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Research Article

IN SILICO ANALYSIS OF SOME MICROBIAL AMIDASES FOR THEIR AMINO ACID AND PHYSIOCHEMICAL PARAMETERS

Krishan Gopal[1], Nikhil Sharma[1] and TC Bhalla[1,2]*
[1]Sub-Distributed Information Centre, Himachal Pradesh University, Summer- Hill Shimla, India -171005
[2]Department of Biotechnology, Himachal Pradesh University, Summer- Hill Shimla, India -171005

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Abstract: Amino acid sequences of amidases were retrieved from respective databases and *in silico* analysis for different physiochemical properties and substrate specificity has been done. Multiple Sequence Alignment (MSA) and statistical analysis of amino acid sequences has revealed significant differences among aliphatic and signature amidases in terms of conserved motif that plays a vital role in substrate binding and catalytic function. MSA has revealed that in aliphatic amidases the conserved amino acid residues involved in catalytic function are position specific which remains within catalytic traid of Cys-166, Glu-59, Lys-134, while signature amidase contains signature motif GGSS (S/G) GS and catalytic traid of Ser-171, Ser-195, Lys-96 which are not position specific. Statistical analysis has revealed that these two groups also differ in physiochemical properties. In contrast to aliphatic amidases, signature amidase have significantly higher number of amino acid residues & molecular mass, theoretical pI, charged (negative and positive) residues, aliphatic index and grand average of hydropathicity. Present investigation revealed that the amino acid residues i.e. Cys, Met, Tyr, Asn, Ile, Trp, Glu and Gly in aliphatic amidases, while in case of signature amidases amino acids i.e Leu, Pro, Ser, Ala and Val have been found to play an important and significant role both in enzyme catalysis, substrate specificity as well as in structural stability.

Key words: Amidase; Aliphatic amidase; Signature amidase; Amino acid; Substrate specificity; Multiple alignments.

INTRODUCTION

Amidases (EC 3.5.1.4) are ubiquitous enzymes belongs to the family of hydrolases (acylamideamidohydrolase subclass). Amidases are amide bond-cleaving enzymes thus plays important role in the hydrolysis of various endogenous as well as foreign aliphatic and aromatic amides. These enzymes catalyze the hydrolysis of carboxylic acid amides to free carboxylic acids and free ammonium by transferring an acyl group to water. [1,2,6] So they are responsible for reduction of nitrogen and production of ammonia. The reactions catalyzed by these complex enzymes, are of primary interest for large scale production of acrylamide, nicotinamides and acrylic acid in industry.[3] Many microbial amidases have been purified and characterized. From previously reported studies, it becomes obvious that they exhibit fairly wide substrate specificity and can be assigned to phylogenetically unrelated families on the basis of amino acid sequence[4]. Amidases are further divided into four groups or subfamilies based on their physiological properties and catalytic activity function which includes aliphatic amidases that are structurally related to the nitrilase-superfamily enzymes, aromatic amidases belonging to the signature family, acyl transferase and ureases.[5]

The first type among aliphatic amidases includes which hydrolyze short-chain aliphatic amides[6] the second one includes aliphatic amidases that hydrolyze mid-chain amides and arylamides[1] and which are coupled with the nitrilase/cyanide hydratases involved in nitrile metabolism. The bacterial amidases from the nitrilase/cyanide hydratase family are supposedly sulfhydryl enzymes.[7,4] These enzyme have a conserved catalytic triad *i.e.* Glu, Lys, Cys responsible for catalysis mechanism,[8,4,9] where Cys166 act as a nucleophile in the catalytic function.[7] These enzyme have wide spectrum activity and do not contain the central conserved GGSS signature that is common to all other amidase enzymes. These enzymes are typically homohexamers of approximately 230 kDa.[6]

In contrast, signature amidases are characterized by the presence of invariant conserved GGSS(S/G)GS signature motif in their primary sequence[7] and contains catalytic triad *i.e.* Ser, Ser, Lys.[10] This family may also contain asparagines and serine in their catalytic site.[11] Signature amidases has a highly conserved linear sequence of approximately fifty amino acid long rich in serine and glycine residues.[5] Signature amidases usually exhibit wide substrate specificity[12,13] and are active towards aliphatic as well as aromatic amides. They also shows stereospecificity towards amides of α_substituted carboxylic acids.[13,14] They are typically homodimers having a native molecular weight of approximately 110 kDa.[15] Unlike aliphatic amidases, which form either homotetrameric or homohexameric[16] complexes in solution, most of the characterized signature amidases exist as homodimeric or homooctameric[5] in their active form. Microbial amidases with altered substrate specificity have attracted growing interest in the last decade as they have great potential to detoxify the industrial effluents containing toxic amides such as acrylamide and formamide etc.[17] Amidases can be used for the development of a biosensor to investigate the acrylamide contamination in food samples such as potato chips and breakfast cereals.[18]

***Corresponding Author:**
Prof. TC Bhalla,
Sub-Distributed Information Centre,
Himachal Pradesh University,
Summer- Hill Shimla--171005, India

Additionally, immobilized amidase can be used efficiently for production of acrylic acid from acrylamide, by converting a toxic ambient and hazardous contaminant into widely used industrial raw material. Amidases also find application as biocatalysts in organic synthesis, and also in the manufacturing of therapeutic agents.[19]

These first two subfamilies are substrate specific for their catalytic activity and still are not sufficiently investigated. Therefore the detailed comparison of these two amidases is of great interest: it is necessary to understand the differences in physiochemical properties that contribute for substrate specificity, amino acids involved in catalytic function and their evolutionary relationship. Comparison of physiological properties and identification of the active site will certainly help in elucidating its unique specificity for each amide substrate as well as its reaction mechanism which subsequently help us to understand its physiological functions. In present investigation a number of potential amino acid sequences from related organisms has been retrieved from respective databases and *in silico* analysis of physiochemical properties and MSA of aliphatic and signature amidases has been done from the retrieved amino acid sequences indicating that certain conserved amino acids at certain positions besides the catalytic triad are also responsible for substrate specificity of amidases.

MATERIALS AND METHODS

Data collection and tools:

Specificity of amidases towards aliphatic and aromatic substrates was investigated using BRENDA, (http://www.brenda-enzymes.org/) an enzyme information system contains all the information regarding biological reaction catalyzed by a particular protein. Amino acid sequences of twenty six microorganisms which were manually curated or reviewed with experimentally proved substrate specificity were retrieved from SwissProt available at ExPASy proteomics server (http:/www.expasy.org/sprot/) in FASTA format for analysis (table 1). A search for homology among various aliphatic and signature amidases belonging to same E.C. number (E.C.3.5.1.4) was carried out by performing BLAST similarity search tool at NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ProtParam tool (http://expasy.org/tools/protparam.html) available on ExPASy proteomic server, was used for atomic composition and comparison of various physiochemical parameters among aliphatic and signature amidases. Clustal W was used for multiple sequence alignment. Protein calculator, Compute pI/Mw and ProtScale[20] were also used to calculate/deduce different physiochemical properties of aliphatic and signature amidases from the protein sequences.

The molecular weights (Kda) of the various aliphatic and signature amidases were estimated by the addition of average isotopic masses of amino acid in the protein and deducting the average isotopic mass of one water molecule. The pI of aliphatic and signature amidases was calculated using pK values of amino acid according to Bjellqvist *et al.*,[21] The extinction coefficient of aliphatic and signature amidases were calculated by using the following equation.[22]

E (Prot) = Numb (Tyr) *Ext (Tyr) + Numb (Trp) *Ext (Trp) + Numb (Cystine) *Ext (Cystine)

ProtParam (ExPASy) tool[20] is used to calculate the values of aliphatic index of various aliphatic and signature amidases. The instability index and grand average of hydropathicity (GRAVY) were estimated following earlier published method [23,20] respectively.

Table.1: Substrate specificity of selected amidases with their SwissProt accession numbers.

Microorganisms	Substrate Specificity	Accession No. (ExPASy)
Alcanivorax borkumensis SK2	Aliphatic	Q0VN20
Delftiaacidovorans DSM 14801	Aliphatic	A9C011
Bacillus stearothermophilus BR388	Aliphatic	Q9RQ17
Bradyrhizobium japonicum USDA 110	Aliphatic	Q89VS2
Helicobacter pylori	Aliphatic	O25067
Marinobacteraquaeolei (ATCC 700491 / DSM 11845 / VT8)	Aliphatic	A1U7G1
Burkholderiacenocepacia AU 1054	Aliphatic	Q1BP24
Nocardia farcinica	Aliphatic	Q5Z1U0
Pseudomonas aeruginosa PAC142	Aliphatic	P11436
Pseudomonas putida F1	Aliphatic	A5W2C0
Rhodococcus erythropolis	Aliphatic	Q01360
Thioalkalivibrio sp. HL-EbGR7	Aliphatic	B8GQ39
Variovoraxparadoxus S110	Aliphatic	C5CWZ4
Aspergillusoryzae	Signature	Q12559
Aspergillusnidulans	Signature	P08158
Pseudomonas chlororaphis B23 (S-Es)	Signature	P27765
Pseudomonas putida	Signature	O69768
Rhodococcus rhodochrous	Signature	P84650
*Rhodococcus erythropolis N-774**	Signature	P22984
Sulfolobus solfataricus DSM 5833 (S-Es)	Signature	P95896
Saccharomyces cerevisiae	Signature	P22580
Mycobacterium tuberculosis	Signature	P63492
Schizosaccharomycespombe	Signature	O59805
Synechocystis sp (strain PCC 6803)	Signature	Q55424
Mycobacterium bovis	Signature	P63495
Archaeoglobusfulgidus	Signature	O28325

*Reference sequence for signature amidase

Statistical analysis:

P-value was calculated for both physiochemical parameters (number of amino acids, molecular weight, instability index, aliphatic index *etc*) as well as twenty different amino acids by using ProtParam tool. An analysis of variance (ANOVA) was used to calculate different physiochemical parameters for each study with the statistical packages '*Assistat version-7.6 beta 2012*'. *F*-tests were applied to determine the statistical significance. Tukey test was applied for all significant effects over the pairwise comparison of mean responses. Sequence alignment of amino acids

between aliphatic and signature amidases was performed by using Clustal W program.

RESULTS

The present investigation reports the comparison of various physiochemical parameter as well as substrate specificity among two different classes of amidases on the basis of their amino acid analysis. Thirteen amino acid sequences from each group were selected and significant difference have been recorded. The result of analysis of physiochemical properties and amino acids content have been tabulated in table 2a &2b.The total number of amino acid residues in these amidases differed substantially as the number of amino acids were found to be less in aliphatic amidases (339-348 amino acids) in comparison to signature amidases (466-548 amino acids). On other hand molecular weight in case of signature amidases (49080.9-61410.1) were found to be significantly higher as compared to that of aliphatic amidases (37712.9-39085.4) i.e. 1.4 folds higher than aliphatic amidases. It was further analyzed that theoretical pI of aliphatic amidases ranges between 4.94-6.20 than that of signature amidases ranges in between 5.25-6.68. The average content of negatively as well as positively charged species were significantly higher and is found to be more in signature amidases i.e. 1.3 and 1.4 fold high respectively. Non-significant difference have been recorded for extinction coefficient and instability index as aliphatic amidases have lesser average values for extinction coefficient and instability index than that of signature amidases. It was further observed that the values of aliphatic index for aliphatic amidases varied in between 71.76-77.32 while it was found to be 85.68-96.55 for signature amidases and highly significative difference have been recorded. The average value of aliphatic index for signature amidases were found to be higher i.e.1.2 fold higher than aliphatic amidases. The values of grand average of hydropathicity lies in between -0.446 to -0.279 in case of aliphatic amidases while for signature amidases it lies in between -0.089 to + 0.048 and was found 3.5 fold higher than aliphatic amidases as the values of GRAVY were more negative in aliphatic.

Table.2a: Comparative analysis of physiochemical parameters of aliphatic and signature amidases

Parameters	Substrate specificity	Microorganisms													P-Value
		1	2	3	4	5	6	7	8	9	10	11	12	13	
No. of amino acids	**Aliphatic**	348.0	345.0	348.0	346.0	339.0	348.0	341.0	345.0	346.0	347.0	345.0	347.0	344.0	**244.72****
	Signature	545.0	548.0	505.0	466.0	517.0	520.0	504.0	549.0	462.0	533.0	506.0	473.0	453.0	
Molecular weight	**Aliphatic**	38359.4	38564.7	39085.4	38441.7	37712.9	38460.5	38161.3	38183.0	38494.7	38784.0	38200.1	38825.8	38195.4	**201.03****
	Signature	60098.4	60228.5	54047.2	50162.1	54735.2	54574.4	55655.3	61410.1	49080.9	58704.5	54254.1	50916.1	50062.7	
Theoretical pI	**Aliphatic**	4.96	5.69	5.55	5.65	6.20	5.06	5.72	4.98	5.30	5.38	4.94	5.36	5.44	**4.36***
	Signature	6.23	5.84	5.54	6.29	5.30	5.35	5.94	5.25	6.68	5.82	5.47	6.62	4.89	
Negatively charged species (Asp+Glu)	**Aliphatic**	47.0	44.0	46.0	43.0	41.0	47.0	43.0	48.0	45.0	46.0	48.0	47.0	44.0	**30.117****
	Signature	66.0	68.0	52.0	53.0	55.0	58.0	64.0	67.0	48.0	63.0	45.0	50.0	68.0	
Positively charged species (Arg+Lys)	**Aliphatic**	31.0	35.0	36.0	35.0	38.0	33.0	35.0	31.0	35.0	36.0	32.0	36.0	34.0	**35.823****
	Signature	60.0	58.0	37.0	49.0	38.0	41.0	59.0	54.0	47.0	56.0	37.0	48.0	51.0	
Extinction coefficients (M-1cm-1) at 280 nm	**Aliphatic**	57465.0	54945.0	57340.0	64790.0	58955.0	57465.0	64330.0	60320.0	58830.0	60320.0	60320.0	61935.0	53330.0	**0.368 ns**
	Signature	86665.0	85050.0	60780.0	45755.0	50810.0	40630.0	63050.0	77070.0	46660.0	83685.0	61350.0	47900.0	57090.0	
Instability index	**Aliphatic**	32.92	38.09	34.81	38.05	41.47	28.78	39.82	31.57	36.71	36.08	30.15	37.58	46.73	**0.052 ns**
	Signature	34.66	39.18	30.44	41.05	34.26	32.76	29.60	35.79	34.99	42.31	44.36	40.31	38.57	
Aliphatic index	**Aliphatic**	68.16	69.54	72.87	73.65	77.35	68.13	71.76	76.90	74.74	77.32	74.96	69.14	70.93	**180.44****
	Signature	88.81	89.36	85.68	87.94	87.97	85.81	90.14	90.0	93.46	94.22	89.86	96.55	91.70	
Grand average of hydropathicity (GRAVY)	**Aliphatic**	-0.333	-0.397	-0.404	-0.348	-0.347	-0.360	-0.371	-0.279	-0.332	-0.346	-0.319	-0.446	-0.324	-----
	Signature	-0.240	-0.237	-0.118	-0.162	0.048	-0.037	-0.197	-0.201	0.001	-0.152	0.027	0.018	-0.089	

Aliphatic Amidases: (1) *Alcanivoraxborkumensis* SK2 **(2)** *Delftiaacidovorans* DSM 14801 **(3)** *Bacillus stearothermophilus* BR388 **(4)** *Bradyrhizobiumjaponicum* USDA 110 **(5)** *Helicobacter pylori* **(6)** *Marinobacteraquaeolei* (ATCC 700491 / DSM 11845 / VT8) **(7)** *Burkholderiacenocepacia* AU 1054 **(8)** *Nocardia farcinica* **(9)** *Pseudomonas aeruginosa* PAC142 **(10)** *Pseudomonas putida* F1 **(11)** *Rhodococcus erythropolis* **(12)** *Thioalkalivibrio sp.* HL-EbGR7 **(13)** *Variovoraxparadoxus* S110.

Signature Amidases: (1) *Aspergillusoryzae* **(2)** *Aspergillusnidulans* **(3)** *Pseudomonas chlororaphis* B23. (S-Es) **(4)** *Pseudomonas putida* **(5)** *Rhodococcus rhodochrous* **(6)** *Rhodococcus erythropolis* N-774 **(7)** *Sulfolobussolfataricus.DSM* 5833 (S-Es) **(8)** *Saccharomyces cerevisiae* **(9)** *Mycobacterium tuberculosis* **(10)** *Schizosaccharomycespombe* **(11)** *Synechocystis sp.* (strain PCC 6803) **(12)** *Mycobacterium bovis* **(13)** *Archaeoglobusfulgidus.*

** Significative at a level of 1% of probability ($p < 0.01$) ;*Significative at a level of 5% of probability ($.01 =< p < 0.05$);**ns** Non-significative ($p >= 0.05$)

Results of amino acid analysis of two types of amidases are shown in table 2b. The comparison of individual amino acid composition has revealed that in case of aliphatic amidases, percentage composition of Cys, Glu and Tyr (2.2, 1.3 and1.6) were high and found to be highly more significative. In contrast, the content of Leu, Pro, Ser were found to be highly more significative in signature amidases *i.e.* 2.0, 1.4 and 1.4 fold higher than aliphatic amidases. It was further observed that the content of Asn, Met, Trp, Gly, Ile for aliphatic amidases were highly significative while in case of signature amidase the content of Val, Ala were found to be significantly more. The content of Gln, His, Lys were non significantly higher in aliphatic amidases and the content of Thr, Asp, Phe, Arg were also found to be non-significantly higher in signature amidases.

Multiple sequence alignment with the Clustal W program of all the sequences obtained from the database search has been done to detect the identity score and the conserved amino acids subsequences regions between aliphatic and signature amidases (Fig 1). In nitrilase related aliphatic amidase, amino acid residues involved in catalytic function remains within a catalytic traid of Cys-166, Glu-59, Lys-134, and found to be position specific conserved in all diverged sequence taken. Beside this catalytic traid other conserved amino acid residues were Gly-51, Trp-60, Gly-64, Ile- 65, Met-66, Try-67, Gly-126, Glu-127, Ile-128, Gln-130, Tyr-132, Arg-133, Trp-138, Glu-142, Trp-144, Tyr-145, Gly-158, Ile-164, Gly-169, Asn-170, Tyr-171, Glu-173, Ile-174, Trp-175, Arg176, Gly-191, Tyr-192, Met-193. While signature amidase contains central conserved motif GGSS and catalytic active residues were Lys-96, Ser-171, Ser-195, along with several other distinct amino acids, such as, Gly-189, Asp-191, Gly-193, Gly-194, Arg-197, Pro-199 were also found to be conserved. In signature amidases conserved motif as well as catalytic function residues were not position specific except in selected reference sequence. Result obtained from the present study revealed that the amino acid residues i.e. Cys, Glu, Tyr, Trp, Gly, Asn, and Met in aliphatic amidases, while in case of signature amidases amino acids *i.e.* Ser, Asp, Arg, Leu, and Pro also accounts for the substrate specificity.

Table2b: Amino acid comparison of aliphatic and signature amidases

Amino Acids	Substrate specificity	Microorganisms													P-Value
		1	2	3	4	5	6	7	8	9	10	11	12	13	
Ala (A)	Aliphatic	9.5	7.2	6.9	8.7	7.4	8.9	7.3	9.3	9.2	8.9	9.6	6.9	8.4	5.58 *
	Signature	10.6	9.9	12.3	11.4	12.4	13.3	6.0	4.0	15.6	8.8	10.7	13.5	8.2	
Arg (R)	Aliphatic	4.0	5.2	4.0	4.3	3.5	4.0	6.7	5.2	6.1	5.8	4.6	4.9	5.2	1.44 ns
	Signature	5.1	4.2	4.8	7.3	5.0	6.0	4.2	3.8	9.3	3.9	3.8	9.1	4.0	
Asn (N)	Aliphatic	4.3	4.9	4.9	4.6	4.4	4.9	4.1	2.3	4.0	4.0	4.1	3.7	4.7	10.02 **
	Signature	3.7	4.2	3.2	1.1	2.5	2.7	3.8	5.3	1.3	3.8	3.0	2.5	2.4	
Asp (D)	Aliphatic	6.3	5.2	4.0	5.2	5.6	6.6	6.2	7.2	5.5	6.1	7.0	6.3	5.8	0.01 ns
	Signature	5.7	5.3	6.3	5.6	6.2	7.3	6.3	6.7	6.7	6.4	4.7	5.5	4.6	
Cys (C)	Aliphatic	2.9	2.9	2.3	2.3	3.2	2.9	2.3	2.6	2.6	2.6	2.3	2.9	2.6	79.51 **
	Signature	1.1	0.7	1.6	1.5	1.5	0.8	0.8	2.2	0.9	1.3	1.6	0.2	1.1	
Gln (Q)	Aliphatic	3.4	2.6	3.4	2.6	4.7	3.2	2.6	4.1	4.0	4.3	3.5	3.5	2.6	1.97 ns
	Signature	2.8	2.7	4.2	3.9	1.4	1.7	1.4	2.7	3.9	3.0	5.7	2.3	1.3	
Glu (E)	Aliphatic	7.2	7.5	9.2	7.2	6.5	6.9	6.5	6.7	7.5	7.2	7.0	7.2	7.0	9.46 **
	Signature	6.4	7.1	4.0	5.8	4.4	3.8	6.3	5.5	3.7	5.4	4.2	5.1	10.4	
Gly (G)	Aliphatic	9.8	9.9	9.5	9.5	9.4	9.8	10.3	9.6	9.8	9.2	9.6	9.5	10.5	12.10 **
	Signature	6.8	7.5	10.3	8.4	9.3	10.2	9.3	6.2	8.9	6.2	8.9	8.2	8.6	
His (H)	Aliphatic	2.3	3.2	2.9	2.6	2.1	2.3	2.9	2.9	2.0	2.3	2.3	2.6	2.6	2.76 ns
	Signature	2.8	2.6	3.4	2.4	2.9	3.1	1.4	1.5	1.5	1.7	1.4	2.3	0.4	
Ile (I)	Aliphatic	5.7	6.4	8.9	6.4	6.2	5.7	6.2	5.2	6.1	6.9	7.0	6.9	7.0	9.06 **
	Signature	5.9	6.2	4.2	3.4	4.3	4.6	6.5	7.5	3.9	5.1	4.5	2.7	7.1	
Leu (L)	Aliphatic	4.6	4.6	3.7	4.9	5.6	4.3	4.7	6.1	5.8	5.5	4.6	4.3	4.9	170.16**
	Signature	8.4	8.2	10.1	9.7	8.5	7.7	10.3	10.4	10.0	10.7	9.9	11.0	8.6	
Lys (K)	Aliphatic	4.9	4.9	6.3	5.8	7.7	5.5	3.5	3.8	4.0	4.6	4.9	5.5	4.7	0.23 ns
	Signature	5.9	6.4	2.6	3.2	2.3	1.9	7.5	6.0	0.9	6.6	3.6	1.1	7.3	
Met (M)	Aliphatic	5.5	4.6	4.0	4.3	2.1	4.9	4.7	3.2	4.3	4.0	3.8	3.7	5.2	32.81**
	Signature	1.7	1.8	3.2	2.1	2.7	1.9	4.4	2.4	1.7	2.4	2.0	1.7	2.0	
Phe (F)	Aliphatic	2.6	2.9	3.2	2.0	3.2	2.9	2.3	2.6	2.6	2.3	2.6	2.6	3.2	1.27 ns
	Signature	2.6	2.6	2.0	1.7	2.5	3.1	2.8	4.0	2.8	2.1	4.3	3.4	5.1	
Pro (P)	Aliphatic	4.6	5.2	4.6	5.2	5.0	4.3	5.3	4.6	4.6	4.6	4.3	4.9	4.7	65.71**
	Signature	5.9	5.7	6.1	6.7	7.2	6.5	6.5	5.6	7.1	7.1	8.7	6.6	7.5	
Ser (S)	Aliphatic	4.9	4.6	3.7	5.2	4.4	4.3	3.5	4.6	4.6	4.6	4.1	5.2	4.9	19.74**
	Signature	6.4	6.2	5.3	6.0	5.2	6.2	6.5	9.7	5.6	6.2	6.9	5.3	4.6	
Thr (T)	Aliphatic	4.9	4.6	6.3	4.0	3.8	5.5	6.2	4.9	3.8	3.2	5.2	5.8	4.1	3.89 ns
	Signature	5.0	5.3	5.1	7.5	8.3	7.5	4.4	6.0	4.8	5.6	4.4	5.9	4.2	
Trp (W)	Aliphatic	1.7	1.4	1.7	1.7	1.8	1.7	2.1	1.7	1.7	1.7	1.7	1.7	1.5	12.57 **
	Signature	1.8	1.8	1.0	1.1	0.8	1.0	1.2	1.6	1.3	1.9	1.1	1.3	1.3	
Tyr (Y)	Aliphatic	4.6	5.2	4.6	6.1	5.0	4.6	5.0	5.2	4.9	5.2	5.2	5.5	4.9	57.12 **
	Signature	3.9	3.6	4.4	2.6	3.7	2.3	4.0	3.3	1.9	3.6	3.0	2.1	3.5	
Val (V)	Aliphatic	6.3	6.7	5.7	7.2	8.3	6.9	7.6	8.1	6.6	6.9	7.0	6.3	5.5	5.86 *
	Signature	7.7	8.0	6.1	8.8	8.9	8.5	6.3	5.6	8.2	8.3	7.9	10.1	7.7	

** Significative at a level of 1% of probability ($p < 0.01$); * Significative at a level of 5% of probability ($.01 =< p < 0.05$); **ns** Non-significative ($p >= 0.05$)

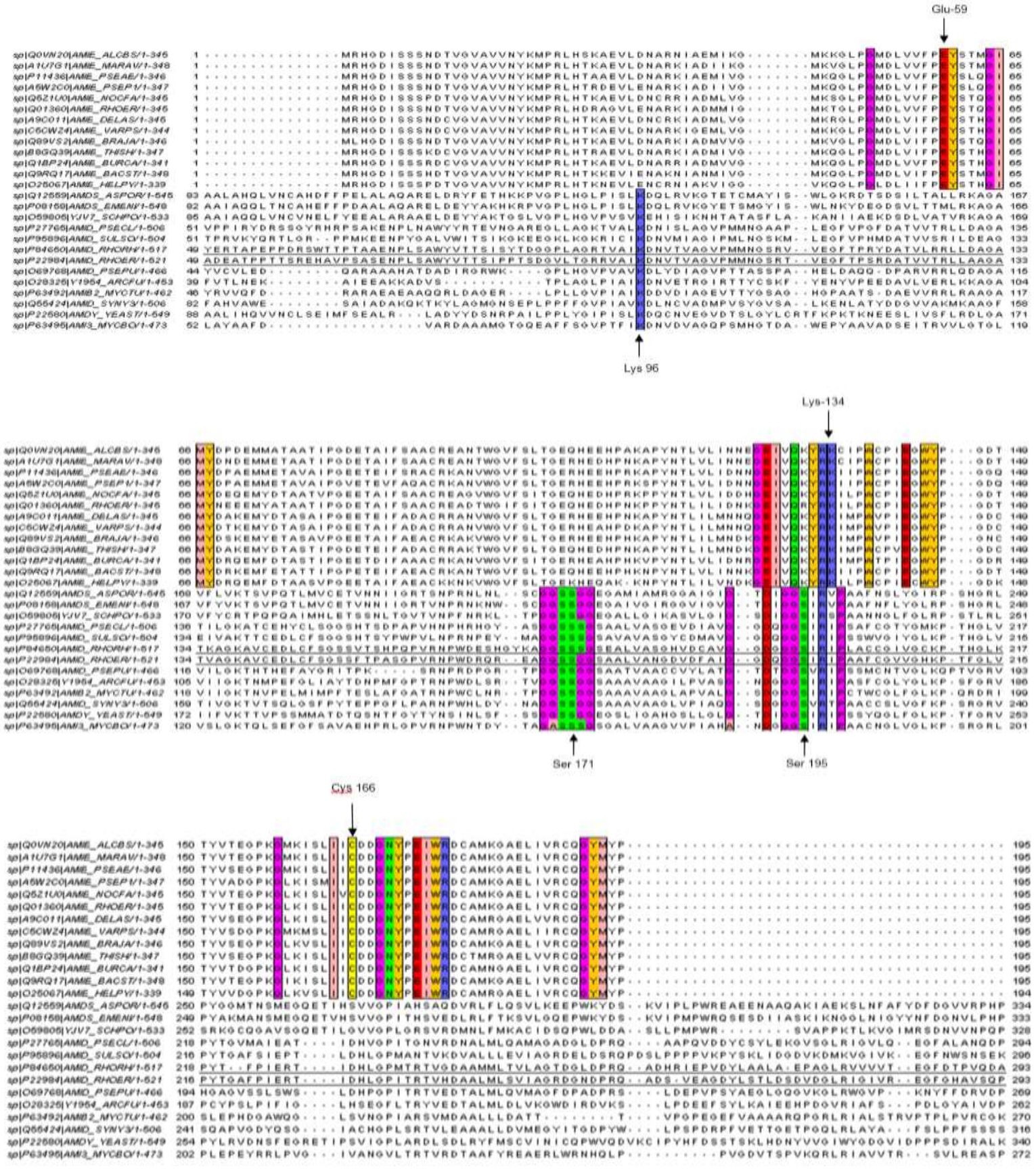

Figure 1: Deduced amino acid sequences alignment of aliphatic and signature amidases.
#Reference sequence P22984 (Rhodococcus erythropolis N-774). Colored-shade represents the conserved amino acids.

DISCUSSION

In the present investigation an attempt has been made to differentiate between aliphatic amidases and signature amidases on the basis of their physiochemical properties. It has been observed that these two classes of amidases remarkably differ towards their substrate specificity as well as for catalytic mechanism. Highly significant difference in total number of amino acids and molecular weight revealed that these two parameters are playing some role towards the specificity of aliphatic and signature amidases. The values of theoretical pI significantly affect the solubility as well as specificity of these amidases to some extent. Insignificant difference has been recorded for extinction coefficient and instability index indicating that these properties does not relate to the substrate specificity of amidases. Significantly higher values of aliphatic index and GRAVY in signature amidases indicated that they are thermally more stable and less hydrophilic than aliphatic amidases.

From the present analysis it has been observed that the percentage composition of individual amino acid and other conserved amino acid residues besides the catalytic triad significantly contributes towards the substrate specificity of these two groups of amidases. Chemical composition of twenty different amino acid also contribute to substrate specificity and physiochemical properties of the proteins.[24,25] Multiple sequence alignment has shown unique difference among aliphatic and signature amidases. MSA has been shown that the sequences belonging to first group contains several position specific conserved motifs. In our study amino acid residues *i.e.* Cys, Glu, Trp, Tyr and Gly are found to be critical residues in enzyme catalysis as well as in structural stability of the aliphatic amidases where Cys and Glu remains within catalytic traid of enzyme. Moreover site-directed mutagenesis of Cys residue has shown that Cys166 acted as the nucleophile in the catalytic mechanism.[1,26] Substitution of Glu59 to Val resulted in the destabilization of enzyme structure and loss of activity indicated that Glu is responsible for structural stability.[27] However Trp, Tyr and Gly is also found to be conserved around catalytic triad at different positions. Substitution of Trp-138 to Gly results in altered substrate specificity, stability and confirmation of the enzyme.[28] Alteration in Trp144 also results in low affinity towards urea and hydroxyurea.[29,30] It has also been reported that catalytic traid lies together with Met193, Tyr60, Trp138, Glu142, Gly191, and Tyr192.[31] Where Gly-191 & Tyr192 is responsible for H bond formation of an intermediate to acetohydroxamic acid during acyl transferase activity. In present study amino acids *i.e.* Met, Asn, Ile and Gln are also found to be conserved around the catalytic triad and it may be concluded that these residues may also play some role in specificity as well as catalytic mechanism of aliphatic amidases.

Whereas amino acid residues like Ser and Lys are crucial for the specificity of signature amidases because these residues are found absolutely conserved and present in active site of enzyme. However, site directed mutagenesis has shown that the substitutions of Ser195 to Ala result in the loss of specificity.[11] While Lys-60 act as a general base and found to play an important role in stabilizing the resulting oxyanion of serine residue.[32] Furthermore we have reported that Asp, Arg and Pro have been found to be absolutely conserved near to one of the catalytic triad residue i.e. Ser-195, indicating that these residues are crucial for the specificity as well as for catalysis mechanism. Substitutions of Asp to Asn at position 191 result in the loss of specificity[11] and Arg-197 is crucial for transition state stabilization.[33]

CONCLUSION

All the selected sequences in each group are 100% homologous and more than 30% identical with respect to reference sequence indicating that they share a common pattern of ancestry but represent distantly related diverse functions. Present study has clearly distinguished the aliphatic and signature amidases. In contrast to aliphatic amidases, signature amidases have significantly higher number of amino acid residues, molecular mass, higher values for theoretical pI, aliphatic index and grand average of hydropathicity, In contrast to aliphatic amidase it may be further concluded that signature amidases are more stable and amino acids involved in catalytic mechanism along with other conserved amino acids are not position specific in signature amidases which points towards the functional diversity in these two closely related class of amidases. The results obtained from the present study will be certainly helpful in current as well as for future Biotechnological application in the identification and elucidation of aliphatic/signature amidases.

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REFERENCES

1. Fournand D, Arnaud A. Aliphatic and enantioselective amidases: from hydrolysis to acyl transfer activity, J Appl Microbiol, 2001, 91, 381–393.

2. Kelly JL, Kornberg HL. Purification and properties of acyltransferases from *Pseudomonas aeruginosa,* Biochem J, 1964, 93, 557-568.

3. Banerjee A, Sharma R, Banerjee UC. The nitrile-degrading enzymes: current status and future prospects, Appl Microbiol Biotechnol, 2002, 60, 33–44.

4. Novo C, Farnaud S, Tata R, Clemente A, Brown PR. Support for a three-dimensional structure predicting a Cys-Glu-Lys catalytic triad for *Pseudomonas aeruginosa* amidase comes from site-directed mutagenesis and mutations altering substrate specificity, Biochem J, 2002, 365, 731–738.

5. Chebrou H, Bigey F, Arnaud A, Galzy P. Study of the amidase signature group, Biochim Biophys Acta, 1996, 1298,285-293.

6. Makhongela HS, Glowacka AE, Agarkar VB, Sewell BT, Weber B, Cameron RA, Cowan DA, Burton SG. A novel thermostable nitrilase superfamily amidase from *Geobacillus pallidus* showing acyl transfer activity, Appl Microbiol Biotechnol, 2007, 75, 801–811.

7. Farnaud S, Tata R, Sohi MK, Wan T, Brown PR, Sutton BJ. Evidence that cysteine-166 is the active-site nucleophile of *Pseudomonas aeruginosa* amidase: crystallization and preliminary X-ray diffraction analysis of the enzyme. Biochem J, 1999, 340, 711-714.

8. Brenner C. Catalysis in the nitrilase superfamily, Curr Opin Struct Biol, 2002, 12, 775–782.

9. Pace HC, Hodawadekar SC, Draganescu A, Huang J, Bieganowski P, Pekarsky Y, Croce CM, Brenner C. Crystal structure of the worm NitFhit Rosetta stone protein reveals a Nit tetramer binding two Fhit dimers. Curr Biol, 2000, 10, 907–917.

10. Valina AL, Mazumder SD, Bruice TC. Probing the Ser-Ser-Lys catalytic triad mechanism of peptide amidase: computational studies of the ground state, transition state, and intermediate, Biochem, 2004, 43, 15657-72.

11. Kobayashi M, Fujiwara Y, Goda M, Komeda H, Shimizu S. Identification of active sites in amidase: Evolutionary relationship between amide bond- and peptide bond-cleaving enzymes, Proc Natl Acad Sci, 1997, 94, 11986-11991.

12. Fournand D, Bigey F, Arnaud A. Acyl transfer activity of an amidase from *Rhodococcus* sp. R312: Formation of a wide range of hydroxamic acids. Appl Environ Microb, 1998, 64, 2844-2852.

13. Hirrlinger B, Stolz A, Knackmuss, HJ. Purification and property of an amidase from *Rhodococcus erythropolis* MP50 which enantioselectively hydrolyses 2-aryl-propionamides, J Bacteriol, 1996, 178, 3501-3507.

14. Kotlova EK, Chestukhina GG, Astaurova OB, Leonova TE, Yanenko AS, Debabov VG. Isolation and primary characterization of an amidase from *Rhodococcus rhodochrous* M8, Biochem, 1999, 64, 384–389.

15. Cameron RA, Sayed M, Cowan DA. Molecular analysis of the nitrile catabolism operon of the thermophile *Geoacillus pallidus* RAPc8B, Biochim, Biophys Acta, 2005, 1725, 35–46.

16. Pertsovich SI, I Guranda DT, Podchernyaev DA, YanenkoAS, Švedas VK. Aliphatic amidase from *rhodococcus rhodochrous* M8is related to the nitrilase/cyanide hydratase family, Biochem (Moscow), 2005, 70, 1280-1287.

17. Nawaz MS, Khan AA, Seng JE, Leakey JE, Sittonen PH, Cerniglia CE. Purification and characterization of an amidase from acrylamide-degrading *Rhodococcus* sp, Appl Environ Microbiol, 1994, 60, 3343–3348.

18. Zhang Y, Zhang G, Zhang Y. Occurrence and analytical methods of acrylamide in heat-treated foods: review and recent developments, J Chromatogr A, 2005, 1075, 1–21.

19. Madhavan NK, Roopesh K, Chacko S, Pandey A. Comparative study of amidase production by free and immobilised Escherichia coli cells, Appl Biochem Biotechnol, 2005, 120, 97–108.

20. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein, J Mol Biol, 1982, 157, 105-132.

21. Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez JC, Frutiger S, Hochstrasser D. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences, Electrophoresis, 1993, 14, 1023-1031.

22. Gill SC, von Hippel PH, Calculation of protein extinction coefficients from amino acid sequence data, Anal Biochem, 1989, 182, 319-326.

23. Guruprasad K, Reddy BVB, Pandit MW. Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence, Protein Eng, 1990, 4, 155-161.

24. O'Reilly C, Turner PD. The nitrilase family of CN hydrolyzing enzymes – A comparative study, J Appl Microbiol, 2003, 95, 1161-1174.

25. Yeom SJ, Kim HJ, Lee JK, Kim DE, Oh DK. An amino acid at position 142 in nitrilase from *Rhodococcus rhodochrous* ATCC 33278 determines the substrate specificity for aliphatic and aromatic nitrile, Biochem J, 2008, 415, 401-407.

26. Kumaran D, Eswaramoorthy S, Gerchman S. E, Kycia H, Studier F W, Swaminathan S. Crystal structure of a putative CN hydrolase from yeast, Protein, 2003, 52, 283–291.

27. Karmali A, Tata R, Brown PR. Substitution of Glu-59 by Val in amidase from *Pseudomonas aeruginosa* results in a catalytically inactive enzyme, Mol Biotechnol, 2000, 16, 5-16.

28. Karmali A, Pacheco R, Tata R, Brown P R. Substitutions of Thr-103-Ile and Trp-138-Gly in amidase from *Pseudomonas aeruginosa* are responsible for altered kinetic properties and enzyme instability, Mol, Biotechnol, 2001, 17, 201-212.

29. Betz JL, Clarke PH. Selective evolution of phenylacetamide utilizing strains of *Pseudomonas aeruginosa,* J Gen Microbiol, 1972, 73, 161-174.

30. Tata R, Marsh P, Brown PR. Arg-188 and Trp-144 are implicatedin the binding of urea and acetamide to the active site of the amidase from *Pseudomonas aeruginosa*, Biochem Biophys Acta, 1994, 1205, 139-145.

31. Andrade J, Karmali A, Carrondo MA, Frazao C. Structure of amidase from *Pseudomonas aeruginosa* showing a trapped acyl transfer reaction intermediate state, J Biol Chem, 2007, 282 , 19598-19605.

32. Shin S, Lee TH, Ha NC, Koo HM, Kim SY, Lee HS, Kim YS, Oh1BH. Structure of malonamidase E2 reveals a novel Ser-cisSer-Lys catalytic triad in a new serine hydrolase fold that is prevalent in nature, EMBO J, 2002, 21, 2509-2516.

33. Labahn J, Neumann S, Buldt G, Kula MR, Granzin J. An alternative mechanism for amidase signature enzymes, J Mol Biol, 2002, 322, 1053–1064.

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