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Expression of LEKTI domains 6–9′ in the baculovirus expression system: recombinant LEKTI domains 6–9′ inhibit trypsin and subtilisin A

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Abstract

The precursor lympho-epithelial Kazal-type-related inhibitor (LEKTI), containing two Kazal-type and 13 nonKazal-type domains, is an efficient inhibitor of multiple serine proteinases, among them plasmin, subtilisin A, cathepsin G, elastase, and trypsin. To gain insight into the structure and function of some of these domains, a portion of the cDNA coding for LEKTI domains 6–9′ was cloned and expressed in Sf9 cells using the baculovirus expression vector system (BEVS). Through a single purification step using a Co^{2+} column, 3–4 mg of purified recombinant LEKTI-domains 6–9′ (rLEKTI6–9′) with the predicted molecular mass of 34.6 kDa was obtained from the cell pellet of a 1-L culture. Unlike full-length LEKTI, rLEKTI6–9′ inhibited trypsin and subtilisin A but not plasmin, cathepsin G, or elastase. The inhibition of trypsin and subtilisin A by rLEKTI6–9′ occurred through a noncompetitive mechanism, with inhibitory constants (K_i) of 356 ± 12 and 193 ± 10 nM, respectively. On the basis of the K_i values, rLEKTI6–9′ was determined to be a more potent trypsin inhibitor and a less potent subtilisin A inhibitor than the full-length LEKTI. In contrast to LEKTI domains 6–9′, recombinant LEKTI domain 6 does not inhibit subtilisin A but competitively inhibited trypsin with a K_i of 200 ± 10 nM. Taking LEKTI6-9′ as an example, the BEVS should facilitate the structure–function analysis of naturally occurring processed LEKTI forms that have physiological relevance.

Keywords: LEKTI; *SPINK5*; Baculovirus; Serine proteinases; Noncompetitive inhibition; LEKTI domains

Two previously unknown polypeptides of 55 and 68 amino acid residues (relative molecular masses of 6478 and 7665 Da, respectively) have been isolated from human blood ultrafiltrate and the former was shown to represent domain 1 and the latter to represent domain 6 of a large precursor protein termed lympho-epithelial Kazal-type-related inhibitor (LEKTI), which contains 15 potential serine proteinase inhibitory domains [1]. More recently, a protein corresponding to domain 5 of LEKTI was isolated from human blood filtrate [2,3]. In addition, a 30-kDa partial LEKTI fragment corresponding to a LEKTI fragment comprising about four domains and starting with domain 8 was isolated from human epidermal keratinocyte conditioned media [4]. Two of the 15 LEKTI domains (domains 2 and 15) resemble typical Kazal-type serine proteinase inhibitors, as deduced from their primary structure and characteristic pattern of six cysteine residues. The other 13 domains share partial homology to Kazal-type inhibitors but lack one of the three conserved Kazal-type disulfide bridges. In addition, all 15 LEKTI domains contain a

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longer sequence stretch (12 or 13 residues) between the first two cysteine residues than is found in typical Kazal-type inhibitors (6–9 residues) [5].

Since LEKTI domains 1, 5, and 6 circulate in human blood, they represent naturally occurring processed forms of LEKTI. No proteinase is known to be inhibited by domain 1, whereas domain 6 has been shown to inhibit trypsin efficiently but temporarily, with an apparent 50% inhibitory concentration of approximately 150 nM [1–3]. Domains 5, 8, and 15 also have been shown to inhibit trypsin [2]. However, trypsin is unlikely to be the natural target proteinase of any of the LEKTI domains given the expression pattern of LEKTI. Recently, the three-dimensional structures of domains 1 and 6 were elucidated [5]. Despite sharing the same disulfide pattern and a sequence identity of about 35% from the first to the fourth cysteine, the two proteins show different structures in this region, providing an explanation for their different behaviors against trypsin [5].

The name "LEKTI" was given by one of the original groups who cloned this gene to reflect the observed pattern of expression in both epithelial tissue and leukocytes [1]. Later, Chavanas et al. [6] identified *SPINK5* (serine protease inhibitor Kazal-type 5) as the defective gene in Netherton syndrome (NTS) and showed that *SPINK5* in fact encodes LEKTI. A total of 34 *SPINK5* mutations have been reported in patients, all of which create premature termination codons of translation [6–12]. NTS is a genetic disorder characterized by congenital ichthyosis, hair shaft abnormalities, immune deficiency, elevated IgE, and failure to thrive. Recently, Komatsu et al. [10] reported that patients with Netherton syndrome have elevated trypsin-like hydrolytic activity in their stratum corneum associated with over-desquamation of corneocytes [9]. This finding hints at a natural target proteinase for LEKTI or its domains.

We recently identified *SPINK5* as one of the genes downregulated in head and neck squamous cell carcinomas [13]. We also cloned *SPINK5* cDNA from biopsy material representing two normal human oral mucosa tissues [14]. Subsequently, we produced recombinant LEKTI (rLEKTI) containing 15 domains and showed that it inhibits multiple serine proteinases among them human plasmin, cathepsin G, elastase, trypsin, and subtilisin A but not chymotrypsin [14].

In the present study, using the BEVS, we purified rLEKTI domains 6–9′ in a single polypeptide (rLEKTI6–9′) and rLEKTI single domain 6 and tested for their proteinase inhibitory activities. Our results demonstrated that rLEKTI6–9′ inhibits trypsin and subtilisin A but not plasmin, cathepsin G, or elastase. In contrast to LEKTI domains 6–9′, recombinant LEKTI domain 6 does not inhibit subtilisin A but inhibits trypsin.

Materials and methods

Materials

Fall Army worm cell line *Spodoptera frugiperda* (Sf9), low-melting point agarose, cellFECTIN, pFASTBAC-HTb, pCRII-TOPO, *Escherichia coli* competent DH10BAC, cabbage looper egg cell line, *Trichoplusia ni-5B1-4* (High Five), Bac-to-Bac Baculovirus Expression System, and ultimate serum-free insect medium were obtained from Invitrogen (Carlsbad, CA). Polymerase chain reaction reagents, recombinant furin, and restriction endonucleases were obtained from New England Biolabs (Beverly, MA). TALON Co^{2+} Resin was obtained from Clontech (Palo Alto, CA). Insect-XPRESS media and fetal bovine serum were obtained from BioWhittaker (Walkersville, MD). YM10 Centriplus was obtained from Millipore (Bedford, MA). Precast sodium dodecyl sulfate–polyacrylamide electrophoresis gels, protein assay kit, and prestained markers were obtained from Bio-Rad (Hercules, CA). Bovine serum albumin was obtained from Kabi Pharmacia (Franklin, OH). Dithiothreitol, glycerol, and papain were obtained from Roche Molecular Biochemicals (Indianapolis, IN). 5-His monoclonal antibody and 6-His-tagged protein ladder was obtained from Qiagen (Valencia, CA). Human plasmin, human cathepsin L, human cathepsin S, human trypsin, human cathepsin G, human chymotrypsin, and human neutrophil elastase (HNE) were obtained from Athens Research & Technology (Athens, GA). Subtilisin A was obtained from Calbiochem–Novabiochem (San Diego, CA). Succinyl-Ala-Ala-Pro-Phe-*para*-nitroanilide (Succ-AAPF-*p*NA), succinyl-Ala-Ala-Val-*p*NA (Succ-AAV-*p*NA), and d-Val-Leu-Lys-*p*NA (VLK-*p*NA) were obtained from Sigma Chemical (St. Louis, MO). H-Glu-Gly-Arg-*p*NA (EGR-*p*NA) and benzyloxycarbonyl-Phe-Arg-*p*NA (Z-FR-*p*NA) were obtained from Bachem Bioscience (King of Prussia, PA). Methoxy-Succ-Arg-Pro-Tyr-*p*NA (MeO-Succ-RPY-*p*NA) was obtained from Chromogenix Instrumentation Laboratory SpA (Milan, Italy).

Construction of LEKTI 6–9′ composite virus

A partial LEKTI cDNA clone in PCRII-TOPO vector containing the LEKTI cDNA sequence between nucleotides 1113 and 1794 (encoding part of domain 6, all of domains 7 and 8, and part of domain 9) was subcloned into pFASTBAC-HTb as a 0.70-kb *Hin*dIII fragment. To extend the 5′ end of this clone, it was digested with *Pst*I and ligated into a 0.43-kb *Pst*I fragment PCR amplified by using a human LEKTI full-length cDNA and two LEKTI primers (forward: 5′-*ctgcag* TGAATCTGGAAAAGCAACC-3′; reverse: 5′-*ctgcag* ATTTCCTTTGCAGC-3′). This new construct (designated pFASTBAC HTb-LEKTI6-9′) contains the LEKTI

cDNA sequence between nucleotides 1066 and 1794 encoding all of domains 6–8 and part of domain 9. We determined the sequence of the pFASTBAC HTb-LEKTI6-9′ DNA using either vector- or cDNA-specific primers and found no mutation as compared to published LEKTI sequences (GenBank/EMBL Accession Nos. AJ228139 and AF086524). Sf9 cells were transfected with this plasmid DNA, and LEKTI6-9′ composite viruses were generated and titrated as described elsewhere [14].

Affinity purification using TALON cobalt resin

Sf9 cells were infected with the recombinant virus using a multiplicity of infection (MOI) of 5 plaque-forming units/cell. Three days after infection, the cell pellet was harvested and the recombinant protein was selectively purified from the cell lysate using Co^{2+}-charged-Sepharose affinity column (TALON) as described previously [15]. Typically, the cell pellet was resuspended at a ratio of $1.0–1.5 \times 10^7$ cells/ml in a lysis buffer containing 10 mM Tris–HCl (pH 8.0); 130 mM NaCl; 10 mM NaF; 10 mM sodium phosphate; 10 mM sodium pyrophosphate; 1.0% (v/v) Triton X-100; 1.0 μg/ml each of leupeptin; pepstatin; and aprotinin; and 5.0 μg/ml Pefabloc SC plus. The suspension was kept on ice for 1 h. The lysed cell suspension was sonicated (three 15-s pulses at 80 W) and the cell debris was removed by centrifugation. The cell-free extract was diluted with 2× binding buffer containing 40 mM Tris–HCl (pH 8.0); 260 mM NaCl; 2.0% (v/v) Triton X-100; and 10% (v/v) glycerol and loaded onto a 1.0×5-cm column containing 0.6 ml TALON Superflow equilibrated in binding buffer. The column was washed with binding buffer until the absorbance at 280 nm was less than $0.005\ M^{-1}\ cm^{-1}$. The protein was then step-eluted with 150 mM imidazole in binding buffer. The fractions (eluates 3–10) containing rLEKTI6–9′ were pooled and then concentrated using a YM10 Centriplus. For long-term storage (−80 °C), glycerol was added to a final concentration of 15%. Protein was quantified using the Bio-Rad Protein Assay Kit II.

Proteinase activity and inhibition of proteinases

Proteinase assay buffers and solvents for the substrates were as described previously [14]. Proteinase inhibitory activity was inferred from the ability of rLEKTI6–9′ to block the cleavage of small, chromogenic peptide substrates as determined by spectroscopy [14]. Briefly, proteinases were individually incubated with various concentrations of rLEKTI6–9′ for 10 min at 22 °C, and proteinase activity was then initiated by adding 10–20 μl of the appropriate *p*NA substrate to assay buffer in a total volume of 1 ml. The change of absorbance at 405 nm (baseline 0.008, $E_{405} = 8.8 \times 10^{-3}\ M^{-1}\ cm^{-1}$) was followed up to 10 min using a spectrophotometer (Beckman Instruments, Fullerton, CA). The rate changes (Δ_A 405/min) of inhibited and control reactions were determined from the velocity plots. Activities were expressed relative to control incubations from which LEKTI6–9′ was excluded.

Classification of the inhibitory mechanism of rLEKTI6–9′

Initial velocities of the release of *p*NA from the peptide substrates were measured as a function of several fixed substrate concentrations (100–1000 μM) for two rLEKTI6–9′ concentrations. Nonlinear and linear regression analyses of experimental data were performed using GraphPad Prism v. 3.02 for Windows (GraphPad Software, San Diego, CA). The (K_i) of inhibition was calculated from the slope of the inhibited reaction ($K_M/V_{max} \times (1 + [I]/K_i)$ as described previously [14,16].

Other methods

SDS–PAGE and immunoblotting were done as described previously [14]. Recombinant clones were analyzed by the DNA sequencing core facility at University of Texas, M.D. Anderson Cancer Center (Houston, TX). Internal amino acid sequence analyses of the purified rLEKTI6–9′ were performed at the Baylor College of Medicine, Protein Chemistry Core Laboratory (Houston, TX).

Results and discussion

Expression and purification of rLEKTI6–9′ using immobilized metal affinity chromatography

Since being introduced in 1983, the baculovirus expression vector systems (BEVS) have become widely used in studies on eukaryotic proteins [17–19]. The higher yield of recombinant proteins than that provided by bacterial and yeast expression systems and the presence of post-translational modifications make the BEVS popular for expressing eukaryotic proteins [20,21]. Recently, we reported the expression and purification of precursor LEKTI containing 15 domains using a BEVS [14]. The identification of several processed forms of LEKTI protein [1–4] as well as the presence of multiple consensus sequences for furin cleavage in the whole LEKTI molecule has led us to identify the individual proteinase inhibitory activity within the LEKTI polypeptide.

In the present study, to gain insight into the structure and function of some of these domains, recombinant baculovirus encoding LEKTI domains 6–9′ was constructed as outlined (Fig. 1A). Sf9 cells were infected with the purified virus for 72 h. The amount of fusion

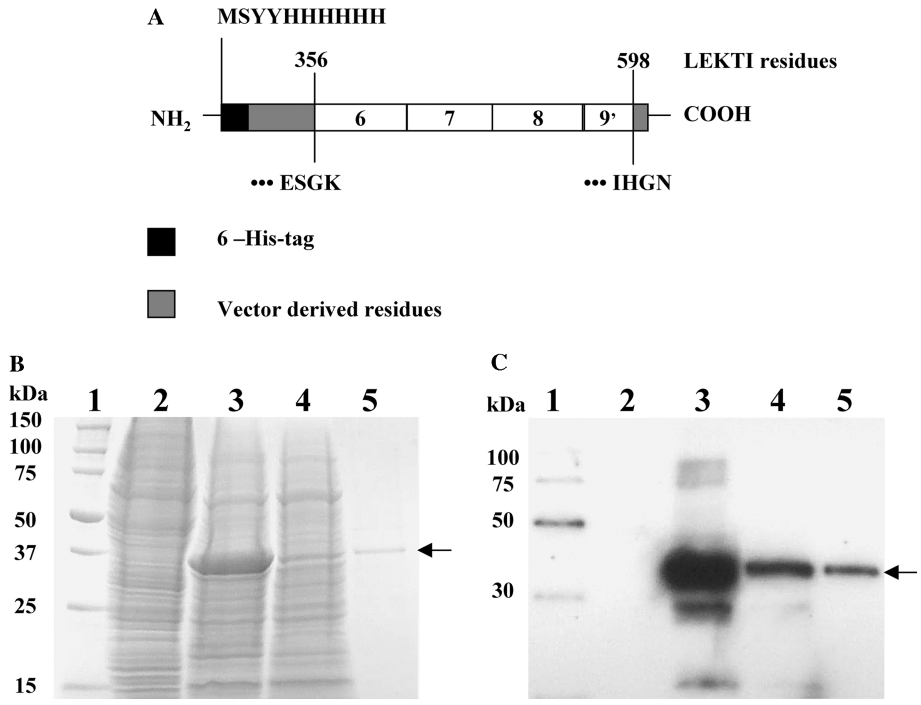

Fig. 1. (A) Baculovirus expression construct for LEKTI domains 6–9′. Amino acids are shown by their single-letter codes. The N- and C-terminal amino acid residues of the LEKTI domains 6–9′ are indicated. (B) Cell lysates from 1-L Sf9 cell cultures (~438 mg) were subjected to metal-affinity and gel-filtration chromatography. A total of 5–15 μl of each sample were resolved by 10% SDS–PAGE. Lane 1, prestained molecular mass markers; lane 2, total fraction of cell lysate from control (60 μg); lane 3, total fraction of cell lysate from infected cells (50 μg); lane 4, soluble fraction of cell lysate from infected cells (50 μg); and lane 5, purified LEKTI domains 6–9′ (1.5 μg) after metal-affinity and gel-filtration chromatography. Positions of molecular weight are noted to the left of the gel and blot. The location of rLEKTI6–9′ is indicated (arrow). (C) Western blot analysis with 5-His mAb. The lanes correspond to those in (A), except for lane 1, which contained 6-His protein ladders.

protein present in the cell lysate was estimated by immunodetection with 5-His mAb. Based on the level of expression, the optimal time for harvest was 72 h after infection and the MOI was 5 plaque-forming units/cell. The rLEKTI6–9′ produced in the present study (Fig. 1A) contains LEKTI domain 6 (residues 356–425), LEKTI domain 7 (residues 426–489), LEKTI domain 8 (residues 490–560), and LEKTI partial domain 9 (residues 561–598) 56 vector-derived residues including six histidine residues at its N-terminus and 11 vector-derived residues at its C-terminus, respectively. The predicted M_r of this polypeptide is 34577.51 Da.

After Sf9 cells were infected with recombinant baculovirus, a 34.5-kDa protein in the total cell lysate (arrow) was resolved using a 7.5% polyacrylamide gel under reducing conditions (Fig. 1B, lane 3). This band was not seen in the mock infection (Fig. 1B, lane 2). According to a Western blot analysis, ~20% of the rLEKTI6–9′ was found in the soluble fraction of the cell lysates after lysis of the Sf9 cells (Fig. 1B, lane 4, and Table 1), suggesting that only the soluble fraction exhibits the necessary disulfide connectivities. Since there was a 6-His tag at the N-terminus, a TALON metal affinity chromatography column containing immobilized Co^{2+} was used in the purification of the recombinant protein. Upon purification from the soluble fraction, a single band of 34.5-kDa protein was rendered visible by Coomassie brilliant blue R-250 staining in the pooled imidazole eluates (Fig. 1B, lane 5). Therefore, pure target protein was obtained using a single step of

Table 1
Purification of rLEKTI6–9′

Purification step	Volume (ml)	Total protein (mg/L culture)	Recombinant protein[a] (mg/L culture)	Purification factor (fold)[b]	Yield (%)[c]
Total cell lysate	42	441	27.16	1	100
Soluble cell lysate	40	388	6.79	—	25
TALON flow-through	40	390	ND	—	—
Imidazole eluates	9	4.28	4.28	103	16

[a] Estimated from band density on Western blots (Fig. 1C).
[b] Calculated based on total protein.
[c] Calculated based on rLEKTI6–9′.

purification. The 34.5-kDa protein was further suggested to be rLEKTI6–9′ by its reactivity with 5-His mAb (Fig. 1C, lane 5). Internal amino acid sequencing of this protein band confirmed it to be bona fide rLEKTI6–9′. The yield at each step of the isolation procedures is shown in Table 1. Approximately 4.0 mg of pure rLEKTI6–9′ was obtained from the Sf9 cell pellet of a 1-L culture. These results suggest that 6-His-tagged rLEKTI can be efficiently expressed and selectively purified from insect cells.

Disulfide status of rLEKTI6–9′ expressed in Sf9

Based on the cDNA-derived amino acid sequence, three of these domains (6, 7, and 8) should form both the conserved disulfide bonds (1–4 and 2–3). Though partial domain 9′ lacked the COOH-terminal Cys^{600} and Cys^{603}, the first two cysteines of domain 9′ Cys^{567} and Cys^{581}, could potentially form a nonnative disulfide bond with each other. To determine whether rLEKTI6–9′ contained disulfide bonds and whether protein aggregates were present in our preparation, rLEKTI6–9′ was analyzed by SDS–PAGE under both reducing and nonreducing conditions. One-dimensional SDS–PAGE performed after reducing and nonreducing sample preparation showed that disulfide-containing polypeptides migrated differently than their reduced counterparts. The rLEKTI6–9′ separated by SDS–PAGE clearly migrated faster under nonreducing conditions (Fig. 2A, lanes 3 and 4) than under reducing conditions (Fig. 2A, lanes 1 and 2), providing evidence of disulfide bonds in the recombinant protein. In addition, no larger molecular bands representing rLEKTI aggregates were rendered visible by Coomassie brilliant blue R-250 staining in the nonreducing SDS–PAGE. However, a few aggregates were detected by the more sensitive technique of immunoblotting (Fig. 2B, lane 4). This finding clearly indicates that most of the rLEKTI6–9′ expressed in the insect cells contained intramolecular disulfide bonds and the Sf9 produced rLEKTI6–9′ can be used to screen for proteinase inhibitory activity.

rLEKTI6–9′ contains only trypsin and subtilisin A inhibitory activities and the inhibition is noncompetitive

Since our previous work demonstrated that the baculo-produced recombinant precursor LEKTI inhibits multiple serine proteinases but not cysteine proteinases, we tested rLEKTI6–9′ against a similar panel of serine and cysteine proteinases (Table 2). The striking result is that rLEKTI6–9′ had a strong inhibitory effect only on trypsin and subtilisin A. Unlike precursor LEKTI [14], rLEKTI6–9′ had only mild inhibitory effects on plasmin, elastase, and cathepsin G at concentrations of 100, 300, and 1000 nM, respectively. In addition, like precursor LEKTI [14], rLEKTI6–9′ did not inhibit chymotrypsin, papain, and several cysteine proteinases. Since the LEKTI gene (*SPINK5*) is mainly expressed in mucous epithelia and associated glands, as well as in lymphoid organs, the digestive enzyme trypsin is unlikely to be a physiological target proteinase of LEKTI6–9′. However, because subtilisin A, which belongs to the S8 family of serine proteinases [22,23], is also a target of rLEKTI6–9′, LEKTI might well play a role in antimicrobial protection. Subtilisin-like proprotein convertases (SPCs) are expressed in the epidermis, and many have been shown to be responsible for converting precursor proteins into their biologically active forms [9,24–27]. Three point

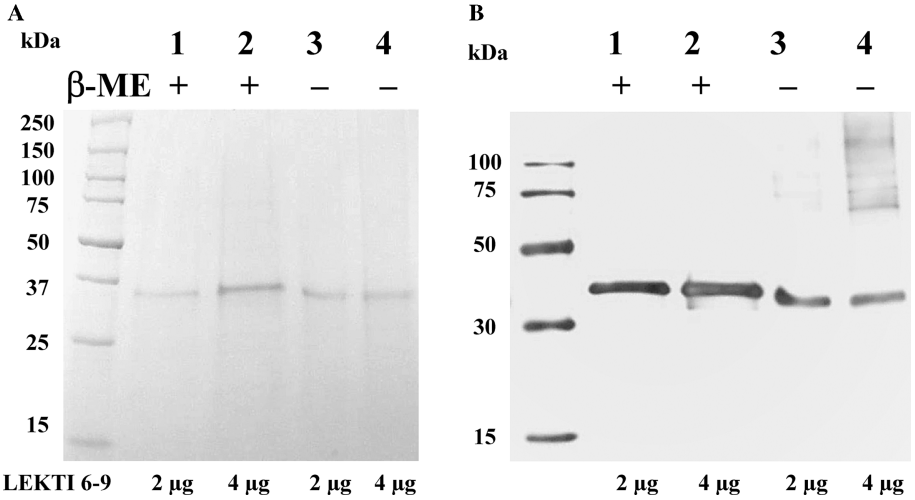

Fig. 2. Disulfide status of rLEKTI6–9′ expressed in Sf9 cells examined using SDS–PAGE (A) and Western blotting (B). Lanes: 1, prestained molecular mass markers (A) or 6-His protein ladders (B); 1 and 2, sample prepared under reducing conditions (5% β-mercaptoethanol); and 3 and 4, sample prepared under nonreducing conditions. Electrophoresis was performed in 10% gels at a constant 200 V for 2 h and protein was rendered visible by Coomassie brilliant blue R-250 staining. Positions of molecular mass markers are noted to the left of the gels. Proteins from a companion gel were transferred to nitrocellulose and immunoblotted with 5-His mAb.

Table 2
Inhibitory profile of rLEKTI6–9′

Proteinase (final concentration)	rLEKTI6–9′[a] (nM)	I/E[b]	Inhibition[c] (%)	Substrate (final concentration)
Trypsin (50 nM)	1000	20	97	EGR-pNA (1.0 mM)
Subtilisin A (36 nM)	600	17	90	Succ-AAPF-pNA (1.0 mM)
HNE (20 nM)	1000	50	40	Succ-AAV-pNA (1.0 mM)
Cathepsin G (12 nM)	300	25	30	Succ-AAPF-pNA (2.0 mM)
Plasmin (17 nM)	100	5.9	25	VLK-pNA (0.8 mM)
Chymotrypsin (10 nM)	500	50	0	MeO-Succ-RPY-pNA (1.0 mM)
Papain (10 nM)	500	50	0	Z-FR-pNA (1.0 mM)
Cathepsin k (20 nM)	1000	50	0	Z-FR-pNA (1.0 mM)
Cathepsin L (20 nM)	1000	50	0	Z-FR-pNA (1.0 mM)
Cathepsin S (20 nM)	1000	50	0	Z-FR-pNA (1.0 mM)

[a] Proteolysis was followed spectrophotometrically by incubating substrate and rLEKTI6–9′ together.
[b] I/E = rLEKTI concentration/proteinase concentration.
[c] Percent inhibition = $100 \times [1 - (\text{velocity in the presence of inhibitor/velocity of uninhibited control})]$.

mutations in *SPINK5* leading to single amino acid exchanges have been identified in patients with atopy, atopic dermatitis, or Netherton syndrome, all these mutations namely N368S, D386N, and E420K occurring in LEKTI domain 6 [10,28,29]. The effects of these mutations on the target proteinases are not known. Recently, we have identified three published and two novel *SPINK5* mutations in NTS patients [30]. Our results demonstrated that, as a result of these mutations, there is a dramatic lack of LEKTI in the epidermis of the respective NTS patients [30]. The striking lack of LEKTI protein in all NTS patients suggests nonsense-mediated decay of mutant truncated LEKTI mRNA, a rapid proteolytic breakdown of truncated LEKTI protein, or possibly, both. Our present results clearly show that domains 6–9′ are sufficient for subtilisin A inhibition. However, it remains to be seen if other domains in LEKTI could also inhibit subtilisin A. If so, then domains 6–9′ may not be essential for subtilisin A inhibition. Nevertheless, on the basis of our findings [14,30] and the present results, it is tempting to speculate that the total absence of LEKTI protein in NTS patients might unleash the activity of a multitude of target proteinases.

To classify the type of inhibition, the kinetic constants (K_M and V_{max}) of trypsin and subtilisin A were determined for their respective chromogenic peptides in the presence of two rLEKTI6–9′ concentrations (Table 3). The K_i value was determined from the Lineweaver–Burk double-reciprocal plots, and the inhibition type was inferred from Lineweaver–Burk double-reciprocal (Figs. 3A and B) and Eadie–Hofstee plots (Figs. 3C and D). As shown by the Lineweaver–Burk double-reciprocal plots, proteinase inhibition by rLEKTI6–9′ was clearly noncompetitive (Figs. 3A and B). Using either the slope or the ordinate intercept of the inhibited reaction, the K_i values of rLEKTI6–9′ for each proteinase were determined to be identical. The K_i values for trypsin were 356 and 396 nM determined at rLEKTI6–9′ concentrations 200 and 400 nM, respectively (Table 3). Similarly, the K_i values for subtilisin A were 199 and 193 nM at rLEKTI6–9′ concentrations 150 and 300 nM, respectively. The corresponding Eadie–Hofstee plots ($V/[S]$ versus V) show that the apparent K_M values (the slope of the plots) remained fairly constant as rLEKTI6–9′ concentration increased, and that the apparent V_{max} values (y-intercept) decreased markedly as rLEKTI6–9′ concentration increased (Figs. 3C and D). This kinetic analysis indicates that the inhibition was strictly noncompetitive, suggesting the existence of a binding site for LEKTI6–9′ distinct from the catalytic center. This type of inhibition is distinguished from competitive inhibition, which alters only apparent K_M, and from

Table 3
Effects of rLEKTI6–9′ on reaction kinetics of trypsin and subtilisin A

Proteinase	rLEKTI6–9′ (nM)	K_M[a] (μM)	V_{max}[a] (μmol/mg/min)	Number (n)	K_i[b] (nM)	r^2
Trypsin (50 nM)	0	357 ± 48	3.30 ± 0.17	3	—	0.9928
Trypsin (50 nM)	200	370 ± 63	2.20 ± 0.17	3	356 ± 12	0.9857
Trypsin (50 nM)	400	385 ± 44	1.80 ± 0.06	3	396 ± 28	0.9883
Subtilisin A (36 nM)	0	286 ± 25	7.50 ± 0.67	3	—	0.9712
Subtilisin A (36 nM)	150	344 ± 26	5.40 ± 0.36	3	199 ± 38	0.9832
Subtilisin A (36 nM)	300	346 ± 37	3.50 ± 0.21	3	193 ± 10	0.9789

[a] K_M and V_{max} were calculated from data shown in Fig. 3 by nonlinear regression analysis as described in Materials and methods. Values are reported as an average value ± SEM.
[b] K_i of rLEKTI6–9′ was calculated from the slope ($K_M/V_{max}(1 + [I]/K_i]$) of inhibited reactions. Values are reported as an average value ± SEM.

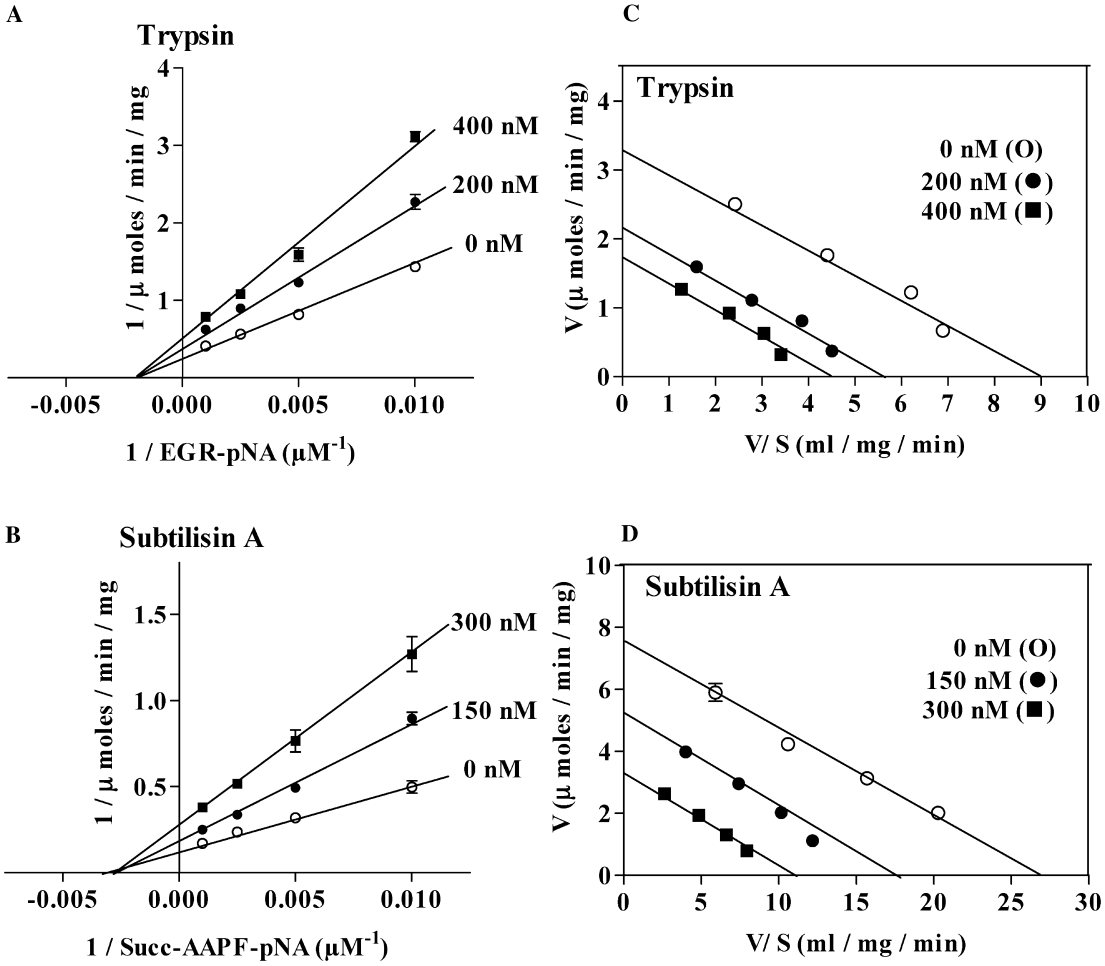

Fig. 3. Lineweaver–Burk (A,B) (1/*S* against 1/*V*) and Eadie–Hofstee (C,D) (V_0 against V_0/S) analyses of hydrolysis of chromogenic peptide by trypsin (A,C) and subtilisin A (B,D) in the presence of rLEKTI6–9′. The solid lines in the Eadie–Hofstee plots represent the linear regression fits. The plot is diagnostic for competitive, noncompetitive, and uncompetitive types of inhibition. (A) The substrate was EGR-pNA (0.1–1.0 mM); (B) the substrate was Succ-AAPF-pNA (0.1–1.0 mM).

uncompetitive inhibition, which alters both apparent K_M and apparent V_{max}.

Moreover, the inhibitory potency of rLEKTI6–9′ against trypsin ($K_i = 356 \pm 12$ nM) was significantly higher than that of precursor LEKTI against trypsin ($K_i = 849 \pm 55$ nM). In contrast, the inhibitory potency of rLEKTI6–9′ against subtilisin A ($K_i = 193 \pm 10$ nM) was significantly lower than that of precursor LEKTI against subtilisin A ($K_i = 49 \pm 3$ nM). Whether C-terminal extension of partial domain 9 into complete domain 9 would improve its K_i for subtilisin A remains to be investigated.

Multiple-domain LEKTI6–9′ acts as a noncompetitive inhibitor, whereas single LEKTI domain 6 acts as a competitive inhibitor

Two features of the fragment LEKTI6–9′ are very interesting: (1) the trypsin and subtilisin A inhibition is permanent and (2) the inhibition is noncompetitive. From a structural point of view, domain 6 has been suggested to belong to a sub-family of Kazal-type inhibitors, as there are some common features [5]. We made recombinant LEKTI domain 6 in our laboratory and tested it against trypsin activity under identical conditions (i.e., same incubation time of 10 min) as tested for LEKTI6-9′. rLEKTI domain 6 did not inhibit subtilisin A activity. However, consistent with the published results [1], our results showed that it behaved like a competitive inhibitor but nonpermanent inhibitor, with a K_i of 200 ± 10 nM (results not shown). Since the sequences of domains 6 and 7 are quite similar, especially in the middle part of the protein, we suspect that isolated single domain 7 would show trypsin-inhibitory activity. Domain 8 has a slightly different amino acid sequence around the canonical loop. In our opinion, the possibility that domain 8 inhibits trypsin or subtilisin A permanently is quite unlikely but cannot be excluded.

The nonpermanent inhibitory activity of domain 6 is mainly ascribed to the fact that it lacks the third

disulfide bond found in classical Kazal-type inhibitors and maybe also to its additional amino acid residues between the first two cysteines [5]. Domain 15, which contains three disulfide bonds and has 12 rather than 13 amino acid residues between the first two cysteines, was shown to be a permanent inhibitor of trypsin [2,3]. The authors concluded that the differences in the inhibitory mechanisms between domain 6 and single domain 15 might be due to a higher rigidity of the latter. In agreement with these findings, the three-dimensional structure of domain 6 demonstrates that it binds to the proteinase in a canonical and substrate-like manner [5].

On the basis of these earlier results and our current finding that multiple-domain LEKTI6–9′ is a permanent and noncompetitive inhibitor of trypsin and subtilisin A, we offer two possible explanations. (1) The domains might stabilize each other, reducing the inherent flexibility of domains with only two disulfide bonds (as found for domain 6) and thus shifting the partly substrate-like behavior to a more inhibitor-like behavior. (2) One domain might bind with its canonical loop in the active site of the proteinase, whereas the other domain binds outside the active site, further stabilizing the interactions between the proteinase and the inhibitor. However, we like to point out that point 1 only gives an explanation for the differences concerning permanent and nonpermanent inhibitory activity and it does not provide an explanation for the noncompetitive behavior of LEKTI6–9′. Only point 2 provides a possible explanation for a noncompetitive inhibition. Experiments are in progress to distinguish between these possibilities.

We have recently shown that the furin-induced overexpression of precursor LEKTI in HEK293T cells generates proteins of 110, 75, 50, 45, 35, and 25 kDa, whose sizes match those of proteins identified in normal human epidermal keratinocyte conditioned media (A. Jayakumar et al., manuscript in preparation). This finding suggests that larger multidomain fragments of LEKTI are generated spontaneously. Taking LEKTI6–9′ as an example, the BEVS should facilitate the production and identification of proteinase targets of these naturally occurring processed LEKTI forms that have physiological relevance.

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References

[1] H.J. Magert, L. Standker, P. Kreutzmann, H.D. Zucht, M. Reinecke, C.P. Sommerhoff, H. Fritz, W.G. Forssmann, LEKTI, a novel 15-domain type of human serine proteinase inhibitor, J. Biol. Chem. 274 (1999) 21499–21502.

[2] H.J. Magert, P. Kreutzmann, L. Standker, M. Walden, K. Drogemuller, W.G. Forssmann, LEKTI: a multidomain serine proteinase inhibitor with pathophysiological relevance, Int. J. Biochem. Cell. Biol. 34 (2002) 573–576.

[3] H.J. Magert, P. Kreutzmann, K. Drogemuller, L. Standker, K. Adermann, M. Walden, H. John, H.C. Korting, W.G. Forssmann, The 15-domain serine proteinase inhibitor LEKTI: biochemical properties, genomic organization, and pathophysiological role, Eur. J. Med. Res. 7 (2002) 49–56.

[4] A. Ahmed, P. Kandola, G. Ziada, N. Parenteau, Purification and partial amino acid sequence of proteins from human epidermal keratinocyte conditioned medium, J. Protein Chem. 20 (2001) 273–278.

[5] T. Lauber, A. Schulz, K. Schweimer, K. Adermann, U.C. Marx, Homologous proteins with different folds: the three-dimensional structures of domains 1 and 6 of the multiple Kazal-type inhibitor LEKTI, J. Mol. Biol. 328 (2003) 205–219.

[6] S. Chavanas, C. Bodemer, A. Rochat, D. Hamel-Teillac, M. Ali, A.D. Irvine, J.L. Bonafe, J. Wilkinson, A. Taieb, Y. Barrandon, J.I. Harper, Y. de Prost, A. Hovnanian, Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome, Nat. Genet. 25 (2000) 141–142.

[7] F.B. Muller, I. Hausser, D. Berg, C. Casper, R. Maiwald, A. Jung, H. Jung, B.P. Korge, Genetic analysis of a severe case of Netherton syndrome and application for prenatal testing, Br. J. Dermatol. 146 (2002) 495–499.

[8] E. Bitoun, C. Bodemer, J. Amiel, Y. de Prost, C. Stoll, P. Calvas, A. Hovnanian, Prenatal diagnosis of a lethal form of Netherton syndrome by SPINK5 mutation analysis, Prenat. Diagn. 22 (2002) 121–126.

[9] E. Bitoun, S. Chavanas, A.D. Irvine, L. Lonie, C. Bodemer, M. Paradisi, D. Hamel-Teillac, S. Ansai, Y. Mitsuhashi, A. Taieb, Y. de Prost, G. Zambruno, J.I. Harper, A. Hovnanian, Netherton syndrome: disease expression and spectrum of SPINK5 mutations in 21 families, J. Invest. Dermatol. 118 (2002) 352–361.

[10] N. Komatsu, M. Takata, N. Otsuki, R. Ohka, O. Amano, K. Takehara, K. Saijoh, Elevated stratum corneum hydrolytic activity in Netherton syndrome suggests an inhibitory regulation of desquamation by SPINK5-derived peptides, J. Invest. Dermatol. 118 (2002) 436–443.

[11] C. Stoll, Y. Alembik, D. Tchomakov, J. Messer, E. Heid, N. Boehm, P. Calvas, A. Hovnanian, Severe hypernatremic dehydration in an infant with Netherton syndrome, Genet. Counsel. 12 (2001) 237–243.

[12] E. Sprecher, S. Chavanas, J.J. DiGiovanna, S. Amin, K. Nielsen, J.S. Prendiville, R. Silverman, N.B. Esterly, M.K. Spraker, E. Guelig, M.L. de Luna, M.L. Williams, B. Buehler, E.C. Siegfried, L. Van Maldergem, E. Pfendner, S.J. Bale, J. Uitto, A. Hovnanian, G. Richard, The spectrum of pathogenic mutations in SPINK5 in 19 families with Netherton syndrome: implications for

mutation detection and first case of prenatal diagnosis, J. Invest. Dermatol. 117 (2001) 179–187.
[13] H.E. Gonzalez, M. Gujrati, M. Frederick, Y. Henderson, A. Jayakumar, P.W. Spring, K. Mitsudo, H.W. Kim, G.L. Clayman, Identification of 9 genes differentially expressed in head and neck squamous cell carcinoma, Arch. Otolaryngol. Head Neck Surg. 129 (2003) 754–759.
[14] K. Mitsudo, A. Jayakumar, Y. Henderson, M.J. Frederick, Y. Kang, M. Wang, A.K. El Naggar, G.L. Clayman, Inhibition of serine proteinases plasmin, trypsin, subtilisin A, cathepsin G, and elastase by LEKTI: a kinetic analysis, Biochemistry 42 (2003) 3874–3881.
[15] A. Jayakumar, W.Y. Huang, B. Raetz, S.S. Chirala, S.J. Wakil, Cloning and expression of the multifunctional human fatty acid synthase and its subdomains in *Escherichia coli*, Proc. Natl. Acad. Sci. USA 93 (1996) 14509–14514.
[16] A. Jayakumar, W. Epstein, E.M. Barnes Jr., Characterization of ammonium (methylammonium)/potassium antiport in *Escherichia coli*, J. Biol. Chem. 260 (1985) 7528–7532.
[17] G.E. Smith, M.D. Summers, M.J. Fraser, Production of human beta interferon in insect cells infected with a baculovirus expression vector, Mol. Cell. Biol. 3 (1983) 2156–2165.
[18] J. Cooley, B. Mathieu, E. Remold-O'Donnell, R.J. Mandle, Production of recombinant human monocyte/neutrophil elastase inhibitor (rM/NEI), Protein Exp. Purif. 14 (1998) 38–44.
[19] G.A. Hastings, T.A. Coleman, C.C. Haudenschild, S. Stefansson, E.P. Smith, R. Barthlow, S. Cherry, M. Sandkvist, D.A. Lawrence, Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival, J. Biol. Chem. 272 (1997) 33062–33067.
[20] V.A. Luckow, M.D. Summers, High level expression of nonfused foreign genes with *Autographa californica* nuclear polyhedrosis virus expression vectors, Virology 170 (1989) 31–39.
[21] V.A. Luckow, M.D. Summers, Signals important for high-level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors, Virology 167 (1988) 56–71.
[22] A.M. Khatib, G. Siegfried, M. Chretien, P. Metrakos, N.G. Seidah, Proprotein convertases in tumor progression and malignancy: novel targets in cancer therapy, Am. J. Pathol. 160 (2002) 1921–1935.
[23] A.M. Khatib, G. Siegfried, A. Prat, J. Luis, M. Chretien, P. Metrakos, N.G. Seidah, Inhibition of proprotein convertases is associated with loss of growth and tumorigenicity of HT-29 human colon carcinoma cells: importance of insulin-like growth factor-1 (IGF-1) receptor processing in IGF-1-mediated functions, J. Biol. Chem. 276 (2001) 30686–30693.
[24] N. Komatsu, M. Takata, N. Otsuki, T. Toyama, R. Ohka, K. Takehara, K. Saijoh, Expression and localization of tissue kallikrein mRNAs in human epidermis and appendages, J. Invest. Dermatol. 121 (2003) 542–549.
[25] F. Logeat, C. Bessia, C. Brou, O. LeBail, S. Jarriault, N.G. Seidah, A. Israel, The Notch1 receptor is cleaved constitutively by a furin-like convertase, Proc. Natl. Acad. Sci. USA 95 (1998) 8108–8112.
[26] D.J. Pearton, W. Nirunsuksiri, A. Rehemtulla, S.P. Lewis, R.B. Presland, B.A. Dale, Proprotein convertase expression and localization in epidermis: evidence for multiple roles and substrates, Exp. Dermatol. 10 (2001) 193–203.
[27] S.J. Duguay, W.M. Milewski, B.D. Young, K. Nakayama, D.F. Steiner, Processing of wild-type and mutant proinsulin-like growth factor-IA by subtilisin-related proprotein convertases, J. Biol. Chem. 272 (1997) 6663–6670.
[28] A.J. Walley, S. Chavanas, M.F. Moffatt, R.M. Esnouf, B. Ubhi, R. Lawrence, K. Wong, G.R. Abecasis, E.Y. Jones, J.I. Harper, A. Hovnanian, W.O. Cookson, Gene polymorphism in Netherton and common atopic disease, Nat. Genet. 29 (2001) 175–178.
[29] E. Bitoun, A. Micheloni, L. Lamant, C. Bonnart, A. Tartaglia-Polcini, C. Cobbold, T. Al Saati, F. Mariotti, J. Mazereeuw-Hautier, F. Boralevi, D. Hohl, J. Harper, C. Bodemer, M.D. Alessio, A. Hovnanian, LEKTI proteolytic processing in human primary keratinocytes tissue distribution and defective expression in Netherton syndrome, Hum. Mol. Genet. 2 (2003) 2417–2430.
[30] M. Raghunath, L. Tontsidou, V. Oji, F. Schürmeyer-Horst, A. Jayakumar, G. Beljan, H. Ständer, J. Smolle, G.L. Clayman, H. Traupe, *SPINK5* mutations: missing lekti and aberrant expression of Elafin, transglutaminases and human β-defensin 2 in Netherton Syndrome, J. Invest. Dermatol. (in press).