

# *tyrB-2* and *phhC* genes of *Pseudomonas putida* encode aromatic amino acid aminotransferase isozymes: evidence at the protein level

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**Abstract** Two *Pseudomonas putida* aminotransferases (ArAT I and ArAT II) that exhibit activity toward L-tryptophan were purified 104- and 395-fold using a six-stage purification procedure involving ammonium sulfate fractionation and chromatographic separation on phenyl-Sepharose, Sephadex G-100 superfine, DEAE-cellulose and Protein-Pack Q8 HR columns. Mass spectrometry analysis resulted in the identification of 27 and 20 % of the total ArAT I and ArAT II amino acid sequences. In addition, N-terminal sequence fragments of ArAT I and ArAT II were determined using the Edman degradation method. Based on the analyses performed, the studied proteins were identified as products of the *tyrB-2* and *phhC* genes, and the presence of these genes in the investigated bacterial strain was confirmed using molecular biology methods. Extensive analysis of the substrate specificities of ArAT I and ArAT II revealed that both enzymes most efficiently catalyzed reactions involving aromatic amino acids and 2-oxoacids followed by dicarboxylic compounds. The best substrates for ArAT I and ArAT II were L-phenylalanine and phenylpyruvate. Based on these results, the studied proteins were classified as aromatic amino acid aminotransferase isozymes.

**Keywords** Aromatic amino acid aminotransferase · *Pseudomonas putida* · *phhC* · *tyrB-2*

## Introduction

*Pseudomonas putida* is an aerobic, rod-shaped, gram-negative bacterium that lives in water and soil, particularly in association with plant roots, and along with *P. fluorescens* is considered the most important species within the *Pseudomonas* genus in the aspect of plant growth stimulation (Botelho and Mendonça-Hagler 2006; Khakipour et al. 2008). Of the various mechanisms of plant growth promotion by *P. putida* and other bacteria, production of indole-3-acetic acid through the indole-3-pyruvate (IPyA) pathway is thought to play a central role in this process (Lambrecht et al. 2000; Patten and Glick 2002; Spaepen et al. 2007). The first reaction of the IPyA pathway, where L-tryptophan is converted to IPyA, is catalyzed by an aromatic amino acid aminotransferase (EC 2.6.1.57, ArAT), which is a pyridoxal phosphate-dependent enzyme that reversibly transfers the amino group of aromatic amino acids (L-phenylalanine, L-tryptophan and L-tyrosine) onto 2-oxoglutarate. The proteins with ArAT activity, particularly toward L-tryptophan, and the genes encoding ArATs were therefore recently studied in various bacteria species, including *Azospirillum brasilense*, *Enterobacter cloacae* and *Pantoea agglomerans* (Koga et al. 1994; Sergeeva et al. 2007; Ge et al. 2009; Castro-Guerrero et al. 2012) as well as in some plants (Simpson et al. 1997; Phillips et al. 2011; Won et al. 2011). In our previous methodological paper, we reported the detection of two aminotransferases that were capable of transaminating L-tryptophan also in *P. putida* (Szkop et al. 2012). However, until now, there were no experimental data concerning the primary structure of the proteins exhibiting ArAT activity or the identity of the genes encoding them in *P. putida*. The only data available resulted from computational analyses of the recently sequenced genomes of several *P. putida* strains

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(e.g., KT2440, Nelson et al. 2002; Bird-1, Matilla et al. 2011; ND6, Li et al. 2012). Based on gene sequence similarity, the putative functions of the two genes, which were designated as *tyrB* (NCBI Gene ID: 1043062) and *tyrB-2* (NCBI Gene ID: 1045962), were predicted to encode ArAT proteins. Considering that a variety of bacterial aminotransferases exist in a single microorganism and that these enzymes usually possess broad and overlapping substrate specificities, the biochemical approach for their identification is required (Xing and Whitman 1992). Particularly that on the basis of primary structure homology ArAT has been classified along with alanine, aspartate and histidyl-phosphate aminotransferases in one subgroup whose representatives exhibit similar enzymatic properties (Metha et al. 1993). Moreover, it was shown in diverse bacterial species that activity toward aromatic amino acids and 2-oxoacids exhibits to some extent also branched-chain amino acid aminotransferase (Whitaker et al. 1982; Xing and Whitman 1992; Yvon et al. 2000). Therefore, the objective of the present study was to purify, identify and properly classify aminotransferases exhibiting activity toward L-tryptophan in the *P. putida* bacterium.

## Materials and methods

### *Pseudomonas putida* strain and culture conditions

The *Pseudomonas putida* strain A used in this study was isolated from wheat rhizosphere and was identified using the ribotyping method by Bliert S.A. DNA-Gdańsk, Poland. The 16S rRNA gene sequence of this strain is deposited in the DNA Data Bank of Japan under accession number AB667903. The bacteria were cultured in 1 l of King B liquid medium (King et al. 1954) while shaking (150 rpm) at room temperature for 48 h. The bacterial culture was centrifuged (20,000×g; 12 min), and the bacterial pellet was washed two times and resuspended in 50 mM phosphate buffer (pH 8.1) containing 5 μM pyridoxal phosphate (PLP) and 5 mM 2-mercaptoethanol (2-ME) (extraction buffer) before being broken with ultrasonication. The crude bacterial extract was centrifuged (20,000×g; 12 min), and the obtained supernatant (homogenate) was used as the starting material for enzyme purification.

### Purification of enzymes

All purification steps were performed at 4 °C. The proteins in the homogenate were fractionated with ammonium sulfate (40–60 % saturation). The collected protein precipitate was dissolved in the extraction buffer, and after centrifugation (20,000×g; 12 min), it was loaded onto a phenyl-Sepharose column (2 × 15 cm) equilibrated with 50 mM

phosphate (pH 8.1) and 0.3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The bound proteins were eluted with a linear gradient (0–100 %) of 50 mM phosphate (pH 8.1) supplemented with 2 μM PLP and 1 mM 2-ME. The fractions exhibiting L-tryptophan:2-oxoglutarate activity were pooled and concentrated to a volume of approximately 12 ml using an Amicon concentrator (Millipore Corporation; Bedford, MA, USA) supplied with a PM 10 membrane. The concentrated preparation was separated by gel filtration on a Sephadex G-100 superfine column (2.6 × 85 cm) equilibrated with 10 mM phosphate (pH 8.1) containing 2 μM PLP and 1 mM 2-ME (buffer A). The fractions with L-tryptophan:2-oxoglutarate activity were pooled, concentrated to a volume of approximately 10 ml and applied to a DEAE-cellulose column (1 × 10 cm) equilibrated with buffer A. Proteins bound to the resin were eluted with a linear gradient (0–100 %) of 0.1 M phosphate buffer (pH 6.8) containing 2 μM PLP and 1 mM 2-ME. The fractions corresponding to the two peaks with L-tryptophan:2-oxoglutarate activity (the first peak corresponded to ArAT II activity, and the second peak corresponded to ArAT I activity) were separately pooled, concentrated to a volume of approximately 4 ml and rechromatographed on the Sephadex G-100 superfine column under the same conditions. Finally, each preparation was subjected to anion exchange chromatography on a Protein-Pack Q8 HR column attached to an HPLC system (Waters Corporation; Milford, MA, USA). For the preparation of ArAT I, the column was equilibrated with 40 mM phosphate buffer (pH 8.1) supplemented with 2.5 % (w/v) sucrose, 2 μM PLP and 1 mM 2-ME. For the preparation of ArAT II, the column was equilibrated with 10 mM phosphate buffer (pH 8.1) supplemented with 2.5 % (w/v) sucrose, 2 μM PLP and 1 mM 2-ME. In both cases, the bound proteins were eluted with a linear gradient (0–100 %) of 0.15 M phosphate buffer (pH 6.8) containing 2.5 % (w/v) sucrose, 2 μM PLP and 1 mM 2-ME. The active fractions were pooled, enriched with sucrose to a final concentration of 20 % (w/v) and stored at –80 °C.

### Aminotransferase activity assays

L-Tryptophan:2-oxoglutarate activity during purification was determined using a method previously developed in our laboratory (Szkop et al. 2012). One unit (U) of enzyme activity was defined as the release of 1 μmol of IPyA per minute. Specific activity was expressed in U per mg of protein. The protein concentration was determined using the method of Bradford (1976). Enzyme specificity analysis towards 2-oxoacids was performed using the above-mentioned method except that 2-oxoglutarate was replaced with various 2-oxoacids at 0.25 or 3 mM concentration in the reaction mixture. Controls omitting one of the

substrates were run with each assay. Specificity of the enzymes towards amino acids was determined using the RP-HPLC-fluorescence method based on L-glutamate (the amino acid product of 2-oxoglutarate transamination) quantification. Here also, controls omitting one of the substrates were run with each assay. The reaction mixture consisted of 0.1 M phosphate buffer (pH 8.0), 2.5 or 10 mM amino acid, 3 mM 2-oxoglutarate, 10  $\mu$ M pyridoxal phosphate and enzyme preparation. The reactions were performed at 35 °C in a total volume of 0.5 ml and were terminated by the addition of 125  $\mu$ l of 2 M borate buffer (pH 12.5) containing 0.7 M 2-ME. The samples were centrifuged and filtered (Amicon Ultra 0.5 ml centrifugal filters Z677094, Sigma–Aldrich, Inc.; St. Louis, MO, USA), and the filtrate was derivatized with orthophthalic aldehyde (Van Eijk et al. 1993) and analyzed using HPLC (binary HPLC 1525 pump, Waters). Separations were performed on C18 columns (Symmetry 4.6  $\times$  150 mm, 5  $\mu$ m, Waters) fitted with a C18 guard column (Symmetry 3.9  $\times$  20 mm, 5  $\mu$ m, Waters) and fluorimetric detection ( $\lambda_{\text{ex}} = 338/\lambda_{\text{em}} = 425$  nm, Model 474, Waters) was used. The samples were automatically derivatized through the autosampler (Model 717plus, Waters) in the following manner. First, 100  $\mu$ l of orthophthalic aldehyde solution (15 mg ml<sup>-1</sup> (v/v) in 50 % methanol) was added into 450  $\mu$ l of the filtered reaction mixture. After two cycles of mixing and a 3-min delay, 100  $\mu$ l of the sample was injected on the column. Chromatographic separations were performed with 60 mM phosphate buffer (pH 6.8; eluent A) and 80 % acetonitrile (eluent B) using two different gradient programs. A shorter program (the mobile phase started with eluent A:eluent B at 100:0 % and varied to 0:100 % and 100:0 % in 12 and 16 min, respectively) was used when the transamination reaction was performed with the participation of all of the analyzed amino acids except L-aspartate. In the latter case, a longer program was applied (the mobile phase started with eluent A:eluent B at 100:0 %, and it was varied to 70:30, 0:100 and 100:0 % in 18, 25 and 31 min, respectively). The flow rate of the mobile phase was 1 ml min<sup>-1</sup>. The concentration of L-glutamate produced in the course of the transamination reactions was quantified based on the prepared calibration curve.

#### Electrophoretic separation and enzyme activity staining

Electrophoresis under native and denaturing conditions was performed in a discontinuous system (4 and 10 % stacking and resolving gels were used, respectively) according to the method of Laemmli (1970). The protein bands in the gels after SDS-PAGE were stained with Colloidal Coomassie Brilliant Blue (Roth; Karlsruhe, Germany) according to the manufacturer's protocol. The gels were calibrated with

Unstained Protein Molecular Weight Marker (Fermentas; Vilnius, Lithuania). The *aromatic amino acid:2-oxoglutarate* activity bands after native PAGE were visualized according to the method described by Pedraza et al. (2004) with modifications. Gels after electrophoresis were incubated in a mixture containing 0.1 M phosphate buffer (pH 8.0), 10 mM L-phenylalanine, 10 mM L-tryptophan or 2.5 mM L-tyrosine, 3 mM 2-oxoglutarate, 2  $\mu$ M PLP, 0.6 mM NBT and 0.1 mM PMS until activity bands appeared. Gels were washed with distilled water and photographed. When the gels were stained for *L-aspartate:2-oxoglutarate* activity, 8 mM L-cysteine sulfinic acid was used as the amino acid substrate (Stejskal 1994).

#### Protein identification

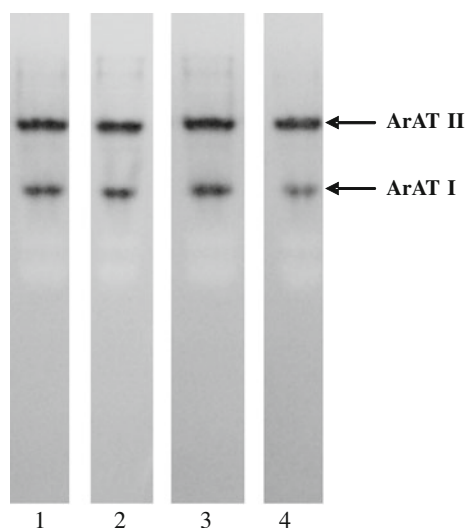
Proteins were identified by mass spectrometry (Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland) and by N-terminal sequencing using the Edman degradation method (Biocentrum; Kraków, Poland). For mass spectrometry analysis, the protein bands obtained in SDS-PAGE were excised and digested with trypsin. The resulting peptides were separated on a nanoAcquity UPLC (Ultra Performance LC) and analyzed with an Orbitrap-based mass spectrometer. N-terminal sequencing of the proteins was performed from PVDF membranes using an automated sequencer Procise 491 system (Applied Biosystems, Foster City, CA, USA).

#### Obtaining a partial sequence of the genes encoding the studied enzymes

Bacterial genomic DNA was isolated using the Genomic DNA Purification Kit (Fermentas). The following primers were used for PCR: F<sub>ArAT I</sub> (5'-GTGTTCAAACATGTC GATGC-3'), R<sub>ArAT I</sub> (5'-TTCGAGAACGAGTTGCTG AC-3'), F<sub>ArAT II</sub> (5'-ATGCACTTCGGAGCCATC-3') and R<sub>ArAT II</sub> (5'-GACCATGTACACGCTGTGCT-3'). These primers were designed based on the nucleotide sequence of putative ArAT from *P. putida* KT2440 (NCBI Gene ID: 1045962; *tyrB-2*) and from *P. entomophila* L48 (NCBI Gene ID: 4086633; *phhC*). The PCR amplifications were performed using Pfu polymerase (Fermentas) using the following program: 10 min at 95 °C, 30 cycles of 1 min at 95 °C, 1.5 min at 56 °C and 4 min at 70 °C and a final extension step for 10 min at 72 °C. The resulting PCR products were ligated into a pJET1.2/blunt cloning vector (Fermentas), amplified in *Escherichia coli* TOP10 cells and sequenced in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (Institute of Biochemistry and Biophysics, Polish Academy of Sciences). The obtained nucleotide sequences were analyzed using bioinformatics tools.

## Results and discussion

Zymographic analysis of the proteins from the crude bacterial extract revealed the presence of two aminotransferases capable of catalyzing the reaction involving aromatic amino acids and L-aspartate as substrates. According to their electrophoretic mobility, the enzymes were designated as ArAT I and ArAT II (Fig. 1). ArAT I and ArAT II were subjected to a six-step purification procedure that consisted of ammonium sulfate fractionation (40–60%), hydrophobic interaction chromatography on phenyl-Sepharose, gel filtration chromatography on a Sephadex G-100 superfine and anion exchange chromatography on



**Fig. 1** Native Page analysis of proteins from the crude bacterial extract. The gels were stained for *L-phenylalanine:2-oxoglutarate* (a), *L-tyrosine:2-oxoglutarate* (b), *L-tryptophan:2-oxoglutarate* (c) and *L-aspartate:2-oxoglutarate* (d) activity

DEAE-cellulose as well as gel filtration rechromatography and anion exchange chromatography on a Protein-Pack Q8 HR (HPLC). The most effective step was the anion exchange chromatography on a DEAE-cellulose column, which permitted the separation of the studied proteins and resulted in an approximate 8-fold purification of ArAT II and a 2.5-fold purification of ArAT I. The purification procedure resulted in a final 104-fold purification of ArAT I and 395-fold purification of ArAT II with a yield of 0.95 and 1.71% and specific activities of 14.7 and 55.7 U mg<sup>-1</sup> protein, respectively (Table 1). Highly purified ArAT I and ArAT II were used for the determination of their substrate specificity, which is the main criterion for enzyme classification. For this purpose, we used thirteen L-amino acids and nine 2-oxoacids with various side chain structures. Among the amino acids tested, both enzymes most efficiently catalyzed the reactions involving aromatic amino acids and L-aspartate (Table 2). The best substrate for ArAT I and ArAT II was L-phenylalanine, and the activities of both isozymes toward L-tryptophan, L-aspartate and L-tyrosine ranged from 56 to 90% of the maximum activity at 10 mM amino acid concentration. Activity of ArAT I toward the other tested amino acids did not exceed 10% of the maximum activity. ArAT II was characterized by a broader substrate specificity and catalyzed relatively efficiently also reactions involving L-histidine (38% of the maximum activity at 10 mM concentration), L-isoleucine and L-leucine (17 and 14% of the maximum activity at 10 mM concentration, respectively). Among the studied 2-oxoacid substrates, ArAT I and ArAT II exhibited the highest activities toward aromatic and dicarboxylic 2-oxoacids; however, different results were obtained depending on the 2-oxoacid concentration. At 0.25 mM 2-oxoacid concentration, both isozymes converted

**Table 1** Summary of the ArAT I and ArAT II purification procedure

| Purification step  | Total activity (U) | Total protein (mg) | Specific activity (U × mg <sup>-1</sup> protein) | Purification (fold) | Yield (%) |
|--|--------------------|--------------------|--|---------------------|-----------|
| Homogenate   | 189.2              | 1338               | 0.141  | 1                   | 100.0     |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (40–60%) | 95.70              | 419.6              | 0.228  | 1.617               | 50.58     |
| Phenyl-Sepharose   | 49.85              | 75.34              | 0.662  | 4.695               | 26.35     |
| Sephadex G-100 Superfine                                 | 36.30              | 28.20              | 1.287  | 9.128               | 19.19     |
| DEAE-cellulose   |                    |                    |  |                     |           |
| ArAT I   | 7.260              | 2.380              | 3.050  | 21.63               | 3.837     |
| ArAT II  | 12.04              | 1.250              | 9.630  | 68.30               | 6.363     |
| Sephadex G-100 superfine                                 |                    |                    |  |                     |           |
| ArAT I   | 4.760              | 0.987              | 4.822  | 34.20               | 2.516     |
| ArAT II  | 7.530              | 0.359              | 20.97  | 145.89              | 3.980     |
| Protein-Pack Q8 HR                                       |                    |                    |  |                     |           |
| ArAT I   | 1.792              | 0.122              | 14.69  | 104.17              | 0.947     |
| ArAT II  | 3.229              | 0.058              | 55.67  | 394.82              | 1.707     |

**Table 2** Substrate specificity of ArAT I and ArAT II toward selected amino acids and 2-oxoacids

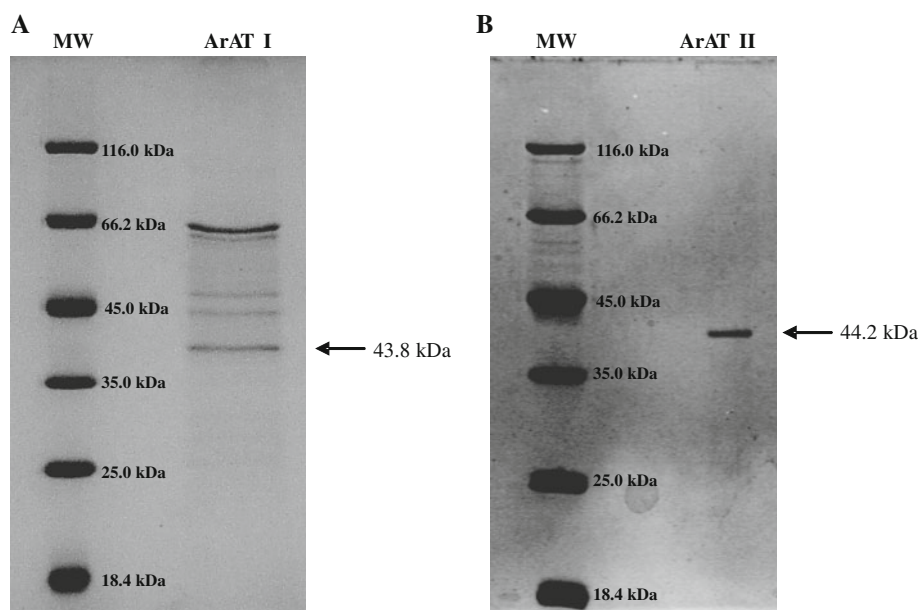
| Substrate<br>L-Amino acid | Relative activity (%) |             | Substrate<br>2-Oxoacid          | Relative activity (%) |             |
|---------------------------|-----------------------|-------------|---------------------------------|-----------------------|-------------|
|                           | 2.5 mM                | 10 mM       |                                 | 0.25 mM               | 3 mM        |
| <b>ArAT I</b>             |                       |             |                                 |                       |             |
| Phenylalanine             | 100.0 ± 4.4           | 100.0 ± 4.1 | Phenylpyruvate                  | 100.0 ± 5.1           | 95.0 ± 4.4  |
| Tyrosine                  | 90.4 ± 4.5            | –           | <i>P</i> -hydroxyphenylpyruvate | 83.0 ± 5.3            | 61.2 ± 3.8  |
| Tryptophan                | 69.2 ± 3.8            | 73.1 ± 3.9  | 2-Oxoglutarate                  | 16.7 ± 1.0            | 75.4 ± 4.2  |
| Aspartate                 | 61.4 ± 2.9            | 64.3 ± 4.0  | Oxaloacetate                    | 13.2 ± 0.8            | 100.0 ± 4.6 |
| Valine                    | 8.2 ± 0.4             | 3.7 ± 0.2   | 2-Oxo-3-methylvalerate          | 5.2 ± 0.3             | 1.3 ± 0.1   |
| Histidine                 | 8.1 ± 0.4             | 4.0 ± 0.2   | 2-Oxo-4-methylvalerate          | 4.3 ± 0.2             | 3.5 ± 0.2   |
| Isoleucine                | 7.1 ± 0.5             | 9.5 ± 0.6   | Pyruvate                        | 4.3 ± 0.2             | 3.2 ± 0.1   |
| Leucine                   | 6.2 ± 0.4             | 8.2 ± 0.4   | 2-Oxoisovalerate                | 3.8 ± 0.2             | 3.1 ± 0.2   |
| Asparagine                | 0.8 ± 0.0             | –           | Imidazole-pyruvate              | 3.2 ± 0.2             | 2.1 ± 0.1   |
| Alanine                   | –                     | 2.1 ± 0.1   |                                 |                       |             |
| Serine                    | –                     | 0.7 ± 0.0   |                                 |                       |             |
| Cysteine                  | –                     | 0.0         |                                 |                       |             |
| Lysine                    | –                     | 0.0         |                                 |                       |             |
| <b>ArAT II</b>            |                       |             |                                 |                       |             |
| Phenylalanine             | 100.0 ± 5.4           | 100.0 ± 5.6 | Phenylpyruvate                  | 100.0 ± 5.7           | 63.9 ± 3.5  |
| Tyrosine                  | 83.2 ± 5.2            | –           | <i>P</i> -hydroxyphenylpyruvate | 99.0 ± 5.4            | 25.5 ± 1.3  |
| Aspartate                 | 62.8 ± 3.9            | 78.8 ± 4.8  | Oxaloacetate                    | 33.3 ± 1.9            | 100.0 ± 4.9 |
| Tryptophan                | 37.2 ± 2.1            | 55.9 ± 3.2  | 2-Oxoglutarate                  | 18.8 ± 0.9            | 78.6 ± 4.2  |
| Histidine                 | 24.9 ± 1.2            | 37.5 ± 2.1  | 2-Oxo-3-methylvalerate          | 4.7 ± 0.2             | 1.3 ± 0.1   |
| Isoleucine                | 14.9 ± 0.8            | 17.0 ± 0.9  | 2-Oxo-4-methylvalerate          | 4.5 ± 0.3             | 10.1 ± 0.4  |
| Leucine                   | 14.1 ± 0.7            | 13.8 ± 0.8  | Pyruvate                        | 3.6 ± 0.2             | 8.7 ± 0.4   |
| Valine                    | 7.3 ± 0.3             | 6.1 ± 0.6   | 2-Oxoisovalerate                | 3.4 ± 0.1             | 1.4 ± 0.1   |
| Alanine                   | –                     | 1.2 ± 0.1   | Imidazole-pyruvate              | 3.0 ± 0.2             | 31.1 ± 1.6  |
| Asparagine                | 0.2 ± 0.0             | –           |                                 |                       |             |
| Serine                    | –                     | 0.5 ± 0.0   |                                 |                       |             |
| Cysteine                  | –                     | 0.0         |                                 |                       |             |
| Lysine                    | –                     | 0.0         |                                 |                       |             |

The values are the mean ± SD from two independent experiments, each performed in duplicate. –, not tested

phenylpyruvate and *p*-hydroxyphenylpyruvate with the highest rate. When 3 mM 2-oxoacid was used, ArAT I and ArAT II catalyzed the reactions involving oxaloacetate most efficiently. The observed phenomenon was associated with the occurrence of progressive substrate inhibition when aromatic 2-oxoacids were used at concentrations above 0.25 mM, which indicated a high affinity of the aminotransferases towards these substrates (Paris and Magasanik 1981). For dicarboxylic 2-oxoacids, progressive substrate inhibition was observed when the concentration of these compounds exceeded 3 mM (data not shown). Activity of ArAT I toward other tested 2-oxoacids did not exceed 5 % of the maximum activity, whereas ArAT II catalyzed with a relatively high rate reaction involving imidazole-pyruvate (31 % of the maximum activity at 3 mM concentration). These results clearly show that the best substrates for ArAT I and ArAT II were aromatic amino acids and 2-oxoacids followed by dicarboxylic

compounds, which allowed for the classification of the studied aminotransferases as ArAT isozymes. High activities toward dicarboxylic substrates characterized also ArATs isolated from other sources. The activity of ArAT from *Escherichia coli* toward L-aspartate reached 65 % of the maximum activity determined with L-tyrosine (Powell and Morrison 1978), and activity of ArAT from *Brevibacterium linens* toward L-aspartate reached 50 % of the maximum activity determined with L-phenylalanine (Lee and Desmazeaud 1985). For comparison, aspartate aminotransferases studied in parallel with ArATs by Powell and Morrison (1978) and Lee and Desmazeaud (1985) were characterized by considerably different relative activities toward amino acid substrates. Aspartate aminotransferase from *E. coli* catalyzed the reaction involving aromatic amino acids at a rate of approximately 20 % of the maximum activity reached with L-aspartate, and the same enzyme from *B. linens* catalyzed the transamination

**Fig. 2** SDS-PAGE of the final ArAT I (a) and ArAT II (b) preparations. Approximately 3  $\mu$ g (ArAT I) and 0.5  $\mu$ g (ArAT II) of protein were loaded. WM, molecular weight standards:  $\beta$ -galactosidase (116.0 kDa), albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98 I (25.0 kDa) and  $\beta$ -lactoglobulin (18.4 kDa)

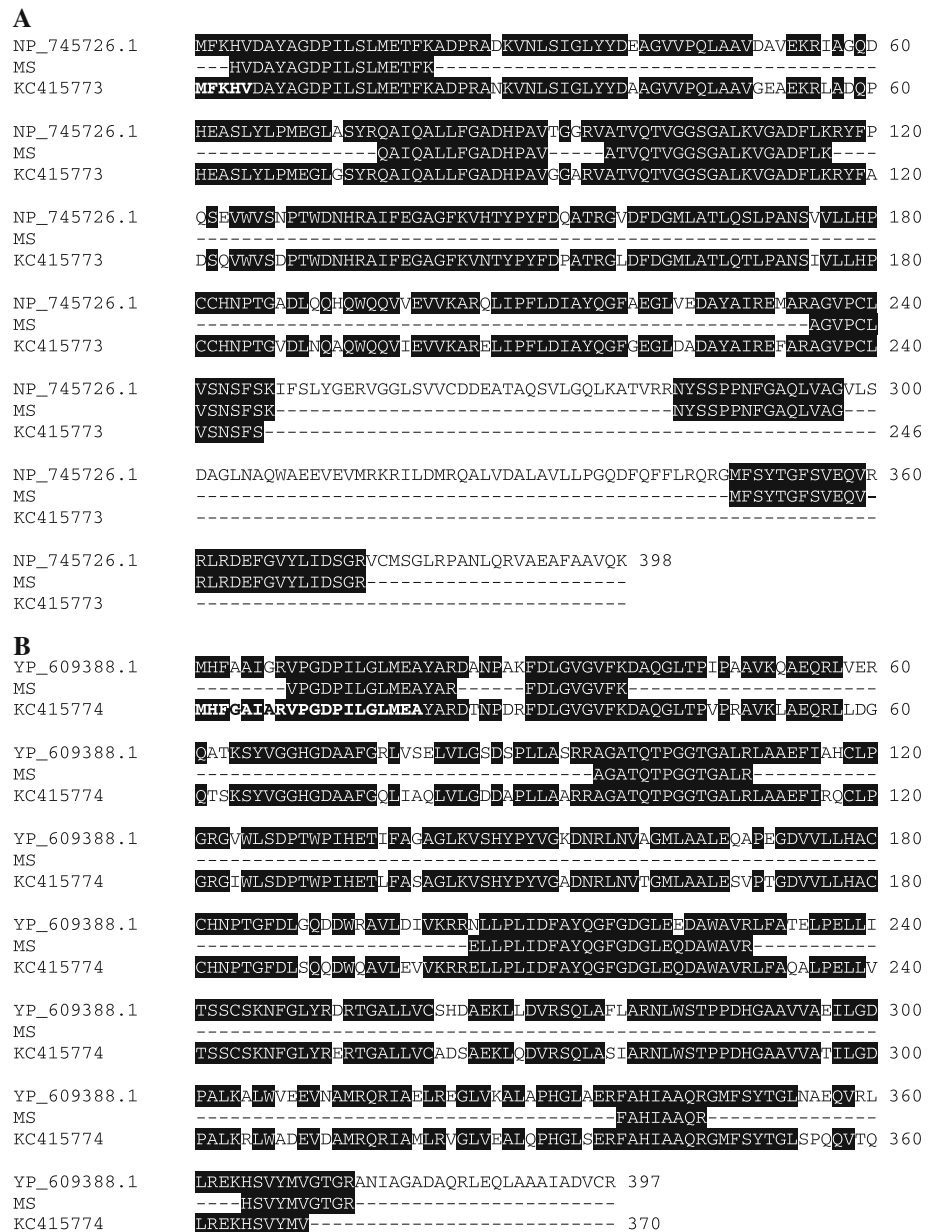


reactions with the participation of various aromatic amino acids six to ten times less efficiently than reactions involving L-aspartate. Similar kinetic properties characterized also aspartate aminotransferase from *Pyrococcus furiosus* (Ward et al. 2002).

The final preparations of ArAT I and ArAT II were subjected to SDS-PAGE. Such an analysis of the ArAT II preparation revealed a single protein band with a molecular mass of approximately 44.2 kDa (Fig. 2b). The identity of this protein band was determined by mass spectrometry. As a result, five peptides with 100 % identity and one peptide with 92 % identity with sequence fragments of putative ArAT from *P. entomophila* L48 (NCBI RefSeq protein accession no: YP\_609388.1; NCBI Gene ID: 4086633; *phhC*) were obtained (Fig. 3b). The identified peptides constituted 20 % of the total enzyme sequence. For ArAT I preparation, SDS-PAGE analysis showed the presence of five visible protein bands (Fig. 2a). All of these bands were separately excised from the gel and analyzed using mass spectrometry. Examination of the protein band with a molecular mass of approximately 43.8 kDa resulted in the identification of seven peptides with 100 % identity to sequence fragments of a putative ArAT from *P. putida* KT2440 (NCBI RefSeq protein accession no: NP\_745726.1; NCBI Gene ID: 1045962; *tyrB-2*) (Fig. 3a). The identified peptides constituted 27 % of the total enzyme sequence. Analysis of other four protein bands did not result in the identification of any peptides similar to any putative ArAT sequence (data not shown). Densitometric analysis of the gel showed that the protein band corresponding to ArAT I constituted approximately 24 % of the total protein content in the final enzyme preparation. To further establish the

identity of the studied isozymes, N-terminal fragments of ArAT I and ArAT II sequences were determined using the Edman degradation method. For ArAT II, an unambiguous 20 amino-acid sequence (MHFGAIARVPGDPILGLMEA) was obtained. This sequence showed a 90 % identity with the N-terminal sequence of YP\_609388.1 protein (18 amino acids identical, Gly in place of Ala at position 4 and Ala in place of Gly at position 7). For ArAT I, a five amino acid sequence with an uncertain amino acid at position three: MF(K+G)HV was obtained. With the adoption of the presence of Lys (K) at that position, this sequence showed 100 % identity with the N-terminal sequence of NP\_745726.1 protein. The consistent results obtained by mass spectrometry and Edman degradation analysis of the ArAT I and ArAT II amino acid sequences allowed the selection of appropriate nucleotide sequences from the NCBI database, which were used as a template to design the primers for PCR. For ArAT I, the primers were designed on the basis of the nucleotide sequence of the *tyrB-2* gene encoding a putative ArAT from *P. putida* KT2440; for ArAT II, the primers were designed based on the nucleotide sequence of the *phhC* gene, which encodes a putative ArAT from *P. entomophila*. As a result, PCR products of 740 and 1,110 bp were obtained and sequenced. These sequences, which represented 62 % (*tyrB-2*) and 93 % (*phhC*) of the total gene sequences, have been deposited in the GeneBank under accession numbers KC415773 and KC415774, respectively. It should be noted that for both enzymes, we observed 100 % identity between all amino acid-sequence fragments obtained by biochemical analysis of the purified proteins and amino acid sequences deduced from the obtained nucleotide sequences. These results demonstrate

**Fig. 3** The alignment of the deduced amino acid sequences of ArAT I (a) and ArAT II (b) with putative ArAT proteins and peptides obtained by mass spectrometry analysis. NP\_745726.1 and YP\_609388.1, deduced amino acid sequences of putative ArAT from *P. putida* KT2440 and *P. entomophila* L48, respectively. MS, peptides derived from mass spectrometry analysis. KC415773 and KC415774, deduced amino acid sequences of ArAT I and ArAT II (studied enzymes), respectively. N-terminal fragments of ArAT I and ArAT II determined using the Edman degradation method are shown in *bold font*. Identical amino acids within aligned sequences are presented on a black background



that the *tyrB-2* and *phhC* genes of the studied *P. putida* strain, taxonomy of which was confirmed using the precise ribotyping method, encode aromatic amino acid aminotransferase isozymes. The function of the *tyrB-2* gene in bacteria of the *Pseudomonas* genus was predicted on the basis of its sequence homology, and the function of the *phhC* gene has been described only in *P. aeruginosa* (Gu et al. 1998). Moreover, to the best of our knowledge, this is the first report demonstrating the presence of the *phhC* gene in the genome of *P. putida*. In other strains of these bacteria, which have been previously sequenced, the only genes encoding proteins with predicted ArAT function were *tyrB* and *tyrB-2*. The fact that only two ArAT isozymes were detected in the studied bacterial strain may

indicate a lack of the *tyrB* gene in this strain or that the authentic function of this gene is different.

**Concluding remarks**

In this paper, we demonstrate evidence based on biochemical approaches for the determination of previously predicted function of the proteins and genes that two *P. putida* aminotransferases, whose substrate specificity allowed for their classification as ArAT isozymes, are encoded by the *tyrB-2* and *phhC* genes. The physiological importance of the existence of two ArAT isozymes, especially in the aspect of their contribution to IAA

biosynthesis by the *P. putida* species, remains to be shown and is a subject of our current study.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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