Cloning and Expression of the *Momordica charantia* Trypsin Inhibitor II Gene in Silkworm by using a Baculovirus Vector

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MCTI-II (*Momordica charantia* trypsin inhibitor II) isolated from bitter gourd (*Momordica charantia* LINN.) seeds is one of the serine protease inhibitors of the squash family. We cloned cDNA that encodes MCTI-II and constructed an expression system for MCTI-II by using a baculovirus vector. The recombinant baculovirus was inoculated to early fifth-instar larvae of the silkworm (strain: Shunrei × Shougetsu). Four days after infection, the hemolymph of silkworm larvae was collected and the recombinant protein was purified. Two kinds of expressed MCTI-II protein were obtained. An amino acid sequence analysis of the two proteins indicates that both were similar to the authentic inhibitor, except for the addition of a tripeptide derived from the vector at the N-terminus. One of the two inhibitors (MCTI-II A) resulted in a single PTH-amino acid in each Edman degradation cycle, while the other (MCTI-II B) resulted in two PTH-amino acids, suggesting the occurrence of cleavage of the reactive site. The inhibitory activities of MCTI-II expressed toward trypsin are examined in terms of the *K*_i value, these being 6.4 × 10⁻¹⁸ m for MCTI-II A and 5.2 × 10⁻¹⁸ m for MCTI-II B.

**Key words:** trypsin inhibitor; squash; expression; baculovirus; silkworm

Protease inhibitors are ubiquitous in the animal, plant, and microorganism kingdom,¹⁻³ and constitute an important group of proteins. Plant protease inhibitors are drawing strong attention and interest because of their roles in the defense strategy of plants against insect or microorganism predators by reducing the digestibility and nutritional quality of the leaves.⁴⁻⁵ Serine protease inhibitors are found in many plants, especially in their seeds. They are classified into several classes according to the primary structure, position of the reactive site, and disulfide bridges.¹⁶ Among them, squash family protease inhibitors are small peptides consisting of only 27–32 amino acid residues with three disulfide bridges.

MCTI-II (*Momordica charantia* trypsin inhibitor II) isolated from bitter gourd seeds (*Momordica charantia* LINN.) belongs to the squash family,⁷,⁸ MCTI-II inhibits trypsin,⁷,⁸ and factors Xa and XIIa of the blood coagulation cascade.⁹ The inhibitor consists of only 28 amino acid residues and is stable at high temperature.⁸ The positions of three disulfide bridges are known, and the reactive site of the inhibitor is located at an Arg²-Ile⁶ peptide site.¹¹ MCTI-II seems an attractive model to reveal the interaction between protease and an inhibitor, and is regarded as an ideal subject for structure-function studies because of its compact structure.

We attempted the expression of MCTI-II by using a baculovirus vector. The advantage of the baculovirus expression vector system is the large-scale expression of foreign gene products owing to the strong polyhedrin promoter.¹⁰ Furthermore, this system achieves proper folding, disulfide bond formation, oligomerization, and post-translational modification of the overexpressed recombinant proteins.¹¹ A system utilizing the *Autographa californica* nuclear polyhedrosis virus (AcNPV) and *Spodoptera frugiperda* IPLB-Sf21-PE (Sf21) cell line is widely used for the production of many heterologous proteins, because of the ease of maintaining a large-scale culture of Sf21 cells in a serum-free medium. Another baculovirus gene expression system, utilizing the *Bombyx mori* nuclear polyhedrosis

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Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; BAE, N-Benzoyl-L-arginine ethyl ester hydrochloride; BAPA, N-Benzoyl-L-arginine-κ-p-nitroanilide; BmNPV, *Bombyx mori* nuclear polyhedrosis virus; FCS, fetal calf serum; HyNPV, hybrid NPV between AcNPV and BmNPV; HyMCTI-II, recombinant HyNPV carrying MCTI-II cDNA; MCTI-II, *Momordica charantia* trypsin inhibitor II; PTH, phenylthiohydantoin; Sf21, *Spodoptera frugiperda* cell line IPLB-Sf21-PE; TFA, trifluoroacetic acid; TGT, towel gourd trypsin inhibitor
virus (BmNPV), has the advantage of being inexpensive and producing a high level of heterologous proteins in silkworm larvae with bearing convenience. The hybrid virus (HyNPV) of AcNPV and BmNPV, which can infect both S. frugiperda and silkworm, is particularly attractive. The advantages of the gene expression system utilizing HyNPV are supported by the rapid preparation of recombinant viruses in SF21 cells and the inexpensive and high production of the protein in silkworm larvae.  

This paper describes the cloning and expression of the MCTI-II gene in silkworms by using a hybrid baculovirus.

Materials and Methods

Materials. Bitter gourd seeds (Momordica charantia Linn., cultivar, futo-reishi) were purchased from Takii Seed Co. (Kyoto, Japan). A DNA extraction kit and perfect-match PCR enhancer were from Stratagene (La Jolla, CA, USA), and the primers were synthesized by Funakoshi (Tokyo, Japan). Taq DNA polymerase, the BamHI restriction enzyme and DNA ligation kit Ver. 2 were purchased from Takara Shuzo (Kyoto, Japan). Agarose-ME was from Nacalai Tesque (Kyoto, Japan), and β-agarase and Sea Plaque GTG agarose were from FMC Bio Products (Rockland, ME, USA). The original TA cloning kit containing the pCR 2.1 vector was a product from Invitrogen (Groningen, Netherlands), the DNA sequencing kit was from PE Applied Biosystems (Foster City, CA, USA), and baculovirus transfer vector pAcGP67A was from Pharmingen (San Diego, CA, USA). DNA of HyNPV was prepared according to the method described previously.  

Fetal calf serum (FCS), the TC-100 medium and lipofectin were from Gibco BRL (Gaithersburg, MD, USA). SP-Toyopearl 650M was purchased from Tosoh Co. (Tokyo, Japan), and the HiLoad16/10 S-Sepharose high-performance column was from Pharmacia (Uppsala, Sweden). Trypsin from bovine pancreas (type III) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), N-benzoyl-L-arginine p-nitroanilide (BAPA), and N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE-HCl) were from Peptide Institute (Osaka, Japan), and methanesulfonic acid (4 µL) containing 0.2% 3-(2-aminoethyl)indol was from Wako Pure Chemical Industry (Osaka, Japan). Authentic MCTI-II was purified from bitter gourd seeds according to the method of Hamato et al.  

Cell line and insect. The Spodoptera frugiperda IPLB-Sf21-AE (Sf21) cell line was used and maintained in the TC-100 medium containing 10% (v/v) heat inactivated FCS and 0.26% tryptose broth in a tissue flask at 27°C.

Fifth-instar larvae of a hybrid strain of silkworm (Shunrei × Shougetsu) were used in this study, the larvae being reared on mulberry leaves at 27°C.

Isolation of bitter gourd genomic cDNA. The bitter gourd was grown on a farm, and the fruit before maturation was harvested to collect immature seeds. These immature seeds were lyophilized and crushed in liquid N₂. Genomic DNA (380 µg) was isolated from powder (0.5 g) of the seeds with a DNA extraction kit.

cDNA cloning of MCTI-II. The coding gene of inhibitor MCTI-II was amplified by PCR. The mixed primers were designed according to the amino acid sequence of MCTI-II (Arg1-Met4 and Cys10-Gly19) as follows: sense primer, 5'-ACGTGATCCACG (A/C/G/T)AT(A/C/T)TG(G/T)CC(A/C/G/T)CG(A/C/G/T)AT(A/C/T)TGATG3' which contained a BamHI restriction enzyme site (underlined); antisense primer, 5'-TGCAAGATCCTCA(A/C/G/T)CC(A/C/G)CA(G/T)TG(A/C/G/T)CC (A/G)TC(A/C/G/T)AC(A/G)CA(A/G/T)AT (A/G)CA-3' which contained a complement of the stop codon and a BamHI restriction enzyme site (underlined) in the non-coding region. Genomic DNA of bitter gourd was used as a template. A 25-µl amount of the PCR mixture contained 2.5 µl of a 10-fold PCR buffer (100 mM Tris-HCl at pH 8.3 containing 500 mM KCl and 15 mM MgCl₂), 2 µl of 2.5 mM dNTP, 0.5 µl of Taq DNA polymerase (2.5 U), 0.5 µl of the perfect-match PCR enhancer, 1 µl of each primer (20 pmol), and 1 µl of the template (1 µg). The reaction was run for 5 cycles (denaturing at 95°C for 0.3 min, annealing at 37°C for 0.3 min, and extending at 70°C for 1 min) and then for 25 cycles (denaturing at 95°C for 0.5 min, annealing at 55°C for 0.3 min, and extending at 70°C for 1 min) by a thermalcycler (Gene Amp PCR System 2400, PE Applied Biosystems). The PCR product was analyzed by electrophoresis on 2% agarose gel and was purified by extracting from 0.8% agarose gel (Sea Plaque GTG agarose) by degradation with agarase and precipitation with ethanol.

The PCR product was subcloned into the pCR 2.1 vector with a TA cloning kit, and its sequence was determined by the dyeoxy-chain termination method with a nucleotide sequence analyzer system (model 377, PE Applied Biosystems). Plasmid pCR-MCTI-II thus prepared was used for the subsequent experiments.

Construction of the transfer vector. Plasmid pCR-MCTI-II was digested with BamHI to isolate the MCTI-II cDNA fragment. This fragment was cloned into the dephosphorylated BamHI site of pAcGP67A (the baculovirus transfer vector). The recombinant transfer vector was constructed to ex-
press MCTI-II under the control of the baculovirus polyhedrin promoter. The pAcGP67 vector contains the signal sequence of acidic glycoprotein 67 of the baculovirus, so MCTI-II was expected to be secreted. Under these conditions, the tripeptide, Ala-Asp-Pro, derived from the signal sequence of the vector is considered to have been added at the N-terminus of MCTI-II.

Construction of the recombinant baculovirus. SF21 cells cultivated in the TC-100 medium containing 10% (v/v) FCS and 0.26% tryptose broth were transfected with 2 µg of circular viral DNA from HyNPV and 15 µg of recombinant transfer vector DNA by using lipofectin. After incubating at 27°C for five days, the culture medium was collected and subjected to a plaque assay. Recombinant HyNPV (HyMCTI-II) was selected by picking up polyhedral negative plaque. Three rounds of viral amplification were performed to obtain a high-titer stock solution.

Expression of recombinant MCTI-II. Sixty fifth-instar larvae of silkworm at the early stage (strain: Shunrei×Shougetsu) were inoculated with 50 µl of the stock solution of HyMCTI-II by subcutaneous injection. The larvae were reared at 27°C for 4 days on mulberry leaves, before the legs of the larvae were cut off and the hemolymph (15 ml) was collected.

Purification of recombinant MCTI-II. Recombinant MCTI-II was purified from the hemolymph of larvae that had been inoculated with HyMCTI-II. The hemolymph was diluted 10-fold with distilled water, and heated at 95°C for 10 min to remove the heat-labile protein. The sample was centrifuged at 15,000 rpm for 20 min, and the resulting supernatant was collected as a crude sample.

Step 1: SP-Toyopearl 650M column chromatography. The crude sample was subjected to SP-Toyopearl 650M column chromatography (1.5 × 30 cm) performed with a medium-pressure liquid chromatography (BPLC-6000FC, Yamazen). The column was equilibrated with a 10 mM sodium acetate buffer (pH 3.5). The protein was eluted with a 150-min linear gradient from 0 to 0.5 M NaCl in the same buffer at a flow rate of 3 ml/min, with a fraction volume of 3.0 ml.

Step 2: HiLoad S Sepharose high-performance column chromatography. The active fraction from SP-Toyopearl chromatography was loaded into a HiLoad™ 26/10 S Sepharose high-performance column that had been equilibrated with a 10 mM sodium acetate buffer (pH 3.5) and was eluted with a 100-min linear gradient from 0 to 1 M NaCl in the same buffer at a flow rate of 6 ml/min with an FPLC instrument (Pharmacia). The fraction volume was 3.7 ml.

Step 3: First reversed-phase column chromatography. The fraction containing trypsin inhibitory activity was subjected to reversed-phase HPLC (L-7000, Hitachi) with an Inertsil ODS-3 column (GL Sciences, 4.6 × 250 mm). The column was equilibrated with 0.1% TFA and eluted with a 100-min linear gradient of acetonitrile from 0 to 80% in the equilibrating solvent at a flow rate of 1 ml/min.

Step 4: Second reversed-phase column chromatography. The active factions were further separated by reversed-phase HPLC (L-7000, Hitachi) with the Inertsil ODS-3 column (GL Sciences, 4.6 × 250 mm). The column was this time equilibrated with a 10 mM ammonium acetate buffer at pH 5.6. The protein was eluted with a linear gradient from 0 to 60% of acetonitrile in the equilibrating buffer for 100 min at a flow rate of 1 ml/min, protein being monitored at 280 nm.

Assay for trypsin inhibitory activity. Trypsin inhibitory activity was assayed by using BAPA or BAEE as a substrate by the method of Shibata et al. 13). The trypsin inhibitory activity of each chromatographic fraction was assayed with BAPA, while estimation of the inhibition units and titration of the inhibitory activity were done with BAEE. One unit of inhibition toward trypsin is defined as the amount of the inhibitor which inhibited 1 nmol of the enzyme.

The reaction was carried out in a 50 mM Tris-HCl buffer at pH 8.0 containing 0.02 M CaCl2 in the assay with BAEE. An appropriate amount of the inhibitor was incubated with bovine pancreatic trypsin (0.021 mg) in 2 ml of the reaction buffer for 4 min at 30°C. The reaction was started by adding 100 µl of 10 mM BAEE-HCl. Release of the ethyl group was monitored by measuring the change in absorption intensity at 253 nm with a spectrophotometer (U-3300, Hitachi).

In the assay using BAPA, the reaction was carried out in a 50 mM Tris-HCl buffer at pH 7.5 containing 0.02 M CaCl2. An appropriate amount of the inhibitor was incubated with bovine pancreatic trypsin (0.021 mg) in 1 ml of the reaction buffer for 5 min at 30°C. The reaction was carried out for 10 min after adding 1 ml of 1 mM BAPA, and then stopped by adding 200 µl of 30% acetic acid. The release of p-nitrophenol was measured by the increase of absorbance at 410 nm.

In order to determine the inhibition constant (K_i) value, the residual trypsin activity was assayed with BAEE in the presence of various concentration of the inhibitor as already described. The residual enzyme activity was then plotted against the inhibitor concentration, and the K_i value was calculated by the
method of Green and Work.\textsuperscript{14)}

\textbf{Mass spectrometric analysis.} The mass number of the inhibitor was analyzed in the positive mode with a laser ionization TOF mass spectrometer (MALDI II, Kratos). Sinapinic acid was used as the matrix reagent, and the accelerating voltage was 20 kV.

\textbf{Amino acid sequence analysis.} The amino acid sequence analysis was performed with an automatic gas phase sequencer (PPSQ-10, Shimadzu Co.).

\section*{Results and Discussion}

\textbf{Cloning of MCTI-II cDNA}

cDNA of MCTI-II was cloned by PCR amplification with the synthesized sense and antisense primers and genomic DNA of bitter gourd as the template. These primers were designed according to the amino acid sequence of MCTI-II. Electrophoresis of the amplified PCR product indicated that cDNA of about 110 bp had been obtained. After introducing the PCR product into the pCR 2.1 vector, the nucleotide sequence of the PCR product was analyzed with the results shown in Fig. 1. These results show the amino acid sequence that was deduced from the DNA sequence of the PCR product to be identical with the amino acid sequence of authentic MCTI-II. This indicates that cDNA of MCTI-II had been successfully cloned. Only one squash family inhibitor, TGTI-II from towel gourd seeds, has been analyzed for its cDNA sequence.\textsuperscript{15)} Since mixed primers were used for cloning MCTI-II cDNA, the whole nucleotide sequence cannot be compared with that of TGTI-II. The nucleotide sequence of the region encoding Trp-Gln\textsuperscript{19} shows 56.4\% identity with that of TGTI-II. The homology of the whole amino acid sequence between MCTI-II and TGTI-II was 60.7\%, and the genomic DNA of TGTI-II also showed no intervening sequence.

\textbf{Construction of the recombinant virus}
The recombinant virus, HyMCTI-II, was constructed by using the recombinant transfer vector. First, MCTI-II cDNA was introduced into the pAcGP67 vector at the dephosphorylated BamHI site which has a polyhedrin promoter, the signal sequence of acidic glycoprotein 67 of the baculovirus, and the non-coding region of polyhedrin. The obtained transfer vector was co-transfected with virus

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Ion-exchange Chromatography of the Crude Sample. The hemolymph of silkworm larvae inoculated by HyMCTI-II was subjected to SP-Toyopearl 650M column chromatography after removing the heat-labile protein. The elution conditions are described in the Materials and Methods section. --- absorbance at 280 nm; ----- trypsin inhibitory activity.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Ion-exchange Chromatography of the Active Fraction from SP-Toyopearl Chromatography. The fraction containing trypsin inhibitory activity from SP-Toyopearl 650M chromatography was loaded into a HiLoad S Sepharose column. The elution conditions are described in the Materials and Methods section. Two active fractions, A and B, were collected. --- absorbance at 280 nm; ----- trypsin inhibitory activity.}
\end{figure}

\begin{verbatim}
R I C P R I W M E C I 10
MCTI-II CCG ATA TGT CCG CCG ATA TGG ATG GAA TGC 30
*** ***
TGTI-II GGA ATT TGG CCA AGA ATC CTG ATG CCC TGC 60
G I C P R I L M P C
K R D S D C M A Q C 20
MCTI-II AAA CGC GAC TCC GAT TGC ATG GCT CAG TGT 60
** * *** ** *** *** ** * *
TGTI-II AAG ACC GAC GAT GAC TGC ATG CTT GAC TGT TGT
X T D D D D C M L D C
I C V - D G H C G 28
MCTI-II ATA TGG GTT ---- GAC GGT CAC TGG GGA 84
TGTI-II CCG TGC CTG TCC AAC GGC TAT TGT GGT
R C L S H G Y C G
\end{verbatim}

\section*{Fig. 1. Nucleotide and Deduced Amino Acid Sequences of MCTI-II DNA.}
The nucleotide and deduced amino acid sequences of MCTI-II DNA are compared with those of TGTI-II. Upper and lower lines respectively show the sequences of MCTI-II and TGTI-II. Identical nucleotides in the region encoding Trp-Gln\textsuperscript{19} are indicated by asterisks, and identical amino acids in whole sequence are in boldface.
DNA of HyNPV in Sf21 cells. The recombinant HyMCTI-II virus was recovered from the cell medium and purified by three-round plaque purification.

Sf21 cells cultivated in the TC-100 medium containing 10% (v/v) FCS and 0.26% tryptose broth were infected with the wild type or recombinant virus. At 72 h postinfection, polyhedra were observed in the cells that had been infected with the wild type of virus, HyNPV. However, no polyhedra were apparent in the cells that had been infected with HyMCTI-II. This suggests that the polyhedrin gene of virus DNA had been successfully replaced by DNA encoding MCTI-II in the recombinant HyMCTI-II virus.

Expression of MCTI-II in silkworms

Sixty silkworm larvae were inoculated with HyMCTI-II. At 4 days postinfection, the legs of the silkworms were cut off and the hemolymph was collected. Recombinant MCTI-II was purified from the hemolymph. The collected hemolymph (15 ml) was diluted to 150 ml with distilled water and heated at 95°C for 10 min to remove heat-labile protein. After centrifugation, the supernatant was obtained as a crude sample. In this supernatant, 334 units of trypsin inhibitory activity was recovered. The supernatant was subjected to SP-Toyopearl chromatography (Fig. 2). The fraction containing trypsin inhibitory activity was collected and subjected to HiLoad S chromatography. Two peaks with inhibitory activity, A and B, were obtained (Fig. 3). Peaks A and B were each subjected to the first reversed-phase chromatography. The chromatographic patterns of peaks A and B were shown in Fig. 4. The peaks (A and B) with inhibitory activity were then subjected to the second reversed-phase chromatography in the same column with different buffer conditions (data not shown). Thus, two recombinant types of MCTI-II, A and B, were purified. The yields of MCTI-II A and B were 16.3 nmol (4.9%) and 18.5 nmol (5.5%), respectively.

Characterization of recombinant MCTI-II A and B

The molecular weights of recombinant MCTI-II A and B were determined by mass spectrometry. MCTI-II A and B had molecular weights of 3,503 and 3,522, respectively, and no other mass peak was apparent in either mass analysis. These facts indicate

Fig. 4. Fractionation of A and B by Reversed-phase Column Chromatography.

The solution of A and B in Fig. 3 was loaded into an Inertsil ODS-3 column. The elution conditions are described in the Materials and Methods section. The peak containing trypsin inhibitory activity is marked with an arrow. A), A in Fig. 3; B) B in Fig. 3.

Fig. 5. Covalent Structures of Expressed MCTI-II A and B.

The arrow indicates the reactive site toward trypsin.
that both inhibitors were pure. The amino acid sequences of expressed MCTI-II A and B were determined as shown in Fig. 5. It was found that the tripeptide (Ala-Asp-Pro) derived from the vector had been added to the N-terminus of both inhibitors, indicating that the expressed inhibitor had been processed and cleaved in its signal sequence as expected in the silkworm larvae. MCTI-II A had the same amino acid sequence as that of authentic MCTI-II, except for the addition of the tripeptide. However, from the sequence analysis of MCTI-II B, two PTH-amino acids were identified at each cycle of the Edman degradation. These correspond to sequences Ala4-Arg8 and Ile6-Gly31 of authentic MCTI-II, indicating that the reactive site of MCTI-II B had been cleaved. This cleavage might have occurred to a limited degree by inherent trypsin-like protease in the silkworm body or during purification.

The $K_c$ values toward bovine pancreatic trypsin that had been calculated from the titration data were $6.4 \times 10^{-10}$ M and $5.2 \times 10^{-10}$ M for MCTI-II A and B, respectively. Compared with the $K_c$ value ($1.3 \times 10^{-10}$ M) of authentic MCTI-II, the expressed MCTI-II inhibited trypsin less efficiently than authentic MCTI-II. An X-ray crystallographic study of Cucurbita maxima trypsin inhibitor I (CMTI-I) on the complex formed with bovine $\beta$-trypsin has shown the amino terminal region from Arg1 to Glu6 (corresponding to Arg1 to Glu8 in MCTI-II) to be the primary binding segment, with weak additional binding resulting from a few residues in the carboxyl terminal region from His23 to Gly29 (corresponding to Gly25 to Gly28 in MCTI-II). This indicates that the structure of the amino terminal region might have affected the inhibitory activity and that the expressed MCTI-II might have been a weaker inhibitor due to the addition of the tripeptide at the N-terminus. The $K_c$ values for MCTI-II A and B were almost the same. However, MCTI-II B required a longer time (10 min) to form the complex with trypsin than MCTI-II A did. This might have been caused by the greater mobility of the Arg residue at the reactive site due to cleavage of the reactive site peptide bond.

In this study, we cloned the MCTI-II gene, expressed it successfully in the silkworm body and purified recombinant MCTI-II. We have demonstrated that recombinant MCTI-II was obtained in two forms due to cleavage of the reactive site bond, perhaps as a result of inherent trypsin-like protease in the silkworm body. The cleavage site of the signal sequence and the amino acid sequence of recombinant MCTI-II were as expected. It is suggested that recombinant MCTI-II was successfully expressed and secreted into hemolymph by the signal sequence.

References