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Removal of DNA contaminants from therapeutic protein preparations

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ABSTRACT

Mammalian cell culture-derived biotechnical products for therapeutic use have risks associated with cell substrate DNA. Regulatory authorities in the USA and Europe have set stringent limits for this contaminant, requiring orders of magnitude reduction from fermenter harvests to purified products. This paper addresses the relevant unit processes (*e.g.*, cell separation, affinity chromatography and ion-exchange chromatography) utilized for purification of mammalian cell culture-derived products as they pertain to removal of DNA contaminants.

INTRODUCTION

A wealth of knowledge is available regarding the production of therapeutic proteins from fermentation culture fluid. The starting solution is usually dilute in product and an efficient purification procedure is necessary. The purification strategy is designed to meet US Food and Drug Administration (FDA) guidelines, which specify the allowable limits for extraneous proteins such as DNA contaminants. A combination of several unit operations could produce a product with a desirable DNA content. This paper addresses the removal efficiency of DNA in each downstream processing step.

EXPERIMENTAL

^{32}P R3 DNA was provided by Dr. S. Chan (Molecular Biology, Pharmaceutical Division, Miles). After nick translation, the material was passed through a Sephadex G-50 column to remove free ^{32}P dNTPs. The specific activity was $1.25 \cdot 10^8$ cpm/μg. Experimental samples were precipitated with trichloroacetic acid prior to counting in a scintillation counter.

The dot blot hybridization procedure developed by Dr. S. Cho (Molecular Biology, Pharmaceutical Division, Miles) was used. In brief, DNA samples were exposed to a protease to digest proteins including histones. Protein was removed by phenol extraction and any residual phenol was removed by chloroform extraction. NaOH was then used to denature the double-stranded DNA into single strands. After neutralization with acid and buffer, the samples were serially diluted and dot blotted on to nitrocellulose paper. The nitrocellulose was then incubated with a hybridization mixture containing denatured host cell ^{32}P DNA probe for hybridization of the probe to blotted sample DNA. Hybridized DNA was exposed to X-ray film and the amount of DNA was determined by comparison with the intensity of standard DNA hybridization dots.

An antibody column was prepared by the procedure Roy *et al.* [1]. Chromatography was conducted on a 3×1.5 cm I.D. column at a linear velocity of 80 cm/h.

Bioprocessing aid (Biocryl BPA-1000) was

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purchased from Supelco (Bellefonte, PA, USA). The filter aid was used directly from the bottle as supplied by the manufacturer. It was sampled as a homogeneous 9.8% solution after vortex mixing. Each experiment was conducted with a volume equivalent to 100 ml of fermentation fluid.

All ion-exchange and size-exclusion chromatographic resins were products of Pharmacia LKB Biotechnology (Piscataway, NJ, USA). Fractions were collected on a Superrac system. Absorbances at 280 nm were monitored with a Uvicord UV detector and recorder.

Low-pressure chromatographic columns were products of Bio-Rad (Hercules, CA, USA). Ion exchange was conducted in a 4×1.5 cm I.D. column at a linear velocity of 100 cm/h. Protein was eluted with an NaCl gradient of 0–0.6 *M* in 0.02 *M* Tris–0.02 *M* KH_2PO_4 buffer (pH 8). Size exclusion was conducted on a 52×1.0 cm I.D. column at a linear velocity of 15 cm/h.

For the ionic strength-dependent binding analysis, DEAE-Sepharose was equilibrated in an appropriate buffer (0.01 *M* Tris–0.001 *M* EDTA–0.1–0.5 *M* NaCl) overnight. Aliquots of the DNA spike (50 μl) were added to a series of screw-capped microcentrifuge tubes containing 300 μl of resins [1:1 (w/w) with buffer] with increasing increments of NaCl concentration. A solution of 50 μl of DNA spike and 300 μl of buffer served as the control. After 1 h of equilibration with occasional and gentle vortex mixing to assure complete adsorption, the gel slurry was centrifuged at 8000 *g* for 15 min, and the supernatant was removed to check for radioactivity. The percentage of unbound radioactivity was calculated by the ratio of activity in the supernatant and the activity in the control.

Solution conductivity was monitored with a CDM83 conductivity meter (Radiometer America, Westlake, OH, USA).

Total protein was determined by absorbency measurement at 280 mm.

Urease and blue dextran was purchased from Sigma (St. Louis, MO, USA). Albumin stabilized by N-acetyl-DL-tryptophan is a product of Miles (Clayton, NC, USA).

RESULTS AND DISCUSSION

Cell separation

Prechromatographic polishing of tissue culture fluid with a bioprocessing filter aid has been demonstrated to remove DNA [2]. Such a bioprocessing aid is a water-insoluble suspension of an acrylic polymer containing a quaternary amino functionality. In the experiment shown in Fig. 1, various concentrations of BPA-1000 were added to tissue culture fluid containing an equal amount of spiked ^{32}P DNA. After mixing for 15 min and centrifugation at 8000 *g* for 15 min, the supernatant was assayed for product yield and ^{32}P DNA removal. About 80% of a recombinant product was obtained and 20% of ^{32}P DNA was cleared in the supernatant at BPA concentrations of less than 50 ppm. At concentrations greater than 200 ppm, the ^{32}P DNA clearance was greater than 80% but there was a significant loss of product. Hence the recovery is dependent on the ratio of filter aid to product.

Immunoaffinity chromatography

Immunoaffinity is an effective technique for purifying a specific protein from a complex of

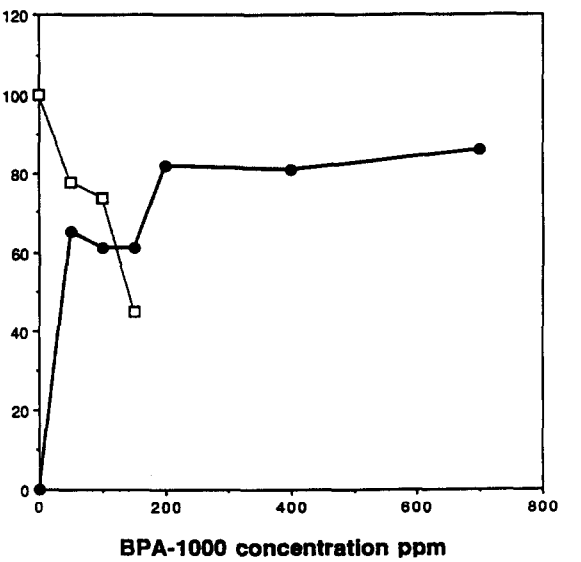

Fig. 1. Effect of increasing concentrations of filter aids on cell separation of tissue culture fluid. □ = Product yield (%); ● = ^{32}P-labeled DNA removed (%). Concentration in ppm (w/w).

many components. Other than adsorption to non-specific binding sites on the support resin, DNA should stay in the flow-through and the wash. In order to assess clearance across the step, total radioactivity before the antibody column step was designed to contain more than 1×10^7 cpm after a ^{32}P DNA concentrate was added to the protein solution. As shown by the three experiments in Fig. 2, recoveries of the spiked activities in the eluate fraction were insignificant relative to the unbound fraction. It could also be calculated that more than 99.99% of the radioactivity was removed across the antibody column step (range 99.99445–99.9987%).

Size-exclusion chromatography

Protein fractionation by size-exclusion chromatography is a standard technique scalable to a process level. Proteins of considerably different sizes, *e.g.*, albumin with a molecular mass of 65 000 and urease with a molecular mass of >240 000 could be resolved into distinct peaks (Fig. 3). Because of its extended configuration and its susceptibility to chain scission, DNA could contain molecules of widely different molecular masses. Accordingly, when a DNA concentrate was applied to a Sephacryl S-5000 HR (useful molecular mass fractionation range $4 \cdot 10^4$–$2 \cdot 10^7$) column previously calibrated with blue dextran, albumin, urease and N-acetyl-DL-tryptophan, it was resolved into two main peak areas, one containing the high- and the other the low-molecular-mass fractions (Fig. 3). The high-molecular-mass front was significantly displaced to the right, suggesting that early-eluting components were present in the DNA, which was not observed as they eluted near the exclusion limit of the column. This heterogeneous nature of the DNA molecule agrees with the observation that size-exclusion chromatography could not completely segregate DNA contaminant from the recombinant product (unpublished data). In other words, protein and DNA of equivalent hydrodynamic size could elute simultaneously. The similarity in size does not indicate identical molecular mass, as protein has a compact dense structure as opposed to the elongated or a more open structure of DNA.

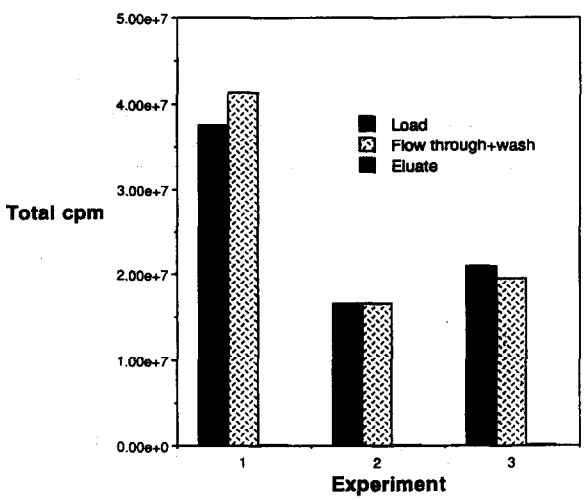

Fig. 2. Clearance of ^{32}P-labeled DNA across an antibody column. Product and DNA spike were loaded, washed and eluted from an antibody column (3 × 1.5 cm I.D.). Recoveries of spike DNA were expressed as total cpm in the load, flow-through + wash and the eluate, as shown.

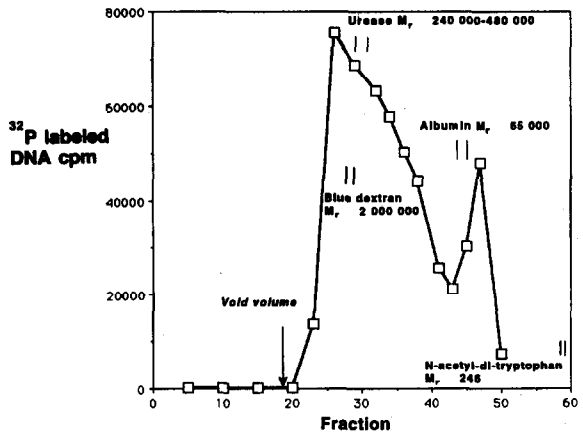

Fig. 3. Size-exclusion chromatography of DNA in a Sephacryl S-500 HR column (52 × 1 cm I.D.) calibrated with two molecular markers, urease and albumin. Equilibration buffer was 0.01 *M* Tris–0.1 *M* NaCl–0.001 *M* EDTA (pH 7.5). Fraction size = 0.74 g.

Ion exchange

The monomeric units of DNA are nucleotides that consist of a phosphate, a sugar and a purine and pyrimidine base. All of the phosphate res-

idues are negatively charged. Therefore, DNA is highly negatively charged and is expected to have an extremely strong affinity for positive charged surface such as anion-exchange resins [3]. In a model mixture studied, albumin and spiked DNA could be eluted separately under a sodium chloride gradient. As shown in Fig. 4, a significant amount of radioactivity was detected after the albumin absorbance at 280 nm had returned to the baseline. Approximately 90% of the spiked activity was obtained when the column was purged with 2 *M* NaCl. The ^{32}P activity seen at lower NaCl concentrations could be related to DNA of lower molecular mass, as demonstrated in the following experiment. In this study, the high- and the low-molecular-mass DNA peak fractions obtained by size-exclusion chromatography were contacted with DEAE-Sepharose at different NaCl concentrations. Based on the supernatant radioactivity, the high-molecular-mass DNA bound more tightly to the resin at NaCl concentrations below 0.5 *M* (Fig. 5). This is consistent with the surface charge of the molecules, *i.e.*, larger DNA molecules are expected to contain more negatively charged phosphate residues. Confirmation of this observation was reported by Hanna *et al.*, [4] who showed early elution of small DNA fragments in an anion-exchange column.

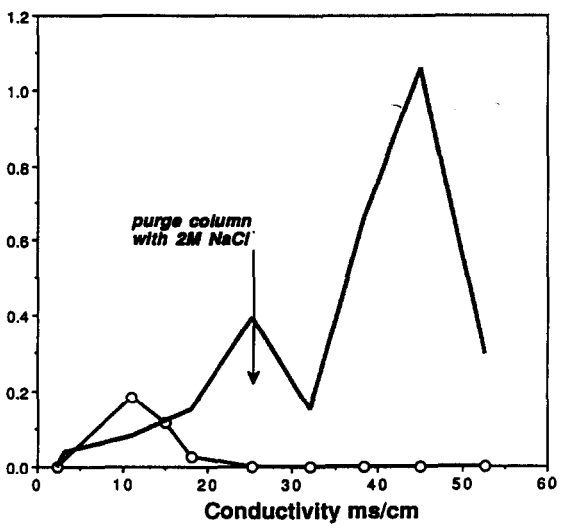

Fig. 4. Clearance of ^{32}P-labeled DNA across an anion-exchange column (4 × 1.5 cm I.D.). Product was eluted with an NaCl gradient from 0 to 0.6 *M* in 0.02 *M* Tris–0.02 *M* KH_2PO_4 buffer prior to the 2 *M* NaCl purge. ○ = Absorbance at 280 nm; other line = ^{32}P-labeled DNA divided by 10 000.

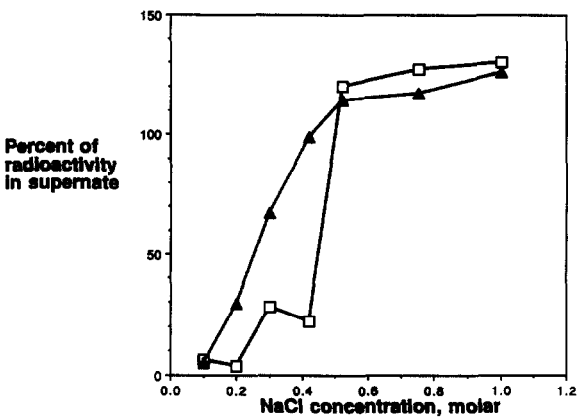

Fig. 5. Binding of ^{32}P-labeled DNA to anion-exchange resins at increments of NaCl concentrations. □ = High-molecular-mass fraction; ▲ = low-molecular-mass fraction.

DNA clearance across the process

The capacity to produce a biologically active recombinant protein with DNA level below a target level is highly dependent on the number of steps and specific step employed. In the example shown in Fig. 6, each circle represented the

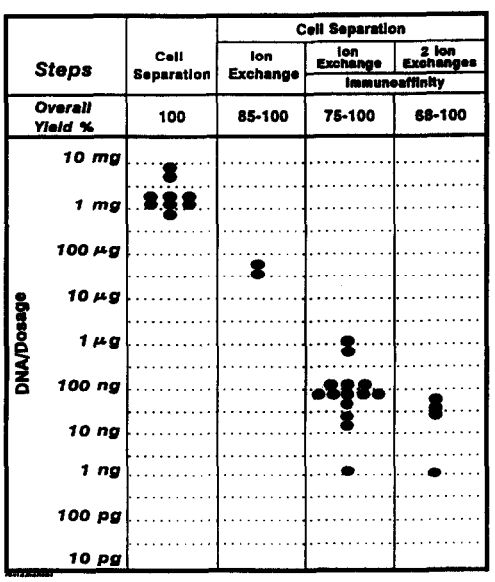

Fig. 6. DNA contaminants per dosage as a function of product yield and number of process steps.

result of a preparative experiment where DNA concentration was measured by dot blot hybridization. Progressively lower DNA levels correlated with more steps in the process. Although additional steps will improve the overall clearance, they will be counterbalanced by a potential loss in product yield. Assuming an average step yield of 90%, it could be calculated that no more than six steps would be permitted in order to obtain an overall yield of *ca.* 50%. If a 50% yield is required of an economically viable process, it is essential that in constructing a purification process there is a proper selection of step or steps to achieve this goal while keeping the DNA at <10–100 pg per injection dose (FDA guidelines [5]).

CONCLUSIONS

Excellent clearance of cell substrate DNA from protein was obtained by immunoaffinity owing to the specificity of the monoclonal antibody for the product in question. Anion-exchange chromatography was also effective in achieving a substantial decrease in cell substrate DNA because of the high negative charges in the phosphate groups. Large-sized DNA obtained by size-exclusion chromatography appears to bind more tightly than smaller sized DNA, presumably owing to the increased surface charge of the molecules. Constructing a process requires a proper combination of steps to achieve a good product yield and proper DNA clearance.

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