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NOTE

Expression and purification of the recombinant mustard trypsin inhibitor 2 (MTI2) in *Escherichia coli*

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The mustard trypsin inhibitor 2, MTI2, was expressed in *Escherichia coli*. A specific procedure for its production and purification is described. The recombinant protein was recovered by protein extraction from the insoluble fraction, then renatured and purified by ion exchange and gel filtration chromatography. Finally, the inhibitory activity against trypsin was also determined.

[**Key words:** Heterologous expression; Serine proteinase inhibitor; *Sinapis alba L.*; Trypsin inhibition; *Escherichia coli*]

TEXT

Proteinase inhibitors (PIs) are widely expressed in plants, especially in the seeds of *Cruciferae*, *Leguminosae*, *Solanaceae* and *Gramineae* (1). Their expression is regulated at the transcriptional level in response to different conditions, e.g., germination (2) or insect attack (1, 3). Specifically, these inhibitors act against proteolytic enzymes inside the guts of insects and parasites. For this reason, their potential role in agriculture as natural insecticides has been studied for years. Moreover, these proteins show anti-inflammatory and anti-carcinogenic activity (4), suggesting their potential clinical use. The most studied and characterized PIs are the serine proteinase inhibitors, which were initially isolated from leguminous seeds. These inhibitors can be classified into various families, including the Bowman–Birk, the Kunitz, and the potato-type inhibitor I family (1). During previous work on the isolation of the high molecular weight protease inhibitor, MTI1, from mustard seeds (*Sinapis alba*), a new member of the inhibitor family has been identified and described as the mustard trypsin inhibitor II (MTI2) (5). The sequence of MTI2 has been determined (6): the protein consists of 63 residues with eight cysteines, presumably organized in four disulfide bridges. MTI2 is effective against *Lepidoptera*, as has been demonstrated by feeding insect larvae with the transgenic plants expressing this inhibitor (7, 8). The design of natural insecticides based on MTI2 has been hampered by the limited amount of inhibitor in mustard seeds, and by the long and complex purification procedure. For these reasons, previous studies attempted to express MTI2 using the eukaryotic systems (9). Specifically, the over-expression of MTI2 precursor in *Pichia pastoris* yielded 160 mg/L of inhibitor, 75% of which was purified (9). In contrast, using the same type of yeast, His-tagged and mature MTI2 were produced at 40 and 70 mg/L, respectively, and purification of these inhibitor forms corresponded to recoveries equal to 25 and 60% (9). To obtain secretion of the mature MTI2 in *P. pastoris*, the α factor secretion signal was fused to the N-terminus of the inhibitor (9). Remarkably, it is known that processing of the secretion signals is frequently aberrant in both yeasts (including *P. pastoris*) and filamentous fungi, leading to the production of the target protein composed of a mixture of differently-processed forms (10–12), where this is likely the case for mature MTI2 over-expressed in *P. pastoris* (9).

In this study, we describe the expression of the mustard trypsin inhibitor precursor (containing six additional amino acids at the C-terminus), devoid of any tag or secretion signal, in a prokaryotic system. In particular, MTI2 was over-expressed in two different strains of *E. coli*; the recombinant protein was found in the insoluble fraction of protein extracts and its solubilization was studied in detail to identify the most efficient procedure for its recovery. Moreover, the refolding and purification conditions were experimentally optimized to obtain a homogeneous recombinant trypsin inhibitor.

E. coli BL21(DE3) [F^- *ompT*, *gal*, *dcm*, *lon*, *hsdS*$_B$(r_B^- m_B^-), λDE3] and BL21AI [F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) *araB*::*T7RNAP-tetA*] were used as hosts for the expression of MTI2. The synthetic gene coding for the MTI2 precursor, optimized for the *E. coli* codon usage (Entelechon GmbH, Germany, sequence: ATGGACTCCGAATGCCT-GAAAGAGTACGGTGGTGATGTTGGCTTCCCGTTTTGTGCTCCACG-

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TATCTTCCCGACTATCTGCTATACCCGTTGCCGTGAAAACAAAGGTG-CAAAAGGCGGTCGTTGTATCTGGGGTGAAGGTACCAACGTGAAA-TGCCTGTGTGACTACTGCAACGACTCTCCGTTCGATCAGATTCT-GCGTGGTGGTATCTAA) was cloned without any tag into the pET9a vector (Novagen, Germany) using the NdeI and BamHI enzymes (New England Biolabs, USA). The pET9a vector is based on the T7 RNA polymerase system that allows the production of high levels of recombinant proteins compared to other traditional systems. BL21 (DE3) and BL21AI *E. coli* strains were tested as the recipients for the expression. These differ in the type of chromosomal promoter controlling the T7 RNA polymerase messenger synthesis: the *lac*UV5 (IPTG inducible) or the *ara*BAD promoter (arabinose inducible), respectively. Following the electroporation of the pET9a-MTI2 construct, single colonies of BL21 (DE3) and BL21AI were grown overnight at 37 °C in 5 mL of the appropriate medium (LB or MagicMedia, Invitrogen). Cultures were diluted (1:500) and inducers (arabinose 13 mM or IPTG 1 mM) were added when populations reached an OD_{600} of approximately 0.7–0.8. Aliquots were harvested by centrifugation (4000 *g* for 20 min) at different times of induction. Total protein extracts were loaded onto the 15% SDS-PAGE gel, and analyzed relative to the 7-kDa target protein. We tried different growth conditions (length of induction time and choice of medium) to identify an optimized protocol for higher expression yields. Specifically, to compare the expression levels, cultures were induced for 1 h, 4 h or overnight using LB or MagicMedia medium. The recombinant protein was always expressed and clearly visible on the SDS-PAGE gel (Fig. 1A), independently of the length of induction. In addition, the expression level was comparable in both *E. coli* strains tested (data not shown). Most of the recombinant MTI2 was recovered from the insoluble fraction (Fig. 1A). To solubilize the protein from the cell pellet and to optimize its recovery, different conditions were assayed. Specifically, 6 M urea was used as a denaturant in combination with the reducing agent (20 mM β-mercaptoethanol), at different temperatures (room temperature, 45, or 65 °C) and pH (8, 10, or 12) (Fig. 1B). The higher recovery of MTI2 from the insoluble fraction was obtained with a solubilization solution containing 50 mM Tris–HCl, 100 mM NaCl, 6 M urea, 20 mM β-mercaptoethanol, pH 8, under shaking conditions, at 45 °C for 3 h (Fig. 1B). The following extraction procedure was used to estimate the MTI2 expression level: protein concentration of extracts was determined, and the relative amount of inhibitor was assessed using SDS-PAGE by comparing the intensity of a band produced by a known amount of pure MTI2 with those detected with solubilized extracts. A detailed analysis (four independent experiments) revealed that the growth medium significantly affected MTI2 yields, which were equal to 55 ± 16 and 258 ± 56 mg/L using LB or MagicMedia, respectively.

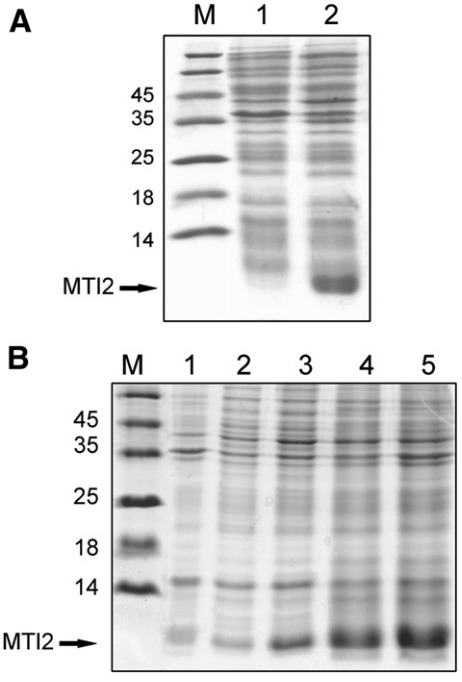

FIG. 1. SDS-PAGE of the recombinant MTI2. (A) Total protein extracts from a bacterial culture induced overnight. Lane M, standard proteins marker (kDa); lane 1, soluble protein fraction obtained after centrifugation; lane 2, insoluble protein fraction obtained from the pellet. (B) Solubilization of MTI2 from the insoluble pellet. Lane M, standard proteins marker; lane 1, 50 mM Tris–HCl pH 8, 100 mM NaCl, 6 M urea, glycerol 50% (v/v); lane 2, 50 mM Tris–HCl pH 10, 100 mM NaCl, 6 M urea, 20 mM β-mercaptoethanol; lane 3, 50 mM Tris–HCl pH 12, 100 mM NaCl, 6 M urea, 20 mM β-mercaptoethanol; lane 4, 50 mM Tris–HCl pH 8, 100 mM NaCl, 6 M urea, 20 mM β-mercaptoethanol for 1 h at 45 °C; lane 5, 50 mM Tris–HCl pH 8, 100 mM NaCl, 6 M urea, 20 mM β-mercaptoethanol for 3 h at 45 °C. Arrows indicate the band corresponding to the recombinant MTI2 (approximately 7 kDa).

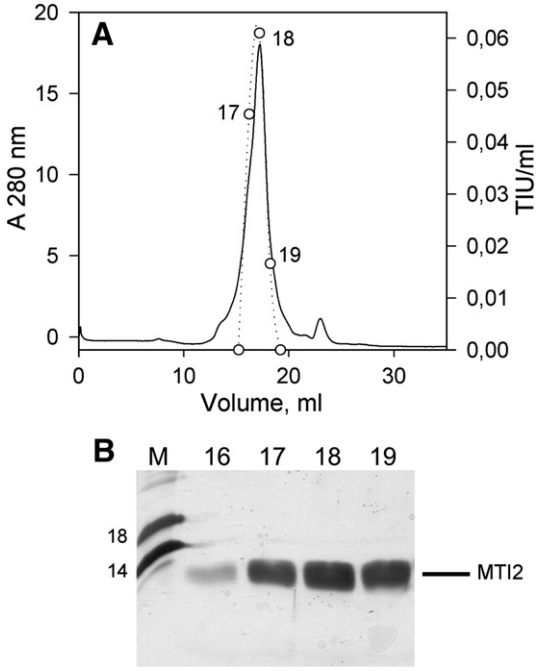

FIG. 2. (A) Profile of a Superose 12 gel filtration of the active fractions from the MONO Q ion-exchange chromatography. The column was equilibrated and eluted with 20 mM Tris–HCl, 0.2 M NaCl, pH 8. The flow rate was 0.4 mL/min and 1 mL fractions were collected. Protein concentration was monitored at 280 nm (solid line) and trypsin inhibition activity was determined (dotted line). Open circles indicate inhibitor units, TIU/mL, determined for each fraction. (B) SDS-PAGE of Superose 12 active fractions 16–19. Lane M, molecular weight markers (18 and 14 kDa); fractions 17 and 18 were pooled, concentrated and desalted.

For the large-scale production, a 500 mL culture was grown under the conditions described above. Cells were harvested by centrifugation and pellets were resuspended in 1/10 of volume of the lysis buffer (50 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM EDTA). Cells were then sonicated on ice (8×15 s pulses with 15 s intervals, at 15 W with a Sonicator 3000, Misonix) and centrifuged for 30 min at 4500 *g*. The soluble fraction was removed and two pellet washes were performed with the lysis buffer containing 1% (w/v) Triton X-100. Pellets were finally resuspended in 50 mL of the solubilization buffer described above, and subsequently centrifuged. Using this procedure, the target protein was obtained in a denatured, inactive form. To refold MTI2, the solubilized sample was diluted in 50 mM Tris–HCl, pH 8, to decrease the urea concentration from 6 to 3 M. Following this first step, the refolding process was performed by dialysis with cellulose mem-