.6	72.6
Pharmacia Q FF	1	0.41	40	16.4		
	2	0.45	50	22.5	38.9	38.9

[a] Percent recovery based on 100 mg protein in each column load by RP-HPLC.

RNAse/ml resin, CM FF—50 mg RNAse/ml resin, Q FF—120 mg HSA/ml resin, and DEAE FF—110 mg RNAse/ml resin. Samples were collected from the column loads, the flow through during loads, the pre-elution wash, the fractions during elution and post elution wash, and were submitted for extensive analysis for product concentration and the above mentioned host cell impurities. In order to avoid protease effects on product and assays, the samples were frozen immediately and thawed just prior to assay.

2.2. Results and discussion

Fig. 2 shows the chromatograms corresponding to the SP FF, CM FF, Q FF, and DEAE FF, respectively. For all the cases, the chromatograms show several peaks. As discussed later, analysis by RP-HPLC and enzyme-linked immunosorbent assay (ELISA) shows that while some of these peaks contain mostly product, some are primarily formed by the presence of the large quantities of HCP and/or nucleic acids.

Table 1 illustrates results from analysis by RP-HPLC of the chromatographic performance of the four columns under the selected operating conditions. To keep the data presentation simple, fractions were pooled as two pools for each column, pool 1 representing the early eluting part of the peak and pool 2 the later part.

The poor performance of the SP and CM columns is also corroborated by the almost 4-fold lower absorbance seen in the chromatograms obtained with the SP and CM columns as compared with those with Q and DEAE columns in Fig. 2. This can be explained to some extent by the lower binding capacity of the cation exchange columns in comparison to the anion exchange columns. Evidently, the $\approx$1000-fold increase in the HCP and DNA have a more deleterious impact on the performance of the cation exchange columns than in the anion exchangers (as discussed in Section 3 later).

This was the case in spite of loading the product on the columns at less than half of the known capacity and at a fifth to tenth of the manufacturer's published dynamic capacity. While the columns were loaded according to the concentration of the product in the load material, the actual protein loading (including the HCP) was significantly greater than the recommended column capacity. As some HCP species were chromatographically similar to the product, some product was displaced, thereby decreasing product yield. Apparently, this phenomenon was less significant with the Q and DEAE columns, where the recoveries compare very well with published values in literature [4]. The deleterious impact of the presence of host cell impurities in large concentrations on the yield of a chromatography step has been previously reported [14]. These data are consistent with the

STANDARD CURVE

X Product mg/mL	Y Peak Area	Y Calc.	% Bias
0.02	801.72	783.01	98
0.04	1677.82	1598.89	95
0.08	3135.50	3230.63	103
0.15	6053.95	6086.17	101
0.3	12234.90	12205.20	100

Slope:	40793.50
Intercept:	-32.86
Correl:	0.9999

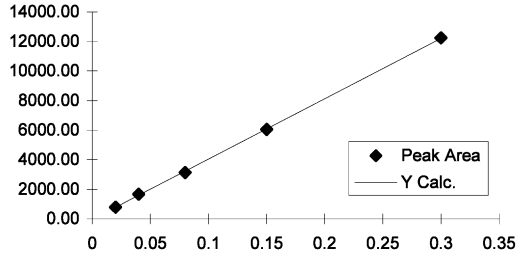

Fig. 3. Standard curve for RP-HPLC analysis.

general understanding that mostly the HCPs are acidic.

3. Analytical

3.1. Reversed-phase HPLC

Each fraction collected from the columns was dissolved in a solubilization solution (0.8 M Tris, 0.4 M DTT, 4% SDS) and analyzed on a Vydac C18 column. The mobile phase was 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile. Samples were loaded and eluted in a gradient of acetonitrile and monitored at 214 nm. This separation mode resolved the product from the other impurities such as nucleic acids, HCPs, and other components which is evident by the elution time versus a reference standard and a typical ratio of 214/280 nm versus a reference standard. Sample results were interpolated from a standard curve prepared from reference standard.

Fig. 3 illustrates the standard curve for the RP-HPLC analysis. It is evident from the linearity of the curve that despite the large amount of the HCP and DNA in the in-process samples, the method is capable of performing accurate analysis in the concentration range under consideration. The results of testing are shown in Fig. 4. The RP-HPLC, being a resolving assay, shows only the product signal which, contrasted to the UV 280 nm trace in Fig. 2, illustrates the interfering signals at 280 nm, presumably nucleic acids, HCP, and other components that absorb at this wavelength. An interesting observation from Fig. 3 is that all cases either exhibit dual peaks or peaks with shoulders. This is most likely due to early eluting protein species (from ion-exchange) that are higher molecular weight product aggregates or complexes of product and other components such as DNA or RNA giving it an apparently higher molecular weight. These high molecular weight species are dissociated by the denaturing RP-HPLC analysis.

It follows from comparison of Fig. 4A and B that the SP FF and CM FF columns behave in a similar fashion and the product elutes in fractions 13–15 predominately, which corresponds to the second and smaller peak in the A280 (absorbance at 280 nm) profiles in Fig. 2A and B. Interestingly, the larger peak in the A280 profiles in Fig. 2A and B does not contain much product due to presence of other impurities (discussed later).

The profiles in Fig. 4C and D are different for the Q FF and DEAE FF columns, respectively. Analysis by RP-HPLC shows that the product mainly elutes in the fractions 11–16 for the Q FF column and fractions 8–11 for the DEAE FF column. This corresponds to the valley between the two major peaks in the A280 (absorbance at 280 nm) profile in Fig. 2C and D and leads to the conclusion that the A280 peaks themselves are formed by one or several of the host cell impurities and/or DNA.

Further, the product concentration is much higher with the anion exchangers then for cation exchangers, and this is indicated by the higher scale of the Y-axis in plots C and D for the anion exchange resins (700 mAU for Q-FF and

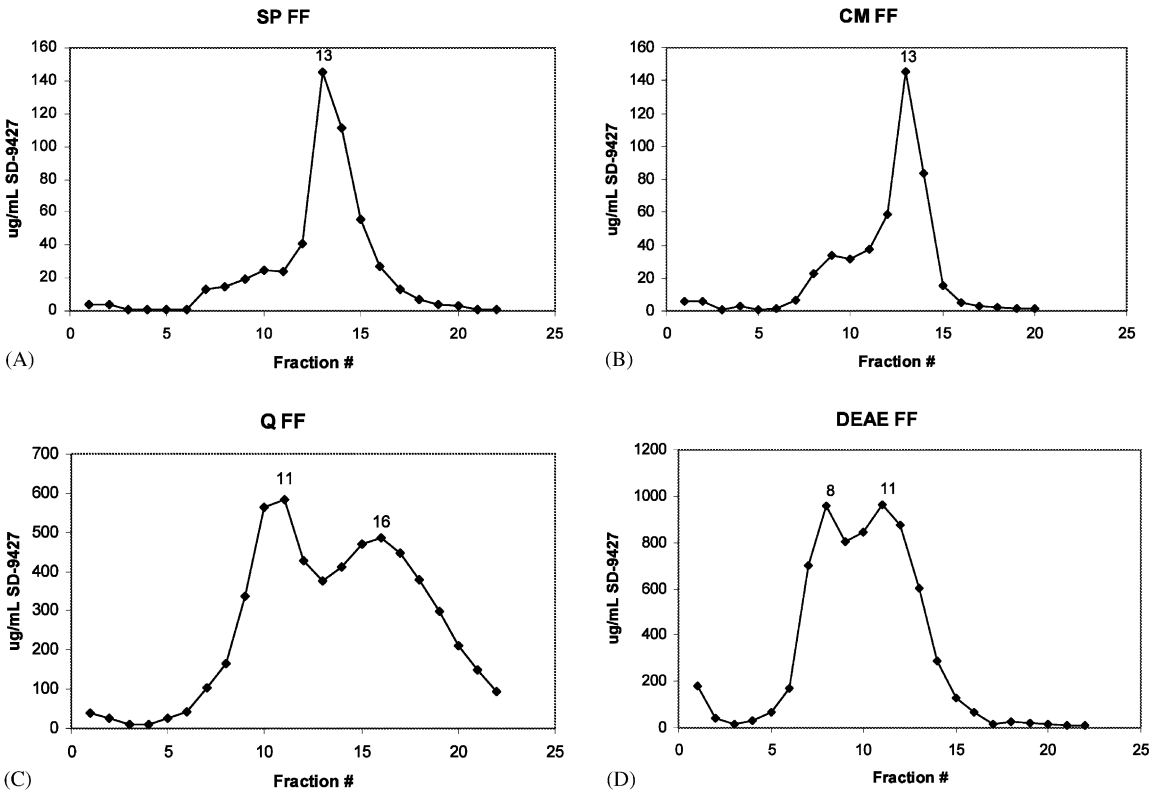

Fig. 4. RP-HPLC analysis of pools for different columns.

1200 mAU for DEAE-FF) compared with plots A and B (160 mAU for both SP-FF and CM-FF).

3.2. Host cell protein

This method required the preparation of HCP to be used as an immunogen and assay standard, and purified antibodies from the serum of animals immunized with the Host Cell Protein extract. A "null" cell was prepared, identical to the *E. coli* master cell except the gene encoding the product is not present. This material was fermented under typical expression conditions, and the extract used to immunize rabbits. Rabbit serum was screened for titer and specificity to HCP, pooled, and purified by affinity chromatography on a column where HCP had been immobilized. The purified antibodies were split and a portion biotinylated. An assay was developed where the capture antibody is immobilized on a 96 well microplate, non-specific sites are blocked with an inert protein solution, samples are applied, the biotinylated antibody applied, followed by a solution of streptavidin–horseradish peroxidase conjugate. The complex is reacted with hydrogen peroxide and *o*-phenylaminediamine. The plates were monitored at 490 nm in a plate reader. Samples were diluted and results obtained by interpolation by a standard series performed in the same plate.

Host cell protein analysis is very sensitive, capable of ng/ml measurements, and uses highly specific antibodies that measure HCP in complex samples [1,3]. Because the HCP content was on the order of mg/ml for these samples, several million-fold dilution was required to obtain results in range, as shown for the case of IBS in Fig. 5. At greater dilutions, a maximum signal is achieved (the antibody concentration on the plate is limiting), then a dose-dependent signal, followed by

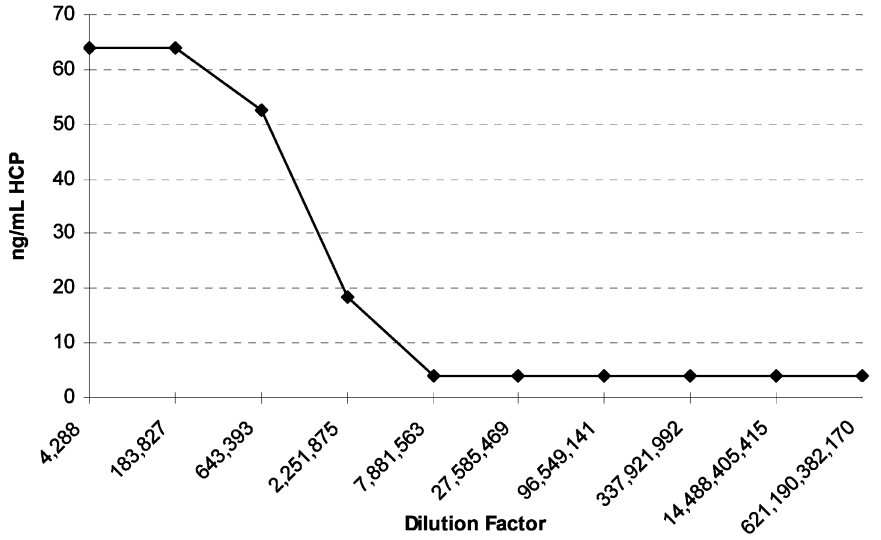

Fig. 5. HCP analysis of IBS.

levels that are below detection (4 ng/ml). Once the linear range was found, more dilutions were tested over a smaller range, to match signal levels in the standard. The criteria for a valid test were (1) spike recovery of 75–125% and (2) results in the linear range of the standard curve. The starting material (IBS), individual fractions from each column, and the two pools from each column were tested in this manner. These dilutions were spiked to prove the absence of interfering substances, and results in range averaged. The IBS contained 38 mg/ml of HCP. Since the concentration of product in the slurry was 15 mg/ml, the ratio of HCP/product was 2.53/1.

The individual fractions were diluted until in range, spiked to show 75–125% recovery, and an average calculated. Fig. 6 shows the performance of all four Pharmacia columns with regards to separation of the HCP species. Since the load concentration of HCP is approximately twice that of product (loaded at 0.2 mg/ml), it is was about 0.4 mg/ml, or 400 000 ng/ml when units changed. It can be seen that HCP are distributed across the range of the elution, a phenomenon consistent with the fact that HCP are of enormous variety and heterogeneity [4]. Some general conclusions may be drawn from the results presented in Fig. 6:

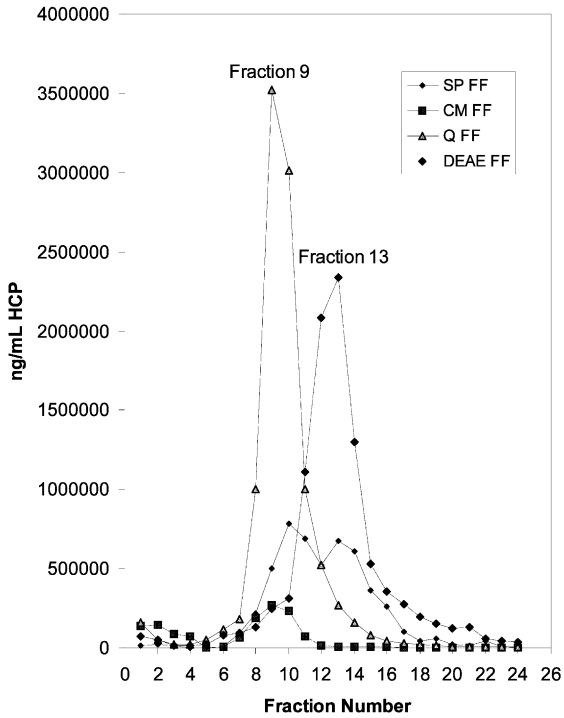

Fig. 6. Host cell protein analysis of fractions from pharmacia resins.

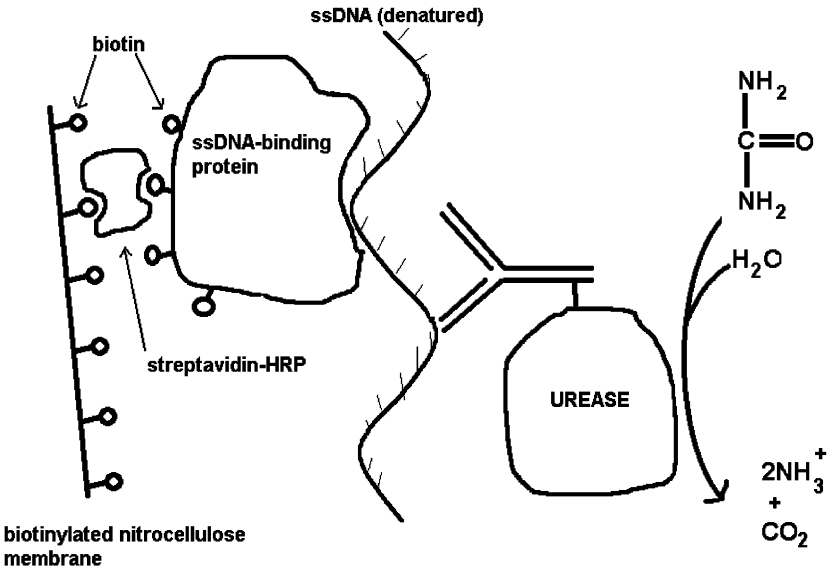

Fig. 7. Assay mechanism to quantitate DNA.

the anion-exchangers (Q, DEAE) retain much more of HCP than the cation-exchangers. This corroborates with the general understanding that most HCP are acidic species which bind more strongly to the anion exchangers.

3.3. Residual DNA

Samples were diluted and digested with Proteinase K for 4 h at 55 °C in order to digest the protein to very small peptide fragments. Samples were then heat denatured at 105 °C in order to separate the DNA strands to single stranded DNA (ssDNA), and then chilled on ice for 15 min to prevent annealing. The samples were then combined with a labeling solution (Threshold® reagent from Molecular Devices) comprised of streptavidin and two affinity ligands which bind to the DNA: (1) biotinylated ssDNA-binding and (2) an ssDNA binding monoclonal antibody conjugated to urease. Samples were incubated for 1 h at 37 °C and then filtered through a nitrocellulose membrane impregnated with biotin. The complex (shown in Fig. 7 above) is captured on the membrane, exposed to a solution of urea in a specially designed reader, and changes in potential measured. The signal is measured proportionally to the content of complex containing the target analyte, DNA. Thus, residual DNA is measured quantitatively, and sample results interpolated from a standard curve analyzed in the same run.

DNA was tested in the IBS and samples by very large dilutions, in order to get within the working range of the assay (pg). The chemistry of the DNA-binding reaction involves two ligands (ssDNA binding protein and antibody conjugate), both of which must bind to the same strand of DNA in order for capture and detection [3]. Because of this, the test is not suitable when there are excessive quantities of DNA, since the law of mass action results in single-labeled species that either cannot be captured or cannot be detected. This phenomenon manifests itself by giving no signal when (1) there is a very large quantity of DNA in the sample or (2) the amount is below range. Therefore, a negative test could mean either. For this reason, spiking studies are done to prove whether a negative test is below range or above range (if excessive DNA is present, the spike will be zero). This is illustrated below in Fig. 8, where, for the IBS, lower dilutions give no signal and higher dilutions show a dose-response range

Table 2
Clearance of host cell impurities by different chromatography resins

	Protein (mg/ml)	HCP (mg/ml)	HCP/Protein	Fold change	HCP log clearance	DNA (mg/ml)	DNA/Protein	Fold change	DNA log clearance
SP FF									
Pool 1	0.027	0.44	16.3	0.2	−0.8	1.61E−06	0.00006	1230	3.1
Pool 2	0.035	0.37	10.6	0.2	−0.6	2.78E−06	0.00008	922	3.0
CM FF									
Pool 1	0.04	0.16	4.0	0.6	−0.2	6.13E−06	0.00015	479	2.7
Pool 2	0.029	0.01	0.3	7.3	0.9	5.22E−06	0.00018	407	2.6
DEAE FF									
Pool 1	0.478	1.40	2.9	0.9	−0.1	0.000375	0.00078	93	2.0
Pool 2	0.469	1.00	2.1	1.2	0.1	5.32E−05	0.00011	646	2.8
Q FF									
Pool 1	0.762	1.10	1.4	1.8	0.2	0.000484	0.00064	115	2.1
Pool 2	0.782	0.50	0.6	4.0	0.6	5.83E−05	0.00007	984	3.0

*Clearance based on starting levels of contaminants in IBS of 2.53 mg HCP per mg protein and 0.07 mg DNA per mg of protein.

until the sample is diluted below range. Samples were prepared and tested until specific criteria were achieved, indicative of a valid result: (1) spike recovery of a known concentration of DNA was recovered at least 50–150% and (2) the result was in the linear portion of the standard curve.

This process was repeated for both pools from each resin study. Individual fractions were not tested because of the low throughput and substantial cost of the DNA test. Each pool was averaged from results in range and is reported in Table 2 and shown in Fig. 9. The ratio of DNA to product for the cation exchangers is similar in the two pools, suggesting that the large peak in the A280 profiles is not comprised of DNA or DNA/product complex and is either host cell protein, non-DNA nucleic acid (e.g. RNA), or product aggregate. The low residual DNA eluting from the SP FF and CM FF columns is consistent with its strong negative charge which prevents its binding to cation-exchange resins which are negatively charged under the chosen operating conditions. The negatively charged nucleic acid contaminants are most likely removed in the flow-through.

The anion exchange columns were not as effective in clearing the DNA from the rest of the species [15]. Due to the positively charged surface, the negatively charged DNA bind tightly to the anion exchangers. This results in a 8–10X greater DNA concentration in the first pool from the DEAE and Q columns, suggesting it elutes differently than the product and this accounts for the better log clearance in Pool 2. The IBS was found to contain 1.1 mg/ml of DNA. Since the product concentration of this sample was 15 mg/ml, the ratio of DNA/product was 0.07/1.

Further, a comparison of the analysis of HCP and DNA in Figs. 6 and 9 and the respective chromatograms in Figs. 2 and 4 leads to some interesting observations. These observations are illustrated in the schematic diagram shown in Fig. 10 to show the elution of the various species for the four resins under consideration. The trace in the background is the RP-HPLC profile for the respective resin, with the bars representing the position of elution for the HCP and the DNA. As mentioned above, it appears that almost all the resins exhibit poor selectivity between the product

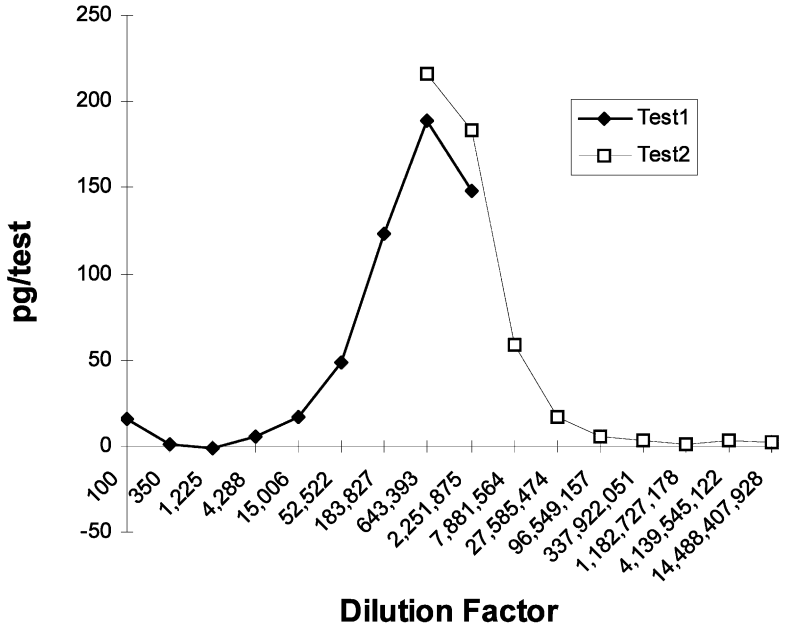

Fig. 8. DNA in inclusion body sample, dilution series.

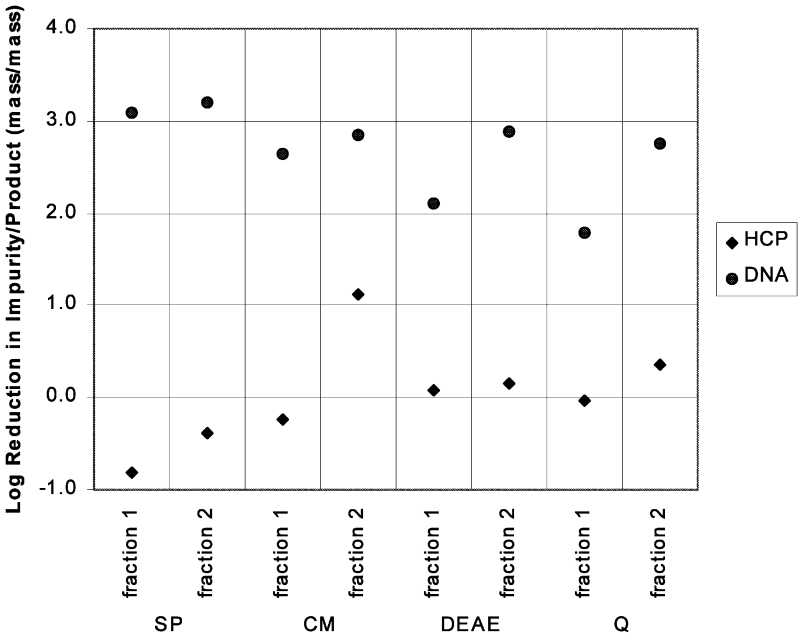

Fig. 9. Clearance of DNA and host cell protein by chromatography resin.

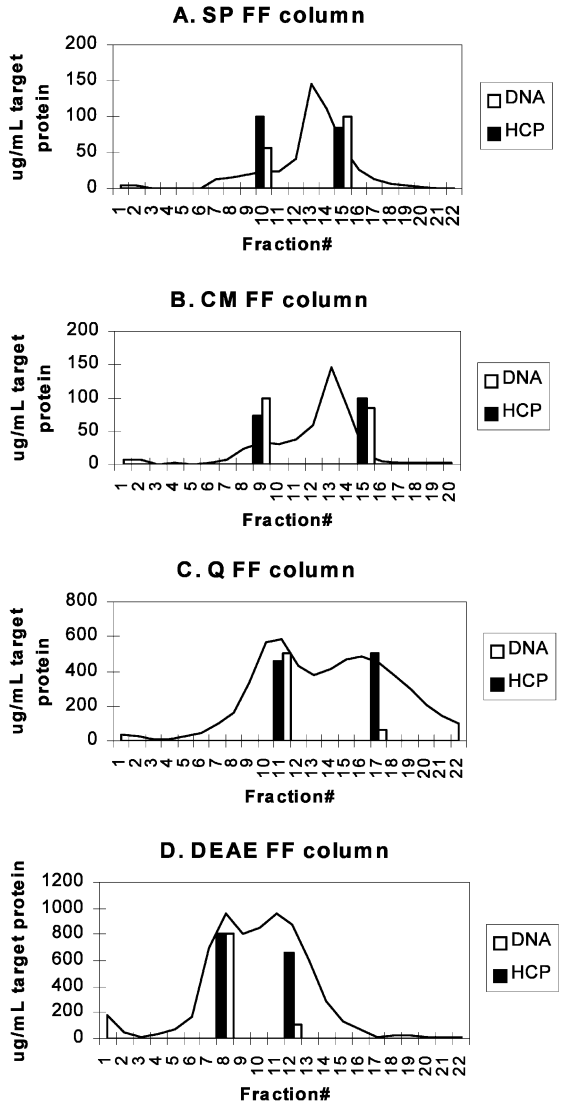

Fig. 10. Schematic illustration of chromatographic elution of different species.

and the HCP. Similar observations have been reported in the literature [4]. However, under the chosen conditions of host cell impurity concentrations in the load, the anion exchanger columns show a much higher retention of the various species as compared with the cation-exchanger columns for reasons mentioned above. The cation-exchangers (Fig. 10A and B) show non-product UV280 nm absorbing large peaks in Fig. 2. These peaks are resolved from the product to some degree. The HCP and DNA elute over a wider range than the product, suggesting the potential for their removal by fractionation. The anion exchangers (Fig. 10C and D) do not show this separation of HCP from the target protein, which elutes as two broad peaks. DNA, however, is eluted as the first peak, suggesting that fractionation in anion exchangers may be useful to remove DNA. Other studies, where the load of HCP is much less, have shown better selectivity and purification options (not shown).

4. Conclusions

The primary goal of this study was to understand the impact of the presence of large quantities of impurities on performance of the different analytical methods. This was of interest since similar samples may be encountered when working with non-inclusion body systems, such as periplasmic expressions, or in cases where the upstream unit operations under-perform in IB cleaning. Three analytical assays, namely, RP-HPLC, ELISA, and Threshold Total DNA Assay, are commonly utilized to characterize samples from crude process streams. These assays are commonly used for quantification of a protein product, HCP and residual DNA, respectively. The IBS used for this study contained about 15 mg/ml product, 38 mg/ml HCP and 1.1 mg/ml DNA. Thus, the relative amounts of HCP and DNA in the IBS was excessive, and about 10^3 times greater than typical because the cells and IB were not processed with the normal number of washing steps during isolation.

The challenge of such crude samples was surmounted by (1) pre-treatment to dissociate aggregates and/or complexes by denaturation for HPLC; (2) digestion of interfering protein by proteolysis for DNA analysis; and [3] significant dilution for HCP and DNA. In particular, the reagents used for residual DNA are prone to a false positive when excess DNA is present, and this was corrected by application of spike recovery to establish a valid test. Not discussed are other less sensitive tests for DNA. Threshold® DNA analy-

sis was selected because its great sensitivity is typically valuable to establish clearance factors when the eluted fraction has very low DNA. It follows from Figs. 3, 5 and 8 that all three methods under consideration were surprisingly robust in analysis of these crude samples.

The large amounts of impurities that were present in the samples, however, had an expected deleterious impact on the ion-exchange chromatography itself, particularly for cation exchangers. This is shown by poor recovery of product, ineffective removal of the HCP, and modest removal of residual DNA, as summarized in Table 1.

Table 2 presents the clearance of host cell impurities in mg impurity/mg product, respectively. The data are further presented graphically in Fig. 9. The finding that IBS contained a ratio of HCP/product of 2.53 suggested that at least 5–6 logs of clearance would be required to reduce levels to an amount consistent with processing of biotechnology protein products. Since single chromatography step could only reduce HCP by $<$ 1 log, at least 5–6 steps would be required to achieve the target levels. This determination points out the limitations of chromatography and the necessity of using other means, such as filtration and washing of the cells and IB during upstream processing to remove HCP. The finding that IBS contained a ratio of DNA/product of 0.07 suggests that at least 6 logs of clearance would be required to reduce levels to an amount consistent with the history of biotechnology protein products. Since a single chromatography step could reduce the amount by 2–3 logs, it is reasonable to assume that 2 steps would be adequate for DNA removal. As with HCP, much of DNA can be removed by upstream processing steps mentioned above, and it would be unusual to begin with a level this high.

Finally, the findings in this study show the limitations of a single chromatography step for removal of the HCP when they are present at such high concentrations, and point out the need for good upstream processing to reduce the levels of contaminants prior to chromatography. The testing methods described are necessary to characterize these processes.

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