1 **INCORPORATION OF LIPOSOMES CONTAINING SQUID TUNIC ACE-**
2 **INHIBITORY PEPTIDES TO FISH GELATIN**

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10 ** This centre has implemented a Quality Management System which fulfils the requirements
11 of the ISO standard 9001:2000.

13 **Running Title:** Liposomes containing squid tunic ACE-inhibitory peptides added to fish
14 gelatin

ABSTRACT

BACKGROUND: hydrolysates from collagen of the jumbo squid *Dosidicus gigas* tunics have shown excellent angiotensin I converting enzyme (ACE) inhibitory activity, however, peptides directly included in food systems may suffer a decrease in activity, which could be minimized by loading them into nanoliposomes.

RESULTS: a fraction of peptides with molecular weights <1 kDa, obtained from hydrolyzed squid tunics, with reasonably high ACE-inhibitory activity (half maximal inhibitory concentration $IC_{50} = 0.096 \text{ g}\cdot\text{L}^{-1}$), was encapsulated in phosphatidylcholine nanoliposomes. The peptide concentration affected the encapsulation efficiency and the stability of the resulting liposomes, which remained with a high zeta potential value (-54.3 mV) at least for one week at the most convenient peptide concentration. The optimal peptide concentration was established at $1.75 \text{ g}\cdot\text{L}^{-1}$. Liposomes obtained with this peptide concentration showed an encapsulation efficiency of 53 %, a zeta potential of -59 mV, an average diameter of 70.3 nm, and proved to be stable in a pH range between 3 and 7 at 4°C .

CONCLUSION: liposomes containing ACE-inhibitory peptides were incorporated in fish gelatin without detriment to the rheological properties and thermal stability of the resulting cold-induced gel. The ACE-inhibitory activity of the peptide fraction, which was not affected by the encapsulation process, conferred the bioactive potential to the nanoliposome-containing gelatin gel.

KEYWORDS: Peptides; Squid collagen; ACE-inhibitory activity; Nanoliposomes; Fish gelatin

40 INTRODUCTION

41 Liposomes are colloidal vesicular structures composed of one or more lipid bilayers that are
42 used as carriers of drugs, peptides, proteins, plasmid DNA, antisense oligonucleotides or
43 ribosomes, for pharmaceutical, cosmetic, and biochemical purposes ¹. The phospholipid-based
44 liposomes have the ability to act as carriers for both lipophilic and hydrophilic drugs and have
45 been used as a potential drug delivery system in tumor targeting, gene and antisense therapy,
46 genetic vaccination, immunomodulation, and for topical products ^{2,3}. In the field of food
47 industry, liposomes may offer a potential solution to enhance the efficacy and stability of
48 bioactive substances such as proteins, enzymes, vitamins, antioxidants, carotenoids, minerals
49 and flavors which could be included in the formulation of functional foods ⁴⁻⁷.

50 Gelatin is a natural polymer widely used in the industry in a broad variety of products, and
51 commonly used for pharmaceutical and medical applications because of its biodegradability
52 and biocompatibility in physiological environments ⁸. Gelatin itself may be considered a
53 highly-digestible dietary food, ideal as a complement in certain types of diet. Most
54 commercial gelatins are derived from mammalian sources, mainly pigskin and cowhide, but
55 due to socio-cultural and health-related concerns, marine gelatins are increasingly demanded
56 ⁹. For technological purposes, gelatin can be used as an ingredient to improve the elasticity,
57 consistency and stability of foods, and therefore its functionality depends largely on the
58 rheological properties ¹⁰. Upon cooling, gelatin chains in solution give rise to the formation of
59 networks, result of the ability of gelatin molecules to re-form the native collagen structure ¹¹.
60 The extent of cross-links and hydrogen bonds formed greatly influences both the gel strength
61 and the thermal stability, which are the main quality indicators of a commercial gelatin ¹².
62 Nevertheless, the gel properties may be affected by the presence of other components, such as

63 low molecular weight compounds or other food biopolymers that can interact with gelatin
64 chains ¹³.

65 The use of lipids for reinforcement of gel systems has been reported for different protein
66 matrices suggesting that, in non-interactive systems, lipid droplets might fill pores in the gel
67 matrix without actively interacting with the protein-based network ¹⁴. There is evidence that it
68 is possible to incorporate liposome-loaded bioactive compounds into the pores of the gel
69 matrix in gelatin systems improving their release over prolonged periods of time ². However,
70 scarce information is available regarding the effect of liposomes on the rheological properties
71 of gelatin gels ¹⁵, especially when focused as a functional food with potential bioactive
72 properties.

73 Antihypertensive peptides are peptide molecules which may lower blood pressure when
74 ingested through inhibition of vasoactive enzymes such as the angiotensin converting enzyme
75 (ACE) ¹⁶. Synthetic ACE-inhibitors, such as Captopril, Enalapril, etc. are being used
76 extensively in the treatment of hypertension and heart failure in humans. However, these
77 compounds are believed to produce certain side effects such as coughing, taste disturbances,
78 skin rashes or angioneurotic edema ¹⁷. Therefore, much work has concentrated on finding
79 natural sources of ACE-inhibitors, such as food proteins, which though less potent than the
80 synthetic ones are without known side effects. Reasonable potent ACE-inhibitory
81 hydrolysates and peptides have been obtained from diverse collagenous materials from marine
82 origin ¹². Specifically, hydrolysates from collagen of the jumbo squid *Dosidicus gigas* tunics
83 have shown excellent antioxidant and ACE-inhibitory activities ^{18,19}. *Dosidicus gigas* is the
84 largest ommastrephid squid and it is endemic to the eastern Pacific ²⁰. The big size of the
85 mantle together with the fact that it is usually marketed as cleaned and peeled mantle, imply
86 the generation of large amounts of collagenous wastes, which after convenient and eco-

friendly enzymatic hydrolysis, could yield high value-added products in the form of bioactive peptides, with promising health benefits for nutritional or pharmaceutical applications. However, within the organism, a large number of peptides are degraded before reaching its target ²¹. Liposome entrapment represents a way to stabilize bioactive compounds against a range of environmental and chemical agents. Peptides directly included in food systems may suffer a decrease in activity as a result of proteolytic degradation or interactions with food components, which could be minimized by loading them into nanoliposomes ²². An important area of functional food development concerns the encapsulation of bioactive compounds in liposomes to protect them from a range of environmental, enzymatic and chemical changes that may lead to the loss of their bioactivity. Gelatin is considered as a model system for the incorporation of peptide-containing liposomes in this study, since it is widely used in the food industry, both as a final product in a wide variety of desserts and also as an ingredient in processed food due to its well-known binding properties or hydrogel formation.

The aims of this work were: (i) to encapsulate a low molecular weight peptide fraction (< 1 kDa) with ACE-inhibitory activity from jumbo squid tunics into phosphatidilcoline nanovesicles at the most suitable concentration, and (ii) to study the gelling behavior and potential antihypertensive activity of a gelatin gel incorporated with the peptide-containing liposomes.

MATERIALS AND METHODS

Materials

Frozen tunics of jumbo squid (*Dosidicus gigas*) were provided by PSK Océanos, S.A. (Vigo, Spain), Angiotensin-I converting enzyme (ACE; EC 3.4.15.1, 1 U), hippuryl-L-histidyl-L-Leucine (HHL), and hippuric acid (HA) were purchased from Sigma-Aldrich (St. Louis, MO,

110 USA). Esperase (EC 232.752.2, 8.0 L) was supplied by Novozymes (Bagsvaerd, Denmark).
111 NaOH was obtained from Panreac (Madrid, Spain). Crude soybean lecithin was provided by
112 Solae S.A. (Esteio, Brazil). A commercial warm water fish gelatin was purchased from Lapi
113 Gelatin S.P.A. (Empoli, Florence, Italy).

114 **Enzymatic hydrolysis**

115 Lyophilized squid tunics were subjected to hydrolysis with Esperase using a pH-stat (TIM
116 856, Radiometer analytical, Villeurbanne, France), with an enzyme-substrate ratio of 1:100
117 (w:w) at the optimal conditions for enzymatic activity (pH 8, 60 °C) for 3h. The pH of the
118 reaction was kept constant by continuous adding of a 0.1 N NaOH solution to the reaction
119 medium. Afterwards, the enzymes were inactivated by heating the mixture at 90 °C for 10
120 min.

121 **Ultrafiltration**

122 The hydrolysate obtained from squid tunics was fractionated by ultrafiltration with molecular
123 weight cut-off (MWCO) membranes of 1 kDa (Omega 1 kD Centramate™ cassette medium
124 screen channel, Pall Corporation, Madrid, Spain). The resulting permeate was freeze-dried
125 and stored at -80 °C until analysis (maximum one week). The peptide fraction obtained was
126 designated as P1.

127 **Purification of crude soybean lecithin**

128 The crude soybean lecithin (10 g) was dissolved in 50 mL of ethyl acetate (99.8 %; Merck,
129 Darmstadt, Germany). Then, distilled water (2 mL) was added slowly and under agitation,
130 resulting in the formation of two phases. The upper phase was separated from the lower phase
131 and discarded. The lower phase was washed with 30 ml of acetone (99.9 %) twice. The

precipitate was vacuum-filtered and dried in a desiccator. The product obtained was partially purified phosphatidylcholine (PC).

Liposome production

The encapsulation of the peptide fraction was carried out following the film hydration method, as reported previously²³. Phosphatidylcholine (0.15 g) was dissolved in 10 mL of chloroform (99 %) in a round-bottom flask and the organic solvent was removed by rotary evaporator until a thin film was formed on the walls. Traces of organic solvents were removed by storage in desiccator under vacuum for 18 h. P1 was dissolved in phosphate buffer (10 mM, pH 7) at varying concentrations, and 5 mL were added to disperse the resulting dried lipid film. Six concentrations of P1 between 0.5 and 3 g·L⁻¹ were tested, taking into account the zeta potential and the entrapment efficiency of the encapsulated product to determine the optimum peptide concentration. These mixtures were vortexed above their phase transition temperature (60 °C) to produce multilamellar lipid vesicles (MLVs). Sonication of the preparation (in order to reduce the size and homogenize liposomes) was carried out in an ultrasonic cell disrupter (Model Q700, Qsonica sonicators, Newton, CT, USA) with a power rating of 700 W (100 % amplitude), where the MLV were exposed to five cycles of sonication for 1 min, followed by 1 min stopped to allow sample cooling. The peptide fraction encapsulated in the nanovesicles at the selected concentration of 1.75 g·L⁻¹ was designated as LP1. The peptide-loaded liposome suspension (LP1) was filtered through 0.22 µm membrane (Millex-GP Syringe Filter Unit, Millipore, USA).

Entrapment efficiency

Sephadex filtration method was performed to separate free peptides (P1) from encapsulated peptides (LP1). A glass-column (350 mm high x 7 mm diameter) was packed with 8 mL of

drained resin Sephadex® G-100 (Pharmacia Biotech, Uppsala, Sweden). The column was eluted with 10 mM phosphate buffer pH 7.0 as mobile phase. Fractions of 0.5 mL were collected for measurement. Prior to the start of the test, 10 ml of a suspension with empty liposomes (without peptides) was passed through the column to avoid liposome disruption during further elution. Entrapment efficiency (EE) was evaluated by determining the amount of protein in each one of the collected fractions by the Lowry method ²⁴. Data were analyzed with Origin 8SR0 data analysis software version 8.0724 (OriginLab Corporation, Northampton, MA 01060 USA). Data obtained were plotted and areas of curves representing P1 and LP1 concentration were determined (output timing of each of them were determined in a previous analysis). The EE was calculated using the following equation:

$$EE = (LP1 \text{ area} / (\text{free P1 area} + LP1 \text{ area})) \times 100.$$

Characterization of nanovesicles

Liposome size and polydispersity index (PDI) were determined by Dynamic Light Scattering (DLS) in a Zetasizer nano-ZS (Malvern Instruments Ltd, Worcestershire, UK), using Laser Doppler Electrophoresis (LDE). Average diameter was provided as z-average. Both measurements were carried out after dilution of the formulations in 10 mM phosphate buffer, to avoid particle aggregation. To determine the liposomal stability at pH 7 as a function of time, DLS measurements were performed on the newly prepared liposomes (t0) and after one week of storage at 4 °C (t1).

Stability of liposomes at different pH

The liposomal solution (10 µL) was diluted into 10 mL of different buffers and stored at 4 ± 0.5 °C. Hydrochloric acid-potassium chloride buffer 0.05 M was used at pH 1 and 2; citrate-phosphate buffer 0.05 M was used at pH 3, 4, 5 and 6; phosphate buffer 0.05 M was used at

178 pH 7 and 8. After 24 h of incubation with the different buffers, the zeta-potential was
179 measured as described above.

180 **Incorporation of nanovesicles in gelatin**

181 Fish gelatin was dissolved at 40°C at 66.7 g·L⁻¹ in phosphate buffer (10 mM, pH 7)
182 containing free (P1) or encapsulated peptide fraction (LP1) at increasing concentrations
183 ranging from 0.175 to 1.75 g·L⁻¹. Thus, P1 or LP1 concentrations (g·kg⁻¹ dry gelatin) were a)
184 2.62 g·kg⁻¹, b) 6.56 g·kg⁻¹, c) 13.12 g·kg⁻¹ and d) 26.24 g·kg⁻¹. The same gelatin solution
185 without added peptides (P1 or LP1) was used as control (G).

186 **Dynamic oscillatory study**

187 Viscoelastic properties were performed on a Bohlin CRS-10 controlled stress rheometer
188 rotary viscometer (Bohlin Instruments Ltd, Gloucestershire, UK) using a cone-plate geometry
189 (cone angle 4 °, gap = 0.15 mm). A dynamic frequency sweep was done at 4 °C by applying
190 an oscillation amplitude within the linear region (strain $\gamma = 0.005$) over the frequency range
191 0.1-10 Hz. The elastic modulus (G' ; Pa) and viscous modulus (G'' , Pa) were plotted as a
192 function of angular frequency. To characterize the frequency dependence of G' over the
193 limited frequency range, the power law²⁵ was used:

$$194 \quad G' = G_0' \omega^n$$

195 where G_0' is the energy stored and recovered per cycle of sinusoidal shear deformation at an
196 angular frequency of 1 Hz, ω is the angular frequency and n is the power law exponent that
197 should be near-zero for gels exhibiting an ideal elastic behavior²⁶.

198 The dynamic temperature sweep was done by cooling and heating from 5 to 40 °C and back to
199 40 °C, at a scan rate of 1 °C·min⁻¹, frequency of 0.5 Hz and oscillating target strain $\gamma = 0.005$.
200 The elastic modulus (G' , Pa) was plotted as a function of temperature.

201 **ACE-inhibitory activity**

202 Reverse-phase high performance liquid chromatography (RP-HPLC) was used to determine
203 the ACE-inhibition capacity of the non-encapsulated (P1) and encapsulated (LP1) peptide
204 fraction, according to the method described by Alemán et al ²⁷. The total reaction volume was
205 230 μ L, made up of 50 μ L of 5 mM HHL (Hippuryl-L-Histidyl-L-Leucine), 160 μ L of 0.025
206 U/mL of ACE and 20 μ L of sample at different concentration (all prepared with 100 mM
207 potassium-phosphate buffer, containing 300 mM NaCl, pH 8.3). The mix was incubated at 37
208 °C for 2 h in a water bath with continuous agitation at 160 shakes·min⁻¹ (OSL 200, Grant
209 Instruments Ltd., Cambridge, England) and the reaction was quenched by addition of 100 μ L
210 0.1 M HCl. The released hippuric acid (HA) was quantified by RP-HPLC (model SPE-
211 MA10AVP, Shimadzu, Kyoto, Japan) on a C18 column (Tracel excel, 120 ODSA 5 μ m,
212 Teknokroma, Barcelona, Spain). The injection volume was 50 μ L and the flow rate 0.8
213 mL·min⁻¹ using an acetonitrile gradient from 20 to 60% in 1 g·L⁻¹ trifluoroacetic acid (TFA)
214 (v/v) for 26 min. The HA and HHL were monitored at 228 nm and eluted at 8.30 and 15.70
215 min, respectively. The IC₅₀ value was defined as the concentration of peptide fraction (g·L⁻¹)
216 required to reduce the HA peak by 50 % (indicating 50 % inhibition of ACE activity). The
217 IC₅₀ of a synthetic ACE inhibitor, Enalapril©, was also determined for comparative purposes.
218 All determinations were carried out at least in duplicate.

219 **Statistical analysis**

Statistical analysis was performed using a STATGRAPHICS Centurion XV version 15.2.06 program (StatPoin, Inc. Warrenton, VA, USA). One-way analysis of variance (ANOVA) was performed, and Tukey HSD test was used for determining statistical differences. The level of significance of the difference was set at $p \leq 0.05$. In the case of the zeta-potential measurement at different peptide concentrations and measurement times (newly produced liposomes $t0$ and liposomes after one week at 4°C storage $t1$), two-way ANOVA was performed. All experiments were run in triplicate ($n=3$).

RESULTS AND DISCUSSION

Encapsulation of peptides

Concentrations between 0.5 and 3 g·L⁻¹ of the < 1 kDa peptide fraction (P1), obtained from the squid tunics after hydrolysis with Esperase²⁷, were tested for encapsulation into partially purified soybean phosphatidylcholine (PC) vesicles. The entrapment efficiency (EE) of P1 in liposomes at pH 7 is shown in Figure 1. Values of EE between 44.0 % for a peptide concentration of 3 g·L⁻¹ and 52.5 % for 2 g·L⁻¹ were obtained, which contradicts the idea that increasing concentrations of peptides may induce greater encapsulation efficiency. Beyond a certain peptide concentration liposomes are likely saturated and do not support an increased load. Entrapment around 50 % represents an intermediate value among the different EE reported in the literature for PC-based liposomes encapsulating peptides, being possible to find values from < 20 % to > 90 %²⁸⁻³¹. The EE greatly depends on the lipid composition and concentration, the preparation method, the chemical properties of peptides to be encapsulated, as well as on the electrostatic and hydrophobic interactions between phospholipids and peptides^{32,33}. The peptide fraction P1 has around 53 % of hydrophobic amino acid residues³⁴,

indicating that a considerable part of peptides is located in the inner aqueous phase of the liposome, while the other, slightly more abundant, will be locked within the lipid bilayers mainly through hydrophobic interactions^{35,36}.

The zeta potential of the newly produced liposomes (t0) was hardly modified as a function of the peptide concentration, with values ranging from - 45 to - 51 mV (Figure 2).

In order to monitor the stability of liposomes over time, the zeta potential analysis was repeated after one week at 4 °C (t1). In this case, the stability was significantly higher ($p \leq 0.05$) at 1.5 and 2 g·L⁻¹, without significant differences between them ($p > 0.05$). The rest of the peptide concentrations tested showed a significant ($p \leq 0.05$) loss of liposome stability with time (Figure 2), which would decrease the aptitude of peptide-loaded liposomes to be included in the formulation of a functional food. The zeta potential is a measure of the liposomal surface charges and it is used as an index of particle stability³⁷. When the zeta potential is higher than 30 mV or lower than -30 mV, it is possible to obtain a physically stable nanosuspension stabilized only by electrostatic repulsions³⁸. All the treatments resulted in highly stable liposomes at t0, but only the vesicles with P1 concentrations of 1.5 and 2 g·L⁻¹ maintained the stability at the highest level one week later. This indicates that these concentrations were the most appropriate to obtain very stable liposomes containing the peptide fraction. In a previous work, PC nanovesicles entrapping a peptide fraction from sea bream scales showed zeta potential of -40.8 mV²³. Similarly, the zeta potential of nisin-loaded liposomes prepared with the same lipid used in this study was of -52.3 mV³¹. Surface charge of liposomes depends greatly on phospholipid composition. PC liposomes normally show properties of uncharged or slightly anionic monolayers at near neutral pH values. For instance, empty PC vesicles have been reported to have zeta potential values of -2.3 mV³⁹ or -8.3 mV⁴⁰. The

268 liposomes prepared in the present study without peptide entrapment showed zeta
269 potential of -44.37 mV at pH 7, which strongly suggests that the partially purified PC
270 used may contain other lipids or impurities that may influence the membrane charge ³⁰.
271 Indeed, in agreement with Taylor et al. ⁴⁰, the liposomal surface charge changed little
272 with the inclusion of the peptide fraction.

273 The diameter of the liposomes with increasing concentrations of peptides is shown in
274 Figure 3. Average diameters between 69 and 110 nm were obtained, depending on the
275 peptide concentration tested; however differences were found statistically not
276 significant ($p \leq 0.05$) in most cases. Therefore, no definite relationship between particle
277 size and peptide concentration could be established.

278 According to Taylor et al. ⁴⁰, the size of liposomes is not necessarily related to the
279 amount of encapsulated peptide. Average sizes of around 100 nm have been also
280 described for PC liposomes containing peptides of a considerably higher molecular
281 weight (~ 14 kDa lysozyme and ~ 3.5 kDa nisin) than the peptide fraction of the present
282 work (<1 kDa) ^{22,33}, which suggests that the molecular weight of peptides is not a key
283 factor in the size of liposomes.

284 There is evidence that the smaller the size of liposomes, the more efficient the
285 compound-delivery into the organism ^{41,42}, although the transport also depends on the
286 surface properties of the lipid used ²². The size of the liposomes depends largely on the
287 sonication conditions used and on the lipid species ^{43,44}. In this case, the sonication
288 treatment was optimized in a preliminary study and both the amplitude and duration of
289 sonication were the same in all cases.

From the EE and zeta potential results, it could be concluded that the most suitable peptide concentration for encapsulation in PC-liposomes was between 1.5 and 2 g·L⁻¹. In order to find a more accurate concentration, liposomes were produced using a peptide concentration of 1.75 g·L⁻¹. With this concentration, new nanovesicles (LP1) were obtained with EE of 53.2 ± 1.3 %, zeta potential of -58.8 ± 2.9 mV and average diameter of 70.3 ± 6.0 nm (Figure 4).

The particle size distribution was uni-modal, with a polydispersity index of 0.215 ± 0.021, which was very acceptable and similar to that previously reported for nisin-containing liposomes²². The peptide concentration of 1.75 g·L⁻¹ was then selected as the most appropriate for further experiments.

Effect of pH on liposome stability

The stability of liposomes in food systems can be affected by numerous factors; among them, the pH may play a relevant role. The pH stability of the encapsulated peptide fraction was determined by dissolving LP1 in different buffers between pH 1 and 8 for 24 hours at 4.0 ± 0.5 °C. Table 1 shows the zeta potential values after incubation at different pH values.

Liposomes at pH 1 precipitated immediately, indicating that the system became extremely unstable and formed large aggregates. Certain instability was also observed at both pH 2 and pH 8, showing zeta potential values around -22.5 mV. The nanovesicles proved to be stable at pH ranging from 3 to 7, which is more common in most food systems. This is a very important characteristic, as it indicates that the liposomes obtained have high capacity to retain the encapsulated material in a wide pH range. According to Taylor et al.⁴⁰, the possible chemical interaction of peptides with the phospholipid membrane may exert a stabilizing

effect on nanovesicles. The highest zeta potential was obtained at pH 7 (-54.3 mV), therefore this pH was considered the most suitable to ensure liposomal stability.

Incorporation of liposomes into gelatin

In order to evaluate the degree of interference caused by the addition of LP1 in the gelling behavior of gelatin, dynamic oscillatory studies were carried out to determine the viscoelastic properties of gelatin with increasing LP1 concentrations. For comparison purposes, gelatin solutions without added peptides (G) and with the non-encapsulated peptide fraction (P1) were also characterized. Despite the intrinsic pH of the gelatin solution was around 5, the gelatin system was adjusted to pH 7 in order to favor liposome stability. The G' and G'' frequency dependence of the gelatin solutions with encapsulated (LP1) and non-encapsulated (P1) peptides at 5°C, is shown in Figure 5.

All the samples showed a typical gel-like behavior after a brief cold maturation period, as denoted by $G' > G''$ values. The gelatins containing either LP1 or P1 showed slightly higher G' and G'' values throughout the frequency range than the gelatin without added peptides (G), being indicative of certain gel reinforcement. No definite variations were found between gelatin with LP1 and P1 up to peptide concentrations of $13.1 \text{ g}\cdot\text{kg}^{-1}$. However, at the highest concentration ($26.2 \text{ g}\cdot\text{kg}^{-1}$), a remarkable increase in G' values at frequencies $> 1\text{Hz}$ were clearly observable with the non-encapsulated peptides (P1). Moreover, this sample exhibited also noticeable higher G'' values than the others. All the samples showed G' values which successfully fitted the power law ($R^2 = 0.92\text{--}0.99$). The Power law exponent n' is related to structural stability and protein network conformation: the higher the n' values, the higher the instability of the matrix against frequency changes. For gels exhibiting an ideal elastic behavior n' should be near-zero. The n' value obtained for the gelatin solution without peptides was 0.12. Increasing concentrations of LP1 did not alter the frequency dependence of

the resulting gelatin gels, since n' values were 0.11 in all cases. Thus, the structural stability of the gel network was practically unaffected by the presence of the nanovesicles. Similarly, no remarkable changes in the gel stability were found when the non-encapsulated peptide fraction (P1) at moderate concentration (from 2.62 to 13.12 g·kg⁻¹) was included, as denoted by the corresponding n' values ranging between 0.10 and 0.11. However, P1 increased considerably the frequency dependence ($n' = 0.19$) at the highest concentration, indicating that the incorporation of the non-encapsulated peptides beyond a certain concentration might be detrimental to the gel stability, probably due to interactions with the gelatin polypeptide chains.

The gelling behavior of gelatin solutions with both LP1 and P1 at the highest peptide concentration (26.2 g·kg⁻¹) was determined as a function of temperature, by performing a cooling ramp from 40 to 5 °C and a subsequent heating ramp from 5 to 40 °C (Figure 6). The cold-induced increase in G' was more evident in the samples containing LP1 and P1 than in the gelatin without added peptides (G), being gelling and melting temperatures unaffected in any case. Despite peptide addition induced certain gel reinforcement, the highest increase was observed in the liposome-containing gelatin (LP1). In this connection, Thakur et al.⁴⁵ reported the well dispersed oil droplets in oil-in-water gelatin emulsions to cause an increase in gel strength. A slight increase in the tolerance of cross-linked gelatin gels to tensile stress by incorporating liposomes has been also reported^{46,47}. As in the present work, these authors observed that the presence of liposomes did not affect the gel thermal stability.

ACE-inhibitory activity

The concentration of P1 required to inhibit 50% of the ACE activity (IC_{50} value) was 0.096 ± 0.005 g·L⁻¹. This peptide fraction was more potent ACE-inhibitor than various previously reported squid gelatin hydrolysates, which showed IC_{50} values in the concentration range of

0.34-0.89 g·L⁻¹ ²⁷. The main difference with the study of Alemán et al. ²⁷ consists in that the dry tunics were directly subjected to enzymatic hydrolysis without a previous gelatin extraction. The high ACE-inhibitory activity showed by P1 may be related to the high content of hydrophobic amino acids (537 residues per 1000 residues) and proline residues (64 residues per 1000 residues). Specifically, Pro seems to be one of the most effective amino acids for increasing ACE inhibitory activity and has been identified in many of the naturally occurring ACE peptide inhibitors, especially those from collagenous sources ¹². It is worth noting that no inhibitory activity was found in the mixture of gelatin with the encapsulating material in the absence of squid tunic peptides. When the encapsulated peptide fraction (LP1) was incorporated into the gelatin solution, the IC₅₀ value, calculated per weight of peptides, was 0.0905 ± 0.0085 g·L⁻¹. Therefore, it was concluded that neither the encapsulation process nor the presence of gelatin significantly affected the ACE inhibitory activity of the peptide fraction (p > 0.05). Furthermore, the encapsulation in liposomes allowed the peptide fraction to preserve the biological activities in the gelatin gel. In order to evaluate the potential antihypertensive effect of the liposome-containing gelatin gel (at the highest peptide concentration tested), the IC₅₀ value was calculated considering the total amount of water and dry solids (gelatin, PC and P1) in the formulation, and it was found to be 50.8 ± 4.8 g·L⁻¹. Taking into account that the IC₅₀ of Enalapril© is 0.0027 g·L⁻¹ ²⁰ and that this drug is marketed in preparations of 2.5, 5, 10 and 20 mg, it would be needed one portion of around 100 g of gelatin gel with LP1 to emulate the 5 mg dose of Enalapril©.

CONCLUSIONS

A low molecular weight (<1 kDa) peptide fraction from squid tunics with high ACE-inhibitory activity was successfully encapsulated into PC-based nanoliposomes, which proved

to be stable in a pH range between 3 and 7. The nanoencapsulated peptide fraction was incorporated into a gelatin system without detriment to the rheological properties and thermal stability of cold-matured gels. The ACE-inhibitory activity of the peptide fraction was not affected by the encapsulation procedure and was maintained in the fish gelatin system, which could be offered as a nutraceutical gel dessert of marine origin.

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REFERENCES

1. Ulrich A S, Biophysical aspects of using liposomes as delivery vehicles. *Bioscience reports* **22**: 129-150 (2002).
2. Mufamadi M S, Pillay V, Choonara YE, Du Toit LC, Modi G, Naidoo D, Ndesendo VM, A review on composite liposomal technologies for specialized drug delivery *Journal of Drug Delivery* ID 939851 (2011).
3. Verma P, Ram A, Jha A, Mishra A, Thakur A, Phosphatidylcholine: a revolution in drug delivery technology. *International Journal of Pharma Science and Research* **1**: 1-12 (2010).
4. Mozafari MR, Khosravi-Darani K, Borazan GG, Cui J, Pardakhty A, Yurdugul S, Encapsulation of food ingredients using nanoliposome technology. *International Journal of Food Properties* **11**: 833-844 (2008).
5. Mozafari MR, Johnson C, Hatziantoniou S, Demetzos C, Nanoliposomes and their applications in food nanotechnology. *Journal of Liposome Research* **18**: 309-327 (2008).
6. Gibbs BF, Kermasha S, Alli I, Mulligan CN, Encapsulation in the food industry: A review. *International Journal of Food Sciences and Nutrition* **50**: 213-224 (1999).
7. Tan C, Xue J, Lou X, Abbas S, Guan Y, Feng B, Zhang X, Xia S Liposomes as delivery systems for carotenoids: Comparative studies of loading ability, storage stability and in vitro release. *Food and Function* **5**: 1232-1240 (2014).
8. Young S, Wong M, Tabata Y, Mikos AG, Gelatin as a delivery vehicle for the controlled release of bioactive molecules. *Journal of Controlled Release* **109**: 256-274 (2005).

9. Karim AA, Bhat R, Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. *Food Hydrocolloids* **23**: 563-576 (2009).
10. Stainsby G, Gelatin gels, In *Advances in Meat Research, Collagen as a Food*, AM Pearson, TR Dutson, AJ Bailey, Eds. Van Nostrand Reinhold Company Inc., New York, USA,; Volume 4, pp. 209-222 (1987).
11. Ledward DA, Gelation of gelatin, In *Functional Properties of Food Macromolecules*, JR Mitchell, DA Ledward, Eds., Elsevier Applied Science Publishers, London, UK, pp 171-201 (1986).
12. Gómez-Guillén MC, Giménez B, López-Caballero ME, Montero MP, Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids* **25**: 1813-1827 (2011).
13. Harris P, Normand V, Norton IT, Gelatin, In *Encyclopedia of Food Sciences and Nutrition*, 2nd ed; B Caballero, Ed. Academic Press, Oxford, UK, pp. 2865-2871 (2003).
14. Mor Y, Shoemaker CF, Rosenberg M, Compressive Properties of Whey Protein Composite Gels Containing Fractionated Milkfat. *Journal of Food Science* **64**: 1078-1083 (1999).
15. Sakai H, Sato A, Takeoka S, Tsuchida E, Rheological properties of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma-substitute solutions. *Langmuir* **23**: 8121-8128 (2007).
16. Chen Q, Xuan G, Fu M, He G, Wang W, Zhang H, Ruan H, Effect of angiotensin I-converting enzyme inhibitory peptide from rice dregs protein on antihypertensive activity in spontaneously hypertensive rats. *Asian Pacific Journal of Clinical Nutrition* **16**(1): 281-285 (2007).
17. Atkinson AB, Robertson JIS, Captopril in the treatment of clinical hypertension and cardiac failure. *Lancet* **2**: 836-839 (1979).
18. Alemán A, Giménez B, Montero P, Gómez-Guillén MC, Antioxidant activity of several marine skin gelatins. *LWT - Food Science and Technology* **44**: 407-413 (2011).
19. Alemán A, Giménez B, Pérez-Santín E, Gómez-Guillén MC, Montero P, Contribution of Leu and Hyp residues to antioxidant and ACE-inhibitory activities of peptide sequences isolated from squid gelatin hydrolysate. *Food Chemistry* **125**: 334-341 (2011).
20. Markaida, U, Sosa-Nishizaki O, Reproductive biology of jumbo squid *Dosidicus gigas* in the Gulf of California, 1995-1997. *Fisheries Research* **54**. 63-82 (2001).
21. Palm C, Netzereab S, Hällbrink M, Quantitatively determined uptake of cell-penetrating peptides in non-mammalian cells with an evaluation of degradation and antimicrobial effects. *Peptides* **27**: 1710-1716 (2006).
22. Malheiros PDS, Sant'Anna V, Barbosa MDS, Brandelli A, Franco BDGDM, Effect of liposome-encapsulated nisin and bacteriocin-like substance P34 on *Listeria monocytogenes* growth in Minas frescal cheese. *International Journal of Food Microbiology* **156**: 272-277 (2012).

23. Mosquera M, Giménez B, Mallmann da Silva I, Ferreira Boelter J, Montero P, Gómez-Guillén MC, Brandelli A. Nanoencapsulation of an active peptidic fraction from sea bream scales collagen. *Food Chemistry* **156**: 144-150 (2014).
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry* **193**: 265-275 (1951).
25. Friedrich C, Heymann L. Extension of a model for crosslinking polymer at the gel point. *Journal of Rheology* **32**: 235-241 (1988).
26. Zhou N, Mulvaney SJ. The Effect of Milk Fat, the Ratio of Casein to Water, and Temperature on the Viscoelastic Properties of Rennet Casein Gels. *Journal of Dairy Science* **81**: 2561-2571 (1998).
27. Alemán A, Pérez-Santín E, Bordenave-Juchereau S, Arnaudin I, Gómez-Guillén M C, Montero P. Squid gelatin hydrolysates with antihypertensive, anticancer and antioxidant activity. *Food Research International*, **44**: 1044-1051 (2011).
28. Mishra D, Mishra P K, Dubey V, Nahar M, Dabadghao S, Jain N K. Systemic and mucosal immune response induced by transcutaneous immunization using Hepatitis B surface antigen-loaded modified liposomes. *European Journal of Pharmaceutical Sciences* **33**: 424-433 (2008).
29. Vyas S P, Singh R P, Jain S, Mishra V, Mahor S, Singh P, Gupta P N, Rawat A, Dubey P. Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B. *International Journal of Pharmaceutics* **296**: 80-86 (2005).
30. Malheiros PDS, Sant'Anna V, Micheletto YMS, Da Silveira NP, Brandelli A. Nanovesicle encapsulation of antimicrobial peptide P34: Physicochemical characterization and mode of action on *Listeria monocytogenes*. *Journal of Nanoparticle Research* **13**: 3545-3552 (2011).
31. Malheiros PDS, Micheletto YMS, Silveira NP, Brandelli A. Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin. *Food Research International* **43**: 1198-1203 (2010).
32. Riaz M. Liposomes preparation methods. *Pakistan Journal of Pharmaceutical Sciences* **19**: 65-77 (1996).
33. Were LM, Bruce BD, Davidson PM, Weiss J. Size, Stability, and Entrapment Efficiency of Phospholipid Nanocapsules Containing Polypeptide Antimicrobials. *Journal of Agricultural and Food Chemistry* **51**: 8073-8079 (2003).
34. Mosquera M, Giménez B, Ramos S, López-Caballero ME, Gómez-Guillén MC, Montero P. Antioxidant, ace-inhibitory and antimicrobial activities of peptide fractions obtained from dried giant squid tunics. *Journal of Aquatic Food Product Technology* **2014**. In press.
35. Laridi R, Kheadr EE, Benech R, Vuilleumard JC, Lacroix C, Fliss I. Liposome encapsulated nisin Z: Optimization, stability and release during milk fermentation. *International Dairy Journal* **13**: 325-336 (2003).
36. Teixeira ML, dos Santos J, Silveira NP, Brandelli A. Phospholipid nanovesicles containing a bacteriocin-like substance for control of *Listeria monocytogenes*. *Innovative Food Science and Emerging Technologies* **9**: 49-53 (2008).

37. Hans ML, Lowman AM. Biodegradable nanoparticles for drug delivery and targeting. *Current Opinion in Solid State and Materials Science* **6**: 319-327 (2002).
38. Müller RH, Jacobs C, Kayser, O. Nanosuspensions as particulate drug formulations in therapy: Rationale for development and what we can expect for the future. *Advanced Drug Delivery Reviews* **47**: 3-19 (2001).
39. Bailey AL, Sullivan SM. Efficient encapsulation of DNA plasmids in small neutral liposomes induced by ethanol and calcium. *Biochimica et Biophysica Acta – Biomembranes* **1468** (1-2): 239-252 (2000).
40. Taylor TM, Gaysinsky S, Davidson PM, Bruce BD, Weiss J. Characterization of antimicrobial-bearing liposomes by ζ -potential, vesicle size and encapsulation efficiency. *Food Biophysics* **2**: 1-9 (2007).
41. Nagayasu A, Uchiyama K, Kiwada H. The size of liposomes: a factor which affects their targeting efficiency to tumors and therapeutic activity of liposomal antitumor drugs. *Advanced Drug Delivery Reviews* **40**: 75-87 (1999).
42. Ramezani M, Khoshhamdam M, Dehshahri A, Malaekheh-Nikouei, B. The influence of size, lipid composition and bilayer fluidity of cationic liposomes on the transfection efficiency of nanolipoplexes. *Colloids and Surfaces B: Biointerfaces* **72**: 1-5 (2009).
43. Honary S, Zahir F. Effect of process factors on the properties of doxycycline nanovesicles. *Tropical Journal of Pharmaceutical Research* **11**: 169-175 (2012).
44. Silva R, Little C, Ferreira H, Cavaco-Paulo A. Incorporation of peptides in phospholipid aggregates using ultrasound. *Ultrasonics Sonochemistry* **15**: 1026-1032 (2008).
45. Thakur G, Mitra A, Basak A, Rousseau D, Pal K. Characterization of oil-in-water gelatin emulsion gels: Effect of homogenization time. Proceedings of the International Conference on Systems in Medicine and Biology, Boston, USA, July, 2010, Oxford University Press, *Bioinformatics Journal* **26**, pp. 305-308 (2010).
46. DiTizio V, Ferguson GW, Mittelman MW, Khoury AE, Bruce AW, DiCosmo F. A liposomal hydrogel for the prevention of bacterial adhesion to catheters. *Biomaterials* **19**: 1877-1884 (1998).
47. DiTizio V, Karlgard C, Lilge L, Khoury AE, Mittelman MW, DiCosmo F. Localized drug delivery using crosslinked gelatin gels containing liposomes: Factors influencing liposome stability and drug release. *Journal of Biomedical Materials Research* **51**: 96-106 (2000).

Table 1. Zeta-potential of partially purified PC-liposomes (10 mM phosphate buffer) encapsulating the < 1 kDa squid tunic peptide fraction (LP1) at different pH values. Different letters (a,b,c) indicate significant ($p \leq 0.05$) differences (ANOVA, Tukey HSD test, $n=3$).

pH	Zeta-potential (mV)
8	-22,77 $\pm$ 1,88 ^a
7	-54,30 $\pm$ 4,59 ^b
6	-30,27 $\pm$ 0,85 ^c
5	-32,23 $\pm$ 0,29 ^c
4	-30,07 $\pm$ 1,02 ^c
3	-41,77 $\pm$ 3,25 ^d
2	-22,50 $\pm$ 0,62 ^a
1	-2,06 $\pm$ 0,13 ^e

Figure 1.

Entrapment Efficiency (%) of the < 1 kDa squid tunic peptide fraction into partially purified PC-liposomes (10 mM phosphate buffer, pH 7) at different peptide concentrations. Different letters (a,b,c,...) indicate significant ($p \leq 0.05$) differences (ANOVA, Tukey HSD test). Error bars represent standard deviation (n=3).

Figure 2.

Zeta-potential of partially purified PC-liposomes (10 mM phosphate buffer, pH 7) encapsulating the < 1 kDa squid tunic peptide fraction at different peptide concentrations. t0: newly produced liposomes; t1: liposomes after one week at 4 °C storage. Different letters (a,b or A,B...) indicate significant ($p \leq 0.05$) differences (ANOVA, Tukey HSD test) regarding peptide concentration. Significant differences ($p \leq 0.05$) regarding storage time are indicated by an asterisk (*). Error bars represent standard deviation (n=3).

Figure 3.

Diameter of partially purified PC-liposomes (10 mM phosphate buffer, pH 7) encapsulating the < 1 kDa squid tunic peptide fraction at different peptide concentrations. Different letters (a,b) indicate significant ($p \leq 0.05$) differences (ANOVA, Tukey HSD test). Error bars represent standard deviation (n=3).

Figure 4.

Size distribution of partially purified PC-liposomes (10 mM phosphate buffer, pH 7) encapsulating the <1 kDa squid tunic peptide fraction at $1.75 \text{ g}\cdot\text{L}^{-1}$ peptide concentration (LP1). Error bars represent standard deviation ($n=3$).

Figure 5.

Elastic modulus (G') and viscous modulus (G''), as a function of the angular frequency, of gelatin solutions containing the encapsulated (LP1) (10 mM phosphate buffer, pH 7) and non-encapsulated (P1) peptide fraction at 5°C. Peptide (P1 or LP1) concentrations ($\text{g}\cdot\text{kg}^{-1}$ of dry gelatin) were G: $0\text{ g}\cdot\text{kg}^{-1}$, Ga: $2.62\text{ g}\cdot\text{kg}^{-1}$, Gb: $6.56\text{ g}\cdot\text{kg}^{-1}$, Gc: $13.12\text{ g}\cdot\text{kg}^{-1}$ and Gd: $26.24\text{ g}\cdot\text{kg}^{-1}$.

Figure 6.

Elastic modulus (G') and viscous modulus (G''), as a function of temperature, of gelatin solutions containing the encapsulated (LP1) (10 mM phosphate buffer, pH 7) and non-encapsulated (P1) peptide fraction at $26.2 \text{ g}\cdot\text{kg}^{-1}$ dry gelatin.