Protein Sequence Analysis, Cloning, and Expression of Flammutoxin, a Pore-forming Cytolysin from Flammulina velutipes

MATURATION OF DIMERIC PRECURSOR TO MONOMERIC ACTIVE FORM BY CARBOXYL-TERMINAL TRUNCATION*

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Flammutoxin (FTX), a 31-kDa pore-forming cytolysin from Flammulina velutipes, is specifically expressed during the fruiting body formation. We cloned and expressed the cDNA encoding a 272-residue protein with an identical N-terminal sequence with that of FTX but failed to obtain hemolytically active protein. This, together with the presence of multiple FTX family proteins in the mushroom, prompted us to determine the complete primary structure of FTX by protein sequence analysis. The N-terminal 72 and C-terminal 107 residues were sequenced by Edman degradation of the fragments generated from the alkylated FTX by enzymatic digestions with Achromobacter protease I or Staphylococcus aureus V8 protease and by chemical cleavages with CNBr, hydroxylamine, or 1% formic acid. The central part of FTX was sequenced with a surface-adhesive CNBr, hydroxylamine, or 1% formic acid. The central part of FTX was sequenced with a surface-adhesive CNBr, hydroxylamine, or 1% formic acid. The central part of FTX was sequenced with a surface-adhesive CNBr, hydroxylamine, or 1% formic acid. The central part of FTX was sequenced with a surface-adhesive CNBr, hydroxylamine, or 1% formic acid. The central part of FTX was sequenced with a surface-adhesive CNBr, hydroxylamine, or 1% formic acid.

Pore-forming cytolytic proteins are distributed in a wide variety of eukaryotic and prokaryotic organisms (1, 2). Complement, perforin from the cytotoxic T-cells, α-hemolysin from Staphylococcus aureus, streptolysin O from Streptococcus pyogenes, aerolysin from Aeromonas hydrophila, and some others have been intensively studied in terms of pathophysiologival functions (2–6). The self-assembling, pore-forming cytolyins are illustrative molecules for the study of the assembly, membrane insertion, and molecular architecture of transmembrane pores (3–6). Several cytolytic proteins have been isolated from the basidiocarpst of both toxic and edible mushrooms, and their pore-forming properties as well as cardiototoxicity and cytoxicity were studied (7–10). Although the physiological function of the mushroom cytolsyns remains enigmatic, recent studies have implied the involvement of hemolytic proteins in the fruiting initiation of some mushrooms. The Aa-PrI gene, which encodes a putative 16-kDa protein, has been shown to be specifically expressed in the fruiting initiation of the edible mushroom Agrocybe aegerita (11). Aegerolysin was isolated as a 17-kDa hemolytic protein from the basidiocarp of A. aegerita, and it was preferentially detected in the primordia and immature fruiting bodies of the mushroom (12).

Lin et al. (9) isolated a cardiototoxic and cytolytic 22-kDa protein from the basidiocarp of the edible mushroom Flammulina velutipes, and designated it flammutoxin (FTX).1 Later, Bernheimer and Oppenheim (13) purified a hemolytic protein of 32 kDa from the same mushroom and referred to it as FTX on the assumption that the FTX of Lin et al. (9) derived from the 32-kDa FTX by partial proteolysis. We isolated FTX as a 31-kDa single hemolysin of F. velutipes, determined the N-terminal 28 residues, and studied the molecular basis of the cytolytic action of the protein (14). Our results showed that FTX assembles into a ring-shaped oligomer with outer and inner diameters of 10 and 5 nm, respectively, which forms membrane pores with a functional diameter of 4–5 nm and causes an osmotic burst of human erythrocytes (14). By using planar lipid bilayers, we showed that FTX forms a cation-selective, voltage-gated channel with a diameter of 4–5 nm (15).

Watanabe et al. (16) purified a 30-kDa transepithelial electrical resistance-decreasing protein from the basidiocarp of F. velutipes (9).

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB015948.

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1 The abbreviations used are: FTX, flammutoxin; rFTX, recombinant FTX; rFTX271, recombinant FTX consisting of 271 residues; rFTX251, recombinant FTX consisting of 251 residues; TPCK, N-tosyl-L-phenylalanyl-chloromethylketone; PE, S-pyridylethylated; HPLC, high performance liquid chromatography; RP-HPLC, reversed phase HPLC; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry.
velutipes, which increased tight junctional permeability of human intestinal Caco-2 monolayers. The N-terminal amino acid sequence of the purified protein was identical with that of FTX reported by us (14, 16). Watanabe et al. (16) cloned a cDNA encoding a 272-residue protein (AB012289) and concluded that the cloned cDNA encodes the transepithelial electrical resistance-decreasing protein, because N-terminal sequence and molecular mass of the predicted protein coincided with those of the purified protein (16). However, they did not express the cloned cDNA. Concurrently with their cloning, we cloned a cDNA encoding the same protein (GenBankTM accession number AB015948) and expressed the cDNA in Escherichia coli but failed to obtain hemolytically active recombinant protein. Taken together with the fact that F. velutipes produces multiple FTX family proteins with N-terminal sequences similar to that of FTX (described below), it remained uncertain that the cloned cDNAs encode FTX or the transepithelial electrical resistance-decreasing protein. This prompted us to determine the complete primary structure of FTX by protein sequence analysis.

Sakamoto et al. (17) studied expression of genes in different developmental stages of F. velutipes and cloned C1 cDNA (GenBankTM accession number AB030006), which was specifically expressed during the fruiting body formation. A search on the DDBJ/GenBankTM/EBI nucleotide sequence data bases indicated that the C1 cDNA is identical with the cDNAs cloned by us and by Watanabe et al. (16). The same group also analyzed chronological expression of proteins in F. velutipes by using two-dimensional electrophoresis and showed that four 30–32-kDa proteins, which had N-terminal sequences similar to that of FTX, were abundantly expressed in the fruiting bodies of F. velutipes (17). Furthermore, cDNAs identical with FTX cDNA were cloned from Hericium erinaceum, Agrocybe chaixii, Pleurotus eryngii var. felureae, Coprinus comatus, and Ganoderma lucidum, which cover different families of Basidiomycetes (GenBankTM accession numbersAY281063–AY281067). Thus, FTX and/or FTX family proteins are produced by F. velutipes and other mushrooms during the fruiting body formation.

In this study, we determined the complete primary structure of FTX by protein sequence analysis. As a result, protein and nucleotide sequences were in accord except for the lack of the initial Met and the C-terminal 20 residues in protein. Based on the sequence information obtained, we constructed expression vectors for the transcription of cDNAs encoding FTX and/or the transepithelial electrical resistance-decreasing protein. To obtain the purified protein (16), we failed to express the cloned cDNA. Concurrently with their cloning, we cloned a cDNA encoding the same protein (GenBankTM accession number AB015948) and expressed the cDNA in E. coli but failed to obtain hemolytically active recombinant protein. Taken together with the fact that F. velutipes produces multiple FTX family proteins with N-terminal sequences similar to that of FTX (described below), it remained uncertain that the cloned cDNAs encode FTX or the transepithelial electrical resistance-decreasing protein. This prompted us to determine the complete primary structure of FTX by protein sequence analysis.

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Construction of Expression Plasmids—The FTX cDNA was inserted into the NcoI site of pT7c99A (Amerham Biosciences) to produce rFTX with the same N-terminal sequence as that of FTX. The pUF1 was digested with EcoRI and HindIII, the resultant EcoRI-HindIII fragments were inserted into pT7c99A, and the cloned pT7c99A was designated pTFS. The pTFS was digested with NcoI, blunted, and digested with SalI. To amplify the DNA segment from the 4th to the 413th nucleotide of the FTX cDNA (which corresponds to the N-terminal 135 amino acid residues of FTX, the nucleotide numbering is according to Fig. 7), a PCR was performed using the pUF-N as the template and the following primers. The sense primer was 5'-ATTCGATGATGCTAGCTCCGACCAAGTTGGAGGATCCT-3' (where the underline indicates the NcoI site and the nucleotide numbering is according to Fig. 6), and the antisense primer was 5'-GGTGTCGACTCCGTAGAAATCGAAATCTCGATGACACGA-3' (where the underline indicates the HindIII site and a stop codon, respectively, followed by the nucleotide numbering is included; the nucleotide numbering is according to Fig. 6), and the antisense primer was 5'-ATTCGATGATGCTAGCTCCGACCAAGTTGGAGGATCCT-3' (where the underline indicates the NcoI site and the nucleotide numbering is according to Fig. 6), digested with EcoRI and HindIII, and ligated with the SalI-digested pTFS possessing the blunt end of the NcoI site. The resultant plasmid for expression of FTX precursor was designated pTXT272.

To construct an expression system for mature FTX, the DNA segment, which contained the FTX cDNA fragment from the 337th to the 758th nucleotide and a stop codon, was amplified by a PCR using pTFS as the template and the following primers. The sense primer was 5'-GTCATCTGCGTGAATACTGAAATCTCGACCAAGTTGGAGGATCCT-3' (where the underline indicates the SalI site and the nucleotide numbering is according to Fig. 7), and the antisense primer was 5'-ATTCGATGATGCTAGCTCCGACCAAGTTGGAGGATCCT-3' (where the underline indicates the HindIII site and a stop codon, respectively, followed by the nucleotide numbering is included; the nucleotide numbering is according to Fig. 6), digested with NcoI and HindIII, and ligated with the double-digested pTFS272 with PSTI and HindIII. The resultant plasmid was designated pTXT252.

DNA Sequencing—The cycle-sequencing reaction was performed with Sequi Therm Long Read cycle sequencing kits containing M13 forward and reverse IR-dye primers (Epicenter Technologies, Madison, WI). The DNA sequencing system (Li-Cor model 4000L, Li-Cor Inc., Lincoln, NE) was used for sequencing. The resultant sequencing data were analyzed using the GENETYX software package. A similarity search for nucleotide sequences was performed on the DDBJ/GenBank/EMBL nucleotide sequence databases.

Expression, Renaturation, and Purification of rFTX—E. coli DH5α cells harboring pTXT272 or pTXT252 were grown at 37 °C in 2× YT medium (1% Bacto Trypticase, 1% Bacto Yeast extract, and 0.5% NaCl, Difco) with ampicillin (100 μg/ml). When optical density at 660 nm of the culture reached 0.4, isopropyl-β-D-thiogalactoside was added at 1 μM concentration, and the cultures were incubated at 37 °C for 20 min. The precipitates obtained were suspended in 20 mM sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl, 2 mM ascorbic acid, and 0.05% (w/v) glutaraldehyde at 20 °C for 20 min and subjected to Western immunoblotting using anti-FTX serum as described (14).

Assembly of rFTX into Membrane Pore Complexes—Complexes formation by rFTX was assayed as described (14). rFTX (0.5–2.0 μg) was incubated with human erythrocytes (1.0 × 10⁸ cells/ml) in 1 ml of Tris-buffered saline at 25 °C for 30 min. The erythrocytes were washed twice with 5 mM Tris–HCl buffer, pH 7.2, and the erythrocyte membranes obtained were solubilized in 2% (w/v) SDS at 25 °C for 5 min. The solubilized membranes were subjected to Western immunoblotting using anti-FTX serum.

Isolation and Electron Microscopy of Pore Complexes—Pore complexes of rFTX were isolated and analyzed by electron microscopy as described (10, 14). Human erythrocytes (3 × 10⁷ cells) were incubated with rFTX271 or rFTX251 (50 μg of each) in 50 ml of Tris-buffered saline at 25 °C for 30 min. Erythrocyte membranes were collected and solubilized with 2% SDS (w/v) at 25 °C and loaded onto a 10–40% (w/v) sucrose gradient in 10 mM Tris–HCl buffer (pH 7.2) containing 0.1% SDS. Centrifugation was performed using a Beckman SW40Ti rotor at 32,000 rpm for 19 h at 4 °C. Fractions were analyzed by Western immunoblotting using anti-FTX serum. Fractions containing the pore complexes were stained negatively with 1% (w/v) sodium phosphotungstic acid (pH 7.2) and examined under an electron microscope H-8100 (Hitachi, Tokyo, Japan) at an acceleration voltage of 80 kV.

A biomolecular interaction assay for rFTX271 (1 μg/ml) was treated with TPCK-treated trypsin (10 μg/ml) in 20 mM Tris–HCl buffer (pH 8.0) at 37 °C for 0–24 h. Small portions were withdrawn and mixed with soybean trypsin inhibitor (final concentration, 100 μg/ml) and were subjected to SDS-PAGE or hemolytic assay. For MALDI-TOF MS, rFTX271 (28 μg/ml) was treated with TPCK-treated trypsin (0.4 μg/ml) at 37 °C for 4 or 24 h, and small portions were immediately withdrawn and analyzed as described above.

RESULTS

Protein Sequence Analysis of FTX—The strategy used for determination of the complete amino acid sequence of FTX is summarized in Fig. 1. The complete sequence was established on the sequence information of the peptides generated by enzymatic and chemical cleavages of PE-FTX, together with the results of the amino acid compositions and molecular masses of the peptides. The N-terminal 72 residues and the C-terminal 107 residues (i.e. −70% of the whole sequence) were sequenced by automated Edman degradation of intact PE-FTX and the fragments arising from PE-FTX by enzymatic digestions with Chromobacter protease I or S. aureus V8 protease and chemical cleavages with CNBr, hydroxylamine, 1% formic acid (or at methionin, Asn-Gly, or Asp-Pro bonds). The central part of FTX was obtained by analyses of subdigest peptides derived from a surface-adhesive 7-kDa tryptic peptide (7Tk) or a CNBr fragment (M2) by digestion with thermolysin, S. aureus V8 protease, or endoproteinase Asp-N. Some overlaps were provided by peptides obtained from T7k by 12 M HCl treatment. The molecular masses of FTX and several selected peptides were determined by MALDI-TOF MS to confirm the sequences obtained by automated Edman degradation.

CNBr Cleavage of PE-FTX—Cyanogen bromide fragments arising from PE-FTX (13 nmol) were first fractionated by CPC using tandem columns of two TSKgel G2000SWXL (Fig. 2A), and the fractions I, II, and III obtained were further fractionated by RP-HPLC on an Aquapore RP-300 column. Fragments M1/M6 and M3/M4/M5 were isolated from the fractions I and III, respectively (Fig. 2, B and C). Fragment M2 was desalted with a column of Sephadex G-25 fine. Isolated fragments were analyzed for their compositions and sequences (Fig. 1 and Table I). Fragments M2, M3, M4, M5, and M6 yielded 20, 11, 13, 12, and 27-residue sequences, respectively, and M6 was lytic unit was defined as the amount of FTX, which caused 50% hemolysis under the conditions described.

Cross-linking of rFTX with Glutaraldehyde—rFTX271 or rFTX251 (final concentration of each protein 5.0 or 4.6 μg/ml, respectively) was treated with 0.05% (w/v) glutaraldehyde at 20 °C for 20 min as described (25). The glutaraldehyde-treated rFTX was heated in the presence of 2% (w/v) SDS and 5% mercaptoethanol at 100 °C for 5 min and subjected to Western immunoblotting using anti-FTX serum as described (14).
suggested to be the C-terminal fragment because of the lack of homoserine (Table I). The amino acid sequence of M2 overlapped with that of intact PE-FTX (Fig. 1), suggesting that M2 spans the central part of FTX based on the molecular size (i.e., 15 kDa).

Digestion of PE-FTX with Achromobacter Protease I or S. aureus V8—As shown in Fig. 2D, nine peptides, K1–K9, were resolved from an Achromobacter protease I digest of PE-FTX (4.5 nmol) by RP-HPLC on an Aquapore RP-300 column. Sequence analysis of peptide K4 revealed a new 26-residue sequence. K5 extended the sequence of M3 and overlapped with M4. Peptide K6 provided overlaps M4–M6. The peptide K9 overlapped with M6 and extended the sequence (Fig. 1 and Table II).

A digest of PE-FTX (4.6 nmol) by S. aureus V8 protease was fractionated by GPC using a TSKgel G2000SWXL column (Fig. 3A), and the fractions I–V obtained were further fractionated by RP-HPLC on an Aquapore RP-300 column (Fig. 3B and results not shown). Eight peptides, E1–E8, were isolated by RP-HPLC and were subjected to compositional and sequential analyses. As shown in Fig. 1, the E6 peptide obtained from the fraction II overlapped with the peptides K4 and K5, and the other peptides were assigned to the N- and C-terminal regions already established as above.

Cleavage of PE-FTX with Hydroxylamine or 1% Formic Acid—Because the M2 peptide arising from PE-FTX by the cleavage with CNBr contains an Asn–Gly bond (Fig. 1), PE-FTX (6.2 nmol) was cleaved with hydroxylamine to obtain a peptide starting from Gly43. The cleaved sample was fractionated by GPC using tandem columns of two TSKgel G2000SWXL and one TSKgel G3000SWXL, and three fractions (i.e., NG0, NG1, and NG2) were obtained (Fig. 3C). The apparent molecular masses of the fractions suggested that NG1 would be the peptide starting from Pro60. The cleaved sample was desalted on a column of Sephadex G-25 fine. An automated Edman degradation of the NG1 yielded the 22-residue sequence starting from the Gly43, which overlapped with the sequence of M2 (Fig. 1).

Because the NG1 peptide contains an acid-labile Asp–Pro bond (Fig. 1), PE-FTX (6.4 nmol) was cleaved with 1% formic acid to obtain a peptide starting from Pro60. The cleaved sample was fractionated by GPC using tandem columns of two TSKgel G2000SWXL and one TSKgel G3000SWXL, and three fractions were obtained (Fig. 3D). The apparent molecular masses of the fractions suggested that DP1 would be the peptide starting from Pro60, whereas D0 and D2 would be the intact molecule and an N-terminal fragment of FTX, respec-
tively. The DP1 fragment was passed through a column of Sephadex G-25 fine and subjected to automated Edman degradation. As a result, the DP1 peptide gave the 25-residue sequence starting from Pro60 (although there was some ambiguity in the sequence; Fig. 1).

Isolation of a Surface-adhesive Peptide T7k from the Central Part of FTX and Its Fragmentation with Thermolysin, S. aureus V8 Protease, or 12M HCl—Since no peptide representing the central part of FTX was recovered from any proteolytic digest, those missing peptides must be lost by adsorption during the digestion or separation. To test these possibilities, PE-FTX was digested with trypsin, and the supernatant was subjected to GPC using tandem columns of two TSKgel G2000SWXL. B and C, RP-HPLC for the fractions I and III in A on an Aquapore RP-300 column. Peptides were eluted with a linear gradient of acetonitrile (0–80%) D, RP-HPLC for the Achromobacter protease I digest of PE-FTX on an Aquapore RP-300 column. Peptides were eluted with a linear gradient of acetonitrile (0–50%) for 40 min, followed by another linear gradient of acetonitrile (50–80%) for 5 min. The upper and lower lines in B–D indicate the absorbance of the effluent at 215 and 290 nm, respectively.

Fig. 2. Fractionation of the peptides arising from PE-FTX by the cleavage with CNBr or Achromobacter protease I. A, GPC for the peptides arising from PE-FTX by the cleavage with CNBr using tandem columns of two TSKgel G2000SWXL. B and C, RP-HPLC for the fragments I and III in A on an Aquapore RP-300 column. Peptides were eluted with a linear gradient of acetonitrile (0–80%). D, RP-HPLC for the Thermolysin, S. aureus V8 Protease, or 12M HCl digest of PE-FTX on an Aquapore RP-300 column. Peptides were eluted with a linear gradient of acetonitrile (0–50%) for 40 min, followed by another linear gradient of acetonitrile (50–80%) for 5 min. The upper and lower lines in B–D indicate the absorbance of the effluent at 215 and 290 nm, respectively.

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Isolation of a Surface-adhesive Peptide T7k from the Central Part of FTX and Its Fragmentation with Thermolysin, S. aureus V8 Protease, or 12M HCl—Since no peptide representing the central part of FTX was recovered from any proteolytic digest, those missing peptides must be lost by adsorption during the digestion or separation. To test these possibilities, PE-FTX was digested with trypsin, and the supernatant was subjected to GPC using tandem columns of two TSKgel G2000SWXL and one TSKgel G3000SWXL. Again, no large (>3-kDa) fragment was observed (results not shown). From the amino acid compositions of FTX and the sequences already placed, only a few trypsin-cleavable residues were left unsequenced. The tube was washed with 6 M guanidine hydrochloride and applied to the same column, and this time a double peak at 7–8 kDa appeared (Fig. 4A). The N-terminal sequences of these two fragments (both having Thr-Thr-Glu-Thr-Val-Trp-Ser-Tyr-Asp-Asn-Ser-Gln-) overlapped with that of DP1 and proved that they are the missing peptides. By MALDI-TOF MS using α-cyano-4-hydroxycinnamic acid as a matrix, mass (M + H)+ of T7k was estimated to be 7656 Da (results not shown).

T7k (3 nmol) was cleaved with 12 M HCl at room temperature for 15 h, and the peptides generated were fractionated by RP-HPLC using a TSKgel Super-ODS column (Fig. 4B). Sequence analyses for the peak fractions indicated that fractions 35, 53, 55, and 56, respectively, contained the peptides having new sequences (T7k-HCl-35, T7k-HCl-53, T7k-HCl-55, and T7k-HCl-56; Fig. 1): Thr-Leu-Ser-Ile-Thr-Asn-His-Ala (856.95 Da), Ser-Ile-Thr-Ile-Pro-Gly-Val-Gly-gly (798.91 Da), Ser-Ser-Phe-Glu-Ile-Ser-Ile-Ser-Thr-Glu (1100.17 Da), and Ser-Phe-Glu-Ile-Ser-Ile-Ser-Thr-Glu (1013.09 Da), where the calculated molecular masses of the peptides are indicated in parentheses and the glycine starting with a lowercase letter was tentatively identified. By MALDI-TOF MS using α-cyano-4-hydroxycinnamic acid as a matrix, masses (M + H)+ of the peptides in fractions 35, 55, and 56 were estimated to be 858.7, 1101, and 1015 Da, respectively, which confirmed the respective sequences of the peptides. The amino acid composition of fraction 53 coincided well with that expected from the sequence (results not shown).
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Amino acid compositions of CNBr peptides (M1–M6)

Results are expressed as residues per peptide by amino acid (aa) analysis or, in parentheses, from the sequence (Fig. 1).

|   | M1  | M2  | M3  | M4  | M5  | M6  |
|---|-----|-----|-----|-----|-----|-----|
| Asp/Asn | 2.8 (1/2) | 12.3 (6/7) | 0.7 (1/0) | 1.6 (1/1) | 1.4 (0/2) | 0.8 (1/0) |
| Glu/Gln | 5.2 (2/3) | 16.2 (11/5) | 0.9 (1/0) | 4.0 (3/1) | 2.1 (2) |
| Ser | 2.2 (2) | 18.9 (22) | 1.2 (1) | 2.6 (3) | 1.6 (1) | 3.9 (3) |
| Gly | 4.2 (3) | 14.2 (10) | 1.6 (1) | 0.7 (1) | 1.0 (1) | 1.0 (1) |
| His | 1.0 (1) | 0.5 (1) | 0.9 (1) | 0.7 (1) | 1.0 (1) | 1.0 (1) |
| Arg | 1.6 (1) | 4.5 (4) | 0.9 (1) | 1.0 (1) | 4.7 (5) |
| Thr | 1.2 (1) | 19.9 (26) | 1.9 (2) | 0.7 (1) | 1.0 (1) | 1.0 (1) |
| Ala | 4.0 (4) | 5.2 (4) | 1.0 (1) | 1.0 (1) | 1.0 (1) |
| Pro | 3.2 (2) | 6.4 (6) | 1.7 (1) | 2.7 (2) | 4.2 (3) |
| Tyr | 1.0 (1) | 3.4 (3) | 1.8 (2) | 1.2 (2) | 1.7 (2) | 0.8 (1) |
| Val | 1.7 (2) | 8.5 (5) | 1.0 (1) | 0.6 (1) | 0.8 (1) |
| Met | PE-Cys | 0.0 (1) | 0.0 (1) | 0.0 (1) | 0.0 (1) |
| Ile | 0.8 (1) | 7.7 (10) | 2.0 (2) | 0.6 (1) | 0.6 (1) |
| Leu | 2.5 (2) | 8.0 (8) | 1.0 (1) | 1.1 (1) | 1.1 (1) |
| Phe | 1.9 (2) | 2.0 (2) | 1.0 (1) | 1.0 (1) | 1.0 (1) |
| Trp | + (2) | + (6) | + (2) | + (6) | + (6) |
| Lys | 1.7 (2) | 3.4 (5) | 2.0 (3) | 3.2 (4) |
| Total residues | (32) | (144) | (12) | (16) | (13) | (30) |
| Yield (%) | 19.8 | 9.1 | 10.5 | 30.4 | 23.9 | 12.3 |

* Homoserine.
* Each composition was calculated on the basis of the integral value of the residue marked.
* The plus sign indicates the presence of absorbance at 290 nm.

The peptides arising from T7k (3 nmol) by thermolysin digestion were fractionated by RP-HPLC on a Nova-Pak C18 column (Fig. 4C). Sequence analyses of three selected peptides (peptides 37, 62, and 63) yielded 13-, 16-, and 12-residue sequences, respectively (Fig. 1). Amino acid sequence of the peptide in the fraction 62 (T7k-Th-62) overlapped with the N-terminal sequence of T7k. The peptide in fraction 63 (T7k-Th-63) overlapped with T7k-HCl-53 and T7k-HCl-55. The peptide in fraction 72 through 142 was established. To fill the gap left between T7k and K4, M2 peptide (3 nmol) was digested with endoproteinase Asp-N. The digest was fractionated by GPC on a TSKgel G2000SWXL column, followed by RP-HPLC using a Nova-Pak C18 column (results not shown). Analyses for amino acid compositions and sequences indicated that fraction 20 contained the peptide, Asp-Ser-Thr-Lys-Thr-Lys-Ser-Lys-Glu-His-Thr-Leu-Thr-Asn-Thr-Trp, which overlapped with T7k-Th-62 and with T7k-Th-63. The mass (M + H) of the T7k-E-rec peptide was estimated by MALDI-TOF MS using α-cyanocinnamic acid as a matrix, and the value 3078.8 Da (results not shown) obtained was in good agreement with the calculated mass (3078.4 Da) (Fig. 1). Thus, the continuous sequence from residue 72 through 142 was established. To fill the gap left between T7k and K4, M2 peptide (3 nmol) was digested with endoproteinase Asp-N. The digest was fractionated by GPC on a TSKgel G2000SWXL column, followed by RP-HPLC using a Nova-Pak C18 column (results not shown). Analyses for amino acid compositions and sequences indicated that fraction 20 contained the peptide, Asp-Ser-Thr-Lys-Thr-Lys-Ser-Lys-Glu-His-Thr-Leu-Thr-Asn-Thr-Trp, which overlapped
with the C terminus of T7k and K4, resulting in the completion of the 251-residue sequence (Fig. 1).

**Molecular Mass of FTX and Heterogeneity of Its C Terminus**—The sequence information of the intact PE-FTX and the peptides generated by enzymatic and chemical cleavages of PE-FTX established the 251-residue sequence of FTX with a calculated mass of 27,814.75 Da. However, the molecular mass of an FTX preparation (that gives a single band corresponding to 31 kDa on SDS-PAGE; results not shown) was estimated to be 27,563 by MALDI-TOF MS (Fig. 5). The observed mass value for the FTX preparation coincided well with the calculated mass of the truncated molecule lacking Val250 and Lys251. MALDI-TOF MS for another FTX preparation revealed two peaks corresponding to (M + H)+ 27,491 or 26,654 Da (results not shown), which coincided well with the calculated (M + H)+ of the FTX molecules with a truncation of the C-terminal 3 or 11 residues, respectively. SDS-PAGE for the FTX preparation revealed two bands corresponding to 31 and 29 kDa (results not shown). Furthermore, the lower molecular mass fractions of CNBr-cleaved PE-FTX were fractionated by RP-HPLC on an Aquapore RP-300 column. MALDI-TOF MS for the resolved peaks suggested the presence of the C-terminal peptides ranging from Glu218 to Lys251, Thr249, Leu248, or Pro247 (results not shown). Thus, FTX...
molecules purified from mushroom had heterogeneous C-terminal residues.

Expression of rFTX of Precursor and Mature Forms and Their Molecular Properties—As described under “Experimental Procedures,” we amplified an ~1-kbp DNA by reverse transcription-PCR using a sense primer corresponding to the N-terminal 10 residues of FTX and an antisense oligo(dT) primer and analyzed the 5′-end of the cloned cDNA by the 5′-rapid amplification of cDNA end. The cloned cDNA consisted of 819 nucleotides, encoding 272 amino acid residues (Fig. 6; GenBank™ accession number AB015948). Based on the sequence determined by Edman degradation, we concluded that the cloned cDNA encodes a FTX precursor with the initial Met and additional C-terminal 20 residues (Figs. 1 and 6). Therefore, we constructed expression systems to produce rFTX of the precursor and mature forms. Briefly, the FTX cDNA was inserted into the NcoI site of pTrc99A to construct pFTX272, and the PstI-HindIII segment of pFTX272 was replaced with the DNA fragment corresponding to the C-terminal region of FTX to construct pFTX252 (Fig. 7A). Both the precursor and mature forms of rFTX were expressed as insoluble proteins in E. coli DH5α, and no hemolytic activity was detected in the bacterial lysates. The insoluble rFTX fraction was unfolded in 8 M urea and refolded by a stepwise dialysis against 10 mM Tris-HCl buffer (pH 8.5) containing 4, 2, 1, 0.5, or 0 M urea. Apparent molecular masses of rFTXs of precursor and mature forms were estimated to be 33 or 31 kDa, respectively, on SDS-PAGE (Fig. 7). The insoluble rFTX fraction was unfolded in 8 M urea and refolded by a stepwise dialysis against 10 mM Tris-HCl buffer (pH 8.5) containing 4, 2, 1, 0.5, or 0 M urea. Apparent molecular masses (M_C) of rFTXs of precursor and mature forms were estimated to be 33 or 31 kDa, respectively, on SDS-PAGE (Fig. 7) and results not shown). These values coincided well with that of FTX (results not shown), suggesting that the initial Met was cleaved in the E. coli cells. Molecular masses (M + H) of the precursor and mature forms were estimated to be 29,866 and 27,854 Da, respectively, by MALDI-TOF MS (Fig. 9C and results not shown). These values coincided well with the calculated masses (M + H) of 29,966 and 27,816 Da, for the FTX molecules with or without the C-terminal 20 residues, respectively. Thus, rFTXs of the precursor and mature forms consisted of 271 or 251 residues and were designated rFTX271 and rFTX251, respectively. Molecular sizes of native rFTX271 and rFTX251 were analyzed by GPC using a TSKgel G3000SW column. As shown in Fig. 7C, rFTX271 and rFTX251 were eluted at the positions corresponding to 32 or 35 kDa, respectively, suggesting that rFTX271 was a dimer in solution, whereas rFTX251, like natural FTX (14), existed as a monomer in solution. To confirm this conclusion, a chemical cross-linking experiment was performed using a lower concentration of rFTX (150 nM); rFTX271 (5.0 μg/ml) or rFTX251 (4.6 μg/ml) was treated with 0.05% (w/v) glutaraldehyde, followed by Western immunoblotting. As shown in Fig. 7D, a band corresponding to 66 kDa was formed upon the treatment of rFTX271 with glutaraldehyde, whereas no band corresponding to dimers was visible with rFTX251 under the same conditions.

**Maturation of a Mushroom Cytolysin by C-terminal Truncation**—A hemolytic assay indicated that rFTX251 lysed human erythrocytes in a dose-dependent manner at concentrations of 0.2–2.0 μg/ml, and the hemolytic activity of rFTX251 was comparable with that of natural FTX (Fig. 8A). In contrast, rFTX271 exhibited no significant activity at high concentrations of up to 100 μg/ml (Fig. 8A). Oligomer formation by rFTX was tested by the experiments including solubilization of rFTX-treated human erythrocytes with 2% SDS at 25 °C and Western immunoblotting. As shown in Fig. 8B, two immunostained bands corresponding to 31 or 180 kDa were visible when human erythrocytes were incubated with rFTX271 but failed to form an oligomer there. The membranes of the rFTX251-treated erythrocytes were solubilized with 2%
SDS and fractionated by a sucrose density gradient ultracentrifugation, and the fractions obtained were subjected to Western immoblotting using anti-FTX serum. As a result, the immunostained band corresponding to 180 kDa was detected in the fractions of 20–25% (w/w) sucrose (results not shown), as shown in the previous study (14). Electron microscopy for the fractions indicated the presence of the ring-shaped structures with outer and inner diameters of 10 and 5 nm, respectively (Fig. 8C). Thus, monomeric rFTX251, like natural FTX (14), bound to and assembled into a ring-shaped pore complex on human erythrocytes, whereas dimeric rFTX271 bound to the cells but failed to form a pore complex.

To study the maturation process of FTX, rFTX271 was treated with TPCK-treated trypsin at 37 °C for 0–24 h at an enzyme/substrate weight ratio of 1:10, followed by SDS-PAGE and hemolytic assay for the trypsin-treated protein. As shown in Fig. 9A, rFTX271 was cleaved by trypsin to form the band corresponding to 31 kDa, and thereafter the 29-kDa molecules were generated by the prolonged incubation. Hemolytic activity of the reaction mixture increased in tandem with the conversion of rFTX271 (33 kDa) to the 31- and 29-kDa molecules (Fig. 9, A and B). To determine the cleavage site(s), rFTX271 was cleaved with TPCK-treated trypsin at 37 °C for 4 or 24 h in an enzyme/substrate weight ratio of 1:70, and the reaction mixtures were subjected to MALDI-TOF MS. Without the trypsin digestion, molecular mass ((M+H)+) of rFTX271 was determined to be 29,972.3 Da, which coincided well with the calculated value (29,964.2 Da) of the protein (Fig. 9C, lower panel). When treated with trypsin for 4 or 24 h, rFTX271 gave peaks corresponding to (M+H)+ of 29,971/27,819/26,774 Da or 29,929/27,781/26,737 Da, respectively (Fig. 9C, middle and upper panels). The (M+H)+ of 27,819 and 27,781 coincided well with the calculated mass of FTX (27,815 Da), and the (M+H)+ of 26,774 and 26,737 Da were essentially the same as the calculated mass of a truncated molecule without C-terminal 30 residues (26,776 Da). The intensity of the peaks corresponding to the 251- or 241-residue molecules increased in a time-dependent manner, probably reflecting a conversion of rFTX271 to the 251-residue molecule and thereafter to the 241-residue molecule. A shoulder of the peak of (M+H)+ 26,737 suggested the cleavage between Lys240 and Lys241, producing a 240-residue molecule by the prolonged trypsin digestion (Fig. 9C, upper panel). Furthermore, the molecular size of the trypsin-treated rFTX271 was estimated by GPC using a TSKgel G3000SW.
column. As shown in Fig. 7C (trypsin-treated rFTX271), the trypsin-treated rFTX271 gave a large peak corresponding to 35 kDa as well as a small peak corresponding to 62 kDa. The trypsin-treated rFTX271 also exhibited hemolytic activity at a level of 5000 units/mg protein (results not shown). Thus, dimeric rFTX271 was converted to hemolytically active monomers by cleavage of the linkage between Lys 251 and Met 252.

**DISCUSSION**

In this study, we established the complete amino acid sequence of FTX from the sequence information obtained by Edman degradation of intact PE-FTX and the peptides arising from PE-FTX by several enzymatic and chemical cleavages, together with the compositional analyses and MALDI-TOF MS for the peptides. As a result, FTX consisted of 251 residues, indicating that the cloned cDNA encoded a FTX precursor with the initial Met and an additional C-terminal 20 residues. Analyses of the molecular and pore-forming properties of rFTX showed that dimeric FTX precursor was converted by the cleavage at the linkage between Lys 251 and Met 252 to monomers, which bound to and assembled into an SDS-stable, ring-shaped pore complex on human erythrocytes. Activation of protoxin by a C-terminal truncation has been reported to several pore-forming cytolysins including aerolysin from *A. hydrophila* (26, 27), *Clostridium septicum/H9251*-toxin (28), enterobin from the Brazilian plant *Enterolobium contortisiliquum* (29), and *Pseudomonas aeruginosa* cytotoxin (30), and all of the precursors were shown to be dimers in solution. Previous studies showed that aerolysin, *C. septicum/H9251*-toxin, and enterobin remain dimers after the C-terminal truncation, and the processed dimers bind to and assemble into pore complexes on the target cells (26–29). In contrast, the precursors of FTX and *P. aeruginosa* cytotoxin are converted to active monomers by the cleavage of the C-terminal 20 residues (this study) (30). Thus, the C-terminal segment of FTX precursor is involved in the formation of stable dimer. According to an analysis of the hydropathy profile using the algorithm of Kyte and Doolittle (31) and a secondary structure prediction using the algorithm of Garnier et al. (32), the 7-residue segment containing the cleavage site (i.e. Lys 251–Met 252) forms a hydrophilic α-helix (Fig. 10A), which disappears by the removal of the C-terminal 20 residues (Fig. 10B). Because the linkage between Lys 251 and Met 252 is highly sus-
the analysis of the central part, the heterogeneity in the C-structure (Fig. 10). Upon contact with membrane, FTX forms relatively large peptides of this region were recovered only in recovery of the peptides of this region from enzyme digests. The sticky nature of this region prevented formation. The terminal segment of FTX precursor contributes to dimer membranes. Further study is needed to elucidate how the C-terminal proaerolysin would mask hydrophobic patches of the toxin x-ray crystallographic analysis (33), the C-terminal region of certain conformation of the protein in a dimer. Based on the FTX precursor may be required for stabilization of a part of the molecule. The sticky nature of this region prevented completion of the sequence analysis. We concluded that FTX consists of 251 residues on the basis of the fact that no peptide extending from Lys251 was detected among the peptides arising from PE-FTX by the cleavage with CNBr (Fig. 2, A–C, and Table I), Achromobacter protease I (Fig. 2D and Table II), or S. aureus V8 protease (Fig. 3, A and B, and results not shown). The conclusion is consistent with the finding that rFTX271 was cleaved by trypsin preferentially at the linkage between Lys251 and Met252 (Fig. 9C), irrespective of the presence of two more Lys residues in the C-terminal segment (i.e. Lys254 and Lys260; Fig. 6). The resultant 251-residue protein, FTX, may be thereafter cleaved by carboxypeptidase(s) to generate heterogenous C termini in the mushroom and/or in the purification steps. Furthermore, some FTX preparations exhibited another band corresponding to 29 kDa on SDS-PAGE/Western immunoblotting, and cleavage at the linkages between Lys244 and Thr242 or between Lys240 and Lys241 was indicated by MALDI-TOF MS (Fig. 9C). The amount of the 29-kDa molecules increased during storage at 4 °C in the absence of protease inhibitors, producing more heterogeneity in the C terminus. Incidentally, the presence of the C-terminally truncated molecular species may interpret, at least in part, the higher hemolytic activity of natural FTX, compared with that of rFTX251 (i.e. FTX and rFTX251 caused 50% hemolysis at the protein concentration of 0.5 or 0.7 μg/ml, respectively) (Fig. 8A), because C-terminally truncated FTX with an apparent mass of 29 kDa lysed human erythrocytes 3-fold more efficiently than intact FTX.2

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