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ACE inhibitory tetrapeptides from *Amaranthus hypochondriacus* 11S globulin

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ABSTRACT

Amaranth seed is a valuable source of dietary protein with very high nutritional quality, and recently its potential as a nutraceutical has been proposed. The aim of this work was to provide experimental evidence for the presence of anti-hypertensive peptides in globulin 11S, one of the major constituents of the seed, by means of an *in-silico* based peptide library screening method. A three-dimensional model of globulin 11S was built, upon which anti-hypertensive peptides were mapped via a database-driven method. Solvent accessibility was evaluated for each potential peptide, and two potent and exposed tripeptides were detected: IKP and LEP. An N-terminal extension of these two peptides was built using the globulin 11S primary sequence information, and ACE inhibitory behaviour was simulated by automated ligand–protein docking. The occurrence of two inhibitory tetrapeptides, ALEP and VIKP, was predicted and experimentally validated by an in vitro ACE inhibition assay that showed IC50 values of 6.32 mM and 175 μM, respectively. This study is the first to provide experimental proof of the anti-hypertensive value of Amaranth. Furthermore, this is the first time that a peptide docking approach is used to find ACE-inhibitory peptides from a food protein source.

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1. Introduction

In recent years, Amaranth (genus *Amaranthus*) has emerged as an attractive source of vegetal protein due to its high nutritional value. Its seed proteins possess an unusually well-balanced amino-acidic composition with higher sulphur amino acid content than legume proteins and higher aromatic and hydrophobic amino acid contents than cereals (Gorinstein et al., 2002). Globulins are the most abundant fraction of Amaranth protein isolates, their main constituents being the 11S globulin (amarantin) and the structurally related globulin-P (Quiroga et al., 2007). While its nutritional and functional value has been thoroughly studied (Aphalo et al., 2004; Bressani et al., 1989; Colla et al., 2006; Escudero et al., 2004; Marcone, 1999; Silva-Sánchez et al., 2004), much less is known about its nutraceutical potential.

Bioactive peptides are important components of functional foods, since it is generally acknowledged that specific sequences within the parent food proteins can provide physiological benefits once they are released either by in vivo digestion, microbial fermentation, or in vitro enzymatic hydrolysis (Hartmann and Meisel, 2007).

Anti-hypertensive peptides inhibit angiotensin I-converting enzyme (ACE) and thus reduce blood pressure in vivo by hindering formation of angiotensin II, a potent vasoconstrictor (Kubota et al., 2002). The use of non-peptidic vasopeptidase inhibitors has proved successful in the treatment of chronic hypertension (Sagnella, 2002). In the past years, it has been shown that food proteins comprise a source of natural anti-hypertensive peptides with in vivo activity, milk proteins being the most thoroughly studied (Murray and FitzGerald, 2007).

Recently, the presence of encrypted bioactive peptides in several Amaranth seed storage proteins was shown (Silva-Sánchez et al., 2008). In this work, the occurrence of anti-hypertensive peptides in Amaranth 11S globulin is further assessed, using a more complete database and including structural information that gives insight into the actual probability of the encrypted peptides being released by proteolysis. Moreover, the possibility of the occurrence of as yet non-described anti-hypertensive peptides is raised by in-silico simulation of the interaction between ACE and novel potential peptide inhibitors using automated docking. Predictions are also evaluated by in vitro ACE inhibition assays.

2. Results and discussion

2.1. Structural analysis

2.1.1. Molecular modeling of Amaranth 11S globulin

In order to relate the presence of encrypted anti-hypertensive peptides in the primary sequence of 11S globulin from Amaranth (*A. hypochondriacus*) with structural information, a 3D model was constructed using homology modeling Fig. 1. From the two

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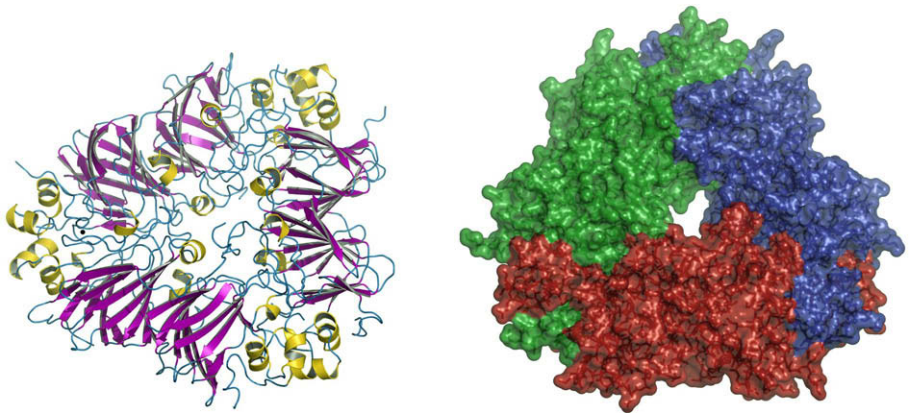

Fig. 1. Amaranth 11S globulin three-dimensional model. Ribbon (A) and surface (B) representations of the homotrimer are shown. (A) Alpha-helix portions are colored in yellow, pleated beta-sheets in magenta. The beta-rich cupin fold can be easily distinguished between the alpha-helix segments that contribute to inter-subunit interactions. (B) Each subunit of the trimer was colored differently. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

independent 11S globulin sequences available, GenBank Accession Nos. ABM66807 and CAA57633, the first was chosen for analysis after confirming by global alignment that both sequences were identical except for point differences in 4 of their 487 amino acid residues.

The FFAS03 system (Jaroszewski et al., 2005) was used for choosing the template, which assigned the G1 soy glycinin as the most representation structure (PDB Accession No. ***1FXZ***, score −99.5). The structure highlighted gaps in positions 1–9, 92–109, 179–197, 228–232, 249–296, and 471–476. A structural alignment of the following three candidates, ***2CV6***, ***2D5F***, and ***2EVX***, all with FFAS score lower than −70, indicated that the absence of information in such zones is consistent and is associated to high beta factor values. Since this is a common characteristic 3D structures of storage globulins (Barre et al., 2005), the initial election of the 1FXZ structure as template was maintained.

After modeling the homotrimeric structure with subsequent refinement by energy minimization, the model fitness was evaluated by several criteria using the Eval3D server, which yielded positive values for the Verify3D (Lüthy et al., 1992) and the Eval23D structural quality assessment (Gracy et al., 1993) throughout the polypeptide chain. This confirmed that the the model was both structurally and energetically coherent, as determined by the dpf function of the Modeller program (calculated automatically by T.I.TO.) and the Eval3D tools, respectively.

As expected, the final model shows that the structure of the 11S globulin has a fold characteristic of other storage globulins and, more generally, of the cupin superfamily. Each subunit of the homotrimer has two central cupin domains formed exclusively by anti-parallel beta-sheets. Such domains are located in the center of the subunit and are flanked by zones rich in alpha-helix secondary structure which are involved in the interactions between subunits.

2.1.2. Structural characterization of encrypted ACE-inhibitory peptides

After obtaining the structural model, the potential Accessible Solvent Area (ASA) values were examined for each residue, together with gaining possible information about secondary structure. In the zones in which 3D information could not be obtained due to template gaps, the ASAP predictive method based on sequence information (Yuan et al., 2006; Yuan and Huang, 2004) was used. The location of encrypted anti-hypertensive peptides in the structure was established using the Biopep database (Dziuba et al., 1999) and the database published by Wu (Wu et al., 2006). Results are shown in Fig. 2.

As shown in the Figure, potential ACE-inhibitory peptides are present both in zones with defined secondary structure as well as in more relaxed loop regions and random coil zones. The frequency of solvent exposure variation along the polypeptide is considerably lower than the frequency of oscillation of the anti-hypertensive potential. As a consequence, peptides with inhibitory potential are found both in exposed (ASA > 0.3) and in buried zones (ASA < 0.3). Also, there is a lack of "hot spots" of potential ACE inhibitory activity, as reported for some milk proteins. Overall, 61% of inhibitory peptides are located in the protein core and, in average, exhibit a higher inhibitory potency than exposed peptides (log [IC50] of 2.22 vs. 2.6).

These results indicate that there are potentially beneficial bioactive peptides in very diverse structural contexts. Since they can exert their effect only after being released from the original polypeptide by proteolysis, one of the most useful structural parameters to evaluate their probable in vivo action is solvent exposure. Peptides located in the protein surface will be more likely to constitute protease substrates, which would enable them to exert a physiological action. Such observation is also applicable to enzymatic hydrolysates of proteins and protein isolates.

Taking this fact into account, two tripeptides with high anti-hypertensive potency (log [IC50] < 1) and solvent exposure were identified: IKP and LEP. The location of these peptides in the structural context of the 11S globulin from Amaranth is shown in Fig. 3. It can be appreciated that these peptides are located in opposite sides of the trimer, that a significant portion of both is exposed to the solvent, and that they are not associated to zones of defined secondary structure.

2.2. Library screening by ligand–protein docking

To explore the potential existence of anti-hypertensive peptides from Amaranth 11S globulin not yet identified, an in-silico simulation of binding to ACE was performed by means of virtual library screening. The library was constructed by performing N-terminal extensions of the inhibitory peptides IKP and LEP, using the 11S globulin sequence. This procedure yielded 9 peptides containing 4–9 amino acid residues. In theory such peptides can be released from 11S globulin by proteolysis, and have the additional advantage of being exposed to the solvent. Their postulated inhibitory activity is based on studies on the structural requirements of ACE-inhibitory peptides showing that the C-terminal di and tripeptide sequence is a determinant for high-affinity binding (Fitz-Gerald and Meisel, 2000; Pripp et al., 2004). This led us to speculate

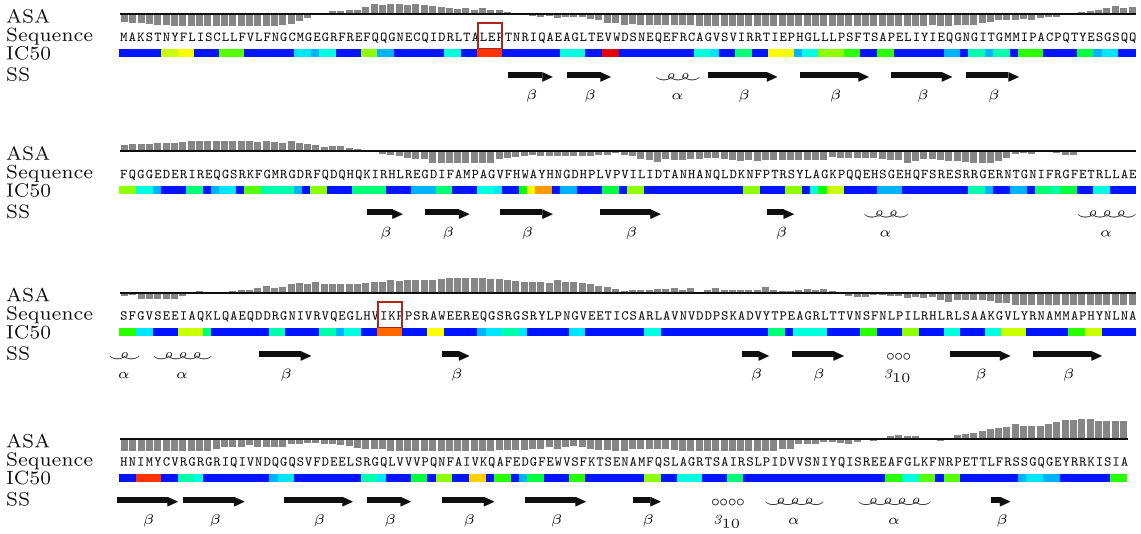

Fig. 2. Accessible surface area (ASA) and anti-hypertensive potential (IC50) of Amaranth 11S globulin. ASA values for each residue are shown in the vertical top bars. Values above 0.3 (exposed) are shown as positive, below 0.3 are shown as negative. Anti-hypertensive power is depicted in the bottom bar as a temperature gradient, where 'hot' colors represent high ACE inhibitory character. Below, secondary structure elements (SS) are drawn. It can be seen that tripeptides IKP and LEP (boxed) have both high ASA and anti-hypertensive power values.

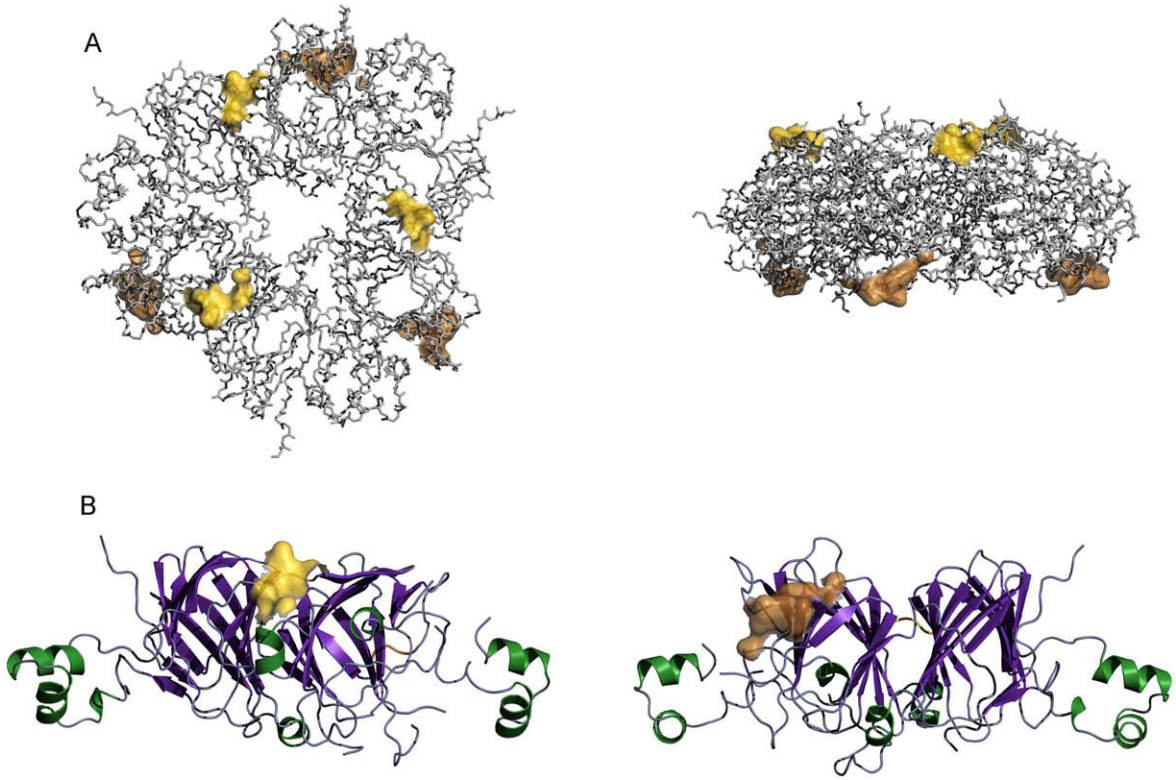

Fig. 3. Mapping of IKP (brown) and LEP (yellow) tripeptides in the Amaranth 11S globulin model. A: Stick diagram. Tripeptide surfaces are shown. It can be seen that they are located in opposite faces of the trimer, and that both are exposed to the exterior. B: Ribbon diagram. Secondary structure elements are displayed as in Fig. 1. Both tripeptides are in zones without any defined secondary structure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that this small peptide library, whose components have a proven inhibitory sequence in their C-terminus, may contain as yet unknown ACE-inhibitory peptides.

To test this hypothesis, the interaction free energy and the binding mode between each peptide and the 1O8Z structure corresponding to human ACE (Natesh et al., 2003) were theoretically calculated. Results are shown in Fig. 4, which depicts the values of free energy for the formation of the ACE-peptide complex for each component of the library. As a positive control, docking was also performed for the KP, IKP, EP, and LEP peptides with proven ACE inhibitory activity, and the non-peptide inhibitor Captopril.

In every case, the binding mode of control ligands with ACE agreed with experimental data; the union is established between the Zn^{2+} heteroatom of the active site of the metalloproteinase

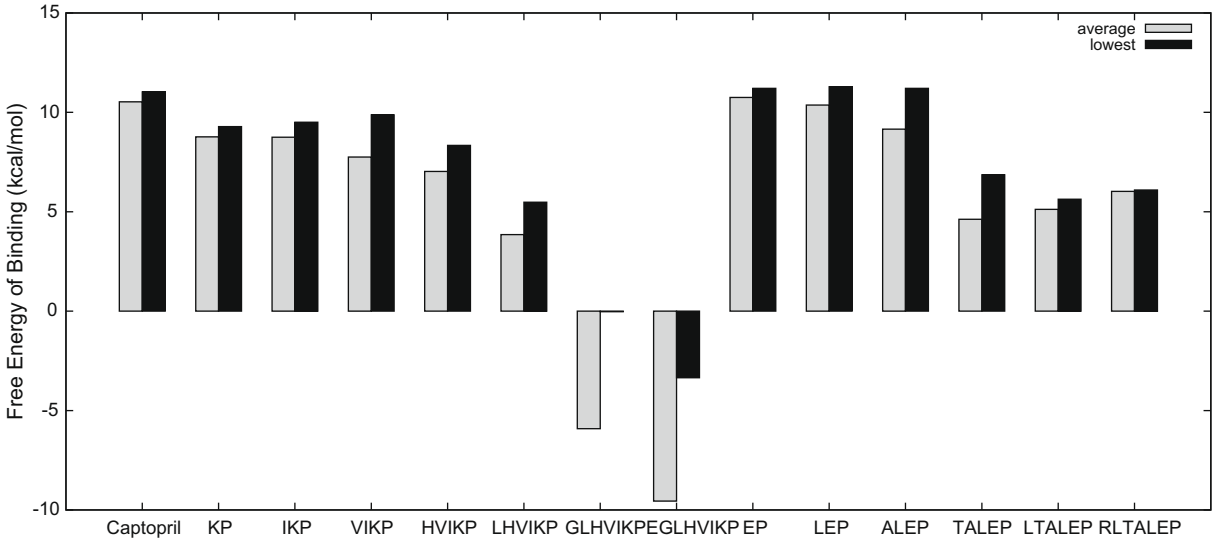

Fig. 4. Predicted free energy of binding of the peptide library. For each ligand, the lowest energy among all 50 runs (black) and the average energy of the highest scoring cluster (gray) are shown. Captopril, KP, IKP, EP, and LEP are positive controls. The predicted binding energy monotonously decreases with increasing length. The best new inhibitory peptide then is the corresponding tetrapeptide for each series: VIKP and ALEP.

and the 2-carboxyl-pyrrolidinic group (corresponding to the C-terminal proline) or the sulphydryl in the case of control peptides and Captopril, respectively.

For Captopril, the predicted interaction energy agrees with measured values of the inhibition constant: 6.35 nM vs. 1.2 nM (Michaud et al., 1997). For control peptides, the prediction is quantitatively less accurate, the predicted Ki being about one order of magnitude lower than the real one. Nevertheless, it is known that docking methods are less successful for predicting the binding mode than for predicting the inhibition constant (Sousa et al., 2006).

Notwithstanding, the change trend of these values with the N-terminal extension of the peptide, the binding mode, and the cluster histograms of the docked structures can be taken as indicative data of the potential inhibitory activity of a peptide by comparison with data predicted for peptides with proven inhibitory activity.

Seven of the nine evaluated peptides were good ligands, with interaction energy comparable to that of control inhibitory peptides (Fig. 4). In every case, they interacted through ionic bonds established with the free coordination sites of the Zn^{2+} of the active site. This ionic bridge accounted for a large part (roughly 50%) of the total free energy of binding, especially for shorter peptides.

In peptide collections based on IKP or LEP, a negative correlation was found between the ΔG of binding and the number of residues in the ligand. This finding is in line with the fact that about 90% of the peptide ACE inhibitors reported to date possess six residues or less (Dziuba et al., 1999).

For each series, the uncharacterised peptides with highest probabilities of anti-hypertensive activity were those of four residues in length: VIKP and ALEP. In both cases, the theoretical free energy of interaction had a magnitude comparable to that of the parental peptide. While VIKP seems to display a binding mode similar to IKP and Captopril, ALEP seems to bind to Zn^{2+} through its glutamic acid residue in its most frequent binding mode (see Fig. 5). These two tetrapeptides were chosen as candidates for in vitro inhibitory assays.

2.3. In vitro ACE inhibition assay

To confirm experimentally the results obtained by virtual screening, the ACE inhibitory activity of VIKP and ALEP peptides obtained by chemical synthesis was studied in vitro. IC50 (Inhibitory Concentration 50) curves were drawn, which permitted establishment of the inhibitor concentration necessary to reduce by 50% the peptidyl-transferase activity of ACE. The assay was performed in the presence of the synthetic substrate Hippuryl Histidyl Leucine (HHL), whose hydrolysis product, hippuric acid, can be measured spectrophotometrically. As a positive control, the IC50 curve of Captopril was determined (Fig. 6). A non-linear fit using the cooperative Hill function gave an apparent inhibition constant of (10.1 ± 0.5) nM and a cooperativity index or Hill slope of 2.0 ± 0.3. These values are in close agreement with those determined by others (Michaud et al., 1997; Vermeirssen et al., 2002).

As shown in Fig. 5, both ALEP and VIKP inhibited ACE activity in the conditions of the assay (IC50 6.32 mM and 175 μM, respectively). In the case of ALEP, these data indicate an inhibitory potency three orders of magnitude lower than its N-terminal tripeptide LEP. In contrast, VIKP exhibited an inhibitory activity comparable to that of IKP and of acceptable magnitude for a peptide inhibitor. There have been reports of β-casein derived tetrapeptides with IC50 values ranging from 10^1 to 5×10^2 μM (Dziuba et al., 1999; Geerlings et al., 2006; Silva et al., 2006). Previous work has also detected the presence of ACE-inhibitory peptides derived from soybean (Gouda et al., 2006) and sunflower (Megías et al., 2004) 11S globulins, respectively.

The actual difference in affinity between the assayed tetrapeptide and its C-terminal tripeptide cannot be quantitatively described solely by looking at the theoretical free energy of binding. The fairly higher IC50 of ALEP, as compared to LEP, could be explained by the fact that its lowest energy binding mode to the active site of ACE involves an ionic bridge between the carboxyl moiety of its asparagine residue and the Zn^{2+} of the enzyme, while LEP does so by means of its C-terminal proline. Alternatively, Pripp found a positive but low correlation ($R^2 = 0.28$) between predicted and actual inhibitory power, and particularly stressed the need of experimental confirmation of predictions via in-silico simulations (Pripp, 2007). This implies that while accurate affinity constant predictions cannot be expected, the library screening method can be helpful in reducing the amount of putative inhibitory peptides to be assayed by either an in vitro or in vivo method.

Limitations in the in-silico approach used here are mainly those tied to automated ligand–protein docking. Chief among these is the

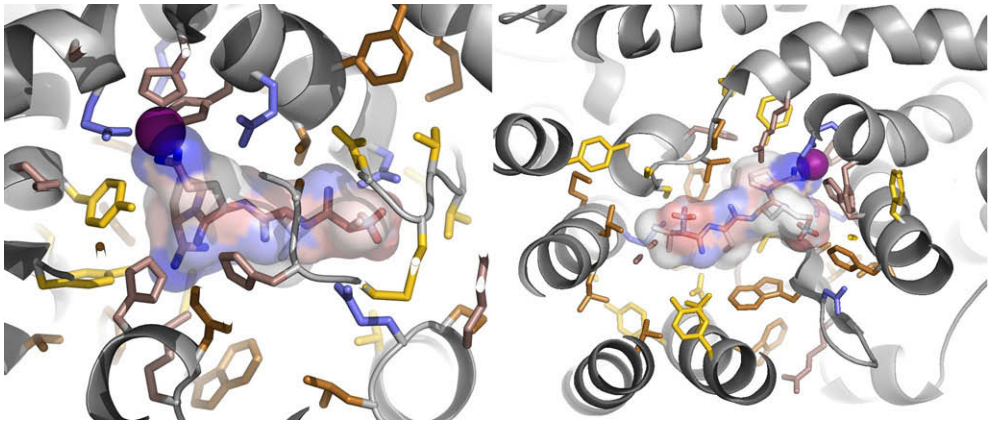

Fig. 5. Binding mode of ACE:ALEP (left) and ACE:VIKP (right) complexes corresponding to the most frequent conformation. Macromolecular side chains closer than 7 Å to the ligand are drawn as sticks and colored by their residue type: polar (yellow), hydrophobic (brown), acidic (pink) and basic (blue). The Zn^{2+} atom is colored purple. The ligand is shown in both stick and surface representations, and colored by its partial charge in a per-atom basis, where blue represents negative charge and red, positive. VIKP interacts with the Zn^{2+} ion via its C-terminal carboxyl moiety, while ALEP does so by means of its glutamic acid R group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

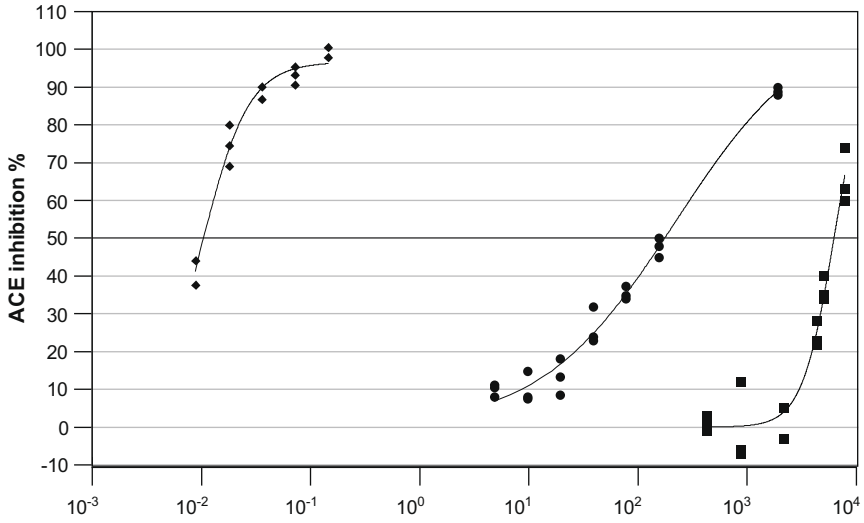

Fig. 6. ACE inhibition curves of Captopril (♦), VIKP (●), and ALEP (■). Continuous lines represent the non-linear regression using Hill's equation for each data set.

less than ideal distinction between good and bad inhibitor candidates. This is related to the fact that free energy of binding calculation is still the limiting factor of docking algorithms due to the use of simplified scoring functions necessary to reach a compromise between high accuracy and low computation time (Sousa et al., 2006). Other sources of inaccuracies are the lack of an explicit solvent model that could contemplate specific water mediated bonds, and fine aspects of interaction such as entropy and whole macromolecular motions for induced fit contributions to affinity (Sousa et al., 2006; Hetényi and Spoel, 2002).

Also, the relative importance of the overall surface accessibility of the encrypted peptides is bound to the assumption that a peptide encrypted in a solvent exposed surface devoid of tight secondary structure will have greater chances of being released by enzymatic hydrolysis by means of easier access and enhanced molecular flexibility (Fontana et al., 2004; Novotný and Bruccoleri, 1987). This, however, does not rule out the possibility that buried zones might be a valuable source of ACE-inhibitory peptides for conditions in which high degree of hydrolysis are achieved, or considers the specificity of any particular enzyme.

Another possible source of error is the usage of not yet perfect empirical structure–activity relationships to reduce the size and complexity of the peptide library. This step can be completely left out, albeit with the consequence of having to perform screening over a considerably larger collection of peptide candidates.

The peptides described in this work can also be found in other seed storage proteins, most notably *Glycine max* glycinin and *Chenopodium quinoa* 11S globulin (Table S1, Supplementary Data).

3. Concluding remarks

It can be concluded that both ALEP and VIKP are tetrapeptides with ACE inhibitory activity in vitro. These peptides are encrypted in the primary sequence of 11S globulin from *Amaranthus hypochondriacus* and are located in regions devoid of defined secondary structure and with high exposure to the solvent. The latter two characteristics increase the probability of their release by proteolysis both in the gastrointestinal tract and in enzymatic hydrolysates of either 11S globulin or Amaranth protein isolates. To our knowledge, this is the first time that a food-derived ACE inhibitory

peptide is found exclusively via an *in-silico* analysis through library screening and automated docking.

4. Experimental

4.1. Chemicals

All chemicals and solvents were of analytical grade and were obtained from either Merck or Sigma. The substrate Hippuryl Histidyl Leucine was acquired from MP; rabbit lung purified angiotensin I-converting enzyme was obtained from Sigma. Synthetic peptides VIKP and ALEP were custom-synthesized, HPLC purified to more than 97% purity, and analysed by HPLC–MS by SBS Genetech (China).

4.2. Molecular modeling of Amaranth 11S globulin

Amaranth 11S globulin sequences (GenBank Accession Nos. ABM66807 and CAA57633) were obtained from the Entrez database through the NCBI website (http://www.ncbi.nlm.nih.gov/sites/entrez). Multiple amino acid sequence alignments were carried out with ClustalX (Thompson et al., 1997) and edited manually when required. Template candidates were selected with the FFAS03 server (Jaroszewski et al., 2005) and structurally aligned with VMD's MultiSeq plugin (Eargle et al., 2006; Humphrey et al., 1996). Homology-based molecular modeling was done using T.I.T.O. server, (Labesse and Mornon, 1998) frontend of the Modeller program (Sali and Blundell, 1993). The model was further refined by steepest descent energy minimization with GROMACS (Spoel et al., 2005). Model quality was checked by means of the Eval23D server (Gracy et al., 1993). Cartoons were drawn using PyMol (DeLano, 2002).

4.3. Accessible surface area calculation

ASA values for each residue were calculated by the GETAREA server (Fraczkiewicz and Braun, 1998) at http://www.chem.ac.ru/Chemistry/Soft/GETAREA.en.html using the three-dimensional model for Amaranth 11S globulin. To cover the model's discontinuities, ASAP (Yuan et al., 2006; Yuan and Huang, 2004) was used with primary sequence information.

4.4. Anti-hypertensive peptide mapping

Amaranth 11S globulin was annotated using information from the Biopep database (Dziuba et al., 1999) as well as that published by Wu (Wu et al., 2006). Annotation was done automatically by means of a script that read from this updated database and wrote down the encrypted peptide positions and IC50 values as features in a standard GenBank sequence file. Annotated Amaranth Globulin 11S was then processed by downstream scripts that returned ASA and IC50 values as a function of residue number. Scripts were written in the Perl language with the aid of the BioPerl modules (Stajich et al., 2002) and are available upon request. Sequence and feature display was done with the TeXshade package (Beitz, 2000).

4.5. Library screening by ligand docking

Peptide library was manually built using Ghemical (Stone et al., 2001). The target molecule used was that of human ACE complexed with lisinopril (PDB Accession code ***1O86***) (Natesh et al., 2003). Ligand and target molecule preparation were done as described by Hetényi and Spoel, 2002. Rigid automated docking was performed using Autodock's Lamarckian Genetic Algorithm (Morris et al., 1998). For each ligand, 50 runs were done with a population size of 150 individuals and a maximum of 10,000,000 energy evaluations per generation. Result files were automatically parsed to extract the clustering histogram information, the binding mode and free energy change of both the lowest energy and most common complexes (if different). Clustering of final docked conformations was done using an RMSD tolerance of 2.0 Å.

4.6. ACE inhibition assay

Angiotensin converting enzyme inhibition assay was performed using the method of Cushman and Cheung, 1971 as modified by Kim et al., 1999. Briefly, 20 μL of peptide sample was added to 0.1 M potassium phosphate containing 0.3 M NaCl and 5 mM Hippuryl Histidyl Leucine (HHL), pH 8.3. ACE (5 mU) was added and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.1 mL 1 M HCl. The hippuric acid formed was extracted with ethyl acetate, heat evaporated at 95 °C for 10 min, redissolved in distilled water, and measured spectrophotometrically at 228 nm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.04.006.

References

Aphalo, P., Castellani, O.F., Martinez, E.N., Añón, M.C., 2004. Surface physicochemical properties of globulin-P amaranth protein. J. Agric. Food Chem. 52, 616–622.

Barre, A., Borges, J., Rougé, P., 2005. Molecular modeling of the major peanut allergen Ara h 1 and other homotrimeric allergens of the cupin superfamily: a structural basis for their IgE-binding cross-reactivity. Biochimie 87, 499–506.

Beitz, E., 2000. TEXshade: shading and labeling of multiple sequence alignments using LATEX2 epsilon. Bioinformatics 16, 135–139.

Bressani, R., Elias, L.G., Garcia-Soto, A., 1989. Limiting amino acids in raw and processed amaranth grain protein from biological tests. Plant Foods Hum. Nutr. 39, 223–234.

Colla, E., Sobral, P.J.D.A., Menegalli, F.C., 2006. *Amaranthus cruentus* flour edible films: influence of stearic acid addition, plasticizer concentration, and emulsion stirring speed on water vapor permeability and mechanical properties. J. Agric. Food Chem. 54, 6645–6653.

Cushman, D.W., Cheung, H.S., 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol. 20, 1637–1648.

DeLano, W., 2002. The PyMOL User's Manual.

Dziuba, J., Minkiewicz, P., Nalecz, D., Iwaniak, A., 1999. Database of biologically active peptide sequences. Nahrung 43, 190–195.

Eargle, J., Wright, D., Luthey-Schulten, Z., 2006. Multiple Alignment of protein structures and sequences for VMD. Bioinformatics 22, 504–506.

Escudero, N.L., Arellano, M.L.D., Luco, J.M., Giménez, M.S., Mucciarelli, S.I., 2004. Comparison of the chemical composition and nutritional value of *Amaranthus cruentus* flour and its protein concentrate. Plant Foods Hum. Nutr. 59, 15–21.

FitzGerald, R.J., Meisel, H., 2000. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. Br. J. Nutr. 84 (Suppl 1), S33–S37.

Fontana, A., de Laureto, P.P., Spolaore, B., Frare, E., Picotti, P., Zambonin, M., 2004. Probing protein structure by limited proteolysis. Acta Biochim. Pol. 51, 299–321.

Fraczkiewicz, R., Braun, W., 1998. Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. J. Comput. Chem. 19, 319–333.

Geerlings, A., Villar, I.C., Zarco, F.H., Sánchez, M., Vera, R., Gomez, A.Z., Boza, J., Duarte, J., 2006. Identification and characterization of novel angiotensin-

converting enzyme inhibitors obtained from goat milk. J. Dairy Sci. 89, 3326–3335.
Gorinstein, S., Pawelzik, E., Delgado-Licon, E., Haruenkit, R., Weisz, M., Trakhtenberg, S., 2002. Characterisation of pseudocereal and cereal proteins by protein and amino acid analyses. J. Sci. Food Agri. 82, 886–891.
Gouda, K.G.M., Gowda, L.R., Rao, A.G.A., Prakash, V., 2006. Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (glycine max). J. Agric. Food Chem. 54, 4568–4573.
Gracy, J., Chiche, L., Sallantin, J., 1993. Improved alignment of weakly homologous protein sequences using structural information. Protein Eng. 6, 821–829.
Hartmann, R., Meisel, H., 2007. Food-derived peptides with biological activity: from research to food applications. Curr. Opin. Biotechnol. 18, 163–169.
Hetényi, C., Spoel, D.V.D., 2002. Efficient docking of peptides to proteins without prior knowledge of the binding site. Protein Sci. 11, 1729–1737.
Humphrey, W., Dalke, A., Schulten, K., 1996. VMD: visual molecular dynamics. J. Mol. Graph. 14, 33–38.
Jaroszewski, L., Rychlewski, L., Li, Z., Li, W., Godzik, A., 2005. FFAS03: a server for profile–profile sequence alignments. Nucleic Acids Res. 33, W284–W288.
Kim, Y.K., Yoon, S., Yu, D.Y., Lönnerdal, B., Chung, B.H., 1999. Novel angiotensin-I-converting enzyme inhibitory peptides derived from recombinant human alpha s1-casein expressed in *Escherichia coli*. J. Dairy Res. 66, 431–439.
Kubota, E., Dean, R.G., Balding, L.C., Burrell, L.M., 2002. Inhibition of peptidases in the control of blood pressure. Essays Biochem. 38, 129–139.
Labesse, G., Mornon, J., 1998. Incremental threading optimization (TITO) to help alignment and modeling of remote homologues. Bioinformatics 14, 206–211.
Lüthy, R., Bowie, J.U., Eisenberg, D., 1992. Assessment of protein models with three-dimensional profiles. Nature 356, 83–85.
Marcone, M.F., 1999. Possible nutritional implications of varietal influence on the 7S/11S seed globulin ratios in amaranth. Plant Foods Hum. Nutr. 54, 375–380.
Megías, C., Yust, M.D.M., Pedroche, J., Lquari, H., Girón-Calle, J., Alaiz, M., Millán, F., Vioque, J., 2004. Purification of an ACE inhibitory peptide after hydrolysis of sunflower (*Helianthus annuus* L.) protein isolates. J. Agri. Food Chem. 52, 1928–1932.
Michaud, A., Williams, T.A., Chauvet, M.T., Corvol, P., 1997. Substrate dependence of angiotensin I-converting enzyme inhibition: captopril displays a partial selectivity for inhibition of N-acetyl–seryl–aspartyl-lysyl–proline hydrolysis compared with that of angiotensin I. Mol. Pharmacol. 51, 1070–1076.
Morris, G.M., Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K., Olson, A.J., 1998. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. J. Comput. Chem. 19, 1639–1662.
Murray, B.A., FitzGerald, R.J., 2007. Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production. Curr. Pharm. Des. 13, 773–791.
Natesh, R., Schwager, S.L.U., Sturrock, E.D., Acharya, K.R., 2003. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. Nature 421, 551–554.
Novotný, J., Bruccoleri, R.E., 1987. Correlation among sites of limited proteolysis, enzyme accessibility and segmental mobility. FEBS Lett. 211, 185–189.
Pripp, A., 2007. Docking and virtual screening of ACE inhibitory dipeptides. Eur. Food Res. Technol. 225, 589–592.
Pripp, A.H., Isaksson, T., Stepaniak, L., Sørhaug, T., 2004. Quantitative structure–activity relationship modeling of ACE-inhibitory peptides derived from milk proteins. Eur. Food Res. Technol. 219, 579–583.
Quiroga, A.V., Martínez, E.N., Añón, M.C., 2007. Amaranth globulin polypeptide heterogeneity. Protein J. 26, 327–333.
Sagnella, G.A., 2002. Vasopeptidase inhibitors. J. Renin Angiotensin Aldosterone Syst. 3, 90–95.
Sali, A., Blundell, T.L., 1993. Comparative protein modeling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815.
Silva, S.V., Pihlanto, A., Malcata, F.X., 2006. Bioactive peptides in ovine and caprine cheeselike systems prepared with proteases from *Cynara cardunculus*. J. Dairy Sci. 89, 3336–3344.
Silva-Sánchez, C., González-Castañeda, J., León-Rodríguez, A.D., Rosa, A.P.B.D.L., 2004. Functional and rheological properties of amaranth albumins extracted from two Mexican varieties. Plant Foods Hum. Nutr. 59, 169–174.
Silva-Sánchez, C., Rosa, A.P.B.D.L., León-Galván, M.F., Lumen, B.O.D., León-Rodríguez, A.D., Mejía, E.G.D., 2008. Bioactive peptides in amaranth (*Amaranthus hypochondriacus*) seed. J. Agric. Food Chem. 56, 1233–1240.
Sousa, S.F., Fernandes, P.A., Ramos, M.J., 2006. Protein–ligand docking: current status and future challenges. Proteins 65, 15–26.
Spoel, D.V.D., Lindahl, E., Hess, B., Groenhof, G., Mark, A.E., Berendsen, H.J.C., 2005. GROMACS: fast, flexible, and free. J. Comput. Chem. 26, 1701–1718.
Stajich, J.E., Block, D., Boulez, K., Brenner, S.E., Chervitz, S.A., Dagdigian, C., Fuellen, G., Gilbert, J.G., Korf, I., Lapp, H., et al., 2002. The bioperl toolkit: perl modules for the life sciences. Genome Res. 12, 1611–1618.
Stone, J., Gullingsrud, J., Grayson, P., Schulten, K., 2001. A system for interactive molecular dynamics simulation. Paper presented at: 2001 ACM Symposium on Interactive 3D Graphics (ACM SIGGRAPH).
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Vermeirssen, V., Camp, J.V., Verstraete, W., 2002. Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. J. Biochem. Biophys. Methods 51, 75–87.
Wu, J., Aluko, R.E., Nakai, S., 2006. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure–activity relationship study of di- and tripeptides. J. Agric. Food Chem. 54, 732–738.
Yuan, Z., Huang, B., 2004. Prediction of protein accessible surface areas by support vector regression. Proteins 57, 558–564.
Yuan, Z., Zhang, F., Davis, M.J., Bodén, M., Teasdale, R.D., 2006. Predicting the solvent accessibility of transmembrane residues from protein sequence. J. Proteome Res. 5, 1063–1070.