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Prevention of *in vitro* oxidation of low density lipoproteins (LDL) by amaranth peptides released by gastrointestinal digestion

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ABSTRACT

The objective of this work was to analyze the capacity of amaranth peptides generated by gastrointestinal digestion to prevent LDL oxidation. A simulated gastrointestinal digest from protein isolate (***Id***); ***Id*** fractions separated by gel filtration FPLC, and synthetic peptides (products of digestion) were evaluated. The evolution of conjugated dienes (CD) and, for most active samples, TBARS, fluorescent compounds (FC) evolution and electrophoretic mobility (EM) during Cu^{+2}/H_2O_2-induced-LDL-oxidation were evaluated. ***Id*** was able to increase the lag time and to decrease the propagation rate for CD and FC formation; however, EM was not modified. Most active FPLC fractions (0.59–1.4 kDa) attained a complete inhibition of CD, and partial or total prevention of FC formation and electrophoretic changes. Peptides evaluation indicated that the presence of histidine, hydrophobic and aromatic residues would be important in the inhibition of Cu^{+2}/H_2O_2-induced LDL oxidation. The most active were cationic or neutral peptides.

1. Introduction

It has been demonstrated that amaranth (*Amaranthus mantegazzianus*) peptides obtained after simulated gastrointestinal digestion can exert antioxidant activity (Orsini Delgado, Galleano, Añón, & Tironi, 2015). In this way, gastrointestinal digests from amaranth protein isolates have demonstrated the ability to act through different reactive species present in the human body, especially against peroxyl and hydroxyl radicals and peroxynitrites. On the other hand, through chromatographic methods, active fractions have been isolated from the digest and some peptides that could be identified and synthetized have demonstrated the ability to scavenge peroxyl radicals (Orsini Delgado, Nardo et al., 2016). Besides, *in vitro* assays using Caco2-TC7 monolayers have demonstrated a potential ability of some antioxidant amaranth peptides to cross the intestinal epithelium (Orsini Delgado, Añón et al., 2016). Finally, the addition of a minor proportion of amaranth protein isolate (2.5% w/w) to the diet (containing 1% w/w cholesterol) of Wistar rats improved the antioxidant status of plasma and liver, and produced a reduction in plasma and liver cholesterol (Lado, Burini, Rinaldi, Añón, & Tironi, 2015).

Oxidation of low-density lipoproteins (LDL) in the arterial wall is crucial at the first stages of atherosclerotic plaque development due to some especial characteristics of oxidized LDL, such as their ability to be rapidly taken up by macrophages to form foam cells, their chemoattractant property for circulating monocytes and T cells, and their immunogenic and cytotoxic effects to various cell types, including endothelial cells and resulting in loss of endothelial integrity, among others (Young & McEneny, 2001). LDL oxidation does not take place in the circulation because of the considerable antioxidant defense of serum and the content of alpha-tocopherol in LDL, but it occurs in the arterial subendothelial space where LDL may be exposed to cell-derived oxidants (Yoshida & Kisugi, 2010). *In vivo* oxidation of LDL could be initiated by both enzyme-mediated and non-enzymatic mechanisms, while some studies have concluded that different mechanisms may predominate at different stages of plaque development. Lipid peroxidation occurs through a free radical chain reaction, first including the formation of conjugated dienes that react with molecular oxygen to form peroxyl radical and then hydroperoxide resulting in chain propagation (minimally oxidized LDL). Finally, lipid hydroperoxides fragment to shorter-chain aldehydes, which may bind to ε-amino groups of apo B-100 (highly oxidized LDL) increasing the net negative charge and the recognition by the scavenger receptor (Yoshida & Kisugi, 2010; Young & McEneny, 2001).

Dietary intervention is actually considered an important means of prevention and treatment of cardiovascular risk factors. Due to the relationship between oxidative modification of LDL with atherosclerosis and cardiovascular disease, a higher dietary

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proportion of antioxidant compounds, such as polyphenols, vitamins, and carotenoids would be beneficial (Chu & Liu, 2004; Gammone, Riccioni, & D'Orazio, 2015; Loffredo, Perri, Nocella, & Violi, 2016). Information about the action of dietary peptides against the LDL oxidation is scarce. Decker, Ivanov, Zhu, and Frei (2001) demonstrated that carnosine and histidine inhibited Cu^{2+}-promoted LDL oxidation. Copper-chelating peptide fractions purified from an extensive chickpea protein hydrolysate have shown similar capability, the antioxidant effect being mainly due to the formation of copper chelates *via* the imidazole ring of the amino acid histidine (Torres-Fuentes, Alaiz, & Vioque, 2014). Casein peptides with positive charges presented excellent inhibition effect on LDL oxidation and were proposed as natural antioxidants for the prevention of human atherosclerosis (Wang, Wang, Huo, & Li, 2016).

The objective of the present work was to evaluate the *in vitro* capacity of amaranth peptides generated by a simulated gastrointestinal digestion process to prevent the oxidation of LDL. Structure-activity relationships of the peptide sequences were also studied.

2. Materials and methods

2.1. Chemicals

Pepsin 1:15,000 5X NF standards and porcine Pancreatin 4X-100USP units/mg came from MP Biomedicals LLC (Solon, OH, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), blue dextran, N-Hippuryl-His-Leu, tyrosin and 2,4,6-trinitrobencenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human insulin (Humulin, 100 U/mL) was obtained from Lilly (France). All the other reagents were of analytical grade.

2.2. Samples

2.2.1. Protein Isolate (I)

Amaranth plants (*Amaranthus mantegazzianus*) were grown at Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, Córdoba (Argentina). Flour was obtained by grinding whole seeds in an Udy mill, 1 mm mesh, screened by 0.092 mm mesh, and defatted by extraction with hexane during 24 h at room temperature. Amaranth protein isolates were obtained from the defatted flour by extraction at pH = 9, isoelectric precipitation (pH = 5), neutralization and freeze-drying (Martinez & Añón, 1996). Isolates presented the following composition: 78.40 ± 0.05 g proteins/100 g, 8.3 ± 0.2 g carbohydrates/100 g; 8.55 ± 0.06 g water/100 g, 2.6 ± 0.1 g ash/100 g, and 1.2 ± 0.1 g lipids/100 g.

2.2.2. Simulated gastrointestinal digest (Id)

The simulated gastrointestinal digestion had previously been optimized for amaranth protein isolate (Orsini Delgado, Tironi, & Añón, 2011). A brief outline of the protocol follows: the sample (***I***) was initially treated with a pepsin solution (0.1 equi/L HCl, 0.03 mol/L NaCl, pH = 2, pepsin/protein ratio = 1/10 w/w, 37 °C, agitation, 60 min). The pepsin digest was adjusted to pH 6 and a pancreatin solution was added (0.1 mol/L $NaHCO_3$, pH = 6, pancreatin/protein ratio = 1/10 w/w, 37 °C, 60 min). The enzyme activity was stopped by heating at 85 °C for 10 min and suspensions were freeze-dried. ***Id*** presented the following composition: 67 ± 2 g proteins/100 g, 10.9 ± 0.7 g carbohydrates/100 g; 6.4 ± 0.5 g water/100 g, 15.6 ± 0.8 g ash/100 g, and 1.0 ± 0.5 g lipids/100 g. The hydrolysis degree (HD) of ***Id*** was measured by reaction of free amino groups with TNBS (Adler-Nissen, 1979), according to previous works (Orsini Delgado et al., 2015).

2.2.3. Gel filtration chromatography

A soluble fraction from ***Id*** was obtained by suspension of 10 mg/mL of the freeze-dried sample in 32.5 mmol/L Na_2HPO_4/2.6 mM NaH_2PO_4 (pH = 7.8), agitation (500 rpm, 1 h, 37 °C) and centrifugation (10,000*g*, 10 min, room temperature). It was separated by gel filtration FPLC using a Superdex 30 prep grade (GE Healthcare, optimal separation range < 10 kDa) column and an AKTA Purifier (GE Healthcare Bioscience AB) equipment. The chromatographic conditions were: room temperature; mobile phase: 35 mM phosphate buffer, pH = 7.8; flow: 0.8 mL/min; injection volume: 2 mL; column total volume (V_t = 110 mL). Calibration was performed using Blue Dextran for exclusion volume (V_0 = 38.9 mL), human insulin (5.8 kDa), peptide LAGKPQQEHSGEHQ (1.54 kDa), peptide GDRFQDQHQ (1.13 kDa), N-Hippuryl-His-Leu (0.43 kDa) and tyrosin (0.18 kDa), obtaining the following expression for the molecular mass (MM) determination: log MM = 1.21–2.31 × K_{av} (r^2 = 0.9859), where $K_{av} = (V_e - V_0)/(V_t - V_0)$, V_e = elution volume. Detection was performed at 210 nm. The fractions (1.6 mL) were then collected. Protein concentration in the soluble and gel filtration fractions was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.2.4. RP-HPLC

A C18 (5 μm ST 4.6/250 (Agilent) column was used to analyze the components in some FPLC gel filtration fractions using a Waters System HPLC (Waters Corp., Milford, MA) equipped with a diode array detector. Solvent A was a mixture of water and acetonitrile (98:2) with trifluoroacetic acid TFA (650 μL/L), and solvent B was a mixture of water and acetonitrile (35:65) with TFA (650 μL/L). Filtered (0.45 μm) samples were eluted with a linear gradient of solvent B in A (0–100% in 55 min, flux rate: 1.1 mL/min). Separation was carried out at 40 °C. Detection was performed at 210 and 280 nm.

2.3. LDL oxidation assays

2.3.1. Preparation of LDL

The plasma of 5 healthy 12 h-fasted volunteers was obtained by venepuncture and immediate centrifugation (1000*g*, 20 min, 4 °C) of the citrated blood. Pooled plasma was stored at −80 °C until use. Volunteers gave their informed consent; the principles of the Declaration of Nüremberg, Helsinski and Tokio were respected. The experimental protocol was approved by the Research Ethical and Bioethical Committee (COBIMED) (Facultad de Ciencias Médicas, Universidad Nacional de La Plata, Argentina). Equal volumes (100 μl) of plasma and LDL precipitating reactive (ByoSystems S. A., Barcelona, Spain) were mixed and LDL fraction was obtained according to manufacturer instructions. Precipitated LDL was washed with saline solution, newly centrifuging. LDL was suspended in PBS (500 rpm, 25 °C, 15 min, Temomixer Eppendorf). Protein concentration in the LDL suspension was determined by the Lowry method (Lowry et al., 1951).

2.3.2. LDL oxidation

In order to optimize the LDL oxidation reaction, preliminary experimental runs based on published works (Scheffer et al., 2000; Scoccia, Molinuevo, McCarthy, & Cortizo, 2001) were performed in our lab. The final protocol follows: 100 μL LDL (1 mg protein/mL), 55 μL sample or buffer (control), 15 μL H_2O_2 (0.3% w/v in PBS) and finally, 30 μL 100 μM $CuSO_4$ (recently prepared solution in MilliQ water) were mixed and incubated at 37 °C for 4 h with agitation.

2.3.2.1. Conjugated dienes (CD) evolution. During the incubation period (in UV 96-well flat-bottom plate, Corning LS, USA), CD was monitored by reading the absorbance at 234 nm every 5 min (SYNERGY HT–SIAFRT multidetection microplate-reader, Biotek Instruments, USA), plotting $\Delta Abs = Abs_{t=t} - Abs_{t=0}$ as a function of the incubation time. Trolox was used as a known antioxidant. Different concentrations of soluble fractions of ***I*** and ***Id***, gel filtration fractions from ***Id*** and synthetic peptides were evaluated in their capacity to prevent LDL oxidation. For each assay measurements were performed at least by triplicate. Curves were fitted using the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) by Non Linear Regression according to the Sigmoidal Dose-Response (variable slope) equation: $Y = bottom + (top-bottom)/(1 + 10\text{^}(X_{50}-X) * Hill\ Slope))$, where Y = ΔAbs, X = t, $X_{50} = t_{50}$ (time at which $\Delta Abs = ½\ \Delta Abs_{max}$), top = ΔAbs_{max}, Hill Slope = propagation rate (PR), bottom was constrained to a constant value of 0. Lag Time (LT) was considered as the time at which $\Delta Abs = 0.1\ \Delta Abs_{max}$.

2.3.2.2. Thiobarbituric acid reactive substances (TBARS). After incubation, oxidation reaction was stopped with EDTA (1.5 mg/mL). Sample (40 µl) was mixed with 100 µl of 20% w/v trichloroacetic acid (pH 3.5) and 100 µl of 0.78% w/v TBA solution. After incubation at 90 °C, 45 min, and centrifugation (2000*g*, 5 min), fluorescence (λ_{exc} = 485 nm, λ_{em} = 528 nm, SYNERGY HT–SIAFRT, Biotek Instruments, USA) was measured.

2.3.2.3. Fluorescence evolution. Reaction mixtures (in black plates) were prepared and incubated as was indicated in Section 2.3.2. Fluorescence (λ_{exc} = 360 nm, λ_{em} = 460 nm, SYNERGY HT–SIAFRT, Biotek Instruments, USA) was read every 5 min during 4 h in order to evaluate the interaction between lipid oxidation products and lysine residues (Cominacini et al., 1991). For each assay measurements were performed at least by triplicate. Similarly to CD evolution, curves (ΔF vs t) were fitted according to the Sigmoidal Dose-Response (variable slope) (see Section 2.3.2.1).

2.3.2.4. Agarose gel electrophoresis. After 4 h of incubation, oxidation reaction was stopped by addition of EDTA (final concentration = 1.5 mg/mL). Electrophoretic mobility was measured in Tris/Glicine, pH 8.3 on 0.5% agarose gel using a B2-BP Owl (Thermo Scientific, USA) equipment. Electrophoresis was performed at a constant voltage of 65 V (initial intensity of 40 mA) for 45 min followed by 90 V for 15 min. Bands were revealed with Coomasie Brillian Blue. Mobility was expressed as percentage respect to native LDL (Carnevale et al., 2007; Trostchansky et al., 2001).

3. Results and discussion

3.1. Characterization of samples

The gastrointestinal digest from amaranth protein isolate (***Id***) presented a hydrolysis degree (HD) of 63 ± 6% according to the TNBS method, corresponding to a mean length of the peptide chain (MLP = 100/HD%; Adler-Nissen, 1986) of 1.6 amino acids. When ***Id*** (67% protein w/w) was solubilized in 35 mM phosphate (pH = 7.8) buffer (10 mg ***Id***/mL), the soluble peptide concentration was 5.8 ± 0.6 mg/ml, corresponding to a high solubility value (86.5%).

The gel filtration chromatogram of soluble ***Id*** (Fig. 1) shows a very high amount of peptides with MW between 2.1 and 0.2 kDa (fractions 21–38) corresponding to peptides with 2–19 amino acids. Also, a considerable amount of smaller molecules could be observed (fractions 39–44), probably corresponding to free amino acids.

3.2. Activity against LDL oxidation

It is known that LDL can be oxidized *in vivo* by different mechanisms. *In vitro*, LDL can be oxidatively modified in the presence of transition metals such as iron and copper and this cell-free oxidized LDL is physically-, chemically- and biologically indistinguishable from LDL oxidized by a cellular system (Jialal & Devaraj, 1996). One of the most-often-used procedures for measuring the resistance of LDL to *in vitro* oxidation is the determination of the lag

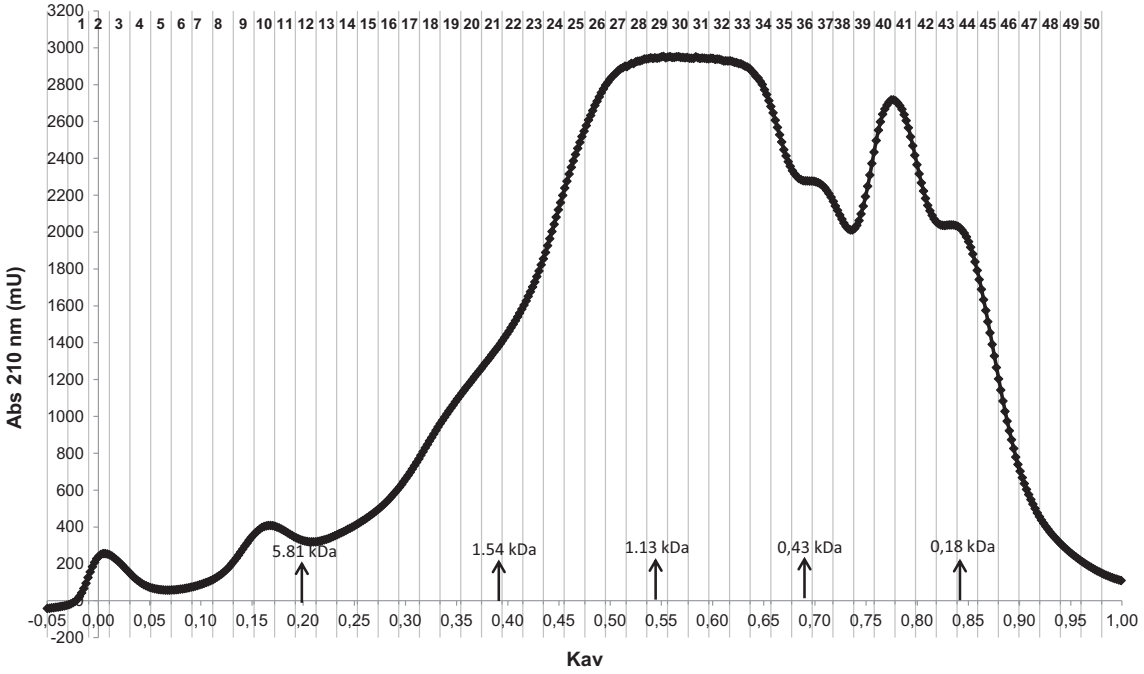

Fig. 1. Gel filtration FPLC (Superdex 30) chromatogram for digested amaranth protein isolate (***Id***) showing the collected fractions (1–50).

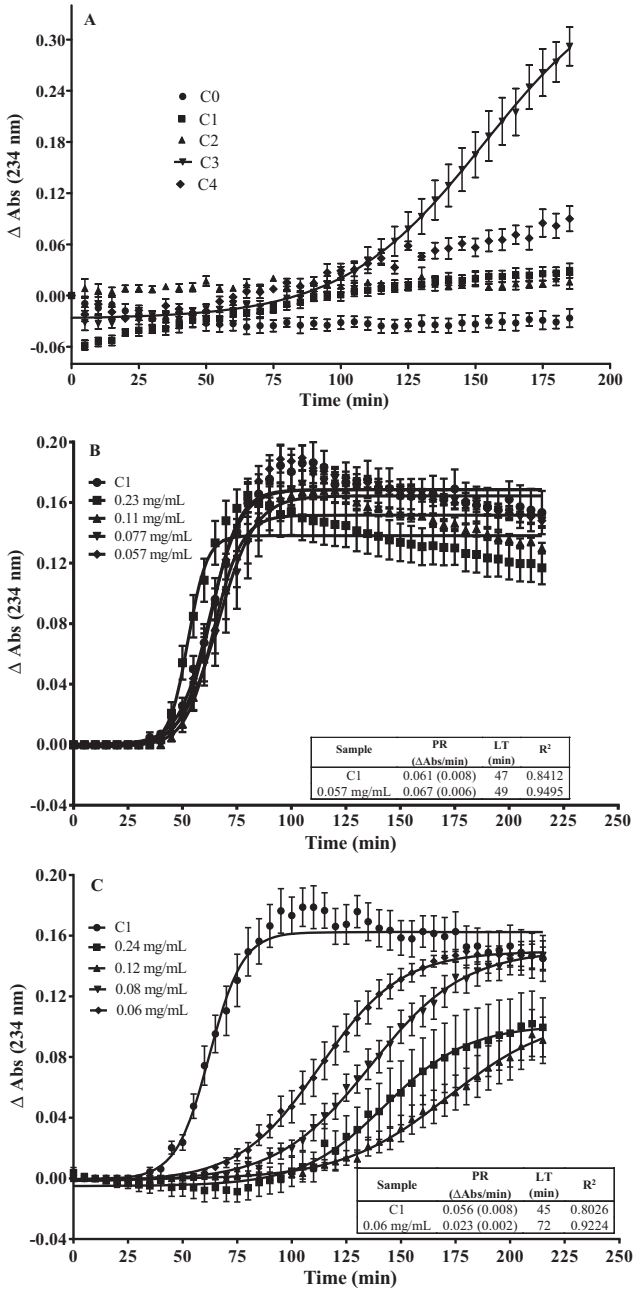

Fig. 2. Conjugated dienes (CD) evolution for: (A) control systems: C0 = LDL + buffer; C1: LDL + Cu^{+2}; C2: LDL + H_2O_2; C3: LDL + Cu^{+2} + H_2O_2; C4 = LDL + Cu^{+2} + H_2O_2 + -Trolox 0.1 μM. (B) Systems in the presence of different protein concentrations of amaranth protein isolate (***I***). (C) Systems in the presence of different protein concentrations of digested amaranth protein isolate (***Id***). LT and PR values are shown in the box.

time (LT) for the formation of conjugated dienes (CD), initiated by catalytic amounts of transition metal ions (Esterbauer, Jurgens, Quehenberger, & Koller, 1987; Scheffer et al., 2000). Besides, metal ions catalyze the *in vivo* formation of $OH^{\cdot}$ radical in the presence of hydrogen peroxide (H_2O_2); this radical is one of the most potent biological oxidants (Halliwell & Gutteridge, 1999). In the present work, the LDL oxidation assay was optimized using a combination of Cu^{+2}/H_2O_2. Fig. 2A shows the evolution of CD for control systems (without any antioxidant molecule) in the presence of Cu^{+2}, H_2O_2 or Cu^{+2}/H_2O_2. The kinetics of LDL oxidation presented an initial lag phase (no increments in the absorbance), followed by a propagation phase with a maximum slope corresponding to the propagation rate (PR) that starts presumably after the endogenous antioxidants have been consumed; finally a plateau phase is reached (Jialal & Devaraj, 1996). As shown in Fig. 2A, no formation of CD was observed during the assay time for the control system in the absence of Cu^{+2} and H_2O_2 (C0), as well as in the system where only H_2O_2 was added (C2). When only Cu^{+2} was added as an oxidation inducer, a mild absorbance increment was observed (C1, Fig. 2A). Diene production is dependent on reaction conditions such as LDL and Cu^{+2} concentrations, temperature and LDL isolation methodology (Kleinveld, Hak-Lemmers, Stalenhoef, Demacker, 1992). Even when identical conditions are used to oxidize the LDL, the products could differ significantly, depending upon the fatty acid composition and antioxidant status of the starting LDL preparation (Levitan, Volkov, & Subbaiah, 2010). These facts could explain the lack of CD formation in the assay time; it is probably that the initiation of oxidation would require longer times under the present reaction conditions. However, in the presence of Cu^{+2}/H_2O_2, a typical CD kinetic curve was registered (C3, Fig. 2A), presenting lag and propagation phases along the assayed time (180 min). Consecutive action of several oxidizing agents and enzymes is more likely to be involved in the generation of *in vivo* oxidized LDL. Free radicals and nonradicals (H_2O_2 among them) oxidizing agents have been shown to be present in the atherosclerotic lesions (Levitan et al., 2010). Accordingly, the combination Cu^{+2}/H_2O_2 was selected as oxidation inducers and the assay time was extended to 240 min in order to complete the plateau phase of the curve. Trolox, a water-soluble analog of vitamin E, was assayed using this protocol and no CD formation was observed with concentrations in the range of 0.1–5 μM (C4, Fig. 2A), indicating that in the presence of a chain-breaking antioxidant, radicals may be scavenged and LDL oxidation prevented.

At first, the activity of ***Id*** against LDL oxidation was analysed in comparison with ***I*** in order to evaluate the effect of the gastrointestinal digestion process. The presence of ***I*** (0.055–0.23 mg protein/mL) did not produce modifications in the oxidation kinetic with respect to the control system (Fig. 2B). However, when ***Id*** was added to the reaction mix, strong effects were registered, such as considerable increments in the LT and a decrement in the PR (Fig. 2C). These responses were dependent upon the protein concentration. In the case of the most diluted sample (0.06 mg/ml) a sigmoidal curve could be fitted with a good correlation factor; LT value increased in about 70% and PR value was about 40% of the value for the control system. For more concentrated samples, sigmoidal model correlations were poor because the oxidation was largely retarded, but it can be clearly seen in the Fig. 2C that LT increased as a function of the protein concentration, achieving increments of about 150% with respect to the control (C1).

After corroborating that the whole gastrointestinal digest was able to prevent or retard LDL oxidation, peptide fractions (16–39) separated from ***Id*** according to their molecular weights (gel filtration FPLC) were evaluated. Different behaviours were observed among them. Fractions 16–21 (supplementary material, Fig. 1SA) and fractions 29–32 (supplementary material, Fig. 1SB) produced an increment in the LT (1–2.5 times with respect to the control) and a decrement in the PR (2–3 times with respect to the control) (Table 1). Fractions 33–39 exhibited lower LDL-antioxidative effects; some of them (F33–F36) presented an increment in the LT, while others (F37–F39) lowered these values. Additionally, increments in the PR were registered for some samples (F35–F39, supplementary material, Fig. 1SC), a different response from the

Table 1
Kinetic parameters obtained from sigmoidal dose-response equation for the CD evolution in systems with the presence of gel filtration FPLC fractions, separated from digested amaranth proteins (***Id***).

Sample	Cprot (mg/mL)	MW (kDa)	PR (ΔAbs/min)	PR/PRc%	t_{50} (min)	LT (min)	LT/LTc%	R^2
F16	0.13 (0.01)	2.8–2.5	0.037 (0.004)	48	95 (1)	70 (4)	206	0.933
F17	0.27 (0.3)	2.5–2.2	0.028 (0.004)	37	123 (2)	89 (7)	265	0.874
F18	0.19 (0.07)	2.2–1.95	0.025 (0.002)	32	136 (2)	97 (5)	288	0.941
F19	0.232 (0.006)	1.95–1.7	0.018 (0.004)	24	138 (5)	85 (17)	253	0.701
F20	0.31 (0.08)	1.7–1.5	0.017 (0.003)	22	147 (5)	92 (14)	272	0.777
F21	0.32 (0.03)	1.5–1.4	0.017 (0.004)	23	167 (7)	113 (20)	334	0.674
F29	0.03 (0,03)	0.59–0.52	0.028 (0.002)	36	139 (1)	105 (3)	311	0.949
F30	0.10 (0.01)	0.52–0.46	0.026 (0.001)	34	149.1 (0.9)	112 (3)	333	0.967
F31	0.054 (0.001)	0.46–0.41	0.027 (0.001)	36	151 (1)	116 (3)	345	0.962
F32	0.011 (0.006)	0.41–0.36	0.034 (0.003)	44	120 (1)	92 (2)	272	0.934
F33	0.09 (0.01)	0.36–0.32	0.059 (0.005)	77	80.5 (0.8)	64 (2)	191	0.939
F34	0.24 (0.04)	0.32–0.29	0.070 (0.006)	92	72.7 (0.6)	59 (2)	175	0.952
F35	0.270 (0.002)	0.29–0.25	0.086 (0.009)	111	64.4 (0.6)	53 (2)	158	0.934
F36	0.32 (0.04)	0.25–0.22	0.099 (0.008)	129	52.7 (0.4)	43 (1)	128	0.971
F37	0.009 (0.004)	0.22–0.20	0.12 (0.01)	154	40.5 (0.4)	32 (1)	96	0.958
F38	–	0.20–0.18	0.10 (0.02)	133	39.6 (0.9)	30 (3)	90	0.843
F39	–	0.18–0.16	0.072 (0.009)	94	31.6 (0.9)	18 (3)	55	0.863
F22 (1/5)	0.016 (0.007)	1.4–1.2	0.049 (0.005)	63	70 (1)	50 (3)	149	0.902
F22 (1/2)	0.08 (0.07)		0.021 (0.003)	27	134 (3)	88 (9)	259	0.855
F23 (1/5)	0.06 (0.01)	1.2–1.1	0.049 (0.006)	64	79 (1)	59 (4)	176	0.869
F23 (1/2)	0.14 (0.01)		0.024 (0.003)	31	128 (3)	89 (8)	262	0.872
F24 (1/5)	0.018 (0.01)	1.1–0.95	0.042 (0.004)	54	79 (1)	56 (4)	165	0.901
F24 (1/2)	0.044 (0.003)		0.024 (0.003)	32	135 (2)	96 (6)	284	0.907
F25 (1/5)	0.06 (0.02)	0.95–0.85	0.033 (0.004)	43	89 (2)	60 (5)	179	0.848
F25 (1/2)	0.2 (0.1)		0.024 (0.008)	32	139 (6)	100 (18)	295	0.527
F26 (1/5)	0.038 (0.001)	0.85–0.75	0.034 (0.004)	45	86 (2)	58 (5)	171	0.862
F26 (1/2)	0.094 (0.003)		0.020 (0.002)	26	144 (3)	97 (9)	287	0.876
F27 (1/5)	0.04 (0.03)	0.75–0.65	0.041 (0.005)	54	90 (2)	67 (4)	198	0.861
F27 (1/2)	0.09 (0.06)		0.018 (0.003)	23	163 (4)	109 (13)	323	0.821
F28 (1/5)	0.012 (0.009)	0.66–0.59	0.045 (0.004)	59	89 (1)	68 (3)	201	0.919
F28 (1/2)	0.03 (0.02)		0.021 (0.004)	27	190 (6)	145 (15)	428	0.736

Results were obtained by fitting three measures. The standard error in the calculation of each parameter is expressed in parentheses.
F22–F28 (not diluted): parameters are not shown because the data did not present good correlation with the sigmoidal dose-response equation (CD formation inhibited or strongly retarded).

rest of the fractions (Table 1). These results indicate that free amino acids did not have the capacity to prevent lipid oxidation of the LDL. Finally, in the case of fractions 22–28 (0.59–1.4 kDa), no formation of CD was registered along the whole incubation period, indicating that LDL-lipid-oxidation was completely inhibited in the presence of these samples (Fig. 3A). It is important to note that the activity of the different fractions was not directly related to the total peptide concentration; for example, the fractions exhibiting the greatest antioxidant capacity (i.e., 22 to 28, C = 0.16, 0.28, 0.088, 0.31, 0.19, 0.18 and 0.06 mg/mL, respectively) were not the most concentrated samples (Table 1), indicating that the higher antioxidant effect was associated with the kind of peptides contained in these samples and not necessarily with the total concentration. For fractions 22–28, dilutions (1/2 and 1/5) were assayed, obtaining good fitting to the sigmoidal model in almost all cases; with the exception of sample F25(1/2), which produced an important retardation in the CD formation. For each sample, the antioxidant effect (increment in LT and decrease in PR) was dependent upon the peptide concentration (Table 1, Fig. 3).

In order to obtain more information about the possible action mechanisms of the amaranth peptides, other parameters related to lipid and protein oxidation in the LDL particles were measured. Polyunsaturated fatty acids of LDL lipids are the major targets of oxidizing agents, being the hydroperoxy derivative of phospholipids and the rearrangement of double bonds to form conjugated dienes the first oxidation products. Further oxidation results in short-chain aldehyde or carboxy derivatives, 4-hydroxynonenal (HNE) and malondialdehyde (MDA) among them (Levitan et al., 2010). TBA-reactive substances (TBARS), such as MDA and other substances, were measured at the end of the incubation period. ***Id*** at two concentrations (0.12 and 0.24 mg/mL) were not able to reduce them respect to the control system without antioxidants (Fig. 4A). The aldehydes may form adducts with the lysine residues of apo B. This protein modification generates fluorophores, which fluoresce strongly at 430 nm with excitation at 360 nm (Cominacini et al., 1991). Time course of fluorescence is shown in Fig. 4B for systems containing ***Id*** at two different peptide concentrations. In the absence of ***Id*** (C1 in Fig. 4B) fluorescence presented, similarly to CD evolution, an initial inhibition period and a second propagation period during which the fluorescence rose quickly. Data could be fitted to the sigmoidal dose-response (variable slope) equation (Fig. 4B). Comparing CD (Fig. 2) and fluorescence time evolution for C1 (Fig. 4B), we can note that, although LT was lower for fluorescence, CD achieved the maximal value before 100 min of incubation while fluorescent compounds formation continued up to about 200 min. These observations are in agreement with the fact that CD are intermediate products of the oxidative chain reaction while fluorescent compounds may represent at least one of the products of the final step, thus possibly matching the overall rate of apo-B modification (Cominacini et al., 1991). In presence of ***Id***, the appearance of fluorescent compounds was delayed as a function of the peptide concentration (LT increment of about 319% for 0.12 mg/mL, and 477% for 0.24 mg/mL) (Fig. 4B). The modification of the protein molecule results also in alteration of the electrophoretic mobility. LDL has a negatively charged surface and migrates to the anode in agarose gel electrophoresis under non denaturing conditions. Oxidation renders LDL more negatively charged, possibly because of derivatization of lysine residues of

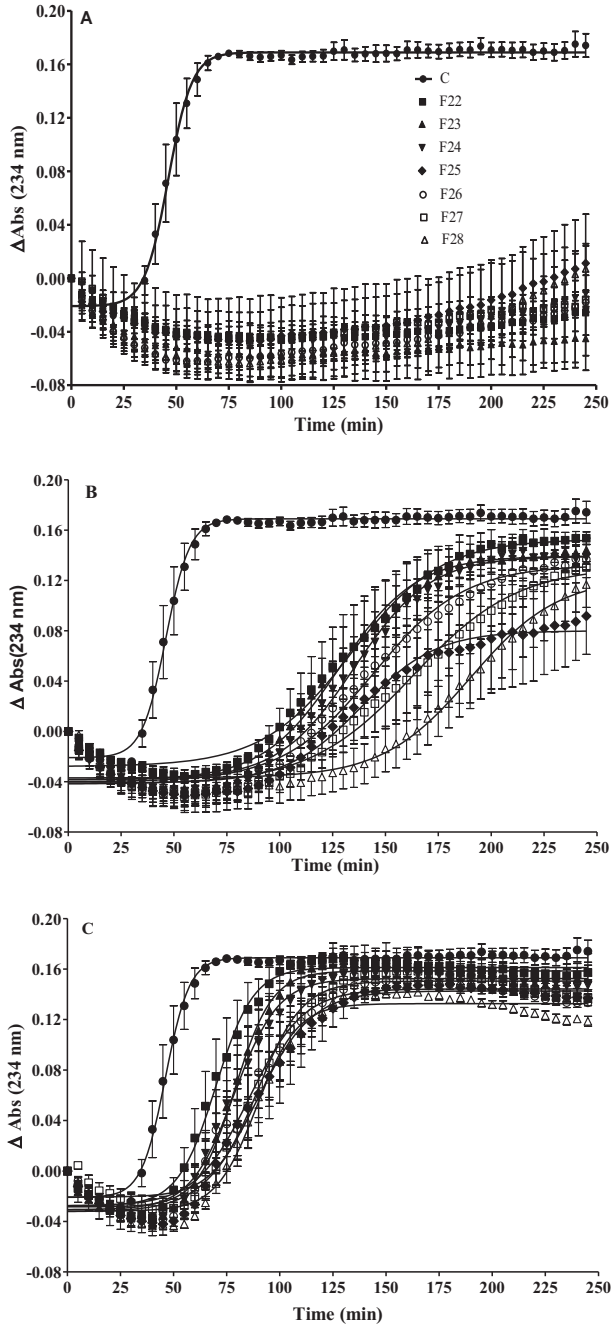

Fig. 3. Conjugated dienes (CD) evolution for ***gel filtration FPLC fractions*** (22–28) from digested amaranth protein isolate (***Id***): (A) without dilution; (B) dilution 1/2; (C) dilution 1/5.

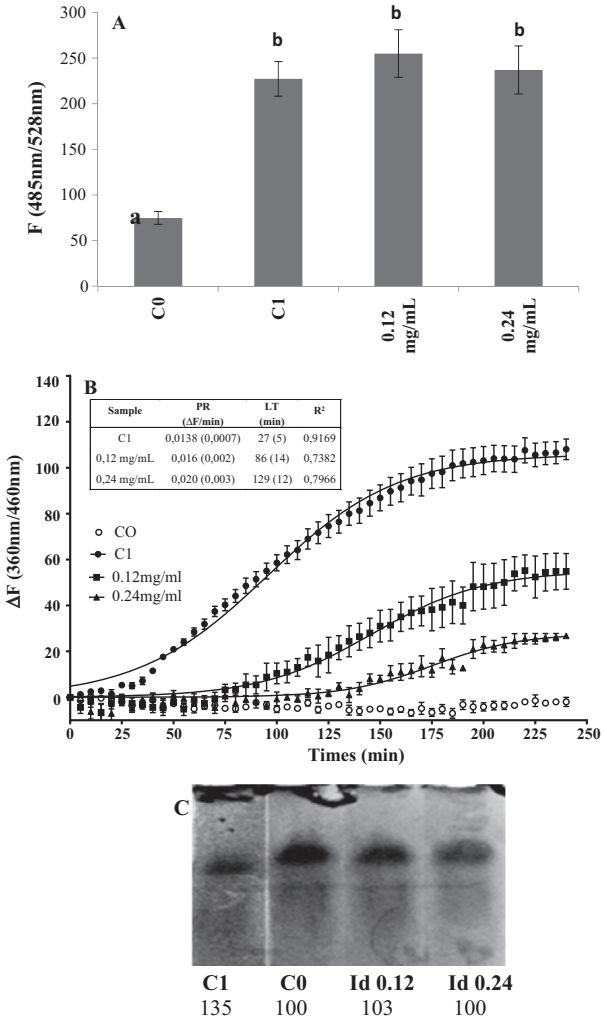

Fig. 4. Antioxidant activity of ***Id***: (A) TBA assay after 4 h of incubation at 37 °C; (B) fluorescence evolution; (C) agarose gel electrophoresis (Mobility% respect to native LDL (C0) is shown below the gel picture).

apo B-100 by some reactive aldehydes, or direct reaction of the reactive oxygen species that converts histidine and proline residues to negatively charged aspartate or glutamate (Jialal & Devaraj, 1996). Non radical oxidants such as H_2O_2, hypochlorite, and peroxynitrite tend to modify the proteins directly, especially the cysteine, methionine and tyrosine (Levitan et al., 2010). This fact could be evidenced in the present systems where Cu^{+2}/H_2O_2-oxidized-LDL (C1 in Fig. 4C) migrated longer than native LDL (C0, Fig. 4C). In presence of ***Id*** (0.12 and 0.24 mg/mL), LDL mobility was similar than for native LDL (Fig. 4C).

Gel-filtration fractions (22–28) with the greatest capacity to inhibit CD formation were also evaluated. Significant ($p < 0.05$) diminution of TBARS at the end of the incubation time respect to the system without antioxidants (C1) were registered for all the fractions, being more notorious for fractions 24 and 25 (Fig. 5A). It is important to remark that no CD formation was registered in presence of these fractions (22–28, Fig. 3A). A possible explanation to this apparent discordance between CD and TBARS results could be the difference in the sensibility of both methods due to CD were detected by absorbance measures while TBARS were detected by fluorescence measures. Also, it could not be discarded the fact that H_2O_2 has demonstrated to produce some artifacts in the TBA test performed in other systems (Cecchini, Aruoma, & Halliwel, 1990). Other more probable cause could be that TBA reaction is not specific for lipid oxidation products since sugars and amino acids may also form TBA adducts (Jialal & Devaraj, 1996). Fractions 22–28 were also very actives to prevent the formation of fluorescent compound. Fractions 27 and 28 presented a delayed beginning of the fluorescence increment (LT increment of 447 and 422% respect to the control system C1, respectively, Fig. 5C). Both effects were stronger in the case of fractions 22, 23, 24, 25 and 26, which completely inhibit the formation of fluorescent compounds (Fig. 5B and C). Results from agarose gel electrophoresis showed that fractions could totally (fractions 24, 25 and 26) or partially prevent mobility changes (in case of fractions 22, 23, 27 and 28 small increases in mobility were recorded) (Fig. 5D).

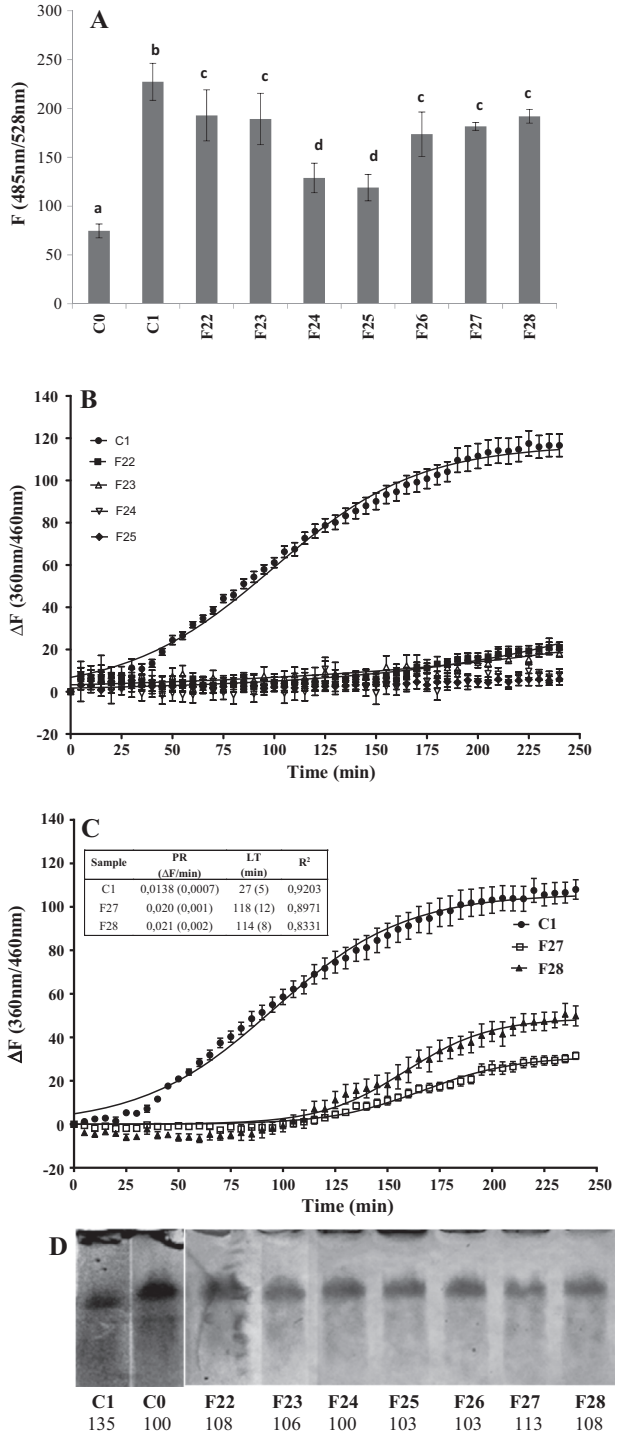

Fig. 5. Antioxidant activity of ***gel filtration FPLC fractions*** (22–28): (A) TBA assay after 4 h of incubation at 37 °C; (B) and (C) fluorescence evolution (LT and PR values are shown in the box); (D) agarose gel electrophoresis (Mobility% respect to native LDL (C0) is shown below the gel picture).

In a work from our lab (Orsini Delgado, Añón et al., 2016) fractions from a simulated gastrointestinal digest (***Id***) were separated by preparative RP-HPLC and some potential active peptides from the amaranth 11S globulin were identified by LC-MS/MS in fractions with high antioxidant activity (ORAC method). Based on previous information about structure-activity relationships, 10 of those peptides were synthesized, which are shown in Table 2. In the present work, the activity of these peptides (0.2 mg/mL) against LDL oxidation was evaluated. Respect to CD evolution, P1 (TEVWDSNEQ) showed a minor effect on the LP value, while P8 (AWEEREQGSR), P5 (YLAGKPQQEH) and P2 (IYIEQGNGITGM) produced only a decrease in the PR value (Table 2). P6 (LQAEQDDR) and P9 (AVNVDDPSK) presented some minor effect on both parameters (Table 2). However, in the case of P7 (HVIKPPSRA) CD formation was strongly retarded. Also, a very important increment in the LT and a decrease in PR for P3 (GDRFQDQHQ), and lesser effects in the case of P10 (KFNRPETT) and P4 (LAGKPQQEHSGEHQ) were registered (Table 2, Fig. 6A). The effect of the peptide concentration (0.04–1 mg/mL) on CD evolution was evaluated for the three most active peptides (P7, P3, and P10). In all cases, concentrations of 1 mg/mL produced a total inhibition of the LDL oxidation in the assay time, and both, the LT increment as well as the PR decrease, were dependent upon the peptide concentration (Table 2). Peptides with the greatest capacity to prevent CD formation (P3, P7, and P10, 0.2 mg/mL) were also able to significantly ($p < 0.05$) reduce the TBARS fluorescence; the effect was greatest for P3 and P10 (Fig. 6B). In other hand, P3 and P7 were able to completely inhibit the fluorescent products formation during the incubation time; P10 produced an increment of LT (315%) (Fig. 6C). In the agarose gel electrophoresis, these peptides prevent changes in the LDL mobility respect to native LDL (Fig. 6C).

Orsini Delgado et al. (2015) demonstrated that gastrointestinal digest from amaranth proteins have the ability to inhibit the formation of OH**.** radicals by the Fenton reaction (by metal chelators peptides) as well as to neutralize these radicals (chain-breaking by peptides that donate a hydrogen atom). These action mechanisms could be also relevant in the retardation of lipid and protein oxidation of LDL by ***Id*** and apply to the subject study of this work. Taking into account CD (Fig. 2C), TBARS (Fig. 4A), fluorescence (Fig. 4B), and electrophoresis (Fig. 4C) results, it is possible to affirm that ***Id*** was able to retard the beginning of the lipid and protein oxidation but not to completely inhibit these processes. Protein modification was delayed but it occurred in some extent as evidenced the fluorescence increase; however, these protein modifications were not enough to change the electrophoretic mobility.

FPLC fractions 22–28 (0.59–1.4 kDa) were the most active ones in the prevention and/or retardation of LDL oxidation. The antioxidant activity of gel filtration FPLC fractions by the ORAC (capacity to scavenge $ROO^{\cdot}$ radicals) and HORAC (capacity to prevent the formation of $OH^{\cdot}$) methods has been previously established (Orsini Delgado, Añón et al., 2016; Orsini Delgado, Nardo et al., 2016). In comparative terms, some of the most active fractions against LDL oxidation had also presented the highest activity by ORAC and HORAC assays (fractions 25–28), while fractions 22–24 showed lower activity by both methodologies. In addition, fractions of low molecular weights (39–42) that had presented a very high ORAC but low HORAC activities, did not present a good capacity to prevent LDL oxidation. Previous and present results indicate that fractions with molecular weights in the range of 0.59–1.4 kDa have the highest antioxidant capacity and that they could prevent LDL oxidation both by prevention of the formation of OH**.** (metal chelation) and scavenging of these radicals. It can be note that although all these fractions were able to completely inhibit CD formation (Fig. 3A), some of them were not capable to completely inhibit the fluorescence increment (fractions 27 and 28) (Fig. 5C) or completely prevent changes in electrophoretic mobility (fractions 22, 23, 27 and 28) (Fig. 5D). A possible explanation for these results would be the occurrence of some direct effect of the oxidants on the protein molecule, without the participation of lipid oxidation products, and these protein modifications would be prevented in

Table 2
Kinetic parameters obtained from sigmoidal dose-response equation for the CD evolution in systems with the presence of synthetic peptides.

Sample	C (mg/mL)	Sequence	t_{50} (min)	PR (ΔAbs/min)	PR/PR_C%	LT (min)	LT/LT_C%	R^2
C			37.0 (0.6)	0.18 (0.03)		32 (2)		0.919
P1	0.2	TEVWDSNEQ	44.0 (0.9)	0.18 (0.06)	101	39 (3)	122	0.888
P2	0.2	IYIEQGNGITGM	38.8 (0.9)	0.15 (0.04)	82	32 (3)	102	0.898
P4	0.2	LAGKPQQEHSGEHQ	76.1 (0.9)	0.055 (0.005)	31	59 (3)	186	0.973
P5	0.2	YLAGKPQQEH	44.6 (0.8)	0.070 (0.008)	39	31 (2)	98	0.962
P6	0.2	LQAEQDDR	50.6 (0.8)	0.13 (0.03)	71	43 (2)	136	0.936
P7	0.2	HVIKPPSRA	nd	nd	nd	nd	nd	nd
P8	0.2	AWEEREQGSR	43.2 (0.9)	0.11 (0.02)	59	34 (2)	108	0.935
P9	0.2	AVNVDDPSK	45.9 (0.7)	0.14 (0.03)	79	39 (2)	124	0.941
P3	0.04	GDRFQDQHQ	67 (2)	0.06 (0.01)	31	50 (6)	168	0.860
	0.1		139 (3)	0024 (0004)	13	98 (10)	332	0.755
	0.2		209 (9)	0.021 (0.005)	12	164 (19)	517	0.840
	1		nd	nd	nd	nd	nd	nd
P10	0.04	KFNRPETT	51 (2)	0.12 (0.05)	66	42 (5)	143	0.739
	0.1		75 (2)	0.07 (0.02)	40	62 (6)	210	0.702
	0.2		108 (1)	0.032 (0.003)	18	78 (4)	247	0.972
	1		nd	nd	nd	nd	nd	nd
P7	0.04	HVIKPPSRA	51 (2)	0.12 (0.05)	71	31 (1)	105	0.872
	0.1		103 (2)	0.045 (0.008)	25	81 (6)	274	0.872
	0.2		nd	nd	nd	nd	nd	nd
	1		nd	nd	nd	nd	nd	nd

Results were obtained by fitting three measures. The standard error in the calculation of each parameter is expressed in parentheses.
nd: not determined because the data did not present good correlation with the sigmoidal dose-response equation (CD formation inhibited or strongly retarded).

presence of some of the fractions (24, 25 and 26) but not in presence of others (22, 23, 27 and 28). In order to evaluate the composition of these fractions, they were analysed by RP-HPLC. Chromatograms showed that the fractions that were able to completely inhibit lipid and protein oxidation (24, 25 and 26) presented both hydrophilic and hydrophobic molecules; meanwhile, fractions 22 and 23 presented a higher content of hydrophobic molecules but lower proportion of hydrophilic molecules, and fractions 27 and 28 showed a contrary situation (higher content of hydrophilic compounds but very low proportion of hydrophobic molecules) (data not shown). These observations suggest that a combination of peptides with different characteristics would enlarge the possible action mechanisms and the ability to act in different environments. For example, the more hydrophilic molecules could prevent LDL oxidation by taking up the water soluble free radicals while other more hydrophobic peptides could interact in the water-lipid interface and scavenge lipid or protein radicals (Viana et al., 1996).

It has previously been detected that among the 10 peptides analysed, four of them presented high ORAC activity, namely: P8 > P5–P2–P1, followed by P4 > P7–P3 which presented a much lower activity. P6, P9 and P10 did not present activity in the ORAC assay with concentrations up to 1 mg/mL (Orsini Delgado, Añón et al., 2016; Orsini Delgado, Nardo et al., 2016). Comparing previous and present results, it is possible to observe that the peptides with the highest scavenging (ORAC) activities (i.e., P8, P5, P2) produced an effect mainly in the PR of LDL oxidation. All of these peptides present at least one bulky aromatic residue in their sequences (Table 2). Besides, the most active peptides against Cu^{+2}/H_2O_2-induced LDL oxidation (P7, P3 and P10) did not have high radical scavenging activity by ORAC assay. They present the following characteristics: P7 is a basic peptide that contains 3 basic residues (H, K and R) and 6 hydrophobic residues (V, I, P (2) and A); P3 is a neutral peptide that contains 2 acidic residues (D), 2 basic residues (R and H) and 1 hydrophobic residue (F); P10 is a basic peptide that contains 1 acidic residue (E), 2 basic residues (K and R) and 2 hydrophobic residues (F and P). Two of these peptides (P7 and P3) present histidine in their sequences, in different locations of the molecules (Table 2). It has been informed that histidine residues can chelate metal ion, quench active oxygen, and scavenge $OH^{\cdot}$ radical, these properties being attributed to its imidazole group (Megías et al., 2008; Udenigwe & Aluko, 2012). All of the most active peptides against LDL oxidation (i.e., P7, P3 and P10) contain hydrophobic and/or aromatic amino acids. Hydrophobic amino acids (e.g., leucine (L), proline (P)) are important for the enhancement of the antioxidant properties of peptides, since they can increase the accessibility of the antioxidant peptides to hydrophobic targets, as can be LDL in this case. Moreover, the electron-dense aromatic rings of phenylalanine (F), tyrosine (Y), and tryptophan (W) residues can contribute to the chelation of pro-oxidant metal ions whereas F can also scavenge OH**.** radicals to form more stable para-, meta-, or ortho-substituted hydroxylated derivatives (Udenigwe & Aluko, 2012). In this way, besides the presence of chelating amino acids such as histidine, other structural characteristics could modify the capacity of peptides to interact with LDL particles in order to be more capable of preventing their oxidation. The latter is demonstrated by the fact that peptides that contain histidine, showed differences in the behaviour against LDL oxidation, and this behaviour did not present a direct correlation with the scavenging capacity of oxygen-reactive species (ORAC) measured in systems where the peptides reacted directly with these species. Wang et al. (2016) informed that negatively charged peptides may decrease the initiation rate of LDL oxidation by reacting with the Cu^{+2} via a metal chelating mechanism; in our case, acidic peptides P1, P2, P6, P8 and P9 produced some slight effects on LT and/or PR in the CD formation (Table 2). These authors also reported that positively charged fractions with high content of basic amino acids (arginine, lysine and histidine) may be responsible for the high inhibition ability and that may act as chain terminators and prevent the propagation of LDL oxidation by hydrogen donation and lipid radical scavenging. That would be consistent with our results since the most active peptides detected in the present work are cationic (P7 and P10) or neutral (P3) peptides.

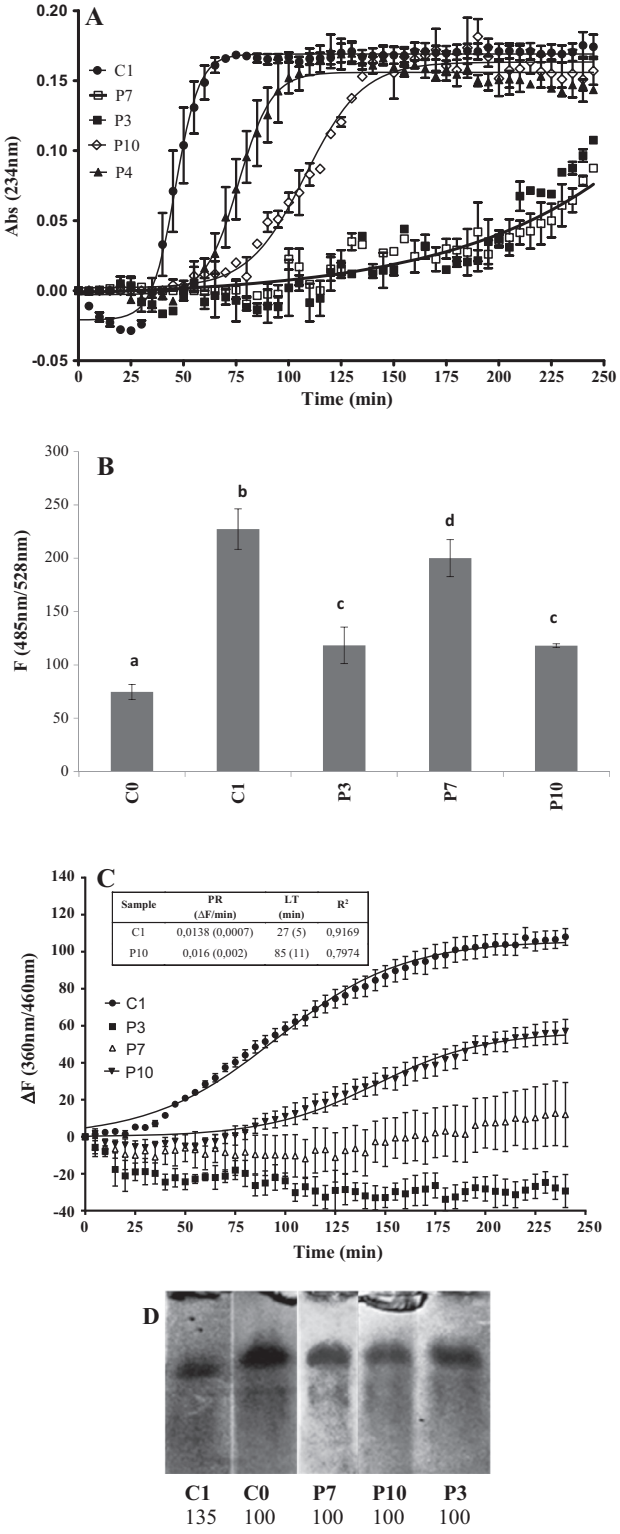

Fig. 6. Antioxidant activity of ***peptides*** (P3, P4, P7 and P10): (A) Conjugated dienes (CD) evolution; (B) TBA assay after 4 h of incubation at 37 °C; (C) fluorescence evolution (LT and PR values are shown in the box); (D) agarose gel electrophoresis (Mobility% respect to native LDL (C0) is shown below the gel picture).

4. Conclusions

The present work studied the capacity of amaranth peptides generated by simulated gastrointestinal digestion to inhibit or retard the oxidation of LDL particles. The whole amaranth protein digest ***Id*** was able to retard the onset and decrease the propagation rate but not completely inhibit the CD and fluorescent compounds formation in the assayed protein concentration range; however, electrophoretic mobility of LDL was not modified. Separation by gel filtration allowed the identification of the most active fractions (0.59–1.4 kDa), attaining a complete inhibition of CD, partial or total prevention of fluorescence compounds formation and electrophoretic changes with peptide concentrations that were similar to those used for ***Id***. Also, pure peptides were evaluated indicating that the presence of histidine as well as of hydrophobic and aromatic residues would be important in the inhibition of Cu^{+2}/H_2O_2-induced LDL oxidation. The most active peptides were cationic or neutral peptides. The present results suggest that, following the gastrointestinal digestion process, ingested amaranth proteins would be able to produce peptides with the capacity to prevent the *in vivo* LDL oxidation. According to their physicochemical characteristics, different peptides could act by different mechanisms and/or in different environments (water bulk, water-lipid interface) preventing lipid and/or protein oxidation of LDL. These peptides should be able to pass the intestinal barrier and resist serum peptidases. In that sense, preliminary assays in our lab have showed that some of the molecules in the most antioxidant fractions would be able to be absorbed. Further studies are currently underway in order to confirm this hypothesis. Furthermore, *in vivo* action after amaranth ingestion in an animal model will be evaluated.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.04.032.

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