Accurate Disulfide Formation in Escherichia coli: Overexpression and Characterization of the First Domain (HF6478) of the Multiple Kazal-Type Inhibitor LEKTI

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The human hemofiltrate peptide HF6478, a putative serine proteinase inhibitor, which is part of the precursor protein LEKTI, was cloned, overexpressed, and purified. HF6478 contains two disulfide bridges with 1-4, 2-3 connectivity, sharing partial homology to Kazal-type domains and other serine proteinase inhibitors. It was expressed as thioredoxin (Trx) fusion protein, and disulfide formation occurred in the oxidative cytoplasm of Escherichia coli Origami (DE3) strain which carries a trxB⁻/gor522⁻ double mutation. The soluble fusion protein was purified using metal-chelating affinity chromatography. Cleavage of the Trx fusion protein with factor Xa and subsequent purification yielded the final product in amounts sufficient for structural studies. Characterization of recombinant HF6478 was done by amino acid sequencing, mass spectrometry, capillary zone electrophoresis, and CD spectroscopy. Taking the blood filtrate peptide HF6478 as example, we present a strategy which should facilitate the expression of different extracellular proteins in the E. coli cytoplasm.

Proteinase inhibitors are essential tools for in vivo regulation of endogenous proteinases. Many pathological effects originate from misregulated endogenous proteinases or proteinases encoded by bacteria, viruses, or parasites (1), showing the important function of their inhibitors. Thus, proteinase inhibitors become more and more important as drugs against currently untreatable diseases. The most prominent example for this kind of therapy is probably the rationally designed HIV-1 envelope proteinase inhibitors (2), although the observed numbers of resistances against several of these drugs are increasing (3).

HF6478 (hemofiltrate peptide with molecular weight 6478 Da) seems to be a serine proteinase inhibitor according to sequence homologies with other inhibitors. It is isolated from human blood and is part of the precursor protein LEKTI that exhibits 15 potential inhibitory domains (4). The amounts of HF6478 (55 amino acids) isolated from human blood were sufficient for amino acid sequencing and mass spectrometry (4). Further studies like inhibition assays and the determination of the three-dimensional protein structure require higher amounts of HF6478, making expression of the recombinant protein (rHF6478) necessary.

Here we report cloning, overexpression, and purification of rHF6478 as thioredoxin fusion protein, using an Escherichia coli trxB⁻/gor522⁻ double mutant that allows disulfide formation in its oxidative cytoplasm (5, 6). After cleavage of the fusion protein, subsequent

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2 Abbreviations used: BCA, bicinchoninic acid; CD, circular dichroism; CZE, capillary zone electrophoresis; EK, enterokinase; Trx, E. coli thioredoxin; HIV, human immunodeficiency virus; HF6478, hemofiltrate peptide with molecular weight 6478 Da; rHF6478, recombinant HF6478; LB, Luria-Bertani; IPTG, isopropyl β-D-thiogalactoside; PMSF, phenylmethylsulfonyl fluoride; SEC, size-exclusion chromatography.

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purification yields rHF6478 in amounts sufficient for NMR spectroscopy. The recombinant protein has been analyzed by mass spectrometry, CZE, and circular dichroism (CD) spectroscopy. The disulfide connectivity of rHF6478 was determined by CZE, using a chemically synthesized version of the peptide with known disulfide pattern as reference.

**MATERIALS AND METHODS**

**Construction of the Expression Vector pET-32a-Xa-hf6478**

A fragment of the LEKTI cDNA encoding the peptide HF6478 was amplified by PCR (7) using a forward primer which introduces a KpnI site and a sequence encoding a factor Xa cleavage site at the 5' end of the gene (GGAGGAAGCTTGGATCCATGAAAGCTTGATGACGAGATG). The reverse primer contains a Xhol site at its 5' end (GGAGGAGTCTAGATCTAGAGATG). The fragment was inserted into the T7 DNA polymerase-based expression vector pET-32a (Novagen). The resulting pET-32a-Xa-hf6478 is able to produce the peptide HF6478 with an NH2-terminal E. coli thioredoxin fusion (Trx-tag, Novagen). The two proteins are connected by a 43-amino-acid linker containing six histidine residues and a factor Xa cleavage site resulting in the fusion protein Trx-Xa-HF6478.

**Preparation of Soluble Extracts**

A starter culture of 50 ml LB containing 200 μg/ml ampicillin, 15 μg/ml kanamycin, and 12.5 μg/ml tetracycline was inoculated with the E. coli strain Origami (DE3)/pET-32a-Xa-hf6478 and grown overnight at 37°C. The starter culture was used to inoculate 1 liter of LB medium (containing 200 μg/ml ampicillin, 15 mg/ml kanamycin, and 12.5 μg/ml tetracycline) at an OD600 of 0.1. Cultures were grown to an OD600 of 0.8, and the expression of the recombinant protein was induced by addition of IPTG to 1 mM. After 3 h, cells were harvested and resuspended in 20 ml of buffer A (50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole) with 0.5 mM PMSF, 0.2 μg/ml lysozyme, 0.2 mg/ml DNaseI, and 2 mg/ml benzamidine. The cells were lysed by three 30-s sonications (T = 4°C; Labsonic U sonicator at 150 W intensity) followed by overnight freezing at −80°C. After thawing, the extract was clarified by centrifugation for 60 min at 40,000 rpm, at 4°C in a TFF 70.38 rotor.

**Purification of Recombinant HF6478**

The soluble extract (20 ml) was loaded on a Ni2+-NTA column (3-ml bed vol, Qiagen, batch column) which was equilibrated in buffer A. The column was washed with buffer A and 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 30 mM imidazole, and the absorbed proteins were eluted with 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 200 mM imidazole. The fractions containing the fusion protein (as verified by SDS-PAGE) were dialyzed against 50 mM Tris/HCl, pH 8.0, 100 mM NaCl to reduce the high imidazole concentrations. The protein concentration was estimated using the BCA assay (Pierce) (8).

**RESULTS AND DISCUSSION**

**Purification of Recombinant HF6478**

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 (7) and was cloned into pET-32a. As host strain for the expression of recombinant HF6478, E. coli Origami (DE3) (Novagen), a trxB/gor522 double mutant was used, allowing disulfide formation in the oxidative cytoplasm of this strain (5, 6). Thus, recombinant HF6478 with an NH2-terminal E. coli thioredoxin fusion was expressed by the strain E. coli Origami (DE3)/
Expression of the Trx fusion protein (Trx-Xa-HF6478; 212 amino acids; 23.4 kDa) was induced by 1 mM IPTG with an expression level of 40% of total cellular proteins (Fig. 1a, lane 2) as deduced by densitometric analysis of Coomassie-stained SDS-PAGE (data not shown). To avoid reduction of disulfide bridges and renaturation of denatured fusion protein from inclusion bodies, only the soluble fraction of cell lysate was used for further purification, containing fusion protein at 28% of the soluble fraction (Fig. 1a, lane 3). Trx-Xa-HF6478 was purified by one-step Ni²⁺-chelating affinity chromatography (9) with a purity of 66% (Fig. 1a, lane 4). A total of 27 mg of recombinant Trx fusion protein was obtained from 2.4 g wet wt cells (1 liter of culture).

Enterokinase (EK) and factor Xa are, according to their specificity (EK, DDDDK↓; factor Xa, IEGR↓), the only commercially available proteinases that cleave the fusion protein to rHF6478 without additional amino acids at its NH₂-terminus. Since we were unable to cleave the thioredoxin domain from a fusion protein containing the EK cleavage site, as in the original pET-32a vector construct, a modified Trx fusion protein containing a factor Xa cleavage site was used. Compared to the EK cleavage site, sequences similar to that recognized by factor Xa are more prevalent, leading to a higher probability of secondary cleavage (10, 11). The fusion protein (Trx-Xa-HF6478) contains Arg 157 of the factor Xa cleavage site and Arg 74 as part of the thioredoxin sequence, as well as arginines 129 and 143, located between His-tag and factor Xa cleavage site, as potential secondary cleavage sites. As the sequence of HF6478 does not contain any arginine residues, the overall yield of the peptide would not have been influenced by an unspecific activity of factor Xa. After incubation with the enzyme, a strong decrease in intensity of the fusion protein band (Fig. 1a, lane 5) was observed, showing that cleavage of Trx-Xa-HF6478 was possible.

In addition to the expected bands of apparently 17 kDa (Trx-tag) and 7 kDa (rHF6478) (Fig. 1a, lane 5, marked by arrow), two bands with apparent molecular masses of 15 and 14 kDa (Fig. 1a, lane 5) were obtained, indicating that secondary cleavage has indeed occurred after Arg 129 and Arg 143. Size-exclusion chromatography (SEC) to remove secondary cleavage products resulted in substantial loss of rHF6478 (data not shown), necessitating a different purification strategy. As part of all larger peptide fragments (comprising amino acids 1–129, 1–143, and 1–157), the His-tag allowed a second Ni²⁺-chelating affinity chromatography step to purify rHF6478 from secondary cleavage products (Fig. 1, lane 6). After SEC to remove small cleavage fragments (comprising amino acids 129–143, 129–157, and 143–157; Fig. 1b), rHF6478 with apparent homogeneity was obtained (Fig. 2b). Starting with 27 mg of Trx fusion protein containing 7.4 mg of rHF6478, the total of 3 to 3.6
rHF6478 was estimated to be 7 kDa, in agreement with the molecular weight obtained by electrospray mass spectrometry (6477.69 ± 0.68 Da) as well as with the calculated value of 6477.59 Da.

When expression of His-tagged HF6478 was carried out in E. coli BL21 (DE3), all of the three possible disulfide isomers were formed, making isolation of the correct isomer necessary. In contrast, expression of rHF6478 as Trx fusion protein in E. coli Origami (DE3) resulted in a uniform product. In order to determine its disulfide pattern, we took advantage of the significantly different retention times of the three possible disulfide isomers in RP-HPLC (data not shown) and CZE. Coinjection (Fig. 2c) of rHF6478 with a synthetic version of the peptide with selectively synthesized disulfides (Fig. 2a) resulted in a strong single peak, indicating a consistent 1-4, 2-3 (Cys 8–Cys 44; Cys 22–Cys 41) connectivity for both peptides suggested to be the native form in human blood filtrate.

The CD spectrum of rHF6478 from 185 to 250 nm (Fig. 3) is typical for a protein containing both α-helix and β-sheet with a broad minimum between 208 and 222 nm as well as an intense positive band at 195 nm. Evaluation of the spectrum resulted in an estimate of 19.8% α-helix and 33.2% β-sheet (12). As HF6478 contains two disulfide bridges, as well as the side chains of aromatic amino acid residues that influence CD spectra (13), the exact contents of regular secondary structural elements of the peptide will be known only after determination of the detailed 3D structure.

CONCLUSIONS

When extracellular proteins are expressed in conventional E. coli strains, missing or wrong disulfide bridges

mg of recombinant HF6478 obtained after cleavage and subsequent purification corresponds to an overall recovery yield of 40 to 48%. Isotopic labeling, as required for heteronuclear NMR experiments, can be performed in minimal medium, leading to similar overall recovery yields.

Characterization of Recombinant HF6478

The NH₂-terminal sequence of rHF6478 (KNEQDEMCH−) was verified by sequencing. Using SDS–PAGE, the apparent molecular weight of

FIG. 2. CZE analysis of rHF6478 (as described under Materials and Methods). (a) Synthetic HF6478 with selectively synthesized disulfide bridges (Cys 8–Cys 44 and Cys 22–Cys 41). (b) Recombinant HF6478 (SEC-pooled fractions corresponding to the hatched area in Fig. 1b). (c) Coinjection of recombinant with synthetic HF6478.

FIG. 3. The CD spectrum of rHF6478 is typical for a protein containing both α-helix and β-sheet. The spectrum was recorded on a Jasco J-810 spectrometer at room temperature with 30 μM rHF6478 in H₂O, pH 4.5.
often cause formation of insoluble aggregates (14). Expression of the putative serine proteinase inhibitor HF6478 in the oxidative cytoplasm of E. coli Origami (DE3), however, resulted in soluble, properly folded protein with accurately formed disulfides. Thus, the presented strategy is most likely generally useful for the cytoplasmic expression of different extracellular proteins in E. coli.

REFERENCES