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Note

Purification of core salt-extracted *E. coli* 30S ribosomal proteins by high-performance liquid chromatography under non-denaturing conditions

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High-performance liquid chromatography (HPLC) mainly in the reversed-phase and ion-exchange modes has been widely applied to the preparation of ribosomal proteins[1–5]. The principal disadvantage of these methods is the use of denaturing conditions in the extraction process or/and elution.

Generally, proteins are extracted with LiCl–urea or with 66% acetic acid and separated by ion-exchange HPLC using 6 *M* urea or by reversed-phase HPLC using 0.1% trifluoroacetic acid in elution buffers. Various studies have shown that secondary and especially tertiary structures are very sensitive to the purification process[6]. It was found that proteins prepared by salt extraction show a large proportion of the α-helix, but are not very different from urea-treated proteins[7,8]. More recently, NMR studies and proteolysis experiments have demonstrated that salt-extracted ribosomal proteins have a better defined tertiary structure than urea-treated proteins[9]. Therefore, to determine the secondary or tertiary structures of ribosomal proteins, it is useful to have samples that are as native as possible. The principal means of purifying these proteins so as to be as native as possible is the salt extraction method described by Dijk and Littlechild[10], but unfortunately this purification procedure consumes large amounts of 30S subunits and also is time consuming.

In order to avoid these problems, we have developed a method using HPLC and salt-extracted proteins that allows the simpler preparation of "core" proteins in pure and native form. This method does not require large amounts of material and needs few concentration and purification steps (one gel permeation and one ion-exchange HPLC). In addition, it allows the direct use of the collected fractions for spectroscopic studies (UV, fluorescence, etc.), owing to the simplicity of the buffers used.

EXPERIMENTAL

Chemicals

Urea was for biochemical use (Merck) and all other chemicals were of analytical-reagent grade (Merck).

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Buffers

The following buffers were used: TSM (0.01 *M* Tris–0.03 *M* succinic acid–0.01 *M* $MgCl_2$, pH 8), TMK (0.03 *M* Tris–0.02 *M* $MgCl_2$–0.35 *M* KCl, 0.006 *M* β–mercaptoethanol, pH 7.4), buffer A (0.05 M ammonium acetate–6 *M* urea, 0.006 *M* β-mercaptoethanol, pH 5.6), buffer B (buffer A–0.5 *M* NaCl), buffer C (0.05 *M* ammonium acetate, pH 5.6) and buffer D (buffer C–1 *M* NaCl).

Isolation of core particles

The 30S subunits of *Escherichia coli* MRE 600 ribosomes were isolated as described previously by zonal sucrose gradient centrifugation[11] and stored in TSM buffer at −80°C in small aliquots (200 A^a_{260}/ml).

The method of preparing core particles described by Venyaminov and Gogia[12] was used with some modifications. The 30S subunits (200 A_{260}) were fractionated by 3.5 *M* LiCl into two main groups (1 ml of 30S subunits solution was mixed with the same volume of 7 *M* LiCl). The two groups are[12,13] the core particles, containing mainly S4, S7, S8, S15, S16 and S17 in association with 16S RNA and in reduced amounts S6, S11, S18 and S19, and the split proteins, *i.e.* S1, S2, S3, S5, S9, S10, S12, S13, S14, S20, S21 and S6, S11, S18, S19. Instead of centrifugation (300 000 *g*, 10 h), we preferred to use gel permeation HPLC to collect the core particles. Electrophoresis and urea ion-exchange HPLC showed that core particles prepared by centrifugation and gel permeation HPLC possess the same composition of proteins, except S1 and S2, which were often recovered with the last core particle fractions, and S11, which was found in very small amounts. The core particle fractions were then pooled (5 ml) and concentrated to 1 ml in a Centricon 10 centrifugate (Amicon) before extraction.

Extraction of core proteins

Extraction of core proteins was effected with TSM–4.5 *M* LiCl–6m*M* β-mercaptoethanol for 20 h at 4°C. The precipitate of RNA was removed by centrifugation (5000 *g*, 5 min) and the supernatant equilibrated in the *ad hoc* buffer (buffer A or buffer C) through a PD 10 column (Pharmacia) before application to the ion-exchange column. To check the total extraction, the precipitate was dissolved in acetate buffer (pH 5.4) and re-extracted with 3.5 *M* LiCl–4 *M* urea. Fig. 1 shows that the extraction can be considered to be complete.

Identification of ribosomal proteins

Polyacrylamide gel electrophoresis at pH 4.5[14] was used in combination with urea ion-exchange HPLC to assess the identity of the chromatographic fractions.

Chromatography

Chromatography was performed using a Pharmacia fast protein liquid chromatography (FPLC) system. The absorbance of the eluate was monitored with a Pharmacia UV-1 detector (254 or 280 nm) or a Waters Assoc. Model 480 spectrophotometer. In all instances, separations were carried out at room temperature. Samples with a volume greater than 1 ml were injected with a Pharmacia superloop.

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[a] One A_{260} unit is the amount of substance in 1 ml of a solution giving an absorbance of 1.0 when measured at 260 nm with a path length of 1 cm.

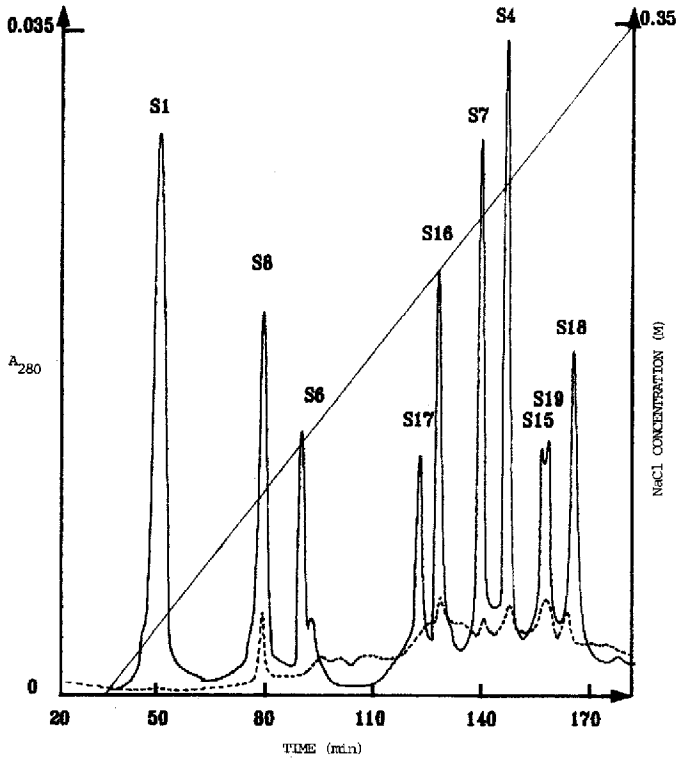

Fig. 1. Ion-exchange HPLC in 6 *M* urea of re-extracted proteins from core particles (dashed line). The CM column was eluted at pH 5.6 in buffer A by a salt gradient of NaCl (0–0.35 *M*). The gradient began with 100% eluent A for 30 min and was then linearly ramped to 100% eluent B over 150 min. Proteins were identified by comparison with their usual elution order (solid line).

Gel filtration

Gel permeation HPLC was performed using a Pharmacia Superose 12 column (30 cm × 10 mm I.D.). Generally, sample solutions (200 A_{260} per 2 ml) were eluted at a flow-rate of 0.5 ml/min in TMK.

Ion exchange

The ion-exchange column was an LKB Ultropac TSK CM-3SW (150 × 7.5 mm I.D.). Samples (50 A_{280} per 3 ml) in buffer C containing 0.15 *M* NaCl to prevent precipitation were eluted at a flow-rate of 1 ml/min with buffers C and D. The gradient shape is shown in Fig. 2. Changing the gradient at 0.4 *M* increases the resolution in the range 0.4–0.65 *M* where many proteins are eluted. Fractions of 1 ml were collected and could be used directly for spectroscopic studies (the typical A_{280} was *ca.* 0.01).

Spectroscopy

Absorbance spectra were recorded on a Varian Cary 2200 spectrophotometer interfaced with a PC AT computer.

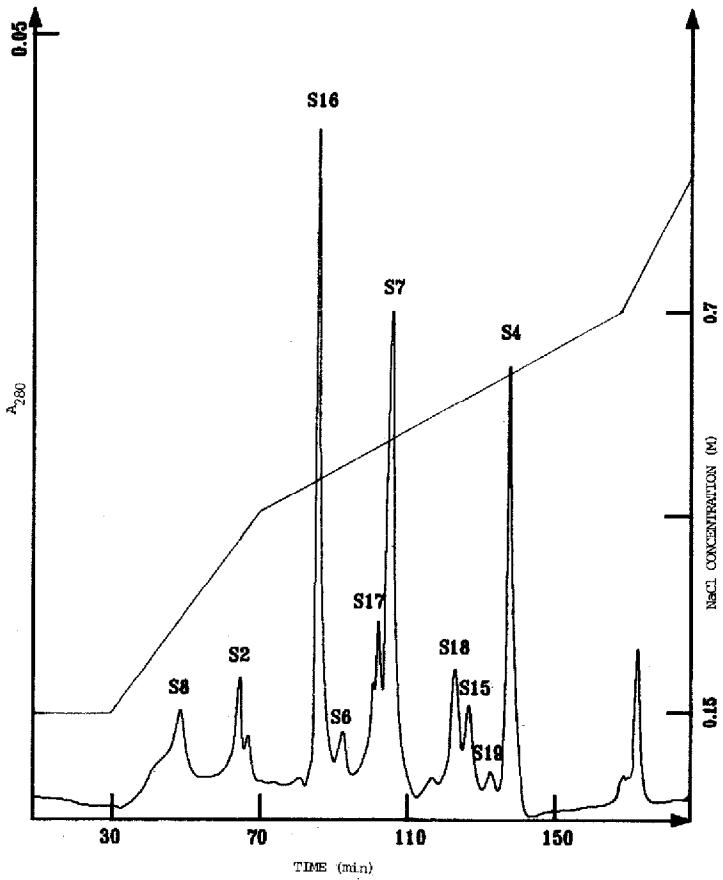

Fig. 2. Ion-exchange HPLC of core proteins under non-denaturing conditions. 50 A_{280} of core proteins in 2–3 ml of buffer C with 0.15 *M* NaCl was applied to the CM column. Following a 30-min equilibration with buffer C–0.15 *M* NaCl, gradients were applied from 0.15 to 0.4 *M* over 40 min, from 0.4 to 0.7 *M* over 100 min and from 0.7 to 1 *M* over 50 min at a constant flow-rate of 1 ml/min. Proteins were identified by rechromatography in 6 *M* urea and by polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Core particles were isolated by gel filtration HPLC instead of ultracentrifugation, which is time consuming and requires more material. Only 30 min is needed to separate core particles from split proteins.

Normally, the manufacturer's instructions recommend the use of sample volumes of not more than 200 μl for the best resolution. Routinely, we loaded the column with 2 ml of solution without any problems, because core particles migrate in the void volume.

Ion-exchange HPLC was carried out as described under Experimental. Fig. 2 shows the different peaks corresponding to the core proteins.

The principal results are summarized in Table I. Although the gradients were

TABLE I

CHARACTERISTICS OF ION-EXCHANGE HPLC OF CORE PROTEINS UNDER NON-DENATURING CONDITIONS

Elution conditions as in Fig. 2

	S8	*S6*	*S17*	*S16*	*S7*	*S4*	*S15*	*S19*	*S18*
Buffer A:									
Elution order	1	2	3	4	5	6	7	8	9
NaCl concentration (*M*)	0.1	0.125	0.19	0.205	0.225	0.24	0.26	0.265	0.28
Yield[a]	1	0.94	0.65	0.7	0.61	0.56	N.D.[e]	N.D.	0.94
Buffer C:									
Elution order	1	3	6	2	4	9	7	8	5
NaCl concentration (*M*)	0.27	0.48	0.51	0.46	0.52	0.63	0.59	0.605	0.58
Recovery[b] ± 0.1	0.9	0.15	0.6	1.5	1	0.95	1	N.D.	0.5
Yield[c] ± 0.2	0.9	0.15	0.4	1	0.6	0.5	>0.5	N.D.	0.5
Classical yield[d]	0.35	0	0.1	0.13	0	0.26	0	0.19	0

[a] Previous results[3]. The yield of each protein is calculated for 200 A_{260} units of 30S using a protein concentration as determined by absorbance measurements at 280 or 230 nm.

[b] Relative recovery defined as the ratio of the peak areas under non-denaturing and denaturing conditions.

[c] Absolute yield obtained by multiplying the yield with buffer A by the recovery with buffer C.

[d] Previous results[10].

[e] N.D. = not determined.

different, we observed an enhancement of ionic strength when proteins were eluted under non-denaturing conditions. Protein recovery efficiencies were measured by comparing the peak areas obtained under denaturing and non-denaturing conditions. For the main core proteins (S8, S7, S4, S15), the recoveries were very close to 100%; S16 is the only exception with a recovery greater than in urea. It has been shown previously[3,4] that recovery efficiencies using urea are greater than 50%, so we can conclude that there is minimal loss of material using this procedure.

Fractionation into two groups by salt washing has been used by many workers with different conditions[10,12,13,15]. The composition of the extract depends on the salt concentration but also on Mg^{2+} concentration. The principal problem was to obtain two groups with a stable composition. We chose salt washing with 3.5 *M* LiCl because we obtained groups with a more reproducible composition than with salt washing in medium low in Mg^{2+}. LiCl is an inorganic denaturant that causes only partial unfolding. Various studies on proteins other than ribosomal proteins showed that 5 *M* is the upper limit of LiCl concentration that can be employed for salt washing a subunit without denaturation[16,17].

To prevent interfering absorbances, we used the simplest buffers without urea and β-mercaptoethanol. The rapidity of the chromatography allows optical measurements to be made with a very low diffusion level. In this medium, samples were usually stable for few hours.

The recovery efficiencies are good except for S6, which is poorly soluble. The reason why the recovery of S16 is greater than in urea is not known. We thought it

might be a mixture of S16 and S17 owing to bad LiCl dissociation, but gel electrophoresis and urea HPLC showed a high purity of this protein. All the yields obtained with this method are greater than those obtained by the classical method. In particular, good yields of S7, S15 and S18, which were not recovered by the other method, were obtained.

Separations can be made without any interferences in the pH range 5.4–7. Ion-exchange and gel permeation HPLC consume only small amounts of materials, so it is easy to prepare fresh samples on demand, whereas classical methods which require large samples, need conservative methods for preparation of the samples.

REFERENCES

1 A. R. Kerlavage, L. Kahan and B. S. Cooperman, *Anal. Biochem.*, 123 (1982) 342.
2 A. R. Kerlavage, C. J. Wietzmann, T. Hasan and B. S. Cooperman, *J. Chromatogr.*, 266 (1983) 225.
3 P. J. Flamion and J. P. Schreiber, *Anal. Biochem.*, 147 (1985) 458.
4 M. Capel, D. Datta, C. R. Nierras and G. R. Craven, *Anal. Biochem.*, 158 (1986) 179.
5 R. M. Kamp, Z. J. Yao, A. Bosserhoff and B. Wittman-Lievold, *Hoppe-Seyler's Z. Physiol. Chem.*, 364 (1983) 1777.
6 C. A. Morrison, E. M. Bradbury, J. A. Littlechild and J. Dijk, *FEBS Lett.*, 81 (1977) 435.
7 H. G. Wittmann, J. A. Littlechild and B. Wittmann-Liebold, in G. Chambliss, G. R. Craven, J. Davies, K. Davis, L. Kahan and M. Nomura (Editors), *Ribosomes, Structure, Functions and Genetics*, University Park Press, Baltimore, 1980, p. 51.
8 J. Dijk, J. A. Littlechild, A. M. Freund, J. Pouyet, M. Daune and S. W. Provencher, *Biochim. Biophys. Acta*, 874 (1986) 227.
9 J. A. Littlechild, A. Malcolm, K. Paterakis, I. Ackermann and J. Dijk, *Biochim. Biophys. Acta*, 913 (1987) 245.
10 J. Dijk and J. A. Littlechild, *Methods Enzymol.*, 59 (1979) 481.
11 S. J. S. Hardy, C. G. Kurland, P. Voynow and G. Mora, *Biochemistry*, 8 (1969) 2897.
12 S. Yu Venyaminov and Z. V. Gogia, *Eur. J. Biochem.*, 126 (1982) 299.
13 V. D. Vasiliev and V. E. Koteliansky, *FEBS Lett.*, 76 (1977) 125.
14 P. S. Leboy, E. C. Cox and J. G. Flaks, *Proc. Natl. Acad. Sci. U.S.A.*, 52 (1964) 1367.
15 T. Itoh, E. Otaka and S. Osawa, *J. Mol. Biol.*, 33 (1968) 109.
16 S. Maruyama, K. Kuwajima, K. Nitta and S. Sugai, *Biochim. Biophys. Acta*, 494 (1977) 343.
17 F. Ahmad, *J. Biol. Chem.*, 258 (1983) 11143.