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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 twofold and the substitution of Pro at C-terminal position by Arg increased the activity twofold, with an IC_{50} of LHLPLR as low as 1.8 μM.

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]. Gómez-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

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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. Materials and methods

2.1. Peptide synthesis

Eight peptides: LHLPLP, LHLPLPL, 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.

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 °C. 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 de percentage of ACE-inhibitory activity was: $100 \times (C - S)/(C - B)$. 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 °C 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 °C 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 °C for 10 min in a water bath, followed by cooling to room temperature. Each sample was stored at −20 °C 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 min 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 10,000 (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 0.1 M Na-phosphate and 0.3 M NaCl (pH 8.3) was incubated with ACE (6 mU/mg of peptide) for 3 h at 37 °C. The reaction was stopped by heating at 95 °C for 15 min. To verify the stability of peptides to ACE, the mixture was analyzed by HPLC–MS/MS, as described above.