Homologous Proteins with Different Structures: Solution Structures of LEKTI Domains 1 and 6

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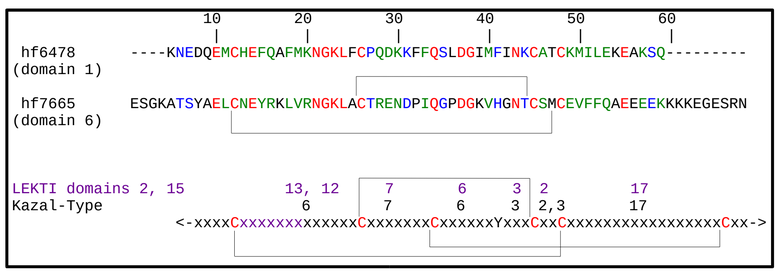
We have determined the threedimensional structures of the potential proteinase inhibitors HF6478 and HF7665, which were isolated from human blood filtrate (1). These proteins are part of the larger precursor protein LEKTI containing 15 potential serine proteinase inhibitory domains. As 13 of the 15 domains share partial homology to typical Kazal-type inhibitors but lack one of the three conserved disulfide bonds, they may represent a novel type of serine proteinase inhibitors. Structure determination as well as further inhibitory assays of different domains of LEKTI shall prove this hypothesis. Structural characterizations of recombinant HF6478 (1st domain) as well as of synthetic HF7665 (6th domain) were carried out by CD- and multidimensional NMR-spectroscopy. In spite of sharing the same disulfide connectivity pattern and sequence identity of about 35% from the first to the fourth cysteine, the two proteins show different threedimensional structures. The order and arrangement of the secondary structural elements - two α-helices and a small β-sheet - as well as the overall structure of HF7665 (domain 6) bear resemblance to proteinase inhibitors of the Kazal-type, but differs from those of HF6478 (domain 1), which shows three helices and a β-hairpin, but in different sequence stretches and in a different order. Also the putative binding loop of HF6478 (domain 1) has a non-typical conformation compared to known proteinase inhibtitors (2). In contrast to HF7665, which inhibits efficiently the serine proteinase trypsin, no proteinase was found so far to be inhibited by HF6478.

Methods and results

Sequence alignment



Sequence alignment of LEKTI domains 1 and 6. Both peptides show a sequence identity of 35% from the 1st to the 4th Cysteine (additional 32% of the residues show high similarity). Comparison with typical Kazal-type inhibitors shows that the third disulphide bond (3-6 connectivity) is missing. In addition, there is a longer sequence stretch from the 1st to the 2nd Cysteine residue (13 instead of 6 residues). Identical residues (red) and residues with strong (green) or low similarity (blue) are marked by colour.

Sample preparation

The gene fragment coding for the peptide HF6478 was combined with a sequence encoding a factor Xa cleavage site at its 5´ end, using standard PCR techniques and was cloned into pET-32a. For the expression of recombinant HF6478 an *E. coli* strain with a *trx*B$^-$/*gor*522$^-$ double mutation was used, allowing disulfide formation in its oxidative cytoplasm (3, 4).

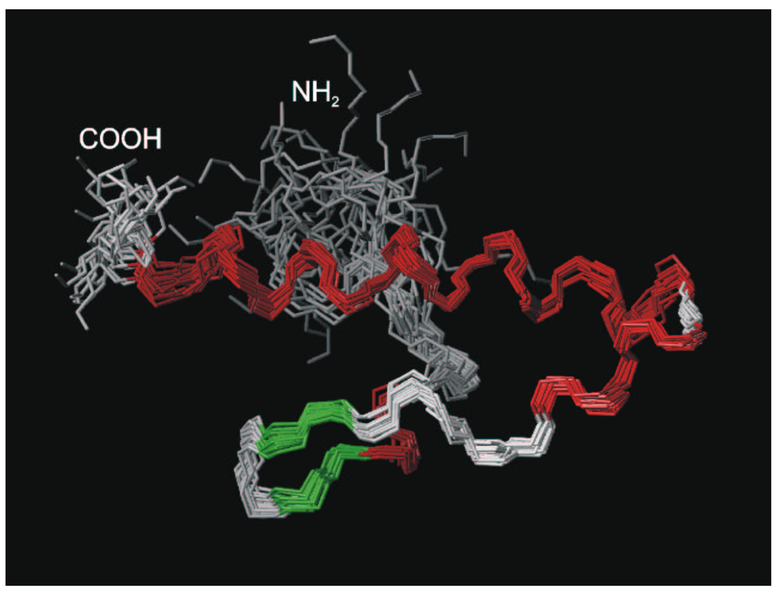
The ^{15}N labeled protein was purified (5) from cells grown on M9 minimal media. Sample conditions for ^{15}N labeled HF6478 were 1.5 mM protein in H_2O/D_2O (9:1, v/v), pH 4.5. Sample conditions for unlabeled chemically synthesized HF7665 were 2 mM protein in H_2O/D_2O (9:1,v/v), pH 4.2.

Structure determination

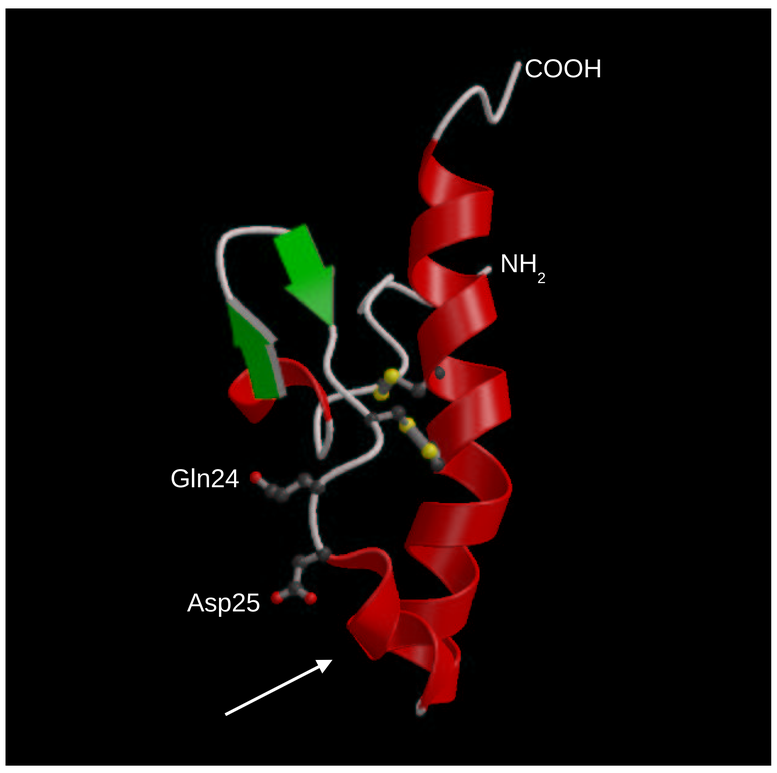
All NMR spectra were recorded on a Bruker DRX600 spectrometer at 25°C. The following spectra were recorded for the backbone and sidechain assignment of HF6478: ^{1}H,^{1}H,^{15}N-TOCSY- and NOESY-HSQC, ^{15}N,^{15}N,^{1}H-HMQC-NOESY-HSQC, HNHA, ^{1}H,^{15}N-HSQC spectra. To assign aromatic amino acid side chains, 2D-TOCSY and NOESY spectra of an unlabeled sample were used. Structural restraints for HF6478 were achieved from ^{1}H,^{1}H,^{15}N-NOESY-HSQC, HNHA, and 2D-NOESY spectra. Resonance assignment of HF7665 was possible using 2D DQF-COSY, Clean-TOCSY, and NOESY spectra of an unlabeled protein sample. Restraints for the structure calculation of HF7665 were obtained from 2D-NOESY spectra. The solution structures were calculated with XPLOR 3.851, using a modified simulated annealing protocol.

The structure of HF6478 (domain 1)

Experimental restraints used for the final structure calculation		**Atomic RMSDs from the avarage structure** (backbone/*heavy atoms*)	
Intraresidual NOEs	102	Overall[a]	1.80 Å/*2.45 Å*
Interresidual NOEs		Residues 7 - 52	0.57 Å/*0.99 Å*
sequentiell	203	secondary	
medium-range	162	structure[b]	0.47 Å/*0.83 Å*
long-range	56		
Dihedral angle restraints		[a] residues	1 - 55
J (^{15}N,$^{\alpha}$H)	14	[b] residues	12 - 16, 18 - 19, 26 - 30, 32 - 52



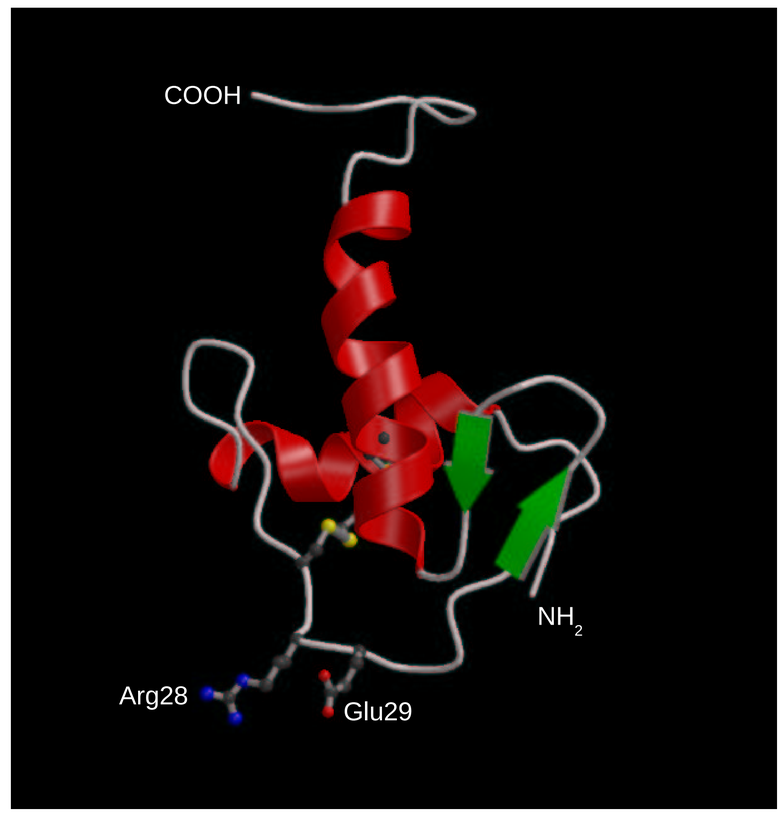
Backbone overlay of the 21 accepted structures (generated with SYBYL 6.5 (Tripos Inc., St. Louis, MO)). Helical elements are displayed in red, the hairpin structure is coloured in green.



Schematic drawing of a representative structure of HF6478, indicating regular secondary structural elements. The putative loop region of HF6478 (P3 to P3´; comprising amino acids Cys22 to Lys27) features an unusual main chain conformation compared with canonical loops of other serine proteinase inhibitors (e.g., the backbone conformation of the P2´ and P3´ residues is helical (marked by arrow) instead of the typical parallel β-sheet conformation ($-140° < \varphi < -99°$; $70° < \psi < 120°$; (2)). Disulfide bridges (Cys8-Cys44; Cys22-Cys41) and sidechains of the putative P1-P1´ residues are shown as stick representation. (The figure was created with MOLSCRIPT (6) and Raster3D (7))

Structural characterization of HF7665

Secondary structure of HF7665: summary of sequential and medium-range NOEs and chemical shift indices (HA CSI). The relative strength of the NOEs, classified as weak, medium and strong from cross-peak intensities of 2D-NOESY spectra, is indicated by the strength of the horizontal bars. Positive and negative chemical shift indices are typical for β-sheets and α-helices (8), as denoted by rectangles above and below the axis. The secondary structure of HF7665, determined by molecular dynamics calculations, is depicted in the bottom line.



A family of 40 structures was calculated by restrained molecular dynamics calculations, using 470 distance constraints, obtained from analysis of the NOE connectivities. One representative structure of HF7665 is shown in the MOLSCRIPT (6) and Raster3D (7) view, indicating regular secondary structural elements. The structure features a canonical loop like proteinase binding site (P3 to P3´; comprising amino acids Cys26 to Asp31). The sidechains of the P1-P1´ residues are exposed (as shown in stick representation), disulfide bridges (Cys12-Cys48; Cys 26-Cys45) are indicated.

Comparison of both structures and conclusions

The 3D-structures of both peptides show common features with other serine proteinase inhibitors, like a hydrophobic core, as well as the exposed sidechains of the putative protease recognition site (P1 and P1' residues).

The arrangement of secondary structural elements is comparable for both structures corresponding to the long COOH-terminal and shorter NH_2-terminal α-helix, but significantly differs in the central region, where HF6478 shows another short helix whereas HF7665 features a short β-sheet. This main difference leads to completely diverse conformations of the binding loops of both peptides: HF7665 possesses a canonical loop like conformation (2) that has to be validated by structure refinement, whereas the main chain conformation of the putative loop region (from P3 to P3´) of HF6478 is unusual, which could explain the missing inhibitory activity.

Since HF7665 shows a protein fold comparable to known Kazal-type inhibitors, but features some characteristic differences, like an extended COOH-terminal and an additional NH_2-teminal α-helix, as well as a missing disulfide bond, we assume that this peptide may be indeed a member of a novel type of serine proteinase inhibitors.

References

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