Preparation of Antioxidant Enzymatic Hydrolysates from α-Lactalbumin and β-Lactoglobulin. Identification of Active Peptides by HPLC-MS/MS

BLANCA HERNÁNDEZ-LEDESMA, ALBERTO DÁVALOS, BEGOÑA BARTOLOMÉ, AND
LOURDES AMIGO*

Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

We have investigated the antioxidant activity of hydrolysates from whey proteins bovine α-lactalbumin (α-La) and β-lactoglobulin A (β-Lg A) by commercial proteases (pepsin, trypsin, chymotrypsin, thermolysin, and Corolase PP). Corolase PP was the most appropriate enzyme to obtain antioxidant hydrolysates from α-La and β-Lg A (ORAC−FL values of 2.315 and 2.151 μmol of Trolox equivalent/ mg of protein, respectively). A total of 42 peptide fragments were identified by HPLC-MS/MS in the β-Lg A hydrolysate by Corolase PP. One of the sequences (Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile) possessed radical scavenging (ORAC−FL value of 2.621 μmol of Trolox equivalent/μmol of peptide) higher than that of butylated hydroxyanisole (BHA). Our results suggest that whey protein hydrolysates could be suitable as natural ingredients in enhancing antioxidant properties of functional foods and in preventing oxidation reaction in food processing.

INTRODUCTION

Reactive oxygen species (ROS) have been implicated in the etiology of various degenerative diseases, including cardiovascular disease, cancer, diabetes, cataracts, neurodegenerative disorders, and aging (*1*, *2*). The body has its own defense system against ROS on the basis of antioxidant enzymes (i.e., SOD, CAT, and GPx) and endogenous antioxidants (i.e., glutathione). Oxidative stress occurs when ROS overload the body's antioxidant defenses or when the antioxidant defense system loses its capacity for response (e.g., elderly people) and can lead to damage of vital cellular components. Enhancement of the body's antioxidant defenses through dietary supplementation would seem to provide a reasonable and practical approach to reducing the level of oxidative stress, and there is a wealth of evidence to support the effectiveness of such a strategy in vitro (*3*). This hypothesis has stimulated human intervention studies concerning well-known dietary antioxidants (i.e., vitamins E and C) and other less-known compounds with potential health-promoting effects (i.e., carotenoids and polyphenols), as well as research into new food ingredients with antioxidant potential (*4*).

Several studies have described the antioxidant activity of proteins from several animal and plant sources, such as milk proteins (*5*, *6*), maize zein (*7*), and wheat gliadin (*8*) and secretory peptide hormones (*9*). Amino acids have also been accepted to exhibit antioxidant activity, which is greater when they are incorporated in dipeptides (*10*). Thus, the antioxidant activity of peptides generated from the hydrolysis of various proteins, such as egg-white albumin (*11*), lecithin-free yolk protein (*12*), soy protein (*13*), soluble elastin (*14*), and milk casein (*15*) has been reported. Recent studies have described the antioxidant activity of whey protein hydrolysates (*16*, *17*). However, there are no data about the antioxidant properties of the individual peptides released after whey protein hydrolysis.

Cheese whey is an abundant liquid byproduct of the dairy industry. β-Lactoglobulin (β-Lg) and α-lactalbumin (α-La) are the main whey proteins, corresponding to 60% and 25% of them, respectively. The high nutritional, functional, and biological value reported for its proteins has resulted in their use in different foods, drugs, and cosmetics. Recently, new biological activities of whey proteins and peptides derived from them have been described, such as the angiotensin-converting enzyme inhibitory, antithrombotic, and antimicrobial activity (*18*−*20*).

A wide range of both in vivo and in vitro methods are currently used to assess the antioxidant activity of a compound/ mixture, all of which have certain advantages and limitations (*21*). The oxygen radical absorbance capacity (ORAC) method measures the scavenging activity of a compound against peroxyl radicals and is one of the few methods that combines both inhibition percentage and inhibition time of the antioxidant activity of the reactive species into a single quantity (*22*). Recently, this method has been improved by the use of fluorescein (ORAC−FL) as fluorescent probe (*23*) and has been adapted to a conventional fluorescence plate reader (*24*).

In this paper, we have investigated the antioxidant properties of peptides obtained from whey proteins by enzymatic hydrolysis, with the final aim of using these as antioxidant ingredients. For this purpose, pure whey proteins (bovine α-La and β-Lg

* Corresponding author. Tel.: +34 91 5622900; fax: +34 91 5644853; e-mail: amigo@ifi.csic.es.

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A) were hydrolyzed by different proteases of digestive and microbial origin, and the radical scavenging activity of the hydrolysates was determined by the ORAC−FL approach. Furthermore, different peptide fractions were isolated from a hydrolysate of β-Lg A by Corolase PP, their main peptides identified by tandem mass spectrometry (HPLC-MS/MS), and the subsequent synthetic peptide sequences evaluated for antioxidant activity.

MATERIALS AND METHODS

Chemicals. Bovine α-La and β-Lg A, and fluorescein disodium (FL), and amino acid standards [Val (V), Ile (I), Asp (D), Thr (T), Leu (L), Glu (E), Ala (A), Met (M), Tyr (Y), His (H), Phe (F), Trp (W), Cys (C), Asn (N), Lys (K), Gly (G), Gln (Q), and Pro (P)] were purchased from Sigma Chemical (St. Louis, MO). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azobis (2-methylpropionamide)-dihydrochloride (AAPH), and amino acid standard Arg (R) were obtained from Aldrich (Milwaukee, WI). Amino acid standard Ser (S) was purchased from Merck KGaA (Darmstadt, Germany).

Pepsin (EC 3.4.4.1.; 1:60 000, 3400 U/mg protein), trypsin (EC 3.4.21.4.; Type I; 10 900 U/mg protein), and chymotrypsin (EC 3.4.21.1.; Type I−S, 44 U/mg protein) from bovine pancreas and thermolysin (EC 3.4.24.4.; 72 U/mg protein) from *Bacillus thermoproteolyticus rokko* were purchased from Sigma. Corolase PP from pig pancreas glands was purchased from Röhm (Darmstadt, Germany).

Enzymatic Hydrolysis of α-La and β-Lg A. Hydrolysis of α-La and β-Lg A was carried out in citrate buffer (pH 2.5) for pepsin and in Tris-HCl buffer (pH 8.0) for the pancreatic proteases and thermolysin. α-La and β-Lg A (1 mg/mL) were incubated with the enzyme (1:20, enzyme-to-substrate ratio, w/w) for 24 h at 37 °C. After incubation, enzymatic reactions were stopped by heating at 95 °C for 15 min, except for thermolysin that was stopped by addition of EDTA (20 mM, final concentration). Hydrolysates by pepsin were adjusted to pH 7.5 with 1 M NaOH.

The hydrolysates were centrifuged at $10000g$ for 30 min, the supernatants were removed, and an aliquot was frozen and kept at −20 °C until use. Another aliquot of the hydrolysates was subjected to ultrafiltration through a hydrophilic 3000 Da cutoff membrane (Centripep, Amicon, Inc., Beverly, MA). The 3 kDa-permeates were freeze-dried and kept at −20 °C until use.

Freeze-dried 3 KkDaDa-permeates were reconstituted with distilled water. The protein content of the hydrolysates and of the permeates was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL) using bovine serum albumin as standard protein.

ORAC Assay. The ORAC-fluorescein (ORAC−FL) assay was based on that proposed by Ou et al. (*23*) and modified by Dávalos et al. (*24*). 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 FL (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 80 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 FL 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

$$AUC = 1 + \sum_{i=1}^{i=80} f_i/f_0$$

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:

$$net\ AUC = AUC_{antioxidant} - AUC_{blank}$$

The regression equation between net AUC and antioxidant concentration was calculated. The slope of the equation was used to calculate the ORAC−FL value by using the Trolox curve obtained for each assay. Final ORAC−FL values were expressed as μmol of Trolox equivalent/mg of protein for hydrolysates and permeates, and as μmol of Trolox equivalent/μmol of compound for synthetic amino acids and peptides.

Fractionation of Antioxidant Hydrolysates by RP-HPLC. Semipreparative RP-HPLC analysis was carried out on a Waters System HPLC (Waters Corp., Milford, MA), equipped with two pumps (module Delta 600), a pump controller (module 600), an autosampler (module 717), and a diode array detector (module 996) in combination with an automatic fractions collector (module II). The data processing software was Program Millenium, version 32 (Waters).

The 3 kDa-permeate from the β-Lg A hydrolysate by Corolase PP was applied to a column Prep Nova-Pack HR C_{18}, 60 Å, 4 μm, 7.8 × 300 mm (Waters) with a C_{18} cartridge as guard column. Solvent A was a mixture of water and trifluoroacetic acid (TFA) (1000:1, v/v), and solvent B contained acetonitrile and TFA (1000:0.8, v/v). The injection volume was 250 μL. The peptides were eluted with a linear gradient of solvent B in A going from 0% to 20% over 60 min, at a flow rate of 4 mL/min. Detection was carried out at 214 and 280 nm. Five fractions (corresponding to 10 min each one) were collected from 35 separate RP-HPLC runs, pooled, dried under vacuum, and redissolved in distilled water. Protein concentration and antioxidant activity were determined for each fraction.

Analysis by Online RP-HPLC-MS/MS. RP-HPLC separation of the five fractions (F1−F5) obtained from the 3 kDa-permeate of β-Lg A hydrolysate by Corolase PP was performed on an Agilent HPLC system connected online to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany), according to the method of Hernández-Ledesma et al. (*25*). The flow rate was 0.8 mL/min. Peptides of fractions F1 and F2 were eluted with a linear gradient of solvent B in A going from 0 to 8% over 60 min. Peptides of fractions F3 and F4 were eluted with a linear gradient from 0 to 5% of solvent B in solvent A over 5 min followed by a 65-min gradient of solvent B in A from 5 to 15%. Peptides of fraction F5 were eluted with a linear gradient from 0 to 10% of solvent B in solvent A over 5 min followed by a 65-min gradient of solvent B in A from 10 to 20%.

The flow was split postdetector by placing a T-piece (Valco, Houston, TX) connected to a 75-μm i.d. peek outlet tube of an adjusted length to give approximately 20 μL/min of flow entering directly into the mass spectrometer via the electrospray interface. Nitrogen was used as nebulizing and drying gas and operated with an estimated helium pressure of 5×10^{-3} bar. The capillary was held at 4 kV. Spectra were recorded over the mass/charge (m/z) range 100−1500. About 25 spectra were averaged in the MS analyses and about 5 spectra in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 5000 (i.e., 5% of the total signal) and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp going from 0.35 to 1.4 V. Using Data Analysis (version 3.0; Bruker Daltoniks), the m/z spectral data were processed and transformed to spectra representing mass values. BioTools (version 2.1; Bruker Daltoniks) was used to process the MS(n) spectra and to perform peptide sequencing.

Peptide Synthesis. Peptides were prepared by the conventional Fmoc solid-phase synthesis method with a 431A peptide synthesizer (Applied Biosystem Inc., Überlingen, Germany), and their purity was verified by analytical RP-HPLC-MS/MS.

RESULTS AND DISCUSSION

Radical Scavenging Activity of Enzymatic Hydrolysates. **Table 1** reports the protein concentration and radical scavenging activity (ORAC−FL values) for the hydrolysates and their corresponding 3 kDa-permeates obtained from α-La and β-Lg A by enzymatic digestion with pepsin, trypsin, chymotrypsin,

Table 1. Protein Concentration and Radical Scavenging Activity (ORAC–FL Values) of the Hydrolysates and Their Corresponding Permeates (*F* < 3 kDa) from α-Lactalbumin and β-Lactoglobulin by Pepsin, Trypsin, Chymotrypsin, Corolase PP, and Thermolysin

	α-lacta lbumin				β-lactoglobulin			
	protein concn[a] (mg/mL)		ORAC–FL value (μmol of Trolox equiv/mg of protein)		protein concn[a] (mg/mL)		ORAC–FL value (μmol of Trolox equiv/mg of protein)	
enzyme	hydrolysate	*F* < 3 kDa	hydrolysate	*F* < 3 kDa	hydrolysate	*F* < 3 kDa	hydrolysate	*F* < 3 kDa
pepsin	0.911 ± 0.010	0.466 ± 0.001	1.065 ± 0.056	0.790 ± 0.017	0.627 ± 0.006	0.245 ± 0.001	0.701 ± 0.033	0.821 ± 0.007
trypsin	0.932 ± 0.038	0.415 ± 0.001	1.031 ± 0.046	0.942 ± 0.011	0.771 ± 0.015	0.472 ± 0.014	0.979 ± 0.022	0.667 ± 0.012
chymotrypsin	0.645 ± 0.007	0.653 ± 0.012	2.528 ± 0.078	1.755 ± 0.051	0.842 ± 0.003	0.571 ± 0.011	1.378 ± 0.032	1.508 ± 0.039
Corolase PP	0.546 ± 0.049	0.496 ± 0.012	2.954 ± 0.106	2.315 ± 0.080	0.423 ± 0.042	0.408 ± 0.024	2.151 ± 0.051	1.897 ± 0.019
thermolysin	0.539 ± 0.016	0.487 ± 0.016	2.039 ± 0.018	1.365 ± 0.030	0.620 ± 0.028	0.446 ± 0.038	1.657 ± 0.038	1.519 ± 0.005

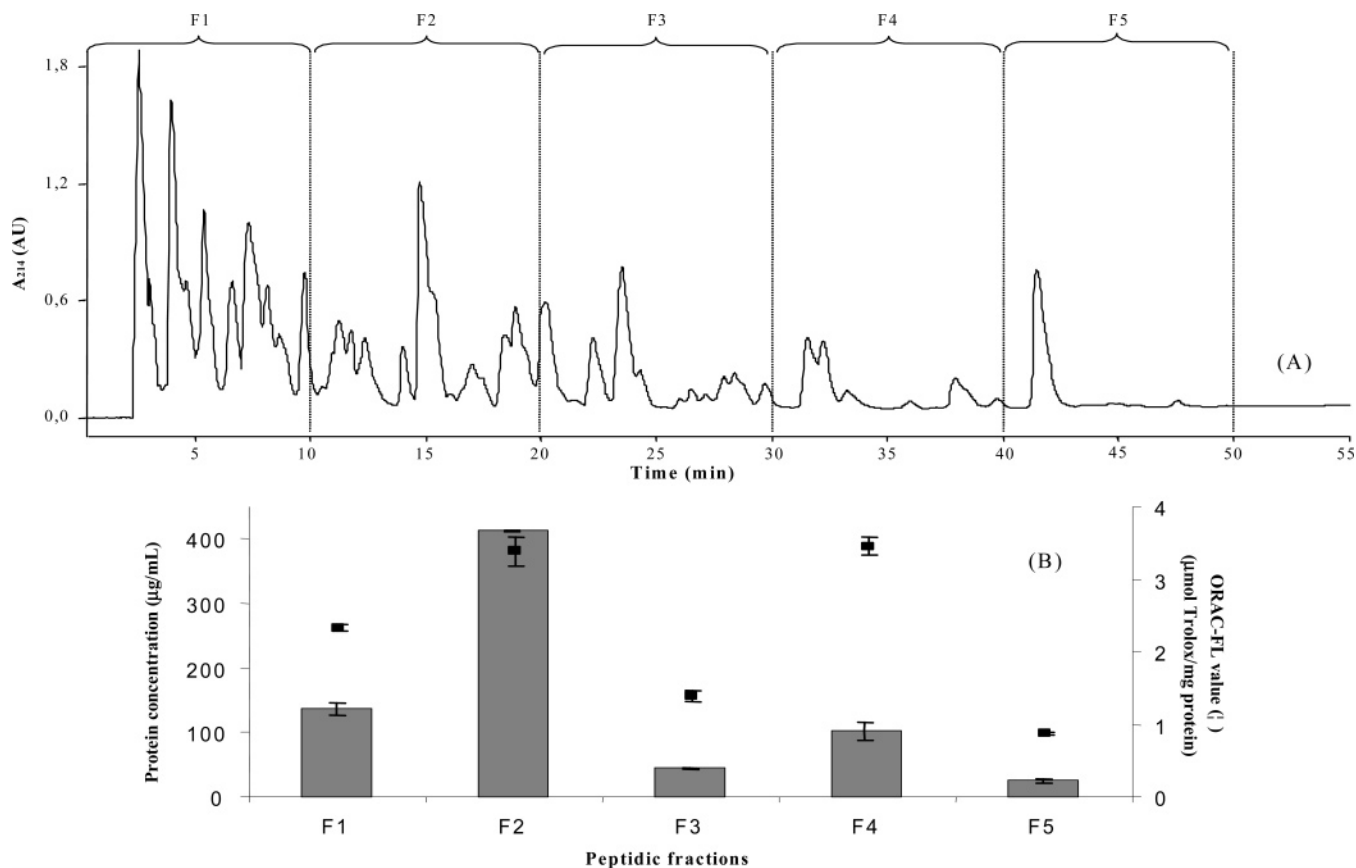[a] Results are presented as the mean (*n* = 3) ± SD.

Figure 1. (**A**) Fractionation by preparative RP-HPLC of the 3 kDa-permeate obtained from the β-lactoglobulin A hydrolysate by Corolase PP. Collected fractions are termed with F followed by a number. (**B**) Protein concentration (bars) and ORAC–FL values (■) of the collected fractions from the preparative RP-HPLC system. Vertical bars represent standard errors (*n* = 3).

Corolase PP, and thermolysin. Corolase PP and thermolysin acting on α-La and Corolase PP acting on β-Lg A conducted at the lowest protein concentration in the hydrolysates. This fact was due to the greatest degradation of the whey proteins. Corolase PP is a complex mixture of enzymes that acts synergistically on whey proteins resulting in extensive degradation. Low enzymatic specificity of thermolysin would explain its action on whey proteins. For these two proteases, the protein concentration of the 3 kDa-permeates was slightly lower than that corresponding to the hydrolysates (**Table 1**), indicating that the peptides from whey proteins released by Corolase PP and thermolysin were, mainly, of small size. A previous analysis of the peptides released from bovine β-Lg AB after hydrolysis with thermolysin revealed that peptide size was between 390 and 855 kDa (*18*).

The ORAC−FL values for the hydrolysates obtained from α-La and β-Lg A varied from 0.667 to 2.954 μmol Trolox

equivalent/mg of protein (**Table 1**). These values were higher than that obtained for a hydrolysate from crude egg protein by pepsin (0.381 μmol Trolox equivalent/mg of protein) (*26*). The hydrolysates obtained from α-La by Corolase PP and by chymotrypsin (2.954 and 2.528 μmol Trolox equivalent/mg of protein, respectively) and that from β-Lg A by Corolase PP (2.151 μmol Trolox equivalent/mg of protein) showed the highest antioxidant activity. Differences in the radical scavenging activity among hydrolysates were attributed to differences in the size and amino acid sequence of the peptides released by the proteases, which exhibited different specific activity on whey proteins. Chen et al. also found differences in the antioxidant activity of hydrolysates from soy β-conglycinin depending on the protease used (*13*). The range of variation of the ORAC−FL values for the 3 kDa-permeates (from 0.667 to 2.315 μmol Trolox equivalent/mg of protein) was close to that reported above for the corresponding hydrolysates, which indicated that
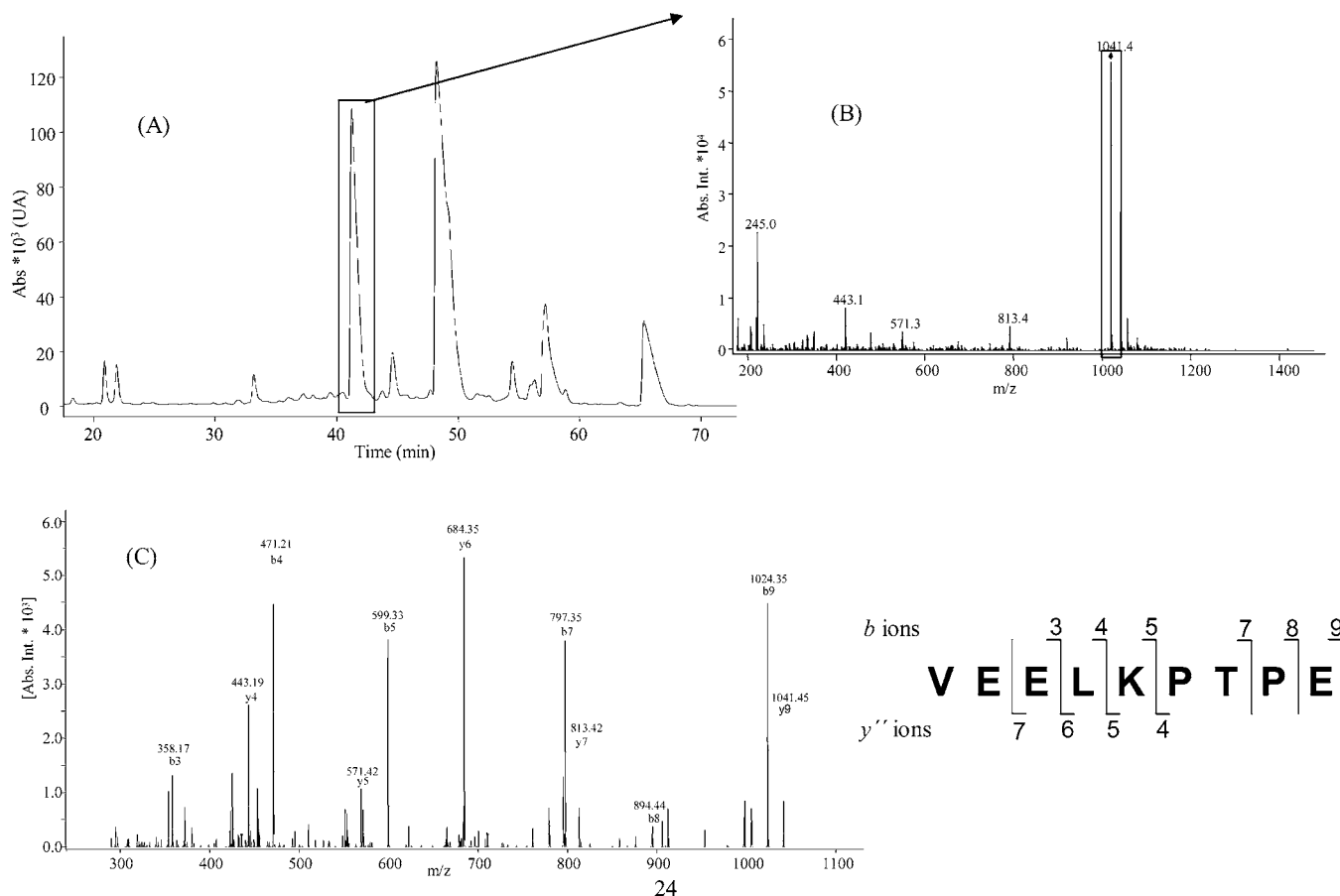
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Figure 2. (**A**) UV-chromatogram of the fraction F4 of the 3 kDa-permeate obtained from the β-lactoglobulin A hydrolysate by Corolase PP. (**B**) Mass spectrum of the selected chromatographic peak in (**A**). (**C**) Tandem mass spectrum of ion *m/z* 1041.4. Following sequence interpretation and database searching, the MS/MS spectrum was matched to β-lactoglobulin A f(43–51). The sequence of this peptide is displayed with the fragment ions observed in the spectrum. Fragment ions are labeled according to the nomenclature proposed by Roepstorff and Fohlman (*28*). For clarity, only the *b* and the *y''* fragment ions are labeled.

the 3 kDa-fraction was mainly responsible for the antioxidant activity found in the whole hydrolysate (**Table 1**). For instance, it was calculated that the antioxidant activity (μmol Trolox equivalent/mL of solution) of the 3 kDa-permeates obtained from α-La and β-Lg A by Corolase PP represented 71% and 85% of the total activity of their corresponding hydrolysates. A previous study on egg white proteins also revealed that the 3 kDa-permeate retained most of the antioxidant activity found in the total hydrolysate by pepsin (*26*). Other authors have reported that accessibility to the oxidant–antioxidant test systems is greater for small peptides and amino acids than for large peptides and proteins (*9*). Short peptides exhibiting antioxidant activity have been isolated and characterized from casein (*15*) and protein sources such as soy protein (*13*).

Although the antioxidant activity of the 3 kDa-permeate from α-La by Corolase PP was slightly higher than that from β-Lg A, we selected the latter as material for further peptide identification studies. β-Lg is the major whey protein, so identification of potential active peptides derived from this dairy industry byproduct should initially concentrate on those derived from β-Lg.

Purification and Characterization of Antioxidant Peptides. The 3 kDa-permeate from the β-Lg A hydrolysate by Corolase PP was subjected to preparative RP-HPLC (**Figure 1A**). The total chromatogram was divided into five different fractions (F1–F5). Enough material of each fraction was collected in successive analysis to determine radical scavenging activity and protein concentration (**Figure 1B**). Antioxidant activity of F1,

F2, and F4 (ORAC–FL values of 2.327, 3.384, and 3.449 μmol Trolox equivalent/mg of protein, respectively) (**Figure 1B**) was higher than that of the 3 kDa-permeate (1.897 μmol Trolox equivalent/mg of protein). Antioxidant activity of the rest of the fractions (F3 and F5) was lower, with ORAC–FL values of 1.389 and 0.875 μmol Trolox equivs/mg of protein, respectively (**Figure 1B**). Identification of the possible fragments responsible for the antioxidant activity was carried out for the different fractions.

For peptide identification, the five fractions were subjected to RP-HPLC coupled online to a mass spectrometer. As an example, **Figure 2A** shows the UV-chromatogram obtained for F4. The mass spectrum of one selected peak is shown in **Figure 2B** and the MS/MS spectrum of a single charged ion with *m/z* 1041.4 and the amino acid sequence of the identified peptide with the major fragment ions is shown in **Figure 2C**. Following sequence interpretation and database searching, the MS/MS spectrum was matched to β-Lg A f(43−51). All peptides of the total ion chromatogram with a signal >5000 units were considered for peptide sequencing. The strategy of identifying the peptides by matching the tandem mass spectra to selected peptides with a given mass allowed the unambiguous identification of most peptides included in these fractions. In this process, certain mass signals and their corresponding fragmentation spectra obtained by MS/MS could not be matched with any single peptide fragment, such as for disulfide-containing peptides. A total of 42 peptide fragments derived from β-Lg A were identified: 10 peptides in F1, 15 in F2, 8 in F3, 4 in F4, and 5

Table 2. Peptides Identified by MS/MS in Fractions Collected by Preparative RP-HPLC of the 3 kDa-Permeate of the Hydrolysate from β-Lactoglobulin A by Corolase PP

fraction	obsd mass	calcd mass	fragment	sequence
F1	273.1	273.13	f(36–38)	SAP
	636.2	635.34	f(148–152)	RLSFN
	268.1	268.11	f(20–21)	YS
	346.2	346.19	f(15–18)	VAGT
	420.1	420.19	f(27–30)	SDIS
	442.1	443.31	f(70–73)	KIIA
	553.3	552.29	f(98–101)	DYKK
	768.4	768.37	f(96–101)	DTDYKK
	432.2	432.19	f(52–55)	GDLE
	454.2	454.19	f(19–21)	WYS
F2	570.3	570.30	f(47–51)	KPTPE
	681.3	680.37	f(142–147)	ALPMHI
	698.4	699.39	f(123–128)	VRTPEV
	609.3	609.33	f(143–147)	LPMHI
	713.3	713.40	f(43–48)	VEELKP
	445.3	445.25	f(83–86)	KIDA
	645.3	645.30	f(155–159)	QLEEQ
	667.3	668.38	f(145–149)	MHIRL
	559.8	560.33	f(79–83)	PAVFK
	927.3	927.54	f(87–94)	LNENKVLV
	913.2	914.60	f(77–84)	KIPAVFKI
	572.4	572.35	f(71–75)	IIAEK
	658.2	658.28	f(50–55)	PEGDLE
	444.2	444.26	f(71–74)	IIAE
	650.3	651.31	f(42–46)	YVEEL
F3	1050.2	1049.54	f(131–139)	EALEKFDKA
	803.3	803.32	f(125–131)	TPEVDDE
	550.4	550.35	f(100–103)	KKYL
	528.4	528.33	f(76–80)	TKIPA
	874.2	874.36	f(125–132)	TPEVDDEA
	659.3	659.28	f(110–115)	SAEPEQ
	814.4	814.44	f(43–49)	VEELKPT
	984.3	984.48	f(47–55)	KPTPEGDLE
F4	468.2	468.20	f(18–20)	TWY
	1062.4	1062.54	f(82–90)	FKIDALNEN
	1040.4	1040.54	f(43–51)	VEELKPTPE
	673.3	674.29	f(85–90)	DALNEN
F5	1225.4	1226.56	f(19–29)	WYSLAMAASDI
	805.5	804.44	f(1–7)	LIVTQTM
	677.3	677.34	f(149–154)	LSFNPT
	699.4	699.46	f(67–72)	AQKKII
	772.4	771.44	f(52–58)	GDLEILL

in F5 (**Table 2**). The fragments contained between 2 and 11 residues and some of them were further synthesized and tested for their antioxidant activity.

Radical Scavenging Activity of Amino Acids and Peptide Fragments. To synthesize the most potentially active peptide fragments reported above, the ORAC–FL of their constitutive amino acids were determined. Trp (ORAC–FL value of 4.649 μmol Trolox/μmol of amino acid), Tyr, and Met (1.574 μmol Trolox/μmol of amino acid and 1.126 μmol Trolox/μmol amino acid, respectively) showed the highest antioxidant activity (*26*), followed by Cys (0.1492 μmol Trolox/μmol of amino acid), His (0.073 μmol Trolox/μmol of amino acid; 26), and Phe (0.0025 μmol Trolox/μmol of amino acid). The rest of the amino acids analyzed (Arg, Asn, Gln, Asp, Pro, Ala, Val, Lys, Ile, Thr, Leu, Glu, and Gly) did not exhibit antioxidant activity by this method (ORAC–FL value < 0.00001 μmol Trolox/μmol of amino acid). The high antioxidant activity of Trp and Tyr may be explained by the capacity of the indolic and phenolic groups, respectively, to serve as hydrogen donors. It is, therefore, likely that the oxygen radical quenches the Trp-indolic and Tyr-phenolic hydrogen (H$^+$), resulting in the formation of more stable indoyl and phenoxyl radicals. Met is prone to oxidation to Met sulfoxide and Cys donates the sulfur hydrogen. There-

fore, the radical scavenging activity found in the 3 kDa-permeate obtained from β-Lg A hydrolysate by Corolase PP was attributed to the presence of Trp, Tyr, Met, and His in the identified peptides, but not to Cys.

On the basis of their amino acid composition, three sequences were selected as the most potentially active peptides: Met-His-Ile-Arg-Leu (from F2), Tyr-Val-Glu-Glu-Leu (from F2), and Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile (from F5). They were synthesized and their radical scavenging activity determined. A high antioxidant activity was found for Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile (ORAC–FL value of 2.621 μmol Trolox/μmol of peptide). Compared to known antioxidants of plant origin, this radical scavenging activity was approximately 1.7- and 4-fold lower than those of *p*-coumaric acid and the flavonoid quercetin, respectively, measured under the same conditions (*24*). However, the antioxidant activity of this peptide was slightly higher than that of butylated hydroxyanisole (BHA) (2.43 μmol Trolox/μmol BHA) (*24*). BHA is currently used in the food industry as a synthetic antioxidant, although its potential adverse effects have stimulated its replacement by new natural antioxidants. These results suggest that hydrolysates from whey proteins could be used as natural antioxidants in enhancing antioxidant properties of functional foods and in preventing oxidation reaction in food processing. Protein hydrolysates can be obtained from a low-cost source (i.e., cheese whey). Besides, they have an extra nutritional value in comparison to other antioxidants from plants, although studies about their antigenicity should be done before any proposal for human use (*27*). The antioxidant activity of Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile would be due to the residues of Trp, Tyr, and Met, although the ORAC–FL value of the peptide was lower than that of Trp (see above). To explain this result, the radical scavenging activity of an equimolar amino acid mixture [Trp, Tyr, (Ser × 2), Leu, (Ala × 3), Met, Asp, and Ile] was determined. The ORAC–FL value obtained (4.311 μmol Trolox/μmol of amino acid mixture) indicated that the peptidic bond or structural peptide conformation attenuated the antioxidant activity of the constitutive amino acids. Besides, the presence of other amino acids may lead to antagonist effects among them, reducing their own radical scavenging activity.

Met-His-Ile-Arg-Leu and Tyr-Val-Glu-Glu-Leu also showed radical scavenging activity (ORAC–FL values of 0.306 μmol Trolox/μmol of peptide and 0.799 μmol Trolox/μmol of peptide, respectively), which was attributed to the respective presence of Met and Tyr, although the antioxidant activity of these amino acids on their own was higher (see above). Contrary to previous findings for Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile, the ORAC–FL value of the equimolar mixture [Try+Val+(Glu × 2)+Leu] (0.427 μmol Trolox/μmol of amino acid mixture) was approximately 2-fold lower than that of Tyr-Val-Glu-Glu-Leu, indicating in this case that the peptidic bond or structural peptide conformation enhanced the antioxidant activity of the constitutive amino acids. As seen for Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile, the presence of other amino acids may lead to antagonist effects among them, reducing their own radical scavenging activity.

In conclusion, this paper reports for the first time the antioxidant properties of peptides obtained from whey proteins by enzymatic hydrolysis. Corolase PP is the most appropriate enzyme to obtain antioxidant hydrolysates from α-La and β-Lg A. Identification of 42 peptides obtained from the hydrolysate of β-Lg A with Corolase PP was successfully carried out by HPLC-MS/MS. Of special interest is the peptide Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile which has a stronger anti-

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oxidant activity than BHA. Present results also suggest that peptide conformation can lead to both synergistic and antagonistic effects in comparison to the antioxidant activity of the amino acids on their own. Further research about the structure/ activity relationship in peptides and about the synergistic and antagonistic effects among the amino acids will be carried out in our laboratory.

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