Chapter 1 Introduction

1.1 *Vibrio* species

In the last four decades, researches on taxonomic, environmental, virulent, and medical aspects of *Vibrio* species (*Vibrio* spp.) have expanded greatly. Members of the genus *Vibrio* are defined as Gram-negative, rod or curved rod-shaped, halophilic facultative anaerobes (Fig. 1). They are considered as the natural inhabitants of marine and estuarine environments worldwide (Morris and Black, 1985).

Vibrio spp. are considered opportunistic pathogens in both aquacultured species and human. Vibriosis is the major disease caused by *Vibrio* spp. in shrimp aquaculture, resulting in high mortality and severe economic loss in all producing countries. On the basis of phenotypic data, the major species causing vibriosis in shrimp are *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, and *V. parahaemolyticus* (Goarant *et al.*, 1999). The *V. anguillarum*, *V. damsela*, and *V. carchariae* are major vibriosis-causing species in fish.

Fig. 1. Scanning Electron Microscopy (SEM) of *V. alginolyticus* intermediary morphologies (Albertini *et al.*, 2006).

Vibrio infection is becoming an important public health problem in the United States. Since 1988, the Centers for Disease Control and Prevention (CDC) has maintained a database of reported *Vibrio* isolates and infections from humans. CDC estimates that approximate 8000 *Vibrio* infections and 60 deaths occur annually in the United States (Mead *et al.*, 1999). However, states are not required to submit reports of *Vibrio* isolates and the true number of *Vibrio* isolates may be greater than reported. There are at least twelve pathogenic *Vibrio* species recognized to cause human illness (Table 1) (Janda *et al.*, 1988; Levine and Griffin, 1993). Three species of most medical significance are *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*. Other species, including *V. alginolyticus*, *V. fluvialis*, *V. furnissii*, *V. hollisae*, and *V. metschnikovii*, have been associated with gastroenteritis (Rippey, 1994). *V. cincinnatiensis*, *V. damsela*, and *V. carchariae* have not been associated with gastroenteritis, but on rare occasions are pathogenic to human.

Table 1. Association of *Vibrio* spp. with different clinical syndromes.

	Clinical Syndrome[a]		
Species	**Gastroenteritis**	**Wound Infection**	**Septicemia**
V. alginolyticus	+[b]	++	
***V. cholerae* O1**	++		
***V. cholerae* non-O1**	++	+	+
V. cincinnatiensis			
V. damsela		++	
V. fluvialis	++	(+)	(+)
V. furnissii	++		
V. hollisae	++	(+)	(+)
V. metschnikovii	(+)		
V. mimicus	++	(+)	(+)
V. parahaemolyticus	++	+	(+)
V. vulnificus	+	++	++

[a] Data from Levine and Griffin,1993.
[b] ++ = common presentation, + = less common presentation, and (+) = rare presentation.

Vibrio infections are seasonal and the number of patients has a highest peak during summer months (Hlady and Klontz, 1996). Transmission of many *Vibrio* infections is often implicated in food-borne disease which is primarily through the consumption of undercooked seafood, or exposure of wounds to warm seawater in coastal areas (Morris and Black, 1985; Levine and Griffin, 1993; Marano *et al.*, 2000). Pathogenic vibrios cause three major syndromes of clinical illness: gastroenteritis, wound infections, and septicemia (Blake *et al.*, 1980). The most common clinical presentation of *Vibrio* infection is self-limited gastroenteritis, but wound infections and septicemia may also occur (Table 1) (Levine and Griffin, 1993). Most people with gastroenteritis or septicemia consumed raw seafood especially oysters in 7 days before their illness onset. Furthermore, patients with liver disease are at particularly high risk for morbidity and mortality associated with these infections (Hlady and Klontz, 1996).

1.2 *Vibrio alginolyticus*

V. alginolyticus (Fig. 1) first recognized by Miyamoto *et al.* (Miyamoto *et al.*, 1961) and named *Oceanomonas alginolytica* was renamed *V. alginolyticus* by Sakazaki at 1968 (Sakazaki, 1968). *V. alginolyticus* is very similar to *V. parahaemolyticus* on their biochemical properties and also isolated from similar types of marine samples. Acetoin production and fermentation of arabinose are important features which distinguish *V. alginolyticus* from *V. parahaemolyticus* (Molitoris *et al.*, 1985). *V. alginolyticus* was found with worldwide distribution (Schmidt *et al.*, 1979) and had been isolated from coastal water, sediments, and seafood taken from the temperate and tropical areas (Molitoris *et al.*, 1985). As other *Vibrio* spp., *V. alginolyticus* appears on a seasonal basis that was rarely found in winter and abundant in summer (Molitoris *et al.*, 1985). Studies by Baross and Liston showed that the minimum growth temperature for *V. alginolyticus* is 8℃ (Baross and Liston, 1970).

Grouper culture has become an alternative and important activity since penaeid shrimp culture industry collapsed in the late 1980's in Taiwan. The production of farmed grouper in Taiwan has increased more than two times from 5052 tonnes in 2001 to 12,103 tonnes in 2004. Vibriosis caused by pathogenic *V. alginolyticus* is a common problem in the intensive culture of grouper with a gastroenteritis syndrome (swollen intestine containing yellow fluid) (Lee, 1995). In addition to grouper culture, *V. alginolyticus* was also considered important in fish and shrimp culture pathogenicity on account of previous data from the field studies (Licciano *et al.*, 2005) and a disease outbreak of shrimp farming in 1996 (Lee *et al.*, 1996).

V. alginolyticus was first recognized as human pathogen in 1973 (Zen-Yoji *et al.*, 1973). In recent years, several studies have reported the clinical infection caused by *V. alginolyticus* (Levine and Griffin, 1993; Reina Prieto and Heravs Palazon, 1993; Gahrn-Hansen and Hornstrup, 1994) which poses a risk to humans who are in contact with it. Most human infections caused by *V. alginolyticus* were accounted for the consumption of raw or undercooked seafood obtained from fish, shellfish, shrimps, or squid (Rippey, 1994). As other *Vibrio* spp., the major clinical syndromes of illness *V. alginolyticus* causes are gastroenteritis, wound infections, and septicemia. Wound infections through waterborne transmission are the most frequently *V. alginolyticus* infections and account for 71% of its total infections (Rose *et al.*, 2001). Gastroenteritis was a rare presentation of *V. alginolyticus* infection, but it accounted for 12% of infections (Hlady and Klontz, 1996). Other cases of diseases including ear infections, central nervous system disease and osteomyelitis have also been reported (Opal and Saxon, 1986; Matsiota-Bernard and Nauciel, 1993). Thus, prevention, early detection and initiation of treatment of *V. alginolyticus* infections are very important to keep fish and human healthily.

1.3 Biofilm

Most microbes, either prokaryotic or eukaryotic, Gram-positive or Gram-negative, pathogenic or non-pathogenic in the natural environments live in surface-attached organized communities called biofilms rather than as single free-swimming planktonic cells (Costerton *et al.*, 1987; Costerton *et al.*, 1995). Biofilms were mainly composed of polysaccharides and other extracellular structures including flagella and pili (Pratt and Kolter, 1998) and curli (Olsen *et al.*, 1989; Vidal *et al.*, 1998). These components are involved in initial adhesion of bacteria to a solid surface or in subsequent steps of biofilm formation. Biofilms can harbor different species of microorganisms to establish mutual interactions through complex chemical signaling known as quorum sensing (Fuqua *et al.*, 1994; Costerton *et al.*, 1995). A mature biofilm can display complex structural and functional architecture, with cells located in different areas showing different biochemical and morphological properties (Lawrence *et al.*, 1991; Costerton *et al.*, 1994).

Microorganisms growing in biofilm are more resistant to antibiotics, biocides, desiccation, oxidative stress, and both protozoa predation and bacteriophages attack than their planktonic counterparts (Hoyle and Costerton, 1991; Weiner *et al.*, 1995; Finlay and Falkow, 1997; Stewart, 2001). These observations strongly suggest that biofilms can be considered as a “resistance form” of growth, able to withstand stress conditions more efficiently than planktonic cells. Thus, the dynamics of biofilm formation might facilitate the transmission of pathogens by providing a stable protective environment and acting as a nidus for the dissemination of large numbers of microbes (Hall-Stoodley and Stoodley, 2005). For this reason, understanding the mechanisms of biofilm formation is undoubtedly important for exploring effective strategies to control harmful biofilm formation and promote beneficial biofilm formation.

The clinically relevant bacteria including *V. cholerae*, *Escherichia coli*, and *Pseudomonas aeruginosa* are model organisms for biofilm studies. Genetic and microscopic studies of biofilm formation have suggested that development of a mature biofilm involves the following three stages: the planktonic stage, the monolayer stage and the biofilm stage (Fig. 2) (O'Toole *et al.*, 2000). Free-swimming planktonic cells encountering a surface become transiently attached to it. The permanent immobilization of these cells on the surfaces results in the formation of the monolayer. Induction of extracellular matrix biosynthesis by cells in the monolayer leads to the development of a multi-layered biofilm (Whiteley *et al.*, 2001; Schembri *et al.*, 2003; Zhu and Mekalanos, 2003).

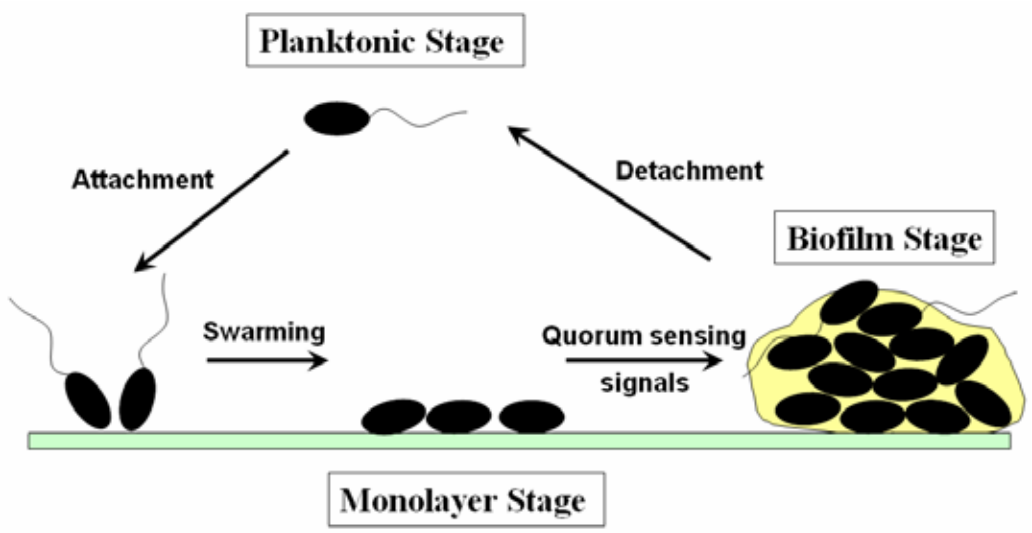


Fig. 2. Model of the development of a mature biofilm from planktonic cells. Biofilm formation is initiated by the attachment of individual cells to a surface, followed by their surface migration, replication to form microcolonies, and quorum-sensing regulated differentiation into the mature biofilm encased in polysaccharide. Subsequent detachment and dispersal of organisms completes the cycle.

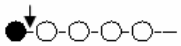
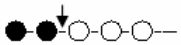
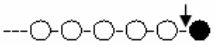
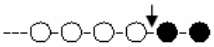
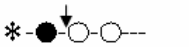
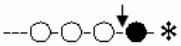
Biofilm-growing cells display different patterns of gene expression when compared with planktonic cells (Prigent-Combaret *et al.*, 1999). Expression of determinants for initial adhesion is likely to take place already in planktonic cells in response to environmental

conditions (Pratt and Kolter, 1998; Prigent-Combaret *et al.*, 1999). The adhesion event itself stimulates the expression of biofilm-specific genes, such as the *algC* operon in *Pseudomonas* spp. (Davies and Geesey, 1995). As cells start growing on the colonized surface, the increase of their local concentration leads to activation of quorum-sensing-dependent genes, several of which encode proteins involved in the production of the exopolymeric substance of mature biofilm (Miller and Bassler, 2001). In contrast, several genes might be repressed through the biofilm forming process. The expriments performed by Brombacher *et al.* show that the expression of *pepD* negatively affects biofilm formation (Brombacher *et al.*, 2003). Therefore, the peptide-hydrolyzing enzyme PepD might be involved in bacterial biofilm formation.

1.4 Proteolytic Enzymes

The term proteolytic enzymes also commonly termed proteases, peptidases, and proteinases were named for all the enzymes that hydrolyze peptide bonds. However, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) recommends the term peptidase as the general term for these enzymes. Proteolytic enzymes with different peptide bond hydrolyzing activities could be classified into several groups (Table 2) (Rao *et al.*, 1998) or in clans and families.

Table 2. Classification of peptidases.

Peptidase	Mode of action[a]	EC No.
Exopeptidase		
Aminopeptidase		3.4.11
Dipeptidyl peptidase		3.4.14
Tripeptidyl peptidase		3.4.14
Carboxypeptidase		
Serine-type carboxypeptidase		3.4.16
Metallocarboxypeptidase		3.4.17
Cysteine-type carboxypeptidase		3.4.18
Peptidyl dipeptidase		3.4.15
Dipeptidase		3.4.13
Omega peptidase		3.4.19
		3.4.19
Endopeptidase		
Serine endopeptidase		3.4.21
Cysteine endopeptidase		3.4.22
Aspartic endopeptidase		3.4.23
Metalloendopeptidase		3.4.24
Threonine endopeptidase		3.4.25
Endopeptidase of unknown catalytic mechanism		3.4.99

[a] Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and asterisks signify the blocked termini. Arrows show the sites where the enzymes act.

In general, exopeptidases cleave one or a few amino acids from the *N*- or *C*-terminus of the polypeptide chain, and endopeptidases cleave internally. The exopeptidases that act at a free *N*-terminus liberate a single amino acid residue (aminopeptidases) or a dipeptide (dipeptidyl peptidases) or a tripeptide (tripeptidyl peptidases). Those acting at a free *C*-terminus liberate a single residue (carboxypeptidases) or a dipeptide (peptidyl dipeptidases). Other exopeptidases are specific for dipeptides (dipeptidases) or remove terminal residues that are substituted, cyclized or linked by isopeptide bonds (peptide linkages other than those of α-carboxyl to α-amino groups) (omega peptidases). The endopeptidases are divided on the basis of catalytic mechanism into aspartic endopeptidases, cysteine endopeptidases, serine endopeptidases, threonine endopeptidases, and metalloendopeptidases.

Homologous peptidases are able to be classified more detailed into families and clans. Families are grouped by statistically significant similarity in amino acid sequence to the typical sample especially at its catalytically active part. Each peptidase family is also named two letters with a letter denoting the catalytic type followed by a sequentially assigned number, for example A1, A2, A3, etc., which A represents the catalytic type of aspartic. The recognized catalytic types are aspartic, cysteine, metallo, serine, threonine, and unclassified which depends upon the chemical nature of the groups responsible for catalysis. Clans are groups of homologous families for which there is evidence of similarity in three-dimensional structures or the arrangement of catalytic residues. The name of a clan is identified with two letters that the first represents the catalytic types followed by a serial second capital letter, for example CA, CD, CE, etc., which C represents the catalytic type of cysteine (Rawlings *et al.*, 2006).

1.4.1 Metallopeptidase

The peptidase required metal ion for its catalytic activity was named metallopeptidase. Metallopeptidases are the most diverse of the four main type of peptidase, with 15 clans and more than 30 families identified to date (Rawlings and Barrett, 1995; Rawlings *et al.*, 2006). In these enzymes, the nucleophilic attack on a peptide bond is mediated by a water molecule, which is also observed in aspartic and glutamic peptidases (Rawlings and Barrett, 1993). The water molecule was activated by a divalent metal cation, usually zinc but sometimes cobalt, manganese, nickel or copper.

Metallopeptidases can be divided into two board types depending on the number of metal ions required for catalysis. In many metallopeptidases, only one metal ion is required, but in some families they require two metal ions that act together or so called "co-catalytically". All of the known metallopeptidases in which cobalt or manganese is essential require two of cobalts or manganeses for their catalytic activity, and some of the zinc-dependent metallopeptidases are also included. However, all the metallopeptidases containing nickel require only one for catalysis. All known co-catalytic metallopeptidases are exopeptidases which include aminopeptidases, carboxypeptidases, dipeptidases, and tripeptidases, whereas metallopeptidases with only one catalytic metal ion might be exopeptidases or endopeptidases.

In the metallopeptidases, the metal ion is held in place by amino acid residues, usually three in number. These residues are His, Glu, Asp, or Lys and at least one other residue is required for catalysis, which might play an electrophilic role. Of the known metallopeptidases and their crystallographic studies, around half contain an HEXXH motif to form part of the metal-binding site (Rawlings and Barrett, 1995).

1.4.2 Peptidase family M20

According to the MEROPS database (http://merops.sanger.ac.uk), metallopeptidases were classified into 15 clans including MA, MC, MD, ME, MF, MG, MH, MJ, MK, MM, MN, MO, MP, MQ, and M-. The peptidases in clan MH contains a variety of co-catalytic zinc-dependent peptidases binding two atoms of zinc per monomer, which is held by five amino acid ligands (Rawlings *et al.*, 2006) and inhibited by the general metal chelator ethylenediamine-tetraacetic acid (EDTA).

The peptidase clan MH is further classified into four families: M18, M20, M28, and M42. Proteins of peptidase family M20 were characterized as water bound by two zinc ions ligated by five residues in the order His/Asp, Asp, Glu, Glu/Asp, and His at the active site. An additional Asp and a Glu residue are thought to be also important for catalysis and occur adjacent to metal-binding resides (Rawlings and Barrett, 1995). In general, the general active site residues arrangement of metal-binding residues with the addition of two catalytic residues (bold) would be His/Asp, **Asp**, Asp, **Glu**, Glu, Glu/Asp, His. The Asp residue between two catalytic residues binds both metal ions. However, there are variations in the individual subfamilies that can be seen in their amino acid sequence alignments. Peptidase family M20 can be devided into 4 subfamilies, M20A, M20B, M20C, and M20D, which their active site residues were different among subfamilies. The type protein of peptidase family M20C is aminoacylhistidine dipeptidase.

1.4.3 Aminoacylhistidine dipeptidase

Aminoacylhistidine dipeptidases (EC 3.4.13.3, also Xaa-His dipeptidase, X-His dipeptidase, carnosinase, and PepD) are zinc-containing metallopeptidase, which catalyze the cleaving and release of an *N*-terminal amino acid, usually neutral or hydrophobic residue, from Xaa-His dipeptides or polypeptides (Fig. 3) (Rawlings and Barrett, 1995).

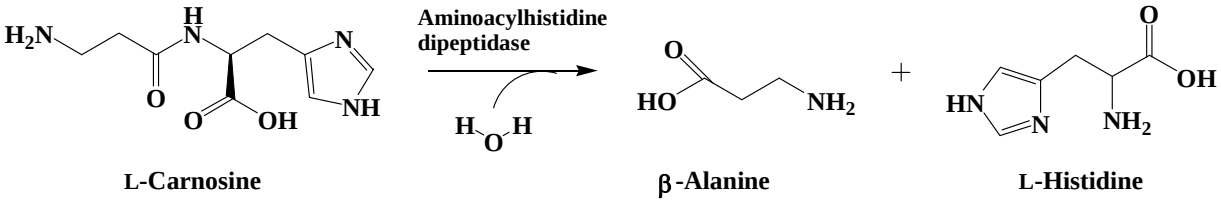


Fig. 3. An enzymatic reaction catalyzed by aminoacylhistidine dipeptidase. Aminoacylhistidine dipeptidase catalyzes the hydrolysis of a dipeptide L-carnosine (β-Ala-L-His) into two amino acids.

This gene is found extensively among the prokaryotes and eukaryotes. In 1974, the first direct proof of aminoacylhistidine dipeptidase activity to hydrolyze an unusual dipeptide L-carnosine (β-Ala-L-His) in bacteria was obtained with *Pseudomonas aeruginosa* (van der Drift and Ketelaars, 1974). In the following years, this carnosine-hydrolyzing enzyme is found from a number of bacterial species (Bersani *et al.*, 1980), but only PepD of *Escherichia coli* have been characterized genetically and biochemically (Schroeder *et al.*, 1994).

The *E. coli pepD* encodes a 52 kDa protein and is active as a homodimer with molecular mass of 100 kDa (Klein *et al.*, 1986). The pure enzyme had a pH and temperature optimum at pH 9.0 and 37℃, respectively. *E. coli* PepD appears to be a metallopeptidase with board substrate specificity, which was activated by Co^{2+} and Zn^{2+}, and deactivated by metal chelators (Schroeder et al., 1994).

In general, dipeptidases are involved in the final breakdown of protein degration fragments produced by other peptidases or the final dipeptide breakdown for amino acid utilization. The same result was observed in PepD-deficient mutant of *E. coli* (Miller *et al.*, 1978) and *S. typhimurium* (Kirsh *et al.*, 1978), which indicates that PepD hydrolyzes dipeptide as an amino acid source. However, the biological impact of PepD still remains unclear.

1.4.4 Peptidase V

The Peptidase V (PepV) from *Lactobacillus delbrueckii* ssp. *lactis* DSM 7290 was originally identified as a carnosinase cleaving L-carnosine as a source of histidine by the *E. coli* mutant strain UK197 (*pepD*, *hisG*) (Vongerichten *et al.*, 1994). Lactobacilli are organisms with multiple amino acid auxotrophies making them critically dependent on their proteolytic abilities to efficiently degrade milk protein casein and used as starter cultures in dairy fermentations. Deletion of the dipeptidase *pepV* gene from *Lactococcus lactis* resulted in significantly decreased growth rates but did not reduce the final cell density (Hellendorn *et al.*, 1997).

L. delbrueckii pepV is 1413 nucleotides in length and consists of a 470 residues polypeptide corresponding to a protein with predicted molecular mass of 52 kDa. It has been characterized as a relatively unspecific dipeptidase cleaving a variety of dipeptides, especially those wih the unusual β-alanyl residue in the *N*-terminus, but also catalyzing the removal of the *N*-terminal amino acid from a few distinct tripeptides (Vongerichten *et al.*, 1994). Interestingly, PepV is related not only to peptidases but also to acetylornithine deacetylase (ArgE, EC 3.5.1.16) and succinyldiaminopimelate desuccinylase (DapE, EC 3.5.1.16), and has recently been described as a member of the aminoacylase-1 family (Biagini and

Puigserver, 2001). These enzymes share the characteristics of hydrolyzing amide bonds in a zinc- (or cobalt-) dependent manner.

PepV is also recognized as a metallopeptidase since full inhibition with metal chelating agent 1,10-phenanthroline or EDTA could be detected. This result is confirmed by the crystal structure of PepV, which two zinc ions is associated in one monomer protein (Jozic *et al.*, 2002). The 3D structure of PepV protein consists of two distinc domains, named the lid (lower) domain and the catalytic (upper) domain (Fig. 4A). The latter consists of residues from Met1 to Gly185 and from Ser388 to Glu468, whereas the lid domain comprises the residues from Glu186 to Gly387. Zinc ions are located in the catalytic domain and involved in the interactions with His87, Asp119, Glu154, Asp177, and His439 (Fig. 4B) (Jozic *et al.*, 2002).

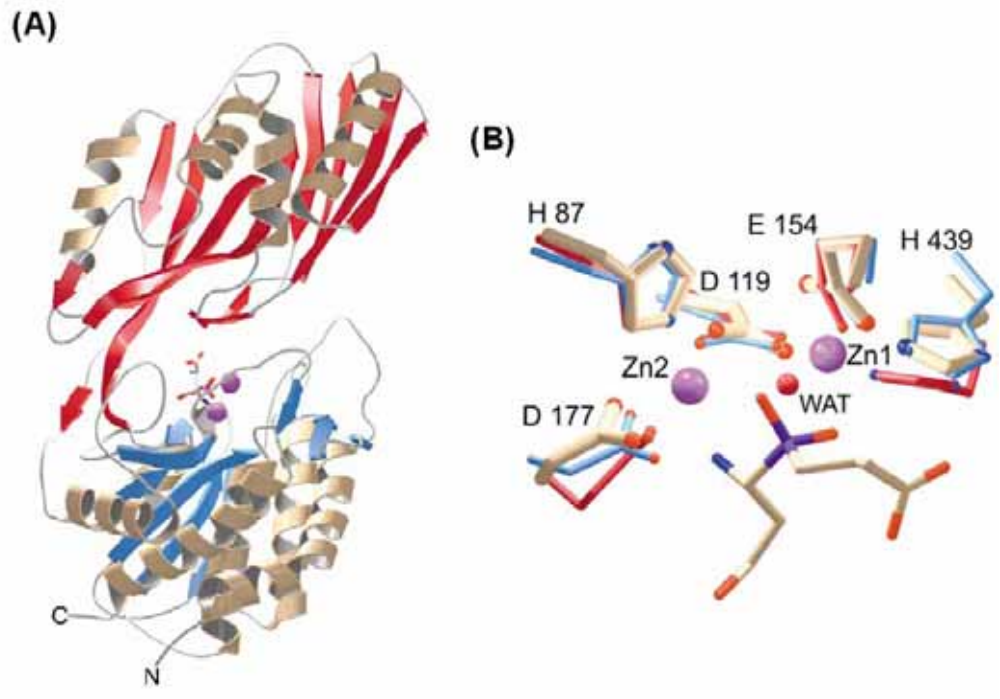


Fig. 4. (A) Ribbon diagrams of the crystal structure of PepV and (B) Stereo view of zinc-binding residues of PepV. The stereo view of PepV was superimposed with the residues of AAP (blue) and CPG2 (red). The co-crystallized inhibitor AspΨ[PO_2CH_2]AlaOH is depicted as a stick model (beige). Two zinc ions of PepV are represented as purple spheres. The catalytic water molecule (WAT) of CPG2 is depicted in red

1.4.5 Carnosinase

The enzymes with L-carnosine hydrolyzing activity were also observed in mammals named carnosinase in general. The unique substrate of carnosinase, L-carnosine, is abundant in skeletal muscles of most vertebrates. It also detected in brain and cardiac muscle, but not in several other organs, such as kidney, liver, and lung (Jackson and Lenney, 1996). Carnosine represents the archetype of a series of histidine-containing dipeptides in mammals, such as homocarnosine (γ-Amino-butyryl-His), carcinine, *N*-acetylcarnosine, and anserine (Fig. 5). The complete role of these dipeptides is still unknown, even though their function has been studied intensively in recent years. Available studies indicate that carnosine has a range of antioxidant or cytoprotective properties (Boldyrev *et al.*, 2000) to act as a cytosolic buffer (Vaughan-Jones *et al.*, 2006), an antioxidant (Decker *et al.*, 2000), and an antiglycation agent (Seidler, 2000). Moreover, homocarnosine can represent a GABA reservoir, mediating the antiseizure effects of GABAergic therapies (Petroff *et al.*, 2001).

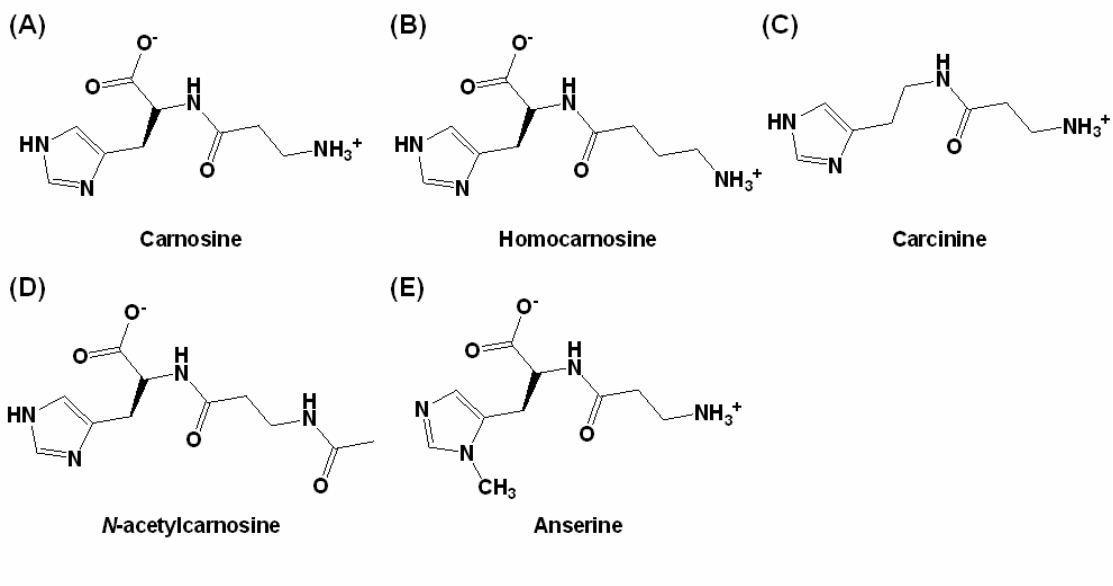


Fig. 5. Common histidine-containing dipeptides present in mammals.

There are at least two isoforms of carnosinase have been characterized so far. The first enzyme is a cytosolic form (also named tissue carnosinase, CN2, EC 3.4.13.18) and the second one is known as serum carnosinase (CN1, EC 3.4.13.20) (Teufel *et al.*, 2003). In the previous study, serum carnosinase is assumed to be involved in some important pathological conditions. Decreased concentrations of serum carnosinase have been observed in patients with Parkinson's disease, multiple sclerosis, or after a cerebrovascular accident (Wassif *et al.*, 1994). It was also suggested that monitoring of serum carnosinase might be useful to predict the clinical outcome of patients with acute stroke (Butterworth *et al.*, 1996). Dificiency of human carnosinase has been associated with neurological deficits including intermittent seizures and mental retardation (Perry *et al.*, 1967; Murphey *et al.*, 1973).

Experimental evidence shows that tissue carnosinase acts as an ubiquitous nonspecific dipeptidase with broad substrate specificity and strongly inhibited by bestatin (Lenney *et al.*, 1985; Teufel *et al.*, 2003). Serum carnosinase is characterized by distribution in the plasma and brain of mammals, and hydrolysis of both anserine and homocarnosine (Jackson *et al.*, 1991; Teufel *et al.*, 2003). Serum carnosinase is present in solution as a homodimer, despite the monomer appears to be catalytically self-sufficient (Lenney *et al.*, 1982). The nature of metal ion in serum carnosinase remains unknown and could be activiated by Cd^{2+} and citrate ions (Lenney *et al.*, 1982). Study on serum carnosinase was also approached by computational analysis that suggests a therapeutic usefulness of either inhibiting by L-carnosine analogues (e.g., in diabetes) or activating the enzyme by the rational design of citrate-like, non-toxic allosteric modulators (e.g., in homocarnosinosis) (Vistoli *et al.*, 2006).

1.4.6 Other Carnosine-Hydrolyzing Enzymes

There are also some other proteins reported to have the dipeptidase activity on L-carnosine. BapA from *Pseudomonas* sp. proposed as β-Ala-Xaa dipeptidase (EC 3.4.13.-) was found to hydrolyze peptide bonds of β-alanyl dipeptides (β-Ala-Xaa) (Komeda and Asano, 2005). Pep581 from *Prevotella* species (*Prevotella* spp.) shared similar sequence identity of 47% with *E. coli* PepD and has a calculated molecular weight of 53.2 kDa. Pep581 hydrolyzed both dipeptides and single amino acid from the *N*-terminus of tri- and oligopeptides, which differs from PepD enzymes (Walker *et al.*, 2005). Anserinase (Xaa-methyl-His dipeptidase, EC 3.4.13.5) mainly catalyzing the hydrolysis of *N*α-acetylhistidine in all poikilothermic vertebrates is also active on carnosine.

1.5 Research Goal

L-carnosine, an unusual dipeptide, is benefited to organisms at several physiological aspects. However, the enzyme named aminoacylhistidine dipeptidase which exists extensively among parkaryotes and eukaryotes could hydrolyze it into two amino acids. Both the biological importance and function of aminoacylhistidine dipeptidase and L-carnosine are less known.

Bacterial adhesion to fish external surfaces is generally the initial step for colonization (Alexander and Ingram, 1992). Based on this phenomenon, *V. alginolyticus*, an important pathogen to human and fish, might infect fish with the biofilm formation on the intestine. Since PepD affects the biofilm formation, it could be a promising target to control bacterial biofilm formation and infection. Besides, bacterial DapE (EC 3.5.1.18) and ArgE (EC 3.5.1.18), the same members of peptidase family M20 with PepD, are now both considered as the potential targets of antimicrobial agents. Therefore, PepD could also be another potential target for antimicrobial treatments.

Since the latent importance of bacterial aminoacylhistidine dipeptidase in biological aspect, additional with the studies on it is simply in the genetic and biochemical aspect from *E. coli* and *S. typhimurium*, we perform a study on a putative aminoacylhistidine dipeptidase gene from *V. alginolyticus* genome through sequence identification, gene expression and protein purification, and biochemical properties characterization. Besides, due to neither investigations on its functional residues nor crystal structure are reported, a study of the functional residues of *V. alginolyticus* PepD through site-directed mutagenesis analysis are also performed.