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PREPARATIVE ISOLATION OF PLASMID DNA WITH SEDIMENTATION FIELD FLOW FRACTIONATION

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SUMMARY

Sedimentation field flow fractionation (SFFF) can be used to isolate plasmids preparatively from crude cellular lysates. Total purification time is about 3/4 day, including lysate preparation. The purity and yield of plasmids isolated by SFFF appear to be at least equivalent to those prepared by traditional methods. Molecular-weight data also are supplied rapidly by SFFF without the need for standards.

INTRODUCTION

Sedimentation field flow fractionation (SFFF) has proved to be a powerful technique for separating and determining the mass and size distribution of a variety of colloids and particulates with diameters from 0.005 to 2 μm [1—4]. A wide range of soluble macromolecules and various colloids of about 10^6—10^{13} molecular weight (MW) also can be separated by SFFF. Whole bacteria, various intact viruses, viral DNAs, mammalian RNA, liposomes, and a wide variety of other biomolecules of interest can also be fractionated and characterized [5—9].

The ability of SFFF to fractionate DNAs without damage due to shear and without change in biological properties has been demonstrated [9]. In this work, it was shown that plasmids and other DNAs could be isolated in essentially quantitative yields. A unique feature of SFFF is that the molecular weights of components can be determined without reference to standards, since this mass-separating method is based on physical first principles [1].

The present study is the first describing the applicability of SFFF in a

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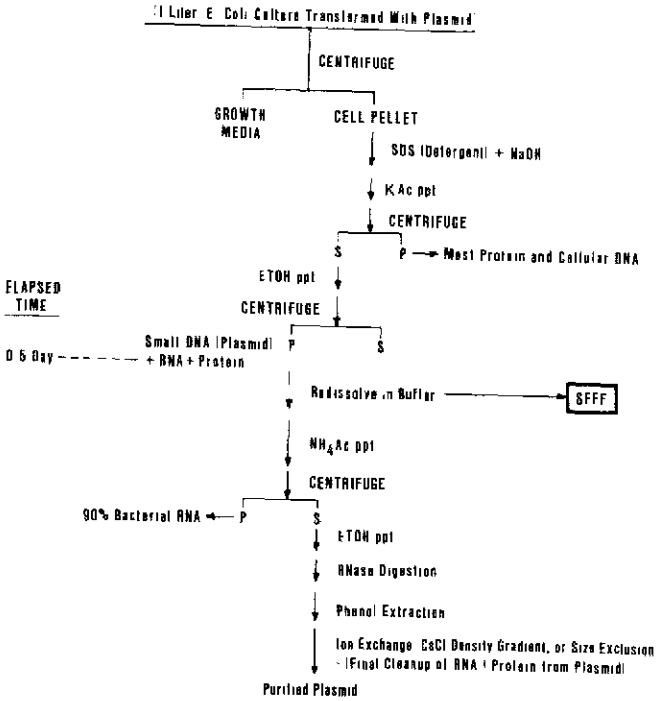

Fig. 1 Typical procedure for preparative isolation of bacterial plasmids. SDS = sodium dodecyl sulfate; KAc = potassium acetate; ppt = precipitation; S = supernatant; P = precipitate.

practical recombinant DNA procedure, namely, the preparative isolation of plasmids from crude cellular lysates, as well as the analytical measurement of molecular weights of plasmids in such systems. Two plasmids were chosen: a standard laboratory cloning vehicle (pBR322) grown in a standard laboratory strain *Escherichia coli*, and pDM24D (pDP5009), a plasmid found in a natural isolate of an unclassified anaerobic thermophile. The first case compares the preparative ability of SFFF for plasmids that have been successfully purified by traditional techniques. The second case was attempted to test the ability of SFFF to purify plasmids that are especially difficult to isolate.

Conventional preparative purification of bacterial plasmids from the cultured cells in which they are grown involves differential precipitations and centrifugations, followed by higher resolution methods such as ion-exchange chromatography, size-exclusion chromatography or density-gradient centrifugation. A scheme typical of those in current use is illustrated in Fig. 1 [10, 11].

EXPERIMENTAL

SFFF equipment and techniques

Equipment and techniques used for this work have been described previously [3, 4, 7, 9]. Separations were carried out with SFFF instrumentation consisting of a modified Model L5-50B ultracentrifuge (Beckman Instruments, Fullerton, CA, U.S.A.) equipped with a high-speed, water-cooled rotating

face seal, a Model 850 microprocessor-controlled solvent metering system (Du Pont Biomedical Products, Wilmington, DE, U.S.A.), a Varichrom UV spectrophotometric detector (Varian Instruments, Walnut Creek, CA, U.S.A.), a Valco Instruments remote air-actuated microsampling valve (Valco Instruments, Houston, TX, U.S.A.), and a MINC-023 computer (Digital Equipment Corporation, Maynard, MA, U.S.A.). The SFFF apparatus was equipped with the floating plastic channel design as previously described [4]. The density of DNA was taken as 1.71 g/ml.

Mobile phase reagents

Ultrapure Tris buffer and sodium chloride were obtained from ICN Pharmaceuticals (Cleveland, OH, U.S.A.) and Fisher Scientific (Fairlawn, NJ, U.S.A.), respectively.

Preparation of pBR322 bacterial lysate

Crude plasmid preparations were made by a procedure initially described by Birnboim and Doly [10] and later modified by Ish-Horowicz and Burke [11]. Unamplified *E. coli* HB101 cells containing the plasmid pBR322 were harvested from 1 l of suspension by centrifugation, and resuspended in 20 ml of a 25 m*M* Tris · HCl pH 8.0–50 m*M* Na_2EDTA—1% glucose mixture. Resuspended cells then were treated with 40 ml of a 0.2 *M* sodium hydroxide—1% sodium dodecylsulphate (Bio-Rad Labs., Richmond, CA, U.S.A.) mixture, and gently mixed and incubated at 5°C for 10 min. The suspension then was gently mixed with 30 ml of pre-chilled 3 *M* potassium acetate—2 *M* acetic acid, pH 4.8 mixture and incubated for 10 min. This solution was centrifuged at 12 000 *g* for 30 min. The pellets were discarded and the supernatant was filtered through cheesecloth. The plasmid was precipitated from the supernatant by adding 2 vols. of cold (—20°C) ethanol, letting the solution stand for 20 min, and centrifuging the precipitate for 20 min at 12 000 *g*. The supernatant was discarded and the plasmid pellet was redissolved in 6.6 ml of 10 m*M* Tris—1 m*M* EDTA, pH 8 (TE). This final solution was used for SFFF experiments.

Preparation of pDM24D bacterial lysate

Crude plasmid DNA was isolated by a modification of the procedure of Clewell and Helinski [12]. Frozen cell paste (1 g) was suspended in 6 ml TE containing 10 mg/ml lysozyme. The suspension was flash-frozen in a dry ice—ethanol bath for 15 min, and allowed to thaw at 37°C for 15 min. Sodium dodecyl sulfate (1.8 ml of 20% solution), EDTA (1.2 ml of 0.5 *M* solution), and sodium chloride (2.4 ml of 5.0 *M* solution) were then added, mixed throughly, and allowed to stand at 4°C overnight. The resulting precipitate was pelleted at 39 000 *g* at 4°C for 45 min. The amber-colored supernatant was used for SFFF experiments (see Fig. 1).

Gel chromatography of pDM24D lysate

A 1 × 33 cm column of Sephadex G200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated with TE. A sample of 3 ml crude lysate was introduced and chromatographed with a 15-cm pressure head at a flow-rate of

0.4 ml/min. The elution profile was monitored by UV absorption, and 0.8-ml fractions were collected and analyzed for nucleic acid content by agarose gel electrophoresis.

Agarose gel electrophoresis

Agarose gel analysis of plasmid preparations was performed by using either 0.7% (for pBR322) or 1% (for pDM24) agarose gels at 30 V (constant) in 89 m*M* Tris—89 m*M* boric acid—2.5 m*M* sodium EDTA, pH 8.3 (TBE buffer). Gels were stained with ethidium bromide and visualized by UV light, as described by Maniatis et al. [13].

THEORY

The theoretical basis for SFFF has been detailed previously [1—3, 5, 14]. Basically, SFFF separations are carried out in a thin, open channel (e.g., ca. 250 μm) with a continuously flowing single carrier liquid, under the influence of an external centrifugal force field generated in a centrifuge. The laminar flow profile established within this thin channel causes the mobile phase velocity to reach a maximum in the center of the channel and approach zero at the walls. The centrifugal force field that is applied at right angles to the channel flow forces larger or denser particles into slower flow streams nearer the wall, so that they elute later than smaller or less dense particles. In this way, components elute in the order of increasing mass or particle density in the form of high-resolution fractograms that are similar to chromatograms.

SFFF is a separation method based on physical first principles. Giddings and co-workers [1, 2] have shown that the molecular weight of a species is directly related to retention under a particular set of operating variables. Thus, by measuring retention, and knowing the operating variables by which this retention was obtained, it is feasible to calculate the mass (molecular weight) or particle size of a species. No standards are required for this molecular-weight measurement.

At a constant rotor speed (gravitation force) [1]:

$$M = \frac{R_0 T}{\lambda \omega^2 r W(\Delta\rho/\rho_s)}$$

where, R_0 = gas constant [8.31 × 10^7 g × cm^2/(sec^2 × deg × mol)]; T = absolute temperature (Kelvin); M = mass or molecular weight of the soluble species or particle dispersion; ω = centrifuge speed (rad/sec); r = radial distance from the centrifuge rotating axis to the SFFF channel (cm); W = channel thickness (cm); $\Delta\rho$ = density difference between sample component and mobile phase (g/cm^3); ρ_s = density of the sample component (g/cm^3); and the dimensionless retention parameter $\lambda \simeq V_0/6V_R$, for retained peaks eluting at a retention volume V_R that is at least twice that of the channel void volume V_0. In the case of time-delay, exponential field-decay SFFF programming (called TDE-SFFF), retention becomes a function of time and varies according to the particle field strength at that time. Thus, in programmed separations by TDE-SFFF, molecular weights of components over a wide mass range can be determined in a single experiment [3, 14].

RESULTS

pBR322 Plasmid isolation

In this experiment a 1-l culture of *E. coli* containing the plasmid pBR322 was taken through initial steps which removed the cellular debris, most of the protein and large bacterial DNA (MW ca. 10^9). This process produced 6.6 ml of clear cellular lysate containing the desired plasmid, plus large amounts of contaminating RNAs and small amounts of protein (Fig. 1). This procedure required one-half day of work. To determine if the plasmid might be isolated from this crude lysate without further purification, an analytical SFFF fractionation was performed on a 100-μl aliquot of the lysate. For this separation an initial rotor speed of 30 000 rpm (70 000 *g*) was utilized with a programmed exponential decrease in force field, using an exponential time delay/decay constant of 12.0 min (see ref. 14). These conditions were chosen so that the plasmid would be well separated from the unretained RNA and proteins eluting at the channel dead volume V_0. Fig. 2 shows the analytical fractogram which was obtained on this material by time-delayed exponential decay SFFF using the instrumental techniques previously described [4].

In the separation of Fig. 2 a single retained peak was obtained, completely resolved from the unretained fraction (V_0). Total separation time was about 1 h. The calculated molecular weight of the material in this peak, based on retention, agreed closely with the reported literature value for this plasmid ($2.8 \cdot 10^6$). It should be noted that the trailing edge of the plasmid peak also contained a small concentration of the plasmid dimer that also is produced

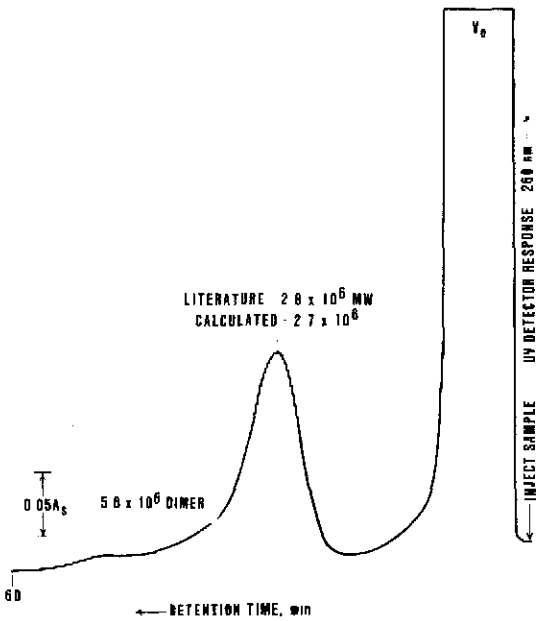

Fig. 2. Analytical separation of crude pBR322 lysate. Conditions: sample: 100 μl of crude lysate; channel thickness: 0.024 cm; initial rotor speed: 30 000 rpm (70 000 *g*); delay/exponential decay time constant τ: 12.0 min; relaxation: 15 min; mobile phase: 0.01 *M* Tris—0.1 *M* sodium chloride, pH 7.6; flow-rate: 0.5 ml/min; detector : UV, 260 nm.

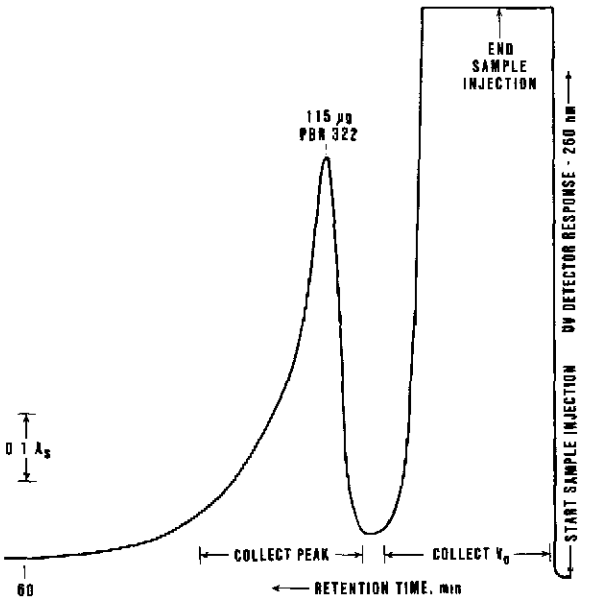

Fig. 3. Preparative SFFF isolation of pBR322. Conditions same as for Fig. 2, except for sample: 3.3 ml of crude lysate; rotor speed: 32 000 rpm (80 000 *g*), constant.

within the bacteria. If desired, separation of the monomer and dimer forms of the plasmid could have been accomplished by using appropriate operating conditions with a longer separation time.

Based on these results, instrument conditions were optimized for a preparative isolation; no change in SFFF equipment was required. For the preparative run shown in Fig. 3, 3.3 ml of the crude lysate (about one-half of the total sample) was injected into the channel. Relative to the analytical separation in Fig. 2, the initial force field was increased to 32 000 rpm (80 000 *g*), and separation was by constant force field conditions, to provide optimum resolution of the unretained components in V_0 from the plasmid. Both the retained plasmid peak and unretained V_0 fraction were collected. Quantitation of the retained plasmid peak by UV absorption at 260 nm (using E = 20, 0.1%, 1.0 cm) indicated that 115 µg of pBR322 had been isolated.

Although yields vary significantly with culture conditions, plasmid type, and bacterial strain, it was anticipated that 1 l of unamplified HB101 cells should produce approximately 300 µg of pBR322 with conventional isolation procedures. Since only one half of the total lysate was preparatively fractionated (Fig. 3), it would appear that the yield of purified pBR322 by SFFF fractionation is better than 75%. It is interesting to note that the total purification time using SFFF was about 3/4 day, including the time required to prepare the lysate.

The purity of the materials isolated in the preparative fractionation was demonstrated by agarose gel analysis (Fig. 4) as described in Experimental. This test showed that the lysate starting material not only contained large amounts of RNA, but also some cellular DNA as well as the expected monomeric and dimeric forms of pBR322. The unretained V_0 peak showed cellular RNA (MW

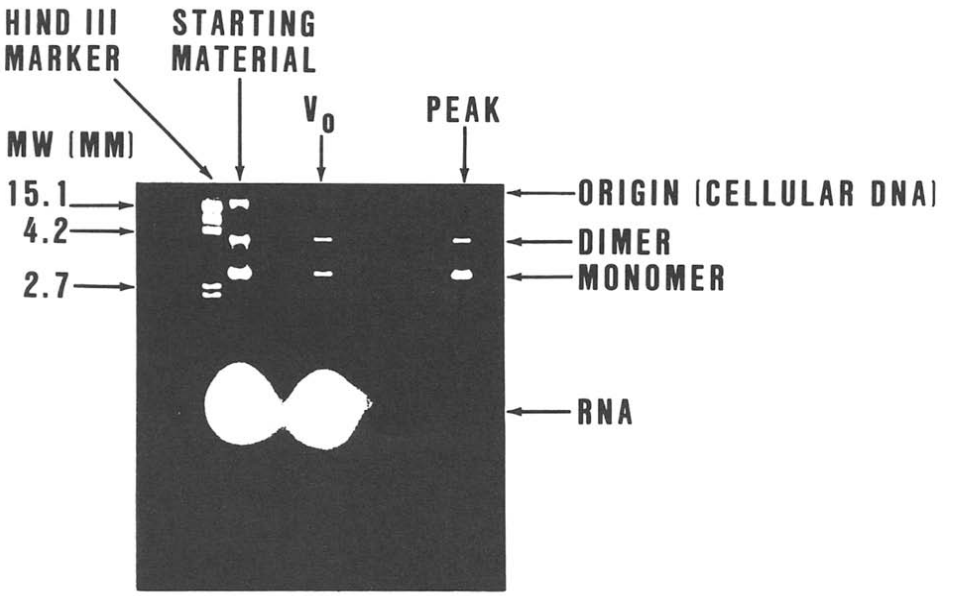

Fig. 4. Agarose electrophoresis gel of pBR322 preparative fractions. Conditons: 0.7% agarose gel separation as described in ref. 13; sample: about 0.5 μg each.

$< 1 \cdot 10^6$). Lower-molecular-weight protein contaminants also eluted in this unretained fraction, although these materials cannot be visualized by the technique used in this gel analysis. Also present in the unretained V_0 fraction were small amounts of the plasmid monomer and dimer, resulting from incomplete initial relaxation (equilibration) of the sample prior to initiating mobile phase flow for the separation. The isolated retained peak of Fig. 3 was shown to contain only the two forms of pBR322. (When the fractionation in Fig. 3 was terminated, the cellular DNA of much higher molecular weight was still retained within the channel and was later eluted by allowing the rotor to come to rest while purging with several channel volumes of mobile phase.)

Isolation of pDM24D

SFFF also has demonstrated utility for isolating plasmid from bacterial lysates of a much cruder composition than that for pBR322. An unclassified thermophilic anaerobe contained a plasmid of interest, designated pDM24D (MW ca. $1.5 \cdot 10^6$) [15]. In the case of this organism, host cellular DNA and proteins cannot be removed effectively by standard selective precipitation techniques such as those described in Fig. 1. Therefore, the resulting pDM24 cellular lysate of deep amber color contained much more cellular DNA and protein than the clear lysate obtained for pBR322. An analytical SFFF fractionation of a 250-μl aliquot of this crude lysate and the agarose gel electrophoresis of fractions collected from this separation are shown in Figs. 5 and 6, respectively.

In gel electrophoresis, fraction 1 (Fig. 6) seen as a shoulder on the unretained V_0 peak in Fig. 5) contained the various conformational forms of the plasmid (supercoiled to relaxed), as determined by comparison to a sample of pDM24D

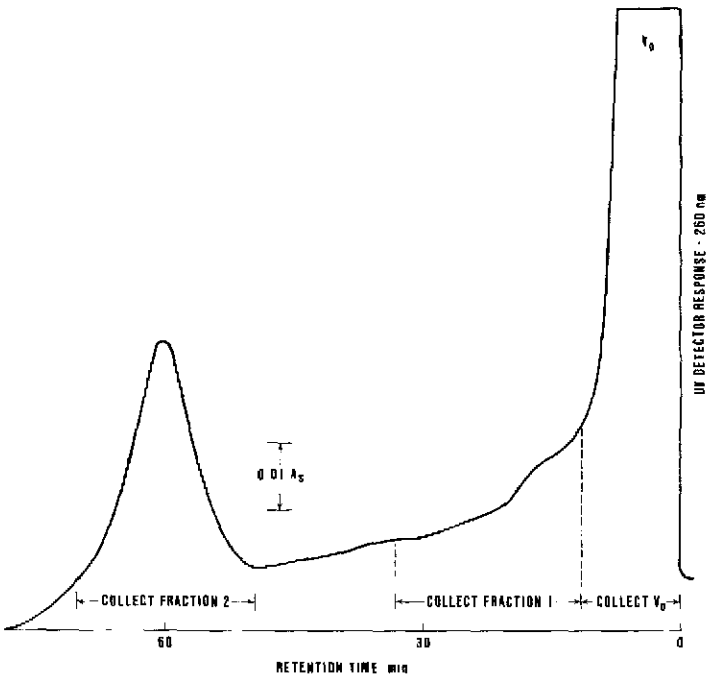

Fig. 5. Analytical separation of crude lysate containing pDM24D. Conditions: same as for Fig. 2 except for initial rotor speed: 32 000 rpm (80 000 *g*); delay before exponential speed decay: 20.0 min, exponential decay constant τ: 6.0 min; relaxation: 5 0 min.

previously purified by a four-day cesium chloride density gradient [15]. Most of the plasmid in fraction 1 was in the relaxed form. This is consistent with the appearance of the starting lysate, which shows a much greater concentration of relaxed plasmid than supercoiled forms. The wide band of material in the starting lysate more strongly retarded on the gel than the relaxed plasmid is believed to be of chromosomal origin [15]. The RNA separated in the unretained fraction V_0 can be seen at the bottom edge of the gel of the appropriate lane in Fig. 6. Also found in the V_0 peak was a small amount of the pDM24D (mostly in the relaxed form). This result was expected since the plasmid was not baseline-separated from the V_0 peak under the operating conditions used (Fig. 5). Fraction 2 (Fig. 5) contained components which co-migrated with the chromosomal material present in the crude lysate and were very weakly stained by ethidium bromide. Plasmid molecular-weight standards (Fig. 6) suggested that the molecular weight of the supercoiled pDM24D was approximately $1.5 \cdot 10^6$.

Based on these studies it appeared that an effective isolation of pDM24D from contaminating components should be feasible by SFFF, particularly if some of the lower-molecular-weight protein contaminants could be at least partially removed prior to SFFF. Accordingly, a fresh lysate was prepared and passed through a Sephadex G200 size-exclusion column (exclusion limit, 600 000 MW). The portion of the exclusion volume containing the plasmids was collected and the retarded fraction containing smaller proteins was discarded.

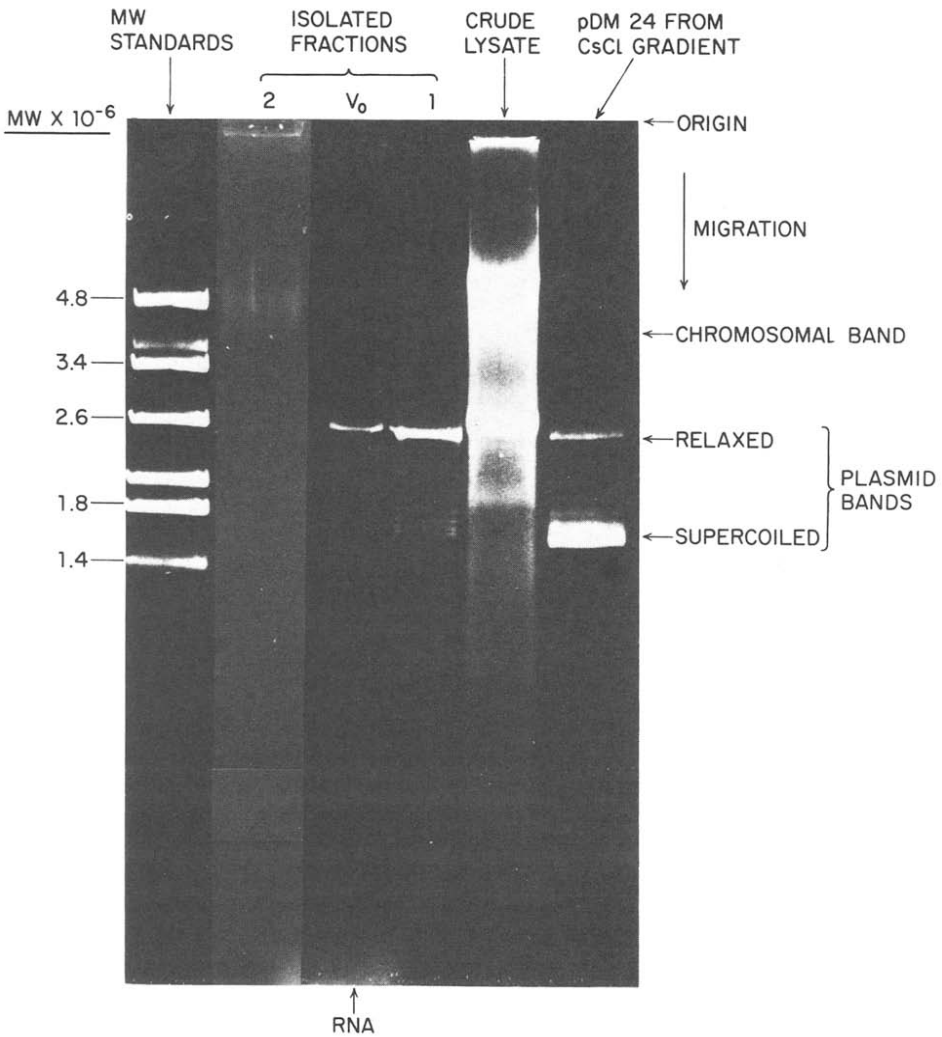

Fig. 6. Agarose electrophoresis gel of pDM24D fractions. Conditions: same as for Fig. 4 except for 1% agarose gel. Note: the lane containing fraction 2 is from a second photo of the same gel, but exposed a longer time in order to bring out the weakly stained band.

The preparative SFFF separation shown in Fig. 7 was carried out on a 1.4-ml aliquot of eluate from the G200 column. To enhance the separation of the plasmid from the V_0 peak, the separation was carried out with constant force field at 32 000 rpm (80 000 *g*) following a 5-min relaxation. In this single run of about 40 min, 42 μg of plasmid were isolated, as determined by UV spectrophotometric assay. Gel electrophoresis confirmed both the identity of this material and the purification performed by SFFF. The observed SFFF molecular weight of $1.3 \cdot 10^6$ for the isolated plasmid fraction (Fig. 7) compares favorably with the $1.5 \cdot 10^6$ MW value estimated by gel electrophoresis. Note that the single peak for the various pDM24D conformers in Fig. 7 results from the fact that SFFF separates strictly by mass. Supercoiled and relaxed plasmid forms will elute at the same time, since retention in SFFF is not influenced by particle shape, except in very extreme cases [16].

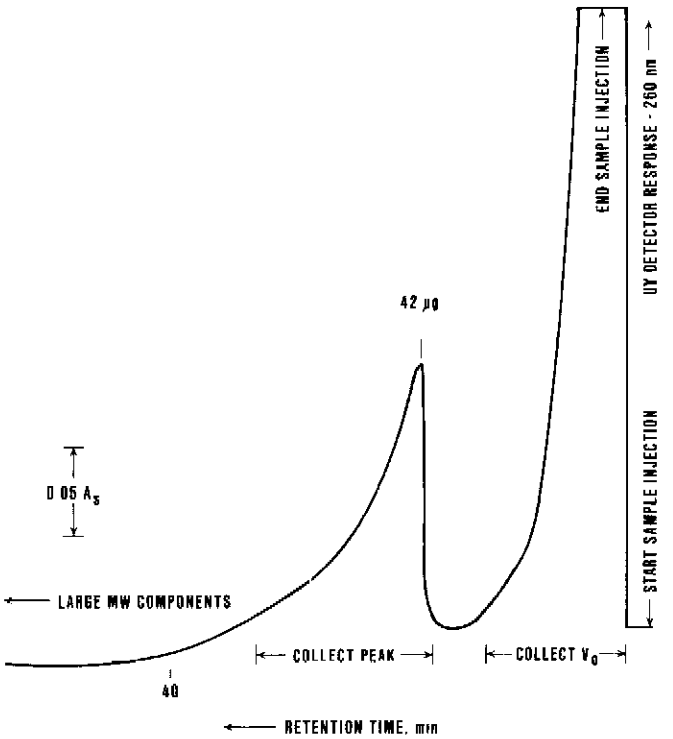

Fig. 7. Preparative isolation of Sephadex G200-purified pDM24D lysate Conditions: same as for Fig. 3 except for sample: 1.4 ml of crude lysate.

DISCUSSION

These results demonstrate the utility of SFFF for rapid preparative purification of plasmid recombinant vectors from crude bacterial lysates. Although the maximum sample load for any single preparative run depends on the nature of the sample and the degree of resolution required, these experiments suggest that for cellular lysates milligram quantities of plasmid can be isolated promptly, ready for use in recombinant DNA studies.

Yields in SFFF separations can be maximized by optimizing operating conditions. In the case of cellular lysates, initial force field should be at a sufficient level to ensure adequate separation from lower-molecular-weight components (e.g., RNA, proteins). Although the mobile phase flow-rate usually is not critical for closed-form DNA molecules, very fragile, linear DNA (e.g., λ-DNA) should be fractionated at relatively low flow-rates to insure accurate retention and mass determination [16]. Relaxation time must be long enough to ensure correct retention and minimum loss of retainable components in the V_0 peak [17].

The ability of SFFF to rapidly and accurately estimate the molecular weights of purified nucleic acids and other biological materials has been demonstrated previously [8, 9]. Results presented in this paper show that

accurate molecular weights also can be determined in the more complex mixtures actually encountered in recombinant DNA studies.

This work and previous studies [8, 9] have shown that SFFF has many advantages for the fractionation of nucleic acids and other biological compounds. (1) The gentleness and speed of SFFF fractionation result in little risk of loss of biological activity. (2) Resolution and sensitivity are more than adequate for many biological separation problems. Baseline resolution of non-interacting components can be achieved with a 20% difference in mass. (3) Separation time is short and generally not dependent on the sample molecular weight range. If the approximate molecular weight range of the sample is known, instrument conditions can be conveniently and rapidly established, so that a single component of interest differing either slightly or widely in molecular weight from contaminants often can be separated within about 1 h. (4) Preparative isolations can be carried out in essentially the same time as analytical runs, since complete recovery of fractionated material is accomplished simply by collecting the eluent as it exits the detector. (5) Molecular weight information is supplied rapidly and accurately by on-line analysis. (6) Isolated materials generally are ready for use without further treatment. (7) The purity and yield of materials appear to be at least equivalent to those prepared by traditional methods.

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