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ARTICLE

Identification of Novel Antioxidative Peptides Derived from a Thermolytic Hydrolysate of Ovotransferrin by LC-MS/MS

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Ovotransferrin is a glycoprotein well-known for its iron-binding property. Ovotransferrin was reported to have antioxidative properties, but the presence of antioxidant peptides within the protein has not been reported. The purpose of the study was to characterize the antioxidant peptides within ovotransferrin. Ovotransferrin was sonicated and hydrolyzed by thermolysin, and peptides from the hydrolysate were fractionated by ion-exchange fast protein liquid chromatography and reversed-phase high-performance liquid chromatography. Fourteen peptides derived from ovotransferrin were characterized using LC-MS/MS, and their oxygen radical absorbance capacity (ORAC) values were determined using synthetic peptides. Two tetrapeptides (Trp-Asn-Ile-Pro and Gly-Trp-Asn-Ile) showed the highest antioxidant activity. Interestingly, the addition of amino acid residues to either the N or C terminus of the two peptides decreased the antioxidant activity, suggesting that the motif of Trp-Asn-Ile is responsible for the high antioxidant activity.

KEYWORDS: Ovotransferrin; antioxidant peptides; LC-MS/MS; thermolysin

INTRODUCTION

Oxidation of biomolecules such as proteins and lipids has been identified as a free radical mediated process, which exerts deleterious impacts on food and biological systems (*1*). The use of antioxidants is one of the effective methods of prevention against oxidation. In addition to being antioxidant enzymes, proteins can launch multipronged approaches to prevent oxidation, including scavenging of reactive oxygen species and free radicals, inactivation of reactive intermediaries, chelation of prooxidative transition metals, and alteration of physical properties of food systems (*1*). The overall antioxidant activity of a protein could be further enhanced by disruption of its structure to increase the proximity of amino acid residues for scavenging free radicals and chelating prooxidative metals. Upon structural disruption, antioxidative amino acids, such as Cys, Met, Trp, Tyr, Phe, and His, can be exposed to function more effectively. Partial denaturation by heat treatment of proteins such as skim milk and β-lactoglobulin was reported to increase antioxidant activity (*1*). Hydrolysis of proteins by either acids or enzymes is the most widely used method to increase the exposure of antioxidant amino acids. Acid hydrolysis is less expensive and relatively simple, but it is more difficult to control and amino acid damage may occur. In contrast, enzymatic hydrolysis is less harsh and more tractable and does not cause amino acid damage (*2*). Enzymatic hydrolysis is still the predominant method to prepare bioactive peptides from proteins; fermentation is another way to hydrolyze proteins but is considered to be less efficient (*2*).

Milk, soy, and egg proteins are the major sources of bioactive peptides (*3*). Recently, antioxidative peptides from milk (*4*) and soy proteins (*2*) have been reviewed. However, studies on antioxidative peptides from laying hen eggs are still limited. Two peptides, YAEERYPIL and SALAM, with oxygen radical absorbance capacity (ORAC) values of 3.8 and 2.7 μmol, respectively, were characterized from an egg white protein pepsin hydrolysate (*5*). An ovalbumin pepsin hydrolysate was demonstrated to scavenge superoxide anion and hydroxyl radicals as well as to inhibit oxidation of linoleic acid (*6*). Additionally, it inhibited D-galactose-induced decrease of antioxidant enzymatic activities (superoxide dismutase (SOD), glutathione peroxidase, and catalase) and increased malondialdehyde (MDA) levels in the serum and liver of aged mice. Antioxidant peptides were also reported from egg yolk proteins (*7*). Egg yolk hydrolysates showed strong antioxidant activity in a linoleic acid oxidation system and on cookies containing linoleic acid (*8*, *9*). Egg yolk hydrolysates were also shown to suppress discoloration of β-carotene and to scavenge superoxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and hydroxyl free radicals. Phosvitin, a phosphoprotein known as an iron carrier in the egg yolk, and its 1 kDa cutoff tryptic digests were found to inhibit hydroxyl radical formation in the Fenton reaction system more effectively than iron-binding proteins such as ferritin, transferrin, and ovotransferrin, possibly by chelating iron ions (*10*). The antioxidant activity of tryptic digests of this protein was further studied by Xu et al. (*11*), who reported that the tryptic digests of phosvitin exhibited strong capability of inhibiting lipid oxidation of linoleic acid and scavenging DPPH free radicals.

Ovotransferrin accounts for 13% of total egg white proteins, second only to ovalbumin (*12*). It is a disulfide-rich (15 disulfide bonds) single-chain glycoprotein containing 686 residues with a molecular mass of 78 kDa (*13*). As a member of the transferrin family, it is capable of binding two Fe^{3+} ions per molecule and is

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responsible for the transfer of ferric ions from the hen oviduct to the developing embryo (*13*, *14*). Due to its high iron-binding capacity, ovotransferrin is used as a nutritional ingredient in iron-fortified products (*13*). In addition to its well-known antimicrobial and antiviral activities (*13*, *15*), ovotransferrin was reported to retard oxidation in turkey meat (*16*). A recent study also showed that ovotransferrin is an SOD mimic protein with potent superoxide anion scavenging activity under the natural xanthine/xanthine oxidase (X/XOD) coupling system (*17*). However, the presence of antioxidant peptides within ovotransferrin has not been reported. In the present study, an ovotransferrin hydrolysate was prepared by thermolysin, fractionated by cation exchange and reversed phase chromatographic methods, and characterized by LC-MS. Fourteen peptides derived from ovotransferrin are identified, and the structure−activity relationship is discussed.

MATERIALS AND METHODS

Materials. Egg ovotransferrin (Ovotransferrin 200) was obtained from Neova Technologies (Abbotsford, BC, Canada). Thermolysin, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), bovine serum albumin (BSA), sodium hydroxide, and formic acid were obtained from Sigma Canada (Oakville, ON, Canada). Ammonium acetate, ammonium carbonate, HPLC-grade acetonitrile, fluorescein, potassium monobasic phosphate, hydrochloric acid, and trifluoroacetic acid (TFA) were obtained from Fisher Scientific Canada (Ottawa, ON, Canada). Peptides (>95% purity with LC-MS verified structures) used in the study were synthesized by Genscript (Piscataway, NJ).

Sample Preparation. Ovotransferrin hydrolysates were prepared by either thermolysin, pepsin, or a sequential combination of thermolysin and pepsin. Twenty grams of ovotransferrin was dispersed in deionized water to make a 5% (w/v) slurry. After heating at 80 °C in a water bath for 15 min, the protein slurry was cooled to 60 °C and adjusted to pH 8 for thermolysin digestion or to 37 °C and pH 2 for pepsin digestion. The temperature and pH were maintained constant by a water bath and a Brinkmann Titrando 842 (Brinkmann Instrument Inc., Mississauga, ON, Canada), respectively, during the digestion. For sonication pretreatment, the ovotransferrin slurry (5%, w/v) was subjected to sonication in four pulses (60 Hz for 30 s for each pulse) using Sonic 300 (ARTEK Systems Corp., Farmingdale, NY), prior to enzymatic digestion for 3 h as above. For the sequential digestion with thermolysin and pepsin, ovotransferrin was first digested by thermolysin for 3 h followed by pepsin for another 3 h, under the conditions mentioned above. After digestion, the enzymatic reactions were terminated by raising the temperature to 95 °C for 15 min. Hydrolysates were then separated by centrifugation at 10000*g* for 30 min at 4 °C, freeze-dried, and used for ORAC assay.

Cation Exchange Chromatography. The hydrolysate prepared from sonicated thermolysin was further passed through a MWCO 3 kDa membrane (Millipore, Billerica, MA), and the permeate obtained was loaded onto a 100 mm × 16 mm i.d., 90 μm, HiPrep 16/10 SP FF column (GE Healthcare, Uppsala, Sweden) operated by an ÄKTA explorer 10S system. The peptides were eluted using 10 mM ammonium acetate, pH 4.0 (buffer A), and 0.5 M ammonium carbonate (buffer B) from 0 to 8% B over 8 column volumes at a flow rate of 5 mL/min. The elution was monitored at 280 nm, and fractions were collected and freeze-dried for the peptide content and the ORAC assay.

Reversed-Phase Chromatography. The active fractions from the above cation exchange chromatography were further fractionated by a 150 mm × 10 mm i.d., 5 μm, XBridge C_{18} column (Waters Inc., Milford, MA) run on a Waters 600 HPLC system. Samples were automatically injected by a Waters 2707 autosampler at a volume of 900 μL and were eluted by acetonitrile containing 0.1% trifluoroacetic acid (mobile phase B) from 2 to 32% at a flow rate of 5 mL/min. The elution was detected at 220 nm, and fractions were collected every 2 min by a Waters Fraction Collector III controlled by Empower version 2 software. The fractions were concentrated using a vacuum-rotary evaporator at 35 °C and used for the peptide content and the ORAC assay.

Peptide Content Assay. The peptide content was measured by use of an OPA reagent solution (Pierce Biotechnolgoy, Rockford, IL). Forty microliters of each sample or BSA standards (0−1 mg/mL) was mixed with 100 μL of the OPA reagent solution in microplate wells. The microplate was immediately placed in a Fluoroskan Ascent microplate reader (Thermo Electron Corp., Waltham, MA) with 355-P excitation and 460-P emission filters, and the fluorescence was recorded. The peptide concentration was calculated on the basis of the standard curve derived from the BSA standards.

Table 1. Antioxidant Activity of Various Enzymatic Hydrolysates with or without Sonication Pretreatment

sample treatment	ORAC[a] (μmol/mg)
control	0.21 ± 0.04 d
thermolysin without sonication	0.49 ± 0.01 c
thermolysin with sonication	1.95 ± 0.02 a
pepsin without sonication	1.42 ± 0.05 ab
pepsin with sonication	1.67 ± 0.06 ab
thermolysin + pepsin without sonication	1.14 ± 0.04 b
thermolysin + pepsin with sonication	1.53 ± 0.03 ab

[a] Means ± standard deviations (n = 3; each with duplicate measurements) without a common letter differ significantly ($P < 0.05$).

ORAC-FL Assay. The ORAC assay using fluorescein as fluorescent probe was developed by Ou et al. (*18*) and modified by Davalos et al. (*19*). Briefly, 80 mM AAPH and 200 nM fluorescein in 75 mM phosphate buffer at pH 7.4 were prepared for each experiment. For each run, 20 μL of sample and 80 μL of phosphate buffer (or 100 μL of Trolox standard solutions at final concentrations of 1−8 μM) were placed in the wells of a 96-well microplate, followed by the addition of 50 μL of the fluorescein solution. The mixture was preincubated for 15 min at 37 °C. After the addition of 50 μL of AAPH solution, the microplate was immediately placed in a Fluoroskan Ascent microplate reader (Thermo Electron Corp., Vantaa, Finland) with 485-P excitation and 538-P emission filters, and the fluorescence was recorded every minute for 100 min. All readings were recorded using Fluoroskan Ascent software. The area under the curve of fluorescence decay (AUC) was calculated using Graphpad Prism software (trial version). Regression equations between AUC and antioxidant concentrations were calculated for all of the samples. The ORAC value was calculated by dividing the slope of sample regression curve by the slope of Trolox regression curve. Final ORAC values were expressed as micromoles of Trolox equivalent (TE) per milligram of protein or peptide.

Analysis by Online RP-UPLC-MS/MS. Identification of peptides in the two most active fractions from the RP-HPLC separation were carried out by a Waters ACQUITY UPLC system connected online to a Waters Micromass Q-TOF Premier Instrument. The samples were separated on a 150 mm × 75 μm i.d., 3 μm, Waters Atlantis dC_{18} UPLC column using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in aceonitrile). Five microliters of sample was injected to a 5 μm trapping column for 2 min at a flow rate of 10 μL/min using 99% solvent A, followed by a gradient from 99% A to 90% A over 5 min, to 70% A over 30 min, to 60% over 3 min, and to 5% A over 1 min at a constant flow rate of 0.350 μL/min. The flow rate was increased to 0.500 μL/min, held at 5% A for 2 min, increased to 98% A over 1 min, held for another 27 min, and then decreased to 0.350 μL/min over 1 min. The flow entered directly into the mass spectrometer via a nanoLockspray ionization source in a positive ion mode (capillary voltage of 3.80 kV and source temperature of 100 °C). Spectra were recorded over the mass/charge (m/z) ranges of 100−1000 in MS mode and 50−1500 in MS/MS mode. The signal threshold to perform auto-MS/MS in the data-dependent acquisition was 20 counts/s in total ion current, and the precursor ions were isolated within a range of m/z 3.0. Instrumental control and data analysis were performed using MassLynx software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.). Peaks Viewer 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used in combination with manual de novo sequencing to process the MS/MS data and to perform peptide sequencing.

Statistical Analysis. All data were analyzed by one-way analysis of variance (ANOVA) using Statistical Analysis System software (version 9.0, SAS Institute, Cary, NC). Significant differences ($p < 0.05$) were determined using Duncan's multiple-range test (*20*) and ranked according to significant differences.

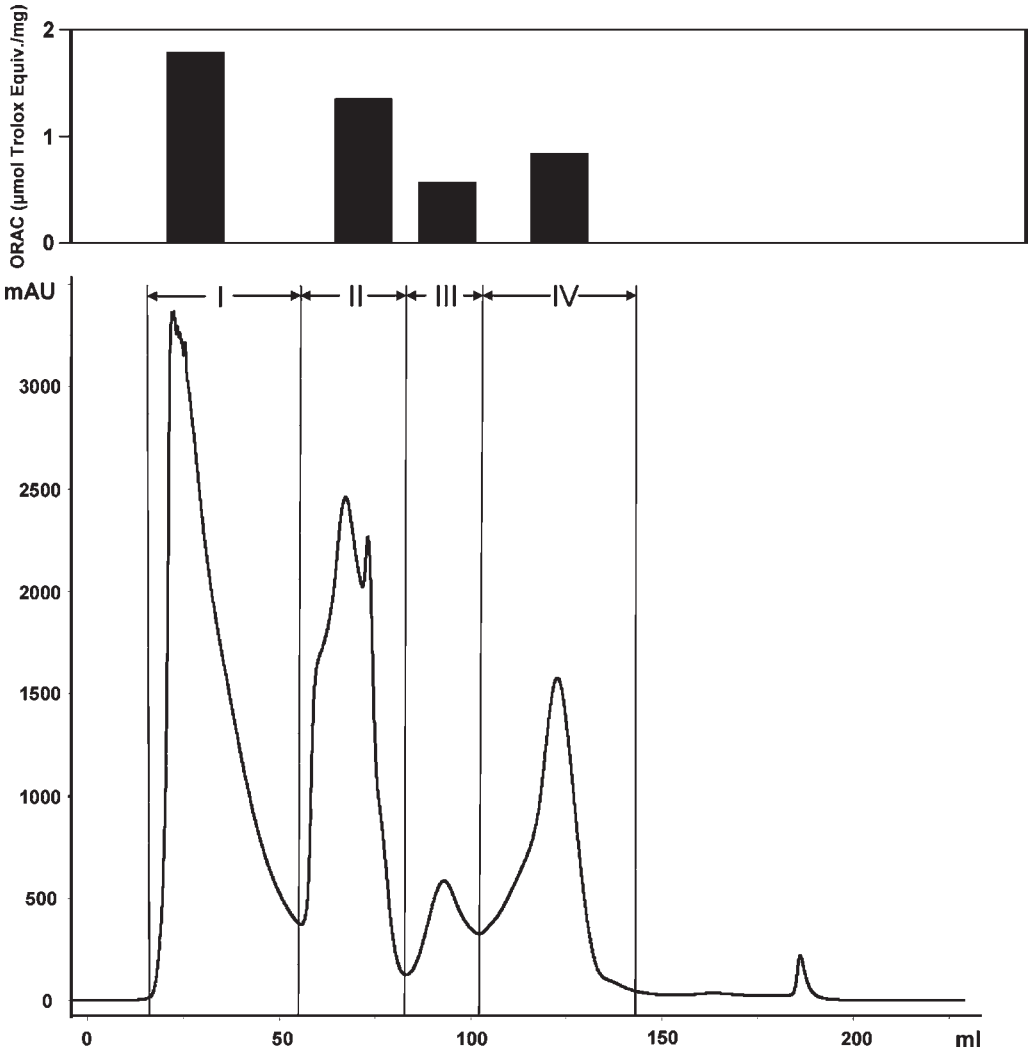

Figure 1. Fractionation by FPLC of the 3 kDa permeate obtained from ovotransferrin by thermolysin. Collected fractions are labeled using Roman numerals (I, II, III, and IV). ORAC-FL values of the fractions are represented by the histogram in the upper panel.

RESULTS AND DISCUSSION

Antioxidant Activity of Enzymatic Hydrolysates. As reported previously (*1*), enzymatic hydrolysis can significantly increase the antioxidant activity of proteins. Our results showed that hydrolysis of ovotransferrin increased ORAC values significantly from 0.21 to 0.49 μmol of TE/mg after thermolysin digestion (**Table 1**); sonication pretreatment further increased the antioxidative value from 0.49 to 1.95 μmol of TE/mg, indicating that sonication may be essential for releasing peptides responsible for antioxidant activity using thermolysin. A similar effect was observed for preparing potent angiotensin converting enzyme inhibitory peptides (*21*). It is interesting to note that sonication did not affect the potency of antioxidant activity of the pepsin hydrolysate or the thermolysin−pepsin hydrolysate (**Table 1**). Considering that pepsin itself can potentiate cleavage between disulfide bonds (*22*, *23*), it seems that sonication may have a similar effect on disulfide bonds, because sonication was previously demonstrated to disrupt disulfide bonds (*24*, *25*), which was conducive to the release of bioactive peptides during enzymatic digestion. The antioxidant activity reported here was comparable to that of an egg white pepsin hydrolysate (*5*) and those of vegetable and fruit extracts (*26*). Because thermolysin sonicated hydrolysate showed the most potent activity, this sample was used for further fractionation to elucidate the active peptides.

Fractionation and Characterization of Antioxidant Peptides. The 3 kDa permeate from ovotransferrin hydrolysate digested by thermolysin was subjected to a two-step fractionating procedures, first by strong cation exchange chromatography on FPLC followed by reversed-phase chromatography on HPLC. Four fractions were collected from the cation exchange chromatography, as shown in **Figure 1**. Peptides contained in the first two eluting peaks (fractions I and II) had higher antioxidant activity than those eluting in the later peaks (fractions III and IV). In agreement with previous papers (*27*−*29*), the acidic fraction (fraction I) obtained from cation exchange chromatography exhibited higher activity than neutral or basic fractions. The two active fractions (I and II) were further separated by reversed-phase chromatography, as shown in **Figure 2**. Both fractions showed quite complex peak profiles in the chromatograms. Consistent with the fact that fraction I had a slightly higher ORAC value than fraction II, subfractions from fraction I (I-1−I-22) were shown to have higher ORAC values than subfractions from fraction II (II-1−II-19) in general. Seventeen of 22 subfractions from fraction I had ORAC values of >4.0 μmol of TE/mg, whereas 9 of 19 subfractions from fraction II had ORAC values of ≥ 4.0 μmol of TE/mg. Among them, I-17 and I-18 were the two most potent fractions, with ORAC values of 11.7 and 12.3 μmol/mg, respectively. Therefore, most fractions obtained from the RP-HPLC in the present study had much higher ORAC values than

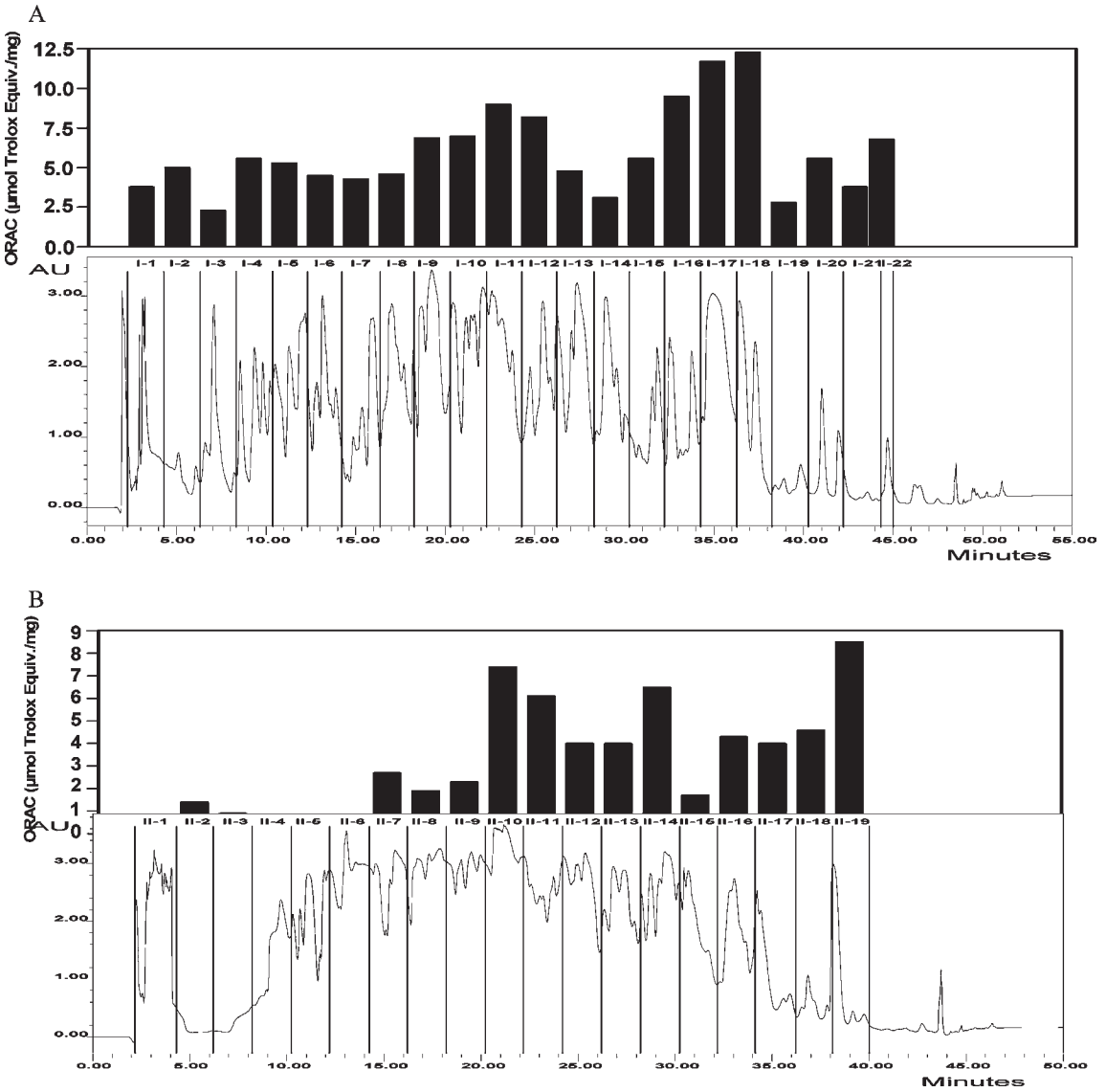

Figure 2. Fractionation by preparative RP-HPLC of the active fractions from FPLC: (**A**) fraction I; (**B**) fraction II. Mean ORAC-FL values of the fractions are represented by the histogram in the upper panel.

Table 2. Peptides Identified in the Active Chromatographic Fractions (I-17 and I-18)

	m/z	*q*[a]	obsd mass	calcd mass[b]	peptide sequence	fragment
						ovotransferrin
1	996.4	2	1991.8	1991.92	IA(deaminationN)NEADAISLDGGQVFEAG	f(68−87)
2	935.9	2	1870.8	1870.83	PIAAEVYE(OxH)TEGSTTSY	f(95−111)
3	879.4	2	1757.8	1757.79	IAAEVYEHTEGSTTSY	f(96−111)
4	928.3	1	928.3	928.49	AGWNIPIGT	f(142−150)
5	657.3	1	657.3	657.34	AGWNIP	f(142−147)
6	542.2	1	542.2	542.27	AGWNI (b-ion)	f(142−146)
7	529.2	1	529.2	529.28	WNIP	f(144−147)
8a	802.8	2	1604.6	1604.74	AIEWEGIESGSVEQA	(156−170)
8b	813.8	2			AIEWEGIESGSVEQA monosodium adduct	
9	767.3	2	1533.6	1533.71	IEWEGIESGSVEQA	f(157−170)
10	953.4	1	953.4	952.47	LSKAQSDFG	f(286−294)
11	628.3	1	628.3	628.30	LGFEY	f(339−343)
12	791.3	1	791.3	791.36	LGFEYY	f(339−344)
13	629.3	1	629.3	629.34	VIPMGL	f(494−499)
14	545.8	2	1090.6	1090.61	LVEKGDVAFI	f(550−559)
						ovalbumin
I	584.3	1	584.3	584.37	ILELP	f(230−234)
II	469.2	1	469.2	469.30	ILEL (b-ion)	f(230−233)

[a] Charge state of the precursor ion. [b] Monoisotopic mass.

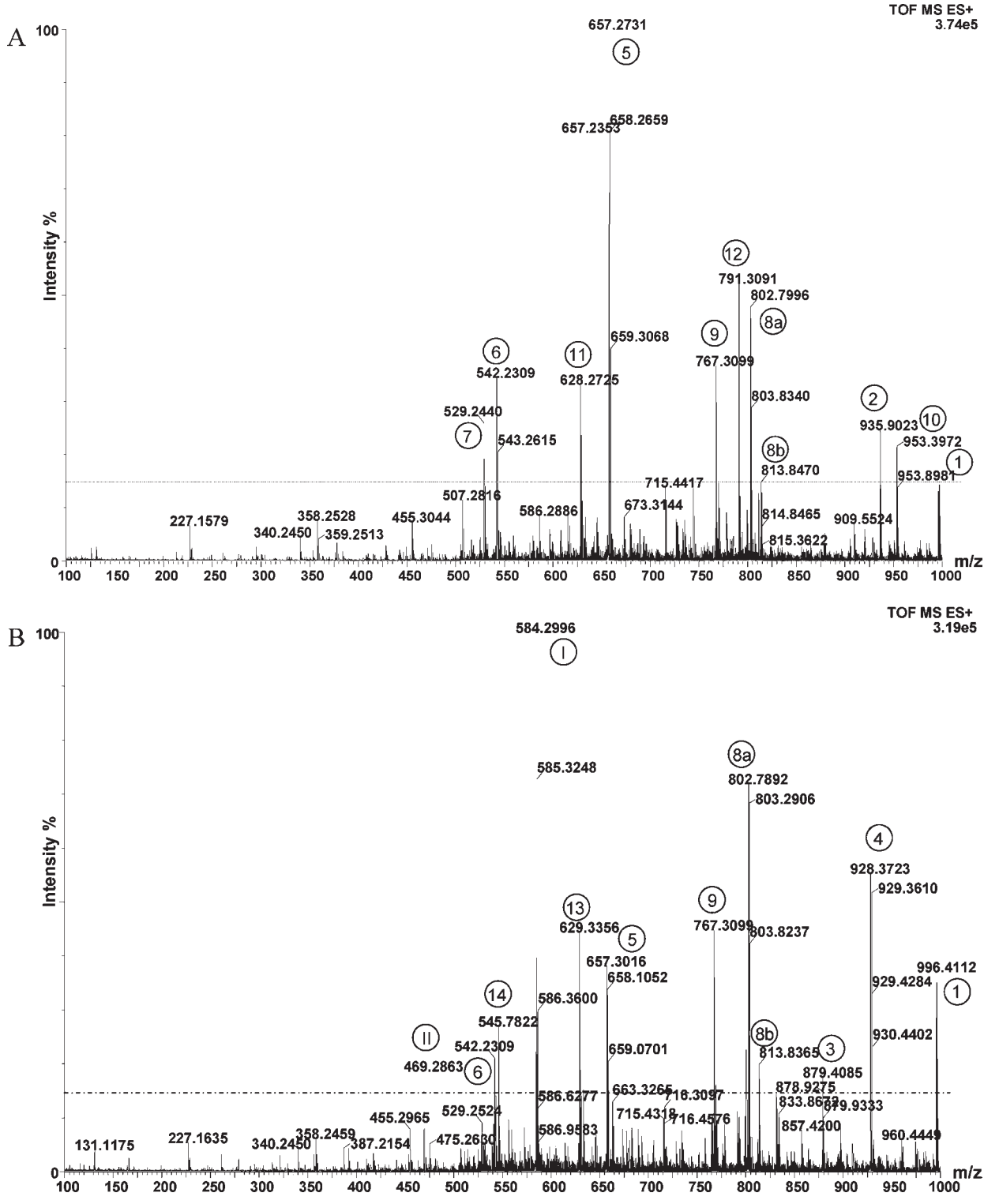

Figure 3. Averaged LC-MS spectra of the active fractions from RP-HPLC: (**A**) I-17; (**B**) I-18. The dashed line represents the cutoff of ion intensity (15%) for choosing parent ions for peptide sequencing.

the fractions obtained from the pepsin hydrolysate of crude egg white in a previous study, in which all fractions gave an ORAC value of < 4.0 μmol of TE/mg (*5*).

To identify the most potent peptides in the acquired fractions, fractions I-17 and I-18 were further characterized by LC-MS/MS. The mass spectra for both fractions are displayed in **Figure 3**, where the identified peptides are numbered. An intensity cutoff of 15% of the base peak intensity was set to filter out small ions. Peptides having intensity above the cutoff were sequenced either by Peaks Viewer software or de novo sequencing. A few peptide ions in fraction I-17, such as *m*/*z* 542.2, 657.3, 767.3, 802.8, and 813.8, recurred in fraction I-18, suggesting the overloading of these ions in fractionation. Sixteen peptides identified are listed in **Tables 2**. Among them, 14 originated from ovotransferrin and two from ovalbumin, possibly the most contaminating protein in the ovotransferrin product. Interestingly, two b-ions (*m*/*z* 542.2 and 469.2) were identified, corresponding to the $(M + H)^+$ ions of peptides AGWNI and ILEL after water loss, respectively.

C-Terminal addition of Pro rendered the observation of complete $(M + H)^+$ ions for peptides AGWNIP (m/z 657.3) and ILELP (m/z 584.3). Interpretation of their tandem mass spectra is shown in **Figure 4**. It should be noted that observation of b-ions in the single MS is rare but possible when in-source dissociation occurs, especially in proline-containing peptides (*30*). Therefore, AGWNI and ILEL b-ions were possibly generated from in-source dissociation of AGWNIP and ILELP, respectively.

All 14 peptides originating from ovotransferrin were chemically synthesized and their antioxidant activities measured (**Table 3**). The majority of the identified peptides showed high ORAC values of >3 μmol of TE/μmol. Two peptides (LSKAQSDFG and LVEKGDVAFI) had negative ORAC values, suggesting that they have prooxidant activity. Some peptide fractions from protein hydrolysates have been shown to be prooxidative (*31*). A tetrapeptide, WNIP, showed the highest

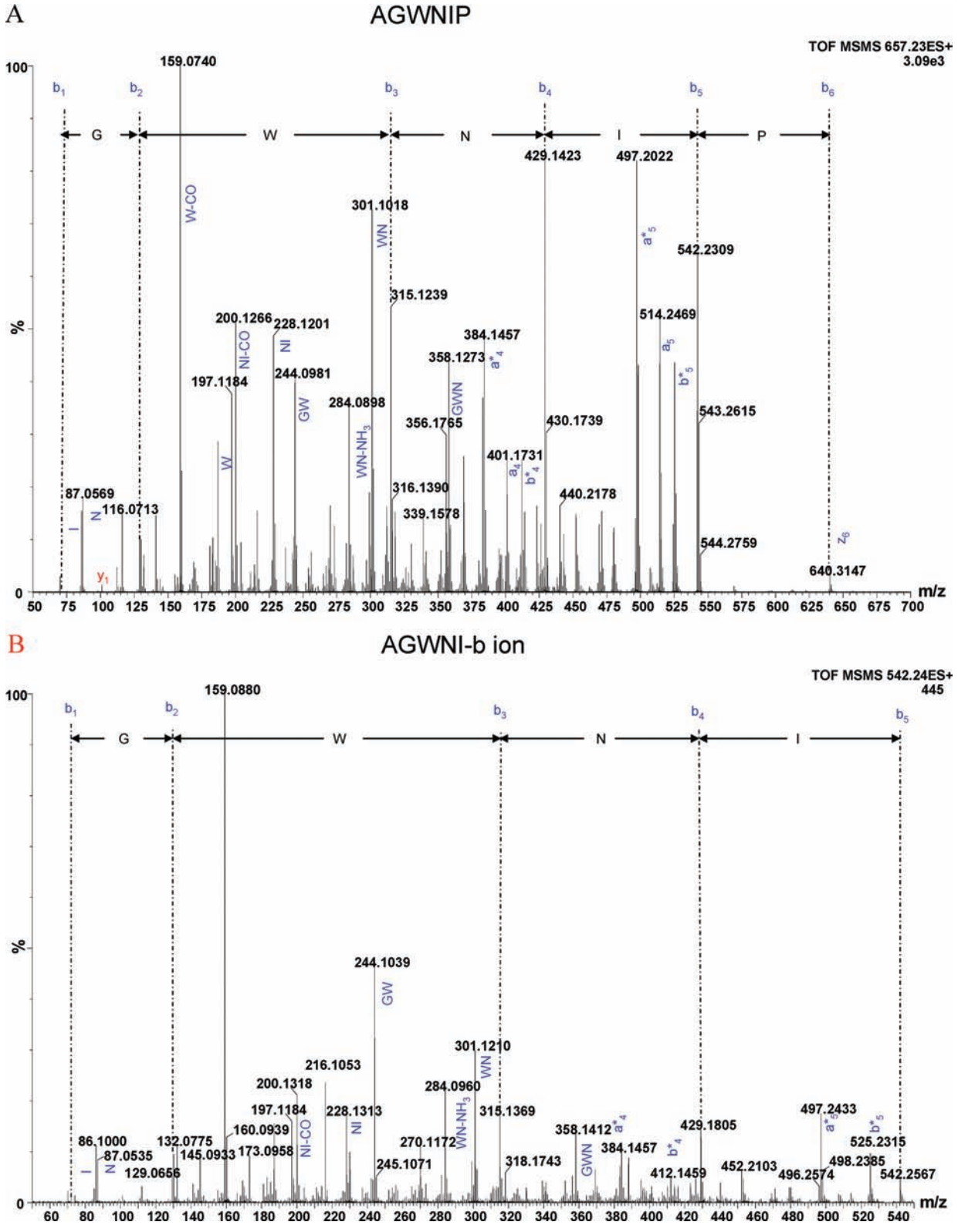

Figure 4. Continued

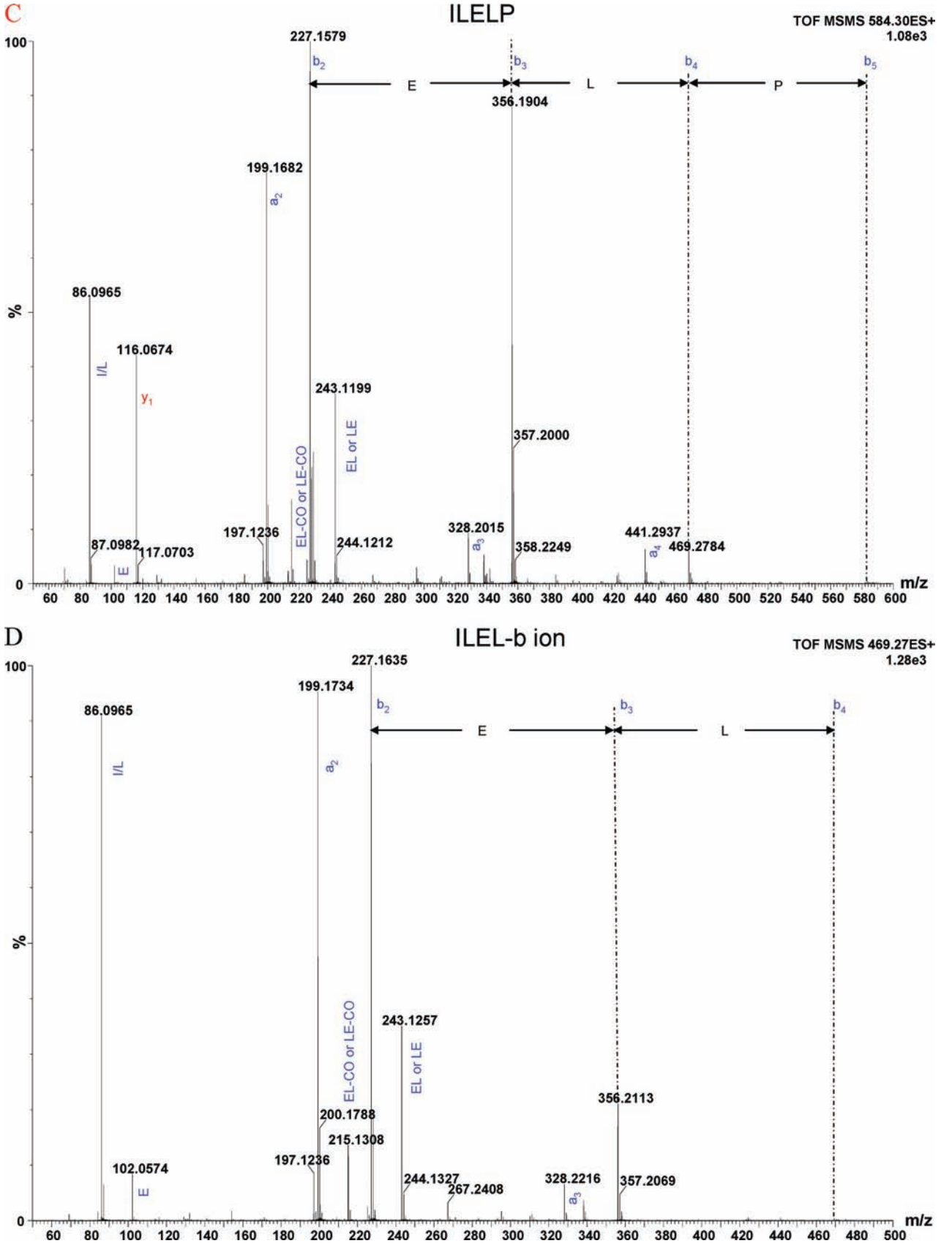

Figure 4. Interpretation of LC-MS/MS spectra of base peak ions and two b-ions identified in the MS spectra shown in **Figure 3**. Ions at *m*/*z* 657.3 (**A**) and 542.2 (**B**) represent AGWNIP and AGWNI (b-ion) derived from ovotransferrin, whereas ions at *m*/*z* 584.3 (**C**) and 469.3 (**D**) are ILELP and ILEL (b-ion) derived from contaminating ovalbumin.

ORAC value (15.47 μmol/μmol). Compared to known antioxidants of plant origin, this activity was close to that of (+)-catechin (14.9 μmol/μmol) (*19*), measured under the same conditions. To examine how the context of the peptide fragment

Table 3. Physiochemical Properties and Antioxidant Activity of Synthetic Peptides

no.	peptide sequence	amino acids	p*I*	ORAC (μmol/μmol)
1	IANNEADAISLDGGQVFEAG	20	3.43	0.43 ± 0.05
2	PIAAEVYEHTEGSTTSY	17	4.24	1.68 ± 0.03
3	IAAEVYEHTEGSTTSY	16	4.24	2.39 ± 0.17
4	AGWNIPIGT	9	5.57	5.25 ± 0.26
5	AGWNIP	6	5.57	7.64 ± 0.83
6	AGWNI	5	5.57	8.55 ± 0.60
7	WNIP	4	5.52	15.47 ± 0.68
8	AIEWEGIESGSVEQA	15	3.58	0.95 ± 0.46
9	IEWEGIESGSVEQA	14	3.58	0.73 ± 0.16
10	LSKAQSDFG	9	5.84	−1.40 ± 0.69
11	LGFEY	5	4.00	9.79 ± 0.55
12	LGFEYY	6	4.00	7.25 ± 0.71
13	VIPMGL	6	5.49	2.89 ± 0.15
14	LVEKGDVAFI	10	4.37	−0.07 ± 0.03
15	GWNIP	5	5.52	6.19 ± 0.64
16	GWNI	4	5.52	13.90 ± 1.05

embedded in the protein sequence affected its antioxidant activity, two more structurally related peptides (GWNIP and GWNI) were synthesized and tested by the ORAC assay. The fact that GWNI and WNIP had similar ORAC values (15.47 vs 13.90 μmol/μmol) indicated that WNI might be the core motif responsible for their high antioxidant activity. N-Terminal Trp was previously shown to be important in a group of structurally related antioxidative peptides (*32*). Addition of Ala to the N-terminus of GWNI (to make AGWNI) or addition of either Gly or Ala-Gly to the N-termius of WNIP (to make GWNIP or AGWNIP) decreased the antioxidant activity by half (**Table 3**). C-Terminal extension of the peptide AGWNIP (to AGWNIPIGT) did not decrease the antioxidant activity further. It was shown that under the same conditions for the ORAC assay, Trp showed the highest antioxidant activity among all amino acids, followed by Tyr and Met and then by Cys, His, and Phe. Other amino acids (Arg, Asn, Gln, Asp, Pro, Ala, Val, Lys, Ile, Ser, Thr, Leu, Glu, and Gly) did not exhibit antioxidant activity (*5*, *32*, *33*). Therefore, the presence of Trp in the structurally related peptides mentioned above is probably the driving force for their antioxidant activity. Similarly, the presence of Tyr and Phe can account for the high antioxidant activity observed with another group of structurally related peptides, LGFEY and LGFYY. However, contrary to this line of prediction, the presence of an extra Tyr at the C-terminus brought the antioxidant activity down slightly from 9.79 to 7.25 μmol/μmol. Our results indicated that the size of the peptide sequence was equally critical as the presence of some favorable amino acids in determining its activity and shorter peptides showed more potent antioxidant activity in our cases. Peptide conformation can lead to both synergistic and antagonistic effects in comparison with those exerted by the amino acid mixture (*3*). Consistent with our observations, deletion of amino acid residues or sequences was shown to enhance antioxidant activity. For example, WY had a higher antioxidant activity than WYS (*32*), whereas YPI had less activity than YPIL (*5*). It is generally believed that bioactive peptides can contain 2−20 amino acid residues (*34*). The potent antioxidative peptides identified in this study fall into this range (**Table 3**). In addition, all of them were acidic peptides, with p*I* values below 6, consistent with the fact that they originated from acidic fractions in cation exchange chromatography. It should be pointed out that the antioxidative properties of peptides varies depending on the assay system, and the discussion above was based on available data obtained with the ORAC-FL assay under the same conditions.

In conclusion, our study reports for the first time the antioxidant properties of peptides obtained from ovotransferrin by enzymatic hydrolysis. Ovotransferrin is known for its antibacterial and immunomodulating activity, but its antioxidant activity has not been well studied before. The sequences of the antioxidant peptides identified in the present study are novel. The most potent peptide sequence was identified to be WNIP. We also demonstrated that WNI is the likely core motif for the potency of antioxidant activity. Although this motif has not previously been reported for antioxidative peptides, it was present in prolyl endopeptidase inhibitory peptides derived from snake (*35*). It remains to be seen whether this motif can be present in other antioxidative peptides derived from different proteins and/or sources of origin.

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