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Isolated Glycosaminoglycans from Cooked Haddock Enhance Nonheme Iron Uptake by Caco-2 Cells

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This study continues previous research to confirm that glycosaminoglycans (GAGs) exert a positive effect on promoting iron uptake by Caco-2 cells. Cooked haddock was digested with papain, and GAGs were further purified on the basis of their sulfur content. Reverse phase chromatography (RP-HPLC) and digestion with chondroitinase ABC (Chase) (50 mU/mg) were used to approach the identification of the GAGs. $FeCl_3$ was mixed with the purified GAGs, and Fe uptake was measured by ferritin formation using an in vitro digestion/Caco-2 cell model. The identificative analyses suggest that chondroitin/dermatan sulfate-related structures promote Fe uptake by Caco-2 cells; however, this effect was lower (40%) than that observed with whole fish muscle. Chase eliminated the positive effect on Fe uptake. These results indicate that specific GAGs may contribute to the enhancing effect of meat on Fe absorption. Further in vivo studies addressing these aspects of the meat factor are needed.

KEYWORDS: Glycosaminoglycans; Fe uptake; meat factor; Caco-2

INTRODUCTION

It has been well documented that meat consumption enhances nonheme iron absorption in in vitro models (*1, 2*), animals (*3, 4*), and humans (*5, 6*). For more than 30 years, much research effort has been directed at defining the components in meat that enhance Fe uptake. In general, the results are inconclusive, and no specific compound has been isolated from meat that clearly enhances Fe absorption. In this way, Hurrell et al. (*6*) indicated that the meat effect is mainly due to the protein component of muscle tissue but also that it is unlikely that a single peptide is involved. Furthermore, the possibility that no protein compounds in muscle tissues might be involved in the enhancing effect of meat on Fe absorption was suggested (*6*). In an in vitro study, Huh et al. (*2*) indicated that acidic and sulfated carbohydrates such as glycosaminoglycans (GAGs) contribute to the promoting effect of fish muscle on Fe uptake by Caco-2 cells. However, Storcksdieck et al. (*7*) could not produce an enhancing effect with two purified GAGs (sodium hyaluronate and chondroitin sulfate) on nonheme Fe absorption in young women when added at 0.1% relative to the total amount of diet. Presently, a single promoter of Fe absorption has not yet been characterized from meat, and further research is needed.

The GAGs are nonprotein components in muscle tissue that might bind Fe (*8*), and those have been previously used in the treatment of Fe deficiency in animal models (*9*). Proteoglycans (PG) are one of the major components of the extracellular matrix in connective tissue (*10, 11*). PGs are a family of macromolecules in which one or more sulfated polysaccharide chains (GAGs) are covalently linked to a protein core (*12*). The GAGs are straight-chain polysaccharides composed of repeating disaccharide units of hexosamine (D-glucosamine or D-galactosamine) and uronic acid (D-glucuronic acid or L-iduronic acid) or galactose (in keratan sulfate). Seven different structures for GAGs have been described: hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate and heparins, and keratan sulfate. The GAGs are structurally diverse and can be present in several biological forms (*13*). Their particular structures allow different electrostatic interactions with different biological constituents (*12*). The PGs are biologically active compounds the function of which is determined structurally (*13*).

Several selective extraction procedures have been previously employed to conduct the isolation and fractionation of GAGs from porcine (*14*) and bovine muscles (*10*), those reporting different extraction efficiencies. The method described by Nakano et al. (*14*) exhibited highest extraction efficiency and was based on an enzymatic (papain) digestion of the tissue and subsequent complexation of GAGs by cetylpyridium before their purification and fractionation according to their sulfation pattern. On the other hand, data on the content and structure of PG in fish muscle are scarce and just beginning to appear in the literature (*15, 16*). Recent studies evaluated the relative com-

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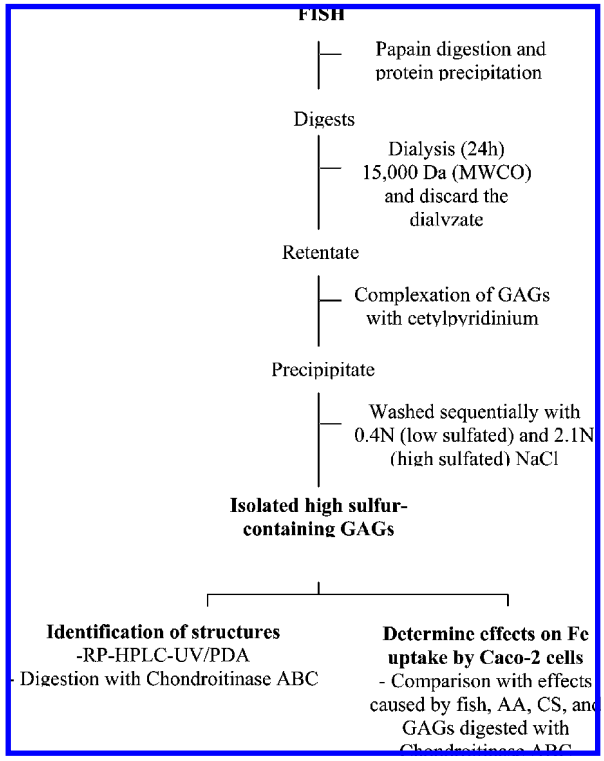

Figure 1. Schematic representation of the study.

position of GAGs in connective tissues of spotted wolfish (*15*) and cod skeletal muscle (*15, 16*).

Although human studies are the definitive tool to evaluate Fe bioavailability, in vitro methods can provide effective insight to factors that influence Fe uptake in vivo. In this sense, a recent expert consensus concluded that the Caco-2 cell culture model is useful for predicting the correct direction of intestinal response to enhancers and inhibitors of Fe absorption in humans, but not its magnitude (*17*). In vitro studies in conjunction with human trials are a valuable tool to improve understanding of the occurring processes at the intestinal level.

In view of the above, the objectives of the present study were (1) to apply a selective method to isolate and purify sulfur-containing GAGs from cooked fish muscle and (2) to determine if GAGs promote Fe uptake by Caco-2 cells. **Figure 1** summarizes the steps followed in our study.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All glassware used in the sample preparation and analyses was treated with 10% (v/v) of HCl concentrated (37%) for 24 h and then rinsed with 18 MΩ cm deionized water before being used.

Instruments. Iron and sulfur concentrations were determined with an inductively coupled argon plasma emission spectrometer (ICP-ES, model 61E Trace Analyzer, Thermo Jarrell Ash Corp., Franklin, MA). Other equipment used included a spectrophotometer (DU 520 UV−vis, Beckman Coulter, Palo Alto, CA) and an automatic γ counter Wizard 3 Wallac 1480 (Perkin-Elmer, Norwalk, CT).

Preparation of Fish. Fresh fillets of haddock were purchased in local markets of Ithaca, NY, and all visible fat and skin were removed. The samples (100 g) were cut into small pieces, mixed with 55 mL of 18 MΩ deionized water, and then microwaved (1000 W) for 1.5 min, stirred, and microwaved for an additional 1.5 min. The cooked slurry was 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 until use.

Isolation of Sulfated GAGs from Fish. A previously reported method to isolate and fractionate sulfated carbohydrates from porcine skeletal muscle was applied (*14*). Four independent aliquots (5 g) of the lyophilized fish were digested with papain (catalog no. 76216, Sigma) (4 μg/mg of tissue) in 50 mL of 0.1 M sodium phosphate buffer (pH 7.2), containing 0.005 M EDTA, 0.005 M cysteine hydrochloride, and 0.02% (w/v) sodium azide, at 65 °C for 24 h. After the addition of trichloroacetic acid (final concentration of 7% w/w, relative to fish muscle), the mixtures were held at 4 °C overnight, and the protein precipitates were removed by centrifugation (12857*g*/4 °C/30 min). The supernatants were transferred to dialysis (15000 Da molecular weight cutoff, MWCO) tubings, previously boiled in distilled water for 10 min, and dialyzed in a beaker (1 L) for 24 h and then in cold distilled water for another 24 h. Cetylpyridinium chloride was added dropwise (final concentration of 5 mM) to the retentate to precipitate GAG as a cetylpyridinium−GAGs complex (*18*). The mixtures were centrifuged (12857*g*/4 °C/30 min) to separate the soluble components and precipitate. The precipitate was sequentially washed with 5 mL of 0.4 and 2.1 M NaCl to isolate the low- and high-sulfated GAG fractions, respectively (*19*). To the high-sulfated fraction was added potassium thiocyanate (*20*) in a molar ratio with cetylpyridinium (1:1), and the resulting precipitate was removed by filtration (Whatman no. 41). The filtrate was dialyzed (15000 MWCO) in deionized water for 24 h, and the retentates were freeze-dried.

Enzymatic Digestion with Chondroitinase ABC. Samples (5 mg) of the purified fraction from fish muscle were dissolved in 2 mL of phosphate-buffered solution (PBS, pH 7.2) [NaCl (140 mM), KCl (2.7 mM), Na_2HPO_4 (6.4 mM), H_2KPO_4 (1.5 mM)]. Chondroitinase ABC from *Proteus vulgaris* (C2905, Sigma) was reconstituted in 3 mL of PBS. Appropiate volumes of enzyme solution were added to the sample solutions to obtain 50 mU/mg, final concentration. Then mixtures were incubated (37 °C) for 1 h, and the resulting products were subjected to an in vitro digestion procedure (see below) and tested for activity in Fe uptake experiments.

HPLC Analysis of Purified Fraction. The analysis was carried out on a HPLC system (Waters, Milford, MA) consisting of a 600E multisolvent pump, a 717 plus autosampler, and a 996 photodiode array detector set at 254 nm, operated using a Empower software. The separation was performed on an Alltima HP C18 AQ 5 μm column (Alltech) 250 × 4.6 mm. The gradient program started with 100% of solvent A (70:30 v/v, water/acetronitrile; HPLC grade) increasing linearly to 100% solvent B (1% acetronitrile in 20 mM phosphate buffer adjusted to pH 2.20 with concentrated H_3PO_4) in 15 min. The column was equilibrated using the initial conditions for 5 min.

Samples (20 mg) of purified fraction from cooked haddock and chondroitin sulfate C (Sigma, C4384) were redisolved in 1 mL of PBS (Sigma, P4417). From each, two sets of samples were prepared, and just one of them was mixed with chondroitinase ABC (50 mU/mg of dry residue). Then, both sets of samples were incubated at 37 °C for 1 h. Afterward, appropriate aliquots of PBS were added to adjust the volume to 2 mL. From these solutions, aliquots (45 μL) were diluted in 1 mL of Milli-Q water. Then, samples were filtered through 0.20 μm (MillexGN, Millipore), and 100 μL of the filtrates was injected into the HPLC system. UV absorbance at 210 nm was used to detect eluting compounds. Total running time for each analysis was 20 min. Shark cartilage chondroitin-6-sulfate (as chondroitin sulfate C; Sigma, C4384) was used as a standard.

Cell Cultures. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passage 25−33. Cells were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco) under conditions previously described (*21*).

The cells were used for Fe uptake experiments at 13 days postseeding. For the assays, Caco-2 cells were seeded at 50000 cells/cm^2 in collagen-treated six-well culture plates (Costar, Cambridge, MA) and were grown with DMEM. On the day prior to the in vitro digestion experiment, the DMEM was removed and washed with 2 mol of minimal essential medium (MEM), and then the cells with another 2 mol of MEM were returned to the incubator.

In Vitro Digestion. To simulate the human gastrointestinal digestion process, the method described by Glahn et al. (*21*) was applied. Porcine pepsin (P-7000, Sigma), pancreatin (P1750, Sigma), and bile extract (B8631, Sigma) (glycine and taurine conjugates of hyodeoxycholic and

other bile salts) were demineralized with Chelex-100 (Bio-Rad Laboratories, Hercules, CA) before use.

Aliquots of the fish (0.3 g), chondroitin sulfate C (Sigma, C4384) (5 mg), and high-sulfated fraction (5 mg), digested or without any previous treatment with chondroitinase ABC, were subjected to in vitro digestion. All samples were mixed with $FeCl_3$ (High Purity Standards, Charleston, SC) (final concentration of 41.7 μmol/L as Fe). A digest containing $FeCl_3$ (41.7 μmol/L) and ascorbic acid (1.0 mmol/L) was used as a positive control. Peptic and intestinal digestions were conducted on a rocking platform shaker placed in an incubator (37 °C/5% CO_2/95% relative humidity). After the gastric step (pepsin/pH 2/1 h), the intestinal digestion (pancreatin−bile extract/pH 6.9−7/2 h) was carried out in the upper chamber of a two-chamber system in six-well plates. The upper chamber was formed by fitting the bottom of an appropriately sized Transwell insert ring (Corning) with a 15000 Da MWCO (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). The dialysis membrane was held in place with a silicone ring (Web Seal, Rochester, NY). In the bottom chamber, 1 mL of MEM for cell uptake experiments was placed. Then an aliquot (1.5 mL) of the intestinal digest was pipetted into the upper chamber and incubated for 2 h. Then, the inserts were retired and an additional 1 mL of MEM was added. The cell cultures were incubated for 22 h at 37 °C with gentle rocking.

Ferritin Analysis. Exactly 24 h after the start of the Fe uptake period, the medium covering the cells was removed and the cells were washed twice with a 1 mL volume of PBS (pH 7.4) (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl). The ferritin concentration and total protein concentration were determined on an aliquot of the harvested cell suspension with a one-stage sandwich immunoradiometric assay (FERIRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad), respectively. Therefore, the ratio of ferritin/total cell protein expressed as nanograms of ferritin per milligram of protein was used as an index of the cellular iron uptake.

Mineral Determination. The iron (Fe) (*22*) and sulfur (S) contents in cooked fish and the isolated fractions during extraction procedure were determined by ICP-ES. Aliquots of the purified fraction (10 mg) were acid digested in 1.0 mL of HNO_3 with 1.5 mL of $HClO_4$ at 120 °C for 1 h and then at 220 °C until $HClO_4$ fumes were observed. The samples were diluted with 5% HNO_3 to 6 mL. The instrument was calibrated with 10% $HClO_4$ as the low standard and 1 μg/g Fe and 40 μg/g S in a multielement standard as the high standards. The Fe and S were determined using the 238.2 and 180.7 nm lines, respectively. In the present study, working solutions from a commercial standard of Fe (High Purity Standards, Fe 1000 μg/mL, catalog no. 100026-2) were prepared in 0.1% HCl aqueous solutions and analyzed in parallel to the samples for quality assurance−quality control of the methodology used.

Statistical Analysis. Two experiments with all treatments were conducted on separate days. Each treatment was performed in triplicate on both days. A one-factor analysis of variance (ANOVA) and the Tukey test (*23*) were applied to determine statistical differences in ferritin concentrations quantified in treated cell cultures. A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus version 5.1 (Rockville, MD) was used for the statistical analysis.

RESULTS AND DISCUSSION

Isolation of GAGs from Fish. The sulfur, iron, and total protein contents in purified fraction from cooked haddock are shown in **Table 1**. In the present study, an average weight for dry residue of 55.1 ± 6.7 mg/g of lyophilized fish was recovered from the papain digest. The sulfur-containing purified fraction accounted for 0.56% of the total amount of fish extracted. The latter compounds were acidic, as concluded from the decreased initial pH value of Milli-Q water (pH 6.5) up to 5.6 when aliquots were dissolved (5 mg in 2 mL). The isolated fraction contained 0.072 mg of protein/mg of dry residue. The sulfur content was 6.2-fold higher in the obtained fraction relative to the initial fish content (**Table 1**). The intrinsic Fe concentration of dry residues in the purified fraction obtained was not detectable (**Table 1**). The observation that these compounds are sulfated and acidic fits the characteristics of GAGs (*24*).

Table 1. Sulfur and Iron Contents in Cooked Haddock and Fractions Obtained from Cooked Haddock[a]

sample	μmol/g of dry residue		protein (mg/mg of dry residue)
	S	Fe	
cooked fish	103.0 ± 1.3	0.12 ± 0.01	na[b]
0.4 M NaCl fraction	74.3 ± 1.5	nd[c]	0.142
2.1 M NaCl fraction	619.5 ± 74.3	nd	0.072

[a] Values are expressed as mean ± standard deviation ($n = 5$). [b] na, not analyzed. [c] nd, not detectable.

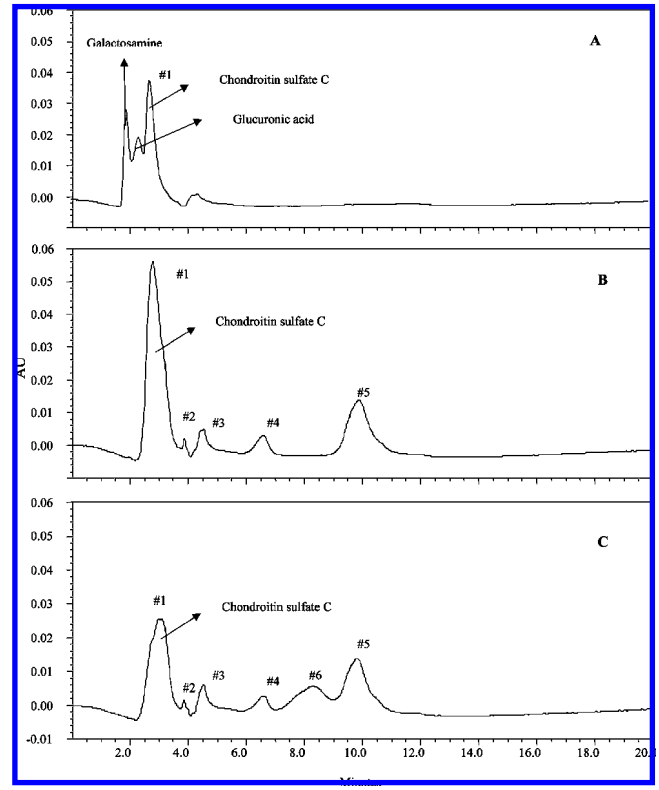

Figure 2. Typical HPLC-RP-PDA/UV chromatograms of chondroitin sulfate C (**A**) and dry residue from 2.1 M NaCl fraction before (**B**) and after (**C**) digestion with chondroitinase.

Further characterization of the purified fraction was needed to narrow the identity and nature of the sulfur-containing fraction obtained. The typical reverse phase HPLC chromatograms obtained for aliquots of the isolated fraction, before and after being digested with chondroitinase ABC, are shown in **Figure 2**. The samples were digested with chondroitinase ABC, which is a bacterial lyase that degrades specifically chondroitin sulfate (CS), dermatan sulfate (DS), and hyaluronan glycosaminoglycans (*25*). In the present study, reverse phase HPLC analysis of nontreated aliquots from the isolated fraction with chondroitinase ABC revealed five different peaks (**Figure 2B**). The main peak matched the elution time of the chromatographic signal recorded for chondroitin sulfate C (**Figure 2A**). After digestion with chondroitinase ABC (**Figure 2C**), only peak 1 (eluted with a retention time of 2.8 min) was decreased, but none of the other peaks (2−5) were altered in terms of either retention time or integration area. These results suggest that compounds of the main peak (1) have structures related to GAGs. Chondroitinase ABC catalyzes the hydrolysis of polysac-

charides containing 1,4-β-D-hexosaminyl and 1,3-β-D-glucuronosyl or 1,3-α-L-iduronosyl linkages; thus, it acts on chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate and would act slowly on hyaluronate (*25, 26*). This fact suggests that chondroitin/dermatan sulfate-related structures are present in the purified fraction.

Some studies have reported differences in the proportion of total content of GAGs in porcine (*14*) and bovine muscles (*9*) accounting for 0.35 and 0.14% by weight, respectively. These methods differ mainly in the extraction procedure used, which included an enzymatic (papain) digestion step (*14*) or not (*9*) to release the GAGs from tissue muscle prior to purification. However, limited information exists on GAG composition in fish muscle. To the best of our knowledge there are no studies related to GAG composition on haddock muscle and the stability of these compounds during cooking. Recent studies evaluated the relative composition of GAGs in connective tissue of spotted wolfish (*15*) and cod skeletal muscle (*15, 16*). Of the total GAGs present, Tingbø et al. (*15*) reported a different proportion of CS/DS in wolfish (58%) than cod (25%). The latter authors showed that heparan sulfate exhibited an inverse relationship with CS/DS and accounted for 14 and 43% of the total GAGs in wolfish and cod, respectively. Keratan sulfate (KS) constitutes the lowest proportions reported in cod (3%) and wolfish (12%) relative to CS/DS proportions (*15*). It is important to mention that a different expression of CS- and KS-proteoglycans between cod and bovine muscle has also been reported (*16*).

Another aspect to consider is that with the exception of hyaluronic acid, which is nonsulfated, all GAGs are sulfated and covalently attached to proteins as proteoglycans (*26*). Hannesson et al. (*16*) reported that CS and KS do not exist in the cod and bovine tissues as free chains, but are linked to different protein cores. They also demonstrated by using Western blot analyses that the CS epitopes in both cod and bovine muscle were carried by a variety of proteoglycans of different molecular sizes. In the present study, the low protein content quantified in dry residues from purified fraction would be in agreement with the presence of proteoglycans. This would also explain the presence of peaks in our HPLC analysis that were not degraded by chondroitinase ABC (**Figure 2**). The variety and different sizes of protein cores attached to GAG with different molecular sizes (*16*) would explain the isolation of GAGs by using a dialysis membrane of 15000 Da MWCO. It is not possible to say with certainly whether the proteoglycans isolated reflect the true structures present in the tissue, the conditions during extraction procedure, or the action of papain (cysteine protease that cleaves peptide bonds of basic amino acids, leucine, or glycine) may cleave part of the protein core carrying GAGs.

Effect of GAGs on Nonheme Fe Uptake Assays. The amount of cooked haddock used (0.3 g) in the present study was established in our own previous work (*2*), in which different aspects of digestion required to initiate the promoting effects on Fe uptake by Caco-2 cells were evaluated. The effects of fish, isolated GAG-containing fraction, and chondroitin sulfate C on nonheme Fe uptake by Caco-2 cell culture model are shown in **Figure 3**. All samples, except that named "fish", provided an equal amount of Fe in the in vitro system (10 μg), which was extrinsically added. The haddock fillets provided an excellent source of muscle tissue with minimal intrinsic Fe concentration (0.12 ± 0.01 μmol of Fe/g of cooked dry fish powder). The sample containing whole fish (fish/Fe) enhanced Fe uptake by Caco-2 cells, as concluded from the increased ferritin concentrations quantified in exposed cultures relative

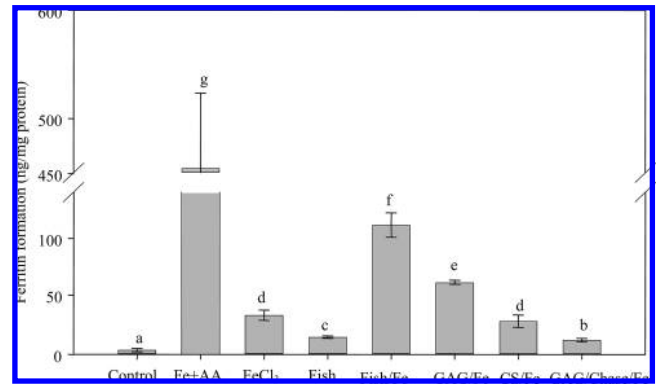

Figure 3. Ferritin levels in Caco-2 cells exposed to fish and dry residue from 0.4 and 2.1 M NaCl fractions. When indicated, samples were mixed with $FeCl_3$ (final concentration = 41.7 μmol/L) and subjected to in vitro digestion. Values are expressed as mean ± standard deviation ($n = 6$). Different letter over the bars indicate statistical ($p < 0.05$) differences.

to the exposure to $FeCl_3$ alone. In the same way, the GAG-containing sample (GAG/Fe) caused greater ferritin formation than the addition of $FeCl_3$ alone, increasing to 85.4 ± 19.4%. However, the increase on ferritin formation in those cultures exposed to GAG/Fe resulted in 60.0 ± 4.0% of that produced by Fish/Fe. After digestion of sample GAG/Fe with chondroitinase ABC (GAG/Chase/Fe), the enhancing activity observed was significantly ($p < 0.05$) decreased with respect to undigested fraction (GAG/Fe), although Fe concentration added within both samples was the same. These results suggest a potential role for GAGs in enhancing Fe uptake by Caco-2 cells. We can assume that the enhancing effect observed on Fe uptake was not due to the low protein content (0.036 mg) loaded in the upper chamber in the in vitro system within the GAG-containing purified fraction. This fact is plausible because in both samples, GAG/Fe and GAG/Chase/Fe, the amino acid contents are similar. Another observation is that chondroitin sulfate C (from shark cartilage) solution had no effect on increasing Fe uptake relative to the addition of $FeCl_3$ alone. As previously reported, the biological functionality of GAGs is determined by their assembling into specific proteoglycans (*11, 12*); thus, PG probably may be responsible of the enhancing effect observed on Fe uptake. Taken together, these results might suggest the indispensable role of peptides as part of specific PG structures in the enhancing effect of GAGs on Fe uptake.

Although many studies have addressed the enhancing effect of meat on Fe uptake (*27, 28*), the nature of the promoter has not yet been clearly characterized. Huh et al. (*2*) suggested that acidic carbohydrates derived from GAGs in the extracellular matrix could contribute to the enhancing effect on Fe uptake by Caco-2 cells. In the latter study, a fraction rich in carbohydrates, which preliminary analysis via ^1H NMR indicated to contain heparin-like-GAGs as major constituents of the fractions, enhanced Caco-2 cell Fe uptake. The results obtained in the present study are in agreement with our previous data (*2*), evidencing that GAGs exert an enhancing effect on Fe uptake by Caco-2 cells. Furthermore, in both of our studies, the protein contents in the active fractions were notably low, <2.3 g/100 g of sample (*2*) and 7.2 g/100 g of isolated fraction here.

Previous studies indicated that the enhancing effect on Fe uptake caused by muscle tissue is mainly due to the proteins/peptides (*3, 5, 6*). Recently, Hurrell et al. (*6*) suggested that the meat effect is due to the protein component of beef and porcine muscle tissue but also that it is unlikely that a single peptide is involved. The authors do not exclude the possibility that a nonprotein enhancer of Fe absorption was concentrated in the

chicken protein isolates and suggested that glycosaminoglycans may be potential nonprotein components of muscle tissue that could bind Fe (*6*). Nevertheless, this statement is in conflict with a recent in vivo study that nonheme Fe absorption in young women was not influenced by GAGs (hyaluronate and CS) (*7*). The lack of effect of sodium hyaluronate and CS on enhancing Fe uptake was confirmed by the Caco-2 model when using the same diets, those sent by Dr. Storcksdieck to our laboratory (unpublished results). According to our results, no statistical ($p > 0.05$) differences between both samples and relative to the controls in ferritin concentration were detected. However, a significant 50% increase in Caco-2 cell associated ^{58}Fe in the presence of sodium hyaluronate relative to the addition of chondroitin sulfate was quantified. This observation is explained by the nonspecifically bound Fe to cellular surface proteins, as we previously reported (*29, 30*). Otherwise, ferritin formation by intestinal cells occurs only in response to Fe that which has been taken up being a more reliable indicator of Fe uptake. An interesting aspect to take into account in the in vivo study (*7*) is that the authors claimed to use an amount (360 mg) of GAGs 2−3 times higher than that provided by daily consumption of a typical serving of meat (∼150 g/day). This calculation was based on the previous reported GAGs content in beef muscle (0.14%) (*9*). Otherwise, a higher content of GAGs (0.35%) in porcine muscle has been reported (*14*). On this basis, and considering the content of GAGs reported in porcine muscle (0.35%), the daily consumption of ∼150 g meat/day stated in the in vivo study (*7*) would provide 525 mg of GAGs, which is 1.5-fold higher than that considered by Storcksdieck et al. (*7*). In the present study, the content of GAGs estimated in cooked haddock is 0.56% (sulfur-containing compounds), which indicates a higher efficiency of the extraction procedure used to isolate GAGs relative to beef and porcine muscle. Thus, the ratio GAGs/Fe used in the present study would be achievable on the basis of a daily consumption of a typical serving of meat (∼150 g/day). In addition to the different estimated daily intakes of GAGs, there is a different structural composition of the commercially available chondroitin sulfate C (Sigma), which was used in the in vivo study (*7*), relative to the GAGs extracted from cooked haddock. All of the aforementioned reasons could be responsible for and explain the differences observed between the results reported by Storcksdieck et al. (*7*) and those observed in in vitro studies.

In summary, GAGs were efficiently extracted from cooked haddock muscle after tissue digestion with papain, and their subsequent isolation with cetylpyridinium and fractionation according to their high sulfur content were successful. Taken together, the RP-HPLC analysis and digestion with a selective enzyme for GAGs such as chondroitinase ABC suggest that chondroitin/dermatan sulfate-related structures were present in the purified fraction. When their activity on promoting Fe uptake to Caco-2 cells was evaluated, the purified GAG-containing fraction produced a positive effect on cell ferritin concentration. From these results, the participation of GAGs as part of the factors responsible for the enhancing effect of meat on nonheme Fe uptake should not be ruled out. However, further in vivo studies are needed to confirm their effect and determine the mechanism(s) involved in the meat effect.

ABBREVIATIONS USED

Fe+AA, positive control containing $FeCl_3$ (41.7 μmol/L) and ascorbic acid (1.0 mmol/L); fish, cooked haddock (no extrinsically added Fe); GAG, high-sulfur fraction obtained in 2.1 M NaCl fraction mixed with $FeCl_3$ (41.7 μmol/L); CS, chondroitin sulfate C (Sigma) mixed with $FeCl_3$ (41.7 μmol/L); GAG/Chase, high-sulfur purified fraction digested with chondroitinase ABC (50 mU/mg, 1 h, 37 °C) and mixed with $FeCl_3$ (41.7 μmol/L).

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