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Biochemical characterization of human kallikrein 8 and its possible involvement in the degradation of extracellular matrix proteins

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## **Abstract**

Human kallikrein 8 (*KLK8*) is a member of the human kallikrein gene family of serine proteases, and its protein, hK8, has recently been suggested to serve as a new ovarian cancer marker. To gain insights into the physiological role of hK8, the active recombinant enzyme was obtained in a pure state for biochemical and enzymatic characterizations. hK8 had trypsin-like activity with a strong preference for Arg over Lys in the P1 position, and its activity was inhibited by typical serine protease inhibitors. The protease degraded casein, fibronectin, gelatin, collagen type IV, fibrinogen, and high-molecular-weight kininogen. hK8 also converted human single-chain tissue-type plasminogen activator (65 kDa) to its two-chain form (32 and 33 kDa) by specifically cleaving the peptide bond Arg<sup>275</sup>-Ile<sup>276</sup>. This conversion resulted in a drastic increase in the activity of the activator toward the fluorogenic substrate Pyr-Gly-Arg-MCA and plasminogen in the absence of fibrin. Our findings suggest that hK8 may be implicated in ECM protein degradation in the area surrounding hK8-producing cells.

*Keywords:* human kallikrein 8; enzymatic characterization; extracellular matrix proteins; tissue-type plasminogen activator

## 1. Introduction

The tissue kallikreins are a subgroup of trypsin- and chymotrypsin-like serine proteases that are characterized by their homology to true tissue kallikrein, which is encoded by *KLK1/Klk1* gene in many species. These proteases share a high degree of sequence and structural similarity [1-3].

In recent studies, at least 15 genes encoding serine proteases have been identified in the human glandular kallikrein locus on chromosome 19q13.3-q13.4 [4-6]. Detailed mapping of the human kallikrein gene locus revealed that *KLK4-14* are located telomeric to *KLK2*, which encodes human glandular kallikrein 2 (hK2), while *KLK15* is located between *KLK1*, which encodes classical tissue kallikrein, and *KLK3*, which encodes prostate-specific antigen (PSA) [7]. Twelve of these fifteen *KLK* genes—i.e., all but *KLK1-3*—were discovered in recent years. Existing evidence suggests that all of the new *KLK* genes encode functional proteins. However, our knowledge of the *KLK* gene products is very limited at present. The chemical identities and molecular properties of human kallikrein 6 (hK6) [8-10], and, more recently, hK13 [11] and hK5 [12] have been determined.

*KLK8* (also known as *neuropsin/ovasin*) was cloned from a human skin cDNA library as a homologue of mouse neuropsin [13]. In the mouse, *KLK8* mRNA is localized at high concentration in the skin and brain, and the gene product has been shown to play an important role in normal hippocampal function [14]. *KLK8* mRNA was also detected in the hippocampus of normal human brains. Interestingly, the *KLK8* transcript level in the hippocampus of patients with Alzheimer's disease was found to be elevated 11.5-fold compared to that in controls, suggesting that *KLK8* may play a role in

neurodegeneration [15]. Furthermore, human *KLK8* mRNA and its protein (hK8) have been shown to be more highly expressed in ovarian cancer tissues than in normal ovarian tissue [16, 17]. These findings led to the proposal that hK8 protein may serve as a new ovarian cancer marker [17, 18]. However, despite the increasing biological and clinical interest in hK8, there have been no reports describing the biochemical and enzymatic properties of this protein. In the present study, active recombinant hK8 was produced and characterized. The present data indicate that hK8 is a trypsin-like enzyme that degrades some extracellular matrix (ECM) proteins, such as collagen type IV and fibronectin. The enzyme is also capable of converting single-chain tissue-type plasminogen activator (tPA) to its two-chain protein. These results suggest that hK8 plays a role in the turnover of ECM proteins.

## 2. Methods

### 2.1. *hK8* expression vector construction

The expression vectors for hK8 were constructed by inserting its cDNA (DDBJ/EMBL/GenBank databases AB009849) including the mature enzyme region (amino acids 33-260) with an enteropeptidase cleavage site at the 5' end into the *KpnI* and *EcoRI* sites of pET30a. To amplify the insert, two oligonucleotide primers were synthesized: the sense primer 5'-CGGGGTACCGACGACGACGACAAGGTGCTGGGGGGTTCAT-3', which contained the *KpnI* site, the enteropeptidase susceptibility site DDDDK, and the 5 NH<sub>2</sub>-terminal residues (Val<sup>33</sup> to His<sup>37</sup>) of mature hK8, and the antisense primer 5'-CCGGAATTCTCAGCCCTTGCTGCC-3', which included the *EcoRI* site and the 4

COOH-terminal residues (Gly<sup>257</sup> to Gly<sup>260</sup>) of the mature enzyme. PCR was performed with KOD plus DNA polymerase (Toyobo, Osaka, Japan) using human adult ovary total RNA (Stratagene, La Jolla, CA) as a template. The PCR conditions were 3 min at 94°C for heating, followed by 30 cycles of 30 sec at 94°C for denaturing, 30 sec at 60°C for annealing and 1 min at 68°C for extension. The reaction product was sequentially digested with *Kpn*I and *Eco*RI, gel purified and ligated in frame between the *Kpn*I and *Eco*RI sites of pET30a. The orientation and sequence of the cDNA in the vector (pET30a-hK8) were confirmed by DNA sequencing.

## 2.2. Preparation of active recombinant hK 8

The ligated vector (pET30a-hK8) was transformed to *E.coli* strain BL21. The cells were grown at 37°C, induced with isopropyl-1-thio- $\beta$ -D-galactoside. Cells were harvested by centrifugation and lysed by freeze-thawing. The materials were washed twice with 0.5% Triton X-100 and solubilized by being dissolved and then incubated in 50 mM Tris-HCl (pH 7.8) containing 6 M urea and 0.5 M NaCl for 12 h at room temperature. The solubilized materials were directly applied to a Ni<sup>2+</sup>-chelate column (5 ml volume) (Novagen, Madison, WI) previously equilibrated with 50 mM Tris-HCl (pH 7.8) containing 0.5 M NaCl and 6 M urea. The column was washed extensively with the above buffer containing 20 mM imidazole (100 ml), and then eluted with 50 mM histidine in the same buffer containing 0.5 M NaCl and 6 M urea. Eluted proteins were dialyzed against 50 mM Tris-HCl (pH 8.0) and stored at -20°C.

The enzymatically inactive fusion protein thus produced was activated with recombinant enteropeptidase from the medaka fish (*Oryzias latipes*), a small freshwater

teleost. Medaka enteropeptidase was immobilized on CNBr-activated Sepharose 4B (Sigma, St. Louis, MO) prior to use according to the manufacturer's protocol. hK8 was incubated with the immobilized enteropeptidase for 16 h at room temperature. After removing the immobilized protease by filtration, the activity of hK8 was assayed by a 4-methylcoumaryl-7-amide (MCA)-containing synthetic substrate, Pro-Phe-Arg-MCA (Peptide Institute, Osaka, Japan). The molecular cloning, expression, and characterization of medaka enteropeptidase will be reported elsewhere.

### 2.3. Enzyme activity assay

Unless otherwise stated, hK8 activity was determined at 37°C with Pro-Phe-Arg-MCA according to the method of Barrett [19], with the slight modifications reported in our previous study [20]. The release of fluorophore 7-amino-4-methyl coumarin (AMC) was measured by spectrofluorometry using an excitation wavelength of 370nm and an emission wavelength of 460nm.

Active hK8 was preincubated with various protease inhibitors at 37°C in 0.1 M Tris-HCl buffer (pH 8.0). After incubation for 15 min, enzyme activity was measured using Pro-Phe-Arg-MCA as substrate.

### 2.4. Kinetic parameters

Kinetic parameters were determined for various MCA substrates. Initial velocities, extrapolated from the plot of product versus time, were transformed into double-reciprocal plots [21]. Maximum velocities ( $V_{max}$ ),  $K_m$ , and  $k_{cat}$  values were obtained from the intercepts of these plots.

The active rhK8 concentration was determined using the active site titrant *p*-nitrophenyl-*p*'-guanidinobenzoate HCl [22].

### *2.5. Digestion of protein substrates*

Forty micrograms of human plasma fibronectin (Chemicon, Temecula, CA), porcine type 1 gelatin (Daiichi Fine Chemicals, Toyama, Japan), mouse laminin (Biomedical Technologies Inc., Stoughton, MA), human fibrinogen (Merk Biosciences, Tokyo, Japan), human high-molecular-weight kininogen (Calbiochem), and casein (Sigma) were incubated at 37°C in 200 µl of 50 mM Tris-HCl buffer (pH 8.0) with 4 µg of hK8. Aliquots of 20 µl mixtures were taken at various time points (0 to 18 h), and mixed with 5 µl of SDS-PAGE sample buffer. The mixtures were boiled and aliquots of 20 µl samples were subjected to SDS-PAGE under reducing conditions. After electrophoresis, gels were stained with 0.25% Coomassie Brilliant Blue.

Twenty-five micrograms of FITC-conjugated bovine collagen type I and type IV (Yagai Corporation, Yamagata, Japan) were incubated with hK8 (0.25 µg) at 33°C for 16 h in 100 µl of 50 mM Tris-HCl buffer (pH 8.0). After incubation, the reactions were terminated by adding 300 µl of stopping reagent (42% ethanol, 50 mM Tris-HCl (pH 9.5), and 0.2 M NaCl). Samples were incubated on ice for 10 min, and then centrifuged at 10,000 g for 10 min. Aliquots of the resulting supernatants were diluted to a volume of 3.0 ml with distilled water, and the fluorescence was measured at an excitation wavelength of 459 nm and an emission wavelength of 520 nm.

### *2.6. Conversion of single-chain tPA to two-chain tPA*

Ten micrograms of recombinant human single-chain tPA (American Diagnostica Inc., Greenwich, CT) were incubated at 37°C with active hK8 (1 µg) in 200 µl of 0.1M Tris-HCl buffer (pH 8.0). After incubation, 20 µl of the reaction mixture was incubated with aprotinin (2 µg) for an additional 10 min at 37°C. The mixtures were then assayed for the enzyme activity using Pyr-Gly-Arg-MCA as a substrate. This procedure enabled the determination of two-chain tPA activity specifically.

Samples of human tPA treated with active hK8 were separated by SDS-PAGE under reducing conditions, and transferred to polyvinylidene difluoride membrane (PVDF) (Millipore, Bedford, MA). The blotted membrane was incubated with mouse anti-human tPA monoclonal antibody (Oncogene Research Products, San Diego, CA) at 1:500 dilution and subsequently with sheep anti-mouse IgG antibody conjugated with horseradish peroxidase (Amersham Biosciences, Buckinghamshire, England). Immunoreactive signals were detected using an ECL Western blot detection kit (Amersham Biosciences).

### *2.7. Plasminogen activation*

Ten micrograms of recombinant human plasminogen (Calbiochem) was incubated at 37°C with sctPA (1 µg) in the presence or absence of active hK8 (0.1 µg) in 200 µl of 0.1 M Tris-HCl buffer (pH 8.0). As additional controls, plasminogen alone or hK8 alone was incubated. After incubation, 20 µl of the reaction mixture was used for the enzyme activity assay using the synthetic substrate Boc-Glu-Lys-Lys-MCA for generated plasmin. Assays with MCA substrate were conducted as described as above.

### 3. Results

#### 3.1. Substrate specificity of hK8

The recombinant protein was expressed in *E.coli* (BL21) transformed with pET30a-hK8, and the enzyme was purified as described in the methods. SDS-PAGE analysis of the purified fusion hK8 protein showed a single band of 35.5 kDa under reducing conditions (Fig. 1). Treatment of the protein with immobilized medaka enteropeptidase successfully produced active hK8 with a molecular mass of 31.5 kDa under the same conditions (Fig. 1). Autocatalytic activation of the fusion protein was not observed.

Active recombinant hK8 was stable at -20°C; the initial enzyme activity was retained for at least 2 months with no detectable change in the electrophoretic pattern.

The enzyme activity of recombinant hK8 was detected at pH range 7-10 in a typical bell shape, and the optimum pH was found to be 8.5 (data not shown).

Fluorogenic peptide substrates having an MCA leaving group were used to determine the kinetic parameters (Table 1). Among the substrates tested, Pro-Phe-Arg-MCA was the best substrate for hK8 based on its  $k_{cat}/K_m$  value. Z-Val-Val-Arg-MCA and Boc-Val-Pro-Arg-MCA were hydrolyzed by the enzyme. hK8 detectably cleaved the substrates Boc-Leu-Lys-Arg-MCA and Boc-Phe-Ser-Arg-MCA. The enzyme reproducibly hydrolyzed a lysine-containing substrate Boc-Val-Leu-Lys-MCA to a similar extent, whereas another available lysine-containing substrate Boc-Glu-Lys-Lys-MCA was resistant to the enzyme hydrolysis. Generally, the  $K_m$  values were fairly constant with all the substrates tested, indicating that differences

in the catalytic efficiency of the enzyme ( $k_{\text{cat}}/K_m$ ) for individual substrates were largely due to the differences in  $k_{\text{cat}}$  values.

The substrates Suc-Leu-Leu-Val-Tyr-MCA, Suc-Ala-Ala-Pro-Phe-MCA, and Ac-Ile-Glu-Thr-Asp-MCA were not hydrolyzed by hK8 at all.

In short, hK8 is an enzyme with primary specificity for *L*-arginine peptide bonds, and with marginal specificity for *L*-lysine peptide bonds.

### 3.2. Effects of inhibitors on hK8

The effects of various protease inhibitors on amidolytic activity of hK8 were examined using Pro-Phe-Arg-MCA as a substrate (Table 2). The enzyme activity was strongly inhibited by serine protease inhibitors, such as diisopropylfluorophosphate (DFP), antipain, aprotinin, leupeptin, benzamidine, and soybean trypsin inhibitor (SBTI). No detectable inhibition was observed with  $N^{\alpha}$ -*p*-tosyl-*L*-lysine chloromethyl ketone (TLCK),  $N^{\alpha}$ -*p*-tosyl-*L*-phenylalanine chloromethyl ketone (TPCK), pepstatin, *o*-phenanthroline, or E-64.

### 3.3. Digestion of protein substrates by hK8

Casein was extensively and rapidly degraded by hK8, yielding a number of fragments with lower molecular masses (Fig. 2a). Efficient but rather specific proteolytic activity of hK8 was found against human plasma fibronectin (Fig. 2b). The pattern of fibronectin digestion by hK8 is apparently different from that of its murine counterpart [24], but similar to that observed with murine Klk21 [20].

Gelatin type I was efficiently degraded by hK8 (Fig. 2c). Collagen type I was

resistant to the enzyme, while collagen type IV was cleaved by hK8 (Fig. 2d).

Human kallikrein 8 could digest fibrinogen by rapidly cleaving the B $\beta$  chain, and slightly cleaving the A $\alpha$  chain. However, the  $\gamma$  chain remained intact (Fig. 2e). This degradation pattern was distinct from that of human thrombin; thrombin degraded the A $\alpha$  chain rapidly and the  $\gamma$  chain slowly, while the B $\beta$  chain was not degraded (data now shown).

High-molecular-weight kininogen was also a substrate of hK8 (Fig. 2f).

#### *3.4. Effect of hK8 on single-chain tPA*

Human single chain tPA, which has little or no activity as assayed with the substrate Pyr-Gly-Arg-MCA, was activated by human kallikrein 8 (Fig. 3a). The tPA activity increased rapidly within the initial 2 h and then leveled off after 6 h. Western blot analysis of the reaction mixtures indicated that the activation underlies the production of two-chain tPA (33 and 32 kDa) from its single chain precursor molecule (65 kDa) in a time-dependent manner with a fixed amount of enzyme sample (Fig. 3b). Prolonged incubation resulted in no decline of activated tPA activity, suggesting that hK8 activated tPA by cleaving a specific peptide bond at the presumed activation site with no further hydrolysis. In contrast, the tPA activity caused by trypsin activation first increased to a level comparable to that observed with hK8 and declined thereafter (data not shown). This was probably due to extensive hydrolysis of the activated tPA by trypsin. These results indicate that proteolytic cleavage at a single internal peptide bond underlies the activation of tPA by hK8. In order to determine the cleavage site, the NH<sub>2</sub>-terminal amino acid sequence of the product was analyzed. Two sets of sequences

(Ile-Lys-Gly-Gly-Leu-Phe-Ala-Glu- and Ser-Val-Gln-Val-Ile-X-Arg-Glu-) were obtained with tPA treated with hK8 for 6 h. The former corresponds to the internal sequence of sctPA (Ile<sup>276</sup> to Glu<sup>283</sup>), and the latter to the NH<sub>2</sub>-terminal sequence (Ser<sup>1</sup> to Glu<sup>8</sup>) [25]. These results clearly indicate that the Arg<sup>275</sup>-Ile<sup>276</sup> peptide bond in sctPA was specifically cleaved by hK8.

hK8 had no effect on plasminogen; incubation of plasminogen with the enzyme for 6 h did not generate plasmin. SDS-PAGE analysis of plasminogen incubated with hK8 showed no degraded polypeptides (data not shown).

The generation of plasmin from plasminogen by single-chain tPA was examined in the presence or absence of hK8. A rapid, dramatic increase of the plasmin activity was observed when plasminogen, single-chain tPA and hK8 were present in the reaction mixture, while the activity increased slowly in the absence of hK8 (Fig. 3c). There was no detectable plasmin activity when plasminogen was incubated with hK8 only. The presence of trace amounts of active tPA in the single-chain tPA preparation probably led to the plasmin generation in the incubation of plasminogen with only sctPA. These results clearly indicate that the production of plasmin from plasminogen by tPA is detectably accelerated in the presence of hK8.

#### **4. Discussion**

The present study provided the first characterization of hK8 using recombinant protein. As expected, hK8 was demonstrated to be a protease with trypsin-like specificity. The enzyme primarily hydrolyzes MCA substrates containing Arg at the P1 position. In addition, hK8 slightly hydrolyzed Boc-Val-Leu-Lys-MCA, but not

Boc-Glu-Lys-Lys-MCA. Regarding the action of hK8 on the Lys-containing substrate, we should note that a crystal structural study [26] on the mouse homologue of hK8, neuropsin, revealed S1-pocket specificity for both Arg and Lys due to the unique conformations of the kallikrein loops G and H.

The present study demonstrated the ability of hK8 to hydrolyze a variety of protein substrates, suggesting its involvement in various biological processes. Previous studies have established that hK8 is detectable in various tissue extracts and biological fluids, including breast milk, amniotic fluid, seminal plasma, and serum [27]. More interestingly, Kishi et al. reported that hK8 protein expression is higher in ovarian cancer tissue extracts, serum, and ascites fluids, than in benign and normal tissues [17]. They also suggested that hK8 is likely secreted by tumor cells in ovarian cancer. Another study reported that the *KLK8* gene was expressed more highly in ovarian carcinomas than in normal ovarian tissue [16]. In this context, it is of particular interest to note our present finding that hK8 is capable of degrading fibronectin and collagen type IV. Fibronectin is an ECM protein that serves as a general adhesive molecule, linking cells to one another or to other substrates such as collagen and proteoglycans. Existing evidence indicates that this adhesion protein is a major ECM component expressed in the ovary [28]. Collagen type IV is a component of the basement membrane. Generally, the degradation of both fibronectin and collagen type IV is thought to be a critical step for cancer cells to invade and metastasize. These considerations tempt us to speculate that hK8 may be involved in the invasive and metastatic process of ovarian cancer cells.

Another interesting finding of the present study is that single-chain tPA is a substrate

of hK8. Specific cleavage at the peptide bond Arg<sup>275</sup>-Ile<sup>276</sup> in single-chain tPA by the enzyme produced two-chain tPA. It has previously been shown that single-chain tPA has only limited plasmin-producing activity unless the cofactor fibrin is present, whereas the two-chain form of tPA itself exhibits full activity in the absence of fibrin [29]. Therefore, hK8 could initiate the operation of the tPA/plasmin system in the absence of fibrin. Indeed, our current data indicate that the active serine protease plasmin was promptly produced upon incubation of plasminogen with single-chain tPA in the presence of active hK8. It is generally agreed that plasmin catalyzes the degradation of proteins in basement membranes and ECM and thus facilitates cancer cell invasion into the surrounding tissue [30-32]. Since plasminogen is abundantly present in the tissue fluid, hK8 may trigger activation of the tPA/plasmin system if cancer cells synthesize and secrete both sctPA and hK8. It would be interesting to examine whether this assumption is correct using ovarian cancer cells producing hK8 protein [17].

We found that hK8 degrades both fibrinogen and HMW-kininogen, but the biological significance of this result is not known at present.

We previously reported the presence of an enzyme capable of converting single-chain tPA to two-chain tPA in the preovulatory human follicular fluid [23]. Judging from its characteristics, we assumed that the enzyme might be a tissue kallikrein. Since we were unable to detect immunoreactive materials in follicular fluid by Western blotting using anti-hK8 antibodies (our unpublished observations), hK8 is not likely to be the enzyme responsible for the activation of single-chain tPA in follicular fluid.

In conclusion, the present report provides enzymatic and biochemical characteristics

of recombinant hK8. Our data indicate that hK8 may play a role in the degradation of ECM proteins in the area surrounding the hK8-producing cells, not only via its own activity but also through the activity of the plasmin it produces by converting single-chain tPA to two-chain tPA.

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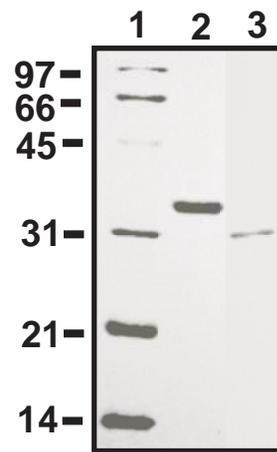
## Figure Legends

Fig. 1 Purity of purified recombinant hK8. Purified hK8 was subjected to SDS-PAGE analysis under reducing conditions and visualized by Coomassie Brilliant Blue R-250 staining. Lane 1, molecular weight marker (kDa); lane 2, hK8 fusion protein; lane 3, active hK8.

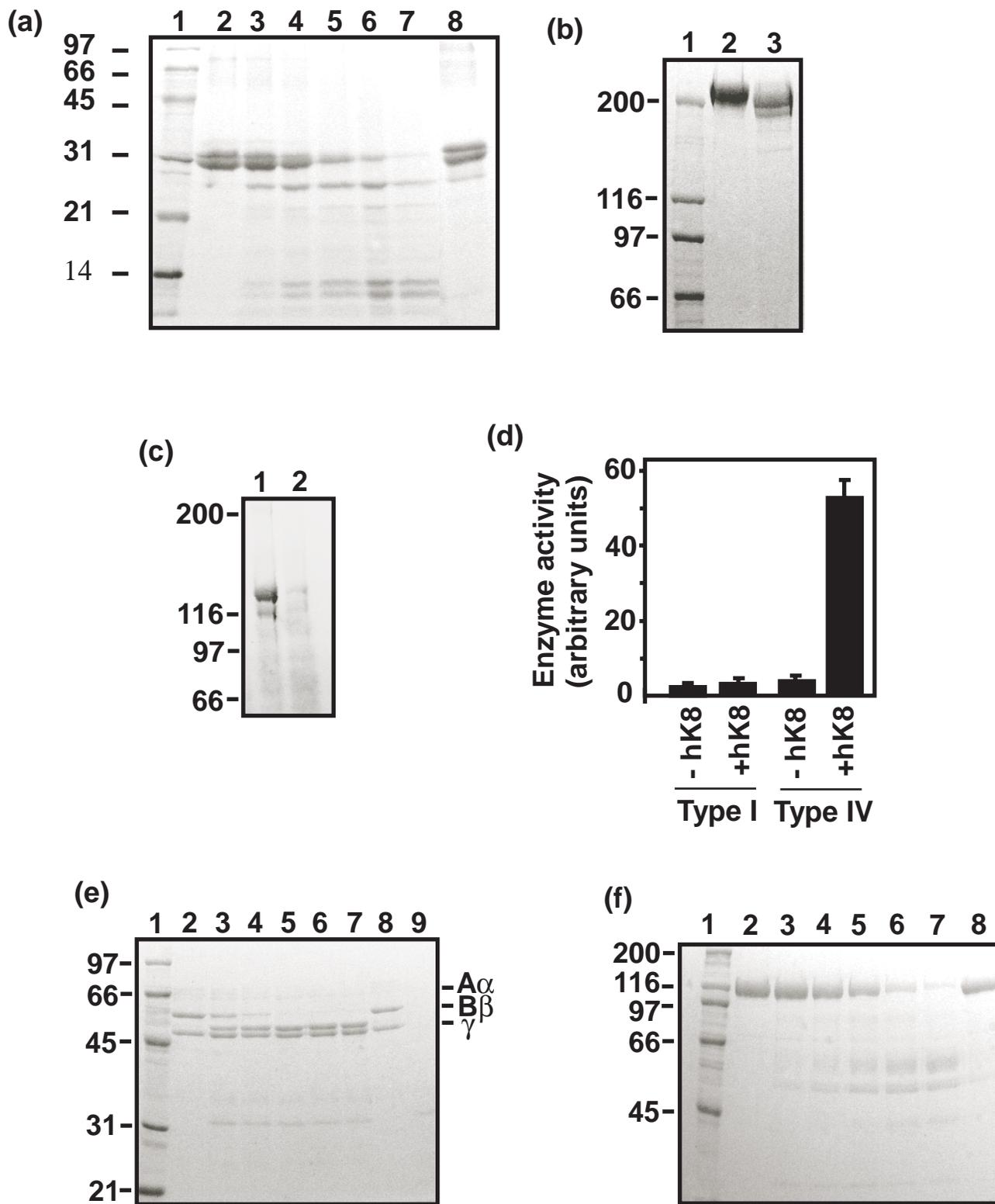
Fig. 2. Action of hK8 on protein substrates. (a) SDS-PAGE of casein incubated with hK8. Lane 1, molecular weight marker (kDa). Casein was incubated with hK8 for 0 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), 6 (lane 6), or 8 h (lane 7). The protein was incubated without hK8 for 1 h (lane 8). (b) SDS-PAGE of fibronectin incubated with hK8. Lane 1, molecular weight marker; lane 2, fibronectin incubated without (lane 2) or with hK8 (lane 3) for 18 h. (c) SDS-PAGE of gelatin incubated with hK8. gelatin incubated without (lane 1) or with hK8 (lane 2) for 18 h at 37°C. (d) Effects on collagens type I and type IV. FITC-conjugated collagens were incubated without (-hK8) or with hK8 (+hK8) for 16 h, and the fluorescence intensities of the supernatants were measured. (e) SDS-PAGE of fibrinogen incubated with hK8. Lane 1, molecular weight marker (kDa). Fibrinogen was incubated with hK8 for 0 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), 6 (lane 6), or 8 h (lane 7). Fibrinogen alone (lane 8) or hK8 alone (lane 9) was incubated for 1 h. Positions corresponding to polypeptides A $\alpha$ , B $\beta$ , and  $\gamma$  of fibrinogen are shown at right. (f) SDS-PAGE of high-molecular-weight kininogen incubated with hK8. Lane 1, molecular weight marker (kDa). High-molecular-weight kininogen was incubated with hK8 for 0 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 4 (lane 6), or 6 h

(lane 7). Lane 8, incubation for 6 h without hK8.

Fig. 3. Effect of hK8 on tPA. (a) Human single-chain tPA was incubated alone (○) or with hK8 (●) at 37°C. Aliquots of the incubation mixture were taken at the indicated times, and the activity of activated tPA toward Pyr-Gly-Arg-MCA was selectively determined by assaying in the presence of aprotinin. As a control, incubations of hK8 alone (▲) were conducted. (b) Human single-chain tPA was incubated with hK8 in the same buffer as in (a). Aliquots of the incubation mixture were taken at 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), and 6 h (lane 6) and subjected to SDS-PAGE/Western blot analysis under reducing conditions. The positions of single-chain tPA (65 kDa) and tctPA (33 and 32 kDa) are indicated at right. (c) Effect of hK8 on plasmin generation in the tPA/plasmin system. Plasminogen was incubated at 37°C with sctPA in the absence or presence of hK8. Plasminogen alone or hK8 alone was incubated as additional controls. Aliquots of the reaction mixtures were taken at the indicated times for plasmin activity assay using Boc-Glu-Lys-Lys-MCA. ●, plasminogen + single-chain tPA + hK8; ○, plasminogen + single-chain tPA; ▲, plasminogen + hK8; □, plasminogen alone; ×, hK8 alone.

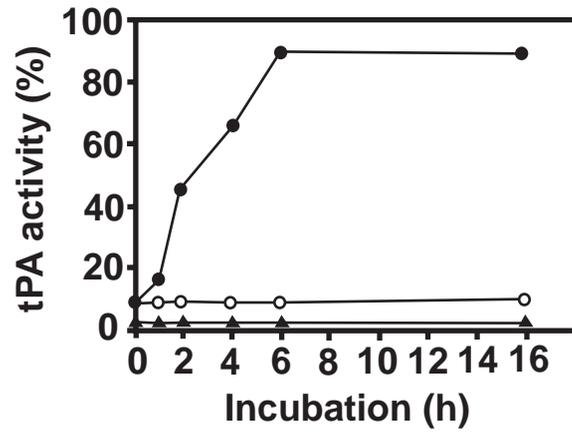


**Figure 1**  
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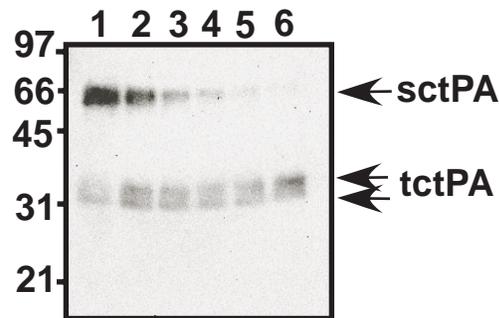


**Figure 2**  
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(a)



(b)



(c)

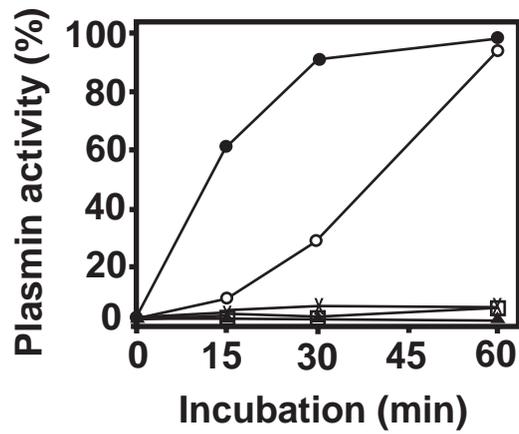


Figure 3  
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Table 1

Kinetic parameters of hK8 as measured on MCA-containing substrates

Substrates	$V_{\max}$	$K_m$	$k_{\text{cat}}$	$k_{\text{cat}}/K_m$
	$\mu\text{mol}/\text{min}/\text{mg}$	mM	$\text{min}^{-1}$	$\text{mM}^{-1}\cdot\text{min}^{-1}$
Pro-Phe-Arg- MCA	7.1	0.07	180	2600
Z-Val-Val-Arg-MCA	5.4	0.07	140	2000
Boc-Val-Pro-Arg-MC	3.9	0.07	98	1400
Boc-Leu-Lys-Arg-MCA	2.6	0.10	65	650
Boc-Phe-Ser-Arg-MCA	1.6	0.07	40	570
Boc-Val-Leu-Lys-MCA	1.9	0.10	48	480
Boc-Glu-Lys-Lys-MCA	ND*			
Suc-Leu-Leu-Val-Tyr-MCA	ND			
Ac-Ile-Glu-Thr-Asp-MCA	ND			
Suc-Ala-Ala-Pro-Phe-MCA	ND			

ND\*, Not detected.

Table 2  
Effects of protease inhibitors on hK8 activity

Inhibitor	Concentration	Inhibition (%)
DFP	2.0 mM	100
Antipain	0.2 mM	100
Aprotinin	0.01 mg/ml	98
Leupeptin	0.2 mM	95
SBTI	0.1 mg/ml	75
	0.2 mg/ml	87
Benzamidine	0.2 mM	18
	1.0 mM	83
E-64	0.5 mM	6
<i>o</i> -Phenanthroline	2.0 mM	7
Pepstatin	0.1 mM	0
TPCK	0.2 mM	3
TLCK	0.2 mM	8

DFP, diisopropyl fluorophosphate; SBTI, soybean trypsin inhibitor;

TPCK, *N*<sup>α</sup>-*p*-tosyl-L-phenylalanine chloromethyl ketone; TLCK,

*N*<sup>α</sup>-*p*-tosyl-L-lysine chloromethyl ketone