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Article

The discovery of synthetic proteolytic peptide

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Abstract: After screening nearly 1000 synthetic peptides, a synthetic peptide termed JAL-AK22 (KYEGHWYPEKPYKGSGFRCIHI) derived from the BoxA domain in Tob1 protein was found to activate both unfolded and folded proMMP-7. In addition, JAL-AK22 showed auto-proteolytic activity. Interestingly, the smaller derivative of JAL-AK22 termed JAL-TA9 (YKGSGFRMI) also possessed auto-proteolytic activity and cleaved 2 fragment peptides (MMP18-33 and MMP18-40) derived from the prodomain of proMMP-7 under physiological conditions. These proteolytic activities were inhibited by AEBSF, a serine protease inhibitor. Our results demonstrate that a small synthetic peptide consisting of only 9 amino acids has serine protease-like activity and activates proMMP-7 by cleaving the prodomain region. We thus propose calling small peptides possessing with protease-like activity Catalytides (catalytic peptides). We expect that our findings will stimulate the development of novel Catalytides and related applications.

Keywords: Catalytide, serine protease-like peptide, Tob1

1. Introduction

We reported the microplate assay method for screening the possible inhibitors of MMPs (MMPIs), which are thought to be critically involved in oncogenesis, using with activated recombinant matrix metalloproteinases (actMMPs) and a fluorescence quenching substrate (3163-v) [1-3]. To prepare activated MMP-7 (actMMP-7) from unfold proMMP-7 (u-proMMP-7) expressed in *Escherichia coli*, refolding step and proteolytic cleavage of prodomain were necessary. The activity of actMMP-7 was monitored with fluorescence intensity produced from 3163-v; thus, the fluorescence intensity reflects the level of cleavage and thereby u-proMMP-7 activation (Scheme 1a). We thought that the chaperone-like peptide could be screened by the microplate assay method. After screening nearly 1,000 peptides, some peptides were found to activate u-proMMP-7 (Scheme 1b). The most potent peptide is termed JAL-AK22 (KYEGHWYPEKPYKGSGFRCIHI), comprising 22 amino acids derived from the BoxA domain in the Tob1 protein (Figure S1a), a membrane-type tyrosine kinase, was found to activate u-proMMP-7, in contrast to the initial expectation (data not shown). The protein encoded by Tob1 is a member of the Tob/BTG family consisted of BTG1, BTG2/Tis21/PC3, ANA/BTG3, BTG4/PC3B and Tob2. The N-terminus region of Tob/BTG family is a highly conserved homology domain that contains BoxA, B and C. According to recent reports, these proteins display antiproliferative activity in a variety of cell types and are involved in the regulation of tumorigenesis [4-6]. However, the role of the BoxA domain of Tob/BTG family protein is still unclear, and there have been no reports of protease activity. Enzymes are generally macromolecular proteins, acting alone or in large complexes. To the best of our knowledge, protease-like activity has never been reported in very small synthetic peptides. In this study, we describe the identification of a small protease-like peptide termed as Catalytide.

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2. Results

2.1. Screening of proteolytic peptide, JAL-AK22

2.1.1. Screening of proteolytic peptide

Our first aim of this study was to find the chaperone-like peptide, which could affect the refolding of u-proMMP-7 [7]. The reaction mixture containing u-proMMP-7 and fluorescence substrate (3163-v) was incubated at 37 °C in assay buffer. The fluorescence intensity was measured with excitation at 320 nm and emission at 390 nm (Scheme 1b). After screening nearly 1000 synthetic peptides in our peptide library, the synthetic peptide derived from BoxA region of Tob1 protein termed JAL-AK22 (KYEGHWYPEKPYKGSGFRCIHI) (Figure S1a) was found as the activator of u-proMMP-7 (data not shown). However, it was unclear whether JAL-AK22 affected the refolding (Scheme 1b) or cleavage (Scheme 1c) of the prodomain of proMMP-7. To clarify this question, we next analyzed the effect of JAL-AK22 on the activation of folded proMMP-7 (f-proMMP-7), which was prepared by renaturation of u-proMMP-7 (Scheme 1a). As shown in Figure 1, JAL-AK22 significantly enhanced the activation of f-proMMP-7 but slightly reduced the activity of preactivated MMP-7 (actMMP-7) activated by trypsin (Scheme 1a). The f-proMMP-7 alone showed much slower activation apparently due to spontaneous auto-catalysis. These results indicated that JAL-AK22 could activate f-proMMP-7 by proteolytic cleavage of proMMP-7 rather than refolding of u-proMMP-7 [8].

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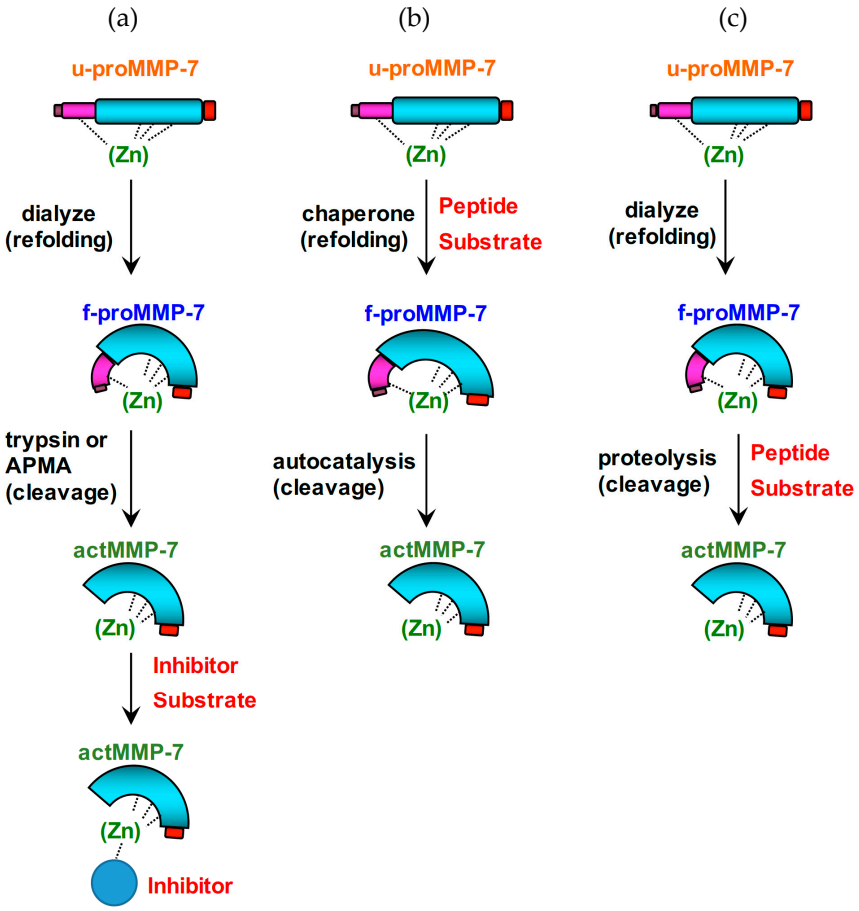

Scheme 1. Screening and identification of proteolytic peptides (a) Screening of MMP inhibitor (b) Screening of chaperone-like peptide (c) Screening of proteolytic peptide

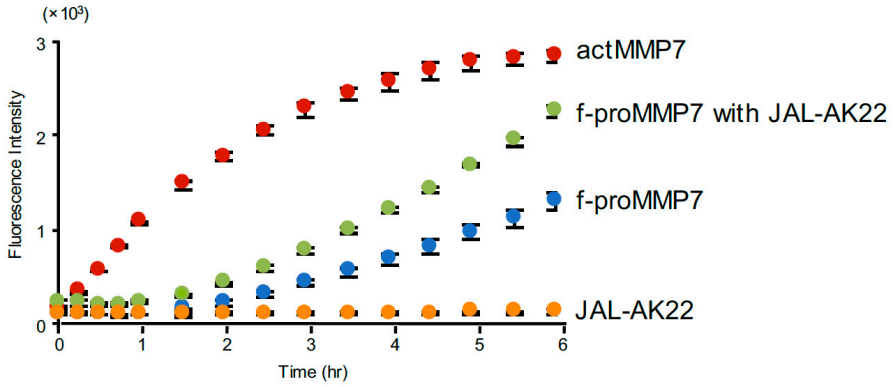

Figure 1. Activation of folded proMMP-7 by JAL-AK22

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2.1.2. Auto-proteolytic activity

In a preliminary analysis of the reaction mixture described above, we noticed a decrease in the JAL-AK22 level after incubation and thus decided to examine its auto-proteolytic activity with or without human serum albumin (HSA). JAL-AK22 was incubated alone at 37 °C in PBS (pH 7.4), and the incubation mixture was analyzed by HPLC (Figure 2a). The original amount of JAL-AK22 decreased in a time-dependent manner, strongly suggesting that JAL-AK22 has auto-proteolytic activity. The peaks (1-4) in the chromatogram that appeared after 5 days were collected (Figure 2b). By MS analyses, 7 kinds of fragment peptides derived from JAL-AK22 were identified in the reaction mixture (Table 1).

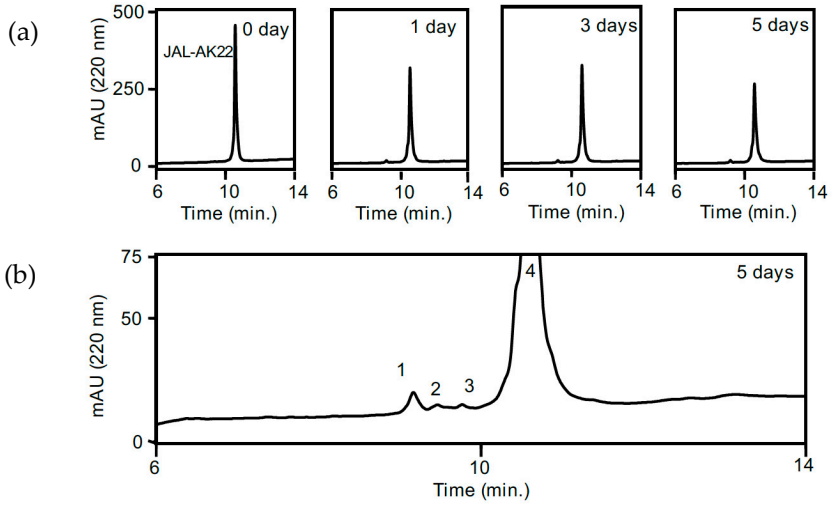

Figure 2. Identification of auto-proteolysis activity of JAL-AK22 (a) time dependent analysis (b) peak collection

Table 1. Identification of fragment peptides of JAL-AK22

Peak	Fragment	Theoretical MS	Experimental MS
1	KYEGHWYPEKPYK	1723.84	1722.8665
1	KYEGHWYPEKPYKGS	1867.89	1866.9345
2	KYEGHWYPEKPYKGSGFR	2228.08	2228.1138
3	KYEGHWYPEKPY	1595.74	1595.7560
4	EKPYKGSGFRC (YKGSGFRCIHI) I	2661.35	2661.3766
4	HWYPEKPYKGSGFRC (RCIH) IHI	2742.35	2742.3270
4	KYEGHWYPEKPYKGSGFRCIHI (dimmer)	5386.64	5386.6256

On the other hand, 8 kinds of fragment peptides were identified in the reaction mixture including HSA (Figure 3 and Table 2). These data indicated that JAL-AK22 might possess the auto-proteolytic activity. Interestingly, the decreasing ratio of original JAL-AK22 was higher than that of JAL-AK22 alone (Figure S2). It suggests that the auto-proteolytic activity might be enhanced in the presence of HSA. The cleavage sites of JAL-AK22 in this reaction are summarized in Figure 11.

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Taken together, we concluded that JAL-AK22 has proteolytic activity and could activate proMMP-7 through proteolytic removal of the prodomain region [6].

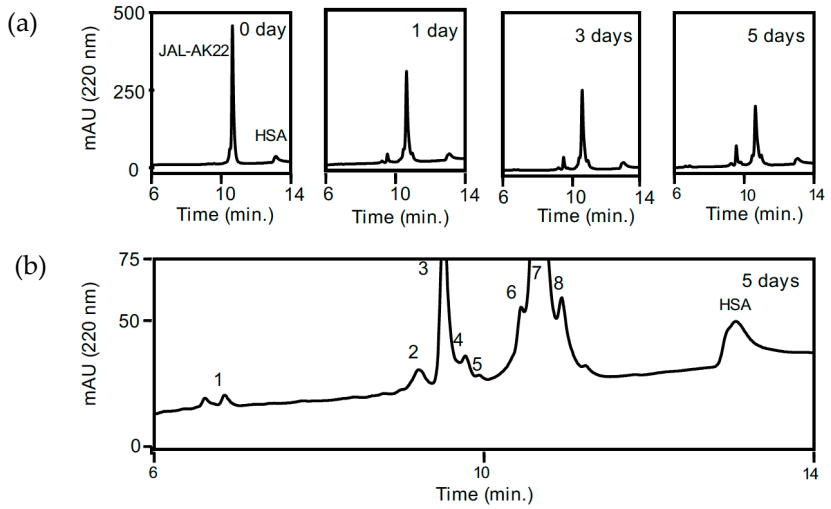

Figure 3. Identification of auto-proteolysis activity of JAL-AK22 with HSA (a) time dependent analysis (b) peak collection

Table 2. Identification of fragment peptides of JAL-AK22 with HSA

Peak	Fragment	Theoretical MS	Experimental MS
1	KYEGH	632.29	631.3216
2	KYEGHWYPEKPYK	1723.84	1722.9005
2	KYEGHWYPEKPYKGS	1867.89	1866.8650
3	KYEGHWYPEKPYKGSGFR	2228.08	2228.1402
4	KYEGHWYPEKPY	1595.74	1595.7788
4	YPEKPYKGSGFRC (SGFRC) I	2210.05	2210.1325
5	EKPYKGSGFRC (KPYKGSGFRCIHI) IHI	3136.64	3136.8076
6	HWYPEKPYKGSGFRC (RCIH) I	2742.35	2742.3695
7	YEGHWYPEKPYKGSGFRC (RC) I	2591.20	2591.3565
7	KYEGHWYPEKPYKGSGFRCIHI (dimmer)	5386.64	5386.7384
8	KYEGHWYPEKPYKGSGFRC (CIHI) IHI	3176.57	3176.6497

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2.2. Identification of proteolytic peptide, JAL-TA9

2.2.1. Auto-proteolytic activity

MS analysis of JAL-AK22 indicated that the Cys residue containing fragment peptides produced by auto-protelytic activity formed the homo/hetero dimers (Table 1 and 2). This reaction is not suitable to identify the proteolytic activity by MS analysis, thus we planed to determine the minimum sequence required for the auto-proteolytic activity. The additional 9 peptides were synthesized and examined the auto-proteolytic activity in the same manner (Figure S2). JAL-TAK22 (KYEGHWYPEKPYKGSGFRMIHI), substituted Cys residue to Met, showed the similar auto-proteolytic activity to JAL-AK22. In addition, JAL-TAY11 (YKGSGFRMIHI) showed more potent than JAL-AK22. The proteolytic activities of the shorter peptides comparison of JAL-TA9 (YKGSGFRMI) were low. As a result, a 9-mer peptide termed JAL-TA9 was found to show the highest auto-proteolytic activity and further investigated for its proteolytic activity (Figure S3b).

The optimal reaction conditions for JAL-TA9 were analyzed by monitoring the auto-proteolytic activity. JAL-TA9 showed potent auto-proteolytic activity between pH 7.0 and 7.5 in the presence of HSA in phosphate buffer (Figure S4a). Judging from the effects of various buffers including PBS, Tris-HCl (pH 7.5), phosphate buffer (pH 7.5), assay buffer (pH 7.5) [1, 3], PBS was selected for the following experiments (Figure S4b). Among the temperatures tested (4 °C, room temperature, 37 °C and 70 °C), the maximum activity was observed at 37 °C in PBS (pH 7.4) (Figure S4c).

JAL-TA9 (final conc., 0.2 mM) was incubated alone in PBS at 37 °C, and the reaction mixture was analyzed for 5 days by HPLC. The level of JAL-TA9 was decreased in time dependent manner, along with the formation of several peaks in the HPLC chromatogram (Figure 4a). On day 5, we corrected the 10 peaks (J1 - J10) including JAL-TA9 (J9) (Figure 4b) and identified 8 peptides and 1 amino acid as derivatives from JAL-TA9 (Table 3).

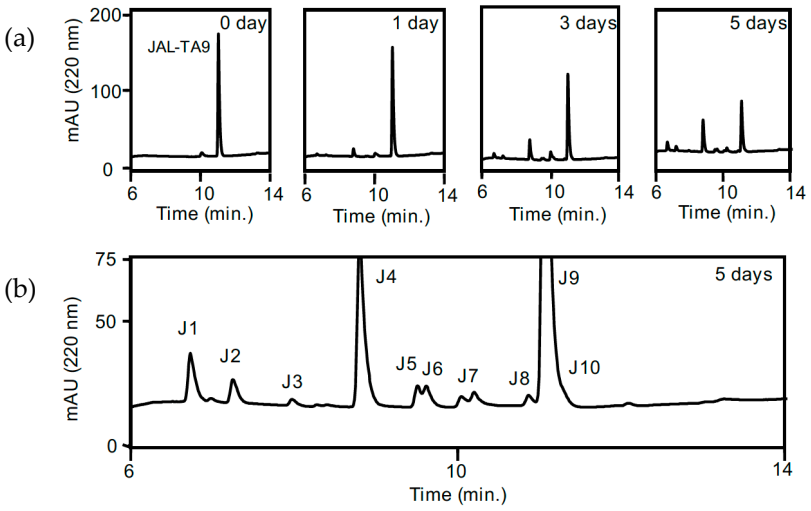

Figure 4. Identification of auto-proteolysis activity of JAL-TA9 (a) time dependent analysis (b) peak collection

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Table 3. Identification of fragment peptides of JAL-TA9

Peak	Fragment	Theoretical MS	Experimental MS
J1	YKGSG	510.24	510.2548
J2	Y	181.19	181.0511
J3	FR	321.18	321.1800
J4	YKGSGFR	813.41	813.4260
J5	YKGSGF	657.31	657.3236
J6	MI	262.14	262.1452
J7	YKGSGFRM	944.45	944.6563
J8	KGSGFRMI	894.47	894.4824
J9	YKGSGFRMI	1057.54	1057.5705
J10	FRMI	565.30	565.3011

We next examined the auto-proteolytic activity of JAL-TA9 in the presence of HSA (final conc., 0.025 % w/v). The reaction mixture was analyzed every 1 hr for 6 hrs by HPLC (Figure 5a). As expected, similar to JAL-AK22, the auto-catalytic activity of JAL-TA9 was enhanced in presence of HSA (Figure S2b and c). The level of JAL-TA9 was decreased, along with the formation of several peaks in the HPLC chromatogram (Figure 5a). After 1 hr of incubation, the peak corresponding to JAL-TA9 had decreased and almost disappeared after 6 hrs, with the concomitant detection of several peaks in the chromatogram (Figure 5b). MS analysis revealed that the new peaks corresponded to new peptides derived from JAL-TA9 (Table 4). The cleavage sites of JAL-TA9 in the presence or absence of HSA are summarized in Figure 11. The specificity for these cleavage sites appeared to be less strict and could not be defined definitively.

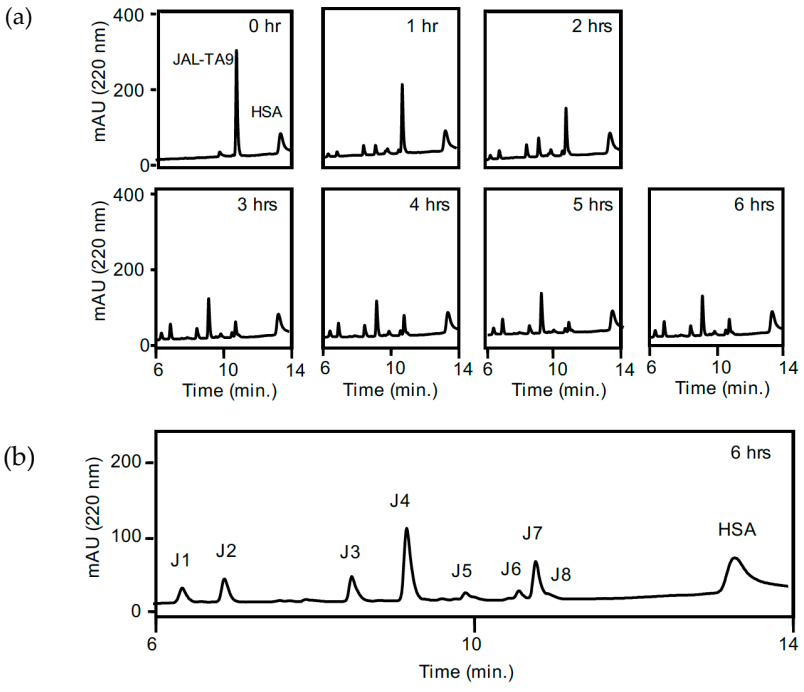

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Figure 5. Identification of auto-proteolytic activity of JAL-TA9 with HSA (a) time dependent analysis (b) peak collection

Table 4. Identification of fragment peptides of JAL-TA9 with HSA

Peak	Fragment	Theoretical MS	Experimental MS
J1	YKGSG	510.24	510.2548
J2	RMI	148.24	418.2364
J3	KGSGF	494.25	494.2612
J4	YKGSGF	658.32	658.3178
J5	YKGSGFRM	944.45	944.4804
J6	KGSGFRMI	894.47	894.4902
J7	YKGSGFRMI	1057.54	1057.5651
J8	GFRMI	622.33	622.3900

Furthermore, we evaluated the auto-proteolytic activity of JAL-TA9 after filtration of the reaction mixture to deny that these phenomena were due to the contamination of some bacteria or another hydrolytic enzymes. The reaction mixture including HSA was filtered using MILLEX-GV (Millipore, 0.22 μm Filter Unit) and TERUMO Syringe (TERUMO, 26G, 1mL) to remove contaminates and then analyzed by the same manner to the reaction mixture without filtration. The chromatograms obtained after incubation for 1 day were the same to those obtained without filtration (Figure S5). These data proved that the auto-proteolytic activity of JAL-TA9 was not due to contamination of bacteria.

In the case of incubation with 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), a serine protease inhibitor, the fragment peptide produced by auto-proteolysis of JAL-TA9 was not appeared on the chromatogram (Figure 6). Peak C in Figure 6b was identified as a complex with JAL-TA9 by MS/MS analysis (Figure 7). The peaks marked with a star (*) were not peptides. These data clearly indicated that JAL-TA9 also possessed proteolytic activity and cleaved its own sequence, suggesting that JAL-TA9 can act as a serine protease [9-11].

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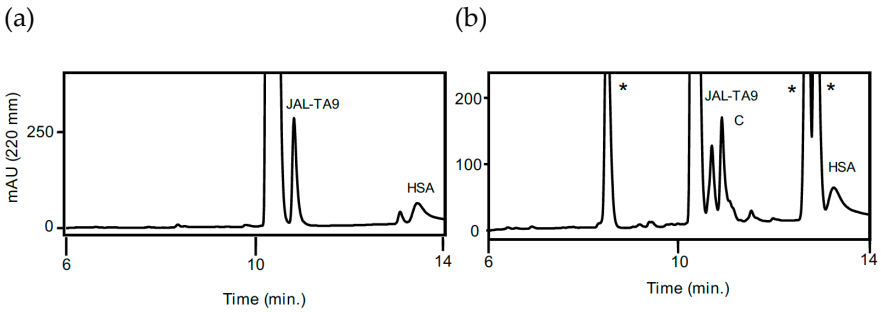

Figure 6. Chromatograms of the reaction mixture with AEBSF (a) day0 (b) day1, C: YKGS (AEBSF) GFRMI, Theoretical MS: 1240.76, Experimental MS: 1240.6283

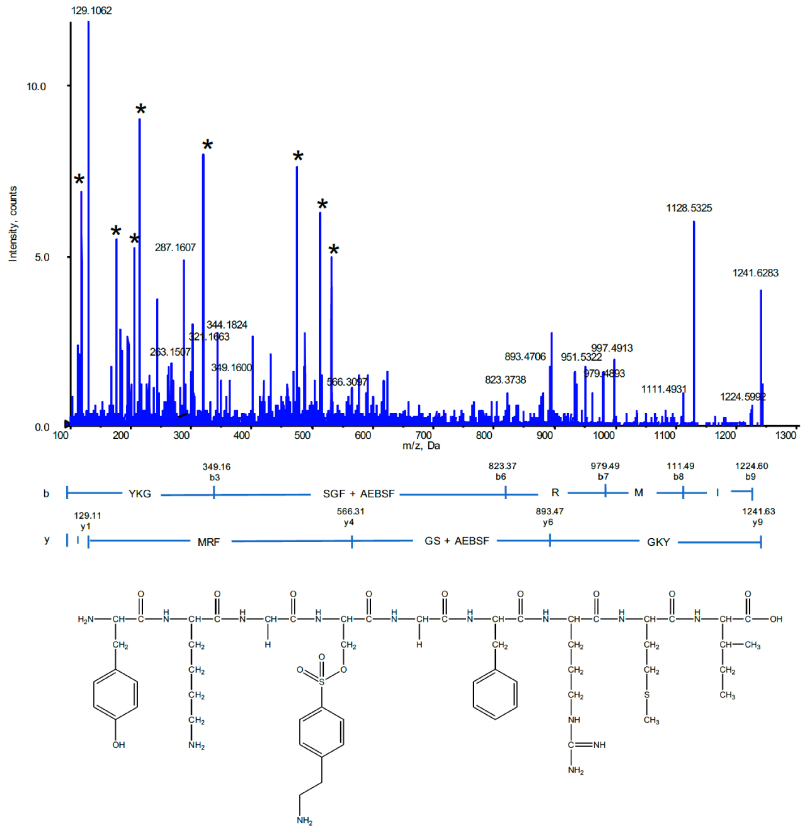

Figure 7. MS/MS analysis of the complexes with JAL-TA9 and AEBSF

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2.2.2. Proteolysis of the fragment peptides derived from the prodomain of MMP-7

We next examined the proteolytic activity of JAL-TA9 against the fragment peptides derived from the prodomain of MMP-7, which contained the cleavage site during the activation of proMMP-7 *in vivo*. Two synthetic fragment peptides derived from the prodomain of MMP-7 (Figure S1b), MMP18-33 (LPLPQEAGGMSELQWE) and MMP18-40 (LPLPQEAGGMSELQWEQAQDYLK), were incubated with JAL-TA9 in the presence of HSA under similar conditions to those described in Figure 3.

Judging from the chromatograms obtained by time dependent analysis, MMP18-33 was stable for 3 days with HSA in PBS (Figure 8a). On the other hand, many peaks were appeared after 1 day incubation in the presence of JAL-TA9 (Figure 8b). To identify these peaks, each peak was collected (Figure 8c) and 6 peaks (M1-6) were identified to the derivatives from MMP18-33 (Table 5). MMP18-40 was also examined according to the same manner, and 7 derivative peptides were identified (Figure 7S). Their cleavage sites were determined (Figure 11).

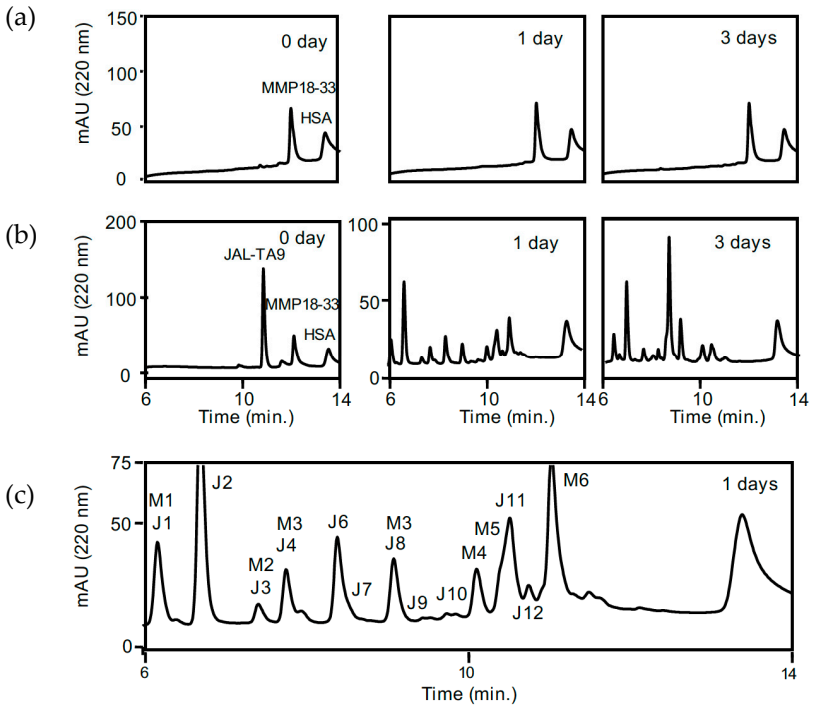

Figure 8. Proteolytic cleavage of MMP18-33 by JAL-TA9 (a) MMP18-33 alone (b) MMP18-33 with JAL-TA9 (c) peak collection

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Table 5. Identification of fragment peptides of MMP18-33

Peak	Fragment	Theoretical MS	Experimental MS
M1	SEL	347.17	347.2215
M2	EAGGM	463.17	463.2255
M3	LPQ	356.21	356.3482
M4	LPLPQ	566.34	566.4081
M5	SELQWE	790.35	790.4393
M6	LPLPQEAG	710.36	709.4516
M6	LPLPQEA	766.42	766.4808

We also evaluate the proteolytic activity of JAL-TA9 after filtration of the reaction mixture with HSA as well as the auto-proteolytic activity (Figure 5S). The chromatograms profiles obtained time dependent analysis were the same to those obtained without filtration (Figure 9). These data indicate that the proteolysis of MMP18-33 was not caused by the contamination of some bacteria but caused by JAL-TA9.

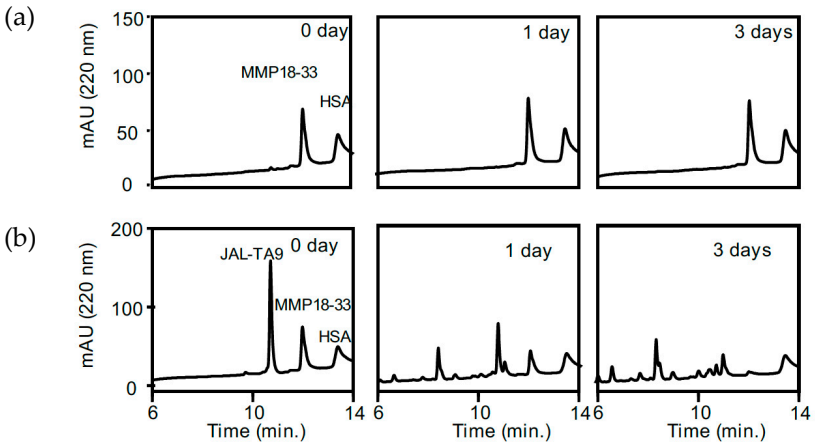

Figure 9. Proteolytic activity of MMP18-33 by JAL-TA9 with filtration (a) MMP18-33 alone (b) MMP18-33 with JAL-TA9

We next examined the inhibitory activity of AEBSF against cleavage reactions (Figure 10). MMP18-33 and MMP18-40 were incubated with JAL-TA9 in the presence of AEBSF, and the reaction mixtures were analyzed by HPLC. On the basis of MS analyses, C1 and C2 in Figure 10b were

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identified as the complex of JAL-TA9 and AEBSF, and C3 was identified as the complex of AEBSF and MMP18-40 (Figure S8). The peaks marked with a star (*) were not peptides and might be the AEBSF products. These data indicated that cleavage reactions of proMMP-7 fragment peptides by JAL-TA9 were inhibited by AEBSF, similar to the auto-proteolysis (Figure 6).

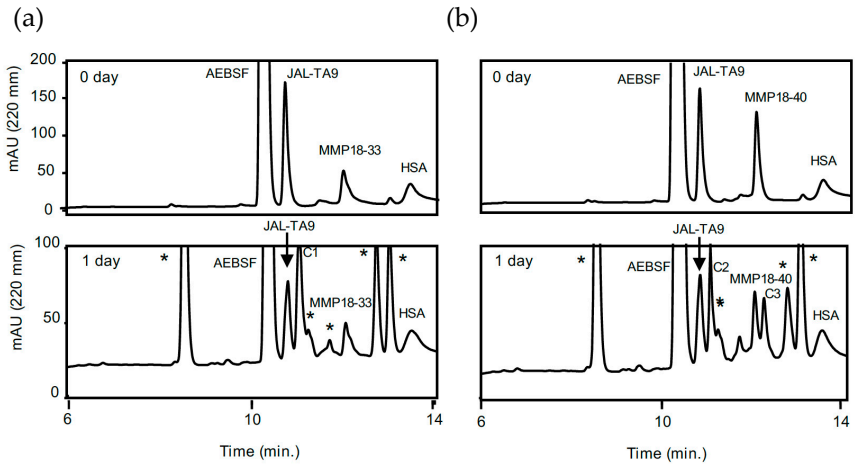

Figure 10. Proteolytic cleavage of proMMP-7 fragment peptides by JAL-TA9 in the presence AEBSF (a) MMP18-33 (b) MMP18-40

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As shown in Figure 11, many cleavage sites by auto-proteolysis were identified with or without HSA. In addition, N-terminus regions of both synthetic peptides were cleaved at preferential sites, suggesting some specificity. However, the specificity did not appear to be absolute and thus could not be defined definitively, as in the case of auto-proteolysis.

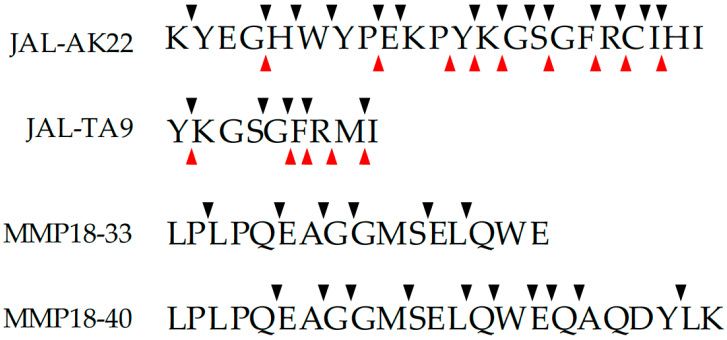

Figure 11. Comparison of cleavage sites in the presence (▼) or absence of HSA (▲)

The *K*m and Vmax values of the cleavage reactions of these peptides by JAL-TA9 were 0.17 mM and 0.55 nmol/hr against MMP18-33 and 0.15 mM and 0.78 nmol/hr against MMP18-40, respectively (Figure 12 and S8). These values were lower than those of known protein enzymes such as chymotrypsin probably due to the absence of a substrate binding site.

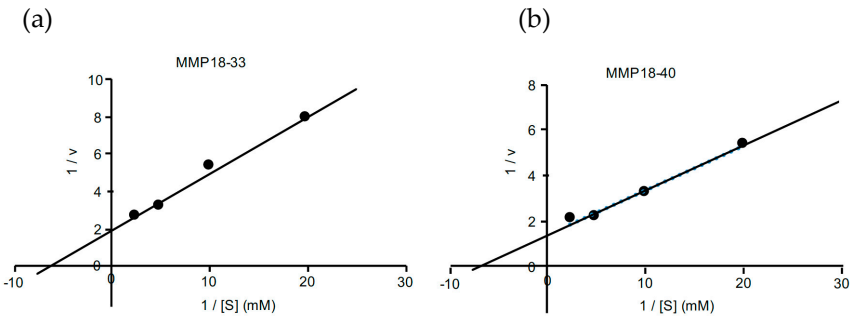

Figure 12. Lineweaver-Burk plot (a) MMP-18-33 (b) MMP-18-40

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242 **3. Discussion**

243 It is the general conception that proteolytic activity is the peculiar to a protein. We also did not
244 have conviction about the existence of proteolytic peptide, therefore we started this project to find a
245 chaperon peptide which could refold the unfolded proMMP-7. In this study, we identified the
246 synthetic peptide possessing the proteolytic activety. It is very exciting and amazing. The high rate
247 of this reaction shows that JAL-TA9 had high activity, potentially higher than that of JAL-AK22,
248 which had not completely degraded even after 5 days of incubation (Figure 3 and 5).

249 The most important question is an apprehension about the contamination of some bacteria. The
250 proteolytic activity obtained from the reaction mixture after filtration proved that the proteolysis
251 was not caused by the contamination of some bacteria but caused by JAL-TA9 (Figure 5S and Figure
252 9). In addition, JAL-TA9 showed the proteolytic activity after boiling with hot water or treatment
253 with organic solvents such as CH_3CN, CH_3OH, DMSO and TFA (data not shown). These data
254 supported the existence of the proteolytic activety of JAL-TA9 itself.

255 The aim of using HSA at first is to monitor the cleavage reaction against the major protein in
256 blood. We also examined the proteolytic activity of JAL-TA9 against 5 native proteins, γ-globulin
257 (γ-G), rabbit immuno-globulin G (IgG), cytochrome C (Cyt-C), lysozyme (Lys) and HSA, by
258 SDS-PAGE with silver staining and HPLC, and none were cleaved by JAL-TA9 (Figure S10). These
259 data suggest that JAL-TA9 may not display serious side effects. Interestingly, HSA enhanced the
260 auto-proteolytic activity of JAL-AK22 and JAL-TA9. However, the effect of HSA is not well
261 understood, but may involve the conformational stabilization of JAL-AK22 and JAL-TA9. AEBSF
262 inhibits the proteolytic activity of JAL-TA9 [9], suggesting the special conformation composed of
263 catalytic triad to show the serine protease-like activity [10, 11]. The conformational study by NMR
264 supported the formation of catalytic triad [12]. This conformation may be very flexible and stabilized
265 by the interaction of HSA. The kinetic parameters indicated that the proteolytic activety of JAL-TA9
266 is weaker than that of protein enzyme such as chymotrypsin due to lack of binding site (Figure 12).
267 In contrast, JAL-TA9 is easy to handle comparison of protein enzyme because of its stability.

268 Although Tob/BTG proteins are reported as the unstable proteins and difficult to purify
269 probably due to their instability [5], whether any Tob protein itself has proteolytic activity remains
270 unknown. Taken together our finding, it is suggested that Tob/BTG proteins may have proteolytic
271 activity. In addition, our study suggests the possibility that other peptide enzymes derived from
272 degradable and unstable proteins could inform research strategies not only in the field of
273 enzymology but also in molecular biology. The remaining important question is what the substrate
274 of JAL-TA9 is. We now almost finish the investigation of the substrate specificity [13] and
275 structure-activity relationship [14].

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4. Materials and Methods

4.1 Chemical synthesis of peptides

Peptides were synthesized from Fmoc-protected L-amino acid derivatives according to the method described by Kojima *et al* [15]. using an automated peptide synthesizer (model 433A, Applied Biosystems, California, U. S. A., 0.1 mmol scale with preloaded resin). After deprotection according to the manufacturer's protocol, each peptide was purified using reversed-phase HPLC (Capcell Pak C18 column, SG, 10 or 15 mm i.d. x 250 mm; Shiseido Co., Ltd. Japan) with a linear elution gradient from 0.1 % trifluoro acetic acid (TFA) to 50 % or 70 % CH_3CN containing 0.1 % TFA over 30 min. The flow rate was set at 3.0 or 6.0 mL/min. The primary peak fractions were collected and then lyophilized. The purity of the synthetic peptides and the progress of the enzymatic reaction were confirmed by analytical reversed-phase HPLC (Capcell Pak C18 column, MGII, 4.6 mm i.d. x 150 mm; Shiseido Co. Ltd., Japan) at a flow rate of 1.0 mL/min with a linear elution gradient from 0.1 % TFA to 70 % CH_3CN containing 0.1 % TFA. The column eluate was monitored with a photodiode-array detector (SPD-M20A; Shimadzu, Japan). Each purified peptide was characterized by ESI-MS using a Qstar Elite Hybrid LC-MS/MS system [15].

4. 2 Preparation of recombinant MMP-7 and identification of proteolytic activity

The recombinant human proMMP-7 was prepared according to our previous report [1]. Briefly, proMMP-7 was overexpressed in *Escherichia coli* as a recombinant zymogen (31 kDa), the C-terminus of which bears artificial hexa-histidines. The insoluble fraction of the *E. coli* extract including u-proMMP-7 was purified in a single step using Ni-NTA resin after solubilization of the precipitates with 8 M urea. The resin-bound recombinant protein was refolded into a f-proMMP-7 by reducing the urea concentration in a stepwise manner. The f-proMMP-7 is activatable by *p*-amino-phenylmercuric acetate (APMA) or trypsin in an autocatalytic manner. The proteolytic activity of the actMMP-7 was confirmed by a microplate reader assay using the fluorescence quenching substrate, 7-methoxycoumarin-4-yl-acetyl-Pro-Leu-Gly-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2 (3163-v: Peptide Institute, Osaka, Japan). Briefly, 60 µL of assay buffer for MMP-7 (final conc.: 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 10 mM $CaCl_2$; 5 µM $ZnCl_2$; 0.05 % Brij-35; 3 mM NaN_3) containing an appropriate amount of MMP-7 (30 µL) and 20 µL of a 1 mM peptide solution (final conc., 0.2 mM) was preincubated in each well of a 96-well microplate at 37 °C for 15 min, and then 180 µL of the fluorescence quenching substrate (3163-v, final conc., 2.5 µM) was added to initiate the proteolytic reaction. The fluorescence intensity was measured at 320 nm for excitation and 390 nm for emission every 15 min for 2 hrs or every 30 min for 6 hrs without interrupting the reaction using a fluorescence microplate reader (FP8300, JASCO, Tokyo, Japan). To identify the proteolytic activity of JAL-TA9, u-proMMP-7 or f-proMMP7 was used in place of actMMP-7 (Scheme 1).

4. 3 Analysis of proteolytic activity and determination of cleavage sites

JAL-TA9 (final conc., 0.2 mM) was individually incubated with or without the fragment peptide derived from the prodomain of MMP-7 (final conc., 0.05 mM) in the presence or absence of human serum albumin (HSA) (final conc., 0.025 % w/v) in PBS (pH 7.4) at 37 °C. A portion of the reaction mixture was analyzed in a time-dependent manner on the analytical HPLC system described above. The peak fractions monitored at 220 nm were collected into microtubes (Eppendorf Safe-Lock Tubes, 1.5 mL).

After lyophilization, the appropriate quantity of 36 % CH_3CN containing 0.1 % HCOOH was determined based on the chromatographic peak height and added with stirring by an automatic mixer. The cleavage site were determined by ESI-MS using the flow injection method with 70 % CH_3CN containing 0.1 % HCOOH on a Qstar Hybrid LC-MS/MS system (ABI). The flow rate was set at 0.1 mL/min [15].

4.4 Kinetic parameters

The enzymatic activity of JAL-TA9 was measured using MMP18-33 or MMP18-40 as substrates at 4 different final concentrations (50, 100, 200 and 400 μM). JAL-TA9 was incubated with MMP18-33 or MMP18-40 in the same manner as in the determination of the proteolytic activity described above. The reaction mixture (10 μL) was analyzed on an analytical HPLC at the initial time and after 24 hrs of incubation. The peak heights of MMP18-33 and MMP18-40 gave linear fits on the Lineweaver-Burk plots.

5. Conclusions

JAL-TA9 possesses serine protease-like activity [9-11] and activates proMMP-7 by cleaving the prodomain region. To the best of our knowledge, protease-like activity has never been reported in such small synthetic peptides, and we therefore propose that these peptides with protease-like activity, such as JAL-TA9, be called Catalytides (catalytic peptides). We expect that our findings will stimulate the development of novel Catalytides and related applications.

6. Patents

T. Yamamoto, T. Akizawa 2016 NOVEL PEPTIDE Patent Pending No US62/275,599, and 2016 NOVEL HYDROLASE-LIKE PEPTIDE AND ITS USE Patent Pending No JP2016-068496

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: Amino acid sequences of Tob1 and MMP-7, S2: Auto-proteolytic activity of JAL-AK22 and JAL-TA9, S3: Structure-activity relationship of JAL-TA9 derivative peptides, S4: Determination of the optimal conditions for the auto-proteolytic activity of JAL-TA9, S5: Auto-proteolytic activity of JAL-TA9 with or without filtration, S6: Cleavage reactions of MMP-7 fragment peptides by JAL-TA9, S7: Identification of cleavage sites , S8: Identification of the complex with JAL-TA9 and AEBSF, S9: Kinetic parameters, S10: Proteolytic activity of JAL-TA9 against native proteins.

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Author Contributions: T.A. were responsible for experimental design and data interpretation. T.A. and R.N. mainly contributed to writing and reversing the manuscript. M.T. was conducted screening of proteolytic peptide. R.N. and A.K. were conducted all experiments, especially HPLC analysis and determination of cleavage sites. R.N., M.K. and Y.H. were contributed to MS analysis. M.K. and R.N. analyzed JAL-TA9 conformation by Computer modeling.

Conflicts of Interest: The authors declare no conflict of interest.

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