溶藻弧菌胺醯組胺酸雙胜肽酶之序列與生化特性分析及其功能性胺基酸之研究

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摘要

溶藻弧菌是一種重要的伺機性病原體，主要於人體及養殖水產引起弧菌症。弧菌屬細菌能自我形成具保護功能之生物膜，使其病原體更易入侵和感染生物體，而生物膜的存在被認為可能與慢性疾病的產生和疾病的大流行有關。胺醯組胺酸雙胜肽酶(PepD, EC 3.4.13.3)為胜肽酶家族 M20 中的一員，被研究認為影響細菌生物膜的形成。過去對於細菌中胺醯組胺酸雙胜肽酶的研究很少，只針對其序列和部分生化特性進行探討，並無其生理角色或活性區胺基酸相關之研究。本論文從溶藻弧菌 ATCC 17749 中首次發現胺醯組胺酸雙胜肽酶之基因，並對其進行序列分析、生化特性及活性區胺基酸之研究。此基因之開放讀碼區(ORF)序列共有 1473 個鹼基對，可轉譯出一條長 490 個胺基酸的蛋白質，計算其分子量約為 53.6 kDa。此胺基酸序列和其他弧菌屬之 PepD 蛋白質序列比對有非常高之相似度，同時與大腸桿菌及鼠傷寒桿菌相似度達 63%。將溶藻弧菌 *pepD* 基因殖入 pET-28a(+)質體中，表現 N 端帶有 His-tag 之重組蛋白，並利用 Ni-NTA 親和層析管柱純化之。純化出的蛋白質可水解雙胜肽 L-carnosine 及其他特定 Xaa-His 雙胜肽，但無水解三胜肽之活性。經酵素動力學研究，溶藻弧菌 PepD 蛋白對雙胜肽 L-carnosine 之 K_m 與 k_{cat} 值分別為 5.38 mM 與 0.132 s^{-1}。研究發現溶藻弧菌 PepD 重組蛋白於 pH 7.4 及 37℃環境下有最佳活性。此酵素活性可被金屬螯合劑 EDTA 及雙胜肽類似物抑制劑 bestatin 所抑制，推測溶藻弧菌 PepD 蛋白為金屬雙胜肽酶。經序列分析預測溶藻弧菌 PepD 蛋白上胺基酸位置 His80、Asp82、Asp119、Glu149、Glu150、Asp173 及 His461 為活性區胺基酸。將其分別定點突變為 H80A、D82A、D119A、E149A、E150A、D173A

及 H461A 後，突變蛋白皆失去原有之活性。此外，以 PepV 蛋白結晶結構為模板做出溶藻弧菌 PepD 蛋白之同源模擬，顯示出相同之活性區胺基酸。因此，根據本論文實驗結果將首次提出胺醯組胺酸雙胜肽酶活性區胺基酸之分佈情形與其可能扮演之功能。

Sequence Identification, Biochemical Characterization, and Functional Residues Analysis of Aminoacylhistidine Dipeptidase from *Vibrio alginolyticus*

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Abstract

Vibrio alginolyticus is one of the important opportunistic pathogens causing vibriosis in aquacultured species and human. *Vibrio spp.* are examples to form a stable protective biofilm that might facilitate the transmission of pathogens. Aminoacylhistidine dipeptidase (PepD, EC 3.4.13.3), a member of peptidase family M20, was considered to be involved in bacterial biofilm formation. The researches on bacterial PepD were less known and only investigated genetically and biochemically. A newly defined aminoacylhistidine dipeptidase from *Vibrio alginolyticus* ATCC 17749 was characterized via the determination of the corresponding gene sequence, biochemical properties and identification of the active site residues. The cloned fragment contained an ORF of 1473 bp, encoding a 490 amino acid residues protein with a calculated molecular weight of 53.6 kDa. The deduced amino acid sequence shared high sequence identity with PepD from various *Vibrio spp.* and both 63% from *Escherichia coli* and *Salmonella typhimurium*. The *pepD* gene of *V. alginolyticus* was cloned into the pET-28a(+) expression vector and expressed as a $(His)_6$-PepD fusion protein. Following the Ni-NTA chromatographic purification, the purified enzyme displays catalytic activity on digestion of an unusual dipeptide L-carnosine (β-Ala-L-His) with K_m 5.38 mM and k_{cat} 0.132 s^{-1} and other Xaa-His dipeptides, but not histidine-containing tripeptides. Expressed PepD was observed with optimal activity at pH 7.4 and 37°C. The enzymatic activity was inhibited

by a dipeptide analogue inhibitor bestatin and metal-chelating agent EDTA, indicating PepD as a metallo-dipeptidase. Sequence analysis revealed that His80, Asp82, Asp119, Glu149, Glu150, Asp173, and His461 are probable the active site residues of *V. alginolyticus* PepD. The results derived from site-directed mutagenesis showed that the mutants of H80A, D82A, D119A, E149A, E150A, D173A, and H461A lose their full activities as compared with wild-type PepD. The homology model of *V. alginolyticus* PepD obtained on the basis of *L. delbrueckii* PepV structure exhibits the similar active site pocket as predicted. Therefore, the results obtained from this study present, for the first time, the investigation of the putative active site residues of bacterial aminoacylhistidine dipeptidase and their possible roles in catalysis.