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Angiotensin-converting enzyme inhibition studies by natural leech inhibitors by capillary electrophoresis and competition assay

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A protocol to follow the processing of angiotensin I into angiotensin II by rabbit angiotensin-converting enzyme (ACE) and its inhibition by a novel natural antagonist, the leech osmoregulator factor (LORF) using capillary zonal electrophoresis is described. The experiment was carried out using the Beckman PACE system and steps were taken to determine (a) the migration profiles of angiotensin and its yielded peptides, (b) the minimal amount of angiotensin II detected, (c) the use of different electrolytes and (d) the concentration of inhibitor. We demonstrated that LORF (IPEPYVWD), a neuropeptide previously found in leech brain, is able to inhibit rabbit ACE with an IC_{50} of 19.8 μM. Interestingly, its cleavage product, IPEP exhibits an IC_{50} of 11.5 μM. A competition assay using *p*-benzoylglycylglycylglycine and insect ACE established that LORF and IPEP fragments are natural inhibitors for invertebrate ACE. Fifty-four percent of insect ACE activity is inhibited with 50 μM IPEP and 35% inhibition with LORF (25 mM). Extending the peptide at both N- and C-terminus (GWEIPEPYVWDES) and the cleavage of IPEP in IP abolished the inhibitory activity of both peptides. Immunocytochemical data obtained with antisera raised against LORF and leech ACE showed a colocalization between the enzyme and its inhibitor in the same neurons. These results showed that capillary zonal electrophoresis is a useful technique for following enzymatic processes with small amounts of products and constitutes the first evidence of a natural ACE inhibitor in invertebrates.

Keywords: capillary electrophoresis; invertebrate; leech; natural angiotensin-converting inhibitor.

In mammals, angiotensin-converting enzyme (ACE) is a well known zinc-metallopeptidase that converts angiotensin I to the potent vasoconstrictor angiotensin II and degrades bradykinin, a powerful vasodilator, both for regulation of vascular tone and cardiac functions [1,2]. Synthetic substrates were developed for the determination of ACE activity in various biological fluids, mostly human plasma, for the diagnosis of sarcoidosis and other granulomatous diseases [3]. After the successful use of captopril, the first ACE inhibitor in the treatment of hypertension, a number of molecules have been synthesized and used in the treatment of congestive heart failure and for preventing cardiac impairment after myocardial infarction [2–4]. The development of this class of anti-hypertensive drugs benefited from structural data on carboxypeptidase active sites [5]. In the last two decades, the ACE gene has been cloned allowing the identification of two isoenzymes: somatic ACE resulting from gene duplication and primarily expressed in endothelial cells, and the germinal or testicular ACE, resulting from the transcription in the male reproductive system from intragenic promoter of a hydrophobic C-terminal peptide for membrane-anchoring, specifically cleaved by a metalloprotease to release soluble forms of both isoenzymes [6]. Recently, a new ACE, termed ACE2, has been characterized [7–9]. The ACE2 gene maps to defined quantitative trait loci on the X chromosome in three different rat models of hypertension, suggesting ACE2 as a candidate gene for hypertension [7–9]. As mice deficient in both ACE2 and ACE show completely normal heart function, it appears that ACE and ACE2 negatively regulate each other. The mechanisms and physiological significance of the interplay between ACE and ACE2 have not yet been elucidated, but it may involve several new peptides and peptide systems [7–9].

Moreover, the recent work of Dive and colleagues [10] showed that the cleavage of angiotensin I and bradykin by somatic ACE appear to obey to different mechanisms. *In vivo* experiments in mice demonstrated that the selective inhibition of either the N- or C-domain of ACE by inhibitors prevents the conversion of angiotensin I to angiotensin II, while bradykin protection requires the

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Abbreviations: AII-amide, angiotensin II-amide; a-AI, anti-angiotensin I; ACE, angiotensin-converting enzyme; AP, aminopeptidase; LORF, leech osmoregulator factor; Neb-ODAIF, *N. bullata* ovary-derived ACE interactive factor.

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inhibition of the two ACE active sites. The conversion of angiotensin I seems to involve the two active sites of ACE, free of inhibitor. These findings suggest that the gene duplication of ACE in vertebrates may represent a means for regulating the cleavage of angiotensin I differently from that of bradykin, implicating natural inhibitors [10]. In this context, research of natural ACE inhibitors [11,12] seems to be a promising way for discovering novel pharmaceutical drugs to treat cardiovascular diseases [5,13]. Moreover, the discovery of such molecules in different animal models would allow a variety of such natural ACE inhibitors to be identified.

In insects, ACE substrate/inhibitor peptides have been characterized from *Neobellieria bullata* ovaries. One of them is a peptide of 1312.17 Da named the *N. bullata* ovary-derived ACE interactive factor (Neb-ODAIF: NKLKPSQ WISL) [14,15]. It interacts with both insect and human ACE and shows high sequence similarity to a sequence at the N-terminal part of dipteran yolk polypeptides [16]. Two peptides are active towards human somatic ACE, the Neb-ODAIF(1–9) and its shorter form Neb-ODAIF (1–7). K_m values of Neb-ODAIF and Neb-ODAIF(1–9) or human somatic ACE (sACE) are 17 and 81 μM, respectively. Additionally, Neb-ODAIF(1–7) (NKLKPSQ) also interacts with sACE (K_m = 90 μM) [14–16].

In leeches, the central nervous system is known to influence water balance [17,18]. In the rhynchobdellid leech *Theromyzon tessulatum* genital maturity is concomitant with a phase of water retention reflected by an increase in mass of the animals and correlated to a cœlomic accumulation of yolk proteins [19]. The neuropeptide (IPEPYVWD) named leech osmoregulator factor (LORF) seems to be implicated in this biological phenomenon [20,21]. Its amount greatly increases during this stage of the leech life span. When injected into leeches, it increases the animal mass [20,21]. However, its mode of action is as yet unclear. LORF has been isolated from the CNS of *T. tessulatum* [20] as well as from sex ganglia [21] and in rat CNS [22].

In this context, in order to check the ability of LORF to act on water balance through ACE activity inhibition, we developed a quick, reproducible, highly sensitive test of angiotensin I processing by ACE and its inhibition in a one-step analysis by capillary zonal electrophoresis. Thus, we report for the first time in invertebrate the existence of a novel ACE inhibitor, the LORF peptide and its cleavage product IPEP.

Materials and methods

Chemical

Angiotensin I (DRVYIHPFHL: AI), angiotensin II (DRVYIHPF: AII), FMRF-amide, rabbit ACE were obtained from Sigma.

Peptide synthesis

LORF (IPEPYVWDamide, IPEPYVWD), IPEP, YVWD, IP, YVWDamide and GFEIPEPYVWD were synthesized according to classical Fmoc chemistry on *p*-alkoxybenzyl alcohol resin on a 25-μmol scale with a ABI 432A. Conventional side chain-protecting groups were used 2,3,5,7,8-pentamethylchroman 6-sulfonyl (Arg), triphenylmethyl (Cys, Asn and Glu), *t*-butoxycarbonyl (Lys) and *t*-butyl (Ser and Tye). Briefly, a standard Fmoc deprotection was used in conjunction with benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate/*N*-hydroybenzotriazole/diisopropyethylamine. Coupling reactions were allowed to proceed for 15 min. After two dimethylformamide washings, a second coupling with the same excess of reagents was routinely performed. At the end of the synthesis, the resin was washed with dichloromethane and ether and dried under nitrogen. The final trifluoroacetic acid cleavage was performed in the same reaction vessel with 5 mL of reacting buffer (100 μL trisopropylsilane, 100 μL ethanedithiol and 1.8 mL trifluoroacetic acid) for 150 min. At the end of this time, the peptide was drained in a 40-mL polypropylene centrifuge tube previously filled with 25 mL of cold ether. The peptide was then centrifuged, and the pellet was washed twice with ether. After the second centrifugation, the pellet containing the reduced peptide was dissolved in 0.1 M ammonium acetate buffer (pH 8.5) at a concentration of 35 $mg \cdot L^{-1}$ and was allowed to refold by air oxidation for 17 h at room temperature under constant stirring. The refolded peptide was purified by semi preparative reversed-phase chromatography (Aquapore RP300 column, 250 × 7.0 mm) with a linear gradient of acetonitrile 1% min^{-1} in acidified water (0.1%) at a flow rate of 1 $mL \cdot min^{-1}$.

Inhibitory kinetic studies by capillary zonal electrophoresis

Assays of ACE activities were carried out with 12.5 μU ACE incubated with 30 μM angiotensin I in absence or in presence of 10–40 μM inhibitors in Tris/NaCl (100 μM Tris/HCl, pH 8.4) with a total volume of 100 μL. Reactions were incubated for 45 min at 37 °C and were terminated by addition of 1% trifluoroacetic acid (v/v). The internal standard FMRF-amide was added and samples were centrifuged at 20 000 ***g*** for 10 min at 4 °C. Supernatants were collected and dried by speed-vac. Finally, 30 μL sterile water was added on the pellet and peptides were analyzed by capillary zonal electrophoresis.

Samples (2 nL) were injected under vacuum into a PACE 5000 capillary electrophoresis system (Beckman) equipped with a silica capillary (length 57 cm, internal diameter 75 μm). Separation from anode to cathode was carried out in phosphate buffer (25 mM pH 2.5) during 17 min at a voltage of 25 kV and a temperature of 25 °C. The capillary effluent was monitored by absorption at 214 nm. Retention time of each peptide was determined under these migration conditions [23]. The quantification of peptides was carried out by capillary zonal electrophoresis [24].

Competition assay

The ACE competition assay is based on the ACE activity assay using a simple radio assay for angiotensin-converting enzyme [14,15,25]. Briefly, ACE-activity in diluted fly hemolymph is measured with a synthetic, tritiated ACE substrate *p*-[32]benzoylglycylglycylglycine (Sigma) (= standard condition). Adding 10 μM final concentration of captopril (Sigma) served as a negative control. Only the

activity that could be inhibited by captopril was regarded as ACE activity. To find out if a peptide is an inhibitor for ACE, different concentrations of this peptide were added to the standard condition setup. Addition of an ACE inhibitor or an ACE substrate results in competition with the tritium-labelled substrate for ACE and appears as a reduction in ACE activity [25].

Kinetics of degradation

Kinetic parameters were determined from the regression line fitted to the data plotted as $1/V$ vs. 1/[S]. Correlation coefficients were greater than 0.99 [26,27].

Colocalization between enzyme and inhibitor

Antisera. Polyclonal antisera anti-(LORF-amide) and anti-ACE were raised in rabbits using the synthetic LORF-amide or leech ACE N-terminal region (GLPESPGF) coupled to human serum albumin according to the glutaraldehyde method [28]. No cross-reaction with LORF was obtained. The specificity of ACE antiserum has been described elsewhere [29]. In brief, 20% of cross-reaction with rabbit ACE was observed.

Immunohistochemistry. Animals were anesthetized with 0.01% chloretone. Leeches *T. tessulatum* were fixed overnight at 4 °C in Bouin–Holland fixative (+ 10% $HgCl_2$ saturated solution). They were then embedded in paraffin and then sectioned at 7 μm. After removal of paraffin with toluene, the sections were successively treated either with the anti-(LORF-amide) or with the anti-ACE diluted 1 : 800 and with goat anti-(rabbit IgG) IgG conjugated to horseradish peroxidase as described elsewhere [30]. The specificity of the antisera were tested by preabsorbing the antisera overnight at 4 °C with the respective homologous antigen at a concentration of 500 $\mu g \cdot mL^{-1}$ pure antiserum.

Results and discussion

In order to perform a highly and reproducible test allowing the quantification of the ACE hydrolysis activity in absence or presence of selective inhibitor using capillary zonal electrophoresis, several parameters have to be established. Fig. 1 shows the capillary zonal electrophoresis profile of FMRF-amide (internal standard), angiotensin II, angiotensin I and LORF α-amidated. Each peptide possesses a specific retention time permitted it identification. No peak related to ACE has been observed because of the enzyme elimination by acidic precipitation before the centrifugation. The peak area is proportionnal to the peptide concentration as shown in Fig. 2.

In order to determine optimal digestion duration, time-dependent angiotensin II formation from angiotensin I was measured (Fig. 3). After 75 min digestion, the amount of angiotensin II produced by ACE remains constant and 70% of the angiotensin I is cleaved in 40 min by ACE (12.5 μM). No influence of ionic concentration of the digestion buffer was observed on ACE activity (Fig. 4). Taken together, the optimal digestion conditions were determined to be 30 μM of angiotensin I, 12.5 mM ACE in Tris/NaCl 100 mM for 40 min at 37 °C. Under

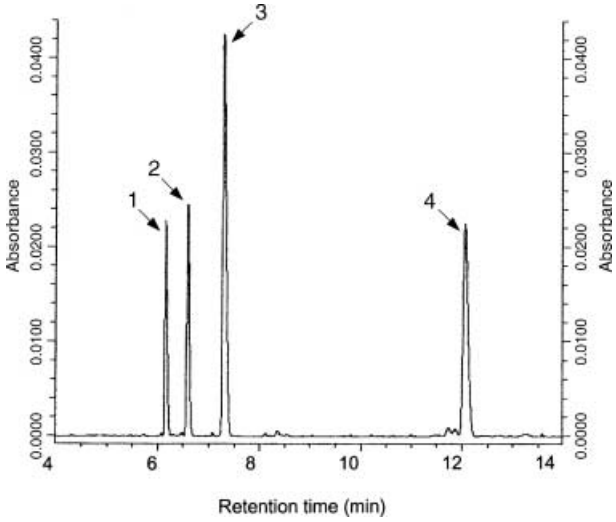

Fig. 1. Capillary zonal electrophoresis migration profile. 1, FMRF-amide; 2, angiotensin I; 3, angiotensin II; 4, LORF-amide. ACE did not appear because the enzyme is eliminated after acidic precipitation and centrifugation.

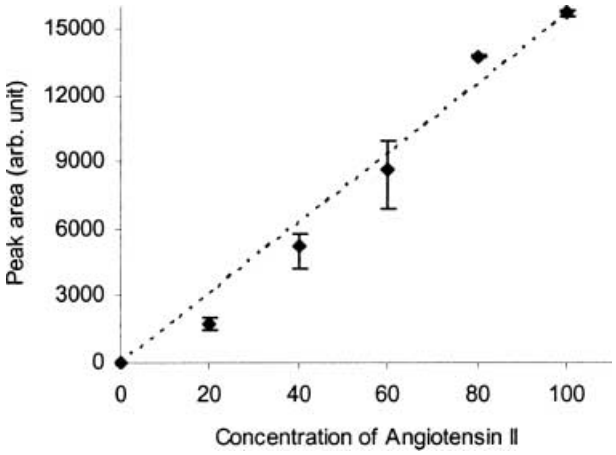

Fig. 2. Different concentration of angiotensin II detected by capillary zonal electrophoresis. Each concentration was measured four times.

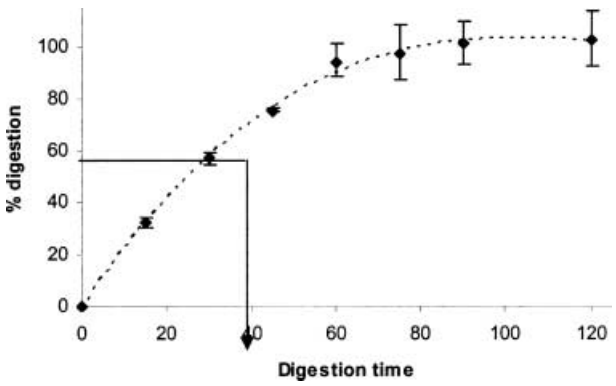

Fig. 3. Determination of the optimal digestion time condition. Thirty micromolar angiotensin I digested by 12.5 μU of rabbit ACE. The experiments were conducted six times.

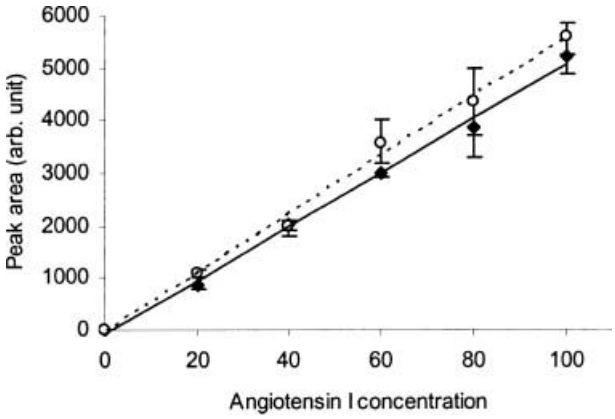

Fig. 4. Influence of the ionic concentration of the digestion buffer on ACE activity. Different concentrations of angiotensin I were digested during 40 min in either Tris/NaCl 50 μM or Tris/NaCl 100 μM buffers. The experiments were conducted six times. ○, 100; ●, 50.

these conditions, the specific activity measured was 5.75 nmol·min^{-1}·g^{-1} enzyme which is in line with the specific activity found for human ACE with Hyppuryl-His-Leu as a chromogenic substrate (10 nmol·min^{-1}·g^{-1}) [31].

Taking the above parameters into account, the inhibitory effect of LORF (data not shown), LORF α-amidated (Fig. 5) and the cleavage products of LORF (IPEP (Fig. 6A), YVWD) were tested. LORF and it α-amidated form, found in the leech brain, have the same inhibitory activity towards rabbit ACE. LORF and LORF α-amidated present an IC_{50} of 19.8 μM and a K_i of 55 μM. Interestingly, the cleavage product of LORF, IPEP presents an IC_{50} of 11.5 μM (Fig. 6) whereas, the YVWD has no inhibitory activity (data not shown). The LORF inhibition is compared to IPEP inhibiton in Fig. 6B. The IC_{50}s are in the same range as various previously described endogenous ACE inhibitors [11] as well as the ones found in insects [25]. The *N. bullata* ovary-derived ACE interactive factor (Neb-ODAIF: NKLKPSQWISL) interacts with human ACE at a km of 17 μM. Additionally, Neb-ODAIF(1–7)

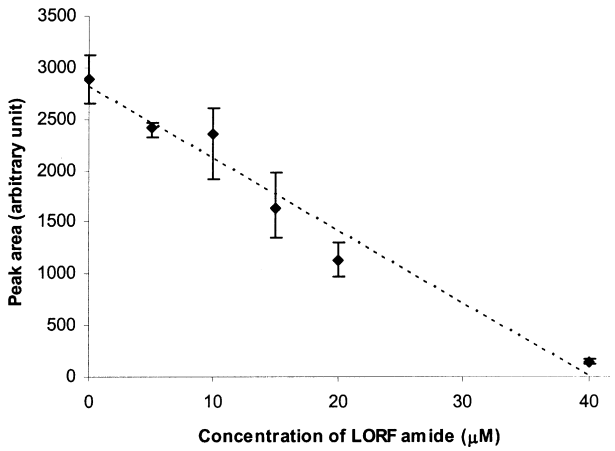

Fig. 5. Digestion of angiotensin I (30 mM) by ACE in presence of different amounts of LORF (10–40 mM).

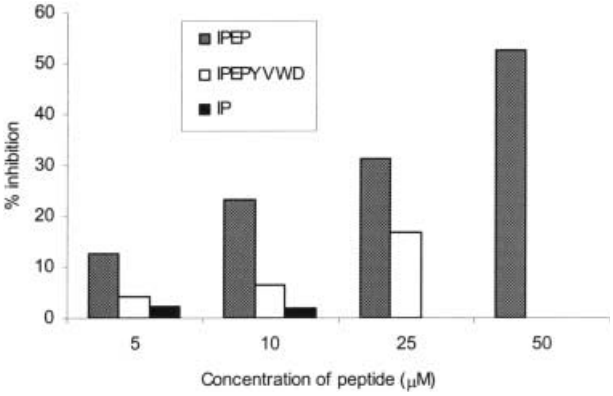

Fig. 7. ACE competition assay. IPEP (50 μM, 25 μM, 10 μM and 5 μM); IPEPYVWD (25 μM, 10 μM and 5 μM); IP (10 μM and 5 μM) were incubated with 1 μM *p*-[32]benzoylglycylglycylglycine and fly hemolymph.

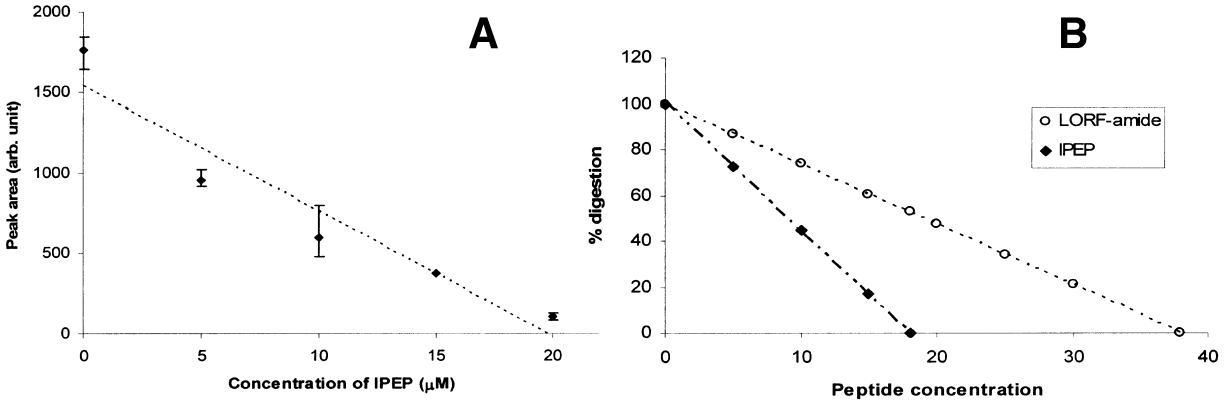

Fig. 6. Digestion of angiotensin I (30 mM) by ACE in presence of different amounts of IPEP (10–20 mM) (A) and comparison of LORF inhibition and IPEP inhibition (B).

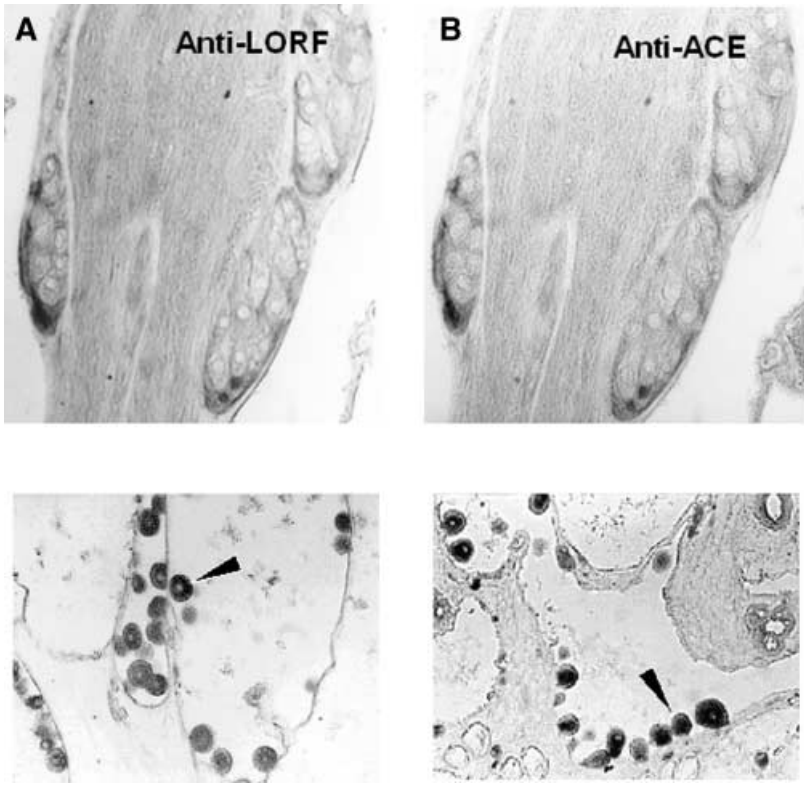

Fig. 8. Immunolabelling of same neurons with anti-LORF (A) and anti-(leech ACE) (B) in brain and in coelomocytes of the leech *Theromyzon tessulatum*.

(NKLKPSQ) also interacts with sACE at a $K_{(m/i)}$ of 90 μM [14,15].

A competition assay using *p*-[32]benzoylglycylglycylglycine and insect ACE was performed with LORF and IPEP. 36% inhibition is found with IPEP (25 μM) and 18% with LORF (25 μM) (Fig. 7). However, LORF appears stable under the experimental conditions as no cleavage and/or degradation was observed upon incubation with ACE suggesting that LORF behaves as a true inhibitor and not as a competitive substrate like that found in insects [16,25]. Moreover, the IC_{50} value obtained for LORF is similar to the one found for other natural ACE inhibitors, i.e. the nonclassical opioid family like hemorphins [11].

Taken together, the inhibitory effect of LORF towards ACE could explain the anti-diuretic effect of this peptide in leeches. Injected into leeches, LORFs increase the animal weight. Moreover, the immunocytochemical data show a colocalization of LORF α-amidated and leech ACE in same neurons and in the coelomocytes (Fig. 8) confirming the role of LORF as a leech ACE inhibitor and its involvement in water balance control. These data are in line with previous studies demonstrating that LORF level increased at stage 3 corresponding to a high water retention in the animal and gametogenesis [19]. Similarly, ACE as well as angiotensin II levels decrease at this stage of the animal [26,32,33]. These data show that yolk proteins are a natural source of ACE inhibitors in invertebrates; ovohemerythrin is a potential source of LORF [34] and ACE is implicated in the modulation of the reproduction. Such a hypothesis is supported by the data found in *N. bullata* [15,16] and in the blood sucker insect mosquito *Anopheles stephensi* [35,36]. In the female mosquito, after a blood meal, ACE activity increases fourfold with much of the enzyme finally accumulating in the ovaries. Addition of two selective inhibitors of ACE, captopril and lisinopril, to the blood meal reduced the size of the batch of eggs laid by females in a dose-dependent manner, with no observable effects on the behaviour of the adult insect. The almost total failure to lay eggs after feeding on either 1 mM captopril or 1 mM lisinopril, did not result from interference with the development of the primary follicle, but was due to the inhibition of egg-laying. As very similar effects on the size of the egg-batch were observed with two selective ACE inhibitors, belonging to different chemical classes, these suggest that these effects are mediated by the selective inhibition of the induced mosquito ACE, a peptidase probably involved in the activation/inactivation of a peptide regulating egg-laying activity in *A. stephensi* [35,36].

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