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Isolation of angiotensin converting enzyme from testes of *Locusta migratoria* (Orthoptera)

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Abstract. By means of a tracer assay using a labeled synthetic angiotensin converting enzyme (ACE) substrate hippurylglycylglycine, we have detected high ACE activity in the testes of the African migratory locust, *Locusta migratoria*. Lower, but significant, ACE activity was observed in midgut and hemolymph. In a two-step purification procedure involving anion exchange and gel permeation chromatography, we have purified *Lom*ACE from the locust testes. The enzyme of approximately 80 kDa shows substantial amino-acid sequence homology with ACE from both vertebrate and invertebrate origin. The ACE identity of the purified enzyme was further confirmed by cDNA cloning of the *Locusta* ACE fragment, which, after *in silico* translation, revealed a mature protein of 623 amino acids with a large structural similarity to other known ACE proteins.

INTRODUCTION

As a part of the renin-angiotensin system (RAS), the Zn^{2+} metalloprotease angiotensin converting enzyme, or ACE (dipeptidyl carboxypeptidase I, EC 3.4.15.1), is an important factor in the regulation of blood pressure and fluid and electrolyte homeostasis of mammals. By cleaving off its C-terminal dipeptide, ACE converts the decapeptidic angiotensin I into the potent vasoconstrictor angiotensin II. In addition, ACE inactivates the potent vasodilator bradykinin by the sequential removal of two C-terminal dipeptides (Corvol et al., 1995). ACE functionality is, however, not restricted to blood pressure regulation. The hemoregulatory peptide N-Ac-Ser-Asp-Lys-Pro, which is an inhibitor of haematopoietic stem cell proliferation, is inactivated through hydrolysis by ACE (Rousseau et al., 1995; Azizi et al., 1996). ACE also exerts a very broad *in vitro* substrate specificity, hydrolyzing various peptides such as bradykinin, substance P, luteinising hormone-releasing hormone, angiotensin I and angiotensin II (Hooper, 1991). It is, in addition to its important role in blood pressure, known to be involved in developmental processes. Its exact function in these processes is still under investigation (Ganong, 1995; Vinson et al., 1997; Kessler et al., 2000).

Mammalian ACE exists in two isoforms. The somatic form (sACE) is composed of two highly similar domains, each containing one similar catalytic site. They are called the N- and C-domain, according to their relative position within the protein. The testicular isoform (tACE) has only one domain and is homologous to the C-domain of sACE (Howard et al., 1990; Ehlers et al., 1992). While sACE is widespread throughout the body (Hooper et al., 1991), tACE expression is limited to maturing sperm and spermatozoa (Velletri, 1985; Pauls et al., 1999). sACE is responsible for blood pressure regulation, but the function of tACE remains uncertain, although a role in male fertility is hypothesized (Kessler et al., 2000; Hagaman et al., 1998).

ACE homologues have recently been discovered in several insect species (Isaac & Lamango, 1994; Loeb et al., 1998; Schoofs et al., 1998; Ekbote et al., 1999; Zhu et al., 2001a, b; Vandingenen et al., 2002). Although we have indicated that the *Neobellieria bullata* trypsin modulating oostatic factor (*Neb*TMOF) is a putative *in vivo* substrate for *Neb*ACE (Vandingenen et al., 2001), the exact physiological relevance of insect ACE remains obscure (Isaac et al., 1998; Veelaert et al., 1999; Seinsche et al., 2000). Cloning and/or purification of ACE from the fruitfly *Drosophila melanogaster* (AnCE) (Cornell et al., 1995), the housefly *Musca domestica* (Lamango et al., 1996), the buffalo fly *Haematobia irritans exigua* (*Hie*ACE) (Wijffels et al., 1996), and the silk worm *Bombyx mori* (*Bm*ACER or ACE-related) (Quan et al., 2001) have contributed to the molecular and biochemical characterization of insect ACE, which is composed of only one domain and shares structural and enzymatic properties with tACE and the C-domain of sACE.

Because most of what is known about insect ACE characteristics originates from dipteran ACE homologues, we have conducted both the purification of *Lom*ACE from testes of *L. migratoria* and the cloning of its cDNA. The obtained data were used to compare the structural characteristics of orthopteran ACE with those of its dipteran and mammalian counterparts. The purified or expressed *Lom*ACE will be used in future investigations concerning the functions of insect ACE.

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MATERIAL AND METHODS

Handling of insects

Locusts were reared as described (Ashby et al., 1972). For collection of hemolymph, animals were anaesthetized with CO_2 and a leg was amputated. Hemolymph was drawn from the bleeding wound with a pipette and immediately diluted tenfold in ice-cold Hepes buffer (0.05 M Hepes; pH 8.0; 0.3 M NaCl; 0.06 M $(NH_4)_2SO_4$) to prevent coagulation. After centrifugation of the pooled hemolymph (13,000 rcf, 5 min, 4°C), supernatant (designated as hemolymph sample) was stored at –80°C. Each locust typically yielded 20–100 µl of (undiluted) hemolymph.

For dissection of testes and midgut, animals were anaesthetized and head, legs and wings were cut off. The ventral side of the body was slit open; midgut and testes were removed and rinsed with *Locusta* Ringer solution (9.82 g/L NaCl; 0.48 g/L KCl; 0.19 g/L NaH_2PO_4; 0.25 g/L $NaHCO_3$; 0.73 g/L $MgCl_2$; 0.32 g/L $CaCl_2$; pH 6.5). Tissues were pooled and homogenized in Tris buffer (Tris-HCl; pH8.2; 20mM). The homogenate was centrifuged (10,000 rcf, 30 min, 4°C) repeatedly until pelleting stopped. Supernatants were stored at –80°C.

ACE activity assay

ACE activity measurements were based on the method proposed by Ryan et al. (1977) and modified as in Vandingenen et al. (2001). Typically, reactions were conducted in a final volume of 100 µl in HEPES (see above) buffer. 40 µl of sample, to which 10 µl of buffer was added, was incubated at 37°C with 50 µl of tritiated substrate [2 µM buffered [^{3}H]benzoylglycylglycylglycine (^{3}H-hippurylglycylglycine, Amersham)] (1). In a negative control, 10 µl of HEPES buffer (see above) containing 100 µM captopril (Sigma) was added (instead of 10 µl buffer), resulting in a complete inhibition of ACE activity (2). After a desired incubation time, reactions were terminated by adding 1 ml of 0.1 M HCl. The reaction product (^{3}H-hippurate) was separated from the unhydrolyzed substrate (^{3}H-Hip-Gly-Gly) by extraction with 1 ml of ethyl acetate. The two phases were mixed by vortexing and the layers were separated by centrifugation (2000 rpm, 20 min, and 4°C). 500µl of the organic phase, containing the ^{3}H-hippurate, was added to 4 ml of scintillation fluid and counted for 2 min in a liquid scintillation counter (Beckman). For accurate measurement of the initial amount of radioactivity added, an equal volume (50 µl) of tritiated substrate was added directly to 4 ml of scintillation fluid and counted (3). Counts per minute are a measure for ACE activity as they express the amount of tritiated ^{3}H-hippurate formed. The ACE activity was expressed as % hydrolysis of the radioactive substrate: % hydrolysis = [cpm(1) – cpm(2)] × 2 × 100 / cpm(3). Captopril is a selective and specific ACE inhibitor and, in this calculation, only the enzymatic activity that could be fully inhibited by captopril was defined as ACE activity. The factor × 2 was added because only half of the total volume of the organic phase was added to the scintillation fluid.

Synthetic peptides

Synthetic human angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) and bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) were purchased from Sigma and stored at –20°C in a stock concentration of 400 µM in HEPES buffer (see above).

Protein purification

52 testes equivalents in Tris-HCl buffer, pH 8.2, 20 mM were applied to a column (16 cm × 1.2 cm) of Q Sepharose Fast Flow (Pharmacia), equilibrated with the same Tris buffer. Proteins were eluted with a linear gradient of NaCl (0–0.4 M). The flow rate amounted to 30 ml/hr; fractions of 3 ml were collected. The column was washed with Tris buffer containing 1.0 M NaCl.

Active fractions were pooled and concentrated on a Diaflo PM 10 membrane (Amicon), cut-off 10 kDa, using a 90 mm Hi-Flux cell (Millipore). The retentate was applied on a column (100 cm × 2.5 cm) of Ultrogel AcA34 (IBF), fractionation range of 20–350 kDa. Elution was performed with Tris-HCl buffer, pH 8.2, 100 mM at a flow rate of 23 ml/h in fractions of 2.5 ml. The column was calibrated with a standard protein mixture, consisting of dimers of subunits (450 kDa) of *Helix pomatia* hemocyanin, dimers (134 kDa) and monomers (67 kDa) of bovine serum albumin, ovalbumin (43 kDa) and myoglobin (17 kDa).

The absorbance of eluted fractions was followed at 230 nm with a Perkin Elmer UV/VIS spectrometer lambda 20. For detection of ACE activity in the fractions, each fraction was diluted 4:5 in a 5× Tris buffer (Tris-HCl; pH 8.2; 20 mM or 100 mM; 1.5 M NaCl; 1 M $(NH_4)_2SO_4$; 50 µM $ZnCl_2$) and incubated for 12 hr in an ACE activity assay. The assay buffer was a Tris buffer (Tris-HCl; pH 8.2; 20 mM or 100 mM) containing 0.3 M NaCl; 0.2 M $(NH_4)_2SO_4$ and 10 µM $ZnCl_2$.

For visualization of protein profiles, 300 µl of each fraction was dried, dissolved in 20 µl of sample buffer containing SDS and 2-mercaptoethanol and subjected to SDS-PAGE (Laemmli, 1970). The components of a low molecular weight calibration kit from Amersham were used as protein size markers. Gels were stained with Coomassie Brilliant Blue.

Protein identification

Tryptic in-gel digestion. The protein band was excised from the Coomassie Blue-stained SDS-PAGE gel and sliced into small pieces, which were destained with two changes (20 min each) of 50 µl 50% acetonitrile (ACN) containing 50 mM NH_4HCO_3. With one change of 50% ACN, pieces were dehydrated until they became opaque white (± 5 min). The pieces were dried and covered with 20 µl of 10 mM dithiothreitol (DTT), 100 mM NH_4HCO_3, reduced at 56°C for 1 h and cooled to room temperature. The DTT was replaced with 20 µl of 55 mM iodoacetamide, 100 mM NH_4HCO_3 and the pieces were incubated in the dark for 45 min at room temperature with occasional vortexing. Pieces were washed for 10 min with 50 µl of 100 mM NH_4HCO_3, dehydrated for 10 min by addition of 50 µl of 50% ACN, swollen for 10 min by rehydration in 50 µl of 100 mM NH_4HCO_3 and shrunk for 10 min by addition of 50 µl of 50% ACN. The liquid phase was removed and pieces were dried in a vacuum centrifuge for 5 min. Twenty-five µl of digestion buffer (50 mM NH_4HCO_3; 5 mM $CaCl_2$; 25 ng trypsin/µl buffer) were added and the pieces were incubated for 45 min in an ice-bath. The supernatant was removed, replaced by 5 µl of 50 mM NH_4HCO_3 5 mM $CaCl_2$ and the pieces were incubated overnight at 37°C. Subsequently, the gel pieces were washed for 20 min with one change of 20 µl of 20 mM NH_4HCO_3 and peptides were extracted with three changes (20 µl and 20 min for each change) of 5% formic acid in 50% ACN at room temperature and dried.

Desalting and concentrating. The peptide mixture was redissolved in 50 µl of 2% ACN, 0.1% trifluoroacetic acid (TFA) and desalted by means of a ZipTip (Millipore), which is a pipette tip containing 1 µl of C_{18} beads at the orifice. The entire mixture was loaded onto the ZipTip in 10 µl batches. Subsequently, the tip was washed with 0.1% TFA to remove salts and the peptides were eluted with 3 µl of ACN/water/formic acid (70.0/29.9/0.1, *v/v/v*).

CID analysis with ESI-TOF MS. Nanoflow electrospray ionization quadrupole orthogonal acceleration time-of-flight mass spectrometry was performed on a Q-Tof system (Micro-

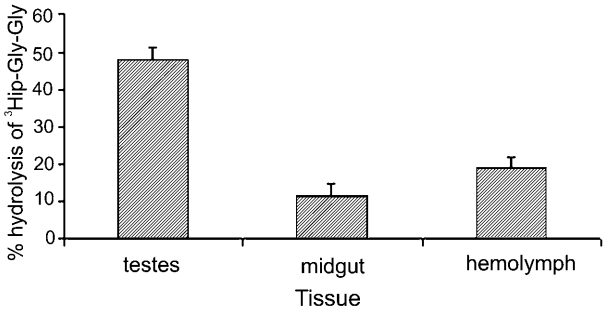

Fig. 1. ACE activity in midgut (2 eq./ml), hemolymph (100 µl/ml) and testes (0.04 eq./ml) samples of *L. migratoria*, expressed as the % ^{3}Hip-Gly-Gly hydrolyzed after 6 hr of incubation at 37°C in HEPES buffer. Each sample represents a pool of at least 10 animals. Values are a mean of 3 independent measurements. Standard deviation is indicated.

mass, UK). Two µl of the desalted and concentrated sample was loaded in a metal-coated capillary (Protana L/Q nanoflow needle). This sample was sprayed at a typical flow rate of 30 nl/min, giving extended analysis time in which we acquired an MS spectrum, as well as several MS/MS spectra. During MS/MS or tandem mass spectrometry, fragment ions are generated from a selected precursor ion by collision-induced dissociation (CID) (Morris et al., 1996). Because not all peptide ions fragment with the same efficiency, the collision energy was typically varied between 20 and 35 eV so that the parent ion was fragmented into a satisfying number of different daughter ions. Needle voltage was set at 900 V, cone voltage was 35 V. The obtained fragmentation spectra were combined and transformed into their singly charged state by treatment with the Max-ent3 software (Masslynx 3.5 software; Micromass, UK). Amino acid sequences were determined by calculating the mass differences between adjacent b-type ions and/or y"-type ions.

cDNA cloning

Messenger RNA was isolated from *Locusta* testes according to the "Quickprep mRNA purification Kit" (Pharmacia). 0.8 µg of mRNA was used for single stranded cDNA synthesis (Marathon, Clontech), of which 0.1 µl was used in the PCR reaction. A first *Locusta* specific ACE (*Lom*ACE) cDNA fragment was obtained by PCR with two degenerate primers (SP1F : 5' CAY YTN YWN GGN AAY ATG TGG GC 3' and SP2R : 5' RTC NCC NAC NGC YTC RTG RAA CN 3') based on the consensus sequences deduced from the open reading frames of ACE sequences of several organisms. The reaction had an annealing temperature of 50°C and contained the following components : 2µg of each primer, 1× advantage 2 polymerase mix (Clontech), 0.5mM dNTP's, 40 mM Tricine-KOH, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 µg/ml BSA. The PCR products were separated on a 1.2% agarose gel, cloned into the PCR 2.1 TA-cloning vector (Invitrogen) and sequenced. Double stranded cDNA synthesis and further RACE reactions were performed according to the Marathon protocol with 3' and 5' RACE primers (P4 : 5' GAC ATC TCG GTT CCC TTC CCT GGA AAG C 3' and M1 : 5' GCT GAG GCA TGG CAT ATC AGT TCT CTC CC 3') based on the *Lom*ACE fragment.

RESULTS

ACE activity in different tissues of *L. migratoria*

Testes, midgut and hemolymph samples could effectively hydrolyze the ACE substrate ^{3}Hip-Gly-Gly (Fig. 1). Testicular tissue clearly contains the highest ACE activity, while hemolymph and midgut exhibit substantially less ACE activity.

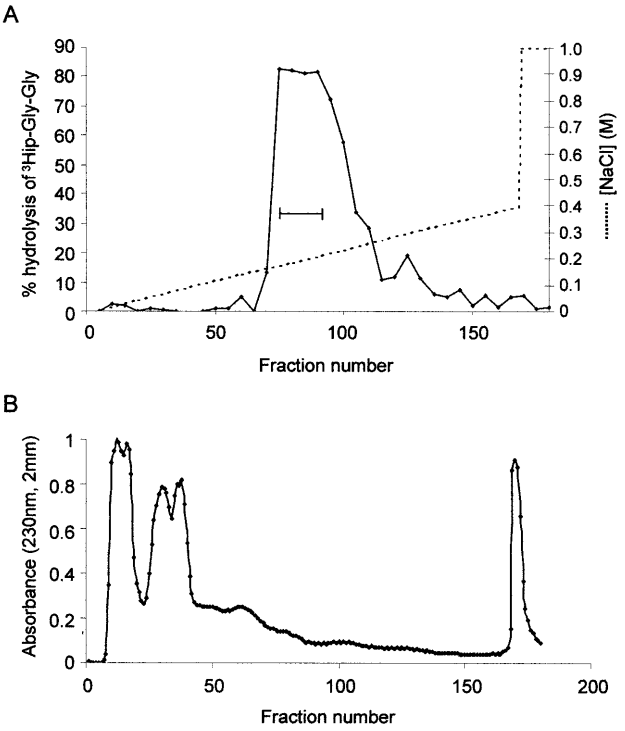

Fig. 2. Chromatography on Q-Sepharose Fast Flow of testes sample of *L. migratoria*. A – profile of ACE activity. The fractions indicated with a bar were pooled for further investigation; B – absorbance at 230 nm, 2 mm path length.

Purification and identification of Lom-ACE from the locust testes

A 52 equivalent testes sample was subjected to anion exchange chromatography (Fig. 2). The majority of ACE activity eluted from fraction 75 to 95, corresponding to a NaCl concentration ranging from 0.18 M to 0.22 M (Fig. 2A). The maximum hydrolysis level reached ≈ 80% and the peak had a slight trailing tendency. As shown by the absorbance measurements, the bulk of the proteins present in the testes sample eluted in the first 50 (0–0.12 M NaCl) and the last 10 fractions (rinsing of the column with 1.00 M NaCl), while considerably fewer proteins eluted in the other fractions, especially in the region of ACE activity (Fig. 2B).

Active fractions of the Q-Sepharose chromatography (fractions 75–94) were pooled and concentrated by ultrafiltration. A hundred-fold dilution of the retentate (containing proteins > 10 kDa) exhibited 65% hydrolysis of ^{3}Hip-Gly-Gly, while the filtrate was completely devoid of ACE activity. Part of the retentate (2 ml, corresponding to about 1/3 of the material) was submitted to gel chromatography on Ultrogel AcA34 (Fig. 3). ACE activity was only present in fractions 80 to 90. This activity was, however, not stable and decreased within 5 days. As indicated by the absorbance profile at 230 nm (data not shown), the bulk of the protein material in the retentate eluted at the salt volume of the column (proteins < 20 kDa). From its elution volume, the M_r of the ACE protein was estimated

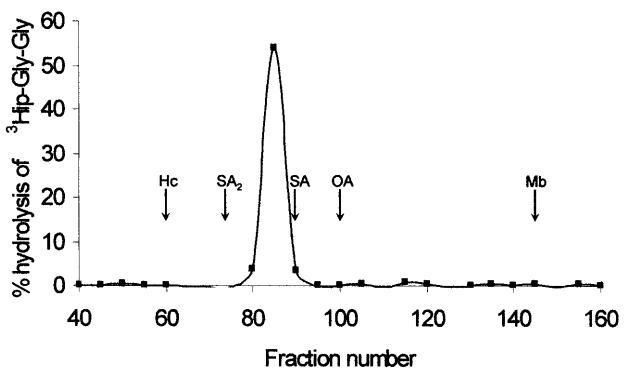

Fig. 3. ACE activity profile of gel permeation Ultrogel AcA34 chromatography of pooled active fractions of the Q-Sepharose chromatography. The elution positions of calibration proteins are indicated by arrows: Hc, hemocyanin (eluting at the void volume); SA_2 – serum albumin dimers; SA – serum albumin monomers; OA – ovalbumin; Mb – myoglobin.

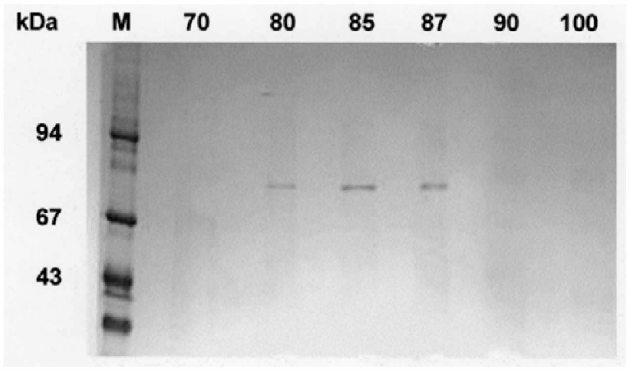

Fig. 4. SDS-PAGE (5–15% polyacrylamide) of fractions of gel permeation chromatography. M – protein size markers. Active fractions (80–87) reveal a ≈ 78 kDa protein band of apparent homogeneity in fraction 85. The gel was stained with Coomassie Blue.

to be 81, 000, making use of a plot of log M_r *versus* elution volume constructed with calibration proteins (Andrews, 1965). This value was in fair agreement with the result obtained by SDS-PAGE analysis. Indeed, the active fractions from the Ultrogel column revealed a ≈ 78 kDa protein band of apparent homogeneity (Fig. 4). Non-active fractions did not contain this protein band.

To investigate whether this protein band corresponded to the enzyme that exerts ACE activity, the band in fraction 85 was excised from the SDS-PAGE gel and trypsinolyzed. The eluted peptides were subjected to a CID analysis with ESI-TOF MS. Amino acid sequences of 10 of the 12 identified peptide fragments were 100% identical with sequences within the *Lom*ACE amino acid sequence as predicted by the cDNA cloning of *Lom*ACE (Fig. 5). This alignment proves that the enzyme isolated from locust testes exerting ACE activity was visualized on an SDS-PAGE as a single protein band of ≈ 78 kDa, of which the amino acid sequence displays sequence similarities with ACE orthologues from animal species of vertebrate and invertebrate origin.

The two other peptide fragments display no significant similarity with ACE sequences (data not shown).

Structure of the LomACE sequence

Sequence analysis of the RACE-PCR products revealed an ACE fragment of 623 AA's, which represents the full mature protein sequence. Comparison with ACE protein sequences from several invertebrate organisms predicts that ≈ the first 10–20 AA's, representing the signal peptide, are missing (Fig. 6). Several attempts to gain this 5' sequence information failed. However, the full sequence of the mature ACE protein is identified. There is a good consensus site for cleavage of the presumed signal peptide between AA 3 and 4 (L-D), which results in a mature protein of 623 AA's, with a calculated mass of 72 kDa deduced from its longest ORF. There are 4 possible consensus sites for N-glycosylation, from which 3 are conserved in *Drosophila* AnCE. The region with the highest similarity to other ACE proteins is situated around the conserved active site (His^{357}, His^{361}, Glu^{385}). The aspartic acid residue, Asp^{389}, believed to have a role in the positioning of the first zinc binding residue and the Glu^{358}, which is involved in catalysis (Corvol et al., 1995), are also conserved in the *Locusta* Ace sequence.

DISCUSSION AND CONCLUSIONS

ACE activity, defined as a captopril-inhibitable dipeptidyl carboxypeptidase activity towards ^{3}H-Hip-Gly-Gly, was detected in testes, midgut and hemolymph of the African migratory locust, *Locusta migratoria.* These results are in agreement with the general tissue distribution of ACE in insects. Although this distribution is not identical in different species, ACE seems to be concentrated in certain tissues, including reproductive tissues, brain, midgut and hemolymph (Cornell et al., 1995; Ekbote et al., 1999; Vandingenen et al., 2001; Vandingenen et al., 2002), suggesting that the enzyme is of physiological importance in these tissues. Indeed, numerous reports substantiate the involvement of insect ACE in reproduction, pro-hormone processing and regulation of peptide titers (Isaac et al., 1994; Tatei et al., 1995; Wijffels et al., 1996; Lamango et al., 1997). Since ACE activity was highest in the testes, which is consistent with the former observation that very high ACE activity is found in locust adult testis (Isaac et al., 1998), this particular tissue was chosen for isolation of the enzyme.

An ≈ 80 kDa enzyme displaying ACE activity was isolated from testes of *L. migratoria* by means of sequential anion exchange and gel permeation chromatography, two techniques that were also included in the purification procedure of ACE from leech and buffalo fly (Laurent & Salzet, 1996; Wijffels et al., 1996). Because 10 tryptic digestion products of this protein were 100 % identical to the amino acid *Lom*ACE sequence as predicted by the partial cDNA cloning and displayed significant similarity with ACE sequences from other organisms, this dipeptidyl carboxypeptidase was identified as *Lom*ACE. The presence in the digest of two remaining peptide fragments, which could not be aligned with the ACE sequences, could indicate the presence of (an) equally sized contaminating protein(s) co-eluting with *Lom*ACE

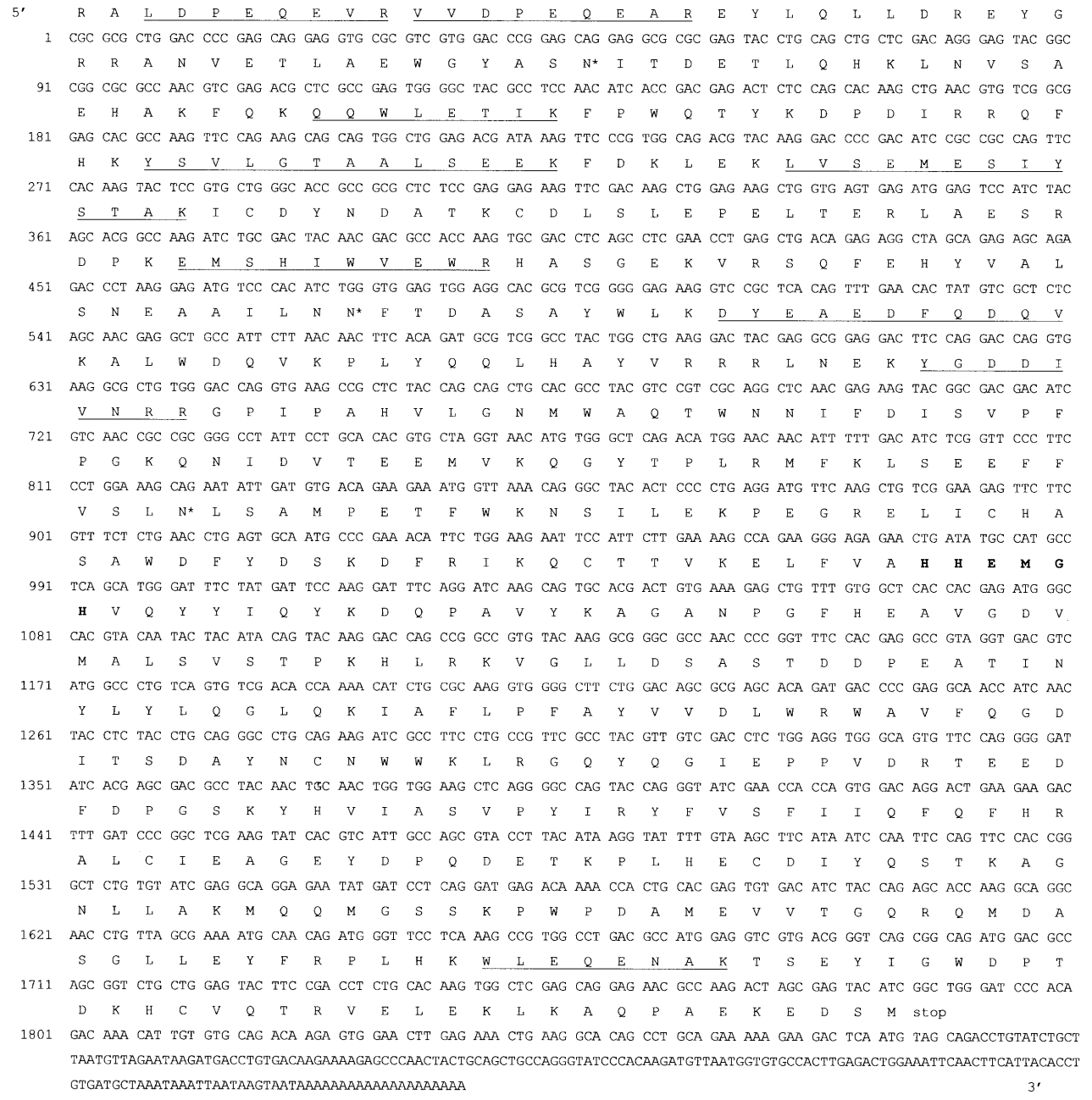

Fig. 5. Nucleotide and deduced amino acid sequence of the *Locusta migratoria* angiotensin converting enzyme. The 10 peptides of the tryptic digest of the ≈ 78 kDa isolated protein are underlined. Possible N-glycosylation sites are indicated with an asterisk. The active site is shown in bold.

or represent an external contamination in the sample which is picked up in the highly sensitive ESI-TOF MS procedure.

The purification of *Lom*ACE from a testes sample, homogenized without the use of detergents and deprived of membranes, suggests that *Lom*ACE is a soluble enzyme, as is the case for all insect ACEs studied to date (Cornell et al., 1995; Lamango et al., 1996; Wijffels et al., 1996). Mammalian ACE on the contrary is, due to the presence of a C-terminal membrane anchor, mainly expressed as a membrane-bound ecto-enzyme, although a soluble form can be found in body fluids (Soubrier et al., 1993). *Lom*ACE is also much smaller than mammalian sACE (140-180 kDa), but is similar in size to dipteran ACE (≈ 67 kDa) (Cornell et al., 1995; Lamango et al., 1996; Wijffels et al., 1996) and the deglycosylated forms of tACE (76-84 kDa) (Ehlers et al., 1992) and ileal ACE (≈ 68 kDa, a mammalian ACE homologue found in ileal fluid and consisting of only the N-terminal domain) (Deddish et al., 1994), suggesting that, exactly as dipteran ACE, *Lom*ACE is a single-domain protein.

The success of the purification process was confirmed by the cDNA sequence information encoding the complete mature *Lom*ACE protein of 623 amino acids. The active site regions of mammalian and invertebrate ACE are conserved in the deduced amino acid sequence of

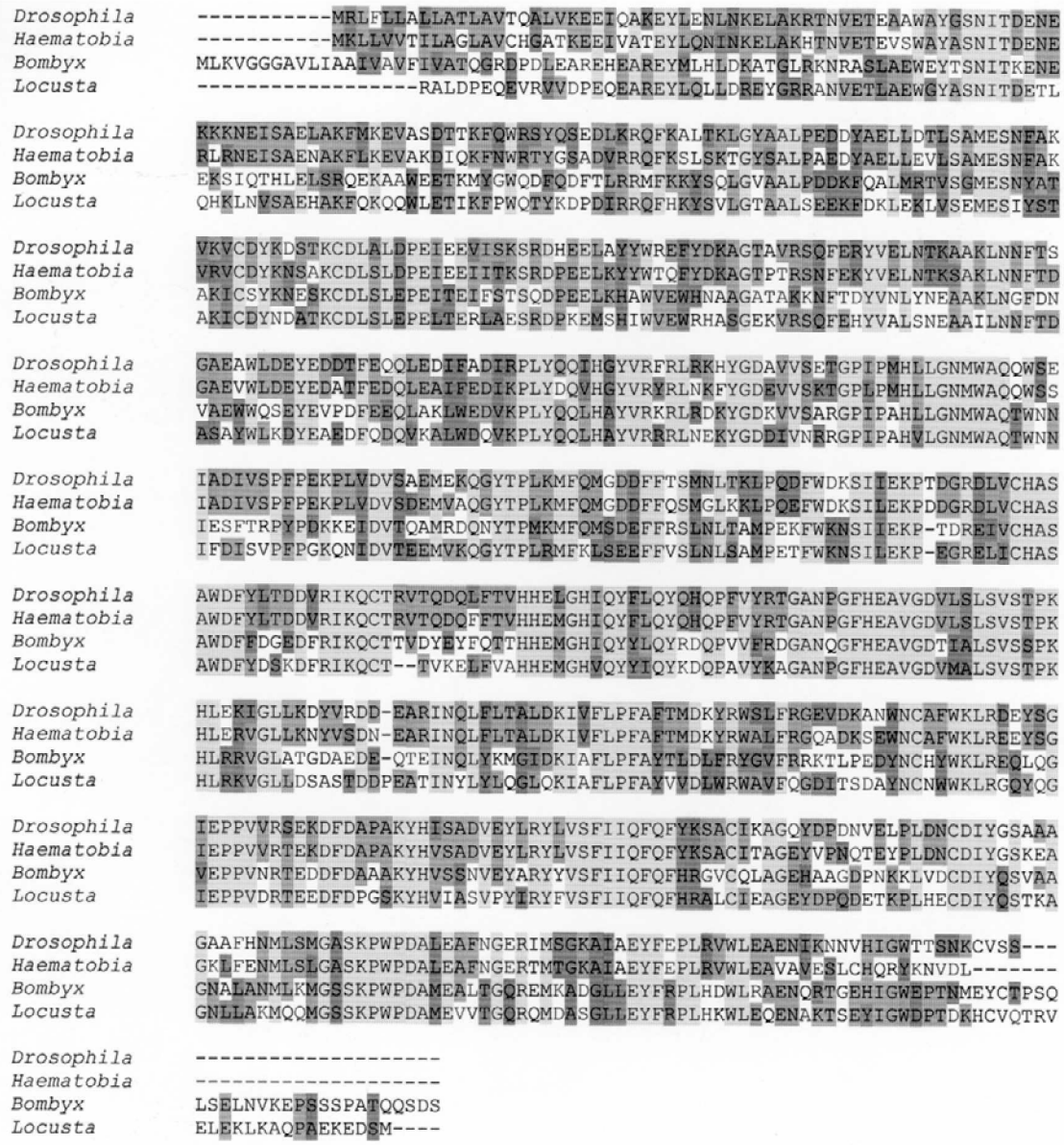

Fig. 6. Alignment of *Lom*ACE with ACE homologues from *Bombyx mori* (*Bm*ACER, accession AB026110.1) *Drosophila melanogaster* (AnCE, accession NM_057698.3) and *Haematobia irritans* (accession Q10715).

*Lom*ACE. The predicted translation product showed the largest identity and similarity with the *Bombyx mori* homologue of ACE. The difference in protein size between the purified band on the SDS-PAGE gel and the size as predicted by the deduce amino acid sequence can be attributed to the 3 N-glycosylation sites present in the *Lom*ACE sequence. Based upon the available information of invertebrate ACE sequences (Fig. 6), we expect a secretion signal at the 5' end of *Lom*ACE. We did not succeed in getting the full sequence information, so only the last two amino acids before the predicted cleavage site of this signal peptide (confirmed by N-terminal sequencing of the *Lom*ACE protein) are identified. However, sufficient cDNA information has been retrieved to identify the complete mature protein sequence and can be used in recombinant expression experiments.

In conclusion, a ≈ 80 kDa soluble ACE homologue was purified to apparent homogeneity from testes of *L. migratoria* that shared high structural similarity with dipteran ACE, *Bm*ACER, the C-domain of mammalian sACE and with mammalian tACE. Whether these similarities imply any functional conservation remains to be determined. The presence of *Lom*ACE activity in testes, hemolymph and midgut, however, suggests a physiological role for ACE in these tissues. The exact function of insect ACE remains unclear and will be investigated in future experiments using the purified ACE or the expression of recombinant *Lom*ACE.

Abbreviations. ACE – angiotensin converting enzyme; ACER – ACE-related; ACN: acetonitrile; AnCE – angiotensin converting enzyme (*Drosophila melanogaster*); *Bm* – *Bombyx mori*; CID – collision induced dissociation; DTT – dithiothreitol; ESI-TOF – electrospray ionization-time of flight;

Hie – Haematobia irritans exigua; Hip-Gly-Gly – Hip-purylglycylglycine; *Lom – Locusta migratoria*; *Neb – Neobellieria bullata*; sACE – somatic ACE; tACE – testicular ACE; TFA – trifluoroacetic acid; TMOF – trypsin modulating oostatic factor.

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