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Bioactivity of hydrolysates obtained from bovine casein using artichoke (*Cynara scolymus* L.) proteases

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ABSTRACT

The objective of this work was to obtain casein hydrolysates with aspartic proteinases present in extracts from the artichoke flower (*Cynara scolymus* L.) and evaluate their antioxidant, antimicrobial, and angiotensin-I converting enzyme (ACE) inhibitory activity in vitro. The casein hydrolysates produced by the action of *C. scolymus* had elevated antihypertensive and antioxidant activity due to their high hydrophobic peptide content (93.84, 96.58, and 90.54% at 2, 4, and 16 h of hydrolysis, respectively). Hydrolysis time and molecular weight (<3 kDa) had a significant influence on the hypertensive and antioxidant activity of the hydrolysates, which were greater at hydrolysis times of 4 and 16 h and corresponding to the <3 kDa fractions. The <3 kDa fraction of the 16 h hydrolysate had an ACE inhibitory activity with a half-maximal inhibitory concentration (IC_{50}) of 71.77 μg peptides per mL; DPPH and $ABTS^{\bullet+}$ radical scavenging activities of 6.27 μ*M* and 6.21 m*M* Trolox equivalents per mg of peptides, respectively; and iron (II) chelation activity with an IC_{50} of 221.49 μg of peptides per mL. Antimicrobial activity against *Enterococcus faecalis* was also observed in the hydrolysates. From the peptide sequences identified in the hydrolysates, we detected 22 peptides (from the BIOPEP database) that were already in their bioactive form (AMKPWIQPK, AMKPWIQPKTKVIPYVRYL, ARHPHPHLSFM, DAQSAPLRVY, FFVAPFPEVFGK, GPVRGPFPII, KVLPVPQK, LLYQEPVLGPVRGPFPIIV, MAIPPKKNQDK, NLHLPLPLL, PAAVRSPAQILQ, RELEELNVPGEIVESLSSSEESITR, RPKHPIKHQ, RPKHPIKHQGLPQEVLNENLLRF, SDIPNPIGSENSEK, TPVVVPPFLQP, VENLHLPLPLL, VKEAMAPK, VLNENLLR, VYPFPGPIH, VYQHQKAMKPWIQPKTKVIPYVRY, VYQHQKAMKPWIQPKTKVIPYVRYL) and are reported to display antioxidant, antimicrobial, and ACE inhibitory activity. We also identified 12,116, 14,513, and 25,169 peptide sequences in the hydrolysates at 2, 4, and 16 h, respectively, that were contained in the primary sequence, and these are reported to display ACE inhibitory, antioxidant, dipeptidyl peptidase IV inhibition, antithrombotic, opioid, immunomodulation, antiamnesic, anticancer, chelating, and hemolytic bioactivity.
Key words: angiotensin-I converting enzyme (ACE) inhibitor, antioxidant, antimicrobial, artichoke, casein bioactive peptide

INTRODUCTION

The potential capacity of bioactive peptides to reduce the risk of chronic disease and promote health has aroused the interest of both the scientific community and industry (Hernández-Ledesma et al., 2011). After their liberation through chemical or enzymatic hydrolysis (Korhonen and Pihlanto, 2006; Hernández-Ledesma et al., 2014), these peptides have specific biological activities (e.g., antihypertensive, antioxidant, opioid, antithrombotic, immunomodulative, antimicrobial). On the other hand, the AA sequence in a particular peptide encodes the information that provides a range of properties. A single peptide sequence could also exhibit different biological properties (Agyei et al., 2016).

Methods adopted to produce bioactive peptides ultimately influence the biological effects of these peptides, as well as their antioxidant and antimicrobial properties (Nongonierma and FitzGerald, 2018). The most common method for obtaining bioactive peptides is through enzymatic hydrolysis, which mainly uses enzymes of animal origin; the number of vegetable proteases used is still limited (Xie et al., 2008; Hernández-Ledesma et al., 2014). The aspartic proteases (cardosins) obtained from cardoon flowers of the species *Cynara cardunculus* have been used as a milk coagulant in cheesemaking with sheep's milk (Tejada and Fernández-Salguero, 2003) and goat's milk (Tejada et al., 2006; Tejada et

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al., 2008). In all cases, greater proteolytic activity on the caseins and a greater percentage of hydrophobic peptides were observed than when other enzymes were used (Agboola et al., 2004; Tejada et al., 2008). A direct relationship between the concentration of peptides in the hydrolysates and their level of bioactivity has been established (Mao et al., 2011; Arruda et al., 2012; Ibrahim et al., 2017). Furthermore, hydrophobic peptides have been observed to have greater bioactivity. In this way, hydrophobic peptides have shown a higher antioxidant activity (Ren et al., 2008; Zhang et al., 2012), because they can scavenge more free radicals. They also have greater angiotensin-I converting enzyme (**ACE**) inhibitory activity (Kitts and Weiler, 2003; Hernández-Ledesma et al., 2008; Alvarado and Guerra, 2010), because ACE has a greater affinity with competitive or inhibitory substrates that contain hydrophobic AA residues (Cheung et al., 1980). This is also one of the principal factors that gives peptides their antimicrobial activity (Nguyen et al., 2011). As well, hydrophobicity might improve the epithelial transport and bioavailability of the casein peptides (Wang and Li, 2018).

Cynarases, which are very similar to cardosins with elevated proteolytic activity (Sidrach et al., 2005; Llorente et al., 2014), have been extracted and defined from the artichoke flower (*Cynara scolymus* L.). As an unused agricultural byproduct, the artichoke flower is much more abundant and easier to acquire than the wild cardoon flower. Extracts of this flower could be used to hydrolyze casein and obtain peptides with bioactive potential, which could serve as useful ingredients in future foods. However, no studies have evaluated the capacity for bioactive peptide production of casein proteases extracted using *C. scolymus* species.

The objective of this work was to obtain casein hydrolysates with aspartic proteinases in extracts from the artichoke flower (*C. scolymus*) and to evaluate their ACE inhibitory, antioxidant, and antimicrobial activities in vitro. As well, we identified the sequences of the peptides in the hydrolysates and checked them with those collected in BIOPEP database (Minkiewicz et al., 2008) to detect different reported bioactivities in the hydrolysates obtained.

MATERIALS AND METHODS

Obtaining the Freeze-Dried Extract of C. scolymus

We used mature artichoke flowers (*C. scolymus*) from the Murcia Region of Spain. We obtained the freeze-dried enzymatic extract by following the procedure described by Tejada and Fernández-Salguero (2003). Briefly, the flower styles and stigmas of *C. scolymus* were macerated in water at a proportion of 1:5 (wt/vol) for 24 h. The aqueous extract was sieved, centrifuged (4,000 × *g* for 5 min), and filtered. The resulting permeate was freeze-dried and stored at −20°C until it was used.

Obtaining the Hydrolysates

To evaluate the effect of hydrolysis time on bioactivity, we obtained 3 casein hydrolysates, at 2 (**CH2**), 4 (**CH4**), and 16 (**CH16**) h. Each was obtained 3 times under the previously established optimal hydrolysis conditions (Bueno-Gavilá, 2017). A solution of commercial total bovine casein (carbohydrate- and fatty acid–free; Calbiochem, Calbiochem EMD Chemicals Inc., San Diego, CA) was prepared at 1% (wt/vol) in distilled water, adjusting the pH to 6.2 with 1 *M* NaOH. The hydrolysis reaction occurred at 50°C at a concentration of enzymatic protein extract of 23 μg/mL of casein solution. The reaction was stopped at 2, 4, or 16 h by increasing the temperature to 100°C for 10 min, and the pH was adjusted to 4.6 with HCl (1 *M*). Then, the hydrolysates were centrifuged at 4,000 × *g* for 20 min, and the supernatant was collected and filtered through a 0.45-μm nylon filter. Finally, the pH was adjusted to 7 with NaOH (1 *M*) and distributed into Falcon tubes for storage at −20°C until use. To obtain the molecular weight fraction <3 kDa, we used Amicon Ultra-15 centrifuge filters of regenerated cellulose (3.000 NMWL; Merck Millipore, Burlington, MA). The filtration was carried out by centrifuge in refrigerated conditions at 4°C at 4,000 × *g* for 40 min. The resulting permeate was stored in Falcon tubes and frozen at −20°C until use.

Peptide Determination

The quantity of peptides was determined by triplicate with each of the hydrolysates after precipitating the proteins through trichloroacetic acid at 5% in a proportion of 1:2 (vol/vol) and centrifuging at 3,200 × *g* for 20 min. The Kjeldahl method (AOAC, 1990) was used to determine the nitrogen in the supernatant, and peptide concentration was calculated using a conversion factor of 6.38. The hydrolysate peptides that were soluble in water were separated using HPLC (Shimadzu LC-10AD; Shimadzu, Kyoto, Japan) in reverse phase following the method of González de Llano et al. (1994). We determined the hydrophilic nature of the peptides based on their retention time. In this way, the hydrophilic peptides were estimated to appear in the peptide portion eluted between the AA tyrosine and tryptophan, and the hydrophobic peptides to appear after tryptophan. To identify the reference AA, we used commercial templates (l-Tyr, +99%, Acros Organics,

Fairlawn, NJ; and L-Trp ≥98%, Sigma-Aldrich, Madrid, Spain).

ACE Inhibitory Activity

The ACE-inhibitory activity was determined following the spectrophotometric method of Cushman and Cheung (1971), modified by Miguel et al. (2004). First, 40 μL of the hydrolysate sample and 2 mU of ACE (EC 3.4.15.1; Sigma-Aldrich) were added to 100 μL of hippuryl-histidyl-leucine (**HHL**; Sigma-Aldrich) dissolved in a borate buffer of 0.1 *M* and NaCl 0.3 *M* at pH 8.3. Following incubation at 37°C for 30 min, 150 μL of HCl 1 *M* was added to stop the reaction. The hippuric acid produced was extracted by adding 1,000 μL of ethyl acetate and the subsequent separation and evaporation of the organic phase. The residue obtained was resuspended in distilled water, and its absorbance measured at 228 nm. The control was carried out using distilled water in place of hydrolysate. The blank was carried out by adding HCl before the enzyme to avoid producing the reaction. The percentage of ACE inhibitory activity was determined using the following formula:

$$\text{ACE inhibitory activity (\%)} = (A_{control} - A_{sample})/(A_{control} - A_{blank}) \times 100,$$

where $A_{control}$ is the absorbance of hippuric acid formed from the action of non-inhibiting ACE, A_{sample} is the absorbance of the hippuric acid formed from the action of ACE in the presence of the hydrolysate, and A_{blank} is the absorbance of non-reacting HHL. The half-maximal inhibitory concentration (**IC_{50}**) value was defined as the concentration of peptides required to inhibit 50% of the ACE activity.

Antioxidant Activity

DPPH Radical Scavenging Activity. We determined the 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) radical scavenging activity (**RSA**) following the method of Bersuder et al. (1998). First, 1,000 μL of hydrolysate dissolved in ethanol (1:1, vol/vol) was mixed with 125 μL of a solution of DPPH (Sigma-Aldrich) in ethanol (0.02% wt/vol). This was then incubated in darkness for 1 hour and centrifuged at 10,000 × *g* for 2 min. Absorbance was measured at 517 nm. The percentage of antioxidant activity was determined using the following formula:

$$\text{DPPH RSA (\%)} = (A_{control} - A_{sample})/A_{control} \times 100,$$

where $A_{control}$ is the absorbance of the DPPH with water in the place of hydrolysate, and A_{sample} is the absorbance of the DPPH with the hydrolysate. The antioxidant activity was calculated as Trolox equivalent antioxidant capacity (**TEAC**; μ*M* Trolox/mg of peptides; Sigma-Aldrich). The TEAC against DPPH of the casein hydrolysates was extrapolated from the following linear equation: $y = 3.479x + 0.029$ ($R^2 = 0.997$).

ABTS Radical Scavenging Activity. We determined the 2,2'-azino-bis-(3-ethiylbenzothiazoline-6-sulfonic acid) (**ABTS**) RSA using the method of De Gobba et al. (2014a). First, to produce ABTS radicals ($ABTS^{\bullet+}$), we prepared a solution of ABTS diammonium salt (19.4 m*M*; Sigma-Aldrich) with potassium persulfate (6.7 m*M*) in distilled water, and this was left to react for 14 h. The $ABTS^{\bullet+}$ solution was diluted with buffered phosphate (10 m*M*, pH 7.4) 350 times to achieve an absorbance of 0.6 to 0.7. Following this, 200 μL of the $ABTS^{\bullet+}$ working solution was mixed with 50 μL of the hydrolysate at various concentrations, and absorbance was measured at 405 nm over 30 min. The last measurements were used to calculate the $ABTS^{\bullet+}$ RSA (%) by applying the following formula:

$$ABTS^{\bullet+} \text{ RSA (\%)} = 100 - (100 \times A_{sample}/A_{control}),$$

where $A_{control}$ is the absorbance of the $ABTS^{\bullet+}$ solution with water instead of the hydrolysate, and A_{sample} is the absorbance of the $ABTS^{\bullet+}$ solution with the hydrolysate. The half-maximal scavenging concentration (SC_{50}) was the concentration of peptides required to scavenge 50% of the $ABTS^{\bullet+}$. The TEAC value of the hydrolysates (m*M* Trolox equivalents/mg of peptides) was also calculated from the following linear equation: $y = 6.728x + 1.891$ ($R^2 = 0.997$).

Iron (II) Chelating Activity. The iron (II) chelating capacity was determined according to the method of Wu et al. (2007), with some modifications. A 100-μL aliquot of Cl_2Fe (75 μ*M* in water) was mixed with 25 μL of the hydrolysate at different concentrations and incubated for 10 min. Then, 100 μL of FerroZine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid; 500 μ*M* in water; Sigma-Aldrich] was added, and absorbance was measured at 560 nm. The chelation activity was calculated by applying the following formula:

$$\text{Iron (II) chelating activity (\%)} = 100 - (100 \times A_{sample}/A_{control}),$$

where $A_{control}$ is the absorbance of the control with distilled water, and A_{sample} is the absorbance in the presence of the hydrolysate. The IC_{50} was defined as

the peptide concentration needed to inhibit 50% of the iron–FerroZine complex formation.

Antimicrobial Activity

The antimicrobial activity of each of the hydrolysates obtained was determined from the growth curve of each strain being studied according to the method of Hill et al. (2013) using a 96-well microplate. We used 2 gram-negative species (*Enterococcus faecalis* and *Escherichia coli*) and 2 gram-positive species (*Listeria innocua* and *Pseudomonas fluorescens*) to compare the possible antimicrobial activity against the 2 types of microorganisms. The bacterial strains were obtained from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo), of which the following species were used for the trials: *L. innocua* (CCUG 15531), *E. faecalis* (NCIMB 775), *E. coli* (NCIMB 9484), and *P. fluorescens* (NCIMB 9046). The cultivation media used were peptone water (Panreac-Cultimed, Barcelona, Spain); tryptic soy broth (Scharlau, Sentmenat, Spain); and plate count agar (Scharlau). For the positive inhibition controls, we used a gentamicin (Sigma-Aldrich) of ≥95% purity (5 mg/mL).

Once the microorganism broth cultures (tryptic soy broth) were prepared at the required concentration (previously tested), 150 μL of the inoculum was mixed with 150 μL of the hydrolysate sample, and the absorbance was measured (650 nm) at 10-min intervals over 15 h at the incubation temperature for each microorganism. The data obtained were processed using DMFit 3.5 for the growth curve adjustment according to the model developed by Baranyi and Roberts (1994), obtaining the most representative kinetic parameters (duration of the latency phase and maximum growth rate), as well as maximum growth.

Peptide Identification

Protein Digestion. Proteolytic digestion was performed by adding trypsin (Promega, Madison, WI), 12.5 ng/μL of enzyme in ammonium bicarbonate 25 m*M*, and incubating at 37°C overnight. Protein digestion was stopped by adding trifluoroacetic acid at 1% final concentration. Digest samples were dried in a vacuum concentrator (SpeedVac; Thermo Fisher Scientific, Waltham, MA).

Nano Liquid Chromatography-Tandem MS Analysis. Briefly, nano liquid chromatography (nLC) was performed using a Dionex Ultimate 3000 nano ultra-performance liquid chromatograph (Thermo Fisher Scientific) with a C18 75 μm × 50 Acclaim Pepmam column (Thermo Fisher Scientific). Previously, peptide mix was loaded in a 300 μm × 5 mm Acclaim Pepmap precolumn (Thermo Fisher Scientific) in 2% acetonitrile/0.05% trifluoroacetic acid for 5 min at 5 μL/min. Peptide separation was performed at 40°C for all runs in. Mobile phase buffer A was composed of water and 0.1% formic acid. Mobile phase buffer B was composed of 20% acetonitrile and 0.1% formic acid. Samples were separated at 300 nL/min. Mobile phase buffer B was increased from 4 to 45% B for 60 min; then from 45 to 90% B for 1 min; followed by a 5-min wash at 90% B and a 15-min re-equilibration at 4% B. Total time of chromatography was 85 min.

Eluting peptide cations were converted to gas-phase ions by nano electrospray ionization and analyzed on a Thermo Orbitrap Fusion (Q-OT-qIT; Thermo Fisher Scientific). The mass spectrometer was operated in positive mode. Survey scans of peptide precursors from 400 to 1,500 m/z were performed at 120K resolution (at 200 m/z) with a 5 × 105 ion count target. Tandem MS was performed by isolation at 1 Th with the quadrupole, collision-induced dissociation fragmentation with normalized collision energy of 35, and rapid scan MS analysis in the ion trap. The automatic gain control ion count target was set to 102, and the max injection time was 75 ms. Only precursors with a charge state of 2–6 were sampled for tandem MS . The dynamic exclusion duration was set to 15 s with a 10-ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3-s cycles, meaning the instrument would continuously perform tandem MS events until the list of nonexcluded precursors diminished to 0 or 3 s, whichever was shorter.

Data Analysis. The raw data were processed using Proteome Discoverer (version 2.1.0.81, Thermo Fisher Scientific). The MS2 spectra were searched with the SEQUEST HT engine against a database from UniProt (Uniprot_yourorganism_Aug2016; UniProt Consortium, 2018). Both non-enzyme digestion and oxidation of methionine as variable modification were set up in database searching. Precursor mass tolerance was 10 ppm, and product ions were searched at 0.6 Da tolerances. Peptide spectral matches (**PSM**) were validated using percolator based on q-values at a 1% false discovery rate. With Proteome Discoverer, peptide identifications were grouped into proteins according to the law of parsimony and filtered to a 1% false discovery rate.

Bioactivity Analysis in the Peptides Identified. Analysis of the data obtained from identification of the hydrolysate peptides was carried out using R (version 3.4.0; https://www.r-project.org/). The peptides and their respective quantification were identified (with high confidence and without post-translational modifications) from each sample through the PSM. The quantification values were normalized, paying at-

Table 1. Effect of hydrolysis time on peptide concentration and hydrophobicity of bovine casein hydrolysates from *Cynara scolymus* proteases[1]

Item[2]	Hydrolysis time (h)			P-value[3]		
	2	4	16	H	MW	H × MW
Peptides (mg/mL)						
Total hydrolysate	3.12 ± 0.45[a]	2.89 ± 0.29[a]	4.28 ± 0.31[d]	≤0.05	≤0.01	>0.05
<3 kDa	1.93 ± 0.30[bc]	1.64 ± 0.30[c]	2.24 ± 0.22[ab]			
HI (%)						
Total hydrolysate	6.15 ± 2.10[a]	3.42 ± 0.39[a]	9.46 ± 1.63[c]	≤0.01	≤0.05	>0.05
<3 kDa	10.63 ± 2.91[a]	10.11 ± 0.40[b]	20.69 ± 0.69[d]			
HO (%)						
Total hydrolysate	93.84 ± 1.54[b]	96.58 ± 5.86[c]	90.54 ± 4.65[c]	≤0.05	≤0.01	>0.05
<3 kDa	89.4 ± 32.25[a]	89.89 ± 7.07[a]	79.31 ± 7.07[bc]			
HO/HI						
Total hydrolysate	15.25 ± 4.01[c]	28.23 ± 1.94[d]	9.57 ± 2.28[b]	≤0.01	≤0.01	0.059
<3 kDa	8.40 ± 2.07[b]	8.89 ± 0.71[b]	3.83 ± 0.42[a]			

[a–d]Values within a row with the same superscript letters were not significantly different (LSD test, $P > 0.05$).

[1]Values represent mean ± SE (n = 3).

[2]<3 kDa = hydrolysate fraction of molecular weight <3 kDa; HI = hydrophilic peptides; HO = hydrophobic peptides; HO/HI = hydrophobic and hydrophilic peptide ratio.

[3]H = hydrolysis time; MW = molecular weight; H × MW = hydrolysis time × molecular weight interaction.

tention to the total PSM for all peptides in the sample. In this way, the quantification of a single peptide was comparable between different samples.

We also carried out a search for each of the peptides identified in the BIOPEP bioactive peptide database (Minkiewicz et al., 2008). Two types of searches were carried out: identification of biopeptides in a bioactive state in the sample; and identification of potential biopeptides that contained bioactive sequences in their primary structure.

Statistical Analysis

Statistical analyses were conducted using SPSS (version 21; IBM Corporation, Armonk, NY). We performed ANOVA and Tukey tests to determine the effect of hydrolysis time and the molecular weight of the peptides in the various defined parameters. In the study of the antibacterial activity of hydrolysates, we used 1-way ANOVA and the Dunnett test to identify differences between the means of the samples and the control, establishing a confidence level of 95%.

RESULTS AND DISCUSSION

Total Peptides and Hydrophilic and Hydrophobic Peptides of Bovine Casein Hydrolysates

As observed in Table 1, the peptide concentration at 16 h of hydrolysis was significantly ($P \leq 0.05$) higher than at 2 and 4 h, both in the total hydrolysate (**TH**) and in the fraction below 3 kDa. Similar results were obtained in other studies, where casein was hydrolyzed with proteases of vegetable origin, such as pomiferin from *Maclura pomifera* latex (Corrons et al., 2017), enzymes from the latex of *Jacaratia corumbensis* (Arruda et al., 2012), or proteinases from *Ficus carica* latex (Di Pierro et al., 2014); enzymes of animal origin, such as pepsin and pancreatin (Petrat-Melin et al., 2016); or proteases of microbial origin, such as AS1.398 neutral protease from *Bacillus subtilis* (Jiang et al., 2010) and fermentation with *Bifidobacterium longum* (Chang et al., 2013).

The quantity of hydrophobic (**HO**) and hydrophilic (**HI**) peptides increased both in TH and in <3 kDa fractions, and were higher after 16 h of hydrolysis. Corrons et al. (2017) obtained similar results when hydrolyzing bovine casein with latex extract from *M. pomifera*. The principal peptides were hydrophobic, with percentages in the total hydrolysates of 93.84 ± 1.5%, 96.58 ± 5.9%, and 90.54 ± 4.6% at 2, 4, and 16 h, respectively. The greatest increase in HO, both in TH and in <3 kDa peptides, was produced during 2 to 4 h of hydrolysis. Nevertheless, the principal increase in the HI was produced from 4 to 16 h. In the TH, more HO was observed than in the <3 kDa fractions. The highest (HO/HI) ratio was observed at 4 h of hydrolysis, both in the TH and the <3 kDa fractions, diminishing considerably at 16 h of hydrolysis due to a greater concentration of hydrophilic peptides. In the same way, De Gobba et al. (2014b) and Corrons et al. (2017) observed that the concentration of hydrophilic peptides increased with time during hydrolysis. We found no studies on the concentration of HO and HI peptides in casein hydrolysates. The works carried out on cheese have shown that when proteases from *C. cardunculus* were used as a coagulant, a greater percentage of hydrophobic peptides was observed than when

using other enzymes (Agboola et al., 2004; Tejada et al., 2008).

As observed in other studies (Alvarado and Guerra, 2010; Nguyen et al., 2011; Zhang et al., 2012), we would expect that casein hydrolysates obtained with *C. scolymus*, with a high content of peptides (mostly hydrophobic), would be highly bioactive.

ACE Inhibitory Activity of Bovine Casein Hydrolysates

Figure 1 shows the percentages for ACE inhibitory activity of the bovine casein TH with extract of *C. scolymus* flower at different hydrolysis times, as well as their <3 kDa fractions at a concentration of 125 μg/mL. The ACE inhibition in the TH increased over the time of hydrolysis ($P \leq 0.01$), particularly at 16 h. This must have been due to a major enzyme action resulting in shorter sequence peptides, which can better accommodate the ACE active site (Natesh et al., 2003). At <3 kDa, we observed no significant differences between hydrolysis times. In other studies, ACE inhibition also increased with the proteolysis time of casein hydrolysates (Mao et al., 2007; Jiang et al., 2010; Welderufael et al., 2012; De Gobba et al., 2014b; Corrons et al., 2017). However, Otte et al. (2007) found no significant differences in ACE inhibitory activity between 3 and 24 h of hydrolysis, similar to the findings for the <3 kDa fraction of our study. Some authors have observed that, after a specific hydrolysis time, ACE inhibitory activity does not increase, and may even decrease as a result of extensive proteolytic degradation of ACE inhibitory peptides (Mao et al., 2007; Ong et al., 2007; Jiang et al., 2010; Welderufael et al., 2012).

The ACE inhibitory activity we observed in the casein hydrolysates obtained from *C. scolymus* was higher than that observed in other studies. In hydrolysates with alcalase, Mao et al. (2007) detected ACE inhibition of 79.5% and 85.4% for their <6 kDa ultrafiltrate at a final concentration of 4 mg/mL, which was much greater than that used in our study (0.125 mg/mL). In a pancreatic casein hydrolysate, Wu et al. (2013a) observed 73.5% ACE inhibitory activity with a concentration 8 times greater than that used in our study.

The molecular size of the peptides had a significant influence on ACE inhibition ($P \leq 0.01$), with the <3 kDa fractions responsible for the main action in all cases, although we found no statistically significant differences between the ACE inhibitory TH activity of CH16 (55.05 ± 3.69%) and its <3 kDa fraction (61.91 ± 3.22%). These results were in accordance with those observed in other casein hydrolysate studies (Mao et al., 2007; Miguel et al., 2009; Jiang et al., 2010; De Gobba et al., 2014b; Corrons et al., 2017). As previously stated, this may have been because the smallest peptides were more accessible to the ACE active site (Natesh et al., 2003).

The IC_{50} obtained from the hydrolysates at 16 h were 117.04 ± 3.3 μg/mL and 71.77 ± 11.8 μg/mL from the TH and the <3 kDa fractions, respectively. These values were lower (indicating greater activity) than those found in studies of casein hydrolysates using different enzymes. Using an extract of *M. pomifera*, Corrons et al. (2017) obtained an IC_{50} value of 1.72 mg/mL for their <3 kDa hydrolysate fraction. Wu et al. (2013b) observed an IC_{50} of 2.36 mg/mL in a fraction of α casein between 5 and 10 kDa and an IC_{50} of 4 mg/mL in another fraction of β casein, using a protease of *Lactobacillus casei* ssp. c*asei.* Through the N "Amano" protease of *Bacillus subtilis,* Welderufael et al. (2012) obtained an IC_{50} value of 285 μg/mL. Using alcalase, Mao et al. (2007) observed IC_{50} values of 290 μg/mL and 250 μg/mL, respectively, in 2 isolated peptides (PPEIN and PLPLL). With neutral protease AS1.398, Jiang et al. (2010) obtained an IC_{50} of 684 μg/mL for TH and 461 μg/mL for its <3 kDa fraction. The casein hydrolysates obtained from the *C. scolymus* L. flower extract demonstrated potent ACE inhibitory activity in vitro, greater than that observed in other studies. This could have been due to the greater proteolytic action of the aspartic proteinases present, thereby producing a greater number of peptides, most of which were hydrophobic.

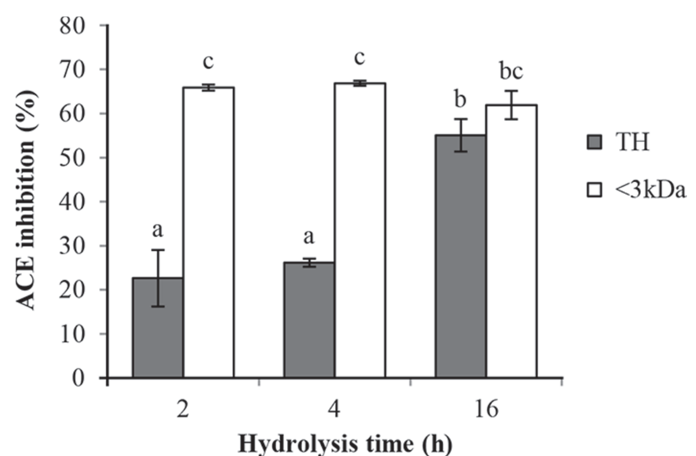

Figure 1. Angiotensin-I converting enzyme (ACE) inhibitory activity (%) of bovine casein total hydrolysates (TH) and their low-molecular-weight fractions (<3 kDa). Values are mean ± SE (n = 3). Bars with the same letter (a–c) were not significantly different (LSD test, $P > 0.05$).

Antioxidant Activity and Iron (II) Chelating Activity

The hydrolysates obtained with *C. scolymus* showed a high DPPH RSA, expressed as Trolox equivalents (μM)/milligram of peptides (Figure 2). Hydrolysis time had a significant influence on the DPPH RSA ($P < 0.05$), with higher values observed in CH4 than in CH2 and CH16 TH. Mao et al. (2011) observed a similar evolution with increased DPPH RSA for up to 7 h of hydrolysis, followed by a decline. The high CH4 activity may have been due to the high content of HO peptides and the highest HO/HI ratio observed in this hydrolysate. In fact, a positive correlation can be observed between these parameters (0.84 and 0.78, respectively for HO and HO/HI; $P < 0.05$). Other studies have also observed that peptides with a high hydrophobic amino acid content increase the antioxidant activity of the hydrolysates (Ren et al., 2008; Zhang et al., 2012). On the other hand, differences in DPPH RSA were observed between the TH and its <3 kDa fractions. The <3 kDa fractions displayed greater activity than the TH ($P \leq 0.05$) in the same way as in other studies, which found that the low-molecular-weight peptides had a higher antioxidant capacity (Jeon et al., 1999; Kim et al., 2007; Timón et al., 2014; Irshad et al., 2015).

It has also been observed that the hydrolysates display an elevated level of antioxidant activity in the presence of the $ABTS^{\bullet+}$ radical (Figure 3). Hydrolysis time significantly ($P \leq 0.01$) affected this activity, producing a decrease in SC_{50} and at the same time an increase in Trolox equivalents (mM)/milligrams of peptides (indicating greater antioxidant potency). As a consequence, CH16 <3 kDa was the hydrolysate with the greatest antioxidant capacity against $ABTS^{\bullet+}$. Other studies also observed an increase in the $ABTS^{\bullet+}$ RSA by the hydrolysates over the hydrolysis time (Gómez-Ruiz et al., 2008; Kumar et al., 2010, 2016; Chang et al., 2013; De Gobba et al., 2014b; Petrat-Melin et al., 2016). Molecular weight had a significant effect on the $ABTS^{\bullet+}$ RSA ($P \leq 0.05$), although a variation in function over the hydrolysis time was observed. In this way, the TH displayed greater activity ($P \leq 0.05$) than the <3 kDa fraction up to 4 h of hydrolysis (CH2 and CH4). Nonetheless, CH16 <3 kDa showed an antioxidant action that was significantly higher ($P \leq 0.05$) than CH16 TH. In other studies, a greater activity in the low-molecular-weight fractions (<3 kDa and <1 kDa) has been observed (Chang et al., 2013; Shanmugam et al., 2015). The SC_{50} value of CH16 TH was similar to that observed by De Gobba et al. (2014b) and much lower (meaning greater activity) than that detected in casein hydrolysates obtained from different enzymes (Kitts, 2005; Rossini et al., 2009).

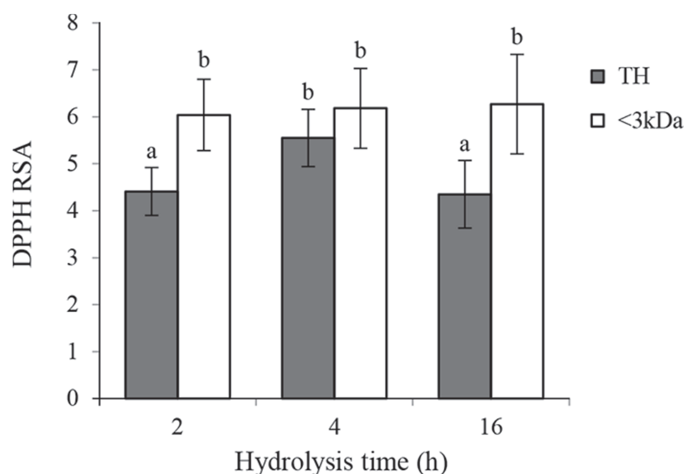

Figure 2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA; μM Trolox equivalents/mg of peptides) of bovine casein total hydrolysates (TH) and their low-molecular-weight fractions (<3 kDa). Values are mean ± SE (n = 3). Bars with the same letter (a, b) were not significantly different (LSD test, $P > 0.05$).

The iron (II) chelating activity from the *C. scolymus* casein hydrolysates was significantly affected as much by hydrolysis time as by the molecular weight of the hydrolysates (Figure 4). After 4 h of hydrolysis, we observed an increase in both the TH and in the <3 kDa fraction. In this respect, Hidalgo et al. (2015) also observed that in bovine sodium caseinate hydrolysates, chelating activity increased over the hydrolysis time up to 3 h. The CH4 TH demonstrated the greatest level of iron (II) chelating activity. This hydrolysate showed the greatest HO/HI ratio and the greatest concentration of hydrophobic peptides, and Pownall et al. (2010) described that the presence of aromatic and hydrophobic amino acids in the peptides may contribute to higher chelating activity. Alternatively, De Gobba et al. (2014b) did not detect any iron (II) chelating activity in casein hydrolysates from *Arsukibacterium ikkense* proteases.

The bovine casein hydrolysates produced with proteases extracted from the artichoke flower displayed important antioxidant activity, although it varied by the method used, because the scavenging mechanism differs from one radical to another (Li et al., 2013). In our study, the method used to determine the $ABTS^{\bullet+}$ RSA was much more sensitive than the method for the DPPH RSA, because the hydrolysates showed an activity equivalent to Trolox that was 1,000 times greater (mM vs. μM Trolox equivalents/mg of peptides) against $ABTS^{\bullet+}$ RSA, similar to the findings of other authors (Chang et al., 2013; Li et al., 2013; Kumar et al., 2016).

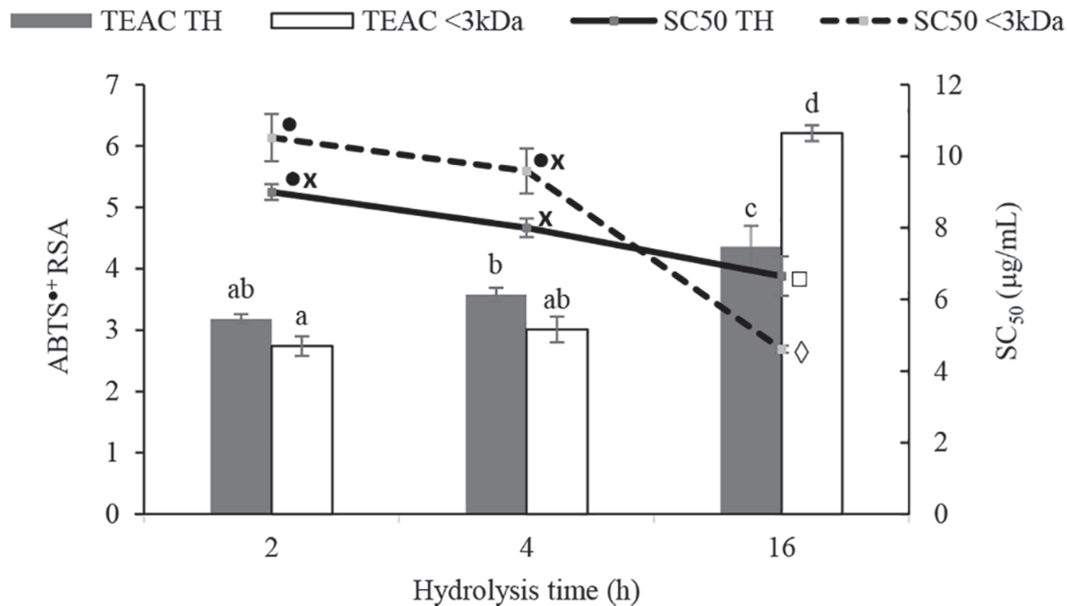

Figure 3. 2,2'-Azino-bis-(3-ethiylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity (RSA) of bovine casein total hydrolysates (TH) and their low-molecular-weight fractions (<3 kDa). Bars show Trolox equivalent antioxidant capacity (TEAC; m*M* Trolox equivalents per mg of peptides) and lines represent half-maximal scavenging concentration (SC_{50}; μg/mL). Values are mean ± SE (n = 3). Bars with the same letters (a–d) and lines with the same symbol (●, ×, □, ◊) were not significantly different (LSD test, $P > 0.05$).

Antimicrobial Activity

The hydrolysates we obtained showed relevant antimicrobial activity only on *E. faecalis*. Table 2 shows the effect on the parameters of bacterial growth, lag phase, growth rate, and maximum growth for this bacteria. We observed a significant inhibitory effect with respect to the control curves ($P \leq 0.01$) in the lag phase and maximum growth parameters with the 3 types of TH. We found a significant increase ($P \leq 0.01$) in the lag phase when the activity of the <3 kDa fractions were analyzed at CH2 and CH16. The TH displayed greater antimicrobial activity than the <3 kDa fractions, similar to the findings of Kumar et al. (2016). These differences between the antimicrobial activity of TH and their fractions, which has also been observed in other studies, may have been due to their ionic nature, the presence of different peptides of various sizes and charges, and their concentration (Andreu and Rivas, 1998; Gennaro and Zanetti, 2000; Kustanovich et al., 2002; Gobbetti et al., 2004).

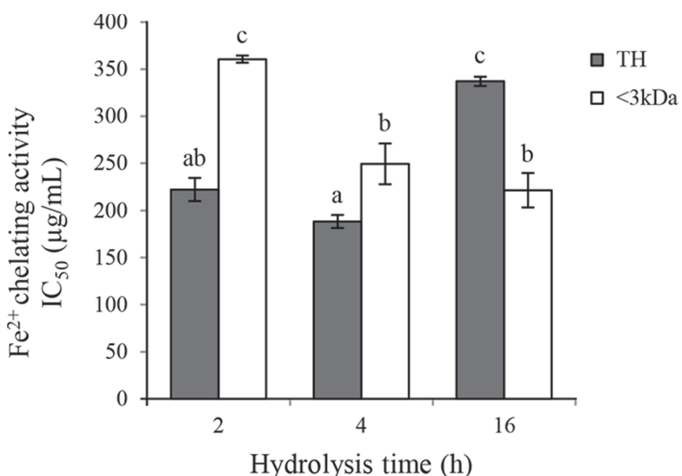

Figure 4. Iron (II) chelating activity [half-maximal inhibitory concentration (IC_{50}); μg/mL] of bovine casein total hydrolysates (TH) and their low-molecular-weight fractions (<3 kDa). Values represent mean ± SE (n = 3). Bars with the same letters (a–c) were not significantly different (LSD test, $P > 0.05$).

In the rest of the microorganisms studied, we observed a significant increase in lag phase only in *E. coli* ($P \leq 0.05$) with CH2 TH. In the gram-positive microorganisms, we observed an increase in lag phase only in *P. fluorescens* (data not shown). The scant antimicrobial activity observed may have been due to the low final concentration of hydrolysates in our study (an average of 1.7 mg/mL for the TH and 0.97 mg/mL for its ultrafiltrates). For the same reason, De Gobba et al. (2014b) did not find antimicrobial activity in their casein hydrolysates. Arruda et al. (2012) observed antimicrobial activity only when using hydrolysate concentrates between 62.5 and 250 mg/mL; the 50 mg/mL concentration was active only on *Staphylococcus aureus*. On the other hand, Sedaghati et al. (2016) reported that several fractions of the plasmin digest of bovine

Table 2. *Enterococcus faecalis* antibacterial activity (in absorbance units, UAbs) of bovine casein hydrolysates from *Cynara scolymus* proteases[1]

Molecular weight	Parameter	Control	Hydrolysis time (h) 2	4	16
Total hydrolysate	Lag phase (min)	301.62 ± 5.67	831.22 ± 27.29*	900.00 ± 0.00*	876.77 ± 11.83*
	Growth rate (mUAbs/min)	1.19 ± 0.04	0.75 ± 0.48	0.01 ± 0.00	1.34 ± 0.70
	Maximum growth (UAbs)	0.403 ± 0.01	0.084 ± 0.04*	0.009 ± 0.00*	0.095 ± 0.05*
<3 kDa[2]	Lag phase (min)	295.55 ± 22.01	625.42 ± 53.39*	417.43 ± 19.74	526.74 ± 32.37*
	Growth rate (mUAbs/min)	1.45 ± 0.14	2.25 ± 0.45	1.67 ± 0.15	1.29 ± 0.13
	Maximum growth (UAbs)	0.454 ± 0.01	0.348 ± 0.04*	0.407 ± 0.02	0.332 ± 0.02*

[1]Values represent mean ± SE (n = 3).

[2]Hydrolysate fraction of molecular weight <3 kDa.

*Samples and control were significantly different (Dunnett test, $P \leq 0.01$).

β-casein (at a concentration of 0.45 mg/mL) reduced *E. coli* growth and completely stopped the growth of *S. aureus*, *L. casei,* and *Lactobacillus acidophilus*. Growth inhibition was also observed against *L. innocua* in bovine casein hydrolysates using a purified protease of *Lactococcus lactis* ssp. *lactis* BR16 (1 mg/mL), at hydrolysis times of 4 to 24 h, but no activity was obtained against the other tested bacteria (*Micrococcus luteus*, *Salmonella enteriditis,* and *E. coli*; Bougherra et al., 2017). Almi-Sebbane et al. (2018) reported that only the <1 kDa fraction of the peptic hydrolysate of camel milk β-casein (at 1 mg/mL) exhibited *L. innocua* inhibition; it was not significantly affected in the presence of the >1 kDa fraction. However, *S. aureus* growth was affected by the <10 kDa fraction, and *E. coli* growth was inhibited in the presence of all hydrolysate fractions (including the >10 kDa fraction) and the native β-casein.

Table 3. Peptide sequences identified in bovine casein hydrolysates from *Cynara scolymus* proteases that were registered in the bioactive peptide database (BIOPEP)[1]

Sequence (origin hydrosylate)	ID	Origin	MM	Activity	IC_{50} (μ*M*)
AMKPWIQPK (2 h, 4 h)	7497/3395	α_{S2}-CN	1,098.36	ACE inhibitory	600
AMKPWIQPKTKVIPYVRYL (16 h)	3030	α_{S2}-CN	2,331.87	Calmodulin binding	—
ARHPHPHLSFM (2 h)	7797	β-CN and κ-CN	1,329.54	Antioxidative	—
DAQSAPLRVY (2 h, 4 h, 16 h)	9112	β-LG	1,119.23	ACE inhibitory	13
FFVAPFPEVFGK (2 h, 4 h)	7800	α_{S1}-CN	1,384.63	ACE inhibitory	18
	8311			Anticancer	—
GPVRGPFPII (16 h)	8286	β-CN	1,052.27	Antioxidative	—
	8169			ACE inhibitory	—
KVLPVPQK (4 h, 16 h)	7876	β-CN f(169–176)	908.14	Antioxidative	—
LLYQEPVLGPVRGPFPIIV (4 h, 16 h)	8174	β-CN f(1–28)	2,107.55	Immunomodulating	—
MAIPPKKNQDK (16 h)	3292	κ-CN f(106–116)	1,269.52	Antithrombotic	—
	2796			Platelet inhibitory	—
NLHLPLPLL (4 h, 16 h)	8315	β-CN	1,029.28	Anticancer	—
PAAVRSPAQILQ (2 h, 4 h, 16 h)	8271	κ-CN f(64–75)	1,250.45	Antibacterial	—
RELEELNVPGEIVESLSSSEESITR (2 h, 4 h)	3055	Phosphopeptide β-CN f(1–25)	2,803.03	Immunomodulating	—
RPKHPIKHQ (2 h, 4 h, 16 h)	7483	α_{S1}-CN f(1–9)	1,140.35	ACE inhibitory	13
RPKHPIKHQGLPQEVLNENLLRF (2 h, 4 h, 16 h)	3035	Isracidin/α_{S1}-CN f(1–23)	2,764.22	Antibacterial	—
	8171			Immunomodulating	—
SDIPNPIGSENSEK (2 h, 4 h, 16 h)	8335	α_{S1}-CN f(195–208)	1,486.55	Antibacterial	—
TPVVVPPFLQP (4 h)	3503	β-CN f(80–90)	1,193.44	ACE inhibitory	749
VENLHLPLPLL (4 h, 16 h)	8317	β-CN	1,257.53	Anticancer	—
VKEAMAPK (2 h)	7796	β-CN	873.07	Antioxidative	—
VLNENLLR (4 h)	8334	α_{S1}-CN f(30–37)	970.13	Antibacterial	—
VYPFPGPIH (4 h)	2614	β-CN	1,026.18	Antiamnestic	110
VYQHQKAMKPWIQPKTKVIPYVRY (16 h)	3965	α_{S2}-CN f(183–206)	3,002.59	Hemolytic	—
	3435			Antibacterial	—
	3033			Calmodulin binding	—
VYQHQKAMKPWIQPKTKVIPYVRYL (16 h)	3034	α_{S2}-CN f(183–207)	3,115.75	Calmodulin binding	—
	3964			Hemolytic	—
	5469			Antibacterial	—

[1]ACE = angiotensin-converting enzyme; IC_{50} = half-maximal inhibitory concentration; ID = BIOPEP sequence identification number; MM = molecular mass (Da).

Identification of the Bioactive Peptides Present in Casein Hydrolysates

We searched for the sequences identified in the different casein hydrolysates in the BIOPEP database (Minkiewicz et al., 2008). We detected 2 types of biopeptides: those in their bioactive form in the casein hydrolysates (Table 3); and potentials, which contain bioactive sequences in their primary structure (Figure 5).

Some of the 22 peptides detected in their bioactive form were found in the 3 hydrolysates: DAQSAPLRVY and RPKHPIKHQ with ACE inhibitory activity (IC_{50} 13 μM); PAAVRSPAQILQ and SDIPNPIGSENSEK with

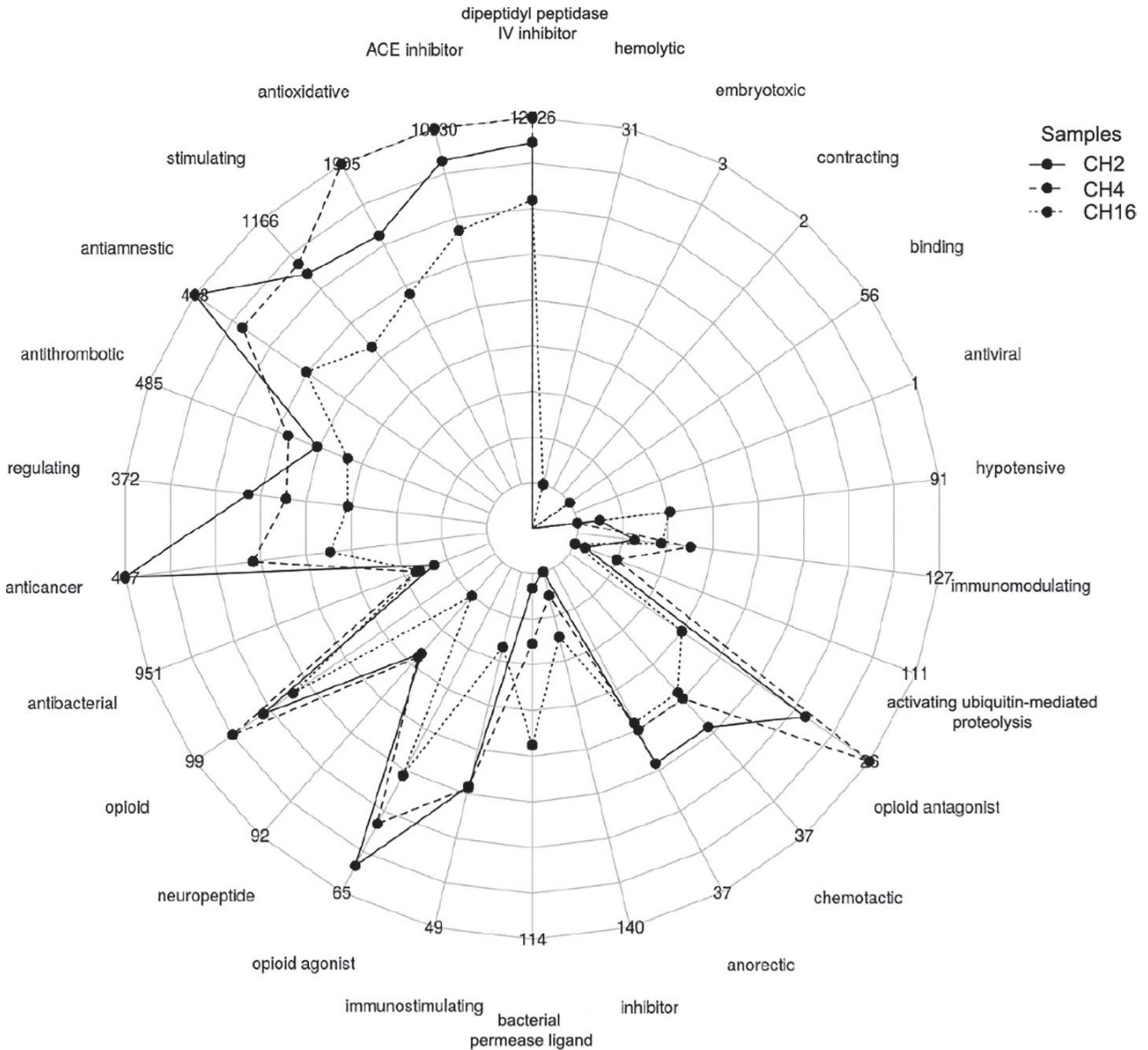

Figure 5. Quantification of potential bioactive peptides in bovine casein total hydrolysates using a *Cynara scolymus* water-soluble extract at different hydrolysis times: 2 h (CH2), 4 h (CH4), and 16 h (CH16), considering their peptide spectral matches (PSM) in liquid chromatography–mass spectrometry and taking into account the presence of demonstrated bioactive sequences in their primary structure. The identified peptides and their respective quantification for each sample were extracted. The quantification values were normalized according to the total PSM of all peptides in the samples. In this way, quantification of the same peptide between samples was comparable. ACE = angiotensin-I converting enzyme.

antibacterial activity; and RPKHPIKHQGLPQEVLNENLLRF with antibacterial and immunomodulatory activity. The CH2 and CH4 hydrosylates shared 3 peptides: AMKPWIQPK with ACE inhibitory activity (IC_{50} 600 μ*M*), FFVAPFPEVFGK with ACE inhibitory activity (IC_{50} 18 μ*M*) and anticancer activity, and RELEELNVPGEIVESLSSSEESITR with immunomodulating activity. The CH4 and CH16 hydrosylates contained 4 common peptides: KVLPVPQK with antioxidant activity, LLYQEPVLGPVRGPFPIIV with immunomodulating activity, and NLHLPLPLL and VENLHLPLPLL with anticancer activity. We identified other peptides that appeared in only 1 of the hydrolysates. We identified the sequences ARHPHPHLSFM and VKEAMAPK with antioxidant activity in CH2. In CH4, we identified TPVVVPPFLQP with ACE inhibitory activity (IC_{50} 749 μ*M*), VLNENLLR with antibacterial activity, and VYPFPGPIH with antiamnesic activity (IC_{50} 110 μ*M*). In CH16, we identified AMKPWIQPKTKVIPYVRYL with calmodulin binding activity, GPVRGPFPII with antioxidant activity, MAIPPKKNQDK with platelet and antithrombotic activity, and VYQHQKAMKPWIQPKTKVIPYVRY and VYQHQKAMKPWIQPKTKVIPYVRYL with calmodulin binding, hemolytic, and antibacterial activities. In casein hydrolysates obtained using other vegetable enzymes, other peptides that were different from ours have been obtained. Using bovine casein hydrolysates from the latex of *M. pomifera,* Corrons et al. (2017) identified 2 peptides with ACE inhibitory activity (YQEPVLGPVRGPFIIV and RFFVAPFPE). In a bovine β-casein hydrolysate with latex extract from *Ficus carica*, Di Pierro et al. (2014) identified peptides with antioxidant activity (DMPIQ, LPLPLL, VPYPQR, DKIHPFA, GPFPIIV, KVLPVPQK, QPHQPLPPT, and YPFPGPIPNS).

If we consider the number of peptides with potential bioactivity in a qualitative manner (number of different peptide sequences identified without taking their abundance into consideration), we identified 25,169 different sequences in CH16, 14,513 in CH4, and 12,116 in CH2. Figure 5 shows the distribution of peptides with potential activity for the casein hydrolysates at CH2, CH4, and CH16, grouped according to the type of activity observed in their sequence fragments. Although CH16 contained more different sequences, the greatest quantity of peptides with potential activity was observed in CH4, considering their quantitative abundance in the sample relating to the PSM, and highlighting its ACE inhibitory and antioxidant activity, its dipeptidyl peptidase IV inhibition, and its stimulant, antithrombotic, opioid, and immunomodulatory activities. The CH2 stood out because of its potential antiamnesic, anticancer, opioid agonist, anorexigenic, and chemoattractant activities. The CH16 stood out because of its greater potential for binding the bacterial permease and its hypotensive, chelating, and hemolytic activities.

CONCLUSIONS

Bovine casein peptides obtained from extracts of *C. scolymus* demonstrated an elevated level of ACE inhibition and antioxidant activity in vitro compared with the findings of similar studies using enzymes of different origin (vegetable, animal, or bacterial). As well, the hydrolysates showed *E. faecalis* antibacterial activity in vitro. The hydrolysis time of 16 h yielded hydrolysates with a greater concentration of peptides. Low-molecular-weight fractions of <3 kDa obtained at a hydrolysis time of 16 h showed a greater level of ACE inhibition and higher antioxidant activity than the total hydrolysate. We identified 22 bioactive peptides, highlighting those that had demonstrated ACE inhibitory activity, antioxidant activity, and antimicrobial activity. We also identified a large number of peptides with putative activity and that possessed fragments of bioactive sequences, particularly sequences related to ACE inhibition, dipeptidyl peptidase IV inhibition, and antioxidant activity.

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