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Influence of the Extent of Hemoglobin Hydrolysis on the Digestive Absorption of Heme Iron. An In Vitro Study

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This study was designed to assess the interactions of heme with peptides produced by enzyme hydrolysis of hemoglobin, and their relationship with heme iron absorption. Bovine hemoglobin was hydrolyzed by pepsin or by subtilisin, which differ in their hydrolysis processes. The hydrolysis rate ranged from 0 (native hemoglobin) to 15%. Heme solubility and heme−peptides interactions were compared to iron absorption by the Ussing chamber model, at intestinal pH (7.5) . Increasing hemoglobin hydrolysis enhanced iron absorption; the highest value was reached between 8 and 11% hydrolysis, whatever the enzyme used. Comparing the products of hydrolysis of the two enzymes showed that heme iron absorption depends not only on its solubility, but relies mainly on the balance between the strength of heme−peptides and the polymerization rate of heme.

INTRODUCTION

Iron deficiency is a major nutritional problem in both developed and developing countries. Iron absorption of nonheme iron is usually poor and depends on digestive interactions (*1*); moreover, supplementation with iron salts causes side effects such as diarrhea, nausea and vomiting, constipation, and epigastric pain (*2*).

Heme iron is provided by meat and blood-derived foods, and it is more efficiently absorbed than nonheme iron: when provided within a meal its absorption rate is about three times greater than that of control salts (*3*) and is less affected by digestive interactions (*3*, *4*). Hemoglobin was already used with success for food fortification (*5*, *6*). The large range of absorption rates of heme iron depends on whether hemoglobin or heme is used; hemoglobin absorption is usually less than iron absorption from muscle (*7*−*9*). Heat-induced denaturation affects also heme iron availability (*10*). In addition, the low iron concentration of hemoglobin (0.35%) limits its use in this native form.

Concentrated heme issued from hemoglobin hydrolysis and purification yields poorly absorbed iron (*6*, *11*, *12*), because pure heme iron is poorly soluble at the low gastric pH. The presence of peptides produced by peptic hydrolysis of globin is needed to prevent the formation of large insoluble heme polymers. Heme iron is absorbed as an intact metalloporphyrin across the intestinal brush border by a specific transporter and is cleaved inside the enterocyte by a heme oxygenase (*13*). It then enters the common enterocyte pool of inorganic iron. Therefore, it appears that interactions occurring during the luminal phase of heme iron digestion could be important determinants of its absorption (*4*, *11*).

The present work studied these interactions to yield a concentrated heme iron with a good bioavailability. Hemoglobin digestion was performed with pepsin or subtilisin, which differ in their mechanisms of hydrolysis.

The in vitro heme−heme and heme−peptide interactions were correlated with iron absorption by rat duodenum in the Ussing chamber model (*14*).

MATERIALS AND METHODS

Ussing Chamber. Before the experiments the diffusion cells (Marty Technologies, Marcilly-sur-Eure, France) were washed with dilute (1/100) nitric acid (Merck Laboratories, Nogent-sur-Marne, France) to prevent contamination. Ringer Lavoisier solution (Laboratoires Chaix et Marais, Nancy, France) was used for luminal and serosal solutions. It is made of (mmol/L) Na^+ (139), K^+ (2.7), Ca^{2+} (1.8), $HCO3^-$ (2.4), and Cl^- (141.4), at pH 6.55. Initial luminal iron concentration was 100 μmol/L. D-Glucose (20 mmol/L) was added to the serosal medium to help maintain tissue viability, and D-mannitol (20 mmol/L) was added to the luminal solution to keep the osmolarity at 300 mOsmol/L on each side of the mucosa (*14*). The pH was adjusted to 7.5 by addition of HCl. Solutes were circulated by gas lift controlled by valves (O_2 95%/CO_2 5%) to which an antifoaming agent was added (Silicone 414, Rhodorsil, France).

The diffusion cells and the media were preheated to, and maintained at, 37 °C by a block heater.

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Animals. Adult female Sprague−Dawley rats ($n = 6$/group), 200−250 g, were obtained from Caen University farm. They were housed at 20−22 °C in a room with controlled lighting. The rats had free access to a maintenance diet for adult animals (UAR, Villemoisson-sur-Orge, France; [Fe] = 240 mg/kg diet) and deionized water.

Experimental Design and Sample Analysis. The protocol used was previously described (*14*, *15*). Briefly, animals were fasted overnight, and were killed by intracardiac injection of Pentobarbital (Doléthal, Vétoquinol). The first centimeter of duodenum was used for the experiment and placed in the Ussing chamber; the next centimeter was used to measure the basal mucosal content of iron.

Integrity and viability of intestinal segment were controlled by histological preparation and by determination of transepithelial electrical resistance (TEER) across the membrane (Millicell-ERS, Millipore) through experience. The resistance (ohms- cm^2) of the two diffusion chamber mediums (without mucosa) was subtracted from the values obtained in the presence of intestinal membrane (*16*).

At the end of the experiment (2 h), the integrity of the mucosa was checked by histology (results not shown). Luminal and serosal media were analyzed before and at the end of the experiment. The two half-cells were rinsed with diluted nitric acid until no iron could be desorbed from cell walls or digestive membrane.

The pieces of duodenum were dried in an oven until their weight was stable and were digested by incubating with 1 mL of nitric acid (65%) for 24 h.

Iron concentration was measured in luminal and serosal media and in duodenal tissues (experimental and control) by atomic absorption spectrometry (Perkin-Elmer 1100B).

Iron uptake and absorption were calculated as follows: %Tot, iron uptake (%) during the experiment, calculated as iron content of the experimental mucosal minus content of control loop; and %S, iron absorption (% initial amount) defined as iron transferred from the luminal to the serosal medium across mucosa during the experiment.

Hemoglobin Hydrolysis. Bovine hemoglobin was prepared from centrifuged red cells provided by Veos Novo (Zwevezele, Belgium). Hemoglobin solution was adjusted to 5% (w/v) with 10 mM HCl. Hemoglobin concentration was determined according to the cyanmet-hemoglobin method (17). The pH was then adjusted to the appropriate hydrolysis pH with HCl (1 mol/L) or NaOH (1 mol/L).

Hydrolysates were prepared at pilot plant scale at the Laboratoire de Technologie des Substances Naturelles (Lille University, France), as previously described (*18*). Enzymes were used at their optimal pH.

Pepsin hydrolysis was carried out at pH 3 and 40 °C by addition of porcine pepsin (pepsin A, EC. 3.4.23.1, from porcine stomach mucosa, Sigma Chemical Co., St. Louis, MO) with an enzyme/protein ratio of 132 000 UI/g. pH was maintained constant by using a pH-stat (Titroline alpha, Schott Geräte, Hofheim, Germany). The reaction was stopped by adjusting the solution to pH 8 with 1 mol/L NaOH. Subtilisin hydrolysis (Subtilisin Carlsberg, EC. 3.4.21.14, Gist Brocades, Seclin, France) was performed at pH 10 and 40 °C with an enzyme/protein ratio of 0.36 U Anson/100 g. The enzyme was inactivated by addition of phenylmethylsulfonylfluoride (Sigma; 5 μL at 100 mmol/L in 2 mL of hydrolysate).

An increasing level of hemoglobin hydrolysis from 0% (native hemoglobin) to 15% was obtained for two enzymes. The rate of hydrolysis (DH) defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds, was determined by the Adler−Nissen method (*19*). Amino groups released during hydrolysis were assayed by the trinitrobenzenesulfonate acid method (TNBS).

Assessment of Heme and Peptide Solubility. Heme−peptide interactions in hemoglobin are very strong. Adjusting pH to 2 by addition of 1 mol/L HCl assessed the stability of interactions between peptides. The solutions were then shaken and centrifuged at 5000 rpm for 15 min. Heme concentration of each supernatant was determined by the pyridine hemochromagen method (*18*, *20*).

Native hemoglobin and heme monomers present one absorption band at 400 nm: the Soret band (*21*). The ultraviolet (UV) spectrum of hemoglobin hydrolysates allows observation of the association states of heme. They were recorded on a Lambda 5 UV/Vis spectrometer (Perkin-Elmer), at 340−500 nm of wavelength. A 25-μL aliquot of hemoglobin solution or hydrolysates was diluted with 5 mL of 50 mM phosphate buffer pH 7.5 (*18*).

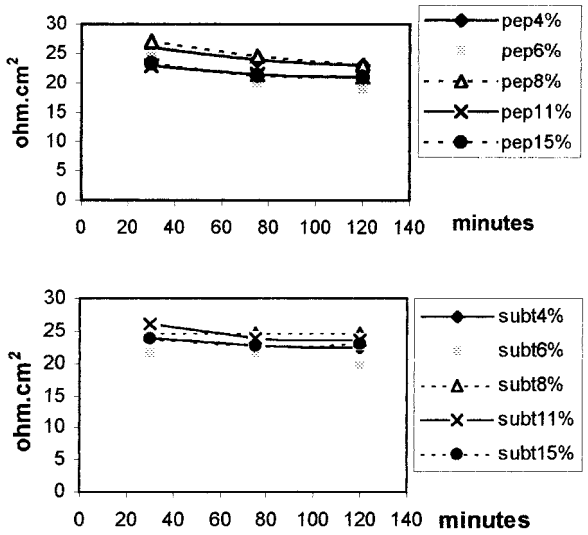

Figure 1. Mucosa transepithelial electrical resistance (TEER): pepsin hydrolysis, left panel; subtilisin hydrolysis, right panel; results are given as mean values (ohms cm^2). No significant change (ANOVA $p < 0.05$) in TEER was observed through the experience and between groups.

Statistical Analysis. Results are expressed as means ± 1 SD. The absorption rate of iron provided by the two kinds of hydrolysates from two enzymes was compared by Student's *t* test at each hydrolysis rate. The influence of the rate of hydrolysis was studied by an ANOVA test between groups and post hoc analysis using the Fischer exact *t* test. The level of significance was set at $p < 0.05$.

RESULTS

Figure 1 shows the evolution of transepithelial electrical resistance (TEER) throughout the experience. No significant difference was observed between groups, nor significant changes with time.

Table 1 shows the results of iron uptake and absorption across the mucosa, depending on the rate of hemoglobin hydrolysis.

Iron uptake from peptic hydrolysates increased significantly from a DH of 4% hydrolysis to a DH of 8−11% and decreased thereafter to the native hemoglobin level. For the subtilisin hydrolysates, the increase of uptake occurred from a DH of 6% hydrolysis up to a DH of 8−11%, and decreased above 15% hydrolysis; however it remained significantly higher than that from hemoglobin. The highest iron uptake was observed between 8 and 11% hydrolysis for both enzyme hydrolysates. Iron uptake from hydrolysates produced by subtilisin was significantly higher at 15% hydrolysis than that from peptic hydrolysates.

Iron absorption from every hydrolysate was higher than that of native hemoglobin, except for the 11% DH peptic hydrolysate. At 15% hydrolysis, iron from subtilisin hydrolysate displayed a better absorption than peptic hydrolysate.

The changes of the Soret bandwidth and of heme solubility determined at pH 2 as a function of DH are shown respectively in **Figures 2** and **3**. The relative increase of the Soret bandwidth illustrates the polymerization of heme that occurs during the course of hemoglobin hydrolysis; the heme solubility at pH 2 decreases as the strength of the heme−peptide association weakens.

In the course of subtilisin hydrolysis, heme polymerization remained low up to a DH of 7%; it then progressively increased up to a DH of 14%. During peptic hydrolysis, heme polymer-

Table 1. Iron Uptake (%Tot) and Absorption (%S)

DH [a]		0 (Hb)	4	6	8	11	15
%Tot[b]	peps	1.57 ± 0.29	2.94 ± 0.59	3.83 ± 1.13	4.51 ± 1.16a	4.19 ± 0.83a	2.54 ± 0.58*b
	subt	1.56 ± 0.29	2.87 ± 0.38	3.00 ± 0.39	5.55 ± 1.56c	6.02 ± 1.91c	3.77 ± 0.32*
%S[c]	peps	1.15 ± 0.39	2.20 ± 0.44d	1.76 ± 0.40d,e	2.64 ± 0.35d	1.71 ± 0.28e	1.77 ± 0.51*d, e
	subt	1.15 ± 0.39	2.42 ± 0.50d	2.21 ± 0.6d	2.40 ± 0.44d	2.08 ± 0.47d	2.69 ± 0.28*d

[a] Heme hydrolysates: pepsin (peps) vs subtilisin (subt) hydrolysis at different hydrolysis rates (DH, percent of native hemoglobin). Student *t* test between samples with the same DH: *, $p = 0.02$. [b] %Tot, iron uptake by mucosa (% initial luminal amount). ANOVA followed by Fischer exact *t* test within each row: pepsin, ANOVA = 0.0014; subtilisin: ANOVA < 0.001. Online a, different from 4 and 15% DH ($p < 0.001$); b, different from 6% DH ($p = 0.03$); c, different from every other group ($p = 0.02$). [c] %S, iron absorption; iron transferred from luminal to serosal compartment (% of initial luminal amount). ANOVA followed by Fischer exact *t* test within the same row: pepsin, ANOVA $p=0.0002$; subtilisin: ANOVA $p < 0.0001$. Online d, different from Hb ($p < 0.04$); e, different from DH 8% ($p < 0.01$).

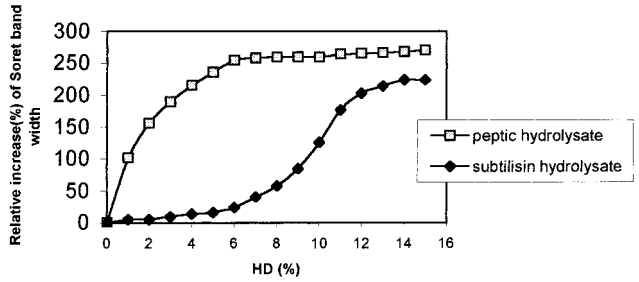

Figure 2. Increase of Soret bandwidth during hydrolysis of hemoglobin by pepsin and subtilisin. Intensities of Soret band are expressed relative to that of native hemoglobin.

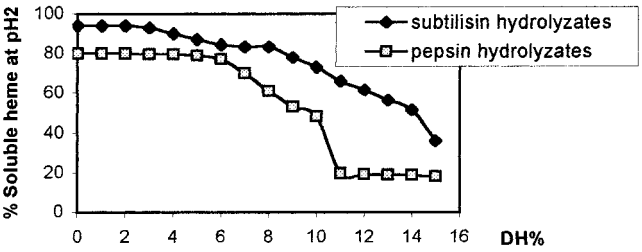

Figure 3. Heme–peptides interactions during hemoglobin pepsin and subtilisin hydrolysis. The interactions were quantified by the aptitude of peptides to maintain heme in solution at pH 2.

ization started with hydrolysis and reached its maximum for a DH of 6%. It can be noticed that at a DH of 15%, heme of the subtilisin hydrolysate was less polymerized than that of peptic hydrolysate.

During hydrolysis by subtilisin or pepsin, the strength of the heme−peptide associations was slightly altered up to a DH of 6%, although it was slightly weaker in peptic hydrolysates than in subtilisin hydrolysates (**Figure 3**). However, from about 8% of hydrolysis and above, heme−peptide interactions were dramatically weakened. Whatever the DH, they were significantly stronger in the subtilisin hydrolysates than in peptic ones.

DISCUSSION

Dietary heme is somewhat protected from nutrient interactions in the digestive tract (*6*, *7*, *9*, *22*, *23*). Peptides and amino acids produced by meat hydrolysis can enhance the absorption of heme and nonheme iron (*7*, *11*, *24*); nevertheless, pure heme issued from hemoglobin hydrolysis is poorly absorbed because it forms large, insoluble polymers at low pH (*11*).

Optimizing the interactions between heme and peptides could improve the absorption of hemoglobin-derived heme iron; providing a heme iron complex could contribute to that effect inasmuch as the most efficient site of iron absorption is the proximal digestive tract, before a significant hydrolysis of dietary proteins can occur (*25*).

The present study aimed at more precisely defining the interactions between the products of enzyme hydrolysis of hemoglobin and their effects on heme iron absorption. The Ussing chamber model uses a full-thickness piece of mucosa, including the presence of adherent mucus layer which has been shown to play a key role in the absorption process of iron (*25*). This model was previously used in absorption studies of minerals and peptides (*15*, *26*, *27*). The absorption and metabolism of heme iron are known to occur in the rat mucosa such as that in humans, even if the absorption of heme iron is lower in the rodent (*13*, *28*−*31*).

The results of the present study showed that increasing the hydrolysis level of hemoglobin was associated with an enhancement of iron uptake; the highest uptake rate was reached between 8 and 11% of globin hydrolysis, whatever the enzyme used. Although the uptake of iron provided by the subtilisin hydrolysate was significantly higher than that of peptic at 15% hydrolysis.

The interactions between heme and peptides produced by hemoglobin hydrolysis gave some information on the luminal phase of its digestion, which depends on both the type of enzymatic hydrolysis and the rate of globin hydrolysis. The mechanisms of heme iron solubility and availability differ between the two enzymes: the peptic hydrolysis leads to weak heme−peptide interactions and to an intense polymerization of the heme; on the contrary, after subtilisin hydrolysis a low polymerization rate of, but strong, heme−peptides interactions are observed.

These results suggest that heme iron uptake by intestinal cells could be affected by two phenomena: the occurrence of monomers or polymers of heme and the strength of the heme−peptide interactions.

Hemoglobin is hydrolyzed by subtilisin along a "one-by-one" mechanism (*19*). It releases low-molecular-weight peptides throughout the reaction. So, subtilisin hydrolysates were made of free proteins (or large polypeptides) and small peptides. One can assume that up to about a 6% hydrolysis rate (DH), heme was solubilized as monomers by native globin (or very large peptides). At higher DH, the concentration of globin chains was not sufficient and heme was solubilized by small peptides which could not prevent its polymerization.

At pH 3, hemoglobin is hydrolyzed by pepsin according to a "zipper" mechanism (*19*). Pepsin hydrolysis produced a fairly continuous distribution of intermediate size peptides which were degraded to small peptides. From the onset of the peptic hydrolysis, heme was carried as polymers by these peptides. Therefore, heme polymerization was lower in subtilisin hydrolysates than in peptic hydrolysates. Moreover, the strength of heme−peptide interactions was weaker in peptic hydrolysates, especially at high DH.

The comparison between biochemical and absorption data suggests that the formation of soluble heme−peptide complexes was not sufficient to enhance heme iron absorption, because globin-bound iron was poorly absorbed. On the other hand, the weak binding of heme to low-molecular-weight peptides, which occurs in the subtilisin hydrolysates, enhanced iron absorption. Providing dietary heme iron in such a hydrolyzed form in the diet can be of interest, because during the time of duodenal digestion which is allowed for iron absorption no such interactions are likely to occur at a significant rate.

These observations give strength to the role played by mucin in the absorption of minerals. It binds and keeps soluble monomeric ionic and complexed iron (*32*). The pH gradient of the adherent mucus layer may facilitate cellular uptake of iron by dissociating the soluble complexes and enhancing heme−enterocyte membrane interaction (*33*). Therefore, the mucus layer could act as coarse filter preventing the diffusion of large heme−globin complexes and could enhance the access of heme from the heme−peptide complexes to the apical membrane of the enterocyte.

The differences observed between the absorption rate of iron between the two hydrolysates suggest that the profile of peptides is likely to influence both the digestive and the enterocyte phases of absorption of heme iron. Amino acids such as cysteine are known to enhance the enterocyte transfer of both nonheme and heme iron (*15*, *34*) possibly because free iron released by heme oxygenase enters a common cellular pool. On the other hand, the constancy of mucosal permeability assessed by TEER throughout the experiment suggests that no significant iron absorption occurred by the passive paracellular pathway; it confirms that in basal conditions tight junctions do not leak heme−peptide (*27*).

Several steps are critical for the regulation of heme iron absorption. The uptake and heme oxygenase activity are the main determining factors (*13*). Heme splitting is enhanced in iron-deficient animals; therefore, increasing heme uptake by intestinal epithelium could provide more substrate to heme oxygenase and enhance heme iron bioavailability.

CONCLUSION

This study assessed the influence of two hemoglobin hydrolysis processes of the absorption of heme iron. The results emphasize the influence of the surrounding peptides on the digestive metabolism of heme iron, which depends on heme solubility and polymerization and on the strength of heme−peptides interactions. They suggest that both the luminal and the enterocyte phases can be critical for the regulation of heme iron absorption. Providing heme iron in a preformed complex could help improve its absorption during the short duodenal transit time.

Although the in vitro model reported here used peptides that were in a digested form, further in vivo studies are needed to confirm these observations.

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