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Preparation Method for *Escherichia coli* S30 Extracts Completely Dependent upon tRNA Addition to Catalyze Cell-free Protein Synthesis

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Abstract A simple method for depleting *E. coli* S30 extracts of endogenous tRNA has been developed. An ethanolamine-Sepharose® column equilibrated with water selectively captured the tRNA molecules in *E. coli* S30 extracts. As a result, S30 extracts filtered through this column became completely dependent upon the addition of exogenous tRNA to mediate cell-free protein synthesis reactions. We anticipate that the procedures developed and described will be particularly useful for *in vitro* suppression reaction studies designed to introduce unnatural amino acids into protein molecules.

Keywords: cell-free protein synthesis, unnatural amino acid, *in vitro* suppression, tRNA, chloramphenicol, acetyltransferase, erythropoietin

INTRODUCTION

Site-specific incorporation of unnatural amino acids provides a versatile platform for exploring and manipulating protein function. Since the first report of Noren *et al.* [1], *in vitro* suppression of a purposely introduced nonsense codon has been widely used to introduce unnatural amino acids into protein structures. Examples of this approach include the incorporation of unnatural amino acids with photoactive [2-5], fluorescent [6-9], spin-labeled [10], and chemically reactive side chains [11,12]. The last decade has witnessed significant evolution of nonsense suppression methodologies, in particular is the introduction of *in vivo* suppression technology [13,14]. It is expected that engineered proteins with unnatural amino acids will soon be produced at preparative scale. Nonetheless, since this technique requires the establishment of an orthogonal pair of tRNA/aaRS for each unnatural amino acid to be exclusively inserted, the throughput pace for screening an unnatural amino acid for a desired performance is severely limited. Therefore, *in vitro* suppression techniques using chemically misacylated tRNA molecules still hold an advantage as a more rapid and simple approach to the parallel preparation of modified proteins.

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It seems feasible to adopt the strategy of using the *in vitro* suppression method at the stage of screening to predict and produce the selected species in properly engineered cells.

Different versions of *in vitro* suppression technologies have been developed for the incorporation of unnatural amino acids into protein structures. For example, tRNA species that read the triplet UAG stop codon can be chemically acylated with unnatural amino acids and added into an *in vitro* translation reaction to generate proteins with a substituted amino acid residue. In addition to the stop codons, certain sense codons and more-than-three-base codons have also been shown to anchor the mischarged tRNA [15-20]. In this method, however, the insertion of unnatural amino acids is always in competition with their reading by endogenous tRNA, which reduces the yield of the desired product [21]. Such competition can in principle be avoided if the endogenous tRNA can be eliminated from the S30 extract and substituted with an exogenous tRNA pool containing a selective appropriate tRNA species. In line with such a view, Jackson *et al.* [22] recently developed a method to remove endogenous tRNAs from wheat germ extracts (WG) or rabbit reticulocyte lysates (RRL). Briefly, the extracts were run through a column of epoxy-activated Sepharose® 6B and the resulting extracts exhibited a strong dependence upon exogenously added tRNAs for translating mRNAs [22].

In the experiments, however, direct application of their protocols to the preparation of tRNA-depleted *E. coli* S30 extracts was not particularly successful since the *E. coli* tRNA molecules failed to bind to the resin under identical ionic conditions. Instead, we found it necessary to reduce the ionic strength of the equilibration buffer to allow efficient binding of the tRNAs. In our study we determined that the tRNA molecules of *E. coli* S30 extracts can be separated on the resin simply by using pure water as the equilibration buffer. The filtered extracts were unable to catalyze, *in vitro*, transcription/translation reactions unless supplied with a freshly prepared mixture of tRNAs. The tRNA-depleted S30 extracts are expected to enhance the efficiency of *in vitro* suppression reactions designed to introduce unnatural amino acids into protein structures.

MATERIALS AND METHODS

Materials

ATP, GTP, UTP, CTP, creatine phosphate, creatine kinase, and the *E. coli* total tRNA mixture were purchased from Roche Applied Science (Indianapolis, IN, USA). The epoxi-activated Sepharose® 6B and L-[U-^{14}C] leucine (11.9 GBq/mmol) were obtained from Amersham Biosciences (Uppsala, Sweden). All other reagents were purchased from Sigma and used without additional purification. The standard S30 extract was prepared from the *E. coli* strain BL21 (DE3) as described earlier [23,24].

Chromatographic Removal of Endogenous tRNAs

Epoxy-activated Sepharose® 6B was thoroughly washed with autoclaved DDW to remove preservatives. For the preparation of ethanolamine-Sepharose, the gel beads were resuspended in 1 M ethanolamine (5 mL of ethanolamine solution per 1 mL of swollen gel), incubated with gentle agitation overnight at room temperature and extensively washed with water (ED – as water is important to the paper, try to detail whatever water it is that is being referred to). Depending on the experiment design, columns of 3 mL bed volume were equilibrated with Buffer A (25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10 mM HEPES-KOH, pH 7.4), S30 buffer (14 mM MgOAc, 60 mM KOAc, 10 mM Tris-Cl, 1 mM DTT), or autoclaved DDW.

Analysis of tRNA

The total RNAs of filtered S30 fractions were recovered by a single extraction with phenol:chloroform:isoamyl alcohol (25:24:1) followed by ethanol precipitation in the presence of 0.3 M sodium acetate. The precipitated RNA pellet was dissolved in nuclease-free water and the RNA samples then analyzed on a 7 M urea/12% polyacrylamide (PAG) gel. RNA bands on the gel were visualized using a methylene blue stain.

Cell-free Protein Synthesis Reactions

The standard reaction mixture used for cell-free protein synthesis reactions consisted of the following components in a total volume of 15 μL: 57 mM of Hepes-KOH (pH 8.2), 1.2 mM of ATP, 0.85 mM each of CTP, GTP, and UTP, 2 mM of DTT, 0.64 mM of cAMP, 90 mM of potassium glutamate, 80 mM of ammonium acetate, 12 mM of magnesium acetate, 34 μg/mL of L-5-formyl-5,6,7,8-tetrahydrofolic acid , 0.5 mM each of 20 amino acids, 2% PEG 8000, 67 mM of creatine phosphate (CP), 5.6 μg/mL of creatine kinase, 10 μM of L-[U-^{14}C] leucine (11.3 GBq/mmol, Amersham Biosciences), 0.1 μg plasmid DNA, 4 μL of the S30 extract. 0.17 mg/mL of *E. coli* total tRNA mixture (from stain MRE600) was added according to the experiment needs. The cell-free synthesized protein was quantified by measuring TCA-precipitated radioactivity using a liquid scintillation counter (WALLAC 1410), as described elsewhere [23-28].

The size of the cell-free synthesized protein was analyzed by Western blot after running the reaction samples on 16% Tricine-SDS-polyacrylamide gel, as described by Schägger and von Jagow [29].

RESULTS AND DISCUSSION

Preparation of tRNA-depleted S30 Extracts from *E. coli*

The S30 extracts were prepared from the *E. coli* strain BL21 (DE3) as described in the Materials and Methods. To remove endogenous tRNA, the S30 extracts were loaded onto a column packed with a 3 mL bed volume of ethanolamine-Sepharose® 6B resin. The residual tRNA content in the filtrate fractions was analyzed on 7 M urea/12% polyacrylamide gels by methylene blue staining. At the same time, 200 μL fractions of the column-filtered extracts were collected and assayed for their activity to direct protein synthesis from pK7CAT plasmids. As shown in Fig. 1, when the columns were equilibrated with Buffer A that had been used for the preparation of tRNA-depleted WG and RRL as described in the works of Jackson *et al.* [22], most of the endogenous tRNA was found in the flow-through fractions indicating that the *E. coli* tRNAs did not effectively bind to the activated resin under these conditions. Interestingly, the filtered S30 extracts exhibited substantially reduced activity for protein synthesis. When compared to the standard S30 extract, only 53% amount of the cell-free synthesized protein was measured (Fig. 1B).

We assumed that the decline of translational activity was due to a non-optimal ionic condition of the equilibration buffer. When we substituted the equilibration buffer for the one used to prepare the S30 extract (S30 buffer: 14 mM MgOAc, 60 mM KOAc, 10 mM Tris-HCl, 1 mM DTT), the column still failed to retain the *E. coli* tRNAs, but the flow-through fractions exhibited a translation activity level nearly comparable to unfiltered S30

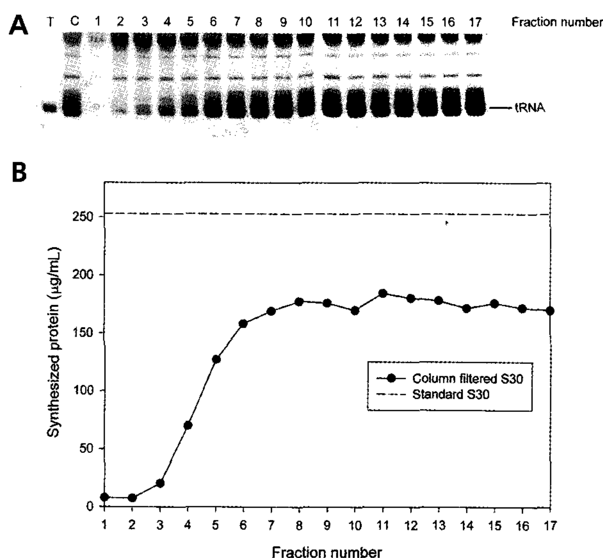

Fig. 1. Residual tRNA amounts and the translation activity of an S30 extract that has been filtered through an ethanolamine-Sepharose® 6B column using Buffer A as equilibration buffer. An *E. coli* S30 extract was passed through a 3 mL bed volume of ethanolamine-Sepharose® column that has been equilibrated with Buffer A. Fractions approximating 200 μL were collected and assayed for residual tRNA content and their ability to mediate protein synthesis from the pK7CAT plasmid. (A) RNA molecules were separated on a 12% polyacrylamide gel containing 7 M urea under denaturing conditions and stained with methylene blue. Lane T, standard tRNA mixture from the MRE600 strains; lane C, total RNAs extracted from *E. coli* S30 extract. (B) Radioactivity of the [^{14}C] leucine-labeled protein was measured as described in the Materials and Methods. The dashed line indicates the mean expression level of CAT expressed in the standard *E. coli* S30 extracts.

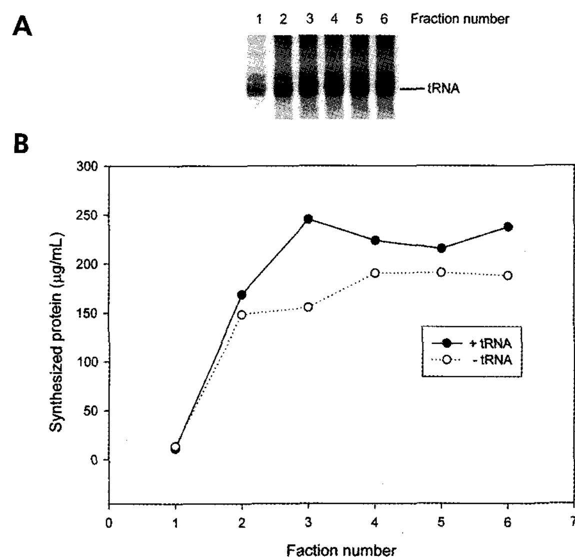

Fig. 2. Residual tRNA amount and the translation activity of an S30 extract that has been filtered through an ethanolamine-Sepharose® column using the S30 buffer as equilibration buffer. (A) RNA molecules were separated on a 12% polyacrylamide gel containing 7 M urea under denaturing conditions and stained with methylene blue. (B) Each flow-through fraction was assayed for the incorporation of [^{14}C] leucine in the presence or absence of a commercial *E. coli* total tRNA mixture (Roche Applied Science). After a 2-h incubation at 37°C, 15 μL samples were withdrawn and their content of TCA-insoluble radioactivity measured as described in the Materials and Methods.

extracts (Fig. 2).

In their description of the process for preparing the tRNA-depleted extracts from WG and RRL, Jackson *et al.* found that the presence of monovalent ions interfered with the binding of tRNAs to the ethanolamine-Sepharose® resin [22]. Therefore, they reduced the concentration of monovalent ion (potassium) in the equilibration buffer to achieve an acceptable separation of the tRNAs. Since the *E. coli* tRNAs were not separated onto the resin, however, even at the low concentration of potassium ion, we chose to equilibrate the resin with pure water and assess any effect upon separation. As shown in Fig. 3A, most of the endogenous tRNA was captured by the activated Sepharose® beads when equilibrated with water. Based on the relative intensity of the stained tRNA bands, approximately 95% of the total endogenous tRNA was estimated to be eliminated by filtering the S30 extracts through a column of water-equilibrated Sepharose® beads.

tRNA-dependent Cell-free Protein Synthesis Using Filtered S30 Extracts

The tRNA-depleted S30 extracts were examined for their ability to mediate protein synthesis with or without the addition of fresh tRNA. As shown in Fig. 3B, when the pK7CAT plasmid was incubated in filtered extracts along with the other necessary components for cell-free protein synthesis (see Materials and Methods), the TCA-insoluble radioactivity level measured from the reaction was not significantly higher than background. Instead, a substantial amount of protein synthesis was observed upon the addition of exogenous tRNAs to the reaction mixture. Approximately 90% of the original activity was seen to be restored when 0.17 mg/mL of a tRNA mixture was added to the filtered extract.

Western blot analysis also clearly showed that protein synthesis in the filtered S30 extracts was completely dependent upon the presence of exogenous tRNA (Fig. 4). In cell-free synthesis experiments for human erythropoietin (hEPO), while the standard S30 extract supported expression of full-length hEPO even in the absence of additional tRNAs, virtually no protein bands were observed with filtered extracts unless the extracts were supplemented with exogenous tRNAs.

In conclusion, we have identified a process of preparation for *E. coli* extracts wherein the translation activity was completely and totally dependent upon the addition of exogenous tRNA.

A cell-free protein synthesis system that is so dependent upon exogenously added tRNA will be useful to enable

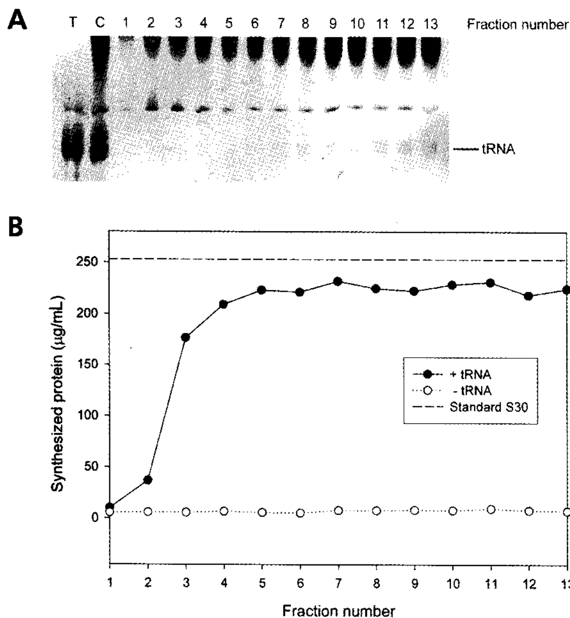

Fig. 3. Residual tRNA amount and the translation activity of an S30 extract that has been filtered through an ethanolamine-Sepharose® column using pure water as equilibration buffer. (A) RNA molecules were separated on a 12% polyacrylamide gel containing 7 M urea under denaturing conditions and stained with methylene blue. Lane T, standard tRNA mixture from the MRE600 strains; lane C, total RNAs extracted from *E. coli* S30 extract. (B) Effect of tRNA addition on the recovery of translation activity of the filtrate *E. coli* lysate. Each of the flow-through fractions was assayed for incorporation of [^{14}C] leucine in the presence or absence of a commercial *E. coli* total tRNA mixture. The reaction mixture was incubated at 37°C for 2 h. Fifteen μL samples were withdrawn and the [^{14}C] leucine-labeled radioactivity of the protein measured as described in the Materials and Methods.

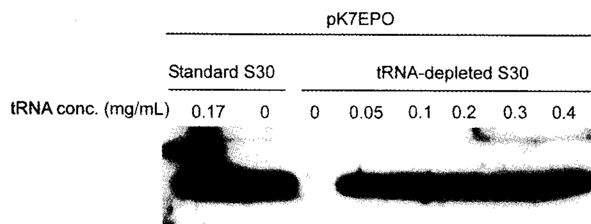

Fig. 4. Expression of human erythropoietin in the column-filtered S30 extract. The pK7EPO plasmid was incubated with the standard S30 extract or the column-filtered S30 extract with varying concentrations of a tRNA mixture. After an incubation of 2 h, 2 μL of the reaction mixture was applied to a 16% Tricine-SDS-PAGE gel and analyzed by Western blot.

further studies of the effect of the relative abundance of tRNA species upon the various events of protein synthesis.

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