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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUES TO THE SEPARATION OF RIBOSOMAL PROTEINS OF DIFFERENT ORGANISMS

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SUMMARY

The ribosomal proteins from *Escherichia coli*, *Bacillus stearothermophilus* and *Methanococcus vannielii* were separated by size-exclusion, ion-exchange and reversed-phase high-performance liquid chromatography (HPLC), employing new column materials, different gradient systems, and preparative columns, respectively. The purity of the isolated proteins was analysed by one- and two-dimensional gel electrophoresis and by direct micro-sequencing.

The separation of ribosomal proteins could be improved by employing propanol gradients in combination with Vydac reversed-phase columns. From the *E. coli* ribosome, fifteen S and twenty-three L proteins were isolated in sequencer purity by this method. In addition, ion-exchange HPLC was proven to be useful for isolating ribosomal proteins under native conditions: six S proteins and sixteen L proteins from *E. coli* could be purified. Some of these proteins were not isolated by the reversed-phase procedures, *e.g.* proteins L9, L14 and L21.

INTRODUCTION

The *Escherichia coli*, *Bacillus stearothermophilus* and *Methanococcus vannielii* ribosomes contain two subunits, with about 20 and 30–40 proteins, respectively[1–3]. The proteins differ considerably in molecular mass and hydrophobicity. The classical methods of separating these protein mixtures include purification by gel filtration, CM-, DEAE- and phospho-cellulose ion-exchange chromatography[4]. These methods can only be employed if relatively large amounts of ribosomal subunits are available for protein purification. However, in the case of ribosomes from organisms, such as Archaebacteria, the amounts available are very limited, and new methods have to be applied to separate their proteins. High-performance liquid chromatographic (HPLC) methods are now widely applied to protein purifications[5–7], which were adapted to the separation of ribosomal protein mixtures[8–11]. Most purified proteins were obtained by employing reversed-phase techniques[8], but ion-exchange HPLC is also suitable for resolving this complex protein mixture[11].

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In this paper we show that similar results can be obtained with analytical and preparative reversed-phase columns. New gradients employing propanol instead of acetonitrile were applied, and their advantages are described. Further, we show that ion-exchange columns are useful for the purification of ribosomal proteins. We compare the different HPLC techniques for the separation of ribosomal proteins derived from different sources and discuss the advantages and limitations of the methods.

EXPERIMENTAL

Materials

30S and 50S subunit proteins from *Escherichia coli* K12, strain A19, were prepared and stored in 2% acetic acid, as described[4]. Growth of *Bacillus stearothermophilus* strain 799 and preparation of ribosomes were as described recently[2]. TP30 and TP50 from *Methanococcus vannielii* DSM 1224 were a gift from Dr. A. Böck and G. Schmid (University, Munich, F.R.G.).

The organic solvents employed as mobile phases for the HPLC separations were Uvasol or LiChrosolv grade (Merck, Darmstadt, F.R.G.). Trifluoroacetic acid (Fluka, Buchs, Switzerland) was redistilled from $CaSO_4 \cdot 0.5H_2O$ over a 30-cm column filled with glass rings. All other chemicals were pro analysis grade.

Size-exclusion chromatography

The size-exclusion HPLC system consisted of a HPLC pump, Model 6000A (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne injection valve, No. 7120 (Rheodyne, Berkeley, CA, U.S.A.) and a variable-wavelength UV detector, Jasco Uvidec-100-II (Biotronik, Munich, F.R.G.). The separation was performed on a TSK 2000 SW column, purchased from Varian, particle size 10 μm, pore size 125 Å, column size 500 × 7.5 mm I.D. The eluent, 0.1 *M* ammonium acetate buffer (pH 4.1), was made from ammonia and acetic acid.

Reversed-phase chromatography

For these separations, the liquid chromatograph 850 (DuPont, Wilmington, DE, U.S.A.), equipped with a variable-wavelength spectrophotometer, Model 852 (DuPont) and an automatic sampler (Wisp 710A, Waters Assoc.), was employed. Reversed-phase separations were performed on Vydac TP-RP [C_{18}, particle size 10 μm, pore size 300 Å, column sizes 250 × 4.6 mm I.D. and 250 × 8.0 mm I.D.; the support was from ChromPak (Müllheim, F.R.G.) and the steel columns from Knauer (Berlin, F.R.G.)] and on Ultrapore RPSC [short alkyl chains, particle size 5 μm, pore size 300 Å, column size 75 × 4.6 mm I.D., purchased from Beckman (Munich, F.R.G.)].

Proteins were eluted at 35°C with gradients made from buffer A [0.1% trifluoroacetic acid (TFA) in water] and buffer B (0.1% TFA in 2-propanol or acetonitrile).

Ion-exchange chromatography

The experiments with cation-exchange columns were performed with the following equipment: the gradient liquid chromatograph, Model 334, equipped with two HPLC pumps (Altex 110A); an Altex injection valve, Series 210; and a variable-wavelength UV detector, Model 165; all purchased from Beckman (Munich,

F.R.G.). The ion exchanger was Spherogel TSK IEX-530 CM [particle size 10 μm, pore size 125 Å, column size 300 × 4.6 mm I.D., purchased from Beckman (Munich, F.R.G.)]. All aqueous buffers were prepared with deionized water from a Mill-Q water purification system (Millipore, Bedford, MA, U.S.A.). The aqueous buffers were filtered through a 0.45-μm type HAWP filter and the organic solutions through Durapore HVPL 0.45-μm filter (both from Millipore). For more details see ref. 9.

Gel electrophoresis

One-dimensional electrophoresis was performed according to ref. 12 and two-dimensional electrophoresis according to ref. 13.

Micro-sequencing

Sequencing of the proteins was performed manually or automatically after attachment to *p*-phenylenediisothiocyanate-activated amino glass by the 4-N,N(dimethylamino)-azobenzene-4′-isothiocyanate–phenylisothiocyanate double coupling method[14,15]. The released 4-N,N-dimethylaminoazobenzene-4′-thiohydantoin amino acids were identified by thin-layer chromatography or isocratic HPLC[16]. Large proteins were degraded in the Berlin Sequencer by liquid-phase sequencing, employing a program with a repeated coupling and cleavage at each degradation cycle[17]. Identification of the released phenylthiohydantoin (PTH)-amino acids was made on-line[17], employing isocratic, recycling HPLC[18].

RESULTS AND DISCUSSION

Size-exclusion chromatography

The 30S and 50S subunit proteins from *E. coli* were separated on a TSK G 2000 SW column under conditions as reported[9]. Samples of 100–200 μg were injected for analytical runs and 5–10 mg for preparative runs.

The results on the size-exclusion HPLC column show that the separation of ribosomal proteins on TSK 2000 SW depends not only on the molecular mass but also on the net charge of the ribosomal proteins (for details of physical properties of these proteins see Table I).

Typical elution profiles are presented in Fig. 1a and b. The TP30 and TP50 proteins migrated in six and seven peaks, respectively. The first peak of TP30 contained largely the acidic proteins S1, S2 and S6. In the case of TP50, the first proteins that were eluted (independently of the molecular masses) were the acidic proteins, L7/L12 and L9. Negatively charged silanol-groups adsorb basic molecules and repulse acidic proteins. This may explain, why the acidic proteins are eluted earlier than the larger and more basic ones. After the elution of the negatively charged proteins the remaining proteins were separated according to size.

Ribosomal protein recoveries on TSK 2000 SW were very high, over 90% for most of the proteins. The pooled fractions contaning proteins were rechromatographed on reversed-phase columns to obtain pure proteins, as given below.

Reversed-phase chromatography

The most reproducible reversed-phase chromatographic separations of ribosomal proteins were obtained on Vydac TP-RP (self packed) and Ultrapore RPSC columns (packed by the distributor).

TABLE I

PHYSICAL PROPERTIES OF *ESCHERICHIA COLI* RIBOSOMAL PROTEINS

Protein	*Residues*	*Molecular mass*	*Hydrophobicity (%)**	*Charge*	
				*Positive (%)***	*Negative (%)****
S1	557	61159	31.0	14.5	18.5
S2	240	26613	30.9	15.8	13.8
S3	232	25852	28.5	20.7	10.8
S4	203	23137	25.7	22.2	11.8
S5	166	17515	28.8	15.6	7.8
S6	135	15704	25.2	17.7	22.2
S7	177	19732	27.0	19.8	10.7
S8	129	13996	30.3	15.5	11.7
S9	128	14569	25.7	22.7	10.1
S10	103	11736	30.9	19.5	13.6
S11	128	13728	21.8	20.2	6.2
S12	123	13606	21.9	25.2	5.7
S13	117	12968	26.6	24.8	10.2
S14	98	11191	23.5	26.5	9.2
S15	87	10001	26.6	24.1	11.6
S16	82	9191	29.3	20.7	11.0
S17	83	9573	33.7	24.1	13.2
S18	74	8896	21.7	25.7	6.8
S19	91	10299	27.5	27.5	8.8
S20	86	9553	19.7	27.9	5.8
S21	70	8369	20.0	34.3	12.8
L1	233	24599	28.7	15.5	10.7
L2	272	29730	23.1	23.2	7.7
L3	209	22258	29.2	16.2	10.0
L4	201	22087	30.5	17.5	12.0
L5	178	20171	30.9	18.0	13.5
L6	176	18831	26.8	16.5	10.3
L7	120	12220	29.2	11.6	18.3
L8	=	L7 + L10			
L9	148	15696	31.1	13.5	13.6
L10	165	17737	27.8	15.8	12.7
L11	141	14874	27.0	13.4	8.6
L12	120	12178	29.2	11.6	18.3
L13	142	16019	26.0	22.6	10.5
L14	123	13541	33.4	19.5	9.0
L15	144	14981	25.7	19.4	7.7
L16	136	15296	30.2	22.8	7.4
L17	127	14365	25.2	22.9	10.3
L18	117	12770	23.2	21.4	10.3
L19	114	13002	29.9	22.9	10.5
L20	117	13366	26.6	26.6	5.1
L21	103	11565	31.1	21.4	11.6
L22	110	12227	29.1	24.5	11.8
L23	99	11013	30.2	23.2	13.1
L24	103	11185	29.2	22.3	10.7
L25	94	10694	28.8	21.3	12.8
L26	=	S20			

TABLE I (*continued*)

Protein	*Residues*	*Molecular mass*	*Hydrophobicity (%)**	*Charge*	
				*Positive (%)***	*Negative (%)****
L27	84	8993	21.5	26.2	9.6
L28	77	8875	27.3	27.3	7.8
L29	63	7274	30.3	20.6	12.7
L30	58	6411	31.0	22.4	8.6
L31	62	6971	24.1	22.6	11.3
L32	56	6315	17.9	30.3	7.2
L33	54	6255	24.1	31.5	9.3
L34	46	5381	21.8	37.0	0

* Hydrophobicity values (%) are based on the mole percentages of Val, Ile, Leu, Phe, Met and Trp in the molecule.

** Positive charges are based on Arg, Lys and His.

*** Negative charges on the presence of Asp and Glu.

An amount of 30 μg TP30 or TP50 was injected for analytical runs and 2 mg for preparative runs (column size 250 × 4.6 mm I.D.) or 10 mg (column size 250 × 8.0 mm I.D.). Two different gradient systems were applied:

(a) TFA and acetonitrile;

(b) TFA and 2-propanol.

Four S proteins could be isolated in purified form on the Vydac column with acetonitrile[9]; propanol gradients are superior, as shown in Fig. 2: fifteen S proteins obtained are of sequencer purity. Further, this solvent is more suitable for the separation of ribosomal proteins under native conditions and it is less dangerous. The purity of the pooled fractions of Fig. 2 was further tested by two-dimensional gel electrophoresis.

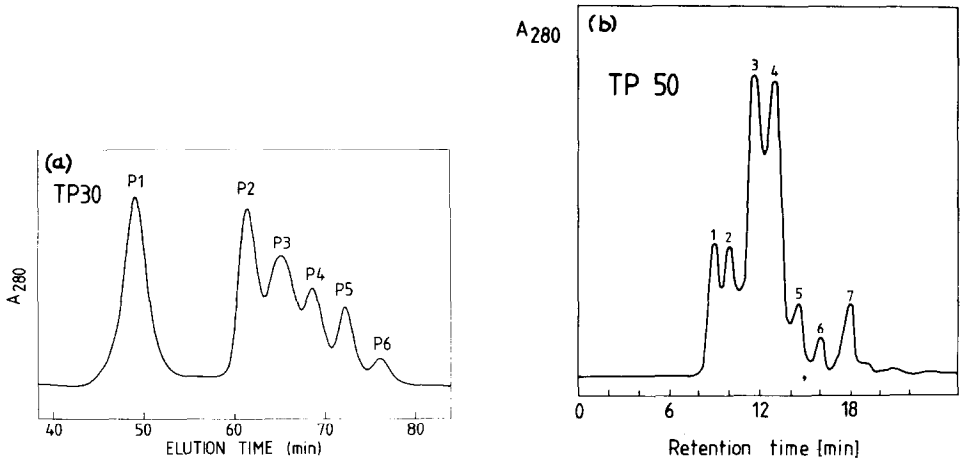

Fig. 1. Group separation of 30S (a) and 50S (b) ribosomal proteins from *E. coli* by HPLC on TSK 2000 SW. An amount of 1 mg protein mixture was injected in 100 μl 2% acetic acid in water. The eluent was 0.1 *M* ammonium acetate, pH 4.1. Flow-rates: 0.2 ml/min (30S) and 1.0 ml/min (50S) at room temp.; the eluate was monitored at 280 nm, 0.02 a.u.f.s.

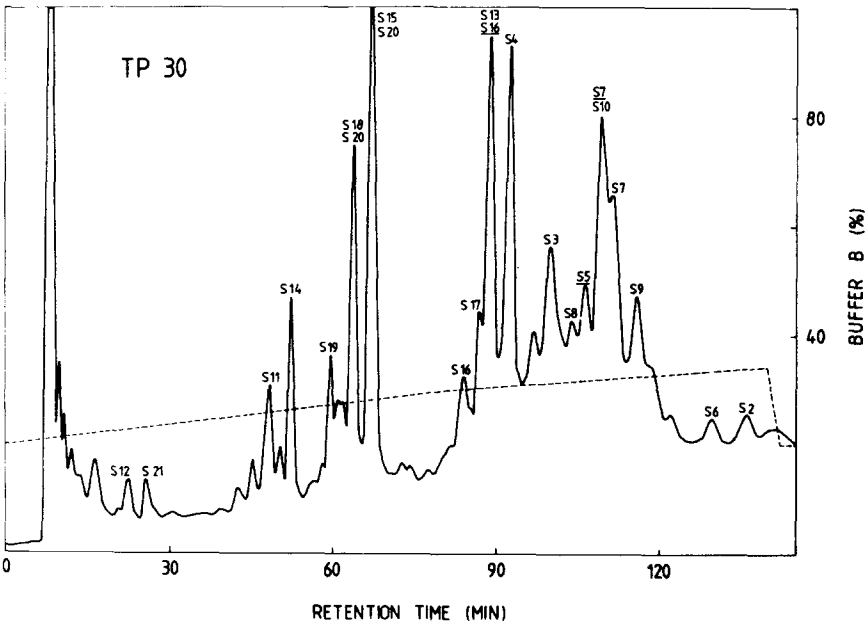

Fig. 2. Purification of 30S ribosomal proteins from *E. coli* on Vydac TP-RP. An amount of 1 mg TP30 was injected in 100 μl 2% acetic acid in water. The eluents were: buffer A, 0.1% aqueous TFA; buffer B, 0.1% TFA in 2-propanol. Linear gradient: 20% B to 30% B in 80 min, 30% B to 35% B in 60 min, 35% B to 20% B in 5 min, and reconditioning for 30 min at initial conditions. The eluate was monitored at 220 nm, 0.64 a.u.f.s.; flow-rate 0.5 ml/min, temperature 35°C.

Propanol was also tested for the separation of 50S proteins on Vydac TP-RP and Ultrapore RPSC columns (see Fig. 3). Also, in this case, this solvent was superior to acetonitrile: a total of twenty-three *E. coli* L proteins were found to be pure. Further, we observed more protein complexes in the fractions from runs with acetonitrile than with propanol (see Fig. 3). For example, protein L2 migrated with other proteins; it was found in seven different fractions and could not be isolated in purified form with acetonitrile. On the other hand, propanol gradients produced pure protein L2.

The use of acetonitrile as an organic modifier often causes proteins to unfold and denature. Changes in secondary or tertiary structure alters the retention time of the proteins. This problem can be avoided using propanol as eluent. This suggests that the chromatographic behaviour of proteins depends not only on amino acid sequences, but also on their secondary and tertiary structure.

In Fig. 3 the results of the 50S separation on analytical (4.6 mm I.D.) and preparative (8.0 mm I.D.) Vydac columns are presented. Fig. 4 represents the isolation on Ultrapore RPSC. The same elution profile was obtained for analytical and preparative runs, but it was necessary to use higher flow-rates in case of the preparative columns. The majority of the experiments were carried out on Vydac columns, as it is possible to obtain this packing material for self-packing of analytical or preparative columns. This gives reproducible results over a long period of time. The

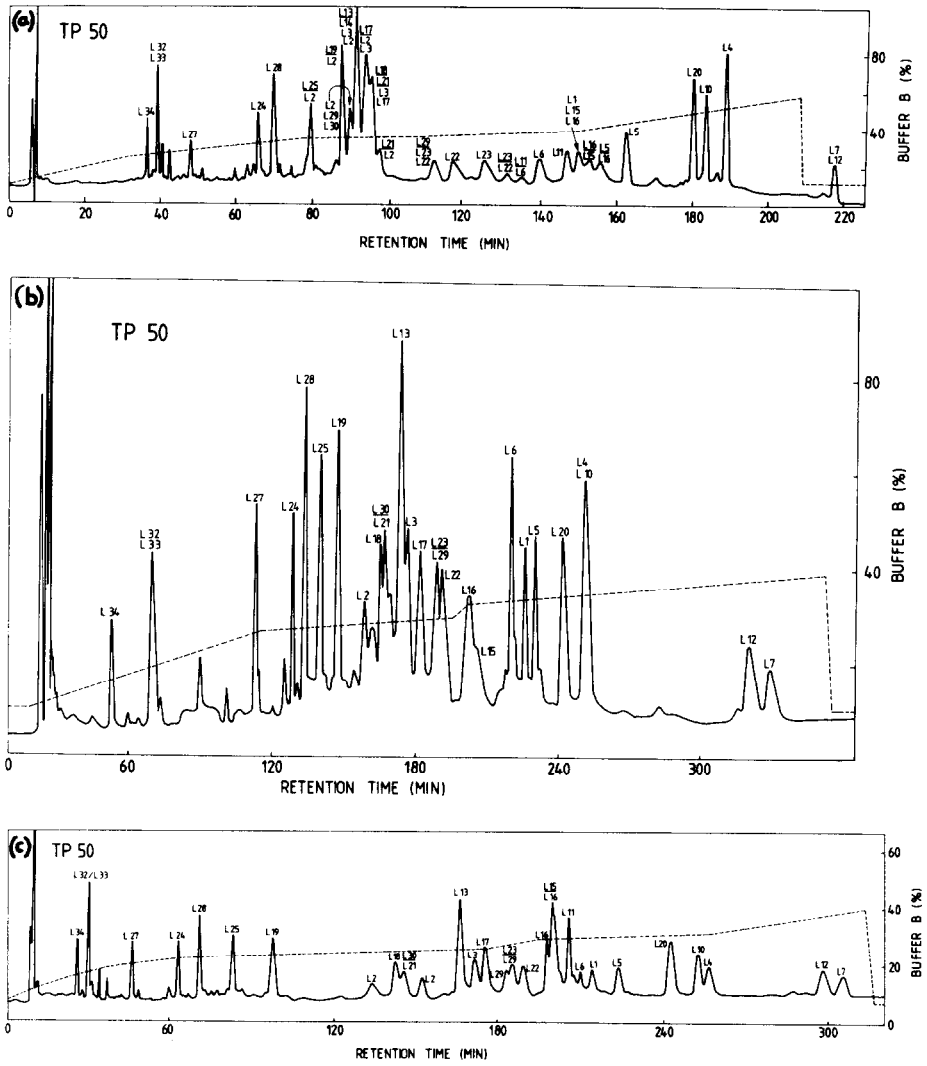

Fig. 3. Separation of 50S ribosomal proteins from *E. coli* on Vydac TP-RP. (a) Analytical column, 250 × 4.6 mm I.D.; 2 mg TP50 was injected in 200 μl 2% acetic acid. The eluents were: buffer A, 0.1% aqueous TFA; buffer B, 0.1% TFA in acetonitrile. The gradient applied was: 10% B to 25% B in 30 min, 25% B to 35% B in 45 min, 35% B to 36% B in 30 min, 36% B to 40% B in 40 min, 40% B to 55% B in 60 min, 55% B to 10% B in 5 min, reconditioning for 30 min at initial conditions; the eluate was monitored at 220 nm, 0.64 a.u.f.s. Flow-rate 0.5 ml/min, temperature 35°C; recorder speed 5 mm/min. (b) Column, sample load, flow-rate, monitoring and temperature as in (a). Eluents as in (a) except that acetonitrile was replaced by 2-propanol. Recorder speed was 1 mm/min. The gradient applied was: 10% B to 27% B in 100 min, 27% B to 30% B in 80 min, 30% B to 33% B in 5 min, 33% B to 38% B in 170 min, 38% B to 10% B in 5 min and reconditioning for 30 min at initial conditions. (c) Semipreparative column, 250 × 8.0 mm I.D.; 10 mg TP50 dissolved in 500 μl 2% acetic acid were injected. The buffers were as in (b). The gradient applied was: 10% B to 27% B in 100 min, (curve −3, Dupont 850 programmer) followed by a linear gradient 27% B to 30% B in 80 min, 30% B to 33% B in 10 min, 33% B to 35% B in 60 min, 35% B to 40% B in 60 min, 40% B to 10% B in 5 min. The eluate was monitored at 220 nm, 1.28 a.u.f.s.; flow-rate 1.0 ml/min, temperature 35°C; recorder speed was 2 mm/min.

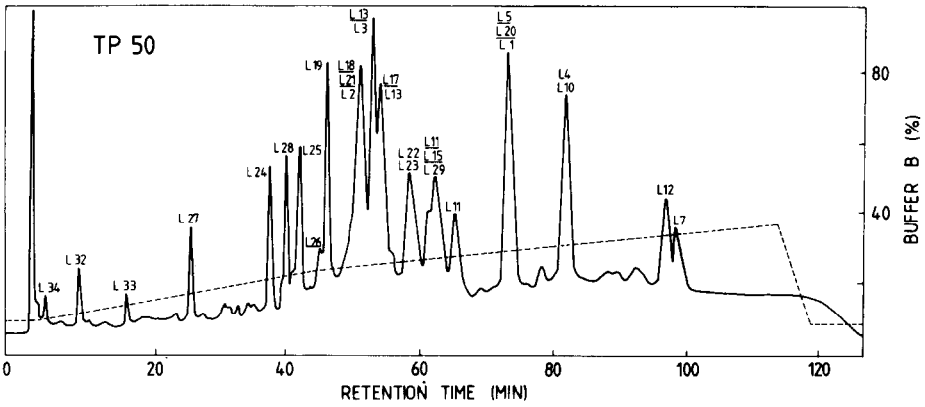

Fig. 4. Purification of TP50 from *E. coli* on Ultrapore RPSC. Sample load, eluent, measurements, flow-rate and temperature as in Fig. 3b. The gradient applied was: 10% B to 25% B in 40 min, 25% B to 40% B in 70 min, 40% B to 10% B in 5 min.

application to the separation of ribosomal proteins from *B. stearothermophilus* and *M. vannielii* on the Vydac columns is illustrated in Figs. 5 and 6.

Reversed-phase HPLC with volatile buffers allows direct micro-sequencing[9,10]. Further, the fractions obtained could be dried in the cup of the liquid-phase sequencer, thus avoiding losses that might occur due to precipitation or transfer steps.

Ion-exchange chromatography

In order to isolate those proteins which could not be obtained pure from re-

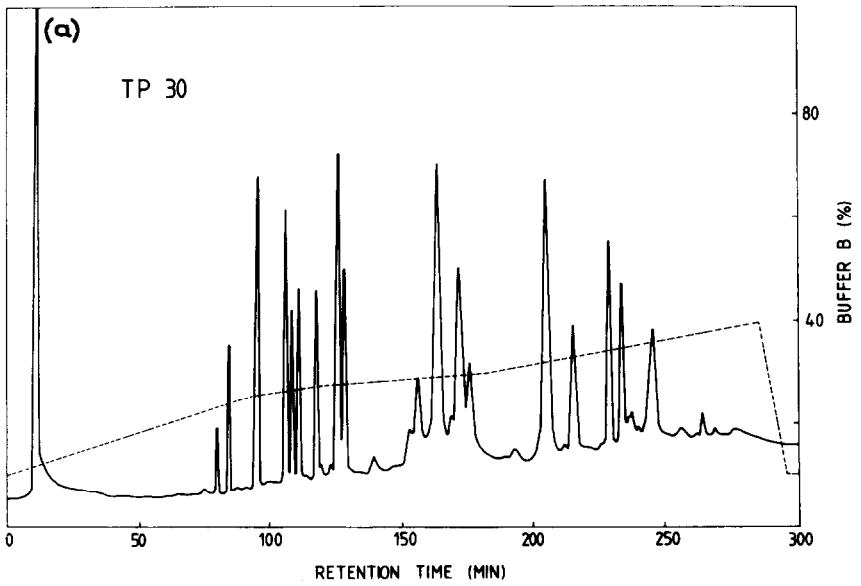

Fig. 5.

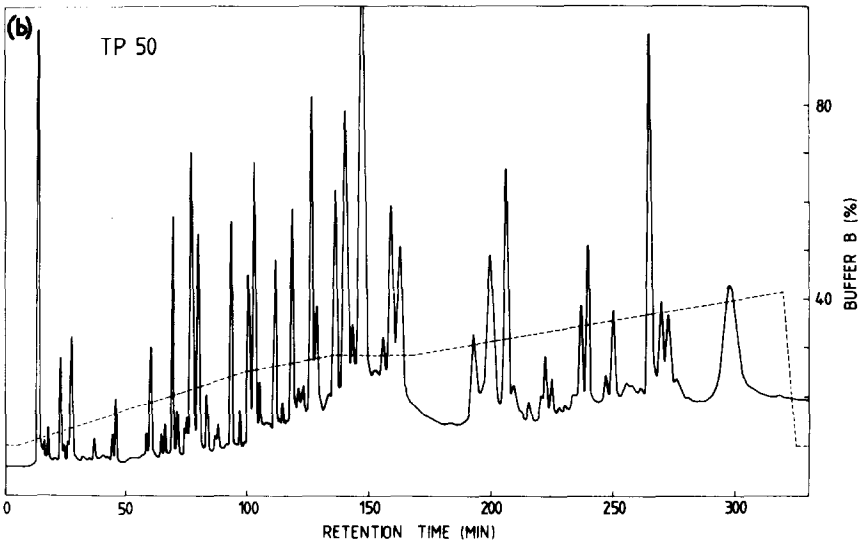

Fig. 5. Separation of *Bacillus stearothermophilus* proteins on Vydac TP-RP. The purification conditions were as in Fig. 2 for 30S (a) and Fig. 3b for 50S (b). The gradient applied for TP30 was: 10% B to 25% B in 90 min, 25% B to 27% B in 20 min, 27% B to 30% B in 75 min, 30% B to 40% B in 100 min, 40% B to 10% B in 10 min. Gradient for TP50 was: 10% B to 25% B in 90 min, 25% B to 29% B in 40 min, hold at 29% B in 30 min, 29% B to 31% B in 30 min, 31% B to 41% B in 130 min, 42% B to 10% B in 5 min.

versed-phase or size-exclusion columns, we applied ion-exchange HPLC with salt gradients. This is important for isolating ribosomal proteins under native conditions. High urea concentrations have to be chosen to guarantee sufficient recoveries of the proteins; 5 *M* urea was found optimal. Ion-exchange chromatography complemented the other methods, and the combination of all three techniques enabled the isolation of almost all ribosomal proteins.

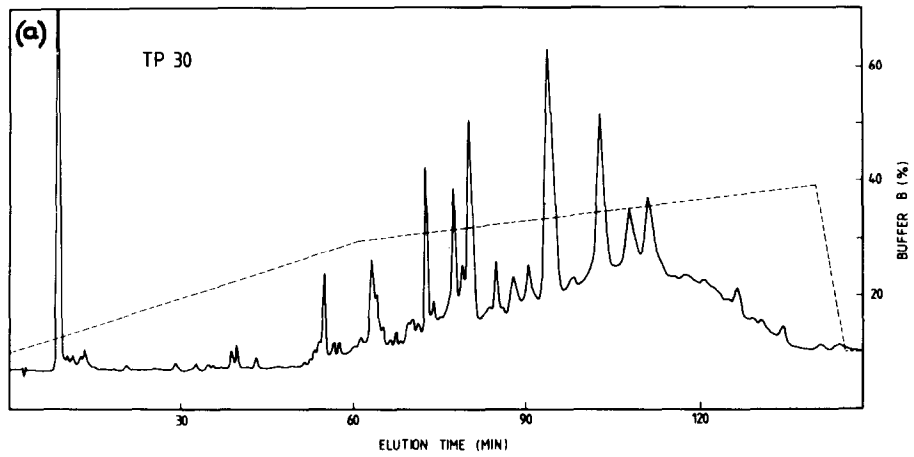

Fig. 6.

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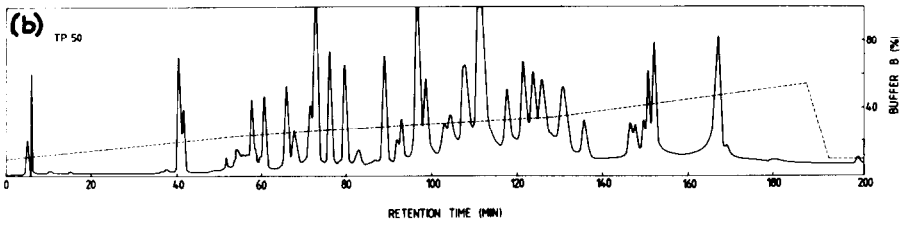

Fig. 6. Separation of TP30 (a) and TP50 (b) ribosomal proteins from *Methanococcus vannielii* on Vydac TP-RP. Sample load, eluents, measurements, flow-rate and temperature as in Fig. 2 (30S) and Fig. 3b (50S). The linear gradient applied for TP30 was: 10% B to 30% B in 60 min, 30% B to 33% B in 30 min, 33% B to 40% B in 50 min, 40% B to 10% B in 5 min. The gradient applied for TP50 was: 10% B to 25% B in 60 min, 25% B to 30% B in 30 min, 30% B to 35% B in 40 min, 35% B to 55% B in 60 min, 55% B to 10% B in 5 min. The correlation of the individual proteins to the different peaks obtained in the chromatogram, their purity check and N-terminal sequence will be described in a separate paper[19].

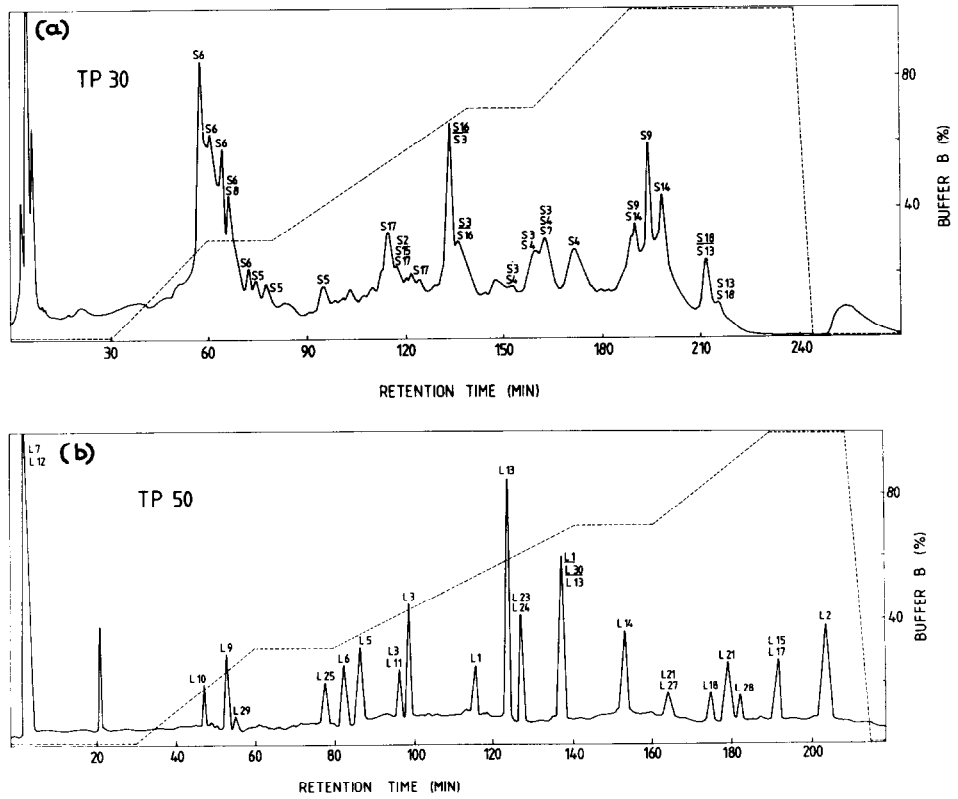

Fig. 7. Purification of 30S (a) and 50S (b) proteins from *E. coli* on Ultrasphere TSK IEX-530 CM column. Amounts of 3 mg TP50 and 2 mg TP30 were injected in 200 µl 2% acetic acid in water. The eluents were: buffer A, 0.01 *M* sodium phosphate in 5 *M* urea, pH 7.0; buffer B was made from buffer A and 0.3 *M* potassium chloride. The gradient applied for TP30 was: hold at 0% B in 30 min, 0% B to 30% B in 30 min, hold at 30% B in 20 min, 30% B to 70% in 60 min, hold at 70% B in 20 min, 70% B to 100% B in 30 min, hold at 100% B in 50 min, 100% B to 0% B in 5 min and reconditioning for 60 min at 0% B. The gradient employed for separation of TP50 was the same besides a hold at 100% B for 20 min. Measurements were made at 280 nm, 0.02 a.u.f.s.; flow-rate 1.0 ml/min; temperature ambient.

Fig. 7 demonstrates the separation of ribosomal proteins from *E. coli* on the Ultrasphere TSK IEX-530 CM column. Protein fractions obtained from the CM-TSK column were precipitated with 50% trichloroacetic acid and then identified by one- or two-dimensional gel electrophoresis and manual micro-sequencing.

CONCLUSIONS

The results demonstrate that reversed-phase and ion-exchange HPLC are very useful means of separating ribosomal proteins. Elution of these proteins with gradients made from propanol are superior to those made from acetonitrile or methanol. The purity of the protein fractions was investigated by one- and two-dimensional gel electrophoresis and by manual and automatic microsequencing. According to these criteria, eighteen proteins from the 30S subunits and thirty-one proteins from 50 S particles of *E. coli* were obtained pure by the described chromatographic techniques. The ion-exchange HPLC procedure, successfully applied in this paper to the separation of ribosomal proteins, resulted in six S and fourteen L proteins from *E. coli* which were purified to micro-sequence purity. Protein recoveries were calculated directly from amino acid analyses. Best recoveries were found after size-exclusion HPLC with up to 90% recovery. The yields after reversed-phase chromatography were lower, between 25 and 83%[9,10]. The final yields in case of the ion-exchange column were considerably lower due to further losses at the desalting step. This can be circumvented by the application of preparative HPLC runs, where more material is applied.

The elution of the ribosomal proteins from reversed-phase columns depends on hydrophobicity, protein size, and net charge (see Table I). The hydrophilic basic and small proteins are eluted first, whilst the acidic or hydrophobic ones are retarded. Reversed-phase chromatography is the most suitable method for the separation of ribosomal proteins. UV detection at 220 nm allows very high sensitivity at the picomole level. The limitation of this method is that the use of acetonitrile as an organic modifier causes migration of some proteins in multiple peaks. On the other hand, purification of some proteins is possible only by ion-exchange chromatography. This chromatographic technique is disadvantageous, since the lowered sensitivity at 280 nm does not allow detection of some proteins, which contain little or none of the aromatic amino acids tryptophan and tyrosine. We observed that some small and very basic proteins, L31–L34 and S19–S21, were not eluted by applying salt gradients (0–0.3 *M* potassium chloride) of classical ion-exchange columns. We found, that ribosomal proteins are adsorbed more strongly on HPLC column materials than on CM-cellulose or CM-Sephadex. Consequently, buffers of higher ionic strength are necessary for eluting these protein mixtures.

Ion-exchange chromatography is more suitable for the isolation of functionally active proteins or for rechromatography after reversed-phase separation. The combination of the three techniques enables purification of most of the ribosomal proteins to sequencer purity.

The separation methods elaborated for *E. coli* ribosomal proteins were applied to the purification of proteins from other ribosomal sources. The results show that the gradient must be optimised for each protein mixture. Examples of the separation of ribosomal proteins, derived from *B. stearothermophilus* and methanogenic bac-

teria, are presented in Figs. 5 and 6. Recently, these procedures have been extended to the isolation of ribosomal protein of different bacteria, and the proteins were subjected to N-terminal sequence studies by means of a liquid- and solid-phase sequencer. These results will be presented elsewhere[19].

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