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Interactions between Bovine β-Lactoglobulin and Peptides under Different Physicochemical Conditions

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The aim of this study was to determine if peptides could interact with β-lactoglobulin (β-LG) and what the physicochemical conditions promoting their interaction with the protein are. The binding of negatively charged (β-LG 125−135 and 130−135), positively charged (β-LG 69−83 and 146−149), and hydrophobic (α_{S1}-CN 23−34 and β-LG 102−105, both bioactive peptides) peptides to bovine β-LG was determined using an ultrafiltration method under different physicochemical conditions: pH 3.0, 6.8, and 8.0; buffers of 0.05 and 0.1 M; 4, 25, and 40 °C; β-LG/peptide ratios of 1:5 and 1:10. At pH 3.0, none of the peptides interacted with β-LG at any temperature, buffer molarity, or β-LG/peptide ratio probably due to electrostatic repulsions between the highly protonated species. At pH 6.8 and 8.0, charged peptides β-LG 130−135, 69−83, and 146−149 bound to β-LG under some physicochemical conditions, possibly by nonspecific binding. However, both hydrophobic peptides probably bind to the inner cavity (β-barrel) of β-LG, provoking the release of materials absorbing at 214 nm. Given the known biological activities of the hydrophobic peptides used in this study (opioid and ACE-inhibitory activities), their binding to β-LG may be relevant to a better understanding of the physiological function of the protein.

KEYWORDS: Bovine β-lactoglobulin; peptide; protein−peptide interactions; hydrophobicity

INTRODUCTION

β-Lactoglobulin (β-LG) is a small globular protein found in milks of many mammals, but it has not been found in human milk (*1*−*3*). In cow milk, its concentration varies throughout the lactation period, reaching 18−20 mg/mL in the first colostrum and stabilizing at 4 mg/mL after the two first weeks postpartum (*4*). Bovine β-LG exists in various oligomeric states as a function of pH, temperature, concentration, ionic strength, and genetic variant (*5*). At the pH of bovine milk (pH 6.8) and room temperature, the protein is a dimer of ∼36800 Da, but at pH values <3 and >8, it dissociates into monomers (*6*). Increasing temperature (5−76 °C) or decreasing ionic strength also shifts the monomer/dimer equilibrium toward the monomeric form of β-LG (*7*, *8*). The monomer contains 162 amino acid residues, and its conformation is stabilized by the presence of the two intrachain disulfide bridges Cys66−Cys160 and Cys106−Cys119 (*9*). The protein also contains a sulfhydryl group (Cys121) that reacts abnormally during the Tanford transition, which is a reversible and pH-dependent conformational change that bovine β-LG undergoes between pH 6.5 and 8.0 (*9*, *10*).

Bovine β-LG has a calyx fold consisting of an eight-stranded antiparallel β-barrel typical of the lipocalin protein superfamily (*11*) and shares the ability to bind a variety of small hydrophobic molecules (*12*). One side of the calyx is formed by β-strands A−D, whereas the other side is composed of the terminal portion of β-strand A together with the strands E−H (*10*). The second β-sheet is largely covered by a three-turn α-helix (residues 129−142) and parts of the N- and C-terminal regions (*13*). The base of the β-barrel is closed by the N-terminal loop, which crosses the molecule (*10*). The β-LG molecule also has a ninth β-strand (I), which is involved in dimer formation together with the AB loop (*9*, *10*).

Apart from its nutritional value, the true physiological function of β-LG is still unclear. The ligand-binding properties of β-LG, coupled with its structural similarity to retinol-binding protein, suggest a transport role for this protein (*14*). β-LG has been reported to bind saturated fatty acids, polyunsaturated molecules, and aromatic groups, with at least three independent binding sites having been identified (*12*). For retinoids and fatty acids, β-LG has two plausible sites: one in the calyx formed by the β-barrel (*15*) and the other in an external hydrophobic pocket between the α-helix and the β-barrel (*16*). However, there is no unequivocal evidence for this external binding site (*10*). Qin et al. (*13*) and Wu et al. (*17*) have shown that the central cavity is likely the primary binding site for hydrophobic ligands based on the cocrystallization of palmitic acid at that site. The third binding site, distinct from both the internal cavity and external hydrophobic pocket, is located on the outer surface close to the parallel stack of Trp19/Arg 124 (*12*). This site is likely to be responsible for binding aromatic ligands such as

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Table 1. Physicochemical Characteristics of the Six Peptides under Study

peptide	sequence of amino acids	MW[a] (Da)	charge at pH 3.0	charge at pH 6.8	charge at pH 8.0	isoelectric point[a]	$H\phi_{av}$[b] (kcal/residue)
β-LG 125–135	TPEVDDEALEK	1245.3	+1	–4	–4	3.83	0.85
β-LG 130–135	DEALEK	703.7	+1	–2	–2	4.14	0.78
β-LG 69–83	KKIIAEKTKIPAVFK	1714.2	+5	+4	+4	10.18	1.68
β-LG 146–149	HIRL	537.7	+2	+1	+1	9.76	1.53
α_{S1}-CN 23–34	FFVAPFPQVFGK	1383.6	+1	+1	+1	8.75	1.79
β-LG 102–105	YLLF	554.7	0	0	0	5.52	2.58

[a] Theoretical mass and isoelectric point were obtained from ExPASy Molecular Biology Server. [b] Average hydrophobicity was calculated according to the method of Bigelow (*42*).

p-nitrophenyl phosphate and 5-fluorocytosine (*12*), ellipticine (*18*), or protoporphyrin (*19*), but this has yet to be confirmed experimentally.

Most previous studies have focused on interactions between β-LG and retinoid compounds, fatty acids, or aromatic ligands. The binding capacity of β-LG for peptides has not been yet studied. The aim of this work was thus to study β-LG−peptide interactions, to determine the impact of peptide chemistry (mass, charge, and hydrophobicity) on these interactions, and to evaluate the effect of physicochemical conditions on the interactions.

MATERIALS AND METHODS

Materials. Bovine β-lactoglobulin (variants A and B, 3× crystallized) was obtained from Sigma Chemical Co. (St. Louis, MO), whereas the six peptides under study were synthesized by the Service de sequence de peptides de l'est du Québec (Sainte-Foy, PQ, Canada). All peptides were water-soluble with a high degree of purity (75−95%), and their physicochemical characteristics are presented in **Table 1**. The six peptides were selected according to their mass, charge, and hydrophobicity. Also, two of these peptides were chosen because of their bioactive activity. β-LG 102−105 and α_{S1}-CN 23−34 are recognized as ACE-inhibitory peptides, and β-LG 102−105 is also an opioid peptide (*20*). For chromatographic analysis, HPLC grade water (18 MΩ, Modulab, Fisher Scientific) was used to prepare buffers and mobile phases, which were also filtered through 0.22 μm filters (Millipore, Bedford, MA). All other reagents were of analytical grade.

Interactions β-LG—Peptide. Interactions between β-LG and peptides were studied using a modification of the ultrafiltration method of Wang et al. (*21*) under different physicochemical conditions: pH 3.0, 6.8, and 8.0; buffers of 0.05 and 0.1 M; 4, 25, and 40 °C; β-LG/peptide molar ratios of 1:5 and 1:10. β-LG (100 and 200 μM) and peptide (1000 μM) solutions were prepared in glycin (pH 3.0), phosphate (pH 6.8), or Tris (pH 8.0) buffers adjusted to molar concentrations of 0.05 or 0.1 M. Selection of pH values was based on the monomeric form of β-LG at pH 3.0 (*3*), dimeric form prevailing at the pH of milk (*10*), and pH value (pH 8.0) above the Tanford transition (pH ∼7) and below the polymerization of the molecule (*9*). For all conditions, 150 μL each of β-LG and peptide solution were mixed in an Eppendorf tube and incubated overnight at the temperature under study. Solutions were then transferred to a Microcon YM-10 centrifugal filter device with a MWCO of 10000 Da (Millipore) and centrifuged at 9500*g* for 35 min (25 and 40 °C) or for 45 min (4 °C) using a Micromax centrifuge (IEC, Needham Heights, MA). Filtrates containing unbound peptide were transferred to a new Eppendorf tube, sealed, and kept frozen until quantification by RP-HPLC analysis.

Peptides Quantification. To verify the release of compounds from the Microcon membranes and the total retention of β-LG by the membranes and to estimate peptide adsorption to the membranes, the following solutions were analyzed by RP-HPLC: (1) blanks for each buffer at 0.1 M filtered at 25 °C; (2) β-LG solution (200 μM, 150 μL) in each buffer (150 μL) at 0.1 M incubated overnight at 4, 25, or 40 °C and filtered; (3) solutions of each peptide (1000 μM, 150 μL) in each buffer (150 μL) at 0.1 M incubated overnight at each temperature and filtered; (4) solutions of each peptide (1000 μM, 150 μL) in each buffer (150 μL) at 0.1 M incubated overnight at 25 °C and not filtered. For all physicochemical conditions studied, unbound peptide in β-LG/peptide mixtures was estimated relative to peptides in filtrates of pure peptide solutions without β-LG. From HPLC analysis, the peptide content of samples was determined from the peak area of the peptide, and the percentage of unbound peptide was calculated as follows:

$$\frac{\text{peak area for peptide contained in filtrate isolated from } \beta\text{-LG:peptide solution}}{\text{peak area for peptide contained in filtrate isolated from pure peptide solution}} \times 100$$

RP-HPLC Analysis. RP-HPLC analysis was performed using an HPLC Beckman System Gold (Mississauga, ON, Canada) equipped with two pumps (model 126P), a variable UV−visible detector (model 166P) operating at 214 nm, and an automatic injector Hewlett-Packard (series 1100, Agilent Technologies, Palo Alto, CA). Data acquisition and chromatographic analysis were controlled with Beckman System Gold Nouveau software (version 1.6). RP-HPLC analyses were performed using a Nova-Pak C_{18} column (4 μm, 3.9 i.d. × 150 mm) from Waters Co. (Millipore, Milford, MA) connected to a guard column (4 μm, 3.9 i.d. × 20 mm) filled with the same matrix. Column temperature was 39 °C, flow rate 1 mL/min, solvent A 0.11% (v/v) TFA in water, and solvent B 60% (v/v) acetonitrile plus 40% (v/v) water and 0.10% (v/v) TFA. An aliquot (20 μL) of peptide solution was injected into the column, and elution was obtained with a linear gradient from 0 to 60% of solvent B for 10 min, from 60 to 100% solvent B for 2 min, and from 100 to 0% B for 2 min.

Statistical Analysis. Results were analyzed as a four-variable pH × °C × M × β-LG/peptide ratio (3 × 3 × 2 × 2) factorial experiment. Statistical analysis was performed by SAS (Statistical Analysis System) using the quadruple interaction as error term. Only physicochemical conditions providing significant effect ($p \leq 0.05$) are presented in this paper. A peptide was considered to interact with β-LG only when the amount of unbound peptide was <90%. This value was determined from the coefficient of variation (<9.8%) obtained for duplicate analysis performed for 12 different physicochemical conditions.

RESULTS AND DISCUSSION

The physicochemical characteristics of the six peptides studied are presented in **Table 1**. Five of them corresponded to fragments of bovine β-LG, and one comes from α-$_{S1}$ casein (α_{S1}-CN). These peptides were selected according to their mass (538−1714 Da), charge at the three pH values studied, and average hydrophobicity (0.78−2.58 kcal/residue). **Figure 1** illustrates RP-HPLC chromatograms of 0.1 M Tris buffer blank (pH 8.0) filtered on Microcon membrane (**Figure 1A**), filtered β-LG (100 μM) in the same buffer (**Figure 1B**), and peptide β-LG 130−135 (500 μM) in the same buffer before (**Figure 1C**) and after Microcon filtration (**Figure 1D**). RP-HPLC chromatograms revealed the presence of a peak in all filtered samples (**Figure 1A**,**B**,**D**), corresponding to glycerin, which is used as a wetting agent in the Microcon membrane. Fortunately, it eluted at a retention time (∼13.2 min) different from those of the peptides studied and did not interfere with peptide quantification. The filtered buffer (**Figure 1A**) and β-LG

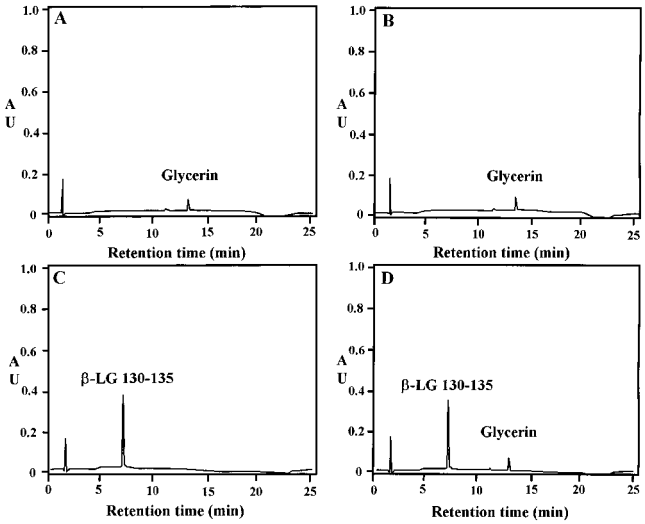

Figure 1. RP-HPLC chromatograms for Microcon-filtered 0.1 M Tris buffer (A), Microcon-filtered β-LG (100 μM) in the same buffer (B), and peptide β-LG 130–135 (500 μM) in the same buffer before (C) and after (D) Microcon filtration. All filtrations were done at 25 °C.

Table 2. Effect of pH, Temperature, and Buffer Molarity on the Binding of Peptide β-LG 130–135 to β-Lactoglobulin[a]

pH	temp (°C)	buffer molarity (M)	unbound peptide (% ± SE)
3.0	4	0.05	99.8 ± 1.2
		0.10	104.9 ± 1.8
	25	0.05	105.9 ± 0.8
		0.10	104.7 ± 1.3
	40	0.05	98.6 ± 0.5
		0.10	118.8 ± 1.3
6.8	4	0.05	98.0 ± 2.6
		0.10	94.6 ± 3.3
	25	0.05	89.7 ± 3.0
		0.10	108.9 ± 0.1
	40	0.05	87.4 ± 4.1
		0.10	103.3 ± 0.5
8.0	4	0.05	95.4 ± 0.8
		0.10	107.8 ± 0.9
	25	0.05	79.6 ± 3.5
		0.10	64.6 ± 0.5
	40	0.05	93.2 ± 0.0
		0.10	94.2 ± 2.4

[a] pH × T × μ interaction was significant at $p = 0.01$.

solution (**Figure 1B**) had practically identical chromatographic profiles, indicating that no β-LG (18.4 kDa as monomer) crossed the 10 kDa MWCO membrane and thus did not interfere with quantification of unbound peptide. Finally, adsorption of the peptide to the Microcon membrane was estimated by comparing peaks such as those obtained for β-LG 130−135 illustrated in **Figure 1C**,**D**. For all peptides and physicochemical conditions studied, adsorption ranged from 1.1 to 8.9% (results not shown). Quantification of unbound peptide was thus based on peak areas obtained with Microcon-filtered peptide solutions.

Tables 2−**7** give the values for peptide binding to β-LG. Only the peptides and physicochemical conditions providing significant effect ($p \leq 0.05$) are presented in these tables. **Table 2** presents the effect of pH, temperature, and buffer molarity on the binding of peptide β-LG 130−135 to β-LG. At pH 3.0, neither this peptide nor any other interacted (>90% unbound peptide) with β-LG at any temperature, buffer molarity, or β-LG/peptide ratio (results not shown). At this pH, β-LG and all of the peptides are positively charged (**Table 1**) except for the peptide β-LG 102−105, which remains neutral at this pH, making interactions between peptides and β-LG difficult due to electrostatic repulsion. Using a fluorescence technique, Busti et al. (*5*) observed the absence of interaction between β-LG and alkyl sulfonate ligands at pH 3.0, whereas Frapin et al. (*22*) observed a lower affinity for palmitate below pH 4.5. Furthermore, Dufour et al. (*23*) have shown that the strength of retinol

Table 3. Effect of Buffer Molarity on the Binding of Peptides β-LG 69–83 and β-LG 146–149 to β-Lactoglobulin

peptide	buffer molarity (M)	unbound peptide (% ± SE)
β-LG 69–83[a]	0.05	78.0 ± 4.4
	0.10	87.0 ± 4.0
β-LG 146–149[b]	0.05	89.2 ± 1.0
	0.10	98.9 ± 1.3

[a] $p = 0.005$. [b] $p = 0.0002$.

Table 4. Effect of pH and β-LG/Peptide Ratio on the Binding of Peptide β-LG 69–83 to β-Lactoglobulin[a]

pH	β-LG/peptide	unbound peptide (% ± SE)
3.0	1:5	104.4 ± 1.7
	1:10	103.2 ± 2.0
6.8	1:5	71.8 ± 3.0
	1:10	82.4 ± 2.9
8.0	1:5	59.0 ± 2.5
	1:10	74.2 ± 4.8

[a] pH × R interaction was significant at $p = 0.03$.

Table 5. Effect of pH, Temperature, and β-LG/Peptide Ratio on the Binding of Peptide α_{S1}-CN 23–34 to β-Lactoglobulin

factor		unbound peptide (% ± SE)
pH[a]	3.0	102.2 ± 1.0
	6.8	85.7 ± 2.7
	8.0	86.4 ± 2.3
temp (°C)[b]	4	98.9 ± 1.7
	25	89.0 ± 2.7
	40	86.5 ± 3.4
β-LG/peptide[c]	1:5	89.1 ± 1.9
	1:10	93.7 ± 1.9

[a] $p = 0.0006$. [b] $p = 0.002$. [c] $p = 0.02$.

Table 6. Effect of pH and Temperature on the Binding of Peptide β-LG 102–105 to β-Lactoglobulin[a]

pH	temp (°C)	unbound peptide (% ± SE)
3.0	4	108.0 ± 1.4
	25	105.2 ± 1.5
	40	101.4 ± 1.2
6.8	4	100.0 ± 1.1
	25	81.8 ± 4.2
	40	75.1 ± 4.0
8.0	4	105.0 ± 0.7
	25	81.1 ± 2.9
	40	83.2 ± 3.5

[a] pH × T interaction was significant at $p = 0.01$.

Table 7. Effect of pH and β-LG/Peptide Ratio on the Binding of Peptide β-LG 102–105 to β-Lactoglobulin[a]

pH	β-LG/peptide	unbound peptide (% ± SE)
3.0	1:5	105.3 ± 1.7
	1:10	104.3 ± 1.5
6.8	1:5	80.7 ± 6.0
	1:10	90.5 ± 3.6
8.0	1:5	86.1 ± 5.8
	1:10	93.4 ± 4.0

[a] pH × R interaction was significant at $p = 0.03$.

binding to β-LG is pH-dependent with higher binding at pH 7−8 than at pH 3. The monomeric form of β-LG, which occurs at pH 3.0, also exhibits a highly structured β-sheet core similar to that of the dimer at physiological pH, in addition to disordered loop regions and terminal segments (*24*, *25*). Fogolari et al. (*25*) have observed sizable differences within the structured regions at the β-barrel−α-helix interface and at the CD loop and flanking residues. They suggest that these differences, together with disordering in the loops and terminal regions, lead to relevant changes in surface electrostatic properties, which may be of consequence to the binding of protonated ligands at low pH.

As shown in **Table 2**, values >100% were generally measured for unbound peptide at pH 3.0, indicating that larger amounts of peptide crossed the Microcon membrane in the presence of β-LG than in its absence. This effect was observed for almost all measurements done at pH 3.0 (**Tables 2** and **4**−**7**) and for a few other specific conditions: pH 6.8, 0.1 M, 25 and 40 °C; pH 8.0, 0.1 M, 4 °C (**Table 2**); and pH 8.0, 4 °C (**Table 6**). The values >100% for unbound peptides may be due to different adsorption of peptides onto the Microcon membrane in the presence of β-LG and/or the facilitation of peptide passage through the Microcon membrane by Donnan exclusion phenomena, which is the electrostatic exclusion of ions (co-ions) carrying the same charge at the membrane surface (*26*). Because the calculation of unbound peptide was based on the peptide content of Microcon-filtered solutions, higher exclusion of peptides from the membrane in the presence of β-LG could lead to values >100% for unbound peptide.

At pH 6.8 and 8.0 (**Table 2**), peptide β-LG 130−135 is negatively charged (−2) and interacts with β-LG (<90% unbound peptide). At pH 6.8, 10 and 13% binding was measured for the lower molar concentration of the phosphate buffer (0.05 M) at 25 and 40 °C, respectively, while at pH 8.0, peptide binding increased from 20 to 35% at 25 °C by increasing molar concentration of the Tris buffer from 0.05 to 0.1 M (**Table 2**). At pH 6.8, β-LG exists mainly as dimers, which dissociate gradually into monomers at pH 8.0 (*27*). Also, increasing the temperature from 25 to 40 °C or decreasing the ionic strength would have contributed to shifting the equilibrium from the dimeric to the monomeric form (*7*, *8*). Because binding of the peptide β-LG 130−135 to β-LG seems favored by the formation of monomers under some physicochemical conditions, the binding site may be near the region involved in dimer formation (*10*). This region of β-LG contains two Arg residues (40 and 148), which are positively charged at pH 6.8 and 8.0 and may interact electrostatically with the negative charges of peptide β-LG 130−135. This peptide could also bind to any other positively charged region at the surface of the protein. In contrast, the other negatively charged peptide, β-LG 125−135, did not bind at all under any of the physicochemical conditions studied (result not shown). Peptide β-LG125−135 is longer (11 residues) than peptide β-LG 130−135 (6 residues), contains a proline residue at position 126, and probably does not sterically fit the binding groups at the dimerization site or at other positively charged site on the protein.

Table 3 presents the effect of buffer molarity on the binding of positively charged peptides β-LG 69−83 and β-LG 146−149 to β-LG. For both peptides, temperature had no significant effect on the amount of unbound peptide. Binding was significantly increased ($p \leq 0.005$), however, at the lower molar concentration of the buffers from 13 to 22% for peptide β-LG 69−83 and from 1.1 to 10.8% for peptide β-LG 146−149. The significant effect of buffer molarity on the binding of positively charged peptides to β-LG indicates that the force involved in this attraction is electrostatic. In aqueous solution, globular proteins such as β-LG can be represented as solid spheres with a hydrodynamic radius dependent on pH (*28*). At any pH, charged residues are grouped in small clusters of either positive or negative charge, creating dipolarity within the molecules (*29*) and allowing them to engage in electrostatic attractions to each other. Increased ionic strength provides shielding against long-distance electrostatic attraction by the presence of ions (salt). In effect, a diffuse double layer of counterions is formed around the clusters of charges on the surface of the protein (*29*). Increasing the concentration of counterions increases the charge density of the double layer, which in turn decreases electrostatic attraction (*30*). The peptide β-LG 146−149 is composed of four amino acids, and increased ionic concentration likely causes the total screening of its one positive charge, thus limiting its interaction with β-LG. For the longer peptide β-LG 69−83 (15 residues), which bears more positive charges (+4), the higher ionic concentration is probably insufficient to totally screen the electrostatic attraction between the peptide and the protein.

For the peptide β-LG 69−83, a significant interaction ($p = 0.03$) was also found between pH and β-LG/peptide ratio (**Table 4**). In fact, binding of this peptide to β-LG increased with both pH and β-LG/peptide ratio. At the lower β-LG/peptide ratio (1:10), 18 and 26% of peptide β-LG 69−83 bound to the protein at pH 6.8 and 8.0, respectively, whereas at the higher β-LG/peptide ratio (1:5), 28 and 41% of bound peptide were measured. Increasing the pH from 6.8 to 8.0 increased the net negative charge of the protein, whereas peptide β-LG 69−83 remained positively charged (**Table 1**), allowing electrostatic attractions between both molecules.

Results obtained for the binding of hydrophobic peptides α_{S1}-CN 23−34 and β-LG 102−105 to β-LG are presented in **Tables 5**−**7**. For both hydrophobic peptides, no interaction was observed at pH 3.0 (**Tables 5**−**7**) and 4 °C (**Tables 5** and **6**), and buffer molarity had no impact on interaction (results not shown). The amount of bound peptide varied from 11 to 25% for both peptides, the higher values being obtained at pH 6.8, 40 °C, and the higher β-LG/peptide ratio (1:5). Thus, its seems that hydrophobic interactions are the driving force for the binding of hydrophobic peptides to β-LG and that the hydrophobic inner cavity of the protein could be the binding site. According to Qin et al. (*13*) and Wu et al. (*17*), the inner cavity of β-LG is lined exclusively with hydrophobic side chains that allow the binding of retinol or palmitic acid, but this cavity is large enough to bind substantially larger and longer molecules. The binding of hydrophobic peptides α_{S1}-CN 23−34 (12 residues) and β-LG 102−105 (4 residues) thus appears to be possible.

Figure 2 illustrates RP-HPLC chromatograms obtained for the quantification of peptide β-LG 102−105. As observed in

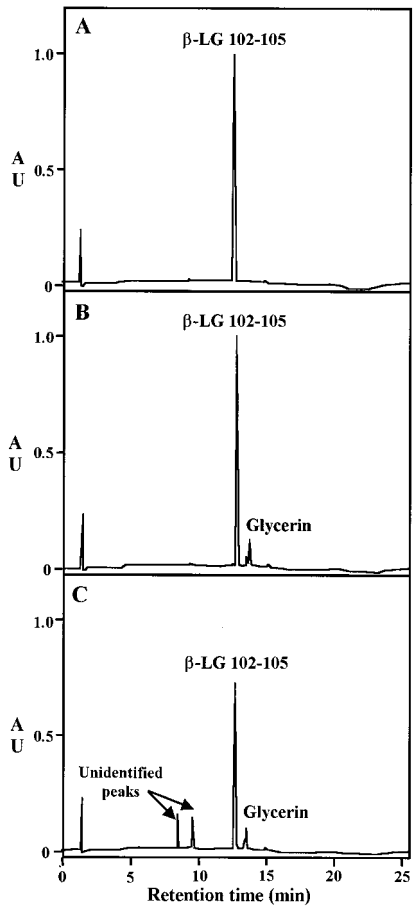

Figure 2. RP-HPLC chromatograms for peptide β-LG 102–105 (500 μM) in 0.1 M Tris buffer (A), the same solution after filtration on Microcon membrane (B), and Microcon-filtered β-LG/β-LG 102–105 solution (100:500 μM) in the same buffer (C). All filtrations were done at 25 °C.

Figure 1, the glycerin peak appears on the chromatograms of Microcon-filtered solutions (**Figure 2B**,**C**). After contact between β-LG and the peptide β-LG 102−105 (**Figure 2C**), a smaller peptide peak was observed than for the peptide solution alone (**Figure 2B**), indicating peptide binding to protein. New peaks (unidentified peak) appeared at retention times of 8.6 and 9.7 min. These unidentified peaks were observed only on chromatograms of hydrophobic peptides (β-LG 102−105 and α_{S1}-CN 23−34) and only after their interaction with the protein (results for α_{S1}-CN 23−34 not shown). The binding of hydrophobic peptides to β-LG thus caused the release of material that adsorbed at 214 nm. Because these materials were eluted earlier than the hydrophobic peptides, they are probably less hydrophobic than the peptides themselves.

Using X-ray diffraction methods, Qin et al. (*9*) observed a trail of electron density inside the calyx of β-LG and beneath the EF loop (85−90) at pH 6.2, whereas no ordered electron density was observed at pH 7.1 or 8.2. Because no potential ligand was included in the protein purification, the authors could not eliminate the possibility that this density was a part of a hydrocarbon chain. In our study, it is possible that hydrophobic peptides having high affinity for the protein displaced ligands already bound in its inner cavity under specific physicochemical conditions (pH 6.8−8.0, 25−40 °C). Because the hydrophobic peptides used in this study are included in the family of bioactive milk peptides (*20*, *31*), this observation may be relevant to the biological function of β-LG. In fact, peptides β-LG 102−105 and α_{S1}-CN 23−34 are both recognized as ACE-inhibitory peptides and could be involved in the control of hypertension (*20*, *32*, *33*). Peptide β-LG 102−105 is also recognized as an opioid peptide (*20*, *32*, *33*).

Although bovine β-LG is included in the lipocalin family (*11*, *34*) and its ability to bind a variety of small hydrophobic molecules is well documented, the actual physiological function of β-LG remains unclear. Bovine β-LG is stable at low pH (*3*) and resistant to gastric digestion (*35*−*37*) and reaches the intestine essentially intact (*38*). As proposed by Pérez and Calvo (*39*), these properties make this protein a good candidate for use as a carrier for small hydrophobic molecules in controlled delivery applications. This hypothesis is supported by the pH control of the inner binding site, underlying the so-called Tanford transition (*40*), and implies a carrier role for β-LG. As observed by Qin et al. (*9*), the EF loop (residues 85−90) forms a lid on the calyx of the protein, which is closed at pH 6.2 and open at pH 7.1 and 8.2, and this conformational change accounts for the physical and chemical pH-dependent properties of β-LG and has functional implications for the reversible binding and release of ligands. This controlled release of ligands may be of practical use in pharmaceuticals and functional food applications for delivering bioactive molecules to the right environment, protecting them against oxidation or degradation, or shielding them from hostile environments (*41*).

On the basis of this study, peptides can be now added to the three groups of ligands reported to bind β-lactoglobulin and classified by Sawyer et al. (*12*) as (1) the saturated fatty acid group, (2) the polyunsaturated molecule group, and (3) the aromatic group. Binding of charged peptides seems to be nonspecific, whereas hydrophobic peptides probably bind to the inner cavity of the protein, provoking the release of material absorbing at 214 nm. Although binding sites have been proposed in this study, these results need to be confirmed by other methods. Experiments are in progress using mass spectrometry to identify the material released from the protein following the binding of hydrophobic peptides.

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