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Partial purification and characterization of the proline-specific dipeptidyl peptidase (DPP) IV enzyme from Drosophila melanogaster

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Partial Purification and Characterization of the Proline-Specific Dipeptidyl Peptidase (DPP) IV Enzyme from *Drosophila melanogaster*

A Thesis presented to the Faculty of the Department of Biology,
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in partial fulfillment of the requirements for the Honors degree in Biology.

By

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ABSTRACT

Dipeptidyl peptidase (DPP) IV is an N-terminal prolyl endopeptidase that is proline specific. In *Drosophila*, DPP RNA expression was shown to be highest in the late larval stage compared to the early hours after pupation. The enzyme was purified 2.5-fold from the soluble fractions of an homogenate prepared from the integument adhering to cuticles of third instar larvae via ion-exchange chromatography. Further purification by gel filtration resulted in a loss of activity. The partially purified enzyme was used to investigate the catalytic properties of *Drosophila* DPP. *Drosophila* DPP has a pH optimum of 8.5 and a temperature optimum of 44.5°C (assayed on the chromogenic substrate Gly-Pro-β-Naphthylamide). The apparent molecular mass of *Drosophila* DPP is about 216kDa. Like the mammalian, blowfly, and cockroach DPP, *Drosophila* DPP is classified a serine protease and is inhibited by several selective and nonselective mammalian inhibitors.

INTRODUCTION

Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5) [CD26] is a cell surface endopeptidase that was first discovered by Hopsu-Havu and Glenner in rat liver homogenates, and has since been identified in many other organisms (Mentlein, 1999). DPP IV is an N-terminal prolyl endopeptidase that is proline specific, which means that DPP IV can cleave a dipeptide from the unmodified N-terminal end of a peptide or protein when the penultimate amino acid is a proline (and with lower activity, an alanine) and the third amino acid is neither proline nor hydroxyproline (EXPASY, June 6, 2002). The DPP IV enzyme is classified as a serine protease because it contains a serine recognition site (G-X-S-X-G) and a catalytic triad that consists of asparagine, histidine, and serine (EXPASY, June 6, 2002).

DPP IV has been reported in many mammalian tissues, frogs, chickens, cockroach, the blowfly, bacteria, and yeast (Yaren and Naider, 1993; Martensen et al., 1998; Nassel et al., 2000). DPP IV is a multi-functional enzyme, with functions that may differ between tissues or species. In general, the enzyme has a major role as a regulatory protease in several species.

Evidence for the Existence of DPP IV in *Drosophila melanogaster*

Evidence for the existence of DPP IV in *Drosophila* was first reported in a transgenic fly containing the gene for flounder antifreeze protein (AFP). The transgenic flies produced hsp70-AFP fusion gene transcripts. The transcripts were translated and an AFP of similar size to pro-AFP was secreted into the hemolymph. Edman degradation sequencing of pure pro-AFP revealed that its N-terminus began "two amino acids in from the predicted signal peptide cleavage point" (Peters et al., 1993). Also present was a second amino acid sequence that began "two amino acids further into the 'pro' sequence" (Peters et al., 1993). Thus, dipeptidyl

aminopeptidase IV activity in the AFP transgenic *Drosophila* produced pro-AFP proteins. That is, it processed the preAFP protein (the AFP gene product) by removal of an N-terminal Xaa-Pro sequence just as it was presumed to be processed in the flounder (Peters et al., 1993).

DPP IV in *Drosophila melanogaster*

Previous work reported by Chihara gave further evidence of the existence of DPP IV in *Drosophila melanogaster*. The third instar larval cuticle of the wild type fly reveals five major proteins (LCP1-5) and five so-called minor proteins (LCP 2a, LCP6-9) on nondenaturing polyacrylamide gel electrophoresis (PAGE) (Figure A; Chihara et al., 1982). An ethane methane sulfonate (EMS)-induced mutant, referred to as *omega*, was identified as a recessive modifier of the third instar larval cuticle protein 5 (LCP5) by its altered mobility on PAGE (Chihara and Kimbrell, 1986). The protein pattern of *omega* showed a shift in the migration of LCP5 to a new position above LCP4. The band at position 5 was gone. The band above LCP4 was designated *omega* and was later interpreted as being an unmodified protein 5 due to a failure to remove the dipeptide Arg-Pro- from the N-terminal end of the LCP5 protein, thus causing the shift of the mature protein on non-denaturing gels (Chihara and Kimbrell, 1986).

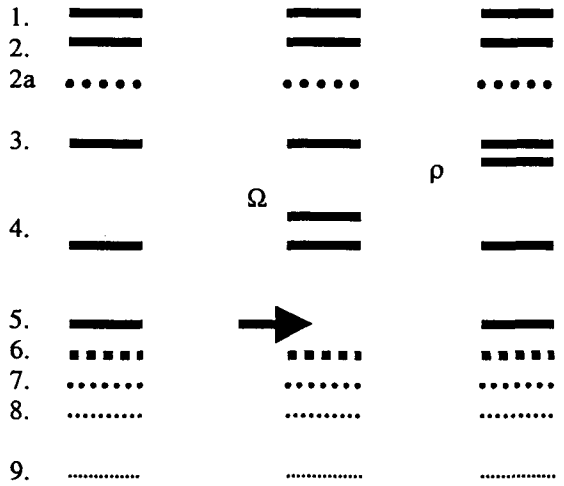


Figure A. Schematic of 15% PAGE separation of third instar cuticle proteins. The major bands are depicted as the darkest bands (1-5). The *omega* protein banding pattern is characterized by the appearance of a band between LCP3 and 4, (Ω) and a lack of a band at position 5 (arrow). ρ is the co-dominant band induced by EMS in the same screen (after Chihara et. al., 1986).

Further evidence for putative enzyme activity in third instar larvae is from microarray analysis done by White et al., as seen on the online database Metamorphosis: 534 Regulated Genes Database (White KP, *et al*, 1999). The microassay analysis shows the transcription of LD21715 – an EST now known to be a complete cDNA of the omega gene (Berkeley Drosophila Genome Project, 2002)

The *omega* mutation most likely yields a dipeptidyl peptidase that does not function normally. Partial sequencing of the cuticle protein genes revealed that the "unmodified wild-type 5 protein" of *omega* is different from the wild-type 5 protein (Chihara and Kimbrell, 1986). The difference was then shown to be at its N-terminal end of an additional two amino acids (Arg-Pro), which is recognized as a recognition motif for DPP IV. Thus the LCP5 protein is processed much as the Flounder AFP protein. The signal peptide is removed and then the Arg-Pro dipeptide is removed by DPP IV activity (Chihara, C. 1999a,b).

DPP IV Activity in Insects

Thus far, DPP IV activity has been reported in the blowfly, *Calliphora vicina*, and also the cockroach *Leucophaea maderae*. In *C. vicina* DPP IV enzyme cleaves the asp-pro-N-terminus of the ecdysiostatic peptide trypsin-modulating oostatic factor (Neb-TMOF). TMOF was isolated from the ovaries of the fleshfly *Neobelleria bullata*. Neb-TMOF is a regulatory peptide that inhibits ecdysone biosynthesis in the ring glands of the blowfly. The actions of many regulatory peptides like Neb-TMOF can be terminated by specialized exo- and endopeptidases such as DPP IV. Neb-TMOF is not protected against the attack of DPP IV because it [Neb-TMOF] contains a proline at the second position at the N-terminal end. Therefore DPP IV is a regulatory protease that inactivates Neb-TMOF in flies, thus allowing ecdysone biosynthesis to occur (Martensen et al., 1998).

Nassel et al. (2000) studied the peptides known as tachykinin-related peptides (TRPs) in the cockroach *Leucophaea maderae* and how they [the Lem-TRPs] are affected by DPP IV. TRPs are regulatory peptides that have multiple actions in the nervous system and on various types of muscle. As with the Neb-TMOF peptides, the TRPs also have a characteristic amino terminal Xaa-Pro sequence. After several studies, it was reported that DPP IV activity is important for degradation and clearance of neuropeptides from the extracellular space and hemolymph of insects and also as a digestive enzyme in the midgut.

The significance of DPP IV in *Drosophila melanogaster* is not yet clear. Studies of the *omega* mutant fly which is significantly deficient in DPP IV activity have suggested that DPP IV has pleiotropic effects. The *omega* fly lacks almost all DPP IV activity present in the wild type fly. In the fruit fly, as with the blowfly and the cockroach, DPP IV is thought to play a role as a regulatory protease that processes several important peptides that likely include some immunity

peptides, drosocin, diptericin and metchnikowin in particular. Like other regulatory peptides, these peptides, when processed by DPP IV enzyme, have an N-terminal X-proline dipeptide removed during conversion of the propeptide to the mature protein.

The mutant *omega* fly displays several defects. Early studies reported that the *omega* homozygote results in a developmental delay during the larval stage. Also, the males show a decrease in fertility levels and a decrease in their ability to successfully fertilize females. *Omega* mutants also have a loss of a post-translational processing of larval cuticle protein 5, which is, therefore, a natural substrate for the enzyme DPP IV (Pineda, 1997).

Characterization of *Calliphora* and *Leucophaea* DPP IV

In the Blue Blowfly *Calliphora vicina*, the DPP IV enzyme activity was highest in the late larval stage. Martensen et al (1998) purified DPP IV activity 240-fold from the soluble fractions of pupae of mixed age. The enzyme was characterized based on several catalytic properties as an invertebrate homologue of mammalian DPP IV. The blowfly DPP IV has a molecular mass of 200 kDA and showed an optimal pH of 7.6 to 8.0 and an optimal temperature of 40°C. DPP IV cleaved the substrate Gly-Pro-4-nitroanilide and other substrates with penultimate Pro or Ala (with lower activity). The enzyme liberated Xaa-Pro dipeptide from the N-terminus of several bioactive peptides that included substance P, neuropeptide Y, and peptide YY. Like the mammalian enzyme, the blowfly DPP IV belongs to the serine class of proteases. However, the blowfly DPP IV was not inhibited by several selective or nonselective inhibitors of its mammalian counterpart. For instance, the substrate derivatives Lys-tetrahydropyrrol and ε-Z-Lys-Pro are good inhibitors of mammalian DPP IV and exhibit only a moderate inhibitory activity on blowfly DPP. Also the peptide antibiotic bacitracin, a good mammalian DPP IV

inhibitor, was ineffective on fly DPP. Effective inhibitors of blowfly DPP are the serine protease inhibitors, phenylmethanesulfonyl fluoride (PMSF) and Pefabloc, and zinc ions, which are also nonselective inhibitors of mammalian DPP.

The blowfly results suggest that DPP IV functions as a regulatory enzyme that hydrolyzes TMOF in flies. Furthermore, DPP IV is thought to be a regulatory enzyme that hydrolyzes other bioactive peptides, in various invertebrates, that have an N-terminal Xaa-Pro sequence (Martensen et al., 1998).

The cockroach *Leucophaea maderae* has several neuropeptides, including LemTRP-1, that are potential substrates for DPP IV. In order to investigate its effects on such neuropeptides the enzyme was partially purified and characterized. The enzyme was partly purified by one chromatographic step from the brain and midgut of *L. maderae*. The enzyme cleaved substrate Gly-Pro-4-nitroanilide. The highest enzyme activity was obtained from the membrane fraction of the intestine. Ten times less activity was obtained from the membrane fraction of the brain. The soluble fractions of both tissues also reported enzyme activity but at levels much less than either of the membrane fractions. The solubilized intestinal DPP IV activity revealed a molecular mass of 75 kDa and an optimal pH of 8.5. Diprotin A, a tripeptide with the sequence Ile-Pro-Ile, was found to be an efficient competitive inhibitor of the cockroach DPP IV enzyme. Unlike the mammalian and blowfly DPP the cockroach DPP IV was less affected by zinc ions and the serine protease inhibitor PMSF (Nassel, et al., 2000).

Preliminary *D. melanogaster* DPP IV Studies

Preliminary studies were performed using crude enzyme extract obtained from wild-type *Drosophila* third instar larvae. A kinetics assay revealed that the Km with the crude extract on substrate Gly-Pro-pNA was 20μM. The crude enzyme preparation yielded a broad range pH optimum of 7.5-8.5 for the substrate Gly-Pro-pNA. Two DPP IV inhibitors were tested on the crude extract – PMSF and $ZnCl_2$. The $ZnCl_2$ (2mM) eliminated the enzyme activity while PMSF (2mM) decreased the DPP IV activity by 78% (Song, 2000).

MATERIALS AND METHODS

Fly Stocks:

Drosophila melanogaster were grown at 25°C on standard agar-molasses-corn meal-yeast media, containing 10% Tegosept and 0.6% propionic acid and supplemented with live yeast in 1/2 pint plastic bottles.

The wild type strain is "Oregon R" and has been maintained by Dr. Chihara's lab for many years.

Population Cage Protocol:

Young flies were used to fill the cages (1-3 days post emergence). A box (20 x 10 x 5cm) with a 2cm layer of prepared food was kept in the cage without the cover to feed the flies. Eggs were collected on the second day after the cage had been set up. Two food boxes (a 20 x 10 x 5cm plastic box with a cover that had a silk screen window) containing the media described above and supplemented with a ring of light yeast paste were placed in the cage without covers on the second day. Eggs were collected for 3-4 hours. After two days, the larvae were fed with a thick paste of yeast mixed with water. The covers were then replaced and the boxes were incubated at 25°C for 4-5 days. Two new boxes were placed in the cage. Eggs were collected until the next morning and then incubated at 25°C for 4-5 days. After two days of incubation, the larvae were fed with a thick yeast paste and the box was taped around the edge of the cover to prevent the larvae from escaping. Third instar larvae were collected by washing them out from the boxes with water into beakers.

Preparation of Third Instar larvae Epidermal Cell Crude Extract

Third instar larvae were collected from the food boxes after the 4th or 5th day of incubation when larvae had climbed out of the food. The larvae were rinsed with water containing added table salt, which served as a density-dependent separation to float the larvae and separate them from the food particles. Once the larvae was collected, free of food particles, they were rinsed several times with water until all of the salt was gone. An ice platform was set-up using a Styrofoam box filled with ice and then covered with a glass plate. A sheet of aluminum foil was placed over the glass plate. A spoonful of larvae were spread over the foil. Another piece of foil or a piece of Saran wrap was used to cover the larvae. A solid brass, heavy cylinder-shaped metal rolling pin (~1.814kg, 10.5cm x 6cm) was rolled over the larvae several times to extrude the insides of the larvae. The carcasses were washed off the piece of foil with ice cold 1X *Drosophila* Ringer's solution until the wash was clear and all visible fat body and discs were gone from the foil (under the dissecting microscope). The carcasses were filtered through a piece of silk screen and homogenized in an ice-cold blender (Waring blender, 7010 model 31BL91, 50-60Hz) for 90 seconds at high speed in Buffer 1 (0.5mM phenylthiourea, 0.38M sucrose, 0.1M Tris-HCl, pH 8.5), using 10ml buffer per 250 larval carcasses. The carcasses were assumed to have cuticles, adhering epithelial cells, and probably some muscle tissue. The homogenate was centrifuged (Beckman J2-21 Centrifuge, JA-20 Rotor) for 3 hours at 18,000 rpm at 4°C. The supernatant was collected and labeled "cytosol fraction" and stored at -70°C. The pellet was rinsed in Buffer 1 and centrifuged (Eppendorf Centrifuge 5402) at 14,000 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellet was extracted with Buffer 2 (Buffer 1 plus 1% Triton-X) using 500µl per 250 larval carcasses by manually homogenizing the pellet with a pestle for 2 minutes on ice. The homogenate was centrifuged at 14,000 rpm at 4°C

for 30 minutes. The supernatant was collected and labeled "membrane fraction" and stored at -70°C.

Preparation of Crude Enzyme Extract from Pupae

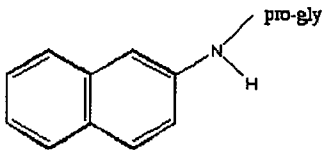
Pupae were collected from the food boxes after the 5th day of incubation. The food in the boxes was first removed. Since the pupae adhere to the sides of the boxes, the boxes were filled with water, which served to solubilize the substance that adheres them to the walls. After 5-10 minutes the pupae were collected and then homogenized in an ice-cold blender (Waring blender, 7010 model 31BL91, 50-60Hz) for 90 seconds at high speed in Buffer 1 using 10ml buffer per 250 pupae. The homogenate was assayed for enzyme activity.

ENZYME ASSAYS

Substrates and Reagents: Chromogenic substrates, inhibitors, and peptides were purchased from Bachem (Bubendorf, Switzerland). The ingredients for buffers were purchased from Sigma. Human DPPIV was a generous gift from Dr. Hans-Ulrich Demuth of Probiodrug Gesellschaft für Arzneimmittelforschung mbH and from Sigma.

Standard end-point assay for DPP IV cytosol enzyme extracts (Martensen, et. al., 1998): The substrate Gly-Pro-β-Naphthylamide (Figure B, page 11) was dissolved in dimethylsulfoxide (DMSO) to a concentration of 100mM or 200mM. These stock solutions can be stored at -20°C. For Gly-Pro-β-Naphthylamide, 50µl of 5mM substrate solution (diluted in 0.1M Tris-HCl buffer, pH 8.5 from the stock solution; prepared fresh for each assay; final substrate concentration was 0.5mM) was incubated with 50µl of 0.1M Tris-HCl, pH 8.5 (1ml 1M Tris-HCl, pH 8.5, 9ml distilled water) and 400µl of enzyme (or appropriate amounts after

concentration) for a total volume of 500µl for 1.5 hours at 45°C. The reaction was terminated with 500µl of a termination buffer (2M sodium acetate, 10% (wt/vol) Triton X-100, 0.5 mg/ml of Fast Garnet GBC salt (Sigma, Germany), pH 4.19-4.21 by addition of acetic acid) for a final volume of 1ml. The absorbance was measured against a blank (450µl 0.1M Tris-HCl, 50µl 5mM Substrate, 500µl termination buffer) at 525nm using the Spectronic 21D spectrophotometer. Human DPP IV (Demuth, Probiodrug) served as a positive control for the assay.



gly-pro-β-naphthylamide

Figure B: Structural Formula for Substrate

pH optimum: The optimal pH of DPP IV was determined according to the standard end-point assay but using as buffer a mixture of 750mM Bis-Tris, 750mM Bicine, 750mM Hepes adjusted to desired pH values with NaOH or HCl. Before the enzyme sample was added, 1-3µl (consistent for each assay) of the substrate-buffer mix were aliquoted to test the pH of each using pH paper (all reactions were prepared in duplicates). After incubation, the pH of each solution was again tested in the same manner as before the incubation. (Martensen et al., 1998).

Temperature optimum: The temperature optimum was determined using the standard end-point assay at appropriate temperatures (all reactions were prepared in duplicates).

Effects of Inhibitors (Martensen, et. al., 1998): The enzyme was incubated with inhibitor using the standard end-point assay for DPP IV at the concentrations indicated. Values obtained for inhibitor reactions were compared to a control assay in which water replaced the inhibitor.

Bio-Rad Protein Assays

The protein concentration of all samples was determined by the Bio-Rad protein assay kit containing Coomassie Brilliant Blue G-250 dye and bovine serum albumin (BSA) as standard according to the microassay procedure.

Purification of DPP IV

Third instar larvae were collected, homogenized, and separated into membrane and soluble fractions as described above. The soluble fraction was applied onto a column (3cm o.d. x 23.5cm) of DEAE-Sepharose Fast Flow (Pharmacia Biotech) equilibrated with 0.1M NaCl in 20mM triethanolamine-HCl buffer, pH 8.0. The column was washed with 500ml of this buffer and eluted first with a linear gradient of 0.1-0.25M NaCl in 20mM triethanolamine-HCl (250ml of each buffer), pH 8.0 and then with a linear gradient of 0.25-0.5M NaCl in 20mM triethanolamine-HCl pH 8.0 (250ml of each buffer). Fractions of 8-9ml were collected and every second tube was tested for dipeptidyl peptidase activity. Active fractions (no. 21-54; tot. vol. 285ml) were combined and concentrated by ultrafiltration (Jumbosep Centrifugal Concentrators, Pall Gelman Sciences) to a volume of 2ml. The concentrate was applied onto a Superdex S-200 gel filtration column (2cm o.d. x 69cm). The column was washed with 200ml of 0.1M NaCl in 20mM triethanolamine-HCl buffer, pH 8.0 and eluted with the same buffer. Fractions of 2ml

were collected and every fourth tube was tested for dipeptidyl peptidase activity. The active fractions (no. 28-40; tot. vol. 26ml) were pooled and used for characterization analysis (Martenson et al. 1998).

Molecular Mass Determination

To determine the approximate native molecular mass of the partially purified enzyme from the soluble fraction of third instar larvae, the sample was applied onto a Superdex S-200 gel filtration column at time zero (T_o). The column was eluted with 150ml of 0.1M NaCl in 20mM triethanolamine-HCl buffer, pH 8.0. Fractions of 1.6ml were collected and every third tube was tested for DPP activity.

The void volume of blue dextran and the elution volumes of molecular mass marker proteins were obtained in the same run according to the procedure outlined in the SIGMA Gel Filtration Molecular Weight Markers Kit (Sigma).

SDS Protein Gels

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Mini-PROTEAN 3 electrophoresis apparatus (Bio-Rad) with a 4-15% precast polyacrylamide gel in Tris-HCl buffer (10 wells, 30µl; Bio-Rad). Gels were run at room temperature (~25°C) in 1X Tris/Glycine/SDS buffer at 200-250 volts until the dye fronts ran off the gel. High Molecular Weight Range SigmaMarker proteins were used as standards (Sigma). Gels were stained in Coomassie stain (0.01% Coomassie R-250, 10% acetic acid, 5% methanol) and then destained (10% acetic acid, 5% methanol).

RESULTS

Purification of Dipeptidyl Peptidase from Pupae of *Drosophila*

A pupal extract was prepared from young pupae between 5 and 24 hrs post pupation. The total amount of protein (in mg) was very high, while the enzyme activity (in relative abs units) of the crude pupae extract was very low. The low enzyme specific activity (0.025 abs units/mg protein) may be due to degradation by nonspecific proteases in the pupal hemolymph.

Purification and Molecular Properties of Dipeptidyl Peptidase IV from *Drosophila*

A larval extract was prepared from late third instar larval as described in the methods. The enzyme was partially purified 2.5-fold from the soluble fraction of third instar larval epithelium by ion exchange chromatography (Table 1). Further purification by gel filtration chromatography resulted in partial denaturation of the enzyme yielding a loss of specific activity. (Table 1). The apparent molecular mass of the partially purified enzyme (after gel filtration) was determined by gel chromatography to be about 216 kDa (Fig. 1).

Table 1. Purification of DPP IV From Third Instar Larvae of *Drosophila**

Preparation Step	Total Activity (Abs Units)	Total Protein (mg)	Specific Activity (abs U/mg)	Yield (%)	Purification (-fold)
Homogenate	2147	602	3.6	100	1
Soluble fraction	2135	439	4.9	99	1.4
DEAE-Sepharose	486	55	8.8	23	2.5
Superdex 200	37	14	2.6	2	0.7

*Substrate: 0.5mM Gly-Pro-β -Naphthylamide in 0.1M Tris-HCl, pH 8.5, 37°C

A second extract of the enzyme was prepared from larval carcasses. Purification of the soluble fraction by ion exchange chromatography resulted in partial loss of the enzyme yielding a

purification fold of only 0.8 (Table 2). The eluted enzyme activity was pooled and concentrated 225-fold.

Table 2. Purification of DPP IV From Third Instar Larvae of *Drosophila**

Preparation step	Total activity (abs Units)	Total protein (mg)	Activity (abs units/mg)	Yield (%)	Purification (-fold)
Homogenate	219	75	2.9	100	1
Soluble fraction	73	39	1.9	33	0.6
Membrane fraction	43	12	3.6	20	1.2
DEAE – Sepharose	1.7	0.73	2.3	1	0.8

*Substrate: 0.5mM Gly-Pro-β -Naphthylamide in 0.1M Tris-HCl, pH 8.5, 37°C

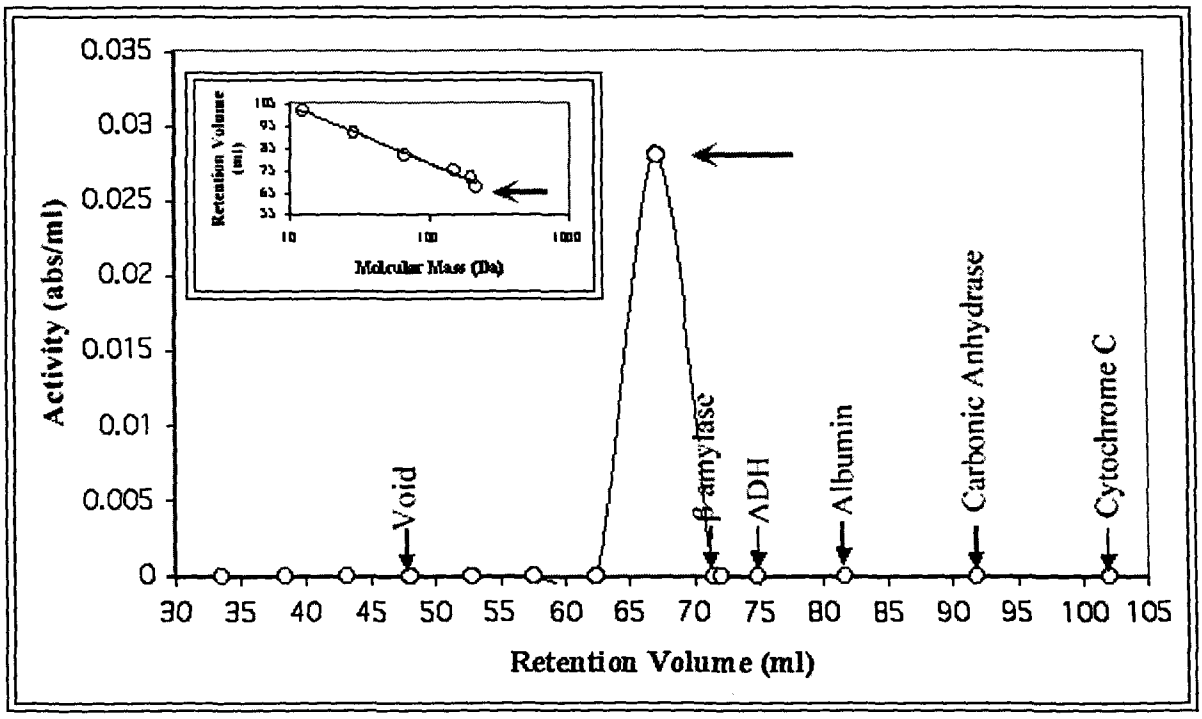


Figure 1. Molecular mass of dipeptidyl peptidase (DPP) from the soluble fraction of *Drosophila*. Partially purified enzyme was applied to a Superdex S-200 column. From the eluate, 2ml fractions were collected and assayed for DPP activity. The retention volumes of molecular mass marker proteins were obtained in the same run. The apparent molecular mass of DPP was about 216 kDa.

pH Optimum

pH Optimum – Membrane Fraction

The crude cell homogenate of third instar larvae yielded a broad range pH optimum of 7.5-8.5 for DPP IV activity on the substrate Gly-Pro-β-Naphthylamide (Song, 2002).

Activity in the membrane fractions had a pH optimum of 7.9-8.5 (Fig. 2). Data for the pH optimum assay can be seen in Table 3.

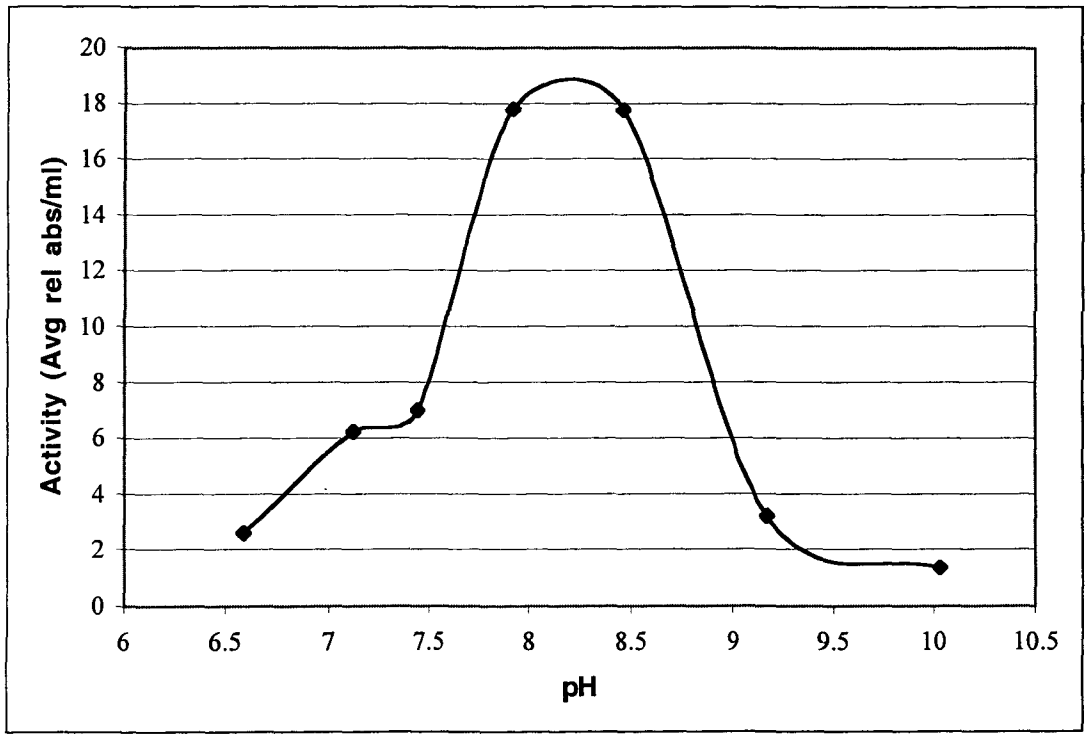


Figure 2. Effect of pH on the cleavage of Gly-Pro-β-Naphthylamide by the membrane fraction from homogenate of third instar larvae.

Table 3. Data for the pH optimum of membrane DPP IV*

Reaction Tubes	A525nm	Activity (rel abs/50µl)	Activity (rel abs/µl)	Activity (rel abs/ml)	**average Activity (rel abs/ml)**
pH 6.59 A	1.206	0.125	0.0025	2.50	
pH 6.59 B	1.215	0.134	0.00268	2.68	**2.59**
pH 7.12 A	1.391	0.310	0.0062	6.20	
pH 7.12 B	1.394	0.313	0.00626	6.26	**6.23**
pH 7.44 A	1.402	0.321	0.00642	6.42	
pH 7.44 B	1.460	0.379	0.00758	7.58	**7.00**
pH 7.92 A	1.951	0.870	0.0174	17.40	
pH 7.92 B	1.990	0.909	0.01818	18.18	**17.8**
pH 8.46 A	1.962	0.881	0.01762	17.62	
pH 8.46 B	1.976	0.895	0.0179	17.90	**17.8**
pH 9.17 A	1.243	0.162	0.00324	3.24	
pH 9.17 B	1.239	0.158	0.00316	3.16	**3.2**
pH 10.03 A	1.154	0.0732	0.00146	1.46	
pH 10.03 B	1.146	0.0651	0.0013	1.30	**1.4**

*DPP IV was incubated in a 75mM Tris-Bis Bicine Hepes buffer adjusted to different pH values and with 0.5mM Gly-Pro-β-Naphthylamide at 45°C for 1.5 hours. Duplicate measurements of each reaction were recorded.

[a]The relative absorbance per 50µl is the difference between the absorbance of the reaction at 525nm and the absorbance of the blank.

[b]The average relative absorbance per ml is an average of the two duplicates. These averages were used to generate the pH optimum curve in Figure 2.

Preliminary pH Optimum – Partially Purified Cytosol Fraction

The Gly-Pro-β-Naphthylamide activity with the partially purified enzyme (Superdex fractions 28-40) from the cytosol yielded a pH optimum of 8.5 (Fig. 3). The pH of the partially purified enzyme is similar to the pH range of the membrane fraction (Fig. 2).

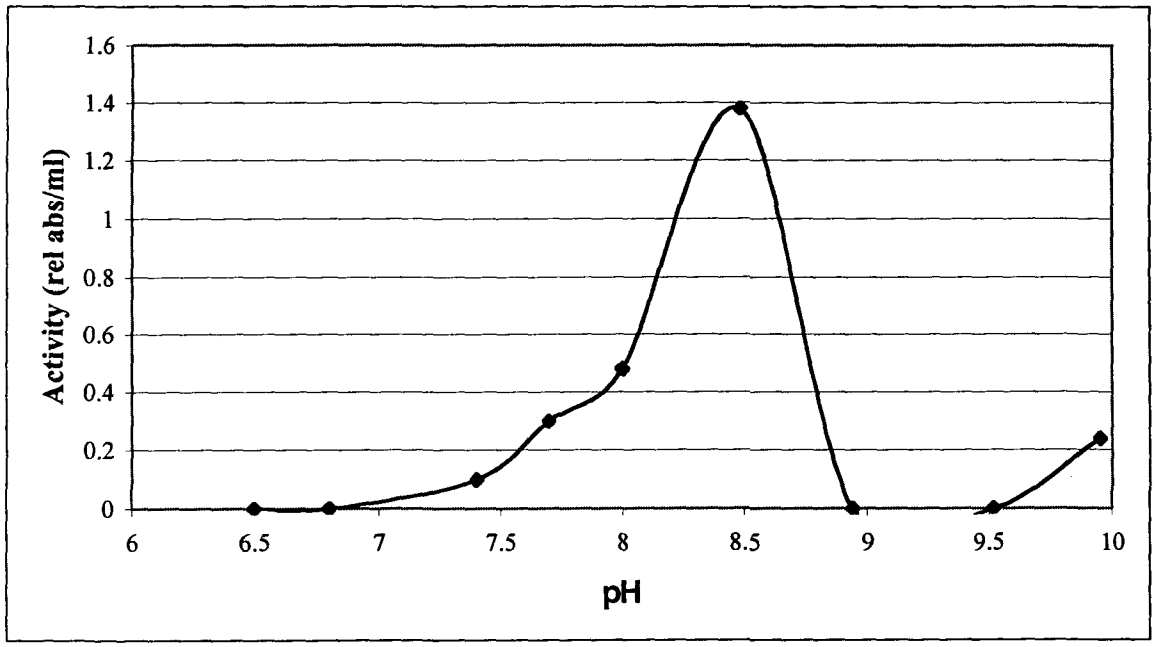


Figure 3. Effect of pH on the cleavage of Gly-Pro-β-Naphthylamide by the cytosol.

Temperature Optimum

Temperature Optimum – Membrane Fraction

In the membrane fraction, the enzyme preparation gave a temperature optimum of 45°C (Fig. 4)

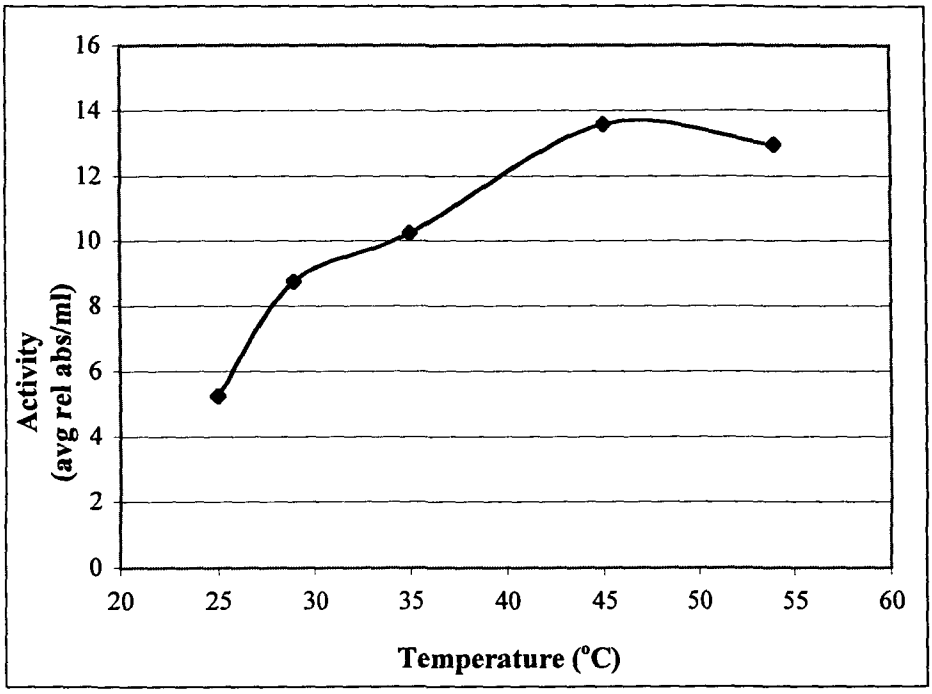


Figure 4. Effect of temperature on the cleavage of Gly-Pro-β-Naphthylamide by DPP IV in the membrane fraction from homogenates of third instar larval carcasses.

Temperature Optimum –Cytosol Fraction

The cytosol fraction had a temperature optimum in the region of 44.5°C (Fig 5). The temperature optimum of this enzymatic reaction is similar to the optimum of the membrane fraction (Fig. 4).

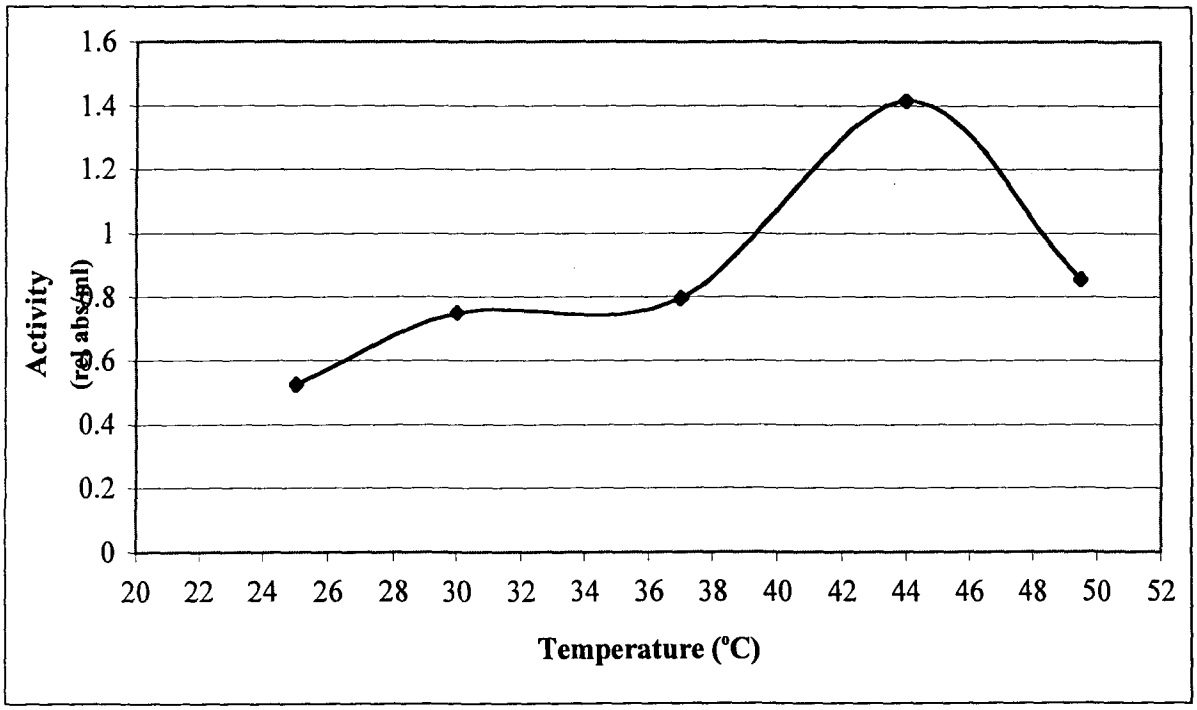


Figure 5. Effect of temperature on the cleavage of Gly-Pro-β-Naphthylamide by DPP IV in the partially purified cytosol sample.

Inhibitors

Earlier studies revealed that enzyme activity in the crude enzyme preparation was decreased by 78% by the inhibitor phenylmethanesulfonyl fluoride (PMSF) (Song, 2000), and that the *Drosophila* DPP IV activity in the crude enzyme extract was completely eliminated by the nonselective, mammalian DPP IV inhibitor $ZnCl_2$ (Song, 2000).

I have extended these studies to show that the membrane fraction enzyme was sensitive to diprotin A, a slowly cleaved substrate with high affinity for mammalian DPP IV. The influence of diprotin A on *Drosophila* DPP IV activity is shown in Table 4.

Table 4. Influence of Diprotin A on the Membrane Fraction Enzyme Activity.

Inhibitor	Concentration (mM)	Residual activity (% of control)
Diprotin A[1]	0.1	22
Diprotin A	0.2	4.6

[1]Ile-Pro-Ile is a slowly hydrolyzed competitive, but high affinity substrate for mammalian DPP IV

Drosophila DPP IV was compared to blowfly DPP, cockroach (*L. maderae*) DPP, and mammalian DPP IV in its sensitivity to inhibitors (Table 5). *Drosophila* DPP IV, like the blowfly, cockroach, and mammalian DPP IV, is classified as a serine protease. One example of a serine protease inhibitor is PMSF. The *Drosophila* enzyme, like the blowfly and the mammalian DPP IV is also inhibited by PMSF. However, the cockroach DPP IV was not sensitive to PMSF (Nassel, 2000). The blowfly and the mammalian DPP IV activity are also inhibited by zinc ions, while the cockroach DPP IV was not. Diprotin A, however, proved to be an effective inhibitor for both the cockroach and the *Drosophila* enzyme, while it exhibited no inhibitory effect on the blowfly enzyme. From Table 5 it is clear that the *Drosophila* DPP IV is more similar to the blowfly than the cockroach enzyme.

Table 5. Comparison of the sensitivity of *Drosophila,* Blowfly, and Cockroach DPP IV to Inhibitors.

	Inhibitor	Concentration (mM)	Residual Activity (% of control)	Inhibitor Spectrum
Drosophila	PMSF	2.5	22	Serine Proteases
Blowfly[a]		2	0	
Cockroach[b]		1	80	
Drosophila	$ZnCl_2$	2	0	Nonselective, mammalian DPP IV
Blowfly[a]		1	0	
		0.1	34	
Cockroach[b]		1	88	
		0.1	100	
Drosophila	Diprotin A[c]	0.1	22	Mammalian DPP IV, Xaa-Pro-
		0.2	4.6	
Blowfly[a]		0.1	96	
Cockroach[b]		1	12	

[a] Blowfly data is from Mentlein, et. al., 1998

[b] Cockroach data is from Nassel, et. al., 2000

[c] Ile-Pro-Ile is a slowly cleaved but high affinity substrate (acting as a competitive inhibitor) for mammalian DPP IV.

SDS-PAGE Analysis of Crude Homogenates and Cytosol Fractions

In the homogenate prepared from third instar larvae there are several proteins visible by SDS-PAGE (Fig. 6 A & B). The proteins range from 100kDa to less than 36kDa in size. The cytosol fractions prepared from these homogenates appear much the same on the SDS-PAGE (Fig. 6 A & B). In the crude pupae extract, like the homogenates, there are several proteins visible by SDS-PAGE (Fig. 6 B) but unlike the homogenates, there are large proteins of about 205kDa in size and also more small proteins.

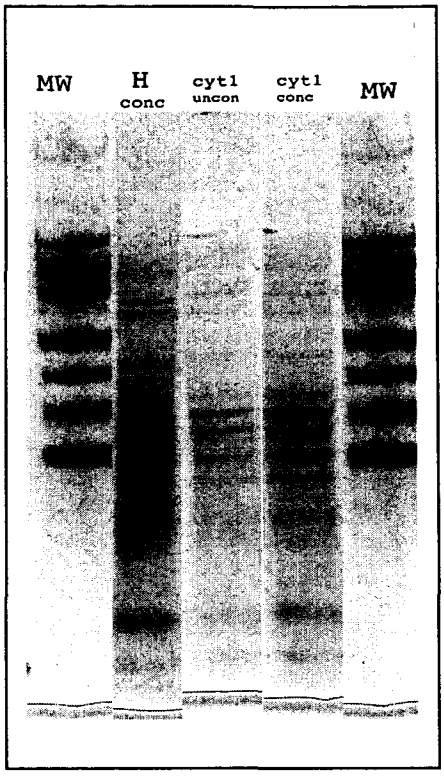


Figure 6 A.

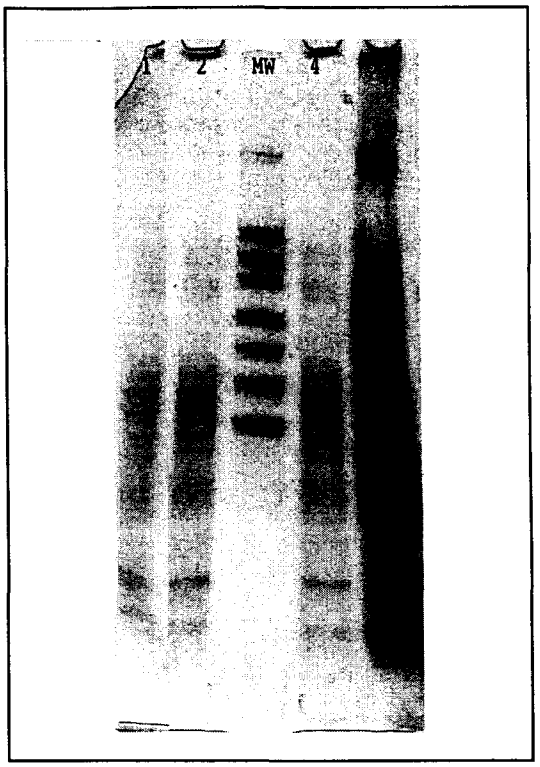


Figure 6 B.

Figure 6. SDS-PAGE separation of larval and pupae homogenate samples and cytosol samples prepared from the larval homogenate. A. Lane MW: High Molecular Weight Range SigmaMarker protein standards (MW in kDa recorded along side gel); H conc.: Homogenate 1 from third instar larvae concentrated 2-fold by ultrafiltration; cyt1 unconc.: Cytosol fraction 1 from Homogenate 1 unconcentrated; cyt1 conc.: Cytosol fraction 1 concentrated 2-fold. B. Lane 1: Cytosol fraction 2 from Homogenate 2 of third instar larvae unconcentrated; 2: Cytosol fraction 2 concentrated 2-fold; MW: High Molecular Weight Range SigmaMarker protein standards; 4: Homogenate 2 concentrated 2-fold; 5: Crude pupae extract concentrated 2-fold.

SDS-PAGE Analysis of Membrane Fractions and Partiallly Purified DPP

Preliminary SDS-PAGE separation of the partially purified DPP revealed a very large protein of about 220kDa in size (not shown). Initially it was thought that this large protein was the enzyme present as a dimer held together by disulfide bonds, explaining why it had not separated into its respective monomers on the denaturing gel. When the partially purified enzyme was run with and without β-mercaptoethanol on SDS-PAGE, the large protein was visible in the sample without β-mercaptoethanol and it had disappeared in the sample with β-

mercaptoethanol (Fig. 7 A). Although close in size to the enzyme, the large protein is assumed to be a protein that co-purified with the enzyme during the purification process. The small proteins of less than 36kDa in size may be degradative products – which may explain some of the loss of activity we see in the purification process.

In the membrane fraction of the homogenate prepared in the second attempt to purify the enzyme there were several proteins visible by SDS-PAGE (Fig. 7 B).

The enzyme activity eluted from the DEAE purification step of the second attempt was pooled and concentrated 225-fold and run on a denaturing gel (Fig. 7 B). There is one small molecular weight protein visible on SDS-PAGE that appears to have co-purified with the enzyme during the purification process. This is an indication that although there was a loss in activity there was an obvious separation of proteins from the enzyme during the DEAE process.

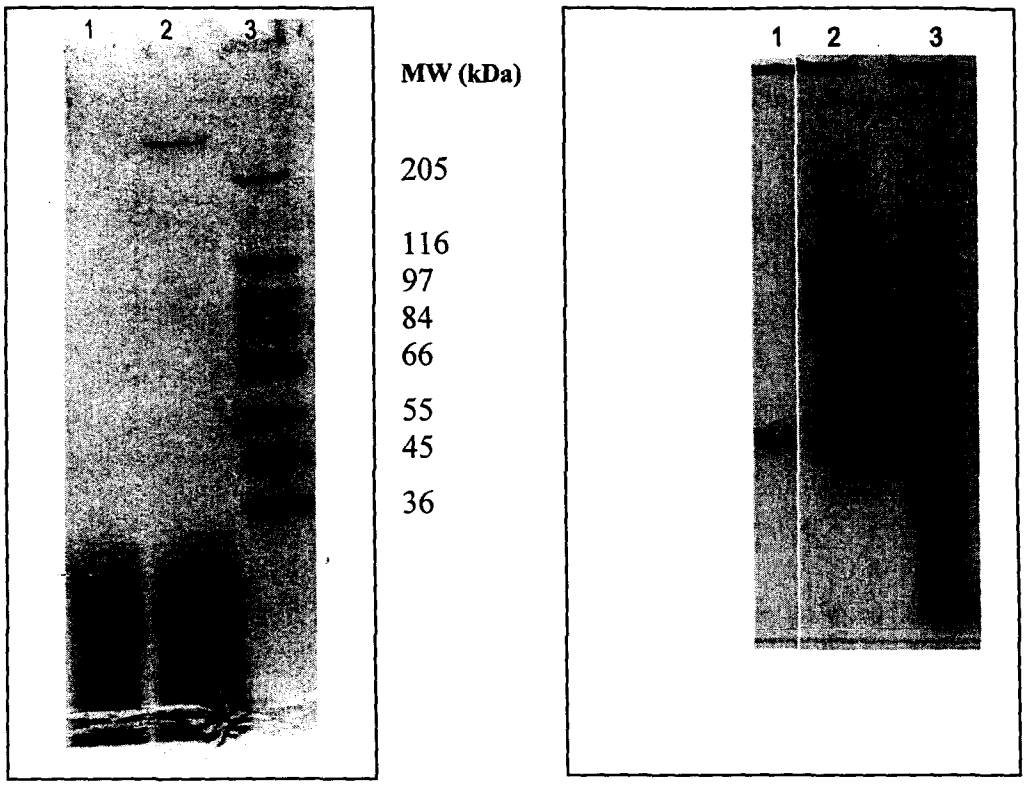


Figure 7 A. Figure 7 B.

Figure 7. SDS-PAGE separation of partially purified DPP and membrane fractions prepared from homogenates of third instar larvae. A. Lane 1: Partially Purified DPP (0.7-fold; 1st attempt) with b-Mercaptoethanol; 2: Partially purified DPP (0.7-fold; 1st attempt) without b-Mercaptoethanol; 3: High Molecular Weight Range SigmaMarker protein standards. B. Lane 1: DEAE eluted enzyme activity concentrated 225-fold by ultrafiltration; 2: High Molecular Weight Range SigmaMarker protein standards; 3: Membrane fraction (2nd attempt).

DISCUSSION

DPP IV Activity in *Drosophila* extracts.

The partially purified enzyme activity found in the tissue adhering to the third instar larval cuticles of *Drosophila* has properties characteristic of proline-/alanine- specific DPP , specifically DPP IV. Evidence that the partially purified enzyme is a DPP IV-like enzyme is based on its substrate specificity, membrane association, and weakly alkaline pH optimum (Mentlein, 1999). The fruit fly DPP IV, like mammalian, cockroach, and blowfly DPP IV, is specific for substrates such as Gly-Pro-β-Naphthylamide and Gly-Pro-4-Nitroanalide (Song, 2000). Fruit fly DPP IV activity is present in its highest concentrations in the membrane fraction of this tissue, similar to the mammalian DPP activity.

The fruit fly DPP IV exhibited catalytic properties characteristic of the mammalian, blowfly, and cockroach DPP IV, as well as some differences. DPP IV characteristically has a weakly alkaline pH between the range of 7.5-8.5. The fruit fly DPP IV has a pH optimum of 8.5, the same as the pH optimum of the cockroach DPP IV (Nassel et al, 2000). Blowfly DPP IV, however, has a pH optimum in the range of 7.5-8.0 (Martensen et al, 1998).

Like the mammalian DPP IV, the fruit fly DPP IV is efficiently inhibited by the competitive inhibitor Diprotin A (Ile-Pro-Ile) so is the cockroach enzyme but not the blowfly DPP IV (Martensen et al, 1998, Nassel et al, 2000). The fruit fly enzyme is also inhibited by other inhibitors of mammalian DPP IV, specifically, $ZnCl_2$ and the serine protease inhibitor PMSF. Like the human and fruit fly DPP IV blowfly DPP IV activity is sensitive to $ZnCl_2$ and to PMSF but the cockroach activity is not inhibited by these inhibitors (Nassel et al, 2000).

The native molecular mass of the fruit fly DPP IV was determined to be approximately 216 kDa, by gel filtration. The putative molecular weight of DPP IV from the Flybase database

should be between 89873 and 90222D depending on the position of the first methionine translated. Thus the DPP IV seems to function as a dimer. This is similar to the molecular masses of the native mammalian (also a dimer) and blowfly DPP IV that were determined to be 220 kDa (Mentlein, 1999) and 200 kDa (Martensen et al, 1998), respectively.

FUTURE RESEARCH

Future work will focus on attempting to further purify this enzyme from the membrane fraction of the homogenate prepared from the carcasses of third instar larvae. If successful, characterization of the enzyme will follow. In addition to finding pH and temperature optima and determining the effects of inhibitors, an analysis (via reverse-phase high performance liquid chromatography) of the enzyme's ability to hydrolyze peptides such as trypsin-modulating oostatic factor (TMOF), bradykinin, and several bioactive peptides including substance P and peptide YY will be performed. Such an analysis will provide further information about the exact nature of the Drosophila enzyme.

In this paper I have shown that the *Drosophila* enzyme has all the major characteristics of a DPP IV serine protease but that there are differences in the enzyme compared to various insects and human tissue, sharing some characteristics with the insects and others with the human enzyme.

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Appendix:

Suggestions And Protocols For Continuation Of Enzyme Purification And Characterization.

Protocol for Enzyme Purification (in general):

To isolate DPP IV from larvae.

1. Prepare larvae by washing away any excess food etc. in several washes of water and salt.
2. Roll out the insides of the larvae on a foil covered glass plate over a platform of ice.
3. Wash with several volumes of ice cold 1X Ringer's solution.
4. Homogenize the cuticles with adhering tissue in the Waring Blendor (according to procedures outlined in this manual) in buffer "1" – made 0.1M NaCl. 10ml/250 carcasses.
5. Spin down the cuticle and save supernatant as "soluble fraction."
6. Rehomogenize the membrane fraction in buffer "2" made 0.1M NaCl. 500µl/250 carcasses label "membrane fraction."
7. Store both fractions in the -70 freezer. Assay both for activity.
8. Apply membrane fraction to DEAE-Sepharose Fast Flow column equilibrated with 0.1M NaCl in 20mM triethanolamine (TEOLA) -HCl buffer, pH 8.
9. Wash column with 400-500ml of 0.1M NaCl in 20mM TEOLA-HCl buffer, pH 8 buffer.
10. Elute with a linear gradient: 1st 0.1-0.25M NaCl in 20mM TEOLA, pH 8; 2nd 0.25-0.5M NaCl in 20mM TEOLA, pH 8. Or 0.1-0.5M NaCl in 20mM TEOLA, pH 8.
11. Collect fractions of 8-9 ml if doing two separate gradients; 6ml fractions if doing one gradient. Test for DPP activity (for fruit fly cyto active fractions were 21-54; for trial columns with mem. active fractions were in 2nd half of gradient).
12. Combine active fractions (should come out in 2nd half of gradient) and concentrate by ultrafiltration to a volume of 2ml
13. Apply concentrate to Superdex S-200 gel filtration column.
14. Elute column with 0.1M NaCl in 20mM TEOLA-HCl, pH 8 and collect 2 ml fractions.
15. Pool peak activity fractions (fruit fly cyto active fractions were 28-40) and concentrate to 1ml by ultrafiltration.
16. Repeat step 13 if necessary.
17. Use the partially purified enzyme for characterizing. Determine the following:
    - pH optimum
    - temp optimum
    - effects of inhibitors (Diprotin A, PMSF, $ZnCl_2$)
    - Hydrolysis of peptides (TMOF, Bradykinin, substance P, Peptide YY) via rp-HPLC
    - Apparent molecular weight
    - run on SDS gels

18. If time permits, run an aliquot on Mono Q column using a 30ml gradient of 0.1-0.4M NaCl in 20mM TEOLA-HCl, pH 8 at a flow rate of 1ml/min – collect fractions of 0.5ml [blowfly activity eluted as a broad peak with two maxima in fractions 20-29].

Columns Required:	Matrix
3cm (o.d.) x 23.5cm	DEAE-Sepharose Fast Flow (Pharmacia)
2cm (o.d.) x 69cm	Superdex S-200

Solutions/Buffers:

- 1X *Drosophila* Ringers
- Buffer 1 and Buffer 2 for Maxi preparation
- Gly-Pro-b-Naphthylamide (substrate, labeled S3) for enzyme assays
- 0.1M Tris-HCl, pH 8.5 buffer for enzyme assays
- Termination Buffer for enzyme Assays
- 750mM Tris-Bis, Bicine, Hepes at desired pH's for pH optimum assays
- Column buffers
  - 0.1M NaCl in 20mM TEOLA-HCl buffer, pH 8
  - 0.25M NaCl in 20mM TEOLA-HCl buffer, pH 8
  - 0.5M NaCl in 20mM TEOLA-HCl buffer, pH 8
  - 0.4M NaCl in 20mM TEOLA-HCl buffer, pH 8
- 0.1mg/ml BSA for protein assays
- inhibitors at desired pH (usually b/n 0.1M to 1M final concentrations)
- peptides (stock solutions 5mM and final concentrations in reaction 50μM)

Protocols:

Maintaining Wildtype (+/+) Stocks:

1. Maintain stock bottles of wildtype flies throughout the project.
2. Flies from stock bottles should be transferred to new bottles every 2 weeks in the following manner:
   - Obtain a new food bottle from the refrigerator
   - Drain the food bottle of any excess liquid that may have accumulated while in the fridge
   - Add a small amount of dry yeast (do not add too much)
   - Place a kimwipe into the food using a pencil (as Dr. C. if unclear)
   - Transfer flies from stock bottle to a new food bottle (have Dr. C. teach you technique for transferring flies to a new bottle)

3. When you have at least 20 bottles of flies, preferably young flies, begin a food cage.
   - place a fly box (a 20 x 10 x 5cm plastic box with a cover that had a silk-screen window) into the cage containing the standard agar-molasses-corn meal-yeast media along with a ring line of yeast paste (yeast mixed with water).
   - Leave this box in for 24 hours and then throw it out (note do these steps in the early morning around 7 or 8am)
   - Place two new food boxes in the cage (around 7 or 8am) and collect eggs for 3-4 hours.
   - After 3-4 hours take out the two boxes, cover them, and then store in the 25°C incubator for 4-5 days.
   - Place two new boxes in the cage and take out early the next morning (around 8 or 9am).
   - Cover these boxes and then store at 25°C for 4-5 days.
   - After 2 days the larvae were fed with a thick paste of yeast and the boxes were taped around the edge of the cover to prevent the larvae from escaping.
   - On the 3rd day of incubation prepare the following solutions for larvae prep:

     1X ringers
     Buffer 1
     Buffer 2

   - After 4-5 days larvae were collected when they climbed out of the food (see Preparation of third instar larvae crude extract protocol).

Preparation of Third Instar Larvae Epidermal Cell Crude Extract

Note: To avoid food contamination, focus on collecting larvae that are on the cover, along the walls of the food box and on the surface of the food.

1. Rinse the larvae with water into a beaker.
2. If a lot of food was collected, the larvae will have to be rinsed in water with enough table salt (the salt creates a density-dependent separation to float the larvae and separate them from the food particles). This procedure may have to be repeated several times depending on how much food contamination.
3. Once the larvae are separated from the food, the small silk screen funnels can be used to collect the larvae in a separate beaker. Then the larvae must be rinsed with water several times to rid of the salt.
4. With the clean larvae, do a second cleaning (under a dissecting scope) to remove any pupae and remaining food particles.
    - pour a few larvae into a large Petri dish, place under the dissecting scope, and pick-out pupae and food with forceps
    - once clean, collect the larvae in a small amt. of water (too much water will drown them) and keep them on ice
5. Collect all larvae onto a paper towel to dry them, then transfer them to a weigh dish (some larvae will stick to the paper towel, if you have plenty of larvae don't worry about them).
6. Weigh the larvae to determine the # of larvae collected (one larvae is approximately equal to 2g).
7. Put the larvae on ice in the weigh dish.
8. If not already done, set up an ice platform.
    - a Styrofoam box filled with ice and then covered with a glass plate
    - place a sheet of aluminum foil over the glass plate
    - spread a spoonful of larvae over the foil
    - place a piece of Saran wrap over the larvae
    - use a solid brass, heavy cylinder-shaped metal rolling pin to roll over the larvae several times to extrude the insides of the larvae (cont. rolling until you no longer hear a "squishing" noise)
    - wash the carcasses from both the foil and Saran wrap into a beaker with **ICE COLD** 1X ringer's solution
    - filter the carcasses through a piece of silk screen (do NOT throw away carcasses)
9. Homogenize the larvae in an ice-cold blender.
    - rinse larvae with buffer 1
    - pour buffer 1 into blender (10ml buffer/250 larval carcasses)
    - begin on "low" speed and then immediately begin blending on "high" speed for 90 seconds
    - collect homogenate into a beaker or other storage container using a silk screen filter (discard skins)

- aliquot about 2ml of the homogenate for enzyme and protein assays and for running on a gel; centrifuge the homogenate or store at -70°C (be sure to store in small aliquots otherwise it will take hours to defrost it for later use)

Assaying the Homogenate

1. Assay 400µl of the homogenate for enzyme activity according to the standard end-point assay
2. Assay 10-30µl of the homogenate for protein
3. Concentrate 1ml of the homogenate by ultrafiltration.
   - concentrate at least 2-fold
   - assay for protein and enzyme activity
   - apply the concentrate onto an SDS gel

Centrifuging the Homogenate

1. Centrifuge the homogenate in using the Beckman J2-21 centrifuge and the JA-20 rotor for 3 hours at 18,000 rpm at 4°C.
2. Collect the supernatant and label it "cytosol fraction."
   - aliquot 2ml for assaying (in the same manner as was done for the homogenate)
   - store the remaining cytosol fraction, in small aliquots, at -70°C
3. Rinse the pellet with Buffer 1 and then centrifuged at 14000 rpm at 4°C for 5 minutes.
4. Discard the supernatant and extract the pellet with Buffer 2 in proportion of 500µl per 250 larval carcasses by manually homogenizing the pellet with a pestle for 2 minutes on ice.
5. Centrifuge in Beckman centrifuge (use the green eppendorf holders specific for the JA-20 rotor) at 18,000 rpm at 4°C for 30 minutes to an hour.
6. Collect the supernatant and label it "membrane fraction."
   - aliquot for assays
   - store the remaining in small aliquots at -70°C

Assaying the Membrane Fraction

1. Assay 50µl for enzyme activity
2. Assay 2-4µl for protein
3. Run 10µl of membrane (unconcentrated) on an SDS gel

*NOTE: Prepare a table of purification as follows:

Table. 1. Purification of DPP IV from Third Instar Larvae of *Drosophila*

Preparation step	Total activity (abs units)	Total protein (mg)	Activity (abs units/mg)	Yield (%)	Purification (-fold)
Homogenate					
Soluble Fraction					
Membrane Fraction					
DEAE-Sepharose					
Superdex 200					
Superdex 200 (2nd)*					
Mono Q*					

*these steps may not be necessary

Standard Enzyme Assay

Prepare the following:

- 0.1M Tris-HCl buffer, pH 8.5 by diluting 1M Tris-HCl stocks (may need to pH the 0.1M solution)
- 5mM S_3 (Gly-Pro-β-Naphthylamide) by diluting 200mM stock solutions (may need to prepare 200mM and 100mM stocks; the substrate is stored in -20°C)
- Termination Buffer (recipe for 10ml)
  - 5ml 2M sodium acetate (be sure that the pH is b/n 5-6)
  - 1ml Triton-X
  - 5mg Fast GBE
  - pH to 4.19-4.21 with acetic acid
  - bring to 10ml with dH_20

1. Prepare a Blank Blank (in eppendorf tubes)
   - 50µl of 5mM S3
   - 450µl of Tris buffer
   - 500µl of termination buffer

2. Prepare a Blank at T_0
   - 50µl of 5mM S3
   - Xµl of Tris buffer*
   - 500µl of termination buffer
   - Xµl of sample (homog., cyto, or membrane)*

   *The amount of Tris, enzyme, and substrate should total a volume of 500µl

3. Prepare enzyme reactions
   - 50µl of 5mM S3
   - Xµl of Tris Buffer
   - Xµl of sample
   - Incubate samples for 1.5 hours in a 42°C water bath
   - Terminate reaction with 500µl of term buffer

NOTE: total volume for all tubes is 1ml

4. Prepare a Human Control (using the 1:100 dilution in -20°C freezer) using 1-3ul of the sample. Prepare the same as in step 3.
5. Determine the abs of each reaction and all blanks at 525nm. Use the relative absorbance to determine the total abs units in your sample.

Protein Assay

1. Prepare a Standard Curve using 0.1mg/ml BSA (in -20°C) as follows:
    a. Label 5 eppendorf tubes:
        -0mg BSA, 2mg (20µl of 0.1mg/mlBSA), 4mg (40µl of BSA), 8mg (80µl of BSA), and 10mg (100µl of BSA)
        -to these tubes add sterile glass dH20 to bring the volume of each tube to 800µl
        -bring the final volume to 1ml with 200µl of Bio-Rad reagent (in refrigerator)
        NOTE: When the BSA has been added, immediately add the Bio-rad and vortex

    b. Let mix stand for 5-10 minutes
    c. Determine the absorbance of each at 595nm

2. Prepare unknown tubes in the same manner using the appropriate amounts of your sample (homogenate, cyto, membrane, etc.)

3. Using the Standard curve determine the linear regression equation using excel. Use the equation and the relative absorbances of each unknown sample determine the amount of protein (mg/ml).

Purification Steps

I. DEAE-Sepharose column (to be done in cold room)
   A. Make the following buffers:
      1. 0.1M NaCl in 20mM Triethanolamine (TEOLA), pH 7.5-8
      2. 0.25M NaCl in 20mM TEOLA, pH 7.5-8 (only necessary if doing two gradients)
      3. 0.5M NaCl in 20mM TEOLA, pH 7.5-8
   B. Wash the column with 300ml of 0.1M NaCl in 20mM TEOLA using the pump; check pH of column (should be close to pH of the buffer, if not wash more buffer through)
   C. Defrost the sample (if have large volumes may want to begin defrosting them night before in the refrigerator).
   D. Set-up fraction collector to column.
      1. Fraction collector holds 80 tubes (determine amount of tubes needed for entire run). Label tubes with #.
      2. Determine the # of drops needed to obtain desired volume/fraction (if doing 2 gradients collect 8-9ml/fraction; if doing single gradient collect 6ml/fraction)
   E. Load sample using the pump (adjust speed to 2-3 minutes per fraction). Mark time sample loaded and sample volume.
   F. Set-up the gradient maker:
      1. Fill both wells with 250-275ml of buffer in each (make sure all valves are closed).
         -if doing 2 gradients: 0.1-0.25M NaCL and then 0.25-0.5M NaCl in 20mM TEOLA
         -if one gradient: 0.1-0.5M NaCl in 20mM TEOLA
      2. Put stirrer on outflow side, plug in and turn on.
      3. Open outflow valve first, then immediately open connector valve.
   G. After sample is loaded, connect the pump to the gradient maker, and to the column.
   H. Turn on the pump.
   I. Monitor the first 10-20 tubes to be sure that the volume per fraction is consistent. If everything is okay monitor the column every 30-40minutes.
   J. Once all fractions have been collected, aliquot 1ml for assaying. Store the remainder of the fraction in 15ml conical tubes in the -70°C freezer.
   K. Assay every 2nd or 3rd tube for enzyme and protein.

II. Concentrate active fractions
   A. Pool active fractions for concentration by ultrafiltration.
      1. aliquot 1ml for assaying and SDS protein gels

B. Concentrate the active fractions using either the Pall Filtron Macrosep centrifugal concentrators or the Nalgene centrifuge filters to a volume of 2ml. (see operating instructions provided; use centrifuge in Gen. Bio. Lab at 4200rpm)
C. Assay both the retentate (concentrate) and the filtrate for enzyme activity and protein content.

III. Gel Filtration Column (to be done in cold room)
A. Prepare 0.1M NaCl in 20mM TEOLA buffer, pH 7.5-8.
B. Wash the column with 200-300ml of this buffer (use pump).
C. Set-up the fraction collector:
1. Label tubes with # (need ~100tubes).
2. Determine the # of drops needed to obtain 2ml/fraction
D. Set-up pump to a rate of 2min/fraction
E. Load concentrate with pump. Note time loaded and volume.
F. Once sample is loaded, wash through 0.1M NaCl in 20mM TEOLA buffer, pH 7.5-8 until ~100 tubes have been collected.
G. When column is done, aliquot 1ml (or less) of each fraction for assaying and store the remainder in 15ml conical tubes in -70°C freezer.
H. Assay ever 2^{nd} or 3^{rd} tube.
I. Pool active fractions. Use these active fractions for characterizing the enzyme.
J. If activity is not being lost with each column run, it may be a good idea to run a second gel filtration column.

Temperature Optimum Assay

Do this assay according to the standard enzyme assay at appropriate temperatures.

Temperatures should range b/n 20°C and 60°C. There is a water bath in Dr. C.'s lab and several heating blocks in the Biotech lab that can be used to perform this assay.

pH Optimum Assay

Preparation for this assay

1. Make 75mM Tris-Bis, Bicine, Hepes buffer at desired pH (5-10ml should be enough) dilute from 1M stock (may need to make another stock solution)
   -pH should range b/n 6-10
2. Filter sterilize the buffers.
3. Set-up reaction tubes (duplicates of each) by first adding the substrate and the Tris buffer. Before adding the enzyme aliquot 1-3µl (consistent for each assay) of the substrate-buffer mix to test the pH using the pH paper. Add the sample and then incubate for 1.5 hours.
4. After incubation, the pH of all tubes (including blanks) was determined in the same manner as above. Record these as final pH values.
5. Determine A525nm
6. To determine an optimum pH generate a curve of Activity (avg. rel abs/ml) vs. pH.

Inhibitor Assay

The enzyme is to be incubated with inhibitor at desired concentrations according to the standard enzyme assay. Values obtained for inhibitor reactions were related to a control assay in which water replaced the inhibitor.

Hydrolysis of Peptides

See Dr. Chihara.

Note: make stocks of peptides at a concentration of 5mM; final concentration in reaction tubes of each peptide should be 50µM

Protein Gels

1. Use the Bio-Rad MINI-Protean 3 apparatus (can be found on shelf above vortex labeled "mini-protean[?]") and Bio-Rad ready-made 4-15% Tris gels (in fridge).
2. For these purposes, it is most useful to use the high range MW protein standards; use 10µl per gel.

3. Determine amount of each sample you should load.
4. Add SDS dye to each sample.
5. Set-up apparatus. Add 1X Tris/Glycine/SDS buffer.
6. Load samples.
7. Run gel at 250V for 30 minutes or until dye fronts run off the gel.
8. Stain gel for 20 minutes.
9. Destain for 20 minutes or overnight if necessary.
10. Examine the gel on the light box.
11. Scan the gel (see Dr. C.).
12. Dry gel.