Identification of Functional Regions in the Rhodospirillum rubrum L-Asparaginase by Site-Directed Mutagenesis.

ARTICLE *in* MOLECULAR BIOTECHNOLOGY · NOVEMBER 2014

Impact Factor: 1.88 · DOI: 10.1007/s12033-014-9819-0 · Source: PubMed

10 AUTHORS, INCLUDING:

Alexander V Veselovsky
Institute of Biomedical Chemistry, Moscow
45 PUBLICATIONS **440** CITATIONS

Mol Biotechnol (2015) 57:251–264
DOI 10.1007/s12033-014-9819-0

RESEARCH

Identification of Functional Regions in the *Rhodospirillum rubrum* L-Asparaginase by Site-Directed Mutagenesis

M. V. Pokrovskaya · S. S. Aleksandrova · V. S. Pokrovsky · A. V. Veselovsky · D. V. Grishin · O. Yu. Abakumova · O. V. Podobed · A. A. Mishin · D. D. Zhdanov · N. N. Sokolov

Published online: 5 November 2014

Abstract Site-directed mutagenesis of *Rhodospirillum rubrum* L-asparaginase (RrA) was performed in order to identify sites of the protein molecule important for its therapeutic and physico-chemical properties. Ten multi-point mutant genes were obtained, and five recombinant RrA variants were expressed in *E. coli* BL21(DE3) cells and isolated as functionally active highly purified proteins. Protein purification was performed using Q-Sepharose and DEAE-Toyopearl chromatography. Overall yield of the active enzymes was 70–80 %, their specific activity at pH 7.4 and 37 °C varied of 140–210 U/mg. L-Glutaminase activity did not exceed 0.01 % of L-asparaginase activity. All RrA mutants showed maximum enzyme activity at pH 9.3–9.5 and 53–58 °C. Km and Vmax values for L-asparagine were evaluated for all mutants. Mutations G86P, D88H, M90K (RrAH), G121L, D123A (RrAI) caused the loss of enzyme activity and confirmed the importance of these sites in the implementation of catalytic functions. Removal of four residues from C-terminal area of the enzyme (RrAK) resulted in the enzyme instability. Mutations D60K, F61L(RrAD), and R118H, G120R(RrAJ) led to the improvement of kinetic parameters and enzyme stabilization. Substitutions E149R, V150P (RrAB) improved antineoplastic and cytotoxic activity of the RrA. A64V, E67K substitutions, especially in combination with E149R, V150P (RrAE), considerably destabilized recombinant enzyme.

Keywords Biotechnology · L-Asparaginase · Site-directed mutagenesis · Physico-chemical properties of L-asparaginases

M. V. Pokrovskaya · S. S. Aleksandrova · V. S. Pokrovsky · A. V. Veselovsky · D. V. Grishin (✉) · O. Yu. Abakumova · O. V. Podobed · A. A. Mishin · D. D. Zhdanov · N. N. Sokolov
Institute of Biomedical Chemistry, ul. Pogodinskaya 10, Moscow 119121, Russia
e-mail: molbiol_ibm@inbox.ru

M. V. Pokrovskaya
e-mail: ivan1190@yandex.ru

S. S. Aleksandrova
e-mail: v-aleksandrov@yandex.ru

V. S. Pokrovsky
e-mail: vadimpokrovsky@yandex.ru

A. V. Veselovsky
e-mail: veselov@ibmh.msk.su

O. Yu. Abakumova
e-mail: olabakumo@yandex.ru

O. V. Podobed
e-mail: ovpodobed@gmail.com

A. A. Mishin
e-mail: a56m@mail.ru

D. D. Zhdanov
e-mail: zhdanovdd@mail.ru

N. N. Sokolov
e-mail: Nikolai.Sokolov@ibmc.msk.ru

Introduction

L-Asparaginase or L-asparagine amidohydrolase for more than 40 years have been applied for therapy of acute lymphoblastic leukemia, lympho- and reticulosarcoma [1]. Since 2006 L-asparaginases are included into the international nosological system and the European Medicines Agency [http://www.ema.europa.eu/docs/en_GB/document_library/Other/2009/10/WC500004053.pdf, Accessed July 5, 2011].

The antineoplastic action was demonstrated for several L-asparaginases from different microorganisms [2–11],

however, at the present only enzymes from *Escherichia coli* (EcA) and *Erwinia chrysanthemi* (ErA) are generally used in clinical practice.

The exact antineoplastic mechanism of L-asparaginases action is still unknown. Hypothetically, this action is based on enzymatic hydrolysis and decrease of the concentration of exogenous L-asparagine, which is essential for growth of tumor cells, owing to lack of their own system of this amino acid biosynthesis. It was established that L-asparagine hydrolysis occurs in two steps via an intermediate beta-acyl-enzyme complex [12, 13].

Therapeutic usage of L-asparaginases is limited by adverse effects, associated with the L-glutaminase activity (hepatotoxicity, nephrotoxic effect, acquired hypofibrinemia, CNS depression), and immunogenicity of this protein [14–19].

Attempts to reduce EcA L-glutaminase activity by site-directed mutagenesis are accompanied by significant decrease of enzyme activity on L-asparagine [20]. Currently, only bacterial enzymes from *Wolinella succinogenes* (WsA) [21], *Helicobacter pylori* (HpA) [22], *Bacillus licheniformis* [23] *Pyrococcus furiosus* [24], and *Rhodospirillum rubrum* (RrA) [25], with low L-glutaminase activity (no more than 1 % of the L-asparaginase activity) are known.

Bacterial L-asparaginase exert their activity only in the form of a homotetramer, since the active sites of these enzymes are located in the interface of subunits. [26–29]. However, it was reported that L-asparaginase from *Pyrococcus furiosus* is natively in dimeric form [24].

Structural and functional investigations of short chain L-asparaginases are absent.

RrA subunit has small molecular weight (18 kDa; monomer contains 172 amino acids), enzyme is non-toxic, immunologically differs from EcA and ErA preparations, and hypothetically, can be used for replacement therapy in case of development of hypersensitivity toward L-asparaginases generally used in clinical practice [25]. However, the antineoplastic action of RrA is lower as compared to EcA (our unpublished data), that is possibly explained by its pH optimum, differing from pH of the blood.

The purpose of this study was to identify functionally important regions of RrA by site-directed mutagenesis with prospect of improvement therapeutic and physico-chemical properties of this protein.

Materials and Methods

Reagents

Chemicals of p.a. quality were used. The suppliers were: Difco-Laboratories (Detroit, USA), Merck (Darmstadt, Germany), Amersham Biosciences (Freiburg, Germany), Serva (Heidelberg, Germany), Pharmacia (Uppsala, Sweden), Bio-Rad (Hercules, California, USA), Fluka (Buchs, Switzerland), Sigma-Aldrich (St.-Louis, USA), and Millipore Corporation (Schwalbach, Germany).

The water used in the preparation of all reagents was purified by Milli-Q Water System (Millipore Corporation, Bedford, MA, USA).

Enzymes and reagents for PCR were purchased from SibEnzyme (Novosibirsk, Russia).

Bacterial Strains and Culture Conditions

E. coli Strains

XL-blue (Stratagen, Heidelberg, Germany) and BL21(DE3) (Novagen, Madison, WI, USA) were cultured according to conditions described earlier [25]. Inductor (lactose) was added to expressed culture at density of $A_{600} \sim 1.0$ to a final concentration of 0.2 %. The yield of wet biomass was about 6 g/L.

Preparation of RrA Mutants

RrA gene cloned into the vector pET23a (Novagen, Madison, WI, USA) [25].

To generate mutants, we carry out the QuikChange Site-Directed Mutagenesis with *S*ingle-*P*rimer *R*eactions *I*n *P*arallel [30] to overcome the problem of primer–primer annealing observed with the double primer PCR by the following protocol:

1. Amplify the parental whole plasmid containing cDNA insert in two separate PCR reactions containing either forward or reverse primer.
2. Combine the two single-primer PCR products in one test tube and denature to separate the newly synthesized DNA from the plasmid template DNA.
3. Cool the tube gradually to allow reannealing of the complementary strands.
4. Digest methylated non-mutated DNA (i.e., the parental plasmid) with DpnI.
5. Transformation into *E. coli* XL-blue competent cells was conducted by CaCl2 method.

Oligonucleotides were matched by programs NCBI/Primer-BLAST (Primer3), OligoAnalyzer 3.1, jPCR Tool & FastPCR and synthesized by DNA Synthesis (Moscow, Russia) and stored in aqueous solution at −20 °C. The list of oligonucleotides used for mutagenesis is presented in Table 1.

All molecular biology procedures were performed according to standard protocols [31]. PCR was conducted according to the TaqSE-DNA-polymerase manufacturer's protocol recommended by SibEnzyme company at lower annealing temperature to 54–51 °C.

Table 1 Oligonucleotides used for mutagenesis

No.	Oligonucleotides*	Change site
1.	CGGGTGGGGTTAAGCGCCGCGG CCGCGGCGCTTAACCCCACCCG	*Sfr*3031
2.	GCTATGCACGGCAGGCCTTTCGACCCG CGGGTCGAAAGGCCTGCCGTGCATAGC	*Pce*I
3.	CCGAGGTCGACCGCAAAGCG CGCTTTGCGGTCGACCTCGG	*Sal*I
4.	GGATAGCCTGAAGTTAACCGAGG CCTCGGTTAACTTCAGGCTATCC	*Hpa*I
5.	CCCACCCCACTCACACCAAGGTTGAAACC GGTTTCAACCTTGGTGTGAGTGGGGTGGG	*Bsp*191 (no)
6.	CCGGCCACGTGAGGGGAAGCG CGCTTCCCCTCACGTGGCCGG	*Psp*CI
7.	CGGCGGGACCAGCACTAAACACTATCGC GCGATAGTGTTTAGTGCTGGTCCCGCCG	*Bsa*291 (no)
8.	GGTCGTCGGCCAGCTGTTCGTCGCC GGCGACGAACAGCTGGCCGACGACC	*Pvu*II
9.	GTTCGAGCCCACCTAGGACCAGGAGTAG CTACTCCTGGTCCTAGGTGGGCTCGAAC	*Asp*A21

* Nucleotide sequences for the recognition by restriction enzymes are underlined. Mutations were confirmed by DNA sequencing according to Sanger method [32]

Primary mutant selection was performed on plasmid DNA minipreps by virtue of appearance of new restriction site or the removal of existing site in RrA gene.

Protein Purification

All purification stages were performed at +4 °C. Biomass was suspended in ten volumes of buffer "A" (10 mM sodium phosphate buffer, 1 mM Glycine, 1 mM EDTA, pH 7.8) and destroyed by ultrasound treatment. Cell debris and unbroken cells were removed by centrifugation (35,000×*g*, 30 min). Supernatant, containing the enzyme, was applied to Q-Sepharose column (1.5 × 44 cm) equilibrated with the same buffer. Protein was eluted with a linear gradient of 0–1.0 M NaCl. Column fractions were examined for protein content by spectroscopy at 280 nm and enzyme activity. The active fractions were combined and chromatographed on DEAE-Toyopearl 650 m (2.5 × 37 cm) analogously to Q-Sepharose—procedure. Buffers with different pH were used for the different mutant forms of L-asparaginase.

Electrophoresis and Protein Determination

Protein concentration was determined by the method of Sedmak [33] with bovine serum albumin as the standard. For purity tests of enzyme preparations, SDS–polyacrylamide gel electrophoresis was carried out as previously described [34].

Enzyme Activity Assay

One unit of L-asparaginase activity is defined as the enzyme amount, releasing 1 μM of ammonia per minute under experimental conditions. The ammonia content was evaluated by the direct Nesslerization [35, 36]. L-Glutaminase activity was measured by the same procedure using L-glutamine as substrate. Specific enzyme activity was expressed in U/mg protein.

Effect of pH and Buffer Composition on RrA Mutants Activity

There is not any information about influence of buffer composition on asparaginases activity, so we use buffers that cover a wider pH range, have an overlapping optimal buffer pH ranges and constant buffer capacity in these ranges, recommended by Dawson and Elliot [37]: sodium acetate (pH 3.0–6.0), sodium phosphate (pH 6.0–8.0), Tris–HCl (pH 7.0–9.0), borate (pH 9.0–11.0).

The Influence of pH on Enzymatic Activity of RrA Mutants was examined at 37 °C.

Effect of Temperature on RrA Mutant's Activity

Influence of temperature on enzyme activity and stability after heating for 3 min was assayed in 0.1 M sodium phosphate buffer at pH 7.4 at temperatures ranging from 30 °C to 80 °C.

Stability of RrA Mutants in the Presence of Urea

Stability of RrA mutants was investigated after 1 h incubation in phosphate buffer at pH 7.4 and borate buffer at pH 9.35 in the presence of 0–8.0 M of urea. Residual enzymatic activity was defined as described before [35, 36]. The activity of enzyme examined at 37 °C in the corresponding buffer in the absence of urea was taken as 100 %.

Influence of Ionic Strength on Activity of RrA Mutants

Dependence of the specific activity of target protein on ionic strength was investigated in 0.1 M sodium phosphate buffer at pH 7.4 for a KCl concentration ranging from 0 to 6.0 M.

RrA Mutants Stability after "Freezing and Thawing"

Enzyme stability was estimated after 2–3-fold "freezing-thawing" cycles as described Jameel F. et al., [38] in 0.1 M

sodium phosphate buffer pH 7.4 and also upon storage at +4 °C.

Determination of Kinetic Parameters of Enzymes

K_m values definition was carried out by the rate of ammonia formation at L-asparagine enzymatic hydrolysis. L-Asparaginase activity was determined in 0.1 M sodium phosphate buffer, pH 7.5.

One millilitre of reagent mix contained 0.04–1 units of L-asparaginase and 20–250 μmol of L-asparagine. Samples were incubated at 37 °C for 2 min.

Reaction was stopped by addition of 0.5 ml of 20 % of trichloroacetic acid. On the next step, 0.25 ml of the clear supernatant, 2 ml of water, and 0.25 ml of a Nessler reagent were brought in other tube. The optical absorbance was measured at A_{500}.

The corresponding concentrations of L-asparagine in the same buffer without enzyme addition were used as control non-enzymatic hydrolysis of L-asparagine. Graphical processing of the obtained data and calculation of K_m and V_{max} were carried out by means of Microsoft Excel program by a method of the double-reciprocal (also known as the Lineweaver–Burk method) plot.

Determination of L-Asparaginases Cytotoxic Activity

Cell line of the human acute T cell leukemia (Jurkat) from a collection of Orekhovich Institute of Biomedical Chemistry of the Russian Academy of Medical Sciences was used. Jurkat cells were cultured according to techniques described previously [39]. MTT-test was applied to determination of cytotoxic antineoplastic activity of L-asparaginases [39, 40]. Statistical calculation of the results and diagram plotting were carried out by means of the Microsoft Excel program. Differences were considered reliable in the range of $p \leq 0.05$.

Oligomeric L-Asparaginase Structure

To determine the quaternary enzyme structure, the method of size-exclusion chromatography (SEC) was applied. Research was performed on Bio-gel P150 column 1.0 × 100 cm in 0.1 M Na-phosphate buffer, pH 7.25 with 0.02 % NaN_3, elution at 4.5 mL/h, fractions of 0.45 mL were collected. The column was calibrated with proteins of known molecular masses: bovine serum albumin—monomer (66.5 kDa) and dimer (133 kDa), egg albumin (44.5 kDa), horse-heart myoglobin (17 kDa). Column free volume (V_0) was determined as blue dextran elution volume, column total volume (V_t) as p-nitrophenol elution volume. Protein was detected by absorption at 280 nm, L-asparaginase activity was detected as described above.

Table 2 Multipoint mutant forms of RrA

The designation of mutations	Protein
RrA	[25]
RrA_F	I17S, D18T, D20H
RrA_G	D32Q, P33L
RrA_D	D60K, F61L
RrA_C	A64V, E67K
RrA_H	G86P, D88H, M90K
RrA_E	E149R, V150P, A64V, E67K
RrA_I	G121L, D123A
RrA_J	R118H, G120R
RrA_B	E149R, V150P
RrA_K	E149R, V150P, I168T, deleted sequence DDQE(169–172)

K_{av}, the partition coefficient, characterizing the protein mobility, was calculated according to the formula [41]:

$$K_{av} = V_e - V_0 / V_t - V_0,$$

where V_e is the elution volume, V_0 the free column volume (20.5 ml), and V_t is the total column volume (73 ml).

V_e of calibrant proteins were defined as peaks of A280 on the elution curves, V_e of asparaginase mutants were defined as peaks of the activity elution.

Calibration curve was made via plotting K_{av} against logM, where M is the molecular mass of the calibrant.

Molecular Modeling

For design of RrA 3D model, the RrA protein sequence (Uni Protcode Q2RMX1) was searched for closest enzyme homologue possessing known spatial structure in the RCSB Protein Data Bank (PDB). A sequence alignment was performed by DNASIS (*Hitachi Software Engineering Co.*) and ClustalW (*Kyoto University Bioinformatics Center*). The three-dimensional structure of L-asparaginase *Rhodospirillum rubrum* monomer was designed using web-server SWISS-MODEL (http://swissmodel.expasy.org/) by homology modeling method [42]. Analysis of the structure was performed using SYBYL8.1. program (Tripos, Inc, USA).

Results and Discussion

Preparation of Mutants of RrA

To study protein structure–function relationships, site-directed mutagenesis of RrA gene inserted into pET23 vector was performed. The aim was to replace several negative charged and uncharged residues at the different regions of protein. Ten multipoint RrA mutants were

constructed and confirmed by DNA sequencing. Activity of enzyme variants was determined. The list of obtained mutations is presented in Table 2.

L-Asparaginase activity mutants RrA_I, RrA_H, RrA_F and RrA_G in *E. coli* expression system after biomass disruption by sonication did not exceed 2 U/ml.

Mutation RrA_K is characterized by nonsense codon appearance, removal of terminal amino acids (DDQE), and by I168T replacement in recombinant protein sequence. It caused the instability and loss of enzyme activity during freezing–thawing, sonication, and storage at +4 °C. Protein precipitation by ammonium sulfate (60 % of saturation) [37] caused its irreversible inactivation.

Active and stable mutants (RrA, RrA_D, RrA_C, RrA_J, RrA_B and RrA_E) were investigated further.

Protein Purification

Wild type RrA and its mutants are intracellular enzymes, since they lack signal peptide. This fact established using Signal P 4.0 program was taken into consideration for protein purification [43]. All procedures started with biomass disruption using sonication and obtaining of crude intracellular extract and were optimized for each RrA mutant. Cellular extraction and protein purification on Q-Sepharose column was a general procedure for all of RrA variants and effectively eliminated bulk of ballast proteins. RrA and RrA_D variants were eluted at NaCl concentrations range of 0.3–0.5 M; RrA_E, RrA_C and RrA_J—0.4–0.7 M; RrA_B—0.6–0.8 M.

For final purification of recombinant proteins, column Toyopearl-DEAE 650 m was used. Chromatography was made in buffer A (for RrA, RrA_J and RrA_B at pH 7.8; for RrA_E, RrA_D and RrA_C at pH 6.1). RrA, RrA_B, RrA_D, RrA_C, and RrA_J variants were eluted at concentrations range of NaCl 0.2–0.3 M; 0.125–0.3 M; 0.4–0.5 M; 0.7–0.85 M; and 0.5–0.75 M, accordingly, but RrA_E mutant was not bound with the sorbent. The purified L-Asparaginase preparations were desalted and concentrated on Amicon Millipore Ultracel 10 kDa filters, then enriched with glucose (up to 0.5 %) and lyophilized. Enzyme samples were stored in the freezer at −20 °C. During the enzyme purification, specific activity increased to 140–210 U/mg. Results of a 12 % SDS-PAGE electrophoresis of the purified RrA variants are presented in Fig. 1.

The major band with MW = 18−20 kDa is evident in all RrA purified preparations with target enzyme content corresponding to 70–80 % of the total protein (as calculated by Gel-Proanaluser 3.1.00.00 (Media Cybernetics, USA)).

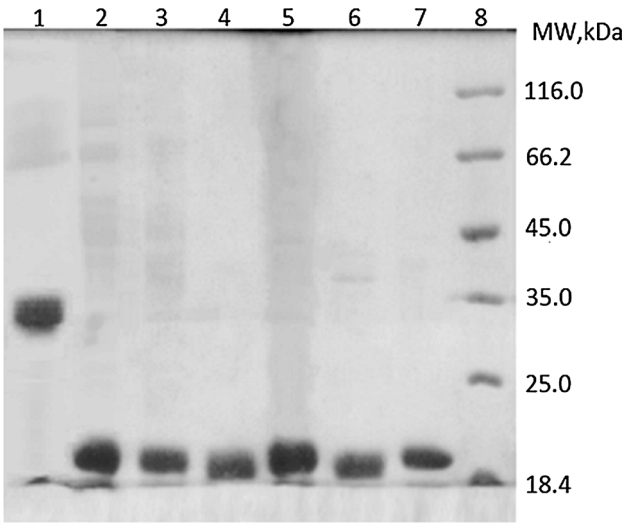

Fig. 1 SDS-PAGE analysis of expressed and purified recombinant RrA variants. 1, L-Asparaginase *E. coli* (Medak); 2, RrA_D; 3, RrA_C; 4, RrA_E; 5, RrA_J; 6, RrA_B; 7, RrA; 8, Protein molecular weight marker # SM0431 (Fermentas)

Theoretical pI Values of Mutants

RrA—5.05; RrA_F—5.55; RrA_G—5.19; RrA_D—5.39; RrA_C—5.37; RrA_H—5.56; RrA_I—5.19; RrA_J—5.21; RrA_B—5.37; RrA_K—5.61 (ProtParam, "http://www.expasy.org").

Effect of pH and Buffer Composition on RrA Mutants Activity

Dependence of mutants RrA activity from pH is shown in the Fig. 2.

As shown in Fig. 2, pH dependence of activity of all RrA variants has a bell-shaped form. The pH optimum of native RrA and its mutant forms did not change and remained within 9.2–9.5. RrA_C only had a reduced pH—9. However, mutants with almost identical activity at physiological pH values strongly differ in the activity at optimum values. So the enzyme activity of RrA_D at pH 9.3 exceeds activity at pH 7.4 by 3.1 times, activity of RrA_C and RrA_E—by 2.6 times, while the native enzyme RrA, RrA_J and RrA_B are identical on this activity relation—2.9. In optimal conditions, the activity of RrA_D overlaps activity of RrA_E and RrA_C twice.

In contrast to RrA variants, the HpA and EcA recombinant enzymes and others full-size microbial L-asparaginases show catalytical activity in the wide pH range [22, 44, 45]. Dependence of the original RrA activity and its mutant forms on the pH almost identical response curves of K_m on pH for L-asparaginases ErA and *E. coli* with a sharp maximum in a narrow pH range 9.5–10 [46–48]. Similar response curves of K_m on pH for L-asparaginases ErA,

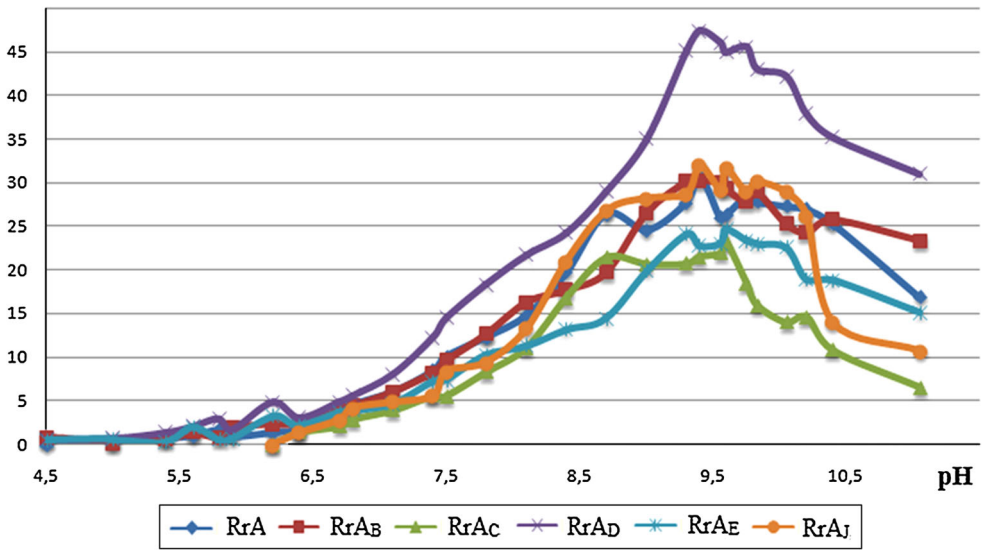

Fig. 2 Dependence of activity of RrA mutants from pH (Color figure online)

E. coli, and original RrA and its mutant forms activity on the pH with a sharp maximum in a narrow pH range 9–10 may be due to considerable weakening of the enzyme-substrate affinity in the narrow pH range, caused by changing of charge in the active site in this pH interval or reflects conformation changing of a protein molecule [46]. The aspartic acid is protonated in an acidic environment and has a bigger affinity for the active site, acting as a competitive inhibitor. In an alkaline environment, it shifts to the deprotonated form (aspartate) lead to decreasing affinity. As a result, asparagine predominantly binds to the active site at higher pH.

Buffers without amino groups (acetate, phosphate, and borate) did not affect RrA mutants activity. To the contrary, Tris-buffer increased the activity of all variants up to 1.5–2.5 times [data not shown]; it can be assumed the existence of nonspecific allosteric site of the enzyme. Interaction with it can induce the conformational alteration in protein structure and, correspondently, change the catalytic property of enzyme.

Tris can affect metal binding sites of some enzymes and influence on affinity of the enzyme for substrate and catalytic efficiency [49–51]. Monovalent cations (Na+ and K+) increased the L-asparaginase activity of *Bacillus licheniformis,* whereas divalent cations and thiol reagents inhibit its activity [23, 49].

Temperature Effect on L-Asparaginase Activity of RrA Mutants

The temperature optimum of enzymes and denaturation temperature were defined in 0.1 M sodium phosphate buffer at pH 7.4 and temperature of 30–80 °C. Variants RrA, RrA_B, RrA_E, and RrA_J were characterized by

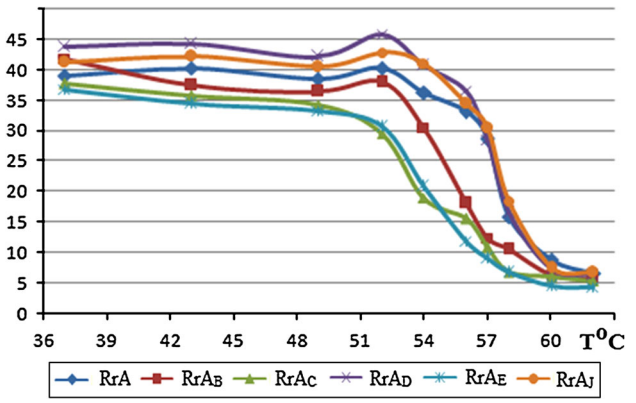

Fig. 3 Temperature effect on the RrA mutants activity (Color figure online)

temperature optimum of action—54–56°, RrA_D—58 °C. RrA_C has a optimum of action at 53 °C. At 37 °C, activity of variants RrA was not less than 60 % of the maximum. Increased hydrolysis of L-asparagine at higher temperatures was, probably, due to the decrease of the activation energy of the reaction. Loss of enzyme activity at a temperature above 57 °C is explained by thermal denaturation of protein.

Enzyme denaturation temperature was determined by the standard method of evaluation of residual specific activity after heating substrate-free enzyme preparation for 3 min at 37–70 °C. Obtained data are presented in Figs. 3 and 4.

As shown in Fig. 4, preliminary incubation at temperatures up to 53 °C, practically, did not changed the activity of recombinant RrA variants, but heating above 53 °C led to their inactivation. L-Asparaginase renaturation to the catalytically active form was not found.

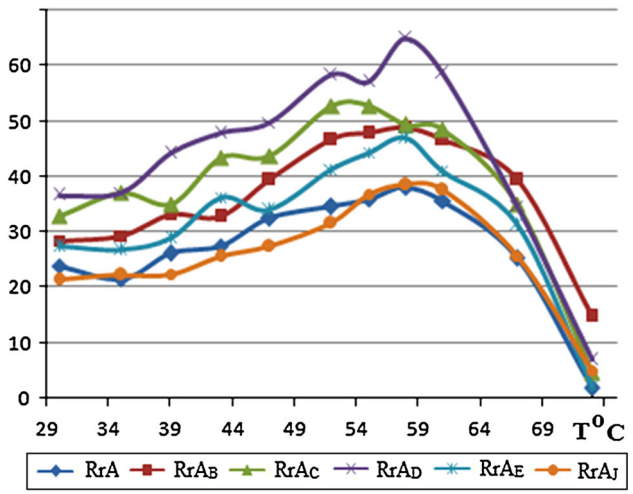

Fig. 4 Residual activity of RrA mutants after a thermal denaturation (Color figure online)

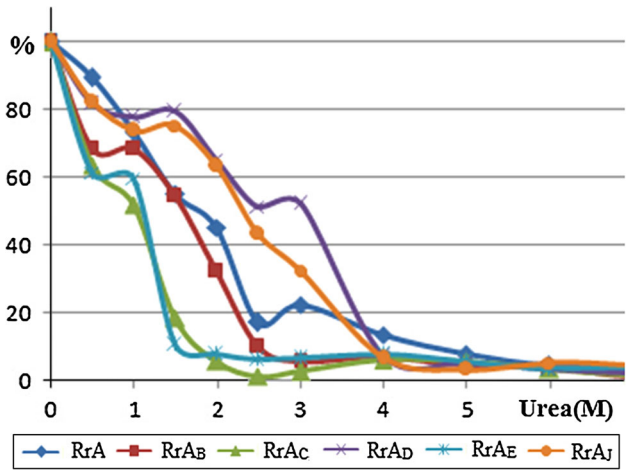

Fig. 5 Residual enzyme activity of RrA variants–urea concentration diagram (pH 7.4) (Color figure online)

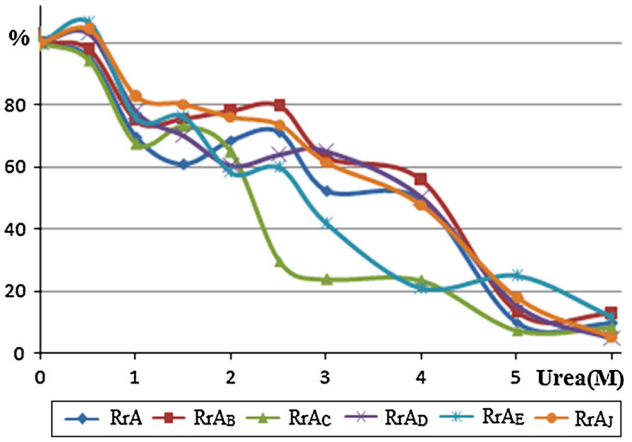

Fig. 6 Residual enzyme activity of RrA variants–urea concentration diagram (pH 9.35) (Color figure online)

The same was observed for EcA and HpA, however, they were more stable at higher temperatures (59 °C) [22]. N281D (ErA) mutation imparted less stability to the enzyme at elevated temperatures compared to Wild-Type Enzyme [52].

Enzyme Specificity

All tested mutants showed high selectivity for L-asparagine, however, some activity for D-asparagine was noticed: 0.9; 1.2; 1.3; 1.4; 1.6; and 1.6 % from L-asparaginase activity for RrA_E, RrA_C, RrA, RrA_J, RrA_D, and RrA_B variants, respectively. L- and D-glutaminase activity did not exceed 0.01 % of L-asparaginase activity.

There were several reports about residues that determine glutaminase activity. Mutants of Gly11, His87, Gly88, Gly57, Gln 59, His183, His197 and Asn248 of EcA2 and Asn281 of ErA showed reduced glutaminase activity [20, 27, 46, 52]; D-asparaginase activity also decreased. Too low-glutaminase activity of RrA did not allow us to determine the participation of any residues in this activity.

RrA Mutants Stability after "Freezing and Thawing"

Enzymes RrA, RrA_B, and RrA_E retained more than 68 %, RrA_C—78 % of native enzyme RrA activity after 5–6-fold cycles of "freezing-thawing" or storage at +4 °C for 2 months. RrA_D and RrA_J did not show any inactivation in these conditions.

Gladilina [22] reports, after 50 repeated cycles of thawing-freezing of HpA retains at least 80 % of the specific activity of the initial value. Stability of the recombinant enzyme was the same as the stability of asparaginase EcA (Medac). Enzyme derived from ErA loses its activity upon exposure to consecutive freeze–thaw cycles. A total protein assay suggested that the loss of some enzyme activity was a result of the dissociation enzymes tetramers into monomers, some of which were cleaved into small fragments. The shortened monomers then aggregate and precipitate [38].

Stability of RrA Mutants in the Presence of Urea

The curves of the residual L-asparaginase activity of RrA variants in the presence of urea are shown in Figs. 5 and 6 at pH 7.4 and 9.35, respectively.

RrA and RrA_B had almost identical urea denaturation profile. RrA_D and RrA_J were the most stable at physiological pH values in the presence of urea. Residual activity of RrA_D, RrA_J, RrA, and RrA_B in 2–3 M urea solution at pH 9.35 was 2–7 times higher than at pH 7.4. The fact that RrA_C and RrA_E have normal activity but greatly reduced stability in the presence of urea especially at pH

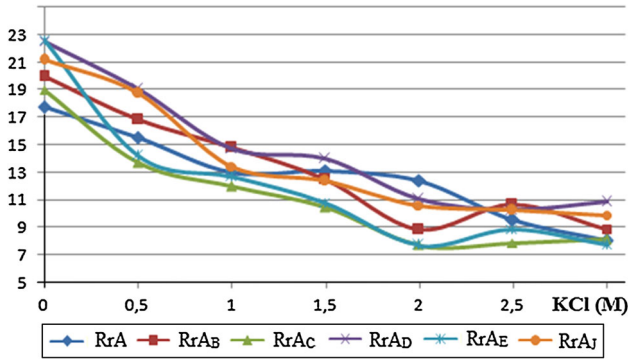

Fig. 7 Influence of solution ionic strength on RrA mutants activity (Color figure online)

7.4.suggests that residues A64, E67 can participate in the stabilization of the oligomereric protein.

Note that L-asparaginases HpA and EcA retained their activity in 3 M urea solution [22]. S122A(EcA2) mutant exhibited little change in its kinetic properties and showed normal stability in urea solutions, on the other hand, the stability of a D124A mutant was strongly impaired even in the absence of denaturants [53, 54]. Urea could not induce unfolding and inactivation L-asparaginase from Pyrococcus furiosus; however, with guanidine hydrochloride (GdnCl) a two-state unfolding pattern was observed [24].

Residue His87(EcA) as His85 (RrA) in homology models later in the manuscript are located in the interior of the protein, adjacent to the active site. The effects of the H87A as H197L mutation EcA on both activity and stability in urea are moderate and similar. Replacement G86P, D88H, M90K (RrA_H) drastically reduces the activity of the protein. In such a case, the environment of His85 should be rather sensitive to conformational changes of the active site. Sensitivity of RrA variants to the presence of even 1 M urea at pH 7.4 is indicative of weak hydrogen bonds in the protein and importance of its structural organization for the implementation of physiological functions.

Influence of Ionic Strength on Activity of RrA Mutants

Unlike full-size L-asparaginases (HpA and EcA, etc.), which activity practically did not change upon the increase of ionic strength at pH 7.4, RrA mutants showed distinctly dependence of their activity on the solution ionic strength: the higher was the ionic strength of the solution, the lower was activity. Results of the influence of ionic strength on RrA variants activity are shown in Fig. 7.

KCl concentration increasing up to 2.5–3 M led to more than twice enzyme activity decline. Dependence RrA mutants activity from the ionic strength indicates the prevalence of electrostatic interactions in binding of a substrate molecule to the active center.

Table 3 Kinetic properties of RrA mutants

RrA variants	K_m, L-asparagine (μM)	V_{max}, L-asparagine (μM/min)	Specific activity (U/mg)
RrA	280 ± 112	5.9 ± 0.5	173
RrA_D	237 ± 70	9.7 ± 0.5	170
RrA_E	707 ± 107	6.9 ± 1.4	210
RrA_C	717 ± 98	6.5 ± 1.2	209
RrA_B	756 ± 118	6.3 ± 1.2	140
RrA_J	337 ± 73	7.3 ± 1.4	173

Kinetic Properties of Purified RrA Variants

Kinetic properties of RrA variants in reaction with L-asparagine were defined in 0.1 M Na-phosphate buffer (pH 7.5). Obtained data are summarized in Table 3.

Tabular data show that at pH 7.4, intragenic mutations, which increased pI of the studied protein, led to rise in *V*max- toward L-asparagine for all mutants, especially for RrA_D (1.7 times higher as compared to RrA). Specific activity of RrA_B at pH 7.4 was reduced by 19 %, RrA_E and RrA_C increased up by 19 % compared to RrA, RrA_D, and RrA_J did not change. Note, that at pH 9.35, the specific activity of RrA_D and RrA_J exceeded the specific activity of the native RrA 1.7 and 1.3 times, respectively.

K_m values (280 ± 112, 237 ± 70, 337 ± 73 μM) for RrA, RrA_D and RrA_J were practically identical. K_m of RrA_B increased 2.7 times, and of mutants RrA_E, RrA_C—2.5 times compared to RrA.

Mutants T89 V and T89S (3ECA) showed comparable or even lower Michaelis constants, as compared to the wild type, but the activity of mutant T89 V was dramatically decreased, whereas mutant T89S had residual activity about 20 % of the wild type enzyme [12].

None of the mutations H87A, H87L, H87K, H183L, H197L (EcA2), substantially affected the K_m, for L-aspartic acid fl-hydroxamate or impaired aspartate binding [47].

ErA deamidation at the Asn41 and Asn281 sites did not affect enzyme specific activity (1,062 and 924 U/mg, respectively, as the wild type (908 U/mg)). However, a double mutant (N41D N281D) had an increased specific activity (1,261 U/mg). The N41D mutation conferred a slight increase in the catalytic constant (k_{cat} 657 s−1) compared to the wild-type (k_{cat} 565 s−1), which was further increased in the double mutant, (k_{cat} 798 s−1). The increased α-helical content observed with the N41D mutation by circular dichroism spectroscopy correlates with the difference in k_{cat}, but not Km [52].

Since L-glutaminase activity of studied variants was very low (<0.01 % from L-asparaginase activity), the authentical measurement by the same procedure of

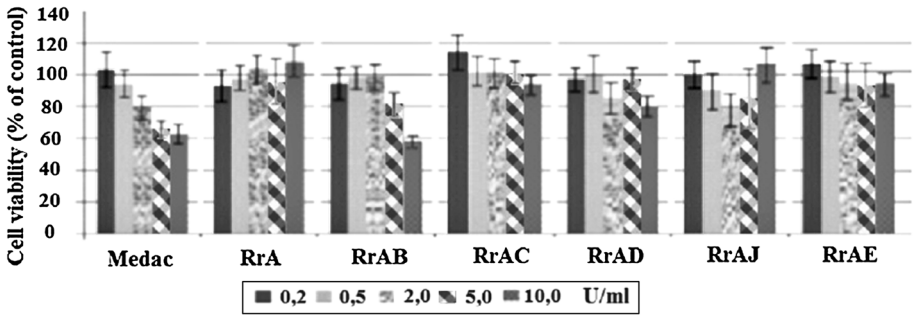

Fig. 8 Comparative analysis of the antineoplastic and cytotoxic activity of commercial L-Asparaginase Medac and various mutants of RrA (Jurkat cells) ($n = 4$, $p \leq 0.05$)

L-glutamine hydrolysis kinetic parameters for RrA variants was not possible.

Antineoplastic and Cytotoxic Activity of RrA L-Asparaginase Mutants.

The results of determination of antineoplastic and cytotoxic activity for RrA and its mutant forms (RrA_B, RrAc, RrA_D, RrA_E and RrA_J) are presented in Fig. 8. Commercial L-asparaginase Medac (Germany) from *E. coli* was used as control.

Our results showed that incubation with increasing amount of RrA_B in the medium led to dose-dependent decrease of viable metabolically active tumor cells number in comparison to control. Antineoplastic and cytotoxic activity of the RrA_B mutant is quite comparable to Medac L-asparaginase by dosage of 2 U/ml and above, while initial RrA, RrAc, RrA_D, RrA_E and RrA_J variants significantly did not influence the growth of Jurkat cells.

It should be noted that the initial L-asparaginase RrA, which was inactive against Jurkat cells showed some cytotoxic antitumor activity for other tumor cell lines [25].

Structural and Functional Studies

The K_{av} coefficient characterizing mobility of RrA and its active mutant forms RrA_D, RrA_E, RrA_C, RrA_B, RrA_J was about 0.071–0.074 (V_e in the range 24.2–24.4 mL) and corresponded to the molecular weight about 80 kDa. These data testify that the studied enzyme showed the maximum activity mainly in a tetrameric form, like enzymes with higher molecular sizes (*E. coli, Erw. carotovora, Erw. chrysanthemi*, etc.).

Available literature lacks structural and functional investigations of low-molecular weight short chain L-asparaginases [55]. The presence of the conservative motif THGTDTMVETA (RrA 84–94) in most studied L-asparaginases irrespective of their length and intracellular localization proposes the common structural/functional properties for this group of enzymes (Fig. 9).

An extensive site-directed mutagenesis study of *E. coli* asparaginase II [28, 56–59] together with the information

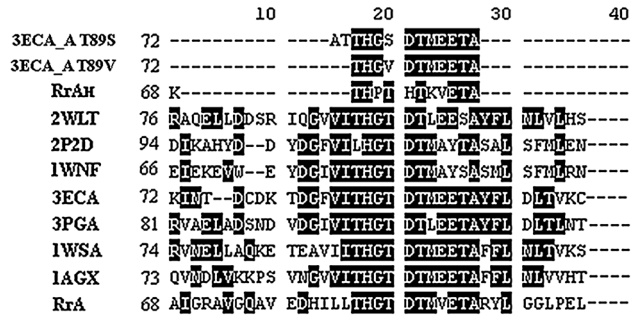

Fig. 9 Alignment of conservative fragments of L-asparaginases amino acid sequences. 3ECA_A T89 V, *Escherichia coli* L-asparaginase mutant; 3ECA_A T89S, *Escherichia coli* L-asparaginase mutant; RrA_H, *Rhodospirillum rubrum* L-asparaginase mutant (G86P, D88H, M90K), 2WLT, *Helicobacter pylori* L-asparaginase; 2P2D, *Escherichia coli* cytoplasmic L-asparaginase; 1WNF, *Pyrococcus horikoshii* L-asparaginase; 3ECA, *Escherichia coli* native L-asparaginase (*used In Cancer Therapy*); 3PGA, *Pseudomonas* L-Asparaginase and L-glutaminase; 1WSA, *Wolinella succinogenes* L-asparaginase; 1AGX, *Acinetobacter glutaminasificans* L-Asparaginase and L-glutaminase; RrA, *Rhodospirillum rubrum* native L-asparaginase

from X-ray crystallography [12] revealed that Tyr 25, Thr 12, Thr 89, and Lys 162 play a catalytic role, while Asp 90, Ser 58, Asn 248, Ala 114, and Glu 283 assist substrate binding by hydrogen bonding.

Both hydroxyl groups in position 89 and in position 12 are absolutely required for catalysis and are closest to the side chain carboxylate of bound aspartate and thus the most likely candidate for the catalytic nucleophile [12, 56]. Mutations G86P, D88H, M90K (RrA_H), I17S, D18T, D20H (RrA_F) changed the charge and configuration of recombinant protein adjacent to the catalytic threonines 87, 89, and 16, respectively (Table 4), that dramatically decreased the enzyme activity and consistent to the importance of this regions in implementation of enzymatic reactions.

In the active center of *E. coli* asparaginase II, there are two groups of three amino acids each ('triads'). One of the putative triads contains Thr 89, Lys 162, and Asp 90, while the other one is formed by Thr 12, Tyr 25, and Glu 283 [20]. The residues in the triad are also connected through hydrogen bonds. These structures resemble the catalytic

Fig. 10 Alignment of amino acid sequences of L-Asparaginases from *Rh. rubrum* (Q2RMX1) and *P. horikoshii* (O57797)

```
Q2RMX1     1      LRIFTAGGTIDKDYRLEENGLVVGDPFVAEVLKTAR--LAGAVSIV
O57797     2    mr--ililgmggtiasvkge--rgyesals-vskilklagisseakiear

Q2RMX1     45   ALSRKDSLDFTEADREAIGRAVGQA--VEDHILLTHGTDTMVETARYLGG
O57797     46   dlmnvdstliqpsdwerlakeiekevweydgivithgtdtmaysasmlsf

Q2RMX1     93   LPELAGKTVVLSGAMVPGRVGGSDAAFNIGFACAAALMLAPGVYIAMHGK
O57797     97   mlrnppipivltgsmlpiteknsdapfnlrtalefvklgirgiyiafngk

Q2RMX1     143  VFDPAKTRKNR--GLGRFEPIDDQ-------------------------
O57797     146  vxlgvraskirsmgfdafesinypnvaeikddklrilhipdfygdeffsd

Q2RMX1          ------------------------------------------------
O57797     196  ikyepkvlviklipglsgdivrealrlgykgiilegygvggipyrgtdlf

Q2RMX1          ------------------------------------------------
O57797     246  evvssiskripvvlttqaiydgvdlqrykvgrialeagvipagdmtkeat

Q2RMX1          ---------------------------------
O57797     296  itklmwilghtknieevkqlmgknitgeltrvs
```

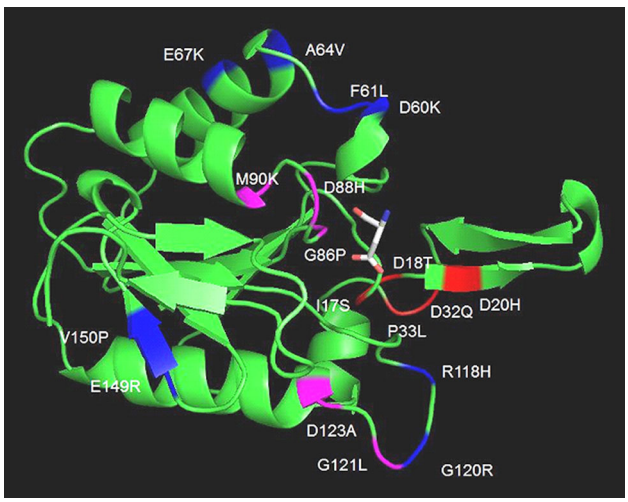

Fig. 11 Position of amino acid substitutions inducted in the structure of L-Asparaginase *Rh. Rubrum.* Substitutions in the active mutant forms investigated in the work were marked by the *blue color*. The mutations leading to the loss of enzymatic activity were marked by the *red* (RrA_F, RrA_G) and *magenta* (RrA_I, RrA_H) *color* (Color figure online)

Table 4 Comparison of amino acid residues forming the active centers of *Rh. rubrum* and *E. coli* L-asparaginases

Rh. rubrum	*E. coli*
GLY15	GLY11
THR16	THR12
LEU17	ILE13
LYS19	VAL27
ASP57	GLY57
SER58	SER58
LEU59	GLN59
His85	His87
GLY86	GLY88
THR87	THR89
ASP88	ASP90
THR89	THR91
ALA113	ALA114
Met114	Met115
LYS158	LYS162

triad of the serine proteinase which is made up by an aspartate, a histidine, and a serine residues.

For the RrA-depended L-asparagine hydrolysis, it is important the existence of aspartic acid not only in the position "88", but also in the position "123", since the RrA_I mutant (G121L, D123A) lost the enzymatic properties. Despite the remoteness in amino acid sequence of these mutations from each other, it is possible to assume that these groups closer together and come in threonine-rich enzyme active center by a protein folding.

Structural changes of the enzyme mutants RrA_D (D60K, F61L) and RrA_J (R118H, G120R) led to the improvement of kinetic parameters and enzyme stabilization. Probably, the reason of similar positive changes in the elimination of the steric hindrance in the case of mutations RrA_D during oligomerization. Reduction of negative charges near areas of contact of both subunits may lead to reduces repulsion monomers generally negatively charged, and increase stability the whole macromolecule. Rigidity and stability of the protein globule packing increases by means of additional salt linkage and the saturation of the protein molecule nonpolar contacts.

A64V, E67K substitutions, especially in combination with E149R, V150P (RrA_E), considerably destabilized recombinant enzyme. It is possible that exact local conformational instability at physiological pH values is the reason of low kinetic parameters and low biological activity.

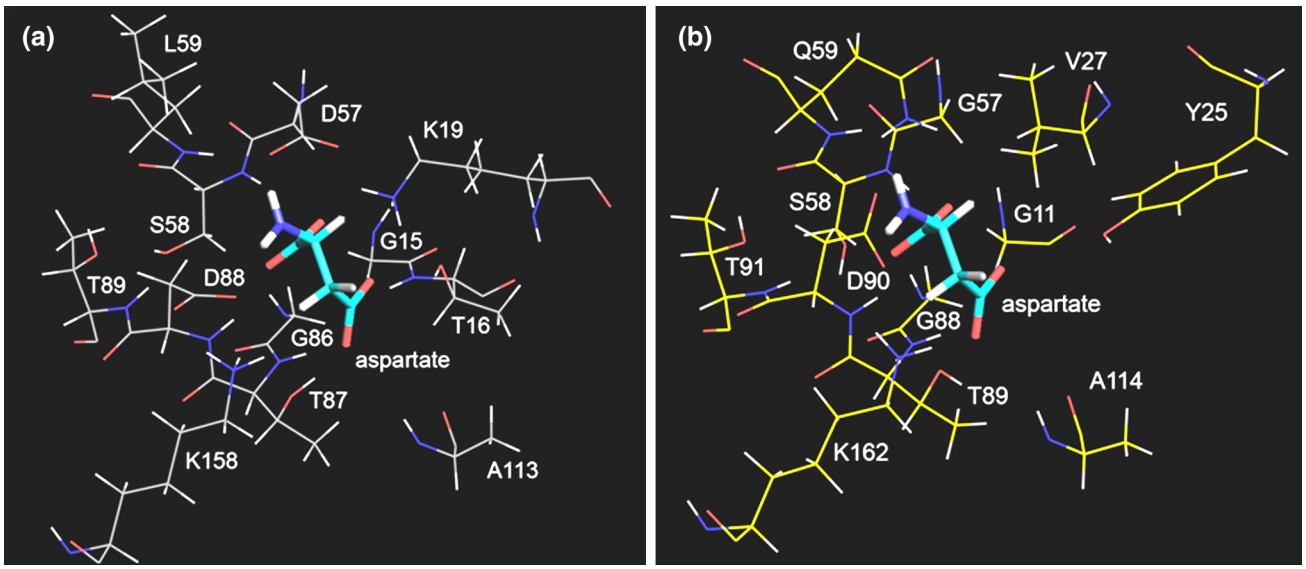

Fig. 12 Structures of active center of L-Asparaginases *Rh. rubrum* (**a**) and *E. coli* (**b**). It is shown residues that surround aspartate within 5 Å

Computer Modeling of the Spatial Structure of L-Asparaginase *Rh. rubrum*

Search of *Rh. rubrum* L-asparaginase homologs with known spatial structure on the RCSB PDB server showed that L-asparaginase of I type from *Pyrococcus horikoshii* (PDB 1wls code, UniProt O57797 code) is the closest to RrA. *P. horikoshii* L-asparaginase is represented by the homodimer, each subunit of which consists of two domains: N-terminal domain (amino acid residues 1–198) and C-terminal domain (amino acid residues 199–328). Alignment of amino acid sequences of proteins from *Rh. rubrum* and *P. horikoshii* showed (Fig. 10) that sequence of *Rh. rubrum* L-asparaginase can be aligned with the N-terminal domain of the another enzyme. The sequence identity was about 28 %. Such identity lies on the lower limit, for spatial structure modeling by the homology method.

The designed model of 3D-structure of monomer of L-asparaginase *Rh. rubrum* (Fig. 10) showed the satisfactory QMEAN4 global scores that indicated plausibility of the designed model. In addition, the reliability of this model was estimated by means of the ProCheck program ("http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) [58]. Our analysis showed that in the designed model of L-asparaginase *Rh. rubrum* more than 80 % of amino acid residues are in the most favored regions on the Ramachandran plot, and only four residues, located in loops, are in disallowed regions. All these residues are located in unstructured regions of protein. One of these residues (Asp32) was mutated in RrA_G. In designed model, the unfavorable conformation of this residue is stabilized by salt bridge with Arg116.

Position of the mutated residues in monomer structure of *Rh. rubrum* L-asparaginase is presented in Fig. 11.

Substitutions in the active mutant forms investigated in the work were marked by the blue color. The mutations leading to the loss of enzymatic activity were marked by the red (RrA_F, RrA_G) and magenta (RrA_I, RrA_H) color.

In addition, comparison of the structure of active centers in *Rh. rubrum* and *E. coli* L-asparaginases showed that many amino acid residues forming the active centers in the N-domain of *E. coli* and *Rh. rubrum* L-asparaginases coincided (including catalytic threonines T16 *Rh. rubrum* and T12 *E. coli*) (Table 4, Fig. 12). Only two amino acids from the C-domain of other subunit participate in formation of the *E. coli* L-asparaginase active center that also confirmed the constructed model adequately reflects RrA folding. Nevertheless, our experimental data have showed that in solution the RrA is mainly in tetramer form, thus a question of what region(s) of this L-asparaginase are participate in oligomer formation remains open. Analysis of position of mutated residues showed that it could be residues D60 and F61 from RrA_D and R118 and G120 from RrA_J. Both these mutants have increased rate of reaction and were more stable for urea. Also, it is possible to assume that C-terminal amino acid residues of RrA are involved in this process, since the removal of four C-terminal amino acid residues led to destruction of the quaternary structure, i.e., these amino acids can quite participate in the doubling subunit binding. It is necessary to emphasize that the active site of all of L-asparaginases is formed by interaction of two subunits.

Calculation of the electrostatic potential on a surface of the designed model of L-asparaginase from *Rh. rubrum* showed an existence of a large amount of negatively charged regions and limited number of clusters having an opposite charge. Following the reduction L-asparaginase activity at increasing ionic strength of the solution, we can assume that the electrostatic interaction contributes greatly to the formation of the enzyme oligomer.

Conclusion

Site-directed mutagenesis gene of *Rh. rubrum* L-asparaginase (RrA) allows to identify several regions important for its biological and physico-chemical properties.

Ten multipoint mutant genes were obtained, and five recombinant RrA variants were expressed in *E. coli* and isolated as functionally active proteins. Loss of asparaginase activity under changes G86P, D88H, M90K (RrA_H), I17S, D18T, D20H (RrA_F), G121L, D123A(RrA_I), D32Q, P33L(RrA_G) confirms the importance of these regions for obtaining active protein in *E. coli* expression system. Mutations D60K, F61L(RrA_D) and R118H, G120R(RrA_J) led to the improvement of kinetic parameters and enzyme stabilization. Substitutions E149R, V150P (RrA_B) improved antineoplastic and cytotoxic activity of the RrA. A64V, E67K substitutions, especially in combination with E149R, V150P (RrA_E), considerably destabilized recombinant enzyme. The attempts to shift pH optimum to physiological values were unsuccessful.

Antineoplastic and cytotoxic activities of RrA forms do not show direct relationship with the kinetic properties or stability of studied enzyme, indicating that antitumor action of L-asparaginase, related not only to the decrease of the concentration of exogenous L-asparagine.

Acknowledgments We express our sincere gratitude to Dr. Mikhail A. Eldarov (Centre "Bioengineering" RAS, Moscow, Russia) for his participation in the discussion, constructive suggestions, useful critique of this research work as well as for help with translation of this article. Dr. Vasiliy N. Lazarev's (Scientific Research Institute of Physical–Chemical Medicine, Moscow, Russia) valuable support and assistance in DNA sequencing and Prof. Ekaterina Kolesanova's (Orekhovich Institute of Biomedical Chemistry, Moscow, Russia) help with protein oligomerisation studies are greatly acknowledged.

References

1. Avramis, V. I., & Tiwari, P. N. (2006). Asparaginase (native ASNase or pegylated ASNase) in the treatment of acute lymphoblastic leukemia. *International Journal of Nanomedicine, 1*(3), 241–254.
2. Howard, J. B., & Carpenter, F. H. (1972). L-Asparaginase from *Erwinia carotovora*. *Journal of Biological Chemistry, 247*(4), 1020–1030.
3. Khushoo, A., Pal, Y., & Mukherjee, K. J. (2005). Optimization of extracellular production of recombinant asparaginase in *Escherichia coli* in shake-flask and bioreactor. *Applied Microbiology and Biotechnology, 68*(2), 189–197.
4. Ferrara, M. A., Severino, M. B., Mansure, J. J., Martins, A. S., Oliveira, E. M., Siani, A. C., et al. (2006). Asparaginase production by a recombinant Pichia pastoris strain harbouring Saccharomyces cerevisiae ASP3 gene. *Enzyme and Microbial Technology, 39*(7), 1457–1463.
5. Onishi, Y., Yano, S., Thongsanit, J., Takagi, K., Yoshimune, K., & Wakayama, M. (2011). Expression in *Escherichia coli* of a gene encoding type II L-asparaginase from *Bacillus subtilis*, and characterization of its unique properties. *Annals of Microbiology, 61*(3), 517–524.
6. Schwartz, J. H., Reevesi, J. Y., & Broome, J. D. (1966). Two L-asparaginases from *E. coli* and their action against tumors. *Proceedings of the National Academy of Sciences, 56*(5), 1516–1519.
7. Wink, P. L., Bogdawa, H. M., Renard, G., Chies, J. M., Basso, L. A., & Santos, D. S. (2010). Comparison between two *Erwinia carotovora* L-asparaginase II constructions: cloning, heterologous expression, purification, and kinetic characterization. *Journal of Microbial & Biochemical Technology, 2*(1), 13–19.
8. Duval, M. (2002). Comparison of *Escherichia coli* asparaginase with *Erwinia*-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organisation for Research and Treatment of Cancer-Children's Leukemia Group phase 3 trials. *Blood, 99*(8), 2734–2739.
9. Hawkins, D. S., Park, J. R., Thomson, B. G., Felgenhauer, J. L., Holcenberg, J. S., Panosyan, E. H., et al. (2004). Asparaginase pharmacokinetics after intensive polyethylene glycolconjugated L-asparaginase therapy for children with relapsed acute lymphoblastic leukemia. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research, 10*(16), 5335–5341.
10. Kotzia, G. A., & Labrou, N. E. (2005). Cloning, expression and characterisation of Erwinia carotovora L-asparaginase. *Journal of Biotechnology, 119*(4), 309–323.
11. Kotzia, G. A., & Labrou, N. E. (2007). L-Asparaginase from *Erwinia chrysanthemi* 3937: cloning, expression and characterization. *Journal of Biotechnology, 127*(4), 657–669.
12. Palm, G. J., Lubkowski, J., Derst, C., Schleper, S., Röhm, K. H., & Wlodawer, A. (1996). A covalently bound catalytic intermediate in *Escherichia coli* asparaginase: crystal structure of a Thr-89-Val mutant. *FEBS Letters, 390*(2), 211–216.
13. Avramis, V. I., & Panosyan, E. H. (2005). Pharmacokinetic/Pharmacodynamic relations of asparaginase formulations the past, the present and recommendations for the future. *Clinical Pharmacokinetics, 44*(4), 367–393.
14. Pui, C. H., Burghen, G. A., Bowman, W. P., & Aur, R. J. (1981). Risk factors for hyperglycemia in children with leukemia receiving L-asparaginase and prednisone. *Journal of Pediatrics, 99*(1), 46–50.
15. Evans, W. E., Tsiatis, A., Rivera, G., Murphy, S. B., Dahl, G. V., Denison, M., et al. (1982). Anaphylactoid reactions to *Escherichia coli* and *Erwinia* asparaginase in children with leukemia and lymphoma. *Cancer, 49*(7), 1378–1383.
16. Priest, J. R., Ramsay, N. K., Steinherz, P. G., Tubergen, D. G., Cairo, M. S., Sitarz, A. L., et al. (1982). A syndrome of thrombosis and hemorrhage complicating L-asparaginase therapy for childhood acute lymphoblastic leukemia. *Journal of Pediatrics, 100*(6), 984–989.
17. Sahu, S., Saika, S., Pai, S. K., & Advani, S. H. (1998). L-asparaginase (Leunase) induced pancreatitis in childhood acute lymphoblastic leukemia. *Journal of Pediatric Hematology/oncology, 15*(6), 533–538.

18. Feinberg, W. M., & Swenson, M. R. (1988). Cerebrovascular complications of L-asparaginase therapy. *Neurology, 38*(1), 127–133.
19. Vrooman, L. M., Supko, J. G., Neuberg, D. S., Asselin, B. L., Athale, U. H., Clavell, L., et al. (2010). *Erwinia* asparaginase after allergy to *E. coli* asparaginase in children with acute lymphoblastic leukemia. *Pediatric Blood & Cancer, 54*(2), 199–205.
20. Derst, C., Henseling, J., & Röhm, K. H. (2000). Engineering the substrate specificity of *Escherichia coli* asparaginase II. Selective reduction of glutaminase activity by amino acid replacements at position 248. *Protein Science, 9*(10), 2009–2017.
21. Distasio, J. A., Nredrerman, R. A., Kafkewitz, J., & Goodman, D. (1976). Purification and characterization of L-asparaginase with anti-lymphoma activity from *Vibrio succinogenes*. *Journal of Biological Chemistry, 251*(22), 6929–6933.
22. Gladilina, Iu A, Sokolov, N. N., & Krasotkina, Iu V. (2009). Cloning, expression and purification of *Helicobater pylori* L-asparaginase. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 3*(1), 89–91.
23. Mahajan, R. V., Kumar, V., Rajendran, V., Saran, S., Ghosh, P. C., & Saxena, Kumar R. (2014). Purification and characterization of a novel and robust L-asparaginase having Low-glutaminase activity from *Bacillus licheniformis*: in vitro evaluation of anticancerous properties. *PlosOne, 9*(6), 1–8.
24. Bansal, S., Gnaneswari, D., Mishra, P., & Kundu, B. (2010). Structural stability and functional analysis of L-asparaginase from *Pyrococcus furiosus*. *Biochemistry (Moscow), 75*(3), 375–381.
25. Pokrovskaya, M. V., Pokrovskiy, V. S., Aleksandrova, S. S., Anisimova, N Iu, Adrianov, R. M., Treshchalina, E. M., et al. (2012). Recombinant intracellular *Rhodospirillum rubrum* L-asparaginase with low L-glutaminase activity and antiproliferative effect. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 6*(2), 123–131.
26. Mezentsev, Yu V, Molnar, A. A., Gnedenko, O. V., Krasotkina, Yu V, Sokolov, N. N., & Ivanov, A. S. (2007). Oligomerization of L-asparaginase from Erwinia carotovora. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 1*(1), 58–67.
27. Aghaiypour, K., Wlodawer, A., & Lubkowski, J. (2001). Do bacterial L-asparaginases utilize a catalytic triad Thr-Tyr-Glu? *Biochimica et Biophysica Acta, 1550*(2), 117–128.
28. Aung, H. P., Bocola, M., Schleper, S., & Röhm, K. H. (2000). Dynamics of a mobile loop at the active site of *Escherichia coli* asparaginase. *Biochimica et Biophysica Acta, 1481*(2), 349–359.
29. Prakasham, R. S., Hymavathi, M., Rao, C. S., Arepalli, S. K., Rao, J. V., et al. (2010). Evaluation of antineoplastic activity of extracellular asparaginase produced by isolated Bacillus circulans. *Applied Biochemistry and Biotechnology, 160*(1), 72–80.
30. Edelheit, O., Hanukoglu, A., & Hanukoglu, I. (2009). Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. *BMC Biotechnology, 9*(61), 1–8.
31. Green, M. R., Sambrook, J., & Peter, MacCallum (2012). *Molecular cloning: a laboratory manual* (4th Edn), Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press ISBN 978-1-936113-41-5.
32. Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, 74*(12), 5463–5467.
33. Sedmak, J. J., & Grossberg, S. E. (1977). A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Analitical Biochemistry, 79*(1–2), 544–552.
34. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685.
35. Wriston, J. C., & Yellin, T. O. (1973). L-Asparaginase: a review. *Advances in Enzymology and Related Areas of Molecular Biology, 39*, 185–248.
36. Wade, H. E., Robinson, H. K., & Philips, B. W. (1971). Asparaginase and glutaminase activities of bacteria. *Journal of General Microbiology, 69*(3), 299–312.
37. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1986). *Data for biochemical research (third edn)*. OUP, Oxford: Oxford Science Publications. 580.
38. Jameel, F., Bogner, R., Mauri, F., & Kalonia, D. (1997). Investigation of physicochemical changes to L-asparaginase during freeze-thaw cycling. *Journal of Pharmacy and Pharmacology, 49*(5), 472–477.
39. Abakumova, O., Podobed, O. V., Karalkin, P. A., Kondakova, L. I., & Sokolov, N. N. (2012). Antitumor activity of L-asparaginase from *Erwinia carotovora* against different human and animal leukemic and solid tumours cell lines. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 6*(4), 306–315.
40. Abakumova, O., Podobed, O. V., Borisova, A. A., Sidoruk, K. V., Aleksandrova, S. S., Omelyaniuk, N. M., et al. (2009). Antitumor activity of L-asparaginase from Yersinia pseudotuberculosis. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 3*(2), 198–201.
41. Winzor, D. J. (2011). Gel filtration: a means for estimating the molecular mass of proteins. *Biochemical Journal, 1*, 1–3.
42. Arnold, K., Bordoli, L., Kopp, J., & Schwede, T. (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modeling. *Bioinformatics, 22*(2), 195–201.
43. Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). Signal P 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods, 8*(10), 785–786.
44. Liboshi, Y., Papst, P. J., Hunger, S. P., & Terada, N. (1999). L-Asparaginase inhibits the rapamycin-targeted signaling pathway. *Biochemical and Biophysical Research Communications, 260*(2), 534–539.
45. Balcao, V. M., Mateo, C., Fernandez, L., Lafuente, R., Malcota, F. X., & Guisan, J. M. (2001). Structural and functional stabilization of L-asparaginase via subunit: immobilization on to highly activated supports. *Biotechnology Progress, 17*(3), 537–542.
46. Libinson, G. S., & Mikhalev, A. V. (1976). Relationship between the magnitude of Km and pH for L-asparaginase. *Biochemistry (in Russian), 41*(1), 149–152.
47. Wehner, A., Harms, E., Jennings, M. P., Beacham, I. R., Derst, C., Bast, P., et al. (1992). Site-specific mutagenesis of *Escherichia coli* asparaginase II. None of the three histidine residues is required for catalysis. *European Journal of Biochemistry, 208*(2), 475–480.
48. Shifrin, S., Luborsky, S. W., & Grochowski, B. J. (1971). L-Asparaginase from Escherichia coli B. Physicochcmical studies of the dissociation process. *Journal of Biological Chemistry, 246*, 7708–7714.
49. Leung-Toung, R., Li, W., Tam, T. F., & Karimian, K. (2002). Thiol-dependent enzymes and their inhibitors: a review. *Current Medical Chemistry, 9*(9), 979–1002.
50. Hethey, J., Lai, J., Loutet, S., Morgan, M., & Tang, V. (2002). Effects of Tricine, Glycine and Tris buffers on alkaline phosphatase activity. *Journal of Experimental Microbiology and Immunology, 2*, 33–38.
51. Ma, Lan, Thomas, T., Tibbitts, S., & Evan, R. K. (1995). *Escherichia coli* alkaline phosphatase: X-ray structural studies of a mutant enzyme (His-412 + Asn) at one w of the catalytically important zinc binding sites. *Protein Science, 4*(8), 1498–1506.
52. Gervais, D., & Foote, N. (2014). Recombinant deamidated mutants of *Erwinia chrysanthemi* L-asparaginase have similar or

increased activity compared to wild-type enzyme. *Molecular Biotechnology, 56*(10), 865–877.
53. Derst, C., Henseling, J., & Rohm, K. H. (1992). Probing the role of threonine and serine residues of *E. coli* asparaginase II by site-specific mutagenesis. *Protein Engineering, 5*(8), 785–789.
54. Schubert, D., Derst, C., & Röhm, K. H. (1996). Abstract. *Annual ABRF Meeting: Biomolecular Techniques*, San Francisco, CA.
55. Sanches, M., Krauchenco, S., & Polikarpov, I. (2007). Structure, substrate complexation and reaction mechanism of bacterial asparaginases. *Current Chemcial Biology, 1*(1), 75–86.
56. Harms, E., Wehner, A., Aung, H. P., & Rohtn, K. H. (1991). A catalytic role for threonine-I2 of *E. coli* asparaginase TI as established by sitedirected mutagenesis. *FEBS Letters, 285*(1,8), 55–58.
57. Derst, C., Wehner, A., Specht, V., & Rohm, K.-H. (1994). States and functions of tyrosine residues in *Escherichia coli* asparaginase II. *FEBS European Journal of Biochemistry, 224*, 533–540.
58. Laskowski, R. A., MacArthur, M. W., Moss, D. S., & Thornton, J. M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. *Journal of Applied Crystallography, 26*(2), 283–291.
59. Wehner, A., Derst, C., Specht, V., Aung, H. P., & Rohm, K. H. (1994). The catalytic mechanism of *Escherichia coli* asparaginase II. *Hoppe-Seyler's Z. Physiol. Chem., 375*, 108.