



Effects of gastrointestinal pH conditions on the stability of the β -lactoglobulin/vitamin D₃ complex and on the solubility of vitamin D₃

Fatoumata Diarrassouba^a, Gabriel Remondetto^b, Li Liang^c, Ghislain Garrait^d,
Eric Beyssac^d, Muriel Subirade^{a,*}

^a Chaire de Recherche du Canada sur les Protéines, les Bio-systèmes et les Aliments Fonctionnels, INAF/STELA, Université Laval, Québec, QC, Canada

^b Centre de Recherche et Développement, Agropur Coopérative, 4700 Armand Frappier, St-Hubert, QC, Canada J3Z 1G5

^c State Key Lab of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, China

^d ERT-CIDAM, Lab Biopharmacie, Faculté de Pharmacie, 28 Place Henri Dunant, 63001 Clermont-Ferrand, France

ARTICLE INFO

Article history:

Received 27 August 2012

Received in revised form 12 February 2013

Accepted 13 February 2013

Keywords:

β -Lactoglobulin

Vitamin D₃

Stability

Solubility

Fluorescence spectroscopy

U-HPLC

pH-induced structural transition

ABSTRACT

β -Lactoglobulin (β lg) is the major bovine milk protein with important biological and functional properties including a transport role for small hydrophobic ligands. However, β lg is prone to structural changes triggered by modification of its environment such as pH variation. An unfavorable environment during formulation, manufacture or storage of food products or transit in the gastrointestinal track can have a dramatic impact on the stability of the β lg/ligand complex, resulting in the deterioration of its binding ability and premature release of the ligand. This can impair the biological properties of the ligands and reduce their beneficial effects on health. In the present study, vitamin D₃ (D₃) was used as a nutraceutical ligand model to study the pH-stability of the β lg/D₃ complex as well as the consequences of the complex formation on the solubility of the vitamin. Fluorescence spectroscopy was used to monitor the stability of the β lg/D₃ complex at pH 1.2, 2.0, 3.0, 5.0, 6.8, 7.0 and 8.0. U-HPLC was used to evaluate the solubility of D₃ by preparing the β lg/D₃ complex in different ratios using a static concentration of D₃ and increasing concentrations of β lg. The binding of D₃ to β lg was not significantly affected by pH. Furthermore, the data allowed determination of the fractional residual fluorescence representing the fraction of β lg not bound to the ligand, which indicated that the β lg/D₃ complex remained stable at all pH values. Therefore, D₃ might be retained during formulation and storage of food products at different pH values and during passage in the stomach, which has important implications for the food industry. The solubility of D₃ was also significantly increased as a result of binding to β lg, which confirms its role as a carrier to improve the uptake of D₃ and consequently the beneficial effects of D₃ on health.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

As a member of the lipocalin superfamily, the role of the major bovine milk protein β -lactoglobulin (β lg) as a versatile carrier for small bioactive molecules has long been established (Kontopidis, Holt, & Sawyer, 2002, 2004; Liang & Subirade, 2012; Liang, Tajmir-Riahi, & Subirade, 2008; Wang, Allen, & Swaisgood, 1997, 1999; Yang, Guan, Liu, et al., 2008; Yang, Chen, Chen, Wu, & Mao, 2009). The ligands can bind to four sites on the β lg molecule including the central calyx formed by the β -barrel, the surface hydrophobic pocket in the groove between the α -helix and the β -barrel, the outer surface near tryptophan (Trp)19–arginine (Arg)124, close to the entrance of the β -barrel, and the monomer–monomer interface of the dimer (Liang

& Subirade, 2012; Liang et al., 2008; Yang, Guan, Liu, et al., 2008). Evidence suggests that after binding to β lg, hydrophobic ligands are better protected against oxidative degradation and the solubility of some of them is improved (Hattori, Watabe, & Takahashi, 1995; Liang et al., 2008). As such, β lg protects retinol and β -carotene from oxidative degradation (Hattori et al., 1995). The solubility of resveratrol and α -tocopherol is improved after binding to β lg, which is advantageous for the fortification of foods with low fat content (Liang & Subirade, 2012; Liang et al., 2008). However, slight structural alterations can dramatically affect the binding capacity of β lg and trigger spontaneous release of the ligand, which can impede the protective effects of the carrier protein. Indeed, whereas high temperature leads to the unfolding of the protein, disallowing binding of ligands to the calyx, pH plays an important role in controlling the opening–closing of the EF loop or ‘calyx cap’, thus moderating the entrance and release of ligands that bind inside the central cavity (Hattori et al., 1995; Liang & Subirade, 2012; Ragona et al., 2003). The EF loop (residues 85–90) folds over the entrance of the calyx to form the closed conformation

* Corresponding author at: Faculté des Sciences de l'Agriculture et de l'Alimentation, Pavillon Paul Comtois, 2425 Rue de l'Agriculture, Université Laval, Québec, QC, Canada G1V 0A6. Fax: +1 418 656 3353.

E-mail address: Muriel.Subirade@fsaa.ulaval.ca (M. Subirade).

at pH lower than 6.5 and at pH above 7.0 it adopts the open conformation to expose the interior of the calyx (Qin et al., 1998).

It has been established that moderate to considerable pH-induced structural transitions of β lg occur between pH 2.0 and 8.0, while above pH 9.0, β lg undergoes significant and irreversible structural modification dubbed base-induced unfolding of the protein (Sakurai & Goto, 2007; Taulier & Chalikian, 2001; Uhrínová et al., 2000). Despite significant structural modifications, the overall conformation of the protein is conserved extraordinarily well from pH 2.0 to 8.0 (Uhrínová et al., 2000). Uhrínová et al. stated that the closed conformation of the EF loop at acidic pH confirms the physiological role of β lg as a transporter of small molecule ligands since bound ligands might be protected in the acidic stomach and later be released within the basic small intestine when the EF loop is in the 'open' conformation (Uhrínová et al., 2000). On the contrary, Ragona et al. indicated that native β lg cannot be considered as such a carrier through the alimentary tract since fatty acids are released at low pH (Ragona et al., 2000). On the other hand, Liang and Subirade proved that whereas acidification caused the release of α -tocopherol bound to the internal cavity, it had no influence on that bound to a site at the surface of β lg (Liang & Subirade, 2012). The discrepancies between these different studies can be ascribed to binding studies using dissimilar methods and pH values, the smallest variation of which has important effects on the structure of the protein (Yang, Guan, Liu, et al., 2008). Most importantly, the binding capacity of β lg alone is evaluated under various pH conditions rather than the stability of the β lg/ligand complex, which is problematic considering that formation of the β lg/ligand complex is expected to take place before or after incorporation in the intended food matrix or oral administration system (Yang, Guan, Liu, et al., 2008). Furthermore, in most cases, studies are conducted using pH values that are not representative of those used during the manufacturing and storage of food products and/or conditions prevailing in the gastrointestinal tract. These issues can lead ultimately to the instability of the β lg/ligand complex, deterioration of the binding ability of the protein, premature release and consequent impairment of biological properties of the ligand, and a reduced beneficial effect on health.

In the current study, vitamin D₃ (D₃) was used as a nutraceutical ligand model. This hormone-like vitamin has sparked interest, especially after its non-skeletal actions were revealed (Holick, 2005; Lal, Pandey, & Aggarwal, 1999). Classical actions of D₃ on calcium and phosphorus homeostasis regulation have long been established. Recently, a number of beneficial effects on health have been suggested, including the regulation of both innate and adaptive immune response, effects on the cardiovascular system, lung immunity and respiratory diseases, and protection against bacterial infections, inflammatory bowel diseases, breast and colon cancers, leukemia, tuberculosis, multiple sclerosis, various autoimmune disorders and degenerative diseases (Ardizzone et al., 2011; Bikle & Gerald, 2011; Gorham et al., 2005; Hansdottir, Monick, & Gerald, 2011; Hewison & Gerald, 2011; Hines, Jorn, Thompson, & Larson, 2010). Milk, fruit juices, and various food products are enriched with D₃ in high latitude countries to compensate for the significant decrease of the level of this vitamin, particularly during winter (Calvo & Whiting, 2003; Calvo, Whiting, & Barton, 2004). However, fortification practices seem less effective than supplementation in preventing D₃ deficiency among vulnerable populations when exposure to sun is reduced, making the enhancement of the uptake of D₃ a goal of public health agencies (Calvo & Whiting, 2006). As such, the U.S. Department of Health and Human Services and the Department of Agriculture encourage individuals within the high-risk groups higher daily intakes of vitamin D of about 25 μ g or 1000 IU of vitamin D per day in order to reach and maintain serum 25-hydroxyvitamin D values at 80 nmol/L, which was suggested to be the optimal level of the biomarker (U.S. Department of Health and Human Services & U.S. Department of Agriculture, 2005). Moreover, the incorporation of fat-soluble vitamins like D₃ is challenging for the food industry, especially for formulation of beverage or foods with high water content. Therefore, increasing the solubility of D₃

would be highly valued considering the ever-increasing demand for healthy food products with low fat content.

Two binding sites for D₃ have been clearly identified on β lg, one within the central calyx formed by the β -strands, the other as an exosite located at the pocket between the α -helix and the β -barrel (Yang, Guan, Liu, et al., 2008). Therefore, β lg has been suggested as a good carrier candidate for improved D₃ oral delivery (Yang, Guan, Liu, et al., 2008; Yang et al., 2009). However these studies were conducted at pH 8.0, where β lg is found in its monomeric form, which does not usually represent the regular pH of food products (Yang, Guan, Liu, et al., 2008; Yang et al., 2009). Additionally, the impact of pH on the binding capacity of β lg was evaluated whereas the stability of the β lg/vitamin D₃ (β lg/D₃) complex at different pH values still remains unknown. Therefore, the current study aims at investigating the stability of the β lg/D₃ complex under various pH conditions, including that prevailing in the gastrointestinal track, and the impact of complexation with β lg on the solubility of D₃.

2. Material and methods

2.1. Materials

β lg (B variant, purity $\geq$ 90% PAGE) from bovine milk and D₃ (purity $\geq$ 98%, HPLC) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and used without further purification. Methanol (MeOH, HPLC grade) and Trifluoroacetic Acid (TFA) were purchased from EMD International (Gibbstown, NJ, USA).

2.2. Sample preparation

β lg stock solution was made daily by dissolving in Milli-Q water to obtain concentrations of 200 μ M, measured by spectrophotometer (HP 8453 UV–visible, spectrophotometer, Palo Alto, CA) using a molar extinction coefficient of 17,600 M⁻¹ cm⁻¹ at 278 nm (Liang et al., 2008). Stock solutions of D₃ concentrated at 100 ppm were prepared daily by dissolving 20 mg of the vitamin in 50 mL of methanol followed by a dilution with Milli-Q water to an initial concentration of 40 μ M, which represents less than 2% of methanol after mixing with β lg solutions (Bilodeau et al., 2011). The β lg/D₃ complex was prepared by mixing β lg and D₃ solutions in varying proportions and samples were incubated for about two hours at room temperature prior to analysis. All samples containing D₃ were protected from light by using amber tinted conical tubes.

2.3. Influence of the pH on the stability of the β lg/D₃ complex

The effects of pH (1.2, 2.0, 3.0, 5.0, 6.8, 7.0 and 8.0) on the stability of the complex were determined after complex formation. The pH of the β lg/D₃ complex-containing solutions was adjusted by adding either 0.5 M HCl or 0.1 M NaOH. The samples were kept at room temperature and allowed to stabilize for at least 10 min after pH adjustment before proceeding to fluorescence spectroscopy measurements. All solutions were prepared in triplicate for each pH condition. Steady state fluorescence spectroscopy was used to confirm the binding of D₃ to β lg and to determine the influence of pH on the stability of the complex. Additionally, synchronous fluorescence spectra of β lg were obtained by recording the fluorescence spectra resulting from the difference between the excitation wavelength and the emission wavelength at 60 nm intervals, which corresponds to changes of the polarity around tryptophan residues (U.S. Department of Health and Human Services & U.S. Department of Agriculture, 2005). A Cary Eclipse fluorescence spectrophotometer (Varian Inc.) was used to measure the intrinsic fluorescence of β lg concentrated at 10 μ M after complex formation at a constant ratio of [β lg]/[D₃] of 1/2 (Yang, Guan, Liu, et al., 2008). Emission spectra were recorded from 290 to 550 nm and from 300 to 550 nm with an excitation wavelength of 280 and 290 nm, respectively (Liang et al., 2008). Spectral

resolution was 5 nm for both excitation and emission wavelengths. Water and ligand backgrounds were subtracted from the raw spectra.

Since titration experiments have established that D₃ binds strongly to both the central hydrophobic calyx and the exosite of β lg, the fractional residual fluorescence can be used to study the stability of the β lg/D₃ complex (Wang et al., 1997; Yang, Guan, Liu, et al., 2008; Yang et al., 2009). The fractional residual fluorescence ($F_{\max}/F_0$) was used to determine the fraction of the total protein fluorescence that was not quenched by D₃, and thus the fraction of β lg not bound to the ligand. F is the fluorescence intensity of β lg versus wavelength, $F_{\max}$ is the intensity at the emission maximum ($\lambda_{\max}$), and F_0 is the intensity for β lg at $\lambda_{\max}$ and is proportional to concentration. Thus a low ratio indicates strong binding of the ligand while a ratio of 1 (100%) indicates no binding at all (Liang & Subirade, 2012). All fluorescence measurements were made in triplicate and the values of $F_{\max}$ and F_0 were computed from the fluorescence intensity at $\lambda_{\max}$ for each pH point.

2.4. Influence of the β lg/D₃ complex formation on the solubility of vitamin D₃

The capacity of β lg to influence the solubility of D₃ as a consequence of the formation of the β lg/D₃ complex was investigated by ultra-high performance liquid chromatography (U-HPLC). Different ratios of β lg to D₃ were prepared using D₃ at a static final concentration of 20 μ M and increasing concentrations of β lg (0, 1.25, 2.5, 5, 10, 20, 30, 60 and 80 μ M). The mixtures were protected from light and kept at 4 °C overnight. The concentration of D₃ was determined after complex formation by U-HPLC, which was performed on a Phenomenex Kinetex 2.6 μ m 2.1 $\times$ 75 mm C18 column (Torrance, California, USA) using an Agilent 1260 HPLC Series system equipped with a diode Array detector (Agilent Technologies, Palo Alto, CA, USA). The gradient elution was 0–2 min, 20–65% B; 2–2.25 min, 65–100% B; 2.25–4.85 min isocratic 100% B; 4.85–5 min, 100–20% B; 5–8 min isocratic 20% B. Mobile phases were A: H₂O + 0.1% TFA and B: acetonitrile + 0.1% TFA (v/v). The mobile phase flow rate was 0.4 mL $\times$ min⁻¹. After separation on the Kinetex column, detection was achieved by setting the detector at 280 nm for the protein and 265 nm for D₃.

2.5. Statistical analysis

Statistical analysis was performed using the software SAS version 12.0. ANOVA and the Least Significant Difference (LSD) were used to determine differences among means and the significance level was fixed at $p < 0.05$. All measurements were made at least in triplicate.

3. Results and discussion

3.1. Influence of the pH on the interaction between β lg and D₃

The pH values were specifically selected to be within a range which would not cause appreciable structural modification of the native β -barrel conformation of β lg and also below 9.0, above which an irreversible base-induced unfolding transition occurs leading to the loss of structural integrity of the protein (Taulier & Chalikian, 2001). Unlike previous studies of the effects of pH on the D₃ binding capacity of β lg, the β lg/D₃ complex was first formed and then exposed to specific values of pH. The intrinsic fluorescence spectra for β lg alone and the β lg/D₃ complex were obtained at the different pH values using excitation wavelengths at 280 and 290 nm. The excitation at 290 nm is typically that of Trp19 which is in a non-polar environment and contributes to about 80% of the total fluorescence of β lg, while excitation at 280 nm provides information on the polarity of the environment of both tyrosine (Tyr) and Trp (Liang et al., 2008). Changes in the fluorescence emission spectra can arise from the binding of ligands in the vicinity of the fluorophores, and thus be used to monitor formation of the

complex and resulting energy transfer between the protein and the ligand (Wang et al., 1997).

Fig. 1 clearly shows the binding of D₃ to β lg, with strong quenching of the intrinsic fluorescence of β lg at all pH values. The highest quenching was observed at pH 5.0, the extent of the quenching being about 32.7% and 26.3% at 280 nm and 290 nm, respectively (Fig. 1c and d). Furthermore, the synchronous fluorescence of β lg alone and the β lg/D₃ complex at intervals of 60 nm, which provides information on the vicinity of the Trp amino acid, also confirms that maximum quenching occurs at pH 5.0 (Fig. 2a and b). This finding may be explained by the fact that pH 5.0 is close to the isoelectric point ($pI = 5.2$) of β lg where octamers are formed by the tetramerization of the protein dimer, implying a decreased electrostatic repulsion and increased hydrophobic interactions between the monomers of the protein (Casal, Köhler, & Mantsch, 1988; Lefèvre & Subirade, 2000). Since β lg binds D₃ essentially through hydrophobic forces, it could be suggested that the auto-association of the monomers reinforces the binding (Yang et al., 2009). Although an increased interaction between the monomers of β lg can contribute to the internal quenching of the fluorescence intensity of the protein (Liang & Subirade, 2012), both Figs. 1 and 2 show that at pH 5.0, the quenching of the fluorescence intensity is much more important for the β lg/D₃ complex than for β lg alone. Therefore, it can be assumed that at pH 5.0, D₃ binds more closely to β lg, and more specifically to Trp.

The quenching of the fluorescence intensity of β lg by D₃ at pH below 5.0 (1.2, 2.0 and 3.0) was greater than at higher pH values (6.8, 7.0 and 8.0). This difference is probably due to the dissociation of dimers into monomers at lower pH, with subsequent reduction of the internal quenching (Liang & Subirade, 2012). Interestingly, the quenching of the fluorescence intensity of β lg was minimal at pH 8.0, with a decrease of only 24% and 13.6% at 280 nm and 290 nm, respectively. Reduced quenching at high pH may be due to several factors. At pH 8.0, the monomers are formed because of increased electrostatic repulsion and the EF loop, that is the gate of the central calyx, is in the open conformation, which is confirmed by the blue shift observed at higher pH (Table 1). This means that the Trp residue is in a more hydrophobic environment caused by the presence of D₃. It is known that the decrease in polarity in the vicinity of the Trp residue is accompanied by a blue shift in the emission maximum ($\lambda_{\max}$) to shorter wavelengths and an increase in fluorescence intensity (Liang et al., 2008). Additionally, Taulier and Chalikian observed that both the volume and compressibility of β lg decreased at acidic pH, whereas at pH above 7.5 there is a loosening in the interior packing of β lg (Taulier & Chalikian, 2001). On the other hand, at pH < 3.0, $\lambda_{\max}$ for the β lg/D₃ complex is similar to that of β lg in the same conditions, confirming that the EF loop is in the close conformation (Table 1). At pH 1.2, structural changes affecting the tertiary structure of β lg are responsible for the slight shift of $\lambda_{\max}$ (Taulier & Chalikian, 2001). At pH > 3.0, $\lambda_{\max}$ shifts to shorter wavelengths (blue shift), confirming that the EF loop is in the open conformation.

At acidic pH where the EF loop is the close conformation, it can be assumed that the interaction between D₃ and the protein increases as shown in Fig. 3A. In summary, the higher quenching at low pH is possibly due to a tighter packing of β lg around D₃ than at high pH. Although the extent of quenching of the fluorescence intensity of β lg is pH-dependent, the interaction between β lg and D₃ does not seem to be affected by pH (Figs. 1 and 2). Rather, the differences in the fluorescence intensity of β lg result from slight structural changes, also called structural transitions, which are triggered by modification of the pH values (Qin et al., 1998). These structural transitions are categorized into distinct classes including the transition below pH 2.0, the dimer-to-monomer transition occurring between pH 4.5 and 2.0, the native dimeric (N) to the acidic (Q) form transition which occurs between pH 6.0 and 4.5 (and includes the pH 5.0 transition), the Tanford (N–R) transition occurring between pH 7.0 and 8.0, and the transition at pH 9.0 or above where β lg undergoes an irreversible base-induced unfolding (Taulier & Chalikian, 2001). Given that the interaction of β lg with vitamin D₃ is

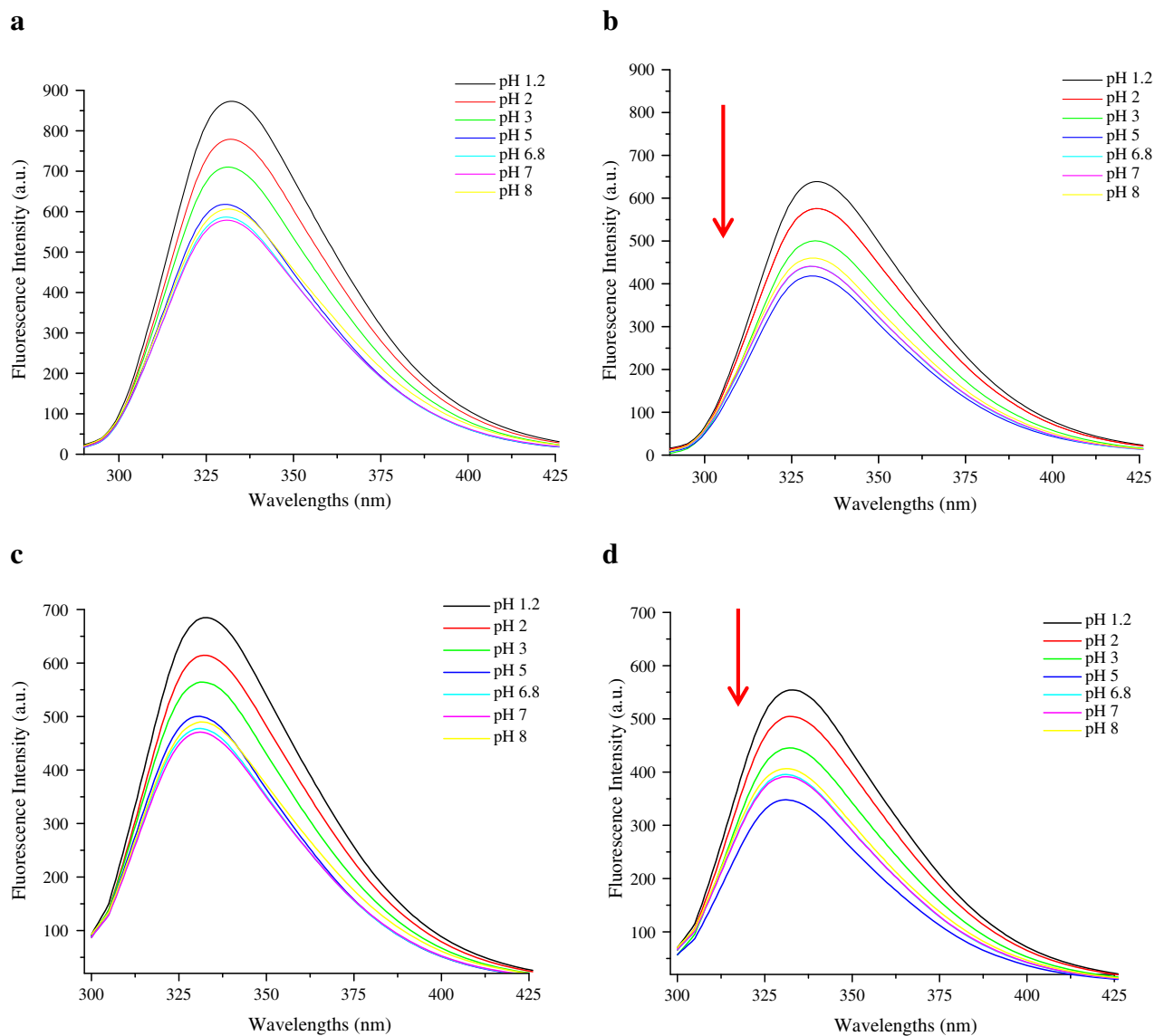


Fig. 1. Fluorescence intensity of β lg (a and c) and the β lg/ D_3 complex (b and d) at different pH values (1.2, 2.0, 3.0, 5.0, 6.8, 7.0 and 8.0). Excitation at 280 nm (a and b) and at 290 nm (c and d).

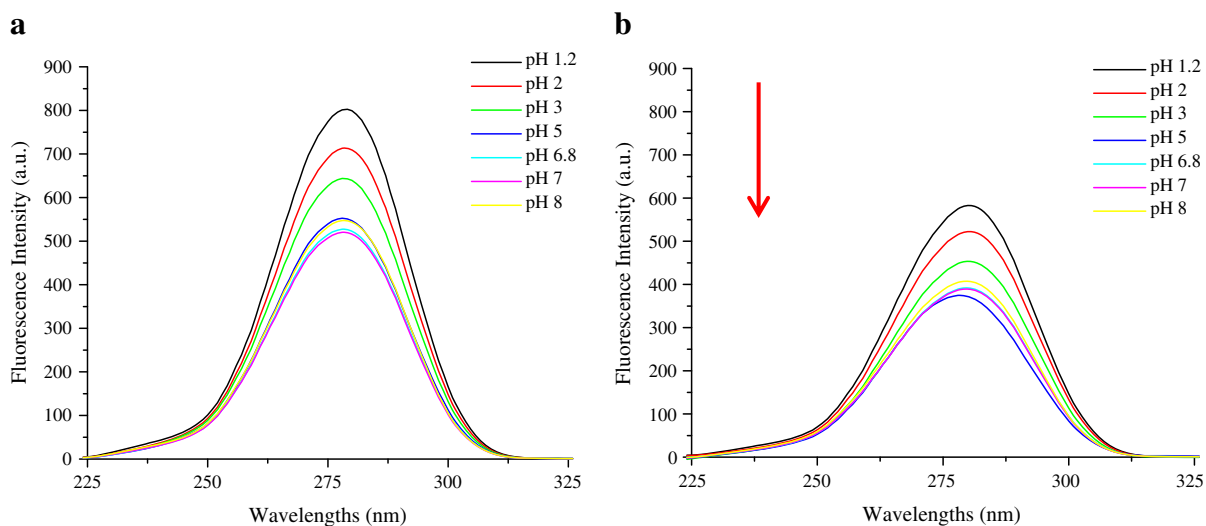


Fig. 2. Synchronous fluorescence of Trp using $\Delta\lambda = 60$ nm of β lg in absence (a) and presence of vitamin D_3 (b).

Table 1
Influence of pH on the emission maximum wavelength of β lg and the β lg/D₃ complex.

pH	λ_{max} (nm)	
	β lg	β lg/D ₃ complex
1.2	333	332
2.0	334	334
3.0	331	331
5.0	331	329
6.8	332	330
7.0	331	329
8.0	332	330

not significantly affected by pH, it is possible to evaluate the stability of the β lg/D₃ complex in light of the reported pH-induced structural transitions of β lg.

3.2. Influence of pH on the stability of the β lg/D₃ complex

The values of pH were chosen to represent those of the gastrointestinal tract (pH 1.2 and 6.8), acidic food (pH 2.0 and 3.0), the isoelectric point of the protein (pH 5), neutral (pH 7.0), and alkaline (pH 8.0) conditions.

At pH 1.2, D₃ was not released from its binding sites on β lg (Figs. 1, 2 and 3). The quenching of the fluorescence intensity of β lg by D₃ was maximal, probably due to the decrease in volume and compressibility of the protein below pH 1.5, which might provide a more

compact structure to β lg, as previously reported (Taulier & Chalikian, 2001). According to these authors, this structural transition continues at pH values less than 1.0, although no explanation was suggested. Given the fact that the EF loop is still in the 'closed conformation', there might be a repositioning, rather than a release, of D₃ inside the hydrophobic cavity of the protein closer to the fluorophores. Consequently, the quenching of the fluorescence intensity is high at acidic pH, especially at pH 1.2 compared to more alkaline pH ($p = 0.0002$), as shown in Fig. 3A. Moreover, fluorescence quenching is higher at 280 nm than at 290 nm probably due to the contribution of both Trp and Tyr amino acids.

The dimer-to-monomer transition of β lg between pH 4.0 and 2.5 is accompanied by a decrease in internal quenching and enhancement of protein fluorescence (Fig. 1a and c) (Liang & Subirade, 2012). The pH 4.0 to 2.5 transition also includes the β lg transition from the monomeric (M) to the acidic form (Q) at pH 3.0 (Sakurai & Goto, 2007; Taulier & Chalikian, 2001). In the present study, the β lg/D₃ complex remains stable during the pH 4.0 to 2.5 transition (Figs. 1, 2 and 3). Fig. 3A shows that the binding capacity of β lg was not significantly changed from pH 2.0 to 3.0 and was comparable to pH 1.2, although slightly less inferior (Fig. 3A). This result is consistent with changes induced by the pH 4.0 to 2.5 transition (Sakurai & Goto, 2007). Indeed, the structural modification results into a different orientation of the α -helix, thus affecting only the surface electrostatic properties of β lg. D₃ binds to both the internal cavity and surface site, which consists of a pocket formed between the α -helix and the core of the β -barrel, mainly through hydrophobic interactions (Taulier & Chalikian, 2001; Yang, Guan, Liu, et al., 2008; Yang et al., 2009). The binding of D₃ is therefore unaffected by the pH 4.0 to 2.5 structural transition.

The structural transition from the native dimeric form (N) to the Q form occurs in the pH 6.0 to 4.5 range and includes the pH 5.0 transition, which is described by Taulier and Chalikian (2001). This transition is accompanied with a slight expansion of the hydrodynamic volume of β lg and has minor impact on its structure (Sakurai & Goto, 2007; Taulier & Chalikian, 2001). Precipitates of octamers form at a pH close to the isoelectric point of β lg (from pH 3.9 to 5.0) (Sakurai & Goto, 2007). The findings of the present study suggest that the tetramerization of β lg leading to the formation of octamers can be advantageous for the binding of D₃. In fact, when excited at 280 nm, the behavior of the β lg/D₃ complex is similar from pH 2.0 to pH 5.0 whereas, when excited at 290 nm, the binding capacity of β lg is significantly higher at pH 5.0 (Fig. 3A). This finding confirms that the self-association of β lg contributes to the increased internal quenching, and most importantly, provides evidence that D₃ is more closely bound to the Trp residues as discussed above.

The well-known Tanford (N–R) transition occurs between pH 7.0 and 8.0 (Bertone-Johnson et al., 2005; Ragona et al., 2000; Sakurai & Goto, 2007; Taulier & Chalikian, 2001; Uhrínová et al., 2000). The data generated in the current study indicate that the β lg/D₃ complex remains stable when the pH is varied from pH 6.8 to pH 8.0 (Fig. 3A). The Tanford transition produces an alteration in the secondary and tertiary structure of β lg, mainly represented by the opening–closing the calyx due to the protonation of the carboxyl group of glutamate side chain of residue 89 (Glu89) located within the EF loop 85–90. Glu89 is inaccessible at pH 6.2, but above pH 7.0, becomes protonated, exposed and accessible to the solvent provoking the displacement of the loop EF which opens the interior of the calyx (Qin et al., 1998; Uhrínová et al., 2000). In the present study, the shift to the Tanford transition can be seen in Fig. 3. From pH 6.8 to 8.0, the β lg/D₃ complex presented similar behavior, confirming that the movement of the EF loop leading to the 'open' confirmation started at pH 6.8. This finding is important since the N–R transition was suggested to occur above pH 7.0 (Qin et al., 1998). Furthermore, the binding of D₃ to β lg was already demonstrated when the protein is in a dimeric form at pH 7.0 and when the dimer dissociates into monomers at

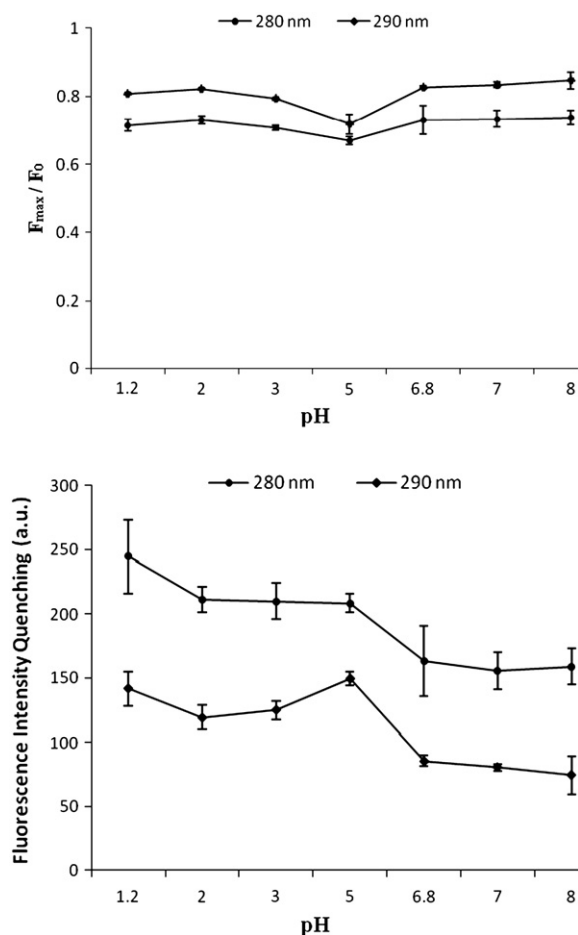


Fig. 3. A. Vitamin D₃ binding capacity of β lg at different pH values (1.2, 2.0, 3.0, 5.0, 6.8, 7.0 and 8.0). B. Fractional residual fluorescence of β lg. F_{max} is the intensity at the emission maximum (λ_{max}) and F_0 is the intensity for β lg at λ_{max} . Excitation at 280 and 290 nm.

pH 8.0 (Wang et al., 1997; Yang, Guan, Liu, et al., 2008). The pH of the intestine is around pH 6.8, indicating that the β lg/D₃ complex might also remain stable during transit in the gastrointestinal tract. However, whereas β lg is resistant to peptic digestion in the stomach, it is susceptible to proteases prevailing in the intestine (Pérez & Calvo, 1995). A possible explanation for the intestinal susceptibility of β lg might be the exposure of Tyr amino acids which are sensitive to tryptic and chymotryptic hydrolysis, as the result of the conformation transition at pH greater than 7.0 (Chobert, Dalgalarondo, Dufour, Bertrand-Harb, & Haertlé, 1991; Pérez & Calvo, 1995). Conversely, β lg showed increased stability toward urea unfolding when both sodium dodecyl sulfate and palmitic acid bound the internal cavity of the protein (Creamer, 1995). Moreover, the hydrophobic interaction between these ligands and β lg was enhanced and the exclusion of water from the cavity provided further stability to the protein (Creamer, 1995; Loch et al., 2012). It was also shown that surfactant *n*-alkylsulfonate ligands stabilize the monomeric structure of β lg (Busti, Scarpeci, Gatti, & Delorenzi, 2005). Consequently, it can be suggested that D₃ binding β lg to both the internal cavity and the surface site through hydrophobic interaction might stabilize the protein. This assertion remained to be proven with further research.

3.3. Influence of pH on the fractional residual fluorescence

The fractional residual fluorescence (FRF) used to determine the proportion of β lg not bound to the ligand provided further proof that D₃ remains bound to β lg at all studied pH values (Fig. 3B). This suggests that the acid stability of the β lg/D₃ complex might result from the fact that the EF loop remains in the 'closed conformation' when the pH is rapidly decreased or in the 'open conformation' when the pH is increased. In fact, a static concentration of β lg and D₃ was used to initially form the β lg/D₃ complex before quickly proceeding to pH modification to acidic or alkaline conditions. Except at pH 5.0, the proportion of bound β lg was similar from pH 1.2 to 8.0, with about 30% bound protein. At pH 5.0 the proportion of bound protein was significantly higher with about 40% of β lg bound to D₃ ($p = 0.02$). D₃ was shown to bind at two binding sites on β lg at pH 7.0 as well as at pH 8.0 (Wang et al., 1997; Yang, Guan, Liu, et al., 2008). In the current study, since the FRF at pH 7.0 and 8.0 had profiles similar to those at lower pH, it can be suggested that D₃ remains at the two different binding sites when the pH is lowered. Comparable results were observed with the β lg/resveratrol complex, for which the FRF at pH 2.0 was similar to that at pH 7.4, indicating that the complex is stable at acidic pH (Liang & Subirade, 2012).

3.4. Influence of the β lg/D₃ complex formation on the solubility of D₃

U-HPLC measurements were used to determine the impact of complexation with β lg on the solubility of D₃ (Fig. 4). A fixed concentration of D₃ of 20 μ M was incubated in the presence or absence of increasing concentration of β lg (1.25 to 80 μ M). In the absence of β lg, 10% of D₃ was degraded after storage overnight at 4 °C. However, the solubility of D₃ increased steadily when the concentration of β lg was increased from 1.25 to 10 μ M before reaching a plateau at somewhat higher concentrations of the protein (Fig. 4). This result is consistent with previous D₃- β lg binding studies using titration methods, which have shown that the maximal binding of D₃ with β lg was reached at a 2:1 ratio (Wang et al., 1997; Yang, Guan, Liu, et al., 2008; Yang, Guan, Yang, et al., 2008). It was also reported that complexing with β lg significantly increased the hydrosolubility of resveratrol (Liang et al., 2008). This beneficial effect is of importance in the food industry where formulation of food products with low fat content can be challenging. It is also worth noting that a U-HPLC method was developed and optimized in the present study to identify and determine the concentrations of both D₃ and β lg in a single run.

Overall, the findings indicate that the β lg/D₃ complex remains stable during pH-induced structural transitions occurring between pH 1.2 and

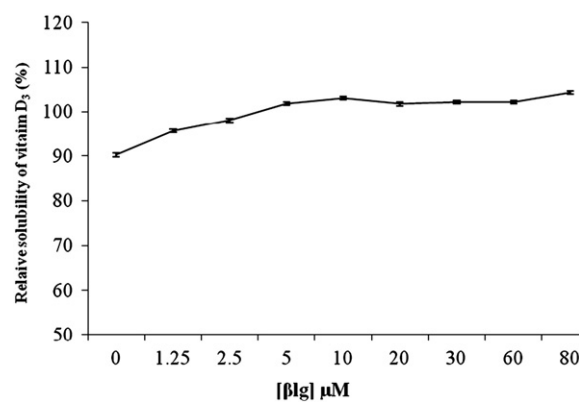


Fig. 4. Relative solubility of vitamin D₃ in the presence and absence of β lg. A static concentration of vitamin D₃ of 20 μ M was incubated overnight at 4 °C with increasing concentration of β lg (0, 1.25, 2.5, 5, 10, 20, 30, 60 and 80 μ M).

8.0. This pH range encompasses that of the gastrointestinal track, the pH of acidic foods and beverages, transitional values (3.0 and 4.0) and that near the pI of β lg (5.0), and neutral and alkaline values. These results are in good agreement with the physiological significance of the closing of the EF loop at acidic pH — that is, the putative transport and protective role of β lg on small hydrophobic ligands in the acidic stomach. Additionally, at pH near the pI of β lg, the tetramerization of β lg which leads to the formation of octamers could be advantageous for the binding of D₃. Furthermore, D₃ might be retained during formulation and storage of various solid and liquid foods, such as D₃-containing processed dairy products, which can provide added value to foods. The present study generates new information on the pH-stability of the β lg/D₃ complex, which is valuable for future applications in the food industry. Solubility and subsequent absorption are key determinants for the bioavailability of fat soluble nutrients (Fairweather-Tait & Southon, 2003; Vieth, 2008; Wagner et al., 2008; Wood, 2005). Therefore, the findings of the current work have implications for the health care system, given that increased solubility of D₃ implies enhancement of its absorption and bioavailability and hence, improved health benefits.

References

- Ardizzone, S., Cassinotti, A., Bevilacqua, M., Clerici, M., Porro, G. B., & Gerald, L. (2011). Vitamin D and inflammatory bowel disease. *Vitamins & hormones*, Vol. 86. (pp. 367–377): Academic Press.
- Bertone-Johnson, E. R., Chen, W. Y., Holick, M. F., Hollis, B. W., Colditz, G. A., Willett, W. C., et al. (2005). Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. *Cancer Epidemiology, Biomarkers & Prevention*, 14, 1991–1997.
- Bikle, D. D., & Gerald, L. (2011). Vitamin D regulation of immune function. *Vitamins & hormones*, Vol. 86. (pp. 1–21): Academic Press.
- Bilodeau, L., Dufresne, G., Deeks, J., Clément, G., Bertrand, J., Turcotte, S., et al. (2011). Determination of vitamin D₃ and 25-hydroxyvitamin D₃ in foodstuffs by HPLC UV–DAD and LC–MS/MS. *Journal of Food Composition and Analysis*, 24, 441–448.
- Busti, P., Scarpeci, S., Gatti, C. A., & Delorenzi, N. J. (2005). Binding of alkylsulfonate ligands to bovine β -lactoglobulin: Effects on protein denaturation by urea. *Food Hydrocolloids*, 19, 249–255.
- Calvo, M. S., & Whiting, S. J. (2003). Prevalence of vitamin D insufficiency in Canada and the United States: Importance to health status and efficacy of current food fortification and dietary supplement use. *Nutrition Reviews*, 61, 107–113.
- Calvo, M. S., & Whiting, S. J. (2006). Public health strategies to overcome barriers to optimal vitamin D status in populations with special needs. *Journal of Nutrition*, 136, 1135–1139.
- Calvo, M. S., Whiting, S. J., & Barton, C. N. (2004). Vitamin D fortification in the United States and Canada: Current status and data needs. *American Journal of Clinical Nutrition*, 80, 1710S–1716S.
- Casal, H. L., Köhler, U., & Mantsch, H. H. (1988). Structural and conformational changes of β -lactoglobulin B: An infrared spectroscopic study of the effect of pH and temperature. *Biochimica et Biophysica Acta (BBA) — Protein Structure and Molecular Enzymology*, 957, 11–20.
- Chobert, J.-M., Dalgalarondo, M., Dufour, E., Bertrand-Harb, C., & Haertlé, T. (1991). Influence of pH on the structural changes of β -lactoglobulin studied by tryptic hydrolysis. *Biochimica et Biophysica Acta (BBA) — Protein Structure and Molecular Enzymology*, 1077, 31–34.
- Creamer, L. K. (1995). Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of bovine beta-lactoglobulin. *Biochemistry*, 34, 7170–7176.

- Fairweather-Tait, S. J., & Southon, S. (2003). Bioavailability of nutrients. In C. Editor-in-Chief: Benjamin (Ed.), *Encyclopedia of Food Sciences and Nutrition* (2nd ed.) (pp. 478–484). Oxford: Academic Press.
- Gorham, E. D., Garland, C. F., Garland, F. C., Grant, W. B., Mohr, S. B., Lipkin, M., et al. (2005). Vitamin D and prevention of colorectal cancer. *The Journal of Steroid Biochemistry and Molecular Biology*, 97, 179–194.
- Hansdottir, S., Monick, M. M., & Gerald, L. (2011). Vitamin D effects on lung immunity and respiratory diseases. *Vitamins & hormones*, Vol. 86. (pp. 217–237): Academic Press.
- Hattori, M., Watabe, A., & Takahashi, K. (1995). beta-Lactoglobulin protects beta-ionone related compounds from degradation by heating, oxidation, and irradiation. *Bioscience, Biotechnology, and Biochemistry*, 59, 2295–2297.
- Hewison, M., & Gerald, L. (2011). Vitamin D and innate and adaptive immunity. *Vitamins & hormones*, Vol. 86. (pp. 23–62): Academic Press.
- Hines, S. L., Jorn, H. K. S., Thompson, K. M., & Larson, J. M. (2010). Breast cancer survivors and vitamin D: A review. *Nutrition*, 26, 255–262.
- Holick, M. F. (2005). Vitamin D: Physiology, dietary sources and requirements. In B. Caballero (Ed.), *Guide to nutritional supplements* (pp. 456–465).
- Kontopidis, G., Holt, C., & Sawyer, L. (2002). The ligand-binding site of bovine β -lactoglobulin: Evidence for a function? *Journal of Molecular Biology*, 318, 1043–1055.
- Kontopidis, G., Holt, C., & Sawyer, L. (2004). Invited review: β -Lactoglobulin: Binding properties, structure, and function. *Journal of Dairy Science*, 87, 785–796.
- Lal, H., Pandey, R., & Aggarwal, S. K. (1999). Vitamin D: Non-skeletal actions and effects on growth. *Nutrition Research*, 19, 1683–1718.
- Lefèvre, T., & Subirade, M. (2000). Molecular differences in the formation and structure of fine-stranded and particulate β -lactoglobulin gels. *Biopolymers*, 54, 578–586.
- Liang, L., & Subirade, M. (2012). Study of the acid and thermal stability of β -lactoglobulin–ligand complexes using fluorescence quenching. *Food Chemistry*, 132, 2023–2029.
- Liang, L., Tajmir-Riahi, H. A., & Subirade, M. (2008). Interaction of β -lactoglobulin with resveratrol and its biological implications. *Biomacromolecules*, 9, 50–56.
- Loch, J. I., Polit, A., Bonarek, P., Olszewska, D., Kurpiewska, K., Dziedzicka-Wasylewska, M., et al. (2012). Structural and thermodynamic studies of binding saturated fatty acids to bovine β -lactoglobulin. *International Journal of Biological Macromolecules*, 50, 1095–1102.
- Pérez, M. D., & Calvo, M. (1995). Interaction of β -lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: A review. *Journal of Dairy Science*, 78, 978–988.
- Qin, B. Y., Bewley, M. C., Creamer, L. K., Baker, H. M., Baker, E. N., & Jameson, G. B. (1998). Structural basis of the Tanford transition of bovine β -lactoglobulin. *Biochemistry*, 37, 14014–14023.
- Ragona, L., Fogolari, F., Catalano, M., Ugolini, R., Zetta, L., & Molinari, H. (2003). EF loop conformational change triggers ligand binding in β -lactoglobulins. *Journal of Biological Chemistry*, 278, 38840–38846.
- Ragona, L., Zetta, L., Fogolari, F., Molinari, H., Pérez, D. M., Puyol, P., et al. (2000). Bovine β -lactoglobulin: Interaction studies with palmitic acid. *Protein Science*, 9, 1347–1356.
- Sakurai, K., & Goto, Y. (2007). Principal component analysis of the pH-dependent conformational transitions of bovine β -lactoglobulin monitored by heteronuclear NMR. *Proceedings of the National Academy of Sciences*, 104, 15346–15351.
- Taulier, N., & Chalikian, T. V. (2001). Characterization of pH-induced transitions of β -lactoglobulin: Ultrasonic, densimetric, and spectroscopic studies. *Journal of Molecular Biology*, 314, 873–889.
- U.S. Department of Health and Human Services, & U.S. Department of Agriculture (2005). Dietary guidelines for Americans, 2005. In D. o. A. (USDA) (Ed.), (6th ed.). *Adequate nutrients within calorie needs*, Vol. 2013, Washington, DC: U.S. Government Printing Office.
- Uhrinová, S., Smith, M. H., Jameson, G. B., Uhrin, D., Sawyer, L., & Barlow, P. N. (2000). Structural changes accompanying pH-induced dissociation of the β -lactoglobulin dimer. *Biochemistry*, 39, 3565–3574.
- Vieth, R. (2008). Vitamin D. In H. Editor-in-Chief: Kris (Ed.), *International Encyclopedia of Public Health* (pp. 532–537). Oxford: Academic Press.
- Wagner, D., Rousseau, D., Sidhom, G., Pouliot, M., Audet, P., & Vieth, R. (2008). Vitamin D-3 fortification, quantification, and long-term stability in Cheddar and low-fat cheeses. *Journal of Agricultural and Food Chemistry*, 56(17), 7964–7969.
- Wang, Q., Allen, J. C., & Swaisgood, H. E. (1997). Binding of vitamin D and cholesterol to β -lactoglobulin. *Journal of Dairy Science*, 80, 1054–1059.
- Wang, Q., Allen, J. C., & Swaisgood, H. E. (1999). Binding of lipophilic nutrients to beta-lactoglobulin prepared by bioselective adsorption. *Journal of Dairy Science*, 82, 257–264.
- Wood, R. J. (2005). Bioavailability. In C. Editor-in-Chief: Benjamin (Ed.), *Encyclopedia of Human Nutrition* (2nd ed.) (pp. 195–201). Oxford: Elsevier.
- Yang, M. C., Chen, N. C., Chen, C. -J., Wu, C. Y., & Mao, S. J. T. (2009). Evidence for β -lactoglobulin involvement in vitamin D transport in vivo—Role of the γ -turn (Leu–Pro–Met) of β -lactoglobulin in vitamin D binding. *FEBS Journal*, 276, 2251–2265.
- Yang, M. -C., Guan, H. -H., Liu, M. -Y., Lin, Y. -H., Yang, J. -M., Chen, W. -L., et al. (2008). Crystal structure of a secondary vitamin D3 binding site of milk β -lactoglobulin. *Proteins: Structure, function, and bioinformatics*, 71, 1197–1210.
- Yang, M. C., Guan, H. -H., Yang, J. -M., Ko, C. -N., Liu, M. -Y., Lin, Y. -H., et al. (2008). Rational design for crystallization of β -lactoglobulin and vitamin D3 complex: Revealing a secondary binding site. *Crystal Growth & Design*, 8, 4268–4276.