Carbohydrate Fractions from Cooked Fish Promote Iron Uptake by Caco-2 Cells[1]

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ABSTRACT The objective of this study was to isolate and characterize the meat factor(s) that enhances nonheme iron bioavailability using various analytical and in vitro cell culture techniques. Nonheme iron bioavailability was measured via radiolabeled iron uptake or ferritin formation in Caco-2 cells. Fish haddock fillet was cooked and lyophilized to be used as the muscle tissue of choice because of its low intrinsic iron content. It was demonstrated that the low pH of the stomach (pH 2.0) was the primary factor responsible for initiating the enhancing effect of fish on nonheme iron uptake. Subsequently, cooked fish samples were titrated with HCl to pH 2.0 and incubated for 1 h without digestive enzymes to release the factor(s) from the fish. The supernatant of this acidic digest was then used as a starting material for the meat factor isolation procedures. Fractions generated through Sephadex G-25 size exclusion increased Caco-2 cell iron uptake approximately 9-fold. Subsequent chromatography of these fractions via C18 reverse-phase HPLC were conducted, and enhancing activity was observed only in the "injection peak." This observation coupled with protein measurement and amino acid composition analysis revealed that the active fractions contained negligible amounts of proteins or amino acids. Active fractions were highly enriched with carbohydrates. Subsequent chromatography via high performance anion exchange chromatography with pulsed amperometric detection yielded 3 active peaks that increased Caco-2 cell iron uptake 3.4- to 4.9-fold. Our results indicate that specific carbohydrates contribute to the enhancing effect of meat on iron uptake by the enterocyte. These carbohydrates may be oligosaccharides originating from glycosaminoglycans in the extracellular matrix of muscle tissue. J. Nutr. 134: 1681–1689, 2004.

KEY WORDS: • *meat factor* • *iron* • *in vitro digestion* • *Caco-2 cells* • *oligosaccharides*

The consumption of muscle tissues (meats) enhances nonheme iron absorption in humans (1–7), animals (8,9), and in vitro (10–14) models. This effect was first published in the late 1960s, when investigators observed that iron absorption from vegetable foods was improved with veal or fish muscle (4,15). Since the enhancing effect from muscle tissues was found, many attempts have been made to determine how muscle tissues enhance nonheme iron absorption (3,6,10–12,16–20). However, the enhancing effect of muscle tissues remains elusive, and thus is commonly called the "meat factor."

Several mechanisms have been proposed for the "meat factor." Some researchers believe that enhancement of nonheme iron absorption is indirectly facilitated via gastric factor(s). For instance, there is some evidence that consumption of meat stimulates the secretion of gastric acid, which may provide a more acidic environment in which to solubilize nonheme iron (21). This evidence is supported by the finding that iron-deficient patients with achlorhydria absorb less iron than those with a normal acid output (22,23). However, there are many in vitro studies showing the enhancement of nonheme iron uptake with meats, supporting the fact that dietary factors are responsible for the meat effect. Some researchers believe that certain amino acids and peptides released from muscle proteins by proteolytic digestion enhance iron absorption by reducing dietary iron to the more absorbable ferrous (Fe^{2+}) form, or by forming soluble iron complexes that are easily taken up by mucosa cells (3,17,19,24). However, one study reported that dialysis products from either digested or nondigested meat homogenates increased soluble iron at pH 7 in vitro, which indicates that proteolytic digestion is not necessary for iron solubilization by meat (25). Cysteine or cysteine-containing peptides were considered as probable candidates for the promotional effect of meat on iron absorption because they were shown to reduce ferric (Fe^{3+}) iron to the more bioavailable ferrous (Fe^{2+}) iron (26). Also, in vivo and in vitro studies showed enhanced nonheme iron availability in the presence of cysteine (3,19,24). However, the observation that different sources of animal proteins (e.g., chicken egg albumin) with about the same amino acid composition as muscle protein did not similarly enhance nonheme iron absorption (1,2) does not support the role of the sulfhydryl group as a major part of the meat factor. Histidine was also implicated as part of the meat effect in several studies (17,28,29); however, the results are inconclusive.

We utilized the Caco-2 cell line as a screening tool for the

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following reasons: *1*) the number of samples or treatments in animal or human studies is seriously limited relative to in vitro systems; *2*) bioavailability estimates based on soluble or dialyzable iron were shown to be inadequate for assessing iron bioavailability in some cases (13,30,31); *3*) the Caco-2 cell system could allow us to focus on dietary factors, instead of physiologic factors such as gastric acid secretion; and *4*) the Caco-2 cell iron uptake from meat (e.g., beef, chicken, and fish) was shown, in previous studies (13,30), to be 3- or 5-fold higher than that from other protein sources, which closely matched those of many in vivo studies. Also, an in vitro digestion technique was employed to conduct simulated peptic digestion followed by intestinal digestion in the presence of Caco-2 monolayers (14,32–34), which allowed us to determine digestion conditions that induce the meat effect. Furthermore, several different unique approaches were applied to the digestion and fractionation procedures so that the isolation procedures could be simplified and feasible for this study.

In the present study, isolation and characterization of the "meat factor" were performed via a combination of in vitro digestion, Caco-2 cell culture, and subsequent chromatographic purification techniques.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, all reagents were purchased from Sigma Chemical.

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 50,000 cells/cm^2 in collagen-treated 6- or 24-well plates (Costar). The cells were grown in DMEM with 10% v:v fetal bovine serum (FBS,[3] GIBCO), 25 mmol/L HEPES (Sigma), 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 were replaced every 2 d. The cells were used in the iron uptake experiments at 14 d postseeding. Under these conditions, the amount of cell protein measured in each well was highly consistent from well to well within each culture plate.

Meat samples. Fish was selected for this study as the muscle tissue of choice because it has a relatively low total iron concentration (~0.12 μmol Fe/g cooked powder) with minimal levels of heme iron (<0.01 μmol heme Fe/g cooked powder). Fish was therefore considered optimal because it provided an excellent source of muscle tissue with minimal iron contamination. The sample of fish was prepared from frozen fillets of haddock. It was purchased locally and all visible fat and skin were removed. A total of 200 g of the fillet was cut into 1.5- to 2.5-cm cubes, placed in 200 mL of deionized water, and then homogenized in a blender (Waring Products). The slurry was microwaved for 3 min and stirred at 1.5-min intervals. The cooked slurry was homogenized again in a blender with a 10-s pulse once, poured into ice cube trays, and frozen at −20°C. The frozen slurry was lyophilized and ground; the resulting powder was stored at −20°C.

Preparation of acidic extracts. Lyophilized cooked fish powder (0.9 g) was mixed with 30 mL of 0.01 mol/L HCl, and brought to pH 2.0 with 5 mol/L HCl. It was incubated on a rocking platform shaker (Reliable Scientific) at 37°C in an incubator with 5% CO_2 and 95% air for 1 h. After the incubation, the mixture was centrifuged (IEC Model HN-SII Centrifuge) at 4000 × *g* for 30 min. The supernatant was collected and lyophilized.

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. The swollen resin was transferred to 0.01 mol/L HCl at pH 2.0 for equilibration, and transferred onto a column (diameter, 1 cm; length, 32 cm, Flex-column chromatography column, Kontes). To equilibrate the column, 0.01 mol/L HCl was eluted overnight. The low rate was set at 1.0 mL/min. A 150-mg sample of the acidic extract was dissolved in 1.5 mL of 0.01 mol/L HCl at pH 2.0. The sample solution was applied to the column and eluted with 0.01 mol/L HCl at pH 2.0 in combination with a peristaltic pump (EP-1 Econo Pump, Bio-Rad Laboratories). Fractions (F_S) of 2.0 mL were collected into tubes with the aid of a fraction collector (Medel 328, Instrumentation Specialties). The elution of iron was monitored by the measurement of total iron using an inductively coupled argon plasma emission spectrometer (ICAP Model 61E Trace Analyzer, Thermo Jarrell Ash) after dry-ashing. The elution of protein and protein digestion products was monitored using a Bio-Rad DC protein assay kit, which is a commercial semimicro adaptation of the Lowry assay (Bio-Rad Laboratories).

Reverse phase-HPLC purification with protein and peptide C18 column. The fractions (F_R), collected from the gel chromatography, were applied to reverse-phase HPLC (RP-HPLC) on a protein and peptide C18 column (analytical: 4.6 mm i.d. or semipreparative column: 10 mm i.d. × 250 mm length, Vydac) equilibrated using water with 0.1% trifluoroacetic acid (TFA, v:v) at various flow rates. The linear gradient of 100% acetonitrile (ACN) (in 0.1% TFA) was applied from 0 to 60%. Elution was monitored at 210 nm. Each peak was collected and then evaporated with a spin evaporator (Labconco).

HPAEC-PAD purification with a CarboPac PA1 column. High performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) purification was performed using a CarboPac PA1 (Dionex) column (9 × 250 mm). Separation was achieved with a 100 mmol/L NaOH mobile phase at a constant flow rate of 3 mL/min. On-line desalting was performed with an anion micro-membrane suppressor (AMMS) using 0.3 mol/L TFA for neutralization. The AMMS was placed between the PAD detector cell and the fraction collector. The 3 major peaks between 5 and 10 min were collected from repeated injections; like fractions were pooled and then lyophilized. The purity of each fraction (F_H) was determined by HPAEC-PAD analysis as above except that an analytical CarboPac PA1 column (4 mm i.d.) was used without AMMS with a flow rate of 0.8 mL/min.

^{1}H NMR analysis of HPAEC-PAD fractions. HPAEC-PAD fractions with iron uptake activity were analyzed by ^{1}H NMR. Samples were exchanged twice with D_2O (99.96%; Cambridge Isotopes) and were dissolved in 50 μL of D_2O. Proton spectra were acquired with a 400-MHz Varian INOVA spectrometer at 25°C utilizing a Varian gHX Nano Probe (spinning at 2000 Hz). To decrease the intensity of the water peak, presaturation experiments were performed. The number of transients collected varied from 512 to 2048 scans with a sweep width of 6400 Hz, a total recycle time of 2.6 s, and 32,000 data points.

In vitro digestion/Caco-2 cell iron uptake on the 6-well culture plates. The 6-well culture plates coupled with inserts carrying a dialysis membrane with a molecular weight cutoff of 15 kDa (Spectra/Por Regenerated Cellulose, Spectrum Medical Industries) were prepared according to the procedure of Glahn et al. (14) except for the use of 0.5 mol/L HCl instead of 70% ethanol to eliminate mineral contamination from the dialysis membrane fastened to the insert ring. Briefly, the day before starting the in vitro digestion, DMEM (GIBCO) was aspirated from the cells and replaced with 2 mL of MEM (GIBCO). Plastic inserts were formed by fitting the bottom of an appropriately sized Transwell insert ring (Costar) with a dialysis membrane. After fastening the dialysis membrane to the insert ring, the entire unit was soaked in 0.5 mol/L HCl and kept in sterile water until use. The next day, the cells were washed with 2 mL of MEM and then covered by a fresh 1-mL aliquot of MEM before the intestinal digestion period. The inserts with attached dialysis membranes were aseptically placed in the wells of cell plates and companion plates (6-well plates devoid of cells used to measure the bottom chamber iron dialyzed across the membrane), thus creating the two-chamber system.

Details of the in vitro digestion method were described elsewhere (14). This study introduced a slight modification to the preparation of

[3] Abbreviations used: AA, ascorbic acid; ACN, acetonitrile; AMMS, anion micro-membrane suppressor; FBS, fetal bovine serum; F_H, HPAEC-PAD fraction; F_R, C18 RP-HPLC fraction; F_S, Sephadex G-25 fraction; HLGAG, heparin-like glycosaminoglycan; HMBC, heteronuclear multiple bond correlation; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid.

the digests to determine the critical aspect of digestion required to initiate the promoting effects of fish on intestinal nonheme iron uptake. In the first series of experiments (**Fig. 1**), digests containing $FeCl_3$ (41.7 μmol/L) with or without fish (300 mg) were subjected to varying degrees of digestion to determine the cumulative effects of low pH, pepsin, and the pancreatin/bile digestion on iron availability in the presence of the cooked fish muscle tissue. The first two digest conditions involved no low pH treatment, pepsin digestion, or pancreatin/bile digestion of the samples. For these digests, $FeCl_3$ and $FeCl_3$ + fish were placed in tubes with 10 mL of 130 mmol/L NaCl, 5 mmol/L KCl, and 5 mmol/L PIPES. The pH was adjusted to 7.0 and the samples were incubated on a rocking shaker at 37°C for 1 h. After 1 h, these digests were brought to a total volume of 15 mL, and a 1.5-mL aliquot was placed in the upper chamber of the designated culture wells. The culture plates were then incubated at 37°C for 2 h on a rocking shaker. These digests were labeled "Fe" and "Fe/Fish." The next pair of digests was identical to the first pair, except that the pH was initially adjusted to 2.0 with HCl and they were incubated at 37°C for 1 h. Then, these digests were adjusted to pH 7.0 with 1 mol/L $NaHCO_3$ before adjustment to a total volume of 15 mL. These digests were labeled "Fe/pH2" and "Fe/Fish/pH2." The next set of digests was identical to the previous set, except pepsin was added during the 1 h incubation period at pH 2.0. These digests were labeled "Fe/Pep" and "Fe/Fish/Pep." The final set of digests was subjected to the complete in vitro digestion process; thus, pancreatin and bile extract were added after pepsin digestion at pH 2.0 and titration to pH 7.0. As for the other digests, the total volume was adjusted to 15 mL. The second series of experiments (**Fig. 2**) was designed to reproduce the results of the first series. In addition, we measured total soluble iron and the total soluble ferrous (Fe^{2+}) iron in each preparation at the start of the pancreatin/bile digestion period. The digest conditions were identical to those listed above, with the addition of two other digests. A "blank" sample contained only the 130 mmol/L NaCl, 5 mmol/L KCl, and 5 mmol/L PIPES, at pH 7.0. No adjustments in pH were made to this sample, and it was incubated under the same conditions as the other digests. The second added condition was "Fe + AA." For this sample, $FeCl_3$ (41.7 μmol/L) was mixed with ascorbic acid (AA, 1.0 mmol/L) at pH 2.0, then adjusted to pH 7.0 and incubated in identical fashion to the other digests.

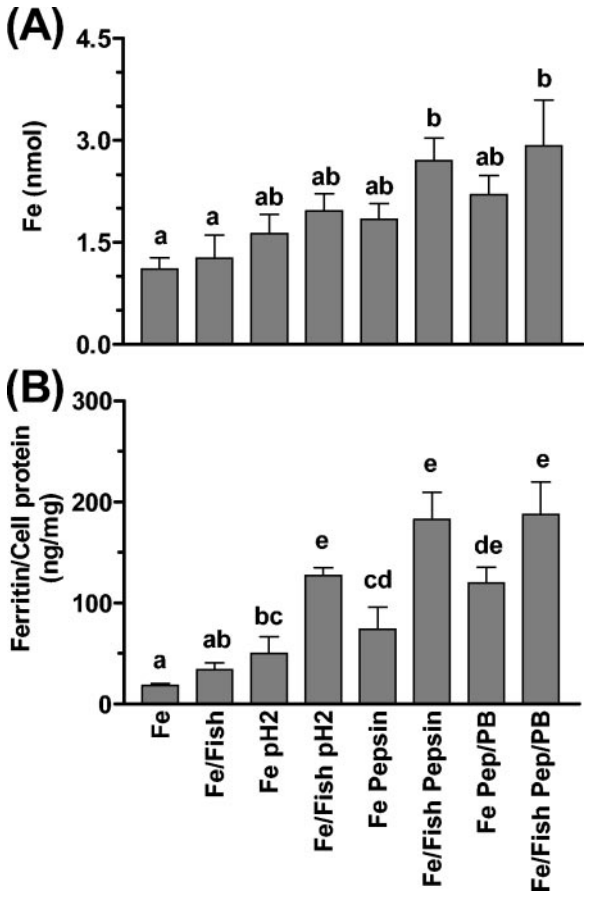

FIGURE 1 Measurement of bottom chamber iron (*A*) and Caco-2 cell ferritin formation (*B*) for the determination of the component of digestion required to induce the enhancing effect of fish on nonheme iron uptake. Digestion conditions tested the following factors on nonheme iron uptake: *1*) the presence of fish (Fe, Fe/Fish); *2*) the effect of low gastric pH (Fe pH2, Fe/Fish pH2); *3*) the effect of low gastric pH with pepsin (Fe Pepsin, Fe/Fish Pepsin); and *4*) the effect of complete in vitro digestion (Fe Pep/PB, Fe/Fish Pep/PB). Abbreviation used: Pep, pepsin; PB, pancreatin/bile. The *upper panel* represents the amount of iron measured in the bottom chamber with no cells present at the end of the intestinal digestion period. The *lower panel* represents Caco-2 cell ferritin formation measured 24 h after the start of the intestinal digestion period. Values are means ± SEM, $n = 5$. Means without a common letter differ, $P < 0.05$.

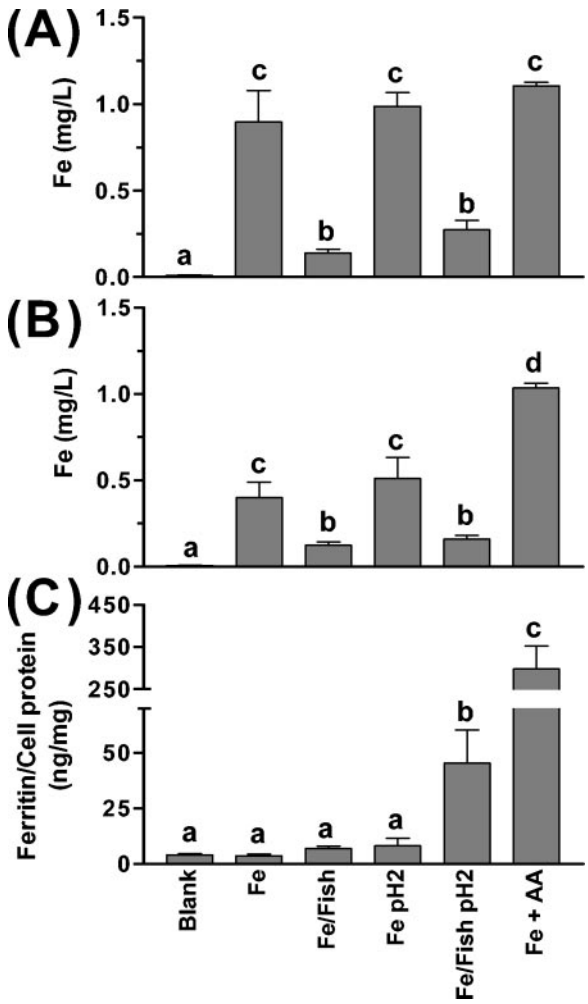

FIGURE 2 Measurement of total soluble iron (*A*), soluble ferrous (Fe^{2+}) iron (*B*), and Caco-2 cell ferritin formation (*C*) from digests of $FeCl_3$ and fish (Fe/Fish, no digestion; Fe/Fish pH2, low gastric pH treatment). Digests containing no added fish (Fe and Fe pH2) were used as controls compared with Fe/Fish and Fe/Fish pH2, respectively. A digest containing iron and 1.0 mmol/L ascorbic acid (Fe + AA) was used as a positive control. The total amount of iron placed in each digest was 2.32 μg/mL, except for the blank digest which received no added iron. Values are means ± SEM, $n = 4$. Means without a common letter differ, $P < 0.05$.

At the start of the intestinal digestion period, a 1.5-mL aliquot of the digest was placed into the upper chamber. The plate was covered and incubated at 37°C for 2 h on top of a rocking platform (model RP-50, Laboratory Instruments). After the 2-h incubation, the inserts were carefully removed and an additional 1 mL MEM was added to each lower chamber. The bottom chamber contents of the companion plates were transferred to tubes and stored at −20°C for future iron analysis. The cell plates were then placed back in the incubator for a further 22 h (24 h from start of intestinal digestion) to allow time for ferritin formation.

Harvesting of Caco-2 cell monolayer for ferritin analysis. The cells were harvested as described by Glahn et al. (14). Briefly, 24 h after the start of the intestinal digestion period, the medium covering the cells was removed and the cells were washed twice with a 2 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 2-mL volume of deionized water was placed on the cell. 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, Lab-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.

Iron uptake on the 24-well culture plates. To study the effect of fraction samples on iron uptake, 24-well culture plates were used with the addition of radiolabeled iron (**Figs. 3**, **4**, and **Table 1**). The cell medium of a 24-well plate was replaced with 0.5 mL MEM. The next day, before the uptake experiment, the cell layer was washed with 37°C MEM at pH 7, and 0.5 mL MEM was placed on each monolayer.

For the iron uptake experiments, a radiolabeled iron solution was prepared by combining $^{59}FeCl_3$ (iron-59, iron chloride in 0.5 mol/l hydrochloric acid, NEN Life Science Products) with $FeCl_3$ (1040 mg Fe/L $FeCl_3$ standard in 0.1 mol/L HCl). The amounts of added ^{59}Fe and $FeCl_3$ standard solutions were adjusted to provide ~11 kBq/mL of uptake solution and an iron concentration of 10 μmol/L. The samples for the iron uptake experiment were constituted in 300 μL of 0.01 mol/L HCl (pH ~2.0). The prepared radiolabeled iron solution (15 μL) was added into the sample solutions, and then 30 μL of 1.5 mol/L NaCl and 455 μL MEM were added subsequently in the order listed. For starting the uptake, 500 μL of the prepared uptake solution was placed onto the appropriate Caco-2 cell monolayer. At the end of the 2 h uptake, the cell monolayer was harvested for quantification of ^{59}Fe via gamma counting. As described above, fish muscle tissue contained very little intrinsic iron (~0.12 μmol Fe/g cooked fish powder). Accordingly, all fraction samples used in iron uptake experiments contained an equal amount of iron, which was almost entirely (>96%) extrinsically added nonheme iron (10 μmol/L of $FeCl_3$).

Harvesting of Caco-2 cell monolayer for ^{59}Fe measurement. The cells on 24-well culture plates were washed once with 0.5 mL of a "rinse" solution at pH 7. The rinse solution was then aspirated and 0.5 mL of a freshly prepared "removal" solution was placed on the cell monolayer for 10 min to remove surface-bound iron. The removal solution consisted of the above rinse solution with an additional 5 mmol/L sodium hydrosulfite and 1 mmol/L bathophenanthrolene

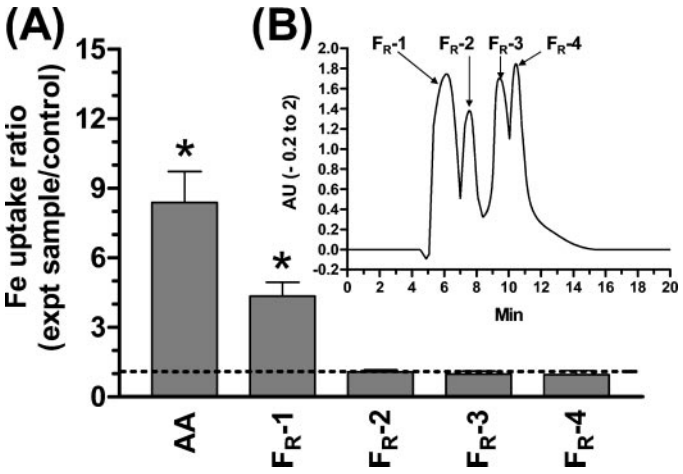

FIGURE 4 Measurement of Caco-2 cell iron uptake (*A*) from the 4 fractions (F_R-1, 2, 3, and 4) collected by RP-HPLC. The collected fractions were reconstituted into 300 μL of 0.01 mol/L HCl solution. All uptake solutions were designed to contain 10 μmol/L $FeCl_3$ as an iron source. The uptake solutions containing $FeCl_3$ and $FeCl_3$ with ascorbic acid (Fe:AA = 1:20) without any fraction were used as baseline (dashed line) and positive (AA) controls, respectively. The inset figure (*B*) represents a typical RP-HPLC chromatogram of the mid-fractions (F_S-10, 11) prepared from fish Sephadex G-25 fractionation. Values are means ± SEM, $n = 4$ for AA and F_R-1; $n = 2$ for F_R-2, 3, and 4). *Different from the baseline control, $P < 0.05$.

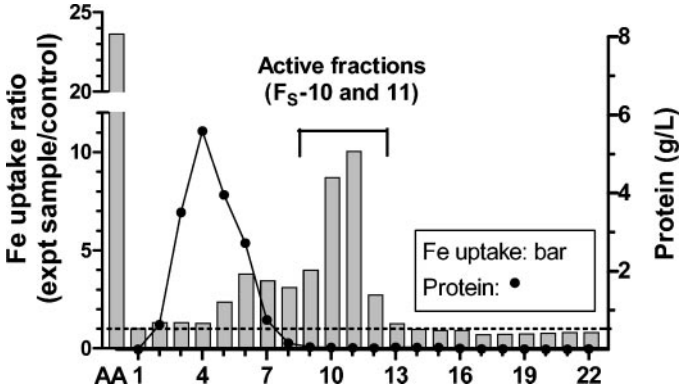

FIGURE 3 Results of a typical radiolabeled iron uptake screening experiment and protein concentrations from the fish Sephadex G-25 fractions (F_S-1 to 22). All uptake solutions were designed to contain 10 μmol/L of $FeCl_3$ as an iron source. The control uptake solution (dashed line) received only 10 μmol/L of $FeCl_3$. The uptake solutions containing $FeCl_3$ with ascorbic acid (Fe:AA = 1:20) were used as a positive (AA) control. Uptake levels were expressed as fractions of the value of the baseline control. Elution of protein products is indicated by the measurement of total protein in the fractions using a semimicro adaptation of the Bio-Rad DC protein assay kit.

TABLE 1

Radiolabeled iron uptake in Caco-2 cells from the 3 fractions of muscle collected by HPAEC-PAD[1]

Fraction	Radiolabeled iron uptake	
	Trial A[2]	Trial B[3]
	%	
Control[4]	2.3	3.9
F_H-1	7.9	6.4
F_H-2	7.9	7.4
F_H-3	11.3	16.3

[1] Radiolabeled iron uptake in Caco-2 cells. The collected fractions for the peaks were dried and then reconstituted in 300 μL of 0.01 mol/L HCl solution. All uptake solutions were designed to contain 10 μmol/L of $FeCl_3$ as an iron source.

[2] Each fraction was isolated from fish muscle, and equivalent amounts (2.3 mg) of the fraction samples were used for iron uptake.

[3] Fractions were prepared from white breast chicken muscle by the identical procedures for fish muscle, and the retention times of the fractions matched those from fish.

[4] The uptake solution containing $FeCl_3$ without any fraction was used as a baseline control.

disulfonic acid, to ensure removal of nonspecific bound iron from Caco-2 cell monolayers without damaging the brush border membrane (35). After the removal period, the removal solution was aspirated and the cell monolayer was washed with 0.5 mL of the "rinse" solution. After aspirating the rinse solution, 1.0 mL of a 0.5 mol/L NaOH solution was placed on each monolayer to solubilize each monolayer, and then the cells were scraped from the plate. The cell suspension was transferred into scintillation vials for counting in a gamma counter.

Analyses. All glassware used in the sample preparation and analyses were rinsed with 10% HCl and deionized water before use to avoid mineral contamination. Caco-2 cell protein was measured on samples that had been solubilized in 0.5 mol/L NaOH, using a Bio-Rad DC protein assay kit, which is a commercial semimicro adaptation of the Lowry assay (Bio-Rad Laboratories). Protein in the meat samples and fractions was determined using the method described above. ^{59}Fe was counted in an automatic gamma counter (Packard Auto-Gamma model 5530, Packard Instruments). An immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-IRON II Serum Ferritin Assay, RAMCO Laboratories). A 10-μL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL, was used for each ferritin measurement. Analyses of the iron content of the experimental solutions, samples, and digests were conducted using an inductively coupled argon plasma emission spectrometer (ICAP Model 61E Trace Analyzer, Thermo Jarrell Ash Corporation) after dry-ashing. A colorimetric assay using ferrozine as the colorimetric reagent was used to quantitate the total soluble iron and soluble ferrous iron present in the digests (36). Centrifugation for 10 min in a microcentrifuge at 15,000 $\times$ *g* was used to separate the insoluble iron in each sample. Samples of the each digest were collected immediately at the start of the 2-h incubation of the digests. The colorimetric assay was conducted within minutes of this time point. Amino acid analyses of samples were conducted by HPLC using the Waters PicoTAG method (Waters Chromatography Division).

Statistical analysis. Data were analyzed by one-way ANOVA after testing for normality and equal variance with Prism software (GraphPad Software). Samples that had unequal variances were transformed logarithmically and analyzed with one-way ANOVA. Dunnett's post-test was used to compare the means to the control; Tukey's post-test was used to compare pairs of means. Differences were considered significant at $P < 0.05$.

RESULTS

Determination of digestion conditions to induce the meat effect. The results on the digestion step(s) required to induce the enhancing effect of fish on nonheme iron uptake, as measured by ferritin formation, are summarized in Figures 1 and 2. The amount of bottom chamber iron tended to increase with the addition of each digestive step, but no striking differences were observed (Fig. 1). The addition of fish with the pH maintained at 7.0 did not affect ferritin formation (Fe vs. Fe/Fish). Reducing the pH to 2.0 for 1 h increased ferritin formation by 171% from digests without fish (Fe vs. Fe pH 2.0). The addition of fish coupled with incubation at pH 2.0 for 1 h increased ferritin formation by an additional 154% (Fe pH 2.0 vs. Fe/Fish pH 2.0). No further significant increases in ferritin formation were observed with the addition of pepsin and pancreatin enzymes.

Additional experiments designed to reproduce the results of Figure 1 and also provide measurements of total soluble iron and soluble ferrous (Fe^{2+}) iron are summarized in Figure 2. Once again, incubation at pH 2.0 with fish significantly increased ferritin formation severalfold (i.e., 452%; Fe pH 2.0 vs. Fe/Fish pH 2.0). The presence of fish, with no incubation at pH 2.0, decreased total soluble iron by 84%, and soluble ferrous iron by 69%. Acidifying the digests to pH 2.0 did not change the amount of total soluble iron and soluble ferrous iron. Again, ferritin formation was dramatically increased when the fish was incubated with the iron at pH 2.0, even though the presence of fish decreased total soluble iron by 72% and soluble ferrous iron by 69%.

Based on the results of Figures 1 and 2, cooked fish muscles were digested without treatment of digestive enzymes under acidic conditions (pH 2.0) for 1 h. This was the initial and primary step in the digestion process to show an enhancement of nonheme iron uptake. Additional steps in the digestion process (i.e., Fe/Fish Pepsin and Fe/Fish Pep/PB) did not produce any additional significant increases. These results indicate that acid extract of fish muscle represents an initial material for further purification procedures. Lyophilization of the acid extract gave an ~21% yield (i.e., recovery on a per mass basis) of the cooked fish powder.

Fractionation of acid extract. Sephadex G-25 gel chromatography of the acid extract generated 22 fractions. Aliquots of these fractions were subjected to radiolabeled iron (^{59}Fe) uptake experiments to determine which fractions had an enhancing effect on iron uptake in Caco-2 cells. The analysis of the fractions revealed that enhancing activity was obtained from fractions 5–12 (Fig. 3), with the highest enhancement typically occurring in fractions 10 (F_S-10) and 11 (F_S-11) by 7.7- and 9.0-fold, respectively. Protein analysis results revealed only trace amounts of proteins or peptides in the fractions showing a high iron uptake enhancing effect (Fig. 3). This fractionation experiment, including iron uptake and protein analysis, was repeated numerous times, with a consistent appearance of the enhancing effect and protein levels similar to those observed in Figure 3.

Injection of the above active fractions onto a C18 protein and peptide column with ACN gradient (0–60%) in water with 0.1% TFA (v:v) yielded several peaks. Each peak was collected and tested using the radiolabeled iron uptake method with Caco-2 cells, where it was found that activity remained in the "injection peak" (data not shown). To further separate the injection peak, the collected injection peak was loaded onto the same column without ACN gradient at a lower flow rate (Fig. 4). Four fractions [2 distinct fractions (F_R-1 and F_R-2) and 2 fractions (F_R-3 and F_R-4) that eluted closely] appeared on the chromatogram (Fig. 4). The iron uptake results showed that for F_R-1, the injection peak still exhibited enhancing activity by 430%; however, the others had no activity (Fig. 4). Because almost all peptides exhibit some retention via reverse-phase C-18 chromatography, this observation was additional evidence that the enhancing factor was not a peptide.

Amino acid profile of active fractions. Amino acid analysis was conducted on F_R-1 collected from the RP-HPLC. Because the elution profile did not vary between runs, the collected samples from several runs were pooled for the analysis. The analysis revealed that only 2.2 g/100 g of the sample was derived from proteins or peptides, which indicated that the active component(s) was not a peptide(s). The following amino acids were present in concentrations < 0.1 μmol/g: Arg, Asx (i.e., Asp + Asn), Glx (i.e., Glu + Gln), His, Ala, Ile, Val, Cys (detected as a disulfide and reported as a free thiol), Gly, Ser, Thr, and Tyr. The following amino acids were detected at levels > 0.1 μmol/g: Lys (0.32 μmol/g); Leu (0.33 μmol/g); Met (0.44 μmol/g); Phe (0.25 μmol/g); and Pro (2.19 μmol/g).

HPAEC-PAD purification of active fractions. In accord with these findings, it was decided to further separate the active peak obtained from the previous RP-HPLC purification using the HPAEC-PAD with a CarboPac PA1 column. The chromatogram was divided into 3 parts, 0–3 min, 3–10 min, and 10–30 min, and each part was collected (**Fig. 5**). To desalt the mobile phase, an in-line AMMS was used before the

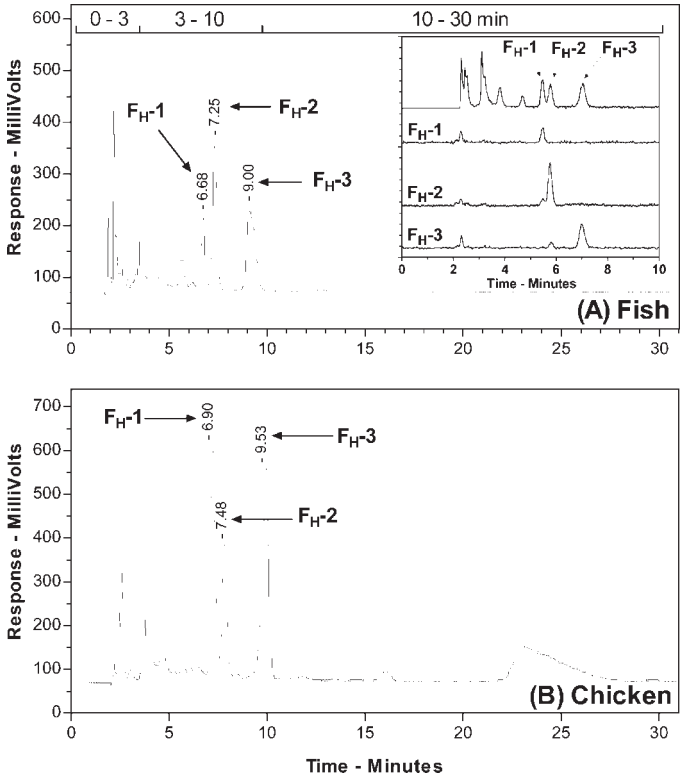

FIGURE 5 HPAEC-PAD of the fish (*A*) and chicken (*B*) fraction collected from RP-HPLC purification. The active fractions (i.e., F_R-1) were injected onto a semipreparative CarboPac PA1 (9 mm i.d.) column and eluted with 100 mmol/L NaOH at 3 mL/min. The peaks (i.e., F_H-1, F_H-2, and F_H-3) were collected and then reinjected onto an analytical CarboPac PA1 column (4 mm i.d.) to determine fraction purity (*inset in panel A*).

fraction collector. Following the desalting step, the 3 parts collected and pooled from multiple HPAEC-PAD runs were subjected to a radiolabeled iron uptake test with Caco-2 cells, and only the second part showed a significant increase in the radiolabeled iron uptake (data not shown). Based on this result, the 3 major peaks in the second part of the HPAEC-PAD chromatograms, labeled F_H-1, F_H-2, and F_H-3, were collected and desalted as described above. Table 1 shows the results of iron uptake screening test for the 3 peaks. The screening result revealed that F_H-1 and F_H-2 demonstrated 1.7 and 1.9-fold higher activity, respectively, than the control containing iron alone. Furthermore, activity of F_H-3 was 4.2-fold higher than the control. In Table 1, the Caco-2 cell iron uptake experiment was performed only once on the purified fractions collected from HPAEC-PAD due to the limited amount of each fraction available. However, this observation was confirmed by an additional Caco-2 cell test using the purified fractions from chicken muscle (Table 1). The HPAEC-PAD fractionation was repeated, producing a sample (<1 mg) that was a combination of F_H-1 and F_H-2 isolated from fish meat as above, and that had 3.4-fold higher activity than the control (Fe alone).

NMR analysis of active fractions. Preliminary NMR analysis indicated that the fractions with iron uptake activity are derived from heparin-like glycosaminoglycans (HLGAGs). The F_H-1/F_H-2 sample with 3.4-fold higher iron uptake activity revealed a multiplet at 7.817 ppm with a coupling constant ($^3J_{H,H}$) value of 9.12 Hz. This chemical shift and coupling constant agree with the NH values (7.82 ppm, 9.40 Hz) reported for a de-*N*-sulfated, re-*N*-acetylated heparin (37). However, the anomeric proton chemical shift of the F_H-1/F_H-2 sample (5.234 ppm) did not agree with the *N*-acetyl-glucosamine value (5.11 ppm) reported for de-*N*-sulfated, re-*N*-acetylated heparin (37). The anomeric proton chemical shift of the F_H-1/F_H-2 sample was close to that reported for the α-L-iduronic acid residue of trisulfated heparin (5.22 ppm) (37). However, the $^3J_{H,H}$ we measured for the F_H-1/F_H-2 anomeric proton chemical shift was 3.9 Hz, which was higher than the $^3J_{H,H}$ = 2.9 Hz reported for the anomeric proton of the iduronic acid residue of trisulfated heparin (37). The F_H-1/F_H-2 anomeric proton appeared to be more closely related to 6-*O*-sulfated, *N*-acetyl-glucosamine or disulfated-glucosamine (5.24 or 5.25 ppm and $^3J_{H,H}$ = 3.5 Hz) (37), which are monosaccharide constituents of HLGAGs. The F_H-3 sample collected at the same time as the F_H-1/F_H-2 sample had a broad doublet at 7.402 ppm, an acetyl proton at 2.081 ppm, and a lone anomeric proton at 4.643 ppm (β-configuration). The F_H-3 sample with 4.2-fold higher iron uptake activity than the control (first HPAEC-PAD fractionation) was analyzed by NMR before the nanoprobe was available; therefore, the spectral resolution was not as good. However, a 2-D NMR heteronuclear multiple bond correlation (HMBC) experiment revealed correlation between chemical shifts at 8.09 ppm (^{1}H) and 171.23 ppm (^{13}C). An acetyl proton was also observed at 2.01 ppm in spectra for this sample. Mulloy et al. (37) reported that the chemical shifts for 6-*O*-sulfated, *N*-acetyl-glucosamine were 8.13 ppm (NH) and 177.30 ppm (acetyl CO), respectively, which could explain the HMBC results. This provides further evidence that the fractions with iron uptake activity were derived from HLGAGs.

DISCUSSION

Numerous studies were conducted over the past 3 decades to identify the meat factor(s); however, the mechanism(s) of the meat effect and the factor(s) contributing to the effect have not been conclusively defined. The fractionation and isolation conditions used in this study were designed to surmount several difficulties that many scientists have experienced in attempts to characterize the meat factor.

Muscle tissue is generally considered to be a good source of iron because of the high content of heme and nonheme iron. The intrinsic heme iron is likely to be broken down into nonheme iron released from porphyrin rings during cooking (38). Thus, it is expected that meat samples prepared by cooking and digestion contain a high level of nonheme iron (6), which could be a difficult problem in interpreting results due to intrinsic iron contamination. Fish muscle tissue was used in this study because it has a relatively low total iron concentration; therefore, fish muscle served as an adequate source of muscle tissue and presumably the meat factor(s), with minimal intrinsic iron contamination to control iron levels in fractions.

Most previous attempts to identify the meat factors using beef (12,17,39) or chicken (12,18,39,40) overlooked the intrinsic iron contamination, which might complicate interpretation of results because each treatment must have contained different amounts of iron. Even though treatment blanks (extrinsic iron omitted) to determine the contribution of intrinsic iron were prepared, the different concentration of iron could result in differences in the percentage of iron availability. In other words, the observed increase in the percentage of iron uptake potentially can be attributed to the lower iron concentration, not to the difference in treatments (4,17,41). In addition, the present study used fish acid extract as a starting

material for isolating the meat factor(s) rather than whole fish muscle tissues (12,18,39,40); this also helped to exclude contamination from heme iron because the solubility of heme iron is extremely low at an acidic pH (42). Also, many previous studies suggested that the meat factor was associated with the intrinsic iron in meat (43–46). Thus, they tried to determine the concentration and distribution of iron in meat by radiolabeling and fractionating the meat. However, a primary component of the meat effect is to promote absorption of extrinsic iron from meals (1,2,15,47). In this study, we sought to follow fractions with activity, i.e., we did not follow iron in meat, by preparing muscle tissue with minimal iron contamination. This approach effectively eliminated the difficulties that could occur in interpretation of our data.

Another important feature of our study is that we could simplify digestion conditions without digestive enzymes. Considerable attempts to isolate the meat factors were based on an in vitro digestion method with digestive enzymes (17,40). In those cases, it was difficult to exclude the contamination of the digestive enzymes from meat. The results of Figures 1 and 2 demonstrate that there was no significant difference in ferritin formation between Fe and Fe/Fish, suggesting that fish did not have an enhancing effect on nonheme iron uptake when it was incubated at pH 7.0 for 1 h. The observation agrees with the previous results stating that the iron absorption-enhancing component of beef was not present in a water extract (2). In Figure 1, it was also indicated that the addition of pepsin and pancreatin-bile (Fe/Fish Pep and Fe/Fish Pep/PB) did not significantly increase ferritin formation compared with Fe/Fish pH 2.0, which clearly suggested that the enhancing effect of fish on nonheme iron uptake was due primarily to the incubation of the fish muscle tissue with iron at pH 2.0 for 1 h. This observation is particularly useful because there is no need to add pepsin and pancreatin-bile, which could make the isolation procedures of the meat factor(s) more complicated. Based on the results of Figures 1 and 2, it was thought that at least some, perhaps not all, of the meat factor(s) should be present in the digest after incubating a fish sample for 1 h at pH 2.0. Thus, the fish muscle tissue was digested under the acidic condition (pH 2.0 for 1 h) without digestive enzymes, and the supernatant of the acidic digest was used as a starting material for the meat factor isolation procedures.

Some results that conflict with the above findings can be found in previous in vitro studies on the meat factors. For example, it was reported that the solubility of added iron was significantly increased only by the acid-insoluble fraction (18). However, the discrepancy between our results and those of Slatkavitz and Clydesdale can be readily explained with substantial evidence that iron solubility could not be a good indication of bioavailable iron (17,31,48). The results of Figure 2 also obviously indicate that total soluble iron is not indicative of iron availability. For example, the digest containing fish incubated with iron at pH 2.0 had 72% less total and 69% less soluble iron than the digest without fish (Fe/pH2); yet the ferritin levels in the cells were >450% greater, indicating that the iron was much more available. Similar effects were observed in Figure 1, where dialyzable iron (i.e., bottom chamber Fe) did not correlate with ferritin values.

Another advantage of this approach over other meat factor studies can be found in the fractionation at acidic pH. Once the acid extract of the fish sample was obtained, the sample solutions were kept acidic at pH 2.0 before the iron uptake period. Also, all isolation steps including fractionation and sample preparation for screening experiments were conducted under the acidic environment conditions. The acidic condition simulates a gastric environment in which the meat factor(s) could interact with nonheme iron and maintain its enhancing effect on iron uptake. For example, AA is a strong enhancer of nonheme iron absorption because of the reducing and/or chelating effects of nonheme iron. However, it should be recognized that ferric iron is easily reduced by AA only when the pH is <6.0 (49). Also, it is generally agreed that the initial interactions of food with iron at the low pH of the stomach are the critical factors that determine iron bioavailability (50). Our preliminary experiments indicated that AA could not increase iron uptake with Caco-2 cells when it was added and incubated with iron at neutral pH, which clearly shows the importance of low pH in creating or maintaining the activity of iron uptake enhancers, such as AA and the meat factor.

The observation that low pH plays a role in iron absorption has interesting physiologic implications. Ingestion of a meal stimulates gastric acid secretion; however, upon ingestion, overall gastric pH typically rises from a pH of 1–2 to a pH of 4.5–6, depending on the foods present in the meal (51,52). Gastric pH begins to return to preingestion values within 60 min, reaching pH values of 2–3 within 2 h after meal ingestion (51,52). Certainly the transient rise in gastric pH is due to the buffering capacity of the meal. It is very likely that segments of the meal in the gastric lumen experience transient moments of lower pH before peristaltic mixing, particularly those closest to the acid secretion along the wall of the stomach. We speculate that these moments are the ones most responsible for iron solubilization and interaction of iron with food components. Achlorhydria is commonly associated with iron deficiency anemia (22,23); thus, it may be that a certain level of acid secretion is necessary to induce adequate iron solubility and hence allow for the possibility of iron absorption in the intestinal tract.

Size exclusion chromatography is one of the most widely used separation techniques. The Sephadex G-25 resin (fractionation range ~1000–5000 Da) was selected for the initial step of the fractionation because small peptides released during proteolytic digestion were thought to be one of the candidates for the "meat factor" (18,53). The results of Figure 3 indicate that proteins were not responsible for the 7- to 9-fold enhancement of radiolabeled iron uptake in Caco-2 cells, and the active factors are low-molecular-weight compound(s). These results are interesting because there have been very few reports mentioning that nonprotein components of meat improve nonheme iron uptake. Carpenter and Mahoney (25) found that low-molecular-weight (<6000–8000 Da) homogenates from either digested or nondigested meat increased soluble iron, which indicates that proteolytic digestion of meat is not necessary for iron solubilization. On the basis of their results, it was speculated that the meat factor(s) was possibly a nonprotein, low-molecular-weight compound(s).

In Figure 4, the retention time of F_R-1 was ~6 min, which supports our hypothesis once again that the meat factor(s) in the fraction was neither protein nor peptides because proteins and peptides normally elute out later (>6 min) under these chromatographic conditions (Enslow, W., personal communication). In addition, the finding was confirmed by the amino acid analysis result showing that negligible amounts of amino acids were present in F_R-1. It was particularly surprising because the result did not agree with previous works suggesting that cysteine (3,19,20,29,54) and histidine (17,29,54) were among the most probable enhancers of the bioavailability of nonheme iron

Because the above results clearly indicated that the factor isolated from the fish acid extract was neither proteins nor peptides, we conducted a test to determine whether the active

fractions contained carbohydrates. Using the phenol-sulfuric acid assay, which is a typical colorimetric test for carbohydrates, positive results occurred in our active fraction, F_R-1 (data not shown). Therefore, we utilized a Dionex system set up specifically for carbohydrate analysis using HPAEC-PAD (55–58). Results of HPAEC-PAD analysis of F_R-1 from C18 RP-HPLC showed that the 3 major peaks, labeled F_H-1, F_H-2, and F_H-3, were eluted (Fig. 5). From the iron uptake results (Table 1), it appears that all 3 of the eluted compounds increased nonheme iron uptake, particularly F_H-3. In addition, identical results of Sephadex G-25 size exclusion chromatography, RP-HPLC, HPAEC-PAD purification, as well as iron uptake data by Caco-2 cells (Table 1) occurred with chicken muscle tissue. This observation indicates that the factors are not unique to fish muscle and are present in other meats.

The NMR data collectively suggest that the active components of the meat factor are low-molecular-weight carbohydrate (i.e., oligosaccharides, disaccharide, and monosaccharide) degradation products of HLGAGs. The structures of these polysaccharides are very complex, consisting of 32 different plausible combinations of a uronic acid (α-L-iduronic acid or β-D-glucuronic acid) 1–4-linked to a α-D-glucosamine which can be *O*-sulfated at up to 3 positions as well *N*-sulfated or *N*-acetylated (59). Therefore, it is likely that the different HPAEC-PAD fractions consist of HLGAGs with different levels of sulfation or different uronic acids. Although all 3 HPAEC-PAD peaks had iron uptake activity, the elucidation of the actual structure of the HLGAG with the optimal iron uptake activity will be the subject of a future publication.

We speculate that the meat factors isolated in this study are released from the extracellular matrix in skeletal muscle under acidic digestion conditions and that they enhance iron uptake by the intestinal epithelial cells. In skeletal muscle, there exists the extracellular matrix, which is an organized structure located outside cells, composed of proteins and polysaccharides produced by the cells (60). Proteoglycans, one major component of the extracellular matrix, contain a central core protein to which one or more glycosaminoglycan chains are attached. With our acidic treatment of the cooked fish sample, it is unlikely that protein digestion occurred adequately because there was no proteolytic digestive enzyme in the digest. Because only 10–20% of protein digestion takes place in the stomach due to the action of pepsin and the majority of the protein digestion occurs in the intestine (61), we contend that our digestion condition without digestive enzymes would not catalyze the hydrolysis of proteins. In addition, one of the important features of pepsin digestion is its ability to digest the protein collagen, which is affected little by other digestive enzymes (61). Collagen is a major constituent of the intercellular connective tissue of meats; therefore, it is likely that digestion of the collagen fibers facilitates penetration of the digestive enzymes into the tissue for digestion of cellular meat proteins. Consequently, the acidic treatment without pepsin activity may poorly digest the fish sample. Thus, we can speculate that oligosaccharides from partially fragmented glycosaminoglycans may be readily liberated from the extracellular matrix under the acidic conditions, and the released carbohydrates may interact with iron, resulting in more iron uptake. It is likely that muscle proteins/peptides remain mostly intact under these acidic conditions, which represent relatively mild conditions of digestion.

The results of Figure 2 may provide some valuable clues concerning the mechanism involved in the enhancement of nonheme iron availability by meat. The results indicate that the enhancing effect of meat was not due to increased amounts of ferrous iron. Thus, in contrast to AA, fish does not appear to enhance iron uptake by reducing ferric iron to the more available ferrous state. These results are interesting because it was proposed that the meat enhances nonheme iron uptake by creating more available ferrous iron in the intestinal lumen, presumably due to factors such as cysteine that may reduce ferric iron to the ferrous form (19). Consumption of cysteine with meals promoted iron uptake about 2-fold in humans, but did not have an effect when combined with the meal before ingestion and subjected to cooking, presumably because the cooking oxidizes the cysteine to cystine (19,20). Our fish sample was cooked before digestion; thus it appears that the factors are to some extent resistant to cooking, or are created during the cooking period. Therefore, it is unlikely that the factor is a reducing agent because reducing compounds are generally unstable at high temperature. Also, it is possible that the meat factor(s) may serve on the luminal surface to render insoluble ferric iron available for transport into the intestinal mucosal cell either through the iron transporter or some other mechanism. Recently Simovich et al. (62) reported that a large proportion of the iron transporter (i.e., divalent metal transporter-1 and mobilferrin) was extracellularly associated with the luminal mucin (highly glycosylated molecules produced by the intestinal goblet cells), and the mucin might act to increase surface area and extend into the luminal space to capture essential nutrients that otherwise might pass beyond the absorptive surface. Accordingly, the meat factor(s) may act like the mucin in our Caco-2 cell system, thus allowing the cells to take up more iron, possibly insoluble ferric iron, that otherwise does not reach the iron transporter. Also, iron bioavailability to human infants from human milk is greater than that from cow's milk on a fractional basis. Although the factor(s) responsible for higher iron bioavailability in human milk compared with cow's milk have not yet been identified, there is evidence that the enhancing factor in human milk is primarily present in the low-molecular-weight whey fraction ($<$10 kDa), which may include most of human milk oligosaccharides (63). Perhaps these oligosaccharides also promote iron uptake, which would also explain why human milk has high fractional iron absorption.

In conclusion, this study publishes a unique approach to investigate the "meat factor." We observed that the promotional effect of muscle tissue on iron uptake was initiated by the low pH conditions of the stomach. This observation could effectively eliminate several obstacles that have made the identification of the factor(s) in meat difficult. Although the structural basis for the active compounds remains unclear, our present findings strongly suggest that low-molecular-weight carbohydrates of muscle tissue are responsible for the increasing nonheme iron uptake in Caco-2 cells. Further studies to determine the structures of the active compounds and investigate how the compounds play a role in the enhancement of nonheme iron absorption are being conducted. These studies may provide valuable insight into the mechanism(s) involved in the "meat factor," which has remained a mystery for the past 35 years.

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