Amsacta moorei Entomopoxvirus Encodes a Functional Esterase (*amv133*) with Protease Activity

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Key Words

Amsacta moorei entomopoxvirus · Lipase · Esterase · Protease · *amv133*

Abstract

Objectives: Lipolytic genes have been investigated in several viral genomes, and some of them show enzyme activity which can be used for various functions including the production of DNA replication metabolites, rescue from endosomes, and membrane fusion. *Amsacta moorei* entomopoxvirus (AMEV) replicates in nearly the entire insect body, especially in the adipose tissue. One of the open reading frames (ORFs) in the AMEV genome, *amv133*, encodes a putative lipase enzyme. In this study, we therefore investigate the enzyme activity of *amv133*. **Methods:** *amv133* was aligned with known lipase genes and their homologs in entomopoxviruses. Expressed proteins were partially purified and assayed for lipase, esterase and protease. **Results:** We found that *amv133* contains all the domains required for a functional lipase enzyme and that it shows a significant similarity with homologs in other entomopoxviruses. Since there is a similarity of the catalytic triad between lipases and serine proteases, we also investigated the protease activity of *amv133*. Lipase, esterase and protease assays showed that *amv133* encodes a functional esterase enzyme with protease activity. **Conclusion:** The current data show that *amv133* is a

conserved gene in all entomopoxvirus genomes sequenced so far and might contribute greatly to degrading the lipids or proteins and hence improve the virus infection.

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Introduction

Amsacta moorei entomopoxvirus (AMEV) is the type species of betaentomopoxvirus encompassing Lepidoptera (moths) and Orthoptera (grasshoppers) [1]. Therefore, AMEV could be used as a microbiological control agent against some harmful insects in agriculture [2]. AMEV is the best and most studied entomopoxvirus because it is one of the few insect poxviruses which can be readily grown and manipulated in tissue culture [3, 4]. Its replication takes place in various larval tissues, but it prefers fat body cells [5], which are the major site of lipid storage [6]. A complete genome analysis of AMEV showed that it has 232,392 base pairs [7] and contains 294 open reading frames (ORFs) [2]. *amv133* is one of the ORFs and encodes a putative triacylglycerol lipase [7].

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) and esterases (carboxylic ester hydrolases; EC 3.1.1.1), collectively known as lipolytic enzymes, are characterized by their ability to hydrolyze hydrophobic long- and short-chain carboxylic acid esters [8]. Most of these hydrolases

contain the consensus sequence Gly-X-Ser-X-Gly (X = any amino acid) around the active site of serine and function via a Ser-Asp-His catalytic triad [9]. Some lipases share a common Ser-Asp/Glu-His catalytic triad with serine proteases [10, 11]. Some of the serine proteases, such as chymotrypsin and subtilisin, show lipase activity; however, not all lipases tested show serine protease activity, except commercially available porcine pancreatic lipase [11–13].

Lipase-like genes have been found in several viral genomes showing lipase activity, including *p37* from vaccinia virus [14], *vp1* from adeno-associated virus [15, 16] and *vp1* from parvovirus [17, 18], and they have several different functions, such as facilitating membrane fusion and escape from endosomes. *vlip* from Marek's disease virus [19] and *orf19* from *Heliothis virescens* ascovirus [20] do not show lipase activity. Ectromelia virus [21] and *Melanoplus sanguinipes* entomopoxvirus (MSEV) [22] have *amv133*-similar genes that are thought to play a role in the viral inflammatory response [7]. Furthermore, 5 different entomopoxviruses have homologs to *amv133* [2]; however, none of them were characterized as showing lipase-encoding activity or any other type of activity.

Proteases are toxic to insects because they degrade the peritrophic matrix [23], and serine proteases have been shown to increase the viral speed with which the host is killed [24]. *amv133* has a catalytic triad of serine proteases.

The importance of lipid metabolism for viral growth and examples of other viral lipase genes suggest that *amv133* could be an active lipase gene. Also, because of the similarity of the catalytic triad between serine proteases and lipases, it is thought that *amv133* may also show protease activity. In our study, we investigated the lipase, esterase and protease activities of *amv133* and found that *amv133* encodes an active esterase with protease activity.

Materials and Methods

Prediction Studies

The amino acid sequence of *amv133* was predicted using the ExPASy-PROSITE program and aligned with other lipase family members using the MEGA 5.05 and GeneDoc programs.

Virus and Cells

An aliquot of AMEV and the *Lymantria dispar* (IPLB-LD-652) cells were kindly provided by Basil Arif (Laboratory for Molecular Virology, Great Lakes Forestry Centre, Canada).

The IPLB-LD-652 cells were maintained in 45% Excell-420 (SAFC Biosciences), 45% Grace's Insect Medium (Gibco), which

is serum-free, and 10% fetal bovine serum (Gibco) at 28°C. An infection of IPLB-LD-652 cells with AMEV was performed as follows: cells were seeded in T75 tissue culture flasks at 1×10^5 cells/cm² and allowed to attach at 28°C for 2 h. The medium was removed, and the virus was added at 0.1 multiplicity of infection. The flasks were incubated at 28°C and gently rocked for 2 h to allow for virus attachment. At the end of the incubation period, a fresh culture medium was added and the flasks were incubated at 28°C for 5 days. Cells were centrifuged at 1,000 g for 15 min to remove cell debris. The titer of enveloped virus stock was determined as described in previous studies [25].

DNA Extraction from AMEV

In order to extract DNA, IPLB-LD-652 cells were infected with AMEV at 0.1 multiplicity of infection, and the virus was harvested as described above. The virus stock was centrifuged at 18,000 rpm for 30 min. The resulting pellet was dissolved in 9 ml TE buffer (10 mM Tris, pH 8; 1 mM EDTA). 30 µl β-mercaptoethanol, 100 µl proteinase K, 400 µl 5 M NaCl and 1 ml 10% SDS were added, and the mixture was incubated at 37°C for 5 h. Then the mixture was incubated overnight at 4°C. 15 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the mixture which was then allowed to rotate at the lowest speed in a rotary mixer for 10 min. Then it was centrifuged at 300 g for 10 min. The supernatant was transferred to a clear tube, and 15 ml of phenol:chloroform:isoamyl alcohol was mixed and centrifuged at the same conditions. Sodium acetate (1/10 volume of 3 M solution, pH 7) and absolute ethanol (2.5 volume) were added, and the mixture was incubated on ice for 30 min and then centrifuged at 16,000 g for 15 min. After drying the pellet, it was dissolved in 200 µl dH₂O. The DNA concentration was measured by NanoDrop (Thermo Scientific).

Cloning, Expression and Purification of *amv133*

amv133 was amplified directly from the genomic DNA of AMEV using 2 pairs of primers (LIP-*NdeI*-Fw: 5'-CCATATG ACAATATTTGAAATATTAATATGG-3', LIP-*BamHI*-Rv: 5'-C GGATCCCTATTAATTTCTAAATATTTTAG-3'; LIP-*BamHI*-Fw: 5'-CGGGATCCACAATATTTGAAATATTAATATGGATA ATTG-3'; LIP-*EcoRI*-Rv: 5'-CGGAATTCTTAAATTTCTAA ATATTTTAGATTATCAATATTG-3') designed according to the *amv133* sequence of the AMEV complete genome sequence [7]. 0.5 µl Phusion DNA polymerase (NEB, Ipswich, Mass., USA) was used to perform PCR reactions with 10 ng AMEV genomic DNA (260/280 ratio: 1.9) as the template and with 10 µl 5x Phusion HF buffer, 1 µl 10 mM dNTP, 2.5 µl 10 µM forward primer, 2.5 µl 10 µM reverse primer and 32.5 µl nuclease-free water in a final volume of 50 µl. The PCR conditions were as follows: 1 initial denaturation step at 98°C for 30 s, 35 cycles at 98°C for 10 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, except for the final cycle where the extension proceeded for 5 min. The PCR fragment was cleaned from the agarose gel using the Nucleospin extraction II kit (Machery-Nagel) following the manufacturer's instructions, cloned into pJET1.2 vector (Thermo Scientific) and sequenced (Macrogen).

For the bacterial expression of *amv133*, the 864 base pair fragment was released from pJET1.2 and digested with *NdeI* and *BamHI* restriction enzymes. Then it was ligated with pET28a(+) (Novagen) and digested with *NdeI* and *BamHI*. The ligation products transformed into *Escherichia coli* BL21(DE3)pLysS (Novagen). 10 clones were inoculated into 2 ml Luria Bertani (LB) broth

medium containing 50 mg/ml kanamycin and 10 mg/ml chloramphenicol containing an overnight culture. The overnight cultures were transferred to a 5-ml LB broth medium and the optical density was adjusted to 0.1 at 600 nm. The cultures were grown until OD₆₀₀ was between 0.3 and 0.5 and induced with isopropyl β-D-1-thiogalactopyranoside at the final concentration of 1 mM. After 3 h of incubation at 30°C, the cultures were chilled on ice for 30 min, followed by a spindown at 5,000 rpm for 10 min at 4°C. The pellets were resuspended in 500 μl ice-cold PBS and incubated with 10 mg/ml lysozyme at 30°C for 15 min, followed by sonication with Labsonic M (Sartorius; 80% amplitude, 0.6 cycle for 5 min) to release intracellular proteins. The cell-free extract was centrifuged at 10,000 rpm for 30 min to remove cell debris. Supernatants were analyzed by Western blot using the anti-His antibody conjugated with alkaline phosphatase. The clone that showed the highest level of expression was used for protein expression in 1 liter LB broth medium using the same procedure as above except for a pellet resuspended in 2 ml ice-cold PBS for activity experiments.

For the baculovirus expression of *amv133*, the Bac-to-Bac Baculovirus Expression System (Invitrogen) was used. The 864 base pair fragment was released from pJET1.2 digested with *Bam*HI and *Eco*RI restriction enzymes, and then it was ligated with pFastBacHTB digested with *Bam*HI and *Eco*RI. The ligation product transformed into *E. coli* JM101 (NEB). pFastBacHTB vectors carrying *amv133* were isolated from one of the positive clones, and then they transformed into *E. coli* DH10Bac cells to regenerate bacmid expressing *amv133* (AmBac133). AmBac133 was confirmed via PCR using a pair of primers, M13Fw and M13Rv, and extracted. *Spodoptera frugiperda* cells (2×10^6 cells/ml) were transfected with the AmBac133 using Cellfectin (Invitrogen) transfection reagent according to the Bac-to-Bac manual. The transfected cells were incubated at 27°C until the cells showed visible signs of infection (usually 72 h or more). The expression of *amv133* was confirmed by Western blot analysis using anti-His monoclonal antibody conjugated with alkaline phosphatase (1:5,000; Sigma). Recombinant baculovirus expressing *amv133* (vAmBac133) were amplified using T75 tissue culture flasks, and the virus titer was determined by endpoint dilution assay.

S. frugiperda cells were adapted to suspension culture by the following procedure: 2.5×10^6 *S. frugiperda* cells were transferred to a 125-ml Erlenmeyer glass flask together with 20 ml SFM 900 medium containing 5% fetal bovine serum and incubated at 28°C and rocked at 100 rpm for 48 h, after which the cells were subcultured. For protein production, 100 ml of a medium containing 2×10^6 cells/ml were infected with vAmBac133 at 5 multiplicity of infection, and cells were harvested 78 h after infection and washed with PBS containing protease inhibitor solution (Roche). The resulting pellet was frozen at -80°C and resuspended in 5 ml PBS. The protein purification was performed using the MagneHis™ Protein Purification System (Promega) following the manufacturer's instructions. The protein concentration was determined by Bradford's [26] method with bovine serum albumin as the standard. Extraction was confirmed by Western blot analysis using anti-His monoclonal antibody (1:5,000; Sigma).

Lipase/Esterase Activity Assay

A standard esterase activity assay was performed using *p*-nitrophenyl butyrate (C4) as a substrate [27], dissolved in acetonitrile at a concentration of 10 mM. To prepare the substrate solution, *p*-nitrophenyl butyrate, ethanol and 50 mM Tris-HCl (pH 8.0), were

added to a final composition of 1:4:95 (v/v), respectively. The standard reaction mixture was composed of 900 μl substrate solution, 270 μl buffer (50 mM Tris-HCl, pH 8.0) and 30 μl (1 μg) purified proteins. The enzyme was replaced with the buffer in cases of a blank. The mixtures were incubated at 28°C for 20 min, and then the reactions were stopped by adding 200 μl 10% SDS solution. The enzyme activity was measured by monitoring the change in absorbance at 405 nm in a SpectraMax M2 (Molecular Devices) plate reader, representing the amount of released *p*-nitrophenol. The experiment was repeated 3 times.

For the lipase activity assay, previously described methods [28] were used. In brief, 1.5 g agar was added to 100 ml Tris-Cl (pH 8.8) along with 8 mg Victoria blue B dye and 1.5 ml of a 20% olive oil in polyvinyl alcohol (2% polyvinyl alcohol in water) emulsion or triolein (Sigma). The mixture was autoclaved and then poured onto a 35-mm plate. Wells were cut using a rivet sterilized in 100% ethanol. The concentrations of the samples were adjusted to fall between those of the controls. Each well was loaded and the plate incubated at 28°C for 24 h. The experiment was repeated 3 times.

Protease Activity Assay

Proteolytic activity was assayed according to the method of Murata et al. [29]. In brief, the enzyme solutions or controls (650 ng) were added to 2% (w/v) 400 μl casein solution (dissolved in 200 mM Tris-HCl buffer, pH 7) and incubated at 28°C for 30 min. The reaction was stopped by adding 440 μl 10% trichloroacetic acid and allowed to stand for 30 min. The mixture was centrifuged at 1,500 g for 15 min, and an aliquot (1 ml) was mixed with 2.5 ml of 0.4 M sodium carbonate and 0.5 ml Folin-Ciocalteu reagent 1:2 diluted with water. The assay was read at 660 nm. To inhibit protease activity of *amv133*, two different conditions were employed before substrate addition: (1) 0.1% SDS followed by heating at 98°C for 1 h, and (2) 1 mM PMSF.

In all the experiments, a nonenzymatic protein, the 337L gene from the Chilo iridescent virus expressed in the same vectors, was also purified and the concentration calculated, to be used as a negative control. The experiment was repeated 3 times. The results of the enzyme activities were compared with the control group using the paired sample *t* test (SPSS for MAC, version 21.0.0.0).

Results

Alignment Analysis of *amv133*

The amino acid sequence of *amv133* has a nucleophilic elbow (GXSG motif, X = any amino acid) between the 176th and 180th amino acid, which confers enzyme activity in lipases and esterases [9]. Also, it includes a putative catalytic triad, which contains serine, aspartic acid and histidine amino acid at position 178, 232 and 270 respectively, with a high overall degree of amino acid similarity to known lipases (fig. 1). Among these amino acid sequences, which are used in these alignments, only the lipase protein encoded by *Rhizopus niveus* and *H. virescens* ascovirus have been tested for lipase activity. It was determined that *H. virescens* ascovirus lipase is not active in

Fig. 1. Alignment of *amv133* at the active site of triacylglycerol lipases. AMEV = *Amsacta moorei* entomopoxvirus, accession No. NP_064915.1; Bacillus sp. = *Bacillus* sp., accession No. WP_010195194.1; Celegans = *Caenorhabditis elegans*, accession No. NP_504600.3; Cowpox = CPV putative lipase, accession No. CAA64113.1; Hv-ascovir = *Heliothis virescens* ascovirus, accession No. YP_001110872; Ipomoea = *Ipomoea nil*, accession No. U55867;

MSEV = *Melanoplus sanguinipes* entomopoxvirus strain Tucson, accession No. NC_001993; Rhizopus = *Rhizopus niveus*, accession No. D12680; Synecho = *Synechocystis* sp., accession No. NP_442511.1; ACEV = *Anomala cuprea* entomopoxvirus, accession No. YP_009001656.1. The ExPASy-PROSITE signature is PS00120. Triangles indicate catalytic triad.

vitro [20] but that *R. niveus* has an active lipase [30]. The remaining proteins have not been tested for lipase activity in vitro yet.

amv133 was also aligned with 5 betaentomopoxviruses (fig. 2). There is a significant similarity between these entomopoxviruses. All of them include a conserved se-

quence of GHSLG, which is a nucleophilic elbow, and a catalytic triad composed of serine, aspartic acid and histidine amino acid.

There was no significant similarity between the protein sequence of *amv133* and known proteases. However, *amv133* has the catalytic triad of serine proteases.

Fig. 2. Alignment of *amv133* among betaentomopoxviruses. AMEV = *Amsacta moorei* entomopoxvirus 'L' strain Moyer, accession No. NC_002520; AHEV = *Adoxophyes honmai* entomopoxvirus 'L' strain Japan, accession No. HF679131; CBEV = *Choristoneura biennis* entomopoxvirus 'L' strain Canada, accession No.

HF679132; CREV = *Choristoneura rosaceana* entomopoxvirus 'L' strain Canada, accession No. HF679133; MySEV = *Mythimna separata* entomopoxvirus 'L' strain China, accession No. HF679134. The triangles indicate the catalytic triad.

Fig. 3. Expression of *amv133* in a bacterial expression system. The asterisk indicates an approximately 32-kDa protein belonging to *amv133*. A = Unpurified protein of *amv133*; B = purified protein of *amv133*; M = molecular weight (kDa).

Fig. 4. Expression of *amv133* in a baculovirus expression system. The asterisk indicates an approximately 32-kDa protein belonging to *amv133*. A = Purified protein of *amv133*; B = unpurified protein of *amv133*; M = molecular weight (kDa).

amv133 Encodes Functional Esterase Gene with Protease Activity

To test the enzyme activity of *amv133*, a bacterial vector system (fig. 3) and a baculovirus vector system (fig. 4) were used to express *amv133*. Expressed protein was partially purified by using His-tag and used for enzyme as-

say. For the negative control, a nonenzymatic 337L gene of Chilo iridescent virus was also expressed in the same vectors and partially purified using His-tag. An equal amount of purified 337L and *amv133* were used for a lipase, esterase and protease activity assay. In the test results, *amv133* did not show any lipase activity, but showed

Fig. 5. Esterase activity of *amv133* expressed in bacterial and baculovirus vector systems. Bars show standard errors. **a** Activity of the protein produced via a baculovirus vector system. **b** Activity of the protein produced via a bacterial vector system.

Fig. 6. Protease activity of *amv133* expressed in bacterial and baculovirus vector systems. Bars show standard errors. **a** Lipase activity of the protein produced via a baculovirus vector system. **b** Lipase activity of the protein produced via a bacterial vector system.

a 3.7- and 2-fold higher esterase activity than control groups in baculovirus and bacterial vector systems, respectively (fig. 5).

Protease activity of *amv133* was 7.7- and 2.9-fold higher than control protein expressed in baculovirus and bacterial vector systems, respectively (fig. 6). Protease activity can be inhibited in the presence of PMSF, which is a specific inhibitor of serine proteases [31]. Therefore, the results indicate that *amv133* shows serine protease activity. Control and test results were compared statistically and showed that the results varied in comparison to the control group ($p < 0.05$).

Discussion

So far, thymidine kinase [32], DNA topoisomerase [33], NAD⁺-dependent DNA ligase [34], superoxide dismutase [35], inhibitor of apoptosis [36, 37], *p33* [38],

poly(A) polymerase [3] and DNA photolyase [39] genes of the AMEV have been functionally examined. In this study, we examined for the first time the lipase, esterase and protease activity of *amv133* from AMEV.

We observed the GX SXG (X = any amino acid) motif, called the nucleophilic elbow, which contains lipase active serine amino acid in the amino acid sequence of *amv133*. This serine amino acid is also a component of the catalytic triad that is composed of serine, aspartic acid and histidine at the position of the 178th, 232nd and 270th residues of the *amv133* amino acid sequences. These characteristics are found in lipolytic enzymes. The alignment between *amv133* and known lipases indicates that the GHSLG motif was present in all aligned sequences (fig. 1). Furthermore, the catalytic triad was common not only to the lipases and esterases, but also to the known active center of serine proteases [9]. Based on the fact that some serine proteases exhibit lipase activity, protease assays were performed for *amv133*, and it was found that *amv133* ex-

hibits protease activity, too. The protease activity of *amv133* can be inhibited in the presence of PMSF, and this shows that *amv133* encodes a serine protease. *amv133* also showed esterase activity, but not lipase activity.

AMEV replication takes place in various larval tissues, but it prefers fat body cells [5], which are the major site of lipid storage [6]. Lipids play an essential role on both the viral and the cellular sides and in key features such as virion structure, target membrane and cell receptor recognition, viral membrane fusion, viral replication, assembly, egress and entry [40]. Furthermore, the vaccinia virus carries an active lipase gene that is essential for membrane fusion and cell-to-cell transmission [14]. *amv133* has lipolytic activity and thus it might also have such a function.

Furthermore, *amv133* also shows protease activity. Viral proteases showed insecticidal activity by degrading the peritrophic matrix and increasing the speed of insect death [23, 24]. *amv133* may contribute to viral infection through such a function. Apart from this, a lipid-carrying protein (lipoprotein), apolipophorin, which inhibits the bacterial infection [41] and is one of the host immune-related proteins against virus infection [42], stimulates the immune activation [43] and is

essential for lipid transport in insects for energy sources and/or lipid storage [44, 45]. *amv133* shows both esterase and protease activities, so it may have some functions to degrade these lipoproteins; therefore, viruses can escape from the host immune response and improve the infection.

Conclusions

In summary, *amv133* shows esterase and protease activity and may be essential for membrane fusion, like in the case of the vaccinia virus lipase gene, or important for host immune modulation, as is suggested for the lipase-like genes of either Marek's disease virus [19] or ectromelia virus [21], by degrading lipoproteins. Our data raises the possibility that *amv133*-like genes may be such a component in entomopoxviruses.

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