Functional Expression and Characterization of Dipeptidyl peptidase IV from the Black-Bellied Hornet Vespa basalis in Sf21 Insect Cells

Sheng-Kuo HSIEH,¹ Jason T.C. TZEN,¹,²,³ Tzong-Yuan WU,⁴ Ying-Ju CHEN,⁴ Wei-Hung YANG,² Chun-Fa HUANG,² Feng-Chia HSIEH,⁵ and Tzyy-Rong JINN²†

¹ Graduate Institute of Biotechnology, National Chung-Hsing University, Taichung, 40227, Taiwan
² School of Chinese Medicine, China Medical University, Taichung, 40402, Taiwan
³ Agricultural Biotechnology Research Center, Academia Sinica, Taipei, 11529, Taiwan
⁴ Department of Bioscience Technology, Chung Yuan Christian University, Chung Li, 32023, Taiwan
⁵ Division of Biopesticides, Agricultural Chemicals and Toxic Substances Research Institute, Wufong, Taichung, 41358, Taiwan

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† To whom correspondence should be addressed. Tel: 886-4-22053366; Fax: 886-4-22032295; E-mail: jinn@mail.cmu.edu.tw
maturation of mastoparan B, the major toxin peptide in the venom of *Vespa basalis*, requires enzymatic cleavage of its prosequence presumably via sequential liberation of dipeptides. The putative processing enzyme, dipeptidyl peptidase IV, was expressed as a glycosylated His-tag fusion protein (rDPP-IV) via the baculovirus expression system. rDPP-IV purified by one-step nickel-affinity chromatography was verified by Western blot and LC-MS/MS analysis. The $k_{\text{cat}}/K_m$ of rDPP-IV was determined to be in the range of 10-500 mM$^{-1}$S$^{-1}$ for five synthetic substrates. The optimal temperature and pH for rDPP-IV were determined to be 50°C and pH 9. Enzymatic activity of rDPP-IV was significantly reduced by 80 and 60% in the presence of sitagliptin and phenylmethylsulfonyl fluoride respectively.

**Key words**: dipeptidyl peptidase IV; mastoparan B; prosequence processing; sitagliptin; *Vespa basalis*

Mastoparan toxins are a class of mast-cell degranulating peptides found in the venoms of many vespid species.\(^1\) Mastoparan B, a cationic tetradecapeptide, is the major peptide isolated from the venom of black-bellied hornet (*Vespa basalis*), the most dangerous species of vespine wasps found in Taiwan.\(^2\) Besides its anti-bacterial activity, mastoparan B possesses potent hemolytic activity and pore-forming ability attributable partly to the toxicity of this hornet venom.\(^3\) Unlike other vespid mastoparan toxins, mastoparan B is capable of inducing short-term hypotension in rats.\(^4\) Since various biological actions of mastoparan B are probably exerted through different mechanisms, the hypotensive effect may be useful in developing this peptide into an anti-hypertension agent if its potency can be enhanced and hemolytic activity abolished.
Recently, we obtained a cDNA fragment encoding the precursor polypeptide of mastoparan B.\textsuperscript{5} According to the deduced amino acid sequence, mastoparan B can presumably be processed from a precursor polypeptide comprising a signal sequence, an anionic prosequence of 11 conservative dipeptides, the mature sequence of mastoparan B, and a C-terminal glycine. Post-translational processing and modification might include cleavage of the N-terminal signal sequence, removal of prosequence, and C-terminal amidation. The N-terminal signal sequence responsible for endoplasmic reticulum (ER) targeting is presumably cleaved by a signal peptidase after translocation into ER lumen. Instead of being cleaved by a specific protease in one step, the prosequence is proposed to be removed via sequential liberation of dipeptides catalyzed by dipeptidyl peptidase IV (DPP-IV) for mature mastoparan B.\textsuperscript{6} DPP-IV is a glycosylated serine protease that selectively removes dipeptides from the N-terminus of peptides with proline or alanine in the penultimate position.\textsuperscript{7} Because DPP-IV is an important membrane protein expressed in various cell types, DPP-IV was cloned and expressed by an expression system for further study and application.\textsuperscript{8,9} In our previous study, a cDNA fragment encoding a DPP-IV from Vespa basalis putatively responsible for the processing of the prosequence was obtained.\textsuperscript{5} Whether this putative DPP-IV possesses enzymatic activity of dipeptidyl peptidase has not been verified. In this study, we employed the baculovirus expression system to express V. basalis DPP-IV as a His-tagged fusion protein (rDPP-IV) that was purified by nickel-affinity chromatography. Enzymatic properties of the purified rDPP-IV were characterized.

\textbf{Materials and Methods}

\textit{Insect cells.} The insect cell line IPLB-Sf21-AE (Sf21, Invitrogen, Carlsbad, CA) used in this study was originally isolated from ovarian tissue of Spodoptera frugiperda (fall armyworm). The cells were routinely cultured at 26°C in TNM-FH basal medium.
(Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Rockford, IL). The Sf21 cells were passaged twice a week, and fresh media were provided every other day.

Generation of recombinant baculovirus. The Dpp-IV gene (accession No. DQ661743), encodes 776 amino acids. It was amplified using primers 5’CGGGATCCATGGTTCCACTACGAAGTTTCgTA3’ (BamHI site underlined) and 5’ACGAATTCTCAAGTGATGGTGATGGGTGGTGAGCGTGAGACAGATTGAA3’ (EcoRI site underlined). Plasmid pAcP_{10}DPPIV was constructed from transfer vector pAcUW21 (PharMingen, San Diego, CA), in which the Dpp-IV gene was introduced into the BamHI and EcoRI restriction sites, and thus directed by the P_{10} promoter (Fig. 1). The accuracy of pAcP_{10}DPPIV was confirmed by PCR and automated sequencing. The expected recombinant DPP-IV protein (rDPP-IV) possessed a 6His-tag fused to its C-terminus. A recombinant baculovirus, vAcP_{10}DPPIV, was generated from Sf21 insect cells by co-transfection with pAcP_{10}DPPIV and linear AcRP23.LacZ DNA (PharMingen) following the manufacturer’s protocol. A single recombinant baculovirus was selected after 3 rounds of plaque assay, and propagated in Sf21 cells. Plaque titration of the virus was determined following the standard protocol described by O’Reilly et al. The titer of virus stock was $2.32 \times 10^9$ plaque-forming units (pfu) per mL for recombinant baculoviruse vAcP_{10}DPPIV.

Expression and collection of rDPP-IV in Sf-21 cells. Sf21 cells were first seeded in cell culture flasks ($5 \times 10^6$ cells/25 cm$^2$ flask). After attachment of the cells, the medium was removed and the cells were infected with vAcP_{10}DPPIV at multiplicities of infection (m.o.i) of 1, 5, 10, or 15 for 48, 72, 96, or 120 h. After infection, the cells were precipitated by centrifugation at $10,000 \times g$ for 15 min, and the culture medium containing the majority of rDPP-IV was collected.
Purification of rDPPIV. Purification of 6His-tagged rDPP-IV under native conditions was carried out at 4°C by nickel-chelated affinity chromatography (Ni-NTA, Novagen, Darmstadt, Germany) following the manufacturer’s protocol. A culture medium of infected Sf21 cells containing rDPP-IV was dialyzed against a binding buffer (50 mM NaPO₄, 0.5 mM NaCl, pH 8.0), combined with 5 mL of Ni-NTA slurry in the binding buffer, and incubated with agitation overnight at 4°C. The slurry was poured into a His-bind quick column and drained. The column was washed with 10 volumes of the binding buffer and 6 volumes of wash buffer (500 mM NaCl, 20 mM Tris-HCl, and 60 mM imidazole, pH 7.9), and then eluted by adding 5 ml of the binding buffer supplemented with 250 mM imidazole. The eluted rDPP-IV was collected and dialyzed with 1 x PBS buffer prior to storage and further analysis.

SDS-PAGE, Western blot and glycoprotein staining. Protein concentrations were determined with a protein assay kit (Bio-Rad., Hercules, CA) using 1 serial dilutions of bovine serum albumin (BSA) to plot a standard curve. Samples were prepared by mixing 15-μL aliquots with equal volumes of 2× sample buffer, and all samples were boiled for 5 min and stored at 4°C prior to electrophoresis. SDS-PAGE was conducted in a 6% polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue R250. For western blot analysis, proteins resolved on SDS-PAGE were transferred to a PVDF membrane (PerkinElmer, Wellesley, MA) in a Bio-Rad Trans-Blot system following the manufacturer’s instructions. The membrane was subjected to immunodetection using mouse anti-His IgG (GE Healthcare, New York, NY) (1:500) and goat anti-mouse IgG-horseradish peroxidase (Jackson ImmunoResearch., West Grove, PA) (1:1,000) as primary and secondary antibodies respectively. Immunological detection was done using an enhanced chemiluminescence western blotting luminal reagent (Perkin Elmer, Wellesley, MA) and quantified with a Fujifilm LAS-3000 chemiluminescence detection system (Fuji Photo Film, Tokyo). A glycoprotein staining assay was conducted by SDS-PAGE as described by the
manufacturers of the Gelcode glycoprotein staining kit (Thermo Fisher Scientific,
Rockford, IL).

**LC-MS/MS of rDPP-IV.** The expected protein band of rDPP-IV resolved on
SDS-PAGE was manually excised from the gel and ground into pieces. The gel pieces
were washed twice with 50% acetonitrile and 20 mM NH₄HCO₃ for 15 min. The
protein in the gel was then reduced at 56°C for 15 min in 10 mM dithiothreitol and
alkylated with 10 mM iodoacetic acid in 10 mM ammonium bicarbonate, followed by
overnight in-gel digestion at 37°C with 0.1 µg of TPCK-treated modified porcine
trypsin (Promega, Madison, WI) in 10 mM ammonium bicarbonate. The supernatant
containing the resulting tryptic peptide was combined with those extracted twice from
the gel pieces with 50% acetonitrile/1% formic acid and subjected to LC/MS-MS
(UltiMate 3000, Bruker Daltonics, Dionex, MA) at the Biotechnology Center at China
Medical University, Taiwan.

**Determination of the enzymatic activity of rDPP-IV.** The enzymatic activity of
rDPP-IV was measured by the release of p-nitroanilide (pNA) from a synthetic
substrate, Ala-Pro-pNA, Gly-Pro-pNA, Glu-Pro-pNA, Glu-Ala-pNA, Lys-Ala-pNA,
Lys-Pro-pNA, or Ser-Pro-pNA (final concentration 0.3 mM) in 20 mM Tris, 20 mM
KCl, 0.1 mg/mL BSA, and 1% DMSO, pH 7.4, following a method reported
previously. One unit of rDPP-IV activity was defined as the amount of enzyme that
liberates 1 µmol p-NA per min at 37°C. To measure the expression levels of rDPP-IV
at various m.o.i. values, the substrate solution was mixed with 30 µL of reaction
buffer and then incubated with 70 µL of the culture medium from
vAcP₁₀DPPIV-infected Sf21 cells for 30 min at 37°C. Enzyme activity was measured
at 405 nm by ELISA reader. The optimal temperature and pH were determined
using the above-mentioned enzyme assay. The reaction mixture was adjusted to
desired temperature (ranging from 25 to 55°C) at pH 7.4 and desired pH (ranging
from 5 to 10) at 37°C with Ala-Pro-pNA as substrate. At the same time, PBS incubated with Ala-Pro-pNA substrate was used as an internal control. For the inhibition assay, purified rDPP-IV was incubated with 1.0 mM iodoacetamide (a cystein protease inhibitor), 1.0 mM sitagliptin (a DPP-IV inhibitor), or 1.0 mM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor).12-14) All the assays were done in triplicate, and the resulting values were averaged and analyzed by one-way ANOVA using JMP 5.01 (JMP, a business unit of SAS, 1989-2002 by SAS Institute, Cary, NC).

Results

Expression of rDPP-IV in Sf21 cells

A recombinant plasmid, pAcP10DPPIV, was constructed (Fig. 1) to generate a recombinant baculovirus, vAcP10DPPIV, for the production of V. basalis DPP-IV in Sf21 cells. To examine the expression of rDPP-IV, Sf21 cells were infected with vAcP10DPPIV or vAcMNPV (wild-type virus) at m.o.i. = 5 for 72 h. After infection, proteins extracted from the culture medium were resolved on a SDS-PAGE gel. Compared with proteins in the culture medium of uninfected Sf21 cells and of cells infected with vAcMNPV, an extra protein band of approximately 95 kDa was found in the culture medium of Sf21 cells infected with vAcP10DPPIV and was immunologically recognized with the anti-6His antibody (Fig. 2). While the majority of rDPP-IV was found in the culture medium, only a minor amount of it was detected within the Sf21 cells (data not shown).

Optimal conditions for rDPP-IV production in infected Sf21 cells

To explore the optimal expression conditions for rDPP-IV production, Sf21 cells were infected with vAcP10DPPIV at m.o.i. of 1, 5, 10, or 15 for 72 h and at m.o.i. of 10 for 48, 72, 96, or 120 h. The expression level of rDPP-IV was monitored by measuring its dipeptidyl peptidase activity in a culture medium of infected Sf21 cells. The results
indicated that the optimal expression condition for rDPP-IV production in Sf21 cells was an m.o.i. of 10 for 96 h (Fig. 3). As expected, no dipeptidyl peptidase activity was detected in the culture medium of uninfected Sf21 cells (data not shown).

**Affinity purification, glycostaining and LC/MS-MS confirmation of rDPP-IV**

The rDPP-IV in the culture medium of Sf21 cells infected with vAcP10DPPIV in the optimal condition was subjected to further purification by nickel-chelated affinity chromatography. As revealed by SDS-PAGE and Western blot, the purified rDPP-IV (about 95 kDa) recognized by the anti-6His antibody was the predominant protein representing 90% of the total proteins eluted from the Ni-NTA column (Fig. 4A). In addition, we found that the dimeric rDPP-IV (190 kDa) was weakly detected by the anti-6His antibody (Fig. 4A). Glycoprotein staining assay indicated that rDPP-IV was glycosylated (Fig. 4B). To confirm the correctness of rDPP-IV expressed in Sf21 cells, the affinity-purified 95 kDa protein was subjected to LC-MS/MS analysis. Five peptide fragments cumulatively corresponding to 68 amino acid residues of DPP-IV protein sequence were identified in the LC-MS/MS analysis. The predicted N-terminal membrane anchoring segment, transmembrane domain, 5) is shown in Fig. 4C. Taken together, these results indicate that the affinity-purified 95 kDa protein was rDPP-IV correctly expressed as a glycosylated His-tag fusion protein in Sf21 cells. By calculation, the recovery yield of this one-step Ni-NTA affinity column purification was 14.6% with 5.5-fold purification (Table 1). Approximately 6.4 mg of purified rDPP-IV was obtained per liter suspension culture containing $1 \times 10^9$ infected Sf21 cells.

**Enzymatic activity of purified rDPP-IV**

Among the five synthetic substrates examined in this study, Ala-Pro-pNA and Glu-Pro-pNA were hydrolyzed most and least efficiently by rDPP-IV respectively (Table 2). The $k_{cat}/K_m$ values in a range of 10-500 mM$^{-1}$·S$^{-1}$ for the seven synthetic...
substrates (Table 2).

The optimal temperature and pH for the enzymatic cleavage of Ala-Pro-pNA by rDPP-IV were determined to be 50°C and pH 9 (Fig. 5). The optimal activity was similar to a purified DPP-IV from porcine kidney.\(^{15}\) As expected as for the inhibition assay, the enzymatic activity of rDPP-IV was significantly reduced by 80 or 60% in the presence of sitagliptin (a DPP-IV inhibitor) or PMSF (a serine protease inhibitor), but was not apparently affected by iodoacetamide (a cysteine protease inhibitor) (Fig. 6).

**Discussion**

The venoms of wasps and bees contain a variety of lytic peptides, such as mastoparan and melittin.\(^{16,17}\) Regardless of the drastic difference in both size and sequence between mastoparan B and melittin, precursor polypeptides of these two toxin peptides share a comparable structural organization of a signal sequence, an anionic prosequence of 11 conservative dipeptides, the cationic toxin peptide, and a C-terminal glycine.\(^5\) Similarly, proline and alanine were exclusively found in alternate positions of the prosequences of the precursor polypeptides of mastoparan B and melittin (Fig. 7). On the basis of the observation that DPP-IV from pig kidney released dipeptides from the N-terminus of promelittin, the prosequence was proposed to be removed via sequential liberation of dipeptides presumably catalyzed by a DPP-IV in the venom gland of honeybee *Apis mellifera*.\(^{18}\) Though dipeptidyl peptidase activity was detected in the extract of venom gland of honeybee, neither DPP-IV was purified and characterized nor was its corresponding gene cloned and analyzed.

According to previous reports, human DPP-IV is not only a dimeric protein, but also is a membrane-bound glycoprotein.\(^8,19,20\) In this study, we found that purified His-tagged *V. basalis* DPP-IV is mainly monomeric form, but the dimeric form (190 kDa) was also weakly detected by the anti-6His antibody (Fig. 4A). This is consistent
with the human DPP-IV expressed in Sf9 insect cells and then purified by nickel-chelated affinity chromatography. Hence, we predict that expressed *V. basalis* rDPP-IV also possesses dimeric conformation via the baculovirus expression system. We will further study the expressed rDPP-IV its structure and function. In this study, we successfully expressed and purified *V. basalis* DPP-IV as a glycosylated His-tagged fusion protein via the baculovirus expression system. Additionally, the purified rDPP-IV was found to possess enzymatic activity of dipeptidyl peptidase *in vitro* and the optimal activity of rDPP-IV was at 50°C and pH 9 (Fig. 5), similar to previously reports. On the basis of some reports, we speculate that the optimal activity for rDPP-IV involves important physiologic mechanisms of *V. basalis*, such as defense or antagonist of the external stress.

For further determine the biological activity of *V. basalis* rDPP-IV in order to remove the prosequence of mastoparan B by specific cleavage of alanine or proline residues in the penultimate position, three synthetic substrates, Glu-Pro-pNA, Glu-Ala-pNA, Lys-Ala-pNA, were also selected to determine the enzyme activity of rDPP-IV. Results indicated that the expressed rDPP-IV possesses functional potential in sequential liberation of dipeptide from the prosequence of mastoparan B (Table 2). This also confirms that prosequence in the precursor protein of mastoparan B is removed via sequential liberation of dipeptides during post-translational processing.

The sequential liberation of dipeptides has been proposed to be a mechanism by which the premature release of a peptide is prevented as it can rupture membrane by interacting with phospholipids. Such a ticketing mechanism might possess a built-in time scale to guarantee a clean temporal and spatial separation between export and activation of toxin peptides. In addition, the negatively charged residues predominantly found in prosequences (Fig. 7) are presumably involved in ionic interactions with the cationic toxin peptides in the transit conformation of their proproteins. In this study, the negatively charged Glu-Pro-pNA was hydrolyzed least efficiently among the seven synthetic substrates examined, by rDPP-IV (Table 2).
The relatively weak activity of rDPP-IV on Glu-Pro-pNA appears to agree with the proposed mechanism, in which post-translational processing is slowed deliberately.

The deduced amino acid sequence of *V. basalis* DPP-IV with a theoretical molecular mass of 89 kDa contains eight potential N-glycosylation sites mainly present in the β-propeller domain of its modeling structure. Although N-linked glycosylation of DPP-IV does not contribute significantly to its peptidase activity, it is generally accepted that glycosylation of DPP-IV is a prerequisite for its enzymatic activity and correct protein folding. In this study, the observed molecular mass (95 kDa) of rDPP-IV on SDS-PAGE was slightly higher than that (90 kDa) calculated from the amino acid sequence of this recombinant His-tag fusion protein. The size difference presumably resulted from post-translational glycosylation of rDPP-IV during synthesis of it via the baculovirus expression system. Possibly, the glycosylation of rDPP-IV found in this study might be the key factor that led to the success of functional expression of this processing enzyme.

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**References**


Figure legends

**Fig. 1.** Schematic Representation of the Construction of the Recombinant Baculovirus Transfer Vector, pAcP_{10}DPPIV, with Polyhedron (polh) Promoter Expressing rDPP-IV.

**Fig. 2.** SDS-PAGE and Western Blot Analysis of rDPP-IV Secreted in the Culture Medium of Infected Sf21 Cells. Culture media were collected from uninfected Sf21 cells and those cells infected with wild-type virus or vAcP_{10}DPPIV at m.o.i. of 5 for 72 h. Proteins in the culture media were resolved on a 6% SDS-PAGE gel (left panel). A duplicate gel was transferred to a PVDF membrane and then subjected to immunoblotting with detection by anti-6His antibody (right panel). Arrows indicate the positions of rDPP-IV.

**Fig. 3.** Optimal Infection Conditions for rDPP-IV Production in Sf21 Cells. To determine optimal conditions, rDPP-IV expression was examined by infecting Sf21 cells with vAcP_{10}DPPIV at m.o.i. of 1, 5, 10, or 15 for 72 h (A), and at m.o.i. of 10 for 48, 72, 96, or 120 h (B). Enzymatic activity of dipeptidyl peptidase in the culture medium was measured to determine the expression level of rDPP-IV.

**Fig. 4.** Analysis and Identification of Affinity-Purified rDPP-IV. (A) The expressed rDPP-IV was purified by nickel-chelated affinity chromatography. Purity of rDPP-IV was examined by SDS-PAGE and Western blot. Bold arrows indicate the positions of monomeric rDPP-IV and broken arrow indicate the position of dimeric rDPP-IV. (B) Glycostaining of the purified rDPP-IV. BSA was used as positive control. The arrow indicates the position of the glycosylated rDPP-IV. (C)
Identification of rDPP-IV by LC-MS/MS analysis. Five peptide fragments of rDPP-IV identified in the LC-MS/MS analysis are shown in bold.

**Fig. 5.** Optimal pH and Temperature for rDPP-IV.

The enzymatic activity of rDPP-IV was detected at pH 7.4 to measure optimal temperature (A) and at 37°C to measure optimal pH (B) with Ala-Pro-pNA as substrate.

**Fig. 6.** Effects of Three Inhibitors, Iodoacetamide, Sitagliptin and PMSF on the Enzymatic Activity of rDPP-IV.

These data were obtained from three replicated experiments and are shown as means ± standard derivation.

**Fig. 7.** Sequence Comparison of the Anionic Prosequences of 11 Conservative Dipeptides in the Precursor Proteins of Mastoparan B and Melittin.

Proline and alanine residues found at alternate positions of prosequences are boxed. Negatively charged residues (D and E) are shown in bold.