β -Lactoglobulin microparticles obtained by high intensity ultrasound as a potential delivery system for bioactive peptide concentrate

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Abstract This work attempted to assess the effect of high intensity ultrasound (HIUS) upon development of bio-based delivery systems, from β -lactoglobulin (β -Lg) gelled microparticles, for encapsulation of a bioactive peptide concentrate (PepC). Solutions of 150 g L⁻¹ of commercial β -Lg and 30 g L⁻¹ PepC, at various pH values (3.0, 4.0 and 5.5), were accordingly subjected to gelation for 30 min using a dry bath kept at 80 °C. The gelled systems were then exposed to HIUS at 0–4 °C, and the effect of processing time (2.5–20.0 min) was ascertained. Laser light scattering and confocal microscopy were used to characterize the particle size distribution, prior to and immediately after HIUS treatment. Gels obtained at pH 5.5 and 4.0 were harder than those obtained at pH 3.0. Ultrasound treatment of gels produced an important reduction in particle mean diameter as sonication time elapsed. Confocal microscopy indicated that application of HIUS led to almost round and monodispersed particles, at both pH 5.5 and 4.0. The peptide encapsulation efficiency was assessed by chromatography and accompanied by assay for

bioactivity, after precipitation of the encapsulated material and analysis of the soluble peptides therein.

Keywords Whey protein · Whey peptide concentrate · Microencapsulation · High intensity ultrasound

Abbreviations

HIUS	High intensity ultrasound
β -Lg	β -Lactoglobulin
PepC	Peptide concentrate
D ₃₂	Particle mean diameter
T _{gel}	Gelation temperature
DLS	Dynamic light scattering

Introduction

There has been a growing interest in the use of foods with bioactive peptides as agents against human chronic diseases and maintenance of human well-being (Özen et al. 2014; Udenigwe and Aluko 2012). A peptide concentrate (Pep C) obtained from whey, as described by Tavares et al. (2012a), is a promising candidate for nutraceutical additives—due to its major potential as ingredient in functional foods specifically designed for treatment of hypertension and ulcers, or to serve as anti-inflammatory adjuvant or antinociceptive agent (Tavares et al. 2011a, b, c, 2012b, 2013). In particular, PepC possesses several types of (previously confirmed) bioactivities, namely anti-hypertensive—with decrease in systolic blood pressure up to ca. 20 mmHg (Tavares et al. 2012b). Remember that pathways for synthesis of vasoregulator compounds exist that are effective upon blood pressure in the human body; control of such routes has traditionally focussed on the renin-angiotensin system, via inhibition of

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the angiotensin converting enzyme (ACE). To simplify the study, model validation has to be performed via quantification of inhibition of ACE—one of the most studied antihypertensive mechanisms (López-Fandiño et al. 2006). However, a major issue to be addressed is the passage of such peptides through the gastrointestinal tract—as they can undergo modifications, namely induced by hydrolysis, thus reducing their physiological activity. Hence, it is desirable to increase peptide bioavailability under the harsh conditions prevailing throughout digestion—and encapsulation of such sensitive compounds appears to be a suitable solution to overcome said limitation.

β -Lactoglobulin (β -Lg) may represent an excellent bio-based material to encapsulate peptides; in addition to GRAS nature, it has a high nutritional value, and is a major protein constituent (i.e. ca. 50–55% of whey fraction of bovine milk). This protein represents the primary gelling agent of whey proteins as it possess different binding sites, most of which lie in the vicinity of a Trp residue (Liang and Subirade 2012)—thus allowing binding of hydrophobic bioactive molecules via either primary amino groups, or ionic and hydrophobic binding (Wang et al. 1997; Zimet and Livney 2009). These features, coupled with its stability at low pH and high resistance to proteolytic degradation in the stomach (Jiang and Liu 2010), turn β -Lg into a promising material to produce biocompatible carriers for oral administration of sensitive compounds. Microencapsulation has been widely proven to improve delivery of bioactive compounds into foods (Champagne and Fustier 2007). Hence, several microencapsulation technologies have been developed for this purpose, and may promote successful delivery of bioactive ingredients to the gastrointestinal tract (Champagne and Fustier 2007).

One of the technologies that attracted a growing interest in recent years is high intensity ultrasound (HIUS). This is a novel, robust, green and rapid technology suitable for scaleup, and susceptible to enhance efficiency of bioactive peptide delivery. HIUS operates with a high frequency range (18–100 kHz) and wide power range (10–1000 W cm⁻²), and takes advantage of physical and chemical phenomena that are fundamentally different from those applied in conventional extraction, processing or preservation techniques (Arzeni et al. 2012). HIUS has been increasingly utilized in food science applications, e.g. microparticulation, emulsification, homogenization, crystallization, sterilization, degassing and extraction (Martini et al. 2010), because it offers several advantages; these include more effective mixing and micro-mixing, faster energy and mass transfer, reduced thermal and concentration gradients, reduced temperature, faster start-up, increased rate of production, and elimination of processing steps (Arzeni et al. 2012; Chemat et al. 2011).

Several studies have indeed explored the effect of HIUS upon functionality of biopolymers, by altering their molecular characteristics. For instance, HIUS was successfully applied to bovine serum albumin (Gülseren et al. 2007), whey protein isolates (Krešić et al. 2008), soy protein isolate (Karki et al. 2009) and egg white (Arzeni et al. 2012)—where it may bring about changes in protein structure (e.g. protein surface hydrophobicity, charge, solubility, binding, emulsifying and foaming capacity) that affect functionality. However information focussing on the potential effects of HIUS upon microparticles made from β -Lg is scarce, despite this protein being widely used in the food industry.

Therefore, the aim of the present work was to evaluate the impact of HIUS upon development of a bio-based delivery system, based on β -Lg microparticles, for encapsulation of a bioactive concentrate. The ratio of target peptide was also optimized, and the pH selected so as to mimic the environmental conditions prevailing in food (where the peptide is expected to be used).

Materials and methods

Materials

β -Lg was purchased from Danisco Foods International (USA), with a reported composition on a dry-weight basis of: $97.0 \pm 0.1\%$ (w/w) protein, $< 0.5\%$ (w/w) lactose, $0.3 \pm 0.1\%$ (w/w) fat, $2.5 \pm 0.2\%$ (w/w) ash and $5.2 \pm 0.5\%$ (w/w) moisture. PepC was obtained from a whey protein concentrate (WPC) from bovine sweet whey, and was characterized elsewhere (Tavares et al. 2012a)—leading to the following composition on a dry-weight basis: $72.9 \pm 0.7\%$ (w/w) protein and $27.3 \pm 0.9\%$ (w/w) lactose. WPC was subjected to several purification steps aimed at concentrating up to 86% protein (besides 10% lactose and 2% lipids), on a total solids mass basis. A hydrolyzate was then produced via incubation of WPC with a commercial aqueous (crude) extract from dried flowers of *Cynara cardunculus* (Formulab, Maia, Portugal). The enzyme/substrate (E/S) ratio was set at 1.6%(v/v), using 15 g protein L⁻¹ of enzyme solution and 40 g protein L⁻¹ of WPC solution. This mixture was incubated at pH 5.2 and 55 °C (previously found to be the optimum processing conditions); hydrolysis was quenched by 7 h, via heating at 95 °C for 15 min. This step was followed by ultrafiltration, aimed at separating the target peptide from non-hydrolyzed protein; PepC was then freeze-dried and kept at 4 °C prior to use. PepC possessed peptides with molecular weight ranging between 500 and 1200 Da, and exhibited an IC₅₀ (50% inhibitory concentration) of $23.6 \pm 1.1 \mu\text{g mL}^{-1}$ in terms of ACE-inhibitory activity (Tavares et al. 2011a, b).

Sample preparation

Three replicates of each solution: 30 g L⁻¹ PepC, 150 g L⁻¹ β-Lg and mixture of 150 g L⁻¹ β-Lg with 30 g L⁻¹ PepC were prepared in deionized water; pH was then adjusted to 3.0, 4.0 and 5.5, via addition of 1 N NaOH or 1 N HCl (as appropriate). Prior to use, all solutions were passed through filters with 0.22 μm pore width for filter-sterilization. In the case of PepC solutions, they were further filtered through 0.02 μm pore width filters (Millipore Corporation).

Sol–gel transition

The sol–gel transition of samples containing β-Lg and mixture of β-Lg with PepC, prepared at various pH values (i.e. 3.0, 4.0 and 5.5), was carried out according to Relkin et al. (1998), with modifications. Briefly, 2 mL of each sample was distributed through a set of six test tubes, which were hermetically sealed and heated in a dry bath (Thermoline dribath, Barnstead) at 100 °C. The inner temperature was controlled through a thermometer inserted into one of the tubes. A tube chosen at random was taken every minute, to check whether any deformation occurred in the meniscus upon tilting; the minimum temperature when this took place, as assessed by the naked eye, was recorded as the gelation temperature (T_{gel}). The average of at least two determinations was taken as a datum point. The gels were then produced in a dry bath at 85 °C for 30 min.

High-intensity ultrasound (HIUS) treatment

The gels, containing β-Lg and PepC, were dispersed in an equal volume of citrate buffer (0.01 M) at pH 3.0 or 4.0, or phosphate buffer (0.01 M) at pH 5.5, in order to obtain the final concentration of 75 and 15 g L⁻¹ of β-Lg and PepC, respectively, suitable for ultrasound application. Although the charge and chemical profile of the buffer itself influences protein properties, (distinct) buffers were required owing to the strong buffering capacity of the protein solutions themselves. The solutions were sonicated for 0.0, 2.5, 5.0, 10.0, 15.0 and 20.0 min, using an ultrasonic processor Vibra-Cell Sonics (model VCX 750, USA), operated at 20 kHz and 20% amplitude—with a 13 mm-high-grade titanium alloy probe, connected to a 3 mm-tapered microtip. The sonicated volume in each batch was 10 mL, using a 15 mL-glass test tube, which was glycerine-jacketed in the temperature range 0–4 °C using a continuous circulation bath (Poly-stat, Cole-Parmer, USA). Friction increases temperature of the dispersion as sonication time elapses, so the bath ensured that sonication took place within 20–30 °C.

Particle size distribution

Two instruments were used to measure particle size distribution, depending on the particle size (Gordon and Pilosof 2010). For particle sizes ranging from 0.1 to 1000 μm, a Mastersizer 2000E (Malvern Instruments Ltd., UK), equipped with a Hydro 2000 M/MU provided with a He–Ne laser (633 nm), was used. The measurement angle was set at 90°, and the volume-surface mean diameters were determined through the following equation:

$$D_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$$

where n_i is the number of droplets of diameter d_i ; D_{32} thus provided a measure of the mean diameter, i.e. where most particles fell (Jambrak et al. 2009).

The mean hydrodynamic diameter of particles ranging from 0.3 to 6000 nm was determined by dynamic light scattering (DLS), in a Zetasizer Nano-ZS Model ZEN3600 (Malvern Instruments Ltd., UK), equipped with a He–Ne laser (633 nm), using a digital correlator. Samples of 1 mL were poured into disposable polystyrene cuvettes with a path length of 12 mm, and measurements were carried out at a fixed scattering angle of 173° and at 25 °C. The values represent average ± standard deviation of ten measurements.

The size of particles before any treatment was assessed by a nanosizer instrument and analyzed without dilution, while the size of gelled particles submitted to HIUS treatment was assessed by Mastersizer.

Confocal microscopy analysis

Confocal laser scanning microscopy (Olympus Fluoview, FV300, USA), provided with a He–Ne laser (543 nm), was used to visualize the distribution of the mixture of β-Lg (150 g L⁻¹) and PepC (30 g L⁻¹), at various pH values, and after 0.0 and 20.0 min of HIUS treatment. The mixture of β-Lg and PepC was labelled with Rhodamine B Isothiocyanate—RBITC (Sigma-Aldrich, USA), and observed using PLAN APO 60X and 100X objectives. Briefly, 0.0015 g of Rhodamine solution (3 mg of dye in 1 mL of 100 mM sodium carbonate, pH 9.3) was added to the protein solution and incubated at 20 °C for 1 h. Unattached dye was removed by dialysis (cut off 100–500 Da; Spectra/Por CE; Spectrum laboratories; USA). After staining protein solutions, microparticles were prepared as described before (see “High-intensity ultrasound (HIUS) treatment” section). In order to identify each protein, the laser was adjusted to red (RBITC-labeled GMP) mode, which yielded an excitation wavelength at 561 nm.

High-performance liquid chromatography (HPLC)

In order to determine whether the PepC peptides were trapped in the β -Lg particles, the sonicated samples were subjected to centrifugation at $10,000\times g$ for 10 min—to separate the supernatant (supernatant I) from the pellet. The latter was dissolved in 50 mM Tris-HCl buffer (pH 6.8), containing 12.5 mM ethylenediamine tetracetic acid (EDTA), 10 mM dithiothreitol (DTT), 20 g L^{-1} sodium dodecyl sulphate (SDS), 100 g L^{-1} glycerol and 10 g L^{-1} β -mercaptoethanol to release the encapsulated PepC peptides, with concomitant disruption of any β -Lg aggregates. The solution obtained was centrifuged at $10,000\times g$ for 10 min—to separate the supernatant (supernatant II) from this second pellet.

Both supernatants, as well as the PepC sample, were subjected to reverse phase chromatography (RP-HPLC). The separation was carried out in a HP Agilent 1100 System (Agilent Technologies, Waldbronn, Germany), equipped with quaternary pump, automatic injector and internal degasser. A wavelength absorbance detector was set at 214 nm, and a ChemStation software was used for data acquisition and control (all 1100 Series, Agilent Technologies). The samples were injected at 50 μL , and separated using a Hipore[®] RP318 C₁₈ column ($250\times 4.6\text{ mm}$ in inner diameter, and 5 μm of particle size) from Bio-Rad Laboratories (Richmond, CA, USA). Samples were then eluted using 0.37%(v/v) trifluoroacetic acid (Sigma) in milli-Q water (solvent A), and a mixture of acetonitrile (Sigma) and 0.27%(v/v) trifluoroacetic acid (solvent B). Elution took place at a flow rate of 0.8 mL min^{-1} , under a linear gradient (from 0 to 45%) of solvent B in A within 90 min; absorbance of solvent was monitored continuously at 214 nm.

In vitro ACE-inhibitory activity determination

To ascertain the efficiency of PepC microencapsulation, the ACE-inhibitory activity, of both supernatants I and II, was quantified via a fluorimetric assay as per Tavares et al. (2011a). Toward this purpose, a non-linear fit to the data was performed to estimate the IC₅₀ value (using GraphPad Prism 5 as tool); data were expressed as mean $\pm$ standard deviation.

Results and discussion

Interactions between PepC and β -Lg in aqueous solutions

The interactions between PepC and β -Lg in aqueous solutions were determined by DLS, by analyzing the size

distributions of single β -Lg, PepC and their mixture, before thermal or ultrasound treatment.

The size distribution curves in volume obtained for PepC (30 g L^{-1}), β -Lg (150 g L^{-1}) and mixture of β -Lg (150 g L^{-1}) and PepC (30 g L^{-1}) dispersions, at various pH, values are shown in Fig. 1. All solutions exhibited monomodal particle size distributions. At pH 3.0 (Fig. 1a), PepC and β -Lg isolated or mixed showed similar size distributions, in nanometer range, i.e. below 10 nm. At pH 4.0 (Fig. 1b), PepC, β -Lg and their mixture displayed different size distributions: PepC (peak centered at 1 nm) showed the lowest particle sizes when compared to other protein solutions, and a reduction when compared to that at pH 3.0. This peak was followed by the protein mixture solution (peak centered at 10 nm), and entails an increase when compared to that at pH 3.0; however both peaks are present in the nanometer range. For β -Lg at pH 4.0, the peak appeared in micro scale range (i.e. $> 1000\text{ nm}$). This increase vis a vis with the values found at pH 3.0 may be derived from the pH change. At pH near the pI of β -Lg (4.6–5.2), the protein starts to aggregate, so the volume of aggregates of larger sizes ($> 1000\text{ nm}$) increase—see Fig. 1. Close to pI, the proteins contain essentially equivalent positive and negative charges, and interaction between charges with opposite sign may be implicated in aggregation—besides hydrophobic interaction and hydrogen bonding (Nicolai et al. 2011).

At pH 5.5 (Fig. 1c), PepC and the mixture of proteins remained essentially unchanged—with peaks centered at 3 and 8 nm, respectively, when compared to that at pH 4.0. On the other hand, the size of β -Lg particles (in volume) decreased to nanometer range (peak centered at 10 nm). This may be explained by β -Lg being completely reversed when pH is increased or decreased away from the pI. The rate of aggregation is maximum at ca. pH 4.6 (Majhi et al. 2006), and negligible at pH 5.5 (Mehalebi et al. 2008).

At both pH values, the mixed solution showed a size distribution of ca. 10 nm, where the peak corresponding to PepC does not appear anymore. This can be taken as evidence of molecular interaction between PepC, with molecular weight range of 500–1200 Da, and β -Lg. It has recently been shown by DLS that β -Lg strongly interacts with such other peptides as caseinomacropeptide (Martinez et al. 2012).

The amino-acid sequence and 3-dimensional structure show that β -Lg is a lipocalin, a widely diverse family; and that most of it binds to small hydrophobic ligands, thus allowing it to act as a specific transporter. There is enough evidence that the binding site for hydrophobic ligands lies within the central calyx of the protein structure (Flower et al. 2000).

Fig. 1 Particle size distribution of (light gray line) PepC (30 g L^{-1}), (dark gray line) β -Lg (150 g L^{-1}), and (black line) mixture of β -Lg (150 g L^{-1}) and PepC (30 g L^{-1}) solutions before thermal and HIUS treatments, at various pH values: **a** 3.0, **b** 4.0 and **c** 5.5, performed with a Zetasizer Nano-ZS equipment

Gelation

The determination of T_{gel} showed that PepC solutions (30 g L^{-1}) did not undergo gelation, and that gels obtained with β -Lg or β -Lg containing PepC exhibited T_{gel} values of 80 ± 0.9 , 80 ± 1.4 and 75 ± 0.7 °C, at pH 3.0, 4.0 and 5.5, respectively. Although gelation temperatures at a given pH were not affected by presence of PepC, the time required to reach that temperature varied, depending on pH. For pH 3.0, it decreased twofold, whereas for pH 4.0 and 5.5 it increased twofold or threefold, respectively. Stronger and self-supporting β -Lg with PepC gels were obtained at pH 4.0 and 5.5 (Fig. 2); within this pH range, close to the isoelectric point of β -Lg, protein associates through formation of disulfide bonds, due to reduction of electrostatic repulsion. Under these conditions, aggregation

Fig. 2 Morphology of gels obtained from mixture of β -Lg (150 g L^{-1}) and PepC (30 g L^{-1}), at various pH values

is accelerated by heat, and leads to formation of a white opaque strong gel composed of micrometer-sized particulate random aggregates (see Fig. 2) (Nicolai et al. 2011; Ramos et al. 2014).

On the other hand, only a soft gel was obtained at pH 3.0, composed of finely stranded nanometer-thick networks, with transparent or translucent appearance and rubbery texture (see Fig. 2). It was formed under conditions where intermolecular electrostatic repulsion is dominant, known to occur at pH values far from the pI (i.e. pH 3.0). This piece of evidence may be explained by the fact that disulfide bonds may form at this pH, but only to a low extent, or even not form at all—due to a very low reactivity of thiol groups (Liu et al. 1994).

Taking into account the potential interactions between PepC and β -Lg in aqueous solution (results presented above), coupled with the gelation results, one infers that the isoelectric point of β -Lg can be slightly changed following addition of PepC. This effect is especially apparent at pH 4.0 (Fig. 1); the particle size of β -Lg containing PepC is lower than that of plain β -Lg solution, which could point at a change in the isoelectric point of β -Lg. Moreover, the data obtained for gelation of β -Lg corroborates this observation: i.e. the time and temperature required to gel by the solution composed by β -Lg and PepC were higher compared to those of the β -Lg solution.

As mentioned above, decreases of electrostatic repulsion promote rearrangement of its structure to octamers, close to the isoelectric point of β -Lg—thus favoring gelation; this is consistent with the lower T_{gel} value found at pH 5.5.

Size distribution after HIUS treatment of gels

The physical and structural properties of proteins may sometimes change due to mechanical reasons. Ultrasonic vibrations cause partial denaturation that significantly affects the physical and structural properties of proteins. Previous studies have shown that ultrasound (Bryant and McClements 1998; Jambrak et al. 2008), and HIUS in particular (Arzeni et al. 2012; Gülseren et al. 2007; Karki et al. 2009; Krešić et al. 2008) can significantly change the

structural properties of whey proteins. However, only few studies have actually focussed on size reduction of protein clusters, e.g. soy protein isolates and concentrates (Jambrak et al. 2009) and whey proteins (Gordon and Pilosof 2010).

The results of particle size distribution (Fig. 3) showed that application of HIUS for 2.5–20.0 min sonication time reduced the average diameter of gels composed by β -Lg and PepC at pH 4.0 and 5.5. At pH 3.0, the test was also performed; however, the experimental data obtained were not reproducible, besides being recorded below the detection limit of the Mastersizer instrument (so they are not presented here).

At pH 4.0, application of HIUS for 2.5 min produced a reduction in average diameter of 8.5 μ m—with a typical decrease from 10.8 ± 0.05 to 1.13 ± 0.2 μ m (see Fig. 3a); however, no decrease with sonication time was found. At pH 5.5, the average diameter was reduced by 46.1 μ m, i.e. from 54.9 ± 0.3 down to 8.8 ± 0.1 μ m (see Fig. 3b) after sonication for 2.5 min; however, a continuing decrease was observed up to 10.0 min of treatment. At this pH, the D_{32} reduction was higher because the size of aggregates was greater—so their reduction was faster, and more visible when compared to small ones (Lu et al. 2002). The small size of particles at pH 4.0 also explains why their size was not further decreased by long sonication times (Fig. 3a).

The results of particle size distribution obtained at different sonication times (Fig. 4) indicate that HIUS treatment caused a decrease in particle size (for all sonication times, their distribution was narrowed); this may lead to an increase of its free surface, as reported elsewhere (Jambrak et al. 2014).

At pH 4.0 (Fig. 4a), the particles in the original gelled sample (before HIUS) exhibited a bimodal distribution—yet with a dominant peak at ca. 10 μ m. By 2.5 min of sonication or longer (i.e. 2.5–20 min), the distribution remained bimodal, but was characterized by a smaller size range (< 10 μ m). On the other hand, the particles in the original gelled sample (before HIUS) exhibited a high range of sizes (5–1000 μ m) at pH 5.5 (Fig. 4b), which was reduced to monomodal dispersion upon application of sonication (as can be observed at all sonication times), with

Fig. 3 Particle average diameter (D_{32}) of mixture of β -Lg (150 g L^{-1}) and PepC (30 g L^{-1}) solutions at pH **a** 4.0 and **b** 5.5, within sonication time, performed with a Mastersizer 2000E equipment

Fig. 4 Particle size distribution of mixture of β -Lg (150 g L^{-1}) and PepC (30 g L^{-1}) solutions at pH **a** 4.0 and **b** 5.5, after (gray dotted line) 0.0, (light gray dashed line) 2.5, (dark gray dashed line) 5.0, (gray line) 10.0, (black dotted line) 15.0 and (black dashed line) 20.0 min of HIUS treatment, performed with a Mastersizer 2000E equipment

a size distribution within 40–60 μm after 2.5 min of sonication, or 4–20 μm after 10.0 min of sonication.

HIUS-induced structural changes in proteins are associated to partial cleavage of intermolecular hydrophobic interactions, rather than peptide or disulphide bonds. When particles are subjected to shear stress, increases occur in the speed of aggregation as an outcome of increase in collision rate (Jambrak et al. 2014). As the sonication time of HIUS treatment increases, the particle size is reduced and the particle size distribution is narrowed down (Fig. 4). By reducing the size of particles, the free surface of the material increases. In this case, the particles are reduced because of cavitation forces; this includes disruption of agglomerates and aggregates. Ultrasonic cavitation is indeed quite effective in breaking up agglomerates, aggregates and even smaller particles, thus overcoming the prevailing van der Waals's forces (Jambrak et al. 2014).

The most effective treatment was at pH 4.0 and lasted for 2.5 min, and at pH 5.5 for 10.0 min of sonication—once the smaller particles obtained under these conditions may resist more effectively than larger ones; according to Iida et al. (2008), chances for damage by cavitation decrease due to shorter relaxation times. The particles obtained from gels at pH 5.5 may be the most useful for microencapsulation of the peptide extract, due to their higher uniformity in particle size.

Morphological characterization after HIUS treatment

Apart from reducing particle size, application of HIUS led to round and almost monodisperse particles at pH 5.5 and pH 4.0, as can be seen in the confocal images labelled as Fig. 5—where the protein particles appear red-stained in the presence of rhodamine. The higher fraction of round particles is consistent with that observed for other materials treated with HIUS (Li et al. 2007; Rajan and Pandit 2001).

At pH 3.0, the whole image appears red, thus indicating formation of very small particles not observable under confocal microscopy. Moreover, the HIUS treatment completely disintegrated the soft gel at this pH, as apparent from inspection of Fig. 2; this accounts for the difficulty in monitoring particle size via light scattering with the Mastersizer instrument (minimum size of 0.1 μm).

The results obtained through confocal microscopy analysis are consistent with the data reported above, encompassing D_{32} (see Fig. 3) and particle size distribution (see Fig. 4) over sonication times—as determined by light scattering.

Efficiency of encapsulation of PepC

In order to determine whether PepC peptides became entrapped in the gelled β -Lg particles, supernatants from

Fig. 5 Confocal micrographs of particles obtained from mixture of β -Lg (150 g L^{-1}) and PepC (30 g L^{-1}) at various pH values after 0.0 and 20.0 min of HIUS treatment

sonicated samples at pH 4.0 or 5.5 (supernatant I), as well as supernatants from β -Lg particles containing PepC treated with a mixture of disrupting agents to liberate bound PepC (supernatant II) were subjected to RP-HPLC. A typical example is depicted in Fig. 6. Absence of a peptide profile of PepC in the supernatant suggests that peptides were mostly bound to gelled β -Lg microparticles. This result was somehow expected, since PepC and β -Lg interact in aqueous solution even prior to gelation or microparticulation (Fig. 1). Similar results were found for gelled microparticles at pH 4.0, consistent with data pertaining to ACE-inhibitory activity of supernatant I—with IC_{50} values above $1000 \mu\text{g mL}^{-1}$. Note that values of this order of magnitude do not indicate ACE-inhibitory activity—thus suggesting that peptides responsible for biological activity of PepC were not present in supernatant I, at least in active form(s).

Regarding the HPLC profile (see Fig. 6) of particles treated with a mixture of disrupting agents used to release PepC (supernatant II), some peaks corresponding to the

PepC profile itself are apparent—yet they are characterized by low intensity; this further indicates that PepC was entrapped and strongly bound to gelled β -Lg microparticles. However, supernatant II possessed ACE-inhibitory activity, of 96.7 ± 12.4 and $68.3 \pm 7.5 \mu\text{g mL}^{-1}$ for pH 4.0 and pH 5.5, respectively. When compared with the activity before encapsulation ($23.6 \pm 1.1 \mu\text{g mL}^{-1}$) and the value obtained for supernatant I, one realizes that most bioactivity was preserved in the extracts.

Even though the focus of the present work was an evaluation of the impact of HIUS upon development of bio-based delivery systems, a final word is deserved by the putative feasibility of delivering pepC from the β -Lg microparticles under physiological conditions. Teng et al. (2015) claimed that β -Lg resists peptic digestion, so pepC release during the early phase of digestion (i.e. mouth and stomach) is not likely; release will eventually occur later in digestion, but only after trypsin and pancreatin—secreted by the pancreas into the intestinal fluid, have a chance to act upon β -Lg, along with a pH increase up to 7.5.

Fig. 6 RP-HPLC peptide profiles of supernatants obtained from mixture of β -Lg (150 g L^{-1}) and PepC (30 g L^{-1}), at pH 5.5 after 20.0 min of HIUS treatment, as compared to PepC profile

Furthermore, once the integrity of the microparticle is disrupted, the molecules of β -Lg tend to form a polymerized dimer—with drug-binding sites located in regions readily accessible to the intestinal aqueous fluid (Chen and Subirade 2015; Izadi et al. 2016), thus promoting release of otherwise bound pepC.

Conclusion

The feasibility of encapsulating a peptide concentrate possessing biological activity in micron-sized β -Lg particles via gelation and further HIUS treatment was experimentally assessed. β -Lg interacted with the peptide concentrate in solution and upon gelling, and HIUS proved an effective technique to control size and shape of the resulting protein particles. The particle size distribution has shown that HIUS treatment caused a decrease in particle size and narrowed their distribution. HIUS-induced structural changes in proteins are associated with partial cleavage of intermolecular hydrophobic interactions, rather than peptide or disulphide bonds. Accurate selection of pH and sonication time may allow for control and optimization of size of particles, and even their shape. Ultrasound appears indeed as a robust and economically feasible option for delivery of peptide drugs.

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