The endopeptidase activity and the activation by Cl^- of angiotensin-converting enzyme is evolutionarily conserved: purification and properties of an angiotensin-converting enzyme from the housefly, *Musca domestica*

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A soluble 67 kDa angiotensin-converting enzyme (ACE) has been purified by lisinopril-Sepharose affinity column chromatography from adult houseflies, *Musca domestica*. The dipeptidyl carboxypeptidase activity towards benzoyl-Gly-His-Leu was inhibited by captopril (IC_{50} 50 nM) and fosinoprilat (IC_{50} 251 nM), two inhibitors of mammalian ACE, and was activated by Cl^- (optimal Cl^- concentration 600 mM). *Musca* ACE removed C-terminal dipeptides from angiotensin I, bradykinin, [Leu^5]enkephalin and [Met^5]enkephalin and also functioned as an endopeptidase by hydrolysing dipeptideamides from [Leu^5]enkephalinamide and [Met^5]enkephalinamide, and a dipeptideamide and a tripeptideamide from substance P. *Musca* ACE was also able to cleave a tripeptide from both the N-terminus and C-terminus of luteinizing hormone-releasing hormone, with C-terminal hydrolysis predominating. Maximal N-terminal tripeptidase activity occurred at 150 mM NaCl, whereas the C-terminal tripeptidase activity continued to rise with increasing concentration of Cl^- (0–0.5 M). *Musca* ACE displays properties of both the N- and C-domains of human ACE, indicating a high degree of conservation during evolution of the substrate specificity of ACE and its response to Cl^-.

INTRODUCTION

Angiotensin-converting enzyme (EC 3.4.15.1, ACE, peptidyl dipeptidase A) is a zinc metallopeptidase that is found in a wide variety of mammalian tissues, principally as a membrane-bound ectoenzyme [1]. ACE is responsible for the removal of the C-terminal dipeptide from angiotensin I to generate the powerful vasoconstrictor octapeptide, angiotensin II [2,3]. The same enzyme also degrades and inactivates the vasodilatory peptide, bradykinin (BK), by the sequential removal of dipeptides from the C-terminus [2,3]. Angiotensin I and BK are not the only substrates for ACE: other small peptides including [Leu^5]enkephalin, [Met^5]enkephalin, substance P (SP), neurotensin, cholescystokinin 8 and luteinizing hormone releasing hormone (LHRH) are also hydrolysed by this enzyme *in vitro* [1,4,5]. Although typically functioning as a carboxydipeptidase, ACE can also perform as an endopeptidase removing dipeptideamides from peptides with an amidated C-terminus (e.g. SP [6–9], [Leu^5]enkephalinamide, [Met^5]enkephalinamide [9] and cholecystokinin [10]). With SP, ACE hydrolysis occurs at two peptide bonds, resulting in the release of a tripeptideamide in addition to the dipeptideamide [6–9]. LHRH is an even more unusual substrate because ACE cleaves an N-terminal tripeptide in addition to a C-terminal tripeptideamide [11].

In mammals, ACE exists as two main forms, a somatic ACE (sACE) and a testicular ACE (tACE) [12,13]. sACE consists of a single polypeptide chain of molecular mass 140–180 kDa with two similar domains (N- and C-domains), both of which contain the consensus active site sequence of HEXXH found in metallopeptidases [14–19]. The testicular isozyme, which is unique to the testis, is a smaller protein of 90–110 kDa and has a single active site, the bulk of which is identical to the C-domain of sACE [20–23]. There is evidence that the N- and C-domains of human ACE have different physiological roles. On the basis of the properties of recombinant N- and C-domains of human ACE *in vitro*, it has been proposed that the C-domain is most likely to be responsible for much of the ACE activity on the surface of endothelial cells [13,19]. The two domains display differences in their interactions with peptide substrates and their enzymic activity is affected differently by Cl^- [19,24–26]. A specific role for the N-domain in the catabolism of the haemoregulatory peptide, *N*-acetyl-Ser-Asp-Lys-Pro, has recently been proposed [27]. This peptide has been co-localized with human sACE and is selectively and efficiently hydrolysed *in vitro* by recombinant human N-domain. The N-domain also appears to be responsible for much of the N-terminal tripeptidase activity towards LHRH by human sACE, although the catalytic efficiency of this reaction is low [24,25]. The two domains of human and rat sACE can also be distinguished by differences in the relative potency of inhibitors and dissociation rates for the ACE–inhibitor complexes [25,26,28].

We have recently shown that membranes prepared from heads of *Musca domestica* possess dipeptidyl carboxypeptidase activity that is inhibited by a number of inhibitors of mammalian ACE [29]. The molecular mass of a soluble form of the housefly ACE was estimated at 80 kDa by gel-filtration chromatography, suggesting the existence of a single-domain form of the enzyme in insects. Recently, a cDNA for insect ACE has been cloned

Abbreviations used: ACE, angiotensin-converting enzyme; BK, bradykinin; Bz, benzoyl; LHRH, luteinizing hormone releasing hormone; MALDI-MS, matrix-assisted laser desorption mass spectrometry; MGTA, DL-2-mercaptoethyl-3-guanidinoethylthiopropanoic acid; sACE, somatic ACE; SP, substance P; tACE, testicular ACE; TFA, trifluoroacetic acid.

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from *Drosophila melanogaster* embryo cDNA library that codes for a single-domain 615 amino acid protein with high levels of similarity to both the N- and C-domains of mammalian sACE [30].

We now report the single-step purification of a soluble 67 kDa ACE from *M. domestica* by using a lisinopril-Sepharose affinity column, and we describe the substrate specificity of the enzyme. The insect enzyme, like mammalian ACEs, possesses endopeptidase activity in addition to the typical dipeptidyl carboxypeptidase activity. Other properties that are evolutionarily conserved are activation by Cl^- and the higher affinity of the enzyme for BK compared with the other peptide substrates examined.

MATERIALS AND METHODS

Chemicals

Sepharose CL-4B, 4-butanediol diglycidyl ether, sodium borohydride, glycine, *N*-hydroxysuccinamide, *NN′*-dicyclohexylcarbodi-imide, angiotensin I, captopril (SQ 14225; D-3-mercapto-2-methylpropanoyl-L-proline), benzoyl (Bz)-Gly-His-Leu and peptides were obtained from Sigma Chemical Co. 6-[*N*-(*p*-Aminobenzoyl)]caproic acid and 1,4-dioxane were obtained from Aldrich Chemical Co. Lisinopril (MK 521; *N*-(*S*)-1-carboxy-3-phenylpropyl-L-lysyl-L-proline) was a gift from Dr. H. Bull, Merck, Sharpe and Dohme Research Laboratories. Fosinoprilat (SQ-27519; *trans*-4-cyclohexyl-1-{[hydroxy(4-phenylbutyl)phosphinyl]acetyl}-L-proline and Phe-Arg were gifts from Dr. N. M. Hooper and Professor A. J. Turner, Department of Biochemistry and Molecular Biology, University of Leeds, U.K. DL-2-Mercaptoethyl-3-guanidinoethylthiopropanoic acid (MGTA) was from Calbiochem Corporation.

Insects

M. domestica were raised under laboratory conditions as described previously [29].

Synthesis of affinity adsorbent

Lisinopril-Sepharose was synthesized by the method of Hooper and Turner [9] except that the final blocking of the reactive groups with glycine was omitted. Instead the gel was left for 4 h at room temperature for the excess *N*-hydroxysuccinamide ester groups to break down.

Enzyme purification

M. domestica were decapitated by shaking in liquid nitrogen and the bodies were separated from the heads and limbs with a sieve. Bodies (18 g) were suspended in ice-cold 50 mM Tris/HCl, pH 7.4 (60 ml) and blended with a fast-rotating (Ultra-Turrax) blade to yield a homogenate that was centrifuged at 1000 ***g*** for 10 min. The pellet was discarded and the supernatant was centrifuged at 40000 ***g*** for 30 min (Beckman J2-21, JA-21 rotor). Ammonium sulphate (final concentration 0.2 M) was added to the 40000 ***g*** supernatant, which was then loaded on to a lisinopril-Sepharose affinity resin (30 ml) that had been equilibrated with the loading/washing buffer (10 mM Tris/HCl, pH 8.3, 0.2 M ammonium sulphate) and was connected to a UV detector set at 280 nm. Aliquots of the enzyme preparation (5 ml) were injected on to the column at 1 ml/min flow rate, until all the enzyme had been loaded. Unbound protein was eluted with the loading/washing buffer, pumped at 4 ml/min until a stable UV absorbance baseline was obtained. The eluting buffer (10 mM Tris/HCl, pH 8.3) was then run through the column at 4 ml/min until a UV-absorbing peak was eluted. During the washing and elution phases of the purification, 4.5 ml fractions were collected and assayed for ACE activity. The protein concentration of each fraction was measured by using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) with BSA as the protein standard. Peak activity fractions were pooled and concentrated by ultrafiltration on Centrifugal Ultrafree-20 (30000 nominal molecular mass limit; Millipore) by spinning at 1500 ***g***. SDS/PAGE [7.5 % (w/v) homogeneous PhastGel; Pharmacia Biotech] was performed with the Pharmacia Phast System (Pharmacia Biotech) and the protein bands were revealed by staining with Coomassie Blue.

Enzyme assays

ACE activity was measured with an HPLC-based assay for the hydrolysis of Bz-Gly-His-Leu [29]. Bz-Gly-Arg, Bz-Gly-Phe and Bz-Gly-Lys were employed as carboxypeptidase substrates. The assay conditions were identical with those used to measure the hydrolysis of Bz-Gly-His-Leu except that a different HPLC solvent system was used to quantify Bz-Gly released from the hydrolysis of Bz-Gly-Lys. This solvent system comprised a 5 min linear gradient (11–30 %) of acetonitrile in 0.1 % orthophosphoric acid. At the end of the gradient the solvent was switched to 60 % acetonitrile in 0.1 % orthophosphoric acid for 3 min.

Hydrolysis of peptides by ACE

Purified enzyme (0.1–0.23 μg of housefly ACE) was incubated with, as individual substrates, angiotensin I, LHRH, SP, BK, [Met^5]enkephalin, [Leu^5]enkephalin, [Met^5]enkephalinamide and [Leu^5]enkephalinamide (final concentration 200 μM) in 0.1 M Tris/HCl, pH 8.3, containing 0.3 M NaCl and 10 μM $ZnCl_2$ or 10 μM $ZnSO_4$ in a total volume of 15 μl. Incubations of with BK, SP or LHRH were performed in the presence of 10 μM MGTA to block the activity of a carboxypeptidase that was a minor contaminant of the purified insect ACE. Reactions were terminated either by boiling or by the addition of 5 μl of 8 % (v/v) trifluoroacetic acid (TFA). Samples were spun at 13000 ***g*** (Micro centaur, MSE) for 2 min and supernatants were diluted by adding 80 μl of 0.1 % (v/v) TFA before analysis by HPLC.

Peptide analysis by HPLC

The HPLC system consisted of Gilson 305 and 306 pumps, a 231 sample injector and an Applied Biosystems 759A UV detector set at 214 nm. Samples were analysed by using either a Pharmacia SuperPac Pep-S column (250 mm × 4 mm; Pharmacia Biotech) packed with 5 μm particles (solvent systems 1–4) or a Shandon Hypersil ODS column (75 mm × 4.6 mm; Shandon Southern Products) packed with 3 μm particles (system 5) at a flow rate of 1 ml/min. The five systems used were as follows.

System 1: a short (1.5 min) linear gradient of increasing acetonitrile (5–16 %) in 0.1 % TFA followed by a linear increase to 38 % acetonitrile in 0.1 % TFA over 10 min.

System 2: a linear gradient of acetonitrile (6–46 %) in 0.1 % TFA over 11 min.

System 3: a linear gradient of acetonitrile (5–43 %) in 0.1 % TFA over 14 min.

System 4: isocratic elution with 5 % acetonitrile in 0.1 % orthophosphoric acid for the first 3 min followed by a linear gradient of increasing acetonitrile (5–28 %) in 0.1 % orthophosphoric acid over 15 min.

System 5: three consecutive gradients (5–27 % in 4.5 min; 27–38 % in 15 min; 38–49 % in 3 min) of acetonitrile in 0.1 % orthophosphoric acid.

At the end of each gradient, the column was washed with 60 %

acetonitrile in 0.1 % TFA or 0.1 % orthophosphoric acid for 2 min before re-equilibration with the initial solvent.

Identification of peptide metabolites

Whenever possible authentic peptides were used as HPLC markers to identify metabolites; however, when the appropriate compounds were not available, peptide fragments isolated by HPLC were subjected to either amino acid analysis or matrix-assisted laser desorption mass spectrometry (MALDI-MS) (Texas Agricultural Station Biotechnology Support Laboratory, Peptide Services, College Station, TX, U.S.A.).

K_m and $V_{max.}$ determinations

The rates of hydrolysis of angiotensin I, BK, BK^{1-7} and the enkephalin peptides at different substrate concentrations were determined by quantifying the rate of formation of angiotensin II, Phe-Arg, BK^{1-5} and Tyr-Gly-Gly, respectively, by HPLC. The extent of hydrolysis was restricted to less than 20 % of the initial substrate and under these conditions the reaction products seemed to be stable to further hydrolysis. The low-level hydrolysis of SP by housefly ACE was dominated by the cleavage of the dipeptideamide to form SP^{1-9} but some SP^{1-7} and Phe-Phe was also formed. The rate of overall hydrolysis of SP was determined by summing the molar quantities of SP^{1-9}, SP^{1-7} and Phe-Phe formed during the course of the reaction. Kinetic data for the overall hydrolysis of LHRH were obtained by measuring the rate of pGlu-His-Trp production. Peak areas from the HPLC analysis of SP metabolites and pGlu-His-Trp were converted into mole quantities by using available information on the relative molar extinction coefficients of peptide fragments and the parent peptides at 214 nm [31].

RESULTS

ACE distribution in the housefly

Before embarking on the purification of ACE from adult *M. domestica* we investigated the relative distribution of ACE in soluble and membrane fractions from the heads and bodies (thorax and abdomen). ACE activity in the 40000 ***g*** supernatants (soluble form) and resuspended pellets (membrane form) of heads and bodies (thorax and abdomen) of *M. domestica* were determined by using Bz-Gly-His-Leu as the substrate (Table 1). The specific activity of the crude soluble ACE from the heads and bodies was much higher than the specific activity of ACE in the corresponding membrane fractions. About 66 % of the total ACE activity in a single fly was in the soluble fraction prepared from bodies, and over 95 % of this activity was inhibited by 10 μM captopril. It was decided to purify the soluble ACE from housefly bodies because this was the most abundant source of the enzyme.

Enzyme purification

It was necessary to include 0.2 M ammonium sulphate in the loading buffer for binding the insect ACE to the lisinopril-Sepharose column. Bound enzyme was eluted as a single UV-absorbing peak by simply washing the column with buffer devoid of ammonium sulphate. This protein peak coincided with the major peak of ACE activity measured with Bz-Gly-His-Leu as the substrate. This procedure resulted in recoveries in excess of 20 % and a purification factor of 710 (Table 2). SDS/PAGE revealed a single protein band with a molecular mass of about 67 kDa (Figure 1).

Co-purification of a carboxypeptidase

The hydrolysis of BK by ACE should yield BK^{1-7} and Phe-Arg as the primary reaction products [1]. However, when affinity-purified housefly ACE was incubated with BK, BK^{1-8} was also detected as a metabolite (results not shown), suggesting that the enzyme, apparently homogenous by SDS/PAGE, was contaminated with carboxypeptidase activity. The carboxypeptidase activity in the purified preparation was assayed with Bz-Gly-Arg, Bz-Gly Lys and Bz-Gly-Phe as substrates and the same assay procedures as employed to measure the hydrolysis of Bz-Gly-His-Leu. Bz-Gly-Arg and Bz-Gly-Lys, but not Bz-Gly-Phe, were hydrolysed (Table 3) and the carboxypeptidase activity was estimated to be less than 10 % of the ACE activity measured with Bz-Gly-His-Leu. The carboxypeptidase was completely inhibited by 10 μM MGTA but was unaffected by 10 μM captopril. For subsequent studies on the metabolism of BK by insect ACE, it was important to include 10 μM MGTA in the incubation mixture.

Effect of NaCl and inhibitors on housefly ACE activity

Fosinoprilat and captopril were potent inhibitors of the insect ACE, with IC_{50} values of 50 and 251 nM for captopril and fosinoprilat respectively. The optimal NaCl concentration for the hydrolysis of Bz-Gly-His-Leu was 600 mM, and at this salt concentration the rate of hydrolysis was 3.2-fold greater than the rate measured in the absence of salt (Figure 2).

Hydrolysis of mammalian peptides

Although mammalian ACE is best known as a dipeptidyl carboxypeptidase dipeptidase cleaving dipeptides from the

Table 1 Distribution of ACE activity in a single fly

Soluble and membrane fractions from heads and bodies (thorax and abdomen) were prepared from three groups of five flies by centrifugation at 40000 ***g*** for 30 min. ACE activity was asayed with Bz-Gly-His-Leu as the substrate [29]. The specific activities are means ± S.E.M. for the enzyme prepared from each of the three groups of flies. One unit of activity is 1 nmol of Bz-Gly-His-Leu hydrolysed per min.

Tissue fraction	Total activity (units per head or units/body)	Total protein (mg per head or mg per body)	Specific activity (units/mg of protein)
Head, soluble	2.23	0.185	12.05 ± 1.14
Head, membranes	0.29	0.153	2.00 ± 0.26
Body, soluble	8.59	0.838	10.25 ± 0.54
Body, membranes	2.00	0.585	3.42 ± 0.31

Table 2 Purification of housefly ACE

One unit of activity is 1 nmol of Bz-Gly-His-Leu hydrolysed per min.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)	Purification factor
Homogenate	10960	1135.5	9.64	100	1
Supernatant	9832	971.5	10.12	90	1
Lisinopril-Sepharose	2620	0.3834	6834	24	709

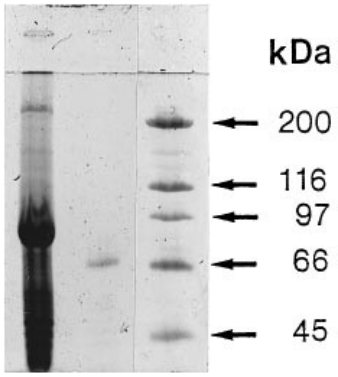

Figure 1 SDS/PAGE of ACE affinity-purified from houseflies

A sample of the crude fly-body extract (lane 1) and approx. 2 μg of affinity-purified ACE (lane 2) were applied to a Pharmacia PhastGel Homogeneous 7.5% polyacrylamide gel, which was then subjected to electrophoresis (Pharmacia Phast System) with SDS buffer strips. Proteins were stained with Coomassie Blue and the molecular mass of the housefly ACE was determined by reference to molecular mass marker proteins (lane 3).

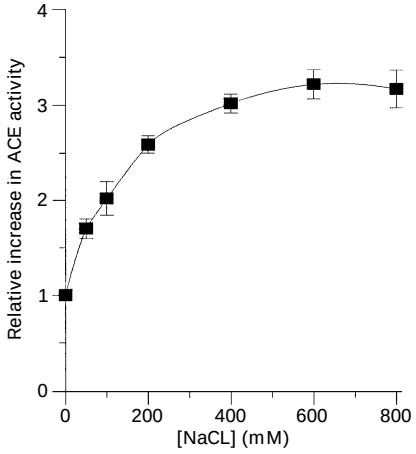

Figure 2 Effect of NaCl on the activity of housefly ACE

The effect of NaCl on ACE activity was determined by incubating housefly ACE with Bz-Gly-His-Leu (1 mM) in 100 mM Tris/maleate buffer, pH 8.0, containing 10 μM $ZnCl_2$. Points are the means $\pm$ S.E.M. for quadruplicate determinations.

C-terminus of angiotensin I, BK and enkephalin peptides, the enzyme can also hydrolyse peptides with amidated C-terminal residues; the hydrolysis need not be restricted to the penultimate peptide bond. The enzymic properties of housefly and mammalian ACEs were compared by studying the hydrolytic activity of *M. domestica* ACE towards a range of mammalian peptides regarded as typical and atypical substrates of human ACE. In all instances peptide metabolism was inhibited (more than 90% inhibition) by 10 μM captopril. The identity of peptide fragments was established by co-chromatography with authentic fragments obtained commercially, by subjecting HPLC-isolated metabolites to MALDI-MS or amino acid analysis.

Hydrolysis of angiotensin I, BK and BK[1–7]

Housefly ACE cleaved the Phe-His bond of angiotensin I releasing the C-terminal dipeptide, His-Leu, and forming angio-

Table 3 Inhibition of the carboxypeptidase activity that co-purifies with affinity-purified housefly ACE

Housefly ACE was incubated with all four substrates under identical assay conditions for the same incubation period. The amount of Bz-Gly formed was measured as described in Materials and Methods.

	Percentage of maximum activity*		
Substrate	Normal assay	+ Captopril (10 μM)	+ MGTA (10 μM)
Bz-Gly-His-Leu	100 $\pm$ 1.07	2.70 $\pm$ 0.27	100.45 $\pm$ 1.00
Bz-Gly-Arg	9.29 $\pm$ 0.20	9.53 $\pm$ 1.05	0.62 $\pm$ 0.03
Bz-Gly-Lys	9.71 $\pm$ 0.68	9.88 $\pm$ 0.99	0
Bz-Gly-Phe	0	0	0

* Values are expressed as a percentage of the maximum hydrolytic activity observed with Bz-Gly-His-Leu. Data are presented as means $\pm$ S.E.M. ($n = 3$).

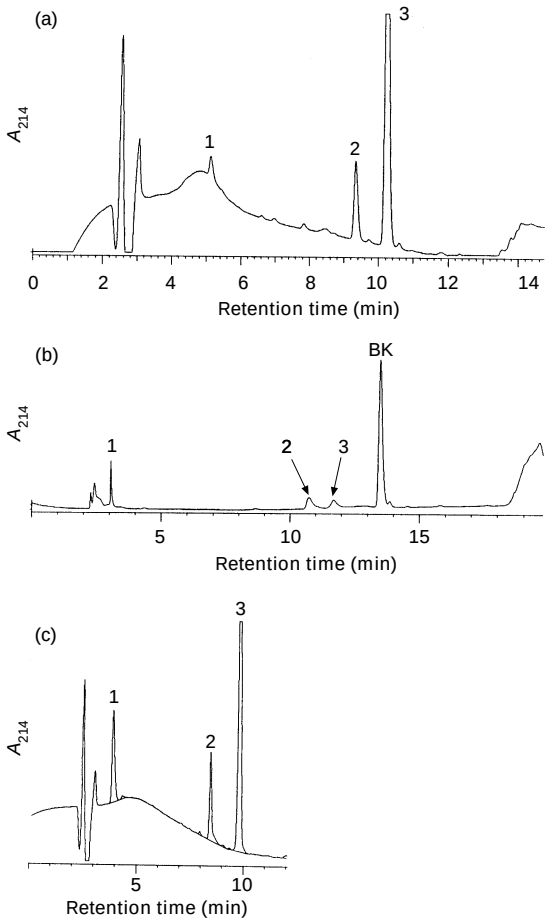

Figure 3 HPLC analysis of the products formed on hydrolysis of (a) angiotensin I, (b) BK and (c) [Leu^5]enkephalin by housefly ACE

Peptides (200 μM) were incubated with housefly ACE (0.12 μg for BK and [Leu^5]enkephalin, 0.23 μg for angiotensin I) in 100 mM Tris/HCl, 10 μM $ZnCl_2$, 0.3 M NaCl, pH 8.3, for 30 min at 37 °C. The hydrolyses of angiotensin I and BK were performed in the presence of 10 μM MGTA. (**a**) His-Leu (1), angiotensin II (2) and angiotensin I (3) were resolved on HPLC system 1. (**b**) Phe-Arg (1), BK$^{1–5}$ (Arg-Pro-Pro-Gly-Phe; 2) and BK$^{1–7}$ (Arg-Pro-Pro-Gly-Phe-Ser-Pro; 3) were resolved on HPLC system 4. (**c**) Tyr-Gly-Gly (1), Phe-Leu (2) and [Leu^5]enkephalin (3) were resolved on HPLC system 5. All peptide fragments were identified by co-chromatography with authentic peptides.

tensin II (Figure 3a). Three major metabolites were formed on incubation of BK with housefly ACE; these were shown to have retention times identical with those of authentic Phe-Arg, BK$^{1–5}$ and BK$^{1–7}$ on HPLC analysis (Figure 3b). This metabolite pattern resulted from cleavage of the Pro^7-Phe^8 peptide bond of BK, followed by hydrolysis of the Phe^5-Ser^6 bond of BK$^{1–7}$ to release BK$^{1–5}$. The incubation of insect ACE with BK$^{1–7}$ showed that this peptide was attacked at the Phe^5-Ser^6 position, giving rise to BK$^{1–5}$, which appeared to be a stable metabolite (results not shown).

Hydrolysis of [Leu^5]enkephalin, [Leu^5]enkephalinamide, [Met^5]enkephalin and [Met^5]enkephalinamide

Housefly ACE attacked all the enkephalin peptides at the same peptide bond (Gly^3-Phe^4) to release Tyr-Gly-Gly and the corresponding C-terminal dipeptide or dipeptideamide (Figure 3c).

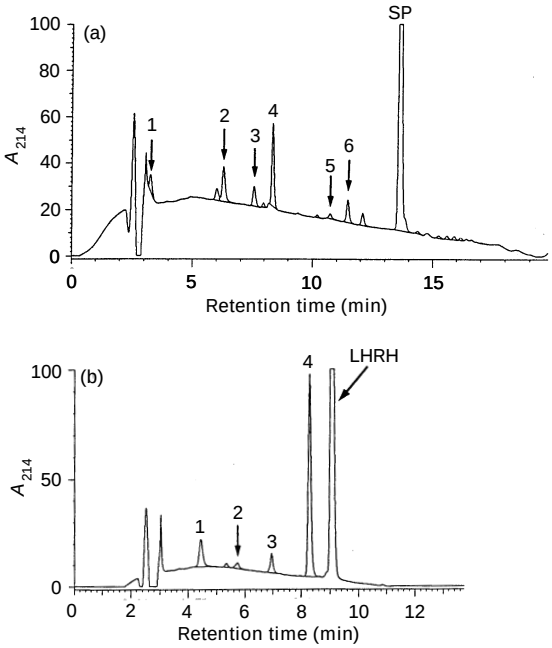

Figure 4 HPLC analysis of the products formed on hydrolysis of (a) SP and (b) LHRH by housefly ACE

(**a**) SP (200 μM) was incubated with housefly ACE (0.23 μg) in 100 mM Tris/HCl, 10 μM $ZnCl_2$, 0.3 M NaCl, 10 μM MGTA, pH 8.3, for 60 min at 37 °C. Reaction products (HPLC peaks 1–6) were resolved by using reversed-phase HPLC (solvent system 3). Peaks 2, 3, 4, 5 and 6 were identified as Phe-Gly, Gln-Phe, SP$^{1–7}$, SP$^{1–9}$ and Phe-Phe respectively by comparison with the retention times of authentic SP fragments and by MALDI-MS. Peak 1 was identified as SP$^{1–5}$ by MALDI-MS only. (**b**) LHRH (200 μM) was incubated with housefly ACE (0.10 μg) in 100 mM Tris/HCl, 10 μM $ZnCl_2$, 0.3 M NaCl, 10 μM MGTA, pH 8.3, for 150 min at 37 °C. Reaction products (HPLC peaks 1–4) were resolved by using reversed-phase HPLC (solvent system 2). Peaks 1, 2 and 4 were identified as Ser-Tyr, Gly-Leu and pGlu-His-Trp by amino acid analysis. MALDI-MS was used to identify Peak 3 as LHRH$^{4–10}$ and to confirm the identity of pGlu-His-Trp.

Hydrolysis of SP

The metabolism of SP by mammalian ACE is unusual in that it can be initiated by the hydrolysis of two peptide bonds, resulting in the formation of SP$^{1–9}$ and SP$^{1–8}$ and the corresponding dipeptide- and tripeptide-amides, respectively. Thereafter, sequential ACE activity removes dipeptides from the C-terminus of these two primary metabolites. HPLC analysis of the metabolites formed on hydrolysis of SP by housefly ACE revealed a number of fragments, of which SP$^{1–7}$, SP$^{1–9}$, Phe-Gly and Phe-Phe were identified (Figure 4a). The presence of SP$^{1–9}$ and SP$^{1–7}$ signifies cleavage at the Gly^9-Leu^{10} and Phe^7-Phe^8 bonds respectively (Table 4). The presence of Phe-Gly is further confirmation of the cleavage of both peptide bonds. The presence of Phe-Phe signifies initial cleavage of SP at the bond between Phe^8 and Gly^9, which releases the C-terminal tripeptideamide Gly-Leu-Met-NH_2, followed by cleavage of the Gln^6-Phe^7 bond by dipeptidyl carboxypeptidase activity. These results show that the housefly enzyme, like mammalian ACEs, has the peculiar property of being able to remove tripeptide- and dipeptide-amides from the C-terminus.

Hydrolysis of LHRH

The metabolites generated by incubating LHRH with housefly ACE (Figure 4b) were identified as Ser-Tyr, Gly-Leu, Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LHRH$^{4–10}$) and pGlu-His-Trp.

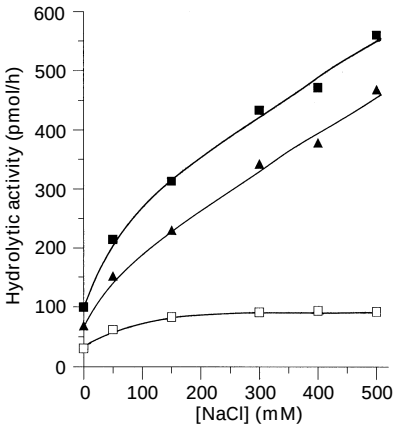

Figure 5 Effect of NaCl on the overall hydrolysis of LHRH (■), N-terminal (□) and C-terminal (▲) tripeptidase activities of housefly ACE towards LHRH

LHRH (200 μM) was incubated with ACE (0.74 μg) in 50 mM Hepes, 10 μM $ZnCl_2$, 10 μM MGTA, pH 7.5, for 180 min at 37 °C in the absence and presence of NaCl (0–0.5 M). $LHRH^{1-3}$ (pGlu-His-Trp) and $LHRH^{4-10}$ (Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) were quantified by HPLC (system 2). $LHRH^{1-3}$ is an end product of both the N- and C-terminal tripeptidase pathways and the amount generated was taken as a measure of the overall rate of hydrolysis of LHRH. $LHRH^{4-10}$ is a primary product of the N-terminal tripeptidase activity only and was stable under the reaction conditions employed. The rate of formation of $LHRH^{4-10}$ was used as a measure of the N-terminal tripeptidase activity. The C-terminal hydrolysis of LHRH was determined by subtracting the N-terminal tripeptidase activity from the total rate of LHRH hydrolysis. The points are means of triplicate determinations and the S.E.M. of each data point is less than 3%.

$LHRH^{4-10}$ was produced by N-terminal tripeptidyl endopeptidase cleavage, which also gave rise to pGlu-His-Trp. The presence of Gly-Leu indicates cleavage between Leu^7 and Arg^8 and between Tyr^5 and Gly^6 (Table 4). It is known from the substrate specificity of mammalian ACE that metabolism initiated at the C-terminus through hydrolysis at Leu^7-Arg^8 leads to the rapid sequential breakdown of the N-terminal fragment ($LHRH^{1-7}$) resulting in the final products Gly-Leu, Ser-Tyr and pGlu-His-Leu [11,24,25]. Therefore the identification of Ser-Tyr in the present study is indicative of dipeptidyl carboxypeptidase activity towards $LHRH^{1-5}$, a fragment not identified in our chromatograms. $LHRH^{4-10}$ is the product of the N-terminal hydrolysis of LHRH and does not appear to be hydrolysed during the course of its generation. When a mixture of 7 μM $LHRH^{4-10}$ and 200 μM LHRH was incubated with the enzyme under normal assay conditions, the amount of $LHRH^{4-10}$ found at the end of the reaction was equivalent to the sum of $LHRH^{4-10}$ added at the start, and the amount of $LHRH^{4-10}$ formed from the hydrolysis of LHRH in the absence of added $LHRH^{4-10}$. Thus $LHRH^{4-10}$ seemed to be a stable product of LHRH metabolism and was therefore used to measure the rate of N-terminal tripeptidase activity. An examination of the molar ratio of pGlu-His-Trp formed from both the N- and C-terminal hydrolysis of LHRH, and the metabolite $LHRH^{4-10}$ formed by N-terminal hydrolysis only, showed that 83% of the LHRH breakdown catalysed by housefly ACE in 0.3 M NaCl was via the C-terminal tripeptidyl endopeptidase pathway.

Effect of Cl^- on the N-terminal and C-terminal hydrolysis of LHRH

The N-terminal tripeptidase activity, measured by the rate of formation of $LHRH^{4-10}$, was activated by Cl^- with a maximum increase of 3-fold achieved at 150 mM NaCl.

The contribution of the C-terminal tripeptidase activity to the metabolism of LHRH was calculated by subtracting the N-terminal activity (nmol of $LHRH^{4-10}$ formed per h) from the total hydrolytic activity (nmol of pGlu-His-Trp formed per h) (Figure 5). Hydrolysis of the C-terminal tripeptide increased steadily with increasing concentration of NaCl (0–0.5 M) (Figure 5). The ratio of the C-terminal tripeptidase activity to that of the N-terminal tripeptidase increased from 3.7:1 in Cl^--free buffer to 7.2:1 in 0.5 M NaCl, revealing the different effects of NaCl on the two hydrolytic pathways.

K_m and $V_{max.}$ values for the hydrolysis of mammalian peptides

BK was by far the best substrate for insect ACE with a low K_m of 9 μM and the highest $V_{max.}/K_m$ ratio (Table 4). BK^{1-7}, [Met^5]enkephalin and [Leu^5]enkephalin were also relatively good substrates compared with angiotensin I, SP and LHRH. The K_m values for [Met^5]enkephalinamide and [Leu^5]enkephalinamide were considerably greater than their non-amidated analogues, demonstrating the importance of a free C-terminus for the binding of the enkephalin peptides at the active site (Table 4). LHRH was the poorest substrate for housefly ACE, as indicated by the very low $V_{max.}/K_m$ ratio.

DISCUSSION

Housefly ACE failed to bind to the lisinopril-Sepharose column in the presence of the buffers routinely employed to purify mammalian ACE [9,32]. However, the presence of 0.2 M am-

Table 4 The substrate specificity of housefly ACE

Initial sites of hydrolysis are indicated by bold arrows (↓) and subsequent cleavage positions are indicated by normal arrows (↓). K_m and $V_{max.}$ values were determined as described in the Materials and methods section. Data are the means ± S.E.M. for triplicate experiments. One unit of activity is 1 nmol of peptide hydrolysed per min.

Peptide	Primary structure and cleavage sites	K_m (μM)	$V_{max.}$ (units/mg protein)	$V_{max.}/K_m$
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe↓-His-Leu	235 ± 21	88 ± 4	0.4 ± 0.04
BK	Arg -Pro-Pro-Gly-Phe↓-Ser-Pro↓-Phe-Arg	9 ± 3	95 ± 3	10.6 ± 3.5
BK^{1-7}	Arg-Pro-Pro-Gly-Phe↓-Ser-Pro	33 ± 7	176 ± 10	5.3 ± 1.1
Leu^5-enkephalin	Tyr-Gly-Gly↓-Phe-Leu	98 ± 15	255 ± 14	2.6 ± 0.4
Leu^5-enkephalinamide	Tyr-Gly-Gly↓-Phe-Leu-NH_2	557 ± 132	888 ± 127	1.6 ± 0.5
Met^5-enkephalin	Tyr-Gly-Gly↓-Phe-Met	53 ± 12	241 ± 17	4.6 ± 1.1
Met^5-enkephalinamide	Tyr-Gly-Gly↓-Phe-Met-NH_2	1056 ± 115	510 ± 41	0.5 ± 0.1
SP	Arg-Pro-Lys-Pro-Gln↓-Gln-↓Phe↓-Phe↓-Gly↓-Met-Leu-NH_2	222 ± 28	171 ± 10	0.8 ± 0.1
LHRH	pGlu-His-Trp↓-↓Ser-Tyr↓-Gly-Leu↓-Arg-Pro-Gly-NH_2	253 ± 10	44 ± 1	0.2 ± 0.01

monium sulphate in the loading buffer facilitated the binding of ACE to the lisinopril-Sepharose by an unknown mechanism, and after removal of the unbound protein, ACE was eluted by simply washing the column with ammonium sulphate-free buffer. The purified enzyme seemed to be a single protein with a molecular mass of 67 kDa on SDS/PAGE and this size estimate was in good agreement with the size of the single-domain protein (68 921 Da) predicted from sequence analysis of a cDNA for *D. melanogaster* ACE [30]. Subsequent analysis revealed that the affinity-purified housefly ACE was contaminated with what seemed to be a small amount of carboxypeptidase M (N)-like activity that cleaved lysine and arginine residues from the C-terminus of BK, Bz-Gly-Arg and Bz-Gly-Lys but not Bz-Gly-Phe, and was inhibited by the carboxypeptidase inhibitor MGTA. It was not possible to remove the carboxypeptidase contaminant from the affinity-purified enzyme by ion-exchange chromatography without serious loss of ACE activity (more than 90 %). Any interference in the assay of ACE activity towards peptide substrates was prevented by incubating housefly ACE with 10 μM MGTA before introducing the substrate.

The substrate specificity of mammalian ACE is complex, with the enzyme displaying endopeptidase activity in addition to the typical dipeptidyl carboxypeptidase activity towards substrates such as angiotensin I and BK [1,4]. Housefly ACE performed as a peptidyl dipeptidase and an endopeptidase by removing dipeptides from the C-terminus of angiotensin I, BK, [Leu^5]enkephalin and [Met^5]enkephalin, and dipeptideamides from [Leu^5]enkephalinamide and [Met^5]enkephalinamide. The much higher K_m values for [Leu^5]enkephalinamide and [Met^5]enkephalinamide compared with the corresponding peptides with a free C-terminus showed that the presence of a C-terminal amide group greatly reduced the ability of the substrate to bind at the active site.

Mammalian ACEs cleave both the dipeptideamide and tripeptideamide from SP, with the tripeptidyl endopeptidase pathway predominating to generate Phe-Phe as one of the most abundant peptide products [6–9]. The dipeptidyl endopeptidase pathway leads to the accumulation of SP$^{1-7}$ by rapid removal of Phe^8-Gly^9 from the initial SP metabolite (SP$^{1-9}$) [6–9]. Thus the ratio of Phe-Phe to SP$^{1-7}$ provides an indication of the relative importance of the dipeptidyl and tripeptidyl endopeptidase activities in the overall hydrolysis of SP. For housefly ACE, the Phe-Phe-to-SP$^{1-7}$ ratio was 0.44:1, indicating that the dipeptidyl endopeptidase and not the tripeptidyl endopeptidase cleavage was the dominant pathway. This value is markedly different from the Phe-Phe-to-SP$^{1-7}$ ratio of 4.0:1 for the N-domain and 1.9:1 for the C-domain of human somatic ACE [24].

Although LHRH is not efficiently hydrolysed by mammalian ACE, this substrate is of interest because of the atypical manner in which it is hydrolysed to release the N-terminal tripeptide as well as the C-terminal tripeptide by endopeptidic attack at the Trp^3-Ser^4 and Leu^7-Arg^8 peptide bonds, respectively [11,24,25,34]. The N-terminal tripeptidase activity of human sACE constitutes about 87 % of the overall hydrolytic activity towards LHRH in 0.3 M Cl^-. In contrast, human tACE (C-domain) hydrolyses LHRH at a much slower rate, with the C-terminal tripeptide endopeptidase activity predominating (more than 96 % of the total hydrolysis of LHRH) over the N-terminal tripeptide endopeptidase cleavage [25], suggesting that the N-domain active site is responsible mainly for the N-terminal tripeptidase pathway and that both the N- and C-domains contribute to the hydrolysis of the Leu^7-Arg^8 bond. This conclusion is supported by a study on the kinetics of LHRH hydrolysis by (i) mutant human sACE protein comprising only one domain (N- or C-domain), (ii) a full-length recombinant protein and (iii) full-length proteins that have the active site of one domain inactivated [24]. Furthermore a naturally occurring N-domain of human ACE has recently been isolated from ileal fluid that hydrolyses the release of the N-terminal tripeptide of LHRH at a much faster rate (6–31 times faster, depending on the Cl^- concentration) than that observed with human tACE [35]. Although housefly ACE is more like human tACE (C-domain) in that the C-terminal tripeptidase activity predominates the overall hydrolysis of LHRH at 300 mM NaCl, the insect enzyme also catalyses the N-terminal endopeptidic hydrolysis of LHRH at a significant level, a property associated mainly with the N-domain of human ACE.

The effect of NaCl on ACE activity depends upon the nature of the substrate and pH at which the experiment is conducted [24,36–39], and is believed to result from conformational changes to the protein resulting from the binding of Cl^- to a critical lysine that stabilizes the enzyme–substrate complex [39]. Optimal Bz-Gly-His-Leu hydrolysis occurs in 10 mM NaCl for the human N-domain and 800 mM NaCl for the C-domain of recombinant human sACE [19,36]. The two domains of human ACE also differ in that the activity of the C-domain, but not the activity of the N-domain, towards Bz-Gly-His-Leu is absolutely dependent on Cl^-. Housefly ACE has a high Cl^- optimum but the presence of Cl^- is not essential for activity and therefore the insect enzyme displays two properties that distinguish the two domains of human ACE.

The overall hydrolysis of LHRH by insect ACE and the predominant C-terminal tripeptidase pathway were activated 5.6-fold and 6.7-fold respectively when the NaCl concentration was increased from 0 to 500 mM, whereas the N-terminal tripeptidase activity was affected to a smaller extent, achieving a maximal increase of 3-fold at about 150 mM NaCl. This activation by NaCl is somewhat similar to the effect of Cl^- on the recombinant C-domain fragment of human sACE, which displays a high Cl^- optimum (400 mM) for the overall degradation of LHRH (mainly the result of C-terminal tripeptidase activity) and a low Cl^- optimum (30 mM) for the N-terminal tripeptidase activity [24]. The mutant full-length sACE ($K^{361,365}$), possessing only a catalytically active C-domain, displays only C-terminal tripeptidase activity, which is maximal at 500 mM NaCl. In contrast, the optimal NaCl concentration for overall LHRH hydrolysis by the wild-type human sACE, the mutant N-terminal fragment, the mutant full-length sACE ($K^{959,963}$), with only a catalytically active N-domain and the naturally occurring N-domain from human ileal fluid, is between 10 and 50 mM [24,35]. Therefore cleavage of the N-terminal and C-terminal tripeptides from LHRH are optimal at low and high Cl^- concentrations respectively, in both human and insect ACEs.

The analysis of the ACE gene from *D. melanogaster* has led to the proposal that the single-domain insect enzyme might resemble an ancestral from of the two-domain ACE found in mammals [30]. If this assumption is correct then the data presented in this study on housefly ACE provide information about the evolution of the functional properties of the two domains of human ACE. The fact that insect ACE displays some of the properties of both the N-domain and the C-domain of the human enzyme is consistent with the divergence of the two active sites after duplication of an ancestral gene with enzymic properties similar to those described for insect ACE.

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