Proc. KSAM International Symposium
June 20-22, 2001, Chonan, Korea

Production of Pediocin by Chemical Synthesis and Bactericidal Mode of Action

Koo, Minseon, Wang June Kim, Dae Young Kwon and Kyung-Hee Min*
Korea Food Research Institute, *Sookmyung Women's University

Abstract

To investigate the mode of bactericidal action for antimicrobial peptide, pediocin, synthetic and mutant pediocins were prepared by direct chemical synthesis. Native pediocin was purified from *Pediococcus acidilactici* M and its conformational structure and bactericidal functions were analyzed and compared to synthetic pediocin. Schematic mode of pediocin actions, how pediocin binds on the target cell membrane, penetrates and makes tunnel are proposed. For these purposes, primary and secondary structures of pediocin was analyzed and disulfide bond assignment was also done. The pediocin purified from *P. acidilactici* M had high effective bactericidal ability against gram positive bacteria, especially *Listeria monocytogenes* and was very stable at extreme pHs and even at high temperatures such as autoclaving temperature (121°C). Pediocin was consisted of 44 amino acids with four cysteines. Novel synthetic peptides were achieved by solid phase peptide synthesis(SPPS) method. To explain the function of cysteine in C-terminal region, mutant pediocin, Ped[C24A+C44A], was synthesized and their structural and biological functions were analyzed. Second mutant pediocin, Ped[K11E], was prepared to explain the function of lysine at 11 of N-terminal part of pediocin, especially loop of β-sheet, and to predict the initial binding site of pediocin.

The native and synthetic pediocins was showed random coil conformation by spectropolarimetry in moderate conditions. This conformation was observed in extreme conditions such as high temperature and low and high pHs, also. Circular dichroism(CD) data also showed the existence of β-turn structure in N-terminal part both native and synthetic pediocins. A structural model for pediocin predicts that 18 amino acids in the N-terminal part of the peptide assume a three-strand β-sheet conformation. This random coil in C-terminal part of pediocin was converted to folding structure, helix structure, in nonpolar solvents such as alcohol and TFE. The disulfide bond between ^{9}Cys and ^{14}Cys was concrete and inevitable, however, evidences of disulfide bond between ^{24}Cys and ^{44}Cys was not. Data of Ped[C24A+C44A], pediocin mutant showed that ^{44}Cys was required during killing the target cells but not inevitable, since Ped [C24A+C44A] still have bactericidal activity but much less than native pediocin. Another pediocin mutant, Ped[K11E], had still bactericidal activity, was controversial to propose that positive charge like as ^{11}Lys in loop or hinge in bacteriocin bound or helped to binding to microorganism with electrostatic interaction between cell membrane especially teichoic acid and positive amino acid nonspecifically. The conformation of pediocin among native, synthetic and mutant pediocins did not show big difference. The conformations between oxidized and reduced pediocin were almost similar regardless of native or synthetic.

Intrudocution

Most of lactic acid bacteria(LAB) produce antagonistic substances that have been identified as bacteriocins(Klaenhammer, 1988; Kim, 1993). Bacteriocins are biologically-active ribosomally synthesized

proteins with inhibitory properties against other bacterial species but not producer organism(Montville *et al.*, 1994). Interests in the bacteriocins of LAB have been increasing because they can serve as natural food preservatives and processing(Kim, 1993; Lewus *et al.*, 1991). Also, bacteriocins are generally regarded as safe(FDA, 1988).

Pediocin, the bacteriocin from *Pediococcus acidilactici,* exhibits an expansive bactericidal host range with included most gram-positive LAB and food borne pathogen (Klaenhammer, 1993). Pediocin is a small heat-stable, non-lanthionine and short cationic polypeptide.. It consists 44 amino acid with four cysteines.

However, how pore complexes formed and to act at the membrane is unclear(Chikindas *et al.*, 1993; Kleanhammer, 1993). The hypothesis that nisin and pediocin-like bacteriocin permeabilize target cell membrane through a multi-step process of binding, insertion, and pore formation provides a conceptual framework for studies on the molecular mechanism of bacteriocin action(Montville & Chen, 1998).

To study of bactericidal mode of action, especially tunnel formation, we prepared native pediocin, synthetic pediocin and its mutants (point substitution of amino acid). Relationships between conformational structure and bactericidal functions were examined with these synthetic peptides and their characteristics were compared to native pediocin.

Materials and Methods

Native pediocin and bacteriocin assay

The bacteriocin producer strain used in this study was *Pediococcus acidilactici* M, isolated from fermented sausage(Kim *et al.*, 1991). *Listeria monocytogenes* ATCC 15313 used as a indicator strain. Pediocin produced by *P. acidilactici* M was extracted from the modified TGE broth by pH-mediated adsorption and desorption process(Biswas *et al.*, 1991; Daba *et al.*, 1994). Purification was done by reversed phase high performance liquid chromatography (Elegado *et al.*, 1997). Bactericidal activity against *L. monocytogenes* was assayed by the agar-well diffusion method of Tagg & McGiven(1971) with some modifications(Kim *et al.*, 1993).

Synthetic pediocin

Synthetic pediocin and its mutant were synthesized by stepwise solid phase peptide synthesis(SPPS) method with acetic acid capping and double coupling. Synthesis started with the cysteine preloaded HMP resin, alanine preloaded HMP resin and was carried out using the Fastmoc strategy on an Applied Biosystems Peptide Synthesizer(model 433A, Perkin Elmer, CA. USA). Purification was done by GPC and RP-HPLC.

Protein chemistry

Protein concentration was determined by BCA(bicinchoninic acid) microassay method (Smith *et al.*, 1985). Bovine serum albumin was used as protein standard for peptide concentration determination(Pierce, Rockford, IL, USA). Amino acid sequence of peptide were determined by Edman degradation (250 pmol of each sample) using a Applied Biosystems 491 Protein Sequencer(Perkin Elmer, CA. USA) with 785A programmable absorbance detector and 140C microgradient system. The sample was loaded using polybrene-coated glass fiber as a support.

Free thiol groups were determined by Ellman's method(Ellman, 1958) with some modification(Rid-

dles *et al.*, 1979). To induce oxidation of free thiol group of cysteine, crude peptide was dissolved in 5 M GuHCl (in 0.2 M Tris-HCl buffer, pH 8.4). Then, under air purging, peptide solution was stirred at 40°C for 6 day. The progress of oxidation was monitored by analytical RP-HPLC (Vydac 218TP54, 5 μm, 4.6250 mm) at 220 nm. Assignment of disulfide bond in pediocin was done by modified method of Henderson *et al.* (1993). Trypsin was used as cleavage enzyme to clarify number and assignment of disulfide bond.

To confirm the purity and mass of peptide, mass spectral analyses were performed by using a electro-spray mass spectrometer(Platform II, Micromass, Manchester, UK). The mass scale was calibrated by using bovine serum albumin. The accuracy was ±0.1 atomic mass. Circular Dichroism(CD) studies for analysing the secondary structures of protein were done by using jasco polarimeter (Model J710, Hachioji, Japan). Measurements were made at wavelength range of 190-250 nm at room temperature, using a cylindrical quartz cell of 1 mm path length (3001 volume).

Results and Discussion

The purified pediocin from *Pediococcus acidilactici* M was high effective to the anti-growth gram positive strain, especially *Listeria monocytogenes* and was very active at extreme pHs and even at high temperatures such as autoclaving temperature (121). Pediocin was consisted of 44 amino acids with four cysteines, i.e., ^{9}Cys, ^{14}Cys, ^{24}Cys and C-terminal ^{44}Cys. Pediocin was successfully synthesized by chemical method and point mutated mutant pediocin were also synthesized. The native and synthetic pediocins showed random coil which was determine by spectropolarimetry in moderate conditions. This random coil was maintained even in extreme conditions such as high temperature and low and high pH. CD data also showed that the existence of a little bit β-turn structure might represents N-terminal part for native and synthetic pediocins. A structural model for pediocin predicts that 18 amino acids in the N-terminal part of the peptide assume a three-strand β-sheet conformation. This random coil in C-terminal part of pediocin was converted to folding structure for example, helix structure, in nonpolar solvents such as alcohol and TFE. The transformed α-helix in C-terminal by low dielectric constant solvent showed typical amphipathic α-helix structure, half of helix barrel was hydrophobic and nother side was hydrophilic. The amphiphatic α-helices is generally considered to be a structural motif that enables interaction with lipid bilayer and enables formation of tunnel with structured barrel. The disulfide bond between ^{9}Cys and ^{14}Cys was concrete and inevitable. However evidences of disulfide bond between ^{24}Cys and ^{44}Cys were not concrete. Thus mutant pediocin I(Ped[C24A+C44A]) was prepared and data of Ped[C24A+C44A] showed ^{44}Cys was required during killing the microbial cells but not inevitable, since mutant pediocin Ped[C24A+C44A] still have bactericidal activity but much less than native pediocin. Also, Mutant pediocin II(Ped[K11E]) still have bactericidal activity, although positive charges of lysine was replaced with negative charge by glutamic acid substitution. It is thought that the initial binding *in vivo* between pediocin and target cell membrane has related to "receptor or recognition site", called protein assisted binding. Because it was known that positive charge like as ^{11}Lys in loop or hinge in bacteriocin helped to binding to microorganism with electrostatic interaction between cell membrane especially teichoic acid and positive amino acid nonspecifically. The conformation of pediocin among native, synthetic and mutant pediocins did not show big difference, only native and synthetic pediocin showed a little bit difference. The conformations between oxidized and reduced pediocin were almost similar regardless of native or synthetic.

From these results, schematic mode of pediocin actions were proposed on the basis of barrel stave model. The initial binding *in vivo* between pediocin and target cell membrane should be related to receptor protein on the cell membrane so called protein assisted binding. The penetration probably occurs in conjunction with conformation transformation. Non-aqueous environment induced conformation changes before or after protein-recognition event. After helix transformation, amphipathic -helix worked for making tunnel during the pore formation as described below. In transformed helix remaining two cysteines(^{24}C, ^{44}C) with free thiol group in C-terminal region might make stable disulfide bond. Aggregation of pediocin in membrane after penetration of pediocin into the membrane results in pore/ tunnel formation with inserted pediocins. Finally, transmembrane potential between in and out of plasma membrane was dissipated by the pores constructed with pediocin mediated tunnel and thus microorganism could not growth anymore.

References

1. Biswas, S. R., P. Ray, M. C. Johnson, and B. Ray. 1991. Influence of growth conditions on the production of bacteriocin, Pediocin AcH, *Pediocin acidilactici* H. *Appl. Environ. Microbiol.* 57, 1265-1267.
2. Chikindas, M. L., M. J. G.-Garcera, A. J. M. Driessen, A. M. Ledeboer, J. N.-meyer, I. F. Nes, T. Abee, W. N. Konings and G. Venema. 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cell. *Appl. Environ. Microbiol.* 59, 3577-3584.
3. Daba, H., C. Lacroix, J. Huang, R. E. Simard, and L. Lemieux. 1994. Simple method of purification and sequencing of bacteriocin produced by *Pediococcus acidilactici* UL5. *J. Appl. Bacteriol.* 77, 682-688.
4. Elegado, F. B., W. J. Kim, and D. Y. Kwon. 1996. Rapid Purification, Partial Characterization, and antimicrobial spectrum of the bacteriocin, Pediocin AcM, from *Pediococcus acidilactici*. M. *Int. J. Food Microbiol.* 37, 1-11.
5. Ellman, G. L. 1958. A colorimetric method for determining low concentrations of mercaptans. *Arch. Biochem. Biophys.* 74, 443-450.
6. Food and Drug Adminstration. 1988. Nisin preparation : Affirmation of GRAS status as a direct human of food ingredient. *Fed. Regist.* 53, 11247.
7. Henderson, J. T., A. L. Chopko, and D. van Wassenaar. 1993. Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC-10. *Arch Biochem. Biophys.* 295, 5-12.
8. Kim, W. J., D. M. Ha, and B. Ray. 1991. Plasmid linkage of bacteriocin production and sucrose fermentation phenotypes in *Pediococcus acidilactici* M. *J. Micro. Biotechnol.* 1, 169-175.
9. Kim, W. J., S. S. Hong, S. K. Cha, and Y. J. Koo. 1993. Use of bacteriocinogenic *Pediococcus acidilactici* in sausage fermentation. *J. Microbiol.* 3, 199-203.
10. Klaenhammer, T. 1993. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* 12, 39-86.
11. Lewus, C. B., A. Kaiser, and T. J. Montville. 1991. Inhibition of food-borne bacterial pathogenby bacteriocins from lactic acid bacteria isolated from meat. *Appl. Environ. Microbiol.* 57, 1683-1688.
12. Montville, T. J., and M. E. C. Bruno. 1994. Evidence that dissipation of proton motive force is a common mechanism of action for bacteriocins and other antimicrobial proteins. *Int. J. Food Microbiol.* 24, 53-74.
13. Montville, T. J., and Y. Chen. 1998. Mechanistic action of pediocin and nisin: recent progress and unresolved questions. *Appl. Environ. Microbiol.* 50, 511-519.
14. Riddles, P. W., R. L. Blakeley, and B. Zerner. 1979. Ellman's reagent: 5,5' dithiobis (2- nitrobenzoic acide)-a reexamination. *Anal. Biochem.* 94, 75-81.

15. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. D. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76-85.
16. Tagg, J. R., and A. R. McGiven. 1971. Assay system for bacteriocins. *Appl. Microbiol.* 21, 943.