Archives of Insect Biochemistry and Physiology 68:171–180 (2008)

Angiotensin-Converting Enzyme-Like Activity in Crab Gills and Its Putative Role in Degradation of Crustacean Hyperglycemic Hormone

J. Sook Chung[1*] and S.G. Webster[2]

Angiotensin-converting enzyme-like enzyme activity (ACELA) was found in *Carcinus maenas* using reverse phase high performance liquid chromatography (RP-HPLC) analysis of degradation kinetics of a synthetic substrate (Hippuryl-histidyl-leucine) and a specific inhibitor (captopril). Gills contained the highest ACELA, then brain, muscle, and testis, respectively, while no activity was detected in the following tissues: hepatopancreas, hindgut, hypodermis, heart, and hemolymph. ACELA present in gill membranes exhibited a K_m of 0.23 mM and V_{max} of 7.6 nmol with synthetic substrate. The enzyme activity was dependent on Cl^- concentration and was markedly inhibited by captopril, lisinopril, and EDTA. Addition of Zn^{2+} to membranes previously treated with EDTA restored 89% activity, suggesting that *C. maenas* ACELA is a Zn^{2+} metalloenzyme. Gill membranes prepared from premolt crabs showed similar levels of ACELA to those of the intermolt animals. Administration of captopril in vivo lengthened the half life of circulating CHH, while in vitro incubation of gill membranes with captopril reduced CHH. These results suggest that *C. maenas* ACELA present in gills is likely to be involved in degradation of this neuropeptide. Arch. Insect Biochem. Physiol. 68:171–180, 2008. .

KEYWORDS: angiotensin-converting enzyme-like activity; crab gills; crustacean hyperglycemic hormone; inactivation of hormone

INTRODUCTION

Neurohormones or hormones in the hemolymph or blood are transient and at low concentration, yet they are crucial for initiating signalling pathway resulting in physiological action. In crustaceans, crustacean hyperglycemic hormone (CHH) is usually released in immediate response to stresses (e.g., hyperthermal, hypoxia, and emersion), but stays only a short time in the hemolymph (Webster, 1996; Chang et al., 1998; Chung and Webster, 2005). The circulating levels of CHHs, molt inhibiting hormone (MIH), and crustacean cardioactive peptide (CCAP) in hemolymph are low, ranging in concentration from $<10^{-11}$ to 10^{-10} M (Webster, 1996; Chang et al., 1998; Chung et al., 1999; Webster and Chung, 1999; Phlippen et al., 2000; Chung and Webster, 2005; Nakatsuji and Sonobe, 2003).

Compared with crustacean neuropeptides (5–10 min), insect neuropeptides, despite relatively smaller size, appear to have much longer half lives in circulation. For example, adipokinetic hormone, hypertrehalosemic hormone, allatostatins, and bombyxin, when injected into various species of

[1]Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland

[2]School of Biological Sciences, Bangor University, Bangor, Gwynedd, United Kingdom

Grant sponsor: Biotechnology and Biological Sciences Research Council, UK.

Abbreviations used: ACE = angiotensin converting enzyme (dipeptidyl carboxypeptidase I, EC 3.4.15.1); AG-I = angiotensin-I; CHH = crustacean hyperglycemic hormone.

*Correspondence to: J. Sook Chung, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD 21202.
E-mail: chung@comb.umbi.umd.edu

Received 1 December 2007; Accepted 4 March 2008

DOI: 10.1002/arch.20247
Published online in Wiley InterScience (www.interscience.wiley.com)

insects, showed half lives of approximately 18–53 min (Rayne and O'Shea, 1992; Strey et al., 1993; Garside et al., 1997; Goldsworthy et al., 2002) and up to 170 min (Suenobu et al., 2004) in hemolymph. Such short half lives of crustacean neuropeptides, compared to insects, imply that crustaceans may have mechanisms to rapidly remove circulating neuropeptides. However, very little is known about enzymes and tissues in crustaceans that may be involved in processes of metabolic inactivation, degradation, or in clearance of circulating neuropeptides.

Much of the information available in invertebrates regarding the degradation of neuropeptides or neurohormones was initially gathered through homologous studies of vertebrate systems. For example, angiotensin-converting enzyme (ACE) activity in invertebrates has been suggested to inactivate neuropeptides (Lamango and Issac, 1994; Laurent and Salzet, 1996a,b; Lamango et al., 1996). Moreover, the wide distribution of ACE activity, including central nervous tissues, reproductive tissues, hemocytes, and hemolymph, may further implicate ACE in a putative role in other physiological actions such as metamorphosis, reproduction, and immunity in corresponding tissues (Vandenbroeke et al., 1997; Hens et al., 2002; Vandingenen et al., 2002; Ekbote et al., 2003; Salzet and Verger-Bocquet, 2001; Macours et al., 2003; Laurent et al., 1997; Leung et al., 1992). In crustaceans, angiotensin-converting enzyme-like activity (ACELA) was initially reported in the gills of the blue crab, *Callinectes sapidus* (Smiley and Doig, 1994) and in the terrestrial crab, *Chasmagnathus granulatus* (Delorenzi et al., 1996).

Our initial clearance experiment to determine a half-life of CHH using [^{125}I]CHH (previously injected into crabs) showed that it was rapidly removed from hemolymph, and accumulated in the gills. This result indicated that gills may play a role in degradation of CHH. Our preliminary observation, together with previous reports that ACE activity was found in gills of other species of crabs (Smiley and Doig, 1994; Delorenzi et al., 1996), led us to investigate ACELA in *C. maenas* gills and its putative role in regulating neuropeptide levels in hemolymph.

Here we report that ACELA is, indeed, present in gills of *C. maenas*. The results obtained from in vitro incubation of CHH with gill membranes and an in vivo half life study suggest that ACELA may be an enzyme involved in regulation of CHH titres in hemolymph, and CHH can be an endogenous substrate for this enzyme.

MATERIALS AND METHODS

Animals

C. maenas were collected with baited traps from the Menai Strait (Wales, UK). They were kept in re-circulated seawater at 12–15°C under ambient light conditions. Adult male crabs at intermolt (carapace width: 45–55 mm) were used for all experiments.

Chemicals

Hippuryl-1-histidyl-1-leucine (Hip-His-Leu), hippuric acid, captopril {1-[(2S)-3-mercapto-2 methylproprionyl]-L-proline}, and lisinopril {(S)-1{N2-(1-carboxy-3-phenylpropyl)-L-lysyl]-L-proline dihydrate} were purchased from Sigma (St. Louis, MO). Angiotensin I was obtained from Novabiochem.

Purification, Quantification, and Iodination of CHH

The methods for isolation, purification, and quantification of *C. maenas* CHH and iodination procedures were as described in Webster (1993). Briefly, the extracts of sinus glands were first isolated on a Phenyl column (4.6 × 250 mm, Waters) using a gradient 30–80% B over 60 min at a flow rate of 1.0 ml/ min. (A = 0.11% trifluoroacetic acid [TFA] in H_2O; B = 0.1% TFA in 60% acetonitrile.) The fractions containing CHH were then pooled and re-run on a C_{18} column (4.6 × 250 mm, Phenomenex) using a gradient 45–60% B over 30 min at same flow rate. Peaks were manually collected. The quantification of neuropeptides was performed by amino acid analysis of hydroly-

sates obtained by gas-phase hydrolysis in vacuo at 150°C for 1 h using azeotropic hydrochloric acid containing a trace of phenol. Vacuum-dried hyrolysates were then quantified for amino acid composition by *o*-phthalaldehyde pre-column derivertization. Then, 300 pmol of CHH was iodinated with 300 μCi of [^{125}I](Amersham) using the Chloramine T method.

Preparation of Membranes

Anterior and posterior gills were dissected from crabs (n = 6), after being pre-chilled on ice for 30 min. After rinsing in ice-cold crustacean saline (Webster and Keller, 1986), gills were homogenized with a Polytron® in ice-cold buffer containing 50 mM HEPES, either in the presence or absence of 300 mM NaCl (pH 8.3). Homogenates were centrifuged at 1,000*g* for 10 min at 4°C and supernatants were further centrifuged at 30,000*g* for 40 min at 4°C. The resulting pellets, once re-suspended in 50 mM HEPES, were estimated for protein concentration using a modified Lowry method (Lowry et al., 1951). Aliquots of membrane protein (120–150 μg/μl) were immediately frozen in liquid nitrogen and kept at –20°C until used.

Treatment of Gill Membranes With Ethyldiaminetetraacetic Acid (EDTA)

Membranes, pre-incubated with 5 mM EDTA in the assay buffer (50 mM HEPES and 300 mM NaCl, pH 8.3) for 30 min at 37°C, were centrifuged at 30,000*g* for 40 min at 4°C. Pellets were washed twice with the assay buffer and the final pellets were re-suspended in the same buffer.

Phase Separation of Membrane Proteins With Triton X-114

Membranes were initially incubated on ice (30 min with occasional shaking) with 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 1.5% (w/v) Triton X-114. Detergent-insoluble material was removed by centrifugation at 30,000*g* for 40 min at 4°C. Portions of the supernatant were gently overlaid on 200 μl of the same buffer containing 0.06% (w/v) Triton X-114 and 6% (w/v) sucrose and incubated for 5 min at 37°C. Phase separation of Triton X-114 was obtained by immediate centrifugation at 13,000 rpm for 5 min at 20°C. The supernatant was collected and the detergent layer was re-suspended in an equal volume of buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.4). Both detergent-rich and detergent-poor fractions were tested for ACELA.

ACE Assay

ACELA was determined by incubating membrane preparations (20 μg/20 μl final incubation volume) in the presence of 3 nmol of Hip-His-Leu in the assay buffer described above, either in the presence or absence of captopril (final concentration at 18.75 μM) for 30 min at 37°C. The enzyme activity was calculated by the estimation of the difference between amounts of remaining Hip-His-Leu at times 0 and 30 min after incubation. At the beginning or end of incubation, samples were acidified with glacial acetic acid (to give a final concentration of 2 N in 100 μl final volume). The samples were mixed and centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant (~100 μl) was applied onto a RP-HPLC connected to a C18 column (Bakerbond WP C18, 4.6 × 250 mm, J.T. Baker Co.), with a gradient of 30–80% B over 30 min (A = 0.11% TFA in H_2O, B = 0.1% TFA in 60% acetonitrile). Absorbance was monitored at 210 nm with a flow rate of 1 ml/ min. The samples of AG-I and CHH were chromatographed under the same conditions.

Degradation of Angiotensin-I and CHH by *C. maenas* ACELA in Gills and Amino Acid Analysis of Peaks

Substrate specificity of *C. maenas* ACELA in gills was tested using synthetic ACE substrate, AG I, and CHH. Each substrate (3 nmol) except CHH (300 pmol) was incubated with a membrane concentration of 40 μg/50 μl at 37°C for 30 min, either in the presence or in the absence of captopril (360

μM). The samples were treated as described above for RP-HPLC separation. The degradation rate of each substrate was calculated by comparing peak areas of substrate in the presence or in the absence of captopril. Peaks were collected manually and analyzed for amino acid composition as described above.

Statistics

Statistical analysis was carried out using either Student's *t*-test or ANOVA (GraphPad InStat version 3.0, GraphPad Software, San Diego, CA).

RESULTS

The Tissue Distribution of ACELA in Crabs

The following tissues from male intermolt *C. maenas* were examined for ACELA: muscle (from walking legs), hypodermis, hindgut, heart, midget gland, brain, hemolymph, and gills. Of these tissues tested, gills displayed by far the highest level of ACELA (Fig. 1). Brains, and to a lesser extent testis and muscle membranes, showed much less enzyme activity than that of gill membranes (Fig. 1). Gill membranes prepared from the edible crab, *Cancer pagurus*, also exhibited ACELA, with approximately 30% of that in *C. maenas* gills.

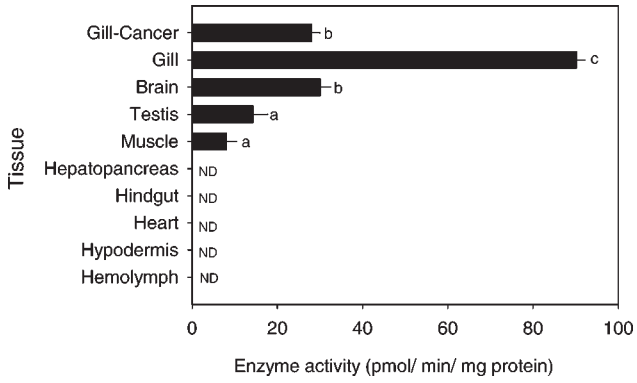

Fig. 1. Tissue distribution of ACELA. Enzyme activity (pmol/min/mg protein) was presented as mean ± 1 SE, n = 5–8. ND = non-detected. Statistical significance was tested using nonparametric ANOVA (Kruskal-Wallis Test).

Gill membranes prepared from premolt crabs showed similar levels of enzyme activity (0.995 ± 0.025 nmol/min/mg protein) to that of intermolt (1.044 ± 0.05 nmol/min/mg protein).

Characteristics of ACELA in Crab Gills

The saturation curve analyses of Lineweaver-Burk plot (Fig. 2) of the hydrolysis of Hip-His-Leu by ACELA in gill preparation gave values of Km, 0.23 ± 0.012 mM and Vmax, 7.6 nmol/min/mg protein, respectively. All substrate concentrations used were well below the Km value and in no case was more than 10% of the substrate consumed during the experimental period. Captopril inhibited ACE activity in a dose-dependent manner with a value of IC_{50} < 1 μM (Fig. 2B).

To determine the effect of Cl^- concentration on the hydrolysis of ACE substrate by gill membrane preparations, NaCl was added to the incubation medium to give final concentrations of Cl^- ranging from 0 to 300 mM. ACELA was highly dependent on Cl^- concentrations. The maximal activity was observed at 25 mM of Cl^-, double the activity compared to that at 0 mM (Fig. 3). Increasing Cl^- concentration to over 300 mM did not change the maximal enzyme activity.

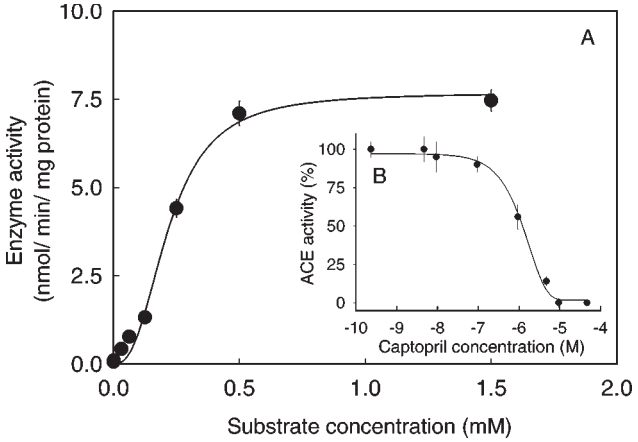

Fig. 2. **A:** Michaelis-Menten plot of the hydrolysis of synthetic ACE substrate by *Carcinus* gill membranes. **B:** ACELA was inhibited by captopril in a dose-dependent manner. Each point in A and B was shown as mean ± 1 SE (n = 4–6).

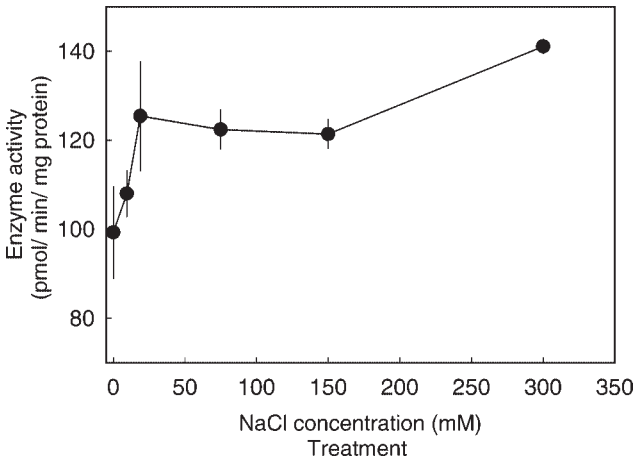

Fig. 3. Effect of Cl^- concentrations on ACELA in gills. Each point was presented as mean ± 1 SE (n = 4–6).

Effect of a Divalent Chelating Agent (EDTA) and Divalent Metal Ions on the ACELA

EDTA (100 μM) -treated gill membranes contained 21% of enzyme activity compared with untreated controls. Inclusion of the following bivalent metal ions (Co^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Zn^{2+}) was carried out to determine whether enzyme activity could subsequently be retrieved from the gill membranes pre-treated with 5 mM of EDTA. The addition of 100 μM of either Zn^{2+} or Co^{2+} restored approximately 89 and 56% of enzyme activity, while Mn^{2+}, Mg^{2+}, or Fe^{2+} application regained less activity (37, 19, and 13%, respectively). In contrast, the remaining ACELA in the membrane pre-treated with EDTA was completely abolished by the addition of 100 μM of Cu^+.

Phase Separation of *C. maenas* ACELA

The phase separation property of Triton X-114 at >30°C in partitioning hydrophobic proteins into the detergent-rich phase has been used for isolating integral membrane proteins. ACELA was estimated after gill membrane was treated with Triton X-114, to determine if this enzyme activity was associated with an integral membrane protein. Approximately 80% of the activity was in the detergent-poor phase, while the remaining activity (20%) was recovered from the detergent-rich phase.

In Vivo Clearance Rate of CHH

Initially, 120 fmol of [^{125}I] CHH (≈200,000 DPM) was injected into crabs. Two minutes after injection (to allow distribution of the injected bolus), the radioactivity in the hemolymph was measured and was considered to be at a reference time 0 (100%). At 5, 10, and 30 min, small hemolymph samples (100 μl) were removed and radioactivity was measured by a gamma counter (Wallac 1470). The hormone was cleared very rapidly, with an estimated half-life of 8 min (Fig. 4). When crabs were first injected with captopril, to give an estimated circulating concentration of 500 μM, followed by hormone injection, the half-life increased to 13 min. At sampling points of 15 and 30 min, crabs injected with captopril showed a significantly high recovery of [^{125}I]CHH in their hemolymphs compared to controls ($P > 0.01$ and $P > 0.05$, respectively). Subsequent dissection of major tissues from these crabs (gills, hindgut, hepatopancreas, testis, heart, muscle, and hypodermis) followed by the

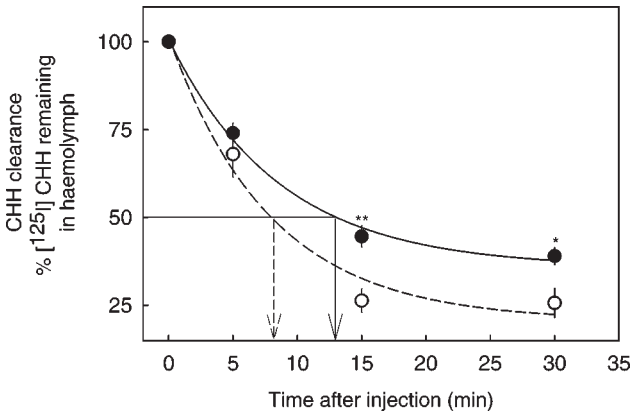

Fig. 4. The clearance of [^{125}I] CHH from hemolymph, in vivo. Animals were injected with [^{125}I]CHH and hemolymphs were removed at the sampling points. The samples were counted for the radioactivity remaining in hemolymphs. Closed circles: In the absence of captopril; Open circles: in the presence of captopril. Each point was shown as mean ± 1 SE (n = 8). Statistical significance was obtained by the comparison between in the absence and the presence of captopril at sampling points of 15 min (**) and 30 min (*), respectively. $^{**}P < 0.005$, $^{*}P < 0.05$ (Student's *t*-test).

measurement of residual radioactivity revealed that almost 98% of the radioactivity was found in gills. The radioactivity was equally distributed over gill pairs, and there was no difference between anterior and posterior gills (Fig. 5).

Degradation of Angiotensin-I and CHH by *C. maenas* ACELA in Gills

To determine substrate specificity of ACELA in *C. maenas* gills, Hip-His-Leu, AG-I, and CHH were incubated with gill membranes in the presence of captopril. The presence of captopril at a concentration of 360 μM reduced the degradations of Hip-His-Leu by 46%, of AG-I, by 13%, and of CHH by 83%. Peaks identified by amino acid analyses show in Figure 6A that *C. maenas* ACELA processed Ag-I (peak 4) to Ag-II (peak 3) by cleaving off His-Leu-OH (peak 1) from the C-terminus. Peak 2 (AG-III), the product of peak 3, was further cleaved by aminopeptidase. Amino acid composition of peak 1 of Figure 6B (Table 1) showed that it was a degraded product of CHH, while peak 2 was identified as intact CHH.

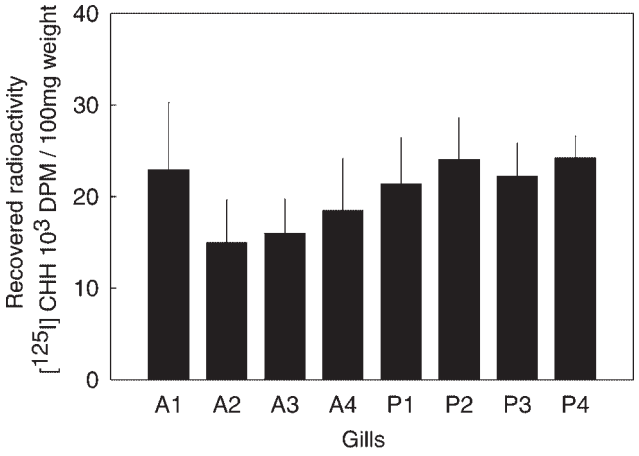

Fig. 5. The distribution of injected [^{125}I] CHH in gills. Gills were collected 30 min after the injection of radioactive CHH. After rinsing and drying, the radioactivity was measured. Anterior gills (4) were presented as A1–A4; posterior gills, P1–P4. Bar was presented as mean ± 1 SE (n = 8). There was no statistical significance (at $P < 0.05$) among the values of [^{125}I] CHH obtained from gills (nonparametric ANOVA, Kruskal-Wallis Test).

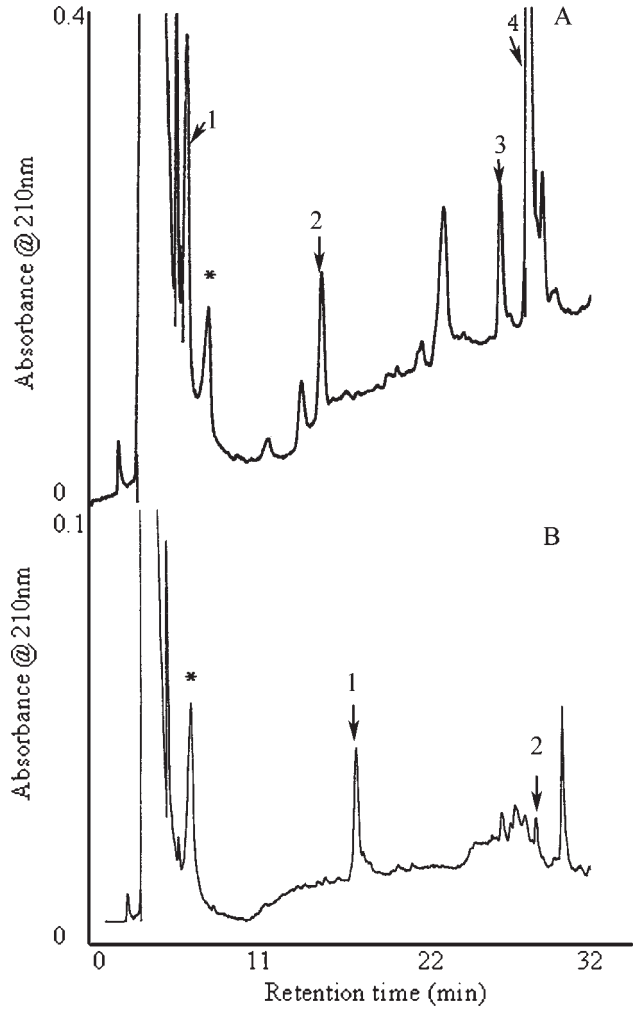

Fig. 6. RP-HPLC chromatogram showed cleavage of AG-I (A) and CHH (B) after incubation with membranes prepared from gills. The results of amino acid composition of each peak are as follows. **A:** Peak 1: His-Leu-OH; Peak 2 (AG-III): Arg-Val-Tyr-Ile-His-Pro-Phe-OH; Peak 3 (AG-II): Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH; and Peak 4: H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH. **B:** Peak 1: Digested CHH; Peak 2: CHH intact. *Captopril. Note that the peaks obtained from each HPLC do not accurately represent amount of product formed due to a difference in extinction coefficients.

DISCUSSION

The present study using the synthetic substrate of mammalian ACE (Hip-His-Leu-OH) and captopril, a selective specific inhibitor, demonstrated ACELA in gill, brains, and testis of the shore crab, *C. maenas*. The highest ACELA was detected in the

TABLE 1. The Amino Acid Composition of Each Peak (Fig. 6A and B)*

	AG peaks (no.)				CHH peak (no.)	
AA	1	2	3	4	1	2
Asx			0.8 (1)	0.8 (1)	3.59	6.9 (13)
Glx					4.5	5.8 (6)
Ser					2.6	4.0 (4)
His	0.9 (1)			0.9 (1)	0.97	0.8 (1)
Thr					1.7	1.8 (2)
Arg		1.1 (1)	1.1 (1)	1.1 (1)	5	4.7 (5)
Gly					1	0.9 (1)
Ala					2.94	4.7 (4)
Tyr		0.8 (1)	0.9 (1)	0.9 (1)	3.96	6.87 (7)
Met					[b]	[b] (4)
Val		0.9 (1)	0.9 (1)	0.9 (1)	1.9	5.7 (6)
Phe		0.9 (1)		0.9 (1)	0.9	2.6 (3)
Ile		0.9 (1)	0.9 (1)	0.9 (1)	1.2	1.2 (1)
Leu	1.1 (1)			1.1 (1)	2.1	5.8 (6)
Lys			0.8 (1)	0.8 (1)	2.3	2.9 (2)

*Amino acids (AA) are presented as three letter abbreviation. Numbers shown in upper layer were obtained from the amino acid analysis of the peaks shown in Figure 6A and B, while those in parentheses are based on the amino acid sequences of peptides and their fragments.
[a]Pro and Cys were not determined.
[b]Met was unable to be calculated.

gill membranes. Enzyme activities in gills of premolt crabs were similar to activities of intermolt animals, suggesting that this enzyme is probably not involved in the molting process. The wide distribution of ACELA in various tissues of *C. maenas* is in agreement with previous results found in the blue crab, *C. sapidus* (Smiley and Doig, 1994). Interestingly in *C. granulatus,* ACELA found in CNS and gills was co-exited with AGII (Delorenzi et al., 1996). Additionally, the gill membranes of the edible crab, *C. pagurus*, also exhibited ACELA, suggesting that ACELA in gills may be a common feature in crab species at least.

Based on our results, the properties of *C. maenas* ACELA were also similar to those of mammalians and invertebrates including houseflies and leeches (Salzet and Verger-Bocquet, 2001, Ekbote et al., 2003; Macours et al., 2003). The identical properties are listed as follows: (1) Zn^{2+} metalloenzyme, (2) captopril and lisinopril sensitive, and (3) Cl^{-}-dependent activity. Furthermore, *C. maenas* ACELA seemed to be a membrane-bound protein, but it could be easily dissociated from the membrane. After phase separation with Triton X-114, most of the enzyme activity was found in the detergent-poor phase, like the one in the leech and housefly (Salzet and Verger-Bocquet, 2001; Laurent et al., 1997). However, ACELA was not detected in *C. maenas* hemolymph, while similar activity was described in hemolymph of *Mytilus edulis* (Leung et al., 1992).

By injection of [^{125}I] CHH into *C. maenas*, it was possible to monitor the clearance rate from hemolymph as well as tissue-specific accumulation of this radiolabelled peptide. The measured half-life of *C. maenas* [^{125}I] CHH in hemolymph was 5–10 min, in agreement with our previous results obtained from non-radiolabelled CHH injection into crabs (Webster, 1996; Chung and Webster, 1996). However, in the presence of captopril, the half-life of this peptide was increased by more than 5 min. Over 99% of the radioactivity was recovered from the gill tissues, in which it was distributed equally between the left and right sides of the gills. Posterior gills showed slightly higher radioactivity than those of anterior gills, but the difference was not statistically different. This implies that the distribution of ACELA is ubiquitous, throughout posterior and anterior gills.

C. maenas ACELA in gills produced AG-II by cleaving the dipeptide from the C terminus of AG-I (Fig. 6A). Interestingly, AG-II was further processed by an aminopeptidase that was co-present in gill membranes, yielding a further product, peak 2 (AG-III). The combination of selective inhibitors of neprilysin (phosphoramidon and 1,10 o-phenanthroline) and aminopeptidase (amastatin) with these substrates showed that each of these peptides can be degraded by more than one enzyme in the tissue membrane extract (Masler et al., 1996; Leung et al., 1992). Surely, synthetic ACE substrate containing a single ACE-specific cleavage site can be the only substrate for ACE, whereas AG-I might be a substrate for neprilysin in the presence of captopril. As a result, the inhibitory effect of captopril on AG-I degradation was much smaller than that of the synthetic substrate. This result is congruent with a finding from the leech (Ekbote et al., 2003), also suggesting the presence of multiple enzymes in the gill membrane preparations.

The highest inhibition (83%) of ACE in the presence of captopril was observed with *C. maenas* CHH

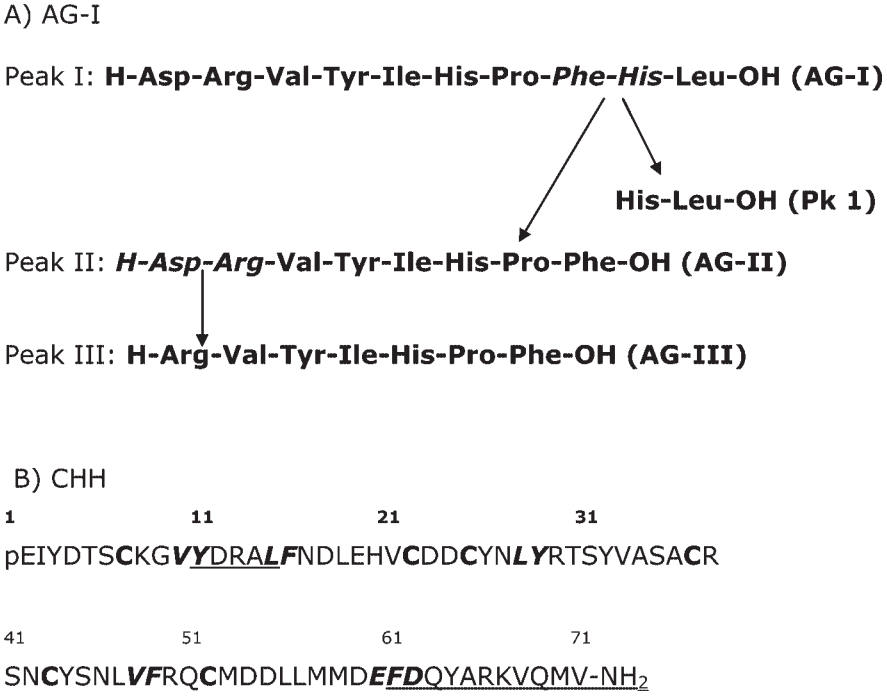

Fig. 7. Proposed degradation pathway of AG-I (**A**) and putative cleavage sites of ACELA in CHH (**B**). Amino acids italicized: cleavage sites; amino acids underlined: parts possibly cleaved.

compared with AG-I and synthetic ACE substrate. Considering the nature of known ACE substrates, CHH would seem an unlikely candidate since it is large (72 amino acid residues), blocked at both N- and C-termini, and has three intradisulphide bridges (Kegel et al., 1989). The solution structures of CHH neuropeptides (CHH and MIH) obtained by circular dichroism consisted of a very high percentage of α-helices and β-strands (Chung and Webster, 1996; Katayama et al., 2003). A close examination of the primary sequence of CHH reveals that there are more than five putative ACE cleavage sites (Fig. 7B), but these putative cleavage sites may not be readily available to ACE since they may not be exposed to the surface of the molecule. Nevertheless, our preliminary data obtained from the amino acid analysis of two peaks of CHH cleaved by ACELA indicated that the cleavage occurred at a part of the C-terminus and potentially the area between disulphide bridges, resulting in fragmented and inactivated CHH. However, we are unable to locate the exact cleavage site of CHH. More work is required to obtain detailed information on the degradation pathway of CHH. Our initial study into the substrate specificity of crab ACELA present in gills agreed with a broad range of enzyme activity including endopeptidase function on a wide substrate specificity (Isaac et al., 1998).

Crustacean gills, which are multifunctional tissues composed of at least six different cell types (Mantel and Farmer, 1983; Lawson et al., 1994), seem to serve as a target tissue of the CHH receptor (Chung and Webster, 2006) as well as a site of degradation. It will be of interest to localize the exact cells that exhibit ACELA.

In conclusion, we propose that ACELA present in the gill membranes of *C. maenas* participates in regulating the levels of circulating neuropeptides. Additionally, since ACELA is found in the gills of following species, *C. maenas, C. pagurus, C. sapidus,* and *C. granulatus*, we believe that this enzyme may be ubiquitously present in all crustaceans with at least a function in the processing/inactivation/degradation of circulating neuropeptides.

ACKNOWLEDGMENTS

This work was supported by a grant to S.G.W. from the Biotechnology and Biological Sciences Research Council, UK.

LITERATURE CITED

Chang ES, Keller R, Chang SA. 1998. Quantification of crustacean hyperlgycemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. Gen Comp Endocrinol 111:359–366.

Chung JS, Webster SG. 1996. Does the N-terminal pyroglutamate residue have any physiological significance for crab hyperglycaemic neuropeptides? Eur J Biochem 240: 358–364.

Chung JS, Webster SG. 2005. Dynamics of in vivo release of molt-inhibiting hormone (CHH) and crustacean hyperglycaemic hormone (CHH) in the shore crab, *Carcinus maenas*. Endocrinology 146:5545–5551.

Chung JS, Dircksen H, Webster SG. 1999. A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. Proc Natl Acad Sci USA 96:13103–13107.

Delorenzi A, Pedreira ME, Romano A, Garcia SY, Pirola CJ, Nahmod VE, Maldonado H. 1996. Angiotensin II enhances long-term memory in the crab *Chasmagnathus granulatus.* Brain Res Bull 41:211–220.

Ekbote UV, Weaver RJ, Isaac RE. 2003. Angiotensin I- converting enzyme (ACE) activity of the tomato moth, *Lacanobia oleracea*: changes in levels of activity during development and after copulation suggest roles during metamorphosis and reproduction. Insect Biochem Mol Biol 33:989–998.

Garside CS, Hayes TK, Tobe SS. 1997. Inactivation of Dipallatostain 5 by membrane preparations from the cockroach *Diploptera punctata*. Gen Comp Endocrinol 108: 258–270.

Goldsworthy GJ, Kodrik D, Comley R, Lightfoot M. 2002. A quantitative study of adipokinetic hormone of the firebug, *Pyrrhocoris apterus.* J Insect Physiol 48:1103–1109.

Hens K, Vandingenen A, Macours N, Baggerman G, Karaoglanovic AC, Schoofs L, De Loof A, Huybrechts R. 2002. Characterization of four substrates emphasizes kinetic similarity between insect and human C-domain angiotensin-converting enzyme. Eur J Biochem 269:3522–3530.

Isaac RE, Coates D, Williams TA, Schoofs L. 1998. Insect angiotensin-converting enzyme: comparative biochemistry and evolution. In: Coast GM, Webster SG, editors. Recent advances in arthropod endocrinology, Society for Experimental Biology Seminar Series. Cambridge: Cambridge University Press. p 357–378.

Katayama H, Nagata K, Ohira T, Yumoto F, Tanokura M, Nagasawa H. 2003. The solution structure of molt-inhibiting hormone from the Kumara prawn *Marsupenaeus japonicus.* J Biol Chem 278:9620–9623.

Kegel G, Reichwein B, Weese S, Gaus G, Peter-Katalinic J, Keller R. 1989. Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas*. FEBS Lett 255:10–14.

Lamango NS, Isaac RE. 1994. Identification and properties of a peptidyl dipeptidase in the house fly, *Musca domestica*, that resembles mammalian angiotensin-converting enzyme. Biochem J 299:651–657.

Lamango NS, Sajid M, Isaac RE. 1996. 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*. Biochem J 314:639–646.

Laurent V, Salzet M. 1996a. Biochemical properties of the angiotensin-converting like enzyme from the leech *Theromyzon tessulatum*. Peptides 17:737–745.

Laurent V, Salzet M. 1996b. Metabolism of angiotensins by head membranes of the leech *Theromyzon tessulatum*. FEBS Lett 384:123–127.

Laurent V, Stefano G, Salzet M. 1997. Presence and biochemical properties of a molluscan invertebrate angiotensin-converting enzyme. Regul Pept 69:53–61.

Lawson SL, Jones MB, Moate RM. 1994. Structural variability and distribution of cells in a posterior gill of *Carcinus maenas* (Decapoda: Brachyura). J Mar Biol Ass UK 74:771–716.

Leung MK, Houston B, Stefano GB. 1992. Degradation of Metenkephalin by hemolymph peptidases in *Mytilus edulis*. Cell Mol Neurobiol 12:367–378.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Macours N, Hens K, Francis C, De Loof A, Hybrechts R. 2003. Molecular evidence for the expression of angiotensin converting enzyme in hemocytes of *Locusta migratoria*: stimu-

lation by bacterial lipopolysaccharide challenge. J Insect Physiol 49:739–746.

Mantel LH, Farmer LL.1983. Osmotic and ionic regulation. In Mantel LH, editor. The biology of crustacea: internal anatomy and physiological regulation, vol. 5. New York: Academic Press. p 53–161.

Masler EP, Wagner RM, Kovaleva ES. 1996. In-vitro metabolism of an insect neuropeptide by neural membrane preparations from *Lymantria dispar*. Peptides 17:321–326.

Nakatsuji T, Sonobe H. 2003. Measurement of molt-inhibiting hormone titer in hemolymph of the American crayfish, *Procambarus clarkii*, by time-resolved fluoroimmunoassay. Zool Sci 20:999–1001.

Phlippen MK, Webster SG, Chung JS, Dircksen H. 2000. Ecdysis of decapod crustaceans is associated with dramatic release of crustacean cardioactive peptide into the hemolymph. J Exp Biol 203:521–536.

Rayne RC, O'Shea M. 1992. Inactivation of neuropeptide hormones (AKH-I and AKH-II) studied in vivo and in vitro. Insect Biochem Mol Biol 22:25–34.

Salzet M, Verger-Bocquet M. 2001. Elements of angiotensin system are involved in leeches and molluscs immune response modulation. Brain Res Mol Brain Res 94:137–147.

Smiley JM, Doig MT. 1994.Distribution and characterisation of angiotensin-converting enzyme-like activity in tissues of the blue crab, *Callinectes sapidus*. Comp Biochem Physiol 108A:491–496.

Suenobu A, Mizoguchi A, Ichikawa T. 2004. Relationship between firing activity of bombyxin-producing neurosecretory cells and hemolymph bombysin titer in the silkworm *Bombyx mori.* Gen Comp Endocrinol 137:219–226.

Strey A, Hayes TK, Isaac RE. 1993. Metabolism of insect hypertrehalosemic hormone in *Blaberus discoidalis* cockroaches. Biochem Soc Trans 21:244S.

Vandenbroeke F, Laurent V, Verger-Bocquet M, Stefano M, Salzet M. 1997. Biochemical identification and ganglionic localization of leech angiotensin-converting enzymes. Brain Res Mol Brain Res 49:229–237.

Vandingenen A, Hens J, Baggerman G, Macours N, Schoofs L, De Loof A, Huybrechts R. 2002. Isolation and characterization of an angiotensin coverting enzyme substrates from vitellogenic ovaries of *Neobellieria bullata.* Peptides 23:1853–1863.

Webster SG. 1993. High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane bound receptors on the Y-organ of the shore crab *Carcinus maenas*. Proc R Soc Lond B 251:53–59.

Webster SG. 1996. Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. J Exp Biol 199:1579–1585.

Webster SG, Chung JS. 1999. Roles of moult-inhibiting hormone and crustacean hyperglycemic hormone in controlling moulting in decapod crustaceans. In: Roubos EW, Bonga SEW, H Vaudry H, DeLoof A, editors. Recent developments in comparative endocrinology and neurobiology, Proceedings of the 19th Conference for European Comparative Endocrinology. Maastricht: Shaker. p 213–216.

Webster SG, Keller R. 1986. Purification, characterization and amino acid composition of the putative molt-inhibiting hormone (MIH) of *Carcinus maenas* (Crustacea, Decapoda). J Comp Physiol B 156:617–624.