Basic nutritional investigation

Nutritional assessment of raw and germinated pea (Pisum sativum L.) protein and carbohydrate by in vitro and in vivo techniques

Gloria Urbano, Ph.D. a,*, María López-Jurado, Ph.D. a, Sławomir Frejnagel, Ph.D. b, Elena Gómez-Villalva a, Jesús M. Porres, Ph.D. a, Juana Frías, Ph.D. c, Concepción Vidal-Valverde, Ph.D. c, Pilar Aranda, Ph.D. a

a Departamento de Fisiología, Instituto de Nutrición, Universidad de Granada, Granada, Spain
b Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland
c Instituto de Fermentaciones Industriales (CSIC), Madrid, Spain

Manuscript received January 1, 2004; accepted April 2, 2004.

Abstract
Objective: We assessed the effect of germinating Pisum sativum L. variant Arvense cv. EsIa for 3 and 6 d in darkness on the chemical composition and nutritive utilization of protein and carbohydrates.

Methods: Nutritional assessment of protein and carbohydrates was based on chemical analysis of raw and germinated pea flours and in vitro and in vivo rat balance methodologies.

Results: Germination caused a notable decrease in α-galactoside content and significant increases in sucrose, glucose, and fructose. The ratio of available starch to total starch increased as a consequence of processing. The content of vitamin B2 increased significantly, whereas no significant change was observed in vitamin B1 content in germinated peas. Protein digestibility assessed with an in vivo technique (apparent digestibility coefficient) or as the percentage of dialyzable nitrogen increased significantly as a result of germination in contrast to what was observed with the in vitro pH-drop methodology. Daily food intake, nitrogen absorption and balance, percentage of retained versus absorbed nitrogen, protein efficiency ratio, and the index of available carbohydrates were significantly improved by germination for 3 d and significantly decreased by germination for 6 d.

Conclusions: Germination of pea seeds for 3 d significantly improves palatability of these seeds and the nutritive utilization of protein and carbohydrates. © 2005 Elsevier Inc. All rights reserved.

Keywords: Peas (Pisum sativum L.); Germination; Nutritional assessment; Protein; Carbohydrates; In vitro; In vivo

Introduction

Peas are of great nutritional importance due to their high content of protein, complex carbohydrates, dietary fiber, minerals, vitamins, and antioxidant compounds. Although peas are widely used in animal nutrition [1], human consumption of peas is lower than that of other traditionally more accepted pulses [1,2]. Nevertheless, in recent years, the wealth of nutrients available from the pea and its beneficial functional properties have prompted increasing interest and demand for this legume for the food preparation oriented to geriatric and infant nutrition [3].

Germination is a technologic application widely used for its ability to decrease levels of antinutritional factors present in legume seeds and improve the concentration and availability of their nutrients [4,5]. However, germination is also a very active and complex metabolic process that may alter the chemical, structural, and organoleptic properties of the seed meal, thus decreasing its nutritive value [6,7].

Among the different techniques used to assess the nutritive utilization of protein, in vitro methodologies are frequently used to predict the potential digestive utilization of protein in different foods and feed ingredients [8,9]. These techniques have the advantages of simplicity, reproducibility, speed, and lower costs. Compared with in vitro techn-
niques, in vivo methodologies are expensive and time consuming and require specialized equipment and personnel; conversely, they allow the researcher to control the physiologic or pathologic status of the experimental animal and offer numerous data related to food intake and metabolic utilization that are not available when using in vitro methods. Therefore, it seems important to compare and correlate the different in vitro and in vivo methodologies, particularly when the food processing conditions are known to cause considerable changes in the chemical structure and composition of the meal, as is the case with processes such as germination [6].

This study examined the effect of germination during short (3 d) or prolonged (6 d) experimental periods on the germination process on the digestive utilization of protein, seeking to correlate an in vivo balance technique with the different in vitro methodologies, pH-drop [10] and nitrogen dialyzability [11].

**Materials and methods**

**Diets**

*Raw pea flour*

Raw pea flour (RP; *Pisum sativum* L. variant Arvense cv. Esla) was obtained from the germ plasm collection of Valladolid (Valladolid, Spain).

**Germination**

The process was carried out on a semi-pilot scale. Pea seeds, 500 g, were soaked in 2500 mL of 0.7% sodium hypochlorite solution for 30 min at room temperature. Seeds were drained, washed to neutral pH, and then soaked in distilled water for 5 h 30 min. Imbibed seeds were germinated at a pilot scale by layering them over a moist filter paper continuously watered by capillarity in a seed germinator (G-120 Snijders, Tilburg, The Netherlands) for 3 d (G3DNL) and 6 d (G6DNL) without light at 20°C and 99% relative humidity. Sprouted seeds were freeze dried and analyzed for moisture, nitrogen, crude protein, and ash content. Crude protein was calculated as nitrogen × 6.25. Insoluble nitrogen and soluble protein and non-protein nitrogen were measured according to the methodology described by Periago et al. [14].

**Determination of available soluble sugars and α-galactosides**

Analysis of glucose, fructose, sucrose, and α-galactosides (raffinose, stachyose, and verbascose) was carried out by high-performance liquid chromatography according to the method described by Frias et al. [15].

**Determination of total and available starch levels**

Total and available starch levels for samples were determined according to the method of Vidal-Valverde et al. [16] by a procedure based on total enzyme digestion of starch to glucose for 3 h and 30 min, respectively, and starch content was calculated by multiplying the glucose content by 0.9.

**Determination of vitamins B1 and B2**

A single extraction procedure for vitamins B1 and B2 was carried out according to the method of Vidal-Valverde et al. [17]. These vitamins were quantified by high-performance liquid chromatography as described previously [18].

**Trypsin inhibitor activity determination**

Trypsin inhibitor activity (TIA) was determined as described in Vidal-Valverde et al. [17].

**Sodium dodecylsulfate polyacrylamide gel electrophoresis**

Proteins were extracted from the pea flours with 50 mM phosphate buffer, pH 7.8, containing 1% sodium dodecylsulfate (SDS) and 1% β-mercaptoethanol. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli [19]. The final concentration of acrylamide in the running gel was 15%. Gels were fixed and stained with 0.2% Coomassie brilliant blue R-250 in methanol:acetic acid:water (5:4:1 v/v/v). Equal amounts of nitrogen were loaded in each lane. The mixture of molecular weight markers (Merck, Darmstadt, Germany) consisted of cytochrome C (12.3 kDa), myoglobin (16.9 kDa), carboanhydrase (30 kDa), ovalbumin (42.7 kDa), albumin (66.25 kDa), and ovotransferrin (78 kDa).

**Endoprotease activity assay in gel**

Pea flours (2 g) were extracted with 20 mL of 50 mM sodium acetate (pH 4.7) containing 1 mM ethylene-diaminetetra-acetic acid and 2 mM cysteine for 1 h at 4°C. The procedure described by Jameel et al. [20] was used to detect endoprotease activity in SDS-PAGE (12%) copolymerized with 0.20% (w/v) gelatin. Equal amounts of nitrogen (12.5 μg) were loaded in each lane. Gels were run at constant current (12 mA) for 4.5 h at 4°C. After electrophoresis, SDS was removed by incubating gels in 2% Triton X-100 for 60 min at room temperature. Gels were then incubated overnight at 45°C in 50 mM sodium acetate (pH 4.94), 2 mM
cysteine, and 1% Triton X-100 or in 50 mM phosphate buffer (pH 6.75), 2 mM cysteine, and 1% Triton X-100. Endoproteolytic activities were developed by staining the gels with 0.2% Coomassie brilliant blue R-250 in methanol: acetic acid:water (5:4:1 v/v/v) and appeared as white regions against a dark blue background.

**In vitro protein digestibility**

Two in vitro methods were tested. The pH-drop multienzyme system described by Hsu et al. [10] and the in vitro method of Miller et al. [11] that uses a pepsin digestion period of 2 h followed by pH equilibration and pancreatin digestion for another 2 h coupled with equilibrium dialysis (Molecular Weight Cut Off [MWCO] 12 000 Da). Dialyzable nitrogen was expressed as a percentage of the total amount of nitrogen in each digestion vial, assuming that the dialyzable component had equilibrated across the dialysis membrane by the time the dialysis bag was removed at the end of the digestion period.

**Biological methods**

**Experimental design and diet**

We used a biological balance technique that recorded changes in body weight and food intake and then calculated nitrogen intake and fecal and urinary nitrogen excretion. Three 10-d experiments, in which raw or germinated peas were the only food source, were carried out. During the first 3 d of experiments, rats were allowed to adapt to the diet and experimental conditions, and the main experimental period comprised the next 7 d, during which body weight and food intake were recorded and feces and urine were collected for analysis.

**Animals**

In each experiment we used 10 young, albino, Wistar rats (five male and five female). Growing animals (recently weaned), with an initial body weight of 100 ± 1.5 g, were housed from day 0 of the experiment in individual stainless-steel metabolic cages designed for the separate collection of feces and urine; cages were located in a room with a 12-h light, 12-h dark period, a temperature of 21 ± 2°C, and an appropriate ventilation system. Throughout the experimental period all rats had free access to doubly distilled water, and the diet was consumed ad libitum. At the end of the experimental period, animals were anesthetized with CO2 and killed by decapitation. The liver and the longissimus dorsi muscle were collected for analysis. Rats were handled at all times in accordance with current European regulations regarding laboratory animals.

**Biological indices**

The following indices and parameters were determined for each group according to the formulas given below:

intake (expressed as dry weight), body weight gain, protein efficiency ratio (weight gain in grams per day/protein intake in grams per day), index of available carbohydrates (weight gain in grams per day/total intake of available carbohydrates in grams per day), apparent digestibility coefficient (ADC), nitrogen retention (nitrogen balance), and percentage of nitrogen retention versus nitrogen absorption (%R/A).

\[
\text{ADC} = \frac{[I - (F + U)]}{I} \times 100
\]

where \( I \) represents intake, \( F \) represents fecal excretion, and \( U \) represents urinary excretion

**Statistics**

Data were subjected to multifactor analysis of variance with Statgraphics Statistical Graphics 5.0 (Statistical Graphics Corporation, Rockville, MD, USA).

**Results**

The RP assayed had an average total nitrogen content of 4.05 ± 0.01 g of nitrogen/100 g of DM, 14.1% of which corresponded to soluble non-protein nitrogen, 75.8% of which corresponded to soluble protein nitrogen, and the remaining 10.1% was insoluble at the basic pH conditions used for extraction (Table 1). Total nitrogen content increased gradually with increasing germination periods, resulting in significant differences among the three pea flours tested. Insoluble nitrogen content decreased significantly during the germination period (32% and 19% decreases in pea flour germinated for 3 d and 6 d, respectively, versus RP). The content of soluble protein nitrogen was significantly decreased by 6 d of germination, with no significant differences being found between 3-d germinated flour and RP. Soluble non-protein nitrogen increased progressively with increasing germination periods (1.7-fold and 2.8-fold in G3DNL and G6DNL, respectively, versus RP).

Fig. 1A shows changes in the SDS-PAGE pattern of proteins from the RP and germinated pea flour in the present study. RP exhibited high-density bands corresponding to the storage proteins convicilin (70 kDa), vicilin (12 to 30 to 35 kDa), α-legumin (39 to 40 kDa), and β-legumin (20 to 25 kDa). Germination for 3 or 6 d led to severe hydrolysis of pea storage proteins, which was reflected in the disappearance or loss of density from the main polypeptide bands and the appearance of a smear of lower-molecular-weight polypeptides that was particularly evident after 6 d of germination. Fig. 1B shows changes in the protease activity of pea flours as a result of germination. Hardly any activity was detected in the RP, whereas a significant increase in endoproteolytic activity could be observed for the pea flours germinated for 3 and 6 d.

The pH of RP was 6.45, which decreased to 6.27 and 6.05
in the G3DNL and G6DNL diets, respectively. Conversely, the titratable acidity expressed as milliequivalents of NaOH/100 g of DM increased from 3.37 in the RP diet to 7.04 and 10.14 in the G3DNL and G6DNL diets, respectively.

Total /H9251-galactoside content was significantly decreased by the germination process (94.6% and 98.4% in G3DNL and G6DNL, respectively). Decreases in raffinose and stachyose content were 63% and 97%, respectively, in the G3DNL diet and 86% and 100%, respectively, in the G6DNL diet compared with the RP diet. No detectable levels of verbascose were observed after either germination period.

The content of sucrose increased by 1.5- to 2-fold in the G3DNL and G6DNL diets compared with RP. Under the experimental conditions of the present study, levels of fructose and glucose, monosaccharides that were not detected in the RP diet, were 0.09 ± 0.01 and 0.07 ± 0.01, respectively.

Table 1

<table>
<thead>
<tr>
<th>Diets</th>
<th>RP</th>
<th>G3DNL</th>
<th>G6DNL</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (g/100 g of DM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.31 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.50 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insoluble</td>
<td>0.41 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28 ± 0.02&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.33 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soluble protein</td>
<td>3.07 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.08 ± 0.02&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.70 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soluble non-protein</td>
<td>0.57 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.95 ± 0.01&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.57 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Available soluble sugars (g/100 g of DM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.12 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.34 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.73 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.51 ± 0.03&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.05 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total available soluble sugars</td>
<td>1.73 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.65 ± 0.03&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.52 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Starch (g/100 g of DM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>42.65 ± 0.58&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39.73 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.49 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Available</td>
<td>36.70 ± 1.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.81 ± 0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.94 ± 0.49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Resistant</td>
<td>3.95 ± 0.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.93 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.55 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total available sugars</td>
<td>40.43 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.45 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.46 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamins (mg/100 g of DM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B1</td>
<td>0.73 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin B2</td>
<td>0.15 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-Galactosides (g/100 g of DM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raffinose</td>
<td>0.56 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.21 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stachyose</td>
<td>2.24 ± 0.06&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.07 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Verbascose</td>
<td>2.39 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>5.19 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.28 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Trypsin inhibitor activity (TIU/mg of DM)</td>
<td>8.69 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.74 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.65 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

DM, dry matter; G3DNL, peas germinated for 3 d in darkness; G6DNL, peas germinated for 6 d in darkness N, nitrogen; ND, not detected; RP, raw peas
* Values are mean ± standard deviation (n = 3). The same superscript letter in the same row indicated no significant difference (P < 0.05).

Fig. 1. (A) Sodium dodecylsulfate polyacrylamide gel electrophoresis of proteins extracted from raw and germinated pea flours. An equal amount of legume nitrogen (1.45 g) was loaded in each lane. (B) Protease staining after sodium dodecylsulfate polyacrylamide gels copolymerized with 0.20% gelatin; 12.5 μg of nitrogen was loaded per well. The gel was incubated in 50 mM of phosphate buffer (pH 6.75), 2 mM cysteine, and 1% Triton X-100 overnight at 45°C. Both gels are representative of three independent analyses. Lane 1, raw pea flour; lane 2, flour made from peas germinated in darkness for 3 d; lane 3, flour made from peas germinated in darkness for 6 d. M, molecular weight markers.
RP, increased significantly to 0.05 and 0.09 g/100 g of DM, respectively, in the G3DNL diet and to 0.12 and 0.34 g/100 g of DM, respectively, in the G6DNL diet. RP had an average total starch content of 42.65 ± 0.58 g/100 g of DM, 90.7% of which corresponded to available starch and 9.3% of which corresponded to resistant starch. Germination resulted in a significant decrease of total and resistant starch content, thus increasing the proportion of available starch with respect to total starch (4.9% and 6.8% increases in G3DNL and G6DNL, respectively).

Germination caused a significant increase in levels of vitamin B2 of the two germinated pea flours tested (80% and 87% increments for 3 and 6 d of germination, respectively). Vitamin B1 content was not affected by the germinating conditions and remained similar to that of RP.

No difference for TIA was observed as a result of germination for 3 d when compared with RP but did increase significantly after 6 d.

Food intake, expressed as grams per rat per day, increased significantly in animals fed the diet of peas germinated for 3 d compared with animals fed RP and decreased significantly in animals fed the diet of peas germinated for 6 d in comparison with the former two groups (Table 2). Intake of protein, total available sugars, and vitamins B1 and B2 was related to food intake and the amount of nutrients present in the diet. Therefore, the highest intake of protein, total available sugars, and vitamins B1 and B2 was found in animals fed the G3DNL diet.

Intake of α-galactosides decreased significantly with longer germination periods, whereas the intake of total starch, available sugars, and TIA increased significantly in the group fed the G3DNL diet and decreased significantly in the group fed the G6DNL diet with respect to the group fed the RP diet.

In vitro protein digestibility assessed by the pH-drop technique decreased slightly ($P < 0.05$) with longer germination periods compared with the RP diet (2.3% and 7.9% in G3DNL and G6DNL, respectively; Fig. 2). In vitro protein digestibility, calculated as the percentage of dialyzable nitrogen, was significantly improved as a result of germination (from 59.5% in the RP diet to 67.2% and 70.9% in the G3DNL and G6DNL diets, respectively), and there were no significant differences between germination periods. In vivo protein digestibility assessed by the ADC increased significantly in groups fed germinated pea diets compared with the group fed the RP diet, and there were no differences between germinated pea groups.

Nitrogen absorption in absolute values was significantly higher in the group fed the diet of peas germinated for 3 d than in those fed the other two diets (Table 3). Metabolic utilization of nitrogen assessed as a balance or %R/A was higher among rats fed the G3DNL diet than among those

### Table 2

<table>
<thead>
<tr>
<th>Nutrient and antinutritional factor intake*</th>
<th>Diets</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RP</td>
<td>G3DNL</td>
<td>G6DNL</td>
</tr>
<tr>
<td>Intake (g of dry matter)</td>
<td>10.72 ± 0.20b</td>
<td>14.23 ± 0.42c</td>
<td>8.55 ± 0.25a</td>
</tr>
<tr>
<td>Intake (g/100 g of rat body weight per day)</td>
<td>9.08 ± 0.24a</td>
<td>10.19 ± 0.29b</td>
<td>8.52 ± 0.19a</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>2.71 ± 0.05b</td>
<td>3.83 ± 0.11c</td>
<td>2.41 ± 0.06a</td>
</tr>
<tr>
<td>Available soluble sugars (g)</td>
<td>0.19 ± 0.00a</td>
<td>0.38 ± 0.01c</td>
<td>0.30 ± 0.01b</td>
</tr>
<tr>
<td>Total starch (g)</td>
<td>4.57 ± 0.09b</td>
<td>5.65 ± 0.17c</td>
<td>3.21 ± 0.09a</td>
</tr>
<tr>
<td>Resistant starch (g)</td>
<td>0.42 ± 0.01b</td>
<td>0.27 ± 0.01b</td>
<td>0.22 ± 0.01a</td>
</tr>
<tr>
<td>Available starch (g)</td>
<td>4.15 ± 0.08b</td>
<td>5.38 ± 0.16c</td>
<td>2.99 ± 0.09a</td>
</tr>
<tr>
<td>Total available sugars (g)</td>
<td>4.33 ± 0.08b</td>
<td>5.76 ± 0.17c</td>
<td>3.29 ± 0.10a</td>
</tr>
<tr>
<td>Vitamin B1 (mg)</td>
<td>0.08 ± 0.00b</td>
<td>0.11 ± 0.00b</td>
<td>0.06 ± 0.00a</td>
</tr>
<tr>
<td>Vitamin B2 (mg)</td>
<td>0.02 ± 0.00c</td>
<td>0.04 ± 0.00b</td>
<td>0.02 ± 0.00a</td>
</tr>
<tr>
<td>Total α-galactosides (g)</td>
<td>0.56 ± 0.01b</td>
<td>0.04 ± 0.00b</td>
<td>0.01 ± 0.00a</td>
</tr>
<tr>
<td>Trypsin inhibitor activity (TIU)</td>
<td>93 148 ± 1775b</td>
<td>124 388 ± 3638b</td>
<td>82536 ± 2396b</td>
</tr>
</tbody>
</table>

G3DNL, peas germinated for 3 d in darkness; G6DNL, peas germinated for 6 d in darkness; RP, raw peas

* Values are mean ± standard error of the mean ($n = 10$) per rat per day. The same superscript letter in the same row indicated no significant difference ($P < 0.05$).
fed the RP and G6DNL diets, and there was no significant differences between the latter experimental groups.

Weight gain (grams per day), growth efficiency coefficient (protein efficiency ratio), and index of available carbohydrates were significantly improved among animals fed the diet of peas germinated for 3 d compared with those that fed the diet of RP or peas germinated for 6 d. There were no differences between the latter two diets (Fig. 3).

No significant differences were found in water or nitrogen content in the liver and longissimus dorsi muscle of the animals given the different diets (Fig. 4).

## Discussion

Total nitrogen content of the peas used for the present study was within the range of values found in the literature [21]. The insoluble nitrogen residue has been described as proceeding from non-covalent interactions or from disulfide bonds between different proteins [21]. The decrease in the level of insoluble nitrogen as a result of the germination process found in the present experiment was similar to that reported by Wanasundara et al. [22] in flax seeds.

Under our experimental conditions, the drastic decrease in α-galactoside content was similar to that described by other investigators in germinated legume seeds such as cowpea [23] and lentil [24]. Monteze Guimaraes et al. [25] suggested that germination contributes to the hydrolysis of galacto-oligosaccharides into mono- or disaccharides that are used as an energy source or hydrocarbon skeleton for the biosynthesis of macromolecules necessary for embryonic development during the early stages of germination. The hydrolysis of galacto-oligosaccharides is catalyzed by α-galactosidase. This enzyme is not found in appreciable amounts in resting seeds and becomes active at the onset of germination. Nnanna and Phillips [23] and Monteze Guimaraes et al. [25] observed that α-galactosidase activity reach maximum values within 3 d of germination in cowpeas; under our experimental conditions, the enzyme also remained active after 3 d, as demonstrated by the decreased α-galactoside content (71.4%) from day 3 to day 6 of germination.

Total starch content in peas underwent a significant decrease during germination, similar to that observed after germination of cowpea [6] and lentils [24]. α-Amylase inhibitors in peas (0 to 16.8 IU/kg of DM) [4] can interfere with starch digestion. However, Alonso et al. [4] and Sathe et al. [26] reported a significant decrease in the levels of α-amylase inhibitors during germination.

As a result of the germination process, legume seeds undergo considerable metabolic changes in their carbohydrate storage, which includes starch and oligosaccharides of the raffinose family that are hydrolyzed, causing a concomitant increase in levels of reducing sugars [27]. This is the reason for the great increment in available soluble sugars,
mainly sucrose, observed after 6 d of germination. Our results are in agreement with those of Balasaraswathi and Sadasivam [28] who described a gradual increase in the amount of reducing sugars from day 0 to day 5 in sunflower seeds and with those of Frias et al. [24] who observed a significant increase in levels of sucrose after 4 d of germination of lentils. These mono- or disaccharides are more easily absorbed by the small intestine of monogastrics.

The proportion of available versus total starch improved as a consequence of the germination process. These results are in agreement with those described by Vidal-Valverde and Frias [27] for lentils and indicate that starch could be better used nutritionally. Reddy et al. [29] suggested that germination of legumes alters their starch properties by increasing gelatinization capacity and solubility. These changes improve accessibility of the starch granule to digestive enzymes, thus improving its digestibility.

Under our experimental conditions, we observed a considerable increment of riboflavin content after 3 d of germination (1.8-fold), whereas no further increment was observed after 6 d, in agreement with what has been described in the literature for peas and lentils [30,31]. The thiamin content of peas was not modified after a prolonged germination period of 6 d, similarly to the findings of Prodanov et al. [31] in lentils and in contrast to what has been described for peas by Sierra and Vidal-Valverde [30] who found decreased levels of thiamin during the germination period.

The TIA content of RP used for the present experiment was within the range of values found in the literature for the pea [21]. The influence of germination on TIA depends on the type of legume and on the conditions and duration of the germination process [32]. Thus, some investigators have found significant decreases in the TIA content of germinated beans [33], whereas others have found no substantial variations in TIA levels of beans [34] or lentils [35] after germination. Under our experimental conditions, the TIA of peas was not affected by 3 d of germination but increased slightly with 6 d of germination due to concentration caused by the loss of DM.

Intake

The duration of the germination period affected the daily food intake of the experimental animals. The increase in food intake by the animals fed the diet of 3-d germinated peas may have been due to the decreased α-galactoside content and to an increment in the amount of palatable carbohydrates such as the available sugars sucrose and fructose derived from the hydrolysis of α-galactosides and the predigestion of starch. In addition, supplementation of 4% olive oil to the diet may have improved its aroma and flavor. The significant decrease in food intake after 6 d of germination (G6DNL), even compared with the RP diet, could be attributed to changes in food texture or properties such as hardness, elasticity, or chewiness [6,36], to the appearance of compounds that may cause the loss of the peas’ organoleptic characteristics [23,37], to changes in the acidity of the diet, and to the appearance of free non-protein amino acids with neurotoxic effects or anorexigenic compounds that could affect the nutritional value and acceptability of the food [7,38,39]. Under our experimental conditions, the influence of these compounds that tended to decrease the palatability of the G6DNL diet was greater than that of the increase in soluble palatable sugars and of the total absence of α-galactoside oligosaccharides.

In vitro and in vivo protein digestibility

Results obtained with the muli-enzyme technique of pH-drop seem to indicate that the RP diet had the highest protein digestibility, followed by the G3DNL diet (2.2% decrease) and the G6DNL diet (7.9% decrease). These results could be due to the appearance of peptides that are resistant to proteolytic enzymes [34]. Further, the pH-drop technique did not take into account the higher titratable
acidity of germinated diets, which is caused by predigestion of storage protein that takes place during germination and is reflected in the observed decrease of soluble protein nitrogen in addition to the increment in the levels of soluble non-protein nitrogen composed of free amino acids, nucleic acids, purine and pyrimidine bases, polyamines, alkaloids, and small peptides. These results agree with the decreased density of polypeptide bands corresponding to the seed storage proteins that was observed in the protein gel as a result of increasing germination periods and the increment in endoprotease activity of the germinated pea flours. The greater buffering capacity of the germinated pea diets interfered with the pH-drop and would be responsible for the lower protein digestibility value obtained. In contrast, the percentage of dialyzable nitrogen showed a higher digestibility of legume protein after 3 and 6 d of germination and maintained a good correlation with the enhanced hydrolysis of legume storage proteins caused by a higher endoprotease activity present in the germinated pea flours, which was active over a wide pH range (4.79 to 6.79), and the increased levels of soluble non-protein nitrogen. These results showed a trend similar to those obtained by the in vivo technique (ADC). Predigestion of pea protein caused by germination and the decrease in tannin content [40] achieved by the process could be responsible for the significant improvement in the digestive utilization of nitrogen because TIA was not affected. Nevertheless, the in vivo method showed a much lower increase in digestive utilization when compared with the in vitro dialyzability system assayed, and it produced a much higher value (Fig. 2), overall indicating that the rat’s digestive system is well able to cope with the protein in a non-germinated pea flour diet.

In vivo digestive utilization of protein from the peas used in the present experiment was high and within the range of values found in the literature [41], similar to that reported for faba beans [42], and higher than that of lentils [43].

Metabolic utilization of protein and carbohydrates

The highest nutritive utilization of nitrogen was obtained for the group fed the diet of 3-d germinated peas versus the groups fed the RP and 6-d germinated pea diets. This is due to the higher daily food intake but also to better metabolic utilization of nitrogen (%R/A) obtained with the G3DNL diet. All these factors produced a higher level of nitrogen retention with which to respond to growth and structural repair necessities because weight gain was significantly higher in this experimental group than in the others.

The protein efficiency ratio, which measures the relation between weight gain and protein consumed, was significantly higher among rats given peas germinated for 3 d than among rats fed raw or 6-d germinated peas. Within the G3DNL group, the higher food intake, the good metabolic utilization of protein, and the better nutritive utilization of carbohydrates would be responsible for improved nitrogen %R/A. Animals did not use protein to fulfill energetic needs as was the case with the other two diets. Further, peas germinated for 3 d without light provided an amount of vitamins B1 and B2 that was higher than the nutrient requirements of the growing rat [44] and that contributed to the improved nutritive utilization of carbohydrates.

Germination for 6 d caused a sharp decrease in food intake and, hence, in net absorption and retention of nitrogen. In addition, it did not improve the metabolic utilization of protein or carbohydrates, as shown by the percentage of retained versus absorbed nitrogen, protein efficiency ratio, and index of available carbohydrates. The caloric malnutrition generated by decreased daily food intake in the group of animals fed the G6DNL diet may have been responsible for these findings.

The nitrogen contents in the muscle and liver of the animals given the diets of raw peas and of germinated peas were similar in all cases and might have been related to the size of the tissues. This would indicate that, for rats in the range of body weights used in the present study, variations will appear in the size of the different tissues but not in their composition.

In conclusion, we recommend short germination periods of 3 d without light, which are optimal for improving the organoleptic and nutritional properties of peas. These germination periods are sufficient to produce an appreciable decrease in the factors responsible for flatulence, thus increasing food intake and improving the utilization of protein and available carbohydrates. Under our experimental conditions, the percentage of dialyzable nitrogen was a better index to predict the potential digestibility of protein because it more accurately reproduced the physiologic process of digestion at acidic pH with pepsin followed by pH adjustment and digestion with pancreatic enzymes at alkaline pH that takes place in the animal. Nevertheless, digestive utilization of protein was found to be more efficient in the digestive tract of the animal. The multienzyme technique of pH-drop may be useful to assess the digestibility of proteins that have not been predigested because this would release free amino acids or peptides with buffering capacity that interfere with the pH-drop on which this methodology is based. Compared with these in vitro methodologies, the biological assay is more complete in providing information about food intake, weight gain, and the metabolic utilization of nitrogen by the animals as complementary results to the digestive utilization of protein.

Acknowledgments

The authors thank Rosa Jiménez and Dr. Carmen Lluch Plá for skillful technical assistance and literature support.
References


