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Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interaction with phytochemicals

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ABSTRACT

Egg proteins are important components for the development of functional foods and nutraceuticals. This study investigated the oxygen radical-scavenging effects of egg white protein ovotransferrin (including the whole protein, thermolysin–pepsin hydrolysate, and its bioactive peptides) and their interactions with four phytochemicals (vitamin C, epigallocatechin gallate (EGCG), caffeic acid, and quercetin), based on the oxygen radical absorbance capacity (ORAC) assay. The results showed that the hydrolysate possessed significantly higher oxygen radical absorbance capacity than the whole protein. The peptide IRW possessed very strong oxygen radical-scavenging effect, which might be attributed to its constitutive amino acid tryptophan (W) and the peptide bond between arginine (R) and W. Both the hydrolysate and the whole protein of ovotransferrin showed negative effects on four phytochemicals. However, the interactions between peptides/amino acids and the four phytochemicals are complex: some are positive, while others are negative. The results suggest that not only the phytochemicals, but also the hydrolysates or the peptides from the egg proteins could be a potential source of natural antioxidants.

1. Introduction

With the current upsurge of interest in the measurement of efficacy and the use of naturally derived antioxidants, functional foods and nutraceuticals have received much attention in recent years. Eggs are economically and nutritionally important since they can form a significant component of the diet and they are also an excellent source of biological active substances (Mine, 2007). Egg proteins can release peptides with physiological benefits for human health by enzymatic hydrolysis during gastrointestinal digestion or food processing (Yoshikawa et al., 2000). Many previous studies have already reported that egg hydrolysates or peptides could exert different biological activities, such as antibacterial, antifungal, antiviral, anticarcinogenic, antimutagenic, antiinflammatory, antihypertensive, as well as antioxidant activities (Erdmann, Cheung, & Schröder, 2008; Miguel et al., 2007; Mine, 2007). Therefore, eggs play a vital role in human nutrition and health.

Reactive oxygen species (ROS; superoxide, hydrogen peroxide, and hydroxyl radicals) and free radical-meditated reactions can cause oxidative damage to cellular structures and functional molecules (i.e., DNA, proteins, and lipids), and therefore lead to many diseases, such as aging, cancer, diabetes, cardiovascular disease, Alzheimer's disease, and other neurodegenerative disorders (Finkel & Holbrook, 2000). Antioxidants are thought to be highly effective in the management of ROS-mediated tissue impairments. Considering the potential adverse effects of the synthetic antioxidants (e.g., BHT), natural antioxidants derived from dietary source have received much attention in recent years (Chen, Pearson, & Gray, 1992). The utilisation of protein hydrolysates or peptides to improve the antioxidant capacity in functional foods presents additional advantages over other natural antioxidants, since they also confer an additional nutritional value (proteins are essential nutrients), as well as other desired functional properties, e.g., good water solubility, emulsion and foaming properties (Hernández-Ledesma, Amigo, Recio, & Bartolomé, 2007). Peptides may be present in foods together with other antioxidants; therefore, it would be interesting to study the interactions of peptides and other non-peptidic antioxidant agents, e.g., phytochemicals.

Egg protein hydrolysates or peptides are some of the sources of natural antioxidants. Some antioxidant peptides were found in lecithin-free egg yolk hydrolysate (Park, Jung, Nam, Shahidi, & Kim, 2001). There are more reports on the antioxidative efficacy of egg white proteins in recent years. Four peptides (Tyr–Ala–Glu–Glu–Arg–Tyr–Pro–Ile–Leu, Ser–Ala–Leu–Ala–Met, Tyr–Gln–Ile–Gly–Leu, and Tyr–Arg–Gly–Gly–Leu–Glu–Pro–Ile–Asn–Phe), included in the protein sequence of ovalbumin, possessed high radical-scavenging activity and ACE inhibitory effect (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004). The egg white protein,

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ovotransferrin, was found to be a superoxide dismutase (SOD) mimic protein with a potent superoxide anion scavenging activity (Ibrahim, Hoq, & Aoki, 2007). Currently, our laboratory has developed an innovative analysis method of peptide production through an integrated bioinformatics and quantitative structure and activity relationship (QSAR) approach; two novel ACE inhibitory peptides, IRW and LKP, were identified and characterised from the thermolysin–pepsin hydrolysate of ovotransferrin (Wu & Majumder, 2009). However, whether these peptides and hydrolysate possess antioxidant capacity is unknown and knowledge on structure–activity relationship of peptides from egg proteins are limited (Wu, Aluko, & Nakai, 2006).

Therefore, the objective of the present study was to investigate antioxidant activity of the egg white protein ovotransferrin, including the whole protein, its hydrolysate, and peptides IRW and LKP, and their interactions with four common phytochemicals (vitamin C, caffeic acid, EGCG, and quercetin) by oxygen radical absorbance capacity (ORAC) assay. The ORAC assay is a reliable test based upon the inhibition of peroxyl-radical-induced oxidation, initiated by the thermal decomposition of azocompounds, such as AAPH (2,2′-azobis(2-methylpropionamide)-dihydrochloride). It is largely used to assess the total antioxidant capacity of proteins, plant or food extracts, and pure antioxidant compounds (Prior & Cao, 1999). In order to explore structure–activity relationship of the peptides, six free amino acids (isoleucine (I), arginine (R), tryptophan (W), leucine (L), lysine (K), and proline (P)) and four di-peptides (IR, RW, LK, and KP) from two major tripeptides were also investigated.

2. Materials and methods

2.1. Chemicals and reagents

Trolox (6-hydroxy-2,5,7,8-tetramethylchromate-2-carboxylic acid), two enzymes (pepsin and thermolysin), butylated hydroxytoluene (BHT), six free amino acids (I, R, W, L, K, and P), and four phytochemicals, ascorbic acid (vitamin C), caffeic acid, epigallocatechin gallate (EGCG), and quercetin, were purchased from Sigma (Oakville, ON, Canada). Fluorescein disodium and AAPH (2,2′-azobis(2-methylpropionamide)-dihydrochloride) were obtained from Fisher Scientific (Ottawa, ON, Canada). Chemically synthetic peptides IR, RW, IRW, LK, KP, and LKP were obtained from GenScript Corporation (Piscataway, NJ, USA), and their purity (>95%) was verified by analytical RP-HPLC–MS/MS. All other chemicals and reagents used in this study were obtained from Sigma and were of the analytical grade.

2.2. Preparation of ovotransferrin hydrolysate

Ovotransferrin was prepared as 5% (w/v, dry weight) slurry in distilled water and sonicated for 120 s at 60 kHz in 4 pulses, 30 s for each pulse. After heating the sample slurry at 80 °C for 2 min with continuous shaking, the temperature was adjusted to 60 °C by putting it into an ice bucket, and the pH was adjusted to 8 by adding 1 N NaOH. After stabilisation, the sample slurries were first digested by thermolysin (25–100 units/mg) at a ratio of 0.25% (w/w enzyme:protein) for 3 h. Then, the pH was adjusted to 2 to inactivate the enzyme by adding 1 N HCl solution. The sample was subjected to further pepsin (4%, w/w) digestion for another 3 h, after the temperature was adjusted to 37 °C. The hydrolysis was terminated by raising the temperature to 95 °C and maintaining it there for 10 min. The whole digests were then centrifugated at 10,000*g* for 25 min. The supernatant and precipitate were collected and stored at −20 °C. All the digestions were carried out using Titrando (Metrohm, Herisan, Switzerland) for maintaining a constant pH during the course of the hydrolysis. The temperature of the sample during digestion was maintained using circulating water bath. Each digest was prepared individually in duplicate (Wu & Majumder, 2009).

2.3. Radical-scavenging activity by the ORAC assay

The radical-scavenging activity was assayed by the improved oxygen radical absorbance capacity (ORAC) method using fluorescein as the fluorescent probe (Dávalos, Gómez-Cordovés, & Bartolomé, 2004; Monagas, Garrido, Lebrón-Aguilar, Bartolome, & Gómez-Cordovés, 2007). Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4), and the final assay mixture was 200 μl. Totally 100 μl of antioxidant (trolox or sample at different concentrations) and 50 μl of fluorescein (50 nM, final concentration) were placed in the well of the black 96-well microplates (96F nontreated, Nunc, Denmark). The mixture was preincubated for 15 min at 37 °C. AAPH solution (50 μl; 20 mM) was added rapidly using a multichannel pipet. The plate was automatically shaken before the first reading, and the fluorescence was recorded every minute for 100 min. A Fluoroskan Ascent microplate reader (Thermo Electron Corporation, Vantaa, Finland) with 485-P excitation and 538-P emission filters, controlled by Fluoroskan Ascent Software Version 2.6 (Thermo Scientific), was used for fluorescence measurement. As blank, phosphate buffer was used instead of the antioxidant solution; six calibration solutions using trolox standard (0, 2, 4, 8, 12, and 16 μM) were carried out in each assay. All the tests were at least performed in triplicate for each sample.

Fluorescence measurements were normalised to the curve of the blank. From the normalised curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=100} f_i/f_0$$

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 the net AUC and the antioxidant concentration was calculated. The ORAC value was calculated from the slope of the sample equation divided by the slope of trolox curve obtained for the same assay. The final ORAC values were expressed as trolox equivalent (TE), i.e., μmol TE/μmol of antioxidant for peptides, amino acids, and phytochemicals, or μmol TE/mg of antioxidant for the whole protein and the hydrolysate of ovotransferrin, as well as all the other samples.

2.4. Positive/negative interaction on antioxidant effects

Positive or negative interaction among the egg protein/peptides and the phytochemicals was investigated followed the method of Hernández-Ledesma et al. (2007). Mixtures of the synthetic protein/hydrolysate/peptides/amino acids and the four phytochemicals (vitamin C, caffeic acid, EGCG, and quercetin) at different molar ratios (0:1, 2:8, 4:6, 6:4, 8:2, and 1:0) (1 μg/ml for the whole protein and hydrolysate of ovotransferrin, 1 μM for the peptides and amino acids; total concentration) were prepared, and their radical-scavenging activity was determined as described above. For the different sample:phytochemical mixtures, the calculated ORAC value was determined as follows:

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

where R is the sample concentration ratio in the sample:phytochemical mixture. Finally, the variation (expressed as percentage) between both the observed and the calculated ORAC values was calculated as follows:

Table 1
The oxygen radical absorbance capacity values (trolox equivalent) of the whole protein/hydrolysate/peptides/amino acids of ovotransferrin and four phytochemicals.

Sample	ORAC value[a]	
	(μmol TE/μmol)	(μmol TE/mg)
Whole protein (ovotransferrin)		0.21 ± 0.03
Hydrolysate (thermolysin–pepsin)		1.69 ± 0.15[c]
IRW	6.63 ± 0.37	14.0 ± 0.78
IR	0.29 ± 0.02[d]	1.00 ± 0.06[d]
RW	10.1 ± 0.54[d]	28.3 ± 1.51[d]
I	0.54 ± 0.04[d]	4.11 ± 0.30[d]
R	0.57 ± 0.03[d,e]	3.25 ± 0.15[d,e]
W	7.22 ± 0.84[e]	35.4 ± 4.11[e]
LKP	1.27 ± 0.09	3.55 ± 0.25
LK	0.34 ± 0.05	1.30 ± 0.18
KP	0.29 ± 0.04	1.19 ± 0.17
L	0.89 ± 0.01	6.78 ± 0.06
K	1.03 ± 0.08	7.03 ± 0.55
P	0.65 ± 0.03	5.68 ± 0.26
Vitamin C	1.65 ± 0.11	9.35 ± 0.63
EGCG	6.42 ± 0.46	14.0 ± 1.00
Caffeic acid	10.4 ± 0.43	57.5 ± 2.39
Quercetin	11.9 ± 0.74	39.4 ± 2.45
BHT (positive control)	5.30 ± 0.26	24.1 ± 1.18
LSD ($p < 0.05$)[b]	0.605	2.301

[a] All the data were expressed as mean ± standard deviation (SD).
[b] *LSD* ($p < 0.05$), least significant difference, was used for comparison between means of various samples.
[c] Indicated $p < 0.001$ compared to the whole protein by *t*-test.
[d] Indicated $p < 0.001$ compared to IRW by Tukey's test.
[e] Indicated $p < 0.001$ compared to RW by Tukey's test.

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

2.5. Statistical analysis

All sample determinations were conducted in triplicate, and the results were calculated as mean ± standard deviation (SD). The fluorescence decay curve was obtained using GraphPad Prism Version 5.02 (GraphPad Software, Inc., CA, USA). Coefficients of determination (R^2) were calculated using Microsoft Excel 2003. Differences between the mean values in Table 1 were compared by least significant difference (*LSD*), calculated using the Statistical Analysis System (SAS Institute, Inc., Cary, NC). One-way analysis of variance (ANOVA) with Tukey's *post hoc* test was used to determine the statistical differences among three or more groups, while the *t*-test was used between two groups. Differences were considered significant with p value <0.05.

3. Results and discussion

3.1. Oxygen radical absorbance capacity of the whole protein, hydrolysate, peptides, amino acids of ovotransferrin, and phytochemicals

The whole protein of ovotransferrin, its hydrolysate, and peptides IRW and LKP were analysed for their radical-scavenging activity by the ORAC assay, a method previously used for studying the antioxidant capacity of pure phenolic compounds and other raw materials (Dávalos et al., 2004; Prior & Cao, 1999). Six free amino acids (I, R, W, L, K, and P), present in the peptides, and four di-peptides (IR, RW, LK, and KP) were also investigated for exploring the structure–activity relationship. In addition, four phytochemicals, vitamin C, EGCG, caffeic acid, and quercetin were investigated as well with BHT as a positive control. Their ORAC values are listed in Table 1.

The whole protein of ovotransferrin only exhibited weak oxygen radical-scavenging effect, giving an ORAC value of 0.21 μmol trolox equivalent (TE)/mg. After digestion by thermolysin and pepsin, the resultant hydrolysate possessed significantly higher ORAC value (1.69 μmol TE/mg) than did the whole protein ($p < 0.0001$) (Table 1), which indicated that digestion is necessary to release potent antioxidant peptides from the parent protein. The antioxidant activity of the hydrolysates seemed to be inherent to the character-

Table 2
Negative antioxidant effects between the whole protein/hydrolysate of ovotransferrin and phytochemicals, at different weight ratios.

Sample	ORAC value (μmol TE/mg)	Sample:phytochemical (1 μg/ml)						Phytochemical
		0:1	2:8	4:6	6:4	8:2	1:0	
Whole protein	Observed	9.35	2.10	1.94	1.67	0.83	0.21	Vitamin C
	Calculated		7.53	5.70	3.87	2.04		
	Variation (%)		−72.1	−65.9	−56.8	−59.4		
Hydrolysate	Observed	9.35	4.09	3.21	3.30	2.48	1.69	
	Calculated		7.82	6.29	4.75	3.22		
	Variation (%)		−47.7	−48.9	−30.5	−23.0		
Whole protein	Observed	14.02	10.4	7.13	4.81	2.93	0.21	EGCG
	Calculated		11.3	8.50	5.73	2.97		
	Variation (%)		−7.8	−16.1	−16.1	−1.4		
Hydrolysate	Observed	14.02	7.36	6.23	5.03	3.59	1.69	
	Calculated		11.6	9.08	6.62	4.15		
	Variation (%)		−36.3	−31.5	−24.0	−13.5		
Whole protein	Observed	57.48	22.9	18.3	11.9	8.64	0.21	Caffeic acid
	Calculated		46.0	34.6	23.1	11.7		
	Variation (%)		−50.3	-47.2	−48.6	−25.9		
Hydrolysate	Observed	57.48	19.9	16.7	11.8	7.15	1.69	
	Calculated		46.3	35.2	24.0	12.9		
	Variation (%)		−57.1	−52.5	−51.0	−44.3		
Whole protein	Observed	39.38	24.6	22.4	15.1	7.80	0.21	Quercetin
	Calculated		31.5	23.7	15.9	8.05		
	Variation (%)		−22.1	−5.7	−5.2	−3.0		
Hydrolysate	Observed	39.38	18.1	13.4	11.0	5.04	1.69	
	Calculated		31.8	24.3	16.8	9.22		
	Variation (%)		−43.2	−44.9	−34.6	−45.4		

istic amino acid sequences of the derived peptides (Pihlanto, 2006). Among the two characterised peptides, IRW, with an ORAC value of 6.63 or 13.98 μmol TE/mg, showed significantly higher oxygen radical-scavenging effect than did LKP, whose ORAC value was 1.27 or 3.55 μmol TE/mg. Among the four di-peptides, RW exhibited the strongest oxygen radical-scavenging effect, with an ORAC value significantly higher than IRW. However, the other three ones, IR, LK, and KP, with ORAC values 0.29, 0.34, and 0.29 μmol TE/μmol, respectively, showed weaker oxygen radical-scavenging effects than did their parent peptides and constituting amino acids (with significant difference to W and K). Several amino acids, such as Y, M, H, K, and W, are generally accepted as antioxidants in spite of their pro-oxidative effects in some cases (Marcuse, 1960; Wang & de Mejia, 2005). Our study also showed that the amino acid W exhibited strong oxygen radical-scavenging effect (ORAC value = 7.22 μmol TE/μmol, significantly different from other amid acids); the ORAC value of the amino acid K was also over 1 μmol TE/μmol. The other four amino acids possessed moderate oxygen radical-scavenging effects, with their ORAC values ranging from 0.54 to 0.89 μmol TE/μmol.

Vitamin C, EGCG, caffeic acid, and quercetin are four common phytochemicals, which possess broad bioactivities, including antioxidant capacity, and play a potential role in cancer prevention (Birlouez-Aragon & Tessier, 2003; Huang, Cai, & Zhang, 2010). Compared with the positive control BHT, a synthetic lipophilic organic compound that is primarily used as an antioxidant food additive, three of the tested four phytochemicals (EGCG, caffeic acid, and quercetin) exhibited significantly higher oxygen radical-scavenging effects, with ORAC values 6.42, 10.35, 11.89 μmol TE/μmol, respectively (Table 1).

3.2. Positive/negative antioxidant effects between phytochemicals and the whole protein/hydrolysate/peptides/amino acids of ovotransferrin

The possible positive/negative antioxidant effect between the four tested phytochemicals and egg protein ovotransferrin (including whole protein, its hydrolysate, and peptides IRW and LKP, as well as four di-peptides and six free amino acids) was investigated at different ratios (weight ratio for the whole protein and hydrolysate, and molar ratio for peptides and amino acids). To determine the ORAC values of the mixtures, the slope of the net AUC versus the concentration curve was divided by the slope of the trolox calibration curve. The observed ORAC value was compared with the calculated ORAC value, and the variation was determined. Data are shown in Tables 2–4.

The oxygen radical-scavenging effect variation seemed negative (from −1.4% to −72.11%) by comparison with the calculated ORAC value for the whole protein of ovotransferrin and its hydrolysate (Table 2), however, the observed ORAC values of the mixtures were greatly increased, corresponding to the increased weight ratios of

Table 3
Antioxidant effects between egg peptide IRW and phytochemicals at different molar ratios.

Sample	ORAC value (μmol TE/μmol)	Sample:phytochemical (1 μM)						Phytochemical
		0:1	2:8	4:6	6:4	8:2	1:0	
IRW	Observed	1.65	2.82	4.68	5.56	8.81	6.63	Vitamin C
	Calculated		2.64	3.64	4.63	5.63		
	Variation (%)		6.8	28.8	19.9	56.5		
IRW	Observed	6.42	7.36	8.37	6.62	6.73	6.63	EGCG
	Calculated		6.46	6.50	6.54	6.59		
	Variation (%)		14.0	28.8	1.1	2.2		
IRW	Observed	10.4	12.1	11.7	10.4	6.95	6.63	Caffeic acid
	Calculated		9.60	8.86	8.11	7.37		
	Variation (%)		25.7	32.5	28.4	−5.6		
IRW	Observed	11.9	9.24	7.78	4.09	3.68	6.63	Quercetin
	Calculated		10.8	9.79	8.73	7.68		
	Variation (%)		−14.8	−20.5	−53.1	−52.0		

Table 4
Interaction among peptide IRW/its amino acids and phytochemicals, at different molar ratios[a].

		I	II	III	IV		I	II	III	IV		I	II	III	IV
Vitamin C	I	+++	+++	+++	+++	IR	++	–	–	–					
	R	+	+++	+++	–						IRW	+	++	+	+++
	W	–	++	++	+	RW	+++	+	–	–					
EGCG	I	–	+	+	++	IR	–	+	––	–					
	R	–––	–––	–	––						IRW	+	++	+	+
	W	–––	+	+	+	RW	––	+	+	+					
Caffeic acid	I	––	––	–	–	IR	+	+++	+++	+++					
	R	––	––	––	–						IRW	++	++	++	–
	W	–	–	–	+	RW	–	––	–	––					
Quercetin	I	–	+++	++	+++	IR	–	–	––	–					
	R	–	+++	+	+						IRW	–	––	–––	–––
	W	–	+++	+++	+++	RW	+	+++	+	++					

I: sample:phytochemical = 2:8.
II: sample:phytochemical = 4:6.
III: sample:phytochemical = 6:4.
IV: sample:phytochemical = 8:2.
[a] Positive effects: "+": 0 < variation (%) ⩽ 20; "++": 20 < variation (%) ⩽ 40; "+++": variation (%) > 40. Negative effects: "–": −20 ⩽ variation (%) < 0; "––": −40 ⩽ variation (%) < −20; "–––": variation (%) < −40.

phytochemicals, which means that these four phytochemicals could enhance the oxygen radical-scavenging effects of the whole protein and the hydrolysate of ovotransferrin. The ORAC values of mixtures of the whole protein/hydrolysate of ovotransferrin and vitamin C were relatively lower than other combinations because the ORAC value of vitamin C was lower than that of other three phytochemicals. Previous studies also demonstrated that some nonpeptidic natural and synthetic antioxidants, such as ascorbic acid, tocopherol, BHT, and BHA (butylated hydroxyanisole), had synergistic effects on the antioxidant activity of the protein hydrolysates and peptides (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Hernández-Ledesma et al., 2007). Chen et al. (1996) considered that the synergistic effect of nonpeptidic antioxidants on the antioxidant activity of the protein hydrolysates had nothing to do with the antioxidant activity of the constituted peptides. Interestingly, although the whole protein of ovotransferrin showed lower ORAC value than did its hydrolysate, its mixtures with caffeic acid, quercetin, and high ratios of EGCG (2:8 and 4:6) exhibited higher ORAC values than its hydrolysate. In addition, the negative variation between the observed ORAC values and the calculated ORAC values was greater for the hydrolysate mixture than for the whole protein mixed with caffeic acid, quercetin, and EGCG. This might be due to the fact that the hydrolysate possessed a lot of different peptides and had a more complex interaction with phytochemicals.

Different behaviours in the oxygen radical-scavenging effects of egg proteins IRW and LKP, as well as their amino acids and di-peptides were observed when they were mixed with the four different phytochemicals. Table 3 shows that for vitamin C, EGCG, and caffeic acid, the observed ORAC values were higher than the calculated ORAC values for nearly all the IRW mixtures tested (except for 8:2 mol/mol of IRW:caffeic acid), which indicated positive effects between both types of antioxidants, peptides and phytochemicals. The varied percentage changed from 6.8 to 56.5 for

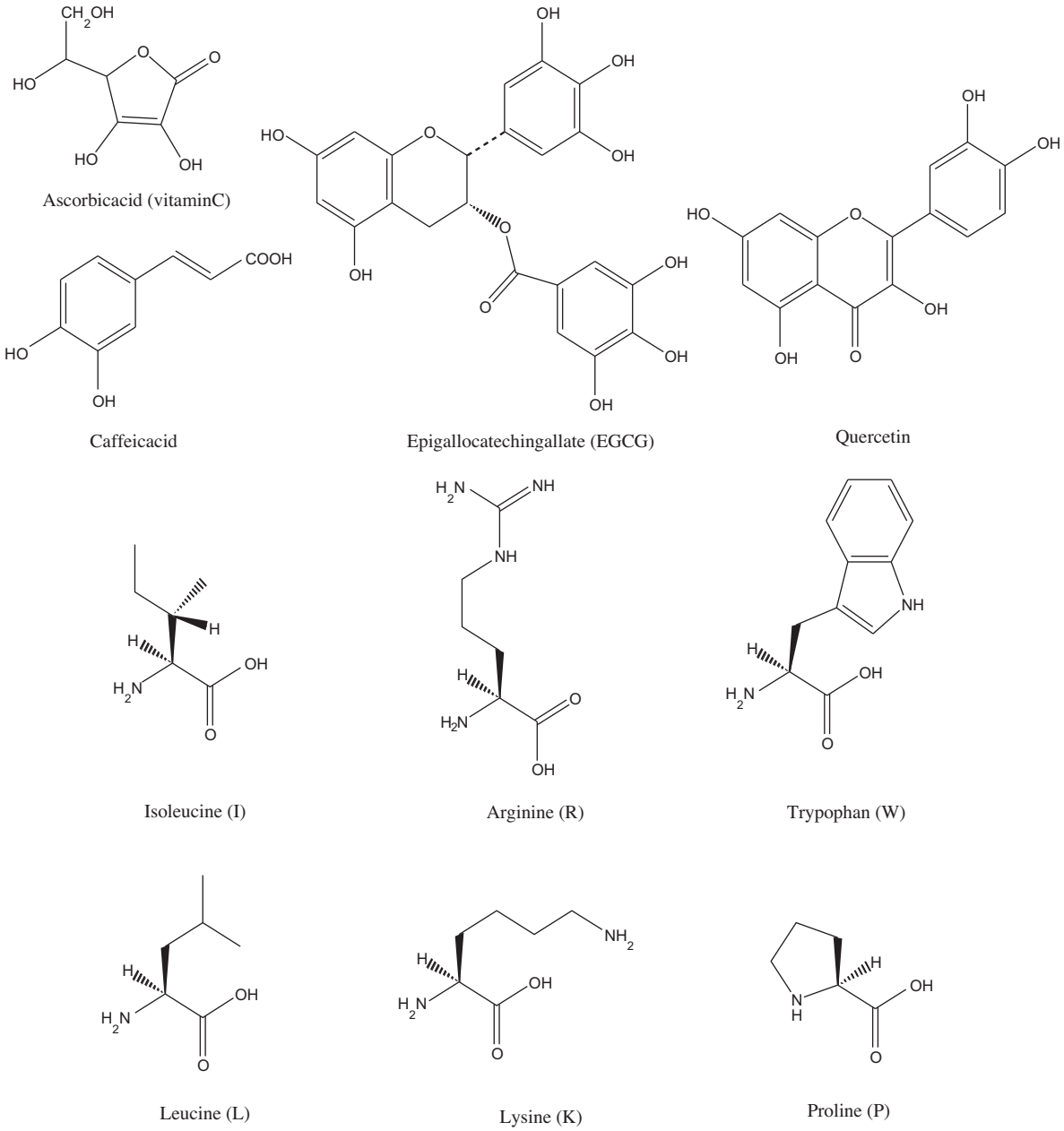

Fig. 1. Structures of four phytochemicals (vitamin C, EGCG, caffeic acid, and quercetin) and six amino acids (I, R, W, L, K, and P).

the IRW:vitamin C mixtures, from 1.1 to 28.8 for IRW:EGCG, and from −5.6 to 32.5 for IRW:caffeic acid, respectively. LKP showed similar effects to those of IRW (data not shown). However, for quercetin, the strongest tested antioxidant phytochemical, the observed ORAC values were lower than the calculated ORAC values for both the IRW and LKP mixtures at all the four different ratios, which suggested negative effects between the antioxidants. The decreased percentage varied from −14.8 to −53.1 for the IRW mixtures, and from −20.5 to −50.4 for the LKP mixtures. The free amino acids and di-peptides also showed different interaction with the four phytochemicals: some were positive, and the others were negative. Table 4 shows that the six amino acids had positive effects for vitamin C or quercetin at most molar ratios but negative effects for caffeic acid. The amino acid I exhibited great positive interaction with vitamin C, with the ORAC variation of all the different molar ratio mixtures above 40%. The greatest positive interaction between amino acid and phytochemical was found in the 6:4 mol/mol W:quercetin mixture (variation = 113.6%), while the greatest negative interaction was found in the 2:8 mol/mol R:EGCG mixture (−74.4%). For peptides mixed with phytochemicals, the greatest positive effect was in the 8:2 mol/mol LK:quercetin mixture (99.7%), while the greatest negative effect was in the 6:4 mol/mol IRW:quercetin (LKP's data not shown in the tables).

3.3. Structure–activity relationship of the peptides and phytochemicals

Six free amino acids and four di-peptides were investigated for their oxygen radical-scavenging effects and their interaction with phytochemicals to try to explore the structure–activity relationship of the peptides. Table 1 shows the significant difference between egg peptide IRW and its di-peptides IR and RW, as well as its amino acids I and R. The high ORAC values of RW and W suggested that the strong oxygen radical-scavenging effect of egg peptide IRW was attributed to the amino acid W and the peptide bond between R and W. Hernández-Ledesma et al. (2007) recently reported that synthetic β-lactoglobulin-derived peptides possessed higher antioxidant activities than did the constituent amino acid mixtures, which confirmed that the peptide bond or the structural peptide conformation improved the hydrogen donor capacity of the amino acid residues and enhanced their antioxidant activity. In addition, the presence of W, Y, and M residues were responsible for the relatively high radical-scavenging activity of these peptides. Among these amino acids, W also showed the strongest antioxidant capacity (Hernández-Ledesma et al., 2007). The distinguishing structural characteristic of W is that it contains an indole functional group (see Fig. 1), which might contribute to its antioxidant activity. Pihlanto (2006) also found that milk-derived antioxidative peptides possessed hydrophobic amino acids, such as P, histidine (H), tyrosine (Y) or W in the sequence. On the other hand, the position of the amino acids within the peptide sequence could play an important role in the antioxidant activity. Saito et al. (2003) constructed series of tripeptide libraries to explore the antioxidative properties of the peptides and found that tripeptides containing W or Y residues at the C-terminus had strong radical-scavenging activities. Chen et al. (1996) compared the antioxidant activity against the peroxidation of linoleic acid of 28 synthetic peptides, and found that the addition of L or P to the N-terminus of HH increased the activity. In the present study, the bioactive egg peptides IRW and LKP possess a W residue at the C-terminus and a L residue at the N-terminus, respectively.

The structures of the four phytochemicals showed that EGCG, caffeic acid, and quercetin all possessed phenolic hydroxyl groups (Fig. 1); the ORAC assay exhibited that these three phytochemicals had very good oxygen radical-scavenging effects. Many previous studies proved that phenolic compounds were responsible for the antioxidant capacity of plant extracts (Huang, Cai, Xing, Corke, & Sun, 2008; Huang et al., 2010). In addition, phenolic compounds or some other antioxidants may exert strong synergistic effects with antioxidative peptides (Chen et al., 1996; Hernández-Ledesma et al., 2007; Saito et al., 2003). The interaction between peptides and phytochemicals seemed complicated and it was very difficult to conclude the structure–activity relationship (Table 4). In this study, egg peptides IRW and LKP showed synergistic effects in terms of their oxygen radical-scavenging effects in combination with the three tested phytochemicals, vitamin C, EGCG, and caffeic acid, but negative effects with quercetin. In addition, different molar ratios of peptides/amino acids to phytochemicals might also affect their interactions. It seemed that synergistic effect had nothing to do with the structures of the peptides, since the interaction of peptides and phytochemicals was not always consistent with that of the constitutive amino acids or di-peptides. For example, the peptide IRW had a positive effect with caffeic acid; however, free amino acids I, R, and W mostly had negative effects with caffeic acid. On the other hand, for quercetin, IRW showed negative effects, but I, R, and W mostly showed positive effects. Interestingly, the di-peptides IR and RW had completely opposite interactions with these two phytochemicals, and IRW's results seemed more similar to that of IR. The difference might be due to the different structures of the phytochemicals. Similarly, LKP and its constitutive amino acids L, K, and P also showed opposite interactions with caffeic acid and quercetin (data not shown).

4. Conclusions

The oxygen radical absorbance capacity assay of the egg protein ovotransferrin confirmed that hydrolysates or peptides from egg proteins are a good resource of natural antioxidant. The oxygen radical-scavenging effects of the hydrolysates seemed to be associated with the peptide sequence. The egg peptide IRW possessed strong oxygen radical-scavenging effect, with an ORAC value of 6.63 ± 0.37 μmol TE/μmol. The C-terminal amino acid W and the peptide bond between R and W might be responsible for the antioxidant activity of this peptide. The results suggested that the egg hydrolysates or peptides could be used as food ingredients aiming to enhance the antioxidant properties of functional foods and prevent oxidation reactions in food processing, which would benefit human nutrition and health. In addition, the egg peptides IRW and LKP showed synergistic effects with the phytochemicals vitamin C, EGCG, and caffeic acid at high molar ratios. However, further study is needed to fully understand the interactions between peptides and phytochemicals, as well as the structure–activity relationship.

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