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Isolation of a cDNA Encoding a Protease from *Perinereis aibuhitensis* Grube

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Abstract The cDNA encoding a protease of *Perinereis aibuhitensis* Grube (PPA) was cloned. The deduced amino acid sequence analysis showed that the protein had 49% identity to the C-terminal amino acid 169–246 of serine protease of *Heterodera glycines*. Northern blotting analysis indicated that the cDNA could hybridize with mRNA of approximately 260 bases isolated from the marine earthworm. The cDNA was amplified by polymerase chain reaction and cloned into pMAL-p2 to construct expression vector pMAL-PPA. pMAL-PPA was introduced into *Escherichia coli* BL21(DE3) and overexpression of PPA fused with maltose binding protein was achieved by isopropyl-β-*D*-thiogalactopyranoside induction. The fusion protein was purified by affinity chromatography on an amylose resin column and ion-exchange chromatography on a diethylaminoethyl-Sepharose 4B column. Rabbits were immunized with the purified protein and antiserum was prepared. The antibody could react with a protein of approximately 9 kDa extracted from the marine earthworm as shown by Western blotting analysis. The activity analysis of the recombinant PPA suggested that it was probably a plasminogen activator.

Key words cDNA; protease; *Perinereis aibuhitensis* Grube

Proteases form a group of enzymes that specialize in the cleavage of peptide bonds, and are found in diverse organisms such as prokaryotes, plants, animals and viruses. Some of the proteases are serine endoproteases that might participate in a number of different physiological functions, such as coagulation, cellular and humoral immunity, fibrinolysis, embryonic development and digestion [1]. Those proteases participating in the digestion process are referred to as trypsins (EC 3.4.21.4) [2].

Perinereis aibuhitensis Grube is a kind of marine clamworm widely distributed along the seaside in Asia. It has been used in Chinese traditional herbal medicine for hundreds of years and as fish bait. Much attention has been focused on the clamworm in recent years because several bioactive peptides have been isolated from it. Pan *et al.* purified a new antimicrobial peptide called perinerin and studied its biochemical properties [3]. The giant hemoglobin from the marine polychaete *P. aibuhitensis* was extensively studied by Tsuneshige *et al*`[4]. A fibrinolytic protein consisting of two chains with a molecular weight of 47.4 kDa and a pI of 4.5 was isolated from *P. aibuhitensis* as reported by Tan *et al.* [5]. As several other proteins with fibrinolytic activity have also been purified from earthworms belonging to the same family as marine earthworm [6–8], *P. aibuhitensis* might be a new source of thrombolytic agents.

Thrombosis is one of the most widely occurring diseases that often causes disability and death. Thrombolysis is an effective way to treat this kind of disease. All of the currently used thrombolytic agents are plasminogen activators, serine proteases that are very efficient in restoring the blood flow [9]. Despite the widespread use of established thrombolytic agents, such as streptokinase, tissue-type plasminogen activator and urokinase-type plasminogen activator, these agents have a number of inadequacies, including resistance to reperfusion, occurrence of coronary reocclusion and bleeding complications. The quest continues for new plasminogen activators with higher potency, more specific thrombolytic activity and fibrin selectivity, and longer half-life [10,11].

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In this article, a cDNA presumed to encode a small protease (PPA) which might be a new plasminogen activator was cloned from the *Perinereis aibuhitensis* Grube clamworm digestive tract and sequenced. The cDNA amplified by polymerase chain reaction (PCR) was cloned into an expression plasmid. The recombinant protein PPA fused with maltose-binding protein (MBP) was expressed in *Escherichia coli*, and the recombinant protein was purified to ascertain the biological activities of PPA.

Materials and Methods

Clamworm and bacterial strains

P. aibuhitensis was collected in the Jiaozhou Bay (Qingdao, China) in June 2004. *E. coli* strain JM109 (Promega, Madison, USA) was used for subcloning and strain BL21(DE3) (Invitrogen, Grand Island, USA) was used for protein expression. *E. coli* was grown in Luria broth (LB) medium with 100 μg/ml ampicillin.

cDNA cloning and sequencing

mRNA isolation from the epithelial cells of clamworm digestive tract and reverse transcription were carried out as described previously [12]. According to the amino acid sequence in the conserved domain of serine proteases published, a cDNA fragment was amplified by PCR using two primers, P1 (5′-GGTGACTCYGGYGGCCCT-3′) and P2 (5′-TTTTTTTTTTTTTTTTTTTT-3′), and the PCR product was cloned into pGEM-T and sequenced. According to the sequence, the 5′ upstream sequence was amplified using 5′ rapid amplification of cDNA ends according to the manufacturer's instructions (Invitrogen) using the primers P3 (5′-AAAGTCGACTTATTGCATGACACTG-3′) and P4 (5′-CGACTGGAGCACGAGGACACTGA-3′). The full-length open reading frame of the cDNA for cloning into the expression plasmid was further amplified with the two primers P5 (5′-AAAGAATTCATGTCTGACGCGGAAG-3′) and P3 using total cDNA as the template. The cycle program used was as follows: 94 ºC for 45 s, 52 ºC for 45 s and 72 ºC for 1 min. PCR product was separated by agarose gel electrophoresis, and the corresponding band (approximately 260 bp) was recovered using an Agarose gel DNA fragment recovery kit (TaKaRa, Dalian, China) and ligated into pGEM-T (Promega) to construct pGEM-TPPA. Those positive colonies were selected by restriction enzyme digestion with *Eco*RI and *Sal*I. The inserted DNA fragment was further confirmed by sequencing using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Courtaboeuf, France).

Northern blotting analysis

Total RNA (2 μg) from epithelial cells of clamworm digestive tract was separated in a denatured agarose gel and transferred to nylon Hybond N^+ membranes (Amersham Biosciences, Piscataway, USA). DNA probes for Northern blotting were ^{32}P-labeled PPA gene amplified by PCR, and hybridization was carried out at 55 ºC. Other procedures of Northern blotting were according to the previous method [13].

Construction of expression vector

The DNA fragment was cut down from pGEM-TPPA with *Eco*RI and *Sal*I, and ligated into pMAL-p2 (New England Biolabs, Ipswich, USA) lineared with the same enzymes to construct recombinant plasmid pMAL-PPA. Positive colonies were ascertained by digestion with *Eco*RI and *Sal*I.

Expression and purification

Plasmid pMAL-PPA was introduced into *E. coli* BL21 (DE3). One transformed colony was inoculated into 50 ml LB medium containing 100 μg/ml ampicillin and shaken vigorously overnight at 37 ºC. The culture was transferred into a 4 liter flask containing 1 liter of rich LB medium (New England Biolabs) supplemented with 100 μg/ml ampicillin and cultured for 3 h in the same way. The culture temperature was then cooled to 30 ºC and isopropyl-β-*D*-thiogalactopyranoside was added to a final concentration of 0.5 mM.

The culture was shaken vigorously at 30 ºC for 6 h and the cells were harvested by centrifugation. Cell pellets were washed with ice-chilled column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM EDTA, 5 mM β-mercaptoethanol) and resuspended in the same buffer. The cells were then sonicated at 0 ºC 30 times, 10 s each time, with a 45 s interval. All the following operations were carried out at 4 ºC. The cell extract was centrifuged at 20,000 *g* for 30 min, and the recombinant protein in the supernatant was absorbed onto an amylose resin column (1 cm×10 cm). After the column was washed with the 100 ml column buffer, the MBP-PPA on the column was eluted with 20 ml elution buffer (column buffer supplemented with 10 mM maltose). The elution was diluted to 100 ml with TSE buffer (20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 5 mM EDTA, 5 mM β-mercaptoethanol) and applied to a diethylaminoethyl (DEAE)-Sepharose 4B column (2 cm×15 cm). The column was eluted with NaCl gradient (from 20 to 1000 mM) in TSE buffer at a flow rate of 0.5 ml/min and 1 ml

fraction was collected. The proteins in each fraction were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Assay of caseinolytic activity

Caseinolytic activity has been widely used to measure the activity of plasminogen activators [14]. For the determination of caseinolytic activity of the purified recombinant protein, 9 ml of TS buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 100 mg agarose and 100 mg skim milk was boiled for 2 min and cooled to 42 ºC, then 50 μl of TS buffer containing 2 mg/ml human plasminogen was added and poured down to a plate. In solidified agarose gel in a plastic dish, 3 mm apertures were holed out and various different samples were placed in them. After incubation for at least 6 h at 37 ºC, the clear zone around the small aperture was estimated for the caseinolytic activity.

To determine the substrate specificity of PPA toward plasminogen, an agarose gel plate was prepared as described above without plasminogen. Samples of MBP-PPA, plasminogen, Factor Xa (Sigma, St. Louis, USA), mixture of MBP-PPA and plasminogen, and mixture of MBP-PPA, Factor Xa and plasminogen were placed into the small apertures. The plate was then incubated at 37 ºC for 6 h, and the clear zone around the small apertures was estimated for the caseinolytic activity.

Preparation of antiserum against MBP-PPA

One Japanese adult male rabbit was immunized with a mixture of 150 μg MBP-PPA and the same volume of complete Freund's adjuvant. The rabbit was again immunized with the same mixture but with only 100 μg MBP-PPA after three weeks. Ten days later, the blood serum was collected from the rabbit's carotid artery.

Western blotting analysis

One gram of *P. aibuhitensis* was mixed with 10 ml TE buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, and 1 mM dithiothreitol) and homogenized with a glass pestle on ice. The homogenate was then centrifuged at 15,000 *g* for 30 min at 4 ºC, and 5 μl of the supernatant was mixed with the same volume of 2×SDS-PAGE loading buffer and boiled for 5 min. After the sample was separated by SDS-PAGE with 18% separating gel, the proteins in the gel were transferred onto polyvinyldifluoridine membranes (Pall, Ann Arbor, USA). The following operation was processed according to the standard protocol using antisera against MBP (New England Biolabs) and MBP-PPA, respectively. Horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulin G was used as the secondary antibody [15].

Results

Cloning of the open reading frame encoding a serine protease

A DNA band of approximately 260 bp was amplified by reverse transcription-PCR [**Fig. 1(A)**]. PCR product was directly cloned into pGEM-T, confirmed by restriction endonuclease digestion and sequencing. The nucleotide and deduced amino acid sequence are shown in **Fig. 2**. The inserted DNA cut down from pGEM-TPPA was cloned into expression vector pMAL-p2 and those positive clones were also confirmed by endonuclease digestion [**Fig. 1 (B)**].

Protein sequence alignment of PPA and serine protease of *Heterodera glycines*

Protein sequence alignment of PPA indicated that this

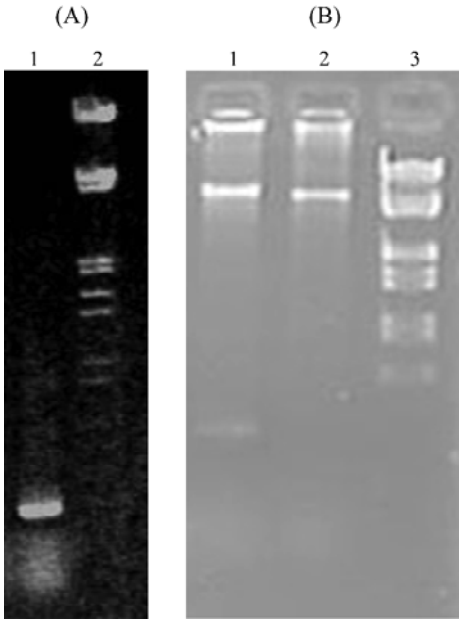

Fig. 1 Polymerase chain reaction (PCR) amplification of the cDNA encoding a serine protease of *Perinereis aibuhitensis* Grube and identification of the positive clone by endonucleases

(A) Total mRNA was extracted from clamworm digestive tract and reverse-transcripted into cDNA. The cDNA was used as the template to amplify the protease gene. 1, PCR product; 2, λDNA/*Eco*RI+*Hin*dIII marker. (B) PCR product cut with *Eco*RI and *Sal*I was cloned into pMAL-p2 lineared with the same endonucleases to construct expression vector pMAL-PPA. 1, pMAL-PPA/*Eco*RI+*Sal*I; 2, pMAL-p2/*Eco*RI+*Sal*I; 3, λDNA/*Eco*RI+*Hin*dIII marker.

```
atggaggtaactgtgttctctcgctctgaatgtgaaacttttggggcagttccatcaatgatggccat
 M  E  V  T  V  F  S  R  S  E  C  E  T  F  W  G  S  S  I  N  D  G  H
gtctgtgtcggtgtcattggctctgctggtgcctgtaacggagactccggtggccctctggcagcatct
 V  C  V  G  V  I  G  S  A  G  A  C  N  G  D  S  G  G  P  L  A  A  S
gaaaaactggttggcgtgacatcatttggactcagtggatgcttcaccagtcgcccatccgtgtacagc
 E  K  L  V  G  V  T  S  F  G  L  S  G  C  F  T  S  R  P  S  V  Y  S
agcattgagaacttcagggagttcatcgacagtgtcatgcaa
 S  I  E  N  F  R  E  F  I  D  S  V  M  Q
```

Fig. 2 Cloned cDNA sequence encoding a serine protease of *Perinereis aibuhitensis* Grube and deduced amino acid sequence

protein was homologous to the C-terminal domains of several serine proteases. This peptide possessed 49% identity to the C-terminal amino acid 169–246 of serine protease of *H. glycines* (SPHG) (**Fig. 3**) [16]. For the positive amino acids, the similarity was up to 65%. There were four cysteine residues encoded by the cloned cDNA, and each had its counterpart in the C-terminal domain of the above serine protease.

```
PPA    3   VTVFSRSECETFWGSS-INDGHVCVGVIGSAGACNGDSGGPLAASEKLVGVTSFGLSGCF   61
SPHG 169   MTVLTNSNCASRWSAAQINNGHICVSS-SSVSACSGDSGGPLVCGSTLVGATSWGQAQCN  227
PPA   62   TSRPSVYSSIENFREFIDS                                            80
SPHG 228   PSYPSVYTRISYFRSWIDS                                           246
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Fig. 3 Protein sequence alignment of a protease of *Perinereis aibuhitensis* Grube (PPA) and serine protease of *Heterodera glycines* (SPHG)

Northern blotting analysis

In order to confirm that the cloned cDNA was indeed an intact gene transcript and not a degraded cDNA fragment, total RNA from the epithelial cells of clamworm digestive tract was analyzed by Northern blotting. The results showed that the probe could hybridize with RNA of 260 bases, and no larger RNA molecules could be detected (**Fig. 4**). It could be inferred from these results that the small cDNA was indeed reverse-transcripted from an intact mRNA molecule.

Protein expression and purification

E. coli BL21(DE3) cells harboring the pMAL-PPA induced by isopropyl-β-*D*-thiogalactopyranoside could overexpress the MBP-PPA. Gel scanner analysis indicated that the amount of recombinant protein could be up to 68% of the total proteins in cells [**Fig. 5(A)**]. Further research results showed that most of the recombinant protein appeared in soluble form and only a very small part in

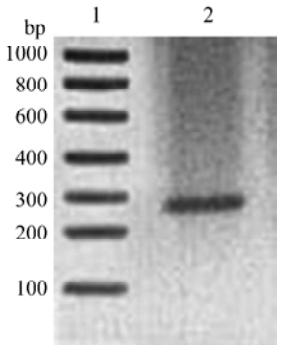

Fig. 4 Northern blot analysis of the total RNA from the epithelial cells of digestive tract of *Perinereis aibuhitensis* Grube

1, ^{32}P-labeled RNA ladder; 2, total RNA.

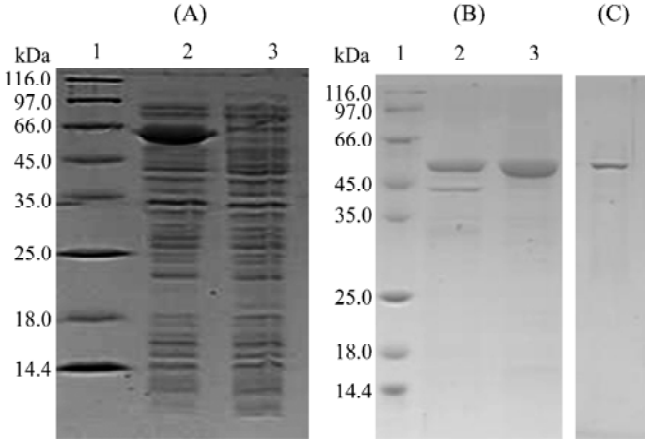

Fig. 5 Overexpression, purification and detection of the protease of *Perinereis aibuhitensis* fused with maltose binding protein (MBP-PPA) by Western blotting in *Escherichia coli* BL21 (DE3)

(A) Isopropyl-β-*D*-thiogalactopyranoside induced overexpression of MBP-PPA in *E. coli*. 1, protein marker; 2, total proteins of *E. coli* BL21(DE3) expressing MBP-PPA; 3, total proteins of *E. coli* BL21(DE3). (B) Purification of MBP-PPA through chromatography on amylose resin column and diethylaminoethyl (DEAE)-Sepharose 4B column. 1, protein marker; 2, proteins eluted from amylose resin column; 3, MBP-PPA eluted from DEAE-Sepharose 4B column. (C) Detection of MBP-PPA by Western blotting.

inclusion bodies (data not shown). After breakage of the cells and removal of insoluble materials of the cell extracts by high-speed centrifugation, the recombinant MBP-PPA in the supernatant was purified through chromatography on an amylose resin column followed by a DEAE-Sepharose 4B column. The protein eluted from the ion-exchange chromatography was homogeneous in SDS-PAGE [**Fig. 5(B)**, lane 3] and had an apparent molecular weight of approximately 51 kDa [**Fig. 5(B)**]. In order to confirm the

eluted protein contained MBP, the protein was analyzed by Western blotting using antiserum against recombinant MBP. **Fig. 5(C)** shows that the purified protein was indeed an MBP fused protein and its molecular weight was much larger than MBP alone (42 kDa).

Western blotting analysis of proteins in *P. aibuhitensis*

To ascertain the actual molecular weight of PPA *in vivo*, total proteins of the clamworm were analyzed by Western blotting. The results showed that two positive bands were detected (**Fig. 6**). One band was very weak with a molecular weight of approximately 30 kDa, whereas the other was very clear with an apparent molecular weight of approximately 9 kDa (**Fig. 6**). The molecular weight of the small band was the same as that of the peptide deduced from the cloned cDNA.

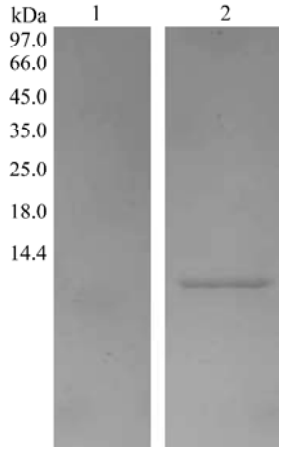

Fig. 6 Western blotting analysis of proteins in a protease of *Perinereis aibuhitensis* Grube (PPA)

Total proteins extracted from *P. aibuhitensis* were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred onto polyvinyldifluoridine membranes. Proteins were detected using antiserums raised against maltose-binding protein (MBP) and MBP-PPA. 1, detected result using antiserum against MBP; 2, detected result using antiserum against MBP-PPA.

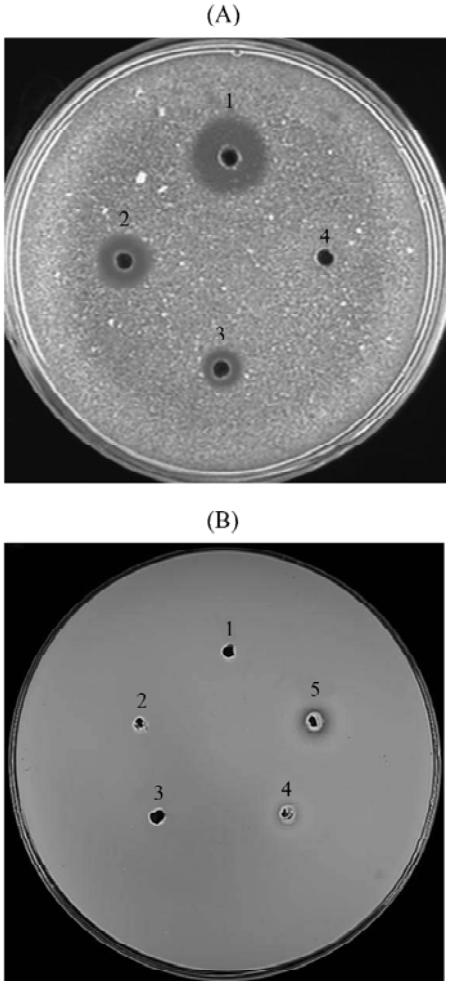

Fig. 7 Comparative analysis of caseinolytic activity of combinations of a protease of *Perinereis aibuhitensis* Grube (PPA), maltose-binding protein (MBP), human plasminogen and Factor Xa

Various samples were put into the small apertures in agarose gel plates with (A) or without (B) plasminogen, and the plates were incubated at 37 ºC for 8 h (A) or 6 h (B). (A) 1, 10 μg recombinant streptokinase; 2, 50 μg recombinant MBP-PPA and 1 unit of Factor Xa; 3, 50 μg recombinant MBP-PPA; 4, 50 μg recombinant MBP. (B) 1, 1 unit of Factor Xa; 2, 2 μl 2 mg/ml human plasminogen; 3, 50 μg recombinant MBP-PPA; 4, 50 μg MBP-PPA and 2 μl 2 mg/ml human plasminogen; 5, 50 μg MBP-PPA, 1 unit of Factor Xa and 2 μl 2 mg/ml human plasminogen.

Caseinolytic activity of the purified protein

The caseinolytic activities of MBP-PPA and the digested product by Factor Xa were assayed with skim milk plates, which were incubated at 37 ºC for at least 6 h. The activities could be detected by the formation of transparent plaques around the wells filled with active proteins. The results showed that both MBP-PPA [**Fig. 7(A)**, sample 3] and MBP-PPA digested by Factor Xa [**Fig. 7(A)**, sample 2] could produce transparent plaques, and the plasminogen activating activity of digested MBP-PPA using Factor Xa was larger than that of intact MBP-PPA. Comparing the activities of digested MBP-PPA with recombinant streptokinase, we could see that the former showed a much lower activity.

To determine the substrate specificity of PPA to plasminogen, we checked the caseinolytic activities of various samples with or without plasminogen. Neither plasminogen alone [**Fig. 7(B)**, sample 2] nor MBP-PPA alone [**Fig. 7(B)**, sample 3] had caseinolytic activity, but

the mixture of the two proteins did [**Fig. 7(B)**, sample 4]. As a protease of MBP-PPA, Factor Xa alone could not degrade casein [**Fig. 7(B)**, sample 1], but could promote the caseinolytic activity of the mixture of MBP-PPA and plasminogen [**Fig. 7(B)**, sample 5]. From these results, we suspected that PPA could specifically activate plasminogen and degrade casein in the agarose gel.

Discussion

To date, a large number of serine proteases with fibrinolytic activity have been found. Many of them have been used in the prevention and treatment of cardiac and cerebrovascular diseases. However, some fibrinolytic proteases are restricted to narrow medicinal uses because of their immunoreactions and short half-life. Usually, the peptides with a smaller apparent molecular weight are less immunogenic than larger proteins. Therefore, the cloning of genes encoding fibrinolytic peptides with low molecular weight would offer some clues to finding new fibrinolytic agents.

In this paper, a short cDNA was cloned and expressed as an MBP-fused protein. The fusion protein showed fibrinolytic activity, although its activity was much lower compared with that of recombinant streptokinase. In our previous study, the gene was expressed in *E. coli* with or without a short His-tag, but both of the expressed products appeared in inclusion bodies (data not shown). In order to get soluble and active recombinant proteins, various refolding measures were taken, but none of them really worked (data not shown). Therefore, it is suspected that the natural form of PPA in *P. aibuhitensis* might be glycosylated.

PPA deduced from the cloned cDNA lacks an N-terminal domain of most serine proteases from other organisms [17]. We initially suspected that we had only cloned a partial cDNA sequence encoding the C-terminal domain of a protease, but the results of Western blotting analysis from this study indicated that there indeed existed a small peptide of 9 kDa *in vivo* (**Fig. 6**). At present, the exact physiological functions in *P. aibuhitensis* are not clear. This study will offer some clues to find peptides of small molecular weights, which might act as new plasminogen activators, and a basis for further investigation to identify new thrombolytic agents with high biological activity by site-directed mutagenesis.

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