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Molecular characterization of a venom acid phosphatase Acph-1-like protein from the Asiatic honeybee *Apis cerana*

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ABSTRACT

Bee venom contains a variety of peptides and enzymes, including acid phosphatases. An acid phosphatase has been identified from European honeybee (*Apis mellifera*) venom. However, although the amino acid sequence is known, no functional information is currently available for bee venom acid phosphatase Acph-1-like proteins. In this study, an Asiatic honeybee (*Apis cerana*) venom acid phosphatase Acph-1-like protein (*Ac*Acph-1) was identified. The analysis of the predicted *Ac*Acph-1 amino acid sequence revealed high levels of identity with other bee venom acid phosphatase Acph-1-like proteins. Recombinant *Ac*Acph-1 was expressed as a 64-kDa protein in baculovirus-infected insect cells. The enzymatic properties of recombinant *Ac*Acph-1, determined using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate, showed the highest activity at 45 °C and pH 4.8. Northern and western blot analyses showed that *Ac*Acph-1 was expressed in the venom gland and was present as a 64-kDa protein in bee venom. In addition, *N*-glycosylation of *Ac*Acph-1 was revealed by tunicamycin treatment of recombinant virus-infected insect Sf9 cells and by glycoprotein staining of purified recombinant *Ac*Acph-1. Our findings show that *Ac*Acph-1 functions as a venom acid phosphatase. This paper provides the first evidence of the role of a bee venom acid phosphatase Acph-1-like protein.

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Introduction

Bee venom is composed of a variety of enzymes, peptides, and biogenic amines (Hoffman, 2006; Hoffman and Jacobson, 1996; Peiren et al., 2005; Son et al., 2007; Winningham et al., 2004). Some bee venom components can cause life-threatening allergic reactions due to an immediate hypersensitivity-induced reaction that leads to anaphylaxis (Fitzgerald and Flood, 2006; Golden, 2007). Honeybee venom allergens are as follows: Api m 1 (phospholipase A_2), Api m 2 (hyaluronidase), Api m 3 (acid phosphatase), Api m 4 (melittin), Api m 5 (dipeptidylpeptidase IV), Api m 6, Api m 7 (CUB serine protease), Api m 8 (carboxylesterase), and Api m 9 (serine carboxypeptidase). Two major components of honeybee venom are melittin and phospholipase A_2 (PLA_2) (Gauldie et al., 1976; Six and Dennis, 2000) and are the most-studied bee venom components because of their anticancer effects (Heinen and da Veiga, 2011). In addition to melittin and PLA_2, other bee venom components have been studied as promising sources of bioactive compounds. Our previous studies demonstrated a novel mechanism by which bumblebee venom affects the hemostatic system via venom serine protease inhibitor-mediated antifibrinolytic activity as well as via venom serine protease-mediated fibrin(ogen) olytic activities (Choo et al., 2010, 2012; Qiu et al., 2011, 2013). Bee venom is considered a rich source of pharmacologically active components, and venom components have been intensively studied as potential compounds on which to base novel pharmaceuticals (Heinen and da Veiga, 2011; Son et al., 2007).

Acid phosphatase is also a bee venom component (Barboni et al, 1987; de Abreu et al., 2010; Hoffman, 1977; Marz et al., 1983; Peiren et al., 2005, 2008) and belongs to a group of enzymes that hydrolyze phosphomonoesters at acidic pH. European honeybee *Apis mellifera* venom acid phosphatase (Acph-1) is an allergen that is able to release histamine and induce wheal and flare reactions in sensitized humans (Barboni et al, 1987; Grunwald et al., 2006; Hoffman, 1977). The gene structure and functional features of *A. mellifera* venom acid phosphatase have been reported (Grunwald et al., 2006; Soldatova et al., 2000), and a three-dimensional model of *A. mellifera* venom acid phosphatase has provided a basis for investigating structure–function relationships (Georgieva et al., 2009). Additionally, mounting evidence has demonstrated the presence of acid phosphatases in the venom of endoparasitoid and ectoparasitoid wasps (Dani et al., 2005; de Graaf et al., 2010; Heavner et al., 2013; Zhu et al., 2008). Data from *Hymenoptera* venom studies indicate that acid phosphatase is a common component of venom.

In addition to venom acid phosphatases, venom acid phosphatase Acph-1-like proteins have also been found in the venoms of hymenopteran insects, including bees (Heavner et al., 2013). Although venom acid phosphatase Acph-1-like proteins from bees can be found in

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database searches, the functional role of bee venom acid phosphatase Acph-1-like proteins remains relatively unexplored. Moreover, no enzymatic property of bee venom acid phosphatase Acph-1-like proteins has been demonstrated. Here, we report the first Asiatic honeybee *Apis cerana* venom acid phosphatase Acph-1-like protein (*Ac*Acph-1) that functions as a venom acid phosphatase. We describe the molecular characterization of this enzyme and demonstrate that it exhibits acid phosphatase activity. In addition, the *N*-glycosylation of *Ac*Acph-1 was evaluated using glycoprotein staining and the *N*-glycosylation inhibitor tunicamycin. The present study provides the first evidence that *Ac*Acph-1 functions as an acid phosphatase.

Materials and methods

cDNA cloning and sequence analysis

A clone encoding *Ac*Acph-1 was selected from a set of expressed sequence tags (ESTs) generated from a cDNA library that was constructed using whole bodies of *A. cerana* (Kim et al., 2013a,b). Plasmid DNA was extracted using a Wizard Mini-Preparation kit (Promega, Madison, WI, USA), and the generated cDNA sequence was analyzed using an ABI310 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The sequenced cDNA was compared using the DNASIS and BLAST databanks (http://www.ncbi.nlm.nih.gov/BLAST). The signal sequence was predicted by SignalP 4.0 software (http://www.cbs.dtu.dk/services/SignalP). MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK) was then used to align the predicted amino acid sequences of GenBank-registered venom acid phosphate Acph-1-like genes.

Recombinant protein expression

A baculovirus expression system (Je et al., 2001) using *Autographa californica* nucleopolyhedrovirus (AcNPV) and the *Spodoptera frugiperda* (Sf9) insect cell line was employed to construct a recombinant virus expressing recombinant *Ac*Acph-1. *AcAcph-1* cDNA was PCR-amplified from *pBluescript-AcAcph-1* using a forward primer (90–110) 5′-GGATCCATGATTTTGCCTCTGTTGTAT-3′ and reverse primer (1296–1316) 5′-GAATTCTTAATGATGATGATGATGATGTACGAACACAGTAATTAC-3′. *AcAcph-1* was engineered to include a His-tag sequence. The PCR cycling conditions were as follows: 94 °C for 3 min, 30 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min), and 72 °C for 5 min. The PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (Perkin-Elmer Applied Biosystems). The isolated *AcAcph-1* fragment was inserted into the *pBacPAK8* vector (Clontech, Palo Alto, CA, USA) to generate an expression vector under the control of the AcNPV polyhedrin promoter. For expression experiments, 500 ng of the construct (*pBacPAK8-AcAcph-1*) and 100 ng of AcNPV viral DNA (Je et al., 2001) were co-transfected into 1.0–1.5 × 10^6 Sf9 cells for 5 h using the Lipofectin transfection reagent (Gibco BRL, Gaithersburg, MD, USA). The transfected cells were cultured in TC100 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) at 27 °C for 5 days. Recombinant baculoviruses were propagated in Sf9 cells cultured in TC100 medium at 27 °C.

Recombinant protein purification and polyclonal antibody production

The recombinant proteins were purified using the MagneHis™ Protein Purification System (Promega). Purified *Ac*Acph-1 (~5 μg) was mixed with an equal volume of Freund's complete adjuvant (a total of 200 μL) and injected into BALB/c mice (Qiu et al., 2011). Two successive injections were administered using antigens mixed with equal volumes of Freund's incomplete adjuvant (a total of 200 μL) at 1-week intervals, beginning one week after the first injection. Blood was collected 3 days after the last injection with antigens only and centrifuged at 10,000 ×g for 5 min. The supernatant antibodies were stored at −70 °C until use.

SDS-PAGE, western blot analysis, and glycoprotein staining

Protein samples were mixed with protein sample buffer (0.0625 M Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, and 0.125% bromophenol blue) and boiled for 5 min. The protein samples were separated using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the gels were fixed and stained with 0.1% Coomassie brilliant blue R-250. A western blot analysis was performed using an enhanced chemiluminescence (ECL) western blot analysis system (Amersham Biosciences, Piscataway, NJ, USA). The protein samples were mixed with sample buffer, boiled for 5 min, and loaded onto a 12% SDS-PAGE gel as described above. The proteins were blotted onto a sheet of nitrocellulose transfer membrane (Schleicher & Schuell, Dassel, Germany), and the membrane was blocked by incubation in a 1% bovine serum albumin (BSA) solution. The membrane was then incubated with the antiserum solution (1:1000 v/v) at room temperature for 1 h and washed in TBST [10 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 0.05% (v/v) Tween 20]. The membrane was incubated with a 1:5000 (v/v) diluted anti-mouse IgG horseradish peroxidase (HRP) conjugate and HRP-streptavidin complex. After repeated washing, the membrane was incubated with ECL detection reagents (Amersham Biosciences) and exposed to autoradiography film. The protein concentrations were determined using Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Glycoprotein staining was performed using Gel Code Glycoprotein Staining Kit (Pierce, Rockford, IL, USA).

RNA extraction and northern blot analysis

The Asiatic honeybee *A. cerana* (*Hymenoptera*: *Apidae*) used in this study was supplied by the Department of Agricultural Biology, National Academy of Agricultural Science, Republic of Korea (Kim et al., 2013a,b). *A. cerana* worker bees were dissected on ice using a stereomicroscope (Zeiss, Jena, Germany). Tissue samples (epidermis, fat body, gut, muscle, and venom gland) were collected and washed with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). Total RNA was isolated from the epidermis, fat body, gut, muscle, and venom gland of *A. cerana* using Total RNA Extraction Kit (Promega). Total RNA (5 μg/lane) was separated using a 1.0% formaldehyde agarose gel, transferred onto a nylon blotting membrane (Schleicher & Schuell), and hybridized at 42 °C with the appropriate probe in hybridization buffer containing 5× SSC (0.75 M sodium chloride and 0.75 M sodium citrate), 5× Denhardt's solution (0.1% each of BSA, Ficoll, and polyvinylpyrrolidone), 0.5% SDS, and 100 mg/mL denatured salmon sperm DNA. *AcAcph-1* cDNA was labeled with [α-^{32}P]dCTP (Amersham Biosciences) using the Prime-It II Random Primer Labeling kit (Stratagene, La Jolla, CA, USA), and the labeled cDNA was used as the probe for hybridization. After hybridization, the membrane was washed three times for 30 min each in 0.1% SDS and 0.2× SSC at 65 °C and then exposed to autoradiography film.

Enzymatic assay

The acid phosphatase enzymatic assay was performed as previously described (Dani et al., 2005; Grunwald et al., 2006; Zhu et al., 2008). Five micrograms of recombinant *Ac*Acph-1 diluted in 10 μL of sterile distilled water (SDW) was added to 180 μL of 5 mM *p*-nitrophenyl-phosphate (*p*-NPP; Sigma, St. Louis, MO, USA) prepared in 0.1 M sodium acetate/acetic acid buffer or phosphate buffer. The amount of *p*-nitrophenol (*p*-NP) released was measured using a microplate reader (Bio-Rad) at 405 nm. The effect of pH on *Ac*Acph-1 activity was determined using sodium acetate/acetic acid buffer (pH 3.6–5.6) and phosphate buffer (pH 5.8–7.2). For the effect of temperature on *Ac*Acph-1 activity, the

reaction solutions were incubated at temperatures ranging from 25 °C to 80 °C for 1 h; the reactions were stopped with the addition of 0.05 M NaOH. The *Ac*Acph-1 activity was expressed as percentages of the maximum activity of the controls.

Tunicamycin treatment

The addition of *N*-linked carbohydrate in infected insect cells was verified by culture in the presence of tunicamycin (5 μg/mL, Sigma) to prevent the reaction (Wei et al., 2005, 2006a,b). Sf9 cells were infected with the recombinant virus expressing *Ac*Acph-1 in a 35-mm diameter dish (1×10^6 cells) and incubated for 2 h at 27 °C. The supernatants were replaced with 5 mL of supplemented TC100 medium containing 5 μg of tunicamycin per mL of medium. After incubation at 27 °C, total cellular lysates were harvested from the infected cells at 2 days post-infection (p.i.) and subjected to 12% SDS-PAGE and western blot analysis.

Results and discussion

AcAcph-1 is a venom acid phosphatase Acph-1-like protein

An *AcAcph-1* cDNA sequence that included the full-length venom acid phosphatase Acph-1-like protein gene was identified by searching a set of *A. cerana* ESTs (GenBank accession number KJ710421). We assigned the signal peptide and the mature protein that was predicted by identifying the signal peptide region using the SignalP software program. *Ac*Acph-1 consists of 408 amino acids, including a 19-amino acid signal peptide and a 389-amino acid mature protein, with a calculated molecular mass and p*I* of 46 kDa and 5.50, respectively (Fig. 1). The predicted *Ac*Acph-1 amino acid sequence includes 7 putative *N*-glycosylation sites of the sequence Asn-X-Ser/Thr. An analysis revealed that the predicted *Ac*Acph-1 amino acid sequence shares high similarity to other bee venom acid phosphatase Acph-1-like proteins, with 92% amino acid sequence identity to that of *A. mellifera*.

A. mellifera venom acid phosphatases have been studied with regard to their biological and enzymatic properties, gene structure, three-dimensional model, and allergenicity (Barboni et al., 1987; Georgieva et al., 2009; Grunwald et al., 2006; Marz et al., 1983). Increasing evidence indicates that acid phosphatase is a common component of *Hymenoptera* venoms (Dani et al., 2005; de Graaf et al., 2010; Heavner et al., 2013; Zhu et al., 2008), and venom acid phosphatase Acph-1-like genes have been cloned from other bees. In this study, we hypothesized that *Ac*Acph-1 is a member of the bee venom acid phosphatase Acph-1-like protein family based on protein sequence identity. As stated above, *Ac*Acph-1 shares high protein sequence identity with other bee venom acid phosphatase Acph-1-like proteins, such as that from *A. mellifera* (92% protein sequence identity), *Bombus impatiens* (69% protein sequence identity), and *Megachile rotundata* (63% protein sequence identity). However, the active site sequence RHGXRXP motif, which is characteristic for bee venom acid phosphatases (Georgieva et al., 2009; Grunwald et al., 2006), is not conserved in the N-terminal region of these venom acid phosphatase Acph-1-like proteins.

AcAcph-1 is a bee venom component and functions as an acid phosphatase

The expression pattern of *Ac*Acph-1 in *A. cerana* worker bees was examined to confirm that it is an *A. cerana* venom gland-derived protein. A northern blot analysis was performed using total RNA prepared from the epidermis, fat body, gut, muscle, and venom gland of *A. cerana* worker bees, showing constitutive expression in the venom gland (Fig. 2A). To further characterize *Ac*Acph-1, recombinant *Ac*Acph-1 was expressed in baculovirus-infected insect cells, and an anti-*Ac*Acph-1 antibody was generated using recombinant *Ac*Acph-1 (64 kDa). A western blot analysis was performed using protein samples prepared from the epidermis, fat body, gut, muscle, and venom gland of *A. cerana* worker bees. Consistent with the northern blot data, the western blot analysis revealed that *Ac*Acph-1 was expressed in the venom gland (Fig. 2B). We next examined the pattern of *Ac*Acph-1 expression to confirm that it is a component of bee venom. The anti-*Ac*Acph-1 antibody was used to demonstrate the presence of *Ac*Acph-1 in the venom gland and venom of *A. cerana* worker bees (Fig. 2B), and *Ac*Acph-1 was detected as a 64-kDa protein in the venom gland and venom. Taken together, these data showed that *Ac*Acph-1 is produced in the venom gland and then stored in the venom sac.

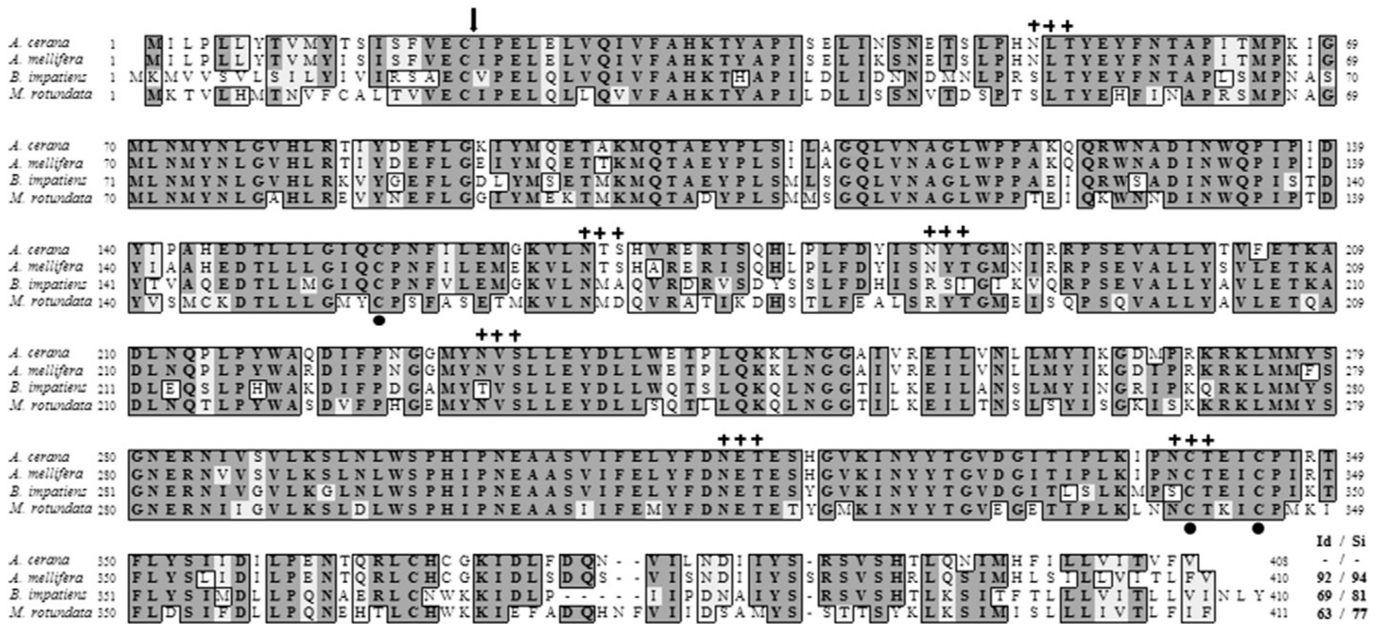

Fig. 1. Alignment of the amino acid sequences for *Ac*Acph-1 and known bee venom acid phosphatase Acph-1-like proteins. A vertical arrow indicates the end of the signal peptide. Potential *N*-glycosylation sites are indicated by crosses. The sources of the aligned sequences are *Apis cerana* (this study, GenBank accession no. KJ710421), *Apis mellifera* (GenBank accession no. XP_006569977), *Bombus impatiens* (GenBank accession no. XP_003486582), and *Megachile rotundata* (GenBank accession no. XP_003706109). The *Ac*Acph-1 sequence was used as the reference for the identity/similarity (Id/Si) values.

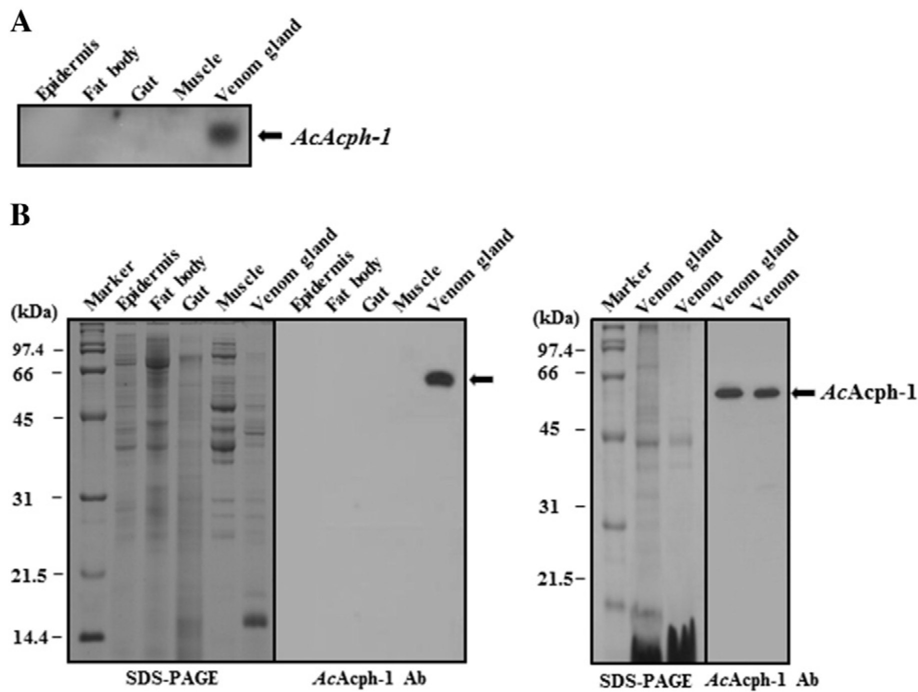

Fig. 2. Expression of *Ac*Acph-1. (A) Expression of *AcAcph-1* in *A. cerana* worker bees. Total RNA was isolated from the epidermis, fat body, gut, muscle, and venom gland of *A. cerana* worker bees. RNA was separated by 1.2% formaldehyde agarose gel electrophoresis, transferred onto a nylon membrane, and hybridized with radiolabeled *AcAcph-1* cDNA. The *AcAcph-1* transcripts are indicated with an arrow. (B) Detection of *Ac*Acph-1 in *A. cerana* venom gland (left) and venom (right). Protein samples prepared from tissues or venom of *A. cerana* worker bees were analyzed by 12% SDS-PAGE and by western blotting with an antibody against recombinant *Ac*Acph-1.

Using *p*-NPP as a substrate, we determined the enzymatic properties of recombinant *Ac*Acph-1. The activity was determined at various temperatures, ranging from 25 °C to 80 °C at pH 4.8, and the optimum temperature of recombinant *Ac*Acph-1 activity was at 45 °C (Fig. 3A). By incubating the enzyme at this optimum (45 °C) for 1 h, the optimum pH of recombinant *Ac*Acph-1 was determined to be in the pH range of 3.6–7.2; pH 4.8 was optimal (Fig. 3B).

As it remains to be determined whether bee venom acid phosphatase Acph-1-like proteins function as acid phosphatases, we evaluated the ability of the *A. cerana* venom Acph-1-like protein to function as an acid phosphatase. Our results demonstrate that *Ac*Acph-1 is a component of bee venom that is expressed in the venom gland. *p*-NPP is hydrolyzed by acid phosphatase (Dani et al., 2005; Grunwald et al., 2006; Zhu et al., 2008), and recombinant *Ac*Acph-1 expressed in baculovirus-infected insect cells exhibited the capacity to utilize *p*-NPP as a substrate, which demonstrated that the protein possesses acid phosphatase activity. In addition, the highest observed activity at 45 °C and pH 4.8 is similar to previous findings. The venom acid phosphatases of *A. mellifera* (Grunwald et al., 2006) and the endoparasitic wasp *Pimpla hypochondriaca* (Dani et al., 2005) exhibit optimal enzyme activities at pH 4.5, and the endoparasitic wasp *Pteromalus puparum* venom acid phosphatase shows optimal enzyme activities at 45 °C and pH 4.8 (Zhu et al., 2008). Given these observations and the enzymatic property of *Hymenoptera* venom acid phosphatases (Dani et al., 2005; Grunwald et al., 2006; Zhu et al., 2008), our results showed that bee venom acid phosphatase Acph-1-like proteins also function as venom acid phosphatases.

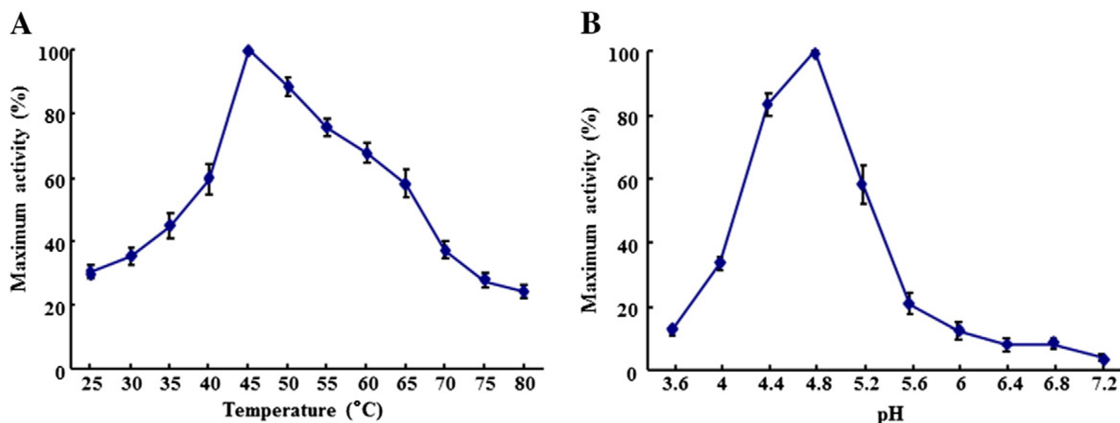

Fig. 3. Enzymatic properties of recombinant *Ac*Acph-1 expressed in baculovirus-infected insect cells. (A) Optimum temperature of recombinant *Ac*Acph-1. The optimum temperature for enzyme activity was determined by incubating recombinant *Ac*Acph-1 in sodium acetate/acetic acid buffer (0.1 M, pH 4.8) containing 5 mM *p*-NPP at a temperature range from 25 °C to 80 °C, with 5 °C increments ($n = 3$). (B) Optimal pH of recombinant *Ac*Acph-1. Recombinant *Ac*Acph-1 was incubated in sodium acetate/acetic acid buffer (pH 3.6–5.6) and phosphate buffer (pH 5.8–7.2) for 1 h at 45 °C prior to measuring the acid phosphatase activity ($n = 3$).

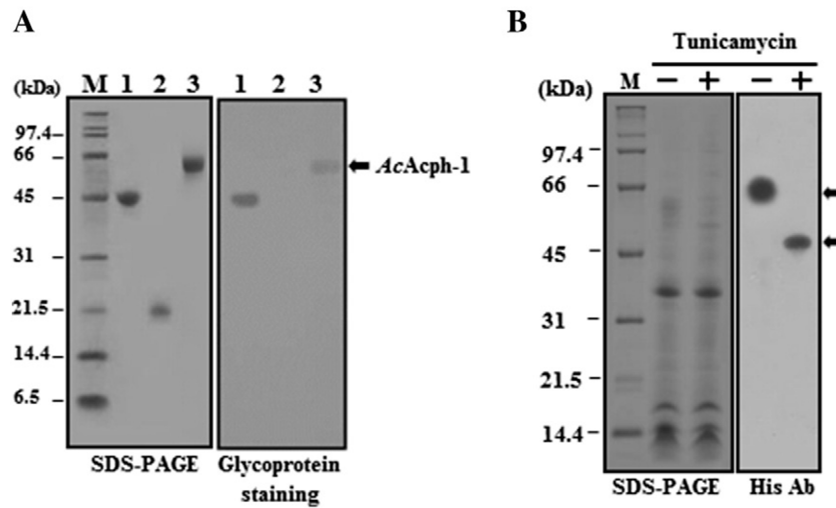

Fig. 4. *N*-glycosylation of *Ac*Acph-1. (A) Glycoprotein staining of *Ac*Acph-1. Purified recombinant *Ac*Acph-1 and control protein samples were subjected to 12% SDS-PAGE (left) and then analyzed by glycoprotein staining (right). Horseradish peroxidase (5 μg), a glycosylated protein, was used as a positive control (lane 1). Soybean trypsin inhibitor (5 μg), a non-glycosylated protein, was used as a negative control (lane 2). *Ac*Acph-1 (lane 3) is indicated with an arrow. A molecular weight standard was used for size comparison (lane M). (B) *N*-glycosylation of *Ac*Acph-1 in baculovirus-infected insect cells. Sf9 cells were infected with recombinant AcNPV (AcNPV-*Ac*Acph-1) and cultured without (−) or with (+) tunicamycin (5 μg/mL). Total cellular lysates were collected 2 days p.i. and then subjected to 10% SDS-PAGE (left) and western blot analysis (right). The *N*-glycosylated (64 kDa) and nonglycosylated (46 kDa) *Ac*Acph-1 proteins are indicated by arrows.

AcAcph-1 is N-glycosylated

The purified recombinant *Ac*Acph-1, which contains an additional six His residues compared to the native protein, was produced as a 64-kDa protein (Fig. 4A), much larger than its predicted molecular mass of 46 kDa. Several putative *N*-glycosylation sites were found in the protein sequence of *Ac*Acph-1 (Fig. 1). Thus, to determine whether *Ac*Acph-1 is indeed glycosylated, glycoprotein staining of recombinant *Ac*Acph-1 was performed. The difference between the predicted molecular mass of 46 kDa for the *Ac*Acph-1 protein and the observed molecular mass of 64 kDa by SDS-PAGE was shown to be due to the presence of carbohydrate moieties (Fig. 4A). To further assess whether the expected addition of *N*-linked carbohydrate moieties to the potential *N*-glycosylation sites of *Ac*Acph-1 occurred, recombinant baculovirus (AcNPV-*Ac*Acph-1)-infected cells were incubated with tunicamycin, a specific inhibitor of *N*-linked oligosaccharide addition, and total cellular lysates were subjected to SDS-PAGE and western blot analysis (Fig. 4B). Fig. 4B shows an apparent shift in the molecular weight of recombinant *Ac*Acph-1 in the tunicamycin-treated Sf9 cells, indicating the presence of 64-kDa and 46-kDa bands corresponding to *N*-glycosylated and nonglycosylated recombinant *Ac*Acph-1, respectively.

Similar to *A. mellifera* venom acid phosphatase, which is a glycoprotein containing four potential *N*-glycosylation sites (Barboni et al., 1987; Georgieva et al., 2009; Grunwald et al., 2006; Marz et al., 1983), the sequence of *Ac*Acph-1 was found to contain seven putative *N*-glycosylation sites. The difference between the recombinant *Ac*Acph-1 protein detected as a band of approximately 64 kDa by SDS-PAGE and the calculated molecular mass of mature *Ac*Acph-1 (46 kDa) is approximately 18 kDa, which could be explained by a possible post-translational modification at the seven potential *N*-glycosylation sites of *Ac*Acph-1. Such phenomena appear to be fairly common in glycoproteins (Wei et al., 2005, 2006a,b).

Conclusions

We provide the first molecular characterization and evidence for the enzymatic property of an Asiatic honeybee venom acid phosphatase Acph-1-like protein. Our results define the role for *Ac*Acph-1 as a bee venom acid phosphatase. The finding that *Ac*Acph-1 exhibits acid phosphatase activity highlights the functional role of bee venom acid phosphatase Acph-1-like proteins.

Acknowledgments

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