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ISOLATION PROCEDURE FOR BACTERIAL DNA BASED ON GEL PERMEATION CHROMATOGRAPHY ON A SEPHAROSE COLUMN

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SUMMARY

Gel permeation chromatography of bacterial lysates, no matter what type of lysis is used, on a Sepharose 4B column equilibrated with buffered 2 *M* NaCl was demonstrated to be generally useful as a simple method for the quantitative and reproducible isolation of high-molecular-weight DNA (average molecular weight 2.2×10^7 daltons). Conventional analyses of this DNA and its re-chromatography on a MAK column and electrophoretic fractionations in polyacrylamide and agarose gels show less heterogeneity and a lower polysaccharide content in comparison with DNA preparations isolated by other complex processes, no RNA contamination and 2–3% of residual proteins. The method is also recommended for bacteria affected by different factors, where classical methods for the quantitative isolation of DNA fail.

INTRODUCTION

Isolation methods for DNA reported so far are very complex processes consisting of many steps (for a review see, *e.g.*, ref. 1). During these time-consuming operations (particularly deproteinizations, precipitations, and fractionations), a considerable danger of molecular weight degradation exists, which is manifested in the higher heterogeneity of the final preparations obtained[2]. The introduction of gel filtration materials and, more recently, agarose gels (Sepharose), mostly used for nucleic acid purifications and separations[3–7], provides an excellent tool for a considerable simplification of DNA isolation processes as well[8,9].

Recently, we have used a column of Sepharose 4B for the separation of components of *Bacillus subtilis* cell lysate[10]. A relatively pure transforming DNA was released. The aim of the work now reported was to characterize the DNA isolated by means of this column and prove that such a method could serve as a general single-step process for the quantitative and reproducible isolation of bacterial DNAs.

EXPERIMENTAL

Sepharose 4B (Pharmacia, Uppsala, Sweden), having an exclusion limit of molecular weight of about 5×10^6 daltons and a fractionation range from 10^5 to 5×10^6 daltons, was used as the most suitable agarose sorbent. Table I shows the column

TABLE I

ISOLATION COLUMN PARAMETERS AND EXPERIMENTAL CONDITIONS

	Column scale	
	Small	*Large*
Bed material	Sepharose 4B, 40–190 μm	
Column length, mm	400	800
Column diameter, mm	25	30
Maximal sample volume, ml	18	50
Maximal weight of wet cells, g	2	8
Eluent	2 *M* NaCl in 0.01 *M* sodium citrate	
Temperature, °C	20–25	20–25
Flow-rate, ml/cm^2·min	0.08–0.14	0.06–0.17
Fraction volume, ml	10	10
Detection wavelength	260 (Unicam)	254 (Uvicord)

parameters and the conditions under which all isolation experiments were conducted.

Different *B. subtilis* (SMYW, W23 Y) and *Escherichia coli* (WP14) strains of our collection were cultivated in liquid glucose–mineral media with appropriate supplements as mentioned elsewhere[11,12]. Radioactively labelled cultures were obtained by cell growth in the same media supplemented with [methyl-^{3}H]thymidine (2.9 Ci/mmole; 1.3 μCi/ml), [^{3}H]uridine (1 Ci/mmole; 0.33 μCi/ml), and ^{14}C-labelled protein hydrolysate (1 mCi/mg; 0.033 μCi/ml), respectively. The labelled thymidine was always added after inhibition of the thymidylate synthesis of the cells by fluorodeoxyuridine (4 μg/ml). Experiments with 5-azacytidine to follow changes after its incorporation into the DNA were carried out under growing conditions described previously[11].

All lysates of the harvested and frozen cells were prepared after thawing either by lysozyme or by sodium dodecylsulphate treatments in the Marmur isolation medium[13] at pH 8, 2 *M* in sodium chloride, and after a short centrifugation (10 min at 15,000 × *g*) used for DNA isolation on the Sepharose column (see Table I).

Besides conventional characterizations of the DNA fractions using UV spectroscopy (Unicam SP 700), chemical colour tests for estimations of protein, RNA, and DNA components (Lowry method, orcinol reagent and diphenylamine reaction, respectively), scintillation measurements of the labelled precursor (Tricarb Model 3375; Packard, Downers Grove, Ill., U.S.A.), sedimentations in an analytical ultracentrifuge (Spinco Model E), and analyses of transforming activity in the case of *B. subtilis* DNA, methods which were described earlier[2,10,11], the degree of purity and further characteristics were obtained by re-chromatography of certain fractions on a three-layered MAK column according to Mandell and Hershey[14] (20 × 170 mm) and by analytical electrophoreses on polyacrylamide (Loening[15]) and agarose gels (a slight modification of the method of Aaij and Borst[16]).

For MAK re-chromatography[14], the fractions from the Sepharose column had to be diluted by 0.013 *M* phosphate buffer at pH 7 to obtain an initial NaCl concentration in the sample of 0.1 *M*. Chromatography was carried out by elution with a linear gradient of 0.1 to 1.1 *M* NaCl and each fraction was monitored at 260 nm by the Unicam SP 700 spectroscope.

No modification of the fractions was needed for the agarose gel electrophoresis[16]. At least 10 μg of DNA in the final 100-μl volume were layered on 0.5% agarose (Serva, Heidelberg, G.F.R.) in electrophoretic tubes (8 × 80 mm) and fractionated in 0.08 *M* Tris at pH 7.7 with 0.04 *M* sodium acetate and 0.004 *M* EDTA for 6 h at room temperature and 2.5 mA/tube. For better visualization of DNA under UV light the gels were then incubated in the dark in 0.05 *M* acetate buffer at pH 4.5 with 0.001 *M* EDTA containing 100 μg/ml ethidium bromide.

Polyacrylamide gel electrophoresis[15] was effected in the same tubes as mentioned above (2.2% gel in 0.04 *M* Tris at pH 7.8 with 0.02 *M* sodium acetate and 0.002 *M* EDTA for 3 h at 10 V/cm and 5 mA/tube) with fractions concentrated by means of Aquacide II (Calbiochem, Luzerne, Switzerland). The 10-μl samples, containing at least 50 μg DNA, were monitored by scanning at 260 nm with the Chromoscan microdensitometer (Joyce, Loebel and Co., Burlington, Mass., U.S.A.).

RESULTS AND DISCUSSION

During the centrifugation of the bacterial lysate, when the concentration of NaCl had been increased up to 2 *M*, part of the cellular proteins was precipitated and sedimented and the clear supernatant, which had a viscosity suitable for the column process, was applied to the Sepharose. Figs. 1 and 2 represent the separation of such supernatants using two types of column (see Table I) both loaded with samples of maximal volume. The difference between these two elution curves is in the shape of the second peak only, which is not decisive for a quality of the isolated DNA being located in the first peak (compare the radioactivity of incorporated thymidine). Complete separation of these two peaks is the sole and sufficient condition for the isolation of high-molecular-weight DNA with a good yield. The characteristics are summarized

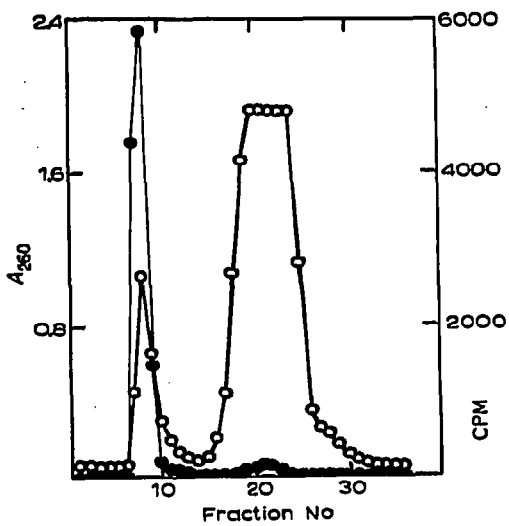

Fig. 1. Gel filtration on a Sepharose 4B column of the *B. subtilis* lysate labelled with [methyl-^{3}H]-thymidine. For the column parameters and the experimental conditions, see Table I (small column). ○, absorbance at 260 nm; ●, radioactivity (cpm). The first peak represents DNA, the second a mixture of proteins, RNAs, and other cell components.

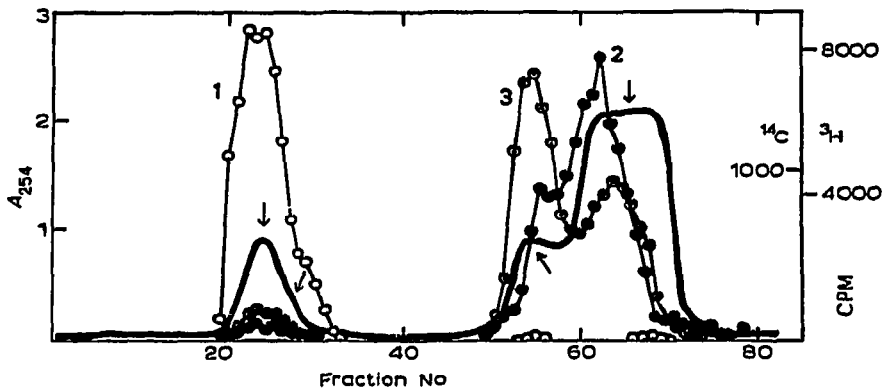

Fig. 2. Tracing by the incorporation of labelled specific precursors of the separation of macromolecules from *B. subtilis* lysate on a Sepharose 4B column. For the column parameters and the experimental conditions, see Table I (large column). ——, optical absorbance at 254 nm. 1 = Radioactivity of [methyl-^{3}H]thymidine; 2 = radioactivity of [^{3}H]uridine; 3 = radioactivity of ^{14}C-labelled amino acids. The arrows indicate the fractions used for further separations and analyses.

in Table II. The reproducibility of the separations is excellent for both *E. coli* and *B. subtilis* lysates (no effect of enzymic or chemical lyses was observed), DNA always being collected in tube Nos. 6–13 and 20–32, respectively, and the second peak starting from tube No. 16 or 50 of the corresponding column (Table I). A possible separation of cellular proteins, RNAs and lysozyme added to the cells corresponding to the distributions of the incorporated precursors in Fig. 2 was described in detail elsewhere[10].

Both UV spectra (shift of minimal wavelength to the longer values and lower $A_{max.}/A_{280}$ ratios) and denaturation curves (higher T_m values) of the DNA fractions obtained from the Sepharose column confirmed the higher content of residual proteins, estimated chemically as 2–3%. Their existence was proved by fraction re-chromatography on a MAK column (Fig. 3), where the first peak represented a mixture of peptides with very low molecular weight and oligosaccharides which had been seen after hydrolysis on paper chromatograms as negligible amounts of glucose, ribose, and glucosamine (see also ref. 2). These admixtures can be found particularly in the latter fractions of the first peak, so that the quality of DNA preparations can be improved

TABLE II

ANALYTICAL CHARACTERISTICS OF ISOLATED DNAs

All values are averages of at least three experiments.

DNA source	$\lambda_{max.}$ *(nm)*	$\lambda_{min.}$ *(nm)*	$\frac{A_{max.}}{A_{min.}}$	$\frac{A_{max.}}{A_{280}}$	T_m *(°C)*	$s_{20,w}$ *(S)*	ΔA *(%)*	*Residual proteins (%)*	*RNA (%)*
*B. subtilis**	260	233	1.92	1.81	89.7	31.7	36	2.5	0
*B. subtilis***	258	229	2.52	2.16	87.3	27.1	35	1.1	2.8
*E. coli**	260	233	1.88	1.71	92.8	33.1	33	3.0	0
*E. coli***	258	230	1.99	1.96	90.5	27.9	30	<1	2.2

* DNAs isolated on the Sepharose column.

** DNAs isolated by the modified Marmur method.

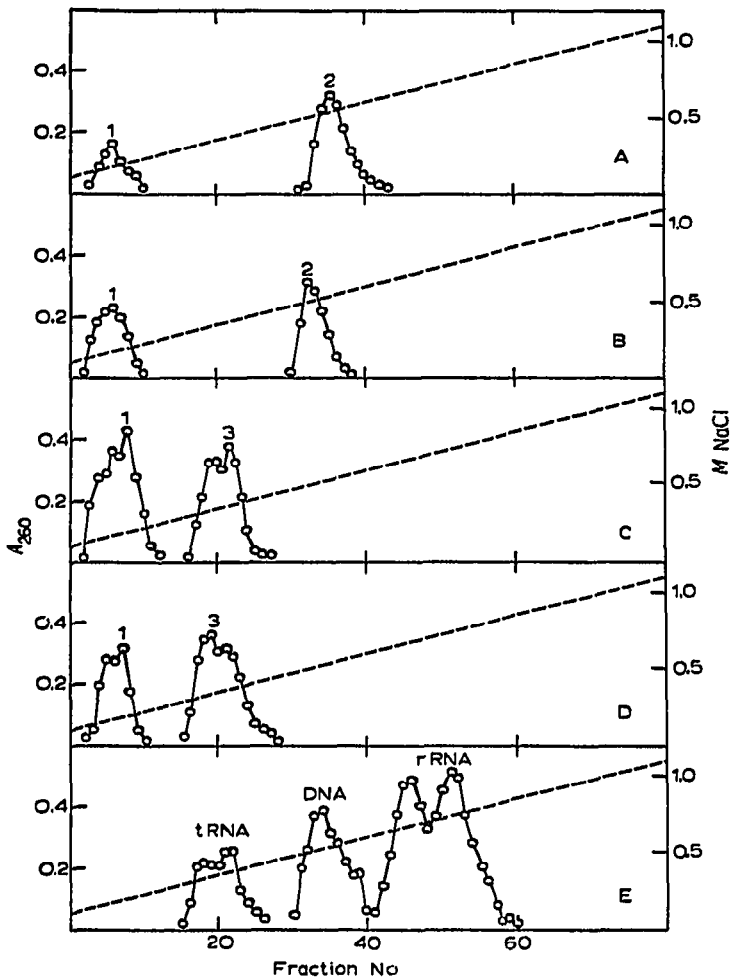

Fig. 3. Re-chromatography of selected fractions from the Sepharose column on the MAK column. The column[14] (20 × 170 mm) was eluted with a linear gradient of 0.1 to 1.1 *M* NaCl (total volume 480 ml) at a flow-rate of 0.14 ml/cm^2·min and at room temperature. Fractions of 6 ml were collected and monitored at 260 nm by a Unicam SP 700 spectroscope. A, B, C, and D represent fraction Nos. 24, 28, 57, and 70 of Fig. 2, respectively; E represents locations of nucleic acid markers under the same conditions. 1 = Peptide and saccharide contaminations; 2 = high-molecular-weight DNA; 3 = RNAs and their degradation products.

by combining fractions from the first two thirds of the peak only (Nos. 20–28). To keep the isolation quantitative, the whole DNA peak can be re-purified by means of MAK re-chromatography when elution with the short gradient of 0.5 *M* to 1 *M* NaCl is sufficient[17]. Especially in the case of *B. subtilis*, where DNA contains usually some amount of teichoic acid, isolation with a Sepharose column, as well as MAK re-chromatography, is highly recommended for obtaining DNA of high purity. A new and promising method for the removal of polysaccharides from DNA based on affinity chromatography with concanavalin A bound to agarose was recently proposed by Edelman[18].

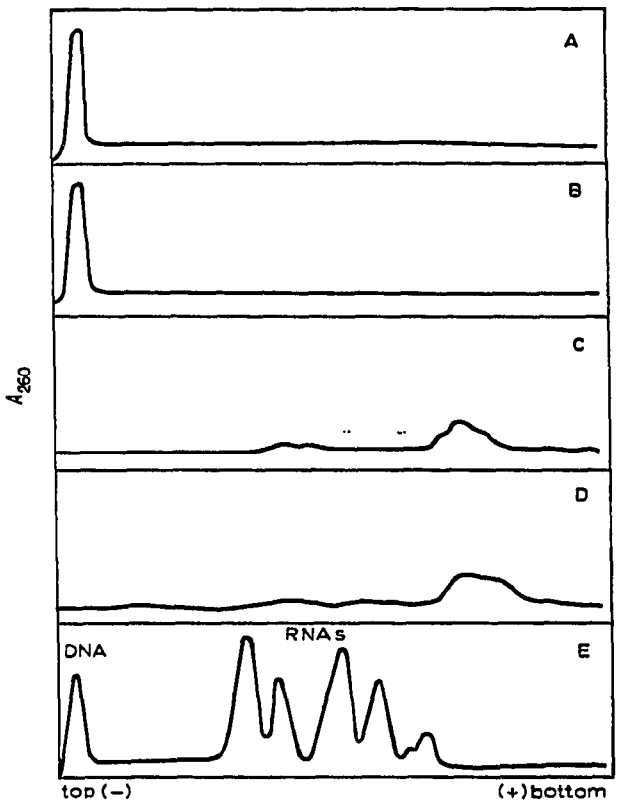

Fig. 4. Polyacrylamide gel electrophoresis of selected fractions from the Sepharose column. Of the fractions 10 μl (marked with arrows in Fig. 2), containing about 1 $A_{260\ nm}$ unit were applied to the gel (2.2%) in tubes of 8 × 80 mm. Electrophoresis was carried out in 0.04 *M* Tris at pH 7.8 with 0.02 *M* sodium acetate and 0.002 *M* EDTA for 3 h at 10 V/cm, 5 mA/tube and room temperature. For explanations of A, B, C, D, and E, see the legend to Fig. 3.

The high degree of purity in respect of RNA admixtures and the high molecular weight and lower heterogeneity of the isolated DNA in comparison with classically isolated DNAs were also proved by electrophoretic analyses on polyacrylamide (Fig. 4) and agarose (Fig. 5) gels. In the first case, the results were in very good accord with separation on the MAK column, confirming that there was no RNA contamination in the first Sepharose peak and that DNA of high molecular weight was present. The same holds for the agarose gel (Fig. 5), where on comparing three DNAs the lower molecular weight heterogeneity of the evaluated preparation is clear.

To show the usefulness of the described method for tracing the fate of DNA in cells where other isolation methods failed, we incorporated 5-azacytidine into *B. subtilis*, which is known to decrease the yield of DNA while its synthesis remains unaffected[11,12]. The lysate of such a bacterial culture is found after its separation on the Sepharose column to have some residual transforming activity in the second peak (Table III) never found in the control culture[10]. The activity was more pronounced with the marker whose transforming activity had been proved to be more dependent on the polynucleotide parts of DNA rich in adenine–thymine (methionine marker;

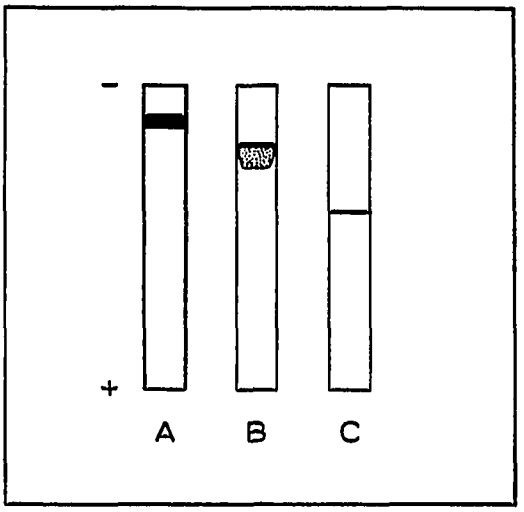

Fig. 5. Agarose gel electrophoresis of different DNA preparations. A 100-μl sample of DNA (10 μg) was applied to 0.5% agarose in tubes of 8 × 80 mm and electrophoresis was carried out in 0.08 *M* Tris at pH 7.7 with 0.04 *M* sodium acetate and 0.004 *M* EDTA for 6 h at 2.5 mA/tube and at room temperature. The DNA location was followed under UV after the incubation of gels in 0.05 *M* acetate buffer at pH 4.5 with 0.001 *M* EDTA and 100 μg/ml ethidium bromide. A = DNA of *B. subtilis* isolated on a Sepharose column; B = the same DNA isolated by the modified Marmur method[2]; C = DNA of T7 phage isolated by a phenol extraction (kindly supplied by dr. Udvardy, Szeged).

TABLE III

TRANSFORMING ACTIVITY OF SELECTED FRACTIONS FROM THE SEPHAROSE COLUMN SEPARATING LYSATES OF *B. SUBTILIS* WITH OR WITHOUT THE INCORPORATION OF 5-AZACYTIDINE

Fraction No.	*Frequency of transformants* obtained with fractions of* control / 5-azacytidine *lysate for marker*		
	Adenine	*Leucine*	*Methionine*
21	2.5×10^{-3}	9.1×10^{-4}	3.1×10^{-3}
	1.2×10^{-3}	8.6×10^{-4}	1.4×10^{-3}
27	2.9×10^{-3}	1.1×10^{-3}	4.3×10^{-3}
	5.2×10^{-4}	2.9×10^{-4}	6.7×10^{-4}
32	2.0×10^{-3}	9.7×10^{-4}	2.9×10^{-3}
	2.1×10^{-4}	1.6×10^{-4}	2.3×10^{-4}
55	0	0	0
	4.0×10^{-5}	4.8×10^{-5}	1.2×10^{-4}
70	0	0	0
	3.2×10^{-6}	4.1×10^{-6}	2.4×10^{-5}

* Average values of at least six independently transformed cultures (*B. subtilis* requiring adenine, leucine and methionine as a recipient strain).

see ref. 17). It may be concluded that massive incorporation of the labile antimetabolite (50% of cytosine residues replaced), preferentially into regions rich in guanine–cytosine, can induce their degradation or fragmentation and decrease the yield of DNA during isolation, *e.g.* by the modified Marmur method[2].

Summarizing the results and experience so far obtained, it can be concluded that the method described represents a direct, simple, time-saving, and gentle procedure for the isolation of relatively pure DNA while preserving its high molecular weight and biological activity. If necessary, further purification can be achieved by MAK column chromatography, which does not affect the integrity of the DNA molecules.

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