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Research Article

DIGESTIBILITY AND ANTI-INFLAMMATORY ACTIVITY *IN VITRO* OF SACHA INCHI (*PLUKENETIA VOLUBILIS* L.) PROTEINS

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ABSTRACT

Objective: The aim of this study was to evaluate the digestibility and anti-inflammatory activity *in vitro* of sacha inchi protein isolate.

Methods: Proteins were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gastric digestibility was evaluated with pepsin at different pHs and at different relation enzyme/substrate. Anti-inflammatory activity *in vitro* of sacha inchi protein isolate was evaluated using denatured protein method with egg albumin.

Results: A yield of 20.88% of protein isolate of defatted sacha inchi flour at pH 4.0 with a 93.1% of protein was obtained. 11S globulins were resistant to gastric and duodenal digestion. Sacha inchi protein isolate at pH 4.0 (1000 µg/ml) presents 78.13% of anti-inflammatory activity *in vitro*.

Conclusions: Sacha inchi seed is a good source of proteins. 11S globulins are resistant to pepsin and pancreatin hydrolysis. Sacha inchi protein isolate has high anti-inflammatory activity *in vitro*.

Keywords: Sacha inchi, Gastric digestion, Duodenal digestion, Globulins and albumins.

INTRODUCTION

Sacha inchi (*Plukenetia volubilis* L.), also named as Inca peanut, is a plant that grows in the wild and is native to the rain forests of the Andean region of South America. This plant belongs to the *Euphorbiaceae* family and is composed of 19 species [1]. Food proteins extracted from plants are important for human and animal nutrition particularly in developing countries where average protein intake is less than required [2]. The production of plant protein isolates is of growing interest to food industry because of the increasing applications of plant proteins in food markets, nutraceutical products, and functional foods [3,4]. It is known that sacha inchi seeds have a high content of oil (54%) and protein (27%) [5,6]. Medicinal plants are considered a rich source of pharmaceutical components for the prevention and treatment of diseases and ailments. Inflammation process is the defense mechanism of the body to eliminate or limit the spread of injurious stimuli and heal the injury, excessive inflammation is associated with onset of diseases such as rheumatoid arthritis, asthma, periodontitis, inflammatory bowel disease, atherosclerosis, Alzheimer's disease, and even cancer such as gallbladder carcinoma [7,8]. Nonsteroidal anti-inflammatory drugs are widely used in the treatment of acute and chronic inflammation, pain, and fever. Their use is associated with adverse effects such as severe gastritis, peptic ulcer, nausea, vomiting, salt and water retention, worsening of renal function in renal or cardiac and cirrhotic patients, and hypersensitivity [9,10]. Therefore, the development of new, economic, potent and safe anti-inflammatory drugs from natural sources is needed, especially for developing countries. The aim of this study is to evaluate the gastrointestinal digestibility and anti-inflammatory activity *in vitro* of sacha inchi proteins (*Plukenetia volubilis* L.).

METHODS

Protein isolate from sacha inchi

Sacha inchi isolate was prepared according to Martıínez and Añón [11] with modifications. The defatted flour was suspended in water in a 1:10 w/v. The suspension was adjusted to pH 8.0 by adding 2 M NaOH. The suspension was stirred during 1 hr and then centrifuged at 4500 g for 30 minutes at 25°C. The supernatant was adjusted to pHs 2.0, 3.0, 4.0, 5.0, and 6.0 with 2 N HCl and centrifuged for 20 minutes at 4500 g. The pellet was suspended in a small volume of water, neutralized with 0.1 M NaOH, and lyophilized and then frozen at −20°C. The content of protein isolate was determined using the bicinchoninic acid (BCA) method.

Gastric digestion

Sacha inchi protein isolate at pH 4.0 was subject to *in vitro* gastric digestions at 5 mg/mL final concentration. The digestions were performed in simulated gastric fluid (0.35 M NaCl) at different pHs: 1.2, 2.0 and 3.2 at 37°C for 120 minutes, with porcine pepsin (EC 3.4.23.1, 4,500 U/mg protein, Sigma–Aldrich), with an enzyme/substrate ratio of 1:20, wt/wt (2000 U/mg) [12,13]. At least four replicates of each digestion assay were performed and compared with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method to ensure the repeatability of the results. Aliquots were taken at 120 minutes for analysis. The digestions were stopped by mixing with the SDS-sample buffer for SDS-PAGE, and subsequently, pH was raised to 7.0 with ammonium bicarbonate to irreversibly inactivate pepsin after 10 minutes of equilibration. The protein concentration of the supernatants was determined by the BCA method.

Duodenal digestion

Duodenal digestions were performed as previously described [14] referenced to a 120 minutes gastric digests re-adjusted at pH 7.0. The following elements were added:

a) 0.125 M bile salt mixture containing equimolar quantities of sodium glycodeoxycholate and sodium taurocholate (Sigma–Aldrich) (6.15 mM final concentration of each salt)
b) 1 M $CaCl_2$ (7.6 mM final concentration)
c) Pancretine (100 U/mg) mixed with 20.3 mM Bis–Tris.

Reactions were carried out at 37°C during 120 minutes and stopped by heating at 80°C during 5 minutes. Hydrolysates were analyzed using SDS-PAGE.

SDS-PAGE

SDS-PAGE of sacha inchi protein isolate was carried out according to the method proposed by Laemmli (1970) [15] using 4-8% and 4-12% polyacrylamide gel in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). Polypeptide bands were stained in Coomassie Brilliant Blue G-250 for 12 hrs. Relative molecular masses of protein were determined by a comparison to molecular weight markers (Bio-Rad, Hercules, CA, USA) and software Quantity One of Chemidoc (Bio-Rad).

Evaluation of *in vitro* anti-inflammatory activity

Anti-inflammatory activity of sacha inchi protein isolate at pH 4.0 was evaluated with the protein denaturation method [16,17]. Diclofenac sodium, a powerful nonsteroidal anti-inflammatory drug was used as a standard drug. The reaction mixture consisting of 2 mL of different concentrations of protein isolate (100-1000 µg/mL) or standard diclofenac sodium (100 and 200 µg/mL) and 2.8 mL of phosphate buffered saline (pH 6.4) was mixed with 2 mL of egg albumin (from fresh hen egg) and incubated at 27°C for 15 minutes. Denaturation was induced by keeping the reaction mixture at 70°C in a water bath for 10 minutes. After cooling, the absorbance was measured at 660 nm using double distilled water as blank. Each experiment was done in triplicate and the average was taken. The percentage inhibition of protein denaturation was calculated using the following formula:

% Inhibition: (At-Ac/Ac) ×100

Statistical analysis

Results are presented as means±standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance. The *post hoc* analysis was performed by the Tukey's test. All tests were considered significant at $p<0.05$. A statistical analysis was performed using the software package Prism 4 for Windows, version 4.3 (GraphPad Software Inc., www.graphpad.com).

RESULTS

Sacha inchi protein isolates were obtained using the isoelectric precipitation method with water as solvent. Using water as solvent, the highest yield was obtained at pH 4.0 with a 20.88% of protein isolate content (Table 1). The content of protein in the protein isolate was determined using the BCA method. Table 2 shows the results of BCA method. The content of protein was extremely high (93.1%) in sacha inchi protein isolate at pH 4. This treatment of precipitation at pH 4 was chosen as the optimum to evaluate digestibility and anti-inflammatory *in vitro* activity.

Gastrointestinal simulation

Sacha inchi protein isolate at pH 4.0 was hydrolyzed with pepsin at 172 U/mg of protein during 60 minutes at 37°C, at pH 1.2, 2.0 and 3.2. It was observed that the isolate of sacha inchi was resistant to the hydrolysis with pepsin at low concentration of enzyme. 7S, 11S, and 2S albumins were resistant to proteolytic digestion (Fig. 1). With regards to the isolate of sacha inchi when assayed with a higher concentration of enzyme (2000 U/mg of protein), it was determined that 11S globulins show a remarkable stability to hydrolysis with pepsin as shown in Fig. 2 and Fig. 3.

7S globulins and 2S albumins were susceptible to pepsin hydrolysis at 2000 U/mg of protein.

On the other hand, a digestion model was assayed in two steps; the first step was named gastric digestion with pepsin at 2000 U/mg of protein at pH 3.0 during 120 minutes at 37°C. The second step was performed by adding a pancreatin solution at pH 7.0 during 120 minutes at 37°C. Using SDS-PAGE method, we observed that 11S globulins continue being resistant to hydrolysis with enzymes. 11S globulins from sacha inchi are resistant to proteolytic digestion with pepsin and with the mix of pepsin and pancreatin.

The reason for choosing sacha inchi protein isolate at pH 4.0 was due to the fact that this treatment presents a higher yield in this study with a higher content of protein. The inhibitory effects of different concentrations of Sacha inchi protein isolate at pH 4.0 were determined using the protein denatured method described by Padmanabhan *et al*., 2012. The results are shown in Table 2. Sacha inchi protein isolate (100-1000 µg/ml) showed a significant inhibition of egg albumin denaturation in a dose-dependent form. The *in vitro* anti-inflammatory

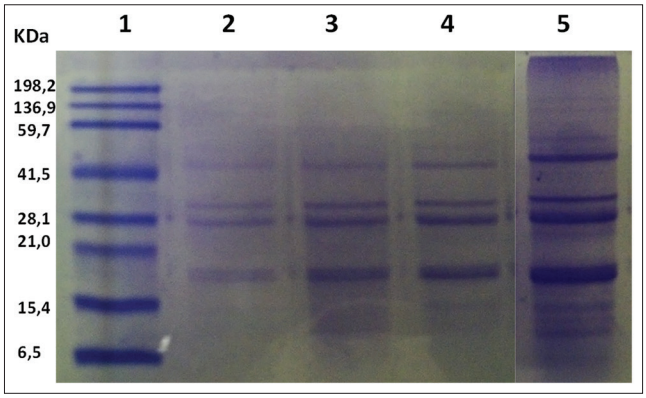

Fig. 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sacha inchi protein isolate of pH 4.0 hydrolyzed with pepsin at pH 1.2; 2.0 and 3.2 (gastric digestion with pepsin at 172 U/M of protein)

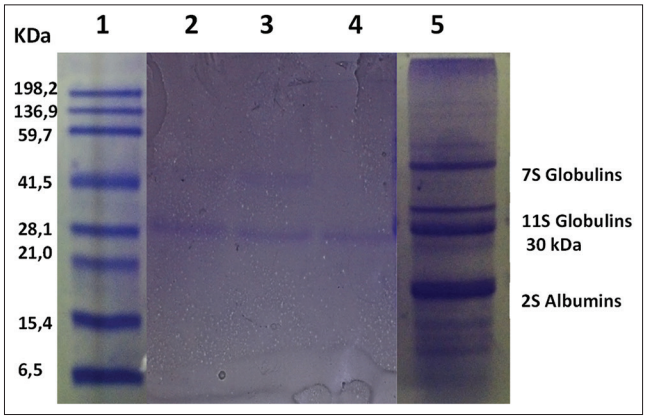

Fig. 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sacha inchi protein isolate of pH 4.0 hydrolyzed with pepsin at pH 1.2; 2.0 and 3.2 (gastric digestion with pepsin at 2000 U/M of protein)

Table 1: Sacha inchi isolate protein yield obtained at different pHs and content of protein in the sacha inchi isolate

Sample	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0
% Isolate with water	15.65±0.8[a]	16.73±1.2[a]	20.88±1.3[a]	18.53±0.2[a]	19.10±0.2[a]
% Protein by BCA	62.8±0.04[a]	89.1±0.004[d]	93.1±0.01[e]	83.6±0.02[c]	70.4±0.02[b]

BCA: Bicinchoninic acid, SD: Standard deviation, results represent the average of three determinations ±SD. Different letters show statistical difference from the control group (p <0.05) ANOVA and Turkey's test

Table 2: *In vitro* anti-inflammatory activity of sacha inchi protein isolate

Treatment	Concentration µg/ml	% Inhibition of denaturation
Protein isolate	100	7.77±0.39[a]
	200	9.27±0.34[a]
	500	18.94±1.09[b]
	1000	78.13±0.44[c]
Diclofenac Sodium	100	72.69±0.05[c]
	200	76.20±2.5[c]

SD: Standard deviation, results represent the average of three determinations ±SD. Different letters show statistical difference from the control group (p <0.05) ANOVA and Turkey's test

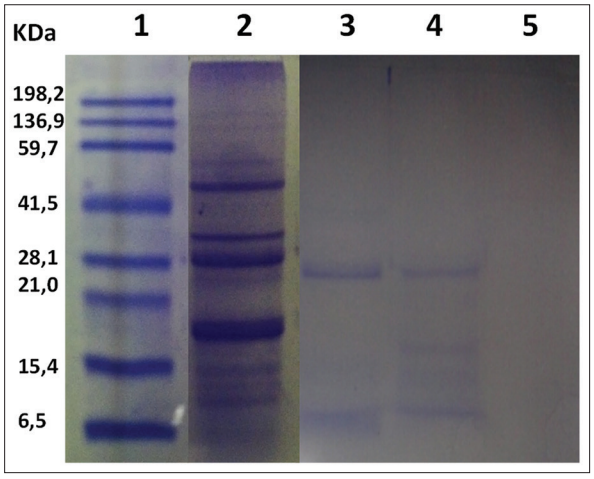

Fig. 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sacha inchi protein isolate of pH 4.0 hydrolyzed with pepsin more pancreatin at pH 7.0 (duodenal digestion)

activity of sacha inchi protein isolate at pH 4.0 (1000 µg/ml) was comparable to the one of the positive control (diclofenac sodium and anti-inflammatory drug) with 78, 13% of inhibition.

DISCUSSION

It is known that plant proteins from legume and nonlegume plants have two of the main classes of storage proteins. These proteins are named 7S and 11S depending on their sedimentation coefficients. 11S globulins are hexamers with molecular weights between 300 and 400 kDa, consisting of two opposed hexagonal rings, each containing three hydrophobically associated pairs of disulfide-linked acidic (29-35 kDa) and basic (18-28 kDa) subunits. 7S globulins are glycoproteins with molecular weights between 150 and 200 kDa [18]. The occurrence of 11S and 7S type storage globulins in angiosperm seeds has been recognized and accepted [19]. 2S albumins have been characterized in different plant proteins for example amaranth, quinoa, lupin seed, soybean and sacha inchi, as they are considered as allergens [20,21]. The water-insoluble 7-11S globulin and the soluble 2S albumin are the two major storage proteins of many plants. A number of plant proteins have been found to present a high resistance to proteolysis in the gastrointestinal tract because of specific structural properties. Major proteins structural properties of legume and other plant proteins that have been reported to affect *in vivo* digestibility such as soybean, cereals, lupin, nuts and wheat proteins. The protein fraction of plant foods with high cysteine content, the albumin fraction, according to classical Osborne classification methodology, has been found to be quite resistant to heat denaturation and proteolytic digestion. Stability is conferred by the presence of a high number of disulfide bonds contained in low molecular weight proteins, such as Bowman-Birk inhibitor (BBI) [22-25]. Unlike the closely related Kunitz trypsin inhibitor, soybean BBI is a small molecule of 8 kDa and seven disulfide bridges. Other very stable and highly active inhibitors of the BBI class have recently been isolated from lentil (*Lens culinaris* L.) and pea (*Pisum sativum* L.) seeds. Lee *et al*. [26] have reported that primary allergen of soybean 11S basic polypeptide presented resistance to hydrolysis with pepsin at pH 2.0. In this study, 11S globulin is resistant to hydrolysis with pepsin and pepsin/pancreatin; this can be due to the high content of cysteine.

CONCLUSIONS

Sacha inchi seeds are an excellent source to obtain proteins isolate. 7S globulin and 2S albumin of sacha inchi were susceptible to gastric and duodenal hydrolysis. 11S globulins were resistant to gastric and duodenal digestion. Sacha inchi protein isolate at pH 4.0 (1000 µg/ml) presents 78.13% of anti-inflammatory activity *in vitro*.

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