## Contents

**Synopsis**

Page No. I-VIII

**List of Figures**

**List of Tables**

### Chapter 1 - Introduction

1. A1. Phosphorylation event in bacteria

2. A2. Hydrolysis of carboxyl phosphate bond – A tale about another Phosphatase

3. A3. Structural aspects of Acylphosphatase in light of its catalytic action


5. A5. Role of Tyrosine phosphorylation in bacteria

6. A6. Bacterial tyrosine kinase (BYK) and Bacterial tyrosine phosphatases (BYP)

7. A7. Low Molecular Weight PhosphoTyrosine Phosphatase

8. A8. Structural and functional aspects of BYP with respect to *Vibrio cholera*O395-

   Low molecular weight phosphotyrosine phosphatases (Vc-LMWPTP)

9. B1. Protein X-ray Crystallography to study the structure-function relationship of the

   aforementioned phosphatases


11. B3. Protein preparation


13. B5. Data processing step


15. B7. Refinement and Phase improvement

16. B8. Validation and deposition of the structure

### Chapter 2 - Materials & Methods

2. Cloning Over-Expression, Purification, Crystallization, Data Collection, Processing and Refinement

3. 2.1. Cloning and Over-expression of the proteins as 6× His-tagged construct
2.1A. Cloning and over-expression of the wild type Vc-AcP and its mutant
2.1B. Cloning and over-expression of the wild type VcLMWPTP-1 and its mutant
2.1C. Cloning and over-expression of the wild type VcLMWPTP-2 and its mutants
2.1D. Polymerase Chain Reaction (PCR)
2.1E. Site directed mutagensis (SDM)
2.1F. Digestion and Ligation
2.1G. Transformation and Analysis
2.1H. Protein over-expression

2.2. Purification of recombinant proteins in E. coli using affinity chromatography and size exclusion chromatography
2.2A. Purification of 6×His-tagged proteins using affinity chromatography
2.2B. Removal of His-tag with thrombin
2.2C. Fast Protein Liquid Chromatography (FPLC) using Sephacryl S-100

2.3. Characterization of protein
2.3A. Dynamic Light Scattering (DLS)
2.3B. Characterization of protein by Transmission Electron Microscopy (TEM)
2.3C. Chemical Cross Linking

2.4. Crystallization of target proteins
2.5. Data Collection and Processing
2.6. Structure determination of the target proteins
2.6A. Molecular Replacement (MR) method
2.6B. Refinement and Model Building step

2.7.Structural analysis

2.8. Enzyme assay
2.8A. Enzyme kinetics of Vc-AcP and its mutant
2.8B. Enzyme kinetics of VcLMWPTPs and their mutants
Chapter 3 - Results & Discussion

3.1. Acylphosphatase (Vc-AcP) ........................................................................................................39
  3.1A. Cloning and Overexpression of Vc-AcP and its mutant ............................................................39
  3.1B. Ni-NTA affinity chromatographic purification of the target proteins .....................................39
  3.1C. Thrombin cleavage standardization and Fast Protein Liquid Chromatography (FPLC) .........40
  3.1D. Dynamic Light Scattering (DLS) profile of Vc-AcP and Vc-AcP-C20R .................................40
  3.1E. Crystallization of Vc-AcP and Vc-AcP-C20R .......................................................................42
  3.1F. Data collection and processing of Vc-AcP and Vc-AcP-C20R ..................................................43
  3.1G. Structure solution of Vc-AcP-C20R ..........................................................................................45
  3.1H. Structure solution of Vc-AcP .....................................................................................................46
  3.1I. Structural analysis of Vc-AcP-C20R: Overall structure of Vc-AcP-C20R monomer ...............48
  3.1J. Crystal structure of Vc-AcP-C20R reveals a trimeric assembly involving a set of unique residues ............................................................................................................................48
  3.1K. Active site structure of Vc-AcP-C20R .....................................................................................50
  3.1L. Nature of electrostatic surface of Vc-AcP-C20R .....................................................................51
  3.1M. Structural analysis of Vc-AcP: Overall crystal structure of Vc-AcP ..........................................52
  3.1N. Arrangement and symmetry of the dodecameric protein cage .................................................54
  3.1O. Nature of electrostatic surface of Vc-AcP .................................................................................57
  3.1P. Role of unique residues in the formation of ordered multimeric structures: An outcome of Multiple Sequence Alignment result for Vc-AcP .................................................................58
  3.1Q. Kinetic Studies of Vc-AcP-C20R .............................................................................................59
  3.1R. Transmission Electron Microscopy (TEM) .............................................................................60
  3.2. Low Molecular Weight Protein Tyrosine Phosphatase-1 (VcLMWPTP-1) ............................61
  3.2A. Cloning and Overexpression of VcLMWPTP-1 and its mutant ..............................................61
  3.2B. Ni-NTA affinity chromatographic purification of the target proteins .....................................61
  3.2C. Thrombin cleavage standardization and Fast Protein Liquid Chromatography ....................62
  3.2D. Determination of oligomers using FPLC .................................................................................63
  3.2E. Enzyme kinetics .......................................................................................................................65
  3.2F. Crystallization of VcLMWPTP-1 .............................................................................................65
  3.2G. Data collection and Processing of VcLMWPTP-1 ..................................................................66
  3.2H. Structure solution of VcLMWPTP-1 .......................................................................................67
  3.2I. Structural analysis of VcLMWPTP-1: Overall structure of VcLMWPTP-1 monomer ............69
  3.2J. Active site architecture of VcLMWPTP-1 .................................................................................70
3.2K. Detection of different conserved ‘Motif’ s in the LMWPTP family - A comparison based on the Multiple Sequence Alignment result.................................................................72

3.2L. Dimerization of VcLMWPTP-1.................................................................................................................75

3.2M. Insight into differential substrate specificity: Diverged surface property of the active site.................................................................77

3.2N. Glutaraldehyde crosslinking to capture the dimeric state in solution.................................79

3.3. Low Molecular Weight Protein Tyrosine Phosphatase-2 (VcLMWPTP-2).........................80

3.3A. Cloning and Overexpression of VcLMWPTP-2 and its mutants....................................................80

3.3B. Ni-NTA affinity chromatographic purification of the target proteins........................................80

3.3C. Thrombin cleavage standardization and Fast Protein Liquid Chromatography..............81

3.3D. Enzyme kinetics........................................................................................................................................81

3.3E. Crystallization of VcLMWPTP-2........................................................................................................82

3.3F. Data collection and Processing of VcLMWPTP-2........................................................................82

Chapter 4- Discussion and Summary

4. Vc-Acp and Vc-AcP-C20R........................................................................................................................85

4.1A. Unique set of residues involved in the tetrahedral cage formation of Vc-AcP and triangular Vc-AcP-C20R.................................................................85

4.1B. Single mutation C20R on Vc-AcP affect the assembly formation........................................86

4.1C. Designer cage- new horizon to carrier molecule..............................................................................89

4.2. VcLMWPTP-1.............................................................................................................................................91

4.2A. VcLMWPTP-1 forms enzymatically active dimer in solution.................................................................91

4.2B. Molecular details of the dimeric crystal structure of VcLMWPTP-1 reveal the altered mode of dimerization ......................................................................................91

4.2C. Electrostatics surface indicates similarity of VcLMWPTP-1 with eukaryotic LMWPTPs.................................................................................................................92

4.3. VcLMWPTP-2.............................................................................................................................................93

References

Publications
List of Figures

Figure 1: Proposed catalytic mechanism for Acylphosphatase

Figure 2: The catalytic mechanism followed by LMWPTPs

Figure 3: Genomic organization of BY-kinases and their cognate phosphotyrosine-phosphatase genes in proteobacteria

Figure 4: The Phase Diagram in Protein Crystallization

Figure 5: Comparison of ‘hanging drop’ and ‘sitting drop’ Vapor Diffusion method

Figure 6a-6b. Cloning and overexpression check of Vc-AcP

Figure 7. 15% SDS-PAGE profile showing the Ni-NTA purification profile of Vc-AcP

Figure 8a-8d. Dynamic light scattering profiles of Vc-AcP and Vc-AcP-C20R

Figure 9a-9d. Dynamic light scattering profiles of Vc-AcP with different oxyanions

Figure 10a-10b. Crystals of Vc-AcP & Vc-AcP-C20R

Figure 11a-11b. Diffraction pattern of Vc-AcP & Vc-AcP-C20R crystals upon exposure to X-rays

Figure 12a-12b. Structure of monomeric AcP and Vc-AcP-C20R

Figure 13a-13b. Molecular details of Vc-AcP-C20R and its active site

Figure 14. Molecular details of the active site Vc-AcP-C20R

Figure 15a-15b. Electrostatic surface view of Vc-AcP-C20R

Figure 16a-16b. Dodecameric assembly of Vc-AcP

Figure 17a-17c. Different faces of Vc-AcP

Figure 18a-18c. Molecular details of the ST-face and sulfate assembly

Figure 19. Symmetry elements in Vc-AcP protein cage with tetrahedral symmetry.

Figure 20a-20c. Electrostatic surface view of different faces of Vc-AcP

Figure 21a-21b. Electrostatic view of exterior and interior of Vc-AcP cage

Figure 22. Multiple sequence alignment of Vc-AcP

Figure 23. Reaction kinetics of acetylphosphate by Vc-AcP-C20R and Vc-AcP

Figure 24. TEM image

Figure 25a-25b. Cloning and overexpression check of VcLMWPTP-1
Figure 26. 15% SDS-PAGE profile showing the Ni-NTA purification profile of VcLMWPTP-1

Figure 27. Thrombin cleavage standardization of VcLMWPTP-1

Figure 28. Existence of oligomeric VcLMWPTP-1 in solution by FPLC

Figure 29. FPLC analysis of VcLMWPTP-1 with different protein and salt concentrations

Figure 30a-30b. Enzyme kinetics of VcLMWPTP-1 and C8S

Figure 31a-31b. Crystals and diffraction pattern of VcLMWPTP-1

Figure 32a-32b. Overall structure and ligand (MOPS) binding at the active site of VcLMWPTP-1

Figure 33. Stereo representation of the active site of VcLMWPTP-1

Figure 34a-34b. Zoomed view of the active site of VcLMWPTP-1

Figure 35. Similarity of binding of MOPS with that of phosphotyrosine binding

Figure 36. Structure based multiple sequence alignment of VcLMWPTP-1 and other LMWPTPs from different organisms

Figure 37a-37d. Comparison of dimeric interface of VcLMWPTP-1 with that of bovine LMWPTP

Figure 38. Comparison of the electrostatic surface around the active site cleft of LMWPTPs from different organisms

Figure 39a-39b. Glutaraldehyde crosslinking of VcLMWPTP-1.

Figure 40a-40b. Cloning and overexpression check of VcLMWPTP-2

Figure 41. 15% SDS-PAGE profile showing the Ni-NTA purification profile of VcLMWPTP-2

Figure 42. Enzyme kinetics of VcLMWPTP-2 and its mutants

Figure 43a-43b. Crystals and diffraction pattern of VcLMWPTP-2

Figure 44. Superposition of 12 monomers of Vc-AcP and monomer of Vc-AcP-C20R

Figure 45. Superposition on Vc-AcP-C20R on Vc-AcP cage

Figure 46. Vc-AcP-C20R superposed on Vc-AcP showing possible clashes which prevent the mutant cage formation

Figure 47. Construction of disulfide-bridged cage.

Figure 48. Schematic representation of programmable designer protein cage
List of Tables

Table 1. Functions of several BYPs from different organisms
Table 2. Function of paired BYP and BYK from several gram-positive and gram-negative bacteria
Table 3: Primer Sequences of wild type proteins
Table 4: Primer Sequences of mutant proteins for the 1st step PCR of SDM
Table 5: Composition of the PCR reaction mixture
Table 6: Calculation of the two-step-PCR reaction mixture
Table 7: Composition of the restriction enzyme double digestion and ligation
Table 8: Buffer compositions for the purification of recombinant proteins
Table 9: Crystallization conditions of recombinant proteins
Table 10: Data collection statistics of all crystals
Table 11: Data collection and processing statistics of Vc-AcP-C20R and Vc-AcP
Table 12: Improvement of Phaser statistics of Vc-AcP-C20R and Vc-AcP
Table 13: Data collection, phasing and refinement statistics of Vc-AcP and Vc-AcP-C20R
Table 14: PISA server data for Vc-AcP-C20R and Vc-AcP
Table 15: Data Collection and processing statistics of VcLMWPTP-1
Table 16: Improvement of Phaser statistics of VcLMWPTP-1
Table 17: Statistics for data collection and refinement of crystal structure of VcLMWPTP-1
Table 18: Motif table
Table 19: Results of VcLMWPTP-1 (one dimer) from the PISA server
Table 20: Data Collection and processing statistics of VcLMWPTP-2