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Short Report

Preliminary Report of NAD$^+$-Dependent Amino Acid Dehydrogenase Producing Bacteria Isolated from Soil

Hamid Shahbaz Mohammadi[1], Eskander Omidinia[*2], Abbas Sahebghadam Lotfi[3] and Reza Saghiri[2]

[1]Young Researcher Club, Dept. of Biochemistry, Science & Research Campus, Islamic Azad University, Tehran; [2]Dept. of Biochemistry, Pasteur Institute of Iran, Tehran 13164; [3]Dept. of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran 14115-115, Iran

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ABSTRACT

Background: Amino acid dehydrogenases (L-amino acid: oxidoreductase deaminating; EC 1.4.1.X) are members of the wider superfamily of oxidoreductases that catalyze the reversible oxidative deamination of an amino acid to its keto acid and ammonia with the concomitant reduction of either NAD$^+$, NADP$^+$ or FAD. These enzymes have been received much attention as biocatalysts for use in biosensors or diagnostic kits to screen amino acid metabolism disorders such as phenylketonuria (PKU), maple syrup urine disease (MSUD), homocystinuria (HCY) and hyperprolinemia. This study was aimed to isolation and screening of novel amino acid dehydrogenases from soil bacteria. **Methods:** The enzyme producing bacteria were selected among L-methionine and L-phenylalanine utilizers isolated from soil by thin layer chromatography, activity staining and confirmed by enzyme assay. Bacterial strains were identified by phenotypic and biochemical characteristics. The steady-state kinetic studies of enzymes were also performed. **Results:** In total of 230 tested strains, four of them were recognized as amino acid dehydrogenase producers that belong to species of *Pseudomonas*, *Citrobacter* and *Proteus*. They exhibited the desired NAD$^+$-dependent dehydrogenase activities toward L-isoleucine, L-methionine, L-cysteine, L-serine and L-glutamine in oxidative deamination reaction. The specific activity of L-isoleucine dehydrogenase, L-methionine dehydrogenase and L-glutamine dehydrogenase for oxidative deamination of L-isoleucine, L-methionine and L-glutamine were 1.59, 1.2 and 0.73 U/mg, respectively. The K_{cat}/K_m ($s^{-1}.mM^{-1}$) values in these strains were as follows: L-isoleucine, 113.6, L-methionine, 62.05 and L-glutamine, 95.83. **Conclusion:** This is the first report of occurrence a specific isoleucine dehydrogenase, glutamine dehydrogenase and methionine dehydrogenase in bacteria. *Iran. Biomed. J. 11 (2): 131-135, 2007*

Keywords: Amino acid dehydrogenase, Amino acid, Bacteria, Enzyme

INTRODUCTION

The amino acid dehydrogenases (L-amino acid: oxidoreductase deaminating; EC 1.4.1.X) are the important groups of coenzyme-dependent enzymes that catalyze the reversible oxidative deamination of an amino acid to its keto acid and ammonia with the concomitant reduction of either NAD$^+$, NADP$^+$ or FAD. The enzyme with dehydrogenase properties is distributed in a number of diverse prokaryotic and eukaryotic organisms [1-5]. Currently, there are 17 different enzymes listed in the 1992 Enzyme Nomenclature Catalog [6]. Amino acid dehydrogenases have been studied widely because of their potential applications in biosensors or diagnostic kits, synthesis of L- amino acids for use in production pharmaceutical peptides, herbicides and insecticides [6-9] and development of coenzyme regeneration systems for industrial processes [6-9]. According to the above demands, studies on molecular cloning such as production of recombinant phenylalanine

*Corresponding Author; Tel & Fax: (+98-21) 6640 2770; E-mail: skandar@pasteur.ac.ir

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dehydrogenase [10-12] and site-directed mutagenesis [13, 14] have also been carried out. These situations have prompted us to study amino acid dehydrogenase producer bacteria among Iranian soil inhabitant micro-organisms. This paper is the first report of occurrence some specific enzymes including isoleucine dehydrogenase, glutamine dehydrogenase and methionine dehydrogenase among bacteria.

MATERIALS AND METHODS

Materials. NAD^+, INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride), phenazine methosulfate were purchased from Sigma-Aldrich Corp. (USA), and nitrocellulose filter was from Advantech (Japan). All other chemicals were of analytical grade. About 30 soil samples were collected from three different regions of Iran including Tehran, Hamadan and Bandar Abbas.

Media and growth conditions. Medium A: 1% L-phenylalanine, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl, 0.01% $MgSO_4.7H_2O$, 0.01% yeast extract in 11 tap water, pH 7.2. Medium B: 0.5% L-methionine, 2 g K_2HPO_4, 1 g NaCl, 0.5 g $MgSO_4.7H_2O$, 5 g yeast extract, 5 g polypeptone in 11 tap water, pH 7.5. Medium C: 1g L-methionine, 0.1g NaCl, 0.2 g K_2HPO_4, 1g polypeptone, 0.5 g yeast extract, 1g Na_2CO_3 in 11 tap water, pH 9.5. For agar plates, the media were supplemented with 2% agar. The soil samples collected from mesophilic habitats of Iran were suspended in 5 ml of different media and were incubated at 37 °C with shaking (130-140 rpm). The isolated bacteria were maintained on agar media.

Enzyme screening methods. Amino acid dehydrogenase producing bacteria were isolated from soil by TLC [15] and enzyme activity staining techniques [10, 14].

Preparation of crude extract. For the preparation of the crude extract, cells were harvested by centrifugation after 48 h, washed and resuspended with 0.1 M potassium buffer (pH 7.0) containing 0.1 mM EDTA and 5 mM 2-mecaptoethanol. The cell suspension was disrupted by ultrasonication and cell debris was removed by centrifugation. The supernatant solution served as the enzyme source.

Enzyme assay. L-amino acid dehydrogenase activity was measured spectrophotometrically at 340 nm with different L-amino acids. The standard enzyme activity in the oxidative deamination was assayed by reduction of NAD^+ in reaction mixture (1.0 ml) containing 10 mM L-amino acid, 100 mM glycine-KCl-KOH buffer (pH 10.4), 2.5 mM NAD^+ and the enzyme solution. One unit of enzyme activity (U) was defined as the amount of enzyme that produced 1 μmol of NADH per min in the oxidative deamination of each amino acid.

Steady-state kinetics. The initial velocity studies for oxidative deamination reactions were performed by varying the concentration of one substrate in the presence of different fixed concentrations of the other substrates [16]. The kinetic parameters were calculated from the secondary plots of intercepts versus reciprocal concentrations of the other substrates.

Protein assay. The total protein concentration was determined by a Bio-Rad protein assay kit.

Taxonomical studies. The identification of bacteria isolated from soil was performed by their phenotypic characteristics including microscopic morphology, gram-staining reactions, colony morphology and growth requirements [17]. The results were summarized in Table 1.

RESULTS AND DISCUSSUION

We here described the isolation and screening of NAD^+-dependent amino acid dehydrogenases which were isolated from soil samples by enrichment culture with methionine or phenylalanine as a sole source of carbon, nitrogen and enzyme inducer. At the end of screening, we isolated four bacteria among 230 strains tested that exhibited the desired dehydrogenase activities toward L-isoleucine, L-methionine, L-serine, L-cysteine and L-glutamine in oxidative-deamination reactions. These types of studies have been used long time in different areas to screen and find novel bacteria or enzymes [1-5, 15, 18-20] but they have been unsuccessful in isolation of the isoleucine and methionine dehydrogenase from bacteria [15]. All isolated strains of our study were gram negative. Whereas others reported that the most amino acid dehydrogenase producing bacteria such as *Bacillus* and *Streptomyces* species were Gram-positive [5-7,

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Table 1. Taxonomic characteristics of isolated bacterial strains.

Characteristic	Finding			
	Strain A	**Strain B**	**Strain C**	**Strain D**
Shape	Rod	Rod	Rod	Rod
Colony morphology	Smooth, round, flat, glossy and 1-2 in diameter	Smooth, round, convex, shiny and 2-4 in diameter	Smooth, round, flat, opaque and 1-2 in diameter	Smooth, round, flat, opaque and 0.5-2 in diameter
Growth at Blood agar	Positive	Positive	Positive	Positive
Growth at MacConkey agar	Positive	Positive	Positive	Positive
Growth at 37°C	Positive	Positive	Positive	Positive
Gram-stain	Negative	Negative	Negative	Negative
Triple Sugar Iron Agar (TSIA)	Alkaline/Acid	Alkaline/Alkaline	Alkaline/Alkaline	Alkaline/Acid
Citrate utilization	Positive	Positive	Positive	Positive
Methyl red test	Positive	Negative	Negative	Positive
Voges-Proskauer test (VP)	Negative	Negative	Negative	Negative
Motility test	Positive	Positive	Positive	Positive
Indole production	Negative	Negative	Negative	Positive
Urea hydrolysis	Negative	Negative	Negative	Positive
Oxidase test	Negative	Positive	Positive	Negative
Catalase test	Positive	Positive	Positive	Positive
Production of H_2S	Negative	Negative	Negative	Negative

9, 10, 18-21]. This finding is a considerable subject among enzyme producer species that will be study later.

As shown in Table 2, at strain A the most activity was for isoleucine dehydrogenase. As a matter of fact, there was no amino acid dehydrogenase activity for L-isoleucine amino acid analogs including L-leucine, L-valine, L-alanine or even L-methionine. And the specific activity for isoleucine dehydrogenase was 1.59 U/mg that higher than other enzyme producing bacteria. It can be concluded that we found an isoleucine dehydrogenase with high substrate specificity for L-isoleucine in oxidative deamination reaction.

According to the taxonomic characteristics, it was identified as *Citrobacter* species (Table 1). The *Km* and V_{max} for L-isoleucine were 0.025 mM and 3.33, respectively (Table 3). Also the specificity constant

Table 2. Various L-amino acid dehydrogenases activities in crude enzyme preparation of isolated bacteria**.**

Amino acids	Specific activity (U/mg)			
	Strain A	**Strain B**	**Strain C**	**Strain D**
Glycine	0.47	0.08	0.00	0.00
D, L-Alanine	0.00	0.00	0.00	0.32
L-Valine	0.00	0.47	0.70	0.25
L-Leucine	0.00	0.36	0.00	0.30
L-Isoleucine	1.59	0.00	0.70	0.36
L-Methionine	0.00	0.36	1.20	0.10
L-Phenylalanine	0.36	0.36	0.00	0.17
L-Tyrosine	0.48	0.00	0.00	0.00
L-Aspartic acid	0.38	0.00	0.00	0.00
L-Glutamaic acid	0.42	0.00	0.00	0.00
L-Cysteine	0.00	0.47	0.73	0.00
L-Serine	0.52	0.47	0.73	0.00
L-Glutamine	0.70	0.47	0.73	0.00
L-Histidine	0.17	0.36	0.36	0.34
L-Asparagine	0.00	0.36	0.00	0.27
L-Arginine	0.32	0.00	0.00	0.17
L-Tryptophan	0.00	0.27	0.00	0.00
L-Lysine	0.38	0.00	0.00	0.00

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Table 3. Steady-state kinetic parameters.

Amino acid	*Km* (*mM*)	V_{max} (μmol.min^{-1}.ml^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1}.mM^{-1})
L- Isoleucine	0.025	3.33	2.84	113.6
L-Methionine	0.034	2.77	2.11	62.05
L- Glutamine	0.012	1.36	1.15	95.83
L- Valine	0.044	6.66	4.95	112.5
L- Cysteine	0.016	1.78	1.29	80.62
L- Serine	0.020	1.88	1.43	71.5

(K_{cat}/K_m) for isoleucine dehydrogenase was 113.6. It should be noted that this is the first report about occurrence of a specific isoleucine dehydrogenase in bacteria. The most activities at strain B were seen for L-valine, L-serine,L-cysteine and L-glutamine. These results showed that there was a valine dehydrogenase which has a nonspecific reaction for L-leucine, also a glutamine dehydrogenase with lower substrate specificity toward L-glutamine and an amino acid dehydrogenase which has equal activity for L-serine and L-cysteine. Different types of valine dehydrogenase have been reported from *Streptomyces* species [6, 7]. The *Km* and V_{max} values for L-valine were calculated to be 0.044 mM and 6.66, respectively. The highest K_{cat}/K_m value in this strain was observed for valine dehydrogenase (Table 3). Therefore, the catalytic efficiency of valine dehydrogenase was better than cysteine or serine dehydrogenase. The taxonomical studies indicated that this strain belongs to *Pseudomonas* species. Nevertheless the highest activity that was seen for L-methionine, L-serine, L-cysteine and L-glutamine belongs to strain C. It can be concluded there was a methionine dehydrogenase enzyme as a result of its higher specific activity. Asano and Tanetani [15] have screened for an L-methionine specific dehydrogenase-producing bacterium from soil and have been unsuccessful.

Therefore, this is the first report on the presence of methionine dehydrogenase in a bacterium. Another considerable enzyme in this strain was a glutamine dehydrogenase with high substrate specificity for L-glutamine because no dehydro-genase activity was seen toward its amino acid analog including L-asparagine. There is a report about occurrence of glutamine dehydrogenase in plants [22]. It is worth to know, on the basis of information from Brunhuber and Blanchard [6], this enzyme has been not reported in bacteria. The taxonomical results in Table 1 showed the bacterium was species of *Pseudomonas.* The *Km* values for L-methionine and L-glutamine were 0.034 and 0.012 mM, respectively. The K_{cat}/K_m or specificity constant values were 95.83 for L-glutamine and 62.05 for L-methionine. As a result, the catalytic efficiency of glutamine dehydrogenase was higher than methionine dehydrogenase.

Strain D showed no novel and considerable dehydrogenase activity toward L-amino acids but it was a gram-negative bacterium which utilized L-amino acids in oxidative deamination reactions. Also this strain was determined as *Proteus* species. Further experiments are in progress to purify these enzymes and determine their other properties.

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