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Defatted *Jatropha curcas* flour and protein isolate as materials for protein hydrolysates with biological activity

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ABSTRACT

Jatropha curcas L. protein hydrolysates were produced by treatment of a non-toxic genotype with Alcalase as well as the digestive enzymes pepsin and pancreatin. The *J. curcas* protein hydrolysate produced with the pepsin–pancreatin system from protein isolate had the highest TEAC value and was shown to undergo single-electron transfer reactions in the $ABTS^{\cdot+}$ reduction assay, demonstrating its antioxidant capacity. Testing of antimicrobial activity in the *J. curcas* protein hydrolysates against seven bacterial pathogens showed no growth inhibitory effect in Gram-negative and Gram-positive bacteria. More ACE-I inhibitory active peptides were produced in the Alcalase hydrolysates obtained from *J. curcas* protein isolate. The protein hydrolysate obtained with Alcalase from defatted *J. curcas* flour as well as from the protein isolate showed the highest inhibitory effect of ADP-induced aggregation of human platelets in platelet-rich plasma. It is expected that the information collated will facilitate new applications of proteins present in *Jatropha* plant.

1. Introduction

The genus *Jatropha* is extremely old and may have already existed 70 million years ago on the ancient continent "Gondwanaland" before it split up to form the individual continents. It is considered to be the most primitive member of the large genus *Euphorbiaceae*. The genus *Jatropha* consists of 165–175 species. *Jatropha curcas* is a shrub or a small to medium sized tree and is native to almost all tropical regions. There are two genotypes of *J. curcas*, toxic and non-toxic. Non-toxic genotype is available only in Mexico (Makkar & Becker, 2009).

The *J. curcas* seeds contain about 300–350 g kg^{-1} oil, which can be used as a fuel directly or, in its transesterified form, as a substitute for diesel. The oil is also used for making candles, lubricants and varnishes, and is used for illumination. Large-scale planting of *Jatropha* has already taken place or is currently planned in India, China, Madagascar, Myanmar and many other developing countries, with the aim of using the oil as biodiesel (Francis, Edinger, & Becker, 2005).

J. curcas seeds are highly toxic to a number of animal species. The toxicity is a scribed to the presence of phorbol esters. Other antinutrients present in high amounts in the seed are trypsin inhibitor, lectins and phytates. In addition to the presence of these toxic and antinutritional factors, the high level of shells in the seed cake obtained after oil extraction by screw press prevents its use in animal diets. The protein quality of the meal obtained from shelled *Jatropha* seeds is high. The levels of essential amino acids (except lysine) in protein of *Jatropha* seed cake are higher than in the FAO reference protein for a growing child of 3–5 years (Makkar, Francis, & Becker, 2008).

In recent years, increased global industrialisation and increased demand for livestock products to meet human food demand have greatly increased the pressure on agricultural land and the environment. A greater need for proteins in the livestock sector has accentuated the search for new protein sources that do not conflict with human food security interests. In the current situation, nonedible oil seeds are the most potential and preferred choice for protein and other nutrients for livestock, provided these could be made free of toxic and antinutritional factors. One promising oilseed plant is *J. curcas* which has advantages over other oilseed plants (e.g., *Pongamia pinnata*, *Simarouba glauca*, *Ricinus communis*, *Azadirachta indica*) because of its wide adaptability enabling it to grow under various agroclimatic conditions, for example, adverse soil conditions, drought areas, marginal lands, arid as well as higher rainfall conditions, and land with thin soil cover (Rao, Rao, & Reddy, 2008). More importantly, *Jatropha* seed oil has gained tremendous interest as a feedstock for biodiesel industries. Large-scale *Jatropha* cultivation projects have been initiated in the past 5–10 years with a projected worldwide cultivation of 12.8 million hectares yielding 2 t/ha of oil by 2015 (Global Exchange for Social Investment market study). In the future, this will result in the availability of high amounts of pressed seed cake or kernel meal as byproducts, which are rich in proteins of high quality. These byproducts can be utilised

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in animal nutrition after detoxification and could also be a source for various bioactive protein molecules having a wide range of activities (Rakshit, Makkar, & Becker, 2010).

Production of *J. curcas* protein isolate from defatted seed meal has been described as a way to reduce the contents of antinutritional and toxic components. Moreover, *J. curcas* protein isolate is a good substrate for the production of protein hydrolysates as a source of bioactive peptides with beneficial biological activities.

The objective of the present study was to hydrolyze defatted flour and protein isolate of *J. curcas* with Alcalase and the pepsin–pancreatin sequential enzymatic system and to identify and quantify any antioxidant, antimicrobial, ACE-inhibitory and antithrombotic activities in the resulting hydrolysates.

2. Materials and methods

2.1. Materials

J. curcas L. seeds were obtained from the September 2006 harvest in Puebla state, Mexico. Reagents were analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, USA), Sigma (Sigma Chemical Co., St. Louis, MO, USA), Merck (Darmstadt, Germany) and Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Angiotensin converting enzyme from rabbit lung (2 units/mg protein) was purchased by Sigma (A6778 Sigma Chemical Co., St. Louis, MO, USA). Gastric porcine pepsin (Pepsin $\geqslant$ 250 unit/mg), and porcine pancreatin (4 $\times$ USP) were purchased by Sigma (P7000, P3292). The Alcalase 2.4L enzyme was purchased from Novo Laboratories (Copenhagen, Denmark). Alcalase 2.4L is an endopeptidase from *Bacillus licheniformis*, with subtilisin Carlsberg as the major enzyme component and a specific activity of 2.4 Anson units (AU) per gram.

2.2. Jatropha curcas flour

Flour was produced from 6 kg *J. curcas* seed by first removing all impurities and damaged seeds. Standard AOAC procedures were used to determine nitrogen (method 954.01), fat (method 920.39), ash (method 925.09), crude fiber (method 962.09), and moisture (method 925.09) contents in the shelled milled seeds (AOAC, 1997). Nitrogen (N_2) content was quantified with a Kjeltec Digestion System (Tecator, Sweden) using cupric sulphate and potassium sulphate as catalysts. Protein content was calculated as nitrogen $\times$ 6.25. Fat content was obtained from a 1 h hexane extraction. Ash content was calculated from sample weight after burning at 550 °C for 2 h. Moisture content was measured based on sample weight loss after oven-drying at 110 °C for 2 h. Carbohydrate content was estimated as nitrogen-free extract (NFE).

Oil extraction from the milled seeds was done with hexane in a Soxhlet system for 8 h (AOAC, 1997, method 920.39). The defatted *J. curcas* flour was milled in a Cyclotec 1093 (Tecator, Sweden) mill until passing through an 80 and 100-mesh screen and AOAC (1997) procedures were used to determine its proximate composition.

2.3. Jatropha curcas protein isolate

The defatted *J. curcas* flour was processed using the wet fractionation method of Betancur-Ancona, Gallegos-Tintoré, and Chel-Guerrero (2004). Briefly, whole flour was suspended in distiled water at a 1:6 (w/v) ratio, pH was adjusted to 11 with 1 M NaOH, and the dispersion was stirred for 1 h at 400 rpm with a mechanical agitator (Caframo Rz-1, HeidolphSchwabach, Germany). This suspension was wet-milled with a Kitchen-Aid mill and the fiber solids were separated from the starch and protein mix by straining through 80- and 150-mesh sieves and by washing the residue five times with distiled water. The protein–starch suspension was allowed to sediment for 30 min at room temperature to recover the starch and protein fractions. The pH of the separated solubilised proteins was adjusted to their isoelectric point (4.5) with 1 M HCl. The suspension was then centrifuged at 1317*g* for 12 min (Mistral 3000i, Curtin Matheson Sci.), the supernatants were discarded and the precipitates were freeze-dried at −47 °C and 13 $\times$ 10^{-3} mbar until use.

2.4. Enzymatic hydrolysis

Defatted *J. curcas* flour and *J. curcas* protein isolate, prepared as previously described were hydrolyzed using a totally randomized design with the treatments being the enzymatic system applied: Alcalase 2.4L FG and the sequential system using pepsin from porcine gastric mucosa (Sigma, P7000-100G) and pancreatin from porcine pancreas (Sigma, P3292-100G). The response variable was degree of hydrolysis (DH).

Hydrolysis was done under controlled conditions (temperature, pH and stirring) in a 1000 mL reaction vessel equipped with a stirrer, thermometer and a pH electrode. Hydrolysis with Alcalase was done according to Yust et al. (2003). Flour or isolate were suspended in distiled water to produce a 5% (w/v) protein solution. This solution was equilibrated at optimum temperature and pH before adding the respective enzyme. Protease was then added to the solution at a ratio of 0.3 UA/g for Alcalase. Hydrolysis conditions were 60, 90 and 120 min at 50 °C and pH 8.0. The pH was kept constant by adding 1.0 M NaOH during hydrolysis. Hydrolysis with the sequential pepsin–pancreatin system was done with a pH-stat method for 60, 90 and 120 min: predigestion with pepsin for 30, 45 and 60 min followed by incubation with pancreatin for 30, 45 and 60 min. Hydrolysis parameters were substrate concentration 5%; enzyme/substrate ratio 1:10; pH 2 for pepsin; pH 7.5 for pancreatin; and 37 °C (Megías et al., 2004; Yang, Marczak, Yokoo, Usui, & Yoshikawa, 2003). The pH was kept constant by adding 1.0 M NaOH or 1.0 M HCl during hydrolysis. In all three treatments the reaction was stopped by heating to 80 °C for 20 min, followed by centrifuging at 9880*g* for 20 min to remove the insoluble portion. Hydrolysates protein content was determined according to Lowry, Rsebrough, Farr, and Randall (1951).

2.5. Degree of hydrolysis

Degree of hydrolysis (DH) was calculated by determining free amino groups with *o*-phthaldialdehyde following Nielsen, Petersen, and Dambmann (2001): DH = $h/h_{tot} * 100$; where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds.

The h_{tot} factor is dependent on raw material amino acid composition and it was determined by reverse-phase high performance liquid chromatography (RP-HPLC) according to Alaiz, Navarro, Giron, and Vioque (1992). Samples (2–4 mg protein) were treated with 4 mL of 6 mol equi L^{-1} HCl, placed in hydrolysis tubes and gassed with nitrogen at 110 °C for 24 h. They were then dried in a rotavapor and suspended in 1 mol L^{-1} sodium borate buffer at pH 9.0. Amino acid derivatization was performed at 50 °C using diethyl ethoxymethylenemalonate. Amino acids were separated using HPLC with a reversed-phase column (300 $\times$ 3.9 mm, Nova Pack C18, 4 mm; Waters), and a binary gradient system with 25 mmol L^{-1} sodium acetate containing (A) 0.02 g L^{-1} sodium azide at pH 6.0, and (B) acetonitrile as solvent. The flow-rate was 0.9 mL min^{-1}, and the elution gradient was: time 0.0–3.0 min, linear gradient A:B (91:9) to A–B (86:14); time 3.0–13.0 min, elution with A–B (86–14); time 13.0–30.0 min, linear gradient A–B (86:14) to A-B (69:31); time 30.0–35.0 min, elution with A–B (69:31).