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Expression and purification of full-length Alanyl-tRNA-synthetase from *Thermus thermophilus* HB27

M. Yu. Rybak[1], A. E. Priss[1], O. I. Gudzera[1], A. O. Kovalchuk[2], I. A. Kryklyvyi[1], M. A. Tukalo[1]

[1] Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03143

[2] ESC "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv
64/13, Volodymyrska Str., Kyiv, Ukraine, 01601
mariia.rybak@gmail.com

Aim. To gain insight into structural and functional properties of alanyl-tRNA,synthetase (AlaRS), we genetically engineered constructs for expression and purification of full-length AlaRS and checked its activity in aminoacylation assays. **Methods.** The genomic DNA for the *AlaS* gene from the *T. thermophilus* (HB 27 strain) was amplified by PCR and cloned into vectors with and without a histidine tag. To optimize conditions for the protein expression in *E.coli* and to develop efficient purification procedure, the molecular biology techniques were applied. AlaRS was purified by affinity and size-exclusion chromatography. The molecular weight of enzyme was determined by gel filtration. **Results.** The expression and purification conditions for recombinant AlaRS were optimized. Approximately 1.5 mg of the pure active recombinant enzyme can be obtained from 1 L of bacterial culture. AlaRS from *T. thermophilus* is a dimer in solution with an experimental MW of 204 kDa. **Conclusions.** The purified recombinant enzyme will be used for further studies on the functional kinetics and structure of the crystal complex with tRNA.

Keywords: aminoacyl-tRNA-synthetase, AlaRS from *T. thermophilus*, expression of recombinant protein, protein purification.

Introduction

Aminoacyl-tRNA-synthetases (aaRSs) attach cognate amino acids (aa) to their tRNAs in a two-step reaction: 1) the activation of amino acid with ATP (generation of aa-AMP) and 2) the transfer of activated amino acid to the 3'-end of tRNA (formation of aminoacyl-tRNA) [1, 2]. By different functional and structural features, these enzymes were divided into classes I and II [3–5]. Alanyl-tRNA synthetase (AlaRS) belongs to class II and consists of four domains: the N-terminal (aminoacylation domain), editing one, the domain of tRNA recognition and the

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C-terminal domain, responsible for oligomerization [6]. The C-Ala domain also has a subdomain, which plays an essential role in aminoacylation and editing [7]. In different organisms, AlaRS may have a quaternary structure of tetramer (*Escherichia coli*) [8] or dimer (*Thermus thermophilus* HB8) [9], or exist as a monomer (*Bombyx mori*) [10]. Noteworthy, this synthetase has a special history of study: on the one hand, AlaRS from *E. coli* is the first synthetase that was cloned, sequenced [11], characterized genetically [12] and biochemically [13, 14], on the other hand, only after 25 years of research the first crystal structure of AlaRS was resolved for the 453-aa N-terminal catalytic fragment of the *Aquifex aeolicus* enzyme [15]. Then the 441-aa catalytic fragment of *E.coli* AlaRS with different leucine-zipper surface mutations in the complex with glycine, L-alanine and L-serine was reported [16]. Later the modified leucine half-zipper technique [17] was used for the *E.coli* AlaRS and the interactions between $tRNA^{Ala}$ and the synthetase were studied by ITC (Isothermal Titration Calorimetry) [18].

The alanyl-system is also interesting in its recognition context. $tRNA^{Ala}$ contains a unique wobble G3:U70 base pair in the acceptor stem which determines its specificity to AlaRS [19]. Recently, the first complex of archaeon AlaRS catalytic fragment from *Archaeoglobus fulgidus* has been reported in 2 states — with $tRNA^{Ala}$ bearing G3·U70 and with variant A3·U70 [20]. Interestingly, human AlaRS was identified to be able to mischarge G4:U69-tRNA, possessing an evolutionary gain-of-function and not being an accidental mistake [21]. The human C-Ala domain evolutionarily lost its prokaryotic tRNA functional role [7] on editing and aminoacylation and is remolded into the DNA-binding protein [22]. Recently, Chong *et al.* have reported three different strategies in the G3:U70 recognition among kingdoms of life [23]. On the other hand, it was shown for *E.coli* AlaRS that G3:U70 pair is an important element for specific interaction with the editing domain of the enzyme [19]. Thus, the precise recognition mechanism abides disputable until the spatial structure of full-length AlaRS with $tRNA^{Ala}$ is solved.

Despite the progress in the field, the mechanisms of aminoacylation and editing by AlaRS remain unknown; the observations of functional and structural properties of synthetases from all kingdoms of life are still relevant. In this study we cloned AlaRS from the *T. thermophilus* HB27 strain (AlaRSTT), developed and compared purification technique of enzyme with and without C-terminal 6-histidine-tag, resulted in a fast 2-step purification procedure.

Materials and Methods

Cloning of alaS gene. Genomic DNA from *T. thermophilus* cells was obtained according to [24]. Based on the sequence information of *alaS* from *Thermus thermophilus* strain *HB27* (P61707 in UniProt [25] and WP_011173855.1 in GenBank [26], 882 amino acid residues) a pair of primers was designed for PCR: 5' forward – **atatg**cgcacggcggagatccgcgagaagttcc and 5' reverse – **aagctt**attaggggaggaggcc-ggggagggcctcccgg, which contained NdeI and HindIII restriction sites (marked respectively). Long-PCR-mix (Pfu+Taq) was used for gene amplification. PCR product was gel purified and further cloning proceeded according to TOPO TA protocol («Invitrogen», USA) into the pCR2.1 vector. Briefly, 6 µl of mixture, consisted of 2/3 volume of purified PCR pro-

duct, 1/6 — pCR2.1 vector, 1/6 — salt solution (200 mM NaCl, 10 mM $MgCl_2$), was incubated at 37ºC for 10 min. Electrocompetent Top10 *E.coli* cells («Invitrogen», USA) were used for the transformation («Bio-Rad» electroporation system). The blue-white screening was performed for selection of Kan^R and Amp^R clones with further restriction analysis for target gene (about 2646bp) detection. Positive clones were confirmed by NcoI and HindIII restriction and visualized on 1.5 % agarose gel. The correct sequence of AlaRSTT gene was confirmed by DNA sequencing.

To create the His-tagged protein construction, C-terminal linker, encoding 6 histidine residues, was added in PCR with 2 primers: 5'-ctccccggcctcctcccctataagcttgcggccgcactcg-3' (AlaRSTT-6His-1) and 5'-cgagtgcggccgcaagcttataggggaggaggccggggag-3' (AlaRSTT-6His-2).

Then both AlaRSTT constructions (with and without His-tag) were excited by NcoI and Hind III Fast Digest («Thermo Scientific») restrictases and ligated into the pET28b vector, previously dephosphorylated by 1 unit of shrimp phosphatase in 1^X reaction buffer («Roche»). Ligation proceeded with 1 unit of T4-DNA-ligase in 1^X ligase buffer («Thermo Scientific») for 20 h at 14ºC, followed by Top10 electroporation and sequencing.

A small-scale test of AlaRSTT expression

The *E.coli* BL21(DE3)Star cells were electroporated with pET28b-AlaRS/His-AlaRS plasmid. The analysis of the expression level was performed in P (phosphate), LB (Lauria-Broth) and TB (Terrific-Broth), supplemented with kanamycin (50 μg/ml). Pre-culture (2 ml) was grown overnight at 37 °C. Culture (10 ml of each medium) was inoculated by pre-culture in 1:100 dilution, at OD_{600} = 0.6 the protein expression was induced by 1 mM IPTG for 3 h. Cells were harvested by centrifugation (10 min, 6000 × g at 4 °C) and resuspended in 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 4 % glycerol, 20 mM β-mercaptoethanol, 1^X cocktail of protease inhibitors («Roche»), 0.5 μg/μl lysozyme, followed by 2 cycles of freezing/defrosting (80 °C, 10 min). To reduce the viscosity, 25 mM $MgCl_2$ and 1 u/μl DNase I were added and left for 15 min at 37 °C. The lysed cells were centrifuged for 30 min at 6000 × g. The supernatant (fraction of soluble proteins) was analysed by 10 % PAGE.

Expression of AlaRSTT and strategy of its purification

Pre-culture (50ml) of *E.coli* BL21(DE3)Star, carrying pET28b-AlaRSTT plasmid, was grown in LB with kanamycin (50 μg/ml) overnight at 37 °C. The cell culture (1 L) was inoculated with pre-culture and grown to OD_{600} = 0.6; after the induction of protein expression with 1 mM IPTG, the culture was grown for 3 hours at 37 °C.

All subsequent steps were conducted at 4 °C. Cells were precipitated at 15 000 x g for 20 min. The precipitate was resuspended in a buffer A (20 mM Tris-HCl pH 8.0, 5 mM DTT, 0.1 mM EDTA, 1 mM PMSF, a tablet of a cocktail of protease inhibitors («Roche»)). Lysozyme was added for better cell lysis (to a final concentration of 1 mg/ml) and incubated for 30 min. Cells were disrupted by sonication 8 × 30 sec with 1 min breaks. The lysate was heated at 70 °C for 35 min, debris was precipitated by centrifugation at 20 000 × g (30 min). The resulting supernatant was applied to a DEAE-Sepharose column (30 ml, «GE Healthcare»), pre-equilibrated with mo-

dified buffer A (0.1 mM PMSF and 0.1 mM EDTA) and further washed with it. AlaRSTT was eluted in the linear gradient concentration of NaCl – from 0 to 350 mM. Collected fractions were analysed by Bradford assay and SDS-PAGE. Fractions containing protein were combined and desalted to the 90 % concentration of $(NH_4)_2SO_4$ (600 g/L). The precipitate was dissolved in a buffer B (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.003 % NaN_3,) and applied to the pre-equilibrated Hi-Load 16/60 Superdex 200 (150 ml, «Pharmacia Biotech») column with flow rate 0.5 ml/min. The isolated fractions were concentrated on 10 kDa Centricon («Merck») at 6000 rpm, analysed spectrophotometrically and electrophoretically, their aminoacylation activity was determined before freezing and storage at -20 °C.

Final protein concentrations were determined by the Bradford assay using Roti®-Quant («Roth») [27]. Light absorption coefficient at 280 nm (ε_{280} = 93530 M^{-1} cm^{-1}) and absorbance of 0.1 % solution (A_{280} (1 mg/ml) = 0.959 unites·mg^{-1}·ml) were calculated from the amino acid sequence of the DTDTT (ProtParam tool, ExPASy, Swiss Port) [28] and used for determination of enzyme concentration.

His-AlaRSTT purification by affinity and size-exclusion chromatography

The production of His-tagged recombinant protein was performed with 0.6 mM IPTG induction during 3 hours at 37 °C in P-medium (1 L). All steps of His-AlaRSTT purification were performed at 4°C. Harvested cells after 30 min centrifugation (15 000 x g) were resuspended in buffer C (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM β-mercaptoethanol, 1^X protease inhibitors cocktail («Roche»)), sonicated 10 × 30 sec with 1 min breaks. Then cell debris was precipitated (20 000 × g, 30 min), heated in water bath (70°C), and followed by centrifugation. The concentration of imidazole («Sigma») was adjusted to 15 mM and the clear supernatant was added to Ni-NTA Sepharose Fast Flow resin (2 ml of 50 % slurry, «GE Healthcare»), pre-equilibrated with the same buffer and incubated for 1 h at 150 rpm. The resin was washed with buffer C, supplemented with 15 mM imidazole followed by washing with solution containing 500 mM NaCl. AlaRSTT was eluted from the column by 250 mM imidazole (as *E.coli* enzyme [17]). Target protein fractions were combined and dialyzed overnight against 1 L of buffer D (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$ (as recommended for *Archaeoglobus fulgidus* AlaRS [29]), 1 mM β-mercaptoethanol), concentrated on 30 kDa Centricon at 6000 rpm to 1 ml volume (4 mg/ml) and loaded to the Hi-Load 16/60 Superdex 200 column, pre-equilibrated with buffer D, supplemented with 150 mM NaCl, 0.1 mM PMSF, 0.003 % NaN_3) with flow rate 1 ml/min. Peak fraction was concentrated on 30 kDa Centricon (before concentration this fraction was 3-fold diluted in 1 mM β-mercaptoethanol and a saturated solution of $(NH_4)_2SO_4$ in proportion 1:20), the purity was analysed by SDS-PAGE.

Gel filtration of proteins

To access the approximate molecular weight of purified AlaRSTT the size-exclusion chromatography on Hi-Load 16/60 Superdex S 200 was performed. The column was equilibrated with 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$ 1 mM β-mercaptoethanol, 150 mM NaCl, 0.1 mM PMSF, 0.003 % NaN_3. All samples ran at 1 ml/

min flow rate, the elution was monitored at 280 nm. An elution volume of each protein sample from the column was calculated; the log (MW) of marker proteins was plotted against the ratio V_e / V_o. Marker proteins, which were used for column calibration, are the following: ferritin dimer (900 kDa), ferritin monomer (450 kDa), β-amilase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carboanhydrase (29 kDa) and cytochrome c (12,4 kDa). The molecular weight of AlaRSTT was determined by comparison of its V_e / V_o index with those of known protein standards. The calibration curve is shown in Fig. 5.

Test of the aminoacylation activity of purified AlaRSs

The reaction mixture (25 μl) contained 100 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, 100 μg/ml BSA, 3 mM ATP, 25 μM [^{14}C]-L-Ala (158 mCi/mmol; Amersham), 5 μg/μl total tRNA, 50 nM AlaRS was conducted at 37 °C for several minutes. Aliquots were removed from the reaction at appropriate time points, quenched by cold 10 % TCA and passed through nitrocellulose filters. Dried filters were analysed by liquid scintillation counting.

Results and Discussion

Expression and purification of the full-length AlaRSTT with problem of its proteolysis

The final protein expression was performed successfully in the LB medium (Fig. 1). Even at the expression step and analysis of protein solubility, we noticed rapid protein degradation, which did not show the dependence on media or buffer composition (Fig.1, 1 — full-length protein, 2a/b — proteolyzed).

The purification procedure combined the anion exhange and size-exclusion chromatographies. The peak fractions, eluted from DEAE-Sepharose with the linear gradient of NaCl (0-350 mM), were desalted by $(NH_4)_2SO_4$ and uploaded on Superdex 200 column (Fig. 2). During both purification steps, the ratio of full-length and proteolyzed forms of AlaRSTT remained constant. Despite the including of proteases inhibitors in all solutions, it was impossible to reduce the level of synthetase degradation. We hypothesize that a short form appeared due to the existence of unstable regions of the protein C-terminal domain, which led to the damage and shortening of the target enzyme. This issue seems to be the main reason AlaRSTT has not been crystalized yet, despite the first report of its purification from the

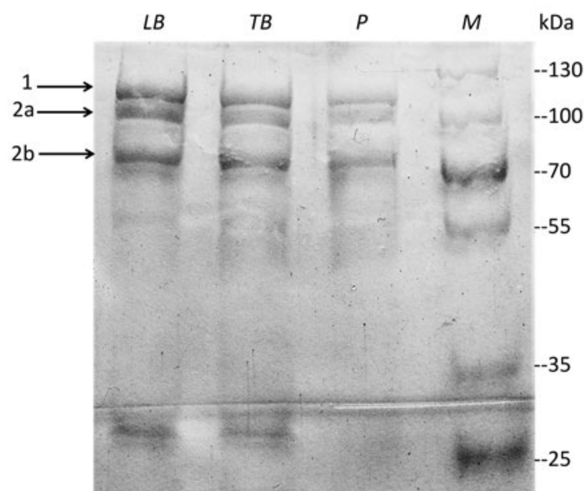

Fig. 1. Test of AlaRSTT expression in LB, TB and P-media after 3h of induction with 1 mM IPTG (soluble fraction). *M* –marker (ThermoFisher, PageRuler™ Prestained Protein ladder 10-170 kDa).

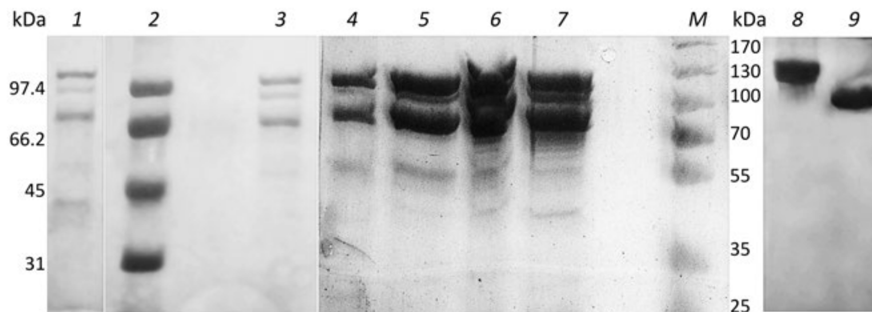

Fig. 2. Purification of AlaRSTT without His-tag by anion exhange and size-exclusion chromatographies. 12.5 % SDS-PAGE: 1 — lysate after heating; 2 — protein marker (BioRad LowRange); 3 — 23rd fraction on DEAE-Sepharose; 4–7 — peak fractions (24–27 respectively) on DEAE-Sepharose, combined for desalting; *M* — molecular weight marker (ThermoFisher, PageRuler™ Prestained Protein ladder 10–170 kDa); 8–9 — concentrated AlaRSTT fractions (8-full-length, 9-short) after Superdex 200 column.

T. thermophilus strain HB8 more than 20 years ago [9]. There is no evidence how to overcome this problem, therefore our work is relevant.

Expression and purification of the full-length AlaRSTT with C-terminal His-tag

To overcome the problem with AlaRSTT degradation, we recloned it with C-terminal His-tag. Surprisingly, it increased the protein stability up to 90 %. At the first purification step (affinity chromatography on Ni-NTA-Sepharose), only about 5-10 % of degraded AlaRSTT were observed (Fig. 3). We tried to elute AlaRS with the imidazole gradient, varied concentrations of NaCl and β-mercaptoethanol in lysis buffer, adding one more purification step after affinity column (DEAE-Sepharose), but it did not help to eliminate the

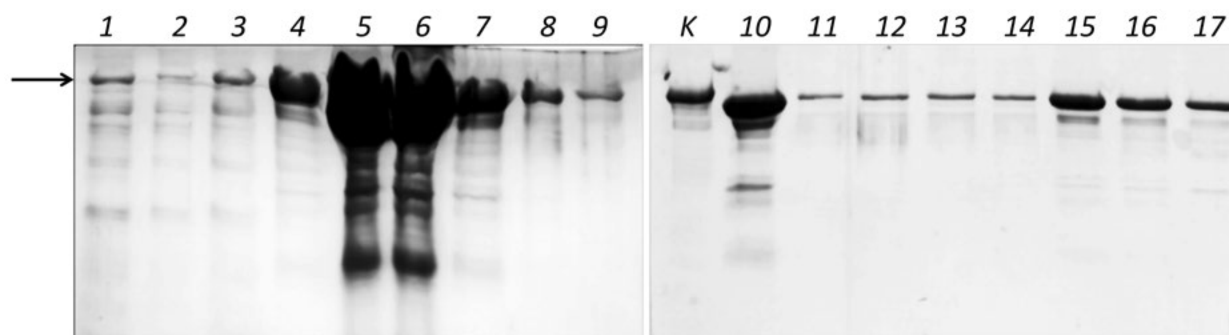

Fig. 3. Two-step purification procedure of His-AlaRSTT: 1 — lysate, uploaded to Ni-NTA-Sepharose column; 2 — fraction of proteins, not bound with Ni-NTA; 3 — wash flow-through fraction; 4–9 — peak fractions, eluted from column; *K* — control, LeuRS from *Mycobacterium tuberculosis* (107 kDa); 10 — combined eluate after dialysis, loaded on Superdex 200 column, 11–17 — fractions on gel-filtration column (1–7 respectively).

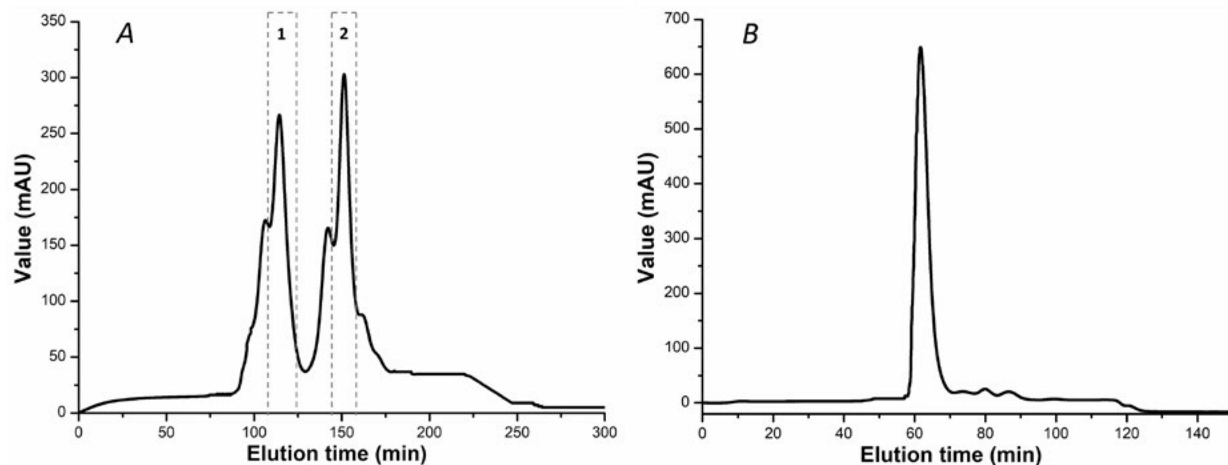

Fig. 4. Purification of AlaRSTT on size-exclusion chromatography on Superdex S 200 column: *A* — Elution profile for AlaRSTT with flow rate 0.5 ml/min (1 — full-length protein, 2 — its shorten form); *B* — for 6^xHis-AlaRSTT with flow rate 1 ml/min.

primary (about 10 %) protein degradation level (data not shown). Nevertheless, the ratio did not increase after overnight dialysis and the second (size-exclusion) chromatography step (Fig. 4). The elution profile of AlaRSTT on the gel-filtration column is represented in Fig. 4 (A — construct without His, B — with C-terminal 6^xHis-tag). The Fig. 4B profile together with the elution of marker proteins were used for the determination of AlaRSTT molecular weight (MW). The theoretically calculated MW of the corresponding monomer structure was 97.48 kDa. Experimentally, it was determined as 204.2 kDa, confirming protein dimeric form. Calibration curve is shown in Fig. 5. The total amount of purified protein was ~1.5 mg from 1L of bacterial culture (~4.2 g of cell pellet). Thus, our results are comparable with the literature data (Table 1).

Table 1. **Comparison of purification steps and total enzyme quantities in *T. thermophilus* HB8 and HB27 strains**

	Total number of purification steps	Purified protein amount from 1g of *E.coli* cells (mg)
His-tagged AlaRSTT (strain HB8)[a]	4	0.46
AlaRSTT (HB27)[b]	2	0.72 (0.24 – full-length; 0.48 – short form)
His-tagged AlaRSTT (HB27)[b]	2	0.36

[a] Literature data, assumed from [9].
[b] Current study

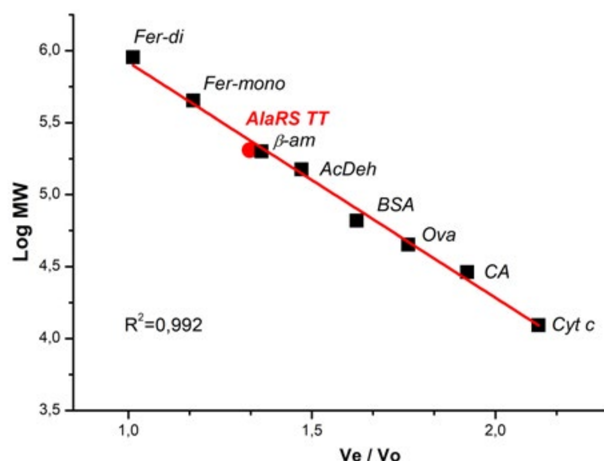

Fig. 5. Determination of AlaRSTT molecular weight by size-exclusion chromatography. Calibration curve on Superdex S 200 column was prepared as described in „Materials and methods" (Fer-di — ferritin dimer; Fer-mono — ferritin monomer; β-A — β-amilase; Cat — catalase; AcDeh – alcohol dehydrogenase; BSA — bovine serum albumin; Ova — ovalbumin; AlaRSTT — alanyl-tRNA-synthetase; CA — carboanhydrase; Cyt.c — cytochrome c)

Aminoacylation activity of purified AlaRSTT

All purified enzymes were tested for their activity with bulk tRNA from *E.coli* and radiolabelled [^{14}C]-L-Ala (Fig. 6). Noteworthy, the shortened form of AlaRSTT had only a 2-fold less initial velocity in comparison to the full-length protein. Thus, aminoacylation domain of AlaRS (C-Ala) worked properly. The full-length AlaRS has a slightly higher velocity, so it will be used for further functional investigations.

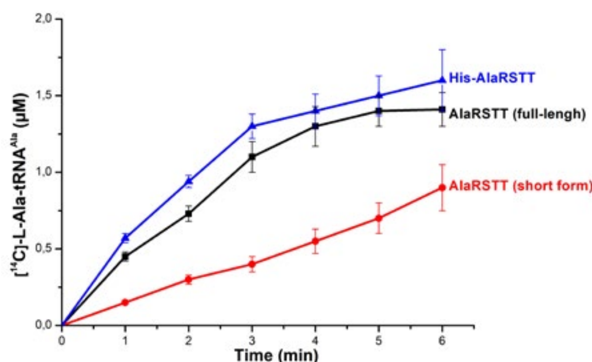

Fig. 6. The initial levels of Ala-tRNA^{Ala} synthesis by AlaRSTT. Experimental details were shown in section "Methods"

Conclusions

The recombinant His-AlaRSTT was expressed in *E.coli* and purified to homogeneity without premixes of degraded forms. For the successful expression and the prevention of protein degradation during purification steps, the affinity tag was attached to the C-end of the target enzyme. AlaRSTT will be used for further structural studies and investigation of its editing mechanism.

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Експресія та очистка повнорозмірної аланіл-тРНК-синтетази *Thermus thermophilus* штаму HB27

М. Ю. Рибак, А. Є. Прісс, О. І. Гудзера,
А. О. Ковальчук, І. А. Крикливий, М. А. Тукало

Мета. Для детального вивчення структурних та функціональних властивостей аланіл-тРНК-синтетази (АлаРС) було створено генно-інженерну конструкцію для експресії та очищення повнорозмірної синтетази та перевірено її активність у реакції аміноацилювання. **Методи.** Ген AlaS з *T. thermophilus* (штам HB27) геномної ДНК ампліфікували за допомогою ПЛР з відповідними праймерами та клонували у вектор з та без гістидинової послідовності. Для оптимізації умов експресії білка в *E.coli* та розробки ефективної процедури очищення були застосовані сучасні методи молекулярної біології. АлаРС очищали методами афінної хроматографії та гель-фільтрації. Молекулярна маса ферменту визначалася на гель-фільтраційній колонці. **Результати.** Умови експресії та очистки рекомбінантної АлаРС були оптимізовані. Близько 1,5 мг чистого рекомбінантного активного ферменту можна одержати з 1 л бактеріальної культури. АлаРС з *T. thermophilus* є димером у розчині з експериментальною масою 204 кДа. **Висновки.** Отриманий рекомбінантний фермент буде використовуватися для подальших експериментів з функціональної кінетики та структурних досліджень кристалічного комплексу з тРНК.

Ключові слова: аміноацил-тРНК-синтетаза, АлаРС T. thermophilus, експресія рекомбінантного білка, хроматографічне очищення білка.

Экспрессия и очистка полноразмерной аланил-тРНК-синтетазы Thermus thermophilus штамма HB27

М. Ю. Рыбак, А. Е. Присс, О. И. Гудзера,
А. А. Ковальчук, И. А. Крикливый, М. А. Тукало

Цель. Для детального изучения структурных и функциональных свойств аланил-тРНК-синтетазы (АлаРС) было создано генно-инженерную конструкцию для экспрессии и очистки полноразмерной синтетазы и проверено ее активность в реакции аминоацилирования. **Методы.** Ген alaS из геномной ДНК *T. thermophilus* (штамм HB27) амплифицировали с помощью ПЦР с соответствующими праймерами и клонировали в вектор с и без гистидиновой последовательностью. Для оптимизации условий экспрессии белка в *E.coli* и разработки эффективной процедуры очистки были применены современные методы молекулярной биологии. АлаРС очищали методами аффинной хроматографии и гель-фильтрации. Молекулярная масса фермента определялась на гель-фильтрационной колонке. **Результаты.** Условия экспрессии и очистки рекомбинантной АлаРС были оптимизированы. Около 1,5 мг чистого рекомбинантного активного фермента можно получить с 1 л бактериальной культуры. АлаРС с *T. thermophilus* является димером в растворе с экспериментальной массой 204 кДа. **Выводы.** Полученный рекомбинантный фермент будет использован для дальнейших экспериментов с функциональной кинетики и структурных исследований кристаллического комплекса с тРНК.

Ключевые слова: аминоацил-тРНК-синтетазы, АлаРС T. thermophilus, экспрессия рекомбинантного белка, хроматографическое очистки белка.

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