1 **The preferential use of a soy-rape seed lecithin blend for the liposomal encapsulation of a**
2 **tilapia viscera hydrolysate**

3 Cindy T. Sepúlveda^a, José E. Zapata^a, Oscar Martínez-Álvarez^b, Ailén Alemán^b, M. Pilar Montero^b,
4 M. Carmen Gómez-Guillén^{b*}

5 ^aDepartment of Food, Faculty of Pharmaceutical and Food Sciences, University of Antioquia,
6 Medellín 050010, Colombia

7 ^bInstitute of Food Science, Technology and Nutrition (ICTAN-CSIC), C/José Antonio Novais 10,
8 Madrid 28040, Spain

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10 *author for correspondence: mc.gomez@csic.es

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12

13 **Abstract**

14 Viscera of tilapia (*Oreochromis* spp.) were used for the production of a protein hydrolysate, which
15 was characterized in terms of proximate composition, molecular weight distribution, amino acid
16 composition and antioxidant activity (ABTS radical scavenging capacity, ferric ion reducing power
17 (FRAP) and ion chelating activity). Liposomes from soy lecithin (SL), rapeseed lecithin (RL), and
18 soy-rapeseed (SRL) lecithin blend (1:1, w/w) were prepared, showing average particle sizes of
19 153, 218 and 193 nm, respectively, and similar ζ potential of -46 mV. Rapeseed lecithin liposomes
20 presented much higher antioxidant activity than with SL. Liposomes made of SRL, having
21 intermediate hydrodynamic and antioxidant properties than the other two, were selected to
22 encapsulate the tilapia hydrolysate at different concentrations (5, 10 and 20 g/100 g, with respect
23 to lecithin). Liposomes loaded with 20 g/100 g of hydrolysate (SRLH20) had smaller particle size
24 and higher ABTS values and iron chelating activity, while liposomes loaded with 5 g/100 g
25 (SRLH5) had higher electronegative ζ potential and entrapment efficiency, which was calculated as
26 a function of total protein content, antioxidant capacity and individual amino acid residues. Charged
27 amino acid residues and alanine were highly entrapped in both SRLH5 and SRLH20. No clear
28 preference for encapsulating hydrophobic or hydrophilic amino acids was observed.

29 Keywords: fish hydrolysate, viscera, liposomes, lecithin, rapeseed.

30

31 **1. Introduction**

32 Tilapia processing generates by-products that are about 60% of the total weight of fish (Robert et
33 al., 2015). Protein hydrolysates from tilapia by-products with high antioxidant and antihypertensive
34 activities have been obtained from frame (Chuesiang & Sanguandeeikul, 2015; Roslan, Yunos,
35 Abdullah, & Kamal, 2014), skin (Thuanthong, De Gobba, Sirinupong, Youravong, & Otte, 2017)
36 and viscera (Gómez et al., 2019). Besides health benefits, protein hydrolysates have known
37 limitations, including hydrophobicity, hygroscopicity, low bioavailability, and unpleasant sensory
38 characteristics for food application (Sarabandi et al., 2019). The encapsulation of bioactive

39 peptides can overcome such limitations, providing stability and protection against extreme
40 conditions of pH, temperature, and high ion concentrations (Yokota, Moraes, & Pinho, 2012).
41 Liposomes are suitable to encapsulate bioactive peptides because they can entrap hydrophilic
42 peptides in the inner aqueous core and hydrophobic peptides in the bilayer; amphiphilic peptides
43 can interact with both the nucleus and the bilayer depending on the polarity of the amino acid
44 residues (Mohan, Rajendran, He, Bazinet, & Udenigwe, 2015). A wide range of marine
45 hydrolysates have been encapsulated within liposomes, including hydrolysates of gelatin or acid-
46 soluble collagen (Hosseini, Ramezanzade, & Nikkhah, 2017; Marín, Alemán, Montero, & Gómez-
47 Guillén, 2018), whitemouth croaker by-product (da Rosa Zavareze et al., 2014), salmon protein (Li,
48 Paulson, & Gill, 2015) or oyster (Xie et al., 2016); however no information is available regarding the
49 liposomal encapsulation of fish viscera hydrolysates.

50 The constituents used for the production of food-grade liposomes could be low-cost if they are
51 produced from lecithin or natural semi-purified phospholipids. Soy and rapeseed are primary
52 sources of lecithin that have been proven to be a good source of essential fatty acids with positive
53 health effects (Butina, Gerasimenko, Bugaets & Kopteva, 2018); however, they are rich in mono-
54 and polyunsaturated fatty acids, which can be prone to oxidation (Wand & Wang, 2008). The
55 presence of natural antioxidants in plant lecithin, as well as the encapsulation of antioxidant
56 peptides, can decrease the production of undesirable oxidation products in liposomes (Taladrid et
57 al., 2017; Marín et al., 2018). There are previous studies comparing the properties of liposomes
58 obtained from soy and rapeseed lecithin, but the mixing of both has not yet been reported (Arab
59 Tehrany et al., 2012; Jiménez, Sánchez-González, Desobry, Chiralt, & Tehrany, 2014).

60 This work aims to select the most suitable source of lecithin (soy, rapeseed, and soy-rapeseed
61 blend) to encapsulate increasing concentrations of a tilapia viscera hydrolysate in liposomes, and
62 to evaluate their structural and antioxidant properties. The entrapment efficiency in terms of total
63 protein content, amino acid composition and antioxidant activity was also evaluated as a function
64 of hydrolysate concentration.

2. Materials and methods

2.1. Materials

The chemicals, including 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate (ferrozine), ferrous chloride, bovine serum albumin, aprotinin, Vitamin B12, hippuril-L-histidyl-L-leucine (HHL), and norleucine were purchased from Sigma-Aldrich. Potassium persulphate and glycine were obtained from Panreac. 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) was obtained from Fluka. All other reagents were of analytical grade.

2.2. Enzymatic protein hydrolysis

Fresh red tilapia viscera were provided by Piscícola el Gaitero in Sopetrán, Colombia. The viscera were packed in polyethylene bags and kept in ice during transportation. Immediately they arrived at the laboratory, they were heated at 90 °C for 20 min to inactivate endogenous enzymes and stored in a plastic vessel at -20 °C. After 24 h, the fat was separated from viscera and removed by cutting the frozen block with a knife. The defatted viscera remained at -20 °C until hydrolysis. Enzymatic hydrolysis was carried out in a Bioflo 310® batch reactor (New Brunswick Scientific Co., Inc. USA) with pH (10) and temperature control (59 °C). The reaction was started by addition of Alcalase® 2.4 L (Novozymes, Denmark) at an enzyme/substrate (E/S) ratio of 1:10 (w/w, wet weight) with 4.6 units of enzymatic activity per gram of protein. The hydrolysis was controlled for 3 h using pH-stat with addition of 2 mol/L NaOH. The enzyme was inactivated heating at 90 °C for 10 min. The hydrolysate was centrifuged at 4500xg for 30 min at 4 °C. The supernatant was freeze-dried and stored at -20 °C. The degree of hydrolysis was 8.0%, according to Adler-Nissen, Eriksen, & Olsen (1983).

2.3. Proximate composition of hydrolysate

Nitrogen content was determined by Dumas method, using a LECO FP-2000 nitrogen/protein analyzer (Leco Corp., Joseph, MI, USA). Protein content was calculated using a protein nitrogen conversion factor of 6.25. Ash was determined gravimetrically in triplicate, according to method

91 950.46 (A.O.A.C, 2005). Total fat was estimated by the Soxhlet method, and total dietary fiber was
92 determined according to method 985.29 (A.O.A.C, 1985). Moisture was determined by difference.

93 2.4. *Molecular weight distribution of hydrolysate*

94 HPLC size-exclusion chromatography (Model SPE_MA10AVP, Shimadzu, Japan) was carried out
95 using a Superdex peptide PC 3.2/30 column (GE Healthcare, Bio-Sciences, Barcelona, Spain) with
96 a fraction range from 0.1 to 7.0 kDa. The dried hydrolysate was solubilized in ultrapure water
97 (10 mg/mL), centrifuged at 9000xg for 5 min, and filtered at 0.45 µm. The mobile phase was 30%
98 acetonitrile (v/v) with TFA 0.01% (v/v), the flow rate was 0.1 mL/min, and the injection volume was
99 10 µL. Bovine serum albumin (67 kDa), aprotinin (6.51 kDa), vitamin B12 (1.34 kDa), HHL (429
100 Da) and glycine (75 Da) were used as molecular weight standards.

101 2.5. *Amino acid composition of hydrolysate*

102 The amino acid composition was determined as described by Alemán, Gómez-Guillén, & Montero
103 (2013) by acid hydrolysis in 6 mol/L HCl at 110 °C for 24 h, using norleucine (Sigma-Aldrich, Inc.)
104 as internal standard. Results were expressed as number of residues per 1000.

105 2.6. *Liposome preparation*

106 Soy lecithin (SL) (Manuel Riesgo S.A., Spain), rapeseed lecithin (RL) (BungeMaxx, The
107 Netherlands) and a mixture of the two lecithins (SRL) (1:1 w/w) were used to prepare empty
108 liposomes as described previously (Taladrid et al., 2017). Briefly, lecithin (5 g/100 mL) suspended
109 in phosphate buffer (pH 7) was gently stirred for 1 min and then kept in a water bath for 2 h at 80
110 °C. The heated dispersion was vortexed to produce multilamellar lipid vesicles and subsequently
111 sonicated using a Q700 sonicator (Qsonica, Newton, CT, EEUU, max 700 W) with a 12.7 mm
112 diameter probe. Sonication was carried out with intensity set at 90% in a pulsed mode for 5 min,
113 with a 60 s stop every min to avoid sample overheating. The soy-rape seed lecithin blend was
114 selected to encapsulate the tilapia hydrolysate following the same procedure, but mixing lecithin
115 with 5 g/100 g (SRLH5), 10 g/100 g (SRLH10) and 20 g/100 g (SRLH20) of hydrolysate (with
116 respect to lecithin content, dry weight, dw).

2.7. Particle properties of liposomes

The particle size (z-average and particle size distribution), polydispersity index (PDI) and ζ potential of liposomal suspensions were determined by Dynamic Light Scattering (DLS) and Laser Doppler Micro-electrophoresis using a Zetasizer Nano S90 (Malvern Instruments, UK). Measurements were made at 25 °C by 10-fold dilution in water as previously described (Marín et al., 2018).

2.8. Antioxidant activity

ABTS free radical scavenging activity was determined as described by Alemán, Giménez, Pérez-Santín, Gómez-Guillén, & Montero (2011). The determination was made in triplicate, and the results were expressed as μmol Trolox equivalents (Trolox Eq.)/g sample (dw).

Ferric ion reducing capacity (FRAP) as described by Alemán et al. (2011) was applied to evaluate the ability of the hydrolysate to reduce ferric iron (Fe^{+3}) present in a complex with 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) up to the ferrous form (Fe^{+2}). The analysis was performed in triplicate, and the results were expressed as μmol Trolox equivalents (Trolox Eq.)/g sample (dw).

Ferrous ion chelating activity was determined as described by Pérez-Santín, Calvo, López-Caballero, Montero, & Gómez-Guillén (2013) with some modifications. A volume of 1 mL of hydrolysate (2 mg/mL) was mixed with 3.7 mL of H_2O and 100 μL of 2 mmol/L FeCl_2 . After 3 min the reaction was started with 200 μL of 5 mmol/L ferrozine. The mixture remained at room temperature for 10 min and the absorbance of the ferrozine ferrous iron complex at 562 nm was determined. Deionizer water was used instead of the sample as blank and a sample control without the addition of ferrozine was also used according to Equation 1. Results were expressed as μmol EDTA equivalents (EDTA Eq.)/g sample (dw).

(1)

139

140 Lipophilic antioxidant was determined by the photochemiluminescence (PLC) assay as described
141 by Pérez-Santín et al. (2013). Results were expressed as μg Trolox equivalents (Trolox Eq.)/mg
142 sample (dw).

143

144 2.9. *Entrapment efficiency*

145 The entrapment efficiency (EE) of the hydrolysate in liposomes was calculated according to
146 Equation 2:

$$(2)$$

147 The amount of encapsulated hydrolysate was calculated as the difference between the total
148 hydrolysate used for liposome preparation and the non-encapsulated hydrolysate. The non-
149 encapsulated hydrolysate was separated from the liposomes by filtration as follows: the liposomal
150 dispersion (diluted with water 1:1) was placed in a 10 kDa filtration tube (Amicon® Ultra-15, Merck
151 Millipore Ltd., Ireland) and centrifuged at 5000xg for 40 min at 4 °C. The non-encapsulated
152 hydrolysate penetrated through the membrane while the liposomes remained in the filter. Both non-
153 encapsulated and total hydrolysate were determined by measuring protein content (by the Lowry
154 method and by total amino acid content) and antioxidant activity (by ABTS and FRAP).

155 2.10. *Statistical analysis*

156 Analysis of variance (ANOVA) and the Fisher minimum significant difference method (LSD) were
157 used to discriminate between the means. The statistically significant difference was established at
158 $P < 0.05$.

159

3. Results and discussion

3.1. *Hydrolysate characterization*

Protein was the major constituent of tilapia viscera hydrolysate with 42.2 ± 1.1 g/100 g, which was low compared to other fish viscera hydrolysates, in which values between 65 and 90% were reported (Abdelhedi et al., 2016; Ovissipour, Abedian Kenari, Motamedzadegan, & Nazari, 2012). The low protein content in the hydrolysate could be attributed to the heat treatment introduced for the endogenous enzymes inactivation prior to hydrolysis, which could result in protein aggregation that would make enzymatic breakdown more difficult (Šližyte, Daukšas, Falch, Storrø, & Rustad, 2005). Fat content was relatively low (3.6 ± 0.1 g/100 g), according to the range of 1.27 to 14.46% reported by Abdelhedi et al. (2016) for different fish viscera hydrolysates, indicating that the physical process used for fat separation was enough to obtain an acceptable residual level. The ash content was 22.9 ± 0.6 g/100 g, which agrees with the value around 25 % reported by Roslan et al. (2014) for a tilapia by-product hydrolysate powder. The addition of alkali to maintain the optimum pH during the hydrolysis process could have contributed to increase the ash content of the final product (Benjakul & Morrissey, 1997). However, considering that the present hydrolysate came from viscera and not from mineralized tissues, such as bones or scales, a possible high mineral content from the fish feed cannot be disregarded. In this connection, a high dietary fiber content (15.3 ± 0.6 g/100 g) was determined in the hydrolysate, probably also from feed accumulated in the viscera. The relatively high moisture (≈ 16 g/100 g) could point to a high hygroscopicity of the freeze-dried hydrolysate, probably favored by the high mineral content.

Table 1 shows the amino acid composition of the tilapia hydrolysate. The most abundant amino acids were Glx, Asx, and Gly, followed in descending order by Ala, Leu, and Ser, while Cys was the least abundant. These results agree with other studies in tilapia (Robert et al., 2015; Roslan et al., 2014). Hydrophobic amino acids represented around 39% of the total, which is relevant for antioxidant activity due to the ability of some of them to act as proton or electron donors (Je, Park, Hwang, & Ahn, 2015). They can also increase the interaction of peptides with target lipids or their

entry into target cells through hydrophobic associations with the membrane lipid bilayer (Girgih et al., 2014).

The hydrolysate molecular weight distribution in Fig. 1 shows the presence of peptides below 6511 Da, with a striking amount around 336 Da that presumably corresponds to dipeptides or tripeptides. According to Lu, Zhang, Sun, Song, & Huang (2019) the majority of food-derived antioxidant peptides have a molecular weight between 500 and 1500 Da. Alemán et al. (2011) also found that hydrolysates most of whose peptides were in the range of 1400 to 500 Da showed the highest antioxidant activity. These results agree with different studies (Intarasirisawat, Benjakul, Visessanguan, & Wu, 2012; Silva, Hernández-Ledesma, Amigo, Netto, & Miralles, 2017) that have shown the ability of Alcalase® to produce low molecular weight hydrolysates.

Table 2 shows the hydrolysate antioxidant activity determined by different methods. The ABTS, FRAP and iron-chelating activity values found in the tilapia hydrolysate were comparable to those reported for hydrolysates from unicorn leatherjacket gelatin (Karnjanapratum & Benjakul, 2015) and rainbow trout by-products (Nikoo, Benjakul, Yasemi, Ahmadi Gavlighi, & Xu, 2019). Iron is an indispensable metal in the metabolism of living beings; however, in its free form Fe^{2+} can be prooxidant and promote the formation of free radicals (Esfandi, Willmore, & Tsopmo, 2019). The chelating activity may be related to the presence of negatively-charged peptides favoring the formation of the complex with metal ions (Nikoo et al., 2019). The chelating activity results agree with the amount of negatively charged amino acids, which is twice that of positively charged amino acids, as shown in Table 1. The tilapia hydrolysate also showed notable lipophilic superoxide radical scavenging capacity, as determined by PCL assay, which could be attributed to the abundance of hydrophobic peptides.

3.2. *Liposome characterization*

The particle properties of liposomal dispersions obtained from SL, RL and the mixture of both (SRL) are shown in Table 3. The mean size was significantly higher ($P < 0.05$) with RL than with SL, while SRL liposomes presented intermediate values. Moreover, liposomes from SL had the

lowest polydispersity index (PDI). Similar hydrodynamic properties were reported for soy lecithin liposomes produced by similar heating and ultrasonication, without including any further partial purification step of phospholipids (Taladrid et al., 2017). SL liposomes showed a typical monomodal narrow particle size distribution, as shown in Fig. 2a, in contrast to RL liposomes, in which the main peak was broader and shifted towards higher particle sizes. The mixture of both lecithins (SRL) (1:1 w/w) led to liposomes with intermediate particle size distribution, suggesting that a good blend was obtained. The differences in hydrodynamic particle properties may be associated with the particular composition in fatty acids and other residual compounds, and with different surface-active properties of each lecithin (Arab Tehrany et al., 2012; da Rosa Zavareze et al., 2014). The high electronegative ζ potential values of the three liposomal dispersions, with no statistical differences, were indicative of high stability. Values of ζ potential ($\geq + 30$ and $\leq - 30$ mV) indicate excellent stability thanks to repulsion charges preventing aggregation due to collisions between adjacent particles (Mohan, McClements, & Udenigwe, 2016). The negative charge is due to the presence of phospholipids with negatively charged hydrophilic heads (Taladrid et al., 2017).

The antioxidant activity of empty liposomes is also shown in Table 3. RL liposomes presented much higher antioxidant activity than with SL, measured by the different methods, but especially by the PCL assay. The SRL mixture led to liposomes with intermediate values. According to Koprivnjak et al. (2008), the amino group of phosphatidylcholine can promote the donation of electrons or hydrogen atoms, which is vital in the mechanism of antioxidant activity. However, phospholipids composed of long chains of unsaturated fatty acids are highly sensitive to oxidation, which may decrease their antioxidant activity (Ramadan, 2012). Arab Tehrany et al. (2012) showed that soy lecithin contains more polyunsaturated fatty acids (PUFA) than canola rapeseed lecithin. More specifically, while linoleic acid predominates in soy lecithin, oleic acid is more abundant in rapeseed lecithin (Jiménez et al., 2014); therefore the low antioxidant activity of SL compared to RL could be related to the presumably high PUFA content of SL (Ramadan et al., 2003). In addition, there may be differences in the content of other lecithin antioxidant components. In this respect, Li, Pedersen, Anankanbil, & Guo (2018) also found the antioxidant activity of rapeseed

lecithin stronger than soy lecithin, attributing the difference to the higher content of phenolic compounds causing a synergistic effect with phospholipids. Both soy and rapeseed lecithins are also known to contain significant amounts of sterols and tocopherols with high antioxidant capacity (Taladrid et al., 2017; Butina et al., 2018).

Since SL liposomes presented lower particle size and polydispersity, and RL higher antioxidant activity, the soy-rapeseed blend (SRL), having intermediate properties, was chosen for encapsulation of the tilapia hydrolysate.

3.3. Characterization of hydrolysate-loaded liposomes

The particle properties and antioxidant activity of SRL liposomes encapsulating different hydrolysate concentrations (SRLH5, SRLH10, SRLH20) are also shown in Table 3. Increasing the concentration of hydrolysate significantly reduced the average size of liposomes, all of which were smaller than the empty liposomes. These results agree with Panja, Khatua, & Halder (2019), who found a substantial size reduction after casein addition in soybean lecithin liposomes. The PDI was not significantly ($P > 0.05$) affected with the increase in hydrolysate concentration. In addition, PDI of SRL liposomes loaded with the hydrolysate at any of the tested concentrations showed no significant ($P > 0.05$) differences with respect to the SRL empty liposomes. These results agree with the particle size distributions presented in Fig. 2b, where profiles of all peptide-loaded liposomes resembled that of empty SRL liposomes, with SRLH20 slightly shifting towards lower particle size. The PDI values in this work were lower than reported for liposomes loaded with anionic and cationic whey peptides (Mohan, Rajendran, Thibodeau, Bazinet, & Udenigwe, 2018), or with a flaxseed protein hydrolysate (Sarabandi et al., 2019).

The ζ potential of liposomes with tilapia hydrolysate maintained a high negative charge, with no significant difference between the empty liposome (SRL) and the one with the lowest hydrolysate content (SRLH5). The decrease in SRLH20 could be due to ionic interactions between the amino groups of positively charged peptides and the negative surface charge of liposomes (Li et al., 2015). A decrease in the ζ potential of liposomes loaded with whey protein hydrolysate was also

265 found by Mohan et al. (2018). However, knowledge about the surface dynamics of the interaction
266 between peptide hydrolysates and liposomes is still limited.

267 SRLH20 had the highest ABTS values compared to SRLH10 and SRLH5 (Table 3). Increasing
268 values were also observed in iron chelating activity for SRLH5, SRLH10 and SRLH20. The
269 presence of hydrolysate noticeably increased ($P < 0.05$) these activities compared to the empty
270 liposomes. In contrast, there were no statistical differences in FRAP activity as a function of
271 hydrolysate concentration, and the values for empty liposomes were slightly lower ($P < 0.05$).
272 Superoxide radical scavenging capacity (PCL assay) decreased with the addition of increasing
273 hydrolysate concentrations, and was higher ($P < 0.05$) in the empty liposomes. The explanation
274 may be that this activity was determined in the lipophilic mode, and therefore the antioxidant
275 components contained in the lecithin mixture accounted for most of the reported activity. Part of the
276 non-encapsulated hydrolysate could be blocking the activity of liposomes by association with the
277 external membrane surface.

278 3.4. *Entrapment efficiency*

279 The entrapment efficiency (EE) in SRLH5, SRLH10, and SRLH20 was compared in terms of
280 protein content (determined by the Lowry method and by the amino acid composition) and
281 antioxidant capacity (FRAP and ABTS assays), as shown in Fig. 3. SRLH5 liposomes had the
282 highest EE by all the test methods, and in general little variation was observed between SRLH10
283 and SRLH20. A study carried out on liposome-in-alginate beads showed that EE decreased
284 significantly when a larger amount of hydrolysate was added (Xie, Lee, Choung, Kang, & Choi,
285 2016). A significant decrease in EE has also been reported with increasing concentration of a
286 peptide fraction in liposomes of partially purified soy phosphatidylcholine (Mosquera et al., 2014).
287 The smaller variations in EE values determined by the FRAP method were consistent with the fact
288 that the differences observed between hydrolysate-loaded and empty liposomes were very slight.

289 The entrapment efficiency (EE) values calculated for each individual amino acid residue in both
290 SRLH5 and SRLH20 are presented in Table 4. All values in SRLH5 were above 70%, indicating

that all residues were highly entrapped. Higher entrapment were found for Glx, Arg, Ala, and Leu residues with values of 92.01%, 89.74%, 87.07% and 86.92% respectively. In contrast, Pro, Cys and Tyr showed the lowest values, suggesting that peptides that include these amino acids in their sequences were more difficult to entrap. The entrapment of the different amino acid residues in SRLH20 varied considerably, the highest being for Arg, Ala, Glx and Ser. It is important to note that charged amino acids, such as Glx (-) and Arg (+), and Ala—which is the most abundant hydrophobic amino acid—registered high entrapment percentages in both SRLH5 and SRLH20. From Table 4, it could be deduced that non-encapsulated peptides in SRLH20 contained more Cys, Leu, Tyr, Val and high total hydrophobic amino acids content. This would explain the considerable decrease of encapsulation efficiency in SRLH20 in terms of ABTS, strongly suggesting that non-encapsulated peptides contribute significantly to the antioxidant activity of the liposomal dispersion in terms of radical scavenging capacity.

4. Conclusions

With the soy-rapeseed lecithin mixture (SRL), liposomes with intermediate particle size, polydispersity and antioxidant activity were obtained, compared to soy and rapeseed lecithin liposomes separately. The lecithin blend allowed getting closer to the best antioxidant properties of rapeseed lecithin liposomes and the smaller size of the liposomes obtained with soy lecithin. The high antioxidant activity provided by the rapeseed lecithin in SRL was further increased by the addition of the tilapia viscera hydrolysate, the highest encapsulation efficiency being found with 5% of hydrolysate. Glx, Arg and Ala were the most entrapped aminoacid residues, regardless of the hydrolysate concentration used.

Acknowledgements

The authors are grateful for the financial support provided by Comité para el Desarrollo de la Investigación en la Universidad de Antioquia (CODI) through sustainability program 2018-2019,

COLCIENCIAS (Project Code 111574558746) and by Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER), through project NANOALIVAL AGL2017-84161.

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480 Legend to figures

481 Fig. 1. Molecular weight distribution of tilapia viscera hydrolysate.

482 Fig. 2. Particle size distribution of liposomes made of (a) soy lecithin (SL), rapeseed lecithin (RL)
483 and soy-rapeseed lecithin blend (SRL); and (b) liposomes made of soy-rapeseed lecithin blend
484 loaded with hydrolysate at 5 g/100 g (SRLH5), 10 g/100 g (SRLH10), and 20 g/100 g (SRLH20).

485 Fig. 3. Entrapment efficiency (EE) of SRLH5, SRLH10, and SRLH20 determined by the total
486 protein content (by Lowry method and by amino acid composition) and by the antioxidant activity
487 (FRAP and ABTS).

488 Error bars represent the standard deviation ($n = 3$). Different letters (a,b,c) indicate significant
489 differences ($P < 0.05$).

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Table 1. Amino acid composition of tilapia viscera hydrolysate.

Amino acid	residues/1000 residues
Asx	109 ± 7
Thr	57 ± 3
Ser	74 ± 4
Glx	117 ± 7
Gly	109 ± 7
Ala	95 ± 6
Cys	6 ± 0
Val	53 ± 3
Met	24 ± 1
Ile	37 ± 2
Leu	77 ± 5
Tyr	35 ± 2
Phe	43 ± 3
His	18 ± 1
Lys	58 ± 3
Arg	31 ± 2
Pro	58 ± 3
EAA	349 ± 21
HAA	387 ± 23
PCAA	107 ± 6
NCAA	226 ± 14

EAA: Essential amino acids (Phe, Met, Lys, Leu, Ile, Val, Thr)

HAA: Hydrophobic amino acids (Ala, Val, Ile, Leu, Phe, Pro, Met)

PCAA: Positively charged amino acids (Arg, His, Lys)

NCAA: Negatively charged amino acids (Asx, Glx)

Values are expressed as mean ± S.D. (n = 2)

Table 2

Antioxidant activity of tilapia viscera hydrolysate by ABTS, FRAP, chelation of Fe²⁺, and photochemiluminescence (PLC).

Activity	Values
ABTS (μmol Trolox Eq./g)	535.9 ± 8.7
FRAP (μmol Trolox Eq./g)	114.8 ± 8.3
Chelation of Fe ²⁺ (μmol EDTA Eq./g)	377.4 ± 0.7
PCL (μg Trolox Eq./mg)	4.3 ± 0.1

Values are expressed as mean ± S.D. (n = 3).

Table 3

Mean particle size (z-average), polydispersity index (PDI), ζ potential and antioxidant activity of empty liposomes made of soy lecithin (SL), rapeseed lecithin (RL), and soy-rapeseed lecithin blend (SRL), and liposomes made of soy-rapeseed lecithin blend loaded with hydrolysate at 5 g/100 g (SRLH5), 10 g/100 g (SRLH10), and 20 g/100 g (SRLH20).

Property	SL	RL	SRL	SRLH5	SRLH10	SRLH20
z-average (nm)	153.3 $\pm$ 0.3 ^a	217.7 $\pm$ 2.4 ^b	193.2 $\pm$ 3.0 ^c	177.2 $\pm$ 2.6 ^d	173.7 $\pm$ 2.2 ^d	147.1 $\pm$ 1.9 ^e
PDI	0.25 $\pm$ 0.02 ^a	0.36 $\pm$ 0.03 ^b	0.33 $\pm$ 0.03 ^{b, c}	0.31 $\pm$ 0.03 ^c	0.30 $\pm$ 0.02 ^c	0.33 $\pm$ 0.03 ^{b, c}
ζ potential (mV)	-45.6 $\pm$ 1.8 ^a	-45.5 $\pm$ 3.0 ^a	-46.4 $\pm$ 1.1 ^a	-45.5 $\pm$ 2.4 ^a	-43.9 $\pm$ 2.2 ^{a, b}	-41.9 $\pm$ 2.6 ^b
ABTS (μ mol Trolox Eq./g)	17.2 $\pm$ 0.08 ^a	27.4 $\pm$ 1.4 ^b	27.7 $\pm$ 0.5 ^b	40.1 $\pm$ 0.4 ^c	52.7 $\pm$ 0.4 ^d	65.6 $\pm$ 0.3 ^e
FRAP (μ mol Trolox Eq./g)	11.7 $\pm$ 0.3 ^a	53.1 $\pm$ 0.8 ^b	41.3 $\pm$ 0.8 ^c	43.4 $\pm$ 0.2 ^{c, d}	46.3 $\pm$ 8.0 ^{c, d}	48.0 $\pm$ 1.9 ^{b, d}
PCL (μ g Trolox Eq./mg)	0.85 $\pm$ 0.03 ^a	58.3 $\pm$ 1.1 ^b	27.1 $\pm$ 0.5 ^c	20.6 $\pm$ 0.3 ^d	19.9 $\pm$ 0.2 ^d	18.2 $\pm$ 0.2 ^e
Chelation of Fe ²⁺ (μ mol EDTA Eq./g)	15.8 $\pm$ 0.2 ^a	21.5 $\pm$ 0.05 ^b	18.8 $\pm$ 0.1 ^c	20.6 $\pm$ 0.04 ^d	21.7 $\pm$ 0.1 ^b	22.1 $\pm$ 0.4 ^e

¹Values are expressed as mean $\pm$ S.D. (n = 3). Different letters (a,b,c,...) indicate significant differences ($p < 0.05$).

Table 4. Entrapment efficiency (%) calculated for each individual amino acid residue in liposomes loaded with hydrolysate at 5 g/100 g (SRLH5) and 20 g/100 g (SRLH20).

Amino acid	SRLH5	SRLH20
Ala	87.07 ± 5.22 ^a	67.51 ± 4.05 ^b
Arg	89.74 ± 5.38 ^a	78.18 ± 4.69 ^b
Asx	83.02 ± 4.98 ^a	52.33 ± 3.14 ^b
Cys	70.83 ± 4.25 ^a	9.52 ± 0.57 ^b
Glx	92.01 ± 5.52 ^a	59.26 ± 3.56 ^b
Gly	77.14 ± 4.63 ^a	44.06 ± 2.64 ^b
His	81.10 ± 4.87 ^a	50.60 ± 3.04 ^b
Ile	84.49 ± 5.07 ^a	46.43 ± 2.79 ^b
Leu	86.92 ± 5.22 ^a	38.70 ± 2.32 ^b
Lys	81.51 ± 4.89 ^a	48.42 ± 2.91 ^b
Met	85.45 ± 5.13 ^a	51.18 ± 3.07 ^b
Phe	86.13 ± 5.17 ^a	42.26 ± 2.54 ^b
Pro	70.97 ± 4.26 ^a	53.62 ± 3.22 ^b
Ser	82.95 ± 4.98 ^a	56.83 ± 3.41 ^b
Thr	81.48 ± 4.89 ^a	43.56 ± 2.61 ^b
Tyr	72.94 ± 4.38 ^a	34.97 ± 2.10 ^b
Val	81.96 ± 4.92 ^a	35.28 ± 2.12 ^b
HAA	82.82 ± 5.00 ^a	48.60 ± 2.87 ^b
HLA	84.01 ± 4.80 ^a	48.84 ± 2.58 ^b
PCAA	84.07 ± 5.05 ^a	56.90 ± 3.54 ^b
NCAA	87.80 ± 5.25 ^a	55.82 ± 3.35 ^b

HAA: Hydrophobic amino acids (Ala, Val, Ile, Leu, Phe, Pro, Met)

HLA: Hydrophilic amino acids (Cys, Gly, Thr, Ser, Tyr, Glx, Asx)

PCAA: Positively charged amino acids (Arg, His, Lys)

NCAA: Negatively charged amino acids (Asx, Glx).

Values are expressed as mean ± S.D. (n = 2). Different letters (a,b) in the same row indicate significant differences ($P < 0.05$).

Figure 1

Figure 2

Figure 3