Iron Availability from Iron-Fortified Spirulina by an in Vitro Digestion/Caco-2 Cell Culture Model

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Iron deficiency, one of the most important nutritional problems in the world, can be caused not only by foods deficient in iron but also by poor availability of dietary iron. Iron food fortification in combination with highly available iron from supplements could effectively reduce this deficiency. The aim of this study was to examine the iron availability from iron-fortified spirulina. We have used an in vitro digestion/Caco-2 cell culture system to measure iron spirulina availability and made a comparison with those of beef, yeast, wheat floor, and iron sulfate plus ascorbic acid as a reference. Iron availability was assessed by ferritin formation in Caco-2 cells exposed to digests containing the same amount of iron. Our results demonstrate a 27% higher ferritin formation from beef and spirulina digests than from digests of yeast and wheat flour. When iron availability was expressed per microgram of iron used in each digest, a 6.5-fold increase appeared using spirulina digest in comparison with meat. In view of this observed high iron availability from spirulina, we conclude that spirulina could represent an adequate source of iron.

Keywords: *Iron availability; spirulina; in vitro digestion; Caco-2 cells; ferritin*

INTRODUCTION

Iron deficiency anemia is a serious widespread issue throughout the world (*1*, *2*) and affects approximatively 20% of the world population, despite the fact that the average daily diet contains iron far in excess of the amount needed for metabolic purposes. This discrepancy is attributed to the low availability of food iron (*3*), as many foods that are potentially good sources of iron are limited by the bioavailability of the iron (*4*). Therefore, an accurate assessement of iron availability in the diet is important. This has become more apparent as rapid advancements in food technology and processing continue to provide an increasing number of new food products.

The aim of this work was to measure the bioavailability of iron from iron-fortified spirulina. The cyanobacteria *Spirulina platensis* (blue-green algae) is commercially available for human consumption. Spirulina represents one of the richest protein sources of plant origin (60−70%) and is a good source of vitamins and minerals (*5*). These microalgae are now used as a health food source for humans (*6*). The simple cultivation technology and the good quality of their protein, as well as the absence of any toxic side effects (*7*, *8*), favor their large-scale production. Moreover, the ability to control the chemical composition of the algae by varying the cultivation conditions makes spirulina the most easily Fe-supplementable vegetable by way of the aquatic environment. The algal iron availability was compared with that of iron sulfate, yeast, wheat flour, and beef.

A most promising technique at present for determining iron availability is the human intestinal epithelial (Caco-2) cell line system (*9*−*11*). These cells sponteanously differentiate after reaching confluency, forming a well-defined brush border on the apical surface and tight cellular junctions (*12*, *13*). They have recently been successfully applied to the study of iron availability in a pioneering work by Glahn et al. (*14*, *15*) and also by Garcia et al. (*16*); moreover, they represent an alternative method that reduces or can replace the use of laboratory animals (*17*).

MATERIALS AND METHODS

Spirulina Iron Fortification. The algae-fortification step was performed at Aquamer S.A. (Mèze, France). Algae (*Spirulina platensis*) were grown in a 130-L photobioreactor under continuous lighting on Zarouk's medium at 22 °C and pH 10.5 in the presence of $FeCl_3$. This medium contained 16.8 g/L $NaHCO_3$, 0.5 g/L K_2HPO_4, 2.5 g/L $NaNO_3$, 1.0 g/L K_2SO_4, 1.0 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.04 g/L $CaCl_2$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.08 g/L EDTA, 2.86×10^{-3} g/L H_3BO_3, 1.81×10^{-3} g/L $MnCl_2 \cdot 4H_2O$, 0.22×10^{-3} g/L $ZnSO_4 \cdot 7H_2O$, 7.9×10^{-5} g/L $CuSO_4 \cdot 5H_2O$, 1.5×10^{-5} g/L MoO_3, and 2.1×10^{-5} g/L Na_2MoO_4 and was supplied with a light aeration (30 L/min) and with addition of 0.03% CO_2. At the end of the growth, the biomass was recovered and filtered through a 20-μm membrane, thoroughly washed with distilled water, frozen, and lyophilized.

Cell Culture. Caco-2 cells originating from human colorectal carcinoma were obtained from the American Type Culture Collection (ATCC, Rockville, MD) at passage 22 and used in

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experiments at passage 45. Caco-2 cells were grown at 37 °C in an atmosphere of 5% CO_2−95% air at constant humidity and in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 1% nonessential amino acids, and 4mM L-glutamine. The medium was changed every other day. The cells were routinely expanded in tissue culture flasks (75 cm^2), and when the cells were 80−90% confluent, they were harvested by treatment with a solution containing 0.25% trypsin and 1 mM EDTA, thoroughly washed, and resuspended in supplemented growth medium. For transport experiments, Caco-2 cells were seeded at a level of 5×10^4 cells/cm^2 in six-well plates (Corning Costar Science Products, Brumath, France). The cells were used in the iron uptake experiments at 28 d postseeding.

In vitro digestion was strictly performed according to the procedure of Glahn et al. (*15*). Briefly, porcine pepsin (800−2500 units/mg of protein), pancreatin (activity, 4xUSP specification), and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were purchased from Sigma (Saint Quentin Fallavier, France) and prepared by a treatment with Chelex-100 (Bio-Rad S.A., Ivry sur Seine, France) in order to remove the Fe and a most of the Zn from the enzyme preparations, as strongly recommended by Glahn et al. (*15*). Peptic and intestinal digestions were conducted on a rocking platform shaker (Rotomix, Bioblock, Illkirch, France) in an incubator at 37 °C with a 5% CO_2−95% air atmosphere maintained at constant humidity. The digestion was carried out in the upper chamber of a two-chamber system in six-well plates, with the cell monolayer cultured on the bottom surface of the lower chamber, as described by Glahn et al. (*15*). The upper chamber was formed by fitting the bottom on an appropiate-sized Transwell insert ring with a 15 000 molecular weight cutoff irradiated dialysis membrane (Spectra/Por 2.1, Spectrum Laboratories Inc., Los Angeles, CA). The dialysis membrane was held in place with a sterilized Teflon ring. After the dialysis membrane was fastened to the insert ring, the entire unit was kept in sterile water until use. To start the peptic digestion, the pH of each sample was adjusted to 2.0 with 5.0 mol/L HCl. The sample was transferred to a 50-mL screw-cap culture tube, 0.5 mL of the pepsin solution was added per 10 mL of sample, and the tube was capped, placed horizontally, and incubated in the rocking shaker for 60 min. For the intestinal digestion step, the pH of the digest was raised to 6.0 by dropwise addition 1 of mol/L $NaHCO_3$. Then, 2.5 mL of pancreatin−bile extract mixture was added per 10 mL of original sample, the pH was adjusted to 7.0 with NaOH, and the volume was brought to 15 mL with 120 mmol/L NaCl and 5 mmol/L KCl.

Experimental Design. Just before the intestinal digestion step, the growth medium was removed from each well, and the cell monomayer was washed twice with Minimum Essential Medium (MEM, Gibco) containing no added iron at 37 °C and pH 7.0. This medium was supplemented with 10 mmol/L PIPES (piperazine-*N*,*N*′-bis-[2-ethanesulfonic acid]), 1% antibiotic−antimicotic solution, hydrocortisone (4 mg/L), insulin (5 mg/L), selenium as sodium selenite (5 μg/L), triiodothyronine (34 μg/L), and epidermal growth factor (20 μg/L). All culture medium components were from Sigma (Saint Quentin Fallavier, France). During the experiment, the cells were covered with 1.0 mL of MEM. A sterilized insert ring, fitted with a dialysis membrane, was then inserted into the well, thereby forming the two-chamber system. Then, a representative 1.5 mL aliquot of the intestinal digest was carefully poured into the upper chamber, the plate was covered, and the sample was incubated on the rocking shaker for 120 min.

At the end of the intestinal digestion, the insert ring and the digest were removed. The solution in the lower chamber was allowed to stand on the cell monolayer, and an additional 1 mL of MEM was added to each well. The six-well plate was then returned to the incubator for a 22-h period at the end of which the cells were harvested for analysis.

Cells Harvesting. The cells were harvested as described by Glahn et al. (*15*), and the iron bound to the cell surface and not taken up by the cells was removed by pipetting 2 mL of a solution containing 140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L PIPES, at pH 7.0, with an additional 5 mmol/L sodium hydrosulfite and 1 mmol/L bathophenanthrolene disulfonic acid (BPDS) onto the cell monolayer for 10 min (*18*). After being washed with the above solution without hydrosulfite and BPDS, the monolayer was washed with 2 mL of deionized water. Then, the plates were sonicated for 15 min at 4 °C, and the cells were scraped, harvested in 2 mL of water for each well, and stored at −80 °C.

Experimental Procedure and Food Sampling. In our experiments, we compared samples of iron sulfate ($FeSO_4 \cdot 7H_2O$), both with (Fe−AA) and without (Fe) ascorbic acid (Sigma Chemical, Saint Quentin Fallavier, France); yeast; wheat flour; and beef with iron-fortified spirulina. Yeast (Selegerm, Cereal, Annonay, France) and wheat flour were purchased at a local market, and iron-fortified spirulina was prepared by Aquamer S.A. (Mèze, France); they contained 9.3 μg, 20.1 μg, and 7.0 mg of Fe/g of sample. Fresh beef was purchased from a local supermarket, and all visible fat and connective tissue was removed; it was finely minced and freeze-dried and found to contain 111.3 μg of Fe/g of sample. Digests of the above samples contained 10 μg of Fe from each sample.

Biochemical Analyses. Caco-2 cell protein was measured by a commercial protein assay (Sigma, Saint Quentin Fallavier, France) according to the method of Smith et al. (*19*) and using bovine serum albumin as the standard. Analyses of the iron content of the solutions and food samples were performed by inductively coupled plasma mass spectrometry (ICP-MS) using a Varian Vista spectrometer (Varian, Les Ulis, France). The Caco-2 cell ferritin content was measured by using a solid-phase two-site immunoradiometric assay kit (Ferritin-CT, CIS bio international, Gif-Sur-Yvette, France) on 25-μL samples of the sonicated cells harvested in 2 mL of water.

Statistical Analysis. For each food sample tested, three six-well plates were run, and this experiment was repeated three times. The data from each food sample were averaged ($n = 54$), and this average value was the data point used in the statistical analysis. Data are given as mean ± SEM; they were analyzed by one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) method for comparing groups using Stat View 4.5 (Abacus Concepts, Inc., Berkeley, CA). A significance level of $p < 0.05$ was adopted for all comparisons.

RESULTS

In our experiments, food sample amounts were prepared in order to supply 10 μg of Fe in each digest; thus, 0.014 g of spirulina, 0.107 g of yeast, 0.497 g of wheat flour, and 0.090 g of beef were used in each digest. Additionally, a blank digest system containing only pepsin, pancreatin, and bile extract without addition of Fe or food was used. Moreover, a digest containing iron sulfate plus 1 mmol/L ascorbic acid (Fe−AA) was used as a reference; another digest of iron sulfate alone (designed Fe) was introduced in the experiment. The amount of iron measured in 1.5 mL of each digest placed in the upper chamber was consistent with the expected iron content of the digests (only 1 μg of iron was loaded in the upper chamber of each culture well); no significant differences appeared among the digests. The cellular ferritin levels measured 24 h after the start of the intestinal digestion are summarized in Figure 1. Ferritin was 7.5-fold higher for iron sulfate in the presence of ascorbic acid than for iron sulfate alone. Iron-fortified spirulina and beef digests led to identical levels of ferritin formation that were significantly higher (about 27%) than those exhibited by yeast and wheat flour. Ferritin formation expressed per microgram of iron loaded in each upper chamber is summarized in Figure 2. The digest of spirulina showed a dramatic increase

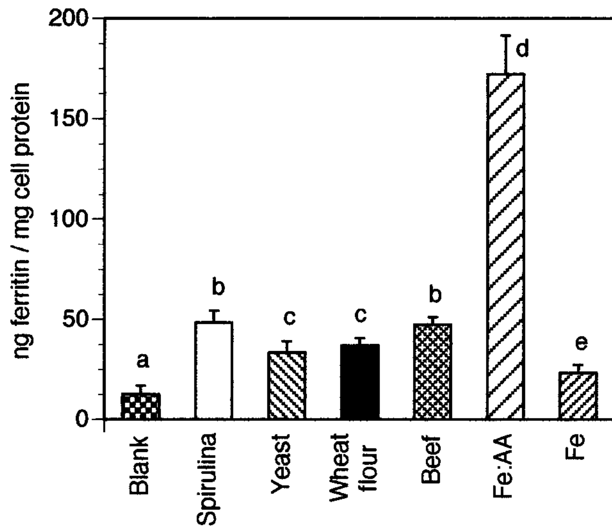

Figure 1. Caco-2 cell ferritin formation 24 h after the start of in vitro digestion. Values are means ± SEM (n = 54). Bars with identical letters are not significantly different ($p < 0.05$).

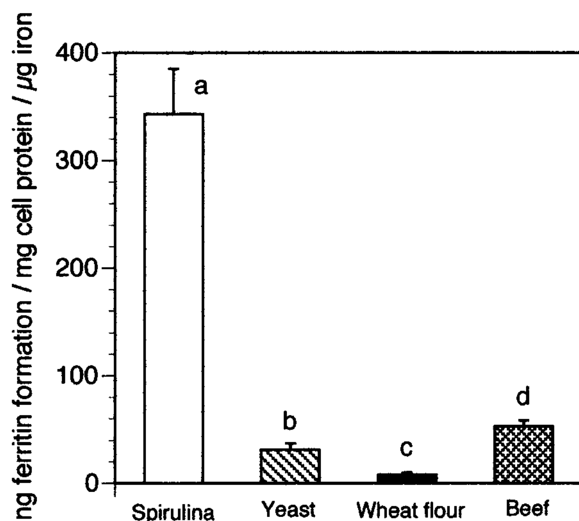

Figure 2. Caco-2 cell ferritin formation 24 h after the start of in vitro digestion and expressed per microgram of iron loaded in each upper chamber. Values are means ± SEM (n = 54). Bars with identical letters are not significantly different ($p < 0.05$).

in ferritin formed in comparison with the other digests and, in particular, showed a ferritin level 6.5 times greater than that from the beef digest.

DISCUSSION

Iron availability can be defined as the amount of ingested iron that enters the body beyond the intestinal cell. Iron that enters the intestinal cell and remains there, being excreted during the cell desquamation process, is not available. Absorbed iron can enter enterocytic iron reserves and can be mobilized in order to satisfy body needs: this represents bioavailable iron. Although there is no clear accepted definition of bioavailability, there is a general consensus that it is the proportion of a given nutrient that the body actually utilizes (*4*).

According to Crosby (*20*), mucosal synthesis of ferritin is normal in states of iron repletion; thus iron entering from the lumen is trapped in mucosal ferritin. This hypothesis led to a possible mechanism by which the ferritin content of the cell could be conditioned by the amount of iron in the cell's environment. Nevertheless, ferritin is a necessary storage protein to prevent damage and compile iron reserves. Thus, it can be believed that mucosal ferritin is closely related to both iron status and absorption (*21*−*23*).

Studies on Caco-2 cells reported a direct correlation between the ferritin formation in these cells and the level of iron content of the culture medium (*9*, *24*); in our study, the starting culture conditions were selected in order to obtain a low cell iron status. To discard any iron contamination by the experimental system, the minimum essential culture medium (MEM) was chosen with no added iron in its composition. An iron analysis of MEM showed 5.4 µg of Fe/L, which was deemed sufficiently free of iron and an acceptable iron level for this system (*15*). In the same way, pepsin and pancreatin−bile solutions that contained iron were treated on Chelex-100 resin to remove the iron, and this experimental step allowed us to remove 94 and 50% of the iron, respectively, leading to an average iron concentration of 63 µg of Fe/L. The removals of zinc from pepsin and the pancreatin−bile mixture were 97 and 68%, respectively. This is an important point because the method used is very sensitive and a minimal iron contamination must be maintained in this system for differences in iron food bioavailability to be detected. Otherwise, zinc might interfere with iron uptake (*25*). On the other hand, Halloran et al. (*26*) reported that the removal of iron from tissue culture media is accompanied by an immediate cessation of cell growth and proliferation.

Among dietary factors, ascorbic acid is one of the most clearly documented enhancers of nonheme iron uptake and bioavailability (*27*, *28*). Here, we have used iron sulfate plus ascorbic acid (Fe−AA) as a reference for iron availability. In comparison with iron sulfate alone in the digest (Fe), the dramatic positive effect of ascorbic acid on the availability of iron as measured by ferritin formation was clearly shown in Figure 1. In this study, it was expected that iron availability would be higher in the beef digest relative to the digests of either yeast, wheat flour, or spirulina. However, ferritin formation from iron-fortified spirulina in Caco-2 cells was not significantly different from that exhibited by the meat digest (Figure 1). An enormous absorption of nonheme iron appeared when ferritin formation was expressed per microgram of iron from food submitted to in vitro digestion (Figure 2), emphasizing significant efficiency differences among the four iron sources. It is unusual to find plant-derived iron that is so highly available. Yet, Johnson and Shubert (*29*) reported that spirulina-fed rats absorbed iron more than or equal to rats fed iron from a ferrous sulfate supplement. Moreover, it has been shown that spirulina is more effective than casein and wheat gluten in improving the iron status in rats during pregnancy and lactation (*30*). It appears, therefore, that spirulina contains a highly available form of iron; it is one of the richest iron foods, supplying 1.5 g of Fe/kg. It has been reported that iron in spirulina is over twice as absorbable as the form of iron found in vegetables and most meats and 60% better absorbed than other iron supplements such as iron sulfate. Algae concentrate mineral and trace elements from aquatic media, and some authors have reported that they are in an organic

form (*31*). In spirulina, phycocyanin is a pigment that represents about 25% of the cellular protein and resembles biliary pigments. We suspect iron to filter into the porphyric structure of such a protein. Moreover, because spirulina proteins are highly digestible and possess a high biological value (*32*), this could represent one explanation for why iron from this microalga is so highly available. Another hypothesis would be that the iron is associated with the protein surface by ionic bonds; this iron pool could be easily released, particularly by peptic digestion. Thus, two iron pools could coexist in fortified spirulina, one containing iron resembling heme iron and another comprising nonheme iron that can be rapidly released. Spirulina fractionation studies are in progress to test this hypothesis.

To conclude, we have demonstrated that iron fortification of spirulina represents a process allowing for a concentrated source of available iron (7.0 g of Fe/kg). Iron deficiency and iron deficiency anemia are still relatively common in toddlers, adolescent girls, and women of childbearing age (*33*). Many other kinds of people should be concerned about iron: athletes whose diet must include enough iron (*34*), blood donors who need to replace their red blood cells, people following diets to control weight, vegetarians, and people in developing countries. Intake of iron-rich spirulina caps might provide another means of iron supplementation beside the addition of bovine hemoglobin to foods (*35*) and bovine lactoferrin to infant formulas (*36*).

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