Structure-based identification and design of ACE-inhibitory peptides from whey proteins

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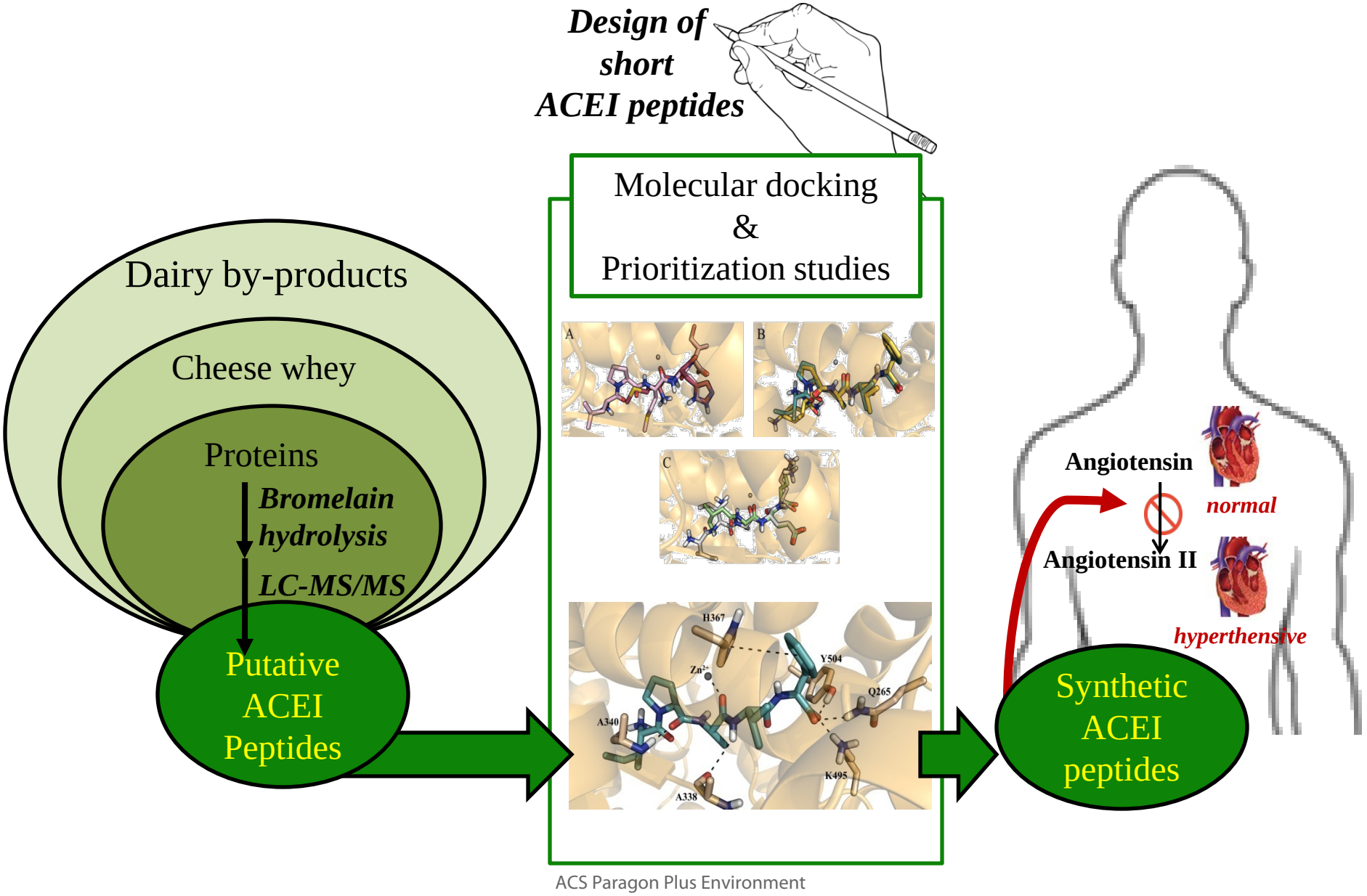
Design of short ACEI peptides
Molecular docking & Prioritization studies
Dairy by-products
Cheese whey
Proteins
Bromelain hydrolysis
LC-MS/MS
Putative ACEI Peptides
A
B
C
H367
Y504
Zn2+
Q265
A340
K495
A338
Angiotensin
normal
Angiotensin II
hyperthensive
Synthetic ACEI peptides

Structure-based identification and design of ACE-inhibitory peptides from whey proteins

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Abstract

Besides their nutritional value, whey protein (WP) peptides are food components retaining important pharmacological properties for controlling hypertension. We herein report how the use of complementary experimental and theoretical investigations allowed the identification of novel ACE inhibitory (ACEI) peptides obtained from a WP hydrolysate and addressed the rational design of even shorter sequences based on molecular pruning. Thus, after bromelain digestion followed by 5kDa cut-off ultrafiltration, WP hydrolysate with ACEI activity was fractioned by RP-HPLC; two out of 23 collected fractions retained ACEI activity and were analysed by LC-MS/MS. In the face of 128 identified peptides, molecular docking was carried to prioritize peptides and to rationally

guide the design of novel shorter and bioactive sequences. Therefore, eleven peptides, consisting of 3-6 aminoacids and with molecular weights in the range from 399 to 674 Da, were rationally designed and then purchased to determine IC_{50} value. This approach allowed to identify two novel peptides: MHI and IAEK with IC_{50} ACEI values equal to 11.59 and 25.08μM, respectively. Interestingly, we also confirmed the well-known ACEI IPAVF with an IC_{50} equal to 9.09 μM.

In light of these results, this integrated approach could pave the way for high throughput screening and identification of new peptides in dairy products. In addition, the herein proposed ACEI peptides could be exploited for novel applications both for food production and pharmaceuticals.

Keywords: *bioactive peptides; bromelain; hypertension; peptide docking; whey proteins.*

Abbreviations:

ACE, Angiotensin I-Converting Enzyme;

WPI, Whey protein Isolate

FAPGG, *N*-[3-(2-furil)-acryloil]-L-phenylalanine-glycyl-glycine

WPIh, Whey Protein Isolate hydrolysate

BLG, ß-Lactoglobulin

ALA , α-Lactoalbumin

SDS-PAGE, Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

MSA, Multiple Sequence Alignment

AnCE, *Drosophila melanogaster* homologous of ACE

1. Introduction

Hypertension is a worldwide pathological condition that dramatically raises the risk of cardiovascular diseases and damage to vital organs such as heart, brain, and kidney causing the death of approximately 7.5 million people every year. A recent study reports that the number of people with hypertension doubled from 594 million to more than 1.1 billion from 1975 to 2015[1]. Although healthy lifestyle still remains the best medicine, a wide arsenal of blood pressure drugs is also available. Many molecules are still in the investigative stage and need long term clinical studies to ascertain their safety and effective efficacy[2]. In this scenario, a supportive role can be played by bioactive peptides as complement to the traditional treatment of hypertension[3]. Evidences for the effectiveness of ACE inhibitory (ACEI) peptides to modulate the cardiovascular system were provided by the finding that isoleucine–proline–proline (IPP), valine–proline–proline (VPP), and isoleucine-tryptophan (IW) from milk and whey proteins decreased blood pressure and reduced activity of plasma ACE in hypertensive and normotensive patients [4,5]. So far, ACEI peptides have been released by food proteins (mainly milk, whey and plants) through both innovative and conventional approaches, including the production of recombinant peptides[6] or enzymatic hydrolysis of the parent protein[7]. However, the problems associated with such procedures (extensive time, cost, and loss of functional properties), have demonstrated the need to develop more straightforward methods to produce ACEI peptides. In this regard, there is very limited information on the relationships between structure and activity (SAR) of food protein-derived antihypertensive peptides[8] that could lead to the discovery of novel active sequences with simplified production process and improved bioavailability. Most food protein-derived peptides with ACEI potential have relatively low molecular weights, generally ranging from dipeptides to pentapeptides of 150 and 800 Da, respectively[9]. This latter characteristic could facilitate their absorption, as intact form, from the intestinal tract into blood circulation promoting antihypertensive effects [10]. Because the primary activity of ACE is to cleave the C-terminal dipeptide of oligopeptide substrates with a wide specificity, the inhibitory activity of ACEI peptides is strongly influenced by their C-terminal

tripeptide sequence. The most potent ACEI peptides contain hydrophobic amino acid residues (e.g. tryptophan, phenylalanine, tyrosine, or proline) at each of the three C-terminal positions that interact with bulky hydrophobic sub-sites of the ACE active site [10]. It was suggested that most naturally occurring ACEI peptides contain Pro, Lys, or aromatic amino acid residues [11]. On the other side, the least favourable C-terminal amino acids of ACEI peptides are the dicarboxylic amino acids [12]. Other SAR studies have pointed out that also residues in the C-terminal with charged side groups influence ACEI potency. Examples are given by the positive charged Lysine (ε-amino group) and Arginine (guanidine group). [8,13]

It must be emphasized that the observed correlations between ACE inhibition and structural properties of peptides, confirmed by QSAR models, are valid for smaller peptides. As peptide length increases (7 or 8 residues), the relationship between C-terminal structure and activity decreased [13].

In the case of enzymatic hydrolysis of food proteins, the chosen enzyme and kind of protein are fundamental to increase the probability of obtaining ACEI peptides. Whey proteins (WPs) are recovered by cheese whey or other dairy industry by-products (ricotta or scotta) that sophisticated strategies of valorisation aim at converting in high value-added products[14-15]. Among functional properties, WPs show in their intact form numerous ACEI peptides[14] due to the presence of hydrophobic amino acids at different terminals, glycine, isoleucine, leucine and valine at N-terminal and proline, tyrosine and tryptophan at the C-terminal. However, biological activities have been associated with protein hydrolysates (mainly peptic and tryptic digests) and less information is available about sequences binding ACE enzyme [16].

Bromelain, a cysteine protease released from pineapple tissues, already exploited in therapeutic applications, has been recently proposed in food industry due both to its technological properties [17] and the ability to release biological peptides from several sources[18.] To date, ACEI peptides obtained from enzymatic treatment of WPs with bromelain have been poorly investigated[19].

Based on this evidence and background, in this work we digested WPs with the enzyme bromelain in order to obtain whey-derived ACEI peptides that are either novel or endowed with improved activity. Then, purified and identified peptides putatively endowed with ACEI activity were assayed against ACE enzyme by an innovative peptide docking protocol to identify binding sequences and elucidate the rationale behind their inhibitory activity. Finally, predicted ACEI peptides were synthesized to validate molecular docking analysis and to determine IC_{50} value.

2. Materials and methods

2.1. Materials and chemicals

Whey proteins isolate (WPI), purchased from Volchem (Grossa di Gazzo, Italy), was composed (as dry matter) of ß-lactoglobulin (49.7 %; BLG), α-lactalbumin (16.6%, ALA), bovine serum albumin (4.6%; BSA), and immunoglobulins (6.5 %) as reported in the technical data sheet. Bromelain from pineapple stem (≥ 3U /mg protein), Angiotensin Converting Enzyme from rabbit lung (ACE, ≥ 2.0 U/mg protein),*N*-[3-(2-furil)-acryloil]-L-phenylalanine-glycyl-glycine (FAPGG) and captopril were purchased from Sigma–Aldrich (Milan, Italy). All chemicals and reagents for the electrophoresis and LC/MS analysis were obtained from Bio-Rad (Bio-Rad Laboratories srl, Milan, Italy) and Carlo Erba Reagents (Cornaredo, Milan, Italia), respectively.

2.2 Hydrolysis of WPI

2.2.1 Enzymatic hydrolysis

WPI (6%) was dissolved in 50 mL of distilled water previously heated to 80 °C. The suspension was adjusted to pH 8.16 with 4 M NaOH solution, and the total volume was finally brought to 100 mL with distilled water. Samples were digested at 37°C with bromelain (ratio Enzyme/Substrate of 1:10 w/w), in a Thermomixer R (Eppendorf, Hamburg, Germany) at 300 rpm for 4 h.[20] WPI without enzyme and dissolved in alkaline water (pH 8.16) was also included as control. All samples were performed in triplicate. During hydrolysis, pH of each sample was measured and eventually

restored to its original value; in order to monitor hydrolysis, at 1h time interval aliquots (1 mL) were also retrieved for protein content determination. At the end of the hydrolysis, samples were heated at 100 °C for 10 minutes to inactivate the enzyme; then, pH was neutralized (pH = 7.0) by using 4 M NaOH or 2 M HCl, depending on the case. Finally, all samples were centrifuged at *13,000 x g*, 4 °C for 15 min; then, supernatants were quantified for residual protein content by the Bradford's method against a calibration curve of BLG (ranging from 0.1 to 1.0 mg/mL)[21]; samples were stored at -20 °C for the following analyses.

2.2.2 SDS-PAGE

The electrophoretic analysis of control samples (WPIc) and WPI hydrolyzates (WPIh) at different time of sampling (0, 1, 2, 3, 4 h of reaction; 7 μg) was performed by sodium sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), using the Criterion™ Cell (Biorad), as previously reported.[22] The Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (10–250 kDa; BioRad) were used as reference. In addition, *α*-lactoalbumin (ALA), and *β*-lactoglubulin (BLG) were loaded (5 μg) on the gel as reference standards. Experiments were carried out in triplicate. Stained gels were scanned with a GS-800 calibrated the Image Scanner III (GE Healthcare, USA).

2.2.3 Ultrafiltration and quantification of free aminoacids (FAA) and small peptides

In order to remove residual undigested proteins and large polypeptides that could cause significant interference with biological assay, the hydrolyzed samples (WPIh) and related controls (WPIc) were filtered on Vivaspin Turbo 4 Ultrafiltration Unit with 5-kDa molecular-weight cut-off membrane (Sartorius AG, Göttingen, Germany) for 40 min at *7,500 x g*. Then, permeate samples were analysed for free amino acids (FAA) and small peptides concentrations by following the method described by Church et al.[23] These values were calibrated against glycine solutions at

different concentrations (0.8 mg/mL a 0.01 mg/mL) and expressed as glycine (Gly)-equivalent concentration. Each analysis was performed in triplicate.

2.3 In vitro assay of ACE inhibitory activity

ACE inhibitory (ACEI) activity of ultrafiltrated WPIh were measured by the spectrophotometric assay described by Donkor et al.[24] In particular, ACE was dissolved at 1 U/μL in phosphate buffer (0.01 M, 0.5 M NaCl, pH 7) and added (3 U) to a solution of reaction buffer (50 mM HEPES, 300 mM NaCl, pH 8.3) containing 0.025 to 5 μg/mL Gly-equivalent of WPIh and WPIc samples and pre-equilibrated at 37 °C. The absorbance at 340 nm was recorded every 10 minutes for 30 min using the Varioskan Flash spectrofluorimeter (Thermo Fischer Scientific, Waltham, MA, USA). The anti-hypertensive agent captopril (0.5 mM), with proved ACEI activity, was included as positive control. At the end of incubation, the extent of inhibition was calculated as follows:

$$ACE\ inibitory\ activity\ \% = \left[1 - \left(\frac{c-d}{a-b}\right)\right] x100$$

Where *a* is the absorbance of the ACE solution in the buffer, *b* is absorbance of the buffer, *c* is absorbance of the ACE solution with the ACEI peptide added in the buffer, *d* is absorbance of the ACEI peptide in the buffer.

2.4 Purification of ACE inhibitory peptides

2.4.1 Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

In order to purify the peptides endowed with ACEI activity, ultrafiltrated WPIh samples were fractionated by Reversed-Phase (RP) Chromatography on Jupiter 4U Proteo 90 A (C12, 250x10.00 mm, 4μm, Phenomenex, Inc, Torrance, CA) mounted on AktaPurifier 10 system (GE Healthcare, Uppsala, Sweden) and maintained at 25°C. UV detection was performed at 220 nm and injection volume was 100 μL. The mobile phases were composed of 0.1 % trifluoroacetic acid (TFA) in MQ water (phase A) and 100 % acetonitrile (phase B). After column equilibration with 10% B for 15

179 min, the separation was performed at 3 mL / min using the following gradient: 28 % B from 0 - 22
180 min, 50%B from 22-32min, 100% B from 32-37 min. Fractions were collected with FRAC-
181 920fraction collector by applying the following peak threshold values: slope sensitivity > 0.1
182 mAU/min, minimum height > 100 mAU; fraction size during peak collection was 1.0 mL. A total of
183 fifteen runs were performed; fractions from each run were pooled and placed in a SpeedVac vacum
184 concentrator (VR1 Hetovac; HetoLab Equipment, Allerød, Denmark) to remove solvent before
185 freeze-drying. Finally, each lyophilized fraction (100-fold concentrate) was dissolved in MQ water
186 and assayed for ACEI activity as described above. Active fractions were diluted two times in MQ
187 and tested again; diluted fractions able to inhibit ACE activity more than 50 % were analysed by
188 mass spectrometry analysis in order to identify peptide sequence.
189
190 *2.4.2 Mass spectrometry analysis and Multiple Sequence Alignment*
191 For peptide identification a system composed of a UHPLC pump provided with an Acclaim™
192 PepMap100 analytical column (internal diameter 1 mm, length 150 mm, 3 μm particle size) coupled
193 with a ESI interface and Linear IonTrap Mass Spectrometer VelosPro™ (Thermo Fisher Scientific,
194 San Jose`, CA, USA) was used. Global proteomics analyses were performed in data-dependent
195 acquisition (DDA) mode. The mass analyser performs two scan events: a full MS scan event (first
196 event) and then 20 most abundant ions were selected for the subsequent MS/MS analysis (second
197 event). The second event is automatically triggered when the ion current of precursor ions exceed
198 specific thresholds. In this frame ion charge screening was activated and only doubly and triply
199 charged ions were typically selected for the subsequent CID activation. All the chromatographic
200 and MS settings are detailed in a previous paper.[22] Raw data were processed via commercial
201 software Proteome Discoverer™ version 1.3 (Thermo Fisher Scientific) and identification was
202 achieved by Sequest search algorithm by interrogating the Uniprot database (DB). Software results
203 were filtered by peptide mass deviation (300 ppm), and peptide confidence (high = 0.01).

In addition, overlapping of the identified peptides was studied by using Clustal W2 multiple sequence alignment (MSA)[25-26] in order to find sequence similarity. The alignment was analysed using the multiple sequence editor Jalview [27]. Default settings were used for both programs.

2.5 Molecular modelling

Molecular modelling studies were structured in two steps. First, we considered for molecular docking analyses only oligopeptides identified in the chromatographic fractions provided with higher experimental ACEI activity. Second, a rational pruning was carried out on the sequences of the top-scored oligopeptides in the attempt to prioritize shorter chains for further experimental studies.

2.5.1 Peptide-protein docking protocol

To perform molecular docking, first of all, a crystal structure of the ACE target protein complexed with a peptide mimetic ligand was searched in the Protein Data Bank (PDB). In this respect, AnCE (*Drosophila melanogaster* homologous of ACE, pdb entry 4AA2 with a resolution of 1.99 Å)[28] complexed with Bradykinin-Potentiating Peptide-b (BPPb) was selected for docking simulations. Such structure was preliminary pre-treated by employing the protein preparation wizard available from Schrödinger Suite 2016-3 for adding missing hydrogen atoms, reconstructing incomplete side chains, and assigning favourable protonation states at physiological pH. Moreover, water molecules were removed and the optimal protonation states for histidine residues were determined. The 3D peptide structures were built by using PyMOL (v1.8)[29] and prepared by using the LigPrep tool implemented in the Schrödinger suite 2016-3. 3D structures with all the possible tautomers and ionisation states at physiological pH were thus generated.

Molecular docking was carried out by employing the peptide docking tool recently available from the Schrodinger suite which adapts Glide, commonly used to dock small organic molecules, to peptide structures. The grid was automatically centred on the cognate ligand of target protein and

GlideScore was used as scoring function. Importantly, Glide was able to nicely reproduce the binding more of the X-ray cognate ligand returning an iRMSD value of 1.37 Å. [30]

2.5.2 Prioritization studies

Top-scored peptides were visually inspected to assess if the obtained poses matched the expected binding mode normally experienced by other food derived ACEI peptides. Particular attention was paid to analyse the occurrence of specific hydrogen bonds and appropriate hydrophobic interactions. In order to compare the binding site of AnCE with those of other ACE human enzymes available from PDB, the X-ray crystal structure of AnCE was aligned to different human X-ray solved enzymes reported in the Protein Data Bank (pdb codes: 4APH, 4APJ, 1O8A, 4CA5, 1O86) and the binding sites were inspected by means of PyMOL (v1.8).

Being known that food derived peptides, provided with ACEI activity, have a peptide length from two to five amino acids[9] and that C-terminal peptide residues for ACE inhibition is of key importance[31], we decided to prune selected peptides accordingly. As a result, the pruning was carried out to identify shorter chains whose residues were conserved among the different docked oligopeptides and by removing N-terminal residues not involved in any molecular interaction. Based on pruning, the new designed shorter peptides were submitted to docking calculations. To prioritize the peptides to progress to experimental biological assays, a pool including the new designed shorter peptides doped with the oligopeptides having LE better than the X-ray cognate ligand was *ad hoc* created. These peptides were also searched in five public databases in order to assess their novelty.

2.6 In vitro assay of ACE-inhibitory activity of predicted peptides

Predicted oligopeptides were purchased (purity > 95%; Gen Script Leiden, Netherlands) and assayed for ACEI activity. Based on previous results, range of peptide concentrations and reference standard captopril was set from 0.125 μg / mL to 20 μg/mL in triplicate measures. The half maximal

inhibitory concentration (IC_{50}) was thus calculated for the most promising bioactive peptides by using the software package GraphPad Prism (v8.0.2) and expressed as μM.

2.7 Statistical analysis

Data are given as mean ± standard deviation (SD). All variables were checked for normality (Shapiro-Wilk test, $P < 0.05$) and their homogeneity of variance analysed by Levene test ($P \geq 0.05$). Differences among groups were assessed by means of Student's t-tests for paired sample (two groups) or one-way ANOVA followed by Tukey HSD or Duncan post hoc tests ($P < 0.05$). Two-way ANOVA was performed to study possible interactions ($P< 0.05$) between two independent variables (digestion and hydrolysis time) on dependent variable (protein concentration). All statistical analyses were performed with the SPSS software package (SPSS, release 22.0 IBM, Armonk, NY, USA).

3. Results and discussions

3.1 Hydrolysis of WPI

A two-way ANOVA was performed to evaluate the effect of digestion and hydrolysis time on protein concentration. No statistically significant interaction was found between the effects of digestion and time course on protein content ($P> 0.05$; **Supplementary Table S1**). By contrast, simple main effects analysis showed that the protein concentration was significantly reduced by average of 98% by the bromelain digestion activity (paired-sample T test; $t(11) = 164.97$, $p< 0.0001$) regardless the timing. However, at 4 h of digestion the protein concentration reached the lowest values in comparison with those found at the previous hydrolysis times ($P = 9.25 \times 10^{-8}$, Tukey *post hoc test*).

These results were confirmed by SDS-PAGE profiles of digested and undigested samples (**Supplementary Figure S1**). In accordance to WPI composition, protein bands corresponding to ALA and BLG showed the highest intensity in the profiles of undigested samples; these latter did

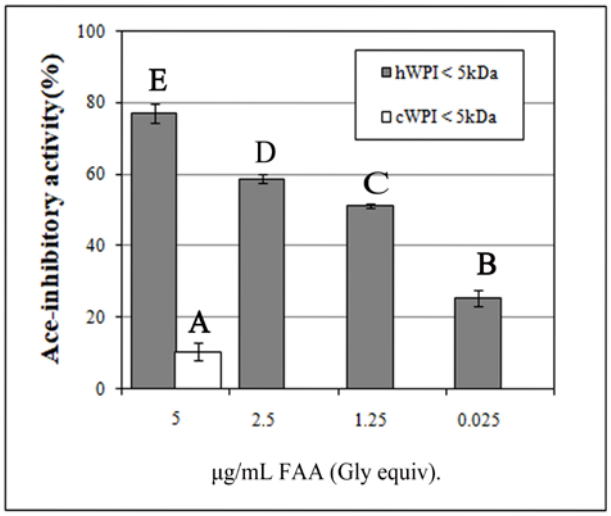
not show significant changes during the incubation in the alkaline solution (**Supplementary Figure S1;** *lanes 8-11*). However, after pH neutralization and centrifugation the reduction of ALA and BLG bands and the appearance of protein oligomers in the undigested WPI were found (**Supplementary Figure S1;** *lane 12)*; the formation of oligomers with high molecular weight could be correlated with gelation process induced by pH lowering, as previously reported[32]; this effect putatively determined the protein amount reduction registered in the final undigested sample (*ca.* 58 %). As regard WPI digested samples, they showed the reduction or disappearance of WP bands, starting from 1 hours of incubation (**Supplementary Figure S1;** *lane 7).* After three additional hours of incubation SDS-PAGE patterns of WPIh showed only the presence of a faint BLG band, whilst ALA was completely hydrolysed (**Supplementary Figure S1;** *lane 6-3*). The high hydrolytic activity of bromelain towards whey proteins and, above all against BLG, was favoured both by heating and alkaline conditions applied during reaction; this latter, indeed, promoted dissociation of globular conformation and gelation reduction and by making protein more susceptible to enzymatic cleavage.[20] By contrast, under lower temperature and pH, WPs were much more resistant to proteolysis by bromelain also for more extended reaction times.[19,33]

3.2 ACEI activity of ultrafiltrated WPIh

ACEI activity of ultrafiltrated WPI is reported in **Figure 1**. In particular, an increase of the concentration from 0.025 to 1.25 µg/mL (expressed as Gly equivalent) caused the doubling of ACEI activity (from 25.4 to 51.2%); by contrast, a further double increase of peptide concentration (up to 2.5 µg/mL) gave a mild (only of *ca.* 7.5%) but significant ($P< 0.001$) activity increment; at the highest concentration (5 µg/mL) ACEI activity was by 77.1%. Conversely, 5 µg/mL of the ultrafiltrated fraction from the undigested WPI showed an ACEI activity (10.4%) statistically ($P<0.001$) much lower than that recorded on the permeate of WPIh diluted to 0.025 µg/mL (**Fig. 1**); thus, no further dilution and subsequent purification steps were performed for this sample. Recently, also Abadía-García et al.[19] reported the ACEI activity of a hydrolysate obtained by the digestion of

whey with bromelain; however, no information on the peptide sequence responsible for this activity was reported.

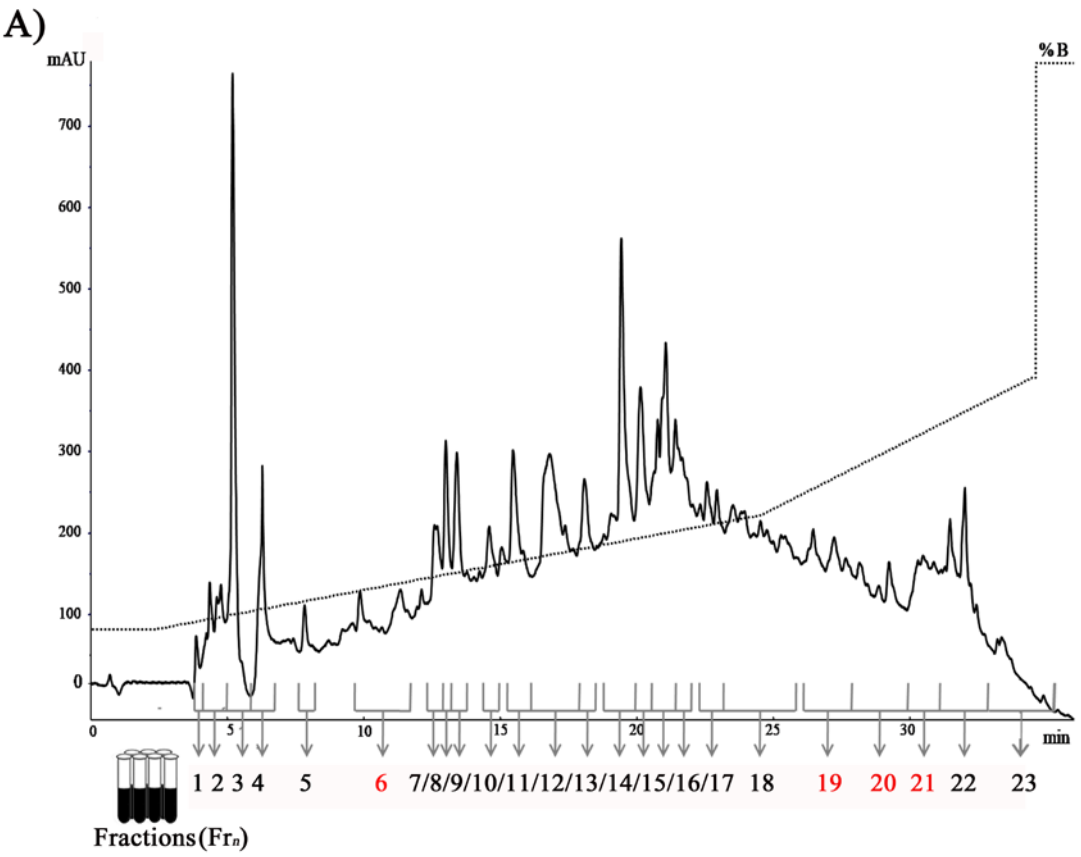
Figure 1. ACEI activity (%) of 5 kDa cut off permeate from WPI digested (WPIh) or not (WPIc) with bromelain. Bars are means ± standard deviation. Peptides and free amino acids (FAA) concentrations in each sample was expressed as Glycine equivalent. Different percentage values of ACEI activity are annotated with the different superscript letters according to Tukey HSD post hoc test (*post hoc* HSD Tukey's test (P< 0.001; N = 3).

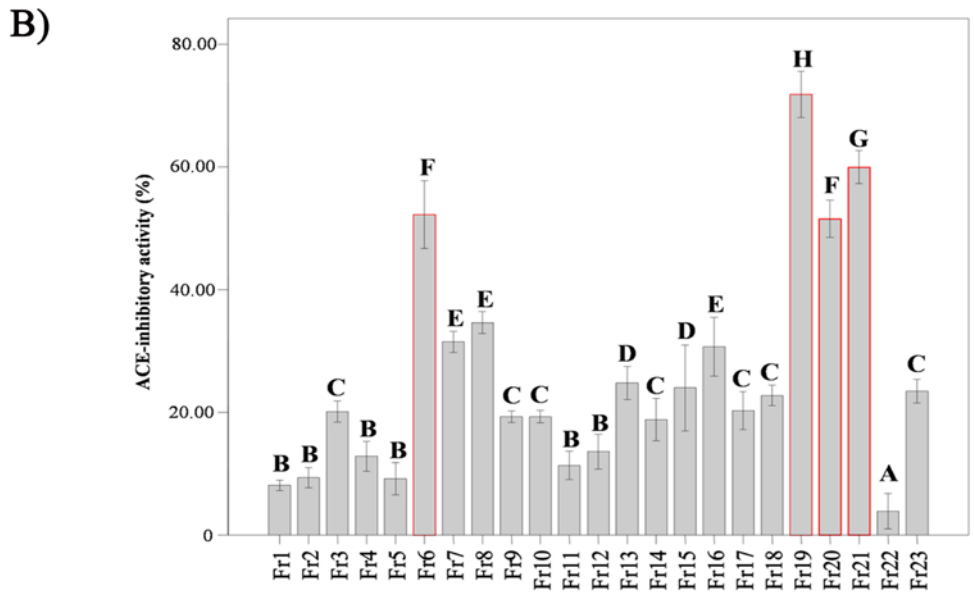


3.3 Identification of peptides endowed with putative ACEI activity

In order to purify ACEI peptides ultrafiltrated WPIh were separated by RP-HPLC. A total of 23 fractions (FR) were obtained (**Fig. 2, panel A**), that were assayed again for their biological activity. RP-HPLC fractions showed an ACEI activity by average of 25.8 %; however only four of them exceeded 50% of activity (**Fig. 2, panel B**). There was a statistically significant difference among all assayed fractions ($F(22, 46) = 98.894, p = 1 \times 10^{-31}$). A Duncan post hoc test revealed that the fraction FR19 showed the highest activity (71.8% ± 3.8) followed by the significantly ($P < 0.05$) lower results on average from the hydrophobic fractions FR20 and FR21 (55.7 % ± 0.2) and hydrophilic FR6 (52.2% ± 5.5).Twice dilution of these fractions showed that only FR6 and FR19

326 retained ACEI activity (61.0 % ±1.1 and 53.0% ±1.8, respectively); ACEI increase of FR6 could be
327 due to the dilution of interfering peptides.
328
329 **Figure 2; panel A)** RP-HPLC of ultrafiltrated WPI hydrolisate and eluted fractions (Fr_n) numbered
330 from 1 to 23; **panel B)** ACEI activity of the different fractions harvested from RP-HPLC analysis.
331 Values are means ± standard deviation. Similar superscript letters represent values with the same
332 ACEI ($P > 0.05$) by Duncan's test. Red marked bars were selected for subsequent analyses.
333





334

335

336 Based on these results, these latter fractions (FR6 and FR19) were analysed by LC-MS/MS. Peptide

337 sequences identified in both fractions are reported in **Supplementary Tables S2 and S3**.

338 In particular, 39 and 89 peptides were identified in FR6 and FR19; among these, 82 peptides

339 belonged to BLG and 11 to ALA and immunoglobulins. The remaining ones were released by

caseins (κ- and β- caseins) that contaminated WPI. As expected, hydrophilic peptides characterized FR6, whereas hydrophobic peptides were eluted at higher retention times (FR19; peptide hydrophobicity > 50 %).

FR6 and FR19-derived peptides were subsequently subjected to Multiple Sequence Alignment Analysis (MSA) as depicted in **Supplementary Figure S2**. MSA analysis combined with neighbour joining clustering allowed to group different peptides harbouring common patterns; in particular, homogenous groups contained from 2 to 12 short peptides (5-19 residues; **Supplementary Figure S3**). The matching of the homogenous peptide sequences with the parental protein (using NCBI COBALT) suggested that the bromelain cleaved if arginine, lysine, and leucine were found in position P1 and if threonine and alanine were in position P1'. These results are partially consistent with the behaviour of bromelain towards other food proteins or specific substrates[20, 34].

Among identified peptide sequences we found ALPMHIR (BLG, f(142–148)), IPAVFK (BLG, (f(78–83)), IIAEK (BLG, f(71–75)). These latter were also obtained by Power et al.[35] registering ACE IC_{50} values ranging from 40 to 150 μM; differently from what described in this work, ACEI peptides were released after the tryptic digestion of purified BLG under extended time of incubation (24 hours). Longer or shorter sequences containing the above-mentioned peptides or their fragments were also identified (e.g. VLDTDYKK, IIAEKTKIPAVF, MHIRL; **Supplementary Tables S2 and S3**); however, to the best of our knowledge, no information on their ACEI activity has been reported previously. The peptide list from **Supplementary Tables S2 and S3** also contained peptides characterized by the ACEI DAQSAPLRVY motif. Despite consolidated *in vitro* ACEI effect of this latter (IC_{50} of *ca*. 10 μM), its orally administration in hypertensive rats showed conflicting data, reporting the significant decrease of diastolic blood pressure[36] or the absence of antihypertensive effects.[37] This ambiguous behaviour could be attributed to the hydrolytic activity by gastrointestinal enzymes responsible for the maintenance or the loss of the peptide site binding the ACE enzyme. Therefore, a deep study of ACE binding sequences could be useful to improve

knowledge on mechanism of action of ACEI peptides and their effects *in vivo.* In addition, naturally occurring peptides are suitable as starting points for pharmacophores or the design of drug-like molecules with incorporated secondary structural elements to increase their efficacy or modulate different physiological processes.[38] In this regard, ACEI peptides, such as herein identified IPAVFK and MHIRL, were previously reported further biological properties (antimicrobial and antioxidant activity)[14]; thus, might be selected also for performing multitarget studies.

3.4 Docking results of ACEI peptides

The AnCE target protein represents a valuable option to explore the important C-domain selective ACEI peptides. In fact, it has been reported that the C-domain is primarily responsible for the *in vivo* conversion of angiotensin I to angiotensin II whereas bradykinin (BK) is cleaved with similar efficiency by both its terminals. Importantly, the single C-domain of AnCE is highly resembling the C-domain of human enzyme. Thus, we are confident that AnCE structure is well suited for structure-based design of C-domain selective inhibitors[28]. We considered for molecular docking an initial number of 128 oligopeptides, coming from the two chromatographic fractions (FR6 and FR19) provided with highest experimental ACEI activity. Aware that oligopeptides containing more than 300 atoms and 50 rotatable bonds cannot be reliably docked[31], 39 oligopeptides were discarded from docking simulations, thus reducing from 128 to 89 the number of oligopeptides to dock. The 89 docked oligopeptides were thus subsequently ranked trading-off ligand efficiency and docking score values[39]. Only peptides showing values of ligand efficiency (LE) or docking scores better than that of the cognate ligand (BPPb), taken as a reference, were further considered.

First of all, our analysis resulted that four out of five oligopeptides provided with LE better than the cognate ligand are included in the FR19, the fraction showing a higher *in vitro* ACEI activity. Only one peptide (that is IIAEK) comes from FR6. Moreover, these peptides belong to BLG in agreement with a study indicating that peptides derived from this WP showed an appreciable ACEI activity. [40]

Regarding the docking results of the 11 new designed peptides (MHI, PMHI, LPMHI, HIRL, NKLV, IAEK, IIAEK, ALNE, VLVL, IPAVF, and KIPAVF), we observed that only three out of 11 showed LE worse than the cognate ligand. The pruning operation output on the new designed peptides is shown in **Supplementary Table S4.**

According to final LE-based final ranking including both unpruned and new designed peptides, we observed that all the eight new designed peptides, with the only exception of IPAVF, are provided with significantly higher LE values compared to the five unpruned peptides (**Table 1**).

Molecular docking showed that these peptides were mostly involved in hydrophobic and polar interactions with the latter being more relevant to better stabilize the peptide-protein complex[41]. We also observed that the docked peptides established polar interactions with the zinc ion of ACE through its third-last residue. Peptide residues can be accommodated in the S1 and S2 binding sites of ACE that are proximal to the zinc ion as previously described by Pina et al.[42]

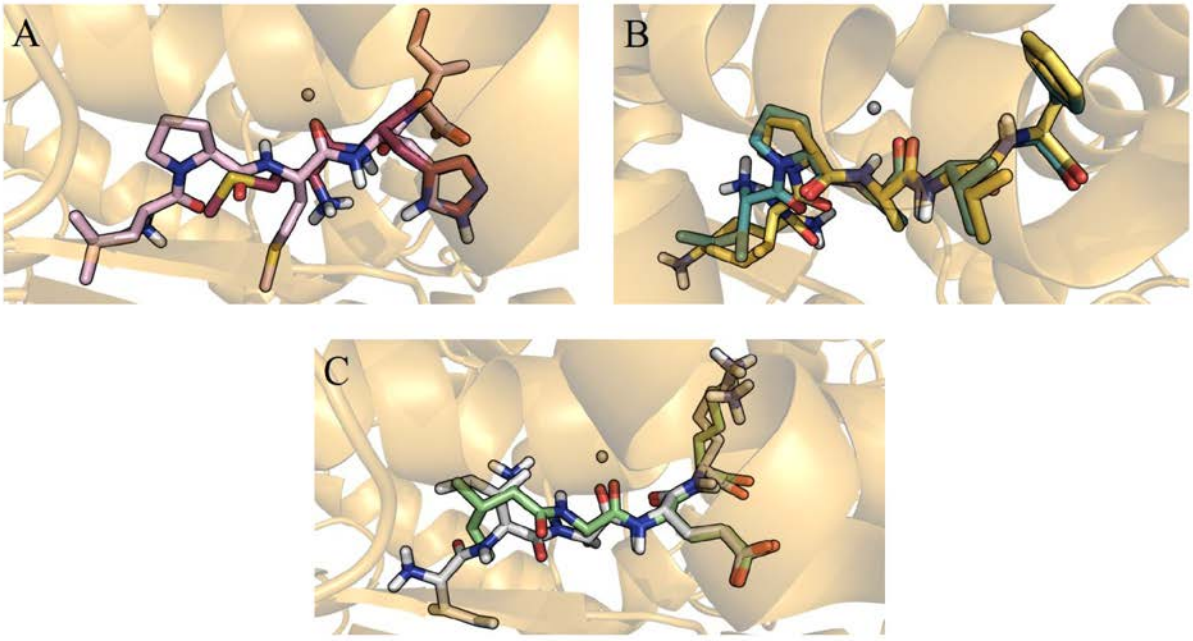
As far as AnCE is concerned, S1 binding site includes A338, E368, and Y507 (corresponding to A354, E384, and Y523, respectively, of human ACE) while the S2 binding site includes Q265, Y504, H337, H497, and K495 (corresponding to Q281, Y520, H353, H513, and K511, respectively, of human ACE). As shown in **Supplementary Table S5**, all the best top-scored docked peptides establish interactions with almost all these AnCE/ACE key amino acids. Interestingly, the COOH terminal is always directed towards the S2 binding pocket by making hydrogen bonds and salt bridges with K495, Y504 and occasionally with Q265. Another key evidence is the interaction with A340 detected in the top-scored poses of all the docked peptides.

Table 1. Ranking of the 16 ACEI peptides based on LE. For sake of clarity, new designed peptides are reported in italic while the cognate ligand taken as reference is reported in bold. For the sake of completeness, the three new designed peptides whose LE value is lower than that of the cognate ligand are also listed.

ACE-I PEPTIDES	LIGAND EFFICIENCY
VLVL	–0.420
MHI	–0.407
NKVL	–0.395
IAEK	–0.388
ALNE	–0.382
PMHI	–0.357
HIRL	–0.351
IIAEK	–0.320
LPMHI	–0.309
IPAVFK	–0.306
KIPAVF	–0.298
MHIRL	–0.298
IPAVF	–0.296
RPKIPP	**–0.288**
ALNENK	–0.242
NENKVL	–0.240
ALNENKVL	–0.223

This seems to have a role in stabilizing the peptide backbone thus increasing the resistance to the enzymatic cleavage.[28] Interestingly, the posing of the new designed peptides and that of the unpruned ones remained almost unchanged irrespective of LE. This holds particularly true when comparing LPMHI and MHI, KIPAVF and IPAVF, IIAEK and IAEK (**Fig. 3**). New designed peptides contain some typical branched aliphatic amino acids (L, I, and V) at the N-terminal residues whose importance for ACEI was already highlighted in recent studies.[9]

Figure 3. Comparison of docked poses of the unpruned and pruned peptides. In particular; superimpositions of LPMHI (pink) and MHI (purple) (**A**); KIPAVF (yellow) and IPAVF (cyan) (**B**); IIAEK (white) and IAEK (green) (**C**) are displayed. Zn^{2+} ion is rendered as a grey sphere.



3.5 ACE inhibitory activity of prioritized peptides

From the databases screening carried out for assessing the novelty of the predicted ACEI peptides, remarkably, 12 out of the total pool of 16 peptides (the 11 new designed peptides in addition to the five best ACEI peptides compatibly with LE ranking) were not matched in any of the searched database (see **Supplementary Table S6**). In order to validate the ACEI activity of the predicted peptides, 11 new designed peptides (MHI, PMHI, LPMHI, HIRL, NKLV, IAEK, IIAEK, ALNE, VLVL, IPAVF, and KIPAVF) with the corresponding sequence were purchased and tested. Among all, only three bioactive peptides (MHI, IAEK, IPAVF) returned a high percentage of ACE inhibition ranging from 63.2 % to 72.5% at a concentration of 5 μg/mL (**Supplementary Table S7**). Remaining peptides showed percentage of inhibition lower than 50% at the higher concentration of 10 μg/mL (**Supplementary Table S7**). Thus, the IC_{50} values were determined only for MHI, IAEK, IPAVF as showed in **Table 2**. It is interesting to underline that the *in vitro* results reflected both the criteria adopted for the pruning operations. On one side the peptides sharing common residues (MHI and IPAVF) and, more importantly, the peptides phasing out the N-terminal residue (IAEK) on the other side (**Supplementary Table S4)**.

447 **Table 2.** IC_{50} values of the designed ACEI peptides.

ACE-I PEPTIDES	IC_{50} (μM)
IPAVF	9.09
MHI	11.59
IAEK	25.08

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450 Among the three most active peptides, IAEK was previously reported by Hernández-Ledesma et
451 al.[43] in a 3kDa permeate obtained after gastro-simulated digestion of milk; however, no ACE
452 activity was found. By contrast, IIAEK, that in our study did not show significant ACEI activity,
453 were reported to exhibit an IC_{50} value of 63.7 μg/mL slightly higher than that observed in our study
454 for the tetrapeptide IAEK.[35]
455 Importantly, the most active designed peptide is IPAVF that is provided with a IC_{50} value of 9.09
456 μM; IPAVF fulfils almost all the chemical requirements and features according to the reported SAR
457 studies.[8–13] Its sequence, in fact, encloses three C-terminal hydrophobic residues (that are Ala, Val
458 and Phe). Moreover, the occurrence of the proline not far from the zinc ion could contribute to the
459 inactivation of the enzyme as elsewhere reported.[11] **Figure 4** displays AnCE interactions of this
460 designed peptide. By contrast, in our study the cleavage of lysine residue determined a reduction of
461 ACEI activity; indeed, for IPAVFK we registered a percentage of inhibition only by 47% also when
462 a concentration of 20 μg/mL was applied. Both IPAVF and IPAVFK peptides were, previously,
463 used at high amounts (3-5 mg/g) for the development of food products endowed with ACE
464 inhibitory activity[44]; IPAVF and IPAVFK were obtained by hydrolysis of whey with neutrase and
465 were precursor of the ACEI peptide IPA (IC_{50}= 50μM). Since biochemical assay was coupled on-
466 line to the HPLC fractionation system separating peptides, interfering molecules could be
467 responsible for the higher IC_{50} value of IPAVF (300 μM) in comparison to that registered in this
468 work for the purchased peptide. The discovery of a known ACEI peptide sequence further supports

469 the strength of our approach enabling to focus on the active sequence and thus to augment
470 knowledge on its function and substrate specificity.
471 In contrast to IPAVF, published data regarding ACEI activity of MHI are referred only for the
472 longer motifs ALPMHIR, ALPMH, ALPM with worse IC_{50} values (42.6, 52.0, and 928.0 μM,
473 respectively).[45] These results further confirmed the predictive strength of molecular docking for the
474 design of new bioactive peptides.
475 The confidence of our results[46] was further strengthen by analysing the Applicability Domain
476 (**Supplementary Figure S4**) derived from a pool of 87 ACEI peptides taken from CHEMBL[47-49]
477 (data are enclosed as **Supplementary File S1**).
478 Diet integration with bioactive peptides may contribute to keeping hypertension under control.
479 Their release from foods through suitable enzyme hydrolysis represents a fascinating approach
480 addressed to sustain the value chain of food supplements. To this regards we developed a hydrolysis
481 protocol of whey with the food grade protease bromelain that allowed to obtain hydrolysate
482 endowed with ACEI activity. Then, the application of theoretical methods based on molecular
483 docking revealed peptides-enzymes interactions and allowed to design 3 new and short peptides
484 (MHI, IAEK, IPAVF) with high *in vitro* ACEI activity ($9 < IC_{50} < 25$ μM). Although further
485 investigation are needed to better characterized bioavailability and *in vivo* effects of these peptides,
486 we demonstrated that the structure-based approach herein adopted could be a valid option for
487 evaluating and improving ACEI potency of whey protein bioactive peptides and for exploitation the
488 herein whey digest in diary process and food supplement production.
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491 **Figure 4.** Molecular interactions between top-scored pose of IPAVF and AnCE. For the sake of
492 clarity; polar and π-π interactions are depicted as black dotted lines; non-polar hydrogen atoms are
493 hidden and Zn^{2+} ion is rendered as grey sphere.
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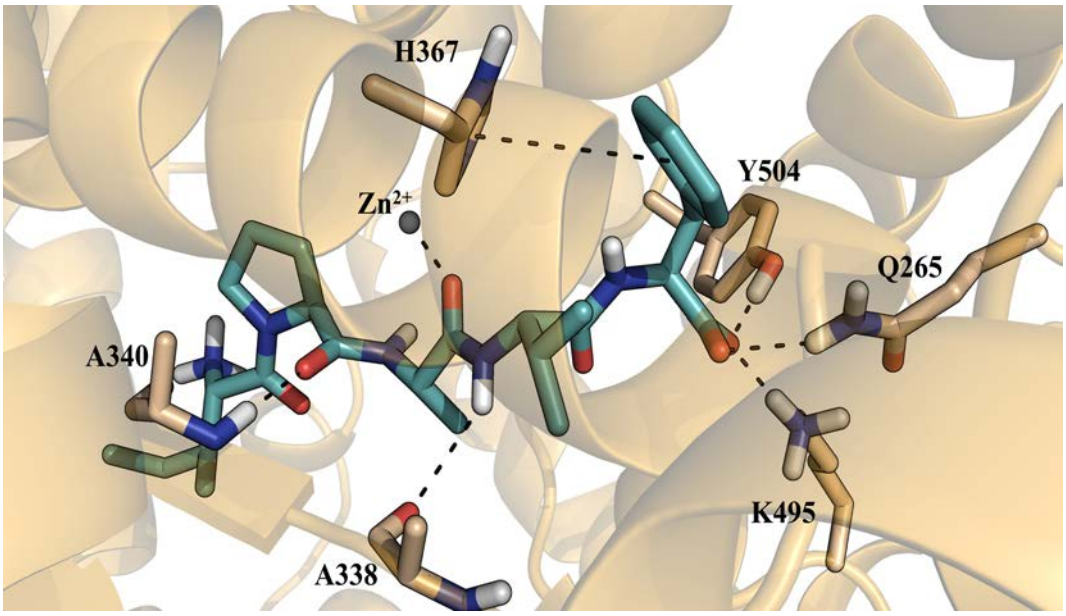

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Declaration of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Supporting Information

SDS–PAGE of whey protein hydrolysates (Figure S1); Multiple alignment sequence of putative ACEI peptides (Figure S2); Neighbour joining tree of putative ACEI peptides sequences (Figure S3); Applicability Domain of ACEI peptides (Figure S4); Protein concentrations of whey proteins obtained before and after bromelain hydrolysis (Table S1); List of identified peptides putatively endowed with ACEI activity (Tables S2 and S3); Pruning operations of the docked peptides (Table S4); AnCE-interactions of the best ACEI peptides (Table S5); Match for predicted ACEI peptide sequence queries in databases publicly available (Table S6); ACEI activity percentages of predicted ACEI peptides (Table S7).

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