

## Iron absorption from experimental infant formulas based on pea (*Pisum sativum*)-protein isolate: the effect of phytic acid and ascorbic acid

Lena Davidsson\*, Triantafillia Dimitriou, Thomas Walczyk and Richard F. Hurrell

Laboratory for Human Nutrition, Institute of Food Science, Swiss Federal Institute of Technology (ETHZürich), PO Box 474, CH-8803 Rueschlikon, Switzerland

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Infant formula based on pea (*Pisum sativum*)-protein isolate has been suggested as an alternative to soyabean formula in countries where soyabean is not a native crop, or when soyabean protein cannot be used due to allergic reactions or intolerances. In the present study, Fe absorption from experimental infant formulas based on pea-protein isolate was measured in healthy non-anaemic young women. The influence of phytic acid and ascorbic acid on Fe absorption was evaluated, using a stable-isotope technique based on incorporation of Fe stable-isotope labels into erythrocytes 14 d after administration. Geometric mean Fe absorption increased from 20.7 (+1SD 41.6, –1SD 10.3) % to 33.1 (+1SD 58.6, –1SD 18.7) %; ( $P < 0.0001$ ;  $n$  10) after enzymic degradation of virtually all phytic acid. Doubling the molar ratio Fe : ascorbic acid from 1 : 2.1 to 1 : 4.2 in the infant formula with native phytic acid content also increased Fe absorption significantly ( $P < 0.0001$ ;  $n$  10); geometric mean Fe absorption increased from 14.8 (+1SD 32.1, –1SD 6.8) % to 22.1 (+1SD 47.2, –1SD 10.4) %. These results confirm the inhibitory and enhancing effects of phytic acid and ascorbic acid respectively on Fe absorption, but also indicate relatively high fractional Fe absorption from the pea-protein-based formulas. After adjusting for differences in Fe status, our data indicate that Fe absorption from dephytinised pea protein might be less inhibitory than dephytinised soyabean protein as measured in a previous study (Hurrell *et al.* 1998).

### Pea protein: Phytic acid: Ascorbic acid: Iron: Stable isotopes

Most infant formulas are based on cow's milk, although commercial products based on soyabean-protein isolate are available in many countries. Alternative protein sources, such as pea (*Pisum sativum*) protein, have been suggested as interesting novel raw materials for infant formula production in countries where soyabean is not a native crop, or in situations where a vegetable-protein-based formula is desirable and soyabean protein cannot be used due to allergic reactions or intolerances. Infant formulas based on soyabean protein have been used for a long time, and the nutritional status of infants fed soyabean formula has been well documented and found to be equivalent to infants fed cow's milk formula. However, the bioavailability of nutrients, especially minerals and trace elements, has been reported to be lower than that from milk-based formulas (Golden & Golden, 1981; Sandström *et al.* 1983; Gillooly *et al.* 1984).

An important factor contributing to the reduced bioavailability of minerals and trace elements from

soyabean formula is the relatively high concentration of the metal chelator, phytic acid, present in the soyabean-protein isolate. Phytic acid is an inhibitor of mineral and trace element absorption, and is a particularly strong inhibitor of Fe absorption (Hurrell *et al.* 1992). The negative effect of phytic acid on Fe absorption has been shown to be dose-dependent in human subjects (Hallberg *et al.* 1989; Siegenberg *et al.* 1991). In addition to the influence of phytic acid, soyabean protein *per se* has been demonstrated to inhibit Fe absorption (Lynch *et al.* 1994). Fe absorption, however, depends on the balance between dietary inhibitors (e.g. phytic acid) and enhancers of absorption. Ascorbic acid is a potent enhancer of Fe absorption that can counteract the inhibitory effect of phytic acid (Siegenberg *et al.* 1991). We have recently demonstrated a significant ( $P < 0.05$ ) increase in Fe absorption by infants and adults from soyabean formula after the degradation of phytic acid, or by doubling the concentration of ascorbic acid in

**Abbreviation:** Hb, haemoglobin.

\* **Corresponding author:** Lena Davidsson, fax +41 1 704 5710, email davidsson@ilw.agrl.ethz.ch

the native phytic-acid-containing formula (Davidsson *et al.* 1994; Hurrell *et al.* 1998).

The aim of the present study was to evaluate Fe absorption in adult women from experimental infant formulas based on pea protein. The effect of phytic acid degradation and the effect of an increased ascorbic acid content were investigated, using a stable-isotope technique based on erythrocyte incorporation of Fe stable-isotope labels 14 d after administration.

## Materials and methods

### Infant formulas

Semper AB (Stockholm, Sweden) produced experimental infant formulas based on pea-protein isolate for the study. Except for the protein source, the overall composition and processing of the experimental products were similar to those for a commercial soyabean infant formula (Soja Semp; Semper AB). Two different experimental products were prepared as liquid 'ready-to-feed' formulas without any added Fe or ascorbic acid. Commercial pea-protein isolate (Provital Industrie SA, Warcoing, Belgium) was used for production of an experimental infant formula with the native content of phytic acid (product 1), while product 2 was based on dephytinised pea-protein isolate. Dephytinised pea-protein isolate was prepared by the addition of commercial phytase (Phytase Novo L; Novo Nordisk, Copenhagen, Denmark) to commercial pea-protein isolate, using a process similar to that previously described for soyabean isolate (Davidsson *et al.* 1994). The microbiological safety and the nutritional composition of the products were assured before release from the factory according to the control system used for commercial infant formulas.

### Subjects

Twenty healthy women (ten per study) were recruited for the study from the student population and staff at the Swiss Federal institute of Technology and the University of Zürich. Exclusion criteria included pregnancy or lactation as well as subjects with gastrointestinal disorders or metabolic diseases. No medication (except oral contraceptives) or vitamin–mineral supplements were allowed during the study. Volunteers were fully informed about the aims and the procedures of the study both verbally and in writing and written informed consent was obtained. The study protocol was reviewed and approved by the Ethical Committee at the University Hospital, Zürich.

### Study protocol

A baseline venous blood sample (7 ml) was drawn in EDTA-treated Vacutainer tubes (Becton Dickinson Vacutainer Systems, Europe, Meylan, France) for determination of haemoglobin (Hb) and ferritin on the first day of the study. Body weight and height were measured and a questionnaire regarding previous illnesses, medication and food habits was completed. Administration of labelled test meals was done in the morning on four consecutive days, after an overnight fast. Women were randomly allocated to study 1 or 2. The standardised study protocol was identical to that previously used by us and each labelled test meal consisted of 217 g liquid infant formula (Hurrell *et al.* 1998). The two different test meals (A and B) within each study were administered in the order ABAB or BABA. Five women in each study started with test meal A and the other five women with test meal B. Fe stable-isotope labels and ascorbic acid (Merck, Darmstadt, Germany) were added to the liquid test meals as solutions immediately before intake (Table 1). The administrations of labelled test meals were done under close supervision by the investigators. No food or fluid was allowed for 3 h following intake of labelled test meals. A second blood sample (7 ml) was drawn on day 19 for analysis of Fe stable-isotope composition and Hb.

### Blood analysis

Blood samples were analysed for Hb by cyanomethaemoglobin technique (Sigma kit; Sigma, St Louis, MO, USA) and for Fe isotopic composition (see p. 61). Plasma was separated from an aliquot of the whole blood and analysed for ferritin (ELISA kit; Ramco, Houston, TX, USA). Quality-control materials for Hb (Dia-HT1-2-3; DiaMed, Cressier sur Morat, Switzerland) and ferritin (Ferritin Assay Control Serum; Ramco) were analysed together with the samples.

### Stable-isotope labels

Isotopically-labelled FeSO<sub>4</sub> solutions were prepared from isotopically-enriched elemental Fe by dissolution of the metal in 0.1 M-H<sub>2</sub>SO<sub>4</sub>. The isotopic composition of Fe in solution was determined by negative-thermal-ionisation MS using a magnetic-sector-field instrument (MAT 262; Finnigan MAT, Bremen, Germany). Fe concentrations of the solutions were determined by isotope-dilution MS *v.* an Fe standard, prepared gravimetrically from an isotopic

**Table 1.** Study protocol for the administration of labelled test meals based on pea (*Pisum sativum*)-protein-isolate infant formula

Study	Test meal	Phytic acid content	Fe stable isotope	Molar ratio Fe : ascorbic acid
1	A	Native	<sup>57</sup> Fe	1 : 2.1
	B	Dephytinised*	<sup>58</sup> Fe	1 : 2.1
2	A	Native	<sup>57</sup> Fe	1 : 2.1
	B	Native*	<sup>58</sup> Fe	1 : 4.2

\* Commercial pea-protein isolate was dephytinised by the addition of commercial phytase (Phytase Novo; Novo Nordisk, Copenhagen, Denmark), as described by Davidsson *et al.* (1994) for soyabean-protein isolate.

reference material (IRM-014; Institute of Reference Materials and Measurements, Geel, Belgium).

Individual doses of isotopic labels (2.0 mg  $^{57}\text{Fe}$  or 2.0 mg  $^{58}\text{Fe}$  per test meal; total dose 4.0 mg  $^{57}\text{Fe}$  and 4.0 mg  $^{58}\text{Fe}$  per subject) were prepared by weighing the corresponding amount of isotopically-labelled Fe solution into Teflon containers, which were flushed with Ar to keep the Fe in the  $\text{Fe}^{2+}$  oxidation state.

#### *Iron isotopic analysis of blood samples*

For all experimental work, the guidelines for trace element analysis were strictly followed, including additional purification of commercial chemicals and reagents and acid-washing of all containers used during trace element analysis. All handling of samples was done under clean laboratory conditions to reduce the risk of sample contamination during analysis.

Each isotopically-enriched blood sample was analysed in duplicate for its Fe isotopic composition under chemical blank monitoring. Whole-blood samples were mineralised using an  $\text{HNO}_3\text{-H}_2\text{O}_2$  mixture (5:2, v/v) and microwave digestion. Sample Fe was separated from the matrix by anion-exchange chromatography following a solvent-solvent extraction step into diethyl ether (Beer & Heumann, 1993; Kastenmayer *et al.* 1994; Walczyk *et al.* 1997). All isotopic analyses were performed using a magnetic-sector-field MS (MAT 262; Finnigan MAT) equipped with a multi-collector system for simultaneous ion-beam detection. For Fe isotope-ratio measurements, the recently-developed negative-thermal-ionisation MS technique (Walczyk, 1997) was used. Fe separated from the sample was loaded on  $\text{BaF}_2$ -coated Re filaments of a double-filament ion source together with AgF to promote the formation of negatively-charged  $\text{FeF}_4^-$  ions. Based on the high enrichment of the isotopically-enriched labels and the low amounts of labels expected to be incorporated into the erythrocytes it was possible to normalise the acquired isotopic data for the natural  $^{54}\text{Fe} : ^{56}\text{Fe}$  isotope ratio (Taylor *et al.* 1992).

#### *Calculation of iron absorption*

Based on the shift of Fe stable-isotope ratios in the blood samples and circulating Fe, the amount of  $^{57}\text{Fe}$  isotopic label and  $^{58}\text{Fe}$  isotopic label present in the blood of each study subject 14 d after test meal administration was calculated. Calculations were based on the principles of isotope dilution, taking into consideration that the Fe isotopic labels were not monoisotopic (Walczyk *et al.* 1997). Circulating Fe was calculated based on blood volume and Hb concentration (Kastenmayer *et al.* 1994). Blood volume calculations were based on height and weight following empirically-derived formulas (Brown *et al.* 1962). For calculation of fractional absorption, 80% incorporation of the absorbed Fe into erythrocytes was assumed.

#### *Food analysis*

Food samples were mineralised by microwave digestion

(MLS 1200; MLS GmbH, Leutkirch, Germany) in a  $\text{HNO}_3\text{-H}_2\text{O}_2$  mixture and analysed for Fe and Ca by electrothermal-flame atomic absorption spectroscopy (SpectrAA 400; Varian, Mulgrave, Australia) using the standard addition technique to minimise matrix effects. Phytic acid analysis *myo*-inositol triphosphate to *myo*-inositol hexaphosphate was performed at the Department of Food Science, Chalmers University of Technology, Göteborg, Sweden, according to the method of Skoglund *et al.* (1997) with some modifications. Liquid samples (10 ml) were extracted with 1 M-HCl (10 ml) for 3 h followed by evaporation of 15 ml extract and dissolution in 0.025 M-HCl (2 ml). The solution was centrifuged and 400  $\mu\text{l}$  supernatant fraction filtrated through Millipore Microcon YM-30 centrifugal filter (Millipore AB, Sundbyberg, Sweden). The filtrate was injected into the HPLC analysis system, consisting of a biocompatible HPLC pump (Waters Model 626; Waters Associates, Milford, MA, USA), 50  $\mu\text{l}$  injector loop, a HPIC Omni Pac PAX-100 (4  $\times$  250 mm) analytical column, a PAX-100 (4  $\times$  50 mm) guard column (Dionex Corp., Sunnyvale, CA, USA), and Waters 486 tunable absorbance detector (Waters Associates).

Protein content was based on analysis of N, using a standard Kjeldahl technique, and applying the factor 6.25 to convert N content to protein.

#### *Statistics*

Paired *t* test was used for the evaluation of Fe absorption in both studies. Values were log transformed before statistical analysis. Results are presented as geometric means and +1SD and -1SD.

#### **Results**

The protein content of the two experimental formulas based on pea protein (native phytic acid *v.* dephytinised) was 20.4 g/l *v.* 18.4 g/l. The corresponding concentrations (mg/l) of Fe and Ca were 3.6 *v.* 3.0 and 343 *v.* 334 respectively in the two products. Phytic acid content was 513  $\mu\text{mol/l}$  *v.* <1  $\mu\text{mol/l}$  respectively in the two products.

Twenty women (mean age 22 (range 20–28) years, mean body weight 55 (range 48–62) kg) participated in the two studies. Mean Hb was 138 (range 123–155) g/l for study 1 and 145 g/l (range 136–150) g/l for study 2. The geometric mean (and range) plasma ferritin concentration was 13 (5–28)  $\mu\text{g/l}$  for study 1 and 10  $\mu\text{g/l}$  (6–27)  $\mu\text{g/l}$  for study 2. Four and six women had low Fe stores, as indicated by plasma ferritin <12  $\mu\text{g/l}$  in studies 1 and 2 respectively.

In study 1 Fe absorption increased significantly after the degradation of phytic acid; geometric mean Fe absorption increased from 20.7 (+1SD 41.6, -1SD 10.3) % to 33.1 (+1SD 58.6, -1SD 18.7) %;  $P < 0.0001$ ). Doubling the molar ratio Fe:ascorbic acid in study 2 also increased Fe absorption significantly; geometric mean Fe absorption increased from 14.8 (+1SD 32.1, -1SD 6.8) % to 22.1 (+1SD 47.2, -1SD 10.4) %;  $P < 0.0001$ .

#### **Discussion**

Recent studies on Fe bioavailability from soyabean

formulas have reported a significant increase in Fe absorption by infants and adults after phytic acid degradation or by doubling the concentration of ascorbic acid in the formula containing native phytic acid (Davidsson *et al.* 1994; Hurrell *et al.* 1998). The results from the present study confirm these earlier observations and clearly demonstrate that Fe bioavailability from infant formula based on pea-protein isolate can also be improved by phytic acid degradation or by increased addition of ascorbic acid. The present study was implemented in women and not in infants since the experimental infant formulas used in the present study have not yet been evaluated in infant growth studies or tested for acceptability in infants. Fe absorption studies in adults, however, have been demonstrated to be a valuable tool for evaluating the influence of phytic acid and ascorbic acid on Fe absorption from infant formulas in infants (Hurrell *et al.* 1998).

Fractional Fe absorption by young women from the experimental infant formulas based on pea-protein isolate was relatively high, compared with earlier data on Fe absorption by young adults from soyabean formulas (Hurrell *et al.* 1998). Using a radioisotopic technique we have previously reported geometric mean Fe absorption values of 2.4 and 6.0 % respectively from soyabean formula with native phytic acid content or dephytinised. After doubling the ascorbic acid concentration, geometric mean Fe absorption increased from 4.1 % to 5.3 % (Hurrell *et al.* 1998). The soyabean formulas contained similar amounts of Fe and ascorbic acid to the pea-protein formulas and were fed in 217 g servings with an identical study design, except that radioisotopes were used instead of stable-isotope labels of Fe. Due to the pronounced inter-individual variation in Fe absorption resulting from differences in Fe status, it is not possible to compare directly data from different studies. Serum ferritin concentration has a close inverse relationship with Fe absorption (Cook *et al.* 1974; Walters *et al.* 1975; Magnusson *et al.* 1981) and several of the women participating in the present study had low plasma ferritin concentrations, while the geometric mean ferritin value for the young women and men participating in the soyabean-formula study was about 35 µg/l (Hurrell *et al.* 1998). Studies can be compared, however, if the Fe absorption values are adjusted to comparable Fe status. Since the formulas based on soyabean- and pea-protein isolates contained different amounts of phytic acid, the most relevant comparison is that between products based on dephytinised protein isolates. Using the model proposed by Cook *et al.* (1991), the individual Fe absorption data from the pea-protein formulas used in study 1 were adjusted to 35 µg ferritin/l, resulting in a geometric mean Fe absorption of 7.8 % *v.* 12.3 % (native phytic acid content *v.* dephytinised). Thus, these results indicate that dephytinised pea protein might be less inhibitory than dephytinised soyabean protein (geometric mean Fe absorption 6.0 %; Hurrell *et al.* 1998).

Lynch *et al.* (1994) reported that soyabean protein *per se* was inhibitory to Fe absorption in human subjects and identified the major protein fraction conglycinin as the inhibitory component. Pea protein contains 55–57 % globulins and 21–26 % albumins compared with 90 %

and 10 % respectively of the corresponding proteins in soyabean (Guéguen & Lemarié, 1996). The major proteins in both soyabean and peas are globulins (type 7S and type 11S), but the globulin fractions are different. The globulins in soyabean are β-conglycinin and γ-conglycinin (7S) and glycinin (11S), while pea protein contains vicilin and convicilin (7S) and leguminin (11S). Pea protein does not contain the conglycinin fraction shown to inhibit Fe absorption in soyabean protein, and this difference in protein composition could explain the higher fractional Fe absorption from dephytinised pea-protein formula indicated by our studies. Additional studies with direct comparisons between dephytinised pea and dephytinised soyabean protein would be required for a final evaluation of the effect of these different protein sources *per se* on Fe bioavailability.

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