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ORIGINAL PAPER

Expression in *Escherichia coli* of the recombinant *Vibrio anguillarum* metalloprotease and its purification and characterization

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Abstract The full length *emp*A gene encoding *Vibrio anguillarum* metalloprotease was amplified by PCR and fused to the expression vector pBAD24. The carboxy-terminal 6xHis-tagged recombinant metalloprotein (rEmpA) was expressed from plasmid pBAD-VAP6his in *E. coli* TOP10 and purified with affinity chromatography using a Ni-NTA column. SDS-PAGE analysis and Western blotting revealed a molecular mass of the mature rEmpA predicted to be 36 kDa. The optimal temperature and pH for the purified rEmpA were 37°C and 8.0, respectively. The enzyme was stable below 30°C and between pH 5.0 and 8.0, respectively. The results show that Ca^{2+}, Na^{+} and Mg^{2+} had an activating effect on the enzyme while Zn^{2+} and Cu^{2+} acted as inhibitors of the enzyme. The purified rEmpA was characterized as a zinc metalloprotease as it was inhibited by zinc- and metal-specific inhibitors, such as 1,10-phenanthroline, EDTA and EGTA. The results indicate that some characteristics of EmpA from marine *V. anguillarum* had been modified after expression and processing in the engineered *E. coli*. The purified rEmpA showed degradation activity towards various kinds of proteins, indicating its potential role in pathogenesis.

Keywords *emp*A gene · *Vibrio anguillarum* · recombinant metalloprotease · expression · characterization

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Introduction

The maricultural industry has been developing very rapidly in last two decades in China and sea-food yields in China have become the biggest in the world (Chi et al. 2006). However, the industry is frequently plagued by bacterial infections, particularly vibriosis mainly caused by *Vibrio anguillarum* and *V. harveyi*, leading to extensive economic losses (Austin and Austin 1999; Teo et al. 2003; Staroscik et al. 2005). So far, much less has been known about the virulence factors that are involved in the pathogenesis of marine *Vibrio* spp. (Teo et al. 2003; Denkin and Nelson 2004; Staroscik et al. 2005). Studies on the pathogenicity of various marine *Vibrio* spp. showed that their extracellular enzymes including protease, haemolysin, phospholipase and chitinase are ones of the virulence factors (Teo et al. 2003; Austin and Austin 1999). In order to confirm that they can play an important role in pathogenicity in marine animals, it is very important to clone and express the genes encoding the extracellular enzymes. It is also significant to purify and characterize the recombinant enzymes from *E. coli* in order to understand their biochemical properties.

Metalloprotease, one of the virulence factors from marine *Vibrio* spp. has received increasing attention in recent years (Teo et al. 2003; Denkin and Nelson 2004; Staroscik et al. 2005). In our previous studies (Chen et al. 2002a; Zhang et al. 2006), *emp*A gene encoding a metalloprotease from *V. anguillarum* W-1 (which is a prevalent pathogen in marine fish in China) was cloned and overexpressed in *E. coli* and its translocation pathway and processing of the enzyme in *E. coli* were investigated. We found that EmpA was exported into the periplasm via the Sec pathway but not secreted into

the medium in *E. coli* MC4100. It represents a great potential for quick purification of EmpA for biotechnological application including preparation of a vaccine against this pathogen. Interestingly, thermal treatment of the whole cells of *E. coli* TOP10 expressing the *emp*A gene provoked a general proteolysis and only few peptides remained in the cellular extracts. Among them, a 36-kDa derivative of EmpA was the most abundant polypeptide. A metalloprotease gene *pap*6 was cloned from *V. harveyi* strain AP6 and found to be 2034 bp. A recombinant 6xhis tagged enzyme was purified and characterized and proved to be a zinc-dependent metalloprotease (Teo et al. 2003). A cysteine protease from a pathogenic Taiwanese strain of *V. harveyi* 820514 is a novel protease of 38 kDa that exhibited lethal toxicity to *Panaeus monodon* (Liu et al. 1997). Alkaline protease has also been extracted from the supernatants of *V. harveyi* (Fukasawa et al. 1988). The optimal temperature and pH for the purified metalloprotease from wild type *V. anguillarum* W-1 were found to be 50°C and 7.0, respectively (Chen et al. 2002b). It was found that the purified metalloprotease from wild type *V. anguillarum* W-1 is a zinc-metalloprotease and plays an important role in pathogenicity in *Lateolabrax japonicus* (Chen et al. 2002b).

In the present study, the cloned *emp*A gene encoding metalloprotease from *V. anguillarum* W-1 was expressed in *E. coli* and the rEmpA was purified and characterized from the engineered *E. coli* cells.

Materials and methods

Bacterial strains, plasmids and media

Vibrio anguillarum W-1 was isolated from infected *Lateolabrax japonicus* in Laizhou Bay, Shandong Province, China. *V. anguillarum* W-1 was grown aerobically in 2216E marine medium containing 20 mg ml^{-1} NaCl at 28°C (Chen et al. 2002a). *Escherichia coli* TOP10 [F^- *mcr*AΔ (*mrr-hsd*RMS-*mcr*BC)Φ80*lac*ZΔ M15Δ*lac*X74 *rec*A1 *deo*R *ara*D139 (*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] cells were used as the cloning expression host and cultivated in Luria–Bertani (LB) broth or LB agar at 37°C. *E. coli* transformants were grown on LB medium containing 100 μg ml^{-1} ampicillin. pBAD24 with L-arabinose inducible P_{BAD} promoter was used as the expression vector, As required, arabinose (2 mg ml^{-1}) was added to LB media (Guzman et al. 1995).

Construction of plasmid

To study rEmpA expression and purification in *E. coli*, the *emp*A gene was amplified from the plasmid pUCM-VAP (Chen et al. 2002a), carrying the *emp*A gene from *V. anguillarum* W-1, with the primers CW25786 (5′-GCGCCATGGAAAAAGTACAACGTC-3′, the underlined bases encode an *Nco*I site) and CW25787 (5′-CGCGTCTAGATTA*gtgatggtgatggtgatg* ATCC AGTCTTAACGTTACACC-3′, the underlined bases encode a *Xba*I site, italic bases encode 6His sequence) by PCR using a Thermocycler (Thermo Hybid31208) with Taq DNA polymerase (Promega, USA). The PCR procedure was as follows: initial denaturation at 94°C for 8 min and 5 cycles of 1 min at 94°C, 1 min at 38°C, 2 min at 72°C, followed by 25 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, followed by additional 8 min at 72°C. The resultant 1854 bp PCR product was digested with *Nco*I and *Xba*I and cloned into pBAD24 vector digested with the same endonucleases. The resultant plasmid was named as pBAD-VAP6his and the expression of the recombinant *emp*A gene was under the tight control of the P_{BAD} promoter, i.e. induced by arabinose (Guzman et al. 1995).

Expression and purification of rEmpA

E. coli TOP10 was transformed with pBAD-VAP6his and grown in LB broth with 100 μg ampicillin ml^{-1}. The cells were induced with L-arabinose and incubated with shaking at 37°C until $OD_{600\ nm}$ reached 1.8. After harvesting, the cells were resuspended in Bacterial Protein Extraction buffer offered by the manufacturer (Pierce, USA). The cell lysate was centrifuged and the supernatant was applied onto a Ni-NTA spin column. The purification of rEmpA was carried out according to the protocols offered by the manufacturer (Pierce, USA). The column was washed four times with the washing buffer to remove non-specific proteins. The 6xHis-tagged rEmpA was eluted with the elution buffer. The eluted 6XHis-tagged fusion protein was assayed by SDS-PAGE.

Assay of protease activity

The recombinant EmpA (rEmpA) protease activity in the cellular fractions was assayed according to Inamura et al. (1985). Briefly, 100 μl of the purified EmpA protease was added to 100 μl of azocasein (5 mg/ml, Sigma) in 200 mM Tris–HCl buffer (pH 8.0) and additional volume of 20 mM Tris–HCl (pH 8.0) buffer was added to make final volume of 500 μl. The mixture

was incubated at 37°C for 20 min. The reaction was terminated by adding 1.75 ml of 5.0% (w/v) trichloroacetic acid and left for 5 min on ice, followed by centrifugation at 5000 g and 4°C for 10 min. The supernatant was neutralized by adding 2.25 ml of 0.5 M NaOH, and the absorbance at 440 nm was measured using a spectrophotometer. One unit of protease activity is defined as the amount of enzyme that causes an increase of 0.001 absorbance unit at 440 nm. 20 mM of Tris–HCl (pH 8.0) buffer was used as a control.

Electrophoresis, Western blotting analysis and zymography

SDS-PAGE was performed according to Sambrook et al. (1989) using 5% stacking gel and 12% resolving gel. The purified rEmpA was separated on SDS-PAGE gel and electrotransferred onto Immnuno-Blot nitrocellulose membrane (Millipore) for Western blotting. Immuno-Blot analysis was performed according to Kurien and Scofield (2003). The Western blots were probed with anti-EmpA antiserum (dilution 1:1,000). The secondary antibody and the substrates were used to determine immuno-labeled bands according to the manufacture's instructions.

Polyclonal anti-EmpA antisera were prepared by immunizing rabbits with the purified metalloprotease EmpA from the secreted fractions of *V. anguillarum* W-1 following standard protocols (Harlow and Lane 1988). The secondary antibody is goat anti-rabbit horseradish peroxidase (HRP)-labelled IgG purchased from WuHan BoShiDe Biotechnology, China.

The protease activity was also visualized as transparency bands on background when polyacrylamide gels containing 2% (w/v) immobilized gelatin were immersed in 100 mM PBS buffer for about 4 h as described by Martone et al. (1999). In order to determine protein digestion, the purified rEmpA was incubated with different proteins (50 μg) including collagen, gelatin, shrimp proteins and casein at 37°C 4 h. Digestion products were analysed on a 12% SDS-PAGE gel.

Preparation of shrimp proteins

Two grams of the shrimp meat were homogenized in a DY89-I Type Electric Glass Homogenizer (Xinzhi, Zhejiang, China) and homogenization proceeded for 1 h on the ice. The cell debris was removed by centrifugation at 12,000 rev min^{-1} and 4°C for 30 min and the supernatant obtained was used as shrimp proteins.

Effects of pH and temperature on rEmpA activity and stability

The effect of pH on the enzyme activity was determined by incubating the purified rEmpA at pH values between 4.0 and 13.0 using the standard assay conditions. The buffers used were 20 mM acetate buffer (pH 4.0–5.0) 20 mM glycine–NaOH buffer (pH 6.0–7.0) and 20 mM Tris–HCl buffer (pH 8.0–13.0). The pH stability was tested by 30 min pre-incubation of the purified rEmpA in appropriate buffers that had the same ionic concentrations at different pH values ranging from 4.0 to 13.0 at 0°C. The remaining activities of rEmpA were measured immediately after this treatment with the standard method as mentioned above. The optimal temperature for activity of rEmpA was determined at 0°C 20°C, 30°C, 37°C, 50°C, 60°C and 80°C in the same buffer as described above. Temperature stability of the purified rEmpA was tested by pre-incubating the enzyme at different temperature ranging from 0°C to 80°C for 30 min, residual activity was measured as described above immediately. Here, pre-incubated sample at 0°C was used a reference to calculate the residual activity.

Effects of different metal ions on rEmpA activity

To examine effects of different metal ions on rEmpA activity, enzyme assay was performed in the reaction mixture as described above with various metal ions at the final concentrations of 0.1 or 10 mM, respectively. The activity assayed in the absence of metal ions was defined as control. The metal ions tested include Zn^{2+}, Cu^{2+}, Mg^{2+}, $Ca^{2+}$and Na^{+}.

Effects of protein inhibitors on rEmpA activity

The effects of protease inhibitors (1,10-phenanthroline, EGTA, EDTA, PMSF, SDS and urea at 10 mM final concentration) on rEmpA activity were measured in the reaction mixture as described above. The purified enzyme was pre-incubated with the respective compounds for 30 min at 0°C, followed by the standard enzyme assay as described above. The activity assayed in the absence of the protein inhibitors was defined as control.

Results and discussion

Construction of the recombinant plasmid pBAD-VAP6his

The PCR product obtained in Materials and methods was ligated into a pBAD24 vector. The resultant

plasmid was named as pBAD-VAP6his. After sequencing, the size and sequence of the cloned gene with 18 bp encoding 6 histidine residues were found to be identical to those (Accession number: AY046320) reported in our previous study (Chen et al. 2002a; Zhang et al. 2006).

Expression and purification of rEmpA-his

The full length *emp*A encoding *V. anguillarum* metalloprotease was amplified by PCR and fused to the expression vector pBAD24 vector. The carboxy-terminal 6xHis-tagged rEmpA was expressed from plasmid pBAD-VAP6his in *E. coli* TOP10 and purified by affinity chromatography using a Ni-NTA column. rEmpA was purified to homogeneity with a 1.29-fold increase with a yield of about 12.46% as compared to the crude cell lysate and had a specific activity of $17.5 \pm 0.1 \times 10^4$ U mg^{-1} (Table 1). SDS-PAGE analysis showed that there was only one single protein band from the finally concentrated elute (Fig. 1A) and Western blotting predicted a molecular mass of the mature rEmpA of 36 kDa (Fig. 1B), suggesting that the His-tag was likely to have been part of a C-terminal propeptide which was cleaved during the maturation of rEmpA. Therefore, it is likely that rEmpA was processed into its mature protein by cleavage of the signal peptide, the N-terminal propeptide and C-terminal propeptide (Miyoshi et al. 2001, 2002; Zhang et al. 2006). The purified rEmpA was proteolytically active as demonstrated by gelatin zymography (Fig. 1C).

Optimum temperature and thermal stability

The rEmpA protease activity measured as a function of temperature from 0 to 80°C shows that the activity was the highest at 37°C (Fig. 2). The optimal temperature for enzyme activity of the recombinant *V. mimicus* metalloprotease (rVMC61) from *E. coli* ranges from 30 to 40°C and preincubation at temperatures above 45°C results in a decrease of enzyme activity, indicating that rVMC61 is a thermally unstable enzyme (Lee et al. 1998, 2003). Teo et al. (2003) reported that the optimum temperature for the recombinant protein pap6 from *V. harveyi* was also at 37°C. However, the optimal temperature for the metalloprotease from wild type strain of *V. anguillarum* W-1 (Chen et al. 2002b) is 50°C. This means that some biochemical characteristics of the enzyme had been modified after expression and processing in *E. coli*. The thermostability was investigated by re-incubating the enzyme in the same buffer as described in Materials and methods for 30 min and the remaining activity was determined. As shown in Fig. 2, the enzyme was stable up to 30°C, but inactivated rapidly at temperatures above this. The results in Fig. 2 also show that the enzyme was inactivated totally at 37°C. This means that the rEmpA became very unstable at temperatures higher than 30 °C. However, the enzyme activity of recombinant pap6 from *V. harveyi* is still retained over a wide temperature range of between 4 and 65°C (Teo et al. 2003). Indeed, most metalloproteases isolated from different *Vibrio* spp. showed decreased activities at temperatures above 50°C (Teo et al. 2003).

Optimum pH and pH stability

The rEmpA protease activity was measured at various pH values in buffers with the same ionic concentrations. The results in Fig. 3 show that maximum activity was observed at pH 8.0. In contrast, the maximum activity of the metalloprotease from wild type strain of *V. anguillarum* W-1 was pH 7.0 (Chen et al. 2002b) whereas optimal caseinolytic activity of Pap6 from *V. harveyi* was detected in an alkaline medium of pH 9.0 (Teo et al. 2003) and rVMC61 from *V. minicus* has maximal activity at pH 8.0 and is stable over a range of pH 6.0–10.0, retaining at least 80% of its original activity (Lee et al. 2003). To investigate pH stability of the purified enzyme, the enzyme preparation was incubated at 0°C for 30 min in various buffer solutions that had the same ionic concentrations and residual activity was measured at pH 8.0. The activity profile of the enzyme was stable just between pH 5.0 and 8.0 (Fig. 3). When pH value was higher than 8.0 or lower than 5.0, the enzyme activity was decreased significantly. These results suggest that the enzyme was sensitive to change of pH.

Effects of different cations on activity of rEmpA

As indicated in Table 2, Ca^{2+}, Na^{+} and Mg^{2+} had an activating effect on the enzyme, with Ca^{2+} showing the highest rank (139.5%). The results were identical to

Table 1 Purification of rEmpA protein

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Fold
Total cell lysate	2.08	$28.08 \pm 1.9 \times 10^4$	$13.5 \pm 0.9 \times 10^4$	100	1
Ni-NTA chromatography	0.20	$3.5 \pm 0.02 \times 10^4$	$17.5 \pm 0.1 \times 10^4$	12.46	1.29

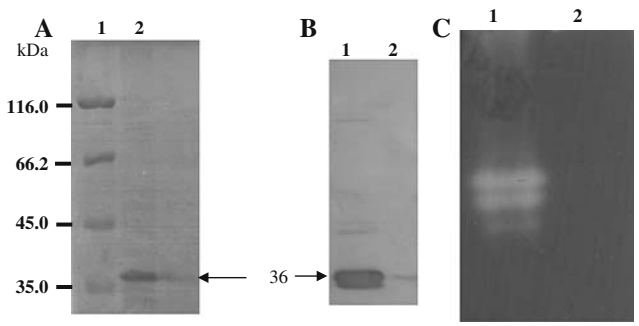

Fig. 1 SDS-PAGE, Western Blotting and gelatin-gel zymography of the purified rEmpA. **A:** SDS-PAGE analysis of the purified rEmpA through a Ni-NTA column. Electrophoresis was carried out on a 12% polyacrylamide gel. Lane 1, Molecular mass marker proteins (β-galactosidase 116.0 kDa, Bovine serum albumin 66.2 kDa, Ovalbumin 45 kDa and Lactate dehydrogenase 35 kDa); Lane 2, The purified rEmpA. **B:** Western Blotting analysis using anti-EmpA antibodies; Lane 1, Purified rEmpA; Lane 2, TOP10/pBAD24 induced by arabinose was used as the negative control. **C:** Proteolytic activity was detected using a 10% Native-PAGE with 2% (w/v) gelatin; Lane 1,Purified rEmpA; Lane 2, TOP10/pBAD24 induced by arabinose was used as the negative control

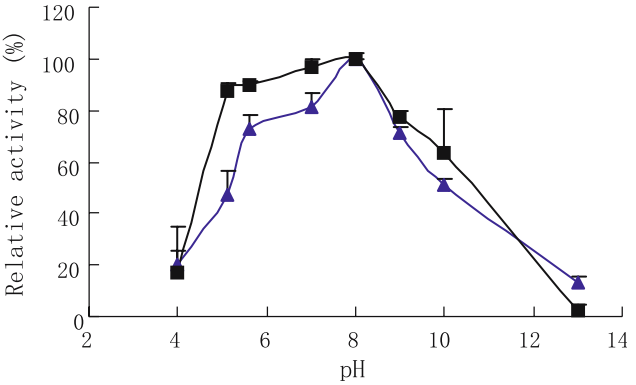

Fig. 3 Effects of pH on activity and stability of rEmpA protease. Optimal pH (▲); pH stability (■). The effect of pH on the enzyme activity was determined by incubating the purified rEmpA between pH 4.0 and 13.0 using the standard assaying condition as mentioned in Materials and methods. The pH stability was tested by 30 min pre-incubation of the purified rEmpA in appropriate buffers at different pH values ranging from 4.0 to 13.0 at 0°C. The remaining activities of rEmpA were measured immediately after this treatment with the standard method as mentioned in Materials and methods

those obtained from the purified metalloprotease of wild type of *V. anguillarum* W-1 (Chen et al. 2002b). The results demonstrate that the enzyme was Ca^{2+}-, Na^{+}- and Mg^{2+}-dependent. In contrast, Teo et al. (2003) found that the addition of Mg^{2+} (0.1–10 mM) and Ca^{2+} (0.1–10 mM) did not significantly affect enzyme activity of Pap6 from *V. harveyi*. However, $Zn^{2+}$and Cu^{2+}acted as inhibitors in decreasing the activity of rEmpA, with Cu^{2+} showing the lowest rank

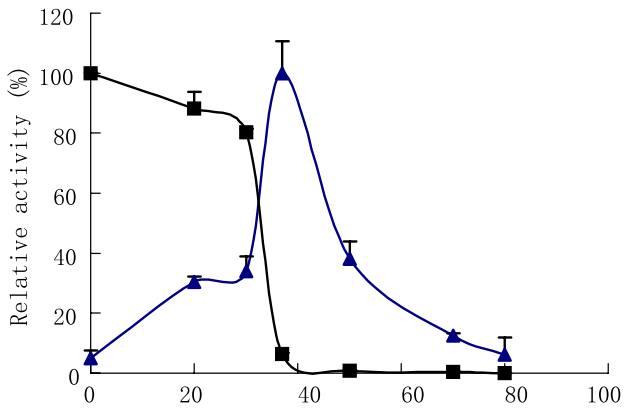

Fig. 2 Effect of temperature on the activity and stability of rEmpA. The optimal temperature (▲); the thermal stability of the enzyme (■). The optimal temperature for activity of rEmpA was determined at 0°C–80°C in the same buffer as described in Materials and methods. Temperature stability of the purified rEmpA was tested by pre-incubating the enzyme at different temperature ranging from 0°C to 80°C for 30 min, residual activity was measured as described in Materials and methods immediately. Here, pre-incubated sample at 0°C was used a reference to calculate the residual activity

(1.1%) (Table 2). These results were identical to those obtained from the purified metalloprotease of wild type of *V. anguillarum* W-1 (Chen et al. 2002b).

Effects of protein inhibitors on the activity of rEmpA

Table 3 depicts the effects observed in the presence of protein inhibitors of the enzyme. Like the metalloprotease from wild type strain of *V. anguillarum* W-1, PMSF (10 mM) had no effect on the enzyme activity. This means that serine residues were not essential for the enzyme active sites. The purified rEmpA was characterized as a zinc metalloprotease as it was inhibited by zinc and metal-specific inhibitors, such as 1,10-phenanthroline, EDTA and EGTA (Table 3). The activity of the metalloprotease from wild type strain of *V. anguillarum* W-1 was also completely inhibited by 1.0 mM of EDTA (Chen et al. 2002b). Activity was partiallly inhibited by 10 mM SDS and 10 mM urea had a weak influence on the protease activity (Table 3).

These results obtained by Teo et al. (2003) suggested that Pap6 from *V. harveyi* is also a zinc-dependent metalloprotease. They also found that the zinc-specific metal chelators 1,10-phenanthroline (10 mM) and EGTA (10 mM) strongly inhibited enzyme activity and caused an almost total loss of enzyme activity, whilst EDTA (10 mM) reduced the enzyme activity by almost 50%.

Table 2 Effect of metal ions on the activity of rEmpA

Metal ions	Concentrations (mM)	Relative activity (%)
None		100
$ZnSO_4$	0.01	102.0 ± 3.5
	0.1	97.0 ± 5.6
	1	5.9 ± 0.7
	10	4.4 ± 2.3
$CaCl_2$	10	139.5 ± 18.1
	1	109.1 ± 1.8
$MgSO_4$	10	136.0 ± 15.0
	1	107.7 ± 0.6
NaCl	10	112.1 ± 10
	1	108.6 ± 0.5
$CuSO_4$	10	1.1 ± 0.5
	1	98.3 ± 0.7

The enzyme activity assay was performed in the reaction mixture as described in Materials and methods with various metal ions, respectively. The relative activity assayed in the absence of metal ions was regarded as 100%

Substrate specificity of rEmpA

Digestion of various protein substrates by the recombinant rEmpA was examined. The results in Fig. 4 show that rEmpA was able to degrade collagen, gelatin, shrimp proteins and casein (Fig. 4A–D). These results imply that the rEmpA may result in destructive tissue damage in marine animals (Denkin and Nelson 2004). This means that the recombinant *V. anguillarum* metalloprotease was still able to use collagen, gelatin, shrimp protein and casein as substrates and may play the potential role in pathogenesis in marine animals.

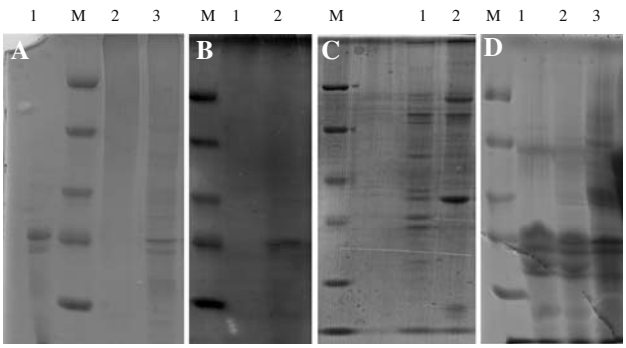

Fig. 4 Analysis of protein digestion by rEmpA. Each substrate (50 μg) was incubated in the absence or in the presence of rEmpA at 37°C for 4 h. **A:** Lane1, purified rEmpA; lane M, molecular weight protein marker; lane 2, collagen; lane 3, incubated collagen with rEmpA; **B:** lane 1, gelatin; lane 2, incubated gelatin with rEmpA; **C:** lane 1, incubated shrimp protein with rEmpA; lane 2, shrimp protein. **D:** lane 1, lane 2, incubated casein with rEmpA; lane 3, casein

Table 3 Effect of protein inhibitors on the activity of rEmpA

Inhibitors	Concentrations (mM)	Relative activity (%)
None		100
1,10-Phenanthroline	10	17.4 ± 4.3
EDTA	10	36.6 ± 5.5
EGTA	10	13 ± 2.5
PMSF	10	101 ± 1.2
SDS	10	65 ± 0.2
Urea	10	96.8 ± 2.3

The purified enzyme was pre-incubated with the respective compounds for 30 min at 0°C, followed by the standard enzyme assay as described in Materials and methods. The relative activity assayed in the absence of the protein inhibitors was regarded as 100%

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