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Recombinant production, purification and biochemical characterization of domain 6 of LEKTI: a temporary Kazal-type-related serine proteinase inhibitor

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Abstract

Lympho-epithelial Kazal-type-related inhibitor (LEKTI) is a 15-domain serine proteinase inhibitor which is of pathophysiological relevance for skin diseases and atopy. Domains 2 and 15 of LEKTI contain six cysteine residues and match the Kazal-type inhibitor motif almost exactly. The other 13 domains seem to be Kazal-type derived but lack the cysteines in positions 3 and 6 usually conserved within this family of inhibitors. Here, we report the recombinant production and comprehensive biochemical characterization of the 7.7 kDa LEKTI domain 6 (LD-6). Testing a selected number of different serine proteinases, we show that both native and recombinant LD-6 exhibit a significant but temporary inhibitory activity on trypsin. Furthermore, the relation of LEKTI domain 6 to Kazal-type inhibitors is confirmed by determining its disulfide bond pattern (1–4/2–3) and its P_1 site located after the second Cys residue of LD-6. The established strategy for the recombinant production of LEKTI domain 6 will enable further investigation of its mode of action and its physiological role.
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Keywords: Recombinant production; Purification; Characterization; LEKTI, domain 6; Serine proteinase inhibitor

1. Introduction

Endogenously produced proteinase inhibitors are responsible for the control of various proteinases involved in regulatory processes such as prohormone conversion, zymogen activation, tissue remodeling, cell cycle regulation and many others. Disproportion in the ratios of proteinases to inhibitors caused by genetic defects, inflammation or certain harmful impacts may lead to severe pathological effects [1]. Thus, proteinases and their specific inhibitors represent worthwhile targets for the development of drugs [2,3].

Depending on their reactive center and the catalytic type of action, proteinases are subdivided into serine and threonine, cysteine, aspartate and metalloproteinases [4]. In the case of the inhibitors, an even higher number of different families exists. For instance, about 20 different types of serine proteinase inhibitors are already described [1,5,6]. The most common mechanism of their inhibitory effect is based on an exposed canonical site loop which fits well into the substrate binding pocket of the proteinase. The kinetics of hydrolysis of the specific target peptide bond between two amino acids positioned within the reactive site loop at positions termed P_1 and P_1' is, however, usually slower than for non-inhibitory substrates [7,8].

Some inhibitors, in particular those of the Kazal-type [9,10], exhibit two or more inhibitory domains which may be specific for different proteinases. Their P_1 sites are located typically at the second position after the second cysteine residue in the C-terminal direction [11]. For example, the ovoinhibitor occurring in avian egg white consists of seven Kazal-type domains with at least five reactive sites: two for trypsin, two for chymotrypsin and one for porcine pancreatic elastase [12]. Another remarkable member of the Kazal-type inhibitors is the thrombin inhibitor rhodniin from the assassin bug *Rhodnius prolixus*. It is composed of two Kazal-type domains of which only one acts in a manner typical for canonical inhibitors. In contrast, the second domain

Abbreviations: LD-6, LEKTI domain 6; EK, enterokinase; HF7665, hemofiltrate peptide with a molecular weight of 7665 Da; LEKTI, lympho-epithelial Kazal-type-related inhibitor; CZE, capillary zone electrophoresis; L-BAPNA, $N\alpha$-benzoyl-L-arginine *p*-nitroanilide

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binds to the fibrinogen recognition exosite of thrombin by electrostatic interaction [13].

Starting with the isolation of two hitherto unknown peptides from human hemofiltrate, we cloned and characterized a novel 15-domain serine proteinase inhibitor termed lympho-epithelial Kazal-type-related inhibitor (LEKTI) (SWISS-PROT entry name: ISK5_HUMAN) which includes two putative Kazal-type domains. These domains exhibit six cysteine residues probably leading to the typical 1–5/2–4/3–6 disulfide bond pattern of Kazal-type inhibitors. The remaining 13 domains lack the cysteines in positions 3 and 6, which, if they descend from Kazal-type domains, should result in a final 1–4/2–3 disulfide bond pattern [14]. Interestingly, other groups recently demonstrated that certain mutations within the LEKTI gene (*SPINK5*) are associated with skin diseases such as Ichthyosis linearis circumflexa (Netherton syndrome) [15] and atopic dermatitis as well as with atopic disorders in general [16–18]. In addition, a maternally inherited gene polymorphism was detected in the region encoding LEKTI domain 6 (position 1258: G → A). It may lead to a predicted amino acid exchange of glutamic acid to lysine in position 65 of the LEKTI domain 6 (LD-6) peptide and is significantly linked to an increased risk for atopy [17]. Therefore, LEKTI domain 6 represents a potential drug candidate for the treatment of the mentioned disorders [19,20].

In this paper, we report the recombinant production of LEKTI domain 6 in *Escherichia coli* as a homogeneous product. We further describe the analysis of its inhibitory activity against a selected number of typical serine proteinases. The biochemical and functional data obtained revealed that LEKTI domain 6 is a temporary inhibitor of trypsin. Using mass spectrometric analysis and Edman degradation, we demonstrate that LEKTI domain 6 exhibits a disulfide bond pattern and a P_1 site expected for an inhibitor descending from Kazal-type domains but differing by the loss of one of the original three disulfide bonds. This finding confirms the assumption that LEKTI domain 6 represents a novel type of inhibitory motif related to the classical Kazal-type.

2. Experimental

2.1. Construction of the expression vector pTrcHisB-hemofiltrate peptide with a molecular weight of 7665 Da (hf7665)

A partial LEKTI cDNA fragment encoding the peptide LD-6 was amplified by PCR using cDNA from human vaginal epithelium, a 5′-phosphorylated sense primer (*p*-GAATCTGGAAAAGCAACCTCATATGC), and an antisense primer (CCGTATGGTACC*GAATTC*TTACTAGTTCTTGATTCGCCTTCCTTC) possessing two stop codons (underlined) and an *Eco*RI recognition sequence (printed in italics) at its 5′-terminus. After hydrolysis with *Eco*RI, the fragment was ligated site-directed with the expression vector pTrcHisB (Invitrogen, Karlsruhe, Germany), which was first treated with *Bam*HI, then with nuclease S1 (blunting of the *Bam*HI-generated ends), and subsequently with *Eco*RI. The resulting plasmid pTrcHisB-*hf*7665 is suitable for production of recombinant LD-6 as a fusion protein in the *E. coli* strain TOP10 (Invitrogen, Karlsruhe, Germany). The primary product possesses an amino-terminal oligo-histidine (His_6) tag which is connected to the LD-6 peptide by a 21-amino-acid linker containing the enterokinase cleavage site and the Xpress™ epitope for easy detection with an Anti-Xpress™ antibody (Invitrogen).

2.2. Recombinant production of LEKTI domain 6 and preparation of soluble cell extracts

A starter culture of 50 ml LB medium containing 100 μg/ml ampicillin was inoculated with the *E. coli* strain TOP10/pTrcHisB-*hf*7665 resulting from transformation of *E. coli* TOP10 with the expression vector construct pTrcHisB-*hf*7665. After growth overnight at 37 °C, the culture was used to inoculate 1 l of fresh LB/ampicillin medium (dilution 1:50). When reaching an OD_{600} of 0.4, production of the recombinant peptide was induced by adding IPTG to a final concentration of 1 mM. After 5 h, cells were harvested and frozen overnight at −20 °C.

The cells were lysed by adding 20 ml of decomposition buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM Pefabloc, 0.20 mg/ml lysozyme, 0.2 mg/ml DNaseI), incubated on ice for 20 min and finally sonificated (three steps, 8 W, 30 s each; VibraCell, Sonics & Materials Inc., Danbury, CT, USA). Crude cell debris was then removed by centrifugation of the extract for 60 min at 16,000 × *g* and 4 °C.

2.3. Purification of recombinant LEKTI domain 6

For purification of recombinant LD-6, an equal volume of start buffer (20 mM phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4–7.6) was added to the extract which was subsequently filtered through a 0.45 μm filter to remove residual cell debris and other insoluble material. The filtrate was loaded onto a Ni^{2+}-HiTrap chelating column with 1 ml bed volume (HisTrap™-column, Amersham Biosciences, Freiburg, Germany) preequilibrated with starting buffer. The column was washed with 50 ml of starting buffer and the absorbed peptides/proteins were eluted with elution buffer (20 mM phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4–7.6). Fractions containing the fusion protein were identified by a standard Western blot using the commercially available Anti-Xpress™ antibody (Invitrogen, Karlsruhe, Germany). Positive fractions were pooled and dialyzed against enterokinase buffer (50 mM Tris/HCl, pH 8.0, 1 mM $CaCl_2$).

The His-tag was removed by adding 0.1% (v/v) Tween-20 to the dialyzed protein solution followed by incubation at 37 °C with enterokinase (EnterokinaseMax™, Invitrogen, Karlsruhe, Germany) for 16–19 h according to the manufacturer's instructions. After addition of an equal

volume of phosphate–NaCl buffer (20 mM phosphate, 0.5 M NaCl, pH 7.4–7.6), the sample was loaded onto a Ni^{2+}-HiTrap chelating column (HisTrap^{TM}-column, Amersham Biosciences, Freiburg, Germany) preequilibrated with phosphate–NaCl buffer. The column was washed with phosphate–NaCl buffer and starting buffer and the collected fractions containing LD-6 were pooled. The final purification of the recombinant peptide was performed by RP-HPLC (Jupiter, Phenomenex C18, 30 nm, 5 μm, 250 × 10 mm) with acetonitrile/TFA (solvent A 0.07% TFA; solvent B 80% CH_3HN, 0.05% TFA; gradient 20–50% solvent B in 60 min). Fractions containing the desired peptide, as verified by mass spectrometry, were combined and lyophilized.

2.4. Analysis of recombinant LEKTI domain 6

NH_2-terminal amino acid sequencing was performed with a Procise 494 sequencer (Applied Biosystems, Weiterstadt, Germany) by Edman degradation with on-line detection of phenylthiohydantoin amino acids using the standard protocol recommended by the manufacturer.

Molecular masses of proteins were determined by MALDI-TOF–MS (Voyager DE mass spectrometer, Perseptive/Vestec, TX, USA) as described [21] or by ESI–MS using the Sciex API 100 single quadrupole mass spectrometer (Sciex, Biomolecular Mass Analyzer, Perkin-Elmer, Langen, Germany) with electrospray ionization supported by the MassChrom 1.1 software. Flow injection was carried out at 5 μl/min.

Capillary zone electrophoresis (CZE) was performed on a P/ACE system MDQ (Beckman Coulter, Fullerton, CA, USA) supported by the accompanying system 2.2 software. A fused silica capillary (50 cm × 75 μm, Beckman Coulter, Fullerton, CA, USA) was used in combination with a 0.1 M sodium phosphate buffer (0.02% (w/v) hydroxypropyl-methylcellulose), pH 2.5 at 25 °C. The separation was performed with a constant voltage of 20 kV for 20 min and substances were detected at 214 nm using an integrated DAD.

2.5. Determination of the disulfide bridges of LEKTI domain 6

Native LD-6 was isolated in milligram amounts from human hemofiltrate by the use of cation exchange and different reversed phase chromatographic steps as described for other hemofiltrate peptides [22]. The isolation was directed by the molecular mass of LD-6 determined by MALDI-TOF–MS. To analyze the disulfide bond pattern of LD-6, cleavage of the native peptide was performed by the endoproteases Asp-N and Lys-C (sequencing grade from Roche diagnostics, Mannheim, Germany) and by cyanogen bromide. For cyanogen bromide cleavage, 30 μg of the lyophilized peptide was incubated for 4 h with a cyanogen bromide gas produced by the reaction of 320 mg CNBr with 70% (v/v) formic acid. For Asp-N digestion, the peptide was incubated using a peptide/enzyme ratio of 1/100 (w/w) for 8 h at 37 °C in sodium phosphate buffer (pH 8.0, 50 mM) as recommended by the manufacturer. For Lys-C digestion, incubation was performed using a peptide/enzyme ratio of 1/100 (w/w) for 2 h at 37 °C in Tris/HCl buffer (pH 8.5, 25 mM; EDTA 1 mM) according to the manufacturer's instructions. The resulting fragments were separated by analytical RP-C18 HPLC and characterized by ESI–MS and sequence analysis.

2.6. Trypsin inhibition assay

Trypsin inhibition assays were performed with 3.5 μg/ml bovine trypsin (Sigma, Deisenhofen, Germany) in trypsin buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 50 mM $CaCl_2$, 0.01% (v/v) Triton X-100) and 250 μM *N*α-benzoyl-L-arginine *p*-nitroanilide (L-BAPNA) (Sigma, Deisenhofen, Germany) as a chromogenic substrate. Hydrolysis of L-BAPNA at 25 °C was monitored with a spectrophotometer DU 640 (Beckman Coulter, Fullerton, CA, USA) by the change in absorbance at 405 nm. Analysis of the temporary inhibitory activity was performed by mixing trypsin and 1 μM inhibitor in trypsin buffer, subsequent incubation for a time period of 0–120 min at 25 °C, and measuring residual enzyme activity as described above. Inhibition assays with a selected number of other commercially available serine proteinases (chymotrypsin, leukocyte elastase, chymase, thrombin, tissue plasminogen activator, tryptase, plasmin, tissue kallikrein, factor Xa, plasma kallikrein, and urokinase) were performed in a similar way but using different suitable chromogenic substrates.

2.7. Determination of the P_1–P_1' site of LEKTI domain 6

Two hundred and thirty-eight micrograms of native LD-6 was incubated with 1.25 μg trypsin sequencing grade (Roche Molecular Biochemicals, Mannheim, Germany) in 200 μl of 50 mM Tris/HCl buffer pH 8.0 containing 100 mM NaCl. Samples were taken after 10, 30, 60 and 180 min of incubation and analyzed by HPLC using a Nucleosil C18 PPN column (Macherey-Nagel, 5 μm, 100 Å, 250 × 2 mm i.d., 0.2 ml/min, UV detection at 215 nm) with a solvent system consisting of solvent A: 0.1% aqueous TFA (v/v) and solvent B: 0.05% TFA in acetonitrile/water (4:1) (v/v), gradient 10–70% solvent B in 60 min. The main peak from the chromatogram and the front shoulder were separated and analyzed by ESI–MS and Edman degradation as specified above.

3. Results and discussion

3.1. Recombinant production and purification of LEKTI domain 6

In order to enable easy purification of recombinant LD-6 as a fusion protein by the well established Ni^{2+}-chelating

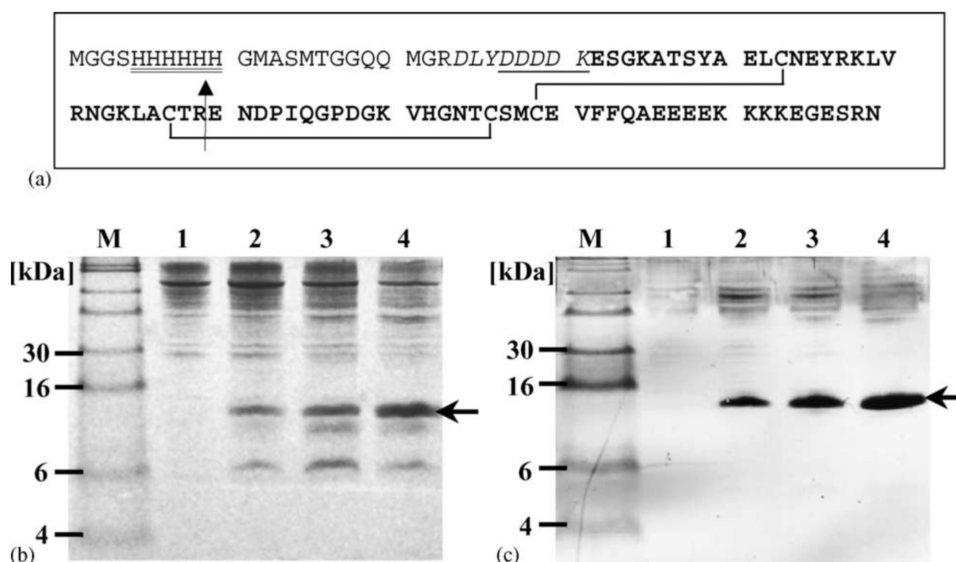

Fig. 1. Heterologous expression of the recombinant fusion protein His-EK-rHF7665. (a) Amino acid sequence of the recombinant fusion protein His-EK-rHF7665 exhibiting 99 amino acid residues and a calculated molecular weight of 11.1 kDa. The sequence of the isolated human LD-6 peptide is marked with bold letters and the enterokinase recognition site is underlined. In addition, the two disulfide bridges are indicated. The poly-histidine tag (His-tag) for affinity purification is underlined twice and the Xpress™ epitope for the detection of the recombinant peptide with the Anti-Xpress™ antibody is indicated by italics. The putative P_1–P_1' site is marked with an arrow. (b) SDS-PAGE stained with Coomassie and (c) His-tag specific Western blot of the His-EK-rHF7665 fusion protein produced in transformed *E. coli* TOP10. Lane M: pre-stained molecular weight marker in kDa; lane 1: extract of non-induced cells; lanes 2–4: cell extracts taken 1.0, 2.5, and 5.0 h after induction. Extracts from equal numbers of cells were applied in each lane. The peptide His-EK-rHF7665 is marked by an arrow.

affinity chromatography [23], we employed the pTrcHisB expression system. The vector exhibited an IPTG-inducible Trc promoter as well as regions encoding a His_6-tag, an Xpress™ epitope for product detection by a specific antibody, and a recognition site for the serine proteinase enterokinase (Fig. 1a). The latter enzyme was used for proteolytic removal of the N-terminally located His-tag and the Xpress™ epitope after purification of the fusion protein [24]. cDNA encoding LEKTI domain 6 was generated and cloned into pTrcHisB as described above. The resulting expression vector, termed pTrcHisB*hf*7665, was used for transformation of the *E. coli* TOP10 strain. Aliquots of the culture of transformed *E. coli* TOP10 were taken at various time points after induction and analyzed by SDS-PAGE (Fig. 1b) and Western blots (Fig. 1c). Since an optimum amount of fusion protein His-EK-rHF7665 was detected 5 h after induction, this time point was chosen for harvesting the cells. His-EK-rHF7665 was isolated from the soluble fraction of the cell lysate by one-step Ni^{2+}-chelating affinity chromatography [23] using a HisTrap™ column. As monitored by mass spectrometry, complete cleavage of His-EK-rHF7665 could be achieved with enterokinase. The detached fusion part of the protein containing the His-tag and the Xpress™ epitope was then removed by a second Ni^{2+}-chelating affinity chromatography step. Final purification of the peptide was performed by RP-HPLC as described above. From 1 l of culture, after the application of several chromatographic purification steps and lyophilization, we finally obtained an amount of 630 μg homogeneous recombinant LEKTI domain 6.

3.2. Structural analysis of recombinant and native LEKTI domain 6

In order to verify the correct amino acid sequence of recombinant LEKTI domain 6, we performed Edman degradation from the N-terminus up to the proline residue in position 32. The partial amino acid sequence obtained (ESGKATSYAELNEYRKLVRNGKLATRENDP) exactly matched the expected sequence. Using MALDI-TOF–MS and ESI–MS, the apparent molecular mass of recombinant LD-6 was determined to be 7666.5 and 7665.9 Da, respectively. These data are in good agreement with the molecular mass of 7665.5 calculated for the native peptide.

To determine the disulfide pattern of native LEKTI domain 6, the peptide was reduced and the resulting free thiol

Table 1
Disulfide bond pattern of LEKTI domain 6

Sequenced fragments	Calculated mass (Da)	Determined mass (Da)
ATSYAELCNEYRK – CEVFFQAEEEEK (disulfide-linked)	3033.3	3033 ± 0.8
VHGNTCSM[a] – LACTRENDPIQGPDGK (disulfide-linked)	2511.7	2511 ± 0.8

The native peptide LD-6 was cleaved by endoproteinase Lys-C and subsequently by cyanogen bromide as described in Section 3. The resulting fragments analyzed by ESI–MS and sequencing are listed.

[a] Homoserine-lactone.

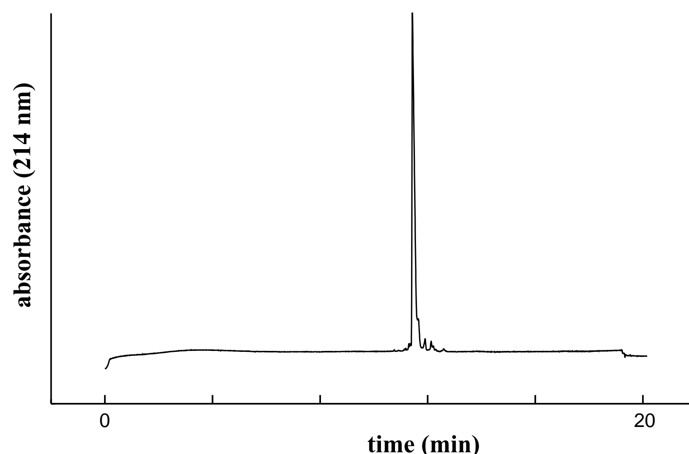

Fig. 2. Analysis of the LEKTI domain 6 peptides by capillary zone electrophoresis. Co-injection of native and recombinant LD-6. CZE analysis was performed with a peptide concentration of 100 μM.

functions were amidoalkylated as described. The molecular mass of the alkylated LD-6 was determined to be 7899 Da indicating four cysteine residues (mass increase of 58.5 Da per cysteine). Cyanogen bromide cleavage of the native peptide did not lead to a fragmentation but to a decrease in the molecular weight to 7636 as determined by mass spectrometry. These results indicate the hydrolysis of the peptide bond between Met47 and Cys48 and cyclization of methionine into homoserine-lactone.

Proteolytic cleavage of native LD-6 with endoprotease Asp-N did not lead to the generation of two separable peptides but to an increase in the molecular mass to 7683 Da. This finding is due to hydrolysis of the peptide bond Asn30-Asp31 of LD-6 which is located between the second and third cysteine residue. Thus, a 1–2/3–4 connection between the four cysteines is not present in LEKTI domain 6. Endoproteinase Lys-C digestion and subsequent cyanogen bromide cleavage resulted in the generation of several fragments which were subsequently analyzed by mass spectrometry and Edman degradation (Table 1). Sequencing of a fragment with MW 3033 Da yielded two amino acid residues per cycle which could be assigned to amino acid residues Ala5-Lys17 and Cys48-Lys59 of LD-6. Another fragment with MW 2511 Da resulted in a double sequence comprising Val40-Met47 and Leu24-Lys39 of LEKTI domain 6. The data obtained are compatible with disulfide linkages between Cys12–Cys48 and Cys26–Cys45, respectively. Taken together, the data prove a 1–4/2–3 disulfide bond pattern of LD-6 which exactly matches a Kazal-type-related peptide.

Since disulfide isomers of peptides possessing disulfide bonds usually exhibit different migration times during capillary zone electrophoresis [25], we used this technique to compare the disulfide bond pattern of recombinant LD-6 with that of native LEKTI domain 6 (Fig. 2). Co-injection of both peptides indeed resulted in a single peak (Fig. 2) indicating an identical disulfide pattern of both peptides. Thus, it is conceivable that the described recombinant production resulted in a peptide absolutely identical to native LEKTI domain 6. Since LD-6 is representative of most of the 15 domains of LEKTI and mutations within the gene region encoding LEKTI domain 6 are described to be linked to Netherton syndrome and different forms of atopy [16–18], this peptide appears to be a worthwhile object for

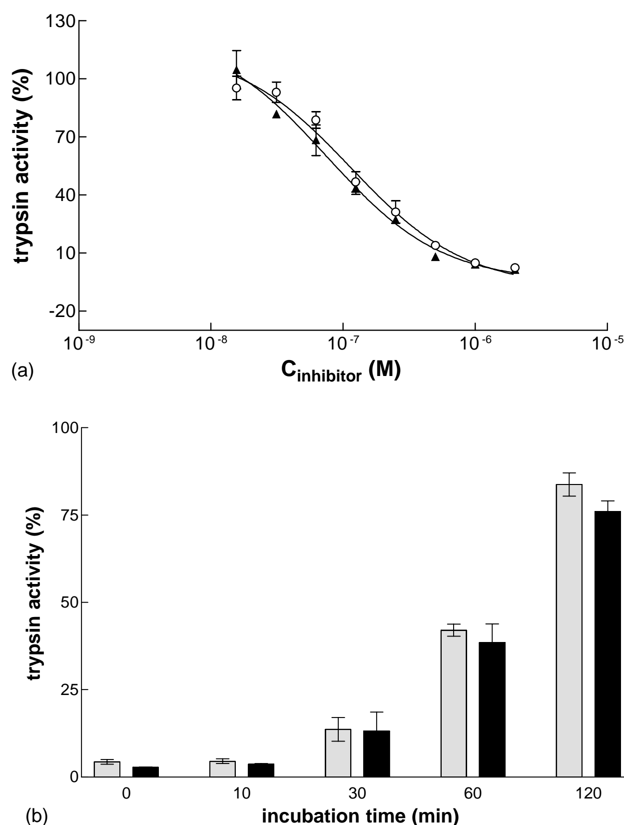

Fig. 3. Inhibition of trypsin by native and recombinant LD-6. (a) Inhibition of trypsin by native LD-6 (○) and recombinantly produced peptide (▲) in concentrations ranging from 16 nM to 2 μM. (b) Temporary inhibition of trypsin by native (□) and recombinant (■) LD-6 after a preincubation of 1 μM inhibitor and 3.5 μg/ml trypsin. For details see experimental procedures.

further intensive investigation. Such investigation requires the availability of sufficient amounts of the peptide. However, proteins and peptides heterologously expressed in *E. coli* often form insoluble aggregates caused by lacking or inaccurate disulfide bridges [26]. Even in the case of solubility of the recombinantly produced peptide, disulfide isomers may occur. For instance, as described by Lauber and co-workers, the heterologous expression of LEKTI domain 1 in *E. coli* BL21 resulted in different peptide isoforms partially exhibiting unexpected patterns of disulfide bridges [27]. In contrast, the expression of LEKTI domain 6 in the cytoplasm of *E. coli* TOP10 resulted exclusively in soluble homogeneous folded peptide exhibiting the same disulfide pattern as the native peptide.

3.3. Inhibitory activities of recombinant and native LEKTI domain 6

To determine the inhibitory activity of LD-6, it was analysed with the commercially available serine proteinases chymotrypsin, leukocyte elastase, chymase, thrombin, tissue plasminogen activator, tryptase, trypsin, plasmin, tissue kallikrein, factor Xa, plasma kallikrein, and urokinase. Among these enzymes only trypsin was inhibited by the recombinant LD-6 as well as by the native peptide with similar potency. At a concentration of 3.5 μg/ml trypsin, both peptides exhibit a uniform IC50 value of approximately 100 nM (Fig. 3a).

For investigation of their stability against tryptic proteolysis, 1 μM of each peptide was incubated for 4 h with trypsin under the standard assay conditions. In this case, native and recombinant LD-6 again behaved similarly, and approximately 50% of the initial trypsin activity was recovered after 60 min of incubation (Fig. 3b). After 3–4 h of incubation, any inhibitory effect of the two peptides was lost (data not shown), indicating their complete hydrolysis. The rather short lifetime of LEKTI domain 6 may be due to the lack of one of the three disulfide bridges which are conserved in typical Kazal-type inhibitors [14].

The entire LEKTI comprises a total of 15 domains, 12 of which are related to domain 6 by their amino acid sequences and the number of only four cysteine residues resulting in

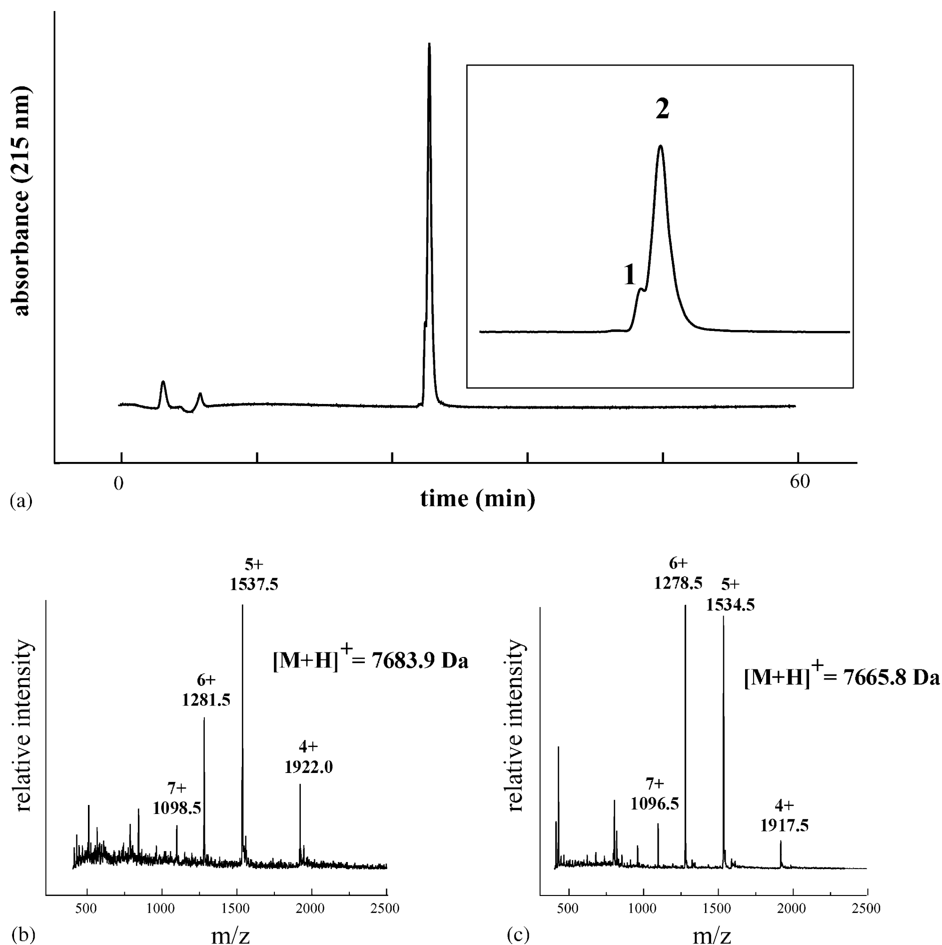

Fig. 4. Verification of the P_1–P'_1 site of native LD-6. (a) The peptide was incubated with trypsin and analyzed by HPLC after 180 min. The inset shows a zoom of the peak. (b) ESI–MS of the collected peak 1 of the HPLC. (c) ESI–MS of the collected peak 2 of the HPLC. HPLC parameters: Nucleosil C18 PPN column (Macherey-Nagel, 5 μm, 100 Å, 250 × 2 mm i.d., 0.2 ml/min, UV detection at 215 nm) with a solvent system consisting of solvent A: 0.1% aqueous TFA (v/v) and solvent B: 0.05% TFA in acetonitrile/water (4:1) (v/v), gradient 10–70% solvent B in 60 min.

two disulfide bridges. Thus, we assume that this multidomain inhibitor depending on certain physiological requirements is responsible for a rapid but temporary inhibition of one or more serine proteinases which still remain to be identified.

3.4. Verification of the putative P_1–P_1' sites of LEKTI domain 6

The P_1 site of a typical Kazal-type inhibitor is located at the second position in the C-terminal direction after the second cysteine residue [11]. To verify the presumed P_1–P_1' sites Arg28-Glu29 of LD-6, the native peptide was incubated with trypsin and analyzed by HPLC at various points in time. Cleavage of the peptide by trypsin was detectable in the HPLC chromatogram as a front shoulder of the LEKTI domain 6 peak evolving after 10 min of incubation (Fig. 4a). The determined molecular weight of the peptide concealed under the shoulder (Fig. 4b) is exactly 18 Da higher than the mass of the initial LD-6 (Fig. 4c).

This difference corresponds to the molecular mass of water and indicates the hydrolysis of one peptide bond. Sequence analysis of the cleaved protein revealed double peaks during Edman degradation that could be assigned to two different parts of the amino acid sequence of LD-6. One of the sequences obtained corresponds to the amino-terminus of the native peptide (ESGKATSYAEL), the other sequence starts with the presumed P_1-site and the following C-terminal amino acids (ENDPIQGPDGK). This experimental confirmation of the P_1-site provides further evidence for the relation of the two disulfide bridged LEKTI domains to Kazal-type inhibitors.

4. Conclusion

In summary, the heterologous production of LD-6 in *E. coli* TOP10 resulted in a homogeneous product exhibiting the same biochemical and functional characteristics as the native peptide. By performing trypsin inhibition assays, we demonstrated that LEKTI domain 6 acts as a serine proteinase inhibitor. Using the biochemical analysis described, we demonstrated that the positioning of the disulfide bonds of LEKTI domain 6 exactly corresponds to that of two of the three disulfide bonds occurring in typical Kazal-type domains. In addition, the determined P_1 site is identical to the P_1 site expected for a Kazal-type-related inhibitor. These data as well as the occurrence of two typical Kazal-type domains within the putative LEKTI precursor protein strongly indicate that LD-6 and the 12 related LEKTI domains represent a new type of inhibitory motif which is related to the classical Kazal-type. Furthermore, the established strategy for the recombinant production of LEKTI domain 6 will enable further investigation of its mode of action and its physiological role.

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