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Milk Peptides Increase Iron Dialyzability in Water but Do Not Affect DMT-1 Expression in Caco-2 Cells

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In vitro digestion of milk produces peptide fractions that enhance iron uptake by Caco-2 cells. The objectives of this study were to investigate whether these fractions (a) exert their effect by increasing relative gene expression of DMT-1 in Caco-2 cells and (b) enhance iron dialyzability when added in meals. Two milk peptide fractions that solubilize iron were isolated by Sephadex G-25 gel filtration of a milk digest. These peptide fractions did not affect relative gene expression of DMT-1 when incubated with Caco-2 cells for 2 or 48 h. Dialyzability was measured after in vitro simulated gastric and pancreatic digestion. Both peptide fractions enhanced the dialyzability of iron from ferric chloride added to PIPES buffer, but had no effect on dialyzability from milk or a vegetable or fruit meal after in vitro simulated gastric and pancreatic digestion. However, dialyzability from milk was enhanced by the addition of a more concentrated lyophilized peptide fraction.

KEYWORDS: Milk peptides; iron bioavailability; Caco-2 cell cultures; gel filtration; dialysis

INTRODUCTION

Milk peptides, formed during enzymatic digestion, may exhibit a range of biological effects including opiate, antithrombotic, antihypertensive, immunomodulatory, and metal-binding properties (*1*, *2*). However, they have not been fully investigated for their potential effect on iron absorption. Bovine milk provides iron of low bioavailability and may inhibit the absorption of nonheme iron present in a meal (*3*−*5*), but small peptides released during proteolytic digestion are thought to have a promoting effect on iron absorption (*6*−*10*). There is little knowledge on the properties of peptides released during the gastrointestinal digestion of milk. In a previous study we showed that following a simulated gastrointestinal digestion of milk, peptide fractions, isolated by gel filtration, increased iron uptake by Caco-2 cells. The enhancing effect of these peptide fractions was significant when compared to the effect of nonfractionated milk digest or the effect of other peptide fractions (*11*). These peptidic fractions need to be further explored.

Milk peptide fractions may constitute an ingredient that, when added in foods, may enhance the absorption of iron. This application may be of particular interest in formulating foods targeted to infants and children. Milk may be fortified with iron (*12*) at relatively high levels (*13*), which may induce indirect negative effects (*14*). An alternative approach may be to lower the amount of added iron while employing factors that would enhance iron absorption. Milk peptides are a plausible candidate for this role and need to be further investigated for their effect on iron bioavailability when added to foods. One way of predicting the effect of milk peptide fractions, as well as other dietary factors, on iron bioavailability is by measuring iron dialyzability following a simulated gastrointestinal digestion procedure (*5*). This in vitro methodology has limitations, recognized and considered in the interpretation of results, but offers a reliable, rapid, and inexpensive first step in assessing iron bioavailability in a food or meal (*15*).

The objectives of the present study were to test the following hypotheses: (a) Milk peptides produced during in vitro digestion of milk enhance iron absorption by increasing relative gene expression of DMT-1 (divalent metal transporter 1, an intestinal Fe transporter) in Caco-2 cells, and (b) milk peptides enhance iron dialyzability when added to meals. Peptide fractions formed by simulated gastrointestinal digestion of milk and isolated by gel filtration from milk were compared for their effects expression of DMT-1 in a Caco-2 cell system. In addition, peptide fractions were added in a series of meals that included milk, a vegetable meal, and a fruit meal preparation. Iron dialyzability was compared for predicting iron bioavailability of the selected meals in the presence of milk peptide fractions.

MATERIALS AND METHODS

Glassware. All glassware was washed, soaked overnight in 1 N HCl, and rinsed with distilled deionized 18 MΩ H_2O before use to avoid mineral contamination.

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Chemicals. Unless otherwise stated, all reagents were purchased from Sigma-Chemicals, St. Louis, MO.

Preparation of Milk Digest. A sample of 10 mL of cow's milk, fresh, pasteurized, 0% fat, commercially obtained, or water (control), pH adjusted to 2.8 with HCl (6 mol/L), was digested in vitro under simulated gastrointestinal conditions (*11*). At the end of the in vitro digestion, the digest was collected, centrifuged at 9000*g* for 15 min, and placed in a refrigerator at 4 °C.

Fractionation of Milk Digest. Sephadex G-25 resin (5 g) was soaked in deionized water overnight at 4 °C and then decanted to remove fine particles that did not settle [fractionation range, $M_r = (1-5) \times 10^3$]. The hydrated resin was transferred to PIPES buffer 0.075 mol/L, pH 5.7, for equilibration and transferred onto a column (diameter, 1 cm; length, 32 cm, Flexcolumn chromatography column, Kontes). To equilibrate the column, 0.075 mol/L PIPES, pH 5.7, was eluted overnight. The flow rate was set at 0.5 mL/min (peristaltic pump EP-1 Econo Pump, Bio-Rad Laboratories). The supernatant from the milk digest, 0.25 mL, was applied to the column and eluted with 0.075 mol/L PIPES, pH 5.7. Fractions of 1.0 mL were collected into tubes with the aid of a fraction collector (Medel 328, Instrumentation Specialties). Vitamin B_{12} (MW = 1579), monitored spectrophotometrically, was employed as a standard at 0.1 mg/mL to estimate the molecular weight of the eluted fractions. Each fractionation was repeated twice. All fractions were tested for their ability to maintain Fe in a soluble form. Each fraction (0.5 mL) was mixed with 0.1 mL of $FeCl_3$, 10 μmol/L in 0.01 mol/L HCl, and incubated for 15 min at room temperature at pH 5.7. Soluble iron was determined spectrophotometrically, with ferrozine (*12*). Fractions eluted at 26 and 38 min (named "fraction A" and "fraction B", respectively) were the most potent in the formation of soluble iron. These have been identified in previous experiments as the fractions that may enhance iron uptake from Caco-2 cells (*11*). The concentration of protein in the eluted fractions was monitored at 280 nm. A standard curve, generated from a bovine serum albumin standard, was employed for the determination of the protein concentration. Fraction A contained 0.26 mg/mL and fraction B 0.4 mg/mL peptides. These fractions were collected and stored at −18 °C until further testing. Fractions obtained from repeated chromatographic fractionations were pooled before the Caco-2 cell or the in vitro digestion experiments. In particular, pooled fractions were used for the experiments on *DMT-1* expression (pooled from 5 fractionations), dialyzability (pooled from 4 fractionations), or dialyzability involving lyophilized samples (pooled from 12 fractionations).

Effect of Fractions from Milk Digest on DMT-1 Gene Expression. *Cell Cultures.* Caco-2 cells were obtained from the American Type Culture Collection at passage 17 and used in experiments at passage 30−35. Cells were seeded at a density of 50000 cells/cm^2 in collagen-treated 24-well plates (Costar Corp., Cambridge, MA). The cells were grown in DMEM with 10% v/v fetal bovine serum (GIBCO), 25 mmol/L HEPES, and 1% antibiotic antimycotic solution (GIBCO). The cells were maintained at 37 °C in an incubator with a 5% CO_2/95% air atmosphere at constant humidity, and the medium was replaced every 2 days. The cells were used at 14 days postseeding. Under these conditions, the amount of cell protein measured in each well was highly consistent from well to well within each culture plate.

Incubation of Milk Fractions with Caco-2 Cells. A 0.5 mL aliquot from the selected fractions A and B or control (MEM, media with no bovine serum albumen added and therefore containing no iron) was combined with 0.5 mL of MEM and transferred to a microcentrifuge tube. Iron (as ferric chloride) was added to each sample to bring the final concentration to 20 μmol/L. The MEM on top of the cells was removed by aspiration. After the contents of each microcentrifuge tube had been vortexed, 1 mL of this solution was placed on top of the cells. The cells were incubated for 2 or 48 h. Cell protein was assessed using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA), based on the Lowry assay.

Total RNA Isolation and Reverse Transcription. At the end of each experimental period cells were harvested and immediately transferred to liquid nitrogen. Total RNA was isolated from cell cultures using a commercial kit (Rneasy Midi-Maxi kit, Qiagen) and reverse-transcribed using oligo (dT) and Superscript II reverse transcriptase (MBI, Fermentas Inc.).

Gene Expression Analysis of DMT-1. First-strand cDNAs were synthesized from 5 μg of total RNA from each cell culture well using oligo $(dT)_{18}$ as primers in the presence of MLV reverse transcriptase (Fermentas Inc.) for 1 h at 42 °C. PCR was carried out with primers chosen from a fragment of the previously published human gene sequences; *DMT-1* (forward, 5′-GGT GTT GTG CTG GGA TGT TA-3′; reverse, 5′-AGT ACA TAT TGA TGG AAC AG-3′). Ribosomal 18S was used to normalize the results, with primers from the human small intestine ribosomal 18S mRNA (forward, 5′-GAA CTA CGA CGG TAT CTG ATC GTC T-3′; reverse, 5′-CCG CCC GTC CCC GCC GGT TGC CTC T-3′). Determination of the linear phase of the PCR amplification was performed with Tfi-DNA polymerase (Access RT-PCR system, Promega) with pooled aliquots removed at 15, 20, 25, 30, 35, 40, 45, 50, and 55 cycles. Amplification of the *DMT-1* gene was performed for 32 cycles for *DMT-1*. The reaction consisted of denaturation (95 °C, 30 s), annealing (48 °C, 1 min), and extension (72 °C, 1 min); ribosomal 18S was amplified at 30 cycles under identical conditions in a different tube. Ribosomal 18S (350 bp) and human duodenal *DMT-1* (350 bp) PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using a Gel-Pro analyzer version 3.0 (Media Cybernetics, LP).

In Vitro Digestion of Food Samples in the Presence of Fractions from Milk Digest. *Sample Preparation.* For the study of the effect of fractions A and B on iron dialyzability, 1.9 mL of fraction A or fraction B or 0.075 mol/L PIPES buffer, pH 5.7 (control) was adjusted to pH 2.8 with HCl 6 mol/L. Subsequently, 0.1 mL of iron, as ferric chloride in HCl 0.01 mol/L, was added so as to bring the final concentration to 0.20 mmol/L. For the study of the effect of fractions A and B on iron dialyzability from milk, 0.204 g of dried milk, 0% fat (Regilait, Saint-Martin-Belle-Roche, France), was added to 1.9 mL of fraction A or fraction B or 0.075 M PIPES, pH 5.7, buffer. The pH of the mixtures was adjusted to 2.8 with 6 N HCl first and then with 1 N for the final pH adjustment. Iron, 0.1 mL, as ferric chloride in HCl 0.01 mol/L, was added so as to bring the final concentration to 0.20 mmol/L. For the study of the effect of fractions A and B on iron dialyzability from infant meals, 0.5 g of fruit meal or vegetable meal (Nestle, Vevey, Switzerland) was mixed with 1.4 mL of fraction A or fraction B or 0.075 M PIPES, pH 5.7 (control). The pH of the mixtures was adjusted to 2.8, and 0.1 mL of iron (as ferric chloride) was added so as to bring the final concentration to 0.20 mmol/L.

For the study of the effect of lyophilized fractions A and B on iron from milk, 7 mL of 0.075 M PIPES, pH 5.7, or 7 mL of fraction A or fraction B was put in the freezer at −18 °C for 72 h and subsequently lyophilized. The solid residue was transferred to a well of a six-well plate. Cow's milk (1.9 mL, pH 2.8) and 0.1 mL of iron, as ferric chloride in HCl 0.01 mol/L, were added so as to bring the final concentration to 0.20 mmol/L.

In Vitro Digestion. All samples were digested with a newly introduced modification of the in vitro digestion procedure (*15*). This method is a modification of the dialyzability method (*5*). Briefly, 2 mL of final volume was transferred to or prepared in wells in a six-well plate. Pepsin suspension (0.1 mL) was added to each well, and the plates were covered with a plastic lid. The plates were placed in a shaking incubator maintained at 37 °C and incubated for 2 h. At the end of this incubation, cylindrical inserts (Costar) with a piece of dialysis membrane (molecular weight cutoff of 6000−8000, Spectrum Laboratories, Rancho Dominguez, CA) fastened to one end with an elastic band were placed in each well (*16*) in such a way that the membrane was in contact with the digest in the well. The insert was filled with 2 mL of PIPES buffer, pH 6.3. The buffer diffused through the membrane, thereby gradually adjusting the pH of the samples from 2.8 to 6, as shown previously (*15*). After 30 min, the insert was slightly lifted, 0.5 mL of a pancreatin−bile salt mixture was added to the samples, the insert was replaced, and the incubation was continued for another 2 h. At the end of this incubation period, the insert holding the dialysis membrane was removed. The fraction held on the dialysis membrane, named the dialysate, containing soluble compounds of low molecular weight was transferred to centrifuge tubes. Dialysates were centrifuged at 10000*g* for 20 min, and the iron concentration was measured in the supernatants spectrophotometrically with ferrozine (*12*).

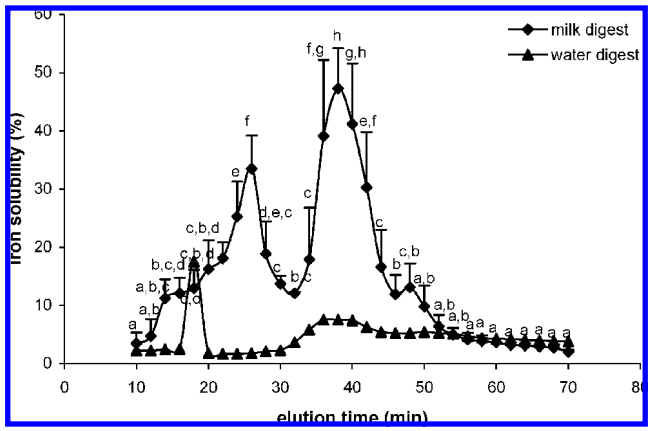

Figure 1. Iron solubility in fractions of a milk digest eluted through a Sephadex G-25 gel filtration column incubated with ferric chloride. Soluble iron was determined spectrophotometrically (means ± standard deviation, $n = 2$). Water digest was run once. Different letters suggest statistical difference: $P < 0.05$.

Amino Acid Determination in Selected Fractions Using GC-FID. Fractions A and B isolated through gel filtration were transferred into a vial insert and lyophilized in a vacuum concentrator. Acid hydrolysis of the protein in the sample followed, using vapor phase hydrolysis, to obtain conjugated amino acids in free form (EZFaast GC-FID for amino acid analysis, Phenomenex, Aschaffenburg, Germany). *N*-Alkoxycarbonyl ester derivatives of amino acids were analyzed on a GC-FID system (Fisons 8000 Series). Aliquots of the derivatized amino acids (2 μL) were injected at 1:15 split ratio at 250 °C into a Zebron column (ZB-AAA, 10 and 0.25 mm in diameter) programmed from 110 to 320 at 32 °C/min. Helium was used as a carrier gas at flow rate of 1.5 mL/min. The detector temperature was 320 °C. Quantification was performed using mixtures of amino acid standards and internal standard (NVal) provided by the kit.

Statistical Analysis. Data were analyzed by one-way ANOVA. Values of $P < 0.05$ were considered to be significant. Differences among samples were tested with the LSD test. Means were considered to be significantly different at the 95% confidence interval (*17*). Analysis of data was carried out with the program Statgraphics Statistical Graphics System, version 2.1 (Statgraphics, Rockville, MD).

RESULTS

The fractionation of a milk digest, with the aid of gel chromatography, produced two major peaks, eluting at 26 and 38 min, that enhanced iron solubility after a short incubation with $FeCl_3$ (**Figure 1**). The void volume was eluted at 15 min (data not shown) which corresponds to elution of peptides of molecular weight equal to or higher than 5000, according to the fractionation range of Sephadex G-25, which is 1000−5000. Vitamin B_{12} (MW = 1579) was eluted at 32 min (data not shown), thereby providing a relative indication on the molecular weight of the eluted fractions. Therefore, the molecular weight of the most promising peptides was between 5000 and 1579 (fraction A, eluted at 26 min) and between 1579 and 1000 (fraction B, eluted at 38 min).

Fractions A and B were analyzed for their amino acid contents (**Table 1**). In particular, in fraction A, the amino acids found in highest concentration were LEU, PRO, GLU, PHE, VAL, LYS, and HIS, which were determined at 1.65, 1.25, 1.55, 0.91, 0.73, 1.63, and 0.63 μmol/mg of sample, respectively. In fraction B, the amino acids found in highest concentration were LEU, PHE, and TYR at 0.74, 0.50, and 0.45 μmol/mg of sample, respectively, followed by PRO, MET, and GLU at 0.12, 0.11, and 0.19 μmol/mg of sample respectively.

Fractions A and B have been identified in previous experiments as the fractions that may enhance iron uptake from Caco-2 cells (*11*). These two fractions were compared for their effect on *DMT-1* expression, an intestinal iron transporter, when incubated with Caco-2 cells for 2 or 48 h (**Figure 2**). Relative *DMT-1* gene expression did not significantly differ between treatments and controls. There was, however, an increase in the expression of *DMT-1* from cells incubated for 48 h in comparison with those incubated for 2 h, but this increase was not significant ($P > 0.05$).

Fractions A and B were subsequently tested for their effect on iron dialyzability. Both fractions increased iron dialyzability by >2-fold in comparison to the control when added to PIPES buffer. Fraction B had a stronger effect than fraction A (**Table 2**). However, when added in milk, fractions A and B did not exhibit any effect on iron dialyzability, except when fraction A was added after lyophilization (**Table 2**). Subsequently, fractions were tested for their effect on iron dialyzability from two typical infant meals. There was no effects of milk peptide fraction A or B on iron dialyzability from a fruit meal or a vegetable meal (**Table 2**).

Table 1. Amino Acid Composition in Fractions of a Milk Digest Eluted through a Sephadex G-25 Gel Filtration Column[a]

amino acid	fraction A (μmol of amino acid/mg of sample)	fraction B (μmol of amino acid/mg of sample)
ALA	0.31	0.05
GLY	0.23	0.08
VAL	0.73	0.07
NVAL	0.15	0.10
LEU	1.65	0.74
ILE	0.23	0.02
THR	0.29	0.02
SER	0.33	0.03
PRO	1.25	0.12
ASP	0.39	0.06
MET	0.18	0.11
HYP	0.01	nd
GLU	1.55	0.19
PHE	0.91	0.50
LYS	1.63	0.09
HIS	0.63	0.04
HLY	0.02	nd
TYR	0.36	0.45
TPR	0.01	nd
C−C	nd	nd

[a] Amino acid determinations were performed with GC-FID. Abbreviations: ALA, alanine; GLY, glycine; VAL, valine; LEU, leucine; ILE, isoleucine; THR, threonine; SER, serine; PRO, proline; ASN, asparagine; ASP, aspartic acid; MET, methionine; HYP, 4-hydroxyproline; GLU, glutamic acid; PHE, phenylalanine; GLN, glutamine; LYS, lysine; HIS, histidine; HLY, hydroxylysine; TYR, tyrosine; TRP, tryptophan; C−C, cystine; NVAL, norvaline, used as internal standard, nd, not detected.

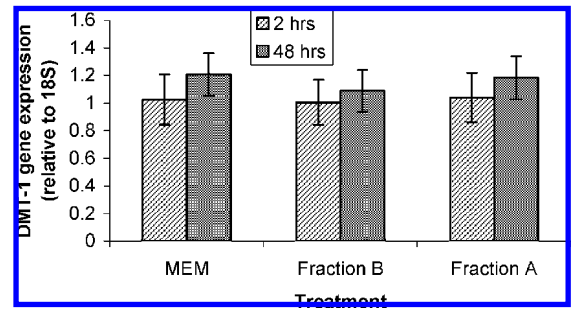

Figure 2. Relative gene expression of divalent metal transporter 1 (*DMT-1*) in Caco-2 cells incubated for 2 or 48 h with fractions of milk digest isolated by Sephadex G-25 gel filtration (means ± standard deviation, $n = 6$). Differences are not significant ($P > 0.05$).

Table 2. Dialyzable Iron Formed after in Vitro Digestion in a Series of Samples in the Presence of Milk Peptide Fractions (Fractions A and B) Separated by Gel Filtration from Milk Digested in Vitro[a]

	treatment	dialyzable iron (%)
control	PIPES	11.1 ± 2.6 a
	PIPES + fraction A	20.4 ± 2.3 b
	PIPES + fraction B	26.1 ± 0.4 c
milk	milk + PIPES	6.4 ± 3.7 a
	milk + fraction A	6.1 ± 1.8 a
	milk + fraction B	6.9 ± 1.5 a
	milk + lyophilized PIPES	7.7 ± 1.5 a
	milk + lyophilized fraction A	10.3 ± 1.9 b
	milk + lyophilized fraction B	8.7 ± 1.7 a
meals	fruit meal + PIPES	49.6 ± 0.7 a
	fruit meal + fraction A	45.1 ± 3.5 a
	fruit meal + fraction B	48.7 ± 6.3 a
	vegetable meal + PIPES	29.5 ± 2.6 b
	vegetable meal + fraction A	32.95 ± 1.61 b
	vegetable meal + fraction B	32.33 ± 0.15 b

[a] Results are expressed as percentage of iron added as $FeCl_3$ before incubation (means ± standard deviation, $n = 3$). Values with different letters within the same treatment group (control, milk, or meal) are significantly different: $P < 0.05$.

DISCUSSION

The first finding reported herein was that two milk peptide fractions, isolated after in vitro digestion of cow's milk, enhance the solubility of iron at pH values similar to those expected in the intestinal lumen (**Figure 1**). We observed in the past that these fractions also increased the uptake of iron by Caco-2 cells (*11*). The molecular weights of the most promising peptides were between 5000 and 1579 (fraction A, eluted at 26 min) and between 1579 and 1000 (fraction B, eluted at 38 min). Small peptides released during proteolytic digestion are thought by others to have a promoting effect on iron absorption (*6*−*10*). For example, casein peptides isolated from α_{s1}-casein, with amino acids 43−58, 59−79, amd 43−79, peptides isolated from α_{s2}-casein, with amino acids 1−25 and 46−70, and peptides isolated from β-casein, with amino acids 1−25 and 33−48, have been studied in various systems for their enhancing effect on iron absorption (*18, 19*).

Fractions A and B were analyzed for their amino acid contents (**Table 1**). Differences in the nature of the two fractions were observed, which may be further identified in the future. In particular, in fraction A, all amino acids were found in higher concentration in comparison with fraction B. In fraction A, among the amino acids, LEU, PRO, GLU, PHE, LYS, and HIS were found at highest concentration. This may be related to the difference in the MW of fractions A and B. Presumably the iron solubilization, particularly by fraction B, may be due to the presence of amino acids as well as other putative unidentified factors. For example, in the past it has been suggested that in other protein sources, in particular fish and chicken muscle, glycosaminoglycans are effective stimulants of iron uptake in Caco-2 cells (*20*).

The second finding was that these two selected milk peptide fractions did not affect the expression of *DMT-1* mRNA in Caco-2 cells (**Figure 2**). DMT-1 is the major intestinal iron transporter, identified among a number of genes involved in iron metabolism. In Caco-2 cells, treatment with iron for 72 h down-regulates *DMT-1* mRNA expression (*21, 22*). It is not known, however, whether milk constituents exert an indirect effect on the expression of *DMT-1*. In this experiment, it was hypothesized that iron, in the presence of milk peptides, reaches the DMT-1 in a chelated form and therefore produces changes to the *DMT-1* expression, similar to those seen in iron repletion depending on the availability of the chelated iron. Consequently, gene expression analysis was included to determine whether the milk peptide fractions might have affected the expression of the gene encoding for DMT-1.

Our finding suggests that the milk digest fractions did not affect gene encoding for DMT-1 in Caco-2 cells, although in these cells an increase in iron uptake was observed in a previous study when milk peptide fractions were present (*11*). Various explanations may be offered for this observation: First, DMT-1 may not be the only transporter involved in iron uptake by the Caco-2 cells. Although DMT-1 has been reported as the main iron transporter in the intestine, recently, at least for heme iron, other transporters have been discovered, which appear to function independently of DMT-1 through different mechanisms that are still not fully investigated (*23, 24*). Second, the pretreatment procedure applied to the cell cultures may have affected *DMT-1* gene expression. In particular, cells were exposed to MEM for 24 h. The absence of iron in the media may have induced up-regulation of the *DMT-1* gene expression in all treatment groups, masking thereby a potential effect on the *DMT-1* gene expression in the presence of milk peptide fractions (*25*). Third, exposure of cells to the treatment for 48 h may not be sufficient for inducing measurable changes in *DMT-1* mRNA (*21, 22*). Finally, it must also be mentioned that, although Caco-2 cells have been used for studying the expression of *DMT-1* as an indicator of adaptation of cells to iron treatment, it has been suggested that regulation of iron transport in the enterocyte may be related mostly to alterations to villus structure (*26*). This suggests a potential limitation of Caco-2 cell monolayers, or in vitro studies in general, particularly in the study of iron transport regulation.

The third finding was that the milk peptide fractions, isolated after the in vitro digestion of milk (**Figure 1**), enhance iron solubility in fractions of a milk digest and subsequently may preserve their enhancing properties after subjection to a second in vitro digestion. This finding is supported by results that show that milk peptides enhance iron dialyzability (**Table 2**), thereby demonstrating that the properties of the peptide fractions are not affected by the physicochemical transformations occurring under conditions that simulate the gastrointestinal environment. This finding encourages further investigation of the properties of milk peptides in food systems as a potential functional ingredient.

The fourth finding was that the milk peptide fractions do not enhance iron dialyzability from milk or from a commercial fruit or vegetable infant meal (**Table 2**). This finding is supported by results that show that the presence of milk peptides did not have any effect on the dialyzability of iron in any of the products tested, despite the fact that on a weight basis the fractions constituted a great percentage of the total ingredients. It therefore appears that milk peptides have properties that are not strong enough to overcome the effect of other dietary factors. Therefore, when milk peptides are added in water, an increase of 2-fold is observed on iron bioavailability. However, when added in milk, which is a food that inhibits iron bioavailability (*3, 4*), no enhancing effect of milk peptides is observed. Moreover, when milk peptides are added in the fruit or vegetable meals which, although cooked, may be high in ascorbate, a well-known enhancer of iron dialyzability, no additional effect of milk peptides is observed. Iron dialyzability, evaluated after the in vitro digestion of meals, has been employed as an index for the prediction of iron bioavailability (*15, 27*).

The fifth finding was that milk peptides when concentrated through lyophilization may produce a significant effect on the enhancement of iron dialyzability from milk. In particular, a small but significant effect was observed in samples containing fraction A only. This finding encourages further investigation of methods that allow the concentration of milk peptide fractions, which may be further useful in potential applications in formulating new products.

One plausible explanation for the lack of observed effect on fraction B may be related to the lower content of all amino acids in fraction B in comparison to fraction A (**Figure 2**). Some of these amino acids have been studied for their effect on iron bioavailability, in particular, the amino acids VAL, LEU, PRO, GLU, LYS, and HIS. The effect of histidine as a potential enhancer of nonheme iron bioavailability has been suggested previously (*28*). In particular, it was suggested that the enhancement of nonheme iron absorption by beef may be due to peptides produced during gastrointestinal digestion and that histidine content may be important, whereas a weak trend was also found in lysine content. Furthermore, the amino acids GLU, LEU, VAL, and PRO are also contained in the caseinophosphopeptide (1−25) of β-casein, which was reported to have an enhancing effect on iron uptake by Caco-2 cells (*29*).

The methodological approach used in this study for the isolation of milk peptide fractions begins with an in vitro digestion process. It appears that this approach has the advantage of producing peptides or other constituents that preserve their properties when subjected to a second in vitro digestion process, suggesting that they may be resistant to further proteolytic action. This property has to be further investigated in vivo, as it is a major issue in the exploitation of potential applications in foods. Nevertheless, generating milk peptides through in vitro digestion may produce valuable fractions and therefore deserves further exploitation in comparison to other casein enzymatic hydrolysis approaches or isolation of casein fractions through ultrafiltration. This has been recently suggested by other researchers as well (*30*).

In conclusion, our results suggest that during the in vitro digestion of milk, peptide fractions that enhance iron solubility are formed and may be isolated. In a Caco-2 cell model and specific test conditions, these peptide fractions do not affect expression of *DMT-1*. Milk peptides preserve their enhancing properties after in vitro digestion, but they have a limited effect on iron dialyzability from milk and fruit and vegetable meals. However, lyophilized peptide fractions may enhance iron dialyzability in milk. The findings in this study suggest that it may be possible to identify in milk, digested under gastrointestinal conditions, factors that under certain conditions may enhance iron absorption. These milk peptide fractions may be further investigated with the aim of finding potential applications in new products.

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