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Characterization of an Isopentenyl Diphosphate Isomerase involved in the Juvenile Hormone pathway in *Aedes aegypti*

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Abstract

Isopentenyl diphosphate isomerase (IPPI) is an enzyme involved in the synthesis of juvenile hormone (JH) in the corpora allata (CA) of insects. IPPI catalyzes the conversion of isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate (DMAPP); afterwards IPP and DMAPP condense in a head-to-tail manner to produce geranyl diphosphate (GPP), this head-to-tail condensation can be repeated, by the further reaction of GPP with IPP, yielding the JH precursor farnesyl diphosphate. An IPPI expressed sequence tag (EST) was obtained from an *Aedes aegypti* corpora-allata + corpora cardiaca library. Its full-length cDNA encodes a 244-aa protein that shows a high degree of similarity with type I IPPIs from other organisms, particularly for those residues that have important roles in catalysis, metal coordination and interaction with the diphosphate moiety of the IPP. Heterologous expression produced a recombinant protein that metabolized IPP into DMAPP; treatment of DMAPP with phosphoric acid produced isoprene, a volatile compound that was measured with an assay based on a solid-phase micro extraction protocol and direct analysis by gas chromatography. *A. aegypti* IPPI (AaIPPI) required Mg^{2+} or Mn^{2+} but not Zn^{2+} for full activity and it was entirely inhibited by iodoacetamide. Real time PCR experiments showed that AaIPPI is highly expressed in the CA. Changes in AaIPPI mRNA levels in the CA in the pupal and adult female mosquito corresponded well with changes in JH synthesis (Li et al., 2003). This is the first molecular and functional characterization of an isopentenyl diphosphate isomerase involved in the production of juvenile hormone in the CA of an insect.

Keywords

Mosquito; IPP isomerase; juvenile hormone; corpora allata; *Aedes*

INTRODUCTION

Isoprenoids are a large family of organic compounds derived from C_5 isoprene units. They are present in Archaea, Bacteria and Eukarya and have essential roles in signal transduction, electron transport, photosynthesis and regulation of development and reproductive cycles (Sacchetti and Poulter, 1997; Ramos-Valdivia et al., 1997; Satterwhite, 1985; Spurgeon and Porter, 1981; Bonano et al., 2001). An important building block of all isoprenoids is the compound isopentenyl pyrophosphate (IPP) (Ramos-Valdivia et al., 1997). Almost all organisms synthesize IPP by the condensation of three units of acetyl-CoA through the

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mevalonate pathway (MVA) (Ramos-Valdivia et al., 1997). In contrast, in some prokaryotes and plant plastids, IPP production is accomplished via the 2-C-methyl-D-erythritol-4-phosphate pathway (MEP) (Rohmer, 1999).

The synthesis of isoprenoids includes the conversion of IPP to the electrophile dimethylallyl pyrophosphate (DMAPP), catalyzed by isopentenyl diphosphate isomerase (IPPI). IPPI generates DMAPP by the isomerization of the carbon-carbon double bond of IPP via the stereoselective antarafacial transposition of hydrogen (Durbecq et al., 2001). There are two types of IPPIs, type I is found in almost all eukaryotes while type II has only been observed in prokaryotes (Ramos-Valdivia et al., 1997). The optimal functioning of IPPI type I requires a divalent metal cation (Mg^{2+} or Mn^{2+}) (Durbecq et al., 2001). Zinc has also been identified as an essential cofactor for the activity of recombinant IPPI type I from *Escherichia coli* (Carrigan and Poulter, 2003). Type II IPPI requires a reduced flavin coenzyme in addition to a divalent metal cation (de Ruyck et al., 2008).

In the corpora allata (CA) of insects, IPPI is involved in the synthesis of juvenile hormone (JH) (Belles, et al., 2005; Goodman and Granger, 2005); IPP and DMAPP can condense in a head-to-tail manner to produce geranyl diphosphate (GPP). This type of head-to-tail condensation can be repeated by the further reaction of GPP with IPP, yielding the JH precursor farnesyl diphosphate (Goodman and Granger, 2005). In insect species, IPPIs have been only partially characterized from extracts of the silkworm *Bombyx mori* larvae (Koyama et al., 1985). We cloned an IPPI expressed in the CA of the mosquito *Aedes aegypti* (Noriega et al., 2006). Heterologous expression produced a recombinant protein that metabolizes IPP into DMAPP. The protein requires Mg^{2+} or Mn^{2+} but not Zn^{2+} for full activity and is entirely inhibited by alkylation of the catalytic cysteine residue by iodoacetamide. Real time PCR experiments showed that IPPI mRNA levels in the CA match the changes in JH synthesis.

MATERIAL AND METHODS

2.1. Insects

Aedes aegypti of the Rockefeller strain were reared at 28 °C and 80% relative humidity under a photoperiod of 16 h light: 8 h dark. Mated adults were offered a cotton pad soaked in 3% sucrose solution. The cotton pad sucrose-fed adults are referred to as sugar fed. Four-day-old female mosquitoes were fed porcine blood equilibrated to 37 °C. Adenosine triphosphate was added to the blood meal to a final concentration of 1 mM immediately before use (Noriega et al., 1999).

2.2. Chemicals

Isoprene was purchased from Sigma-Aldrich (St Louis, MO). IPP and DMAPP were obtained from Echelon Biosciences (Salt Lake City, UT).

2.3 Identification of the *A. aegypti* IPP isomerase cDNA

An IPPI expressed sequence tag (EST) was obtained from an *A. aegypti* corpora-allata + corpora cardiaca library, constructed and sequenced as previously described (Noriega et al., 2006). The IPPI EST sequence was queried against the *A. aegypti* database at VectorBase (Lawson et al., 2009); it revealed a single identical sequence (Accession number: AAEL006144). The *A. aegypti* IPPI (AaIPPI) cDNA was PCR-amplified from cDNA obtained from the thorax of mosquitoes (containing the corpora allata) using the following two primers:

Forward: AaIPPI-F, 5'-CATATGTCTCTTCTGGCTCGCTT-3'.

Reverse: AaIPPI-R, 5' - GCGGCCGCTCAAATCGTTCGATTCGTTG-3'.

2.4. Expression and purification of recombinant AaIPPI

The coding region of the AaIPPI cDNA was ligated into the pET-28a(+) expression vector (Novagen, Gibbstown, NJ). *E. coli* strain BL21 (DE3) were transformed with the construct and expressed as previously described (Mayoral et al., 2009). Recombinant protein containing a C-terminal His-tag was purified with a cobalt column (Pierce, Rockford, IL) and desalted using a PD-10 column (Amersham, Pharmacia Piscataway, NJ) (Mayoral et al., 2009). The purified protein was concentrated (225 ng/ μ L) using Centricon YM-10 centrifugal filters (Millipore, Billerica, MA), aliquoted and stored at -20°C until use.

2.5. IPPI enzymatic assay

IPPI activity was determined by conversion of DMAPP to isoprene by treatment with phosphoric acid, followed by gas chromatographic (GC) analysis of isoprene (Fisher et al., 2001; Bruggemann and Schnitzler, 2002) (Fig. 1). We modified previously described methods by using a solid-phase micro extraction protocol (SPME) to adsorb the volatile isoprene for direct GC analysis. A sample containing 93 μ L of assay buffer (0.4 M Tris, 1 mM DTT, 10mM MgCl_2 , pH 8.0), 5 μ L of IPP (1 $\mu\text{g}/\mu\text{L}$) and 2 μ L of the recombinant AaIPPI (200 ng/ μ L) was incubated at 35°C for 1 h and the reaction was terminated by the addition of 30 μ L of phosphoric acid (14.8 M). After incubation the solution was transferred to a 2-mL glass vial sealed with a silicone septum vial (Agilent) and the needle of the holder carrying the SPME fiber was introduced through the septum. The SPME holder and the 30 μm divinylbenzene-carboxen-polydimethylsiloxane (DVD-CAR-PDMS) fibers used to absorb isoprene were supplied by Supelco (Bellefonte, PA). The vial was placed on a heating block at 50°C and the fiber was exposed to the atmosphere in the vial (Supplementary Fig. 1). After 1 hour of incubation at 50°C , the fiber was retracted into the needle. The holder was removed and transferred to the GC equipment. The needle was then introduced into the injection port of the GC equipment preheated at 200°C ; isoprene was immediately desorbed from the fiber and the GC run started.

GC determinations of isoprene were performed on a Trace Gas Chromatography Ultra apparatus (Thermo Electron Corporation, Waltham, MA) equipped with a non-polar capillary column DB-5MS (30 m \times 0.25 mm i.d.; film thickness 0.25 μm) (Agilent, Santa Clara, CA) and a flame ionization detector. The output was recorded using a ChromQuest version 4.1 data system. Helium was employed as the gas carrier at a flow rate of 1 mL/min. The injector and detector temperatures were 200°C and 230°C , respectively. The column oven temperature was isothermally held at 30°C . The amounts of isoprene detected were expressed as GC peak areas without using corrections factors. Prior to re-use, the fiber was conditioned for 10 min in the GC injection port at 200°C .

For the kinetic studies, substrate concentrations ranged from 10 to 100 μM in a reaction mixture of 100 μL , containing 400 ng of recombinant enzyme. Reactions were incubated for 1 h at 35°C . The kinetic values were calculated by nonlinear regression analysis of the Michaelis-Menten equation using GraphPad Prism software 3.03. A Lineweaver-Burk plot was derived from transformation based upon nonlinear regression analysis.

2.6. RNA extraction and Real Time-PCR

Corpora allata-corpora cardiac complexes (CA-CC) (20 pairs per replicate) were dissected in a drop of sterile DNA-RNase free phosphate buffered saline (PBS). Total RNA was isolated using RNA-binding glass powder as previously described (Noriega and Wells, 1993). RNA samples were treated with rDNase I using DNA-free™ kit (Ambion, Austin, TX, US) according to the manufacturer's recommendations. Reverse transcription was carried out

using the SuperScript[®] III first strand synthesis kit. Real-time PCR was performed in a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, US) using TaqMan[®] Gene Expression Assays together with TaqMan[®] Universal PCR Master Mix (Applied Biosystems). The primers and probes for the house keeping gene 60S ribosomal protein rpL32 (Accession number: AAEL003396) and the AaIPPI gene are included in Supplementary table 1. Primer/probes were synthesized by Applied Biosystems and reactions were carried out in 20 μ L volume according to the manufacturer's recommendations for Custom TaqMan[®] Gene Expression Assays. Reactions were run in triplicate using 1 μ L of cDNA per reaction. Standard curves to quantify relative gene copy number were made from ten-fold serial dilutions of plasmids containing rpL32 or AaIPPI (from 300,000 to 30 copies of a plasmid per reaction). Real-time data were collected by 7300 System SDS Software and analyzed in Microsoft Excel. Transcript levels were normalized with rpL32 mRNA levels in the same sample. Relative transcript levels are expressed as a number of copies of transcript per 10,000 copies of rpL32.

2.7. Phylogenetic analysis

IPPI sequences were obtained from GenBank and VectorBase (Lawson et al., 2009) databases and used for the alignments and phylogenetic analysis. We aligned the IPPI sequences using ClustalW (Higgins et al., 1994). A Maximum-Likelihood tree was built using MEGA software version 5.1 (Tamura et al., 2011) with a bootstrapping of 1000 (Felsenstein, 1985). Partial deletion method was selected for the gap/missing data in the software.

2.8. Modeling *A. aegypti* IPPI

The tertiary structure model of the AaIPPI was created using the protein structure homology-modeling server Swiss-Model v.8.05 (Arnold et al., 2006; Schwede et al., 2003) using as template the Human IPPI (2i6kA) that has 46.6% identity with AaIPPI. The QMEAN6 (Benkert et al., 2011) score obtained for the model obtained was 0.720, the secondary structure agreement 80.0% and the solvent accessibility agreement 82.3%.

3. RESULTS

3.1. Molecular characterization of *A. aegypti* IPPI

A single orthologue gene to the AaIPPI EST was found in the genome of *A. aegypti* (VectorBase) (Lawson et al., 2009). The IPPI gene is located on supercontig 1.191, and it is composed of three exons interrupted by two introns with lengths of 4,158 and 9,351 bp. It encodes a 244 amino acid protein with an estimated molecular weight of 28.5 kDa. Analysis of the AaIPPI sequence revealed the presence of a typical Nudix hydrolase superfamily domain (Nudix: NUCleoside Diphosphate linked to some other moiety X) (Fig. 2). Residues previously described as important for metal coordination and interaction with the diphosphate moiety of the IPP are well conserved in AaIPPI; including two important motifs that have been associated with IPPI type I functionality: a TNACCSHPL motif containing a conserved cysteine residue and a WGEHEIDY motif that contains a conserved glutamate residue (Fig. 2).

IPPI orthologues were searched in other species of insects. An alignment of the AaIPPI with related insect proteins is shown in the Supplementary Fig. 2. A cladogram of the phylogenetic relationship of the insect's IPPI sequences was generated (Supplementary Fig. 3).

3.2. Establishment of an improved protocol to measure IPPI activity

We developed a convenient and simple IPPI activity assay based on the non-enzymatic acidic hydrolysis of DMAPP, which generates the volatile compound isoprene (Fisher et al., 2001). Our modification was to introduce the use of solid-phase micro extraction (SPME) to adsorb the volatile isoprene that can be later directly quantified by GC (Fig. 1). Optimal conditions for hydrolysis were explored; a temperature of 50 °C was chosen because at this temperature isoprene was efficiently adsorbed by the SPME fiber. Higher temperatures could also be used, but heating the sample over 50 °C caused spontaneous hydrolysis of IPP and unwanted production of isoprene. At 50 °C formation and adsorption of isoprene derived from DMAPP was very effective and generation of isoprene from IPP was minimalized (Supplementary Fig. 4). Analysis of different reaction times showed that most of the isoprene formed from DMAPP was recovered within 1 hour. Using these conditions three standard curves were constructed by non-enzymatic hydrolysis of mixtures containing 1) increasing amounts of DMAPP, 2) increasing amounts of IPP and 3) increasing amounts of DMAPP and decreasing amounts of IPP (Supplementary Fig. 4). We used a mix of IPP and DMAPP to mimic the situation of enzymatic reactions in which increasing amounts of IPP were transformed into DMAPP, but still there was a mix of both compounds in the solution that was hydrolyzed by phosphoric acid. A linear increase in non-enzymatic generation of isoprene formation was observed as the amount of DMAPP was increased and IPP decreased (Supplementary Fig. 4). The DMAPP standard curve was adjusted for IPP interference and developed into a corrected DMAPP standard curve that was used for product quantification (Supplementary Fig. 4). Under these conditions, the SPME method was sensitive and reproducible.

3.3. AaIPPI activity in the presence of cofactors and inhibitors

To analyze the effect of metal cofactors, Mg^{2+} in the form of $MgCl_2$ was added to samples to obtain final concentrations of 5, 10 and 20 mM. There was a 2-fold increase in activity when 10 mM was added; increasing the concentration to 20 mM also stimulated AaIPPI activity but it was less pronounced (Fig. 3). Buffer without metal cofactor was used as control (0.4 M Tris, 1 mM DTT, pH 8.0). The same process was repeated with Mn^{2+} in the form of $MnCl_2$ to produce concentrations of 0.02, 0.05, 0.1 and 0.2 mM. There was a 2.5-fold increase in activity when 0.1 mM was added; increasing the concentration to 0.2 mM also stimulated AaIPPI activity but it was less marked (Fig. 3). The effect of Zn^{2+} as a cofactor was analyzed with the addition of $ZnCl_2$ to produce concentrations ranging from 50 μM to 10 mM (50 μM , 100 μM , 200 μM , 1 mM, 5 mM and 10mM). Addition of Zn^{2+} had no effect on AaIPPI activity. The 0.1 mM Mn^{2+} samples yielded the highest rate of IPP isomerization, thus this concentration was chosen to examine the effect of the irreversible inhibitor iodoacetamide. The inhibitor was added to the sample reactions at concentrations between 1 μM and 5 mM. The inhibitor was very effective; 1 μM iodoacetamide caused a relative inhibition of ~ 70%, with complete inhibition at 100 μM (Fig. 4).

3.4. Kinetic properties of recombinant AaIPPI

Kinetic constants were measured for purified recombinant AaIPPI. A plot of the initial velocity of DMAPP production versus IPP concentration was hyperbolic (Fig. 5). The values for K_m and V_{max} were calculated by non-linear regression analysis. A Lineweaver-Burk double reciprocal plot was derived from transformation based upon nonlinear regression analysis (Supplementary Fig. 5). The K_m was 276.8 μM and V_{max} was 0.6 μmol of substrate (IPP) converted to product (DMAPP) per minute per mg of enzyme. The equilibrium constant, $K_{eq} = [IPP]/[DMAPP] = 0.6$ was uniform over the logarithmic phase of the enzymatic reaction (Fig. 5).

3.5. Tissue distribution and developmental expression of AaIPPI mRNA

Real time PCR was used to analyze the expression of AaIPPI mRNA in adult female and male tissues. AaIPPI mRNA expression was higher in the female CA-CC. Lower levels of mRNA transcripts were detected in the ovary, hindgut and brain of the female, as well as the testis and accessory glands of the male (Fig. 6). AaIPPI mRNA was also detected in the female heart, midgut, fat body and Malpighian tubules. In addition, the levels of the AaIPPI transcripts were studied in the CA-CC during female pupal and adult development. In the female CA-CC, the AaIPPI mRNA levels were low in the early pupae, started increasing 6 hours before adult eclosion and reached a maximum 24 hours after female emergence. Blood feeding resulted in a decrease in transcript levels. The pattern of changes of AaIPPI mRNA resembled the changes in JH biosynthesis (Fig. 7).

4. DISCUSSION

4.1. Molecular and functional characterization of an insect IPPI

We have molecularly and functionally characterized an *A. aegypti* IPPI involved in the synthesis of JH in the CA. An IPPI expressed sequence tag (EST) was obtained from an *A. aegypti* corpora-allata + corpora cardiaca library, constructed and sequenced as previously described (Noriega et al., 2006). The AaIPPI 244 amino acid sequence is slightly longer than the 182 amino acid long IPPI from *E. coli*, considered to represent the basic IPPI core necessary for IPP isomerization (Durbecq et al., 2001). The extra 62 amino acid present in *A. aegypti* IPPI are mostly an N-terminus extension. Analysis of the AaIPPI sequence revealed the presence of a typical Nudix hydrolase superfamily domain. Members of the Nudix family are found in a wide variety of organisms and they are capable of hydrolyzing organic pyrophosphates. Nudix family proteins are characterized by the conserved motif, G-X5-E-X7-R-E-U-X-E-E-X2-U, where X is any residue and U denotes a large hydrophobic side chain (Midvan et al., 2005). The corresponding sequence within the IPPIs contains a similar but changed conserved motif, G-X3-A-X2-R-R/K-U-X2-E-L-G-U (Fig. 2) (Bonano et al., 2001). The Nudix motif forms a loop- α helix-loop structure which functions as a versatile Mg^{2+} -binding and catalytic site (Lin et al., 2008). This motif is part of a shared α/α sandwich or Nudix fold containing additional regions which differ, depending on the detailed mechanism and specificity of the enzyme (Midvan et al., 2005; Lin et al., 2008). AaIPPI exhibits a compact globular α/β structure very similar to that described for human IPPI (Zheng et al., 2007) (Supplementary Fig. 6). AaIPPI shows a high degree of similarity with IPPIs from other organisms, especially in two important motifs that have been associated with IPPI type I functionality: a TNACCSHPL motif containing a conserved cysteine residue and a WGEHEIDY motif that contains a conserved glutamate residue (Fig. 2) (Zheng et al., 2007). The antarafacial stereochemistry of the isomerization is consistent with an active site containing two residues located on opposite faces of the allyl moiety in IPP, both residues are indispensable to accomplish the protonation/deprotonation steps during IPP isomerization; in the sequence of AaIPPI these two residues have been identified as C₁₀₅ and E₁₅₆, located face-to-face close to the bottom of the active site pocket. W₂₁₃ and Y₁₆₈ are two additional active site residues with important roles in catalysis that are located at the bottom of the deeply buried active site cavity. These four residues are highly conserved among the insect IPPIs (Supplementary Fig. 2). *E. coli* type I IPPI requires two divalent metal atoms for activity. In its native resting form, *E. coli* type I IPPI seems to be a zinc metalloprotein, but this zinc can be replaced by a Mn^{2+} (Carrigan and Poulter, 2003). The divalent metal is located in a hexacoordinate His3Glu2 pocket, stabilizes the N-terminus of the enzyme, and is a part of the catalytic machinery for protonating the carbon-carbon double bond in IPP. In addition to zinc and manganese, the enzyme-substrate complex contains an atom of magnesium that facilitates substrate binding through the diphosphate moiety (Lee and Poulter, 2006). Residues previously described as important for metal

coordination and interaction with the diphosphate moiety of the IPP are well conserved in AaIPPI (Fig. 2), as well as in other insect IPPIs (Supplementary Fig. 2).

Measuring the enzymatic activity of IPPI is challenging because substrate (IPP) and product (DMAPP) are isomers, excluding the possibility of using spectrophotometric assays. The most common assay for IPPI is a radiometric technique based on the acid lability of DMAPP and ^{14}C radiolabeled precursors (Satterwhite, 1985). Alternatively, IPPI activity has been determined by acidic hydrolysis of DMAPP, which is converted into isoprene, a volatile compound that can be analyzed in the head space of a vial by adsorption in alumina and subsequent desorption in the GC equipment (Lehning et al., 1999; Fisher et al., 2001; Bruggemann and Schnitzler, 2002). We developed a novel method that takes advantage of the differential lability of DMAPP under acidic conditions as compared with IPP, as well as the efficacy of SPME fibers to adsorb the volatile isoprene (Hyspler et al., 2000; Ignea et al., 2011). The method is much simpler and the use of the fiber avoids the necessity of a GC instrument equipped with a headspace auto sampler or similar devices in the injector to get desorption of adsorbed isoprene (Bruggemann and Schnitzler, 2002).

The SPME-GC protocol was employed to assay the effects of three metal cofactors and an irreversible inhibitor on the AaIPPI. For every IPPI type I studied, addition of Mn^{2+} results in a stronger activation than addition of Mg^{2+} (Ramos-Valdivia et al., 1997; Zheng et al., 2007). AaIPPI activity was also stimulated by addition of Mn^{2+} or Mg^{2+} , with Mn^{2+} having a stronger activation than Mg^{2+} . The maximum activity was detected at $100\ \mu\text{M}$ Mn^{2+} , a value similar to the optimum concentration of manganese for full activity of a partially purified IPPI from *Bombyx mori* (Koyama et al., 1985). Interestingly, the enzyme's catalytic activity did not reach a plateau after passing an optimal cofactor concentration threshold, but its activity decreased at higher concentrations of cofactor. This observation may be explained through the formation of a complex between the metal cofactor and the substrate, thus the availability of the substrate is reduced with increasing concentrations of cofactor, as reported in other enzymes requiring metal cofactors (Hallcher and Sherman, 1980). A stimulatory effect of Zn^{2+} on IPPI has so far only described for *E. coli* type I (Lee and Poulter, 2006). Our studies showed that Zn^{2+} is not a required cofactor for mosquito IPPI.

Iodoacetamide has been previously described as a robust inhibitor of IPPIs (Reardon and Abeles, 1986); with the mechanism of inhibition occurring from protonation of the thiol group of the catalytic cysteine residue (Reardon and Abeles, 1986). It was also very effective on AaIPPI, with complete inhibition at $0.1\ \text{mM}$, a concentration in the range previously described for other IPPIs (Ramos-Valdivia, et al., 1997). IPPIs are critical enzymes very well conserved during evolution, with identical structure and mechanism of action; therefore is not surprising that metal requirement and inhibitor activity are also conserved in insect IPPIs. Most reported K_m values for IPP isomerases fall in the range of $1\text{--}10\ \mu\text{M}$ for a diverse group of organisms that include *Claviceps purpurea*, *Penicillium sp.*, *Narcissus pseudonarcissus*, *Cinchona robusta*, as well as samples from porcine and avian livers (Ramos-Valdivia et al., 1997), however, higher K_m values in the range of $22\text{--}74\ \mu\text{M}$ have been reported for partially purified IPPI (Reardon and Abeles, 1986; Koyama et al., 1996; Bruggemann and Schnitzler, 2002; Philips et al., 2008). Comparisons of K_m between purified and recombinant enzymes are not always straightforward. Discrepancies between the kinetic properties of purified and recombinant IPPI from the same species have been reported (Ramos-Valdivia et al., 1997).

The equilibrium constant of the isomerization of IPP to DMAPP by AaIPPI ($K_{\text{eq}} = 0.6$) suggest that 60% of the isopentenyl pyrophosphate was converted to dimethylallyl pyrophosphate. K_{eq} values ranging from 0.36 to 6.69 have been reported from a variety of

taxa (Shah et al., 1965; Lutzow and Beyer, 1988; Bruggemann and Schnitzler, 2002; Jonnalagadda et al., 2012), although comparisons are difficult since they were obtained using different experimental conditions.

4.2. Role of IPPI on JH synthesis in mosquitoes

IPPI is a key enzyme of the mevalonate pathway, a metabolic route that gives origin to a great diversity of compounds in insects, including heme, ubiquinone, dolichol and isopentenyl adenine (Belles et al., 2005). In addition, isoprenoids from the mevalonate pathway are involved in the prenylation of membrane-bound proteins that play an important role in cell signaling; as well as in synthesis of defensive secretions, pheromones and JH (Belles et al., 2005). AaIPPI transcripts were expressed in all adult female mosquito tissues evaluated; suggesting that AaIPPI might be involved in many metabolic pathways. The higher levels of IPPI mRNA were detected in the CA; because transcript levels are expressed as the ratio IPPI/ribosomal proteins, and the CA has very low cell number when compared to other tissues, the remarkable differences in the IPPI/rpl32 ratios between CA and the rest of the tissues should not be misinterpreted as AaIPPI strict specificity of expression in the CA (Nouzova et al., 2011). Transcript levels were also markedly elevated in the reproductive tissues of female and male. IPPI mRNA levels are expressed almost exclusively in the CA of *Bombyx mori* 4th instar larvae, with relative low levels in other tissues (Kinjoh et al., 2007).

We found a good correlation between AaIPPI mRNA expression in the CA and JH synthesis (Li et al., 2003). AaIPPI mRNA levels were very low in the CA of early pupae and JH synthesis is suppressed during pupae development. In adult females, CA AaIPPI transcript levels and JH synthesis reached maximum values during the first day after eclosion. In addition, changes in AaIPPI transcripts in CA of sugar-fed and blood-fed female mosquito were also in good agreement with the changes in JH synthesis in the CA (Li et al., 2003). Studies on *Bombyx mori* also showed a good agreement between JH biosynthesis and expression of IPPI in the CA of larvae, pupae and adult female (Kinjoh et al., 2007).

In summary, this is the first functional characterization of an isopentenyl diphosphate isomerase involved in the production of juvenile hormone in the CA of an insect. AaIPPI has the typical structure and functional features of members of the family described in other groups of organisms. Changes in IPPI mRNA levels in the CA in the pupa and adult female mosquito correspond well with the changes in JH synthesis, suggesting that AaIPPI transcript fluctuations are at least partially responsible for the changes of JH biosynthesis observed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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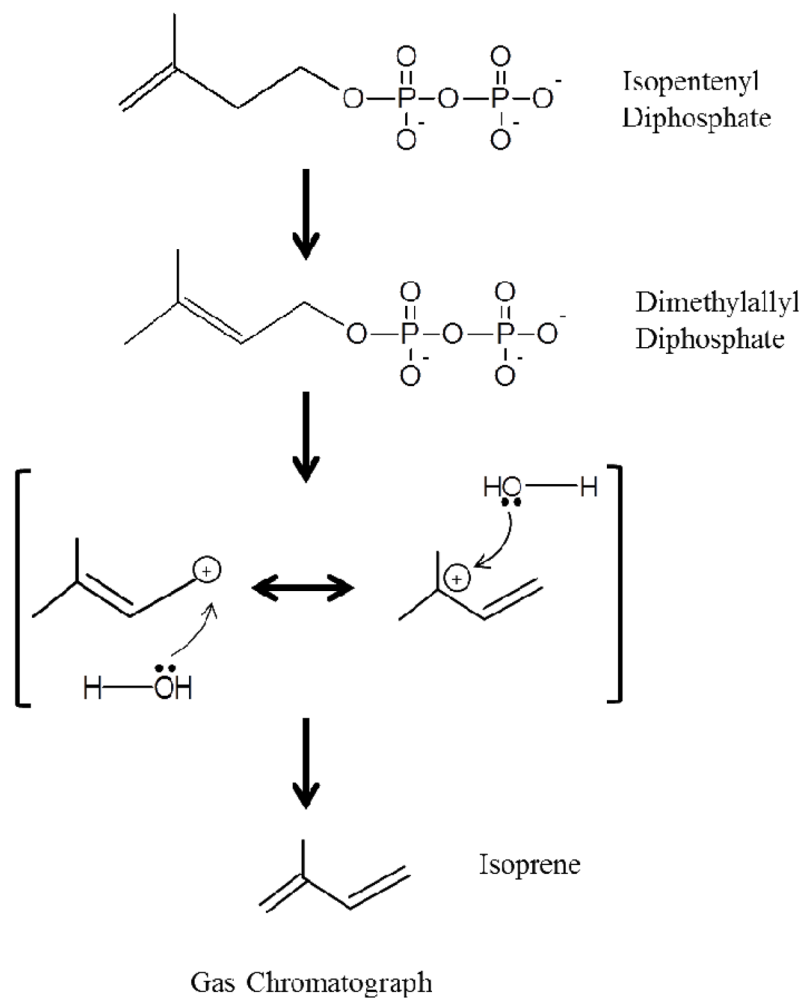
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- We characterized an isopentenyl diphosphate isomerase from the mosquito *Aedes aegypti*.
- The protein catalyzes the isomerization of IPP into DMAPP.
- AaIPPI is highly expressed in the corpora allata (CA).
- The pattern of changes of AaIPPI mRNA in the CA resembles the changes in JH biosynthesis.

**Figure 1. IPPI assay outline**

Isomerization of IPP to DMAPP is catalyzed by IPPI. During the reaction an electrophilic attack by a proton from water occurs on the IPP double bond to yield the carbocation intermediate, afterwards the C-2 pro-R hydrogen of the intermediate is stereospecifically eliminated. DMAPP is converted to isoprene by acidic hydrolysis and dehydration using phosphoric acid. Volatile isoprene is adsorbed using a SPME fiber and detected by gas chromatography.

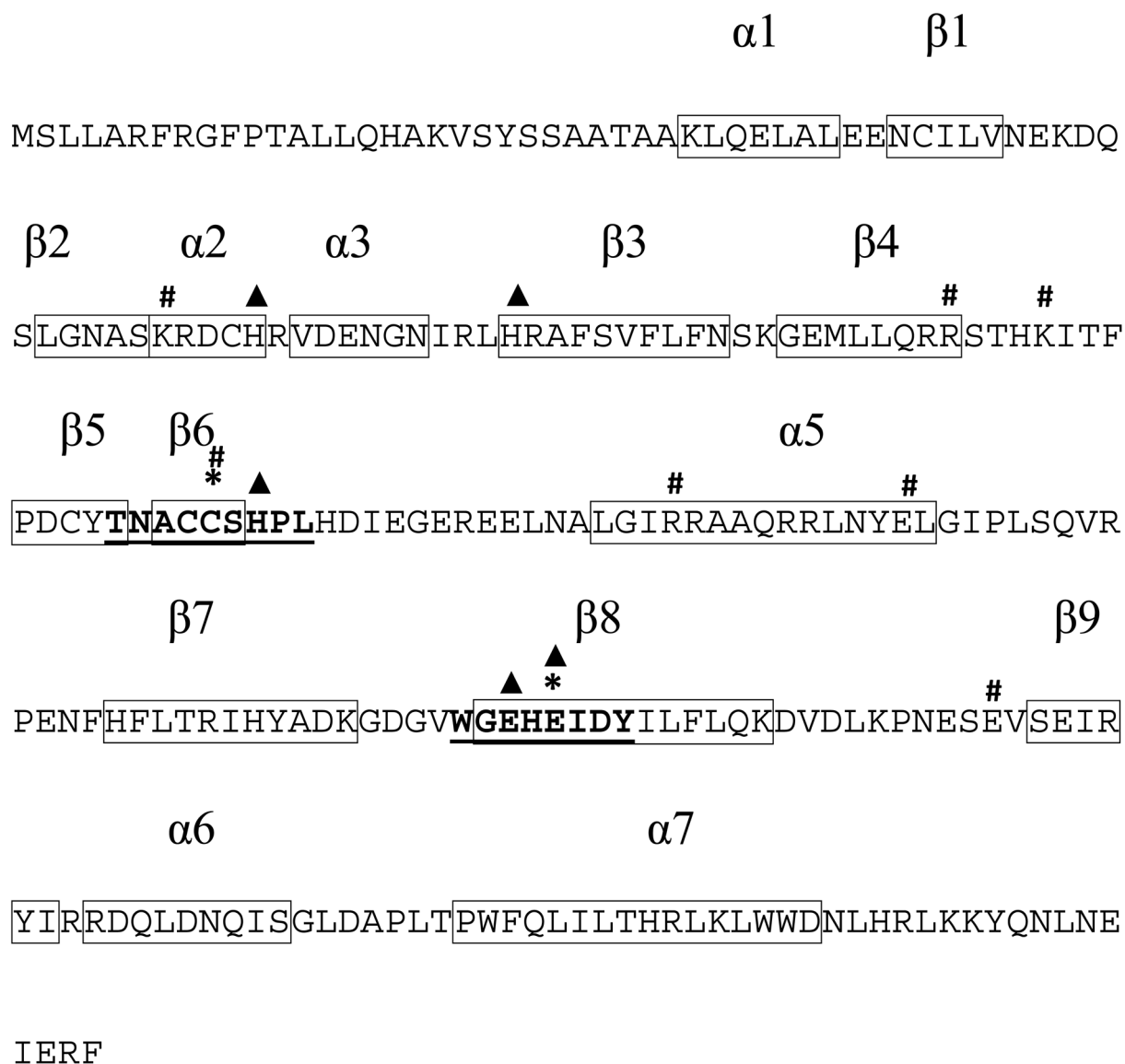


Figure 2. AaIPPI amino acid sequence

Predicted α -helices and β -sheets are labeled on top and shown inside a box. IPPI conserved sequence motifs are highlighted in bold and underlined. (▲): Residues important for metal coordination. (*): Residues important for catalysis. (#): Residues important for interaction with the diphosphate moiety of the substrate. The putative Nudix motif is highlighted by a grey box.

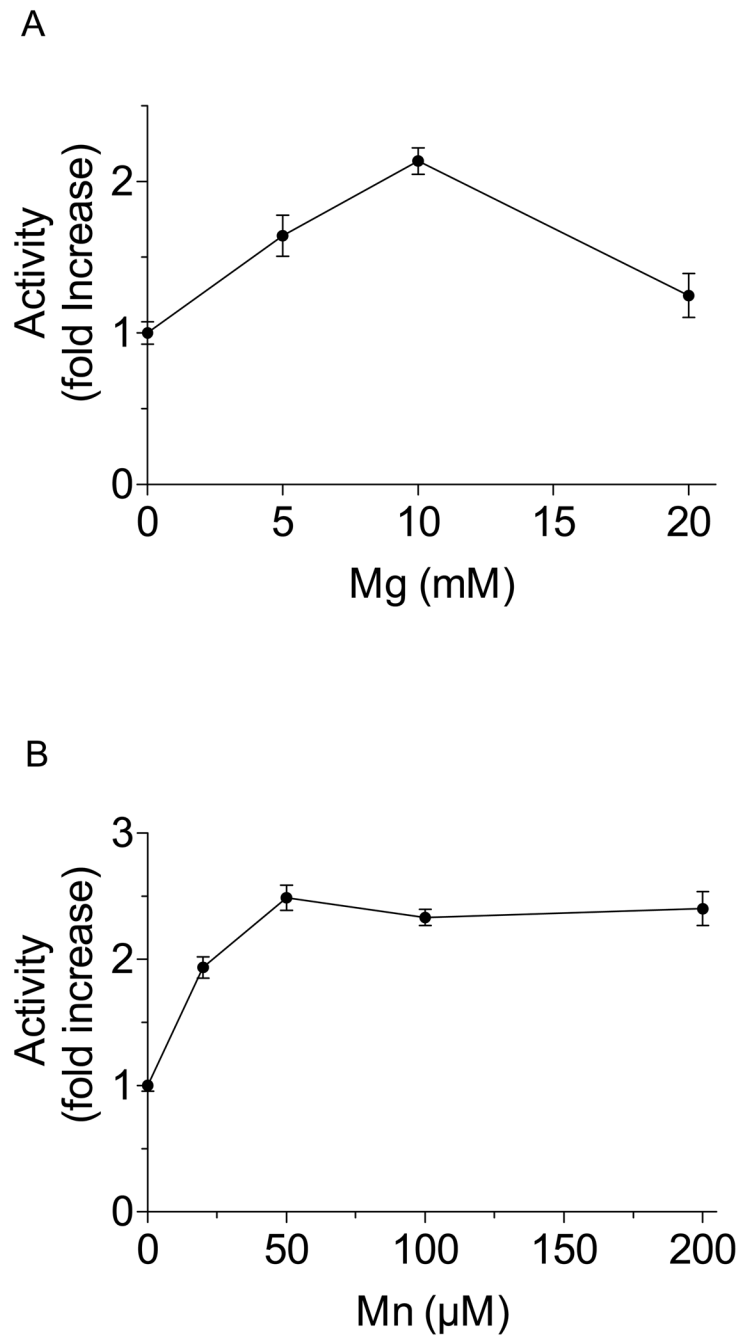


Figure 3. Effect of metal cofactors

Different concentrations of Mg²⁺ (A) or Mn²⁺ (B) were added to samples and IPPI activity was analyzed. Activities are expressed as increases relative to the activity of the control without metal cofactor (fold increase). Each data point is the mean \pm S.E.M of 2–4 independent replicates.

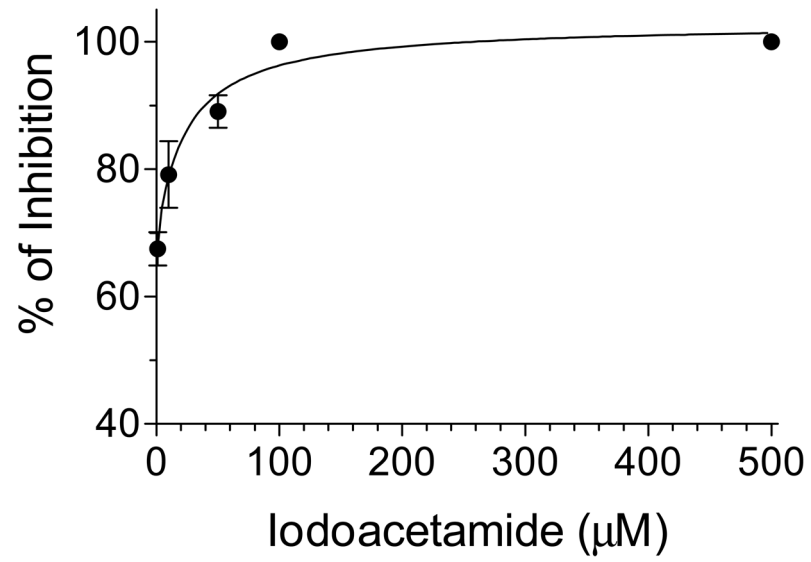


Figure 4. Iodoacetamide inhibition of AaIPPI

Percentages of inhibition increased as increasing concentrations of iodoacetamide were added to the sample up to 500 μM . Controls had no inhibitors. Each data point is the mean \pm S.E.M of 2 independent replicates.

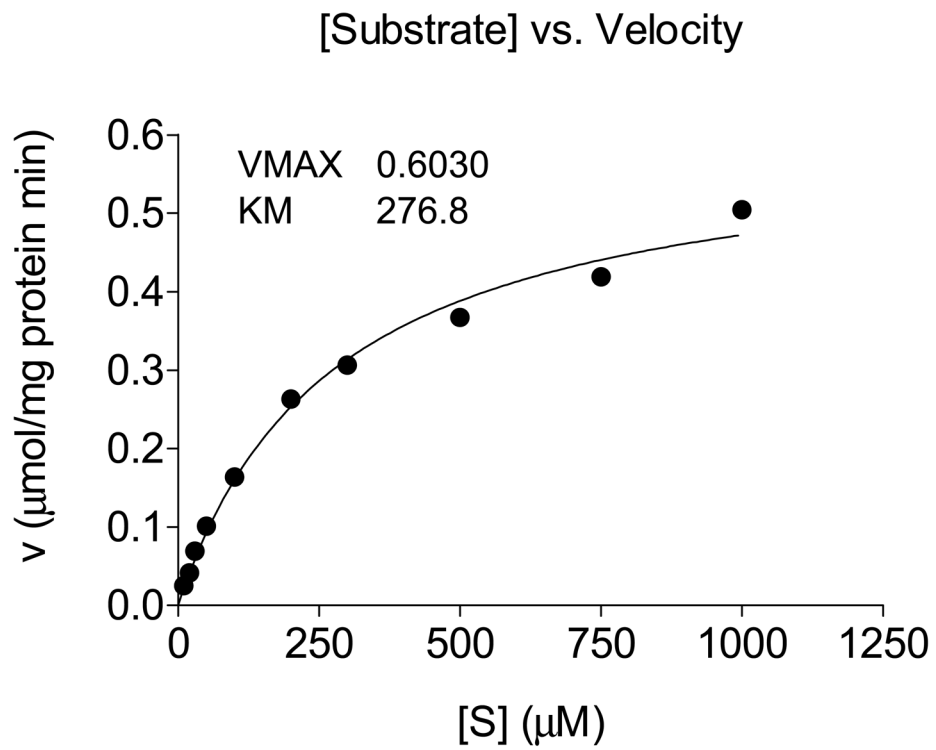


Figure 5. Saturation kinetics of AaIPPI

Plot of initial velocity of AaIPPI activity versus IPP concentration. Each data point is the mean \pm S.E.M of 3 independent replicates.

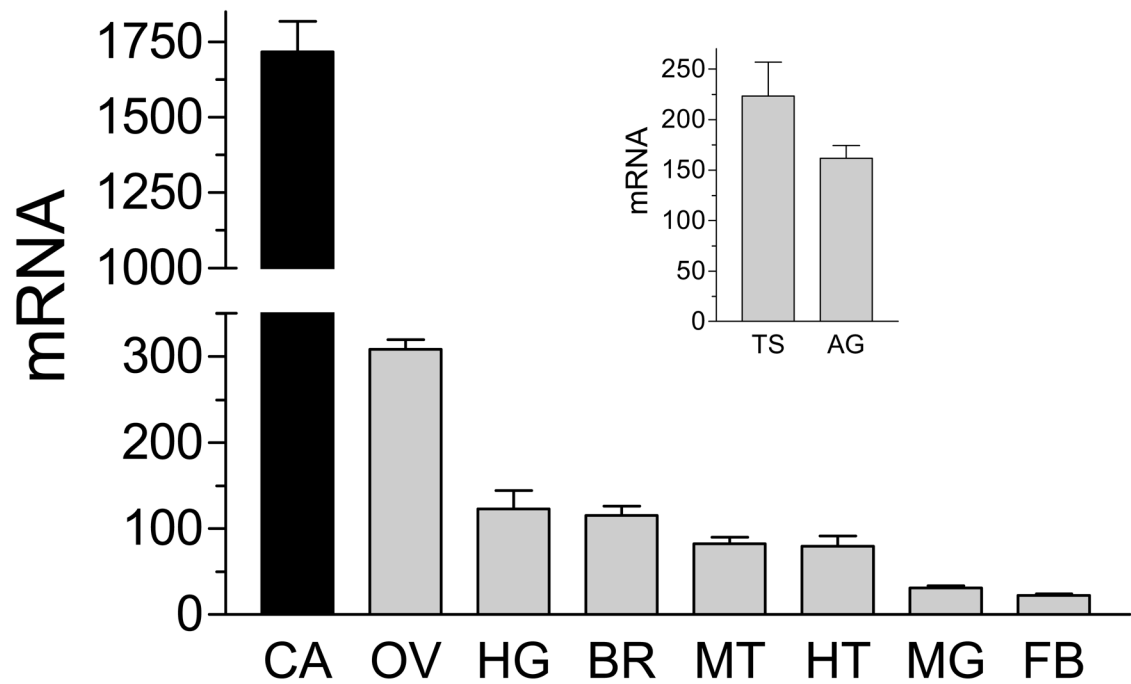


Figure 6. Tissue specific expression of AaIPPI mRNA

All female tissues were dissected from three-day old sugar-fed females. CA: corpora allata-corpora cardiaca; OV: ovary; HG: hindgut; BR: brain; MT: Malpighian tubules; HT: heart; MG: midgut and FB: fat body. The insert shows the male testis (TS) and accessory glands (AG). AaIPPI mRNAs are expressed as copy number/10,000 copies of rpl32 mRNA. Each RT-PCR data point is mean \pm S.E.M of two independent biological replicates of 10 tissue samples. These studies are an expansion of studies published by Nouzova et al. (2011).

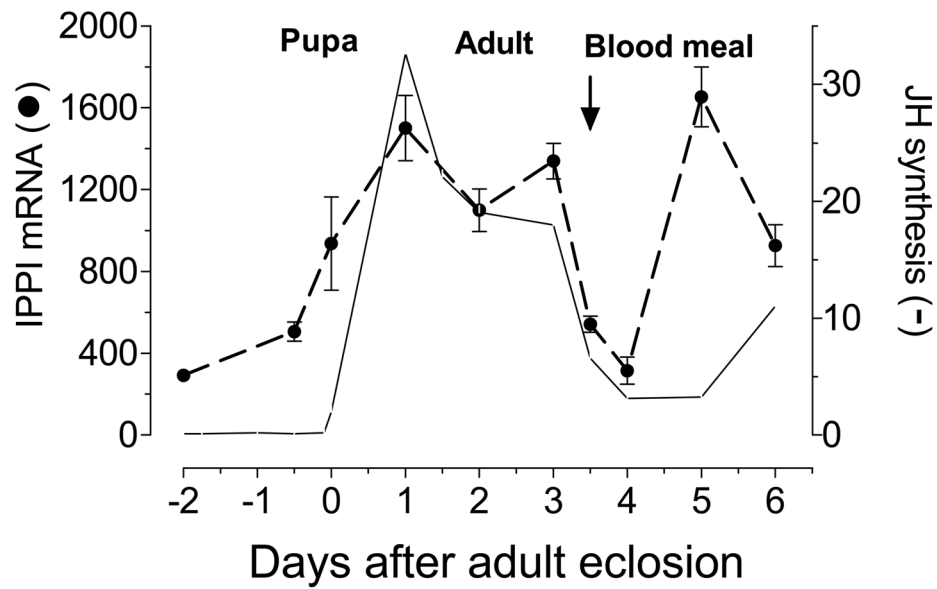


Figure 7. Developmental expression of AaIPPI mRNA in the CA

Expression of AaIPPI mRNA in CA-CC of pupa, sugar-fed and blood-fed adult female. AaIPPI mRNA is expressed as copy number of AaIPPI mRNA/10,000 copies of rpL32 mRNA. Each RT-PCR data point is mean \pm S.E.M of two independent biological replicates of 20 CA-CC. JH biosynthesis values are based on Li et al. (2003) and are expressed as fmol/CA/h. These studies are an expansion of studies published by Nouzova et al. (2011).