Eur. J. Biochem. 237, 414–423 (1996)

Cloning and characterisation of angiotensin-converting enzyme from the dipteran species, *Haematobia irritans exigua*, and its expression in the maturing male reproductive system

Gene WIJFFELS, Clare FITZGERALD, Joanne GOUGH, George RIDING, Chris ELVIN, David KEMP and Peter WILLADSEN

CSIRO Division of Tropical Animal Production, Queensland, Australia

(Received 20 October 1995/28 December 1995) – EJB 95 1712/4

The angiotensin-converting enzymes (ACE) are involved in the regulation of the specific maturation or degradation of a number of mammalian bioactive peptides. A carboxydipeptidase similar to mammalian ACE has now been identified in the adult stage of the haematophagous fly, *Haematobia irritans exigua* (buffalo fly), a close relative of the horn fly of North America. The enzyme was purified by lectin-affinity chromatography and ion-exchange chromatography and migrated as a doublet of 70 kDa upon reducing SDS/PAGE. Unlike mammalian ACE, the fly carboxydipeptidase (HieACE) is not membrane bound. The amino acid sequence of an internal peptide from HieACE and a conserved amino acid region present in all mammalian ACE were used to design degenerate oligonucleotide primers suitable for PCR. A DNA fragment amplified from adult buffalo fly cDNA was used to identify a cDNA clone that encoded the enzyme. The cDNA sequence encodes a carboxydipeptidase with 41–42% amino acid identity to the mammalian testicular ACE. The active-site regions of mammalian ACE are conserved in the deduced amino acid sequence of HieACE. Enzymatically, HieACE is very similar to its mammalian counterparts, with comparable K_m and V_{max} values for the synthetic substrate, benzoylglycylglycylglycine, and similar patterns of inhibition by EDTA, ACE inhibitor peptide and captopril. HieACE also specifically activates angiotensin I to angiotensin II and degrades other mammalian ACE substrates such as bradykinin, substance P and cholecystokinin-8. In the adult fly, HieACE is expressed in the compound ganglion and in the posterior region of the midgut. Similar to the mammalian system, expression of this enzyme is induced in the maturing male reproductive system, which suggests conservation of ACE function in these species.

Keywords: angiotensin-converting enzyme; *Haematobia irritans*; Diptera neuropeptide; male reproductive system.

The angiotensin-converting enzymes (ACE) are ubiquitous in mammalian systems, where they fulfil a plethora of critical biochemical functions. As their name implies, ACE are involved in the regulation of blood pressure through the conversion of angiotensin I to angiotensin II. The endothelial form of these carboxydipeptidases is the target for a new class of antihypertensive drugs based on the specific ACE inhibitor, captopril [1, 2]. However, ACE also degrade many regulatory peptides in several different tissues including the lungs, kidneys, small intestine, chorioid plexus and lymphocytes (reviewed in [3]). Substrates for these enzymes include peptides such as bradykinin, neurotensin, encephalin, luliberin, substance P, substance K, gastrin and cholecystokinin [4–8]. Generally the tissue form of ACE is membrane bound although there is evidence for release of active ACE into plasma and seminal fluid [9–11]. Somatic ACE is a large enzyme of 180 kDa. It has two catalytic domains, both of which contain a bound Zn^{2+}. A second, smaller form of ACE, testicular ACE (110 kDa), occurs in the developing and mature testes. While this ACE is also membrane bound, it has only one catalytic site and is extensively modified with O-linked glycans in its unique N-terminal domain [12]. This alternative form of ACE arises by exon splicing of the mRNA transcript of the ACE gene but the function of testicular ACE in sperm and Leydig cells is unknown [13–15].

Studies of invertebrate physiology have revealed many novel regulatory peptides and peptides similar to mammalian neuropeptides [16–19]. However, little work has been conducted on the biochemical regulation of these neuropeptides and it is possible that an ACE-like enzyme may play a role. An enzyme activity similar to ACE has been reported in membrane preparations of crude extracts of the heads of the house fly (*Musca domestica*) [20]. The amino acid sequences of peptides derived from the cattle tick (*Boophilus microplus*) vaccine antigen, Bm91, were similar to those of mammalian ACE [21] and the predicted amino acid sequence of the Bm91 cDNA has 43% identity with human testicular ACE sequences [22]. Most recently, a *Drosophila* ACE-like protein has been cloned which also has 43% identity with the human testicular ACE sequence [23, 24]. The enzymatic characteristics of this protein, purified from *Drosophila* eggs, are similar to those of mammalian ACE [23]. The enzyme is expressed throughout embryogenesis, and lethal gene mutants die in larval or pupal stages [24].

Correspondence to G. Wijffels, CSIRO Division of Tropical Animal Production, Private Bag 3, PO Indooroopilly, Queensland, Australia 4068

Fax: +61 07 3214 2882.

Abbreviations. ACE, angiotensin-converting enzyme; HieACE, *Haematobia irritans exigua* ACE.

Enzyme. Angiotensin I-converting enzyme (EC 3.4.15.1).

Note. The novel nucleotide sequence data reported here have been submitted to the Genbank sequence data bank and are available under accession number L43965.

We now describe the isolation, characterisation, tissue expression and sequence of an ACE-related enzyme (HieACE) expressed by the adult stage of *Haematobia irritans exigua*. This dipteran species, known as the buffalo fly in Australia, is closely related to the horn fly (*H. irritans irritans*) of the North American continent. These species are pests of cattle herds on both continents and cause economic losses of $900 million per annum.

MATERIALS AND METHODS

Materials. Newly emerged adult flies were collected and frozen at −70 °C. Unless otherwise stated, all reagents were analytical grade. Chromatographic supports and equipment were supplied by Pharmacia.

Enzymatic assay. The carboxydipeptidase activity of the various preparations was assessed by the hydrolysis of the synthetic substrate, benzoylglycylglycylglycine [25]. The reaction was monitored by release of [^{3}H]benzoylglycine from a trace addition of [^{3}H]benzoylglycylglycylglycine (Amersham). The assay was modified and conducted in microfuge tubes [26].

Purification of HieACE from adult buffalo fly. Frozen flies (100−120 g, 40000−50000 flies) were washed sequentially in 70% (by vol.) ethanol (−20 °C), distilled water (4 °C) and 100 mM Hepes, pH 7.2. The flies were deposited in 1200 ml 100 mM Hepes, pH 7.2, 2 μM leupeptin, 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride and homogenised for 3 min. The resulting suspension was passed through a nylon mesh to remove cuticular material and the filtrate clarified by centrifugation (900×*g*, 20 min, 4 °C). The supernatant was decanted and the pellets extracted with 250 ml 100 mM Hepes, pH 7.2, and clarified. The supernatants of both extractions were pooled (1500 ml), filtered through tissue paper, and subjected to high-speed centrifugation (160000×*g*, 30 min, 4 °C). The resulting supernatant was adjusted to 0.1% Brij 35, 2 mM $MnCl_2$ and 2 mM $CaCl_2$. The solution was passed through a 20-ml concanavalin-A−Sepharose column. The column was washed with 100 mM Hepes, pH 7.2, 0.1% Brij 35, 2 mM $MnCl_2$, 2 mM $CaCl_2$, 2 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride (buffer A) and proteins were eluted with 0.5 M α-methyl mannopyranoside in buffer A (1 ml/min). The eluted material was applied to a 20-ml DEAE-Sepharose column. This column was extensively washed with 50 mM Tris/HCl, pH 7.5, 0.1% Brij 35, 2 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride (buffer B) and with buffer B containing 0.1 M NaCl. The enzyme was eluted with 100 ml buffer B containing 0.2 M NaCl. The fractions that contained enzyme activity were pooled, concentrated and buffer exchanged into 50 mM Tris/HCl, pH 8.5, 0.1% Brij 35 (buffer C). The concentrate was loaded onto a Mono Q HR 5/5 column (1 ml), equilibrated with buffer C, and the column was developed with a discontinuous gradient from 0 to 0.2 M NaCl in buffer A over 20 min and from 0.2 M to 0.5 M NaCl in buffer A over 60 min. Elution of proteins was monitored at 280 nm. Fractions (0.5 ml) were assayed for enzyme activity and analysed by SDS/PAGE. Fractions that contained enzyme activity were pooled, concentrated and buffer exchanged into 50 mM Tris/HCl, pH 7.0. The concentrate was applied to an AX300 anion-exchange cartridge (Applied Biosystems Inc.) equilibrated in the same buffer. Proteins were eluted with a continuous gradient from 0 to 0.5 M NaI in 50 mM Tris/HCl, pH 7, over 100 min and their elution was monitored at 280 nm. Fractions (0.5 ml) were collected and assayed as previously described. The protein content of the various pools of fractions was determined by the Pierce Protein Assay (Pierce) with BSA as a standard.

Amino acid sequencing. The N-terminal sequence of HieACE was determined by sequencing of the reduced and alkylated purified protein (20 μg). The protein was applied in 100% trifluoroacetic acid to the filter of a Model 471A sequencer (Applied Biosystems Inc.). HieACE (50 μg) was carboxyamidomethylated in 8 M urea and digested with endoproteinase Lys-C (Boehringer Mannheim) [27]. Peptides were purified by reverse-phase chromatography and sequenced [28].

PCR. All oligonucleotide primers were synthesised by means of an Oligo 100 DNA Synthesiser (Beckman Instruments) and PCR were conducted in an Omnigene Temperature Cycler (Hybaid). The DNA probe used to screen the cDNA library was produced by PCR with degenerate sense and antisense oligonucleotide primers designed from amino acid sequences. Their DNA sequences were 5′-AA($^{T}_{C}$)TT($^{T}_{C}$)GA($^{A}_{G}$)AA($^{A}_{G}$)TA($^{T}_{C}$)-GTNGA($^{A}_{G}$)($^{C}_{T}$)NAA($^{T}_{C}$)AC-3′ and 5′-TGGCCCACAT($^{A}_{G}$)TTNC-CNA($^{A}_{G}$)NA($^{A}_{G}$)($^{A}_{G}$)TG-3′, respectively. PCR were performed with 10 ng adult buffalo fly cDNA [29] in 100 μl PCR buffer (Promega) containing 4 mM $MgCl_2$, 0.5 mM each dNTP, 2 μg of each of the oligonucleotide primers and 2.5 U Amplitaq *Taq* polymerase (Cetus). The PCR proceeded for 40 cycles: 94 °C, 2.5 min; 60 °C, 2.5 min; 72 °C, 2.5 min. A dominant species of approximately 250 bp was detected by agarose-gel electrophoresis. The fragment was purified with the Gene Clean II kit (BIO 101 Inc.) according to the manufacturer's instructions and ligated into the pGEM-T vector (Promega) by means of T4 DNA ligase (Boehringer). Processing of recombinant clones was performed as described [29].

After confirmation that the predicted amino acid sequence was similar to that of mammalian ACE, the 250-bp fragment was labelled by incorporation of digoxigenin-11-dUTP (Boehringer) by means of PCR following the manufacturer's recommendations. The labelling PCR was performed with 10 ng purified DNA fragment and the above oligonucleotide primers for 40 cycles: 94 °C, 2.5 min; 55 °C, 2.5 min; 72 °C, 2.5 min).

Library screening. Adult buffalo fly cDNA [28] was cloned into λgt11 by means of the Amersham λgt11 cloning system. The cDNA library contained 500000 independent clones and was amplified to 10^9 plaque-forming units. Aliquots (5×10^5 plaque-forming units) of the λgt11 cDNA library were plated out and a nylon-filter (Amersham) lift was prepared from each plate. The filters were screened with the digoxigenin-labelled probe as described [30]. Randomly selected positive plaques were rescreened until individual clones were purified. The λgt11 plasmid DNA of six clones was purified by means of the LambdaSorb Phage Adsorbent (Promega) according to the manufacturer's instructions.

DNA sequencing. The inserts from the three positive λgt11 clones were obtained by means of PCR with primers that flank the cloning site. The sense and antisense primers were 5′-GGT-GGCGACGACTCCTGGAGCCCGTC-3′ and 5′-TTTGACAC-CAGACCAACTGGTAATGG-3′, respectively. The reaction conditions, isolation of PCR products and DNA sequencing were as described above. The largest insert (2.6 kb) was obtained from clone H8. DNA sequencing of the H8 λgt11 insert was performed on both strands. As sequence became available, sequential primers were synthesised to allow the entire insert to be sequenced in both directions.

Enzyme characterisation. To decrease the amount of enzyme required for enzymatic characterisation, the sensitivity of the assay was enhanced by increasing the specific activity of the substrate to 600 μCi/mmol and by reducing the substrate concentration to 6.4 mM. To determine the pH optimum, the substrate was prepared in 200 mM Hepes or 200 mM acetic acid at the indicated pH. The enzyme (10 ng) was incubated in the Hepes and acetic acid buffers for 1 h. Control hydrolysis tubes

(no enzyme) were prepared at each pH in each buffer. For the inhibition titrations, the enzyme (80 ng) was incubated (10 min, room temperature) with a range of concentrations of each of the inhibitors: ACE inhibitor peptide (Sigma), captopril (Sigma) and EDTA. After addition of substrate, the reaction proceeded for 1 h at 37 °C.

Digestion of ACE substrates. Reactions (20 µl) contained 25 ng rabbit lung ACE (Sigma) or 20 ng HieACE. Digestion of three known ACE substrates (200 µM), angiotensin I (Sigma), substance P and sulfated cholecystokinin-8, was in performed in 0.6 M Na_2SO_4, 100 mM Hepes, pH 7.5. The degradation of bradykinin is reported to be more efficient in conditions of lower ionic strength [31] and therefore these reactions were performed in 100 mM NaCl, 100 mM Hepes, pH 7.5. The purity of oligopeptides (Auspep) was verified by reverse-phase HPLC. Digests were performed at 37 °C and stopped by storage at −20 °C. Control tubes that contained 2 nmol captopril were analysed in parallel. The 20-µl reaction mixture was injected onto a C_8 reverse-phase cartridge (Brownlee Aquapore RP-300 100 mm cartridge; Applied Biosystems Inc.) equilibrated in 0.1% trifluoroacetic acid. Peptides were eluted with a linear gradient from 0 to 80% acetonitrile (in 0.1% trifluoroacetic acid) over 10 min. Elution of peptides was monitored at 214 nm. Eluted peptides from the angiotensin I and bradykinin digests were collected, dried by vacuum centrifugation and sequenced.

SDS/PAGE analyses and immunoblots. SDS/PAGE gels were silver stained [32]. For the deglycosylation experiment, 150 ng HieACE was treated with 20 mU N-glycosidase F (Boehringer) as described [21]. Immunoblots were performed as described [33] with the following changes: the nitrocellulose was incubated for 1 h (37 °C) with ovine anti-HieACE serum (1:2000) then with the secondary antibody conjugated to horseradish peroxidase (1:1000) for 1 h (37 °C). Blots were developed with 4-chloro-naphthol or (where indicated) by means of a more-sensitive mixed-chromogen-substrate system [34].

Analysis of individual organs was performed on *unfed* flies (those which had emerged overnight from pupal cases and allowed access to water) and fed flies (those which had access to bovine blood containing 0.2 mg/ml gentamycin and 10 U/ml heparin for varying times. Insect tissues were dissected from adult buffalo flies (mixed sex), placed in chilled 100 mM Hepes, pH 7.2, 2 µM leupeptin, 1 µM pepstatin, 1 mM phenylmethylsulfonyl fluoride and stored at −70 °C. On thawing, the buffer was removed and replaced with 100 µl reducing-SDS/PAGE sample buffer. Due to their smaller mass, the salivary glands and compound ganglia were suspended in 50 µl sample buffer. The suspensions were heated to 100 °C for 10 min, homogenised, treated at 100 °C for a further 10 min and loaded onto the SDS/polyacrylamide gels. Some midguts, after dissection, were cut into three parts: the cardia, anterior midgut and posterior midgut. The gut sub-portions were homogenised and treated as described above. The results of the immunoblots represent the total immunoreactive protein content of the specific organ.

The ovine antiserum to HieACE was generated by immunisation of sheep with 30 µg purified HieACE with two injections, 4 w apart, in Freund's complete adjuvant and Freund's incomplete adjuvant (Sigma), respectively. The 'Australian code of practice for the care and use of animals for scientific purposes' was followed during the generation of HieACE ovine antiserum.

RESULTS

Purification of HieACE from adult buffalo fly. The purification of an ACE-related enzyme from adult buffalo fly extracts was followed by means of the benzoylglycylglycylglycine assay which is used to detect and measure the activity of this class of carboxydipeptidase [25]. By means of this assay, it was found that, unlike the related mammalian ACE, the buffalo fly activity was almost entirely soluble (94.5%) in the absence of detergents, and the remaining activity was detected in the membrane fraction of whole fly homogenates. The soluble activity bound to concanavalin-A−Sepharose and further purification was performed upon a series of anion-exchange resins: DEAE-Sepharose, a MonoQ FPLC column and an AX300 HPLC cartridge. The active fractions that eluted from the MonoQ column were purified 2300-fold from the low-speed supernatant and had a specific activity of 2 µmol · min^{-1} · mg^{-1}. The enzyme was further purified on an AX300 cartridge, and the resulting preparation had a specific activity of approximately 14 µmol · min^{-1} · mg^{-1} and was purified 20000-fold from the low-speed supernatant. Typically, 150−300 µg purified HieACE was isolated from 100−120 g of frozen whole flies.

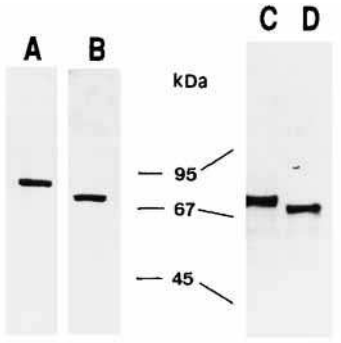

Fig. 1. SDS/PAGE analysis of purified HieACE. Silver-stained gels of 150 ng HieACE. (a), reduced; (b), non-reduced; (c), incubated at 37 °C without N-glycosidase F; and (d), incubated at 37 °C with 20 mU N-glycosidase F.

Upon non-reducing SDS/PAGE, the purified protein migrated as a doublet of 67 kDa. The two components appeared to differ by 1−2 kDa but were approximately equimolar according to their staining intensites (Fig. 1). Under reducing conditions the doublet migrated more slowly, at approximately 70 kDa, which suggests the presence of internal disulfide bonding (Fig. 1). When the reduced, denatured HieACE was digested with N-glycosidase F, the enzyme was reduced in size by 2.5−3 kDa (Fig. 1). The change in molecular mass was unlikely to be due to proteolytic digestion since HieACE incubated without N-glycosidase F failed to change in mobility (Fig. 1). To provide further evidence that there was no membrane form of HieACE, a lysate of whole adult buffalo fly was prepared in a buffer containing Triton X-114. The integral membrane proteins were separated by a method that exploited the detergent-phase-separation properties of Triton X-114 [35]. Immunoblot analysis did not detect any material reactive with the ovine antiserum to HieACE (data not shown). The small amount of activity detected in the membrane pellet in the usual extraction procedure was probably trapped in the bulk precipitate.

Enzymic analyses of HieACE. A bell-shaped pH/activity curve was obtained in Hepes, with maximum activity at pH 7.5 (Fig. 2). This established suitable pH and buffer conditions for the study of the catalytic activity of the enzyme. The enzyme behaved differently in acetic acid, and a pH optimum could not be determined in the acetic acid buffer over the pH range tested (pH 3−6.5). Under optimal conditions (200 mM Hepes, pH 7.5), the average K_m of HieACE for benzoylglycylglycylglycine was 5 mM and the average V_{max} was 42 µmol · min^{-1} · mg^{-1} enzyme (calculated from two determinations of Michaelis-Menten plots;

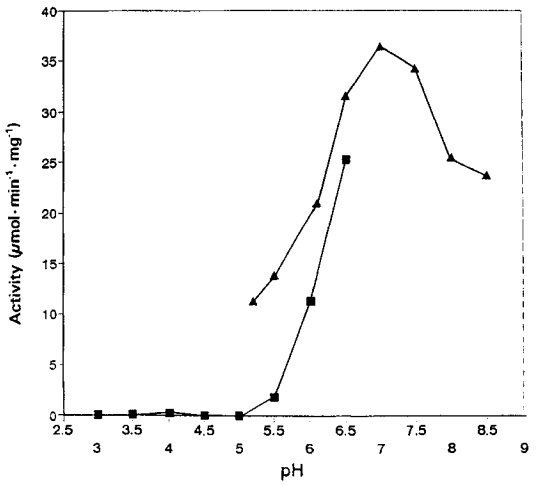

Fig. 2. pH/activity curves of HieACE. The activity of HieACE (10 ng) was determined by means of the benzoylglycylglycylglycine assay in 200 mM acetic acid (■) and 200 mM Hepes (▲) buffers. HieACE (10 ng) was incubated for 60 min at 37°C.

Table 1. Inhibition of HieACE by ACE inhibitors. HieACE (80 ng) was incubated for 10 min with EDTA, ACE inhibitor peptide (Glp-Trp-Arg-Pro-Gln-Ile-Pro-Pro) or captopril. HieACE activity was assessed by means of the benzoylglycylglycylglycine assay (see Materials and Methods). Values represent the results of two independent experiments.

Inhibitor	Concentration	Inhibition
	nM	%
EDTA	50	99, 97
ACE inhibitor peptide	1	97, 95
Captopril	0.05	90, 87

data not shown). High concentrations of Na_2SO_4 were required for optimal activity against this substrate. High-ionic-strength solutions, in the presence of low levels of NaCl, tend to promote high activities of ACE [36]. The effects of standard inhibitors of mammalian ACE were also tested on HieACE in two independent series of titrations over a wide range of concentrations (1 mM to 1 pM) of each of the inhibitors. The inhibition assays revealed that the catalytic activity of HieACE was sensitive to EDTA, ACE inhibitor peptide (Glp-Trp-Arg- Pro-Gln-Ile-Pro-Pro) and captopril (Table 1). The activity of 80 ng (approximately 1 pmol HieACE) was ablated by 50 nM EDTA, 1 nM ACE inhibitor peptide, and 50 pM captopril. At these concentrations, the inhibitor/enzyme molar ratios are 1000:1, 20:1 and 1:1, respectively, which demonstrates the efficiency of captopril as an inhibitor of this enzyme.

N-terminal and peptide sequences. Direct N-terminal sequencing of purified HieACE revealed two proteins in approximately equimolar amounts. The proteins are related to each other; one sequence lacks the initial three residues of the other sequence (Fig. 4). These sequences did not exhibit similarity to the N-terminal sequences of the putative mammalian ACE counterparts. Two peptides derived from an endoproteinase Lys-C digest of purified HieACE were also sequenced (Fig. 4). The sequence of peptide Hie6 could be aligned to a region approximately 200 residues from the N-termini of mature mammalian testicular ACE polypeptides (Fig. 4).

Cloning of HieACE. A sense oligonucleotide primer was designed from the peptide Hie6 sequence, and a site highly conserved in all ACE proteins was used to design an antisense primer (Figs 3 and 4). In the testicular ACE polypeptides, this site is 75 residues (225 bases) downstream of the region that exhibits homology to peptide Hie6. PCR with these oligonucleotide primers and adult buffalo fly cDNA generated a single species of 242 bp. The DNA sequence, the predicted amino acid sequence and the similarity of HieACE to other ACE molecules are shown in Figs 3 and 4. This region of the predicted HieACE polypeptide has similarity to testicular ACE with 45–47% amino acid identity.

The primary screen of an adult buffalo fly λgt11 cDNA library with the DNA fragment detected 150 positive plaques (0.03% of the library), consistent with the abundant expression of this enzyme in adult flies. The cDNA inserts of three clones were obtained by PCR with primers that flanked the λgt11 cloning sites. The insert sizes were 1.6, 1.8 and 2.6 kbp. The largest insert (from clone H8) was sequenced on both strands, and the smaller inserts were shown to be truncated forms of the H8 insert (data not shown).

HieACE is similar to mammalian testicular ACE. The H8 λgt11 cDNA clone contained a 2664-bp insert which consisted of a 70-bp 5′ untranslated region, an ORF of 1833 bp and a 761-bp 3′ untranslated region. There was a polyadenylation signal (AATAAA) 107 bp from the stop codon but no polyadenylated tail (Fig. 3). The coding region consists of 611 amino acids and the molecular mass of the predicted protein is 70.5 kDa.

Direct N-terminal sequencing of purified HieACE yielded two related N-termini (Fig. 4). The N-terminus of mature HieACE is taken as the N-terminus of the longer sequence (Seq. 1; Fig. 4), and thus defines a 17-residue signal sequence which does not appear to be similar to the leader sequences of human ACE or ACE-related enzymes of the cattle tick (Bm91) and *Drosophila* (Fig. 4). The calculated molecular mass of the mature unmodified protein (69 kDa) agrees with the apparent mobility of the deglycosylated protein upon reducing SDS/PAGE. The N-terminal sequences (residues 1–25), the sequence of peptide Hie6 (residues 162–173) and the sequence of peptide Hie8 (residues 58–67) can be aligned with the predicted HieACE polypeptide sequence (Fig. 4).

Overall, the protein sequence has approximately 41% amino acid identity with mammalian testicular ACE (Fig. 4). The BLAST algorithm [37] gave a very low Expect value of 7.1×10^{-182} for a comparison of the whole mature HieACE protein sequence with human testicular ACE which indicates highly significant similarity. The BLAST program also compared the N-terminal, central and C-terminal segments of HieACE with human testicular ACE (data not shown). There is little conservation near the N-terminal region (residues 4–116) with amino acid identity and positive amino acid conservation of only 22% and 48%, respectively. Over the central portion of the HieACE sequence (amino acids 126–514), similarity to ACE was very high. When compared with human testicular ACE, there was 50% amino acid identity and 68% positive amino acid conservation. Similarity decreases slightly toward the C-terminal region (residues 519–576) with amino acid identity and positive amino acid conservation of 46% and 63%, respectively.

Consistent with the limited conservation near the N-terminus, the regions that flank the first cysteine pair (Cys116 and Cys124) are not similar to the human testicular ACE, although the cysteines themselves are conserved. The only conserved region in the N-terminal region is a potential N-linked glycosylation site, although it is unknown whether this site is glycosylated in any species. There are only two other potential N-linked glycosylation sites. In the latter part of the molecule, the regions with the highest similarity are near the remaining conserved cysteines (Cys319, Cys337, Cys505 and Cys523). Cys450 is dis-

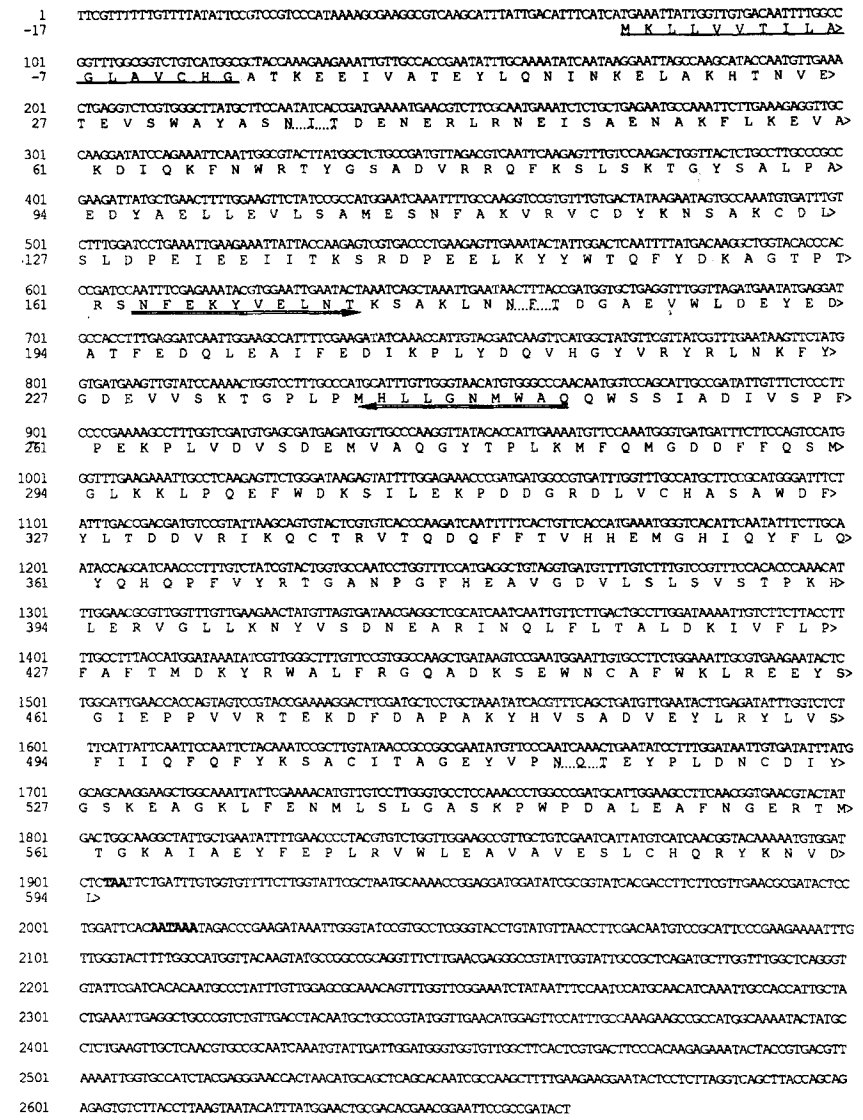

Fig. 3. The DNA sequence of the H8 λgt11 clone and the predicted amino acid sequence of the ORF. The leader sequence is underlined and potential N-linked glycosylation sites are indicated by the dotted line. The stop codon and polyadenylation site are in bold. The PCR-oligonucleotide-primer sites used to generate the 242-bp cDNA-library screening probe are indicated by the arrows.

placed in HieACE compared of location of the analogous cysteine in the mammalian sequences (Cys496; Fig. 4). Furthermore, a cysteine is located near the C-terminus (Cys585) which gives HieACE a total of eight cysteines. The mature mammalian testicular ACE have seven cysteine residues.

Consistent with the enzymatic similarity of HieACE with mammalian ACE, the amino acids which have been identified as important catalytic residues are conserved. The ACE Zn^{2+}-binding-site motif (His-His-Glu-Met-Gly-His) is intact and the third Zn^{2+}-coordinating residue, a nearby glutamic acid [38], is also conserved (Glu358). A tyrosine suggested to be involved in enzymatic stability in mammalian ACE [39, 40] is also conserved in HieACE (Tyr167).

The HieACE amino acid sequence has 59.3% identity with the recently published sequence of the *Drosophila* ACE-like enzyme [23, 24]. Two of the three glycosylation sites are also conserved. Both polypeptides have a cysteine residue close to the C-terminus, although these regions show low similarity (Fig. 4). The cattle tick (*B. microplus*) ACE-like enzyme, Bm91, is a slightly larger protein (86 kDa) and retains only 35% identity with the two dipteran enzymes.

Digestion of mammalian ACE substrates. The ability of HieACE to process known ACE substrates was examined. Rabbit lung ACE (170 fmol, 0.0027 U) and HieACE (230 fmol) were incubated with angiotensin I and bradykinin. Digestion products were resolved by reverse-phase HPLC, collected and their identity confirmed by amino acid sequencing. All reactions were inhibited by addition of 2 nmol captopril. Fig. 5A represents the reverse-phase HPLC chromatograms of the 90-min digests of angiotensin I by rabbit lung ACE and HieACE. Mammalian ACE released a single resolvable product, namely angiotensin II [41]. Similarly, HieACE converted angiotensin I to angiotensin II (as determined by amino acid sequencing). There were no further digestion products even after incubation for several hours. Digestion of bradykinin by rabbit lung ACE released a single product, the anticipated seven-amino-acid peptide of degraded bradykinin (Fig. 5B; [42]). HieACE released a product that eluted at the same position on reverse-phase HPLC (Fig. 5B) and this product was identified by amino acid sequencing as the seven-residue peptide of degraded bradykinin.

Digestions were also performed on two other ACE substrates: substance P and cholecystokinin-8. Proteins similar to

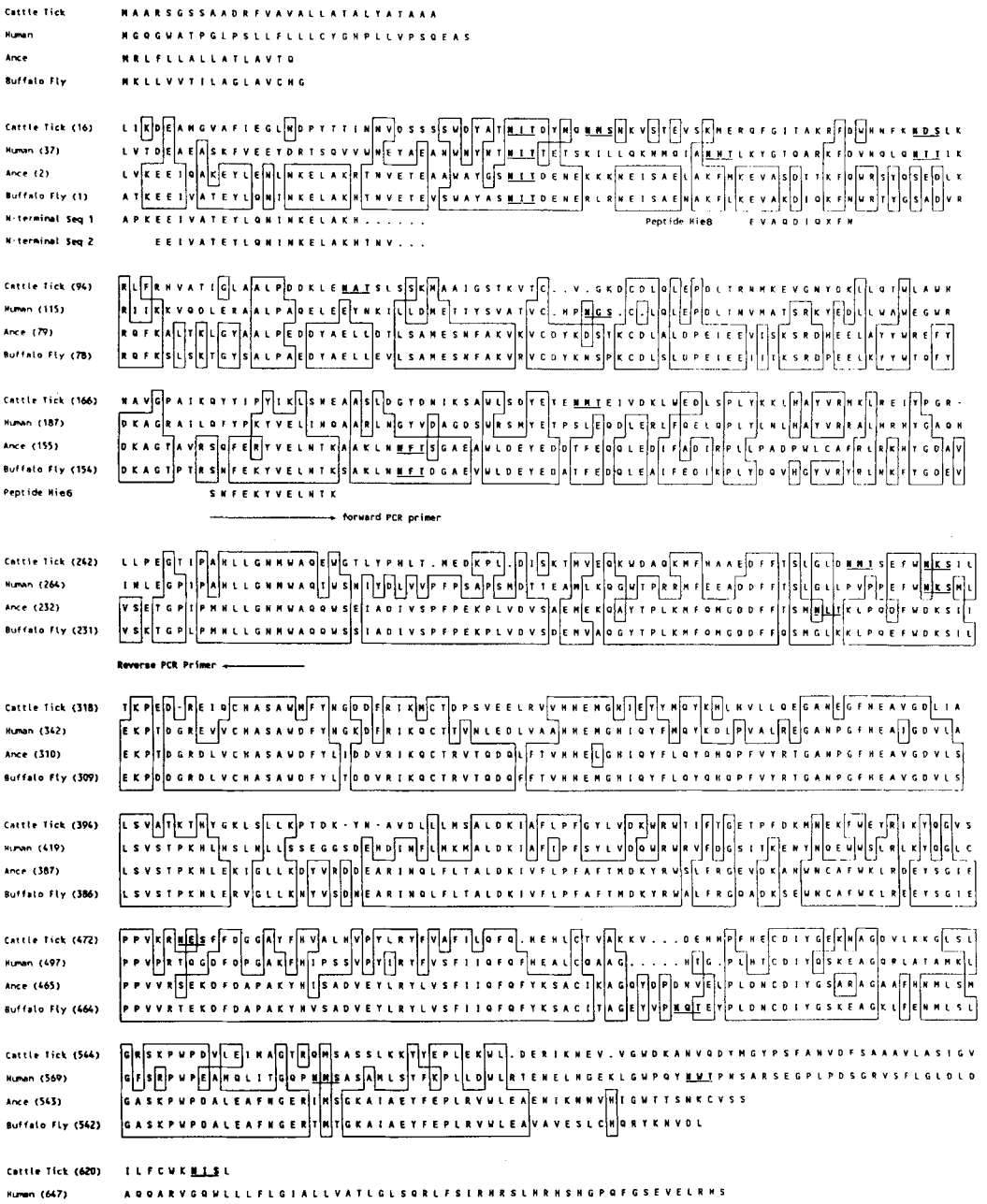

Fig. 4. Alignment of the predicted HieACE amino acid sequence with the sequences of the cattle tick ACE-related protein, Bm91 [22], human testicular ACE (S05238 [14, 15]) and the *Drosophila* ACE-related protein, AnCE (U25344 [23, 24]). Potential N-linked glycosylation sites are in bold and underlined in all sequences. Numbers in brackets indicate the position of the first residue of each sequence. The leader sequences of each protein are shown at the top. The sequences obtained by direct amino acid sequencing of the N-terminus and purified internal peptides of HieACE are also included in the alignment.

these two mammalian neuropeptides are found in insects, namely the leucokinin and allostatin peptide families [16–19]. Substance P and cholecystokinin-8 are amidated, and sulfated neuropeptides and their digestion by different ACE is complex and results in numerous products and variable kinetics [5, 8, 43–45]. HieACE digested both these substrates. The chromatograms of the digests of each substrate by the rabbit lung ACE and HieACE revealed products of similar mobility upon reverse-phase HPLC (Fig. 5C and D). The reaction rates of HieACE against substance P and cholecystokinin-8 were different to those of rabbit lung ACE.

Location of HieACE in adult buffalo fly. The major tissue sources of HieACE was determined by means of an immunoblot of crude SDS-solubilised homogenates of tissues dissected from newly emerged unfed adult buffalo fly. When the immunoblots were probed with ovine antiserum specific for HieACE, the antigen was detected in only one tissue, namely the midgut (Fig. 6A). There was no reactivity detected in other parts of the digestive system (the salivary glands and hindgut), the reproductive organs, the Malpighian tubules or the brain. The midgut was more closely examined by dissection of each midgut into three parts: the cardia, anterior midgut and posterior midgut. These tissues were probed for HieACE, and the posterior midgut contained most of the enzyme with a small amount detected in the anterior midgut (Fig. 6B), although the reactivity in the anterior midgut was variable in other experiments. When a more sensitive peroxidase-substrate system [34] was used, HieACE

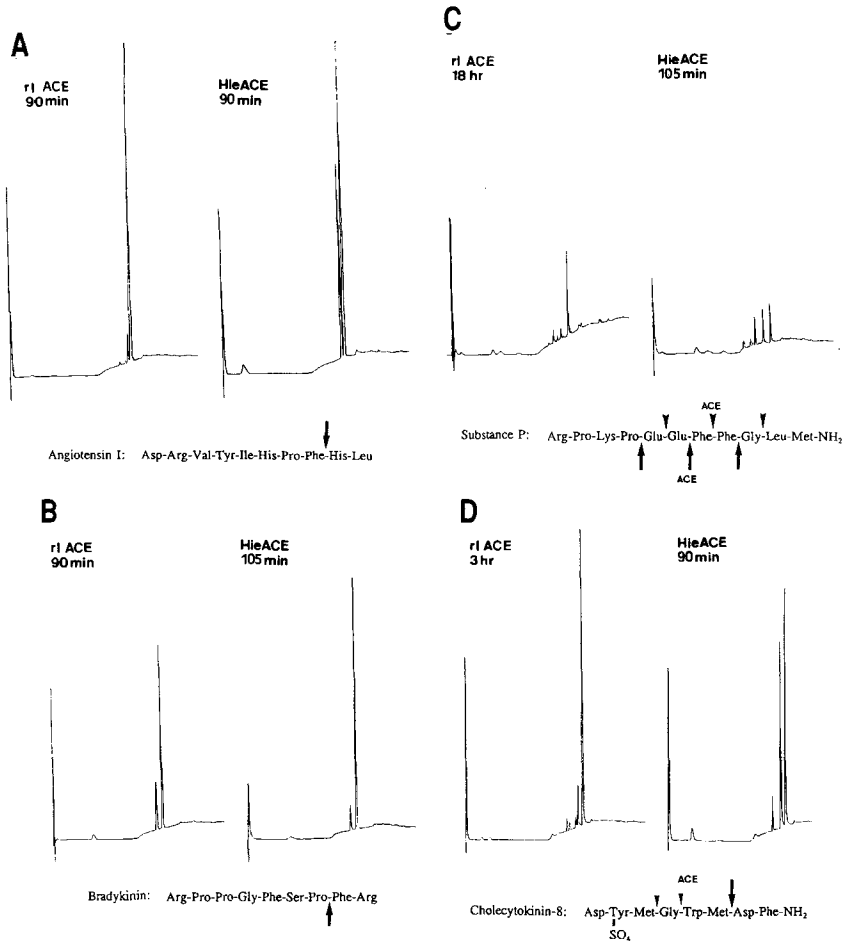

Fig. 5. Reverse-phase HPLC chromatogram of the digestion of ACE substrates by rabbit lung ACE (rl ACE) and purified HieACE. The substrates (4 nmol), angiotensin I (A), bradykinin (B), substance P (C) and cholecystokinin-8 (D) were treated with 3 mU rabbit lung ACE (rl ACE) and 20 ng HieACE for the indicated times. (Reaction conditions are described in Materials and Methods). Reaction components were resolved by reverse-phase HPLC, and in the case of the angiotensin I and bradykinin digests, the peptide products were collected and amino acid sequenced. The common HieACE and ACE cleavage site on angiotensin I and bradykinin are given below the relevant set of chromatograms. The ACE cleavage sites on substance P and cholecystokinin-8 are also presented with the relevant set of chromatograms.

was also detected in the compound ganglia (Fig. 6B) while other tissues tested negative for HieACE expression. Significantly, the only protein species detected by the anti-HieACE serum was the 70-kDa protein, which suggests that, unlike in mammals, there is no larger form of HieACE.

We investigated whether the expression of HieACE was more widespread or increased in fed, and thus metabolically active, flies. Tissues were collected 1 h and 26 h after feeding. At the first time-point, expression of HieACE was restricted to the midgut (data not shown). However, tissues collected 26 h after feeding showed strong expression of HieACE in the posterior midgut and the testes and/or associated tubules but not in the ovaries or other tissues analysed (Fig. 7A). A time-course experiment was performed to determine when HieACE expression was induced in the testes. The testes of flies fed for 1.5 h did not express HieACE. After 2 h feeding, expression was detected and persisted for at least 26 h (Fig. 7B).

DISCUSSION

An enzyme with ACE-like carboxydipeptidase activity and ACE-related sequence has been isolated from whole adult extracts of the haematophagous fly, *H. irritans exigua*. The buffalo fly carboxydipeptidase, termed HieACE, is a soluble enzyme that consists of two species of approximately 70 kDa. Direct N-terminal sequencing of the purified HieACE revealed two similar proteins in near equivalent quantities. The second sequence lacked the three initial residues of the first sequence. While the N-terminal sequences were not similar to mammalian ACE, the sequence of an internal peptide isolated from a digest of HieACE was aligned to the mammalian testicular ACE sequences. A cDNA clone of 2.6 kbp was isolated from an adult buffalo fly cDNA library. The ORF of 1833 bp encodes a mature protein of 69 kDa with overall amino acid identity to mammalian testicular ACE of 41–42%. Similarity is highest around the cysteines and the residues involved in the catalytic site.

The mammalian testicular ACE are mature proteins of 704–710 amino acids, whereas HieACE has a mature polypeptide of only 594 residues. The fly enzyme lacks the heavily glycosylated 36–42 amino acid N-terminal extension and the transmembrane and cytoplasmic regions that are present in mammalian testicular ACE. However, the absolute conservation of the three pairs of cysteines and the catalytic site suggests that the domain structure of mammalian ACE will be maintained in HieACE. One of the potential N-linked glycosylation sites, very close to the N-terminus, is also conserved. The other two potential N-linked glycosylation sites are not located in comparable positions in the mammalian counterparts. Human testicular ACE

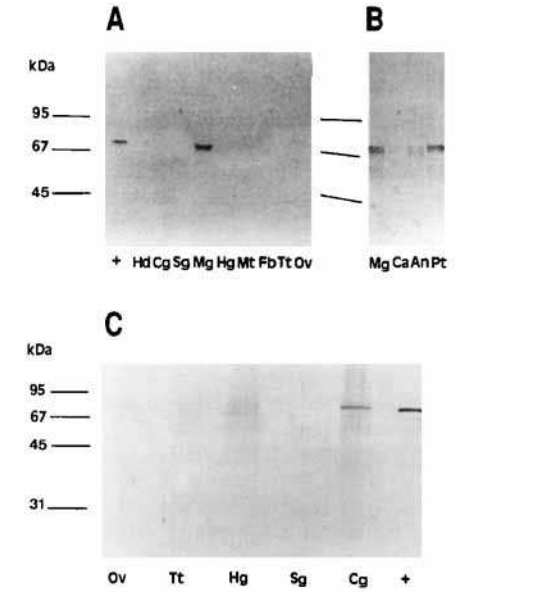

Fig. 6. Immunoblot analysis of the expression of HieACE in various tissues dissected from newly emerged unfed adult flies. (A) SDS homogenates of compound ganglia (Cg) and salivary glands (Sg) from ten flies and entire head (Hd), midgut (Mg), hindgut (Hg), Malphigian tubules (Mt), the fat body (Fb), testes (Tt) and ovaries (Ov) from two flies were resolved in 15% reducing SDS/PAGE. (+), 500 ng purified HieACE. (B) SDS homogenates containing 10 tissue equivalents each of whole midgut (Mg), cardia (Ca), anterior midgut (An), and posterior midguts (Pt). (C) SDS homogenates of ovaries (Ov), testes (Tt), hindgut (Hg), salivary glands (Sg) and compound ganglia (Cg) from two flies were probed with the antiserum. The blots were probed with ovine antiserum specific for HieACE and binding of the secondary antibody conjugated to horseradish peroxidase was visualised by means of 4-chloronaphthol except for Panel (C) where, to increase sensitivity, a mixed-chromogen system was used [34].

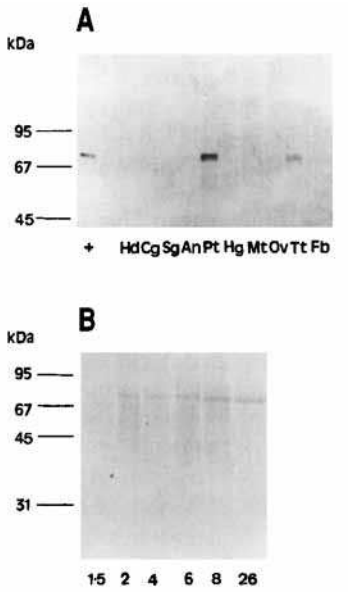

Fig. 7. Immunoblot analysis of the expression of HieACE in various tissues dissected from newly emerged fed adult flies. The immunoblots were performed as described for Fig. 6. The immunoreactivity of the antiserum was detected with 4-chloro-naphthol and the mixed chromogen system [34] for (A) and (B), respectively. (A), SDS homogenates were prepared from tissues collected from flies 26 h after feeding. Homogenates of compound ganglia (Cg) and salivary glands (Sg) from ten flies, and of entire head (Hd), anterior midgut (An), posterior midgut (Pt), hindgut (Hg), Malphigian tubules (Mt), ovaries (Ov), testes (Tt) and the fat body (Fb) from two flies were resolved in 15% reducing SDS/PAGE. (+), 500 ng purified HieACE. (B) SDS homogenates were prepared from testes collected from flies fed for times indicated. The homogenate of one tissue was loaded in each lane except for the 1.5-h time point where the homogenate of two tissues were loaded.

is extensively modified with O-linked glycans [12] and N-linked glycans (17 kDa) [14].

Enzymatic analyses of HieACE revealed optimal activity against benzoylglycylglycylglycine at approximately pH 7, which, like that of mammalian ACE, was dependent on high concentrations of anions for maximal activity. The K_m value compares well with the apparent K_m of 5 mM obtained for human ACE [25]. HieACE was also inhibited by EDTA, an ACE inhibitor peptide and captopril, standard inhibitors of ACE [31, 46]. The potential of HieACE to cleave ACE substrates was assessed by incubation of angiotensin I and bradykinin with this enzyme. HieACE exhibited rapid and specific cleavage of a single C-terminal dipeptide from angiotensin I, analogous to the rabbit lung ACE. HieACE also specifically degraded bradykinin to its seven-residue product. Mammalian ACE can also digest sulfated and amidated peptides. Preliminary results indicated that HieACE also can digest amidated and sulfated neuropeptides, such as substance P and cholecystokinin. These peptides have been identified as ACE substrates [5, 8]. While a detailed analysis of the digestion of mammalian ACE substrates would increase the understanding of the specificity of HieACE, identification of its substrates would be relevant to an understanding of the biological function of this enzyme in insects.

Candidate substrates might be those peptides with sequence similarity to mammalian ACE substrates. The allostatins, an important group of insect neuropeptides, have a C-terminal tripeptide sequence (Phe-Gly-Leu) that is also found in substance P [19]. A second group of insect neuropeptides, the sulfakinins, has amino acid sequence similarity to the mammalian neuropeptides, cholecystokinin and gastrin. The similarity includes their sulfated tyrosine and amidated C-terminus [16–18] and, like their mammalian counterparts, these peptides affect gut motility. By means of immunoblot analysis, abundant expression of HieACE was detected in the posterior midgut, and small amounts of HieACE were detected in the compound ganglion of the newly emerged unfed adult buffalo fly. In mammals, somatic ACE is expressed by the gut epithelium [47]. Whether it functions as a digestive enzyme, or to regulate neuropeptides active in gut motility, or to regulate neuropeptides involved in the absorption of water and Na^+ is unknown [3]. The posterior midgut of adult flies is similarly involved in terminal digestion of ingested proteins and peptides, and in water and Na^+ balance. Mammalian somatic ACE is also associated with several neural pathways [3], hence the appearance of low levels of HieACE in extracts of compound ganglion might be anticipated.

In haematophagous flies, maturation of the male reproductive system in the newly emerged fly is dependent on the first blood meal [48–50]. 26 h after the first blood feed, expression of HieACE was detected in the maturing male reproductive system (while expression in the posterior midgut was unaltered). The expression of HieACE in the male reproductive system was detected 2 h after the first blood meal and persisted thereafter. Mammalian testicular ACE is expressed in developing and mature testes (reviewed in [3]). The role of ACE in mammalian reproduction is not known since both forms of ACE are present; somatic ACE is found in the prostate and epididymis, and testicular ACE is detected in sperm and the Leydig cells. The cells that express HieACE in the testes and/or associated structures have yet to be determined, so it is unknown whether HieACE

expression parallels that of somatic ACE or of testicular ACE, or both in the male fly. A recent study of mice in which the ACE gene was deleted revealed no significant defect in the testes or sperm of homozygote male mutant mice. However, the sperm seem unable to fertilise ova [51]. It would be of interest to determine whether HieACE has a similar role in the reproduction of flies.

Until recently, there has been little or no evidence that proteins similar to ACE exist in invertebrates. There is now ample evidence that ACE-related enzymes are well represented in arthropod species. The sequences of peptides derived from the cattle tick carboxydipeptidase, Bm91, which is also membrane bound, and its predicted amino acid sequence show high similarity with regions of mammalian testicular ACE [21]. An enzyme with ACE-like activity has also been detected in the adult house fly, *M. domestica*, although this protein appears to be membrane bound [20]. We have now described an ACE-related enzyme, HieACE, of the adult stage of the haematophagous fly, *H. irritans exigua*. An ACE-related enzyme of *Drosophila* was isolated and cloned from the egg stage of the life cycle [23] and the gene was found to be a target of a homeobox regulatory gene [24]. The evolutionary relationship of mammalian ACE and the *Drosophila* ACE-related enzyme has been discussed [23]. The two dipteran ACE-related enzymes are very similar with 79% overall amino acid identity and these data will assist in the estimation of evolutionary time-scales [23]. The two dipteran ACE-like enzymes have only 35% identity with the *B. microplus* ACE-related enzyme, Bm91. Other species of flies have proteins similar to HieACE. Immunoblots with ovine anti-HieACE serum have detected 65−70 kDa proteins in the lysates of the adult and larval stages of the sheep blowfly (*Lucilia cuprina*) and the larval stages of the secondary strike fly, *Chryosoma rufiiciens*, and *Drosophila* (Gough, J., unpublished results). It is likely that the dipteran ACE-like enzymes are expressed in all stages of the life cycle.

In summary, the ACE-like enzyme of the buffalo fly has strong similarities to mammalian ACE. HieACE has similar activity against angiotensin I, bradykinin, substance P and cholecystokinin-8 and is sensitive to ACE inhibitors. HieACE consists of a single catalytic domain and hence is similar to testicular ACE, although it differs by its smaller size and lack of cell-membrane association or extensive glycosylation. Like somatic ACE, it is expressed in the gut and nervous tissues, and like mammalian testicular ACE, it is found in the maturing male reproductive system. It is possible that the function of the mammalian testicular ACE and the dipteran ACE in testes are conserved. Studies in transgenic mice suggest a role in fertility [51] and *Drosophila* transheterozygote mutants of the ACE gene exhibit male sterility [24]. However, as with the mammalian ACE, its exact function in many tissues and the substrates in these tissues, have to be determined.

The authors are grateful for the assistance of Mrs Dell Dunn in preparing this manuscript and for the advice and critical comments of Dr Ross Tellam and Mr David Waltisbuhl. We gratefully acknowledge the contribution of the Meat Research Corporation in their support of the Buffalo Fly Vaccine Project.

REFERENCES

1. Cushman, D. W., Cheung, H. S., Sabo, E. F. & Ondetti, M. A. (1977) Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids, *Biochemistry 16*, 5484−5491.
2. Cushman, D. W., Cheung, H. S., Sabo, E. F., Rubin, B. & Ondetti, M. A. (1979) Development of specific inhibitors of angiotensin I converting enzyme (kininase II), *Fed. Proc. 38*, 2778−2782.
3. Ehlers, M. R. W. & Riordan, J. F. (1989) Angiotensin-converting enzyme: new concepts concerning its biological role, *Biochemistry 28*, 5311−5318.
4. Soffer, R. L. (1976) Biochemical properties of angiotensin-converting enzyme, *Annu. Rev. Biochem. 45*, 73−94.
5. Skidgel, R. A., Engelbrecht, S., Johnson, A. R. & Erdös, E. G. (1984) Hydrolysis of substance P and neurotensin by converting enzyme and neutral endopeptidase, *Peptides (Elmsford) 5*, 769−776.
6. Skidgel, R. A. & Erdös, E. G. (1985) Novel activity of human angiotensin I converting enzyme: release of the NH_2- and COOH-terminal tripeptides from luteinizing hormone-releasing factor, *Proc. Natl Acad. Sci. USA 82*, 1025−1029.
7. Kase, R., Sekine, R., Katayama, T., Takagi, H. & Hazato, T. (1986) Hydrolysis of neo-kyotorphin (Thr-Ser-Lys-Tyr-Arg) and [Met]-enkephalin-Arg^6-Phe^7 by angiotensin-converting enzyme from monkey brain, *Biochem. Pharmacol. 35*, 4499−4503.
8. Dubreuil, P., Fulcrand, P., Rodriguez, M., Fulcrand, H., Laur, J. & Martinez, J. (1989) Novel activity of angiotensin-converting enzyme. Hydrolysis of cholecystokinin and gastrin analogues with release of the amidated C-terminal dipeptide, *Biochem. J. 262*, 125−130.
9. Das., M., Hartley, J. L. & Soffer, R. L. (1977) Serum angiotensin I-converting enzyme: isolation and relationship to the pulmonary enzyme, *J. Biol. Chem. 252*, 1316−1319.
10. Yokoyama, M., Takada, Y., Iwata, H., Ochi, K., Takeuchi, M., Hiwada, K. & Kokubu, T. (1982) Correlation between angiotensin-converting enzyme activity and histologic patterns in benign prostatic hypertropy tissue, *J. Urol. 127*, 368−370.
11. Strittmatter, S. M., Theile, E. A., De Souza, E. B. & Snyder, S. H. (1985) Angiotensin-converting enzyme in the testis and epididymus: differential development and pituitary regulation of isozymes, *Endocrinology 117*, 1374−1379.
12. Ehlers, M. R. W., Chen, Y. N.-P. & Riordan, J. F. (1992) The unique N-terminal sequence of testis angiotensin-converting enzyme is heavily O-glycosylated and unessential for activity or stability, *Biochem. Biophys. Res. Commun. 183*, 199−205.
13. Kumar, R. S., Kusari, J., Roy, S. N., Soffer, R. L. & Sen, G. C. (1989) Structure of testicular angiotensin-converting enzyme: a segmental mosaic enzyme, *J. Biol. Chem. 264*, 16754−16758.
14. Lattion, A.-L., Soubrier, F., Allegrini, J., Hubert, C., Corvol, P. & Alhenc-Gelas, F. (1989) The testicular transcript of the angiotensin I-converting enzyme encodes for the ancestral, nonduplicated form of the enzyme, *FEBS Lett. 252*, 99−104.
15. Howard, T. D., Shai, S. Y., Langford, K. G., Martin, B. M. & Bernstein, K. E. (1990) Transcription of testicular angiotensin-converting enzyme (ACE) is initiated within the 12th intron of the somatic ACE gene, *Mol. Cell. Biol. 10*, 4294−4302.
16. Nachman, R. J., Holman, G. M., Haddon, W. F. & Ling, N. (1986) Leucosulfakinin, a sulfated insect neuropeptide with homology to gastrin and cholecystokinin, *Science 234*, 71−73.
17. Nachman, R. J., Holman, G. M., Cook, B. J., Haddon, W. F. & Ling, N. (1986) Leucosulfakinin-II, a blocked sulfated insect neuropeptide with homology to cholecystokinin and gastrin, *Biochem. Biophys. Res. Commun. 140*, 357−364.
18. Veenstra, J. A. (1989) Isolation and structure of two gastrin/CCK-like neuropeptides from the American cockroach homologous to the leucosulfakinins, *Neuropeptides 14*, 145−149.
19. Woodhead, A. P., Stay, B., Siedel, S. L., Khan, M. A. & Tobe, S. S. (1989) Primary structure of four allatostatins: neuropeptide inhibitors of juvenile hormone synthesis, *Proc. Natl Acad. Sci. USA 86*, 5997−6001.
20. Lamango, N. S. & Isaac, R. E. (1994) Identification and properties of a peptidyl dipeptidase in the housefly, *Musca domestica*, that resembles mammalian angiotensin-converting enzyme, *Biochem. J. 299*, 651−657.
21. Riding, G. A., Jarmey, J., McKenna, R. V., Pearson, R., Cobon, G. S. & Willadsen, P. (1994) A protective concealed antigen from *Boophilus microplus*: isolation, localisation and possible function, *J. Immunol. 153*, 5158−5166.
22. Cobon, G., Willadsen, P., Kemp, D., Tellam, R., inventors; Biotech Australia Pty Ltd., CSIRO assignees. Tick antigen. PCT/AU94/00463. 1994.

23. Cornell, M. J., Williams, T. A., Lamango, N. S., Coates, D., Corvol, P., Soubrier, F., Hoheisel, J., Lehrach, H. & Isaac, R. E. (1995) Cloning and expression of an evolutionary conserved single-domain angiotensin converting enzyme from *Drosophila melanogaster*, *J. Biol. Chem. 270*, 13613–13619.
24. Tatei, K., Cai, H., Ip, Y. T. & Levine, M. (1995) Race: a *Drosophila* homologue of the angiotensin converting enzyme, *Mech. Dev. 51*, 157–168.
25. Ryan, J. W., Chung, A., Ammons, C. & Carlton, M. L. (1977) A simple radioassay for angiotensin-converting enzyme, *Biochem. J. 167*, 501–504.
26. Jarmey, J. N., Riding, G. A., Pearson, R., McKenna, R. V. & Willadsen, P. (1995) Carboxydipeptidase from *Boophilus microplus*: a 'concealed' antigen with similarity to angiotensin converting enzyme, *Insect Biochem. Mol. Biol. 25*, 969–974.
27. Stone, L. S., LoPresti, M. B., Crawford, J. M., DeAngelis, R. & Williams, K. R. (1989) Enzymatic digestion of proteins and HPLC peptide isolation in *A practical guide to protein and peptide purification for microsequencing* (Matsudaira, P. T., ed.) pp. 33–47, Academic Press Inc., New York.
28. Willadsen, P., Riding, G. A., McKenna, R. V., Kemp, D. H., Tellam, R. L., Nielsen, J. N., Lahnstein, J., Cobon, G. S. & Gough, J. M. (1989) Immunologic control of a parasitic arthropod. Identification of a protective antigen from *Boophilus microplus*, *J. Immunol. 143*, 1346–1351.
29. Elvin, C. M., Whan, V. & Riddles, P. W. (1993) A family of serine protease genes expressed in adult buffalo fly (*Haematobia irritans exigua*), *Mol. & Gen. Genet. 240*, 132–139.
30. Casu, R. E., Pearson, R. D., Jarmey, J. M., Cadogan, L. C., Riding, G. A. & Tellam, R. L. (1994) Excretory/secretory chymotrypsin from *Lucilia cuprina*: purification, enzymatic specificity and amino acid sequence from mRNA, *Insect Mol. Biol. 3*, 201–211.
31. Bünning, P. & Riordan, J. F. (1983) Activation of angiotensin converting enzyme by monovalent anions, *Biochemistry 22*, 110–116.
32. Rabilloud, T., Carpentier, G. & Tarroux, P. (1988) Improvement and simplification of low background silver staining of proteins by using sodium dithionite, *Electrophoresis 9*, 288–291.
33. Wijffels, G. L., Salvatore, L., Dosen, M., Waddington, J., Wilson, L., Thompson, C., Campbell, N., Sexton, J., Wicker, J., Bowen, F., Friedel, T. & Spithill, T. W. (1994) Vaccination of sheep with purified cysteine proteinases of *Fasciola hepatica* decreases worm fecundity, *Exp. Parasitol. 78*, 132–148.
34. Young, P. R. (1989) Enhancement of immunoblot staining using a mixed chromogenic substrate, *J. Immunol. Methods 121*, 295–296.
35. Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution, *J. Biol. Chem. 256*, 1604–1607.
36. Dorer, F. E., Kahn, J. R., Lentz, K. E., Levine, M. & Skeggs, L. T. (1976) Kinetic properties of pulmonary angiotensin-converting enzyme. Hydrolysis of hippurylglycylglycine, *Biochim. Biophys. Acta 429*, 220–228.
37. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) Basic local alignment search tool, *J. Mol. Biol. 215*, 403–410.
38. Williams, T. A., Corvol, P. A & Soubrier, F. (1994) Identification of two active site residues in human angiotensin I-converting enzyme, *J. Biol. Chem. 269*, 29430–29434.
39. Soubrier, F., Hubert, C., Testut, P., Nadaud, S., Alhenc-Gelas, F. & Corvol, P. (1993) Molecular biology of the angiotensin I converting enzyme. I. Biochemistry and structure of the gene, *J. Hypertens. 11*, 471–476.
40. Riordan, J. F., Chen, Y.-N. P., Kleemann, S. G. & Banning, P. (1991) Peptide inhibitors and the active site(s) of angiotensin converting enzyme, *Biomed. Biochim. Acta 50*, 809–814.
41. Soffer, R. L. (1981) *Biochemical regulation of blood pressure*, John Wiley & Sons, New York.
42. Cushman, D. W. & Ondetti, M. A. (1980) Inhibitors of angiotensin-converting enzyme for treatment of hypertension, *Biochem. Pharmacol. 29*, 1871–1877.
43. Thiele, E. A., Strittmatter, S. M. & Snyder, S. H. (1985) Substance P and substance K as possible endogenous substrates of angiotensin converting enzyme in the brain, *Biochem. Biophys. Res. Commun. 128*, 317–324.
44. Cascieri, M. A., Bull, H. G., Mumford, R. A., Patchett, A. A., Thomberry, N. A. & Liang, T. (1984) Carboxyl-terminal tripeptidyl hydrolysis of substance P by purified rabbit lung angiotensin-converting enzyme and the potentiation of substance P activity *in vivo* by captopril and MK-422, *Mol. Pharmacol. 25*, 287–293.
45. Yokosawa, H., Endo, S., Ogura, Y. & Ishii, S.-I. (1983) A new feature of angiotensin-converting enzyme in the brain: hydrolysis of substance P, *Biochem. Biophys. Res. Commun. 116*, 735–742.
46. Ondetti, M. A., Rubin, B. & Cushman, D. W. (1977) Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active hypertensive agents, *Science 196*, 441–444.
47. Cushman, D. W. & Cheung, H. S. (1971) Concentrations of angiotensin-converting enzyme in tissues of the rat, *Biochim. Biophys. Acta 250*, 261–265.
48. Stoffolano, J. G. (1974) Influence of diapause and diet on the development of gonads and accessory reproductive glands of the black blowfly, *Phormia regina* (Meigen), *Can. J. Zool. 52*, 981–988.
49. Foster, W. A. (1976) Male sexual maturation of the tsetse flies *Glossina morisitans* Westwood and *G. austeni* Newstead (Dipt., Glossinidae) in relation to blood feeding, *Bull. Entomol. Res. 66*, 389–399.
50. Anderson J. R. (1978) Mating behaviour of *Stomoxys calcitrans*: effects of a blood meal on the mating drive of males and its necessity as a prerequisite for proper insemination of females, *J. Econ. Entomol. 71*, 379–386.
51. Krege, J. H., John, S. W. M., Langenbach, L. L., Hodgin, J. B., Hagaman, J. R., Bachman, E. S., Jennette, J. C., O'Brien, D. A. & Smithies, O. (1995) Male-female differences in fertility and blood pressure in ACE deficient mice, *Nature 375*, 146–148.