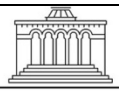
Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia



Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

• Փորձարարական և տեսական հոդվածներ • Экспериментальные и теоретические статьи •
• Experimental and theoretical articles •

Biolog. Journal of Armenia, 4 (65), 2013

ISOLATION AND IDENTIFICATION OF STRAIN WITH D-AMINOACYLASE ACTIVITY: PRELIMINARY CHARACTERIZATION OF THE ENZYME

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The results of BLAST analyses of partial nucleotide sequence of 16S ribosomal RNA gene of newly isolated strain N 6.1, possessing D-aminoacylase activity, revealed maximum of 83 % identity with Bacterial and Archaeal databases, indicating that the isolated bacteria belongs to a new species and even the new genus. The enzyme was more than 850 times purified with 2.7 % overall yield as a result of 4 stage purification scheme. The purified D-aminoacylase showed optimal activity at 40^0C and pH 7.5-7.8. The enzyme retained 50 % of its initial activity when incubated 20 min at 40^0C, and in these conditions it was more stable at pH 8.5-9.5.

Strain N6.1 – purification of D-aminoacylase – temperature optimum – pH optimum – thermostability – pH stability

Նոր անջատված D-ամինաացիլազային ակտիվությամբ N 6.1 շտամի 16S ռիբոսոմային ՌՆԹ-ի գենի մասնակի նուկլեոտիդային հաջորդականության BLAST անալիզի արդյունքները առավելագույնը 83 % նմանություն են ցույց տալիս բակտերիալ և արքեալ տվյալների բազաների հետ, վկայելով, որ անջատված բակտերիան պատկանում է նոր տեսակի, և նույնիսկ նոր ցեղի: Կիրառված 4 փուլերով մաքրման սխեմայի արդյունքում անջատված ֆերմենտը ավելի քան 850 անգամ մաքրվել է 2,7 % ընդհանուր ելքով: Մաքրված D-ամինաացիլազը օպտիմալ ակտիվություն է ցուցաբերում 40^0C ջերմաստիճանում և pH 7,5-7,8 տիրույթում: Ֆերմենտը պահպանում է իր սկզբնական ակտիվության 50 %-ը 20 րոպե 40^0C ջերմաստիճանում պահելիս և այս պայմաններում առավել ակտիվ է pH 8,5-9,5 տիրույթում:

Շտամ N6.1 – D-ամինաացիլազի մաքրում – ջերմաստիճանային օպտիմում – pH օպտիմում – ջերմակայունություն – pH կայունություն

Результаты БЛАСТ анализа частичной нуклеотидной последовательности гена 16S рибосомной РНК вновь выделенного штамма, обладающего D-аминоацилазной активностью, показали максимум 83 % идентичности с бактериальными и архейными базами данных, свидетельствующих о том, что выделенные бактерии принадлежат к новому виду и даже новому роду. Фермент был более чем 850 раз очищен с выходом 2,7 % в результате 4-стадийной схемы очистки. Очищенная D-аминоацилаза показала оптимальную активность при 40^0C и pH 7,5-7,8. Фермент сохранил 50 % своей первоначальной активности после инкубации при 40^0C 20 мин, и в этих условиях он был более стабилен при pH 8,5-9,5.

Штамм N6.1 – очистка D-аминоацилазы – температурный оптимум – оптимум pH – термостабильность – pH-стабильность

Bio-catalysis and enzyme technologies are contemporary and intensively developing directions of biotechnology. Among others, biotransformation technologies are considered more promising for obtaining the biologically active substances (BAS), including

L- and D-amino acids and their derivatives, having permanently increasing need in world market.

D-Amino acids are important intermediates in the preparation of semi-synthetic antibiotics [18], therapeutic drugs [3] pesticides [17], and bioactive peptides [21]. They occur in microorganisms, plants and animals and their physiological roles have been investigated [20]. It has been reported that D-amino acids can be introduced into the bacterial cell wall and have regulatory roles in the bacterial kingdom [10]. Recently, an increasing number of studies on natural D-amino acid-containing compounds have been reported. For example, a D-phenylalanine derivative lacking either a sulfonylurea or benzamido moiety, is used in diabetes type-2 treatment [1].

Despite D-aminoacylases were known long ago [5, 19], the researches of biotechnological value appeared much afterward. L-Aminoacylases are often used to obtain optically active amino acids from their racemates. From the point of view of obtaining of D-amino acids from their racemic mixtures by enantioselective separation, D-aminoacylases are more interesting. N-acyl-D-amino acid amidohydrolase (D-aminoacylase, EC 3.5.1.81) is an important enzyme for industrial applications and liberates D-amino acids from the corresponding N-acyl-D-amino acids. They have been found in a variety of microorganisms including *Alcaligenes* [12], *Pseudomonas* [15], *Variovorax* [7], *Stenotrophomonas* [13], *Streptomyces* [16], *Bordetella* [2], *Defluvibacter* [6] and *Microbacterium natoriense* [8, 9].

The aim of this study is identification of strain with D-aminoacylase activity and preliminary physicochemical and catalytic characterization of corresponding aminoacylase.

Materials and methods. *D-Aminoacylase producing* microorganisms were isolated by the modified procedure of Tsai at all [19]. Soil extract was spread onto agar plates containing isolation medium, which was composed of 0.5 % N-acetyl-D-valine (the sole carbon and nitrogen source), 0.1 % KH_2PO_4, and 0.025 % $MgSO_4$ **.**$7H_2O$ (pH 7.2), and was solidified with 1.5 % agar. The cells were grown at the 30^0C up to colony formation. Strains able to grow on this medium were selected and cultivated at 30^0C on fresh agar plates with the same medium with N-acetyl-DL-valine. The cells were collected, and the intracellular D-aminoacylase activity was measured after the cells were treated with toluene.

The nucleotide sequence determination was done by Eurofins Genomic & Sequencing Services department.

For enzyme characterization the cells grown on N-acetyl-DL-valine were collected by centrifugation and disrupted by ultrasonic treatment during 30 min at 4^0C (Labsonic 2000, B. Braun, Germany) in solution A (20 mM HEPES, pH 7.2, 0.1 mM PMSF). The cell debris was removed by centrifugation for 20 min at 20000 g (centrifuge K-24, Germany).

The enzyme purification was performed at 4^0C according to the four-stage scheme presented below. After cells ultrasonic disruption and centrifugation the obtained crude enzyme preparation was subjected to anion-exchange chromatography on DEAE-Toyopearl. The extract was applied to the column (2.5 × 20 cm) and was washed with two column volumes of solution A (0.1 % sodium azide, 0.1 mM PMSF, 20 mM HEPES, pH 7.2). Proteins were eluted with a linear gradient of sodium chloride concentration (0-0.4 M) prepared in the same solution A (V=500 ml). The active fractions were combined.

Obtained enzyme preparation was further purified on a hydroxyapatite (prepared according to Mazin et al. [11]) column (2.5 × 7 cm). The enzyme preparation was applied to the column, and adsorbed proteins were eluted with a linear gradient of concentration of phosphate buffer, pH 7.2 (0-0.3 M, V = 200 ml) prepared on solution B (0.1 % sodium azide, 0.1 mM PMSF).

Combined active fractions were concentrated and simultaneously purified by gradual centrifugal ultra filtration through 100 and 30 kDa Millipore MWCO devices.

In the fourth stage enzyme extract was subjected to second anion-exchange chromatography on DEAE-Toyopearl resin. The extract was applied to the column (1.5 × 10 cm) and was washed with two column volumes of solution A. Proteins were eluted with a linear gradient of sodium chloride concentration (0.1-0.3 M) prepared in the same solution A (V = 60 ml). The active fractions were combined and concentrated.

The enzyme activity was measured at 30^0C by modified orthophthalic aldehyde method [4] in the reaction mixture with 200 µl final volume, containing 10 mM N-acetyl-D-methionine, 50 mM HEPES, pH 7.2, and necessary quantity of the enzyme. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 µmol of D-amino acid per min. The concentration of protein was measured by the method of Groves and Davis by the absorption in the ultraviolet region [14].

In enzyme purification, determination of pH and temperature optima, temperature stability and pH depended temperature stability experiments the activity of enzymes was measured at specified conditions by described above method.

The reaction mixture for determination of the optimal pH contained 200 mM borate, 200 mM phosphate and 200 mM citrate buffer with specified pH. For determination of pH depended temperature stability the enzyme incubated in borate, HEPES and citrate containing buffer (each of 20 mM) with specified pH for 20 min at 40^0C. After that the residual activity was measured after adjustment of pH to 7.2. In both experiments the final pH was determined in situ.

To study the thermostability of D-aminoacylase, the enzyme samples were incubated at different temperatures for 20 min at pH 7.2. After the incubation, the samples were chilled in a water bath and the residual activity was measured.

Results and Discussion. As a result of screening of soil samples the bacterial strains possessing D-aminoacylase activity, named N 6.1, was selected. N 6.1 was Gram-negative, motile bacteria able to grow under aerobic conditions. The results of 16S rDNA sequence, covering 1002 nucleotides, are presented in fig. 1.

```
ORIGIN
        1 GTTACGACTT CACCCCAGTC ATGAGACCTA CCGTGGTCGT CGCCCCCCTT CCGGGGTGCA
       61 AACTACTTCT GGTGAAACCC CCTCCCTGGT GAGACGGGGG GTGGGGACAA GACCGGGGAA
      121 CGTATTCTCC GCCACATGGT TATCCACCAT TAATAACGAT TCACACTTCC TTCACTGGGT
      181 TTAGTTACTG AAATCCAGAC TAACAGAGAG TTTCTGGGAT GGGATCCCCC CCACGGGTTG
      241 GCGACCCTCT GTCCCGACCA TTGTATGACG TGTGAAGCCC TACCTGTAAA GGCCGTGAAG
      301 ACTTACTTTC CTCCCCCCCT TCCTCCCGTT TGTCTCACGC CGGCTCTTTC TAATGACCTT
      361 TCACCCTAAC TAGCGACAAA GGTTGCACTC GTTGCGGGAC TTATCCCAGA ATCTCACCAC
      421 CCTCTCTGAC GACAGCCATA CAACACCTGT GTTCCGGTTC TCTTGCGAGC ACTACCAAAT
      481 CTCTTTCTCA TTCCATATAT GGTATGGGTA GGTAAGGTTT TTCGTTCTTG CATCTAATTA
      541 ATAAATATCA TCCACCGCTT GTGCGGGTCC CCGGCCATTC CTTTGAGTTT TAATCTTGCG
      601 ACCGTACTCC CCACTCCGTC AACTTCACGC TTTATGCTGC GCTACCAAGG ACCGAAGGCC
      661 CCGACAGAAC CTTGACATCG TTTAGGGCGT GGACTACGTG GACTATCTAA GCCTGTTTGC
      721 TCCCCACGCT TTCACGCATG AGCGTCAGTG TTATCCCACA CAGGCTGCCT TCGCCATCTT
      781 CTGTTCCTGT GCATATCTAC GATATTTCAC TGCTACACGC GCTAATTCCA CCACTCTCTG
      841 ATCCACTCTA GCTCGGTAGT CAGCAATGCA TCTTCCAAAA TTAAGCTCTG GGATTTGCAC
      901 ATCTTTCTTT CCGACCCGAC TGAGCACGCT ATTACGTCCA TTAATCCCAG ATTTATCGCG
      961 TAGCACCCTA CGCACTTAAC GCAGCTAGCT GGGACTGCAA GG
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Fig. 1. 16S ribosomal RNA gene sequence of N6.1 strain, carrying D-aminoacylase activity.

The results of BLAST analyses of presented nucleotide sequence with database of 16S ribosomal RNA gene sequences of Bacteria and Arcahaea revealed maximum of 83 % identity with more than 29 gaps and high degree of coverage for 100 BLAST hits. The results for 9 BLAST hits (including 29 and 30 Alcaligenes faecalis strains among 100 BLAST hits) are presented in tab. 1. The presented data indicate that on the base of BLAST analyses we can conclude about screening from soil samples the aerobic bacteria with D-aminoacylase activity, which belongs to a new species and even the new genus. The precise determination of full 16S ribosomal RNA gene sequence is needed for final clarification of the problem.

Strain N6.1 was cultivated at 30^0C on induction medium with N-acetyl-DL-valine. The cells grown on N-acetyl-DL-valine were collected by centrifugation and disrupted by ultrasonic treatment as mentioned above. The obtained crude enzyme preparation was used for D-aminoacylase purification. The typical results of purification are presented in tab. 2.

Table 1. The homology between N6.1 strain's 16S rRNA partial nucleotide sequence and 9 BAST hits of Bacterial and Archaeal 16S rRNA database.

N	Source of 16S ribosomal RNA	Identities	Gaps	NCBI Reference
1	*Achromobacter insolitus strain LMG 6003*	831/999 (83%)	27/999 (2%)	NR_025685.1
2	*Bordetella hinzii LMG 13501*	831/999 (83%)	27/999 (2%)	NR_027537.1
3	*Bordetella avium strain ATCC 35086*	831/999 (83%)	27/999 (2%)	NR_041769.1
4	*Achromobacter xylosoxidans strain: DSM 10346*	830/999 (83%)	27/999 (2%)	NR_044925.1
5	*Achromobacter xylosoxidans A8 strain A8*	829/999 (83%)	27/999 (2%)	NR_074754.1
6	*Achromobacter denitrificans strain DSM 30026*	829/1000 (83%)	29/1000 (2%)	NR_042021.1
7	*Bordetella holmesii strain CDC F5101*	829/1000 (83%)	29/1000 (2%)	NR_029173.1
8	*Alcaligenes faecalis strain IAM12369*	808/1005 (80%)	37/1005 (3%)	NR_043445.1
9	*Alcaligenes faecalis subsp. parafaecalis strain G*	781/971 (80%)	30/971 (3%)	NR_025357.1

Table 2. Purification of D-aminoacylase isolated from strain N6.1

Purification steps	Volume, ml	Protein, mg/ml	Specific activity, U/mg	Yield, %
Crude extract	31.0	8.55	0.10	100.0
DEAE- Toyopearl	36.0	0.58	1.66	130.7
Hydroxyapatite	14.0	0.67	2.61	92.4
100-30 kDa cut off	0.93	0.55	12.09	23.3
DEAE- Toyopearl	0.17	0.049	87.10	2.7

It can be seen from tab. 2 that as a result of purification the specific activity of the enzyme increased more than 850 times with overall yield of 2.7 %. Nevertheless, the obtained preparation was not homogenous, as judged by native PAGE in anionic conditions.

Some catalytic properties of partially purified new D-aminoacylase were studied.

The results of determination of temperature and pH optima are presented in fig.1.

It is obvious, from obtained data, that the enzyme demonstrates the highest activity at 40^0C (fig.2. A). Enzyme showed highest activity at the range of pH 7.5-7.8 (fig.2. B).

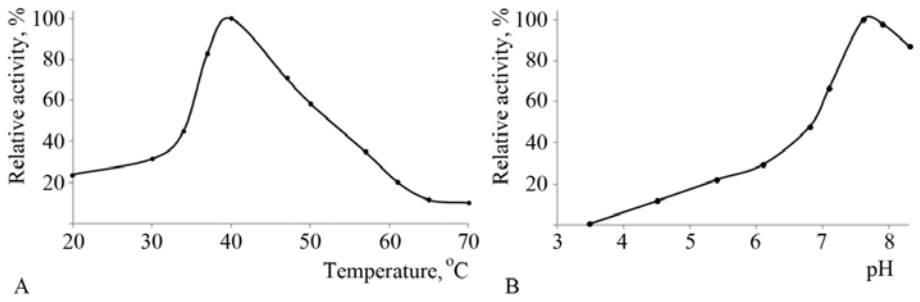

Fig. 2. The temperature (A) and pH (B) optimums of enzyme

The thermal stability and its dependence of pH for obtained enzyme preparation were also studied. The results are presented in fig. 3.

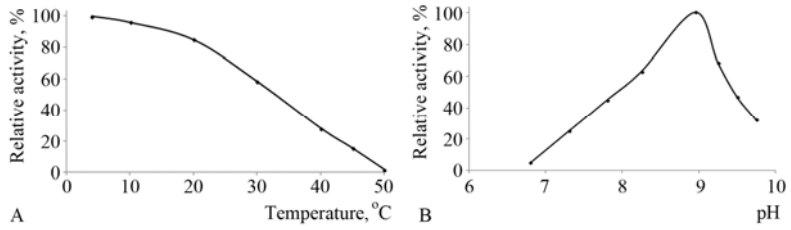

Fig. 3. The thermal stability (A) and its pH dependence (B) for partially purified D-aminoacylase.

It can be seen from fig. 3(A), that in the case of 20 min incubations at different temperatures D-aminoacylase retains its stability until up to 20^0C. Afterward the enzymes activity gradually decreases, achieving half of its initial activity at 35^0C. From fig. 3(B) it's follows, that the enzyme is more thermostable (when incubated at 40^0C and at mentioned pH during 20 min) at the range of pH 8.5-9.5.

Acknowledgments.

The author expresses her gratitude to Armine Margaryan, Yerevan State University, for support in processing of sequence data.

This work was supported by State Committee Science MES RA, in frame of the research projects № 0462 and №11-2i381.

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Received on 06.07.2013