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Iron Availability from Whey Protein Hydrogels: An in Vitro Study

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The influence of whey protein hydrogel microstructure, filamentous versus particulate, on iron delivery was studied under different conditions, including simulated gastrointestinal conditions. Experiments were initially conducted to determine the impact of pH and enzymes on iron release. The results show that different iron release profiles can be obtained from filamentous and particulate gels. Particulate gels released more iron than filamentous gels at acidic pH, but the opposite was observed at alkaline pH. In the presence of pepsin at pH 1.2 or pancreatin at pH 7.5, both gel types showed increased protein hydrolysis, but only filamentous gels showed increased iron release, suggesting that matrix structure plays an important role in iron delivery. A dissolution test was carried out under gastrointestinal conditions to mimic the in vivo dissolution process. Filamentous gel released most of its iron during the intestinal phase of a simulated digestion, hence protecting iron during its transit in the gastric zone. Absorption of iron by the Caco-2 system, used to estimate intestinal absorption, revealed that filamentous gels favored intracellular iron absorption. These results suggest that filamentous gels show promise as matrices for transporting iron and promoting its absorption and therefore should be of major interest in the development of innovative functional foods.

KEYWORDS: Iron availability; whey proteins hydrogels; in vitro release; Caco-2 cells

INTRODUCTION

Iron deficiency is one of the most significant nutritional problems in the world (*1*). It affects close to 2 billion people (*1*, *2*). The ideal strategy to resolve this problem is to improve the diet to include a large variety of iron-rich foods and to increase dietary iron absorption (*3*−*5*). However, the incorporation of iron into complex systems such as foods raises a variety of problems, including its oxidation and precipitation, which result in lower bioavailability (*6*−*8*). Given that the presence of amino acids in the intestines is required to increase iron bioavailability (*9*), the use of suitable protein gels may offer an effective strategy for protecting iron and increasing dietary iron absorption from functional foods.

Whey proteins, also known as the serum protein of the principal byproduct of cheese manufacture, are widely used in food products because of their high nutritional value and ability to form gels (*10*, *11*), emulsions (*12*, *13*), and gelled emulsion (*14*). In a previous study, we have shown that cold-induced gelation of β-lactoglobulin, the major whey protein, can be achieved by adding Fe^{2+} ions to a preheated protein suspension (*15*). This method, adapted from that of Barbut and Foegeding (*16*), requires a heating step to denature and polymerize whey proteins into soluble aggregates, followed by a cooling step and the subsequent addition of a ferrous salt, which results in the formation of a network via Fe^{2+}-mediated interactions of soluble aggregates. Two types of gels have been obtained, depending on iron/protein ratios: (1) "filamentous" gels, composed of more or less flexible linear strands, making up a regular network characterized by elastic behavior and high resistance to rupture, and (2) "particulate" gels, composed of large and almost spherical aggregates, characterized by less elastic behavior and lower rupture resistance (**Figure 1**). The former are created by linear aggregation of structural units maintained by hydrophobic interactions, whereas the latter are produced by random aggregation of structural units essentially controlled by van der Waals interactions (*17*). These gels are well characterized at the macroscopic (*15*) and molecular level (*17*). However, nothing is known on the impact of gel microstructure on iron release. This point is of primary importance because only what is released can be absorbed.

The aim of this paper was to study the influence of whey protein hydrogel microstructure (filamentous vs particulate) on iron delivery under various physicochemical conditions, including gastrointestinal conditions. Experiments were initially conducted to determine the impact of different pH values and enzymes on iron release from filamentous or particulate gels.

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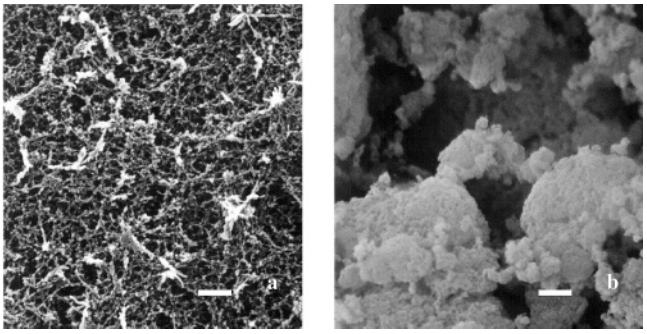

Figure 1. Scanning electron micrographs of β-lactoglobulin cold gels produced in the presence of (**a**, **left**) 10 mM iron (filamentous gels) and (**b**, **right**) 30 mM iron (particulate gels) (*15*). Bar corresponds to 1 μm.

A medium that simulated the gastrointestinal environment was then used to test the gels under conditions mimicking in vivo dissolution. Finally, a Caco-2 cell system was used to imitate the intestinal wall (*18*, *19*) and to estimate the intestinal absorption of iron (*20*, *21*). The results are discussed on the basis of gel microstructure and are linked to their sensitivities to environmental conditions.

MATERIALS AND METHODS

Gel Preparation. Suspensions of β-lactoglobulin (Davisco International, Inc., Le Sueur, MN; 98.2% protein by semimicro-Kjeldahl analysis using an N factor of 6.38) were prepared in double-distilled water at 9.5% protein (w/v), adjusted to pH 7, heated at 80 °C for 30 min, cooled, and then held at room temperature (24 °C) for 4 h. After dilution to 6% protein, 1 M $FeSO_4$ solution (reagent grade, Analar, BDH Inc., Toronto, Canada) was added to obtain an Fe^{2+} concentration of 10 or 30 mM, thus producing filamentous or particulate gels, respectively, as shown in **Figure 1** (*15*).

Dissolution Experiments. Dissolution studies were performed according to Pharmacopoeia official methods (*22*, *23*). Paddle apparatus II, shown in ref *23*, was used with a temperature circulator/controller, both from Distek Inc. (North Brunswick, NJ). Agitator speed was set at 60 rpm, and the temperature was maintained at 37 ± 0.5 °C.

An extraction cell (*22*), made of chemically inert material, consisting of a flat circular support disk and a membrane-retaining annular cover attached by screws, was placed inside the paddle and vessel assembly. The distance between the paddle blade and the top surface of the disk assembly was 25 ± 2 mm throughout the test. A 3 g disk of tested gel was held by the membrane in the central cavity of the cell, the dimensions of which were 32 mm in diameter and 2.6 mm deep. The membrane, which consisted of a single layer of high-strength 100% natural fiber paper (Papeterie Cascadec, France) of large pore size, was thus held between the gel sample and the dissolution medium, but did not affect the intrinsic gel release properties. Located on the side of the extraction cell was a tubing outlet for periodic withdrawal of liquid samples (15 mL) by means of a syringe. The dissolution volume was maintained constant at 900 mL by adding warm fresh medium after each sampling, and dilution was taken into account when concentrations were calculated. These conditions allowed us to maintain sink conditions.

Gastric Acid Medium Preparation. All chemicals and enzymes were obtained from Sigma Chemical Co., St. Louis, MO. The simulated gastric fluid (*24*) consisted of 2.0 g of sodium chloride, 7.0 mL of 37% hydrochloric acid, and 1000 mL of double-distilled water. The final pH was 1.2. Dissolutions were carried out with or without pepsin (3.2 g, 3200 units/mg of protein) to evaluate digestion by acid and enzyme separately. Dissolution of the different gels was followed for 6 h.

Intestinal Alkaline Medium Preparation. The simulated intestinal fluid (*24*) consisted of 6.8 g of monobasic potassium phosphate dissolved in 250 mL of double-distilled water and added to 190 mL of 0.2 N sodium hydroxide and 400 mL of double-distilled water. Dissolutions were carried out with or without pancreatin (10.0 g, activity equivalent to USP specifications) added to this mixture. The pH was adjusted to 7.5 ± 0.1 using 0.2 N sodium hydroxide, and the final volume was then brought to 1000 mL with double-distilled water. Dissolution of the different gels was followed for 6 h.

In Vitro Gastrointestinal Assays. The following protocol was applied to simulate the succession of pH conditions and enzymatic activities encountered after ingestion. The gels were subjected to gastric conditions with pepsin (pH to 1.2) for 30 min, and the pH was then adjusted to 7.5 ± 0.1 with 1 N sodium hydroxide and monobasic potassium phosphate. Pancreatin was then added as described above to transform the simulated gastric conditions into intestinal conditions. Gel dissolution was followed for 6 h with periodic sample withdrawal.

Dissolution Data Analysis. Each experiment was performed on six cells, and the results were expressed as mean, standard deviation (SD), and coefficient of variance (CV%). Analyses of the iron content of the solution were done by standard atomic absorption spectrometry technique using a Perkin-Elmer 3300 spectrometer (Perkin-Elmer, Shelton, CT). Iron released was calculated as

$$[Fe^{2+}]_{\%} = \frac{[Fe^{2+}]_{free}}{[Fe^{2+}]_{total}} \times 100$$

where $[Fe^{2+}]_{free}$ is the free iron concentration in mg/L/g of gel, $[Fe^{2+}]_{total}$ is the total iron concentration in mg/L/g of gel, and $[Fe^{2+}]_{\%}$ is the iron concentration in percentage.

Protein detection was carried out by pyrochemiluminescent measurement of elemental nitrogen (*25*) using an Antek nitrogen analyzer 7000 (Antek, Houston, TX) and a glycine calibration curve (*25*). Nitrogen released was calculated as

$$[\text{nitrogen}]_{\%} = \frac{[\text{nitrogen}]_{free}}{[\text{nitrogen}]_{total}} \times 100$$

where $[\text{nitrogen}]_{free}$ is the free nitrogen concentration of the gels in the solution, $[\text{nitrogen}]_{total}$ is the total nitrogen concentration in the gel, and $[\text{nitrogen}]_{\%}$ is the released nitrogen concentration in percentage.

ANOVA tests were performed to find significant differences between gastric and intestinal treatments as well as between gel structures using the Statgraphics Plus Professional v 4.1 program (Manugistics, Inc., Rockville, MD). The means obtained were used for data interpretation and modeling.

Release Kinetics. To characterize the mechanism of iron release from the gels, the release data were fitted to the following equations using regression analysis:

zero-order equation

$$\frac{dM_t}{dt} = k \quad (1)$$

where k is the constant, t is time, and M_t is the amount of iron released at time t, and

first-order equation

$$\frac{dM_t}{dt} = k(M_0 - M_t) \quad (2)$$

where k is the constant, t is time, and M_0 and M_t are the amounts of iron released at time 0 and t, respectively.

Simulated Intracellular Iron Absorption on Intestinal Wall. *Culture Cell Preparation.* All cell culture media and reagents were obtained from Gibco, Grand Island, NY. Caco-2 cells originating from a human colorectal carcinoma were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Caco-2 cells were grown at 37 °C in an atmosphere of 5% CO_2−95% air at constant humidity in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal calf serum, 1% nonessential amino acids, and 1% streptomycin/penicillin. The cells were routinely propagated in tissue culture flasks (75 cm^2) and harvested at 80−90% confluence by treatment with

0.25% trypsin in 1 mM EDTA, thoroughly washed, and replaced in supplemented growth medium.

Iron Intracellular Absorption. Caco-2 cells seeded at 7×10^4 cells/cm^2 in tissue culture flasks (75 cm^2) were used in iron intracellular absorption experiments after 20 days of culture. Each flask was washed carefully with PBS, and 5 mL of gastrointestinal dissolution filtrate (0.45 μm) was added. Flasks of washed cells without dissolution filtrate were treated as reference samples. The cells were then incubated for 2 h and washed again with PBS, and 5 mL of DMEM supplemented with 20% fetal calf serum was added before the culture flasks were returned to the incubator for 22 h. Cells were then harvested in 2 mL of PBS buffer and sonicated for 20 min at 4 °C for iron and ferritin analysis (*26*). Iron content was determined using an atomic absorption spectrometer with graphite electrode (Perkin-Elmer 3300), whereas ferritin content was measured on 25 μL aliquots using a microparticle enzyme immunoassay (MEIA) method (Abbott AxSYM System for ferritin detection, Abbott Park, IL). An internal blood standard was used to test the precision of the method.

RESULTS

Data Quality. Each experiment was performed on six cells, and dissolution data analysis revealed good reproducibility. Data variability was low, as estimated from the coefficient of variance, which was <10% in all experiments for the random aggregated gel. For the filamentous gel, variability was higher in the gastric medium but always remained below 15% (data not shown). The effect of pH and pepsin on the gel structure may have contributed to this difference. The sink conditions and membrane integrity were both maintained throughout the experiment.

Impact of Physicochemical Conditions on Iron Release. *pH-Sensitive Iron Delivery.* The release rate of molecules from hydrophilic matrices, such as proteinaceous hydrogels, is known to be affected by pH variations. This is due to the presence of acidic (e.g., carboxylic) or basic (e.g., ammonium) groups in the polypeptide chains, which either accept or release protons in response to changes in the pH of the medium (*27*). As a result, pH-responsive polymeric networks have been extensively studied (*28*) and have been one of the most frequently used systems for the development of controlled release formulations for oral administration. The difference between the acidic pH in the stomach and the neutral pH in the intestine is large enough to result in differing release profiles. The release of iron from particulate and filamentous gels in the course of matrix degradation was therefore investigated under physiological pH conditions, namely, gastric pH 1.2 and intestinal pH 7.5.

Figure 2 shows iron and nitrogen released by degradation at pH 1.2 (**a**, **b**) and pH 7.5 (**c**, **d**) of the two types of β-lactoglobulin gel. Under acidic conditions, iron release presents a significant statistical difference ($p < 0.05$) between the two gel types (**Figure 2a**). After 360 min, release from filamentous gels (65%) is lower than from particulate gels (85%), suggesting that the latter are more sensitive to acidic conditions. Iron release from filamentous gels follows a zero-order model (eq 1, $r^2 = 0.9285$), whereas its release from particulate gels can be fitted to a first-order equation (eq 2, $r^2 = 0.9394$). These results indicate that the release of iron is not dependent on iron concentration in the case of filamentous gels, whereas it is for particulate gels. In comparison to iron release, the release of soluble nitrogen from the two types of gels was minimal. Patterns were statistically similar from 0 to 120 min but significantly different between 120 and 360 min (**Figure 2b**). These results demonstrate that in acidic conditions, the release of iron from the gels was strongly dependent on gel microstructure. The influence of gel degradation is difficult to observe because of the very limited degree obtained under these conditions.

Increasing the pH to 7.5 changed the release profiles, as illustrated in **Figure 2c**. For both types of gel, the iron release profiles were biphasic, characterized by an initial relatively rapid release phase (first 30 min) followed by a slower sustained release in the case of filamentous gels but no additional release from particulate gels. After 360 min, the release of iron was 32% from filamentous gels and 10% from particulates gels. Compared to acidic conditions (**Figure 2a**), iron release in alkaline conditions was significantly lower, regardless of gel type. However, contrary to the observations in acidic conditions, iron release from filamentous gels was greater than from particulate gels. Soluble nitrogen release (**Figure 2d**) had a pattern similar to that observed under acidic conditions (**Figure 2b**), indicating that neither condition (pH 1.2 or 7.5) produced more than minimal gel degradation. The results obtained at pH 7.5 again demonstrate that the release of iron from the gels was strongly dependent on gel microstructure, whereas the impact of gel degradation is difficult to ascertain because of the limited degree obtained, indicating that iron release under this condition must have been determined mainly by factors affecting diffusion.

Enzyme-Sensitive Iron Delivery. Among the factors that modulate the release properties of hydrogels, those that promote gel breakdown play a major role. In orally administered products, the most important hydrogel degradation mechanism, particularly in protein-based hydrogels, is hydrolytic breakdown by gastrointestinal tract (GIT) digestive enzymes, primarily pepsin at pH 1.2 and pancreatin at pH 7.5.

Figure 3 shows iron and soluble nitrogen release from filamentous and particulate gels in the presence of pepsin at pH 1.2 (**a**, **b**) and in the presence of pancreatin at pH 7.5 (**c**, **d**). In the presence of pepsin, the profile of iron release from particulate gels was strikingly similar to that obtained in the absence of enzyme (**Figure 2a**), whereas release from filamentous gels was substantially increased. The profiles did not differ significantly for the two types of gel ($p > 0.05$), in contrast with the result obtained in the absence of enzyme at the same pH. After 360 min, about 80 and 100% of the iron was released from particulate and filamentous gels, respectively. The presence of pepsin considerably increased the degradation of both particulate and filamentous gels (**Figure 3b**) compared to the results obtained in the absence of enzyme (**Figure 2b**). In the presence of pancreatin (**Figure 3c**), the iron release profile was comparable to that obtained in the absence of enzyme (**Figure 2c**), exhibiting an initial rapid release phase followed by a slower second phase, with release limited at ~10% for particulate gels while progressing from about 30 to 50% after 360 min for filamentous gels. This result was obtained in conjunction with extensive matrix breakdown in both gels (**Figure 3d**), albeit considerably less breakdown than obtained with pepsin. These results nevertheless suggest that enzymatic proteolysis, by either pepsin or pancreatin, substantially affects the release of iron from filamentous but not particulate gels.

Iron Delivery under Gastrointestinal Conditions. Because iron should be released as close as possible to its absorption window (i.e., intestinal wall), it is crucial to control its release along the gastrointestinal (GI) tract. Hydrogel susceptibility to GI conditions was therefore studied using a two-step proteolysis performed at 37 °C, including pepsin predigestion at pH 1.2 for 30 min to simulate gastric conditions, followed by digestion by pancreatin at pH 7.5 for 6 h to simulate intestinal conditions (*29*).

Figure 4 presents the time courses for total iron (**a**) and soluble nitrogen (**b**) released from the hydrogels during the

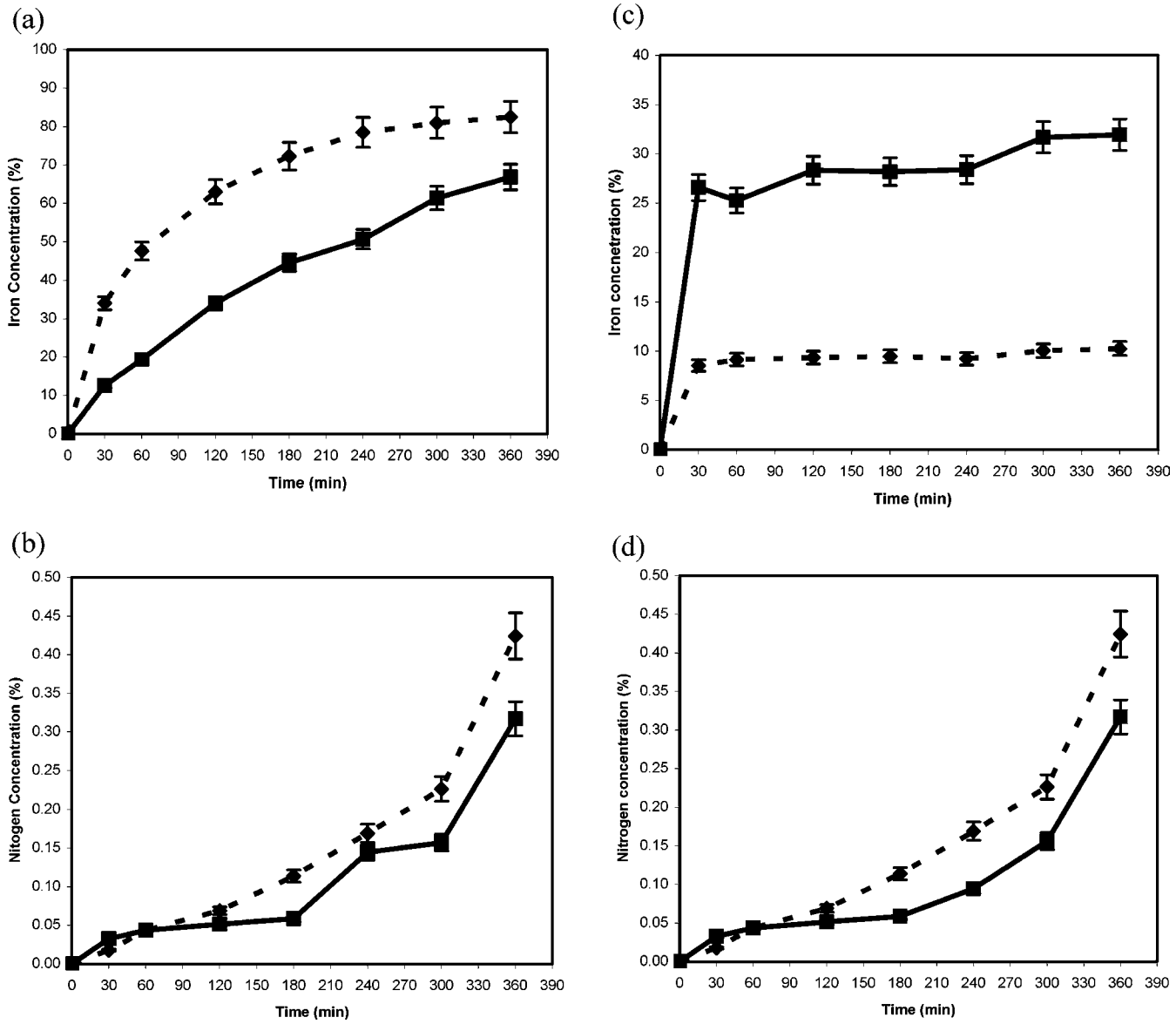

Figure 2. Iron and soluble nitrogen release from filamentous (solid line) and particulate (dashed line) β-lactoglobulin gels at pH 1.2 (**a**, **b**) and pH 7.5 (**c**, **d**).

gastric step (first 30 min) and intestinal step (the next 6 h). At the end of the gastric step, ~20% of the iron had been released from both particulate and filamentous gels (**Figure 4a**). This burst-release was followed by a plateau in the case of particulate gel, whereas a linear release followed in the case of filamentous gels, reaching a cumulative release of 80% at the end of the intestinal step. However, iron release was not correlated with matrix degradation for either filamentous or particulate gels (**Figure 4a**,**b**). These results corroborate the above conclusion that gel structure, and not proteolysis, modulates iron release.

The experiments described thus far do not indicate, however, whether iron absorption occurs in the intestinal wall. Studies of iron diffusion into the intracellular cellular medium were therefore carried out. Because Caco-2 cell monolayers behave similarly to human intestinal mucosa (*18*, *26*), these were exposed to the solutions obtained from the simulated GI digestions. **Figure 5** shows the intracellular iron concentrations obtained with the reference treatment and exposure to the filtrates from filamentous and particulate gel digestions. Although the reference sample was obtained from Caco-2 cells that were not exposed to gel hydrolysis filtrate, it contained iron (0.157 ± 0.031 mg/L). Statistical analyses indicated a significant difference ($p < 0.05$) between the reference sample and the filtrate-exposed cells for filamentous gel, but none with respect to particulate gels. These results are complementary to those obtained in the in vitro release experiment, indicating that the iron released from filamentous gels provides significantly higher cellular absorption than that obtained from particulate gel. They suggest greater bioavailability of iron released from filamentous gels compared to particulate gels. However, it is premature at this stage to predict the bioavailability of iron (i.e., the amount of ingested iron absorbed into body tissues) in such a matrix, because iron that is adsorbed to the intestinal cell may remain there and be excreted by cell desquamation and, therefore, not be bioavailable (*26*). Consequently, it is necessary to use a marker of iron activity.

According to Crosby (*30*), mucosal synthesis of ferritin is normal under conditions of iron repletion. Iron entering from the lumen is thus engaged in mucosal ferritin. This hypothesis suggested a mechanism by which the ferritin content of cells would be determined by the amount of active (bioavailable)

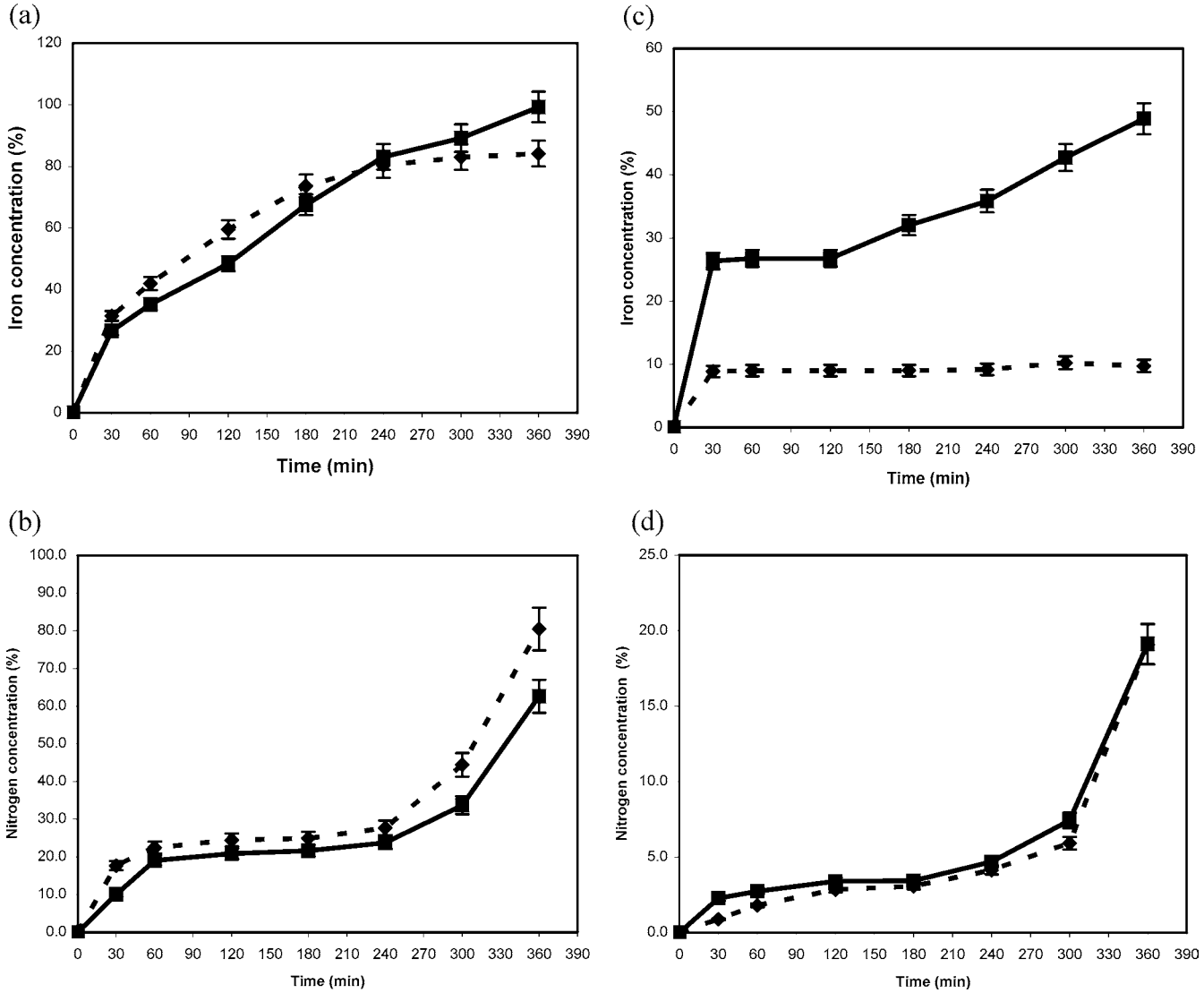

Figure 3. Iron and soluble nitrogen release from filamentous (solid line) and particulate (dashed line) β-lactoglobulin gels in the presence of pepsin at pH 1.2 (**a**, **b**) and pancreatin at pH 7.5 (**c**, **d**).

iron in the cell's environment. Mucosal ferritin levels may thus be closely linked to iron bioavailability (*26*, *30*, *31*).

Figure 6 displays the intracellular ferritin (ng/mL) obtained from Caco-2 cells given the reference treatment or exposed to filtrates from filamentous and particulate gel digestion. Cells exposed to the product of filamentous gel digestion contained a higher level of ferritin (with significant difference, $p < 0.05$) than those exposed to the product of particulate gel digestion. This tendency reflects that obtained for iron (**Figure 5**) and again suggests that iron released from filamentous gels is more bioavailable than that released from particulate gels, supporting the hypothesis of a role for gel structure in iron bioavailability.

DISCUSSION

In a previous work (*17*), we showed that particulate gels are produced by random aggregation of unit structures inside which iron is entrapped, whereas filamentous gels are created by linear aggregation of structural units that form filaments with iron located at the outer surface. Microstructural analysis shows that particulate gel formation leads to larger gel pore sizes than obtained by filamentous gel formation (*15*). The impact of gel microstructure on iron release revealed in the present study will now be discussed.

The two gel types are distinguished by their sensitivities to pH and enzyme activity as well as by their responses to GI tract conditions.

First, the gels differ by their behavior in acidic (pH 1.2) and basic (pH 7.5) conditions. The release of iron is greater in acidic conditions than in basic conditions, suggesting that the latter induce stronger interactions between gels and iron. This may be explained by the net charge of the protein molecules. As the pH differs from the p*I* (5.2), the net charge of the whey protein molecules increases (positive below p*I*, negative above p*I*). In acidic conditions, the greater release of iron may be attributed to electrostatic repulsive forces between positive charges on the polypeptide chains, produced by the protonation of amine groups, and the positively charged ferrous ions. Iron release is facilitated by the pores in the gel microstructure, which are larger in particulate gels than in filamentous gels (**Figure 1**). Conversely, above the p*I*, polypeptide chains negatively charged due to ionized carboxyl groups can interact strongly with ferrous ions, limiting their release. The initial rapid release observed at pH 7.5 may be due to iron located at and near the gel surface. In the case of particulate gels, the release of iron is much less, due to its location inside aggregates that associate to form networks (*17*). It can therefore be

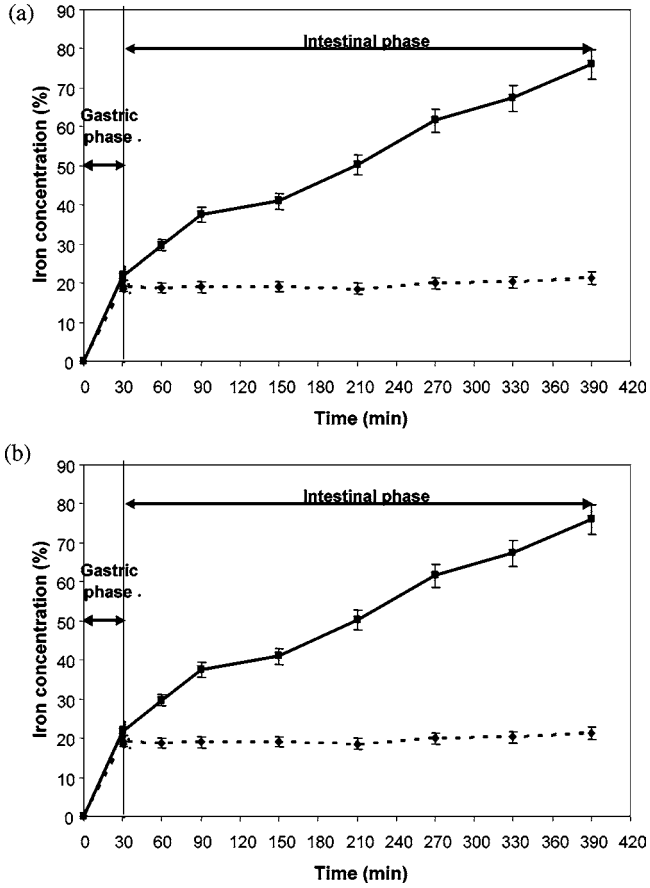

Figure 4. Impact of simulated GI conditions on iron release (**a**) and soluble nitrogen release (**b**) from filamentous gels (solid line) and particulate gels (dashed line).

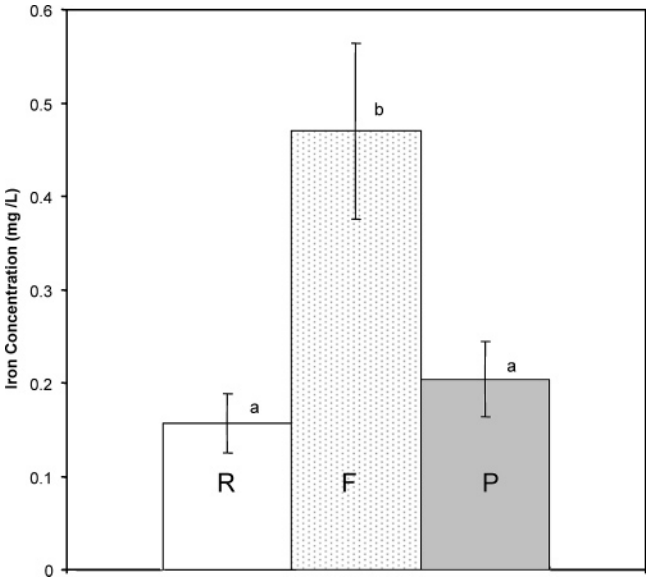

Figure 5. Iron concentration (mg/L) inside Caco-2 cells: (**R**) reference solution; (**F**) solution from simulated GI digestion of filamentous gels (10 mM Fe^{2+}); (**P**) solution from simulated GI digestion of particulate gels (30 mM Fe^{2+}). Bars with different letters are significantly different ($p < 0.05$).

concluded that the presence of ionizable and/or ionized groups along with pore size govern the pH-sensitive iron release mechanism.

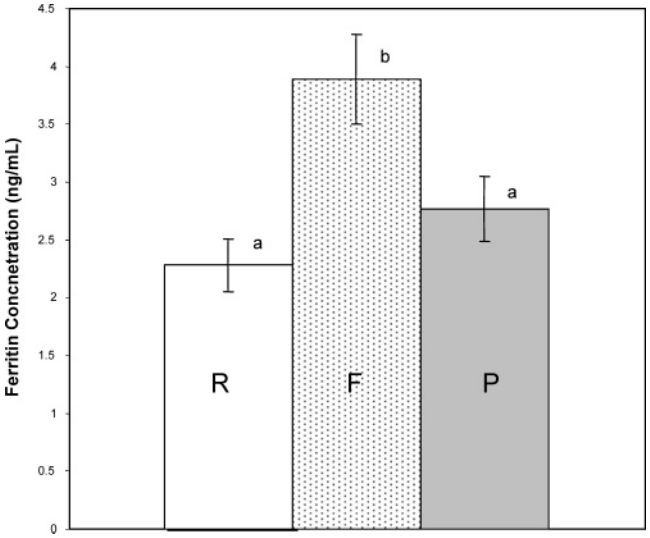

Figure 6. Ferritin concentration (ng/mL) inside Caco-2 cells: (**R**) reference solution; (**F**) solution from simulated GI digestion of filamentous gels (10 mM Fe^{2+}); (**P**) solution from simulated GI digestion of particulate gels (30 mM Fe^{2+}). Bars with different letters are significantly different ($p < 0.05$).

Second, the addition of digestive enzymes reveals that in gastric (pH 1.2 plus pepsin) and intestinal (pH 7.5 plus pancreatin) conditions, matrix digestion is considerable in comparison to the degree obtained without enzymes. The release of iron from filamentous gels is influenced by the presence of digestive enzymes, whereas the release of iron from particulate gels is not influenced by either gastric or intestinal enzymes. These behaviors corroborate the proposition that iron localization inside the matrix plays a major role, because iron is entrapped within the random aggregates of particulate gels but located at the outer surface of the linear aggregates of filamentous gels, facilitating its release.

The last point concerns the behavior of gels under GI conditions, that is, a gastric step (first 30 min) followed by an intestinal step. In the gastric step, a small amount of iron (20%) is released from both gel types, indicating that either filamentous or particulate networks have the desired property for oral delivery of iron, because a significant fraction of it remains in the gels in the low-pH environment of the stomach. However, filamentous gels release larger amounts of iron in the intestine, where most intracellular iron uptake occurs, thus favoring increased iron absorption.

In conclusion, this research shows that different iron release profiles can be obtained depending on the microstructure of the gels in which the mineral is entrapped. Filamentous gels, prepared in the presence of 10 mM Fe^{2+}, differ from particulate gels, prepared in the presence of 30 mM Fe^{2+}, with respect to their response to different conditions, especially to pH and enzymes, providing means of controlling iron release. Furthermore, the results reveal that more iron is released and available for absorption into the intestinal wall from filamentous gels than from particulate gels, indicating that incorporation of excess iron is not necessary to increase its bioavailability. Therefore, filamentous gels constitute an excellent matrix for transporting iron and promoting its absorption. These characteristics may be useful for site-specific controlled biomolecule delivery, a strategy that is widely used in the pharmaceutical field (*27*, *32*, *33*), and could find a broad range of applications in the development of innovative functional foods.

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