ISOLATION OF TRANSFORMING DNA FROM *Bacillus subtilis* BY GEL FILTRATION ON SEPHAROSE 4B

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A method of isolation of transforming DNA from *Bacillus subtilis* is described. The cells are lyzed by lysozyme, the concentration of NaCl is increased to 2M, and the lysate is subjected to gel filtration on a column of Sepharose 4B. The elution profile obtained shows two peaks (A_{260}). The former represents highly polymerous transforming DNA, approximately 2 to 3% of proteins, and traces of RNA. Proteins and other components of the original lysate, as well as lysozyme, are contained in the second peak. The method can be used also for bacterial species not lyzed by lysozyme; in this case the lysis is effected by detergents.

The main problem involved in the isolation of DNA is complete deproteinization with simultaneous retainment of intact DNA molecules. The isolation procedures (*e.g.*[1-3]) regarded to date as classical and using detergents and organic solvents, provide excellent deproteinization of the preparation, however, they are relatively time-consuming and also impair to a certain degree the DNA. During the past few years the development of the isolation techniques has been focused predominantly on two procedures: first, on the centrifugation in density gradients, used in preparative scale experiments as centrifugation in zonal rotors[4,5], and second, on gel filtration[6,7] whose applicability increased with the introduction of agarose columns.

This paper describes a simple procedure of obtaining relatively pure high molecular weight DNA from bacteria by gel filtration on Sepharose 4B in solutions of high ionic strength.

EXPERIMENTAL

Cultivation of bacteria. Bacillus subtilis prototrophic strains W23 and SMYW from the collection of this Institute were used. The bacteria were grown in the liquid glucose-mineral medium of Spizizen[8] with the admixture of 0·15% of beef extract, 0·15% of yeast extract, and 0·5% of peptone. The strains used for the experiments with specific labelling of DNA and RNA by the radioactive precursors were grown in the minimum medium only. 5-Fluorodeoxyuridine[9] (4 μg/ml) was added to the medium to increase the incorporation of [^{3}H]-thymidine. Thymidine-[methyl-^{3}H] (specific activity 2·9 Ci/mM, Institute for Research, Production, and Use of Radioisotopes, Prague) was added to the medium up to a final concentration of 1·3 mCi/l of medium. The labelled precursor was diluted so that the total concentration of thymidine in the medium might be 1 to 2 mg/l. Similarly, in experiments with the labelling of the culture with [^{3}H]-uridine (specific activity

1·0 Ci/mM, Radiochemical Centre, Amersham), the medium contained 0·33 mCi/l. The bacteria for the labelling of proteins were cultivated in the minimal medium with a mixture of ^{14}C-labelled amino acids (Institute for Research, Production, and Use of Radioisotopes, Prague) containing together 0·033 mCi/l (33 μg/l). The cells were always harvested during the stationary phase of growth by a centrifugation, washed with 0·15M-NaCl containing 0·1M-EDTA at pH 8·0, and frozen.

The isolation of DNA from Bacillus subtilis was carried out by the following standard procedure (procedure 1 in Table I): The wet bacteria were suspended in the solution of 0·15M-NaCl and

TABLE I

Certain Modifications of Standard method of DNA Isolation from *B. subtilis* on Sepharose 4 B Column (see text for detailed description)

Procedure	Lysozyme	SDS	RNase	Pronase	Chloroform–isoamyl alcohol	Ethanol	Sepharose 4B
1	+	−	−	−	−	−	+
2	−	+	−	−	−	−	+
3	+	+	+	+	−	−	+
4	+	+	−	−	+	+	+
5	+	+	+	+	+	+	+
6	+	+	−	−	+	+	−
7[a]	+	+	+	−	+	+	−

[a] The isolation was carried out by the modified procedure of Marmur[3,5].

TABLE II

Certain Characteristics of Fractions Chosen for Transformation Experiments (Fig. 1)

Fraction No	Peak	λ_{max}	λ_{max}	$\frac{A_{max}}{A_{min}}$	$\frac{A_{260}}{A_{280}}$	$s_{20,w}$	Transforming activity
20	I	260	237	2·06	1·87	32·5	+
22		259	234	1·97	1·86	31·1	+
27		259	238	1·38	1·49	30·6	+
53	IIa	278	250	—	—	—	—
57		278	250	—	—	—	—
65	IIb	260	241	1·29	—	—	—
69		258	—	—	—	—	—
75		261	229	2·71	3·86	—	—

0·1M-EDTA at pH 8·0; the total volume of the suspension was 10 ml. Lysozyme (10 mg, Nutritional Biochemicals Corp.) was added to the suspension. The mixture was gently homogenized in Potter-Elvehjem homogenizer and incubated 1 h at 37°C with constant slow shaking (Dubnoff bath). The lysis of the culture manifested itself by a strong increase of the viscosity of the suspension. A 5M-NaCl solution (6·7 ml) containing 0·025M sodium citrate was added to the lysate and the mixture was stirred. The lysate was centrifuged twice 10 min at 12000 *g* to remove the rest of the unlyzed cells and a part of the precipitated proteins. The entire volume of the supernatant (usually 15 to 15·5 ml) was applied to a 3 . 80 cm column of Sepharose 4B, equilibrated in 2M-NaCl containing 0·01M sodium citrate. The same solution was used for the elution (0·4 ml/min, fraction volume 10 ml). The fractions corresponding to the first peak were pooled and dialyzed against SSC.

We have also assayed different modifications of this method (Table I): dodecyl sulfate instead of lysozyme was used for the lysis of the bacteria. The original suspension of the bacteria was heated at 60°C and 1 ml of 25% solution of sodium dodecyl sulfate at the same temperature was added. The lysate was cooled down to room temperature after 10 min. (procedure 2). In certain cases the lysis of the bacteria was carried out stepwise with both lysozyme and sodium dodecyl sulfate. In procedure 3 and 5, the concentration of NaCl in the lysate was increased to 2M (by 5M-NaCl in 0·025M sodium citrate), 1·5 mg of RNase (Reanal, Budapest) was added, and the mixture was incubated 45 min at 37°C. The RNase solution (12·5 ml/mg in 0·15M-NaCl, pH 5·0) was heated at 80°C for 10 min before use to inactivate DNase which might have been present[3]. In procedure 3, 90 mg of pronase (Calbiochem, Los Angeles) was also added to the lysate and the mixture was incubated 9 h at 37°C. In cases where the preparation was purified by organic solvents before the gel filtration, the concentration of NaCl in the lysate was raised to 2M, an equal volume of the mixture chloroform–isoamyl alcohol (24 : 1, v/v) was added, and the mixture was shaken 20 min in a laboratory shaker. The mixture was then centrifuged, the upper layer was carefully separated, and DNA precipitated by the addition of an equal

TABLE III

Transforming Activity of Lysate of *B. subtilis* SMYW after Gel Filtration on Sepharose 4B

The fraction numbers are those used in Fig. 1 and Table II. An aliquot was taken from each tube and submitted to the transformation experiment according to[15].

Fraction No	% of transformants			
	ade	leu	met	thr
20	0·16	0·32	0·21	0·25
22	0·24	0·43	0·27	0·31
27	0·20	0·41	0·25	0·27
53	0	0·01	0[a]	0·01
57	0	0[a]	0[a]	0[a]
65	0	0	0	0
69	0	0	0	0
75	0	0	0	0

[a] Less than 0·01%.

volume of ethanol. The crude DNA was dissolved in 9 ml of dilute SSC and 6 ml of 5M-NaCl in 0·025M sodium citrate was added to the obtained solution (procedure 4). For comparative experiments, a sample of DNA was also prepared by the modified procedure of Marmur[5]; still another sample was prepared by following the same method up to the first precipitation with ethanol (procedures 7 and 6, respectively).

Analytical examination of the DNA preparation. The absorbance of the fractions at 260 nm was measured in Unicam SP 700 spectrophotometer. The thermal denaturation curves were determined in SSC by the continuous method[10]. The radioactivity was measured with 0·1 ml aliquots of the sample placed on aluminum planchets in a gas-flow counter (Frieseke und Hoepfner). The sedimentation analysis was effected in Spinco-Model E analytical ultracentrifuge at 42 040 rev./min. The molecular weights were determined from the obtained sedimentation coefficients $s_{20,w}$ by using the equations of Eigner and Doty[11]. The quantity of DNA in the preparations was determined from the values of absorbance at 260 nm and by the color test using diphenylamine[12]. The content of contaminating RNA was determined by the orcinol method[13] and the content of proteins by the method of Lowry[14].

Determination of transforming activity of DNA. The transformation experiments with *B. subtilis* SMYW DNA were carried out according to Šrogl[15]. A quadruple *B. subtilis* mutant ade$^-$, leu$^-$, met$^-$, thr$^-$ served as recipient strain.

RESULTS AND DISCUSSION

The *B. subtilis* lysate is resolved into two parts by gel filtration on Sepharose 4B (Fig. 1). The first peak of the elution profile (*I*) contains relatively pure highly polymerous DNA whose molecules exceed in their size the exclusion limit of the gel used. The range of analytical values obtained with ten preparations isolated as described above is the following: $s_{20,w}$ 30·1–31·7; λ_{max} 259–261; λ_{min} 232–235; A_{max}/A_{min} 1·85–1·99 and A_{max}/A_{280} 1·71–1·88; T_m 89·7–91·3°C; protein content 2–3%, traces of RNA. The second peak contains the remaining cell components and lyso-

TABLE IV

Certain Analytical Values of DNA Samples Prepared by Different Modifications of Standard Isolation Procedure (Table I)

Sample No	λ_{max}	λ_{min}	A_{max}/A_{min}	A_{max}/A_{280}	% of proteins	$s_{20,w}$	m.w.
1	259	233	1·99	1·87	2·2	31·1	$2{\cdot}06 \cdot 10^7$
2	259	234	1·95	1·86	2·3	30·5	$1{\cdot}96 \cdot 10^7$
3	262	235	1·93	1·85	1·9	29·7	$1{\cdot}83 \cdot 10^7$
4	263	238	1·74	1·68	2·1	28·5	$1{\cdot}66 \cdot 10^7$
5	262	233	1·93	1·85	1·1	27·4	$1{\cdot}50 \cdot 10^7$
6	262	235	1·89	1·65	2·9	—	—
7	259	230	2·29	2·08	0·7	29·3	$1{\cdot}77 \cdot 10^7$

zyme which has been added. This second peak can be resolved further by a fine separation into additional components (Fig. 1). The distribution of some analytical values across the two peaks obtained is given in Table II.

Most of the experiments were carried out on a 3 . 80 cm column at a flow rate of 0·4—1·2 ml/min. The changes in the flow rate did not affect the course of the elution. The maximum sample volume which could be applied to the column was 50 ml (lysate of 7 g of wet cells). A larger volume of the sample resulted in impaired filtration whereas at higher concentration of the cells the lysis was incomplete and an undesired increase of the viscosity of the solution was observed. Small quantities of the sample, up to 2 g of wet cells (15—16 ml of lysate) can be separated on a 2·5 . 40 cm column at a flow rate of 0·4—0·5 ml/min. The fact that the two peaks described above

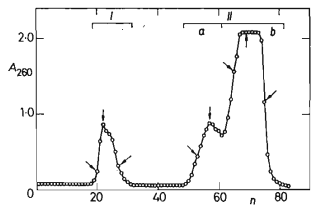

FIG. 1

Lysate of *B. subtilis* SMYW prepared by Standard Procedure, After Gel Filtration on Sepharose 4B Column (3 . 80 cm)

Flow rate 0·40 ml/min, fraction volume 10 ml. The arrows designate fractions used for detailed analysis and transformation experiments.

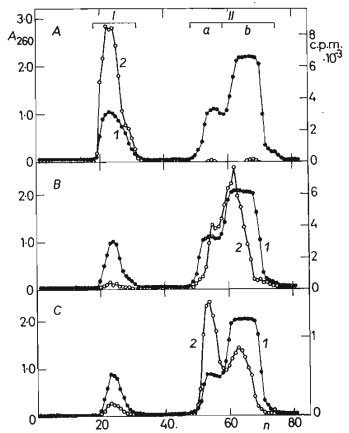

FIG. 2

Lysate of *B. subtilis* SMYW, Prepared by Standard Procedure, after Gel Filtration on Sepharose 4B Column (3 . 80 cm)

Flow rate 0·40 ml/min, fraction volume 10 ml. A culture labelled with [^{3}H]-thymidine, B with [^{3}H]-uridine, C with mixture of amino acids containing ^{14}C. *1* absorbance at 260 nm *2* radioactivity (c.p.m.).

are separated in the column indicates that the column can be used for the separation of the given quantity of the lysate. The increase of the bed volume or of the column dimensions while the volume of the sample remains constant is without any effect on the quality of the final preparation.

In an effort to verify the distribution of DNA, RNA, and proteins during gel filtration of the *B. subtilis* lysate, we made experiments with cultures with incorporated [^{3}H]-thymidine, [^{3}H]-uridine, and a mixture of ^{14}C-amino acids respectively (Fig. 2). We found that DNA was contained practically entirely in peak I (Fig. 2*a*), in accordance with the results of the transformation experiments (Table III). By contrast, RNA is contained in peak II (Fig. 2*b*), and so are the proteins (Fig. 2*c*). Interest deserves the distribution of proteins in peak II: part IIa represents predominantly cellular proteins whereas part IIb, containing considerably more mass, is probably for the most part the lysozyme added. This is indicated also by the observation that the difference between parts IIa and IIb is disappearing when the cells are lyzed by sodium dodecyl sulfate only; the mass of entire peak II is smaller than that of peak I, compared to experiments in which lysozyme is used.

As obvious from Table II, which shows the distribution of certain analytical values across peaks I and II, the quality of the final DNA preparation is markedly better when the tail fractions of peak I are removed before the pooling of fractions. Another procedure of improving the quality of the DNA preparation is its rechromatography on the MAK-column (ref.[16], removal of residual proteins). The transforming activity of the selected fractions from peaks I and II (Fig. 1) is summarized in Table III.

When characterizing the isolation methods described we tried to combine certain operations to improve the quality of the resulting DNA preparation. Some of these experiments are described schematically in Table I. The corresponding analytical values for DNA preparations obtained from the first peak of the gel filtration elution profile are given in Table IV. On the whole we can say that the standard procedure itself, as described in Experimental, cannot be improved markedly by any of the operations given. The use of pronase significantly improves the deproteinization, yet the molecular weight of the resulting preparation is decreased at the same time. Moreover, the sample is considerably contaminated by another protein and this fact must be taken into account before the size of the column is chosen. When pronase is used in combination with the extraction of the sample by the mixture chloroform–isoamyl alcohol and with ethanol precipitation, the deproteinization is considerably improved yet DNA comes into undesirable contact with organic solvents resulting in an additional decrease of molecular weight of the final preparation and in an unrequired prolongation of the isolation period. A positive fact is that both lysozyme and sodium dodecyl sulfate can be used for the lysis of the cells; this is important when the latter isolation method is used for bacteria resistant to lysozyme. The DNA preparations obtained by all these modified procedures examined showed transforming activity.

REFERENCES

1. Kay E. P. M., Simmons N. S., Dounce A. L.: J. A. Chem. Soc. *74*, 1724 (1952).
2. Kirby K. S.: Biochem. J. *66*, 495 (1957).
3. Marmur J.: J. Mol. Biol. *3*, 208 (1961).
4. Williamson R.: Anal. Biochem. *32*, 158 (1969).
5. Zadražil S., Mach O., v. Reid E.: *Separation with Zonal Rotors*, p. 91. Wolfson Bioanalytical Centre, University of Surrey, Guilford 1970.
6. Loeb J. E., Chauveau J.: Biochim. Biophys. Acta *182*, 225 (1969).
7. Pivec L., Štokrová J.: FEBS Letters *14*, 157 (1971).
8. Spizizen J.: Proc. Natl. Acad. Sci. US *44*, 1072 (1958).
9. Zadražil S., Jurovčík M., Fučík V.: This Journal *37*, 3148 (1972).
10. Boublík M., Pivec L., Šponar J., Šormová Z.: This Journal *30*, 2645 (1965).
11. Eigner J., Doty P.: J. Mol. Biol. *12*, 549 (1965).
12. Giles K. W., Myers A.: Nature *206*, 93 (1965).
13. Mejbaum W.: Z. Physiol. Chem. *258*, 117 (1939).
14. Lowry O. H., Rosenbrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).
15. Šrogl M.: *Thesis*. Czechoslovak Academy of Sciences, Prague 1969.
16. Šatava J., Zadražil S.: Unpublished results.

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