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Purification and characterisation of blarinasin, a new tissue kallikrein-like protease from the short-tailed shrew *Blarina brevicauda* – comparative studies with blarina toxin

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Running Title : *Kallikrein-like Protease from the Short-tailed Shrew*

Abstract

A new tissue kallikrein-like protease, blarinasin, has been purified from the salivary glands of the short-tailed shrew *Blarina brevicauda*. Blarinasin is a 32-kDa N-glycosylated protease with isoelectric values ranging between 5.3 and 5.7, and an optimum pH of 8.5 for enzyme activity. The cloned blarinasin cDNA coded for a pre-pro-sequence and a mature peptide of 252 amino acids with a typical catalytic triad for serine proteases and 43.7-54.0% identity to other mammalian tissue kallikreins. Blarinasin preferentially hydrolyzed Pro-Phe-Arg-4-methylcoumaryl-7-amide (MCA) and N-*tert*-butyloxycarbonyl-Val-Leu-Lys-MCA, and preferentially converted human high molecular weight kininogen (HK) to bradykinin. The activity of blarinasin was prominently inhibited by aprotinin (K_i = 3.4 nM). Similar kallikrein-like protease, a lethal venom blarina toxin, has previously been purified from the salivary glands of the shrew *Blarina* and shows 67.9% identity to blarinasin. However, blarinasin was no toxic in mice. Blarinasin was a most abundant kallikrein-like protease and represented 70-75% of kallikrein-like enzymes in the salivary gland of *B. brevicauda*.

Key words: Amino acid sequence analysis; Blarinasin; Blarina toxin; Salivary glands; Short-tailed shrew; Tissue kallikrein

The American short-tailed shrew *Blarina brevicauda* (Say, 1923) is one of the few venomous mammals in the world (Dufton, 1992). This shrew produces a potent salivary venom that may cause paralysis and death due to respiratory failure and circulatory malfunctions in mouse-sized animals (Pucek, 1968; Pearson, 1942). *B. brevicauda* eats insects and also vertebrates, including some larger than themselves, such as murid rodents and frogs (Churchfield, 1990; Babcock, 1914). Hence, this shrew may use venom to catch its prey.

According to the fossil record, the Insectivora may one of the longest established mammalian orders and probably that from which other placental orders are ultimately descended (Crompton *et al.*, 1978; Crompton and Jenkins, 1979). Thus, enzymes produced by insectivores are expected to be more primitive than their counterparts in higher mammals, and may provide important information concerning enzyme structure and function in the mammals. Few serine proteases from the Insectivora have been identified. A 30 kDa tissue kallikrein from the salivary gland of the Eastern Atlantic mole *Scalopus aquaticus* has been partially purifed (Richards *et al.*, 1996), and several serine proteases, such as factor X and neutrophil elastase, have been cloned from a spleen cDNA library of the platypus *Ornithorhynchus anatinus* (Poorafshar *et al.*, 2000).

Due to the interesting ecology of shrews, we have sought to isolate biologically active substances from the shrew *B. brevicauda*. Recently, we have purified and characterised the blarina toxin (BLTX), a lethal mammalian venom from the submaxillary and sublingual glands (Kita *et al.*, 2004). It consists of a mature peptide of 253 amino acids with a high degree of identity with tissue kallikrein. Mice administered BLTX developed irregular respiration, paralysis and convulsions before dying. BLTX converts kininogens to kinins and caused vasodilation, though the mechanisms underlying its

lethal toxicity have not been characterised. On the course of BLTX purification, we found a new kallikrein-like protease named blarinasin from *B. brevicauda* salivary glands. We report here the purification, characterisation and molecular cloning of blarinasin and discuss its structure and enzymatic properties compared to those of BLTX.

Blarinasin was purified from extracts of the shrew *B. brevicauda* submaxillary and sublingual glands (salivary glands) through a series of standard chromatographic procedures. BLTX fraction identified by toxicity was completely separated from blarinasin by a Mono-Q anion exchange column chromatography on the course of purification (Kita *et al.*, 2004). Enzymatic activity was assessed by measuring the proteolysis of Pro-Phe-Arg-4-methyl-coumaryl-7-amide (MCA). Proteolytic activity coincided with a protein peak on analytical gel permeation, anion-exchange, and hydrophobic high performance liquid chromatography. Starting from 17.1 mg of shrew salivary glands, 32.3 μg of purified blarinasin was obtained with a total recovery of 4.7% and a 5.58-fold purification from the crude extract. Blarinasin was unstable and lost its activity under gel permeation chromatography at room temperature as well as by freezing and thawing. Purified blarinasin yielded a single protein band with a molecular mass of approximately 32 kDa under reducing conditions on SDS/PAGE (Figure 1A). A comparison of the 2D-PAGE pattern of the crude *B. brevicauda* salivary gland extract with a purified blarinasin revealed that blarinasin migrated as 8 major spots with similar molecular sizes and different isoelectric points between 5.3 and 5.7 (arrows **4** to **11**, Figure 1B). BLTX migrated as 3 spots (arrows **1** to **3**) with higher isoelectric points than most of the blarinasin spots on 2D-PAGE. Based on the specific activity of blarinasin and the 2D-PAGE pattern of extracted salivary glands, blarinasin was estimated to be 70% to 75% of the total kallikrein activity found in salivary glands. By comparison, BLTX

(Kita *et al.*, 2004) accounts for approximately 5-fold less activity than blarinasin. Blarinasin, up to 20 $mg \cdot kg^{-1}$, *i.p.*, was not toxic in mice, whereas the LD_{50} of BLTX was approximately 1 $mg \cdot kg^{-1}$ (data not shown).

Blarinasin cDNA was cloned from *B. brevicauda* submaxillary and sublingual glands by PCR using degenerate oligonucleotides designed from the sequences of the N-terminus, tryptic peptide fragments, and the consensus sequences of active sites of serine proteases. The isolated cDNA consisted of 962 bp and contained a single putative open reading frame of 843 bp with a proposed initiation codon of ATG at nt 42 and a 3'-untranslated region of 78 bp with a polyadenylation signal (AATAAA) and a poly(A) tail. The deduced amino acid sequence of blarinasin, the N-terminal 15 residues of amino acid sequence starting from an I-V-G-G serine protease motif and the sequences of 7 trypsin cleavage fragments (K1-K7), are shown in Figure 2. Blarinasin was composed of 281 amino acids with a pre-pro-sequence and an active mature protein composed of 252 amino acids. The predicted molecular mass of blarinasin was 28.0 kDa. Due to the presence of one predicted N-glycosylation site (Asn^{122}), purified blarinasin was treated with *N*- and/or *O*-glycosidase under denaturing conditions. After treatment with N-glycosidase, the 32 kDa blarinasin protein band shifted to a single 27-28 kDa band on SDS/PAGE, being consistent with the molecular mass of the mature polypeptide backbone of blarinasin calculated (data not shown).

The optimum pH for blarinasin enzyme activity was 8.5, identical to that reported for tissue kallikreins (Mason *et al.*, 1983; Fukushima *et al.*, 1985), and nearly equal to that of BLTX (Kita *et al.*, 2004). Table 1 shows the substrate specificities of blarinasin and BLTX. Blarinasin preferentially hydrolysed substrates with an Arg or Lys at the P1 position and a hydrophobic amino acid at the P2 position. Of the substrates tested,

Boc-Val-Leu-Lys-MCA was the best substrate with specific activity of 6.82 U/mg protein. Boc-Glu-Lys-Lys-MCA, Boc-Glu(OBzl)-Ala-Arg-MCA, Boc-Val-Pro-Arg-MCA, Z-Phe-Arg-MCA and Pro-Phe-Arg-MCA, which are synthetic substrates for plasmin, factor XIa, α-thrombin, plasma kallikrein, and tissue kallikrein, respectively, were the second best substrates, although the activities with these substrates were 3% to 5% of that with Boc-Val-Leu-Lys-MCA. Substrates with hydrophobic amino acids, such as Tyr, Phe, Ala, and Pro, at P1 position were also hydrolysed, but at only 0.1% to 1% that with Boc-Val-Leu-Lys-MCA. On the other hand, BLTX showed a narrow substrate specificity and both Pro-Phe-Arg-MCA and Boc-Val-Leu-Lys-MCA were the best substrates with specific activities of 267.4 and 244.1 mU/mg protein, respectively.

The activity of blarinasin was markedly inhibited by aprotinin at the concentration of 0.1 μM (K_i = 3.4 × 10^{-9} M), moderately inhibited by secretory leukoprotease inhibitor (SLPI, K_i = 8.7 × 10^{-8} M), leupeptin, benzamidine, phenylmethanesulfonyl fluoride (PMSF) at the concentration of 1 μM, and not inhibited by EDTA or α_1-protease inhibitor (α_1PI) (Table 2). The inhibitor spectrum of blarinasin shows the significant similarity with that of BLTX.

Blarinasin converted human high molecular weight kininogen (HK) into a major 58-63 kDa protein fragment and various smaller peptides in a time-dependent manner on SDS/PAGE analysis (data not shown). The major peptide generated after HK hydrolysis by blarinasin was bradykinin (BK) (Figure 3), being similar to those generated by BLTX and porcine pancreas kallikrein (PPK).

Amino acid sequence of mature blarinasin bore the highest identity of 67.9% with BLTX, and identities of 54.0, 54.0, 53.6, 54.0, 44.0, 43.7, 32.9, and 30.0 % with,

respectively, monkey glandular kallikrein from the crab-eating macaque *Macaca fascicularis* (Lin *et al.*, 1993), dog pancreas kallikrein from *Canis familiaris* (Gauthier *et al.*, 1994), human tissue kallikrein 1 (hK1) (Fukushima *et al.*, 1985), human prostate specific antigen (PSA) (Watt *et al.*, 1986), mouse tissue kallikrein 1 (mGK1) (Mason *et al.*, 1983), rat urinary kallikrein 1 (RUK1) (Swift *et al.*, 1982), human plasmin (Malinowski *et al.*, 1984), and human plasma kallikrein (huPK) (Chung *et al.*, 1986) (Figure 4). Blarinasin has highly conserved residues flanking the residues of the catalytic triad (His^{72}, Asp^{138}, and Ser^{233}), and 10 highly conserved cysteine residues that may form disulfide bonds and stabilise the catalytic pocket. It has a characteristic motif (residues 114-132) containing a unique insertion of 10 residues, D^{123}TYYDEISLG^{132}. BLTX has a non-homologous insertion of 10 residues, L^{123}TFFYKTFLG^{132}, in the same position. To our knowledge, these characteristic insertions near the position of the presumed catalytic triad (Asp^{138}) are rare, and have been shown only in the sequences of gila toxin (GTX) and horridum toxin, venoms from the Mexican lizard *Heloderma horridum* (Utaisincharoen *et al.*, 1993). The pre-pro-sequence of blarinasin also showed high similarity to those of mammalian tissue kallikreins listed in Figure 4 except for a unique insertion of 6 residues, P^{20}GPSIE^{25}.

Blarinasin hydrolysed Boc-Val-Leu-Lys-MCA most rapidly, and its specific activity with this substrate was 27.9-fold higher than that of BLTX. On the other hand, BLTX preferentially hydrolysed Pro-Phe-Arg-MCA and the specific activity of BLTX was 1.37-fold higher than that of blarinasin. The former substrate for blarinasin and its analogues, such as D- and/or DL-Val-Leu-Lys-pNA, are known as general substrates for plasmin. The substrate specificity of blarinasin is similar to those of hK1 (Fujimoto *et al.*, 1990), PPK (Blaber *et al.*, 1989), and RUK1 (Bedi, 1982). These observations suggest

that a lysine at the P1 site is favoured for blarinasin. As with BLTX, aprotinin and SLPI efficiently inhibited the activity of blarinasin. In contrast, UTI, benzamidine, and PMSF moderately inhibited the activity of blarinasin at concentrations of 1 μM, but these inhibitors inhibited the activity of BLTX a little (Kita *et al.*, 2004). Despite the similarities between the amino acid sequences and the enzymatic properties of blarinasin and BLTX, blarinasin did not show any toxicity against mice, as observed with other mammalian tissue kallikreins.

In general, tissue kallikreins are acidic N- and O- glycoproteins with molecular heterogeneity. Both blarinasin and BLTX showed micro-heterogeneous by N-glycosylation. Analysis of blarinasin and BLTX by SDS/PAGE revealed different molecular size bands (32 and 35 kDa), though, after deglycosylation, these bands were shifted to a single band with a molecular size of 27-28 kDa. Based on the typical N-glycosylation motif (Asn-Xaa-Ser/Thr), blarinasin has a putative N-glycosylation site at Asn^{122} (Figure 2), while BLTX possesses two. The difference in the glycosylation may play a role in the different toxicity and enzymatic properties of blarinasin and BLTX.

Among lizard venoms, GTX and horridum toxin are classified as kallikrein analogues because of their amino acid sequences and protease activities (Utaisincharoen *et al.*, 1993; Hendon and Tu, 1981; Datta and Tu, 1997). The LD_{50} values of GTX and horridum toxin injected are 2.5 $mg \cdot kg^{-1}$ body weight, *i.v.*, but lethality may be synergistically increased approximately 8-fold when combined. In contrast, the toxicity of BLTX was not influenced by blarinasin. Although the *in vivo* substrates of these two kallikrein-like proteases have not been clarified, blarinasin may be a typical tissue kallikrein-like protease secreted in the *B. brevicauda* salivary glands, similar to other mammalian tissue kallikreins. On the other hand, BLTX cleaves substrates that result in

products that cause paralysis and respiratory failure.

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Table 1 Comparison of the Substrate Specificities of Blarinasin and Blarina Toxin.

	Relative activity (%)	
Substrate	Blarinasin	BLTX
Pro-Phe-Arg-MCA	100	100
Arg-MCA	8.6	0.0
Boc-Ala-Gly-Pro-Arg-MCA	9.0	19.4
Boc-Gln-Ala-Arg-MCA	30.6	7.8
Boc-Gln-Arg-Arg-MCA	3.5	0.0
Boc-Gln-Gly-Arg-MCA	10.0	0.0
Boc-Glu-Lys-Lys-MCA	153.2	18.0
Boc-Glu(OBzl)-Ala-Arg-MCA	162.0	35.0
Boc-Glu(OBzl)-Gly-Arg-MCA	172.5	5.3
Boc-Gly-Arg-Arg-MCA	26.6	0.0
Boc-Leu-Lys-Arg-MCA	0.0	10.7
Boc-Leu-Shr-Thr-Arg-MCA	4.0	0.0
Boc-Leu-Thr-Arg-MCA	39.5	2.2
Boc-Phe-Ser-Arg-MCA	55.6	8.1
Boc-Val-Leu-Lys-MCA	3482.2	91.3
Boc-Val-Pro-Arg-MCA	167.7	49.5
Bz-Arg-MCA	12.1	0.0
Glt-Gly-Arg-MCA	1.2	0.0
Suc-Ala-Ala-Pro-Phe-MCA	13.1	0.0
Suc-Leu-Leu-Val-Tyr-MCA	4.4	0.0
Z-Phe-Arg-MCA	119.3	24.3
Suc-Ala-Pro-Ala-MCA	33.9	0.0
Suc-Gly-Pro-MCA	28.2	0.0
Boc-Gly-Lys-Arg-MCA	0.0	3.9
Boc-Ile-Glu-Gly-Arg-MCA	0.0	0.0
Boc-Leu-Arg-Arg-MCA	0.0	0.0
Z-Arg-Arg-MCA	0.0	0.0

Enzyme activity using peptidyl-MCA substrates was analysed as previously described (Kido *et al.*, 1992). Reactions were initiated by adding enzyme samples to 0.1 mM substrate in 50 mM Tris/HCl buffer, pH 8.5, in a total volume of 0.5 ml. Relative activities are expressed as percentages of the activity toward Pro-Phe-Arg-MCA (blarinasin, 7.02 mU/ml; BLTX, 5.68 mU/ml). Boc = *t*-butoxycarbonyl; Z = benzyloxycarbonyl; Bz = benzoyl; Suc = succinyl.

Table 2 Comparison of the Inhibitory Effects of Protease Inhibitors and EDTA on the Proteolytic Activity of Blarinasin and Blarina Toxin.

Inhibitor	Concentration (μM)	Residual activity (%)	
		Blarinasin [a]	BLTX [b]
None	–	100	100
Aprotinin	0.1	19	22
Leupeptin	1	44	65
KSTI	1	88	67
α_1-Protease inhibitor	1	96	113
UTI	1	80	108
SLPI	1	26	50
Benzamidine	1	48	78
PMSF	1	40	85
EDTA	10,000	94	99

Blarinasin and BLTX were pre-incubated with various inhibitors for 5 min at 37 °C and enzyme reaction was started by addition of substrate in 50 mM Tris/HCl buffer, pH 8.5. Residual activity was calculated as a percentage of that of the enzyme without inhibitor. KSTI = Kunitz-type soybean trypsin inhibitor; SLPI = secretory leukoprotease inhibitor; PMSF = phenylmethanesulfonyl fluoride; UTI = urinary trypsin inhibitor.

[a] Substrate, Boc-Val-Leu-Lys-MCA.

[b] Substrate, Pro-Phe-Arg-MCA.

Figures

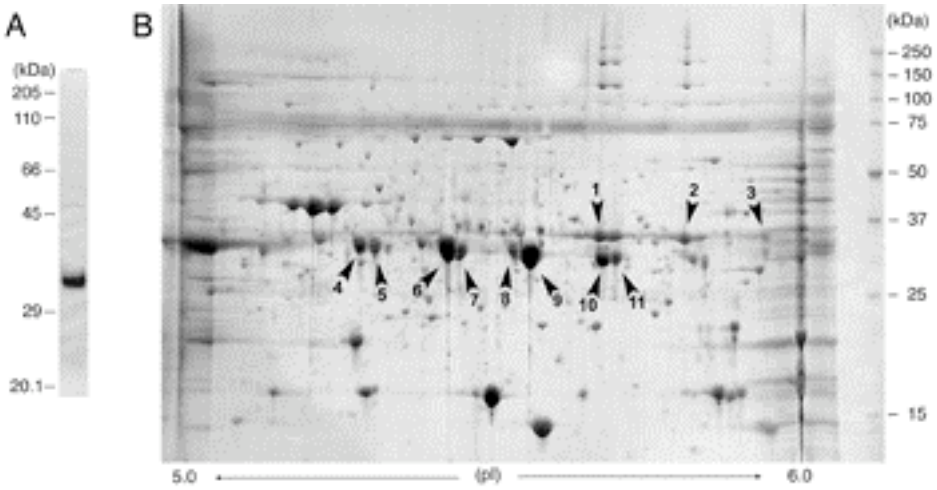

Figure 1 SDS/PAGE Analysis of Purified Blarinasin (A) and 2D-PAGE Analysis of the Extracts of *Blarina* Salivary Glands (B).

(A) SDS/PAGE analysis of blarinasin under reducing conditions. Purified blarinasin (180 ng) was run on 12.5% polyacrylamide gel, and protein bands are shown by silver staining. (B) Analysis of the extracts of *Blarina* salivary glands by 2D-PAGE. Cysteine residues were carbamidomethylated with iodoacetamide. The protein bands were stained with SYPRO Ruby and analysed at excitation and emission wavelengths of 532 nm and 610 nm, respectively. Arrows **1** to **3**, and **4** to **11** indicate the spots of BLTX and blarinasin, respectively.

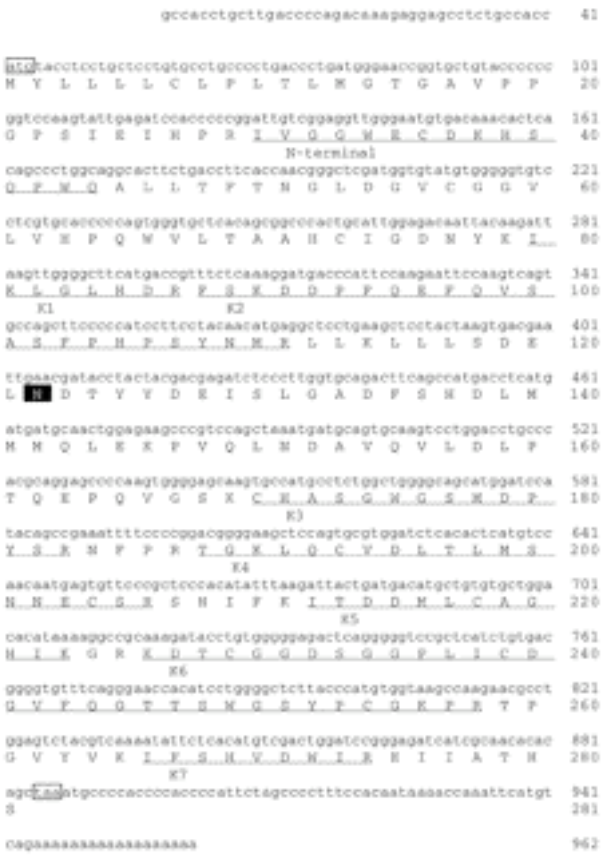

Figure 2 Nucleotide Sequence of cDNA and Deduced Amino Acid Sequence of Blarinasin.

Analysed amino acid sequences of the N-terminal 15 residues of the purified blarinasin and the tryptic peptides (K1-K7) are underlined. The translation-initiation site (ATG) and stop codon (TAA) are boxed. The putative *N*-glycosylation site in the blarinasin sequence is highlighted in black. Isolation of the total RNA from the submaxillary and sublingual glands of *B. brevicauda*, preparation of first-strand cDNA template, and RT-PCR using degenerate oligonucleotides designed from conserved amino acid sequences within the active sites of mammal kallikreins were carried out as described previously (Kita *et al.*, 2004). The 22 independent clones were sequenced, 7 matched the sequences corresponding to the amino acid sequences within blarinasin. BLTX and

one minor tissue kallikrein isoform were obtained from the other clones. Two cycles of 3'-rapid amplification of cDNA ends (RACE) were carried out. A reaction mixture for first cycle contained two primers KLK-gene1 and Oligo-dT, and used the blarinasin cDNAs described above as templates. The reaction mixture for second cycle contained the two primers KLK-gene2 and Oligo-dT and the PCR products of the first reaction. Nucleotide sequences of KLK-gene1 and KLK-gene2, the gene specific primers of blarinasin, and Oligo-dT are 5'- GGCAGCATGAATCCATACAGC -3', 5'- ATGAGTGTTCCCGCTCCCAC -3', and 5'- CGCAGGAATTTTTTTTTTTTTTT -3', respectively. For the 5'-RACE reaction, PCR was performed using a universal primer mix (long; 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3', short; 5'-CTAATACGACTCACTATAGGGC -3'), and the gene specific primer, KLK-gene3, whose sequence is 5'- GATCTCGTCGTAGTAGGTATCGTTCA -3'. The full-length blarinasin cDNA was confirmed by PCR, using KLK-gene4 and KLK-gene5. Nucleotide sequences of KLK-gene4 and KLK-gene5, are 5'- GCCACCATGTACCTCCTGCTCCT -3' and 5'- GCATTTAGCTGTGTGTTGCGAT -3', respectively. The PCR conditions were as follows: an initial denaturing at 94°C for 3 min, followed by 30 cycles of 30 sec of denaturing at 94°C, 30 sec of annealing at 60°C, and 60 sec of extension at 72°C, and a final extension at 72°C for 7 min. The amplified DNA fragments were subcloned and 7 independent clones were sequenced. All clones revealed an identical sequence.

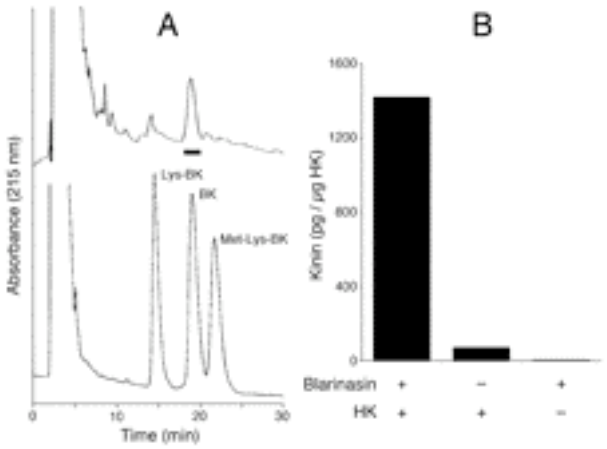

Figure 3 Kinin-releasing Activity of Blarinasin.

HK at a dose of 10 μg was incubated with 400 ng of blarinasin in 50 mM Tris·HCl, pH 8.5, at 37°C for 1 h as previously described (Kita *et al.*, 2004). (A) After incubation, products in the reaction mixture were separated by HPLC. The upper and lower traces show the peptides released from HK after incubation with blarinasin and standard kinins (50 ng each), respectively. The solid bar shows BK. (B) Kinin concentrations in the reaction mixtures were determined with a competitive enzyme-linked immunosorbent assay (ELISA) using a Markit-M bradykinin kit (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan).

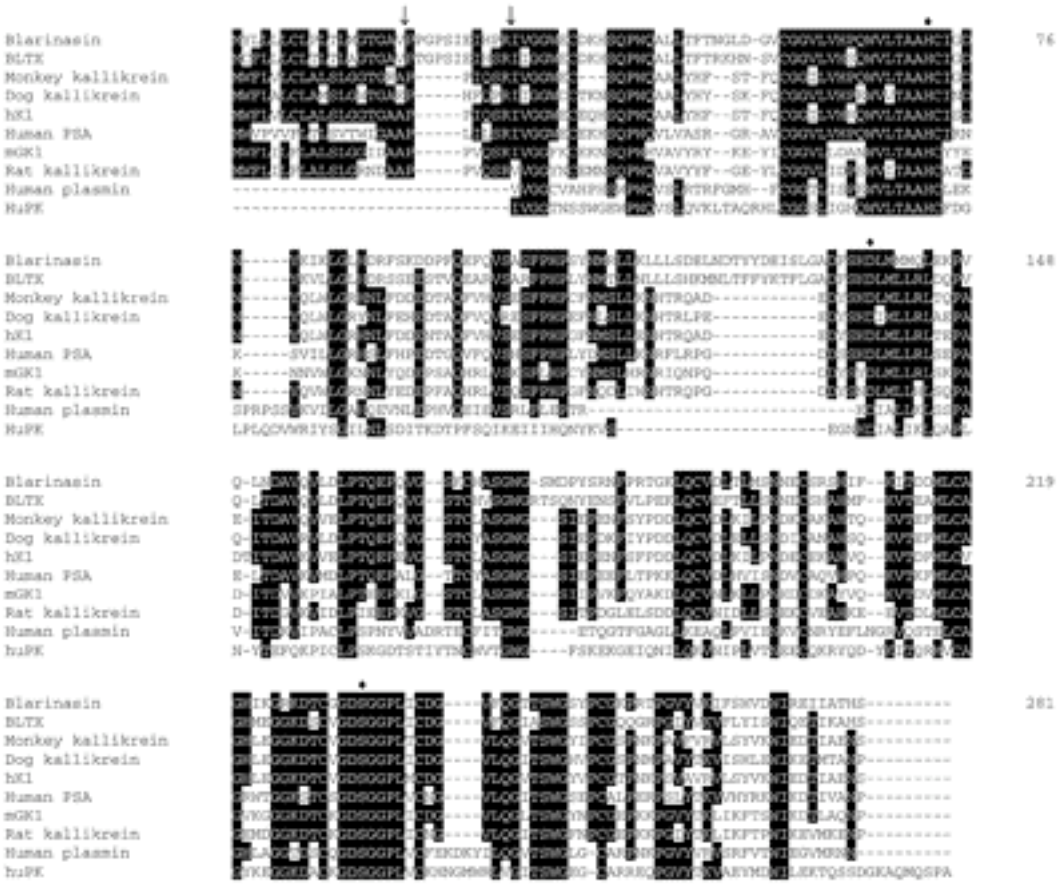

Figure 4 Structure-based Sequence Alignment of Active Forms of Blarinasin, BLTX, and Several Proteases Contained in Tissue Kallikrein Family.

Putative catalytic site residues are marked with a diamond (♦). Arrow shows putative processing sites of pro- and mature forms of blarinasin, BLTX, and various kallikreins. Amino acid sequences that are identical in >5 of the 10 sequences are highlighted in black. BLTX = blarina toxin; Monkey kallikrein = glandular kallikrein 1 from the crab-eating macaque *Macaca fascicularis*; Dog kallikrein = pancreas kallikrein from *Canis familiaris*; hK1 = human tissue kallikrein 1; Human PSA = human prostate specific antigen (tissue kallikrein 3); mGK1 = mouse tissue kallikrein 1; RUK1 = rat urinary kallikrein 1; huPK = human plasma kallikrein.