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Amaranth proteins emulsions as delivery system of Angiotensin-I converting enzyme inhibitory peptides

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ABSTRACT

We analyze the possibility of using the emulsifying properties of amaranth proteins and the bioactivity shown by peptide sequences encrypted in these proteins to formulate functional emulsions with angiotensin I-converting enzyme (ACE) inhibitory activity. For this we formulate O:W emulsions, 20:80, from a mixture in equal parts (50:50) of amaranth protein isolates (API), and hydrolysates (AH) at 1 and 2% protein w/v. Results obtained showed that these emulsions, API50-AH50-1% and API50-AH50-2%, are highly flocculated (Flocculation index: 8.2 and 5.9) and stable at least for 8 days, without evident creaming or coalescence. The components present in the emulsions were capable of inhibiting ACE, in *in vitro* assays (IC_{50} of 0.14 ± 0.02 mg/mL).

API and API50-AH50 emulsions were subjected to a simulated gastrointestinal digestion *in vitro*. The emulsions were susceptible to aggregation and coalescence phenomena during this process, which could be a consequence of the chaotropic action of the bile salt on the interface and the proteolytic and lipolytic action of pancreatin and lipase, respectively ($D_{4.3}$ of original emulsion: 1.22 ± 0.01 μm and $D_{4.3}$ of digested emulsion 79.5 ± 17.1 μm). We also found that amaranth proteins were more resistant to gastric than duodenal digestion.

After the process of simulated gastrointestinal digestion, the inhibition of ACE (IC_{50} of 0.13 ± 0.07 mg/mL) was maintained. This fact evidences the protective effect of the emulsion on the bioavailability of the ACE inhibitory peptides, by either their participation in the formation of the interfacial film and/or their participation in the network of formed flocs.

1. Introduction

Hypertension is a condition of global concern due to its incidence in the adult population (roughly 30%). The increasing prevalence of hypertension can be attributed to an increased life expectancy and to the presence of risk factors associated with a poor diet, high alcohol intake, physical inactivity, overweight and/or exposure to sustained stress conditions (OMS, 2013). Alternatives to decrease the prevalence of this disease are to change the dietary habits and to increase the consumption of functional ingredients that contribute to decrease and/or regulate the blood pressure and/or decrease the risk of developing hypertension (OMS, 2013).

The mechanisms regulating the blood pressure (BP) are complex and involve several metabolic pathways, of which, the more studied is that involving the *in vitro* inhibition of the angiotensin I-converting enzyme. This enzyme participates in the two most important regulatory systems, i.e., the renin-angiotensin (RAS) and the quinine-nitric oxide (QNOS) systems. ACE is a key component of the RAS since not only does it regulate blood pressure, but also the hydro-electrolytic balance (Rice, Thomas, Grant, Turner, & Hooper, 2004).

Over the past years, several studies have demonstrated the presence of bioactive peptides as part of the aminoacid sequence of the amaranth grains storage proteins (Silva Sanchéz et al., 2008). These peptides have, among others, ACE inhibitory activity and are released by enzymatic action or during the gastrointestinal digestion (Fritz, Vecchi, Rinaldi, & Añón, 2011; Martínez-Cruz, Cabrera-Chavez, & Paredes-Lopez, 2014; Tiengo, Faria, & Netto, 2009; Tovar-Pérez, Guerrero-Legarreta, Farrés-González, & Soriano-Santos, 2009; Vecchi & Añón, 2009; Vilcacundo, Martínez-Villaluenga, Miralles & Hernandez-Ledesma, 2018). The ACE inhibitory activity of these peptides has been demonstrated in *in silico*, *in vitro* and *in vivo* assays (Barba de la Rosa et al., 2010; Fritz et al., 2011; Tiengo et al., 2009; Vecchi & Añón, 2009). Amaranth is a high-protein content (15–19%) pseudocereal whose amino acid composition is optimal for human nutrition (Raina &

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Datta, 1992). Not only does amaranth present bioactive properties, but also it displays good emulsifying capacity, as regards formation and stabilization of O:W emulsions (Suárez & Añón, 2018; Ventureira, 2010; Ventureira et al., 2012).

One of the most important challenges for scientists is that the bioactive compounds found in different functional foods reach their target to exert their physiological action. In particular, in the case of bioactive peptides, they must overcome the action of the proteases present in the gastrointestinal digestion process, the absorption at the intestinal level and the plasma (Udenigwe & Fogliano, 2017; Vermeirssen, Camp, & Verstraete, 2004). Conventional emulsions are usually the first study system for the delivery of substances due to their easy preparation and low costs compared to more sophisticated delivery systems. However, they have certain limitations when compared to more sophisticated transport delivery systems, such as their physical instability when exposed to heating, cooling, freezing, drying, extreme pHs and high concentrations of salts (McClements, 2015). O:W emulsions have been used to encapsulate ω-3 fatty acid (Klinkesorn, Sophanodora, & Chinachoti, 2005; Gumus, Decker, & McClements, 2017); ω-6 fatty acid (Fernández-Avila, Arranz, Guri, Trujillo, & Corredig, 2016), and the liposoluble vitamin lycopene (Ribeiro & Schubert, 2006); while W:O have had more limited use.

In the case of ACE inhibitory peptides, an attempt has been made to improve their bioavailability through chemical modification (Mayo, 2000), delivery by genetically engineered GRAS microorganisms (Krüger et al., 2002) and encapsulating synthetic peptides into liposomes (Chen et al., 2003) or protein hydrolysates in carboxymethylated gum/sodium alginate beads (Ruiz Ruiz, Segura Campos, Betancur Ancona, & Chel Guerrero, 2013). Recently Huang, Xiao, Hao, and Yang (2017) have reported that the encapsulation of the antihypertensive peptide Val-Leu-Pro-Val-Pro in sodium alginate-O carboxymethyl chitosan microspheres protects it against simulated gastric digestion, allows its controlled release and does not affect the inhibitory activity of ACE *in vitro*, which is of the order of 48%.

In the present work, we present a simpler way to protect amaranth ACE inhibitory peptides. Amaranth proteins have both physicochemical and biological functions, which could be useful to prepare functional emulsions with the capacity to deliver bioactive peptides. Taking into account these properties we focused on the development and study of bio-functional O:W emulsions, employing mixtures of isolated and hydrolyzed amaranth proteins as tensioactive-stabilising and bioactive agents. This formulation was subjected to a simulated gastrointestinal digestion and the active compounds released were assessed for their ACE inhibitory capacity. We consider that this form is a convenient way to incorporate them into the formulation of functional foods such as dairy products, beverages, among others.

2. Materials and methods

2.1. Amaranth protein isolate (API)

The API was prepared from defatted flour obtained from *Amaranthus hypochondriacus* seeds provided by INDEAR (Institute of Agrobiotechnology of Rosario, Argentina). The procedure described by Martínez and Añón (1996) was employed. This method is based on the alkaline extraction (pH 9.0) of the proteins and their subsequent isoelectric precipitation at pH 5.0. The isolates obtained contained: 84.7 ± 0.1% w/w of proteins, as determined by the Kjeldahl method, 3.3 ± 0.3% w/w of ashes (AOAC, 1990, 923.03 method) and 12% w/w carbohydrates, as determined by difference. All results are expressed in dry base. The moisture content was 4.37 ± 0.58% w/v.

2.2. Hydrolysis

The amaranth hydrolysate (AH) was prepared by treating the API with alcalase (EC 3.4.21.62, Sigma-Aldrich, St. Louis, MO, USA) with specific activity 2.4 Anson units/g in a concentration of 0.16 μL/g isolate, density: 1.25 g/mL. One unit is defined as the amount of enzyme that will release 1.0 μmoL L-tyrosine from hemoglobin per min at 25 °C, pH 7.5. The API was dispersed to a final concentration of 1% w/v in 35 mM phosphate buffer (pH 7.8) and stirred for 1 h at 37 °C before adding the enzyme. Hydrolysis was carried out for 4 h and it was stopped by heating the reaction mixture at 85 °C in a hot water bath for 10 min. Then the slurry was cooled in a bath ice frozen, lyophilized and stored at 4 °C until used. In our laboratory, we have verified that this treatment does not affect the properties of the hydrolysates reaction products. The degree of hydrolysis (DH) was measured by the reaction of free amino groups with orthophthaldehyde, OPA, using a standard protocol applied to amaranth proteins in our laboratory (Sabbione, Ibañez, Martinez, Añón, & Scilingo, 2016). The hydrolysate composition obtained was 48.6 ± 3.0% w/w of proteins, 44 ± 1% w/w. of ash and 7.4 ± 0.3 w/w of carbohydrates determined by the same methods above indicated. All results are expressed in dry base. The moisture content was 9.36 ± 0.27% w/v.

2.3. Preparation of O:W emulsions

API and AH were dispersed at a 50:50 ratio at protein concentrations of 1% w/v and 2% w/v (hereafter: API50-AH50-1% and API50-AH50-2%) in phosphate buffer pH 2 (0.052 M H_3PO_4, 0.048 MKH_2PO_4, 0.052 M NaCl, ionic strength 0.1) and stirred 1 h at room temperature. Emulsions 20:80 O:W prepared with protein dispersions and commercial sunflower oil (δ = 0.918 g/mL, 20 °C) were homogenized in two steps. First, with an ULTRA-TURRAX T25 homogenizer (Janke & Kunkel GmbH, Staufen, German), at 20.000 rpm for 1 min, to produce coarse emulsions; and second, with an ultrasound homogenizer (SONICS Vibra Cell VCX750, Sonics & Materials Inc., CT, USA) at a power level of 50%, applying pulses of 30 s each for 5 min (Suárez & Añón, 2018). The standard tip was immersed 2/3 of the mixture volume in a 28 mm diameter glass placed into an ice bath to reduce heating during homogenization. Temperature was controlled to reduce heating during the both steps of the homogenization process.

2.4. Particle size distribution

The particle size distribution, mean De Brouker diameters ($D_{4,3}$) of the emulsion droplets were determined in freshly prepared emulsions with a Malvern MasterSizer 2S device, (Malvern Instruments Ltd, Worcestershire, UK), according to Suárez and Añón (2018). The variations of $D_{4,3}$ values at different times, in the presence or absence of SDS, was used to calculate the flocculation index (FI), as described by Ventureira et al. (2012). The particle size distribution was analyzed in freshly prepared emulsions and in emulsions subjected to simulated digestion. In freshly prepared emulsions, the determination was performed in absence and in the presence of 1% w/v SDS, whereas digestion products were analyzed in 2% w/v SDS.

2.5. Overall emulsion stability

The stability of the emulsions was determined using a Quick Scan vertical scan analyzer (Beckman–Coulter Inc., CA, USA) according to Suárez and Añón (2018). Samples were loaded into a cylindrical glass measurement glass tube, and the profiles of backscattering percentage (%BS) were monitored all along the tube every day for 8 days as a function of the sample height (total height = 60 mm). Glass tube were stored at 4 °C until the time of measurement. The creaming-flocculation and coalescence-flocculation kinetics were obtained following the variation mean %BS as a function of time in the lower part (10–15 mm height) and the upper part (40–50 mm height) of the cell, respectively.

2.6. Confocal microscopy

An inverted confocal microscopy (LEICA TCS SP5, Mannheim, Germany) equipped with Ar and a He-Ne laser was employed. Emulsions were studied through a non-covalent labeling with rhodamine B (Biopack, Buenos Aires, Argentina) dissolved in distilled water at 0.01% w/v. Excitation and emission wavelengths of 568 nm and 625 nm, respectively, were employed. Images were acquired using a 63× HCX PL APO CS oil immersion objective. A 20 μL sample was smeared on a slide and immediately covered with the rhodamine solution. Samples were incubated 30 min in a sealed container in the dark. Samples were then mounted using a cover slip. Images were analyzed with the LAS AF 2.2.1. build 4842 software provided by the manufacturer.

2.7. ACE inhibitory activity

The ACE inhibition assay was performed according to Hurst and Lowell-Smith (1981) with slightly modifications. Briefly, 25 μL of the soluble fraction of AH, API50-AH50 emulsion or API50-AH50 digested emulsion, were added to a 0.2 M borate buffer solution containing 2.0 M NaCl and 5 mM hyppuryl-histidyl-leucine (HHL), pH 8.3 and ACE (EC 3.4.15.1, 1, Sigma-Aldrich) from rabbit lung with specific activity 1.0 units/mL. The reaction mixture was incubated at 37 °C for 30 min and the enzymatic reaction was stopped by heating the mixture at 90 °C for 15 min. Six hundred μL of a 0.2 mol/L potassium phosphate buffer, pH 8.3, were then added together with 515 μL of a 30 g/kg cyanuric acid (2,4,6-trichloro-*S*-triazine) solution in dioxane. The mixture was vigorously vortexed until the solution became transparent. The hippurate released, which reacted with the cyanuric chloride, was quantified at 382 nm. The IC_{50} value was defined as the protein concentration (mg/mL) in the reaction mixture necessary to inhibit ACE activity by 50%. Captopril was used as positive control of ACE inhibition. The protein content in the supernatant was determined by the Lowry colorimetric method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.8. Simulated gastrointestinal digestion

The emulsions prepared as described in 2.3 were subjected to a simulated digestion procedure according to Malaki Nik, Wright, and Corredig (2011), with modifications. Briefly, 5 mL of the emulsion were incubated at 37 °C before digestion. Twelve mL of simile gastric fluid at 37 °C were then added and the pH was adjusted to 2 with HCl. The mixture was stirred for 1 h. Finally, 12 mL of simile duodenal fluid were added and the pH was adjusted to 7 with NaOH. The mixture was incubated for 1 h at 37 °C with constant stirring at 100 rpm using an orbital shaker (Fbr, Delcolab SRL, Buenos Aires, Argentina). Hydrolysis was stopped by heating the reaction mixture at 85 °C for 10 min in a water bath. Aliquots were taken for the initial emulsion, $E_{i;}$ at the end of the gastric step, Eg; and at the beginning (5 min), $E_{d5,}$ and at the end (60 min), E_{d60}, of the duodenal step in order to determine the particle size of oil droplets, as indicated above.

Reagents used were:

Simile gastric fluid: A solution containing 2.5 mg/mL pepsin (EC.3.4.23.1, MP Biomedicals, CA, USA) 1:15000 5X NF units/min was prepared in a 0.03 M NaCl and 0.1 N HCl solution. The fluid was incubated at 37 °C for 30 min with constant stirring.

Simile duodenal fluid: A solution containing 3 mg/mL pancreatin (EC 232-468-9, MP Biomedicals) 4X-100 USP (One Unit (TAME) hydrolyzes 1 μmol of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per minute at 25 °C, pH 8.1. Conversion of unit: 1 Unit TAME = 19.2 Units USP/NF = 57.5 Units BAEE) units/mg protein and 1.5 mg/mL lipase from porcine pancreas type II (EC 3.1.1.3, Sigma-Aldrich) 100–500 units/mg protein using olive oil-30 min incubation was prepared in a 0.1 M $NaHCO_3$ and 5 mM $CaCl_2$ pH 8.1. The fluid was incubated at 37 °C for 30 min with constant stirring. Porcine bilis (EC 232-369-0, Sigma-Aldrich) was prepared in a 0.1 M $NaHCO_3$ solution, maintained at 37 °C and used a final concentration of 2.5 mg/mL.

2.7.1. Determination of free fatty acids

The degree of lipolysis was determined through the quantification of free fatty acids during the duodenal stage. The quantification of the fatty acids released during the process was carried out by neutralization according to the technique described by the AOAC (940.28 method, (1990)). The lipolytic activity was determined as the percentage of fatty acids released at the time of digestion according to the following equation:

% FFA = ml NaOH x N NaOH x 28.2 / m oil (g)

where:m is the mass of oil present at the moment of incorporating the pancreatic lipase and 28.2 is the conversion factor to express the% FFA in oleic acid.

2.9. Statistical analysis

The statistical analysis was performed with the GraphPad Prism 6.0 (GraphPad Prism Inc., CA, USA). Data were expressed as the mean ± standard deviation of at least two independent experiments. The Analysis of Variance (ANOVA) was employed to compare means between groups through the Least Significant Difference (LSD) test, with a significance level of 0.05.

3. Results and discussion

3.1. Emulsifying properties of amaranth protein and peptides mixtures

3.1.1. Droplet size distribution in emulsions

For the determination of drop size, O:W emulsions were prepared at two protein concentrations, 1 and 2%, respectively. The droplet size distribution corresponding to the API50-AH50-1% (Fig. 1, panel A) and API50-AH50-2% (Fig. 1, panel B) suggested the existence of three and two particle populations, respectively. These populations had a droplet size of 11.2 ± 0.6 and 9.01 ± 0.05 μm, as determined by the $D_{4.3}$ diameters, evidencing a significant reduction in the droplet size as a function of the protein concentration. In the presence of 1% w/v SDS, (Fig. 1), a monomodal distribution was detected, which was characterized by a considerable decrease in the droplet size both in API50-AH50-1% (Fig. 1 panel C) and in API50-AH50-2% (Fig. 1, panel D), to achieve the similar $D_{4.3}$ values for both protein concentrations ($D_{4.3}$ 1.22 ± 0.01 and 1.28 ± 0.01 μm). This finding evidences the existence of flocs of different sizes in the freshly prepared emulsions, which is indicating by the FI values obtained. Table 1 shows the $D_{4.3}$ and FI values corresponding to the API50-AH50-1% and API50-AH50-2% emulsions. These values are compared to results obtained previously for emulsions prepared only with API at two similar total protein concentrations 0.8% and 1.7% w/v (Suárez & Añón, 2018). The presence of peptides of small size, coming from the incorporation of AH in this new emulsions formulation can modify the emulsifying capacity of amaranth proteins. Indeed, for both protein concentrations, the replacement of 50% of API by 50% of AH contributed to the decrease in the droplet size. The presence of proteins/peptides of different sizes and probably more flexible than the non-hydrolyzed amaranth protein, would allow a rapid decrease of the interfacial tension, a greater decrease in the particle size and a decrease in the particle-particle interaction, which is evidenced by the flocculation degree.

3.1.2. Overall stability of emulsions

The stability of emulsions prepared was determined. Fig. 2 shows the destabilization kinetics obtained in the lower (10–15 mm) and upper parts (40–50 mm) of the measurement glass tube of QuickScan. Emulsions kept undisturbed at 4 °C for 8 days to determine their

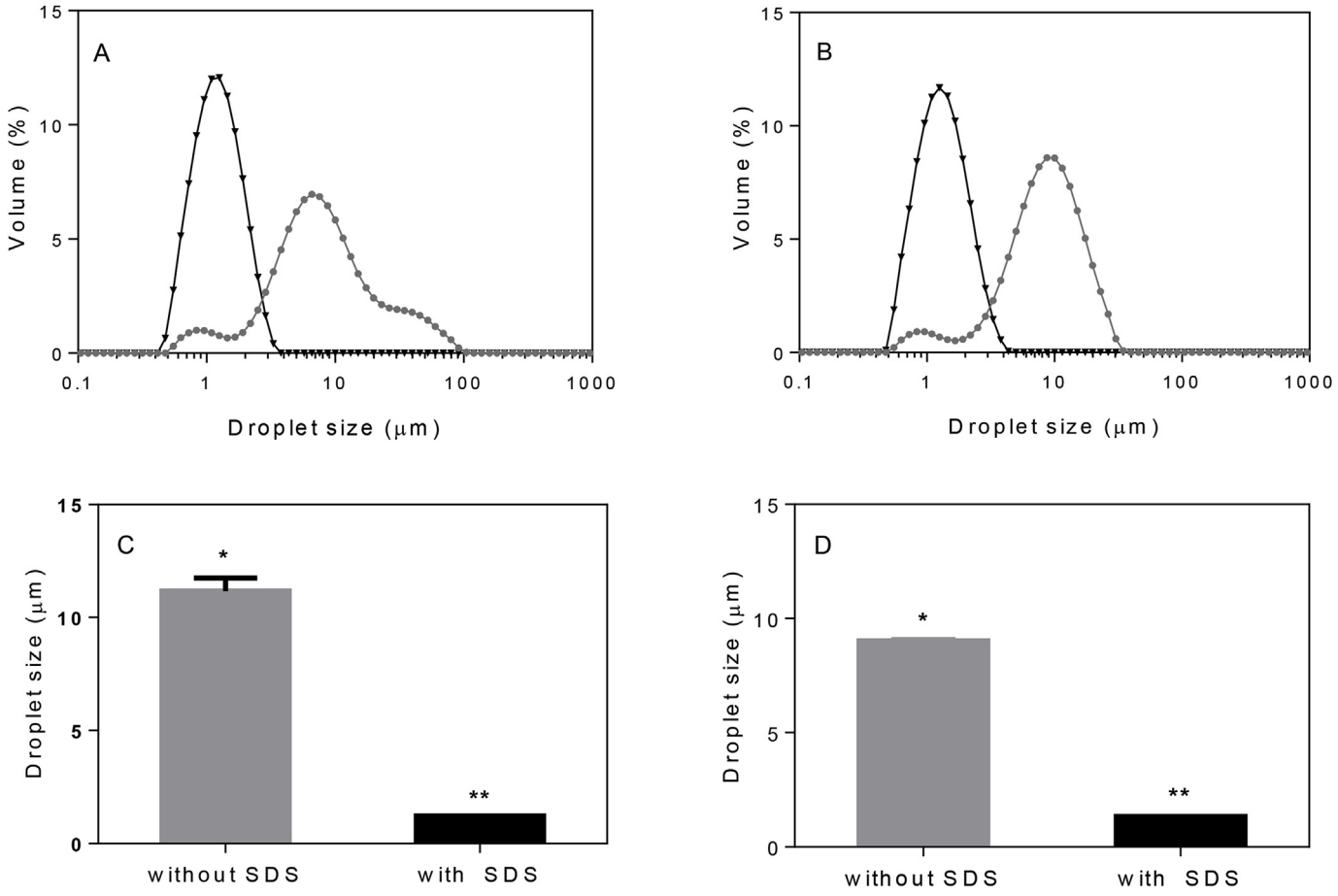

Fig. 1. Droplet size distribution of emulsions with API50-AH50 1% w/v (panel A) and 2% w/v (panel B) and their respective $D_{4,3}$ (panels C and D). Measurements were carried out in the absence (●) and in the presence of SDS (▼).

Table 1
De Broucker average diameter ($D_{4,3}$) and flocculation index (FI) of emulsions with API (0.8 and 1.7% w/v) and API50-AH50 (1.0 and 2.0% w/v).

Protein % w/v	$D_{4,3}$ (μm)	$D_{4,3+SDS}$ (μm)	FI
API			
0.8	18.8 ± 1.6[a]	2.33 ± 0.02[a]	7.1
1.7	20.4 ± 0.1[a]	1.23 ± 0.02[a]	16
API50-AH50			
1.0	11.2 ± 0.6[b]	1.22 ± 0.01[b]	8.2
2.0	9.01 ± 0.05[b]	1.28 ± 0.01[b]	5.9

Results are expressed as the means ± standard deviations of three replicates. Different superscript letters indicate significant differences between API vs API50-AH50 ($p < 0.05$).

stability during creaming and coalescence. The %BS values for the API50-AH50-1% (Fig. 2, panel A) and API50-AH50-2% (Fig. 2, panel B) emulsions, remained constant in the upper part, whereas in the lower part, %BS values decreased by 20% and 12.5% for 1% w/v and 2% w/v protein, respectively. This decrease in the %BS values accounts for the migration of oil droplets towards the upper part the cell. Nevertheless, no changes in light dispersion were detected in the upper part, suggesting that the oil droplets migrated at a low velocity, most probably due to the interference caused the presence of protein flocs in their trajectory or an incipient layer of very compact cream is formed (Table 1). This behaviour was also observed on emulsions elaborated only with API at concentrations where there is sufficient emulsifier to cover the entire droplet surface (Suárez & Añón, 2018). The analysis of the API50-AH50-2% emulsion by confocal microscopy revealed the anchoring of proteins onto the oil droplets and the formation of a protein floc network extending throughout the emulsion (Fig. 2, panels C and D). This network interacted with oil droplets of different size, which evidences the polydisperse nature of the emulsion. Similar phenomena was observed in O:W soy protein isolate emulsions where the flocs formed assist to the emulsion stability (Palazolo, Sobral, & Wagner, 2016; Palazolo, Sorgentini, & Wagner, 2005).

These results clearly support the formulation of stable emulsions using a mixture of native and hydrolyzed amaranth proteins with a good stability during at least 8 days. Since stable emulsions are obtained for both protein concentrations, we select the lowest concentration, 1% w/v, to continue with our study. Therefore, it is important to determine whether this type of emulsions can be employed as an angiotensin I-inhibitory emulsion and to determine its stability during a simulated digestion process.

3.2. Behaviour of amaranth peptides under simulated gastrointestinal digestion

3.2.1. Emulsion stability during simulated digestion

In order to analyze the potential of the API and API50-AH50 emulsions as delivers of bioactive peptides, they were subjected to a simulated digestion process. Firstly, the API-0.8% w/v was tested. Fig. 3 shows the droplet size distribution at different time points during the digestion (E_i, E_g, E_{d5} and E_{d60}). The particle size distribution was assessed in terms of percent volume and $D_{4,3}$ diameters (Fig. 3 insets), since the latter parameter is more representative of the destabilization processes taking place within the emulsion (McClements, 2015).

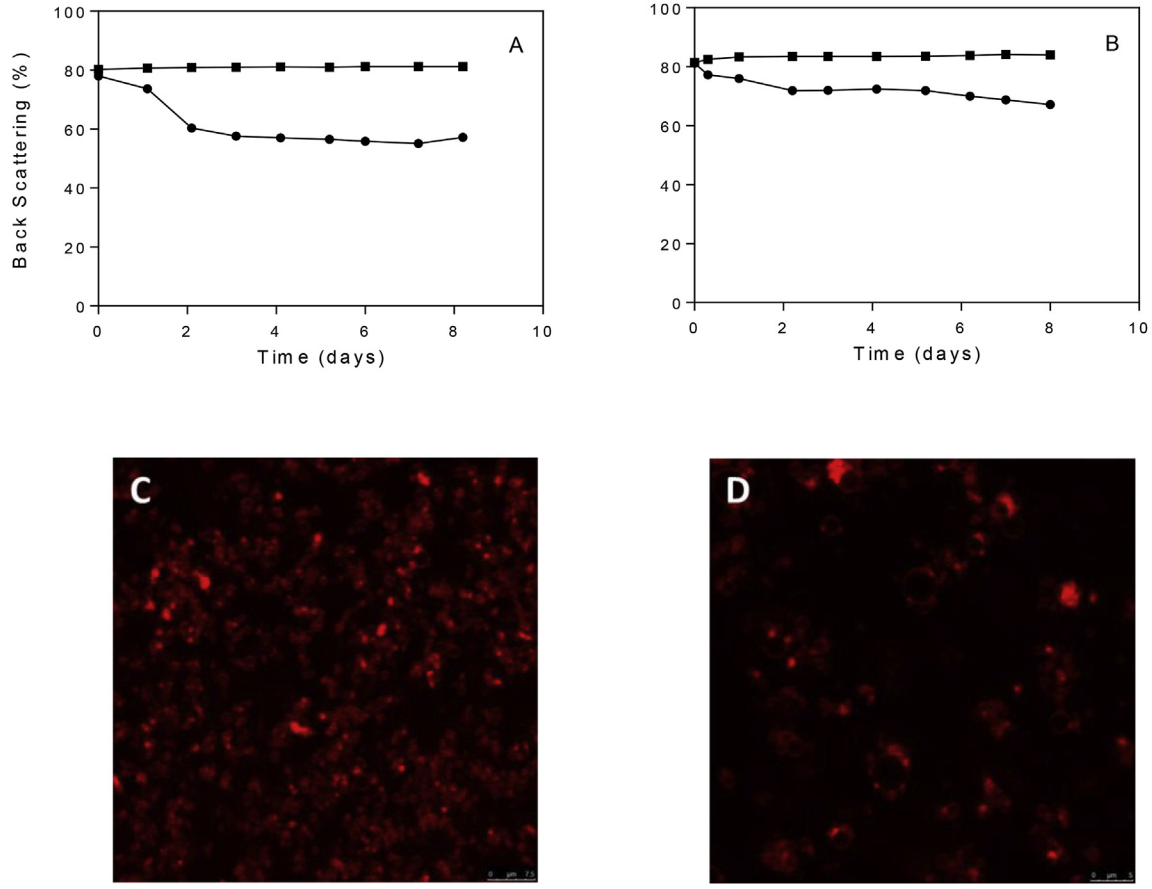

Fig. 2. Variations of the back scattering values, BS%, in zones 10–15 mm (-●-) and 40–50 mm (-■-) versus time for the emulsion: A) API50-AH50 1% w/v; B) API50-AH50 2% w/v. Confocal microscopy of emulsion API50-AH50 2% w/v stained using rhodamine B with different optical zoom magnification, panel C (3.7X) and panel D (5.0X).

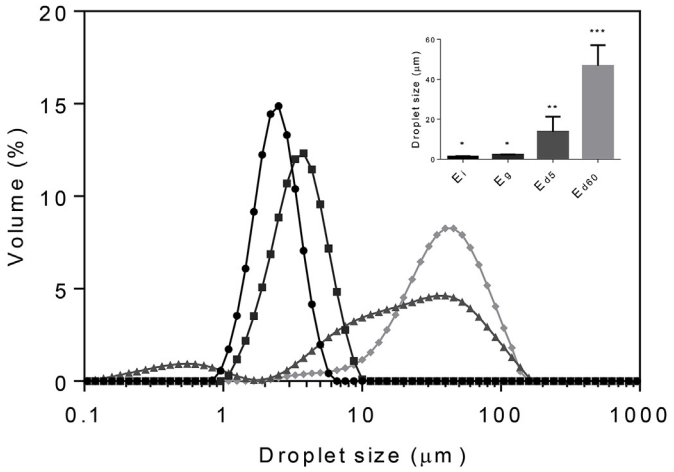

Fig. 3. Droplet size distribution with SDS 2% w/v of emulsion API 0.8% w/v (●), after gastric digestion (■), before (▲) and after (♦) duodenal digestion. Graph is representative of two independent experiments.

Results showed that the droplet size distribution obtained at E_i and E_g were monomodal, with a slight shift towards greater droplet sizes at the end of the gastric digestion that was not statistically significant.

The addition of simile duodenal fluid containing pancreatic enzymes and bile salts at neutral pH (7.0) induced a substantial modification of the profile: for E_{d5}, a minority population with lower particle size (< 1 μm) and another population with high polydispersity (2–200 μm) were identified, with a maximum of 30 μm. Before the addition of the simile duodenal fluid, the protein molecules present in the interface and in the continuous phase of the emulsion have a positive charge according their pI (Suárez & Añón, 2018), and therefore, they repel each other. Upon addition of the simile duodenal fluid, which modifies the pH value (7.0), the positive charge of the amaranth protein change (Suárez & Añón, 2018), but the intermolecular repulsion is still considerable, therefore the increase in the droplets size cannot be attributed to differences in the repulsion strength among droplets. Therefore, such increase must be attributed to the chaotropic effect of the bile salts, which generate interactions with the protein molecules at the interface and displace them to favour the occurrence of aggregation and coalescence phenomena. Bile salts also facilitate the emulsification of lipids preparing the droplets for the enzymatic action (Mun, Decker, Park, Weiss, & McClements, 2006). The existence of the smallest particles within the distribution, (0.1–1 μm) might be attributed to the hydrolysis of oil droplets by lipase. This reaction release of a small number of residual droplets that can remain dispersed due to the presence of molecules with surface activity in the duodenal fluid, such as bile and residual superficially active molecules released during the hydrolytic process (Malaki Nik et al., 2011). The presence of such small particles might also be due to the generation of other colloidal systems caused by the great number of superficially active molecules that are able to disperse the laser employed in the measurement (Gumus et al., 2017).

At the end of the duodenal digestion (E_{d60}), a decrease in the system polydispersity is observed, with a monomodal distribution of droplets (7 and 200 μm) and a mean size of 50 μm and a minor tail of size values ranging from 1 to 10 μm. The increase in the droplet size might be attributed to the proteolytic action of pancreatin at the interfacial film,

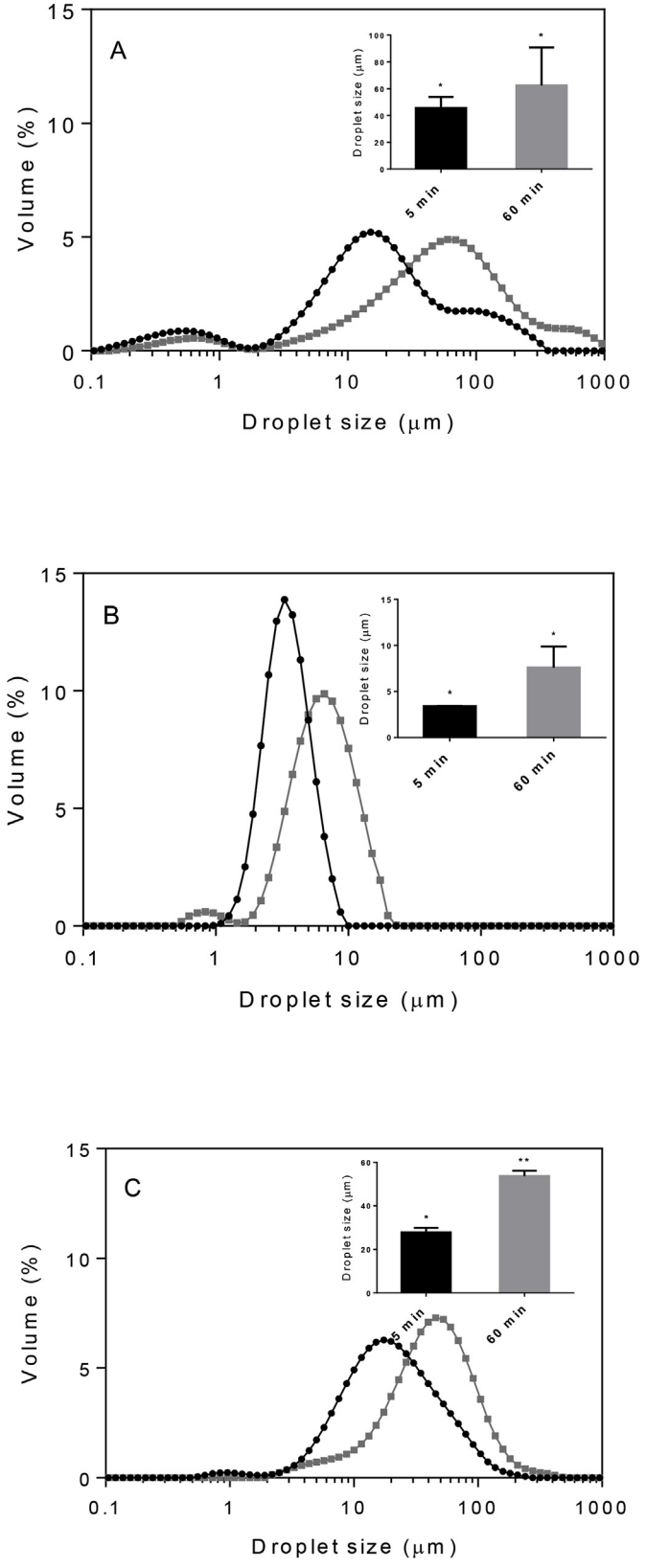

Fig. 4. Droplet size distribution with SDS 2% w/v of emulsion API 0.8% w/v, before (●) and after (■) duodenal digestion, without pancreatin (panel A), lipase (panel B) and bile (panel C). Graph is representative of two independent experiments.

which promotes droplets aggregation due to the gradual loss of surface charges and the reduction in the interfacial thickness (Bellesi, Martinez, Pizones Ruiz-Henestrosa, & Pilosof, 2016; Singh & Sarkar, 2011).

In summary, the considerable increase in the oil droplet size during the simulated digestion [1.6 ± 0.3 μm (E_i), 2.1 ± 0.2 μm (E_g), 18.7 ± 13.5 μm (E_{d5}) and 50.6 ± 8.7 μm (E_{d60})] could be due to changes in the ionic strength and pH values, as well as to the presence of biological components having activity in the interfacial film, such as bile salts and enzymes, among others.

In order to study in detail, the phenomena observed, mainly during the simulated duodenal digestion, several experiments were performed in which the digestion process was carried out in the absence of pancreatin, lipase or bile salts. Results are shown in Fig. 4. When the duodenal digestion was performed without pancreatin (Fig. 4, panel A), a considerable increase in the particle size was observed, as compared with the size observed at the end of the gastric digestion ($D_{4.3}$ 2.1 ± 0.2 μm). This increment occurs rapidly during the digestion ($D_{4.3}$ 45.5 ± 8.4 μm, E_{d5}) to increase thereafter ($D_{4.3}$ 62.5 ± 28.4 μm, E_{d60}). This finding suggests that the presence of bile salts and lipase are enough to cause changes in the system stability, namely, the increase of particle size. The bile present in the duodenal fluid has a key role in the destabilization of the emulsion, leading to the exposure of the lipid surface to favour the enzymatic attack, such as that of lipase. These results are in agreement with those of Mun, Decker, and McClements (2007), who also detected an increase in the $D_{4.3}$ values in emulsions of sodium caseinate and a protein isolate from whey after treating both emulsions with only lipase and bile salts.

In the absence of lipase (Fig. 4, panel B), and even though the droplet size increases when compared to the size obtained at the end of the gastric digestion, ($D_{4.3}$ 2.1 ± 0.2 μm, E_g, $D_{4.3}$, 3.41 ± 0.01 μm, E_{d5} and 7.6 ± 2.3 μm, E_{d60}). This increase is less marked than that obtained with the complete duodenal fluid ($D_{4.3}$ 50.6 ± 8.7 μm) and in the absence of pancreatin ($D_{4.3}$ 62.5 ± 28.4 μm), which indicates that lipase plays a key role in the destabilization of the emulsion.

In the absence of bile salts (Fig. 4, panel C), a considerable increase in droplet size is obtained ($D_{4.3}$, 27.9 ± 20.0 μm E_{d5} and 53.7 ± 2.4, E_{d60}). However, this increase is less marked, as compared to that obtained in the absence of pancreatin. It is thus evident that the absence of bile salts, which have a chaotropic effect in the emulsion interface, did not hamper the enzymatic activity of lipase and pancreatin.

Moreover, the lipolytic reaction was monitored during the duodenal digestion. To this end, the amount of fatty acids released, FFA, during the duodenal digestion was determined. A rapid increase was detected during the first 5 min (% FFA: 22.4 ± 1.0), and these values increased at 60 min (% FFA: 28.8 ± 2.0, E_{d60}) to remain stable up to 2 h after the beginning of the simulated duodenal digestion (% FFA: 29.5 ± 2.3). These findings evidence a rapid release of fatty acids without any induction phase. Other authors have reported that the adsorption of the enzyme onto the interface is immediate, provided the lipase concentration is adequate (McClements & Li, 2010; Mun et al., 2007). The reaction slowing down that was detected after 5 min could be associated to an accumulation of lipolysis products on the droplet surface, thus reducing the enzymatic activity. Furthermore, the lipolysis degree detected is in line with that obtained by Bellesi et al. (2016) and Gumus et al. (2017) in emulsions of soy protein, whey, pea, lentil and broad bean.

The API50-AH50-1% emulsions were subjected to the same process (Fig. 5). As it was mention before the two-protein concentration used for the emulsion preparation showed similar stability during the studying time. This fact and the possibility of developing a functional beverage using amaranth hydrolysate with lower cost were taking into account for chose the emulsion API50-AH50-1% for the bioactive studies. The initial particle size showed a bimodal distribution with $D_{4.3}$ = 1.2 ± 0.0 μm. At the end of the simulated digestion, the particles grouped into three well-defined populations, with $D_{4.3}$ = 79.5 ± 17.1 μm, E_{d60}. The two populations that have a greater

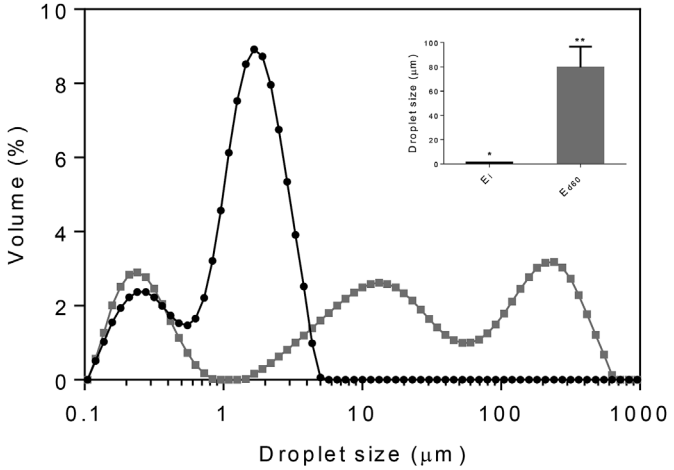

Fig. 5. Droplet size distribution with SDS 2% w/v of emulsion API50-AH50 1% w/v, before (●) and after (■) simulated gastric and duodenal digestion. Graph is representative of two independent experiments.

droplet size than the initial size (1–60 μm and 60–700 μm) are probably the result of destabilization phenomena, such as coalescence. The particle population whose size is less than 1 μm could have arisen as the result of the hydrolysis of oil droplets by lipase, with the release of a number of residual droplets that remain disperse, as stated above. Upon comparing the initial and final states of emulsions prepared with either API or API50-AH50, it can be observed that the droplet size distributions were not equivalent; that is, those emulsions prepared with equal amounts of the amaranth isolate and hydrolysate as tensioactive agent were more sensitive to destabilization processes occurring during the simulated digestion. This phenomenon was evidenced by the appearance of a population of large particles.

3.2.2. ACE inhibitory activity

The amaranth hydrolysate employed to prepare the active emulsion was prepared through an enzymatic digestion with alcalase. The hydrolysis degree obtained was 18.9 ± 0.2%. In order to assess the inhibitory activity of this hydrolysate a commercial ACE was used. The curve of inhibition as a function of protein concentration is shown in Fig. 6**.** The curve was adjusted to the sigmoidal behaviour expected for allosteric enzymes, which bear a subunit/catalytic site and a regulatory fraction interacting with each other. Inhibition curve showed a dose-dependent inhibition of ACE, which confirmed the presence of inhibitory peptides. The inhibition curve parameters are shown in Table 2. The K_h value, which is equivalent to the IC_{50} value, was 0.14 ± 0.02 mg/mL, respectively). The maximum inhibition, which is equivalent to the V_{max} value, 96.2 ± 6.4%, was high whereas the cooperativity index h, which is related to the number of active sites in an enzyme molecule, was positive 1.7 ± 0.3. This result might be due to the presence in the hydrolysate of various peptides with different kinetic behaviour. It must be borne in mind that ACE has two active domains, i.e., C and N, which have different conformational requirements; therefore, to achieve a complete inhibition, peptides with different characteristics could be required (Aluko, 2015; Guang, Phillips, Jiang, & Milani, 2012; Norris & FitzGerald, 2013). The commercial ACE rendered a captopril concentration-dependent inhibition curve. The IC_{50} value was 39.2- ± 11.2 nM. These inhibition values are in line with those reported elsewhere (Sentandreu & Toldrá, 2006; Vecchi & Añón, 2009; Henda et al., 2013).

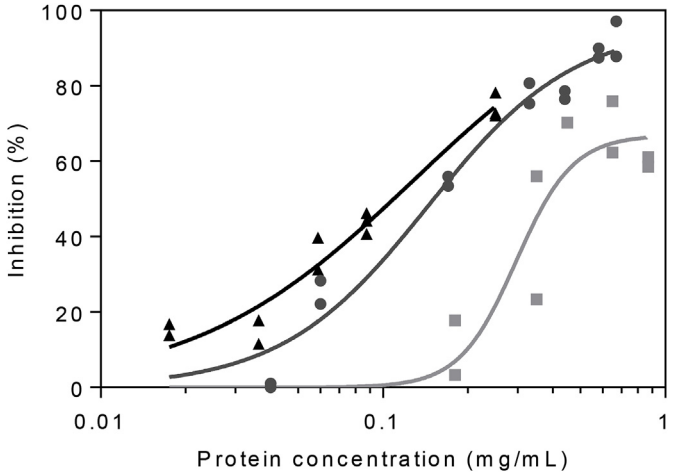

Fig. 6. Inhibition curves using amaranth hydrolyzate (AH, ●), API50-AH50 1% w/v emulsion (E_i, ■) and API50-AH50 1% w/v digested emulsion (E_{d60}, ▲) as inhibitors. Continuous lines represent the non-linear regression using allosteric sigmoidal equation for each data set.

Table 2
Adjustment parameters obtained from the inhibition curves using amaranth hydrolysate (AH), emulsion (E_i) and digested emulsion (E_{d60}) as inhibitors.

SamplesInhibitors	Parameters				
	V_{max}	h	K_h		R
	% Inhibition	–	nM	mg/mL	–
AH	96.2 ± 6.4[a]	1.7 ± 0.3[b]	–	0.14 ± 0.02[a]	0.9779
E_i	66.9 ± 7.1[b]	4.3 ± 2.1[b]	–	0.29 ± 0.03[b]	0.8787
E_{d60}	109 ± 28[a]	1.1 ± 0.3[a]	–	0.13 ± 0.07[a]	0.9717

Different superscript letters indicate significant differences between rows for each parameter ($p < 0.05$). Inhibition (%) = $V_{max}C^h/(K_h{}^h + C^h)$.$V_{max}$ is the maximum inhibition, C (mg/ml) is the concentration of inhibitor used, $K_h = IC_{50}$ and h is the Hill slope.

The ACE inhibitory effect of the API50-AH50-1% emulsions was assessed. The initial protein (E_i) and the digested protein (E_{d60}) were evaluated. The ACE inhibition curves obtained are shown in Fig. 6**.** Both the E_i and the E_{d60} exerted a soluble protein concentration-dependent inhibition. As above, the inhibition curves were also adjusted to an allosteric-sigmoidal behaviour (Table 2**).** The API50-AH50-1% peptides obtained in the E_i phase did not exert a complete inhibition of ACE and interacted with more than an active domain, whereas those peptides obtained at the end of the digestion (E_{d60}) caused a complete inhibition and proved to interact with only one enzyme site, similarly to the behaviour displayed by captopril and the AH. Probably, the presence of a less polydisperse and smaller size population of (poly)peptides favours the enzymatic inhibition. It is clear the ACE activity inhibitory peptides present in the initial emulsion were of a different nature and/or they were in a lower amount than the ones obtained in the final phase of the digestion.

The calculated IC_{50} value for E_i was 0.29 ± 0.03 mg/mL. This value doubles the one obtained when only the active hydrolysate is employed (IC_{50} 0.14 ± 0.02 mg/mL). It should be considered that the estimation of IC_{50} is based on the total protein concentration regardless of the molecular mass of the (poly)peptides present. Considering that, the E_i contains 50% API and 50% AH, and that the compounds present in the API exhibit a low inhibitory effect (Vecchi, 2007), the estimated IC_{50} value would represent mainly the activity of peptides present in the hydrolysate and therefore, the inhibition percentages for E_i and AH would be equivalent. In the case of the emulsion subjected to the gastrointestinal process (E_{d60}) an IC_{50} value 0.13 ± 0.07 mg/mL was obtained. In this case, both the API and AH proteins employed as tensioactive agents underwent digestion, being hydrolyzed to smaller size peptides which exerted ACE inhibitory activity.

The results presented in this work indicate that it was feasible to obtain highly flocculate stable emulsions formulated with a mix of

isolate and hydrolysate of amaranth protein. The fact that formulated emulsions are stable is extremely important if they are part of the formulation of a food and contain bioactive peptides which have to maintain their activity until their absorption. After a simulated digestion process, peptides with angiotensin inhibitory capacity were detected. The bioactive peptides could be part of the interfacial film and/or protected by the network of flocs present in the emulsion, either because it is part of the structure of the floc network or of the continuous phase where this network is immersed. These possibilities are being investigated in our laboratory.

It cannot be ruled out that during the simulated digestion process, novel antihypertensive peptides are generated through the gastric and duodenal enzymatic action.

The results obtained in this work are extremely promising for the production of functional foods, for example functional emulsified beverage, as vegetable milks, dairy products or others emulsified foods, formulated with amaranth proteins/peptides based on proven scientific knowledge.

4. Supporting information description

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