

Notes & Tips

Single-step purification of specific tRNAs by hydrophobic tagging

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Studies on translation frequently require large amounts of purified individual tRNAs. However, few purified tRNAs are commercially available. Individual tRNAs can be easily produced by *in vitro* transcription, but the lack of modifications of the tRNA transcripts may influence or impair their function [1,2]. The purification of specific tRNAs from total tRNA is a laborious process that requires several chromatographic steps [3]. Therefore, developing a simple and rapid method for purification of specific tRNA is of great importance. Here we describe a method for purification of individual tRNAs based on selective tagging of the amino group of specifically charged aminoacyl-tRNAs (aa-tRNAs)¹ with 9-fluorenylmethylsuccinimidylcarbonat (FmocOSu) (Fig. 1), followed by a single chromatographic purification step, using reversed-phase HPLC or hydrophobic interaction chromatography. The method is suitable for all tRNAs and allows up to 20-fold enrichment of a specific tRNA in less than 1 day effective working time. The materials used are readily available at low cost.

We tested the procedure for two different tRNAs from *Escherichia coli*: tRNA^{Ala} and tRNA^{Sec} (the latter being tRNA specific for selenocysteine). As starting material, we used total tRNA, which is commercially available or can be prepared by standard procedures [4]. For the purification of tRNA^{Ala}, we used total tRNA from MRE 600 cells that contained approximately 3% tRNA^{Ala} according to charging with [¹⁴C]alanine in an analytical aminoacylation assay. tRNA^{Sec} is a rare tRNA that is hardly detected in total tRNA (<1%). To increase the amount of tRNA^{Sec} in the initial tRNA preparation, we overproduced tRNA^{Sec}

in BL21(DE3) cells [5] and isolated total tRNA from these cells. tRNA^{Sec} and tRNA^{Ser} in total tRNA can be aminoacylated with serine by seryl-tRNA synthetase [5], yielding Ser-tRNA^{Sec} and Ser-tRNA^{Ser} [6]. (Further conversion of Ser-tRNA^{Sec} to Sec-tRNA^{Sec} requires a number of additional factors and is not addressed here.) Analytical aminoacylation of total tRNA enriched in tRNA^{Sec} with [¹⁴C]serine indicated the presence of approximately 30% tRNA^{Sec/Ser} in total tRNA. For preparative aminoacylation, 50–100 μM total tRNA, 3% (v/v) S100 fraction as a source of aa-tRNA synthetases [7], 3 mM ATP, 50–80 μM ¹⁴C-labeled amino acid (alanine [MP Biomedicals] or serine [Moravic Biochemicals]), and 0.005 U/μl inorganic pyrophosphatase (Sigma) were incubated for 60 min at 37 °C in aminoacylation buffer (50 mM Hepes [pH 7.5], 30 mM KCl, 10 mM MgCl₂, and 2 mM dithiothreitol [DTT]). For the following purification steps, it is crucial to obtain specific charging by only one given amino acid. To avoid charging of other tRNAs, the preparation of aminoacyl-tRNA synthetase must be absolutely free of endogenous amino acids, and this can be achieved by a dialysis step. Alternatively, purified aminoacyl-tRNA synthetases can be used. The aminoacylation efficiency was controlled in an aliquot of the reaction mixture by trichloroacetic acid (TCA) precipitation, filtration through a nitrocellulose filter (Sartorius), and scintillation counting in Quickszint 361 cocktail (Zinsser Analytic). Potassium acetate (pH 4.5) was added to the reaction mixture to a final concentration of 0.3 M, followed by extraction with an equal volume of water-saturated phenol to remove proteins. The aa-tRNA was precipitated from the aqueous phase with 2.5 volumes of cold ethanol, and the pellet of aa-tRNA was dissolved in 0.1 M sodium acetate (pH 4.5) [8].

In principle, individual tRNAs (or aa-tRNAs) can be separated by chromatography on a reversed-phase HPLC column (LiChrospher WP, instruction manual, Merck). However, when large amounts of tRNA are used, the

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¹ Abbreviations used: aa-tRNA, aminoacyl-tRNA; FmocOSu, 9-fluorenylmethylsuccinimidylcarbonat; DTT, dithiothreitol; TCA, trichloroacetic acid; DMSO, dimethyl sulfoxide; Fmoc-aa-tRNA, fluorenyl-9-ylmethoxycarbonyl-aa-tRNA; EF-Tu, elongation factor Tu.

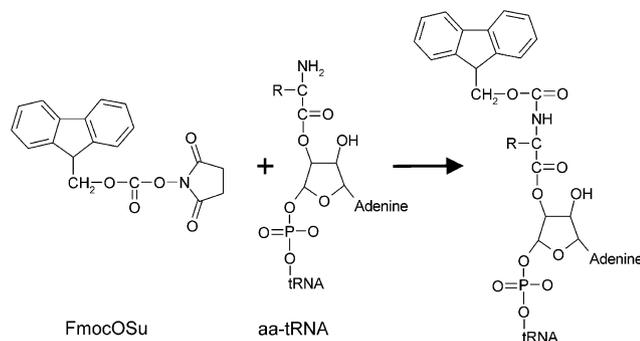


Fig. 1. Formation of fluorenyl-9-ylmethoxycarbonyl-aa-tRNA (Fmoc-aa-tRNA). FmocOSu reacts with the free amino group of aa-tRNA. The hydrophobicity of the resulting Fmoc-aa-tRNA is increased significantly compared with deacylated tRNA or unmodified aa-tRNA.

separation of the individual tRNA peaks is quite poor. To change the retention time of a specific aa-tRNA on the column and thereby improve its separation from other (deacylated) tRNAs, we modified the amino group of aa-tRNA with FmocOSu (Sigma–Aldrich), which adds a large aromatic group to the tRNA, thereby increasing its hydrophobicity and retention time on the column (Fig. 1). Succinimide esters selectively modify the amino group of amino acid and do not react with other amino groups in the tRNA molecule [9]. FmocOSu was dissolved in dimethyl sulfoxide (DMSO), 1 volume of tRNA was mixed with 4 volumes of FmocOSu to final concentrations of 20 μ M aa-tRNA and 35 mM FmocOSu, and the reaction was carried out for 60 min at 0 $^{\circ}$ C, resulting in the formation of fluorenyl-9-ylmethoxycarbonyl-aa-tRNA (Fmoc-aa-tRNA). Potassium acetate (pH 4.5) was added to 0.3 M final concentration, and Fmoc-aa-tRNA was precipitated with ethanol and dissolved in 0.3 M potassium acetate (pH 4.5). To remove unreacted FmocOSu, the sample was phenol-extracted and Fmoc-aa-tRNA was purified by two ethanol precipitations. For the subsequent chromatography, Fmoc-aa-tRNA was dissolved in buffer A (20 mM ammonium acetate [pH 5.0], 10 mM magnesium acetate, and 400 mM NaCl).

Separation of Fmoc-aa-tRNA from total tRNA was achieved by chromatography on reversed-phase HPLC using a LiChrospher WP-300 RP-18 (5 μ m) column (250 \times 10 mm) (Merck) (Figs. 2A and B). The HPLC system allows the efficient separation of Fmoc-aa-tRNA from deacylated tRNAs and residual unmodified aa-tRNA within 2 h. The tRNA mixture (up to 1700 A_{260} units) was applied to the column equilibrated with buffer A, followed by further washing of the column with buffer A at a flow rate of 3 ml/min. The tRNAs were eluted by a linear gradient from buffer A to 100% buffer B (20 mM ammonium acetate [pH 5.0], 10 mM magnesium acetate, 400 mM NaCl, and 30% [v/v] ethanol). The elution profile was monitored by measuring absorption (A_{260}) and scintillation counting of aliquots of eluted fractions to detect Fmoc- 14 C]aa-tRNA. The first small A_{260} peak eluting at 0–15% buffer B contained traces of ATP from the aminoacylation

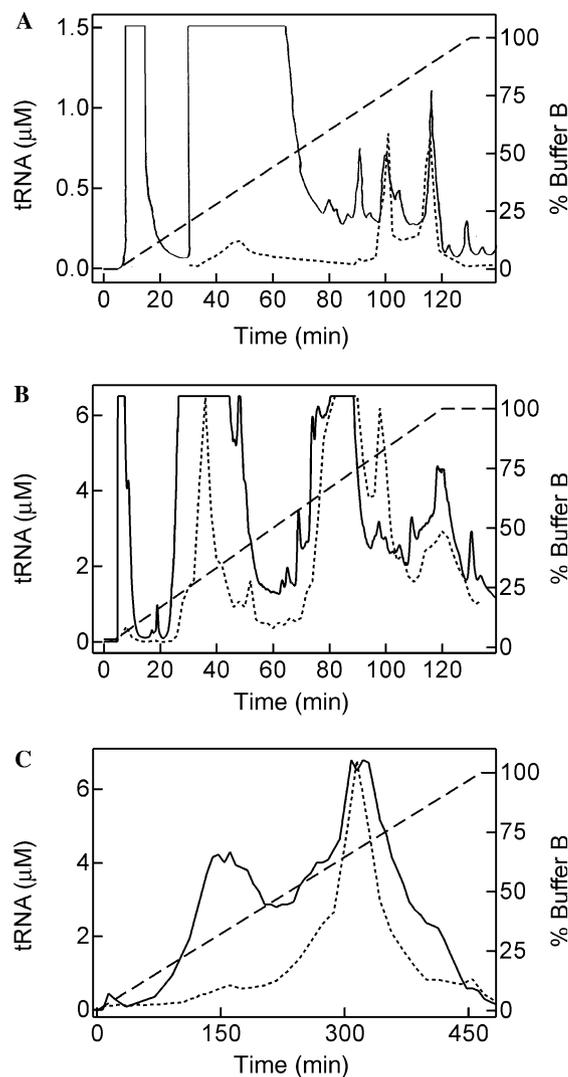


Fig. 2. Separation of Fmoc-aa-tRNA from total tRNA. Elution profiles were monitored by absorption at 260 nm (—) and 14 C radioactivity (---). The increase in buffer B percentage (---) is indicated. (A) Separation of Fmoc-Ala-tRNA^{Ala} from total tRNA by reversed-phase HPLC on a LiChrospher WP300 RP-18 (5 μ m) column (250 \times 10 mm) (Merck). [14 C]Alanine (50 dpm/pmol) was detected by scintillation counting of 50 μ l of each fraction (4.5 ml) in Lumasafe Plus cocktail (Lumac*LSC). (B) Separation of Fmoc-Ser-tRNA^{Sec/Ser} from total tRNA (containing overexpressed tRNA^{Sec}) by reversed-phase HPLC. To measure [14 C]serine (5 dpm/pmol), 50 μ l of each fraction (6 ml) was counted. (C) Separation of Fmoc-Ser-tRNA^{Sec/Ser} from total tRNA on Phenyl Sepharose (75 ml). Here 100 μ l of each fraction (10 ml) was counted to detect [14 C]serine (6 dpm/pmol).

reaction. The second large peak at 20–50% buffer B contained total tRNA. The radioactivity profile indicated in this peak the presence of aa-tRNA that was not modified with FmocOSu. Finally, Fmoc-aa-tRNA eluted at 60–100% buffer B. For either tRNA^{Ala} or tRNA^{Sec/Ser} preparations, two peaks that were well separated from bulk tRNA were found. For Fmoc-Ser-tRNA^{Sec/Ser}, we could show (using analytical assays for the conversion of serine to selenocysteine) that the first of the two peaks contained tRNA^{Sec} (60–90% buffer B), whereas tRNA^{Ser} isoacceptors eluted

in the second peak (90–100% buffer B) [10]. The two peaks of Fmoc-Ala-tRNA^{Ala} presumably contain the two isoacceptors of tRNA^{Ala} [11]; the isoacceptor identity was not analyzed here. The modification efficiency of aa-tRNA with FmocOSu was approximately 70–80%, as estimated from the ratio of ¹⁴C radioactivity in the fractions containing aa-tRNA and Fmoc-aa-tRNA. The fractions containing Fmoc-aa-tRNA were pooled and precipitated with ethanol. As an alternative to reversed-phase HPLC, Phenyl Sepharose 6 Fast Flow (high sub) (GE Healthcare) can be used to separate the modified aa-tRNA from bulk tRNA (Fig. 2C) using a 1.4- to 0-M gradient of ammonium sulfate in 50 mM sodium acetate (pH 6.5) and 10 mM MgCl₂.

To recover deacylated tRNA, Fmoc-aa-tRNA was deacylated by incubation with 1 M Tris-HCl (pH 9.0) at room temperature for 4 h. This procedure resulted in more than 90% deacylation, as verified by analytical reversed-phase HPLC under conditions similar to the preparative HPLC described above. Thus, efficient deacylation can be achieved without using expensive enzymes [12]. Deacylated tRNA was recovered by the addition of potassium acetate (pH 4.5) to 0.3 M and ethanol precipitation. The purified tRNA^{Ala} could be charged by alanine to 60%, corresponding to approximately a 20-fold enrichment in a single chromatographic step. The isolated tRNA^{Sec}, which was purified after overexpression in *E. coli*, also had a 60% acceptor activity.

The current method of tRNA purification has several advantages compared with previously published procedures [9,12–14]. It is generally applicable for all tRNAs without any adaptations in the described protocol because it relies solely on the selective aminoacylation by highly specific aa-tRNA synthetases and the subsequent modification of the amino group. In contrast to a previously published method [9], the ester used in the current work (FmocOSu) is readily available at low cost and is stable. By our method, the tRNA can be rapidly enriched up to 20-fold after only a single purification step. Both chromatographic materials (LiChrospher WP-300 RP-18 and Phenyl Sepharose) are more readily available than the BD cellulose that was used previously for tRNA purification by a similar approach [15]. Compared with other methods, such as affinity chromatography with immobilized elongation factor Tu (EF-Tu) [13] and streptavidin binding of N-biotinylated aa-tRNAs [14], the current method uses chromatographic materials that are significantly less expensive and allows purification in one chromatographic step of tRNAs in very large preparative amounts—up to 1700 A₂₆₀ units on the LiChrospher WP-300 RP-18 column or 4000 A₂₆₀ units/200 ml Phenyl Sepharose. Thus, we have described a fast, inexpensive, and efficient method for the isolation of specific tRNAs of good purity. For more demanding tasks, such as the purification of individual tRNA isoacceptors, the described procedure represents an ideal first step that can be followed by further purification steps using HPLC or hydrophobic chromatography [3].

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