Chapter 2 Materials and Methods

2.1 Bacterial strains, plasmids, animal, and cell

Escherichia coli BL21(DE3)pLysS (Novagen)

Escherichia coli XL1-Blue (Novagen)

Vibrio alginolyticus ATCC 17749 (FIRDI, Taiwan)

pCR®2.1-TOPO (Invitrogen)

pET-28a(+) (Novagen)

Female BALB/c mice (National Science Council, Taiwan)

Mouse myeloma cell line FO (FIRDI, Taiwan)

2.2 Chemicals and Reagents

Acetic acid (Merck)

Acrylamide (GE Healthcare)

Agarose (USB)

α-Ala-L-His (Sigma)

APS (GE Healthcare)

β-Asp-L-His (Sigma)

Bacto™ Agar (DIFCO)

Bestatin (MP Biomedicals)

Bovine Calf Serum (HyClone)

Bromophenol blue (USB)

L-carnosine (ICN Biomedicals, Inc.)

Citric acid (Sigma)

Coomassie® Brilliant blue R 250 (Merck)

Dimethylformamide (Merck)

Dimethyl sulfoxide (MP Biomedicals)

Dodecyl sulfate sodium salt (Merck)

Dulbecco's Modified Eagle Medium (Gibco)

dNTP Set, 100 mM Solutions (GE Healthcare)

Ethylenediamine-tetraacetic acid (Merck)

Freund's Adjuvant, Complete (Sigma)

Freund's Adjuvant, Incomplete (Sigma)

GABA-His (Sigma)

L-glutamine solution 100X, 200mM (biowest)

Glycerol (Merck)

Glycine (Merck)

Gly-Gly-His (Sigma)

Gly-His (Sigma)

Gly-His-Gly (Sigma)

L-histidine (Sigma)

HAT Media Supplement (50X) Hybri-Max® (Sigma)

HT Supplement (100X), liquid (GIBCO)

Hydrogen chloride (Merck)

Imidazole (USB)

IPTG (GeneMark, Taiwan)

Kanamycin sulfate (USB)

LB Broth, Miller (DIFCO)

2-mercaptoethanol (Merck)

Methanol (Merck)

N,*N*'-methylene-bis-acrylamide (Sigma)

Ni-NTA His-Band® Resin (Novagen)

Penicillin-Streptomycin Solution 100X (biowest)

o-phthaldialdehyde (Merck)

Potassium chloride (Merck)

Potassium diphosphate (Merck)

Potassium phosphate (Merck)

Primers (Bio Basic Inc., Taiwan)

Pristane (Sigma)

Restriction enzymes (New England Biolabs)

Sodium azide (Merck)

Sodium chloride (AMRESCO)

Sodium hydroxide (Merck)

SYBR® Green I (Roche)

T4 DNA ligase (Promega)

TEMED (GE Healthcare)

Trichloroacetic acid (Merck)

Tris base (USB)

Tryptic soy broth (ALPHA BIOSCIENCES)

X-gal (GeneMark, Taiwan)

2.3 Kits

BCA Protein Assay Reagent and Albumin Standard (PIERCE)

BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)

GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare)

HMW Native Marker Kit (GE Healthcare)

LMW-SDS Marker Kit (GE Healthcare)

QIAamp DNA Mini Kit (Qiagen)

TOPO TA Cloning® Kit (Invitrogen)

Plasmid Miniprep Purification Kit (GeneMark)

r*Tth* DNA polymerase, XL & XL Buffer II Pack (Applied Biosystems)

2.4 Equipments

25 cm^2 flask (NUNC)

ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems)

Allegra™ 21R Centrifuge (Beckman Coulter)

Avanti® J-E Centrifuge (Beckman Coulter)

Blood Collecting Tubes (Chase Scientific Glass, Inc.)

Centrifuges 5415R (eppendorf)

Colling Circulator Bath Model B401L (Firstek Scientific)

Compact Tabletop Centrifuge 2100 (KUBOTA)

Dri-Bath Type 17600 (Thermolyne)

Durabath™ Water Bath (Baxter)

Econo-Pac Columns (BIO-RAD)

Electrophoresis Power Supply EPS 301 (GE Healthcare)

EPSON® GT-7000 Scanner (EPSON)

F96 MicroWell™ plate (black) (NUNC)

F96 MicroWell™ plate (clear) (NUNC)

Fisher Vortex Genie 2™ (Fisher Scientific)

Fluoroskan Ascent FL Microplate Reader (Thermo)

GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems)

Hoefer® HE 33 Mini Horizontal Submarine Unit (GE Healthcare)

Hoefer® Mighty Small dual gel caster (GE Healthcare)

Kodak Electrophoresis Documentation and Analysis System 120 (Kodak)

Mighty Small II for 8×7 cm gels electrophoresis instruments (GE Healthcare)

Millex®-GS 0.22 μm Filter Unit (Millipore)

Millex®-HA 0.45 μm Filter Unit (Millipore)

Multiskan Ascent Microplate Reader (Thermo)

Orbital shaking incubator Model S300R (Firstek Scientific)

Rocking Shacker Model RS-101 (Firstek Scientific)

Steritop™ 0.22 μm Filter Unit (Millipore)

Ultrasonic Processor VCX 500/750 (Sonics)

US AutoFlow™ NU 4000 Series CO_2 Water-Jacketed Incubator (NuAire)

UV-Visible Spectrophotometer Ultrospec 3100 pro (GE Healthcare)

2.5 Solutions

Blocking buffer

5% non-fat milk in distilled water (dH_2O).

Destain buffer I

Mix 400 mL methanol, 100 mL acetic acid and dH_2O to 1 L. Store at room temperature (RT).

Destain buffer II

Mix 50 mL methanol, 120 mL acetic acid and distilled water (dH_2O) to 1 L. Store at RT.

6X DNA loading dye

0.25% bromophenol blue and 30% glycerol in double distilled water (ddH_2O). Store at -20℃.

IPTG stock solution

Dissolve 4.0863 g IPTG in 10 mL ddH_2O. Filter through 0.22 μm pore size filter and store at -20℃.

Kanamycin stock solution

Dissolve 250 mg kanamycin sulfate in 10 mL ddH_2O. Filter through 0.22 μm pore size filter and store at -20℃.

LB medium

25 g LB Broth was dissolved in 1 L dH_2O and sterilized.

LB plate

25 g LB Broth and 20 g Bacto™ Agar was dissolved in 1 L dH_2O and sterilized. The sterile LB agar was poured and dispersed in petri dishes before it coagulates.

10X Native-PAGE running buffer

Dissolve 144 g glycine and 30 g Tris base in 1 L dH_2O and store at 4℃. Dilute to 1X with dH_2O before use.

5X Native-PAGE sample buffer

8 mg bromophenol blue, 1.7 mL 0.5 M Tris-HCl, pH 6.8, 5 mL glycerol, and 4 mL dH_2O were mixed and stored at -20℃.

OPA reagent (for enzyme kinetics)

Dissolve 50 mg OPA in 5 mL methanol first and then mix with 20 mL borate buffer. The borate buffer was mixed by 0.2 M boric acid (dissolved in 0.2 M potassium chloride solution) and 0.2 M sodium hydroxide solution (50: 50, v/v). The OPA reagent was stored in darkness at 4℃ for no longer than 9 days and prepared at least 90 min earlier before use.

10X PBS buffer

Dissolve 13.7 g Na_2HPO_4, 3.5 g NaH_2PO_4, and 87.7 g NaCl in 1 L dH_2O and store at RT. Dilute to 1X with dH_2O and sterilize before use.

10X SDS-PAGE running buffer

Dissolve 144 g glycine, 30 g Tris base, and 10 g SDS in 1 L dH_2O and store at 4℃. Dilute to 1X with dH_2O before use.

5X SDS-PAGE sample buffer

8 mg bromophenol blue, 1.7 mL 0.5 M Tris-HCl, pH 6.8, 0.5 mL 20% (w/v) SDS, 2 mL 2-mercaptoethanol, 5 mL glycerol, and 4 mL dH_2O were mixed and stored at -20℃.

Stain buffer

Dissolve 1 g Coomassie Brilliant blue R-250 in 500 mL methanol first. Then add 100 mL acetic acid and dH_2O to 1 L final volume. Filter through reused 0.22 μm pore size filter and store at RT.

50X TAE buffer

Dissolve Tris base 242 g, acetic acid 57.1 mL, and 0.5 M EDTA in 1 L dH_2O and adjust to pH 8.5. Dilute to 1X with dH_2O and adjust to pH 7.5-7.8 before use.

10X Western transfer buffer

Dissolve 144 g glycine, 30 g Tris base, and 10 g SDS in 1 L dH_2O and store at 4℃. Dilute to 1X with dH_2O before use.

X-gal stock solution

Dissolve 400 mg X-gal in 10 mL dimethylformamide (DMF) and store in the darkness at -20℃.

2.6 Identification of *V. alginolyticus pepD* Gene Sequence

2.6.1 Primer design for *V. alginolyticus pepD* identification

According to the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) databases, identified nucleic acid sequences of aminoacylhistidine dipeptidase (*pepD*) from *V. parahaemolyticus* RIMD 2210633 (BA000031), *Vibrio vulnificus* YJ016 (BA000037), and *Vibrio cholerae* O1 biovar eltor str. N16961 (AE004299) were aligned and analyzed by ClustalW (http://www.ebi.ac.uk/clustalw) to find the conserved sequences among *Vibrio* spp. *pepD* (Appendix 1). The highly conserved 5' and 3'-end nucleic acid sequences of *Vibrio* spp. *pepD* were selected and designed as primer F1 and R1, respectively (Appendix 2).

2.6.2 Extraction of *V. alginolyticus* genomic DNA

Stock of *V. alginolyticus* ATCC 17749 was cultured in 3 mL tryptic soy broth (TSB) medium supplemented with 3 % sodium chloride (NaCl) at 37℃ with continuous shaking for 12 hrs. Cultures (3 mL) were harvested by centrifugation at 13,200 rpm for 30 sec at RT. The supernatant was removed and *V. alginolyticus* genomic DNA was extracted from the resultant pellets using QIAamp DNA Mini Kit, following the manufacturer's guidelines (Qiagen).

2.6.3 PCR amplification of *pepD* from *V. alginolyticus*

V. alginolyticus pepD gene was obtained by polymerase chain reaction (PCR) method. Fifty-microliter reaction mixtures were prepared with 100 ng of *V. alginolyticus* genomic DNA as a template containing 1X XL Buffer II, 1.5 mM $Mg(OAc)_2$ solution, 1 μL (2 U) of r*Tth* DNA polymerase, 0.8 mM each deoxynucleoside triphosphate (dNTP), and a sense (F1) and an antisense (R1) primer each at 0.2 μM. Amplification reactions were performed in the

96-well GeneAmp® PCR System 9700 Thermal Cycler. *V. alginolyticus pepD* DNA was amplified by PCR as recommended by the manufacturer of r*Tth* DNA polymerase (94℃ for 45 sec, 56℃ for 1 min, and 72℃ for 2 min; 29 cycles) (Table 3). Five-microliter of each reaction mixture was combined with 1.5 μL SYBR Green and 1 μL 6X DNA loading dye. The each total 7.5 μL mixed sample was loaded onto a 1% agarose gel with 0.5X TAE buffer, and electrophoresis was performed at 120 Volt for 15 min. The gel was autoradiographied on Kodak film.

Table 3. Reaction conditions and cycling parameters for the PCR reaction.

V. alginolyticus genomic DNA	0.5
Primer F1 (10 μM)	1
R1 (10 μM)	1
dNTP mix (2.5 mM each)	4
3.3X XL Buffer II	15
$Mg(OAc)_2$ (25 mM)	3
ddH_2O	25
r*Tth* polymerase (2 U/μL)	0.5
Total	50 (μL)

Segment	Cycles	Temperature	Time
1	1	94℃	2 minutes
2	29	94℃	45 seconds
		56℃	1 minute
		72℃	2 minute
3	1	72℃	15 minutes
4	1	4℃	pause

2.6.4 DNA sequencing of *V. alginolyticus pepD*

The PCR products of *V. alginolyticus pepD* were subcloned into pCR®2.1-TOPO vector, following the manufacturer's guidelines (Invitrogen) to obtain pCR®2.1-TOPO-pepD plasmid. A 6 μL reaction containing 1 μL *pepD* PCR product, 0.5 μL TOPO vector and 1 μL salt solution incubated for 15 min at RT. Add *E. coli* XL1-Blue competent cells and incubate on ice for 15 to 20 min. The cells were transformed by heatshock method for 30 sec at 42℃ following 2 min on ice. The transformed cells were shaked at 200 rpm in 1 mL Luria-Bertani (LB) medium for 1 hr at 37℃ incubator and propagated on LB plates containing 25 μg/mL kanamycin (LB_{Kan}), 40 μL 40 mg/mL 5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside

(X-gal) and 10 μL 1 M isopropyl-1-thio-β-D-galactoside (IPTG). Incubate plates overnight at 37℃. Pick white colonies and culture in 3 mL LB medium containing 25 μg/mL kanamycin overnight at 37℃. The plasmid DNA was isolated by Plasmid Miniprep Purification Kit, following the manufacturer's guidelines (GeneMark).

The plasmids obtained were checked by restriction enzymes, PCR method and nucleotide sequencing. Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method with only one forward or reverse primers described on Appendix 2. Sequencing reactions were carried out with BigDye® Terminator v3.1 Cycle Sequencing Kit and the reaction mixtures were run on the ABI PRISM® 3100 Genetic Analyzer, following the manufacturer's guidelines (Applied Biosystems). Due to the clone being longer than the limits of accuracy for the sequencer, internal primers (including F2, F3, R2, and R3) were used to sequence the complete clone.

The *E. coli* cells containing desirable plasmid were kept as frozen stock in LB medium supplemented with 10-50 % (v/v) glycerol at -20℃ and -80℃.

2.6.5 Sequence analysis of *V. alginolyticus pepD*

Protein translation was carried out using ExPASy Translation tool (http://ca.expasy.org/tools/dna.html). The molecular weight and theoretical p*I* of protein were calculated by ProtParam (http://ca.expasy.org/tools/protparam.html). Homology searches of the DNA and amino acid sequences were carried out using the BLAST program from NCBI (http://www.ncbi.nlm.nih.gov/blast). The alignments of DNA and protein sequences were performed by ClustalW (http://www.ebi.ac.uk/clustalw).

2.7 Cloning and Expression of *V. alginolyticus pepD*

2.7.1 Construction of *E. coli* expression vector containing *pepD* gene

Plasmids pCR®2.1-TOPO and pET-28a(+) were used as a cloning and expression vector, respectively. *V. alginolyticus pepD* DNA was amplified by PCR method following steps described on 2.6.3 with primer F1 replaced by N1 which has an additional *Nde*I restriction site on its 5'-end. PCR products were also subcloned into pCR®2.1-TOPO vector following steps described on 2.6.4. The *pepD* gene was excised from pCR®2.1-TOPO-pepD by *Nde*I/*Not*I digestion at 37℃ for 3 hrs (Table 4). The each resultant 10 μL digestion mixture was performed electrophoresis as described on 2.6.3. The excised DNA was purified by GFX™ PCR DNA and Gel Band Purification Kit following the manufacturer's guidelines (GE Healthcare) and subcloned into the pET-28a(+) plasmid by ligation using T4 DNA ligase at 4℃ overnight (Table 4) (Fig. 7). The ligation mixture was then used to transform *E. coli* XL1-Blue competent cells following steps described on 2.6.4 with differences on incubation at 42℃ for 50 to 60 sec and spread on LB_{Kan} plates at 37℃ overnight to obtain pET-28a(+)-pepD plasmids for protein expression. The desired pET-28a(+)-pepD plasmid was checked by restriction enzymes, PCR and nucleotide sequencing methods as described above. The *E. coli* cells containing desirable plasmid were kept as described on 2.6.4.

Table 4. Reaction conditions of pCR®2.1-TOPO-pepD digestion and pET-28a(+)-pepD ligation.

pCR®2.1-TOPO-pepD	**1**		
NEBuffer 3	**1**	**pET-28a(+)**	**1**
10X BSA	**1**	***pepD* DNA**	**1**
***Nde*I (20 U/ μ L)**	**0.5**	**10X buffer**	**1**
***Not*I (10 U/ μ L)**	**0.5**	**T4 DNA ligase (3 U/ μ L)**	**1**
ddH_2O	**6**	**ddH_2O**	**6**
Total	**10 (μ L)**	**Total**	**10 (μ L)**

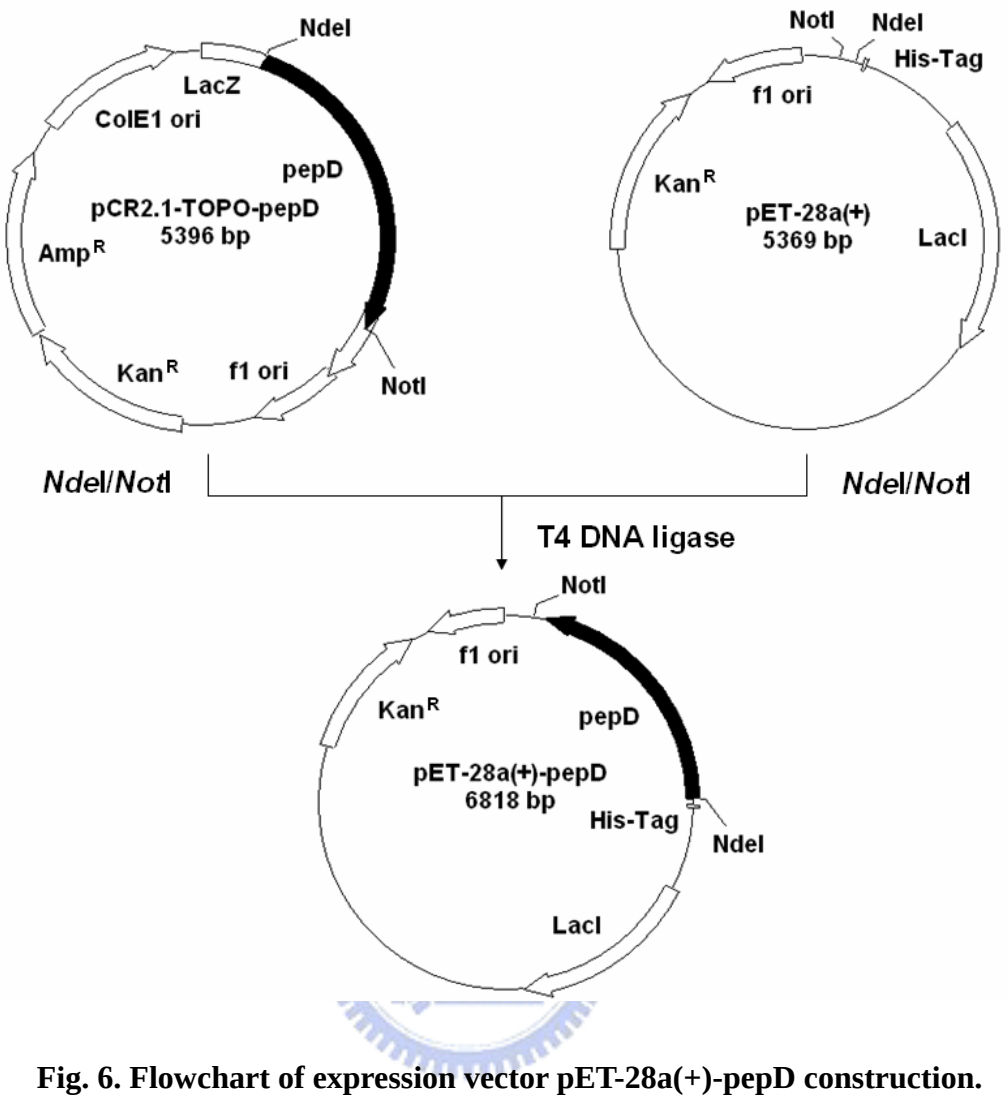


Fig. 6. Flowchart of expression vector pET-28a(+)-pepD construction.

2.7.2 Expression of *V. alginolyticus pepD* gene in *E. coli*

The pET-28a(+)-pepD plasmid was transformed into *E. coli* BL21(DE3)pLysS competent cells by heatshock method following steps described on 2.7.1 and spread on LB_{Kan} plates. Picked *E. coli* cells harboring pET-28a(+)-pepD were subcultured in 3 mL LB_{Kan} medium, shaken at 37℃ for an appropriate number of hrs, and poured into 300 mL LB_{Kan} medium. After OD_{600} reaches 0.5-0.6, 150 μL 1 M IPTG was added to the final concentration 0.5 mM to induce the expression of PepD. The culture was then incubated for 16 hrs to express PepD. As a control, *E. coli* cells transformed with pET-28a(+) plasmid without *pepD* gene were treated in the same way.

After 16 hrs incubation at 37℃ with rotary shaking, the cells were harvested by centrifugation at 6,500 rpm for 30 min at 4℃. The supernatant was removed and the bacterial pellets were washed twice with 5 mL 20 mM Tris-HCl, pH 7.4 to remove the traces of medium. The harvested cells were resuspended in 20 mL 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4 (Buffer A) containing 20 mM imidazole. The resuspended cells were ruptured by sonication on ice with pulse on 2 sec and pulse off 1 sec for the total sonication time 3 min at 30% energy using a sonicator. Repeat the sonication step on ice for at least 3 times. For the removal of intact cells and cell debris, the cell lysate was then centrifuged at 10,000 rpm for 30 min at 4℃. The supernatant was collected and used for further purification. The *E. coli* cells containing desirable plasmid were kept as frozen stock described on 2.6.4.

2.8 Purification of Expressed *V. alginolyticus* PepD

2.8.1 Purification of PepD by affinity chromatography

The supernatant containing expressed PepD was purified by affinity chromatography on Ni-NTA His-Band® Resin. A 20 mL plastic column was packed with 1 mL Ni-NTA resin and pre-equilibrated with 10 mL Buffer A containing 20 mM imidazole (10 bed volumes) by gravity force. After filtered with 0.45 μm pore size filter, the supernatant was loaded onto Ni-NTA column and washed with 5 bed volumes of Buffer A containing 20 mM imidazole. The expressed PepD was step eluted successively with 5 bed volumes of Buffer A containing 40, 100, 200, 300, and 500 mM imidazole. The eluted fractions were collected for SDS-PAGE analysis and enzymatic activity assay. The fractions with PepD activity were collected and dialyzed with 2 L 50 mM Tris-HCl, pH 7.4 for 3 hrs and following 3 L overnight. The purified proteins were kept frozen at -80℃ ready for the following assay. PepD could be stored at -80℃ without loss of activity for more than one month. Internal amino acid sequencing of the purified enzyme was performed at Academia Sinica. Protein concentration was determined by

BCA method.

2.8.2 Protein concentration determination

The protein concentrations of purified fractions were measured by BCA Protein Assay Reagents. Each 20 μL sample was mixed with 200 μL BCA™ Working Reagents (BCA™ Reagent A：BCA™ Reagent B = 50：1) and incubated in F96 MicroWell™ plate at 37℃ for 30 min. The absorbances of samples were measured at 562 nm on Multiskan Ascent Microplate Reader. A 2 mg/mL bovine serum albumin (BSA) stock and successive dilutions (1.5, 1.0, 0.75, 0.5, 0.25, 0.125, 0.025 mg/mL) treated in the same way as described above were served as standards.

2.8.3 SDS-PAGE and Native-PAGE analysis

The purified fractions were electrophoresed on a 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Table 5) to check the expression level, purity, and determination of the molecular weight. Each 10 μL sample was mixed with 2.5 μL 5X SDS-PAGE sample buffer. The mixed samples were incubated at 95℃ for 5 min following on ice for 5 min to denature proteins. The electrophoresis was performed with 1X SDS-PAGE running buffer at 90 Volt for 30 min following 120 Volt for 1.5 hrs. The proteins was stained with stain buffer containing Coomassie Brilliant blue R-250 for 30 min and destained with destain buffer I (methanol/acetic acid/water = 4: 1: 5, v/v/v) for 20 min and destain buffer II (mathanol/acetic acid/water = 1.2: 0.05: 8.75) overnight. The gel was scanned on EPSON® GT-7000 Scanner.

Native-PAGE was performed in the similar way to SDS-PAGE analysis with the absence of SDS and denaturing treatment. The purified and dialyzed proteins fractions were

electrophoresed on a 7.5% Native-PAGE (Table 5) to check the native form of PepD. Each 10 μL sample was mixed with 2.5 μL 5X Native-PAGE sample buffer. The mixed samples were performed immediately with iced 1X Native-PAGE running buffer at 90 Volt for 3 hrs with 10℃ circulating water bath. The proteins were stained and destained in the same way as SDS-PAGE analysis.

Table 5. Solutions and volumes for preparing SDS-PAGE and Native-PAGE separating gel and stacking gel.

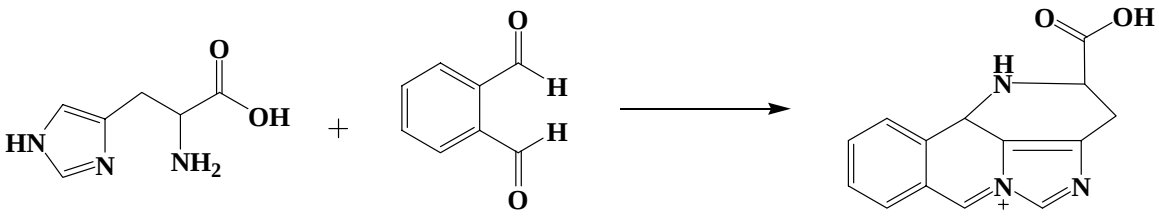
	Separating gel						Stacking gel
	7.5%	10%	12%	12.5%	15%	20%	4%
30% acrylamide / 1% *N*, *N*'-methylenediacrylamide (mL)	8.75	11.67	14	14.6	17.5	23.33	1.7
1.5 M Tris-HCl, pH 8.8 (mL)	8.75	8.75	8.75	8.75	8.75	8.75	-
1 M Tris-HCl, pH 6.8 (mL)	-	-	-	-	-	-	1.25
10% SDS (mL)[a]	0.35	0.35	0.35	0.35	0.35	0.35	0.1
TEMED (mL)	0.028	0.014	0.014	0.014	0.014	0.014	0.01
10% Ammonium persulfate (APS)[b] (mL)	0.35	0.35	0.35	0.35	0.35	0.35	0.1
ddH_2O (mL)	16.8	13.88	11.55	11	8.05	2.22	6.8
Total (mL)	35	35	35	35	35	35	10

[a] Replace SDS with ddH_2O when preparing Native-PAGE.
[b] Recommended to prepare freshly and mix at last.

2.9 Characterization of Expressed *V. alginolyticus* PepD

2.9.1 Enzymatic Activity Assay of PepD

PepD activity was assayed according to a method described by Bando *et al.* (Bando *et al.*, 1984) and Teufel *et al.* (Teufel *et al.*, 2003) on the basis of measurement of histidine derived with *o*-phthaldialdehyde (OPA) (Fig. 8). Briefly, substrate hydrolysis was carried out in a 200 μL final volume containing 80 μL 50 mM Tris-HCl buffer, pH 7.4, 100 μL 2 mM L-carnosine (dissolved in 50 mM Tris-HCl, pH 7.4) and 20 μL of purified enzyme (0.5 mg/mL). The reaction was initiated by addition of substrate and stopped by adding 50 μL of 1% trichloroacetic acid (TCA) after 60 min incubation at 37℃. Liberated histidine was derivatized by adding 50 μL of 5 mg/mL OPA dissolved in 2 M NaOH and 15 min of incubation at 37℃ in darkness. Fluorescence of OPA-derivated L-histidine was measured using Fluoroskan Ascent FL (λ_{Exc}: 355 nm and λ_{Em}: 460 nm). The reaction with L-histidine and L-carnosine only solution were treated in the same way to serve as the positive and negative control, respectively. All reactions were carried out in triplicate.



L-Histidine **OPA** **(Schiff base)**

Fig. 7. Formation of a Schiff base by L-histidine and OPA.

2.9.2 pH optimum

The effect of pH on the enzymatic stability and activity was determined at 37℃ by performing according to the activity assay procedure (see 2.9.1) but at various pH values in the following buffer systems (50 mM) were used: citric acid (pH 4, 5 and 6) and Tris-HCl (pH

6, 7, 7.4, 8.5, 9 and 9.5). The substrate hydrolyzed at highest rate was defined as 100 %.

2.9.3 Temperature optimum and thermostability

The effect of temperature on the enzymatic activity was determined in 80 μL 50 mM potassium phosphate (KPi) buffer, pH 7.4 by performing according to the activity assay procedure (See 2.9.1) but at various temperature including 4, 10, 25, 37, 50, 60, and 70℃. To estimate the thermostability, each 20 μL enzyme (0.5 mg/mL) was pre-incubated for 30 min in 80 μL 50 mM KPi buffer, pH 7.4 at various temperatures as described above. The reaction mixtures were initiated by adding 100 μL 2 mM L-carnosine following the activity assay procedure (see 2.9.1). The substrate hydrolyzed at highest rate was defined as 100 %.

2.9.4 Substrate specificity

PepD activities toward different substrates were determined under the reaction condition described on 2.9.1. Various Xaa-His dipeptides, including β-Ala-L-His (L-carnosine), α-Ala-L-His, Gly-His, β-Asp-L-His, and γ-Amino-butyryl-His (GABA-His, homocarnosine) and two histidine-containing tripeptides, Gly-Gly-His and Gly-His-Gly, were used. The activity on L-carnosine was defined as 100 %.

2.9.5 Inhibitor profile

Several inhibitors, benzamidine, ethylenediamine tetraacetic acid (EDTA), bestatin, and *N*-ethylmaleimide (NEM), were prepared as stock solutions in 50 mM Tris-HCl, pH 7.4. 80 μL inhibitors with different concentrations were pre-incubated with 20 μL enzyme (0.5 mg/mL) for 30 min at 37℃. The reaction was initiated by the addition of 100 μL 2 mM L-carnosine following the activity assay described on 2.9.1. The substrate hydrolyzed rate without inhibitor was defined as 100 %.

2.9.6 Enzyme kinetics

For determination of V_{max}, K_m, and k_{cat} of *V. alginolyticus* PepD, the method described by Csámpai *et al.* (Csámpai *et al.*, 2004) was modified to use. The substrate hydrolysis was carried out in the same method as described on 2.9.1 with different concentrations of L-carnosine (1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005, and 0.0025 mM) added to initiate enzymatic reations. After 15 min incubation at 37℃, liberated histidine was derivatized by adding 100 μL OPA reagent (see 2.1.5) for 5 min incubation at 37℃ in darkness. Fluorescence was measured using Fluoroskan Ascent FL (λ_{Exc}: 355 nm and λ_{Em}: 460 nm). Reactions were carried out in triplicate.

Each 200 μL various concentration of L-histidine solution (0.125, 0.05, 0.025, 0.01, 0.005, 0.0025, 0.001, 0.0005, 0.00025, and 0.0001 mM) derivatived with 100 μL OPA reagent for 5 min were detected as method described above to serve as standards.

2.10 Mutagenesis Analysis of *V. alginolyticus pepD*

2.10.1 Site-directed mutagenesis on *V. alginolyticus pepD*

Site-directed mutagenesis was performed by a Quick-change method to create all mutants. Each reaction mixtures were prepared with 100 ng of pET-28a(+)-pepD plasmid (wild-type) as a template, 1X *Pfu* polymerase buffer, 0.8 mM of each dNTP, 1 μL of each 12.5 μM primer (see Appendix 2), 1 μL (2.5 U) *Pfu* polymerase and ddH_2O to the final volume of 50 μL (Table 6). The PCR mutagenesis reaction was performed in the 96-well GeneAmp® PCR System 9700 Thermal Cycler as recommended by the manufacturer of *PfuUltra*™ High-Fidelity DNA polymerase(95℃ for 30 sec, 52℃ for 1 min, and 72℃ for 8 min; 18 cycles) (Table 6). The PCR products were incubated with *Dpn*I for 3 hrs at 37℃ to selectively digest the methylated parent plasmids, and the resulting products were analyzed by 0.8%

agarose gel electrophoresis (see 2.6.3). The productive reactions were transformed into *E. coli* XL1-Blue competent cells (see 2.7.1), with selection for resistance to kanamycin, and the successful mutagenesis was confirmed by restriction enzymes and DNA sequencing of plasmid. The desired mutant plasmids were transformed and expressed in *E. coli* BL21(DE3) pLysS as described on 2.7.2.

Table 6. Reaction conditions and cycling parameters for the PCR mutagenesis reaction.

pET-28a(+)-pepD plasmid	0.5
Primer 1 (12.5 μM)	1
Primer 2 (12.5 μM)	1
dNTP mix (2.5 mM each)	4
10X *Pfu* polymerase buffer	5
ddH_2O	37.5
Pfu polymerase (2.5 U/μL)	1
Total	50 (μL)

Segment	Cycles	Temperature	Time
1	1	95℃	2 minutes
2	18	95℃	30 seconds
		52℃	1 minute
		72℃	8 minutes
3	1	72℃	10 minutes
4	1	4℃	pause

2.10.2 Circular dichroism (CD) spectroscopy

The effects of single mutant on PepD secondary structures were analyzed by monitoring CD spectra. The CD spectra of wild-type and mutated PepD were recorded every 1 nm between 200 and 300 nm using a quartz cuvette of 1 mm path-length in a Jasco J-715 spectropolarimeter. Each protein sample was prepared in 50 mM Tris-HCl, pH 7.4 at a protein concentration of 0.3-0.6 mg/mL. The denatured wild-type PepD protein was prepared by incubating at 95°C for 5 min and on ice for another 5 min. The results were scanned 4 times and averaged.

The spectra were corrected by subtracting the spectrum of buffer alone and coverted to mean residue ellipticity (MRE) (Yiallouros *et al.*, 2002) using the equation: $[\theta]_{MRE} = (MRW \times \theta_{obs}/c \times d)$, where θ_{obs} is the observed ellipticity (in millidegrees) at the respective wavelength,

MRW is the mean residue of the enzyme (MRW = M/n, M = 53548.8 g/mole, n = 490 amino acid residues), d is the cuvette path-length in cm, and c is the protein concentration in mg/mL.