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

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ABSTRACT

High-performance ion-exchange chromatography was applied to the separation of proteins from the 30S ribosomal subunit under non-denaturing conditions. It was shown that a single chromatographic step only allows the purification of nine proteins. To increase the number of separated proteins, a prefractionation step was added that depends on the physical characteristics of the proteins to be purified. Sixteen out of 21 proteins could be purified by using prefractionation (gel permeation and lithium chloride salt washing). This method is well suited to preparing fresh samples on demand for optical studies owing to the simplicity of the buffers used and the amounts of proteins recovered in the eluted peaks (0.05–0.1 mg/ml).

INTRODUCTION

The extraction and separation methods used in the preparation of isolated proteins are of crucial importance for the study of their structures, in particular for the 30S ribosomal proteins which have a prominent role in 16S rRNA and 30S subunit conformations [1–6]. Structural studies require a biological material that is as native as possible without denaturing during extraction, purification and preservation processes for sample preparation. To achieve this goal, it is now well established that proteins have to be extracted from 30S subunits by LiCl salt washing [7,8] (instead of with 3.5 *M* LiCl–4 *M* urea or 66% acetic acid, which are harsh extraction conditions). It has been shown, by nuclear magnetic resonance (NMR) and circular dichroism studies, that salt-extracted 30S ribosomal proteins have a better defined tertiary structure than urea-treated proteins [9,10]. Dijk and Littlechild [11] published a purification procedure involving traditional chromatographic methods, based on this salt extraction. Unfortunately, it required large amounts of 30S subunits, large volumes of solvents and was also time consuming.

In recent years, high-performance liquid chromatographic (HPLC) techniques have been developed, and numerous separations of 30S ribosomal proteins have been published using high-performance size-exclusion chromatography (HPSEC) [12,13], high-performance ion-exchange chromatography (HPIEC) [14,15] and re-

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versed-phase HPLC (RP-HPLC) [16–18]. HPSEC separations are generally performed under native conditions but lead to poor resolution in contrast to HPIEC and RP-HPLC, which offer higher resolution but under denaturing conditions, by using urea for HPIEC and trifluoroacetic acid (TFA) mixed with solvents for RP-HPLC. Apart from HPSEC, no HPLC separation of total 30S ribosomal proteins has been carried out under non-denaturing conditions.

We recently described the use of HPLC in the ion-exchange mode for the separation of eight "core" *E. coli* 30S ribosomal proteins (S4, S7, S8, S15, S16, S17, S18 and S19) under non-denaturing conditions [19]. In contrast to the conventional preparation [11], we have shown that HPIEC is a fast preparation method using small amounts of material with only a few purification steps. In this paper, we demonstrate the advantage of using two chromatographic steps to prepare pure proteins from specific groups (*e.g.*, high-molecular mass protein group or core proteins) with acceptable yield and purity and without the need to recover the other groups.

EXPERIMENTAL

Chemicals

Spectroscopic-grade TFA and HPLC-grade acetonitrile were purchased from Merck (Darmstadt, Germany) and Aldrich-Chimie (Strasbourg, France) respectively. Urea was for biochemical use (Merck) and all other chemicals were of analytical-reagent grade (Merck).

Buffers

The following buffers were used: TSM [0.01 *M* tris(hydroxymethyl)aminomethane–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 (pH 5.6)] and buffer B (buffer A + 1 *M* NaCl).

Isolation of ribosomal proteins

The 30S subunits of *E. coli* MRE 600 ribosomes were isolated by zonal sucrose gradient centrifugation as described previously [20] and stored in TSM at −80°C in small aliquots (200 A_{260} units/ml).

Extraction of total proteins (TP30) was carried out on 30S subunits with 4.5 *M* LiCl–6 m*M* β-mercaptoethanol for 20 h at 4°C. The precipitate of 16S rRNA was removed by centrifugation (5000 *g*, 5 min). It has been shown that the residual protein in the precipitate is less than 1.5% [19]. The supernatant is used as it is for application to the permeation column, or equilibrated in buffer A with 0.15 or 0.25 *M* NaCl using a Pharmacia PD10 column (Pharmacia–LKB, Uppsala, Sweden) before application to the ion-exchange column.

Fractionation of 30S subunits into two main groups (core particles and split proteins) was done with 3.5 *M* LiCl–6 m*M* β-mercaptoethanol as described previously [19,21]. Both groups can be recovered in two different ways. For quantitative preparations (up to 15 mg of 30S), centrifugation (300 000 *g*, 10 h) was used and the resulting pellets of core particles were dissolved in TSM, whereas split proteins remained in the supernatant. HPSEC was preferred for analytical preparations: in a single run of 2 h, core particles which migrate in the void volume and split proteins

were separated. Before application to the ion-exchange column, the core proteins were extracted and treated as for TP30 whereas the split proteins were equilibrated in buffer A with 0.15 or 0.25 *M* NaCl using the Pharmacia PD10 column.

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 or a Waters Assoc. Model 480 spectrophotometer at 254 or 280 nm. All separations were carried out at room temperature. Sample volumes greater than 1 ml were injected with a 10-ml superloop obtained from Pharmacia.

HPSEC

HPSEC was performed using a Pharmacia Superose 12 column (300 × 10 mm I.D.) or a Pharmacia Superdex XK 16/70 column (60 × 1.6 cm I.D.). Generally, sample solutions were eluted in TMK or in buffer A with 0.15 or 0.25 *M* NaCl at flow-rates of 0.5 ml/min for the Superose column and 1.0 ml/min for the Superdex column. The volumes of the collected fractions were 0.5 ml for the Superose column and 1 ml for the Superdex column. Before application to the ion-exchange column, the collected fractions were pooled and concentrated in Sartorius Centrisart (cut-off 5000) to 1–5 ml if the recovered volumes were greater than 10 ml.

HPIEC

HPIEC was carried out using a LKB Ultropac TSK CM-3SW (150 × 7.5 mm I.D.). Samples in buffer A containing 0.15 or 0.25 *M* NaCl to prevent precipitation were eluted at a flow-rate of 1.0 ml/min using a gradient from buffers A and B. The gradient shape is shown in Fig. 1. Changing the gradient shape at 0.4 *M* increases the resolution in the range 0.4–0.65 *M* where most of proteins are eluted.

RP-HPLC

RP-HPLC was performed using a Pharmacia ProRPC HR 5/10 column (5-μm silica, 300 Å pore size, C_1/C_8-bonded phase) (100 × 5 mm I.D.). Solvent I was 0.1% TFA in water (pH 2.0) and solvent II was 0.1% TFA in acetonitrile–water (1:1, v/v). Proteins were eluted at room temperature using a linear gradient from 30% to 90% II in 120 min at a flow-rate of 0.2 ml/min. Before injection, the column was equilibrated with 30% II, and then the fractions from HPIEC were loaded directly onto the column. The optimum protein concentration injected was 0.1 mg per 100 μl.

Identification of ribosomal proteins

RP-HPLC was used in combination with polyacrylamide gel electrophoresis (PAGE) at pH 4.5 [22] and urea HPIEC [14] to assess the identity of chromatographic fractions.

Degrees of purification and yields

Degrees of purification and yields were calculated from spectrophotometric measurements. The amounts of proteins recovered were determined from absorbances determined from peak areas on the chromatograms or by direct measurements of eluted peaks on a Varian Cary 2200 spectrophotometer (Varian, Mulgrave, Australia).

The protein concentrations were determined by using the molar absorptivities of each protein calculated from the composition of tryptophans, tyrosines and cysteines in the corresponding protein and with respective values of the molar absorptivities of 5600, 1400 and 127 l/mol · cm at 278 nm [23]. It has been shown that these residues are the only ones that contribute significantly to absorbance over the range 276–282 nm [23] and that the molar absorptivities calculated on this basis are very close to the measured values, with an average relative standard deviation of 3.8% [24]. Purification yields were calculated from ion-exchange chromatograms with reference to the amount of 30S subunits used. Degrees of purity were determined from reversed-phase chromatograms by comparing the amounts of all the other eluted proteins with the amount of wanted protein.

RESULTS AND DISCUSSION

TP30 were extracted from subunits with 4.5 *M* LiCl, which is the upper limit of LiCl concentration that can be employed for salt washing without denaturation of proteins [25,26]. The 21 salt-extracted 30S proteins were resolved into sixteen major peaks by using HPIEC with buffers A and B. Samples ranging from 50 to 200 μg of proteins gave typical chromatograms such as that presented in Fig. 1. Peak fractions were analysed by both RP-HPLC and one-dimensional PAGE. Under non-denaturing conditions, the elution was performed with a ionic strength 20% higher than that under denaturing conditions for the same duration of elution. The elution orders for cation-exchange HPLC on carboxymethyl (CM) supports eluted with 6 *M* urea buffers [13,17] are very similar to the conventional orders. Under non-denaturing conditions, a more pronounced difference exists (Table I). Because HPLC produces selective and rapid separations, it was expected that a single run would be sufficient to purify the major part of total ribosomal proteins but, as can be seen from Table I, only three peaks are pure (S4, S5 and S8). Attempts to increase the resolution by using a shallower gradient were unsuccessful. As has been reported previously using traditional methods [11], HPIEC offers higher resolution than classical chromatography, in which proteins disturb the chromatography, mainly S2, S3, S5 and S17 which are not eluted as single peaks, owing to complex formation or partial precipitation. It was found that only S4, S8, S10, S14 and S16 can be recovered with acceptable degrees of purity and yields by this method (see Table III).

To isolate the other proteins in a simple way with only two purification steps we carried out an initial fractionation of TP30 into two groups of various compositions according to the selected proteins. The choice of the prefractionation method is very important because it determines the final yield of the prepared proteins (see Table III). Two methods are generally used to split TP30 [11,21], HPSEC and LiCl salt washing, which are poorly selective and just allow the concentrations of the wanted proteins to increase in one of the groups to the detriment of the other. We shall give two examples to illustrate the purification methods with prefractionation.

Fractionation of TP30 by HPSEC is well suited to purifying the ribosomal proteins with a molecular mass ranging from 15 000 to 40 000 daltons. Fig. 2A shows a typical elution profile obtained from the Pharmacia Superose 12 column (the Superdex column gave similar results), where TP30 migrate in three groups (G1, G2 and G3). Table II presents the elution characteristics and the composition of the three

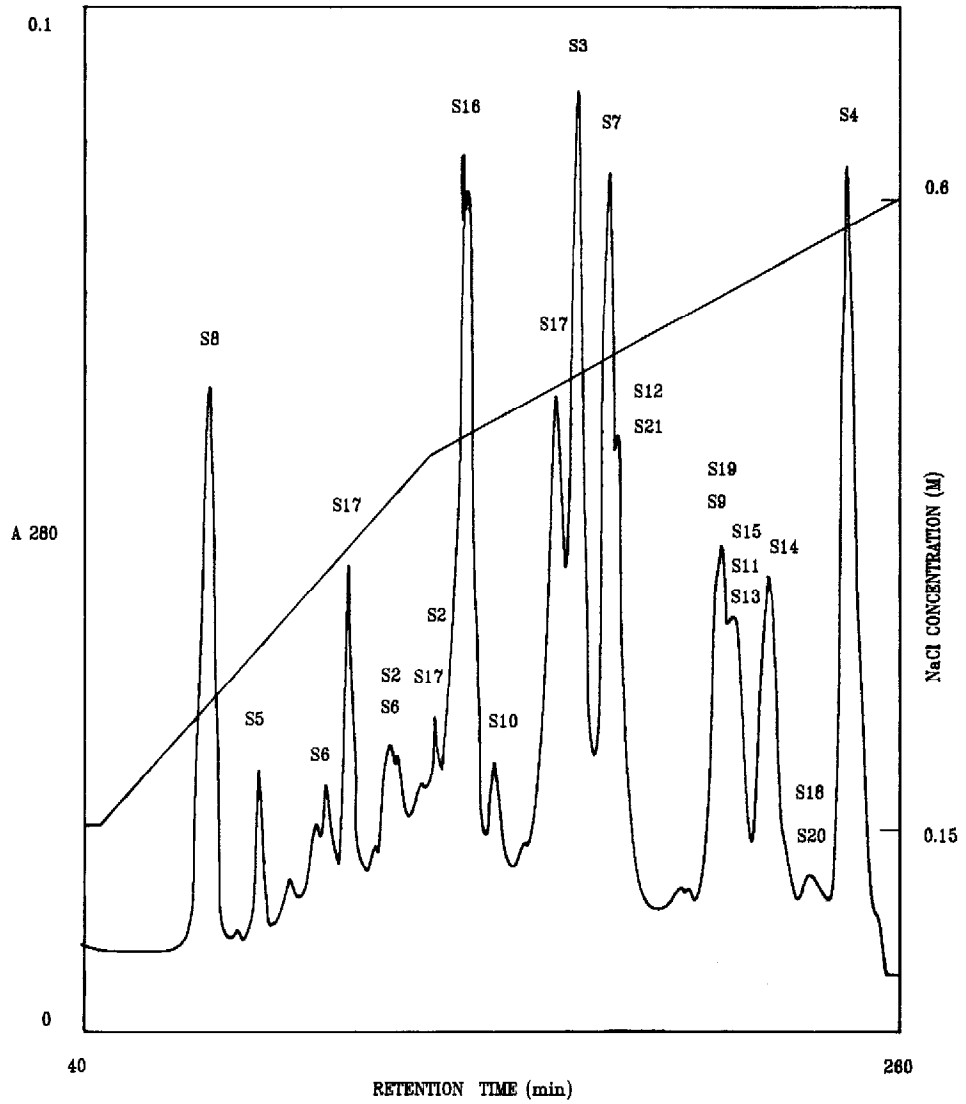

Fig. 1. HPIEC on TSK CM-3SW (150 × 7.5 mm I.D.) of TP30 under non-denaturing conditions. 50–200 μg of TP30 in 5–10 ml of buffer A with 0.15 *M* NaCl were applied to the CM column. Following a 30-min equilibration with buffer A–0.15 *M* NaCl, gradients were applied from 0.15 to 0.4 *M* over 50 min and from 0.4 to 0.6 *M* over 140 min. Note that absorbance scale (A_{280}) was changed to 0.2 unit after 145 min (elution time of S16).

selected groups, the first group consisting only of protein S1. Proteins are eluted roughly according to their molecular masses (M_r), with some exceptions. The principal anomaly is the presence of S7 (M_r = 19 732 [21]) in the third group, which is mainly constituted by proteins with molecular masses ranging from 8000 to 14 000 daltons. It should be noted that the resolution on silica-based columns such as TSK G2000 SW seems higher than that on organic-based material such as Pharmacia Superose or

TABLE I

CHARACTERISTICS OF ION-EXCHANGE HPLC OF TP30 PROTEINS UNDER NON-DENATURING CONDITIONS

Elution conditions as in Fig. 1.

Proteins	Peak No.															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Main[a]	S8 (1)	S5 (3)	S6	S17	S2 (2)	S17	S16 (5)	S10 (1)	S17	S3 (4)	S7	S19	S15	S14 (6)	S18	S4 (7)
Others			S2	S2 S6	S17	S2	S2	S3 S5	S3 S5	S17 S14	S21 S12	S15 S9	S14 S13	S18	S20	
NaCl (%)[b]	23	26	32	34	37	40	41	42	45	46	47	51	52	54	56	57

[a] Previous results in parentheses [11].

[b] NaCl concentration in buffer B as reference.

Superdex [27]. It has been demonstrated [28] that the elution on a silica-based column not only depends on the protein sizes but also is strongly affected by the electrostatic interactions between the proteins and the support. This could explain the observed differences in resolution and elution order between the two types of supports, but here the difference is mainly due to the extraction methods for TP30, which was native in this work and denaturing in the studies of Kamp and Wittmann-Liebold [27].

Groups G2 and G3 were then rechromatographed by HPIEC as described above, to obtain purified proteins. Fig. 2B and C show the elution profiles of groups G2 and G3, respectively. Group G2 gives S2, S3, S4, S5 and S6 with degrees of purity better than 90% calculated from reversed-phase chromatograms and a small amount of S19–S13 complex. Group G3, mainly constituted of S7, S10, S14, S15, S16, S17, S18, S19 and S20, is more complicated to resolve because many proteins migrate together, *e.g.*, S7, S21, S12 and S19, S15, S13. If proteins of group G3 are wanted, a more suitable prefractionation must be applied using HPSEC to simplify the ion-exchange HPLC, by only collecting the fractions where the wanted proteins are eluted.

Fractionation of TP30 by LiCl salt washing was performed with concentrations of LiCl ranging from 1.5 to 3.5 *M*, which allowed the removal of increasing amounts of proteins from the subunits [7,8]. The major problem is that the composition of the groups is not well defined because it depends not only on LiCl concentration but also on Mg^{2+} concentration and ribosome subunit preparation. In addition, this fractionation is not selective and several proteins are present in two groups in various amounts. An example is 3.5 *M* LiCl treatment of 30S subunits. The two resulting groups are the core particles which are constituted of 16S rRNA in association with S4, S7, S8, S15, S16 and S17 and in reduced amounts with 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. Small amounts of core proteins S4, S7, S16 and S17 were always recovered in the split fraction. As shown previously (Fig. 3 [19]), from the group of core proteins we can purify S8, S17, S16, S7, S4, S15, S19 and S18 by using one HPSEC and one HPIEC step, but the purification of the other proteins from the split group was not greatly facilitated by this procedure owing to the bad selectivity of salt washing. To purify

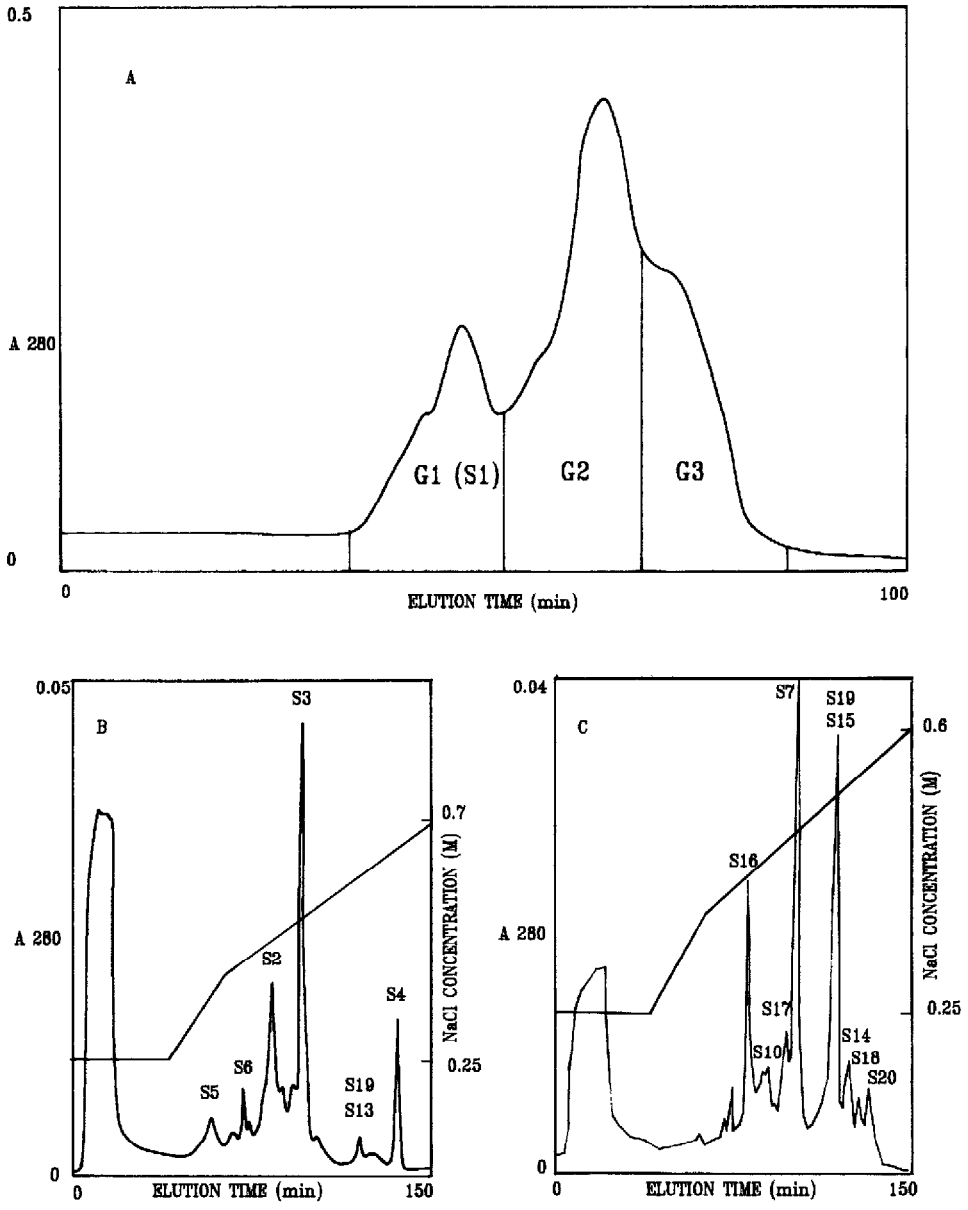

Fig. 2. (A) HPSEC of TP30 on Pharmacia Superdex XK 16/70 (60 × 1.6 cm I.D.) under non-denaturing conditions. About 1–10 mg of TP30 extract were injected in 4 ml of 4.5 *M* LiCl–TMK mixture. The proteins were eluted in TMK or buffer A with 0.15 *M* NaCl at a constant flow-rate of 1.0 ml/min. (B) Ion-exchange HPLC of the G2 proteins under non-denaturing conditions. 1–10 ml of concentrated G2 proteins in buffer A with 0.25 *M* NaCl were applied to the CM column. The gradient was started with eluent A–0.25 *M* NaCl over 30 min and then linearly increased to 0.4 *M* over 40 min and to 0.6 *M* over 80 min. (C) Ion-exchange HPLC of the G3 proteins under the same conditions in (B).

TABLE II

CHARACTERISTICS OF GEL PERMEATION HPLC OF TP30 UNDER NON-DENATURING CONDITIONS

Protein (molecular mass)	Elution volume (ml)													
	G1	G2					G3							
	52[a]	62	68	70	73	75	77	79	81	83	85	87	89	91
S1 (61 159)	★★[b]													
S2 (26 613)		★[b]	★★	★★										
S2 (25 852)			★★	★★	★★									
S4 (23 138)			★★	★★	★★									
S5 (17 514)			★	★★	★★	★								
S6 (15 200)			★	★★	★★	★								
S7 (19 732)								★	★★	★★	★			
S8 (12 196)						★	★★	★★	★					
S9 (14 569)				★	★	★	★	★	★	★				
S10 (11 736)									★	★★	★★	★		
S11 (13 788)						★	★★	★★	★					
S12 (13 608)											★	★★	★★	★
S13 (12 968)			★	★	★	★	★	★	★					
S14 (11 200)					★	★★	★★	★						
S15 (10 000)											★	★★	★★	★
S16 (9192)								★	★★	★★	★			
S17 (9573)								★	★★	★★	★			
S18 (8896)				★	★★	★★	★							
S19 (10 239)					★	★★	★★	★						
S20 (9553)									★	★★	★★	★		
S21 (8369)							★	★★	★★	★				

[a] Elution volume corresponding to the maximum value of A_{280}.

[b] The presence of large amounts of protein is indicated by two asterisks and of smaller amounts by one asterisk.

proteins of the split group more easily, a lower LiCl concentration must be used during the salt-washing step to reduce the number of split proteins and the concentration value adjusted with respect to the wanted proteins.

Table III lists the isolation conditions, yields and degrees of purity of sixteen proteins of the 30S subunit. It is clear that the core proteins are the easiest to prepare under non-denaturing conditions because of their good solubilities (1–5 mg/ml) and weak interactions between them, a single HPIEC run on TP30 can give S4, S8, S10, S14 and S16 and also S3, S5 and S17 with a degree of purity better than 90% but with poor yields because of co-migrations, the pre-fractionation by gel permeation leads to the purification of S1, S2, S3, S4, S5 and S6 with a lower yield than by direct HPIEC owing to the loss of material during HPSEC and the additional concentration step before the ion-exchange separation, the prefractionation by LiCl salt washing allows the purification of core proteins with yields greater than those obtained by the classical method and S9, S11, S12, S13 and S21 were not recovered in a pure form under these conditions.

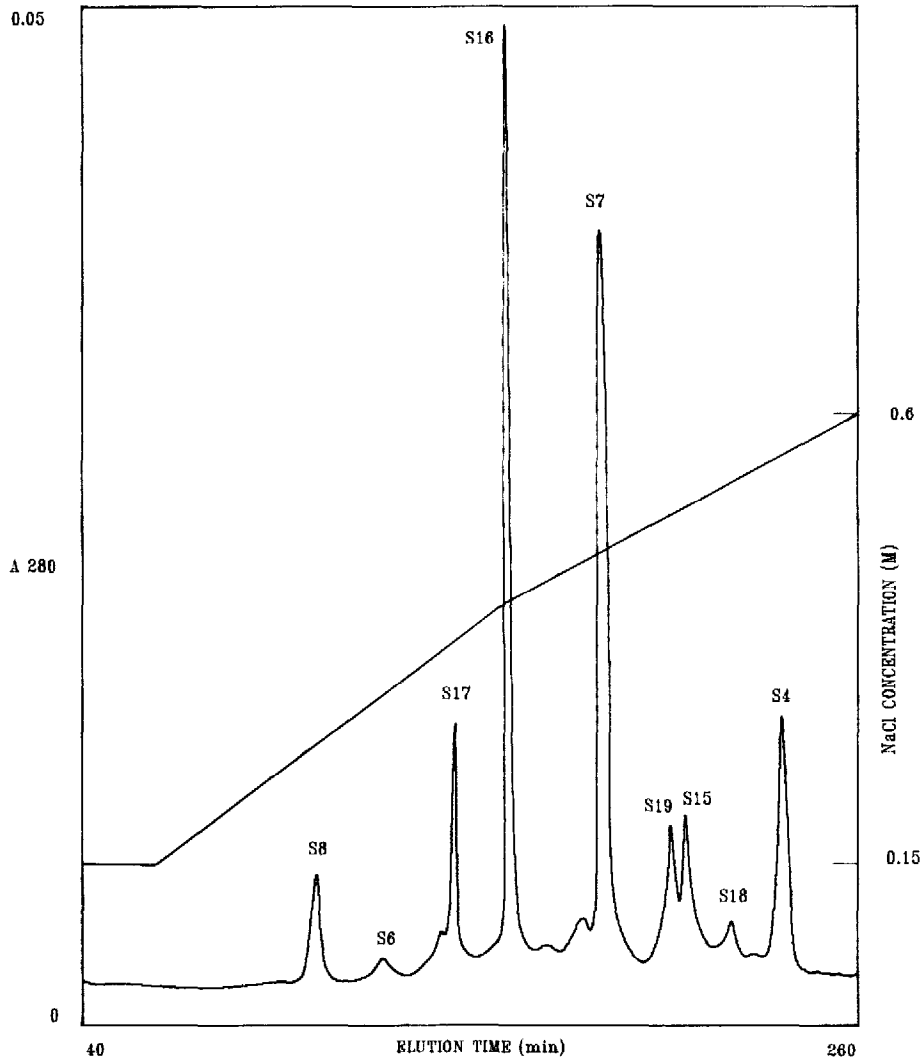

Fig. 3. HPIEC of core proteins under non-denaturing conditions. 50 A_{280} of core proteins (obtained by centrifugation) in 2–3 ml of buffer A with 0.15 *M* NaCl were applied to the CM column. An elution gradient identical with that in Fig. 1 was used.

CONCLUSIONS

We have shown that it is possible to prepare fresh samples of sixteen 30S proteins under non-denaturing conditions. Under these conditions, HPIEC is the most suitable technique for the isolation of 30S proteins. RP-HPLC was only used here as an analytical technique. Although the selectivity and rapidity of HPIEC improved the separation in comparison with conventional chromatographic methods, this was not sufficient to eliminate completely co-migration along the column owing to complex

TABLE III

SEPARATION METHODS FOR TP30 WITH YIELDS AND DEGREES OF PURITY

Protein	Ion-exchange on TP30		Size-exclusion + ion exchange (groups G1, G2, G3)		Salt washing[a] + ion exchange (core proteins)	
	Yield (%)	Purity (%)	Yield (%)	Purity (%)	Yield (%)	Purity (%)
S1			24	>95		
S2			23	>95		
S3	25	>90	26	>95		
S4	70	>95	15	>95	50	>95
S5	25	>95	10	>95		
S6			5	>95	15	>95
S7	20	>90			60	>95
S8	95	>95			90	>95
S9						
S10	44	>95				
S11						
S12						
S13						
S14	70	>95				
S15					>50	>90
S16	90	>95	22	>95	100	>95
S17	25	>90			40	>95
S18					50	>95
S19					40	>90
S20			27	>95		
S21						

[a] Previous results [19].

formation and partial precipitation. It was found that prefractionation of the proteins into groups by salt washing or by HPSEC improves significantly the fraction purity, but this does not lead to major simplification of the procedure, but only allows the composition of one group to be made suitable in relation to the proteins to be purified. Protein recoveries are generally higher than those obtained by the conventional method (see Table III), with values ranging from 10 to 90%. Further, it was possible to purify several proteins such as S7, S15 and S18 that are not recovered by traditional methods in good yield.

Samples prepared by this method can be used directly for optical studies, in particular for circular dichroism spectroscopy, owing to the simplicity of the buffers used. The amounts recovered, ranging from 0.05 to 0.1 mg/ml, allow the direct use of the peak fractions for these optical studies, whereas NMR studies requiring larger amounts of proteins need a preparative-scale separation.

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