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Original Research Article

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Running title: Differences in KLKs between mouse strains

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**Abstract**

Several studies have reported differences in physiological and pathological phenotypes between different strains of experimental mice, such as environment-based behavior, skin damage, damage in response to toxins and nervous system injury. However, the mechanisms underlying these differences have not yet been fully elucidated. We have been studying the function of kallikrein-related peptidases (KLKs), serin proteases known to serve a variety of functions. In this study, we focused on differences in KLKs between C57BL/6 mice and 129 mice. Among 13 KLKs genes examined, 12 KLKs showed differences in the mRNA coding region sequence and 7 KLKs showed different deduced amino acid sequences of their proteins when comparing C57BL/6 and 129 mice. KLK6 protein from 129 mice had six amino acid differences compared with that from C57BL/6 mice. KLK6 protein from 129 mice showed reduced SDS-PAGE mobility compared with that from C57BL/6 mice. Moreover, recombinant KLK6 protein from 129 mice had a higher optimum pH and more than 15 times higher hydrolytic enzymatic activity for several substrates than that from

C57BL/6 mice. These results suggest that KLKs may contribute to the genetic basis of the differences between mouse strains.

**Keywords**

129 mouse; C57BL/6 mouse; enzymatic activity; kallikrein-related peptidase, mouse strains, protease.

## *Introduction*

Many experimental animals are used to study gene function and disease mechanisms and in drug discovery. Mice are the most widely used experimental mammal and several strains of mice are used in many experiments. C57BL/6 mice are the most popular experimental strain. 129 mice are another popular strain, and 129 substrains have often been used to create transgenic and gene-targeted mice [1]. It has been shown there are many differences in physiological and pathological characteristics between mouse strains, such as environmental-based behavior [2,3], the reaction of skin to some reagents [4,5], susceptibility to virus [6], asbestos-induced lung fibrosis [7] and renal ischemia-reperfusion injury [8]. It has been also reported that 129 and C57BL/6 mice show different phenotypes in nervous system disorders such as behavioral response to drugs [9,10], sensitivity to toxic agents [11,12], and degree of damage in response to spinal cord injury (SCI) [13,14]. However, the factors that cause these phenotypic differences between the two strains have not yet been fully elucidated.

Kallikreins and kallikrein-related peptidases (KLKs) are trypsin-like serine proteases, and 15 family members have been reported in humans [15,16]. KLKs are known to serve a variety of physiological functions such as regulation of blood pressure, semen liquefaction and skin desquamation [17,18]. KLKs also contribute to a wide range of pathological processes, especially tumorigenesis, angiogenesis and metastasis [17,18,19].

KLK6/protease M/neurosin is a major KLK expressed in the central nervous system (CNS). KLK6 is constitutively expressed in oligodendrocytes in the CNS and its expression is up-regulated after SCI and in experimental autoimmune encephalomyelitis (EAE) [20,21,22,23]. *Klk6*$^{-/-}$ mice showed a smaller number of oligodendrocytes and a smaller amount of myelin proteins in their developing spinal cords than wild-type (WT) mice. Moreover, *Klk6*$^{-/-}$ mice showed lower expression of myelin basic protein (MBP) in the damaged spinal cord than WT mice after SCI [24]. These results suggest that KLK6 has functional roles in oligodendrocyte development and myelin formation in the developing spinal cord, and in remyelination of damaged spinal cord after SCI.

KLKs may be part of the genetic basis for the differences between C57BL/6 mice and 129 mice. We therefore investigated the mRNA sequences of *Klk*s in these two strains. We found several differences in the mRNA sequences of *Klk*s and their putative protein sequences between C57BL/6 mice and 129 mice.

## *Materials and Methods*

### *Animals*

C57BL/6 mice, *Klk8*$^{-/-}$ mice [25,26,27], and BALB/c mice were bred in our laboratory and in the Animal Laboratory for Medical Research in Asahikawa Medical University. 129X1/SvJ mice and ICR mice were purchased from Japan SLC (Shizuoka, Japan). All experiments were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Asahikawa Medical University and the guidelines in the U.S. National Institutes of Health, the Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the numbers of animals used and their pain and suffering.

### *Sequence analysis of KLK mRNA*

We analyzed the sequences of *Klk* mRNAs from three 129X1/SvJ mice, one BALB/c mouse, one ICR mouse and two *Klk8*$^{-/-}$ mice. Mice were euthanized by decapitation under deep ether anesthesia. Spinal cord (for *Klk6* and *Klk8*), kidneys (for *Klk1*) and tongue (for *Klk5*, *Klk7*

and *Klk9–15*) were removed. Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and aliquots containing 2 μg of total RNA were used for reverse transcription (RT) reaction with AMV-reverse transcriptase (Promega, Madison, WI, USA) to produce cDNA, according to the manufacturer's instructions. cDNAs of *Klk* mRNAs, including the coding regions, were amplified by PCR using the primer pairs shown in Supplementary Table. The PCR products were purified using MagExtractor (Toyobo, Osaka, Japan) and the DNA sequence was analyzed using a BigDye Terminator v1.1 Cycle Sequencing Kit and 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

***Western blot analysis***

We analyzed KLK6 protein by western blot analysis from two C57BL/6 mice, two 129X1SvJ mice, two *Klk8*$^{-/-}$ mice, one BALB/c mouse and one ICR mouse. Mice were euthanized by decapitation under deep ether anesthesia, and the spinal cord was removed from each mouse and homogenized in assay buffer (50 mM Tris-HCl, 0.1% SDS, 0.5% sodium deoxycholate,

1% Nonidet P40, and 150 mM NaCl, pH 8.0). The homogenates were clarified by centrifugation at 19,000 × g for 5 min at 4 °C, and the supernatant fraction was used in subsequent experiments. After determination of the protein concentration (Micro BCA Protein Assay, Thermo Scientific, Rockford, IL, USA), 20 μg of protein was loaded onto each lane of a 12.5 % polyacrylamide gel for SDS-PAGE. After electrophoresis, proteins were transferred onto an Immobilon-P membrane (Merck, Damstadt, Germany). Immunoreaction was carried out using anti-KLK6 (rabbit polyclonal, 1:500) [23] and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6C5, mouse monoclonal, Life Technologies, 1:10,000) antibodies overnight at 4 °C. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK, 1:5,000) for 1 hr at room temperature, and visualized using the ECL Prime Western Blotting Detection Kit (GE Healthcare) and luminescent Image Analyzer (LAS-3000, FujiFilm, Tokyo, Japan).

***Production of recombinant pro-KLK6***

cDNA encoding prepro-KLK6 was obtained from mouse spinal cords using RT-PCR as mentioned above, and was inserted into a pcDNA3.1B(+) vector (Life Technologies) to produce recombinant KLK6 with a *myc* epitope and polyhistidine tag at the C-terminus. The vector was transfected into HEK293 cells using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany), and cells stably expressing transfected genes were then selected using G418 disulfate salt (Sigma, Saint Louis, MO, USA). To identify HEK293 cells expressing high levels of pro-KLK6 protein, we screened the amount of pro-KLK6 protein in the culture media by western blot analysis using an anti-c-Myc antibody (9E10, mouse monoclonal, Merck, 1:200). The recombinant pro-KLK6 was purified from the culture media of the HEK293 cells using His GraviTrap (GE Healthcare), concentrated, and the buffer changed to activation buffer (50 mM Tris-HCl, 0.05% Brij-35, pH 8.0) using Amicon Ultra Centrifugal Filters (Ultracel 30K, Merck). Samples were then stored at −80 °C until used for further experiments. The concentration of

KLK6 protein in each solution was estimated by SDS-PAGE and Coomassie Brilliant Blue R-250 (ICN Biomedicine, Aurora, OH, USA) staining, using bovine serum albumin as a standard. The molecular weight of recombinant pro-KLK6 was analyzed by western blot analysis using anti-c-Myc antibody (9E10, Merck) as mentioned above.

***Enzymatic activity assay***

For determination of optimal pH of the reaction buffer, 40 ng recombinant pro-KLK6 was activated by incubation with $1.0 \times 10^{-3}$ AU/mL lysyl endopeptidase (Wako, Osaka, Japan) in the activation buffer (50 mM Tris-HCl, 0.05% Brij-35, pH 8.0) at 30 °C for 30 min. Activated KLK6 was then incubated with 100 μM solutions of various peptidyl MCA compounds (*t*-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine 4-methylcoumaryl-7-amide (Boc-VPR-MCA), *t*-butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine 4-methylcoumaryl-7-amide (Boc-FSR-MCA), *t*-butyloxycarbonyl-L-glutainyl-L-alanyl-L-arginine 4-methylcoumaryl-7-amide

(Boc-QAR-MCA), succinyl-L alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-methylcoumaryl-7-amide (Suc-AAPF-MCA), and succinyl-L leucyl-L-leucyl-L-valyl-L-tyrosine 4-methylcoumaryl-7-amide (Suc-LLVY-MCA), Peptide Institute Inc., Osaka, Japan) in 0.1 M Tris-HCl (pH 5.2–9.8) at 37 °C for 30 min. The reaction was terminated by addition of 0.1 M sodium acetate and 0.1 M monochloroacetic acid. The amount of 7-amino-4-methylcoumarin (AMC) released was measured using a fluorescence spectrometer (F-4500, Hitachi, Tokyo, Japan) at 360 nm for excitation and 440 nm for emission. For determination of kinetic constant, 80–120 ng recombinant pro-KLK6 from C57BL/6 mice (KLK6-B6) and 20–30 ng recombinant pro-KLK6 from 129X1/SvJ mice (KLK6-129) were used. The recombinant pro-KLK6 was incubated with lysyl endopeptidase (Wako) as mentioned above. Activated KLK6 was incubated with the substrates at various concentrations (6.25, 12.5, 25, 50, 100 and 200 μM) at 37 °C for 5 min and AMC released was measured as mentioned above. The $k_{cat}$ and *Km* were calculated from the data set of initial reaction rate versus substrate concentration by non-linear regression analysis (Image J,

National Institute of Mental Health, Bethesda, MD, USA).

***Statistical analysis***

The significance of differences in enzymatic activity between the two recombinant KLK6 proteins was assessed using the Student's *t*-test.

***Results***

***mRNA and amino acid differences in KLKs***

We determined mRNA sequences of *Klk*s to establish whether there were any differences in the *Klk* gene sequences between C57BL/6 mice and 129 mice. We sequenced the cDNA for *Klks* from 129X1/SvJ mice, which are a commercially available substrain of 129 mice, and compared these with database sequences for C57BL/6 mice (GenBank, http://www.ncbi.nlm.nih.gov/genbank/). The sequences of *Klk* mRNAs determined in this study were registered in DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/index-e.html, Supplementary Table). The sequences of *Klk4* and *Klk7* mRNA from 129 substrains have already been registered in GenBank and we used these data for comparison (Supplementary Table). The results are shown in Table 1. The coding region of *Klk13* mRNA from 129X1/SvJ mice was not different from that of C57BL/6 mice. The coding regions of *Klk1*, *Klk7*, *Klk8*, *Klk11*, and *Klk14* mRNA from 129X1/SvJ mice had one or more differences compared with those from C57BL/6 mice, but did not have any differences in the deduced

amino acid sequences of their proteins. The sequence of *Klk4* mRNA from 129/SvJ mice had five different nucleotides in the coding region, resulting in one different amino acid residue of the deduced protein. *Klk5, Klk6, Klk9, Klk10, Klk12,* and *Klk15* mRNA from 129X1/SvJ mice had one or more different nucleotides resulting in differences in the deduced amino acid sequence of their proteins. Of these, *Klk6* mRNA from 129X1/SvJ mice had the most changes in the deduced amino acid residues of the protein.

***Two different KLK6 in several mouse strains***

We focused on *Klk6*, because this had the greatest number of different deduced amino acid residues in the protein when comparing mRNA sequences from C57BL/6 mice and 129X1/SvJ mice. First, we analyzed the KLK6 protein from spinal cord samples using SDS-PAGE and performed immunoblot assays. We observed differences in the mobility of KLK6 derived from the two mouse strains (Fig. 1A). KLK6 protein from 129X1/SvJ mice showed reduced SDS-PAGE mobility compared with that from C57BL/6 mice. KLK6 from

C57BL/6 mice was approximately 28.2 kDa and that from 129X1/SvJ mice was 29.4 kDa. We also analyzed KLK6 from BALB/c mice and ICR mice. The sequence of the *Klk6* mRNA coding region in these two strains is identical to that in C57BL/6 mice, and KLK6 protein from these strains showed the same SDS-PAGE mobility as that from C57BL/6 mice (Fig. 1A). Moreover, we examined KLK6 from *Klk8*$^{-/-}$ mice. *Klk8*$^{-/-}$ mice were created using the gene from the 129/Sv genomic library and ES cells derived from 129/Ola mice [25], therefore these mice should have the same variant of KLK6 as 129X1/SvJ mice. As expected, *Klk8*$^{-/-}$ mice had the identical sequence in the coding region of *Klk6* mRNA to 129X1/SvJ mice, and KLK6 protein from *Klk8*$^{-/-}$ mice showed the same SDS-PAGE mobility as that from 129X1/SvJ mice (Fig. 1A).

The difference in SDS-PAGE mobility between KLK6 protein from 129X1/SvJ mice and that from C57BL/6 mice (1.2 kDa in weight) was greater than the difference predicted based on the amino acid sequences (0.052 kDa in weight). To confirm whether the differences of amino acid sequence caused the difference in SDS-PAGE mobility of KLK6 protein derived

from the two mouse strains, we produced recombinant KLK6 protein with a *myc* epitope and a polyhistidine tag and evaluated their mobility via SDS-PAGE and western blot analysis. We detected two bands in each recombinant KLK6 (Fig. 1B). A strong lower band was presumed to be pro-KLK6, and a weaker upper band was presumed to be prepro-KLK6, which had leaked from dead cells. The weights of these recombinant KLK6 proteins from C57BL/6 mice (KLK6-B6, Fig. 1B, left) were approximately 32.0 kDa (pro) and 35.4 kDa (prepro), whereas the weights of the recombinant KLK6 proteins from 129X1/SvJ mice (KLK6-129, Fig. 1B, right) were 33.5 kDa (pro) and 36.4 kDa (prepro). KLK6-129 showed reduced SDS-PAGE mobility compared with KLK6-B6, and this difference was similar to the result of SDS-PAGE analysis using spinal cord samples (Fig. 1A). These results suggest that the difference in amino acid sequence caused the difference of mobility of KLK6 protein in SDS-PAGE.

***Difference in enzymatic activity of KLK6***

Because the amino acid sequence of KLK6 protein differed between C57BL/6 and 129X1/SvJ mice, we investigated whether the enzymatic activity was also different. Uncleaved recombinant KLK6 proteins showed no enzymatic activity, as has been observed in other KLKs [28,29]. The prosequence of the recombinant KLK6 protein was therefore cleaved by lysyl endopeptidase, and the enzymatic activity of activated KLK6 protein was measured by hydrolysis activity using various peptidyl MCA compounds as substrates. First, we identified the optimum pH of the reaction buffer for substrate hydrolysis. For Boc-VPR-MCA, a substrate for α-thrombin and the best substrate yet known for KLK6, the optimum pH for KLK6-B6 was 8.0. In contrast, the optimum pH for KLK6-129 was 8.5, and good enzymatic activity was maintained under more alkaline conditions (Fig. 2A). For Boc-FSR-MCA and Boc-QAR-MCA, substrates for trypsin, the optimum pH for KLK6-B6 was 8.0, and that for KLK6-129 was 9.0 (Fig. 2B and C). We could not detect any enzymatic activity of either KLK6 protein for Suc-AAPF-MCA or Suc-LLVY-MCA, substrates for chymotrypsin. In the following experiments, we used the reaction buffer with the optimum

pH levels determined in these experiments.

Next, we quantitatively assessed the KLK6 enzymatic activity for the substrates examined above. For all substrates of which we could detect the hydrolysis reaction, KLK6-129 showed about 70% lower *Km*, more than four times higher $k_{cat}$ and more than 15 times higher $k_{cat}/Km$ than KLK6-B6 (Table 2). These results suggest that KLK6-129 has significantly higher enzymatic activity than KLK6-B6.

***Discussion***

In this study, we identified mRNA sequence differences in several *Klk* genes when comparing C57BL/6 mice and 129 mice (Table 1), some of which resulted in one or more different amino acid residues in the deduced protein. We considered that these amino acid differences may cause different enzymatic activity, resulting in some phenotypic differences between the mouse strains. Because KLK6 showed the most changes in the amino acid residues and we previously reported the functional roles of KLK6 in CNS [24], we focused on KLK6 and studied further. However, it is possible that other KLKs may show different enzymatic activity between the mouse strains. Therefore, we also need to study the enzymatic activity of other KLKs further to elucidate the involvement of KLKs in the phenotypic differences between mouse strains.

KLK6 protein from 129X1/SvJ mice showed reduced SDS-PAGE mobility compared with that from C57BL/6 mice (Fig. 1A). This difference of KLK6 mobility (1.2 kDa) was greater than the difference predicted based on the amino acid sequence differences (0.052 kDa).This

result strongly suggests that KLK6 protein from 129X1/SvJ mice may more glycosylated than that from C57BL/6 mice. Human KLK6 is reported to have N-glycosylation site at position $Asn^{132}$ [30], however, there are no report on glycosylation of mouse KLK6. KLK6 from C57BL/6 mice has putative N-glycosylation site at position $Asn^{190}$, and this site was not changed in KLK6 from 129X1/SvJ mice. Moreover, none of the different amino acid residues in KLK6 from 129X1/SvJ mice constituted the putative N-glycosylation site. In contrast, KLK6 from 129X1/SvJ mice had a new serin residue substituted for a prolin residue at position 54, and this serine residue may be glycosylated, contributing to the observed difference in mobility. Further study will be needed to better elucidate the cause of the difference in mobility.

Recombinant KLK6 protein from 129 mice (KLK6-129) had a higher optimum pH and more than 15 times higher enzymatic activity than that from C57BL/6 mice (KLK6-B6) (Fig. 2 and Table 2). However, none of the variations in KLK6-129 occurred at the reported catalytic active sites or substrate binding sites [30]. These differences may therefore affect secondary

or tertiary structure, leading to the modification of catalytic activity or substrate binding. Among the six different amino acids, $Leu^{68}$, $Gln^{69}$, and $Ile^{71}$ in KLK6-B6 comprise one β-strand, which is important for the spatial position of $His^{57}$, the essential residue shaping the active site [30]. The difference of these three amino acids may influence the secondary structure of this β-strand and thus the high enzymatic activity of KLK6-129. KLK6-129 may be a useful tool for understanding the mechanism of KLK6 catalytic activity.

The high enzymatic activity of KLK6 of 129 mice may cause pathological phenotypes in 129 mice. 129S3Svimj mice demonstrate greater autonomic dysreflexia after SCI than C57BL/6 mice [14]. 129T2Sv/EMS mice show larger areas of lesion and cavitation in damaged spinal cords after SCI than either C57BL/6 mice or BALB/c mice [13]. These reports suggest that the response to SCI was different between C57BL/6 mice and 129 mice. We previously reported that KLK6 is up-regulated in the spinal cord and has functional roles in the expression of myelin protein after SCI [22, 24]. The functions of KLK6 may cause the different response to SCI between C57BL/6 mice and 129 mice. The phenotypic differences

between the mouse strains were also reported in skin damages. 129/SvJ mice are more sensitive to 12-*O*-tetraecanoylphorbol-13-acetate (TPA)-induced inflammation and hyperplasia of skin than C57BL/6 mice [5]. 129/J mice are more sensitive to 7, 12-dimethylbenzanthracene (DMBA)-induced skin ulceration than C57BL/6 mice [4]. These reports suggest the different response to skin inflammation between C57BL/6 mice and 129 mice. Kishibe et al. reported that the expression of KLK6 is up-regulated in the epidermis after TPA application, suggesting the involvement of KLK6 in the inflammatory cascade by KLK family in skin [31]. The function of KLK6 may cause the different response to skin inflammation between C57BL/6 mice and 129 mice. The functional analysis of KLK6 in these phenotypic differences between 129 mice and other strains may provide new therapeutic approach to SCI and skin inflammation.

129 mouse substrains have commonly been used in transgenic and gene targeting experiments [1]. The gene-targeted mice produced from 129 mouse-derived ES cells are often backcrossed to C57BL/6 mice to change their genetic background. Consequently, most

genes derived from the 129 mouse genome are displaced by those derived from the C57BL/6 mouse genome. We have previously reported several studies using *Klk8*$^{-/-}$ mice [25,26,27]. As mentioned above, *Klk8*$^{-/-}$ mice were created using the gene from the 129/Sv genomic library and 129/Ola mice-derived ES cells [25], and were backcrossed to C57BL/6 mice through at least five generations. However, because of the close genetic proximity of the *Klk6* gene to the *Klk8* gene on chromosome 7 (≈ 35 Kb), the *Klk6* gene derived from 129 mice was inherited together with the *Klk8*-targeting construct, and the *Klk6* gene of 129 mice was expressed in *Klk8*$^{-/-}$ mice despite the genetic background being derived from C57BL/6 mice. The results in this study showing the expression of KLK6 protein from 129 mice in *Klk8*$^{-/-}$ spinal cord (Fig. 1A) and high enzymatic activity of KLK6-129 (Table 2) suggest the possibility that the highly active KLK6 protein from 129 mice may cause the additive phenotype of *Klk8*$^{-/-}$ mice in spinal cord damage such as SCI and EAE [26,27]. It has also been reported that the gene derived from 129 ES cells affects the phenotype of the gene-targeted mice despite backcrossing onto a C57BL/6 background [32]. It is important to

be aware of the potential influence of several genes derived from ES cell lines in gene targeting experiments.

***Conclusion***

We have reported differences in mRNA and deduced amino acid sequences of *Klk* genes between mouse strains, and have studied the enzymatic activities of KLK6. KLK6 from 129 mice had higher enzymatic activity than KLK6 from C57BL/6 mice. Further study of the differences between mouse strains may provide new information to understand the contribution of KLK6 in physiological and pathological conditions. Finally, analysis of the highly active KLK6 from 129 mice may be useful for understanding the mechanism of KLK6 catalytic activity and the development of novel KLK6-targeting drugs.

**Abbreviations**

KLK, kallikrein-related peptidase; CNS, central nervous system; SCI, spinal cord injury; EAE, experimental autoimmune encephalomyelitis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCA, 4-methylcoumaryl-7-amide; Boc-VPR-MCA, *t*-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine 4-methylcoumaryl-7-amide; Boc-FSR-MCA, *t*-butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine 4-methylcoumaryl-7-amide; Boc-QAR-MCA, *t*-butyloxycarbonyl-L-glutaminyl-L-alanyl-L-arginine 4-methylcoumaryl-7-amide; Suc-AAPF-MCA, succinyl-L alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-methylcoumaryl-7-amide; Suc-LLVY-MCA, succinyl-L leucyl-L-leucyl-L-valyl-L-tyrosine 4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin.

***Conflict of interest***

There is no conflict of interest in this study.

***Acknowledgment***

All authors contibuted to design this research. Dr. Tanaka contributed to production of recombinant KLK6. Dr. Murakami contributed to all experiments in this study. We are grateful to Mr. K. Hazawa for his technical assistance.

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***Figure legends***

**Figure 1.**

Representative image of western blot analysis of KLK6 protein from spinal cord tissue using anti-KLK6 antibody (A) and purified recombinant KLK6 using anti-c-Myc antibody (B). (A) Lane 1, wild-type C57BL/6 mice; lane 2, *Klk8*$^{-/-}$ mice; lane 3, 129X1/SvJ mice; lane 4, BALB/c mice; lane 5, ICR mice. GAPDH was used as an internal control. (B) Left, KLK6-B6; right, KLK6-129. 500 ng protein was loaded onto each lane. The upper and lower bands were presumed to be prepro-KLK6 and pro-KLK6 respectively.

**Figure 2.**

Optimum pH for KLK6-B6 and KLK6-129 for hydrolysis of Boc-VPR-MCA (A), Boc-FSR-MCA (B), and Boc-QAR-MCA (C). Then, 100 μM of the substrates indicated were incubated with 40 ng of activated recombinant KLK6 at 37 °C for 30 min and AMC intensity was measured. Graphs represent the mean percentage of the maximum intensity ± S.D. for

three individual experiments in reaction buffer at each pH.

| KLK | Nucleotide difference [a, c] | Amino acid difference [b, c] |
|---|---|---|
| KLK1 | G126A | No |
| KLK4 | G44A, C117T, T477C, C609G | No |
| | T752C | I251T |
| KLK5 | G228A, C738T, G810C | No |
| | A10G | T4A |
| | A137G | T46S |
| KLK6 | C160T | P54S |
| | C202A | L68M |
| | C205G | Q69E |
| | A211T | I71F |
| | A383G | K128R |
| | G509A | R170H |
| KLK7 | G24C, G75A, T420C, C711T | No |
| KLK8 | C579G, C666T | No |
| KLK9 | G133A | R45Q |
| KLK10 | A348T, A396G, T444C | No |
| | A581G | K194R |
| KLK11 | A174C, C705T | No |
| KLK12 | T75C, A684G | No |
| | A353G | H118R |
| | T725C | I242T |
| KLK13 | No | No |
| KLK14 | T166C, A177G, G363A, T714C | No |
| KLK15 | C51T, T120C, G126T, T129C, T273C, C288T, T315C, T390C, T399C, C705A | No |
| | C474A | H158Q |
| | G583A | V195I |

| | G751A | V251M |
|---|---|---|

**Table 1.** Nucleotide and amino acid differences of KLK family members between C57BL/6 mice and 129 mice. The accession numbers of *Klk* mRNAs of C57BL/6 mice and 129 mice were described in Supplementary Table.

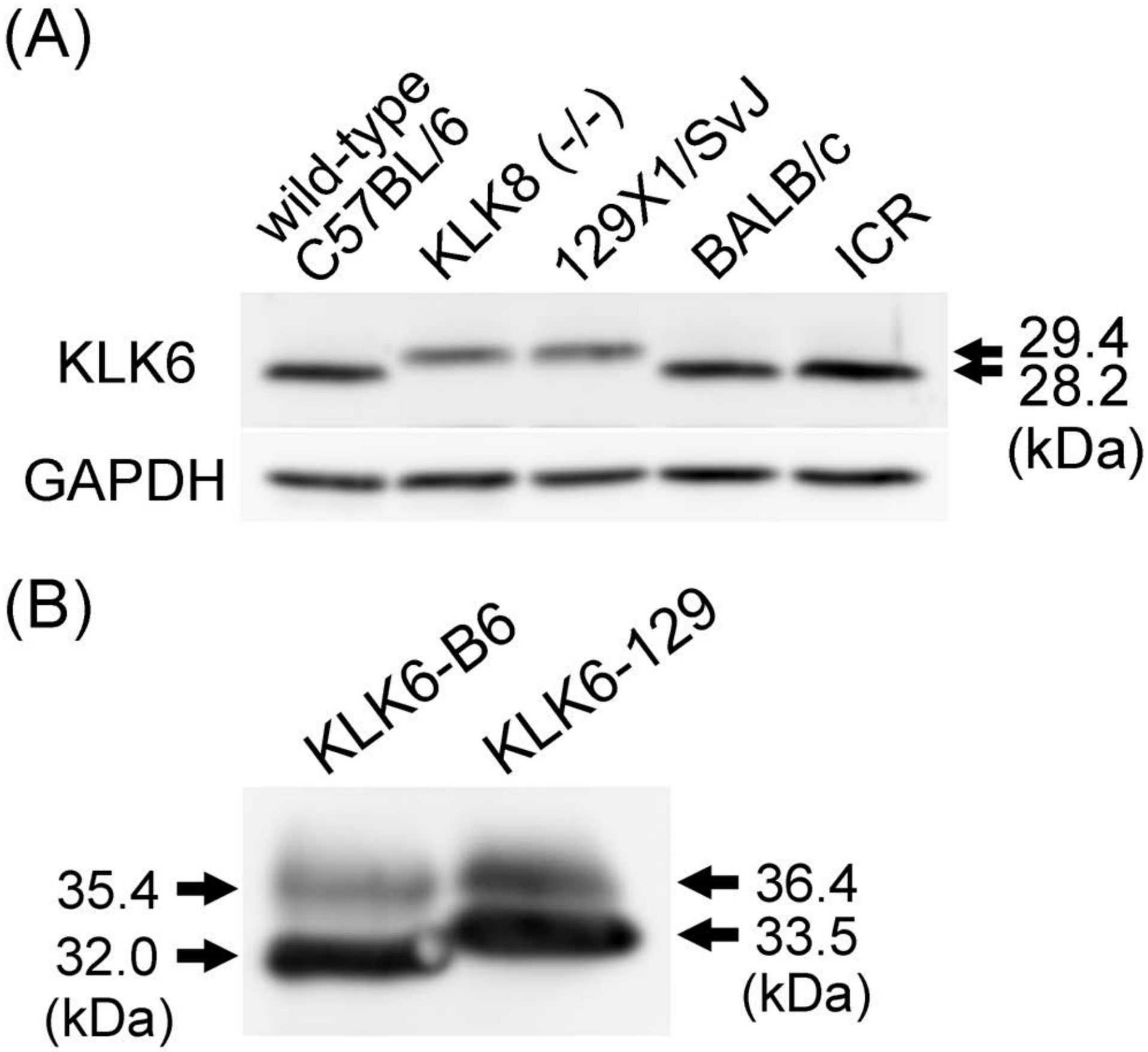
[a] The numbers in the "Nucleotide difference" column are the relative positions from the putative translation start site, defined as 1.

[b] The numbers in the "Amino acid difference" column are the relative positions from the methionine residue of the putative translation start site.

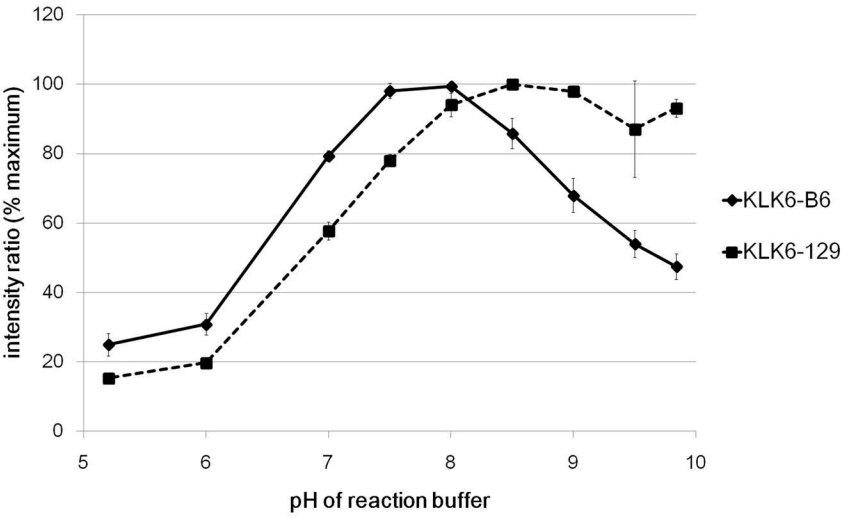
[c] "No" indicates that there is no difference in mRNA or amino acid sequence.

| Substrate | *Km* (μM) | | | $k_{cat}$ ($min^{-1}$) | | | $k_{cat}$/Km ($\mu M^{-1}min^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | KLK6-129 | KLK6-B6 | 129 / B6 (%) | KLK6-129 | KLK6-B6 | 129 / B6 (%) | KLK6-129 | KLK6-B6 | 129 / B6 (%) |
| Boc-VPR-MCA | 80.5 ± 2.3 | 300 ± 41 | 26.8 | 719 ± 33 | 178 ± 52 | 404 | 8.94 ± 0.66 | 0.585 ± 0.138 | 1528 |
| Boc-FSR-MCA | 34.8 ± 1.5 | 147 ± 23 | 23.7 | 368 ± 23 | 85.0 ± 16.9 | 433 | 10.6 ± 0.8 | 0.582 ± 0.123 | 1821 |
| Boc-QAR-MCA | 218 ± 22 | 664 ± 14 | 32.8 | 844 ± 105 | 132 ± 38 | 639 | 3.87 ± 0.18 | 0.197 ± 0.042 | 1964 |

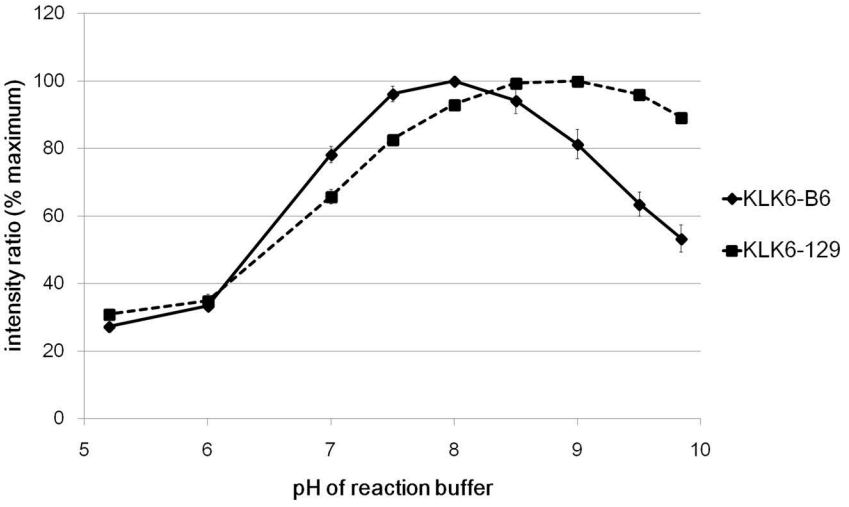
**Table 2.** Kinetic constants of KLK6-B6 and KLK6-129 for various peptidyl MCA compounds. 80–120 ng (KLK6-B6) or 20–30 ng (KLK6-129) of activated recombinant KLK6 was incubated with 6.25–200 μM of the substrates indicated at 37 °C for 5 min and AMC intensity was measured. Each value for KLK6-B6 and KLK6-129 represents the mean ± S.D. for three individual experiments. The differences in the values between KLK6-B6 and KLK6-129 were all significant ($p < 0.01$).


(A)
wild-type C57BL/6
KLK8 (-/-)
129X1/SvJ
BALB/c
ICR
KLK6
GAPDH
29.4
28.2
(kDa)
(B)
KLK6-B6
KLK6-129
35.4
32.0
(kDa)
36.4
33.5
(kDa)

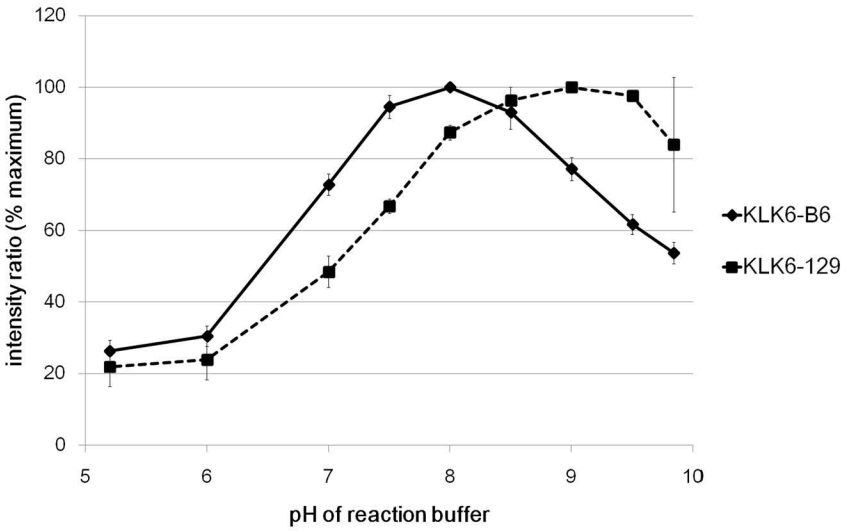
(A) Boc-VPR-MCA




(B) Boc-FSR-MCA




(C) Boc-QAR-MCA



| *Klk* | Primer pairs | Primer position [a] | Accession No. (C57BL/6 mice) | Accession No. (129 mice) |
|---|---|---|---|---|
| *Klk1* | Forward ; AGCTCCAAGATCACTGCCTGCA | -42 to -21 | NM_010639 | AB981036 |
| | Reverse ; CATTTGCTGCATGGTGGGCTT | 813 to 834 | | |
| *Klk4* | — | — | BC100716 | AF198031 [b] |
| *Klk5* | Forward ; CAGAACCACTTAGCCTCGAC | -47 to -28 | NM_026806 | AB981037 |
| | Reverse ; ACATAGAGCAAGTTCTGGAGAGACT | 969 to 993 | | |
| *Klk6* | Forward ; GCAGCCTTCTGTCAGTCTGC | -164 to -145 | NM_011177 [c] | AB981038 |
| | Reverse ; AGCTGGACATCTGAAGGCTCT | 826 to 846 | | |
| *Klk7* | Forward ; AGACACTGGTCTCCGGATCAA | -87 to -67 | NM_011872 | AF339930 [b] |
| | Reverse ; CTTTACAGGGATGCCACGAT | 804 to 823 | | AB981039 |
| *Klk8* | Forward (1) ; AAGACAGCTCCGGAAACACCTCCT | -106 to -83 | NM_008940 | AB981040 |
| | Reverse (1) ; ACACACCAGGATCAGTCCCTGT | 773 to 794 | | |
| | Forward (2) ; CCCACTGCAAAAAACAGAAG | 215 to 234 | | |
| | Reverse (2) ; GCCAATGGTGGTGTTTATTGAC | 811 to 832 | | |
| *Klk9* | Forward (1) ; AATCACAGGGCGGCCCCAAC | -103 to -84 | NM_028660 | AB981041 |
| | Reverse (1) ; GCACAGCTGGAGTCAGCCGG | 363 to 382 | BC109326 | |
| | Forward (2) ; GTGGAGCTGACACCCGTGCC | 50 to 69 | | |
| | Reverse (2) ; GGGCGGAGTTTCATCATGGGGCGT | 870 to 887 | | |
| *Klk10* | Forward ; TTTGCAGCCCACACTGCAGGGCAA | -103 to -80 | NM_133712 | AB981042 |
| | Reverse ; GGGACTGCTAGCTTCCTTGGGGTA | 948 to 971 | | |
| *Klk11* | Forward (1) ; TTGTCCCACATCTGACTAGGGA | -107 to -86 | NM_001177373 | AB981043 |
| | Reverse (1) ; AACTGGGGGCTGGACGTGGT | 523 to 542 | | |
| | Forward (2) ; GTGGGGCAACCCTCATCGCC | 218 to 237 | | |
| | Reverse (2) ; ATTCCAGGCCCCAGGTCGAT | 984 to 1003 | | |
| *Klk12* | Forward ; TTCCTGGCAGACCCTGGAGG | -29 to -10 | NM_027097 | AB981044 |
| | Reverse ; AGGAGACCTTGGTGCTCCGA | 794 to 813 | | |
| *Klk13* | Forward (1) ; TTAGGCCTGGCACCTATGGTGGCGT | -133 to -109 | NM_001039042 | AB981045 |
| | Reverse (1) ; GCCAGGTCGGTTGGGCTGTC | 722 to 741 | BC063763 | |
| | Forward (2) ; CCAGCTCAGCAGCCACGTCC | 405 to 424 | | |
| | Reverse (2) ; GGAGGCGGGCAAATGAACAC | 836 to 855 | | |
| *Klk14* | Forward ; CCTTCCACTTGCTCTCTGCA | -49 to -30 | NM_174866 | AB981046 |
| | Reverse ; GCACAGAGACAGCTGGGGAT | 774 to 793 | | |
| *Klk15* | Forward (1) ; CAGATCTCTAAGACCCGGAC | -31 to -12 | NM_174865 | AB981047 |
| | Reverse(1) ; CCCAGCCTGACACCACGCAG | 405 to 424 | BC106965 | |
| | Forward (2) ; TGTCTGCAGCGCAGGATGGC | 32 to 51 | | |
| | Reverse(2) ; GTCAGAGTCCCAGGTGGTCC | 794 to 813 | | |

**Supplementary Table** Primers pairs and accession numbers of KLKs.

[a] The numbers in the “Primer position” column are the relative positions from the putative translation start site, which is defined as “1”.

[b] The accession numbers for the *Klk4* and *Klk7* genes in 129 substrains have already been registered in GenBank.

[c] The indicated accession number is for *Klk6* mRNA from BALB/c mice, and the sequence is identical to C57BL/6 mice, as described in the manuscript.