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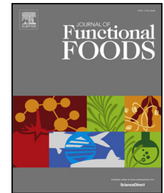

Identification of renin inhibitors peptides from amaranth proteins by docking protocols

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ABSTRACT

The objective of this work was to develop a new protocol to predict with greater confidence peptides as potential inhibitors of the renin enzyme. For this, free, friendly and rigorous servers developed specifically for peptides as ligands were used. Six peptides (SFNLPILR; FNLPILR; SFNLPIL; QAFEDGFEWVSFK; AFEDGFEWVSFK and VNVDDPSKA) identified in an amaranth hydrolysate obtained with alcalase (hydrolysis degree 21% ± 4) were used. Two positive (angiotensinogen and IRLIIVLMPILMA) and one negative (a tridecapeptide of alanine) controls were included in the analysis. A protocol was designed to include two consecutive stages was performed using CABS-dock server (http://biocomp.chem.uw.edu.pl/CABSdock) and FlexPepDock server (http://flexpepdock.furmanlab.cs.huji.ac.il/). Peptides SFNLPILR, FNLPILR and AFEDGFEWVSFK inhibited the enzyme *in vitro*. The heptapeptide FNLPILR was the most potent inhibitor, with an IC_{50} of 0.41 mM.

1. Introduction

In mammals, blood pressure (BP) is regulated by the renin angiotensin aldosterone system through two main enzymes: renin and angiotensin converting enzyme (ACE) (Zhou et al., 2017). The first drugs developed for the control and regulation of BP focused on inhibition of ACE and in blocking receptors that interact with angiotensin II, the product released by this enzyme. However, renin inhibition would be a better target to control BP since this enzyme acts on a single substrate, in the first step of enzymatic cascade thus controlling the rate of the reaction that triggers the increase in BP (Duprez, 2006). Nevertheless, the development of renin inhibitors has not been as successful because they are not effective in inhibiting the enzyme *in vivo*. Until now only one effective inhibitor of renin has been developed (aliskiren, IC_{50} = 0.6 nM) (Wood et al., 2003).

In addition to pharmacological treatment, one of the strategies used to reduce BP is to encourage changes in habits, for example, by incorporating into diet ingredients that contribute to preserving and maintaining health. Bioactive peptides generated during processing and preparation of food or generated from gastrointestinal digestion of food proteins are products that have been widely studied, especially in terms of their antihypertensive effect. The studies related with antihypertensive peptides derived from food sources focused on the possible action on ACE, and also on renin inhibition (Aluko, 2019; Pihlanto & Mäkinen, 2017).

Amaranth, an Andean crop with high quality proteins, has been studied as a source of bioactive peptides (Orsini Delgado et al., 2016; Quiroga, Barrio, & Añón, 2015; Sabbione, Nardo, Añón, & Scilingo, 2016; Tovar-Pérez, Lugo-Radillo, & Aguilera-Aguirre, 2018). Particularly the antihypertensive activity of amaranth peptides has been studied both *in vitro* and *in vivo,* assays in which it has been demonstrated that they act on ACE plasma, renin plasma and the vascular system (Aphalo, Martínez, & Añón, 2015; Barba de la Rosa et al., 2010; Fritz, Vecchi, Rinaldi, & Añón, 2011; Quiroga, Aphalo, Ventureira, Martínez, & Añón, 2012; Suárez, Aphalo, Rinaldi, Añón, & Quiroga, 2020; Vecchi & Añón, 2009). In addition, these peptides have been incorporated into different matrices such as emulsions and cookies with potential antihypertensive activity (Sabbione, Suárez, Añón, & Scilingo, 2019; Suárez & Añón, 2019). In a previous work, we identified the amino acid sequence of six peptides (SFNLPILR, FNLPILR, SFNLPIL, QAFEDGFEWVSFK, AFEDGFEWVSFK and VNVDDPSKA) in an amaranth alcalase hydrolysate (hydrolysis degree 21% ± 4) as renin inhibitors (Quiroga, Aphalo, Nardo, & Añón, 2017).

Despite the advances in obtaining and purifying bioactive peptides, one of the most important challenges in this area remains to identify the most active of a set of potential active sequences. Bioinformatics and computational chemistry have different useful tools to address this problem. Biomolecular simulation techniques are fundamental tools to

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understand the type of molecular interactions and analyze the correlation between structure-activity. However, not all simulations are suitable or applicable to all systems, among other reasons because the equations that support the method and/or those that were timely developed lose their validity (Bottaro & Lindorff-Larsen, 2018). Besides the application of more rigorous simulation methods in complex systems requires a lot of time and calculation capacity.

Among the techniques of molecular simulations, molecular docking is the most used to predict the predominant binding mode of ligands with a protein. However, its use with peptides continues to be a challenge for the scientific community (Ciemny et al., 2018; Tu, Cheng, Lu, & Du, 2018). Many authors use docking techniques to complement their experimental tests, because they are freely available and have been successful with small peptides (2-4 residues), but their use with larger sequences leads to false positives. The main difficulty of docking peptides as ligands is due to peptides, even short ones, are often highly flexible, with few rigid parts, such as double bonds or ring systems.

The main objective of this work was to test new free, friendly and rigorous servers developed specifically for docking with peptides to predict with greater confidence the best peptides that could act as enzyme inhibitors. We used six peptides that we previously identified (Quiroga et al., 2017) to perform a docking protocol of two consecutive stages. First, CABS-dock server was used to explore the possible sites of interaction on renin surface, obtain the peptide-protein complexes and analyze the residues involved in the interaction. In the second stage, FlexPepDock server was used to estimate the energy of peptide-protein interaction (evaluated by Rosetta score) starting from the CABS-dock complexes where the peptide was located in the active site. We included in the analysis as positive controls angiotensinogen (DRVYIHPFHLVIHN) and the peptide IRLIIVLMPILMA (IC_{50} = 6.5 mM) (Fitzgerald et al., 2012) and as a negative control a tridecapeptide of alanine.

2. Materials and methods

2.1. CABS-dock server

The CABS-dock server (http://biocomp.chem.uw.edu.pl/CABSdock, accessed on February of 2019) (Kurcinski, Jamroz, Blaszczyk, Kolinski, & Kmiecik, 2015) performs flexible protein-peptide docking. CABS-dock automated protocol merges *coarse grained* simulation with all-atom local optimization of selected reconstructed models. Starting from crystalline structure of renin and amino acid sequence of putative ligands, this server perform docking search for the binding site allowing for full flexibility of the peptide and small fluctuations of the receptor backbone. For each peptide and controls, we use the structure PDB 2V0Z (chain C) of renin with 50 cycles of Monte Carlo simulation. Complementary analyzes of the resulting structures were done with VMD (Humphrey, Dalke, & Schulten, 1996).

2.2. FlexPepDock server

FlexPepDock server was employed to find high-resolution modeling of protein-peptide interactions (London, Raveh, Cohen, Fathi, & Schueler-Furman, 2011). This server, freely available at http://flexpepdock.furmanlab.cs.huji.ac.il/ (accessed on February of 2019), is based on Rosetta modeling software (version 3.2). It uses Monte Carlo minimization approach to stabilize peptide backbone and rigid body orientation respectively. Initially, peptide structure was refined in 200 independent FlexPepDock simulations, which included 100 stimulations in high-resolution mode and 100 stimulations in low-resolution mode with pre-optimization followed by high-resolution refinement. Finally, FlexPepDock created 200 models for each analysis and they were further ranked based on their Rosetta generic full atom energy score (London et al., 2011).

The structures used for this stage of refinement were obtained from the analysis performed with CABS-dock server. Previously, residues in pdb file were renumbered according FlexPepDock needs. Three independent replicates were made for each peptide, specifying in 200 the number of low resolution structures and the number of high resolution structures. Complementary analyzes of the resulting structures were done with VMD (Humphrey et al., 1996).

2.3. Synthetic peptides

Peptides SFNLPILR (P1), FNLPILR (P2), AFEDGFEWVSFK (P5) and VNVDDPSKA (P6) were synthesized, HPLC purified to more than 95% purity, and analyzed by HPLC-MS by the company ChinaPeptides Co. Ltd. (Shanghai, China).

2.4. Renin inhibition assay

In vitro inhibition of human recombinant renin was performed with Renin Inhibitor Screening Assay Kit (Cayman Chemical, MI, USA) according to their instructions. Substrate and assay buffer were added to the following wells: (a) background, (b) 100% initial activity, (c) background peptide inhibitor sample and (d) peptide inhibitor. The enzymatic reaction was initiated by the addition of renin to (b) and (d) treatment wells. The microplate was shaken for 10 s and was incubated at 37 °C for 15 min. The fluorescence intensity (FI) was then recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm in a microplate reader (Biotek Synergy HT, Winooskii, VT, USA).

The IC_{50} values were calculated by dose–response curves. The logarithmic values of three (P1) or five (P2 and P5) different sample concentrations (mg/mL) against the inhibitory activity (%) were plotted. The IC_{50} values were calculated by dose–response curves using a nonlinear regression sigmoidal curve fit functions (normalized response) to P1 and P5 in GraphPad prism 6.01 (Graphpad Software Inc., San Diego, CA, USA). As for P2 we have a complete data set that defines a sigmoidal curve, a nonlinear regression sigmoidal curve fit functions was used.

2.5. Kinetics of renin inhibition

Preliminary studies of kinetics of renin inhibition were performed using 2.5, 3.5, 5, and 10 μmol/L substrate in the absence and presence of P2 at 0.25 mM and 1 mM.

3. Results and discussion

Human renin (EC 3.4.23.15) is a 335 amino acid, glycosylated aspartic protease composed of two β-sheet domains with a cleft between them (Pihlanto & Mäkinen, 2017; Yuan, Wu, Aluko, & Ye, 2006). The active site is located in this cleft and is formed by distinct binding pockets which recognize the side chains of angiotensinogen, called S1, S2, S3, S1′ and S2′ sites, and the non-substrate $S3^{SP}$ (S3 subpocket) site (Rahuel et al., 2000). Each domain provides one of the catalytic residues Asp32 and Asp215 (numbering based on the pepsin sequence), which are located around the center of the cleft to promote the cleavage of the Leu10–Val11 peptide bond on angiotensinogen. It has been crystallized alone and linked to different ligands (Tawada et al., 2016). For this study, we used the crystalline structure that has been co-crystallized with aliskiren (PDB 2V0Z), the only commercial inhibitor of this enzyme. Our research was focused on identifying the sequences responsible for renin inhibition by an amaranth hydrolysate previously evaluated (Quiroga et al., 2017). With this objective and taking into account the relevance of *in silico* tools and bioinformatics approach on the study of bioactive peptides, we try out new servers specifically developed for docking peptide-protein which are friendly with experimental scientists and have fast implementation.

CABS-dock server was used to perform a global docking on the entire surface of renin, without restriction on peptide binding site. This

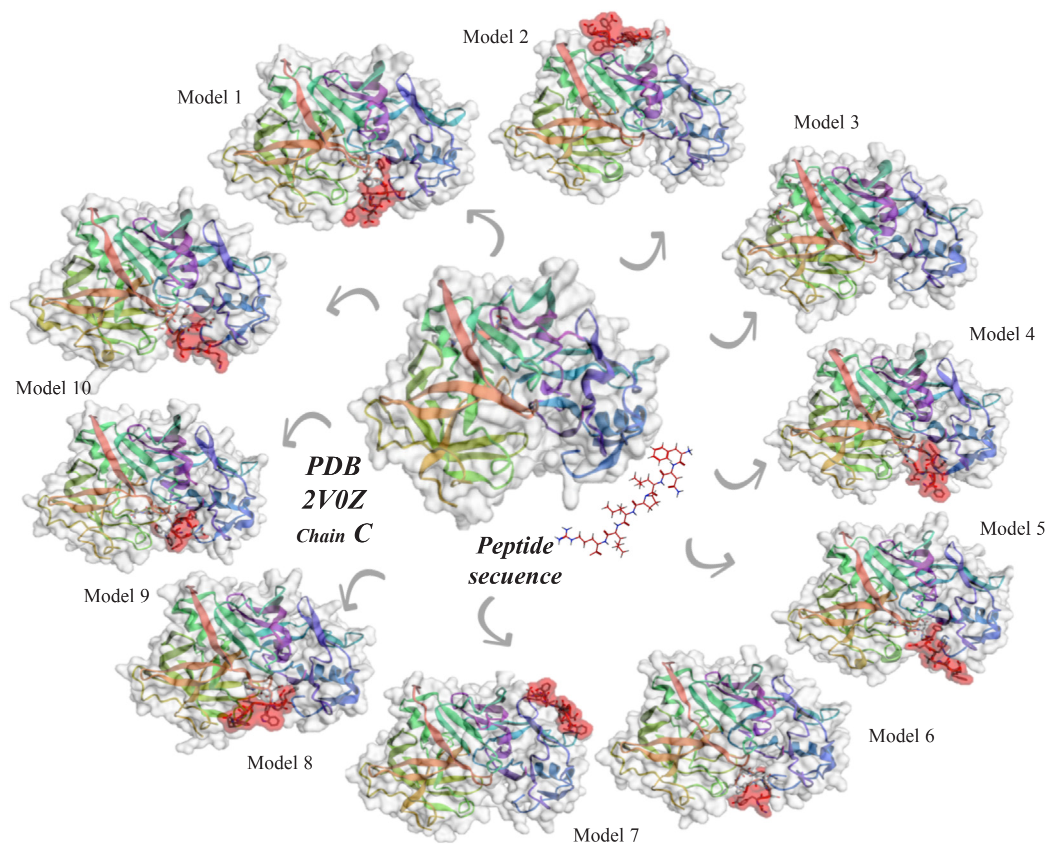

Fig. 1. Structure of the 10 finals models generated of CABS-dock results using P5 (AFEDGFEWVSFK) as a ligand. The peptide evaluated is show in red colour.

Table1
Details of CABS-dock results.

Peptide	Number of the ten finals models docked at active site of renin	Detail of interaction with renin of model selected to refine with FlexPepDock
P1 – SFNLPILR	3 (models 1, 2, 3) and 1 near outside (model 4)	Model 1 interacts with Asp32 and Asp215.
P2 – FNLPILR	3 (models 3, 4, 5)	Model 4 shows the interaction with Asp215.
P3 – SFNLPIL	3 (models 1,2,3)	Model 2 interacts with Asp32 and Asp215. The other two have no interaction with catalysts.
P4 – QAFEDGFEWVSFK	2 near outside	None of the models located near of the active site interacts with catalytic residues.
P5 – AFEDGFEWVSFK	7 (models 1, 2, 5, 6, 7, 9 and 10)	Model 1 and model 5. Both models interact with Asp32 and Asp215, through different residues. They present opposite orientations in the cleft of the active site.
P6 – VNVDDPSKA	3 (models 1, 3, 8) and 2 near outside	Model 3 witch interacts with Asp32 and Asp215.
Angiotensinogen – DRVYIHPFHLVIHN	1 (model 5)	Model 5 witch interacts with Asp32 and Asp215.
CONTROL – IRLIIVLMPILMA	1 (model 1)	Model 1 and model 9. Both models interact with Asp32 (model 1 by Arg2 and model 9 by Ile1) and Asp215 (model 9 by Ile4 and Leu3; model 1 by Ile4 and Ile5).
CONTROL – AAAAAAAAAAAAA	1 (model 3)	Model 3, shows the interaction with Asp215 by Ala13.

docking algorithm returns, for each peptide evaluated, 10 models of the complex peptide-protein numbered according to the cluster density, which is the ratio of number of elements in each structural cluster, and the backbone root mean square deviations (RMSD) of cluster members (a measure of the structural difference). Results show that all peptides evaluated were capable of interacting with renin through different sites on its surface. As an example, the Fig. 1 shows the structure of corresponding results obtained using P5 peptide for docking. Peptide P5 residues were located at the active site in 7 of the 10 final models proposed. In contrast, for angiotensinogen and controls, peptides were located at the active site only in one of the 10 final models. Table 1 summarizes the number of finals models in which peptides were docked at the active site of renin.

As expected, we observed different binding patterns depending on the peptide evaluated. To characterize the binding sites detected and analyzed if they are the same for different peptides, contact profiles were constructed. For all final models obtained, renin residues located at a distance of 4.5 Å of each peptide were counted. These results are shown in Fig. 2. This representation allows us to graphically analyze the most frequent residues in peptide-renin interaction. Analyzing this

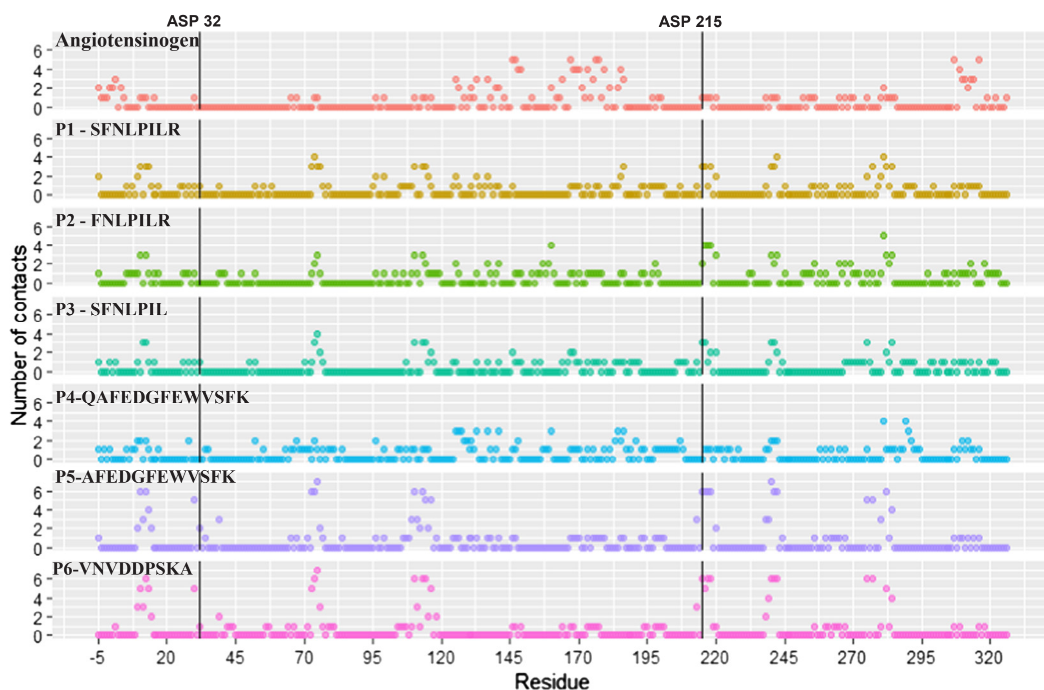

Fig. 2. Number of contacts (pairs of peptide/receptor residues closer than 4.5 Å) of the 10 finals models of peptide-renin generated by CABS-dock as a function of renin residue. The catalytic residues Asp32 and Asp215 are marked with black lines.

profile we observe some renin residues (such as 9, 10 12 and 13; 73, 74 and 75; 240, 241 and 242; and 281, 282 and 283) which participate in interactions in at least one model of each peptide evaluated, regardless of its sequence. These regions could be protein-protein recognition motifs located on renin surface.

Considering that the amaranth hydrolysate, from which our ligands were isolated behaved as a competitive renin inhibitor (Quiroga et al., 2017), we continued our studies with the model in which peptides were located in the active site. Interaction energies were calculated using the FlexPepDock server. Peptides often lack a distinct fold in their unbound state, and go through simultaneous binding and folding upon encountering their target protein receptor. The docking algorithm of FlexPepDock takes this into account and performs a high-resolution structural and energy refinement of the peptide in the binding site. This method has been tested in a large reference set of protein-peptide complexes and has been shown to generate high resolution models (London et al., 2011). This is more suitable for modeling protein-peptide interactions, than a global docking protocol such as that performed by the CABS-dock. FlexPepDock is based on *coarse grained* simulations (Kmiecik et al., 2016) and needs a protein peptide complex, where the ligand must be located at the active site of the enzyme (or interaction site). For this reason, we started from the models generated with CABS-dock. Table 1 also details the model selected to perform this stage, according to the interactions observed. Peptide P4 was discarded in this second stage because neither was docked at the active site nor in its vicinity, probably due to steric impairments as a result of its size and/or the lack of recognition motif in its sequence.

As an output, FlexPepDock server gave the 10 best predicted models for each peptide-protein complex, ordered according to Rosetta score. This score in Rosetta corresponds to weighted sums of energy terms, some of which represent physical forces such as electrostatics and van der Waals' interactions, while others represent statistical terms such as the probability of finding the torsion angles in Ramachandran space (Alford et al., 2017). In addition, if the user prefers, the output details the value score terms, like energy on the interface, for each model. Table 2 summarizes FlexPepDock results.

All docking performed presented positive values of Rosetta score. This parameter is correlated with peptide-protein interaction energy and therefore negative values are expected. The positive results obtained were probably due to internal clashes in the receptor structure (renin) that are not relieved by the classic FlexPepDock protocol, because this only optimizes the side chains on the receptor in the vicinity of the ligand. Although much of the energy function in Rosetta is physics-based, it also has certain statistical terms to favor structures that look like known protein structures. A lower scoring structure is more likely to be closer to the real/native structure. The score correlates with energy, but do not have a direct conversion to physical energy units like kcal/mol. When we analyzed the different components of the Rosetta score, the energy at the interface and the terms of peptide scoring, were effectively negative. These values confirm that interaction of evaluated ligands and renin was possible and favorable, although the overall score are positive. This highlights the "problem of scoring function" of docking methods (Meng, Zhang, Mezei, & Cui, 2011; Rentzsch & Renard, 2015). FlexPepDock requires a "good" complex (peptide-receptor) to perform energy refinement. Taking this into consideration, to verify that positive scores were due to structural problems, as internal clashes of laterals chains of renin, in the starting structure, we performed new dockings of each peptide and controls. In this case we performed stage of energy minimization using NAMD with force field CHARMM27. After that, a decrease in Rosetta scores was observed, but they still showed values greater than zero.

Considering that the main objective of this study was to identify from the list of six peptides which ones were the best renin inhibitors, we proceeded to select peptides to be synthesized for *in vitro* evaluation based on all results found in both global and local docking. Peptides P1 (SFNLPILR) and P5 (AFEDGFEWVSFK) were synthesized because they presented the best interaction energy with renin according to FlexPepDock and both have the highest number of models coupled to the active site according to CABS-dock results. To validate the predictive value of protocols used in this study, P2 (FNLPILR) and P6 (VNVDDPSKA) were also synthesized.

Experiments performed with the synthetic peptides confirmed that, three of the four peptides synthesized interact with renin and modulate its activity. Fig. 3 shows the percentage of renin inhibition caused by different concentrations of P1, P2 and P5. These peptides inhibited the enzyme in a dose response manner, IC_{50} values are shown in the table

Table 2
Description of FlexPepDock results.

Peptide		FlexPepDock with CABS-dock models		FlexPepDock with CABS-dock models after energy minimization of the complex	
		Rosetta Score	Interface energy	Rosetta Score	Interface energy
P1 – SFNLPILR	Model 1	1074 ± 22	−13 ± 2	246 ± 1	−18 ± 1
P2 – FNLPILR	Model 4	1341 ± 10	−12.7 ± 0.4	358 ± 4	−14 ± 3
P3 – SFNLPIL	Model 2	1177 ± 2	−15.5 ± 0.2	255 ± 2	−12.1 ± 0.4
P5 – AFEDGFEWVSFK	Model 1	1336 ± 4	−15 ± 0.5	331 ± 4	−19 ± 3
P5 – AFEDGFEWVSFK	Model 5	1072 ± 11	−22 ± 4	267 ± 0.1	−25 ± 2
P6 – VNVDDPSKA	Model 3	1254 ± 21	−18 ± 1	317 ± 5	−20 ± 1
Control – IRLIIVLMPILMA	Model 9	1110 ± 21	−13 ± 2	281 ± 1	−25.6 ± 0.4
Control – Angiotensinogen	Model 5	1160 ± 23	−24 ± 7	375 ± 6	−30.5 ± 0.3

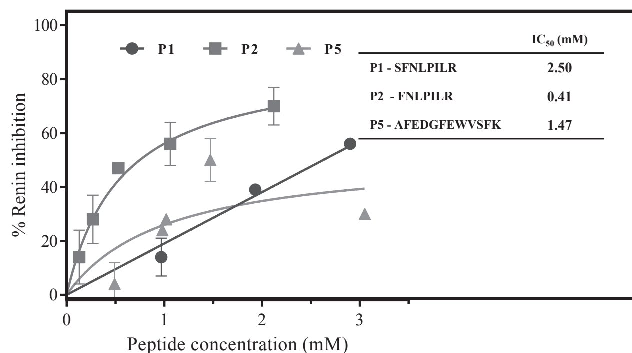

Fig. 3. Renin inhibitory activity of peptides analyzed. P1: SFNLPILR, P2: FNLPILR and P5: AFEDGFEWVSFK. P6 showed no activity in range tested (0.13–2.90 mM). Inset table: IC_{50} values (mM) of peptides assayed.

inserted in Fig. 3. P2 was the most potent inhibitor with an IC_{50} equal to 0.41 mM, while P6 was inactive at all concentrations tested. These results did not totally agree with score values obtained with FlexPepDock, which predicted a higher affinity for P1 followed by P5 and P2. This inaccuracy has been reported by other authors (Lammi, Zanoni, Aiello, Arnoldi, & Grazioso, 2016), although in many cases the results are not published and reflect the problem of scoring function that docking techniques have in common (Bottaro & Lindorff-Larsen, 2018).

Peptide P2 has a better IC_{50} value than other reported renin-inhibitory peptides from different food sources as pea protein hydrolysate, microalgae palmaria palmata, rapeseed and flaxseed (Fitzgerald et al., 2012; García-Mora et al., 2017; He, Malomo, Girgih, Ju, & Aluko, 2013; Li & Aluko, 2010).

Based on these results, the kinetics of renin inhibition was studied for P2 to elucidate the mechanism of action (Fig. 4). Although results are preliminary, this peptide behaved as a competitive inhibitor of the enzyme. While some of the reported peptides interact with the active site as mixed inhibitors, most peptides reported in the literature are noncompetitive suggesting the existence of other sites on the enzyme that could interact with inhibitory peptides and modulate their activity through conformational changes. Usually, human angiotensinogen is represented only with its 14 N-terminal (the segment which is recognized by renin) but it is a 485 amino acid protein. This simplification is useful in different studies, like ours, but it should be noted that the recognition mechanism between both molecules is somewhat more complex and probably is mediated by the association of angiotensinogen with renin outside the active cleft. To confirm this behavior we need to analyze a higher number of substrate and peptide concentrations.

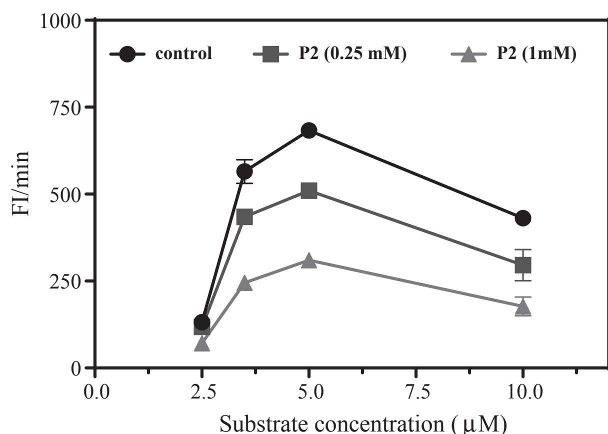

Fig. 4. Kinetics of renin inhibition by P2 (FNLPILR) at varying concentrations of renin substrate (2.5–10.0 μM).

Fig. 5A shows the best poses of peptides and controls performed with CABS-dock. For P1, P2 and P5 Fig. 5B details the structure of renin-peptide complex and includes 2D diagrams of interactions of amaranth peptides with renin residues corresponding to the best FlexPepDock pose. Although P1 and P2 differ only in one residue, according the docking studies performed they adopt different orientation to interact with renin. While P1 orients its N-terminal towards catalytic residues, P2 interacts through the R residue located at its C-terminal. P2 mainly establishes two hydrogen bonds, one by the amino group of the side chain of R residue with the acid group of Glu103 of renin, and another through the α-amino of L with the carboxyl of Asp215 of renin. Also contribute to the interaction a π-interaction through side chain of the L residue and Tyr75 of renin. On the other hand, the dodecapeptide P5 adopts a turn conformation, and directs its residue W towards the catalytic residues. This agree with Udenigwe, Li, and Aluko (2012) who previously reported that the most potent dipeptide predicted by QSAR analysis have a W residue at C-terminal. Although P5 (AFEDGFEWVSFK) and P1 (SFNLPILR) contain in their sequence the SF motif, reported as a renin inhibitor by Udenigwe et al. (2012), it seems that in a greater amino acid sequence as in P5 the preference of renin predominates for bulky amino acids as W. It is probably the existence of a major structural restriction of recognition. Finally, the difference in activity between P1 and P2 is probably due to the specific orientation that both peptides adopt at the renin active site; while P1 interacts by SF motif, P2 do it by LR motif. This agree with the values of renin inhibition reported by Udenigwe et al. (2012) when they evaluated these sequences as dipeptides.

4. Conclusion

In this work we have identified a heptapeptide (FNLPILR) as an inhibitor of the renin enzyme, demonstrating that amaranth proteins contain encrypted different sequences capable of inhibiting renin *in vitro*. CABS-dock server was used as a first approximation to generate the peptide-renin complex and identify those sites most likely to interact. FlexPepDock server was used to refine the interaction energy of the complex. We introduced and discussed the application of adequate free software to use in the identification of bioactive peptides with some

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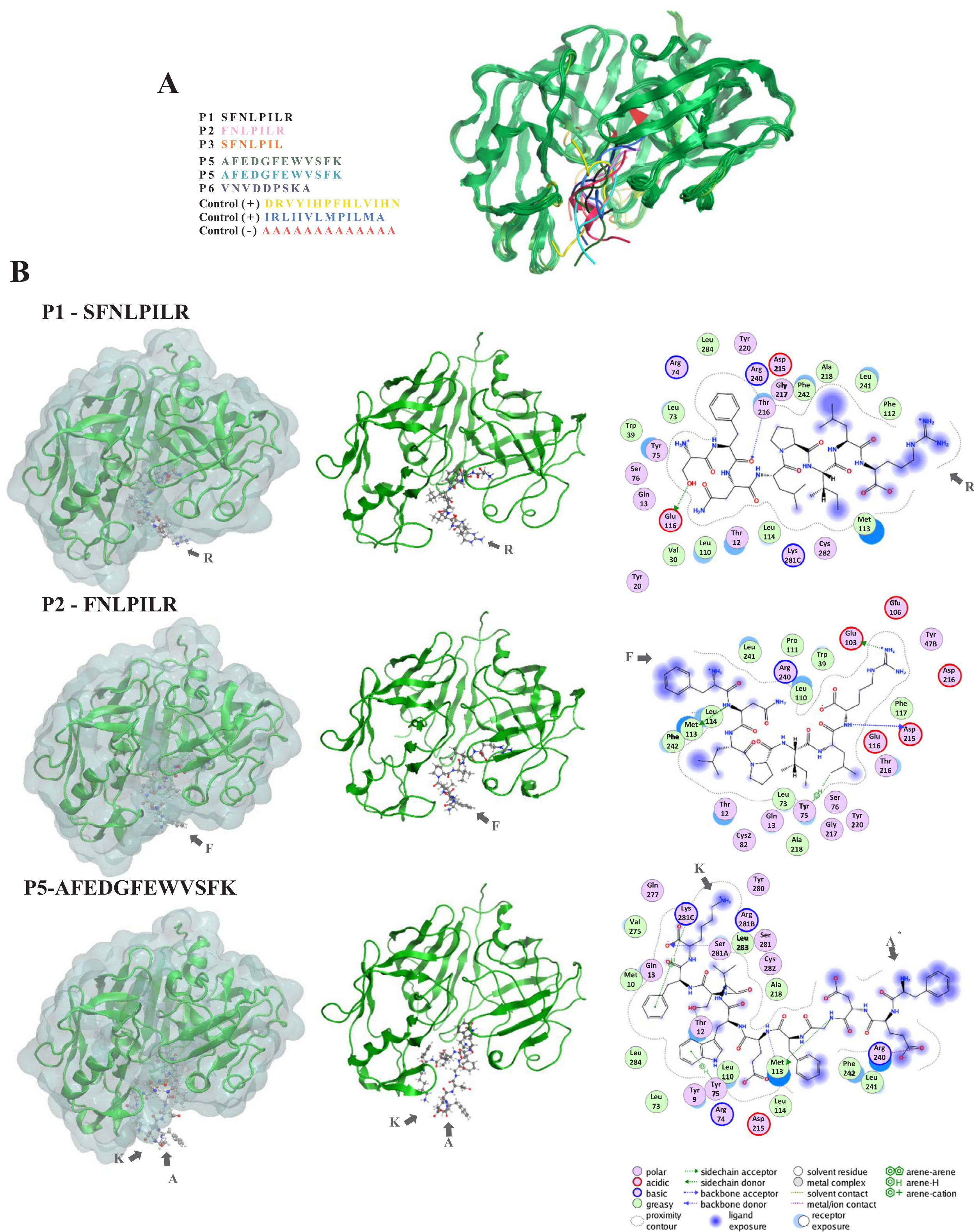

Fig. 5. A. Structural alignment of renin complexes with peptides obtain by CABS-dock server. Peptides P1, P2, P3, P4, P5, P6 and controls are shown in the color scale detail in the same figure. B. Conformation details at the active site of renin for P1, P2 and P5. The complexes are shown in three different ways for each peptide. In the first, molecular surface of renin is represented in cyan color. The second is the same structure without the surface. The third one is a 2D diagram chemical structure of P1, P2 and P5 and binding pockets of renin corresponding to the interaction observed on the best FlexPepDock model. Due to software restrictions, P5 structure is represented without its first residue (Ala) and is indicated by an arrow with an asterisk where it should be. This residue is located completely outside of renin active structure.

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additional advantages: fast implementation and friendly with experimental scientists. Recently CABS-dock incorporates new extensions for adding a refinement step and a stand-alone version (available at https://bitbucket.org/lcbio/cabsdock/src/master/). These improvements can probably result in an increase in the success rates of this docking protocol with bioactive peptides from food proteins.

By far docking with peptides remains to be a challenge for the scientific community, and this is a trend area of bioinformatics. The protocol used in this work employs new servers specifically developed for this purpose. However, we must also be pragmatic in the way biomolecular simulations are used. Like experiments, simulations are not perfect; the analysis of results often requires specialized knowledge and its use without *in vitro* validation can lead to uncertain results.

Ethics statement

This research did not include any human subjects and animal experiments.

Declaration of Competing Interest

The authors declare no conflict of interest.

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