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Measuring Angiotensin-I Converting Enzyme Inhibitory Activity by Micro Plate Assays: Comparison Using Marine Cryptides and Tentative Threshold Determinations with Captopril and Losartan

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ABSTRACT: To determine the angiotensin-I converting enzyme (ACE) inhibitory activity of marine cryptides, different methods were tested. ACE inhibition was measured using two synthetic substrates, (*N*-[3-(2-furyl) acryloyl]-Phe-Gly-Gly (FAPGG) and *N*-hippuryl-His-Leu hydrate salt (HHL)), and a natural one, angiotensin-I. The IC_{50} value (defined as the concentration of inhibitory molecule needed to inhibit 50% of the ACE activity) of the reference synthetic inhibitor captopril was in the nanomolar range (1.79−15.1 nM) when synthetic substrates were used, whereas it exhibited IC_{50} of micromolar range (16.71 μM) with angiotensin-I. We chose losartan, an antagonist of angiotensin-II receptor as negative control for the ACE inhibition. Losartan was also able to inhibit ACE whatever the substrate tested, with IC_{50} of micromolar range (17.13−146 μM). We defined this value as a limit above which molecules are not showing *in vitro* ACE inhibitory activity. Val-Trp (VW), Val-Tyr (VY), Lys-Tyr (KY), Lys-Trp (KW), Ile-Tyr (IY), Ala-Pro (AP), Val-Ile-Tyr (VIY), Leu-Lys-Pro (LKP), Gly-Pro-Leu (GPL), Ala-Lys-Lys (AKK), and Val-Ala-Pro (VAP) were tested as inhibitors of ACE with synthetic and natural substrates. IC_{50} displayed were substrate-dependent. With FAPGG as substrate, IW, VAP, KY, IY, AP, AKK, and VIY show IC_{50} values over the IC_{50} value of losartan and should not be considered as inhibitors of ACE. VY, VW, KW, and LKP exhibited IC_{50} value lower than the IC_{50} value of losartan for all substrates tested and were thus considered as good candidates for effectively decreasing hypertension. It appears that the comparison of IC_{50} is not consistent when IC_{50} values are obtained with different substrates and different methods. *In vitro* ACE inhibitory activity assays should always include various ACE substrates and references such as captopril and a negative control to obtain data reliable to discriminate ACE inhibitory peptides.

KEYWORDS: *angiotensin-I converting enzyme (ACE), ACE inhibitory peptides, captopril, losartan, marine cryptides*

INTRODUCTION

A great number of ACE-inhibitory peptides have been isolated from food proteins.[1] They are actively searched and studied since they are considered as an interesting therapeutic approach in the treatment of high blood pressure. Indeed, ACE plays a central role in the regulation of blood pressure through the production of the vasoconstrictor, angiotensin-II, and the degradation of the vasodilator, bradykinin. Therefore, ACE inhibitory peptides may have the ability to lower blood pressure *in vivo* by limiting the vasoconstrictive effects of angiotensin-II and by potentiating the vasodilatory effects of bradykinin. ACE inhibitory peptides are mainly identified after a careful screening of an enzymatic digest of proteins. After each step of purification, biologically active fractions are characterized in term of concentration needed to inhibit commercial ACE until the identification of one or more active peptides. Rational design after docking could be used as suggested by Wang et al. to identify a novel ACE inhibitory peptide.[2] However, docking may not be a fully reliable predictive tool since activity did not correlate in all cases with theoretical findings.[3] Measurements of the ACE inhibitory activity using *in vitro* tests is a cornerstone in the research of new potentially antihypertensive peptides before *in vivo* evaluation.

The first method introduced by Cushman and Cheung uses the substrate *N*-α-hippuryl-L-histidyl-L-leucine (HHL). This substrate was developed on the analogy of the natural substrate angiotensin-I.[4] Holmquist et al. have synthesized new substrate *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) and have introduced, for the first time, a new continued kinetic method for determining the ACE activity in serum.[5] With hydrolysis, the resulting product has a lower absorbance than the substrate at 345 nm; thus, the resulting absorbance is negative, and there is a drop in the absorbance level. FAPGG has a faster hydrolysis process compared to that of HHL. Considering kinetic parameters, FAPGG has a $K_m = 3{\cdot}10^{-4}$ M and a $k_{cat} = 19000\ min^{-1}$ which compare favorably with $K_m = 2.4{\cdot}10^{-3}$ M and $k_{cat} = 15600\ min^{-1}$ for HHL.[6]

Comparison of biological activity is traditionally made on behalf of IC_{50}, the concentration of peptide needed to inhibit 50% of the enzyme activity. Once the IC_{50} is measured, the value is often used to compare the level of the biological activity of hydrolysates or peptides from one work to another. The IC_{50} of a reference molecule like captopril, a synthetic ACE inhibitor, is sometimes mentioned in order to indicate that the test performed is efficient.[7] IC_{50} strongly depends on

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various reaction parameters such as nature and concentration of substrate, volume, enzyme quantity, the mode of inhibition of inhibitor used, and detection methods of reaction products. Comparison between IC_{50} of hydrolysate or peptides could lead to biased conclusions if all parameters are not considered.

Until now, synthetic substrates such as HHL, FAPGG, and *o*-aminobenzoylglycyl-*p*-nitrophenylalanylproline (Abz-Gly-Phe-(NO2)-Pro; Abz) are used for measuring ACE activity and *in vitro* inhibition.[8] Rational choice of substrate depends on the equipment available to separate or detect reaction products after ACE hydrolysis: HPLC, spectrophotometer, or spectrofluorometer is respectively needed when HHL, FAPGG, or Abz is used. To a lesser extent, natural substrates, angiotensin-I and bradykinin, were also utilized to examine ACE activity. Ruiz-Giménez et al.[9] tested inhibition of ACE by heptapeptides such as PACEI 50L (RKWHFLW) and PACEI 52L (RKWHLFW) with angiotensin-I and bradykinin as substrates and compared with HHL. They observed that none of heptapeptides tested inhibited ACE activity when bradykinin was used as substrate and higher ACE inhibition values when angiotensin-I was the substrate compared to HHL.

Most of the research proposed on new ACE inhibitory peptides implied the use of the synthetic ACE substrate probably because of previously published methods and convenience.

The objective of the present work is to investigate the sense of standard values for ACE inhibitory tests when synthetic and natural substrates are used. Since eventually all peptides could inhibit ACE, we decided to add a non-ACE inhibitor but an angiotensin-II receptor blocker to tentatively establish the threshold IC_{50} values for a non-ACE inhibitor. We chose losartan because of its wide use as therapeutic agent.[10] In addition to substrate dependence of the inhibitory effect of captopril, we examined the ability of six dipeptides, Val-Trp (VW), Val-Tyr (VY), Lys-Tyr (KY), Lys-Trp (KW), Ile-Tyr (IY), and Ala-Pro (AP), and the following five tripeptides: Val-Ile-Tyr (VIY), Leu-Lys-Pro (LKP), Gly-Pro-Leu (GPL), Ala-Lys-Lys (AKK), and Val-Ala-Pro (VAP) to inhibit ACE when FAPGG, HHL, and human angiotensin-I were each used as a substrate for rabbit lung ACE. They were mainly found in fish products and coproducts as well as other marine resources such as wakame where KY was also found.[11] They all were chosen for their low, previously calculated IC_{50}'s and their shortness (they are neither prodrugs nor substrates). They were found in chicken, sardine, or sea bream, indicating that they are also ubiquitous.[12] Their previous IC_{50}'s were evaluated mainly using HHL as substrate and were between 0.32 and 16 μM.[12,13] In our study, IC_{50}'s were calculated using FAPGG, HHL, and angiotensin-I as substrates.

Moreover for each protocol, strengths, weaknesses, opportunities, and threats (SWOT) analysis of ACE inhibitory measurement using HHL, FAPGG, and angiotensin-I was performed.

MATERIALS AND METHODS

Materials. Angiotensin-I converting enzyme (from rabbit lung), captopril, human angiotensin-I, *N*-[3-(2-furyl) acryloyl]-Phe-Gly-Gly (FAPGG), losartan, and *N*-α-hippuryl-L-histidyl-L-leucine hydrate salt (HHL) were purchased from Sigma Aldrich (Saint Quentin Falavier, France). Synthetic peptides were purchased at >90% purity from Altergène (Bischeim, France). Purity of each peptide was checked by HPLC after solubilization in adequate buffer. Their molecular masses and amino acid sequences were determined using ESI-MS.

ACE Activity Assays. *ACE Inhibition Using HHL As Substrate.* The method was adapted from a previous study with slight modifications:[14] 30 μL of ACE 60 mU/mL in 50 mM borate buffer, 300 mM NaCl, pH 8.3 were mixed in the 96-well micro titer plate with 20 μL of the same buffer and incubated for 5 min at 37 °C. Ten microliters of the inhibitor solution (captopril, losartan, or peptide) at various concentrations (0.001−2000 μM) was added, and the reaction was initiated with the addition of 50 μL HHL (5 mM) in assay buffer. After incubation for 30 min at 37 °C, the reaction was terminated by the addition of 100 μL of 1 M HCl. Blank samples allowing the calculation of 100% activity were prepared by replacing the inhibitor solution with assay buffer. Well content was separated on a Waters (Milford, USA) system with a W2707 autosampler, a W600 pump,and a W996 PDA detector with a C18 column (Kinetex 2.6 μ C18 100A 100 mm × 4.6 mm Phenomenex (Le Pecq, France) protected with a "krushgard". Detection was performed at a wavelength of 228 nm. The flow rate was at 0.5 mL·min^{-1} and the elution was isocratic with 25% acetonitrile, 0.1% TFA and 75% water for 6 min. A delay of 5 min was respected between each run. Retention time of HHL and HA were 2.27 and 1.57 min, respectively. The ACE inhibition (in percentages) was calculated according to the following equation:

$$\text{ACE inhibition (\%)} = [1 - (\text{A inhibitor}/\text{A blank})] \times 100 \quad (1)$$

where A inhibitor and A blank are peak areas corresponding to HA for the inhibitor sample and the blank sample, respectively.

The IC_{50} value was defined as the concentration of inhibitor in the test required to reduce the product peak area by 50% and was calculated using a nonlinear regression from a plot activity vs inhibitory concentration fitted from SigmaPlot 10.0 software.

ACE Inhibition Using FAPGG As Substrate. The FAPGG degradation method was modified from Cushman et al. and was performed in a 96-well microplate.[7] One hundred and fifty microliters of FAPGG (0.87 mM in 50 mM Tris HCl (pH 7.5)) containing 300 mM NaCl (assay buffer) was mixed with 10 μL of the assay buffer and 10 μL of inhibitor solution, and the mixture was incubated at 37 °C for 5 min. A blank sample was prepared by replacing the inhibitor solution with the assay buffer. The reaction was started by the addition of 10 μL ACE (127.5 mU/mL) in the assay buffer. The micro plate was shaken 20 s, and FAPGG degradation was followed at 340 nm for 5 min with a Molecular Devices Versa max spectrophotometer.

The degree of ACE inhibition (in percentage) was calculated according to the following equation:

$$\text{ACE inhibition(\%)} = [1 - (\text{slope inhibitor}/\text{slope blank})] \times 100$$

where "slope inhibitor" and "slope blank" were the slope of the curve degradation of FAPGG vs time.

The IC_{50} value was defined as the concentration of inhibitor required to reduce the slope by 50% in the well and calculated as previously described in section ACE Inhibition Using HHL As Substrate.

ACE Inhibition Using Angiotensin-I As Substrate. In a microplate, 20 μL of sodium borate buffer 50 mM, 300 mM NaCl, pH 8.3 (assay buffer) were left standing at 37 °C for 5 min. Thirty microliters of ACE (127.5 mU/mL) in the assay buffer, 10 μL of sample, and 20 μL of angiotensin-I (5 mM) were sequentially added to initiate the reaction. The microplate was incubated at 37 °C for 30 min. Blank samples were prepared by replacing the inhibitor solution with assay buffer. The reaction was terminated by adding 100 μL of 1 M HCl. The angiotensin-II liberated by ACE was quantified by the system previously described for the HHL test. A gradient of acetonitrile containing 0.1% TFA from 25 to 50% in 7 min followed by one min at 50% then 25% in 2 min was used. Peak areas allowed quantifying angiotensin-II. Retention times were 1.68 and 2.45 min for angiotensin-I and angiotensin-II, respectively. The degree of ACE inhibition (in percentages) was calculated according to the eq 1 where A inhibitor and A blank are peak areas corresponding to angiotensin-II for inhibitor and blank sample, respectively. The IC_{50} value was calculated as previously described in part ACE Inhibition Using HHL As Substrate.

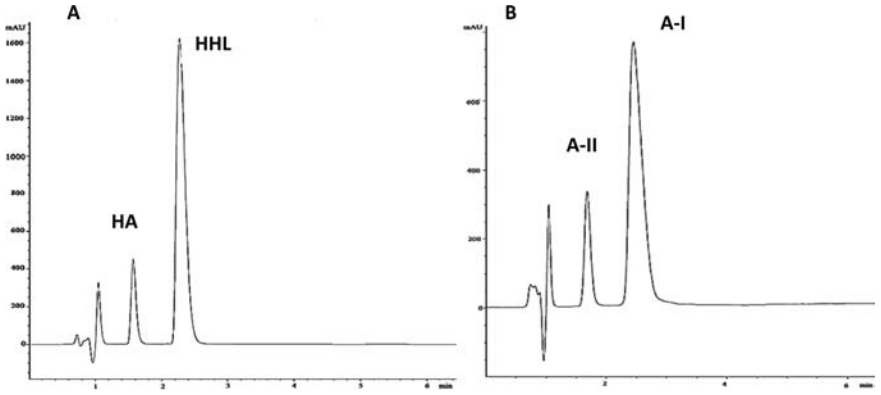

Figure 1. HPLC analysis of hydrolysis products of HHL(A) and angiotensin-I (A-I) (B) respectively in HA and angiotensin-II (A-II) by ACE, Column C18 (100 mm × 4.60 mm). (A) HA was eluted at 1.57 min followed by HHL (at 2.27 min), UV detection at 228 nm. (B) The angiotensin-II was eluted at 1.68 min followed by angiotensin-I (at 2.45 min), UV detection at 215 nm.

Table 1. Inhibitory Concentration (μM) of Captopril, Losartan, and Peptide Leading to 50% of Angiotensin-I-Converting Enzyme Inhibition[a]

ACE substrate drug/peptide sequence	FAPGG	HHL	angiotensin-I
captopril	0.00179 ± 0.0003	0.0151 ± 0.005	16.71 ± 1.9
losartan	**17.13 ± 1.4**	**49.61 ± 3.7**	**146 ± 11**
VY	1.64 ± 0.2	0.067 ± 0.009	0.22 ± 0.01
VW	10.15 ± 2	0.48 ± 0.02	18.26 ± 1.3
KW	12	1.7 ± 0.02	108.33 ± 3.88
KY	**85 ± 7**	0.08 ± 0.002	3.18 ± 0.2
IY	**140 ± 8**	0.88 ± 0.08	1.03 ± 0.06
LKP	13 ± 2.5	0.076 ± 0.006	0.41 ± 0.03
AKK	0.090 ± 0.002	0.178 ± 0.01	**420.89 ± 2.3**
VIY	**714.77 ± 8.65**	2.27 ± 0.78	127.97 ± 0.65
GPL	3.31 ± 0.4	**1020 ± 9**	**1074 ± 7**
VAP	**52.54 ± 9.15**	0.639 ± 0.12	**458.2 ± 3.62**
AP	**135.22 ± 13.38**	18.97 ± 1.71	**437.72 ± 4.00**

[a]Results were obtained with FAPGG, HHL, and angiotensin-I as substrate. Bold numbers indicate value higher than those obtained with losartan. All values are statistically significant with a p value <0.005.

Statistical Analysis. For all measurements, a minimum of six replicates was taken for data analysis. Data are expressed as the mean ± SD. Differences among groups were evaluated by one-way ANOVA using the software Origin 6.0. A p value of <0.005 was considered statistically significant.

RESULTS AND DISCUSSION

Determination of ACE Inhibitory Activity of Captopril and Losartan with FAPGG, HHL and Angiotensin-I As Substrates. The inhibitory activity of captopril was measured with two synthetic substrates, FAPGG and HHL, and a natural one, angiotensin-I. While FAPGG degradation was followed spectrophotometrically, ACE hydrolyzed HHL and angiotensin-I in HA and angiotensin-II, respectively, and both were separated on HPLC. A typical chromatogram is presented in Figure 1. Retention times were between 1 and 3 min, and the reaction product was eluted before the substrate.

Calculated IC_{50}'s are presented in table 1. The lowest IC_{50} value was obtained for captopril using the FAPGG as substrate (1.79 nM ± 0.3), while with HHL and angiotensin-I, values were higher from 10 to 10000 times (respectively 15.1 nM ± 0.5 and 16.71 μM ± 1.9). Compared to literature data, previous IC_{50} values obtained with FAPGG as substrate were 13.9 ± 0.6 nM in spectrophotometric cuvettes with an FAPGG concentration of 50 μM and a final volume of 1.2 mL.[15] Vermeirssen et al.[16] calculated an IC_{50} of 1.56 nM, while Murray et al.[17] reported values from 9.1 to 39.4 nM with enzyme concentration varying from 155 mU/mL to 222 mU/mL and an FAPGG concentration of 0.8 mM. Lahogue et al.[18] found 1.75 nM when FAPGG degradation by ACE was followed by HPLC. All these values were in the nanomolar range. Captopril should be used as reference molecule for each work, aiming for an IC_{50} within the nanomolar range. Extemporaneous captopril solution should also be used to obtain reliable data.

The losartan, which is not an ACE inhibitor but a competitive antagonist of the ATI receptor, exhibited comparatively to captopril very high IC_{50} values from 17.13 μM when FAPGG was used as substrate to 146 μM with angiotensin-I. Losartan is heterocyclic, like captopril. Its conformation could explain its inhibition potential toward ACE indicating that some molecules with similar conformation could also inhibit ACE. In our study, we defined IC_{50} values obtained with losartan as reference values for noninhibitory molecules.

FAPGG and HHL are convenient tools for ACE studies as they permit measurements of the ACE activity even in human serum. Such substrates were then employed to measure ACE inhibition by various kinds of molecules in screening tests. In this study, we add to synthetic substrates, human angiotensin-I. This peptide was also hydrolyzed by commercial rabbit lung ACE in our test condition. We noted that the ratio difference

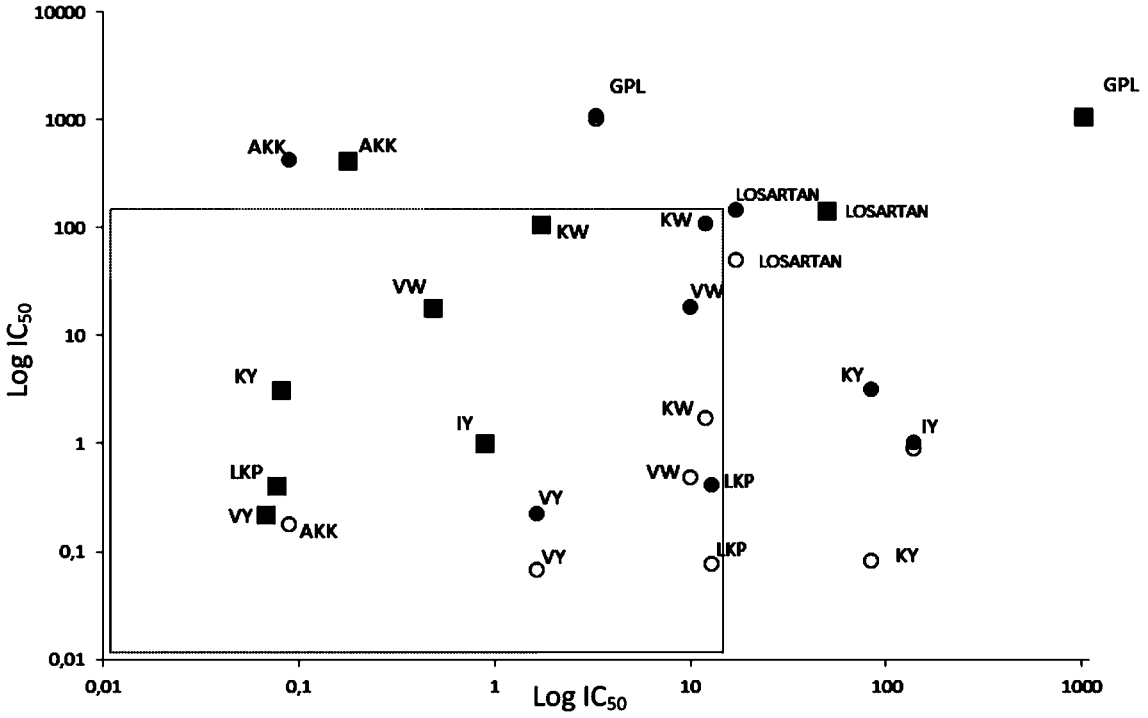

Figure 2. Correlation analysis of log IC_{50} using angiotensin-I, FAPGG, or HHL as substrate. ■: angiotensin-I vs HHL; ○: HHL vs FAPGG; ●: angiotensin-I vs FAPGG. Square depict an IC_{50} zone lower than that of losartan.

between IC_{50} of captopril and losartan for angiotensin-I ACE degradation is only 8-fold, whereas the difference between FAPGG and HHL are 10000- and 3300-fold, respectively. Affinity of the enzyme for each substrate differs. The two active domains of ACE are subtly different in substrate specificity, leading to substrate differential hydrolysis.[19] Indeed, it was demonstrated that the C-domain active site can hydrolyze angiotensin-I and HHL more efficiently, while the N-domain active site preferentially hydrolyzes other substrates such as captopril, lisinopril, and fosinoprilat.[20] Thus, comparison of IC_{50}'s should also include values from inhibitors other than captopril.

Our results point out once again the substrate dependence of ACE in the measurement of the inhibitory effect and the importance of indicating the substrate used when IC_{50} values are mentioned or compared to each other. In addition to reference value with a well-established ACE inhibitor such as captopril, the level of the biological activity should also be modulated by a noninhibiting molecule and data from *in vivo* tests.

ACE Inhibitory Activities of Synthetic Di- and Tripeptides. Val-Tyr (VY), Val-Trp (VW), Lys-Trp (KW), Lys-Tyr (KY), Ile-Tyr (IY), Leu-Lys-Pro (LKP), Ala-Lys-Lys (AKK), Val-Ile-Tyr (VIY), Gly-Pro-Leu (GPL), Val-Ala-Pro (VAP) and Ala-Pro (AP), were all previously identified as ACE inhibitors from fish products or marine coproducts hydrolysates. We measured their IC_{50} using FAPGG, HHL and angiotensin-I as substrates. Results are presented in Table 1. When FAPGG or HHL are used as substrates, IC_{50}'s calculated for peptides are, as expected, higher than the IC_{50} of captopril. However, when angiotensin-I is substrate, captopril has an IC_{50} value equal or higher than those measured for peptides. VW's IC_{50} was almost equal to captopril's IC_{50}, whereas those of VY, KY, IY, and LKP were lower.

Comparing HHL and angiotensin-I, our results are in accordance with those previously published by Ruiz-Giménez, et al.[9] who indicated that synthetic heptapeptides IC_{50} values obtained with angiotensin-I were higher than those obtained with HHL.

Various peptides exhibited IC_{50}'s higher than the IC_{50} of our noninhibitor losartan (gray colored in the table). For example KY, IY, VIY, VAP and AP could not be considered as potent *in vitro* ACE inhibitors when FAPGG is used as substrate. When HHL and angiotensin-I were used, the IC_{50} values of GPL and AKK and GPL were also respectively higher than the one of losartan. The use of IC_{50} to define biological activity is a practical tool to compare peptides when measurements were done under the same conditions (same substrate, same enzyme quantity, buffer, means of detection, etc.), but it has obvious limits if references are not mentioned.

No correlation between IC_{50} measured on HHL, FAPGG, and angiotensin-I was observed when the three substrates were compared (data not shown). Figure 2 presents the log of IC_{50} values of each peptide obtained with HHL, FAPGG, and angiotensin-I. Values inside the square are considered as interesting for ACE inhibition since they are all lower than those obtained for losartan.

VY appears to be the most valuable peptide of our study. Its IC_{50} values are the lowest no matter the substrate used, and are all less than those of losartan.

The peptide VY was first identified in sardine hydrolysate and exhibited IC_{50} of 10 μM.[21] Other value of 16 μM was found later using HHL as substrate.[22] Interestingly, this peptide can be absorbed into the human circulatory blood system.[23] VY was also found in Sea Bream scale and correspond to the sequence of the angiotensin 3−4 fragment. Thus, VY is ubiquitous since it was found in various species, and is a promising natural tool for ACE inhibition.

HHL as ACE substrate	
Strenghts Various references in literature. IC_{50} calculated on reaction product (HA) after HPLC separation. Cost (23,2 euros/25mg).	Weaknesses HPLC equipement needed with microplate reader and column. Analysis time : 11min (6min. + 5min. condition column) per well. Synthetic substrate.
Opportunities Analysis time could be reduced optimizing elution condition.	Threats Peptide tested eluted with HA.

FAPGG as ACE substrate	
Strenghts Various references in literature. Analysis time (5min) for a whole microplate. Equipment required simple (spectrophotometer). Safe (no solvent needed). Cost (41 euros/25mg).	Weaknesses IC_{50} measured from a reaction slope from substrate consumption. Synthetic substrate.
Opportunities Convenient for screening.	Threats slight OD variation. reproductibility.

Angiotensin-I as ACE substrate	
Strenghts Natural substrate.	Weaknesses HPLC equipement needed with microplate reader and column. Analysis time : 11min (6min. + 5min. condition column). Cost (Angiotensin-I: 622.5 euros/25mg).
Opportunities Analysis time could be reduced optimizing elution condition. Cost reduced optimizing test condition and detection.	Threats Peptide tested eluted with reaction product or substrate.

Figure 3. Strengths, weaknesses, opportunities, and threats of ACE tests using HHL, FAPGG, and angiotensin-I as substrates.

The following three peptides able to inhibit ACE with IC_{50} value lower than those of losartan are VW, KW, and LKP. Considering LKP, it was first evidenced as the result of the hydrolysis by ACE of LKPMN, a longer peptide extracted from dried bonito hydrolysate. It had an IC_{50} of 0.32 μM measured on HHL.[24] It was also evidenced in ovotransferin hydrolysates.[25] VW was identified in salmon with an IC_{50} of 2.5 μM when HHL was used as substrate and HA extracted by ethyl acetate.[26,27]

KW was found in sardine[21] and in the muscle tissue of the chicken leg.[22] Since values obtained with FAPGG and angiotensin-I are close to the value measured for losartan, it seems less interesting as an ACE inhibitor.

In our study, all experiments were done using a micro plate in order to easily test numerous peptides with various concentrations. Figure 3 presents a SWOT analysis of all tests performed and all substrates used; the "FAPGG test" was the fastest and the easiest. Taking into account the time needed to prepare the plate, the reading, data collection, and treatment, IC_{50} can be obtained for two or more inhibitors within 30 min. FAPGG is reasonably cheap, and a spectrophotometer is quite easy to use. This test is useful for screening fractions obtained from hydrolysate or to quickly evaluate ACE inhibitory potential and compare biological activities from one batch to another. Even though it has these strengths, it should be accompanied by a biological activity verification using other substrates or *in vivo* studies.

With HHL and angiotensin-I as substrate, we used HPLC to separate reaction products. One experiment needs at least 15 min of analysis due to column equilibration. The whole micro plate analysis needs a day. The use of HPLC gives the opportunity to observe separated reaction products on the chromatogram. Thus, it is possible to determine if peptides were cleaved by ACE and were substrates or if they were only inhibitors. Of course, the basic equipment needed is an equipped HPLC, the use of which requires more investment. Considering angiotensin-I, it is the most expensive substrate, the use of which should be reserved to confirm the biological activity of purified peptides previously selected after ACE inhibition tests on synthetic substrates.

Peptides have a potential for use as functional ingredients in nutraceuticals and pharmaceuticals due to their effectiveness in both prevention and treatment of hypertension in addition to their nutritive value. Their main advantage could be the absence of side effects since they seem to be well tolerated by the body. *In vitro* tests to assess the inhibitory activity were carried out with three different substrates: the synthetic substrates, HHL and FAPGG, and the natural substrate, angiotensin-I. The inhibition values obtained for captopril, the inhibitor of reference for losartan, our noninhibitory molecule, and for peptides were clearly substrate dependent. The IC_{50} values of the dipeptides and tripeptides tested on the HHL and FAPGG substrates were between the IC_{50}'s of captopril (the reference inhibitor) and losartan (our negative control). With angiotensin-I as substrate, peptides exhibited IC_{50} values equal or lower than the captopril IC_{50}. Angiotensin-I could be one of the substrates to use to evaluate ACE inhibition *in vitro* for known molecules. The synthetic substrates HHL and FAPGG could be reserved for screening of fractions almost when they are used in micro plate tests. To the inhibitory potency, indicated by IC_{50}, the substrate used should be mentioned as well as results obtained for reference molecules. The results of our study suggest that *in vitro* tests are useful for screening fractions or hydrolysate, *in vivo* tests are always needed since peptide bioavailability and collateral actions are not fully understood. Further studies (including those on bradykinin and other

bioactive peptides) to add data on marine ACE inhibitors are undergoing. Characterization of kinetic parameters of ACE with all the substrates mentioned will also strengthen the knowledge for this well-used enzyme.

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Notes

The authors declare no competing financial interest.

ABREVIATIONS USED

ACE: angiotensin-I converting enzyme
HHL: *N*-α-hippuryl-L-histidyl-L-leucine
FAPGG: *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine
HA: hippuric acid

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