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Osvaldo E. Sponton, Adrián A. Perez, Carlos Carrara, Liliana G. Santiago

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1 **Effect of limited enzymatic hydrolysis on linoleic acid binding properties of β -Lactoglobulin**

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3 Osvaldo E. Sponton, Adrián A. Perez, Carlos Carrara & Liliana G. Santiago*,

4
5 Grupo de Biocoloides, Instituto de Tecnología de Alimentos, Universidad Nacional del Litoral,

6 1 de mayo 3250 (3000) Santa Fe, Argentina

7
8 * Corresponding author. Tel.: Tel.: +54 342 4571252 ext. 2602

9 E-mail address: lsanti@fiq.unl.edu.ar (L. G. Santiago).

10 **Abstract**

11 β -Lactoglobulin (BLG) is a member of lipocalin family, proteins with ability to bind small
12 hydrophobic ligands, such as retinol, vitamins and fatty acids. Moreover, BLG is susceptible to
13 protease action producing a wide range of polypeptides depending on the hydrolysis degree (HD).
14 In the present work, the effect of limited enzymatic hydrolysis on fatty acid binding properties of
15 BLG was studied. Linoleic acid (LA) was used as a model fatty acid. Limited enzymatic hydrolysis
16 was performed using α -chymotrypsin immobilized on agarose microparticles. BLG hydrolysates
17 were produced at HD: 1, 3 and 5%. In order to determine the influence of HD on BLG molecular
18 weight SDS-PAGE was used. BLG structural modification and LA binding properties were
19 monitored by means of fluorescence spectroscopic techniques. The increase in HD produced: (i) a
20 BLG degradation and a molecular weight distribution of BLG hydrolysates and (ii) an increased
21 exposition of buried hydrophobic residues, however it was observed a decrease in surface
22 hydrophobicity possibly due to a deterioration of hydrophobic protein domains. It was observed that
23 enzymatic hydrolysis treatment produced a decrease in BLG ability for binding LA. It was
24 concluded that limited enzymatic hydrolysis could deteriorate the specific site on BLG structure
25 necessary for binding LA.

26

27 **Key words:** β -Lactoglobulin, Enzymatic hydrolysis, α -Chymotrypsin, Fluorescence Spectroscopy,

28 Linoleic Acid, Binding Properties.

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29

30 1. Introduction

31

32 Milk whey proteins are widely used in food industry for their nutritional and functional
33 properties such as foaming, emulsifying and gelling (Bolder, Vasbinder, Sagis, & van der Linden,
34 2007; Essemine, Hasnia, Carpentiera, Thomas, & Tajmir-Riahia, 2011; Perez, Carrara, Carrera
35 Sánchez, Rodríguez Patino, & Santiago, 2012a,b). Generally, functional properties of these
36 commercial preparations could be explained in terms of β -Lactoglobulin (BLG) which constitutes
37 the main protein fraction of milk whey proteins (Bolder et al., 2007, Croguennec, O’Kennedy, &
38 Mehra, 2004). BLG is a globular protein with 162 aminoacids residues, 18.3 kDa and its pI is ~5.1
39 (Bhattacharjee & Das, 2000; Bolder et al., 2007; Fennema, 1996). At pH higher than 7.5 or lower
40 than 2.0 BLG shows a monomeric structure. At pH between 2.0-3.7 and 5.1-7.5 BLG form dimers,
41 while at pH between 3.7-5.1 BLG forms octamers. Two disulphide bounds (Cys106–Cys119,
42 Cys66–Cys160) stabilize the BLG globular structure (Cheison, Lai, Leeb, Kulozik, 2011). In this
43 conformation, hydrophobic domains are mainly buried into the protein minimizing free energy,
44 while hydrophilic domains are located at the protein-water interphase (Kato & Nakai, 1980;
45 Fennema, 1996).

46 Another interesting functional property of BLG is its ability for solubilization and transport of
47 bioactive lipophilic nutrients such as retinol, fatty acids and vitamin D (Considine, Patel, Singh,
48 Creamer, 2005; Qin, Creamer, Baker, & Jameson, 1998; Wang, Allen, & Swaisgood, 1999). This
49 feature allows classifying BLG in a super family of carriers of small hydrophobic molecules called
50 *Lipocalins*. Lipocalins have a common tridimensional structure which consists of eight stranded
51 antiparallel β -sheets. Aminoacid residues of antiparallel β -sheets are adjacent in space but not in
52 sequence and they form a hydrophobic β -barrel (Frapin, Dufour, & Haertle, 1993; Sawyer &
53 Kontopidis, 2000). Crystal structure of bovine BLG presents a similarity with plasmatic retinol-

binding proteins and flavor-binding proteins (Fessas, Lametti, Schiraldi, & Bonomi, 2001; Frapin et al., 1993). Moreover, it is well known that BLG has two lipophilic ligand binding sites: a central hydrophobic β -barrel (or *calyx*) and a superficial *pocket* (Cheison et al., 2011; Fennema, 1996; Wang, Allen, & Swaisgood, 1998, 1999). Superficial *pocket* is located close to dimer contact region, between an α -helix (sited laterally to the *calyx*) and the *calyx* surface (Cheison et al., 2011; Frapin et al., 1993; Wang et al., 1998, 1999). Usually fatty acids binds at BLG *pocket*, while others molecules (as retinol) binds to BLG *calyx*. Thus, BLG could simultaneously link two lipophilic molecules (Frapin et al., 1993; Wang et al., 1999).

Currently, there is an increased interest on the development of several kinds of biopolymer nano and microparticles with applications in foods, cosmetics and pharmaceutical products. Therefore, binding properties of BLG could allow the development of biopolymer nanoparticles for encapsulation of bioactive lipophilic compounds (ω -3 fatty acids, linoleic acid, carotenoids or vitamins) in order to protect them from deterioration induced by UV radiation, oxygen, moisture, etc. (Jones & McClements, 2011; Matalanis, Jones, & McClements, 2011; McClements & Li, 2010)

On the other hand, BLG is sensitive to the action of proteases. Enzymatic structural modifications could mainly include a decrease in protein molecular weight and an increased exposition of buried hydrophobic sites (Caessens, Daamen, Gruppen, Visser, & Voragen, 1999; Hernández-Ledesma, Ramos, Recio, & Amigo, 2006; Madsen, Ahmt, Otte, Halkier, & Qvist, 1997). Moreover, it has been reported that BLG hydrolysis with trypsin could promote disulfide links between polypeptides (Caessens et al., 1999). Limited enzymatic hydrolysis (at lower hydrolysis degrees) could modify BLG functionality, such as emulsifying, foaming and gelling properties (Caessens, Visser, Gruppen, & Voragen, 1999; Perez et al., 2012a,b). Nevertheless, the incidence of limited enzymatic hydrolysis of BLG on fatty acid binding properties has been not evaluated yet. In this context, the aim of the present work was to evaluate the effect of limited enzymatic hydrolysis on the BLG ability for binding a model polyunsaturated fatty acid, linoleic acid (LA). Our hypothesis

was to modify the BLG hydrophobic characteristics by enzymatic hydrolysis in order to observe if the hydrolyzed BLG have an improved ability for binding LA. This information could be interesting for the design of encapsulation systems using BLG hydrolysates as carriers.

2. Materials and methods

2.1. Materials

BLG sample was supplied by Danisco Ingredients (Brabrand, Denmark). Its composition was: protein $92 \pm 2\%$ (BLG $> 95\%$, α -lactalbumin $< 5\%$), fat $< 0.2\%$, ash 1.9% y moisture 4.8% . This sample was used without previous purification. Linoleic acid (LA) sample was purchased from Sigma (USA) and 1-anilino-8-naphthalene sulfonic acid (ANS) was obtained from Fluka Chemie AG (Switzerland).

2.2. Limited enzymatic hydrolysis

Limited enzymatic hydrolysis was performed using α -chymotrypsin II (EC 3.4.21.1) (Sigma, USA). Enzyme was immobilized on agarose microparticles (40 mg/g of support). The use of immobilized enzyme allows the enzyme recuperation after enzymatic hydrolysis, for example, through a filtration process. Thus, residual enzyme in the obtained hydrolysate samples is minimized. Enzymatic reaction conditions were pH 8 and 50°C . Hydrolysates samples were obtained at different hydrolysis degrees (HD): 1% (H1), 3% (H3) y 5% (H5). HD was monitored by mean of pH-stat method and the HD was calculated according to Spellman, McEvoya, O'Cuinnb, & FitzGerald, (2003). The composition of BLG hydrolysates was: (i) H1: protein 88.09%, moisture 6.30%, ash 4.20%, and others (possibly fat and/or lactose) 1.41%; (ii) H2: protein 88.00%, moisture

7.30%, ash 4.31%, and others 0.39%; and (iii) H3: protein 85.90%, moisture 7.43%, ash 5.50%, and others 1.17%. Further experimental considerations about limited enzymatic hydrolysis procedure are described in Perez et al., (2012a).

2.3. SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970) with some modifications. Model electrophoretic cell Mini Protean II (Bio-Rad Laboratories, USA) was used. SDS-PAGE was applied using a discontinuous gel system. Stacking and separation gels were prepared using 4 and 13 % acrylamide concentration, respectively. Experiments were carried out in Tris-Gly buffer at pH 8.3. Samples were dispersed in Tris-Gly buffer containing SDS and bromophenol blue (advancing front indicator). Protein dispersions (~3 mg/ml) were heated during 5 minutes at 95°C in order to allow SDS fixation. The following molecular weight markers (LMW Kit, VWR International, USA) were used: α -lactalbumin (α -LA), 14.4 kDa; trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 45 kDa; bovine serum albumin (BSA), 66 kDa; phosphorylase B, 97 kDa. Other experimental conditions were: voltage 150 V, maximum intensity 45 mA and power 6.75 W. Gels were revealed with Coomassie Brilliant Blue R-250.

2.4. Fluorescence spectroscopy

Fluorescence measurements were performed using a Hitachi 2000 fluorescence spectrophotometer (Japan). For this BLG and its hydrolysates samples were dispersed in 0.05 M phosphate buffer pH 7, at 1% wt protein concentration. Then, hydrolysate dispersions were filtered using a cellulose ester filter (0.22 μ m) in order to eliminate possible protein aggregates. Intrinsic fluorescence spectra (due to Trp fluorescence emission) were obtained at $\lambda_{\text{ex}} = 295$ nm and the Trp

129 fluorescence emission was evaluated between 310-430 nm. In all cases the maximum fluorescence
 130 intensity (FI_{Trp}) and its wavelength ($\lambda_{M Trp}$) were registered (Perez, Carrara, Carrera Sánchez,
 131 Rodríguez Patino, & Santiago, 2009).

132 On the other hand, extrinsic fluorescence spectra were obtained as a measure of protein
 133 surface hydrophobicity. In this technique, ANS was used as a fluorescence probe. For this, 100 μ l of
 134 ANS solution (8 mM) were added to 2 ml of protein dispersion. Spectra were obtain at $\lambda_{ex} = 350$ nm
 135 and the ANS fluorescence emission was registered between 400-560 nm (Perez et al., 2009). For
 136 each spectrum the maximum fluorescence intensity (FI_{ANS}) and its wavelength ($\lambda_{M ANS}$) were
 137 registered. All fluorescence experiments were performed in triplicate.

138

139 **2.5. Linoleic acid binding experiments**

140

141 Binding experiments were carried out following the variation of Trp emission fluorescence
 142 intensity of proteins upon conjugation with LA. Emission spectra were obtained between 310-360
 143 nm ($\lambda_{ex} = 295$ nm) using a Hitachi 2000 fluorescence spectrophotometer (Japan). Conjugation
 144 process was monitored by the increase in maximum emission fluorescence intensity (FI_{Trp}) (Frapin
 145 et al., 1993). For this, 2 ml of protein solution were titrated with increasing volumes (0-26 μ l) of 4
 146 mM LA in ethanolic solution. Small increments (2 μ l) of LA solution were added (with a
 147 micropipette) in the tube containing protein solution. Proteins (BLG and its hydrolysates) were
 148 dispersed at 0.08% wt in 0.05 M phosphate buffer pH 7. Tubes were vortexed for 2 minutes prior to
 149 analysis. At the end of the various titrations the ethanol concentration amounted to less than 2 %,
 150 and was therefore assumed to have no effect on protein conformation (Cogan, Kopelman, Mokady,
 151 Shinitzky, 1976). In all cases, fluorescence intensity of the blank solution (buffer+LA) was
 152 subtracted from fluorescence intensity measurements of the LA/protein conjugates. Titration curves
 153 were represented by Trp relative fluorescence intensity (RFI_{Trp}) defined by the ratio between the

154 FI_{Trp} at a given LA concentration and FI_{Trp} at zero LA concentration. Binding experiments were
155 performed in triplicate at room temperature (25°C).

156 From titration curve for native BLG was obtained the LA binding parameters, such as
157 binding site number (n) and apparent dissociation constant (K_d'), according to the method
158 developed by Cogan et al. (1976). This parameters was obtained by mean of lineal regression from
159 the first point since the origin of $P_0.\alpha$ vs. $R_0.\alpha/(1-\alpha)$ plot according to:

$$P_0.\alpha = \frac{1}{n} \cdot \frac{R_0.\alpha}{1-\alpha} - \frac{K_d'}{n} \quad (1)$$

161

162 where P_0 is the total protein concentration (μM), R_0 is the total LA concentration, α is the fraction of
163 ligand binding sites in free protein molecules and is given by (Cogan et al., 1976):

$$\alpha = \frac{RFI_{Trp Max} - RFI_{Trp}}{RFI_{Trp Max} - RFI_{Trp 0}} \quad (2)$$

165

166 where $RFI_{Trp 0}$, RFI_{Trp} and $RFI_{Trp Max}$ are relative fluorescence intensity at zero LA concentration, at a
167 given concentration and at the LA saturation, respectively.

168

169 2.6. Statistical analysis

170

171 One way analysis of variance (ANOVA) was carried out using StatGraphics Plus 3.0
172 software, and statistical differences among systems were determined using LSD test at 95%
173 confidence level.

174

175 3. Results and discussion

176

177 3.1. SDS-PAGE

178

179 The effect of limited enzymatic hydrolysis, in the range of HD: 0-5%, on the BLG
 180 electrophoretic pattern (SDS-PAGE) under non-reducing conditions is presented in Figure 1. Figure
 181 shows the BLG used as substrate of α -chymotrypsin hydrolysis (lane B) in comparison with its
 182 hydrolysates: H1 (lane C), H3 (lane D) and H5 (lane E). It was observed that limited enzymatic
 183 hydrolysis modified BLG electrophoretic pattern. The increase in HD caused a shift of the BLG
 184 band (lane B) towards regions of lower molecular weight (lanes C-D). The intensity of lane E was
 185 lower than lanes B-D suggesting a more pronounced BLG degradation (molecular weight
 186 distribution) at the highest HD evaluated. Panyam & Kilara (1996) reported that the resultant
 187 protein hydrolysate could contains low molecular weight peptides as well as higher molecular
 188 weight peptides and unhydrolyzed proteins, depending on the specificity of the enzyme,
 189 environmental conditions and extent of hydrolysis.

190

191 **3.2. Intrinsic fluorescence spectroscopy**

192

193 Some BLG conformational characteristics could be determined by intrinsic fluorescence
 194 measurements (Albani, 2004; Bhattacharjee & Das, 2000). BLG has two Trp residues: Trp19 and
 195 Trp61 (Bhattacharjee & Das, 2000; Wang et al., 1998). Trp19 is located in the base of central
 196 hydrophobic *calyx*, while Trp61 is found close to protein surface (Wang et al., 1998). BLG intrinsic
 197 fluorescence is mainly due to Trp19 (~70% of the fluorescence) while Trp61 is a minor contributor
 198 due to the adjacent disulphide link Cys160-Cys166 which decreases its fluorescence (Bhattacharjee
 199 & Das, 2000; Fessas et al., 2001). Figure 2a shows the effect of HD (0-5%) on the BLG intrinsic
 200 fluorescence emission spectrum. It was noted that α -chymotrypsin hydrolysis caused a significant
 201 increase in BLG FI_{Trp} ($p < 0.05$). This behaviour is better observed in Figure 2b. Due to enzymatic
 202 hydrolysis, Trp19 situated into central *calyx* could be more exposed to aqueous medium and more

203 available to emit fluorescence. Moreover, this behaviour was consistent with the increased $\lambda_{M\ Trp}$
 204 with HD (as can be seen in Figure 2b). Red shift (displacement to the higher wavelengths) could
 205 indicate that Trp19 would be close to protein surface in major contact with polar aqueous medium
 206 (Albani, 2004). As can be seen in Figure 2b, the effect of enzymatic hydrolysis on protein intrinsic
 207 fluorescence (FI_{Trp}) does not follow a defined tendency (increase or decrease) because the enzyme only
 208 recognizes hydrophobic aminoacids both on the intact protein and on the released polypeptides (Galvão,
 209 Pinto, Jesus, Giordano, & Giordano, 2009). In the range of HD evaluated, neither the evolution of the
 210 protein intrinsic fluorescence intensity with the increase in HD nor the state of the hydrophobic site in this
 211 molecular species can be predicted. On the other hand, there are not significant differences between FI_{Trp}
 212 for H1 and H5; while FI_{Trp} for H3 was significantly lower than FI_{Trp} for H1 and H5 ($p<0.05$). This
 213 last result, could suggest a lower amount of Trp capable to emit fluorescence in H3 (Albani, 2004).
 214 Therefore, enzymatic hydrolysis could generate a heterogeneous population of molecular species that could
 215 interact with each other forming aggregates due to protein hydrophobic interactions between the
 216 increased exposed hydrophobic areas (Fennema, 1996) changing protein intrinsic fluorescence.
 217 However, as it can be seen in Figure 1, protein aggregation was not detected in electrophoresis
 218 neither for H3 nor for the other hydrolysates probably due to SDS could dissolve the formed
 219 hydrophobic aggregates.

220

221 3.3. Extrinsic fluorescence spectroscopy

222

223 Protein surface hydrophobicity could be determined by using ANS fluorescence probe. This
 224 probe binds to protein in a non-covalent way by means of hydrophobic or electrostatic interactions.
 225 When ANS binds to surface hydrophobic domains of protein its fluorescence emission intensity
 226 increases with respect to ANS alone in aqueous medium (Albani, 2004). The effect of HD (0-5%)
 227 on the BLG extrinsic fluorescence emission spectrum is presented in Figure 3a. It was noted a

gradual decrease in FI_{ANS} with the increased HD. These results are better observed in Figure 3b. This behaviour could indicate that ANS is bound to BLG in less proportion with the increase in HD (Albani, 2004). Bovine α -chymotrypsin is a serine protease that hydrolyzes peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met, Leu) on the carboxyl end of the bond (Galvão et al, 2009). Therefore, a limited enzymatic treatment with α -chymotrypsin could substantially decrease the number of protein hydrophobic domains available for ANS union or it might be presumed that the reduction of FI_{ANS} caused by the increasing degree of hydrolysis is proportional to the reduction of intact protein. In addition, it was observed that the increase in HD practically not changed $\lambda_{M\ ANS}$ with exception of H3. Limited enzymatic hydrolysis at 3% caused a significant decrease ($p<0.05$) in $\lambda_{M\ ANS}$ (blue shift) which could suggest that ANS is bound to a more hydrophobic environment on the protein (Albani, 2004). This result would be consistent with the idea of H3 aggregate formation via hydrophobic interactions, as it was discussed in the previous section. Panyam & Kilara (1996) reported that interactions facilitated by surface hydrophobic groups can lead to a reduction in the effective hydrophobicity.

242

243 3.4. Linoleic acid binding experiments

244

245 3.4.1. Binding properties of BLG

246

247 Trp fluorescence intensity increases when fatty acids bind to BLG superficial *pocket*
248 (Albani, 2004; Cogan et al., 1976; Frapin et al., 1993; Wang et al., 1999). Therefore, fluorescence
249 technique could be used in order to study the effect of α -chymotrypsin hydrolysis on the LA
250 binding properties of BLG. Figure 4 shows RFI_{Trp} of native BLG as a function of LA concentration
251 (0-47 μ M). The insert in Figure 4 corresponds to $P_0 \cdot \alpha$ vs. $R_0 \cdot \alpha / (1 - \alpha)$ used to obtain n and K_d' . It was
252 observed that the increase in LA concentration caused an increase in BLG RFI_{Trp} . This behaviour

could be explained considering an increase in LA-protein conjugate amount in solution (Cogan et al., 1976; Frapin et al., 1993). Therefore, it can be concluded that LA bound to the BLG *pocket*. In this study, it was found that LA bound to BLG with a binding site number (n) 0.89 ± 0.01 and an apparent dissociation constant (K_d') $6.40 \pm 3.17 \times 10^{-9}$ M. According to these results, there is only one LA binding site on BLG. These results are consistent with Frapin et al. (1993) who reported the following values: $n=0.83 \pm 0.08$ and $K_d'=1.90 \pm 0.01 \times 10^{-7}$ M. As it was described above, in this work it was used a commercial BLG sample without previous purification. Therefore, discrepancies in K_d' values could be attributed to the different pre-treatment of BLG samples.

3.4.2. Binding properties of BLG hydrolysates

Figure 5 presents the effect of HD (0-5%) on the BLG RFI_{Trp} as a function of LA concentration (0-47 μ M). Data were plotted as averages and standard deviations. It was observed that the increase in LA concentration caused an increase in RFI_{Trp} of proteins (BLG and its hydrolysates). Binding parameters (n and K_d') for LA-hydrolysate conjugates could not be obtained due to BLG hydrolysates showed a wide molecular weight distribution and so it is not possible to calculate P_0 (μ M). However, binding properties were studied from RFI_{Trp} vs LA concentration plots (Figure 5), taking into account that the increase in RFI_{Trp} corresponds to an increase in LA-protein conjugate amount in solution (Cogan et al., 1976; Frapin et al., 1993). It can be noted that limited enzymatic hydrolysis modified the LA binding properties of BLG. In spite of the small differences between RFI_{Trp} values, significant differences, mainly at the higher LA concentration, were observed. At the higher LA concentration, protein binding properties decrease in the following order: BLG>H1=H5>H3. Therefore, it could be observed that limited enzymatic hydrolysis at 3% (H3) presented the minimum LA binding ability. This finding is consistent with intrinsic and extrinsic fluorescence results discussed in previous sections. The α -chymotrypsin specific cleavage could

278 break the surface hydrophobic domains and consequently the LA binding site on BLG (Wang et al.,
279 1999). The binding of fatty acid to BLG should require a particular spatial disposition of the
280 aminoacid residues in BLG superficial *pocket* (a specific site for binding fatty acids) which could
281 have been attacked by α -chymotrypsin.

282

283 4. Conclusions

284

285 BLG enzymatic hydrolysis produced a major exposure of Trp19 (buried hydrophobic
286 aminoacid in BLG *calyx*) to the aqueous medium. However, the structure (spatial disposition) of
287 hydrophobic domains, especially at BLG *pocket*, could be broken as consequence of α -
288 chymotrypsin cleavage. This effect could produce a deterioration of LA binding properties of BLG.
289 Therefore, it was concluded that BLG limited enzymatic hydrolysis (at low HD) using α -
290 chymotrypsin would not a convenient technological strategy to improve its fatty acid binding ability
291 and consequently for the design of encapsulation systems.

292

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294

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299

300 6. References

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381 Color Figure Legends

382 **Figure 1.** Effect of limited enzymatic hydrolysis on BLG electrophoretic pattern (SDS-
383 PAGE) under non-reducing conditions. Lane A corresponds to molecular weight patterns: α -LA,
384 14.4 kDa; Trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 45 kDa; BSA, 66
385 kDa; phosphorylase B, 97 kDa. Lane B: native BLG (HD = 0 %), lane C: H1 (HD = 1 %), lane D:
386 H3 (HD = 3 %) and E a H5 (HD = 5 %).

387
388 **Figure 2.** Effect of limited enzymatic hydrolysis on: (a) intrinsic fluorescence emission
389 spectrum of BLG, symbols: (—) HD = 0%, (—) HD = 1%, (—) HD = 3%, (—) HD = 5%
390 and (b) maximum fluorescence intensity (FI_{Trp}) and its wavelength ($\lambda_{M Trp}$), represented with ■ and
391 ●, respectively. The error bars correspond to the standard deviation. Protein concentration: 1% wt.

392
393 **Figure 3.** Effect of limited enzymatic hydrolysis on: (a) extrinsic fluorescence emission
394 spectrum of BLG, symbols: (—) HD = 0%, (—) HD = 1%, (—) HD = 3%, (—) HD = 5%
395 and (b) maximum fluorescence intensity (FI_{ANS}) and its wavelength ($\lambda_{M ANS}$), represented with ■
396 and ●, respectively. The error bars correspond to the standard deviation. Protein concentration: 1%
397 wt.

398
399 **Figure 4.** Relative fluorescence intensity of Trp (RFI_{Trp}) vs Linoleic Acid (LA)
400 concentration. The insert shows the application of Eq (1) for the obtention of binding parameters of
401 LA-BLG conjugation. Protein concentration: 0,08% wt (44 μ M).

402
403 **Figure 5.** Effect of limited enzymatic hydrolysis of BLG on Trp relative fluorescence intensity
404 (RFI_{Trp}) as function of Linoleic Acid (LA) concentration. Symbols: (—●—) HD = 0 %, (—▲—) HD =
405 1 %, (—■—) HD = 3 % and (—★—) HD = 5 %. Protein concentration: 0,08% wt.

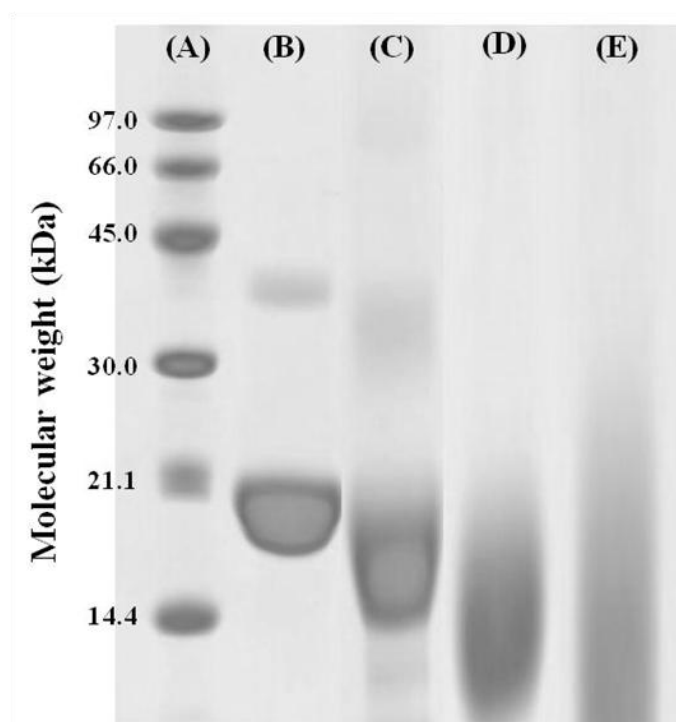
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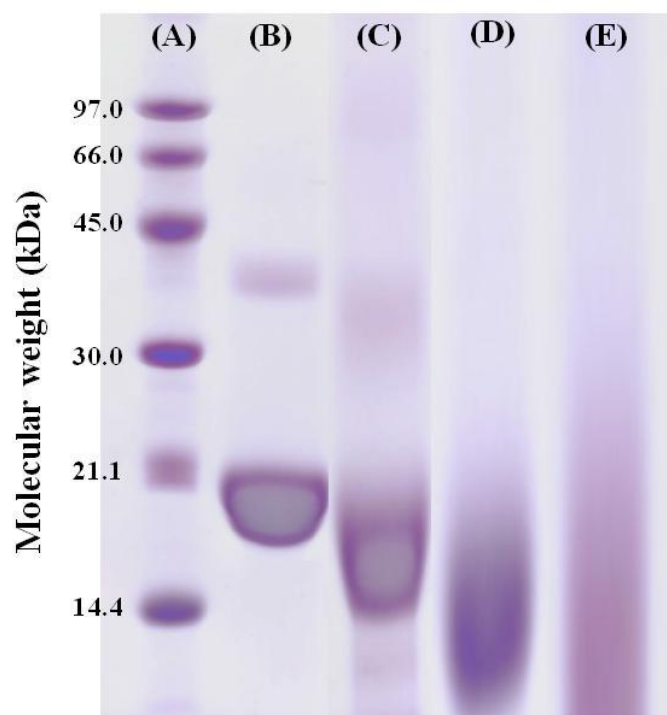
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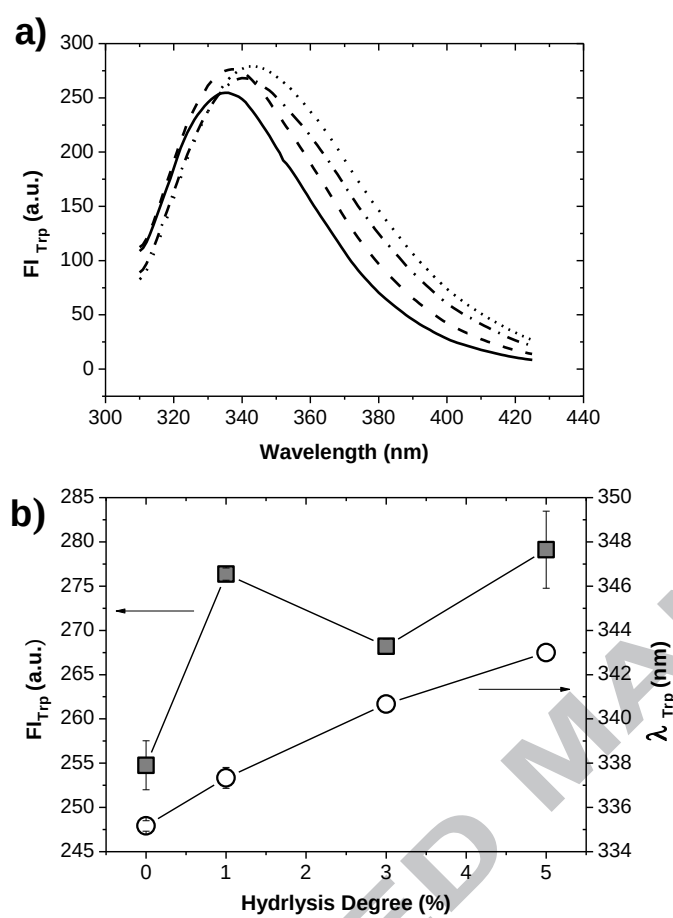


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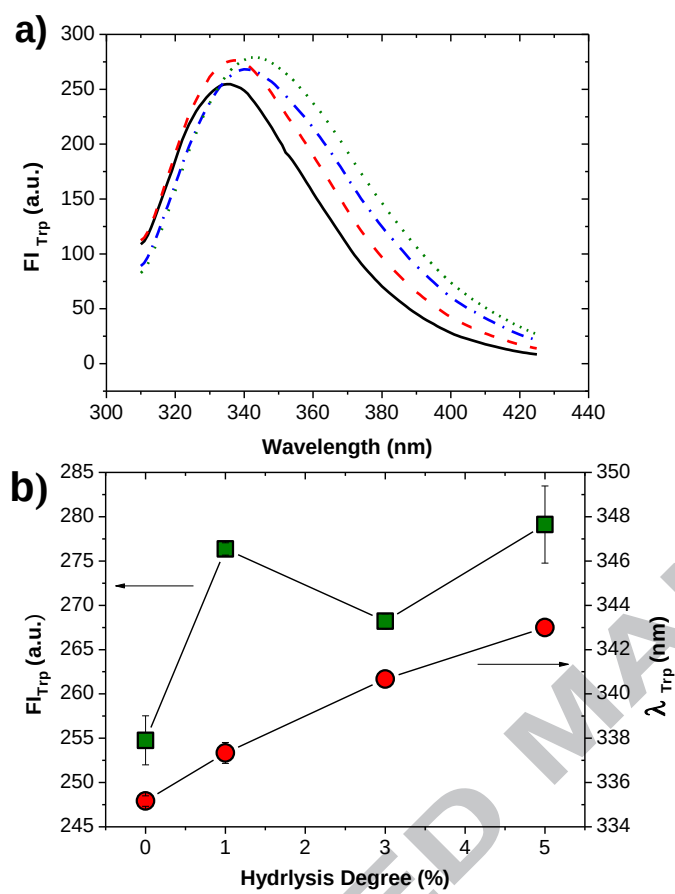


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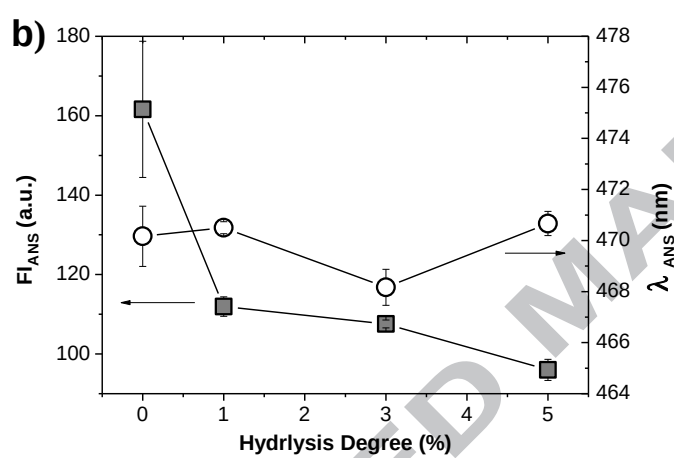
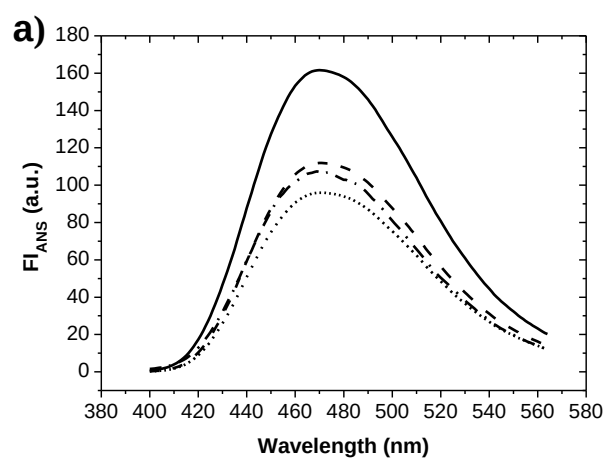


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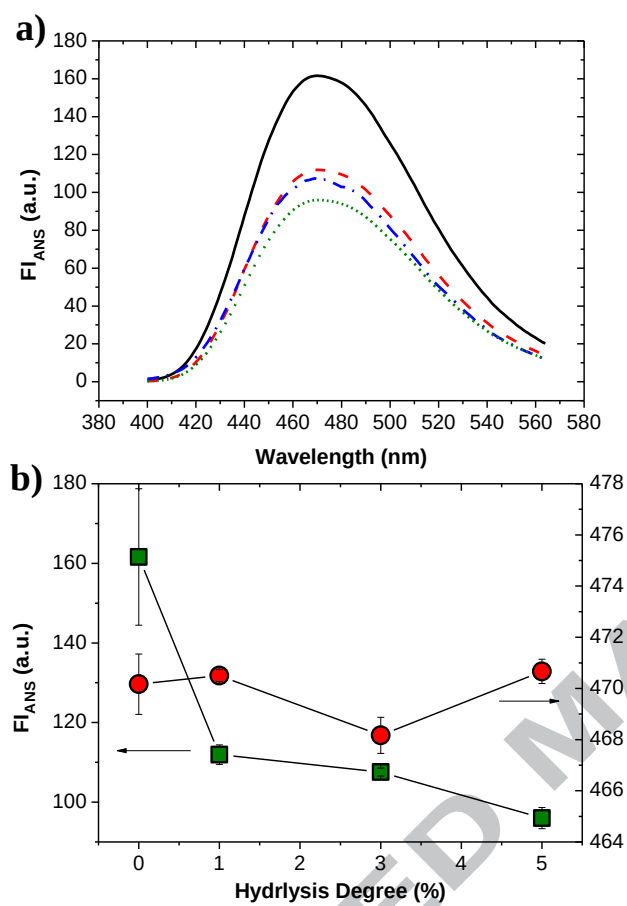


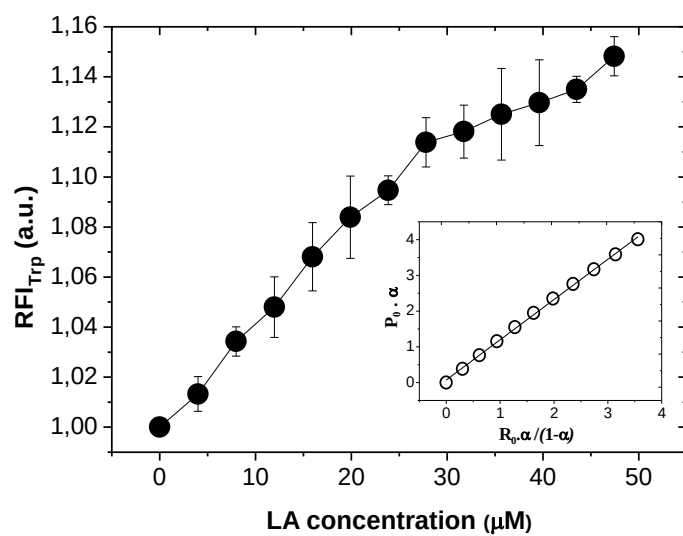
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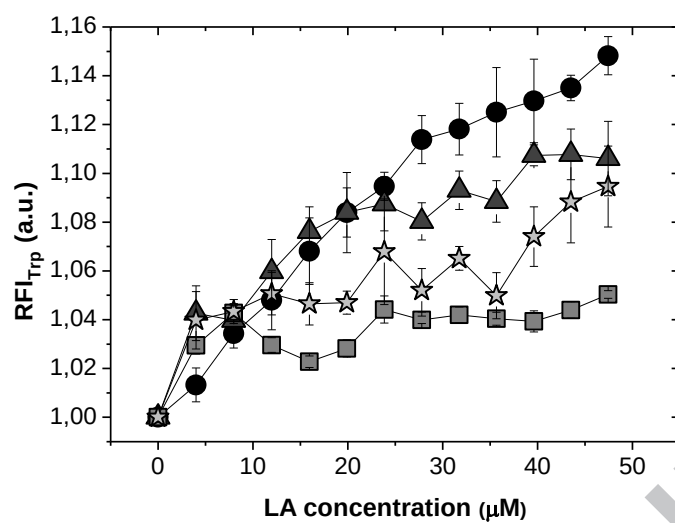
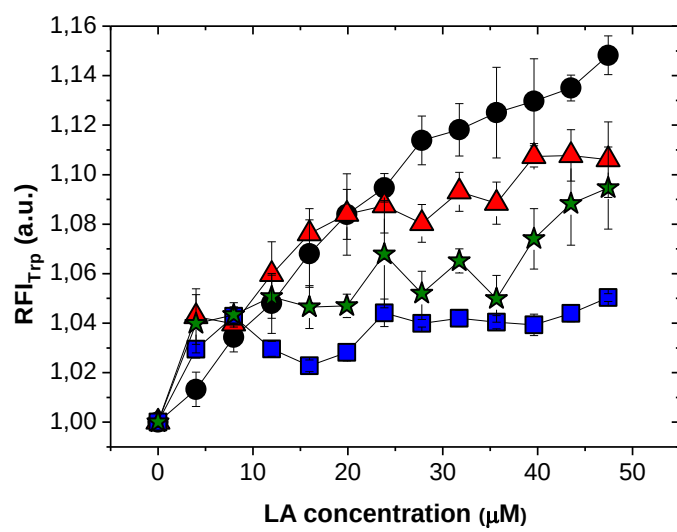
Figure 5

Figure 5



Black and White Figure Legends

Figure 1. Effect of limited enzymatic hydrolysis on BLG electrophoretic pattern (SDS-PAGE) under non-reducing conditions. Lane A corresponds to molecular weight patterns: α -LA, 14.4 kDa; Trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 45 kDa; BSA, 66 kDa; phosphorylase B, 97 kDa. Lane B: native BLG (HD = 0 %), lane C: H1 (HD = 1 %), lane D: H3 (HD = 3 %) and E a H5 (HD = 5 %).

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Figure 5. Effect of limited enzymatic hydrolysis of BLG on Trp relative fluorescence intensity (RFI_{Trp}) as function of Linoleic Acid (LA) concentration.

Symbols: (—●—) HD = 0 %, (—▲—) HD = 1 %, (—■—) HD = 3 % and (—☆—) HD = 5

%. Protein concentration: 0,08% wt.

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410 Highlights

411 Hydrolysis on linoleic acid (LA) binding ability of β -lactoglobulin (BLG) was studied.

412 Hydrolysis produced a molecular weight distribution of BLG hydrolysates. Hydrolysis caused an

413 increased exposure of BLG hydrophobic residues. Hydrolysis produced a reduction of BLG surface

414 hydrophobicity. LA binding ability of BLG decreased with hydrolysis due to LA binding site

415 disruption.

416 .

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418