Characterization of a Novel PepF-Like Oligopeptidase Secreted by *Bacillus amyloliquefaciens* 23-7A†

Shiou-Huei Chao,¹ Tzu-Hao Cheng,¹ Chin-Ying Shaw,¹ Meng-Hwan Lee,¹ Yuan-Hsun Hsu,² and Ying-Chieh Tsai¹* 

Institute of Biochemistry, National Yang-Ming University,¹ and Graduate Institute of Medical Science, Taipei Medical University,² Taipei, Taiwan

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An oligopeptidase from *Bacillus amyloliquefaciens* 23-7A was characterized along with its biochemical activities and structural gene. The protein’s amino acid sequence and enzymatic activities were similar to those of other bacterial PepFs, which belong to metallopeptidase family M3. While most bacterial PepFs are cytoplasmic endopeptidases, the identified PepF<sub>βa</sub> oligopeptidase is a secreted protein and may facilitate the process of sporulation.

In various species and tissues, peptidases of family M3 are involved in peptide degradation, bioactive neural-peptide synthesis, and cleavage of signal peptides (3, 4, 14, 16). This kind of endopeptidase only hydrolyzes oligopeptides that contain no more than 20 amino acid residues. Bacterial PepFs also belong to the M3 family of peptidases. In this report, another PepF-like oligopeptidase was identified in a collagen-degrading strain, *Bacillus amyloliquefaciens* 23-7A. This peptidase, designated as PepF<sub>βa</sub>, was characterized in great detail, and its potential physiological roles were also discussed.

**Bacterial cultivation and enzyme purification.** The microbial strain 23-7A bearing collagenolytic activity was screened from soil in Taiwan. The bacterium was spore-forming, gram-positive, and taxonomically identified as *Bacillus amyloliquefaciens*. The first residue of the mature protein was Ser<sup>31</sup>, Ala<sup>24</sup>-Tyr-Lys, matching sequences from *Bacillus licheniformis* Pz peptidase (1) and *Bacillus subtilis* PepF (8). A forward primer, 5′'-GA(A/G)AA(A/G)CCNGA(A/G)GA(T/C)AA(T/C)CAC-3′, and a backward primer, 5′'-GT(A/G/C)AT(T/C)TC(T/C)TC(A/G/T)ATNGC(A/G/T)TG-3′, were designed based on N-terminal and internal sequences. The PCR consisted of 30 cycles with an annealing temperature of 47°C, and the product was around 350 bp. Using this amplified fragment as a probe, the complete gene of pepF<sub>βa</sub> was cloned from a *B. amyloliquefaciens* 23-7A genomic library. PepF<sub>βa</sub> was 2,010 bp long and encoded a protein of 670 amino acid residues. The molecular mass and pI were calculated as 77,049 Da and 5.58 using the ExpASy molecular biology server (http://tw.expasy.org/). A 23-residue signal peptide was defined by the SignalP prediction server (www.cbs.dtu.dk/services/SignalP) (5). Since the first residue of the mature protein was Ser<sup>31</sup>, Ala<sup>24</sup>-Tyr-Asp-Leu-Thr-Lys-Gly<sup>30</sup> might be considered a prosesequence that will be removed after the enzyme is secreted. The peptide sequence determined by Q-TOF II MS analysis (Micromass; Waters). The digested peptides were extracted from the gel with 5% trifluoroacetic acid/50% acetonitrile. Extracts were lyophilized and resuspended in 1% formic acid for matrix-assisted laser desorption ionization mass spectrometry (MS) and quadrupole time-of-flight (Q-TOF) II MS analysis (Micromass; Waters). One peptide was determined as Leu-Tyr-Ser-His-Ala-Ile-Glu-Glu-Ile-Thr-Lys, matching sequences from *Bacillus licheniformis* Pz peptidase (1) and *Bacillus subtilis* PepF (8). A forward primer, 5′'-GA(A/G)AA(A/G)CCNGA(A/G)GA(T/C)AA(T/C)CAC-3′, and a backward primer, 5′'-GT(A/G/C)AT(T/C)TC(T/C)TC(A/G/T)ATNGC(A/G/T)TG-3′, were designed based on N-terminal and internal sequences. The PCR consisted of 30 cycles with an annealing temperature of 47°C, and the product was around 350 bp. Using this amplified fragment as a probe, the complete gene of pepF<sub>βa</sub> was cloned from a *B. amyloliquefaciens* 23-7A genomic library. PepF<sub>βa</sub> was 2,010 bp long and encoded a protein of 670 amino acid residues. The molecular mass and pI were calculated as 77,049 Da and 5.58 using the ExpASy molecular biology server (http://tw.expasy.org/). A 23-residue signal peptide was defined by the SignalP prediction server (www.cbs.dtu.dk/services/SignalP) (5). 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† Corresponding author. Mailing address: Institute of Biochemistry, National Yang-Ming University, 155, Sec. 2, Li-Nong Street, Pei-Tou, Taipei 11221, Taiwan. Phone: (886) 2-2826-7125. Fax: (886) 2-2826-4843. E-mail: tsaiyc@ym.edu.tw.

‡ Supplemental material for this article may be found at http://aem.asm.org/.

* Corresponding author. Mailing address: Institute of Biochemistry, National Yang-Ming University, 155, Sec. 2, Li-Nong Street, Pei-Tou, Taipei 11221, Taiwan. Phone: (886) 2-2826-7125. Fax: (886) 2-2826-4843. E-mail: tsaiyc@ym.edu.tw.

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2). Pz peptidase is a dimeric metallopeptidase that does not hydrolyze proteins (2). *B. subtilis* PepF was identified as the homologue of PepF1 in *Lactococcus lactis*, a well-characterized enzyme that belongs to the M3 family of oligopeptidases (7, 8, 11).

**Enzymatic properties.** The amount of zinc in PepF<sub>Ba</sub> was determined by inductively coupled plasma mass spectrometry (7500cs; Agilent Technologies). An average of 14.6 ± 0.35 pmol/ml Zn<sup>2+</sup> was obtained for every 7.8 pmol/ml PepF<sub>Ba</sub>, given a molar ratio of 1.87 (Zn<sup>2+</sup>/PepF<sub>Ba</sub>). To further determine the enzymatic properties of the proteins under the effects of various chemical reagents as well as temperature and pH, the synthetic peptide FALGPA was used as a substrate to monitor the activity of PepF<sub>Ba</sub>. EDTA (10 mM) and 1,10-phenanthroline (10 mM) strongly inhibited PepF<sub>Ba</sub> activity, but epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), iodoacetamide, phosphoramidon, and phenylmethylsulfonyl fluoride did not. Like other bacterial PepFs, PepF<sub>Ba</sub> was inhibited by the presence of 10 mM Cd<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>2+</sup>, and Pb<sup>2+</sup> while Ca<sup>2+</sup> and Mg<sup>2+</sup> were able to increase PepF<sub>Ba</sub> activities. Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Co<sup>2+</sup> could also cause the restoration of enzyme activity after EDTA treatment (see Table S2 in the supplemental material). PepF<sub>Ba</sub> was most active at pH 7. It showed the highest activity at 45°C and became thermally inactive at temperatures over 55°C (see Fig. S2 in the supplemental material). At optimal pH, PepF<sub>Ba</sub> was stable at 40°C for 1 h.

**FIG. 1.** PepF<sub>Ba</sub> activity and optical density of *B. amyloliquefaciens* 23-7A grown in basal medium. Total cell numbers ( ■) at the indicated time were measured by optical density at 600 nm (OD600). For spore titer (●) determination, the cultured broth was heated for 20 min at 65°C and then spread at appropriate dilutions on LB agar plates. PepF<sub>Ba</sub> activity (○) was determined by hydrolysis of the peptide FALGPA as described previously (17).

**FIG. 2.** Alignments of the amino acid sequences of PepF<sub>Ba</sub> from *B. amyloliquefaciens* 23-7A [PepF(BAL)_Bamy] with those of other oligopeptidases of the M3 family. The conserved residues are shown in white on a black background. The zinc binding motif is indicated by asterisks. The signal peptide of PepF<sub>Ba</sub> is boxed, and the first residue of mature protein is indicated by an arrowhead. The internal peptide sequence resolved by matrix-assisted laser desorption ionization–time-of-flight MS is shown by an arrow. The sequences are from *B. subtilis* PepF (NCBI accession no. CAB13011, PepF_Bsub) (8), *B. licheniformis* Pz peptidase (NCBI accession no. BAA13561, Pz-pep_Blich) (1), *Streptococcus* PepB (NCBI accession no. AAC44215, PepB_Strepto) (9), and *L. lactis* PepF1 (NCBI accession no. CAA83534, PepF1_Lacto) (11).
Substrate specificity. The oligopeptide substrates shown in Table 1 were incubated with the enzyme in a ratio of 1/100 (wt/wt) in 20 mM Tris-HCl (pH 7.0) containing 10 mM Ca\(^{2+}\) at 37°C. The reactions were stopped by addition of 10 mM EDTA, and the mixtures were subjected to reverse-phase high-performance liquid chromatography using a Cosmosil 5C18-MS column. In the mobile phase, acetonitrile was increased from 0 to 40% of acetonitrile in 40 min, and the eluent was monitored at a wavelength of 214 nm. The recovered eluent was lyophilized, redissolved in water, and then analyzed by Q-TOF II MS (Micromass; Waters). The results indicated that only peptides ranging from 5 to 21 residues in length are cleavable substrates of PepFBa. While PepFBa revealed no hydrolytic activities on bradykinin residues 1 to 5 (five residues), the substrates FALGPA, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz peptide), and N-carboxybenzoyl-Gly-Pro-Leu-Gly-Pro that are 5 residues long were hydrolyzed at a high rate. For FALGPA, the specific activity of PepFBa was 20.7 U/mg, and the \(K_m\) and \(k_{cat}/K_m\) values were \(9.92 \times 10^{-5}\) M and \(3.3 \times 10^6\) M\(^{-1}\) s\(^{-1}\), respectively. The cleavage pattern of these substrates by PepFBa was similar to that of bacterial PepFs, including \(L.\ lactis\) PepF1 (11) and \(S.\ agalactiae\) PepB (9). Nevertheless, analysis of the cleavage sites suggested that the bonds cleaved by PepFBa are more flexible. For most oligopeptidases in the M3 family, the residues in the P1 position of the cleavage site are mainly hydrophobic and basic ones (3, 10, 16). But PepFBa also cleaved acidic residues at the P1 site, such as Glu 17 in oxidized insulin chain A and Glu 4 in neurotensin. Because of its substrate specificity, sequence similarity, and requirement for Zn\(^{2+}\) as a cofactor for enzymatic activity, PepFBa should be assigned to the M3 peptidase family. In this family, PepFBa and the Pz peptidase from \(B.\ licheniformis\) are secreted proteins, while others are all cytoplasmic. Thus, these extracellular peptidases should play distinct roles from canonical bacterial PepFs. PepFBa has sequence similarity with \(B.\ subtilis\) PepF, an enzyme that hydrolyzes intracellular Phr pentapeptides. Phr preproteins are exported outside of cells during the exponential growth phase (6, 18). After processing, active Phr pentapeptides were imported into cells to stimulate the formation of spores (13, 15). The characteristics of Phr pentapeptides are an Arg or Lys in the second residue as well...
as aliphatic or negatively charged residues in the first position (12). As demonstrated in this study, PepF<sub>Ba</sub> is produced extracellularly during the exponential phase. Furthermore, it possesses flexibility in the P1 position and ability to digest substrates with aliphatic residues in the P1’ site and Arg or Lys in the P2’ site, such as Phe<sup>2</sup>-Arg<sup>8</sup> and Gly<sup>10</sup>-Lys<sup>11</sup> (adrenocorticotropic hormone fragment 1-14). It is plausible that PepF<sub>Ba</sub> could facilitate sporulation by processing a pro-Phr into the active pentapeptides. On the other hand, PepFBa may also play a role in the degradation of peptides that are produced by the action of other secreted proteases. Further studies will be necessary to define its physiological functions.

**Nucleotide sequence accession number.** The nucleotide sequence of the *B. amyloliquefaciens* 23-7A PepF<sub>Ba</sub> gene and its encoded amino acid sequence have been deposited in the GenBank nucleotide database under the accession number AF525011.

**REFERENCES**

1. Akiyama, K., K. Mori, and R. Takata. 1999. Cloning and sequencing of the Pz-peptidase gene from *Bacillus licheniformis* N22. J. Biosci. Bioeng. 87:231–233.

2. Asdornnithee, S., E. Himeji, K. Akiyama, T. Sasaki, and R. Takata. 1995. Isolation and characterization of Pz-peptidase from *Bacillus licheniformis* N22. J. Ferment. Bioeng. 79:200–204.

3. Barrett, A. J., M. A. Brown, P. M. Dando, C. G. Knight, N. McKie, N. D. Rawlings, and A. Serizawa. 1992. Thimet oligopeptidase and oligopeptidase M or neurolysin. Methods Enzymol. 248:259–256.

4. Barrett, A. J., and N. D. Rawlings. 1992. Oligopeptidases, and the emergence of the prolyl oligopeptidase family. Biol. Chem. Hoppe-Seyer 373:353–360.

5. Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak. 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340:783–795.

6. Grossman, A. D., and R. Losick. 1988. Extracellular control of spore formation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA 85:4369–4373.

7. Kanamaru, K., S. Stephenson, and M. Perego. 2002. Overexpression of the PepF oligopeptidase inhibits sporulation initiation in *Bacillus subtilis*. J. Bacteriol. 184:43–50.

8. Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Avezedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borris, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuno, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerston, A. Danchin, et al. 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature 390:249–256.

9. Lin, B., W. F. Averett, J. Novak, W. W. Chatham, S. K. Hollingshead, J. E. Coligan, M. L. Egan, and D. G. Pritchard. 1996. Characterization of PepB, a group B streptococcal oligopeptidase. Infect. Immun. 64:3401–3406.

10. Monnet, V. 1995. Oligopeptidases from *Lactococcus lactis*. Methods Enzymol. 248:579–592.

11. Monnet, V., M. Nardi, A. Chopin, M. C. Chopin, and J. C. Gripot. 1994. Biochemical and genetic characterization of PepF, an oligopeptidase from *Lactococcus lactis*. J. Biol. Chem. 269:32070–32076.

12. Perego, M., and J. A. Hoch. 1993. Thimet oligopeptidase—a review of a thiol dependent metallo-endopeptidase also known as Pz-peptidase endopeptidase 24.15 and endo-oligopeptidase. Methods Enzymol. 22:1541–1547.

13. Perego, M., and J. A. Brannigan. 2001. Pentapeptide regulation of aspartyl-phosphate phosphatases. Peptides 22:1541–1547.

14. Rawlings, N. D., and A. J. Barrett. 1995. Evolutionary families of metallo-enzymes. Methods Enzymol. 248:183–228.

15. Solomon, J. M., B. A. Lazazzera, and A. D. Grossman. 1996. Purification and characterization of an extracellular oligopeptidase from *Bacillus subtilis*. J. Bacteriol. 175:6321–6327.