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Isolation, Purification, and Characterization of Phenylpyruvate Transaminating Enzymes of *Erwinia carotovora*

A. M. Paloyan, A. A. Hambardzumyan*, and Gh. P. Halebyan

SPC Armbiotechnology, National Academy of Sciences of Republic of Armenia, Gyurjyan Str. 14, Yerevan 0056, Republic of Armenia; fax: (+037410) 654-183; E-mail: arm_biotech@yahoo.com; arthambardzumyan@gmail.com

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Abstract—Enzymes of *Erwinia carotovora* that transaminate phenylpyruvate were isolated, purified, and characterized. Two aromatic aminotransferases (PAT1 and PAT2) and an aspartic aminotransferase (PAT3) were found. According to gel filtration, these enzymes have molecular weights of 76, 75, and 78 kDa. The enzymes consist of two identical subunits of molecular weights of 31.4, 31, and 36.5 kDa, respectively. The isoelectric points of PAT1, PAT2, and PAT3 were determined as 3.6, 3.9, and 4.7, respectively. The enzyme preparations considerably differ in substrate specificity. All three of the enzymes productively interacted with the following amino acids: L-aspartic acid, L-leucine (except PAT3), L-isoleucine (except PAT3), L-serine, L-methionine, L-cysteine, L-phenylalanine, L-tyrosine, and L-tryptophane. The aromatic aminotransferases display higher specificity to the aromatic amino acids and the leucine-isoleucine pair, whereas the aspartic aminotransferase displays higher specificity to L-aspartic acid and relatively low specificity to the aromatic amino acids. The aspartic aminotransferase does not use L-leucine or L-isoleucine as a substrate. PAT1, PAT2, and PAT3 show the highest activity at pH 8.9 and at 48, 53, and 58°C, respectively.

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Key words: *Erwinia carotovora*, aromatic aminotransferase, purification, subunit content, isoelectric point, substrate specificity

Since 1937 when Braunstein and Kricman discovered the transamination reaction [1] many aminotransferases having different spectra of substrate specificity have been isolated, purified, and studied. Aminotransferases are pyridoxal phosphate dependent enzymes that catalyze the reversible transfer of an amino group from an amino acid to a keto acid molecule. On the base of the molecular structure the aminotransferases are divided into four subgroups. Subgroup I includes aspartate, alanine, tyrosine, histidinol phosphate, and phenylalanine aminotransferases; subgroup II includes acetylornithine, ornithine, O-amino acid, 4-aminobutyrate, and diaminopelargonic aminotransferases; subgroup III includes D-alanine and branched-chain amino acid aminotransferases; subgroup IV includes phosphoserine and serine aminotransferases [2].

Aspartate and aromatic aminotransferases from subgroup Iα are characterized in detail, but only eight members of this subgroup having relatively small difference in the sequence were experimentally characterized based on their substrate specificity [3]. The enzymes of this subgroup are well studied in *Escherichia coli* [4-7], *Bacillus subtilis* [8, 9], *Bacillus* sp. [10], coryneform bacteria [11, 12], *Pseudomonas* [13, 14], *Klebsiella aerogenes* [15], the actinomycete *Amycolatopsis methanolica* [16], *Lactococcus lactis* [17], archaea [18], hyperthermophilic archaea *Thermococcus litoralis* [19], *Pyrococcus horikoshii* [20], and *P. furiosus* [21]. Aromatic aminotransferase from *Trypanosoma cruzi* belonging to subgroup Iγ is also well studied [22, 23]. The sequence of this enzyme has shown to have only 15% homology with the sequence of similar aminotransferases of the Iα subgroup.

Previously we have developed a biotransformation method for obtaining L-phenylalanine using aminotransferases of *Erwinia carotovora* [24]. This work is devoted to isolation, purification, and characterization of the enzymes of *E. carotovora* transaminating phenylpyruvate.

MATERIALS AND METHODS

The strain *E. carotovora* subsp. *carotovora* formerly known as *Erwinia aroideae* (Collection of Micro-

* To whom correspondence should be addressed.

organisms of SPC Armbiotechnology, NAS RA, INMIA No. 8724) was used in this work.

The bacterium was grown on a medium with pH 7.0 containing (%): aspartic acid, 2; yeast extract, 0.1; pyridoxine, 0.01; KH_2PO_4, 0.5; $FeSO_4$, 0.001; $MgSO_4$, 0.05. The cells were grown on circular shakers (200 rpm) at 30°C. The cells were harvested by centrifugation at 5000*g* for 40 min at 4°C (K-26 centrifuge; Germany), washed in solution A (20 mM Hepes-NaOH, pH 7.2, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM mercaptoethanol (ME), 0.05 mM pyridoxal phosphate (PLP)), and after centrifugation were kept at −18°C.

The cells were disrupted by ultrasonic treatment for 20 min at 20 kHz frequency and 300 W power (Labsonic 2000; B. Braun, Germany) in solution A. The cell debris was removed by centrifugation for 20 min at 20,000*g*.

The enzymes were purified at 4°C according to the six-stage scheme presented below. In the second stage the crude enzyme extract obtained in the previous stage was subjected to anion-exchange chromatography on DEAE-cellulose equilibrated with solution A. The extract was applied to the column (2.5 × 20 cm) and was washed with two column volumes of solution A. Proteins were eluted with a linear gradient of sodium chloride concentration (0-0.5 M) prepared in the same solution (500 ml). The active fractions were combined.

The proteins were further purified on a hydroxyapatite column (2.5 × 7 cm) prepared according to Mazin et al. [25]. The combined active fractions were applied to the column, and adsorbed proteins were eluted with a linear gradient of concentration of phosphate buffer (0-0.2 M) prepared in solution B (1 mM EDTA, 0.1 mM PMSF, 5 mM ME, 0.05 mM PLP, pH 7.2).

In the fourth stage again the anion-exchange chromatography on DEAE-Toyopearl was used for effective separation of PAT3 from PAT1 and PAT2. The extract was applied to the column (1.5 × 25 cm), which was then washed with two column volumes of solution A. The adsorbed proteins were eluted with a linear gradient of sodium chloride concentration (0-0.25 M) in buffer A (200 ml).

For separation of PAT1 from PAT2 another chromatography on hydroxyapatite (1.5 × 10 cm) was used. The proteins were applied to the column, which was then washed with two column volumes of solution A, and the adsorbed proteins were eluted with a linear concentration gradient of phosphate buffer (0-0.2 M) prepared in solution B (200 ml). At this stage the chromatography of PAT3 on hydroxyapatite was performed separately according to the method described above.

For the final stage gel filtration on Toyopearl 50 F (1.0 × 65 cm) was used. The aminotransferases were eluted by solution A containing 0.1 M NaCl.

The enzyme activity, if not mentioned specifically, was measured in reaction medium of 200 µl final volume containing 50 mM L-phenylalanine, 25 mM 2-ketoglutarate, 0.05 mM PLP, 0.1 M Hepes-NaOH, pH 7.2, and enzyme preparation of required quantity. The amount of phenylpyruvate generated was determined by absorption in the presence of 1 M NaOH (ε_{320} 17,500 $M^{-1}\cdot cm^{-1}$) [5]. In determination of the pH optimum of enzyme activity the reaction mixture contained 100 mM concentrations of Tris, phosphate, carbonate, and borate. The pH was adjusted by adding concentrated HCl or NaOH. One enzyme unit is defined as the amount of enzyme that catalyzes the synthesis of 1 µmol of product per minute under the mentioned conditions. The concentration of protein was measured by the method of Groves and Davis by the absorption in the ultraviolet region [26].

The molecular weights of the enzymes were determined by gel filtration in a column packed with Toyopearl 50F (1.5 × 54 cm). The column was calibrated using the following protein standards with known molecular weight: bovine serum albumin (67 kDa), egg albumin (45 kDa), chymotrypsinogen (25 kDa), and horse myoglobin (17.8 kDa).

The molecular weights of the enzyme subunits were determined by disc electrophoresis in 12.6% polyacrylamide gel in the presence of SDS by the method proposed by Pharmacia (Sweden) [27]. The mixture of Pharmacia low molecular weight proteins (rabbit phosphorylase B, 94 kDa; bovine serum albumin (BSA), 67 kDa; egg albumin, 43 kDa; bovine erythrocyte carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; bovine α-lactalbumin, 14.4 kDa) was used as molecular weight standards.

The isoelectric points of enzymes were determined by isoelectric focusing in 5% polyacrylamide gel plates using the Pharmacia protocol [27]. The 1.6% solution of LKB ampholyte (Sweden) was used (75% with pH 4.0-6.0 and 25% with pH 3.5-9.0). Filter papers saturated with 0.5 M NaOH and 0.25 M H_2SO_4 were used as electrode buffers. The following wide p*I* spectrum Pharmacia isoelectric point markers were used: trypsinogen (p*I* 9.30), lentil lectin basic group (p*I* 8.65), lentil lectin neutral group (p*I* 8.45), lentil lectin acidic group (p*I* 8.15), myoglobin basic group (p*I* 7.35), myoglobin acidic group (p*I* 6.85), human anhydrase (p*I* 6.55), bovine anhydrase (p*I* 5.85), β-lactoglobulin A (p*I* 5.20), soybean trypsin inhibitor (p*I* 4.55), amyloglucosidase (p*I* 3.50).

The substrate specificity of the enzymes was determined at concentration of amino group donors 50 mM (L-tyrosine, 10 mM), and 25 mM 2-ketoglutarate was used as an amino group acceptor. The concentration of L-glutamic acid was determined qualitatively by thin layer chromatography on silica gel plates (Silufol, Czechia) following Kirchner [28] and quantitatively by means of glutamate dehydrogenase by NAD reduction in Tris-hydrazine buffer.

Table 1. Results of purification of enzymes of *Erwinia carotovora* transaminating phenylpyruvate

Purification stage	Enzyme preparation	Volume, ml	Protein, mg/ml	Specific activity, U/mg	Yield, %
Cellular disintegration	–	150	28	0.04	100
Chromatography on					
DEAE-cellulose	–	124	4.7	0.15	55.7
hydroxyapatite	–	39	4.2	0.43	43.3
DEAE-Toyopearl	PAT1/PAT2	19	2.1	1.03	26.1
	PAT3	18	1.6	0.63	11.2
hydroxyapatite	PAT1	15	0.2	6.9	12.2
	PAT2	17	0.4	2.9	10.9
	PAT3	20	0.2	3.2	8.5
Gel filtration on Toyopearl 50F and concentration	PAT1	2	0.6	10.3	7.4
	PAT2	2	1.2	4.6	6.7
	PAT3	2	0.8	5.2	5.2

In this work following materials and reagents were used: EDTA, PMSF, ME, acrylamide, Hepes, protein molecular weight markers, glutaric acid, and dialysis bags from Serva (Germany); DEAE-cellulose from Whatman (Great Britain); DEAE-Toyopearl and Toyopearl 50F from Toyo Soda (Japan); other reagents were products of CIS countries.

RESULTS

Enzymes that transaminate phenylpyruvate were isolated and purified from cellular extracts of *E. carotovora*. The results of purification are presented in Table 1.

In the fourth stage of purification using ion-exchange chromatography on DEAE-Toyopearl, the PAT3 aminotransferase was completely separated from PAT1 and PAT2 (Fig. 1a). In the fifth stage of purification using hydroxyapatite chromatography of the fraction containing both PAT1 and PAT2, these aminotransferases were separated (Fig. 1b). At this stage PAT3 was subjected to chromatography separately on the same column after its regeneration. In the sixth stage, by separate gel filtration on Toyopearl 50F and subsequent concentration, the specific activity of PAT1, PAT2, and PAT3 enzyme preparations increased 1.5-1.6-fold.

The results of determination of the molecular weights of the enzymes by gel filtration are shown in Fig. 2. It can be seen that molecular weights of PAT1, PAT2, and PAT3 are 76, 75, and 78 kDa, respectively.

The results of disc electrophoresis in the presence of SDS are presented in Fig. 3. The molecular weights of PAT1, PAT2, and PAT3 subunits are 31.4, 31 and 36.5 kDa, respectively. Comparison of these data with the molecular weights of the intact enzymes shows that all three aminotransferases consist of two identical subunits. From Fig. 3 it also follows that if PAT3 is nearly homogeneous, the degree of homogeneity of PAT1 exceeds 80%, and in the PAT2 preparation the foreign component is present that makes up more than 70% of the total protein

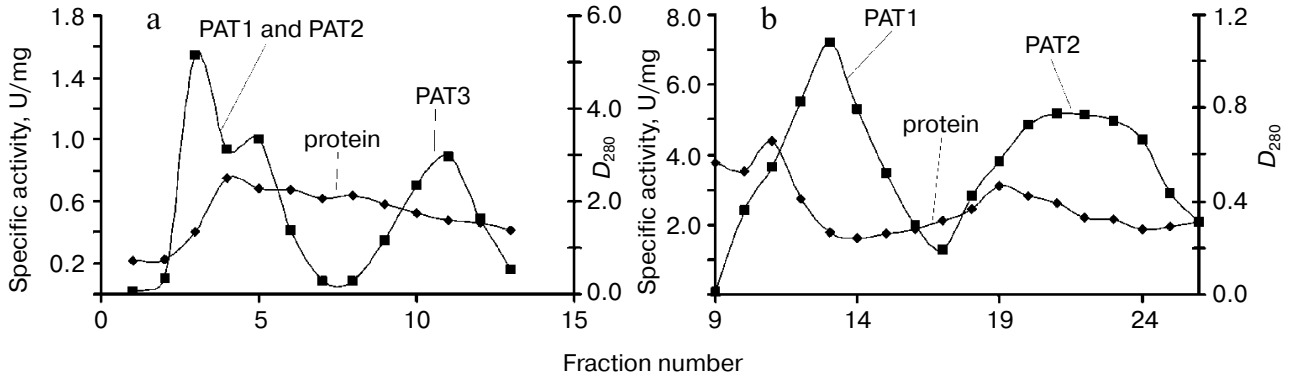

Fig. 1. Elution profiles of aminotransferases of *E. carotovora*: a) separation of PAT3 from PAT1 and PAT2 on DEAE-Toyopearl column; b) separation of PAT1 from PAT2 on hydroxyapatite column (repeated chromatography).

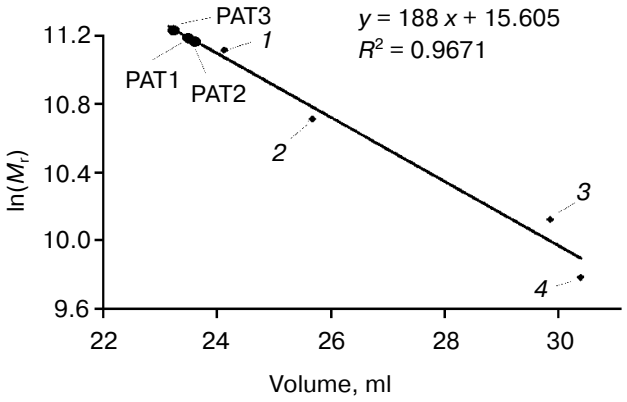

Fig. 2. Determination of molecular weights of enzymes of *E. carotovora*: *1*) bovine serum albumin (67 kDa); *2*) egg albumin (45 kDa); *3*) chymotrypsinogen (25 kDa); *4*) horse myoglobin (17.8 kDa).

of the preparation. The protein bands were identified by native disc electrophoresis in the Laemmli buffer system [27], where a piece of the polyacrylamide gel plate was stained for protein and other piece of the gel was sliced and the aminotransferase activities were determined in the slices (data not presented).

The results of determination of isoelectric points are presented in Fig. 4. The isoelectric points of enzymes PAT1, PAT2, and PAT3 appear to be 3.9, 3.6, and 4.7, respectively. It should be mentioned that here we also observe the same picture of ratio of aminotransferases and foreign proteins in the respective traces (see Fig. 3).

The results of study of substrate specificity of the enzymes in reactions of 2-ketoglutarate amination are shown in Table 2. All of the protein amino acids were examined as amino group donor with the exception of L-glutamic acids. The table presents only the data for those amino acids that were amino group donors.

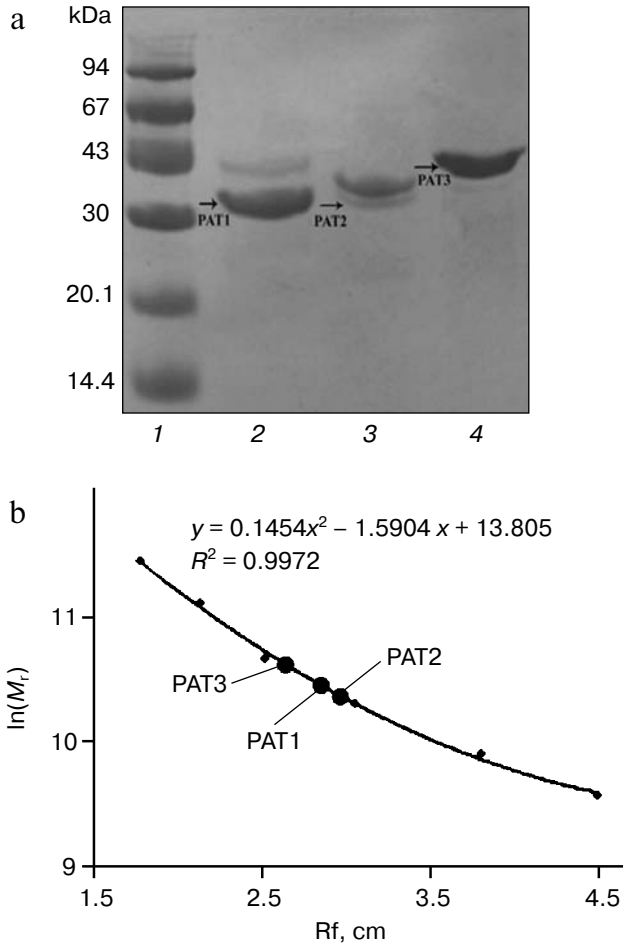

Fig. 3. Determination of molecular weights of subunits of enzymes of *Erwinia carotovora*: a) protein markers (*1*), PAT1 (*2*), PAT2 (*3*), PAT3 (*4*); b) calibration curve for determination of the molecular weights of the subunits.

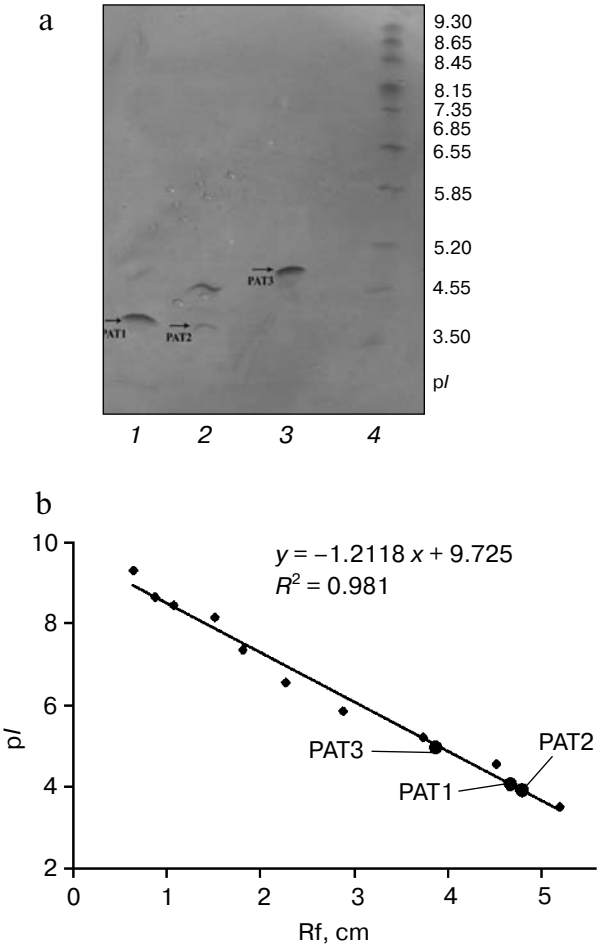

Fig. 4. Determination of isoelectric points of enzymes of *E. carotovora*: a) PAT1 (*1*), PAT2 (*2*), PAT3 (*3*), proteins with known isoelectric points (*4*); b) calibration curve for determination of isoelectric points.

Table 2. Substrate specificity of enzymes of *E. carotovora* transaminating phenylpyruvate with 2-ketoglutaric acid as amino group acceptor

Substrate	Specific activity, U/mg		
	PAT1	PAT2	PAT3
Aspartic acid	2.58	1.09	2.98
Leucine	6.17	3.71	0.00
Isoleucine	1.80	0.55	0.17
Serine	0.94	0.68	0.38
Methionine	6.09	1.98	1.37
Cysteine	7.73	2.49	2.67
Phenylalanine	5.31	1.87	0.75
Tyrosine	3.39	1.12	1.15
Tryptophane	8.91	3.44	2.28

The results show that the enzymes PAT1 and PAT2 are similar in their activity to particular amino acids. They show highest activity with tryptophane, cysteine, methionine, leucine, phenylalanine, aspartic acid, and, in all likelihood they are aromatic aminotransferases [7, 18]. PAT3 shows relatively high activity with aspartic acid, cysteine, tryptophane, methionine, tyrosine, and phenylalanine. It is like aspartic aminotransferase in its inability to use branched chain amino acids as substrates [7, 18]. It is necessary to note that in contrast to the known aspartic and aromatic aminotransferases, the enzymes PAT1, PAT2, and PAT3 showed high activity with cysteine and methionine [7].

The results of determination of temperature optimum of the enzyme preparations are presented in Fig. 5. PAT1, PAT2, and PAT3 show the highest activity at 48, 53, and 58°C, respectively.

The dependences of the activities of aminotransferases on pH are presented in Fig. 6. PAT1, PAT2, and PAT3 show maximum activity at pH 8.9. However, although PAT1 and PAT2 are active over a rather wide range of pH (6.7-9.3), for PAT3 this pH range is narrower (7.7-9.3).

DISCUSSION

From cellular extracts of *E. carotovora* three enzymes were isolated and purified (PAT1, PAT2, and PAT3) which are able to transaminate phenylpyruvate using various donors of amino groups. On the basis of the results on substrate specificity, we consider PAT1 and PAT2 to be aromatic aminotransferases and PAT3 to be an aspartate aminotransferase. These aminotransferases are known to be analogous; they belong to the Iα subgroup of aspartate and aromatic aminotransferases [3] and can interconvert as a result of a limited number of point mutations [29]. Most aspartate and aromatic aminotransferases participating in a metabolism of aromatic amino acids are known also in other bacterial strains. For example, the following aminotransferases are known in bacteria: in *E. coli*, aspartate and aromatic [5, 7]; coryneform bacteria, aspartate and aromatic [11]; *Ps. aeruginosa*, aspartate and three aromatic [13]; *Methanococcus aeolicus*, aspartate and two aromatic [18].

In Table 3 we summarize the physical and chemical characteristics of the studied aminotransferases from *E. carotovora* and compare them to the literature data.

The molecular weights of aspartic and aromatic aminotransferases vary in the range of 50-260 kDa. By this characteristic the aminotransferases of *E. carotovora* are similar to aminotransferases of *E. coli* and *Ps. aeruginosa*. The aromatic and aspartic aminotransferases in coryneform bacteria and archaebacteria have much higher molecular weights. Despite the diversity of molecular weights of aspartic and aromatic aminotransferases, all of

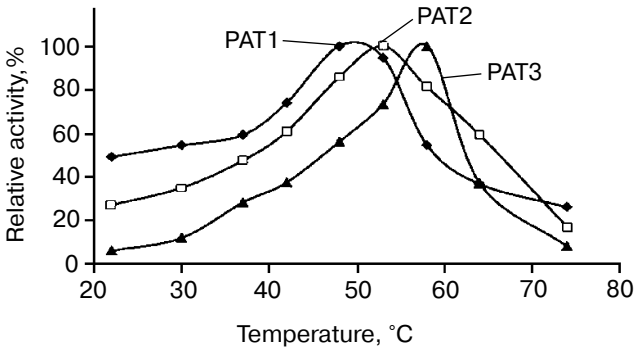

Fig. 5. Temperature optimum of enzymes of *E. carotovora* (100% activity corresponds to 7.4, 6.7, and 5.2 U/mg activities of enzymes PAT1, PAT2, and PAT3, respectively).

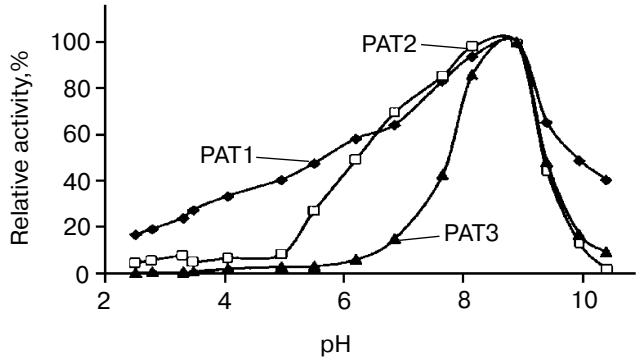

Fig. 6. pH optimum of enzymes of *E. carotovora* (100% enzyme activity corresponds to 7.4, 6.7, and 5.2 U/mg activities for PAT1, PAT2, and PAT3, respectively).

Table 3. Comparison of physical and chemical properties of aspartate and aromatic aminotransferases of different bacterial strains

Strain	Enzyme	Molecular weight, kDa	Subunit molecular weight, kDa	Isoelectric point, p*I*	Optimum pH	Specific activity, U/mg	Literature
E. carotovora	PAT1	76	31.4	3.9	6.7-9.3	10.3	this work
	PAT2	75	31.0	3.6	6.7-9.3	4.6	
	PAT3	78	36.5	4.7	7.7-9.3	5.2	
E. coli	ArAT	88	43	4.6	7.5-8.0	103[a]	[5]
	AspAT	82	42	4.5	8.0-9.0	8.0[a]	
Coryneform	ArAT I	155	–	–	–	0.0004[b]	[11]
(B. flav.. C. glut.)	ArAT II	260	–	–	–	0.003[b]	
Pseudomonas	ArAT I	50	–	–	–	0.0094	[13]
aeruginosa	ArAT II	70	–	–	–	0.0009	
	ArAT III	200	–	–	–	0.0003	
	AspAT	64	–	–	–	0.0310	
Klebsiella aerogenes	ArAT	90	42	4.6	6.0-9.6	725[c]	[15]
	AspAT	–	–	–	–	132[c]	
Methanococcus	ArAT I	150	–	4.4	–	1.1[d]	[18]
aeolicus	ArAT II	90	–	4.9	–	0.04[d]	
	AspAT	162	–	4.4	–	42.7[e]	

[a] Calculated from values of k_{cat}.
[b] Determined by radioisotope method in the reaction of oxaloacetate transamination with phenylalanine.
[c] Value of k_{cat} for the pair of substrates phenylalanine and 2-ketoglutarate.
[d] Value of k_{cat} for the pair of substrates phenylalanine and 2-ketoglutarate (for AspAT the substrates were aspartate and 2-ketoglutarate).
[e] Value of k_{cat} for the pair of substrates aspartate and 2-ketoglutarate.

them are homodimers. The specific activities of known aminotransferases greatly vary, and for the aminotransferases studied by us this parameter is in the intermediate range.

Table 3 also shows that the isoelectric point of the aspartic aminotransferase of *E. carotovora* (p*I* 4.7) coincides with the literature data [5, 18], but the isoelectric points of the aromatic aminotransferases of *E. carotovora* are more acidic (p*I* 3.6 and 3.9).

The temperature optimum of aspartic aminotransferase of *E. carotovora* (58°C) exceeds the temperature optimum of aromatic aminotransferase of *E. carotovora* (49 and 52°C). This fact suggests higher thermostability of the aspartic aminotransferases, which corresponds with the literature [4]. The optimum pH of the studied aminotransferases in *E. carotovora* is around pH 8.0, which is similar to the known aminotransferases of subgroup Iα [5, 7, 8, 12, 14, 15].

The studies of substrate specificity of the aminotransferases in *E. carotovora* allowed their division into two groups. Enzymes PAT1 and PAT2 we referred to aromatic aminotransferase, and PAT3 to aspartate aminotransferases. The studied aromatic aminotransferases differ from the aspartate aminotransferase in substrate specificity (high rate of transamination of aromatic amino acids, lower rate of transamination of aspartic acid, considerable activity in reactions of transamination of L-leucine and L-isoleucine) and in physical and chemical characteristics (low p*I*, high electrophoretic mobility, low temperature optimums, wider pH optima). However, it is necessary to point out that the measured specific activities of PAT2 and PAT3 are underestimated because the purity of PAT2 is only 25-30% and PAT3 was assayed for substrate specificity at pH 7.2, which is lower than optimal by more than one unit. All three of the aminotransferases show high activity with cysteine and methionine. The rate of transamination of L-methionine and L-cysteine by the aminotransferases of *E. carotovora* substantially exceeds the known literature data [7], which will aid in developing biotransformation methods for obtaining these sulfur-containing amino acids.

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