Stability to gastrointestinal enzymes and structure-activity relationship of β-casein-peptides with antihypertensive properties.

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ABSTRACT

Physiological digestion plays a key role in the formation and degradation of angiotensin-converting enzyme (ACE)-inhibitory peptides. In this study, we evaluated the impact of a simulated gastrointestinal digestion on the stability of eight peptides previously identified in fermented milk with antihypertensive activity. Two of these identified peptides with sequences LHLPLP and LVYPFPGPIPNSLPQNIPP, possess ACE-inhibitory activity in vitro and antihypertensive activity in vivo. The results showed that LHLPLP was resistant to digestive enzymes. In contrast, LVYPFPGPIPNSLPQNIPP was totally hydrolyzed and its activity decreased after incubation with pepsin and a pancreatic extract. The peptide LHLPLP was incubated with ACE and was found to be a true inhibitor of the enzyme and to exhibit a competitive inhibitor pattern. A structure-activity relationship study of this peptide was carried out by synthesizing several modified peptides related to the sequence LHLPLP. The substitution of amino acid Leu in the penultimate position by Gly improved the ACE-inhibitory activity two fold and the substitution of Pro at C-terminal position by Arg increased the activity five fold, with an IC_{50} of LHLPLR as low as 1.8 μM.

Keywords: ACE-inhibitory peptides; Fermented milk; Simulated gastrointestinal digestion; Antihypertensive activity; Competitive inhibitors.

1. Introduction

Bioactive peptides derived from dietary proteins may affect the major physiological systems (cardiovascular, digestive, immune and nervous) and promote health well being [15]. Among these, angiotensin converting enzyme (ACE)-inhibitory peptides have been extensively studied due to their capacity to control hypertension, which is estimated to affect one third of the western population (for a review see [17]). Fermentation with highly proteolytic strains of lactic acid bacteria is considered to be a successful strategy to produce ACE-inhibitory and antihypertensive peptides [7]. Our group has just demonstrated the ability of four different *Enterococcus faecalis* strains to produce fermented milk with potent ACE-inhibitory activity and antihypertensive activity in spontaneously hypertensive rats (SHR) [23]. The administration of a fermented product produced with this microorganism exerted a significant blood pressure-lowering effect in these animals after long-term intake [20]. The protein fragments responsible for the ACE-inhibitory and antihypertensive activity were identified. Of special interest were the peptides β-casein f(133-138) with sequence LHLPLP which possess an IC_{50} of 5.5±0.4 μM and exhibit potent antihypertensive activity at a dosage of just 2 mg/kg, and β-casein f(58-76) with sequence LVYPFPGPIPNSLPQNIPP which possess an IC_{50} of 5.2±0.3 μM and showed a significant decreased of blood pressure at a dosage of 6 mg/kg [26].

The potential hypotensive effect of antihypertensive peptides depends on their capacity to reach their target organs intact after oral administration. Few studies have demonstrated the effect of specific peptide sequences on hypertensive animals and in clinical trials with human patients [10,33,34]. However, it is difficult to establish a direct relationship between ACE-inhibitory activity in vitro and antihypertensive activity in vivo. Proteolytic digestive enzymes, absorption through the intestinal tract and serum peptidases are the main barriers in

the human body where bioactive peptides can be activated or inactivated [36]. In this respect, several studies have demonstrated the important role of in vitro gastrointestinal digestion on ACE-inhibitory peptide formation and degradation [11]. Gomez Ruiz et al. [9] showed that the ACE-inhibitory activity of 11 peptides identified in Manchego cheese did not change drastically, except for the peptide TQPKTNAIPY from α_{s2} casein that exhibited an activity 6 times greater after simulated digestion. Roufik et al. [29] studied the proteolytic susceptibility to in vitro digestion of short- and long-chain selected peptides from β-lactoglobulin. They concluded that the bioactivity of short-chain peptides may be preserved during the gastrointestinal process; however the long-chain bioactive peptides would need to be protected from gastrointestinal enzymes. Miguel et al. [19] found that the potent antihypertensive peptide YAEERYPIL from ovalbumin was totally hydrolyzed to the fragments YAEER and YPI, with the latter being responsible for the decrease in blood pressure in SHR.

There are several methods to determine ACE-inhibitory activity in vitro, which are useful to select substances with potential antihypertensive activity in vivo. The spectrophotometric method of Cushman and Cheung [3] is the most commonly used. It is based on the hydrolysis of hippuryl-His-Leu by ACE to hippuric acid (HA) and HL. The extent of HA release from HHL is measured after it is extracted with ethyl acetate, which is a tedious process and can overestimate ACE activity if unhydrolyzed HHL is also extracted [17]. HHL can also be separated and quantified by HPLC to avoid this problem, but it results in a long-time consuming method to evaluate ACE-inhibitory activity [38]. Other methods using as substrate furanacryloyl (FAPGG) have been optimized and validated [37]. Recently, a fluorescence assay which employs o-aminobenzoylglycyl-p-nitrophenylalanylproline as substrate of ACE has been developed by Sentandreu and Toldrá [32]. The fluorescence generated by release of the product (o-aminobenzoylglycine) group is read on a microtiter-plate multiscan

fluorimeter. This method is rapid, simple, sensitive and has many advantages compared to other assays, such as, allowing continuous monitoring of ACE activity, the fact that it involves only one-step reagent, and the main advantage is that a large number of samples can be processed in a short time.

The objective of this study was to evaluate the impact of a simulated gastrointestinal digestion on the stability of eight synthetic peptides previously identified in a fermented milk with antihypertensive activity. The peptides generated after simulated physiological digestion were sequenced by tandem MS. The type of ACE-inhibition for peptide LHLPLP was also evaluated. In addition, studies of structure-activity relationship were carried out by modifying the last three amino acid residues of LHLPLP and evaluating ACE-inhibitory activity.

2. Material and methods

2.1. Peptide synthesis

Eight peptides: LHLPLP, LHLPLL, VVVPPF, LTQTPVVVPPF, VRGPFPIIV, LVYPFPGPIPNSLPQNIPP, VLGPVRGPFP, VLGPVRGPFPIIV, were chemically synthesized to evaluate their resistance to simulated gastrointestinal digestion. Additionally other seven synthetic peptides: LHLPLL, LHLPLR, LHLPAP, LHLPYP, LHLPGP, LHLYLP and LHLWLP, which result from the substitution of the last three amino acid residues of LHLPLP were used for the structure-activity study.

All synthetic peptides were prepared by GenScript Corporation (Piscataway, NJ, USA). The purity of these peptides was verified by analytical RP-HPLC-MS/MS.

2.2. Measurement of ACE-inhibitory activity

ACE-inhibitory activity was measured by fluorescence using the method of Sentrandreu and Toldrá [32] with some modifications. The ACE (peptidyl-dipeptidase A, EC

3.4.15.1) was purchased from Sigma Chemical (St. Louis, MO, USA). ACE working solution was diluted with 0.15 M tris buffer (pH 8.3) containing 0.1 μM $ZnCl_2$ with 0.04 U/mL of enzyme in the final reaction solution. A total of 40 μL of distilled water or this working solution were added to each microtiter-plate well, then adjusted to 80 μL by adding distilled water to blank (B), control (C) or samples (S). The enzyme reaction was started by adding 160 μl of 0.45 mM o-Abz-Gly-p-Phe(NO_2)-Pro-OH (Bachem Feinchemikalien, Bubendorf, Switzerland) dissolved in 150 mM Tris-base buffer (pH 8.3), containing 1.125 M NaCl, and the mixture was incubated at 37 ºC. The fluorescence generated was measured at 30 min using a multiscan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany). Ninety-six-well microplates (Porvair, Leatherhead, UK) were used. Excitation and emission wavelengths were 350 and 420 nm, respectively. The software used to process the data was FLUOstar control (version 1.32 R2, BMG Labtech).

The activity of each sample was tested in triplicate. Inhibitory activity was expressed as the peptide concentration required to inhibit the original ACE activity by 50% (IC_{50}). The formula applied to calculate the percentage of ACE-inhibitory activity was: 100 × (C-S)/(C-B). This parameter was plotted vs. peptide concentration and non-linear adjustment was performance as indicated Quirós et al.[26] to estimate IC_{50}.

2.3. Simulated gastrointestinal digestion

The two stage-hydrolysis process was carried out according to the method of Alting et al. [1] modified by Gómez-Ruiz et al. [9]. The hydrolysates were prepared from an aqueous solution of the synthetic peptide (10 mg/mL). The samples were first hydrolyzed with pepsin (E.C. 3.4.23.1; 1:10,000, 1750 U/mg protein) (Sigma) (enzyme:substrate ratio of 1:50 w/w) for 90 min at 37 ºC at pH 2.0 followed by hydrolysis with Corolase PP® (enzyme:substrate ratio of 1:25 w/w) (Röhm, Darmstadt, Germany) at pH 7-8 and 37 ºC for 240 min. Corolase

PP® is a proteolytic enzyme preparation from pig pancreas glands that, besides trypsin and chymotrypsin, contains numerous amino- and carboxypeptidase activities. Hydrolysis was carried out in a thermally controlled incubator under constant stirring (Unitron, Infors AG, Bottmingen, Switzerland). The reaction was finished by heating at 95 ºC for 10 min in a water bath, followed by cooling to room temperature. Each sample was stored at –20 ºC until further analysis.

2.4. Analysis by on line RP-HPLC-MS/MS

RP-HPLC-MS/MS analysis of the digests of the different peptides was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization source, as previously described [11].

Solvent A was a mixture of water-trifluoroacetic acid (1000:0.37 v/v) and solvent B contained acetonitrile-trifluoroacetic acid (1000:0.27 v/v). Peptides were eluted with a linear gradient of solvent B in A going from 0% to 45% in 60 minutes at a flow rate of 0.8 mL/min. Spectra were recorded over the mass/charge (*m/z*) range 100-1500. About 5 spectra were averaged in the MS and in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 10000 (i.e., 5% of the total signal) and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp going from 0.35 to 1.4 V. Using Data Analysis™ (version 3.0; Bruker Daltonik), the m/z spectral data were processed and transformed to spectra representing mass values. BioTools (version 2.1; Bruker Daltonik) was used to process the MS(n) spectra and perform peptide sequencing.

2.5. Stability of peptides to ACE

The stability of LHLPLP to ACE was determined following the method described by Fujita et al. [8]. Each peptide prepared at 5 mg/mL in 0.1M Na-phosphate and 0.3M NaCl (pH 8.3) was incubated with ACE (6 mU/mg of peptide) for 3 h at 37 ºC. The reaction was stopped by heating at 95 ºC for 15 min. To verify the stability of peptides to ACE, the mixture was analyzed by HPLC-MS/MS, as described above.

2.6. Determination of inhibition pattern on ACE

To investigate the inhibition pattern on ACE, two different concentrations of peptide LHLPLP (0.04 and 0.08 mg/mL) were added to each reaction mixture according to Bush et al. [2]. Enzyme activity was measured with several substrate concentrations (4.5, 3.5, 2.5 and 1.5 mM). The inhibition kinetics of ACE in the presence of peptide was determined by Line-weaver-Burk plots.

3. Results and discussion

3.1. Survival of β-casein-derived peptides to simulated gastrointestinal digestion

With the aim of evaluating the resistance of the eight peptides identified in fermented milk with *Enterococcus faecalis* CECT 5727 to digestive enzymes, a two-stage hydrolysis process which simulated in vivo conditions during physiological digestion was carried out. After incubation with pepsin simulating the stomach digestion process and then with Corolase PP® (trypsin, chymotrypsin, carboxy and aminopeptidases) simulating intestinal conditions, the digests were analyzed by HPLC-MS/MS (Fig. 1) and the fragments released by the action of gastrointestinal enzymes were sequenced. These results are summarized in Table 1. Among the eight peptides, only the potent antihypertensive peptide β-casein (133-138) LHLPLP was resistant to digestive enzymes. This could possibly occur because this peptide contains two proline residues and this amino acid residue makes sequences less susceptible to proteolytic enzymes [13]. Nevertheless, bioavailability studies of the antihypertensive peptide LHLPLP

using Caco-2 cells have shown that LHLPLP is hydrolyzed by cellular peptidases to HLPLP prior to the transport across the intestinal epithelium [27]. Previous results in our laboratory have shown that human β-casein f(125-130) fragment, with sequence HLPLP, was formed after digestion of human milk with pepsin and pancreatin and this peptide also possesses ACE-inhibitory activity (IC_{50} of 21 μM) [12].

The peptides LHLPLPL, VVVPPF, LTQTPVVVPPF and VRGPFPIIV, were partly hydrolyzed and, interestingly, their ACE-inhibitory activity increased after digestion (Table 1). The most significant case was peptide LHLPLPL which lost its C-terminal Leu releasing the active peptide LHLPLP (approx. 32% of the initial concentration).The activity of digested LHLPLPL increased approximately 23 times. In fact, we have found that this sequence possesses a modest antihypertensive activity in SHR, probably due to the transformation of LHLPLPL in the active form, LHLPLP, in vivo [21]. The capacity of VVVPPF and LTQTPVVVPPF to inhibit ACE increased slightly after hydrolysis, probably due to the formation of fragments VVVPP and LTQTPVVVPP, which contain VPP as the C-terminal tripeptide. The sequence VPP has been proven to exhibit a potent ACE-inhibitory activity and antihypertensive effects in both animals and humans [10,24,33,34]. It is noteworthy to point out that although VVVPPF and LTQTPVVVPPF showed low ACE-inhibitory activity (IC_{50} > 1500 μM), they could exert a potential antihypertensive effect in vivo due to the formation of shorter peptides during gastrointestinal digestion. This merits further investigation in animal models. The activity of VRGPFPIIV, which was also partly hydrolyzed, moderately increased 2 fold after hydrolysis. This sequence had shown a slight but not significant decrease of blood pressure in SHR [21]. The generation of active peptides during digestion has been observed by other authors. Maeno et al. [18], found that the sequence KVLPVPE corresponding to β-casein f (169-175), with IC_{50} > 1000 μM, showed potent antihypertensive activity in SHR.

These results suggested that pancreatic hydrolysis released from this peptide the ACE inhibitor KVLPVP (IC_{50} = 5 μM) responsible for the potent activity in vivo.

The peptides with sequences LVYPFPGPIPNSLPQNIPP, VLGPVRGPFP and VLGPVRGPFPIIV were totally hydrolyzed (Table 1). The potent ACE inhibitor LVYPFPGPIPNSLPQNIPP with 19 amino acids, was totally hydrolyzed releasing as a major fragment the sequence LVYPFPGPIPN which includes the peptide YPFPGPIPN, identified by Saito et al. [31] in a Gouda cheese of eight months (IC_{50} of 14.1 μM). As Table 1 shows, the activity of peptide LVYPFPGPIPNSLPQNIPP decreased 18 times after digestion. These results agree with those obtained for the in vivo experiments, because although this sequence has similar in vitro IC_{50} as LHLPLP, its antihypertensive activity measured in SHR was lower than that of LHLPLP. In addition, the maximum decrease in SBP caused by this longer peptide was observed 2 h later than that of LHLPLP (6 h post-administration instead of 4 h post-administration) [26]. Other authors have suggested that the bioactivity of short-chain peptides may be preserved during the gastrointestinal digestion process, but that longer molecules would need to be protected to escape the action of digestive enzymes and exert their physiological effects in the organism [28]. The peptides VLGPVRGPFP and VLGPVRGPFPIIV were totally hydrolyzed (Table 1) and their capacity to inhibit ACE decreased and did not change, respectively.

3.2. Enzymatic studies

In order to determine whether the peptide β-casein f (133-138), LHLPLP, is a true inhibitor or a substrate of ACE, this peptide was incubated with the enzyme and the mixture was analyzed by HPLC-MS/MS. As RP-HPLC chromatograms of LHLPLP before and after incubation with ACE show (Fig. 2A), this peptide was not hydrolyzed by ACE. According to Fujita et al. [8] peptides that are not susceptible to hydrolysis by ACE can be considered to be

true inhibitors. It can, therefore, be concluded that LHLPLP acts as a true inhibitor of the enzyme. Several ACE inhibitory peptides isolated from Manchego cheese were also found to act as true inhibitors of ACE [9]. In contrast, the peptide GFHGTHGLHGF isolated from chicken breast muscle was hydrolyzed with ACE (only 10%) and the ACE-inhibitory activity of the peptide solution after incubation with ACE was lower than the ACE-inhibitory activity of the peptide [30].

The inhibitory pattern of peptide LHLPLP was investigated by carrying out two sets of rate experiments for the peptide at concentrations of 0.04 and 0.08 mg/mL using different substrate concentrations (1.5, 2.5, 3.5 and 4.5 mM) and the results were plotted reciprocally (Fig. 2B). Because the Lineweaver-Burk plot obtained by changing the substrate concentration intersects with the y axis, the inhibition of ACE by LHLPLP corresponds to competitive inhibition. This means that this antihypertensive peptide found in fermented milk with *E. faecalis* binds competitively at the active site of ACE.

3.3. Relationship between the inhibitory activity and structure of LHLPLP

In order to study the relationship between activity and the last three amino acid residues of the sequence LHLPLP, several modified peptides with substitutions of amino acids in the antepenultimate, penultimate and ultimate positions were synthesized and their ACE-inhibitory activity was measured. Structure-activity correlations between different peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate [4]. In contrast, no relationship has been found between the N-terminal structure and ACE-inhibitory activity [25], although the length of the sequence seems to be determinant in the final activity [9,30]. Table 2 summarized the peptides synthesized and their ACE-inhibitory activity expressed as IC_{50} determined by fluorimetric assay.

To investigate the importance of the C-terminal amino acid in ACE-inhibitory activity, two peptides were synthesized. The peptide LHLPLL, where the Pro was replaced by the hydrophobic amino acid Leu, and LHLPLR where Pro was substituted by a positively charged amino acid. The activity of LHLPLL (IC_{50} 3.5 μM) was similar to the activity found for the original peptide LHLPLP (IC_{50} 3.7 μM). Proline as the C-terminal residue has been shown to enhance binding to the ACE [5], although the presence of another hydrophobic amino acid like Leu does not seem to affect the final activity. Other authors have also reported that the presence of Leu as the C-terminal amino acid helps to inhibit ACE [5,9]. The activity of the model peptide LHLPLR with an IC_{50} value of 1.8 μM was twice more potent than the activity of LHLPLP. Structure-activity data suggest that the positive charge on the guanidine or ε-amino group of the C-terminal arginine and lysine side-chains, respectively, contributes substantially to inhibiting potency [6].

The Leu in the penultimate position was substituted by Ala, Gly or Tyr. The inhibitory capacity found for peptide LHLPAP with an IC_{50} of 6.0 μM, was lower to that found for LHLPLP. Other authors have reported that replacement of the terminal sequence Pro-Pro by Ala-Pro in the potent ACE-inhibitory peptide EWPRPQIPP derived from snake venom increased the affinity of this nonapeptide inhibitor for ACE [4]. Captopril (D-3-mercapto-3-methylpropanoyl-L-proline), an ACE inhibitory drug commonly used to treat hypertension is analogous to the dipeptide Ala-Pro and has an IC_{50} of 0.022 μM [16]. On the contrary, the activity of peptide LHLPYP (IC_{50} of 2.3 μM) slightly increases compared to LHLPLP. Kohmura et al. [14] found several potent ACE-inhibitory peptides with the dipeptide C-terminal Tyr-Pro, some with an IC_{50} below 5 μM. However, the nature of the C-terminal amino acid seemed to be most important to the overall binding of dipeptide to the active site of the enzyme, and may not be always strictly the same as contribution of the same amino acid residue in the penultimate position of a substrate [5]. The peptide LHLPGP with an IC_{50}

value of 1.9 μM presented an inhibitory capacity two fold that of LHLPLP. A mixture of peptides with the C-terminal sequence Pro-Gly-Pro was found in a fraction with ACE-inhibitory activity obtained from pig sodium caseinate hydrolyzed with *Lb. helveticus* PR4 proteinase [22].

In order to compare aromatic amino acids with Pro in the antepenultimate position, the peptides LHLYLP and LHLWLP were synthesized where Pro is substituted Tyr and Trp, respectively. These sequences showed IC_{50} values of 18.9 and 9.0 μM respectively, which is five-times and twice lower ACE-inhibitory capacity than LHLPLP. The presence of Pro as the antepenultimate residue, appears to enhance binding to ACE [28,35], although aromatic amino acids in this position also seem to positively affect the inhibitory capacity of peptides.

5. Conclusions

In conclusion, of the eight peptides from milk fermented with *Enterococcus faecalis* CECT 5727, only peptide LHLPLP was resistant to gastrointestinal proteases and this peptide was a true competitive inhibitor of ACE. The sequence LVYPFPGPIPNSLPQNIPP was hydrolyzed by digestive enzymes and its ACE-inhibitory activity decreased. The capacity of peptides LHLPLPL, VVVPPF, LTQTPVVVPPF and VRGPFPIIV to inhibit ACE increased after digestion. These peptides could exert a potential antihypertensive effect in vivo.

Modifications of the sequence LHLPLP to search for a relationship between structure and activity showed that substitution of Leu in the penultimate position by Gly, and the substitution of Pro in the C-terminal position by Arg, can improve the ACE-inhibitory activity in vitro.

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FIGURE CAPTIONS

Figure 1. RP-HPLC chromatograms of peptides after simulated gastrointestinal digestion: (A) LHLPLP (B) LHLPLPL (C) VVVPPF (D) LTQTPVVVPPF (E) VRGPFPIIV (F) LVYPFPGPIPNSLPQNIPP (G) VLGPVRGPFP (H) VLGPVRGPFPIIV.

Figure 2. (A) RP-chromatogram of LHLPLP after incubation with ECA (B) Lineweaver-Burk plot of ACE-activity in presence of LHLPLP (◇) control; (□) peptide at 0.04 mg/mL; (Δ) peptide at 0.08 mg/mL. The Lineweaver Burk plots at varying concentrations of peptide are straight lines of different slope intersecting at common intercept on the y-axis that indicates competitive inhibition.

Table 1

Table 1. Behavior of peptides obtained from 3000 Da-permeate-water soluble extract of milks fermented with *Enterococcus faecalis*. ACE-inhibitory activity of peptides before and after simulated gastrointestinal digestion, and sequence of main peptides released after digestion are shown.

Sequence	IC_{50} (mg/ml) before digestion	IC_{50} (mg/ml) after digestion	Result of the digestion	Fragments released after digestion
LHLPLP	0.004±0.0003	0.004±0.0003	Not hydrolysed	
LHLPLPL	0.341±0.035	0.014±0.0013	Partly Hydrolysed	LHLPLPL; LHLPLP
VVVPPF	>1	0.772±0.071	Partly Hydrolysed	VVVPPF; VVVPP
LTQTPVVVPPF	>1.8	0.428±0.03	Partly Hydrolysed	LTQTPVVVPPF; LTQTPVVVPP; VVPPF; LTQTPV
VRGPFPIIV	0.597±0.055	0.284±0.027	Partly Hydrolysed	VRGPFPIIV; VRGPFPI; GPFPI
LVYPFPGPIPNSLPQNIPP	0.010±0.0006	0.171±0.024	Hydrolysed	LVYPFPGPIPN; YPFPGPIP
VLGPVRGPFP	0.142±0.006	0.588±0.062	Hydrolysed	VLGPV; LGPVR
VLGPVRGPFPIIV	>1	>1	Hydrolysed	VLGPV; GPFPIIV; GPFPI

Table 2. ACE-inhibitory activity of synthetic peptides corresponded to modifications of LHLPLP (amino acid substituted is in bold).

Synthetic Peptide	IC_{50} (μM)
LHLPLP	3.7±0.3
LHLPL**L**	3.5±0.3
LHLPL**R**	1.8±0.3
LHLP**A**P	6.0±0.7
LHLP**Y**P	2.3±0.3
LHLP**G**P	1.9±0.2
LHL**Y**LP	18.9±4.3
LHL**W**LP	9.0±0.8

IC_{50}: Concentration of peptide needed to inhibit the original ACE activity by 50%.

Figure 1

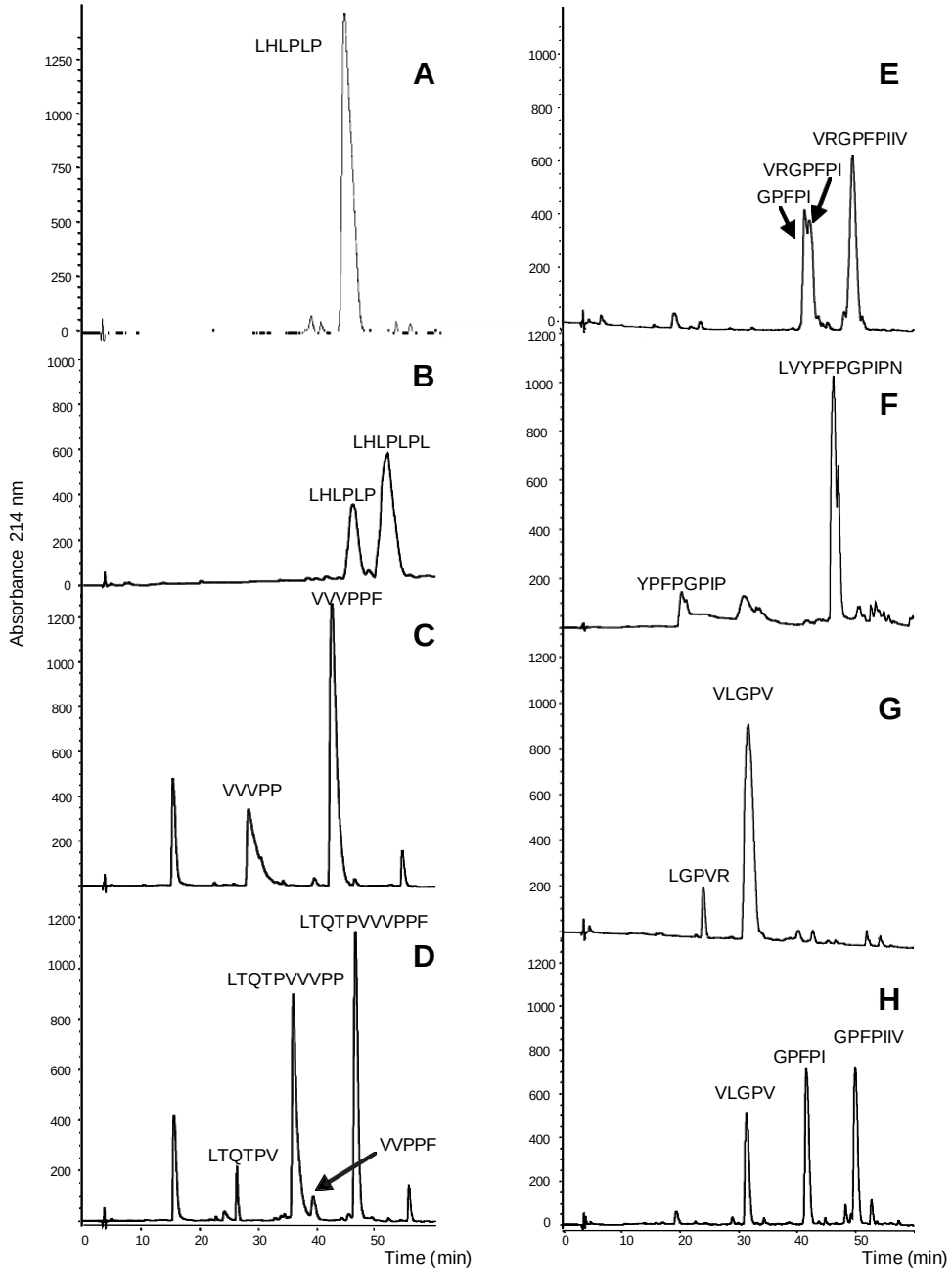
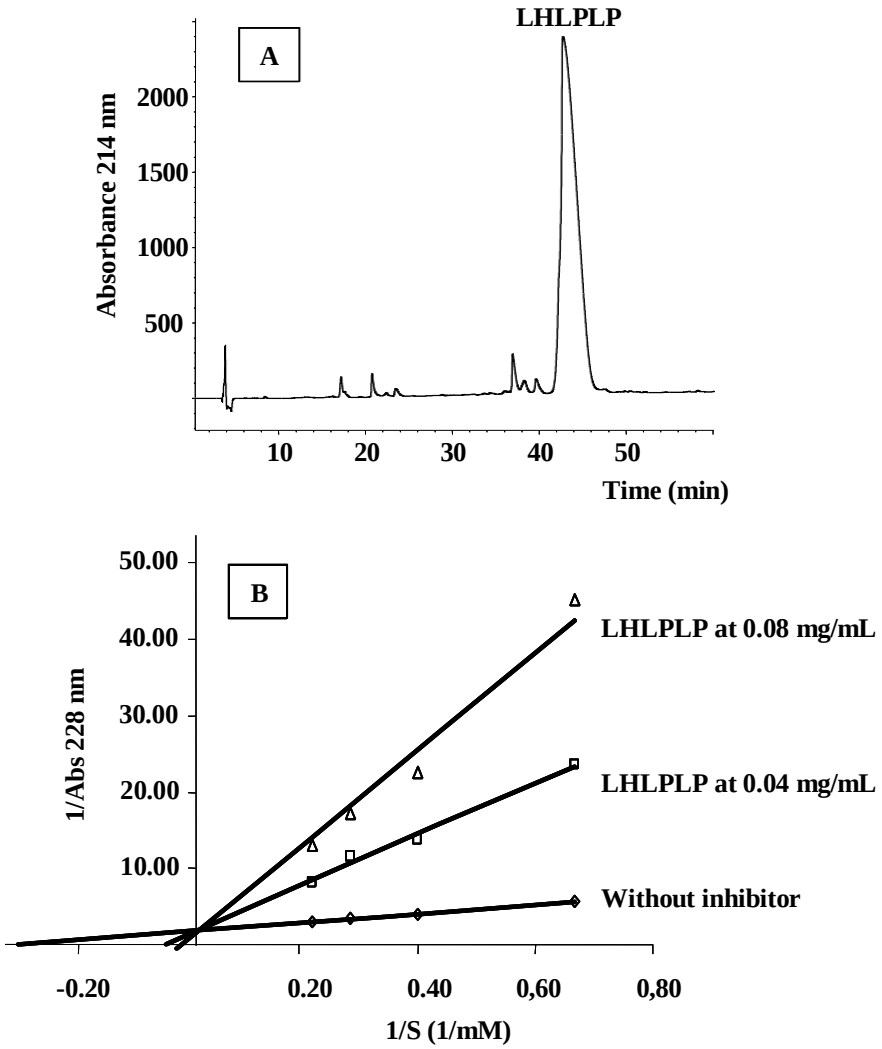

A
LHLPLP
B
LHLPLPL
LHLPLP
C
VVVPPF
VVVPP
D
LTQTPVVVPPF
LTQTPVVVPP
LTQTPV
VVPPF
E
VRGPFPIIV
VRGPFPI
GPFPI
F
LVYPFPGPIPN
YPFPGPIP
G
VLGPV
LGPVR
H
GPFPIIV
GPFPI
VLGPV
Absorbance 214 nm
Time (min)
Time (min)

Figure 2


A
LHLPLP
Absorbance 214 nm
2000
1500
1000
500
10
20
30
40
50
Time (min)
B
50.00
40.00
30.00
20.00
10.00
1/Abs 228 nm
LHLPLP at 0.08 mg/mL
LHLPLP at 0.04 mg/mL
Without inhibitor
-0.20
0.20
0.40
0,60
0,80
1/S (1/mM)