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Peptides Isolated from in Vitro Digests of Milk Enhance Iron Uptake by Caco-2 Cells

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Milk proteins, during digestion, produce a range of biologically active peptides. Among those are peptides that may enhance iron absorption. The objective of this project was to investigate the effect of isolated milk peptides on iron uptake. Cow's milk, 0% fat, was subjected to a modified in vitro digestion process. The milk digest was further fractionated by gel filtration. All eluted fractions as well as β-casein synthetic peptides (a tripeptide and a hexapeptide) were subsequently tested for effects on iron uptake with Caco-2 cell monolayers. Fractions of milk digests obtained through Sephadex G-25 gel filtration had a significant enhancing effect on iron uptake in Caco-2 cells compared to nonfractionated milk digests. Two fractions ($P = 0$) and the hexapeptide ($P < 0.0001$) enhanced iron uptake by up to 3-fold, whereas others and the tripeptide had no effect. These results suggest that selected peptides produced during the in vitro digestion of milk may enhance iron absorption; however, it remains to be demonstrated whether this effect may be nutritionally significant.

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

INTRODUCTION

Milk is a food of high nutritional quality. However, some studies indicate that bovine milk provides iron of low bioavailability and may inhibit absorption of nonheme iron present in a meal (*1, 2*). This has been attributed in part to the protein fraction of milk; most proteins of animal and plant origin, such as casein, egg albumen, or soy protein isolate, inhibit iron absorption. Meat, however, is a well-known enhancer of iron absorption. It has been suggested that peptides formed in the lumen during gastrointestinal digestion of protein bind iron and form complexes; depending on the physicochemical properties of the chelating peptide, the resulting iron complex may be available or not available for absorption. In particular, it has been proposed that peptides of small molecular weight or rich in cysteine may form iron complexes of high bioavailability (*3*). It follows that the effect of various peptides, including milk-derived peptides, on iron absorption is difficult to predict.

Previous results suggest variable effects of milk peptides on iron absorption (*4*). Upon hydrolysis, milk yields some peptides that may exert a positive effect on cation absorption (*5*). Casein, one of the major milk proteins, reduces iron bioavailability (*3, 6*). However, the inhibitory effect of casein is diminished when casein is partially hydrolyzed prior to ingestion; one explanation is that hydrolyzed casein has higher solubility than intact casein under physiological conditions in the gastrointestinal tract; therefore, it may enhance iron bioavailability through the formation of soluble iron chelates (*2, 7*). Caseinophosphopeptides of low molecular weight, formed from α- or β-casein, may chelate iron. This protects iron from further physicochemical changes such as hydrolysis or polymerization associated with decreased iron absorption (*8*). These peptides are produced by enzymatic hydrolysis and have been shown to enhance iron absorption in a rat model (*9, 10*).

Milk peptides, formed during enzymatic digestion, have recently attracted attention because they exhibit a range of biological effects including opiate, antithrombotic, antihypertensive, immunomodulatory, and metal-binding properties. However, peptides that may be produced during the gastrointestinal digestion of milk have not been investigated for properties in relation to iron absorption. Clearly, identifying peptides of multiple functions should result in food applications that exert health-promoting properties.

The overall objective of the present study was to investigate whether milk peptides, produced during the gastrointestinal digestion, may enhance iron uptake. Although the ultimate aim is to demonstrate the effects of these peptides in vivo, knowledge of these potential biological properties in vitro can refine the strategy for in vivo experiments. In this study, the specific objective was to investigate the effect of selected milk peptides on iron uptake under in vitro conditions. Peptidic fractions isolated by gel filtration from milk digested in vitro according

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to a procedure that simulates gastrointestinal digestion as well as synthetic β-casein peptides were compared for iron uptake in a Caco-2 cell system.

MATERIALS AND METHODS

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

Chemicals. Unless otherwise stated, all reagents were purchased from Sigma Chemicals, St. Louis, MO. The tripeptide leucine–leucine–tyrosine (LLY), which corresponds to residues 191–193 of bovine β-casein, and the hexapeptide proline–glycine–proline–isoleucine–proline–asparagine (PGPIPN), which corresponds to residues 63–68 of bovine β-casein, were custom synthesized from Bachem Bioscience Inc. (Weil am Rhein, Germany).

Sample Preparation. A series of samples and controls were studied for their effect on iron uptake by Caco-2 cells. These were milk digested in vitro according to a procedure that simulates gastrointestinal digestion (described below), milk digests fractionated with gel filtration (described below), and two β-casein synthetic peptides, the tripeptide LLY and the hexapeptide PGPIPN. Peptide solutions at 1, 0.5, 0.25, and 0.125 mg/mL in PIPES 0.075 mol/L were prepared (pH 5.7).

In Vitro Digestion. A sample of 10 mL of cow's milk, 0% fat, pH adjusted to 2.8 with concentrated HCl 6 mol/L, was transferred to 100 mL screw cap vials containing 0.5 mL of pepsin suspension (suspended in 0.1 mol/L HCl at 4 g/100 mL), placed in a shaking water bath maintained at 37 °C, and incubated for 2 h in the presence of 0.5 mL of pepsin. At the end of this incubation, the pH of the sample was adjusted from 2.8 to 5.7 with 5 M NaOH. Subsequently, 2.5 mL of a pancreatin–bile salt mixture (0.2 and 1.2 g suspended in 100 mL of 0.1 mol/L $NaHCO_3$) was added to the sample and the incubation continued for another 2 h. At the end of this incubation period, the digest was collected, centrifuged at 9000*g* for 15 min for the precipitation of high molecular weight insoluble compounds and enzymes, and placed in a refrigerator at 4 °C to stop the digestion process.

Sephadex G-25 Column Fractionation. 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 0.075 M PIPES buffer, 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). 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. Vitamin B_{12} (MW = 1579), monitored spectrophotometrically, was employed as a standard to estimate the molecular weight of the eluted fractions. Each fractionation was repeated twice. Fractions collected were stored in the refrigerator for up to 2 h and pooled before the Caco-2 cell experiments.

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). 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 in the iron uptake experiments 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.

Effect of Samples on Caco-2 Cell Fe Uptake. A 0.5 mL aliquot from each eluted fraction, peptide solution, milk digest, ascorbic acid 0.4 mmol/L, or PIPES 0.075 mol/L 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 Fe. 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 24 h to allow ferritin formation, the contents of each well were aspirated, and the cells were washed with a 1 mL volume of the "rinse" solution, consisting of 130 mmol/L NaCl, 5 mmol/L KCl, and 5 mmol/L PIPES, at pH 7.0. The rinse solution was then aspirated, and a 1 mL volume of deionized water was placed on the cells. The plates were then placed on a rack such that the bottom of each plate was in contact with the water of a benchtop sonicator (Elma Transsonic Digital sonicator, Laboratory-Line Instruments), which was kept in a cold room at 4 °C. The cells were sonicated for 15 min, scraped from the plate surface, and stored at −20 °C until analysis for cell protein and ferritin.

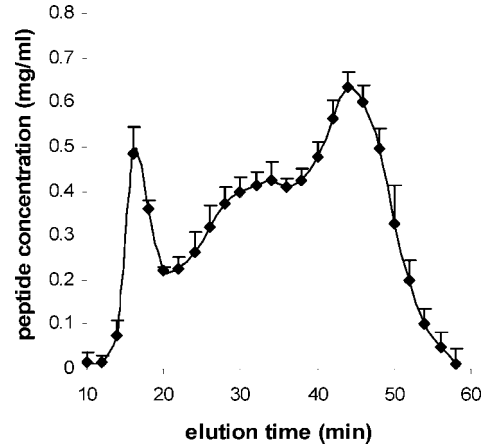

Figure 1. Peptide concentration in fractions of a milk digest eluted through a Sephadex G-25 gel filtration column with PIPES buffer, pH 5.7. Concentration was determined using a standard curve generated from a BSA standard (means ± standard deviation, $n = 2$).

Chemical Analyses. Cell protein was assessed using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA), based on the Lowry assay. Caco-2 cell ferritin was assessed using a one-stage sandwich immunoradiometric assay (FER-IRON II Ferritin Assay; RAMCO Laboratories, Houston, TX).

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 95% confidence interval (Zar, 1999).

RESULTS

The peptidic profile of the fractions of the milk digest generated through gel chromatography is illustrated in **Figure 1**. This fractionation produced two major peaks, eluted at 16 and 44 min (**Figure 1**). The elution time of the first peak at 16 min coincides with the elution time of the void volume and corresponds to peptides of molecular weight 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. The peak at 44 min corresponds to peptides with molecular weight around 1000. All other peptidic fractions eluted between 16 and 44 min and correspond to intermediate molecular weights (1000–5000).

All fractions of the milk digests eluted by the gel filtration column were compared for their effect on iron uptake by Caco-2 cells. Many of the fractions enhanced iron uptake by the cells in comparison to the PIPES control or the unfractionated milk digest ($P < 0.05$). However, only two fractions dramatically increased iron uptake in comparison to the unfractionated milk digest (**Figure 2**). These were the fractions eluted at 26 and 38

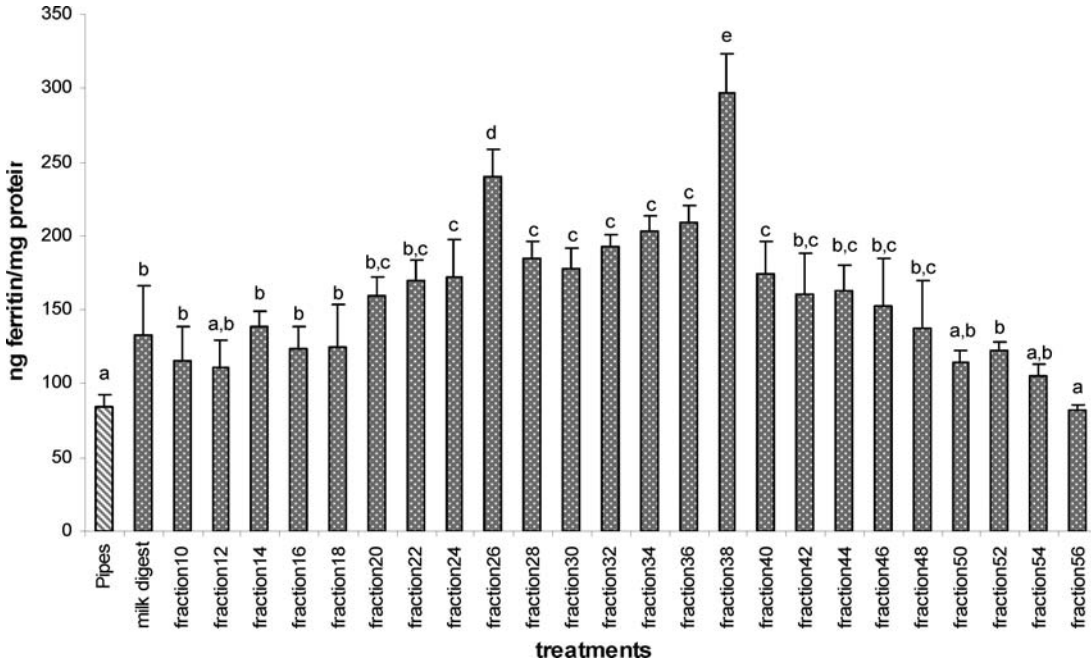

Figure 2. Ferritin formation in Caco-2 cells incubated with iron chloride and peptidic fractions. Peptides were released during an in vitro milk digestion process and fractionated through a Sephadex G-25 gel filtration column (means ± standard deviation, $P < 0.05$, $n = 3$).

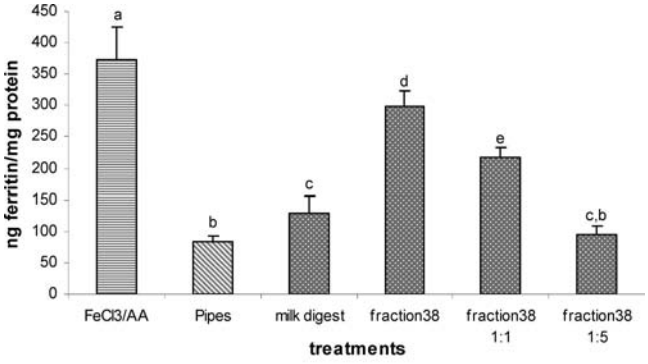

Figure 3. Ferritin formation in Caco-2 cells after incubation with fraction 38 at various dilutions and iron chloride. Peptides were released during an in vitro milk digestion process and fractionated through a Sephadex G-25 gel filtration column (means ± standard deviation, $P < 0.05$, $n = 3$).

min (named "fraction 26" and "fraction 38", respectively). Fraction 38 exhibited a stronger enhancing effect than fraction 26 ($P = 0.0001$). More specifically, in the presence of fractions 26 and 38, the amounts of ferritin synthesized in Caco-2 cells were 240 and 297 of ng ferritin/mg of protein, respectively. This corresponds to 2.8- and 3.5-fold increases in relation to the PIPES control (84.2 ng of ferritin/mg protein, $P = 0$) and 1.8- and 2.2-fold increases in relation to the nonfractionated milk digest (132 mg of ferritin/mg of protein, $P < 0.0001$), respectively. The enhancing effect of ascorbic acid was higher than that of fractions 26 and 38 (**Figure 3**).

Fraction 38, which had the most striking effect, was tested for a dose–response relationship (**Figure 3**). When fraction 38 was diluted at 1:1, a proportionally lower iron uptake by the Caco-2 cells was observed ($P = 0.008$). When it was diluted at 1:5, uptake was not different from that of the control ($P = 0.08$).

The β-casein synthetic peptides PGPIPN and LLY affected iron uptake in a different manner. In the presence of PGPIPN at 1 mg/mL, the amount of ferritin formed was higher in comparison to the control ($P < 0.0001$). At lower concentrations, the effect was lower but still significant in comparison to the control (**Table 1**). The tripeptide LLY did not have an enhancing effect on iron uptake in Caco-2 cells at any of the concentrations tested (**Table 2**).

Table 1. Ferritin Formation in Caco-2 Cells after Incubation with Iron Chloride and the Synthetic Hexapeptide PGPIPN at Various Concentrations (Means ± Standard Deviation, $P < 0.05$, $n = 3$)

treatment	ng of ferritin/mg of protein
PIPES	61.7 ± 8.7 a
hexapeptide, 0.125 mg/mL	87.3 ± 7.2 b
hexapeptide, 0.25 mg/mL	93.9 ± 10.8 b
hexapeptide, 0.5 mg/mL	95.7 ± 7.1 b
hexapeptide, 1 mg/mL	127.0 ± 38.2 c

Table 2. Ferritin Formation in Caco-2 Cells after Incubation with Iron Chloride and the Synthetic Tripeptide LLY at Various Concentrations (Means ± Standard Deviation, $P < 0.05$, $n = 3$)

treatment	ng of ferritin/mg of protein
PIPES	61.7 ± 8.7 a
tripeptide, 0.25 mg/mL	3.8 ± 1.5 b
tripeptide, 0.5 mg/mL	5.6 ± 2.2 b
tripeptide, 1 mg/mL	7.2 ± 1.8 b

DISCUSSION

The most important finding in the present study was that we identified milk-borne peptide fractions that exhibited an enhancing effect on iron uptake. In particular, we found two peptidic fractions that enhanced iron uptake in Caco-2 cells. These peptidic fractions were produced during an in vitro digestion process and were separated with the aid of gel filtration. 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. In particular, two fractions increased iron uptake in Caco-2 cells by >2-fold compared to nonfractionated milk digest ($P < 0.05$). Other fractions, however, did not increase uptake ($P > 0.05$) (**Figure 2**).

We speculate that the isolated peptidic fractions bind iron and render it available either through reduction of ferric iron to the more soluble and bioavailable ferrous form or by forming soluble complexes with ferric iron. It is possible that the active peptides in this peptidic fraction are caseinophosphopeptides. Caseinophosphopeptides, which are strongly phosphorylated peptides obtained by enzymatic digestion of caseins, have shown an enhancing effect on iron availability (*4*, *9*, *11*). Other casein peptides have been studied in various systems for their enhancing effect on iron absorption. These include peptides isolated from α_{s1}-casein, with amino acids 43–58, 59–79, and 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 (*5*, *10*, *12*).

The second important finding was that synthetic peptides of β-casein have variable effects on iron uptake; that is, one enhanced iron uptake, whereas the other had no effect. This finding further encourages the investigation of selected milk peptides as enhancers of iron absorption. More specifically, the hexapeptide PGPIPN exerted an enhancing effect on iron uptake in Caco-2 cells in a dose-responsive manner (**Table 1**). This peptide is rich in proline, and consequently we hypothesized that this effect may be attributed to this particular amino acid. It has been suggested that proline forms stable complexes with iron. The stability of these complexes might be further enhanced by the resistance of proline-rich peptides to digestion due to steric hindrance (*13*). On the contrary, the tripeptide LLY showed no enhancing effect, but it produced ferritin levels that were strikingly lower than those of the PIPES control (**Table 2**). Therefore, it is not clear whether this effect is attributed only to low iron transport. It may be related to the amino acid composition or other structural factors (e.g., size or conformation of the peptide). It is also possible that the tripeptide affects directly the molecule of ferritin, preventing its synthesis or transcription.

The lack of effect in one of the two peptides on iron uptake signifies the important property identified in the other because it suggests that in this experiment the methodology employed was able to detect differences in iron uptake by the cells.

The peptides PGPIPN and LLY have been investigated for their bioactivity in different functions. Specifically, it has been demonstrated that both peptides exert immunoenhancing properties (*14*). The finding that PGPIPN also enhances iron uptake reveals that this peptide may exert multiple functions in the human organism. It is of great interest to identify bioactive peptides with multiple functions in the body. These peptides may be further investigated for applications in foods. According to the findings of this study, the hexapeptide PGPIPN is a promising candidate for this role; in this case, apart from enhancing iron absorption, the peptide PGPIPN also exhibits immunomodulatory properties.

The methodological approach used in this study for the isolation of peptides begins with an in vitro digestion process to generate milk-derived peptides. This approach has the advantage of producing peptides that may be resistant to any further proteolytic action; conditions in the in vitro model include incubation for 4 h at 37 °C, at different pH values, in the presence of peptic and pancreatic enzymes. It may be hypothesized that peptides that have been produced under simulated gastrointestinal conditions are able to resist further degradation if ingested. This property has to be further investigated in vitro and in vivo, as it is a major issue in the exploitation of potential applications in foods (*15*). In this respect, generating milk peptides through in vitro digestion may provide fractions with characteristics different from those of other casein enzymatic hydrolysis approaches or isolation of casein fractions through ultrafiltration. Although these approaches have been proposed and have produced results that have further developed peptide research, gastrointestinal digestion approaches such as the one proposed herein may also generate valuable fractions. This has been recently suggested by other researchers as well (*16*).

In this study, the milk digest was further fractionated using a Sephadex G-25 gel filtration column. The Sephadex G-25 resin (fractionation range = 1000–5000 Da) was selected for the fractionation step because small peptides released during proteolytic digestion are thought to have a promoting effect on iron absorption (*17–19*). The molecular weights of the most promising peptides were between 5000 and 1579 (fraction eluted at 26 min) and between 1579 and 1000 (fraction eluted at 38 min). Interestingly, there seems to be a dose–response relationship between this enhancing effect and the concentration of the peptide that exhibits the effect (**Figure 3**). The Caco-2 cell model applied in this study to assess Fe availability uses ferritin formation by Caco-2 cells as an indicator of Fe uptake. Ferritin formation by Caco-2 cells occurs in response to Fe uptake at concentrations of available Fe greater than that of the culture media to which the cells have been adapted. This model has been used by various scientists to estimate iron availability because it eliminates the need for extrinsic or intrinsic labeling, it has a low cost, and it is easy to use (*20–24*).

The findings in this study are important because they suggest that it may be possible to identify in milk digested under gastrointestinal conditions factors that enhance iron absorption. Milk, a nutritious staple food in the diet of infants and children, provides iron of low concentration and bioavailability and inhibits the bioavailability of nonheme iron from other foods present in the same meal (*1*, *2*). Fresh, ultrahigh-temperature, and condensed cow's milk products and milk-based infant formulas fortified with iron, usually in the presence of ascorbic acid (an enhancer of iron absorption), are available in many countries. Choosing the most appropriate iron fortificant for milk has been a challenge (*25*). In any case, concerns regarding iron fortification at high levels have been expressed (*26*, *27*). An alternative approach, in line with these concerns, is to lower the amount of added iron and to employ milk factors that would enhance iron absorption. Milk peptides are a plausible candidate for this role.

In conclusion, our results suggest that during the in vitro digestion of milk, peptides are produced that increase iron uptake by Caco-2 cells. This suggests that these milk peptides may enhance iron bioavailability. This study presents results that lead to a series of experiments. These include the identification and the amino acid sequence analysis of the active peptides in the peptidic fraction and in vitro and in vivo experiments that may show that these peptides may be employed for the development of functional foods.

ABBREVIATIONS USED

LLY, leucine–leucine–tyrosine; PGPIPN, proline–glycine–proline–isoleucine–proline–asparagine.

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