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Identification and properties of a peptidyl dipeptidase in the housefly, *Musca domestica*, that resembles mammalian angiotensin-converting enzyme

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[D-Ala[2],Leu[5]]Enkephalin was readily metabolized by membranes (40000 *g* pellet) prepared from heads of the housefly, *Musca domestica*, with Gly[3]-Phe[4] being the major site of cleavage. This hydrolysis was only partially inhibited (40 %) by 10 μM phosphoramidon, an inhibitor of endopeptidase-24.11, but was almost totally abolished in the presence of a mixture of 10 μM phosphoramidon and 10 μM captopril, a potent inhibitor of mammalian angiotensin-converting enzyme (ACE). An assay for ACE employing Bz-Gly-His-Leu as the substrate was used to confirm the presence of an ACE-like peptidyl dipeptidase activity in fly head membranes. The peptidase had a K_m of 1.91 mM for Bz-Gly-His-Leu and a pH optimum of 8.2. The activity was inhibited by 100 μM EDTA and was greatly activated by $ZnCl_2$ but not other bivalent metal ions. Captopril, lisinopril, fosinoprilat and enalaprilat, all selective inhibitors of mammalian ACE, were also good inhibitors of the insect enzyme with IC_{50} values of 400 nM, 130 nM, 16 nM and 290 nM respectively. An M_r value of around 87000 was obtained for this enzyme from gel-filtration chromatography, indicating that the insect enzyme is similar in size to mammalian testicular ACE (M_r = 90000–110000) and not the larger form of the enzyme (M_r = 150000–180000) found in mammalian somatic tissues. The fly peptidyl dipeptidase was released from membranes into a soluble fraction by incubating the head membranes at 37 °C but not at 0 °C, suggesting that the insect ACE-like enzyme can be solubilized from cell surfaces through the activity of a membrane-bound enzyme activity. In conclusion, we have shown the existence of a peptidyl dipeptidase in membranes from the heads of *M. domestica*, which has similar properties to those of mammalian ACE.

INTRODUCTION

Peptide hormones synthesized in neurosecretory and glandular cells are regulators of key physiological and developmental events in insects (O'Shea and Schaffer, 1985; Scharrer, 1987; Keeley et al., 1990, 1991). During the last decade there has been a dramatic increase in our knowledge of the structure and diversity of these neurohormones and the precise location of peptidergic cells in the insect neuroendocrine system. The list of peptides that have been isolated and sequenced includes regulators of lipid and carbohydrate metabolism, of juvenile hormone and pheromone biosynthesis, eclosion hormone, diuretic hormones and an array of myotropins (Keeley et al., 1990, 1991; Holman et al., 1990, 1991; Konopinska et al., 1992). Some of these peptides resemble known vertebrate peptides, for example, the leucosulfakinins, locustasulfakinin and drosulfakinins (peptides predicted from *Drosophila* cDNA clones) are related to the cholecystokinin/gastrin family, and the locustatachykinins have structural features in common with the mammalian tachykinin family [see review by De Loof and Schoofs (1990)]. The development of antibodies to insect and vertebrate peptides and their use in immunocytochemical studies has often revealed widespread distribution of these peptides or structurally related molecules [for example, proctolin (Nassel and O'Shea, 1987), adipokinetic hormone (Schooneveld et al., 1983), leucokinin (Nassel and Lundquist, 1991), eclosion hormone (Truman et al., 1990), locustamyotropin (Schoofs et al., 1992), cholecystokinin (Duve and Thorpe, 1984; Nassell et al., 1988), enkephalins (Duve and Thorpe, 1988) and substance P (Sivasubramanian, 1990)] throughout the insect central nervous system and in more than one cell type, including interneurons and other neurons lacking the morphology of neurosecretory cells. These results indicate the extensive use of peptides as neurotransmitters or neuromodulators within the central nervous system of insects.

For neuropeptides to serve as neurotransmitters, there must be an effective mechanism for terminating the peptide signal. In mammals, it is now recognized that plasma-membrane peptidases with their active sites facing the extracellular space, play an important role in the inactivation of neuropeptides in the central nervous system and in peripheral tissues (Turner et al., 1985; McKelvy and Blumberg, 1986). These peptidases have a broad tissue distribution and are able to hydrolyse a variety of peptide substrates. Endopeptidase-24.11 and peptidyl dipeptidase A (angiotensin-converting enzyme; ACE) are two well-characterized peptidases involved in the metabolism of mammalian peptides. Endopeptidase-24.11 is found in post- and presynaptic membranes prepared from substantia nigra of porcine brain where it is co-localized with substance P, suggesting a physiological role for the enzyme in the metabolism of this peptide (Erdos and Skidgel, 1987; Littlewood et al., 1988; Kenny and Hooper, 1991; Barnes et al., 1992, 1993). ACE is a key component of the renin–angiotensin system which leads to the formation of the potent vasoconstrictor, angiotensin II, by cleavage of His-Leu from the C-terminus of angiotensin I (Ondetti and Cushman, 1982). Although best known as a processing enzyme, ACE can also degrade biologically active peptides including bradykinin and enkephalins and can function as an endopeptidase, releasing amidated di- and tri-peptides from

Abbreviations used: ACE, angiotensin-converting enzyme; TFA, trifluoroacetic acid.
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certain C-terminally amidated substrates, e.g. substance P (Hooper, 1991). ACE, like endopeptidase-24.11, has been localized to postsynaptic membranes in porcine brain but the identities of the true physiological substrates for the neural enzyme have not yet been established (Barnes et al., 1992).

In comparison with the mammalian system, relatively little is known about the inactivation of neuropeptides in insects, but it is likely that similar enzyme mechanisms operate at synapses in the insect nervous system. We have shown that synaptic membrane preparations from the locust, *Schistocerca gregaria*, are enriched with neuropeptide-degrading aminopeptidase and endopeptidase activities (Isaac, 1987, 1988). Like endopeptidase-24.11, the insect endopeptidase cleaves peptide bonds comprising the amino group of hydrophobic amino acids and can hydrolyse the enkephalin analogue [D-Ala^2,Leu^5]enkephalin. The endopeptidase activity is inhibited by phosphoramidon and thiorphan, selective inhibitors of mammalian endopeptidase-24.11, and by chelators of bivalent metal ions (Isaac, 1988). The use of [D-Ala^2,Leu^5]enkephalin to measure endopeptidase activity from heads of *Drosophila melanogaster* and the housefly, *Musca domestica*, revealed a second enkephalin-degrading activity which was not inhibited by phosphoramidon (Isaac and Priestly, 1990; Lamango and Isaac, 1993). We now show that the latter activity is a peptidyl dipeptidase which has very similar properties to mammalian ACE. This is the first report of an ACE-like enzyme in an invertebrate.

MATERIALS AND METHODS

Chemicals

Phosphoramidon was obtained from Peninsula Laboratories Europe, St. Helens, Merseyside, U.K. Amastatin, [D-Ala^2,Leu^5]enkephalin, Phe-Leu, Tyr-D-Ala-Gly, captopril and M_r protein standards were from Sigma Chemical Co., Poole, Dorset, U.K. Triton X-114 was purchased from Boehringer-Mannheim Biochimica, Bell Lane, Lewes, East Sussex, U.K. H.p.l.c.-grade solvents were obtained from Rathburn Chemicals, Walkerburn, Scotland, U.K. H.p.l.c.-grade orthophosphoric acid was from Fisons Laboratory Chemicals, Loughborough, Leics., U.K. Lisinopril, enalaprilat, fosinoprilat and pig kidney ACE were gifts from Dr. Nigel Hooper and Professor Tony Turner of the Department of Biochemistry and Molecular Biology, University of Leeds. Protein assay reagents were obtained from Pierce, Pierce and Warriner, Chester, Cheshire, U.K. All other chemicals were of AnalaR grade and were obtained from BDH, Poole, Dorset, U.K.

Insects

M. domestica was raised under laboratory conditions as described by Coulson and Bale (1990).

Enzyme preparation

Adult *M. domestica* were decapitated and the heads were suspended in ice-cold 50 mM Tris/HCl, pH 7.4, and subjected to a fast-rotating (UltraTurrax) blade to break the cuticle. The suspension was then homogenized in a glass homogenizer and the resulting homogenate was centrifuged at 1000 *g* for 10 min (MSE Centaur). The pellet was discarded and the supernatant centrifuged at 40000 *g* for 20 min (Beckman J2-21 centrifuge, JA-21 rotor) to yield a membrane preparation. These membranes were washed by resuspension in 0.5 M NaCl in 50 mM Tris/HCl, pH 7.4, followed by sedimentation at 40000 *g*. The washing procedure was repeated until a colourless supernatant was obtained. The washed membranes were resuspended in 50 mM Tris/HCl, pH 7.4, and stored at −20 °C. Protein was estimated using the bicinchoninic acid/$CuSO_4$ method provided by Pierce with BSA as the protein standard.

Metabolism of [D-Ala^2,Leu^5]enkephalin

Head membranes were incubated with [D-Ala^2,Leu^5]enkephalin (500 μM) in the presence of amastatin (100 μM) and 0.1 M Tris/HCl buffer, pH 7.4, in a total volume of 15 μl. Metabolism was terminated by the addition of 5 μl of 8 % (v/v) trifluoroacetic acid (TFA). Samples were spun at high speed (13000 *g*; Micro Centaur, MSE) for 2 min. Supernatants were diluted by adding 80 μl of 0.1 % (v/v) TFA before analysis by h.p.l.c. (system 1 or system 3). The molar ratio of Tyr-D-Ala-Gly and Phe-Leu was calculated from data given by Stephenson and Kenny (1987) on the quantification of peptides by h.p.l.c.

Assay of ACE activity

The assay was based on the method described by Turner et al. (1989) and used Bz-Gly-His-Leu as the substrate. Bz-Gly-His-Leu (5 mM) was incubated with enzyme in 0.1 M Tris/HCl, pH 8.3, in the presence of 0.3 M NaCl. Unless stated otherwise, 10 μM $ZnCl_2$ was also included in the reaction mixture. Reactions were terminated by adding 5 μl of 8 % (v/v) TFA. Assay tubes were then spun at high speed (13000 *g* for 2 min; Micro Centaur, MSE) and the supernatants diluted by adding 80 μl of 0.1 % (v/v) TFA. The amount of Bz-Gly produced was then measured by h.p.l.c. with u.v. detection (214 nm, system 2).

H.p.l.c.

The following solvent systems were used to elute peptide fragments from a 3 μ Shandon Hypersil ODS column (75 mm × 4.6 mm, internal diameter) with a flow rate of 1 ml/min.

System 1

A series of linear gradients of acetonitrile in 0.1 % (v/v) orthophosphoric acid was used. The acetonitrile concentration increased from 5 to 27 % in 4.5 min, from 27 to 38 % by 15 min and from 38 to 49 % by 18 min. The final solvent composition was maintained for a further 1 min (total run time, 19 min).

System 2

A linear gradient of acetonitrile in 0.1 % (v/v) orthophosphoric acid increasing from 5 to 60 % acetonitrile in 10 min was used. This composition was maintained for a further 2 min.

System 3

A series of linear gradients of acetonitrile in 0.1 % (v/v) orthophosphoric acid was used rising from 16 to 19 % by 2 min, from 19 to 30 % by 5 min and then followed by a step gradient to 60 % which was maintained for a further 3 min before re-equilibration.

Gel-filtration chromatography

Fly head membranes (3 mg of protein/ml) were solubilized by mixing overnight in 50 mM Tris/HCl/1 % (w/v) Triton X-100,

pH 7.4, at 4 °C. The resulting suspension was centrifuged at 40000 *g* (Beckman J2-21 centrifuge, JA-21 rotor) for 3 h to remove detergent-insoluble material. The supernatant was loaded on to a Protein-Pak 300SW column (length, 300 mm; Millipore U.K./Waters Chromatography Division, Harrow, Middx., U.K.), and eluted with 50 mM Tris/HCl, pH 7.4, containing 0.1 % (w/v) Triton X-100 and 0.2 M NaCl at a flow rate of 0.5 ml/min. Fractions (0.25 ml) were collected and assayed for ACE activity. Proteins of known M_r were used to calibrate the column.

Treatment of fly head membranes with EDTA

Membranes were incubated in 50 mM Tris/HCl, pH 7.4, containing 5 mM EDTA for 30 min at 37 °C and recovered by centrifugation at 40000 *g* (Beckman J2-21, JA-21 rotor, 4 °C) for 20 min. The membrane pellet was rinsed with 50 mM Tris/HCl, pH 7.4, and resuspended in the same buffer without EDTA.

Phase separation of membrane proteins with Triton X-114

The method employed in this study is a modification of that described by Bordier (1981). Membranes (0.85 mg of protein) were mixed with 10 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 1.5 % (w/v) Triton X-114 and kept on ice for 30 min with occasional shaking. Detergent-insoluble material was removed by centrifugation at 40000 *g* for 30 min at 4 °C. A portion (250 μl) of the supernatant was gently placed over 200 μl of 10 mM Tris/HCl containing 150 mM NaCl, 0.06 % (w/v) Triton X-114 and 6 % (w/v) sucrose. After incubation at 37 °C for 5 min to induce the phase separation of Triton X-114, samples were immediately centrifuged at high speed on a Micro Centaur (13000 *g*, 5 min, 20 °C). The supernatant was collected, and the detergent layer resuspended in an equal volume of buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.4). Both detergent-rich and detergent-poor fractions were assayed for ACE activity.

RESULTS

Hydrolysis of [D-Ala^2,Leu^5]enkephalin by fly head membranes

Phosphoramidon (10 μM), a selective inhibitor of endopeptidase-24.11, only partially inhibited the formation of Tyr-D-Ala-Gly and Phe-Leu on incubation of [D-Ala^2,Leu^5]enkephalin with fly head membranes (Lamango and Isaac, 1993; Figures 1a and 1b), whereas a mixture of 10 μM phosphoramidon and 10 μM captopril, a potent inhibitor of mammalian ACE, almost completely abolished the appearance of these two fragments (Figure 1c). Tyr-D-Ala-Gly and Phe-Leu were detected by reversed-phase h.p.l.c. in almost equimolar amounts (molar ratio of 1.00:1.17 for Tyr-D-Ala-Gly/Phe-Leu), and both fragments were inhibited to the same extent by phosphoramidon and captopril, indicating that the formation of Tyr-D-Ala-Gly resulted from the hydrolysis at Gly^3-Phe^4 and not from carboxypeptidase activity. Inhibition curves for phosphoramidon, captopril and mixtures of the two inhibitors were generated by measuring the rate of formation of Tyr-D-Ala-Gly over a range of inhibitor concentrations (Figure 2). Maximum inhibition by captopril of around 70 % was achieved at 10 μM inhibitor concentration, indicating that much of the cleavage of the Gly^3-Phe^4 bond by the insect membranes resulted from the activity of an insect ACE-like enzyme. Incubation of membranes with [D-Ala^2,Leu^5]enkephalin in the presence of mixtures of phosphoramidon and captopril clearly showed that the combined effects of these inhibitors are additive, with 90 % inhibition of the Gly^3-Phe^4 cleavage occurring with a mixture of 10 μM phosphoramidon and 10 μM captopril (Figure 2).

Assay of ACE-like activity of fly head membranes

An assay for mammalian ACE (Turner et al., 1989) employing Bz-Gly-His-Leu as the substrate was used to measure the insect peptidyl dipeptidase activity. Enzyme activity was linear with time, had a K_m of 1.91 mM for Bz-Gly-His-Leu, a $V_{max.}$ of 7.61 nmol of Bz-Gly/min per mg of protein and a pH optimum of 8.2 in 0.1 M Tris/HCl (pH range, 6.2–9.2). The presence of NaCl in the assay buffer stimulated the insect ACE-like activity by around 100 %.

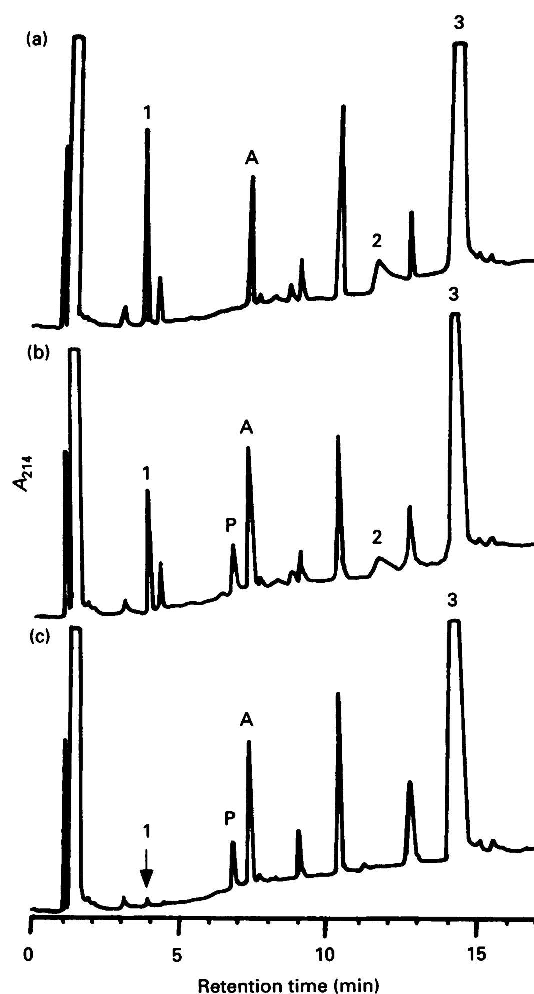

Figure 1 Metabolism of [D-Ala^2,Leu^5]enkephalin by membranes from heads of *M. domestica*

[D-Ala^2,Leu^5]enkephalin (500 μM) was incubated with membranes in the presence of the aminopeptidase inhibitor, amastatin, and the metabolites Tyr-D-Ala-Gly (1) and Phe-Leu (2) were resolved from [D-Ala^2,Leu^5]enkephalin (3) by h.p.l.c. (system 1) with u.v. detection at 214 nm. Incubations were carried out in the absence (**a**) and presence of either 10 μM phosphoramidon (**b**) or a mixture of 10 μM phosphoramidon and 10 μM captopril (**c**). The u.v.-absorbing peak at 10.5 min is an unidentified metabolite whereas other peaks are derived from the membrane preparation, the inhibitors (P, phosphoramidon; A, amastatin) and minor metabolites.

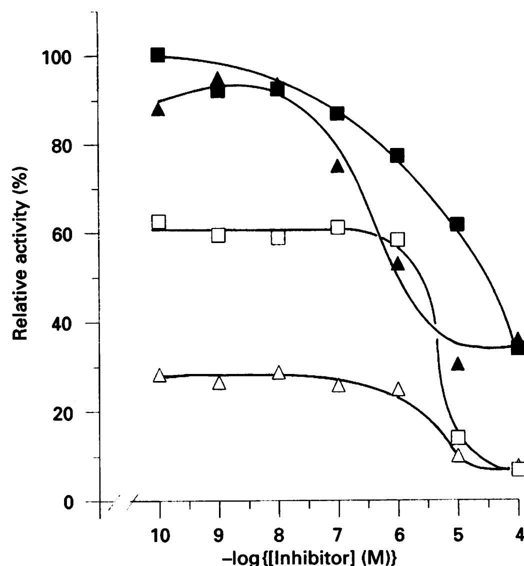

Figure 2 Inhibition of the hydrolysis of [D-Ala^2,Leu^5]enkephalin by phosphoramidon and captopril

Inhibition of the cleavage of the Gly^3-Phe^4 bond of [D-Ala^2,Leu^5]enkephalin by different concentrations of phosphoramidon (■) and captopril (▲) was determined. Peptidase activity was measured by quantifying the peptide product Tyr-D-Ala-Gly by h.p.l.c. (system 3) as described in the text. The effect of mixtures of phosphoramidon and captopril on peptidase activity was also investigated by keeping the concentration of one of the components constant at 10 μM while the concentration of the second inhibitor was varied. Data are the means of triplicate assays (S.E.M. < 5%). □, Phosphoramidon (10 μM) and captopril (10^{-10}–10^{-4} M); △, captopril (10 μM) and phosphoramidon (10^{-10}–10^{-4} M).

Table 1 Effect of bivalent metal ions on the peptidyl dipeptidase activity of EDTA-treated housefly membranes

Fly head membranes were incubated at 37 °C with 5 mM EDTA in 50 mM Tris/HCl, pH 7.4. After 30 min, the membranes were recovered by centrifugation and the pellet was washed in EDTA-free 50 mM Tris/HCl, pH 7.4, before resuspension in the same EDTA-free buffer. Enzyme activity was measured using Bz-Gly-His-Leu as the substrate (see text for details), and metal ions (100 μM) were added to the assay mixture as either the chloride or sulphate salts. The results are expressed as the mean of triplicate assays (S.E.M. < 5%) and are relative to the activity of untreated membranes.

Metal ion	Relative activity (%)
None	7
Zn^{2+}	590
Mn^{2+}	25
Fe^{2+}	9
Co^{2+}	40
Cu^{2+}	8
Mg^{2-}	9
Ca^{2+}	7

Effect of EDTA and bivalent metal ions on the ACE-like activity of fly head membranes

The peptidyl dipeptidase was inhibited by 100 μM EDTA (82% inhibition) and 1 mM 1,10-phenanthroline (50% inhibition). The ability of bivalent metal ions to recover the enzyme activity was tested by incubating EDTA-treated membranes, which had been washed to remove excess EDTA, with Bz-Gly-His-Leu in the presence of different metal ions (Table 1). The addition of 100 μM $ZnCl_2$ resulted in a massive activation (590% activation relative to the activity of untreated membranes) of the EDTA-treated enzyme. No other bivalent metal ions gave a similar level of stimulation, although Co^{2+} and Mn^{2+} did recover part (40% and 25% respectively) of the activity lost on treating the membranes with EDTA. When untreated and EDTA-treated membranes were incubated with Bz-Gly-His-Leu in the presence of various concentrations of $ZnCl_2$, maximum peptidyl dipeptidase activity was achieved with 10 μM and 100 μM $ZnCl_2$, for untreated and EDTA-treated enzyme respectively, with higher concentrations of $ZnCl_2$ resulting in inhibition. The Zn^{2+}-stimulated activity of EDTA-treated membranes was completely inhibited by 10 μM captopril.

Effect of inhibitors of mammalian ACE on the fly ACE-like peptidase

A number of active-site-directed inhibitors of mammalian ACE were tested against the insect enzyme. Captopril, lisinopril, fosinoprilat and enalaprilat were all good inhibitors of the peptidyl dipeptidase of *M. domestica*, with fosinoprilat being the most potent of these compounds (Figure 3). IC_{50} values of 400 nM, 130 nM, 16 nM and 290 nM for captopril, lisinopril, fosinoprilat and enalaprilat respectively were obtained from the inhibition curves.

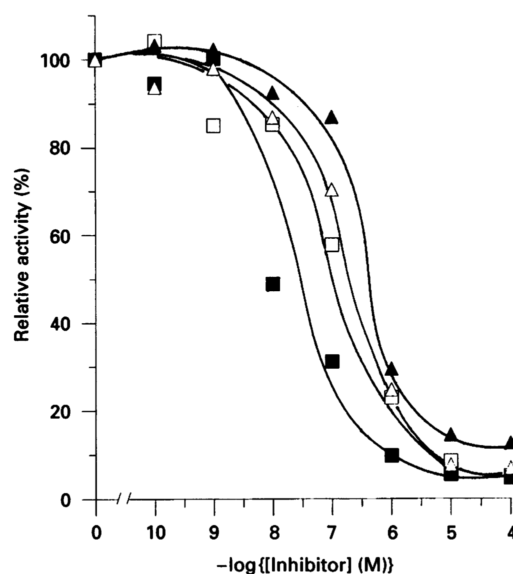

Figure 3 Inhibition of *Musca* peptidyl dipeptidase by inhibitors of mammalian ACE

Inhibition curves for captopril (▲), lisinopril (□), fosinoprilat (■) and enalaprilat (△) were generated by assaying for peptidyl dipeptidase activity in the presence of different concentrations of inhibitor using the substrate Bz-Gly-His-Leu (see the text for details). Data are expressed relative to uninhibited activity and are the means of triplicate assays (S.E.M. < 5%).

Gel-filtration chromatography of the fly ACE-like peptidase

The peptidyl dipeptidase was solubilized by incubating fly head membranes with Triton X-100, and the resulting soluble preparation was loaded on to a TSK 300 gel-filtration column. Elution of the insect enzyme activity was monitored and com-

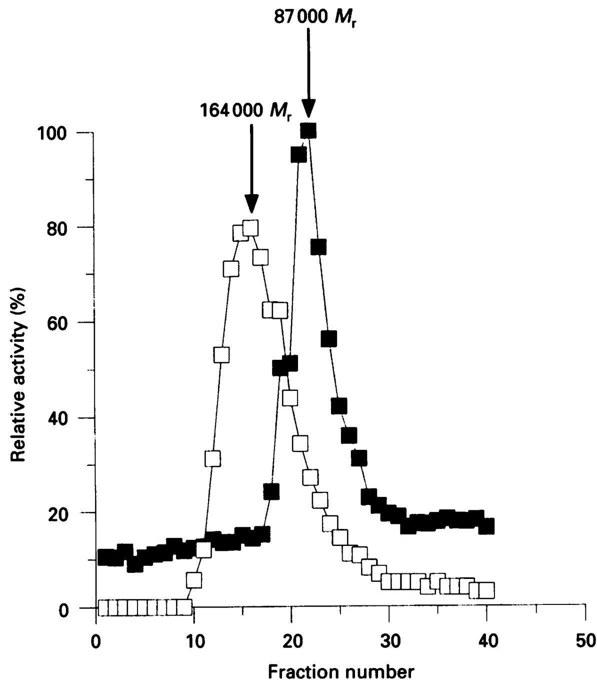

Figure 4 Gel-filtration chromatography of *Musca* peptidyl dipeptidase

Membrane proteins from heads of *M. domestica* were solubilized in Triton X-100 as described in the text and were loaded on to a Protein-Pak 300SW column (300 mm) which was eluted with 50 mM Tris/HCl/0.2 M NaCl/0.1% Triton X-100 (pH 7.3). Fractions (0.25 ml) were collected and assayed for peptidyl dipeptidase activity as described in the text (■). In a separate run, pig kidney ACE was applied to the Protein-Pak 300SW column and was eluted and assayed in an identical manner (□). The column was calibrated with M_r marker proteins (not shown). Identical results were obtained when the experiment was carried out on three separate occasions.

pared with the chromatography of trypsin-solubilized pig kidney ACE (Hooper et al., 1987). The insect and pig enzymes were clearly resolved on this column with elution volumes corresponding to apparent M_r values of 87000 and 164000 respectively (Figure 4).

Phase separation of fly ACE-like enzyme into Triton X-114

The fly head membranes used throughout this study were washed repeatedly with ice-cold 10 mM Tris/HCl/0.5 M NaCl, pH 7.3, to remove peripheral membrane proteins and to ensure, as far as was possible, that the peptidyl dipeptidase being studied was an integral membrane protein. Confirmation that the insect ACE-like enzyme was an integral membrane protein was sought by subjecting the washed membranes to phase separation with Triton X-114. Surprisingly, only 20% of the total activity was found to have partitioned into the detergent-rich phase, suggesting that only a small proportion of the insect enzyme behaved as a typical integral membrane protein.

Effect of temperature on the integrity of the membrane-bound ACE-like peptidase from flies

To investigate whether the insect peptidyl dipeptidase could be converted into a soluble form during the phase-separation experiment, membranes were incubated at either 0 °C or 37 °C in 0.1 M Tris/HCl, pH 7.3, over a 3 h period. At various time intervals, samples were removed, membranes pelleted by centrifugation (40000 *g* for 3 h at 4 °C) and the resulting supernatant assayed for peptidyl dipeptidase activity. Around 30% of the membrane-bound activity was released into the 40000 *g* supernatant after a 3 h incubation at 37 °C (Figure 5). When membranes were kept at 0 °C, very little peptidyl dipeptidase activity was solubilized over the same time period.

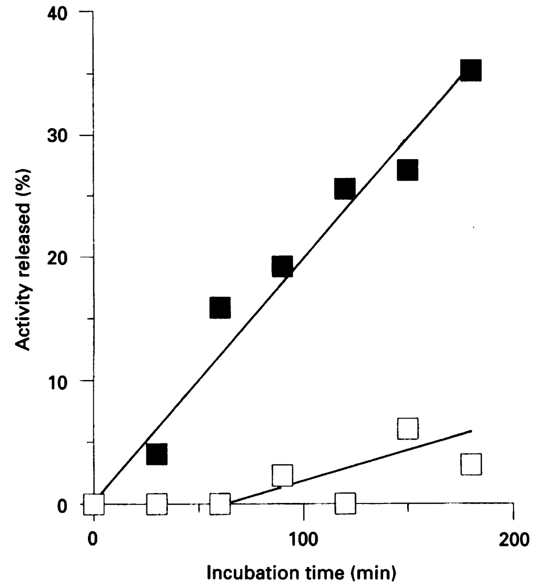

Figure 5 Solubilization of peptidyl dipeptidase activity from *Musca* head membranes in the absence of detergent

Membranes that had been extensively washed with 0.5 M NaCl/50 mM Tris/HCl, pH 7.4, to remove peripheral membrane proteins, were incubated separately at 37 °C (■) and 0 °C (□) in 10 mM Tris/HCl, pH 7.4. Samples were removed at time intervals and the membranes were sedimented by centrifugation (40000 *g*, 60 min at 4 °C). Peptidyl dipeptidase activity released into the resulting supernatant was assayed and the results are expressed as a percentage of the total activity. There was no loss of total peptidyl dipeptidase activity during the course of the experiment. Data are the means of three experiments (S.E.M. $< 5\%$).

DISCUSSION

Fly heads are a convenient source of enriched insect neural tissue and we have previously used heads of the housefly, *M. domestica*, to prepare membranes for the study of a phosphoramidon-sensitive metalloendopeptidase that has similar properties to those of mammalian endopeptidase-24.11 (Lamango and Isaac, 1993). The failure of phosphoramidon to inhibit all of the fly endopeptidase activity towards [D-Ala^2,Leu^5]enkephalin has led to the discovery of a second peptidase which is characterized by its sensitivity to the ACE inhibitor, captopril. The almost total inhibition of the hydrolysis of [D-Ala^2,Leu^5]enkephalin at the Gly^3-Phe^4 bond by a mixture of 10 μM phosphoramidon and 10 μM captopril confirmed the existence of two peptidases in these insect membranes. These results are similar to the data obtained by Turner and Dowdall (1984) who investigated the metabolism of [D-Ala^2,Leu^5]enkephalin by membranes prepared from the electric organ of *Torpedo marmorata* and showed that 30% of the hydrolytic activity at the Gly^3-Phe^4 bond could be attributed to endopeptidase-24.11 with the remainder attributed to a captopril-sensitive peptidyl dipeptidase.

The fact that [D-Ala^2,Leu^5]enkephalin was cleaved by two distinct membrane peptidases yielding identical products meant that this substrate was not suitable for use in further studies on the insect ACE-like enzyme. Bz-Gly-His-Leu is a commonly used

substrate for assaying ACE activity in mammals (Strittmatter et al., 1985; Turner et al., 1987, 1989) and was found to be a convenient substrate to measure the insect enzyme with no apparent interference from other peptidases. The K_m of 1.91 mM for the hydrolysis of Bz-Gly-His-Leu by the insect membrane enzyme is very similar to K_m values obtained for solubilized mammalian endothelial and testicular ACE with the same substrate (Cheung et al., 1980; El-Dorry et al., 1982). Inhibition of the insect peptidyl dipeptidase by metal chelators such as EDTA and 1,10-phenanthroline signifies a requirement for a metal atom for catalysis. Re-activation of enzyme treated with EDTA, by the addition of 100 μM $ZnCl_2$, but only partially or not at all by other bivalent metal ions, indicates that zinc may be the preferred active-site metal. $ZnCl_2$ also activated peptidyl dipeptidase activity that had not been treated with EDTA, which is probably due to the absence of the metal ion from a large proportion of the enzyme molecules. The reason for this is not clear but it is possible that the zinc is lost during tissue homogenization and preparation of the membranes. These data suggest that the fly peptidyl dipeptidase, like mammalian ACE, probably belongs to the zinc metallopeptidase family of enzymes.

The inhibition of the *Musca* peptidyl dipeptidase by mammalian ACE inhibitors such as captopril, fosinoprilat, enalaprilat and lisinopril (Thorsett and Wyvratt, 1987; Ehlers and Riordan, 1989), stresses the similarity of the insect enzyme to mammalian ACE. The inhibitors tested in this study displayed inhibition potencies approximately 100-fold less than those obtained for pig kidney ACE (Turner et al., 1987), except for fosinoprilat which was equally potent to the insect and mammalian enzymes. These observed differences in potency of captopril, lisinopril and enalaprilat are probably due to differences in the enzyme subsites that interact with the side groups of the inhibitors.

Mammalian ACE occurs in two distinct forms, somatic ACE (M_r = 170000–190000) and the testicular enzyme which has an M_r of around 100000 and is only produced by developing spermatozoa (Cushman and Cheung, 1971). The cloning and characterization of cDNA clones for human (Soubrier et al., 1988), mouse (Bernstein et al., 1989), rabbit (Thekkumkara et al., 1992) and bovine (Shai et al., 1992) somatic ACE has shown that the somatic enzyme comprises two highly homologous domains with two putative active sites. cDNAs for human (Ehlers et al., 1989; Lattion et al., 1989), rabbit (Kumar et al., 1989) and mouse (Howard et al., 1990) testicular ACE predict a single active-site region that has a sequence identical with the C-domain of the corresponding somatic form of the enzyme. The two forms of mammalian ACE are encoded in a single copy of the ACE gene, and transcription of the testicular enzyme has been shown to be under the control of an intragenic testis-specific promoter (Langford et al., 1991). It has been proposed that the non-duplicated testicular isoenzyme corresponds to an ancestral gene, which has given rise to the mammalian gene through gene duplication (Lattion et al., 1989; Langford et al., 1991). With this in mind, it is of some interest to note that the insect ACE-like peptidase has an apparent M_r of around 87000, which is close to the size of testicular ACE. The results of the phase-separation experiments using Triton X-114 and the temperature-dependent solubilization of the fly peptidyl dipeptidase suggests that the enzyme is easily solubilized from membranes and that it is difficult to keep the protein in its membrane-bound form. Therefore, although Triton X-100 was included in the elution buffer of the gel-filtration column, it is unlikely that the insect ACE-like peptidase was eluted as a protein–detergent micelle complex. It has been shown that an EDTA-sensitive protease is associated with plasma-membrane preparations from mammalian lung and kidney, and that this enzyme releases a hydrophilic form of ACE from cell surfaces by cleavage of the ACE protein close to its membrane anchor (Hooper et al., 1987; Oppong and Hooper, 1993). A similar protease may exist in insect membranes and be responsible for the solubilization of the ACE-like enzyme seen in the present study.

The physiological role of the ACE-like peptidase will be defined by its cellular localization and its substrate specificity, but at present we have very little information, apart from the fact that the peptidase will hydrolyse [D-Ala^2,Leu^5]enkephalin and Bz-Gly-His-Leu. To our knowledge, no components of the renin–angiotensin system have been found in insects, and it therefore seems unlikely that this peptidyl dipeptidase functions as an ACE. The activity of the insect enzyme may not be restricted to the hydrolysis of peptides with a free C-terminus and may extend to the metabolism of the many insect peptides that have their C-termini blocked by amidation. For such peptides, an amidated C-terminus is invariably required for full biological activity, and thus hydrolysis close to the C-terminus will lead to inactivation.

In conclusion, we have identified an insect peptidyl dipeptidase that has biochemical properties similar to mammalian ACE, and which is similar in size to testicular ACE. The insect enzyme might more closely resemble the ancient form of ACE, which occurred before duplication of the ACE gene which is present in mammals.

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