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Isolation and purification of isoacceptor $tRNA_1^{Ser}$ and $tRNA_2^{Ser}$ from *Thermus thermophilus*

I.A. Krikliviy, O.P. Kovalenko, O.I. Gudzera, A.D. Yaremchuk, M.A. Tukalo

The Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Academician Zabolotny Str., Kyiv, 03143, Ukraine

mtukalo@imbg.org.ua

Serine aminoacylation system is unique as it is the only system where synthetase of class II recognizes tRNA with a long variable stem (type II tRNA), which is of great interest to study the mechanisms of tRNA recognition by corresponding aminoacyl-tRNA synthetase. Current paper presents the methodological approaches for obtaining purified isoacceptor tRNA1Ser and tRNA2Ser in milligram quantities from extremal thermophil Thermus thermophilus for crystallography. The developed method of tRNASer purification employs the chromatography on benzoylated diethylaminoethylcellulose and high-performance liquid chromatography on anion-exchange column Spherogel TSK DEAE 5PW, and reverse phase column Ultrapore C8 using HPLC equipment (Beckman).

Keywords: tRNA, chromatography, high-performance liquid chromatography, extremal thermophil Thermus thermophilus, benzoylated diethylaminoethylcellulose.

Introduction. Serine aminoacylation system (SerRS and $tRNA^{Ser)}$ guarantees correct including of serine amino acid into a protein molecule which has six corresponding codons of genetic code. Characteristic features of this system are as follows: i) seryl-tRNA synthetase is one of three ARSases, which do not recognize anticodon of tRNA in prokaryotes; ii) $tRNA^{Ser}$ has a long variable arm which provides a considerable difference of this system from others.

Structural and functional characteristics of seryl-tRNA synthetase allow referring it to class II [1, 2]. The mechanisms of enzymes interactions with cognate tRNA have radical distinctions for ARSases of classes I and II. Similar to $tRNA^{Leu}$ and prokaryotic $tRNA^{Tyr}$, $tRNA^{Ser}$ has a long variable arm and is classified as type II tRNA [3]. The length of the variable arm of type II tRNA may be 20 nucleotides instead of 4-5 in type I tRNA. The presence of a long variable arm provides considerable difficulty in the study of three-dimensional structure of type II tRNA. Type I $tRNA^{Phe}$ was one of the first biological macromolecules, the three-dimensional structure of which was studied by X-ray analysis [4], however, an attempt to obtain crystals of free tRNA with a long variable arm, which would allow determining its three-dimensional structure, have yet been unsuccessful. Thus, serine aminoacylation system is unique as it is the single system where class II synthetase recognizes type II tRNA, which has a special interest to the study of mechanisms recognition and aminoacylation of $tRNA^{Ser}$ by seryl-tRNA synthetase.

Our research on the interaction between tRNA with a long variable arm, and cognate synthetases ($tRNA^{Ser}$ and $tRNA^{Leu}$ from bovine liver and mammary gland, respectively) by the methods of chemical modification

in solution [5-10] allow revealing important information indicating that these tRNA should differ for both three-dimensional structures and orientations towards enzymes.

Consequently, to clarify the mechanisms of their functioning, as the main link of genetic code realization in the cells of living organisms, it is necessary to carry out a detailed study on three-dimensional structures of tRNA and their complexes with cognate aminoacyl-tRNA synthetases by crystallographic methods.

The success in using X-ray analysis depends on the quality of crystals, which should be well packed and resistant to X-rays, as experimental exposition may continue for several hours. Therefore, preparations of the highest purity are needed to grow crystals of bio-macromolecules and their complexes. Any contaminations can disturb crystal structure and decrease resolution of the method.

At present we possess a set of powerful and highly effective methods (chromatography in different variants, electrophoresis, sedimentation, etc.), the use of which, at first glance, may seem easy to solve the problems of obtaining individual bio-macromolecules of high purity. Nevertheless, all transfer RNAs have slight resemblance to three-dimensional structure (L-form), which determines their similar behavior in interactions with different chromatographic carriers. $tRNA^{Ser}$ content in general tRNA pool is ~2%, but it is very heterogeneous in structural respect. This heterogeneity is conditioned by both degeneration of genetic code (6 anticodon) and the degree of maturation (post-transcriptional modification of nucleotide bases and their transformation into minor components). Moreover, macromolecules with identical primary structure may have different conformations. Seryl-tRNA synthetase recognizes all various $tRNA^{Ser}$ forms and may form complexes with them in the crystallization process. Obtained crystals will be heterogeneous, unavailable for analysis and have high level of loosening. We isolated $tRNA^{Ser}$ preparations of bovine liver, sufficiently purified (over 90%) from other tRNA using only two chromatographic stages on benzoylated DEAE-cellulose (both in presence and absence of Mg^{2+}) [11], but they had considerable structural heterogeneity and were not promising in experiments on crystallization. Thus, while isolating and purifying tRNA, we should have selected $tRNA^{Ser}$ macromolecules more carefully than it is performed by seryl-tRNA synthetase itself.

A serine aminoacylation system from extremal thermophil *Thermus thermophilus* was chosen as object of structural research. These microorganisms live in hot springs with the temperature of 65-80°C, therefore, their macromolecules may have structural peculiarities providing their higher resistance to external influence and better adequacy for structural research.

Current paper presents methodological approaches for obtaining two highly purified isoacceptors $tRNA^{Ser}$ in quantities, sufficient for the study of their primary structures, physical-chemical and functional features, obtaining crystals of $tRNA^{Ser}$ complexes with cognate SerRS and the elaboration of models of three-dimensional structures of complexes on the basis of experimental data of X-ray analysis.

Materials and methods. The following materials were used in the work: benzoylated DEAE-cellulose (BD-cellulose) ("Serva", Germany), NaCl, $MgCl_2$ ("Fisher", USA), tris, phenylmethylsulfonilfluoride ("Calbiochem", USA), ammonium acetate (analytical grade), 2-mercaptoethanol (Merk, Germany), GF/C filters, diethylaminoethylcellulose (Whatman, UK), isopropyl alcohol (analytical grade), ^{14}C-serine (120 Cu/mol) (Institute of Research, Production and Application of Radioactive Isotopes, Czech Republic). All other reagents were of analytical grade. Solutions were prepared with redistilled water. The following equipment was used: centrifuge K-70 (Germany), spectrophotometer Specord UVVIS (Germany), chromatographic equipment (Gold System), high pressure chromatographic columns Spherogel TSK DEAE 5PW, Ultrapore C-3, Ultrapore C-8 (Beckman, USA), scintillation counter Rackbeta (LKB, Sweden).

The cells of *T.thermophilus* HB-8 and HB-27 were grown in the fermenter with the volume of 300 liters in the nutrient medium, containing 5 g/l peptone, 2 g/l yeast extract, and 2 g/l NaCl at the temperature of 75°C. Culture growth was controlled according to absorption increase at A_{590} to 1.8.

The cells were collected by centrifugation, washed from the residues of nutrient medium, frozen, and kept till use.

Crude tRNA was obtained from *T. thermophilus* by phenol extraction of RNA from bacterial cells and deproteinization by mixtures of phenol:chlorophorm (1:1) and chlorophorm:isoamyl alcohol (9:1). Phases were separated on K-70 centrifuge. To purify tRNA from proteins and contaminations of nucleotide and polysaccharide nature, DEAE-cellulose was used. In the final supernatant pH value was obtained by adding dry tris to 7.5, applying it on DEAE-cellulose in a beaker, the mixture was stirred by a glass stick and placed into the refrigerator for sediment formation (the operation was repeated several times). After RNA binding, DEAE-cellulose was washed by 0.14 M NaCl in tris-HCl buffer, pH 7.5, with subsequent introduction into the chromatographic column, then it was washed with 0.3 M NaCl to remove protein admixtures. tRNA was eluated by 1 M NaCl, precipitated by 2.5 of the volume of ethyl alcohol in the presence of 2% of potassium acetate. The precipitate, formed in the course of 17-20 hours at 4°C, was collected by K-70 centrifuge.

The enzyme for tRNA testing in the purification process was isolated from the same biomass. The cells were collected by centrifugation, washed from the admixtures of nutrient medium, and disrupted using French Press in 40 mM tris-HCl buffer, pH 7.9, containing 2 mM dithiothreitol, 0.1 mM EDTA, 1 mM phenylmethylsulfonil fluoride, 5 mM MgCl2, 1 mM NaN3. Obtained extract was centrifuged for 2 hours at 105 000 g, and the supernatant was applied to the column with DEAE-cellulose, equilibrated by 20 mM tris-HCl buffer, pH 7.8, containing 0.2 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonil fluoride, 5 mM $MgCl_2$, 0.5 mM NaN_3. The column was washed by loading buffer with subsequent elution of protein by 0.3 M NaCl in the same buffer. Obtained protein solution was used as a crude enzyme in aminoacylation reactions.

The acceptor activity of tRNA on different stages of isolation was defined according to the maximum level of forming aminoacyl-tRNA in aminoacylation reaction using ^{14}C-serine and crude preparation of aminoacyl-tRNA synthetase. Standard reaction mixture in the volume of 0.25 ml contained 0.1 mM tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.2 mM ATP, 5-15 mM ^{14}C-serine, and 1 mg/ml of crude *T. thermophilus* ARSases. 50 mkl tRNA solution was taken from investigated fractions, added 200 mkl of reaction mixture and incubated during 7 minutes at 65°C. The reaction was stopped by adding 500 mkl of cooled 10% solution of trichloroacetic acid. The inclusion of ^{14}C-serine to aminoacyl-tRNA was determined using scintillation counter.

The individual tRNAs were isolated, using several chromatographic stages on columns with BD-cellulose, Spherogel TSK DEAE 5PW and Ultrapore C3, Ultrapore C8 ("Beckman", USA), as well as equipment for high pressure liquid chromatography (HPLC) ("Gold System" of "Beckman" company, USA). Fractions, active in aminoacylation reaction, were determined after each chromatographic stage, with their subsequent being combined, precipitated by ethanol, and used for further purification.

Results and Discussion. Chromatography on the column with benzoylated DEAE-cellulose [12] was used on the first stage of isolating preparations of individual $tRNA_1^{Ser}$ and $tRNA_2^{Ser}$ according to [13]. Transfer RNA, active in the aminoacylation reaction with serine, was found only in fractions, eluated by 1.5 M NaCl and 10% ethyl alcohol. Selected fractions were pooled, precipitated by ethanol, dried and kept in the refrigerator until further use. tRNA loss on this column was about 20%. Fractions, containing serine activity, were about 10% of material, eluated from the column.

Further purification was carried out by chromatography on high pressure anion-exchange column Spherogel TSK DEAE 5PW (2.1 x 15 cm) using HPLC equipment ("Gold System"). Chromatography was carried out using the following buffer solutions: buffer A contained 0.05 M tris-HCl, pH 7.5, 0.2 M NaCl, 0.01 M $MgCl_2$, and 10% 2-isopropanol; buffer B differed in NaCl content – 1.0 M. After applying tRNA the column was washed with buffer A, concentration of buffer B was increased to 11% in 5 min, and then eluated with the gradient of buffer B concentration which was formed according to curve 4, programmed in "Gold System", while increasing the content of buffer B to 25% in 60 min. The flow rate was 5 ml/min at room temperature (20-25°C).

It is noteworthy that chromatography on DEAE 5PW column at temperature, lower than 20°C, provides

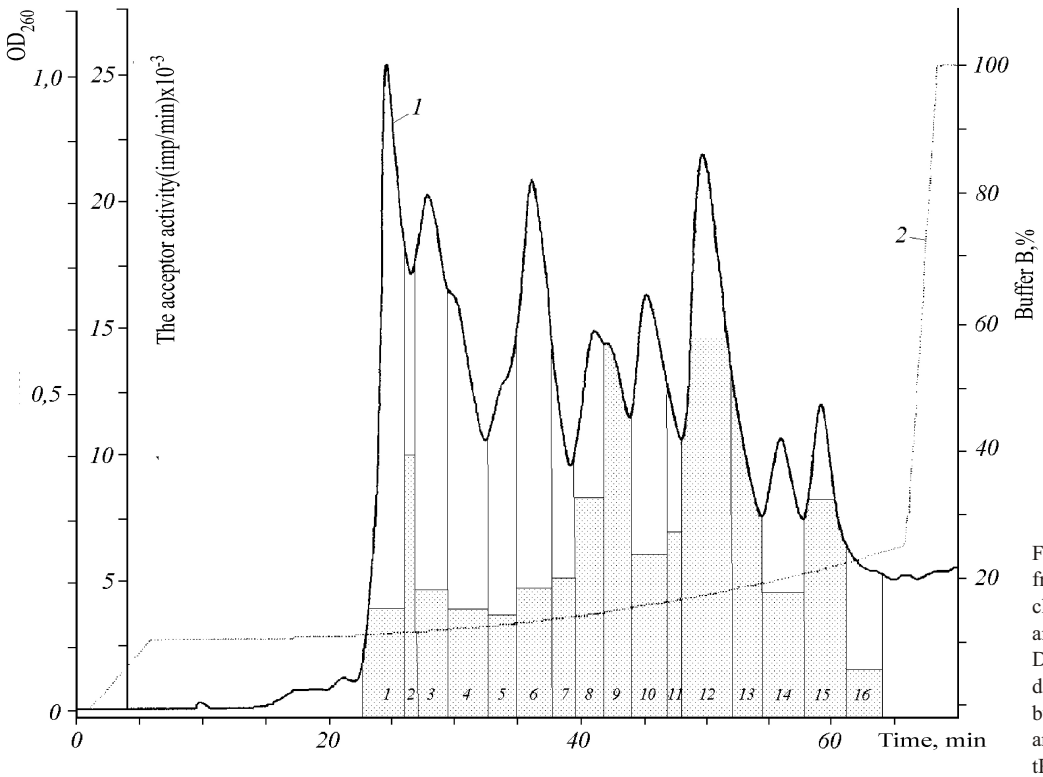

Fig.1. Separation of serine tRNA from *T. thermophilus* using chromatography on high pressure anion-exchange Spherogel TSK DEAE 5PW column: 1 – optical density at 260 nm; 2 –gradient of buffer B concentration. Stained area represents the activity level of $tRNA^{Ser}$ fractions 1-16 in aminoacylation reaction.

worse separation. Fractions were collected in the process of chromatography and acceptor activity $tRNA^{Ser}$ was assayed. In a typical chromatogram, presented in Fig.1, $tRNA^{Ser}$ has four evident well-separated peaks. Fractions groups 8, 9, and 12, 13 were selected for further purification of individual $tRNA^{Ser}$. Fractions 8, 9 contained $tRNA_2^{Ser}$ with about 50% purity degree, while $tRNA_1^{Ser}$ was in fractions 12, 13 and had over 65% purity degree. Other fractions were not used for further purification. $tRNA^{Ser}$ amount was considerably less in these fractions and they had 10-20% purity degree.

The last $tRNA^{Ser}$ chromatography was carried out on high-pressure column Ultrapore C8 (1 x 20 cm) using "Gold System". While selecting chromatography conditions, columns Ultrapore C3 and Ultrapore C8 as well as some components for buffer systems (phosphates, sodium format, ammonium sulfate, and organic solvents – methanol, 2-propanol) were used. We have decided to use column C8. Buffer A contained 0.05 M ammonium acetate and 0.01 M $MgCl_2$; buffer B – 10% 2-isopropanol. The gradient was formed similarly to chromatography on Spherogel TSK DEAE 5PW column (Fig. 2, 3). Column was eluated at a flow rate 4 ml/min at room temperature (20-25°C). It should be mentioned that chromatography on reversed-phase columns Ultrapore C3 and Ultrapore C8 depends on temperature even more than while using Spherogel TSK DEAE 5PW, and if the temperature drops to 15°C, the use of these columns was ineffective. After chromatography of fractions 8, 9 and 12, 13, obtained on Spherogel TSK DEAE 5PW, individual $tRNA_1^{Ser}$ (Fig.2) and $tRNA_2^{Ser}$ (Fig.3) were obtained with over 95% purity degree. High purity of obtained $tRNA_1^{Ser}$ and $tRNA_2^{Ser}$ preparations was confirmed in experiments of determining their primary structures. It is noteworthy that if 4-4.5 g of summary tRNA preparation were applied to the column with BD-cellulose on the first stage, we received 3-3.5 mg $tRNA_1^{Ser}$ and 2-2.5 mg $tRNA_2^{Ser}$, which proves that obtaining bio-macromolecule preparations of high purity degree is a very laborious process with great loss

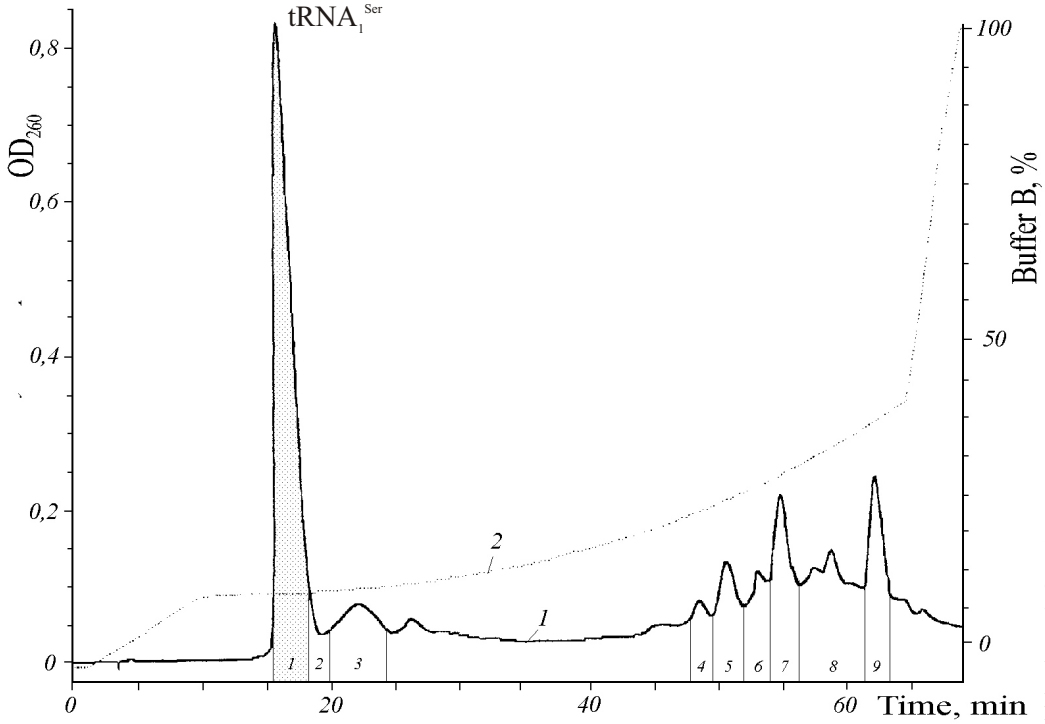

Fig.2. Purification of *T. thermophilus* isoacceptor $tRNA_1^{Ser}$ on reversed-phase Ultrapore column C8: 1 – optical density at 260 nm; 2 –gradient of buffer B concentration. Stained peak contains $tRNA_1^{Ser}$ with the purity of ~ 1600 pM/1 A260.

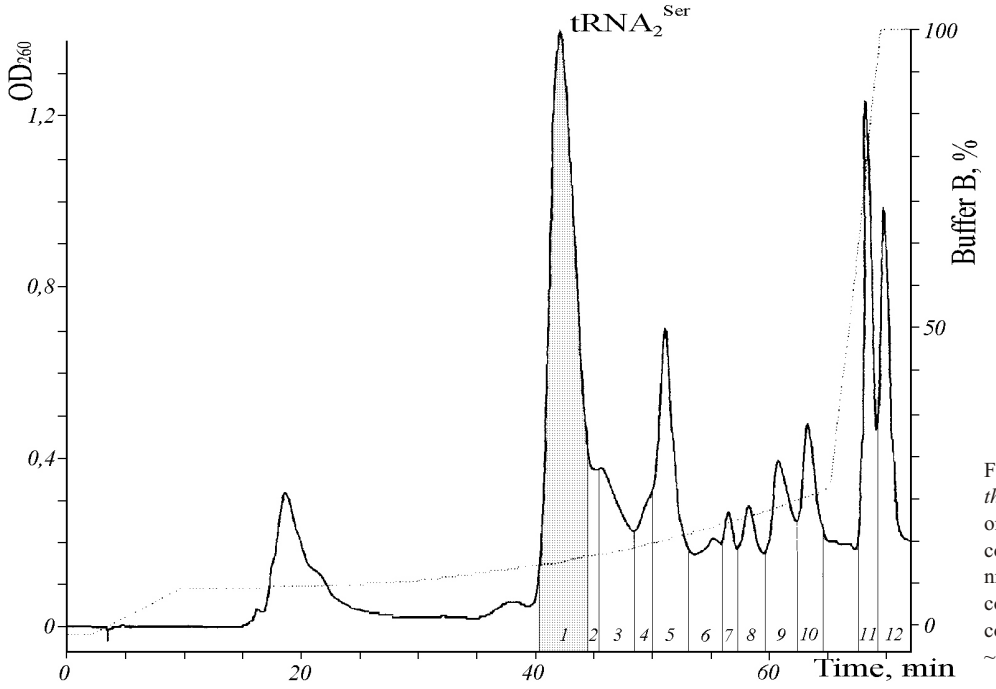

Fig.3. Purification of *T. thermophilus* isoacceptor $tRNA_2^{Ser}$ on reversed-phase Ultrapore C8 column: 1 – optical density at 260 nm; 2 – gradient of buffer B concentration. Stained peak contains $tRNA_2^{Ser}$ with the purity of ~ 1570 pM/1 A260.

of material on different stages of purification, however, in other cases their structural and functional characteristics cannot be determined, and structural research cannot be carried out.

The study on primary structures of obtained $tRNA_1^{Ser}$ and $tRNA_2^{Ser}$ (Fig.4) revealed that they differ in nucleotide sequences and the greatest discrepancies concern the sites of anticodon stem, variable arm, and

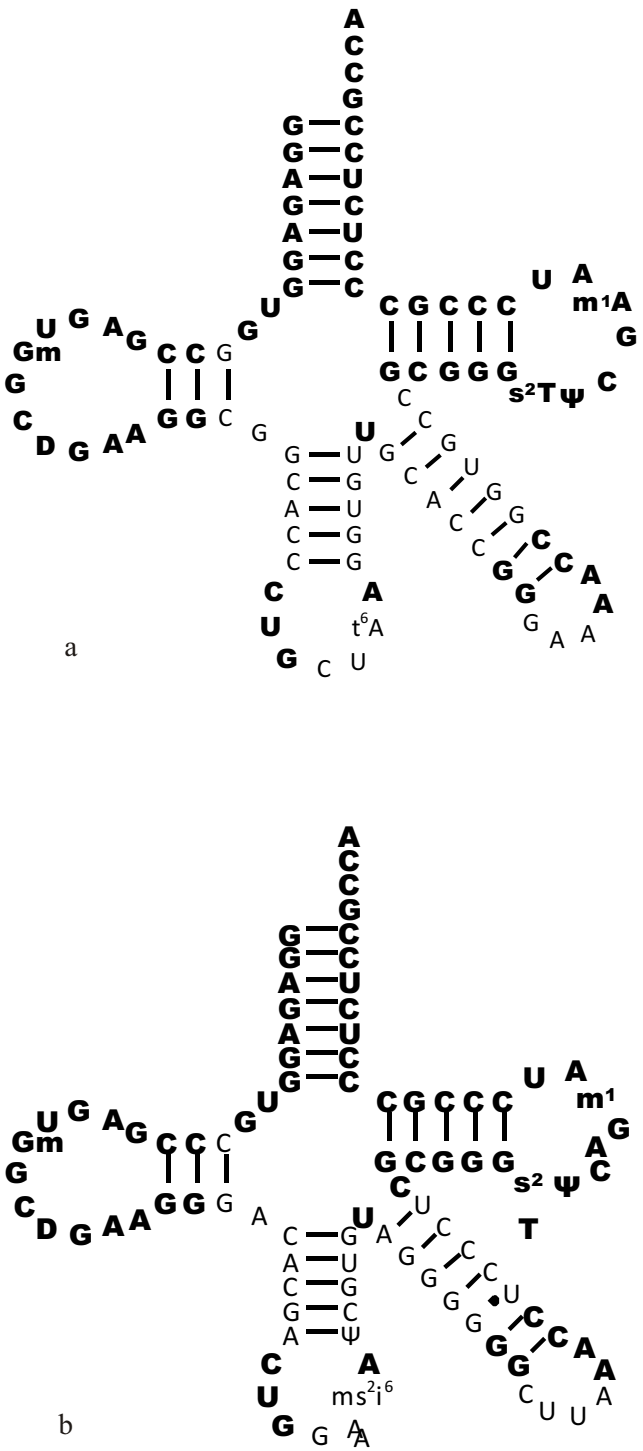

Fig.4. Structures of *T. thermophilus* isoacceptor $tRNA_1^{Ser}$ (a) and $tRNA_2^{Ser}$ (b). Nucleotides, common for $tRNA_1^{Ser}$ and $tRNA_2^{Ser}$ are in bold.

anticodon loop. They also have different length of variable branches –19 and 20 nucleotides for $tRNA_1^{Ser}$ and $tRNA_2^{Ser}$, respectively [14]. It should be noted that structural differences of these isoacceptor $tRNA^{Ser}$ are in the sites, which are the most probable to have interaction with carriers and condition different chromatographic behaviour and separation. Both tRNA were also used to obtain crystals of complexes with homologous SerRS [15]. Obtained crystals differ in space group and resolution, which is more important. Thus, crystals of SerRS: $tRNA_1^{Ser}$ complex had monocline form with resolution 3.5 A; all the efforts of growing crystals of higher quality and building a model of three-dimensional structure of the complex with better resolution were unsuccessful. The crystals of SerRS: $tRNA_2^{Ser}$ complex had orthorhombic form, were more resistant to the influence of X-rays and allowed building a three-dimensional model of the complex with 2.8 A resolution.

The study on structures of SerRS complexes with $tRNA^{Ser}$ and low molecular substrates using X-ray analysis allowed first building the model of three-dimensional tRNA structure with a long variable arm [16], determining structural components of tRNA and the enzyme, involved in complex formation, comparing them with the data, obtained while studying complex formation in solution and, finally, we proposed the model of aminoacylating $tRNA^{Ser}$ by homologous amino acid [17]. Structural research revealed a considerable role of a long variable arm of $tRNA^{Ser}$ in its being recognized by homologous seryl-tRNA synthetase. This information was obtained only due to isolation and purification of *T. thermophilus* isoacceptor $tRNA^{Ser}$.

Recently some approaches to tRNA isolation with high activity level in preparative quantities have been proposed [18]. The authors used the method, combining reversed-phase chromatography and reverse biochemical modification of tRNA. The aim of modification is to introduce additional needed structural elements into tRNA which will change its chromatographic behaviour in comparison with non-modified macromolecules.

In case of isolating Escherichia coli $tRNA^{Ile}$, at first the authors aminoacylated crude tRNA by ^{14}C-isoleucine, and after three chromatographic stages

on Delta Pack C4 column (Waters) and deaminoacylation reaction obtained $tRNA^{Ile}$ with the activity of 1400 pM/1 A_{260}. Nevertheless, while obtaining *Saccharomyces cerevisiae* $tRNA_i^{Met}$, the suggested approach did not work due to instability of methionil-tRNAiMet and its destruction in the course of chromatography. Deeper modification of $tRNA_i^{Met}$ with its transformation into fMet-$tRNA_i^{Met}$ was necessary using enzymes catalyzing methylation and formylation of eukaryotic initiator tRNA. $tRNA_i^{Met}$ with 1700 pM/1 A_{260} purity was obtained after chromatography on Nucleosil C4 column, demodification, and repeated chromatography on the same column.

Therefore, suggested approaches expand the possibility of isolating higher-purified tRNAs of needed specificity. However, they cannot be used in cases of obtaining isoacceptor tRNA in a pure form. Besides, using enzymes for modification is always connected with the risk of their containing admixtures of nucleases, which may change tRNA structure.

Our method of purifying *T. thermophilus* isoacceptor $tRNA^{Ser}$ allows obtaining preparations of high purity degree, completely suitable for structural research. The only disadvantage of this method is considerable loss of material on different stages of purification and low output of final product.

И. А. Крикливый, О. П. Коваленко, О. И. Гудзера, А. Д. Яремчук, М. А. Тукало

Выделение и очистка изоакцепторных форм $тРНК_1^{Ser}$ и $тРНК_2^{Ser}$ из *Thermus thermophilus*

Резюме

Сериновая аминоацилирующая система является уникальной в том понимании, что это единственная система, в которой синтетаза 2-го структурного класса узнает тРНК с длинной вариабельной ветвью (тРНК 2-го типа), что вызывает особый интерес при изучении механизмов узнавания тРНК соответствующей аминоацил-тРНК синтетазой. Изложены методические подходы к получению методами хроматографии высокоочищенных изоакцепторных $мРНК_1^{Ser}$ и $мРНК_2^{Ser}$ из экстремального термофила T. thermophilus в миллиграммовых количествах, необходимых для структурных исследований. Разработанный метод очистки мРНКSer включает хроматографию на бензоилированной ДЭАЭ-целлюлозе, высокоэффективную жидкостную хроматографию на анионобменной колонке Spherogel TSK DEAE 5PW и обратнофазовой колонке Ultrapore C8 с использованием оборудования HPLC.

Ключевые слова: мРНК, хроматография, высокоэффективная жидкостная хроматография, экстремальный термофил Thermus thermophilus, бензоилированная ДЭАЭ-целлюлоза.

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