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Effects of germination on the composition and nutritive value of proteins in *Pisum sativum*, L

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Abstract

The effects of germination for 2, 4 or 6 d, with and without light, on the proteolytic activity, the contents of soluble protein and non protein nitrogen, and the amount of available starch of *Pisum sativum*, L, as well as their nutritive utilisation by growing rats were studied. Food intake increased significantly when the peas were germinated for 2 or 4 d. This improvement was correlated with the reduction of factors responsible for flatulence. Digestive utilization of nitrogen was similar (among all the groups fed germinated-pea flour) to raw-pea flour. The values for nitrogen balance, percentage of retained to absorbed nitrogen, protein efficiency ratio, and index of available carbohydrates were significantly higher among the animals that consumed peas allowed to germinate for 2 or 4 d than among the animals given the raw-pea or 6-day-germinated pea diets. We conclude that germination of peas for 2 d would be sufficient to significantly improve the palatability and nutritive utilisation of protein and carbohydrates from *Pisum sativum*, L. The presence or absence of light during the germination process did not affect the results achieved.

Keywords: *Pisum sativum*; Germination time; Light; Protein; Nutritive value; Growing rats

1. Introduction

The pea (*Pisum* sativum, L.) is a legume with great nutritional potential due to its high protein content and it has been suggested as an alternative protein source to soybean in countries where the former legume is not a native crop, or in situations where soybean cannot be used due to allergic reactions or intolerances (Davidsson, Dimitriou, Walczyk, & Hurrel, 2001). However, the potential benefits might be limited by the presence of antinutritional factors, including trypsin inhibitor activity (TIA) (Urbano et al., 2003; Vidal-Valverde et al., 2003), phytic acid, and α-galactoside oligosaccharides (Urbano et al., 2003; Vidal-Valverde et al., 2002). With the aim of improving the nutritive value of legumes, preparation techniques have been developed to significantly raise the bioavailability of their nutrients. Such techniques include germination, a complex metabolic process during which the lipids, carbohydrates, and storage proteins within the seed are broken down in order to obtain the energy and amino acids necessary for the plant's development (Ferreira, Melo, & Teixeira, 1995; Jachmanian, Perifanova-Nemska, Grompone, & Mukherjee, 1995; Podestá & Plaxton, 1994; Ziegler, 1995). The presence of light advances the metabolic changes that take place during the different stages of germination, and so it is necessary to evaluate how these changes influence the bioavailability of essential nutrients. Germination also affects the

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antinutritional factors of the legume, although there is some disagreement as to the ultimate consequences, because the effect depends on the type of legume and on the conditions and duration of the germinating process (Savelkoul, Van der Poel, & Tamminga, 1992). Thus, various authors (Ibrahim, Habiba, Shatta, & Embaby, 2002; Mbithi-Mwikya, Van Camp, Rodrıguez, & Huyghebaert, 2001) have found significant reductions in TIA content, while others (Chang & Harrold, 1988; Frias, Diaz-Pollan, Hedley, & Vidal-Valverde, 1995) found no substantial variations in TIA levels in beans and lentils after germination periods of up to 6 d. Batra, Vasishta, and Dhindsa (1986), on the other hand, observed that the TIA content in certain varieties of pigeon pea rises slightly after 3 d germination but then falls sharply between 3 and 6 d germination.

With regard to the functionality of the nutrients, Nnanna, Phillips, McWatters, and Hung (1990), Bau Uwaegbute, Iroegbu, and Eke (2000), Uwaegbute et al. (2000) reported that long germination periods have a negative effect on the organoleptic properties of legume seeds. Mbithi-Mwikya, Van Camp, Yiru, and Huyghebaert (2000) reported that germination for periods exceeding 48 h produces considerable losses of dry matter through respiration.

The objective of this study was to establish optimal conditions of light and germination period to decrease the dietary factors that could negatively affect the intake and nutritive utilization of protein, with the aim of obtaining pea flours with improved functional value for use by the food industry.

2. Materials and methods

2.1. Legume

Pisum sativum, L. var. Arvense cv. Esla was from the germplasm collection of Valladolid (Spain). The pea seeds were crushed, milled to a fine powder (0.18 mm sieve), and then lyophilized.

2.2. Germination

The process was carried out in a semi-pilot scale. 500 g of pea seeds were soaked in 2500 ml of 0.7 g/l sodium hypochlorite solution for 30 min at room temperature. Seeds were then drained and washed to neutral pH, and then soaked in distilled water for 5 h 30 min. Finally, imbibed seeds were germinated, on a pilot scale, by layering them over a moistened filter paper continuously watered by capillarity in a seed germinator (G-120 Snijders, The Netherlands) for 2, 4 or 6 d with or without light (G2DL, G2DNL, G4DL, G4DNL, G6DL, G6DNL) at 20 °C, and 99% relative humidity. Sprouts were freeze-dried and ground to pass a 0.18 mm sieve for chemical and biological analysis. The periods of germination were scheduled with the aim of making the time needed to prepare the pea diets after sprouting to be the same in all the experimental groups, thus minimizing the variability caused by different storage periods. All the experimental diets were supplemented with 40 g/kg olive oil prior to being fed to the animals in powder form.

2.3. Analytical methods

2.3.1. Chemical analyses

The moisture content of the different pea diets was determined by drying to constant weight in an oven at 105 ± 1 °C. Total nitrogen was determined according to Kjeldahl's method. Crude protein was calculated as $N \times 6.25$. Insoluble nitrogen and soluble protein and non-protein nitrogen were measured using the methodology described by Periago, Ros, Martínez, and Rincón (1996). The different pea flours (0.5 g) were thrice extracted with 10 ml of 0.02 N NaOH during 60 min and the three extracts pooled. Insoluble material was removed by centrifugation at 3000 rpm for 20 min. The supernatant was mixed with 20 ml of 300 g/l TCA and the mixture stirred for 15 min at 4 °C. Protein was removed by centrifugation at 3000 rpm for 15 min. Total nitrogen was measured in the insoluble material (insoluble nitrogen), protein pellet (protein nitrogen) and supernatant (non protein nitrogen) using the Kjeldahl method. Available starch was determined after extraction and hydrolysis of the sample with amyloglucosidase for 30 min (Vidal-Valverde & Frias, 1992).

2.3.2. SDS-PAGE

Proteins were extracted from the pea flour with 50 mM phosphate buffer, pH 7.8, containing 1% sodium dodecyl sulphate (SDS) and 1% β-mercaptoethanol (1:72 w/v) for two consecutive times during 60 min. The extracts were pooled and centrifuged at 3000 rpm for 30 min, and the supernatant collected and assayed for nitrogen content. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was done according to the method of Laemli (1970). The final concentration of acrylamide in the separating gel was 13%. Equal amounts of nitrogen (2.84 μg) were loaded in each lane. The gels were fixed and stained with 0.2% Coomassie brilliant blue R-250 in methanol–acetic acid–water (5:4:1 v/v/v). The mixture of molecular weight markers (Merck) consisted of cytochrome C (12.3 kDa), myoglobin (16.9 kDa), carboanhydrase (30 kDa), ovoalbumin (42.7 kDa), albumin (66.25 kDa) and ovotransferrin (78 kDa).

2.3.3. Endoprotease activity assay in gel

Pea flours (2 g) were extracted with 20 ml of 50 mM Na acetate pH 4.7 containing 1 mM EDTA and 2 mM

cysteine for 1 h at 4 °C. The procedure described by Jameel, Reddy, Rhodes, and McFadden (1984) was used to detect endoprotease activity in SDS–PAGE (12%) co-polymerized with 0.15% (w/v) gelatin. Equal amounts of nitrogen (12.5 μg) were loaded in each lane. Gels were run at constant current (10 mA) during 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 Na acetate, pH 4.94/2 mM cysteine/1% Triton X-100, or in 50 mM phosphate buffer, pH 6.75/2 mM cysteine/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.

2.3.4. Biological methods

We used a biological balance technique, recording changes in body weight and food intake and then calculating nitrogen intake and faecal and urinary nitrogen excretion. Seven ten-day experiments, in which raw or germinated pea flour was the only food source, were carried out. During the first 3 d of experiments, the rats were allowed to adapt to the diet and experimental conditions, and the main experimental period was the next 7 d, during which body weight and food intake were recorded and faeces and urine were collected for analysis.

For each experimental diet, 10 young albino Wistar rats (a total of 70 animals) were used. The growing animals (recently weaned), with an initial body weight of 75.9 ± 1.0 g, were housed from day 0 of the experiment in individual stainless steel metabolic cages designed to minimize food spoilage and avoid mixing of food with drinking water, and for separate collection of faeces and urine; the cages were located in a room with a 12 h light/dark period, at a temperature of 21 ± 2 °C, fitted with an appropriate ventilation system. Throughout the experimental period, all rats had free access to double distilled water and the diet was consumed ad libitum. At the end of the experimental period the animals were anaesthetized with CO_2 and killed by decapitation. The liver and the *Longissimus dorsi* muscle were collected for analysis. All experiments were undertaken according to the Directional Guides Related to Animal Housing and Care (ECC, 1986).

The following indices and parameters were determined for each group according to the formulas given below: intake expressed as dry weight in grammes per day (calculated by weighing the amounts of diet given, refused and spilled, refusals were measured daily), total available sugar intake (sum of available starch and available soluble sugar intake), body weight gain, protein efficiency ratio (PER; weight gain in grammes per day/protein intake in grammes per day); index of available carbohydrates (IAV; increase in body weight in grammes per day/intake of total available carbohydrates in grammes per day); apparent digestibility coefficient (ADC) (1); nitrogen retention (nitrogen balance) (2), and percent nitrogen retention/nitrogen absorption (%R/A) (3):

$$\mathrm{ADC} = 100 \times (I - F)/I, \tag{1}$$

$$\mathrm{Balance} = I - (F + U), \tag{2}$$

$$\%R/A = 100 \times [I - (F + U)]/(I - F), \tag{3}$$

where I is the intake, F the faecal excretion, and U is the urinary excretion.

The intake of available soluble sugars, vitamin B_1, vitamin B_2, TIA, α-galactosides, and inositol phosphates, from raw and germinated pea flour, was been calculated using the composition data for these same flours previously published by Vidal-Valverde, Pascual-Montaner, Diaz-Pollan, Vicente, and Frias (1998) and Vidal-Valverde et al. (2002).

2.4. Statistical analyses

One-way analysis of variance was applied to the data by the use of Statgraphic Statistical Graphics 2.1 System Software (Statistical Graphics Corporation, Rockville, MD, USA). Differences between means were compared with Tukey's HSD test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Chemical analyses

Table 1 describes the concentration of total nitrogen found in the samples of pea flour, both raw and after germination for periods of 2, 4, or 6 d, in the presence of light and in darkness. The mean value for raw-pea flour was 43.4 ± 0.2 g N/kg DM, of which 14.8% corresponded to soluble non-protein nitrogen and 75.4% to soluble protein nitrogen, while the other 9.6% remained insoluble under the basic pH conditions utilised for nitrogen extraction. The concentration of total nitrogen was similar in all the samples except after 6 d germination, when there was a slight fall (of 3.21%). The content of insoluble nitrogen fell by 35% when the peas were germinated for 2 d (G2DNL, G2DL), and fell by 50% when the germination period was 4 d (G4DNL, G4DL) or 6 d (G6DNL, G6DL). The soluble protein nitrogen content decreased steadily with longer periods of germination, with no significant differences measured between the presence and absence of light (G2DNL 2.99%, G2DL 3.24%), (G4DNL 7.1%, G4DL 6.7%), (G6DNL 12.6%, G6DL 13.2%). The quantity of soluble non-protein nitrogen increased progressively with longer periods of

Table 1
Chemical composition of raw and germinated pea flours

	Total nitrogen (g/kg DM)	Insoluble nitrogen (g/kg DM)	Protein nitrogen (g/kg DM)	Non-protein nitrogen (g/kg DM)	Available starch (g/kg DM)
Raw pea flour germination	43.4 ± 0.3[a]	4.16 ± 0.1[a]	32.7 ± 0.2[a]	6.45 ± 0.3[a]	386 ± 7.7[a]
2 d					
No light	43.3 ± 0.3[a]	2.69 ± 0.1[b]	31.7 ± 0.3[b]	8.60 ± 0.2[b]	389 ± 7.8[a]
zLight	43.4 ± 0.2[a]	2.70 ± 0.2[b]	31.6 ± 0.3[b]	8.59 ± 0.3[b]	389 ± 7.9[a]
4 d					
No light	43.4 ± 0.2[a]	2.08 ± 0.2[c]	29.5 ± 0.3[c]	11.8 ± 0.3[c]	372;± 7.4[a]
Light	43.4 ± 0.3[a]	2.09 ± 0.2[c]	29.5 ± 0.2[c]	11.5 ± 0.2[c]	374 ± 7.3[a]
6 d					
No light	42.0 ± 0.3[b]	2.10 ± 0.2[c]	25.8 ± 0.2[d]	13.8 ± 0.4[d]	341 ± 6.8[b]
Light	42.0 ± 0.3[b]	2.09 ± 0.2[c]	25.6 ± 0.3[d]	14.0 ± 0.4[d]	348 ± 6.9[b]

[a–d] Values are means ± SEM ($n = 3$). Different superscripts within the same column indicate significant differences $P < 0.05$.

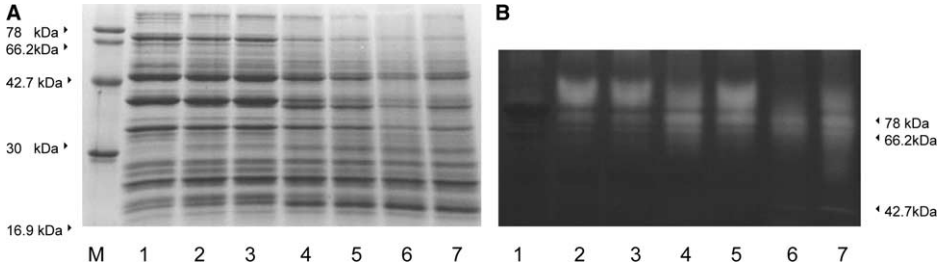

Fig. 1. SDS–PAGE and protease staining of protein extracts from raw and germinated pea flours. (a) SDS–PAGE of the proteins extracted from raw and germinated pea flours. An equal amount of nitrogen (2.84 μg) was loaded in each lane. M, molecular weight markers; 1, RP (raw pea); 2, G2DNL (germination for 2 d without light); 3, G2DL (germination for 2 d with light); 4, G4DNL (germination for 4 d without light); 5, G4DL (germination for 4 d with light); 6, G6DNL (germination for 6 d without light); 7, G6DL (germination for 6 d with light). (b) Protease staining following SDS–PAGE in gels co-polymerized with 0.15% gelatin. 12.5 μg of nitrogen were loaded per well. The gel was incubated in 50mM phosphate buffer pH 6.75/2 mM cysteine/1% Triton X-100 overnight at 45 °C. 1, RP, 2, G2DNL, 3, G2DL, 4, G4DNL, 5, G4DL, 6, G6DNL, 7, G6DL. Both gels are representative of three independent analyses.

germination, in parallel with the decrease in the insoluble and protein nitrogen.

The SDS–PAGE pattern of proteins from raw pea flour (Fig. 1(a)) exhibited high-density bands corresponding to the storage proteins convicilin (70 kDa), vicilin (12–30–35 kDa), α-legumin (39–40 kDa) and β-legumin (20–25 kDa). Germination for 2, 4 or 6 d led to hydrolysis of pea storage proteins, which was reflected in the disappearance or loss of density from the main polypeptide bands, together with the appearance of a smear of lower molecular weight polypeptides that was particularly evident after 6 d of germination. Hardly any endoproteolytic activity could be observed in the raw pea flour at pH 6.7 (Fig. 1(b)) or pH 4.7 (data not shown). Clear protease activity bands were apparent after 2-d, germination and persisted throughout 6-d germination. The protease activity had a broad pH optimum, from 4.94 to 6.75. Nevertheless, maximum enzyme activity was obtained at pH 6.75.

3.2. Biological analyses

The amount of nutrients consumed, expressed as g DM/day (Table 2) increased significantly with the diet of peas that were processed for 2 or 4 d (G2DNL, G2DL, G4DNL, G4DL), with respect to the animals given the raw-pea flour, but fell significantly among the animals that consumed peas germinated for 6 d (G6DNL, G6DL). No significant differences in consumption were found between the animals given the diet of peas germinated for 2 or 4 d. The quantities of protein, total available sugars, vitamin B_1 (Table 2) and TIA (Table 3) consumed were related to the total amount of diet consumed and to the contents of nutrients and antinutritional factors in the diets. The amount of nitrogen and therefore of protein consumed was significantly lower among the animals given the raw-pea diet or the diet of peas germinated for 6 d (G6DNL, G6DL), with respect to the other experimental groups (G2DNL, G2DL, G4DNL, G4DL). The same pattern was observed for total available sugars, vitamin B_1 and TIA.

The amount of soluble carbohydrates consumed increased after the peas were germinated for 4 or 6 d, with no significant differences being recorded between the results of these germination periods in the presence or absence of light.

The amount of vitamin B_2 consumed was significantly greater when the peas were germinated for 2, 4

Table 2
Nutrient intake from raw and germinated pea flour diets

	Food intake (g DM/day)	Protein intake (g/day)	Available soluble sugars (g/day)	Total available sugars (g/day)	Vit B_1 (mg/day)	Vit B_2 (mg/day)
Raw pea flour germination	9.23 ± 0.33^{a}	2.5 ± 0.09^{a}	0.16 ± 0.006^{a}	3.72 ± 0.13^{a}	0.068 ± 0.002^{a}	0.014 ± 0.0005^{a}
2 d						
No light	11.85 ± 0.18^{b}	3.21 ± 0.05^{b}	0.17 ± 0.003^{a}	4.78 ± 0.07^{b}	0.084 ± 0.001^{b}	0.028 ± 0.0004^{b}
Light	10.71 ± 0.33^{bc}	2.90 ± 0.09^{c}	0.15 ± 0.005^{a}	4.32 ± 0.13^{bc}	0.080 ± 0.002^{b}	0.026 ± 0.0008^{b}
4 d						
No light	10.84 ± 0.28^{bc}	2.94 ± 0.08^{bc}	0.33 ± 0.008^{b}	4.37 ± 0.11^{bc}	0.080 ± 0.002^{b}	0.033 ± 0.0008^{c}
Light	10.17 ± 0.32^{c}	2.76 ± 0.09^{ac}	0.30 ± 0.009^{c}	4.10 ± 0.13^{c}	0.080 ± 0.002^{b}	0.040 ± 0.0012^{d}
6 d						
No light	7.11 ± 0.21^{d}	1.93 ± 0.06^{d}	0.32 ± 0.009^{bc}	2.74 ± 0.08^{d}	0.050 ± 0.001^{c}	0.028 ± 0.0008^{b}
Light	7.99 ± 0.18^{d}	2.17 ± 0.05^{d}	0.30 ± 0.007^{bc}	3.08 ± 0.07^{d}	0.060 ± 0.001^{a}	0.028 ± 0.0006^{b}

[a–d] Values are means ± SEM of ten Wistar rats. Different superscripts within the same column indicate significant differences $P < 0.05$.

Table 3
Antinutrient intake from raw and germinated pea flour diets

	TIA (TIU/day)	α-Galactosides (g/day)	Inositol phosphates (mg/day)
Raw pea flour germination	68855 ± 2494^{ab}	0.48 ± 0.02^{a}	36.8 ± 1.33^{a}
2 d			
No light	94089 ± 1421^{c}	0.04 ± 0.0006^{b}	24.8 ± 0.37^{b}
Light	79146 ± 2429^{d}	0.03 ± 0.001^{bc}	24.0 ± 0.74^{b}
4 d			
No light	94308 ± 2409^{c}	0.03 ± 0.0008^{bc}	17.5 ± 0.45^{c}
Light	75258 ± 2332^{ad}	0.03 ± 0.0008^{bc}	15.8 ± 0.49^{c}
6 d			
No light	45736 ± 1341^{e}	0.009 ± 0.0003^{c}	8.8 ± 0.26^{d}
Light	62384 ± 1392^{b}	0.008 ± 0.0002^{c}	9.6 ± 0.21^{d}

[a–e] Values are means ± SEM of ten Wistar rats. Different superscripts within the same column indicate significant differences $P < 0.05$.

or 6 d, in relation to the consumption of raw peas. The intake of α-galactosides and of inositol phosphates fell significantly as the germination time increased, with no significant differences being caused by the light conditions.

Digestive utilization of protein, assessed by the apparent digestibility coefficient (ADC) was the same among all the groups studied (Table 4). The quantity of nitrogen absorbed increased significantly among the animals given the diet of peas germinated for 2 or 4 d, with or without light, with respect to the diet of raw peas. The animals given the peas germinated for 6 d (G6DNL, G6DL) presented the lowest values of nitrogen absorption.

Nitrogen retention or balance (Table 4) was significantly higher among the rats that consumed peas germinated for 2 or 4 d (G2DNL, G2DL, G4DNL, G4DL) than among those given raw peas, and decreased significantly when the peas were germinated for 6 d (G6DNL, G6DL). The metabolic utilisation of nitrogen (%R/A) was similar for the diets of raw peas and for those germinated for 2 or 4 d (G2DNL, G2DL, G4DNL, G4DL), while it fell significantly after a 6 d germination (G6DNL, G6DL).

Weight gain (g/day) and protein efficiency ratio (PER) (Table 5) were significantly higher among the rats fed peas germinated for 2 or 4 d, with or without light, than those consuming raw-pea flour, with no significant differences being found between the two germination times. Germination for 6 d, with or without light, led to less weight gain and a lower PER, with significant differences being found, not only from the animals given peas germinated for 2 or 4 d, but also from those consuming raw peas.

The index of alimentary efficiency of available carbohydrates (IAV) (Table 5) was significantly higher among the animals that consumed germinated-pea flour (G2DNL, G2DL and G4DNL, G4DL) with respect to the control group (raw peas) and the group given peas germinated for 6 d, with or without light.

The group of animals fed the raw pea flour had average water and nitrogen contents of 708 and 116 g/kg and 734 and 147 g/kg in liver and *longissimus dorsi* muscle, respectively. No significant differences were found in the water or nitrogen contents in the liver and *longissimus dorsi* muscle among the different experimental groups studied.

Table 4
Digestive and metabolic utilization of nitrogen

	Nitrogen intake (mg/day)	Fecal nitrogen (mg/day)	Urinary nitrogen (mg/day)	Nitrogen absorbed (mg/day)	ADC (%)	Nitrogen balance (mg/day)	R/A (%)
Raw pea flour germination	400 ± 14.6^{a}	55.7 ± 2.1^{a}	171.1 ± 12.5^{ac}	345 ± 13.6^{a}	86.0 ± 0.5^{a}	174 ± 2.0^{a}	50.9 ± 1.7^{a}
2 d							
No light	514 ± 7.8^{b}	70.8 ± 2.4^{b}	223 ± 4.4^{b}	443 ± 7.1^{bc}	86.2 ± 0.4^{ab}	220 ± 3.3^{bc}	49.7 ± 0.4^{a}
Light	465 ± 14.3^{c}	60.2 ± 2.7^{a}	196 ± 8.2^{ab}	404 ± 12.1^{c}	87.1 ± 0.3^{ab}	208 ± 6.0^{c}	51.6 ± 1.0^{a}
4 d							
No light	509 ± 12.9^{bc}	61.3 ± 1.2^{a}	216 ± 5.6^{b}	448 ± 12.5^{b}	87.9 ± 0.3^{b}	233 ± 8.8^{b}	51.8 ± 0.8^{a}
Light	491 ± 11.0^{bc}	62.1 ± 1.9^{ab}	202 ± 6.5^{b}	429 ± 10.1^{bc}	87.4 ± 0.3^{ab}	220 ± 5.1^{bc}	51.3 ± 0.7^{a}
6 d							
No light	299 ± 8.8^{d}	40.7 ± 2.1^{c}	163 ± 5.3^{c}	258 ± 7.7^{d}	86.4 ± 0.6^{ab}	95.1 ± 3.0^{d}	36.9 ± 0.6^{b}
Light	334 ± 7.5^{d}	46.7 ± 2.6^{ac}	176 ± 8.3^{ac}	288 ± 6.1^{d}	86.1 ± 0.6^{ab}	112 ± 7.1^{d}	39.0 ± 2.4^{b}

[a–d] Values are means ± SEM of ten Wistar rats. Different superscripts within the same column indicate significant differences $P < 0.05$.

Table 5
Weight gain and nutritional indices of rats fed the diets of raw or germinated pea flour

	Weight gain (g/day)	PER	IAV
Raw pea flour germination	2.29 ± 0.14^{a}	0.93 ± 0.08^{a}	0.63 ± 0.05^{a}
2 d			
No light	4.05 ± 0.15^{bc}	1.27 ± 0.06^{b}	0.85 ± 0.04^{b}
Light	3.77 ± 0.07^{c}	1.3 ± 0.25^{b}	0.88 ± 0.02^{b}
4 d			
No light	4.30 ± 0.2^{b}	1.47 ± 0.08^{b}	0.99 ± 0.05^{b}
Light	4.11 ± 0.27^{bc}	1.5 ± 0.11^{b}	1.01 ± 0.08^{b}
6 d			
No light	1.25 ± 0.15^{d}	0.64 ± 0.07^{c}	0.45 ± 0.05^{c}
Light	1.67 ± 0.09^{d}	0.77 ± 0.04^{c}	0.54 ± 0.03^{c}

[a–d] Values are means ± SEM of ten Wistar rats. Different superscripts within the same column indicate significant differences $P < 0.05$.

4. Discussion

4.1. Chemical analyses

Total and non-protein nitrogen (NPN) contents of peas used for the present experiment were within the range of values found in the literature (Alonso, Orúe, Zabalza, Grant, & Marzo, 2000; Chen & Thacker, 1978; Nikokyris & Kandylis, 1997). The fall in the level of insoluble nitrogen was similar to that reported by Wanasundara, Shahidi, and Brosnan (1999a). This insoluble nitrogen has been described as proceeding from non-covalent interactions or from disulphide bonds between different proteins (Alonso et al., 2000).

The NPN fraction of legumes is composed of free amino acids, nucleic acids, puric and pyrimidinic bases, polyamines, alkaloids and small peptides. Germination for 2, 4, or 6 d, with or without light, caused an increase in non-protein nitrogen and a substantial decrease in protein nitrogen due to the hydrolysis of storage proteins that released peptides and free amino acids, increasing the non-protein nitrogen that might be solubilised in the NaOH and cannot be precipitated by TCA used for NPN determination. Similar results have been obtained by Chen and Thacker (1978) and Wanasundara et al. (1999a) in pea and flax seeds. The higher amount of soluble non-protein nitrogen obtained with increasing germination periods was correlated with the reduced density of polypeptide bands from the seed storage proteins observed in the protein gel, and the increment in protease activity of germinated pea samples.

From these results, it is to be expected that the predigestion of the legume proteins resulting from the germination process, together with a higher protease activity present in the germinated-pea flours, would produce an improvement in the digestive utilisation of the afore mentioned nutrient.

4.2. Biological analyses

4.2.1. Intake

Soaking the peas, followed by 2 d germination, led to a reduction of 73% in the intake of α-galactosides and of almost 100% in that of tannins (López-Amorós, 2000),

improving food intake at a similar rate to that observed by Orúe, Butrón, Ibáñez, Alonso, and Marzo (1998). These results agree with those obtained with other legumes, such as the broad bean (Fernandez, Lopez-Jurado, Aranda, & Urbano, 1996) and the chickpea (Nestares et al., 1993) after soaking in water and in a basic solution. After a 4 d germination (G4DNL, G4DL), food intake levels were high and similar to those recorded after 2 d, probably due to the high intake of palatable sugars that may have compensated for the initial appearance of compounds responsible for the loss of the peas' organoleptic characteristics. Such compounds include free fatty acids released by the germination process (Wanasundara, Wanasundara, & Shahidi, 1999b) that are liable to become oxidised and originate volatile compounds which in turn produce rancid flavours; also produced are non-protein amino acids, especially homoserine and some heterocyclic compounds (Rozan, Kuo, & Lambein, 2000, 2001), that could affect the acceptability of the food and its nutritional value. Changes in food texture or properties, such as hardness, elasticity or chewiness, could also diminish its acceptability (Njintang, Mbofung, & Waldron, 2001; Nnanna et al., 1990) while microbial contamination processes could give rise to disagreeable smells and flavours (Nnanna & Phillips, 1988; Uwaegbute et al., 2000) and biogenic amines (Shalaby, 2000; Simon-Sarkadi & Holzapfel, 1995) caused by fermentation.

The significant decrease in food intake after a 6 d germination (G6DNL, G6DL), even compared to the diet of raw-pea flour, can be attributed to the fact that the compounds responsible for the loss of the peas' organoleptic characteristics are present in considerable concentrations, given that the intake of palatable sugars is very high, and that of α-galactosides and tannins minimal.

4.2.2. Digestive and metabolic utilisation of protein and carbohydrates

The digestive utilization of pea protein was high, and within the range of values described in the literature (Orúe et al., 1998), similar to those reported for broad beans (Fernandez et al., 1996) and much higher than those found for chickpeas, lentils and haricot beans (Nestares et al., 1993; Nestares, Barrionuevo, Urbano, & López-Frías, 2001; Urbano et al., 1995). Phytic acid has been reported to form complexes with protein which then become more resistant to proteolytic degradation (Cheryan, 1980). Under our experimental conditions, a reduction in phytic acid intake of 32.6% after a 2 d germination, and of 44.7% after 6 d, did not increase protein digestibility, probably because a greater reduction is necessary for the improvement to become apparent.

When the peas were soaked and germinated for 2, 4 or 6 d, with or without light, the trypsin inhibitor content was unchanged (Vidal-Valverde et al., 1998), in contrast to the findings of other authors (Ibrahim et al., 2002; Mbithi-Mwikya et al., 2001). This result would explain why digestive utilization remained constant, with more than 80% of the nitrogen consumed being absorbed, in every case. Nevertheless, other authors have reported a fall in TIA content as a result of germination, the ADC of the protein remaining unchanged (Orúe et al., 1998).

The pre-digestion of the protein of the legume caused by the different germination conditions, as described in the chemical composition, was not reflected in the biological experiments. The appearance of glycosylated peptides, as a result of germination, which are resistant to proteolytic enzymes (Chang & Harrold, 1988) could be responsible for the fact that, after a 2, 4, or 6 d germination, with or without light, the ADC values were not found to increase. Nevertheless, as ADC remained high throughout all the experiments (87% vs. the 92% of a casein–methionine control diet) (Fernandez et al., 1996), and nitrogen intake improved significantly after a 2 or 4 d germination (G2DNL, G2DL, G4DNL, G4DL), the animals possessed a large amount of absorbed nitrogen with which to respond to the necessities for growth and structural repair. This higher nitrogen absorption was accompanied by a greater nitrogen balance and weight gain among these groups.

The greater retention of nitrogen by the animals consuming the diet of peas germinated for 2 or 4 d (G2DNL, G2DL, G4DNL, G4DL) again reflects the significantly increased intake, with a high %R/A nitrogen (average of 50%). The deficiency in sulfur-containing amino acids, common to all legume seeds, could be the cause of the lower %R/A found in raw and germinated pea diets when compared to a casein–methionine diet (54%) (Fernandez et al., 1996).

After a 6 d germination (G6DNL, G6DL), a significant decrease in the amount of retained nitrogen was observed, which was related to the significant reduction in intake but also to the significant fall in the %R/A to 36.9–39%. Thus, increased germination times may cause changes within the matrix of germinated peas, such as the presence of higher amounts of non-protein amino acids or the 14% reduction in soluble protein nitrogen, and a deterioration of protein quality that negatively affect its biological value.

The protein efficiency ratio (PER), which measures the relationship between the weight gain and protein consumed, was significantly higher among the rats given peas germinated for 2 or 4 d (G2DNL, G2DL, G4DNL, G4DL) than among rats of the control group (RP). If there were any correlation between the increase in protein intake and weight gain, this index would have remained constant, and so the increase observed must be attributed, not only to a better utilization of nitrogen but also to a better utilization of the carbohydrates provided by the legume. This is reflected by the significant rise in the IAV, which measures the relationship between

weight gain and the amount of available carbohydrates consumed. The peas that were germinated for 2 or 4 d, with or without light (G2DNL, G2DL, G4DNL, G4DL), provided a good supply of vitamins B_1 and B_2 (Vidal-Valverde et al., 2002), exceeding the nutritional requirements of growing rats (NRC, 1995). These vitamins contribute to the optimum nutritive utilization of carbohydrates. Germination of the peas for 6 d did not improve the nutritive utilization of protein or of carbohydrates, as was shown by the nitrogen %R/A, the PER and the IAV.

The nitrogen contents in the muscles and livers of the animals given the diets of raw peas and of germinated peas (for 2, 4 or 6 d) were similar in all cases, and might be related to the size of the tissues.

In conclusion, short germination periods of 2 or 4 d, with or without light were optimal for improving the organoleptic and nutritional properties of peas. These germination periods are sufficient to produce an appreciable reduction in the factors responsible for flatulence, thus increasing intake and improving the utilisation of available proteins and carbohydrates. Such germination periods for peas produce sufficient advantages for this food to be recommended for geriatric and infant nutrition.

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