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Compaction Agent Protection of Nucleic Acids during Mechanical Lysis

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Mechanical lysis is an efficient and widely used method of liberating the contents of microbial cells, but the sensitivity of large nucleic acids to shear damage has prevented the application of mechanical lysis to DNA purification. It is demonstrated that polycationic compaction agents can protect DNA from shear damage and allow chromosomal and plasmid DNA purification by mechanical lysis. In addition to being substantially protected during mechanical lysis, the compacted DNA can be separated with the insoluble cell debris, washed, and selectively resolubilized, yielding a substantially purified DNA product. An additional benefit of this method is that lysate viscosity is greatly reduced, allowing the use of much smaller processing volumes when compared with traditional lysis methods used in nucleic acid purification.

Introduction

With numerous DNA vaccines and gene therapy products now in clinical trials, there is considerable demand for improved large-scale DNA separation techniques (*1*−*3*). One major area of concern during large-scale DNA production is the initial cell lysis step (*4*−*6*). Current lysis techniques for DNA production require caustic solutions, enzymes, and/or heat to liberate DNA from bacterial cells (*7*−*9*). In addition, each of these lysis methods requires the use of large volumes to keep viscosity within manageable limits.

Intracellular proteins have long been recovered by mechanical lysis methods including homogenization, bead milling, and sonication. These induce lysis by shear, impingement, cavitation, pressure shock, and combinations of these mechanisms (*10*−*12*). Large nucleic acids such as plasmid and chromosomal DNA, however, are mechanically sheared and fragmented by standard mechanical lysis techniques at strain rates of approximately 10^5-10^6 s^{-1} (*13*−*15*).

We have previously shown that condensation/compaction of DNA can enhance its adsorption on chromatographic media at low ionic strength (*16*) and can serve as the basis for selective precipitation and fractionation of DNA and RNA (*17*−*19*). Compaction employs synthetic versions of small, natural polycations such as spermine and spermidine (*20*, *21*) to induce reversible conformational changes and/or precipitation of nucleic acids. This technique has been shown to produce highly purified DNA with high efficiency (*22*). Compaction agents can also be used to eliminate nucleic acids from lysates containing desired nonnucleic acid products (e.g., proteins and polysaccharides), reducing lysate viscosity and eliminating the need for nucleases or additional nucleic acid removal steps (*23*).

It is shown herein that compaction-agent-induced protection by condensation of plasmid and chromosomal DNA enhances plasmid DNA yields after mechanical lysis; the precipitated DNA can readily be selectively redissolved away from insoluble cell debris.

Experimental Methods

E. coli strain JM109 containing plasmid pCMV sport β gal (Gibco BRL) was grown in a 20-L Applikon fermenter, harvested, pelleted by centrifugation, and stored at −80 °C. "Compaction protection buffer" consisting of 0.5% w/v Brij 58 with added spermidine (15−30 mM) in 20 mM Tris HCl at pH 8.0 was added directly to frozen cell mass at 7−10 mL per gram of wet cells (100−150 mL total volume per French cell press cycle, resulting optical density ca. 80−100). Control experiments were also conducted with a buffer containing 0.5% w/v Brij 58 in 20 mM Tris HCl at pH 8.0 (no spermidine). The nonionic detergent Triton X-100 (1% w/v) performed equivalently to 0.5% w/v Brij 58.

The mixture was vortexed to disperse the cells, allowed to stand for 4 min, and mechanically lysed using a French press (PC-160, SLM Aminco) at a pressure of 4,000−12,000 psig (vide infra). The lysed cells were then further processed by one of two alternative methods.

A. Protected Lysis Only. The initial approach was to add 1 volume of 1.2 M NaCl in 20 mM Tris HCl, pH 8.0 to the spermidine-protected lysate in order to resuspend the plasmid into the supernatant while leaving the majority of cell contents insoluble. The mixture was centrifuged (10,000 × *g*, 15 min), and the supernatant was decanted. For electrophoresis, the supernatant was desalted by addition of 0.7 volumes of 2-propanol, 1-h incubation at −20 °C, and centrifugation at 10,000 × *g* for 15 min. The resulting pellet was then resuspended in TAE (40 mM Tris-HCl, pH 8.0, 40 mM acetic acid, and 1 mM EDTA) and analyzed by gel electrophoresis.

B. Protected Lysis with Further Separation. In the second approach, the lysate was centrifuged at 10,000 × *g* for 15 min (with the DNA still compacted and insoluble). The supernatant was discarded, and the compaction-precipitated DNA/cell debris pellet was mixed with stripping solution (50% ethanol, 600 mM NaCl with 10 mM EDTA; 1 mL per 10 mL initial lysate) to remove the spermidine from the pellet while keeping the DNA

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insoluble. This suspension was then vortexed and recentrifuged at 10,000 × *g* for 5 min. The pellet was then washed with 70% ethanol, and the DNA was selectively resuspended in 1 X TAE and analyzed by gel electrophoresis.

Pure Component Experiments. Purified plasmid DNA (pCMV sport β gal) at 50 μg/mL was resuspended in 10 mM Tris at pH 8.0, with and without 10 mM spermidine (10 mL total volume per run). The samples were then passed through a French press (PC-160, SLM Aminco) at an average pressure of 4,000 and 10,000 ± 1,000 psig three times with samples (1.5 mL) collected after each pass for both the spermidine-containing samples and the control.

Assays. Analytical anion-exchange (AEX) chromatography (24) was performed using a Rainin HPLC system. Samples were diluted in 0.5 M NaCl in 20 mM bis-tris propane at pH 8.0 containing 0.5 μg/mL RNase I (Roche). Samples were then injected onto a Polymer Labs column (PL-SAX, 15−20 μm bead diameter, 4000 Å pore size, 50 mm × 4.6 mm, part no. 1951-9999), and peaks were detected at 260 nm. A linear gradient was run at 4 mL/min from 0.6 to 0.8 M NaCl in 20 mM bis-tris propane at pH 8.0 to elute the plasmid DNA (*25*). To measure the amount of damage in DNA samples, the ratio of the second and third peaks in the chromatogram (nicked/linear and supercoiled plasmid DNA) are used to calculate a relative damage ratio (RDR, = third peak area/(sum of areas of second and third peaks)). The RDR is based on the ability of HPLC-based anion-exchange chromatography to resolve plasmid DNA conformers and genomic DNA by differences in charge density (*24*). The more compact supercoiled DNA forms have a charge density higher than that of open circle plasmid DNA and linear DNA. In addition, damaged DNA is still measurable (*26*) via the RDR analysis even when the percentage of supercoiled DNA is not measurable by densitometric analysis of electrophoretic gels. An RDR of 1 refers to an intact plasmid DNA sample, whereas an RDR of 0 refers to a completely degraded plasmid DNA sample. This ratio tracks with the percentage of supercoiled DNA but also is affected by the overall level of intactness of the degraded DNA samples.

Gel electrophoresis employed 0.8% E-gels (Invitrogen) run at 60 V for 30 min. Each well was loaded (approximately 3 ng of DNA per well) with 20 μL of sample. Gels were imaged on a Gel Doc 1000 (Bio-Rad), and bands were integrated to obtain the approximate percentage of supercoiled plasmid DNA.

Results and Discussion

To test compaction's protective effect on plasmid DNA, purified plasmid pCMV sport β gal was passed through a French cell press (with and without 10 mM spermidine) for three cycles, with samples taken after each pass analyzed for relative damage ratio (RDR) by HPLC (Figure 1). After a single pass at either 4,000 or 10,000 psig, the relative damage ratio of uncompacted plasmid DNA was reduced to zero, implying complete degradation of the plasmid DNA. At both 4,000 and 10,000 psig, compacted DNA substantially survived even multiple passes through the French press.

Figure 2 illustrates results from a French press lysis of *E. coli* cells at 11,000 psig backpressure. Lane 1 shows the product of the control lysis (11,000 psig) with no compaction agent present, and Lane 2 shows the nucleic acids after lysis in compaction protection solution (30 mM spermidine). In contrast to the control, a significant fraction of the compacted plasmid remained intact after lysis. The compaction protection solution also condenses and partially protects chromosomal DNA from being fragmented into contaminants of a broad size distribution

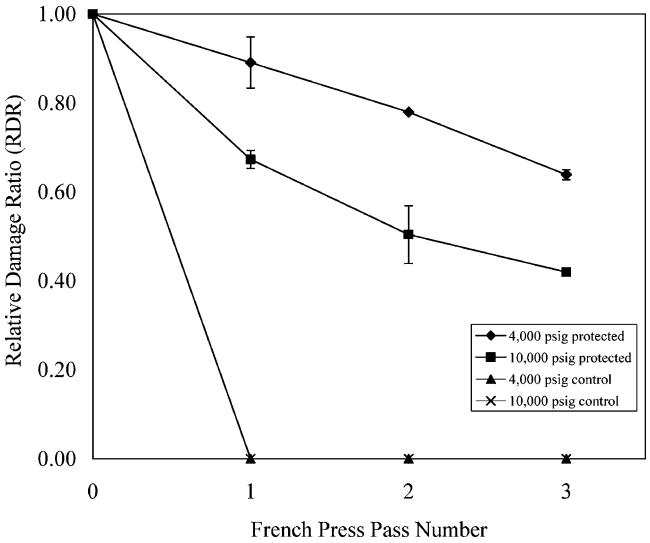

Figure 1. Pure component lysis experiments with 50 μg/mL plasmid DNA processed through a French press (PC-160, SLM Aminco) at 4,000 and 10,000 psig in 10 mM Tris HCl with and without 10 mM spermidine. The *X*-axis shows the number of passes and the *Y*-axis shows the relative damage ratio (RDR, see Methods).

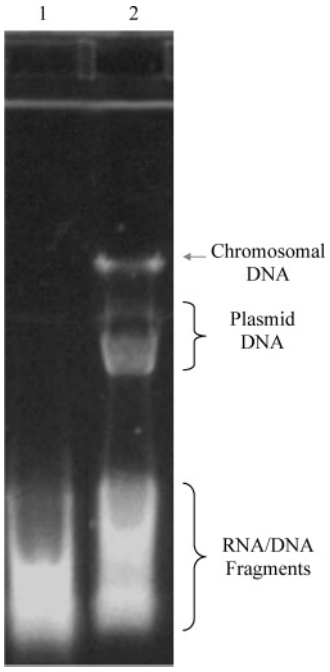

Figure 2. Electrophoresis (0.8% E-gel, Invitrogen) showing compaction protection of nucleic acids during French press lysis at an average pressure of 11,000 psig. Lane 1 is the control unprotected French pressed lysate (in a buffer containing 0.5% Brij 58 in 20 mM Tris HCl at pH 8.0, "Buffer A"), and Lane 2 is the spermidine-protected lysate (lysed in Buffer A + 30 mM spermidine). For analysis of these crude lysates, each sample was precipitated with 0.7 volumes of 2-propanol and resuspended in TAE.

(Figure 2). This is a significant advantage, as these random fragments are difficult to separate completely from plasmid or chromosomal DNA using traditional nucleic acid separation methods. In addition, the compacted DNA solutions all had a viscosity equivalent to water until resuspension (*23*). This reduced viscosity is a very useful side benefit of compaction and greatly enhances the processibility of the plasmid DNA.

To minimize the degradation of plasmid DNA during cell lysis, a reduced shear-force lysis was examined. Lysis at 4,000 psig showed no apparent reduction in the amount of liberated

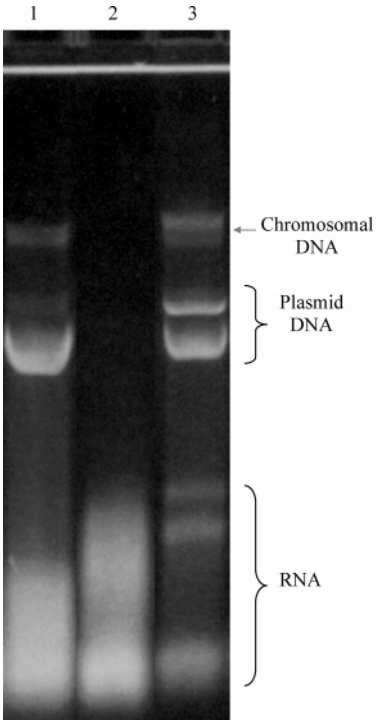

Figure 3. Integrated compaction lysis/purification of plasmid DNA. Lane 1 is the spermidine protected lysate (cells in Buffer A + 15 mM spermidine) after lysis by French press at an average pressure of 4,000 psig; Lane 2 is the RNA-rich supernatant from centrifugation of the lysate in Lane 1 (for analysis the samples in Lanes 1 and 2 were precipitated with 0.7 volumes of 2-propanol and resuspended in TAE); Lane 3 is the plasmid-enriched pellet of the centrifuged Lane 1 lysate after compaction agent removal by washing with 50% ethanol with 600 mM NaCl and 10 mM EDTA, followed by a 70% ethanol wash and resuspension in TAE.

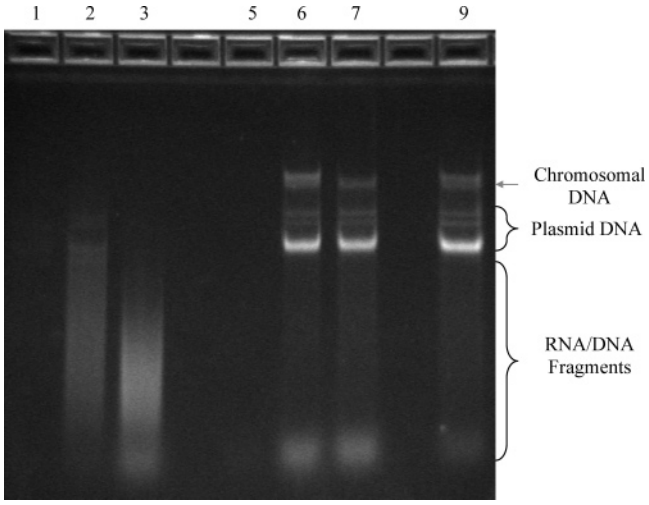

Figure 4. Electrophoretic analysis of reduced shear lysis compaction-protected samples with controls. All samples were precipitated with 0.7 volumes of 2-propanol and resuspended in 1 X TAE before electrophoresis on a 0.8% E-gel (Invitrogen) at 60 V for 30 min. Lane 1 is the prelysis cell suspension control in Buffer A (no spermidine) prior to French press (no free DNA); Lane 2 is the lysate resulting from the cell suspension of Lane 1, after one pass through a French press at ~4,000 psig (no spermidine); Lane 3 is the resulting lysate after two passes through a French press at ~4,000 psig (no spermidine); Lane 4 is blank; Lane 5 is the microbial suspension in Buffer A + 15 mM spermidine prior to French press; Lane 6 is the lysate resulting after 1 pass of the cell suspension from Lane 5 through a French press at ~4,000 psig (with 15 mM spermidine); Lane 7 is the lysate resulting after 2 passes through a French press at ~4,000 psig (with 15 mM spermidine); Lane 8 is blank; and Lane 9 is the sample from Lane 7 after compaction agent removal (by stripping with 50% ethanol, 600 mM NaCl and 10 mM EDTA), a 70% EtOH wash, and resuspension.

DNA and reduced DNA damage. Because compaction protection involves the condensation of nucleic acids, the method can be combined with our previous work on selective compaction precipitation (*17*) into an efficient lysis/partial purification protocol. In Figure 3, Lane 1 is the spermidine-protected lysate (cells in 0.5% Brij 58 in 20 mM Tris HCl at pH 8.0, with 15 mM spermidine) after lysis by French press at an average pressure of 4,000 psig; Lane 2 is the RNA-rich supernatant from centrifugation of the lysate in Lane 1 (for analysis the samples in Lanes 1 and 2 were precipitated with 0.7 volumes of 2-propanol and resuspended in TAE); Lane 3 is the plasmid-enriched centrifugation pellet of the Lane 1 lysate, after compaction agent removal by washing with 50% ethanol with 600 mM NaCl and 10 mM EDTA, followed by a 70% EtOH wash and resuspension in TAE. As seen in Lane 3, this process efficiently produces a product somewhat lower in RNA but higher in genomic DNA than a standard alkaline lysis, though the genomic DNA is of relatively restricted size distribution. An added benefit of including the purification step along with lysis is that the DNA remains precipitated until the purified material is resuspended. Viscosities, therefore, remain low throughout the purification, allowing the lysis to take place in a greatly reduced volume.

Figure 4 compares results obtained without (Lanes 1−3) and with (Lanes 5−7) spermidine compaction. Comparison of results following either one or two passages through the French press at 4,000 psig allowed us to demonstrate that cell lysis is largely complete after a single pass. Lanes 1 and 5 are, respectively, the control and 15 mM spermidine-containing cell suspensions prior to mechanical lysis (no free DNA is observed). Lanes 2 and 3 are the first and second pass lysates without spermidine added prior to lysis. The plasmid DNA is almost completely destroyed after one pass; the reduction in molecular weight seen between Lanes 2 and 3 suggests that degradation of the DNA continues upon the second pass. Lanes 6 and 7 show the first and second lysis pass products of the Lane 5 cell suspension containing 15 mM spermidine, followed by resuspension of the compacted DNA by addition of NaCl. These two lanes correspond to the "Protected Lysis Only" method described in Experimental Methods (vide supra). There is no discernible difference between the products of the first and second protected passes, suggesting both effective protection and also that lysis is essentially complete after one pass. Lane 9 is the product of the "Protected Lysis with Further Separation" method (vide supra) and is the product of the second spermidine-protected pass (Lane 7, without the addition of NaCl) after centrifugation (to obtain cell debris/DNA pellet), washing with stripping solution, washing with 70% ethanol, and resuspension in TAE. This resulting product is substantially pure DNA, because major contaminants (i.e., protein and RNA) remain unprecipitated and are largely discarded in the supernatant, with the residual liquid removed in the washing steps.

These samples were also analyzed by anion-exchange HPLC and densitometric analysis of the gel in Figure 4 to obtain the percentage of supercoiled DNA. The results are shown in Table 1. These data demonstrate that the plasmid DNA is not degraded from pass to pass during compaction-protected mechanical lysis, as the relative amount of supercoiled plasmid DNA is unchanged between protected passes. In the absence of spermidine, the supercoiled plasmid DNA is completely degraded after two passes at 4,000 psig, demonstrating the effectiveness of the compaction protection.

Table 1. Anion-Exchange HPLC Assays of the Samples in Figure 4, Showing Total DNA and Relative Damage Ratio (RDR) for Each Sample and Percentage of Intact Supercoiled DNA Determined by Gel Electrophoresis (% SC pDNA)[a]

sample	total DNA (mg/mL)	RDR (AEX)	SC pDNA (%)
uncompacted, after first pass	0.55	0.14	N/A[b]
uncompacted, after second pass	0.51	0.02	N/A
compacted, after first pass	0.48	0.57	78
compacted, after second pass	0.51	0.53	76
compacted, after stripping and wash	0.47	0.58	77

[a] Uncompacted samples were lysed without the addition of spermidine, and compacted samples were lysed with 15 mM spermidine. Samples were lysed with two separate passes, with the compacted sample subjected to a final stripping (to remove the compaction agent) and wash (last row). Pre-lysis samples (supernatant of the cell suspension before lysis) were below the limit of quantitation of the assay (<0.01 g/L) demonstrating that no DNA escaped from cells by pre-lysis leakage. [b] N/A = not applicable.

In addition to the French press, we have also successfully tested the application of compaction protection to other mechanical cell lysis techniques. Glass beads have been used in a vortexer mini-prep application to release plasmid and genomic DNA from both fresh and frozen *E. coli* paste. In addition, sonication of bacterial cells in the compaction protection solution has been found to effectively release intact DNA (results not shown).

Conclusions

Protection by compaction-agent-induced condensation can allow mechanical liberation of nucleic acids from cells, utilizing existing equipment for protein purification. This lysis method not only simplifies the medium- to large-scale purification of large nucleic acids but also avoids the high viscosities encountered when nucleic acids are released into a small volume of liquid. Potential further applications include protection of nucleic acids during sterile filling, for storage, and in protection of nucleic acids during heat- or chemical-based lysis. Compaction protection may also find applications in the purification of yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), and other shear-sensitive nucleic acid molecules.

Similar techniques might be applied to polysaccharides, shear-sensitive proteins, and other shear-sensitive biomolecules using affinity precipitants that are specific for these compounds.

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