Characterization and Evaluation of the Activity of Dipeptidyl Peptidase IV from the Black-Bellied Hornet Vespa basalis

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Abstract—Characterization and evaluation of the activity of Vespa basalis DPP-IV, which expressed in Spodoptera frugiperda 21 cells. The expression of DPP-IV was confirmed by SDS-PAGE, Western blot analyses, LC-MS/MS and measurement of its peptidase specificity. One-step purification by Ni-NTA affinity chromatography and the total amount of rDPP-IV recovered was approximately 6.4 mg per liter from infected culture medium; an equivalent amount would be produced by 1×10^7 infected SF21 insect cells. Through the affinity purification led to highly stable rDPP-IV enzyme was recovered and with significant peptidase activity. The rDPP-IV exhibited classical Michaelis-Menten kinetics, with kcat/Km in the range of 10-500 mM^-1S^-1 for the five synthetic substrates and optimum substrate is Ala-Pro-pNA. As expected in 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 PMSE (a serine protease inhibitor), but was not apparently affected by iodoacetamide (a cysteine protease inhibitor).

Keywords—Dipeptidyl Peptidase IV; Phenylmethylsulfonyl fluoride; Serine protease; Sitagliptin; Vespa basalis

I. INTRODUCTION

Dipeptidyl-peptidase IV (DPP-IV) is a glycosylated serine protease that selectively removes dipeptides from the N-terminus of peptides or proteins, which preferentially cleaves at their N-terminal and liberates either X-Pro or X-Ala dipeptide [1]. As we known that DPP-IV plays an important role for the processing of precursors of bioactive peptides and proteins in various tissues [2]. As reports, human DPP-IV modulates the maturation processing of functional peptide, such as hormones, chemokines and neuropeptides [3]. DPP-IVs except were found in animal tissue, were found in bacteria, Xenopus laevis, Drosophila melanogaster, Vespula vulgaris (V. vulgaris) and Vespa basalis (V. basalis) etc. [4-8]. Among these DPP-IVs, a DPP-IV was cloned from the venom gland of V. basalis in our previously study [8]. Noteworthy, V. basalis DPP-IV has not been fully elucidated and characterized in the present. By contrast, V. vulgaris DPP-IV has been clearly demonstrated that it modulates the maturation processing of melittin, which is a principal active component of bee venom (apitoxin) and is a powerful stimulator of phospholipase A2 [7].

The V. basalis DPP-IV cDNA fragment (GenBank Accession No. DQ661743) which comprises 2905 nucleotides, including a 5’ untranslated region of 116 nucleotides, an open reading frame of 2328 nucleotides, and a 3’ untranslated region of 461 nucleotides [8].

The deduced amino acid sequence showed that the molecular weight is 89-kDa within the coding region. V. basalis DPP-IV contains nine potential N-glycosylation sites mainly present in the β-propeller domain of the molecule. Although, the previously report demonstrate that N-linked glycosylation of DPP-IV does not contribute significantly to its peptidase activity [9], but it has been generally accepted that glycosylation of DPP-IV is a prerequisite for enzyme activity and correct protein folding [10]. To provide the purpose of this study for characterization and evaluation of V. basalis DPP-IV, we intended to employ the baculovirus expression vector system (BEVS), a powerful eukaryotic expression system, to express and purify active V. basalis DPP-IV in Spodoptera frugiperda cells.

II. MATERIALS AND METHODS

A. Insect cells

The insect cell line used in this study was SF21 (Invitrogen, USA), which was originally isolated from the ovarian tissue of Spodoptera frugiperda (fall armyworm). The cells were routinely cultured at 26 °C in TNI-FH basal medium (Sigma, USA) and supplemented with 10% fetal bovine serum (FBS, Hyclone). The SF21 cells were passaged 2 times weekly, and fresh media was provided every 2 days.

B. Generation of recombinant baculovirus

The recombinant baculovirus pAcP6-DPPIV was generated from the plasmid pAcP6-DPPIV containing the DPPIV gene (accession No. DQ661743) fused with an additional 6His tag at the carboxyl terminus. The DPPIV gene was amplified by using the primers: 5’CGGATCCATGGTCCACTCGAACGTTCGA3’ (BamHI site underlined) and 5’ACGAATTCTCAGTGATGTTGCTGGTGAGACGCAGATGGAA3’ (EcoRI site underlined). Plasmid pAcP6-DPPIV was constructed from the transfer vector pAcuW21 (PharMingen, San Diego, CA), in which the DPPIV gene was introduced into BgII and EcoRI restriction sites, and thus directed by the P10 promoter. The resulting plasmid, pAcP6-DPPIV, was confirmed by PCR and automated sequencing. The recombinant baculovirus vAcP6-DPPIV was generated from SF21 insect cells by co-transfection with plasmid pAcP6-DPPIV DNA and linearized AcRPA23.LacZ DNA (PharMingen), as suggested by the manufacturers. A single recombinant baculovirus was selected after three rounds of plaque assay. The recombinant baculovirus was propagated in SF21 cells, and plaque titration of the virus was determined according to the standard protocol described by O’Reilly et al. [11]. A titer of virus stock was
2.32x10^8 plaque-forming units (pfu) per ml for the recombinant baculovirus vAcP_9DPP IV.

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

The cells were infected with the recombinant baculoviruses vAcP_9DPP IV 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 x 30 s. The culture supernatant was clarified by centrifugation at 10,000 x g for 15 min at 4 °C and was then filtered with a 0.22-mm 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 x 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 detection system (Tokyo, Japan).

E. Purification of rDPP IV

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 NaPO_4, 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 quantified using a protein assay kit (Bio-Rad Lab., Hercules, CA) and the computer program AlphaImagerTM2000 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% acetoni triole and 10 mM NH_4HCO_3 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% acetoni triole / 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, 20 mM KCl, 0.1 mg/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 umol 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 vAcP_9DPP IV-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

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 Vespuella 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].

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, pAcP_{10}DPPIV, was constructed (Fig. 3) to generate a recombinant baculovirus, vAcP_{10}DPPIV, 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 vAcP_{10}DPPIV 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 vAcP_{10}DPPIV-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 vAcP_{10}DPPIV-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 vAcP_{10}DPPIV-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.
fractions were collected for determined the optimization of rDPP-IV expression 10 purified from the soluble extracts of vAcP
-1 extrapolated, to be 0.22 mM and 0.0006 S
of analyses show that near maximal activity (7.43 ± 0.24 nmol product for med)
addition of substrate. After an initial rapid increase in activity, basalis
9 The rDPP-IV expression rates were 6.4 mg protein per liter
chromatography [15] and then was confirmed by LC/MS/MS.

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).

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REFERENCES


