Hydroxyapatite chromatography of phage-display virions

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Hydroxyapatite column chromatography can be used to purify filamentous bacteriophage—the phage most commonly used for phage display. Virions that have been partially purified from culture supernatant by two cycles of precipitation in 2% polyethylene glycol are adsorbed onto the matrix at a density of at least \( 7.6 \times 10^{13} \) virions (about 3 mg) per milliliter of packed bed volume in phosphate-buffered saline (PBS; 0.15 M NaCl, 5 mM NaH_2PO_4, pH-adjusted to 7.0 with NaOH). The matrix is washed successively with wash buffer I (150 mM NaCl, 125 mM phosphate, pH 7.0), wash buffer II (2.55 M NaCl, 125 mM phosphate, pH 7.0), and wash buffer I, after which virions are desorbed in desorption buffer (150 mM NaCl, 200 mM phosphate, pH 7.0), and the matrix is stripped with stripping buffer (150 mM NaCl, 1 M phosphate, pH 7.0). About half of the applied virions are recovered in desorption buffer. Western blot analysis shows that they have undetectable levels of host-derived protein contaminants that are present in the input virions and in virions purified by CsCl equilibrium density gradient centrifugation—the method most commonly used to prepare virions in high purity. Hydroxyapatite chromatography is thus an attractive alternative method for purifying filamentous virions, particularly when the scale is too large for ultracentrifugation to be practical.

INTRODUCTION

Filamentous bacteriophage of the Ff class—strains F1, Fd, and M13 and their derivatives—infect Escherichia coli cells displaying the F pilus and are the most common vectors for phage display (1). The virion consists mostly of a tubular array of thousands of major coat-protein subunits surrounding the circular single-stranded viral DNA, which makes up only 12% of the particle mass; five copies each of four minor coat proteins cap the ends. Because the net charge is strongly negative and the DNA is completely inaccessible from the outside, the intact particle can be regarded physicochemically as a long, thin, proteinaceous polyanion.

In many research and applied contexts, it is necessary to prepare these particles to a high degree of purity. Early steps of virion purification exploit two peculiar properties of filamentous phage. First, because they are secreted from the host cells without lysing them, virions can be separated from the vast bulk of host components simply by removing intact cells from culture medium by low-speed centrifugation. Second, because of their filamentous shape, they can be precipitated from culture supernatant by adding polyethylene glycol (PEG) to a concentration of 2%, which is too low to precipitate most remaining contaminants, such as naked DNA (2).

Further purification of PEG-precipitated virions is most commonly achieved by CsCl equilibrium density gradient centrifugation, which concentrates virions into a nonflocculent, weakly light-scattering band with a sharp upper boundary and diffuse lower boundary. Some contaminants are visible as flocculent and nonflocculent light-scattering bands well separated from the main virion band in these circumstances. However, there is no significant difference between the buoyant density of virions and that of a particularly important contaminant class: host-derived proteins (density about 1.3 g/mL in both cases). Separation from these contaminants relies instead on size differences: at the centrifugal forces used, virions form a relatively sharp band because they are large (molecular weight 16–24 MDa, depending on genome size), while most contaminant proteins are distributed diffusely throughout the gradient because they have much lower molecular weights. Polymeric host-derived proteins such as flagella and type I fimbriae that have been shed into the culture medium would be poorly separated from virions on this basis, however. Moreover, size differences can only be fully exploited if the number of virions is kept very small, less than about 10^14 virions (equivalent to approximately 100 mL of culture) per 13-mL centrifuge tube. Because of their limited solubility, larger numbers of virions form broad bands subtending as much as 20% of the tube volume (and thus encompassing at least 20% of the contaminating proteins). Many nonproteinaceous contaminants, such as PEG and the E. coli lipopolysaccharide (LPS), are also too small to be concentrated into a band during centrifugation and will presumably contaminate the virions substantially even if their densities are very different from 1.3.

Zakharova and co-workers (3) have recently shown that size-exclusion chromatography (SEC) in Sephacryl S-500 resin (nominal size-exclusion limit: 20 MDa) can serve as an effective alternative to CsCl equilibrium density gradient centrifugation for purification of PEG-precipitated filamentous virions. Virions emerge in the void volume while protein contaminants emerge as a broad included peak. It is likely (but not proven) that other contaminants, including PEG and LPS, are likewise retarded relative to the void volume. The capacity of this method, approximately 10^13 particles on a 30-mL column, is even lower than the equilibrium centrifugation method.

This article explores hydroxyapatite (HA) chromatography as yet another alternative for the purification of PEG-precipitated filamentous virions. Chromatography on this mineral matrix, which has the formula \([Ca_3(PO_4)_2]_2 \cdot OH_2\], has long been used as a method for fractionating proteins and nucleic acids (4–8). Basic proteins
elute in relatively low NaCl concentrations (approximately 0.15 M) while acidic proteins are eluted by moderate phosphate concentrations but not by even very high concentrations of NaCl (9–11). By adjusting the concentrations of NaCl and phosphate, it is often possible to separate the desired protein from major contaminants. HA chromatography has already been used to purify a few viruses (12–14) and, as reported in this paper, can likewise be used to separate filamentous virions from major contaminants. A key advantage of HA chromatography is its extremely high capacity; even the small-scale demonstration purifications described here (10.8-ml bed volume in a low-pressure column) yielded 4 × 10¹⁴ purified virions, and it would be easy and inexpensive to scale up by orders of magnitude.

Colonies of K91BlueKan cells (15) harboring phage fd-tet (17) were propagated in NZY medium supplemented with tetracycline at 20 μg/mL (15); fd-tet virions were partially purified from culture supernatant by two successive PEG precipitations (15), using PBS as the dissolving buffer. The yield was about 10¹² virions/mL of culture, and the final concentration was 1.64 × 10¹³ virions/mL; it was this virion preparation that was subjected to HA chromatography.

Virions were quantified spectrophotometrically (18). Infectious units of fd-tet were quantified as tetracycline transducing units (TUs) as previously described (15); infectivity is defined as the number of TU per virion and is generally around 5% for undamaged fd-tet phage.

HA Column Chromatography

HA (Bio-Gel® HTP DNA-grade; Bio-Rad Laboratories, Richmond, CA, USA; modal hydrated particle size, 30–40 μm) was suspended and hydrated in PBS at a concentration of 1 g dry weight per 20 mL buffer; fines were removed by multiple cycles of suspension in buffer, settling, and aspiration, as recommended by the manufacturer; the final packed volume was approximately 2.3 mL/g of original dry matrix. The slurry was packed at a flow rate of 95 mL/h to a bed height of 2.2 cm in a 2.5-cm diameter chromatography column fitted with a flow adaptor and equilibrated with at least 10 column volumes of PBS. Virions (50 mL at 1.64 × 10¹³ virions/mL) that had been partially purified by two PEG precipitations were pumped into the column at a flow rate of 19 mL/h, after which the column was developed successively with wash buffer I, wash buffer II, desorption buffer, and stripping buffer, all at a flow rate of 95 mL/h; by the end, the bed height had compacted to 1.75 cm. Fractions of 4.34 mL were collected throughout; the absorbance of each fraction was measured at 269 nm—the absorption maximum for filamentous virions. Fractions were pooled as specified in the Results section and their virion content quantified spectrophotometrically. Three pools containing substantial numbers of phage were concentrated by ultrafiltration through Centriplus® centrifugal devices with a molecular weight cutoff of 30 kDa (Millipore, Billerica, MA, USA) and dialyzed against multiple changes of PBS for analysis by gel electrophoresis and Western blot analysis.

Analysis of Contaminants by Gel Electrophoresis and Western Blot Analysis

Because type 1 fimbriae are a plausible contaminant of virions, samples were heated in acid to depolymerize fimbriae (if present) prior to gel electrophoresis (19). Accordingly, a 19-μL portion of each sample in PBS or water was mixed with 1 μL 0.3 M HCl to lower the pH below 2.0 and heated for 3–5 min at 95°–100°C. It was then mixed with 41 μL of a premix consisting of 1 mL 1.5× sample buffer [62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate (SDS), 125 μg/mL bromphenol blue], 10 μL 1 M Tris-HCl, pH 9.1, and 10 μL 335 mM Tris(2-carboxyethyl)phosphine (neutralized to pH 7.25 with NaOH), and again heated as described above. Portions of the samples (28 μL), along with molecular weight markers, were loaded into the wells of a 4%–20% acrylamide gradient Tris-SDS minigel (ISC BioExpress, Kaysville, UT, USA) and electrophoresed at 100 V using 25 mM Tris, 192 mM glycine, 0.05% SDS as the running buffer. After electrophoresis, the gel was electroblotted onto supported nitrocellulose in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol] essentially as previously described (20). The nitro-

Table 1. Relative Advantages and Disadvantages of Three Purification Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Hydroxyapatite Chromatography</td>
<td>• High purity</td>
<td>• Poor predictability</td>
</tr>
<tr>
<td></td>
<td>• Very high capacity</td>
<td>• Very difficult to accommodate multiple clones</td>
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<tr>
<td></td>
<td>• Very inexpensive</td>
<td>• Moderate purity</td>
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<tr>
<td>Size-Exclusion Chromatography</td>
<td>• High purity</td>
<td>• Somewhat difficult to accommodate multiple clones</td>
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<tr>
<td></td>
<td>• Excellent predictability</td>
<td>• Moderately expensive</td>
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<tr>
<td>CsCl Centrifugation</td>
<td>• Excellent predictability</td>
<td>• Moderate purity</td>
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<tr>
<td></td>
<td>• Easy to accommodate multiple clones</td>
<td>• Low capacity</td>
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<td></td>
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<td>• Expensive ultracentrifuge required</td>
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RESULTS AND DISCUSSION

The starting virions for the HA chromatography experiments reported here were fd-tet particles (molecular weight, 23.5 MDa; virion length, 1.3 μm; genome size, 9183 bp) that had been partially purified from culture supernatant by two successive PEG precipitations (see the Materials and Methods section). Preliminary experiments (data not shown) revealed that they could be adsorbed to the HA matrix in PBS (0.15 M NaCl, 5 mM NaH₂PO₄, pH-adjusted to 7.0 with NaOH), were not desorbed at NaCl concentrations as high as 2.55 M, but could be largely desorbed at phosphate concentrations between 150 and 200 mM. Accordingly, a step gradient chromatography schedule was devised in which virions are adsorbed to the matrix in PBS; the matrix is washed extensively in wash buffers I and II containing NaCl at 0.15 and 2.55 M, respectively, in 125 mM phosphate; virions are desorbed in desorption buffer containing 0.15 M NaCl in 200 mM phosphate; and the matrix is “stripped” in stripping buffer containing 0.15 M NaCl in 1 M phosphate. Figure 1 shows the results of such an experiment, in which 8.2 × 10¹⁴ virions (total mass, 32 mg) from 800 mL of culture were chromatographed on an HA column with an initial bed volume of 10.8 mL.

As can be seen in the graph, all input virions were adsorbed to the HA matrix in PBS, thus concentrating them to 7.6 × 10¹³ virions/mL of bed volume. Half the adsorbed virions could be desorbed in desorption buffer (pool C), a yield of purified virions that is comparable to that obtained by CsCl equilibrium density gradient centrifugation, and that would be entirely acceptable for large-scale production. Another 39% were recovered in other fractions (pools A, B, and D–F), leaving about 12% of the input unaccounted for. No loss of infectivity (Solutions and Standard Methods) was incurred during the purification (data not shown).

Gorbunoff and Timasheff (9–11) emphasize two types of interaction between proteins and the HA matrix. Basic groups interact with bound negative charges primarily through electrostatic interactions that are sensitive to Debye-Hückel shielding in high concentrations of NaCl. Carboxyl groups, in contrast, interact with bound calcium ions primarily through coordination bonds, which are insensitive to NaCl but can be displaced by ions such as phosphate that themselves coordinate with calcium. The chromatographic profile in Figure 1 indicates that filamentous virions interact primarily through coordination of carboxyl groups with matrix-bound calcium. That would explain why NaCl, even at 2.55 M, fails to desorb the virions, while phosphate at modest concentrations does desorb them. The dominance of carboxyl group interactions is not surprising in view of the structure of the major coat protein (21). In the aggregate, the 3900 major coat-protein subunits in an fd-tet virion have 19,500 exposed carboxyl groups, along with 3900 exposed ε-amino groups, and 3900 exposed weakly basic α-amino groups.

The elution profile in Figure 1 shows considerable chromatographic heterogeneity. When virions were eluted with a continuous linear gradient of phosphate concentration from 5 to 1000 mM, they emerged as a highly asymmetric peak with a very long tail, accounting in the aggregate for less than half the input (data not shown). When a mixture of fd-tet virions with 9183 bp and fd virions with 6408 bp was fractionated using a continuous linear phosphate concentration gradient, no discernible resolution between the two types of virion was achieved (data not shown). When virions eluted in the desorption buffer (Figure 1, pool C) were rechromatographed by the same procedure, much the same elution profile resulted (data not shown),
indicating that heterogeneity is not due to heterogeneity in intrinsic properties of virions.

An explanation for these peculiar behaviors might lie in the very large number (19,500) of carboxyl groups on each particle, which plausibly leads to what we will call “once-on/once-off” kinetics. Under optimal binding conditions (i.e., low phosphate concentration, as in PBS), thousands of coordination interactions are possible between each virion and matrix-bound calcium, thus greatly encouraging adsorption. Indeed, in view of the large size of the virion (molecular weight, 23.5 MDa; length, 1.3 μm), it is likely that adsorption kinetics are entirely diffusion-limited. Even a very weak initial attachment of a polyanion through a single coordination bond positions it to make thousands of additional coordination bonds with the matrix—a “zipping” that would be nearly instantaneous and that would bind the virion irreversibly. Desorption of such a multiply-attached polyanion requires that the phosphate concentration be raised sufficiently to displace thousands of coordination bonds per particle. These bonds will have a range of binding strengths because of accidents of the geometry of interaction. Although the overall binding-strength distribution will be indistinguishable from one particle to another, we speculate that the very strongest interactions—those that determine the threshold phosphate concentration for desorption—differ significantly but randomly among the particles. In these circumstances, different phosphate concentrations will be required to desorb different particles, regardless of particle size. If this account is correct, it is not surprising that the distribution of the strongest bonds depends on the exact conditions of adsorption, which would explain why the fraction of particles desorbed at a given phosphate concentration were different in the step and gradient elution experiments. Once a particle has been released into bulk solvent, readsoption to the matrix is highly improbable (so the theory goes). That’s because the phosphate concentration is now relatively high, vastly reducing the density of available calcium ions on the matrix. According to once-on/once-off kinetics, therefore, the range of phosphate concentrations at which adsorption is feasible is far separated from the range at which desorption is feasible, with the consequence that adsorption and desorption are singular events for each virion. It is random particle-to-particle dispersion in the desorption threshold that accounts for their chromatographic heterogeneity. In contrast, for the ordinary protein molecules studied by Gorbunoff and Bernardi, which are about 1000 times smaller than virions, the conditions for adsorption and desorption overlap extensively, each molecule experiencing many adsorption and desorption events during its transit through the matrix bed. The overall behavior of such a molecule will thus be an average over many adsorption and desorption events—an average that will be monodisperse in a population of identical molecules.

DNA molecules, like filamentous phage particles, are large polyanions with thousands of groups able to coordinate with matrix-bound calcium ions; they, too, would therefore be expected to exhibit once-on/once-off kinetics. In accord with this view, the chromatographic behavior of DNA molecules is largely independent of size (4). However, DNA molecules do not exhibit chromatographic heterogeneity comparable to that shown here for filamentous phage particles. That would suggest, according to the theory propounded here, that there is less molecule-to-molecule dispersion in the strength of the strongest bonds attaching DNA molecules to the HA matrix than in the strength of the bonds attaching phage.

The Western blot in Figure 2 shows that several E. coli protein contaminants were detected in the input virions, in pools A and F, and in CsCl-purified virions; but almost none in pool C, the major fraction of purified virions. Not all possible contaminants will be detected in this manner, of course, but those that are presumably represent a broad cross-section of host cell proteins, with no particular bias with regard to HA elution profile. The results in Figure 2 therefore indicate that HA chromatography can be effective at removing many host-derived contaminants, including contaminants that are present in CsCl-purified virions. In some applications, such as biomedical applications, the higher purity achievable in this manner may be crucial. It should be borne in mind, however, that potential nonproteinaceous contaminants are not detected by Western blot analysis, including LPS and the PEG used in the prechromatography stage of purification. LPS would be of particular concern in some applications because it is likely to adsorb to HA through its phosphate groups (its elution profile is unknown) and because of its high toxicity.

The crystalline HA matrix granules chosen for this study are small (modal

![Figure 2. Western blot analysis of Escherichia coli host protein contaminants in various virion preparations.](image-url)
swollen diameter 30–40 μm) and impermeable. They are designed to have a high capacity for large DNA molecules, and for that reason seem likely to also have a high capacity for filamentous virions. To avoid inordinate back pressure caused by the small size of the matrix granules, the matrix was packed into a short column (2.2 cm) with a relatively large diameter (2.5 cm), which allowed a flow rate of 19.4 cm/h to be sustained with an ordinary peristaltic pump. Simply by increasing column dimensions, the scale of purification could easily be increased manyfold beyond the practical limitations of ultracentrifugation. Newer permeable beaded ceramic forms of HA (Bio-Rad Laboratories) would circumvent the problem of high back pressure; they were avoided in this study, however, because their pores (nominal diameter 0.05–0.1 μm) are very small compared with virion length (1.3 μm).

Virions that have been affinity-selected from phage-display libraries differ from the fd-tet virions studied here in that they display a foreign guest peptide on their outer surface. The displayed peptide’s copy number can range from 1 to several thousand per virion, and its length from a few amino acids to hundreds. According to the once-once-off theory proposed above, in some clones the guest peptide may happen to associate more strongly with the HA matrix than any endogenous groupings on the virion surface and thus to dominate the overall chromatographic behavior of the virion. For this reason, the optimal chromatography conditions may vary substantially from one phage clone to another.

The relative advantages and disadvantages of HA chromatography, SEC, and CsCl equilibrium density gradient centrifugation as methods for purifying virions are summarized in Table 1. HA chromatography seems most suitable for purification of one or a very small number of phage clones on a scale that is too great for ultracentrifugation or SEC, or to a particularly high degree of purity. Under such circumstances, the labor required to set up and run a column and analyze the fractions and to find optimal adsorption, washing, and desorption conditions may be well warranted. When multiple clones are to be processed, high-purity is not required, the purification scale is modest, and a suitable ultracentrifuge and rotor are available, CsCl equilibrium density gradient centrifugation will probably continue to be the standard final stage of virion purification.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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