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DPPH Radical Scavenging Activity of a Mixture of Fatty Acids and Peptide-Containing Compounds in a Protein Hydrolysate of *Jatropha curcas* Seed Cake

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ABSTRACT: *Jatropha curcas*, a tropical plant, has great potential commercial relevance as its seeds have high oil content. The seeds can be processed into high-quality biofuel producing seed cake as a byproduct. The seed cake, however, has not gotten much attention toward its potential usefulness. This work was aimed to determine the antioxidant activity of different fractions of a protein hydrolysate from *J. curcas* seed cake and to elucidate the molecular structures of the antioxidants. Seed cake was first processed into crude protein isolate and the protein was hydrolyzed by Neutrase. The hydrolysate obtained from 1 h of Neutrase hydrolysis showed the strongest antioxidant activity against DPPH radical (2,2-diphenyl-1-picrylhydrazyl). After a purification series of protein hydrolysate by liquid chromatography, chemicals acting as DPPH radical inhibitors were found to be a mixture of fatty acids, fatty acid derivatives, and a small amount of peptides characterized by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

KEYWORDS: *Jatropha curcas, protein hydrolysate, purification, antioxidant activity, characterization*

INTRODUCTION

Three of the most pressing needs in today's world include the production of environmentally acceptable biofuel, the production of food for people and animals in developing countries, and the discovery of bioactive compounds in foods that improve health. In all three categories, the *Jatropha curcas* plant is beneficial.[1,2] *J. curcas* is a semievergreen shrub or small tree of the Euphorbiaceae family that is easy to grow under adverse soil conditions. It can be planted in tropical countries such as India, China, Thailand, Madagascar, Myanmar, and other developing countries.[3] The seeds contain 27−40% (w/w) oil which is used for the production of soap, cosmetics, and particularly fuel.[1,3,4] After oil extraction, the seed cake contains a high amount of protein, approximately 19−27% (w/w).[5] The seed proteins contain all of the dietary essential amino acids (except lysine) at levels higher than the FAO/WHO recommendations for the five-year-old child.[6] However, the seeds are toxic to humans and animals due to the presence of harmful phorbol esters and antinutritional factors such as trypsin inhibitor, lectin, saponin, and phytate, which restrict their unprocessed use for human food or animal feed.[5] Recently, three methods have been developed to eliminate these noxious compounds: physical methods involving heat treatment and ionizing radiation,[7,8] chemical treatments using solvent extraction, bleaching agents, degumming agents, deodorization agents, and alkalis,[7,9] and biological treatments by bacteria or fungal fermentation.[10−12] The detoxified seed cake is rich in high-quality protein and can be utilized as a good quality food for humans and animals.[2] Thus, *J. curcas* seed cake protein could be an alternative protein source, which is greatly needed for humans and animals in developing countries.

A protein hydrolysate obtained from defatted *J. curcas* seed cake has been shown to express biological activities such as antihypertension, antioxidation, and metal-chelation.[13,14] However, the chemical compounds responsible for such activities have not yet been identified. In this study, we aimed to characterize bioactive compounds in *J. curcas* protein hydrolysate responsible for its antioxidant properties.

MATERIALS AND METHODS

Chemicals. Screw press defatted *J. curcas* seed cake was provided by Ladda Co. (Thailand). Chemical reagents were analytical grade and purchased from Fischer Scientific (Loughborough, UK). Neutrase 0.8L, purchased from Sigma-Aldrich (Steinheim, Germany), is a Novozyme product containing 4% (w/w) neutral protease obtained from *Bacillus amyloliquefaciens* with a specific activity of 0.8 Sigma U/g. DPPH (2,2-diphenyl-1-picrylhydrazyl) was purchased from Sigma-Aldrich (Steinheim, Germany). Reagents for tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (tricine-SDS-PAGE) and Seeblue plus2 prestained molecular weight standard were purchased from Bio-Rad (Hercules, CA, USA). Molecular weight standards for gel filtration chromatography: β-amylase (210000 Da), carbonic anhydrase (29000 Da), alcohol dehydrogenase (15000 Da), and cytochrome C (12500 Da) were purchased from GE Healthcare (Uppsala, Sweden).

Protein Isolation from *J. curcas* Seed Cake. The seed cake was ground and then dried in a vacuum oven at 55 °C until its weight was constant. Proximate analysis was carried out according to the Association of Official Analytical Chemists.[15] Phorbol ester and antinutritional factors, including phytic acid, trypsin inhibitor, lectin,

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and saponin, were quantified following the methods outlined by Saetae et al.[16] Phorbol ester was removed from the seed cake by ethanol extraction according to the method of Saetae and Suntornsuk.[9] The detoxified seed cake was kept in a freezer at −20 °C until use.

Proteins were isolated from the seed cake by the method of Makkar et al.[17] with some modifications. The detoxified seed cake was suspended in distilled water (1:10, w/v), and the pH of the suspension was adjusted to 11.0 by 1 M NaOH. The suspension was stirred at 100 rev/min at 30 °C for 3 h and was then centrifuged at 8500*g* at 25 °C for 15 min. The supernatant was collected. The supernatant was then adjusted to pH 4.0 (isoelectric point) using 1 M HCl. The mixture was centrifuged at 8500*g* at 25 °C for 15 min. The precipitate was collected and washed with distilled water (pH 7.0) three times and then freeze-dried for 24 h. The crude protein isolate was stored in a freezer at −20 °C until use.

Protein Hydrolysis. The crude protein isolate was suspended in 0.1 M phosphate buffer, pH 7.0, at the ratio of 1:100 (w/v). The suspension was mixed with Neutrase solution prepared at 1 U/mL at a ratio of 100:1 (v/v). Hydrolysis was carried out at 50 °C on a reciprocal shaker set at 100 rev/min for 6 h. The protein hydrolysate was sampled at 0, 1, 2, 3, 4, 5, and 6 h. The reaction was terminated by heating at 100 °C for 10 min. The pH of the respective samples was neutralized to 7.0, followed by centrifugation at 8500*g* at 25 °C for 15 min. The supernatant was collected and analyzed for soluble protein, free amino acids, degree of hydrolysis, and DPPH radical inhibitory activity. As a control, a mixture of the protein isolate and 0.1 M phosphate buffer, pH 7.0, was carried out under the same conditions.

Protein Purification. *Gel Filtration.* A protein hydrolysate (10 mg protein) expressing the highest DPPH radical inhibitory activity was purified by a gel filtration prepacked column (Superose 12 10/300 GL, GE Healthcare, Uppsala, Sweden) attached to an ÄKTA-FPLC system (GE Healthcare, Uppsala, Sweden). The column was eluted with distilled water, pH 7.0, at a flow rate of 1 mL/min for 35 min. The elution was monitored at 280 nm absorbance and the mass was estimated using molecular weight standards. Fractions (1 mL) were collected and analyzed for DPPH radical inhibitory activity and soluble protein. The samples with the highest antioxidant (DPPH radical) activity were pooled, freeze-dried, and stored at −20 °C.

Ion Exchange Chromatography. A freeze-dried sample (30.0 mg) after gel filtration was dissolved in 10 mL of 0.1 M Tris-HCl buffer, pH 9.0. The solution was then added into 100 g of swollen diethylaminoethyl (DEAE) resin (Sepharose CL-6B, Sigma-Aldrich, Steinheim, Germany) saturated with the 0.1 M Tris-HCl buffer, pH 9.0. The mixture was stirred at 100 rev/min at 25 °C for 1 h and then filtered through a 0.45-μm filter paper. The filtrate (unbound fraction) was collected. To wash off the bound fraction, the resin was mixed with 100 mL of 0.1 M Tris-HCl buffer, pH 9.0 containing 1 M NaCl, and the mixture was stirred at 100 rev/min for 30 min. The resin was filtered through a 0.45 μm filter paper. The filtrate (bound fraction) was collected. The remaining resin was washed by 0.1 M Tris-HCl buffer, pH 9.0, containing 1 M NaCl twice as described above. The bound fractions were pooled. The bound and unbound fractions were determined for soluble protein and DPPH radical scavenging activities. The fraction with the most potent antioxidant activity was freeze-dried and stored at −20 °C.

Reverse Phase HPLC. The freeze-dried DEAE sample (5.0 mg) was dissolved in 10 mL of solution containing 97% (v/v) solution A (Milli-Q water with 0.1% (v/v) trifluoroacetic acid) and 3% (v/v) solution B (acetonitrile with 0.08% (v/v) trifluoroacetic acid). The pH of the sample solution was adjusted to 3.6 using 0.1 M HCl. The sample solution was centrifuged at 10000*g* at 25 °C for 1 min and 100 μL of the supernatant was applied to a reverse phase HPLC C8 column (Luna, 5 μm C8 (2) 100 Å, 250 × 4.6 mm, Phenomenex, Torrance, CA, USA) maintained at 25 °C using an Agilent 1200 liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA). The column was eluted by a linear gradient of the mobile phase as follows: (solutions A/B (as %, v/v)), 97/3 at 0−35 min, 70/30 at 36−44 min, 30/70 at 45−47 min, 10/90 at 48−51 min, and 97/3 at 52−60 min. The elution was monitored by absorbance at 214 nm. Fractions were manually collected and pooled. The pooled fractions were determined for DPPH radical inhibitory activity and protein content. The fraction with the highest DPPH radical inhibitory activity was freeze-dried and stored at −20 °C.

Strong Cation Exchange (SCX) Chromatography. The freeze-dried sample (3.0 mg) obtained from the reverse phase HPLC was dissolved in 1 mL of 40 mM ammonium formate buffer, pH 3.6, and then centrifuged at 10 000*g* at 25 °C for 1 min. The solution of 100 μL was applied to a strong cation exchange (SCX) cartridge (Alltech, MI, USA). The cartridge was washed by 300 μL of 40 mM ammonium formate buffer, pH 3.6, containing 10% (v/v) methanol and subsequently by 300 μL of 40 mM ammonium formate buffer, pH 3.6. The wash fractions were collected. The cartridge was eluted using 300 μL of 0.1−1.0 M ammonium formate buffer, pH 3.6, in a stepwise gradient, and the fraction of each gradient was collected. Protein content and DPPH radical inhibitory activity were determined on all fractions. The fraction with the highest DPPH radical inhibitory activity was freeze-dried and stored at −20 °C.

Characterization. *Molecular Weight Determination by SDS-PAGE.* The protein isolate, protein hydrolysate, and collected fractions from all purification steps were determined for molecular weight by SDS-PAGE.[18] Each sample (200 μg) was dissolved in 50 μL of deionized water and then centrifuged at 10000*g* at 25 °C for 10 min. A sample solution (20 μL) was dissolved with 10 μL of loading buffer containing 4% (w/v) sodium dodecyl sulfate (SDS), 12% (w/v) glycerol, 2% (v/v) mercaptoethanol, and 0.01% (v/v) bromophenol blue in 50 mM Tris-HCl buffer, pH 6.8. The mixture was boiled for 10 min, and 20 μL of mixture was loaded onto polymerized gels with a 16.5% acrylamide resolving gel and a 4% acrylamide stacking gel. The SDS-PAGE experiment was run at 80 V for 3 h using 0.1 M Tris-HCl buffer containing 0.1 M tricine and 0.1% (w/v) SDS as cathode buffer, and 1 M Tris-HCl buffer, pH 8.9, as anode buffer. The gel was fixed in a fixing solution (40% (v/v) methanol and 10% (v/v) acetic acid) for 30 min and stained by 0.025% (w/v) Coomassie blue G 250 in 10% (v/v) acetic acid for 1 h. The gel was destained by shaking the gel in 10% (v/v) acetic acid for 2 h, replacing the destaining solution every 30 min. The Seeblue plus2 prestained marker with molecular weight of 4−250 kDa was used as a protein reference.

Molecular Weight Determination by Electrospray Ionization Mass Spectrometry (ESI-MS). The SCX fraction expressing the strongest DPPH radical inhibitory activity (100 μg) was dissolved in 50 μL of MS grade water and then its pH was adjusted to achieve a final pH of 8.5 by 10 mM ammonium formate buffer, pH 9.5. The solution was centrifuged at 13000*g* at 25 °C for 30 min. The supernatant (2 μL) was injected into an Agilent 1200 liquid chromatography system (Agilent Technologies). The isocratic mobile phase was composed of 0.1% (v/v) formic acid, 50% (v/v) acetonitrile, and 50% (v/v) water at a flow rate of 100 μL/min. The LC eluent was directly infused into an Agilent 6520 quadrupole time-of-flight (Q-TOF) mass spectrometer. Ions were generated by electrospray ionization (ESI), cleaned of solvent by a nitrogen flow of 5.0 L/min and analyzed in negative ion mode (temperature of 300 °C, 30 psi, capillary voltage of 3300 V, fragmentor voltage of 145 V, and skimmer voltage of 55 V). The intensity of negative ions was recorded in the range of 100−1700 m/z and analyzed using Agilent Mass Hunter Workstation Qualitative Analysis software version B.03.01 (Agilent Technologies).

Amino Acid Profile. The amino acid compositions of the protein hydrolysates and the SCX fractions were determined according to the method of Mengerink et al.[19] using precolumn derivatization with orthophthalaldehyde (OPA) (Sigma-Aldrich, Steinheim, Germany). Samples of 0.2−10.0 mg were each hydrolyzed with 1 mL of 6 M HCl containing 0.1% (v/v) phenol at 110 °C for 24 h and the digested sample was dried by a centrifugal vacuum evaporator (SAVANT SC250EXP, Thermo Scientific, Waltham, MA, USA). The dry sample was resuspended in 2 mL of 0.067 M citrate buffer, pH 2.2, and then sonicated and vortexed. The mixture was filtered through a 0.2-μm syringe filter into a new vial. The solution (100 μL) was added to 880 μL of 0.1 M HCl and 20 μL of internal standard, norvaline. A sample solution of 5 μL was injected onto an Agilent Poroshell C18 (2.1 × 150 mm, 3 μm) column, controlled by an Agilent 1200SL instrument

and eluted at 40 °C with a linear gradient of mobile phase A (10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, and 0.5 mM NaN_3) and mobile phase B (acetonitrile, methanol, and HPLC grade water at a ratio of 45:45:10) set at the mobile phases of A and B (A/B, % v/v): 0/0 at 0.0−16 min, 52/48 at 17.0 min, 0/100 at 18.0−25.0 min, and 0/0 at 26.0−28.0 min. The flow rate was operated at 0.4 mL/min. The fluorescence excitation and emission wavelengths were set at 230 and 450 nm, respectively. All amino acids except cysteine, tryptophan, and proline were determined.

Structure Elucidation by NMR. For 1- and 2-D proton, 1-D phosphorus, and carbon spectra, the fraction (5 mg) was dissolved in D_2O (10%) and analyzed in a Shigemi tube. Spectra were acquired at 25 °C on a Bruker Avance 700 MHz spectrometer (Bruker, Rheinstetten, Germany) operating at 700.13 MHz, four radio frequency channels, and gradient pulse capabilities. The following probe heads, a cryoprobe (CPTCI 1H-13C/15N/D Z-GRD) and a room-temperature probe (PAQXI 1H/31P/D-13C/15N XYZ-GRD), were used. One-dimensional proton (^{1}H) spectra were acquired with excitation sculpting to suppress the abundant water signal and 32 768 data points, 2048 scans, 16.0845 ppm spectral width (11261.262 Hz), and 1.0 s pulse delay time. One-dimensional carbon (^{13}C) spectra were recorded with 65536 data points, 20 480 scans, 240.6607 ppm spectral width (42373.883 Hz), 2.0 s delay time or 3072 scans, 399.9793 ppm spectral width (70422.539 Hz), and 2.0 s delay time. One-dimensional phosphorus (^{31}P) spectra were acquired with 65536 data points, 5120 scans, 352.8536 ppm spectral width (100000 Hz), and 2.0 s delay time. All two-dimensional spectra were acquired with excitation sculpting for water suppression and 4096 × 128 data points, 13.9482 ppm × 4 ppm sweep width. The following two-dimensional spectra, TOCSY (60 ms mixing times, 1024 scans, 1.5 s delay time), NOESY (200 ms mixing time, 1024 scans, 1.0 s delay time), and COSY (320 scans, 1.1 s delay time), were recorded. Diffusion ordered spectroscopy (DOSY) experiments were performed with 3−9−19 water suppression and 32768 data points, 4096 scans, 40.0084 ppm sweep width, little DELTA of 1 ms, big DELTA of 0.1 s, and 12 gradients from 2 to 95% (logarithmic scale).

NMR spectra were processed with Topspin v 2.1 (Bruker-Biospin, Rheinstetten, Germany). DOSY data were fitted to exponential functions by iteration, and the diffusion constants of protons were calculated for a range of chemical shifts. Disentanglement of the overlapped spectra from DOSY data gave a measure of the size distribution and identity of compounds present in the sample.

Chemical Analysis. *Soluble Protein.* Soluble protein in the samples obtained after protein hydrolysis and in the fractions obtained from gel filtration and DEAE-ion exchange chromatography was determined according to the method of Lowry et al.[20]

Free Amino Acid Content and Degree of Hydrolysis. Free amino acid content and degree of hydrolysis were measured by the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method.[21] The degree of hydrolysis (%) was defined as the percentage of peptide bonds cleaved, releasing α-amino acid. It was calculated by $100(h/h_{tot})$, where h was the concentration of α-amino acid released during protein hydrolysis (mg equivalents of leucine/g protein) and h_{tot} was the concentration of total amino acid (complete hydrolysis) in 1 g of protein (mg equivalents of leucine/g protein).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity. DPPH radical scavenging activities of the hydrolyzed samples and their purified fractions were determined by the method of Maurya and Devasagayam.[22] The bleaching of DPPH radical was monitored at 517 nm. Ascorbic acid (10 mM) was the positive control, while the buffer solution was the negative control. All reactions were performed in the dark. The IC_{50}, defined as the minimal concentration of protein (mg/mL) that reduced DPPH radical by 50%, was determined by linear regression. The inhibitory activity (%) was calculated as equal to $(1 - A_{Sample}/A_{Control}) \times 100$; where A_{Sample} and $A_{Control}$ were the absorbance of the sample and control, respectively. The ascorbic acid equivalent antioxidant coefficient (AEAC = [inhibition (%) − b]/m) was calculated by linear regression analysis from a graph of % inhibition versus ascorbic acid concentration (mM) where b was the intercept and m was the slope.

Statistical Analysis. Results are given as the means ± standard deviations of three replications. The analyses of variance (ANOVA) and mean comparison (Duncan's multiple range tests) were undertaken using the SPSS software version 11.5 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Chemical Compositions of *J. curcas* Seed Cake, Detoxified Seed Cake, and Seed Cake Protein Isolate. In this study, *J. curcas* seed cake contained 22.7% protein, 25% fat, 10.4% ash, and 13.5% fiber (Table 1). The chemical composition of the seed cake was variable as indicated in earlier reports.[14,16,17,23] This may result from different cultivars, growth conditions (climate and soil), oil extraction methods, and oil extraction efficiency. After ethanol extraction of the seed cake, the amount of fat in the detoxified seed cake was reduced by 54% from the original seed cake (Table 1) since fat was well solubilized in 90% (v/v) ethanol. A change in fiber content but unchanged protein and ash contents were also found in the detoxified seed cake. The results were similar to those in the previous report of Saetae and Suntornsuk.[24]

Table 1. Chemical Composition of Seed Cake, Detoxified Seed Cake, and Seed Cake Protein Isolate from Detoxified *J. curcas* Seed Cake[a]

chemical	seed cake[b]	detoxified seed cake[b]	seed cake protein[b]
protein (% w/w)	22.7 ± 0.1a	23.1 ± 0.5a	82.0 ± 4.2b
fat (% w/w)	25.0 ± 0.1c	11.5 ± 0.1b	4.7 ± 0.4a
ash (% w/w)	10.4 ± 0.1b	10.4 ± 0.1b	2.0 ± 0.0a
fiber (% w/w)	13.5 ± 1.0c	11.5 ± 0.3b	0.2 ± 0.0a
phorbol esters (μg/g)[c]	119.9 ± 17.9c	1.1 ± 0.0b	0.0 ± 0.0a
phytic acid (mg/g)	16.1 ± 1.2c	12.9 ± 0.1a	13.9 ± 0.1b
trypsin inhibitor (TIU[d]/g)	23.3 ± 2.2a	25.7 ± 0.1b	29.5 ± 0.4c
lectin (HU[e]; mg/mL)	0.4 ± 0.0a	1.6 ± 0.0b	3.1 ± 0.0c
saponin (mg/g)[f]	0.1 ± 0.0b	0.0 ± 0.0a	0.0 ± 0.0a

[a]Different letters (a, b, c) in the same row indicate a significant difference ($P < 0.05$). [b]Mean ± SD (on dry matter basis). [c]Equivalent to phorbol-12-myristate-13-acetate. [d]TIU, trypsin inhibitor units [e]HU, heamagglutination units. [f]Equivalent to diosgenin.

The seed cake contained phorbol esters (119.9 μg/g) and the antinutritional factors: trypsin inhibitor (23.3 TIU/g), phytic acid (16.1 mg/g), lectin (0.4 mg/mL), and saponin (0.1 mg/g) as shown in Table 1. Phorbol esters, phytic acid, and saponin contents found in the present study were much lower than those reported by Makkar et al.,[8] Saetae and Suntornsuk,[9] Saetae et al.,[16] and Xiao et al.[23] However, the determined trypsin inhibitor, lectin, and saponin contents were much higher than those reported by Xiao et al.,[23] Makkar et al.,[8] and Saetae et al.,[16] respectively. After ethanol extraction of the seed cake, the amounts of all toxic compounds especially phorbol esters and saponin were reduced (Table 1) as found by Xiao et al.[23] and Saetae and Suntornsuk.[24]

In this work, the seed cake proteins were isolated by isoelectric precipitation with a protein recovery of approximately 36%. The isolate had 82% protein, 5% fat, 2% ash, and a small amount of fiber (Table 1). The protein recovery was lower than that reported by Makkar et al.[17] The efficiency of protein recovery depended on pH, temperature, and extraction time.[16,17] A lower protein content in the precipitate was found

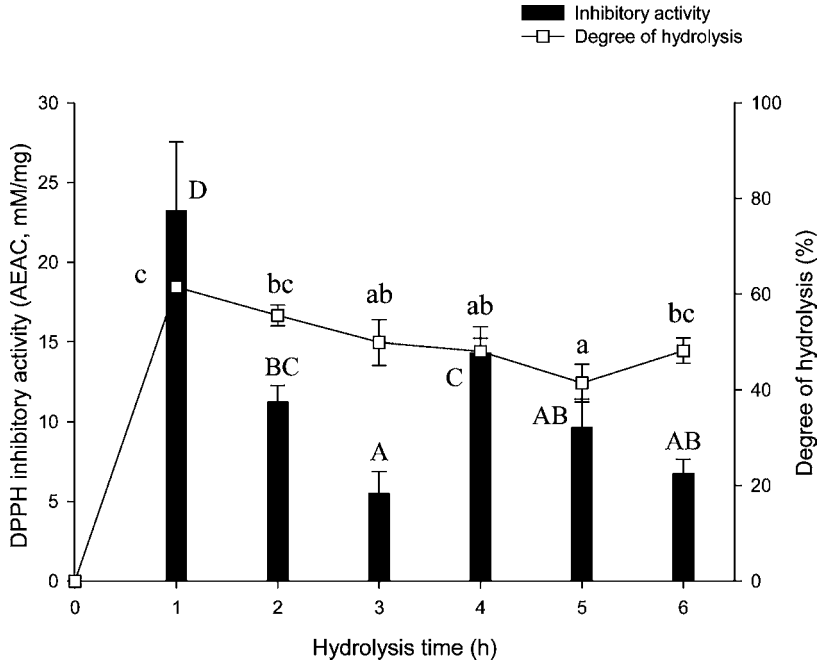

Figure 1. Degree of hydrolysis (□) and DPPH radical inhibitory activity (bar) of the protein hydrolysate during the hydrolysis of *J. curcas* protein by Neutrase. Different lower case letters (a, b, c...) indicate a significant difference at a specific time on the line ($P < 0.05$). Different capital letters (A, B, C...) indicate a significant difference between bars ($P < 0.05$).

Table 2. Antioxidant Activity (DPPH Radical) in Different Fractions of *J. curcas* Protein Hydrolysate

step	weight (mg)	protein (mg/mL)	DPPH radical inhibition (IC_{50}, mg/mL)	yield[a] (%)	purification[b] (fold)
protein hydrolysate	1525.1 ± 1.5	10.00 ± 0.00	3.31 ± 0.00	100	0
gel filtration (fraction C)	211.2 ± 1.0	0.25 ± 0.00	0.42 ± 0.01	2.5	7.9
DEAE sepharose (unbound fraction)	90.1 ± 3.8	0.10 ± 0.00	0.37 ± 0.00	1.0	8.9
RP-HPLC (fraction F4)	19.2 ± 2.0	0.03 ± 0.00	0.26 ± 0.00	0.3	12.9
strong cation exchange (fraction S4)	19.2 ± 0.1	0.03 ± 0.00	0.19 ± 0.00	0.3	17.0

[a]Yield (%) = (protein in purified fraction (mg/mL) × 100)/(protein in hydrolysate (mg/mL)). [b]Purification (fold) = (IC_{50} of hydrolysate)/(IC_{50} of purified fraction).

compared to the reports of Marrufo-Estrada et al.,[14] Saetae et al.,[16] and Makkar et al.,[17] possibly due to the different protein isolation conditions used. Moreover, the phorbol esters in the protein isolate were completely removed and other toxic compounds were significantly reduced after protein isolation except for phytic acid and trypsin inhibitor as shown in Table 1. The increase in the amounts of determined trypsin inhibitor and phytic acid in the isolate after protein isolation may have been due to an interaction between the soluble trypsin inhibitor and phytic acid leading to precipitation of the resulting complex. The amounts of antinutritional factors in the protein isolate were below the upper safe limits for animals.

Protein Hydrolysis and DPPH Radical Inhibitory Activity. The amount of soluble protein found after Neutrase hydrolysis of *J. curcas* protein isolate gradually increased up to 1 h of hydrolysis and slightly declined thereafter (data not shown). This reflected the release of soluble proteins after hydrolysis with subsequent breakdown to peptides and possibly amino acids. Free amino acid content slowly increased during the first hour of hydrolysis and then remained constant (data not shown). The maximal free amino acid content of approximately 1.2 mg/mL was reached at 1 h of Neutrase hydrolysis. The low amount of free amino acids found in the hydrolysate was consistent with Neutrase being an endoprotease, which typically produces short chain peptides with trace amounts of free amino acids.[25]

The degree of hydrolysis and DPPH radical inhibitory activity as a function of the time of hydrolysis of *J. curcas* protein are shown in Figure 1. The degree of hydrolysis reached its maximum of 60% within 1 h. After that, the degrees of hydrolysis remained relatively constant with increasing hydrolysis time ($P > 0.05$) except at 5 h as illustrated in Figure 1. A reduction of degree of hydrolysis at some point might be due to catalysis of some free amino acids by amino acid transaminase and decarboxylase or decarboxylase and deaminase found in storage seeds.[26] In addition, after the highest hydrolysis rate is reached, the rate typically decreases with increasing hydrolysis time as a result of substrate limitation for hydrolysis, enzyme inhibition by an end product, and a decline in peptide bonds available for hydrolysis. The hydrolysis profile of this study was similar to that of enzymatic hydrolysis of *J. curcas* protein and flour reported by Gallegos-Tintoré et al.[13] and Marrufo-Estrada et al.[14] However, the degree of hydrolysis by Neutrase in the presently reported study was much higher than that produced by Alcalase and a sequential system of pepsin−pancreatine reported by Gallegos-Tintoréet al.[13] and Marrufo-Estrada et al.,[14] respectively. Differences in the degree of hydrolysis are likely a function of the enzyme

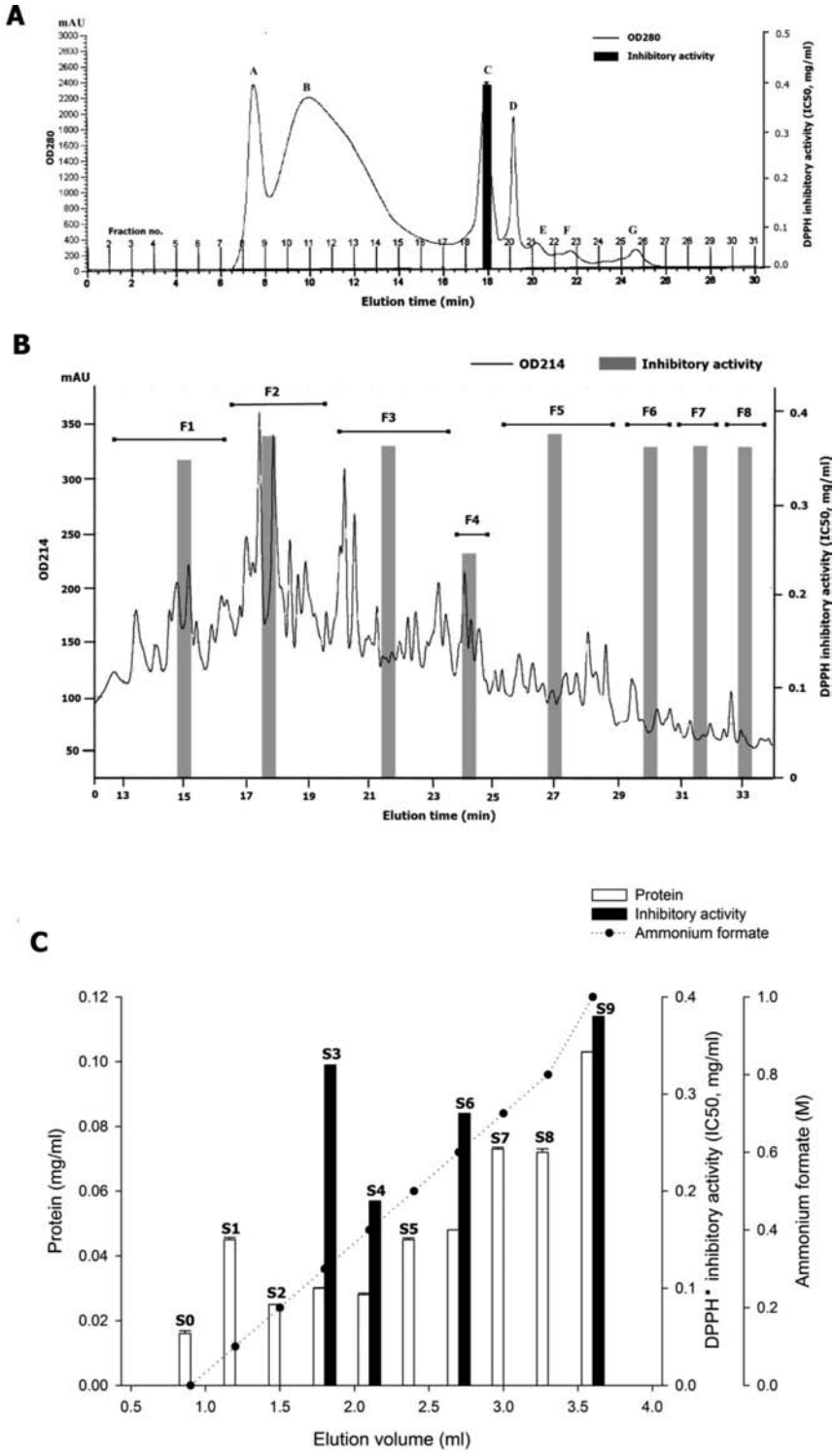

Figure 2. Elution profiles of antioxidants from protein hydrolysate obtained by gel filtration (A), reverse phase HPLC (B), and strong cation exchange cartridge (SCX) (C).

type, enzyme concentration, ratio of enzyme and substrate, hydrolysis time, and hydrolysis conditions used.

In the present study, DPPH radical inhibitory activity of the hydrolysate reached its maximum of 23 mM/mg after 1 h of hydrolysis (Figure 1). The IC_{50} value of the hydrolysate was approximately 3.3 mg/mL (Table 2). Antioxidant activities (DPPH radical inhibitory, superoxide and hydroxyl radical inhibitory, ferric reducing, copper and iron chelating activities) have been associated with the enzymatic hydrolysate of various proteins. Protein hydrolysates from *J. curcas* flour and its defatted flour produced by Alcalase hydrolysis showed anti-DPPH radical activity, ferric reducing ability, and copper and iron chelating activities.[13] A Neutrase hydrolysate of rice endosperm protein showed DPPH radical, superoxide, and hydroxyl radical scavenging activity,[27] while hydrolysates obtained from Alcalase, Neutrase, pepsin, papain, α-chymo-

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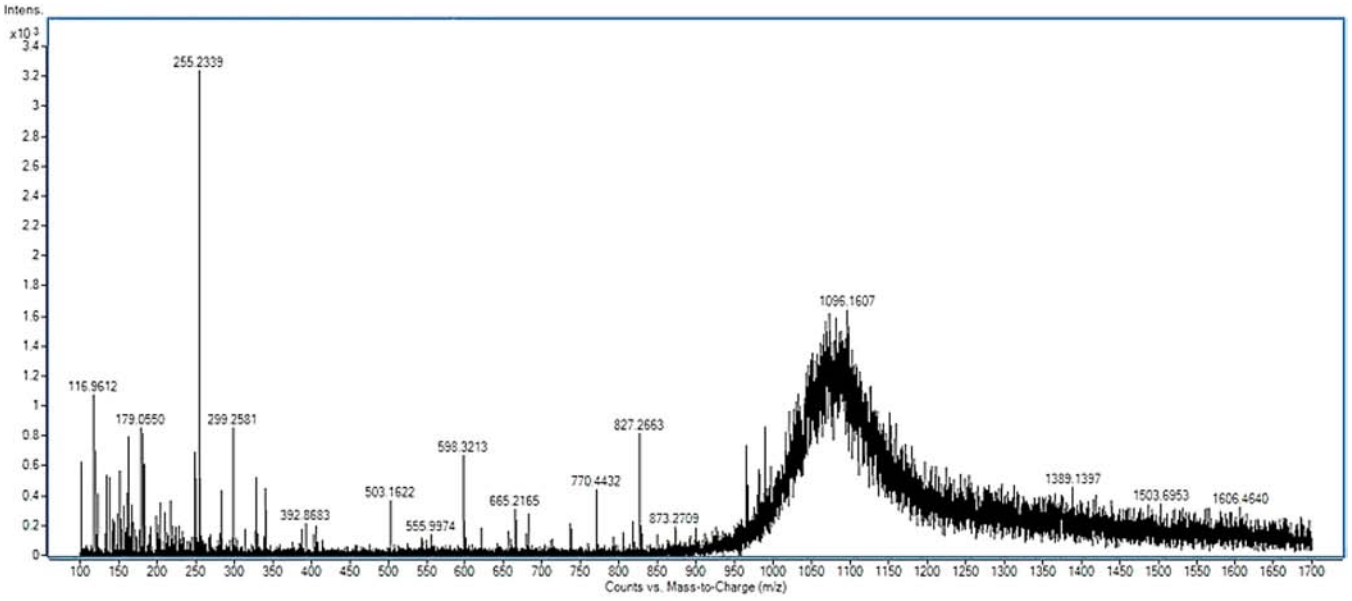

Figure 3. Mass spectrum of antioxidants in the fraction S4 by ESI-mass spectrometry (Q-TOF) in negative ion mode.

trypsin, and trypsin hydrolysis of bullfrog skin inhibited lipid peroxidation, DPPH radical, superoxide, peroxyl, and hydroxyl radicals.[28]

Protein Purification. The hydrolysate found after 1 h of Neutrase hydrolysis was initially purified by gel filtration chromatography. The chromatogram showed four major peaks (fractions A−D) and three small peaks (fractions E−G) at an optical density of 280 nm as shown in Figure 2A. However, fraction C was the only fraction expressing DPPH radical inhibitory activity (IC_{50} of 0.42 mg/mL) (Table 2). It was observed that this step removed most proteins from the hydrolysate and improved its purity by 8 fold (Table 2).

Fraction C was further purified using an anion exchange resin, DEAE Sepharose. Its unbound fraction had stronger DPPH radical inhibitory activity than its bound fraction. The unbound fraction showed an antioxidant activity (IC_{50}) of 0.37 mg/mL (Table 2). It would contain positive charges or nonpolar compounds which could not bind to the resin. This step slightly increased the DPPH radical inhibitory activity from the prior step (Table 2).

The unbound fraction from the DEAE Sepharose was further purified using reverse phase HPLC. Its chromatogram is shown in Figure 2B. The profile of the fraction showed complex mixtures grouped into eight fractions (F1−F8). All groups had DPPH radical inhibitory activities. However, the fraction F4 gave the highest DPPH radical inhibition (IC_{50} of 0.26 mg/mL) (Table 2). The fraction F4 was subsequently applied into a strong cation exchange cartridge (SCX) and eluted and separated into ten fractions as shown in Figure 2C. Four fractions, S3, S4, S6, and S9, possessed the DPPH radical inhibitory activities. The fraction S4 was the most active fraction with an IC_{50} of 0.19 mg/mL with a 17-fold increase in purity (Table 2). The fraction S4 was strongly bound in the SCX cartridge and eluted by 0.4 M ammonium formate buffer indicating the presence of positive charge compounds.

Molecular Weight Determination. *Tricine SDS-PAGE.* The protein isolate, protein hydrolysate, and the most active fractions from all purification steps were subjected to protein molecular weight analysis by SDS-PAGE with tricine buffer. The protein isolate had a wide range of protein molecular weights between 5 and 210 kDa (data not shown). The protein bands found in the protein isolate were similar to those reported by Peralta-Flores et al.[29] The storage proteins extracted from defatted *J. curcas* kernel meal were glutelins (27 and 33 kDa), globulins (less than 20, 30, and 70 kDa), albumins (less than 30 kDa), and prolamins (14.2 and 15.5 kDa). Glutelins, globulins, and albumins were the major *J. curcas* proteins able to be recovered at approximately 57%, 27%, and 11%, respectively, with prolamins at only 1%.[29]

The protein hydrolysate had high intensity bands at low molecular weight proteins (less than 12.5 kDa) but not at the higher molecular weights of 20−40 kDa (data not shown). No protein bands were observed in the fraction C of the gel filtration, the unbound fraction from DEAE Sepharose, the fraction F4 from reverse phase HPLC, or the fraction S4 from the SCX cartridge. The very low amounts of protein in the samples could not be resolved by tricine SDS-PAGE.

Electrospray Ionization Mass Spectrometry (ESI-MS). Fraction S4 was analyzed for molecular mass by ESI-MS in negative ion mode. Its spectrum is shown in Figure 3. The complex mass spectrum would indicate a complex sample. The peak at 255.2339 (m/z) may reflect the presence of palmitic acid, C16:0 (256.24 Da). The presence of palmitic acid in the fraction would not be unexpected because it is one of the main constituents in *J. curcas* seed oil (approximately 18%, w/w).[1] Palmitic acid was possibly co-isolated with the seed protein during protein extraction and retained after purifications. Fraction S4 was the most effective DPPH inhibitor with a large amount of fatty acid present, possibly palmitic acid. For further clarification, NMR spectroscopy was undertaken.

Structure Elucidation by NMR. The ^{31}P NMR spectra (data not shown) did not detect any phosphorus in the sample. Thus RNA, DNA, or phosphorylated entities were not present. The ^{1}H NMR spectra (Figure 4A, horizontal and vertical projections) showed peaks at remarkably different intensities. This would suggest that the sample contained a mixture of compounds at different concentrations. There were relatively few peaks in the whole spectrum indicating the presence of relatively few different chemical environments surrounding the protons. Thus, the mixture seemed to have been composed of

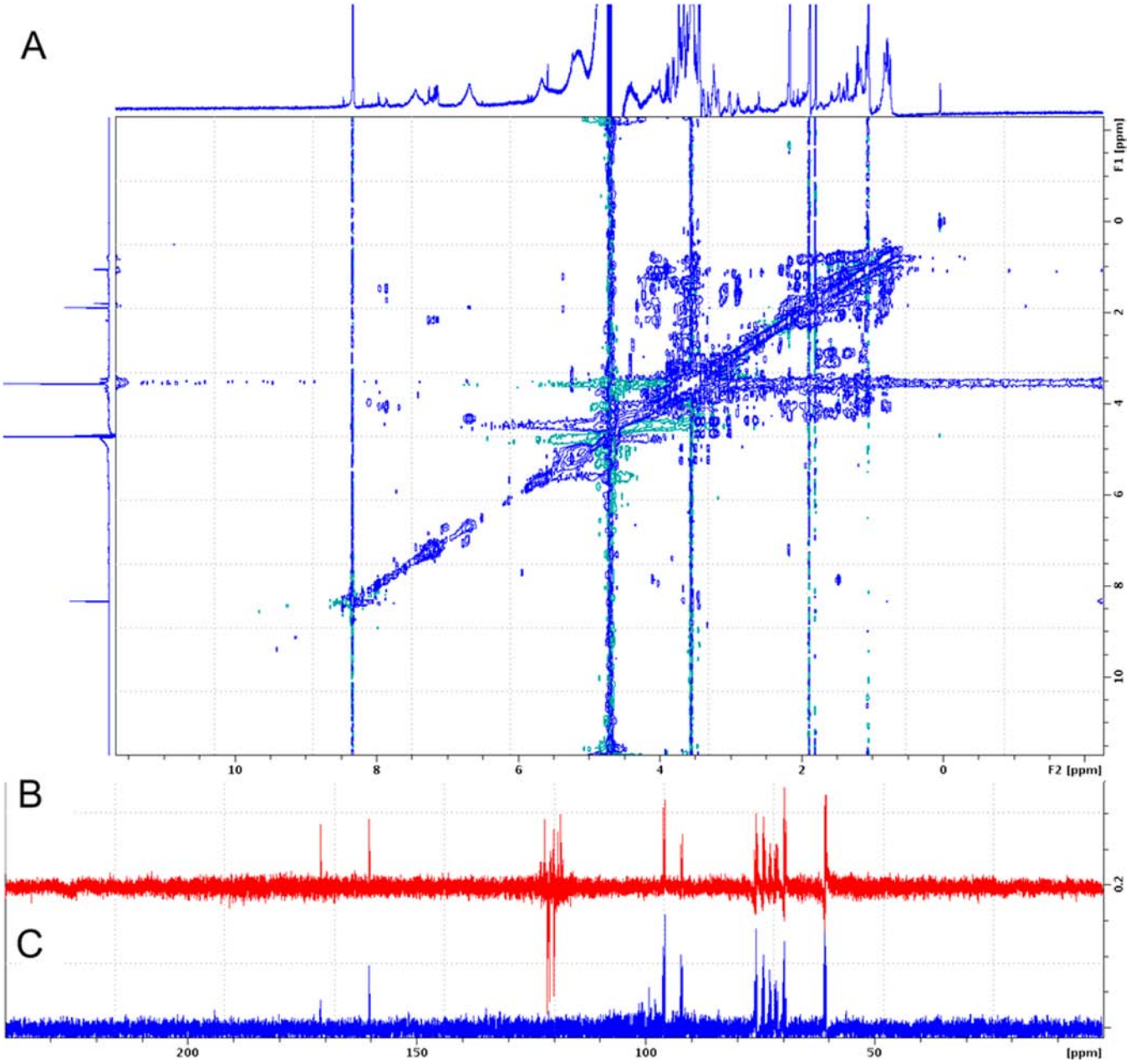

Figure 4. 2-D TOCSY spectrum of the fraction S4 with the projections from 1-D ^{1}H experiments showing a range of different sample intensities (A), ^{13}C NMR spectrum with 20 480 scans (B), and ^{13}C NMR spectrum with 3072 scans (C).

relatively simple compounds. The scarcity of peaks in the "aromatic region" (6−9 ppm) would suggest that there were not many different compounds having aromatic entities and/or containing protons bonded to nitrogen in the sample. In the ^{13}C NMR spectra (Figure 4C), there were no peaks in the aromatic carbon region (110−145 ppm), so aromatic entities did not seem to be present in the mixture. Moreover, peaks populated between 163 ppm and 190 ppm shown in Figure 4B and C could be attributed to carbonyl carbon regions of peptides or proteins. Curiously, in the ^{13}C NMR spectra (Figure 4B and C), there were no peaks below 40 ppm (methylene and methyl carbons) that would be expected from saturated fatty acids. Thus, their concentration had to be very small to escape detection after 20480 scans but ^{13}C NMR is about 6000 times less sensitive at natural abundance than ^{1}H NMR. Unfortunately, the NOESY and COSY spectra (data not shown) had a low signal-to-noise ratio, in spite of the long acquisition times. The more sensitive TOCSY spectra showed cross peaks (Figure 4A) that were consistent with a mixture of fatty acids and derivatives. Cross peaks between the "aromatic region" of the protons and protons in the low aliphatic region (below 2 ppm) suggested attachment of NH groups to fatty acids.

To identify the compounds in the mixture, DOSY experiments were done. The analysis of these diffusion measurements showed that the solution contained a mixture of molecules with different correlation times (between 2×10^{-10} and 2×10^{-9} s) and thus different molecular masses of around 100 Da, 200 Da, 300 Da, 600 Da, and 2 kDa (suggested from DOSY analysis of the proton chemical shifts at 7.23, 8.16, 2.136, 7.843, and 7.125 ppm and 1.549 and 1.328 ppm, respectively). Interestingly, few peaks corresponded to one diffusion constant. This was also

consistent with the presence of relatively simple compounds, such as fatty acids. The ethylene protons at around 5.2 ppm in the ^{1}H NMR spectra confirmed the presence of unsaturation. Some compounds in the mixture might have been the form of $HN-CO-C{=}C-O-CH_2-CH_3$ and several compounds of the form $R-C{=}O-NH$ and $R-C{=}C-CH_3$, as well as fatty acids with double and triple bonds. The compounds near 300 Da might have been linoleic acid ($CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$, 280.45 Da), and/or oleic acid ($CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$, 282.46 Da) which were the two predominant fatty acids in *J. curcas* seed oil at around 48% (w/w) and 28% (w/w), respectively.[1,30] The 600 Da compounds might have been diglyceride derivatives attached to amino groups. Diglycerides are also abundant in *J. curcas* seed oil.[1] The compound at around 2 kDa had a low concentration and probably had a simple structure, with long CH_2 chains, presumably derived from common triglycerides. The ^{13}C NMR spectra (Figure 4B and C) showed peaks at 60.45, 60.59, and 60.85 ppm that might be attributed to epoxy ring carbons supported by the 2.9 ppm peak in the ^{1}H NMR spectra, or associated metabolites with an opening of the oxirane ring supported by the 3.5 ppm peak in the ^{1}H NMR spectra. Furthermore, in the ^{13}C NMR spectra (Figure 4B and C), the series of peaks between 69 and 77 ppm could be assigned to carbinol carbons (−COH−), the four peaks between 90 and 100 ppm to carbon triple bonds (C≡C) and the peak at 160.28 ppm to carbon double bonds with amino groups ($-CO-NH_2$). However, the peaks around the center of the spectrum were from instrumental artifacts due to a slight imbalance in Quadrature detectors (Figure 4B). Thus, there was evidence for the presence of fatty acid derived epoxides (and esters) in the sample likely derived from linoleic acid. Although the physiological benefits of long-chain polyunsaturated fatty acids have been known for years, it was discovered recently that the epoxides from fatty acids are even more potent bioactive compounds.[31,32] In addition, the typical inhibitory mechanism of DPPH radical scavenging activity is involved in the donation of electrons or hydrogens to the radicals.[33] It is reasonable to assume that polyunsaturated fatty acids containing numerous unsaturated bonds would be, at least in part, responsible for DPPH radical scavenging activity among the compounds in the protein hydrolysate derived from *J. curcas* seed cake.

As NMR spectroscopy is a relatively insensitive technique, it cannot be excluded that bioactive compounds of a very high potency at very low (submicromolar) concentrations exist in the sample. Mass spectrometry can potentially detect compounds at lower concentrations than NMR does, but the technology relies on the ability of the compounds to be ionized. In this study, the interference among some compounds in the sample might have been an issue. The presently reported result on the composition of the mixture making up fraction S4 must be regarded as tentative and needs confirmation. Several unresolved contradictions remain. Fraction F4 appeared to be polar and to contain positively charged materials. This is inconsistent with the conclusion that the material is fatty acid based. LC-MS analysis of fraction S4 gave a predominant peak tentatively identified as palmitic acid, but the NMR analysis did not confirm the presence of methylene or methyl groups.

Amino Acid Analysis. The hydrolysate contained high amounts of glutamine (56.68 mg/g) and arginine (43.32 mg/g) (data not shown). This observation agreed well with the amino acid composition of *J. curcas* protein hydrolysate reported by Gallegos-Tintoré et al.[13] The purified fraction S4 had a lower amino acid concentration than the hydrolysate. Amino acid data for the fraction S4 corresponded well with the NMR result indicating that the purified fraction contained a low concentration of NH-residues. In this study, the purified fraction S4 had a high proportion of anionic amino acids; aspartic acid, cationic amino acids; arginine and lysine, polar, noncharged amino acid glutamine, and a hydrophobic amino acid; leucine, with trace amounts of glycine, valine, isoleucine, phenylalanine, histidine, and tyrosine (data not shown). Anionic and cationic amino acids can become electron donors and acceptors stabilizing DPPH radical. Moreover, the hydrophobic amino acids, tyrosine and leucine, have been reported to be potent hydrogen donors.[27,34]

In conclusion, the production of a protein hydrolysate expressing antioxidant activity against the DPPH radical was successfully achieved by the Neutrase hydrolysis of proteins isolated from *J. curcas* seed cake. After several purification steps, the antioxidant was considered likely to be a mixture of compounds containing NH-residues, fatty acids, and fatty acid derivatives such as fatty acid derived epoxides. Compounds from *J. curcas* protein hydrolysates may serve as a source of alternative natural antioxidants for applications in the medical, food, and cosmetic industries.

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Notes

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