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Antioxidant activity of hydrolysates and peptide fractions of glutelin from cocoa (*Theobroma cacao* L.) seed

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ABSTRACT

Glutelin fraction from cocoa almond was hydrolyzed with alcalase for the production of hydrolysates. These were then fractionated by ultrafiltration to obtain peptides with a molecular weight (M_w) lower than 3000 Da. The antioxidant activity (AOX) of the hydrolysates and peptides was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}), and oxygen radical absorbance capacity (ORAC) assays. Ultrafiltered peptide fraction with the highest AOX was purified by size exclusion chromatography, by which four main peaks were identified with a M_w between 535 and 2959 Da. The peptide fraction P1 ($M_w = 2959$ Da) showed the highest capacity to scavenge DPPH[•] and ABTS^{•+} radicals with radical scavenging activity DPPH[•] and ABTS^{•+} with EC_{50} values of 237.48 and 19.29 $\mu\text{g/mL}$, respectively, which were similar to those obtained with glutathione. These results show that enzymatic treatment of cocoa glutelin comprises an attractive bioprocess for the production of peptide fractions with AOX, which could be included in the design of functional foods; moreover, they show an alternative use of cocoa.

Actividad antioxidante de hidrolizados y fracciones peptídicas de glutelina de la semilla de cacao (*Theobroma cacao* L.)

RESUMEN

La fracción de glutelina de la almendra de cacao se hidrolizó con alcalasa para la producción de hidrolizados, los cuales se fraccionaron por ultrafiltración para obtener péptidos con un peso molecular (M_w) menor a 3000 Da. La actividad antioxidante (AOX) de los hidrolizados y péptidos se evaluó mediante los ensayos de 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) y capacidad de absorción del radical oxígeno (ORAC). La fracción peptídica obtenida por ultrafiltración con la mayor AOX se purificó por cromatografía de exclusión molecular, mediante la cual se identificaron cuatro principales picos con un M_w entre 535 y 2959 Da. La fracción peptídica P1 ($M_w = 2959$ Da) mostró la mayor actividad reductora de radicales DPPH[•] y ABTS^{•+} con valores EC_{50} de 237.48 y 19.29 $\mu\text{g/mL}$, respectivamente, los cuales fueron similares a los obtenidos con glutatión. Estos resultados muestran que el tratamiento enzimático de glutelina de cacao es un bioproceso atractivo para la producción de fracciones peptídicas con AOX, que podrían incluirse en el diseño de alimentos funcionales. Además, proporcionaría un uso alternativo del cacao.

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semilla de cacao; glutelina; ultrafiltración; péptidos bioactivos; electroforesis; radicales libres; actividad antioxidante

Introduction

Free radicals, antioxidants, and oxidative stress are common terms when exploring the mechanisms involved in the origin of a vast number of diseases (chronic degenerative, neurodegenerative, and some types of cancer). Within this context, free radicals are continuously produced in the human organism by means of biochemical reactions, which occur as part of the cell metabolism and which are associated with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS); both of them are responsible for oxidative damage to macromolecules, such as DNA, lipids, carbohydrates, and proteins (Valko et al., 2007). Likewise, there is an antioxidant defense system limiting the noxious effects of these radicals; this system can remove reactive species through the use of enzymatic (superoxide dismutase, glutathione (GSH) peroxidase, and catalase) and non-enzymatic antioxidants (antioxidant vitamins, GSH, trace elements, coenzymes, and cofactors) (Cai et al., 2015). Therefore,

disequilibrium between the generation of free radicals and antioxidant defenses gives rise to a process denominated oxidative stress, which can induce cellular damage, trigger physiological alterations, and foster pathological processes. Thus, when the endogenous defense system fails to protect the body against reactive radicals, an external supply of antioxidants is needed (Sarmadi, Ismail, & Hamid, 2011).

Cocoa seeds derive from the fruits of the tropical tree *Theobroma cacao* L. (*Sterculiaceae* family). Various subspecies – including Criollo, Forastero, and Trinitario – are cultivated in Central West Africa, Central America, South America, and Asia (Bertazzo, Comai, Brunato, Zancato, & Costa, 2011). The main component of the cocoa seed is fat (47–55%), which is widely used in the chocolate-manufacturing industry, as well as in pharmaceuticals and cosmetics (Rusconi & Conti, 2010). Likewise, the second most abundant component in the cocoa seed is protein (10–15%), which is constituted by four fractions: albumin (ALB), globulin (GLO), prolamin (PRO), and

glutelin (GLUT) (Abecia-Soria, Pezoa-García, & Amaya-Farfan, 2005). In addition, cocoa seed is recognized as a rich source of alkaloids and polyphenols with biofunctional properties (Abbe, Amin, Chong, Muhajir, & Hasbullah, 2008). Sensory and technological properties of chocolate and cocoa-based products depend on the fermentation, drying, and roasting of cocoa seeds (processed cocoa); during these, changes occur in the protein composition. Several studies have reported different proportions of protein fractions after fermentation, with an increase or decrease on the protein fractions' ratio (Amin, Jinap, & Jamilah, 1997; Preza et al., 2010; Voigt & Biehl, 1993); Globulins suffer extensive degradation during fermentation and are responsible for the formation of precursor compounds (oligopeptides and amino acids) producing the aroma and flavor of the final products (Amin, Jinap, Jamilah, Harikrisna, & Biehl, 2002; Biehl, Brunner, Passern, Quesnel, & Adomako, 1985). Moreover, Abecia-Soria et al. (2005) reported that the content of albumins decreases during roasting; hence, fermented cocoa seeds present a particular protein composition. Although abundant information on the chemical composition and biochemical characterization of the proteins of cocoa seed can be found in scientific literature, studies on their antioxidant properties are scarce. Preza et al. (2010) reported that proteins from unfermented and semi-fermented cocoa seeds showed antioxidant and antitumor activities, while Sarmadi et al. (2011) reported that cocoa autolysates exhibit antioxidant and angiotensin converting enzyme (ACE) inhibitory activities. For the above, the aim of this study was to assess the antioxidant activity (AOX) of protein hydrolysates and peptides obtained from cocoa seed.

Materials and methods

Raw material

Cocoa pods (*Theobroma cacao* L., variety Forastero) harvested in May 2014 by traditional methods were obtained from plantations in the municipality of Comalcalco, State of Tabasco, Mexico. Fruits were depodded and fresh seeds fermented at room temperature in wooden boxes (100 cm³) for 6 days, washed with water to eliminate remaining mucilage and air-dried. Afterwards, the coats were then removed and the cocoa almonds were crushed and stored at -18°C.

Preparation of acetone dry powder (AcDP)

Crushed cocoa almonds (3–4 kg) were defatted with petroleum ether for 24 h and alkaloids partially extracted with chloroform for 8 h in a Soxhlet apparatus. To remove polyphenols, AcDP was prepared according to Kirchhoff, Biehl, and Crone (1989). AcDP obtained was evaporated to dryness and milled using an IKA mill (IKA®A11 basic). Then the flour was sieved through a 150-µm mesh and stored at -18°C until further analysis.

Protein fraction extraction

Extraction of soluble proteins was performed according to the method by Voigt and Biehl (1993), with some modifications. AcDP was extracted successively with 10 mmol L⁻¹ Tris-HCl (pH 7.5 containing 2 mmol L⁻¹ EDTA), 0.5 mol L⁻¹ NaCl (containing 2 mmol L⁻¹ EDTA and 10 mmol L⁻¹ Tris-HCl pH 7.5), 70% (v/v) ethanol, and 0.1 mol L⁻¹ NaOH to obtain

ALB, GLO, PRO, and GLUT fractions, respectively. All of these solutions contained sodium ascorbate (5 mmol L⁻¹). Suspensions (1:10 w:v) were stirred for 4 h at 4°C and centrifuged at 10,000 × g for 20 min at 4°C. Each supernatant was dialyzed (M_w 14 kDa cutoff) against deionized water for 48 h at 4°C, with a change every 24 h. The content of dialysis tubes was centrifuged as previously stated. Finally, solutions were lyophilized and stored at -18°C. Protein concentration was determined according to the method by Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard.

Determination of AOX

DPPH[•] radical scavenging activity (DRSA)

DRSA was determined according to the method by Chen, Yang, Sun, Niu, and Liu (2012), with some modifications. A 0.5 mL aliquot of sample solution (600 µg mL⁻¹) was added to 0.5 mL of DPPH[•] radical (0.1 mmol L⁻¹) in methanol solution. The mixture was shaken vigorously and incubated for 30 min in the dark at room temperature; absorbance was measured at 515 nm. Methanol, instead of DPPH[•], was used for the blank, while deionized water, instead of sample, was used for the control. DRSA was calculated by the following equation:

$$\text{DRSA (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}}/A_{\text{control}})] \times 100$$

ABTS^{•+} radical scavenging activity (ARSA)

ARSA was determined according to the method by Re et al. (1999), with some modifications. The ABTS^{•+} radical cation was generated by mixing ABTS stock solution (7 mmol L⁻¹) with potassium persulfate (2.45 mmol L⁻¹) and the resulting mixture was allowed to rest in the dark at room temperature for 12 h prior to use. The ABTS^{•+} radical solution was diluted in 0.15 mol L⁻¹ phosphate buffered saline (PBS) pH 7.4 (~ 1:35) to obtain an absorbance of 0.70 ± 0.02 at 734 nm. About 3 mL of this diluted solution was mixed with 150 µL of sample (600 µg mL⁻¹). Absorbance was read from 1 and up to 6 min after the initial mixing, under conditions of darkness. PBS, instead of ABTS^{•+}, was used for the blank, while deionized water, instead of sample, was used for the control. ARSA was calculated by the following equation:

$$\text{ARSA (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}}/A_{\text{control}})] \times 100$$

Oxygen radical absorbance capacity (ORAC) assay

ORAC was determined according to the method by Dávalos, Gómez-Cordovés, and Bartolome (2004). The reaction was performed at 37°C in 75 mmol L⁻¹ PBS pH 7.4 and the final assay mixture (200 µL) contained fluorescein (FL, 120 nmol L⁻¹, 120 µL), 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH) (40 mmol L⁻¹, 60 µL), and sample (600 µg mL⁻¹, 20 µL). The microplate was automatically shaken before the first reading and fluorescence (λ_{exc}: 485 nm, λ_{em}: 535 nm) was measured every minute for 120 min (Tecan Safire plate reader controlled by Magellan® software). Final results were calculated by monitoring the fluorescence net decay curve of FL. Trolox (0–10 µmol L⁻¹, final concentration) was employed to generate a standard curve and the Trolox equivalent antioxidant capacity (TEAC, expressed as µmol TE/mg protein) was obtained for each sample.

Preparation of protein hydrolysates

As depicted in Table 1, ALB, GLO, PRO, and GLUT showed *in vitro* AOX with significant differences ($p < 0.05$). Among protein fractions, GLUT showed the highest AOX and was found to be the predominant protein fraction (81.88%). Based on this, GLUT was used to obtain hydrolysates. GLUT was hydrolyzed with alcalase (EC 3.4.21.62, protease from *Bacillus licheniformis*, specific activity 2.4 Anson units g^{-1} , Sigma-Aldrich, Co., St. Louis, MO, USA) using an enzyme: substrate ratio (E:S) of 1:10 (w:w) at pH 7.5 and 50°C. Hydrolysis was carried out for 5, 15, 30, 45, 60, 90, and 120 min. At the end of it, the sample was heated to 95°C for 10 min to inactivate the enzyme. All samples were centrifuged at $5000 \times g$ for 10 min at 4°C. Supernatants were denominated GLUT-H, lyophilized, and stored at -18°C .

Degree of hydrolysis (DH)

The DH was determined by the reaction of free amino groups with the 2,4,6-trinitrobenzenesulfonic acid reagent (Adler-Nissen, 1979). L-Leucine (0–2.5 mM) was used to generate a standard curve. DH values were calculated using the following equation:

$$\text{DH (\%)} = \left[\frac{(L_t - L_0)}{(L_{\text{max}} - L_0)} \right] \times 100$$

where L_t is the amount of free amino groups released after hydrolysis, L_0 is the amount of free amino groups in GLUT, and L_{max} is the total amount of free amino groups in GLUT obtained after total hydrolysis.

Ultrafiltration

For peptide separation, GLUT-H (5–120 min) was fractionated through an ultrafiltration membrane with a M_w cutoff ($M_w\text{CO}$) of 3000 Da (Millipore, Bedford, MA, USA). For each hydrolysis, ultrafiltered peptide fractions were obtained and denominated GLUT-P ($M_w < 3000$ Da).

The AOX of GLUT-H and GLUT-P was assessed under the same experimental conditions previously described (DPPH, ABTS, and ORAC assays).

Electrophoresis

Polyacrylamide gel electrophoresis (SDS-PAGE)

GLUT and GLUT-H profiles were carried out according to the method by Laemmli (1970), using a 12% separating gel (w/v polyacrylamide) and a stacking gel (4% w/v polyacrylamide).

Gels were run in mini-slabs (Bio-Rad, Mini Protean® III Model, Hercules, CA, USA) at 200 V for 45 min.

Tricine-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE)

GLUT-P profiles were carried out according to the method by Schagger (2006), employing 16%, 10%, and 4% (w/v polyacrylamide) separating, spacing, and stacking gels, respectively. Gels were run at 90 V for 3 h.

Size exclusion chromatography

The GLUT-P that showed the highest AOX was separated using fast protein liquid chromatography on a GE Healthcare, FPLC System, equipped with a molecular exclusion Superdex column (G75HR 10/30 GL). The column was equilibrated and eluted with 20 mmol L^{-1} Tris-HCl, 150 mmol L^{-1} NaCl, pH 7.8, at a flow of 0.5 mL min^{-1} , collecting 1 mL fractions. Peptides were detected by their absorbance at 214 nm. The column was calibrated by using the M_w -GF-70 gel filtration market kit for protein (Sigma-Aldrich®, Co., St. Louis, MO, USA). Peptide fractions were collected to assess AOX, while GSH was employed as standard. The EC_{50} value, defined as the effective concentration of the peptide ($\mu g mL^{-1}$) required to scavenge 50% of radical activity (DPPH• or ABTS^{•+}), was calculated from a linear regression plot of scavenging activity percentage versus peptide concentration. TEAC value ($\mu mol TE mg^{-1}$ sample) was also determined for each peptide fraction.

Statistical analysis

Analyses were carried out by triplicate and results were reported as mean \pm standard deviations. Significant differences among treatments were determined by analysis of variance and Fisher's least significant difference (LSD) tests ($p < 0.05$) using the Statgraphics® Centurion XVI statistical software (StatPoint Technologies Inc., Bedford, MA, USA, 2010).

Results and discussion

Protein fractions content in cocoa seed

To avoid irreversible denaturation of proteins by the oxidation of polyphenol products during protein fractioning, as well as an effect on the results, AcDP was obtained from cocoa almond. Employing the qualitative test (with HCl), it was confirmed that AcDP was polyphenol-free. Afterward, ALB,

Table 1. Protein content and antioxidant activity of protein fractions obtained from cocoa seed.

Tabla 1. Contenido de proteína y actividad antioxidante de fracciones proteínicas obtenidas de la semilla de cacao.

Protein fraction	Protein ratio		DPPH assay	ABTS assay	ORAC assay
	(g 100 g^{-1} AcDP)	(%)	DRSA (%)	ARSA (%)	TEAC ($\mu mol TE mg^{-1}$ protein)
ALB	0.65 \pm 0.03 ^b	8.99 \pm 0.11 ^b	4.33 \pm 0.21 ^c	5.19 \pm 0.09 ^b	0.15 \pm 0.02 ^b
GLO	0.52 \pm 0.02 ^b	7.19 \pm 0.09 ^c	6.06 \pm 0.39 ^b	4.48 \pm 0.08 ^{b,c}	0.12 \pm 0.02 ^b
PRO	0.14 \pm 0.01 ^c	1.93 \pm 0.06 ^d	3.22 \pm 0.28 ^d	4.19 \pm 0.07 ^c	0.07 \pm 0.01 ^c
GLUT	5.92 \pm 0.34 ^a	81.88 \pm 1.28 ^a	13.48 \pm 0.65 ^a	8.62 \pm 0.30 ^a	0.28 \pm 0.02 ^a

Notes: Values are shown as mean \pm standard deviation of triplicate determinations.

Means with different superscripts, in the same column, are significantly different ($p < 0.05$).

Final concentrations of protein fractions were 300, 30, and 60 $\mu g mL^{-1}$ in DRSA, ARSA, and ORAC assays, respectively.

Notas: Los valores se muestran como media \pm desviación estándar de las determinaciones por triplicado.

Medias con diferentes superíndices en la misma columna son significativamente diferentes ($p < 0,05$).

Las concentraciones finales de las fracciones proteínicas fueron 300, 30 y 60 $\mu g mL^{-1}$ en los ensayos DRSA, ARSA and ORAC, respectivamente.

GLO, PRO, and GLUT proteins were obtained at the concentrations depicted in Table 1. Total content of protein was $7.23 \text{ g } 100 \text{ g}^{-1} \text{ AcDP}$, with GLUT being the predominant protein fraction – the latter with a value of $5.92 \text{ g } 100 \text{ g}^{-1} \text{ AcDP}$, significantly higher ($p < 0.05$) than the values obtained for other proteins ($0.14\text{--}0.65 \text{ g } 100 \text{ g}^{-1} \text{ AcDP}$)– and with the following percentage distribution: GLUT ($81.88 \pm 1.28\%$) > ALB ($8.99 \pm 0.11\%$) > GLO ($7.19 \pm 0.09\%$) > PRO ($1.93 \pm 0.06\%$). Several studies performed in unfermented and fermented cocoa seed have reported a different distribution of protein fractions. Some authors have reported a higher content of ALB and GLO (14–52%) (Lerceteau, Rogers, Petiard, & Crouzillat, 1999; Voigt & Biehl, 1993), while others have reported a higher content of GLUT (30–56%) (Preza et al., 2010; Zak & Keeney, 1976). This difference in the content of the several protein fractions is influenced by numerous factors, such as seed variety, fractionation method, batch size, fermentation, and storage conditions. Regarding fermentation, a degradation of ALB and GLO fractions has been related to the formation of precursor compounds (oligopeptides and amino acids) responsible for the characteristic aroma of cocoa (Amin et al., 2002; Romero-Cortes et al., 2013; Voigt & Biehl, 1993; Zak & Keeney, 1976). In the present study, cocoa almond was used, obtaining a low content ($<1.0 \text{ g}$) of ALB and GLO, which was different from that obtained from non-fermented seed (3.23 and 2.36 g for ALB and GLO, respectively). Thus, degradation of these proteins occurred during fermentation. In this case, GLUT was degraded at a lower proportion, with a product of 5.92 g, amounting to 7.76 g in the non-fermented seed (data not shown).

AOX of protein fractions

Currently, a large number of analytical methods are available to determine the *in vitro* AOX of different molecules. Among them there are free radical generation methods, which have been widely used to measure the antioxidant effect as a first step for *in vivo* tests to validate this activity (McDonald-Wicks, Wood, & Garg, 2006). Moreover, AOX was assessed through assays that measure DPPH[•] or ABTS^{•+} scavenging, as well as the ORAC value. As depicted in Table 1, all proteins showed AOX, with values ranging from 3.22% to 13.48% DRSA, from 4.19% to 8.62% ARSA, and from 0.07 to $0.28 \mu\text{mol TE mg}^{-1} \text{ protein}$, for the DPPH, ABTS, and ORAC assays, respectively. These values fall within the range reported for proteins of vegetal and animal origin, like soy, amaranth, bovine casein, and whey protein (9–17.74% DRSA; 3.1–37.2% ARSA; $0.05\text{--}0.5 \mu\text{mol TE mg}^{-1} \text{ protein}$, ORAC value) (Del Mar Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, & Recio, 2011; Di Pierro, O’Keeffe, Poyarkov, Lomolino, & FitzGerald, 2014; Jiménez-Ruiz et al., 2013; Tironi & Añón, 2010). Also, for the three test methods used in the present study, GLUT showed the highest AOX ($p < 0.05$), as compared with the values obtained in non-fermented and semi-fermented cocoa seed (Preza et al., 2010). According to these results, GLUT is the protein fraction of the cocoa seed (Forastero variety) with the highest antioxidant potential (*in vitro*). Since GLUT was the predominant protein fraction and the one with the highest AOX, it was chosen for further obtention of enzymatic hydrolysates.

DH and electrophoretic profiles

In this study, GLUT hydrolysis was accomplished with alcalase, which has been frequently used to obtain protein hydrolysates and bioactive peptides, since it is a protease of microbial origin that presents broad specificity and moderate temperature and pH conditions. Additionally, it has been reported that hydrolysis with alcalase generates a higher AOX of hydrolysates and derived peptides (Sarmadi & Ismail, 2010). Figure 1 depicts the DH values obtained for the combinations of time and the E:S ratio assayed for GLUT hydrolysis with alcalase. DH values were 6.31–19.91%, pointing out that in 8 of 9 assayed times, a DH >10% was obtained. The DH reached depends significantly on the reaction time (t), highlighting that in the interval between 5 and 60 min, increases in this variable boost the DH. However, in the range between 60 and 120 min, DH did not increase significantly ($p < 0.05$). Moreover, maximal DH achieved for GLUT was 19.91% ($t = 120 \text{ min}$); it is noteworthy that this was achieved at a relatively low time interval, which may have been due to the E:S ratio employed.

Figure 2(a) depicts the electrophoretic SDS-PAGE profiles of GLUT and GLUT-H. On it, the GLUT profile presents two main bands, which correspond to subunits of 200 and 23 kDa; lesser intensity bands corresponding to the 27 and 14.5 kDa subunits were also found. This polypeptide profile was similar to that reported by other authors in the same cocoa species (Preza et al., 2010; Voigt & Biehl, 1993). Additionally, the intensity of the bands in the electrophoretic profiles of GLUT-H decreased, showing that an increase on the enzymatic reaction time generates a progressive decline on the intensity of the subunits’ bands; this suggests the release of peptide chains of lower M_w . Further, although no significant difference in DH was observed at 60–120 min of hydrolysis, the electrophoretic profiles were different. Hence, it is possible to obtain different electrophoretic profiles with a similar DH. During Tricine-SDS-PAGE electrophoresis, an increasing hydrolysis time generated a progressive degradation of polypeptides, suggesting the formation of peptide chains of lower M_w ($<3.5 \text{ kDa}$), which cannot be retained in the gel (Figure 2(b)).

The hydrolysis of proteins is a process of simultaneous reactions, during which there is a release of peptide species of different M_w . Since bioactive peptides are considered short chains of 2–20 amino acids, separation of the peptides with $M_w < 3000 \text{ Da}$ present in GLUT-H was achieved through ultrafiltration using a 3000-Da $M_w\text{CO}$ membrane aiming to purify peptides produced by hydrolysis with alcalase.

AOX of GLUT-H and GLUT-P

Figure 3(a) shows that all GLUT-H presented DPPH[•] scavenging activity, generating values of 17.63–32.47% DRSA, which were significantly higher ($p < 0.05$) than those found for GLUT (13.48%). The time of hydrolysis exerted a significant effect on DRSA, obtaining the highest percentage at 15 min of hydrolysis (GLUT-H₁₅) and the lowest values between 90 and 120 min. Additionally, all of the ultrafiltrates ($<3000 \text{ Da}$) obtained from GLUT-H (5–120 min) showed a higher DRSA, with values ranging from 40.89% to 48.86%, significantly increasing AOX ($p < 0.05$). The GLUT-P fraction also showed the highest DRSA at 15 min of hydrolysis. In DRSA values obtained in both GLUT-H and GLUT-P fell within the reported

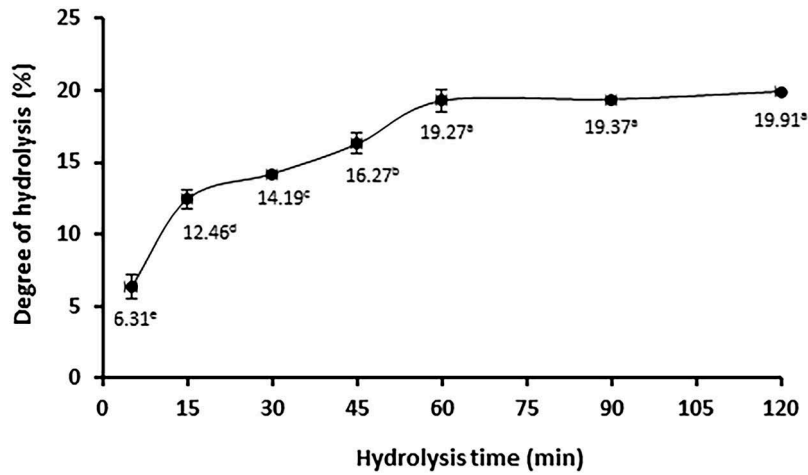


Figure 1. Degree of hydrolysis (%) of GLUT. Results are plotted as mean \pm standard deviation ($n = 3$). Data points with different superscript letters are significantly different ($p < 0.05$).

Figura 1. Grado de hidrólisis (%) de GLUT. Los resultados se representan como media \pm desviación estándar ($n = 3$). Los puntos con diferentes superíndices son significativamente diferentes ($p < 0,05$).

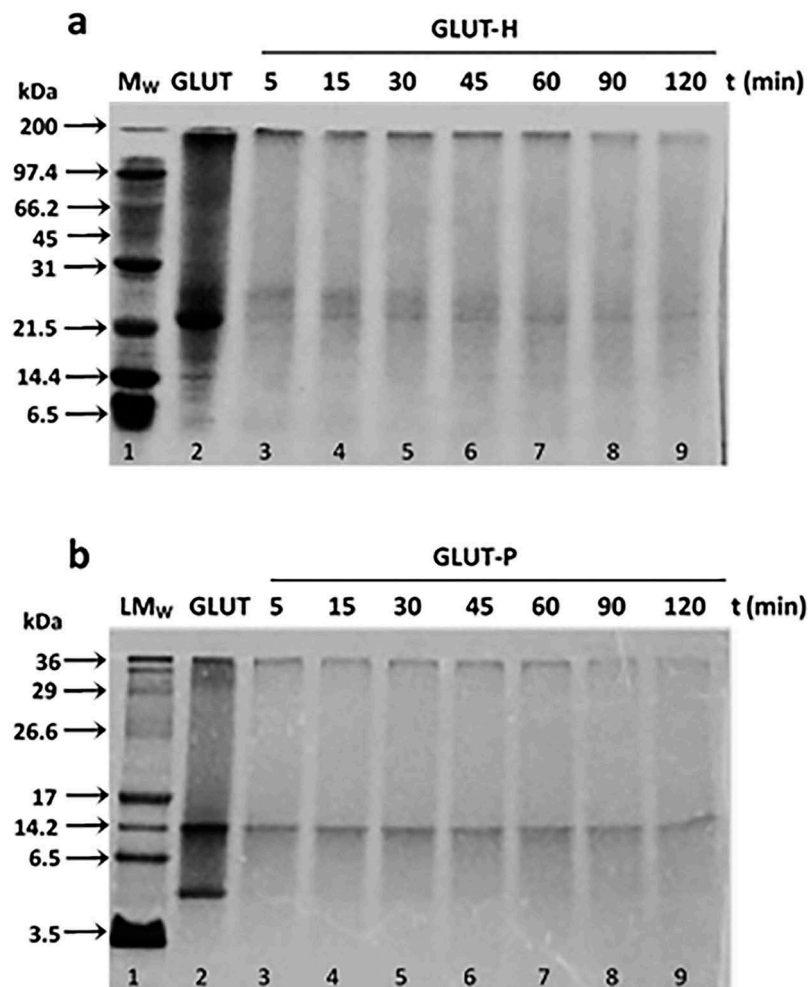


Figure 2. (a) SDS-PAGE profile of GLUT-H. Lane 1: standard (M_w), lane 2: GLUT, lanes 3–9: GLUT-H ($t = 5$ –120 min). (b) Tricine – SDS-PAGE profile of GLUT-P. Lane 1: standard (LM_w), lane 2: GLUT, lanes 3–9: GLUT-P ($t = 5$ –120 min).

Figura 2. Perfil SDS-PAGE de GLUT-H. Línea 1: Estándar (M_w), Línea 2: GLUT, Líneas 3–9: GLUT-H ($t = 5$ –120 min). (b) Perfil Tricine – SDS-PAGE de GLUT-P. Línea 1: Estándar (LM_w), Línea 2: GLUT, Líneas 3–9: GLUT-P ($t = 5$ –120 min).

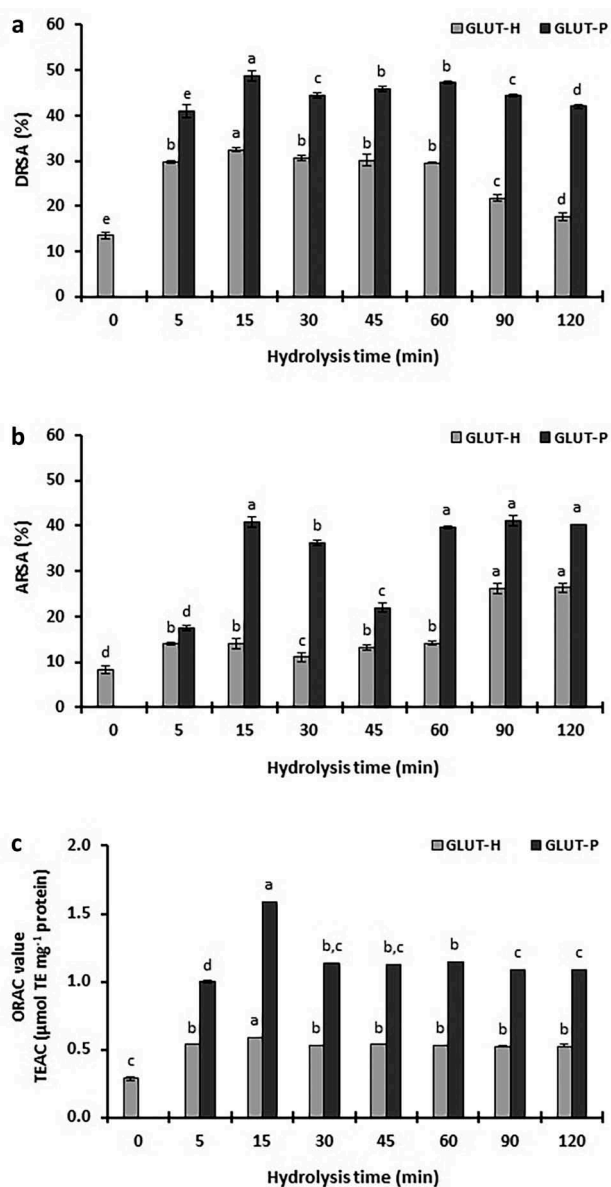


Figure 3. AOX of GLUT-H and GLUT-P. (a) DPPH[•] radical scavenging activity (DRSA, %). (b) ABTS^{•+} radical scavenging activity (ARSA, %). (c) Oxygen radical absorbance capacity (ORAC, TEAC = μmol Trolox equivalents mg⁻¹ protein). Final concentrations of samples were 300, 30, and 60 μg mL⁻¹ in DRSA, ARSA, and ORAC_{FL} assays. Results are plotted as mean ± standard deviation (*n* = 3). Different letters in the same parameter indicate significant differences (*p* < 0.05).

Figura 3. AOX de GLUT-H y GLUT-P. (a) Actividad reductora del radical DPPH[•] (DRSA, %). (b) Actividad reductora del radical ABTS^{•+} (ARSA, %). (c) Capacidad de absorción de radicales de oxígeno (ORAC, TEAC = μmol Trolox mg⁻¹ proteína). Las concentraciones finales de las muestras fueron 300, 30 y 60 μg/mL en los ensayos de DRSA, ARSA y ORAC_{FL}, respectivamente. Los resultados se representan como media ± desviación estándar (*n* = 3). Letras diferentes en el mismo parámetro indican diferencias significativas (*p* < 0,05).

range (10–90%) of hydrolysates and peptide fractions of barley, walnut, and soy (Bamdad, Wu, & Chen, 2011; Chen et al., 2012; Jiménez-Ruiz et al., 2013).

All GLUT-H presented ABTS^{•+} scavenging activity, with values 11.05–26.29% ARSA, which were significantly higher (*p* < 0.05) than those found in GLUT (8.29%). In this case, the hydrolysates obtained at 90 and 120 min depicted a higher scavenging effect, without differences among them (Figure 3 (b)). GLUT-P exhibited a significant increase on ARSA, reaching values 17.38–41.19%; however, the behavior found was

not the same as that of GLUT-H, since the highest scavenging activity was observed in fractions corresponding to 15, 60, 90, and 120 min of hydrolysis, without differences among them; nevertheless, as for the DPPH assay, GLUT-P obtained at 15 min presented a higher percentage of ARSA. Additionally, the obtained ARSA values also fell within the reported range (10–85%) for hydrolysates and peptides from other vegetal sources like amaranth and corn (Soriano-Santos & Escalona-Buendía, 2015; Tironi & Añón, 2010; Wang et al., 2014).

Figure 3(c) shows that GLUT-H also presented a higher radical absorbance capacity of oxygen than GLUT, highlighting that GLUT-H₁₅ depicted the highest ORAC value (0.59 μmol TE mg⁻¹ protein), which was significantly higher (*p* < 0.05) than the one obtained for all other hydrolysates (0.52–0.54 μmol TE mg⁻¹ protein). In this method, GLUT-P also presented an increase in ORAC values (1.0–1.59 μmol TE mg⁻¹ sample), maintaining the same behavior as GLUT-H, being GLUT-P₁₅ the one with the highest AOX. These results are comparable to those reported for soy, whey, and bovine casein (Del Mar Contreras et al., 2011; Di Pierro et al., 2014; Ranamukhaarachchi, Meissner, & Moresoli, 2013).

In this study, GLUT, GLUT-H, and GLUT-P presented AOX through two different mechanisms: electron transfer (reduction of DPPH[•] and ABTS^{•+} radicals) and transfer of hydrogen atoms (through the capture of peroxy radicals). The ORAC_{FL} assay has been widely used in research as a standard method for the assessment of *in vitro* AOX; however, methods that assess the reduction of free radicals also represent a chemically valid and important parameter of potential antioxidant compounds (Sarmadi & Ismail, 2010). On this, AOX of GLUT increased when hydrolyzing with alcalase and when performing the peptide chains separation with *M_w* < 3000 Da, the latter showing the highest AOX. In the three assessment methods used, the ultrafiltered peptide fraction obtained at 15 min (GLUT-P₁₅) showed the highest *in vitro* AOX; hence, it was decided to purify it through size exclusion chromatography.

Purification of antioxidant peptide fractions

GLUT-P₁₅ was separated in four peptide fractions P1, P2, P3, and P4 with *M_w* of 2959 Da, 1856 Da, 997 Da, and 535 Da, respectively (Figure 4(a)). If 120 g/mol is taken as the mean for the *M_w* of an amino acid, the obtained fractions are constituted by peptide chains of 5–25 amino acids, which agrees with previously reported data showing that in general antioxidant peptides contain 2–20 amino acids (Samaranayaka & Li-Chan, 2011). AOX of all fractions was assessed and compared with GSH peptide. Figure 4(b) shows that fraction P1 had the highest DPPH[•] and ABTS^{•+} scavenging capacity (53.33% and 84.47%, respectively). Regarding ORAC value, no significant difference (*p* < 0.05) was found among the four fractions. Therefore, FPLC purification showed an increase in DPPH[•] and ABTS^{•+} scavenging capacity; however, this behavior was not found in the ORAC assay, since even lower ORAC values (1.16–1.27 μmol TE mg⁻¹) than that present in GLUT-P₁₅ (1.58 μmol TE mg⁻¹) were found; this shows a higher activity when peptide chains are linked together than when they are separated. For the ORAC assay, all peptide fractions presented a lower AOX than GSH (Table 2).

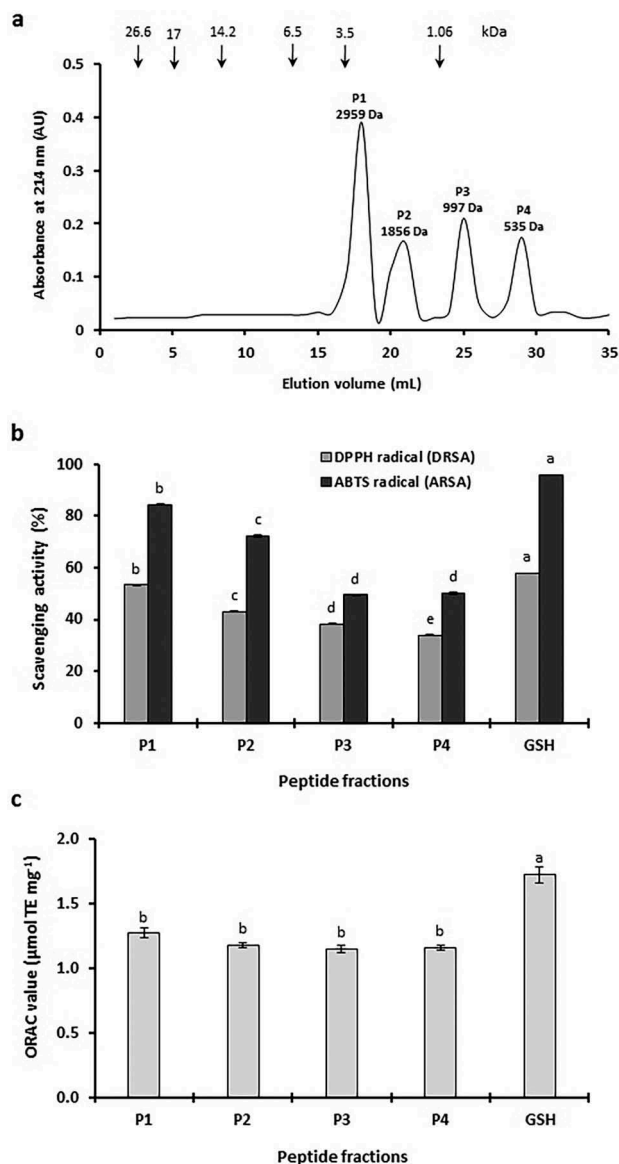


Figure 4. Chromatographic profile of GLUT-P₁₅. (a) FPLC chromatogram. (b) Scavenging activity of DPPH[•] and ABTS^{•+} radicals of each eluted fraction (P1, P2, P3, and P4). (c) ORAC value of each eluted fraction. kDa: M_w of the standard. Results are plotted as mean ± standard deviation (n = 3). Different letters in the same parameter indicate significant differences (p < 0.05).

Figura 4. Perfil cromatográfico de GLUT-P₁₅. (a) Cromatograma FPLC. (b) Actividad reductora de radicales DPPH[•] y ABTS^{•+} de cada fracción eluida (P1, P2, P3 y P4). (c) Valor ORAC de cada fracción eluida. kDa: M_w del estándar. Los resultados se representan como media ± desviación estándar (n = 3). Letras diferentes en el mismo parámetro indican diferencias significativas (p < 0,05).

Additionally, the antioxidant potential of GLUT-P₁₅ peptide fractions was determined by the EC₅₀ value. This parameter is very important since it determines the necessary concentration of an antioxidant to inhibit 50% of the activity of the free radical. Table 2 depicts EC₅₀ values obtained for GLUT, GLUT-H₁₅, GLUT-P₁₅, and FPLC-purified peptide fractions. Lower EC₅₀ values were found in the peptide fractions, where P1 showed the lowest EC₅₀ values: 237.48 ± 1.75 µg mL⁻¹ and 19.29 ± 0.59 µg mL⁻¹ for DRSA and ARSA, respectively. Furthermore, GLUT presented the highest EC₅₀ values: 2225.52 ± 19.25 µg mL⁻¹ and 194.54 ± 1.73 µg mL⁻¹ (DRSA and ARSA, respectively); the same behavior was found for both radicals (DPPH[•] and ABTS^{•+}), with a higher concentration of GLUT and GLUT-H required to achieve 50% scavenging;

Table 2. EC₅₀ and ORAC values of glutelin, hydrolysates, and peptide fractions obtained from cocoa seed.

Tabla 2. Valores EC₅₀ y ORAC de glutelina, hidrolizados y fracciones peptídicas obtenidas a partir de la semilla de cacao.

Sample ^a	DPPH assay ^b		ABTS assay ^b		ORAC assay ^c	
	EC ₅₀ (µg mL ⁻¹)	EC ₅₀ (µg mL ⁻¹)	EC ₅₀ (µg mL ⁻¹)	EC ₅₀ (µg mL ⁻¹)	TEAC (µmol TE mg ⁻¹ protein)	TEAC (µmol TE mg ⁻¹ protein)
GLUT	2225.52 ± 19.25	194.54 ± 1.73	194.54 ± 1.73	194.54 ± 1.73	0.28 ± 0.02	0.28 ± 0.02
GLUT-H ₁₅	1130.76 ± 11.21	109.28 ± 1.16	109.28 ± 1.16	109.28 ± 1.16	0.59 ± 0.04	0.59 ± 0.04
GLUT-P ₁₅	320.66 ± 9.98	33.89 ± 1.05	33.89 ± 1.05	33.89 ± 1.05	1.58 ± 0.18	1.58 ± 0.18
Peptide fractions (FPLC)						
P1	237.48 ± 1.75	19.29 ± 0.59	19.29 ± 0.59	19.29 ± 0.59	1.27 ± 0.06	1.27 ± 0.06
P2	258.54 ± 1.36	22.75 ± 0.68	22.75 ± 0.68	22.75 ± 0.68	1.18 ± 0.02	1.18 ± 0.02
P3	282.53 ± 0.62	28.74 ± 0.66	28.74 ± 0.66	28.74 ± 0.66	1.15 ± 0.03	1.15 ± 0.03
P4	296.28 ± 0.58	28.31 ± 0.54	28.31 ± 0.54	28.31 ± 0.54	1.16 ± 0.02	1.16 ± 0.02
GSH	230.05 ± 0.22	15.51 ± 0.13	15.51 ± 0.13	15.51 ± 0.13	1.72 ± 0.06	1.72 ± 0.06

Notes: Values are shown as mean ± standard deviation of triplicate determinations.

^aGLUT (glutelin); GLUT-H₁₅ (hydrolysates obtained after 15 min of digestion with alcalase); GLUT-P₁₅ (peptide fraction <3000 Da obtained by ultrafiltration from GLUT-H₁₅); P1, P2, P3, P4 (peptide fractions obtained by FPLC); GSH (glutathione).

^bEC₅₀ (sample concentration needed to scavenge 50% of DPPH[•] and ABTS^{•+} radicals' activity).

^cTEAC (Trolox equivalent antioxidant capacity).

Notas: Los valores se muestran como media ± desviación estándar de las determinaciones por triplicado.

^aGLUT (glutelina); GLUT-H₁₅ (hidrolizados obtenidos después de 15 min de digestión con alcalasa); GLUT-P₁₅ (fracción peptídica < 3000 Da obtenida por ultrafiltración a partir de GLUT-H₁₅); P1, P2, P3, P4 (fracciones peptídicas obtenidas por FPLC); GSH (glutación).

^bEC₅₀ (concentración de la muestra necesaria para eliminar el 50% de la actividad de los radicales DPPH[•] y ABTS^{•+}).

^cTEAC (capacidad antioxidante equivalente de Trolox).

however, the concentration required for GLUT-P₁₅ and peptide fractions purified by FPLC decreased. Also, it has been reported that low-M_w peptides depict higher AOX; in the present study, fractions with M_w of 2959 Da (P1) and 1856 Da (P2) were those with the higher antioxidant effect. As previously mentioned, P1 exhibited the highest AOX, reaching EC₅₀ values similar to those obtained with GSH (Table 2); these results suggest that P1 cocoa peptide fraction may have a high antioxidant potential.

Conclusions

The GLUT represented up to 80% of the total of the protein present in the cocoa seed of the Forastero variety and is a good raw material for the production of hydrolysates and peptide fractions with antioxidant capacity. In particular, the peptide fractions with the highest AOX were obtained in a short time (15 min of digestion with alcalase). Partial purified peptides presented EC₅₀ values similar to those of glutathione and ORAC values similar to those found in peptides from other food sources.

This study provides an alternative use for the cocoa seed and can be used as a reference for futures studies to improve the efficiency of the process for its potential application in the design of functional foods.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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