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The first report of kininogen from invertebrates

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Abstract

The hornet possesses highly toxic venom, which is rich in toxin, enzymes, and biologically active peptides. Several bradykinin-like peptides, vespakinins, have been found in wasp venoms since 1970s, but the mode of biosynthesis of these peptides is unknown. In the present study, a vespakinin M was purified from venom of *Vespa magnifica*. Its primary sequence was established as GRPPGFSPFRID. The cDNA encoding the vespakinin M was cloned from the cDNA library of *V. magnifica* venom gland. The cDNA structure of vespakinin M was found to contain a coding region of 168 nucleotides. The encoded precursor of vespakinin M is composed of a signal peptide, an acidic peptide, and a mature peptide of vespakinin M. This is the first kininogen from insects; it is also the first kininogen from invertebrates. The cDNA structure encoding vespakinin M suggests that the generation mode of bradykinin-related peptides in wasp is different from amphibian skin and mammalian blood system.

Keywords: Wasp; Vespakinin; Kininogen; Bradykinin; *Vespa magnifica*

The venoms of arthropods have attracted considerable interest as a potential source of bioactive substances. Wasp venom gland is biochemically, pharmacologically, and physiologically complex organ which fulfills a wide range of functions necessary for wasp survival, including predatoriness, defense, etc. Over the past several decades, studies have focused on the bioactive compounds present in wasp venoms. These compounds include amines, small peptides, and high molecular weight proteins such as enzymes, allergens, and toxins [1,2]. An important family of bioactive compounds having regulatory or hormonal functions from wasps is the bradykinin-like peptide (vespakinin) family that is a counterpart of mammalian and amphibian bradykinins [2]. They participate in a broad spectrum of biological activities and events in pathophysiological conditions [3,4]. The generation of bradykinin in mammalian blood system by the action of kallikrein–kinin system has been well documented [3,4]. Bradykinin is a hydrolysis product by the limited proteolysis of kallikrein on kininogens. There are three types of kininogens in mammalian, high molecular weight, low molecular weight kininogens, and T-kininogens [5–7]. They are single chain glycoproteins consisting of three domains: a bradykinin moiety, an N-terminal heavy chain, and a C-terminal light chain, bridged by a disulfide linkage. Additionally, the heavy chain of kininogen may act as a cysteine proteinase inhibitor. Different from the precursors encoding mammalian bradykinin, most of the precursors (kininogen) encoding amphibian bradykinin are composed of several copies of a peptide segment unit including mature bradykinin plus a spacer peptide [8]. No domains of cysteine proteinase inhibitor were found in amphibian kininogens. Up to date, no kininogen from invertebrates has been reported.

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With a body length of up to 5 cm, *Vespa magnifica* is one of the most dangerous species of vespine wasps, found in Yunnan province of China. It is a kind of social wasp. This hornet is aggressive and predatory. Venoms of this hornet possess paralytic effects on their preys (insects or spiders). The immobilized preys are then used to feed the wasps' larvae. Stings by this hornet generally produce severe pain, local damage, and occasionally death in large vertebrates including human. Herein, we report purification, characterization, and cDNA cloning of vespakinin from *V. magnifica*.

Materials and methods

Wasp venom. The wasps *V. magnifica* were collected in Yunnan, 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 cm), immediately collected, and stored at −20 °C.

Peptide purification. The wasp venom was dissolved in 0.1 M phosphate buffer, pH 6.0. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M phosphate buffer solution, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The absorbance of the elute was monitored at 280 nm. The peaks of interest were submitted to a CM-Sephadex C-25 ion exchange column (Amersham Biosciences, 2.6 × 50 cm) equilibrated with the same buffer. Elution was achieved with a linear NaCl gradient (0–0.8 M) in 0.1 M phosphate buffer, pH 6.0, and the elution was monitored at 280 nm. The final purification of peptides was performed using C_{18} reverse phase high performance liquid chromatography (RP-HPLC, Hypersil BDS C_{18}, 30 × 0.46 cm).

Bioassay. Bradykinin activity was tested by assaying the contractile activity on isolated guinea pig ileum mainly as described [8]. About 10 cm of the distal ileum of guinea pig of either sex (150–250 g body weight) was removed immediately after death and washed with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 5.04 mM D-glucose). Cut segments of 2 cm of the isolated ileum were mounted isotonically, under 1-g load, in 5 ml muscle bath containing Tyrode solution maintained at 37 °C and bubbled with air. PcLab software package was used for the collection and analysis of biological signal (Beijing Microsignalstar Technology Development Co. Ltd.). The peptides were quantified by UV absorbance at 215 and 225 nm using the formula: concentration (mg/ml) = $(A_{215} - A_{225}) \times 0.144$.

Structural analysis. Complete peptide sequences were determined by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Fast atom bombardment (FAB) mass spectrometry was carried out on an Autospec-3000 spectrometer equipped with a high field magnet, using glycerol:3-nitrobenzyl alcohol:dimethyl sulfoxide (1:1:1, v:v:v) as mixed matrix. The ion gun was operated at 25 kV with a current of 1 μA, using Cs^+ as the bombarding gas.

SMART cDNA synthesis. Venom glands ($n = 30$) were dissected from *V. magnifica*. Total RNA was extracted using TRIzol (Life Technologies Ltd.). cDNA was synthesized by SMART™ techniques by using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The first strand was synthesized by using cDNA 3′ SMART CDS Primer II A, 5′-AAGCAGTGGTATCAACGCAGAGTACT (30) N-1N-3′ (N = A, C, G or T; N-1 = A, G or C), and SMART II A oligonucleotide, 5′-AAGCA GTGGTATCAACGCAGAGTACGCGGG-3′. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A, 5′-AAG CAGTGGTATCAACGCAGAGT-3′. Finally, the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI).

Screening of cDNA encoding bradykinin-like peptides. The cDNA synthesized by SMART™ techniques was used as template for PCR to screen the cDNAs encoding vespakinin. Two oligonucleotide primers, S_1 (5′-(A/T)GG(A/T/G)GAAAA(A/T/G)CC(A/T)GGCGG(A/T)CG(A/T/C/G)CC)-3′, in the antisense direction, a degenerate primer designed according to the sequence determined by Edman degradation and primer II A as mentioned in "*SMART cDNA synthesis*" in the sense direction were used in PCRs. The PCR conditions were: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, and 40 s at 72 °C. The DNA polymerase was Advantage polymerase from Clontech (Palo Alto, CA). DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Results and discussion

Purification of bradykinin-like peptide

The wasp venom was fractionated into five fractions by Sephadex G-50 (Fig. 1A). The contractile activity on isolated guinea pig ileum mainly was concentrated in fraction III. The fraction III was collected and applied to a CM-Sephadex C-25 ion-exchange column and 12 fractions were eluted from this chromatography. The contractile activity on isolated guinea pig ileum was concentrated in fractions V (Fig. 1B). The interest fractions were subjected to C_{18} RP-HPLC (Fig. 1C). The fraction V was well separated into more than 30 fractions. The peak indicated by an arrow in Fig. 1C displayed a contractile activity on isolated guinea pig ileum.

Structural characterization

The purified bradykinin-like peptide (indicated by an arrow, in Fig. 1C) was subjected to amino acid sequence analysis by automated Edman degradation. The amino acid sequence of vespakinin M was GRPPGFSPFRID, composed of 12 amino acid residues, and its molecular weight was 1345.5, analyzed by fast atom bombardment (FAB) mass spectrometry. This molecular weight matched well with the theoretical molecular weight (1345.52). There is a conserved PPGF motif as found in other bradykinin-like peptides. Analysis using the ExPASy MW/p*I* tool (http://www.expasy.ch/tools/pi_tool.html) showed that it has a predicted p*I* of 9.6. By BLAST search, the bradykinin-like peptide reported in this paper is the same with vespakinin M found in the venom of *Vespa mandarinia* Smith [9]. Different from vespakinin M containing a 4-hydroxyproline, the proline at the fourth site of the bradykinin-like peptide reported here is not modified.

cDNA cloning

Upon screening of a venom gland cDNA library of *V. magnifica* as indicated, several positive clones containing inserts of around 270-bp were identified and isolated. Both strands of these clones were sequenced (Fig. 2). The complete nucleotide sequence encoding the bradykinin-like peptide and the deduced amino acid sequence are shown in Fig. 2. The overall structure of the cDNA is composed of 267-bp (GenBank Accession No. DQ780005). It was found to contain a coding region of 168 nucleotides. The encoded amino acid sequence corresponds to a polypeptide