Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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Published online: 22 May 2014.

To cite this article: Jiro NAKAYAMA, Yong CAO, Takaaki HORII, Shohei SAKUDA & Hiromichi NAGASAWA (2001) Chemical Synthesis and Biological Activity of The Gelatinase Biosynthesis-Activating Pheromone of Enterococcus faecalis and Its Analogs, Bioscience, Biotechnology, and Biochemistry, 65:10, 2322-2325, DOI: 10.1271/bbb.65.2322

To link to this article: http://dx.doi.org/10.1271/bbb.65.2322

Biosci. Biotechnol. Biochem., **65** (10), 2322–2325, 2001

Note

Chemical Synthesis and Biological Activity of The Gelatinase Biosynthesis-Activating Pheromone of *Enterococcus faecalis* and Its Analogs

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Received April 2, 2001; Accepted May 24, 2001

An 11-residue peptide lactone, termed the gelatinase biosynthesis-activating pheromone (GBAP), triggers the production of the pathogenicity-related extracellular proteases, gelatinase and serine protease, in *Enterococcus faecalis*. In this study, we synthesized GBAP and its analogs and examined their gelatinase biosynthesis-inducing activity. This study on the structure-activity relationship shows that a lactone ring was indispensable for the activity.

Key words: gelatinase biosynthesis-activating pheromone; *Enterococcus faecalis*; peptide lactone; peptide pheromone; quorum sensing

Quorum sensing is a cell density-dependent regulatory system widely spread in various species of bacteria.[1,2] Gram-positive bacteria often use the peptide pheromone as a communication signal for the control of quorum sensing,[3,4] while Gram-negative bacteria often use *N*-acylhomoserine lactone as the signal molecule.[1] In this system, the peptide pheromone is secreted from each cell, accumulated outside the cells, and when its concentration exceeds a threshold level, the expression of one or more specific genes is triggered. Gelatinase and a serine protease, pathogenicity-related extracellular proteases of *Enterococcus faecalis*,[5,6] have recently been found to be controlled by quorum sensing in which their expression is active in the late-log to early-stationary phase.[7] We have isolated a signal molecule, termed the gelatinase biosynthesis-activating pheromone (GBAP), in the quorum sensing system and determined its structure to be a peptide lactone (**5**, see Scheme). GBAP has the novel structure of an 11-amino acid residue cyclic peptide containing a lactone ring between the α-carboxyl group at the C-terminal methionine residue and a hydroxyl group of the serine residue at the third position.

In this study, we synthesized GBAP (**5**), [D-Met[11]]GBAP (**6**), *N*-acetyl GBAP (**10**), linear GBAP (**8**), linear-GBAP amide (**7**) and linear-GBAP methylester (**9**) to identify the structure-activity relationship. The procedure for the synthesis of these peptides is summarized in the Scheme. In order to prepare GBAP, we used a procedure in which Z-undecapeptide (**2**) was cyclized to **3** by intramolecular dehydration and the Z-group of **3** was then removed to afford **5**. **2** was prepared from the fully protected undecapeptide (**1**) that had been manually synthesized by the Fmoc solid-phase synthesis method.[8] **2** was then treated with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) in dimethylacetamide. Figure A shows the result of an RP-HPLC analysis of the reaction mixture. The FABMS analysis suggested peaks 3 and 4 to contain dehydrated products **3** and **4** from **2**, respectively. No peaks corresponding to a dimer or an oligomer were detected, suggesting that intramolecular dehydration had mainly occurred. By treating **3** and **4** with trifluoroacetic acid (TFA) and thioanisole, **5** and **6** were respectively obtained. The FABMS analysis showed that the molecular weights of **5** and **6** were identical to that of GBAP. An amino acid analysis with (+)-1-(9-fluorenyl)ethyl chloroformate to give fluorescent amino acid diastereomers[9] indicated **6** to possess D-Met instead of L-Met, whereas all the amino acids of **5** were indicated to be L-amino acids. Taken together, **5** and **6** were indicated to be GBAP and [D-Met[11]]GBAP, respectively. This implies that the methionine residue at the C-terminus was racemized during the cyclization process. It has been reported that the active ester at the C-terminal α-carboxyl group gives an oxazolone whose ring can be easily opened and that this side reaction causes racemization of the C-terminal amino acid.[10] Synthetic GBAP (**5**) showed the same retention time as that of

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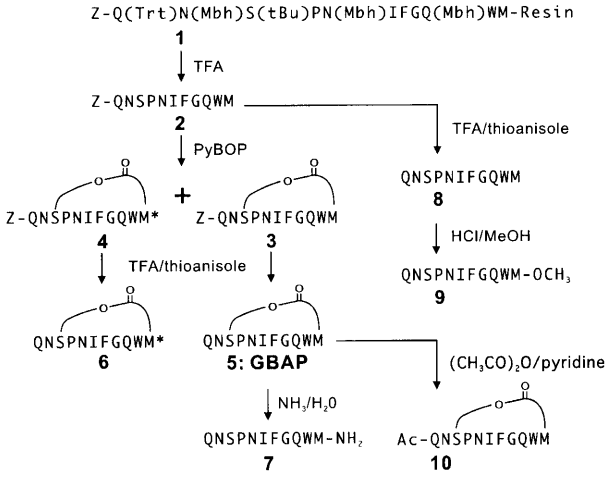
Abbreviations: GBAP, gelatinase biosynthesis-activating pheromone; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; FABMS, fast atom bombardment mass spectrometry; RP-HPLC, reverse phase high-performance liquid chromatography



Scheme. Synthesis of GBAP and Its Analogs.
M*: D-methionine

natural GBAP by reverse phase high-performance liquid chromatography (RP-HPLC) (Figs. B and C) and a mass fragmentation pattern identical to that of natural GBAP from the post source decay spectrum (data not shown). Moreover, it showed the gelatinase biosynthesis-inducing activity ($\Delta A_{540}=0.29$ at 5 nM) as did natural GBAP ($\Delta A_{540}=0.27$ at 5 nM). These results confirmed that synthetic GBAP had the same structure as natural GBAP.

2 was treated with TFA and thioanisole to afford linear GBAP (**8**). **8** was treated with hydrochloric acid-methanol to afford **9**. **5** was treated with acetic anhydride and pyridine to afford **10**. When **5** was treated with 29% aqueous ammonia, linear-GBAP amide (**7**) was obtained together with linear GBAP (**8**).

Table 1 shows results of the gelatinase-biosynthesis inducing assay with GBAP and the analogs that had been prepared. All compounds were assayed at 10 nM and 100 nM. GBAP showed significant activity at 10 nM, and its activity at 100 nM was higher to some extent. [D-Met[11]]GBAP (**6**) showed weak activity at 10 nM, and its activity at 100 nM increased to a similar degree to that of GBAP at 10 nM, indicating that **6** had about 10% activity of GBAP. *N*-acetyl GBAP (**10**) showed weak activity at both 10 nM and 100 nM, indicating that an amino group at the N-terminus contributed to the activity. None of the other compounds showed significant activity, suggesting that the lactone ring was indispensable for the GBAP activity and could not be replaced by a methyl ester or amide without loss of the activity.

GBAP was more stable during culture than sex pheromone cAD1[11] which is a linear oligopeptide.[7] cAD1 is rapidly degraded after the cells enter the stationary phase.[12] cAD1 degradation was not detected in the gelatinase-nonproducing *E. faecalis* strain, indicating that its degradation was due to gelatinase and perhaps also to serine protease.[12] Taken

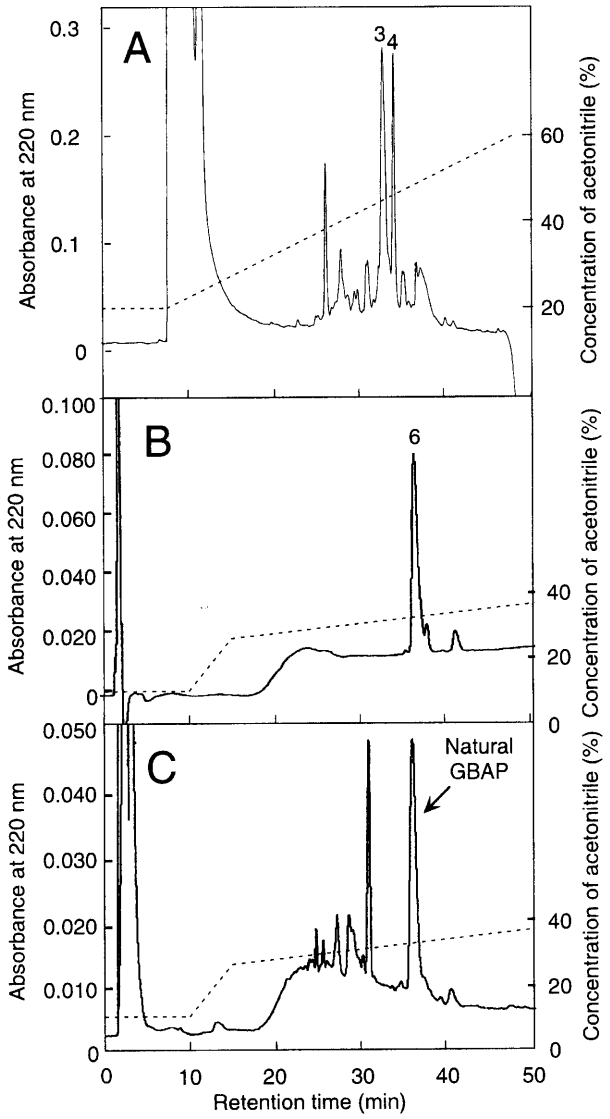


Figure. RP-HPLC data for Products **3** and **4** after the Cyclization Step (A), Synthetic GBAP (**6**) (B), and Natural GBAP (C). The solid line indicates the absorbance at 220 nm, and the dotted line indicates the concentration of acetonitrile. (A) The reaction mixture (0.1 ml) after the cyclization step with PyBOP was injected into a Pegasil ODS column (2.0×25 cm). (B) One microgram of **6** was injected into the Pegasil ODS column (0.46×15 cm). (C) Last purification step of natural GBAP by the Pegasil ODS column (0.46×15 cm).[7]

Table 1. Gelatinase Biosynthesis-Inducing Activity of GBAP and Its Analogs

Compound	Gelatinase-inducing activity (ΔA_{540})*	
	10 nM	100 nM
GBAP (**5**)	0.28±0.06	0.35±0.03
[D-Met[11]]GBAP (**6**)	0.05±0.02	0.28±0.03
N-acetyl GBAP (**10**)	0.11±0.01	0.11±0.03
linear-GBAP (**8**)	0.00±0.01	0.00±0.01
linear-GBAP methylester (**9**)	−0.04±0.03	0.01±0.02
linear-GBAP amide (**7**)	−0.03±0.03	−0.04±0.02

* Each value represents the mean±standard deviation of four samples.

together, it is likely that lactone structure contributes to protection from proteolytic degradation by those extracellular proteases induced by GBAP.

Quorum sensing has recently been considered as a new target for anti-infective therapy;[13] for example, a global inhibitor of virulence in *Staphylococcus aureus* has been designed on the basis of the structures of thiolactone peptide pheromones.[14] Inhibiting GBAP activity, as that staphylococcal inhibitor did, may lead to the development of an anti-infective therapy to prevent virulent *E. faecalis* infection. Using the synthetic technique for the peptide lactone established here, we are now trying to synthesize antagonistic analogues to block GBAP activity.

Experimental

General procedures for the purification and chemical analysis of the synthetic compounds. All synthetic compounds were purified by RP-HPLC with an analytical Pegasil ODS column (0.46 × 15 cm, Senshu Kagaku) or a preparative Pegasil ODS column (2.0 × 25 cm, Senshu Kagaku) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The peptides were monitored during HPLC and their concentrations were calculated by the ultraviolet absorbance at 220 nm. Their molecular masses were ascertained by fast atom bombardment mass spectrometry (FABMS) with a JMS SX102 mass spectrometer (Jeol), using a mixture of glycerol and thioglycerol as the matrix. An amino acid analysis was performed according to the described method[8] with (+)-1-(9-fluorenyl)ethyl chloroformate to give fluorescent amino acid diastereomers which were separated by RP-HPLC with a Docosil-B column (0.46 × 15 cm, Senshu Kagaku). The amino acid sequence was analyzed by a protein sequencer (Applied Biosystems 476A). For the post source decay (PSD) spectrum, 0.5 μl of a sample solution containing approximately 10 ng of a peptide was mixed with 0.5 μl of a matrix solution (50% acetonitrile and 0.1% TFA) saturated with 3,5-dimethoxy-4-hydroxy-cinnamic acid, dried on a plate, and analyzed by a Voyager DE-STR (PE Biosystems) time-of-flight mass spectrometer.

Synthesis of Z-Gln-Asn-Ser-Pro-Asn-Ile-Phe-Gly-Gln-Trp-Met (2). Protected peptide **1**, Z-Gln(Trt)-Asn(Mbh)-Ser(tBu)-Pro-Asn(Mbh)-Ile-Phe-Gly-Gln(Mbh)-Trp-Met-O-Resin (Z = benzyloxycarbonyl, Trt = trityl, Mbh = 4,4-dimethoxybenzhydryl, tBu = *t*-butyl), was manually synthesized by the solid-phase method using the Fmoc strategy with *p*-alkoxybenzyl alcohol resin (0.1 mmol, Kokusan Kagaku) as the solid support. After chain assembly, the peptide was cleaved from the support and the side chain-protecting groups were also deprotected by being treated with 5 ml of a trifluoroacetic acid/phenol/water/ethanedithiol/triisopropylsilane mixture (81.5:5:5:2.5:1) for 3.5 hr. The reaction mixture was concentrated to 1 ml with a rotary evaporator and cold diethyl ether (300 ml) was added. The mixture was kept standing overnight at −20°C. The resulting precipitate was collected by filtration (PTFE, Millipore) and several times washed with cold diethyl ether. The crude peptide on the filter was dissolved in DMSO and loaded into a Sep-pak C_{18} cartridge column (10 g Waters); after washing the column with 50 ml of 10% acetonitrile, Z-undecapeptide **2** was eluted with 50 ml of 80% acetonitrile and evaporated to dryness, to obtained crude **2** (30 mg), FAB-MS *m/z* 1455.5 $(M+H)^+$.

Synthesis of GBAP (5) and [D-Met[11]]GBAP (6). Crude **2** (25 mg) was dissolved in 10 ml of *N,N*-dimethylacetamide, and PyBop (Nova Biochem; 50 mg) and dimethylaminopyridine (50 mg) were added to the solution. The reaction mixture was stirred at room temperature for 8 h under N_2 gas, added to 200 ml of ice-cold water, and loaded into the Sep-pak C_{18} cartridge column. After washing the column with 200 ml of 20% acetonitrile containing 0.1% trifluoroacetic acid, the peptides were eluted with 40 ml of 50% acetonitrile containing 0.1% TFA. Cyclized peptides **3** (1.0 mg) and **4** (0.5 mg) were isolated from the eluate by preparative RP-HPLC. In order to remove N-terminal Z-group, each purified peptide [**3** (1.0 mg) and **4** (0.12 mg)] was dissolved in 5 ml of trifluoracetic acid/thioanisole/*m*-cresol/ethanedithiol/trifluoroacetic acid anhydride (10:1:1:1:0.2) and kept standing for 8 h at room temperature. The reaction mixture was added to 200 ml of ice-cold 5% acetonitrile and loaded into the Sep-pak C18 cartridge column. After washing the column with 200 ml of 16% acetonitrile containing 0.1% trifluoroacetic acid, the peptide was eluted with 40 ml of 50% acetonitrile containing 0.1% TFA. The obtained peptides were further purified by preparative RP-HPLC to afford **5** (0.8 mg) and **6** (45 μg), respectively. The structures of **5** and **6** were ascertained by FABMS, PSD spectrum, amino acid analysis, and amino acid sequencing. **3**: FABMS *m/z* 1437.8 $(M+H)^+$. **4**: FABMS *m/z* 1437.8 $(M+H)^+$. **5**: FABMS *m/z* 1303.7 $(M+H)^+$. **6**: FABMS *m/z* 1303.7 $(M+H)^+$.

Synthesis of linear-GBAP amide (7). **5** (18 μg) was dissolved in 29% aqueous ammonia (50 μl) and kept standing overnight at 37°C. The reaction mixture was added to 500 μl of ice-cold 0.05% TFA, and the resulting solution was neutralized by adding acetic acid and then subjected to RP-HPLC to obtain **7** (7.8 μg), FABMS *m/z* 1320.8 $(M+H)^+$.

Synthesis of linear GBAP (8). The Z group was removed from **2** (0.5 mg) by the same procedure as that described for the syntheses of **5** and **6**. After

purifying the deblocked peptide by RP-HPLC, **8** (125 μg) was obtained, FABMS m/z 1321.7 $(M+H)^+$.

Synthesis of linear-GBAP methylester (9). **8** (62.5 μg) was dissolved in 420 μl of absolute methanol, 30 μl of hydrogen chloride-methanol reagent 5 (Tokyo Kasei Kogyo) was added to the solution, and the mixture was kept standing overnight at 37°C. After purification by RP-HPLC, **9** (15 μg) was obtained, FABMS m/z 1335.7 $(M+H)^+$.

Synthesis of N-acetyl GBAP (10). **5** (18 μg) was dissolved in acetic anhydride (100 μl) and pyridine (100 μl), and kept standing overnight at room temperature. After purification by RP-HPLC, **10** (4.1 μg) was obtained, FABMS m/z 1345.7 $(M+H)^+$.

Assay for gelatinase-biosynthesis inducing activity. The solvents used for dissolving the peptides were removed by lyophilization in the assay tube. An overnight culture of *E. faecalis* OG1RF was inoculated into 3 ml of Todd Hewitt broth (Oxoid) containing each synthetic peptide to be tested to an OD_{660} of 0.01 and were then grown at 37°C. As a control, the bacteria were grown in the medium not containing a synthetic peptide. The culture supernatant was collected 3 h after inoculation and the gelatinase activity in the supernatant was measured according to a previously described procedure with some modifications.[7] The culture supernatant (25 μl) was added to the tube containing 0.5 ml of a preincubated Azocoll solution (0.25 g/50 ml, <50 mesh, Calbiochem), and the mixture was incubated for 4 h at 37°C on a shaker, and was then centrifuged at 1500 × g for 5 min. The supernatant (200 μl) was transferred to duplicated wells of a 96-well ELISA plate (Iwaki 3801-096), and the absorbance at 540 nm was measured by an EIA reader (model 2550, Bio-Rad). The gelatinase biosynthesis-inducing activity is represented by the difference in gelatinase activity between the culture with a peptide and the control culture (ΔA_{540}).

Acknowledgments

We are grateful to Mr. M. Chijimatsu of the Division of Biomolecular Science at Riken for the amino acid analysis.

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