



An anticoagulant serine protease from the wasp venom of *Vespa magnifica*[☆]

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Abstract

Wasp is an important venomous animal that can induce human fatalities. Coagulopathy is a clinical symptom after massive wasp stings, but the reason leading to the envenomation manifestation is still not known. In this paper, a toxin protein is purified and characterized by Sephadex G-75 gel filtration, CM-Sephadex C-25 cationic exchange and fast protein liquid chromatography (FPLC) from the venom of the wasp, *Vespa magnifica* (Smith). This protein, named magnvesin, contains serine protease-like activity and inhibits blood coagulation. The cDNA encoding magnvesin is cloned from the venom sac cDNA library of the wasp. The deduced protein from the cDNA is composed of 305 amino acid residues. Magnvesin shares 52% identity with allergen serine protease from the wasp *Polistes dominulus*. Magnvesin exerted its anti-coagulant function by hydrolyzing coagulant factors TF, VII, VIII, IX and X.

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1. Introduction

The Vespidae family includes hornets (genera *Vespa* and *Dolichovespula*), yellow jackets (genus *Vespula*) and paper wasps (genus *Polistes*). They possess highly toxic venom, which is rich in toxins, enzymes and biologically active peptides (Habermann, 1972; Nakajima, 1984). They are important venomous animal endangering human life, causing fatalities in serious cases. Vespidae venoms have similar venom compositions. The clinical symptoms induced by wasp stings include local reactions (such

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as pain, wheal, edema and swelling), immunological reactions usually leading to anaphylaxis with subsequent anaphylactic shock and systemic toxic reactions caused by large doses of venom, resulting in hemolysis, coagulopathy, rhabdomyolysis, acute renal failure, hepatotoxicity, aortic thrombosis and cerebral infarction (Chao and Lee, 1999; Chen et al., 2004; Evans and Summers, 1986; Korman et al., 1990; Sakhuja et al., 1988; Watemberg et al., 1995). Local reactions may result from biologically active peptides such as bradykinin-like peptides, chemotactic peptides and mastoparans. Allergic reactions induced by wasp venoms have been extensively focused on and studied. The major wasp venom allergens are phospholipase A₁, hyaluronidase, antigen 5 and serine proteases (Habermann, 1972; Nakajima, 1984; Asgari et al., 2003; Yamamoto et al., 2007; Yang et al., 2007). The anticoagulant effect of wasp venom sac extract (VSE) has been reported in several literatures. It is suggested that the anticoagulant effect of wasp VSE was attributed to a proteolytic process involving mainly coagulation factors VIII and IX (Kornberg et al., 1988; Haim et al., 1999). The venom component acting on the coagulation process from wasp has not been characterized still, although a proteolytic enzyme named protease I with anticoagulant activity has been purified from *Vespa orientalis* venom (Haim et al., 1999).

Serine proteinases belong to the trypsin family S1 of clan SA, the largest family of peptidases (Serrano and Maroun, 2005; Halfon and Craik, 1998). Many serine proteases have been found from animal venoms, especially from snake venoms. They can affect the hemostatic system by acting on a variety of components of the coagulation cascade, on the fibrinolytic and kallikrein-kinin systems and on cells (Seegers and Ouyang, 1979; Markland, 1997; Pirkle, 1998). Some serine proteases with allergic or melanization-inhibitory activity have been found from the wasp venom (Asgari et al., 2003; Winningham et al., 2004). No serine protease acting on the hemostatic system has been characterized from wasp venoms. In the current work, an anticoagulant serine protease was purified and characterized from the wasp venom of *Vespa magnifica* (Smith).

2. Materials and methods

2.1. Wasp venom

The wasps *V. magnifica* were collected in Yunan, China. The collected wasps were stimulated by

alternative current (6 V) lasting for 6–10 s. The wasp venom was secreted onto a clean glass plate (50 × 50 cm²), immediately collected and stored at –20 °C.

2.2. Protein purification

Lyophilized wasp venom samples of *V. magnifica* (0.3 g) were dissolved in 10 ml 0.1 M phosphate buffer solution (PBS), pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-75 (Superfine, Amersham Biosciences, 2.6 × 100 cm²) gel filtration column equilibrated with 0.1 M PBS, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml as the purification procedure of the phospholipase A₁ from *V. magnifica* venoms (Yang et al., 2007). The absorbance of the elute was monitored at 280 nm. The anticoagulant activities of fractions were determined as indicated below. The protein peak containing anticoagulant activity was pooled (45 ml) and lyophilized, and re-suspended in 5 ml 0.05 M PBS, pH 6.5, and further dialyzed against 0.05 M PBS (pH 6.5). The product was consequently subject to the cation-exchange CM-Sephadex C-25 column (Amersham Biosciences, 1.6 × 30 cm²). The elution was achieved with a linear NaCl gradient in 0.05 M PBS, pH 6.0, at a flow rate of 1 ml/min as the purification procedure of the phospholipase A₁ from *V. magnifica* venoms (Yang et al., 2007). The peak with anticoagulant activity was collected and finally purified on an AKTA Mono S (1 ml volume, Amersham Biosciences) cationic exchange column equilibrated with 20 mM phosphate buffer solution, pH 6.5, using fast protein liquid chromatography (FPLC, Amersham Biosciences), equilibrated with 0.02 M PBS, pH 6.5. The elution was performed with a linear NaCl gradient in 0.02 M PBS, pH 6.5, at a flow rate of 1 ml/min as illustrated in Fig. 1.

2.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

About 5 µg of purified protein from FPLC was mixed with 10 µl of SDS sample buffer and boiled for 5 min before being loaded onto a 15% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue and destained to allow visualization of the protein (Laemmli, 1970).

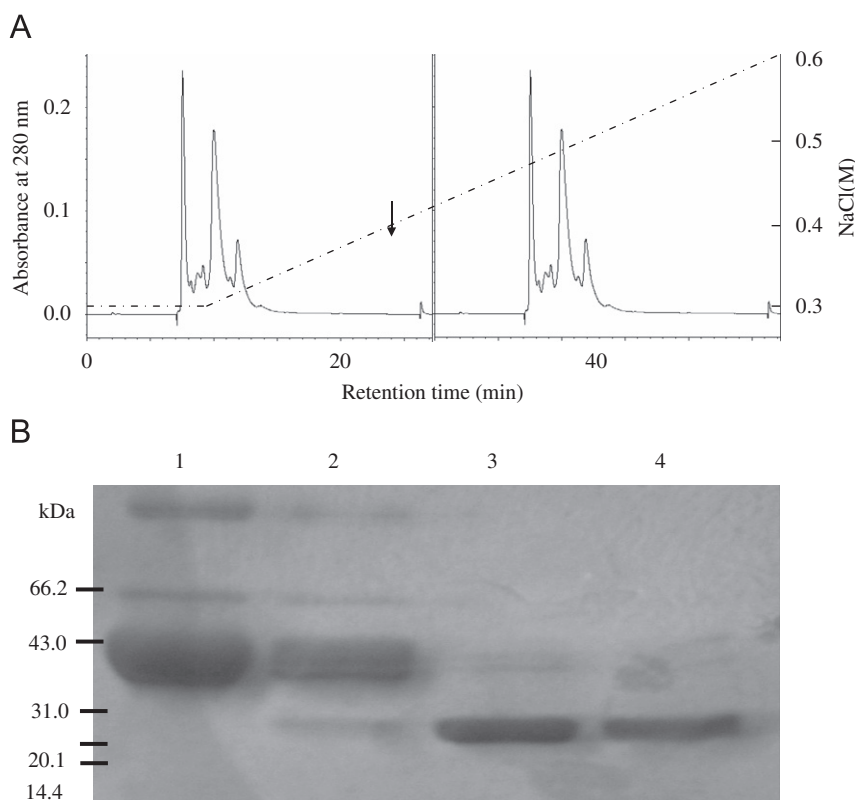


Fig. 1. Purification of magnvesin from *V. magnifica* venom and its SDS-PAGE. (A) The peak with serine protease activity from CM-Sephadex C-25 was applied to Mono S (1 ml volume, Amersham Biosciences) cationic exchange column equilibrated with 20 mM phosphate buffer solution, pH 6.5, using fast protein liquid chromatography (FPLC, Amersham Biosciences), equilibrated with 0.02 M PBS, pH 6.5. The elution was performed with a linear NaCl gradient in 0.02 M PBS, pH 6.5, at a flow rate of 1 ml/min. (B) SDS-PAGE analysis of magnvesin. Lane 1, the peak II of wasp venom from Sephadex G-75 gel filtration (reduction); Lane 2, the last peak from CM-Sephadex C-25 cationic exchange (reduction); Lane 3, purified magnvesin (non-reduction); Lane 4, purified magnvesin (reduction).

2.4. Protein quantification

The protein concentration was determined by a protein assay kit (Bio-Rad, Hercules, CA) with BSA as standard according to the manufacture's instructions.

2.5. Protein sequencing

The amino acid sequences of the N-terminus and partial interior amino acid fragments recovered from the trypsin hydrolysis were determined by the automated Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491.

2.6. Serine protease activity assay

The sample on the hydrolysis of synthetic chromogenic substrates was assayed in 50 mM Tris-HCl, pH 7.8, for substrates S-2238 (H-D-Phe-

Pip-Arg-pNA, Kabi Vitrum, Stockholm, Sweden), S-2302 (H-D-Pro-Phe-Arg-pNA, Kabi Vitrum, Stockholm, Sweden), S-4760 (*N*-Succinyl-Ala-Ala-pNA, Sigma), BTEE (*N*-benzoyl-tyrosine ethyl ester, Sigma), or pH 8.45 for the substrate C-3022 (*N*-CBZ-Gly-Gly-Leu-pNA, Sigma), buffer at 37 °C. The reaction was initiated by the addition of the substrate with a final concentration of 0.5 mM. The formation of *p*-nitroaniline was monitored continuously at 405 nm (for S-2238, S-2302, and S-4760) or 410 nm (for C-3022) or 253 nm (for BTEE) for 2 min.

2.7. SMART cDNA synthesis and clone screening

The total RNA of fresh *V. magnifica* venomous glands was extracted using the TRIzol method (Life Technologies). A SMARTTM PCR cDNA synthesis kit (Clontech) was used to synthesize the cDNA. The first-strand was synthesized with the SMARTTM

IV oligonucleotide, 5'-AAGCAGTGGTATCAA-CGCAGAGTGGCCATTACGGCCGGG-3', and the CDS III/3' PCR primer 5'-ATTCTAGAGGCC-GAGGCGGCCGACATG- d(T)30N₋₁N-3' (N = A, G, C, or T; N₋₁ = A, G or C). The second strand was amplified by using Advantage polymerase and 5' PCR primer IIA, 5'-AAGCAGTGGTATCA-ACGCAGAGT- 3'.

The PCR products were then cloned into pGEM[®]-T Easy vector (Promega), producing a library of about 1.8×10^5 independent colonies. Two primers (a degenerate primer, 5'-At(C/T/A)Gt(A/T/C/G)Gg(A/T/C/G)Gg(A/T/C/G)Gt(A/T/C/G)Ga(A/G)Ac(A/T/C/G)-3' designed according to the sequence determined by Edman degradation and a reverse primer 5'-ATTCTAGAGGCCGA-GGCGGCCGACATG-3' based on the adopter sequence in the cDNA library) were used to screen the magnvesin encoding sequence. The PCR conditions were 2 min at 95 °C, followed by 35 cycles of 10 s at 94 °C, 30 s at 55 °C and 40 s at 72 °C. DNAs extracted from the positive clones were sequenced.

2.8. Anticoagulant assay

Human citrated whole blood was provided by Kunming Blood Center of Yunnan province. Platelet-poor plasma (PPP) was prepared by centrifuging the citrated whole blood twice at 2500g for 15 min at 4 °C and used within 4 h. For the recalcification time assay, certain amount of sample in 0.1 ml 0.9% NaCl was added to 0.1 ml PPP at 37 °C, and the mixture was incubated at 37 °C for 5 min. The time from the addition of 0.1 ml of 25 mM CaCl₂ to the appearance of the first clot was recorded. The plasma aliquots incubated only with 0.1 ml of 0.9% NaCl were tested as controls.

2.9. The in vitro effect of various concentrations of magnvesin on coagulation factors in normal human plasma

Enzyme linked immunosorbent assay (ELISA) kits for coagulant factors are from Unibiolab Beijing, China (Tissue Factor, TF), Assaypro, USA (Factor VIII) and ADI, USA (Factors VII, IX, X), respectively. According to the methods described by Kornberg et al., different concentrations of magnvesin was incubated in normal human plasma at 37 °C for 60 min. Coagulant factors were assayed using ELISA kits according to the manufacturer's instructions.

3. Results

3.1. Purification of serine protease

The wasp venom was divided into four fractions by Sephadex G-75. The peak II (tubes 54–64) was found to exert serine protease activity, and the platelet aggregation and the phospholipase A₁ activities were also concentrated on this peak (Yang et al., 2007). This peak was applied to the CM-Sephadex C-25, eluting with a linear NaCl gradient in 0.05 M PBS, pH 6.0. Five fractions were eluted from CM-Sephadex C-25 cationic exchange chromatography as our previous report (Yang et al., 2007). The last fraction was found to exert serine protease activity. The last fraction was collected and purified by AKTA Mono S cationic exchange column under the conditions indicated in Fig. 1A, and the purified serine protease was indicated by an arrow.

3.2. SDS-PAGE analysis and partial amino acid sequencing

Purified serine protease was named 'magnvesin'. Fig. 1B shows the SDS-PAGE analysis of the purified magnvesin. SDS-PAGE was performed under non-reducing conditions and reducing conditions (5% β-mercaptoethanol). It appeared as a homogeneous protein in both conditions. The purified magnvesin appeared as a single band in both reduced and unreduced conditions, and therefore was thought to consist of a single polypeptide chain of approx. 28 kDa. The N-terminal sequence and several interior sequences were analyzed by automated Edman degradation. Their amino sequences are illustrated in Fig. 2.

3.3. cDNA cloning

Several positive clones, which contained an insert of around 1250 bp, were identified and isolated from the venom gland cDNA library of *V. magnifica*. The complete nucleotide sequence of cDNA (GenBank accession EU267370) and deduced amino acid sequence are shown in Fig. 2. The cDNA encoding protein precursor was composed of 305 amino acid (aa) residues, including a mature magnvesin of 242 aa with a predicted isoelectric point (pI) of 8.9 and a molecular weight of 27.4 kDa. The mature magnvesin has nine half-cystines. The amino acid sequence of mature magnvesin deduced from the cDNA sequence was found to match well with the amino sequence of

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atgaaagtc aaagtcgacgatgacatcgagtagcttttctgtggattaaattcggttcacagtggaatcgatagcatcgaaatgactataaaattc 96
M K V K V D D D I E Y V F C G L N S F T V E S I A S K M T I K F 32
cattcgagatacaactatggaggcaaatctcgatgtattctcagagtagtcaaaggagaatgtagtggttggagaatccgtcgagaatc 192
H S R Y N T Y G G K F R C I L R V V K E E C R C G W K N P S R [I] 64
gtaggcggtgtgaaacaggagtaaatgaatatcctatgatggctggtatagtagcatcttgatacacgttttctttattgttggtgcaactataata 288
[V G G V E T G V N E Y P M M A G] I V H L D T R F L Y C G A T I I 96
actccacaacatgtattaaacggcggtcattgcgttggtgtataaaaagagtttatatttttaggagttattgttggaacataataatcacgg 384
T P Q H V L T A A H C V A L Y K K S L Y I L G V I V G E H N I R 128
acaattaataatacaaaagacgactcgactttacattatcgatgataataatcgatcatcggaattataggccaaaattgaacgatttagcggttatt 480
T I N N T K T T R [L Y L I D D I I V H P N] Y R P K L N D L A V I 160
aaattgcagaagagattagaatttccatgagaattggctccagcttcttcctgttttattacacgtggcgaactttacaggcactgtgtttaca 576
K L Q K R L E Y S M R I G P A C L P F Y Y T W R N F T G T V V T 192
gctgtagggtgggtcatacgaattttatgggtatcaagctcgacgttttaagaaaagtcgatttgcattgaatttcaatgaaggaaatgtatcaag 672
A V G W G H T N F Y G I K S D V L R K V D L H V I S M K E C I K 224
tatcactttctggtacatgtgaagcaactttgtacatttgataggaaggatgcttgcagttcgacagtggtgtccaattttatggcgaat 768
Y H F L A T C K Q L C T F D I G K [D A C Q F D S G G P I L W Q] N 256
ccaagaagcaatcggtttttcttcttggagttatcaattatggaaaaacatgtgcgatgaagctcctggttgaacttaagattactagttat 864
P R S N R V F L L G V I N Y G K T C A D E A P G V N L R V T S Y 288
ttagattttattagaaggtcaaacctgaagaacatactgtcaggcgtatttaattattatacaagacaaagcttgaatgaaaaataagttttt 960
L D F I R R S T P E E T Y C Q A Y * 305
gacttaaatatcaaatataatcttaacacggaatataaagtgatgtcaacaatttgttcgtgacgagtgaaactcagcagcataatctacattal056
tgcttaatatctcaatcatctatttaagcatcgaggtagaataagcgttgatgaaataagcatgataagtgtagcgttataatataag1152
tgtgaatttaacatttaactgaatttaactcacattaaagtgttataatgcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 1234

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Fig. 2. cDNA and the deduced amino acid sequence of magnvesin from *V. magnifica*. The residues boxed were determined by automated Edman degradation. The predicted poly A signal was in italic. *Stop codon.

magnvesin determined by Edman degradation. By BLAST search, magnvesin showed maximal identity (52%) with allergen serine protease from the wasp *P. dominulus* (Winningham et al., 2004) (Fig. 3).

3.4. Amidolytic activity of magnvesin

Amidolytic activity was determined spectrophotometrically using the chromogenic peptide substrates, S2302, S2238, S4760, C-3022 and BTEE. Magnvesin only hydrolyzed S2302 and S2238 as listed in Table 1. Table 1 compares the substrate specificity of the enzyme on these two chromogenic substrates. The studies were carried out by measuring the reaction velocities at different substrate concentrations and by determining the K_m and V_{max} of the enzyme on the chromogenic substrates with Lineweaver–Burk plots (Jin et al., 2001). Magnvesin showed relatively stronger activity on S-2238 than on S-2302.

3.5. Effects of protease inhibitors on magnvesin

The effect of various inhibitors on magnvesin is shown in Table 2. It was not inhibited by EDTA, but partly inhibited by DTT and cysteine. In this test, higher concentrations of DTT and cysteine (10 and 15 mM) were used, but no stronger inhibition was

observed. The result suggested that DTT and cysteine just partially inhibited its enzyme activity. Its enzyme activity was completely inhibited by PMSF and soybean trypsin inhibitor. The results showed that magnvesin was a serine protease and disulfide bonds were necessary for its enzyme activity.

3.6. Anticoagulation activity

The effect of various concentrations of magnvesin on the coagulation process is illustrated in Fig. 4. The addition of magnvesin increased the Ca^{2+} -clotting time of PPP in vitro in a dose-dependent manner (Fig. 4). The Ca^{2+} -clotting time was significantly prolonged by 0.5 μ g/ml magnvesin. At a magnvesin concentration of 4 μ g/ml, the PPP clotting time was longer than 150 min compared with the control (5–6 min). In addition, with an increase of preincubation time of magnvesin with plasma, the clotting time was prolonged (data not shown).

3.7. The in vitro effect of various concentrations of magnvesin on coagulation factors in normal human plasma

As listed in Table 3, the contents of factors TF, VII, VIII, IX and X was reduced gradually from

Vn50	ENSDVTPPPQQAAPVPTTNRSLSTTGINTQENNGCEGVNYYLCSNGTSNNGGVGINIRLGENTCDNYLHVCCSPSNILDRDKRLTPKPKVHRRCGHR	60
Magnvesin	-----IVGG--VETGVNEYPMMAGIVHLDR-----FLYCGATTIPQHVIATAAHCVALLYKSLYI--LGVIVGEHNIRTNNTKTTRLYLID	79
PDSP	-----IVNG--VETEINEFPMMARLIYPSP-----GMVCGGTITPQHIVTAAHCLQKYKRTNVTGHHVVGEHDYTTDTETNVTKRYTIA	79
DPSP	-----VANG--VETAKHEYPSMWGLRLAST-----LPIFCGGTIVSDFRIVTAAHCTVR--QPVASRLALVGEHDLSTASESYAAQHRIL	79
BT	-----IVGG--YTCCGANTVPYQVSLNSGVH-----FCGSLINSQWVVSAAHCVKS-----GIVRLGEDNINNVVEGN--EQFISAS	70
Vn50	NFNGVGRIRISGAENSEAFEGFPMWAVLVSNETSVENTQEEKYHYKGGSLIHRAVLTAAHCLKELNSS--NLLVRAGEWDVTQSEPLPHQDRSVS	157
ELP	-----VQMEAPYQASLWFRGDY-----MCDGVIVSPKTIILTIAQCMGITPEYYPDYKVRAG-----TRCVRAGGEVYTVTA	67
	* * * * *	
Magnvesin	DIIVHPNVRP-----KLNDLAVIKLQKRLEYSMRITGPACLPFYVYTRNFTGTVTAVGWGHTNF--YGIKS--DVLRRKVDLHVISMKECIKYH-----F	164
PDSP	EVTHPNVNS-----HNNDIAIVKTNERFEYSMKYGPVCPFPNVMTRNLTNETVIALGWKLRV--NGQNS--KVLRRKVDLHVIITREQCETHYGA-----A	166
DPSP	SIINHPAYTVTSS--GDLNDIALLOTATAMEWSRGVAPICLPVRQSDSAFQNVVDITGWTGTF--AAAKS--NTLQKATLLTLENSVCRQQYN-----S	169
BT	KSIVHPSVNSN--TLNNDIMLIKLKSAASLSNRVASISLPTSQAS--AGTQCLISGWNTKS--SGTSPYDVLKCLKAPILSDSSCKSAYP-----G	154
Vn50	RIIRHLEFQTR--TGQYDLAILILSEAMEQAENVDIVCLPN--KNEIFDNTRCVASGWGKNVFGNEGEYQVILKRVDLRRVDDHAAQNAIRKTRTGEHF	292
ELP	GIYTHPNYTFSNSGLSDNDFAIIRLKKPMLNEARQAIAIAKENPP--VNAKGFLVGVGRNHL--QDISINFLKAESTVVSRSFCQEKVHK-----	155
	* * * * *	
Magnvesin	LATCKQLCTFD--IGKDAQCFD--SGGPILWQNPNSRVFLLGVINYGKTCAD--EAPGVNLRVTSYLDLFIIRRSTPEETYCQAY	242
PDSP	IANALLCTFD--YGRDAQND--SGGPILWRSPTTDNLILGVVNFGRITCAD--DAPGNAVRTSFMETIHATIGETCYCKAD	244
DPSP	SISASQLCTYDSSGRQDSCQYD--SGGPVILR-QERWYLL--GVISVGRACGQPYGIVNTRITSYLNNWRYVGGAV--C-VR	247
BT	QITSNMFCAGYLE--GKDKSCQGD--SGGPVYCSG--KLQ--GIVSWGSGCAQKNKPGVYTKVNYVSWIKQTIAN-----	223
Vn50	KLEESSICAGGEP--GIDTCKGDGSPVLCPLRNDPTRYSA--GIVSWGVDGEGDGPALYSNVAYAREWIDQQLFAFAD----	369
ELP	PLHANVICTYDRG--DPEKCVGNAGSPLVVDN-----KLV--GLLTWNGDCGK--VYPAVFAAVSQVRFINRIMLQDS-----	223
	* * * * *	

Fig. 3. Alignment of magnvesin with other serine proteases. The identical amino acid residues are indicated by the star (*). Non-identical cysteines are shaded. Conserved active sites (⁴³His, ⁹³Asp and ¹⁸⁵Ser) are bolded. Gaps (-) have been introduced to optimize the sequence homology. *Polistes dominulus* serine protease (PDSP) (Winningham et al., 2004); *Drosophila pseudoobscura* serine protease (DPSP) (Accession: XP_001360288); bovine trypsin (BT) is cited from the paper authored by Bode and Huber, 1978. Vn 50 (venom protein) is reported by Asgari et al., 2003. Elastase-like protein (ELP) is reported by Yamamoto et al. (2007).

100%, 96%, 102%, 99% and 110% without magnvesin to 13%, 5%, 18%, 7% and 33%, respectively, with 2 $\mu\text{g/ml}$ of magnvesin. All the coagulant factors have been hydrolyzed by magn-

vesin. It showed the strongest effect on factor VII among the tested factors.

4. Discussion

A large amount of bioactive substances including amines, toxic peptides and proteins have been identified from wasp venoms. These venom components can act on the cardiovascular system, nervous system and immunological system of mammals. Some vasoactive, inflammatory and thrombogenic substances such as histamine, serotonin, dopamine, leukotrienes, thromboxanes and bradykinin have been found in wasp venoms (Levine, 1976). Serotonin and thromboxanes can induce platelet aggregation and direct vasoconstriction (McMillan et al., 1979). Anticoagulant components have been considered as serine protease and (or) metalloprotease. No anticoagulant serine protease or metalloprotease from wasp venom has been characterized, although an anticoagulant protein (protease I) with proteolytic activity has been purified from *V. orientalis* venom (Haim et al., 1999).

Table 1
Kinetic parameters of magnvesin on chromogenic substrates

Substrate	K_m (μM)	$V_{\max}$ ($\mu\text{M}/\text{min}^{-1}$)
S-2238	52.5	24.7
S-2302	77.9	20.2

Values are the means of four determinations, with S.E. less than $\pm 10\%$

Table 2
Effect of various protease inhibitors on amidolytic activity of magnvesin

Inhibitor	Concentration (mM)	Residual activity (%)
Control		100
Soybean trypsin inhibitor	2	0
PMSF	2	0
EDTA	5	96
DTT	5	39
L-Cysteine	5	42

Phenylmethylsulfonyl fluoride (PMSF) was dissolved in dimethylsulfoxide (DMSO); ethylenediamine tetraacetic acid (EDTA), soybean trypsin inhibitor, cysteine and dithiothreitol (DTT) were dissolved in 50 mM Tris-HCl, pH 7.8, containing 0.1 NaCl. Purified enzyme (2 $\mu\text{g/ml}$) was incubated 30 min at room temperature in 50 mM Tris-HCl, pH 7.8 in the presence or absence of the inhibitors at the indicated concentrations. The initial rate of amidolysis was measured after addition of 200 μl of S-2238. Values are the means of four determinations, with S.E. less than $\pm 10\%$

Table 3
The in vitro effect of various concentrations of magnvesin on coagulation factors in normal human plasma

Concentration ($\mu\text{g/ml}$)	TF	VII	VIII	IX	X
0	100 $\pm$ 7	97 $\pm$ 6	102 $\pm$ 3	99 $\pm$ 5	110 $\pm$ 12
0.5	73 $\pm$ 6	34 $\pm$ 1	61 $\pm$ 10	45 $\pm$ 7	77 $\pm$ 4
1.0	21 $\pm$ 4	13 $\pm$ 5	33 $\pm$ 9	30 $\pm$ 3	52 $\pm$ 8
2.0	13 $\pm$ 6	5 $\pm$ 2	18 $\pm$ 3	7 $\pm$ 4	33 $\pm$ 5

The results are mean $\pm$ S.E of four experiments.

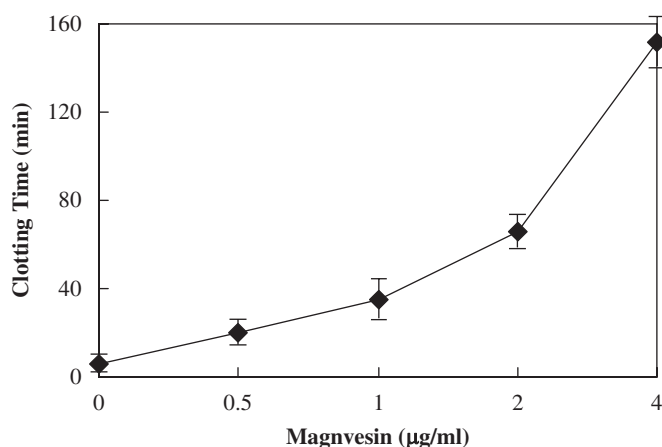


Fig. 4. Dose-dependent anticoagulation activity of magnvesin. Values are the means of four determinations.

In the current study, an anticoagulant serine protease (magnvesin) was purified and characterized from wasp venom of *V. magnifica*. It could strongly inhibit coagulation (Fig. 4). The anticoagulant activity could be inhibited by serine protease inhibitors, for example PMSF. Magnvesin is a basic serine protease with predicted pI of 8.9, which contains 26 basic amino acid residues and 19 acidic amino acid residues. Magnvesin is different from the protease I from *V. orientalis*, which contains 44 basic amino acid residues and 56 acidic amino acid residues (Haim et al., 1999).

The cDNA encoding magnvesin has been screened from the cDNA library of *V. magnifica* venomous glands. In the amino acid sequence of magnvesin, there are nine half-cysteines. It has conserved active sites (⁴³His, ⁹³Asp and ¹⁸⁵Ser) for its enzymatic activity as other serine proteases (Fig. 3). It suggests that they have common catalytic mechanism as other serine proteases, which include a highly reactive serine residue that plays a key role in the formation of a transient acyl-enzyme complex, which is stabilized by the presence of histidine and aspartic acid residues within the active sites (Serrano and Maroun, 2005; Halfon and Craik, 1998). Interestingly, no conserved ⁴³His was found in the elastase from the wasp venoms of *Cyphononyx dorsalis* (Fig. 3).

There are 12 half-cysteines to form six disulfide bonds in most of serine proteases found in vertebrates. Only nine half-cysteines are there in magnvesin. In fact, most of serine proteases from insects also have 8–9 half-cysteines, for example, the allergen serine protease identified from *P. dominulus* wasp venom and the protease from fruit fly (Fig. 3). It appeared that insect serine proteases have different disulfide bond motif from vertebrate serine proteases. By alignment with bovine trypsin, it was found that the four half-cysteines (⁷Cys-¹³⁸Cys, ¹¹⁶Cys-¹⁸³Cys) that form two disulfide bonds in bovine trypsin (Serrano and Maroun, 2005; Bode and Huber, 1978) are absent in insect serine proteases (Fig. 3). One exception is that a serine proteinase homolog venom protein from an endoparasitoid wasp with melanization-inhibitory activity contains 16 cysteines (Asgari et al., 2003) (Fig. 3).

It has been suggested that proteases from wasp venom exert their anticoagulant activity by hydrolyzing coagulant factors such as factors II, V, VII, X, XI and XII (Kornberg et al., 1988; Haim et al., 1999). In our experiments, magnvesin was found to

act on factors TF, VII, VIII, IX and X (Table 3). All the results suggested that wasp venoms can inhibit coagulation by hydrolyzing multiple coagulant factors.

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