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A New Acylamidase from *Rhodococcus erythropolis* TA37 Can Hydrolyze N-Substituted Amides

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Abstract—A new acylamidase was isolated from *Rhodococcus erythropolis* TA37 and characterized. N-Substituted acrylamides (isopropyl acrylamide, N,N-dimethyl-aminopropyl acrylamide, and methylene-*bis*-acrylamide), acid *para*-nitroanilides (4′-nitroacetanilide, Gly-pNA, Ala-pNA, Leu-pNA), and N-acetyl derivatives of glycine, alanine, and leucine are good substrates for this enzyme. Aliphatic amides (acetamide, acrylamide, isobutyramide, *n*-butyramide, and valeramide) are also used as substrates but with less efficiency. The enzyme subunit mass by SDS-PAGE is 55 kDa. Maximal activity is exhibited at pH 7-8 and 55°C. The enzyme is stable for 15 h at 22°C and for 0.5 h at 45°C. The Michaelis constant (K_m) is 0.25 mM with Gly-pNA and 0.55 mM with Ala-pNA. The acylamidase activity is suppressed by inhibitors of serine proteases (phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate) but is not suppressed by inhibitors of aliphatic amidases (acetaldehyde and nitrophenyl disulfides). The N-terminal amino acid sequence of the acylamidase is highly homologous to those of two putative amidases detected from sequenced *R. erythropolis* genomes. It is suggested that the acylamidase together with the detected homologs forms a new class within the amidase signature family.

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Amidases (EC 3.5.1.4) hydrolyze the amide bond between the carbon and nitrogen atoms in molecules of non-protein nature, giving acid and ammonia molecules:

$$R\text{-}CONH_2 \xrightarrow[H_2O]{\text{Amidase}} R\text{-}COOH + NH_3.$$

The modern classification based on homology of amino acid sequence and the presence of conservative motifs recognizes two groups of amidases. The first group forms the family of CN-hydrolases (No. PF00795 in PFAM Database [1]); they contain catalytically active residues of lysine, cysteine, and glutamic acid in the active site [2]. This group consists mainly of aliphatic amidases; their substrates are aliphatic short-chain amides—acetamide, propionamide, and acrylamide [3-5]. The second group forms the amidase family (PFAM No. PF01425); they have two serine residues and one lysine residue in the active site [6, 7]. The totally conservative GGSS motif containing one of the catalytically active serine residues is typical for these amidases. This motif along with approximately 130 other conserved amino acids is called "amidase signature" (AS) sequence, so amidases of latter protein family are called AS amidases [8]. Substrate specificity of AS amidases is wider than that of amidases of the former family. Along with aliphatic amides, AS amidases also hydrolyse aromatic and branched amides—phenylacetamide, 2-phenylpropionamide and its derivatives, mandelamide. These amidases are often stereoselective [7, 9, 10]. Although amidases of both groups hydrolyse the amide bond, they are not homologous in DNA sequences or in amino acid sequences. Amidases of both groups are very promising as catalysts in organic synthesis.

N-Substituted amides are widely used in production of polymers, drugs, nutrients, etc. [11]. However, enzymes capable of hydrolysing N-substituted amides have not yet been adequately investigated. Aliphatic and AS amidases catalyze only hydrolysis of hydroxamic acids

Abbreviations: Ala-pNA, alanine *para*-nitroanilide; DIFP, diisopropyl fluorophosphate; Gly-pNA, glycine *para*-nitroanilide; Leu-pNA, leucine *para*-nitroanilide; NAA, 4′-nitroacetanilide; PMSF, phenylmethylsulfonyl fluoride.

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and cannot hydrolyse other N-substituted amides [5]. Anandamide hydrolases [6, 12], which hydrolyse arachidonoyl ethanolamine and its analogs, are strictly specific to amides of fatty acids. Penicillin acylases [13] hydrolyze only aromatic amides with bulky substituents.

In this work we present the results of isolation and characterization of a new acylamidase from *Rhodococcus erythropolis* TA37 catalyzing hydrolysis of the amide bond in a wide range of N-substituted amides.

MATERIALS AND METHODS

Cultivation of *Rhodococcus erythropolis* TA37. Bacterial culture was grown on mineral medium M3 of the following composition (g/liter): $Na_2HPO_4 \cdot 12H_2O$, 7.0; KH_2PO_4, 3.0; sodium citrate, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.004; pH 7.0-7.2; vitamin mixture, 0.5 ml; acetanilide, 2; NH_4NO_3, 2. Shaker flasks with 100 ml of medium were incubated for 60 h at 30°C.

Estimation of cell acylamidase activity. Cells were washed twice with 0.1 M phosphate buffer, pH 7.5, and resuspended in the same buffer. For activity estimation, 100 µl of cell suspension was mixed with 900 µl of 1 mM 4′-nitroacetanilide (*p*-nitroanilide of acetic acid) and incubated for 20 min at 37°C. The reaction was terminated by heating for 1 min at 100°C. Cells were precipitated by centrifugation, and then *p*-nitroaniline concentration was determined spectrophotometrically at 410 nm (1 cm cuvette, $\varepsilon_{410} = 8900$ liter/mol per cm). The amount of the amidase forming 1 µmol of *p*-nitroaniline during 1 min at 37°C was taken as the activity unit.

Isolation and purification of the acylamidase. To isolate the acylamidase, cells were disintegrated using a Thermo Spectronic extrusion disintegrator at pressure 37 t.p.s.i. The broken cell suspension was centrifuged for 30 min at 20,000*g*; the supernatant was purified by ion-exchange chromatography using a high-speed protein chromatograph from Pharmacia (Sweden). The solution was applied on a column with MonoQ HiTrap equilibrated with 30 mM Tris-HCl, pH 7.5. The enzyme was eluted with a NaCl gradient (0-1 M) at flow rate 0.4 ml/min. Fractions providing active hydrolysis of 4′-nitroacetanilide were pooled and analyzed by SDS-PAGE in 10% polyacrylamide gel [14].

Estimation of activity of the purified acylamidase against 4′-nitroacetanilide (NAA). To measure the enzyme activity, 100 µl of acylamidase solution was mixed with 900 µl of 1 mM NAA solution in 10 mM Tris-HCl, pH 7.5. The mixture was incubated for 20 min at 37°C, the reaction was terminated by heating for 1 min at 100°C, and *p*-nitroaniline concentration was measured as described above.

Study of substrate specificity of the acylamidase. Ability to hydrolyze *p*-nitroanilides of amino acids (Gly-pNA, Ala-pNA, Leu-pNA) was estimated via formation of *p*-nitroaniline; its amount was measured spectrophotometrically analogously to acylamidase activity measurements described above.

Ability of the enzyme to hydrolyze N-acetyl amino acids (N-acetyl glycine, N-acetyl alanine, N-acetyl leucine) was estimated via formation of the amino acid; its amount was measured spectrophotometrically via optical absorption of its colored complex with ninhydrin at 500 nm.

Ability of the enzyme to hydrolyze N-substituted acrylamides (isopropyl acrylamide, N,N-dimethyl-aminopropyl acrylamide, methylene-*bis*-acrylamide) was estimated via decrease in N-substituted amide; its amount was measured spectrophotometrically at 235 nm.

Ability of the enzyme to hydrolyze aliphatic amides was estimated via formation of ammonia; its amount was measured via optical absorption of its colored complex with Nessler reagent.

For all the substrates, the procedure for activity estimation included incubation of a definite amount of the amidase with the substrate (20-40 mM) for 20 min at pH 7.5 and 37°C. The amount of enzyme hydrolyzing 1 µmol substrate during 1 min at 37°C was taken as the activity unit.

Determination of amino acid sequence. For sequencing of the acylamidase, the enzyme was additionally purified after chromatography by SDS-PAGE (10% polyacrylamide gel) and the protein band was applied on PVDF membrane. Protein eluted from the membrane was sequenced (the N-end) via Edman degradation. To determine internal sequences of the acylamidase, the protein was trypsinolyzed. Two peptides were isolated by HPLC, and their N-terminated sequences were also determined.

Reagents. In this work the following reagents were used: $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, sodium citrate, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, acetanilide, acetamide, NH_4NO_3 (all of chemically pure grade) from Reakhim (Russia); 40% glucose in ampules from Semashko Moskhimfarmpreparaty (Russia); 4′-nitroacetanilide, isopropyl acrylamide, N,N-dimethyl-aminopropyl acrylamide, and methylene-*bis*-acrylamide from Aldrich (USA); butyramide from Acros (Belgium). The *para*-nitroanilides of amino acids (Gly-pNA, Ala-pNa, Leu-pNA) and N-acetylamino acids (N-acetyl glycine, N-acetyl alanine, N-acetyl leucine) were kindly provided by Dr. T. L. Voyushina of the Institute for Genetics and Selection of Industrial Microorganisms.

RESULTS AND DISCUSSION

Isolation and purification of the acylamidase. The acylamidase was isolated from biomass of *R. erythropolis* TA37 grown on mineral medium with acetanilide, which induces acylamidase activity. Culture grown in this way

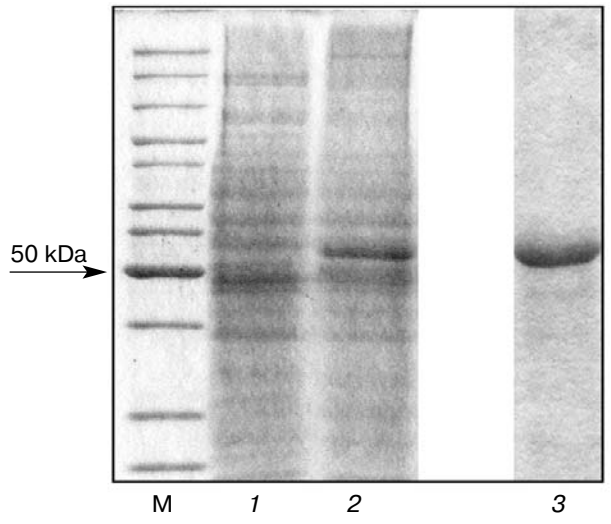


Fig. 1. Electrophoresis in 10% polyacrylamide gel in the presence of SDS demonstrating expression of the amidase and its purification during isolation. Lanes: M, protein markers; *1*) cell-free extract obtained from cells grown without amidase induction on minimal M3 medium containing glucose instead of acetanilide (see "Materials and Methods"); *2*) cell-free extract obtained from cells grown with amidase induction on minimal M3 medium containing acetanilide; *3*) the amidase chromatographically isolated from cell-free extract on HiTrap MonoQ.

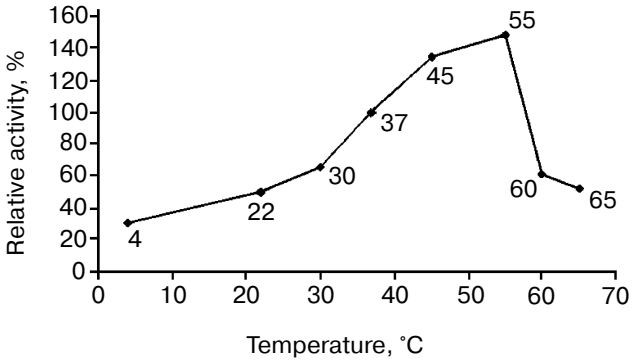


Fig. 2. Temperature dependence of the acylamidase activity. The activity was estimated via NAA hydrolysis according to the standard procedure, only the incubation temperature being varied. The activity value at 37°C (48.3 μmol/min per mg protein) was taken as 100%.

had hydrolytic activity against NAA; thus, the isolation of the acylamidase was monitored via hydrolysis of NAA. The induced acylamidase was detected by SDS-PAGE of cell-free extract as a noticeable band with mass ~55 kDa (Fig. 1, lane *2*). After chromatography of the cell-free extract on MonoQ in a NaCl concentration gradient, the acylamidase was chromatographically and electrophoretically pure (Fig. 1, lane *3*).

Effect of temperature on activity and stability of the acylamidase. The acylamidase activity was studied in the temperature range 4-65°C. The temperature dependence of the acylamidase activity is presented in Fig. 2. The half-life time of the enzyme at various temperatures (Table 1) indicates that its denaturation at temperatures higher than the optimal one (55°C) is very rapid. By the type of temperature dependence, the studied amidase is a typical mesophilic amidase [22-24, 26]. It is noteworthy that a drastic decrease in activity above the optimal temperature is typical for both mesophilic and thermophilic amidases (e.g. from *Sulfolobus solfataricus* [15]).

Effect of pH on activity and stability of the acylamidase. The acylamidase activity was studied in the range pH 5-11. The data are presented in Fig. 3. As shown, neutral pH values are optimal for the studied amidase. To evaluate stability of the enzyme, it was incubated at acidic and basic pH values, and after return to neutral pH the activity was measured again. It appeared that at low pH values the acylamidase is rapidly inactivated, whereas in basic medium inactivation is rather slow. This is evidenced by data on the half-life time of the acylamidase (Table 2).

The type of activity dependence on pH and the stability at various pH values are in accordance with each other and prove that the enzyme is most stable and functional at neutral pH.

Effect of reagents and metal ions on the acylamidase activity. Activity of the acylamidase in the presence of various reagents is presented in Table 3. The studied acylamidase is completely suppressed by inhibitors of serine proteinases – phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DIFP). This is in contrast with the aliphatic amidase from *Rhodococcus rhodochrous* M8 [3], toward which the same inhibitors have no effect. Partial inhibition of the acylamidase by mercury and copper ions is also different from the aliphatic amidase from *R. rhodochrous* M8, toward which these ions have no effect. However, the studied acylamidase is unaffected by sulfhydryl reagents (nitrophenyl disulfides, N-ethyl-

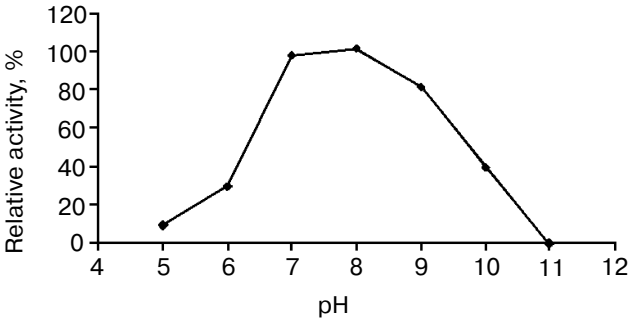


Fig. 3. pH dependence of the acylamidase activity. The activity was estimated via NAA hydrolysis according to the standard procedure, and only the pH of the reaction mixture was varied in the range 5-11. The activity value at pH 8.0 (48.3 μmol/min per mg protein) was taken as 100%.

Table 1. Thermal stability of the acylamidase*

Temperature, °C	Time, h
22	15
37	6
45	0.5
55	5 min

* Stability was determined by measuring the residual activity against NAA according to the standard procedure after incubation at the corresponding temperature. Measurements were dynamic. Half-life time is presented in the second column.

Table 2. pH stability of the acylamidase*

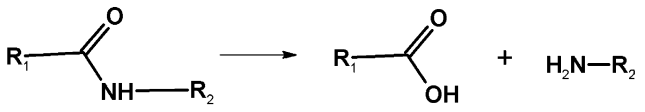
pH	Time
4.5	<5 min
6	14 h
8.5	15 h
10	30 min
11.5	20 min
13	20 min

* Stability was determined by measuring the residual activity against NAA according to the standard procedure after incubation at the corresponding pH value. Measurements were dynamic. The second column presents the half-life of the activity.

maleimide); this is different from the AS amidase from *Pseudomonas chlororaphis* B23 [16], which is suppressed by these reagents.

The effects of inhibitors on the studied amidase is unique and indicates its difference from both aliphatic and AS amidases.

Substrate specificity of the purified acylamidase was studied as its ability to hydrolyze certain N-substituted amides (Table 4). Structural formulae of the studied amides are given in the same orientation for convenience of presentation and discussion. The part of a molecule yielding acid on hydrolysis (Scheme) is depicted at the left, and that yielding amine at the right. So the "left" part is called the amide part and the "right" part is called the N-substituting part.



Scheme of hydrolysis of N-substituted amides

Para-nitroanilides of acids (acid amides substituted with *p*-nitroaniline on the nitrogen atom) are the best substrates for the acylamidase, the rates of hydrolysis of *p*-nitroanilides of acetic acid and glycine being maximal (Table 4). Increased size and hydrophobicity of the amide part of the nitroanilide significantly decreased the amidase activity and its affinity to the substrate.

The rate of hydrolysis of N-acetylated amino acids and N-substituted acrylamides was lower. The preferred substrate among N-acetyl amino acids was N-acetyl alanine, which is intermediate in the size of N-substituted residue between N-acetyl glycine and N-acetyl leucine. Among the tested N-substituted acrylamides, N-isopropylacrylamide appeared to be the preferred substrate. On the whole, the acylamidase hydrolyzes N-substituted amides with higher rate than unsubstituted amides (Table 4). Butyramide is the best substrate among the unsubstituted amides.

Table 3. Effect of reagents on the acylamidase activity

Reagent	Concentration in mixture, mM	Residual activity, %*
Acetaldehyde	2	100
Methylpropylboronic acid	1	24
Bis-2-nitrophenyl disulfide	1	100
Bis-4-nitrophenyl disulfide	1	100
N-Ethylmaleimide	1	100
Iodoacetamide	1	40
EDTA	0.5	100
Dithiothreitol	1	100
β-Mercaptoethanol	0.5	100
$CuSO_4$	10	20
$HgCl_2$	10	65
PMSF	0.5	0
DIFP	$4 \cdot 10^{-6}$	0

* Effect of inhibitors and metal ions on activity was determined via residual hydrolytic activity against NAA. The enzyme solution was preincubated in the presence of the reagent for 10 min at 22°C. Activity without inhibitors according to the standard procedure (48.3 μmol/min per mg protein) (see "Materials and Methods") was taken as 100%. The reaction mixture was preincubated with PMSF and DIFP for 1 h at 22°C. To measure activity, an aliquot of preincubated solution was diluted so as to retain the same concentration of the tested reagent in the reaction mixture.

Table 4. Substrate specificity of the acylamidase

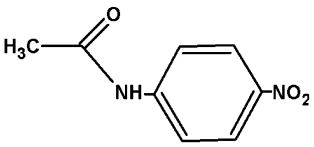
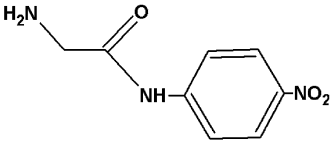
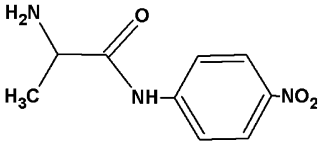
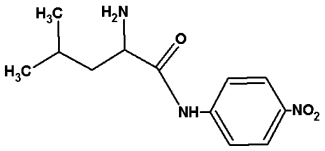
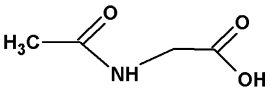
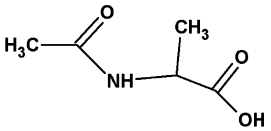
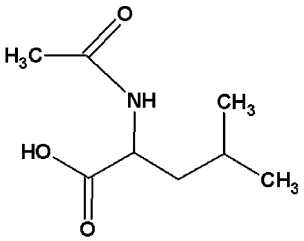
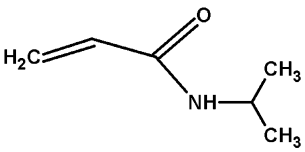
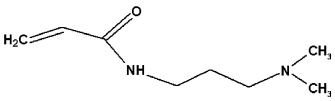
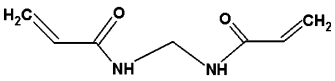
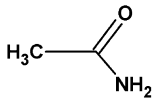
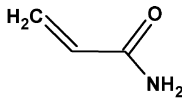
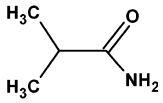
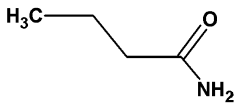
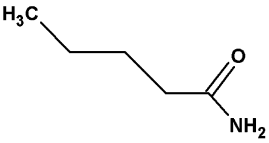
Substrate	Structural formula	Activity, μmol/min per mg protein*	Substrate concentration in reaction medium, mM	K_m, mM
1	2	3	4	5
NAA		72.5	0.3	–
Gly-pNA		104.5	4.4	0.25
Ala-pNA		35.5	1.8	0.55
Leu-pNA		17.0	0.6	0.48
N-Acetyl glycine		0.49	45.5	–
N-Acetyl alanine		1.13	40.7	–
N-Acetyl leucine		0.10	30.8	–
Isopropylacrylamide		3.70	2.0	–
N,N-Dimethyl-aminopropyl acrylamide		1.67	2.0	–
Methylene-*bis*-acrylamide		0.65	2.0	–

Table 4. (Contd.)

1	2	3	4	5
Acetamide		0.26	8.0	–
Acrylamide		0.35	7.0	–
iso-Butyramide		0.66	6.0	–
n-Butyramide		0.91	6.0	–
Valeramide		0.29	5.0	–

* Activity units are matched for 1 μmol substrate hydrolyzed for 1 min at 37°C. Hydrolysis of nitroanilides was determined via appearance of *p*-nitroaniline, N-acetyl amino acids via staining of appearance of free amino acids with ninhydrin, N-substituted acrylamides via change in optical absorption at 235 nm, and unsubstituted amides via appearance of ammonia (see "Materials and Methods").

Data on the ability of amidases to hydrolyze N-substituted amides are few. An aliphatic amidase from *Pseudomonas* sp. MCI3434 [17] hydrolyzing piperazine-2-carboxamide with rate 4.6 μmol/min per mg protein hydrolyzes piperazine-2-*tert*-butylcarboxamide (N-substituted derivative of the above mentioned amide) with rate one order of magnitude lower. AS amidases are more studied in this relation. An amidase from *Arthrobacter* sp. RC100 [18] is able to hydrolyze N-methyl-carbamate with rate 0.4 μmol/min per mg protein, whereas its activity against unsubstituted isobutyramide is three times higher. A mandelamide hydrolase from *Pseudomonas putida* ATCC 12633 [19] is unable to hydrolyze N-ethyl-substituted mandelamide and nitroanilides of mandelamide, and an amidase from *Acinetobacter* sp. 6 [20] hydrolyzes nitroacetanilide with rate $4 \cdot 10^{-3}$ μmol/min per mg protein. An amidase from *Pseudomonas chlororaphis* B23 [16] does not exhibit activity against N-methylacetamide and N-methylbenzamide.

For well-studied AS amidases (malonamidase from *Bradyrhizobium japonicum* [21], amidases from *Sulfolobus solfataricus* [15], *R. rhodochrous* J1 [22], *R. erythropolis* R312 [23], *R. erythropolis* MP50 [24]) hydrolytic activity against N-substituted amides has not been reported.

The spectrum of substrate specificity of the studied amidase indicates that it is principally different from aliphatic amidases. In contrast to them, the acylamidase

Table 5. Degree of identity of putative amidase from *R. erythropolis* SK121 with the nearest homologs*

Microorganism	Degree of identity with amidase from *R. erythropolis* SK121, %	Remarks
R. erythropolis PR4	98	presumed AS amidase
Rhodococcus sp. BH2-N1	75	–"–
Mycobacterium abscessus	48	–"–
Rhodothermus marinus DSM 4252	42	–"–
R. erythropolis MP50	37	[24]
R. erythropolis R312	31	[23]
R. erythropolis AJ270	31	[26]
R. rhodochrous J1	29	[22]

* Amidase sequence from *R. erythropolis* SK121 was taken from the GenBank Database for comparison.

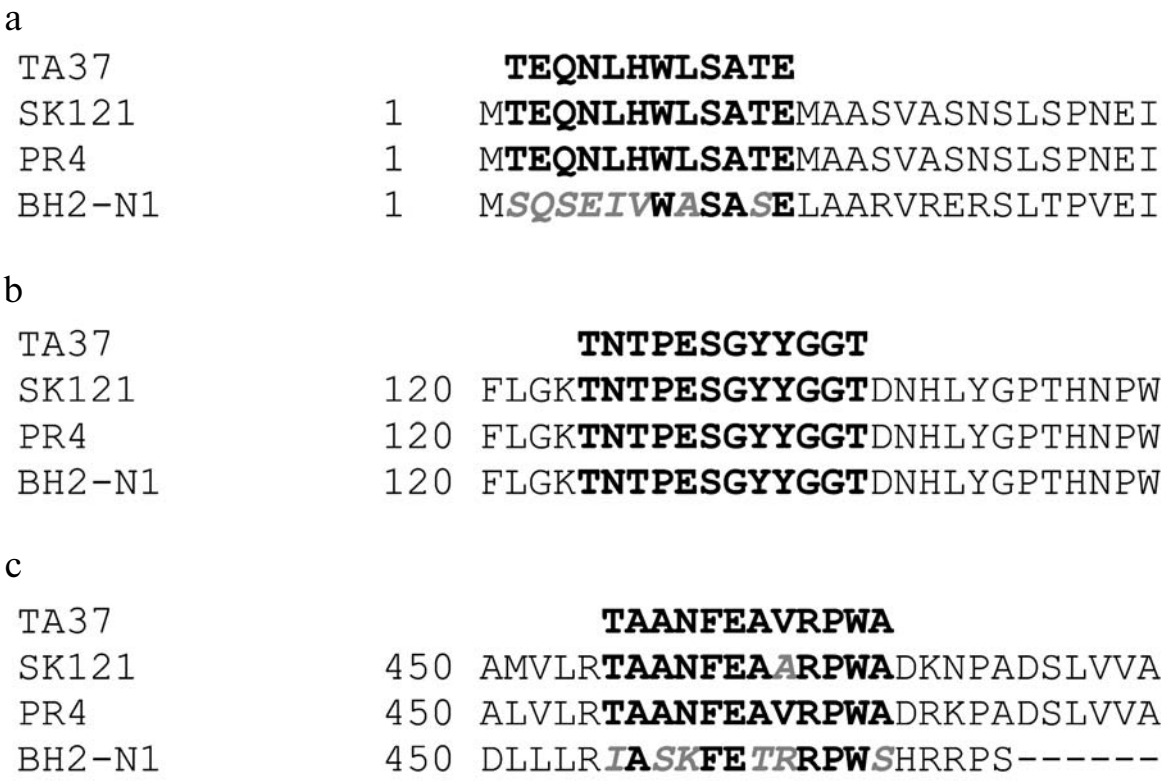


Fig. 4. Alignment of sequences of the acylamidase from *R. erythropolis* TA37 with three putative amidases from *R. erythropolis* SK121, PR4, and BH2-N1 strains. a-c) Aligned fragments of N-ends of the amidases (a), internal peptide II (b), and internal peptide III (c). Differences in amidases from BH2-N1 and PR4 are marked by gray italic (for explanation, see text).

is able to hydrolyze N-substituted amides with higher rate than unsubstituted amides. As for hydrolysis of unsubstituted amides, the acylamidase is close to AS amidases: they also hydrolyze middle-length amides more efficiently than short-chain ones [5, 15]. However, ability to efficiently hydrolyze N-substituted amides distinguishes the acylamidase form *R. erythropolis* TA37 from the known AS amidases.

Determination of partial amino acid sequence. For N-terminal sequencing via Edman degradation, we used protein solution obtained after chromatography; for determination of internal sequences the protein was trypsinolyzed and the peptides purified by HPLC were used for N-terminal sequencing. Amino acid sequences of three peptides were determined: I – TEQNLHWLSATE (N-end of amidase); II – TNTPESGYYGGT (internal peptide); III – TAANFEAVRPWA (internal peptide).

Using the EMBL GenBank Database and BLASTP package [25], we found that the acylamidase fragments are homologous to the products of three genes with unknown functions from *R. erythropolis* SK121, PR4, and BH2-N1 strains (Fig. 4). The acylamidase homologs have the conservative GGSS motif and contain a catalytic triad Ser-Ser-Lys typical for the AS amidase family (PFAM No. 01425). So the gene products from SK121, PR4, and BH2-N1 can be assigned to the AS amidase family. Based on the found homology, we suggest that the studied acylamidase also belongs to the same family.

In this work we have described a new acylamidase from *R. erythropolis* TA37 that has unique substrate specificity. N-Substituted amides, which are bad substrates for aliphatic amidases as well as for AS amidases, are the preferred substrates for this acylamidase. Based on homology and substrate specificity, the studied acylamidase is assigned to the AS amidase family. About 15 enzymes from the AS amidase family are now characterized. It should be noted that about 700 annotated protein sequences from UniProtKB [28] and PFAM Database [1] are assigned to this family; however, they are not yet studied. The amidase studied here together with the identified homologs belong to a group of closely related proteins (Table 5) that form a new class inside the AS amidase family.

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