Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: In vitro effect and stability to gastrointestinal enzymes

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A B S T R A C T

Whey protein concentrate (WPC) was subjected to enzymatic hydrolysis by proteases from the flowers of *Cynara cardunculus*, and the resulting angiotensin-converting enzyme (ACE)-inhibitory effect was monitored. The whole WPC hydrolysate exhibited an IC_{50} value of 52.9 ± 2.9 μg/mL, whereas the associated peptide fraction with molecular weight below 3 kDa scored 23.6 ± 1.1 μg/mL. The latter fraction was submitted to RP-HPLC, and 6 fractions were resolved that exhibited ACE-inhibitory effects. Among the various peptides found, a total of 14 were identified via sequencing with an ion-trap mass spectrometer. Eleven of these peptides were synthesized de novo – to validate their ACE-inhibitory effect, and also to ascertain their stability when exposed to simulated gastrointestinal digestion. Among them, three novel, highly potent peptides were found, corresponding to α-lactalbumin f(16–26) – with the sequence KGYGGVSLPEW, α-lactalbumin f(97–104) with DKVGINYW, and β-lactoglobulin f(33–42) with DAQSAPLRVY; their IC_{50} values were as low as 0.80 ± 0.1, 25.2 ± 1.0 and 13.0 ± 1.0 μg/mL, respectively. None of them remained stable in the presence of gastrointestinal enzymes: they were partially, or even totally hydrolyzed to smaller peptides – yet the observed ACE-inhibitory effects were not severely affected for two of those peptides.

1. Introduction

Cheese whey is a rich by-product in nutritional terms: it indeed possesses high biological value components, excellent functional properties and inert flavor profile, so its upgrade toward production of functional ingredients is in order [38]. In particular, biological activities of whey proteins and their hydrolysates have received the attention of several researchers in recent years [21,22]: studies were indeed conducted on the recovery and characterization of peptides obtained therefrom, and on their impact upon human health – as those peptides may play important physiological functions, as well as modulate a few regulatory processes [8,22,26,28,52]; and also on their use in the formulation of novel nutraceutical ingredients and functional foods [16].

Enzymatic hydrolysis, fermentation or a combination of both have met with success in generating such sort of peptides; the former is still the most common method to produce bioactive peptides – and pepsin, trypsin and chymotrypsin have been the most frequent vectors used therefore [15,16,20]. Less conventional sources of proteolytic enzymes have meanwhile been sought that can cleave the whey protein backbone at specific and less usual sites – and plant rennets constitute an interesting possibility of work toward such an endeavor. This is the case of cardosins, which are aspartic proteinases present in the flowers of *Cynara cardunculus* – a plant related to the common globe artichoke. Among the several bioactive peptides studied, angiotensin-converting enzyme (ACE)-inhibitory peptides have received special attention because of their beneficial effects upon hypertension [31,36,45] – a disorder that constitutes a major public health problem worldwide.

However, effective inclusion of ACE-inhibitory peptides in one's diet requires them to somehow resist gastrointestinal digestion – and afterwards be absorbed in the intestine, to eventually reach the target sites where they will exert their physiological effects in vivo. The structure and bioactivity of short-chain peptides are more easily preserved through gastrointestinal passage than those of their

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long-chain counterparts [43]. Furthermore, peptides absorbed following digestion may accumulate in specific organs, and then exert their action in a systematic and gradual manner [24,25]. However, a few antihypertensive peptides that cannot be absorbed from the digestive tract may still exert their function directly in the intestinal lumen – e.g. via interaction with receptors on the intestinal wall [17,27,29].

Besides carrying out protein degradation to varying extents, gastrointestinal digestion plays also a key role in formation of ACE-inhibitory peptides [30,51]; hence, it is relevant to assess the gastrointestinal bioavailability of any potentially interesting peptides. Several studies have accordingly provided evidence for this realization in Manchego cheese, and in other fermented solutions and infant formulae [10–12,37,40,50]; for instance, a potent antihypertensive peptide was released via gastrointestinal digestion from a precursor with poor ACE-inhibitory effect in vitro [23], and some peptides possess a remarkable intrinsic stability, whereas others are susceptible to unwanted degradation [1,10,40,44] – but whether of those options will apply in each specific case cannot be known in advance.

Finally, it should be emphasized that in vivo tests of promising bioactive peptides should not come into play before careful in vitro models have been tested – as they can provide useful preliminary information on the stability of such peptides upon exposure to the various peptidases and proteinases that they will likely find in the gastrointestinal tract, prior to eventual transport across the intestinal barrier [11,50]. Data generated under conditions that mimic gastrointestinal digestion should still be handled with care, as the degree of hydrolysis of a given peptide depends not only on its size and nature, but also on the presence of other peptides in its vicinity [43] – which would make it difficult to test the required number of possibilities in a limited experimental program.

The aim of the research work described here was to identify the peptides responsible for ACE-inhibitory effects, which were generated by hydrolysis of bovine whey protein concentrate (WPC) brought about by *C. cardunculus* aqueous extracts. The potential effect was first discussed based on their amino acid sequences – as they should fulfill certain patterns. Then, the actual impact of simulated gastrointestinal digestion upon the stability of several synthetic peptides bearing those promising sequences was assayed for – and the peptide fragments thus generated were sequenced by tandem mass spectrometry.

2. Materials and methods

2.1. Isolation and identification of ACE-inhibitory peptides

2.1.1. Preparation of hydrolysate from WPC

A bovine WPC (with 87% protein, 10% lactose and 2%, w/w fat as bulk specifications) was incubated with a commercial crude aqueous extract of *C. cardunculus* (Formulab, Maia, Portugal) – at an enzyme/substrate ratio of 1.6% (v/v), based on the total volume of aqueous cardoon extract (15 g/L, on a protein basis) and WPC solution (40 g/L). This mixture was incubated for 7 h at pH 5.2 and 55 °C, as previously described by Barros and Malcata [4]. The enzymatic reactions were quenched via heating at 95 °C for 15 min. The WPC hydrolysate (containing ca. 73%, w/w protein) was then freeze-dried until further analysis.

2.1.2. Preparation of peptide extracts from hydrolyzed WPC

A solution of 100 $mg_{extract}$/mL of hydrolyzed bovine WPC was prepared by dispersing the hydrolyzed WPC powder in distilled water, and stirring for 1 h at room temperature. A portion of the water-soluble peptide extract underwent ultrafiltration through a hydrophilic 3 kDa cut-off membrane (Centripep, from Amicon, Beverly MA, USA) – and the resulting permeate and retentate were kept at −20 °C until analysis. The overall protein concentration, as well as the ACE-inhibitory effect – expressed as the protein concentration required to inhibit the original ACE activity by 50% (IC_{50}), were calculated for the whole hydrolysate, the permeate and the retentate.

2.1.3. Preparation of fractions of peptide extracts

The peptide extract with a MW below 3 kDa was fractionated by semipreparative, reverse phase, high performance liquid chromatography (RP-HPLC), using a Waters Series 600 HPLC, equipped with the Millennium v. 3.2 software for data acquisition and control (Waters, Mildford MA, USA), in combination with an automatic fraction collector (module II). A Prep Nova Pak® HR C_{18} column (300 mm of length, 7.8 mm of inner diameter, 6 μm of particle size and 60 Å of pore size) from Waters was used, as described in detail by Quirós et al. [41]. Six fractions were selected – corresponding to collections of a few major protein peaks (with cutoffs as close as possible to the baseline, while avoiding peak splitting), and a sufficient amount of each fraction was collected in sequential runs, concentrated by freeze-drying and kept at −20 °C until use. The protein concentration and the ACE-inhibitory effect were calculated for every fraction.

2.1.4. Identification of peptides in low MW fractions

All peptide fractions (F1-F6) obtained from the hydrolysate extract, as well as the chemically synthesized peptides (obtained as described below) and their digests were analyzed using an 1100 HPLC system (Agilent Technologies, Waldbron, Germany) with a Hipore® RP318 C_{18} column (250 mm of length, 4.6 mm of inner diameter and 5 μm of particle size) from Bio-Rad (Richmond CA, USA). The HPLC system was connected online to an Esquire 3000 quadrupole ion trap instrument (Bruker Daltonik, Bremen, Germany), equipped with an electrospray ionization source – as previously described by Hernández-Ledesma et al. [13], to determine the amino acid sequence of the peptide fragments at stake.

The peptides in fraction F1 were eluted via a linear gradient of solvent B in A, going from a previous 5 min under pure A up to 7% (v/v) B within 30 min; the peptides in fractions F2 and F3 were eluted via a linear gradient of solvent B in A, going from a previous 5 min under 5% (v/v) B up to 10% (v/v) within 10 min; the peptides in fraction F4 were eluted via a linear gradient of solvent B in A, going from a previous 5 min under 5% (v/v) B up to 15% (v/v) within 10 min; the peptides in fraction F5 were eluted via a linear gradient of solvent B in A, going from a previous 5 min under 5% (v/v) B up to 20% (v/v) within 30 min; and the peptides in fraction F6 were eluted via a linear gradient of solvent B in A, going from a previous 5 min under 10% (v/v) B up to 30% (v/v) within 40 min. All peptides synthesized (and their digests) were also eluted with a linear gradient of solvent B in A, going from a previous 5 min under pure A up to 45% (v/v) B within 60 min. The eluent produced during the first 5 min of running was disposed off, to avoid contamination of the mass spectrometer with salts. Solvent A was a mixture of water/trifluoroacetic acid (1000:0.37, v/v), whereas solvent B was a mixture of acetonitrile/trifluoroacetic acid (1000:0.27, v/v).

2.2. Measurement of ACE-inhibitory effect

The ACE-inhibitory effect was measured using the fluorimetric assay of Sentandreu and Toldrá [48], as recently modified by Quirós et al. [40]. For this purpose, the protein content of the peptide extracts was determined by Kjeldahl; and that of the fractions collected from the HPLC was estimated by the bicinchoninic acid assay (Pierce, Rockford IL, USA) – using in this case bovine serum

albumin as standard; the protein concentration of the chemically synthesized peptides was based on their actual dry weight. Non-linear fitting to the data was performed to calculate the IC_{50} values, as previously done by Quirós et al. [41].

2.3. Synthesis of ACE-inhibitory peptides

The peptides RELKDL, DKVGINY, KTEIPTIN, RELEEL, DAQSAPLRVY, KGYGGVSL and KGYGGVSLPEW were prepared by conventional Fmoc solid-phase synthesis, using a 431A peptide synthesizer (Applied Biosystems, Überlingen, Germany); whereas DKVGINYW, QVTSTAV, VQVTSTAV and TVQVTSTAV were supplied on demand by GenScript (Piscataway NJ, USA). The purity of the synthesized peptides was checked by analytical RP-HPLC, coupled to an MS device – as described above in Section 2.1.4.

2.4. Simulated gastrointestinal digestion of ACE-inhibitory peptides

A two stage-hydrolysis process parallel to actual human digestion was carried out, as initially suggested by Alting et al. [2] and modified afterwards by Gómez-Ruiz et al. [10]. Each digest was prepared using an aqueous solution of the corresponding synthetic peptide (10 mg/mL). Determination of ACE-inhibitory effect and sequencing of peptide digests were performed as already described in Sections 2.2 and 2.1.4, respectively.

3. Results and discussion

3.1. Identification of ACE-inhibitory peptides

In order to pinpoint peptides bearing a potential ACE-inhibitory effect, the WPC hydrolysate was subjected to ultrafiltration through a 3 kDa cut-off membrane – and the ACE-inhibitory effect of the original and of the two resulting fractions was determined. The results obtained revealed that peptides with a MW below 3 kDa are the main responsible for the ACE-inhibitory effect of the WPC hydrolysate; they exhibited a potent effect characterized by an IC_{50} of 23.6 ± 1.1 μg/mL – i.e. twice that found in the total fraction (52.9 ± 2.9 μg/mL), and almost 40-fold that measured in the retentate (717.0 ± 66.6 μg/mL). Therefore, said fraction below 3 kDa was fractionated by semipreparative RP-HPLC (Fig. 1A). Six fractions (F1–F6) were thus collected throughout elution time – and their ACE-inhibitory effects were accordingly measured (Fig. 1B). Fraction F1 exhibited the highest effect, corresponding to an IC_{50} value of 5.8 ± 0.4 μg/mL; fractions F2, F3, F4 and F6 also showed notable ACE-inhibitory effects, i.e. IC_{50} values lower than 33.0 μg/mL; and fraction F5 came last in terms of effect, but still exhibited a reasonable IC_{50} value of 52.6 ± 4.2 μg/mL.

Analysis of all fractions by HPLC-MS/MS then proceeded, in attempts to pinpoint the fragments more likely responsible for said bioactivity. Knowing the sequence of the precursor proteins in advance, search for the (determined) sequences of our peptides of interest in the WPC hydrolysate fractions was performed in a database of bovine milk proteins – entailing sequence modifications due to genetic variants and post-translational modifications; the mass and partial sequences (i.e. sequence tags) were searched, and matched with the tandem mass spectra, thus allowing unambiguous identification of said peptides.

Peptides accounting for intensities above 5000 units could be claimed to be successfully identified. Typical MS/MS spectra of two peptides are shown in Fig. 2: the MS/MS spectrum of the singly charged precursor ion at m/z 808.4 – which, following sequence interpretation and database searching, was matched to α-La f(97–103), is presented in Fig. 2A (including reference to some *b*- and *y*-type fragments – e.g. fragment ions *b*5, *b*6, *b*7, *y*5 and *y*6). Likewise, the fragmentation spectrum of the singly charged precursor ion at m/z 1192.6 – corresponding to α-lactalbumin f(16–26), is depicted in Fig. 2B (the most prominent fragment ion observed was *b8*, at m/z 762.3, which corresponded to cleavage at the N-adjacent portion of a peptide bond involving Pro). Note that the fragments generated by MS/MS are drastically affected when the peptide has Pro residues in its constitution. In fact, the presence of Pro in a peptide produces intense N-terminal to Pro fragment ions [35]; in our case, said fragment appeared because of the cleavage at the Leu-Pro bond [5].

Following this method for each active HPLC fraction, the major peptide components were sequenced – and are summarized in Table 1. A total of 14 peptides were thus identified: one peptide in F1, one in F3, one in F4, six in F5 and five in F6; of these peptides, seven were released from κ-casein, five from α-lactalbumin, one from β-lactoglobulin and one from β-casein. The reduced number of peptides originated in β-lactoglobulin – despite its being the most abundant protein in whey on a mass basis, can be attributed to the poor susceptibility of this protein to cardosin-mediated breakdown. This observation is consistent with reports [3] of absence of product peptides from β-lactoglobulin hydrolysis effected also via *C. cardunculus*. Only one peptide (in fraction F6) was accounted for by β-lactoglobulin f(33–42); this peptide was also found when a different enzymatic system (i.e. Protease N Amano) was employed [32].

All peptides identified contained between 5 and 11 residues – and selection of peptides for further consideration was based on their C-terminal sequences. Note that the C-domain catalytic site of ACE consists of three subsites – S1, S1′ and S2′, which accommodate the three hydrophobic C-terminal residues of the natural substrate angiotensin I – i.e. F, H and L in this order [6,9]. Hence, it has been claimed that the effect of ACE-inhibitory peptides is promoted when they contain hydrophobic amino acid residues at their three C-terminal positions [18,20,39]. In fact, presence of Pro, Trp, Tyr and Phe at the final [7], Leu at the final [10,14] or Pro as the second before the final [49] C-terminal position have been claimed to be the amino acid residues leading to the strongest ACE-inhibiting effects. According to this rationale, the only peptide sequenced in fraction F1 (i.e. AVEST) would have a reduced probability of exhibiting an ACE-inhibitory effect since it contains only polar, or even charged amino acid residues; hence, other (non-identified) minor peptide(s) would surely exhibit ACE-inhibitory effects that would justify the observed behavior of the whole fraction (but this line of research was not pursued due to limitation of laboratory resources).

Consequently, a total of 11 peptides were chemically synthesized, following their high probability of showing ACE-inhibitory effects – see Table 1. The peptides yielding the lowest IC_{50} values were found in fraction F6; more specifically, two peptides released from α-lactalbumin – viz. KGYGGVSLPEW or f(16–26), and DKVGINYW or f(97–104), and one peptide released from β-lactoglobulin – viz. DAQSAPLRVY or f(33–42), exhibited IC_{50} values as low as 0.70 ± 0.05, 25.4 ± 1.0 and 12.2 ± 1.0 μM, respectively. These results are again consistent with the aforementioned relationships between the primary structure at the C-terminal tripeptide sequence of ACE-inhibitory peptides and their inhibitory effect. Unlike claimed by Gómez-Ruiz et al. [10] and Kim et al. [14], both RELEEL and RELKDL have a Leu residue at their C-termini, yet no relevant ACE-inhibitory effects were observed; Leu is apparently important only if the other two residues are also appropriate. In fact, the low affinity of this peptide for ACE could be explained by the presence of two charged amino acids before the last position in said C-termini – which might bring about an incorrect orientation of the peptide that would hamper binding to the ACE active site, and consequently weaken its inhibitory potential [18].

Similar peptides had been described previously by Otte et al. [34]; α-lactalbumin f(15–26) or LKGYGGVSLPEW, and f(21–26) or

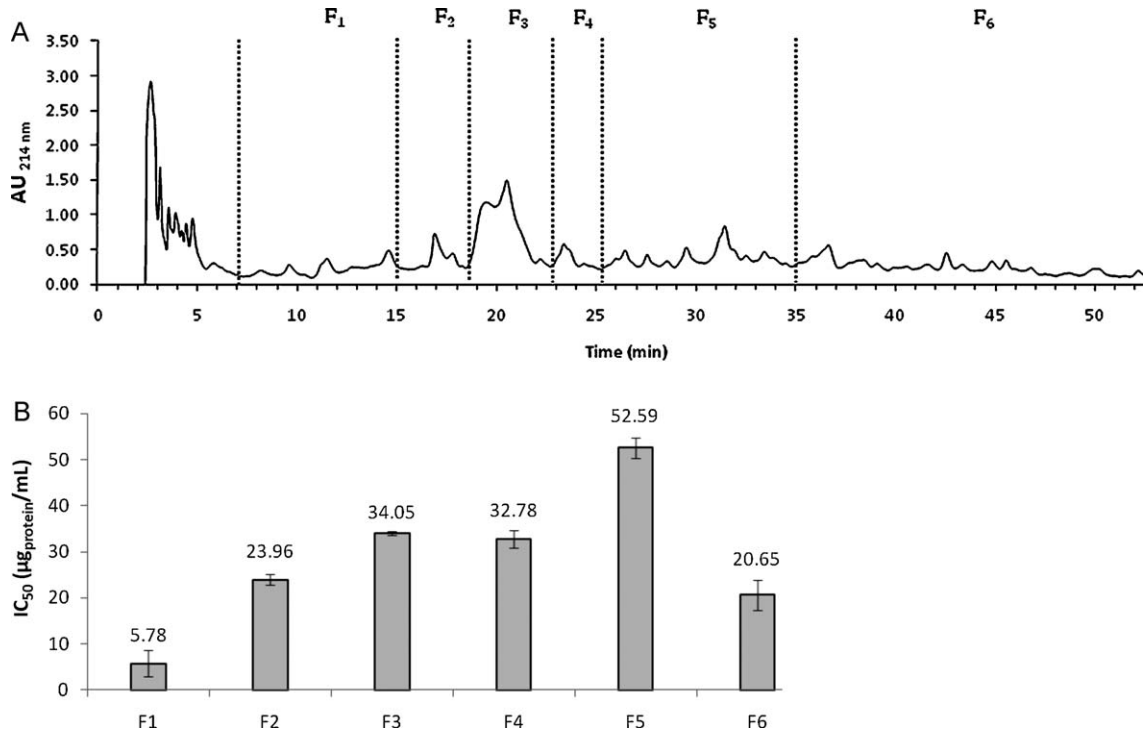

Fig. 1. (A) Typical chromatogram pertaining to fractionation by semi-preparative RP-HPLC of the <3 kDa-permeate obtained from bovine WPC after hydrolysis by *C. cardunculus*, and (B) corresponding ACE-inhibitory effect of the resulting fractions (F1–F6) measured as IC_{50} (data are expressed as mean ± standard deviation, for a minimum of three experiments).

VSLPEW were indeed reported following thermolysin-mediated hydrolysis of bovine α-lactalbumin. Those authors claimed that the considerable inhibition found (i.e. an IC_{50} of 45 μg/mL) was associated with those two Trp-containing peptides – for which IC_{50} values had been determined by Schlothauer et al. [47] to be 5 and 11 μM, respectively. Otte et al. [33] found analogous peptides possessing high ACE-inhibitory effects after hydrolysis of α-lactalbumin also by thermolysin, viz. LKGYGGVSLPEW (with an IC_{50} of 83 μM), YGGVSLPEW (IC_{50} of 16 μM), GVSLPEW (IC_{50} of 30 μM) and VSLPEW (IC_{50} of 57 μM). These results are consistent with our own – as KGYGGVSLPEW (characterized by an IC_{50} of 0.70 ± 0.05 μM) represents a highly potent ACE-inhibitory pep-

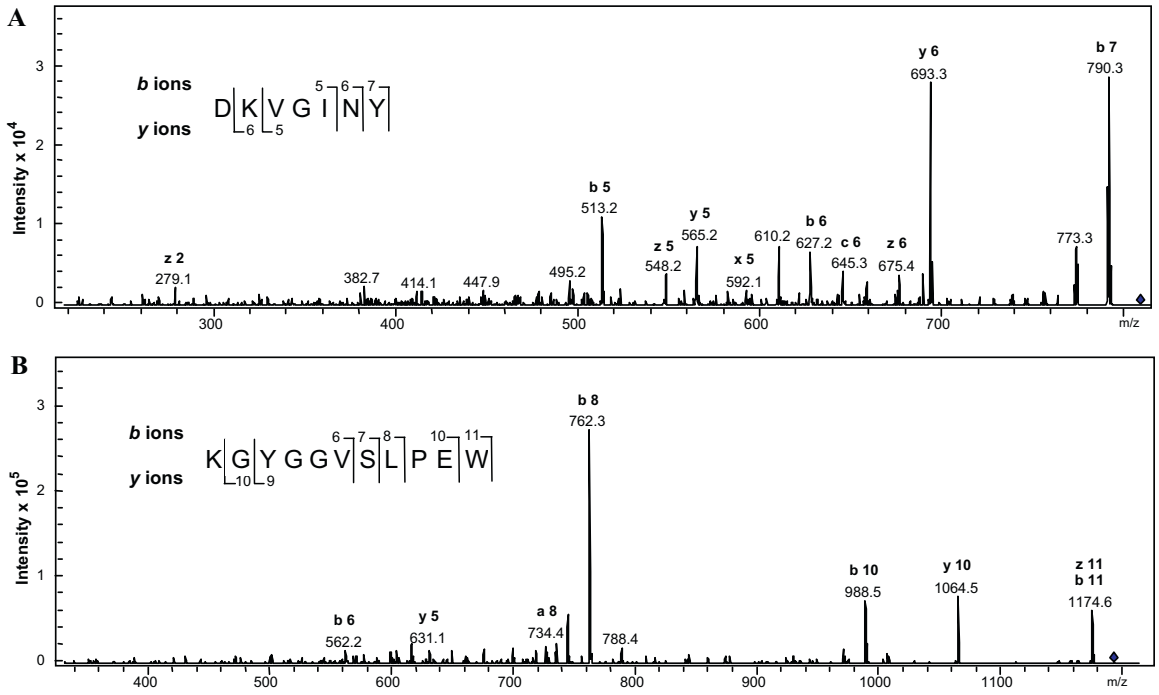

Fig. 2. Typical tandem mass spectra of (A) singly charged *m*/*z* 808.4 ion of fraction F5 and of (B) singly charged *m*/*z* 1192.6 ion of fraction F6. Following sequence interpretation and data base searching, the underlying peptides were identified as α-La f(97–103) and α-La f(16–26), respectively; their sequences are displayed, with the corresponding fragment ions observed in the spectrum – and labeled according to the nomenclature proposed by Roepstorff and Fohlman [42].

Table 1
Sequences of peptides in the RP-HPLC fractions (see Fig. 1A), and corresponding in vitro ACE-inhibitory effect, measured by IC_{50}, of the corresponding synthetic peptides. Identification of the protein fragment within the source protein was via sequence interpretation and data base searching.

Fraction	Ion for MS/MS, expressed as m/z (charge)	Calculated monoisotopic mass (Da)	Observed mass (Da)	Source protein fragment	Amino acid sequence	ACE-inhibitory effect, expressed as IC_{50}[a] (μM; mean ± standard deviation, $n = 3$)
F1	506.3 (+1)	505.27	505.3	κ-CN f(138–142)	AVEST	
F3	1010.6 (+1)	1009.56	1009.6	κ-CN f(107–115)	AIPPKKNQD	
F4	705.4 (+1)	704.37	704.4	κ-CN f(163–169)	QVTSTAV	n.d.
F5	1141.7 (+1)	1140.60	1140.7	κ-CN f(106–115)	MAIPPKKNQD	
F5	804.4 (+1)	803.44	803.4	κ-CN f(162–169)	VQVTSTAV	n.d.
F5	905.6 (+1)	904.49	904.6	κ-CN f(161–169)	TVQVTSTAV	n.d.
F5	773.5 (+1)	772.44	772.5	α-La f(10–15)	RELKDL	n.d.
F5	808.4 (+1)	807.41	807.4	α-La f(97–103)	DKVGINY	99.9 ± 8.1
F5	788.5 (+1)	787.41	787.5	β-CN f(1–6)	RELEEL	n.d.
F6	915.6 (+1)	914.51	914.6	κ-CN f(116–123)	KTEIPTIN	n.d.
F6	1119.6 (+1)	1118.57	1118.6	β-Lg f(33–42)	DAQSAPLRVY	12.2 ± 1.0
F6	780.5 (+1)	779.42	779.5	α-La f(16–23)	KGYGGVSL	300.9 ± 35.5
F6	994.5 (+1)	993.49	993.5	α-La f(97–104)	DKVGINYW	25.4 ± 1.0
F6	1192.6 (+1)	1191.59	1191.6	α-La f(16–26)	KGYGGVSLPEW	0.70 ± 0.05

n.d.: not detected.
[a] Concentration of peptide needed to inhibit 50% of original ACE activity.

tide. Interestingly, when the C-terminal sequence of this peptide (i.e. -PEW) was cleaved, the peptide lose most of its ACE-inhibitory effect: KGYGGVSL exhibited indeed a much higher IC_{50} value, 300.9 ± 35.5 μM.

The peptide DAQSAPLRVY, or β-lactoglobulin f(33–42), had previously been found in Protease N Amano-mediated hydrolysates of bovine β-lactoglobulin; a fragment of this peptide – SAPLRVY, presented an IC_{50} value of 8 μM [32], which is close enough to the one obtained for said peptide in our study (i.e. 12.2 ± 1.0 μM). These results are consistent with the hypothesis that the amino acid residues at the N-terminus do not significantly affect the ACE-inhibitory effect of the peptide. Furthermore, these peptides are more active than ALPMHIR (with an IC_{50} of 43 μM) – the most potent ACE-inhibitory peptide known to date that is derived from β-lactoglobulin [31]. Upon comparison of their primary structures, one concluded that all peptides mentioned possess a hydrophobic amino acid residue before the last position, and a basic amino acid residue in the third position counted from the C-terminus. In addition, peptides DAQSAPLRVY and SAPLRVY have a Tyr as C-terminus – which also favors binding to ACE [7].

Of the remaining peptides generated, some show a high homology with a few ACE-inhibitory peptides already described in the literature; this is the case of TVQVTSTAV, VQVTSTAV and QVTSTAV – all of which share the subsequence VTSTAV, with an IC_{50} value of 52 μM [46]; surprisingly, such homologues do not exhibit a significant ACE-inhibitory effect. The peptide RELEEL was found in water-soluble extracts of Asiago d'Allevo cheeses [19] – and its homologue REQEEL, obtained from ovine β-casein, was also found in the water-soluble extracts of Manchego cheese [10]. However, these peptides did not exhibit any ACE-inhibitory effect – in agreement with our results.

3.2. Stability of ACE-inhibitory peptides to simulated gastrointestinal digestion

The stability of selected ACE-inhibitory peptides when exposed to gastrointestinal enzymes was checked via a two-stage hydrolysis process – which was intended to simulate in vitro the conditions prevailing during human digestion. The digests were also subjected to analysis by HPLC-MS/MS – to identify the fragments released by such enzymes, and to ascertain their ACE-inhibitory activities. The resulting activities were compared with those associated with the source peptides – as summarized in Table 2. (Note that the ACE-inhibitory effects for the various peptides listed in Table 1 are equivalent to those for the same peptides before digestion in Table 2; however, mass units had to be used for the latter so as to

Table 2
Impact of simulated gastrointestinal digestion upon in vitro ACE-inhibitory effect, measured by IC_{50}, of selected peptides in the RP-HPLC fractions.

Amino acid sequence	ACE-inhibitory effect before digestion, expressed as IC_{50}[a] (μg/mL; mean ± standard deviation, $n = 3$)	ACE-inhibitory effect after digestion as IC_{50}[a] (μg/mL; mean ± standard deviation, $n = 3$)	Impact of digestion	Fragments released by digestion
QVTSTAV	n.d.	>500	Partial hydrolysis	QVT; QVTST; QVTSTA; QVTSTAV
VQVTSTAV	n.d.	>500	Partial hydrolysis	STAV; VQVT; VQVTSTAV
TVQVTSTAV	n.d.	>500	Partial hydrolysis	TVQVTST; STAV; TVQVT; TVQVTSTAV
RELKDL	n.d.	>500	Full hydrolysis	KDL; RELKD; RELK; REL
DKVGINY	84.2 ± 3.1	130.8 ± 11.0	Partial hydrolysis	DKVG; IN; DKVGIN; DKVGI; DKVGINY
RELEEL	n.d.	>500	Partial hydrolysis	ELEE; RELEE; RELEEL
KTEIPTIN	n.d.	n.d.	Partial hydrolysis	KTEIPT; KTEIPTIN
DAQSAPLRVY	13.0 ± 1.0	9.0 ± 0.9	Full hydrolysis	DAQSA; SAP; DAQSAP; DAQSAPL
KGYGGVSL	253.6 ± 30.7	336.3 ± 34.9	Full hydrolysis	GGVS; KGY; KGYGGVS; KGYGGV
DKVGINYW	25.2 ± 1.0	254.5 ± 47.0	Partial hydrolysis	NY; DKVGIN; DKVGI; DKVGINYW
KGYGGVSLPEW	0.8 ± 0.1	2.0 ± 0.1	Partial hydrolysis	KGY; KGYGGVS; KGYGGV; GGVSLPE; KGYGGVSLPE; GYGGVSLPE; SLPEW; GGVSLPEW; KGYGGVSLPEW

n.d.: not detected.
[a] Concentration of peptide needed to inhibit 50% of original ACE activity.

allow a better comparability with the results obtained after digestion, when a mix of peptides was present rather than a single, pure peptide.) Of the 11 peptides tested, none could resist the action of pepsin and pancreatic extract without undergoing breakdown to some extent.

The peptide bearing the strongest ACE-inhibitory capacity in the original peptide extract – KGYGGVSLPEW, underwent (partial) hydrolysis to ca. 75%; however, its ACE-inhibitory effect did not change to a significant extent. This could be due to release of such smaller peptides as GGVSLPEW and SLPEW [47] (see Table 2). On the other hand, the peptides RELKDL, DAQSAPLRVY and KGYGGVSL were hydrolyzed in full (Table 2). The already potent ACE-inhibitory peptide DAQSAPLRVY increased moderately its power after said digestion (the IC_{50} value changed from 13.0 ± 1.0 to 9.0 ± 0.9 μg/mL), while releasing SAP, DAQSAP and DAQSAPL as major fragments – which have been proven to possess ACE-inhibitory effects [47]. One should emphasize that these fragments also abide to the heuristic requirements for performance as ACE-inhibitors – since their C-terminal tripeptide sequences contain two or three hydrophobic residues, including Pro at the final (or the one before the final) position. DAQSAPL, in particular, had been identified also by Ortiz-Chao [32] in a β-lactoglobulin hydrolysate, and possesses Leu as C-terminus. It is noteworthy that Pro is present in those three peptides at the final position (or the one just before) – which apparently is an important feature to assure stability against attack by proteolytic enzymes.

The ACE-inhibitory effect of KGYGGVSL decreased upon digestion – from 253.6 ± 30.7 to 336.3 ± 34.9 μg/mL (Table 2); this might be rationalized by the loss of Leu at the C-terminus position. Gómez-Ruiz [10] has accordingly reported that replacement of Leu by Gly at that position produced a notable decrease in the ACE-inhibitory effect of the corresponding peptide. Peptide DKVGINY was in turn fully hydrolyzed by gastrointestinal enzymes (Table 2); and even though its original C-terminal tripeptide sequence was compromised, some of the fragments produced thereby showed a residual ACE-inhibitory effect.

4. Conclusions

Aqueous extracts from the plant *C. cardunculus* could bring about release of peptides from WPC that exhibit potent ACE-inhibitory effects. A peptide mixture generated under optimal processing conditions exhibited an IC_{50} value of 52.9 ± 2.9 μg/mL, but its fraction with MW below 3 kDa had an IC_{50} of 23.6 ± 1.1 μg/mL. Individual peptides in said mixture with potential ACE-inhibitory capacity – as anticipated from their amino acid sequence, were selected for synthesis de novo. Three novel peptides were accordingly identified: α-La f(16–26), with the sequence KGYGGVSLPEW and an outstanding IC_{50} of 0.70 ± 0.05 μM; α-lactalbumin f(97–104), with the sequence DKVGINYW and an IC_{50} of 25.4 ± 1.0 μM; and β-lactoglobulin f(33–42), with the sequence DAQSAPLRVY and an IC_{50} of 12.2 ± 1.0 μM.

The most promising peptides were then exposed to simulated gastrointestinal digestion: no peptide was able to keep its integrity, but even total hydrolysis to smaller peptides did not significantly compromise the overall ACE-inhibitory effect. Peptides DKVGINY and KGYGGVSL decreased their ACE-inhibitory effect upon digestion, whereas QVTSTAV, VQVTSTAV, TVQVTSTAV, RELKDL, RELEEL and DAQSAPLRVY behaved in an opposite way; KGYGGVSLPEW was only partially hydrolyzed, but its ACE-inhibitory capacity remained essentially unchanged. In view of their ACE-inhibitory effects, both before and after gastrointestinal digestion, the peptides KGYGGVSLPEW and DAQSAPLRVY are expected to eventually exhibit antihypertensive effects in vivo.

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