CHROMSYMP. 1377

PURIFICATION OF TRANSFER RNA SPECIES BY SINGLE-STEP ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Anion-exchange high-performance liquid chromatography (HPLC) methods have been developed for the purification and concentration of milligram quantities of tRNA. A Waters Protein Pak DEAE 5PW 150 × 21.5 mm I.D. column was utilized for the separation of tRNA species. The chromatographic conditions chosen created non-denaturing conditions for separating the different species: 0.1 M Tris buffer (pH 7.6) at 25°C, with a 0.25 M to 0.4 M sodium chloride gradient, using a 170-min gradient. The gradient form could be adjusted for optimizing purification (to over 85%) of the tRNA species of interest. The same DEAE packing in a smaller column was found to be effective for concentrating solutions of the purified tRNA. Fifty-fold concentration and recoveries above 90% have been obtained by this method. These methods were successfully applied to the purification of individual tRNA species from both *Escherichia coli* and yeast.

INTRODUCTION

A method of purifying milligram amounts of a single species of tRNA is necessary for biophysical investigations, such as nuclear magnetic resonance (NMR) evaluation of tRNA internal motion as an isolated molecule and while interacing with other biomolecules[1]. However, purification of NMR samples of tRNA by conventional liquid chromatography (LC) requires multiple separations on different columns, and is flawed by moderate resolution and low yields of most tRNA species[2-4]. Reversed-phase chromatography has been found useful, but only if large or strongly hydrophobic functional groups are uniquely present on the tRNA to be purified[3,5,6]. Ion-exchange high-performance liquid chromatography (HPLC) is effective for the separation of small amounts of unfractionated tRNA into single species[7], but non-denaturing conditions are necessary because the column resolves tRNA species on the basis of their secondary and tertiary structures. Here, we describe the successful development of anion-exchange HPLC for the purification of relatively large quantities of tRNA species. With the increased scale necessary to obtain NMR samples,

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the HPLC separation presents an unexpected obstacle: purified tRNA solutions have low concentrations. Efficient concentration of the dilute, purified tRNA is essential, but conventional ethanol precipitation was found ineffective. An effective method for concentrating tRNA was developed, which utilizes the same HPLC column in a manner similar to that of a Bond-Elute coumn[8].

EXPERIMENTAL

HPLC

Chromatography was performed with a Waters 600 multisolvent delivery system (Milford, MA, U.S.A.) and a Waters 480 detector. This quaternary solvent system was ideally suited for loading larger sample volumes. Volumes as large as 500 ml of dilute, purified tRNA were introduced into the system by using one of the solvent lines for sample loading. Data were recorded on a Houston chartrecorder (Austin, TX, U.S.A.). Fractions were collected using a Gilson Model 201 fraction collector (Worthington, OH, U.S.A.).

A Waters Protein Pak DEAE 5PW, 150 × 21.5 mm I.D. column was used for the separation of the tRNA. A smaller, 75 × 7.5 mm I.D. column was used for concentrating the tRNA. The low-salt buffer (reservoir A) for equilibrating and washing the columns, as well as for elution gradients consisted of 0.25 M sodium chloride–0.1 M Tris (pH 7.6). The high-salt buffer (reservoir B) was 0.5 M sodium chloride–0.1 M Tris (pH 7.6). All work was performed at ambient temperatures (20–25°C). The purification of *Escherichia coli* tRNA^{Gln}-2 required optimization of the gradient conditions. These are outlined in Table I. The initial chromatographic conditions ensure adsorption of the tRNA to the packing material. The initial reduced flow-rate is employed to eliminate an observed pressure surge present at the time of injection when higher flow-rates are used. The modest gradient slope from time 5 to 20 min results in the elution of a few tRNA species. The slow gradient change from time 25 to 85 min is to allow the majority of the tRNA species to elute

TABLE I

HPLC SOLVENT AND GRADIENT CONDITIONS FOR SEPARATION OF INDIVIDUAL ISOACCEPTING SPECIES FROM UNFRACTIONATED *E. coli* tRNA

Eluent A, 0.25 M sodium chloride–100 mM Tris (pH 7.6); eluent B, 0.50 M sodium chloride–100 mM Tris (pH 7.6).

Time (min)	%A	%B*	Flow-rate (ml/min)
0	90	10	3.0
5	90	10	4.5
25	76	24	4.5
85	67	33	4.5
100	63	37	4.5
140	0	100	4.5
150	0	100	4.5
170	90	10	4.5

* Gradient A to B was always linear.

with some resolution. It is in this region that additional slowing of the gradient or plateaus can be added to optimize for isolation of other tRNAs. From time 85 to 100 min the slope of the gradient was increased. This increase shortens the run time without greatly affecting the resolution of the tRNA species. The rapid slope of the gradient from time 100 to 140 min removes other tRNA and RNA not of interest in this separation. The remainder of the gradient is used to return the column back to initial conditions. The sample volumes ranged from 600 μl to 2 ml.

For the separation of yeast tRNA^{Phe}, the column was equilibrated with eluent A at a flow-rate of 3 ml/min. The large sample volume (400 ml) was introduced into the system through the third solvent line of the quaternary system at a flow-rate of 4.5 ml/min. After the entire sample had been loaded onto the system, this solvent line was rinsed with an additional 50 ml of eluent A. Eluent A was continued for another 5 min before initiating the gradient. A linear gradient from 100% A to 100% B over 180 min was determined to be optimal for the separation. A column load for separation of 8 mg total RNA was routinely used. When a load of 10 mg was used, a loss of resolution was observed. The high flow-rates (3–4 ml/min) were used to eliminate empirically observed size-exclusion effects observed when this gradient was run at lower flow-rates.

Concentration of the tRNA was most effectively accomplished with the smaller DEAE column. Before introducing the sample into the system, the column was equilibrated with eluent A at a flow-rate of 1.15 ml/min. Sample were loaded onto the system through the third solvent line. Sample volumes ranged from 10 to 200 ml. After the entire sample was loaded onto the system, this solvent line was rinsed with 10–15 ml of eluent A. Eluent A was maintained for approximately another 5 min. Then a 5-min linear gradient, finishing at 100% eluent B was initiated. The column effluent was manually collected in two fractions. The front and tail of the eluate peak were collected as one fraction, containing eluate with absorbance values (A_{254} units) of 0.01 to 0.20 a.u. Eluate with absorbance values above 0.20 a.u. was collected as the second fraction. A column load of 1.2 mg tRNA gave recoveries greater than 95%. When larger loads were used, a reduced recovery was observed.

All reagents and solvents were of HPLC (or electrophoresis) grade. Water was prepared by reverse osmosis, passed through ion exchangers (Technic Lab Five, Seattle, WA, U.S.A.), and subjected to final purification through a DuPont Nylon 66 0.22-μm membrane (Wilmington, DE, U.S.A.). HPLC solutions were sparged with helium.

Gel electrophoresis

Effectiveness of the HPLC separation was evaluated by electrophoresis of the eluates. Fractions from HPLC were routinely analyzed by 7 *M* urea–15% polyacrylamide gel electrophoresis (PAGE). Analyses were typically run long enough so that variations of a single base length could be determined.

Nucleoside analysis

Quantitative and qualitative analysis was performed in the laboratory of Dr. C. Gehrke (Cancer Research Center, Columbia, MO, U.S.A.). The method is well documented[9,10] and the sensitivity is such that sequence changes and modifications to single bases can be easily determined.

Samples

Bulk unfractionated *E. coli* tRNA used during optimization experiments was obtained from Sigma (St. Louis, MO, U.S.A.). The ^{13}C-enriched *E. coli* tRNA sample was isolated from cultures grown in our laboratory. It was a K12 derivative provided by Dr. W. Fiers (Laboratorium voor Moleculaire Biologie, Ghent, Belgium). Into this cell strain was inserted a plasmid, containing the tandem repeat of a gene for tRNA[Gln]-2 under the control of the pL promoter[11]. This gene construction was made by Dr. R. Swanson (Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, U.S.A.). This cell strain also contains the heat-sensitive C1 repressor protein that allows the cloned tRNA to be expressed only by heat induction. The repressor protein binds to the DNA at or near the promotor sequence inhibiting the host's polymerase from read-through of the sequence. All the tRNAs were labeled at the pyrimidines and modified methyl groups by culturing the cells in a medium containing [2-^{13}C]uracil and [methyl-^{13}C]methionine[12]. Yeast tRNA samples were also produced in our laboratory. *Saccharomyces cerevisiae* was grown in a medium containing the ^{13}C-precursor for enrichment of only the methyl carbons[13]. The yeast strain was a well documented methionine auxotroph[12]. The tRNA prepared from cultures grown in our laboratory was isolated by a procedure similar to that published earlier[12].

Aminoacylation

Aminoacylation for Gln and other tRNA from *E. coli,* and Phe and other tRNAs from yeast were accomplished with the homologous synthetases[3,4]. Glutaminyl-tRNA synthetase was obtained from an *E. coli* strain engineered to overproduce the protein. The strain was a gift from Dr. D. Soll (Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, U.S.A.).

RESULTS AND DISCUSSION

After work with the Waters Protein Pak DEAE 5PW column had shown that separation of tRNA into individual species was possible[7], we became interested in developing the methodology for purifying large quantities of tRNA species, in general. We were particularly interested in recovering, in a purified form, large quantities of ^{13}C-enriched tRNA[Gln]-2 from an *E. coli* strain that had been engineered to overproduce that single species of tRNA. Initial work included development of an elution gradient that would yield an acceptable separation of semi-preparative amounts of commercially prepared, unfractionated tRNA. Eluate fractions that contained tRNA[Gln] were identified by aminoacylation. After this methodology had been developed, ^{13}C-enriched, unfractionated tRNA including the overproduced tRNA[Gln]-2 was subjected to it. The presence of the single, overproduced tRNA sufficiently altered the chromatographic profile (A_{254}) as to allow identification of the glutamine tRNA. Aminoacylation of fractions, unique to the ^{13}C-enriched sample, confirmed the glutamine tRNA peak.

The separation gradient was then optimized for the isolation of tRNA[Gln]-2. To evaluate the effectiveness of different gradient conditions, fractions collected from each gradient elution were subjected to 7 *M* urea–15% PAGE. Fig. 1A depicts a chromatogram obtained with the gradient that gave the best quantitative purification
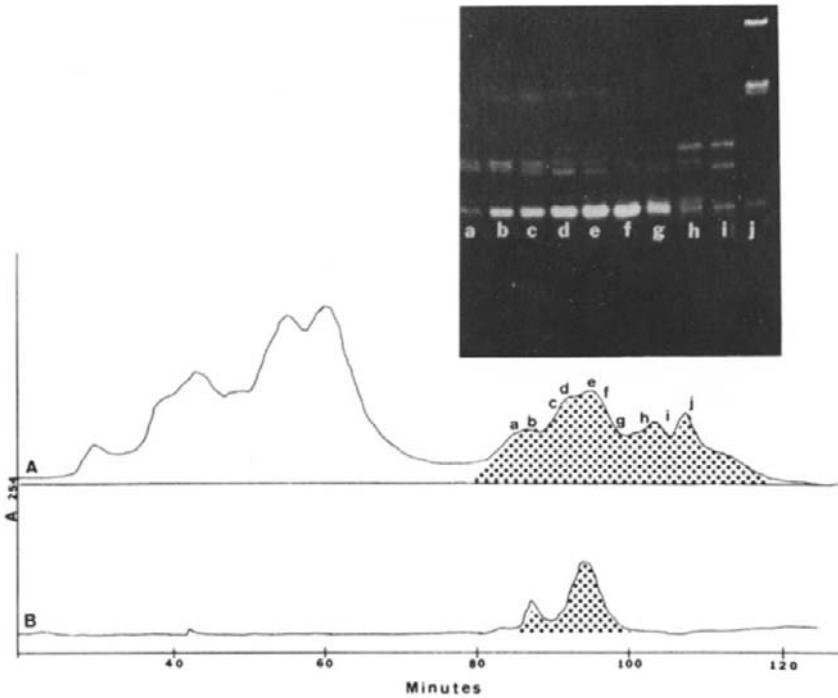
Fig. 1. Separation of *E. coli* tRNAs by anion-exchange HPLC. (A) Unfractionated tRNA (5 mg) from cells engineered to overproduce tRNA^{Gln}-2 was eluted from a DEAE column by the gradient outlined in Table I, Chromatogram A. Inset, results from PAGE analysis of fractions collected in the shaded region of chromatogram A. Lower case letters denote the location within the chromatographic profile at which the fractions were collected. (B) Rechromatography of a sample obtained by pooling fraction (b–g) of chromatogram A. Conditions for B were identical to those for A.

of tRNA^{Gln}-2. The inset in the figure is a photograph of the ethidium bromide-stained gel of the collected fractions. Letters denote the place in the gradient from which the fractions had been taken. When fractions b–g, collected from this separation, were rechromatographed with the same gradient, the elution position in the gradient was retained (see Fig. 1B). Depending on the degree of purity and quantity of sample required, varying numbers of fractions may be pooled. For fractions with high concentrations but unacceptable purity, a second separation with the same gradient has been found to increase the yield of the desired tRNA at a higher purity. A small amount of single-species tRNA with a purity of up to 95% is obtainable from a single gradient elution (Fig. 1, PAGE lane f). PAGE was run on fractions from other peaks in the chromatogram in Fig. 1A. They show separation of single tRNAs with varying degrees of purity. The total RNA recovered from the chromatogram was consistently in excess of 85% of that applied to the column. When a total of 68 mg of unfractionated *E. coli* tRNA was subjected to anion-exchange HPLC, it yielded 7.6 mg of pure tRNA^{Gln}-2. The elution conditions may be modified to optimize the isolation of other tRNA species from *E. coli* or other organisms, as we have done for yeast tRNA^{Phe}.

The method was also applied to the separation of yeast tRNA. Chromatography of unfractionated yeast tRNA resulted in a profile of individual tRNAs similar to that of *E. coli* tRNAs, but required a different salt gradient. This difference in elution condition may be the results of differences in secondary and tertiary structure consistent with NMR observations[13]. Fig. 2 shows a chromatogram of the purification of yeast tRNA^{Phe} from a semi-pure sample of tRNAs. (The tRNA sample originated from fractions of unacceptable purity, collected from chromatography on a benzoylated DEAE cellulose column, a traditional method of tRNA purification)[4].

High flow-rates, shallow gradients, and relatively low loads were found to yield purified tRNA in relatively large volumes (50–200 ml) at low concentrations (5–25 μg/ml). The conventional method for concentrating tRNA is a precipitation with no less than two volumes of ethanol. When the HPLC fractions were treated with ethanol, tRNA was not precipitated. However, the same Protein Pak DEAE column could be used for concentrating tRNA samples. Large volumes of dilute, purified tRNA could be passed through the column and the bound tRNA eluted in small volumes. The only pretreatment of the sample was dilution of the sample (40–60%) with enough HPLC-grade water to lower the ionic strength to reestablish the affinity
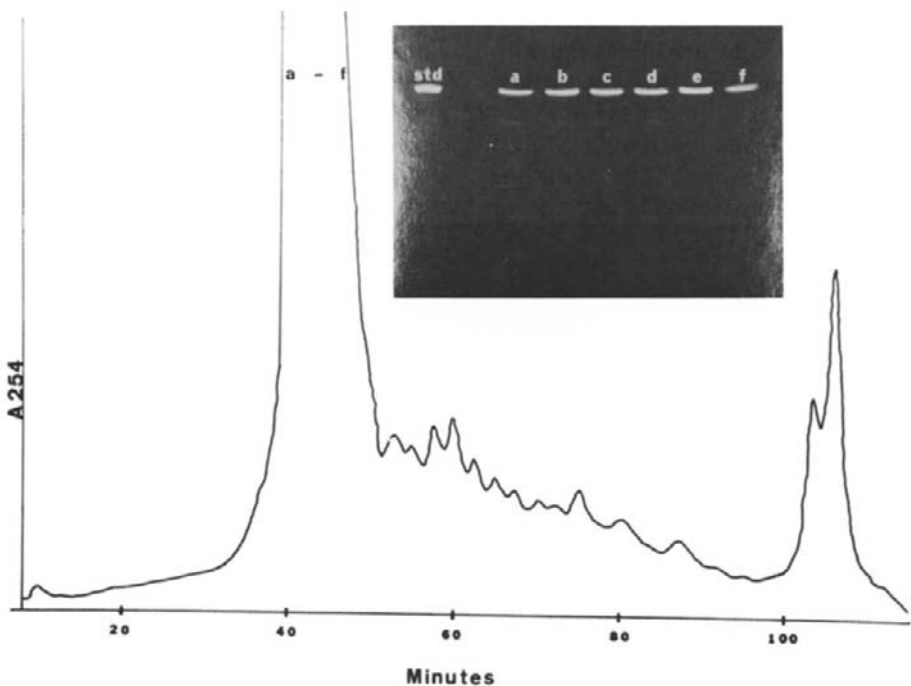

Fig. 2. Chromatogram of the purification of yeast tRNA^{Phe} by anion-exchange HPLC. Yeast tRNA^{Phe} (400 ml) of low purity from benzoylated DEAE chromatography was further purified by HPLC. Inset, PAGE of fractions collected during the purification. Lower case letters denote location in the chromatogram where the fractions were collected. Chromatographic conditions included: (a) column equilibration with 0.25 M sodium chloride–0.1 M Tris (pH 7.6); (b) sample loading at an approximate ionic concentration of 0.15 M sodium chloride; (c) tRNA elution with a 180-min linear gradient from 0.25 M sodium chloride–0.1 M Tris (pH 7.6) to 0.5 M sodium chloride–0.1 M Tris (pH 7.6).
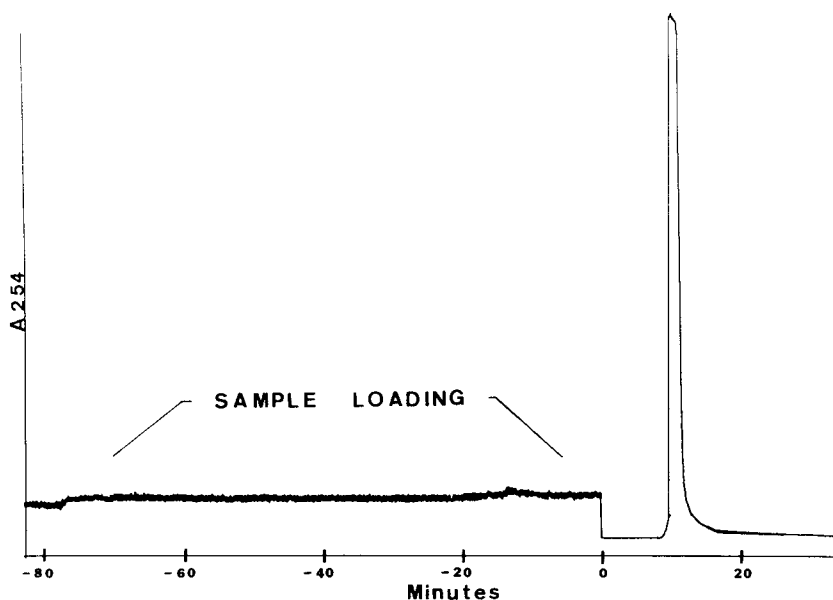
Fig. 3. Concentration of purified tRNA by anion-exchange HPLC. Purified tRNA (1.5 mg in 80 ml) obtained by HPLC, pretreated by dilution (40%) with HPLC-grade water, was sorbed on the DEAE column at low ionic strength (*ca.* 0.2 M sodium chloride) and desorbed in a reduced volume (2.6 ml) by 0.5 M sodium chloride, buffered with Tris.

TABLE II

NUCLEOSIDE COMPOSITION ANALYSIS

Biological activity of tRNA (isolated and concentrated by HPLC) assessed by glutamine aminoacylation: unfractionated tRNA, cpm/A_{260} = 2940; 20-fold purified $tRNA^{Gln}$-2, cpm/A_{260} = 59 600.

Purified tRNA nucleosides	Number of residues per sequence of 75 residues		
	Observed	Modification correction*	Theoretical
C	22.3	22.3	23
U	10.7	9.0	9
G	21.6	21.0	20
A	14.0	13.5	14
dU	1.2	1.2	1
T	1.0	1.0	1
s4U	0.8	1.0	1
Psue	2.4	3.0	3
Um	0.1	1.0	1
Gm	0.4	1.0	1
m2A	0.5	1.0	1
Total	75.0	75.0	75

  * Due to incomplete modification of some of the nucleosides, a correction to normalize to complete modification by subtracting from the corresponding major nucleoside and adding to the modified nucleoside was performed.

of the tRNA to the column. Fifty-fold concentration and recoveries of over 90% have been obtained by this procedure. Fig. 3 is a representative chromatogram of the concentration step. The sensitivity of the detector (A_{254}) was set high during sample loading to make sure that the tRNA was not lost during this step. After the entire dilute sample had been pumped into the column, the sensitivity of the detector was reduced by a factor of 20 in order to keep the tRNA recording on-scale.

The purified tRNAs were found to be structurally intact and biologically active. The biological activity was assessed by aminoacylation. Results of the glutaminyl aminoacylation of unfractionated tRNA and the purified tRNA^{Gln} may be found in Table II. More than twenty-fold increase in activity was observed in the purified species. Purity and structural integrity of the tRNA after HPLC separation was evaluated in three ways. Urea–PAGE confirmed that in addition to high purity, sequence length and integrity present before purification remained unchanged. Major and modified nucleoside analysis was used to determine purity by comparison to that of the known sequence. Table II also lists the results of the nucleoside analysis of 25 μg of purified tRNA^{Gln}-2. The apparent under modification of the end product (Table II) is probably the result of tRNA-modifying enzymes incapable of matching the overproduction of the tRNA transcript during gene induction. Nucleoside analysis of the initial, unfractionated tRNA showed undermodification to be present before purification. Nucleoside analysis of tRNA^{Gln}-2 that had been purified by the methods described here, but obtained from a differently engineered overproducing cell line, was found to be more completely modified[14]. Both ^{1}H and ^{13}C NMR spectroscopy confirmed the integrity and purity of the molecule, as well.

CONCLUSIONS

A single-species tRNA can be successfully purified in milligram quantities with the aid of DEAE anion-exchange HPLC. The tRNA species can be obtained in high purity in one chromatographic step. Increased purity is achieved by rechromatography on the same column. One of the principal obstacles to obtaining high yields of tRNA after purification is overcome by using the DEAE column to concentrate the tRNA solution. Table III outlines the recoveries of tRNA from this purification method, starting with completely unfractionated tRNA and ending with milligram quantities of purified tRNA in a small volume of salt-free solution, suitable for NMR analysis.

TABLE III

STEPWISE AND FINAL RECOVERY OF tRNA AFTER DEAE CHROMATOGRAPHY

Procedure	Step recovery (%)	Cumulative recovery (%)
Initial separation starting with 68 mg	85.0	85.0
Concentration	95.0	80.8
Dialysis	85.0	68.7
Lyophylization	98.0	67.3

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