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 IC₅₀ of 19.8 µM. Interestingly, its cleavage product, IPEP exhibits an IC₅₀ of 11.5 µM. A competition assay using p-benzyglycylglycylglycine 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. Immuno-cytochemical 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 bradykinin 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 bradykinin protection requires the
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 differentially from that of bradykinin, 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 Neobellierella bullata ovaries. One of them is a peptide of 1312.17 Da named the N. bullata ovary-derived ACE interactive factor (Neb-ODAIF: NKLKPSQ) is a peptide of 1312.17 Da named the Neb-ODAIF and its shorter form Neb-ODAIF (1–7). K<sub>m</sub> 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<sub>m</sub> = 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 (IPEPYVWDamide and GFEIPEPYVWD were synthesized according to classical Fmoc chemistry on p-alkoxybenzyl alcohol resin on a 25-μmol scale with an ABI 432A. Conventional side chain-protecting groups were used for the synthesis of the peptides. All amino acids and coupling agents were obtained from Sigma.

**Materials and methods**

**Chemical**

Angiotensin I (DRVYIHPFHL: AI), angiotensin II (DRVYIHPFH: 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 for the synthesis of the peptides. All amino acids and coupling agents were obtained from Sigma.

**Assays of ACE activities**

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 mM 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.

**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 ρ-[32]benzoylglucylglucylglucose (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% chloroetone. 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 horse-radish 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 μg 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
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 IC₅₀ of 19.8 μM and a Kᵢ of 55 μM. Interestingly, the cleavage product of LORF, IPEP presents an IC₅₀ of 11.5 μM (Fig. 6) whereas, the YVWD has no inhibitory activity (data not shown). The LORF inhibition is compared to IPEP inhibition in Fig. 6B. The IC₅₀ values 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: NKLKPQWISL) interacts with human ACE at a kₘ of 17 μM. Additionally, Neb-ODAIF (1–7)

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.

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

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. 7. ACE competition assay. IPEP (50 μM, 25 μM, 10 μM and 5 μM), IPEP/YVWD (25 μM, 10 μM and 5 μM); IP (10 μM and 5 μM) were incubated with 1 μM p-[32]benzoylglycylglycylglycine and fly hemolymph.
(NKLKPSQ) also interacts with sACE at a $K_{m(i)}$ of 90 $\mu$m [14,15].

A competition assay using $p$-[32]benzoylglycylglycine and insect ACE was performed with LORF and IPEP. 36% inhibition is found with IPEP (25 $\mu$m) and 18% with LORF (25 $\mu$m) (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 a-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; ovohemerythin 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 four-fold 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].

Acknowledgements

This work was supported by the CNRS and the MNER. The authors would like to thank Annie Desmons for her skilled technical assistance.

References


