



Impact of germination time on protein solubility and anti-inflammatory properties of *Pisum sativum* L grains

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ABSTRACT

During germination processes take place that modify the major components of the grain, such is the case of proteins that are hydrolyzed to generate peptides that can lead to the generation of bioactivity. The objective of the present work was to germinate grains of *Pisum sativum* to evaluate the effect on the soluble protein content and the anti-inflammatory activity. The grains were subjected to 10 days of germination at 24 °C and relative humidity of 75%. Sprouts were lyophilized, milled, and phenolic compounds were extracted to avoid interferences. Soluble protein content varied significantly during the 10 days of germination. *In vitro* assays indicate that sprouts protein inhibits thermal denaturation of proteins, protease activity, and stabilize cell membranes. The IC50 values indicate that after germination the bioactivity increased between 1.4 and 3.5 times, with respect to the ungerminated grains. Results indicated that *Pisum sativum* sprouts may constitute promising health-promoting foods.

Introduction

Legume seeds such as soybeans (*Glycine max*), beans (*Phaseolus vulgaris*), peas (*Pisum sativum*), lupins (*Lupinus spp.*), and lentils (*Lens culinaris*) are rich sources of protein, carbohydrates, water-soluble vitamins, and minerals, additionally they contain other compounds with nutraceutical potential. In this sense, plant proteins are not only a source of energy and structural compounds such as amino acids, but they can also play a biologically active role as precursors of bioactive peptides with various physiological functions, such as immunomodulatory, antihypertensive, antithrombotic, opioid, etc. There are several alternatives for the bioprocessing of legumes, such is the case of: 1) *In vitro* enzymatic hydrolysis that uses animal, vegetable, or microbial proteases. 2) fermentation using specific microorganisms. 3) germination that activates the metabolism associated with the generation of a new seedling. In all these processes, the native protein of legumes is modified, generating polypeptides, peptides, and free amino acids (Rubio et al., 2014; Ma et al., 2017; Di Stefano et al., 2019).

Inflammation is a process not only evolutionarily conserved but

considered as an immune response, which is characterized by the activation of cells of both an immune and non-immune nature, which protect the host from bacteria, viruses, toxins, and infections, eliminating pathogens and promoting repair and recovery of affected tissues. Depending on whether the inflammation is systemic or local, metabolic, and neuroendocrine changes can occur. Changes in the inflammatory response of short to long duration can cause a decrease in immune tolerance, causing significant alterations in all tissues and organs, as well as in normal cellular physiology, which can increase the risk of various non-communicable diseases (Rizeello et al., 2016; Furman et al., 2019).

Recent studies on the anti-inflammatory effects of various samples such as herbal extracts use different *in vitro* assays. Usually, these studies include a minimum of three *in vitro* assays to evaluate this type of bioactivity, among the most used are protein denaturation and membrane stabilization (Sarveswaran et al., 2017). Protein denaturation has been correlated with the formation of inflammatory disorders like rheumatoid arthritis, diabetes, and cancer (Sangeetha & Vidhya, 2016; Gunathilake et al., 2018a). The lysis of the red blood cell membranes with haemolysis and oxidation of haemoglobin may occur by the results

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of injurious substances exposure to red blood cells. Since human red blood cell membranes are like lysosomal membrane, the inhibition of hypotonicity and heat induced lysis of red blood cell membrane will be taken as a measure of the mechanism of anti-inflammatory activity (Labu et al., 2015; Gunathilake et al., 2018b). Given the importance of legumes for their nutritional contribution, it is important to diversify their consumption alternatives, especially if these alternatives could provide compounds with functional or nutraceutical potential. Considering the previous, the aim of this study was to evaluate the impact of germination on *Pisum sativum* L. grain protein in the release of peptides with anti-inflammatory activity.

Materials and methods

Grains, conditioning, and germination

Pisum sativum L. grains were collected in the state of Hidalgo, 2017, Mexico. According to NMX-FF-038200 200 grains (50 g) were selected. For the conditioning procedure, the methodology described by Ramos and Villanueva (1998) was followed with some modifications. To sanitize the grains sodium chloride at 10% (w/v) was used for 30 min and then silver iodide at 0.32% (w/v) was used for 15 min. After that, grains were left hydrating with distilled water for 160 min and placed on trays covered filter paper saturated with water. Germination was carried out using the methodology developed by Perales-Sánchez et al. (2015). The 200 grains (50 g) were placed in aluminium trays covered with moistened filter paper at saturation and incubated in a germination chamber for 10 days at 25 °C 70% RH and performed in darkness. The raw and sprouted seeds were frozen (-80 °C, 24 h) and stored in plastic bags then were lyophilized (-50 °C, 0.3 mbar, 24 h, Labconco FreeZone 2.5 L-50C) and finally they were milled and sifted (150 µ, Cemotec CM 290) to obtain the flours.

Extraction of phenolic compounds

Phenolic compounds were extracted by solid-liquid extraction with acetone 75% (v/v) in a ratio 1:5 (m/v), flour was subjected to extraction for 30 min at 4 °C by stirring, then centrifuged at 3000 rpm for 10 min at 4 °C (Centrifuge Scilogex D2012). This process was performed five times. The precipitate was recovered for analysis while the supernatant was discarded.

Soluble protein concentrate

The methodology described by Paucar-Mencaho et al., (2010) was followed with some modifications. To 50 mg of freeze-dried flour from ungerminated or germinated grains, a volume of 1.0 mL of buffer was added (0.05 M Tris-HCl, pH 8.2). This mixture was sonicated in an ultrasonic bath (Branson Cpx-952-219r) for 70 min at 24 °C. The samples were centrifuged (Centrifuge Scilogex D2012) at 13,000 rpm for 20 min at 8 °C, the supernatant was recovered, and the precipitate discarded.

Determination of soluble protein concentration

To the Bovine Serum Albumin (BSA) standard or the diluted sample 100 µL of NaOH 2.0 N were added, the mixture was heated in a water bath at 100 °C for 10 min. Then, it cools for 5 min at room temperature and 1.0 mL of the complex reagent (consisting of Na₂CO₃, CuSO₄ and KNaC₄H₄O₆·4H₂O) was added and protected from light. To this mixture, 100 µL of Folin reagent (50 %) was added, allowed to react for 45 min and the absorbance was determined at 750 nm (Thermo Scientific UV-VIS Visible Spectrophotometer Lab Equipment 360-1000 nm 4 nm 721 N) (Lowry et al., 1951). The results obtained were expressed in g equivalent of BSA/100 g of sample.

Inhibition of protein thermal denaturation

The methodology used was described by Khan et al., (2015) with some modifications. The reaction mixture consisted of 500 µL of the sample and 500 µL of 5% (w/v) albumin solution. It was incubated at 37 °C for 20 min, and then the temperature was increased to 70 °C for 5 min. Turbidity was measured spectrophotometrically at 660 nm (Thermo Scientific UV-VIS Visible Spectrophotometer Lab Equipment 360-1000 nm 4 nm 721 N). The percentage inhibition was calculated with Eq. (1):

$$\%PTD = \left(\frac{CA - SA}{CA} \right) * 100 \quad (1)$$

where

PTD = protein thermal denaturation

CA = control absorbance

SA = sample absorbance

Inhibition of proteolytic activity

The methodology developed was described by Naz et al., (2017), with some modifications. To 100 µL of Alcalase solution (protease mixture) at a concentration of 0.06 mg/mL, 100 µL of sodium phosphate buffer (0.2 M, pH 7.4) and 100 µL of the sample were added. At the end of incubation at 37 °C for 5 min, 100 µL of 0.8% (w/v) casein solution was added. A second incubation at 37 °C for 2 min was performed. At the end of the incubation, 200 µL of 10% TCA was added. Subsequently, it was centrifuged (Centrifuge Scilogex D2012) at 3000 rpm for 5 min and monitored spectrophotometrically at 280 nm (Thermo Scientific UV-VIS Visible Spectrophotometer Lab Equipment 360-1000 nm 4 nm 721 N). The percentage of protease inhibition was calculated using equation (2):

$$\%IPA = \left[1 - \left(\frac{CA - SA}{CA} \right) \right] * 100 \quad (2)$$

where

IPA = Inhibition of proteolytic activity

CA = control absorbance

SA = sample absorbance

Cell membrane stabilization

The following methodology was described by Yesmin et al., (2020) with some modifications. For this assay, fresh human blood samples were obtained from healthy volunteers, the samples were available in accordance with the provisions of Mexican regulations (NOM-253-SSA1-2012). From fresh blood sample, 1.0 mL was taken, and 1.0 mL of saline solution (0.9%) was added, this mixture was centrifuged (Centrifuge Scilogex D2012) at 3000 rpm for 10 min. The cell pack (leukocytes, platelets, and erythrocytes) was washed again with saline solution (0.9%), this procedure was performed for a total of 5 times, taking the cell pack at the end. At the end of the washings, the amount of precipitate remaining was measured and reconstituted in a 1:1 ratio with a saline solution (0.9%). From the 1% (v/v) red blood cell solution, 500 µL were taken and 500 µL of the sample were added, this reaction was incubated at 56 °C for 30 min, and then proceeded to stop the reaction in water with ice, later it was centrifuged (Centrifuge Scilogex D2012) at 2500 rpm for 5 min. The absorbance of the supernatant was measured at 560 nm (Thermo Scientific UV-VIS Visible Spectrophotometer Lab Equipment 360-1000 nm 4 nm 721 N). The stabilisation of the red blood cell membrane (5) was calculated with Eq. (3):

$$\%CMS = \left[1 - \left(\frac{CA - SA}{CA} \right) \right] * 100 \quad (3)$$

where

CMS = Cell membrane stabilization

CA = control absorbance

SA = sample absorbance

Scanning electron microscopy

An aliquot of 500 μL of sample from the membrane stabilization assay was taken, then was fixed on a coverslip which contained on its surface porcine gelatine (0.2% w/v in phosphate buffer). After 1.0 mL of glutaraldehyde (2.5% v/v in 0.1 M sodium cacodylate buffer) was added to the sample fixed in the coverslip, was left at room temperature for 2 h and after was storage at 10 °C for 24 h. The remaining glutaraldehyde was removed from the sample washing 5 times with phosphate buffer (pH 7.2) and 1 wash with deionized water. Then the sample was dehydrated with different ethanol solutions (30, 50, 70, 90, and 100% v/v), the solutions were added in increasing order, each wash was performed for 15 min. After the washes, the coverslip was placed in a holder and dried to a critical point (1100 psi, 31 °C). Finally, was placed in a metallizer and taken to observation (ZEISS, Germany).

Statistical analysis

The results obtained with respect to soluble protein and anti-inflammatory activities *in vitro* were analysed by means of a one-way ANOVA and the Tukey method was used for comparison of means.

Results and discussion

Determination of soluble protein

Germination modifies the solubilization of various components present in grains, it can even modify their biological activities. One way to measure the impact of germination on grain reserve proteins is by determining the solubility of nitrogen. Fig. 1 shows the results obtained for the quantification of soluble protein in *P. sativum* sprouts. The soluble protein content was modified statistically significantly ($p < 0.05$), from the beginning of germination the soluble protein content (24.85 g / 100 g of sample) decreased until reaching a minimum content on the seventh day (8.98 g / 100 g of sample). From the eighth day, an increase was again observed until the tenth day (15.34 g / 100 g of sample) without reaching the initial value.

Regarding protein solubility, Paucar-Menacho et al., (2010) reported that during germination of the soybean cultivar BRS-2508, the highest amount of soluble protein (36 g / 100 g of sample) was obtained after 72 h germination at 25 °C. Similarly, in another study De Souza-Rocha et al. (2015) reported a maximum amount of soluble protein of 8.01 g / 100 g of sample for germinated *P. vulgaris* for 72 h at 25 °C for 72 h. According to Badann-Bueno et al. (2020), who evaluated the changes in protein

solubility of the soybean cultivar BRS-257 after different germination times (0, 8, 32, 56, 80, 104 and 176 h), the germination process modified not only the solubility of the proteins but also their bioactivity. According to these authors, the variation in solubility is probably due to the mobilization of protein material for the synthesis of enzymes and new tissues that takes place during germination (Amir et al., 2018). Badann-Bueno et al. (2020), González-Montoya et al., (2018), and Mellen et al., (2018) coincide in reporting that after 18 h of germination, progress in protein hydrolysis releases amino acids and peptides. After this intense metabolic activity, the protein profile of the material is altered, a behaviour that explains what was observed in the protein solubility profile of *P. sativum* sprouts.

Inhibition of albumin denaturation

Protein denaturation has been correlated with the development of inflammatory disorders such as rheumatoid arthritis, diabetes, and cancer (Sangeetha and Vidhya, 2016; Gunathilake et al., 2018a), making it one of the most widely used *in vitro* assays to evaluate anti-inflammatory activity. Fig. 2 shows the results obtained for the evaluation of the Inhibition of albumin thermal denaturation during germination. The inhibition of denaturation was modified statistically significantly ($p < 0.05$), from the beginning of germination (55.63%) decreased until reaching a minimum on the fifth day (21.96%). From the sixth day, an increase was again observed until the tenth day (79.62%) exceeding initial value.

Little is known about the effect that the application of processes such as germination and cooking can have on the anti-inflammatory biological activity of the reserve proteins of legumes. In this regard, Milán-Noris et al. (2018) investigate the anti-inflammatory activity of protein concentrates from germinated and cooked chickpeas (GC and CC, respectively), and reported that anti-inflammatory activity of GC digests was almost 2-fold higher than CC digests ($p < 0.05$), which was associated to greater content of peptides. These authors reported an active peptide fraction from GC digest (IC50 = 93 $\mu\text{g}/\text{mL}$) that contained a total of 24 peptides derived from legumin and vicilin. The results obtained in the present study indicate that the germination of *P. sativum* leads not only to hydrolysis and changes in the solubility of the protein, but also influences the anti-inflammatory activity. It was also observed that the soluble protein content is not a determining factor for biological activity since the maximum value of inhibitory activity of protein denaturation (76.92%) was reached with 1.62 times lower protein content than at the beginning of germination. This indicates that the type and sequence of the released peptides would have a greater influence on the biological activity.

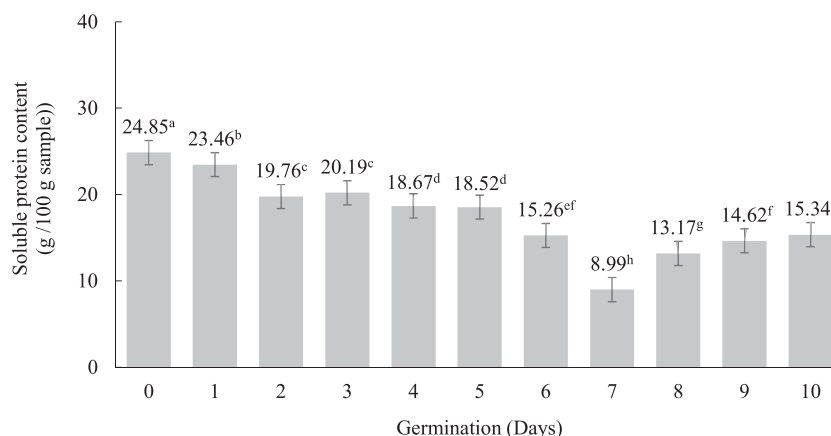


Fig. 1. Effect of germination in soluble protein content.

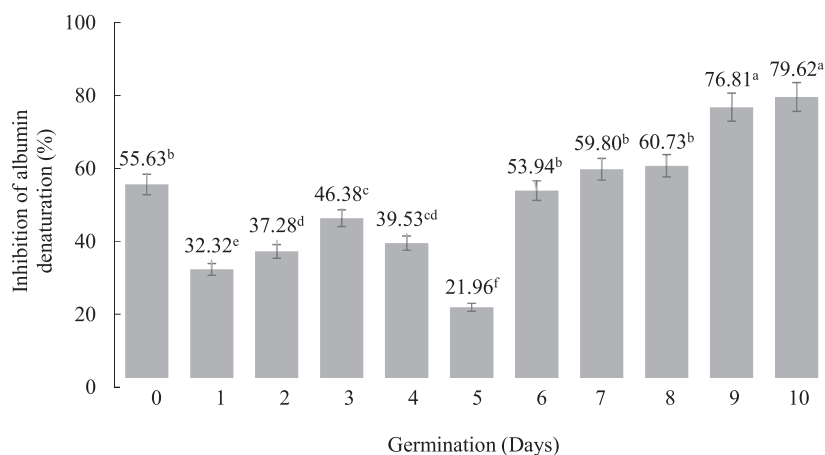


Fig. 2. Effect of soluble protein fraction generated during germination in inhibition of albumin denaturation.

Proteinase inhibitory action

During inflammation, as part of their defensive functions, leukocytes release their lysosomal enzymes, including proteases, causing tissue damage and consequent inflammation (Gunathilake et al., 2018). Neutrophils are a rich source of serine proteinases and are known to be involved in inflammatory reactions. Their contribution has been reported in the pathology of arthritis (Agarwal & Shanmugam, 2019). Therefore, one way to evaluate the anti-inflammatory potential of the proteins generated during the germination of *P. sativum* is the proteinase inhibitory action assay. Albumin was used as a substrate and Alcalase was used as a serine protease enzyme to cleave the peptide linkage of albumin.

Fig. 3 shows the results obtained for the proteinase inhibitory action during germination. The inhibitory action was modified statistically significantly ($p < 0.05$), from the beginning of germination (33.14%) decreased until reaching a minimum on the fourth day (28.58%). From the fifth day, an increase was again observed (41.23%) until the tenth day (33.98%) reaching the same value (statistically) as at the beginning. The results of this assay indicate that throughout the germination process, peptides were generated whose concentration and amino acid sequence allow them to interact as inhibitors of protease activity. This type of enzyme inhibitors can also act on other physiological processes, such is the case of the inhibitor peptides of the angiotensin-converting enzyme, a key enzyme in the treatment of arterial hypertension (Trupti et al., 2021).

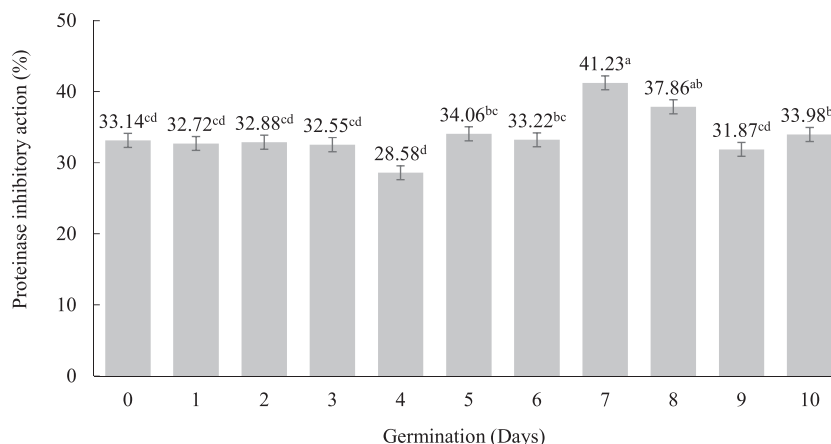


Fig. 3. Effect of soluble protein fraction generated during germination in proteinase inhibitory action.

Cell membrane stabilization

Since human red blood cell membranes are like lysosomal membrane, the inhibition of hypotonicity and heat induced lysis of red blood cell membrane will be taken as a measure of the mechanism of anti-inflammatory activity (Gunathilake et al., 2018b). Fig. 4 shows the results obtained for the evaluation of the cell membrane stabilization denaturation during germination. The percent inhibition of heat-induced haemolysis of red blood cell was modified statistically significantly ($p < 0.05$), from the beginning of germination (53.81%) decreased until reaching a minimum on the sixth day (15.73%). From the sixth day, an increase was again observed until the ninth day (20.86%) without reaching the initial value. The results of this assay indicate that during the germination process the capacity of the proteins to stabilize the membranes of the red blood cells is reduced by almost half compared to the initial day.

Damage to cell membranes makes the cell more susceptible to free radical-induced lipid peroxidation damage. The regulation of the volume of water is also important, the content of which in the cells is controlled by the transporter proteins of the membrane, which also control the movement of sodium and potassium (Gunathilake et al., 2018). To demonstrate that during the red blood cell membrane stabilization test the soluble protein generated during germination acted as membrane protectors, scanning electron microscopy was used (Fig. 5).

The membrane protective capacity of the soluble proteins on day zero and on day 7 was evaluated, because on these days the relatively higher percentages of anti-inflammatory activity are presented for the three mechanisms evaluated. As shown in Fig. 5, when subjected to heat,

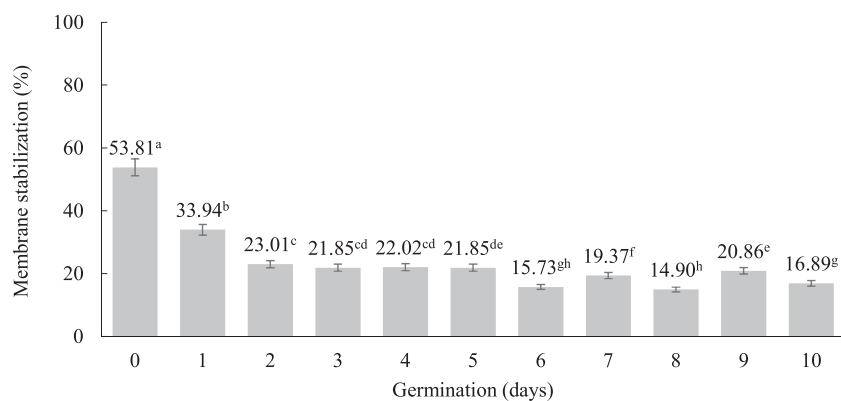


Fig. 4. Effect of soluble protein fraction generated during germination in red blood cell membrane stabilization.

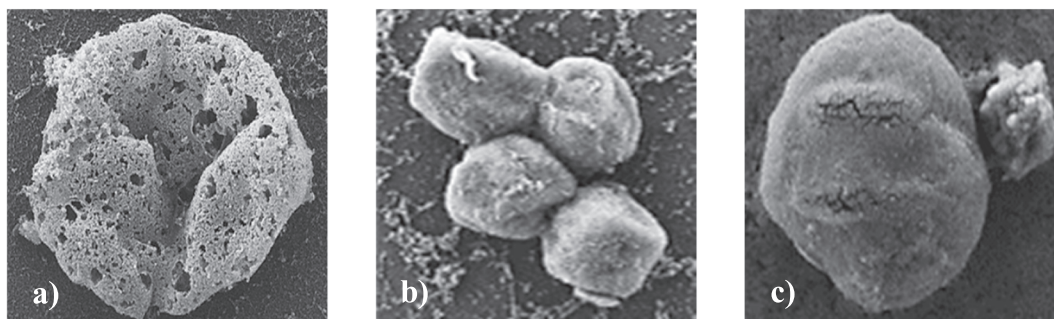


Fig. 5. Scanning electron microscopy (SEM) micrographs of red blood cell during membrane stabilization assay. a) Erythrocytes incubated at 56 °C, b) Erythrocytes incubated with soluble protein nongeminated, c) Erythrocytes incubated with soluble protein germinated for 7 days.

the human erythrocyte membrane undergoes haemolysis, which triggers a series of processes that lead to inflammation (a). One of the many responses to acute inflammation is the activation of leukocytes (mainly neutrophils) and proteases, which when encountered by macromolecules of the red cell membrane destabilize it and can damage other surrounding tissues. However, in Fig. 5, sections b and c, when erythrocytes are found with soluble protein from ungerminated grain and germinated from day seven, they are partially protected from the impact of temperature compared to unprotected cells. Stabilization of cell membranes may retard or inhibit the lysis and subsequent release of the cytoplasmic contents which, minimize the tissue damage and, hence, the inflammatory response. Therefore, substances that contribute significant protection of cell membrane against injurious substances are important in the event of inhibiting the progression of inflammation.

At the end of the germination process and the evaluation of bioactivity, it was possible to select the day on which the maximum values of anti-inflammatory activities occurred. Subsequently, the IC₅₀ values were established. Therefore, the seventh day is the day on which the determination was made and compared with ungerminated grain (day 0). Table 1 shows the IC₅₀ for anti-inflammatory activities.

The anti-inflammatory activity that required the lowest concentration of the sample was the stabilization of the erythrocyte membrane with 0.0214 mg/mL, this means 3.41 times lower concentration than the non-germinated grain. The *in vitro* tests carried out indicate that in the soluble protein generated during the germination of *P. sativum* amino acid sequences can be found with the ability to exert anti-inflammatory effects under three different mechanisms.

Conclusions

The results of the present work indicate that germination has an evident impact on the amount of soluble protein and bioactivity of the sprouts. The amount of soluble protein can be correlated with the

Table 1

IC₅₀ for anti-inflammatory evaluations of proteins concentrates from raw seed and sprout seed for 7 days.

Germination (Days)	Inhibition of albumin thermal denaturation (%)	Proteinase inhibitory action (%)	Cell membrane stabilization (%)
0	2.9470	1.1486	0.0741
7	1.5947	0.7980	0.0214

Concentrations are reported in mg equivalent of BSA/mL of protein concentrate.

presence of peptides from hydrolysis caused by proteases present in grains. *In vitro* studies carried out to corroborate biological functionality indicated that soluble protein contains components with the ability to act as anti-inflammatory agents through various mechanisms. Obtaining sprouts through a controlled process could be an alternative to process *Pisum sativum* grains to obtain minimally processed foods with functional potential or to isolate proteins and peptides with nutraceutical potential.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jorge Carlos Ruiz Ruiz reports was provided by Anahuac University Mayab. None reports a relationship with None that includes: None has patent pending to None. None.

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