Int J Biochem Mol Biol 2012;3(4):384-391
www.ijbmb.org /ISSN:2152-4114/IJBMB1209001

Original Article

Exploring residues crucial for nitrilase function by site directed mutagenesis to gain better insight into sequence-function relationships

Shubhangi Kaushik, Utpal Mohan, UC Banerjee

Biocatalysis and Protein Engineering Group, Department of Pharmaceutical Technology, National Institute of Pharmaceutical Education and Research, Sector 67, S.A.S. Nagar-160 062, Punjab, India

Received September 5, 2012; Accepted December 6, 2012; Epub December 24, 2012; Published December 30, 2012

Abstract: Nitrilases represent a very important class of enzymes having an array of applications. In the present scenario, where the indepth information about nitrilases is limited, the present work is an attempt to shed light on the residues crucial for the nitrilase activity. The nitrilase sequences demonstrating varying degree of identity with *P. putida* nitrilase were explored. A stretch of residues, fairly conserved throughout the range of higher (96%) to lower (27%) sequence identity among different nitrilases was selected and investigated for the possible functional role in nitrilase enzyme system. Subsequently, the alanine substitution mutants (T48A, W49A, L50A, P51A, G52A, Y53A and P54A) were generated. Substitution of the rationally selected conserved residues altered the substrate recognition ability, catalysis and affected the substrate specificity but had very little impact on enantioselectivity and pattern of nitrile hydrolysis.

Keywords: Nitrilase, catalysis, sequence identity, site directed mutagenesis, mutants, conserved residues

Introduction

Nitrilases are well known for their unique ability to efficiently catalyze the nitrile compounds into the corresponding acids with the release of ammonia [1]. Nitrile hydrolysing enzymes are present in various species of bacteria, fungi and plants. Most of the known microbial nitrilases exist in the form of either homo oligomeric spirals or helices [2]. Members of the nitrilase superfamily exhibit conserved catalytic triad of Glu-Lys-Cys where cysteine is involved in imparting the nucleophilic attack on cyano group of nitrile. Nitrilases have been categorised into three classes based on their substrate specificity, which includes aliphatic, aromatic and arylacetonitrilases [3]. Some of the known nitrilases display relaxed substrate specificity indicating the occurrence of the phenomenon of substrate promiscuity in the members of this group of enzyme. In general, the promiscuous nature is believed to be crucial for the enzyme divergent evolution [4].

The members of nitrilase superfamily have been exploited at industrial level for the production of various drugs/drug intermediates, fine chemicals and chiral synthones. They work effectively in mild condition with precise regio-, stereo- and enantioselectivity [5]. In spite of the established industrial importance, the indepth knowledge of the relationships between the sequence, structure and function in nitrilases is still lacking. Furthermore, very limited information regarding the crystal structure of nitrilases is available [6]. However, the structures are available for other members of the nitrilase superfamily like amidases and nitrile hydratases [7-9]. Various research groups have used either site directed mutagenesis or random mutagenesis to explore the nitrilase enzyme system [10, 11]. Mutational studies revealed the specific residues affecting the activity for substituted aromatic nitrile and substrate specificity of nitrilase from *Rhodococcus rhodochrous* [12, 13]. Similarly, the residues affecting the enantioselectivity and amide formation

Table 1. Primers used for construction of alanine substitution mutants

Primer		Sequence of primer	Flanking restriction site	Substitution performed
Forward primer		5'GCG CGG *GAT CCC* ATG CAG ACA AGA AAA ATC GTC CGG 3'	*BamH*I	None
Reverse primers	T48A	5'CAG CCA *GAC GTG* GAA GGG ATA GCC GGG CAG CCA GGC TTC ACC AAA CAC GAT CAG GTC AC 3'	*BmgB*I	ACC to GCC
	W49A	5'CAG CCA *GAC GTG* GAA GGG ATA GCC GGG CAG CGC GGT TTC ACC AAA CAC GAT CAG GTC 3'	*BmgB*I	TGG to GCG
	L50A	5'CAG CCA *GAC GTG* GAA GGG ATA GCC GGG CGC CCA GGT TTC ACC AAA CAC GAT CAG 3'	*BmgB*I	CTG to GCG
	P51A	5'CCA *GAC GTG* GAA GGG ATA GCC GGC CAG CCA GGT TTC ACC AAA CAC 3'	*BmgB*I	CCC to GCC
	G52A	5'CCA *GAC GTG* GAA GGG ATA GGC GGG CAG CCA GGT TTC ACC AAA CAC 3'	*BmgB*I	GGC to GCC
	Y53A	5'CGA CC*A GGC CG*G TGC GCC CAG CCA GAC GTG GAA GGG CGC GCC GGG CAG CCA GGT TTC ACC AAA CAC GAT CAG 3'	*Cfr*101	TAT to GCG
	P54A	5'CC*A GGC CG*G TGC GCC CAG CCA GAC GTG GAA GGC ATA GCC GGG CAG CCA GGT TTC ACC 3'	*Cfr*101	CCC to GCC

The underlined segment represents the codons coding for alanine. The italicized codons represent the restriction site.

were identified for the arylacetonitrilase from *Pseudomonas fluorescens* [14, 15].

In the present study, site directed mutagenesis of the selected conserved residues of *P. putida* nitrilase was performed. The residues of the selected stretch were found to be fairly conserved throughout the higher (96%) to lower (27%) sequence identity. The mutagenesis was carried out to elucidate the possible role of these residues on nitrilase function and to characterize the key residues critical for nitrilase activity.

Materials and methods

Materials

The gel extraction kit, restriction enzymes, T4 DNA ligase, Taq DNA polymerase and DNA molecular weight markers were purchased from MBI fermentas, Germany. The primers used in the study were synthesized at Integrated DNA Technologies, USA. The nitrile compounds and IPTG were purchased from Sigma, Germany. Other chemicals were purchased from Hi Media, Mumbai, India.

Strains used and culture conditions

The arylacetonitrilase gene from *P. putida* MTCC 5110, cloned in pET-21 (b) vector (Novagen) and expressed in *E. coli* BL21 (DE-3), was used for the present study [16-18]. The BL21 cells carrying the construct were initially grown at 37°C for 16 h in a Luria Bertani (LB) medium containing ampicillin (100 µg/ml). After 16 h, 0.1% (v/v) culture was transferred to flasks containing the same medium and induced at 0.6 OD with IPTG (1 mM) for 6 h at 37°C.

Oligonucleotide directed site specific mutagenesis and gene cloning

The forward and reverse primers were designed for the generation of mutants with alanine substitution (**Table 1**). The reverse primers were flanked either with *BmgB*I or *BsrF*I restriction sites (possible nearer restriction sites to the conserved stretch of amino acid) whereas the forward primer was flanked with *BamH*I restriction site. It is to be mentioned that in each reaction the same forward primer was used; only the reverse primer was different depending on the nature of the substitution. PCR was done in Eppendorf Master cycler gradient (Eppendorf AG, Germany) under the following conditions: initial denaturation at 94°C for 10 min followed by cycling conditions, denaturation at 94°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for 1 min 30 sec (repeated for 30 cycles). Final extension at 72°C for 10 min was used to complete the reaction, resulting in amplification of nearly 200 bp in each case.

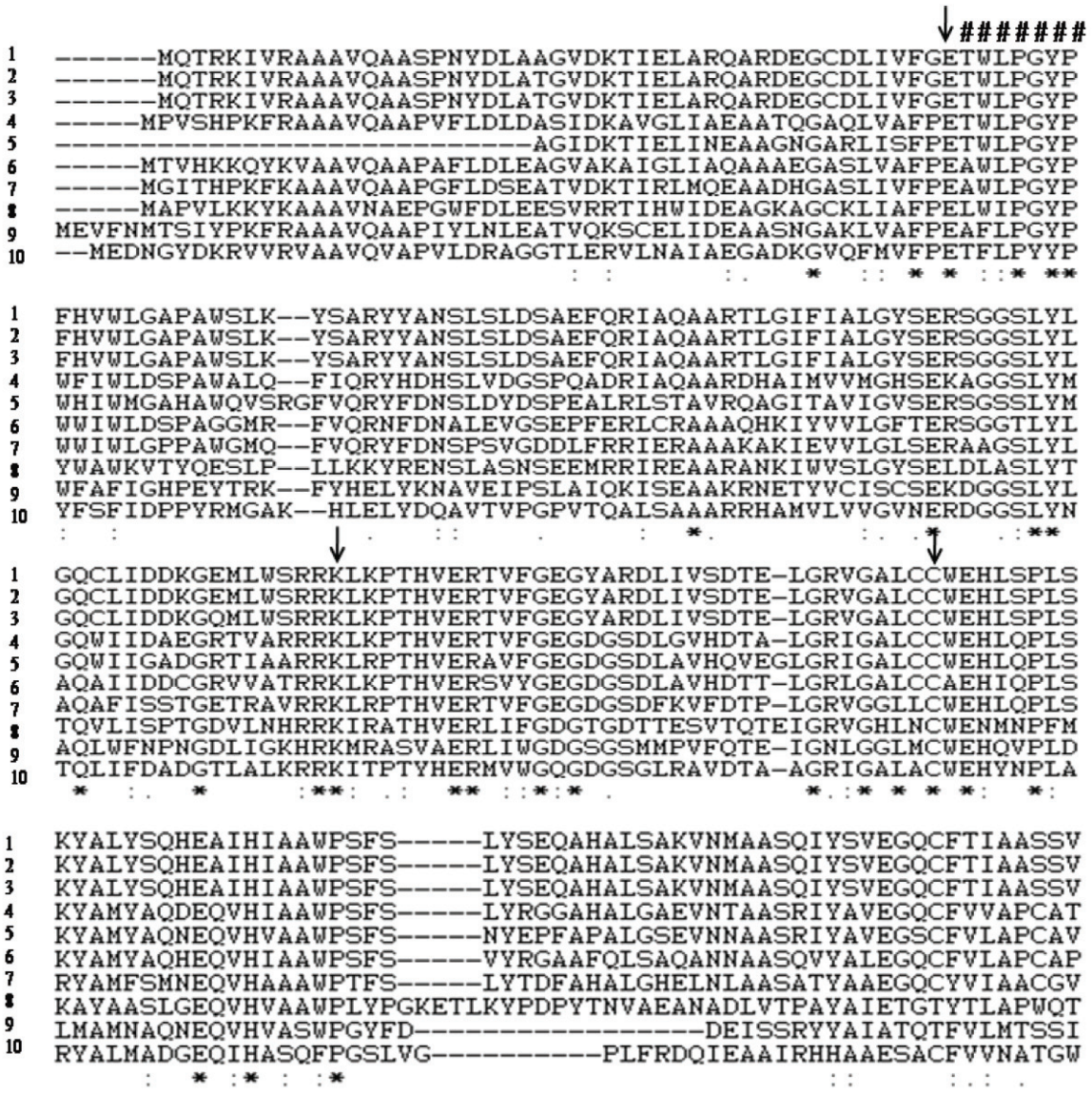

Figure 1. Multiple sequence alignment of *P. putida* nitrilase with different nitrilases of varying sequence identity. 1- *Alcaligenes faecalis* ECU0401 nitrilase (96% sequence identity), 2- *Alcaligenes faecalis* JM3 nitrilase (96% sequence identity) , 3- *Pseudomonas putida* MTCC 5110 nitrilase sequence (Query sequence), 4- *Methylibium petroleiphilum* PM1 nitrilase (49% sequence identity) 5- *Rhodococcus erythropolis* nitrilase (55% sequence identity), 6- *Pseudomonas fluorescens* nitrilase (52% sequence identity), 7- Nitrilase from uncultured organism (54% sequence identity), 8- *Aspergillus clavatus* nitrilase (32% sequence identity), 9- *Bacillus pumilus* nitrilase (33% sequence identity), 10- Nitrilase from *Novosphingobium sp.* (27% sequence identity). The arrows indicate the conserved catalytic triad residues. (*) indicates absolutely conserved residues, (:) indicates strong conservation residues, (#) indicates the residues explored in the present study.

The amplified 200 bp fragments containing individual mutations were restricted using *BmgB*I or *BsrF*I with *BamH*I for 4 h at 37°C and gel extracted. Similarly 909 bp fragment was amplified using nitrilase gene construct cloned in pET-21b as a template and a set of oligonucleotide primers. The forward primer was flanked with *BmgB*I or *BsrF*I restriction site whereas reverse primer was flanked with *Xho*I restriction site. The forward primer sequence was 5' TTC CAC GTC TGG CTG GGC GCA CCG GC 3' and the reverse primer sequence was 5'CGC GGC TCG AGG GAC GGT TCT TGC ACC AGT AGC GTA TC 3'. The *BmgB*I or *BsrF*I with *Xho*I was

Table 2. Kinetic analysis of the alanine substitution mutants

Nitrilase	K_M (mM)	k_{cat} (S^{-1})
PspNit	15.79 ± 2.2	2.99 ± 0.36
T48A	8.56 ±1.1	2.8 ± 0.49
W49A	80.02 ± 3.5	0.77 ± 0.04
L50A	158.6 ± 8.9	1.72 ±0.5
P51A	246 ± 4.2	1.70 ±0.09
G52A	19.8 ± 1.5	0.241 ± 0.02
Y53A	14.06 ± 1.4	0.255 ± 0.04
P54A	6.59 ± 1.0	0.238 ± 0.05

used for restricting the 909 bp PCR fragment and the restricted fragments were gel extracted. The restricted 200 bp (in each case) and 909 bp fragment were ligated using 10 Weiss units of T4 ligase at 22°C for 16 h. The ligated 1kb band generated in each ligation mixture was gel extracted and individually regarded as insert. On the other hand, pET-21(b) vector was also digested with *BamH*I and *Xho*I and gel extracted. The ligation reaction was set up using gel purified vector and insert and the ligation mixture was transformed in the BL21 cells. The presence of insert was confirmed by restriction digestion analysis of isolated plasmids from the respective colonies. The presence of desired mutation was assured by sequencing the mutants at Bioserve, India.

Protein purification

The recombinant cells harbouring either wild type nitrilase or mutant nitrilase were centrifuged individually at 10,000 x g for 30 minute at 4°C and resuspended separately in phosphate buffer (0.05 M, pH 8.0) containing 300 mM NaCl and 15 mM imidazole. Cells were lysed by sonication for 15 min. The resulting cell suspensions were then centrifuged at 10,000 x g for 20 min at 4°C and cell lysate was collected in each case. Meanwhile, the Ni-NTA matrix (Qiagen, USA) was equilibrated with phosphate buffer (0.05 M, pH 8.0) containing 300 mM NaCl and 15 mM imidazole. The generated lysate was filtered through 0.45µ filter and then applied to the equilibrated Ni-NTA column. Flow through was collected and the column was washed with the same buffer containing 20-70 mM imidazole to wash out the loosely bound proteins. The C-terminal His-tagged nitrilase was finally eluted from the column using 250 mM imidazole in phosphate buffer (0.05 mM, pH 8.0) containing 300 mM NaCl. The eluted protein was dialysed against phosphate buffer (0.1 M, pH 7.4). Protein content was estimated by Bradford's method [19]. The purification was assured by the SDS-PAGE (12%) gels.

Measurement of nitrilase activity and kinetic parameters

Nitrilase activity was determined by the modified Berthelot method [20]. The reaction mixture containing 0.1 M phosphate buffer (pH 7.4), 12.5 mM mandelonitrile (in ethanol) and nitrilase enzyme was incubated at 37°C for 20 min and the reaction was stopped by adding 200 µl 1N HCl. For the determination of kinetic parameters, the nitrilase activity was estimated over a range of mandelonitrile concentration (2 to 50 mM) and the parameters were calculated from Lineweaver-Burk plot. For the determination of optimum temperature, nitrilase activity was estimated at various temperatures following the standard assay method.

Determination of rate of conversion and preferentially formed enantiomer

For determining the rate of conversion of mandelonitrile to mandelic acid, the Reversed Phase High Performance Liquid Chromatography (RP-HPLC) was used. The reaction was carried out using 12.5 mM mandelonitrile in ethanol and 20 mg/ml recombinant cells harbouring either wild type or mutant nitrilases in phosphate buffer. The sample was withdrawn after 2 h and the cleared supernatant was used to analyse the mandelic acid formed in the reaction mixture. C18 Waters column (250 ×4.6 mm, 5µm, USA) was used at a flow rate of 0.7 ml/ min with a mobile phase of phosphate buffer (0.01 M , pH 4.6) and acetonitrile (55:45, v/v) as solvent system and absorbance was monitored at 245 nm. The retention times for mandelic acid, mandelamide and mandelonitrile were 2.93, 3.70 and 7.56 minutes respectively.

The chiral HPLC was employed to determine the enantiomers of mandelic acid using CHIRALCEL-OD-H column (250 x·0.46 mm, 5 µm) (Daicel Chemical Industries, USA) at a flow rate of 0.8 ml/min with a mobile phase of hexane, isopropanol and tri fluoro acetic acid (80: 20: 0.2, v/v)

Table 3. Enzyme activities of wild and mutant nitrilases for various nitrile substrates

Substrates		Enzyme activity (μmoles/min.mg)							
		PspNit	T48A	W49A	L50A	P51A	G52A	Y53A	P54A
Aliphatic	Propio nitrile	0.25 ± 0.1	0.012 ± 0.09	N.D.	N.D.	N.D.	0.045 ± 0.03	0.17 ± 0.007	0.035 ± 0.01
	Glutaro nitrile	0.35 ± 0.02	0.12 ± 0.01	N.D.	0.015 ± 0.003	N.D.	0.051 ± 0.03	0.027 ± 0.01	0.064 ± 0.04
	Valero nitrile	1.72 ± 0.1	0.46 ± 0.1	N.D.	0.028 ± 0.007	N.D.	0.011 ± 0.009	0.018 ± 0.006	0.138 ± 0.09
	Crotono nitrile	0.485 ± 0.04	0.099 ± 0.06	N.D.	0.029 ± 0.01	N.D.	0.034 ± 0.007	N.D.	0.03 ± 0.021
Aromatic and aryl-acetonitriles	3-cyano pyridine	0.12 ± 0.069	0.044 ± 0.01	0.021 ± 0.13	0.044 ± 0.003	N.D.	N.D.	0.012 ± 0.01	0.011 ± 0.007
	Benzo nitrile	0.054 ± 0.009	0.099 ± 0.078	0.116 ± 0.007	0.099 ± 0.004	N.D.	N.D.	0.09 ± 0.03	N.D.
	Pyrazine carbonitrile	2.59 ± 0.19	1.236 ± 0.24	0.13 ± 0.012	0.054 ± 0.005	N.D.	0.03 ± 0.006	0.04 ± 0.02	0.26 ± 0.11
	Mandelo nitrile	3.3 ± 0.21	3.76 ± 0.12	0.10 ± 0.005	0.101 ± 0.001	0.09 ± 0.005	0.57 ± 0.1	0.104 ± 0.009	2.1 ± 0.32
	Phenoxy acetonitrile	3.1 ± 0.07	3.3 ± 0.15	0.09 ± 0.014	0.05 ± 0.002	0.048 ± 0.011	1.96 ± 0.21	2.36 ± 0.07	2.85 ± 0.19

N.D.: Not detectable.

Table 4. Conversion rates and enantiospecificity of mutants in comparison to the wild type enzyme

Nitrilase	Conversion rate (%)	Enantiomer formed	ee (%)
PspNit (Wild type)	96.6	*R*	99
$PspNit_{48\text{-}54}A$	3.7	*R*	99
T48A	98.6	*R*	98
W49A	1.4	*R*	86
L50A	1.5	*R*	90
P51A	1.1	*R*	97
G52A	28.1	*R*	89
Y53A	2.4	*R*	91
P54A	53.2	*R*	93

as a solvent system. The retention times for (*S*)-(+) and (*R*)-(-)-isomers were 8.00 and 9.083 min.

Results and discussion

Sequence identity search to select the residues contributing to nitrilase functionality

The nitrilase sequences demonstrating varying degree of identity (96 to 27%) with *P. putida* nitrilase were selected through protein BLAST search. The multiple sequence alignment was performed to identify the residues, apart from the catalytic triad of Glu-Cys-Lys, which remain conserved throughout the selected range of sequence identity. In the present study, a stretch of fairly conserved residues was explored further by site directed mutagenesis (**Figure 1**). Within the selected stretch, the residues 'Pro-Gly-Tyr-Pro'were even found to be fairly conserved in other members of nitrilase superfamily like cyanide hydratases and cyanide dihydratases [3, 21, 22]. Conservation of these residues in other members of nitrilase superfamily apart from the members displaying true nitrilase activity indicates that these are probably the evolutionary or functionally important residues.

Sequential alanine substitution of the selected residues and kinetic analysis

The residues of the selected stretch were individually replaced with alanine in a sequential manner to create the substitution mutants; T48A, W49A, L50A, P51A, G52A, Y53A and P54A. Alanine was selected as a substitution residue of choice as it does not possess the side chain beyond the β-carbon, thus it neither alters the main-chain conformation nor imposes extreme electrostatic effect [23]. The subunit molecular mass of the variant enzymes in SDS/PAGE were approximately 43 kDa like wild type enzyme. These masses were similar to the

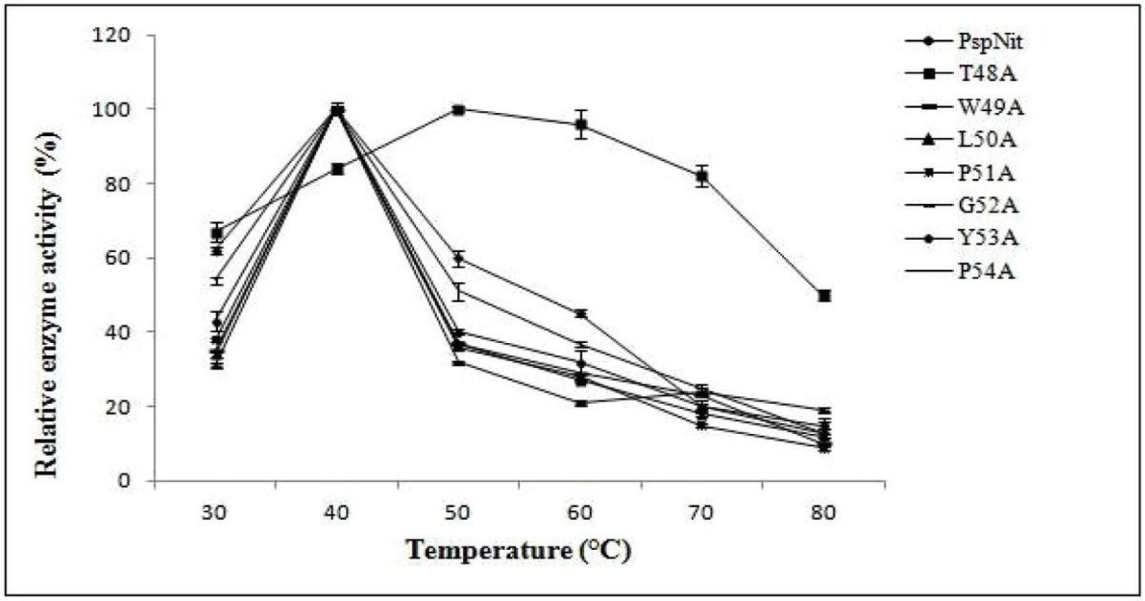

Figure 2. Activity-temperature profile of mutants as compared to wild type enzyme. The relative activity was expressed as a percentage of maximum activity under experimental conditions. Results are represented by mean ± S.D. of two experiments.

theoretically estimated mass of 40 kDa with pI 5.8 as calculated using compute pI/MW tool. Expression levels of the variant nitrilases were not found to be affected by the substitution. Each of the generated mutants retained sufficient activity such that their steady state kinetics could be studied. Kinetic parameters of the purified mutant nitrilases were determined for the hydrolysis of mandelonitrile (**Table 2**). The indepth analysis suggested that amongst the generated mutants, G52A, P54A, Y53A affected the k_{cat} drastically while L50A and P51A substitution largely affected the K_M. G52A, P54A and Y53A substitutions led to almost 12 fold reduction in k_{cat}. Moreover, G52A and Y53A mutants retained K_M comparable to the wild type enzyme but showed substantial reduction in k_{cat}. This suggests that the residues G52A and Y53A are essential for maintaining the proper hydrolytic ability. The residues W49A, L50A, P51A and P54A are crucial for proper substrate recognition, binding and hydrolysis.

Effect of site specific alanine substitution on substrate specificity

The role of the conserved residues in hydrolysing the range of nitriles including aliphatic, aromatic and arylacetonitriles was analysed. The mutants W49A, L50A, G52A, Y53A and P54A showed overall decreased activity for all the tested substrates as compared to the wild type enzyme (**Table 3**). The P51A mutant exhibited very low hydrolytic activity for arylacetonitriles and no activity for the aliphatic and aromatic nitriles. However, unlike other generated mutants, T48A mutant interestingly displayed the ability to catalyze the arylacetonitriles/aromatic nitriles comparable to the wild type nitrilase. A thorough observation of the sequence alignments done by other groups [3, 22] revealed that in few nitrilases, the alanine residue was originally present in the conserved stretch in place of threonine. This suggests that probably in the native organism, the nitrile-hydrolysing activity was supported by the alanine residue. This could be a plausible reason for the observation where the T48A mutant revealed comparable activity to that displayed by the parental enzyme. However, this mutation did not favour the activity for the aliphatic/ heterocyclic nitriles tested in the present study and displayed 50-80% reduction in activity for these substrates with respect to the wild type nitrilase. This observation suggests that T48A mutation has considerably decreased the promiscuous nature of the existing enzyme as compared to the wild type enzyme and thus it may be a determinant residue required for the enzyme to catalyze various nitriles with different functional groups (**Table 3**).

Influence of substitutions on enantiospecificity and other parameters

The effect of these mutations on the conversion of mandelonitrile to mandelic acid was determined through reversed phase HPLC (**Table 4**). All these mutations except T48A showed marked reduction in the conversion rate. Like the wild type enzyme, the mutant enzymes did not show the formation of mandelamide as an intermediate during the conversion of mandelonitrile to mandelic acid. There was no production of mandelic acid when mandelamide was used as a substrate, thus indicating that the substitution of the selected residues did not influence the manner by which the nitrile hydrolysis reaction occurred. Furthermore, the influence of these residues on enantiospecificity was investigated by Chiral HPLC. The chiral analysis demonstrated that all the constructed mutants preferentially formed the (*R*)-enantiomer of the mandelic acid with the enantiomeric excess (ee %) comparable to the wild type enzyme (**Table 4**). These results suggested that although the selected residues proved to be crucial for maintaining the substrate specificity and catalysis, but had very little impact on the enantioselectivity and pattern of nitrile catalysis.

The involvement of selected stretch of conserved residues in the above tested parameters encouraged the investigation of its effect on the ability of the enzyme to withstand thermal stress. Among all the mutations of the conserved stretch, T48A mutation influenced the thermostability. The activity-temperature profile of all the mutants has been shown in **Figure 2**. The T48A mutant exceptionally showed its ability to act at elevated temperatures unlike the wild type enzyme and other mutants. The maximum activity of the T48A mutant was found to be in the range of 50-60°C contrary to wild type and other mutants. Currently, the investigation of the role of this mutation for inducing the ability to act at higher temperature is in progress.

Acknowledgements

SK gratefully acknowledges Department of Biotechnology, Government of India for providing the senior research fellowship to conduct this work.

Address correspondence to: U.C. Banerjee, Biocatalysis and Protein Engineering Group, Department of Pharmaceutical Technology, National Institute of Pharmaceutical Education and Research, Sector 67, S.A.S. Nagar-160 062, Punjab, India. Tel: +91 172 2214682–87; Fax: +91 172 2214692; E-mail address: ucbanerjee@niper.ac.in

References

[1] Pace HC and Brenner C. The nitrilase superfamily: classification, structure and function. Genome Biology 2001; 2: 1-9.

[2] Thuku RN, Brady D, Benedik MJ and Sewell BT. Microbial nitrilases: versatile, spiral forming, industrial enzymes. Journal of Applied Microbiology 2009; 106: 703-727.

[3] O'Reilly C and Turner PD. The nitrilase family of CN hydrolysing enzymes: a comparative study. Journal of Applied Microbiology 2003; 95: 1161-1174.

[4] Hult K and Berglund P. Enzyme promiscuity: mechanism and applications. Trends in Biotechnology 2007; 25: 231-238.

[5] Banerjee A, Sharma R and Banerjee UC. The nitrile-degrading enzymes: current status and future prospects. Applied Microbiology and Biotechnology 2002; 60: 33-44.

[6] Raczynska J, Vorgias C, Antranikian G and Rypniewski W. Crystallographic analysis of a thermoactive nitrilase. Journal of Structural Biology 2011; 173: 294-302.

[7] Barglow KT, Saikatendu KS, Stevens RC and Cravatt BF. Structure of amidase from *Pseudomonas aeruginosa* showing a trapped acyl transfer reaction intermediate state. Journal of Biological Chemistry 2007; 282: 19598-19605.

[8] Song L, Shi J, Xue Z, Wang MX and Qian S. High resolution X-ray molecular structure of the nitrile hydratase from *Rhodococcus erythropolis* AJ270 reveals posttranslational oxidation of two cysteines into sulfinic acids and a novel biocatalytic nitrile hydration mechanism. Biochemical and Biophysical Research Communications 2007; 362: 319-324.

[9] Nakai T, Hasegawa T, Yamashita E, Yamamoto M, Kumasaka T, Ueki T, Nanba H, Ikenaka Y, Takahashi S, Sato M and Tsukihara T. Crystal structure of N-carbamyl-D-amino acid amidohydrolase with a novel catalytic framework common to amidohydrolases. Structure 2000; 8: 729-737.

[10] Kobayashi M, Izui H, Nagasawa T and Yamada H. Nitrilase in biosynthesis of the plant hormone indole-3-acetic acid from indole-3-acetonitrile: Cloning of the *Alcaligenes* gene and site-directed mutagenesis of cysteine resi-

dues. Proceedings of National Academy of Sciences 1993; 90: 247-251.
[11] Schreiner U, Hechera B, Obrowskya S, Waicha K, Klempierb N, Steinkellnera G, Gruberc K, Rozzell JD, Gliedera A and Winkler M. Directed evolution of *Alcaligenes faecalis* nitrilase. Enzyme and Microbial Technology 2010; 47: 140-146.
[12] Yeom SJ, Kim HJ, Lee JK, Kim DE and Oh DK. An amino acid at position 142 in nitrilase from *Rhodococcus rhodochrous* ATCC 33278 determines the substrate specificity for aliphatic and aromatic nitriles. Biochemical Journal 2008; 415: 401-407.
[13] Yeom SJ, Lee JK and Oh DK. A positively charged amino acid at position 129 in nitrilase from *Rhodococcus rhodochrous* ATCC 33278 is an essential residue for the activity with meta-substituted benzonitriles. FEBS Letters 2010; 584: 106-110.
[14] Kiziak C and Stolz A. Identification of amino acid residues responsible for the enantioselectivity and amide formation capacity of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191. Applied and Environmental Microbiology 2009; 75: 5592-5599.
[15] Sosedov O, Baum S, Bürger S, Matzer K, Kiziak C and Stolz A. Construction and application of variants of the *Pseudomonas fluorescens* EBC191 arylacetonitrilase for increased production of acids or amides. Applied and Environmental Microbiology 2010; 76: 3668-3674.
[16] Banerjee A, Kaul P and Banerjee UC. Purification and characterization of an enantioselective arylacetonitrilase from *Pseudomonas putida*. Archives in Microbiology 2006; 184: 407-418.
[17] Naik SC, Kaul P, Barse B, Banerjee A and Banerjee UC. Studies on the production of enantioselective nitrilase in a stirred tank bioreactor by *Pseudomonas putida* MTCC 5110. Bioresource Technology 2008; 99: 26-31.
[18] Kumar S, Mohan U, Kamble AL, Pawar S and Banerjee UC. Cross-linked enzyme aggregates of recombinant *Pseudomonas putida* nitrilase for enantioselective nitrile hydrolysis. Bioresource Technology 2010; 10: 6856-6858.
[19] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976; 72: 248-254.
[20] Kaushik S, Rohamare S, Mohan U and Banerjee UC. Screening strategy for high throughput selection of nitrilase producing microorganisms and mutants for the production of pharmaceutically important drugs and drug intermediates. International Journal of Pharmaceutical Science and Technology 2011; 6: 36-43.
[21] Kiziak C, Klein J and Stolz A. Influence of different carboxy-terminal mutations on the substrate-, reaction- and enantiospecificity of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191. Protein Engineering Design and Selection 2007; 20: 385-396.
[22] Sharma N, Kushwaha R, Sodhi JS and Bhalla TC. *In Silico* analysis of amino acid sequences in relation to specificity and physiochemical properties of some microbial nitrilases. Journal of Proteomics and Bioinformatics 2009; 2: 185-192.
[23] Cunningham BC, Wells JA. High-resolution epitope mapping of hGH-receptor interactions by alanine scanning mutagenesis. Science 1989; 244: 1081-1085.