Cloning, Expression, Purification, and Structural Characterization of the Putative Serine Proteinase Inhibitor HF6478

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Abstract

We have cloned, overexpressed, and purified the putative serine proteinase inhibitor HF6478, which is part of the larger precursor protein LEKTI, exhibiting 15 potential inhibitory domains (1). HF6478 (55 aa) contains two disulfide bridges in a 1−4, 2−3 connectivity, sharing partial homology to Kazal−type domains and other serine proteinase inhibitors. HF6478 was expressed as thioredoxin (Trx)−fusion protein (pET−32a), and disulfide formation occurred in the oxidative cytoplasm of *E. coli* Origami™ (DE3), which carries a *trxB*$^-$/*gor522*$^-$ double mutation. The soluble fusion protein was purified using metal chelating affinity chromatography. Cleavage of the Trx−fusion protein using factor Xa and subsequent purification yielded HF6478, in amounts sufficient for further studies by NMR spectroscopy.

Characterization of recombinant HF6478 was carried out by amino acid sequencing, mass spectrometry, capillary zone electrophoresis (CZE), RP−HPLC, and CD−spectroscopy. Multidimensional NMR studies of the ^{15}N labeled protein, sequential spin system assignment, analysis of the NOE connectivities and coupling constants, and subsequent restrained molecular dynamics calculations resulted in a family of convergent structures. Common features of these structures are a long COOH−terminal helix (Leu32 to Ala52), a short central helix (Lys26 to Phe29), a short NH_2−terminal helix (Phe11 to Phe14), and a hairpin structure (Met15 to Leu20). The disulfide bridges and a hydrophobic core define the relative orientation of these structural elements.

The common disulfide pattern with other inhibitors, the stable conformation provided by the disulfide bridges and the hydrophobic core, as well as the exposed side−chains of the putative P1 and P1' residues of the canonical loop (2) are further indications for the confirmation of the hypothesis of HF6478 being a serine proteinase inhibitor.

Introduction

As part of the large precursor protein LEKTI, HF6478 is a potential serine proteinase inhibitor. To prove this hypothesis, inhibition assays and a structure to function relationship are required. Amounts of HF6478 (55 aa) isolated from human blood filtrate were sufficient only for amino acid sequencing and mass spectrometry (1), making expression of the recombinant protein (rHF6478) necessary.

Here we report cloning, overexpression and purification of rHF6478. Structural features were determined using heteronuclear NMR experiments of the ^{15}N labeled protein.

Results

Purification of Recombinant HF6478

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

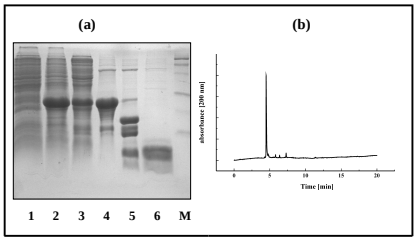

FIG.1: (a) rHF6478 was expressed as thioredoxin fusion protein (Trx−Xa−HF6478) using *E. coli* Origami™ (DE3) as host strain (lane 1 and 2 are before and after induction with 1 mM IPTG, respectively). Trx−Xa−HF6478 was purified from the soluble fraction of cell lysates (lane 3) by one−step Ni^{2+}−chelating−affinity chromatography (lane 4). Cleavage of the fusion protein resulted in four different peptides with molecular masses of apparently 17 kDa, 15 kDa, 14 kDa, and 7 kDa (lane 5), indicating secondary cleavage after Arg 129 and Arg 143. Most of the secondary cleavage fragments could be removed by a second Ni^{2+}−chelating−affinity chromatography step (lane 6; M: molecular weight marker). Small cleavage fragments were removed using size exlusion chromatography (SEC). rHF6478 containing fractions were pooled and analyzed by CZE **(b)**.

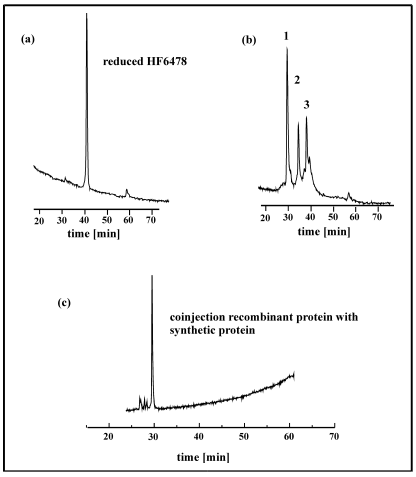

FIG.2: RP−HPLC analysis of HF6478: The reduced peptide **(a)** and the three possible disulfide isomers (1, 2, and 3: obtained after oxidative folding of reduced HF6478) of HF6478 **(b)** show significant differences concerning to their retention times on a C18−column. Coinjection of rHF6478 with a synthetic version of the peptide **(c)** with known disulfide pattern verified a consistent 1−4, 2−3 (Cys 8 − Cys 44; Cys 22 − Cys 41) connectivity for both molecules as expected to be the native form in human blood filtrate.

Structural characterization of HF6478

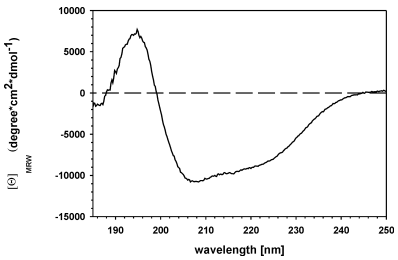

FIG.3: The CD−spectrum of rHF6478 is typical for a protein containing both, α−helices and β−sheets, with a broad minimum between 208 nm and 222 nm as well as an intensive positive band at 195 nm. Evaluation of the spectrum resulted in 19,8% of α−helix and 33,2% of β−sheet (5).

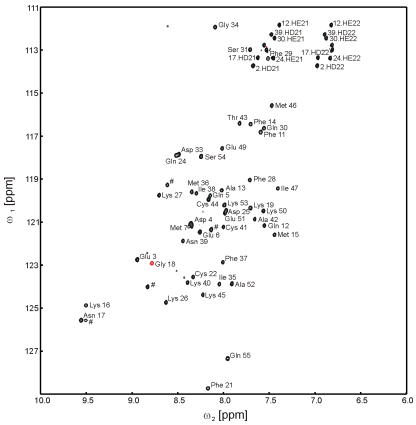

FIG.4: HSQC−spectrum of ^{15}N−rHF6478. The spectrum was recorded at 298 K and pH 4,5. Backbone and sidechain amide proton resonances are labelled (not assigned peaks are marked by #).

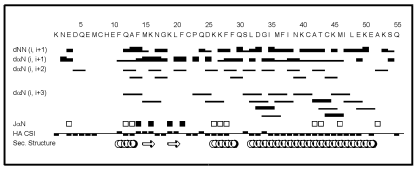

FIG.5: Secondary structure of rHF6478: summary of sequential and medium−range NOEs, vicinal NH−C_αH coupling constants (JαN) and chemical shift indices (HA CSI). The relative strength of the NOEs, classified as weak, medium and strong from cross−peak intensities of the NOESY−HSQC−spectrum, is indicated by the strength of the horizontal bars. Values of JαN < 6 Hz and > 8 Hz, as typical for α−helices and β−sheets, are illustrated as white and black squares, respectively. Positive and negative chemical shift indices are typical for β−sheets and α−helices (6), as denoted by rectangles above and below the axis. The secondary structure of rHF6478, determined by molecular dynamics calculations, is depicted in the bottom line. (The sequential assignment for positions 8 to 10 is missing).

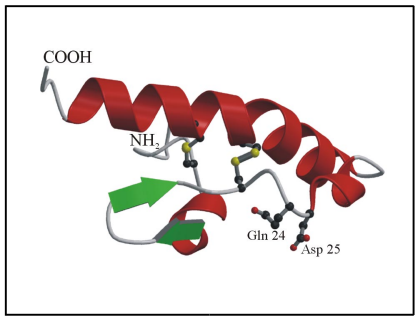

FIG.6: A family of 40 structures was calculated by restrained molecular dynamics calculations (7), using 348 distant constraints, obtained from analysis of the NOE connectivities, and 14 coupling constants. One representative structure of HF6478 is shown in the MOLSKRIPT (8) and Raster3D (9) view, indicating regular secondary structural elements. The disulfide bridges (Cys8−Cys44; Cys 21−Cys 41) and sidechains of the putative P1−P1´ residues are shown as stick representation.

Conclusions

Overexpression of HF6478 in the oxidative cytoplasm of an *E. coli trx*B$^-$/*gor*522$^-$ double mutant and subsequent purification yielded amounts of the peptide sufficient for further studies by NMR spectroscopy. Only one disulfide isomer of recombinant HF6478 was obtained, exhibiting a 1−4, 2−3 connectivity.

The 3D−structure of HF6478 shows common features with other serine proteinase inhibitors, like a hydrophobic core, as well as the exposed side−chains of the putative protease recognition site (P1 and P1' residues). However, compared to the characteristic conformation of the binding loop of known inhibitors, the main chain conformation of the putative P2´ and P3´ sites of HF6478 is unusual. The refinement of the obtained structures will allow a more detailed structural analysis and comparision with other inhibitors, providing information about an inhibitory function of HF6478, as well as further inhibition assays.

References

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