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Transition metal ions induce carnosinase activity in PepD-homologous protein from *Porphyromonas gingivalis*

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ABSTRACT

Aminoacylhistidine dipeptidase (EC 3.4.13.3; also Xaa-His dipeptidase, carnosinase, or PepD) catalyzes the cleavage and release of an N-terminal amino acid, which is usually a neutral or hydrophobic residue, from an Xaa-His dipeptide or degraded peptide fragment. PepD enzyme is found extensively in prokaryotes and eukaryotes, and belongs to the metallopeptidase family M20, a part of the metallopeptidase H (MH) clan. Carnosine is a naturally occurring dipeptide (β-alanyl-L-histidine) present in mammalian tissues that has protective functions in addition to anti-oxidant and free-radical scavenging roles. During bacterial infections, degradation of L-carnosine via carnosinase or PepD-like enzymes may enhance the destructive potential of bacteria, resulting in a pathological impact. This process has been proposed to act in an anti-oxidant manner *in vivo*. In the present study, the recombinant PepD protein encoded by *Porphyromonas gingivalis* TDC60 *pepD* was generated and biochemically characterized. In addition, a recombinant dipeptidase enzyme was found to function not only as an alanine-aminopeptidase, but also as a carnosinase. Furthermore, when carnosine was used as substrate for PepD, the transition metals, Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+} stimulated the hydrolyzing activity of rPepD with β-alanine and L-histidine. Based on its metal ion specificity, we propose that this enzyme should not only be termed L-aminopeptidase, but also a carnosinase.

1. Introduction

Periodontal diseases are complex bacterial-associated inflammatory diseases of supporting tissues of the teeth. The change from a periodontal healthy site to one undergoing destruction is accompanied by an increase in relative abundance of a small number of opportunistic pathogens, in particular *Porphyromonas gingivalis* [1–3]. During related inflammation, proteolytic enzymes are released into periodontal tissue from leukocytes, and activate structural cells of epithelia and connective tissue. In addition, proteolytic enzymes, including collagen-degrading enzymes, elastase-like enzymes, trypsin-like proteases, aminopeptidases, and dipeptidyl peptidases, may be produced by microorganisms [4].

P. gingivalis is a gram-negative, black-pigmented, asaccharolytic anaerobe that relies on fermentation of amino acids for production of metabolic energy. Dipeptidases play a general role in the final breakdown of peptide fragments produced by other peptidases during the protein degradation process [5–8]. Several dipeptidases, and a few oligopeptidases have been implicated in cleavage of the final peptide fragments for amino acid utilization in this bacterium. However, the functional residues of peptidase-related enzymes in *P. gingivalis* are poorly understood.

Carnosine (β-alanyl-L-histidine, see Scheme 1) and related peptides are naturally occurring dipeptides present in mammalian tissues, such as the brain and skeletal muscles [9]. Carnosine has protective functions, in addition to anti-oxidant and free-radical scavenging roles, and has been shown to extend the lifespan of cultured human fibroblast, kill transformed cells, protect cells against aldehydes and an amyloid peptide fragment, and inhibit protein glycation (formation of cross-links, carbonyl groups and

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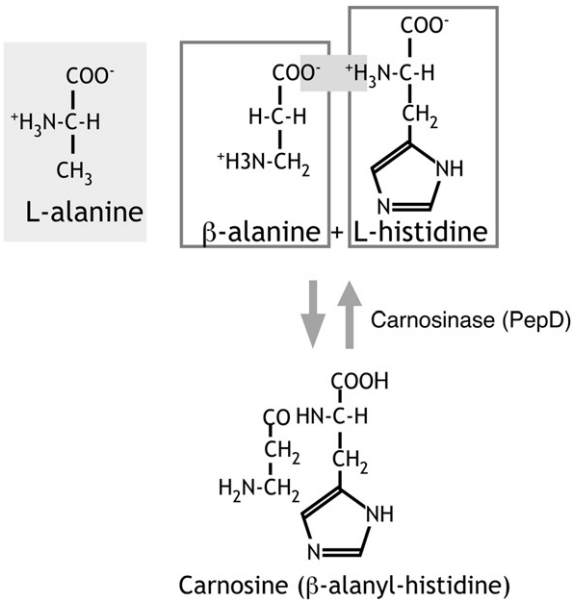

Scheme 1. Chemical structures of compounds containing carnosinase activity (L-histidine, carnosine, β-alanine, L-alanine).

AGEs) and DNA/protein cross-linking in vitro [10]. Although most dipeptides are susceptible to proteolytic degradation, carnosine is resistant to most peptidases, probably due to its β-alanine moiety and N-terminal side. However, certain mammalian tissues that express an enzyme termed carnosinase is able to hydrolyze carnosine into β-alanine and L-histidine [11,12].

Aminoacylhistidine dipeptidase (EC 3.4.13.3; also Xaa-His dipeptidase, carnosinase, or PepD) catalyzes the cleavage and release of an N-terminal amino acid, which is usually a neutral or hydrophobic residue, from an Xaa-His dipeptide or degraded peptide fragment [13]. The PepD enzyme is found extensively in prokaryotes and eukaryotes, and belongs to the metallopeptidase family M20, a part of the metallopeptidase H (MH) clan. This enzyme has generally been identified as a dipeptidase with broad substrate specificity. Such enzymes have shown potential for application as anti-bacterial targets or therapeutic agents for cancer treatment, and may play roles in aging as well as neurodegenerative or psychiatric diseases in humans. On the other hand, in microorganisms, a number of types of aminoacylhistidine dipeptidases were structurally analyzed in *Vibrio alginolyticus* (PepD) [14,15], *Vibrio proteolyticus* aminopeptidase [16], *Streptomyces griseus* aminopeptidase S [17], Pseudomonas sp. carboxypeptidase G2 [18], *Salmonella typhimurium* (PepT) [19], and *Lactobacillus delbrueckii* (PepV) [20]. They were found to play fundamental roles in certain biochemical events, such as protein maturation and degradation, and proposed to be virulence factors in the pathogenesis of disease in humans and some animals. During bacterial infections, the degradation of L-carnosine via carnosinase or PepD-like enzymes was suggested to enhance the destructive potential of bacteria, resulting in a pathological impact [20].

Table 1
Primers for PCR cloning.

Gene name	Primers	Sequence
pepD	Forward	P-aagctttgATGAACATTACAGATCTCAAA
	Reverse	P-aattcttaTTTGGCTGCGGGGATATG

Both forward and reverse primers must be 5′-phosphorylated to clone into the dephosphorylated vector. The underlined ATG and tta in the forward and the reverse primers are the start and terminal codons, respectively. Capital letters indicate target DNA sequence.

In the present study, we present the results of cloning, overexpression, and biochemical characterization of PepD recombinant protein produced from *P. gingivalis* TDC60, as well as a detailed analysis of its substrate specificity and the effects of metals on its enzymatic activity. rPepD from *P. gingivalis* was found to have transition metal-related activity toward β-alanyl dipeptides.

2. Results

2.1. Cloning, and sequence analysis of pepD gene of P. gingivalis TDC60

To clone the *pepD* gene from *P. gingivalis* TDC60, we prepared a DNA fragment for the open reading frame (ORF) of *pepD* using the primer set F-R (Table 1). After cloning with a Profinity eXact cloning kit, we obtained an *Escherichia coli* harbored *pepD* clone (#30-5-14). We then analyzed the PepD protein of *P. gingivalis* TDC60 using NCBI Blast searching, which revealed a high sequence homology to that of other *P. gingivalis* W83 and ATCC33277 (99% identity), and *Porphyromonas* sp (62% identity), *Bacteroides* sp (59–62%), and *Prevotella* sp. (49%), but low with *Lactobacillus* sp. (21% identity). We constructed further distance tree results using the homologous, or PepD-homologous proteins of other bacteria found in our Blast

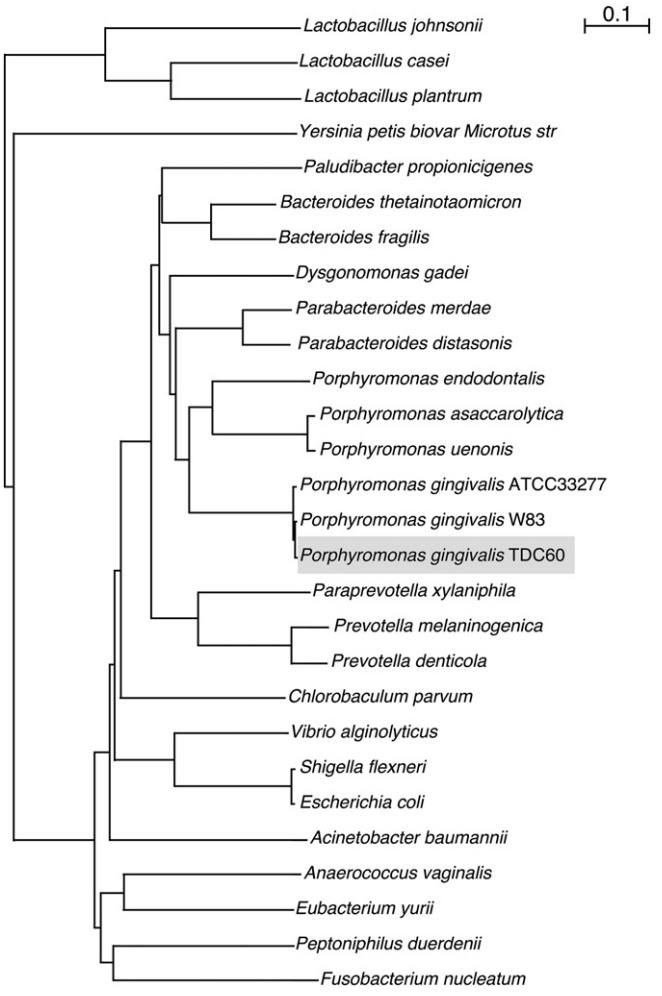

Fig. 1. Distance tree of homologous proteins for PepD from *P. gingivalis* TDC60. After cloning, the nucleotide sequences of *pepD* genes inserted into eXact clones were analyzed using NCBI Blast searching. A distance tree of the results was constructed using the proteins listed by the Blast search.