Purification of *Escherichia coli* 50 S ribosomal proteins by high performance liquid chromatography

Roza Maria Kamp and Brigitte Wittmann-Liebold

Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, D-1000 Berlin 33 (Dahlem), FRG

Received 30 November 1983

The 50 S subunit proteins from the *Escherichia coli* ribosome were purified by size-exclusion, ion-exchange or reversed phase high performance liquid chromatography (HPLC) avoiding any precipitation or desalting procedures during isolation. Best resolution of this complex protein mixture was achieved by reversed phase chromatography on supports with short alkyl chains and C18 hydrocarbon-bonded phases; 23 out of the 32 proteins from the 50 S subunit were purified as shown by two-dimensional gel electrophoresis, amino acid analysis and direct micro-sequencing. Protein recoveries varied between 25 and 84% as determined by amino acid analysis. Ribosomal proteins of other organisms can be separated under similar conditions.

Escherichia coli *ribosome* *50 S subunit protein* *High performance liquid chromatography* *Protein recovery* *Micro-sequencing*

1. INTRODUCTION

The *Escherichia coli* ribosome contains 21 and 32 different proteins within the small and large subunits, respectively [1]. The usual isolation procedures for these proteins include extraction from the subunits with salt or acetic acid, gel filtration, CM-cellulose chromatography and repurification by chromatography on DEAE- or phosphocellulose [2]. Other methods make use of separations by salt extraction in the absence of urea, with the aim of obtaining proteins in their native conformation [3].

New support materials for the separation of high M_r components by high performance liquid chromatography techniques (HPLC) make the rapid separation of proteins possible (review [4]). Separation times can be reduced from days or weeks to hours, and micro-scale separations can be achieved. Recently, this technique was applied to the separation of ribosomal proteins [5,6]. However, in these studies protein yields were estimated by colourimetric measurements only, which are less reliable than amino acid analyses.

We have previously described HPLC-methods for the purification of most of the 30 S ribosomal proteins of the *Escherichia coli* ribosome and determined their purity and protein recoveries by direct micro-sequencing and amino acid analysis [7]. Here we present purification methods for the 50 S subunit proteins.

2. MATERIALS AND METHODS

50 S subunits were isolated from *E. coli* K12, strain A19, and were kindly provided by Dr H.G. Wittmann. Total proteins (TP50) were extracted by acetic acid treatment, dialysed against 20% and 2% acetic acid and directly injected into the HPLC-columns from this solution [7].

Size-exclusion experiments were made on a TSK G2000 SW column (particle diameter 10 μm, pore

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

size 130 Å, column size 300×7.5 mm, purchased from Bio-Rad, Munich) and isocratic elution with dilute ammonium acetate buffers at pH 4.1 and a flow rate of 1.0 ml/min. The maximum amount of protein loaded onto the TSK-column was 5 mg TP50 per injection. The equipment was as given [7].

Reversed phase HPLC was performed employing the following columns:

(i) Vydac TP-RP (C18, particle diameter 5 μm, pore size 300 Å, column size 250×4.6 mm, purchased from Chrompack, Berlin);
(ii) Ultrapore RPSC (short alkyl chain, particle diameter 5 μm, pore size 300 Å, column size 75×4.6 mm, purchased from Beckman, Munich).

In the reversed phase experiments the proteins were eluted with linear gradients made from 0.1% TFA in water and 0.1% TFA in acetonitrile or propanol-2, respectively. For these separations the Liquid Chromatograph 850 (from DuPont Instruments, Wilmington, DE) was used, equipped as in [7]. Flow rates were kept at 0.5–1.0 ml/min and the temperature at 35°C. Measurements were made at 220 nm in the sensitivity range of 0.08–0.64. Protein load for the analytical runs was 20 μg TP50, for preparative runs 1–2 mg total protein mixture.

Fractions of 0.5 ml were collected (Ultrorac 7000, LKB, Bromma) in 1.5-ml plastic tubes (Sarstedt, Nürnbrecht) and stored frozen. As the buffers contained only traces of salts, the protein-containing fractions could be dried quickly (3–4 h in a Speed Vac Concentrator) and identified by SDS-polyacrylamide gel electrophoresis as in [8] and two-dimensional polyacrylamide gel electrophoresis as in [9,10] without recourse to desalting steps or acetone precipitations which often cause loss of protein or insolubilization. Direct micro-sequencing of the protein fractions was performed by the manual DABITC/PITC double coupling method [11], and the ability of the purified proteins to be sequenced was further checked by automated liquid phase degradations in the new Berlin sequencer [12]. For more details see [7].

3. RESULTS AND DISCUSSION

3.1. *Size-exclusion HPLC*

Group separations of the 50 S subunit proteins were made by size-exclusion chromatography on TSK G2000 SW at pH 4.1 under isocratic conditions with a flow rate of 1.0 ml/min. The TP50 mixture gave 7 reproducible peaks (not shown) which were further separated by either of the reversed phase HPLC separations given below.

3.2. *Reversed phase HPLC*

Direct application of the 50 S total protein mixture to the reversed phase columns gave high resolutions, especially with the Vydac TP-RP and Ultrapore RPSC columns (see section 2). However, other reversed phase columns, such as the SynChropak RP-P C18 [6] or Protesil 300 Octyl column (purchased from Whatman, see [13]), although resolving the protein mixture into distinct peaks, resulted in less pure protein fractions, as shown by micro-sequencing.

Different gradient systems made from 0.1% TFA and acetonitrile or propanol-2 were tested. The proteins in the fractions were identified by two-dimensional polyacrylamide gel electrophoresis and their purity checked by manual micro-sequencing. Those fractions which were found pure according to these criteria were further subjected to amino acid analysis and automatic Edman degradation in a liquid phase sequencer.

Good resolution of the 50 S proteins for the Vydac-column was obtained with acetonitrile in a shallow gradient of 3 h 40 min. Eleven proteins were isolated in purified form according to the above criteria, namely proteins L34, L27, L24, L28, L22, L23, L6, L11, L20, L10, and L4 (not shown). Six other proteins were highly enriched containing only traces of other proteins.

Best conditions for the Vydac-column were with the propanol-2 gradient as illustrated in fig.1. Sixteen proteins were isolated in purified form, namely proteins L34, L33, L32, L27, L24, L28, L25, L19, L2, L30, L6, L1, L5, L20, L12 and L7. The same buffers as above have been tested on the Ultrapore RPSC column. Separation with acetonitrile gradients gave 13 pure proteins (L34, L33, L32, L27, L24, L28, L25, L15, L20, L10, L4, L12 and L7, see fig.2) and that with propanol-2 gave 10 purified proteins (L34, L32, L33, L27, L24, L25, L26, L11, L12 and L7 (not shown).

3.3. *Protein recoveries*

Protein recoveries in these investigations were

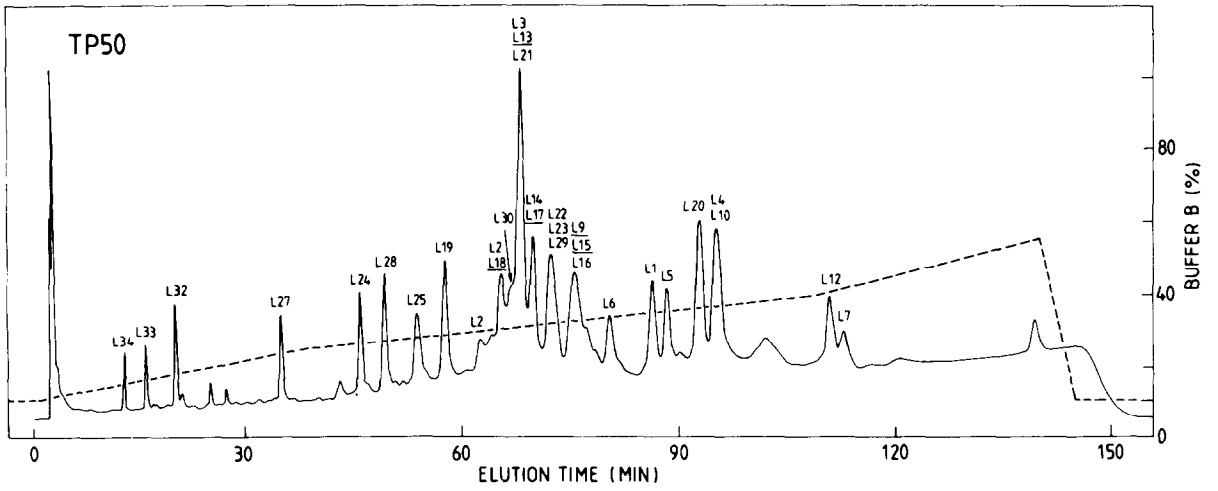

Fig.1. Purification of 50 S ribosomal proteins from *E. coli* by reversed phase HPLC on Vydac TP-RP. Two mg TP50 were injected in 200 μl of 2% acetic acid on Vydac TP-RP (5 μm particle size; 300 Å pore size; column size, 250×4.6 mm of self-packed column; 35°C; flow rate 1.0 ml/min). The eluent was: buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in propanol-2. The gradient applied was: 10% B to 25% B for 40 min; 25% B to 40% B for 60 min; 40% B to 55% B for 30 min; 55% B to 10% B for 5 min; reconditioning was made for 30 min at initial conditions. Measurements were made at 220 nm; range 0.64. The proteins were identified by two-dimensional polyacrylamide gel electrophoresis and micro-sequencing as indicated in the figure.

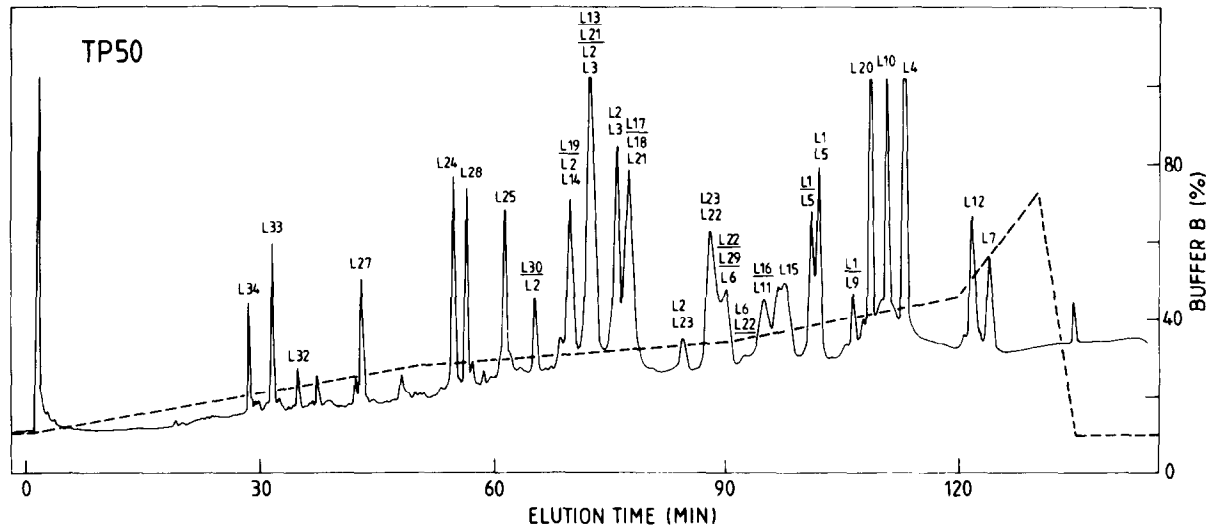

Fig.2. Purification of 50 S ribosomal proteins from *E. coli* by reversed phase HPLC on Ultrapore RPSC. Two mg TP50 were injected as described above into Ultrapore RPSC (5 μm particle size; 300 Å pore size; column size, 75×4.6 mm; 35°C; flow rate 0.5 ml/min). The eluent was: buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile. The gradient applied was: 10% B to 30% B for 50 min; 30% B to 37% B for 40 min; 37% B to 50% B for 30 min; 50% B to 80% B for 10 min; 80% B to 10% B for 5 min; and reconditioning for 30 min at initial conditions. Measurements were made at 220 nm, range 0.64. The identification of the proteins was done as given in fig.1.

Table 1
Recoveries of purified 50 S ribosomal proteins (*E. coli*)

Protein	Yield (%)	HPLC-column
L1	38	Vydac TP-RP
L2	27	Vydac TP-RP
L5	45	Vydac TP-RP
L6	41	Vydac TP-RP
L7	36	Vydac TP-RP
L7	25	Ultrapore RPSC
L11	50	Ultrapore RPSC
L12	33	Vydac TP-RP
L12	25	Ultrapore RPSC
L19	53	Vydac TP-RP
L20	55	Vydac TP-RP
L24	62	Vydac TP-RP
L24	51	Ultrapore RPSC
L25	68	Vydac TP-RP
L27	83	Vydac TP-RP
L27	74	Ultrapore RPSC
L28	59	Ultrapore RPSC
L28	53	Vydac TP-RP
L32	50	Vydac TP-RP
L33	70	Vydac TP-RP
L34	65	Vydac TP-RP

The yields (in %) are based on amino acid analysis of the fractions obtained after chromatography on Vydac TP-RP or Ultrapore RPSC; the elution gradient was made from 0.1% TFA and propanol-2 (see fig.1,2)

calculated from direct amino acid analysis. This gave more reliable results than those obtained by colourimetric determinations. The latter depend on the degree of specific staining which varies from protein to protein. Since volatile buffers for the elution were employed no desalting or precipitation was necessary to determine the protein yields.

The yields varied depending on the support and organic solvent employed. With shallower gradients better recoveries were observed. Total protein recoveries were found higher for short alkyl chain bonded phases, such as Ultrapore RPSC or LiChrosorb RP-2 (see [7]), than for supports coated with longer alkyl side chains, e.g. the Vydac TP-RP material (an C18 phase). On the other hand, resolution of the ribosomal TP50 mixture was better for the latter support. Furthermore, larger pore sizes of the supports gave higher resolution of the complex protein mixture and allowed to elute larger sized hydrophobic proteins. This makes the Ultrapore and Vydac material (both 300 Å) superior to the LiChrosorb RP-2 column (60 Å). Spherical shaped particles of similar size resolved the ribosomal protein mixture better than irregular shaped ones (for details see [7]).

The yields of the ribosomal 50 S proteins were in the range of 25–84%, see table 1, and were highest

Table 2
Recoveries of PTH-amino acid derivatives obtained by liquid-phase sequencing of TP50 proteins isolated by HPLC on Vydac TP-RP

Protein	Applied amounts (nmol/μg)		PTH-amino acid derivatives (in pmol) released in degradation cycle 1–16															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
L32	2.15	13.6	Ala	Val	Gln	Gln	Asn	Lys	Pro									
			337	264	195	197	126	n.d.	42									
L24	2.67	29.9	Ala	Ala	Lys	Ile	Arg	Arg	Asp	Asp	Glu	Val	Ile	Val	Leu	Thr	Gly	
			490	733	200	379	n.d.	n.d.	426	499	n.d.	466	180	260	375	n.d.	53	
L28	2.28	20.2	Ser	Arg	Val	Cys	Gln	Val	Thr	Gly	Lys	Arg	Pro	Val	Thr	Gly	Asn	Asn
			n.d.	n.d.	605	n.d.	250	349	n.d.	192	n.d.	n.d.	50	279	n.d.	190	106	149
L25	2.93	31.3	Met	Phe	Thr	Ile	Asn	Ala	Glu	Val	Arg	Lys	Glu	Gln	Gly	Lys	Gly	Ala
L20	2.28	30.5	Ala	Arg	Val	Lys	Arg	Gly	Val	Ile	Ala	Arg	Ala					
			1016	n.d.	916	400	n.d.	234	756	400	322	n.d.	360					

2 mg TP50 (4.31 nmol) were injected into the Vydac column and elution was performed as given in fig.1. The protein fractions obtained (yields see table 1) were directly applied into the cup of the sequencer (without polybrene) and dried by applying low vacuum under the addition of nitrogen at low speed. After degradation (performed in a cup of 20 mm inner diameter at 2700 rev./min, see [12]) and automatic conversion the released amino acid derivatives were determined on-line by HPLC; injected were 1/10–1/4 aliquot portions into LiChrosorb RP-8 (5 μm); elution was done under isocratic, recycling conditions with 32% acetonitrile in water and 9 mM ammonium acetate at pH 5.3 and measurements performed at 254 nm

for the small hydrophilic proteins. In general, due to the better separation of the TP50 protein mixture, the purified proteins were obtained in larger amounts from the Vydac-column than from Ultrapore RPSC, although the total protein recovery was higher from the Ultrapore material.

Twenty-three proteins of sequencer purity can be obtained by direct injection of the total 50 S protein mixture into two different reversed phase columns. Table 2 presents sequencer results for some of the proteins obtained by isolation on the Vydac column.

On the columns presented here, the elution of the various proteins is dictated by the net charge and hydrophobicity of the proteins with the basic and small proteins eluting first whilst the acidic or hydrophobic proteins, such as L7/L12 or L20 and L10, elute later.

The HPLC-methods described here allow direct isolation of purified proteins in a few hours and in yields higher than obtainable by any of the conventional approaches. Therefore, topographical investigations on the ribosome, protein-chemical studies after chemical modifications and sequence comparison studies of proteins from different organisms are greatly facilitated.

REFERENCES

[1] Wittmann, H.G. (1982) Annu. Rev. Biochem. 51, 155–183.
[2] Wittmann, H.G. (1974) in: Ribosomes (Nomura, M. et al. eds) pp. 93–114, Cold Spring Harbor, Long Island, NY.
[3] Dijk, J. and Littlechild, J.A. (1979) Methods Enzymol. 59, 481–502.
[4] 2nd Int. Symp. on HPLC (1983) J. Chrom. 266.
[5] Dalrymple, P.N., Gupta, S., Regnier, F. and Houston, L.L. (1983) Biochim. Biophys. Acta 277, 157–162.
[6] Kerlavage, A.R., Weitzmann, C.J., Hasan, T. and Cooperman, B.S. (1983) J. Chrom. 266, 225–237.
[7] Kamp, R.M., Yao, Z.-J., Bosserhoff, A. and Wittmann-Liebold, B. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 1777–1793.
[8] Laemmli, U.K. and Faure, M. (1973) J. Mol. Biol. 80, 575–599.
[9] Kaltschmidt, E. and Wittmann, H.G. (1970) Anal. Biochem. 36, 401–412.
[10] Geyl, D., Böck, A.R. and Isono, K. (1981) Gen. Genet. 181, 309–312.
[11] Chang, J.Y., Brauer, D. and Wittmann-Liebold, B. (1978) FEBS Lett. 93, 205–214.
[12] Wittmann-Liebold, B. (1983) in: Modern Methods in Protein Chemistry; (H. Tschesche, ed) pp. 229–266, Walter de Gruyter, Berlin, New York.
[13] Ferris, R.J., Cowgill, C.A. and Traut, R.R. (1983) Fed. Proc. 42, 2426.