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Short communication

A Refolding Strategy for Recombinant Metalloprotease

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The partial cDNA of the *MT-c* clone encoding snake venom metalloprotease was subcloned and expressed in *E. coli*. The expressed metalloprotease was purified by affinity chromatography in the presence of urea, and then successfully refolded into its functional form, retaining metalloprotease activity that hydrolyzes fibrinogen. The simple and convenient refolding strategy established in this work was highly efficient in recovering the recombinant enzyme activity. Experimental evidence suggests that the C-terminal amino acid stretch of 16 residues is a critical sequence for proper folding of the metalloprotease domain.

Keywords: Recombinant metalloprotease, Refolding.

Introduction

Snake venom is a rich source of serine proteases and metalloproteases which induce a variety of toxic effects such as blood coagulation, hemorrhage, and platelet aggregation. Hemorrhage is mostly due to snake venom metalloproteases, Zn^{2+}-dependent enzymes that digest components of the extracellular matrix (ECM) proteins resulting in bleeding (Selitre *et al.*, 1997). However, the exact mechanism of the venom-induced hemorrhage is not fully understood. Hemorrhagic metalloproteases (hemorrhagins) can also digest some blood coagulation factors, including fibrinogen and von Willebrand factor, which increase the hemorrhagic effect (Selitre and Ownby, 1995). Hemorrhagins can be classified into two groups based on their molecular sizes, i.e., low-molecular-mass and high-molecular-mass hemorrhagins. Catrocollastatin from *Crotalus atrox* venom (Zhou *et al.*, 1995) and jararhagin from *Bothrops jararaca* venom (Paine *et al.*, 1992) are high-molecular-mass hemorrhagins. HT-2 from *Crotalus ruber ruber* venom (Takeya *et al.*, 1990) and Ht-b, Ht-c, Ht-d, and Ht-e from *Crotalus atrox* (Hite *et al.*, 1994) are members of the low-molecular-mass hemorrhagins. The low-molecular-mass hemorrhagins are precursors of disintegrins, and it was reported that the disintegrin domain is generated by autoprocessing of the precursor protein in the absence of Ca^{2+} (Takeya *et al.*, 1993). Disintegrins belong to another class of venom components that affect blood homeostasis by inhibiting platelet aggregation (Huang *et al.*, 1991). Although expression of recombinant protein in *E. coli* is convenient due to its low cost and easy handling, the recombinant products in the cells frequently form insoluble inclusion bodies that resist refolding into their native forms. With this difficulty, only limited success has been reported in the expression and refolding of a functional matrix metalloprotease (Itoh *et al.*, 1996). In this work, we have established a strategy to refold a recombinant metalloprotease expressed in *E. coli*.

Methods and Materials

Expression of MT-c protein in *E. coli* In order to obtain the translated mature form of *MT-c* in *E. coli*, expression vector pET-22b-MT-c which carries the cDNA sequence encoding the metalloprotease domain was prepared. The *MT-c* cDNA was amplified by PCR and cloned into the pET-22b Vector (Novagen, Madison, USA). An initiation codon was inserted with the *Nde*I site into the N-terminus of the metalloprotease domain. A histidine hexamer was added to the C-terminus of the metalloprotease domain to facilitate purification of the recombinant protein using a Ni-NTA resin. Plasmid pET22b-MT-c or control plasmid (pET22b) was transformed into *E. coli* strain *BL21* (DE3) pLysS, and transformed cells were grown in LB media with 200 mg/ml ampicillin at 37°C to a cell density of A_{600} = 0.8–1.0. Expression of the recombinant protein was induced by adding 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG) and incubation was continued for 6 h at 37°C.

Purification and refolding of the recombinant protein expressed in *E. coli* Cells were collected by centrifugation and suspended in lysis buffer and then lysed by sonication. Cell lysate

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was centrifuged and washed three times with Tris-EDTA buffer containing 10 mM Tris·HCl (pH 7.6) and 1 mM EDTA. The induced protein was recovered as insoluble inclusion bodies. The precipitate was collected and solubilized in 8 M urea containing 10 mM Tris·HCl (pH 8.0) and 100 mM sodium phosphate. The solubilized protein was mixed with Ni-NTA resin (QIAGEN, Valencia, USA) equilibrated with the same buffer. After incubation for 2 h at 4°C, unbound proteins were washed out with 6 M urea containing 10 mM Tris·HCl (pH 5.9) and 100 mM sodium phosphate. The bound protein was eluted with 6 M urea containing 10 mM Tris·HCl (pH 4.5) and 100 mM sodium phosphate, and then the pH was adjusted to pH 7.5. The purified protein was refolded by passing it through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) equilibrated with phosphate-buffered saline (PBS).

Proteolytic activity of refolded metalloprotease Digestions of blood coagulation-related proteins including factor II, factor X, plasminogen, and fibrinogen were carried out in PBS containing 1 mM $CaCl_2$ at 37°C for 8 h. The ratio of substrate protein to the refolded enzyme was 100/1 (w/w). Enzyme reactions were stopped by heating the reaction mixture at 100°C for 5 min with standard SDS-PAGE sample buffer, and then electrophoresed on SDS gels (Laemmli, 1970).

Results and Discussion

cDNA cloning and sequence analysis By screening the venom gland cDNA library of a Korean snake *(Agkistrodon halys brevicadus)*, we have isolated a cDNA clone having metalloprotease as well as disintegrin sequences and named it *MT-c* (Jeon and Kim, 1998)[a]. Based on the deduced polypeptide structure, it is hypothesized that the enzyme is translated with a signal sequence of 18 amino acids, amino-terminal propeptide of 167 amino acids, a central metalloprotease domain of 218 amino acids, and a disintegrin domain of 73 amino acids (Fig. 1). The deduced amino acid sequence of the *MT-c* metalloprotease domain shows 83% similarity to Ht-d (Hite *et al.*, 1994) and 78% similarity to protrigramin (Huang *et al.*, 1991). *MT-c* also shows similarity to Homosapien meltrin-s (ADAM 12) (Wu *et al.*, 1997) and to *Mus musculus* metalloprotease/disintegrin/cysteine rich (MDC) protein 9 precursor cDNA (Weskamp *et al.*, 1996). The MDC proteins have been implicated in a variety of important cellular processess. Because several MDC proteins contain potential cytoplasmic signaling motifs including a SH3 ligand domain, they may also play a role in signaling pathways or may be regulated through interactions with cytoplasmic proteins (Weskamp *et al.*, 1996). The similarity between the snake venom metalloproteases and mammalian MDC proteins is an exciting finding. It will be interesting to understand the physiological significance of similarities between snake venom toxicology and normal cellular processes.

Expression and refolding of recombinant MT-c proteins The MT-c clone contains a metalloprotease domain with an additional stretch of 16 amino acids in its C-terminus which is known to be the connecting sequence (Chao *et al.*, 1989; Hite *et al.*, 1992) to the disintegrin domain (Fig. 1). There are several reports indicating that the C-termini of the low-molecular-mass metalloproteases purified from snake venoms are –CI(L/I)N(A/K)P (Takeya *et al.*, 1990; Hite *et al.*, 1992), which is consistent with the C-terminal sequence of the MT-c metalloprotease domain, and that the N-termini of the purified disintegrins start with EAGEE (Chao *et al.*, 1989; Kang *et al.*, 1998). Therefore, the connecting sequence 388–403 in between the two domains does not exist in either of the mature proteins (Chao *et al.*, 1989; Hite *et al.*, 1992). In our initial attempts, we were not successful in refolding the recombinant polypeptide chain containing only the metalloprotease domain devoid of the connecting residues. However, we were able to successfully refold the expressed polypeptide containing the additional 16 residues of the connecting sequence in its C-terminus.

Expression of the recombinant MT-c protein, residues 186–403, was induced by the addition of 1 mM IPTG to *E. coli* having the expression vector. When the cell lysate was fractionated and then analyzed by SDS-PAGE, most of the recombinant protein was recovered in the precipitate as insoluble inclusion bodies (Fig. 2). The inclusion bodies were solublized in 8 M urea (pH 8.0) followed by affinity purification with Ni-NTA resin. Binding of the recombinant protein to the Ni-NTA resin through the His6-tag at the C-terminus was specific enough to allow single-step purification of the recombinant polypeptide expressed in *E. coli*. Homogeneous preparation of the recombinant protein retaining metalloprotease activity was successfully recovered by a convenient gel-filtration step, among various methods examined. These results clearly demonstrate that the slightly extended C-terminal sequence, residues 388–403, is a necessary component required for proper refolding of the recombinant metalloprotease into its functionally active conformation. Gradual reduction of urea in the gel-filtration step also appears to be a key point of the refolding strategy, leading to about 40% refolding efficiency. Such a highly efficient refolding is noteworthy when compared with the 2–3% refolding efficiency of the recombinant snake venom protease batroxobin (Maeda *et al.*, 1991). The simple refolding procedure established in this work will facilitate the biochemical analyses of recombinant metalloproteases, and allow an efficient screening system to be developed for investigating the enzyme inhibitors.

Functional characterization of the recombinant metalloprotease Investigation of the recombinant

[a] The cDNA sequence of *MT-c* has been deposited in the GenBank database under accession number AF051790.

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ttgcctgtcttccagccaaatccagcctccaaaATGATCCAAGTTCTCTTGGTGATTATATGCTTAGCAGATTTTCCTTATCAAGGGACC
                                  M  I  Q  V  L  L  V  T  I  C  L  A  A  F  P  Y  Q  G  T   19
                                  ^prodomain start
TCTATAATCCTGGAATCTGGGAACGTGAATGATTATGAAGTTGTCTATCCACGAAAAGTCACTGCATTGCCCAAAGGAGCAGTTCAGCCA
 S  I  I  L  E  S  G  N  V  N  D  Y  E  V  V  Y  P  R  K  V  T  A  L  P  K  G  A  V  Q  P   49
AAGTATGAAGACGCCATGCAATATGAATTTAAAGTGAATGGAGAGCCAGTGGTCCTTCACCTGGAAAAAAATAAAGGACTTTTTTCAAAA
 K  Y  E  D  A  M  Q  Y  E  F  K  V  N  G  E  P  V  V  L  H  L  E  K  N  K  G  L  F  S  K   79
GGTTACAGCGAGACTCATTATTCCCCTGATGGCAGAAAAATTACAACAAACCCTCCGGTTGAGGATCACTGCTATTATCATGGACGCATC
 G  Y  S  E  T  H  Y  S  P  D  G  R  K  I  T  T  N  P  P  V  E  D  H  C  Y  Y  H  G  R  I  109
CAGAATGATGCTGACTCAACTGCAAGTATCAGTGCATGCAACGGTTTGAAAGGACATTTCAAGCATCAAGGTGAGATGTACCTTATTGAA
 Q  N  D  A  D  S  T  A  S  I  S  A  C  N  G  L  K  G  H  F  K  H  Q  G  E  M  Y  L  I  E  139
CCCTTGAAGCTTTCCGACAGTGAAGCCCATGCAGTCTACAAATATGAAAACGTAGAAAAAGAGGATGAGGCCCCCAAAATGTGTGGGGTA
 P  L  K  L  S  D  S  E  A  H  A  V  Y  K  Y  E  N  V  E  K  E  D  E  A  P  K  M  C  G  V  169
ACCCAGACTAATTGGAAATCAGATGAGCCCATCAAGGCCTCTCAGCAACAAAGATTCCCCCAAAGATACATTGAGCTTGTTGTAGTTGCA
 T  Q  T  N  W  K  S  D  E  P  I  K  A  S  Q  Q  Q  R  F  P  Q  R  Y  I  E  L  V  V  V  A  199
                  metalloprotease domain start ^
GATCATGGAATGTTCACGAAATACGACAGCAATTTAGATACTATAAGAACGTGGGTACATGAACTTGTCAACAGTATAAATGAGTTTTAC
 D  H  G  M  F  T  K  Y  D  S  N  L  D  T  I  R  T  W  V  H  E  L  V  N  S  I  N  E  F  Y  229
AGATCTTTGAATATTGATGTCTCACTGACTGAGCTAGAAATTTGGTCCAACCAAGATTTGATCAACGTGCAGTCAGCAGCGGGTGATACT
 R  S  L  N  I  D  V  S  L  T  E  L  E  I  W  S  N  Q  D  L  I  N  V  Q  S  A  A  G  D  T  259
TTGGAAGCATTTGGAGACTGGAGAGAGACAGATTTGCTGAATCGCATAAGTCATGATAATGCTCAGTTACTCACGGCCACTGAATTGGAT
 L  E  A  F  G  D  W  R  E  T  D  L  L  N  R  I  S  H  D  N  A  Q  L  L  T  A  T  E  L  D  289
GGAAACACTATAGGATTGGCTCACGTAGCCAGCATGTGCGACCCGAAGCGTTCTACAGGAGTTGTTCAGGATCATAGTGCAATAAATCTT
 G  N  T  I  G  L  A  H  V  A  S  M  C  D  P  K  R  S  T  G  V  V  Q  D  H  S  A  I  N  L  319
TTGGTTGCAGTTACAATGGCCCATGAGACTGGTCATAATCTGGGCATGAATCATGATGGAAATCAGTGTCATTGCGGTGCTAACTCATGC
 L  V  A  V  T  M  A  H  E  T  G  H  N  L  G  M  N  H  D  G  N  Q  C  H  C  G  A  N  S  C  349
GTTATGGGTGATGTACTAAGCGAAGGAGTTTCCTATGAGTTCAGTGATTGTAGTGAGAATGAATATCAGACGTATCTTACTGATCGTAAC
 V  M  G  D  V  L  S  E  G  V  S  Y  E  F  S  D  C  S  E  N  E  Y  Q  T  Y  L  T  D  R  N  379
CCACAATGCATTCTCAATGAACCCTTGAGAACAGATACTGTTTCAACTCCAGTTTCTGGAAATGAACTTTTGGAGGCCGGAAAAGAATGT
 P  Q  C  I  L  N  E  P  L  R  T  D  T  V  S  T  P  V  S  G  N  E  L  L  E  A  G  K  E  C  409
                                  disintegrin domain start^
GACTGTGGCGCTCCTGCAAATCCGTGCTGCGATGCTGAAACCTGTAAACTGAGACCAGGGCAGCAGTGTGCAGAAGGACTGTGTTGTGAC
 D  C  G  A  P  A  N  P  C  C  D  A  E  T  C  K  L  R  P  G  Q  Q  C  A  E  G  L  C  C  D  439
CAGTGCAGATTTATGAAAGAAGGAACAATATGCCAGGAAGCCAAGGGTGATTGGAATGATGATACCTGCAATGGCATATCTGCTGGCTGT
 Q  C  R  F  M  K  E  G  T  I  C  Q  E  A  K  G  D  W  N  D  D  T  C  N  G  I  S  A  G  C  469
CCCAGAAATGGCTTCTATGGCtaaagaacaatggagatggaaaggtctgcagcaacgggcattgtgttgatgtgaatagagcctactaat
 P  R  N  G  F  Y  G                                                                        476
caacctttgacttctctcagatttgattttggagattcttctttcagaaggtttggcttccctcaagtccaaagagacccatctgcctgc
atccttctagtaaatcacccttagctttcttctccacatttaatctgtttacctttgctgtaatcaaacctttccccaccacaaagct
ccatgggcaagtacaacaccaagggcttatttgctgtcaagaaaaatcaatggccattttaccatttgccaattgcaaagtacatttaat
gcaacaagttctgcctttagagctggtgtattcgaagtcaatgcttcctctcccaaaattttgtgctggctttccaagatgtagctgctt
ccatcaataaactaatattctcattcaaaaaaaaaaaa
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Fig. 1. The cDNA sequence and deduced amino acid sequence of *MT-c*. The cDNA coding regions are shown in uppercase letters, and the 5′- and 3′- untranslated regions in lowercase letters. The predicted amino acid sequence is denoted by one-letter symbols. The polyadenylation signal aataaa is underlined and the 388–403 amino acid stretch is shown in bold type.

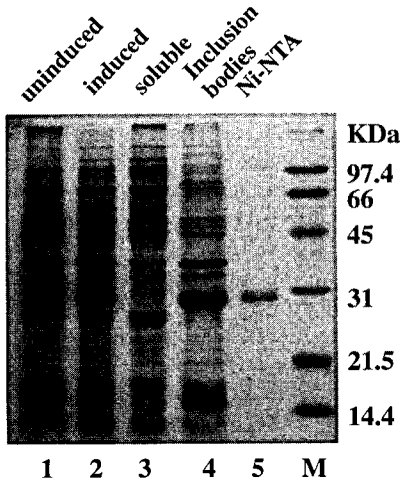

Fig. 2. Purification of recombinant metalloprotease expressed in *E. coli.* Expression and purification of the recombinant MT-c was analyzed on SDS-PAGE: protein extract from uninduced (lane 1) and IPTG-induced (lane 2) *E. coli*, soluble fraction (lane 3) and inclusion bodies (lane 4) from IPTG-induced cells, and recombinant protein purified by Ni-NTA affinity chromatography (lane 5). M, molecular weight markers.

metalloprotease activity was carried out with human blood coagulation-related proteins including factor II, X, fibrinogen, and plasminogen. Prolonged enzymatic reaction of MT-c metalloprotease with each of the above substrates resulted in proteolytic degradation of fibrinogen (Fig. 3, panel A) without cleavage of the other plasma proteins tested (Fig. 3, panel B). The recombinant enzyme exerted no detectable lysis of fibrin clot (data not shown). It was reported that hemorrhagic metalloprotease HR-2a from *T. flavoviridis* can digest dimethylcasein, fibrinogen, and fibronectin (Takeya *et al.*, 1989), and that jararhagin from *B. jararaca* can degrade fibrinogen (Paine *et al.*, 1992). Like these metalloproteases, the recombinant MT-c enzyme was able to digest fibrinogen which is required for platelet aggregation. It was also proposed that fibrinogen degradation is responsible for the metalloprotease-induced abnormalities associated with platelet function (Kamiguti *et al.*, 1994)

Taking these results together, we have demonstrated a simple and ideal strategy that may be generally applicable to refolding recombinant metalloproteases into their functional forms with remarkably high efficiency. Further studies to investigate the *in vivo* functional mechanism related to the metalloprotease activity described in this communication will provide useful information in understanding the physiological significance of the enzyme activity.

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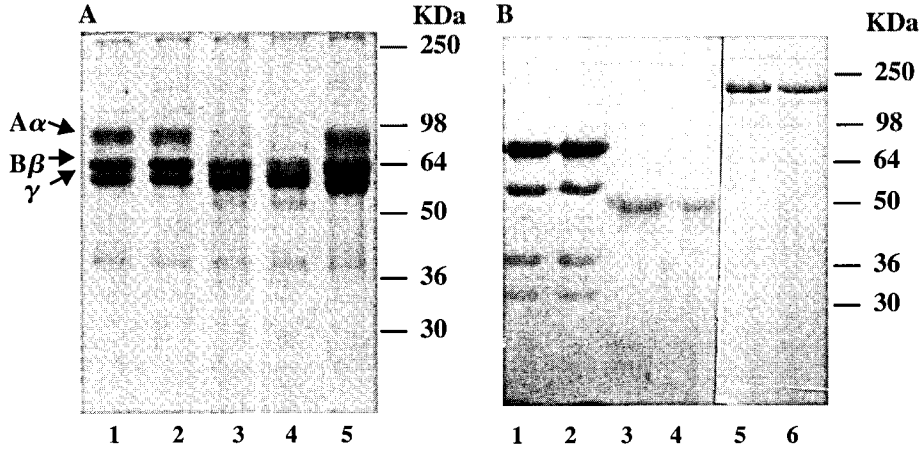

Fig. 3. Proteolytic digestion of the blood coagulation-related proteins by recombinant MT-c. Proteolytic digestions of fibrinogen (Panel A) and other substrates (Panel B) were analyzed on SDS-PAGE. In Panel A, fibrinogen (10 μg, lane 1) was reacted with recombinant enzyme (0.1 μg) for 1 h (lane 2), 4 h (lane 3), and 8 h in the absence (lane 4) or presence (lane 5) of 5 mM EDTA, all at 37°C in PBS containing 1 mM $CaCl_2$. In Panel B, lanes 1, 3, and 5 are 5 μg of factor II, factor X, and plasminogen, respectively, in PBS containing 1 mM $CaCl_2$. Lanes 2, 4, and 6 are 5 μg of factor II, factor X, and plasminogen, respectively, reacted with 0.05 μg of recombinant enzyme at 37°C in PBS containing 1 mM $CaCl_2$.

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