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Molecular characterization of a venom acid phosphatase from the Asiatic honeybee *Apis cerana*

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ABSTRACT

Bee venom contains a variety of toxic components, including enzymes, peptides, and biogenic amines. An acid phosphatase Acph-1-like protein has been identified from Asiatic honeybee (*Apis cerana*) venom. However, no molecular information is currently available for acid phosphatases from *A. cerana* venom. In this study, an *A. cerana* venom acid phosphatase (*Ac*VAP) was identified. The amino acid sequence analysis of the predicted *Ac*VAP protein revealed high identity with other bee venom acid phosphatases. An anti-*Ac*VAP antibody was produced against a recombinant *Ac*VAP (46-kDa) expressed in in baculovirus-infected insect cells. Northern and Western blot analyses showed that *Ac*VAP was expressed in the venom gland and was present as a 46-kDa protein in the secreted bee venom. The enzymatic properties of recombinant *Ac*VAP were determined using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate and exhibited the highest activity at pH 4.8 and 45 °C. Taken together, our data demonstrated that *Ac*VAP functions as a bee venom acid phosphatase.

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Introduction

Bee venom contains a variety of enzyme constituents, including phospholipase A_2, hyaluronidase, acid phosphatase, dipeptidylpeptidase IV, CUB serine protease, carboxylesterase, and serine carboxypeptidase (Hoffman and Jacobson, 1996; Winningham et al., 2004; Peiren et al., 2005; Hoffman, 2006; Son et al., 2007). Phospholipase A_2 (PLA_2) is the most-studied bee venom enzyme constituent given its toxic and anticancer effects (Gauldie et al., 1976; Six and Dennis, 2000; Heinen and da Veiga, 2011).

Acid phosphatase, an enzyme component of bee venom, is an enzyme that hydrolyzes phosphomonoesters at acidic pHs (Hoffman, 1977; Marz et al., 1983; Barboni et al., 1987; Peiren et al., 2005, 2008; de Abreu et al., 2010). Previous studies have demonstrated that the acid phosphatase from European honeybee *Apis mellifera* (Hymenoptera: Apidae) venom is an allergen (Api m 3) that releases histamine and induces wheal and flare reactions in sensitized humans (Hoffman, 1977; Barboni et al., 1987; Grunwald et al., 2006). Molecular and enzymatic properties of *A. mellifera* venom acid phosphatase have been reported, including gene structure, expression, and functional features (Soldatova et al., 2000; Grunwald et al., 2006). A subsequent study generated a three-dimensional model of *A. mellifera* venom acid phosphatase (Georgieva et al., 2009). Furthermore, acid phosphatases have also been reported in the venom of Hymenoptera, such as endoparasitoid, ectoparasitoid wasps (Dani et al., 2005; Zhu et al., 2008; de Graaf et al., 2010; Heavner et al., 2013), and ants (Bonasio et al., 2010).

Recently, we reported molecular characterization of a venom acid phosphatase Acph-1-like protein from the Asiatic honeybee *A. cerana* (Hymenoptera: Apidae) (Kim and Jin, 2014), which is bred locally in Asian countries (Suwannapong et al., 2010; Jung et al., 2014). Furthermore, our previous study demonstrated that *A. cerana* venom acid phosphatase Acph-1-like protein functions as a venom acid phosphatase (Kim and Jin, 2014). However, the molecular characterization of the venom acid phosphatase from *A. cerana* has not been elucidated to date. Here, we describe the molecular cloning and functional characterization of *A. cerana* venom acid phosphatase (*Ac*VAP). We cloned cDNA encoding *Ac*VAP and expressed recombinant *Ac*VAP in baculovirus-infected insect cells. Finally, we demonstrated that *Ac*VAP exhibits acid phosphatase activity as a bee venom component.

Materials and methods

cDNA cloning and sequence analysis of AcVAP

A cDNA encoding *Ac*VAP was cloned from expressed sequence tags (ESTs) generated from a cDNA library using whole bodies of *A. cerana* (Kim et al., 2013a, 2013b). Plasmid DNA extraction was performed using a Wizard Mini-Preparation kit (Promega, Madison, WI, USA), and cDNA sequencing was performed using an ABI310 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Pairwise sequence comparisons of cDNAs were performed using the DNASIS and BLAST databanks (http://www.ncbi.nlm.nih.gov/BLAST). Prediction of the signal peptide sequence of *Ac*VAP was performed using the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP). A multiple amino acid sequence alignment of

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GenBank-registered venom acid phosphate genes was performed using MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK).

Recombinant AcVAP protein expression

Recombinant *Ac*VAP was expressed in the baculovirus expression vector system using *Autographa californica* nucleopolyhedrovirus (AcNPV) and the *Spodoptera frugiperda* (Sf9) insect cell line (Je et al., 2001). The insect Sf9 cells were cultured in TC100 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL). For the construction of the expression vector, *AcVAP* cDNA was PCR-amplified from *pBluescript-AcVAP* using the follow primers: forward primer (1 − 21) 5′-GGATCCATGATGACTTCGCTTATC GAT-3′ and reverse primer (1183–1206) 5′-TCTAGATTAATGATGATG ATGATGATGCTTATTCTCAGTACCCGCTAT-3′. A His-tag sequence was included in the *AcVAP* cDNA sequence to purify recombinant *Ac*VAP using the MagneHis™ Protein Purification System (Promega) (Kim and Jin, 2014). The PCR products were sequenced as described above. The *AcVAP* fragment was inserted into the *pBacPAK8* vector (Clontech, Palo Alto, CA, USA) under the control of the AcNPV polyhedrin promoter. For the production of recombinant AcNPV expressing *Ac*VAP, 500 ng of the vector construct (*pBacPAK8-AcVAP*) and 100 ng of AcNPV viral DNA (Je et al., 2001) were co-transfected into Sf9 cells ($1.0–1.5 \times 10^6$ Sf9 per 35-mm diameter dish) for 5 h using the Lipofectin transfection reagent (Gibco BRL, Gaithersburg, MD, USA). Transfected Sf9 cells were incubated in TC100 medium supplemented with 10% FBS at 27 °C for 5 days. Recombinant baculoviruses expressing *Ac*VAP (AcNPV-*Ac*VAP) were propagated in Sf9 cells cultured in TC100 medium supplemented with 10% FBS at 27 °C.

Recombinant protein purification and polyclonal antibody production

Recombinant *Ac*VAP purified using the MagneHis™ Protein Purification System (Promega, Madison, WI, USA) was used for antibody generation and enzyme assays. For the antibody preparation of *Ac*VAP, Purified recombinant *Ac*VAP (~5 μg per mouse) was into 8-week-old male BALB/c mice (Samtako Bio Korea, Osan, Korea). After the last injection with recombinant *Ac*VAP only, blood was collected at 3 days post-injection and centrifuged at 10,000 ×g for 5 min (Qiu et al., 2011). The supernatant antibodies were stored at −70 °C and used for Western blot analysis.

SDS-PAGE, Western blot analysis, and glycoprotein staining

Protein samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with 0.1% Coomassie brilliant blue R-250. Western blot analysis was performed using an enhanced chemiluminescence (ECL) Western blot analysis system (Amersham Biosciences, Piscataway, NJ, USA) with anti-*Ac*VAP antibodies. The second antibodies were used at a 1:5000 (v/v) dilution of anti-mouse IgG horseradish peroxidase (HRP) conjugate and HRP-streptavidin complex. The amount of recombinant *Ac*VAP proteins was estimated using Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Glycoprotein staining of recombinant *Ac*VAP proteins was performed using the Gel Code Glycoprotein Staining Kit (Pierce, Rockford, IL, USA) according to the supplier's protocol.

RNA extraction and northern blot analysis

Total RNA extraction was performed using Total RNA Extraction Kit (Promega, Madison, WI, USA). Tissue samples (epidermis, fat body,

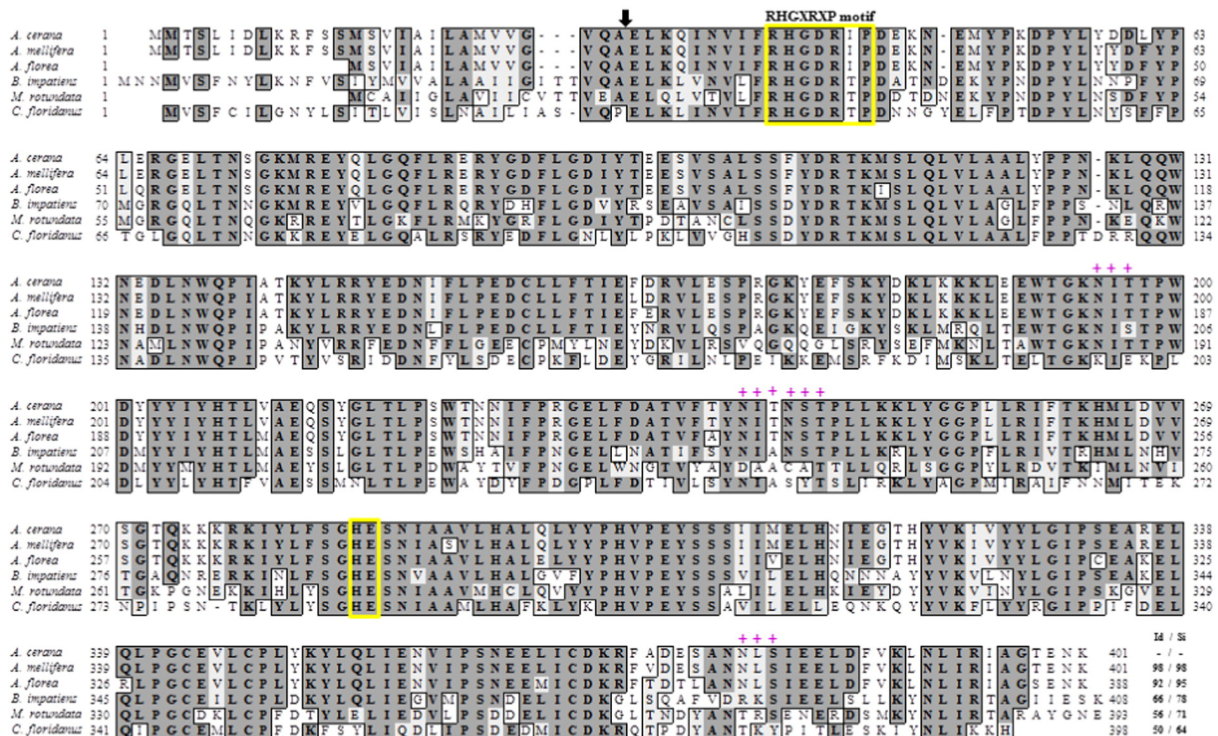

Fig. 1. The alignment of the amino acid sequences for *Ac*VAP and known Hymenoptera venom acid phosphatases. The predicted signal peptide is indicated with a vertical arrow. Amino acids necessary for acid phosphatase activity are boxed. Potential *N*-glycosylation sites are indicated by crosses. The sources of the aligned sequences are as follows: *A. cerana* (this study, GenBank accession no. KJ710422), *A. mellifera* (NM 001013359), *A. florea* (XM 003696461), *Bombus impatiens* (XM 003486535), *Megachile rotundata* (XM 003706060), and *Camponotus floridanus* (GL444789). The *Ac*VAP sequence was used as the reference for the identity/similarity (Id/Si) values.

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