J. Antibiot. 60(8): 519–523, 2007

NOTE

THE JOURNAL OF ANTIBIOTICS

Novel Propeptin Analog, Propeptin-2, Missing Two Amino Acid Residues from the Propeptin *C*-Terminus Loses Antibiotic Potency

Ken-ichi Kimura, Mai Yamazaki, Noriko Sasaki, Tetsuro Yamashita, Shigenori Negishi, Takemichi Nakamura, Hiroyuki Koshino

Received: March 20, 2007 / Accepted: July 13, 2007

Abstract A novel inhibitor of prolyl endopeptidase, the propeptin analog propeptin-2 ($C_{105}H_{130}N_{24}O_{24}$), missing two amino acid residues from the propeptin *C*-terminus was isolated from the fermentation broth of propeptin-producing *Microbispora* sp. SNA-115 grown using a large inoculum. It shows the same enzyme inhibition activity as propeptin against prolyl endopeptidase (Ki=1.5 μM), but its antimicrobial activity against *Mycobacterium phlei* is more than 100-fold lower.

Keywords propeptin, *Microbispora* sp., *Mycobacterium* spp., prolyl endopeptidase

Prolyl endopeptidase (PEP; post-proline cleaving enzyme; official name: prolyl oligopeptidase; EC 3.4.21.26) cleaves peptide bonds at the carboxyl side of proline residues and hydrolyzes biologically active peptides containing proline, such as vasopressin and substance P [1, 2]. It may also be involved in diseases such as Alzheimer's disease and bipolar affective disorder [3~5]. Meanwhile, effective anti-tuberculosis drugs with novel structures and mechanisms of action have not been developed in over 30 years. Moreover, the increase in Human Immunodeficiency Virus (HIV) and cancer patients has resulted in the spread of opportunistic bacteria, such as the *Mycobacterium avium* complex (MAC).

Propeptin (Fig. 1(a)) is an atypical cyclic peptide compound that has an amide bond between the α-amino group of the amino-terminal Gly1 and the β-carboxyl group of Asp9, and consists of 16 L configuration amino acids and 3 glycines [6, 7]. It is produced by some *Microbispora* spp., such as *Microbispora rosea* (IFO14044), *Microbispora rosea* subsp. *nonnitritogenes* (IFO14045) and *Microbispora indica* (IFO14879), but not by *Microbispora parva* (IFO14043) [8]. Propeptin competitively inhibits the enzyme activity of prolyl endopeptidase (Ki=0.7 μM) and also has antimicrobial activity against *Mycobacterium phlei* IFO3158 (Inhibition zone=12~15 mm at 40 μg/disc) [6, 9]. Peptide compounds similar to propeptin have been reported, including anantins (Gly-Asp type) [10, 11], RES-701s (Gly-Asp type)

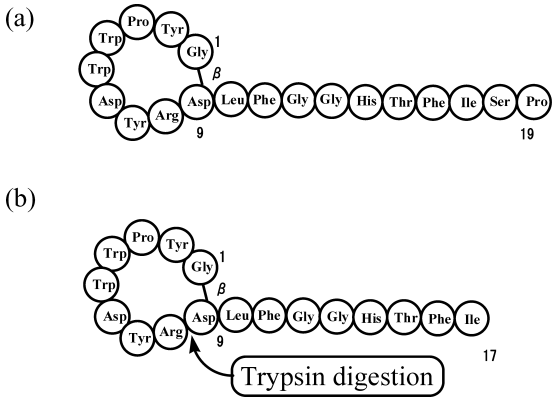

Fig. 1 Structures of propeptin (a) and propeptin-2 (b).

K. Kimura (Corresponding author), **M. Yamazaki, N. Sasaki, T. Yamashita:** Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan, E-mail: kimurak@iwate-u.ac.jp

S. Negishi: Enzyme & Pharmaceuticals Research Laboratory, Godo Shusei Co., Ltd., Matsudo, 271-0064, Japan

T. Nakamura, H. Koshino: RIKEN (The Institute of Physical and Chemical Research), Wako-shi 351-0198, Japan

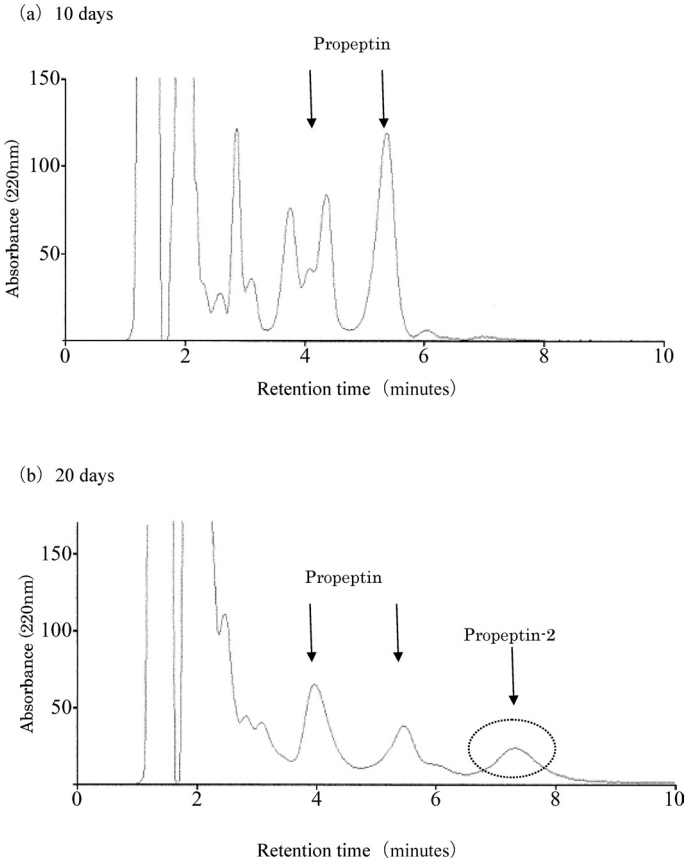

Fig. 2 HPLC traces of mycelium extract by MeOH after (a) 10 days and (b) 20 days of fermentation.

[12～15], microcin J25 (Gly-Glu type) [16～18] and lariatins (Gly-Glu type) [19, 20] with the "lariat protoknot, lasso structure", but they have different biological activities. In addition, the producing strains differ; *Microbispora* spp. (propeptin), *Streptomyces* spp. (anantins and RES-701s), *Escherichia coli* (microcin J25), and *Rohdococcus* sp. (lariatin).

As these cyclic peptide compounds, although related in some aspects, are unique compounds with respect to structure, biological activity and producing strain, we searched for novel propeptins by analyzing the broth from *Microbispora* sp. SNA-115 following changes in fermentation conditions. HPLC detected a new peak in broths grown from large inoculum and/or extended-fermentation experiments, and we subsequently identified a propeptin that was missing two *C*-terminal amino acids (Fig. 1(b)). In this report, we describe the isolation, structural elucidation and biological activity of this novel propeptin analog, designated propetin-2.

The seed medium consisted of 2.0% glucose, 1.0% soluble starch, 0.1% meat extract, 0.4% dried yeast, 2.5% soybean flour, 0.2% NaCl and 0.005% K_2HPO_4 (pH 6.7, before sterilization) in a test tube (13×180 mm, 5.0 ml of the medium, reciprocal shaker (120 rpm)). To determine production of propeptin-2, we analyzed propeptin and propeptin-2 by HPLC during the fermentation period. In a previous paper, propeptin was produced after 2～3 days in a 500-ml Erlenmeyer flask with 70 ml of the medium (200 rpm, rotary shaker) and gradually increased over 5 days [8]. In this experiment, the ratio of propeptin-2 to propeptin gradually increased over 20 days (Fig. 2). Propeptin was detected as two peaks (two conformers) on HPLC analysis, and propeptin-2 was detected as one broad peak [Detector: PDA (JASCO MD-910, SHIMADZU); Column: Capcell Pak 4.6×150 mm (SHISEIDO); Solvent: CH_3CN - 0.1% TFA (40 : 60); flow rate: 1.0 ml/minute]. Propeptin-2 may be produced *via* digestion of propeptin by a specific dipeptidyl carboxypeptidase with angiotensin-converting enzyme-like activity. When sorbose replaced glucose as a carbon source, production of propeptin-2 increased by approximately 5-fold (data not shown).

For isolation of propeptin-2, fermentation was carried out using a large inoculum. Seed culture (500 ml) prepared

as described above (former seed culture was 140 ml to 18 liters [6]), was transferred into 18 liters of the same medium in a 30-liter jar fermenter. Fermentation was carried out at 28°C for 9 days (pH 7.8) and mycelia were obtained by filter press. Isolation of propeptin and propeptin-2 was performed according to published methods [6], except that acetone was used for mycelium extraction and we used an additional BuOH extraction. Propeptin (584.4 mg) and propeptin-2 (114.6 mg) were isolated from the 36 liters of broth as brownish white powders.

The UV spectrum (280 nm (ε 11014)) and optical rotation of propeptin-2 ($[\alpha]_D^{24}=-72.4°$ (c 0.1, MeOH)) were similar to those of propeptin [6], (SHIMADZU UV mini 1240, and JASCO DIP-1000 polarimeter). The ESI-MS spectrum (BRUKER Daltonics Apex-II FT-ICR mass spectrometer) of propeptin-2 indicated a $(M+2H)^{2+}$ ion at m/z 1056. The molecular formula was determined by HR-ESI-MS as $C_{105}H_{130}N_{24}O_{24}$ (Found: $(M+2H)^{2+}$, m/z 1056.4913; Calcd: $(M+2H)^{2+}$, m/z 1056.4918), and a lack of the Ser-Pro residue appeared to be the most logical change to account for the difference in the formula relative to propeptin. Although the ^{1}H- and ^{13}C-NMR signals of propeptin-2 in DMSO-d_6 were not resolved, as in the case of propeptin, the observed signals were similar in pattern to those of propeptin. Structural elucidation of propeptin-2 was performed using the same method as for propeptin [7]. *N*-Terminal sequence analysis was attempted by automated Edman degradation (SHIMADZU PPSQ-21 protein sequencer). As in the case with propeptin, no amino acids were detected in propeptin-2 using Edman procedures. After trypsin digestion of propeptin-2 (30.0 mg, Trypsin TPCK treated (SIGMA, 31000 units), 37°C for 5 hours) to the *C*-terminal arginine, a quasi-linear peptide designated propeptin-2 T (9.6 mg), was isolated by HPLC. The ESI-MS spectrum of propeptin-2 T indicated a $(M+2H)^{2+}$ ion at m/z 1065. The molecular formula was determined by HR-ESI-MS as $C_{105}H_{132}N_{24}O_{25}$ (Found: $(M+2H)^{2+}$, m/z 1065.4975; Calcd: $(M+2H)^{2+}$, m/z 1065.4970), which comprised an additional H_2O to the formula of propeptin-2.

In Edman procedures, although the first amino acid was not detected as it is abnormal (Asp9 is connected to the *N*-terminus of the Gly1 with a chain of 7 amino acids), only the sequence of amino acids 10 (Leu) to 17 (Ile) was detected using propeptin-2 T (Fig. 1(b)). ESI-MS-MS was used to obtained sequence information. ESI-MS-MS analysis of propeptin-2 showed that all fragment ion peaks were consistent with the sequence of amino acids 10 (Leu) to 13 (Gly). No complete sequence information was obtained by ESI-MS-MS analysis of propeptin-2 T due to limitations in fragmentation efficiency. However, the major fragment ions observed in the ESI-MS-MS spectrum of propeptin-2 T were consistent with the amino acid sequence from residues 1 (Gly) to 9 (Asp), as shown in Fig. 1(b) (data not shown).

The complete amino acid composition was obtained following acid hydrolysis. Propeptin-2 was hydrolyzed with 4 N methane sulfonic acid at 90°C for 24 hours and amino acid analysis of the hydrolysate showed the presence of 17 amino acids (molar ratio) (JEOL JLC-500N): Asp (2.40), Thr (0.86), Gly (3.50), Ile (1.01), Leu (1.13), Tyr (1.78), Phe (1.56), His (1.02), Trp (1.52), Arg (1.02) and Pro (0.72). Ser was not detected and Pro was only detected at one molar equivalent in propeptin-2. All amino acids, except Gly, were determined to be L configuration using the enantiomer labeling method (SHIMAZU-CAT model DLAA-1). These data suggest that propeptin-2 lacks two amino acid residues from the *C*-terminus of propeptin (Fig. 1(b)).

Propeptin inhibits the protease activity of prolyl endopeptidase and the growth of *M. phlei* [6, 9]. Prolyl endopeptidase (*Flavobacterium*) and substrate (Z-Gly-Pro-*p*NA) were purchased from Seikagaku Kogyo Co., Ltd. PEP inhibition assay using culture broth was performed, and inhibition activity of propeptins was measured, as described previously [21]. A loopful of *M. phlei* IFO3158 from a mature slant was inoculated into 10 ml of seed medium composed of 3.0% glycerol, 0.5% meat extract, 1.0% peptone and 0.3% NaCl (pH 6.8, before sterilization) in a test tube (13×180 mm). The inoculated tube was incubated at 37°C without shaking for 4 days, and 12.5 ml of medium with 1.0% agar containing 2.5 ml of culture broth was used to prepare one culture plate for antimicrobial assay. Antimicrobial activity was determined using the conventional paper disc method (thickness, 8 mm, ADVANTEC) with incubation at 37°C for 2 days. Propeptin and propeptin-2 exhibited similar inhibition kinetics as PEP, *i.e.*, competitive inhibition, with *Ki* values of 0.7 μM (1.6 μg/ml) and 1.5 μM (3.1 μg/ml), respectively (Fig. 3, Table 1). These results confirm that the Ser-Pro residues are not required for PEP inhibition. In contrast to PEP inhibition, the antimicrobial activity of propeptin-2 against *M. phlei* was not measurable (Table 1). There was no inhibition zone resulting from propeptin-2, even at 4 mg/disc, which represents a decrease in antimicrobial activity of over 100-fold. Thus, the two *C*-terminal amino acids (Ser-Pro) play a critical role in the antimicrobial activity. The Ser-Pro residues of propeptin may thus be involved in drug permeability and/or activity against the molecular targets involved in the antimicrobial activity.

As shown by HPLC analyses of the fermentation broth, the propeptin peak was gradually replaced by the propeptin-2 peak (Fig. 2). Taken together with the

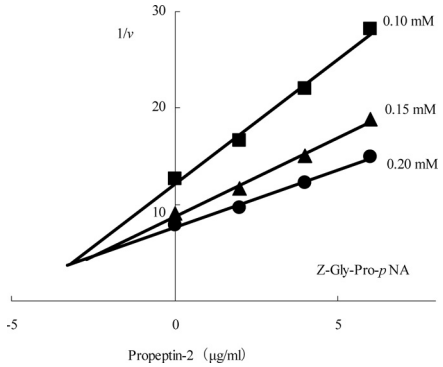

Fig. 3 Dixon plot of inhibition of prolyl endopeptidase by propeptin-2.

1/*v* was defined as 1/*Δ* 410 nm.

Table 1 Biological activities of propeptin and propeptin-2

	PEP inhibition *Ki* (μM)	Antimicrobial activity against *Mycobacterium phlei* Inhibition zone (mm at 40 μg/disc)
Propeptin	0.7	12.9
Propeptin-2	1.5	0

structural data, this suggests that propeptin-2 is an enzyme digestion product of propeptin, possibly catalyzed by a specific dipeptidyl carboxypeptidase. In fact, when isolated propeptin was incubated with either broth filtrate (from 20 days) or commercial angiotensin-converting enzyme, propeptin was apparently converted to propeptin-2 in a time-dependent manner (HPLC data not shown). However, propeptin-2 was not detected in the original fermentation broth under conditions used for production of propeptin. The two *C*-terminal amino acid residues of propeptin may be important for drug permeability through the membrane and/or interaction with the molecular targets of antimicrobial activity. Modification of these amino acid residues may therefore provide novel and more potent antimicrobial compounds. We are also interested in potential proteinase activities in *Microbispora*, particularly dipeptidyl carboxypeptidase-like angiotensin-converting enzymes.

Acknowledgment This work was partially supported by the Nagase Science and Technology Foundation.

References

1. Walter R, Shlank H, Glass JD, Schwartz IL, Kerenyi TD. Leucylglycinamide released from oxytocin by human uterine enzyme. Science 173: 827–829 (1971)
2. Koida M, Walter R. Post-proline cleaving enzyme. Purification of this endopeptidase by affinity chromatography. J Biol Chem 251: 7593–7599 (1976)
3. De Wied D, Gaffori O, Van Ree JM, De Jong W. Central target for the behavioural effects of vasopressin neuropeptide. Nature 308: 276–278 (1984)
4. Aoyagi T, Wada T, Nagai M, Kojima F, Harada S, Takeuchi T, Takahashi H, Hirokawa K, Tsumita T. Deficiency of kallikrein-like enzyme activities in cerebral tissue of patients with Alzheimer's disease. Experientia 46: 94–97 (1990)
5. Williams RSB, Cheng L, Mudge AW, Harwood AJ. A common mechanism of action for three mood-stabilizing drugs. Nature 417: 292–295 (2002)
6. Kimura K, Kanou F, Takahashi H, Esumi Y, Uramoto M, Yoshihama M. Propeptin, a new inhibitor of prolyl endopeptidase produced by *Microbispora*. I. Fermentation, isolation and biological properties. J Antibiot 50: 373–378 (1997)
7. Esumi Y, Suzuki Y, Itoh Y, Uramoto M, Kimura K, Goto M, Yoshihama M, Ichikawa T. Propeptin, a new inhibitor of prolyl endopeptidase produced by *Microbispora* II. Determination of chemical structure. J Antibiot 55: 296–300 (2002)
8. Kimura K, Kanou F, Yoshihama M. A prolyl endopeptidase inhibitor, propeptin production in the various *Microbispora* sp. Actinomycetol 11: 64–68 (1997)
9. Kimura K, Kanou F, Yamashita Y, Yoshimoto T, Yoshihama M. Prolyl endopeptidase inhibitors derived from actinomycetes. Biosci Biotechnol Biochem 61: 1754–1756 (1997)
10. Weber W, Fischli W, Hochuli E, Kupfer E, Weibel EK. Anantin—a peptide antagonist of the atrial natriuretic factor (ANF). I. Producing organism, fermentation, isolation and biological activity. J Antibiot 44: 164–171 (1991)
11. Wyss DF, Lahm H-W, Manneberg M, Labhardt AM. Anantin—a peptide antagonist of the atrial natriuretic factor (ANF). II. Determination of the primary sequence by NMR on the basis of proton assignments. J Antibiot 44: 172–180 (1991)
12. Morishita Y, Chiba S, Tsukuda E, Tanaka T, Ogawa T, Yamasaki M, Yoshida M, Kawamoto I, Matsuda Y. RES-701-1, a novel and selective endothelin type B receptor antagonist produced by *Streptomyces* sp. RE-701. I. Characterization of producing strain, fermentation, isolation, physico-chemical and biological properties. J Antibiot 47: 269–275 (1994)
13. Yamasaki M, Yano K, Yoshida M, Matsuda Y, Yamaguchi K. RES-701-1, a novel and selective endothelin type B receptor

antagonist produced by *Streptomyces* sp. RE-701. II. Determination of the primary sequence. J Antibiot 47: 276–280 (1994)
14. Ogawa T, Ochiai K, Tanaka T, Tsukuda E, Chiba S, Yano K, Yamasaki M, Yoshida M, Matsuda Y. RES-701-2, -3 and -4, Novel and selective endothelin type B receptor antagonists produced by *Streptomyces* sp. I. Taxonomy of producing strains, fermentation, isolation, and biochemical properties. J Antibiot 48: 1213–1220 (1995)
15. Yano K, Yamasaki M, Yoshida M, Matsuda Y, Yamaguchi K. RES-701-2, a novel and selective endothelin type B receptor antagonist produced by *Streptomyces* sp. II. Determination of the primary structure. J Antibiot 48: 1368–1370 (1995)
16. Salomón RA, Farías RN. Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*. J Bacteriol 174: 7428–7435 (1992)
17. Bayro MJ, Mukhopadhyay J, Swapna GVT, Huang JY, Ma L-C, Sineva E, Dawson PE, Montelione GT, Ebright RH. Structure of antibacterial peptide microcin J25: A 21-residue lariat protoknot. J Am Chem Soc 125: 12382–12383 (2003)
18. Rosengren KJ, Clark RJ, Daly NL, Göransson U, Jones A, Craik DJ. Microcin J25 has a threaded sidechain-to-backbone ring structure and not a head-to-tail cyclized backbone. J Am Chem Soc 125: 12464–12474 (2003)
19. Iwatsuki M, Tomoda H, Uchida R, Gouda H, Hirono S, Ōmura S. Lariatins, antimycobacterial peptides produced by *Rhodococcus* sp. K01-B0171, have a lasso structure. J Am Chem Soc 128: 7486–7491 (2006)
20. Iwatsuki M, Uchida R, Takakusagi Y, Matsumoto A, Jiang C-L, Takahashi Y, Arai M, Kobayashi S, Matsumoto M, Inokoshi J, Tomoda H, Ōmura S. Lariatins, novel anti-mycobacterial peptides with a lasso structure, produced by *Rhodococcus jostii* K01-B0171. J Antibiot 60: 357–363 (2007)
21. Kimura K, Kawaguchi N, Yoshihama M, Kawanishi G. Staurosporine, a prolyl endopeptidase inhibitor. Agric Biol Chem 54: 3021–3022 (1990)