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Improvement of iron availability from phytase-treated *Pisum sativum*, L. flour

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Abstract

The effect of dephytinization, using an exogenous microbial phytase under optimal conditions (pH 5.5, 37 °C), and subsequent removal of the soaking solution after processing, on the bioavailability of iron from pea (*Pisum sativum* L.) flour was studied in growing rats by examining the chemical composition of pea flours, and the digestive and metabolic utilization of the above-mentioned mineral. Soaking of the pea flour led to a considerable reduction in the content of iron (33%), whereas a lower reduction in iron content (7%), associated with a higher concentration of total phosphorus, was obtained after additional treatment with phytase, than in the soaked pea flour. The digestive utilization of iron from the raw and soaked pea flours by growing rats was negligible, but increased significantly as a result of phytase treatment. The low iron absorption obtained for the former two dietary treatments during an experimental period of ten days was not reflected in any of the haematological indices (red blood count, haemoglobin, haematocrit) or tissues (femur, heart, kidney) studied, with the exception of the sternum.

Keywords: *Pisum sativum*, L.; Iron; Phytase; Availability; Rats

1. Introduction

Iron deficiency is known to affect a large proportion of the world population and, together with iodine and vitamin A deficiency, is one of the most important micronutrient deficiencies worldwide. Iron deficiency is especially prevalent among specific population groups, such as infants (Lozoff, Wolf, & Jimenez, 1996) menstruating and pregnant women, and populations with a high dietary intake of plant-derived proteins (Hallberg, 2001); it can lead to important health problems and retardation in physical and mental development (Beard & Connor, 2003; Hurrell et al., 2004). Legumes and cereals are dietary sources of iron that are worthy of consideration in view of the high amount of iron that they can provide. Iron in legumes and cereals is present in inorganic form or associated with different seed fractions (such as the storage protein ferritin), structural components, phytic acid or polyphenols in the seed coat. Theil (2004) has reviewed the good availability of ferritin-associated iron from soybean, which results from the ability of this protein to pass largely undigested through the gastrointestinal tract and to cross the mucosal barrier directly into the enterocyte. In contrast, the bioavailability of iron from legumes is usually poor as a result of the presence, in legumes, of non-nutritional components, such as phytic acid or polyphenols, that may interfere with the absorption of this mineral (Stahl, Han, Roneker, House, & Lei, 1999; Tuntawiroon et al., 1991). Furthermore, iron absorption is also dependent on the dietary source, the physiological status of the individual, age and gender. Several technological treatments, e.g. soaking at

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different pH conditions and cooking, germination and fermentation, have been developed with the aim of reducing the amount of non-nutritional components that interfere with iron absorption from legumes and thereby increase its availability (El-Adawy, Rahma, El-Bedawy, & Sobihah, 2000; Porres, Aranda, López-Jurado, & Urbano, 2003; Vidal-Valverde et al., 2002).

In recent years, intensive research has been carried out on novel high-quality vegetable protein sources that may serve as alternatives to soybean in the preparation of dietetic products with high nutritional value. The pea could be one such alternative due to its excellent protein quality and high levels of complex carbohydrates, vitamins and minerals (Savage & Deo, 1989; Vidal-Valverde et al., 2002, 2003). In addition, this legume has the ability to adapt to edaphic and climatic conditions that are not favourable for the growth of soybeans.

The bioavailability of iron, phosphorus, and other nutritionally essential minerals from cereal and legume-based diets can be considerably increased by dephytinization or by the direct supplementation of phytase, either in exogenous form or as phytase-rich dietary ingredients (Hurrell, 2004; Lei & Porres, 2005; Porres, Etcheverry, Miller, & Lei, 2001; Porres, Aranda, López-Jurado, & Urbano, 2005; Stahl et al., 1999).

The main goal of this study was to develop a functional food product from pea flour with high iron availability. This, in addition to the fraction of this mineral associated with ferritin (assuming this mineral fraction to be available for absorption by the growing rat). This product could affect the availability of iron prone to be complexing by phytic acid or polyphenol by the removal of these non-nutritional components after soaking the legume flour, adding phytase, and then discarding the soaking solution. Such novel functional food products hold great potential to improve the nutritional status and may contribute to eradicating the important health problem of iron deficiency.

2. Materials and methods

2.1. Diets

Diets were as follows:

RP: Raw pea flour. *Pisum sativum*, L. var. *esla* from germplasm collection of Valladolid (Valladolid, Spain).

PNP: Soaking treatment without phytase addition. Raw pea flour was incubated in 0.1 N acetic/sodium hydroxide buffer, pH 5.5 at 37 °C for 60 min in a stirring bath with a speed of 350 rpm. The ratio of flour to soaking solution was 1:10 (wt:vol). After incubation, the mixture was centrifuged at 15,300*g* and the supernatant was discarded. The flour was then frozen and freeze-dried.

PP: Soaking treatment with the addition of phytase. Raw pea flour was incubated in 0.1 N acetic/sodium hydroxide buffer, pH 5.5, at 37 °C for 60 min in a stirring bath with a speed of 350 rpm and treated with 800 units of phytase per kg of feed (*Aspergillus niger* phytase, Novo Nordisk, Denmark). One unit of phytase activity is defined as the amount of enzyme that liberates 1 μmol of inorganic phosphorus from sodium phytate per minute at pH 5.5 and 37 °C. The other procedures applied were the same as for the PNP diet.

All the experimental products were supplemented with 5% olive oil prior to feeding the animals.

2.2. Chemical methods

The moisture content of the different pea diets was determined by drying to constant weight in an oven at 105 ± 1 °C. Ash content of diet, feces, and the different tissues assayed was measured by calcination at 500 °C to a constant weight. Samples of ashed material were dissolved in 6 N HCl before analysis. Iron content was determined by atomic absorption spectrophotometry, using a Perkin–Elmer 1100-B spectrophotometer. Phosphorus was measured spectrophotometrically using the technique described by Chen, Toribara, and Warner (1956). Inositol phosphates were determined by HPLC, as reported by Frias, Doblado, Antezana, and Vidal-Valverde (2003). Soluble and insoluble dietary fibre of the samples were quantified according to Prosky, Asp, Schweizer, De Vries, and Furda (1992). Total and available starch levels of samples were determined as in Doblado, Frias, Muñoz, and Vidal-Valverde (2003) by a procedure based on total enzyme digestion of starch to glucose for 3 h and 30 min, respectively. The resistant starch was calculated by difference between total and available starch.

Blood parameters were measured using a KX-21 Automated Hematology Analyzer (Sysmex Corporation, Kobe, Japan).

2.3. Biological methods

2.3.1. Experimental design

A biological balance technique, recording changes in body weight and food intake and then calculating iron intake and fecal iron excretion was used. Three ten-day experiments, in which raw or processed peas were the only food source, were carried out. During the first three days of experiments, the rats were allowed to adapt to the diet and experimental conditions, and the main experimental period was the next seven days, during which food intake and weight gain were recorded and feces were collected for analysis.

2.3.2. Animals

In each experiment, 10 young albino Wistar rats were used (5 males and 5 females). The growing animals (recently weaned), with an initial body weight of 111 ± 1.6 g, were housed from day 0 of the experiment in individual stainless steel metabolic cages designed for the separate collection of faeces; 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 sternum, femur, heart, and kidney were collected for analysis. All experiments were undertaken according to Directional Guides Related to Animal Housing and Care (ECC, 1986).

2.3.3. Biological indices

The following indices and parameters were determined for each group according to the formula given below: intake (expressed as dry weight), body weight gain, and apparent digestibility coefficient (ADC):

$$ADC = [(I - F)/(I)] \times 100$$

where I = Intake, and F = Faecal excretion.

2.4. Statistics

Data were subjected to one way analysis of variance, using Statgraphics Statistical Graphics 5.0 System Software (Statistical Graphics Corporation, Rockville, MDS). Differences between means were compared with Duncan's test. The level of significance was set at $P < 0.05$.

3. Results and discussion

3.1. Chemical analysis of legume flours

The chemical composition of legume seeds is known to be significantly affected by different technological processes (Aranda et al., 2004; Porres et al., 2003, 2005; Vidal-Valverde et al., 2002). The effects vary significantly according to the legume species and variety, the thickness of the seed coat and the length and conditions (temperature, pH) of the soaking process. In addition, the considerable losses in dry matter that can take place as a consequence of processing may have a compensatory effect on the loss of certain nutrients, which could even present higher concentrations with respect to those found in the unprocessed seed.

The total ash, phosphorus and iron contents of the pea seed variety used for the present experiment were within the range of values found in the literature (Koplík et al., 2004; Savage & Deo, 1989) (Table 1). Koplík et al. (2004) have reported that 56% of the total iron content present in the pea was soluble under experimental conditions that are probably optimal for the extraction of the globulin, albumin and glutelin fractions of pea protein. Of that soluble iron, 14% was in inorganic form (ferrous, ferric), whereas 78% would be associated with high molecular weight components (150 kDa, ferritin and other metalloproteins), and the remaining 8% would be associated with seed components of intermediate molecular weight (14 kDa). The remaining iron (44%) was not extractable and could be associated with structural components dietary fibre, to which a significant proportion of protein is bound in peas (Martín-Cabrejas et al., 2003), or complexed by phytic acid in the cotyledons or by phenolic constituents of the seed coat (Moraghan, 2004). From the above-mentioned results of Koplík et al. (2004), it can be concluded that the proportion of iron associated with ferritin is lower in the pea than what has been reported by other authors for the soybean (Ambe, 1994; Theil, 2004).

Table 1
Effects of soaking and treatment with an exogenous microbial phytase on ash, iron, total phosphorus, inositol phosphates and dietary fibre contents of *Pisum sativum* L. var. *esla*[d]

	RP	PNP	PP
Ash (g/100 g DM)	3.01 ± 0.21[b]	1.36 ± 0.16[a]	1.34 ± 0.09[a]
Fe (mg/100 g DM)	6.39 ± 0.08[c]	4.28 ± 0.07[a]	5.94 ± 0.10[b]
Total-P (mg/100 g DM)	312 ± 5.54[c]	152 ± 3.61[a]	201 ± 2.35[b]
IP6 (mg/100 g DM)[f]	339 ± 0.06[c]	75 ± 0.01[b]	25 ± 0.01[a]
IP5 (mg/100 g DM)[f]	40 ± 0.02[a]	69 ± 0.01[b]	41 ± 0.03[a]
IP4 (mg/100 g DM)[f]	ND	73 ± 0.01[b]	51 ± 0.03[a]
IP3 (mg/100 g DM)[f]	ND	ND	57 ± 0.02
Total inositol phosphates (mg/100 g DM)	379	217	174
SDF (g/100 g DM)	3.85 ± 0.16[b]	0.56 ± 0.12[a]	0.65 ± 0.05[a]
IDF (g/100 g DM)	16.7 ± 0.26[a]	17.9 ± 0.32[b]	18.9 ± 0.25[b]
TDF (g/100 g DM)	20.5 ± 0.32[b]	18.4 ± 0.36[a]	19.3 ± 0.37[a]
Resistant starch (g/100 g DM)[e]	3.95 ± 0.65[a]	3.53 ± 0.30[a]	3.98 ± 0.57[a]

[a,b,c] Results within the same row with different superscripts differ significantly ($P < 0.05$).
[d] Values are means ± SD ($n = 3$). RP, raw pea; PNP, no phytase addition; PP, phytase addition; DM, dry matter; IP6, Inositol hexaphosphate; IP%, inositol pentaphosphate; IP4, inositol tetraphosphate; IP3, inositol triphosphate; SDF, soluble dietary fibre; IDF, insoluble dietary fibre; TDF, total dietary fibre.
[e] Results have been previously reported by Urbano et al. (2003).
[f] Results have been previously reported by Frias et al. (2003).

Soaking the legume flour and discarding the soaking solution after the process led to considerable losses in total ash content (54.8%), which were superior to what has been observed for other processes, such as germination, soaking and cooking or α-galactoside oligosaccharide removal (Aranda et al., 2004; Porres et al., 2005; Urbano et al., 2006) in which, nevertheless, the whole legume seed was used. Several factors may have contributed to the 33% reduction in iron content observed after soaking the pea flour: primarily, the pH (5.5) and buffer composition of the soaking solution could have favoured a higher degree of solubility and leaching of inorganic iron that would in turn be discarded with the soaking solution. On the other hand, a high percentage of the iron losses could be associated with the decrease in phytic acid (379 to 217 mg/100 g DM) with high affinity for this mineral as a consequence of the soaking process at pH 5.5, 37 °C for 60 min. A considerable proportion of the phytic acid losses can be attributed to leaching into the soaking solution, although the potential role of endogenous phytase from the pea cannot be ruled out and would be reflected in the increment in the IP5 and IP4 contents with respect to the raw pea flour.

However, it does not appear that endogenous phytase activity plays a major role in phytic acid degradation under the experimental conditions of the present study, which were quite different from the optimal pH and length of incubation period needed for the efficient activity of pea phytase (Fredrikson, Larssson Alminger, Carlsson, & Sandberg, 2001). Other constituents, e.g. ferritin or metalloproteins, may have contributed, although to a much lesser extent, to the loss of iron, given that losses in the soluble protein nitrogen fraction, in which these proteins would be contained, was low as a result of processing conditions (Urbano et al., 2003). The iron associated with structural components would undergo minimal losses, in view of the negligible changes in the composition of insoluble dietary fibre, with a slight increase occurring in the amount of this seed component as a result of the over concentration caused by the loss of other nutrients caused by the soaking process (Urbano et al., 2003).

In both soaking processes (with or without phytase addition), the loss of total phenols could have concurred with a loss of iron due to the affinity of this cation for the above-mentioned non-nutritional components (Lestienne, Besançon, Caporiccio, Lullien-Péllerin, & Tréche, 2005; Tuntawiroon et al., 1991) which can be leached to the soaking solution during processing (López-Amorós, Hernández, & Estrella, 2006).

The addition of exogenous microbial phytase, under conditions optimal for its activity (pH, temperature and length of the incubation period), led to a slight reduction in the contents of IP6, IP5 and IP4 with respect to the soaked pea flour, and to the appearance of IP3 and other inositol phosphates with lower degrees of phosphorylation that could not be detected with the HPLC methodology used in the present study. A lower affinity of phytic acid for iron, and, consequently, higher losses of this mineral into the soaking solution, were expected, as a result of the phytic acid hydrolysis caused by the commercial phytase preparation applied (379 vs. 174 mg/100 g DM in RP and PP, respectively). In contrast, the iron content of the pea flour treated with phytase was higher than that obtained for the non-phytase-treated control, with only a 7% iron loss being detected in the former, compared to the 33% obtained for the latter pea flour product. This could have been caused by insoluble complex formation between iron and phosphate that resulted from phytase-catalyzed hydrolysis of phytic acid during the soaking process (Khare, Hesterberg, & Martin, 2005), and matched the higher amount of total phosphorus in phytase-treated pea flour, compared to the untreated pea flour product (201 vs. 152 mg/100 g DM, respectively).

The soluble, insoluble, and total dietary fibre contents of the pea variety used for the present experiment were within the range of values reported in the literature (Martín-Cabrejas et al., 2003). The decrease in total dietary fibre content was mainly due to the loss in its soluble fraction, composed of uronic acids, pectic substances, gums, mucilages and certain types of soluble hemicelluloses and storage polysaccharides (Martín-Cabrejas et al., 2003; Periago, Ros, Lopez, Martinez, & Rincon, 1993), without any major effect being found in the insoluble dietary fibre component, mainly composed of cellulose and, to a lesser extent, lignin and non-cellulosic polysaccharides, among which it is worth mentioning the resistant starch.

3.2. Biological analyses

3.2.1. Food and Fe intake

The soaking process and the addition of phytase did not lead to any significant modifications in daily food intake among the experimental groups tested. In contrast, daily weight gain was significantly higher for the group of animals fed the soaked pea flour diet, than in the groups fed the raw and phytase-treated pea flour diets (Table 2) (Urbano et al., 2003). Dietary intake of iron, by the animals fed the different food products tested, was sufficient to meet the nutrient requirements of the growing rat (NRC, 1995), even in the case of the soaked pea flour diet, in which iron losses were slightly higher, leading to a numerically lower daily iron intake than in the experimental groups fed the raw and phytase-treated pea flour diets.

3.2.2. Digestive and metabolic utilization

As described by McCance and Widdowson (1937), Hurrell (1997) and Windisch (2002), the regulation of iron homeostasis occurs mainly at the digestive level, with hardly any renal excretion of this mineral taking place. Therefore, taking these facts into consideration, iron absorption would be a term synonymous with bioavailability.

The rat has frequently been used as an experimental model for the study of dietary iron availability, due to its simplicity of handling and certain similarities with the gastrointestinal tract of humans, providing valuable information that has helped to expand scientific knowledge on several dietary factors that play an important role in iron absorption and metabolism in different physiological or

Table 2
Daily food intake, weight gain, and nutritive utilization of Fe

	RP	PNP	PP
Food intake (g/day)	10.72 ± 0.20^{a}	11.20 ± 0.29^{a}	11.3 ± 0.41^{a}
Weight gain (g/day)	1.90 ± 0.11^{a}	3.22 ± 0.24^{b}	1.99 ± 0.38^{a}
Fecal weight (g/day)	1.87 ± 0.05^{a}	1.87 ± 0.03^{a}	2.07 ± 0.06^{b}
Fe intake (μg/d)	690.3 ± 13.1^{b}	483 ± 12.5^{a}	677 ± 21.5^{b}
Fecal Fe (μg/d)	669 ± 35.31^{b}	442 ± 32.74^{a}	425 ± 25.75^{a}
Absorbed Fe (μg/d)	19.5 ± 16.8^{a}	36.8 ± 22.1^{a}	243 ± 17.6^{b}
ADC (%)	2.9 ± 3.8^{a}	7.6 ± 5.2^{a}	37.7 ± 2.4^{b}
Tissue Fe content			
Sternum (mg/100 g DM)	10.7 ± 0.34^{b}	8.85 ± 0.40^{a}	12.8 ± 0.74^{c}
Femur (mg/100 g DM)	12.3 ± 0.33^{b}	11.0 ± 0.41^{a}	10.1 ± 0.27^{a}
Heart (mg/100 g DM)	46.6 ± 2.42^{a}	42.8 ± 1.41^{a}	47.2 ± 2.91^{a}
Kidney (mg/100 g DM)	30.8 ± 0.91^{a}	27.0 ± 0.61^{a}	28.8 ± 2.41^{a}

[a,b,c] Results are means ± SEM of 10 Wistar rats. Means within the same row with different superscripts differ significantly ($P < 0.05$). RP, raw pea; PNP, no phytase addition; PP, phytase addition; ADC, apparent digestibility coefficient.

pathological situations. The availability of different foods and mineral supplements intended for human consumption is usually tested in this animal model in order to study their efficacy compared to a highly bioavailable iron form (Chang, Jo, Hwang, Park, & Kim, 2005), and results have been comparable to those obtained in humans (Mahoney & Hendricks, 1984). However, according to some authors (Reddy & Cook, 1991), the results obtained using the rat as an experimental model should be extrapolated to humans with caution, as iron absorption is far more efficient in this rodent than in humans, whereas the effect of certain dietary components with a proven influence on iron absorption by humans is negligible in the rat.

A marginal Fe deficiency state prior to administering the tested dietary Fe source has been induced by many researchers when conducting biological experiments aimed to assess the availability of this mineral from different foods, individual food components or supplements (Beard, Burton, & Theil, 1996; Chang et al., 2005). Nevertheless, the growing rat with a normal physiological Fe status is also an efficient experimental model due to the greater needs for this nutrient during the exponential growth stage.

Under our experimental conditions, it can be considered that the digestive utilization of iron from the raw pea flour diet was almost nonexistent. Hardly any of the iron ingested by the animals was absorbed, a similar result to what has been reported by other authors (Lynch, Beard, Dassenko, & Cook, 1984; Porres et al., 2005), who found null or very low iron absorption from the pea or other legumes measured by *in vivo* or *in vitro* techniques. The low iron absorption found under our experimental conditions can be attributed to several factors, among which we should note: (1) the presence of seed components that act as inhibitors of iron absorption, such as inositol phosphates, tannins, oxalate or structural components that may form complexes with this mineral, thus interfering with its absorption in the gastrointestinal tract (Lestienne et al., 2005; Siegenberg et al., 1991; Tuntawiroon et al., 1991); (2) the differing behaviour of pea ferritin with regard to iron absorption, compared with iron associated with soybean ferritin, for which Beard et al. (1996) and Davila-Hicks et al. (2004) recorded a reasonable level of absorption derived from the ability of soybean ferritin to pass largely undegraded through the gastrointestinal tract and efficiently cross the mucosal barrier without being affected by the different non-nutritional components present in the soybean that interfere with iron absorption (Theil, 2004); (3) the low levels of seed components with an enhancing effect on iron absorption, e.g. organic acids (Teucher, Olivares, & Cori, 2004) and sulphur-containing amino acids (Layrisse, Martínez-Torres, Leets, Taylor, & Ramírez, 1984); (4) the possible endogenous iron losses in the gastrointestinal tract that would be increased by pea flour experimental diets.

The inhibitory effect of conglycinin protein fraction on iron absorption, reported by Lynch, Dassenko, Cook, Juillerat, and Hurrell (1994) for the soybean, should not have taken place in the present experiment, given that the pea does not contain such a protein fraction, as has been discussed by Davidsson, Dimitriou, Walczyk, and Hurrell (2001), who obtained a higher level of iron availability from infant formulas based on pea protein isolate compared to soybean protein isolate with a similar iron content. On the other hand, it would be expected that, upon dietary fibre fermentation in the large intestine of the rat, iron associated with that seed component would be released. Mineral absorption in the large intestine of the rat has been reported (Lopez et al., 1998), but did not seem to play a major role in iron absorption under the experimental conditions of the present study.

The soaking of pea flour under optimal experimental conditions for the activity of exogenous phytase, without addition of the enzyme, did not improve iron absorption, compared to the raw pea flour diet. This was so, despite a higher amount of potential enhancers of iron absorption, such as the acetate used to buffer the soaking solution that remains within the pea product after the soaking treatment, and the loss of compounds with an inhibitory effect on iron absorption, such as inositol hexaphosphate and possibly phenolic compounds (López-Amorós et al., 2006), caused by the soaking treatment. This low digestive utilization of iron can be attributed to the proportion of inositol phosphates (IP6, IP5, IP4) still remaining in the pea flour after the soaking process that are able to significantly inhibit the absorption of this mineral. In fact, Sandberg and Svanberg (1991) observed that inositol hexaphosphate concentrations in the diet above 0.5 μmol/g can interfere with iron absorption. This was corroborated under our experimental conditions by the significant improvement in iron absorption observed after treatment of the pea flour with exogenous microbial phytase, which led to inositol hexaphosphate levels that were below those reported by the former authors. In addition, the levels of IP5 and IP4 were also lower than those found in the soaked pea flour, although an increase in IP3 content was observed.

It could be hypothesized that the higher net absorption of iron in the phytase-treated than in the untreated pea flour was a consequence of the higher dietary intake of iron by the former experimental group (Table 2). Nevertheless, the digestive utilization of iron, assessed by the apparent digestibility coefficient, an index of the absorbed to ingested iron ratio, also reflects a significant increase in the phytase-treated compared to the untreated experimental group.

Although it has been demonstrated that inositol phosphates with different degrees of phosphorylation have a significant effect on iron absorption, which would not be observed using the individual lower phosphorylated derivatives (Sandberg et al., 1999; Skoglund, Lönnerdal, & Sandberg, 1999), and the sum of total inositol phosphates was similar in the two processed pea flours used in the present experiment, it is to be expected that the availability of this mineral would be higher from the soaked pea flour supplemented with phytase (PP), than from its unsupplemented control (PNP), based on the lower proportion of

Table 3
Blood parameters of the different experimental groups

	RP	PNP	PP
Erythrocyte ($\times 10^6/\mu l$)	7.04 ± 0.22^a	6.75 ± 0.20^a	6.96 ± 0.43^a
Hemoglobin (g/dl)	14.2 ± 0.36^a	12.9 ± 0.39^a	13.1 ± 0.77^a
Hematocrit (%)	40.8 ± 1.1^a	39.0 ± 1.1^a	39.2 ± 2.4^a
MCV (fl)	58.1 ± 0.54^b	57.8 ± 0.46^{ab}	56.4 ± 0.63^a
MCH (pg)	20.3 ± 0.39^b	19.2 ± 0.22^a	18.9 ± 0.30^a
MCHC (g/dl)	35.0 ± 0.52^a	33.2 ± 0.16^a	33.5 ± 0.37^a

[a,b,c] Results are means ± SEM of 10 Wistar rats. Means within the same row with different superscripts differ significantly ($P < 0.05$). RP, raw pea; PNP, no phytase addition; PP, phytase addition, MCV; mean corpuscular volume; MCH, mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin content.

inositol phosphates with a high degree of phosphorylation present in the phytase-treated flour.

In spite of the higher iron availability from the PP dietary product, there was still 70% of iron remaining that was not absorbed under our experimental conditions and this could be associated with structural or undigested components of the pea flour. The results of the present experiment suggest that pea ferritin may not play such an important role in iron absorption as that described for soybean ferritin by Beard et al. (1996) and Davila-Hicks et al. (2004). Nevertheless, further experiments using more specific experimental conditions should be established to settle this question.

Haemoglobin content, haematocrit, red blood cell count, and the other haematological indices studied (Table 3) were within the range of reference values for the animals at this stage of growth (Charles River Technical Bulletin, 1998). The low iron absorption observed in the experimental groups (fed the raw and soaked pea flour products during the 10-day experimental period assayed) was not reflected in any of the haematological indices usually employed for the diagnosis of iron deficiency caused by a low dietary intake of the mineral, such as haemoglobin, haematocrit or red blood cell count (Chang et al., 2005), probably because of the short experimental period assayed, which was not sufficient to affect erythropoiesis. Under our experimental conditions, the Fe content in heart, kidney or femur did not maintain any correlation with the intestinal absorption of this mineral in any of the three experimental groups tested. In the case of a long bone, e.g. femur, some authors have reported significant correlation between Fe availability and bone mineral content (Pallauf, Pippig, Most, & Rimbach, 1999) that was not observed in the present study. Regarding the sternum, our results showed a lack of correlation between availability and storage of Fe in this organ when the absorption of this mineral from the diet was low whereas, for a higher mineral absorption (10-fold), such as that obtained by the PP experimental group, higher Fe availability was related to higher levels of this mineral in the sternum. Thus, the content of Fe in the sternum would be, under our experimental conditions, a better indicator of availability than that in other organs tested.

4. Conclusions

Treatment of pea flour with phytase under optimal conditions for enzyme activity led to a significant improvement of iron absorption by growing rats. The pea flour obtained in this way may be useful for the development of functional food products with improved availability of this mineral.

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References

Ambe, S. (1994). Mössbauer study of iron in soybean hulls and cotyledons. *Journal of Agricultural and Food Chemistry, 42*, 262–267.

Aranda, P., López-Jurado, M., Fernández, M., Moreu, C., Porres, J. M., & Urbano, G. (2004). Bioavailability of calcium and magnesium from faba beans (*Vicia faba* L var *major*), soaked in different pH solutions and cooked, in growing rats. *Journal of the Science of Food and Agriculture, 84*, 1514–1520.

Beard, J. L., Burton, J. W., & Theil, E. C. (1996). Purified ferritin and soybean meal can be sources of iron for treating iron deficiency in rats. *Journal of Nutrition, 126*, 154–160.

Beard, J. L., & Connor, J. R. (2003). Iron status and neural functioning. *Annual Review of Nutrition, 23*, 41–58.

Chang, Y. J., Jo, M. Y., Hwang, E. H., Park, C. U., & Kim, K. S. (2005). Recovery from iron deficiency in rats by the intake of recombinant yeast producing human H-ferritin. *Nutrition, 21*, 520–524.

Charles River Technical Bulletin (1998). *Baseline hematology and clinical chemistry values for Charles River Wistar Rats (CRL:(W)BR) as a function of sex and age*. Wilmington, MA, USA: Charles River Laboratories.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956). Microdetermination of phosphorus. *Analytical Chemistry, 28*, 1756–1758.

Davidsson, L., Dimitriou, T., Walczyk, T., & Hurrell, R. F. (2001). Iron absorption from experimental infant formulas based on pea (*Pisum sativum*)-protein isolate: The effect of phytic acid and ascorbic acid. *British Journal of Nutrition, 85*, 59–63.

Davila-Hicks, P., Theil, E. C., & Lönnerdal, B. (2004). Iron in ferritin or in salts (ferrous sulfate) is equally bioavailable in nonanemic women. *American Journal of Clinical Nutrition, 80*, 936–940.

Doblado, R., Frias, J., Muñoz, R., & Vidal-Valverde, C. (2003). Fermentation of *Vigna sinensis* var. *carilla* flours by natural microflora and Lactobacillus species. *Journal of Food Protection, 66*, 2313–2320.

El-Adawy, T. A., Rahma, E. H., El-Bedawy, A. A., & Sobihah, T. Y. (2000). Effect of soaking process on nutritional quality and protein solubility of some legume seeds. *Nahrung/Food, 44*, 339–343.

European Community Council. (1986). Directional Guides Related to Animal Housing and Care. *Official Bulletin of European Communities*, 18.12.86N L358/1-N L 358/28. Barcelona: European Community Council.

Fredrikson, M., Larssson Alminger, M. L., Carlsson, N. G., & Sandberg, A. S. (2001). Phytate content and phytate degradation by endogenous phytase in pea (*Pisum sativum*). *Journal of the Science of Food and Agriculture, 81*, 1139–1144.

Frias, J., Doblado, R., Antezana, J. R., & Vidal-Valverde, C. (2003). Inositol phosphate degradation by the action of phytase enzyme in legume seeds. *Food Chemistry, 81*, 233–239.

Hallberg, L. (2001). Perspectives on nutritional iron deficiency. *Annual Review of Nutrition, 21*, 1–21.

Hurrell, R. F. (1997). Bioavailability of iron. *European Journal of Clinical Nutrition, 51*, S4–S8.

Hurrell, R. F. (2004). Phytic acid degradation as a means of improving iron absorption. *International Journal for Vitamin and Nutrition Research, 74*, 445–452.

Hurrell, R. F., Lynch, S., Bothwell, T., Cori, H., Glahn, R., Hertrampf, E., et al. (2004). Enhancing the absorption of fortification iron. A sustain task force report. *International Journal for Vitamin and Nutrition Research, 74*, 387–401.

Khare, N., Hesterberg, D., & Martin, J. D. (2005). XANES investigation of phosphate sortion in single and binary systems of iron and aluminum oxide minerals. *Environmental Science and Technology, 39*, 2152–2160.

Koplík, R., Komínková, J., Borková, M., Mestek, O., Kvasnicka, F., & Suchánek, M. (2004). Effect of technological processing and maturity stage of seeds on the content and speciation of phosphorus and trace elements in peas. *Food Chemistry, 87*, 423–432.

Layrisse, M., Martínez-Torres, C., Leets, I., Taylor, P., & Ramírez, J. (1984). Effect of histidine, cysteine, glutathione or beef on iron absorption in humans. *Journal of Nutrition, 114*, 217–223.

Lei, X. G., & Porres, J. M. (2005). Phytases. In W. G. Pond & A. W. Bell (Eds.), *Encyclopedia of animal sciences* (pp. 704–707). New York: Marcel Dekker.

Lestienne, I., Besançon, P., Caporiccio, B., Lullien-Péllerin, V., & Tréche, S. (2005). Iron and zinc in vitro availability in pearl millet flours (*Penisetum glaucum*) with varying phytate, tannin, and fiber contents. *Journal of Agricultural and Food Chemistry, 53*, 3240–3247.

Lopez, H. W., Coudray, C., Bellanger, J., Younes, H., Demigné, C., & Rémésy, C. (1998). Intestinal fermentation lessens the inhibitory effects of phytic acid on mineral utilization in rats. *Journal of Nutrition, 128*, 1192–1198.

López-Amorós, M. L., Hernández, T., & Estrella, I. (2006). Effect of germination on legume phenolic compounds and their antioxidant activity. *Journal of Food Composition and Analysis, 19*, 277–283.

Lozoff, B., Wolf, A. W., & Jimenez, E. (1996). Iron deficiency anemia and infant development: Effects and extended oral iron therapy. *Journal of Pediatrics, 129*, 382–389.

Lynch, S. R., Beard, J. L., Dassenko, S. A., & Cook, J. D. (1984). Iron absorption from legumes in humans. *American Journal Clinical Nutrition, 40*, 42–47.

Lynch, S. R., Dassenko, S. A., Cook, J. D., Juillerat, M. A., & Hurrell, R. F. (1994). Inhibitory effect of a soy-bean protein-related moiety on Fe absorption in humans. *American Journal of Clinical Nutrition, 60*, 567–572.

Mahoney, A. W., & Hendricks, D. G. (1984). Potential of the rat as a model for predicting iron bioavailability for humans. *Nutrition Research, 4*, 913–922.

Martín-Cabrejas, M. A., Ariza, N., Esteban, R., Mollá, E., Waldron, K., & López-Andréu, F. J. (2003). Effect of germination on the carbohydrate composition of the dietary fiber of peas (*Pisum sativum* L.). *Journal of Agricultural and Food Chemistry, 51*, 1254–1259.

McCance, R. A., & Widdowson, E. M. (1937). Absorption and excretion of iron. *Lancet, 2*, 680–684.

Moraghan, J. T. (2004). Accumulation and within seed distribution of iron in common bean and soybean. *Plant and Soil, 264*, 287–297.

National Research Council (1995). *Nutrient requirements of laboratory animals* (5th Revised Edition). Washington, DC: National Academy Press.

Pallauf, J., Pippig, S., Most, E., & Rimbach, G. (1999). Supplemental sodium phytate and microbial phytase influence iron availability in growing rats. *Journal of Trace Elements in Medicine and Biology, 13*, 134–140.

Periago, M. J., Ros, G., Lopez, G., Martinez, M. C., & Rincon, F. (1993). The dietary fiber components and their physiological effects. *Revista Española de Ciencia y Tecnología de Alimentos, 33*, 229–246.

Porres, J. M., Aranda, P., López-Jurado, M., & Urbano, G. (2003). Effect of natural and controlled fermentation on chemical composition and nutrient dialyzability from beans (*Phaseolus vulgaris*, L.). *Journal of Agricultural and Food Chemistry, 51*, 5144–5149.

Porres, J. M., Aranda, P., López-Jurado, M., & Urbano, G. (2005). Nutritional potential of raw and free α-galactosides lupin (*Lupinus albus* Var. *multolupa*) seed flours. Effect of phytase treatment on nitrogen and mineral dialyzability. *Journal of Agricultural and Food Chemistry, 53*, 3088–3094.

Porres, J. M., Etcheverry, P., Miller, D. D., & Lei, X. G. (2001). Phytase and citric acid supplementation in whole-wheat bread improves phytate-phosphorus release and iron dialyzability. *Journal of Food Science, 66*, 614–619.

Prosky, L., Asp, N. G., Schweizer, T., De Vries, J. W., & Furda, I. (1992). Determination of insoluble and soluble dietary fiber in foods and food products: Collaborative study. *Journal of the AOAC International, 75*, 360–367.

Reddy, M. B., & Cook, J. D. (1991). Assessment of dietary determinants on nonheme-iron absorption in humans and rats. *American Journal of Clinical Nutrition, 54*, 723–728.

Sandberg, A. S., Brune, M., Carlsson, N. G., Hallberg, L., Skoglund, E., & Rossander-Hulthén, L. (1999). Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *American Journal of Clinical Nutrition, 70*, 240–246.

Sandberg, A. S., & Svanberg, U. (1991). Phytate hydrolysis by phytase in cereals. *Effects on in vitro* estimation of iron availability. Journal of Food Science*, 56*, 1330–1333.

Savage, G. P., & Deo, S. (1989). The nutritional value of peas (*Pisum sativum*). A literature review. *Nutrition Abstracts Reviews Series A, 59*, 65–88.

Siegenberg, D., Baynes, R. D., Bothwell, T. H., Macfarlane, B. J., Lamparelli, R. D., Car, N. G., et al. (1991). Ascorbic acid prevents the dose-dependent inhibitory effects of polyphenols and phytates on nonheme-iron absorption. *American Journal of Clinical Nutrition, 53*, 537–541.

Skoglund, E., Lönnerdal, B., & Sandberg, A. S. (1999). Inositol phosphates influence iron uptake in Caco-2 cells. *Journal of Agricultural and Food Chemistry, 47*, 1109–1113.

Stahl, C. H., Han, Y. M., Roneker, K. R., House, W. A., & Lei, X. G. (1999). Phytase improves iron bioavailability for hemoglobin synthesis in young pigs. *Journal of Animal Science, 77*, 2135–2142.

Teucher, B., Olivares, M., & Cori, H. (2004). Enhancers of iron absorption: Ascorbic acid and other organic acids. *International Journal of Vitamin and Nutrition Research, 74*, 403–419.

Theil, E. C. (2004). Iron, ferritin, and nutrition. *Annual Review of Nutrition, 24*, 327–343.

Tuntawiroon, M., Sritongkul, N., Brune, M., Rossander-Hultén, L., Pleehachinda, R., Suwanik, R., et al. (1991). Dose-dependent inhibitory effect of phenolic compounds in foods on nonheme-iron absorption in men. *American Journal of Clinical Nutrition, 53*, 554–557.

Urbano, G., Aranda, P., Gómez-Villalva, E., Frejnagel, S., Porres, J. M., Frías, J., et al. (2003). Nutritional evaluation of pea (*Pisum sativum* L.) protein diets alter mild hydrothermal treatment and with and without added phytase. *Journal of Agricultural and Food Chemistry, 51*, 2415–2420.

Urbano, G., López-Jurado, M., Aranda, C., Vilchez, A., Cabrera, L., Porres, J. M., et al. (2006). Evaluation of zinc and magnesium bioavailability from pea (*Pisum sativum*, L.) sprouts. Effect of illumination and different germination periods. *International Journal of Food Science and Technology, 41*, 618–626.

Vidal-Valverde, C., Frias, J., Hernández, A., Martín-Alvarez, P. J., Sierra, I., Rodríguez, C., et al. (2003). Assessment of nutritional compounds and antinutritional factors in pea (*Pisum sativum*) seeds. *Journal of the Science of Food and Agriculture, 83*, 298–306.

Vidal-Valverde, C., Frias, J., Sierra, I., Blázquez, I., Lambein, F., & Kuo, Y. (2002). New functional legume foods by germination: Effect on the nutritive value of beans, lentils and peas. *European Food Research and Technology, 215*, 472–477.

Windisch, W. (2002). Interaction of chemical species with biological regulation of the metabolism of essential trace elements. *Analytical and Bioanalytical Chemistry, 372*, 421–425.