

Research Article

Antioxidant and Chelating Activity of Nontoxic *Jatropha curcas* L. Protein Hydrolysates Produced by *In Vitro* Digestion Using Pepsin and Pancreatin

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Received 14 December 2014; Accepted 24 February 2015

Academic Editor: Henryk Kozłowski

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The antioxidant and metal chelating activities in *J. curcas* protein hydrolysates have been determined. The hydrolysates were produced by treatment of a nontoxic genotype with the digestive enzymes pepsin and pancreatin and then were characterized by fast protein liquid chromatography and reverse phase chromatography. Peptidic fractions with higher radical scavenging activity were analysed by matrix-assisted laser desorption/ionization mass spectrometry. The antioxidant activity was determined by measuring inhibition of the oxidative degradation of β -carotene and by measuring the reactive oxygen species (ROS) in Caco-2 cell cultures. Cu^{2+} and Fe^{2+} chelating activities were also determined. The hydrolysates inhibited the degradation of β -carotene and the formation of ROS in Caco-2 cells. The lower molecular weight peptidic fractions from FPLC had stronger antioxidant activity in cell cultures compared with the hydrolysates, which correlated with a higher content in antioxidant and chelating amino acids. These fractions were characterized by a large presence of peptides with different molecular masses. The hydrolysates exhibited both Cu^{2+} and Fe^{2+} chelating activity. It was concluded that *J. curcas* is a good source of antioxidant and metal chelating peptides, which may have a positive impact on the economic value of this crop, as a potential source of food functional components.

1. Introduction

Jatropha curcas L., a member of the Euphorbiaceae family, is a drought resistant small tree of significant economic importance, because of its various industrial and medicinal uses [1]. Its seeds are a good source of oil that can be used as a diesel substitute. Its oil is also used for manufacturing pharmaceuticals and cosmetics in tropical countries. Although *Jatropha* cake byproduct obtained after oil extraction is rich in proteins, it is not particularly used for feeding animals because of its toxicity, which is attributed to the presence of phorbol esters [2]. However, nontoxic *J. curcas* genotypes that do not contain phorbol esters have been reported in Mexico

[3], where traditional dishes are elaborated using the roasted fruits of these nontoxic genotypes.

The production of protein isolates has been proposed as a method to reduce antinutritional and toxic factors [4]. Protein isolates can be further processed in order to produce protein hydrolysates that have better functional and nutritional properties [5]. In addition, protein hydrolysates are also a source of bioactive peptides that may have beneficial biological effects [6]. Thus, bioactive peptides with antihypertensive, immunomodulatory, opioid, antioxidant, hypocholesterolemic, and metal chelating activity have been described [7].

The chelating properties of bioactive peptides have been related with their antioxidant activity [8]. Various metabolic pathways produce free radical species in living tissues. When these free radicals are generated in excess, or when cellular defences are deficient, biomolecules may be damaged by oxidative stress, a phenomenon that seems to be involved in cellular aging, cardiovascular diseases, cancer, and neurological degenerative diseases [9]. Oxidative reactions may also be responsible for alterations in foods. Thus, unsaturated fatty acids are prone to suffer peroxidative degradation, resulting in adverse effects on nutritional and functional properties. Lipid peroxidation products may even have toxic effects on mammalian cells [10].

Although copper and iron are essential cofactors for a variety of enzymes, their excess may also have deleterious prooxidant effects *in vivo*, by promoting the formation of reactive oxygen species. In addition, they also promote oxidative reactions in foods that have a negative impact on the flavour, texture, nutritive value, and shelf life of food products. Chelating peptides may constitute a powerful tool for preventing these oxidative processes, both in foods and *in vivo* [11]. Among chelating peptides, phosphopeptides derived from milk proteins have been the best characterized. The positive effect of these peptides on the *in vivo* and *in vitro* absorption of minerals such as calcium, zinc, or iron has been reported [12, 13].

The aim of this work was to investigate the antioxidant and chelating properties of *J. curcas* protein hydrolysates obtained from a nontoxic genotype in order to determine their possible use for producing bioactive peptides that could be used for manufacturing of functional foods. In addition, because pepsin and pancreatin are involved in gastrointestinal digestion in human, the results might provide valuable information on the production of bioactive peptides with antioxidant and metal chelating properties, during physiological digestion of *J. curcas* proteins.

2. Materials and Methods

2.1. Materials. *J. curcas* seeds from the August-September 2009 harvest in Huitzilán, Puebla (Mexico), were used to prepare protein hydrolysates. Porcine pepsin and pancreatin, butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 2,2'-azobis(2-amidinopropane) dihydrochloride (ABAP), 2,4,6-trinitrobenzenesulfonic (TNBS) acid, β -carotene, linoleic acid, and Tween 20 were from Sigma (St. Louis, MO); Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, penicillin-streptomycin, and nonessential amino acids solution were purchased from Gibco products (Grand Island, NY). All other reagents were of analytical grade.

2.2. Preparation of Dehulled Defatted Meal and Proximate Composition. *J. curcas* seeds were dehulled by hand using pliers, and the resulting kernels were ground using a domestic blender. Flour was sifted through a 20-mesh screen and then defatted by Soxhlet hexane extraction for 11 hours.

Proximate composition of kernels was determined by AOAC methods [14]: moisture (934.01), fat (920.39), ash (923.03), and crude fibre (962.09). Protein was calculated as nitrogen \times 6.25 and carbohydrate content was estimated as nitrogen-free extract (NFE).

2.3. Preparation of Protein Isolate. The protein isolate was obtained according to Hoover et al. [15] with modifications. A suspension of dehulled defatted meal in water pH 10.5, 1 : 6 (w/v) was stirred for 1 h at room temperature. The suspension was then filtered twice through an 80-mesh screen, and then the solids on the screen were washed using distilled water. The filtrate was strained through a 150-mesh wet screen in order to get rid of the finest particles that still remained in suspension. Starch was eliminated by centrifugation at 487 \times g for 15 minutes, and proteins in the supernatant were precipitated by acidifying the alkaline extract to pH 4.5 using 1 M HCl. Proteins were recovered by centrifugation at 7796 \times g for 15 minutes at 5°C and then freeze-dried.

2.4. Preparation of *J. curcas* Protein Hydrolysates. *J. curcas* protein hydrolysates were prepared according to Megias et al. [12] using a hydrolysis reactor vessel equipped with stirrer, thermometer, and pH electrode. Freeze-dried protein isolate/water (5% w/v) were digested at 37°C using pepsin at pH 2.5 for 60 minutes and then with pancreatin at pH 7.5 for 120 minutes. A 1/20 (w/w) enzyme to substrate ratio was used for both enzymes. Aliquots were taken at different times and hydrolysis was stopped by heat inactivation at 80°C for 20 minutes. The resulting hydrolysates were clarified by centrifugation at 11227 \times g for 30 minutes at 5°C, frozen at -20°C, and stored at the same temperature until further use.

2.5. Protein Determination. Protein content (g kg^{-1}) in protein isolate and hydrolysates was determined by elemental analysis as % nitrogen content \times 6.25 using a LECO CHNS-932 analyzer (St. Joseph, MI).

2.6. Degree of Hydrolysis. The degree of hydrolysis was calculated by measuring the free amino groups (h) by reaction with TNBS [16]. The total number of amino groups (h_t) was determined in samples 100% hydrolyzed by treatment with 6 N HCl at 110°C for 24 h.

The degree of hydrolysis was calculated using the formula $\% \text{ DH} = (h/h_t) \times 100$.

2.7. Amino Acid Analysis. Amino acid analysis was carried out by acid hydrolysis and HPLC, after derivatization with diethyl ethoxymethylenemalonate, according to the method described by Alaiz et al. [17] using D,L- α -aminobutyric acid as internal standard.

2.8. Antioxidant Activity: β -Carotene Bleaching Method. Antioxidant activity was determined by using the β -carotene bleaching method, with modifications as described [18]. A mixture of β -carotene (1 mL, 10 mg/mL in chloroform), linoleic acid (20 mg), and Tween 20 (200 mg) was vigorously stirred by vortexing. After removal of chloroform under a

stream of nitrogen, a clear solution was obtained by mixing in oxygen-sparged distilled water (50 mL). Protein hydrolysates (500 μg) and β -carotene/linoleic acid/Tween 20 solution (200 μL) were added to wells in a 96-well plate and incubated at 50°C in the dark. Negative controls consisted of incubations containing the β -carotene/linoleic acid/Tween 20 solution, in the absence of hydrolysates. Positive controls consisted of incubations containing the β -carotene/linoleic acid/Tween 20 solution and 10 μg of BHT. The peroxidative degradation of β -carotene was monitored by recording absorbance at 450 nm for 135 min using a microplate reader.

2.9. Copper and Iron Chelating Activity. Cu^{2+} chelating activity was determined using the pyrocatechol violet reagent according to Saiga et al. [19]. Hydrolysates (500 μg protein) were added to 96-well plates containing 250 μL of 50 mM sodium acetate pH 6.0, 6.25 μL of 4 mM pyrocatechol violet, and Cu (1 μg , CuSO_4). EDTA (50 μg) was used as a positive control. Absorbance at 632 nm was determined after incubation for 1 minute at room temperature. Copper chelating activity was calculated as follows:

$$\begin{aligned} &\text{Chelating activity (\%)} \\ &= \left(1 - \frac{\text{absorbance of sample at 632 nm}}{\text{absorbance of control at 632 nm}} \right) \times 100. \quad (1) \end{aligned}$$

Fe^{2+} chelating activity was determined by measuring the formation of the Fe^{2+} -ferrozine complex according to Carter [20]. Hydrolysates (500 μg protein) were added to 96-well plates containing 250 μL 100 mM sodium acetate buffer pH 4.9 and 25 μL FeCl_2 solution (0.1 mg/mL water). EDTA (50 μg) was used as a positive control. Absorbance at 562 nm was determined after addition of a ferrozine solution (12.5 μL , 40 mM in water) and incubation for 30 minutes at room temperature. Iron chelating activity was calculated as follows:

$$\begin{aligned} &\text{Chelating activity (\%)} \\ &= \left(1 - \frac{\text{absorbance of sample at 562 nm}}{\text{absorbance of control at 562 nm}} \right) \times 100. \quad (2) \end{aligned}$$

2.10. Fast Protein Liquid Chromatography (FPLC) and Reverse Phase-HPLC. FPLC was carried out in an AKTA-purifier system equipped with a Superdex Peptide column (GE Healthcare); bed dimensions were 10 \times 300–310 mm. Injection volume was 200 μL (10 mg protein hydrolysate/mL) and elution was carried out using 0.75 M ammonium bicarbonate at 1 mL/min. Elution was monitored at 214 nm and molecular mass was estimated using molecular weight standards from Pharmacia: blue dextran (2000000 Da), cytochrome c (12500 Da), aprotinin (6512 Da), bacitracin (1450 kDa), cytidine (246 Da), and glycine (75 Da).

RP-HPLC was carried out by injecting hydrolysates (20 μL , 2 $\mu\text{g}/\mu\text{L}$) in a Discovery BIO Wide Pore C_{18} column (25 cm \times 4.6 mm, 5 μm) (Supelco), using a Beckman-Coulter HPLC system. A linear gradient acetonitrile 0.1% trifluoroacetic acid : water 0.1% trifluoroacetic acid, 0 : 100 to 30 : 70 (v/v) in 60 minutes, at a flow of 1 mL/min and 25°C, was

used for elution. The eluent was monitored for absorbance at 215 nm.

2.11. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) Analysis. RP-HPLC fractions that exhibited strong radical scavenging activity were subjected to MALDI-TOF analysis using a QSTAR XL quadrupole time-of-flight mass spectrometer. The samples were dissolved in 600 μL of acetonitrile at 20% (v/v). To enhance ionization, the matrix α -cyano-4-hydroxycinnamic acid (α -cyano, CHCA) was used in a 1:1 matrix : sample relation. Samples were analyzed in positive ion reflector mode in a range of 500–3000 Da.

2.12. Antioxidant Activity in Caco-2 Cells. Antioxidant activity in Caco-2 cells was determined according to Wolfe and Riu [21] with modifications. Caco-2 cells from the European Collection of Cell Cultures were grown in DMEM, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C and 5% CO_2 . Cells were seeded on 96-well microplates (2×10^4 cells/well). Twenty-four hours after seeding, growth medium was removed and cells were washed with PBS pH 7.4 before incubation for 1 hour in HBSS (100 $\mu\text{L}/\text{well}$), containing protein hydrolysates (100, 200, or 500 μg protein) and DCFH-DA, 25 μM . After incubation with the hydrolysates and DCFH-DA, wells were washed using PBS (100 μL), and HBSS (100 μL) containing 600 μM of ABAP was added. Fluorescence (excitation at 485 nm and emission at 555 nm) was measured after 120 min. Each plate included positive control wells, which consisted of cells treated with DCFH-DA and free radical generator ABAP in the absence of hydrolysates, and negative control wells, which consisted of cells treated with DCFH-DA in the absence of hydrolysates and ABAP. The decrease in cellular fluorescence (fluorescence units), when compared to the control cells, indicated the antioxidant capacity of the hydrolysates.

2.13. Statistical Analysis. A one-way analysis of variance and Fisher's LSD test were used to determine statistically significant differences ($P < 0.05$), using Statgraphics plus version 5.1.

3. Results and Discussion

3.1. Kernels Proximate Composition and Production of *J. curcas* Protein Hydrolysates. Protein content of *Jatropha* kernels ($276.6 \pm 0.8 \text{ g kg}^{-1}$) was similar to that reported for other *J. curcas* genotypes from Mexico (311 g kg^{-1} , for flour from Papantla, Veracruz; 321 g kg^{-1} , for flour from Yautepec, Morelos). Devappa and Swamylingappa [4] reported 248 g kg^{-1} for flour from Karnataka, India. Ash content was $39.4 \pm 0.0 \text{ g kg}^{-1}$, similar to that reported for different genotypes from Mexico (47, 51 g kg^{-1}) and India (41 g kg^{-1}); crude fat was 618.7 g kg^{-1} , slightly higher than other Mexican genotypes (577 g kg^{-1} Papantla, Veracruz; 553 g kg^{-1} Yautepec, Morelos) [2]; however, Devappa and Swamylingappa [4]

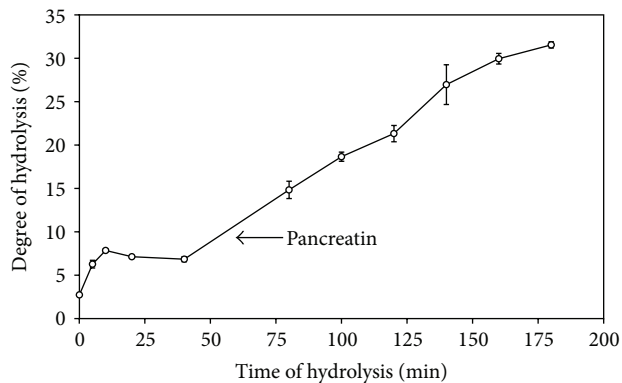


FIGURE 1: Hydrolysis of *J. curcas* protein isolate by treatment with pepsin for 60 minutes followed by treatment with pancreatin for 120 minutes. Data correspond to the average \pm standard deviation of three determinations.

reported that *Jatropha* kernel contained 400–600 g oil kg⁻¹. Crude fiber (55.5 \pm 1.4 g kg⁻¹) was also similar to the content (41.6 g kg⁻¹) reported by these authors.

Preparation of *J. curcas* protein isolate yielded a protein concentration of 901 \pm 10.8 g kg⁻¹, which is similar to that reported by Makkar et al. [1]. Treatment with pepsin for 40 minutes yielded a 6.8% degree of hydrolysis, which did not increase any further during treatment for up to 60 minutes (Figure 1). This result is consistent with previous reports on the use of pepsin to hydrolyze seed proteins [22]. Thus, Torres-Fuentes et al. [23] reported a 9% degree of hydrolysis after treating chickpea protein with pepsin for 60 minutes. Quist et al. [24] obtained a roasted peanut hydrolysate with 9.6% degree of hydrolysis after 30 minutes of pepsin treatment. The final *J. curcas* protein hydrolysate that was produced by treatment with pepsin for 60 minutes, followed by pancreatin for 120 minutes, had 31.5% degree of hydrolysis (Figure 1), which is similar to that reported by Megias et al. [22] for sunflower protein isolates (37%).

Pepsin is the main enzyme generated in the human stomach during food digestion; it has endopeptidase activity and is most efficient in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids, such as Phe, Tyr, and Trp. Pancreatin includes proteases, such as trypsin or chymotrypsin, released by the pancreas in the small bowel. Trypsin is an endopeptidase that is more efficient in cleaving peptide bonds between basic amino acids, such as Arg and Lys; chymotrypsin is an endopeptidase which is more efficient in cleaving peptide bonds between aromatic amino acids, such as Tyr, Phe, and Trp [12, 25–27].

3.2. Fast Protein Liquid Chromatography and Reversed Phase Chromatography. The most prominent feature in the *J. curcas* protein isolate as determined by FPLC was a major protein fraction with an apparent molecular weight of 42.5 \pm 0.96 kDa, corresponding to nonhydrolysed protein, that eluted with the void volume; there were also two species detected with negligible presence and lower molecular weight (Figure 2), probably related to the degree of hydrolysis in the protein

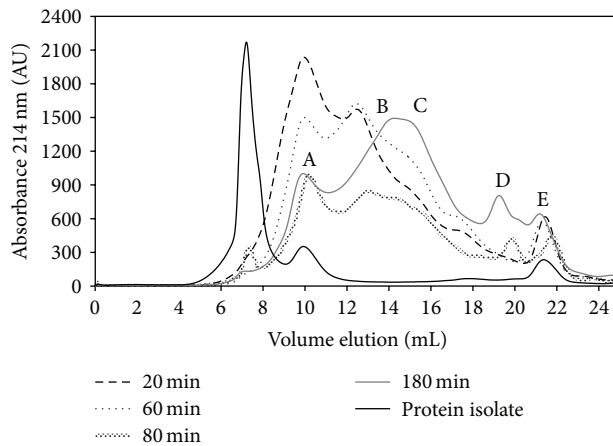
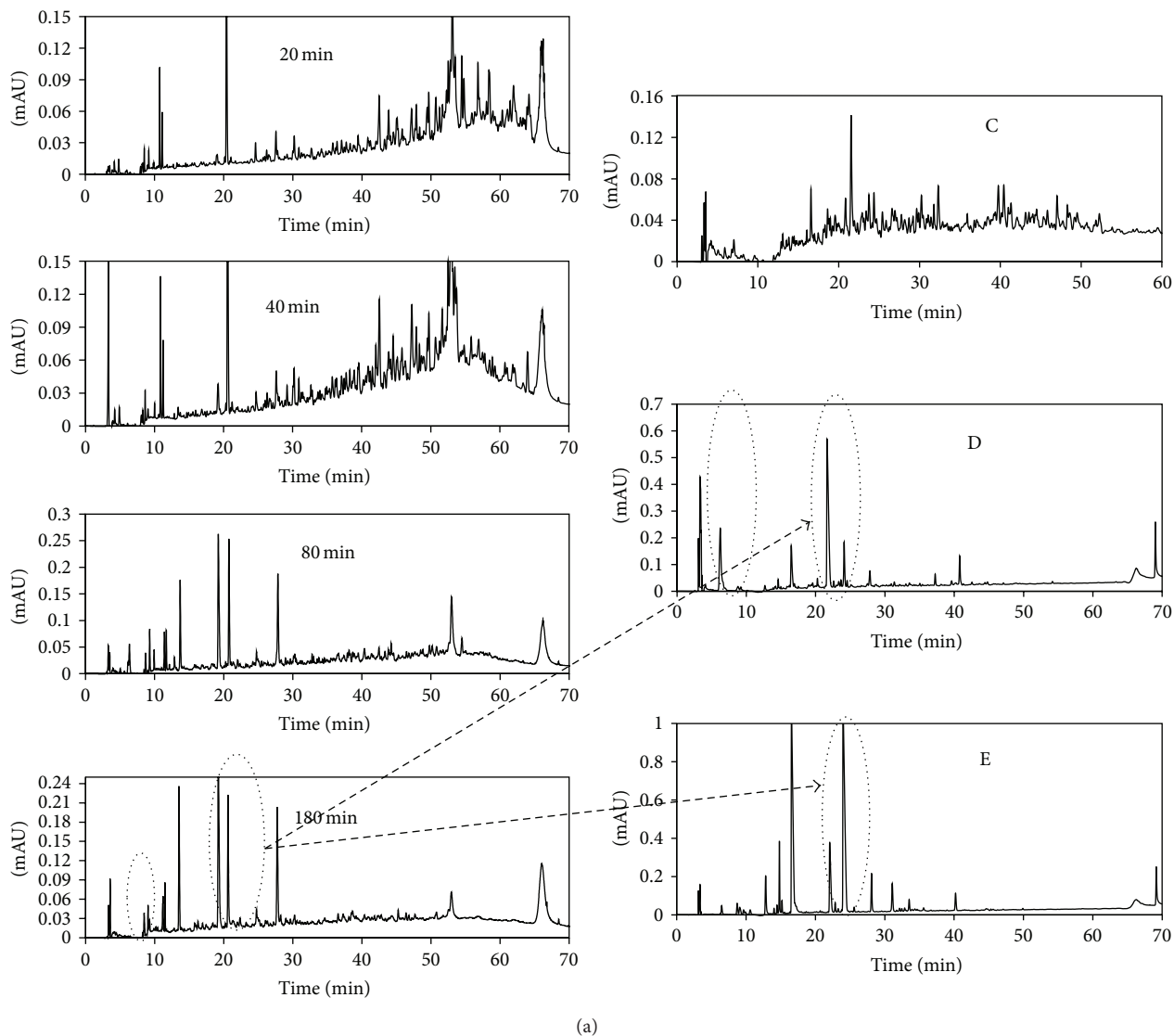


FIGURE 2: Fast protein liquid chromatography of *J. curcas* protein isolate and hydrolysates. Legends refer to total time of hydrolysis with pepsin (20 and 60 minutes) or pepsin followed by pancreatin (80 and 180 minutes, resp.), as shown in Figure 1.

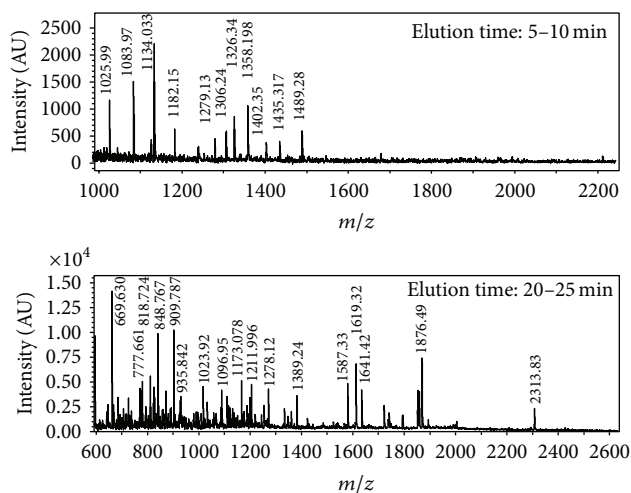
isolate (2.7%). Lower molecular weight fractions appeared in the hydrolysates obtained by treatment with pepsin and pancreatin. Thus, in addition to a common fraction of \sim 11.6 \pm 0.15 kDa, hydrolysis for 20, 60, 80, and 180 minutes yielded hydrolysates with major peptidic fractions, with apparent molecular weight of 3.6 \pm 0.0, 3.4 \pm 0.3, 2.8 \pm 0.0, and 1.5 \pm 0.1 kDa, respectively. Chromatographic analysis using RP-HPLC revealed that the hydrolysates were made up of a complex mixture of peptides (Figure 3(a)). Hydrolysis with pepsin for 20 and 40 minutes resulted in similar peptidic profiles. Additional hydrolysis using pancreatin resulted in increasing amounts of more hydrophilic peptides that elute between 10 and 50 minutes and disappearance of hydrophobic peptides with elution times higher than 50 minutes (Figure 3(a)).

The chromatogram corresponding to 180 min hydrolysate, as shown in Figure 2, was divided into five fractions (A to E), corresponding to peptides eluted between 7.9 and 11.2 mL (fraction A), 11.2 and 14.5 mL (fraction B), 14.5 and 18.6 mL (fraction C), 18.6 and 20.5 mL (fraction D), and 20.8 and 23.5 mL (fraction E). Antioxidant activity was determined using DPPH radical, detecting the highest radical scavenging activity in C, D, and E fractions (data not shown). Antioxidant activity in Caco-2 cells was determined in the lower molecular weight fractions C, D, and E, testing 50 μ g of protein.

3.3. MALDI-TOF-MS Analysis. RP-HPLC fractions that exhibited strong radical scavenging activity (Figure 3(a) marked with circles) were subjected to MALDI-TOF analysis. The peptidic fraction obtained at 5–10 min of elution time in RP-HPLC analysis (Figure 3(b)) contains several peptides with different molecular masses: a peptide with high presence and molecular mass of 1134 Da was detected, followed by two more peptides with moderate presence and molecular masses of 1025, 1083 Da. The peptidic fraction obtained at 20–25 min of elution time showed a high quantity of peptides with different molecular masses (669 to 2313 Da). These peptides



(a)



(b)

FIGURE 3: (a) RP-HPLC analysis of *J. curcas* protein hydrolysates produced by hydrolysis with pepsin (20 and 40 minutes) or pepsin followed by pancreatin (80 and 180 minutes) as shown in Figure 1 and RP-HPLC analysis of lower MW fractions C, D, and E from 180 min hydrolysate separated by FPLC. (b) MALDI-TOF analysis of *J. curcas* peptidic fractions obtained at 5–10 min and 20–25 min of elution time in RP-HPLC analysis.

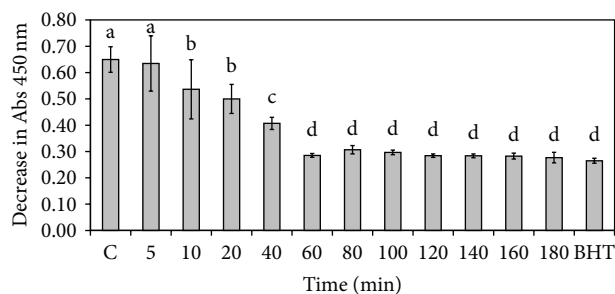


FIGURE 4: Inhibition of β -carotene degradation by *J. curcas* protein hydrolysates. Values are the mean \pm standard deviation of three determinations. Different letters indicate significant differences ($P < 0.05$).

are present in fractions D and E from FPLC (Figure 3(a)). The results were in concert with the general finding that short peptides with 2–10 amino acids exert greater antioxidant potential and other bioactive properties than their parent native proteins or large polypeptides [28].

3.4. Antioxidant Activity: β -Carotene Bleaching Method. Antioxidant activity in the *J. curcas* protein hydrolysates increased with degree of hydrolysis (Figure 4); this was probably because the enzyme hydrolysis may have unfolded the native protein and exposed amino acid residues, which were electron donors and could react with free radicals, convert them to more stable products, and terminate the free radical chain reaction [29]. The highest antioxidant activity was observed in samples produced by hydrolysis from 60 to 180 minutes (treatment with pepsin for 60 minutes and 0 to 120 minutes additional treatment with pancreatin). The inhibition of the oxidative decomposition of β -carotene by these samples was higher than the inhibition caused by the negative control and by the samples that were generated by hydrolysis for 5 to 40 minutes; further treatment with pancreatin did not result in higher antioxidant activity. The activity of these hydrolysates at a concentration of 500 $\mu\text{g}/\text{well}$ as shown in Figure 4 was similar to the activity of 10 μg BHT/ well . These results are comparable to those reported for sunflower protein hydrolysates produced using pepsin and pancreatin [12].

3.5. Metal Chelating Activity. Hydrolysis of the *J. curcas* protein isolates results in an increasing copper chelating activity as shown in Figure 5(a). This activity roughly correlated with the degree of hydrolysis and reached a maximum in the hydrolysates produced by treatment with pepsin for 60 minutes, followed by treatment with pancreatin for 100 to 120 minutes, which chelated 78% of the copper ion in the assay. Thus, protein hydrolysis led to an enhanced Cu^{2+} chelating ability, which may be due to an increased concentration of carboxylic acid groups. These groups promote the conversion of proteins into an anionic form, which facilitates chelation of Cu^{2+} also, an increased exposure of metal chelating amino acid residues, such as histidine, which is a well-known metal chelating amino acid [29, 30]. It is worth mentioning that

J. curcas protein isolate is a good source of histidine (29–35 g kg^{-1}) [1, 31]. Another reason could be a combination of high His content and small peptide size in the hydrolysates [23].

Treatment with pepsin and pancreatin also led to an increase in iron chelating activity although the maximum iron chelating activity, which corresponded to the hydrolysates produced by treatment with pepsin for 60 minutes, followed by treatment with pancreatin for at least 20 minutes, was about three times lower than the maximum copper chelating activity (Figures 5(a) and 5(b)). Peng et al. [29] have also noted that whey protein hydrolysates have a far weaker Fe^{2+} chelating capacity than Cu^{2+} chelating capacity. Kong and Xiong [32] mentioned that this behavior may be related to the higher coordination number of Fe^{2+} (thus, more chelators required) than in Cu^{2+} .

3.6. Antioxidant Activity in Caco-2 Cell Cultures. In order to assess whether the hydrolysates may have antioxidant activity “*in vivo*,” they were assayed using Caco-2 cells treated with ABAP as a free radical generator and DCFH-DA as an indicator of intracellular reactive oxygen species. Treatment with ABAP causes oxidative stress, resulting in the generation of reactive oxygen species. DCFH-DA is taken up by cells and cleaved by nonspecific esterases to form DCFH, which can be oxidized to the highly fluorescent derivative DCF by reactive oxygen species. The hydrolysates produced by treatment solely with pepsin for 60 minutes, or by treatment with pepsin for 60 minutes followed by pancreatin for 120 minutes, were used for these experiments. The former showed antioxidant effect, that was not dependent on protein concentration, while the latter showed a statistically significant ($P < 0.05$) antioxidant effect that was similar at 100 and 200 $\mu\text{g}/\text{well}$ and higher at 500 $\mu\text{g}/\text{well}$ (Figures 6(a) and 6(b)).

Factors other than intrinsic antioxidant activity may have a great impact in the intracellular antioxidant effect of the hydrolysates, as compared to simpler chemical assays. Thus, cell permeability and susceptibility to hydrolysis by cellular exo- and endoproteases could limit or completely prevent antioxidant peptides from getting within cells.

The highest antioxidant activity was detected in fractions D and E (Figure 6(c)); these were especially rich in tyrosine, phenylalanine, arginine, and leucine (Table 1). It has been reported that histidine, tyrosine, and leucine residues are abundant in antioxidant peptides [33]; arginine has also been related to antioxidant activity [12, 34]. Recent studies have shown that tripeptides consisting of Trp or Tyr residues at the C-terminal and Phe at their N-terminal had strong free radical scavenging activity and inhibition of peroxidation of linoleic acid [35]. Moreover, antioxidant peptides often include hydrophobic amino acid residues such as Val or Leu at the N-terminus of the peptides and Pro, His, Tyr, Trp, Met, and Cys in their sequences [36].

Apart from the amino acid composition, the molecular weight of peptides may also determine their antioxidant activity [33]. The majority of the antioxidant peptides derived from food sources have molecular weights ranging from 500 to 1800 Da [36]. For example, in corn gluten meal

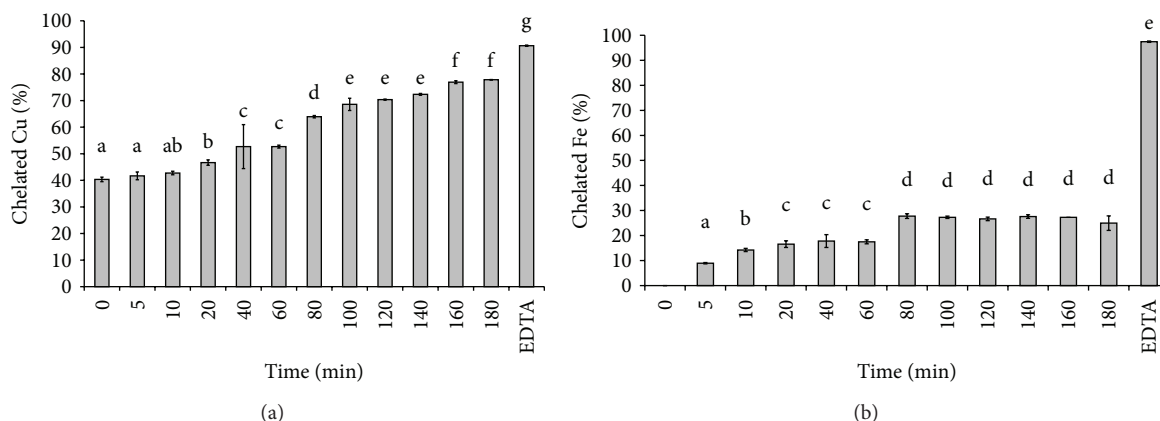


FIGURE 5: (a) Copper chelating activity in *J. curcas* protein isolate and hydrolysates. Values are the mean \pm standard deviation of three determinations. Different letters indicate significant differences ($P < 0.05$). (b) Iron chelating activity in *J. curcas* protein isolate and hydrolysates. Values are the mean \pm standard deviation of three determinations. Different letters indicate significant differences ($P < 0.05$).

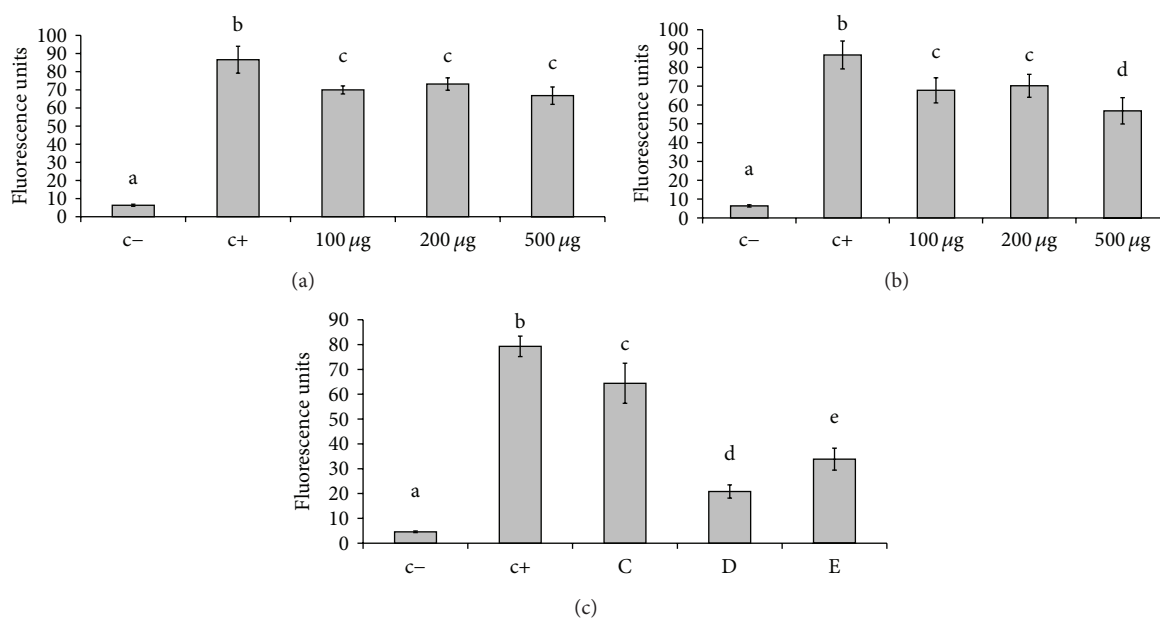


FIGURE 6: Antioxidant activity of increasing concentrations of *J. curcas* hydrolysates in Caco-2 cell cultures treated with the free radical generator ABAP. Formation of reactive oxygen species was determined by oxidation of the fluorescent dye DCFH. Antioxidant effect of hydrolysate produced by treatment solely with pepsin for 60 minutes (a) or treatment with pepsin for 60 minutes followed by treatment with pancreatin for 120 minutes (b). (c) FPLC fractions C, D, and E (50 μ g protein) from 180 min hydrolysate. Values are the mean of data from five wells \pm standard deviation. Experiments were conducted in duplicate on two different days. Different letters indicate significant differences ($P < 0.05$).

hydrolysates, the antioxidant activity of peptides with a MW between 500 and 1500 Da was higher than that of peptides above 1500 Da and below 500 Da [33]. This could explain why fraction D had a stronger antioxidant activity when compared with fraction E.

4. Conclusions

This work has proven the presence of naturally occurring antioxidant and chelating peptides in nontoxic *J. curcas* seeds. These peptides were released from the native proteins by

in vitro treatment, with the digestive enzymes pepsin and pancreatin, which mimics physiological digestion of proteins. Treatment with pepsin for one hour followed by treatment with pancreatin for two hours yielded the highest antioxidant and metal chelating properties. The antioxidant activity was determined using a cell culture system, supporting the view that these bioactive peptides might have activity *in vivo*. *J. curcas* protein digestion with gastrointestinal enzymes generates a broad spectrum of antioxidant peptides of different molecular sizes. This information might increase the economic value of *J. curcas* defatted meal from nontoxic

TABLE 1: Amino acid composition of *J. curcas* protein fractions purified by Fast protein liquid chromatography after 60 min hydrolysis with pepsin and 120 min hydrolysis with pancreatin. Data (g kg⁻¹ protein) represent the average \pm SD of two determinations.

	A	B	C	D	E	FAO (1991)
Asp ^a	143 \pm 8.7	168 \pm 2.5	163 \pm 0.5	102 \pm 28.0	86 \pm 24.0	
Glu ^b	251 \pm 8.8	222 \pm 1.2	167 \pm 0.2	67 \pm 2.9	23 \pm 1.7	
Ser	65 \pm 3.1	67 \pm 1.8	72 \pm 0.1	18 \pm 0.4	13 \pm 1.7	
His	34 \pm 10.8	25 \pm 5.2	32 \pm 1.0	30 \pm 6.0	13 \pm 0.8	19
Gly	64 \pm 0.9	100 \pm 10.6	89 \pm 4.1	16 \pm 0.1	17 \pm 1.0	
Thr	26 \pm 0.1	46 \pm 1.2	40 \pm 0.2	12 \pm 0.4	5 \pm 1.0	34
Arg	133 \pm 13.0	94 \pm 1.8	95 \pm 2.0	233 \pm 6.6	22 \pm 3.0	
Ala	47 \pm 0.2	65 \pm 1.9	76 \pm 1.3	32 \pm 1.0	10 \pm 2.0	
Tyr	32 \pm 0.8	21 \pm 0.7	22 \pm 3.0	53 \pm 0.4	400 \pm 11.1	
Val	17 \pm 0.0	26 \pm 2.1	43 \pm 0.0	24 \pm 1.9	2 \pm 0.4	35
Met	4 \pm 0.1	5 \pm 1.0	7 \pm 3.0	0.0	0.0	
Cys	26 \pm 4.5	17 \pm 2.3	3 \pm 0.7	0.0	0.0	25 ^d
Ile	25 \pm 0.6	27 \pm 0.7	31 \pm 0.1	48 \pm 1.5	7 \pm 2.0	28
Leu	53 \pm 0.0	50 \pm 7.5	82 \pm 1.5	202 \pm 9.8	34 \pm 2.0	66
Phe	22 \pm 3.1	18 \pm 2.3	32 \pm 0.0	142 \pm 7.0	362 \pm 1.5	63 ^c
Lys	58 \pm 0.6	48 \pm 0.2	46 \pm 1.0	22 \pm 0.4	9 \pm 1.8	58
Trp	ND	ND	ND	ND	ND	11
Pro	ND	ND	ND	ND	ND	

^a Asp + Asn. ^b Glu + Gln. ^c Phe + Tyr. ^d Met + Cys. ND: not determined.

J. curcas genotypes, which could be used for producing functional food components.

Abbreviations

ABAP:	2, 2'-Azobis(2-amidinopropane) dihydrochloride
AOAC:	Association of Official Analytical Chemists
BHT:	Butylated hydroxytoluene
DCFH-DA:	2', 7'-Dichlorofluorescein diacetate
DCFH:	Intracellular 2', 7'-dichlorofluorescein
DCF:	Fluorescent dichlorofluorescein
DMEM:	Dulbecco's modified Eagle medium
DPPH:	2,2-Diphenyl-1-picrylhydrazyl
DH:	Degree of hydrolysis
EDTA:	Ethylenediaminetetraacetic acid
FPLC:	Fast protein liquid chromatography
HBSS:	Hanks' balanced salt solution
HPLC:	High performance liquid chromatography
kDa:	Kilodaltons
MALDI-TOF:	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MW:	Molecular weight
PBS:	Phosphate buffer saline
RP-HPLC:	Reverse phase-high performance liquid chromatography
ROS:	Reactive oxygen species
TNBS:	2, 4, 6-Trinitrobenzenesulfonic acid.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by the Mexican National Council of Science and Technology (CONACYT), the National Polytechnic Institute (IPN), the Spanish Ministry of Innovation and Science, through Research Project AGL2007-63580, and the European Union through FEDER funds. Thanks are due to María Dolores García-Contreras and Alicia Chagolla for technical assistance.

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