Isolation and partial purification of tRNA ligase from *Tetrahymena pyriformis*

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The possibility of isolation of tRNA ligase from Protozoa *Tetrahymena pyriformis* has been investigated. The presence of tRNA maturation activities in flow-through fractions of supernatant S-100 chromatography on DEAE-cellulose column has been detected. The chromatography of enzyme preparation on DEAE-cellulose, heparin–agarose and Toyopearl columns resulted in partial purification of the 95 kDa protein. The investigation of the influence of NaCl concentration on the E–pA complex formation showed no inhibitory effect of NaCl in the concentration range 0.4–1 M, suggesting that the E–pA complex is tRNA ligase-pA.

Key words: tRNA ligase, *Tetrahymena pyriformis,* partial purification

***Abbreviations*:** PMSF – phenylmethylsulfonyl fluoride, PEG – polyethylenglycol, EGTA – 1,2 bis(2-aminoethoxy)etan-tetraacetic acid, spermidine – N-(3-aminopropyl)-1,4-butanediamine

INTRODUCTION

tRNAs are synthesized as tRNA precursors in eukaryotes. The maturation of precursors into functionally active molecules of tRNA includes elimination of 5' and 3' additional sequences, modification of nucleosides, elimination of introns, synthesis of 3'-end CCA sequence. Removal of introns by splicing is best studied in yeast.

The tRNA splicing reaction in yeast occurs in three steps catalysed by distinct enzymes. In the first step the pre-tRNA is cleaved at its two splice sites by an endonuclease. The products of this reaction are two tRNA half-molecules and a linear intron with 5'-OH and 3'-cyclicPO_4 ends [1, 2]. The two tRNA half-molecules are the substrate for the ligase reaction, which takes place in three steps. In the first step, phosphodiesterase activity opens the 2',3'-cyclic phosphate at the 3' terminus of the 5'half-molecule. A polynucleotide kinase activity transfers the γ-phosphate of GTP to the 5'-hydroxyl of the 3'half-molecule [3, 4]. tRNA ligase is adenylated at an active site lysine [5] and then the AMP is transferred to the 5'-PO_4 of the substrate. Formation of the 5'–3'-phosphodiester bond proceeds and AMP is released. The phosphate at the spliced junction is derived from the γ-phosphate of GTP, and the phosphate originally at the 5'-splice site remains at the spliced junction as a 2'-phosphate and must be removed to complete reaction. A nicotinamide adenine nucleotide(NAD)-dependent phosphotransferase catalyses the transfer of the 2'-PO_4 to NAD [6, 7].

The enzymes of splicing are present at very low levels (approximately 150–200 molecules per cell), therefore purification of the latter is very complicated [1, 8, 9].

Here we report the identification of tRNA ligase activity (via E-pA complex formation) and partial purification of the enzyme from the ciliated protozoan *Tetrahymena pyriformis*. Ciliophora is one of the earliest diverged and conserved phylum of eukaryotes. Study of tRNAs processing in this organism, which is in progress at our department [10–12], may provide a new information for a better recognition of the splicing mechanism, because even within Eukarya at least four splicing mechanisms are known [1].

MATERIALS AND METHODS

Materials

PMSF, bacitracin, aprotinin, NaCl (Roth), EGTA (Merck), spermidine(Sigma), /8-^{14}C/ATP (Amersham).

***Tetrahymena pyriformis* cultivation.** *Tetrahymena pyriformis* (strain GL, amicronucleus) cells were grown in 6 l of cultivation medium containing 0.6% bacto peptone, 0.6% yeast extract, 1 mM $MgSO_4 \cdot 7\ H_2O$, 0.05 mM $CaCl_2$, 0.01 mM Mor's salt (pH 6.4) at 26 °C for 36 h and then overnight at the same temperature with stirring. The cells were harvested by centrifuga-

tion at 1000 g, washed with 10 mM Tris-HCl pH 8.0, containing 10 mM 2-mercaptoethanol 0.5 mM EDTA, 0.4 mM PMSF.

Preparation of supernatant S-100. The collected cells (apparently 35 g of cell paste) were resuspended in an equal volume of 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 10 mM 2-mercaptoethanol, 20% (w/v) glycerol (buffer A) containing 80 mM NaCl. To this and all subsequent buffers, to prevent protease degradation, the following protease inhibitors were added: 0.4 mM PMSF (freshly prepared as a 200 mM stock solution in isopropyl alcohol), 0.5 mM EGTA, 5 mM spermidine, 20 μg/ml bacitracin, 0.4 μg/ml aprotinin. To produce the cell extract, cell slurry kept in an ice-bath was sonicated 6 times in 10 s bursts with 50 s intervals (destruction of cells was controlled microscopically), then diluted with 30 ml of the same buffer and centrifuged for 1 h 45 min at 100,000 g. All purification procedures were carried out at 4 °C.

Chromatography on DEAE-cellulose column. Supernatant S-100 (65–70 ml) was applied to a DEAE-cellulose column (2,5 × 25 cm) equilibrated with buffer A containing 80 mM NaCl and protease inhibitors. After the end of loading of supernatant S-100 onto a column (45 ml/h), the column was washed with the same buffer (60 ml/h) until A_{280} was below 0.1. Then step NaCl gradient elution was applied (0.12 M, 0.3 M, 0.5 M, 0.8 M NaCl in buffer A).

Chromatography on Heparin-agarose column. Heparin–agarose was prepared by the method of Davison [13]. The packed column (1.5 × 12 cm) was equilibrated with buffer A, containing 0.12 M NaCl and protease inhibitors. Active fractions eluted from the DEAE-cellulose column were pooled and diluted to 0.12 M NaCl concentration and then loaded onto the heparin–agarose column (25 ml/h). Washing was performed with the same buffer (30 ml/h) until no more protein was eluted. Then gradient elution (0.12–0.8 M NaCl) was performed.

Gelfiltration on Toyopearl column. The active fractions from the heparin–agarose column (eluted with 0.8–0.9 M NaCl) were concentrated by saturation with solid $NH_4(SO4)_2$ (30–55%) or by addition of 1.2 vol of 40% (w/v) polyethylene glycol 6000 and incubation for 7 h at 0 °C. The precipitate was collected by centrifugation at 30,000 g for 1 h, resuspended in buffer A, dialyzed *vs* 2 l of buffer A and loaded onto the Toyopearl packed column (1.2 × 35 cm) equilibrated with buffer A. The elution (30 ml/h) was performed with the same buffer.

Determination of tRNA ligase activity. RNA ligases form a covalent E-AMP complex as an intermediate in the ligation reaction. Formation of this intermediate can be used to identify RNA ligase [14]. The RNA ligase activity in the fractions obtained during fractionation of the enzyme preparation from *Tetrahymena pyriformis* was estimated by incubation of enzyme preparation aliquots with /8-^{14}C/ATP at 20 °C for 5 min. The assay mixture contained: 30–45 μl of coctail (0.1 M Tris-HCl pH 8.0, 50 mM $MgCl_2$, 2 mM DTT, in some cases 10% (w/v) PEG 6000), 2.4 μM / 8-^{14}C/ATP, 15–30 μl of enzyme preparation in buffer A in a final volume of 70 μl. After 5 min of incubation 60 μl of assay mixtures was placed on Whatman 3 MM paper strips (1.5 × 1.5 cm), which then were soaked in 5% trichloroacetic acid containing 1% $Na_4P_2O_7$ for 30 min at 0 °C. The volume of TCA was 10–15 ml per strip. Then the strips were rinsed with ice-cold distilled water (50–70 ml per strip), 50 ml of acetone, dried and placed in cuvettes which contained 10 ml of scintillation liquid (4 g PPO and 0.2 g POPOP added to 1 l of toluene) and the radioactivity was measured with an LS-100 C radiospectrometer.

Estimation of enzymatic activities bound with tRNA maturation. The column fractions were incubated with 0.1–1.0 pmol of an internally ^{32}P-labeled substrate (human pre-tRNA^{Tyr}) in assay buffer (20 mM Tris-HCl pH 8.0, 60 mM NaCl, 8 mM $MgCl_2$, 1 mM DTT) for 30 min at 30 °C. The reaction products were analyzed by denaturating PAG electrophoresis and visualized by autoradiography [10].

RESULTS AND DISCUSSION

The enzymes bound with splicing are present in cells of eukaryotes in small amounts (150–200 molecules per cell), therefore isolation and purification of the latter is a very complicated problem [1, 8, 9]. For example, 100 to 300 μg of purified tRNA ligase protein was obtained from 1 kg of yeast cells [9].

There exists a good evidence that tRNA ligase in yeast [2] and in wheat germ [14] is organized into three domains containing, respectively, the ligase, the kinase and the phosphodiesterase activities. The boundaries between these domains are very sensitive to proteases and therefore obtaining the intact 95 kDa protein of tRNA ligase is exceptionally difficult. Successful solving of this problem in the case of tRNA ligase from yeast was achieved by taking the following three measures: the gene was expressed in a protease-deficient strain of *E. coli*, the first steps of isolation were done as rapidly as possible, the mixture of protease inhibitors was included in all extraction and chromatography steps [2].

Tetrahymena pyriformis cells were grown, harvested, homogenized, and the high speed supernatant S-100 was prepared as described in Materials and Methods. The S-100 supernatant was chromatographed over DEAE-cellulose. The elution profile of *Tetrahymena pyriformis* supernatant S-100 chromatography on DEAE-cellulose column is presented in Fig. 1a. One

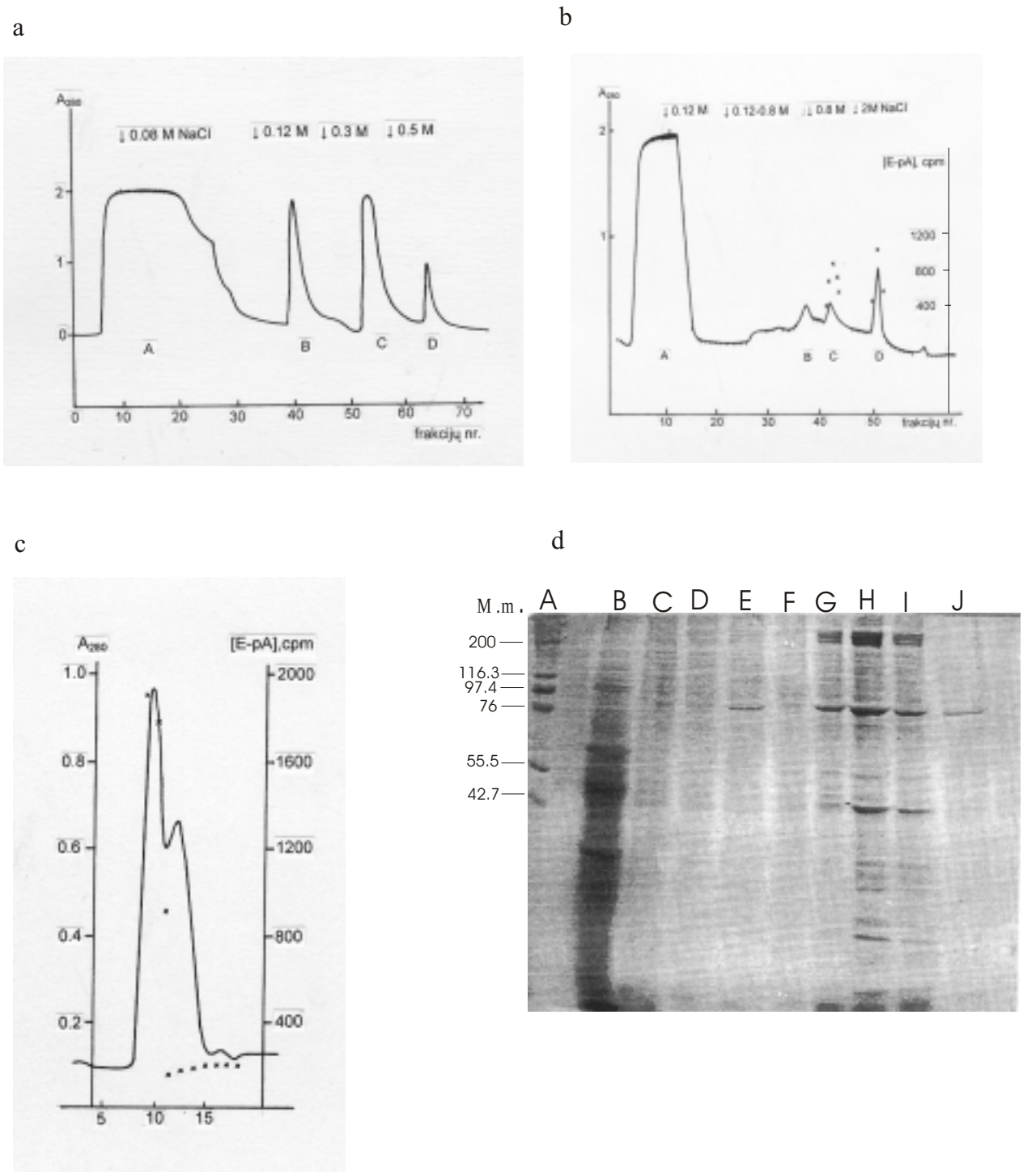

Fig. 1. Elution profiles and purification of tRNA ligase from *T. pyriformis*. a –DEAE cellulose; b – heparin–agarose; c – Toyopearl; d – SDS-PAGE analysis of the tRNA activity fractions (stained with Coomassie brilliant blue) from purification on chromatography columns. Lanes: A – protein standards, B – supernatant S-100, H, I – DEAE–cellulose; G – heparin–agarose; E, J – Toyopearl

can see that a large part of protein applied to the column appears in flow-through (Fig. 1a, peak A). The next peaks are eluted with 0.12 M, 0.3 M, and 0.5 M NaCl in buffer A, respectively. In the opinion of many authors [9, 15, 16], it is difficult to estimate ligase activity at early stages of extract purification because of inhibitors present. They are removed only at the stage of chromatography on a heparin–agarose column [9, 15]. The nature of those inhibitors is unknown. In very rare cases we detected at very low levels tRNA ligase activity by E–pA complex formation in the fractions eluted from DEAE-cellulose column with 0.5 M

NaCl (peak D). To assay for enzymatic activities related to tRNA maturation, the column fractions were incubated with internally ^{32}P-labeled human tRNA^{Tyr} precursor, which was transcribed *in vitro* and gel-purified. This precursor contains a 14 nucleotide 5' leader, 2 nucleotide 3'external sequence and 20 nucleotide intron. Analysis by denaturating PAG electrophoresis and visualization by autoradiography revealed the presence of various products of human pre-tRNA^{Tyr} maturation (Fig. 2a) in reaction mixtures of the DEAE-cellulose column flow-through fractions (Fig. 1a, peak A). The identified products were formed as a result of endonucleolytic cleavage of pre-tRNA^{Tyr} and ligation of tRNA halves and other activities related to tRNA maturation (presence of 5' end and 3' end matured pre-tRNA and native t-RNA^{Tyr} in reaction mixtures

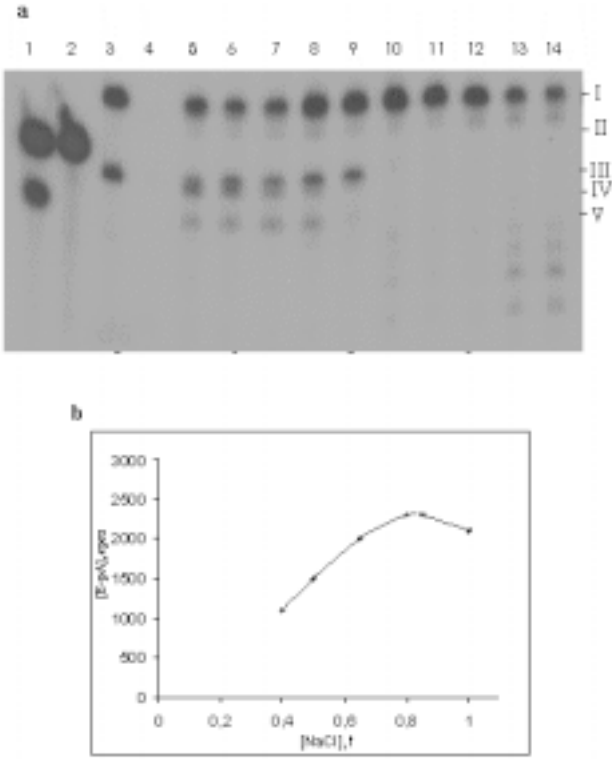

Fig. 2. Assay of pre-tRNA^{Tyr} maturation activities: a – SDS-PAGE analysis of the products of pre-tRNA^{Tyr} maturation after incubation with DEAE–cellulose column peak A fractions. Lanes: 1, II – pre-tRNA^{Tyr} with 5' leader and native CCA end; V – native tRNA^{Tyr}; 2.II – pre-tRNR^{Tyr} with 5' leader and native CCA end; 3.I – pre-tRNA^{Tyr} with 5' leader, 3' leader (2 nt) and introne (20 nt); III – 5' end matured tRNA^{Tyr}; 5–8 – pre-tRNA^{Tyr} with 5' leader, 3'leader and introne incubated with DEAE–cellulose column peak A fractions; I – pre-tRNA^{Tyr} with 5' leader, 3' leader and intron; III – 5' end matured pre-tRNA^{Tyr}; IV – pre-tRNA^{Tyr} 5' and 3' end matured with introne; V – matured tRNA^{Tyr}; 9–14 – pre-tRNR^{Tyr} with 5' leader 3' leader and introne incubated with DEAE cellulose column fraction from peaks B, C, D. b – the influence of the NaCl concentration on the E–pA complex formation

(Fig. 2a, lanes 5–8). No such products were detected by analysis of incubation mixtures of human pre-tRNA^{Tyr} with fractions from peaks B, C, D (lanes 9–14). Similar activities were detected in crude extract from human leukaemia cells [16]. This extract showed a strong splicing endonuclease and ligase activity with human intron-containing pre-tRNA^{Tyr} as a substrate; the processing enzymes were not detectable.

For further purification we applied fractions eluted from the DEAE-cellulose column with 0.5 M NaCl in buffer A. The elution profile of chromatography on the heparin-agarose column is presented in Fig. 1b. The same as in the case of chromatography on the DEAE-cellulose column, the main part of protein applied to the column appears in flow-through fractions (Fig. 1b, peak A). We estimated E–pA complex formation in fractions eluted with buffer A containing 0.8 M NaCl (Fig. 1b, peak C) and observed E–pA complex formation of peak D fractions eluted with a still higher concentration of NaCl in buffer A. It is necessary to pay attention to the fact of E–pA complex formation in the presence of high salt concentration (final concentration of NaCl in activity assay mixtures varied from 0.4 M to 1 M).

T4 DNA ligase is strongly inhibited by NaCl or KCl, if the concentration exceeds 200 mM [17]. We didn't detect inhibition by NaCl (0.4–1 M) in the case of commercial T4 RNA ligase. Data on the influence of NaCl concentration on the E–pA complex formation of the active fractions eluted from the heparin–agarose column are presented in Fig. 2b. As can be seen from presented data, we didn't estimate inhibitory influence of NaCl (in the range of 0.4–1 M final concentration) on the E–pA complex formation of active fractions eluted from the heparin–agarose column. On the contrary, even stimulation of this process by NaCl was observed. This stimulation was most effective when the final concentration of NaCl in the activity assay mixture was 0.8–0.85 M. Concentrated activity-containing fractions from the heparin–agarose column were loaded onto the Toyopearl packed column. The elution profile from the Toyopearl column is shown in Fig. 1c. One can see that the peak of tRNA ligase–pA complex formation activity is in good agreement with the peak of protein concentration profile. E–pA formation activity has never been detected in fractions forming a shoulder of the protein concentration peak (fractions 12–14, Fig. 1c). It is possible that the protein-forming shoulder is a product of tRNA ligase partial degradation. Similar observations were done in the case of purification of tRNA ligase from yeast [9, 15].

Similarly as in the case of isolation of tRNA ligase from other eukaryotes, we came across the phenomenon of instability of enzyme preparations from *T. pyriformis*. Preparations after the heparin–agarose column were stable in the best case within one-two weeks

and after the Toyopearl column only 2–3 days. The lower stability of the preparation after Toyopearl column in comparison with that from heparin–agarose may be explained by a longer time of action of non-inhibited proteases and therefore a more disassembled native structure of the enzyme. On the other hand, the non-identified factors necessary for the maintenance of the active structure of tRNA ligase may be lost in the course of enzyme purification. We observed a positive influence of NaCl on the stability of the enzyme. Active fractions eluted from heparin–agarose column with NaCl of higher concentrations than 1 M were significantly more stable than those eluted with 0.8 M NaCl. We observed a very strong dependence of the initial activity of enzyme preparations upon the presence of protease inhibitors in extraction and chromatography buffers, too. As one can see from data of SDS-PAGE analysis of tRNA activity fractions (Fig. 1d), purification of 95 kDa protein was achieved in the course of chromatographic procedures on various columns.

Further research should be directed to optimization of the conditions for enzyme action, to selection of the enzyme storage conditions in order to raise its stability, to the assay of interactions between the enzyme and the other components of the pre-tRNA maturation system.

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tRNR LIGAZĖS IŠ *Tetrahymena pyriformis* IŠSKYRIMAS IR GRYNINIMAS

S a n t r a u k a

Tirta tRNR ligazės išskyrimo iš *Tetrahymena pyriformis* galimybė. Supernatanto S-100 frakcionavimo DEAE-celiuliozės kolonėlėje frakcijose aptikti pre-tRNR^{Tyr} brendimo aktyvumai. Gryninant fermento preparatą DEAE-celiuliozės, heparin-agarozės ir Toyopearl kolonėlėse gautas dalinai išgrynintas 95 kDa baltymas. NaCl koncentracijos (0,4 M – 1 M) poveikio fermento-pA komplekso susidarymui tyrimų rezultatai leidžia manyti, kad susidaręs kompleksas yra t-RNR ligazė-pA.