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ACE-Inhibitory and Radical-Scavenging Activity of Peptides Derived from β-Lactoglobulin f(19−25). Interactions with Ascorbic Acid

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In this work, the angiotensin-converting enzyme (ACE)-inhibitory and radical-scavenging activities of the β-lactoglobulin (β-Lg)-derived peptides WY f(19−20), WYS f(19−21), WYSL f(19−22), WYSLA f(19−23), WYSLAM f(19−24), and WYSLAMA f(19−25) have been determined. The ACE-inhibitory activity (IC_{50}) varied from 38.3 to 90.4 μM, with the exception of WYS (>500 μM). All β-Lg-derived peptides also exhibited radical-scavenging activity (oxygen radical absorbance capacity (ORAC) values ranged from 4.45 to 7.67 μmol Trolox equivalents/μmol of peptide). The presence and position of amino acids Trp, Tyr, and Met were proposed to be responsible for the antioxidant activity. The equimolar amino acid mixtures of all the peptides showed ORAC values lower than those of the corresponding peptides, indicating that the peptidic bond or the structural conformation had a positive influence on this activity. Finally, positive antioxidant effects of WYS, WYSL, and WYLA with ascorbic acid were observed, whereas WY and WYSLAM showed negative effects, both cases for different molar ratio mixtures. These results should be taken into account in the development of new food ingredients on the basis of peptides from β-Lg.

KEYWORDS: β-Lactoglobulin peptides; ACE-inhibitory activity; radical-scavenging activity; ORAC; ascorbic acid

INTRODUCTION

β-Lactoglobulin (β-Lg) comprises 60% of whey protein. It is known to exert a wide range of nutritional, functional, and biological activities that makes it a potential ingredient for health-promoting foods, drugs, and cosmetics (*1*). In addition to the bioactivities exerted by the native molecule, β-Lg may exhibit further physiological functions because of numerous bioactive peptides that are contained within the protein. Several bioactive sequences derived from this whey protein have been identified and their antihypertensive, opioid, antimicrobial, antithrombotic, mineral-binding, immunomodulant, and hypocholesterolaemic properties have been reported (*2*−*5*).

Free-radical-mediated lipid oxidation is considered to be one of the main limiting factors for the quality and acceptability of foods during processing and storage. Currently, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used to act against free radicals in food and biological systems. However, the potential adverse effects of these synthetic additives have stimulated their replacement by natural antioxidants derived from dietary sources (*6*). Moreover, reactive radicals are implicated in the ethiology of age-associated chronic diseases such as cardiovascular diseases, neurodegenerative disorders, diabetes, and certain types of cancer (*7*). The utilization of protein hydrolysates or peptides to improve the antioxidant activity in functional foods presents additional advantages over other natural antioxidants, since they also confer an additional nutritional value, as well as other desired functional properties. In the past few years, the search for whey-derived peptides with radical-scavenging and lipid peroxidation inhibitory activities is receiving special attention. Recent studies have described the antioxidant activity of whey protein hydrolyzates (*8*, *9*). However, few data on the antioxidant properties of the individual peptides released after whey protein hydrolysis are available. Peptide Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile (WYSLAMAASDI, f19−29) derived from β-Lg after hydrolysis with Corolase PP has shown a relatively large scavenging radical activity (*10*). Since whey peptides may be present in foods together with other antioxidants, all implicated in multiple redox reactions, it would be interesting to study the potential positive effects of these β-Lg-derived peptides with other nonpeptidic antioxidant agents. Positive effects have previously been demonstrated with α-tocopherol and peptides derived from enzymatic hydrolyzates of soybean protein (*11*) and yellowfin sole (*12*).

Antioxidant deficiency has been implicated in the occurrence of hypertension. Antioxidant-rich diets have been shown to

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reduce the blood pressure in spontaneously hypertensive rats (*13*) and hypertensive humans (*14*). Angiotensin-converting enzyme (ACE) inhibitors play an essential role in managing hypertension by improving endothelial dysfunction and have notable effects on oxidative stress (*15*). Captopril, a well-known potent ACE-inhibitory and antihypertensive drug, also exhibits antioxidant properties (*16*). Intracellular antioxidant peptides such as glutathione, carnosine, and others have shown in vitro ACE-inhibitory activity (*17*). This dual (ACE-inhibitory and antioxidant) activity has also been demonstrated for peptides derived from egg-white proteins (*18*) and caseins (*19*). However, to our knowledge, there are no available data about the cross antioxidant and ACE-inhibitory activities of peptides derived from whey proteins.

The aim of this work was to study the multifunctional capacity of peptides derived from β-Lg. Radical-scavenging and ACE-inhibitory activities of synthetic peptides with potentially bioactive sequences (WY, WYS, WYSL, WYSLA, WYSLAM, and WYSLAMA) were determined, and possible structure/activity relationships were proposed. The positive/negative antioxidant effects between these peptides and ascorbic acid (vitamin C), one of the antioxidants commonly used in food processing, were also determined.

MATERIALS AND METHODS

Chemicals and Samples. Fluorescein disodium, amino acid standards [Trp (W), Tyr (Y), Leu (L), Ala (A), Met (M)], hippuryl-histydil-leucine (HHL), and ACE were purchased from Sigma Chemical (St. Louis, MO). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2′-azobis(2-methylpropionamide)-dihydrochloride (AAPH) were obtained from Aldrich (Milwaukee, WI). Amino acid standard Ser (S) was purchased from Merck KGaA (Darmstadt, Germany). Ascorbic acid was obtained from Fluka A. G. (Buchs SG, Switzerland).

Peptides WY, WYS, WYSL, WYSLA, WYSLAM, and WYSLAMA were synthesized by GenScript Corporation (Piscataway, NJ), and their purity was verified by analytical RP-HPLC-MS/MS.

ACE-Inhibitory Activity. ACE-inhibitory activity was measured by the spectrophotometric assay of Cushman and Cheung (*20*), with some modifications. Briefly, 40 μL of each sample was added to 0.1 mL of 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl and 5 mM HHL. ACE (2 mU) (EC 3.4.15.1; 5.1 U mg^{-1}) was added and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.15 mL 1 M HCl. The hippuric acid formed was extracted with ethyl acetate, was heat-evaporated at 95 °C for 10 min, was redissolved in distilled water, and was measured spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate.

The ACE-inhibitory activity was calculated as the peptide concentration needed to cause 50% inhibition of the original ACE activity (IC_{50}).

Antioxidant Activity by ORAC Assay. The oxygen radical absorbance capacity (ORAC) assay using fluorescein as fluorescent probe was based on that proposed by Ou et al. (*21*) and was modified by Dávalos et al. (*22*). Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4), and the final assay mixture (200 μL) contained fluorescein (70 nM), AAPH (12 mM), and antioxidant [Trolox (1−8 μM) or sample (at different concentrations)]. The plate was automatically shaken before the first reading, and the fluorescence was recorded every minute for 98 min. A Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) with 485-P excitation and 520-P emission filters was used. The equipment was controlled by the Fluostar Galaxy software version (4.11−0) for fluorescence measurement. Black 96-well microplates (96F untreated, Nunc, Denmark) were used. AAPH and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4).

All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample. Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=98} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = \mathrm{AUC}_{\text{antioxidant}} - \mathrm{AUC}_{\text{blank}}$$

The regression equation between net AUC and antioxidant concentration was calculated. The slope of the equation was used to calculate the ORAC value by using the Trolox curve obtained for each assay. Final ORAC values were expressed as μmol of Trolox equivalent/μmol of antioxidant. Analysis were carried out in triplicate.

Positive/Negative Antioxidant Effects. Mixtures of synthetic β-Lg-derived peptides and ascorbic acid at different molar ratios (0:1, 0.2:0.8, 0.33:0.66, 0.5:0.5, 0.66:0.33, 0.80:0.2, and 1:0) (1 μM, total concentration) were prepared and their radical-scavenging activity was determined as described above.

For the different peptide:ascorbic acid mixtures, the calculated ORAC value was determined as follows:

$$\text{calculated ORAC} = [\mathrm{ORAC}_{\text{peptide}} \times R] + [\mathrm{ORAC}_{\text{ascorbicacid}} \times (1 - R)]$$

where R is the peptide concentration (expressed as μmol) in the peptide:ascorbic acid mixture. Finally, the % variation between both observed and calculated ORAC values was calculated as follows:

$$\%\ \text{variation} = [(\text{observed ORAC} - \text{calculated ORAC})/\text{calculated ORAC}] \times 100$$

Table 1. ACE-Inhibitory Activity (Expressed as IC_{50}) of the Synthetic β-Lg-Derived Peptides

peptide	fragment	IC_{50}[a] (μM)
WY	f(19–20)	38.3
WYS	f(19–21)	>500
WYSL	f(19–22)	90.4
WYSLA	f(19–23)	86.9
WYSLAM	f(19–24)	56.3
WYSLAMA	f(19–25)	59.9

[a] Peptide concentration needed to inhibit 50% original ACE activity.

RESULTS AND DISCUSSION

ACE-Inhibitory Activity of Peptides Derived from β-Lg. ACE-inhibitory activity (IC_{50} value) of the potentially bioactive synthesized β-Lg-derived peptides is reported in **Table 1**. The dipeptide WY, corresponding to the β-Lg fragment f(19−20), showed potent ACE-inhibitory activity (IC_{50} = 38.3 μM). The addition of a Ser residue at the C-terminal position caused the ACE-inhibitory activity to drop to a negligible value (IC_{50} > 500 μM). Therefore, the amino acid Ser at the C-terminal position seemed to decrease the affinity of the peptide for the active site of ACE. However, if this amino acid was localized at the penultimate and ante-penultimate position in the tetra and pentapeptides (WYSL and WYSLA, respectively), the ACE-inhibitory activity became quantifiable (IC_{50} values of 90.4 and 86.9 μM, respectively). After further addition of a Met residue to the C-terminus of the pentapeptide, the resulting one (WYSLAM) showed an IC_{50} value (56.3 μM) 4-fold lower than that reported by Miguel et al. for the peptide SALAM released from ovalbumin hydrolyzed with pepsin (*23*). Similar ACE-

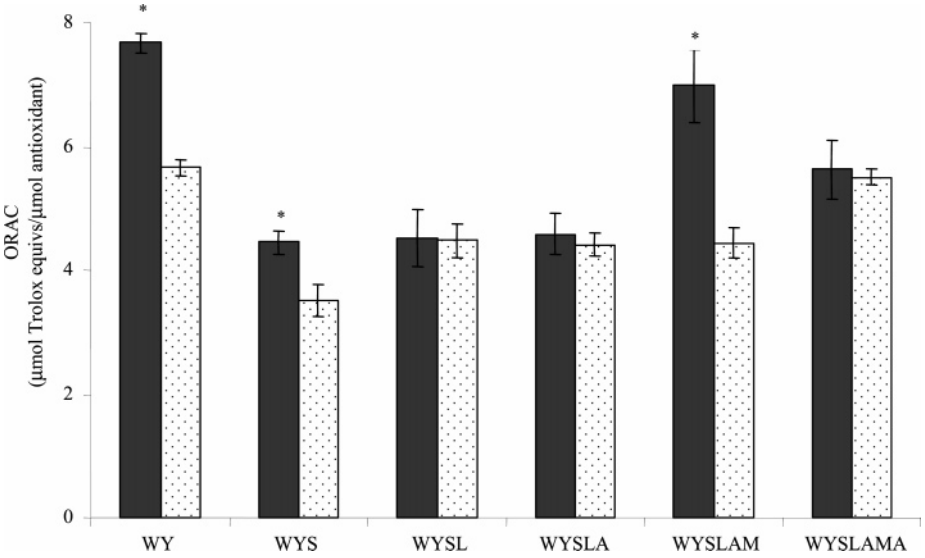

Figure 1. Radical-scavenging activity of the synthetic β-lactoglobulin-derived peptides studied (■) and of their corresponding equimolar amino acid mixtures (□). Error bars represent the SD of the mean ($n = 3$). *, difference between the peptide and its corresponding equimolar amino acid mixture is significant ($p < 0.05$).

inhibitory activity was observed for peptide WYSLAMA ($IC_{50} = 59.9$ μM, **Table 1**).

The ACE-inhibitory activity of the previously characterized peptide WYSLAMAASDI (*10*) was relatively low, with an IC_{50} value of 208.4 μM. The presence of an Asp residue at the C-terminal sequence could be responsible for this moderate activity. Structure−activity data have indicated that ACE has little affinity for substrates of competitive inhibitors with C-terminal dicarboxylic amino acids (*24*). However, more research employing novel strategies, such as molecular modeling, is needed to explain the peptide structural features that determined the ACE-inhibitory activity of these peptides.

Antioxidant Activity of Peptides Derived from β-Lg. The radical-scavenging activity (ORAC values) of β-Lg-derived peptides is shown in **Figure 1**. These values varied from 4.45 (WYS) to 7.67 (WY) μmol Trolox equivs/μmol of peptide. The relatively high-radical-scavenging activity of these peptides was attributed to the presence of Trp, Tyr, and Met residues. The high antioxidant activity of Trp (ORAC value = 4.65 μmol Trolox equivs/μmol), Tyr (ORAC value = 1.57 μmol Trolox equivs/μmol), and Met (ORAC value = 1.13 μmol Trolox equivs/ μmol), as well as their potential mechanism of action, has been reported previously (*10*, *18*). Moreover, the position of these amino acids within the peptide sequence could play an important role in the antioxidant activity of the peptides studied. The influence of the amino acid position on the superoxide anion-scavenging activity of casein-derived peptides has also been described previously (*25*). Moreover, the presence of the amino acid Tyr at the N-terminal has been described as one determinant factor in the radical-scavenging activity of ovalbumin-derived peptides (*18*). In the β-Lg-derived peptides studied, the presence of Tyr and Met at the peptide C-terminal position clearly enhances its scavenging activity against oxygen radicals.

To see if the peptidic bond or structural conformation of the β-Lg-derived peptides studied influenced the antioxidant activity of the constitutive amino acids, the radical scavenging of the corresponding equimolar amino acid mixtures was also assayed (**Figure 1**). The ORAC values of all the analyzed β-Lg-derived peptides (WYSLAMA, WYSLAM, WYSLA, WYSL, WYS, and WY) were higher than those measured for their equimolar amino acid mixtures, although statistically significant differences ($p < 0.05$) were only found for WY, WYS, and WYSLAM (**Figure 1**). This result confirmed that the peptidic bond or structural peptide conformation improves the hydrogen donor capacity of the amino acid residues, enhancing their antioxidant activity.

The ORAC value of the previously characterized peptide WYSLAMAASDI was 2.62 μmol Trolox equivs/μmol of peptide (*10*). This value was lower than those obtained for peptides synthesized and analyzed in the present study, which was attributed to the greater size of WYSLAMAASDI. Therefore, the deletion of sequence AASDI resulted in an important increase in antioxidant activity. The resultant peptide WYSLAM showed an ORAC value of 7.00 μmol Trolox equivs/μmol of peptide (**Figure 1**), almost 3-fold that reported for peptide SALAM (2.66 μmol Trolox/μmol of peptide) (*18*). This result confirms the importance of the presence and position of the Trp and Tyr residues on the radical-scavenging activity of peptide WYSLAM. Moreover, deletion of the Met residue (WYSLA) resulted in an important decrease in radical-scavenging activity (ORAC value = 4.59 μmol Trolox/μmol of peptide) that was similar to that shown by peptides WYSL and WYS (4.51 and 4.45 μmol Trolox/μmol of peptide, respectively) (**Figure 1**). However, deletion of the Ser residue at the C-terminal (WY) resulted in an important increase in radical-scavenging activity. The ORAC value of this dipeptide was the highest determined in this study (7.67 μmol Trolox/μmol of peptide). Moreover, this peptide showed an important ACE-inhibitory activity.

The radical-scavenging activity of all the β-Lg-derived peptides analyzed was between 1.8 and 3.2-fold higher than that of BHA (2.43 μmol Trolox/μmol), measured under the same conditions (*22*). BHA is currently used in the food industry as a synthetic antioxidant, although its potential adverse effects have resulted in this being replaced by new natural antioxidants. Compared to known natural antioxidants of plant origin, the radical-scavenging activity of these β-Lg-derived peptides was similar to that determined for *p*-coumaric acid (4.51 μmol Trolox/μmol of pure compound), chlorogenic acid (5.70 μmol Trolox/μmol of pure compound), and caffeic acid (6.63 μmol Trolox/μmol of pure compound) (*22*). These results suggest that

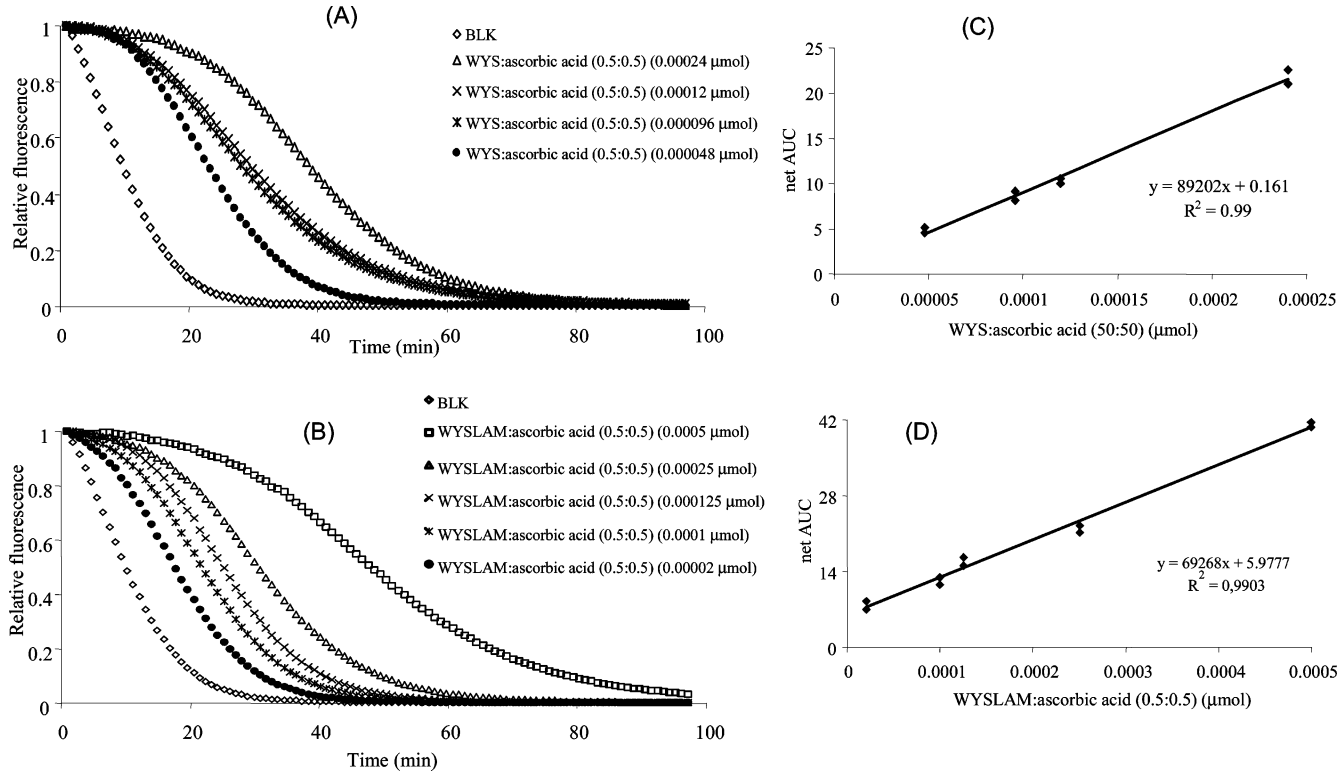

Figure 2. Time course of the reaction of fluorescein with AAPH in the absence (BLK) and in the presence of the mixtures (**A**) WYS:ascorbic acid (0.5:0.5, mol/mol) and (**C**) WYSLAM:ascorbic acid (0.5:0.5, mol/mol). Linear regression of the net area under the curve (net AUC) vs concentration of the mixtures (**B**) WYS:ascorbic acid (0.5:0.5, mol/mol) and (**D**) WYSLAM:ascorbic acid (0.5:0.5, mol/mol) in the assay.

Table 2. Positive/Negative Antioxidant Effects between the Synthetic β-Lg-Derived Peptides and Ascorbic Acid at Different Molar Ratios

		peptide:ascorbic acid						
peptide		0:1	0.2:0.8	0.33:0.66	0.5:0.5	0.66:0.33	0.8:0.2	1:0
WY	ORAC obs[a]	0.781	1.44	2.02	2.36	3.94	4.71	7.67
	ORAC calcd[b]		2.16	3.05	4.23	5.32	6.30	
	% variation		–34	–34	–44	–26	–25	
WYS	ORAC obs	0.781	2.28	2.59	3.32	4.86	5.38	4.45
	ORAC calcd		1.51	1.98	2.62	3.19	3.71	
	% variation		51	30	27	52	45	
WYSL	ORAC obs	0.781	2.08	2.44	3.38	3.56	4.52	4.52
	ORAC calcd		1.53	2.01	2.65	3.24	3.77	
	% variation		36	22	28	10	20	
WYSLA	ORAC obs	0.781	2.31	2.69	2.99	5.31	4.86	4.59
	ORAC calcd		1.54	2.03	2.68	3.28	3.82	
	% variation		50	32	11	62	27	
WYSLAM	ORAC obs	0.781	1.59	2.27	2.25	3.90	4.33	7.00
	ORAC calcd		2.02	2.82	3.89	4.88	5.76	
	% variation		–22	–20	–42	–20	–25	

[a] Observed ORAC value (expressed as μmol Trolox equivs/μmol of peptide). [b] Calculated ORAC value (expressed as μmol Trolox equivs/μmol of peptide).

peptides derived from β-Lg could be used as natural antioxidants to enhance antioxidant properties of functional foods and to prevent oxidation reactions in food processing. The antioxidant activity of these β-Lg-derived peptides indicates that they have potential to enhance product stability by preventing oxidative deterioration. A feasible application of these β-Lg-derived peptides is their addition to muscle foods, since they are subjected to rapid oxidative reactions (*26*) or to food lipid dispersions (*27*). Moreover, if any of these peptides could survive gastrointestinal digestion and it could be absorbed, they might also have physiological implications. Sufficient evidence about the absorption of peptides exists (*28*), and therefore the study of the bioavailability of these peptides in model animals merits further investigation. The extra nutritional and physiological values of the peptides, as well as the low-cost of the cheese whey as a source of bioactive peptides, correspond to additional advantages over natural antioxidants of plant origin.

Positive/Negative Antioxidant Effects of Peptides and Ascorbic Acid. Some nonpeptidic natural and synthetic antioxidants, such as tocopherol, BHT, and BHA, have been demonstrated to enhance the antioxidant activities of protein hydrolysates and peptides (*11*, *29*, *30*). The possible positive/negative antioxidant effects between the synthetic β-Lg-derived peptides studied in this paper and ascorbic acid (vitamin C) at different molar ratios were determined. As an example, **Figure 2A** and **B** depicts the kinetics of the fluorescein/AAPH system in the absence and in the presence of different concentrations of the 0.5:0.5 (mol/mol) mixtures of WYS and WYSLAM with

ascorbic acid, respectively. These mixtures neutralize the peroxyl radicals generated in the system and delay the decay in fluorescence in comparison to the blank (BLK). For all the mixtures tested, the concentration interval, leading to a linear relationship between the net area under the curve (net AUC) and the antioxidant concentration, was determined. As an example, **Figure 2C** and **2D** shows the concentration (μmol in the assay) ranges that ensured linearity between the net AUC and antioxidant concentration for the WYS:ascorbic acid (0.5:0.5, mol/mol) mixture (0.000048−0.00024 μmol in the assay) and WYSLAM:ascorbic acid (0.5:0.5, mol/mol) (0.00002−0.0005 μmol in the assay), respectively. For Trolox, the concentration range was established from 0.0002 to 0.0016 μmol in the assay (figure not shown). To determine the ORAC values of the peptide:ascorbic acid mixtures, the slope of the net AUC versus concentration curve was divided by the slope of the Trolox calibration curve. This observed ORAC value was compared with the calculated ORAC value, and the % variation was determined as indicated in Materials and Methods.

Different behaviors in the total antioxidant activity among the different β-Lg-derived peptides studied were observed when they were mixed with ascorbic acid (**Table 2**). For WYS, WYSL, and WYSLA, the observed ORAC value was higher than the calculated ORAC value for all the different peptide:ascorbic acid mixtures tested, which indicated positive effects between both types of antioxidants. The percentage increase with respect to the calculated ORAC value varied from 27.1 to 52.3 for WYS, from 10.0 to 36.2 for WYSL, and from 11.4 to 61.7 for WYSLA. On the contrary, for WY and WYSLAM, the observed ORAC value was lower than the calculated ORAC value for all the different peptide:ascorbic acid mixtures tested, suggesting negative effects between the antioxidants. The percentage decrease with respect to the calculated ORAC value was 25.2−44.1 for WY and 19.8−42.1 for WYSLAM, and the 0.5:0.5 mol/mol mixture showed the greatest effect.

Ascorbic acid acts as an antioxidant by different mechanisms: quenching of various forms of oxygen, reduction of free radicals, and reduction of primary antioxidant radicals (*31*). In the case of the WYS:ascorbic acid, WYSL:ascorbic acid, and WYSLA:ascorbic acid mixtures, the peptide would act as primary antioxidant (hydrogen donor) and the resulting peptidic radical would then react with ascorbic acid to be regenerated, and the ascorbic acid would be oxidized to dehydroascorbic acid. Positive interactions between other antioxidants (i.e., α-tocopherol) and ascorbic acid have been reported (*32*). However, in the case of WY and WYSLAM, the peptides that showed the highest antioxidant capacity (ORAC $\geq$ 7 μmol Trolox/μmol of peptide), other types of interactions may take place between the peptide and ascorbic acid, probably through the C-terminal amino acids Tyr (WY) and Met (WYSLAM).

Conclusions. ACE-inhibitory and radical-scavenging activities have been confirmed in the β-Lg derived peptides studied, and their magnitude is associated with the peptide sequence. Interactions of the β-Lg-derived peptides with ascorbic acid can enhance/limit their antioxidant efficiency depending on the peptide structure. This should be taken into account in the development of new food ingredients on the basis of peptides from β-Lg. Currently, further research studies are being carried out to determine the mechanism of action as well as the in vivo antioxidant properties of these β-Lg-derived peptides.

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