

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

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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

17 **ABSTRACT**

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

29

30 **KEYWORDS**

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

32

33 **1. Introduction**

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

47 A number of bioactive peptides have already been studied and recent reviews suggest
48 that new research should focus on the application of these functional ingredients to
49 commercial food products (Mohan, Rajendran, He, Bazinet, & Udenigwe, 2015).
50 Functional foods have become popular and commercially successful in some sectors of
51 the food industry, especially in fermented dairy products, partly due to their general
52 acceptance among consumers (Siró, Kápolna, Kápolna, & Lugasi, 2008). However,
53 fortification of these food products with protein hydrolysates is challenging, not only
54 because of their low bioavailability, bitter taste, hygroscopicity and their likelihood of
55 interacting with the food matrix thus altering food texture and colour (Elias, Kellerby, &
56 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

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

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

107

108 **2. Materials and Methods**

109 **2.1. Materials**

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

121

122 **2.2. Preparation of the hydrolysate**

123 The WPC was dissolved in water 5% (w/v) and heated at 90 °C for 10 min. Hydrolysis
124 was carried out in triplicate at 37 °C and pH 8.0 by addition of 1M NaOH for 3 h with
125 constant agitation. Food grade trypsin (Biocatalyst, Nantgarw, UK) was used at an
126 enzyme-to-substrate ratio of 1:20 (w/w). Reactions were stopped by heating at 95 °C for
127 15 min, to ensure the complete inactivation of the enzyme. The hydrolysate was then
128 spray-dried. The inlet temperature of spray drying was maintained at 140 °C and the

129 outlet temperature was between 75 and 100 °C, following the method described in
130 Contreras et al., 2011.

131

132 **2.3. Microencapsulation of the hydrolysate**

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

141

142 **2.4. Morphological characterization of the particles**

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

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151 **2.5. Fourier transform infrared (FT-IR) analysis of the samples**

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

157

158 **2.6. Static *in-vitro* digestion**

159 Dispersions of the free hydrolysate (12 mg/mL) or suspensions of the hydrolysate-
160 loaded microcapsules (40 mg/mL, i.e. the equivalent of 12 mg/mL of hydrolysate) in
161 distilled water were subjected to *in-vitro* gastrointestinal digestion according to the
162 standardized static *in vitro* digestion protocol (Minekus et al., 2014). Simulated salivary
163 fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were
164 prepared according to the reported compositions (Minekus et al., 2014). In the oral
165 phase, the dispersions were mixed with SSF (50:50 v/v) and incubated at 37 °C for 2
166 min in a shaking incubator at 150 rpm. In the gastric phase, the oral digest was mixed
167 with SGF (50:50 v/v) and porcine pepsin (2000 U/mL), and incubated at 37 °C for 2 h in
168 a shaking incubator at 150 rpm). In the duodenal phase, the gastric digest was mixed
169 with SIF (50:50 v/v), porcine bile extract (10 mM) and porcine pancreatin (100 U/mL of
170 trypsin activity), and incubated at 37 °C for 2 h as described above. The pH was initially
171 adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively. After the
172 duodenal phase, the protease inhibitor Pefabloc[®] (1 mM) was added and the digests
173 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 °C. 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 °C 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™) 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 °C (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 Sea₁₈ 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://bioinfo.gp.cnb.csic.es/tools/venny/index.html>).

218

219

220

221 **3. Results and discussion**

222 **3.1. Peptide profile of the hydrolysate**

223 A tryptic whey protein concentrate hydrolysate was produced in order to obtain a range
224 of peptides which would allow the study of the impact of microencapsulation on their
225 resistance to gastrointestinal conditions and their stability against lactic acid
226 fermentation. A total of 47 β -lactoglobulin (β -Lg) peptide sequences were identified in
227 the protein hydrolysate, 27 of which had been previously identified in a tryptic
228 hydrolysate of a β -lactoglobulin enriched whey protein concentrate prepared in a similar
229 way (Martínez-Maqueda, Miralles, Ramos, & Recio, 2013). In addition, 11 α -
230 lactalbumin (α -La) peptide sequences were also found. Cleavages corresponded mostly
231 to specific trypsin sites after Arg and Lys residues, but also peptides with Leu, Phe, Glu
232 and Tyr at the C-terminal position were found. A food-grade trypsin was used in this
233 study, which explains the broad specificity, since this enzyme preparation has
234 chymotrypsin activity. Table 1 summarizes the 58 identified peptides in the hydrolysate.
235 These sequences covered almost the whole protein sequence except those regions
236 containing disulphide bridges, where the identification was impaired. Some of the
237 identified sequences have been reported to exert different bioactivities, such as
238 antimicrobial (β -Lg fragments 15-20, VAGTWY, 78-83, IPAVFK, 92-100,
239 VLVLDTDYK and α -La 1-5, EQLTK) (Pellegrini, Dettling, Thomas, & Hunziker,
240 2001; Pellegrini, Thomas, Bramaz, Hunziker, & von Fellenberg, 1999), ACE-
241 inhibitory(β -Lg fragment 9-14, GLDIQK) (Pihlanto-Leppälä, Rokka, & Korhonen,
242 1998), hypocholesterolemic (β -Lg fragment 70-74, IIAEK) (Nagaoka et al., 2001) and
243 DPP-IV–inhibitory activity (β -Lg fragments 15-20, VAGTWY and 78-82, IPAVF)
244 (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013; Uchida, Ohshiba, &
245 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; Kusonwiriawong,
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

269

INSERT FIGURE 2 ABOUT HERE

270

271 The spectrum of spray-dried chitosan showed a broad band with a maximum at 3386
272 cm^{-1} , attributed to the $-\text{OH}$ and $-\text{NH}$ stretching vibration, and other characteristic bands
273 at 2929 and 2885 cm^{-1} (stretching of $\text{C}-\text{H}$ bonds), 1643 cm^{-1} (Amide I, $\text{C}=\text{O}$ stretching),
274 1561 cm^{-1} (Amide II, $-\text{NH}_2$ bending) and 1076 cm^{-1} ($\text{C}-\text{O}$ stretching of sugar rings)
275 (Bossio, Gómez-Mascaraque, Fernández-Gutiérrez, Vázquez-Lasa, & San Román,
276 2014; Gómez-Mascaraque et al., 2014). The spectrum of spray-dried gelatin also
277 exhibited its most characteristic bands at 3307 cm^{-1} (Amide A), 3078 cm^{-1} (Amide B),
278 1653 cm^{-1} (Amide I), 1542 cm^{-1} (Amide II) and 1244 cm^{-1} (Amide III) (Gómez-
279 Mascaraque, Lagarón, & López-Rubio, 2015). On the other hand, given the protein
280 nature of the hydrolysate, its spectrum showed similar bands as the gelatin one,
281 although centred at slightly different wavenumbers: 3293 cm^{-1} (Amide A), 3079 cm^{-1}
282 (Amide B), 1649 cm^{-1} (Amide I), 1545 cm^{-1} (Amide II) and 1243 cm^{-1} (Amide III).

283 The spectra of the microencapsulated hydrolysate showed the characteristic bands of
284 either chitosan or gelatin and the hydrolysate, generally at intermediate wavelengths due
285 to the contribution of both materials present in the capsules. For instance, one of the
286 bands ascribed to the stretching of $\text{C}-\text{H}$ bonds had its maximum at 2939 cm^{-1} in gelatin
287 and at 2930 cm^{-1} in the free hydrolysate, being centred at an intermediate wavelength of
288 2935 cm^{-1} in the hydrolysate-loaded gelatin microparticles. Similarly, the band centred
289 at 1413 cm^{-1} in chitosan and 1400 cm^{-1} in the free hydrolysate had its maximum at 1407
290 cm^{-1} in the hydrolysate-loaded chitosan capsules. However, certain bands of the
291 encapsulated hydrolysate shifted to higher or lower wavenumbers as compared to both
292 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

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

345 Simulated digestion of the free hydrolysate resulted in a remarkable change in the
346 identified peptides. Their number was reduced by half in the case of β -Lg fragments
347 (Fig. 4a, b). In the case of α -La, with a lower number of peptides, a similar tendency
348 was found. Only two complete sequences from β -Lg (fragments 108-113, ENSAEP and
349 110-115, SAEPEQ) and one from α -La (fragment 63-68, DDQNPH) were resistant to
350 the simulated gastrointestinal digestion. In most cases, peptides identified in the digesta
351 corresponded to fragments from those found in the non-digested sample. The lower
352 number of peptides can be attributed to their degradation to form di- or tri-peptides or
353 free amino acids. Besides, the digesta contained enzyme autolytic fragments and bile
354 salts, giving rise to a much more complex matrix which complicated peptide detection.

355 In the digesta from the hydrolysate-loaded chitosan microparticles, 23 peptides could be
356 identified, 17 of which were similar to those found in the digested free hydrolysate (Fig.
357 4b, c). On the other hand, the digesta from the hydrolysate-loaded gelatin capsules
358 produced a very complex chromatogram where only two peptides from the whey
359 proteins could be identified. The proteinaceous origin of the encapsulation matrix,
360 which was also digested into peptides by the enzymes added during the assay was most
361 probably causing this interference.

362 Summarizing, the results indicated that digestion of the samples modified the peptide
363 profile of the hydrolysate towards lower number of peptides and reduced molecular
364 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

411 **4. Conclusions**

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

435

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443

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580 **FIGURE CAPTIONS:**

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

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

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

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

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

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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	✓	✓

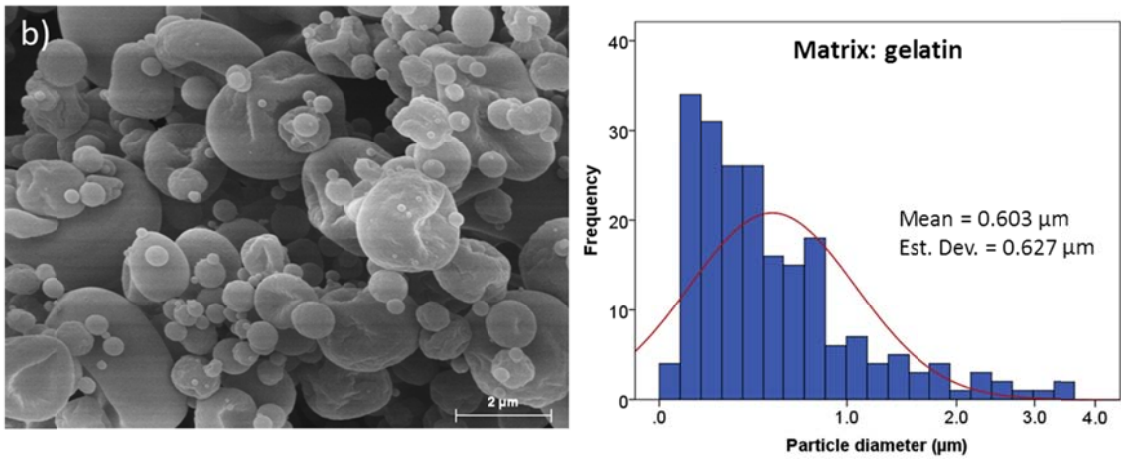
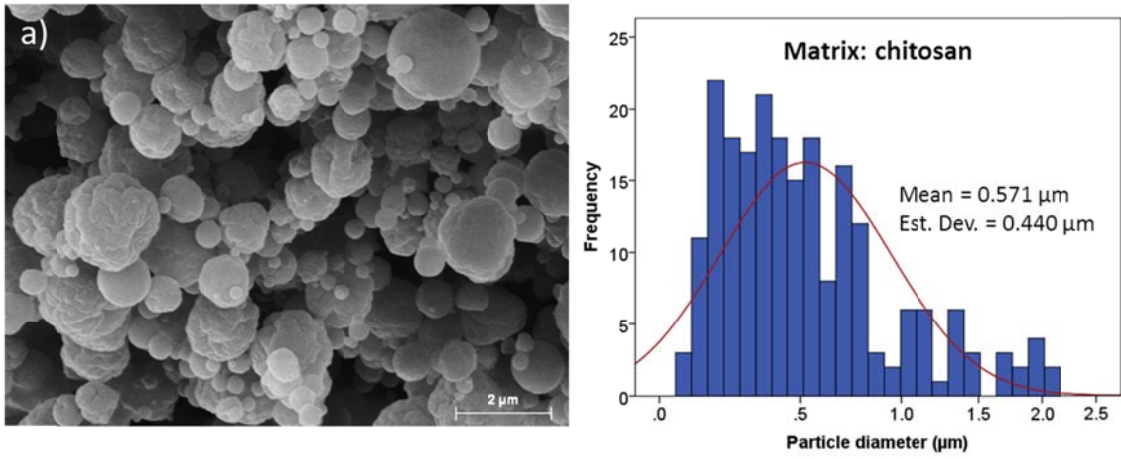
15 – 24	989.5	989.6	LKGYGGVSLP		
32 – 36	563.2	563.2	HTSGY		✓
37 – 43	773.4	773.4	DTQAIVQ	✓	✓
37 – 44	887.4	887.4	DTQAIVQN		✓
45 – 50	727.1	727.3	NDSTEY		✓
51–58	932.5	932.5	GLFQINNK	✓	✓
63–68	724.2	724.3	DDQNPH	✓	✓
94–98	615.4	615.4	KILDK		
99–104	750.3	750.4	VGINYW	✓	✓

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607 **FIGURES**

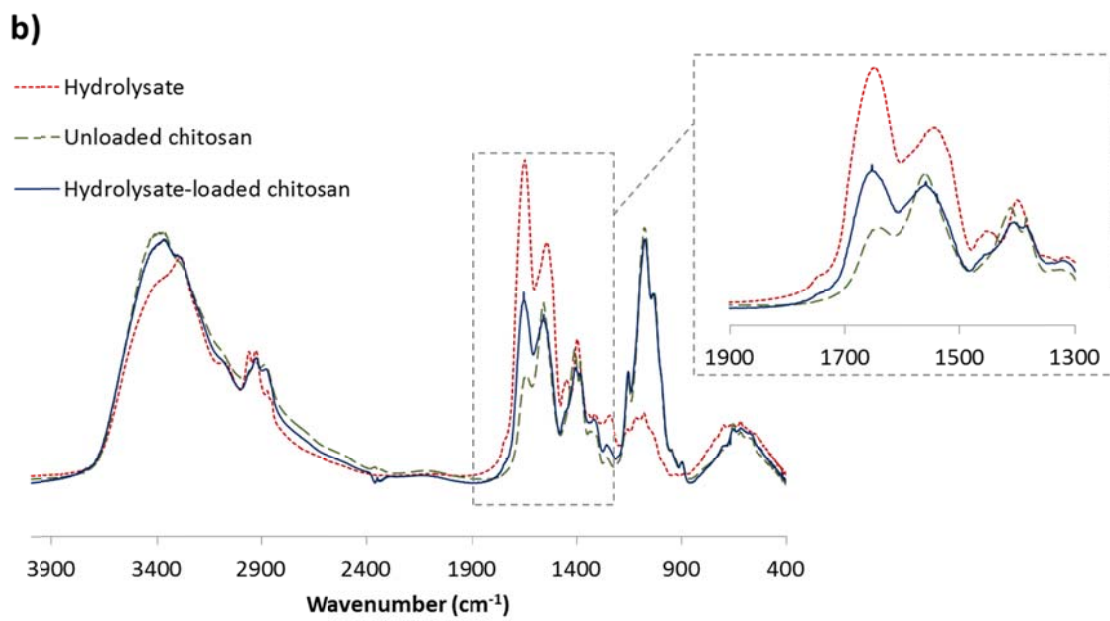
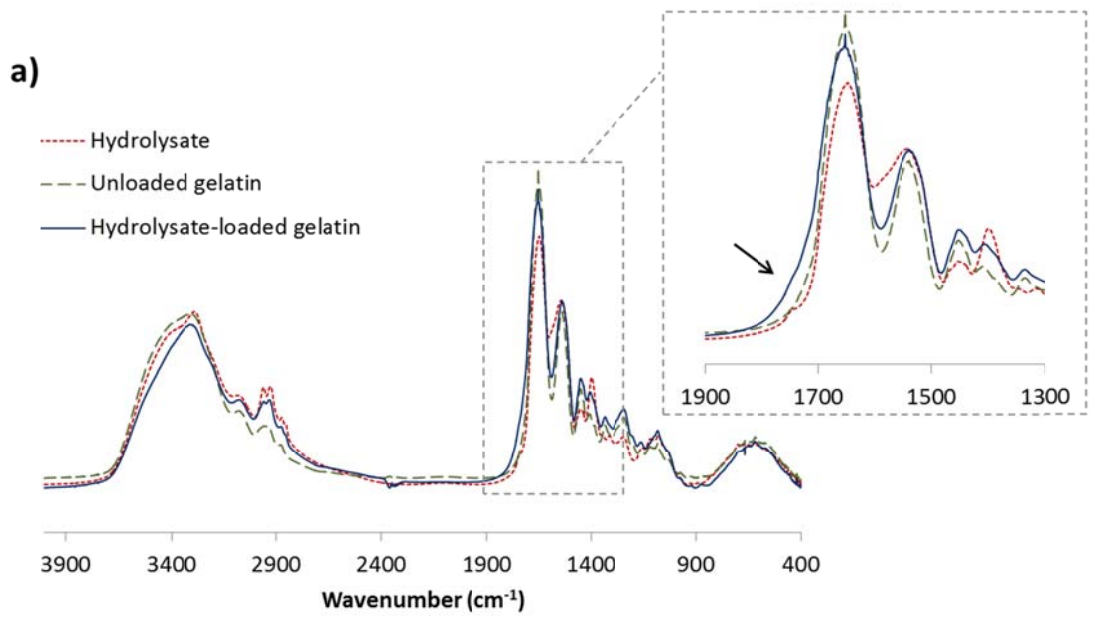
608 Fig. 1:



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611 Fig. 2:

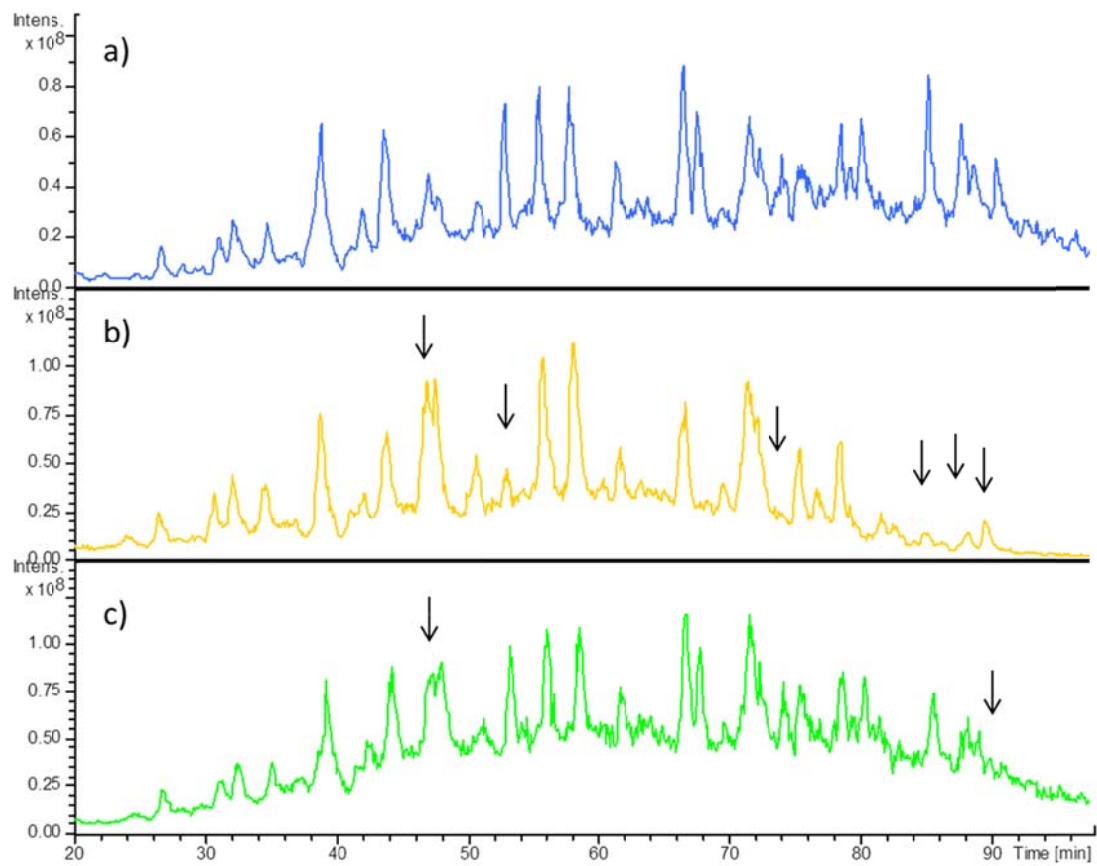


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615 Fig. 3:

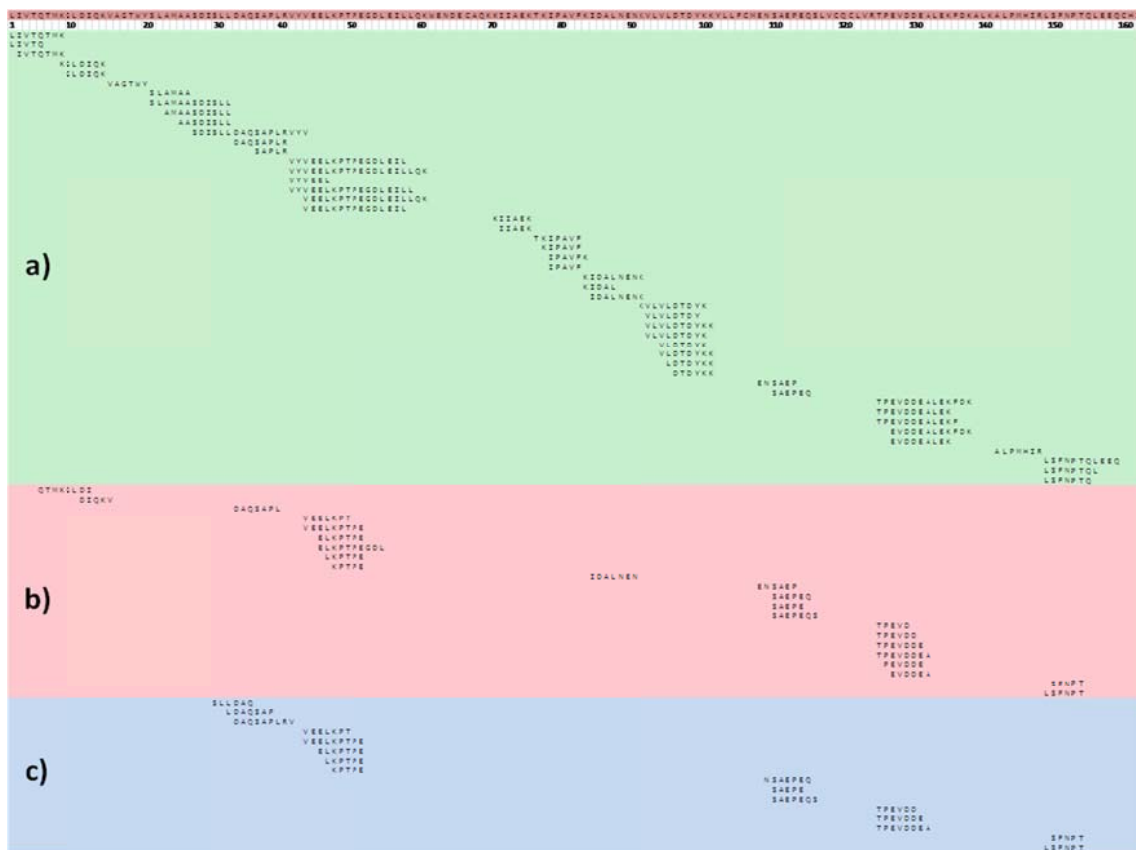


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619 Fig. 4:

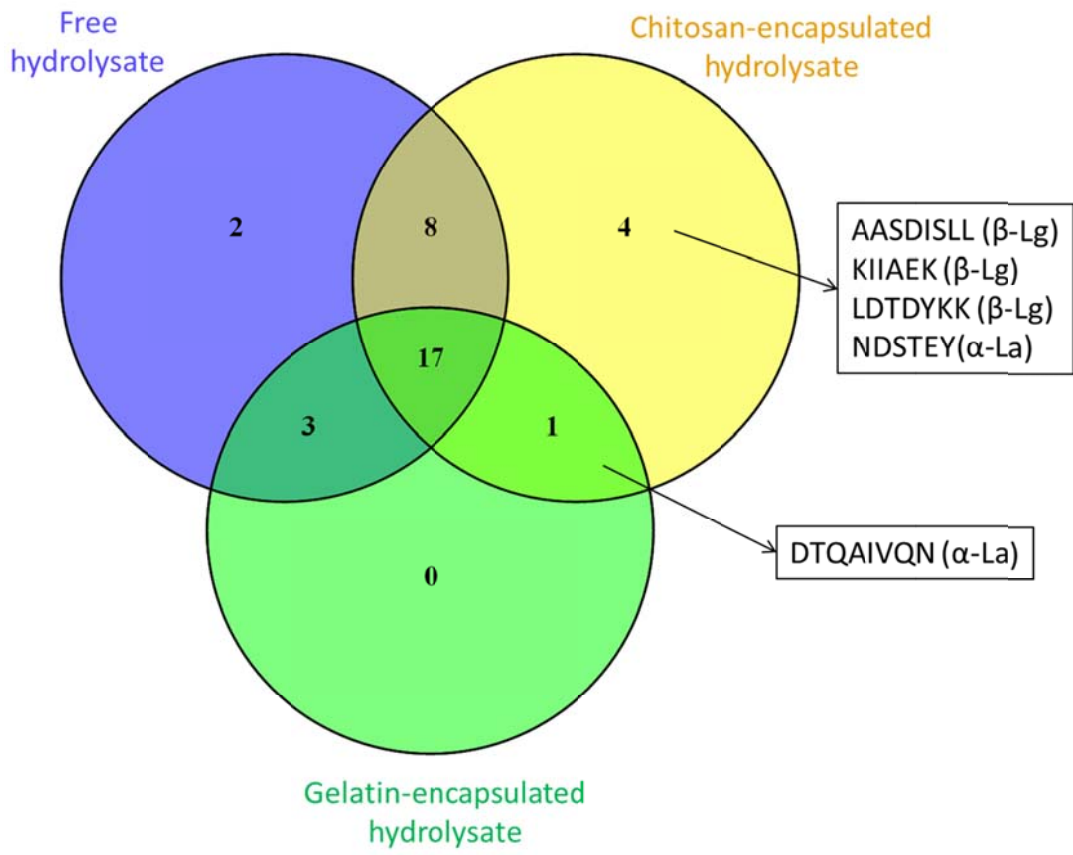


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623 Fig. 5:



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