Chapter 2

Cloning, expression and biochemical characterization of penicillin V acylase from *Pectobacterium atrosepticum* (PaPVA)
2.1. Introduction:

The post-genomics era has led to the sequencing of numerous genes from different microorganisms. The availability of a range of techniques in recombinant DNA technology has also helped clone them and over-express many proteins in soluble form in *E. coli* and other microbial hosts, thus paving the way for development of enzymes for commercial applications. Modifications at the gene level (different constructs, codon optimization) and for growth of host strains (optimization of media and inducers) provide further avenues for maximum protein yields and better stability (Rosano and Ceccarelli 2014).

Penicillin acylases (E.C.3.5.1.11) cleave the acyl side chain of the beta-lactam antibiotics (penicillins and cephalosporins) to generate pharmaceutical intermediates 6-amino penicillanic acid (6-APA) or 7-acetoxy cephalosporanic acid (7-ACA) respectively (Shewale and SivaRaman 1989). Although the penicillin G acylase (PGA) from *E. coli* (*Ec*PGA) has a monopoly in the pharmaceutical industry, pen V acylases (PVA) have been reported to be more suitable for the production of semi-synthetic antibiotics (Shewale and Sudhakaran 1997). However, the use of PVA-Pen V combination in industry is limited by the slightly higher cost of substrate and due to the non-availability of large amount of active acylase enzyme preparations.

Penicillin acylases are members of the Ntn hydrolase protein superfamily (Duggleby et al. 1995; Suresh et al. 1999), characterized by a catalytic N-terminal residue and a common αββα-fold. Although most Ntn hydrolases have similar active site geometry, they share a fairly low sequence and structural homology. For instance, PGAs possess a heterodimeric subunit organization, as do cephalosporin acylases and γ-glutamyl transpeptidases (Castellano and Merlino 2013); while PVAs and bile salt hydrolases (BSH) share a homotetrameric structure (Kumar et al. 2006).

Biophysical and structural characterization of PVAs and BSHs has been so far restricted to Gram-positive bacteria. Structures are available for PVAs from *Bacillus sphaericus* (*Bsp*PVA) (Suresh et al. 1999) and *Bacillus subtilis* (*Bsu*PVA) (Rathinaswamy et al. 2005). *Bsp*PVA can also hydrolyze bile salts like glycodeoxycholate (GDCA) to a small extent (20% of Pen V-hydrolyzing activity),
while BsuPVA is specific for Pen V (Kumar et al. 2006). When trying to classify enzymes of cholylglycine hydrolase group based on substrate preference, Lambert et al. (2008) and Panigrahi et al. (2014) have noted the low sequence homology between those from Gram-positive, Gram-negative bacteria and Archaea, even though they carry out similar reactions. Such studies raise the possibility that PVA homologues from Gram-negative bacteria might show different biochemical and structural characteristics from their counterparts from Bacillus sp. Screening for novel PVA producing bacteria from different sources identified a few organisms that showed a high level of PVA production. The Gram-negative plant pathogenic bacteria, Pectobacterium atrosepticum and Agrobacterium tumefaciens produced PVA with a high specific activity. Hence, it could be definitely considered worthwhile to characterize the active PVA enzymes from such Gram-negative bacteria.

The present chapter describes the cloning, over-expression and detailed biochemical characterization of the penicillin V acylase from P. atrosepticum. The enzyme displayed unusually high expression in soluble form, and exhibited many fold higher specific activity than that of any known PVA reported till date. Characterization of PaPVA and other Gram-negative homologues would help both in industrial application of PVAs and understanding their substrate spectrum.

2.2. Materials and methods:

2.2.1. Materials:

Bile salts, guanidine hydrochloride (Gdn-HCl), Pen V (potassium salt), phenoxy acetic acid (POAA), kanamycin sulphate and HIS-Select matrix were procured from Sigma (USA). ENrich™ SEC 650 column and molecular weight markers were from BioRad. The synthetic substrate 2-nitro 5-(phenoxyacetamido)-benzoic acid (NIPOAB) was synthesized using the method of Kerr (1993) using the Schotten-Baumann reaction from 2-nitrobenzoic acid and phenoxyacetyl chloride (Sigma). Gdn-HCl was prepared as 8M stock and filtered before use. All DNA manipulation enzymes were procured from New England Biolabs (NEB). DNA isolation and
purification kits, cloning plasmids and E. coli strains were from Invitrogen (USA). All media components were procured from Himedia, India. Pectobacterium atrosepticum was obtained from DSMZ, Germany (DSM 30186).

### 2.2.2 Cloning of pva gene from P. atrosepticum:

The pva gene from Pectobacterium atrosepticum (annotated as cholylglycine hydrolase) was amplified from genomic DNA using the primers (restriction sites highlighted):

**PatF** – GGCTAG**CATG**TGACGCGGTTCTATCTGGATCC - PciI

**PatR** - CAATAT**CGAG**GCCCAGCAGATTCAACG - XhoI

PCR was performed in a gradient thermocycler (Applied Biosystems) using conditions: 94°C/5min, 30 cycles of [94°C/30s, 52°C/30s, 68°C/60s] and final extension 68°C/10 min. Restriction digestion was carried out using NcoI and XhoI for plasmid pET 28b (Invitrogen) and PciI and XhoI for insert DNA to generate compatible ends. After 4h at 37°C, the DNA was eluted from 1% agarose gel and ligated at 16°C for 12 h. The ligation mixture was transformed into E. coli DH5α cells and selected on LB agar containing 35µg/ml kanamycin. Colonies were screened for recombinant plasmids using colony PCR and the gene was sequenced using T7 promoter and confirmed to be in-frame to the vector. The plasmid pET28b-PaPVA was then re-transformed into E. coli BL21 star cells for expression using standard calcium transformation procedures (Sambrook et al. 1989). This clone was used for protein preparation, characterization and structural studies.

### 2.2.3 Expression and purification of PaPVA:

E. coli BL21 star cells containing pET28b-PaPVA were grown in LB medium containing 35µg/ml kanamycin at 37°C and 200 rpm for 2-3 h. At O.D.600 ~ 0.6, protein production was induced by adding 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) and culture was transferred to 27°C for overnight incubation (18 h). The cells were harvested by centrifugation at 5000 rpm for 15 min. The cells were resuspended and sonicated in lysis buffer containing 25mM Tris-Cl pH 7.0, 300mM NaCl, 10mM MgCl2 and 2mM β-mercaptoethanol. Sonication was done for 5 x 1min
at 50 W using a Branson Digital Sonifier. The expression of PaPVA enzyme in soluble fraction was confirmed using SDS-PAGE and PVA activity assay.

For purification, 1 g E. coli – PaPVA cells were sonicated in lysis/binding buffer and the clarified supernatant was loaded on a HIS-Select Ni²⁺- affinity column equilibrated with the same buffer. After washing out the unbound proteins, PaPVA was eluted using 250mM imidazole. The eluted protein fractions were dialyzed extensively against 20mM acetate buffer pH 5.2 containing 100mM NaCl and 1mM DTT and stored at 4°C. Protein concentrations were estimated using Bradford method (Bradford 1976) and purity was confirmed using SDS-PAGE (Laemmli 1970).

2.2.4. Determination of molecular weight:

The protein was subjected to SDS-PAGE on a 12% polyacrylamide gel with molecular weight markers (BioRad) and stained using Coomassie Brilliant Blue. The subunit molecular weight was ascertained using Matrix-associated laser desorption ionization-mass spectrometry (MALDI, Perkin Elmer) using a sinapinic acid matrix.

SDS-PAGE (12% gel) was also used to determine the subunit molecular weight. The gel was stained with 0.25% Coomassie brilliant blue R250 in 40% (v/v) methanol and 10% (v/v) glacial acetic acid. Electrophoresis was conducted at 25°C at 90 V (constant voltage) till the bromophenol blue tracking dye reaches the end of the gel. The apparent molecular weight was calculated by comparing the migration of the protein with that of marker proteins of known molecular weights.

To determine the native molecular weight of PaPVA, 200 µl of protein (7 mg/ml) was run on size exclusion chromatography column (ENrich™ SEC column, 10 x 300 mm) using a BioRad NGC™ 10 Medium-pressure chromatography system. A similar experiment was used to test the effect of 1M Gdn-HCl on the enzyme.

2.2.5. PVA Enzyme activity assay:

PVA activity was determined by measuring the amount of 6-aminopenicillanic acid (6-APA) formed from penicillin V using p-dimethyl amino benzaldehyde (Shewale et al. 1987). The reaction was carried out for 5 min with 1.2 µM enzyme and 50 mM
Pen V in 100 mM acetate buffer pH 5.0 at 45°C. The reaction was initiated with the addition of the enzyme in a total reaction volume of 0.5 ml and subsequently quenched with equal volume of citrate phosphate buffer (CPB) pH 2.5. All assays were carried out in triplicates. Colour development was performed using p-dimethyl amino benzaldehyde (pDAB) reagent (0.6% w/v in methanol, 0.01% hydroquinone stabilizer) and the absorbance was read at 415 nm, 2 min after addition of pDAB. One unit (IU) of PVA activity was defined as the amount of enzyme required to liberate 1 µmol of 6-APA per min under the mentioned assay conditions. In the case of NIPOAB used as substrate, the enzyme was added to 1ml of 2 mM NIPOAB (2% DMSO effective concentration).

### 2.2.6. Effect of pH and temperature on PaPVA activity and stability:

The PVA activity was assayed (as detailed above) at different pH values from 4.0 – 9.0 and temperatures (20 – 70°C) to ascertain the optimum conditions for enzyme activity.

*Pa*PVA stability was studied by incubating the protein in 20 mM acetate buffer pH 5.0 for 2 h at different temperatures from 30 to 90°C, and assaying for activity at 45°C after different time intervals. Effect of pH on enzyme stability was studied by incubating the protein in 100 mM buffers of different pH (1-11) for 4 h at 25°C and assaying the residual activity. Buffers used were: HCl-KCl (pH 1-2), acetate (3-6), phosphate (7-8), Tris (8-9) and carbonate-bicarbonate (10-11). All buffers were freshly prepared with pH adjusted at room temperature and filtered before use.

### 2.2.7. Effect of Guanidine hydrochloride on the enzyme:

The enzyme was also incubated with increasing concentrations of Gdn-HCl (0-6M) for 4h to study its unfolding effect. Renaturation experiments were conducted by diluting the Gdn-HCl concentration 10 times and incubating for 1h at 25°C before checking the activity. The effect of 1M Gdn-HCl on the oligomeric nature of *Pa*PVA was also checked using size exclusion chromatography.
2.2.8. Fluorescence measurements:

Conformational changes that occur in proteins in different conditions can be studied using changes in the fluorescence of sensitive fluorophores, like the aromatic amino acids trp, tyr and phe. Fluorescence emission spectra of PaPVA were measured on a Perkin Elmer LS50 B fluorimeter with slit width of 7 nm for both the monochromators. Samples (2 ml) were maintained at constant temperature (± 0.1°C) in a quartz cuvette with the help of a Julabo F 25 circulating cryobath. Samples were excited at 295 nm and the emission spectra were recorded from 310 to 400 nm. All samples were checked for inner filter effect. The fluorescence of buffers, quenchers and various additives were measured at identical wavelengths and corrected for in the observed fluorescence of samples.

2.2.9. Circular dichroism:

Optically active molecules in solution absorb left and right polarized components differentially when circularly polarized light is incident on them, leading to the phenomenon of circular dichroism (CD). Proteins and nucleic acids also display signature CD spectra. The analysis of protein CD spectra in the UV region is commonly used to follow the conformational changes in protein secondary structure, and complements fluorescence spectroscopy and biochemical studies. Far-UV (190-250 nm) CD signals are principally due to absorption by the peptide bond, and can provide an estimation of the secondary structure composition of proteins. Near-UV (250-300 nm) CD signals are contributed by the aromatic amino acids, and are sensitive to the overall tertiary structure of the protein (Kelly et al. 2005).

The enzyme solution filtered through a 0.22 μm membrane and dialyzed against 20 mM acetate buffer at pH 5.0 was used for CD spectroscopy. The CD spectra were recorded on a J-815 spectro-polarimeter with a Peltier Type CD/FL Cell circulating water bath (Jasco, Tokyo, Japan) at 25°C in quartz cuvettes. All spectra were corrected for buffer contributions and converted to mean residue weight ellipticity. Far-UV CD spectra (0.15 mg/ml PaPVA) were recorded in a rectangular quartz cell of 1 mm path length in the range of 190-250 nm at a scan speed of 100 nm/min with a response time of 1 s and a slit width of 1 nm. Near UV spectra (1.2 mg/ml PaPVA)
were recorded in the range of 250-320 nm with a 5 mm path length quartz cell. Each spectrum was recorded as the average of 3 scans.

To understand the conformational changes taking place in the protein structure and their relation to enzyme stability, fluorescence and CD spectra of PaPVA were measured after subjecting the enzyme to different conditions of pH (1-11), temperature (30-90°C) and increasing concentrations Gdn-HCl (0-6M).

2.2.10. Effect of protein modifiers on enzyme activity:

PaPVA (20 µg) was incubated with reducing agent DTT and metal-chelating agent ethylene diamine tetraacetic acid (EDTA) in a 100µl reaction mixture for 30 min at 25°C. Enzyme activity was assayed after incubation; untreated enzyme served as control. The effect of divalent metal ions and detergents was studied using similar experiments. Solvents were incorporated at different concentrations into the assay mixture to study their effect on enzyme activity. All experiments were performed independently in triplicates and results expressed as averages with <5% standard deviation.

2.2.11. PaPVA kinetic parameters:

Kinetic behaviour of PaPVA was determined by assaying the enzyme activity with increasing concentrations of penicillin V as substrate, 5-960mM under the optimum conditions. Preliminary determination of Km and Vmax was done using the method described by Sakoda and Hiromi (1972) and Hill constants were calculated using least squares fitting of ln (v/(vmax-v)) vs ln (S) plots. These initial values were used to fit the data to equation (2.1) using GraphPad Prism version 5.01 (GraphPad software, La Jolla California USA, www.graphpad.com) to determine Km, Vmax, h and Ki of substrate inhibition (Chen and Tanaka 2011).

\[ v = \frac{[V_{max} \cdot S^h]}{[K_{0.5} + S^h + (S^{2h}/K_i)]} \] ....... (2.1)

In case of the synthetic substrate NIPOAB, the hydrolysis of the substrate was followed at 405 nm in a spectrophotometer with increasing substrate concentrations (0.5-12mM).
2.3. Results and Discussion:

While screening organisms for potential PVA producers, we identified a few bacterial species (Table 2.1) that showed high levels of cell-bound activity. Gram negative bacteria like *P. atrosepticum* and *A. tumefaciens* showed much higher activity than other groups including *Bacillus* sp., actinomycetes and yeast. In addition, although PVA enzymes have been isolated from a variety of bacteria and fungi, extensive characterization including structural features is available only for those from *Bacillus sphaericus* (Suresh et al. 1999) and *Bacillus subtilis* (Rathinaswamy et al. 2005, 2012). Based on the sequence homology, the PVAs from Gram-negative bacteria have been shown to cluster separately when compared with their Gram-positive counterparts (Lambert et al. 2008; Panigrahi et al. 2014), and probably possess unique characteristics. In this context, we attempted to clone the genes coding for PVA from Gram-negative plant pathogens and maximize production through heterologous expression. This chapter details the cloning and expression of PVA from *P. atrosepticum* in *E. coli* and biochemical and biophysical characterization of the enzyme.

Table 2.1. PVA producing bacterial strains identified by screening. * PVA activity (IU/g cells) refers to cell-bound activity of culture grown in nutrient broth.

<table>
<thead>
<tr>
<th>Bacterial identification</th>
<th>Strain designation</th>
<th>Source</th>
<th>PVA activity (IU/g cells)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. atrosepticum</em></td>
<td>DSM 30168</td>
<td>DSM</td>
<td>22.5</td>
</tr>
<tr>
<td><em>A. tumefaciens</em></td>
<td>DSM 30205</td>
<td>DSM</td>
<td>19.3</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>ATUAVP1846</td>
<td>Soil (Pune, India)</td>
<td>6.4</td>
</tr>
<tr>
<td><em>Enterobacter hormaechei</em></td>
<td>APS3</td>
<td>Sulphur spring water (Dehradun, India)</td>
<td>3.2</td>
</tr>
<tr>
<td><em>Aeromonas enteropelogenes</em></td>
<td>SPA1</td>
<td>Soil (Pune, India)</td>
<td>14.1</td>
</tr>
</tbody>
</table>
2.3.1. Cloning and expression of pva gene from P. atrosepticum:

The gene ECA3205 from the genome sequence of *Pectobacterium atrosepticum* is annotated in the GenBank database (www.ncbi.nlm.nih.gov) as cholyglycine hydrolase, a group of enzymes which includes penicillin V acylase and bile salt hydrolases. Phylogenetic analysis and biochemical assays confirmed the enzyme as a penicillin V acylase. The *pva* gene contained a 29-amino acid signal sequence that directs the enzyme to the periplasm. This signal peptide is present in most Gram-negative cholyglycine homologs; however, the length and sequence is dependent on the bacterial species. *PaPVA* has only 25% sequence identity with already characterized PVAs from *Bacillus* and BSHs from Gram-positive bacteria, which further the argument for the necessity of studying such enzymes from Gram-negative bacteria.

The *pva* gene was cloned without the periplasmic signal sequence, with a methionine residue added before the N-terminal cysteine (Fig. 2.1a). The recombinant gene was identical to the reported sequence (NCBI accession number NC_004547.2) without any mutation. The protein expressed in *E. coli* with a C-terminal 6X His-tag, in the cytoplasmic soluble fraction; this suggests that the processing of *PaPVA* occurs by the simple removal of methionine in the recombinant *E. coli* and by removal of the periplasmic signal peptide in the wild type *P. atrosepticum*. Ntn hydrolases usually are processed to their active form by post-translational autocatalytic mechanism (Brannigan et al. 1995; Oinonen and Rouvinen 2000). Chandra et al. (2005) prepared various processing-impaired mutants of the PVA enzyme from *B. sphaericus*, which possesses a MLG pro-peptide before the N-terminal cysteine. Mutants with the prosequence retained the tripeptide; however, mutants lacking it appeared to have their initiation formyl methionine (fMet) removed. In the case of BSH from *Clostridium perfringens* (Rossmann 2008), cysteine mutants expressed in mostly insoluble form with the N-terminal methionine intact; neither native nor autocatalytic processing was observed. Structural analysis of *CpBSH* indicated the importance of the position of C1 for nucleophilic attack and the role of R18 and D69 residues in autocatalytic processing. These residues are highly conserved in many cholyglycine
hydrolases characterized so far. Mutagenesis of such residues could shed more light on the processing mechanism of cholyglycine hydrolases in Gram-negative bacteria.

*Pa*PVA was hyper-expressed in *E. coli*, with protein yields comfortably reaching 250 – 300 mg per litre of culture. This is very high compared to model recombinant proteins in *E. coli* (Rosano and Ceccarelli 2014); the enzyme did not accumulate in inclusion bodies even after overnight incubation at 27°C.

![Image of agarose gel and SDS-PAGE gel](image)

**Fig. 2.1.** (a) Restriction digested *Pa*PVA-pET28b on 1% agarose gel (Lane 1 – Invitrogen 10 kb DNA ladder, 2 – *Pa*PVA-pET28b, 3, 4 – recombinant *Pa*PVA-pET28b digested with XbaI/XhoI for 3 h at 37°C). (b) *Pa*PVA on 12% SDS-PAGE gel (Lane 1 – Sonicate of *Pa*PVA expressing BL21 clone, soluble fraction, 2 – purified *Pa*PVA, 3 – BioRad protein molecular weight marker, MW in KDa).

**2.3.2. Purification of *Pa*PVA:**

The protein was purified to homogeneity (Fig. 2.1b) using Ni\(^{2+}\) affinity chromatography. A small amount of precipitate was formed on dialysis, which was clarified by high speed centrifugation. Before characterization, the enzyme was also passed through a size exclusion column to remove any soluble aggregates present. The enzyme (3 mg/ml) stored at 4°C retained 90% of its activity after 30 days. The final yield of protein was 72 mg/ g wet cells, with a specific activity of 430 IU/mg with 50mM Pen V. The catalytic reaction was linear till 10 mins for 1.2 µM enzyme.
PaPVA exhibits many fold greater specific activity for Pen V than other PVAs from Gram-positive bacteria, actinomycetes, fungi and yeast (Shewale and Sudhakaran 1997). Such high activity and increased protein yields make PaPVA a valuable enzyme for use in the pharmaceutical industry. The targeting of such a highly active PVA enzyme to the periplasmic space in *P. atrosepticum* also provides possible pointers to the physiological role of the enzyme. Periplasmic proteins generally interact actively with compounds in the environment, functioning as signaling receptors, binding proteins and hydrolytic or detoxifying enzymes. Valle et al. (1991) have hypothesized the role of penicillin acylases in the environment as scavengers for alternative carbon sources, while Kovacikova et al. (2003) have studied the modulation of *pva* gene expression in *Vibrio cholerae* by bacterial signalling (quorum sensing) mechanism.

2.3.3. Molecular weight determination:

MALDI showed a single peak of 39, 191 Da, corresponding to the subunit molecular weight of PaPVA enzyme (Fig. 2.2). The native molecular weight was estimated to be 154 kDa using size exclusion chromatography, which confirmed a tetrameric subunit association similar to that reported for other PVAs (Suresh et al. 1999). The isoelectric point of the enzyme was 8.4.

Fig. 2.2. PaPVA subunit molecular weight (39.19 kDa) determined by MALDI.
2.3.4. Effect of pH and temperature on PaPVA activity:

The PaPVA enzyme exhibited maximum activity at an optimum pH of 5 (acetate buffer) in the assay (Fig. 2.3a). The enzyme showed a narrow pH spectrum (4-6) of activity and was most active in the acidic pH range. The PVA from B. sphaericus (Olsson et al. 1985) shows a similar optimum pH, while PVAs from Fusarium oxysporum (Lowe et al. 1986) and Streptomyces lavendulae (Torres et al. 2003) are more active at pH 7-9. The optimum temperature for activity of PaPVA was 45°C (Fig. 2.3b); the enzyme showed very little activity when temperatures reached 60°C.

2.3.5. pH stability of PaPVA:

The enzyme was functionally stable in the pH range 3-7 (Fig. 2.4a) with than 80% retention in activity after 4 h. There was significant reduction in activity at pH 9-11, with only 40% of the original activity retained. The enzyme was rapidly inactivated at pH 1-2. The stability of PaPVA and optimum activity in acidic pH signify the potential of the enzyme to be used in large-scale production of 6-APA (Shewale and Sudhakaran 1997).

The fluorescence intensity of the enzyme showed emission maxima (λ_max) at 335 nm. The intensity significantly decreased at extreme pH (1-2 and 9-11),
corresponding to a reduction in enzyme activity (Fig. 2.4b). The far UV CD spectrum of the native PaPVA enzyme at pH 5 (Fig. 2.4c) showed a minimum at 218 nm and was typical of a protein with β-sheet and α-helix structure. Some rearrangement of the structure was evident at pH 1 with minima of 213 nm and significant reduction in negative minima. In the case of CD spectra in the aromatic region (250-320 nm), the enzyme was featureless at pH 1 indicating a loss of structure (Fig. 2.4d). These results agree well with the modulation of PaPVA activity observed at different pH.

![Graphs showing PaPVA stability and CD spectra](image)

**Fig. 2.4.** (a) PaPVA stability at different pH: Residual activity after 4h at 25°C. (b) Fluorescence spectrum of PaPVA at different pH at 25°C. (c) Far-UV CD spectra of PaPVA after 4 h incubation at different pH. (d) Near-UV CD spectra of PaPVA after 4 h incubation at different pH.
2.3.6. Thermal stability of \textit{PaPVA}:

There was a gradual reduction in the enzyme activity with increase in temperature (Fig. 2.5a). The enzyme (40 μg/ml) retained 87% of its original activity at 30°C and 50% activity at 50°C after 2h. \textit{PaPVA} showed significant loss of activity only at 80°C; it lost 90% activity within 15 min. There is a marginal improvement in stability at 60°C over previously reported bacterial PVA/BSH enzymes.

![Graphs showing thermal stability](image)

**Fig. 2.5.** (a) Effect of temperature on \textit{PaPVA} stability: Residual activity after incubation at 30-70°C for different time intervals. (b) \textit{PaPVA} fluorescence intensity at $\lambda_{\text{max}}$ as a function of temperature (incubation time 15 min) (c) Far-UV CD spectra of \textit{PaPVA} after incubation at 30-70°C for 15 min. (d) Near-UV CD spectra of \textit{PaPVA} after incubation at 30-70°C for 15 min.
Chapter 2: Characterization of PaPVA

While the PVAs from *Bacillus* spp. or BSHs show loss of activity and collapse of tertiary structure within 15 min at 60°C (Rathinaswamy et al. 2012), PaPVA was observed to retain at least 80% of original activity till at least 30 min. At higher concentrations (0.4 mg/ml) and in the presence of 100mM NaCl, the PaPVA enzyme was able to retain its full activity up to 6 h at 50°C. This is an additional favourable feature for industrial applicability of PaPVA, besides high yield and specific activity.

Increase in temperature led to a linear reduction of fluorescence intensity of PaPVA (Fig. 2.5b), without any change in $\lambda_{\text{max}}$. The intensity decreased to 40% of the original at 65°C. The PaPVA enzyme contains 8 trp residues per monomer, two of which are situated in the active site. The decrease in intensity might be due to quenching of tryptophan fluorescence and exposure of aromatic residues to the solvent, as a result of thermal inactivation.

The enzyme also showed no significant loss of secondary or tertiary structure till 70°C as revealed by the CD spectra (Fig.2.5 c, d).

2.3.7. Effect of Guanidine hydrochloride on PaPVA:

*PaPVA* showed enhanced activity in the presence of low concentrations of Gdn-HCl (up to 1M) (Fig. 2.6a). Using size exclusion chromatography, we observed that the enzyme formed soluble aggregates in the absence of NaCl; the aggregates disappeared when the protein was incubated in low concentrations of Gdn-HCl till 1M (Fig. 2.6b). Gdn-HCl at low concentrations has been known to confer effective charge shielding (Monera et al. 1994) and prevent aggregation in proteins (Hevehan et al. 1997). In the case of dihydrofolate reductase (Fan et al. 1996), activity enhancement by Gdn-HCl has been attributed to increased conformational flexibility in the active site, thus helping in more favourable binding of the substrate. Thus, it is possible that the increase in *PaPVA* activity could be a consequence of interaction with Gdn-HCl leading to deaggregation and conformational changes at the active site of the enzyme. The enzyme started progressively losing activity after 1.5M Gdn-HCl, and was totally inactivated at concentrations over 2M.
Fig. 2.6. (a) Relative activity of PaPVA after incubation with increasing concentrations of Guanidine hydrochloride (Gdn-HCl, 0-6M) for 4 h. (b) Size exclusion chromatography of PaPVA in 20 mM acetate buffer pH 5 (...) and Gdn-HCl treated enzyme (--). (c) Fluorescence spectrum of PaPVA after incubation with Gdn-HCl for 4 h. (d) Far UV CD spectra of Gdn-HCl treated PaPVA.

The fluorescence $\lambda_{\text{max}}$ shifted to 355 nm (Fig. 2.6c) with increase in Gdn-HCl concentrations above 1.5 M. The 335/355 OD ratio decreased from 1.36 to 0.93 indicating the exposure of tryptophan residues to the polar environment. Unfolding was maximum at 2 M, as indicated (Fig. 2.6d) by the far-UV CD spectra; this correlates well with the complete inactivation of the enzyme from this concentration of the denaturant. However, dilution of Gdn-HCl led to revival of the original enzyme activity within 1h, indicating that unfolding was completely reversible. The duration of treatment with Gdn-HCl and increasing temperatures have been known to influence the yield of protein on renaturation (Kathir et al. 2005).
2.3.8. Effect of protein modifiers on PaPVA activity:

The presence of 1mM DTT enhanced the enzyme activity by 14%, indicating active sulfhydryl group of cysteine present in PaPVA. The enzyme was stored in buffer containing 100 mM NaCl and 1mM DTT to ensure that the protein stays in active state with catalytic cysteine remaining in reduced form. There was no significant change in PaPVA activity in the presence of EDTA (Table 2.2). In the presence of metal ions that bind to sulfhydryl groups (Hg, Ag), the enzyme was completely inactivated within 15 min.

Table 2.2. Effect of additives (Reducing agents, chelating agents and detergents) on PaPVA activity. Enzyme without any modifier served as control (100%).

<table>
<thead>
<tr>
<th>Additives</th>
<th>Concentration</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>1 mM</td>
<td>114.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>123.2 ± 6.1</td>
</tr>
<tr>
<td>βME</td>
<td>1 mM</td>
<td>119.0 ±5.8</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>105.3 ± 3.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>101.1 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>101.1 ±6.3</td>
</tr>
<tr>
<td>CTAB</td>
<td>0.1 %</td>
<td>250.1±4.7</td>
</tr>
<tr>
<td></td>
<td>0.5 %</td>
<td>270.6±0.6</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1 %</td>
<td>180.4±1.3</td>
</tr>
<tr>
<td></td>
<td>0.5 %</td>
<td>185.3±0.5</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.1 %</td>
<td>175.1±4.3</td>
</tr>
<tr>
<td></td>
<td>0.5 %</td>
<td>153.3±2.9</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 %</td>
<td>8.9±1.7</td>
</tr>
<tr>
<td></td>
<td>0.5 %</td>
<td>0</td>
</tr>
</tbody>
</table>

The activity of PaPVA was significantly enhanced by treatment with detergents for 30 min at room temperature. (Table 2.2) Cationic detergent CTAB increased the
enzyme activity by 250%, while mild non-ionic surfactants (Tween 80, Triton) enhanced the activity by 175%, at concentrations higher than their CMCs. Enhancement of PVA activity in *R. aurantiaca* in the presence of detergents has been reported recently (Kumar et al. 2008). Detergents interact with the enzyme surface and play a role in enhancing the solubility and stability of proteins (Neugebauer 2000), and could also modulate kinetic behaviour of enzyme reactions in micelles (Abuin et al. 2007). The anionic detergent SDS, however, rapidly deactivated the *PaPVA* enzyme at 0.1% concentration.

*PaPVA* activity was also enhanced in the presence of organic solvents like isopropanol, acetone, butanone, acetonitrile, hexane and ethyl acetate at 5% (v/v) concentration in the assay mixture (Table 2.3). The protein was stable for 4 h in 10% isopropanol, retaining 80% of its original activity; higher concentrations deactivated the enzyme rapidly (data not shown). Aprotic solvents (DMSO, DMF, dioxan and tetrahydrofuran), and non-polar hydrocarbons (chloroform, dichloromethane) inhibited or deactivated the enzyme.

**Table 2.3. Modulation of *PaPVA* activity in presence of organic solvents. Enzyme with water served as control (100%).**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% (v/v)</td>
</tr>
<tr>
<td>Methanol</td>
<td>74.9 ± 2.1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>82.0 ± 0.8</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>194.6 ± 3.6</td>
</tr>
<tr>
<td>Acetone</td>
<td>106.1 ± 1.6</td>
</tr>
<tr>
<td>Butanone</td>
<td>174.0 ± 2.7</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>165.0 ± 3.4</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>147.5 ± 3.0</td>
</tr>
<tr>
<td>Hexane</td>
<td>113.0 ± 1.8</td>
</tr>
<tr>
<td>DMSO</td>
<td>57.5 ± 1.4</td>
</tr>
</tbody>
</table>
Arroyo et al. (1999) have studied the enhancement of *S. lavendulae* PVA activity and its stability in water-organic co-solvent monophasic systems. Solvents like dioxan, tetrahydrofuran (THF) are hydrophobic with high salvation capacity and thus are strong denaturants. On the other hand, alcohols are highly nucleophilic and could replace water in the deacylation step of Pen V hydrolysis, thus ensuring faster removal of product and enhanced activity (Arroyo et al. 1999, 2000). However, it is to be noted that in PaPVA only isopropanol was observed to increase the enzyme activity.

### 2.3.9. Kinetics of Pen V binding:

PVAs and BSHs reported so far have followed normal Michaelis-Menten (MM) kinetics (Olsson et al. 1985; Rathinaswamy et al. 2012; Kumar et al. 2006). However, substrate binding in PaPVA was more complex with distinct deviation from MM kinetics, showing cooperative behaviour and substrate inhibition (Fig. 2.7a). Chen and Tanaka (2011) have reported a similar behaviour in the case of *Lactococcus lactis* prolidase, an enzyme which hydrolyzes proline-containing dipeptides. The hexameric enzyme Aspartate transcarbamoylase (ATC) has also been reported to show substrate inhibition at pH 7.8-9.1, along with its cooperative nature (Pastra-Landis et al. 1978). PaPVA showed normal allosteric saturation behaviour in the 5-80 mM concentration range, while the 120-960 mM region is due to the inhibition at high substrate concentrations. The intermediate 80-120 mM section exhibits two opposing effects – activity due to normal Pen V binding and inhibition due to possible unproductive binding of the substrate in the enzyme active site. The substrate inhibition in PaPVA was near complete, with the activity nearing zero at very high Pen V concentrations.

The kinetic parameters for PaPVA enzyme (K_{0.5}, K_i, h and V_max) were computed using equation (2.1) as described earlier (Fig. 2.7a). The maximum velocity for PaPVA (V_{max} = 758.5 IU/g) is greater than any other acylase active on Pen V reported so far. The substrate concentration at half-maximum saturation (K_{0.5}) was 40.8 mM, which is comparable to the K_m of BspPVA (40 mM).
Fig. 2.7. Kinetic analysis of PaPVA. (a) $v$ vs $[S]$ curve with Pen V as substrate (5-960 mM); inset: kinetic parameters. (b) $v$ vs $[S]$ curve with synthetic substrate NIPOAB (0.5-12 mM).

PaPVA showed a Hill’s coefficient of 2.04, indicating apparent positive cooperativity. Cooperative behaviour of bile salt hydrolase has been reported in *Lactobacillus salivarius* (Bi et al. 2013), while the enzyme showed curves similar to MM kinetics when DTT was present in the assay mixture. However, in the case of PaPVA, the presence or absence of DTT did not cause any significant change in the allosteric nature of the enzyme. In addition, the occurrence of substrate inhibition ($K_i = 163.1$ mM for PaPVA) is possibly unique to Gram-negative PVS. Substrate inhibition has rarely been reported in any cholyglycine hydrolase so far (Rathinaswamy et al. 2012; Kumar et al. 2006). Kinetic studies using washed cells of PVA producing fungi *Fusarium oxysporum* have indicated weak substrate inhibition ($K_i = 900$ mM) (Lowe et al. 1986). Efforts are being made to further understand the dynamics of Pen V binding and the structural mechanism of cooperative behaviour substrate inhibition in PaPVA. In the case of PGA, Novikov et al. (2013) have proposed that the apparent substrate inhibition is a result of unproductive substrate binding, although it happens at the same active site.

The enzyme rate also showed allosteric behaviour with increasing concentrations of the synthetic substrate NIPOAB (Fig. 2.7b). However, the $v/[S]$ curve couldn’t reach saturation, since the solubility of NIPOAB was restricted to 12mM in 2% DMSO; higher DMSO concentrations proved deleterious for enzyme activity.
In conclusion, the PaPVA isolated from the Gram-negative plant pathogen *P. atrosepticum* shows certain interesting properties so far not reported in PVAs. Both the yield of recombinant protein and the specific activity of the enzyme are very high, auguring well for application in the pharmaceutical industry for 6-APA production. The enzyme exhibited uncharacteristic kinetic behaviour, showing positive cooperativity coupled with substrate inhibition. Further biochemical and structural analysis on PaPVA could help unravel the dynamics of similar enzymes from other Gram-negative bacteria, so as to develop them for industrial applications and also understand the place of such enzymes in microbial physiology.