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Purification, crystallization and preliminary X-ray analysis of an aminoacylhistidine dipeptidase (PepD) from *Vibrio alginolyticus*

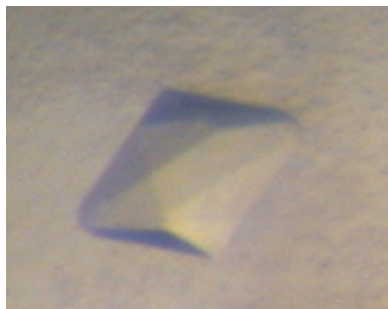
The aminoacylhistidine dipeptidase (PepD) protein encoded by *Vibrio alginolyticus* *pepD* was successfully overexpressed and characterized and the putative active-site residues responsible for metal binding and catalysis were identified. The purified enzyme contained two zinc ions per monomer. The recombinant dipeptidase enzyme, which was identified as a homodimer in solution, exhibited broad substrate specificity for Xaa-His dipeptides, with highest activity towards the His-His dipeptide. The purified protein was crystallized using the hanging-drop vapour-diffusion method. Preliminary crystallographic analysis showed that the crystal belonged to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 80.42$, $c = 303.11$ Å. The crystal contained two molecules per asymmetric unit and the predicted solvent content was 53.4%.

1. Introduction

Vibrio alginolyticus is one of the most common and important pathogens that cause vibriosis in human and marine species (Morris & Black, 1985; Levine & Griffin, 1993; Marano *et al.*, 2000). For example, infection of fish by *V. alginolyticus* results in biofilm formation in their intestine, which leads to fish mortality and potentially significant economic losses (Lee, 1995). Human infection by *V. alginolyticus* is caused by the consumption of raw or undercooked seafood (Blake *et al.*, 1980; Rose *et al.*, 2001). The major clinical symptoms of *V. alginolyticus* infection of humans are wound infection, gastroenteritis and septicaemia (Blake *et al.*, 1980; Hlady & Klontz, 1996; Rose *et al.*, 2001). Thus, prevention, early detection and treatment of *V. alginolyticus* infections are important for the health of humans and marine animals.

Aminoacylhistidine dipeptidase (PepD; EC 3.4.13.3) is a member of metallopeptidase family M20 in the metallopeptidase H (MH) clan (Rawlings & Barrett, 1995). The majority of cocatalytic metallohydrolases are Zn^{2+} -dependent enzymes, but some require Mn^{2+} , Co^{2+} or other divalent metal ions for activity. The PepD enzyme catalyzes the cleavage and release of an N-terminal amino acid (usually a neutral or hydrophobic residue) from an Xaa-His dipeptide or other degraded peptide fragments (Rawlings & Barrett, 1995). The enzyme also exhibits a broad substrate specificity which includes the unusual dipeptides carnosine (β -Ala-His) and homocarnosine (γ -amino-butyl-His) and several distinct tripeptides. These enzymes play fundamental roles in certain biochemical events, such as protein maturation and degradation, tissue repair and cell-cycle control (Chen *et al.*, 2008). The enzymes of the M20 family may also play fundamental roles in aging and neurodegenerative or psychiatric diseases (Teufel *et al.*, 2003) and hence may have great potential for application as antibacterial targets or therapeutic agents (Hellen-doorn *et al.*, 1997).

The *pepD* gene from *V. alginolyticus* contains an open reading frame (ORF) of 1473 nucleotides and codes for a 490-amino-acid protein. Bioinformatic analysis of the *V. alginolyticus* PepD sequence reveals its high homology to those from other *Vibrio* species (94–76% identity) and bacteria (75–63% identity). Sequence-based alignment



of *V. alginolyticus* PepD with peptidase clan MH proteins of known structure, such as *Aeromonas* (*Vibrio*) *proteolyticus* aminopeptidase (AAP; Chevrier *et al.*, 1994), *Streptomyces griseus* aminopeptidase S (SGAP; Greenblatt *et al.*, 1997), *Pseudomonas* sp. carboxypeptidase G2 (CPG2; Rowsell *et al.*, 1997), *Salmonella typhimurium* PepT (Håkansson & Miller, 2002), human aminoacylase 1 (hACy1; Lindner *et al.*, 2003) and *Lactobacillus delbrueckii* peptidase V (PepV; Jozic *et al.*, 2002), showed low sequence identities in the range 7–20% and low sequence similarities in the range 13–34%. Despite the lack of detectable sequence homology, the putative active-site residues for catalysis are relatively conserved in PepD and related di-zinc enzymes from the M20 family (Rowsell *et al.*, 1997; Jozic *et al.*, 2002). His80, Asp119, Glu150, Asp173 and His461 have been predicted to be involved in metal binding, whereas Asp82 and Glu149 are essential for catalysis. These residues were completely conserved apart from Asp173, which is present in homologues with aminopeptidase/dipeptidase specificity, whereas aminoacylase/carboxypeptidases contain a glutamic acid at the same position. Here, we report the cloning and expression of the *V. alginolyticus* *pepD* gene, the purification of the produced recombinant PepD protein and the preliminary X-ray diffraction characterization prior to crystal structure determination to reveal the overall structural features and the spatial locations of the putative active-site residues.

2. Materials and methods

2.1. Molecular cloning of the *V. alginolyticus* *pepD* gene

The *V. alginolyticus* *pepD* gene was amplified from the *V. alginolyticus* ATCC 17749 genomic DNA library. Two primers (F1, 5'-GTGTCTGAGTTCCATTC-3', and R1, 5'-TTACGCCTTTTCAGGAA-3'), based on the highly conserved 5'-end and 3'-end nucleic acid sequences of *Vibrio* spp. *pepD*, were designed to amplify the putative *V. alginolyticus* *pepD* gene. An ORF that contains 1473 nucleotides and codes for a polypeptide chain of 490 amino acids was identified. Sequence analysis indicated a protein with an estimated molecular mass of approximately 53.6 kDa and an isoelectric point of pH 4.7. The nucleotide sequence of the *V. alginolyticus* *pepD* gene was deposited in the GenBank database (accession No. DQ335448).

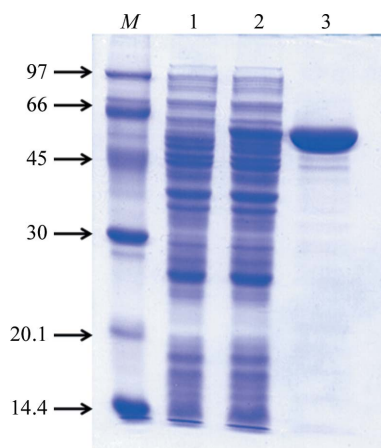


Figure 1
12.5% SDS-PAGE of the purified *V. alginolyticus* PepD protein. Lane M, marker proteins (kDa): phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). Lane 1, crude cell extracts of *E. coli* BL21(DE3)pLysS carrying the pET-28a(+) plasmid. Lane 2, crude cell extracts of *E. coli* BL21(DE3)pLysS carrying the pET-28a(+)-*pepD* plasmid. Lane 3, purified PepD from the Ni-NTA column.

2.2. Production and purification of recombinant *V. alginolyticus* PepD protein

The *V. alginolyticus* *pepD* gene was subcloned into the expression plasmid pET28a(+) and subsequently transformed into *Escherichia coli* BL21(DE3)pLysS cells for recombinant protein production and purification. The production of PepD protein was induced by the addition of 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and continuous incubation for 6 h at 310 K. After 6 h incubation with rotary shaking at 310 K, the cells were collected by centrifugation at 9300g for 30 min at 277 K. The medium was discarded and the cell pellet was resuspended in binding buffer (10 ml) containing 0.5 M NaCl and 20 mM Tris-HCl pH 6.8. The resuspended cells were disrupted by sonication using a 2 s on/1 s off pulsation cycle with a total sonication time of 3 min at 30% energy on ice. The supernatant was collected by centrifugation at 12 000g for 30 min at 277 K. The recombinant PepD protein was purified using an Ni-NTA column and eluted with imidazole. The crude protein containing PepD was loaded onto an Ni-NTA column that had previously been washed with a ten-column volume of buffer A (0.5 M NaCl, 20 mM Tris-HCl pH 6.8) and 60 mM imidazole. The PepD protein was eluted with buffer A containing stepwise increasing concentrations of 100, 200, 300 and 500 mM imidazole. The eluted fractions with PepD enzymatic activity were collected and dialyzed into 50 mM Tris-HCl pH 6.8 before use in crystallization. The purified PepD was concentrated to 10 mg ml⁻¹ using a Centricon (10 kDa molecular-weight cutoff; Amicon Ultra, Millipore) in 20 mM Na HEPES buffer pH 6.8. The purity of the protein was confirmed using 12.5% SDS-PAGE with Coomassie Brilliant Blue R-250 staining.

2.3. Crystallization

Crystallization trials were carried out using the hanging-drop vapour-diffusion method at 291 K and initial screening of crystallization conditions was carried out using Crystal Screens 1 and 2 (Hampton Research) in 48-well plates at 293 K. 1 μ l protein solution (10 mg ml⁻¹) and 1 μ l reservoir solution were mixed in each drop and the drops were equilibrated against 200 μ l reservoir solution.

2.4. X-ray data collection and processing

The protein crystals were initially screened and characterized at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan using synchrotron-radiation X-rays on the SPXF beamline BL13B1 equipped with a CCD (Q315, ADSC) detector. Data

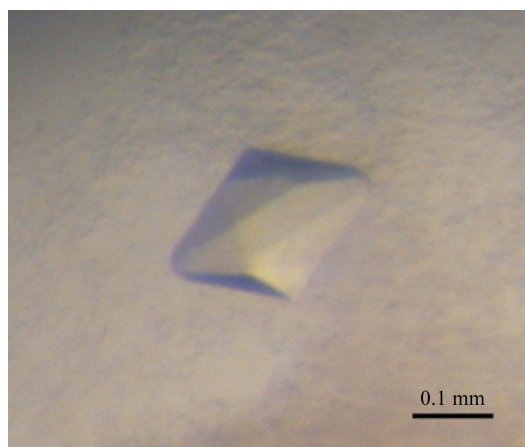


Figure 2
A single crystal of *V. alginolyticus* PepD grown by the hanging-drop method.

Table 1

X-ray diffraction statistics for the PepD crystal.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.00 (SPRING-8 BL12B2)
Temperature (K)	110
Resolution range (Å)	30.0–3.0
Space group	$P6_5$
Unique reflections	54648
Completeness (%)	99.8 (99.9)
$\langle I/\sigma(I) \rangle$	23.8 (6.2)
Average redundancy	4.3
$R_{\text{merge}}^{\dagger}$ (%)	6.1 (32.3)
Mosaicity (°)	0.6
Unit-cell parameters (Å)	$a = 80.42$, $c = 303.11$
No. of molecules per ASU	2
V_M (Å ³ Da ^{−1})	2.63
Solvent content (%)	53.4

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the weighted average intensity for all observations i of reflection hkl .

collection was completed at the Taiwan-contracted beamline BL12B2 equipped with a CCD (Quantum-4R, ADSC) detector at SPRING-8 in Japan. The crystal was transferred into a cryoprotectant solution containing 15% (v/v) glycerol for a few seconds, mounted on a loop (0.2–0.3 mm; Hampton Research) and then flash-cooled in liquid nitrogen. For complete X-ray data collection, 300° of rotation were measured in 1.0° oscillations using X-rays of wavelength 1.00 Å with 15 s exposures and a crystal-to-detector distance of 350 mm at 110 K in a nitrogen stream generated using a cryosystem (X-Stream, Rigaku Inc.). All data were indexed, integrated, scaled and merged using HKL-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

The putative aminoacylhistidine dipeptidase PepD from *V. alginolyticus* was overproduced in *E. coli* and purified. SDS-PAGE of the purified PepD showed a single band with a molecular mass of approximately 54 kDa (Fig. 1). Sequence and structural homologies suggested that the enzyme was a member of the metal-dependent metalloprotease family. Homology comparisons of *V. alginolyticus* PepD, using *L. delbrueckii* PepV as the template, revealed that the putative metal-binding residues are almost superimposed on each other, with the exception of Asp119, which is likely to be the residue that holds the two zinc ions in PepV (Jozic *et al.*, 2002). The conserved Asp119 of PepD aligned with Asp120 of PepV. This residue is adjacent to the metal-binding residue Asp119, whereas Ala118 in PepD is located at the corresponding position to Asp119 in PepV (Wang *et al.*, 2008).

Good-sized diamond-shaped crystals appeared within six months in a condition consisting of 100 mM Na HEPES buffer pH 7.5, 28% (v/v) PEG 400 and 200 mM CaCl₂ and grew to maximum dimensions of 0.3 × 0.2 × 0.1 mm (Fig. 2). The protein crystals were sensitive to the change in precipitant concentration on transfer to the cryoprotectant solution containing 15% (v/v) glycerol. Good-quality crystals were carefully screened and selected for data collection as

they frequently possessed fairly high mosaicities (>1°). The crystals produced diffraction data to 3.0 Å resolution on beamline BL12B2 at SPRING-8. Analysis of the diffraction pattern revealed that the crystals exhibited hexagonal symmetry and systematic absences indicated the space group to be $P6_1$ or $P6_5$. The unit-cell parameters were $a = 80.42$, $c = 303.11$ Å. If we assume the presence of two molecules in the asymmetric unit and a molecular mass of 54 kDa, the calculated solvent content is 53.4% and the Matthews coefficient (V_M) is 2.63 Å³ Da^{−1} (Matthews, 1968), which is within the normal range for protein crystals. The statistics of the collected data are summarized in Table 1. Structure determination by the molecular-replacement method using the aminopeptidase PepV from *L. delbrueckii* (Jozic *et al.*, 2002) as a search model is currently under way.

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