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2.32×10^9 plaque-forming units (pfu) per ml for the recombinant baculovirus vAcP₁₀DPPIV.

C. Expression of rDPP-IV in the Sf-21 cells

The cells were infected with the recombinant baculoviruses vAcP₁₀DPPIV at multiplicities of infection (m.o.i) of 10 for 72 h. The infected cells were harvested and homogenized in lysis buffer containing 500 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% NP-40, and a protease inhibitor cocktail (Sigma, USA), then sonicated with a 3 mm-diameter probe in an ultrasonic processor GE 601 for 6×30 s. The culture supernatant was clarified by centrifugation at $10,000 \times g$ for 15 min at 4 °C and was then filtered with a 0.22-μm filter membrane. Sample identity was analyzed by SDS-PAGE and Western blot with anti-His antibodies, as described below.

D. SDS-PAGE and Western blot analyses

The soluble protein was quantified using a protein assay kit (Bio-Rad Lab., Hercules, CA) with bovine serum albumin (BSA) as a standard. Samples were prepared by mixing 15-μl aliquots with an equal volume of $2 \times$ sample buffer, and all samples were boiled for 5 min and stored at 4 °C prior to electrophoresis. SDS-PAGE was conducted in 15% polyacrylamide gel and visualized by staining with Coomassie brilliant blue G250. For western blot analysis, proteins resolved in SDS-PAGE were transferred to a PVDF membrane (PerkinElmer, Wellesley, USA) in a Bio-Rad Trans-Blot system according to the manufacturer's instructions. The membrane was subjected to immunodetection using a polyclonal mouse anti-His IgG (GE Healthcare, NY, USA) (1:500) and goat anti-mouse IgG-horseradish peroxidase (Jackson ImmunoResearch Lab., PA, USA) (1:1000) as primary and secondary antibodies, respectively. The immunoblotted proteins were visualized using an enhanced chemiluminescence ECL western blotting luminal reagent (PerkinElmer, Wellesley, USA) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

E. Purification of rDPPIV

6His-tagged rDPP-IV was purified using a Ni-NTA affinity column under native conditions. All purification steps were carried out at 4 °C. Infected Sf21 cell culture supernatant and infected larvae extracts containing rDPP-IV were dialyzed by native binding buffer (50 mM NaPO₄, 0.5 M NaCl, pH 8.0). The dialyzed supernatant was combined with 5 ml of 50% Ni-NTA slurry (Novagen, Darmstadt, Germany) in binding buffer and incubated with agitation overnight. The slurry was poured into a His-bind quick column and drained. The column was then washed with 10 volumes of lysis buffer and 6 volumes of wash buffer (500 mM NaCl, 20 mM Tris-HCl, and 60 mM imidazole, pH 7.9) and eluted with native elution buffer (binding buffer plus 250 mM imidazole). Purified samples containing rDPP-IV were verified by SDS-PAGE, and western blot analyses. The purified rDPP-IV sample from infected cell culture supernatant was quantitatively analyzed by scanning and digitizing the immunoblotting membrane using an Alpha-Imager image-analyzing system (Alpha Innotech, San Leandro, CA) and the computer program AlphaImager™2200 version 5.5. One microgram of purified rDPP-IV protein was used as a reference for calculation. Data were collected from triplicate experiments, 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, CA).

F. MALDI-MS of rDPP-IV

The expected protein band of rDPP-IV resolved in SDS-PAGE was manually excised from the gel and ground into pieces. The gel pieces were washed twice with 50% acetonitrile and 10 mM NH₄HCO₃ for 15 min. The protein in the gel was then reduced and alkylated at 56 °C for 15 min in 10 mM dithiothreitol and 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 by 50% acetonitrile / 1% formic acid and subjected to LC/MS-MS (UltiMate 3000, Bruker Daltonics, Dionex, MA, USA) at the Biotechnology Center at China Medical University, Taiwan.

G. Determination of the activity of rDPP-IV

The activity of rDPP-IV was measured by cleavage of the Gly-Pro-pnitroanilide substrate in phosphate buffered saline (20 mM Tris, 20mM KCl, 0.1mg/ml BSA and 1% (w/v) DMSO, pH 7.4). The final concentration of the Gly-Pro-pnitroanilide was 0.3 mM. One unit of rDPP-IV activity was defined as the amount of enzyme that liberates 1 μmol pNA per min at 37 °C. For detect the rDPPIV expression levels at different m.o.i. values, the substrate solution was mixed with 30 μl phosphate buffered saline, and then incubated with 70 μl cells supernatant fraction from vAcP₁₀DPPIV-infected Sf21 cells for 30 min at 37° C. About the inhibition assay was performed with a known protease inhibitor, Iodoacetamide and PMSF. The rDPP-IV activity was measured via a standard kinetic assay, using the chromogenic substrate Ala-Pro-p-nitroanilide dissolved in phosphate buffered saline, pH 7.4. Data are expressed as relative concentrations obtained from ELISA readings under 405 nm with reference to an internal control. All the assays were done in triplicate following the method reported previously [12].

H. Statistical analysis

All statistical analyses were performed and evaluated by one-way ANOVA using JMP 5.01 software (JMP, 1989-2002, by SAS Institute, Cary, CA, USA) while a *P* value of <0.05 was considered to be statistically significant.

III. RESULTS

A. Amino acid sequence analysis of *V. basalis* DPP-IV

We have cloned and sequenced the putative DPP-IV from *V. basalis* venom gland cDNA library [8]. Analysis of the sequence revealed a complete open reading frame of 2328 nucleotides, which encodes a protein of 775 amino acids. While the *V. basalis* DPP-IV sequence was compared with other known DPP-IV amino acid sequences that the identities to *Stenotrophomonas maltophilia* (23.1%), *Aedes aegypti* (37.9%), mouse (31.9%), cow and human (32.5%). Among these other DPP-IV sequence, *V. basalis* DPP-IV compared with *Apis mellifera* (*A. mellifera*) and *Vespula vulgaris* (*V. vulgaris*) DPP-IVs that show significant amino acid sequence identity of 54.4 and 85.8%, respectively (Fig. 1). By contrast, *V. basalis* DPP-IV is more similar to *V. vulgaris* DPP-IV than to

other DPP-IV enzymes. Although the overall homology level is low with other DPP-IVs, the C-terminal part involving the active residues (Ser, Asp, and His) is well conserved in *V. basalis* DPP-IV. From the multiple sequence alignments of DPP-IV of the confirmed active site residues with that of the *V. basalis* DPP-IV, it is apparent that Ser-637, Asp-716, and His-756 represent the catalytic triad in this protein. Furthermore, comparison with the three-dimensional structure reveal that the *V. basalis* DPP-IV is homologous to human DPP-IV (Fig. 2) whose three-dimensional structure has been determined by X-ray crystallography [13,14].

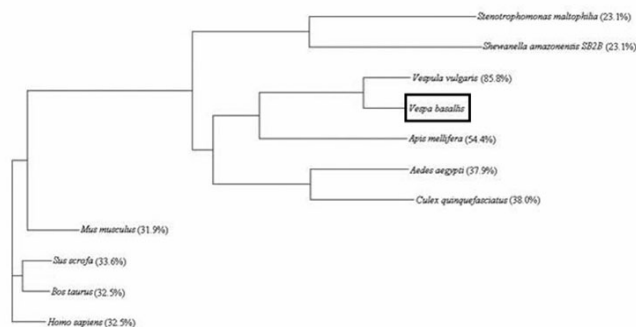


Fig. 1 Dendrogram showing the relatedness of *S. maltophilia*, *S. amazonensis*, *A. mellifera*, *A. aegypti*, *Sus scrofa*, *Bos Taurus*, human, and *Mus musculus* DPP-IVs, and *Vespula vulgaris* to *Vespa basalis* DPP-IV. The amino acid sequences of each protein were aligned using ClustalW. The dendrogram was constructed by the neighbor joining method. Clustering computations were carried out using the Vector NTI Advance™ 9.0 life science software (Invitrogen™, USA)

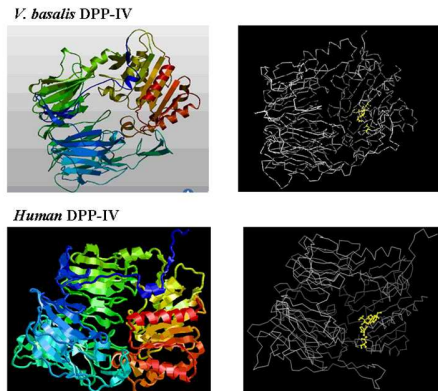


Fig. 2 *V. basalis* DPP-IV and human DPP-IV in three-dimensional structure comparison. The three-dimensional structure of *V. basalis* DPP-IV was modeling by SWISS MODEL Workspace. Backbone stereoscopic conformation with active site was indicated by the atomistic picture (left) and ribbon conformation (right) of DPP-IV 3D structure

B. Expression of rDPP-IV in Sf21 cells

For characterization of *V. basalis* DPP-IV, we intended to employ the baculovirus expression vector system (BEVS) to express active *V. basalis* DPP-IV in *Spodoptera frugiperda* cells. A recombinant plasmid, pAcP₁₀DPP-IV, was constructed (Fig. 3) to generate a recombinant baculovirus, vAcP₁₀DPP-IV, for the production of *V. basalis* DPP-IV in Sf21 cells. To evaluate the expression of rDPP-IV by BEVS, Sf21 cells were infected with vAcP₁₀DPP-IV at m.o.i. = 5 for 72 h. As shown in Fig. 4, an additional and clearly visible protein band of approximately 90 kDa (close to the molecular mass of the

rDPP-IV-6His) was found in the vAcP₁₀DPP-IV-infected culture supernatants on a Coomassie blue stained gel and Western blot analysis, while no protein band was detected in the uninfected culture supernatants and wild type vAcMNPV-infected culture supernatants. In contrast, only a few amount of the rDPP-IV was detected in the vAcP₁₀DPP-IV-infected Sf21 cells pellet (data not shown). The results indicated that the matured form of rDPP-IV was mostly secreted into the culture medium in the vAcP₁₀DPP-IV-infected Sf21 cells. The rDPP-IV expression level was monitored by measuring the specific dipeptidyl-peptidase activity. Expression was optimized by variation of the virus titer and time for infection; the highest specific activity was determined at an m.o.i. 10 and was attained 72 h post-infection (Fig. 5), while uninfected culture supernatants and wild type vAcMNPV-infected culture supernatants do not show the dipeptidyl-peptidase activity.

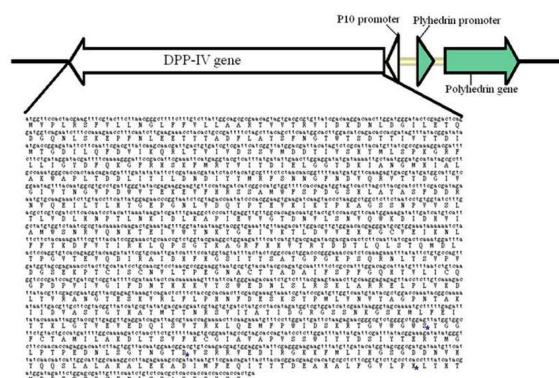


Fig. 3 Schematic presentation of the construction of the baculovirus transfer vectors pAcP₁₀DPP-IV. The sequence of *V. basalis* and its deduced protein with the 6His-tag are shown in the lower panel. Location of active site residues (Ser-637, Asp-716, and His-756) are indicated by stars

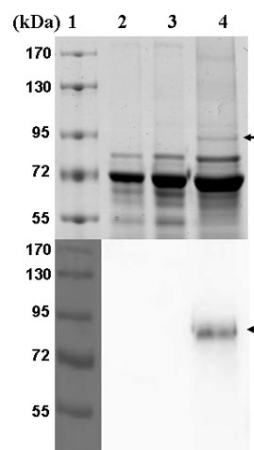


Fig. 4 Detection of secreted form of rDPP-IV protein in Sf21 culture supernatant. Culture supernatant fractions were collected from Sf21 cultures infected with recombinant baculoviruses vAcP₁₀DPP-IV at an m.o.i. of 5 for 72 h and were then separated by 6% SDS-PAGE gel (upper panel) and Western blot analysis using an anti-His₆ antibody (lower panel). Lane 1, marker proteins; Lane 2, uninfected cells; Lane 3, wild type vAcMNPV-infected cells; Lane 4, vAcP₁₀DPP-IV-infected cells. The solid arrows represent the position of the rDPP-IV protein

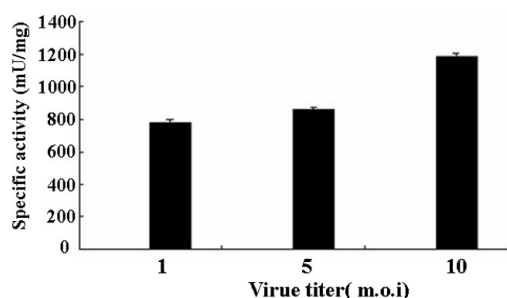


Fig. 5 Optimization of rDPP-IV expression. The vAcP₁₀DPPIV-infected Sf21 cells at different m.o.i.=1, 5, 10 or 15 for 72 h and then culture supernatant fractions were collected for determined the optimization of rDPP-IV expression

C. Assay the activity of rDPP-IV

For analysis the activity of rDPP-IV, the rDPP-IV was further purified from the soluble extracts of vAcP₁₀DPPIV-infected cells culture supernatants by nickel-chelated affinity chromatography [15] and then was confirmed by LC/MS/MS. The rDPP-IV expression rates were 6.4 mg protein per liter suspension culture (1×10^9 cells) (data not shown). The results of analyses show that *V. basalis* rDPP-IV activity exhibited classical Michaelis–Menten kinetics, with K_m and V_{max} extrapolated, to be 0.22 mM and 0.0006 S^{-1} , respectively. *V. basalis* rDPP-IV catalyzed the hydrolysis of Ala–Pro–pNA rapidly, with substantial activity measured within 30min of the addition of substrate. After an initial rapid increase in activity, near maximal activity (7.43 ± 0.24 nmol product formed) measured at 180min (Table I).

TABLE I
SUBSTRATE SPECIFICITY OF THE rDPP-IV

Substrate	K_m (mM)	V_{max} (S^{-1})	K_{cat} (S^{-1})	K_{cat}/K_m ($\text{mM}^{-1} \cdot \text{S}^{-1}$)
Gly-Pro-pNA	1.00	2.98×10^{-4}	52.67	52.41
Ala-Pro-pNA	0.22	5.91×10^{-4}	104.52	465.40
Ser-Pro-pNA	0.56	4.84×10^{-4}	85.54	150.40
Lys-Pro-pNA	0.57	2.74×10^{-4}	48.35	84.04
Glu-Pro-pNA	2.21	1.76×10^{-4}	31.06	14.05

On the other hand, the inhibition assay which 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).

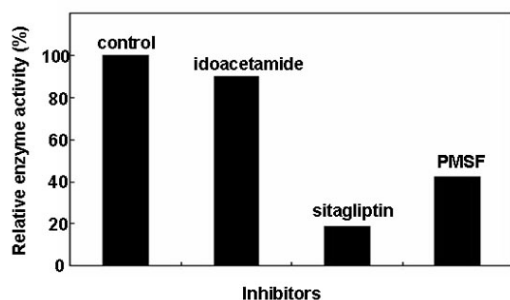


Fig. 6 Effects of three inhibitors, iodoacetamide, sitagliptin and PMSF on the enzymatic activity of rDPP-IV. These data were obtained from three replicated experiments and are shown as means \pm standard derivation (S.D.)

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