Food Chemistry 146 (2014) 577–582

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effect of limited enzymatic hydrolysis on linoleic acid binding properties of β-lactoglobulin

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

Grupo de Biocoloides, Instituto de Tecnología de Alimentos, Universidad Nacional del Litoral, 1 de Mayo 3250 (3000), Santa Fe, Argentina

ARTICLE INFO

Article history:
Received 22 April 2013
Received in revised form 3 September 2013
Accepted 16 September 2013
Available online 25 September 2013

Keywords:
β-Lactoglobulin
Enzymatic hydrolysis
α-Chymotrypsin
Fluorescence spectroscopy
Linoleic acid
Binding properties

ABSTRACT

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

1. Introduction

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

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

* Corresponding author. Tel.: +54 342 4571252x2602.
E-mail address: lsanti@fiq.unl.edu.ar (L.G. Santiago).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.09.089

applications in foods, cosmetics and pharmaceutical products (Fioramonti, Perez, Aríngoli, Rubiolo & Santiago, 2013). 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; Zimet & Livney, 2009).

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, 2012b). 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 polyunsatured 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 ± 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 immobilised on agarose microparticles (40 mg/g of support). The use of immobilised 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, and FitzGeralda (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 min 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 μm) in order to eliminate possible protein aggregates. Intrinsic fluorescence spectra (due to Trp fluorescence emission) were obtained at λ_{ex} = 295 nm and the Trp fluorescence emission was evaluated between 310 and 430 nm. In all cases the maximum fluorescence intensity (FI_{Trp}) and its wavelength (λ_{MTrp}) were registered (Perez, Carrara, Carrera Sánchez, Rodríguez Patino, & Santiago, 2009).

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

2.5. Linoleic acid binding experiments

Binding experiments were carried out following the variation of Trp emission fluorescence intensity of proteins upon conjugation with LA. Emission spectra were obtained between 310 and 360 nm (λ_{ex} = 295 nm) using a Hitachi 2000 fluorescence spectrophotometer (Japan). Conjugation process was monitored by the increase in maximum emission fluorescence intensity (FI_{Trp}) (Frapin et al., 1993). For this, 2 ml of protein solution were titrated with increasing volumes (0–26 μl) of 4 mM LA in ethanolic solution. Small increments (2 μl) of LA solution were added (with a micropipette) in the tube containing protein solution. Proteins (BLG and its hydrolysates) were dispersed at 0.08% wt in 0.05 M phosphate buffer pH 7. Tubes were vortexed for 2 min prior to analysis. At the end of the various titrations the ethanol concentration amounted to less than 2%, and was therefore assumed to have no effect on protein conformation (Cogan, Kopelman, Mokady, & Shinitzky, 1976). In all cases, fluorescence intensity of the blank solution (buffer + LA) was subtracted from fluorescence intensity measurements of the LA/protein conjugates. Titration curves were represented by Trp relative fluorescence intensity (RFI_{Trp}) defined by the ratio between the FI_{Trp} at a given LA concentration and FI_{Trp} at zero LA concentration. Binding experiments were performed in triplicate at room temperature (25 °C).

From titration curve for native BLG was obtained the LA binding parameters, such as binding site number (n) and apparent dissociation constant (K_d'), according to the method developed by Cogan et al. (1976). This parameters was obtained by means of lineal

regression from the first point since the origin of $P_0.\alpha$ vs. $R_0.\alpha/(1-\alpha)$ plot according to:

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

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

$$\alpha = \frac{RFI_{TrpMax} - RFI_{Trp}}{RFI_{TrpMax} - RFI_{Trp0}} \quad (2)$$

where RFI_{Trp0}, RFI_{Trp} and RFI_{TrpMax} are relative fluorescence intensity at zero LA concentration, at a given concentration and at the LA saturation, respectively.

2.6. Statistical analysis

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

3. Results and discussion

3.1. SDS–PAGE

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

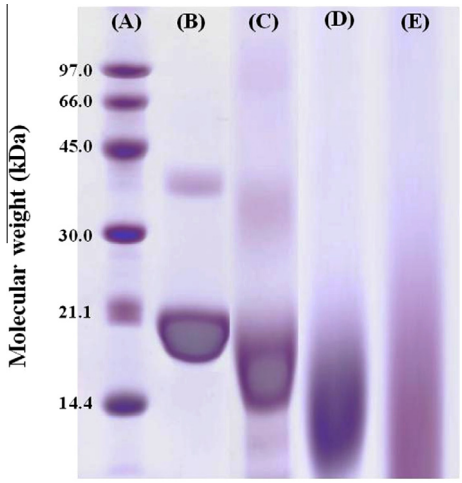

Fig. 1. Effect of limited enzymatic hydrolysis on BLG electrophoretic pattern (SDS–PAGE) under non-reducing conditions. Lane A corresponds to molecular weight patterns: α-LA, 14.4 kDa; Trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 45 kDa; BSA, 66 kDa; phosphorylase B, 97 kDa. Lane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

3.2. Intrinsic fluorescence spectroscopy

Some BLG conformational characteristics could be determined by intrinsic fluorescence measurements (Albani, 2004; Bhattacharjee & Das, 2000). BLG has two Trp residues: Trp19 and Trp61 (Bhattacharjee & Das, 2000; Wang et al., 1998). Trp19 is located in the base of central hydrophobic *calyx*, while Trp61 is found close to protein surface (Wang et al., 1998). BLG intrinsic fluorescence is mainly due to Trp19 (~70% of the fluorescence) while Trp61 is a minor contributor due to the adjacent disulphide link Cys160–Cys166 which decreases its fluorescence (Bhattacharjee & Das, 2000; Fessas et al., 2001). Fig. 2a shows the effect of HD (0–5%) on the BLG intrinsic fluorescence emission spectrum. It was noted that α-chymotrypsin hydrolysis caused a significant increase in BLG FI_{Trp} ($p < 0.05$). This behaviour is better observed in Fig. 2b. Due to enzymatic hydrolysis, Trp19 situated into central *calyx* could be more exposed to aqueous medium and more available to emit fluorescence. Moreover, this behaviour was consistent with the increased λ_{MTrp} with HD (as can be seen in Fig. 2b). Redshift (displacement to the higher wavelengths) could indicate that Trp19 would be close to protein surface in major contact with polar aqueous medium (Albani, 2004). As can be seen in Fig. 2b, the effect of enzymatic hydrolysis on protein intrinsic fluorescence (FI_{Trp}) does not follow a defined tendency (increase or decrease) because the enzyme only recognises hydrophobic aminoacids both on the intact protein and on the released polypeptides (Galvão, Pinto, Jesus, Giordano, & Giordano, 2009). In the range of HD evaluated, neither the evolution of the protein intrinsic fluorescence intensity with the increase in HD nor the state of

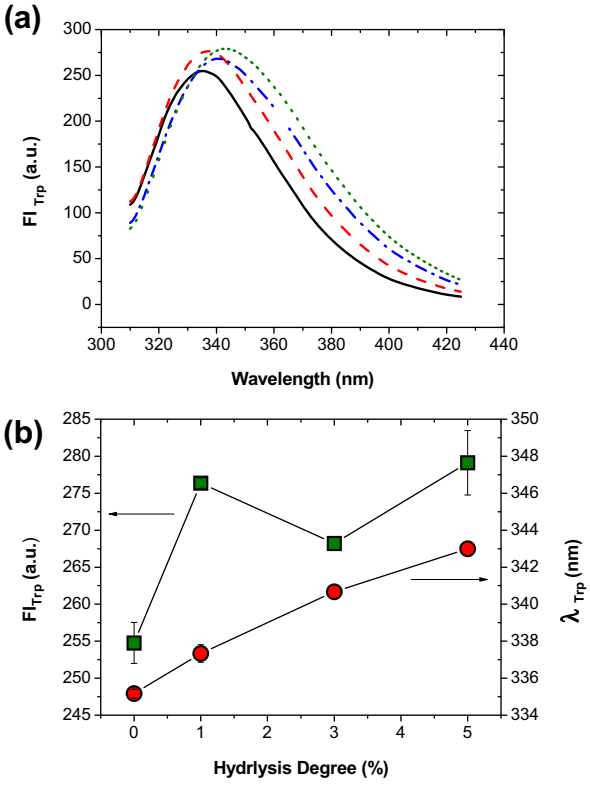

Fig. 2. Effect of limited enzymatic hydrolysis on: (a) intrinsic fluorescence emission spectrum of BLG, symbols: (——) HD = 0%, (— —) HD = 1%, (— · —) HD = 3%, (- - -) HD = 5% and (b) maximum fluorescence intensity (FI_{Trp}) and its wavelength (λ_{MTrp}), represented with ■ and ●, respectively. The error bars correspond to the standard deviation. Protein concentration: 1% wt. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the hydrophobic site in this molecular species can be predicted. On the other hand, there are not significant differences between FI_{Trp} for H1 and H5; while FI_{Trp} for H3 was significantly lower than FI_{Trp} for H1 and H5 ($p < 0.05$). This last result, could suggest a lower amount of Trp capable to emit fluorescence in H3 (Albani, 2004). Therefore, enzymatic hydrolysis could generate a heterogeneous population of molecular species that could interact with each other forming aggregates due to protein hydrophobic interactions between the increased exposed hydrophobic areas (Fennema, 1996) changing protein intrinsic fluorescence. However, as shown in Fig. 1, protein aggregation was not detected in electrophoresis neither for H3 nor for the other hydrolysates probably due to SDS could dissolve the formed hydrophobic aggregates.

3.3. Extrinsic fluorescence spectroscopy

Protein surface hydrophobicity could be determined by using ANS fluorescence probe. This probe binds to protein in a non-covalent way by means of hydrophobic or electrostatic interactions. When ANS binds to surface hydrophobic domains of protein its fluorescence emission intensity increases with respect to ANS alone in aqueous medium (Albani, 2004). The effect of HD (0–5%) on the BLG extrinsic fluorescence emission spectrum is presented in Fig. 3a. It was noted a gradual decrease in FI_{ANS} with the increased HD. These results are better observed in Fig. 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, and 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 λ_{MANS} with exception of H3. Limited enzymatic hydrolysis at 3% caused a significant decrease ($p < 0.05$) in λ_{MANS} (blueshift) 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 and Kilara (1996) reported that interactions facilitated by surface hydrophobic groups can lead to a reduction in the effective hydrophobicity.

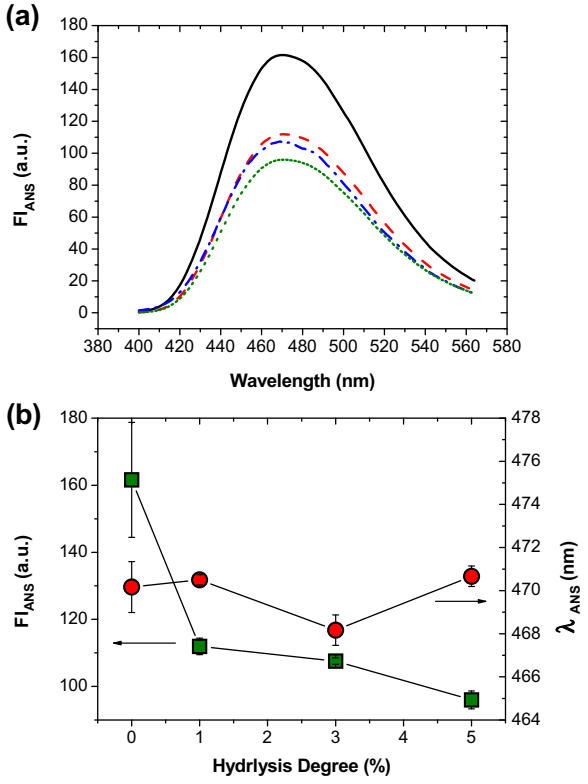

Fig. 3. Effect of limited enzymatic hydrolysis on: (a) extrinsic fluorescence emission spectrum of BLG, symbols: (——) HD = 0%, (– –) HD = 1%, (–·–) HD = 3%, (- - -) HD = 5% and (b) maximum fluorescence intensity (FI_{ANS}) and its wavelength (λ_{MANS}), represented with ■ and ●, respectively. The error bars correspond to the standard deviation. Protein concentration: 1% wt. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Linoleic acid binding experiments

3.4.1. Binding properties of BLG

Trp fluorescence intensity increases when fatty acids bind to BLG superficial *pocket* (Albani, 2004; Cogan et al., 1976; Frapin et al., 1993; Wang et al., 1999). Therefore, fluorescence technique could be used in order to study the effect of α-chymotrypsin hydrolysis on the LA binding properties of BLG. Fig. 4 shows RFI_{Trp} of native BLG as a function of LA concentration (0–47 μM). The insert in Fig. 4 corresponds to $P_0.\alpha$ vs. $R_0.\alpha/(1-\alpha)$ plot used to obtain n and K_d'. It was 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 ± 3.17 × 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

Fig. 5 presents the effect of HD (0–5%) on the BLG RFI_{Trp} as a function of LA concentration (0–47 μM). 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

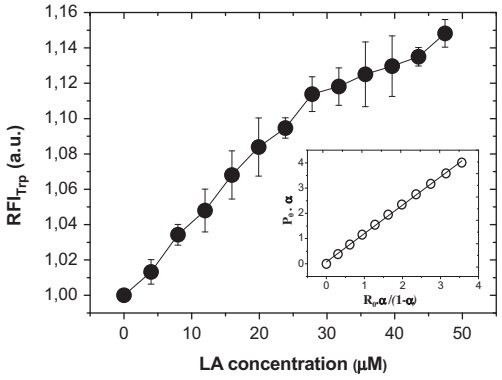

Fig. 4. Relative fluorescence intensity of Trp (RFI_{Trp}) vs. Linoleic Acid (LA) concentration. The insert shows the application of Eq. (1) for the obtention of binding parameters of LA-BLG conjugation. Protein concentration: 0.08% wt (44 μM).

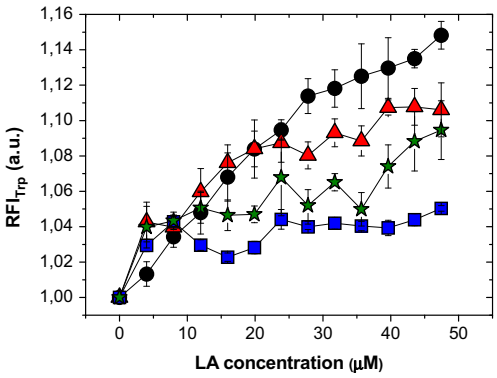

Fig. 5. Effect of limited enzymatic hydrolysis of BLG on Trp relative fluorescence intensity (RFI_{Trp}) as function of Linoleic Acid (LA) concentration. Symbols: (—●—) HD = 0%, (—▲—) HD = 1%, (—■—) HD = 3% and (—★—) HD = 5%. Protein concentration: 0.08% wt. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conjugates could not be obtained due to BLG hydrolysates showed a wide molecular weight distribution and so it was not possible to calculate P_0 (μM). However, binding properties were studied from RFI_{Trp} vs. LA concentration plots (Fig. 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 observed that limited enzymatic hydrolysis at 3% (H3) caused 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 break the surface hydrophobic domains and consequently the LA binding site on BLG (Wang et al., 1999). The binding of fatty acid to BLG should require a particular spatial disposition of the aminoacid residues in BLG superficial *pocket* (a specific site for binding fatty acids) which could have been attacked by α-chymotrypsin.

4. Conclusion

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

Acknowledgements

We acknowledge the financial support of project CAI+D tipo II PI 57–283 (UNL, Santa Fe, Argentina). Authors would like to thank Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET) for the fellowships awarded to Osvaldo E. Sponton and Adrián A Perez.

References

Albani J. R. (2004). *Structure a dynamics of macromolecules: Absorption and fluorescence studies*, Paris: Elsevier, (Chapters 2 & 3).

Bhattacharjee, C., & Das, K. P. (2000). Thermal unfolding and refolding of b-lactoglobulin an intrinsic and extrinsic fluorescence study. *European Journal of Biochemistry, 267*, 3957–3964.

Bolder, S., Vasbinder, A., Sagis, L., & van der Linden, E. (2007). Heat-induced whey protein isolate fibrils: Conversion, hydrolysis, and disulphide bond formation. *International Dairy Journal, 17*, 846–853.

Caessens, P., Daamen, W., Gruppen, H., Visser, S., & Voragen, A. (1999a). β-Lactoglobulin hydrolysis. 2. Peptide identification, SH/SS exchange, and functional properties of hydrolysate fractions formed by the action of plasmin. *Journal of Agriculture and Food Chemistry, 47*, 2980–2990.

Caessens, P., Visser, S., Gruppen, H., & Voragen, A. (1999b). β-Lactoglobulin hydrolysis. 1. Peptide composition and functional properties of hydrolysates obtained by the action of plasmin, trypsin, and *Staphylococcus aureus* V8 protease. *Journal of Agriculture and Food Chemistry, 47*, 2973–2979.

Cheison, S., Lai, M., Leeb, E., & Kulozik, U. (2011). Hydrolysis of β-lactoglobulin by trypsin under acidic pH and analysis of the hydrolysates with MALDI–TOF–MS/MS. *Food Chemistry, 125*, 1241–1248.

Cogan, U., Kopelman, M., Mokady, S., & Shinitzky, M. (1976). Binding affinities of retinol and related compounds to retinol binding proteins. *European Journal of Biochemistry, 65*, 71–78.

Considine, T., Patel, H. A., Singh, H., & Creamer, L. (2005). Influence of binding of sodium dodecyl sulfate, all-trans-retinol, palmitate, and 8-anilino-1-naphthalenesulfonate on the heat-induced unfolding and aggregation of β-lactoglobulin B. *Journal of Agriculture and Food Chemistry, 53*, 3197–3205.

Croguennec, T., O'Kennedy, B., & Mehra, R. (2004). Heat-induced denaturation/aggregation of b-lactoglobulin A and B: Kinetics of the first intermediates formed. *International Dairy Journal, 14*, 399–409.

Essemine, J., Hasnia, I., Carpentiera, R., Thomas, T. J., & Tajmir-Riahia, H. A. (2011). Binding of biogenic and synthetic polyamines to β-lactoglobulin. *International Journal of Biological Macromolecules, 49*, 201–209.

Fennema O. R. (1996). *Food chemistry* (3rd ed.). New York: Marcel Dekker.

Fessas, D., Lametti, S., Schiraldi, A., & Bonomi, F. (2001). Thermal unfolding of monomeric and dimeric β-lactoglobulins. *European Journal of Biochemistry, 268*, 5439–5448.

Fioramonti S.A., Perez A.A., Aríngoli, E.E., Rubiolo, A.C. and Santiago L.G., Design and characterization of soluble biopolymer complexes produced by electrostatic self-assembly of a whey protein isolate and sodium alginate, Food Hydrocolloids, http://dx.doi.org/10.1016/j.foodhyd.2013.05.001

Frapin, D., Dufour, E., & Haertle, T. (1993). Probing the fatty acid binding site of β-lactoglobulins. *Journal of Protein Chemistry, 12*(4), 443–449.

Galvão, C. M. A., Pinto, G. A., Jesus, C. D. F., Giordano, R. C., & Giordano, R. L. C. (2009). Producing a phenylalanine-free pool of peptides after tailored enzymatic hydrolyses of cheese whey. *Journal of Food Engineering, 91*(1), 109–117.

Hernández-Ledesma, B., Ramos, M., Recio, I., & Amigo, L. (2006). Effect of β-lactoglobulin hydrolysis with thermolysin under denaturing temperatures on the release of bioactive peptides. *Journal of Chromatography A, 1116*, 31–37.

Jones, O. G., & McClements, D. J. (2011). Recent progress in biopolymer nanoparticle and microparticle formation by heat-treating electrostatic protein–polysaccharide complexes. *Advances in Colloid and Interface Science, 167*, 49–62.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta, 624*, 13–20.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680.

Madsen, J. S., Ahmt, T. Ø., Otte, J., Halkier, T., & Qvist, K. B. (1997). Hydrolysis of β-lactoglobulin by four different proteinases monitored by capillary electrophoresis and high performance liquid chromatography. *International Dairy Journal, 7*, 399–409.

Matalanis, A., Jones, O. G., & McClements, D. J. (2011). Structured biopolymer-based delivery systems for encapsulation, protection, and release of lipophilic compounds. *Food Hydrocolloids, 25*, 1865–1880.

McClements, D. J., & Li, Y. (2010). Structured emulsion-based delivery systems: Controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science, 159*(2), 213–228.

Panyam, D., & Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. *Trends in Food Science & Technology, 71*, 120–125.

Perez, A. A., Carrara, C. R., Carrera Sánchez, C., Rodríguez Patino, J., & Santiago, L. (2009). Interactions between milk whey protein and polysaccharide in solution. *Food Chemistry, 116*, 104–113.

Perez, A. A., Carrera Sánchez, C., Rodríguez Patino, J., Rubiolo, A., & Santiago, L. (2012a). Effect of enzymatic hydrolysis and polysaccharide addition on the β-lactoglobulin adsorption at the air–water interface. *Journal of Food Engineering, 109*(4), 712–720.

Perez, A. A., Carrera Sánchez, C., Rodríguez Patino, J., Rubiolo, A., & Santiago, L. (2012b). Foaming characteristics of β-lactoglobulin as affected by enzymatic hydrolysis and polysaccharide addition: Relationships with the bulk and interfacial properties. *Journal of Food Engineering, 113*(1), 53–60.

Qin, B. Y., Creamer, L. K., Baker, E. N., & Jameson, G. B. (1998). 12-Bromododecanoic acid binds inside the calyx of bovine β-lactoglobulin. *FEBS Letters, 438*, 272–278.

Sawyer, L., & Kontopidis, G. (2000). The core lipocalin, bovine β-lactoglobulin. *Biochimica et Biophysica Acta, 1482*, 136–148.

Spellman, D., McEvoya, E., O'Cuinnb, G., & FitzGeralda, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal, 13*, 447–453.

Wang, Q., Allen, J. C., & Swaisgood, H. E. (1998). Protein concentration dependence of palmitate binding to β-lactoglobulin. *Journal of Dairy Science, 81*, 76–81.

Wang, Q., Allen, J. C., & Swaisgood, H. E. (1999). Binding of lipophilic nutrients to β-lactoglobulin prepared by bioselective adsorption. *Journal of Dairy Science, 82*, 257–264.

Zimet, P., & Livney, Y. D. (2009). Beta-lactoglobulin and its nanocomplexes with pectin as vehicles for ω-3 polyunsaturated fatty acids. *Food Hydrocolloids, 23*, 1120–1126.