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**The Optimal Activity of a Pseudozymogen Form of Recombinant  
Matriptase under the Mildly Acidic pH and Low Ionic Strength  
Conditions**

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Running head: Activity of Matriptase Zymogen

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Abbreviations: CMK, chloromethylketone; HGF, hepatocyte growth factor; MCA,  
4-methyl-coumaryl-7-amide; r-EK, recombinant enterokinase

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## ABSTRACT

Matriptase is a transmembrane serine protease that is strongly expressed in epithelial cells. The single-chain zymogen of matriptase is considered to have inherent activity, leading to its own activation (*i.e.*, conversion to the disulphide-linked-two-chain form by cleavage after Thr–Lys–Gln–Ala–Arg<sup>614</sup>). Also, there is growing evidence that the activation of zymogen occurs at the cell surface and in relation to the acidification and lowering of ionic strength within cell-surface microenvironments. The present study aimed to provide evidence for the involvement of zymogen activity in its activation in physiologically relevant cellular contexts. For this purpose, the activity of a pseudozymogen form of recombinant matriptase (HL-matriptase zymogen) was examined using acetyl-L-Lys–L-Thr–L-Lys–L-Gln–L-Leu–L-Arg–4-methyl-coumaryl-7-amide as a substrate. HL-matriptase zymogen exhibited optimal activity toward the substrate around pH 6.0. The substrate hydrolysis at the pH value was hardly detected when NaCl was present at a concentration of 145 mM. In a buffer of pH 6.0 containing 5 mM NaCl, the activity of HL-matriptase zymogen was only about 30-times lower than that of the respective two-chain form. These findings suggest that the *in vivo* activation of matriptase zymogen occurs *via* a mechanism involving the zymogen activity.

Keywords: activation cleavage, matriptase, pH and ionic strength dependences, type II transmembrane serine protease, zymogen activity

## INTRODUCTION

Matriptase (also known as epithin, membrane-type serine protease 1, SNC19, suppression of tumorigenecity 14, *etc*) is a member of the type II transmembrane serine protease group, which is characterized by the N-terminal cytoplasmic domain followed by a signal anchor transmembrane domain and the extracellular domain, including a serine protease catalytic domain at the C-terminus (Fig. 1) (1–7). Matriptase is first synthesized as a zymogen comprising 855 amino-acid residues in human, mouse and rat enzymes, which requires processing by cleavage between Arg614 and Val615 (activation cleavage) to generate the disulphide-linked-two-chain fully active enzyme (Fig. 1) (1, 2, 7–9). Two-chain matriptase exhibits activity with trypsin-like specificity, and is known to cleave and activate a number of proteins, including single-chain urokinase-type plasminogen activator and pro-hepatocyte growth factor (HGF) (10–12). The potential substrates, together with the abundant expression in simple epithelial cells such as kidney tubular cells and in keratinocytes (7, 13, 14), suggest that matriptase plays a key role in the establishment and maintenance of epithelial integrity. Indeed, analyses using matriptase knockout mice have indicated the importance for postnatal survival, epidermal barrier function, hair follicle development and thymic homeostasis (15).

Several lines of evidence have indicated that the activation cleavage of matriptase zymogen occurs through a mechanism requiring its own catalytic triad (His656, Asp711 and Ser805) (1, 2, 8, 16–20). Autoactivation where a zymogen is activated by an intramolecular cleavage seems improbable in this protease zymogen, because the catalytic triad is sterically inaccessible to its own activation-cleavage site (16, 17). Rather, the zymogen molecules have been postulated to undergo transactivation where a zymogen interacts with another zymogen, resulting in activation cleavage of each zymogen (1, 17). In either intra- or intermolecular mechanisms of activation cleavage, matriptase zymogen should exhibit catalytic activity. We have found that a pseudozymogen form of rat r-matriptase (designated as HL-matriptase zymogen, refer to Fig. 1) hydrolysed a synthetic peptide substrate, methylsulphonyl-D-cyclohexyl-Tyr-Gly-L-Arg-p-nitroanilide acetate named Spectrozyme tPA<sup>®</sup> (Sp-tPA) (20). This was the first *in vitro* evidence for the catalytic competence of matriptase zymogen. However, it remained uncertain whether hydrolysis of Sp-tPA is indicative of the activation cleavage of matriptase zymogen.

The activation of matriptase zymogen appears to occur in the extracellular environment (*i.e.*, at the cell surface) but not in the intracellular environment. This is evidenced by the observations that (i) immunoreactive two-chain matriptase molecules were detected at cell-cell contacts of a human mammary epithelial line 184 A1N4 treated with sphingosine 1-phosphate (21); (ii) two-chain molecules occur on the cell surface but not in the intracellular environment in monkey kidney COS-1 cells transfected with a full-length rat matriptase cDNA (20); and (iii) no immunoreactive

two-chain molecules were detected in the intracellular compartments of intact epithelial cells (*e.g.*, ductal epithelial cells of the kidney) (22). However, the arrival of matriptase zymogen at the cell surface does not appear to be sufficient for the triggering of the activation. For instance, an *in vitro* study using homogenates of 184 A1N4 cells revealed that matriptase zymogen underwent activation in solutions with mildly acidic pH values [*e.g.*, in sodium phosphate-citric acid (pH 6.0) buffer] but rarely in those with neutral and slightly alkaline pH values [*e.g.*, in sodium phosphate-citric acid (pH 7.4) buffer] (23). In addition, when quiescent 184 A1N4 cells (where only matriptase zymogens were detected) were exposed to phosphate buffer pH 6.0, the activation was strikingly induced (22). Ionic strength also appears to affect the activation of zymogen. For instance, the activation was no longer seen in sodium phosphate-citric acid (pH 6.0) buffer containing 140 mM NaCl (23). These findings suggest that the activation of matriptase zymogen occurs in relation to the acidification and lowering of ionic strength at the surface microenvironment of living cells. However, there is no direct evidence that the *in vivo* activation of matriptase zymogen occurs *via* a mechanism involving the activity of zymogen.

The purpose of the present study is to provide definitive evidence that the activity of matriptase zymogen is involved in its activation in physiologically relevant cellular contexts. This could be indicated if HL-matriptase zymogen exhibited substantial activity under mildly acidic and low ionic strength conditions. In the present study, we examined the activity of the pseudozymogen using Acetyl-L-Lys-L-Thr-L-Lys-L-Gln-L-Leu-L-Arg-4-methyl-coumaryl-7-amide (MCA) (hereinafter abbreviated as

Ac-KTKQLR-MCA) as a substrate. Ac-KTKQLR-MCA is a substrate for proteases with pro-HGF-converting activity (24) and was found to be hydrolysed by an activated form of HL-matriptase zymogen (HL-matriptase enzyme, Fig. 1) (12, 25). Importantly, the amino-acid sequence of this peptidyl-MCA substrate almost matches the P5 to P1 residues of matriptase zymogen: P5(Thr)–P4(Lys)–P3(Gln)–P2(Ala)–P1(Arg) (Fig. 1). The hydrolysis of the substrate is therefore expected to mimic the situation of activation cleavage of matriptase zymogen. In the present study, HL-matriptase zymogen was found to exhibit substantial activity toward the substrate in solutions with mildly acidic pH values and low ionic strength. The finding suggests that the *in vivo* activation occurs *via* a mechanism involving the activity of zymogen.

## MATERIALS AND METHODS

*Materials* – Ac-KTKQLR-MCA was purchased from Peptide Institute (Osaka, Japan). A bovine r-enterokinase (Recombinant Enterokinase<sup>®</sup>) (r-EK) and an immobilized S-protein (S-protein<sup>®</sup> agarose) were purchased from Novagen (Madison, WI). A bovine trypsinogen and a streptavidin conjugated with horseradish peroxidase were from Sigma (St. Louis, MO). Biotinylated EGR-chloromethylketone<sup>®</sup> (L-glutamyl–L-glycyl–L-arginine–chloromethylketone conjugated with biotin) was purchased from Haematologic Technologies (Essex Junction, VT). HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid), MES (2-morpholino-ethanesulphonic acid) and all other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto,

Japan).

*Preparation of HL-Matriptase Zymogen* – We have established a Chinese hamster ovary (CHO)-K1 line expressing a high level of HL-matriptase zymogen (Fig. 1) (26). The clonal line was cultured in a 75-cm<sup>2</sup> plastic flask (Asahi Techno Glass, Tokyo) as described previously (26). After reaching confluence, cells were washed three times with phosphate buffered saline (PBS, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 136 mM NaCl, 2.7 mM KCl, pH 7.4), and 10 ml of Ham's F12 without fetal bovine serum was added to the flask. After 48 h, the conditioned medium was collected, and fresh serum-free medium was added. This was repeated until half of the cells were peeled off. The collected media were centrifuged immediately at 3,000 g for 10 min at 22°C, and the resulting supernatants were stored at - 20°C until use. For purification, 300 ml of the conditioned media was collected using 3 flasks. After thawing, the media were pooled and concentrated to 2.5 ml by ultrafiltration using an Amicon<sup>®</sup> Ultra-15 membrane filter (50,000 MWCO, Millipore, Tokyo branch). The concentrated medium was subjected to gel filtration in a buffer [25 mM HEPES (pH7.5) containing 145 mM NaCl and 0.1% Triton X-100] (hereinafter called purification buffer) using a PD-10 column (GE Healthcare, Tokyo branch) with an elution volume of 3.5 ml. The gel filtrate was divided equally, and each part was transferred to a 2.0 ml microcentrifuge tube in which 62.5 µl slurry of immobilized S-protein was included. The tubes were incubated for 30 min at 22°C with rocking. After brief centrifugation, the supernatants were incubated again with immobilized S-protein as described above. The precipitated slurries were

pooled (250  $\mu$ l) and washed three times with 1.5 ml of purification buffer and twice with 1.5 ml of 10 mM HEPES (pH7.5) containing 5 mM NaCl and 0.01% Triton X-100 (hereinafter called elution buffer). To elute HL-matriptase zymogen molecules, the slurry washed was transferred with 500  $\mu$ l of elution buffer containing 200  $\mu$ M synthetic S-tag peptide (KETAAAKFERQHIDS, synthesized in BEX, Tokyo) to a spin column (Attoprep™, 0.22  $\mu$ m pore size, Atto, Tokyo, Japan) and centrifuged at 5,000 *g* for 1 min at 22°C. The eluate was subjected to gel filtration in elution buffer using an NAP-5 column (GE Healthcare) with an elution volume of 1.0 ml and stored at - 20°C until use.

The concentration of HL-matriptase zymogen was measured semiquantitatively as follows. A 20- $\mu$ l part of elution buffer containing HL-matriptase zymogen was mixed with 5  $\mu$ l of 5  $\times$  Laemmli protein sample buffer (Laemmli buffer) [1  $\times$  Laemmli buffer, 0.05 M Tris-HCl (pH6.8), 10% glycerol, 2% sodium dodecylsulfate (SDS) and 0.005% Bromophenol Blue with dithiothreitol at a final concentration of 12 mM] (27). Various concentrations of bovine trypsinogen (dissolved in distilled water) were also mixed with 5  $\times$  Laemmli buffer: the final concentrations, 17, 33, 67, 133 nM. The mixtures were boiled for 3 min. After cooling to 22°C, samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) followed by silver staining as described previously (20). After drying, the gels were photographed digitally, and the signal intensities of protein bands were analysed by densitometry as described previously (20). The concentration was calculated by comparing the signal density of a 90-kDa band (for HL-matriptase zymogen) with that of a 25-kDa band (for trypsinogen).

*Preparation of HL-Matriptase Enzyme* – To generate HL-matriptase enzyme (Fig. 1), a 500- $\mu$ l part of elution buffer containing 50 nM HL-matriptase zymogen was incubated for 24 h at 22°C with r-EK. The final concentration of r-EK was 1 unit/ml. After incubation, the solution (activation mixture) was stored at - 20°C until use. The concentration of HL-matriptase enzyme occurred in the activation mixture was evaluated by means of SDS-PAGE (12% polyacrylamide) under reducing conditions and Western blotting using a rabbit anti-matriptase catalytic domain antibody (Spr992) (28–30). Practically, the signal density of a 28-kDa band produced from the catalytic domain part of HL-matriptase enzyme was compared with that of a 90-kDa band produced from HL-matriptase zymogen (refer to Fig. 5A). We have confirmed that no 90-kDa band was produced after incubation of HL-matriptase zymogen with r-EK but that the molar concentration of HL-matriptase enzyme is similar to that of HL-matriptase zymogen.

*Hydrolysis of Ac-KTKQLR-MCA by HL-Matriptase Zymogen and Enzyme* – Ac-KTKQLR–MCA was dissolved in dimethylsulphoxide at the concentration of 20 mM and stored at - 20°C until use. Incubation was carried out at 37°C in a 0.5-ml microcentrifuge tube. The reactions were initiated by the addition of 0.5  $\mu$ l of Ac-KTKQLR-MCA to 39.5  $\mu$ l of buffers [*e.g.*, 105 mM MES-NaOH (pH 6.0) buffer containing 5 mM NaCl and 0.011% Triton X-100] containing 45 nM HL-matriptase zymogen or 3 nM HL-matriptase enzyme. The reaction mixtures containing

HL-matriptase zymogen and enzyme were incubated for 48 h and 8 h, respectively. To prevent water evaporation within the tube, the incubation was conducted using a temperature controller (PC805-MI, ASTEC, Fukuoka, Japan). The reactions were terminated by adding 400  $\mu\text{l}$  of 0.1 M monochloroacetic acid in 0.1 M sodium acetate (pH 4.3) buffer, and the absorbance at 370 nm of the product 4-methyl-coumaryl-7-amine was measured. The initial rate of the hydrolysis ( $v_0$ ) was determined using the molar absorption coefficient of  $7.7 \text{ mM}^{-1} \text{ cm}^{-1}$  of 4-methyl-coumaryl-7-amine. In the present study, r-EK was not removed from activation mixtures, because r-EK exhibited no activity toward Ac-KTKQLR-MCA.

*Determination of Kinetic Parameters for Hydrolysis of Ac-KTKQLR-MCA Catalysed by HL-Matriptase Zymogen and Enzyme* – The reactions were initiated by the addition of 2  $\mu\text{l}$  of elution buffer containing 900 nM HL-matriptase zymogen, the buffer containing 60 nM HL-matriptase enzyme, or the buffer alone to 38  $\mu\text{l}$  of buffers [100 mM MES-NaOH (pH 6.0) buffer containing 5.3 mM NaCl and 0.011% Triton X-100 or 100 mM HEPES-NaOH (pH 7.4) buffer containing 153 mM NaCl and 0.011% Triton X-100] containing Ac-KTKQLR-MCA (the final concentrations of the substrate: 0, 1.7, 3.4, 7.8, 15.6, 31.3, 62.5 and 125  $\mu\text{M}$ ). The reaction mixtures containing HL-matriptase zymogen and enzyme were incubated for 48 h and 8 h, respectively. The reactions were terminated, and the absorbance at 370 nm was measured as described above. In order to evaluate the enzyme-catalysed reaction rate, the non-enzymatic spontaneous reaction rate of absorbance increase was subtracted from the reaction rate observed in the

presence of the enzyme. The Michaelis constant ( $K_m$ ) and the rate of maximum turnover ( $k_{cat}$ ) for hydrolysis of Ac-KTKQLR-MCA were determined by fitting the rate data to the Michaelis–Menten equation using KaleidaGraph (Synergy Software, Reading, PA).

*Labelling with a Biotinylated Peptide-Chloromethylketone* – A 2- $\mu$ l part of elution buffer containing 50 nM HL-matriptase or 50 nM HL-matriptase enzyme was mixed with 38  $\mu$ l of buffer containing 25 nM biotinylated EGR-chloromethylketone<sup>®</sup>, and the reaction mixtures were incubated for 3 h at 37°C. After the addition of 5  $\mu$ l of 5  $\times$  Laemmli buffer, the reaction mixtures were boiled for 3 min. Samples were subjected to SDS-PAGE (12% polyacrylamide) followed by Western blotting. Blots were probed with Spr992 or a streptavidin conjugated with horseradish peroxidase.

*Inhibition Assay* – The reactions were initiated by the addition of 2  $\mu$ l of elution buffer containing 900 nM HL-matriptase zymogen or 60 nM HL-matriptase enzyme to 38  $\mu$ l of buffers [100 mM MES-NaOH (pH 6.0) buffer containing 5.3 mM NaCl and 0.011% Triton X-100] containing 125  $\mu$ M Ac-KTKQLR–MCA and biotinylated EGR-chloromethylketone<sup>®</sup> (concentration vary). The reaction mixtures containing HL-matriptase zymogen and enzyme were incubated for 48 h and 8 h, respectively. The reactions were terminated, and the absorbance at 370 nm was measured as described above.

## RESULTS

*Effects of pH on the Hydrolysis of Ac-KTKQLR-MCA Catalysed by HL-matriptase Zymogen and Enzyme* – HL-matriptase zymogen is a secreted variant of r-matriptase consisting of the entire extracellular domain (residues Tyr81 to Val855) (Fig. 1) (20, 26). In this variant, activation-cleavage sequence (Thr-Lys-Gln-Ala-Arg614) is replaced with the enterokinase recognition sequence (Asp-Asp-Asp-Asp-Lys) (Fig. 1). Therefore, HL-matriptase zymogen can be converted to HL-matriptase enzyme by incubation with r-EK (20, 26). In the present study, we used HL-matriptase zymogen and enzyme as models of matriptase zymogen and two-chain fully active matriptase, respectively.

Preliminary experiments indicated that HL-matriptase zymogen hydrolysed Ac-KTKQLA-MCA. In the present study, we first examined the effects of pH on the substrate hydrolysis by HL-matriptase zymogen and enzyme. Note that a non-ionic detergent, Triton X-100, was included in all buffers used in the present study. This is because r-matriptase variants, including HL-matriptase enzyme, display marked loss of activity in the absence of non-ionic detergents.

HL-matriptase zymogen and enzyme were reacted with the substrate in buffers with various pH values (pH 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) and at a fixed concentration of NaCl (5 mM). HL-matriptase zymogen hydrolysed Ac-KTKQLR-MCA with maximal activity at pH 6.0 (Fig. 2, left panel). The Ac-KTKQLR-MCA-hydrolysing activity of HL-matriptase enzyme increased with increasing pH (Fig. 2, right panel). In the buffer at pH 6.0 (hereinafter called MES buffer A), both HL-matriptase zymogen and enzyme

exhibited catalysis properties that fit in the Michaelis-Menten kinetic model. The  $k_{\text{cat}}$ ,  $K_{\text{m}}$  and  $k_{\text{cat}}/K_{\text{m}}$  values were shown in Table 1. It is noteworthy that  $k_{\text{cat}}/K_{\text{m}}$  value is only 27-times lower in the zymogen than in the enzyme.

*Effects of NaCl and KCl on the Hydrolysis of Ac-KTKQLR-MCA Catalysed by HL-matriptase Zymogen and Enzyme* – We next examined the effects of NaCl concentration on the Ac-KTKQLR-MCA hydrolysis by HL-matriptase zymogen and enzyme. They were incubated with the substrate in buffers at pH 6.0 with various concentrations of NaCl (5, 20, 40, 75, and 145 mM). The activity of HL-matriptase zymogen was drastically decreased with increasing concentration of NaCl (Fig. 3, left panel, white bars). It is noteworthy that the pseudozymogen exhibited poor activity in the buffer containing 145 mM NaCl. The activity of HL-matriptase enzyme decreased moderately with increasing concentration of NaCl (Fig. 3, left panel, black bars).

We also examined the effects of NaCl using a buffer at pH 7.4, a pH found in the extracellular milieu. Unlike in MES buffer A, NaCl had no apparent effects on the activity of HL-matriptase zymogen (Fig. 3, right panel, white bars). The activity of HL-matriptase enzyme decreased only in the buffer containing 145 mM NaCl (Fig. 3, right panel, black bars). The kinetic values for HL-matriptase zymogen and enzyme determined using the buffer at pH 7.4 containing 145 mM NaCl are shown in Table 1. The  $k_{\text{cat}}/K_{\text{m}}$  value of HL-matriptase zymogen was 220-times lower than that of HL-matriptase enzyme.

The effects of KCl were also examined. HL-matriptase zymogen and enzyme were incubated with Ac-KTKQLR-MCA in MES buffer A with various concentrations of KCl. Like NaCl, KCl drastically decreased the activity of HL-matriptase zymogen (Fig. 4, white bars). The activity of HL-matriptase enzyme decreased moderately with increasing concentration of KCl (Fig. 4, black bars).

*Binding and Inhibition of HL-matriptase Zymogen with a Biotinylated Peptide-Chloromethylketone* – Synthetic compounds such as peptide-chloromethylketones are known to bind covalently to the active site of serine proteases and to irreversibly inhibit them (31). For instance, a Glu-Gly-Arg-chloromethylketone conjugated with fluorescein has been found to bind to and inhibit a variety of blood coagulation and fibrinolytic serine proteases (32). If such a compound bound to and inhibited HL-matriptase zymogen, the formation of catalytic triad in the pseudozymogen would be indicated.

HL-matriptase zymogen and enzyme were incubated with biotinylated EGR-chloromethylketone<sup>®</sup> (abbreviated hereinafter as biotin-EGR-CMK). Samples were subjected to SDS-PAGE under reducing conditions and Western transfer, and the blot was probed with an antibody raised against catalytic domain of rat matriptase Spr992 (28). Samples of HL-matriptase zymogen and enzyme produced bands at the 90-kDa and 28-kDa positions, respectively (Fig. 5A, blot Spr992). The 90-kDa and 28-kDa bands have been found to represent HL-matriptase zymogen and the catalytic domain of HL-matriptase enzyme, respectively (26, 28, 29). Another blot was probed

with a streptavidin conjugated with horseradish peroxidase. Protein bands at the positions corresponding to 90-kDa and 28-kDa were also produced, although the signal intensity for the 90-kDa band was lower than that for the 28-kDa band (Fig. 5A, blot Avidin-HRP). In addition, the binding of streptavidin conjugated with horseradish peroxidase to biotin-EGR-CMK-untreated HL-matriptase zymogen was not substantial (see Supplementary Fig. 1). These results suggest that some but not all HL-matriptase zymogen molecules may form a catalytic triad analogous to His–Asp–Ser of trypsin-like serine proteases.

Biotin-EGR-CMK inhibited the hydrolysis of Ac-KTKQLR-MCA catalysed by HL-matriptase enzyme with  $IC_{50}$  value of 6  $\mu$ M (Fig. 5B). HL-matriptase zymogen was also inhibited with the compound (Fig. 5B). The  $IC_{50}$  value was calculated as 40  $\mu$ M.

## DISCUSSION

We have found that Sp-tPA is hydrolysed in solutions containing HL-matriptase zymogen (20). However, the pH dependence for hydrolysis of Sp-tPA is quite different from that for hydrolysis of Ac-KTKQLR-MCA. For instance, the hydrolysis of Sp-tPA was detected in solutions at pH more than 6.5 but rarely in solutions at pH less than 6.0 (20). We speculate that the hydrolysis of Sp-tPA in solutions containing HL-matriptase zymogen may be mostly due to a small number of HL-matriptase enzyme molecules that inadvertently occur during assay. Indeed, the pH dependence of HL-matriptase zymogen preparation was not drastically different from those of HL-matriptase enzyme

preparation (20). On the other hand, the hydrolysis of Ac-KTKQLR-MCA in solutions containing HL-matriptase zymogen appears to be mostly due to the pseudozymogen itself. Indeed, the pH and ionic strength dependences of HL-matriptase zymogen preparation are totally different from those of HL-matriptase enzyme preparation (Figs. 2–4). In addition, the pH and ionic strength dependences for the substrate hydrolysis are quite similar to those for the activation of matriptase zymogen in homogenates of 184 A1N4 cells (23). Altogether, we conclude that the assay using Ac-KTKQLR-MCA is more suitable than that using Sp-tPA for evaluating the activity of matriptase zymogen.

It has long been believed that trypsin-like zymogens can exist in two conformations: an inactive conformation and an active conformation (*i.e.*, a matured form-like conformation) (33–36). HL-matriptase zymogen also appears to acquire an active conformation. This is supported by the observation that biotin-EGR-CMK bound to the pseudozymogen (Fig. 5A). The active and inactive conformations in trypsin-like zymogens have been thought to be in equilibrium in favor of the inactive conformation (33–36). The equilibrium constant ( $K_{eq}$ ) can be estimated by comparing the catalytic activities of a zymogen and its respective matured enzyme toward certain substrates. For instance, by measuring  $k_{cat}/K_m$  values for hydrolysis of a peptidyl-amide substrate by trypsinogen and trypsin, the  $K_{eq}$  value for trypsinogen has been estimated to be on the order of  $10^8$  (36). Given the  $k_{cat}/K_m$  values for hydrolysis of Ac-KTKQLR-MCA by HL-matriptase zymogen and enzyme determined in MES buffer A, the  $K_{eq}$  value for the pseudozymogen can be estimated to be  $2.7 \times 10^1$ . This value is comparable to that for single-chain tissue-type plasminogen activator, which is known to have an anomalous

activity (34). Note that the  $K_m$  values for hydrolysis of Ac-KTKQLR-MCA were not substantially different between HL-matriptase zymogen and enzyme (Table 1). This suggests that the architecture of the substrate binding pocket of the pseudozymogen in the active conformation does not differ substantially from that of the enzyme.

Trypsin-like zymogens are activated by proteolytic cleavage at their activation motifs (Arg/Lys-Ile/Val). The  $\alpha$ -amino group of the new N-terminus (*e.g.*, Ile17 in trypsinogen; Val615 in matriptase zymogen) is believed to form a salt bridge with an Asp residue (Asp side chain carboxylate group), adjacent to active-site Ser residue, triggering a conformational change, which produces active enzyme (36). In single-chain urokinase-type plasminogen activator, an Lys residue (Lys320, amino-acid numbering starts from the initial Met residue) has been thought to form a salt bridge with the Asp residue (Asp375), adjacent to the active-site Ser residue (Ser376), to stabilize the active zymogen conformation in the absence of the free N-terminus (Ile179) (37). On the other hand, many of trypsin-like zymogens, including trypsinogen, have a non-basic amino acid in the position corresponding to Lys320 in the plasminogen activator (36, 37). This may explain very little or no intrinsic activities of them. It is noteworthy that matriptase zymogen has an His residue (His752) at the corresponding position (7–9). It is tempting to speculate that His752 in matriptase zymogen interacts with Asp804 for acquisition of the matured form-like conformation. In the present study, we found that at pH 6.0, HL-matriptase zymogen underwent inhibition by NaCl (and KCl) more strongly than did HL-matriptase enzyme (Figs. 3 and 4). One explanation for this is that the putative interaction between His752 and Asp804 in the zymogen form is more sensitive to ionic

strength than that between Val615 and Asp804 in the two-chain fully active form.

The arrival of matriptase zymogen at the cell surface appears to be necessary but not sufficient for activation. It has been thought that the acidification and the lowering of ionic strength within cell-surface microenvironments trigger the activation of matriptase zymogen (22, 23). Such a situation could arise when Na<sup>+</sup>/H<sup>+</sup> exchanger-1 is activated (38). It is tempting to speculate that the activation of matriptase zymogen is triggered in a situation where the exchanger is activated. It is also speculated that sphingosine 1-phosphate induces the activation of matriptase zymogen through a mechanism involving the activation of Na<sup>+</sup>/H<sup>+</sup> exchanger (39). The question arises as to why matriptase zymogen is unable to undergo activation in the intracellular environments. For instance, the lumens of Golgi apparatus and post-Golgi secretory vesicles are under mildly acidic conditions (40). Certain components included in the lumens of intracellular organelles are thought to inhibit the activation. In the present study, we found that KCl decreased the activity of HL-matriptase zymogen (Fig. 4). Potassium ion, fairly abundant in the intracellular environment (41), could be a candidate inhibitory factor.

In summary, we provided evidence that the *in vivo* activation of matriptase zymogen occurs *via* a mechanism involving the zymogen activity. This is supported by the optimal activity of HL-matriptase zymogen toward Ac-KTKQLR-MCA under mildly acidic and low ionic strength conditions. However, HL-matriptase enzyme exhibited the activity toward the substrate higher than did HL-matriptase zymogen. It is therefore likely that the two-chain matriptase molecules mediate the full-scale activation

of zymogen molecules in physiologically relevant cellular contexts. Regardless, the zymogen activity could be essential for the activation of matriptase zymogen in the sense that it triggers the generation of two-chain molecules.

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### **CONFLICT OF INTEREST**

None declared.

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Table 1

Kinetic values for hydrolysis of Ac-KTKQLR-MCA catalysed by HL-matriptase zymogen and enzyme

Protease	pH 6.0, 5 mM NaCl			pH 7.4, 145 mM NaCl		
	$k_{\text{cat}}$	$K_{\text{m}}$	$k_{\text{cat}}/K_{\text{m}}$	$k_{\text{cat}}$	$K_{\text{m}}$	$k_{\text{cat}}/K_{\text{m}}$
	min <sup>-1</sup>	μM	mM <sup>-1</sup> min <sup>-1</sup>	min <sup>-1</sup>	μM	mM <sup>-1</sup> min <sup>-1</sup>
HL-matriptase zymogen	0.1 ± 0.0	32 ± 9	3 ± 2	0.020 ± 0.003	48 ± 12	0.42 ± 0.35
HL-matriptase enzyme	1.4 ± 0.0	17 ± 1	82 ± 10	1.4 ± 0.0	15 ± 1	93 ± 13

The kinetic value is expressed as a mean ± SD of triplicate determinations.

## Figure legends

Fig. 1. **Schematic illustration of the structure of full-length rat matriptase and expression construct.** Matriptase is first synthesized as a single-chain zymogen comprising 855 amino-acid residues (Matriptase zymogen). The amino acid sequence around the matriptase activation cleavage site is indicated in the single-letter code with amino acid numbering at the N-terminal Val residue of the catalytic domain (Val615). The activation cleavage site is indicated by arrowhead. When cleaved, disulphide-linked two-chain matriptase is generated [Two-chain matriptase (fully active)]. HL-matriptase zymogen is a secreted variant of r-matriptase in which the cytosolic domain and the signal anchor (Met1–His80) are replaced with the human immunoglobulin  $\kappa$ -chain signal peptide and S-tag (ST). Enteropeptidase recognition sequences (DDDDK, underlined) and their surrounding sequences are shown in HL-matriptase zymogen. HL-matriptase zymogen is converted to HL-matriptase enzyme *in vitro* by incubation with r-EK. Note that S-tag at the N-terminus can also be removed by incubation with r-EK. The predicted disulphide linkages between two cysteine residues corresponding to Cys604 and Cys731 are shown as SS. TM, transmembrane domain; SEA, sea-urchin sperm protein–enterokinase–agrin domain; C1 and C2, the first and second complement factor 1R–urchin embryonic growth factor–bone morphogenetic protein domains; L1–4, four repeats of low-density lipoprotein receptor class A domain; CD, catalytic domain.

**Fig. 2. Effect of pH on the initial reaction rate in the hydrolysis of Ac-KTKQLR-MCA catalysed by HL-matriptase enzyme and zymogen.**

HL-matriptase zymogen and enzyme were incubated with substrate in 100 mM MES-NaOH (pH 5.0, 5.5, 6.0, 6.5 and 7.0) buffers and in 100 mM HEPES-NaOH (pH 7.5) buffer. In all buffers used, NaCl is included at the concentration of 5 mM. The initial reaction rate ( $v_0$ ) observed at the optimal pH is denoted by  $v_{max}$ . The values shown are means  $\pm$  SE of three separate experiments performed in duplicate.

**Fig. 3. Effect of NaCl concentration on the initial reaction rate in the hydrolysis of Ac-KTKQLR-MCA catalysed by HL-matriptase zymogen and enzyme.**

HL-matriptase zymogen (white bars) and HL-matriptase enzyme (black bars) were incubated with substrate at NaCl concentration range of 5 to 145 mM and fixed pH of 6.0 or 7.4. The initial reaction rates of HL-matriptase zymogen and enzyme in MES buffer A were taken as 100%. The values shown are means of three separate experiments performed in duplicate. The values of the standard error were less than 10 % of mean values.

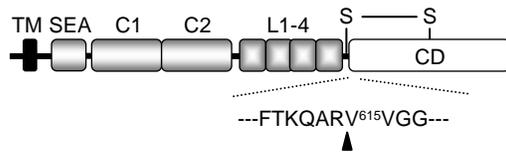
**Fig. 4. Effect of KCl concentration on the initial reaction rate in the hydrolysis of Ac-KTKQLR-MCA catalysed by HL-matriptase zymogen and enzyme.**

HL-matriptase zymogen (white bars) and HL-matriptase enzyme (black bars) were incubated with substrate in MES buffer A containing KCl (the concentration range of 0 to 140 mM). The initial reaction rates of HL-matriptase zymogen and enzyme in MES

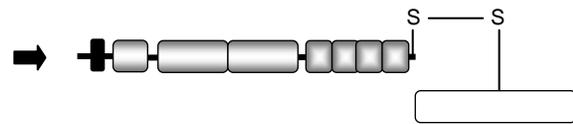
buffer A were taken as 100%. The values shown are means of three separate experiments performed in duplicate. The values of the standard error were less than 10 % of mean values.

**Fig. 5. Binding and inhibition of HL-matriptase zymogen and enzyme with biotin-EGR-CMK.** (A) Binding of the CMK derivative. HL-matriptase zymogen (Z) and enzyme (E) were treated with biotin-EGR-CMK and subjected to SDS-PAGE under reducing conditions. After Western transfer, the blot was probed with an anti-matriptase catalytic domain antibody (Spr992) or a streptavidin conjugated with horseradish peroxidase (Avidin-HRP). The position at which HL-matriptase zymogen labelled with biotin-EGR-CMK migrated is indicated by an arrowhead on the right of the panel. The sizes of the marker proteins were indicated on the left in kDa. (B) Inhibition by the CMK derivative. The initial reaction rates for hydrolysis of Sp-tPA catalysed by HL-matriptase zymogen (white circles) and enzyme (black circles) in the presence ( $v_i$ ) and absence ( $v$ ) of the inhibitor were measured as described in MATERIALS AND METHODS: The values shown are means of three separate experiments performed in duplicate. The values shown are means  $\pm$  SE of three separate experiments performed in duplicate.

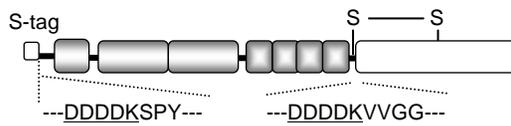
Matriptase zymogen



Two-chain matriptase (fully active)



HL-matriptase zymogen



HL-matriptase enzyme

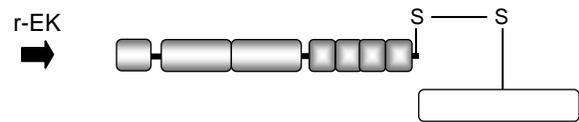


Figure 1

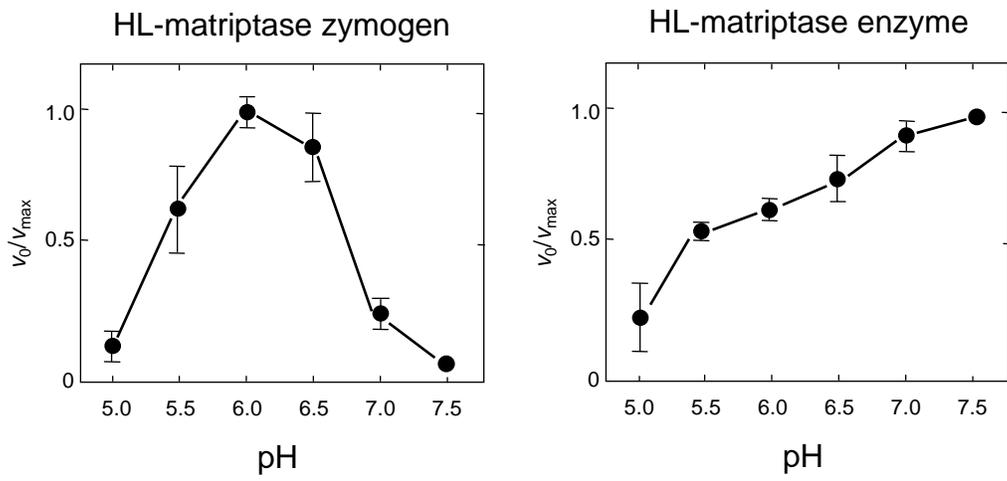


Figure 2

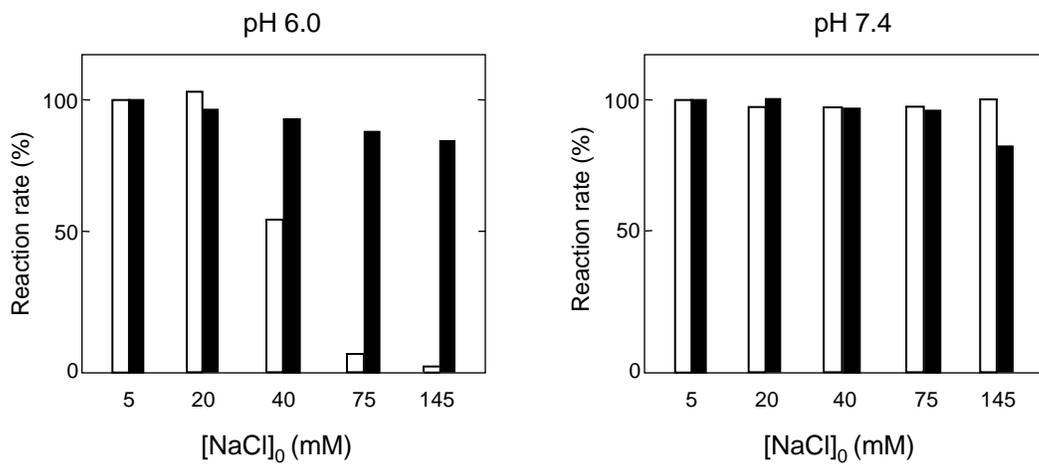


Figure 3

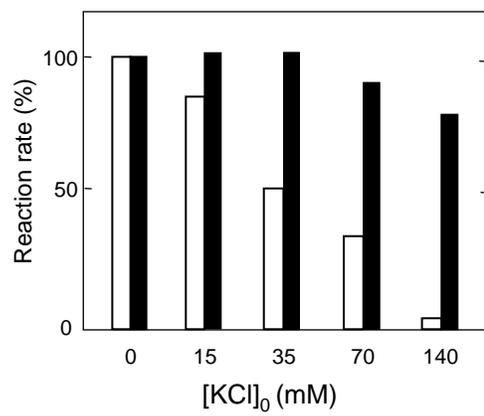
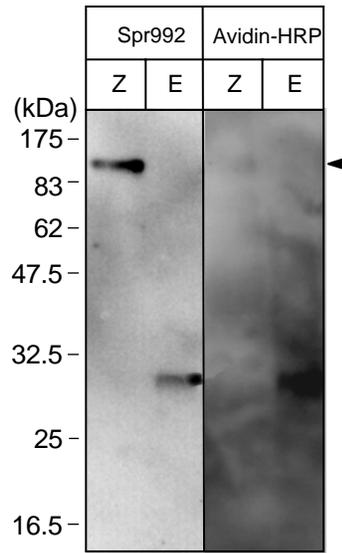
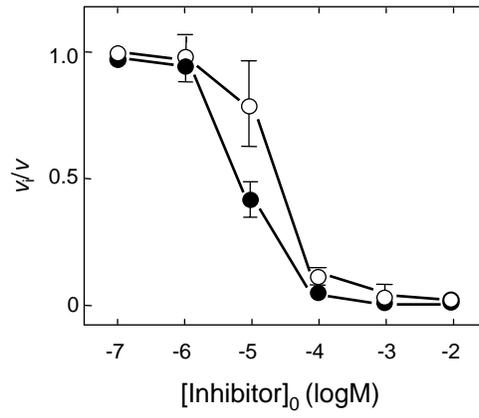


Figure 4

**A****B****Figure 5**