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A Cytosolic form of Aminopeptidase P from *Drosophila melanogaster*: Molecular Cloning and Characterization[1]

Gauri V. Kulkarni and Deepti D. Deobagkar[2]

Molecular Biology Research Laboratory, Department of Zoology, University of Pune, Ganeshkhind, Pune-411007, Maharashtra, India

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Using a functional genomic approach, we have identified and characterized a cytosolic form of aminopeptidase P from *Drosophila melanogaster*. This study represents the first characterization of an insect aminopeptidase P. The complete sequence of a 12.5 kbp genomic clone from *D. melanogaster* showed the presence of a 1,839 bp ORF, encoding a protein of 613 amino acids with a calculated molecular mass of 68.5 kDa. The deduced amino acid sequence was 48% identical and 66% similar to rat and human cytosolic aminopeptidase P. Amino acids important for catalytic activity and the metal binding ligands were found to be conserved between *Drosophila* AP-P and its mammalian homologues. The recombinant enzyme expressed in *Escherichia coli* hydrolyzed the amino terminal Xaa-Pro bond of substance P and bradykinin, revealing its functional identity. Further enzyme characterization showed the enzyme to be a manganese-dependent metallopeptidase. Immunoblot analysis showed that DAP-P is located exclusively in the cytosol and is temporally regulated during *Drosophila* development. In the adult fly, the protein could be detected in gut, testis and ovary, with a high level of expression in brain.

Key words: aminopeptidase P, cytosolic enzyme, *Drosophila*, metalloenzyme, peptidase.

Despite the availability of the complete genome sequence of *Drosophila*, it is a challenge to identify innumerable uncharacterized genes harbored in the sequences and to assign functional roles to them. Powerful computer analysis of integrated databases will not be sufficient to take us from the DNA sequence to biological function, but will provide an important foundation for the design of appropriate experiments. Such functional assignment will not only add to the list of functional genes in *Drosophila*, but will also help in identifying the genes whose products are essential for the development and survival of the organism. Here, we report the cloning of a 1,839 bp ORF from a genomic clone of *D. melanogaster*, encoding a 613aa protein that shows high levels of amino acid sequence homology to the mammalian cytosolic form of aminopeptidase P. This protein was functionally expressed in *E. coli* and biochemically characterized. The temporal expression of this enzyme during *Drosophila* development was also studied. The present work represents the first example of the functional expression and characterization of an insect aminopeptidase P.

Aminopeptidase P (AP-P; X-Pro aminopeptidase; EC 3.4.11.9) has the unique ability to cleave the N-terminal amino acid residue from peptides having proline as the penultimate amino acid residue. Biologically active peptides comprise an important and diverse class of extracellular chemical messengers that mediate a wide range of intercellular interactions. Several bioactive peptides including hormones, neuropeptides, neurotransmitters escape non-specific protease degradation by having an Xaa-Pro motif at their amino termini (*1, 2*). Due to its cyclic nature, proline confers resistance to such peptide bonds so that aminopeptidases with broad specificity cannot act upon such peptides. There are a limited number of peptidases that act on peptide bonds involving a proline residue, such as dipeptidyl peptidase II (DPPII) and dipeptidyl peptidase IV (DPPIV) (which remove Xaa-Pro dipeptides from the N-terminus of peptides), and prolidase (which cleaves the Xaa-Pro bond only in dipeptides), or endopeptidases such as prolyl endopeptidase (which cleaves on the carbonyl side of proline residues within a protein or peptide) (*3–5*). A protease from the human immunodeficiency virus also hydrolyses the Xaa-Pro bond of proteins and peptides (*6, 7*). However, none of these enzymes have been reported to hydrolyze Xaa-Pro bonds located at the N-terminus of peptides and proteins. Therefore, role of AP-P is crucial in this respect. AP-P activity is ubiquitous and has been found in a wide range of organisms including bacteria, yeast and vertebrates (*8–12*). Mammalian AP-Ps exist in membrane-bound and cytosolic forms, which represent two distinct gene products (*9–12*). The cytosolic (soluble) form of aminopeptidase P is found in human leukocytes and rat brain (*13, 14*). Both forms of AP-P can hydrolyze several peptides including bradykinin (a vasodilator) and neurotransmitters such as substance P, peptide YY, and neuropeptide Y (*11, 15–17*). Leucocyte AP-P has been demonstrated to cleave Interleukin-6 (*13*). Neuropeptide families such as FMRFa-

[1] The nucleotide sequence reported here appears in the GenBank®, DDBJ, and EMBL Nucleotide Sequence Databases under the following accession numbers, gi|4583560|emb|AJ131920.2| DME131920.

[2] To whom correspondence should be addressed. Phone: +91-20-5698432, Fax: +91-20-5690087, E-mail: dddeo@unipune.ernet.in

Abbreviations: DAP-P, *Drosophila* aminopeptidase P; Ni^{2+}-NTA, nickel–nitrilotriacetic acid; BLAST, basic local alignment search tool; 6×His, polyhistidine tag.

mide, Neuropeptide Y, and tachykinins have been identified in vertebrates, and, subsequently, their homologues have been identified in *Drosophila*, which is a powerful host for molecular and genetic studies (*18–22*). Immunochemical localization studies of some of these peptides show their cell-specific regulation in *Drosophila* (*23–27*). However, the physiological significance of these neuropeptides, as well as the peptidases that might be involved in their metabolism and regulation in *Drosophila,* remains to be elucidated. The identification of functional aminopeptidase P from *Drosophila* opens up new possibilities for understanding the precise roles played by natural substrates and the significance of aminopeptidase P in their metabolism and regulation.

EXPERIMENTAL PROCEDURES

Materials—The Bluescript SK+ phagemid and Lambda EMBL3 genomic libraries of *D. melanogaster* were obtained from Stratagene (USA). Restriction enzymes were purchased from Life Technologies (USA), New England Biolabs (USA), and Bangalore Genei (India). Sequencing kits were obtained from United States Biochemicals (USA). Taq DNA polymerase, oligonucleotide primers and the Gateway expression cloning system were obtained from Life Technologies (USA). All other fine chemicals and peptides were purchased from Sigma. Ni^{2+}-NTA agarose was obtained from Quiagen (Germany). All chemicals and reagents used were of molecular biology grade.

Cloning and Sequencing of Drosophila Cytoplasmic AP-P—Restriction fragments of a 12.5 kb genomic clone from the *Drosophila melanogaster* EMBL3 genomic library were subcloned into pBluescript SK+ vector. The products were sequenced completely from both ends using T3 and T7 primers, both manually and by using an ABI Prism-377 DNA sequencer. All basic molecular techniques were adapted from Current Protocols (*28, 29*).

Computer Software for DNA and Protein Analysis—DNA and protein sequence analyses were carried out using Vector NTI Suite 5.0 and OMIGA 1.1 software. Sequence similarity searches were conducted using the Basic Local Alignment Search Tool (*30*) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple protein sequence alignment was done using Protein BLAST with the NCBI and OMIGA 1.1 alignment software. Box shading was done manually.

Expression and Purification of Recombinant DAP-P—*Dap*I (5′ AAAAAGCAGGCTCAATCGAAGGTCGTATGAAGAGGAGCACCACCCA 3′) and DapII (5′ GAAGCTGGGTATTAAATGGGCTGAACTTCCT 3′) PCR primers were synthesized and used to amplify the complete coding region of DAP-P from the 12.5 kb genomic clone. The PCR product was purified and cloned in expression vector pDEST17 (Gateway Cloning System) according to the manufacturer's instructions. The recombinant plasmid was transformed into *E. coli* BL21-SI cells. The culture was grown in LB medium in the presence of 100 μg/ml ampicillin without NaCl at 30°C, with shaking. When the OD at 600 nm reached 0.6, protein expression was induced by the addition of 0.3 M NaCl for 3 h. The cells were harvested by centrifugation at 5,000 ×*g* for 10 min at 4°C. The cells were resuspended in bufferA (50 mM Tris, pH 8.0, 300 mM NaCl, 20 mM Imidazole) containing 1mg/ml lysozyme and sonicated at 4°C. The lysate was centrifuged and the supernatant was used for DAP-P purification. The N-terminal His-tag fusion protein was purified by loading the cleared supernatant on a Ni^{2+}-NTA agarose column according to manufacturer's instructions. The fractions containing recombinant DAP-P were identified by SDS-PAGE analysis (*29*). The His-tag was removed by Factor Xa (Pharmacia, UK) digestion as recommended by the manufacturer.

Enzyme Activity Assays—All enzyme activity assays were performed at 37°C. The enzyme activity was assayed using a fluorimetric assay adapted from the method of Simmons and Orawski (*12*). Enzyme (500 ng) with or without His-tag was preincubated in 0.1 M HEPES buffer (pH 8.0), and then incubated with the substrate at a final concentration of 0.1 mM (0.1 ml total reaction volume) at 37°C for different time periods. A 25 μl aliquot of the reaction was mixed with 1 ml of *o*-pthaldealdehyde-2ME reagent, and the fluorescence was read at an excitation of 340 nm and emission of 455 nm on a Perkin Elmer LS50 spectrofluorimeter (Applied Biosystems, USA). The released arginine was quantified using a standard arginine graph. Assay mixtures minus recombinant DAP-P and with only recombinant DAP-P served as controls. The presence of released arginine in the assay mixture was confirmed by TLC (with silica gel–coated TLC aluminium sheets, Merck, Germany) using a butanol:acetic acid:water (4:4:1) solvent system and detection by ninhydrin. The enzyme activity was found to be unaffected by the presence of His-tag. Thus, all further assays were carried out using the recombinant enzyme. For pH optimization, HEPES buffer in a pH range from 6.8 to 9.0 was used. To check the effect of divalent metal ions and EDTA, the enzyme was preincubated with different concentrations of $MnCl_2$/EDTA/metal ions at 37°C for 15 min prior to substrate addition.

Antibody Generation—The recombinant fusion protein purified from *E. coli* was used to raise polyclonal antibodies in rabbits. Two micrograms of the protein in 1 ml phosphate buffered saline (pH 7.0) was mixed with an equal volume of Freund's complete adjuvant (Sigma, USA) and injected subcutaneously at multiple sites. Booster doses were given three times, at 15 days intervals, and serum was collected 1 week after the third and fourth immunizations.

SDS-PAGE and Western Blot Analysis—To check the subcellular localization of DAP-P protein, adults and pupae were ground in liquid nitrogen and homogenized in buffer A (50 mM TrisCl pH 7.4, 10 mM $MgCl_2$, 0.1 mM PMSF, 0.04% BME, 5% glycerol). The homogenate was centrifuged at 10,000 ×*g* for 30 min at 4°C and the supernatant was subjected to centrifugation at 100,000 ×*g* in an ultracentrifuge (Kubota, Japan) for 1 hr at 4°C. The resultant supernatant and pellet were designated S_{100} (soluble proteins) and P_{100} (membrane proteins), respectively. Adult tissues, larvae, embryos, and pupae were homogenized in buffer A and centrifuged at 10,000 ×*g* for 30 min at 4°C, and the supernatants were collected. The protein concentration was determined by the method of Bradford (*31*) with the Bio-Rad protein assay reagent. Bovine serum albumin was used as a standard. The proteins were resolved in 10–12.5% SDS-PAGE (*29*) and transferred to nitrocellulose membranes (Hybond-C, Amersham, UK). The primary anti–DAP-P polyclonal antibody was used at a dilution of 1:3,500. The same blot was also probed with *Drosophila* beta actin antibodies (Amersham) as an internal loading control. The secondary anti-rabbit antibody (HRP-Linked,

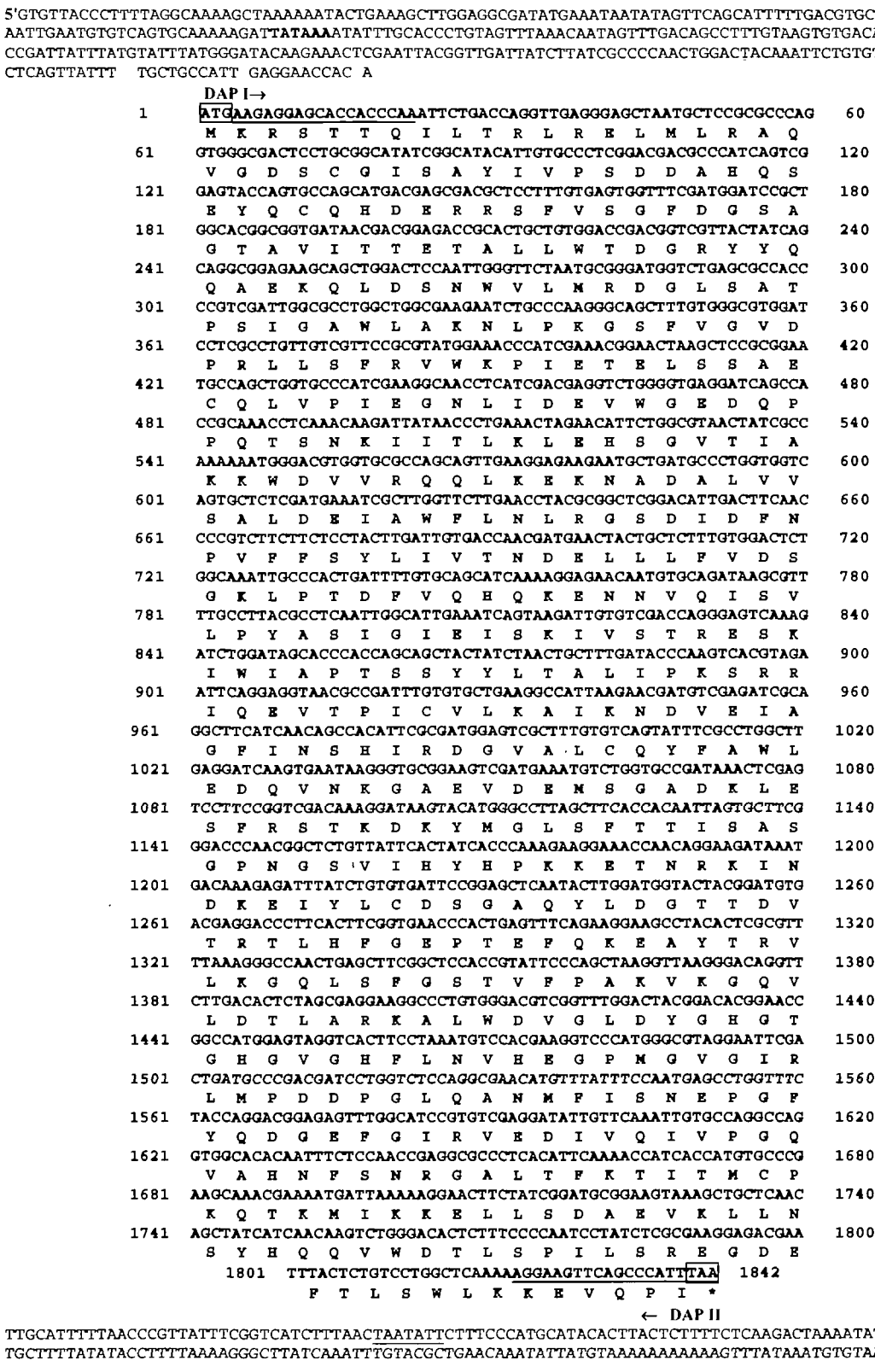

Fig. 1. **Nucleotide and deduced amino acid sequence of *Drosophila* cytoplasmic AP-P.** (GeneBank/EMBL accession number AJ131920) The start and stop codons are shown in boxes. The poly(A) addition signal is underlined. The putative TATA box is indicated by bold letters. The primer sequences, *Dap*I and *Dap*II, which were used to amplify the complete ORF, are marked with arrows.

Sigma, USA) was used at a dilution of 1:1,000. Detection was carried out using an ECL–western blotting detection system (Amersham-Pharmacia, UK).

RESULTS AND DISCUSSION

Sequence Analysis of Drosophila Cytosolic AP-P—When the sequence of the 12.5 kb genomic clone from *D. melanogaster* was analyzed, a part of the sequence showed an uninterrupted open reading frame of 1,839 bp coding for a 613 amino acid protein with a calculated M_r 68,500 (EMBL Acc. No. AJ131920) (Fig. 1). The upstream and downstream sequences showed a putative promoter region at the 5′ end and a polyadenylation signal at the 3′ end. The coding region was PCR-amplified from the genomic clone, and the predicted restriction map was confirmed by digestion with the appropriate restriction enzymes. BLAST search using the deduced amino acid sequence showed homology to aminopeptidase P. Mammalian AP-Ps exist as membrane bound forms, which include a GPI-anchored form and a cytosolic form. Each form is encoded by a separate gene. The *Drosophila* protein sequence shows 48% identity and 66% similarity to rat (R, EMBL Acc. No. AAB95331) and human (H, EMBL Acc. No. AAH05126) cytosolic aminopeptidase P (Fig. 2). Compared with the human membrane-bound form of AP-P, the *Drosophila* sequence shows 40% identity and 59% similarity. Mammalian membrane-bound AP-P is characterized by the presence of hydrophobic signal sequences, including an N-terminal signal peptide and C-terminal GPI anchor addition signal. Neither of these signature sequences is present in the *Drosophila* sequence. Thus, the *Drosophila* protein (DAP-P) shows a high level of sequence homology to the mammalian cytosolic form of AP-P. This gene is localized to band 36A11 of the second polytene chromosome (*Drosophila* Genome Project Gadfly gene-CG6291). The DAP-P sequence also shows 28% identity

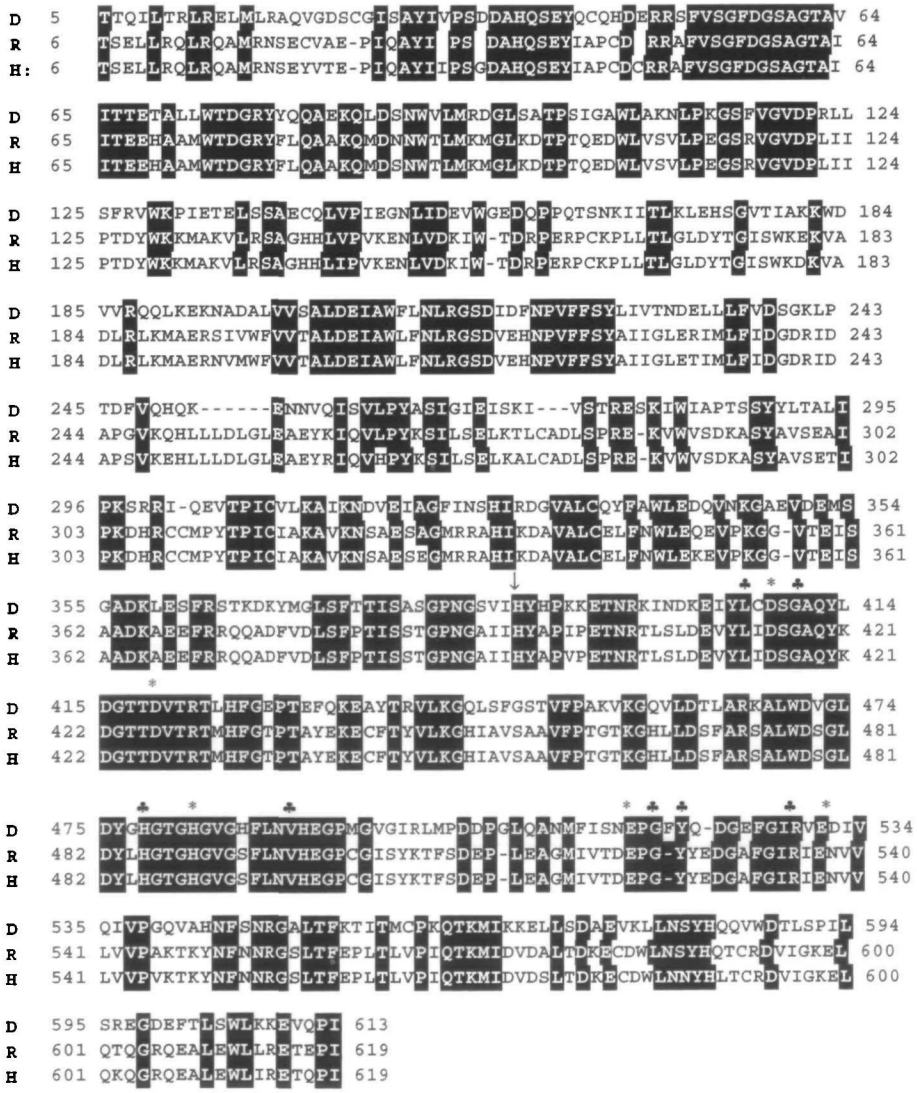

Fig. 2. **Alignment of the amino acid sequence of *Drosophila melanogaster* cytoplasmic AP-P with the rat (R) and human (H) cytosolic AP-P sequences.** Residues identical in all three sequences are box shaded. The conserved putative metal ligands are indicated by *, which are D, D, H, E, and E in all three sequences. The putative proton shuttle is marked with an arrow. Residues that play an important role in proline specificity are marked by ♣, which are L, G, H, V, G, Y, and I in all three sequences. The sequence alignment was done using OMIGA alignment software.

and 45% similarity to *E. coli* aminopeptidase P (EMBL Acc. No. BAA14325). *E. coli* aminopeptidase P and all mammalian AP-Ps share homologous blocks of sequence common to members of the "pita bread fold" family, of which *E. coli* methionine aminopeptidase is a prototype (*32–34*). The structural family of pita bread fold enzymes represents a protein scaffold that can support a diverse set of catalytic functions (*32*). These homologous blocks of sequences contain a proton shuttle, comprising a histidine residue and five metal ion ligands (*32*). The X-ray crystallographic structure of *E. coli* AP-P has been solved and two metal ions are found to be sandwiched in the pita-bread domain. The metal ions are liganded by two Asp, one His and two Glu residues (*33*). The requirement of these residues for catalytic activity of porcine AP-P has been demonstrated by site-directed mutagenesis (*35*). These catalytic residues are also well conserved in mammalian AP-Ps (*34*). The *Drosophila* protein shows conservation of all five putative metal ligand residues at positions 408(D), 419(D), 482(H), 517(E), and 531(E) (Fig. 2). Based on the creatinase (a member of the pita bread family) crystal structure, a histidine residue is thought to be the key catalytic residue, acting as a general acid and base and as a proton shuttle (*32, 33*). DAP-P shows the presence of a putative proton shuttle at position 388 (Fig. 2). Therefore, we suggest that DAP-P is a pita bread fold protein that shows more resemblance to mammalian AP-P. The proline specificity of AP-P and prolidases is characterized by the conservation of seven residues (Leu-258, His-350, Gly-385, Tyr-387, Ile-405, Gly-262, and Val-360 in *E. coli* AP-P) (*32, 33*). These residues are not conserved in creatinase or methionine aminopeptidase. The *Drosophila* protein shows the conservation of these seven residues (Leu-406, His-478, Gly-519, Tyr-521, Ile-528, Gly-410, and Val-490) (Fig. 2). Thus, DAP-P shows similarities to other members of peptidase clan MG in terms of structural properties and the active site configuration.

Expression, Purification, and Activity of DAP-P—To verify that the predicted ORF indeed has aminopeptidase P activity, it was cloned and expressed in *E. coli*. The expressed His-tagged DAP-P was purified to homogeneity from the soluble fraction of bacteria with yields of 6 mg/lit of *E. coli* culture. As indicated by SDS-PAGE, the molecular mass of purified recombinant *Drosophila* AP-P is 71 kDa (Fig. 3). The enzyme activity was assayed using a fluorimetric assay adapted from the method of Simmons and Orawski (*12*). The purified recombinant DAP-P protein hydrolyzed the amino terminal arginine from substance P and bradykinin, typical substrates for mammalian AP-Ps, demonstrating its functional identity. The metalloenzyme nature of the expressed DAP-P was confirmed by its inhibition by the chelating agent EDTA. EDTA, at a concentration of 100 μM inhibited 80% of the enzyme activity (Fig. 4D). The enzyme could hydrolyze substance P in the pH range of 7.2–8.0, with the highest activity at pH 7.6 (Fig. 4C).

The enzyme activity was affected by divalent cations. The effect of a range of concentrations (0.1 μM–10 mM) of Mn^{2+} ions on the hydrolysis of substance P and bradykinin by purified recombinant *Drosophila* AP-P was analyzed. Mn^{2+} stimulated the hydrolysis of substance P in the micromolar range, concentration at which there was no considerable effect on bradykinin hydrolysis. Higher Mn^{2+} concentrations inhibited the enzyme (Fig. 4A). On the basis of

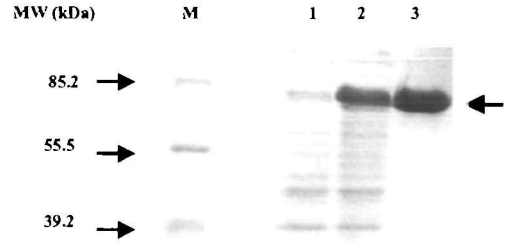

Fig. 3. **Expression and purification of *Drosophila* AP-P from *E. coli*.** The ORF was cloned into expression vector pDEST17, and expression was induced by incubation with 0.3 M NaCl for 3 h. The recombinant N-terminal His-tagged protein was purified on a Ni^{2+}-NTA column and analyzed by 12.5% SDS-PAGE. The gel was stained with Coomasiae Brilliant Blue. Lane M: SDS-PAGE molecular weight markers (Combithek); lane 1: crude cytosolic extract of an uninduced culture; lane 2: crude cytosolic extract of an induced culture showing the induction of DAP-P; lane 3: Ni^{2+}-NTA purified recombinant *Drosophila* protein (arrow).

Mn^{2+} dependence, substrates for pig kidney membrane-bound AP-P were divided into two groups (*36*). Unlike human cytosolic AP-P (*37*), the *Drosophila* enzyme activity towards bradykinin was inhibited by Mn^{2+}. Ca^{2+}, Mg^{2+}, and Co^{2+} stimulated the hydrolysis of substance P at micromolar (10–100 μM) concentrations, but were inhibitory at a concentration of 1 mM. DAP-P activity was inhibited by 56% at 100 μM Cu^{2+}, which decreased further as the concentration of Cu^{2+} ions increased. Ni^{2+} and Zn^{2+} at 1 μM completely inhibited the enzyme activity (Fig. 4B). Zn^{2+} activated the porcine membrane-bound form of AP-P to some extent, in addition to Mn^{2+} (*36*) while the human cytosolic form of AP-P was inhibited by Zn^{2+} (*37*). These differences might reflect the intracellular localization of the enzyme where the Mn^{2+} ion concentration is higher than in the extracellular fluid. Extracellular fluids have a higher concentration of Zn^{2+} ions, which is tolerated by the membrane-bound form, facing the extracellular fluid (*36*). DAP-P activity was found to be inhibited by Zn^{2+} ions. The cloned *Drosophila* AP-P has a pH optimum of 7.6, shows manganese-dependent activity, and is differentially inhibited by Zinc. These properties reflect the similarities between DAP-P and the mammalian cytosolic form of AP-P.

Ca^{2+} and Co^{2+} stimulate the activity of *Drosophila* AP-P in contrast to human AP-P, which is inhibited by these metal ions. The enzyme activity is stimulated by Mn^{2+} ion in a substrate-dependent manner. The effects of divalent metal ions and the inhibition of the *Drosophila* enzyme activity by millimolar concentrations of Mn^{2+} suggest that DAP-P is a Mn^{2+}-dependent enzyme that shares catalytic similarities with mammalian AP-Ps. However, the elucidation of the crystal structure of *Drosophila* AP-P will be helpful in confirming the residues involved in catalysis, metal ligands and the identity of metal ions within the active site.

Expression analysis of Drosophila AP-P—The expression of DAP-P protein in *Drosophila* was analyzed by immunoblotting using polyclonal antibodies produced against recombinant DAP-P. To check the subcellular localization, membrane and cytosolic protein fractions from adults and pupae were used. A single immunoreactive band of DAP-P of the expected size (69 kDa) could be detected in the cytosolic

fractions (S_{100}) of both adults and pupae (Fig. 5). No immunoreactive protein could be detected in the membrane fractions (P_{100}). This observation further confirms the cytosolic localization of DAP-P. The expression of DAP-P in different developmental stages of *D. melanogaster* was analyzed (Fig. 6). The expression appears to be temporally regulated during *Drosophila* development. During embryogenesis, the protein was detected at 3–6 h of embryonic development and its level continued to increase in later stages (Fig. 6A). The amount of DAP-P protein decreased in the larval stages (Fig. 6B, lane c and b), but again increased in the pupae (Fig. 6B, lane a). In adults, the protein could be detected in different tissues including brain, testis and ovary, as well as in the gut (Fig. 6C). Interestingly, the level of DAP-P protein was higher in brain (Fig. 6C, lane c).

Thus, using a functional genomics approach, a functional role was assigned to the 1,839 bp ORF coding for aminopeptidase P. This ORF was cloned and the expressed protein was shown to be catalytically active towards natural peptides including bradykinin and substance P. Immunoblot analysis showed a developmental regulation of DAP-P, suggesting its possible role in *Drosophila* development. Although human cytosolic AP-P has been suggested to play a role in the neural development (*10*), there has been no clear indication of its *in vivo* role in humans. A number of neuropeptides have been described that are present in the insect nervous system. Many mammalian neuropeptides including, FMRFamide peptides, Neuropeptide Y, and some tachykinins, show an N-terminal Xaa-Pro motif, and homologues are also found in *Drosophila* (*18–22*). However, the physiological role of these neuropeptides has not yet been clearly demonstrated. Since DAP-P can hydrolyze sub-

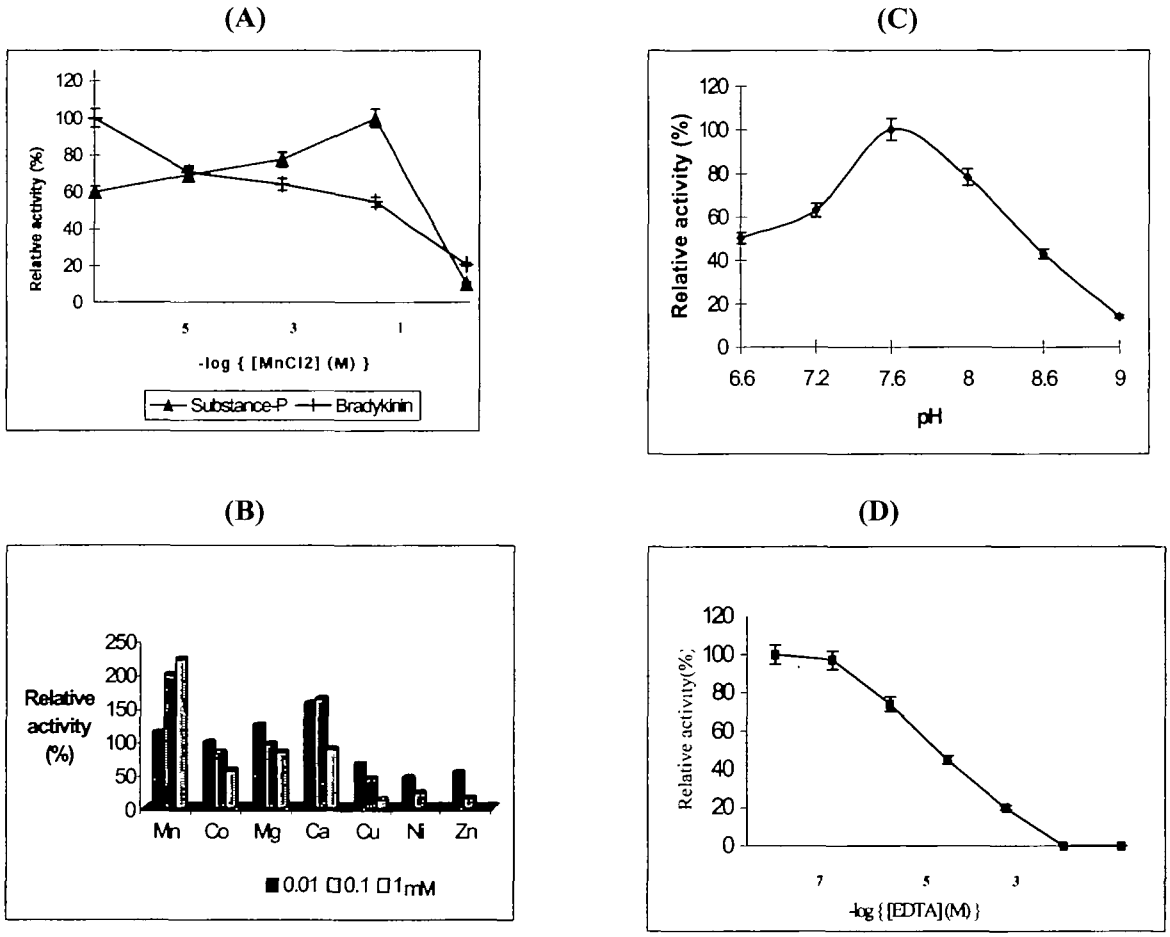

Fig. 4. **Catalytic properties of purified recombinant DAP-P.** (A) Manganese dependence. Purified recombinant aminopeptidase P was incubated with 0.1 mM each of bradykinin and substance P in the presence of various concentrations of $MnCl_2$ in 0.1 M HEPES, pH 7.6, at 37°C. The activity was analyzed and expressed as the percent of the maximum activity observed. Results are the mean values of duplicate determinations at each concentration in a single experiment. Error bars are standard errors of the mean. (B) Effect of divalent metal ions on DAP-P activity. The enzyme was incubated with the indicated concentration of divalent cations for 15 min at 37°C. Upon the addition of 0.1 mM substance P, the samples were incubated at 37°C for 3 h. The activity was analyzed and is expressed as the percent of the activity observed in the absence of added metal ions. Results are the mean values of duplicate determinations at each concentration in a single experiment, which did not differ by more than 6%. (C) Effect of pH. Enzyme activity was assayed in 0.1 M HEPES buffer (pH range 6.6–9.0) in presence of 1 mM $MgCl_2$ and 0.1 mM substance P, and expressed as the percent of the maximum activity observed. Results are the mean of duplicate determinations carried out in three separate experiments. Error bars are standard errors of the mean. (D) Effect of EDTA. Purified recombinant aminopeptidase P was preincubated for 15 min in the presence of various concentrations of EDTA, and then incubated with substance P (0.1 mM) at 37°C for 3 h. The activity is expressed as the percent of activity in the absence of EDTA. Results are the mean of duplicate determinations at each concentration carried out in two separate experiments. Error bars are standard errors of the mean.

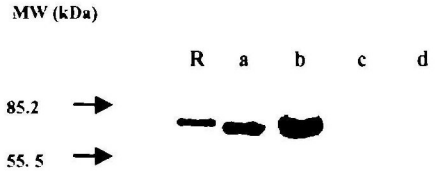

Fig. 5. **Subcellular localization of DAP-P protein in *Drosophila*.** S_{100} (soluble; approx. 20 μg) and P_{100} (membrane; approx. 40 μg) protein fractions from adults and pupae were subjected to 12.5% SDS-PAGE and blotted on a nitrocellulose membrane. The blot was treated with polyclonal antibodies raised in a rabbit against recombinant DAP-P, followed by anti-rabbit HRP conjugate. Detection was with an ECL-Western detection system (Amersham-Pharmacia). The positions of the molecular weight markers are indicated. Lane R, recombinant DAP-P (20 ng); lane a, S_{100} fraction from adults; lane b, S_{100} fraction from pupae; lane c, P_{100} fraction from adults; lane d, P_{100} fraction from pupae.

stance P, a neurotransmitter, and substance P immunoreactive neurons have been detected in *Drosophila* (*21, 22*), it is interesting to speculate that DAP-P might be involved in the regulation of substance P homologues in *Drosophila*. It is noteworthy that DAP-P shows the highest level of expression in adult brain compared to other tissues such as testes, ovaries and gut. Further experiments should elucidate its functional significance in these tissues. The high sequence homology to mammalian cytosolic AP-P, the nature of the metal ion dependence, and immunoblot analysis suggest that this enzyme represents the cytosolic form of AP-P. Since most neuropeptides are extracellular messengers, this form of AP-P might be part of the intracellular signal processing system. A search of the *Drosophila* database (*39*) showed the presence of another AP-P gene with six exons, localized to the 87C region of the third polytene chromosome, and coding for a 704 amino acid product (EMBL Ac. No. AE003696). These forms of AP-P and DAP-P share only 30% identity. Further analysis of this AP-P gene sequence showed the presence of both N- and C-terminal signal sequences typical of the membrane-bound form of AP-P. However, some amino acid residues important in the catalytic site are missing from this sequence (data not shown). The characterization of DAP-P in this study represents the first report of the characterization of an insect aminopeptidase P.

Despite the great divergence of *Drosophila* from mammals, the cytosolic AP-P shows remarkable conservation in its catalytic domains and properties. Further studies of *Drosophila* AP-P (DAP-P) will contribute to the further elucidation of the structure–function relationships of this important enzyme. Also, the further characterization of DAP-P will lead to a better understanding of the regulation and metabolism of Xaa-Pro–containing bioactive peptides in cell signaling, not only in *Drosophila* and other insect systems, but also in the higher vertebrates.

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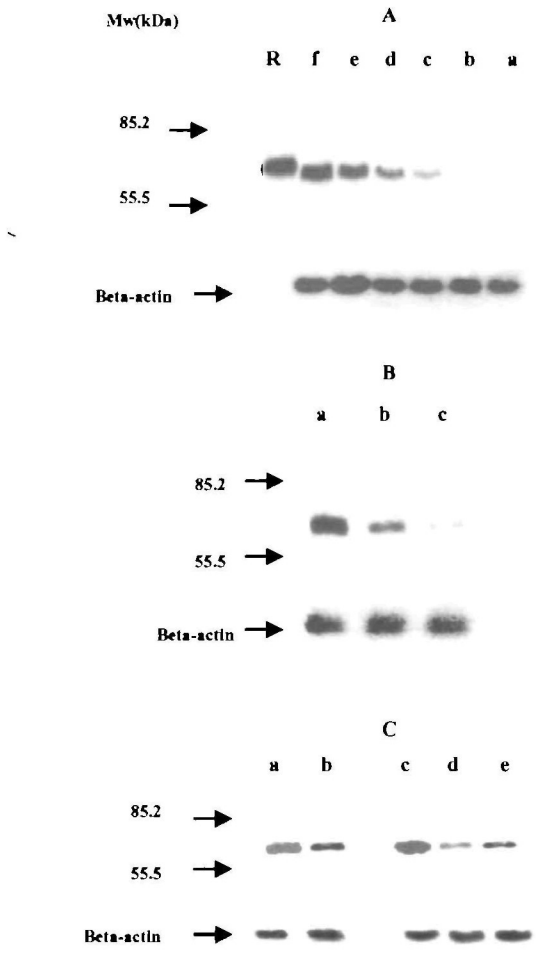

Fig. 6. **Expression of DAP-P protein during *Drosophila* development.** Equivalent amounts of proteins (20 μg) from *Drosophila* at different developmental stages were subjected to 12.5% SDS-PAGE and blotted on a nitrocellulose membrane. The blot was treated with polyclonal antibodies raised in a rabbit against the recombinant DAP-P protein, followed by anti-rabbit HRP conjugate. The same blots were washed and reprobed with *Drosophila* beta-actin antibodies as an internal control. Detection was with an ECL-Western detection system (Amersham-Pharmacia). The positions of the molecular weight markers are indicated. (A) Lane R, recombinant DAP-P (50 ng); lane a, 0–3 h embryos; lane b, 3–6 h embryos; lane c, 6–9 h embryos; lane d, 9–12 h embryos; lane e, 12–16 h embryos; lane f, 16-22 h embryos. (B) Lane a, pupae; lane b, 3rd instar larvae; lane c, 1st and 2nd instar larvae. (C) Lane a, total adult extract; lane b, gut; lane c, brain; lane d, testes; lane e, ovary.

REFERENCES

1. Yaron, A. and Naider, F. (1993) Proline-dependent structural and biological properties of peptides and proteins. *Crit. Rev. Biochem. Mol. Biol.* **28**, 31–81
2. Yaron, A. (1987) The role of proline in the proteolytic regulation of biologically active peptides. *Biopolymers* **26**, S215–S222
3. Mentlein, R. (1988) Proline residues in the maturation and degradation of peptide hormones and neuropeptides. *FEBS Lett.* **234**, 251–256
4. Vanhoof, G., Goossens, F., De Meester, I., Hendriks, D., and Scharpe, S. (1995) Proline motifs in peptides and their biological processing. *FASEB J.* **9**, 736–744
5. Barett, A.J. and McDonald, J.K. (1980) *Mammalian Proteases: a Glossary and Bibliography*, col. 1, pp. 254–256, Academic Press, London
6. Darke, P.L., Nutt, R.F., Brady, S.F., Garsky, V.M., Ciccarone, T.M., Leu, C.T., Lumma, P.K., Freidinger, R.M., Veber, D.F., and Sigal, I.S. (1988) HIV-1 protease specificity of peptide cleavage is sufficient for processing of gag and pol polyproteins. *Biochem.*

Boiophy. Res. Commun. **156**, 297–303
7. Pearl, L.H. and Taylor, W.R. (1987) A structural model for the retroviral proteases. *Nature* **328**, 482
8. Yaron, A. and Mlynar, D. (1968) Aminopeptidase P. *Biophys. Biochem. Res. Commun.* **32**, 658–663
9. Dehm, P. and Nordwig, A. (1970) The cleavage of prolyl peptides by kidney peptidases. Isolation of a microsomal carboxypeptidase from swine kidney. *Eur. J. Biochem.* **17**, 364–371
10. Orawski, A.T., Susz, J.P., and Simmons, W.H. (1987) Aminopeptidase P from bovine lung: solubilization, properties, and potential role in bradykinin degradation. *Mol. Cell. Biochem.* **75**, 123–132
11. Hooper, N.M., Hryszko, J., and Turner A.J. (1990) Purification and characterization of pig kidney aminopeptidase P. A glycosyl-phosphatidylinositol-anchored ectoenzyme. *Biochem. J.* **267**, 509–515
12. Simmons, W.H. and Orawski, A.T. (1992) Membrane-bound aminopeptidase P from bovine lung. Its purification, properties, and degradation of bradykinin. *J. Biol. Chem.* **267**, 4897–4903
13. Rusu, I. and Yaron, A. (1992) Aminopeptidase P from human leukocytes. *Eur.J. Biochem.* **210**, 93–100
14. Harbeck, H.T. and Mentlein, R. (1991) Aminopeptidase P from rat brain: Purification and action on bioactive peptides. *Eur. J. Biochem.* **198**, 451–458
15. Yoshimoto, T., Orawski, A.T., and Simmons, W.H. (1994) Substrate specificity of aminopeptidase P from *Escherichia coli*. comparison with membrane-bound forms from rat and bovine lung. *Arch. Biochem. Biophys.* **311**, 28–34
16. Medeirros, M.S. and Turner, A.J. (1994) Post-secretory processing of regulatory peptides: the pancreatic polypeptide family as a model example. *Biochemie* **76**, 283–287
17. Kim, K.S., Kumar, S., Simmons, W.H., and Brown, N.J. (2000) Inhibition of aminopeptidase P potentiates wheal response to bradykinin in angiotensin-converting enzyme inhibitor-treated humans. *J. Pharmacolo Exp. Ther.* **292**, 295–298
18. Schneider, L.E., Roberts, M.S., and Taghert, P.H., (1991) In situ hybridization analysis of the FMRFamide neuropeptide gene in *Drosophila*. Restricted expression in embryonic and larval stages. *J. Comp. Neurol.* **304**, 608–622
19. Nambu, J.R., Murphy-Erdosh., C., Andrews P.C., Feistner, G.J., and Scheller, R.H. (1988) Isolation and characterization of a *Drosophila* neuropeptide gene. *Neuron* **1**, 55–61
20. Brown, M.R., Crim, J.W., Arata, R.C., Cai, C.C., and Shen, P. (1999) Identification of a *Drosophila* brain-gut peptide related to the neuropeptide Y family. *Peptides* **20**, 1035–1042
21. Nassel, D.R., Lundquist, T., Hoog, A., and Grimelius., L. (1990) Substance P-like immunoreactive neurons in the nervous system of *Drosophila*. *Brain Res.* **507**, 225–233
22. Lundquist, T. and Nassel, D.R. (1990) Substance P, FMRFamide, and gastrin/cholecystokinin-like immunoreactive neurons in the thoraco-abdominal ganglia of the flies *Drosophila* and *Calliphora*. *J. Comp. Neurol.* **294**, 161–178
23. Nichols, R., McCormick, I., and Lim, I. (1999) Regulation of *Drosophila* FMRFamide neuropeptide gene expression. *J. Neurobiol.* **39**, 347–358
24. Nicholas, R., McCormick, I., Lim, I., and Caserta, L. (1995) Cellular expression of the *Drosophila melanogaster* FMRFamide neuropeptide gene product DPKQDFMRFamide. Evidence for differential processing of the FMRFamide polypeptide precursor. *J. Mol. Neurosci.* **6**, 1–10
25. Nichols, R., McCormick, I.B., Lim, I.A., and Starkman, I.S. (1995) Spatial and temporal analysis of the *Drosophila* FMRFamide neuropeptide gene product SDNFMRFamide: evidence for a restricted expression pattern. *Neuropeptides* **29**, 205
26. McCormick, I., Lim, I., and Nichols, R. (1999) Neuropeptide precursor processing detected by triple immunolabeling . *Cell Tissue Res.* **297**, 197–202
27. Nichols, R., McCormick, I., and Lim I. (1997) Multiple antigenic peptides designed to structurally related *Drosophila* peptides. *Peptides* **18**, 41–45
28. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, New York
29. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Tatiana, A., Tatusova., Thomas, L., and Madden. (1999) BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol. Lett.* **174**, 247–250
31. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Anal. Biochem.* **72**, 248–254
32. Bazan, J.F., Weaver, L.H., Roderick, S.L., Huber, R., and Matthews, B.W. (1994) Sequence and structure comparison suggest that methionine aminopeptidase, prolidase, aminopeptidase P, and creatinase share a common fold. *Proc. Natl. Acad. Sci. USA* **91**, 2473–2477
33. Wilce, M C., Bond, C.S., Dixon, N.E., Freeman, H.C., Guess, J.M., Lilley, P.E., and Wilce, J.A. (1998) Structure and mechanism of a proline-specific aminopeptidase from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **95**, 3472–3477
34. Sprinkle, T.J., Caldwell, C., and Ryan, J.W. (2000) Cloning, chromosomal sublocalization of the human soluble aminopeptidase P gene (XPNPEP1) to 10q25.3 and conservation of the putative proton shuttle and metal ligand binding sites with XPNPEP2. *Arch. Biochem. Biophys.* **378**, 51–56
35. Cottrell, G.S., Hyde, R., Lim, J., Parsons, M.R., Hooper, N.M., and Turner, A.J. (2000) Identification of critical residues in the active site of porcine membrane-bound aminopeptidase P. *Biochemistry* **39**, 15129–15135
36. Lloyd, G.S., Hryszko, J., Hooper, N.M., and Turner, A.J. (1996) Inhibition and metal ion activation of pig kidney aminopeptidase P. Dependence on nature of substrate. *Biochem. Pharmacol.* **152**, 229–236
37. Cottrell, G.S., Hooper, N.M., and Turner, A.J. (2000) Cloning, expression, and characterization of human cytosolic aminopeptidase P: a single manganese(II)-dependent enzyme. *Biochemistry* **39**, 15121–15128
38. Copenhaver, P.F. and Taghert, P.H. (1989) Development of the enteric nervous system in the moth. Diversity of cell types and the embryonic expression of FMRFamide-related neuropeptides. *Dev. Biol.* **131**, 70–84
39. Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D , *et al.* (2000) The Genome Sequence of *Drosophila*. *Science* **287**, 2185–2195