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Structure–mechanism relationship of antioxidant and ACE I inhibitory peptides from wheat gluten hydrolysate fractionated by pH

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ABSTRACT

The aims of this study were to assess bioactive properties (ACE inhibition and antioxidant capacity) from wheat gluten hydrolysate peptides fractionated by pH (4.0, 6.0 and 9.0), to determine peptide action mechanism, and to relate it to the secondary structure and functional groups of peptides. Gluten hydrolysate extracts (GHE) were enriched in peptides with medium hydrophobicity and molecular weight (≈60% MH and 5.5 kDa, respectively). Gluten peptides inhibited ACE I by uncompetitive mechanism and a direct relationship between α-helix structure and IC50% value was obtained ($r = 0.9127$). TEAC and cooper chelating activity from GHE 6.5 were the highest and directly correlated with MH peptides. GHE 9.0 had high carotene bleaching inhibition (47.5 ± 0.3%) and reducing power activity (163.1 ± 2.9 mg $S_2O_3^{2-}$ equivalent g^{-1} protein), which were directly related to disulfide bonds content of peptides ($r = 0.9982$ and 0.9216, respectively). pH was a good alternative to select bioactive peptides from wheat gluten hydrolysate.

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1. Introduction

Traditionally, dietary proteins are regarded as a source of energy and essential amino acids, which are needed for growth and maintenance of physiological functions. Recently, interest has been emerging to identify and characterize bioactive peptides from plant and animal sources (Sarmadi & Ismail, 2010). Among dietary vegetable protein sources, wheat gluten becomes highly relevant as it is a by-product of wheat starch process available in large amounts at relatively low cost. Because it is insoluble in water at near-neutral pH and it is viscoelastic when hydrated, gluten is mainly used to enhance the strength of flours for breadmaking and for textured food (Babiker, Fujisawa, Matsudomi, & Kato, 1996; Popineau, Huchet, Larré, & Bérot, 2002). However, the use of these proteins as a source of bioactive peptides is very scarce.

In recent years, a wide range of spectrophotometric assays has been adopted to measure antioxidant capacity of foods. Most of the assays employ the same principle: a synthetic colored radical or redox-active compound is generated, and the ability of the sample to scavenge the radical or to reduce the redox-active compound is monitored by spectrophotometer, applying an appropriate standard to quantify antioxidant capacity (Floegel, Kim, Chung, Koo, & Chun, 2011). Furthermore, there are two types of assays: assays based on hydrogen atom transfer (HAT) reactions (e.g., $ABTS^{+}$) and assays based on electron transfer (ET) like β-carotene-linoleic acid assay (Huang, Ou, & Prior, 2005).

Regarding gluten proteins, few works have focused on producing antioxidant peptides. On the other hand, there are works that have fractionated gluten peptides by ultrafiltration (Cui, Kong, Hua, Zhou, & Liu, 2011; Wang, Zhao, Zhao, & Jiang, 2007). Nevertheless, the use of pH for fractionate wheat gluten hydrolysate has not been addressed. Moreover, there is no information about the relationship between bioactive gluten peptide properties and secondary structure or functional groups.

In recent years, much research has been focused on the generation of ACE I inhibitory peptides derived from food proteins (Segura Campos, Peralta González, Chel Guerrero, & Betancur Ancona, 2013). In this regard, peptides were produced from milk (Mullally, Meisel, & FitzGerald, 1997), maize (Yano, Suzuki, & Funatsu, 1996), wheat germ (Matsui, Li, & Osajima, 1999), etc. These peptides have frequently been reported to act as competitive inhibitors of ACE (Je, Park, Jung, Park, & Kim, 2005; Lee, Qian, & Kim, 2010). However, in recent years, some noncompetitive and uncompetitive ACE-inhibitory peptides have also been isolated. Although some studies have demonstrated the relationship between ACE-inhibitory activities and peptide structures, only few studies discussed about mechanism of their inhibition mode (Chia-Ling, Shih-Li, & Kuo-Chiang, 2012). Therefore, the aims of this study were (i) to assess bioactive properties (ACE inhibition and antioxidant capacity) from peptides of wheat gluten hydrolysate

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fractionated by pH and (ii) to establish the action mechanism and relate it to the secondary structure and functional groups.

2. Materials and methods

2.1. Reagents

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The alkaline protease (Protex 6 L) was provided by Danisco S.A. (Arroyito, Córdoba, República Argentina). Commercial vital gluten was supplied by Molinos SEMINO S.A. (Carcarañá, Santa Fe). Gluten composition in dry basis was moisture: 5.95 g 100 g^{-1} (AACC 44-15A method), protein (Nx5.7): 77.20 g 100 g^{-1} (Kjeldahl—AACC 46-11 method); starch: 13.15 g 100 g^{-1} (Ewers polarimetric method); ether extract: 0.71 g 100 g^{-1} (AACC 30-25 method); and ash: 0.834 g 100 g^{-1} (ICC No. 104—IRAM No. 15851 Standard technique). In order to disperse vital gluten in water and have a uniform suspension, a moderate thermal treatment was carried out according to Drago, González, and Añón (2008). Samples of thermal-treated gluten (TTG) were used as substrate of protein hydrolysis reaction.

2.2. Hydrolysate preparation

The hydrolysate were obtained using 500 mL batch thermostatized reactor. The reaction pH was continuously measured using pH-meter IQ Scientific Instruments and adjusted by adding base (NaOH) with a burette. Substrate concentration was 8 g 100 g^{-1} dispersion. Reaction parameters for hydrolysis reaction were as follows: temperature, 60 °C; pH 9.5; E/S ratio, 600 DU g^{-1} protein, and time, 2.5 h. Once the hydrolysis was finished, the enzyme was inactivated by thermal treatment following the manufacturer guidelines.

Hydrolysis reaction progress was followed by means of the pH-stat technique (Adler-Nissen, 1986), and the degree of hydrolysis (DH) was calculated as follows:

$$\mathrm{DH}\,(\%) = \left(h \times h_{tot}^{-1}\right) \times 100 = B \times N_b \times \alpha^{-1} \times \mathrm{MP}^{-1} \times h_{tot}^{-1} \times 100$$

where h_{tot} is the total number of peptide bonds in the protein substrate (8.3 mEq/g protein), h is the number of peptide bonds cleaved during hydrolysis, B is the base consumption (mL), Nb is the base molarity, α is the average degree of the dissociation of the α-NH groups (at pH 9.0, $\alpha = 1$), and MP is the protein mass (g).

2.3. Preparation of gluten hydrolysate extracts at different pH

In order to obtain the hydrolysate extracts at different pH (4.0, 6.5, and 9.0), a 2 g 100 g^{-1} dry basis solution of the different hydrolysates was prepared (Drago & González, 2001). The pH was achieved by adding 0.8 mol L^{-1} HCl or 0.8 mol L^{-1} NaOH. The samples were stirred for 1 h at room temperature, and then centrifuged during 15 min at 8000×g at room temperature. The supernatant (the extract at each pH) was lyophilized and protein content was determined using LECO micro-analyzer (Leco Corporation, USA). Gluten hydrolysate extracts at different pH levels were, namely, GHE 4.0, GHE 6.5, and GHE 9.0 for pH 4.0, 6.5, and 9.0, respectively.

2.4. Characterization of gluten hydrolysate extracts

2.4.1. Reverse phase HPLC (RP-HPLC)

Gluten hydrolysate extracts were diluted to a protein (Nx5.7) concentration of 2.5 g L^{-1} with buffer, filtrated by 0.22 μm, and analyzed by RP-HPLC using a Sephasil Peptide C8 column of 12 mm ST 4.6/250 (Pharmacia Biotech), together with an auto injector Waters 717 Plus Autosampler—Millipore, a Waters 600 E pump (Multisolvent Delivery System- Millipore), and a diode array detector (Waters 996-Millipore). Peptides were separated and eluted at 1.1 mL min^{-1}, at 60 °C, by using the following buffers: Buffer A: acetonitrile–water 2:98, with 650 mL/l of trifluoroacetic acid (TFA); Buffer B: acetonitrile–water 65:35 with 650 mL min^{-1} of TFA, and detected at 210 nm. Since elution profiles of RP-HPLC can be grouped in categories according to the increasing hydrophobicity of the eluted peptides (Linares, Larré, & Popineau, 2001), chromatogram analysis was carried out by integrating peak areas in three sections of each chromatogram: components of low molecular weight and low hydrophobicity: 0–20 min of elution range (LH); components of medium molecular weight and medium hydrophobicity: 20–40 min of elution range (MH); and components of high molecular weight and high hydrophobicity: 40–60 min of elution range (HH).

2.4.2. Estimation of peptide chain length (PCL)

PCL can be estimated as was decrypted by Adler-Nissen (1986) by means of the following expression:

$$\mathrm{PCL} = \text{Soluble proteins fraction} \times h_{tot} / h$$

where h_{tot} is the total number of peptide bonds in the protein substrate (8.3 mEq/g protein) and h is the number of free amine group.

2.4.3. Determination of thiol groups and disulfide bonds

Thiol groups (—SH) and disulphide bonds (S-S) in gluten hydrolysate extracts were determined colorimetrically with 5.5′-dithiobis (2-nitrobenzoic acid) (DTNB) using the method of Anderson and Wetlaufer (1975), modified by Graveland et al. (1985). For the determination of thiol groups, samples were dissolved in 1 mL of 0.1 mol L^{-1} sodium phosphate buffer pH 7.0 containing 6.0 mol L^{-1} urea, 0.05 M SDS, and 1 mmol L^{-1} EDTA. Then 0.1 mL of 0.01 mol L^{-1} DTNB dissolved in the same buffer was added, and after 5 min, the absorbance was measured at 412 nm. Disulfide bonds were measured after alkaline cleavage. One milliliter of each sample dispersed in the same buffer was mixed with 1 mL 6.0 mol L^{-1} NaOH and incubated for 30 min at 50 °C. The reaction mixture was neutralized by adding 2.0 mol L^{-1} H_3PO_4 (1.0 mL), mixed thoroughly, and the chromophore was developed by adding 0.1 mL of 0.01 mol L^{-1} DTNB solution. Bovine serum albumin was used as standard (Kella & Kinsella, 1985). Disulfide bonds were estimated as:

$$\text{S-S}\,(\mu\text{mol}) = (\text{total SH - free SH}) \times 0.5$$

2.4.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to Laemmli (1970), using a Mini-Protean II Electrophoresis cell (Bio-Rad) equipment with a Model 200/2.0 Bio-Rad source, with 4–15% w/v polyacrylamide gel gradient in 25 mmol L^{-1} Tris–HCl, pH 8.3, 0.18 mol L^{-1} glycine, and 0.1% w/v SDS. The separation was carried out at 180 V for 2 h. Samples were treated with and without β-mercaptoethanol. Gel plates were fixed and stained with a solution containing 0.125% w/v Coomassie blue R-250, 50% v/v methanol, and 10% v/v acetic acid in water and then discolor with 25% v/v methanol and 10% v/v acetic acid.

2.4.5. FTIR analysis

Infrared spectra of gluten hydrolysate extracts were acquired using a Perkin Elmer Spectrum RX FT-IR System (Perkin Elmer, Waltham, MA, USA). A 2 mg sample was mixed with 200 mg KBr and maintained at ambient temperature. Spectra were obtained in the 4000–500 cm^{-1} range by accumulation of 60 scans at 4 cm^{-1} resolution. FTIR spectra were recorded using the Origin software version 7.5 (OriginLab, Northampton, MA, USA). The interpretation of the changes in the overlapping amide I band (1600 –1700 cm^{-1}) components was made possible by deconvolution using the same software. A linear baseline

between 1600 cm^{-1} and 1700 cm^{-1} was formed and the baseline was linearly corrected. All experiments were performed in triplicate.

2.4.6. Fast protein liquid chromatography (FPLC)

Gel filtration chromatography was carried out in an AKTA Prime system equipped with a Superdex 75 column (GE Life Sciences, Piscataway, NJ, USA). Injection volume was 100 μL (10 mg GHE protein mL^{-1}) and elution was carried out using 50 mmol L^{-1} potassium phosphate buffer pH 7.0 plus 150 mmol L^{-1} NaCl at 1 mL min^{-1}. Elution was monitored at 280 nm, and molecular mass was estimated using molecular weight (MW) standards from Pharmacia: ferritin (440,000 Da), conalbumin (75000 Da), carbonic anhydrase (29000 Da), ribonuclease A (13700 Da), and glycine (75 Da).

2.5. Bioactive properties of gluten hydrolysate extract

2.5.1. Angiotensin-converting enzyme activity (ACE) inhibition and inhibition mechanism

Angiotensin-converting enzyme activity inhibition was determined according to Hayakari, Kondo, and Izumi (1978), and antihypertensive activity was expressed as ACE inhibition (%). The antihypertensive activity from gluten hydrolysate extracts was evaluated at 4.5 g L^{-1} proteins. To determine the concentration causing an inhibition of 50% of ACE (IC_{50}%), serial dilutions of gluten hydrolysate extracts from 0 to 10 g L^{-1} protein were made and the data were fitted with the following equation:

$$y = a\text{-} \left(b\, c^{x}\right)$$

where y is the inhibition rate; a, b, and c are the regression parameters; and x is the protein concentration (g L^{-1}). The IC_{50}% value was obtained as follows:

$$IC_{50} = \ln\left[(a-50)\, b^{-1}\right] \ln c$$

To determine inhibition mechanism, different substrate (HHL) concentrations (2.8 mmol L^{-1}, 4.2 mmol L^{-1}, and 8.4 mmol L^{-1}) were incubated with ACE I solution with and without gluten hydrolysate extracts at 5 g L^{-1} of protein. The ACE-inhibitory activity was determined as described before. The kinetics of ACE in the presence of the inhibitory peptides from hydrolysate extracts was determined using Lineweaver–Burk plots according to Ni, Li, Liu, and Hu (2012).

2.5.2. Trolox equivalent antioxidant capacity (TEAC)

To estimate the antioxidant capacity, $ABTS^{+}$ radical cation discoloration assay according to Cian, Martínez-Augustin, and Drago (2012) was used. To determine TEAC, a concentration–response curve for the absorbance (at 734 nm) of $ABTS^{+}$ as a function of concentration (0–2.5 mmol L^{-1}) of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard solution in 0.01 mmol L^{-1} (PBS, pH 7.4) was performed. The absorbance reading was taken at 6 min after mixing. TEAC from gluten hydrolysate extracts was assayed at 2.3 g L^{-1} of protein. To determine the concentration causing an inhibition of 50% (IC_{50}%), serial dilutions of gluten hydrolysate extracts from 0 to 10 g L^{-1} protein were made, and the data were fitted as mentioned before.

2.5.3. Reducing power activity assay

The reducing power activity was determined according to the method of Oyaizu (1986). Gluten hydrolysate extracts were mixed with 0.2 mL of 0.2 mol L^{-1} phosphate buffers (pH 6.6) and 0.2 mL of 1% (w/v) potassium ferricyanide. The concentration of gluten hydrolysate extracts was 5.7 g L^{-1} of protein. The mixture was incubated at 50 °C for 20 min. Then 0.5 mL of 10% (w/v) trichloroacetic acid was added and 0.2 mL of the mixture was mixed with 0.2 mL of distilled water and 40 μL of 0.1% (w/v) ferric chloride. After standing at room temperature for 10 min, the absorbance was measured at 700 nm. The same volume of distilled water instead of the sample was used for the blank. The reducing power activity was expressed as mg $S_2O_3^{2-}$ equivalent g^{-1} protein using a concentration–response curve of $S_2O_3^{2-}$ solution (0–1240 mg L^{-1}).

2.5.4. Copper-chelating activity by assay of β-carotene oxidation

Copper-chelating activity was determined by the assay of β-carotene oxidation according to Megías et al. (2008) with modifications. A solution of β-carotene 10 g L^{-1} in chloroform was prepared. After the addition of 1 mL Tween 20, chloroform was evaporated under nitrogen and then suspended in 0.1 mol L^{-1} pH 7.0 phosphate

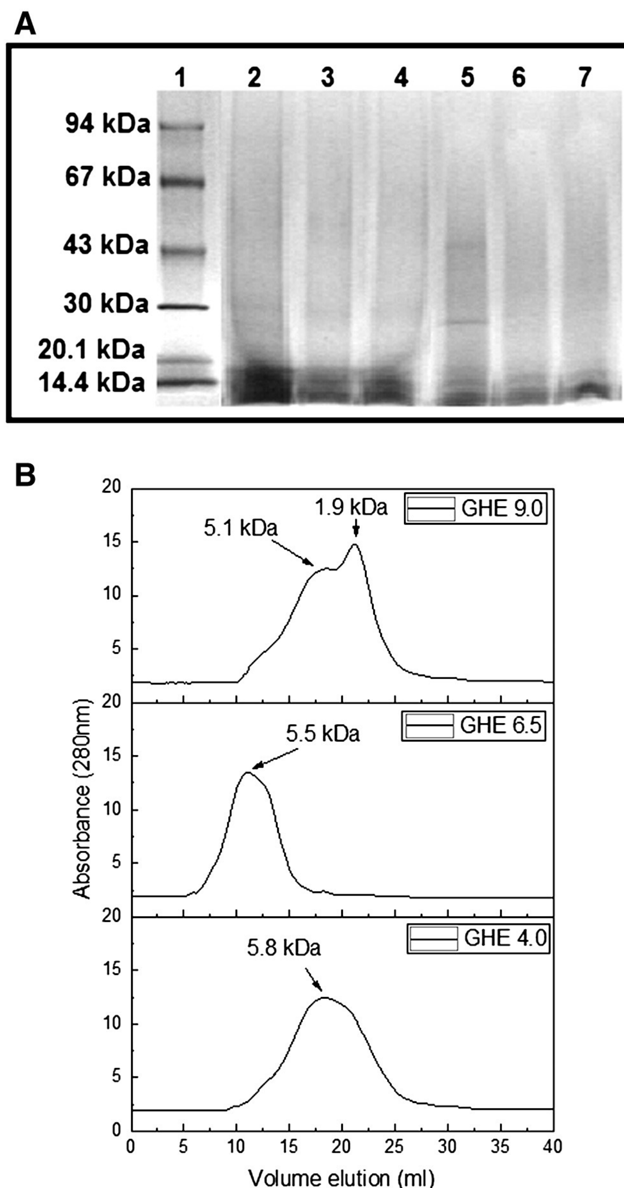

Fig. 1. (A) SDS–PAGE analysis of gluten hydrolysate extract (GHE) obtained at different pHs (4.0, 6.5, and 9.0) with and without β-mercaptoethanol. Lane 1: low molecular weight markers; lanes 2, 3, and 4: GHE 9.0, 6.0, and 4.0 with β-mercaptoethanol, respectively; lines 5, 6, and 7: GHE 9.0, 6.0, and 4.0 without β-mercaptoethanol, respectively. (B) FPLC gel filtration profile of gluten hydrolysate extracts (GHE) obtained at different pHs (4.0, 6.5, and 9.0) with molecular weights lower than 20 kDa. Gel filtration chromatography was carried out with a Superdex 75 column. Injection volume was 100 μL (10 mg protein mL^{-1}). Elution was carried out using 50 mmol L^{-1} potassium phosphate buffer pH 7.0 plus 150 mmol L^{-1} NaCl at 1 mL min^{-1}. The profile shown is representative of several that were carried out.

Table 1
Protein content, thiol group content (−SH), disulfide bonds content (S-S), and average peptide chain length (PCL) from of gluten hydrolysate extracts (GHE) obtained at different pHs (4.0, 6.5, and 9.0).

Sample	Protein (g 100 g^{-1} dw)	S-S (μmol g^{-1} protein)	-SH (μmol g^{-1} protein)	PCL
GHE pH 4.0	81.4 ± 0.5^a	12.7 ± 1.0^a	4.7 ± 0.4^a	15.7 ± 0.8^a
GHE pH 6.5	82.1 ± 0.0^a	11.6 ± 0.8^a	6.5 ± 0.3^a	16.6 ± 0.8^a
GHE pH 9.0	81.9 ± 0.1^a	24.7 ± 1.7^b	14.4 ± 1.1^b	16.2 ± 0.3^a

Results are expressed as mean value ± standard deviation, dw (dry weight). Different letters in the same row mean significant differences between samples ($p < 0.05$), according to Fisher's least significant difference test.

buffer. The assay mixture was as follows: 200 μl β-carotene, 10 μl of 50 μmol L^{-1} $CuSO_4$, and 200 μl sample. The concentration of gluten hydrolysate extracts was 0.2 g L^{-1} of protein. The degradation of β-carotene was monitored by recording the decrease in absorbance at 470 nm.

2.5.5. β-carotene-linoleic acid assay

This assay was carried out according to the method described by Pedroche et al. (2007). One milliliter of 0.2 g L^{-1} β-carotene solution in chloroform was added to a mixture of 20 mg of linoleic acid and 200 mg of Tween 20. Subsequently, the chloroform was eliminated by evaporation and 50 mL of water rich in oxygen obtained by bubbling oxygen during 15 min was added. Aliquots of 2.5 mL of this mixture were added to Pirex tubes containing 100 μL of the sample. Distilled water and 2, 6-di-tert-butil-4-metil-fenol (BHT) solution dissolved in ethanol (800 mg L^{-1}) were used as negative and positive controls, respectively. Samples were incubated at 50 °C, and aliquots at different times were taken (0 to 2 h). Absorbance at 470 nm was determined in every aliquot for following the discoloration of β-carotene. The concentration of gluten hydrolysate extracts was 0.2 g L^{-1} of protein. The results were expressed as β-carotene bleaching inhibition (%) as follows:

$$\beta\text{-carotene bleaching inhibition } (\%) = (Abs_{t=n} / Abs_{t=0}) \times 100$$

2.5.6. Statistical analysis

Results were expressed as mean ± standard deviation and were analyzed by analysis of variance (ANOVA). Means were tested with the Fisher's least significant difference test for paired comparison, with a significance level α of 0.05, using the Statgraphics Plus version 5.1 software (Statgraphics, USA). Simple linear regression was used to find correlation between GHE bioactivities and their secondary structure, hydrophobicity/size, and thiol group. Principal component analysis was performed to show the relationship between bioactive properties and secondary structure/composition from GHE.

3. Results and discussion

3.1. Characterization of gluten hydrolysate extract at different pH

The degree of hydrolysis (DH) of thermal-treated gluten (TTG) obtained by hydrolysis with the protease was 7.35 ± 0.28 %. This medium value of DH can be attributed to the endoprotease activity of bacterial enzyme, which has limited specificity, resulting in DH not higher than 20%–25% (Segura Campos et al., 2013).

The electrophoresis profile of gluten hydrolysate extracts (Fig. 1A) shows components with molecular weight (MW) lower than 14.4 kDa (lines 2 to 4), indicating an effective degradation of gluten proteins by enzymatic hydrolysis process.

GHE 9.0 had components with MW around 43 and 30 kDa (line 5), which correspond to peptides bounded through S–S bridges. These peptides were formed by components lower than 20.1 kDa (line 2), evidenced after β-mercaptoethanol treatment. In contrast, the extracts at pH 4.0 and 6.5 lacked these components corresponding to 30 and 43 kDa (lines 6 and 7). These results are in agreement with thiol groups (−SH) and disulfide bond content (S-S) from extracts (Table 1). In this regard, GHE 9.0 had the highest content of S-S and −SH. This could be explained taking into account that at pH 9.0, the rupture and reduction of intermolecular S-S take place. The pK of the −SH group is about 8.0, and at pH higher than 8.0, thiol group interchange reactions could occur (Mutilangi, Panyam, & Kilara, 1996). Thus, at pH 9.0, it is possible to extract components richer in S-S and SH than at pH 4.0 or 6.5.

Fig. 1B shows the FPLC gel filtration profile of GHE 4.0, GHE 6.5, and GHE 9.0, which have components with molecular weight (MW) lower than 20 kDa. All profiles show a main peak corresponding to MW around 5.5 kDa. This could be attributed to the break pattern of this protease, which generated peptides with medium MW. Moreover, GHE 9.0 profile shows a second peak, which correspond to components of around 1.9 kDa.

Table 2 shows the percentage of each area (LH, MH, and HH) with respect to the total area of the reverse phase HPLC (RP-HPLC) chromatogram. All extracts exhibited the same proportion of protein components. The main components were medium hydrophobicity and medium MW (20–40 min zone). As mentioned before, the break pattern of this protease generates peptides with medium MW (5 kDa), which are soluble and less pH dependent (Cian, Drago, Betancur-Ancona, Chel-Guerrero, & González Rolando, 2010). This agrees with PCL results (Table 1) because PCL values were similar in all extracts (≈16).

Fourier transform infrared (FTIR) analysis was used to investigate secondary structure of peptides from GHE. As shown in Fig. 2A, a typical FTIR spectrum for protein extract was obtained. A peak occurring at 1650 cm^{-1} is characteristic of the Amide I band. This peak is originate from the C = O stretching with some contribution of the N-H vibration (Wang, Su, Jia, & Jin, 2013). Between 1480 and 1575 cm^{-1}, another broad peak (amide II) appears, and it is due to NH bending combined with CN stretching (Georget & Belton, 2006). These results are in agreement with that found by Feeney et al. (2003) for synthetic and recombinant peptides from wheat glutenin.

Table 2
Percentage of each area respect to the total integrated area of RP-HPLC chromatograms and percentage of secondary structure segments of total area of amide I band from gluten hydrolysate extracts (GHE) obtained at different pHs (4.0, 6.5, and 9.0).

Sample	LH (%) (0–20 min)	MH (%) (20–40 min)	HH (%) (40–60 min)	α-Helix and random coil (%)	β-Sheet (%)	Antiparallel β-sheets (%)	β-Turn (%)	α-Helix/β-structure (flexibility)
GHE pH 4.0	10.3	64.6	26.1	22.6	14.1	26.1	25.5	0.89
GHE pH 6.5	11.8	60.1	28.1	26.7	14.9	19.8	29.3	0.91
GHE pH 9.0	12.3	60.9	26.8	10.1	12.2	14.3	28.5	0.35

HH: high hydrophobicity and molecular weight components; MH: medium hydrophobicity and molecular weight components; LH: low hydrophobicity and molecular weight components.

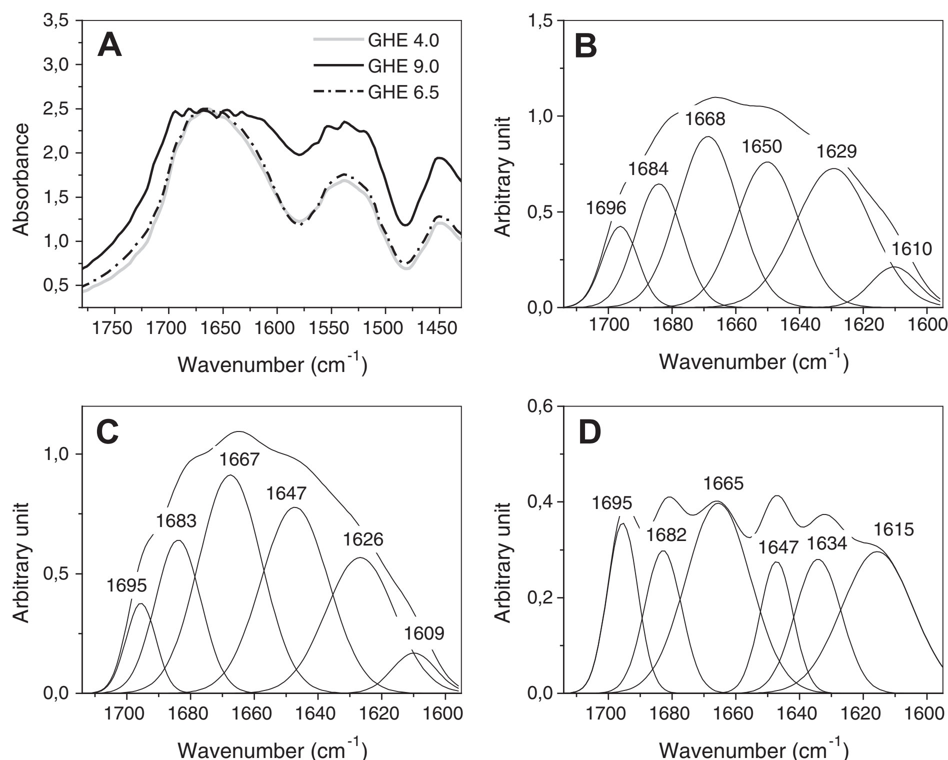

Fig. 2. FTIR spectrum of gluten hydrolysate extracts (GHE) obtained at different pHs (4.0, 6.5, and 9.0) (A). Deconvoluted infrared spectra of amide I band from GHE 4.0, 6.0, and 9.0 (B, C, and D, respectively).

The deconvolution of amide I band showed that it was constituted by at least six components (Fig. 2B, C, and D), which were around at 1696, 1684, 1668, 1650, 1630, and 1615 cm^{-1}, respectively. These six components were all corresponding to the different secondary structure (Wang et al., 2013). The two bands at 1630 and 1684 cm^{-1} were the amide groups involved in the extended β-sheet structure, while the band at 1655 cm^{-1} arose from either, the α-helix, or the random coil structures (Feeney et al., 2003). The 1668 cm^{-1} component can be due to the presence of β-turns, which form the β-spiral structures in the repetitive domain of the high molecular weight glutenins but are also found in gliadins. The 1616 cm^{-1} band is interesting because the 1685/1616 cm^{-1} band pair is very characteristic for intermolecular β-sheet in aggregated proteins. However, bands around 1616–1625 cm^{-1} can also be found in the solution spectra of poly-L-proline or peptides, which contain Pro residues in a hydrated extended chain structure (Feeney et al., 2003). To investigate the contribution of protein secondary structure of amid I band in FTIR spectrum, the percentage of each protein secondary structure was calculated as the ratio of its deconvoluted peak area to the total area of the amide I band (Wang et al., 2013). Secondary structures of GHE obtained from band deconvolution of FTIR spectrum were listed in Table 2. No obvious changes in content of β-sheet and β-turn from GHE were found. However, α-helix and random coil structures content of GHE 9.0 was lower than that obtained for GHE 4.0 and GHE 6.5. This reduction of α-helix structure can be attributed to the deamidation of glutamine at alkaline pH, which is critical in the formation of antiparallel helices of different cereal proteins (Zhang, Luo, & Wang, 2011). Moreover, the molecular flexibility of GHE 9.0, estimated by α-helix/β-structure ratio was lower than that obtained for GHE 4.0 and GHE 6.5. This may be due to the higher S-S and –SH contents of GHE 9.0 (Table 1), which increase molecule stiffness and favor disulfide bridges, respectively (Masci, D'Ovidio, Lafiandra, & Kasarda, 1998).

3.2. Bioactive properties of gluten hydrolysate extracts at different pH

3.2.1. Angiotensin-converting enzyme activity (ACE) inhibition

As shown in Table 3, the IC_{50}% value (amount of peptide required to inhibit 50% of the ACE activity) of GHE 9.0 was lower than those obtained for GHE 4.0 and 6.5. Therefore, this extract exhibits the greatest ACE-inhibitory activity. These results were similar to those found by Thewissen, Pauly, Celus, Brijs, and Delcour (2011) for tryptic wheat gliadin hydrolysates. It is important to note that serine proteases, such as Protex 6 L, tend to produce peptides whose C-terminals are amino acids with large side chains and no charge (aromatic and aliphatic amino acids), such as Ile, Leu, Val, Met, Phe, Tyr, and Trp (Segura Campos et al., 2013). These amino acids residues at C-terminal position generally show high ACE-inhibitory activity (Wijesekara, Qian, Ryu, Ngo, & Kim, 2011). In this sense, high ACE-inhibitory activity from GHE could due to the presence of these amino acids at C-terminal position. The hydrophilic–hydrophobic partitioning in the sequence is

Table 3
Protein concentration required to produce 50% of ACE I inhibition and radical cation ABTS+ (IC50), cooper chelating activity (CCA), carotene bleaching inhibition (CBI), and reducing power activity (RPA) from gluten hydrolysate extracts (GHE) obtained at different pHs (4.0, 6.5, and 9.0).

Sample	ACE I inhibition (IC_{50}: g L^{-1} protein)	$ABTS^{+}$ inhibition (IC_{50}: g L^{-1} protein)	CCA (%)	CBI (%)	RPA (mg $S_2O_3^{2-}$ equivalent g^{-1} protein)
GHE pH 4.0	0.37 ± 0.02^b	1.62 ± 0.06^b	33.8 ± 0.8^a	42.0 ± 1.8^a	150.4 ± 3.8^b
GHE pH 6.5	0.63 ± 0.02^c	1.50 ± 0.01^a	47.0 ± 0.7^c	41.9 ± 1.7^a	139.7 ± 0.8^a
GHE pH 9.0	0.21 ± 0.02^a	1.68 ± 0.03^b	39.8 ± 1.7^b	47.5 ± 0.3^b	163.1 ± 2.9^c

Results are expressed as mean value ± standard deviation. Different letters in the same row mean significant differences between samples ($p < 0.05$), according to Fisher's least significant difference test.

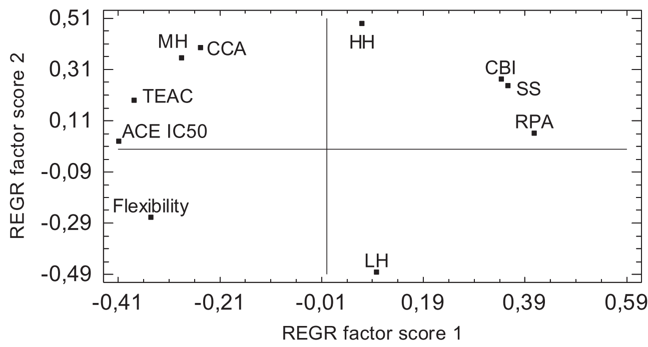

Fig. 3. PCA graphic representation of gluten hydrolysate extracts (GHE) obtained at different pHs (4.0, 6.5, and 9.0), factors one and two. Variable used were percentages of total area from integrated areas of RP-HPLC chromatograms (LH, MH, and HH), flexibility, disulfide bonds content (SS), and correlated to peptide bioactive properties (TEAC, CCA, ACE_{IC50}, RPA, and CBI).

also a critical factor in ACE-inhibitory activity (Sheih, Fang, & Wu, 2009). However, no relationship was found between hydrophobicity/size and IC_{50}% values of GHE (Fig. 3), indicating that sequence of peptides from GHE are mainly responsible of ACE-inhibitory activity. It is noteworthy that a direct relationship between α-helix and IC_{50}% value ($r = 0.9127$) from GHE was obtained. In this context, several authors have suggested that in the case of long peptides (≈5.5 kDa), it is expected that peptide conformation, i.e., the structure adopted in the specific environment of the binding site will influence binding to ACE, and therefore the ACE-inhibitory capacity of these peptides (Alemán, Giménez, Pérez-Santin, Gómez-Guillén, & Montero, 2011).

To clarify kinetically the inhibition mechanism, three sets of rate experiments for each GHE were carried out, and the results are plotted in Fig. 4A. The Lineweaver–Burk plots of GHE peptides showed that they were uncompetitive inhibitors, generating almost parallel lines. These plots suggest that peptides from GHE can bind only to substrate-enzyme complex and decrease the maximum enzyme activity, so it takes longer for the substrate or product to leave the active site (Jao, Huang, & Hsu, 2012). Similar results were found by Nakagomi et al. (1998) for a peptide obtained from tryptic hydrolysate of human plasma.

3.2.2. Antioxidant properties

Antioxidant properties were analyzed using assays with the following mechanisms: scavenging of $ABTS^{+}$ radical (TEAC), reducing power activity assay, copper-chelating activity by assay of β-carotene oxidation, and β-carotene-linoleic acid assay.

Fig. 4B shows $ABTS^{+}$ radical cation discoloration assay, which is a method widely applied to evaluate total antioxidant activity in both lipophilic and hydrophilic samples (Sheih et al., 2009). The higher TEAC value corresponded to GHE 6.5. It was also observed that IC_{50}% value of this extract was lower than those obtained for GHE 4.0 and 9.0 (Table 3), indicating that GHE 6.5 had the greatest $ABTS^{+}$ scavenging activity. As mentioned before, GHE would have mainly components with similar hydrophobicity and molecular size (≈5.5 kDa). Therefore, differences in sequence of peptides obtained by fractionating at different pH could affect the antioxidant properties (Alemán et al., 2011; Quian, Jung, & Kim, 2008). Additionally, a relationship between MH hydrophobicity/size and TEAC from GHE was found (Fig. 3), indicating that components of medium MW and hydrophobicity from GHE are primarily responsible for $ABTS^{+}$ scavenging activity. These data agree with others, which report that radical scavenging mechanism depends on the presence of hydrophobic amino acids (Carrasco-Castilla et al., 2012; Jiménez-Escrig, Alaiz, Vioque, & Rupérez, 2010).

The chelation of metal ions has an antioxidant effect because transition metals, such as iron and copper, catalyze the generation of reactive oxygen species including hydroxyl radical (·OH) and superoxide radical ($O_2 \cdot ^{-}$), leading to the oxidation of unsaturated lipids and promoting oxidative damage at different levels (Saiga, Tanabe, & Nishimura, 2003). Fig. 4C shows the degradation of β-carotene (measured as decrease in absorbance at 470 nm) vs. time. The results for negative control (without Cu^{2+}) show as time reaction increases, absorbance at 470 nm decreases slightly. This may be due to oxidative degradation

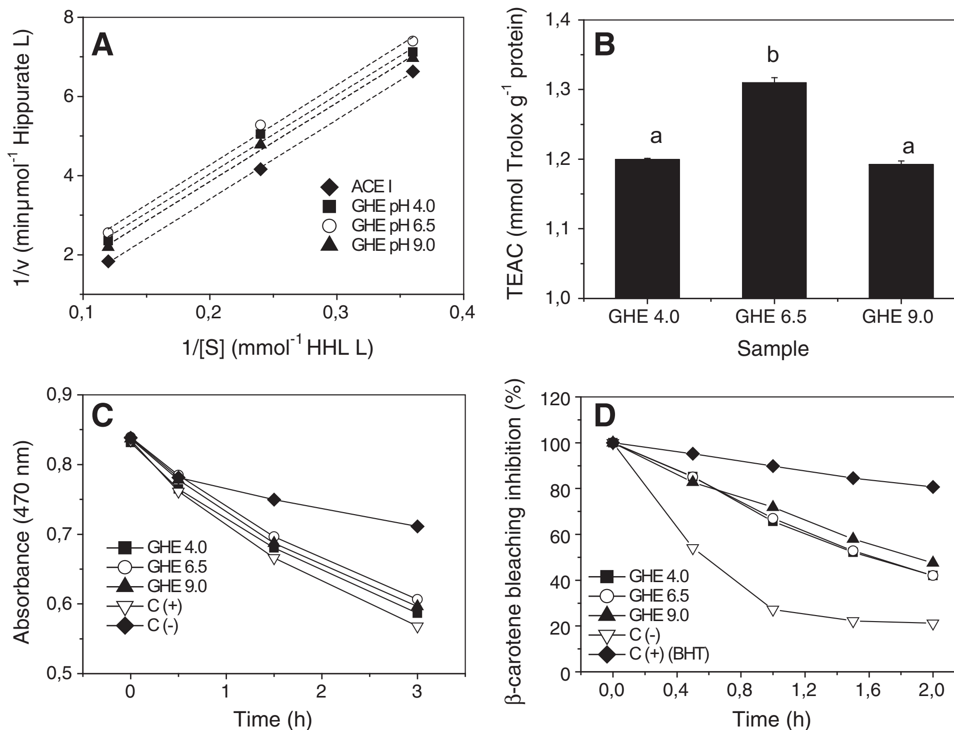

Fig. 4. Lineweaver–Burk plots of the inhibition of ACE I in absence or presence of GHE (A), Trolox equivalent antioxidant capacity (TEAC) (B); copper-chelating activity by assay of β-carotene oxidation vs. time (3 h) (C); and carotene bleaching inhibition (%) vs. time (2 h) of GHE (D).

of β-carotene as a result of autoxidation initiated by light in the presence of oxygen (Megías et al., 2008). However, for positive control (with Cu^{2+}), the absorbance at 470 nm decreases rapidly with the reaction time, indicating the strong oxidation of β-carotene in presence of copper. GHE inhibited β-carotene oxidation in the presence of copper; the higher cooper chelating activity (CCA) corresponded to GHE 6.5 (Table 3). As for $ABTS^{+}$, a relationship between hydrophobicity/size (MH) and CCA from GHE was found (Fig. 3), indicating that peptides of medium MW (≈5.5 kDa) and hydrophobicity are primarily responsible for these properties. In this regard, Megías et al. (2008) reported that hydrophobicity influences peptide binding to cooper.

Inhibition of β-carotene bleaching is show in Fig. 4D. GHE inhibited β-carotene oxidation by scavenging lipid peroxides generated by the autoxidation of linoleic acid promoted by addition of oxygen-sparged distilled water. This result agrees with those reported by Zhu, Su, Guo, Peng, and Zhou (2011) for wheat gluten hydrolysate with Alcalase 2.4 L. GHE 9.0 had higher carotene bleaching inhibition (CBI) than others GHE (Table 3). This could be attributed to −SH/S-S redox couple of peptides. This ratio was higher at pH 9.0 than pH 6.0 and 4.0 (0.58, 0.50, and 0.37, respectively). As mentioned above, at pH 9.0, it is possible to extract components richer in S-S and –SH than at pH 4.0 or 6.5. In this sense, a direct relationship between S-S and −SH and CBI was obtained. The Pearson correlation coefficients were $r = 0.9982$ and $r = 0.9818$ for S-S and –SH, respectively (Fig. 3). As it is known, Met and Cys are two sulfur containing residues that could have redox and antioxidant effects (Quian et al., 2008).

Reducing power activity (RPA) of GHE 9.0 was higher than that obtained for GHE 4.0 and 6.5 (Table 3). As seen in Fig. 3, this increase in RPA was related to disulfide bonds content in each extract. In this regard, a direct relationship between S-S and RPA ($r = 0.9216$) from GHE was obtained. Note that reducing power is a result of the presence of peptides that are electron donors and that could react with free radicals, thus terminating the free radical chain reaction. This activity has been attributed, among other amino acids, to Met and Cys because provide redox couple S-S/−SH to peptides (Carrasco-Castilla et al., 2012). Thus, the higher RPA from GHE 9.0 could be attributed to these amino acid residues.

4. Conclusions

Many bioactive peptides have been discovered from enzymatic hydrolysates of different food proteins, but so far, there has been no research focused on structure–mechanism relationship of antioxidant and ACE I inhibitor peptides from wheat gluten hydrolysates fractionated by pH. It is a good alternative to select bioactive peptides from wheat gluten hydrolysates. We have demonstrated that GHE were rich in medium hydrophobicity and MW peptides (5.5 kDa) with antihypertensive and antioxidant properties mediated by uncompetitive and hydrogen atom/electron transfer (HAT/ET), respectively. Thus, hydrolysis following by pH fractionation could be used as a mean to obtain bioactive peptides from by-product of the wheat starch process, which is available in large amounts and at relatively low cost.

Acknowledgments

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