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Abstract: This paper examines lysozyme (LYS) behavior upon in vitro digestion, mimicking different conditions in the stomach and intestine, and assessing the effect of natural surfactants such as phosphatidylcholine (PC) or bile salts (BS) on hydrolysis and residual immunogenicity of the digests. The hydrolysis pattern of LYS was compared to that of �-lactalbumin (LA). Hydrolysis of LYS only occurred at low pH. PC slightly increased its resistance to pepsinolysis. A similar behaviour was found for LA. Circular dichroism revealed that the more rigid structure of LYS, as compared with that of LA, could protect it from proteolysis at acidic pH and fluorescence spectra suggested that, at acidic pH, both proteins associated to PC films. The gastric digests of LYS showed high IgE-binding capacity using sera from egg-allergic patients. On the other hand, it was found that LYS precipitated under conditions that simulated a duodenal environment mainly due to the presence of BS.

1 **RESEARCH HIGHLIGHTS**

2

3 - LYS is only hydrolyzed at very low pHs (1.2-2) with pepsin at an E:S ratio of 1:20.
4 - The rigid structure of LYS could protect it from proteolysis at acidic pH.
5 - In vitro gastric digestion of LYS led to potential allergenic peptides.

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4 **IMMUNOREACTIVITY OF THE DIGESTS.**

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6 **Running tittle: *IN VITRO* DIGESTION OF LYSOZYME**

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25 **ABSTRACT**

26

27 This paper examines lysozyme (LYS) behavior upon in vitro digestion, mimicking
28 different conditions in the stomach and intestine, and assessing the effect of natural
29 surfactants such as phosphatidylcholine (PC) or bile salts (BS) on hydrolysis and residual
30 immunogenicity of the digests. The hydrolysis pattern of LYS was compared to that of α-
31 lactalbumin (LA). Hydrolysis of LYS only occurred at low pH. PC slightly increased its
32 resistance to pepsinolysis. A similar behaviour was found for LA. Circular dichroism
33 revealed that the more rigid structure of LYS, as compared with that of LA, could protect
34 it from proteolysis at acidic pH and fluorescence spectra suggested that, at acidic pH, both
35 proteins associated to PC films. The gastric digests of LYS showed high IgE-binding
36 capacity using sera from egg-allergic patients. On the other hand, it was found that LYS
37 precipitated under conditions that simulated a duodenal environment mainly due to the
38 presence of BS.

39

40 **Keywords:** lysozyme, α-lactalbumin, in vitro gastric digestion, in vitro duodenal
41 digestion, IgE-binding

42

1. INTRODUCTION

Lysozyme (LYS) is, together with lactoferrin, one of the most extensively studied antibacterial milk proteins (López-Expósito and Recio, 2006). LYS is present in milk from different species, but also in many biological fluids and tissues, such as human milk, tears and saliva. Its bactericidal effect partially lies on its lytic activity on the cell wall of Gram-positive microorganisms. Many attempts have been made to broaden its antimicrobial activity to include Gram-negative bacteria, such as the use of thermal treatments to partially denature the protein, the linkage of hydrophobic ligands to increase its hydrophobicity or the conjugation to polysaccharides through controlled Maillard reaction (Pellegrini et al., 1997). In addition to conformational changes, proteolysis of LYS has been shown to produce peptides able to induce a non-enzymatic bacterial inactivation and thus, active against Gram-positive and Gram-negative bacteria (Mine et al., 2004; Ibrahim et al., 2005). Other biological functions of LYS, such as immunomodulatory, antiviral and anti-inflammatory have been reported (Lesnierowski and Kijowski, 2007).

It can be presumed that milk LYS can act physiologically as a bactericidal agent, although the existence of a definite protective role is not yet known. In fact, the high level of LYS in human milk (which is 100 times higher than that of bovine milk, which contains from 0.13 to 0.32 mg/L of LYS) could be of relevance (Fox and Kelly, 2006). In any case, exogenous LYS is widely added to several cheese varieties to prevent the growth of *Clostridium*, which causes off-flavors and late blowing and its use, in combination with minimal processing techniques, has been proposed to extend the shelf life of milk and dairy products (Sobrino-López and Martín-Belloso, 2008).

67 Hen egg white (probably the richest source of LYS, containing 1-3 g/L) is the main
68 commercial source of LYS used to maintain the quality of food and pharmaceutical
69 products. Members of the LYS superfarmily are related proteins, with similar three
70 dimensional structures but different amino acid sequences, comprising from 123 to 129
71 amino acids. Egg white LYS (Gal d 4, with 14.3 kDa, 129 amino acid residues and four
72 disulphide bridges) has been extensively studied as a model protein for structure and
73 biological properties of LYS from other sources. However, LYS is also a major allergen
74 of egg white, although its allergenic potential has not been studied in depth and no
75 relevant epitopes have been identified as yet. Clinical reactions to egg LYS have been
76 described and anti-LYS IgE antibodies are frequently found in egg allergic patients as
77 markers of sensitization (Mine and Yang, 2008). In fact, the frequent presence of LYS as
78 an additive in dairy products poses a risk to allergic subjects (Iaconelli et al., 2008).

79 It is generally accepted that resistance to digestion is a common feature to food
80 allergens, although it has also been shown that proteolytic fragments produced during
81 digestion may bind IgE and induce allergic responses in sensitized individuals. Besides, in
82 the case of LYS, digestion can be a physiological process to release peptides that
83 contribute to its in vivo defense role. However, available information related to the basis
84 of the resistance of this protein to digestion is limited and, sometimes, contradictory. This
85 can be attributed to the fact that in vitro digestion models use inappropriate amounts of
86 proteases or one-step digestion models, disregarding the complex gastric and duodenal
87 composition and the interactions of proteins with other components, such as lipids
88 (Moreno, 2007).

89 This paper examines LYS behavior upon in vitro digestion, mimicking different
90 conditions in the stomach and upper intestine, and assessing the effect of natural
91 surfactants such as phosphatidylcholine (PC) or bile salts (BS) on hydrolysis and residual

92 immunogenicity of the digests. The structural characteristics of LYS that could play a role
93 on its susceptibility to proteolysis under different conditions were studied and compared
94 to those of its homologous protein bovine milk α-Lactalbumin (LA), which is more
95 susceptible to proteolysis. Because of its broader availability, hen egg LYS, which shows
96 60% homology with human milk LYS, was used. The results obtained regarding egg-
97 white LYS behaviour towards digestion can be relevant to understand its allergenic
98 potential, of importance when added to dairy products, and the contribution of
99 physiological processes to its in vivo defense role that could be common to other members
100 of the LYS family.

101

102

103 2. MATERIALS AND METHODS

104 2.1 Gastric and Duodenal Digestions

105 LYS (L2879, chloride form from chicken egg white Grade VI, ~60000 units/mg
106 protein, EC 3.2.1.17, Sigma-Aldrich, St. Louis, MO, USA) was subjected to in vitro
107 gastric digestions at 5 mg/mL final concentration. The digestions were performed in
108 simulated gastric fluid (SGF, 0.35 *M* NaCl) at different pHs: 1.2, 2.0, 3.2, 4.0 and 4.5, at
109 37ºC for 120 min, with porcine pepsin (EC 3.4.23.1**,** 3440 U/mg protein, Sigma-Aldrich)
110 at an enzyme: substrate ratio (E:S) of 1:20, wt/wt (172 U/mg), considered as a
111 physiological ratio (Moreno et al., 2005a).

112 Gastric digestions in the presence of the natural surfactant phosphatidylcholine (PC,
113 Sigma-Aldrich), were also assessed. Phospholipid vesicles were prepared by dissolving
114 PC in SGF pH 2.0 (9.58 mg/mL), following Martos et al. (2010).

115 At least three replicates of each digestion assay were performed and compared by
116 HPLC and SDS-PAGE to ensure the repeatability of the results. Aliquots were taken at

117 different time points, up to 120 min, for analysis. The digestions were stopped by mixing
118 with the SDS-sample buffer for SDS-PAGE analyses, whereas for RP-HPLC and
119 inhibition ELISA, the pH was raised to 7.0 with ammonium bicarbonate to irreversibly
120 inactivate pepsin and, after 10 min of equilibration, the digestion mixtures were
121 centrifuged at 10000 g and 20ºC for 10 min. The protein concentration of the supernatants
122 was determined by the Kjeldhal method.

123 Bovine α-lactalbumin (LA, Sigma-Aldrich) was also submitted to in vitro gastric
124 digestions without and with PC under the same conditions. In all cases, at least triplicate
125 digestions were conducted.

126 Duodenal digestions were performed as previously described (Moreno et al. 2005a,
127 b; Martos et al. 2010) on the supernatants of the 60 min gastric digests re-adjusted to pH
128 6.5, with the addition of: a 0.125 *M* bile salt (BS) mixture containing equimolar quantities
129 of sodium glycodeoxycholate and sodium taurocholate (Sigma-Aldrich) (6.15 m*M* final
130 concentration of each salt); 1 *M* $CaCl_2$ (7.6 m*M* final concentration); pancreatic porcine
131 lipase (Type VI-S, 111000 U/mg protein, Sigma-Aldrich), at an E:S of 1:3895, wt/wt
132 (28.5 U/mg); pancreatic porcine colipase (Sigma-Aldrich), at an E:S of 1:895, wt/wt;
133 pancreatic bovine trypsin (type I, 10100 U/mg protein, Sigma-Aldrich), at an E:S of
134 1:238, wt/wt (42.5 U/mg); and pancreatic bovine alpha-chymotrypsin (type I-S, 58.3
135 U/mg prot, Sigma-Aldrich), at an E:S of 1:115 wt/wt (0.52 U/mg) in 20.3 m*M* Bis-Tris.
136 The reactions were carried out at 37ºC for either 30 or 60 min and stopped by heating at
137 80ºC for 5 min.

138

139

140 **2.2 Solubility Experiments**

141 The solubility of LYS was examined in SGF at different pHs (1.2, 2, 3.2, 3.6, 3.9,
142 4.7, 5.1, 6.2, 6.4, 6.8, 7.2 and 7.7) as well as in 10 mM phosphate buffer. Solubility of the
143 intact protein was also assessed in 7.6 m*M* $CaCl_2$ and 20.3 m*M* Bis-Tris under simulated
144 duodenal conditions typical of a fasted state (3 m*M* of each BS and 2.4 m*M* PC, pH 7.0),
145 of a fed state (9 m*M* of each BS and 7.2 m*M* PC, pH 6.0) and of an intermediate state (6
146 m*M* of each BS and 4.9 m*M* PC, pH 6.5) (Kostewicz et al., 2002; Kaukinen et al., 2004).
147 This latest condition was similar to the medium used for duodenal digestion. Solubility
148 was determined by SDS-PAGE and/or RP-HPLC analysis following centrifugation
149 (10000 g, 20ºC, 10 min).

150

151 **2.3 SDS-PAGE**

152 SDS-PAGE of the gastric digests was performed using Tris-Tricine ready gels with
153 16.5% acrylamide (Bio-Rad Laboratories, Hercules, CA). Samples were diluted in Tris-
154 Tricine sample buffer (Bio-Rad). Electrophoresis was carried out at 100 V, for 3 h, at
155 room temperature (RT), in Tris-Tricine SDS running buffer (Bio-Rad). Gels were fixed in
156 a 40% methanol and 10% (wt/vol) acetic acid solution, followed by staining with
157 Coomassie Blue G-250 (Bio-Rad). The kaleidoscope prestained standards (Bio-Rad)
158 containing myosin (198 kDa), β-galactosidasa (125 kDa), BSA (88 kDa), carbonic
159 anhydrase (37 kDa), STI (31 kDa), LYS (17 kDa) and aprotinin (7 kDa) was used.
160 SDS-PAGE of the duodenal digests was performed in a PhastSystem equipment
161 (Pharmacia, Uppsala, Sweden) using PhastGel Homogeneous 20 polyacrylamide gels and
162 Phastgel SDS-Buffer Strips (Pharmacia) and following the manufacturer separation and
163 Coomassie staining conditions. The low molecular weight calibration kit for SDS
164 electrophoresis (GE Healthcare, Uppsala, Sweden) containing phosphorylase B (97 kDa),

165 BSA (66 kDa), OVA (45 kDa), carbonic anhydrase (37 kDa), trypsin inhibitor (20.1 kDa),
166 and LA (14.4 kDa), was used.
167
168 **2.4 RP-HPLC**
169 LYS hydrolysates, at a concentration of 2.4 mg/mL, were analyzed in a Hi-Pore®
170 RP-318 (250 x 4.6 mm i.d.) column (Waters, Milford, USA) in a Waters 600 HPLC
171 system. Solvent A was 0.37% (vol/vol) trifluoroacetic acid (Scharlau Chemie, Barcelona,
172 Spain) in double-distilled water and solvent B was 0.27% (vol/vol) trifluoroacetic acid in
173 HPLC-grade acetonitrile (Lab-Scan, Gliwice, Poland). The chromatographic conditions
174 were as in Martos et al. (2010). Detection was at 220 nm and data were processed by
175 using the Empower 2 Software (Waters).
176
177 **2.5 Circular Dichroism Spectroscopy**
178 CD spectra were recorded in the far- (195-260 nm) and near- (250-350 nm) UV
179 regions, using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan) as described
180 in Martos et al. (2010). LYS and LA were dissolved at 0.2 mg/mL for the analysis in the
181 far-UV region and at 2 mg/mL for the near-UV region, either in SGF, pH 2.0, or in 10
182 m*M* phosphate buffer, pH 7.0. PC was added at 0.184 mg/mL or 1.84 mg/mL,
183 respectively, to maintain the protein/phospholipid ratio used during digestion. Buffer
184 blanks were subtracted from each CD spectrum.
185
186 **2.6 Fluorescence Spectroscopy**
187 The interaction between either LYS or LA and PC was studied by fluorescence
188 spectroscopy based on Barbana et al. (2006). Fluorescence spectra between 300 and 380
189 nm (excitation: 280 nm) were recorded at room temperature on a Shimadzu RF-1501

190 spectrofluorophotometer. The binding of PC was measured by following the increase in
191 protein fluorescence. The procedure used for titration of LYS and LA was as described in
192 Martos et al. (2010).
193

194 **2.7 IgG- and IgE- Binding by Inhibition ELISA**

195 The antigenicity of LYS and its gastric hydrolysates was evaluated by inhibition
196 ELISA using commercial IgG antibodies. Single wells of polystyrene microtiter plates
197 (Corning, Cambridge, MA, USA) were coated with 10 µg/mL of LYS solution in 0.01 *M*
198 PBS, pH 7.4, and incubated overnight at 6°C. Afterwards, the plates were washed 3 times
199 with PBS-Tween 20 (PBST) 0.05% using a Microplate Washer (Nunc, Roskilde,
200 Denmark) and PBST 2.5% was used as saturating agent, to avoid non-specific binding.
201 Then, the plate was blocked for 4 h at RT and washed once.

202 Serial dilutions of each sample (not less than 10) were incubated (1:1, vol/vol) at
203 RT during 2 h, with horseradish peroxidase (HRP) conjugated-polyclonal anti-LYS raised
204 in rabbit (Abcam, Cambridge, UK), previously diluted 1:10000 in PBST 0.05%, and 50
205 µL were added to each well. After 2 h of incubation at RT, the plate was washed 3 times
206 and 50 µL of 3,3´,5,5´-tetramethylbenzidine (TMB) substrate (ready-to-use solution;
207 Sigma-Aldrich) were added to each well. Finally, the reaction was stopped with 0.5 M
208 sulfuric acid and the absorbance was measured at 450 nm. A negative control without
209 antibody (native protein in PBST 0.05%) and positive controls (antibody diluted in PBS)
210 were included in each plate.

211 For human IgE-binding, 3 individual serum samples from children with clinically
212 allergic symptoms to egg white proteins were used. The sera were collected from the
213 Maternal and Child Gregorio Marañon Hospital (Madrid, Spain). The patients had specific
214 seric IgE levels towards egg white higher than 100 kU/L, as determined by CAP (GE

215 HealthCare). The procedure described above was followed but, in this case, serial
216 dilutions of each sample (not less than 10) were incubated at RT for 2 h with patient´s sera
217 (1:1, vol:vol), previously diluted in PBST, and 50 µL were added to each well. After 2 h
218 of incubation at RT, the plate was washed 3 times and 50 µL of HRP-conjugated rabbit
219 anti-human IgE (DakoCytomation, Glostrup, Denmark), diluted 1:1000 in PBST 0.05%,
220 were added per well and incubated for 1 h at RT. Then the plate was washed 3 times and
221 the tyramide-biotin and streptavidin-HRP amplification system was used following the
222 instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life
223 Sciences, Walthman, MA, USA). Finally, the plate was washed 4 times and TMB was
224 used as substrate. The reaction was stopped with 0.5 M sulfuric acid and the absorbance
225 was measured at 450 nm. A negative control without serum (native protein in PBST) and
226 positive controls (sera diluted in PBST 0.05%) were included in each plate.

227 Both IgG- and IgE-binding results were statistically processed. A nonlinear
228 adjustment of the data obtained for each dilution was applied for each serum and sample.
229 The adjustment model was a sigmoid curve of inhibition dose-response with variable
230 slope, from which the IC50 (the protein concentration that binds 50% of either IgG or
231 seric IgE) was obtained with the program GraphPad PRISM 4 for Windows (GraphPad
232 software, San Diego, USA). The IgG- and IgE-capacities of the digests were expressed as
233 percentages of the IC50 of the intact protein (as means ± standard errors for n=3).
234 Significant differences ($P<0.05$) were evaluated by one-way analysis of variance
235 (ANOVA).

236

237 3. RESULTS AND DISCUSSION

238 3.1 Effect of the pH on the Gastric Digestion of LYS in vitro.

239 Figure 1 shows the SDS-PAGE pattern of LYS digested with pepsin at an E:S of
240 1:20 (wt/wt), after different hydrolysis times at different pHs, in order to simulate
241 different conditions in the stomach, from the lowest values typical of a fasted state, to
242 higher values characteristic of a fed state or of immature stomach functions. Hydrolysis of
243 the protein only occurred at pH 1.2 and 2.0, with the formation of degradation products of
244 less than 6 kDa that resisted pepsin action for at least 2 h of digestion. Interferences
245 arising from the autodigestion of pepsin were also assessed, but no additional bands were
246 detected in the electrophoretic separations at this enzyme concentration.

247 LYS had disappeared before 60 min of digestion at pH 1.2, while there still was
248 unhydrolysed protein after 120 min at pH 2.0, a pH that prevails in the fasted stage of the
249 stomach of healthy adults. The presence of a partially folded intermediate of LYS,
250 characterized by a significant secondary structure, exposure of non-polar clusters and a
251 disrupted tertiary structure, has been reported at very low pH values (1.5) (Polverino de
252 Laureto et al., 2002). This increased flexibility could be responsible for its increased
253 susceptibility to digestion at pH 1.2. The pH had a very important effect on LYS
254 hydrolysis, despite pepsin exhibits its optimum activity over a broad pH range, between
255 1.2 and 3.5. At pH values equal or higher than 3.2, there was no detectable hydrolysis of
256 the protein, even after 120 min of digestion (Figure 1). The pH in the stomach of infants
257 up to 2 months old is 3.0-4.0 and it can increase after food intake at values above 6.0
258 (Dupont et al., 2009).

259 While it is generally recognized that LYS is resistant to pepsin action (Polverino de
260 Laureto et al., 2002), there is not much information on the proteolytic susceptibility of this
261 protein and some discrepancies exist. Thus, Mine et al. (2004) reported its complete
262 hydrolysis after 60 min of treatment at pH 1.0 and an E:S of 1:25 (wt:wt), while Fu et al.
263 (2002) claimed that it resisted more than 60 min at pH 1.2, at an E:S of 13:1 (wt:wt).

264 Ibrahim et al. (2005) found that 40% of the original LYS was hydrolysed after 120 min of
265 digestion at an E:S of 1:50 (wt:wt) and pH 4.0 (conditions that mimicked the infant
266 stomach), giving three peptides with molecular masses of 7365, 5444 and 4317 kDa with
267 a potent bactericidal activity. According to these authors, this could suggest the important
268 biological role of pepsin hydrolysis of LYS from human milk as a defense system in the
269 stomach of the newborn. However, under our digestion conditions, LYS was completely
270 resistant to pepsin at pH values of 4.0 and 4.5 (Figure 1).

271

272 **3.2 Effect of the Presence of PC on the Gastric Digestion of LYS in vitro.**

273 Figure 2 compares the RP-HPLC pattern of in vitro gastric digestions performed for
274 60 min, at pH 2.0, in the absence and presence of PC, a physiological surfactant secreted
275 by the gastric mucosa and also present in the bile. It should be mentioned that, regardless
276 of the addition of PC, there were signs for protein precipitation on adjustment of the
277 gastric digests to pH to 7.0 to irreversibly inactivate pepsin before the RP-HPLC analyses.
278 The precipitate was removed by centrifugation and it was identified by RP-HPLC as
279 unhydrolysed LYS. While residual LYS precipitated from the gastric digests at pH 7.0
280 solubilization experiments demonstrated that the intact protein was fully soluble in SGF at
281 different pHs up to 7.7, as well as in 10 mM phosphate buffer at pH 7.0 (results not
282 shown).

283 As shown in Figure 2, 6.3 mM PC (a 18:1 molar ratio of PC:protein) did not have an
284 important effect on the susceptibility of LYS to hydrolysis by pepsin although it slightly
285 increased LYS resistance to digestion, decreasing the presence of degradation products,
286 without signs for changes in the fragmentation pattern (Figures 2b and 2c). A similar
287 behaviour has been reported for other proteins, such as LA, whose interaction with PC
288 was shown to retard its proteolysis during gastric digestion, a result that was attributed to

289 the partial penetration of LA into PC vesicles (Moreno et al. 2005b). In fact, when LA
290 was digested with pepsin in the absence and presence of PC, the protective effect of PC on
291 LA proteolysis was confirmed (Figure 3). Thus, in agreement with the results of Moreno
292 et al. (2005b), in the absence of PC, LA was rapidly hydrolysed by pepsin but, when PC
293 was present, there was intact protein remaining after 5 and 15 min. On the other hand,
294 pepsin hydrolysis of other proteins, such as the 2S albumin from Brazil nut (Moreno et al.,
295 2005a), β-Lactoglobulin (LG) (Mandalari et al. 2009; Mazierzanka et al., 2009), or
296 ovalbumin (OVA) (Martos et al., 2010) is not affected by the presence of PC in SGF at
297 pH 2.5 or 2.0.

298 Hen egg white LYS and bovine LA are homologous to each other as they share
299 similar primary structures, but their unfolding profiles and the stabilities of their native
300 conformations are very different (Cawthern et al., 1996; Polverino de Laureto et al.,
301 2002). The biological functions of LYS, such as its antimicrobial and immunomodulating
302 properties, have been attributed to its ability to interact with membrane component
303 phospholipids and to penetrate into lipid bilayers, aspects which have been broadly
304 studied (Gorbenko et al., 2007; Yuan et al., 2007). Similarly, the interaction of LA with
305 phospholipid membranes and vesicles is of interest because membranes are implicated in
306 the protein folding behaviour and in its ability to regulate lactose synthesis (Cawthern et
307 al., 1996).

308 The structural features of LYS and LA in the absence and presence of PC were
309 studied by CD and fluorescence. The far-UV CD spectra of LYS (Figure 4a) showed that
310 the secondary structure of LYS in solution was the same at pH 2.0 and 7.0 and that it was
311 not altered in the presence of PC. These results agree with Witoonsaridsilp et al. (2010),
312 who reported that the secondary structure of LYS entrapped in non-charged and
313 negatively charged liposomes, with various lipid compositions, does not significantly

314 differ from that in buffer solutions at different pHs. The near-UV CD spectrum of LYS at
315 pH 2.0 was the typical of this protein, which does not change with respect to the neutral
316 pH condition (Figure 4b) (Polverino de Laureto et al., 2002). However, in the presence of
317 PC at pH 7.0, a reduction in the signals in the near-UV region was patent, which
318 suggested a disruption of the tertiary structure.

319 The far-UV spectrum of LA at neutral pH displayed two minima of ellipticity near
320 208 and 220 nm (Figure 4c). Comparatively, at pH 2.0, it showed, approximately, a 0.01%
321 of alpha helix and beta strand losses. However, the near-UV spectra showed that the
322 intensity of the dichroic signals in the 250-300 nm region was very low at pH 2.0 in
323 comparison with pH 7.0 (Figure 4d). This is in agreement with previous reports that
324 indicated that, at acidic pH, LA maintains a native-like content of α-helical structure, but
325 its tertiary structure is almost completely disrupted due to the formation of a partially
326 folded state named molten globule (Polverino de Laureto et al., 2002; Moreno et al.,
327 2005b). The enhanced flexibility or local unfolding of LA over LYS at acidic pH
328 probably favors cleavage of the former by pepsin, while LYS is highly resistant.

329 Intrinsic fluorescence emission spectra of LYS at pH 2.0 and 7.0 are shown in
330 Figure 5a. The presence of six tryptophan residues in LYS, with different spectral
331 contributions because of their different polar environments, results in relatively broad
332 fluorescence spectra (Gorbenko et al., 2007). The lower intensity at acidic pH can be
333 attributed to a quenching effect exerted by protonated acidic groups. Upon titration with
334 PC at pH 2.0, the wavelength of maximum emission (λmax) on excitation at 280 changed
335 from 343 nm to 340 nm. Since the transfer of Trp into an environment with a lower
336 polarity usually coincides with a blue shift or the λmax, this suggests that mixing of LYS
337 with PC modified the local environment of the protein making it more hydrophobic,
338 possibly through membrane binding. In fact, a blue shift in the spectrum of LYS has been

339 attributed to a shielding effect from water arising from the penetration of the protein into a
340 phospholipid bilayer (Yuan et al., 2007). When different amounts of PC were titrated into
341 a fixed concentration of LYS at acidic pH the fluorescence intensity decreased regularly
342 (Figure 5c), suggesting an interaction between the phospholipid and the protein that
343 changed the accessibility of Trp residues to the solvent. On the other hand, at neutral pH,
344 fluorescence intensity hardly changed on PC addition.

345 Regarding bovine LA, the longer wavelength of the λmax and the higher
346 fluorescence intensity of the spectrum at pH 2.0, as compared to pH 7.0, supported that
347 the Trp in the molten globule are more accessible to the solvent than in native LA
348 (Svenson et al., 1999) (Figure 5b). In this case, titration experiments showed that the
349 fluorescence intensity progressively changed with the concentration of PC, decreasing at
350 pH 2.0 and increasing at pH 7.0 (Figure 5b and 5d). This suggests that, at pH 2.0, there is
351 a partial insertion of Trp into the apolar phase of the lipid bilayer, while at pH 7.0,
352 association leads to an increase in Trp accessibility. In fact, at acidic pH, LA behaves as a
353 membrane intrinsic protein and penetrates into PC vesicles. Below its isoelectric point
354 (4.8), hydrophobic forces dominate the interaction of LA and PC, with binding being
355 probably reinforced by the unfolding of the protein that increases surface hydrophobicity
356 (Cawthern et al., 1996; Moreno et al., 2005b). Above its isoelectric point, the interaction
357 with the zwitterionic phospholipid is mainly electrostatic, what induce a loose association
358 with the outer surface of the vesicles (Moreno et al., 2005b). Deep insertion of proteins
359 into membranes has been reported to prevent their degradation on incubation with
360 proteases, while membrane association, without loosing protein integrity, may make them
361 more sensitive to proteolytic degradation (Mogensen et al., 2007).

362 While LA attains a molten globule estate at pH 2.0, LYS remains native.
363 Nevertheless, LYS could still interact with zwitterionic phospholipids, such as PC,

364 through hydrophobic and polar interactions that could lead to LYS association to PC
365 films. In fact, and it has been reported that LYS could be entrapped in non-charged PC
366 liposomes (Witoonsaridsilp et al., 2010). However, penetration is difficult once adsorption
367 has occurred, because LYS is excluded from interacting with the hydrophobic portion of
368 the lipid below the chain transition temperature of PC, which is approximately 41ºC
369 (Mudgil et al., 2006). On the other hand, LYS, with an isoelectric point near 11, is a
370 highly electropositive protein, that can easily penetrate anionic phospholipid vesicles,
371 such as formed by phosphatidylserine or phosphatidylglycerol. In this case, LYS binding
372 to anionic vesicles is governed by electrostatic effects that lead to LYS penetration into
373 the lipid phase (Mudgil et al., 2006; Witoonsaridsilp et al., 2010).
374

375 **3.3 Effect of the Presence of BS and PC on the Duodenal Digestion of LYS in vitro**

376 The gastric digests of LYS produced after 60 min at pH 2.0, with and without the
377 addition of PC, once adjusted to pH 7.0 and centrifuged, were subjected to an in vitro
378 duodenal digestion for 30 min with trypsin and chymotrypsin in the presence of BS.
379 Under those conditions, LYS completely disappeared (Figure 2d), despite, according to
380 the literature, trypsin and chymotrypsin hardly hydrolyse the protein (During et al., 1999),
381 or just hydrolyse it partially after overnight incubation at 37ºC (Mine et al., 2004).

382 To elucidate whether the presence of BS could have an effect on LYS solubility, we
383 incubated the intact protein with three different duodenal digestion media, typical of a
384 fasted state, an intermediate state similar to the duodenal mixture we used for digestion,
385 and a fed state, but without proteases and lipases. Figure 6 shows the effect on LYS
386 solubility of the different simulated small intestine conditions.

387 LYS was fully soluble in 7.6 m*M* $CaCl_2$ and 20.3 m*M* Bis-Tris at pH 6.0, 6.5, and
388 7.0, but it precipitated in the presence of BS. The concentration of amphiphilic bile

389 components in the upper intestine, including BS and PC, increases after a meal, while the
390 pH decreases (Kostewicz et al., 2002). As shown in Figure 6, the higher the bile salt
391 concentration the lower the amount of soluble LYS. This indicated that the disappearance
392 of the protein could not be totally attributed to its hydrolysis by duodenal enzymes but, to
393 some extent, to its precipitation with BS in the duodenal medium. It should be noted that
394 the presence of PC partially avoided LYS precipitation, suggesting a positive effect on
395 solubilization of the mixed bile salt-PC micelles present in the duodenal medium
396 (Mandalari et al., 2009). It should be noted that the BS concentration used was always
397 over the critical micelle concentration, 3.5 m*M* (Kaukonen et al., 2004).

398 Although, according to Burnett et al. (2002), BS exert a solubilizing effect,
399 removing proteins adsorbed to emulsions and favoring their resolubilization in aqueous
400 phases, our results show that it is likely that LYS precipitates in the duodenum at pH
401 values, BS and PC concentrations representative of a fed state and, to a lesser extent, of a
402 fasted state. In fact, when LYS is administered orally to human patients (as used for the
403 treatment of chronic sinusitis and to promote expectoration in the case of respiratory
404 disease), food intake negatively affects the uptake of the enzyme, with the maximum
405 levels detected being almost 10-fold lower than those after an identical dose taken after an
406 overnight fast (Hashida et al., 2002).

407 Insolubilization of LYS in the presence of BS could impair its hydrolysis by
408 pancreatic enzymes, affecting its presence in the intestinal tract. Despite its molecular
409 weight, LYS can be effectively absorbed and its uptake occurs preferentially in the upper
410 intestine (Takano et al., 2004). Absorption of the intact protein could be particularly
411 relevant in infants, whose immature gastrointestinal epithelium allows more proteins to
412 cross the barrier into the circulatory system. Furthermore, while LYS precipitation could
413 play a role in the uptake of LYS by intestinal brush border membranes and its presentation

414 to the immune system, it may positively enhance the proportion of antimicrobial agents
415 that can play a defensive role in lower parts of the intestine.
416
417 **3.4 Effect of Digestion on IgG- and IgE- Binding**
418 The IgG and IgE-binding properties of the gastric digests obtained after 60 min
419 were examined by inhibition ELISA. The IgG-binding was estimated with a polyclonal
420 commercial antibody against LYS raised in rabbit, while for IgE-binding we used sera of
421 patients allergic to egg with proved immunoreactivity against LYS.
422 The digests exhibited a substantial residual antigenicity both against IgG and IgE
423 (Figures 7a and 7b), which could only be somewhat attributed to the presence of residual
424 LYS, since, as already mentioned, LYS partially precipitated (around 40-45% as
425 estimated by RP-HPLC) when the pH was adjusted to 7.0 to inactivate pepsin. This
426 indicated that some of the proteolysis products retained a noticeable reactivity against IgG
427 and IgE and that, in fact, the reactivity of the digests was comparatively higher than that
428 of the intact protein. The highest IgG-binding was detected in the gastric digests obtained
429 in the presence of PC ($P<0.05$), which also contained more intact protein than those
430 produced in the absence of PC. Regarding IgE-binding, as expected, the responses were
431 shown to vary depending on the patient's individual susceptibility. In this case, there were
432 no differences between the hydrolysates obtained with or without PC. Overall, these
433 results show that in vitro gastric digestion of LYS led to the formation of peptides which
434 carried an important epitope load and thus, the potential to be allergenic.
435
436 **4. CONCLUSIONS**
437 Digestion of LYS with pepsin was conducted at an enzyme: substrate ratio of 1:20
438 (wt/wt) (172 U/mg), considered as a physiological ratio, under different pHs that

439 simulated various conditions in the stomach. Hydrolysis of the protein only occurred at
440 pH 1.2 and 2.0, typical of a fasted state, with the formation of degradation products of less
441 than 4-5 kDa that resisted pepsin action for at least 2 hours of digestion. At pH values
442 equal or higher than 3.2, typical of a fed state or of immature stomach functions, there was
443 no detectable hydrolysis of the protein, even after 120 min of digestion. The presence of
444 PC, a physiological surfactant, slightly increased LYS resistance to digestion, decreasing
445 the presence of degradation products, and a similar behavior was found for its
446 homologous protein LA. At acidic pH, the enhanced flexibility or local unfolding of LA
447 over LYS, as determined by circular dichroism spectroscopy probably favored the
448 cleavage of the former by pepsin, while LYS was more resistant. Intrinsic fluorescence
449 emission spectra of LYS and LA at pH 2.0, on addition of PC, suggested that the local
450 environment of the proteins became more hydrophobic, possibly through membrane
451 binding. It is, therefore, likely that, at acidic pH, both proteins interact with PC, leading to
452 their association to PC films.

453 IgE-binding experiments using sera of patients allergic to egg showed that in vitro
454 gastric digestion of LYS led to the formation of peptides which carried an important
455 epitope load and could have the potential to be allergenic. The identification of these
456 peptides and their resistance to hydrolysis by pancreatic proteinases are currently being
457 addressed. On the other hand, our results show that unhydrolysed LYS precipitated at pH
458 values, BS and PC concentrations representative of a duodenal fed state (characterized by
459 high BS and PC concentrations and low pH) and, to a lesser extent, of a fasted state. LYS
460 precipitation in the presence of BS could impair its hydrolysis by pancreatic enzymes,
461 either affecting the amount of immunoreactive protein that is absorbed or the proportion of
462 antimicrobial agents that can play a defensive role in lower parts of the intestine.

463

464 **5. ACKNOWLEDGEMENTS**

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469 Madrid, respectively.

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567

7 FIGURE CAPTIONS

Figure 1. SDS-PAGE analysis of LYS digested with pepsin, at a pepsin:protein ratio of 1:20 (w:w), at different pHs and hydrolysis times. Lane 1: molecular mass markers; lane 2: LYS; lanes 3, 4 and 5: LYS digested at pH 1.2 for 0, 60 and 120 min; lanes 6 and 7: LYS digested at pH 2.0 for 60 and 120 min; lanes 8 and 9: LYS digested at pH 3.2 for 60 and 120 min; lanes 10 and 11: LYS digested at pH 4.0 for 60 and 120 min; lanes 12 and 13: LYS digested at pH 4.5 for 60 and 120 min.

Figure 2. RP-HPLC patterns of LYS (a) and its digests under different conditions (b, c, d). In vitro gastric digestions at pH 2.0, for 60 min, in the absence (b); and presence (c) of phosphatidylcholine (PC); and in vitro duodenal digest of (b) for 30 min (d).

Figure 3. RP-HPLC patterns of LA (a, e) and its pepsin digests obtained at pH 2.0 in the absence (a-d); and presence (e-h) of phosphatidylcholine (PC); at different hydrolysis times: 0 (a, e), 5 (b, f), 15 (c, g), and 30 (d, h) min.

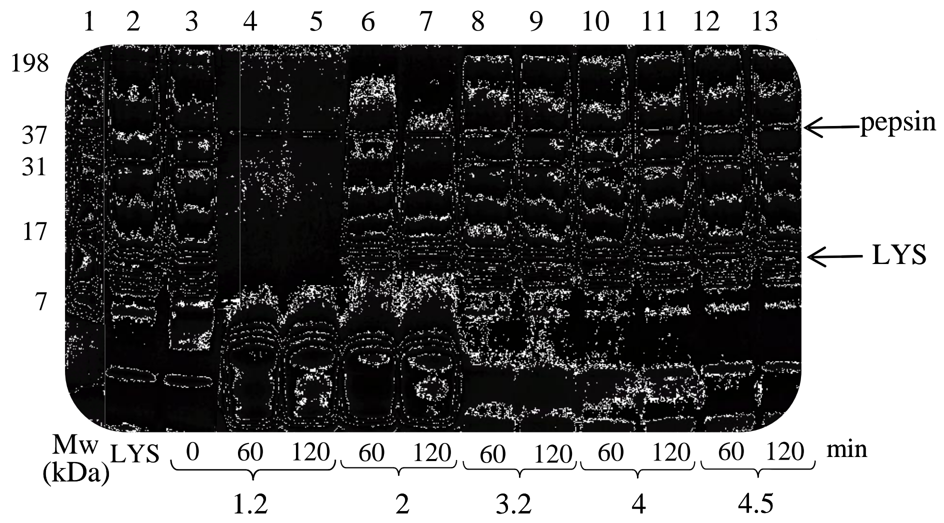
Figure 4. Circular dichroism spectra in the far (a, c) and near (b, d) UV region of LYS (a, b) and LA (c, d), at pH 2.0 and 7.0 in the absence and presence of phosphatidylcholine (PC).

Figure 5. Fluorescence spectra of LYS (a) and LA (b), at pH 2.0 and 7.0, in the absence and presence of phosphatidylcholine (PC), and corrected LYS (c) and LA (d) titration curves with PC at pH 2.0 and pH 7.0.

593 Figure 6. SDS-PAGE analysis of LYS soluble in 7.6 m*M* $CaCl_2$ and 20.3 m*M* Bis-Tris
594 mimicking duodenal conditions typical of a fasted (2, 3, 4); intermediate (5, 6, 7) and fed
595 (8, 9, 10) state. Lane 1: molecular mass markers; lane 2: LYS at, pH 7.0; lane 3: LYS at
596 pH 7.0 with 3 mM of each BS and 2.4 mM PC; lane 4: LYS at pH 7.0 with 3 mM of each
597 BS; lane 5: LYS at pH 6.5; lane 6: LYS at pH 6.5 with 6 mM of each BS and 6.5 mM PC;
598 lane 7: LYS at pH 6.5 with 6 mM of each BS; lane 8: LYS at pH 6.0; lane 9: LYS at pH
599 6.0, with 9 mM of each BS and 7.2 mM PC; lane 10: LYS at pH 6.0 with 9 mM of each
600 BS.
601
602 Figure 7. Inhibition ELISA response against rabbit poyclonal anti-LYS IgG (a) and human
603 IgE (b) of LYS and its pepsin digests obtained at pH 2.0 in the absence (-PC) and presence
604 of phosphatidylcholine (+PC) for 60 min. Human sera were IgE $>$ 100 kU/L. The IgG- and
605 IgE-binding capacities of the digests were expressed as percentages of the IC50 of the
606 intact protein. Significant differences ($P<0.05$) were evaluated by one-way analysis of
607 variance (ANOVA). Different letters above the bars indicate significant differences ($P<$
608 0.05). Error bars correspond to the mean ± standard error (n=3).

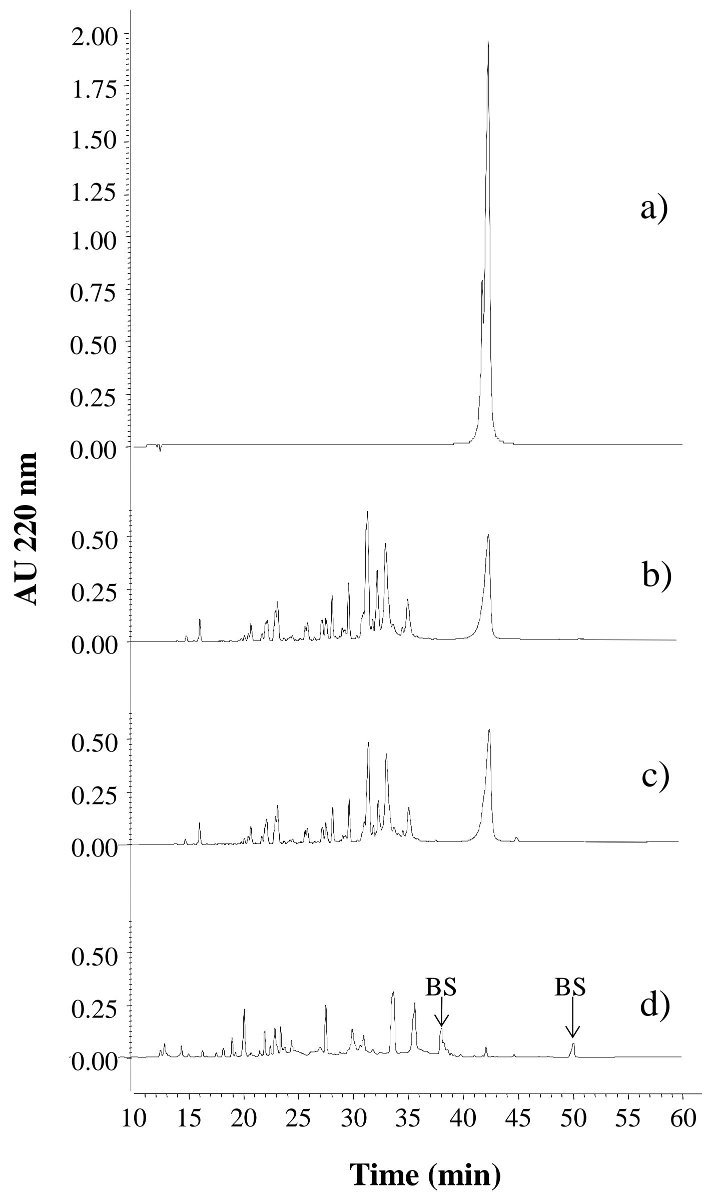
1 **Figure 1. Jiménez-Saiz**

2



3 **Figure 2. Jiménez-Saiz**

4



5 **Figure 3. Jiménez-Saiz**

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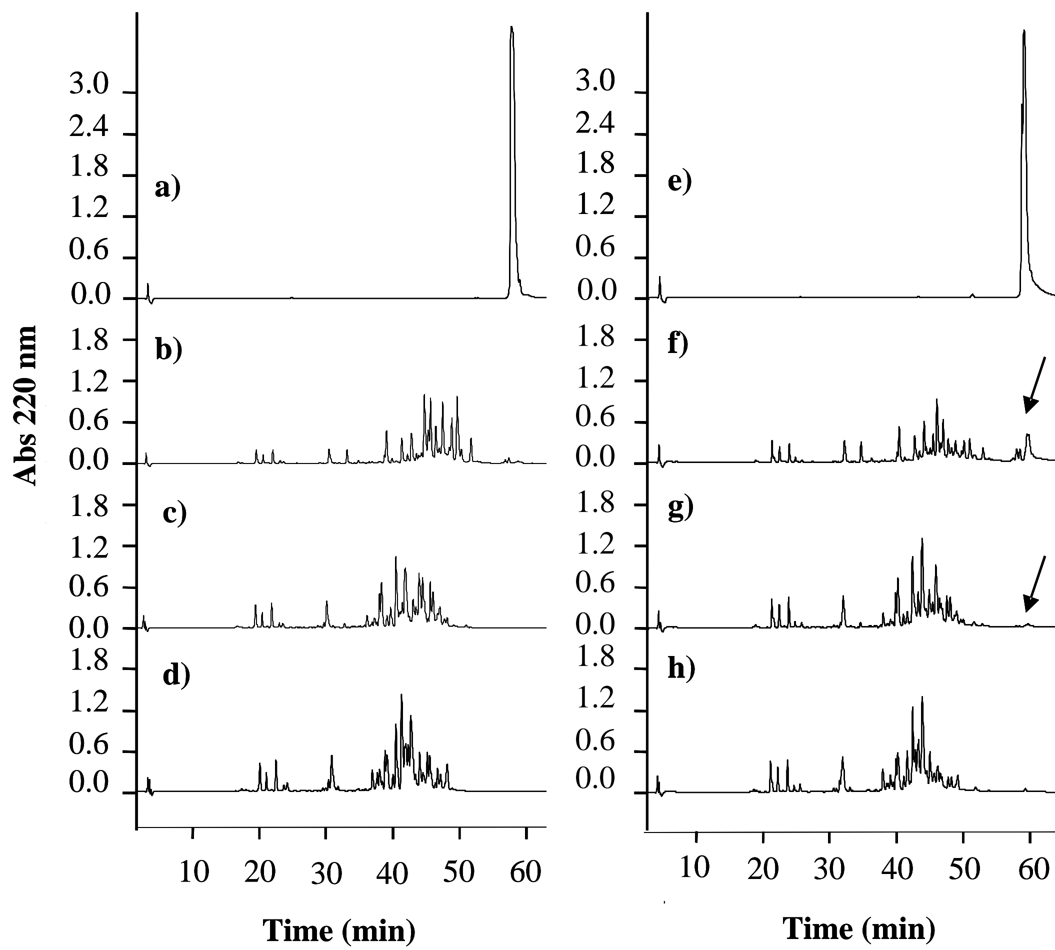
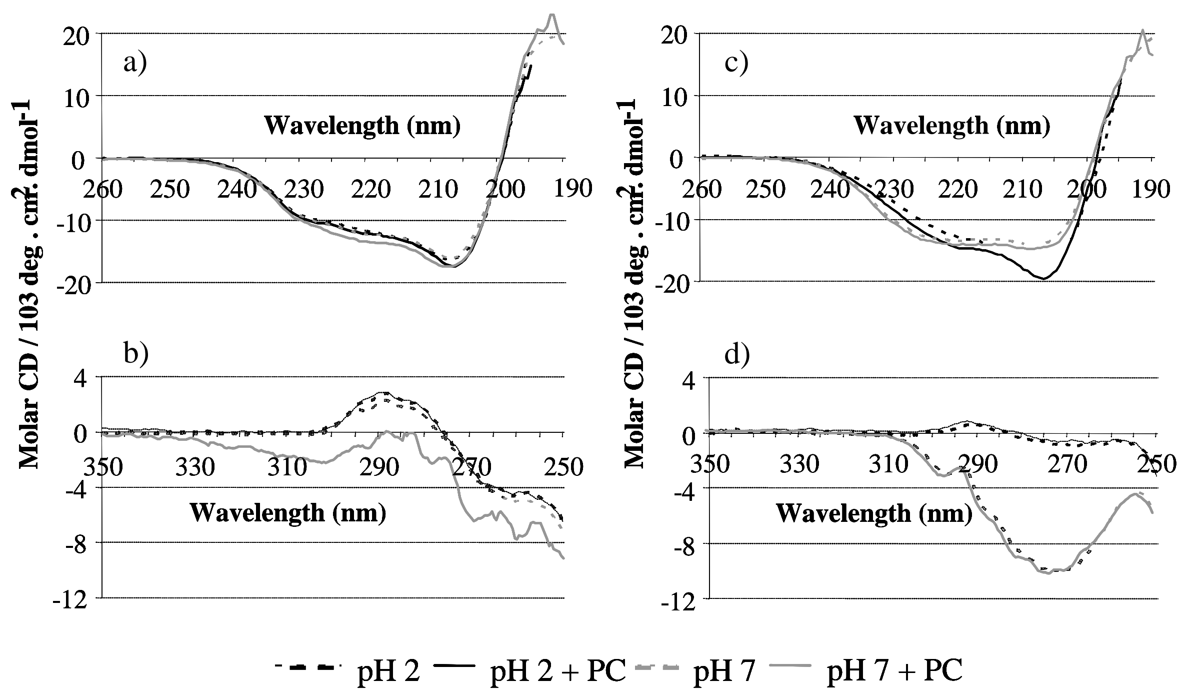


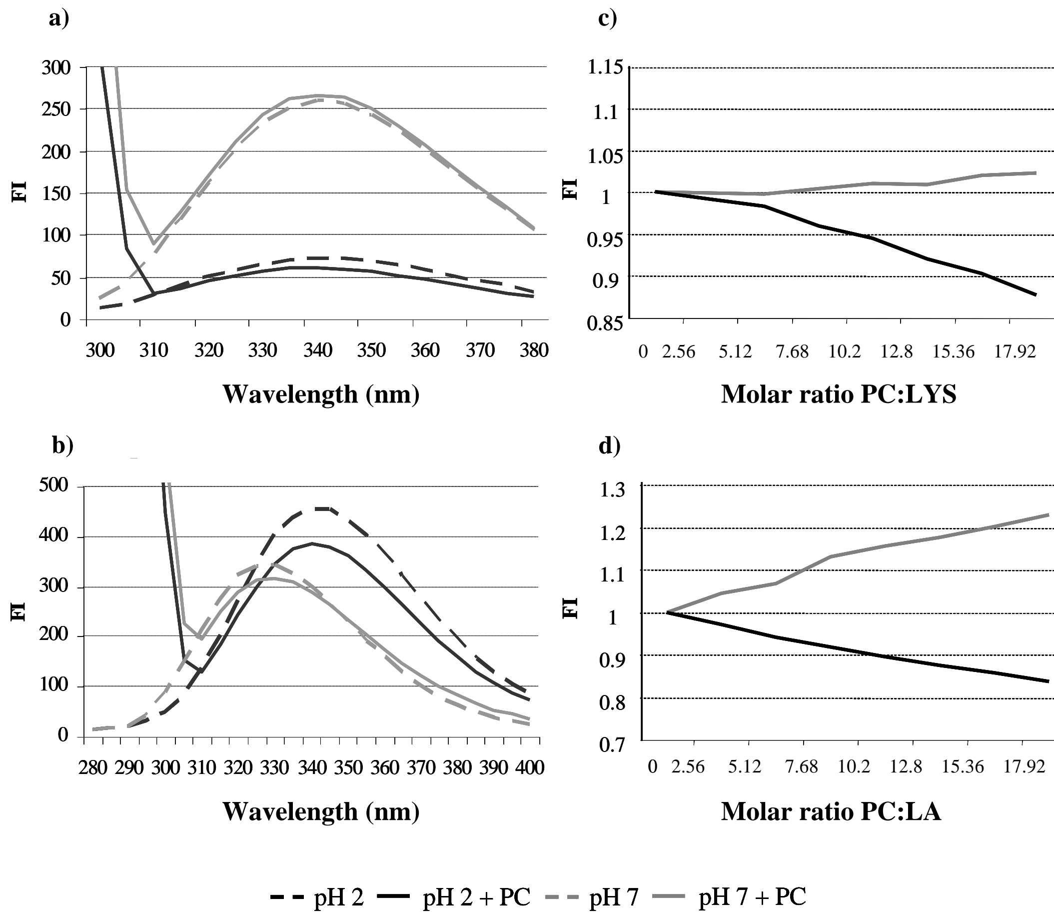
7 **Figure 4. Jiménez-Saiz**

8



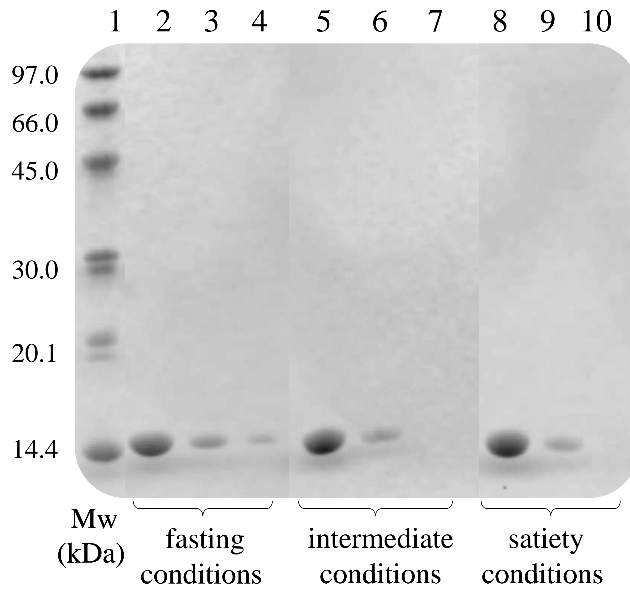
9 **Figure 5. Jiménez-Saiz**

10

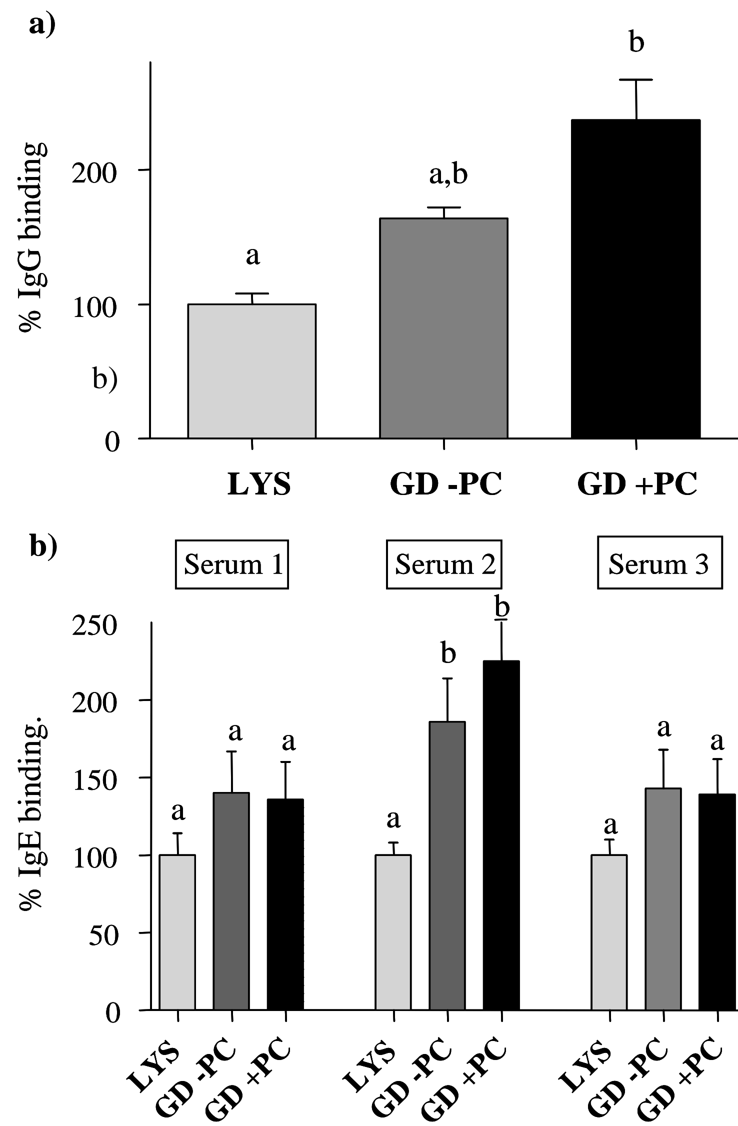


11 **Figure 6. Jiménez-Saiz**

12



13 **Figure 7. Jiménez-Saiz**



Cover Letter

CIAL CSIC UNIVERSIDAD AUTONOMA DE MADRID
Instituto de Investigación en Ciencias de la Alimentación

26th November 2010

Dear Editor,

I am pleased to send you the file of the original paper entitled "**Susceptibility of lysozyme to *in vitro* digestion and immunoreactivity of the digests.**" by Jiménez-Saiz et al., that we would like you to consider for publication in the Food Chemistry journal.

Looking forward to hearing from you.

Sincerely,

Elena Molina

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Campus de la Universidad
Autónoma de Madrid
28049 Madrid