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Antioxidant and chelating activity of *Jatropha curcas* L protein hydrolysates

Running title: Production of *J curcas* hydrolysates

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Abstract

BLACKGROUND:

Antioxidant and chelating activities were determined in protein hydrolysates that were produced by treating a protein isolate of a non toxic genotype of *J. curcas* with the protease preparation, alcalase.

RESULTS:

50 min protein hydrolysate with a degree of hydrolysis of 31.7% showed highest antioxidant and chelating activity. These activities were also determined in six peptidic fractions that were separated by gel filtration chromatography of the 50 minutes hydrolysate. The lower molecular weight peptidic fractions had the highest antioxidant and chelating activities, which correlated with a higher content in antioxidant and chelating amino acids such as tyrosine and histidine.

CONCLUSION:

Results show that *J. curcas* represents a good source of bioactive peptides. This may be important for the revalorization of deffated *J. curcas* flour, a by-product resulting form oil extraction for bio diesel production. This is especially important in third world and developing countries such as Mexico.

Keywords: *J. curcas*, protein hydrolysate, antioxidant activity, chelating activity.

INTRODUCTION

Jatropha curcas L., a member of the *Euphorbiaceae* family, is a drought resistant small tree of great economic importance in developing countries because of its several industrial and medicinal uses[1,2]. The seeds contain around 600 g kg^{-1} oil and 300 g kg^{-1} protein[3]. The seed oil is used for biodiesel production and for manufacturing cosmetics, but the protein rich defatted meal resulting from oil extraction is mostly wasted. This is so because of the presence of toxic phorbol esters in this material.[3] However, non toxic genotypes which do not contain phorbol esters have been reported in Mexico.[1]

The concentration of antinutritional and toxic components in the defatted seed meal is reduced during the preparation of *J. curcas* protein isolates.[4] These protein isolates are a good substrate for the production of hydrolysates with improved functional and nutritional properties, which facilitates the revalorization of numerous oilseeds and grain legumes.[5] In addition, protein hydrolysates are a source of bioactive peptides, which are short chain peptides with beneficial biological activities wich are released from food proteins during hydrolysis.[6] For example, bioactive peptides with antihypertensive, immunomodulatory, opioid, antioxidant, hypocholesterolemic, and metal chelating activity have been described.[6,7]

Oxidative damage due to generation of reactive oxygen species has been related to a variety of diseases, including cancer, cardiovascular disease, and neurodegenerative diseases. Excessive production of oxygen reactive species, and/or decreased antioxidant defenses, lead to damage to different cell components, including lipids, proteins and nucleic acids, and to blood lipoproteins. In foods, the main targets of oxidative reactions are

polyunsaturated lipids. The oxidative alterations of lipids have a negative effect on flavor, texture, nutritive value, and shelf life of food products. Therefore, natural and synthetic antioxidants play a very important role in both health promotion and the conservation of foodstuffs. A variety of antioxidant peptides have been purified from different plant protein hydrolysates.[8,9] Thus, antioxidant peptides generated by hydrolysis of food proteins constitute a new source of functional components that could inhibit deleterious oxidative processes both *in vivo* and in foods. One of the mechanisms for the antioxidant effect of peptides is metal chelation, because transition metals catalyze numerous oxidative reactions.[10] In addition to this, metal chelating peptides are of interest because they can increase the bioavailability of essential trace elements such as calcium, iron and zinc.

The goal of this work was to determine the presence of antioxidant and metal chelating peptides in *J. curcas* protein hydrolysates produced by treatment with the food-grade protease preparation alcalase. The presence of these peptides in *J. curcas* would result in a revalorization of this crop as source of new, high added value bioactive peptides.

MATERIALS AND METHODS

Materials

J. curcas seeds from the September-October harvest in Huitzilán, state of Puebla (Mexico) were used to prepare the hydrolysates. Alcalase 2.4 L (NovoNordisk, Bagsvaerd, Denmark) is a food grade protease preparation from *Bacillus licheniformis* containing subtilisin carlsberg as the major component, and has a specific activity of 2.4 Anson units (AU)/g. Amino acids standards,

2,2-diphenyl-1-picrylhydrazyl (DPPH), D,L-α-aminobutyric acid, trinitrobenzenesulfonic acid (TNBS), β-carotene, linoleic acid and tween 20 were from Sigma (St. Louis, MO). Diethyl ethoxymethylenemalonate was purchased from Fluka (Buchs, Switzerland).

Methods

Preparation of *J. curcas* dehulled defatted meal

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.

Preparation of *J. curcas* protein isolate

Dehulled defatted meal was dispersed in water pH 10.5 1:6 (w/v) and stirred for 1 h at room temperature. The suspension was then filtered twice through an 80-mesh, and the solids on the screen were washed using distilled water. The filtrate was filtered through a 150-mesh wet screen to eliminate the finest particles that still remained in suspension. Starch was eliminated by centrifugation at 487 x *g* for 15 min, and the protein in the supernatant was precipitated by adjusting to pH 4.5 using 1M HCl. Protein was recovered by centrifugation at 7796 x *g* for 15 min and freeze-dried until further use.

Preparation of *J. curcas* protein hydrolysates

J. curcas protein hydrolysates were prepared according to Megías *et al.* [10] using a hydrolysis reactor vessel equipped with a stirrer, thermometer, and pH

electrode. Protein isolates (5 % w/v) were digested with alcalase (0.3 AU/g protein) for 60 min at 50 ^{0}C and pH 8. Aliquots were taken at different times and hydrolysis was stopped by heat inactivation of alcalase at 80 ^{0}C for 20 min. The resulting hydrolysates were clarified by centrifugation at 11227 x *g* for 30 min and were kept frozen at -20 ^{0}C until further use.

Characterization of isolates and hydrolysates

Moisture, fat, ash and crude fiber were determined using the AOAC methods[11] 934.01, 920.39, 942.05, and 962.09, respectively. Polyphenols were determined using the Folin-Ciocalteau method[12] and soluble sugars were determined using the method of Dubois *et al.*[13] Protein was determined by elemental analysis as % nitrogen content x 6.25, using a LECO CHNS-932 analyzer (St. Joseph, MI). Amino acid analysis was carried out by acid hydrolysis and high-performance liquid chromatography (HPLC) after derivatization with diethyl ethoxymethylenemalonate according to the method described by Alaiz *et al.*[14] Tryptophan was determined after basic hydrolysis according to Yust *et al.*[15]

The degree of hydrolysis was calculated by determination of 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 ^{0}C for 24 h.

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

Free radical scavenging activity

Free radical scavenging activity was measured according to Shimada *et al.*[17] with modifications. Protein hydrolysates were added to DPPH in methanol (100

μL, 100 μM) in 96-well plates. Plates were shaken and absorbance at 517 nm was measured after 30 min using a plate reader. Radical scavenging activity (%) was calculated as ($Abs_{control}$ – Abs_{sample}) / $Abs_{control}$ x 100.

Reducing power

Reducing power was determined according to Oyaizu[18] with modifications. This method is based on the reduction of Fe^{3+} to Fe^{2+}, which is recorded by measuring the formation of Perl´s Prussian blue at 700 nm. Protein hydrolysates (50 μL) were added to phosphate buffer (50 μL 0.2 M, pH 6.6) and potassium ferricyanide (50 μL 1% w/v) in 96 well plates, and incubated at 50º C for 20 min. Plates were incubated for another 10 minutes at 50º C after addition of trichloroacetic acid (50 μL 10% w/v) and ferric chloride (10 μL, 0.1% w/v). Absorbance at 700 nm was read using a plate reader.

Antioxidant activity: β-carotene bleaching method

Antioxidant activity was also determined by using the β-carotene bleaching method as described by Marco[19] with modifications. A mixture of β-carotene (1 mL, 10 mg/mL in chloroform), linoleic acid (20 mg), and Tween 20 (200 mg) was vigorously mixed by vortexing and chloroform was removed using a stream of nitrogen. Oxygen-sparged distilled water (50 mL) was then mixed in to obtain a clear solution. Protein hydrolysates (100 μg) and β-carotene solution (200 μL) were added to wells in a 96-well plate and incubated at 50 ºC in the dark. The degradation of β-carotene was monitored by recording the decrease in absorbance at 450 nm using a microplate reader.

Iron and copper chelating activity

Fe^{2+}-chelating activity was determined by measuring the formation of the Fe^{2+}-ferrozine complex according to Carter.[20] Protein hydrolysates were added to 96 well plates containing 250 μL 100 mM Na acetate buffer pH 4.9 and 25 μL of a $FeCl_2$ solution (0.1 mg / mL water). After 30 min, 12.5 μL of ferrozine solution (40 mM in water) was added. Change in color was measured in a plate reader at 562 nm. Iron chelating activity was calculated as follows:

Chelating activity (%) = (1 - absorbance of sample at 562 nm / absorbance of control at 562 nm) x100.

Cu^{2+}-chelating activity was determined using the pyrocatechol violet reagent according to Saiga *et al.* [21] Protein hydrolysates were added to 96 well plates containing 250 μL 50 mM Na acetate pH 6.0, 6.25 μL 4 mM pyrocatechol violet, and Cu (1 μg, $CuSo_4$). After 10 min, change in color was measured in a plate reader at 632 nm. Copper chelating activity was calculated as follows:

Chelating activity (%) = (1 - absorbance of sample at 632 nm / absorbance of control at 632 nm) x100.

Gel filtration chromatography and reverse phase- HPLC

Gel filtration chromatography was carried out in an AKTA-purifier system equipped with a Superdex Peptide column (GE Lifesciences). 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).

Reverse phase HPLC chromatography was carried out by injecting hydrolysates generated at 50 min and 60 min (20 µL, 2 µg/µL) in a Discovery BIO Wide Pore C_{18} column (25cm x 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 min, at a flow of 1 mL/min and 25 ^{0}C, was used for elution, which was monitored at 215 nm.

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 5.1version.

RESULTS AND DISCUSSION

Production and characterization of *J. curcas* protein hydrolysates.

The composition of the protein isolates that were used for the production of hydrolysates is shown in Table 1. Protein concentration was similar to that reported by Selje *et al.*[22] (901 g kg^{-1}). The yield of protein extraction, 26.1 %, was also similar to the yield reported by Selje *et al.*[22] for a toxic Mexican variety of *J. curcas*, 26.5%. Other minor components in the protein isolates were oil, soluble sugars, ashes, and fiber.

Protein isolates were hydrolysed using alcalase, a non-specific alkaline protease with endo-peptidase activity that has been used for producing limited[23] and extensive[24] plant protein hydrolysates. Plant protein hydrolysates produced using alcalase[25] are a good source of bioactive peptides. Fig. 1 shows the

kinetics of hydrolysis of a *J. curcas* protein isolate by alcalase. The rate of hydrolysis leveled off after 5 minutes, time at which a 27 % degree of hydrolysis was achieved. Yust *et al.*[26] reported a 27 % degree of hydrolysis for chickpea legumin hydrolyzed in the same conditions for 30 min. This is the first time that the production of *J. curcas* enzymatic protein hydrolysates has been described. It has been previously reported that production of *J. curcas* protein hydrolysates using strong acids, high temperatures, and long treatment times, resulted in decomposition of essential amino acids. [27]

The molecular weight profile of the isolate and the hydrolysates obtained by treatment with alcalase for 5 and 50 minutes were determined by gel filtration chromatography (Fig. 2). Proteins larger than 7 kDa eluted with the void volume of 7 ml, and the peak at elution volume 27 ml corresponds to salts. A large amount of protein eluted with the void volume in the isolate, while the lower molecular weight fractions increased with hydrolysis time. The maximum absorbances for the 5 and 50 minutes hydrolysates were detected at elution volumes 15.9 and 16.5 mL, respectively, corresponding to estimated molecular weights 1097 and 867 Da.

Reducing power and chelating activity in the hydrolysates.

*R*educing power in the protein hydrolysates was determined as the ability to reduce Fe^{3+} to Fe^{2+}, which indicates the capacity to act as an antioxidant by donating electrons. As shown in Fig. 3A, the reducing power increased after 20 minutes of hydrolysis and reached a maximum after hydrolysis for 40 to 60 minutes.

Free-radical scavenging activity in the hydrolysates was determined using DPPH, a stable free radical commonly used to evaluate antioxidant activity. DPPH radicals are scavenged by proton-donating molecules such as certain antioxidants, resulting in a change of color from violet to yellow that is measured at 517 nm. Free radical scavenging followed a similar pattern than reducing power (Fig. 3B). Thus, scavenging increased starting at 20 minutes and reached a maximum at 50 minutes. Zhu *et al.* [28] also reported 40% DPPH scavenging by a wheat germ protein hydrolysate (1000 μg) that was prepared using alcalase.

It has been reported that plant protein hydrolysates produced using alcalase have metal chelating activity that can inhibit oxidative damage due to reactions catalyzed by the transition metals iron and copper[10]. Iron chelating activity in the hydrolysates increased with hydrolysis time starting at 30 minutes, and reached a maximum at about 50 minutes, following a pattern similar to the changes in reducing power and free radical scavenging with time of hydrolysis (Fig. 4A). The *J. curcas* isolates already presented a 30% copper chelating activity before hydrolysis, and gradually increased to a maximum 70% chelating activity, similar to the maximum iron chelating activity above 60% (Fig. 4B).

Thus, data indicates that the peptides generated after hydrolysis for 20 minutes have a size, structure or sequence that translate in better reducing power, radical scavenging, and chelating activity. The overall amino acid composition of the hydrolysates should not be a factor because it did not change with time of hydrolysis (data not shown). The peptidic profile of the hydrolysates resulting from hydrolysis for 50 and 60 minutes was analyzed by reverse phase HPLC because these hydrolysates had the highest antioxidant and chelating activities.

As shown in Fig. 5, the chromatograms for these two hydrolysates are similar, revealing a complex mixture of many different peptides.

Molecular weight fractionation of the 50 minutes hydrolysate: antioxidant activities, chelating activity, and amino acid composition.

A hydrolysate that was obtained by treatment with alcalase for 50 minutes was subjected to fractionation by gel filtration chromatography in order to determine antioxidant activity in the resulting fractions. The chromatogram as shown in Fig. 2 was divided in six fractions (A to F), corresponding to peptides eluted between 5 and 9.1 mL (fraction A), 9.1 and 11.0 mL (fraction B), 11.0 and 14.7 mL (fraction C), 14.7 and 17.7 mL (fraction D), 17.7 and 20.7 mL (fraction E), and 20.7 and 25 mL (fraction F). Reducing power (Fig. 6A), radical scavenging (Fig. 6B) and inhibition of β-carotene degradation (Fig. 6C) were highest in the lower molecular weight fractions E and F. Li *et al.*[29] also found the highest radical scavenging activity in the smaller peptides of an alcalase hydrolysate that was fractionated by gel filtration chromatography.

Iron chelating activity was also higher in the lower molecular weight fractions E and F (Fig. 7A). However, copper chelation was higher in fractions D and F (Fig. 7B). For the same amount of peptides assayed, chelation of copper was higher than chelation of iron. Similar results have been obtained with chickpea protein hydrolysates fractionated according to molecular weight (unpublished results).

Protein in *J. curcas* seeds is deficient in sulphur amino acids, lysine, and tryptophan as compared to FAO recomendations[30] (Table 2). The amino acid composition of the *J. curcas* protein isolate was similar to that reported before.[4,31] The fractions prepared by gel filtration chromatography of the 50 minutes hydrolysate were also deficient in some essential amino acids. However,

fractions E and F were especially rich in histidine, tyrosine, and phenylalanine. The imidazole ring in histidine residues is directly implicated in metal chelation, and it has been reported that histidine and tyrosine residues are abundant in antioxidant peptides.[32,33] A positive correlation between histidine content and copper chelating activity has been reported in sunflower protein hydrolysates.[34] Data presented in this article indicates that there is a positive correlation between the content in histidine plus tyrosine, and reducing power (r^2 = 0.81), scavenging activity (r^2 = 0.7), inhibition of β-carotene degradation (r^2 = 0.56), and iron chelation (r^2 = 0.61). Fractions containing smaller peptides and higher concentrations of histidine and tyrosine showed the highest antioxidant and chelating activities (Table 2 and Figs. 6 and 7).

CONCLUSIONS

Our results indicate that antioxidant and chelating peptides that are released upon enzymatic hydrolysis are present in *J. curcas* seed proteins. Fractionation by gel filtration chromatography yielded fractions with higher antioxidant and iron chelating activity than the original hydrolysates. Thus, the seeds of *J. curcas* are not only a source of oil for bio diesel production but also a potential source of bioactive peptides. This the first report on the production of bioactive enzymatic protein hydrolysates using the seeds of *J. curcas*. Production of protein hydrolysates enriched in bioactive peptides may result in the revalorization of *J. curcas* defatted meal, which would positively impact the cultivation of this plant in arid regions of developing countries.

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Figure Legends

Fig. 1. Time course of the hydrolysis of *J. curcas* protein isolate by Alcalase. Data correspond to the average ± SD of three determinations.

Fig. 2. Gel filtration chromatography of *J. curcas* protein isolate (time 0), and the hydrolysates that were produced by treatment with alcalase for 5 and 50 minutes.

Fig. 3. Reducing power (A) and free-radical scavenging activity (B) of *J. curcas* protein hydrolysates. Hydrolysates (1000 μg / well in 96 well plates) produced by treatment with alcalase for up to 60 minutes were assayed as described in materials and methods. Data correspond to the average ± SD of three determinations. Different letters indicate significant differences (P< 0.05)

Fig. 4. Percent iron (A) and copper (B) chelating activity of *J. curcas* protein hydrolysates. Hydrolysates (200 μg / well in 96 well plates) produced by treatment with alcalase for up to 60 minutes were assayed for metal chelation as described in materials and methods. Data correspond to the average ± SD of three determinations. Different letters indicate significant differences (P< 0.05)

Fig. 5. Reverse phase HPLC chromatography of hydrolysates obtained by treatment with alcalase for 50 (A) and 60 (B) minutes.

Fig. 6. Reducing power (A), free-radical scavenging activity (B) and inhibition of β-carotene degradation (C) by fractions obtained by gel filtration chromatography of the 50 minutes hydrolysate. Fractions (100 μg / well in 96 well plates) were assayed as described in materials and methods. Data

correspond to the average ± SD of three determinations. Different letters indicate significant differences (P< 0.05)

Fig. 7. Percent iron (A) and copper (B) chelating activity in fractions obtained by gel filtration chromatography of the 50 minutes hydrolysate. *J. curcas* protein hydrolysate fractions (100 μg / well in 96 well plates) were assayed for metal chelation as described in materials and methods. Data correspond to the average ± SD of three determinations. Different letters indicate significant differences (P< 0.05)

Table 1. Chemical composition of *J. curcas* protein isolate.

Compounds	Contents (g kg^{-1})
Moisture	21.6 ± 1.0
Oil	31.9 ± 0.1
Protein	870.0 ± 36.0
Fibre	13.3 ± 0.7
Soluble sugars	22.9 ± 0.1
Poyphenols	2.7 ± 0.0
Ashes	26.0 ± 0.1
Others*	11.6 ± 0.0

*by difference.

Table 2. Amino acid composition of a *J. curcas* protein isolate and fractions obtained by gel filtration chromatography of the 50 minutes hydrolysate. Data (g kg^{-1} protein) represent average ± SD of two determinations.

	JCPI	FA	FB	FC	FD	FE	FF	JCPI*	JCPI†	FAO(1991)
Asp[a]	108±2.0	128±33	142±43	138±44	108±10	88±13	107±0.0	90	125	
Glu[b]	171±3.0	182±2.0	216±12	220±22	200±4.0	137±3.0	98±1.0	188	NR	
Ser	60±0.0	68±2.0	77±5.0	68±1.0	73±2.0	69±1.0	60±6.0	29	52.3	
His	29±0.0	39±2.0	15±0.7	17±0.8	18±4.0	48±7.0	52±1.0	26	35.1	19
Gly	59±0.0	62±3.0	47±1.0	62±7.0	69±6.0	69±4.0	66±1.0	60	51.0	
Thr	43±0.0	43±0.0	30±4.0	46±3.0	50±2.0	44±0.0	36±7.0	30	35.6	34
Arg	123±1.0	139±8.0	160±8.0	113±14	122±1.0	131±8.0	123±1.0	136	141.6	
Ala	56±0.0	67±6.0	45±6.0	58±6.0	70±9.0	79±5.0	44±11	51	54.7	
Tyr	34±1.0	26±2.0	19±1.7	21±2.0	23±4.0	55±4.0	116±37	13	32.0	
Val	47±1.0	37±1.3	33±8.0	39.5±9.5	43±8.5	22±8	28±4.0	60	51.8	35
Met	5±1.0	4.0±0.3	1.0±0.1	5±2.0	0	5.0±0.0	2.5±0.7	13	16.6	
Cys	17±2.0	3.5±0.7	21±3.0	15±4.0	7±1.5	15±1.8	15±0.8	12	13.4	25[d]
Ile	39±0.0	36±4.2	44±1.0	39±4.0	38±3.0	28±5.0	30±1.0	54	44.7	28
Leu	85±1.0	83±8.0	74±5.0	71±6.0	84±3.0	90±3.0	67±23	79	70.8	66
Phe	52±0.0	55±4.0	27±1.0	37±4.0	38±6.0	83±10.0	125±32	55	54.2	63[c]
Lys	43±0.0	28±2.0	49±5.0	52±4.0	57±2.0	38±7.0	31±7.0	42	30.0	58
Trp	8.0±0.0	ND	ND	ND	ND	ND	ND	14	12.3	11
Pro	21±1.0	ND	ND	ND	ND	ND	ND	47	54.5	

[a] Aspartic acid + Asparagine. [b] Glutamic acid + Glutamine. [C] Phe + Tyr. [d] Met + Cys. ND: not determined. NR: not reported. *Devappa and Swamylingappa[4]. †Makkar *et al.* [31]

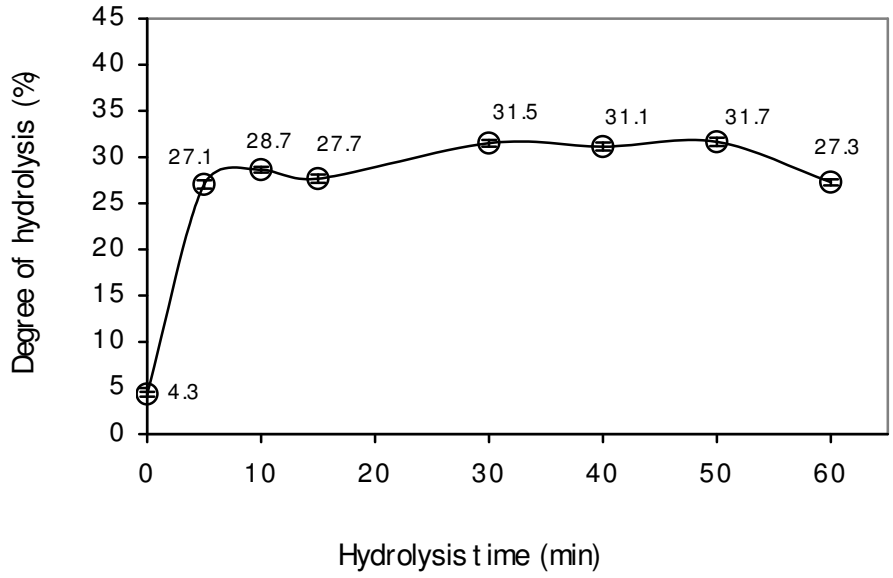
Degree of hydrolysis (%)
Hydrolysis time (min)
4.3
27.1
28.7
27.7
31.5
31.1
31.7
27.3

Fig. 1

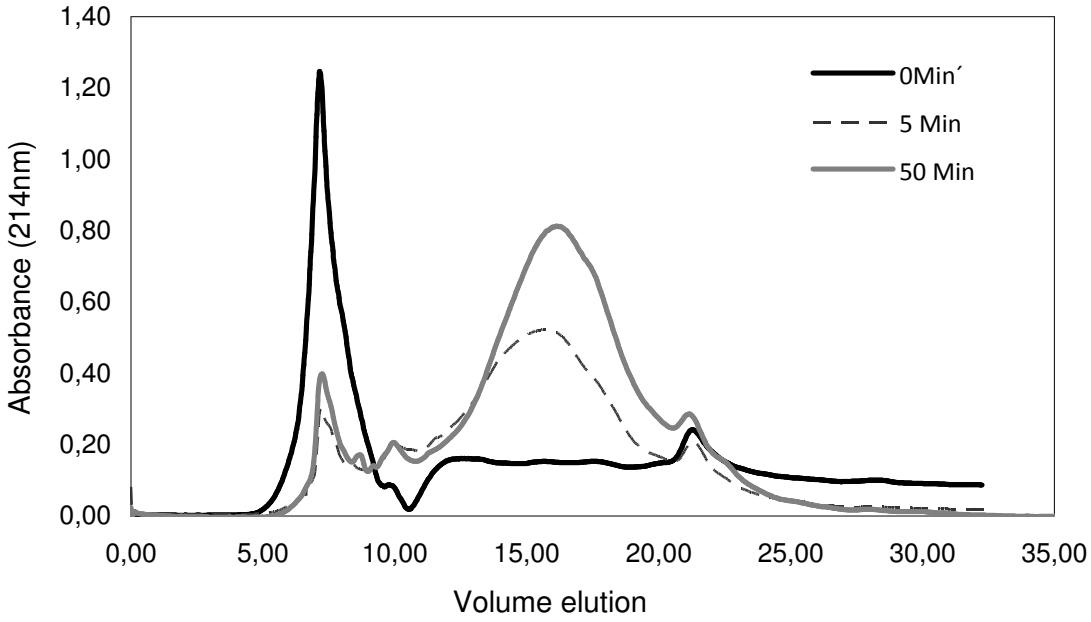
Absorbance (214nm)
Volume elution
0Min´
5 Min
50 Min

Fig. 2

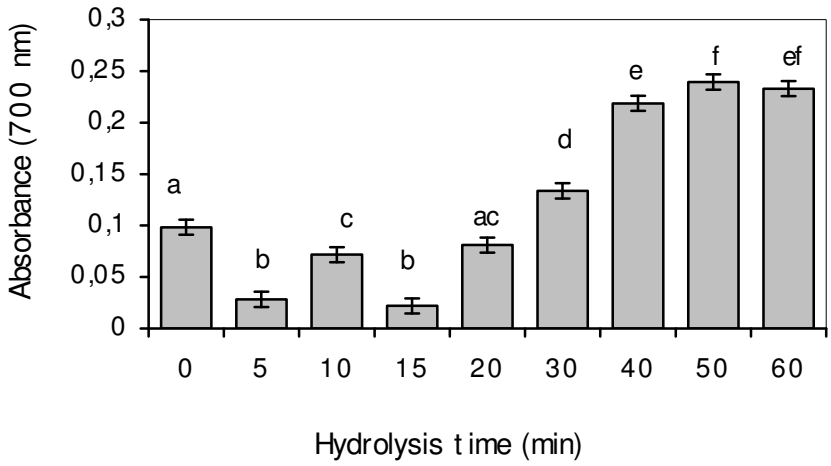

Fig. 3A

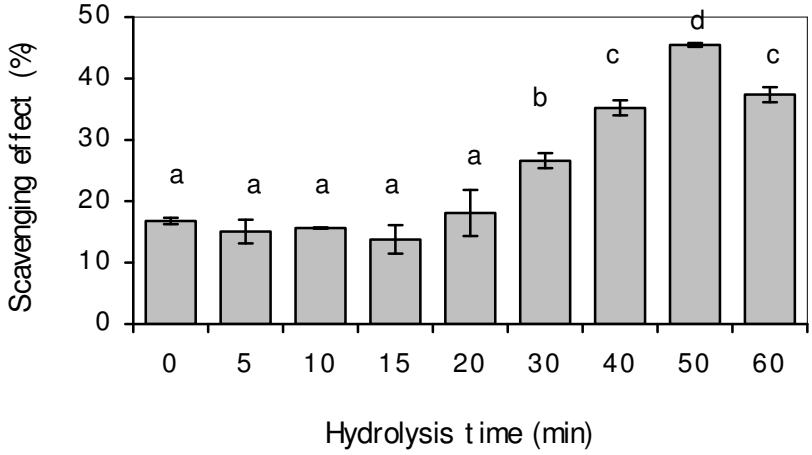

Fig. 3B

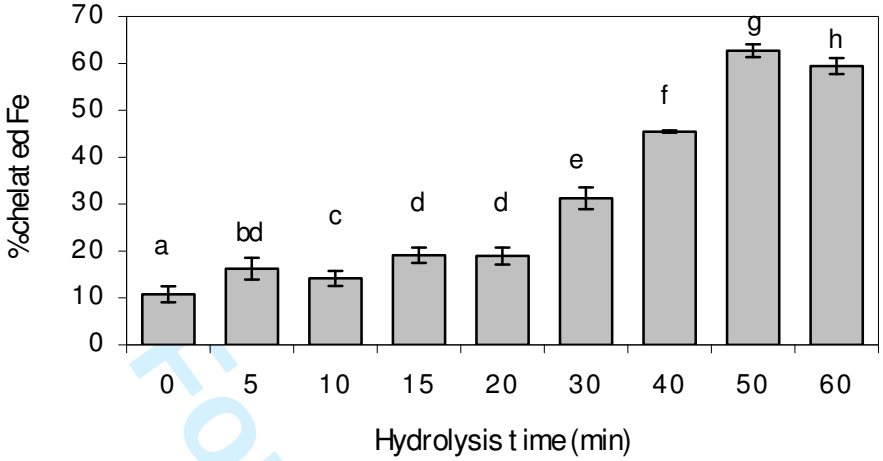

Fig. 4A

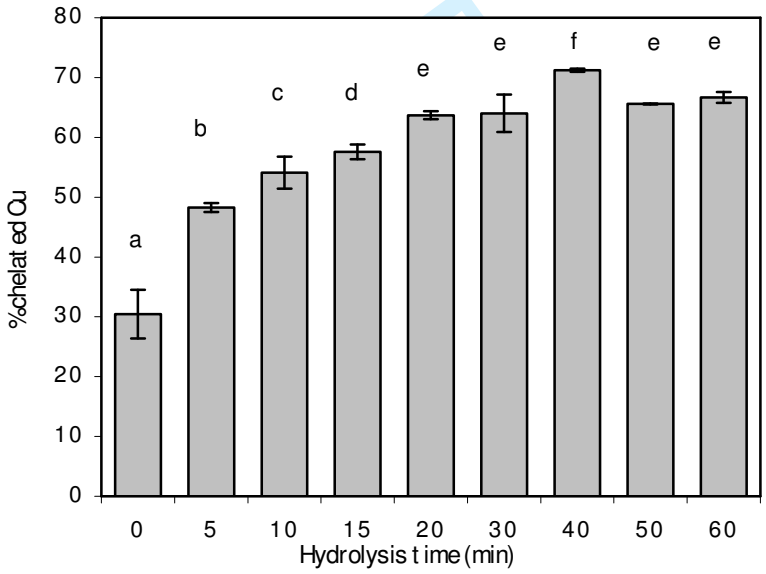

Fig. 4B

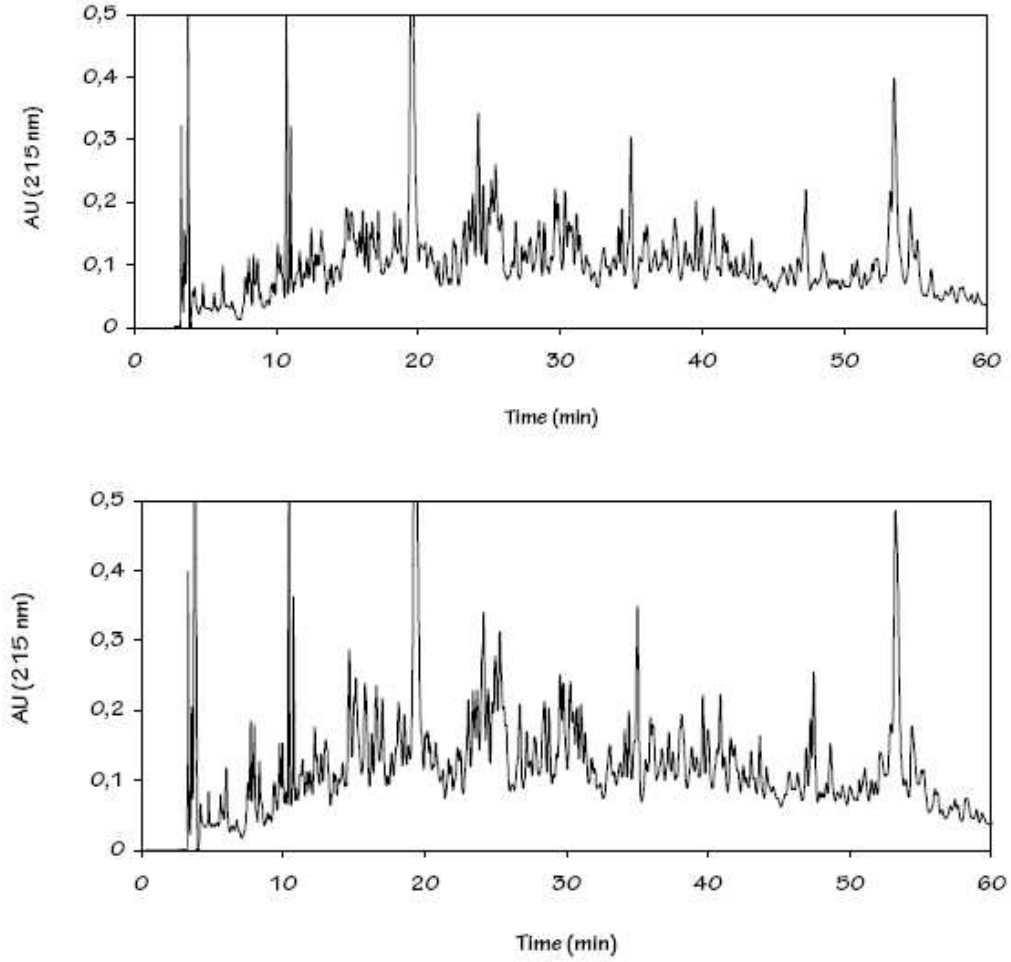

Fig. 5

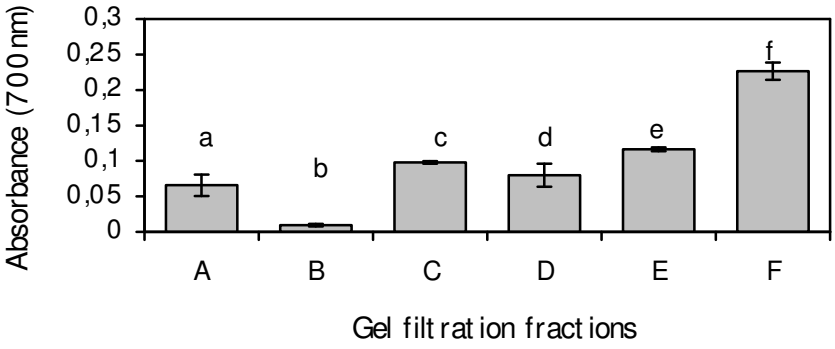

Fig. 6A

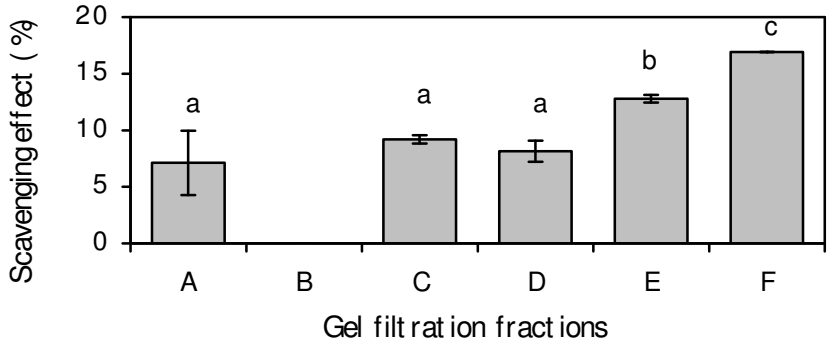

Fig. 6B

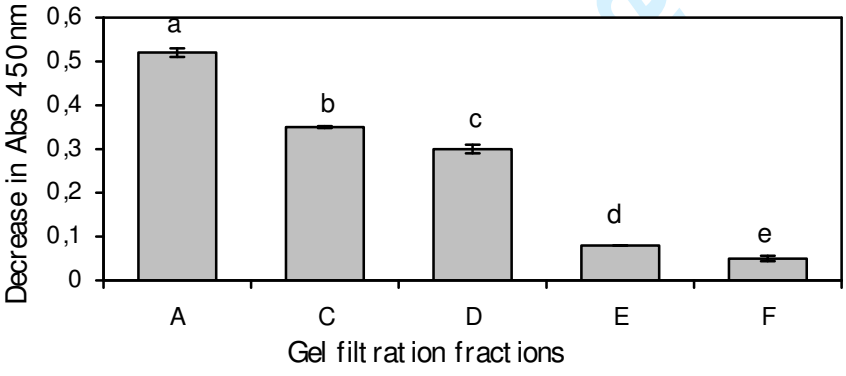

Fig. 6C

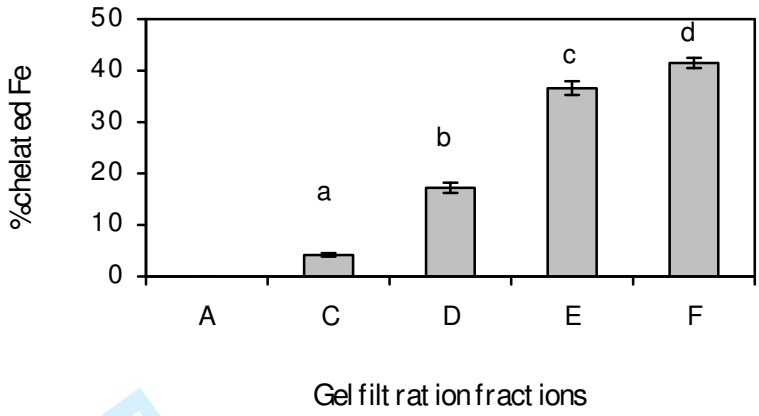

Fig. 7A

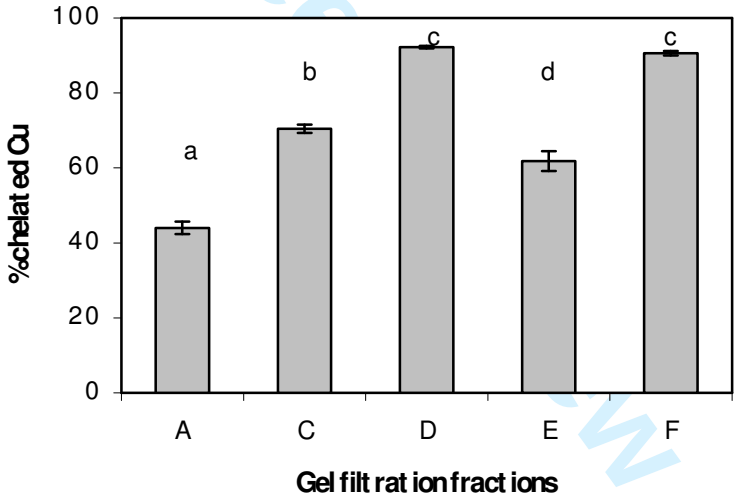

Fig. 7B