"Bottom up" strategy for the identification of novel soybean peptides with ACE-inhibitory activity

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Abstract

IAVPTGVA (Soy1) and LPYP are two soybean peptides, which display a multifunctional behavior showing *in vitro* hypocholesterolemic and hypoglicemic activities. A preliminary screening of their structures using BIOPEP suggested that they might be potential angiotensin converting enzyme (ACE) inhibitors. Therefore, a bottom up-aided approach was developed in order to clarify the *in vitro* hypotensive activity. Soy1 and LPYP dropped the intestinal and renal ACE enzyme activity with IC_{50} values equal to 14.7±0.28 μM and 5.0±0.28 μM (Caco-2 cells), and 6.0±0.35 and 6.8±0.20 μM (HK-2 cells), respectively. In parallel, a molecular modeling study suggested their capability to act as competitive inhibitors of this enzyme. Finally, in order to increase both their stability and hypotensive properties, a suitable strategy for the harmless control of their release from a nanomaterial was developed through their encapsulation into the RADA16 assembling peptide.

Keywords: ACE, peptide encapsulation, bioactive peptides, hypotensive peptides, multifunctional peptides, self-assembling peptides.

INTRODUCTION

Hypertension is one of the main risk factors for the development of cardiovascular diseases.[1] A complex interaction of genetic and environmental factors as well as many other factors (i.e. increased levels of long-term high sodium intake, inadequate dietary intake of potassium and calcium, elevated renin-angiotensin system (RAS) activity, and endothelial dysfunction) are the basis of the pathophysiological development of this disease.[2, 3] In this context, angiotensin converting enzyme (ACE, EC 3.4.15.1), a dipeptidyl-carboxypeptidase expressed in many tissues (lung, kidney, intestine), is a key enzyme for blood pressure regulation, being responsible of the conversion of inactive angiotensin I (Ang) into active Ang II, a vasoconstrictive octapeptide that is accountable for the hypertension progression.[4] The inhibition of this enzyme is therefore considered a successful strategy for lowering high blood pressure.

This is also true in the field of hypotensive food peptides: indeed, several peptides from milk, meat, egg, fish, lupin, and soybean sources have been singled out as inhibitors of the ACE activity.[5-8] Milk proteins have a leading role as a source of ACE inhibitory peptides: in particular, VPP and IPP, two peptides deriving from β-casein and κ-casein, are the most active ACE-inhibitors from any food source;[9] their hypotensive effect has been confirmed *in vivo* in spontaneously hypertensive rats (SHR) fed with sour milk and they are now on the market as ingredients of antihypertensive drinks, such as the Japanese “Calpis” and the Finnish “Evolus”.[10]

IAVPTGVA (Soy1) and LPYP are peptides deriving from the hydrolysis of soybean glycinin with pepsin and trypsin,[11] respectively, which have been demonstrated to be absorbable in Caco2 cell monolayers.[12] More in detail, recent evidences suggest that both peptides are absorbed by differentiated Caco-2 cells as a function of time and that Soy1 is better absorbed than LPYP after 2 h of incubation in the apical side of monolayers.[12] Mature enterocytes represent the first physiological barrier that bioactive food peptides encounter after ingestion, therefore their absorption is a dynamic process which coexists with their metabolic degradation. In light of this observation, some evidences

underline that during its absorption, Soy1 (IAVPTGVA) is partially metabolized by active Caco-2 cells membrane peptidases in three breakdown fragments (AVPTGVA, IAVP, and IAV), which are also absorbed in the same cellular system.[12]

Both peptides have a multifunctional behavior since *in vitro* they are either hypocholesterolemic or hypoglycemic.[12-15] The cholesterol lowering effect is due to the inhibition of 3-hydroxymethylglutaryl coenzyme A reductase (HMGCoAR) and the subsequent activation of the low-density lipoprotein receptor (LDLR) pathway. Moreover, these effects are accompanied by an increase of the phosphorylation level of HMGCoAR on Ser 872 (the inactive form of HMGCoAR), *via* the activation of the adenosine monophosphate-activated protein kinase (AMPK) pathway.[13] The capacity to modulate glucose metabolism and uptake is linked to the activation of the AMPK and protein kinase B (Akt) pathways.[14] The activation of Akt (phosphorylated at Ser 473) leads to the inhibition of glycogen synthase kinase-3β (GSK3), which in turn regulates the glycogen synthase (GS) activity with a modulation of the hepatic glycogen formation. In parallel, the increased protein levels of glucose transporter type 4 (GLUT4) and glucose transporter type 1 (GLUT1) determine an increased attitude of HepG2 cells to clear extracellular glucose. In other experiments, Soy1 and LPYP have been demonstrated to be also capable of inhibiting the activity of dipeptidyl peptidase-IV (DPP-IV), another favorable effect for diabetes prevention.[12, 15]

A preliminary screening of the structures of Soy 1 and LPYP using BIOPEP (www.uwm.edu.pI/biochemia)[16] suggested that they might be compatible with a potential behavior as ACE inhibitors. Hence, the first objective of this work was an evaluation of their ACE-inhibitory activity. Instead of using the traditional *in vitro* assay on the enzyme purified from rat or rabbit (mostly used in literature), our experimentation was based on a cellular assay performed in human intestinal Caco-2 and kidney HK-2 cells, which are among the cells that mostly express this enzyme in the body. In parallel, a molecular modeling study was carried out to investigate their capability to act as competitive inhibitors of ACE, in agreement with previous studies.[17] The *in silico* study was based on a structure-based modeling of both ACE domains (namely, the N-domain and C-domain)

including pharmacophoric analysis, docking simulations, rescoring procedures, and molecular dynamics.

Lastly, since we have previously reported that self-assembling peptide-based nanogels (SAPs) are a viable platform for targeting metabolic diseases with bioactive peptides,[18, 19] thanks to their bona fide properties, well-ordered nanostructures, and biocompatibility; here we provide a smart delivery coating system of Soy1 and LPYP by using RADA16 hydrogel (Ac-RADARADARADARADA-CONH2). The feasibility of this encapsulation strategy was assessed mainly by rheology, ThT binding assay, spectroscopy assay (CD and ATR-FTIR), and release kinetic experiments.

MATERIALS AND METHODS

Materials. All reagents and solvents were from commercial sources. See "Supplementary Information" for further details on materials and methods.

***In vitro* digestion of Soy1 and LPYP.** Pepsin solution (4 mg/mL in NaCl) was added to Soy1 and LPYP (100 μM) at a 1:100 enzyme to substrate ratio (pH 2.0). The digestion was conducted at 37 °C for 90 min under continuous stirring, and then the pH was adjusted to 7.2 with 1 M NaOH in order to inactivate the enzyme. Then, pancreatin (4 mg/mL in H_2O) was added at a 1:50 enzyme to substrate ratio. After digestion at 37 °C for 150 min, the enzyme was inactivated by heating at 95 °C for 10 min. Further details regarding the analysis are available on "Supplementary Information".

Cell culture. Caco-2 cells, obtained from Institut National de la Santé et de la Recherche Médicale (INSERM, Paris), were routinely sub-cultured as previously described.[18] HK2 from ATCC were cultured using Dulbecco Minimum Essential Medium-F12 (DMEM-F12) containing 25 mM glucose, 4 mM stable L-glutamine, 100 U L^{-1} penicillin, 100 μg L^{-1} streptomycin, supplemented with 10% heat-inactivated fetal bovine serum (FBS Hyclone Laboratories, Logan, UT, USA)

ACE activity cell-based assay. Soy1 and LPYP were tested on Caco-2 and HK2 cells (5×10^4/well in black 96-well plates) at 0.1-250.0 μM concentration ranges or vehicle in growth medium for 24 h at 37 °C. For 2D cell culture on RADA16-Soy1 and RADA-LPYP hydrogels, Caco-2 cells were seeded on the surface of the above-mentioned hydrogels at the density of 5×10^4/well. The next day, The ACE inhibitory activity was measured using the ACE1 Activity Assay Kit (Biovision, Milpitas Blvd., Milpitias, CA, USA) following the manufacture's protocol. See See "Supplementary Informations" for further details on Acer activity cell-based assay.

***In silico* modeling.** A molecular modeling approach was used to investigate the interaction of peptides with the N- and C-domain of human ACE from a molecular perspective. In more detail, the computational analysis relied on pharmacophoric modeling followed by docking simulations coupled to rescoring procedures to assess the capability of peptides to fit the catalytic sites of both domains, as previously reported.[17] Then, a 50 nsec dynamic simulations study was applied to assess their capability to persist therein. See "Supplementary Informations" for further details on molecular modeling.

Synthesis and purification of RADA16. As we previously reported[18] RADA16 molecule was synthesized by fluorenylmethoxycarbonyl solid-phase peptide synthesis and purified by HPLC. The purity of lyophilized peptide was tested by single quadrupole mass spectrometry using an Alliance-3100 LC-MS. After lyophilization, RADA16 was dissolved at 1% (w/v) in distilled waters.

Rheological measurement. Rheological measurement was performed by an AR-2000ex Rheometer (TA Instruments, New Castle, DE, USA) with a 20 mm acrylic truncated plate. All peptide samples were tested at the concentration of 1% (w/v) and the sample stage was set to 25 °C. The storage modulus was recorded as a function angular frequency (0.1-100 Hz) at a fixed strain of 1%.

Thioflavin T (ThT) spectroscopy assay. The propensity of assembled peptides to form cross-β fibril structures were performed by using ThT binding assay, as previously described.[18]

Circular dichroism (CD) spectroscopy assay. CD spectra of peptide samples were recorded in continuous scanning mode (190-300 nm) at 25 °C using Jasco J-810 (Jasco Corp., Tokyo, Japan) spectropolarimeter. All spectra were collected using a 1 mm path-length quartz cell and averaged over three accumulations (speed: 10 nm min^{-1}). A reference spectrum of distilled water was recorded and subtracted from each spectrum. The estimation of the peptide secondary structure was achieved by using a literature method.[20]

Fourier transform infrared spectroscopy (FT-IR) analysis. Similar to our previous report,[18] FT-IR analysis was performed on peptides dissolved at a final concentration of 1% (w/v) in distilled water. More details are available on "Supplementary Information".

Kinetic of Soy1 and LPYP peptide release from the nanogels. The peptide leaking from the nanogels as a function of time was measured dissolving the nanogels in PBS and measuring the concentrations of released peptides after 60, 180, and 360 min of incubation by using a method previously described.[19] See "Supplementary Information" for further details on kinetic evaluation of both peptides release.

Cell viability test. Caco-2 cells were seeded on the surface of RADA16-Soy1 and RADA-LPYP hydrogels at the density of 5×10^4/well and cultured for 6 days. Intestinal cell growth was qualitatively evaluated collecting images using Zeiss Axioplan 2 microscope (Oberkochen, Germania). Finally, MTT experiments were carried using method previously reported.[21]

Statistically Analysis. Statistical analyses were carried out by One-way ANOVA using Graphpad

Prism 6 (Graphpad, La Jolla, CA, USA). Values were expressed as means ± s.d. of three independent experiments, each experiment was performed in triplicate; *p*-values < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Soy1 and LPYP inhibit the *in situ* ACE activity on human intestinal Caco-2 and kidney HK-2 cells

Their metabolic propensity to be degraded by peptidases, which are physiologically active along the entire gastrointestinal tract, might dramatically influence the bioactivity of food peptides. Literature provides many studies dealing with the assessment of food bioactive peptide stability to the simulated gastric digestion.[22, 23] In order to in-depth characterize the multifunctional behavior of Soy 1 and LPYP, their stability toward the *in vitro* gastric digestion was assessed by using pepsin and pancreatin. Figure 1 indicates that after co-digestion with these enzymes, LPYP and Soy1 are degraded by only 28.5±1.4% and 27.7±0.3%, respectively. These results highlight that both Soy1 and LPYP are noteworthy stable to the *in vitro* gastric digestion.

Based on these results, in order to investigate the potential hypotensive effect of Soy1 and LPYP, their ability to drop *in situ* the ACE activity was evaluated by using a cell-based assay. In particular, Caco-2 and HK-2 cells ($5x10^4$/well) were treated with Soy1 and LPYP (0.1-250 μM) over night. The following day, cells were lysated and the ACE activity was measured directly in the cell lysates using a fluorescent ACE substrate: in this assay the fluorescent signal is proportional to the enzyme activity. As shown in Figure 2, Soy1 and LPYP reduced the enzyme activity with a dose-response trend in both biological systems (Caco-2 and HK-2 cells). In particular, Soy 1 and LPYP displayed calculated IC_{50} values equal to 14.7±0.28 and 5.0±0.28 μM in Caco-2 cells, respectively (Figure 2A); whereas, the same peptides showed IC_{50} values equal to 6.0±0.35 and 6.8±0.20 μM in HK-2 cells, respectively (Figure 2B).

Literature provides many examples of studies in which different food-derived peptides target the *in vitro* activity of ACE. In all these studies, their biochemical characterization has been carried out using *in vitro* tests employing the purified recombinant ACE enzymes from different animal species, such as pig and rabbit. Although the ACE sequence is highly conserved among species,[24] the only use of biochemical tools involving the purified ACE enzymes and a standard substrate provide only an insufficient characterization of the activity before performing expensive *in vivo* experimental studies. On the contrary, a cell-based assay is certainly more helpful, since it allows the investigation of the enzyme in its natural environment and to account for possible metabolic modifications of the peptide structure and activity. For this reason, two cellular systems, human intestinal Caco-2 and renal HK-2 cells, were chosen to characterize the potential inhibitory activity of Soy1 and LPYP in a more realistic way. In particular, the intestine is the first physiological barrier that peptides from food sources encounter after ingestion and it is well known that intestine and kidney express high level of ACE enzyme, where the ACE and RAS systems play a key role in the blood pressure regulation.[25, 26] Our findings clearly suggest that both soybean peptides show an outstanding ACE inhibitory activity: LPYP displays comparable IC_{50} values in Caco-2 and HK-2 cells, whereas Soy1 is 2-fold more active at renal than intestinal level. This may be explained by the propensity of Soy1 to undergo a metabolic degradation by active peptidases that are expressed in the apical side of intestinal cells. Indeed, a recent study has demonstrated that intestinal cells absorb both LPYP and Soy 1, but during this process, the latter is partially cleaved into shorter peptides (AVPTGVA, IAVP, and IAV).[12]

It is well recognized that soybean proteins contain many bioactive peptides exerting multiple health benefits, i.e. hypocholesterolemic, anti-diabetic, anti-tumor, and hypotensive activity. In this context, LAIPVNKP and LPHF are two ACE inhibitors reported in literature with IC_{50} values of 70 and 670 μM, respectively.[27] Moreover, after the hydrolysis of soybean proteins with pepsin, five peptides have been identified showing *in vitro* and *in vivo* hypotensive activity. In particular, IA shows an IC_{50} value of 153 μM, YLAGNQ (14 μM), FFL (37 μM), IYLL (42 μM), and VMDKPQG (39 μM). Their blood pressure lowering activity has been also confirmed *in vivo* on SHR models.[28] Moreover, peptides

SPYP and WL, obtained from the hydrolysis of soybean glycinin by acid proteinase from *Monascus purpureus*, have been shown to be able to inhibit the ACE activity *in vitro* with IC_{50} values equal to 850 μM and 65 μM, respectively.[27] Surprisingly among the known ACE inhibitory peptides from soybean proteins, SPYP and LPYP are very similar, the only difference relaying on a single amino acid residue. LPYP is 170-fold more potent than SPYP, suggesting that the presence of a hydrophobic amino acid residue with an aliphatic chain (Leu) instead of a polar residue with a hydroxymethyl group (Ser) leads to an impressive potency gain.

Since the literature on ACE inhibitory peptides from food is very extensive, a correlation between their physico-chemical and structural properties with bioactivity is well established. In particular, to produce ACE inhibition, hydrophobic peptides (2-8 amino acid residues) should contain in the N-terminal hydrophobic amino acids, especially those with aliphatic chains such as Gly, Ile, Leu, and Val, and at the C-terminal amino acids with cyclic or aromatic rings (Pro, Tyr, Trp).[29, 30] Many peptides derived from food proteins contain Pro at the C-terminal, a rule concerning mostly short peptides.[31] The ACE inhibitory activity is furthermore improved by the simultaneous occurrence of a C-terminal Pro and an N-terminal branched-side aliphatic amino acid. Indeed, our results are in agreement with these structure-activity relationships. In light of all these observations and in order to get an insight of the binding mode of both peptides with the ACE enzyme, in silico investigation were performed.

Molecular modeling studies

Soy1 and LPYP underwent a molecular modeling study in order to investigate their possible interaction with the N- and C-domain of human ACE at a molecular level. The *in silico* study consisted in the pharmacophoric description of both the catalytic sites of ACE, followed by docking simulations coupled to rescoring procedures to better evaluate the protein-peptide interaction, in agreement with previous studies.[32, 33] The top scored docking pose in each domain was then compared with the respective pharmacophoric fingerprint providing a qualitative structure-activity relationship

analysis. Finally, LPYP, which showed the best IC_{50} value and the highest computational scores (*vide infra*), underwent 50 nanoseconds molecular dynamic simulations to study the geometrical stability of its interaction over time.

As previously reported, the two catalytic sites showed a largely conserved sequence identity and a similar spatial organization of residues, determining a comparable pocket shape and a similar distribution of pharmacophoric properties.[17] Concerning the results of docking simulations and re-scoring procedures, LPYP but not Soy1 seemed able to favorably interact with the two catalytic sites of ACE. Indeed, on one side, LPYP recorded a HINT score of 2570 and 2830 within the N- and C-domain, respectively. On the other side, Soy1 recorded a HINT score of 20 and -374 within the N- and C-domain, respectively. Notably, HINT score relates to the free energy of binding and, specifically, the higher the score the stronger the interaction. Conversely, negative scores, as well as scores proximal to zero, may indicate the lack of appreciable interaction, as previously reported.[32, 34, 35] On this basis, the capability of Soy1 to interact with the two catalytic sites was judged less favorable than the one of LPYP.

The docking analysis poses of LPYP in each ACE catalytic site in respect to their respective pharmacophoric fingerprints provided a molecular rationale to such results. As shown in Figure 3, LPYP engaged both sites *via* a multiple hydrogen bonds network starkly complying with the pharmacophoric fingerprint of the two pockets. Conversely, the N-terminal residues of Soy1 were found not fully matching neither of the two catalytic sites, though the peptide could form hydrogen bonds with its C-terminal residue. Specifically, both hydrophobic-polar and acid-acid interferences were found, as shown in Figure 3B and 3D. These evidences provided a structural rationale explaining the diverse scores recorded by LPYP and Soy1, pointing out the better capability of the former to interact with the catalytic sites of ACE. The low compliance of Soy1 to the catalytic sites of ACE eventually suggested its presumably low capability to inhibit ACE *via* a competitive mechanism with the catalytic site. Nonetheless, this result was in apparent contrast with the experimental evidences reported above stating its ACE inhibitory activity, though of a lower intensity than LPYP. However,

ACE inhibitory peptides may also interact in regions other than the catalytic sites changing the capability of substrates to reach the catalytic core.[36] Therefore, Soy1 might act through mechanisms that do not require the competitive binding at the catalytic sites.

Considering that Soy1 may be hydrolyzed by cells releasing fragments such as AVPTGVA, IAVPT and IAVP[12], these fragments were also submitted to the docking and rescoring procedure to assess their possible capability to fit the catalytic sites of ACE. AVPTGVA, IAVPT, and IAVP recorded 52, 50, and 1956 HINT scores within the N-domain, respectively. Conversely, they recorded 793, 624, and 2164 HINT scores within the C-domain, respectively. In particular, IAVP markedly complied the pharmacophoric requirements of both pockets strongly retracing the mode of binding of LPYP (Figure 3). Moreover, on the basis of the obtained scores, AVPTGVA and IAVPT were found to better satisfy the pocket requirements of the C-domain than those of the N-domain (wherein their interaction was considered not favored due to the low scores recorded). These results might point to their possible preferential interaction and inhibition with the N-domain. This feature might deserve future investigations in order to identify domain-specific inhibitory peptides.

Overall, these results support the full compliance of LPYP as ACE inhibitor *via* competitive mechanisms at the catalytic sites of ACE. Conversely, the capability of Soy1 to interact with the catalytic sites was judged less favored than that of LPYP. However, other non-competitive mechanisms could not be excluded. In addition, Soy1 fragments released by cell peptidases might competitively inhibit ACE concurring to the overall inhibitory potential of Soy1.

Finally, LPYP, which recorded both the best IC_{50} in experimental trials and the highest scores in computational analysis, was submitted to molecular dynamic simulations (50 nanoseconds) to check the capability to persist within the catalytic sites of ACE over time. The interaction of LPYP was found geometrically stable in both ACE domains, as shown by the low RMSD fluctuations of C-alpha (Figure 3G). In addition, the inspection of LPYP trajectories revealed its persistence and stability within both the catalytic sites (Figure 3H), further supporting its capability to inhibit ACE *via* competitive mechanisms.

Supramolecular approach for the development of Soy1 and LPYP based nanogels: mechanical, structural and biological characterizations

SAPs are a promising class of supramolecular nanomaterials for controlled drug delivery applications and beyond. Here we report the feasibility of encapsulating the bioactive peptides Soy1 and LPYP (Figure 4A-B) into self-assembly peptide RADA16. [37]

In order to assess the ability of RADA16 to support the slow release of both Soy1 and LPYP peptide, a 1% (w/v) of RADA16-Soy1 or RADA16-LPYP nanogels were prepared to characterize their viscoelastic properties. Rheological measurements were performed to estimate the elastic response (*G′*) of nanogels, by varying frequencies of applied oscillatory stress at constant strain (0.1-100 Hz, 1% strain). All preassembled solutions showed typical soft hydrogel profiles,[19] featuring a *G′* modulus of ~1800 Pa and ~1100 Pa for RADA16-Soy and RADA16-LPYP respectively (Figure 4C).

The amyloidogenic nature of the nanogels was pursued using a Thioflavin T (ThT) binding assay (Figure 4D). This assay enables evaluation of the amyloidogenic structures and cross-β fibril formation of materials because β-rich structures feature ThT-binding sites. ThT assay resulted in high fluorescence levels, as well as a typical amyloid-binding emission signal (peak at ~490 nm), thus establishing the β-rich amyloidogenic nature of both nanogels. To study the secondary structure of the nanogels in solution, CD spectroscopy was carried out. As expected, both nanogels exhibited a CD signal comprising a negative peak near 215 nm and a positive peak at ~195 nm characteristic of a β-sheet conformation (Figure 4E). To gain further information about the nanogels secondary structure a literature method has been used,[20] which suggested that RADA16-Soy1 has 84% of β-sheet structures, whereas RADA16-LPYP 78%. Thus, the CD spectra are in accordance with ThT binding assay. Furthermore, the β-sheet structural arrangements of RADA16-Soy and RADA16-LPYP were also supported by the ATR-FTIR spectroscopy (Figure 4F), which displayed two peaks at ~1630 cm^{-1} and ~1695 cm^{-1} (Amide I region), and one peak centered around 1530 cm^{-1} (Amide II region) typically associated with β-sheet signatures.

To investigate the capability of Soy1 and LPYP based nanogels to modulate the ACE activity, *in situ* experiments were carried out on human intestinal Caco-2 cells (Figure 5A-D). Briefly, a total of $5x10^4$/well Caco-2 cells were seeded directly on top of the coating-nanogels in which Soy1 and LPYP peptides had been entangled at the concentration of 1.0 µM. Cells were cultured for 6 days in order to evaluate the ability of both soybean peptide-based nanogels to act as cell culture coating. As shown in Figure 5A, Caco-2 cells were able to grow on top of both coating-nanogels without significant morphological variation compared to Caco-2 cells, which grew on top of the RADA16 hydrogel alone. Indeed, as MTT results clearly suggested, no cytotoxicity effects were observed even after 6 days of cell culture (Figure 5B). After 6 days, Caco-2 cells are in a proliferative stage, they reach confluence and even though, they are not fully differentiated in mature enterocytes, they express enough amount of active membrane peptidases, i.e. DPP-IV and ACE.[38] For this reason, this cellular system, which has already been used to monitor the in situ activity of DPP-IV, can be also utilized to evaluate the ACE activity.[39, 40] Based on these results, the kinetic of each peptide release was assessed using a literature method, which is based on chelating the peptide bonds by Cu (II) in alkaline media and monitoring the change of absorbance at 330 nm.[41] Using this method, it was demonstrated that both peptides are released by the coating-hydrogel as a function of time with a different behavior. In details, released Soy1 concentrations are 0.13±0.04, 0.27±0.003, and 0.51±0.08 µg $µL^{-1}$, whereas released LPYP peptide concentrations are 0.46±0.04, 0.84±0.15, 1.22±0.16 µg $µL^{-1}$, respectively, after 60, 180, and 360 min of incubation in PBS (Figure 5C). LPYP peptide is faster released than Soy1 probably because it is less hydrophobic (LPYP hydrophobicity is equal to 6.22 Kcal mol^{-1}, and that of Soy 1 is equal to 8.40 Kcal mol^{-1}). This explains why LPYP may more easily leak from the entangled nanofibrous domains of the hydrogels than Soy1.

Further, the ability of both coating-nanogels to inhibit the ACE activity was evaluated *in situ* on human intestinal Caco-2 cells. Findings clearly underline that both RADA16-Soy1 and RADA16-LPYP maintain their ability to reduce the enzyme activity by 40% and 60%, respectively (Figure 5D). In detail, when Soy1 and LPYP peptides are entrapped in the coating-nanogels at the concentration

of 1 μM, they drop the ACE activity by 34.3±7.6% and 52.2±3.8%, respectively, suggesting a clear improvement of their inhibitory activity. These results are in agreement with the relative activity (data not shown) of each peptide, i.e. LPYP is more active than Soy1. Overall, these results clearly support our hypothesis of developing a suitable smart delivery coating-strategy for the harmless control of ACE inhibitory peptides as a new approach for improving their activity and stability.

Abbreviations

ACE, angiotensin converting enzyme; **Akt,** protein kinase B**; AMC**, amido-4-methylcoumarin hydrobromide; **AMPK**, adenosine monophosphate-activated protein kinase; **BB**, brush border; **CD**, circular dichroism; **DMEM**, Dulbecco's modified Eagle's medium; **DPP-IV**, dipeptidyl peptidase-IV; **FBS**, fetal bovine serum; **FT-IR**, Fourier transform infrared spectroscopy; **GLUT1**, glucose transporter type 1; **GLUT4**, glucose transporter type 4; **GS**, glycogen synthase; **GSK3**, glycogen synthase kinase-3β, **HMGCoAR**, 3-hydroxymethylglutaryl coenzyme A reductase; **PBS**, phosphate buffered saline; **RAS**, renin-angiotensin system; **RFU**, relative fluorescence unit; **RT**, room temperature; **SAPs,** self-assembling peptides; **SHR,** spontaneous hypertensive rats; **TFA**, trifluoroacetic acid; **ThT**, Thioflavin T; **TIS**, triisopropylsilane;

Author Contributions

C.L. conceived the project and designed the experiments. C.L. and C.B. took care all *in situ* and release tests, L.D. performed in silico study, while R.P. and F.G synthesized the RADA16 peptide and carried out all structural and biomechanical experiments. C.L., A.A, G.G., L.D, and R.P wrote the manuscript. All authors critically reviewed the paper and have approved the final article.

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356 **Supporting Information.** Supporting information provides a more detailed description of the
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468

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473 cakes by novel techniques to receive new products and to increase the yield”.

474

475 **FIGURE CAPTIONS**

476 **Figure 1. *In vitro* gastrointestinal digestion**. LPYP (A) and Soy1 (B) were co-digested with pepsin for 90
477 min and pancreatin for 150 min. After digestion, LPYP and Soy1 (D) were degraded by only 28.5±1.4% and
478 27.7±0.3%, respectively vs undigested peptide (UD). Data represent the mean±s.d. of three independent
479 experiments performed in triplicate

480

481 **Figure 2. *In situ* evaluation of the ACE activity**. Soy1 and LPYP reduce *in situ* the ACE activity with a
482 dose-response trend (A) in non-differentiated human Caco-2 cells (IC_{50} values equal to 14.7±0.28 and
483 5.0±0.28 μM, respectively) and (B) in renal HK-2 cells (IC_{50} values equal to 6.0±0.35 and 6.8±0.20 μM,
484 respectively). Data represent the mean±s.d. of three independent experiments performed in triplicate.

485

486 **Figure 3**. **Molecular modeling results**. Protein is represented in cartoon, while peptides and residues involved
487 in polar interactions are represented in sticks. Spheres represent Zn ions. Grey, red, and blue meshes indicate
488 regions sterically and energetically favorable to receive hydrophobic, hydrogen bond acceptor, and hydrogen
489 bond donor groups, respectively. Polar interactions are indicated by yellow dotted lines. The red and black
490 circles indicate hydrophobic-polar and acid-acid interferences. (A) LPYP within C-domain. (B) Soy1 within
491 C-domain. (C) LPYP within N-domain. (D) Soy1 within N-domain. (E) Superimposition of IAVP (yellow) to
492 LPYP (purple) within C-domain. (F) Superimposition of IAVP (yellow) to LPYP (purple) within N-domain.
493 **(**G) RMSD plots of LPYP within the catalytic site of C- and N-domain of ACE. (H) Time-step representation
494 of LPYP trajectories within the N- and C-domain of ACE. The from-red-to-blue color switch indicates the
495 stepwise changes of ligand coordinates over time (50 nanoseconds).

496

497 **Figure 4. Characterization of SAP-nanogels.** A) Cartoon representation and B) chemical structures of
498 RADA16, RADA16-Soy 1 and RADA16-LPYP nanogels. C) Biomechanical characterization of RADA16-
499 Soy1 and RADA16-LPYP nanogels via a frequency sweep test (0.1−100 Hz, 1% strain). D) ThT emission
500 spectra of RADA16-Soy1 and RADA16-LPYP nanogels: their affinity for ThT may be ascribable to the
501 presence of cross-β fibril structures. E) CD spectrum of RADA16-Soy1 and RADA16-LPYP in solution
502 showing the presence of β-sheet assemblies. F) FTIR analysis of RADA16-Soy and RADA16-LPYP with

peaks at ~1630 cm^{-1} and ~1695 cm^{-1} (amide I region) and 1530 cm^{-1} (amide II region) typically associated with β-sheet signatures.

Figure 5. Biological characterization of the nanogels. Photographs of the Caco-2 cells grown on top of RADA, RADA-Soy1, and RADA-LPYP hydrogels for 6 days (A); cell viability tests performed by MTT assay (B); kinetic of peptide release as a function of the time (C); evaluation of the *in situ* ACE-inhibitory effects on human intestinal Caco-2 cells (D). Data represent the mean±s.d. of three independent experiments performed in triplicate; **** $p<0.0001$.

FIGURES

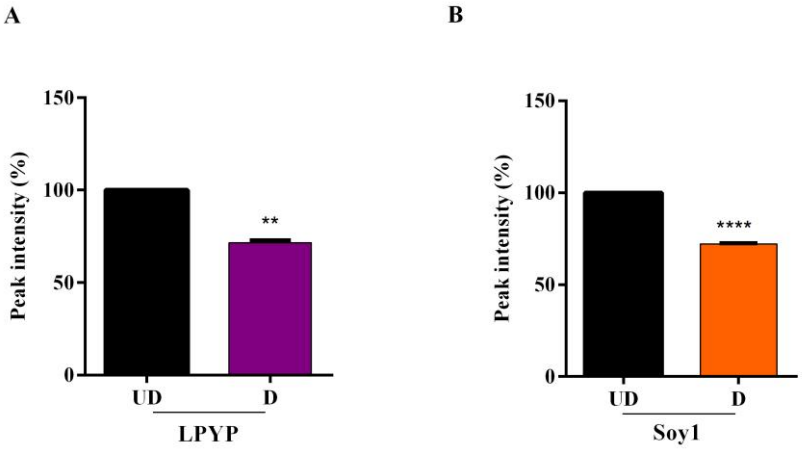


Figure 1

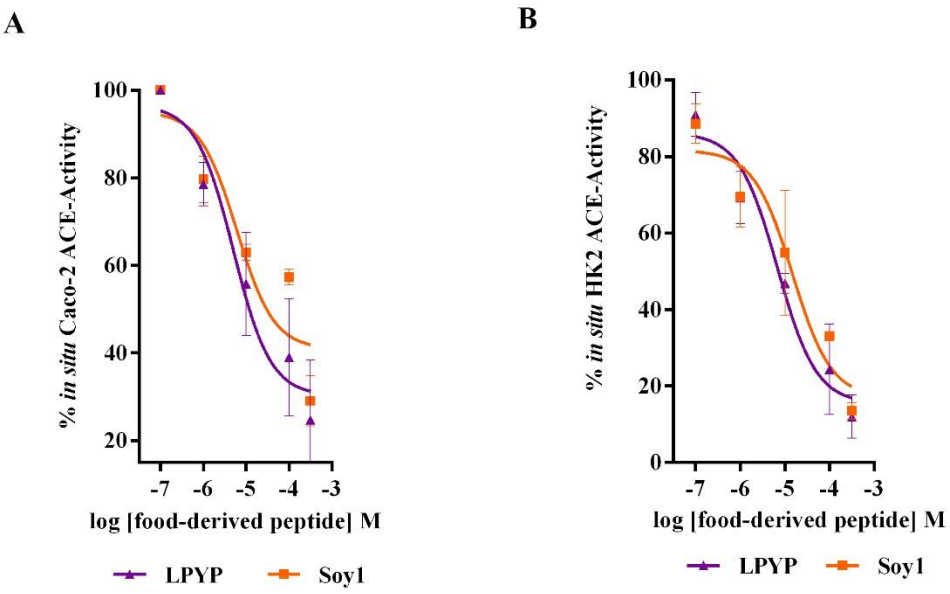

A
B
100
80
60
40
20
0
% in situ Caco-2 ACE-Activity
% in situ HK2 ACE-Activity
-7
-6
-5
-4
-3
log [food-derived peptide] M
LPYP
Soy1

Figure 2

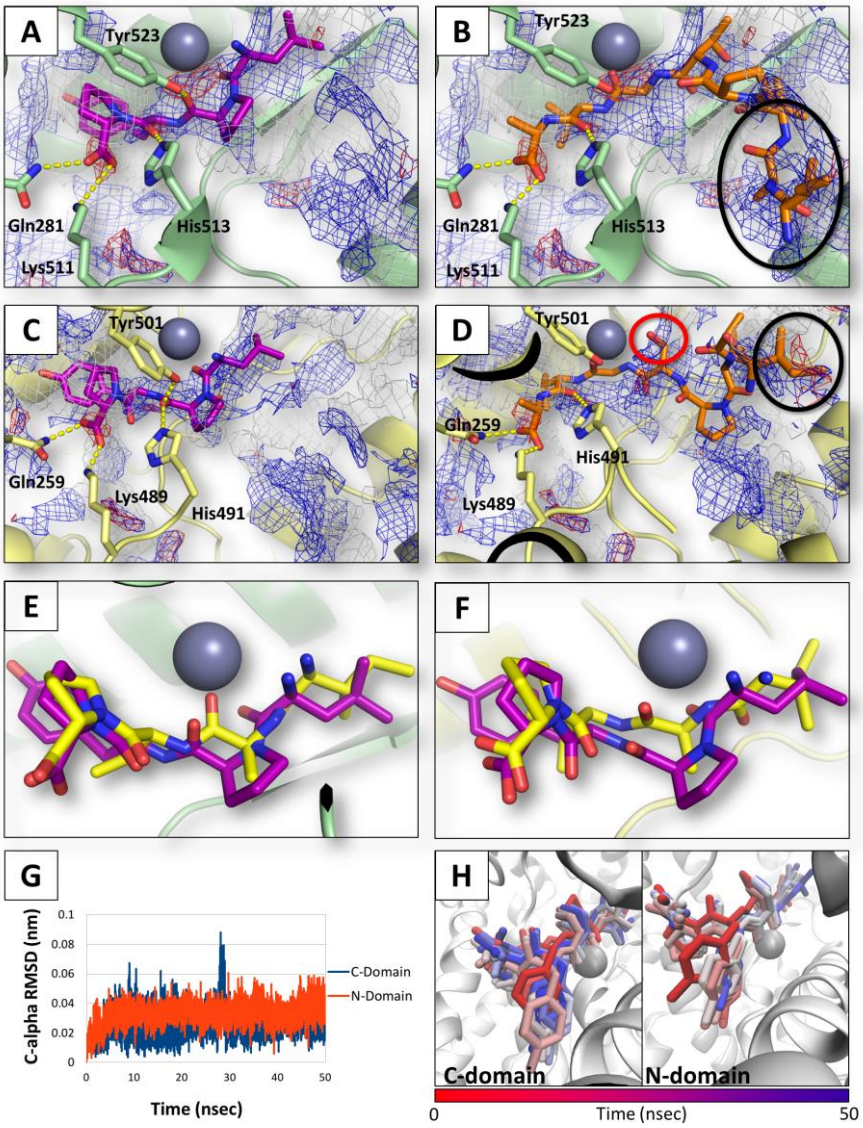

A
Tyr523
Gln281
His513
Lys511
B
Tyr523
Gln281
His513
Lys511
C
Tyr501
Gln259
Lys489
His491
D
Tyr501
Gln259
His491
Lys489
E
F
G
C-alpha RMSD (nm)
0.1
0.08
0.06
0.04
0.02
0
0
10
20
30
40
50
Time (nsec)
C-Domain
N-Domain
H
C-domain
N-domain
0
Time (nsec)
50

Figure 3

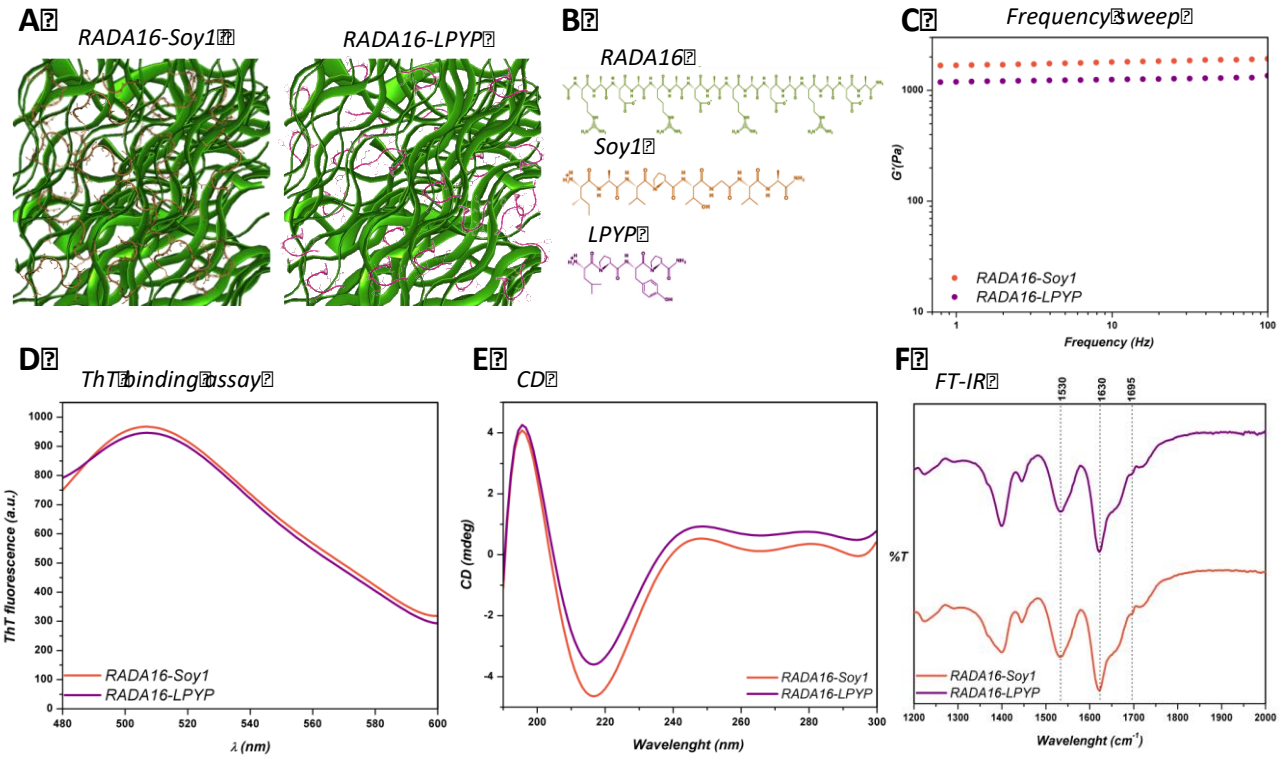

A
RADA16-Soy1
RADA16-LPYP
B
RADA16
Soy1
LPYP
C
Frequency sweep
G'(Pa)
Frequency (Hz)
RADA16-Soy1
RADA16-LPYP
D
ThT binding assay
ThT fluorescence (a.u.)
λ (nm)
RADA16-Soy1
RADA16-LPYP
E
CD
CD (mdeg)
Wavelenght (nm)
RADA16-Soy1
RADA16-LPYP
F
FT-IR
1530
1630
1695
%T
Wavelenght (cm-1)
RADA16-Soy1
RADA16-LPYP

Figure 4

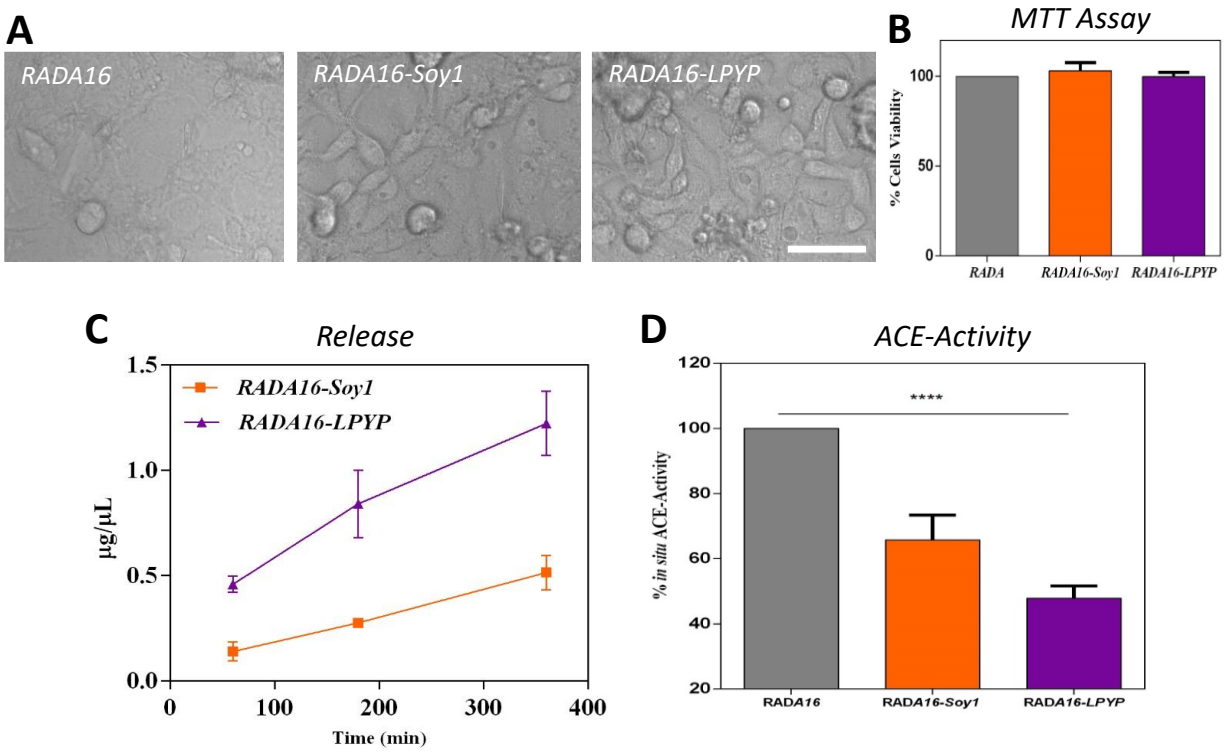
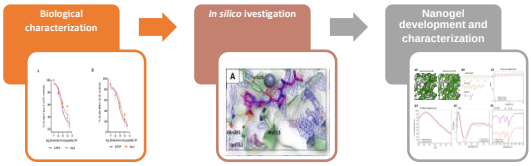

A
RADA16
RADA16-Soy1
RADA16-LPYP
B
MTT Assay
% Cells Viability
100
50
0
RADA
RADA16-Soy1
RADA16-LPYP
C
Release
RADA16-Soy1
RADA16-LPYP
µg/µL
1.5
1.0
0.5
0.0
0
100
200
300
400
Time (min)
D
ACE-Activity
% in situ ACE-Activity
120
100
80
60
40
20
****
RADA16
RADA16-Soy1
RADA16-LPYP

Figure 5


Biological characterization
In silico ivestigation
Nanogel development and characterization

TOC