Chapter 6 Reference

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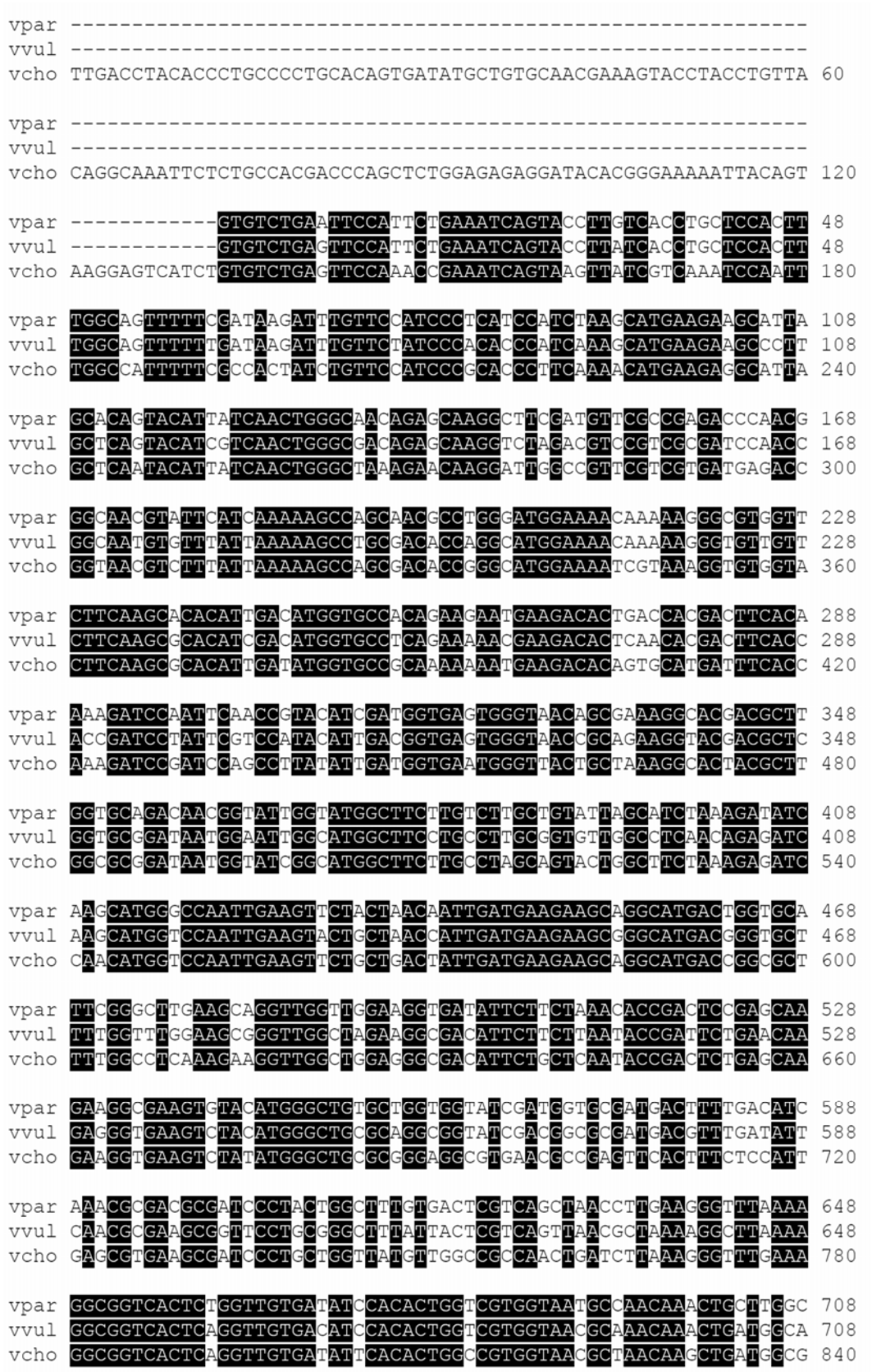
Appendix 1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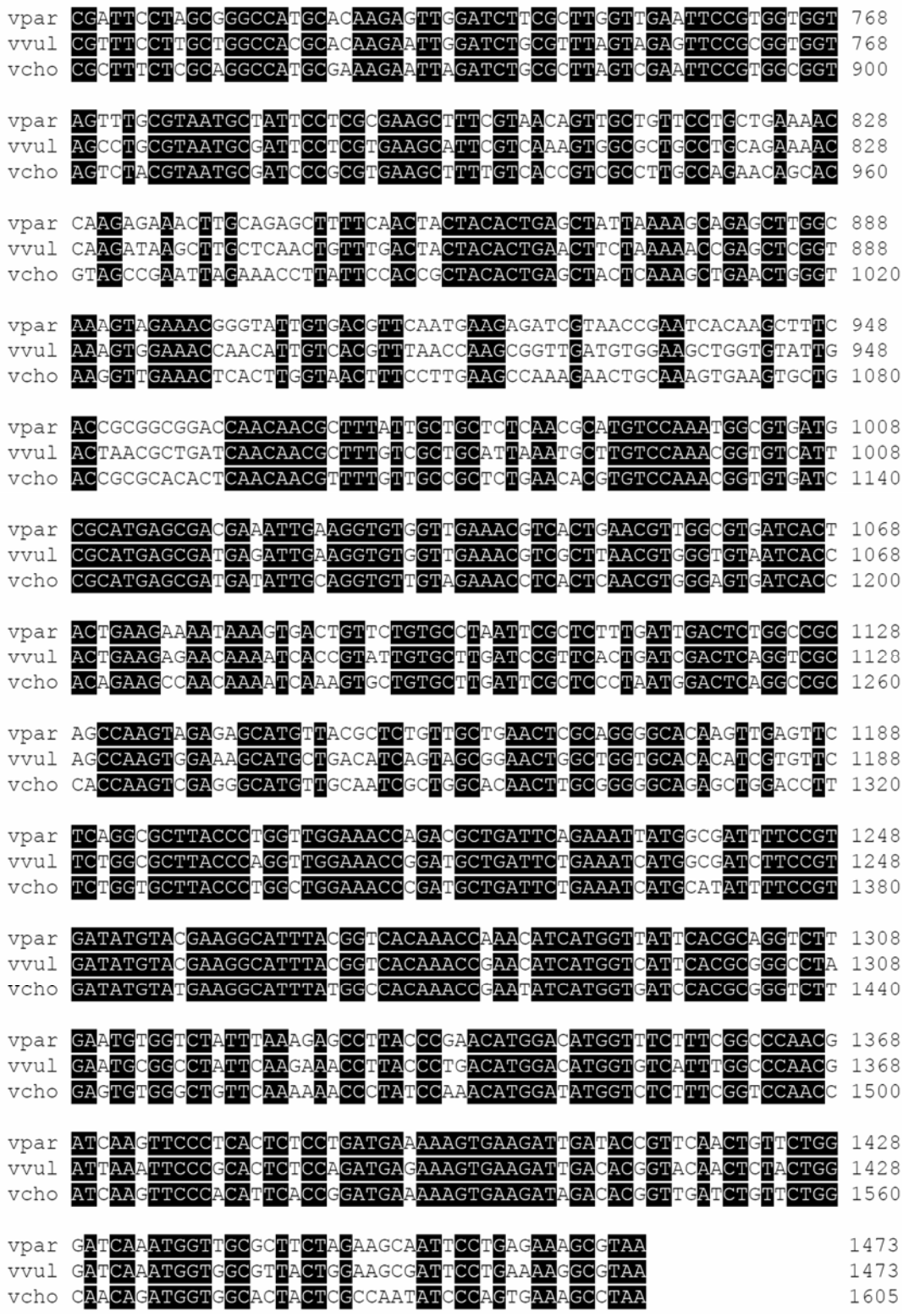


Appendix 1. Nucleic acid sequence alignment of *Vibrio* spp. *pepD* genes. Abbreviations vpar, vvul, and vcho stand for *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, respectively. Conserved nucleic acids among the sequences are marked in black. Dashed lines indicate the gaps introduced for better alignment.

Appendix 2

Appendix 2. Primers used in this thesis.

Sequencing and Expression		
F1 (sense)	5'-GTGTCTGAGTTCCATTC-3'	(1-17)[a]
F2 (sense)	5'-TGGGCGACAGAGCAAGG-3'	(127-143)
F3 (sense)	5'-TCTGGCGCTTACCCAGG-3'	(1189-1205)
R1 (antisense)	3'-AAGGACTTTTCCGCATT-5'	(1457-1473)
R2 (antisense)	3'-CGTAACTTGCGAACAGG-5'	(979-995)
R3 (antisense)	3'-GTGACTAGTGCTGAAGT-5'	(270-286)
N1 (sense)	5'-CGCGGATCCCATATGGTGTCTGAGTTCCATTC-3'	(*Nde*I)[b]
Mutagenesis		
H80A-1	5'-GTGCTTCAAGCAGCGATCGACATGGTGCCAC-3'	(*Pvu*I)[b]
H80A-2	5'-GTGGCACCATGTCGATCGCTGCTTGAAGCAC-3'	(*Pvu*I)
D82A-1	5'-GCACACATCGCCATGGTGCCACAAAAGAACG-3'	(*Nco*I)
D82A-2	5'-CGTTCTTTTGTGGCACCATGGCGATGTGTGC-3'	(*Nco*I)
D119A-1	5'-CGCTCGGGGCAGCTAACGGCATCGGCATGGC-3'	(*Ava*I)
D119A-2	5'-GCCATGCCGATGCCGTTAGCTGCCCCGAGCG-3'	(*Ava*I)
E149A-1	5'-CTGACGATCGATGCAGAAGCAGGCATGACAGG-3'	(*Pvu*I)
E149A-2	5'-CCTGTCATGCCTGCTTCTGCATCGATCGTCAG-3'	(*Pvu*I)
E150A-1	5'-ACTATTGATGAAGCCGCGGGCATGACAGGTGC-3'	(*Sac*II)
E150A-2	5'-GCACCTGTCATGCCCGCGGCTTCATCAATAGT-3'	(*Sac*II)
D173A-1	5'-CCTTCTAAATACAGCTAGCGAACAAGAAGGCG-3'	(*Nhe*I)
D173A-2	5'-CGCCTTCTTGTTCGCTAGCTGTATTTAGAAGG-3'	(*Nhe*I)
H461A-1	5'-CCAACCATCAAGTTCCCTGCTAGCCCAGATGAG-3'	(*Nhe*I)
H461A-2	5'-CTCATCTGGGCTAGCAGGGAACTTGATGGTTGG-3'	(*Nhe*I)

[a] Corresponded sequence position.
[b] Corresponded additional restriction site on primers (underlined).

Appendix 3

Appendix 3. Homology analysis of *V. alginolyticus pepD* amino acids.

Source	Name	Identity	Accession No.
V. parahaemolyticus RIMD 2210633	Aminoacylhistidine dipeptidase	94%	NP_797050
Vibrio alginolyticus 12G01	Aminoacylhistidine dipeptidase	93%	ZP_01260299
Vibrio vulnificus YJ016	Aminoacylhistidine dipeptidase	92%	NP_933645
Vibrio vulnificus CMCP6	Aminoacylhistidine dipeptidase	91%	NP_759335
Vibrio splendidus 12B01	Aminoacylhistidine dipeptidase	88%	ZP_00990223
Vibrio fischeri ES114	Aminoacylhistidine dipeptidase	84%	YP_204119
Vibrio cholerae O1 biovar eltor str. N16961	Aminoacylhistidine dipeptidase	82%	NP_231910
Vibrio cholerae V51	Di- and tripeptidases	82%	ZP_00749022
Vibrio cholerae MO10	Di- and tripeptidases	81%	ZP_00759334
Vibrio cholerae O395	Di- and tripeptidases	81%	ZP_00757002
Vibrio cholerae V52	Di- and tripeptidases	81%	ZP_00746278
Vibrio cholerae RC385	Di- and tripeptidases	81%	ZP_00752195
Vibrio angustum S14	Putative aminoacylhistidine dipeptidase	76%	ZP_01235668
Photobacterium profundum 3TCK	Putative aminoacylhistidine dipeptidase	75%	ZP_01218945
Photobacterium profundum SS9	Putative aminoacylhistidine dipeptidase	75%	YP_129048
Shigella sonnei Ss046	Aminoacylhistidine dipeptidase	63%	YP_309297
Yersinia mollaretii ATCC 43969	Di- and tripeptidases	63%	ZP_00826723
Escherichia coli O157:H7 EDL933	Aminoacylhistidine dipeptidase (peptidase D)	63%	NP_285954

Escherichia coli O157:H7 str. Sakai	PepD	63%	NP_308291
Escherichia coli 53638	Di- and tripeptidases	63%	ZP_00735968
Shigella boydii BS512	Di- and tripeptidases	63%	ZP_00696913
Shigella dysenteriae 1012	Di- and tripeptidases	63%	ZP_00921840
Shigella boydii Sb227	Aminoacylhistidine dipeptidase	63%	YP_406787
Escherichia coli E22	Di- and tripeptidases	63%	ZP_00730150
Escherichia coli B171	Di- and tripeptidases	63%	ZP_00711302
Escherichia coli E110019	Di- and tripeptidases	63%	ZP_00721352
Escherichia coli B7A	Di- and tripeptidases	63%	ZP_00717585
Escherichia coli HS	Di- and tripeptidases	63%	ZP_00704526
Escherichia coli E24377A	Di- and tripeptidases	63%	ZP_00702608
Yersinia pestis Nepa1516	Aminoacylhistidine dipeptidase	63%	YP_646797

Appendix 4

4.1 Production of PepD Monoclonal Antibody

4.1.1 Immunization of mice

The 4-week-old female BALB/c mice were immunized with an antigen that is prepared by emulsifying the purified PepD with Freund's adjuvant. PepD was mixed and homogenized with an equal amount of Freund's complete adjuvant by vortex. Each mouse was given a total 300 μL emulsion containing 125 μg protein with an i.p. (intraperitoneal) injection and 3 s.c. (subcutaneous) injections onto the back. Mice were proceeded secondary and continuous immunization with protein and Freund's incomplete adjuvant mixture with the same volume and method as described above every 10-14 days.

After 3 times of immuniztion, blood samples of each mouse was collected by blood collecting tubes with 0.1% (wt/v) EDTA and centrifuged at 7,000 rpm for 7 min at 4℃ to obtain the supernatant containing polyclonal antibody (pAb) against PepD. The titer of pAb was determined by enzyme-linked immunosorbent assay (ELISA) method (see 2.10.4). Mice with a titer of pAb at a serum dilution of more than 1:1000 were chosen for the following cell fusion experiments. Each chosen mouse was carried out a final booster with the same dose of antigen resuspended only in sterilized phosphate buffered saline (PBS) by 1 i.p. and 1 i.v. (intravenous) injection.

4.1.2 Preparation of myeloma cells

The mouse myeloma cell line FO was used for cell fusion experiment. FO cell stocks in liquid nitrogen were quickly thawed out by 37℃ water bath following centrifugated at 1200 rpm for 5 min. The resultant supernatant was removed, and centrifuged cells were

resuspended by 6 mL pre-warmed Dulbecco's Modified Eagles Medium (DMEM) containing 10% bovine calf serum (BCS) and 1% 100X penicillin-streptomycin stock solution and cultured in 25 cm^2 flasks at 37℃ incubator supplemented with 5% carbon dioxide (CO_2).

The redundant cells were kept as frozen stocks for future usage. In brief, the cells in medium consist of DMEM, 20% fetal bovine serum (FBS) and 7% dimethyl sulfoxide (DMSO) were added to cryo tubes. The tubes were put in a cooler at -80℃ overnight and then stored in liquid nitrogen.

4.1.3 Fusion of myeloma cells with spleen cells

The cell fusion experiment was carried out by fusing previously prepared myeloma cells (FO) (see 2.10.2) with spleen cells from previously immunized mouse (see 2.10.1) to produce hydridoma cells. About 5 days later after final booster immunization, the mouse with highest titer was chosen to be sacrificed with ether. After the mouse sacrificed, the spleen was harvested in laminar flow and squeezed with pre-warmed DMEM (serum and antibiotics free) in a petri dish within a short time to obtain spleen cells. Then the FO cells were mixed with the filtered spleen cells at a ratio around 1:4 to 1:8 and the mixture was centrifuged at 1200 rpm for 5 min. The resultant supernatant was discarded and the pellet was resuspended again by pre-warmed DMEM (serum and antibiotics free) following centrifugation at 1200 rpm for 5 min. Repeat the step described above for one time.

After the last centrifugation, the suspernatant was discarded and the pellet was scattered. Cell fusion was carried out by adding 1 mL pre-warmed polyethylene glycol (PEG)/DMSO solution drop-by-drop to the mixed-cell pellet accompanied with stirring within 1 min and continuously stirring for another minute in water bath at 37℃ followed by adding 10 mL

pre-warmed DMEM (serum and antibiotics free) within 2 min. The cell mixture was then centrifuged at 1000 rpm for 5 min and the resultant supernatant was removed. The pellet was resuspended again by pre-warmed DMEM (serum and antibiotics free) and centrifuged at 1000 rpm for 5 min. Repeat the step described above for one time.

After the last centrifugation, the suspernatant was discarded and the pellet resuspended by HT medium containing DMEM, 20% FBS, 1% 100X penicillin-streptomycin stock solution, 1% 100X L-glutamine stock solution, 1% 100X HT Supplement, and 1% 50X HAT Supplement. The final cell mixture was distributed as 100 μL per well in five 96-well microtiter plates. The plates were cultured at 37℃ incubator supplemented with 5% CO_2. The hybridoma cells were cultured consequently with HY medium which is consist of HT medium without HAT Supplement.

4.1.4 Screening of hybridoma clone by ELISA method

The culture supernatant was collected and assayed by enzyme-linked immunosorbent assay (ELISA) method for screening the hybridoma cells which produce antibodies specific against PepD. Briefly, 100 μL of 0.25 mg/mL purified PepD was initially coated on each well of 96-well microtiter ELISA plate at 4℃ overnight. Unbound proteins were washed out with 1X PBS buffer for 3 times, and the antigen-coated plate was blocked subsequently by adding 350 μL blocking buffer consist of 5% non-fat milk in 1X PBS buffer on each well at RT for 1.5 hrs. Then the blocking buffer was washed out by 1X PBS buffer for 3 times followed by adding 100 μL cell culture supernatant on each well at RT for 1.5 hrs. The plate was then washed with 1X PBS buffer for 5 times and the bound primary antibodies were detected using 100 μL goat anti-mouse IgG conjugated horseradish peroxidase (HRP) at 1: 5,000 dilutions with 1X PBS buffer on each well for 1 hrs at RT. Finally, the plate was washed with 1X PBS

buffer for 5 times and developed by adding 50 μL 3,3',5,5'-tetramethylbenzidine (TMB) solution on each well for 20 min in darkness followed by adding 50 μL 2 M sulferic acid (H_2SO_4) to stop reaction. The absorbance was evaluated by Fluoroskan Ascent ELISA reader at 450 nm. The initial mouse serum-coated and non-fat milk blocked well were performed in the same way to serve as the positive and negative control, respectively.

4.1.5 Limiting dilution of hybridoma clones

The candidate hybridoma clones selected by ELISA method were diluted to obtain single cell line using "limiting dilution" method. In brief, each 10 μL candidate hybridoma cell suspension was mixed with 10 mL pre-warmed HY medium in a petri dish and the mixture was transferred to a 96-well microtiter plate with 100 μL on each well. The wells containing only a single cell clone was cultured, screened, and stocked as described above.

4.1.6 Ascites production in mice

Ascites is an intraperitoneal fluid extracted from mouse, which contains the desired antibodies at high concentrations. Each monoclonal hybridoma cell line with dosage of 10^6 cells in 0.5 mL sterile PBS was i.p. injected into a 6 to 8-week-old female BALB/c mouse which had i.p. injected 0.5 mL Pristane 2 days previously. The ascites was collected 7-10 days later and centrifuged at 7000 rpm for 7 min at 4℃ to obtain the resultant supernatant. The titer and specificity of each ascites were also assayed by ELISA method (see 2.10.4) and Western blot method (see 2.10.7), respectively. The desired ascites was stored with 0.05% sodium azide (NaN_3) added at 4℃ ready to use or at -20℃ with 50% glycerol as a frozen stock.

4.1.7 Western blot analysis

Western blot analysis was initially performed on 12.5% SDS-PAGE as described on 2.8.3. The resultant SDS-PAGE and a nitrocellulose (NC) membrane were soaked instantly in the

transfer buffer for 30 sec. Then the gel was immediately transferred to a NC membrane at 30 Volt for 35 min with a semi-dry blotting apparatus. The NC membrane was blocked with blocking buffer for 1.5 hrs at RT. The membrane was then washed 3 times with 1X PBS buffer and incubated with the primary anti-PepD monoclonal antibody (mAb) at 1: 1,000 dilutions with 1X PBS buffer for 1.5 hrs at RT with gentle shaking, followed by washed 5 times with 1X PBS buffer to remove the unbound primary antibodies. The washed membrane was further incubated with the goat anti-mouse IgG conjugated HRP at 1: 5,000 dilutions with 1X PBS buffer for 1 hrs at RT with gentle shaking. Finally, the membrane was washed with 1X PBS buffer for 5 times. The immunoreactive bands were visualized with a chemiluminescence reagent and the autoradiography film.

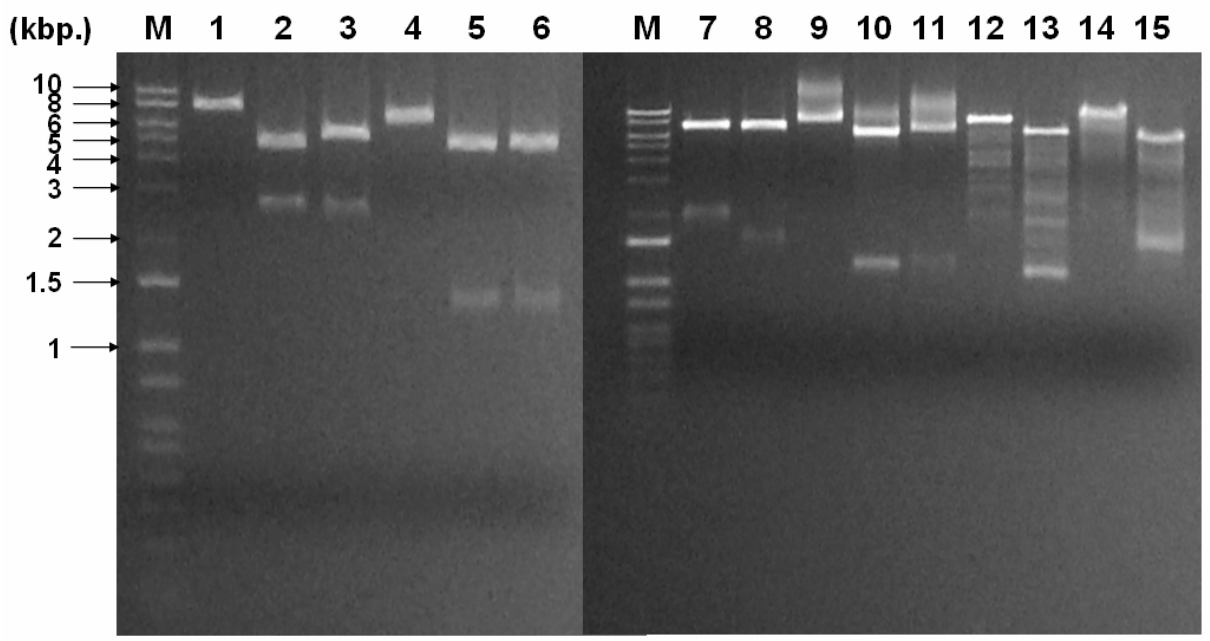
Appendix 5

Appendix 4. The restriction enzyme used in expresson vectors check and the expected size of excised fragment.

Plasmid	R.E.[a] used	Excised frangment
pET-pepD-WT	*Ava*I	-[b]
pET-pepD-D119A	*Ava*I	1.2 kbp.
pET-pepD-D119AE150A	*Ava*I	1.2 kbp.
pET-pepD-WT	*Nco*I/*Not*I	1.6 kbp.
pET-pepD-D82A	*Nco*I/*Not*I	1.4, 0.2 kbp.
pET-pepD-WT	*Nde*I/*Nhe*I	-
pET-pepD-H461A	*Nde*I/*Nhe*I	1.4 kbp.
pET-pepD-WT	*Nhe*I/*Not*I	-
pET-pepD-D173A	*Nhe*I/*Not*I	1.0 kbp.
pET-pepD-WT	*Pvu*I	-
pET-pepD-H80A	*Pvu*I	2.4 kbp.
pET-pepD-E149A	*Pvu*I	2.2 kbp.
pET-pepD-WT	*Sac*II/*Not*I	-
pET-pepD-E150A	*Sac*II/*Not*I	1.1 kbp.
pET-pepD-D119AE150A	*Sac*II/*Not*I	1.1 kbp.

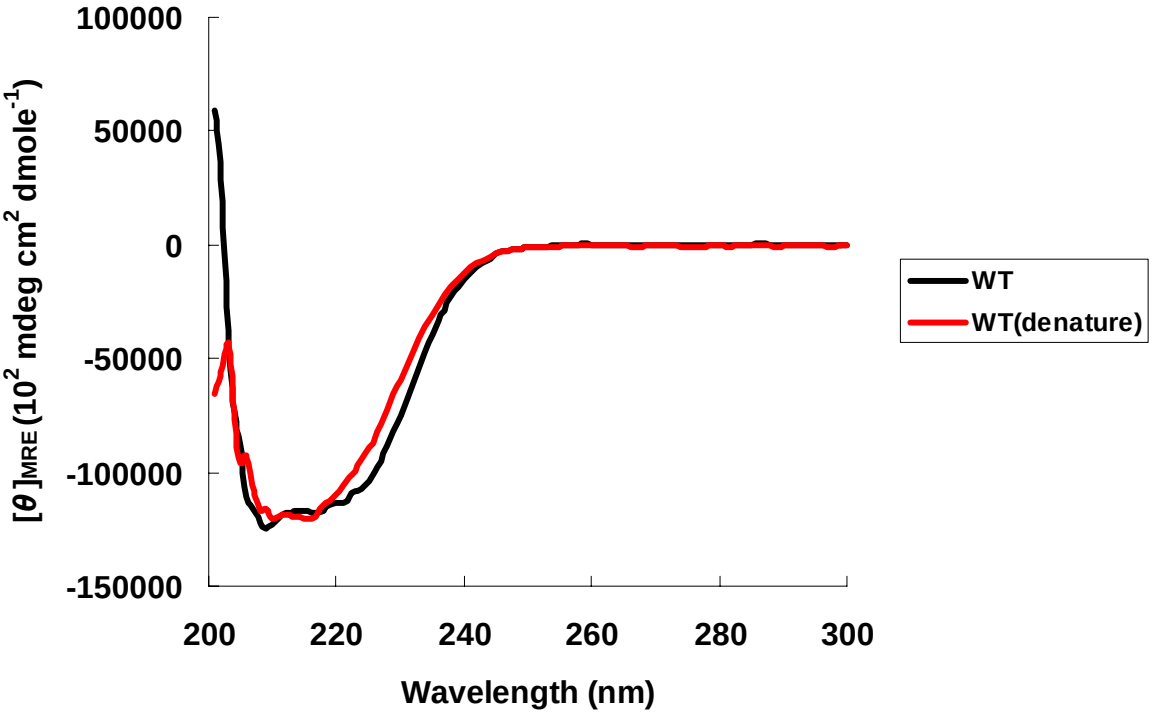
[a] R.E.= restriction enzymes.
[b] No expected DNA fragment was excised.

Appendix 6



Appendix 5. DNA agarose gel electrophoresis of site-directed mutated plasmid checked by restriction enzymes. *Lane M*, 10k-100 bp. DNA marker. *Lane 1*, pET-pepD-WT (*Pvu*I). *Lane 2*, pET-pepD-H80A (*Pvu*I). *Lane 3*, pET-pepD-E149A (*Pvu*I). *Lane 4*, pET-pepD-WT (*Ava*I). *Lane 5*, pET-pepD-D119A (*Ava*I). *Lane 6*, pET-pepD-D119AE150A (*Ava*I). *Lane 7*, pET-pepD-WT (*Nco*I/*Not*I). *Lane 8*, pET-pepD-D82A (*Nco*I/*Not*I). *Lane 9*, pET-pepD-WT (*Sac*II/*Not*I). *Lane 10*, pET-pepD-E150A (*Sac*II/*Not*I). *Lane 1I*, pET-pepD-D119AE150A (*Sac*II/*Not*I). *Lane 12*, pET-pepD-WT (*Nhe*I/*Not*I). *Lane 13*, pET-pepD-D173A (*Nhe*I/*Not*I). *Lane 14*, pET-pepD-WT (*Nde*I/*Nhe*I). *Lane 15*, pET-pepD-H461 (*Nde*I/*Nhe*I).

Appendix 7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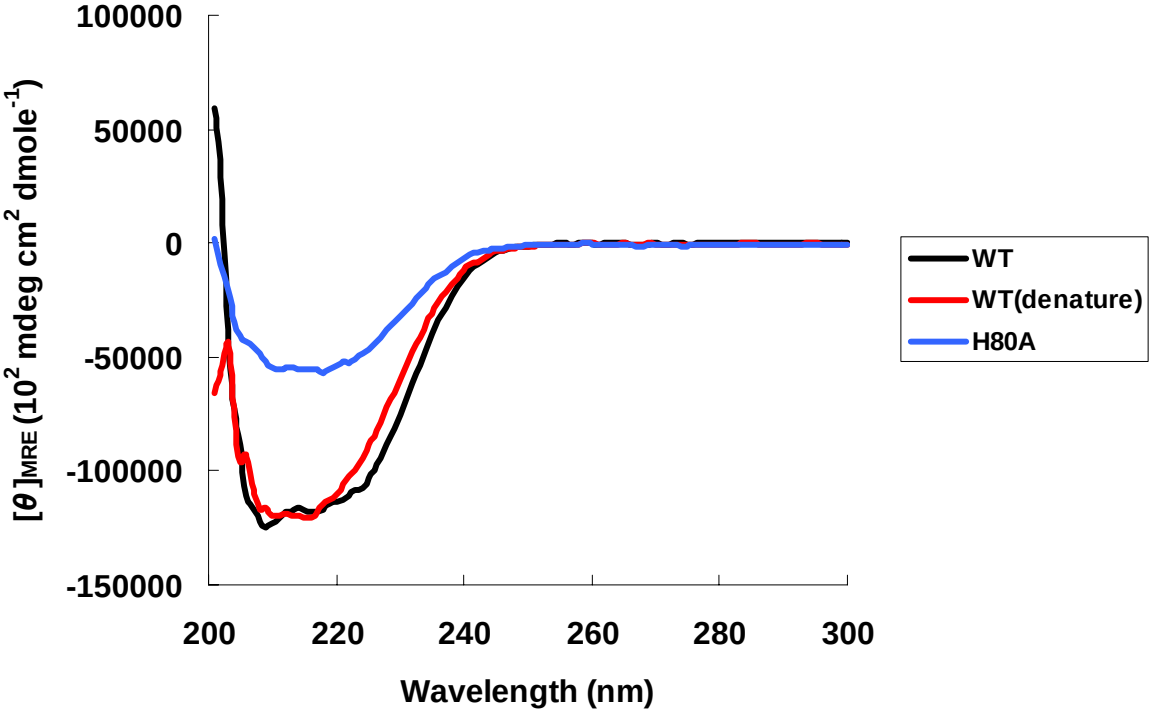
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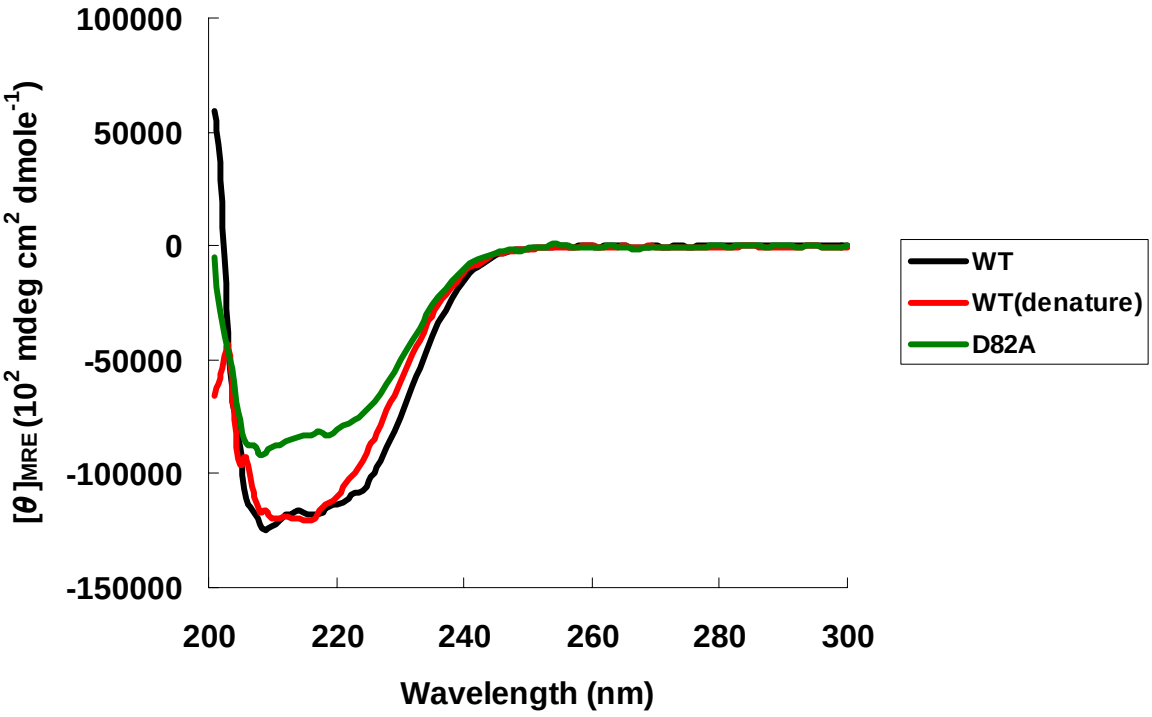
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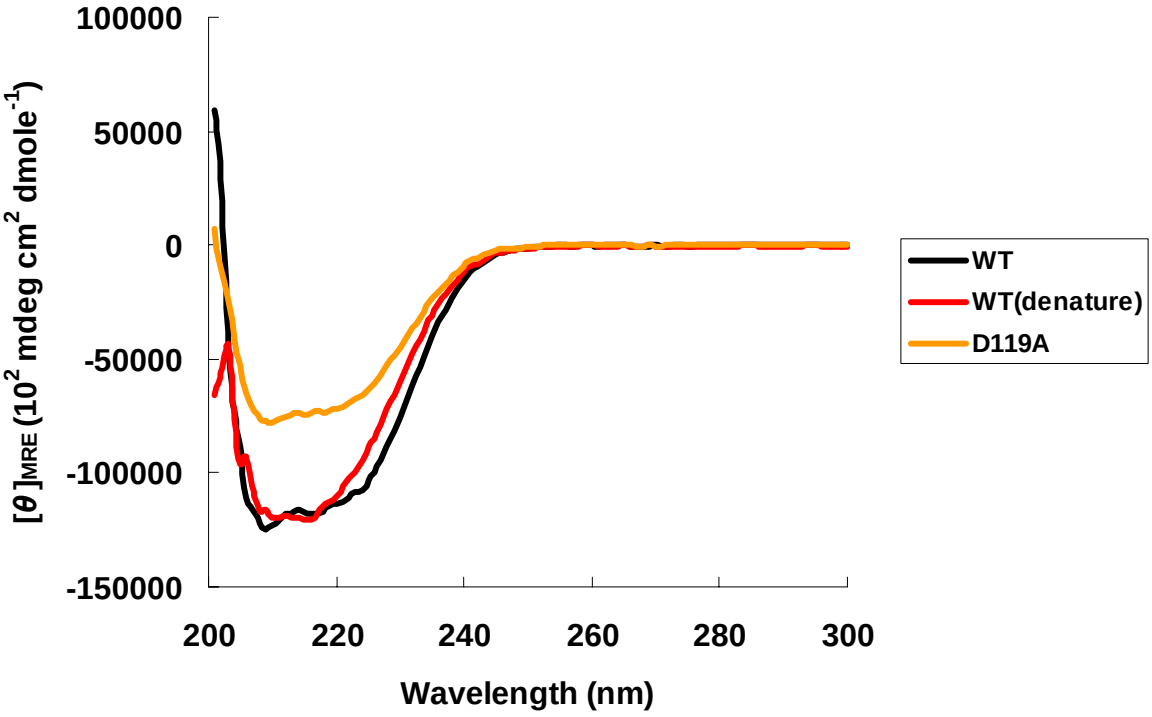
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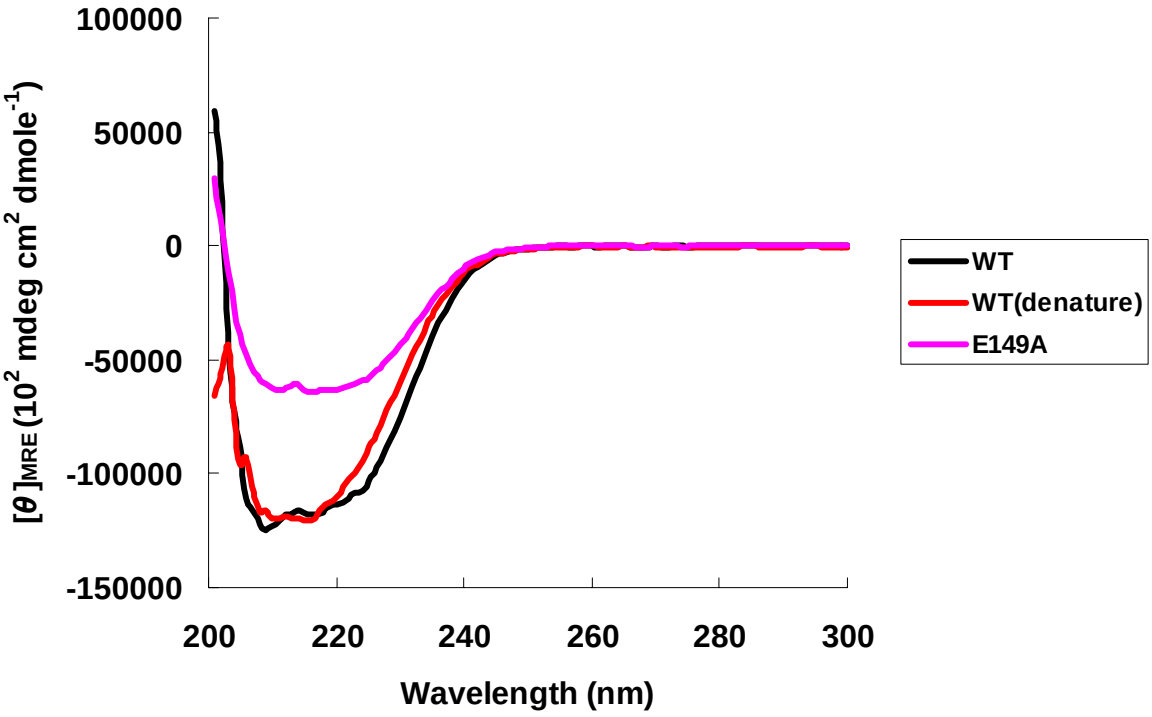
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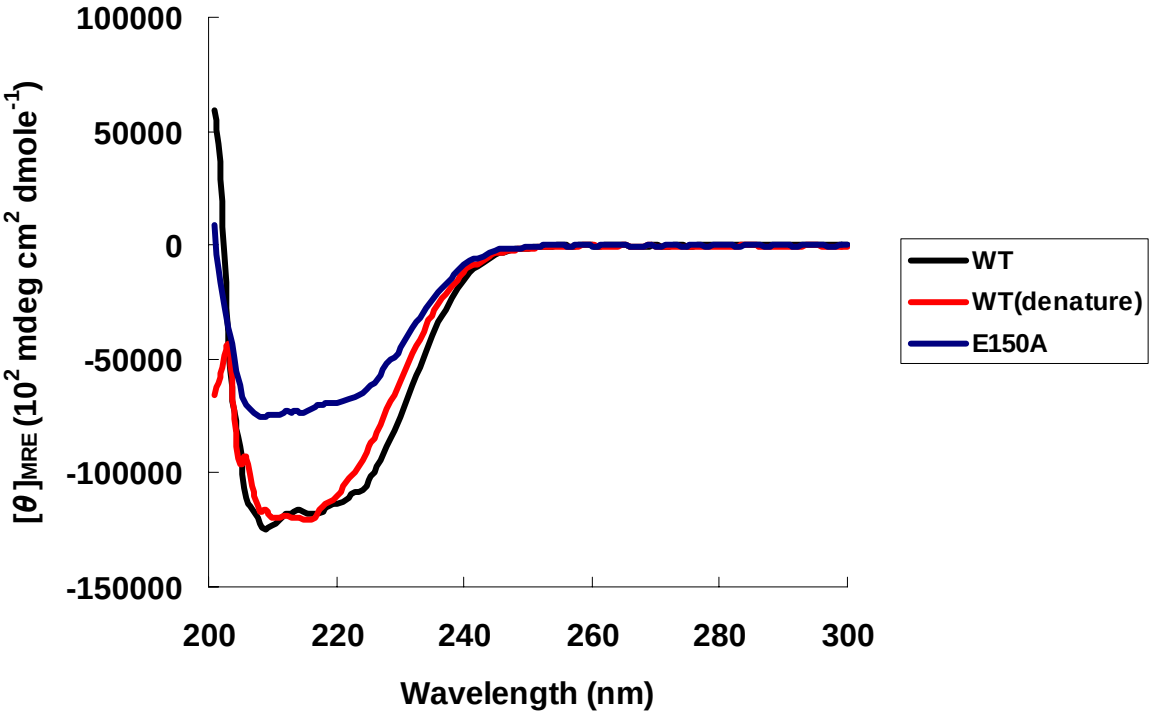
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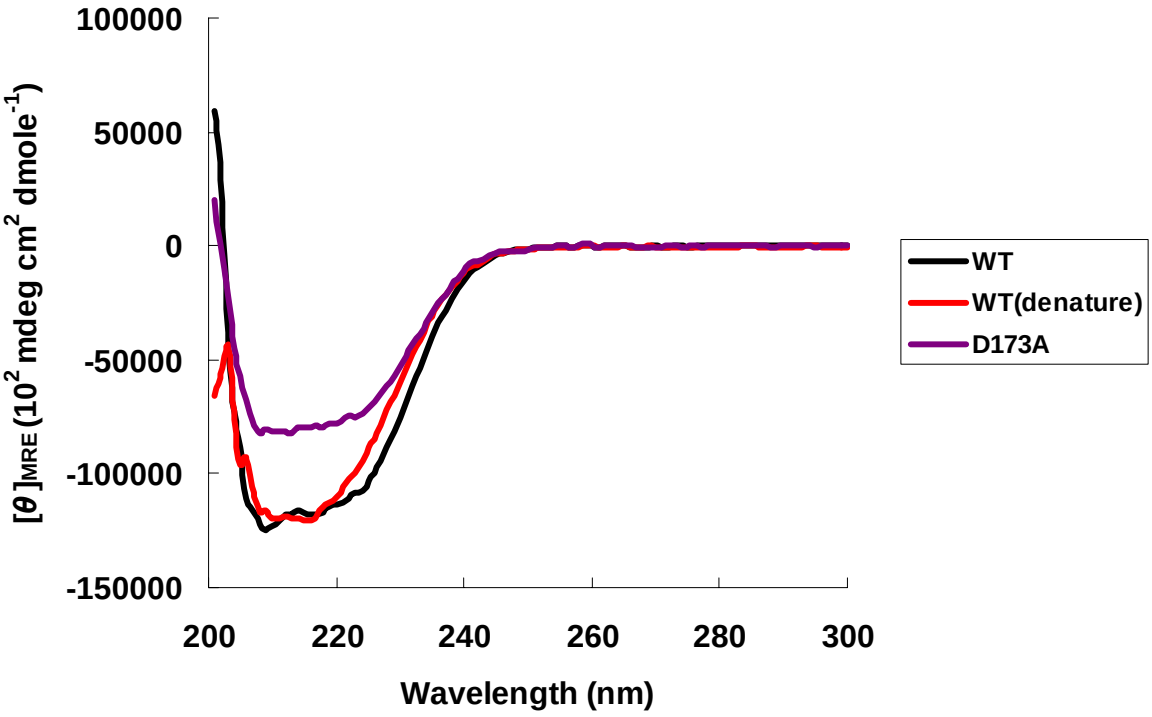
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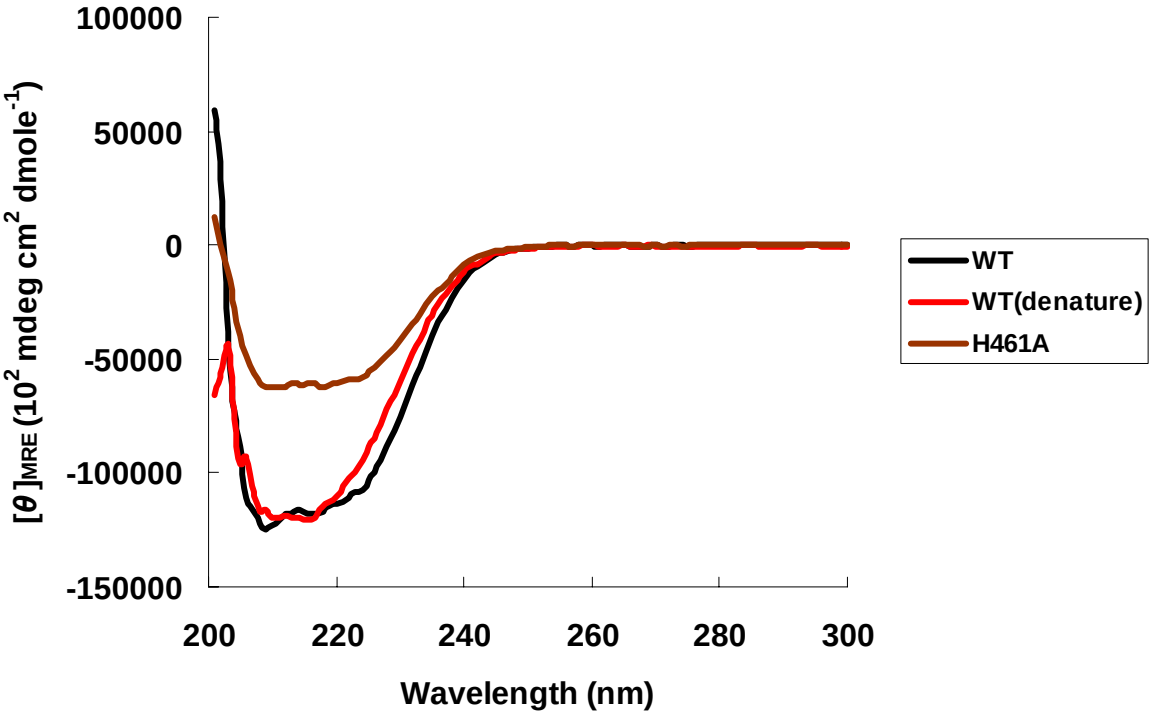
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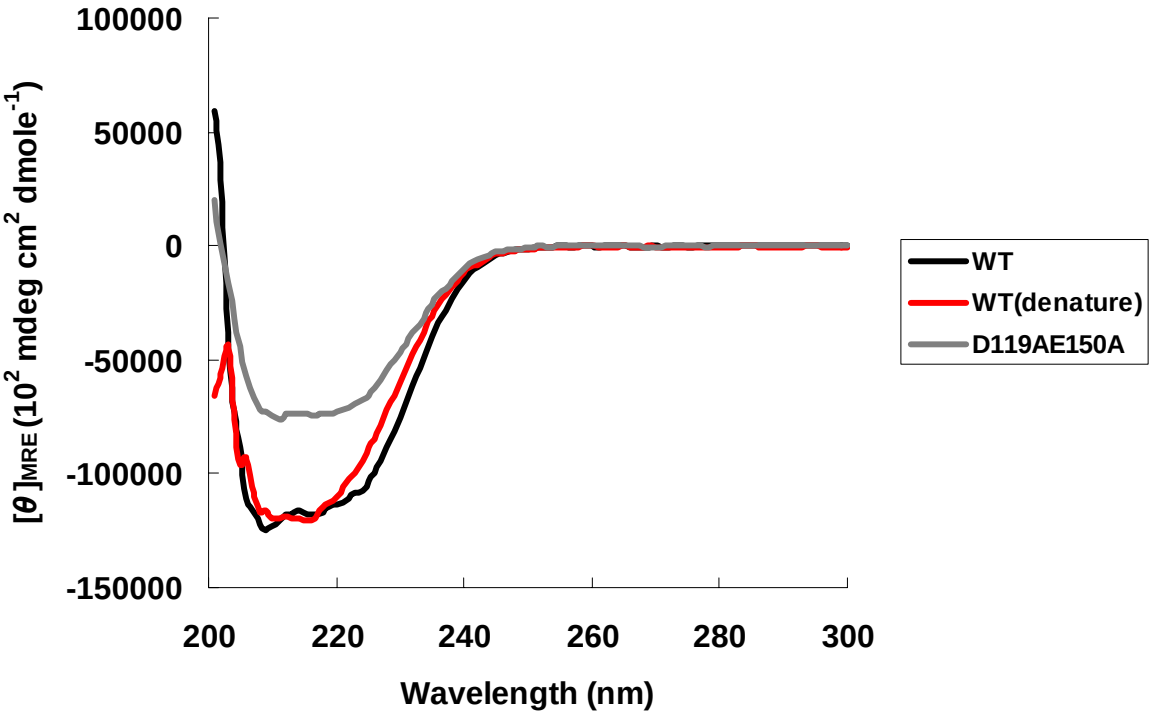
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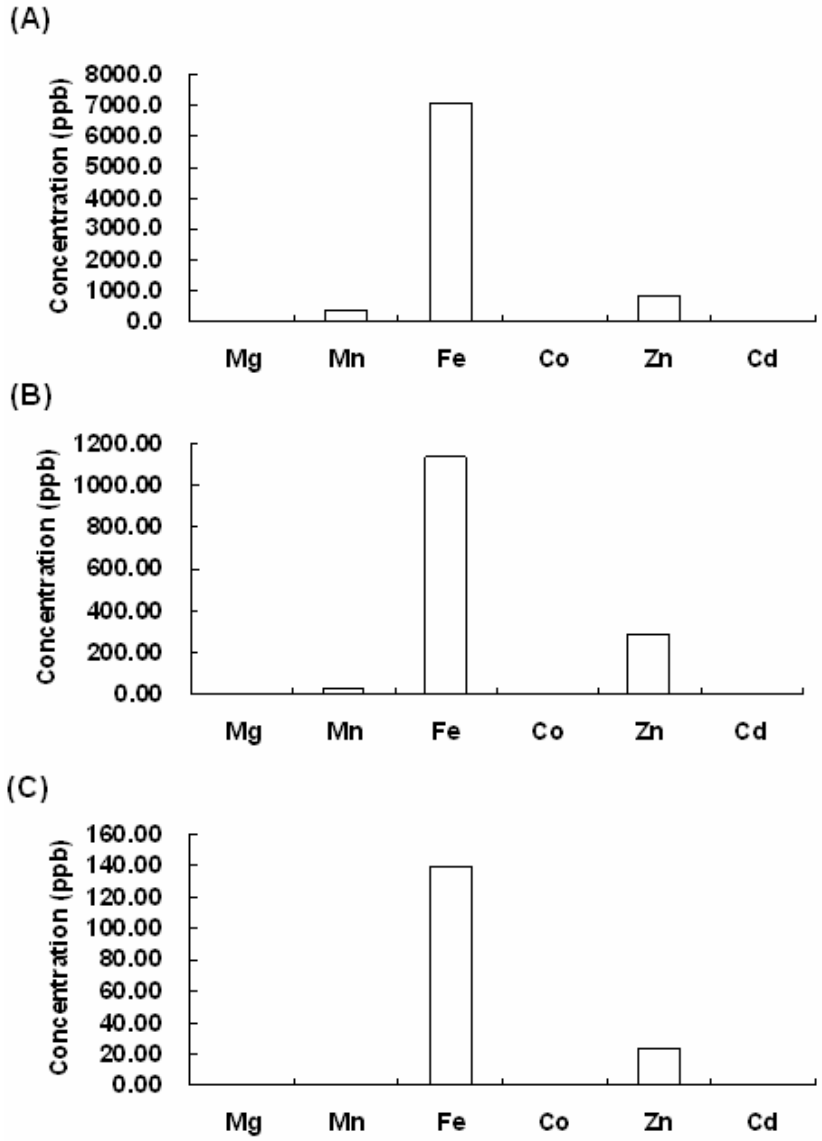
[θ]MRE (10^2 mdeg cm^2 dmole^-1)
WT
WT(denature)
D119AE150A

Appendix 8

Metal element analysis of PepD

Metal element content of purified PepD was determined using an inductively coupled plasma-mass spectrometry (ICP-MS) technique. Briefly, PepD was expressed and purified as described on 2.7, which ddH_2O was substituted for dH_2O through all the experimental steps. The purified and dialyzed fraction was digested by 10% nitric acid and sonicated for 60 min. The following ICP-MS analysis was performed at National Tsing Hua University.



Appendix 7. Metal contents of PepD protein. (A) Fe: Zn = 8.53: 1. (B) Fe: Zn = 3.87: 1. (C) Fe: Zn = 5.81: 1.