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Proceeding Paper

Study of the Antihypertensive Peptides Derived from Alpha-Lactalbumin Hydrolysate after Simulation of Digestion †

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Abstract: Alpha-lactalbumin is a whey protein which is a cheese making industrial residue of high biological value. The antihypertensive capacity of three peptides obtained from the simulated gastrointestinal digestion of alpha-lactalbumin hydrolysates was studied. Alpha-lactalbumin hydrolysis was performed using Alcalase enzyme and subsequently subjected to a simulated digestion process by using pepsin and pancreatin enzymes to mimic digestion conditions. The peptides were identified from a RP-HPLC fractionation of the digest and subsequent identification by mass spectrometry analysis. Three peptides from alpha-lactalbumin sequence were obtained: IWCK-DDQNPH (P1), KFLDDDLTDDIM (P2) and DKFLDDDLTDDIM (P3). The in vitro antihypertensive activity of the peptides was determined by studying the inhibition of angiotensin converting enzyme, with P1 being the only peptide with antihypertensive activity detected by this methodology (IC_{50} = 3.91 ± 0.2 mg/mL). In order to correlate structural (molecular dynamics simulations) and physicochemical properties with potential mechanisms of antihypertensive capacity in silico methods were performed. The peptides P1, P2 and P3 had a negative global charge and were hydrophilic. After molecular modelling, the peptide structures were submitted to a refinement based on an energy minimization and further molecular dynamics simulation to assess their global size and conformational space. After 50 nanoseconds simulation, the global structures, solvated and immersed in an ionic water solution similar to that of blood, were studied in their solvent accessible surfaces. Some secondary structure (alpha-helix) was observed in the P1 peptide but in general, all peptides showed an extended folding. Surfaces were charge code colored and in a visual inspection it could be conjectured that all of them exposed the charge, mainly negative charge, to the solvent surface, in agreement with the GRAVY index which was also evaluated. In conclusion, the structure and amino acid composition of peptide 1 assessed by in silico studies agrees with the antihypertensive activity obtained by the in vitro study.

Keywords: antihypertensive; peptides; molecular dynamics simulations; simulated digestion

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1. Introduction

It has been observed that oxidative stress and the generation of free radicals can be a key factor in the development of Chronic Noncommunicable Diseases (CNCD) and other pathologies [1,2]. CNCDs, mainly cardiovascular diseases, cancer, chronic respiratory diseases, and diabetes, are the main cause of disease and mortality worldwide, causing 75% of annual deaths [3]. Regarding cardiovascular diseases, the greatest risk factor and the most preventable is hypertension. It is estimated that there are currently 1.13 billion people in the world with hypertension, and less than 1 in 5 hypertensive people have adequate control of the disease.

The number of premature deaths from CNCDs is a general concern. About a quarter of global mortality related to CNCDs affects people under 60 years [4]. The risk factors for this type of disease are tobacco use, alcohol consumption, low physical activity and an inadequate diet. The scientific evidence has shown that a healthy diet is essential for human life, and that antioxidants from the diet can help the body reduce oxidative stress and prevent these diseases [5]. This states a significant challenge in the discovery and evaluation of new ingredients with biological activities that contribute to preserving and maintaining general health and well-being.

Among ingredients with biological activities, bioactive peptides are currently a valid alternative to reduce the risk of suffering CNCDs by incorporating them into the formulation of functional foods and nutraceuticals [6]. These are sequences encrypted in proteins of different origin, that after their release by fermentation, hydrolysis catalysed by proteases (in vitro) or during gastro-intestinal digestion (in vivo), may be capable of exerting one or more biological activities, depending on their physicochemical and structural characteristics [1,7].

There is an interest in the search for new protein sources carrying bioactive peptides in their sequence. A source of these proteins are the residues of agro-industrial production as generators of these peptides [8]. The dairy industry related to the manufacture of cheese generates a large contribution of liquid waste, the main one being whey, a by-product of great abundance and low cost in the industry, which has a high nutritional, biological, functional, and technological value [9,10]. Many studies indicate that peptides obtained from α-lactalbumin hydrolysates (the second major whey protein) have bioactive properties [5,11–13].

Molecular simulation can realistically and in detail describe the structure and dynamics of peptides at the atomic level. Thus, current molecular simulations work relatively well to characterize stable peptide conformations and to estimate free folding speeds and energies, although it presents more drawbacks in the case of unfolded or disordered states [14].

The aim of this work was to correlate the antihypertensive activity found in vitro with in silico studies and to evaluate possible structure-activity relationships and possible mechanisms of antihypertensive capacity of the obtained peptides.

2. Materials and Methods

2.1. In Vitro Analysis

2.1.1. α-lactalbumin (α-La) Hydrolysate Obtaining with Alcalase Enzyme

The α-La-hydrolysate (HA) was obtained by the hydrolysis process of α-La with Alcalase enzyme (0.1% *w*/*w*) in a water bath at 30 °C for 1 h with constant stirring, according to the methodology described by Baez et al. [15]. The resulting suspension was frozen and lyophilized to carry out the in vitro digestion simulation.

2.1.2. In Vitro Digestion of α-La-hydrolysate

The in vitro simulation of digestion of α-La hydrolysate (DHA) consisted in the gastric and intestinal phases not including oral phase, according to the methodology described by Baez et al. [15]. At the end of the digestion process, samples were centrifuged

at 13,440 g for 10 min. The supernatant (bioaccesible fraction) was separated for subsequent fractionation into peptides by preparative RP-HPLC chromatography.

2.1.3. Fractionation into peptides by preparative RP-HPLC chromatography

To separate the peptides present in the bioaccesible fraction, RP-HPLC chromatography was performed using a preparative column Sun Fire C8 ST 10/250 with 5 μm diameter particles in a Waters HPLC system equipped with a diode array detector (DAD) according to Baez et al. [15]. 55 fractions were identified, and those corresponding to chromatogram peaks were chosen to be analyzed by LC-MS/MS.

2.1.4. Identification and Synthesis of Peptides

The peptide sequences present in the fractions were identificated using the Mascot software (version 2.3.02) in a Bos taurus database (UniProt, 02/20/2019, 24,088 total sequences). The most common contaminants in proteomics experiments were included in the database. The peptides belonging to the α-lactalbumin sequence found in the different fractions were synthesized by ChinaPeptides Co., Ltd. Shanghai, China.

2.1.5. In Vitro Antihypertensive Activity of Peptides

The study of antihypertensive capacity was carried out using the Angiotensin Converting Enzyme (ACE) inhibition method described by Cushman and Cheung [16] modified by Kim et al. [17]. The IC_{50} (protein concentration required to inhibit ACE activity by 50%) of the three peptides was determined. The assay was performed in triplicate.

2.2. In Silico Analysis

2.2.1. Physicochemical Properties of Peptides

Physicochemical properties such as net charge at physiological pH, isoelectric point, ASAs (Accessible Surface Areas), were evaluated using MOE2015.1 software QSAR package (Molecular Operating Environment Version 2013.1, Chemical Computing Group).

2.2.2. Global Predictors

Additionally, some global indexes were assessed: the GRAVY index was used to assess the hydrophobicity/hydrophilicity; TOXINPRED and CPPPRED to predict toxicity and cellular penetration respectively.

2.2.3. Structures and Related Properties

3D structures of each peptide were generated and refined with MOE. The AMBER12 force field was chosen for all procedures. Energy minimization was performed with a Root Mean Square (RMS) gradient of 0.01 kcal/mol in aqueous phase with Na^+ and Cl^- as counter ions at 0.1 mM and 310 K. In order to make more reliable assumptions about the conformations adopted by these peptides in solution, Molecular Dynamics simulations (50 nanoseconds) using Nanoscale Molecular Dynamics (NAMD, formerly Not Another Molecular Dynamics Program) through MOE's GUI were performed. The trajectories obtained were analyzed by plotting the evolution of total energy (U), Root-Mean-Square Deviation (RMSD), Root-Mean-Square Fluctuation (RMSF) and each peptide's behavior in solution was discussed.

3. Results and Discussion

3.1. In Vitro Analysis

From the bioaccesible fraction of α-La hydrolysate, a fractionation by RP-HPLC was carried out, selecting 13 of the 55 total fractions obtained. It was observed that only fractions 36, 40, 42, 45 and 49 presented peptides encrypted in the α-lactalbumin native protein. Three peptides were identified with molecular weight ranging between 1200 and

1600 Da, all of which were present in the different fractions. These identified peptides were: IWCKDDQNPH (MW: 1254.54 Da), KFLDDDLTDDIM (MW: 1439.64 Da), DKFLDDDLTDDIM (MW: 1554.67 Da), containing 10, 12 and 13 amino acids, respectively. The identified peptides were subsequently chemically synthesized, and their antihypertensive activity was analyzed. From now on, peptides IWCKDDQNPH, KFLDDDLTDDIM and DKFLDDDLTDDIM will be named P1, P2 and P3, respectively.

The antihypertensive activity assay (inhibition of ACE), showed that only P1 presented activity (3.11 mM). In its structure, P1 presents a Pro residue near the C-terminus, Nielsen et al. [18] affirm that Pro in that position improves the binding affinity of the peptide to ACE. P2 and P3 present several Asp residues in their sequence. Nielsen et al. [18] also mentioned that dicarboxylic residues (glutamic acid and aspartic acid) reduce the binding of peptides to ACE, which could justify the absence of activity from these peptides.

3.2. In Silico Analysis

Regarding the physicochemical properties of the peptides, P2 and P3 net charges at physiological pH were the lowest. These peptides differed in only one aminoacid. P3 had an extra aspartate, thus, its charge is one unit lower than P2. P1 presented a negative net charge as well, but closer to neutrality (Table 1).

Table 1. General characteristics and physicochemical properties of peptides. Weight, isoelectric point, and charge at physiological pH were assessed with MOE's QSAR package. GRAVY index was calculated through GRAVY calculator (http://www.gravy-calculator.de/).

Peptide	Sequence	Nº of Amino Acids	Isoelectric Point	Charge at pH = 7.4	Weight (Da)	GRAVY
1	IWCKDDQNPH	10	4.94	−1.4	1254.370	−1.66
2	KFLDDDLTDDIM	12	3.39	−4	1436.558	−0.44
3	DKFLDDDLTDDIM	13	3.3	−5	1550.638	−0.67

The GRAVY value [19] for a peptide or protein shows correspondence between internal regions and hydrophobic regions, and between external and hydrophilic. If this index value is negative, a hydrophilic protein or peptide is expected. On the contrary, if the GRAVY value is positive, a mostly hydrophobic protein or peptide is expected [20], GRAVY values were assessed for P1, P2 and P3. The values obtained were below zero for all cases (Table 1). However, for P1 the value obtained was even lower than for P2 and P3. This confirms that P1 is more hydrophilic than P2 and P3. The length of the peptides analyzed in this work was between 10 to 13 amino acids. Peptides of this size begin to adopt secondary structure [21]. To study this, each peptide conformational space was assessed running Molecular Dynamics simulations of 50 ns in solution using NAMD software. As a result, the three-dimensional structure that P1, P2 and P3 adopted in solution was observed (Figure 1) and their secondary structure was predicted as well. This prediction employed DSSP secondary structure assignments [22], which is the automatic secondary structure assignment algorithm built into MOE.

P2 and P3 were random coils while P1 showed a partial alpha helix structure. For the particular case of P1 the result was consistent with the GRAVY value previously mentioned and there was an agreement between hydrophilicity and the alpha helix formation.

A putative transition from coil to helix for this 10 amino acids peptide in an in silico model was proposed in which an aqueous solution at physiological conditions (0.1 mM NaCl at 310 K) was simulated. It was assessed that the water environment will thermodynamically favor a tendency to adopt a partial alpha helix structure. Then, for the case of P1, from a structural point of view, it could be conjectured that with docking this particular structure in the ACE active site, given the presence of an alpha helix, will expose the more hydrophilic side chains to the binding site.

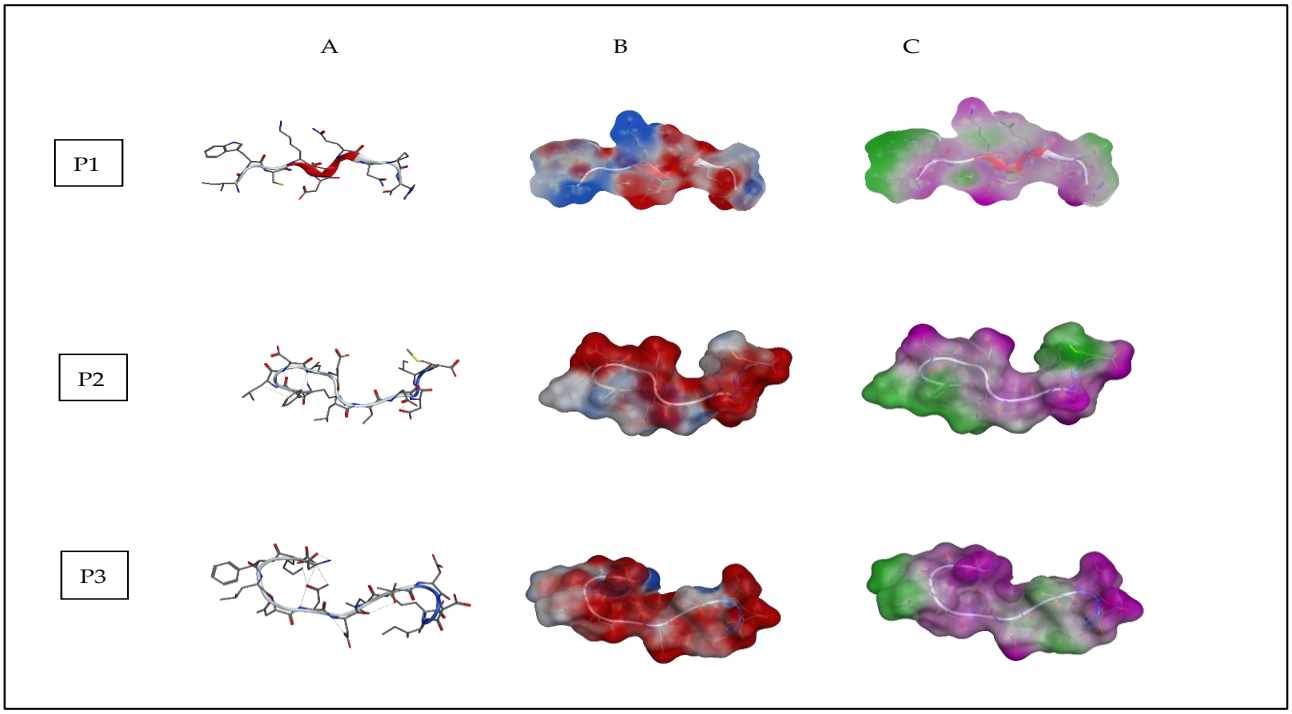

Figure 1. 3D representation of P1 (IWCKDDQNPH), P2 (DKFLDDDLTDDIM) and P3 (DKFLDDDLTDDIM) rendered by MOE showing conformations at 50 ns of the simulation. P1 region that adopts alpha helix structure contains KDDQ residues (**A**) and two molecular surfaces showing electrostatics (**B**) lipophilicity (**C**).

It has been reported that there is a positive correlation between secondary structure of bioactive peptides and their biological activity [18], however little is known about this subject.

Final conformations corresponding to 50 ns of simulation time were presented as colored molecular surfaces according to electrostatics and lipophilicity (Figure 1). P1, P2 and P3 were mostly hydrophilic and negative. Since P2 and P3 only differed in one amino acid, their surfaces were very similar. P1 hydrophilic regions appeared as more external whereas lipophilic areas appeared as more localized and hidden, being this observation coherent with previously discussed results.

Antihypertensive drugs that inhibit ACE such as Lisinopril, Enalaprilat and Captopril establish electrostatic interactions with Zn^{2+} but peptides do not have to necessarily inhibit ACE by establishing the same interactions. Moreover, Nielsen et al. [18], proposed that the presence of Asp and Glu may lower ACE inhibition activity due to the repulsion that those residues generate in the enzyme active site, and P2 and P3 have 5 and 6 Asp (respectively) in their sequence. P1, on the other hand, has a Pro near to the C-terminal, as Angiotensin II does. Besides, P1 has more hydrophobic residues (Ile, Trp and Pro), and this feature has been correlated with ACE inhibition [23]. However, it has been proposed that when Trp is at the N-terminal residue (or when it is proximate to N-terminal) the binding affinity decreases and since P1 has a Trp residue next to its terminal Ile, it may explain the low antihypertensive activity that this peptide showed.

4. Conclusions

Three peptides were obtained from the hydrolysis of alpha-lactalbumin with Alcalase enzyme, and their antihypertensive potential was studied using in vitro and in silico methods. The peptide IWCKDDQNPH (P1) was the only one that presented antihypertensive activity in vitro. The evaluation of P1 structure and amino acid composition through in silico studies showed accordance with what was obtained experimentally in the laboratory.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1.

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