Cloning and isolation of a conus cysteine-rich protein homologous to Tex31 but without proteolytic activity

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We cloned and isolated a cysteine-rich protein, designated Mr30, from Conus marmoreus. Mr30 belongs to the cysteine-rich secretory protein family that is highly homologous to Tex31 previously obtained from Conus textile and reported as a protease responsible for processing of pro-conotoxins. Mr30, purified by a procedure similar to that of Tex31, indeed showed low proteolytic activity. However, further investigations revealed that the detected protease activity actually resulted from a trace amount of protease(s) contamination rather than from Mr30 itself. This finding led us to rethink the role of conus cysteine-rich secretory proteins: they were probably not responsible for the processing of pro-conotoxins as previously deduced, but their real biological functions remained to be clarified.

Keywords  cone snail; cysteine-rich secretory protein; protease; protease inhibitor; covalent modification

The cysteine-rich secretory protein (CRISP) family is highly conserved during evolution, but their functions have not been well defined. The mammalian CRISPs are mainly found in saliva and male reproductive tracts, as well as in human granulocytes and plasma. Their functions are related to sperm-egg fusion and the innate immune system [1–7]. The CRISPs are also present in the venom of snakes and lizards, and some of them could function as ion channel blockers [8–13].

Recently, a novel CRISP, namely Tex31, was isolated from the venom of cone snails, Conus textile [14]. It was reported that Tex31 had protease activity and was responsible for pro-conotoxin processing. To our knowledge, of the CRISPs, only Tex31 was reported as a protease. To confirm this interesting finding, we cloned and isolated a Tex31 homolog, designated Mr30, from Conus marmoreus that were collected from the South China Sea near Sanya City, China. The Mr30 fraction, purified from the venom of C. marmoreus by a procedure similar to that for Tex31, indeed had a low proteolytic activity, but further investigations revealed that the detected activity was actually a result of a trace amount of protease(s) contamination.

Materials and Methods

Materials

The cone snails (C. marmoreus) were collected from the South China Sea near Sanya City. The TRizol RNA isolation agents and a rapid amplification of cDNA ends (3′-RACE) kit were purchased from Invitrogen (Carlsbad, USA). The pGEM-T Easy Vector was the product of Promega (Madison, USA). The Dec-RVKR-chloromethylketone (RVKR-CMK) was from Biomol (Plymouth Meeting, USA). Sepharose 4B and DEAE Sepharose Fast Flow were purchased from Amersham Biosciences (Peapack, USA). Bovine trypsin and aprotinin were purchased from Sigma (St Louis, USA).

Gene cloning

The total RNA was isolated from the venom ducts of C. marmoreus using TRizol agents according to the manufacturer’s manual. The single-strand cDNA was then synthesized from the total RNA templates using the reverse transcriptase and a 3′-end primer, 5′-GGCCACG-
CGCGTCGACTAGTAC(dT)17-3’. Based on the published sequence of Tex31, a 5'-end primer was designed, 5'-ATGTGTTCTAGATGCAGACGTGG-3’. Using the designed 5'-end primer and the 3'-end primer included in the 3'-RACE kit (5'-GGCCAGCGCCGTGACAG-3’), we amplified an approximately 1.0 kb DNA fragment that was subsequently cloned into pGEM-T Easy Vector and sequenced.

**Isolation and purification of Mr30**

Six venom ducts from *C. marmoreus* were homogenized in cold 20 mM Tris-HCl buffer (pH 7.5). After centrifugation (10,000 g for 10 min), the supernatant was loaded onto a DEAE-Sepharose fast flow column (8 mm×55 mm) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was then eluted with step-wise increases of NaCl concentration (150 mM, 300 mM, and 500 mM) in the initial buffer. Fractions were collected and analyzed by SDS-PAGE. The fractions containing Mr30 were pooled and concentrated by ultrafiltration (cut-off molecular weight 10 kDa) then applied to a gel filtration column (Sephacryl S-100 HR, 16 mm×100 mm) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was then eluted with step-wise increases of NaCl concentration (150 mM, 300 mM, and 500 mM) in the initial buffer. Fractions were collected and analyzed by SDS-PAGE. The fractions containing Mr30 were pooled and concentrated by ultrafiltration (cut-off molecular weight 10 kDa) then applied to a gel filtration column (Sephacryl S-100 HR, 16 mm×100 mm; Amersham Biosciences, Peapack, USA) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was eluted with 20 mM Tris-HCl buffer (pH 7.5) at a flow rate of 0.4 ml/min, and fractions were automatically collected (1.4 ml/tube). After analysis using SDS-PAGE, the fractions containing Mr30 were pooled and concentrated by ultrafiltration (cut-off molecular weight 10 kDa). The concentration of the purified Mr30 was quantified by the Bradford method [15]. The purity of the Mr30 was analyzed by SDS-PAGE and HPLC. For SDS-PAGE analysis, 2 µg purified Mr30 was loaded on a 12% SDS gel, and the gel was stained with Coomassie Brilliant Blue R 250 after electrophoresis.

**N-terminal sequencing and mass spectrometry**

For N-terminal sequencing, the purified Mr30 (approximately 50 µg) was transferred to a PVDF membrane and its N-terminal amino acid sequence was determined by Edman degradation on a protein sequencer (Model 491; Applied Biosystems, Foster City, USA). For mass spectrometry, Mr30 and the RVKR-modified Mr30 were further purified by a C3 reverse-phase column (Zorbax 9.4 mm×250 mm; Agilent Technologies, Santa Clara, USA) and eluted by an acetonitrile gradient. The unmodified Mr30 and modified Mr30 (approximately 100 µg) were lyophilized and their molecular masses were measured on a Q-trap mass spectrometer (Applied Biosystems).

**Proteolytic activity assay**

The protease activity was measured using the fluorescent substrate (pGlu-Arg-Thr-Lys-Arg- methylcoumarinamide). The reaction was carried out in 20 mM Tris-HCl buffer (pH 7.5) at 37 °C for 1 min. The pre-warmed fluorescent substrate (at the final concentration of 1 µM) and an appropriate amount of protease were added to the pre-warmed assay solution (total volume of 1 ml), and the increase of fluorescence at 460 nm (excited at 370 nm) was measured by a fluorometer (F-2500; Hitachi, Tokyo, Japan). For the inhibition assay, the inhibitors (final concentrations: RVKR-CMK, 1 mM; aprotinin, 200 µg/ml) were pre-incubated with 10 µg purified Mr30 at 37 °C for 30 min in 20 mM Tris-HCl buffer (pH 7.5) (total 20 µl), then the protease activity was measured as described above.

**Affinity absorption**

Aprotinin was covalently immobilized onto Sepharose 4B resin using 2-chloromethyl-oxirane as a cross-linker. Approximately 1 mg aprotinin was covalently bound to 1 ml Sepharose 4B resin. To absorb the protease(s), 10 µg Mr30 was incubated with 100 µl affinity resin in 20 mM Tris-HCl (pH 7.5) buffer (total volume 1.1 ml) at 4 °C for 2 h. Thereafter, the protease activity of the supernatant was measured as described above and the Mr30 amount in the supernatant was determined by SDS-PAGE and densitometry. For the control experiment, Sepharose 4B was used instead of the affinity resin.

**RVKR-CMK modification**

RVKR-CMK was dissolved in DMSO as a stock solution (20 mM) and stored at –80 °C. The purified Mr30 (1 mg/ml) was first treated with RVKR-CMK (at the final concentration of 1 mM) at 37 °C for 30 min in 20 mM Tris-HCl buffer (pH 7.5). For the control experiment, the same amount of DMSO was used to treat Mr30. Thereafter, part of the modified Mr30 was used for the protease activity assay; the remaining was further purified by C3 reverse-phase HPLC and its molecular mass was measured by mass spectrometry. In the control experiment, trypsin (0.5 mg/ml) was modified by RVKR-CMK, and its molecular mass (before and after modification) and proteolytic activity were measured.

**Results**

**Mr30 cloning from C. marmoreus**

Based on the published cDNA sequence of Tex31, we...
designed several 5'-end primers to amplify the cDNA of Tex31 homolog from the RNA templates isolated from the venom ducts of *C. marmoreus* by 3' RACE, and finally obtained an approximately 1.0 kb fragment. After T-vector cloning, the amplified fragment was sequenced (from eight individual clones). Its open reading frame encoded a polypeptide chain with 289 residues including an N-terminal signal peptide as well as a dibasic cleavage site Fig. 1(A).

The cloned polypeptide contained a polymorphism at position 186. In one isoform (Mr30-1; GenBank accession No. EF493183), this position is a Glu residue (found in four clones), and in the other isoform (Mr30-2; GenBank accession No. 493184) it is a Lys residue (found in four clones). The protein was named Mr30 because it was cloned from *C. marmoreus* with a molecular weight of approximately 30 kDa. The sequence alignment showed
that Mr30 was highly homologous to Tex31 cloned from *C. textile*, their amino acid sequence similarity was as high as 65% [Fig. 1(B)]. The high sequence homology implied that Mr30 and Tex31 probably had similar biological functions.

### Purification of Mr30 from venom of *C. marmoreus*

In addition to the cDNA cloning of Mr30, we also isolated and purified the mature Mr30 protein from the conus venom in sequential steps that were similar to the procedure used for Tex31 purification.

The material extracted from the venom ducts was first applied to a DEAE-Sepharose column and eluted in a step-wise manner (data not shown). The eluted fractions were analyzed by SDS-PAGE [Fig. 2(A)]. The major fraction of Mr30 was eluted by 300 mM NaCl, and the minor frac-

![Fig. 2 Purification of cysteine-rich protein Mr30 from conus venom](image)

(A) SDS-PAGE analysis of the fractions eluted from DEAE-Sepharose column. M, protein marker; 1, crude extract from venom ducts; 2, fraction eluted by 150 mM NaCl; 3, fraction eluted by 300 mM NaCl; 4, fraction eluted by 500 mM NaCl. (B) Gel filtration chromatography. (C) SDS-PAGE analysis of the fractions eluted from the gel filtration column. A sample (20 µl) from each tube was loaded onto a 12% SDS gel, and the gel was stained with Coomassie Brilliant Blue R 250 after electrophoresis. M, protein marker; 22–41, tube number. (D) Purity analysis by HPLC. Mr30 (20 µg) was loaded onto a C3 reverse-phase column and eluted with an acetonitrile gradient (solvent A, 0.1% aqueous trifluoroacetic acid; solvent B, acetonitrile with 0.1% trifluoroacetic acid). Mr30 was eluted from the column at 60% acetonitrile. The UV absorbance at 280 nm was monitored. OD, optical density. (E) Purity analysis by SDS-PAGE. Mr30 (2 µg) was loaded onto a 12% SDS gel, and the gel was stained with Coomassie Brilliant Blue R250 after electrophoresis.
tion was eluted by 500 mM NaCl. The two Mr30 frac-
tions were combined, concentrated by ultrafiltration, and 
applied to a gel filtration column [Fig. 2(B)]. Each eluted 
fraction from the gel filtration column was analyzed by 
SDS-PAGE [Fig. 2(C)]. Mr30 was found in tube num-
bers 36−40, corresponding to the second elution peak in 
Fig. 2(B). These Mr30 fractions were pooled together, 
concentrated by ultrafiltration, and used for purity analysis, 
mass spectrometry, N-terminal sequencing, and proteolytic 
activity assay. As shown in Fig. 2(D,E), the purified Mr30 
showed a major peak on C3 reverse-phase HPLC, and a 
single band on SDS-PAGE. The purity of the Mr30 fraction 
was over 90%.

Characterization of Mr30
The N-terminal amino acid sequencing of the purified Mr30, 
determined by Edman degradation, yielded a single N-
terminal sequence, HAXDSKYSDVTPTHT, where X, 
usually being a cysteine residue, could not be determined 
experimentally. The measured N-terminal sequence of the 
mature Mr30 was identical to that deduced from its cDNA. 
The molecular mass of the purified Mr30 was measured 
and is shown in Fig. 3(A). The measured values (a major 
peak, 29,858 Da, and several small heavier peaks) were 
slightly heavier than its theoretical value, 29,666 Da (186E 
isoform) and 29,665 Da (186K isoform) (assuming 11 
disulfide bonds were formed in the mature Mr30). This 
phenomenon was probably a result of heterogeneous post-
translational modifications that were also observed in 
Tex31 [14] and are very common in cone snails [16]. After 
the protease activity was removed from the Mr30 fraction 
by affinity absorption, little change occurred in the mass 
spectrum of Mr30 (data not shown). Thus the small peaks 
observed in the mass spectrum were not caused by protease 
(s) contamination.

Protease activity assay
As it was reported that Tex31 had proteolytic activity, we 
previously deduced Mr30 to also be a protease because of 
high sequence homology shared with Tex31. As shown in 
Fig. 4(A), the purified Mr30 indeed showed proteolytic 
activity. However, the measured activity of Mr30 was 
extremely low compared with that of trypsin; the specific 
activity of trypsin was approximately 5000-fold that of 
Mr30. We tried adding Ca²⁺ into the assay buffer or 
increasing the pH of the buffer to 8.5 (optimal reaction 
conditions for Tex31), but no significant activity increase 
was observed (data not shown). As shown in Fig. 4(B), 
the activity of Mr30 could be almost completely inhibited 
by aprotinin (a reversible serine protease inhibitor) and
RVKR-CMK (an irreversible serine protease inhibitor). However, N-ethylmaleimide (a cysteine-specific modification reagent) had no effect on the proteolytic activity of Mr30 (data not shown). Reduced glutathione (5 mM) had no effect on its proteolytic activity (data not shown). Although the proteolytic activity was detected in the purified Mr30 fraction, its extremely low specific activity raised the question whether the detected activity really resulted from Mr30 itself or from a trace amount of protease(s) contamination. Although the previous analyses showed the purified Mr30 was homogeneous enough [Fig. 2(D,E)], we could not exclude the possibility that it might still contain a trace amount of protease(s) contamination.

**Affinity absorption**

As aprotinin could completely inhibit the activity of Mr30, as shown in Fig. 4(B), we cross-linked aprotinin onto the Sepharose resin and used the aprotinin resin to remove the possible contaminated protease(s). As shown in Fig. 5(A), after absorbed by the affinity resin, the protease activity of the supernatant became undetectable, whereas the treatment by Sepharose alone had little effect. This suggested that the protease(s) in the Mr30 fraction had been absorbed by the affinity resin. However, as shown in Fig. 5(B), affinity absorption had little effect on the Mr30 concentration in the supernatant. Therefore, the detected proteolytic activity of the Mr30 fraction actually resulted from a trace amount of protease(s) contamination rather than from Mr30 itself.

**RVKR-CMK modification**

As shown in Fig. 4(B), RVKR-CMK could completely inhibit the proteolytic activity of the Mr30 preparation. It is known that RVKR-CMK exerted its inhibitory effect by covalent modification of the His residue of the catalysis triad (composed of Ser, His, and Asp) of serine proteases. If the detected proteolytic activity of the Mr30 preparation was really a result of Mr30 itself, RVKR-CMK could be covalently bound to Mr30 and cause a molecular weight increase (708 Da). Otherwise, the molecular weight of Mr30 would not be changed after the RVKR-CMK treatment. To test the validity of this method, bovine trypsin (a typical serine protease) was used as a model protease. The RVKR-CMK treatment completely inhibited the proteolytic activity of trypsin (data not shown). It also caused a 706 Da increase in the molecular weight of trypsin as revealed by mass spectrometry (for untreated trypsin: measured value, 23,309 Da; theoretical value, 23,311 Da; for RVKR-CMK-treated trypsin: measured value, 24,015 Da; theoretical value, 24,019 Da). The measured molecular weight increase (706 Da) of trypsin was quite consistent with the molecular weight of the inhibitor moiety (708 Da). Therefore, RVKR-CMK was covalently bound to the trypsin molecule. The results of trypsin modification suggested that the method could be used to test whether Mr30 was a protease or not. Although the proteolytic activity of the Mr30 preparation could be completely inhibited by the RVKR-CMK treatment [Fig. 4(B)], the molecular weight of Mr30 itself was not at all increased after the RVKR-CMK treatment, as revealed by mass spectrometry [Fig. 3(B)], implying that RVKR-CMK did not covalently bind to Mr30 itself. Therefore, the RVKR-CMK modification experiment also suggested that the detected protease activity of the Mr30 fraction was actually caused by a trace amount of protease(s) contamination.

**Discussion**

In the present study, the cloning and isolation of a cysteine-rich protein from *C. marmoreus* was reported. The newly obtained Mr30 was highly homologous to Tex31, reported to be inherent in proteolytic activity and concluded to be...
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responsible for pro-conotoxin processing. Mr30 purified by a procedure similar to that of Tex31 also had low proteolytic activity. However, using two independent experiments we showed that the detected activity actually resulted from a trace amount of protease(s) contamination rather than from Mr30 itself.

While we were working on this project, another laboratory also cloned and isolated Mr30 (they named it GlaCrisp) [17]. They found GlaCrisp by chance during their isolation of γ-carboxyglutamate-containing proteins, and they also could not detect any proteolytic activity in their purified GlaCrisp fraction. The purification of GlaCrisp involved an affinity purification step (anti-Gla antibody) that probably removed the protease(s) contamination (without γ-carboxyglutamate modifications) from the GlaCrisp fraction. The fact that Mr30/GlaCrisp had no proteolytic activity led us to rethink the possible role of the conus CRISPs: they are probably not involved in the processing of pro-conotoxins, despite the low protease activity detected in the purified Tex31 fraction. We thought more work (such as affinity absorption and covalent modification) was needed to confirm that the detected proteolytic activity really resulted from Tex31 itself.

Although the purified Mr30 was essentially homogeneous, as analyzed by SDS-PAGE and reverse-phase HPLC [Fig. 2(D,E)], molecular weight heterogeneity was observed in mass spectrometry, and all of the measured molecular weight values were slightly heavier than the theoretical value deduced from its amino acid sequence (Fig. 3). This phenomenon also occurred in Tex31 [14] in that several related isoforms corresponding to different post-translational modifications were present in the mass spectrometry. The molecular weight heterogeneity of Tex31 and Mr30 was probably caused by heterogeneous post-translational modifications. It is known that the conus peptides/proteins contain rich post-translational modifications [16]. In GlaCrisp an N-terminal γ-carboxyglutamate residue had been identified experimentally [17], and other possible modifications, such as hydroxylation, might also occur in GlaCrisp/Mr30 and Tex31.

For both Tex31 and Mr30/GlaCrisp, the mature protein was cleaved after a conserved dibasic site [Fig. 1(B)]. The processing probably involved two sequential steps, the signal peptidase removed the signal peptide (20 or 21 residues), then the furin-like protease recognized the dibasic site and removed the extremely short “pro” sequence (three or four residues).

GlaCrisp/Mr30 is not a protease, but its real biological functions remain unknown. Considering that the CRISPs found in the venom of snakes could function as ion channel blockers [8,11–13], the conus CRISPs might also be ion channel blockers that target the ion channels of prey.

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