



Bioavailability of Iron-fortified Whey Protein Concentrate in Iron-deficient Rats

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ABSTRACT : An iron-fortified whey protein concentrate (Fe-WPC) was prepared by addition of ferric chloride to concentrated whey. A large part of the iron in the Fe-WPC existed as complexes with proteins such as β -lactoglobulin. The bioavailability of iron from Fe-WPC was evaluated using iron-deficient rats, in comparison with heme iron. Rats were separated into a control group and an iron-deficiency group. Rats in the control group were given the standard diet containing ferrous sulfate as the source of iron throughout the experimental feeding period. Rats in the iron-deficiency group were made anemic by feeding on an Fe-deficient diet without any added iron for 3 wk. After the iron-deficiency period, the iron-deficiency group was separated into an Fe-WPC group and a heme iron group fed Fe-WPC and hemin as the sole source of iron, respectively. The hemoglobin content, iron content in liver, hemoglobin regeneration efficiency (HRE) and apparent iron absorption rate were examined when iron-deficient rats were fed either Fe-WPC or hemin as the sole source of iron for 20 d. Hemoglobin content was significantly higher in the rats fed the Fe-WPC diet than in rats fed the hemin diet. HRE in rats fed the Fe-WPC diet was significantly higher than in rats fed the hemin diet. The apparent iron absorption rate in rats fed the Fe-WPC diet tended to be higher than in rats fed the hemin diet ($p = 0.054$). The solubility of iron in the small intestine of rats at 2.5 h after ingestion of the Fe-WPC diet was approximately twice that of rats fed the hemin diet. These results indicated that the iron bioavailability of Fe-WPC was higher than that of hemin, which seemed due, in part, to the different iron solubility in the intestine. (**Key Words :** Whey Protein, Iron, Bioavailability, Anemia, Solubility, Caco-2)

INTRODUCTION

Iron deficiency, with and without anemia, is one of the most significant nutritional problems all over the world (Yip, 1994), affecting approximately 20% of the world population. Because iron deficiency anemia is mainly caused by the insufficient intake of iron, the strategy of iron fortification of food is used worldwide to prevent iron deficiency (Clugston et al., 2002; Demment et al., 2003). However, the incorporation of iron into foods leads to a variety of problems such as its oxidation and precipitation which result in lower bioavailability (Frederic et al., 1981; Hurrell, 1997; Hurrell et al., 1998). It is important to consider not only intake of iron but also its bioavailability to prevent iron deficiency.

Heme iron is used extensively as an iron supplement in the food industry. Heme iron, however, is insoluble in neutral pH, and their absorption and bioavailability have not yet been sufficiently taken into account in its use (Frederic

et al., 1981). Lactoferrin and casein phosphopeptide are widely accepted to be functional proteins or peptides, enhancing iron absorption by improving its solubility in animal intestine, and are applied extensively as iron supplements and ingredients (Kawakami et al., 1988; Soid et al., 2002; Yeung et al., 2002; Jovani et al., 2003; Uchida et al., 2006). However, they are expensive food ingredients due to the high cost of preparation.

The presence of amino acids in the intestines is reported to increase iron absorption (Allen, 2002). Therefore, the use of suitable proteins in the diet may increase the absorption rate of dietary iron. Whey proteins produced as the principal byproduct of cheese or casein manufacturing are widely used as food ingredients such as whey protein concentrate (WPC) or whey protein isolate, because they have high nutritional value and functional properties such as gelling and emulsifying properties (Kim et al., 1989; Kinsella and Whitehead, 1989). More recently, Gabriel et al. (2004) reported that the iron in whey protein hydrogels was superior in intracellular iron absorption in the Caco-2 system that was used to estimate intestinal absorption, because whey protein hydrogels released most of their iron during the intestinal phase of a simulated digestion. In the present study, we prepared iron-fortified whey protein

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Table 1. Composition of iron-fortified whey protein concentrate

Composition	g/kg
Kjeldahl N	48.2
Fat	40.8
Lactose	419.1
Iron	0.89
Calcium	10.07
Magnesium	1.13
Sodium	4.21
Potassium	15.35

concentrate (Fe-WPC) and examined some of its properties. Then the bioavailability of iron-fortified whey protein concentrate was evaluated in comparison with heme iron in iron-deficient rats.

MATERIALS AND METHODS

Materials

Whey of Mozzarella cheese was obtained from a commercial dairy company (Hokkaido Hidaka Milk Products Co., Hokkaido, Japan). Porcine pepsin (800-2,500 units/mg protein), Tosyl-phenylalanine-chlomethyl ketone-treated trypsin and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of Fe-WPC

Cheese whey was concentrated to about 1/5 volume with a Dow Ultrafiltration System (Nakaskov, Denmark) using a GR60PP membrane (cut off, 10 kDa) (Yoon and Jayaprakasha, 2005), 1 M ferric chloride was added to the result in a concentration of iron at 10 mg% in concentrated whey, and lyophilized. The obtained product was called Fe-WPC. The composition of Fe-WPC is shown in Table 1; its iron content was 887 mg/kg.

To evaluate Fe-WPC, gel permeation chromatography using a sephadex G-50 column (2.6×85 cm) was carried out. Each 10 ml of eluent was collected to monitor the absorbance at 280 nm along with iron content. SDS-PAGE was performed using 14% polyacrylamide gels under a reducing condition in the presence of 2-mercaptoethanol according to the method of Laemmli (1970). The gels were stained in Coomassie Blue R-250 for 1 h. Lactoferrin content in Fe-WPC was measured with a commercial kit (Bethyl, Montgomery, TX, USA).

Cell culture

Caco-2 cells were obtained from Riken Cell Bank (Tsukuba, Japan). All cell culture media and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cells were seeded at a density of 50,000 cells/cm² in collagen-treated 6-well plates. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10%

fetal calf serum and antibiotic antimycotic solution. Cells were then maintained at 37°C in an incubator with a 5% CO₂/95% air atmosphere at constant humidity. The medium was changed every 2 d. The cells were used in the iron uptake experiments after 15 d of culture.

In vitro digestion of Fe-WPC and iron uptake into Caco-2 cell

Simulated gastrointestinal dissolution and iron uptake into Caco-2 cell from the Fe-WPC were performed according to the modified method of Raymond et al. (1996, 1998). Sample pH was adjusted to pH 4.0 with 1 N HCl for peptic digestion. Next, 0.5 ml of the pepsin solution that included 0.2 mg pepsin in 10 ml of 0.1 N HCl was added per 10 ml of sample. After incubation at 37°C for 60 min, the pH of the sample solution was raised to 6.0 by 1 N NaHCO₃. Then 2.5 ml of trypsin-bile extract mixture that included 0.05 g trypsin and 0.3 g bile extract in the 25 ml of 0.1 mol/L NaHCO₃ was added per 10 ml of the original sample for intestinal digestion. The pH was adjusted to 7.0 with NaOH, and the sample was incubated for 2 h at 37°C. The volume was made up to 15 ml with 120 mM NaCl and 5 mM KCl, and the simulated gastrointestinal dissolution was ultrafiltered using a Vivaspin (MW cut off 10 kDa) (Vivascience AG, Hannover, Germany).

Before the iron uptake experiment period, the growth medium was removed from each culture well, and the cell layer was washed twice with serum-free Minimum Essential Medium (MEM) at 37°C. This MEM was chosen because it contained no added iron. The serum-free MEM was supplemented with 10 mM PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]), 1% antibiotic antimycotic solution, hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5 µg/L), triiodothyronine (34 µg/L) and epidermal growth factor (20 µg/L). Simulated gastrointestinal dissolution filtrate was added to serum-free MEM to provide the prescribed concentrations of iron. Washed cells without simulated gastrointestinal dissolution filtrate were treated as a reference sample. The cells were next incubated for 24 h, washed five times with PBS, and then harvested in 2 ml of PBS and sonicated for 10 min at 4°C for protein and ferritin analysis. Ferritin content was measured with ferritin enzyme immunoassay test kit (MP Biomedicals, Orangeburg, NY, USA). Cell protein was assessed using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA), based on the Lowry assay.

Diet

All experimental diets were prepared using a commercial low iron diet (AIN-76 diet, Oriental Yeast Co., Ltd., Tokyo, Japan). The AIN-76 diet contained 2.9 mg of iron per kilogram and was used as an Fe-deficient diet. The other three Fe-supplemented diets were prepared by adding

Table 2. Composition of experimental diets

Ingredients	Diets			
	Fe-WPC diet	Hemin diet	Standard diet	Fe-deficient diet
	----- % -----			
Fe-deficient diet ¹	94.888	94.888	94.888	94.888
Fe-WPC ²	5.070			
Hemin ³		0.053		
FeSO ₄ ·7H ₂ O			0.022	
OVA		1.970	1.970	1.970
Corn oil		0.207	0.207	0.207
DL-methionine	0.032			
Lactose·H ₂ O		2.237	2.237	2.237
Citric acid·H ₂ O		0.181	0.181	0.181
NaCl		0.054	0.054	0.054
KCl		0.148	0.148	0.148
MgO		0.010	0.013	0.010
MgSO ₄	0.010	0.010		0.010
CaCO ₃		0.128	0.128	0.128
Sucrose		0.114	0.152	0.167

¹ Oriental Yeast Co., Ltd., Tokyo, Japan.

² Fe-WPC, whey protein concentrate fortified iron.

³ Wako Pure Chemical, Osaka, Japan.

different forms of iron to this Fe-deficient diet to give 45 mg of iron/kg. Composition of the experimental diets is shown in Table 2. In the Fe-WPC diet, Fe-WPC was the sole source of iron. In the hemin diet, hemin from bovine (Nacalai Tesque, Inc. Kyoto, Japan) was used as the sole source of iron. The iron content in the hemin was 85.4 g/kg. In the standard diet, ferrous sulfate was added as the source of iron. The same amount of protein in the Fe-WPC was also added to the other diets. Moreover, the hemin diet and standard diet were prepared to contain amounts of iron, fat, lactose, citric acid, calcium, magnesium, sodium and potassium equal to those in the Fe-WPC diet.

Animals

The animal experiments were conducted in accordance with the guidelines of Kagoshima University. Eighteen 6-wk-old male Wistar rats (Japan SLC, Shizuoka, Japan) were housed individually in metabolic cages in a temperature-controlled room at 23°C with a 12-h light/dark cycle. Rats were given free access to a commercial diet (CE-2, Clea Japan, Tokyo, Japan) and Elix water (treated with an Elix water purification system (Millipore Co., Ltd., Billerica, MA, USA)) for a 1-wk adaptation period. Then the rats were separated into a control group of six rats and an iron-deficiency group of twelve rats. Rats in the control group were given the standard diet throughout the feeding experimental period. Rats in the iron-deficiency group were made anemic by feeding on an Fe-deficient diet without any added iron for 3 wk. After the iron-deficiency period, the iron-deficiency group was separated into an Fe-WPC group and a heme iron group of six rats each. The rats in the Fe-WPC group and heme iron group had a similar mean

hemoglobin level of 11.18 g and 11.04 g/100 ml, respectively. All iron-deficient rats were given free access to either the Fe-WPC diet or the hemin diet, and Elix water for the 20-d recovery period. Blood was obtained weekly from the tail tip to determine the hemoglobin concentration. We conducted iron absorption studies on the Fe-WPC and heme iron groups for 2-d periods starting from day 5 of the anemic recovery period. All feces collected during the 2-d iron absorption period were pooled. After the 20-d recovery period, all rats were deprived of food overnight. Blood was collected from the vena cava inferior under anesthesia with diethyl ether. The animals were killed by venesection from the heart. Liver, heart, and kidney were immediately removed, washed with cold saline, blotted dry using filter paper, and weighed; the liver was stored at -80°C until analysis.

Blood analysis

Hemoglobin concentration was measured by the cyanmethemoglobin method using a colorimetric hemoglobin assay kit (hemoglobin test, Wako Pure Chemical Industries, Osaka, Japan). Hemoglobin regeneration efficiency (HRE) was calculated from the initial and final body weight and hemoglobin concentration of the animals according to the method of Zhang et al. (1989).

Solubility of iron in small intestine

The solubility of iron in the small intestine was examined by the method of Lee et al. (1980, 1992). Eight 6-wk-old male Wistar rats (Japan SLC, Shizuoka, Japan) were housed in individual stainless steel wire-mesh cages in a temperature-controlled room at 23°C with a 12-h light/dark cycle. The rats for meal-feeding experiments were trained to consume 5 g of AIN-76 diet for 1.5 h twice a day (8:00-9:30 and 17:00-18:30) and given free access to Elix water for a 7-d training period. After training, all rats were separated into two experimental groups of four rats each. The experimental groups and diets were the same as shown in Table 2. Rats were fed 5 g of each experimental diet for 1.5 h (8:00-9:30), then sacrificed 1 h after withdrawal of the diet. All experimental rats were anesthetized with diethyl ether and killed by venesection from the heart. The entire small intestinal contents, from proximal duodenum to distal ileum, were thoroughly flushed out with ice-cold saline and made up to a known volume with the same solution. The iron contents in the obtained suspension and supernatant were determined using an atomic absorption spectrophotometer (AAAnalyst 800, Perkin Elmer Inc., Shelton, Conn., USA).

Statistical analysis

The data reported in the tables and figures are expressed

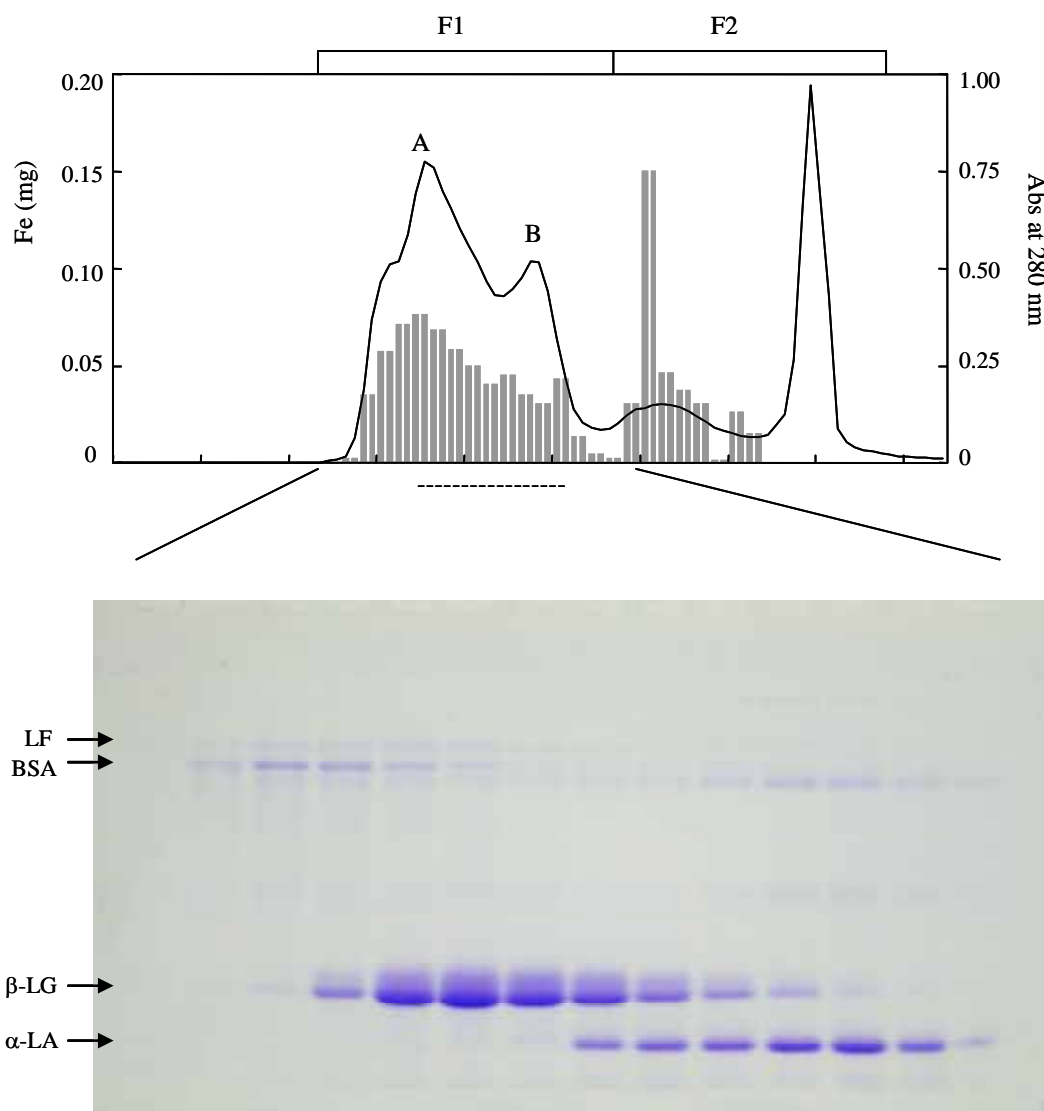


Figure 1. Elution patterns of proteins and iron in Fe-WPC from a Sephadex G-50 column (2.6×85 cm)¹. Line, absorbance at 280 nm. Column, iron content. ¹Fe-WPC, whey protein concentrate fortified iron.

as mean values with standard deviation (SD). Statistical analysis was done by *t*-test at 5% level of probability.

RESULTS AND DISCUSSION

Properties of Fe-WPC

Gel permeation chromatography was performed to characterize the Fe-WPC (Figure 1). The eluate was divided into two fractions. Judging from the properties of the column and molecular weight of whey proteins, the position of fraction 1 corresponds with that of whey protein. SDS-PAGE patterns of fraction 1 indicated that peak 1 and 2 coincided with β -lactoglobulin and α -lactoalbumin, respectively. Seventy-one percent of iron in Fe-WPC was eluted in fraction 1. These results suggest that iron in Fe-WPC exists as a complex with mainly protein such as β -lactoglobulin. Lactoferrin can solubilize over 70 M

equivalent of iron under neutral conditions, which is much higher than the specific iron-binding ability of lactoferrin (Kawakami et al., 1993; Uchida et al., 2006). However, the lactoferrin content in concentrated whey was only 5 mg/100 ml. This suggested that a large part of lactoferrin was recovered as casein fraction during the preparation of whey.

Iron uptake by Caco-2 cell

We examined the bioavailability of iron in Fe-WPC using Caco-2 monolayer combined with *in vitro* gastric and intestinal digestion. Caco-2 cells were exposed to the filtrate obtained from the simulated gastrointestinal dissolution, because Caco-2 cell monolayers behave similarly to human intestinal mucosa (Puyfoulhoux et al., 2001). The increase of ferritin in cells is an evidence that iron has entered the cell because cells produce ferritin in response to increases in intracellular iron (Reymond et al.,

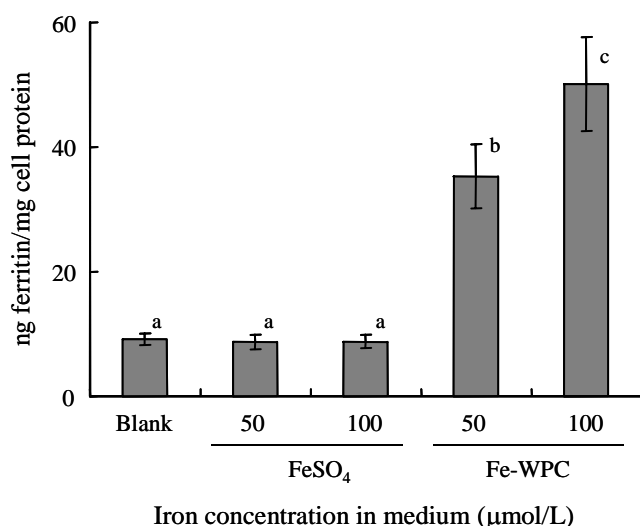


Figure 2. Ferritin levels of Caco-2 cells treated with simulated gastrointestinal dissolution filtrate or Hemin.^{1,2} Samples were added to medium to give prescribed concentrations of iron. Values are means±SD, n = 6. Cells without samples were treated as blank. ¹ Means in column without the same letter are significantly different (p<0.05). ² Fe-WPC, whey protein concentrate fortified iron.

1996, 1998). Therefore, ferritin formation in the cells was used as an indicator of iron bioavailability. Iron uptake into intracellular medium was expressed as a ratio of ferritin and cell protein (ng ferritin×mg cell protein⁻¹), because the factors which promote multiplication of a cell such as lactoferrin were involved in whey (Hagiwara et al., 1995; Pakkanen et al., 1997; Schottstedt et al., 2005). Addition of iron as hemin at the concentration of 50 and 100 µmol/L had no effect on ferritin formation (Figure 2). The ferritin/cell protein ratios in Caco-2 cell with addition of iron as Fe-WPC were significantly higher than that in Caco-2 cell with the addition of iron as hemin at any concentration. This result suggests that a digestion product of the Fe-WPC enhanced iron uptake.

Lactoferrin is well known as a factor enhancing iron absorption (Kawakami et al., 1988; Jovani et al., 2003; Uchida et al., 2006). Concentrated whey used in this study contained 5 mg lactoferrin per 100 ml. Lactoferrin content in Fe-WPC seems to be too low to stimulate the iron absorption. Etcheverry et al. (2004) reported that a low-molecular-weight factor in human and bovine milk whey promoted iron uptake by Caco-2 cells. Some kinds of peptides derived from Fe-WPC may have an ability to enhance iron uptake by Caco-2 cells. Further studies on the absorption mechanism of iron in Fe-WPC are needed.

Bioavailability of Fe-WPC in iron-deficient rats

The hemoglobin concentration in rats fed the Fe-WPC diet increased with time throughout the recovery period, but

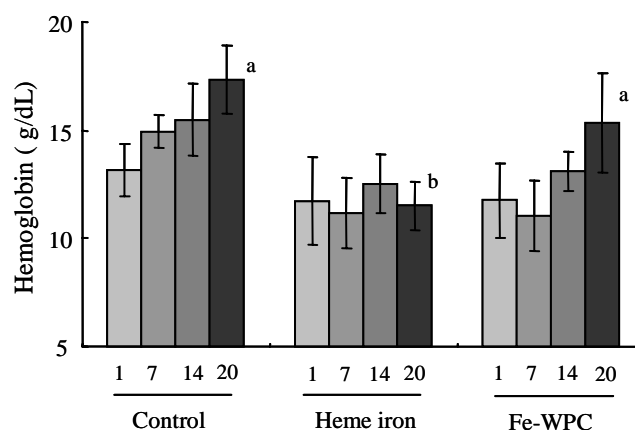


Figure 3. Hemoglobin concentration in rats fed a Fe-WPC diet or a hemin diet¹. Day 1 corresponds to start of recovery period. Values are means±SD, n = 6. Means in column without the same letter are significantly different (p<0.05). ¹ Fe-WPC, whey protein concentrate fortified iron.

it did not change in rats fed the hemin diet (Figure 3). The hemoglobin level in rats fed the Fe-WPC diet at 20 d of the recovery period was significantly higher than in rats fed the hemin diet. While the hemoglobin concentration in rats fed the hemin diet was 66.5% of that in rats fed the control diet, that in rats fed the Fe-WPC diet was regenerated to 88.5% of that in rats fed the control diet. Moreover, the HRE in rats fed the Fe-WPC diet was significantly higher than in rats fed the hemin diet (Table 3). Anemia is a useful index of the severity of iron deficiency, but the serious fatalities due to iron deficiency are more related to a deficiency in tissue iron (Underwood, 1977). Among the body organs, the liver is highest in iron content and is considered to be an organ for iron storage. The liver iron content in rats fed the Fe-WPC diet was significantly higher than that in rats fed the hemin diet. Hemoglobin and liver iron contents are directly influenced by iron absorption. The iron absorption rate in rats fed the Fe-WPC diet at 5 d in the recovery period tended to be higher than that in rats fed the hemin diet (p = 0.054). These results may suggest that iron deposition in the hemoglobin and liver of rats fed the Fe-WPC diet is higher than that in the hemoglobin and liver of rats fed the hemin diet because the iron absorption of Fe-WPC is greater than that of hemin.

Heme iron is relatively well absorbed under various circumstances. Tabayashi et al. (1999) reported that heme iron prevented iron-deficient anemia in pregnant women through its high iron absorption. Hemin, which is produced from hemoglobin by removal of globin, is somewhat inferior in iron absorption to hemoglobin (Martinez and Layrisse, 1971; Hallberg et al., 1979), because globin degradation products are important in maintaining heme iron in a soluble state (Conrad et al., 1966). Isolated heme becomes insoluble, and it would affect the bioavailability of

Table 3. Food intake, body weight gain, iron content in liver, HRE, and apparent iron absorption rate in rats fed Fe-WPC diet and hemin diet

	Diets		
	Control	Fe-WPC diet ¹	Hemin diet
Food intake (g/day)	16.2±2.02 ³	16.2±1.21	16.6±0.86
Iron intake (mg/day)	729±90.9	729±54.5	747±38.7
Body weight (g)	340±20.0	336±16.7	336±22.8
Body weight gain (g/day)	1.98±0.55	2.38±0.59	1.91±0.71
Food efficiency (%)	11.0±2.6	12.4±1.3	10.44±3.4
Organ weight (g/body weight)			
Liver	7.42±1.03	8.05±0.56	7.92±0.73
Heart	1.04±0.11	1.04±0.05	0.92±0.41
Kidney	2.02±0.20	1.98±0.10	2.04±0.21
Iron content in liver (mg/g)	88.4±20.8 ^a	85.9±10.8 ^a	50.4±8.0 ^b
HRE ²	17.0±9.1 ^a	47.2±19.5 ^b	17.5±9.6 ^a
Apparent iron absorption rate (%)		47.2±4.5 ^a	40.3±5.0 ^a

¹ Fe-WPC, whey protein concentrate fortified iron. ² HRE, hemoglobin regeneration efficiency.

³ Mean±SD (n = 6). ^{a, b} p<0.05.

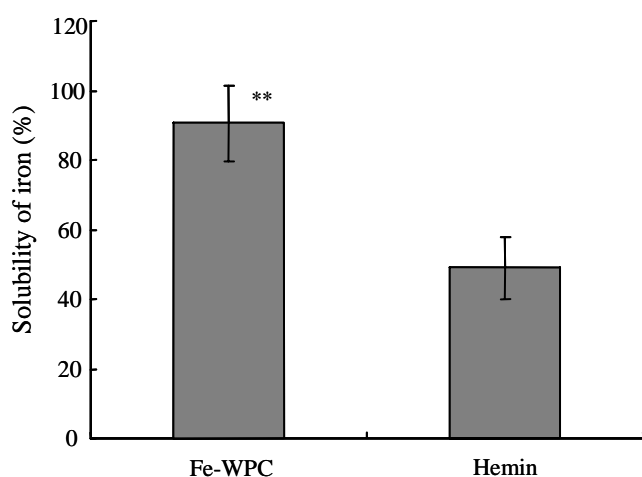


Figure 4. Solubility of iron in small intestinal contents of rats at 2.5 h after ingestion of 5 g of a Fe-WPC diet or 5 g of a hemin diet¹. Values are means±SD, n = 4. ** indicates significant differences with heme iron group (p<0.01). ¹ Fe-WPC, whey protein concentrate fortified iron.

heme iron. It was confirmed by gel permeation chromatography that a large part of iron in Fe-WPC exists as complexes with proteins such as β -lactoglobulin (Figure 1). In addition, all irons in the Fe-WPC were solubilized after *in vitro* digestion, while the iron solubility of hemin was only 15.4%. Whey protein might play an important role in maintaining iron in a soluble state so that the iron bioavailability of Fe-WPC was higher than that of hemin. Lactose and citric acid are contained in Fe-WPC and considered to enhance mineral absorption by increasing solubility in animal intestines. To evaluate the effects of the Fe-WPC on iron bioavailability, the same amount of lactose and citric acid were also added to the hemin diet (Table 2). In this study, iron-deficient rats were fed Fe-WPC or hemin as the sole source of iron. Accordingly, the positive effects

on the iron bioavailability may be not ascribed to lactose and citric acid, but to whey proteins.

Solubility of iron in small intestine

Dietary iron is absorbed in the duodenum and jejunum by active transport, or underneath the small intestine by passive transport. A great mass of iron is assimilated by passive transport when iron concentration in the small intestine is high. Iron solubility in the small intestine is considered to be one of the factors affecting iron absorption. We measured the amount of soluble iron in the small intestinal contents of rats fed the Fe-WPC diet to examine the possible mechanism of iron absorption. The solubility of iron in the small intestine of rats fed the Fe-WPC diet was approximately twice as high as that in rats fed the hemin diet (Figure 4). Our observation that a higher ability to recover from anemia and a higher iron solubility in the intestine in rats fed the Fe-WPC diet support the view that an elevated concentrate of soluble iron during food ingestion enhances iron absorption.

The present results indicate that iron bioavailability of Fe-WPC is higher than that of heme iron due to its high iron solubility in the small intestine. It is difficult for women, especially young females, to take sufficient iron because of dietary food restrictions. It is important to have a good iron source that is absorbed well to prevent anemia. Fe-WPC solution gave almost no iron taste at 25 mg% of iron concentration. Because Fe-WPC has high bioavailability, and its preparation can be carried out easily at low cost, Fe-WPC appears promising as an iron ingredient.

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