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High-performance liquid chromatography of transfer ribonucleic acids on spherical hydroxyapatite beads

YOSHIO YAMAKAWA*
Department of Applied Immunology, National Institute of Health, Kamiosaki 2-10-35, Shinagawa-ku, Tokyo 141 (Japan)
KENJI MIYASAKA and TOSHIHIRO ISHIKAWA
New Venture Business Department, Toa Nenryo Kogyo Co., Hitotsubashi 1-1-1, Chiyoda-ku, Tokyo 100 (Japan)
YUKO YAMADA
Laboratory of Chemistry, Jichi Medical School, Minamikawauchi machi, Kawauchi-Gun, Tochigi 320-04 (Japan)
and
TSUNEO OKUYAMA
Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Fukazawa 2-1-1, Setagaya-ku, Tokyo 158 (Japan)

SUMMARY

High-performance liquid chromatography (HPLC) on newly developed spherical beads of hydroxyapatite was applied to the analysis of purified *E.coli* tRNAs (Val, Met, Tyr and Phe). tRNAs were eluted from the column spearately with appreciable differences in retention time by a 45-min gradient of phosphate buffer (pH 6.8) of concentration from 64 to 123 m*M*; both the retention times and peak areas of respective tRNAs were highly reproducible. Total tRNA ($tRNA^{Total}$) preparations obtained from *E.coli* and *B. subtilis* were also analysed on the column. It is possible even to elute $tRNA^{Total}$ which may, in general, contain 60 or more tRNA species with a relatively shallow gradient such as 75–132 m*M*. The recovery of tRNA from the column was as high as 90%. Owing to the complicated composition, the elution profile of $tRNA^{Total}$ had a wide spread irregular shape but, with a 1-h gradient more than ten peaks were easily detected. When the amino acid-accepting activity of tRNA in the eluate of $tRNA^{Total}$ was determined, for ten specific tRNAs, each activity peak was eluted sharply from the column. In addition, several tRNA activities were eluted in different fractions. This indicates that isoacceptors were separated by the column. The results show that HPLC on hydroxyapatite beads is useful for the purification and characterization of tRNA.

INTRODUCTION

Adsorption chromatography on hydroxyapatite (HAP), introduced by Tiselius *et al.*[1], is sometimes a very effective process for purification of biomacromolecules

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such as proteins and nucleic acids. However, the method was not widely accepted because the synthesis of HAP with reproducible properties was difficult. Currently, spherical beads of HAP, a new type of ceramic which has a rigid structure and uniform quality, are being produced by several manufacturers. Basic studies on some of these new ceramic HAP beads for high-performance liquid chromatography (HPLC) have been reported by Kadoya *et al.*[2]. We have also reported on the easy purification of mouse monoclonal antibodies, especially IgM, using HPLC on HAP beads[3]. The results of the various studies indicate that HPLC on HAP is a valuable method for the purification and analysis of biological polymers. We now report the application of this method to the separation of tRNAs of *E. coli* and *B. subtilis*.

EXPERIMENTAL

tRNAs

Purified *E. coli* tRNAs (Val, Lot No. 42F-05392; f-Met, Lot No. 86F-05121; Tyr, Lot No. 67F–02831; Phe, Lot No. 24F-03761) were purchased from Sigma (St. Louis, NO. U.S.A.). Total tRNAs ($tRNA^{Total}$) was prepared from *E. coli* K12 and *B. subtilis* W168 as described by Zubay[4]. Purified tRNA was dissolved in 5 m*M* phosphate buffer (pH 6.8) at about 0.5 mg/ml and was stored at 5°C. $tRNA^{Total}$ was also dissolved in the same buffer at a concentration of about 1.5–2 mg/ml. The concentration of tRNA was determined spectrophotometrically with the use of the value $E_{1cm}^{0.1\%} = 20$ at 260 nm.

Determination of amino acid-accepting activity

The preparation of crude *E. coli* aminoacyl-tRNA synthetase and the assay of the amino acid-accepting activity of tRNA with ^{14}C-labelled amino acids were carried out as described by Nishimura *et al.*[5].

Columns

Packed columns (10 × 0.75 cm I.D. and 10 × 2.14 cm I.D.) of HAP were obtained from Toa Nenryo Kogyo (Tokyo, Japan). A narrow bore column packed with 2.2-μm HAP beads was used for routine analysis, whereas a large-bore column packed with 5-μm beads was used for semi-preparative purposes.

Apparatus

A Shimadzu LC-6A liquid chromatograph with a two-pump gradient system was used. Elution of nucleic acids was monitored with a SPD-6A UV detector at 260 nm and a Chromatopak C-R6A recorder. Samples were introduced into the column with either a SIL-6A automatic sample injector or a Rheodyne 7125 sample injector. The eluate was collected manually according to the recorder signal.

Elution

Elution of tRNA from the column was performed with a linear gradient of phosphate buffers (pH 6.8) of concentration between (A) 5 m*M* and (B) 300 m*M*. With a gradient from 20 to 45 B, which corresponds to phosphate concentrations from 75 to 138 m*M*, it was possible to elute tRNAs from the column. The analytical column was eluted with a flow-rate of 0.5 ml/min (*ca.* 20 kg/cm^2) and the semi-preparative column at 4.0 ml/min (*ca.* 5 kg/cm^2). Chromatography was carried out at room temperature.

RESULTS

Chromatography of E. coli tRNAs

Fig. 1 shows typical elution profiles of the purified *E. coli* tRNAs on an analytical HAP column. tRNAs were eluted from the column with different retention times by a shallow gradient (20–40% B, corresponding to 64–123 m*M* phosphate) of phosphate buffer (pH 6.8). It was found that $tRNA^{f\text{-}Met}$ and $tRNA^{Val}$ were eluted as relatively sharp peaks whereas $tRNA^{Tyr}$ was eluted as a broad peak and $tRNA^{Phe}$ as two distinct peaks. The phenylalanine-accepting activity in the eluate of $tRNA^{Total}$ of *E. coli* was determined in the same manner, and showed two distinct peaks (see Fig. 6). Chromatography of an equimolar mixture of three tRNAs (f-Met, Val and Phe) was performed successfully using a similar gradient (see Fig. 2b).

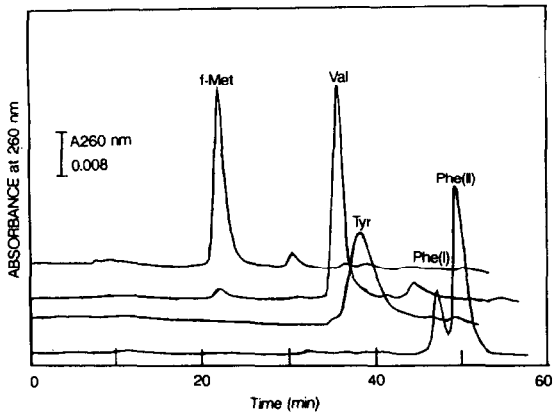

Fig. 1. Chromatography of *E. coli* tRNAs on an HAP column. Four purified tRNAs (about 4 μg) were applied to a column (10 × 0.75 cm I.D.) of HAP which had been equilibrated with 20% B. The column was eluted at a flow-rate of 0.5 ml/min with a 45-min linear gradient of phosphate buffer (pH 6.8) from 20 to 40% B. A, 5 m*M* phosphate buffer pH 6.8); B, 300 m*M* phosphate buffer (pH 6.8).

Reproducibility

To determine the reproducibility of the chromatography of tRNAs on the HAP column, an equimolar mixture of purified tRNAs (f-Met, Val and Phe) was analysed four times on the column. Both the retention times and peak areas of were highly reproducible (Table I).

TABLE I

REPRODUCIBILITY OF RETENTION TIMES AND PEAK AREAS OF PURIFIED *E. coli* tRNAs

tRNA	*Retention time (min)*[a]	*Peak area (μV)*[a]
f-Met	21.89 ± 0.3	38 650 ± 145
Val	32.37 ± 0.4	33 654 ± 1053
Phe I	44.32 ± 0.4	11 589 ± 468
Phe II	46.01 ± 0.3	26 574 ± 724

[a] Means of four runs ± S.D.

Chromatography of tRNA^{Total}

Fig. 2a shows a typical elution profile of *E. coli* tRNA^{Total} on an analytical column when eluted with a 45-min gradient of phosphate buffer from 23 to 45% B. For comparison, the elution profile of a mixture of three purified tRNAs analysed under the same conditions is shown in Fig. 2b. It was possible to elute tRNAs from the column with a shallow gradient of 23–45% B because as much as 95% of the material applied to the column was recovered in the eluates. Comparing these chromatograms, it appeared that tRNA$^{f\text{-}Met}$, tRNA^{Val} and tRNA^{Phe} were eluted in the early, middle and late parts of the tRNA^{Total} chromatogram, respectively. The order of elution of respective tRNAs in tRNA^{Total} from the HAP column was verified by determining the amino acid-accepting activity in the eluate (see Fig. 6). The elution profile of tRNA^{Total}, recorded at 260 nm, had a widespread irregular, shape, as tRNA^{Total} usually contains about 60 species of molecules, including isoacceptors. Therefore, we tried to improve the resolution by extending the gradient time. Comparative chromatograms of *E. coli* tRNA^{Total} obtained with the extended gradient time are shown in Fig. 3. The overall elution profile is still similar but a minor improvement in the resolution was achieved.

Chromatography of *B. subtilis* tRNA^{Total} was also performed under similar conditions, as shown in Fig. 4a. Separation of peaks seemed more evident in comparison with the chromatogram of *E. coli* tRNA^{Total}. To verify the peak positions, several fractions (hatched areas in Fig. 4a) were diluted 3–5-fold with buffer A and rechromatographed under the same conditions. These results are shown in Fig. 4b. The respective fractions were eluted from the column with retention times identical with those obtained in the first chromatography.

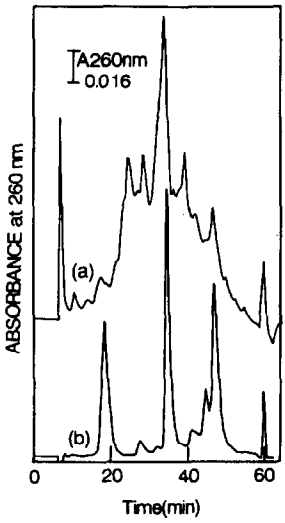

Fig. 2. Chromatography of (a) tRNA^{Total} and (b) a mixture of purified tRNAs of *E. coli* on an HAP column. (a) Total tRNA (about 40 μg) of *E. coli* was applied to a column (10 × 0.75 cm I.D.) of HAP which had been equilibrated with 23% B. The column was eluted at a flow-rate of 0.5 ml/min with a 60-min linear gradient of phosphate by increasing the fraction of buffer B to 43%. Buffers A and B as in Fig. 1. (b) A mixture of three purified tRNAs (f-Met, Val and Phe; 10 μg each) was chromatographed under the same conditions as in (a).

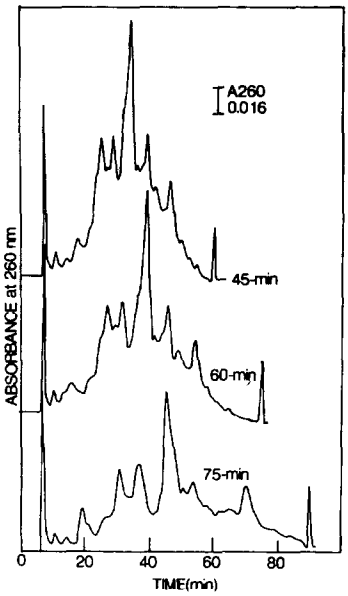

Fig. 3. Effect of extending the gradient time on the resolution of $tRNA^{Total}$ on HAP chromatography. $tRNA^{Total}$ of *E. coli* (about 40 μg) was applied to a column of HAP and eluted at different gradient times as indicated. Conditions in Fig. 2 except for the gradient time.

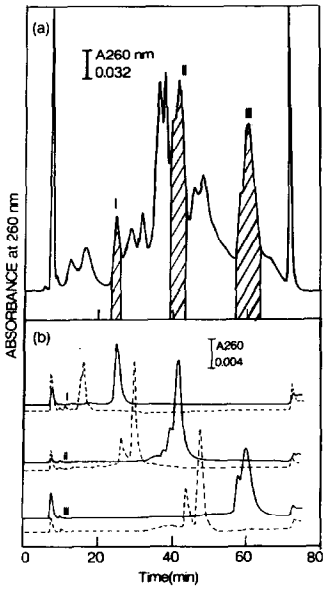

Fig. 4. (a) Chromatography of $tRNA^{Total}$ of *B. subtilis* on an HAP column and (b) rechromatography. (a) $tRNA^{Total}$ (80 μg) of *B. subtilis* was applied to a column of HAP which had been equilibrated with 22% B. The column was eluted at a flow-rate of 0.5 ml/min with a 60-min linear gradient of phophate by increasing the fracton of buffer B to 39%. Buffers A and B as in Fig. 1. (b) Each fraction [hatched in (a)] was diluted 3–5-fold with buffer A and then rechromatographed under the same conditions as in (a); the dashed lines indicate rechromatography performed using buffers containing 0.1 *M* sodium chloride.

Effects of sodium chloride on chromatography

Recently, Lindeberg *et al.*[6] found that the resolution of aminoacyl-tRNAs in HAP chromatography is improved by the addition of 0.1 *M* sodium chloride to phosphate buffers. To confirm this, we performed rechromatography of the same fractions as used in the previous investigation with phosphate buffers including 0.1 *M* sodium chloride. The resulting chromatograms are shown in Fig. 4b (dashed lines). Even though the same phosphate gradient was used, shaper peaks were obtained and the resolution between the main and minor component(s) in the respective fractions was clearly improved. However, it must be emphasized that peaks were eluted at lower phosphate concentrations (about 10 m*M*), which may indicate that the interaction between tRNA and HAP was reduced by sodium chloride. With the expectation of an improved resolution, $tRNA^{Total}$ of *B. subtilis* was again chromatographed in phosphate buffers containing 0.1 *M* sodium chloride. The chromatogram is shown in Fig. 5. Comparison with Fig. 4 indicates that the elution order of the significant peaks was not altered but the peaks became narrower on addition of sodium chloride to the phosphate buffers.

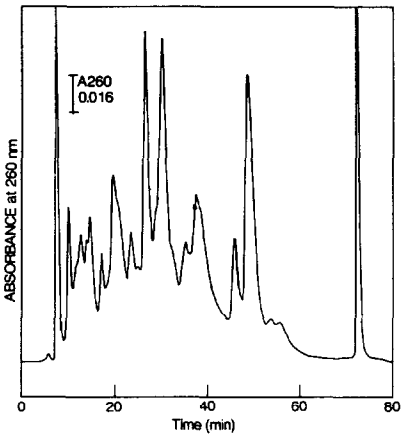

Fig. 5. Chromatography of $tRNA^{Total}$ of *B. subtilis* in phosphate buffer containing sodium chloride. $tRNA^{Total}$ (about 60 μg) of *B. subtilis* was applied to a column of HAP which had been equilibrated with 22% B. The column was eluted as described previously (22–39% in 60 min; flow-rate, 0.5 ml/min) with a gradient of phosphate buffer containing 0.1 *M* sodium chloride. A, 5 m*M* phosphate buffer (pH 6.8) containing 0.1 *M* sodium chloride; B, 300 m*M* phosphate buffer (pH 6.8) containing 0.1 *M* sodium chloride.

Amino acid-accepting activity of tRNAs after separation with the semi-preparative column

To determine the amino acid-accepting activity after separation by chromatography on HAP, 5 mg of *E. coli* $tRNA^{Total}$ were loaded on the 2.14-cm I.D. column and the specific amino acid-accepting activity in the eluates was measured. The results are shown in Fig. 6. The resolution of $tRNA^{Total}$ monitored at 260 nm was poor in comparison with the chromatogram from the analytical column (*cf.*, Fig. 2). However, the amino acid-accepting activity for each tRNA was eluted in a reasonably narrow peak, except for valine-accepting activity. tRNAs such as $tRNA^{Met}$, $tRNA^{Val}$,

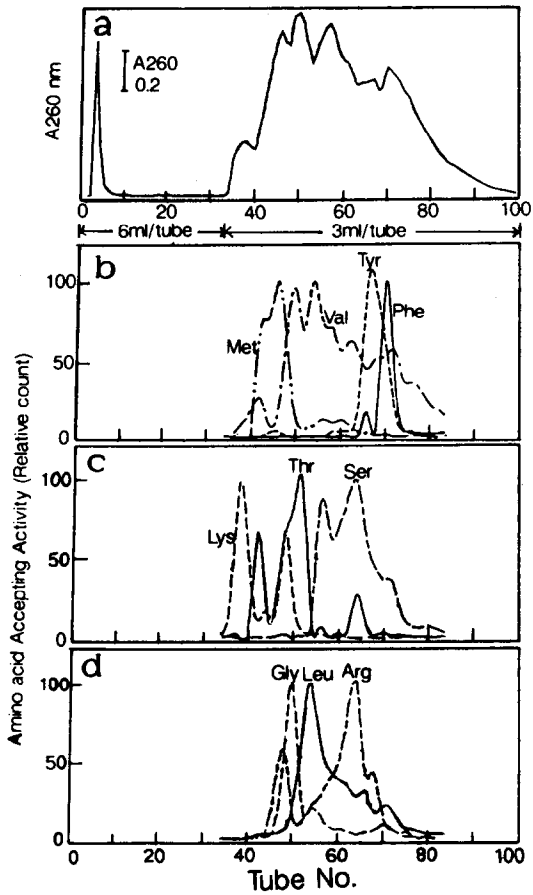

Fig. 6. Chromatography of $tRNA^{Total}$ of *E. coli* on a semi-preparative column of HAP. $tRNA^{Total}$ (5 mg) of *E. coli* was applied to the column of HAP (10 × 2.14 cm I.D.) which had been equilibrated with 20% B. The column was eluted with a 120-min linear gradient of phosphate by increasing the fraction of buffer B to 40%. Fractions of 6 or 3 ml were collected by a fraction collector to determine the absorbance at 260 nm and the amino acid-accepting activities. The buffers did not contain sodium chloride.

$tRNA^{Tyr}$ and $tRNA^{Phe}$ contained in $tRNA^{Total}$ were eluted from the column in the order expected from the results in Fig. 1. Two or more peaks were found for each amino acid-accepting activity, which indicates that isoacceptors of tRNA were separated by the chromatography.

DISCUSSION

As $tRNA^{Total}$ contains various tRNA molecules with physico-chemically similar properties, sevel chromatographic steps with various retention modes were used to purify the desired tRNA. All the results described here indicate that HPLC on spherical HAP beads is useful and effective for the purification and characterization of tRNAs. With a gradient from 75 to 140 m*M* phosphate buffer (pH 6.8), it was

possible to elute tRNAs from the column but fine adjustment of the gradient (only a few percent) might be necessary, depending on the the columns. $tRNA^{Total}$ of baker's yeast was also chromatographed using the same column under similar conditions.

The resolution of tRNAs was improved by the addition of sodium chloride to the phosphate buffer. Further attempts should be made to improve the resolution. From this point of view, it would be interesting to study chromatography at different pH values or with buffers containing reagents that might cause conformational changes in tRNA molecules, such as an alcohol. With divalent cations, for example, the addition of 10 m*M* magnesium chloride should be avoided as the column loses its original properties, as suggested by Lindeberg *et al.*[6]. We also confirmed similar phenomena. In this connection, it is recommended that magnesium chloride, which is occasionally added for stabilization of tRNAs, be removed from the sample prior to chromatography.

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