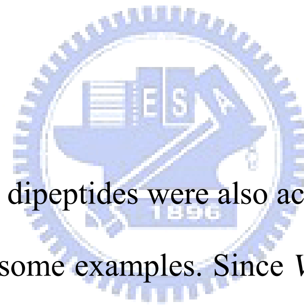
Chapter 4 Discussion and Conclusion

In this study, a putative aminoacylhistidine dipeptidase (*pepD*) gene from *V. alginolyticus* strain ATCC 17749 has been cloned and characterized. *V. alginolyticus pepD* is considered as an aminoacylhistidine dipeptidase that shares both high nucleotide and amino acid sequence identities with *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae* and other bacteria including *E. coli* and *S. typhimurium*. The *V. alginolyticus pepD* encodes a protein with a calculated molecular mass of 53.6kDa. This value agrees closely with the ~55 kDa molecular mass of the expressed PepD in *E. coli* strain with a His-tag on *N*-terminus as estimated by SDS-PAGE, and is also similar to that of previously identified *E. coli pepD* (Klein *et al.*, 1986).

V. alginolyticus PepD was stable at the pH range 6-8.5 and showed maximal activity at pH 7.0-7.4. To our knowledge, the relative acidic or alkaline environment might result in changing the ionization status of some functional residues in protein when their p*K* values were in or near the investigated pH range, but also were the substrate dipeptide as presented by Schroeder *et al.* (Schroeder et al., 1994), which led to the loss of PepD activity on some of the pH profile. The temperature of highest PepD activity, determined at optimal pH, was 37℃. At 70℃, the enzyme still retained 30% of its maximum activity contrast to the loss of full activity when pre-incubated at 70℃ for 30 min, whereas at 10℃, the enzyme remained only 69% of its maximum acitivity contrast to keeping its full activity when pre-incubated at 10℃ for 30 min. The main reason of this phenomenon might result from the enzyme had already hydrolyzed partial L-carnosine before it denatured at higher temperature and could be totally denatured within 30 min. In contrast, the conformation of enzyme was not affected within 30 min at lower temperature, but would decrease its hydrolytic efficiency on substrate when performing at low temperature.

The *V. alginolyticus* PepD hydrolyzed L-carnosine (β-Ala-L-His) as the substrate, which was similar to that exhibited by all of the known PepD. However, the more preference for α-Ala-L-His than L-carnosine as substrate was first identified in bacterial PepD, but the same result could be observed in human cytosolic nonspecific dipeptidase (CN2) (Teufel *et al.*, 2003). Therefore, the amino group in the α or β position of *N*-terminus residue did not affect the recognition and hydrolysis of dipeptide. The Gly-His was another Xaa-His dipeptide able to be cleaved by *V. alginolyticus* PepD. Accordingly, since β-Ala-L-His and GABA-His with the polar amino acid on *N*-terminus were not hydrolyzed by *V. alginolyticus* PepD, we assumed that this enzyme is active on Xaa-His dipeptides which Xaa belongs to small or nonpolar amino acids. The study of substrate specificity on Xaa-His dipeptides was first carried out in bacterial aminoacylhistidine dipeptidase, but had already identified in mammals (Teufel *et al.*, 2003).



The enzymes that degrade dipeptides were also active on tripeptides or polypeptides with the aminopeptidase activity in some examples. Since *V. alginolyticus* PepD had no detectable activities on histidine-containing tripeptides, it is considered to act as a dipeptidase. The same results on substrate specificity of *E. coli* PepD were observed, which neither tripeptides nor GABA-Xaa dipeptides were cleaved. However, *E. coli* PepD was supposed to be a non-strict dipeptidase as a result of exhibiting the deformylase activity toward several formyl amino acids (Schroeder et al., 1994). Other than *E. coli* PepD, comparison of the *L. delbrueckii* PepV and *V. alginolyticus* PepD at the level of substrate specificity, the same substrate of L-carnosine and Gly-Gly-His were examined and shows the identical result. While PepV was capable of hydrolyzing some tripeptides (Vongerichten *et al.*, 1994), PepD acts solely as a dipeptidase. Therefore, according to the results of sequence homology and substrate specificity, the identified dipeptidase gene from *V. alginolyticus* is more likely to be PepD than PepV. In conclusion, PepD from *V. alginolyticus* requires dipeptide substrates exhibiting

a small or nonpolar *N*-terminus with the amino group in both α or β position.

The metal chelator EDTA inhibited *V. alginolyticus* PepD with an IC_{50} of 12 μM, which was similar to the result of human CN2 inhibited by another metal chelating agent 1,10-*o*-phenanthroline at an IC_{50} value of 5 μM (Teufel *et al.*, 2003). The same result was also observed in *E. coli* PepD that both EDTA and 1,10-*o*-phenanthroline exhibited inhibitory activity on β-Ala-L-Met at 1 mM (Schroeder *et al.*, 1994). The other 3 inhibitors, bestatin, benzamidine, and NEM were not examined in the previous studies on bacterial aminoacylhistidine dipeptidase. Bestatin, a compound known to specifically inhibit various aminopeptidases and dipeptidases (Suda *et al.*, 1976), inhibited *V. alginolyticus* PepD at IC_{50} value of 78 μM, which also inhibits two mammal aminoacylhistidine dipeptidases at low concentrations (Teufel *et al.*, 2003). In constrast, two endopeptidase inhibitors have no effects on PepD activity at the recommended concentrations. As a result, *V. alginolyticus* PepD was indicated as a metallo-dipeptidase, which is in good agreement with data on other characterized aminoacylhistidine dipeptidases. Interestingly, based on the result of bestatin inhibition test on *V. alginolyticus* PepD, a two step inhibition phenomenon was considered. The reasons of this phenomenon were probably resulted from a weak binding between PepD and bestatin or the conformational change of PepD caused by bound bestatin.

As indicated as a metallopeptidase, the association of *V. alginolyticus* PepD with metal ions was confirmed using ICP-MS, which indicated that the enzyme was associated with zinc and iron. However, the ratio of them in PepD was not able to be determined conclusively (see Appendix 8). The presence of iron ion was rarely observed in peptidase proteins. According to the iron and zinc at a ratio ranging from 4: 1 to 8: 1, the iron ions have a possibility to form the iron-sulfur cluster (Fe-S cluster) in *V. alginolyticus* PepD. The presence of iron ion was also considered as the external contamination through experimental procedure. The most

direct evidence on the metal content of PepD is determined by crystallography. The expressed *V. alginolyticus* PepD have been characterized as containing one or two zinc ion through the progressive crystallization study.

The kinetics values including k_{cat}, k_{cat}/K_m, with the absence of related report on bacterial PepD and PepV, were compared with mammal aminoacylhistidine dipeptidases. The K_m value and catalytic efficiency (k_{cat}/K_m) of *V. alginolyticus* PepD for L-carnosine were 5.38 mM and 2.45×10^{-2} mM^{-1} s^{-1}, respectively. As compared with that of human carnosinase (CN1) (K_m 1.2 mM and k_{cat}/K_m 8.6 mM^{-1} s^{-1}), PepD catalyzes at a relative low efficiency. Based on the result of higher hydrolysis rate on α-Ala-L-His than β-Ala-L-His as the substrate, PepD could have other better dipeptide substrate or even another totally different enzymatic activity.

According to ProtScale (http://ca.expasy.org/tools/protscale.html), the hydrophilicity plot of the deduced amino acid sequence of *V. alginolyticus pepD* did not show any obvious membrane-spanning domains (data not shown). In addition, no putative signal peptide sequence was noticed upon searching the upstream nucleotide sequence of *pepD* from decoded *V. alginolyticus* 12G01 genome (published at NCBI on march 2006). This suggests that *V. alginolyticus* PepD might be an intracellular enzyme. However, the cellular distribution of PepD in *V. alginolyticus* needs to be further investigated.

The native form of *V. alginolyticus* PepD analyzed by Native-PAGE method shows the relatively less formation of its dimer. However, since a very small amount of protein presents at the position where matches the size of dimerization PepD, we assume a possibility that none of the covalent interaction between PepD proteins are formed and non-covalent interaction is apparently weak. The dimerized PepD might be saperated by electricity through electrophoresis analysis or a substrate is required to stimulate the formation of its dimerization

form. Therefore, the analysis on the native form of *V. alginolyticus* PepD should be excised by gel filtration chromatography method. The *V. alginolyticus* PepD could be present catalytically self-sufficient in solution as a monomer, which is observed in serum carnosinase (Lenney *et al.*, 1982).

The conservation of the active site residues suggests that the hydrolytic mechanism of PepD and PepV might be closely related. Since those 7 amino acids are likely present in the active site of *V. alginolyticus* PepD and was indicated by the loss of activity at their alanine mutants, the putative active site of *V. alginolyticus* PepD could be proposed on the basis of the modeling result and the cystal structures of peptidase family M20. The sidechain ligands of PepD were disposed symmetrically with two zinc ions (Zn1 and Zn2), which Zn1 is coordinated by carboxylate oxygen of Asp119 and Asp173 and by His80. Zn2 is coordinated by the carboxylate oxygen of Glu150 and Asp119 which is different from that of Zn1 and by His461. Thus, each zinc ion was held by three residues, where Asp119 is in fact a bridging ligand between two zinc ions. These two zinc ions, as described by *Jozic et al.*, (Jozic et al., 2002), were considered to play two different roles while hydrolyzing substrates, which were stabilization of the substrate-enzyme tetrahedral intermediate and activation of the catalytic water molecule. Asp82 and Glu149 of PepD were superimposed similarly on PepV Asp89 and Glu153. Asp82 was conserved in all the active enzymes of clan MH and considered to clamp the imidazolium ring of His80. Glu149 served as a general base in catalysis, whereas the water molecule bridged by two zinc ion would act as the attacking hydroxyl ion nucleophile (Rowsell *et al.*, 1997).

In conclusion, a gene cloned from *V.alginolyticus* was predicted as an aminoacylhistidine dipeptidase and named *pepD* because of the high sequence identity with other *Vibrio* species. The further investigation on substrate specificity indicates that *V. alginolyticus* PepD could

use L-carnosine as the substrate and serves simply as a dipeptidase, which is similar to *E. coli* PepD. This enzyme is inactivated by EDTA and bestatin, which confirms it a metallo-dipeptidase in activity. Previous studies have determined bacterial aminoacylhistidine dipeptidase as a metallopeptidase but failed to identify its catalytic residues. Mutagenesis study on PepD has revealed 7 residues that are critical for the peptidase activity and provided clues for the possible active site residues of prokaryotic aminoacylhistidine dipeptidase. As a member of peptidase family M20, our data suggest that the catalytic center of *V. alginolyticus* PepD is depicted as HDDEEDH.