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Interactions between Bovine β-Lactoglobulin A and Various Bioactive Peptides As Studied by Front-Face Fluorescence Spectroscopy

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Front-face fluorescence spectroscopy was used for the first time to study the interactions between bovine β-lactoglobulin variant A (β-Lg A) and various β-Lg-derived bioactive peptides. Fluorescence spectra were recorded for β-Lg A-peptide mixtures at 25 °C and pH 6.8 with an excitation wavelength of 290 nm to characterize the molecular environment of tryptophan (Trp) residues present in the protein but absent in the peptides. Spectra remained unchanged following addition of peptides β-Lg f92−100 and β-Lg f125−135, while Phe−Phe interaction between β-Lg f69−83 molecules interfered with analysis. Addition of β-Lg f102−105 produced a blue shift (3 nm) and a significant increase in fluorescence intensity, while addition of β-Lg f142−148 also caused a significant increase in fluorescence intensity but accompanied by a red shift (3 nm). These results indicate that the polarity of the Trp environment in the β-Lg A structure may be modified differently depending on the peptide added.

KEYWORDS: Bovine β-lactoglobulin A; bioactive peptide; front-face fluorescence spectroscopy; protein−peptide interaction.

INTRODUCTION

β-Lactoglobulin (β-Lg) is the major protein in the whey fraction of milk from ruminants and some nonruminants and is a member of the lipocalycin family (*1*). In recent years, β-Lg has been shown to contain bioactive sequences endowed with antihypertensive, opioid agonistic and antagonistic, immunomodulating, antithrombotic, antimicrobial, mineral-binding, and hypocholesterolaemic properties (*2*−*6*). β-Lg is known to be stable in the acidic environment of the stomach, to resist peptic hydrolysis, and to reach the upper portion of the small intestine mostly intact (*7*−*8*). It was proposed that the high structural and proteolytic stability of β-Lg at low pH (*9*−*11*) and the loss of its tertiary structure at weakly basic pH (*9*, *12*−*14*) could protect its bound ligands in the acidic conditions of the stomach to eventually allow their release in the intestine (*9*, *14*). Therefore, it may become possible to exploit the reversible binding of bioactive peptides by β-Lg in order to provide both protection against enzymes and time-controlled release of these nutraceutical components in the gastrointestinal tract (*14*, *15*).

Bovine β-Lg has a relative molecular mass of 18.3 kDa and its 162-amino acid polypeptide chain is folded into a compact globular conformation stabilized by two intramolecular disulfide bridges. Its core contains an eight-stranded antiparallel β-barrel flanked on one side by an α-helix constituting a hydrophobic pocket (*9*). Bovine β-Lg binds various hydrophobic ligands (*16*) at three potential binding sites, which are the internal cavity (calyx), the external hydrophobic pocket in a groove between the α-helix and the β-barrel, and the outer surface close to Trp_{19}−Arg_{124} (*9*, *10*, *16*−*18*). Most evidence, however, points to the hydrophobic calyx as the major binding site, while the other sites remain the subject of debate (*1*, *19*).

Bovine β-Lg contains fluorophores (aromatic amino acid residues) that allow the protein to display intrinsic ultraviolet fluorescence. Most of the fluorescence emission of β-Lg results from tryptophan (Trp) residues. In fact, Trp is by far the dominant intrinsic fluorophore of β-Lg when excited at wavelengths of 290−300 nm (*20*). β-Lg contains two Trp residues at positions 19 and 61, which are equally capable of absorbing and emitting radiation. However, the molecular environments of these residues differ substantially (*21*). Trp_{19} is located in a hydrophobic environment at the bottom of the calyx, while Trp_{61} is adjacent to β-strand I (residues 145−150), which is involved in β-Lg dimerization and is also close to the Cys_{66}−Cys_{160} disulfide bridge. This bridge is believed to be an effective quencher of Trp fluorescence (*9*, *14*, *21*). Since both of these domains are plausible binding sites (*9*, *10*, *16*−*18*), any change in Trp fluorescence (which is highly sensitive to the environment

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in immediate proximity to Trp residues) most likely reflects their modification.

The maximum emission wavelength of Trp residues in proteins varies from 307 to 353 nm (*22*). This wide variability is due to modifications of the polarity and/or mobility of the immediate environment of the molecule. A maximum fluorescence shifting to shorter wavelengths (blue shift) corresponds to emission by an indole fluorophore in an extremely nonpolar environment inside the protein sphere. Conversely, a red shift or displacement of the maximum toward longer wavelengths indicates that the Trp residue is coming increasingly in contact with bound water and other polar groups (*22*). The shape and maximum emission wavelength (λ_{max}) of the fluorescence emission spectrum thus reflect the average environment of the Trp residues in the molecule. Despite its sensitivity, spectral position (shift) analysis cannot be used to quantify conformational changes in ligand binding because λ_{max} is not a linear response function. Nevertheless, emission intensity at any specific wavelength is a linear response function that can be used to quantify the conformational change resulting from ligand binding (*22*).

Fluorescence spectroscopy has been used to (i) study the conformation of β-Lg at the oil−water interface of emulsions (*23*), (ii) investigate the effect of pH and salt on the association of β-Lg (*24*), and (iii) investigate the binding properties of β-Lg for various molecules. For example, Wang et al. (*25*) studied the binding of retinoids to β-Lg by determining changes in the fluorescence quenching (332 nm) of Trp residues in the protein. High-affinity binding of these compounds was observed at pH 7.0 as reflected by apparent K_d values ranging from 1.7×10^{-8} to 3.6×10^{-8} M. K_d has been determined in the nanomolar range (4.91−6.25 nM) for the binding of vitamin D_2, vitamin D_3, ergosterol, cholesterol, and 7-dehydrocholesterol to β-Lg (*26*). The spectra of the above-mentioned compounds indicate an energy transfer between the ligand and the β-Lg-tryptophanyl residues and that the β-Lg inner cavity (calyx) was the probable binding site. However, other studies have reported small changes in Trp fluorescence or spectral shifts in the fluorescence emission of β-Lg following binding of fluorescent hydrophobic probes, making the estimation of the binding constant difficult (*27*−*29*). In our previous work (*30*), K_d for the β-Lg A/β-Lg f142−148 complex was found to be in the range of 0.5×10^{-3} M. This value is lower than those measured for some endogenous ligands (e.g., retinol), but similar to the values determined for 1−8-anilinonaphthalene sulfonate (ANS) (*31*, *32*) and Ca^{2+} (*16*), which are 1×10^{-3} and 3×10^{-3} M, respectively. The binding constant of β-Lg may be affected by many factors such as pH, concentration, temperature, conformation, or shape of ligand.

In conventional right-angle fluorescence spectroscopy, measurements are carried out in dilute solutions with absorbance below 0.1. At higher absorbance, fluorescence intensity decreases and emission spectra are distorted due to the inner filter effect. To overcome these problems, Parker (*33*) introduced front-face fluorescence spectroscopy (FFFS), which changes the angle of incidence on the sample from 90° to 56° and minimizes artifacts generated by excitation photons reflected from the sample (*34*). This technique allows a quantitative investigation of fluorophores in concentrated or even opaque liquids as well as in powder samples. If absorbance is above 2, more than 99% of the incident intensity is absorbed in the thickness of the sample. Under these conditions, Eisinger and Flores (*35*) have demonstrated using FFFS that the fluorescence intensity is independent of the sample total absorbance and proportional to the fluorophore concentration provided that there is no fluorophore-quenching or energy-transfer phenomena. If total absorbance is kept constant, fluorescence intensity becomes proportional to the fluorophore concentration. FFFS has been used to determine hemoglobin quantitatively in undiluted whole blood (*36*) and to study its aggregation (*37*) and R → T transition (*38*).

Recently, FFFS has been used successfully to (i) investigate network structure and molecular interactions during milk coagulation (*39*), (ii) discriminate between various types of cheese using their intrinsic fluorophores (*40*, *41*), (iii) study the molecular environment of proteins in concentrated and flocculated protein-stabilized emulsions (*42*, *43*), (iv) monitor milk protein denaturation (*44*), (v) quantify the amount of milk proteins loaded on the fat globule interface (*45*), and (vi) characterize ice creams (*46*). These applications suggest that the fluorescence signal is proportional to the molecular environment of the intrinsic probes as well as to the protein−protein, protein−water, and protein−lipid interactions and to the quantity of fluorophore. Although it is possible to monitor and quantify β-Lg−ligand interactions by right-angle fluorescence spectroscopy (*34*, *47*) for absorption less than 0.1, the use of FFFS for this purpose has never been reported.

Known dissociation constants for ligand binding to β-Lg range from 1×10^{-3} to 4.91×10^{-9} M (*16*, *25*, *26*, *31*, *32*). Using isothermal titration calorimetry, we have estimated the K_d for the binding of antihypertensive peptide β-Lg f142−148 to β-Lg A at 0.5 mM (*30*). The purpose of the present work was to use FFFS to investigate conformational changes induced in β-Lg by binding to β-Lg-derived peptides f69−83, f92−100, f102−105, f125−135, and f142−148. These peptides cover a wide range of molecular mass, net charge at pH 6.8, and average hydrophobicity, to help reveal the impact of peptide chemistry on β-Lg A binding affinity, the role of polarity, and the most likely binding site.

MATERIALS AND METHODS

Materials. Bovine β-lactoglobulin (β-Lg) variant A (no. L7880, >92% purity grade) was obtained from Sigma Chemical Co. (St. Louis, MO), while bovine β-Lg AB (no. L3908, >90% purity grade) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Some of the physicochemical characteristics of β-Lg A and B are presented in **Table 1**. The five tryptophan-free peptides selected for this study are normal products of trypsin or trypsin/chymotrypsin hydrolysis of bovine β-Lg: f102−105, f142−148, f92−100, f125−135, and f69−83. They were synthesized by the Service de Séquence de Peptide de l'Est du Québec (Sainte-Foy, PQ, Canada) using a 433A Applied Biosystems peptide synthesizer with fast-MOC chemistry (1.0 mmol scale) using a preloaded Fmoc-Arg(pbf) MBHA resin (Anaspec Inc., San Jose, CA). The trityl side-chain protecting groups were used for histidine. After synthesis, peptides were cleaved and deprotected for 2 h using trifluoroacetic acid and a scavenger (reagent K) and precipitated with *tert*-butyl methyl ether. The precipitated peptides were then dissolved in water and freeze-dried. The crude peptides were purified by reverse-phase HPLC on a preparative C_{18} column. The purity (>80%) and identity of the peptides were confirmed by analytical HPLC and mass spectrometry (matrix-assisted laser desorption ionization time-of-flight). All peptides, except β-Lg f102-105 were water-soluble. Some of their physicochemical characteristics are presented in **Table 2**. All other chemicals were of analytical grade.

Preparation of Solutions. β-Lg AB, β-Lg A, and the peptides were dissolved in phosphate buffer (0.1 M, pH 6.8). Peptide β-Lg f102−105 was dissolved in analytical grade ethanol (95%) because of its high hydrophobicity. The solutions were prepared at room temperature. β-Lg AB and β-Lg A molarities were based on molecular weights of 18 324 and 18 367 Da, respectively.

Table 1. Physicochemical Characteristics of Variants A and B of Bovine β-Lg

protein	amino acid composition[a]	MW[a] (kDa)	charge at pH 6.8[b]	isoelectric point[a]	$H_{\varnothing av}$ [a] (kcal per residue)
β-Lg A	Asp(**11**), Asn(5), Thr(8), Ser(7), Glu(16), Gln(9), Pro(8), Gly(**3**), Ala(**14**), Cys(5), Val(**10**), Met(4), Ile(10), Leu(22), Tyr(4), Phe(4), Lys(15), His(2), Trp(2), Arg(3)	18.36	−9	5.13	1211
β-Lg B	Asp(**10**), Asn(5), Thr(8), Ser(7), Glu(16), Gln(9), Pro(8), Gly(**4**), Ala(**15**), Cys(5), Val(**9**), Met(4), Ile(10), Leu(22), Tyr(4), Phe(4), Lys(15), His(2), Trp(2), Arg(3)	18.28	−8	5.13	1217

[a] These informations were obtained from Farrell et al. (*48*). [b] These values were calculated according to the pK_a of amino acids.

Table 2. Physicochemical Characteristics of the Five β-Lg-Derived Peptides

peptide	amino acid sequence[a]	MW[b] (Da)	charge at pH 6.8	isoelectric point[b]	$H_{\varnothing av}$ [c] (kcal per residue)
β-Lg f102–105	**Y**LL**F**	555.3	0	5.5	2.58
β-Lg f142–148	ALPMHIR	837.5	+1	9.8	1.54
β-Lg f92–100	VLVLDTD**Y**K	1065.2	−1	4.2	1.44
β-Lg f125–135	TPEVDDEALEK	1245.3	−4	3.8	0.85
β-Lg f69–83	KKIIAEKTKIPAV**F**K	1714.2	+4	10.2	1.68

[a] Tyrosine (Y) and phenylalanine (F) are shown in bold. [b] These values were determined using the ExPASy Molecular Biology Server (http://us.expasy.org). [c] Hydrophobicity scale calculated according to the method of Bigelow (*49*).

β-Lg A was titrated by placing 750 μL of 1 mM solution in the 1 mL cuvette of the fluorimeter. Aliquots of 38.5 mM peptide solution were then injected into the cuvette, 5 μL in the case of β-Lg f142−148 or 14 μL for the other peptides, up to a total added volume of 98−100 μL, to reach a final protein−peptide ratio of 1:5. β-Lg solution was also titrated by adding 14 μL aliquots of phosphate buffer or ethanol. Buffer and ethanol (750 μL) were also titrated by adding 14 μL aliquots of peptides up to a total added volume of 98 μL.

Front-Face Fluorescence Spectroscopy Measurements. The fluorescence spectra (intensity, shift in λ_{max}, and changes in width or shape) of β-Lg AB, β-Lg A, free peptides, and mixtures were recorded after the excitation wavelength (λ_{ex}) was set at 290 nm using a FluoroMax-2 spectrofluorometer (Spex-Jobin Yvon, Longjumeau, France) equipped with Data max software and mounted with a variable angle front-surface accessory and front-face illumination. The incidence angle of the excitation radiation was set at 56°. The cell holder temperature was controlled at 25 °C, and the excitation and emission slit widths were set at 1 nm. All spectra were corrected for instrument excitation distortions using a rhodamine cell as a reference channel. All samples were analyzed in duplicate.

RESULTS AND DISCUSSION

Fluorescence Emission Intensity of β-Lg. To determine the appropriate protein concentration for the binding study, Trp emission spectra (305−400 nm) were recorded with λ_{ex} set at 290 nm at increasing β-Lg AB concentrations. **Figure 1** shows that the maximum emission intensity of β-Lg AB decreased as the protein concentration increased from 0.2 to 25 mM. Fluorescence spectra obtained at lower protein concentrations were narrowly distributed on the wavelength scale, while the width of the spectrum obtained at 25 mM was slightly larger. The changes in the shape of the fluorescence spectra coupled with the decrease in fluorescence intensity when the protein concentration increased from 0.2 to 25 mM indicated that association (or possibly polymerization) may occur, thus, shifting the protein equilibrium toward dimer formation at high β-Lg AB concentrations, as supported by Renard et al. (*24*). The proximity of Trp_{61} to the monomer−monomer association interface may have caused quenching. However, dimerization did not seem to cause significant conformational changes around

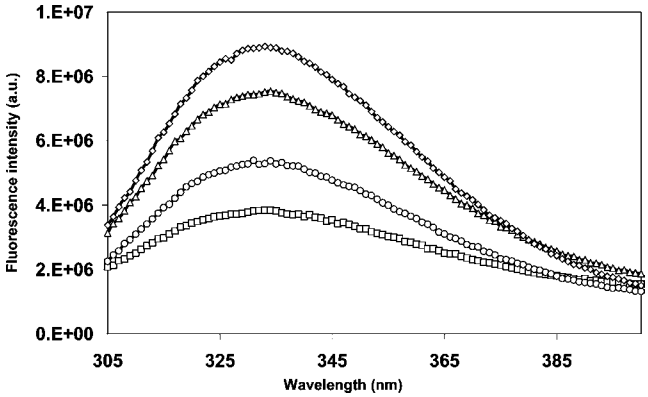

Figure 1. Fluorescence spectra (λ_{ex} 290 nm) of β-Lg AB (pH 6.8, 25 °C) at increasing protein concentrations: 0.2 (◇), 1 (△), 2 (○), and 25 mM (□).

the Trp residues, since λ_{max} remained the same (333−334 nm) at all protein concentrations studied. The use of β-Lg AB aimed at determining the adequate protein concentration for fluorescence study. The quantity of β-Lg A available from the same lot was limited. Therefore, to use the same lot for all experiments, β-Lg A was used only at concentration of 1 mM after results of β-Lg AB showed this concentration to be appropriate. Similar FFFS profiles were obtained at 1 mM for both β-Lg AB and β-Lg A (results not shown). In fact, both proteins are similar in structure. The β-Lg A variant differs from the β-Lg B variant by only two amino acids: Asp_{64} and Val_{118}. These amino acids are substituted by Gly_{64} and Ala_{118} in the B variant (**Table 1**). Renard et al. (*24*) also found that the fluorescence emission of β-Lg AB and β-Lg A are similar, both at λ_{ex} of 295 and 287 nm. They suggest that such a short wavelength maximum indicates very hydrophobic environments around at least one Trp residue, likely Trp_{19}, the major fluorophore, which is located in the hydrophobic calyx of the protein (*21*).

Although the maximum emission wavelength was obtained for 0.2 mM protein, a concentration of 1 mM was chosen because it is reasonably close to the previously determined K_d

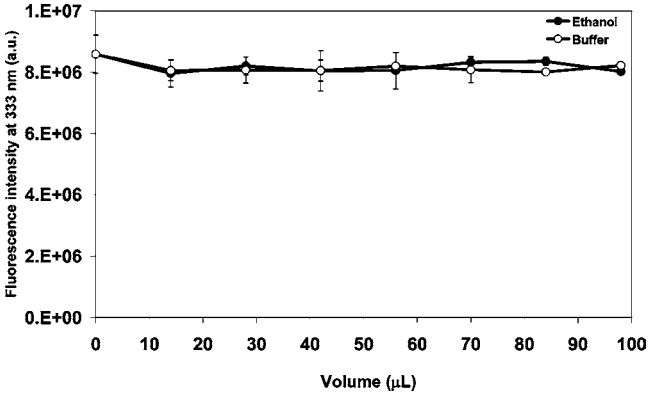

Figure 2. Fluorescence intensity of β-Lg A at λ_{em} 333 nm following the addition of phosphate buffer (○) or ethanol (●).

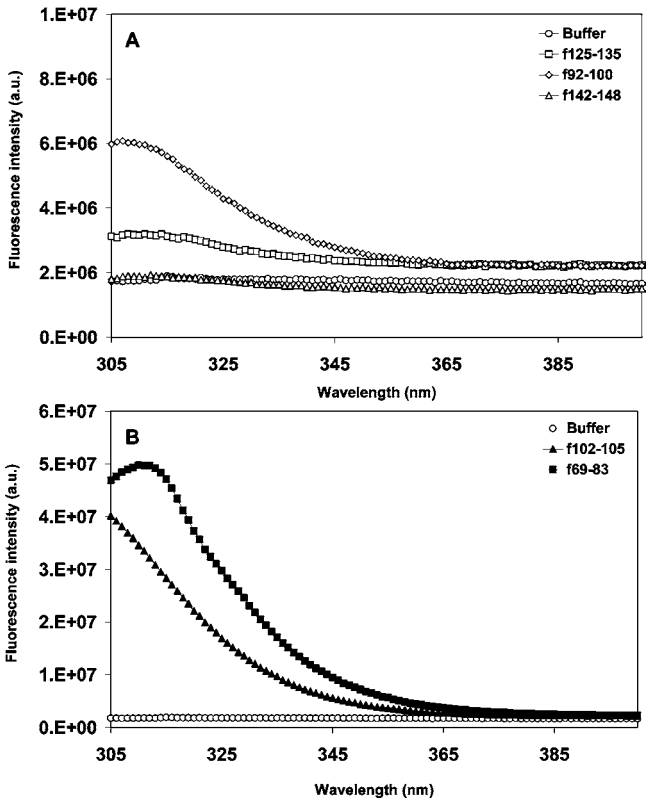

Figure 3. (**A**) Fluorescence spectra (λ_{ex} 290 nm) of phosphate buffer (pH 6.8, 25 °C) titrated with phosphate buffer (○) or free peptide (final concentration of 5 mM): f125–135 (□), f92–100 (◇), and f142–148 (△). (**B**) Fluorescence spectra (λ_{ex} 290 nm) of phosphate buffer (pH 6.8, 25 °C) titrated with phosphate buffer (○) or free peptide (final concentration of 5 mM): f 102–105 (▲) and f69–83 (■).

(0.5 mM) for the binding of β-Lg f142−148 to β-Lg A (*30*) and provides a high protein−peptide ratio (1:5) at the end of the titration.

Fluorescence Emission of Free β-Lg Variant A and Free Peptides. β-Lg A was titrated with phosphate buffer or ethanol (**Figure 2**). Both solvents did not cause any significant change in spectral shape of β-Lg A, and the 333 nm λ_{max} for β-Lg A did not vary as a function of added solvents. Moreover, the fluorescence emission intensity of β-Lg A at λ_{ex} 290 nm remained unchanged. The fluorescence spectra of the five Trp-free peptides were recorded with λ_{ex} set at 290 nm (**Figure 3**). Each 38.5 mM peptide solution was injected into phosphate buffer up to a concentration of 5 mM. None of the peptides showed fluorescence emission characteristic of Trp emission at λ_{ex} of 290 nm. It is well-known that intrinsic protein fluorescence is due to aromatic amino acids, mainly tryptophan. Phenylalanine has a very low quantum yield, and emission by tyrosine in native proteins is often quenched (*50*). The present findings are consistent with these assertions. It has been suggested that excitation at 290 nm does allow selective measurement of fluorescence due only to tryptophan residues because tyrosine does not absorb at this wavelength (*50*). However, the presence of Tyr in peptide f92−100 appeared to contribute to the 305−400 nm emission spectra (**Figure 3A**) but less than peptide f102−105 (**Figure 3B**) which contains, beside a Tyr residue, a Phe residue as well. In fact, the presence of phenylalanine in some peptides (f102−105 and f69−83) appeared to contribute to the 305−400 nm emission spectra (**Figure 3B**). The presence of tyrosine with phenylalanine induced a quenching that could explain the low intensity observed in f102−105 compared to f69−83, in which only phenylalanine is present, in agreement with the findings of Yeboah et al. (*50*).

FFFS is specifically adapted to quantitative investigation of fluorophores in concentrated or even opaque liquids (*35*). The absence of structured spectral emission from free peptides suggests that changes in Trp fluorescence spectra obtained for β-Lg in the presence of Trp-free peptides should be due to conformational or polarization effects induced in the protein (*20*). Peptide β-Lg f69−83 was an exception, exhibiting unexpectedly strong fluorescence emission with λ_{max} at ∼312 nm following excitation at 290 nm (**Figure 3B**). This 15-amino acid peptide contains a single Phe residue, and fluorescence may originate from phenyl−phenyl interactions (*20*) following the formation of peptide aggregates. The λ_{max} of β-Lg f69−83 (312 nm) is different from those of β-Lg alone (333 nm) and of Phe (281 nm).

Fluorescence Emission Intensity of β-Lg A Interacting with Peptides. The fluorescence emission intensity of β-Lg A with λ_{ex} set at 290 nm was investigated for increasing peptide concentrations. Since Trp emission always prevails in the presence of Phe, Tyr, or both Phe and Tyr residues, we expect β-Lg Trp residues to dominate emission upon addition of each Trp-free peptide. Since titration of β-Lg A with buffer or ethanol did not affect the protein fluorescence emission (**Figure 2**), any change in fluorescence intensity, shift in λ_{max}, or change in the shape of the fluorescence spectrum would be due to polarity changes at the molecular level in the vicinity of Trp residues and interpretable as interaction between protein and peptide (*20*) and not due to the addition of solvent(s). The effect of peptide β-Lg f92−100 on the fluorescence emission intensity of β-Lg A at λ_{ex} 290 nm was analyzed. This Trp-free peptide did not cause any significant change in spectral shape or fluorescence intensity, and the 333 nm λ_{max} for β-Lg A did not vary as a function of peptide concentration (**Figure 4**), suggesting that this peptide did not bind to the protein. We have previously demonstrated (*30*) using ultrafiltration that peptide β-Lg f92−100 does not bind to β-Lg A. Although of hydrophobic character (**Table 2**), this nonapeptide has one negative charge at pH 6.8, which may prevent the peptide from interacting with β-Lg A, which is also negatively charged at this pH.

In the case of peptide β-Lg f125−135, the increase in fluorescence intensity following its addition to β-Lg A is slight and appears to be in the same order of magnitude as the variations observed when β-Lg dispersion was titrated with buffer (**Figure 5**). Moreover, the wavelength of maximum emission remained unchanged (λ_{max} 333 nm). Therefore, we cannot conclude unequivocally that this peptide binds to β-Lg

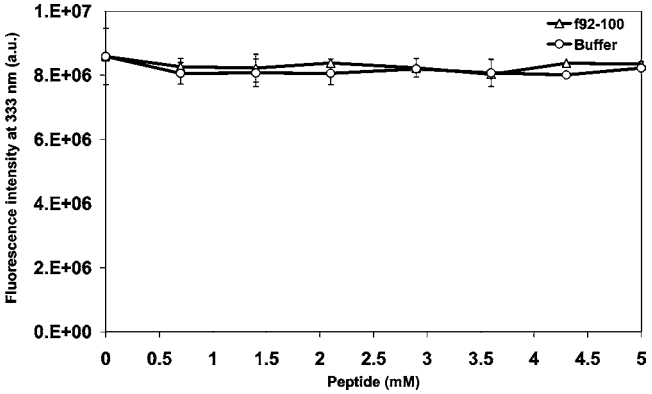

Figure 4. Fluorescence intensity of β-Lg A at λ_{em} 333 nm following the addition of phosphate buffer (○) or β-Lg f92–100 (△).

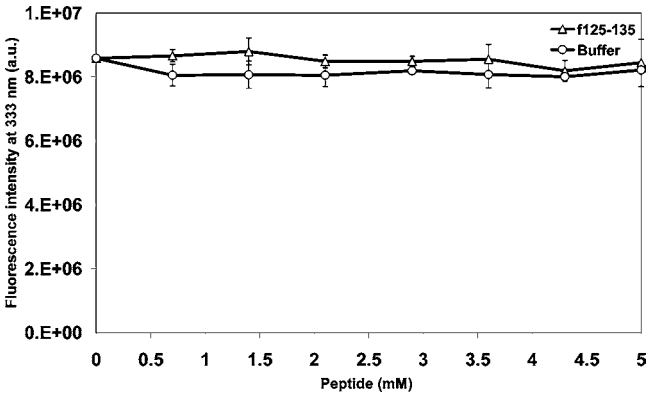

Figure 5. Fluorescence intensity of β-Lg A at λ_{em} 333 nm following the addition of phosphate buffer (○) or β-Lg f125–135 (△).

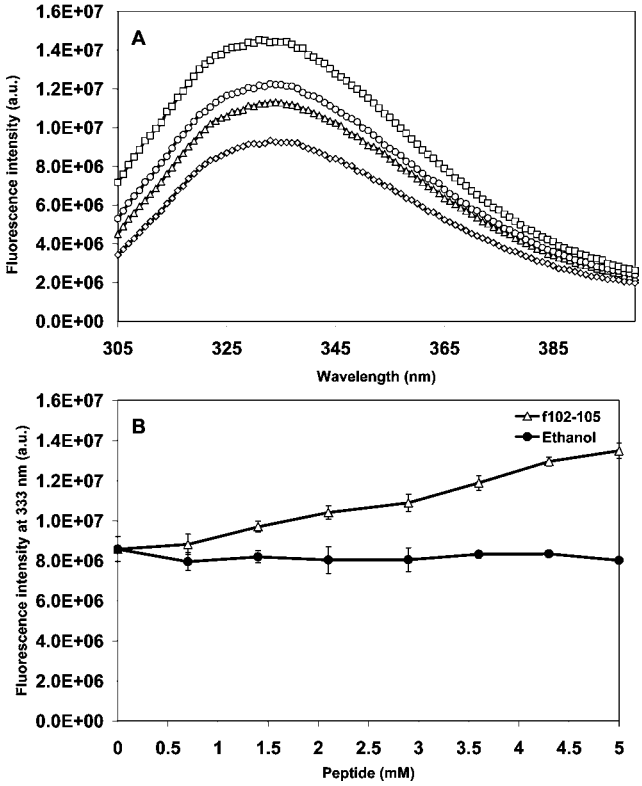

Figure 6. (**A**) Fluorescence spectra of β-Lg A (1 mM, pH 6.8, 25 °C) at λ_{ex} 290 nm following the addition of increasing concentrations of β-Lg f102–105: 0 (◇), 2.1 (△), 3.6 (○), and 5 mM (□). (**B**) Fluorescence intensity of β-Lg A at λ_{em} 333 nm following the addition of ethanol (●) or β-Lg f102–105 (△).

A. We previously found only 0.07 mol of this peptide bound per mole of β-Lg A (*30*). Peptide β-Lg f125−135 (11 amino acids) is hydrophilic (**Table 2**) and has four negative charges at pH 6.8 that could prevent the peptide from interacting with the negatively charged β-Lg at pH 6.8.

Figure 6A shows the effect of the interaction between β-Lg A and peptide β-Lg f102−105 dissolved in ethanol. The fluorescence intensity of β-Lg A titrated with ethanol alone remained unchanged (**Figure 2**), while increasing concentrations of β-Lg f102−105 significantly increased (up to 57% at the end of titration) the fluorescence intensity, suggesting that the protein underwent a conformational change that affected the environment of its Trp residues. The spectrum also showed a blue shift (3 nm), and its shape changed significantly. The shift of λ_{max} to shorter wavelengths indicates that the Trp residues in β-Lg A were in a more hydrophobic environment following interaction with β-Lg f102−105 (*20*, *22*). The increase in the fluorescence intensity and the change in the shape of fluorescence spectra when peptide β-Lg f102−105 was added to β-Lg dispersion indicate conformational changes at the molecular level (e.g., peptide−protein interactions) and strongly suggest that the peptide binds to the protein. In the presence of the two Trp residues of β-Lg, the contribution of peptide β-Lg f102−105 (Trp-free) should be negligible. In fact, it is well-known that when Trp, Tyr, and Phe are present in the same dispersion, Trp emission highly dominates Phe and Tyr emission upon excitation at 290 nm. It has also been shown that the interactions of β-Lg with different ligands induced different effects on the fluorescence spectra of β-Lg recorded after excitation at 290 nm. For example, Dufour and Haertlé (*51*) have shown that the fluorescence intensity at 320 nm (λ_{ex} 290 nm) decreases with the formation of β-Lg−retinol complex. In contrast, the formation of β-Lg−fatty acid complex induces an increase of the fluorescence intensity at 320 nm (λ_{ex} 290 nm) (*52*). The change (decrease/increase) of the fluorescence intensity of β-Lg Trp residues depends on the changes of the environment of β-Lg Trp residues following the formation of the complex; that is, the chemical nature of the ligands and the location of the binding site in the β-Lg molecule induce different changes in the environment of β-Lg tryptophans.

The increase in the fluorescence intensity of β-Lg A resulting from interaction with peptide β-Lg f102−105, along with the shift of λ_{max} to shorter wavelengths while the protein is in a polar solvent, suggests that the immediate environment of Trp became more hydrophobic. Because of the short length of β-Lg f102−105 (four amino acids) and its highly hydrophobic nature (**Table 2**), the hydrophobic calyx near the Trp_{19} residue is a likely binding site. According to Fugate and Song (*53*), the Trp residue is also involved in retinol binding, possibly by complexing the β-ionone ring specifically with the polyene chain. In contrast, Trp_{61} is flanked by Glu and Lys residues near the Cys_{66}−Cys_{160} disulfide bridge (*14*, *54*), and this more hydrophilic environment would probably interfere with binding of such a hydrophobic peptide. However, the contribution of Trp_{61} to the fluorescence intensity cannot be excluded if it moves away from the disulfide bridge following complex formation (*21*, *55*).

Figure 7 shows the effect of the interaction between β-Lg A and β-Lg f69−83. Since the peptide was highly soluble in phosphate buffer and available in large amounts, titration was done to a final concentration of 15.7 mM peptide. A significant increase in fluorescence intensity and a 17 nm blue shift in Trp emission spectra were observed with increasing peptide concentrations, suggesting interaction with β-Lg. However, since the β-Lg f69−83 emission λ_{max} (∼312 nm) in buffer (**Figure**

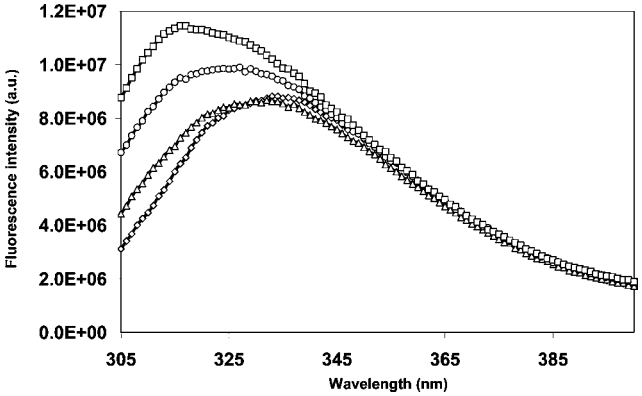

Figure 7. Fluorescence spectra of β-Lg A (1 mM, pH 6.8, 25 °C) at λ_{ex} 290 nm following the addition of increasing concentrations of β-Lg f69–83: 0 (◇), 5 (△), 10.7 (○), and 15.7 mM (□).

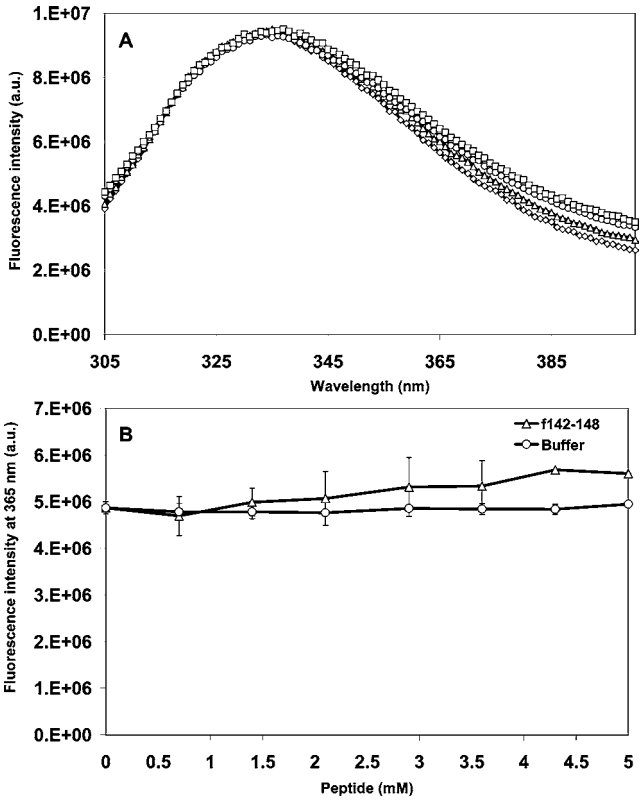

Figure 8. (**A**) Fluorescence spectra of β-Lg A (1 mM, pH 6.8, 25 °C) at λ_{ex} 290 nm following the addition of increasing concentrations of β-Lg f142–148: 0 (◇), 1.25 (△), 2.5 (○), and 5 mM (□). (**B**) Fluorescence intensity of β-Lg A at λ_{em} 365 nm following the addition of phosphate buffer (○) or peptide β-Lg f142–148 (△).

3B) did not match the λ_{max} of Phe (281 nm), its emission spectrum is likely dominated by Phe−Phe interactions, which are quite different from the structured Trp or Phe spectral emissions. Furthermore, since the peptide molecules could also interact with each other (Phe−Phe interactions), we cannot conclude unequivocally that this peptide binds to β-Lg A.

Figure 8A shows the effect of the addition of β-Lg f142−148 on β-Lg A fluorescence. **Figure 8B** shows the fluorescence intensity of β-Lg A at λ_{em} 365 nm as a function of peptide concentration. Titrating the protein with this peptide significantly increased (up to 15% at the end of titration) the fluorescence intensity and produced a 3 nm red shift. The shift was the opposite of that observed following binding of β-Lg f102−105 (**Figure 6**) to β-Lg A under the same conditions. Since β-Lg f142−148 contains no aromatic amino acid residues (**Table 2**) and did not display any fluorescence at λ_{ex} of 290 nm (**Figure 3A**), the change observed in this β-Lg A fluorescence spectrum suggests strongly that β-Lg f142−148 bound to the protein.

The increase in fluorescence intensity can be explained by a more hydrophobic environment surrounding at least one of the Trp residues or by the elimination of Trp carboxyl quenching. However, the red shift suggests that a Trp residue was exposed to the aqueous phase (*20*, *22*). If the peptide bound directly to Trp, the Arg residue at its C-terminal end could explain the red shift. If the peptide bound to the Trp carboxyl, the quenching would be eliminated, which would explain the increase in fluorescence intensity. However, this increase combined with the red shift strongly suggest that both Trp_{19} and Trp_{61} contributed to β-Lg f142−148 binding, a hypothesis supported by our previous results suggesting more than one binding site on β-Lg A for this peptide (*30*) and by studies demonstrating that retinol−protoporphyrin IX and fatty acid−retinoid mixtures can bind simultaneously to β-Lg (*56*, *57*).

In previous studies of interactions between β-Lg and non-fluorescent ligands such as fatty acids (*52*) or ionone and terpenes (*58*), changes in fluorescence emission of β-Lg Trp residues were interpreted as indications of protein−ligand interactions. The observed increases (fatty acids) or decreases (ionone and terpenes) did not exceed 20−30%. Under our conditions, β-Lg displayed a typical fluorescence emission spectrum with λ_{max} 333 nm (**Figure 1**), while the titration patterns of β-Lg A with the five peptides (**Figures 4**−**8**) varied significantly. The most hydrophobic peptide (β-Lg f102−105) produced the greatest change in β-Lg A fluorescence intensity and caused a blue shift of λ_{max}, which was indicative of a more hydrophobic environment surrounding the Trp residues of the protein. β-Lg f142−148 increased Trp emission of the protein and caused a red shift of λ_{max}, suggesting that at least one Trp residue was exposed to the aqueous phase.

The K_d value for the β-Lg−peptide complex could not be determined, since it was impossible to reach a plateau at the end of the titration at the protein concentration used (1 mM). At protein and peptide concentrations equal to K_d, 50% of the ligand is bound to the protein. To bind all or at least 99% of the ligand, the protein concentration has to be much higher (about 100-fold) than the K_d. Nevertheless, on the basis of the titration curves showing that fluorescence intensity changed as a function of the amount of peptide added, the K_d for the complexes may be estimated in the millimolar range. This is in agreement with our previous results using isothermal titration calorimetry, estimating K_d at 0.5 mM for the β-Lg A/β-Lg f142−148 complex (*30*).

On the basis of the above observations of β-Lg A−peptide interactions and on known properties (MW, charge, and hydrophobicity) of the peptides, it is plausible that β-Lg f102−105 bound to the hydrophobic calyx of the protein and that β-Lg f142−148 bound inside the calyx and/or near the dimer formation interface. The results presented here confirm previous evidence of interaction between β-Lg A and certain peptides (*30*, *59*). In the context of the relatively broad and varying specificities of β-Lg A toward different ligands, characterizing its selectivity toward bioactive peptides may lead to a better understanding of its physiological function.

This demonstration of interactions between β-Lg A and bioactive peptides is in response to questions raised by a recent study showing the degradation of some peptides during simulated gastrointestinal digestion (*60*). Since β-Lg A is stable at acid pH and resists the gastric digestion, it could be used to

transport bioactive peptides and protect them against enzymatic degradation in the gastrointestinal tract, allowing them to reach their targets intact and exert their physiological effects in vivo.

ABBREVIATIONS USED

β-Lg, β-lactoglobulin; K_d, constant of dissociation; Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine; FFFS, front-face fluorescence spectroscopy; ANS, 1-8-anilinonaphthalene sulfonate; MW, molecular weight; a.u., arbitrary units.

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