Characterization of recombinant mustard trypsin inhibitor 2 (MTI2) expressed in *Pichia pastoris*

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Abstract The mustard trypsin inhibitor MTI2 was expressed as secretory protein in the yeast *Pichia pastoris*. In order to evaluate the influence of the C-terminal amino acids of the precursor form on the inhibitor activity, the C-terminal precursor and the mature protein were both expressed. A third His-tagged construct was also designed to compare alternative purification procedures. Proteins were efficiently expressed at levels of 40–160 mg/l in shake flasks. Equilibrium dissociation constants demonstrated that the mature protein was a stronger inhibitor of bovine β-trypsin compared to the precursor and His-tagged forms (0.01 nM vs. 0.58 nM and 0.71 nM, respectively). The recombinant proteins were active inhibitors of *Spodoptera exigua* gut proteases.

Key words: Serine protease inhibitor; Recombinant expression; Radial diffusion assay; *Pichia pastoris*

1. Introduction

Protease inhibitors of plants have been classified according to the proteolytic enzymes upon which they act (serine, cysteine, aspartic and metallo-proteases). Inhibitors of serine proteases are the most extensively characterized and they are currently subdivided into at least eight families of structurally different molecules [1].

In seeds of the Crucifera mustard (*Sinapis alba*), two different trypsin inhibitors (MTI and MTI2) have been identified [2,3]. MTI shows some characteristics in common with the soybean trypsin inhibitor of the Kunitz type and has a reported inhibition constant of 2.2 nM for trypsin. MTI2 was the first protease inhibitor which was sequenced from Cruciferae [3]. It is a polypeptide of 63 amino acids, rich in cysteine and glycine residues, showing no structural homology with other known families of plant serine protease inhibitors. Similar protease inhibitors have been purified from rapeseed (*Brassica napus*) seeds [4,5], supporting the hypothesis that they represent a new family of serine protease inhibitors, which could be characteristic of Cruciferae. MTI2 is a potent, heat-stable inhibitor of trypsin with a reported K_i of 0.16 nM [3].

The gene encoding the MTI2 protein is discontinuous with a single intron located upstream of the region encoding the mature protein [6]. The *MTI2* gene encodes a 99 amino acid precursor having 30 and six amino acids at the amino- and carboxy-termini respectively, which are not present in the mature protein. The gene is expressed in seeds toward the end of maturation and in leaves only after wounding.

The 30 amino acids at the N-terminus of the precursor polypeptide contain a cluster of hydrophobic residues flanked by charged amino acids which most likely represents the signal peptide for secretion into the endoplasmic reticulum [7]. The role of the C-terminal portion remains unknown. In this paper we investigate the possibility that the C-terminal peptide keeps the inhibitor inactive.

The possibility that mature MTI2 could constitute a defensive molecule in leaves is suggested by the wound inducibility of the gene, and this has been confirmed by expressing the *MTI2* gene in tobacco and *Arabidopsis thaliana* plants under the constitutive CaMV35S promoter. When the *MTI2* gene was expressed at high levels in transgenic tobacco plants a decrease in leaf surface eaten by *Spodoptera littoralis* larvae together with a reduction in mean larval weight and increased mortality was observed [8].

A broader analysis of MTI2 antimetabolic activity on different insects, of the role of the C-terminal peptide, and of the protein structure has been impeded so far by the small amounts of MTI2 that can be purified from mustard seeds. In order to overcome this problem, we have expressed the MTI2 mature protein and its C-terminal precursor in the methylotrophic yeast *Pichia pastoris*.

2. Materials and methods

2.1. Oligonucleotides

The following primers (Life Technologies) were used for PCR amplifications. Triplets correspond to amino acid codons; restriction sites are underlined (see Fig. 1 for primer locations).

1. 5′-ATG GCC ATG GCA AAA AAA TCA GTT TCT TCG TTC ACC C-3′.
2. 5′-GGGGG GAA TTC CAT CAC CAT CAC CAT CAC GAT AGC GAG TGC CTG AAA GAA TAC GG-3′, restriction site *Eco*RI.
3. 5′-AAAAAAAAA CTC GAG AAA AGA GAG GCT GAA GCT GAT AGC GAG TGC CTG AAA GAA TAC-3′, restriction site *Xho*I. This primer fuses the MTI2 mature protein immediately downstream of the α-factor secretion signal sequence and downstream of the Glu–Ala repeat which is removed by the yeast dipeptidyl aminopeptidase STE 13 (see Pichia Expression Kit manual).

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4. 5′-GGGGGGAAT TCA CTG ATC AAA AGG GCT GTC GTT GCA GTA G-3′, restriction site *Eco*RI.
5. 5′-AAAAAAAAAGCGGCCGC CTA AAT GCC ACC TCT TAG AAT CTG-3′, restriction site *Not*I.
6. 5′-GGCACCTGCAGGAAC TCA AAT GCC-3′, restriction site *Pst*I. The first 15 nucleotides of the primer derive from the 3′ flanking region of the *MTI2* gene [3]. A single nucleotide mutation allows the generation of a *Pst*I restriction site.

2.2. Synthesis of the MTI2 cDNA and subcloning into pPIC9

Poly(A)$^+$ mRNA from immature mustard seeds was prepared as described [6]. RT-PCR was performed using oligonucleotides 1 and 6 as described in [6] with the only difference that amplification was run for 30 cycles of 60 s at 94°C, 60 s at 70°C and 60 s at 72°C. The resulting cDNA was cloned into pUC19. Using this template PCR amplifications were run with three different primer combinations: 2 and 4, 3 and 5, 3 and 4. PCR amplifications were run for 30 cycles of 60 s at 94°C, 60 s at 65°C (for the primer combinations 2/4 and 3/4) or 60°C (for the primer combination 3/5) and 60 s at 72°C. All the reactions were performed using standard conditions: in a final volume of 100 μl, 50 pmol of each primer was added in the presence of 1.5 mM $MgCl_2$, 0.5 mM of each dNTP and 2.5 U Taq polymerase (Promega).

Amplified fragments were digested with the appropriate restriction enzymes and cloned into the pPIC9 vector (Invitrogen).

2.3. Transformation of P. pastoris

About 15 μg of recombinant pPIC9 constructs, previously linearized with *Sal*I, was electroporated into *P. pastoris* strain GS115 (*his4*) cells (Invitrogen) [9] using a Bio-Rad Gene Pulser and plated on MD agar plates without histidine (1.34% YNB (yeast nitrogen base with ammonium sulfate and without amino acids, Difco), 400 μg/l biotin, 1% D-glucose, 1.5% agar). Single colonies were inoculated in 500 μl of BMG medium (100 mM potassium phosphate pH 6.0, 1.34% YNB, 450 μg/l biotin, 1% glycerol). Cultures were grown overnight at 30°C on a plate agitator at 250 rpm. Cells were pelleted and resuspended to $OD_{600} = 1.0$ in 500 μl BMM medium (100 mM potassium phosphate, pH 6.0, 1.34% YNB, 450 μg/l biotin, 0.5% methanol). Cultures were incubated in the same conditions as above and supplemented daily with 50 μl of BMM medium containing 5% methanol for 96 h.

2.4. Selection of high expressors

Trypsin inhibitor activity in miniculture supernatants and in fractions eluted from chromatography columns was evaluated by radial diffusion assay as described in [10]. This method is quantitative over a large concentration range and allows screening of many samples with little effort.

For a quantitative evaluation of inhibitor activity first a reference curve was determined by relating the concentration of active recombinant proteins, evaluated by the benzoyl DL-arginine *p*-nitroanilide (BA*p*NA) assay, with the diameters of the colorless halo [10].

2.5. Fermentation of His-MTI2, Cpre-MTI2 and MTI2 P. pastoris strains

Positive recombinant clones were inoculated in 100 ml of BMGY medium (1% yeast extract, 2% peptone in BMG medium) in a 250 ml flask for 24 h at 30°C in a shaking incubator at 250 rpm. An aliquot of the culture was diluted to $OD_{600} = 1.0$ in a final volume of 500 ml of BMMY (1% yeast extract, 2% peptone in BMM medium) medium. The diluted culture was incubated in a 2 l flask under the same conditions as above for 96 h with a daily supplement of 50 ml of BMMY buffer containing 5% methanol. After induction the culture was stored at 4°C.

2.6. Purification of secreted His-MTI2 protein

Cells from a His-MTI2 culture induced with methanol for 96 h were pelleted by centrifugation at 1500×*g* for 10 min at 4°C. The supernatant was filtered using a 0.22 μm filter (Gelman Sciences) and adjusted to 50 mM Na_2HPO_4, 300 mM NaCl, pH 8.0 (equilibration buffer). Filtered supernatant was loaded onto a column containing 15 ml of Ni-NTA resin (Qiagen), previously equilibrated with equilibration buffer. The column was washed first with 60 ml of equilibration buffer and then with 50 ml of wash buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10% glycerol, pH 6.0). Elution was carried out first with a linear 0–75 mM imidazole gradient and then with a multi-step gradient of 75, 90, 500 mM imidazole in wash buffer at a flow rate of 1 ml/min. The eluant was monitored at 280 nm and all fractions were assayed by radial diffusion assay as described [10].

2.7. Purification of secreted MTI2 and Cpre-MTI2 proteins

Supernatant from cell culture, recovered as described above, was diluted 1:1 with 20 mM sodium citrate, pH 3.0 (equilibration buffer) and loaded onto a HiTrap SP column (Pharmacia), previously equilibrated with the same buffer, at a flow rate of 1 ml/min. The column was then washed with five volumes of equilibration buffer. The adsorbed MTI2 protein was eluted either with a 0–1 M NaCl linear gradient in equilibration buffer or with a 150, 350, 1000 mM NaCl step gradient. Eluant was monitored at 280 nm. All the fractions were analyzed as previously described.

2.8. BApNA and Z-Arg-Arg-pNA assay

A colorimetric assay with the trypsin substrates BA*p*NA (Sigma) or Z-Arg-Arg-*p*NA (Bachem) was used to titrate the trypsin inhibitor activity of purified recombinant proteins. 150 μl of incubation buffer (100 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$) containing bovine β-trypsin at known concentration (in our studies either 0.5 nM (with Z-Arg-Arg-*p*NA as substrate) or 35 nM (with BA*p*NA as substrate)) and a serial dilution of purified proteins was incubated at room temperature for 30 min. The active concentration of bovine β-trypsin was previously titrated by the method of Chase and Shaw [11] using the titrant *p*-nitrophenyl guanidinobenzoate. The residual trypsin activity was measured by following the hydrolysis of the two substrates at 37°C. 50 μl of substrate buffer (100 mM Tris–HCl pH 8.0, 40% DMSO, 4 mM BA*p*NA or 100 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$, 0.1 mg/ml BSA, 1.5 mM Z-Arg-Arg-*p*NA) was added and the adsorbance of the hydrolysis product *p*-nitroaniline [12] was measured at 405 nm.

2.9. Activity towards Spodoptera exigua gut proteases

Protease inhibitor activity of the purified recombinant MTI2 proteins toward *S. exigua* gut proteases was determined with BA*p*NA according to Jongsma et al. [13]. *S. exigua* larvae were fed for 6 days on fresh tobacco leaves replaced every 12 h. Extraction of guts was as described [13]. The potato proteinase inhibitor (PI2) protein [14] used in these assays was produced as a recombinant protein in *P. pastoris* [15].

2.10. Tricine gel analysis

Tricine SDS–PAGE of proteins was according to Schagger and von Jagow [16]. Activity PAGE was performed by a combination of the activity PAGE procedure described in [8] for standard glycine gel and the Tricine SDS–PAGE of proteins [16]. For activity PAGE, gelatin to a final concentration of 0.1% was added to 'spacer' and 'separating' gels described in [16] and SDS omitted. Running and gel treatment conditions were according to [8].

3. Results

3.1. Cloning of MTI2 sequences into the P. pastoris pPIC9 vector

The cDNA of the *MTI2* gene for the whole mature protein (EMBL accession number Y16190) (Fig. 1) was digested with *Pst*I and inserted into pUC19 *Sma*I-*Pst*I sites. The resulting plasmid was designated MTI2cDNA.

Different MTI2 constructs to be expressed in *Pichia* cells were obtained by PCR amplifications of the MTI2cDNA plasmid using the primer combinations 2/4, 3/5 and 3/4.

By cloning the 2/4 amplification fragment into the *Eco*RI site of the *P. pastoris* pPIC9 secretory vector a 6×His-tagged mature MTI2 protein (His-MTI2) was produced. An additional four amino acids (Tyr-Val-Glu-Phe) are expected to be present on the N-terminus of the protein secreted into the medium (see Pichia Expression kit manual).

The expression of the 3/5 amplification fragment allowed the production of the MTI2 protein bearing the last six amino acids of the precursor polypeptide (Cpre-MTI2).

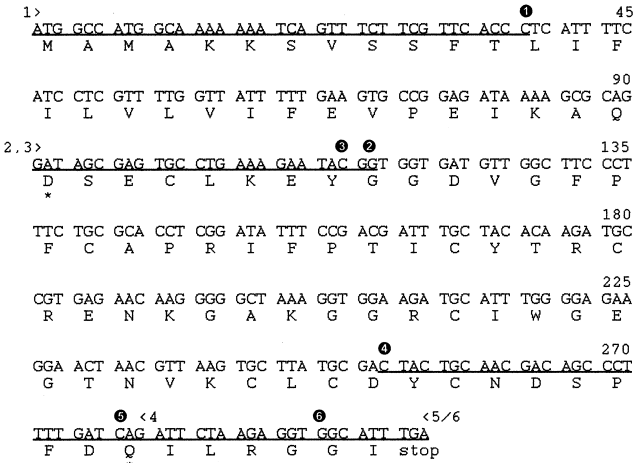

Fig. 1. Sequence of *MTI2* gene cDNA fragment isolated by RT-PCR. Amino acids deduced from the cDNA sequence are given in the single letter code. Sequences corresponding to portions of oligonucleotides used as PCR primers are underlined. Arrowheads indicate 5′ side of the corresponding oligonucleotide and its orientation. Numbers on black background indicate oligonucleotide termini. Complete oligonucleotide sequences are in Section 2. An asterisk indicates the first and last amino acids of the mature protein.

Expression of the 3/4 amplification fragment resulted in the production of the mature MTI2 (MTI2).

3.2. Screening of P. pastoris transformants

His^+ clones showing the highest expression levels were selected by radial diffusion assay [10]. For every transformation, minicultures of 70 His^+ clones were grown and methanol induced in 24 well tissue culture plates. After staining, more than 70% of the colonies for the three different constructs showed inhibitor activity, with levels of expression varying up to four-fold as measured by colorless halo diameters (not shown).

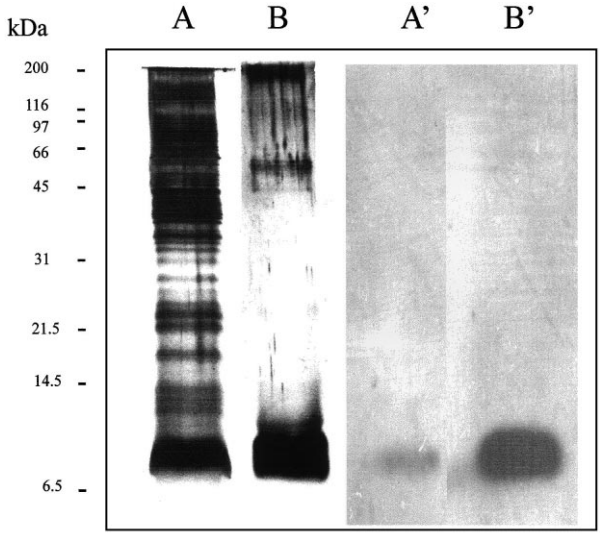

Fig. 2. PAGE analysis of the recombinant MTI2 protein. 0.8 μg from the supernatant of the *Pichia* culture (A) and 0.6 μg from the chromatographic fractions showing the highest trypsin inhibitor activity (B) were electrophoresed through a Tricine SDS–16.5% polyacrylamide gel. Trypsin inhibitors were detected by activity gel: 0.05 μg from the same sources as above was electrophoresed through a Tricine gelatin–16.5% polyacrylamide gel. After electrophoresis, the gel was incubated in a trypsin-containing solution to degrade gelatin. Bands of undegraded blue-stained gelatin indicate the presence of trypsin inhibitors (A′ and B′). The patterns shown for proteins (A and B) and activities (A′ and B′) correspond to the 'separating' phase of the gels. In all the 'spacer' phases neither proteins nor activity could be detected.

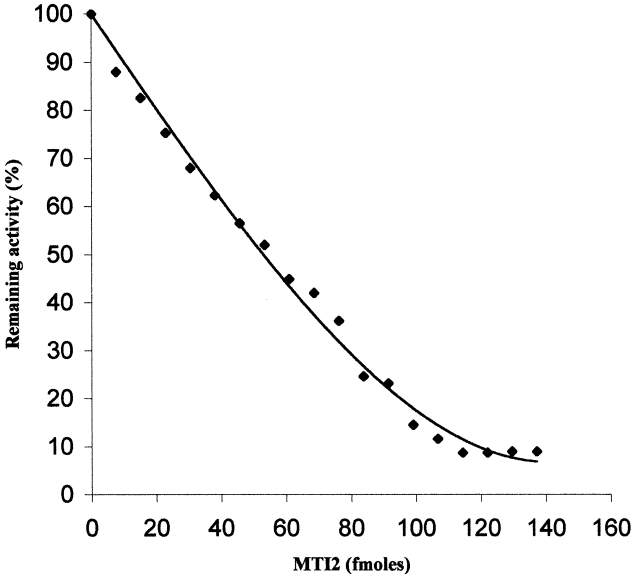

Fig. 3. Titration of the trypsin inhibitor activity of recombinant MTI2. 0.5 nM trypsin was incubated with increasing concentrations of purified recombinant protein. The equilibrium dissociation constant for the inhibitor was determined from this titration curve. The residual trypsin activity was measured by Z-Phe-Arg-*p*NA hydrolysis.

Clones showing trypsin inhibitor activity were also tested by SDS–PAGE. In every instance a thick band of about 7 kDa, which was absent from clones with no activity, was detectable by silver staining (not shown).

3.3. Purification and characterization of His-MTI2

A 500 ml culture of a specific clone selected for the highest expression of the His-MTI2 recombinant protein was grown for 96 h. The concentration of active recombinant protein in the supernatant, determined by the BA*p*NA assay, reached 40 mg/l. Purification of His-MTI2 was performed by affinity chromatography on a Ni-NTA column. The inhibitor activity, revealed by radial diffusion assay, was detectable in fractions eluted by 90 mM imidazole (results not shown). The yield of purified recombinant active protein was estimated to be 11 mg/l.

The apparent dissociation equilibrium constant (K_i) for the recombinant protein and bovine β-trypsin as determined from titration curves with a known amount of protease using the equilibrium method [17] was found to be 0.71 nM (Table 1).

Table 1
K_i for MTI2 proteins when tested with bovine β-trypsin

Inhibitor	K_i (nM)
MTI2 (native)	0.16 [3]
MTI2	0.010 ± 0.002
Cpre-MTI2	0.58 ± 0.06
His-MTI2	0.71 ± 0.09

Values are from at least three separate determinations.

Table 2
Residual trypsin-like activity of *S. exigua* gut proteases after incubation with 100 pmol of MTI2, Cpre-MTI2, PI2 and their combinations (50 pmol each)

Inhibitor	Residual activity (%)
–	100
MTI2	23 ± 6
Cpre-MTI2	24 ± 4
PI2	33 ± 5
MTI2+PI2	23 ± 4
Cpre-MTI2+PI2	23 ± 5

Values are from five independent experiments.

3.4. Purification and characterization of Cpre-MTI2 and MTI2

P. pastoris cultures for two highly expressing Cpre-MTI2 and MTI2 clones were grown and methanol induced as described for the His-MTI2 culture. The concentration of active recombinant MTI2 proteins in the supernatants reached 160 mg/l for Cpre-MTI2 and 70 mg/l for MTI2.

The recombinant proteins were purified from fermentation media by ion exchange chromatography. Radial diffusion assay allowed the identification of positive fractions in the range 300–450 mM NaCl for the linear gradient elution and at 350 mM NaCl for the step gradient elution.

In Fig. 2 gel electrophoresis of the recombinant MTI2 protein before and after chromatography is shown. Identification of proteins with trypsin inhibitor activity on activity gels is also shown (Fig. 2A′,B′).

The amount of purified active recombinant protein recovered from ion exchange chromatography was estimated to be 120 mg for Cpre-MTI2, and 40 mg for MTI2 for 1 l of supernatant from *P. pastoris* culture.

The apparent equilibrium dissociation constants of the two recombinant proteins for bovine β-trypsin were found to differ strongly. The mature protein had a K_i of 0.01 nM, while the presence of six amino acids on the C-terminus reduced the K_i 58-fold (Table 1). Fig. 3 shows the titration curve obtained in the case of the recombinant MTI2 protein.

The activity of recombinant Cpre-MTI2 and MTI2 was also tested against proteases from *S. exigua* guts and compared to the potato trypsin/chymotrypsin inhibitor II (PI2) [14]. *S. exigua* is a widespread and polyphagous lepidopteran pest affecting numerous crops [18]. Table 2 reports the residual protease activity present in *S. exigua* gut extracts after incubation with an excess quantity (100 pmol) of the recombinant proteins Cpre-MTI2, MTI2 and PI2. The combination of MTI2 (or Cpre-MTI2) with PI2 is only as effective as a single inhibitor, indicating that the activity spectrum of the two inhibitor types towards *S. exigua* trypsin-like proteases is similar. Recombinant MTI2 inhibitors appear to be marginally more effective, though, as inhibitors of trypsin-like proteases in this insect.

4. Discussion

In this study we describe the expression in the yeast *P. pastoris* of the mustard trypsin inhibitor MTI2, its C-terminal precursor and a His-tagged form, together with their characterization.

A previous attempt to express MTI2 in a prokaryotic system was partially unsuccessful [19]. The MTI2 was expressed as a fused protein with glutathione *S*-transferase (GST) but at very low yield (0.1 mg/l), most likely as a consequence of the high number of disulfide bonds necessary for the proper folding of the protein.

To improve the expression level of recombinant MTI2 and to facilitate its purification, *P. pastoris* cells were transformed with the secretory expression vector pPIC9 containing the cDNA for the MTI2 protein.

The His-MTI2 construct produced the recombinant protein at about 40 mg/l of cell culture. Purification of this fraction by affinity chromatography on Ni-NTA resin gave a low yield that was never higher than 25% of the active protein present in the culture supernatant. The remaining protein probably remained tightly bound to the resin, since it was not found either in the flow-through or in fractions eluted with buffer containing higher concentrations of NaCl.

With the two constructs Cpre-MTI2 and MTI2, higher levels of protein were obtained in the cultures: 160 mg/l and 70 mg/l, respectively. The differences in the production of the three proteins are probably accidental rather than a result of the specificity or protein sequence of the proteins.

MTI2 proteins produced by Cpre-MTI2 and MTI2 strains were further purified by ion exchange chromatography with satisfactory yields ranging from 60 to 75%.

All three recombinant MTI2 proteins were active toward bovine β-trypsin suggesting that each was properly folded after expression and secretion from *Pichia* cells. The dissociation equilibrium constant of the His-MTI2 molecule was higher than that reported for the mature protein, probably due to the extra N-terminal amino acids present in the protein. The fact that the C-terminal precursor is about 60 times less active than the mature protein could indicate a physiological role for the C-terminal peptide in regulating the inhibitor activity towards endogenous proteases of mustard during sequestration. Under our experimental conditions the recombinant mature MTI2 is 16 times more active than the native protein purified from seeds [3] (Table 1). The difference between the constants measured for native and recombinant mature MTI2 may be the result of differences in the protein sources.

Previous work established the potential defensive role of MTI2 in plant defense against insects [8]. The recombinant proteins were, therefore, also assayed toward trypsin-like activities present in *S. exigua* gut extracts. Under the conditions of the assay reported in Table 2, recombinant MTI2 proteins showed a strong inhibitory activity similar to that of the potato protease inhibitor PI2 [13]. The spectrum of activity of the two inhibitors appears to be similar, because their combination did not increase the level of inhibition. The presence of the C-terminal peptide did not appear to influence the level of inhibition of the trypsin-like gut proteases at the concentrations tested (Table 2). His-MTI2 was not used in the inhibition assays of *S. exigua* gut enzymes owing to its low activity compared to the other MTI2 inhibitors.

The purity level of the MTI2 recombinant protein, as detectable by SDS and activity gels, allows the use of the present material for the formulation of artificial diets in order to assay MTI2 insecticide activity towards a wide range of different phytophagous insects.

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