MATLYSTATINS, NEW INHIBITORS OF TYPEIV COLLAGENASES FROM *Actinomadura atramentaria*

II. BIOLOGICAL ACTIVITIES

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(Received for publication June 5, 1992)

Matlystatin A, the main component of matlystatins, inhibits 92 kDa and 72 kDa typeIV collagenases with IC_{50} values of 0.3 μM and 0.56 μM, respectively, while 7- to 11-fold greater concentrations are required to inhibit thermolysin and aminopeptidase M. The inhibition is reversible and competitive with respect to gelatin. It inhibits the invasion of basement membrane Matrigel by human fibrosarcoma HT1080 dose-dependently with an IC_{50} value of 21.6 μM.

Recent studies on typeIV collagenases reveal that their activity is regulated in a complex way. Two species of typeIV collagenases, with molecular weights of 92 kDa and 72 kDa, exist as different gene products,[1,2] and both are secreted in latent proenzyme forms and activated in extracellular spaces. There are also natural inhibitor proteins of matrix metalloproteinases (MMPs), designated TIMP-1 and TIMP-2.[3~5] They form 1 : 1 ratio complexes with 92 kDa and 72 kDa typeIV collagenase proenzyme, respectively.[2,4,5] They also inhibit activated enzymes, and in this case TIMP-2 is reportedly more potent than TIMP-1.[6] TIMPs inhibit the activation process itself.[7] Although recombinant TIMPs have been extensively studied in animals to treat tumor invasion and rheumatoid arthritis, their effect has a limitation so far, probably because of a high molecular weight nature.[8~10] It is reasonable, therefore, to expect that low molecular weight inhibitors are more effective. As reported in a preceding paper, matlystatins, new inhibitors of typeIV collagenase, have been isolated from *Actinomadura atramentaria*.[11] This paper describes the biological activities of matlystatins.

Materials and Methods

Materials

Thermolysin was purchased from Sigma Chemical Co., U.S.A.; aminopeptidase M from Boehringer Mannheim, Germany; and carbobenzoxy-glycyl-L-leucine-amide and L-leucine-*p*-nitroanilide from Peptide Institute Inc., Osaka, Japan. Human melanoma cells A2058 were generously provided by Dr. K. TRYGGVASON, Oulu University, Oulu, Finland. Human fibrosarcoma cells HT1080 were purchased from the American Type Culture Collection, Rockville, MD, U.S.A.

Purification of Type IV Collagenase

In early experiments, 92 kDa typeIV collagenase was prepared from cultured cells of human melanoma A2058. Cells were cultured in DULBECCO's modified EAGLE's medium (DMEM) supplemented with 1 mM glutamine and antibiotics (100 μg/ml streptomycin, 100 units/ml penicillin) and 10% heat-inactived fetal calf serum. The subconfluent cell cultures were washed with serum-free DMEM, to remove traces of serum, and incubated with serum-free DMEM for 48 hours. The serum-free conditioned medium was applied to a zinc-chelating affinity column preequilibrated in buffer A (50 mM Tris-HCl buffer, pH 7.6, 10 mM $CaCl_2$,

0.2 M NaCl). The column was extensively washed with buffer A and then with buffer A containing 2.5 mM imidazole, and eluted with buffer A containing 25 mM imidazole. The eluate was subjected to 25~80% ammonium sulfate fractionation and dialyzed against buffer A. In more recent experiments, 92 kDa and 72 kDa typeIV collagenases were purified from cultured cells of human fibrosarcoma HT1080. The cells were cultured similarly with human melanoma A2058, except that HAM's F-12 - DMEM 1 : 1 medium was used instead of DMEM. The subconfluent cell cultures were incubated for 5 days in serum-free medium containing 100 units/ml recombinant human TNF α (Genzyme, U.S.A.).[12] The conditioned medium was adjusted to pH 8.0 and passed through DEAE - cellulose preequilibrated in 50 mM Tris-HCl buffer, pH 8.0. The flowthrough was applied to Green A Matrex Gel preequilibrated in buffer B (50 mM Tris-HCl buffer, pH 7.6, 10 mM $CaCl_2$, 0.05% Brij35, and 0.02% NaN_3), and the enzyme was eluted with buffer B containing 1.0 M NaCl. The fractions containing 92 kDa and/or 72 kDa typeIV collagenase were pooled and applied to gelatin-Sepharose (Pharmacia) preequilibrated in buffer B containing 0.5 M NaCl. The column was extensively washed with the same buffer, and the enzyme was eluted using a 0~10% DMSO gradient. This step separated 92 kDa and 72 kDa typeIV collagenase. The activities of typeIV collagenase in these purifications were monitored by gelatin zymogram.

Purification of Stromelysin and TypeI Collagenase

Rabbit synovial fibroblasts have been obtained from New Zealand White Rabbits and cultured according to the method of DAYER *et al.*[13] The cells were cultured under the similar conditions with that of human fibrosarcoma HT1080, except that the cells were used within passage 8 and that serum-free medium was supplemented with 100 ng/ml of 12-*O*-tetradecanoylphorbol 13-acetate, 50 pM recombinant human TNF α and 10 pM recombinant human IL-1β.[14] Stromelysin and typeI collagenase were purified from the conditioned medium by the method of OKADA *et al.*[15]

TypeIV Collagenase Assay

Substrates used were mouse EHS typeIV collagen or typeI collagen-derived gelatin. EHS typeIV collagen was purified by the method of YURCHENCO and FURTHMAYR[16], and was ^{3}H-acetylated[17]. TypeI collagen was purified from rat tail. After ^{3}H-acetylation, it was denatured by heat treatment at 60°C for 30 minutes. 92 kDa typeIV collagenase was activated prior to assay by 1 mM aminophenylmercuric acetate (APMA) at 37°C for 3 hours, and APMA was eliminated by dialysis. The assay was carried out in a total volume of 200 μl containing 50 mM Tris-HCl buffer, pH 7.5, 10 mM $CaCl_2$, 0.15 M NaCl, 0.05% Brij35, 0.02% NaN_3, 2 μg ^{3}H-acetyl typeIV collagen or ^{3}H-acetyl gelatin, and appropriate amounts of the enzyme. For 72 kDa typeIV collagenase, the reaction mixture contained 1 mM APMA. Assays were carried out at 37°C for 0.5~3 hours, and were terminated as described for either typeIV collagen as a substrate[18] or typeI collagen-derived gelatin as a substrate.[19] In all experiments, the reaction was designed to be a linear function of time. *Km* and *Ki* values were obtained by the least square method using a computer program.

Other Enzyme Assays

Other enzymes were assayed by the following methods: Stromelysin by the method of OKADA *et al.*, using ^{3}H-carboxymethyl human transferrin (Cm-Tf) as a substrate at the final concentration of 61 μg/ml;[15] typeI collagenase by the method of CAWSTON and BARRET;[20] thermolysin by the method of KOMIYAMA *et al.*, using carbobenzoxy-glycyl-L-leucine-amide as a substrate;[21] aminopeptidase M by the method of PFLEIDERER, using L-leucine-*p*-nitroanilide as a substrate.[22]

Invasion Assay

The invasion assay was performed by the method of HENDRIX *et al.*[23] using Costar 6.5-mm Transwell chambers equipped with 8.0-μm pore size polycarbonate membranes. The upper surface of the membrane was coated with Matrigel (200 μg of protein, Collaborative Research, U.S.A.). The bottom chamber was filled with 600 μl of a solution containing laminin (100 μg/ml) and fibronecin (50 μg/ml) in DMEM supplemented with 10% NuSerum (Collaborative Research). HT1080 fibrosarcoma cells grown as subconfluent cultures were harvested by a brief treatment with 3 mM EDTA and suspended in DMEM supplemented with 10% NuSerum. Then 2×10^5 cells were seeded on the reconstituted Matrigel in the upper Transwell chamber. After a 72-hour incubation, cells that penetrated through the polycarbonate

membrane were harvested from the bottom chamber by trypsin-EDTA treatment and counted.

Results and Discussion

Antimicrobial Activities

Antimicrobial activities of matlystatins A and B are shown in Table 1. Matlystatin B inhibited the growth of *Bacteroides* most prominently, and its antimicrobial activities were more potent than those of matlystatin A. It may be possible that matlystatin A penetrates the cell membrane less efficiently because of its bulky side chain. Recently YL-01869P, a compound similar to matlystatin B, was isolated from *Streptomyces* as an antimicrobial agent and also shown to inhibit typeI collagenase.[24] Antimicrobial activities of matlystatin B, however, are a little different from those reported for YL-01869P.

Matlystatin Inhibition of TypeIV Collagenases

Table 2 summarizes the concentrations of matlystatin-group compounds required for 50% inhibition (IC_{50}) of 92 kDa and 72 kDa typeIV collagenases, typeI collagenase, and stromelysin. Matlystatin A, the main component of matlystatins, inhibited 92 kDa typeIV collagenase most potently among 5 components. It is also the case for stromelysin. Actinonin, another hydroxamic acid of microbial origin, is reported to inhibit aminopeptidase M.[25] Therefore, inhibitions of aminopeptidase M and thermolysin were also tested, to evaluate the effect on other zinc enzymes. The IC_{50}s of matlysatin A for typeIV collagenases are at the order of 10^{-7} M, while those for thermolysin and aminopeptidase M are at the order of 10^{-6} M. As for stromelysin, it appears to be more sensitive to matlystatins than typeIV collagenases, but *Km* value for ^{3}H-Cm-Tf is very high, and to be precise, it will be necessary to employ native substrates to make a direct comparison of IC_{50} values. In the experiments shown in Table 3, 92 kDa typeIV collagenase was preincubated with varying concentration of matlystatin A at 37°C for 30 minutes, and the concentrations of matlystatin A were reduced to one-tenth at enzyme assay. As indicated, the inhibition of matlystatin A was completely reversible, as it depended on the concentration of the

Table 1. Antimicrobial activity of matlystatins A and B.

Organism	MIC (μg/ml)	
	Matlystatin	
	A	B
Bacteroides fragilis subsp. *fragilis*	12.5	0.012
Staphylococcus aureus 209P JC-1	50	1.5
Bacillus subtilis ATCC 6633	12.5	0.1
Escherichia coli NIHJ JC-2	>100	>100
Streptococcus flexneri IID 642	>100	25
Candida albicans YU 1200	>100	>100
Trichophyton asteroides	>100	>100
T. interdigitale	>100	>100
T. rubrum	>100	>100

Table 2. IC_{50} values of matlystatin group compounds for metalloproteinases.

	IC_{50} (μM)					
	TypeIV collagenase		TypeI collagenase	Stromelysin	Thermolysin	Aminopeptidase M
	92 kDa	72 kDa				
A	0.30	0.56	1.2	0.08	3.30	2.21
B	1.43	1.72	1.2	0.09	3.30	9.95
D	3.64	12.9	28	0.64	16.2	6.70
E	19.3	20.4	59	2.4	18.2	8.88
F	1.82	15.3	5.1	0.27	1.82	14.0

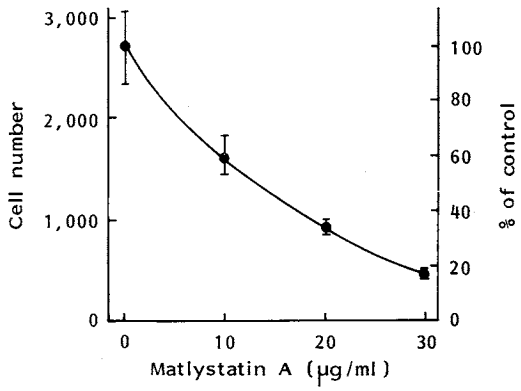
Enzyme assays were carried out as described in Materials and Methods.

Table 3. Reversible inhibition of 92 kDa typeIV collagenase by matlystatin A.

Concentration of matlystatin A (μM)		Relative activity (%)
At preincubation	At enzyme assay	
0	0	100
0	0.17	68.0
0	0.33	46.2
0	0.83	15.1
1.70	0.17	69.2
3.30	0.33	46.2
8.30	0.83	14.5

Enzyme assay was carried out as described in Materials and Methods, except that the enzyme was preincubated with matlystatin A at 37°C for 30 minutes and the concentration of matlystatin A was lowered to one-tenth at enzyme assay.

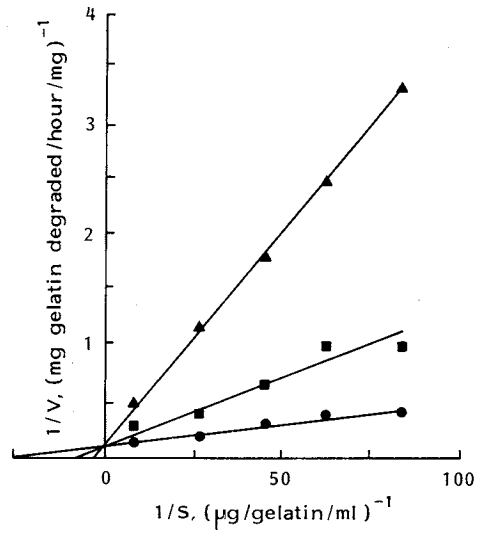
Fig. 2. Inhibition of *in vitro* invasion through basement membrane Matrigel by matlystatin A.



Each point represents the mean of 3 determinations. The vertical bar indicates the standard error.

Fig. 1. Lineweaver-Burk plots for the inhibition of 92 kDa typeIV collagenase by matlystatin A.

● None, ■ 0.30 μM matlystatin A, ▲ 1.25 μM matlystatin A.



The enzyme assay was carried out as described in Materials and Methods, except that the concentrations of ^{3}H-acetyl gelatin were varied as indicated. The points in the reciprocal plots are the experimentally determined values, while the lines are calculated from the fits of these data to the rate equation for competitive inhibition.

inhibitor at enzyme assay, not on the concentration at preincubation. Double reciprocal plots showed that the inhibition was competitive with respect to gelatin (Fig. 1). The *Km* value for gelatin is 40 ng/ml and the *Ki* value for matlystatin A is 1.60×10^{-7} M.

As shown in the preceding paper, matlystatins share a structure containing hydroxamic acid, pentyl or butyl succinic acid, and piperazic acid.[11] In culture broth of the producing strain, the compounds were also found in which hydroxamic acid were reduced, and they were inactive (data not shown). As zinc ion is in the catalytic center of MMPs, it is strongly deduced that matlystatins exert their action mainly by forming a linkage between hydroxamic acid and zinc ion.

Matlystatin A Inhibition of *In Vitro* Invasion Assay

Matlystatin A inhibits the invasion of basement membrane Matrigel by human fibrosarcoma HT1080 dose-dependently with an IC_{50} value of 13.0 μg/ml (21.6 μM) (Fig. 2).

References

1) COLLIER, I. E.; S. M. WILHELM, A. Z. EISEN, B. L. MARMER, G. A. GRANT, J. L. SELTZER, A. KRONBERGER, C. HE, E. A. BAUER & G. I. GOLDBERG: H-ras oncogene transformed human bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen. J. Biol. Chem. 263: 6579~6587, 1988

2) WILHELM, S. M.; I. E. COLLIER, B. L. MARMER, A. Z. EISEN, G. A. GRANT & G. I. GOLDBERG: SV40-transformed human lung fibroblasts secrete a 92 kDa typeIV collagenase which is identical to that secreted by normal human macrophages. J. Biol. Chem. 264: 17213~17221, 1989
3) DOCHERTY, A. J. P.; A. LYONS, B. J. SMITH, E. M. WRIGHT, P. E. STEPHENS, T. J. R. HARRIS, G. MURPHY & J. J. REYNOLDS: Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature 318: 66~69, 1985
4) STETLER-STEVENSON, W. G.; H. C. KRUTZSCH & L. A. LIOTTA: TIMP-2, a new member of the metalloproteinase inhibitor family. J. Biol. Chem. 264: 17374~17378, 1989
5) GOLDBERG, G. I.; B. L. MARMER, G. A. GRANT, A. Z. EISEN, S. WILHELM & C. HE: Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2. Proc. Natl. Acad. Sci. U.S.A. 86: 8207~8211, 1989
6) HOWARD, E. W.; E. C. BULLEN & M. J. BANDA: Preferential inhibition of 72- and 92-kDa gelatinase by tissue inhibitor of metalloproteinase-2. J. Biol. Chem. 266: 13070~13075, 1991
7) HOWARD, E. W.; E. C. BULLEN & M. J. BANDA: Regulation of the autoactivation of human 72-kDa progelatinase by tissue inhibitor of metalloproteinase-2. J. Biol. Chem. 266: 13064~13069, 1991
8) SCHULTZ, R. M.; S. SILBERMAN, B. PERSKY, A. S. BAJKOWSKI & D. F. CARMICHAEL: Inhibition of human recombinant tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells. Cancer Res. 48: 5539~5545, 1988
9) DECLERCK, Y. A.; T.-D. YEAN, D. CHAN, H. SHIMADA & K. E. LANGLEY: Inhibiton of tumor invasion of smooth muscle cell layers by recombinant human metalloproteinase inhibitor. Cancer Res. 51: 2151~2157, 1991
10) ALBINI, A.; A. MELCHIORI, L. SANTI, L. A. LIOTTA, P. D. BROWN, W. G. STETLER-STEVENOSON: Tumor cell invasion inhibited by TIMP-2. J. Natl. Cancer Inst. 83: 775~779, 1991
11) OGITA, T.; A. SATO, R. ENOKITA, K. SUZUKI, M. ISHII, T. NEGISHI, T. OKAZAKI, K. TAMAKI & K. TANZAWA: Matlystatins, new inhibitors of typeIV collagenases from *Actinomadura atramentaria*. I. Taxonomy, fermentation, isolation, and physico-chemical properties of matlystatin-group compounds. J. Antibiotics 45: 1723~1732, 1992
12) OKADA, Y.; H. TSUCHIYA, H. SHIMIZU, K. TOMITA, I. NAKANISHI, H. SATO, M. SEIKI, K. YAMASHITA & T. HAYAKAWA: Induction and stimulation of 92 kDa gelatinase/typeIV collagenase production in osteosarcoma and fibrosarcoma cell lines by tumor necrosis factor α. Biochem. Biophys. Res. Commun. 171: 610~617, 1990
13) DAYER, J.-M.; S. M. KRANE, G. G. RUSSELL & D. R. ROBINSON: Production of collagenase and prostagrandins by isolated adherent rheumatoid synovial cells. Proc. Natl. Acad. Sci. U.S.A. 73: 945~949, 1976
14) MACNAUL, K. L.; N. CHARTRAIN, M. LARK, M. J. TOCCI & N. I. HUTCHINSON: Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinase-1 in rheumatoid human synovial fibroblasts. J. Biol. Chem. 265: 17238~17245, 1990
15) OKADA, Y.; H. NAGASE & E. D. HARRIS, JR.: A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. J. Biol. Chem. 261: 14245~14255, 1986
16) YURCHENCO, P. D. & H. FURTHMAYR: Self-assembly of basement membrane collagen. Biochemistry 23: 1839~1850, 1984
17) GISSLOW, M. T. & B. C. MCBRIDE: A rapid sensitive collagenase assay. Anal. Biochem. 68: 70~78, 1975
18) SALO, T.; L. A. LIOTTA & K. TRYGGVASON: Purification and characterization of a murine basement membrane collagen degrading enzyme secreted by metastatic tumor cells. J. Biol. Chem. 258: 3058~3063, 1983
19) STRICKLIN, G. P.; E. A. BAUER, J. J. JEFFREY & A. Z. EISEN: Human skin collagenase: isolation of precursor and active forms from both fibroblast and organ cultures. Biochemistry 16: 1607~1615, 1977
20) CAWSTON, T. E. & A. J. BARRET: A rapid and reproducible assay for collagenase using [1-^{14}C]acetylated collagen. Anal. Biochem. 99: 340~345, 1979
21) KOMIYAMA, T.; H. SUDA, T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: Studies on inhibitory effect of phosphoramidon and its analogs on thermolysin. Arch. Biochem. Biophys. 171: 727~731, 1975
22) PFLEIDERER, G.: Particle-bound aminopeptidase from pig kidney. Methods Enzymol. 19: 514~521, 1970
23) HENDRIX, M. J. C.; K. R. GEHLSEN, H. N. WAGNER, Jr., S. R. RODNEY, R. L. MISIOROWSKI & F. L. MEYSKENS, Jr.: *In vitro* quantification of melanoma tumor cell invasion. Clin. Exp. Metastasis 3: 221~233, 1985
24) SATO, T.; M. SHIBASAKI, M. MORIOKA, K. SUZUKI & Y. TAKEBAYASHI (Yamanouchi Pharmaceuticals): Jpn. Kokai 157372 ('91), July 5, 1991
25) UMEZAWA, H.; T. AOYAGI, T. TANAKA, H. SUDA, A. OKUYAMA, H. NAGANAWA, M. HAMADA & T. TAKEUCHI: Production of actinonin, an inhibitor of aminopeptidase M, by actinomycetes. J. Antibiotics 38: 1629~1630, 1985