Reverse Phase High Performance Liquid Chromatography of \textit{Escherichia coli} Ribosomal Proteins: Standardization of 70 S, 50 S, and 30 S Protein Chromatograms

**FUNCTIONAL ACTIVITY OF PURIFIED PROTEINS**

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We recently described the use of reverse phase high performance liquid chromatography for the separation of the proteins of the 30 S subunit of \textit{Escherichia coli} ribosomes (Kerlavage et al., Anal. Biochem. 123, 342-348). In the present studies we report improvements in the technique and its extension to the separation of the proteins of the 50 S subunit and of 70 S ribosomes. Using an octadecasilyl silica column and a trifluoroacetic acid/acetonitrile solvent system, the 21 proteins of the 30 S subunit have been resolved into 17 peaks, the 33 proteins of the 50 S subunit into 22 peaks, and the 53 proteins of the 70 S ribosome into 31 peaks. The proteins present in each peak have been identified by polyacrylamide gel electrophoresis, by comparison with previously standardized chromatograms, and by calibration with authentic samples of purified proteins. All of the known ribosomal proteins have been identified on the chromatograms with the exception of L31 and its variant, L31'. Three protein peaks, not corresponding to known ribosomal proteins, have been observed in preparations from the total protein from 50 S subunits and 70 S ribosomes, but the significance of these peaks is unclear.

The reverse high performance liquid chromatography technique has the potential for purifying all ribosomal proteins, as demonstrated by the increase in resolution we obtain when a peak isolated under standard gradient conditions and containing several proteins is reapplied to the column and eluted with a shallower gradient. Its utility in preparing proteins for functional studies is demonstrated by a reconstitution of active 30 S particles using 30 S proteins prepared by reverse phase high performance liquid chromatography.

The \textit{Escherichia coli} ribosome has a sedimentation coefficient of 70 S and consists of two dissociable subunits with sedimentation coefficients of 30 S and 50 S. The 30 S subunit is composed of 16 S RNA and 21 proteins, while the 50 S subunit is composed of 23 S RNA, 5 S RNA, and 33 proteins.

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The proteins derived from the small (30 S) subunit are designated S1-S21 and those derived from the large (50 S) subunit are designated L1-L34. There are a total of 53 unique proteins; L5 is a stable complex of L7/L12 and L10 (1) and S20 and L26 are identical (2). One copy of this protein being partitioned between the 30 S and 50 S subunits (3).

The preparative and analytical separation of ribosomal proteins is of crucial importance to the study of the structure and function of the ribosome. Various approaches requiring purification or analysis of ribosomal proteins have been employed in these studies. These include reconstitution of ribosomal subunits from constituent components with the omission of a single protein, using antibodies to individual proteins (6, 7), affinity labeling of the ribosome with derivatives of its various ligands (8-10), neutron diffraction using selectively deuterated proteins (11), assembly mapping (reviewed in Ref. 12), protein cross-linking (13), and analysis of mutants with altered or missing proteins (reviewed in Ref. 14).

The major problem in separating the ribosomal proteins is that most of them are of similar molecular mass (6,000 to 30,000 Da) and isoelectric point (8 to 12) (15). The classical purification methods for ribosomal proteins include ion exchange and size exclusion chromatography steps. These protocols are quite laborious and yields range from 5% for the smaller, difficult to isolate proteins to 50% for the larger proteins (16-19). The classical analytical methods for the separation of ribosomal proteins have been one- and two-dimensional polyacrylamide gel electrophoresis. One-dimensional electrophoresis performed according to Leboy et al. (20) resolves the 21 proteins of the 30 S subunit into 15 bands and the 33 proteins of the 50 S subunit into 16 bands. For the case of analysis of radioactively labeled proteins, the time required for pouring, running, staining, destaining, and drying the gel and then oxidizing gel slices prior to scintillation counting is quite substantial. In addition, the recovery of covalently incorporated radioactivity is usually below 50% (21). Two-dimensional polyacrylamide gel electrophoresis, performed as introduced by Kaltschmidt and Wittmann (22) or using more recent modifications (23, 24), resolves almost all of the 30 S and 50 S proteins but with an even greater time expenditure and lower recovery of protein.

Reverse phase high performance liquid chromatography is well suited to separating the ribosomal proteins since the basis for retention is hydrophobicity rather than size or charge. RP-HPLC\textsuperscript{c} also has the advantages of speed, high

\textsuperscript{1} The abbreviations used are: RP-HPLC, reverse phase high performance liquid chromatography; TP30, TP50 and TP70, total protein from 30 S and 50 S subunits and 70 S ribosomes, respectively; PAGE, polyacrylamide gel electrophoresis.
recovery yields, and high sensitivity. We previously reported on the separation of TP30 by RP-HPLC into a total of 15 peaks and on the identification of the 30 S proteins within each of these peaks (25). In the present work we report several technical improvements in the RP-HPLC technique, use our improved methodology to separate TP50, TP30, and TP70 into large numbers of peaks, and identify the proteins eluting in each of the separated peaks. Our results clearly demonstrate the superiority of this technique to classical methods for both preparative and analytical separation of ribosomal proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trifluoroacetic acid and HPLC grade acetonitrile were purchased from Fisher. Lyophilization jars were siliconized with Prosil-28 (PCR Chemicals). Poly(U) was purchased from Miles. [3H]Phe-tRNA was prepared from [3H]Phe (400-450 Ci/mol, New England Nuclear) and bulk-stripped tRNA (Grand Island Biological) as described by Ravel and Shorey (26). Coomassie brilliant blue G-250 and bovine serum albumin were purchased from Sigma. All other chemicals were reagent grade.

Purified *E. coli* 50 S ribosomal proteins were a generous gift of Dr. M. Nomura, University of Wisconsin. Ribosomal proteins L7/L12 were a generous gift of Dr. A. Dahlberg, Brown University.

Isolation of Ribosomal Proteins—70 S ribosomes were prepared from *E. coli* Q13 bacteria harvested in mid or late log phase using the modification of the Traub et al. (27) procedure previously described (21). Ribosomal subunits were prepared by sucrose gradient centrifugation as described previously (21) using buffer A (50 mM Tris-HCl (pH 7.5 at 4 °C), 50 mM KCl, 1 mM MgCl₂, 6 mM 2-mercaptoethanol) prior to HPLC injection.

**High Performance Liquid Chromatography**—The HPLC system consisted of one 6000 A pump, one 454 pump, a 600 programmer, and a 662 universal injector, all from Waters Associates. Column eluates were monitored for UV absorbance using a Waters extended wavelength module (214 nm) and a model 440 absorbance detector (280 nm) connected in series. Each was equipped with a 15.5-μl cell with a 1-cm path length. All separations were performed on a Synchronpak RP-3 C₁₈-silica column (6.5 μm silica, 300-Å pore, 4.1 x 250 mm; SynChrom, Inc.).

Proteins were eluted at room temperature using a gradient (as described in Figs. 1, 4, and 6) from 0.1% (w/v) F₃₃COOH in H₂O, pH 2.14 (solvent A) to 0.1% (w/v) F₃₃COOH in CH₃CN (solvent B). The M-45 was used to pump solvent A and the 6000 A was used to pump solvent B at a combined constant flow rate of 0.6 ml/min with column pressure between 1000 and 1500 p.s.i.

**Table I**

| Protein | Relative Retention |
|---------|--------------------|
| TP50    | 0.08               |
| TP30    | 1.00               |
| TP70    | 2.00               |

Since our previous work on the separation of TP30 by RP-HPLC (25), four important technical improvements have been made: (a) utilization of a 300-Å pore C₁₈-silica column (Synchronpak RP-P, SynChrom, Inc.) replacing the 125-Å pore column (μ-Bondpak, Waters Associates) used earlier; (b) changing the gradient shape from linear to convex; (c) addition of 1.0% F₃₃COOH to the acetonitrile solvent; (d) dual wavelength monitoring at 214 and 280 nm. The results of applying our improved procedure to separations of TP50, TP30, and TP70 are discussed in turn below.

**RESULTS AND DISCUSSION**

**50 S Proteins**—Using our improved procedure, the thirty 50 S proteins were resolved into at least 22 peaks as shown in Fig. 1. The relative retentions (α) of the peaks are listed in Table I. Relative retention is the most reliable index for identification of a species. It is dependent only upon the

![Fig. 1. RP-HPLC of TP50.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**FIG. 1.** RP-HPLC of TP50. A solution of TP50 (217 μg) in 10 μl of buffer B was injected onto a Synchronpak RP-P column and eluted with a convex gradient (curve 5, Waters 660 Programmer) of 15 to 45% solvent B in 120 min followed by a 10-min isocratic elution at 45% solvent B and a 60-min linear gradient from 45 to 100% solvent B (dashed line). The upper and lower traces are absorbances at 214 nm and 280 nm, respectively. The proteins present in the lettered peaks are listed in Table I.
temperature and the composition of the column and the mobile phase, and is independent of column dimensions, instrument dead volume, sample size, and flow rate. The identities of the proteins in the peaks, summarized in Table I, were determined by one or more of the following methods: (a) one-dimensional PAGE; (b) two-dimensional PAGE; (c) calibration with authentic samples of purified 50 S proteins. In the analyses using purified proteins, relatively high concentrations of protein standards were applied along with a low concentration of TP50, which served to calibrate the gel. Protein identification is according to Kenny et al. (24).

All of the previously identified TP50 proteins have been located in the HPLC chromatogram shown in Fig. 1 with the exceptions of proteins L31 and L31', the latter described by Fanning and Traut (35). These proteins are stained very weakly with Coomassie G-250, and would have been difficult to identify by the PAGE procedures discussed above. Furthermore, they have previously been isolated in only small amounts (3). Conversely all of the peaks in Fig. 1 have been identified as containing known ribosomal proteins except for peaks D, G, and H. In contrast to the other peaks in Fig. 1, the relative amounts of peaks D, G, and H varied with conditions of TP50 preparation and storage, an indication of their greater susceptibility to degradation. Peak G was the most unstable. When it was lyophilized, redissolved in buffer B, and rechromatographed, no peak eluted in the original position of peak G. Instead, ten earlier eluting peaks were observed. Peaks D and H were unstable to long term storage at -20 °C, but could be stored at -78 °C. On rechromatography of both peaks D and H, the major peaks eluted in the original peak positions, although several minor, earlier eluting peaks were seen with peak D. A composite schematic summary of two-dimensional PAGE runs showing positions due to peaks D, G, and H is shown in Fig. 3. Peak D gave rise to three spots, two of which migrated with the dye front in the area of proteins L32 and L33, and one which was slightly slower in the second dimension. Peak H gave rise to two spots, one migrating near L33 and the other in an area not corresponding to a known ribosomal protein. It remains to be established whether peaks D, G, and H correspond to new 50 S proteins discovered as a consequence of our new HPLC method of analysis, or to degradation products of previously identified 50 S proteins, or to some other procedural artifacts.

30 S Proteins—The improved RP-HPLC procedure described above has resulted in an increased resolution of the twenty-one 30 S proteins into 17 peaks (Fig. 4), as compared with the 15 peaks reported earlier (25). The identities of the proteins present in each peak, as summarized in Table II, were determined by one or more of the following methods: (a) comparison with the previously standardized TP30 chromatogram (25); (b) calibration with authentic samples of purified 30 S proteins; (c) one-dimensional PAGE analysis.

In contrast to the TP50 results, all of the known TP30 proteins have been identified and all of the major peaks...
correspond to known 30 S proteins. The smaller unlabeled peaks seen in Fig. 4 are 50 S proteins, present due to minor contamination of our 30 S subunits by 50 S subunits. The intensity of peak e, corresponding to S20, when compared to peak K (L26) (Fig. 1) confirms the previously reported result that this single protein partitions mostly to the 30 S subunit (3). Two further points should be noted. Proteins S1 and S2 were observed in only small amounts reflecting losses during subunit preparation (3), and the shoulder on peak p (arrow, Fig. 4) contains the protein A described by Subramanian et al. (36).

70 S Proteins—The fifty-three 70 S proteins were resolved into at least 31 peaks by our improved RP-HPLC procedure (Fig. 5). The highly reproducible nature of the chromatograms enabled peak assignment to be made largely on the basis of comparison with the TP30 and TP50 elution profiles. In certain cases, confirmations were obtained using one-dimensional PAGE analysis.

A noteworthy point is that the peak eluting at 135 min during the final steep gradient portion of the chromatogram was found by subsequent two-dimensional PAGE analysis to contain several 50 S proteins, in the relative amounts L1, L9 > L4 > L3, L5, L10 > L15. When this peak was lyophilized, redissolved in buffer B, and rechromatographed, greater than 80% of the absorbance at 214 nm eluted in its original position. Thus, it is likely that this peak contains proteins which are denatured and/or aggregated. It should be emphasized that the amount of any of the proteins found in this peak is small (<10% even for proteins L1 and L9) compared to the amount eluted in the major peak of that protein as defined in Table I. Further studies designed to understand the basis for this apparent partitioning of some ribosomal proteins into two regions of the chromatogram are underway and will be reported elsewhere.

Resolution of Individual Proteins—With our current procedure 16 of 33 known 50 S proteins, 13 of 21 known 30 S proteins, and 18 of 53 known 70 S proteins are fully resolved in single chromatographic runs of TP50, TP30, and TP70, respectively. It is important to emphasize that, where desirable, complete resolution of any given ribosomal protein should be attainable in straightforward fashion by reapplication of

| Peak | Protein | α* |
|------|---------|----|
| a    | S12     | 0.55 |
| b    | S21     | 0.69 |
| c    | S14     | 0.82 |
| d    | S19     | 0.94 |
| e    | S20     | 1.00 |
| f    | S11     | 1.03 |
| g    | S15     | 1.05 |
| h    | S18     | 1.07 |
| i    | S17     | 1.17 |
| j    | S10, S16| 1.38 |
| k    | S4, S8  | 1.43 |
| l    | S3, S13 | 1.48 |
| m    | S5, S9  | 1.69 |
| n    | S6      | 1.72 |
| o    | S7      | 1.78 |
| p    | S2, A   | 2.11 |
| q    | S1      | 2.80 |

*See Footnote a in Table I.
RP-HPLC of E. coli Ribosomal Proteins

Fig. 5. RP-HPLC of TP70. TP70 (442 pg) was dissolved in 16 µl of buffer B, injected onto a Synchropak RP-P column, and eluted with a gradient identical with that in Fig. 1 (dashed line). The upper and lower traces are absorbances at 214 nm and 280 nm, respectively. The upper case letters refer to the TP50 proteins in Table I and the lower case letters refer to the TP30 proteins in Table II. The arrow corresponds to a collection of small amounts of several TP50 proteins (see text).

![Graph showing RP-HPLC of TP70](image)

Fig. 6. Resolution of peak M (Fig. 1). Protein (~70 µg) eluting in peak M (Fig. 1) was lyophilized, redissolved in 10 µl of buffer B, and injected onto a Synchropak RP-P column equilibrated at 35% solvent B. After 15 min at 35% solvent B, a linear gradient was run from 35% solvent B to 40% solvent B in 60 min (dashed line). The upper and lower traces are absorbances at 214 nm and 280 nm, respectively.

![Graph showing resolution of peak M](image)

Fig. 7. Sucrose gradient centrifugation of reconstituted and native 30 S particles. Fractionation profiles for 36-ml sucrose gradient runs for native 30 S subunits (---), subunits reconstituted from acetic acid-extracted proteins (----), and subunits reconstituted from acetic acid-extracted proteins which had been eluted from a Synchropak RP-P column (-----) are shown. The direction of sedimentation is from left to right and the volume is given in milliliters from the top of the gradient.

![Graph showing sucrose gradient centrifugation](image)

TABLE III

| Experiment | 30 S subunit* | Extraction method* | HPLC treatment | Poly(U) | Phe-tRNA<sup>3</sup> binding | Relative Poly(U)-dependent Phe-tRNA<sup>3</sup> binding |
|------------|---------------|--------------------|----------------|---------|-----------------------------|-----------------------------------------------|
| 1          | N             | –                  | –              | –       | –                          | 5.4                                           |
| 2          | N             | –                  | –              | +       | 67.2                        | 1.00                                          |
| 3          | R  AA         | –                  | +              | –       | 21.6                        | 0.26                                          |
| 4          | R  LCU       | +                  | +              | –       | 18.6                        | 0.21                                          |
| 5          | R  LCU       | +                  | +              | –       | 16.0                        | 0.17                                          |
| 6          | R  LCU       | +                  | +              | –       | 13.2                        | 0.13                                          |

* N, native; R, reconstituted.  
* AA, acetic acid; LCU, LiCl-urea.

by their ability to bind Phe-tRNA<sup>3</sup> in a poly(U)-dependent manner. The activity of reconstituted 30 S subunits prepared from proteins extracted using either the acetic acid (16)- or LiCl-urea (17) procedures, and then subjected to HPLC treat-
ment, were compared with the activities of reconstituted 30 S subunits from extracted proteins not subjected to HPLC treatment. The results, presented in Table III, show only a minor loss in activity (<25%) as a result of HPLC treatment. The relatively low Phe-tRNA$^\text{Phe}$ binding for reconstituted 30 S subunits compared to native 30 S subunits was most likely due to partially degraded 16 S RNA.

CONCLUSION

The results presented in this paper establish RP-HPLC as the method of choice for both the analysis and preparation of E. coli ribosomal proteins. By comparison with the methods previously used for these purposes, polyacrylamide gel electrophoresis for analysis and either ion exchange or size exclusion chromatography for preparation, RP-HPLC offers at least equivalent resolution and reproducibility, and is clearly superior with respect to rapidity, recovery yields, and, with the use of the 214 nm monitor, sensitivity. In our own laboratory, we are currently making extensive use of the HPLC technique both to analyze proteins from photoaffinity-labeled ribosomes, and to prepare proteins for reconstitution experiments. Extension of the RP-HPLC method to study eucaryotic ribosomal proteins should be straightforward. It may also be useful in studying other complex cellular components having heterogeneous protein compositions.

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