Compaction agent clarification of microbial lysates

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Received 16 July 2002, and in revised form 30 October 2002

Abstract

Recombinant proteins are often purified from microbial lysates containing high concentrations of nucleic acids. Pre-purification steps such as nuclease addition or precipitation with polyethyleneimine or ammonium sulfate are normally required to reduce viscosity and to eliminate competing polyanions before anion exchange chromatography. We report that small polycationic compaction agents such as spermine selectively precipitate nucleic acids during or after Escherichia coli lysis, allowing DNA and RNA to be pelleted with the insoluble cell debris. Analysis by spectrophotometry and protein assay confirmed a significant reduction in the concentration of nucleic acids present, with preservation of protein. Lysate viscosity is greatly reduced, facilitating subsequent processing. We have used 5mM spermine to remove nucleic acids from E. coli lysate in the purification of a hexahistidine-tagged HIV reverse transcriptase.

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Materials and methods

Cytochrome b562-expressing strain

Escherichia coli BL-21 (DE3) harboring a pET3C-based vector directing expression of rat cytochrome b5 [11] was grown overnight in LB broth containing 50 µg/ml ampicillin, induced with IPTG, and harvested by centrifugation at 3500 g for 20 min. Cells from an original culture volume of 3 L were resuspended in 200 ml of 50 mM Tris, pH 8.0, and recentrifuged. The washed cell paste was weighed and diluted with 2.5 ml lysis buffer (0.1% Triton X-100, 50 mM Tris, pH 8.0) per gram of wet cell paste. Cells were passed through a French press twice at an average pressure of 11,000 psi and then centrifuged at 10,000 g for 25 min to remove cell debris. After centrifugation, the lysate was filtered through Whatmann No. 1 filter paper to remove cell debris further and then aliquoted into separate 5 ml vials.
**Analytical methods**

The viscosities of the centrifuged lysates were determined using an Oswalt Type 200 Viscometer standardized against deionized water at 4°C. The clarified samples were also diluted 200× with buffer to bring both A_{260} and A_{280} below 1.0 on a Beckman DU-7500 spectrophotometer. The total protein concentration was determined using a Biorad protein assay, measured at 595 nm on the Beckman Spectrophotometer. Cytochrome b₅ concentration was calculated using the absorbance of the lysate at 412 nm in conjunction with the cytochrome b₅ extinction coefficient of 130 mM⁻¹ cm⁻¹.

**Lysate clearance**

Spermine (250 mM) was added to each vial to a final concentration of 10 mM and after 30 min the lysate was centrifuged at 10,000 g for 25 min and decanted. The viscosity and absorbances of the clarified lysate were again measured, as described above. Finally, 10 ml of 50 mM, pH 8.0, Tris buffer was added to the pellets to redissolve the precipitated nucleic acids.

It is noteworthy that the compaction agent can successfully be added to the cells, prior to mechanical lysis, thus eliminating a centrifugation step. For the purpose of this work, however, addition of compaction agent after lysis allowed for a more detailed characterization of the nucleic acid pellet.

**HIV reverse transcriptase purification**

We used spermine precipitation of nucleic acids during French press lysis as the first step in purification of a hexahistidine-tagged reverse transcriptase expressed in E. coli [5]. Purification was largely according to the protocol of Le Grice et al. [3,4]. IPTG induced cell pellets of two E. coli strains expressing the two subunits of the enzyme were resuspended together in lysis solution (5 mM spermine, 0.1% Triton X-100, and 25 mM NaPO₄, pH 7.0; 150 ml per liter of culture). The mixed cells were then lysed in a French press cell pre-cooled to 4°C at an average pressure of 11,000 psi and collected on ice. The lysate was centrifuged at 4°C at 10,000 g for 30 min and the supernatant was filtered through Whatmann #1 filter paper for further clarification. The lysate was noticeably less viscous than similar lysates prepared without the addition of spermine and was directly applied to a Talon Superflow IMAC column, which was washed and then eluted with an imidazole gradient. The HIV reverse transcriptase eluted near 100 mM imidazole. While the protein appeared pure by SDS-PAGE, additional DEAE Sepharose and SP Sepharose columns were used to completely eliminate any contaminating nuclelease activities.

### Table 1

Characterization of lysates with and without addition of compaction agents

<table>
<thead>
<tr>
<th>Sample (n = 9)</th>
<th>Viscosity (cP)</th>
<th>Absorbance at 260 nm</th>
<th>Absorbance at 280 nm</th>
<th>260/280 Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without compaction agent</td>
<td>5.37 ± 0.52</td>
<td>1.07 ± 0.09</td>
<td>0.64 ± 0.06</td>
<td>1.67 ± 0.01</td>
</tr>
<tr>
<td>10 mM Spermine added before lysis</td>
<td>1.86 ± 0.06</td>
<td>0.81 ± 0.05</td>
<td>0.56 ± 0.03</td>
<td>1.45 ± 0.01</td>
</tr>
<tr>
<td>10 mM Spermine added after lysis</td>
<td>1.69 ± 0.05</td>
<td>0.73 ± 0.05</td>
<td>0.50 ± 0.02</td>
<td>1.46 ± 0.01</td>
</tr>
<tr>
<td>Redissolved pellet from above sample</td>
<td>0.68 ± 0.04</td>
<td>0.36 ± 0.03</td>
<td>1.89 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>10 mM Spermidine added before lysis</td>
<td>5.33 ± 0.17</td>
<td>1.00 ± 0.05</td>
<td>0.66 ± 0.04</td>
<td>1.52 ± 0.03</td>
</tr>
<tr>
<td>10 mM Spermidine added after lysis</td>
<td>5.15 ± 0.15</td>
<td>0.99 ± 0.02</td>
<td>0.65 ± 0.03</td>
<td>1.52 ± 0.03</td>
</tr>
</tbody>
</table>

The viscosity of the cytochrome b₅-containing sample lysed in the presence of 10 mM spermine was decreased by 65% relative to the control, suggesting that a significant fraction of the nucleic acids were precipitated (Table 1). The data in Table 1 suggest that spermine effectively and selectively precipitates only nucleic acids. The 260 nm absorbance of the sample containing spermine was 25% lower than that of the control sample. Comparing the 260/280 ratio of the samples before spermine was added (1.67) to the 260/280 ratio of the samples after spermine was added (1.46) shows that the amount of nucleic acid precipitated was proportionally higher than the amount of protein precipitated. The A_{260} reduction predicted from a reduction in A_{260} of 0.34 due solely to removal of nucleic acids (using E_{260} = 2.0 × E_{280} for nucleic acids) closely matches that actually observed (0.14 observed vs. 0.17 predicted), further suggesting that the dominant effect of spermine addition is reduction of nucleic acid content, while proteins are left in solution. Not all 260 nm-absorbing species are expected to be removed by the addition of compaction agents due to the presence of non-precipitable nucleotides, cofactors, etc. The selective removal of nucleic acids is illustrated further by Table 2, which presents the total protein concentrations measured in each of the three sets of samples. Before spermine was added to the lysate, the total protein concentration measured 35.2 mg/ml. After adding spermine, the total protein concentration averaged 33.6 mg/ml while the pellet yielded a total of 1.9 mg/ml of pro-
tein. While the background absorbance at 412 nm is low, measurements of cytochrome b5 concentration in crude lysate samples are potentially complicated by changes in molar extinction coefficient with conditions, and the overall mass balance for this single protein did not match as well as that for total protein. Measuring the amount of cytochrome b5 in the lysate at 412 nm generally confirmed these results, however, with 1.35 mg/ml of cytochrome b5 before adding spermine, 1.24 mg/ml after addition of spermine and 0.06 mg/ml in the pellet.

To determine which proteins were most likely to precipitate with the DNA, each sample was analyzed by isoelectric focusing using Pharmacia’s PhastGel system (Fig. 1). In Lanes 1 and 2, samples were diluted 50× and were run using an IEF 3–9 gel with the PhastGel protocol and silver staining. The lysate before adding spermine is shown in Lane 1 and the lysate after adding compaction agent is shown in Lane 2. The similar results from these two lanes support the conclusion from the 280 nm absorbance measurements in Table 2 that little protein is lost upon precipitation. Because the total protein concentration in the pellet was too low to be detected at the loading concentrations used in analyzing the lysate, a second gel was run using various concentrations of the redissolved pellet. As seen in Lanes 3–6, the majority of the small quantity of protein found in the pellet is cationic (top of the gel). This is most likely due to the positively charged proteins binding to the anionic DNA fragments as they are precipitated, suggesting that the spermine does not fully neutralize the nucleic acid charge.

We found relatively little benefit of increasing spermine concentrations above 20 mM. As little as 5 mM spermine can improve lysate properties, but the process is not as effective at these low levels. In contrast to spermine, addition of 10 mM spermidine either before or after lysis did not significantly change the properties of the lysate (Table 1). Subsequent trials, however, showed that at higher concentrations (above 15–20 mM), spermidine can reduce the lysate viscosity.

The HIV RT protocol yielded approximately 12 mg of purified protein from a combined 1.3 L of RT subunit-expressing E. coli cells. The lysate clearance process was also applied successfully to a similar separation of an E. coli-expressed Pseudomonas meta-cleavage enzyme (results not shown).

Discussion

We have found that the addition of inexpensive, synthetic (and therefore virus-free) polycationic compaction agents to cell lysates or to cells before lysis substantially reduces lysate nucleic acid content and viscosity, with no significant loss of protein. The method has been applied to the isolation of several different proteins and has been found to be general and robust. This technique is in many ways an extension of the use of polyethyleneimine (PEI), as described by Burgess et al. [10]. Spermine, however, precipitates low quantities of proteins at low ionic strength (less than 0.1 M NaCl), in contrast to other precipitating agents such as PEI. Finally, in other work we have found that the addition of compaction agents to cells before lysis protects plasmids from shear damage, offering a new approach to plasmid isolation by mechanical lysis (JM, GF, RW, unpublished results).

Acknowledgments

This research was funded in part by grants from the National Science Foundation, the National Space Biomedical Research Institute (NASA Cooperative Agreement NCC-9-58), the Environmental Institute of Houston, and the Robert A. Welch Foundation (Grants 0234101 and 02230101).
We thank Dr. Ronald Giles and Dr. Stuart F.J. Le Grice for providing *E. coli* strains expressing cytochrome *b*₅ and HIV reverse transcriptase, respectively. We also thank Dr. Kishore Mohanty for the use of his Oswald viscometers in these experiments.

References


