Microencapsulation of a whey protein hydrolysate within micro-hydrogels: impact on gastrointestinal stability and potential for functional yoghurt development

Laura G. Gómez-Mascaraque[1], Beatriz Miralles[2], Isidra Recio[2], Amparo López-Rubio[1*]

[1] Food Quality and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

[2] Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM, CEI UAM+CSIC), Madrid, Spain

*Corresponding author: Tel.: +34 963900022; fax: +34 963636301

E-mail address: amparo.lopez@iata.csic.es (A. López-Rubio)

ABBREVIATIONS:
α-La: α-lactalbumin
β-Lg: β-lactoglobulin
FT-IR: Fourier transform infrared spectroscopy
Rt: Retention time
SEM: Scanning electron microscopy
SGF: Simulated gastric fluid
SIF: Simulated intestinal fluid
SSF: Simulated salivary fluid
TIC: Total ion current
WPC: Whey protein concentrate

ABSTRACT

Gelatin and chitosan micro-hydrogels containing a potentially bioactive whey protein hydrolysate were developed through spray drying and the impact of microencapsulation on protection during digestion and peptide stability against lactic acid fermentation during yoghurt manufacturing was assessed. The results showed that the protection exerted by the encapsulation structures during milk fermentation was sequence- and matrix-dependent, being chitosan more effective than gelatin in stabilizing the peptides. However, only 5 out of the 21 fermentation-susceptible peptides identified could be protected through encapsulation within chitosan (1 of which was also protected by gelatin). Moreover, the encapsulation within chitosan microparticles did not substantially affect the peptide profile of the digested hydrolysate, and therefore, the peptide bioaccessibility was not expected to be compromised.

KEYWORDS

Microencapsulation, chitosan, gelatin, hydrolysate, lactic fermentation, peptide

1. Introduction

Biologically active peptides are specific fragments of proteins with 2 to 20 amino acids that have desirable biological activities (de Castro & Sato, 2015). Specifically, bioactive peptides derived from milk proteins have attracted great interest in the field of functional foods (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Korhonen, 2009) because of their potential ability to promote human health by reducing the risk of chronic diseases or enhancing our natural immune system (Korhonen & Pihlanto, 2006; Nongonierma & FitzGerald, 2015). These peptides are inactive within the sequence of the precursor proteins and need to be released (by proteolysis) to exert their physiological functions (Meisel, 1997). Although normal gastrointestinal digestion of milk leads to some release of active peptides, a number of techniques based on fermentation and/or enzymatic hydrolysis have been investigated to produce bioactive peptide-enriched protein fractions (de Castro & Sato, 2015), while adding value to by-products from the food industry (Mora, Reig, & Toldrá, 2014).

A number of bioactive peptides have already been studied and recent reviews suggest that new research should focus on the application of these functional ingredients to commercial food products (Mohan, Rajendran, He, Bazinet, & Udenigwe, 2015). Functional foods have become popular and commercially successful in some sectors of the food industry, especially in fermented dairy products, partly due to their general acceptance among consumers (Siró, Kápolna, Kápolna, & Lugasi, 2008). However, fortification of these food products with protein hydrolysates is challenging, not only because of their low bioavailability, bitter taste, hygroscopicity and their likelihood of interacting with the food matrix thus altering food texture and colour (Elias, Kellerby, & Decker, 2008; Mohan et al., 2015), but also because of their susceptibility to

57 degradation by lactic acid bacteria during fermentation (Paul & Somkuti, 2009; Paul &
58 Somkuti, 2010).

59 Microencapsulation technologies, i.e. processes in which the ingredients of interest are
60 coated with or embedded within a protective matrix (Jiménez-Martín, Gharsallaoui,
61 Pérez-Palacios, Carrascal, & Rojas, 2014) obtaining micron-sized materials, are
62 regarded as an effective approach to overcome the aforementioned limitations (Vaslin,
63 Le Guillou, Hannoucene, & Saint Denis, 2006), and have been successfully used for the
64 preservation of biologically active ingredients in food systems (Munin & Edwards-
65 Lévy, 2011; Santhanam, Lekshmi, Chouksey, Tripathi, & Gudipati, 2015), including
66 protein hydrolysates and peptides (Mohan et al., 2015). Among the numerous
67 encapsulation techniques, spray-drying is the most commonly used one in the food
68 industry (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). It consists of an
69 initial atomization of a formulation containing the protective matrix and the bioactive,
70 and subsequent rapid drying of the obtained droplets using a hot gas stream to produce
71 dry microparticles. Although spray-drying has been extensively applied for the
72 protection of peptides and hydrolysates (Favaro-Trindade, Santana, Monterrey-
73 Quintero, Trindade, & Netto, 2010; Ma et al., 2014; Subtil et al., 2014; Wang, Ju, He,
74 Yuan, & Wang, 2015), there is still lack of information about the impact that
75 encapsulation may have on the functionality and stability of the peptides (Mohan et al.,
76 2015).

77 Both proteins and polysaccharides can be used as protective matrices for the
78 encapsulation of protein hydrolysates by spray-drying (Mohan et al., 2015). However,
79 there is no consensus in the literature regarding the best choice among them, an aspect
80 which should also be explored. In general, hydrogel-forming biopolymers are
81 particularly interesting, as they can be processed in aqueous solutions while preventing

disruption of the produced microparticles in aqueous environments under certain conditions (Gómez-Mascaraque, Méndez, Fernández-Gutiérrez, Vázquez, & San Román, 2014). In this sense, chitosan, a linear polysaccharide obtained by deacetylation of chitin and consisting of β-1,4 linked 2-acetamido-2-deoxy-β-D-glucopyranose units and 2-amino-2-deoxy-b-D-glucopyranose units in a proportion which depends on its degree of deacetylation (Khor & Lim, 2003), is considered a pH-sensitive hydrogel-forming biopolymer (Lim, Hwang, Kar, & Varghese, 2014). On the other hand, gelatin, a protein obtained from partial hydrolysis of collagen and containing repeating sequences of glycine-aa1-aa2, where amino acids aa1 and aa2 are mainly proline and hydroxyproline (Lai, 2013), is considered a thermo-responsive hydrogel-forming biopolymer. Thus, both chitosan and gelatin are edible, naturally-derived and hydrogel-forming biopolymers with potential application in the microencapsulation of protein hydrolysates.

In this work, a whey protein hydrolysate was produced and used as a model peptide-enriched protein fraction to study its microencapsulation by spray-drying within two different biopolymers, a polysaccharide (chitosan) and a protein matrix (gelatin). The implications of its microencapsulation, in terms of protection of the peptides during gastrointestinal digestion and lactic acid fermentation, were studied and the results were compared for both encapsulation matrices. For this purpose, the free and encapsulated hydrolysate were subjected to *in-vitro* gastrointestinal digestion and the peptide profiles were obtained by liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). In addition, commercial UHT low fat milk was supplemented with the microencapsulated and non-encapsulated hydrolysate and fermented to produce yogurts. The protective ability at peptide level of chitosan and gelatin during the assays was compared.

2. Materials and Methods

2.1. Materials

A bovine whey protein concentrate (WPC) was purchased from Friesland Campina Ingredients (Zwolle, The Netherlands). Type A gelatin from porcine skin, with reported gel strength of 175 g Bloom, low molecular weight chitosan, with reported Brookfield viscosity of 20.000 cps, potassium bromide FT-IR grade (KBr), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and bile extract porcine were all obtained from Sigma-Aldrich (Madrid, Spain). 96% (v/v) Acetic acid was purchased from Scharlab (Barcelona, Spain) and Pefabloc® from Fluka-Sigma-Aldrich. All inorganic salts used for the *in-vitro* digestion tests were used as received. Freeze-dried concentrated lactic cultures sachets, under the commercial name of YO-MIX ™, were obtained from Danisco (Sassenage, France). Commercial UHT low fat milk was bought from a local supermarket (Hacendado, Valencia, Spain).

2.2. Preparation of the hydrolysate

The WPC was dissolved in water 5% (w/v) and heated at 90 ºC for 10 min. Hydrolysis was carried out in triplicate at 37 ºC and pH 8.0 by addition of 1M NaOH for 3 h with constant agitation. Food grade trypsin (Biocatalyst, Nantgarw, UK) was used at an enzyme-to-substrate ratio of 1:20 (w/w). Reactions were stopped by heating at 95 ºC for 15 min, to ensure the complete inactivation of the enzyme. The hydrolysate was then spray-dried. The inlet temperature of spray drying was maintained at 140 ºC and the

outlet temperature was between 75 and 100 ºC, following the method described in Contreras et al., 2011.

2.3. Microencapsulation of the hydrolysate

The hydrolysate was microencapsulated within gelatin and chitosan particles by spray-drying. The hydrolysate (30% w/w with respect to the total solids mass) was dispersed in gelatin (10% w/v) or chitosan (2% w/v) stock solutions in acetic acid 20% (v/v). After a 50-fold dilution, the dispersions were fed to a Nano Spray Dryer B-90 apparatus (Büchi, Switzerland) equipped with a 7.0 μm pore diameter cap. The inlet air temperature was set at 90 ºC, the inlet air flow rate was 150 L/min and the pressure 50 mbar. The outlet air temperature was 50 ± 5 ºC. The spray-dried powders were deposited on the collector electrode by means of an applied voltage of 15 kV.

2.4. Morphological characterization of the particles

Samples were sputter-coated with a gold-palladium mixture under vacuum and observed by scanning electron microscopy (SEM) using a Hitachi microscope (Hitachi S-4100) at an accelerating voltage of 10 kV and a working distance of 15-16 mm. Particle diameters were measured from the SEM micrographs using the ImageJ software. Size distributions were obtained from a minimum of 200 measurements.

2.5. Fourier transform infrared (FT-IR) analysis of the samples

The hydrolysate, both in its free form and microencapsulated within the biopolymers, was dispersed in spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the sample at ca. 150 MPa and FT-IR spectra were collected in transmission mode using a Bruker FT-IR Tensor 37 equipment (Rheinstetten, Germany). The spectra were obtained by averaging 10 scans at 1 cm^{-1} resolution.

2.6. Static *in-vitro* digestion

Dispersions of the free hydrolysate (12 mg/mL) or suspensions of the hydrolysate-loaded microcapsules (40 mg/mL, i.e. the equivalent of 12 mg/mL of hydrolysate) in distilled water were subjected to *in-vitro* gastrointestinal digestion according to the standardized static *in vitro* digestion protocol (Minekus et al., 2014). Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the reported compositions (Minekus et al., 2014). In the oral phase, the dispersions were mixed with SSF (50:50 v/v) and incubated at 37 ºC for 2 min in a shaking incubator at 150 rpm. In the gastric phase, the oral digest was mixed with SGF (50:50 v/v) and porcine pepsin (2000 U/mL), and incubated at 37 ºC for 2 h in a shaking incubator at 150 rpm). In the duodenal phase, the gastric digest was mixed with SIF (50:50 v/v), porcine bile extract (10 mM) and porcine pancreatin (100 U/mL of trypsin activity), and incubated at 37 ºC for 2 h as described above. The pH was initially adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively. After the duodenal phase, the protease inhibitor Pefabloc® (1 mM) was added and the digests were snap-frozen in liquid nitrogen for subsequent lyophilisation.

174 Freeze-dried samples were re-suspended in 10 mL of milliQ water and centrifuged for
175 20 min at 1795 g and 4 ºC. The supernatant was then ultracentrifuged using
176 Centriprep® Ultracel® YM-3 centrifugal filter units (Millipore, Cork, Ireland) with a
177 molecular weight cut-off of 3 kDa. The ultracentrifugation was carried out at 3000 g
178 and 4 ºC in three steps of 95 min, 35 min and 10 min, respectively, according to the
179 supplier's instructions. The ultrafiltrates were freeze-dried for storage and re-dissolved
180 in milliQ water prior to HPLC-MS/MS analysis.

181 Samples of the hydrolysate-loaded microcapsules (non-digested) were suspended in
182 acetic acid (20% v/v) under vigorous agitation to dissolve the encapsulation matrices,
183 and subsequently ultrafiltered following the same procedure described above for the
184 digests in order to assess the effective release of the peptides from their encapsulation
185 matrices.

186

187 **2.7. Lactic fermentation of hydrolysate-containing milk**

188 Commercial UHT low fat milk was supplemented with the hydrolysate and fermented
189 to produce peptide-enriched yogurts. For this purpose, 1 sachet of freeze-dried
190 concentrated lactic cultures (YO-MIX ^{TM}) was dispersed in 1 L of milk, and 1 mL of
191 this mixture was further diluted in 1 L of milk. Both the free hydrolysate (200 mg) and
192 the hydrolysate-loaded capsules (667 mg) were dispersed into 15 mL aliquots of the
193 inoculated milk and incubated overnight at 42 ºC (until pH 5 was reached). 'Blank'
194 yogurts (i.e. without hydrolysate) were also produced.

195 The obtained yogurts were then freeze-dried and re-suspended in 10 mL of acetic acid
196 (20% v/v) under vigorous agitation for 5 h in order to dissolve the encapsulation
197 matrices and release the peptides for analysis, as described above. The resulting

198 suspensions were centrifuged to remove solids and the supernatant was subsequently
199 ultracentrifuged using Centriprep® Ultracel® YM-3 centrifugal filter units as described
200 above for the digests. The ultrafiltrates were freeze-dried for storage and re-dissolved in
201 milliQ water prior to HPLC-MS/MS analysis.

202

203 **2.8. HPLC-MS/MS**

204 Samples were analysed on a 1100 series HPLC (Agilent Technologies, Waldbronn,
205 Germany) coupled to an Esquire 3000 ion trap instrument (Bruker Daltonik GmbH,
206 Bremen, Germany). The chromatographic separation was carried out using a
207 Mediterranea Sea_{18} 150 mm × 2.1 mm column (Teknokroma, Barcelona, Spain). The
208 injection volume was 50 µL and the flow rate 0.2 mL/min. A linear gradient from 0 to
209 45% of solvent B (acetonitrile/formic acid 0.1%) and 55% of solvent A (water/ formic
210 acid 0.1%) in 120 min was used. In these analyses, the target mass was set at 750 *m/z.*
211 Spectra were recorded over the mass/charge (*m/z*) range of 200-1500.

212 Data processing was done with Data Analysis™ (version 4.0; Bruker Daltoniks, GmbH
213 Germany). Peptide sequencing was assisted by MASCOT, using a homemade database
214 that includes the most abundant cow's whey proteins. The matched MS/MS spectra
215 were interpreted with BioTools version 3.2, both from Bruker Daltoniks GmbH
216 (Germany). Comparison of peptide profiles were performed with Venny®.
217 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

218

219

220

3. Results and discussion

3.1. Peptide profile of the hydrolysate

A tryptic whey protein concentrate hydrolysate was produced in order to obtain a range of peptides which would allow the study of the impact of microencapsulation on their resistance to gastrointestinal conditions and their stability against lactic acid fermentation. A total of 47 β-lactoglobulin (β-Lg) peptide sequences were identified in the protein hydrolysate, 27 of which had been previously identified in a tryptic hydrolysate of a β-lactoglobulin enriched whey protein concentrate prepared in a similar way (Martínez-Maqueda, Miralles, Ramos, & Recio, 2013). In addition, 11 α-lactalbumin (α-La) peptide sequences were also found. Cleavages corresponded mostly to specific trypsin sites after Arg and Lys residues, but also peptides with Leu, Phe, Glu and Tyr at the C-terminal position were found. A food-grade trypsin was used in this study, which explains the broad specificity, since this enzyme preparation has chymotrypsin activity. Table 1 summarizes the 58 identified peptides in the hydrolysate. These sequences covered almost the whole protein sequence except those regions containing disulphide bridges, where the identification was impaired. Some of the identified sequences have been reported to exert different bioactivities, such as antimicrobial (β-Lg fragments 15-20, VAGTWY, 78-83, IPAVFK, 92-100, VLVLDTDYK and α-La 1-5, EQLTK) (Pellegrini, Dettling, Thomas, & Hunziker, 2001; Pellegrini, Thomas, Bramaz, Hunziker, & von Fellenberg, 1999), ACE-inhibitory(β-Lg fragment 9-14, GLDIQK) (Pihlanto-Leppälä, Rokka, & Korhonen, 1998), hypocholesterolemic (β-Lg fragment 70-74, IIAEK) (Nagaoka et al., 2001) and DPP-IV–inhibitory activity (β-Lg fragments 15-20, VAGTWY and 78-82, IPAVF) (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013; Uchida, Ohshiba, & Mogami, 2011).

246 INSERT TABLE 1 ABOUT HERE

247

248 **3.2. Morphological characterization of the microcapsules**

249 The whey protein hydrolysate was microencapsulated using a protein (gelatin) and a
250 polysaccharide (chitosan) as wall materials by spray-drying in order to compare the
251 suitability of both biopolymers to protect the different peptides during gastrointestinal
252 digestion and against lactic acid fermentation. Fig. 1 shows the SEM micrographs of the
253 hydrolysate-containing spray-dried powders, which exhibited a pseudo-spherical
254 morphology with varying roughness of their surfaces. These heterogeneous shapes are
255 typically observed for spray-dried particles obtained from aqueous solutions (De Cicco,
256 Porta, Sansone, Aquino, & Del Gaudio, 2014; Fu et al., 2011; Kusonwiriyawong,
257 Lipipun, Vardhanabhuti, Zhang, & Ritthidej, 2013) due to the fast evaporation of the
258 solvent. In general, larger microparticles were obtained when gelatin was used as
259 encapsulating matrix, partially due to the lower concentration of the feed suspensions
260 containing chitosan as the wall material, which was a processing requirement due to the
261 high viscosity of the polysaccharide solution.

262
263 INSERT FIGURE 1 ABOUT HERE

264

265 **3.3. FT-IR analysis of the microencapsulated hydrolysate**

266 The spray-dried materials, together with the free hydrolysate, were characterized using
267 FT-IR spectroscopy, and the obtained spectra are shown in Fig. 2.

268

INSERT FIGURE 2 ABOUT HERE

The spectrum of spray-dried chitosan showed a broad band with a maximum at 3386 cm^{-1}, attributed to the –OH and –NH stretching vibration, and other characteristic bands at 2929 and 2885 cm^{-1} (stretching of C–H bonds), 1643 cm^{-1} (Amide I, C=O stretching), 1561 cm^{-1} (Amide II, $–NH_2$ bending) and 1076 cm^{-1} (C–O stretching of sugar rings) (Bossio, Gómez-Mascaraque, Fernández-Gutiérrez, Vázquez-Lasa, & San Román, 2014; Gómez-Mascaraque et al., 2014). The spectrum of spray-dried gelatin also exhibited its most characteristic bands at 3307 cm^{-1} (Amide A), 3078 cm^{-1} (Amide B), 1653 cm^{-1} (Amide I), 1542 cm^{-1} (Amide II) and 1244 cm^{-1} (Amide III) (Gómez-Mascaraque, Lagarón, & López-Rubio, 2015). On the other hand, given the protein nature of the hydrolysate, its spectrum showed similar bands as the gelatin one, although centred at slightly different wavenumbers: 3293 cm^{-1} (Amide A), 3079 cm^{-1} (Amide B), 1649 cm^{-1} (Amide I), 1545 cm^{-1} (Amide II) and 1243 cm^{-1} (Amide III).

The spectra of the microencapsulated hydrolysate showed the characteristic bands of either chitosan or gelatin and the hydrolysate, generally at intermediate wavelengths due to the contribution of both materials present in the capsules. For instance, one of the bands ascribed to the stretching of C–H bonds had its maximum at 2939 cm^{-1} in gelatin and at 2930 cm^{-1} in the free hydrolysate, being centred at an intermediate wavelength of 2935 cm^{-1} in the hydrolysate-loaded gelatin microparticles. Similarly, the band centred at 1413 cm^{-1} in chitosan and 1400 cm^{-1} in the free hydrolysate had its maximum at 1407 cm^{-1} in the hydrolysate-loaded chitosan capsules. However, certain bands of the encapsulated hydrolysate shifted to higher or lower wavenumbers as compared to both components of the particles. Although it is difficult to draw conclusions given the

293 overlapping of most spectral bands and the highly coupled modes in the Amide I and II
294 regions, interactions between the peptides from the hydrolysate and the encapsulation
295 matrices seem to have taken place during the encapsulation process, as inferred from the
296 spectral changes in this area observed in the hybrid capsules (see insets in Fig. 2a and
297 2b). These interactions could, in fact, explain why certain peptides were not detected
298 after dissolving the capsules (Table 1). Crosslinking reactions of proteins have been
299 described upon thermal treatments, as high temperatures lead to protein denaturation,
300 leaving internal thiol and hydrophobic groups exposed and available to form
301 intermolecular disulphide bonds and hydrophobic interactions (Damodaran, 2007;
302 Shimada & Cheftel, 1989). The spray drying process used in this work for the
303 encapsulation of the protein hydrolysate, involving the use of high temperatures, might
304 have thus contributed to promoting this type of crosslinking reactions between the
305 gelatin and the hydrolysate. In fact, an increase in the intensity of the amide band
306 towards greater wavenumbers, related to antiparallel β-sheet interactions (Eissa, Puhl,
307 Kadla, & Khan, 2006; Le Tien et al., 2000) was clearly observed in the hybrid capsules
308 (arrow in inset of Fig. 2a).

309

310 **3.4. Identification of peptides after encapsulation**

311 In order to corroborate the effective encapsulation of the hydrolysate within the two
312 biopolymer matrices, the loaded chitosan and gelatin capsules were subjected to
313 extraction in acidic conditions, dissolving the encapsulation matrices and thus favouring
314 the release of the peptides. The comparison of the total ion current (TIC)
315 chromatograms obtained by HPLC-MS/MS showed little differences indicating an
316 effective release of the hydrolysate from the capsules (Fig. 3). Most of the peptides

317 present in the initial hydrolysate were identified, which demonstrated that the
318 encapsulation procedure did not affect the peptide profile to a great extent (Table 1).
319 Even then, 13 peptides out of 58 from the hydrolysate could not be identified after
320 capsule disruption, probably due to interactions of the peptides with the encapsulation
321 matrices as suggested by the FT-IR results. On the other hand, some peptides were
322 newly found after dissolution, 11 in the case of chitosan capsules and 9 in the case of
323 gelatin capsules, 4 of which were common sequences. Peptide-matrix interactions might
324 have affected peptide identification, specially taking into account that the purification of
325 the samples prior to HPLC-MS/MS analysis included an ultrafiltration step to remove
326 high molecular weight molecules, probably affecting the recovery of the peptides
327 interacting with the matrices. Despite the observed exceptions, it was confirmed that the
328 peptides in the hydrolysate could be released from the microcapsules under suitable
329 conditions. These results are consistent with previous works which had demonstrated
330 that model proteins (such as bovine serum albumin), peptides (e.g. RGVKGPR,
331 KLGPKGPR or SSPGPPVH) or protein hydrolysates (such as atlantic salmon protein
332 hydrolysates) could be effectively released from gelatin and chitosan-based
333 encapsulation structures, respectively, in aqueous systems (He et al., 2016; J. K. Li,
334 1998; Z. Li, Paulson, & Gill, 2015).

335

336 INSERT FIGURE 3 ABOUT HERE

337

338 **3.5. Simulated digestion of the microencapsulated hydrolysate**

339 Although encapsulation may be effective in protecting functional ingredients, it has also
340 been reported that their entrapment within certain microstructures may decrease their

bioaccessibility to a certain extent after ingestion (Roman, Burri, & Singh, 2012). Thus, the microstructures obtained in this work were subjected to *in-vitro* digestion to assess whether the peptides from the hydrolysate would be effectively released during passage through the gastrointestinal tract.

Simulated digestion of the free hydrolysate resulted in a remarkable change in the identified peptides. Their number was reduced by half in the case of β-Lg fragments (Fig. 4a, b). In the case of α-La, with a lower number of peptides, a similar tendency was found. Only two complete sequences from β-Lg (fragments 108-113, ENSAEP and 110-115, SAEPEQ) and one from α-La (fragment 63-68, DDQNPH) were resistant to the simulated gastrointestinal digestion. In most cases, peptides identified in the digesta corresponded to fragments from those found in the non-digested sample. The lower number of peptides can be attributed to their degradation to form di- or tri-peptides or free amino acids. Besides, the digesta contained enzyme autolytic fragments and bile salts, giving rise to a much more complex matrix which complicated peptide detection.

In the digesta from the hydrolysate-loaded chitosan microparticles, 23 peptides could be identified, 17 of which were similar to those found in the digested free hydrolysate (Fig. 4b, c). On the other hand, the digesta from the hydrolysate-loaded gelatin capsules produced a very complex chromatogram where only two peptides from the whey proteins could be identified. The proteinaceous origin of the encapsulation matrix, which was also digested into peptides by the enzymes added during the assay was most probably causing this interference.

Summarizing, the results indicated that digestion of the samples modified the peptide profile of the hydrolysate towards lower number of peptides and reduced molecular weight. Even though a protective effect during digestion was not evidenced, the

365 encapsulation within chitosan microparticles did not alter to a great extent the peptide
366 profile of the digests. Therefore, the peptide bioaccessibility was not expected to be
367 substantially affected by the encapsulation. In fact, previous works have shown the
368 potential of chitosan-based encapsulation structures as effective carriers for oral peptide
369 delivery. Specifically, *in vivo* assays in rats demonstrated an enhanced bioactivity for
370 salmon calcitonin after oral administration of the chitosan-encapsulated peptide (Prego,
371 Garcia, Torres, & Alonso, 2005; Prego, Torres, & Alonso, 2006). Biostability and
372 bioavailability of the peptides are essential to achieve physiological benefits, as they
373 need to reach their targets in an active form in order to exert their bioactivity (Mohan et
374 al., 2015).

375

376 INSERT FIGURE 4 ABOUT HERE

377

378 **3.6. Fermentation assays**

379 Peptide-enriched yogurts were produced by lactic acid fermentation of UHT low fat
380 milk supplemented with the free and microencapsulated hydrolysate. In the yogurts
381 where free hydrolysate had been added, a total of 30 β-Lg and α-La peptide sequences,
382 out of the 51 original, were identified. Thus, a large part of the peptides in the
383 hydrolysate were lost during lactic acid fermentation. It is known that the susceptibility
384 of peptides to living starter cultures depends on the amino acids sequence (Contreras et
385 al., 2011), and thus only some of the peptides were degraded during the fermentation
386 process. None of the peptide sequences identified in the original hydrolysate were
387 detected in a blank yogurt prepared in the absence of hydrolysate.

388 After analysis of the fermented products, five peptides were protected by encapsulation,
389 since they were present in the hydrolysate prior fermentation but not in the yogurt
390 enriched with free hydrolysate (Fig. 5). Four of these sequences were only found when
391 the hydrolysate was encapsulated within chitosan microparticles (β-Lg fragments 25-32,
392 AASDISLL, 70-75, KIIAEK, 95-101, LDTDYKK, and 45-50, NDSTEY), while only
393 one sequence (α-La fragment 37-44, DTQAIVQN) was protected by both types of
394 encapsulation matrices. Two peptides, β-Lg fragments 21-32, SLAMAASDISLL, and
395 36-40, SAPLR, were not observed in the fermented milks containing the encapsulated
396 hydrolysate, fact which could be ascribed either to a low concentration of the peptides
397 in the products or to interactions with the encapsulation matrices, thus hindering release
398 and subsequent identification.

399

400 INSERT FIGURE 5 ABOUT HERE

401

402 As the chemical species within the protein hydrolysates are characterized by their
403 heterogeneity, the protection effect that encapsulation exerted on the protein hydrolysate
404 during milk fermentation was sequence-dependent. Not all the fermentation-susceptible
405 peptides could be stabilized through encapsulation. On the other hand, encapsulation
406 within chitosan protected a greater number of peptides as compared to gelatin. Thus,
407 selecting the most appropriate encapsulation matrix is of utmost importance in order to
408 achieve the protection of selected protein fragments with regard to the intended purpose
409 of the hydrolysate.

410

4. Conclusions

A whey protein hydrolysate was microencapsulated by spray-drying using two different encapsulation matrices, i.e. chitosan and gelatin, obtaining pseudo-spherical particles in both cases. Most of the hydrolysate peptides could be effectively released from the microcapsules by simply dissolving the biopolymeric matrices under acidic conditions. However, 13 peptides could not be identified after capsule disruption, probably due to peptide-matrix interactions which affected peptide recovery during the purification process. *In-vitro* digestion assays were carried out to further assess the release of the peptides during passage through the gastrointestinal tract, given the importance of the bioavailability of the compounds in order to exert their bioactivities. Although no protective effect during digestion was evidenced upon encapsulation within chitosan microparticles, this encapsulation did not substantially alter the peptide profile of the digest as compared to the free hydrolysate, and therefore, peptide bioaccessibility was not expected to be compromised by the encapsulation. Regarding the use of gelatin matrix, the complexity of the chromatogram obtained for the digested samples precluded the identification of the peptides from the hydrolysate and the results were not conclusive. On the other hand, the protection exerted by the encapsulation during milk fermentation was sequence- and matrix-dependent. Only 5 out of the 21 fermentation-susceptible peptides could be stabilized through encapsulation within chitosan, one of which was also protected using gelatin. Overall, chitosan yielded improved results when compared to gelatin regarding peptide protection during milk fermentation, although the most appropriate encapsulation matrix should be selected individually based on the specific target protein fragments, that is, the potentially bioactive peptides present in a hydrolysate.

436 **Acknowledgements**

437 Laura G. Gómez-Mascaraque is recipient of a predoctoral contract from the Spanish
438 Ministry of Economy and Competitiveness (MINECO), Call 2013. The authors would
439 like to thank the Spanish MINECO projects AGL2015-63855-C2-1 and AGL2015-
440 66886-R for financial support. Authors would also like to thank the Central Support
441 Service for Experimental Research (SCSIE) of the University of Valencia for the
442 electronic microscopy service.

443

444 **REFERENCES**

445 Bossio, O., Gómez-Mascaraque, L. G., Fernández-Gutiérrez, M., Vázquez-Lasa, B., & San
446 Román, J. (2014). Amphiphilic polysaccharide nanocarriers with antioxidant properties. Journal
447 of Bioactive and Compatible Polymers: Biomedical Applications, 29, 589-606.

448 Contreras, M. d. M., Sevilla, M. A., Monroy-Ruiz, J., Amigo, L., Gómez-Sala, B., Molina, E.,
449 Ramos, M., & Recio, I. (2011). Food-grade production of an antihypertensive casein
450 hydrolysate and resistance of active peptides to drying and storage. International Dairy
451 Journal, 21, 470-476.

452 Damodaran, S. (2007). Amino Acids, Peptides, and Proteins. In S. Damodaran, K. Parkin & O. R.
453 Fennema (Eds.), Food Chemistry (3rd ed., pp. 321-416). New York: CRC Press.

454 de Castro, R. J. S., & Sato, H. H. (2015). Biologically active peptides: Processes for their
455 generation, purification and identification and applications as natural additives in the food and
456 pharmaceutical industries. Food Research International, 74, 185-198.

457 De Cicco, F., Porta, A., Sansone, F., Aquino, R. P., & Del Gaudio, P. (2014). Nanospray
458 technology for an in situ gelling nanoparticulate powder as a wound dressing. International
459 Journal of Pharmaceutics, 473, 30-37.

460 Eissa, A. S., Puhl, C., Kadla, J. F., & Khan, S. A. (2006). Enzymatic cross-linking of β-lactoglobulin:
461 conformational properties using FTIR spectroscopy. Biomacromolecules, 7, 1707-1713.

462 Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides.
463 Critical reviews in Food science and nutrition, 48, 430-441.

464 Favaro-Trindade, C., Santana, A., Monterrey-Quintero, E., Trindade, M., & Netto, F. (2010). The
465 use of spray drying technology to reduce bitter taste of casein hydrolysate. Food
466 Hydrocolloids, 24, 336-340.

467 Fu, N., Zhou, Z., Jones, T. B., Tan, T. T., Wu, W. D., Lin, S. X., Chen, X. D., & Chan, P. P. (2011).
468 Production of monodisperse epigallocatechin gallate (EGCG) microparticles by spray drying for
469 high antioxidant activity retention. International Journal of Pharmaceutics, 413, 155-166.

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., & Saurel, R. (2007). Applications of spray-drying in Microencapsulation of food ingredients: An overview. Food Research International, 40, 1107-1121.

Gómez-Mascaraque, L. G., Lagarón, J. M., & López-Rubio, A. (2015). Electrosprayed gelatin submicroparticles as edible carriers for the encapsulation of polyphenols of interest in functional foods. Food Hydrocolloids, 49, 42-52.

Gómez-Mascaraque, L. G., Méndez, J. A., Fernández-Gutiérrez, M., Vázquez, B., & San Román, J. (2014). Oxidized dextrins as alternative crosslinking agents for polysaccharides: Application to hydrogels of agarose–chitosan. Acta Biomaterialia, 10, 798-811.

He, S., Mao, X., Zhang, T., Guo, X., Ge, Y., Ma, C., & Zhang, X. (2016). Separation and nanoencapsulation of antitumor peptides from Chinese three-striped box turtle (Cuora trifasciata). Journal of Microencapsulation, 1-11.

Hernández-Ledesma, B., García-Nebot, M. J., Fernández-Tomé, S., Amigo, L., & Recio, I. (2014). Dairy protein hydrolysates: Peptides for health benefits. International Dairy Journal, 38, 82-100.

Jiménez-Martín, E., Gharsallaoui, A., Pérez-Palacios, T., Carrascal, J., & Rojas, T. (2014). Suitability of using monolayered and multilayered emulsions for microencapsulation of ω-3 fatty acids by spray drying: Effect of storage at different temperatures. Food and Bioprocess Technology, 8, 100-111.

Khor, E., & Lim, L. Y. (2003). Implantable applications of chitin and chitosan. Biomaterials, 24, 2339-2349.

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. Journal of Functional Foods, 1, 177-187.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. International Dairy Journal, 16, 945-960.

Kusonwiriyawong, C., Lipipun, V., Vardhanabhuti, N., Zhang, Q., & Ritthidej, G. (2013). Spray-dried chitosan microparticles for cellular delivery of an antigenic protein: physico-chemical properties and cellular uptake by dendritic cells and macrophages. Pharmaceutical Research, 30, 1677-1697.

Lai, J. Y. (2013). Influence of solvent composition on the performance of carbodiimide cross-linked gelatin carriers for retinal sheet delivery. Journal of Materials Science: Materials in Medicine, 24, 2201-2210.

Le Tien, C., Letendre, M., Ispas-Szabo, P., Mateescu, M., Delmas-Patterson, G., Yu, H.-L., & Lacroix, M. (2000). Development of biodegradable films from whey proteins by cross-linking and entrapment in cellulose. Journal of Agricultural and Food Chemistry, 48, 5566-5575.

Li, J. K. (1998). Gelatin nanoencapsulation of protein/peptide drugs using an emulsifier-free emulsion method. Journal of Microencapsulation, 15, 163-172.

Li, Z., Paulson, A. T., & Gill, T. A. (2015). Encapsulation of bioactive salmon protein hydrolysates with chitosan-coated liposomes. Journal of Functional Foods, 19, 733-743.

Lim, H., Hwang, Y., Kar, M., & Varghese, S. (2014). Smart hydrogels as functional biomimetic systems. Biomaterials Science, 2, 603-618.

511 Ma, J.-J., Mao, X.-Y., Wang, Q., Yang, S., Zhang, D., Chen, S.-W., & Li, Y.-H. (2014). Effect of
512 spray drying and freeze drying on the immunomodulatory activity, bitter taste and
513 hygroscopicity of hydrolysate derived from whey protein concentrate. LWT-Food Science and
514 Technology, 56, 296-302.

515 Martínez-Maqueda, D., Miralles, B., Ramos, M., & Recio, I. (2013). Effect of β-lactoglobulin
516 hydrolysate and β-lactorphin on intestinal mucin secretion and gene expression in human
517 goblet cells. Food Research International, 54, 1287-1291.

518 Meisel, H. (1997). Biochemical properties of bioactive peptides derived from milk proteins:
519 Potential nutraceuticals for food and pharmaceutical applications. Livestock Production
520 Science, 50, 125-138.

521 Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou,
522 R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le
523 Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D. J., Menard,
524 O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S. J., Weitschies, W., &
525 Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an
526 international consensus. Food & function, 5, 1113-1124.

527 Mohan, A., Rajendran, S. R., He, Q. S., Bazinet, L., & Udenigwe, C. C. (2015). Encapsulation of
528 food protein hydrolysates and peptides: a review. RSC Advances, 5, 79270-79278.

529 Mora, L., Reig, M., & Toldrá, F. (2014). Bioactive peptides generated from meat industry by-
530 products. Food Research International, 65, 344-349.

531 Munin, A., & Edwards-Lévy, F. (2011). Encapsulation of natural polyphenolic compounds; a
532 review. Pharmaceutics, 3, 793-829.

533 Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., Tadashi, K., &
534 Kuwata, T. (2001). Identification of novel hypocholesterolemic peptides derived from bovine
535 milk β-lactoglobulin. Biochemical and Biophysical Research Communications, 281, 11-17.

536 Nongonierma, A. B., & FitzGerald, R. J. (2015). The scientific evidence for the role of milk
537 protein-derived bioactive peptides in humans: A Review. Journal of Functional Foods, 17, 640-
538 656.

539 Paul, M., & Somkuti, G. (2009). Degradation of milk-based bioactive peptides by yogurt
540 fermentation bacteria. Letters in applied microbiology, 49, 345-350.

541 Paul, M., & Somkuti, G. A. (2010). Hydrolytic breakdown of lactoferricin by lactic acid bacteria.
542 Journal of Industrial Microbiology and Biotechnology, 37, 173-178.

543 Pellegrini, A., Dettling, C., Thomas, U., & Hunziker, P. (2001). Isolation and characterization of
544 four bactericidal domains in the bovine β-lactoglobulin. Biochimica et Biophysica Acta (BBA)-
545 General Subjects, 1526, 131-140.

546 Pellegrini, A., Thomas, U., Bramaz, N., Hunziker, P., & von Fellenberg, R. (1999). Isolation and
547 identification of three bactericidal domains in the bovine α-lactalbumin molecule. Biochimica
548 et Biophysica Acta (BBA)-General Subjects, 1426, 439-448.

549 Pihlanto-Leppälä, A., Rokka, T., & Korhonen, H. (1998). Angiotensin I converting enzyme
550 inhibitory peptides derived from bovine milk proteins. International Dairy Journal, 8, 325-331.

551 Prego, C., Garcia, M., Torres, D., & Alonso, M. (2005). Transmucosal macromolecular drug
552 delivery. Journal of Controlled Release, 101, 151-162.

553 Prego, C., Torres, D., & Alonso, M. J. (2006). Chitosan Nanocapsules as Carriers for Oral Peptide
554 Delivery: Effect of Chitosan Molecular Weight and Type of Salt on the *In Vitro* Behaviour and *In*
555 *Vivo* Effectiveness. Journal of nanoscience and nanotechnology, 6, 2921-2928.

556 Roman, M. J., Burri, B. J., & Singh, R. P. (2012). Release and bioaccessibility of β-carotene from
557 fortified almond butter during in vitro digestion. Journal of Agricultural and Food Chemistry,
558 60, 9659-9666.

559 Santhanam, A., Lekshmi, M., Chouksey, M., Tripathi, G., & Gudipati, V. (2015). Delivery of
560 Omega-3 Fatty Acids into Cake Through Emulsification of Fish Oil-in-Milk and Encapsulation by
561 Spray Drying with Added Polymers. Drying technology, 33, 83-91.

562 Shimada, K., & Cheftel, J. C. (1989). Sulfhydryl group/disulfide bond interchange reactions
563 during heat-induced gelation of whey protein isolate. Journal of Agricultural and Food
564 Chemistry, 37, 161-168.

565 Silveira, S. T., Martínez-Maqueda, D., Recio, I., & Hernández-Ledesma, B. (2013). Dipeptidyl
566 peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate
567 rich in β-lactoglobulin. Food Chemistry, 141, 1072-1077.

568 Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development,
569 marketing and consumer acceptance—A review. Appetite, 51, 456-467.

570 Subtil, S., Rocha-Selmi, G., Thomazini, M., Trindade, M., Netto, F., & Favaro-Trindade, C.
571 (2014). Effect of spray drying on the sensory and physical properties of hydrolysed casein using
572 gum arabic as the carrier. Journal of Food Science and Technology, 51.

573 Uchida, M., Ohshiba, Y., & Mogami, O. (2011). Novel Dipeptidyl Peptidase-4-Inhibiting Peptide
574 Derived From. BETA.-Lactoglobulin. Journal of pharmacological sciences, 117, 63-66.

575 Vaslin, S., Le Guillou, A., Hannoucene, B., & Saint Denis, T. (2006). Protection of bioactive food
576 ingredients by means of encapsulation. Patent application WO2006042861 A1.

577 Wang, Z., Ju, X., He, R., Yuan, J., & Wang, L. (2015). The effect of rapeseed protein structural
578 modification on microstructural properties of peptide microcapsules. Food and Bioprocess
579 Technology, 8, 1305-1318.

FIGURE CAPTIONS:

Fig. 1. SEM images of hydrolysate-loaded spray-dried chitosan (a) and gelatin (b) particles, together with their size distributions. Scale bars correspond to 2 μm.

Fig. 2. Infrared spectra of the hydrolysate together with the (a) gelatin and (b) chitosan spray-dried materials. Insets show magnification of the Amide I and II area of the spectra.

Fig. 3. Total ion current (TIC) chromatograms of the free WPC hydrolysate (a), chitosan-encapsulated hydrolysate (b) and gelatin-encapsulated hydrolysate (c) after matrix dissolution. Arrows indicate differences in the chromatographic profile.

Fig. 4. Peptides from β-Lactoglobulin identified in the hydrolysate before digestion (a), after digestion of the free hydrolysate (b) and after digestion of the hydrolysate-loaded chitosan microcapsules (c).

Fig. 5. Venn diagram of the number of peptides identified in fermented milk fortified with the hydrolysate in its free form, encapsulated in chitosan and encapsulated in gelatin.

602 **TABLES**

603 **Table 1. Peptides identified in the protein hydrolysate and microcapsules with chitosan and gelatin**

Protein	Fragment	Experimental mass	Theoretical mass	Sequence	Detected within the microcapsules (Section 3.4)	
					Chitosan	Gelatin
β–Lg	1 – 5	572.2	572.4	LIVTQ	✓	
	1 – 8	932.5	932.5	LIVTQTMK	✓	✓
	2 – 8	819.4	819.5	IVTQTMK	✓	✓
	8 – 14	800.5	800.5	KGLDIQK	✓	✓
	9 – 14	672.4	672.4	GLDIQK		
	15 – 20	695.2	695.3	VAGTWY	✓	✓
	21 – 26	561.2	561.3	SLAMAA	✓	✓
	21 – 32	1190.6	1190.6	SLAMAASDISLL		
	25 – 32	788.4	788.4	AASDISLL	✓	✓
	21 – 26	562.2	562.3	SLAMAA	✓	✓
	27 – 43	1846.2	1846.0	SDISLLDAQSAPLRVYV		
	23 – 32	990.5	990.5	AMAASDISLL		
	33 – 40	856.4	856.4	DAQSAPLR	✓	✓
	36 – 40	542.3	542.3	SAPLR		
	41 – 46	750.3	750.4	VYVEEL		
	41 – 57	1943.8	1943.0	VYVEELKPTPEGDLEIL		✓
	41 – 58	2057.0	2056.1	VYVEELKPTPEGDLEILL		
	43 – 57	1680.8	1681.0	VEELKPTPEGDLEIL		✓
	43 – 60	2051.0	2050.1	VEELKPTPEGDLEILLQK		✓
	70 – 75	700.4	700.5	KIIAEK	✓	✓
	71 – 75	572.3	572.4	IIAEK	✓	✓
	76 – 82	774.4	774.5	TKIPAVF	✓	✓
	77 – 82	673.4	673.4	KIPAVF	✓	✓
	78 – 82	545.3	545.3	IPAVF	✓	✓
	78 – 83	673.4	673.4	IPAVFK	✓	✓
	83 –87	558.3	558.3	KIDAL	✓	✓
	83 – 91	1043.6	1043.6	KIDALNENK	✓	✓
	84 – 91	915.5	915.5	IDALNENK	✓	✓
	91 – 100	1192.6	1192.7	KVLVLDTDYK		
	92 – 100	1064.6	1064.6	VLVLDTDYK		✓
	92 – 101	1192.6	1192.7	VLVLDTDYKK	✓	✓
	94 – 100	852.4	852.4	VLDTDYK		
	94 – 101	980.5	980.5	VLDTDYKK	✓	✓
	95 – 101	881.4	881.5	LDTDYKK	✓	
	92 – 99	936.4	936.5	VLVLDTDY	✓	✓
	96 – 101	768.3	768.4	DTDYKK	✓	✓
	108 – 113	645.3	645.3	ENSAEP	✓	✓
	110 – 115	659.3	659.3	SAEPEQ		
	125 – 135	1244.6	1244.6	TPEVDDEALEK		✓
	125 – 136	1391.6	1391.6	TPEVDDEALEKF		✓
	125 – 138	1634.6	1634.8	TPEVDDEALEKFDK		✓
	127 – 135	1046.5	1046.5	EVDDEALEK		✓
	127 – 138	1436.4	1436.7	EVDDEALEKFDK		
	142 – 148	837.4	837.5	ALPMHIR*	✓	✓
	149 – 155	805.4	805.4	LSFNPTQ	✓	✓
	149 – 156	918.5	918.5	LSFNPTQL	✓	✓
	149 – 159	1304.6	1304.6	LSFNPTQLEEQ		✓
α–La	1 – 5	617.3	617.3	EQLTK	✓	✓
	12 – 16	615.4	615.4	LKDLK	✓	✓

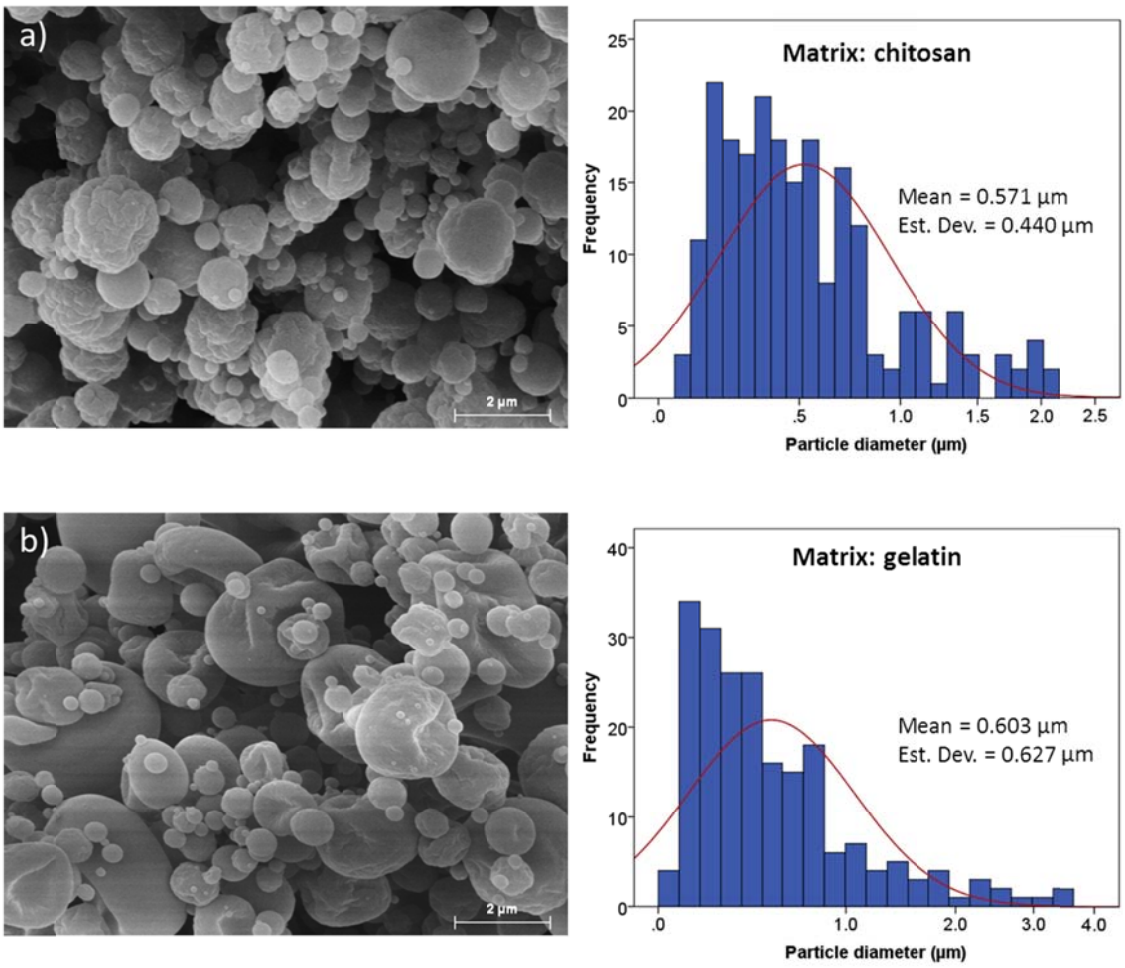
15 – 24	989.5	989.6	LKGYGGVSLP		
32 – 36	563.2	563.2	HTSGY		✓
37 – 43	773.4	773.4	DTQAIVQ	✓	✓
37 – 44	887.4	887.4	DTQAIVQN		✓
45 – 50	727.1	727.3	NDSTEY		✓
51–58	932.5	932.5	GLFQINNK	✓	✓
63–68	724.2	724.3	DDQNPH	✓	✓
94–98	615.4	615.4	KILDK		
99–104	750.3	750.4	VGINYW	✓	✓

604

605

607 **FIGURES**

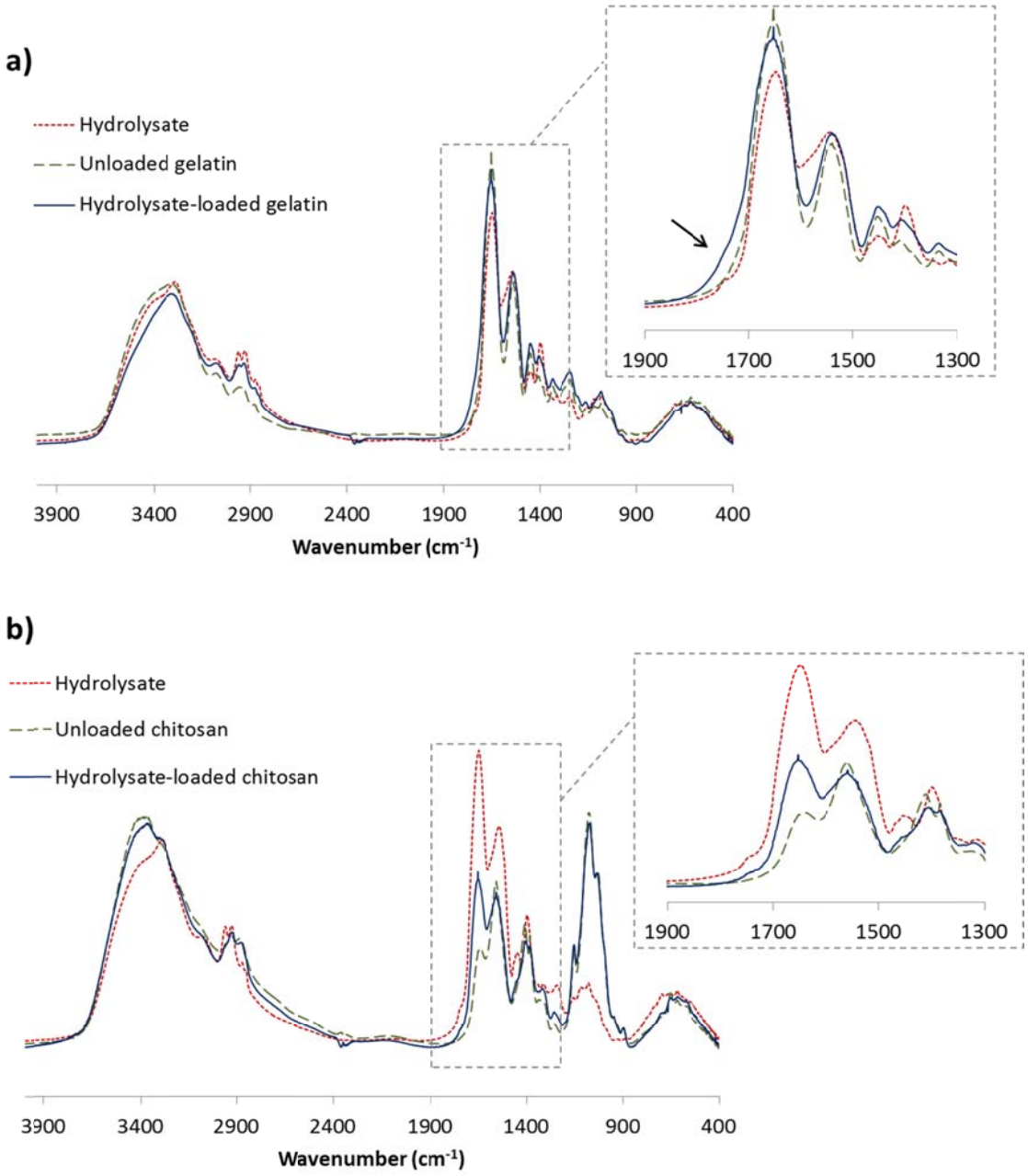
608 Fig. 1:



609

610

611 Fig. 2:

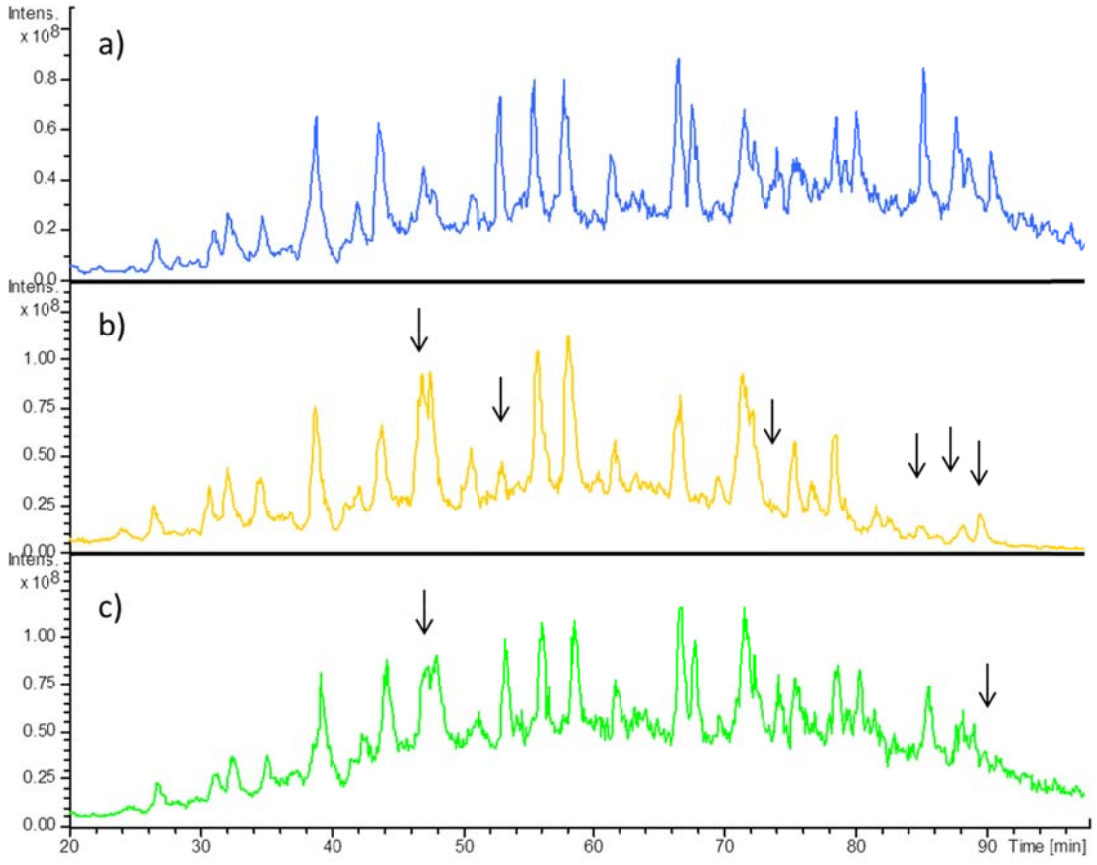


612

613

614

615 Fig. 3:

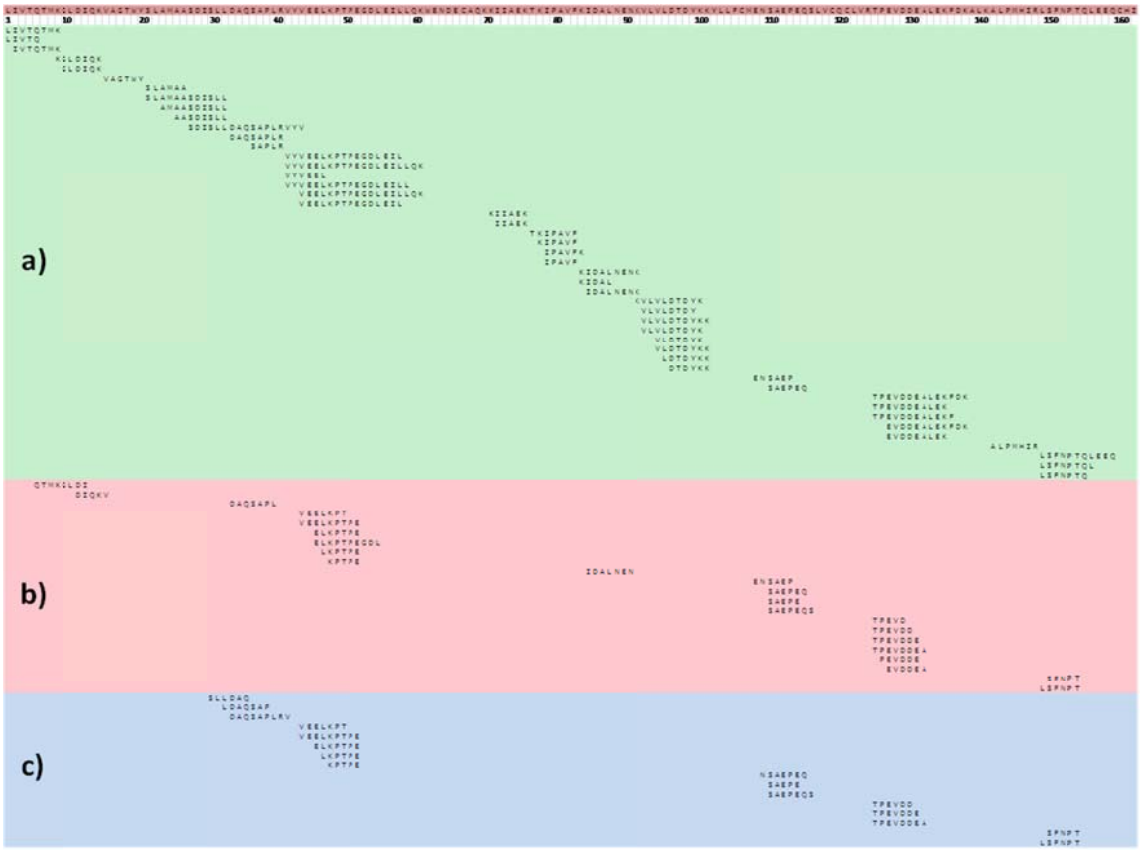


616

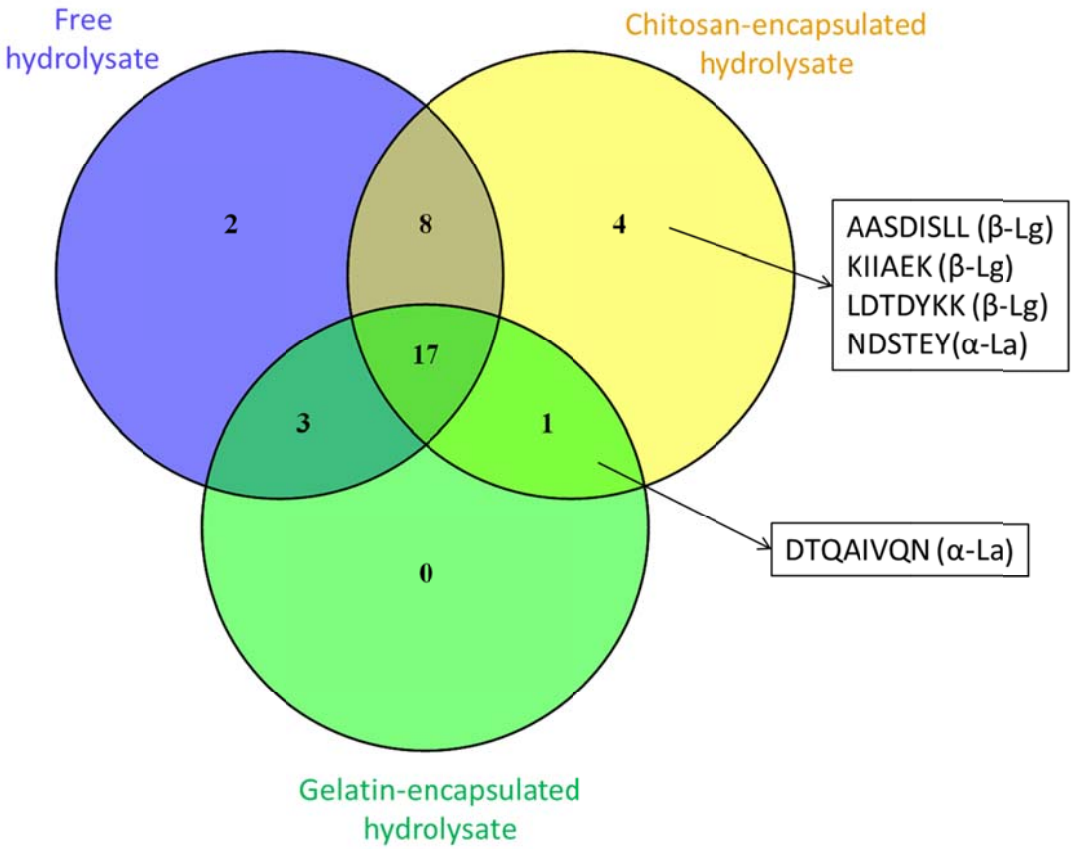
617

618

Fig. 4:



623 Fig. 5:


Free
hydrolysate
Chitosan-encapsulated
hydrolysate
Gelatin-encapsulated
hydrolysate
2
8
4
17
3
1
0
AASDISLL (β-Lg)
KIIAEK (β-Lg)
LDTDYKK (β-Lg)
NDSTEY(α-La)
DTQAIVQN (α-La)

624