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## Enhancing the *in vitro* Fe<sup>2+</sup> bio-accessibility using ascorbate and cold-set whey protein gel particles

A. H. Martin · G. A. H. de Jong

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**Abstract** This paper investigates the possibility for iron fortification of food using a new preparation method for protein gel particles in which iron is entrapped in the presence of ascorbate using cold-set gelation. The effect of ascorbate on the iron-induced cold-set gelation process of whey protein was studied in order to optimize the ratio of iron/ascorbate. Subsequently, the effect of ascorbate on iron bio-accessibility was assessed *in vitro*. Rheology was used to study the protein gel formation, and the stability of the gel particles was determined by measuring the iron and protein content at different pH. *In vitro* studies were performed with the TNO Intestinal Model (TIM). Ascorbate appeared to affect the gel formation process and increased the gel strength of the iron-induced cold-set gels at specific iron/ascorbate ratio. With the Fe–protein gel particles being stable at a broad pH range, the release of iron from the particles was studied as a function of time. The low release of iron indicated a good encapsulation efficiency and the capability of whey protein to keep iron bound at different conditions (pH and presence of calcium). Results obtained with the TIM showed that ascorbate, when added to the protein gel particles, was very successful in enhancing the recovery and absorption of iron. The *in vitro* Fe<sup>2+</sup> bio-accessibility in the presence of ascorbate in iron–protein particles increased from 10% to almost 80%. This suggests that the concept of using protein particles with iron and ascorbate can effectively be used to fortify food products with iron for human consumption.

### Fe-乳清蛋白冷凝胶颗粒中铁的生物利用率

**摘要** 在抗坏血酸存在下制备出一种能包裹铁离子的蛋白凝胶颗粒，并作为食品中强化铁的原料。在抗坏血酸存在的情况下，乳清蛋白凝胶在形成过程中包裹了铁离子。研究了抗坏血酸对铁离子诱导乳清蛋白冷凝胶形成过程的影响，优化了铁离子与抗坏血酸的比例。体外模拟了抗坏血酸对铁离子生物利用率的影响。采用流变学方法研究了蛋白凝胶的形成，以及不同pH、铁离子浓度和蛋白浓度下凝胶颗粒的稳定性，采用TNO肠道模型 (TIM) 体外研究了铁离子的生物利用率。结果表明抗坏血酸影响了蛋白凝胶的形成过程，并且在特定的铁离子

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与抗坏血酸比例下可以形成较硬的Fe-蛋白凝胶。在较宽的pH范围内Fe-蛋白凝胶稳定性较好,铁离子从胶粒中释放量是时间的函数。低的铁离子释放量表明了包埋效果较好和在不同条件下(pH和钙离子存在情况下)乳清蛋白与铁离子结合能力。TIM实验结果表明抗坏血酸能够提高蛋白凝胶的回收率以及蛋白对铁离子的吸附率。在抗坏血酸存在的情况下,Fe-蛋白凝胶颗粒中铁离子生物利用率从10%增加到80%。因此,含有抗坏血酸的Fe-蛋白凝胶颗粒可用于食品中铁强化剂。

**Keywords** Iron bio-accessibility · Ascorbate · Whey protein · Cold-set gelation · In vitro digestion model

**关键词** 铁离子生物利用率 · 抗坏血酸 · 乳清蛋白 · 冷凝胶 · 体外消化模型

## 1 Introduction

Iron deficiency is one of the most prevalent nutritional deficiencies in the world. To address this, iron fortification of food products is used worldwide as this is the most sustainable way to prevent deficiency. However, the addition of iron to complex food systems leads to a variety of negative issues including oxidation, precipitation, and lack of bio-availability. Given that the presence of amino acids in the intestines is required to increase iron bio-availability (Allen et al. 2002), the use of protein gels offers an interesting alternative for protecting iron against oxidation, increasing dietary iron adsorption, and offering masking of off-flavors.

Cold-set gelation of whey proteins can be used to entrap and protect bioactive molecules and micronutrients inside its network. Cold-set gelation is a two-step process. The first step involves unfolding of the protein, usually done by heating at a relatively low salt concentration. The second step consists either of lowering the pH (acid-induced cold gelation) or the addition of salts. In case of the latter, mainly NaCl and CaCl<sub>2</sub> were studied with regard to cold-set gelation (Barbut and Drake 1997; Bryant and McClements 2000; Hongsprabhas and Barbut 1997b; Ju and Kilara 1998). These cold-set gels generally showed higher gel strength and water-holding capacity than gels formed by heat-induced gelation (Hongsprabhas and Barbut 1997b; Hongsprabhas and Barbut 1997a; Ju and Kilara 1998). To a much lesser extent, the mechanism of cold-set gelation as induced by minerals such as Fe<sup>2+</sup> (Remondetto et al. 2002; Remondetto and Subirade 2003) and Mg<sup>2+</sup> (Da Silva and Delgado 2009) has been studied.

Remondetto et al. (2002, 2003) have studied the molecular mechanisms of cold-set gelation of  $\beta$ -lactoglobulin by the addition of Fe<sup>2+</sup>. Depending on the iron concentration used, filamentous or particulate gels are formed which influence the iron delivery (Remondetto et al. 2004). Cold-set gelation by Fe<sup>2+</sup> has not been studied for whey protein isolate (WPI), which is more relevant for industrial applications, and hence, the study on WPI in this paper.

Encapsulation of iron based on cold-set gelation is done by the ferrous iron form (Fe<sup>2+</sup>). It is not known to what extent iron stays in this form when entrapped by whey protein. The uptake of dietary iron in the intestines, however, requires the iron to be in the Fe<sup>2+</sup> form. It is known that ascorbate reduces the rate of Fe<sup>2+</sup> oxidation (Thumser et al. 2010). Moreover, it has been shown that the presence of ascorbate increases iron

absorption in human studies (Lopez and Martos 2004; Moretti et al. 2006; Olivares et al. 1997). Therefore, to minimize the effect of iron oxidation and thus optimize iron bio-accessibility, ascorbate was added during the cold-set gelation process. As ascorbate forms complexes with iron, it is not known whether this would affect the cold gelation process of whey protein as induced by Fe<sup>2+</sup>. The aim of this paper is therefore to study the effect of ascorbate on the iron-induced cold-set gelation process of whey protein and optimize the iron/ascorbate ratio with regard to cold-set gelation. The presence of ascorbate for an optimal binding ratio of Fe/protein together with a new preparation method of the gel particles will be related to the stability of the protein gel particles at different pH and in the presence of Ca<sup>2+</sup>. The stability is determined as the release of iron (both Fe<sup>2+</sup> and Fe<sup>3+</sup>) from the particles. These tests were indicative for the results at gastric conditions obtained with the TNO Intestinal Model (TIM), which were performed with the optimal combination of Fe<sup>2+</sup>/ascorbate/whey protein.

## 2 Materials and methods

### 2.1 Material

WPI (BiPRO) was obtained from Davisco (Minnesota, USA). FeSO<sub>4</sub> was purchased from Fluka (Buchs, Switzerland). CaCl<sub>2</sub> and sodium ascorbate were obtained from Merck (Darmstadt, Germany) and Acros (Geel, Belgium), respectively. Water was purified using a MilliQ system (Millipore Systems, France).

### 2.2 Preparation of protein solutions

WPI was dissolved in MilliQ water at 9% (w/w) and heated for 30 min at 80 °C in a water bath. After heating, the WPI solution was cooled to room temperature and diluted with MilliQ water to 6% (w/w). The pH was set to pH 7 and the WPI solutions were stored on ice until further use. Iron sulfate (1 M) and 1 M Na-ascorbate were prepared and stored on ice until further use. The final concentration of Fe<sup>2+</sup> to induce cold gelation was 10, 20, or 30 mM; final concentrations of ascorbate in the protein gel ranged from 10 to 50 mM.

### 2.3 Gel formation and characteristics

*Gel formation kinetics.* Iron sulfate and ascorbate were mixed prior to addition to pretreated WPI. All ingredients were stored on ice, and mixing was done at cold temperature (<5 °C) just before the start of the experiment. Gelation kinetics were studied as a function of time using a stress controlled AR2000 rheometer (TA Instruments, Etten-Leur, The Netherlands). A concentric cylinder system with a bob diameter of 14 mm was used; the bob was grooved to prevent slip. A temperature range was applied in which the temperature increased from 5 to 25 °C with an increase of 0.5 °C.min<sup>-1</sup>. Subsequently, the measurement was continued at a constant temperature of 25 °C for at least 3 h (strain 10<sup>-3</sup>, 6.27 rad.s<sup>-1</sup>, frequency 1 Hz). The applied strain was within linear region. *G'*, *G''*, and tanδ were measured during both steps as a function of time.

*Large deformation properties.* Protein solutions were gelled overnight at room temperature in a 24-well microtiter plate. A texture analyser (TA Instruments, Etten-Leur, The Netherlands) with a cylindrical Teflon probe (diameter 1 cm) was used to measure the gel strength. The probe was moved with speed of  $1 \text{ mm}\cdot\text{s}^{-1}$  into the gel until a penetration of 10 mm was reached. From the measured force–distance curve, the maximum force  $F_{\text{max}}$  (in Newton) at fracture, the modulus (in Newton per millimeter), and the area beneath the curve (in Newton millimeter) were determined. Each measurement was performed in triplo.

## 2.4 Preparation gel particles

Gels were formed overnight at room temperature via cold-set gelation with  $\text{Fe}^{2+}$ , with or without the addition of ascorbate. The gel was refined into small particles by pressing the gel through a sieve with dimensions  $1 \times 1 \text{ mm}$ . These particles were used for the screening experiments to find the optimal Fe/ascorbate/protein ratio. For further research, the preparation procedure was extended with a washing and a freeze-drying step. The gel particles were washed very quickly with cold-demineralized water with fivefold volume, in order to remove any unbound iron. The washing step was done using a glass filter (G2). This step results in the so-called wet gel particles. Subsequently, these particles were freeze dried by freezing at  $-40 \text{ }^\circ\text{C}$  and drying on a plate. To vary the size of the freeze-dried particles, cryogenic milling was performed (Retch).

## 2.5 Particle size distribution

The size of the gel particles was determined using a Malvern Mastersizer 2000 (Malvern Instruments Ltd, UK) allowing to analyze a size range of  $0.02\text{--}2000 \text{ }\mu\text{m}$ . Particles were dispersed in propane-diol to prevent clustering or dissolution in water.

## 2.6 Protein stability gel particles

Gel particles were tested for their stability at low pH by subjecting the gel particles to a range in pH from 6.5 to 2.0 using 6 M HCl to set the pH. To disperse the gel particles, 1 g of gel was diluted ten times with MilliQ water, mixed shortly with an ultra-turrax, and the pH was set. All dispersions were gently mixed on a roller apparatus for 1 h during which the pH remained constant over the time course of the experiment. A sample was taken and centrifuged for 15 min at 13,000 rpm.

From the supernatant, the protein concentration was determined using the method of Bradford (1976). The amount of protein in the supernatant is a measure for the instability of the gel particles. Supernatant was mixed with Coomassie reagents and the extinction was measured with a spectrophotometer (Versamax, Molecular Devices, UK) at 590 nm. MilliQ water was used as a blank. To determine the maximum possible amount of protein present in the supernatant, unheated protein with iron was used as a reference. As no gel was formed or pellet obtained, all protein should be present in the supernatant.

## 2.7 Fe release from gel particles

To study the encapsulation efficiency and the capability of whey protein to keep Fe<sup>2+</sup> bound, the Fe<sup>2+</sup> release from the protein particles was measured as a function of time (1–24 h). Freeze-dried gel particles were added at 1 mg.mL<sup>-1</sup> concentration to a solution of pH 2, pH 6.5, and pH 6.5 with 1 mg.mL<sup>-1</sup> added CaCl<sub>2</sub>. The pH remained constant over the time course of the experiments.

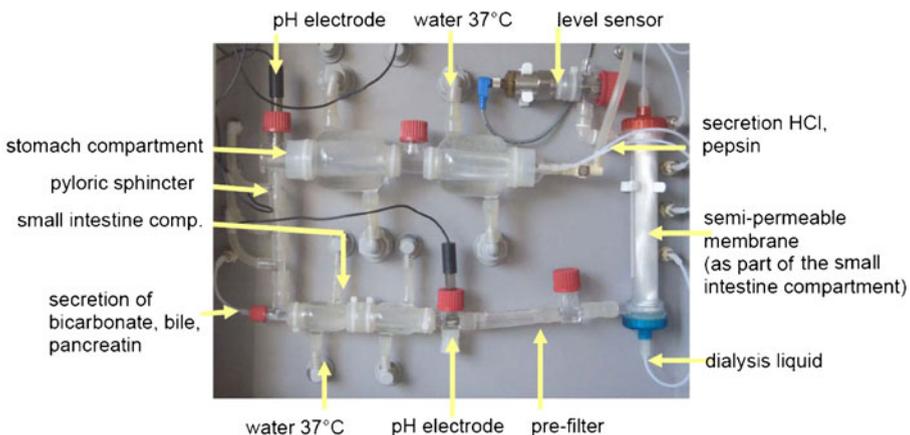
## 2.8 Iron analysis

Fe was determined according to the method as described by Vogel (1988), which is based on a spectrophotometric method with phenanthroline to determine total soluble Fe<sup>2+</sup>. Addition of hydroxylamine in combination with heating at 100 °C was done to reduce present Fe<sup>3+</sup> to Fe<sup>2+</sup>. This procedure enables the determination of total Fe. Extinction of this solution was measured at 510 nm. Values were compared with calibration line made with iron only and the solutions above. Molar extinction coefficient is  $11 \times 10^3$  L.mol<sup>-1</sup>.cm<sup>-1</sup>. Both free Fe<sup>2+</sup> and total Fe content are analyzed. Sensitivity of the method is 0.01 mg.L<sup>-1</sup> Fe.

## 2.9 Tiny-TIM studies

The Tiny-TIM system was used to simulate closely the successive dynamic conditions in the human stomach and small intestine (Minekus et al. 1995). The experiments in the Tiny-TIM (Fig. 1) were performed under simulation of the average physiological conditions in the stomach and small intestine as described for humans (young adults) after the intake of a semi-solid meal, relevant for this type of test products. Four test products were investigated in the Tiny-TIM system: iron; iron and ascorbate; iron and protein; iron, protein, and ascorbate.

Fifty milliliters of each test product was diluted with 250 mL demineralised water, of which 125 mL was injected into the Tiny-TIM system. Protein gels were ground



**Fig. 1** Tiny-TIM system for rapid screening of food products (proteins and carbohydrates) with high predictive quality for the in vivo situation

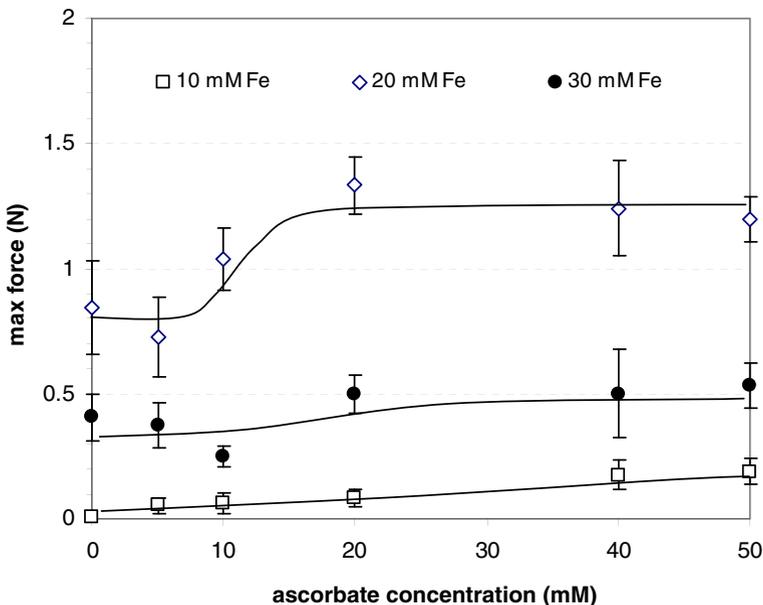
into small gel particles which were diluted as described above. All TIM runs were performed in duplo.

At five different time intervals, the dialysate was collected in 1 h aliquots for 5 h and analyzed for Fe concentration. Residues present in the gastric and intestinal compartment were diluted with rinsing fluid and also analyzed for Fe concentration.

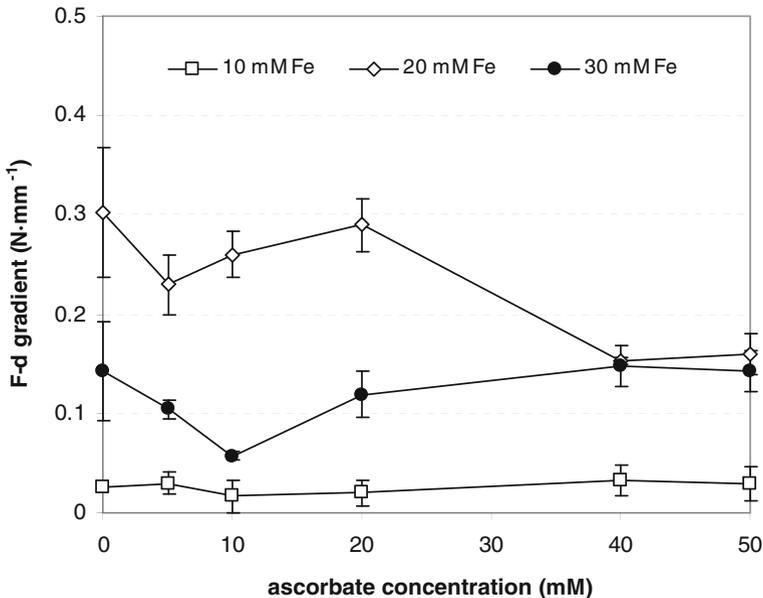
### 3 Results

#### 3.1 Characteristics of iron-induced cold-set gels

To find the optimal protein/Fe/ascorbate ratio, the rheological properties of the gels varying in ratio were screened. The influence of ascorbate on the large deformation properties of iron-induced cold-set whey protein gels was studied by measuring the gel strength of the different gels that varied in iron/ascorbate ratio.  $\text{Fe}^{2+}$  concentration varied from 10 to 30 mM and ascorbate concentration ranged from 0 to 50 mM. Figures 2 and 3 show the results for the maximum force ( $F_{\max}$ ) and the slope ( $F-d$  gradient) that were derived from force–distance ( $F-d$ ) curves. The maximum force refers to the force at which the gel fractures and the  $F-d$  gradient relates to the extent of elasticity, i.e., at which deformation of the gel fractures. In general, gels prepared with 20 mM  $\text{Fe}^{2+}$  were stronger than those made with 10 or 30 mM  $\text{Fe}^{2+}$  with regard to both  $F_{\max}$  and  $F-d$  gradient. At all ascorbate concentrations, gels made with 20 mM  $\text{Fe}^{2+}$  formed the strongest gels, showing that there is an optimum in gel strength with regard to iron concentration. At 10 mM  $\text{Fe}^{2+}$ , it appears that increasing

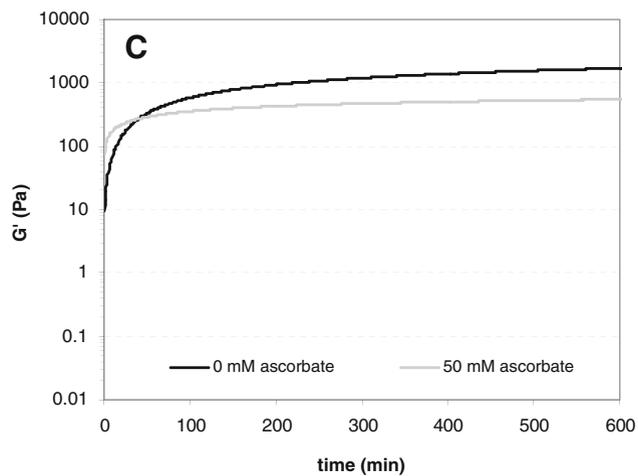
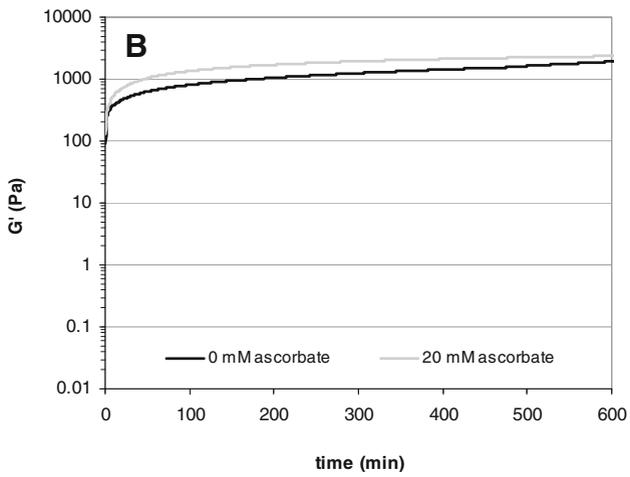
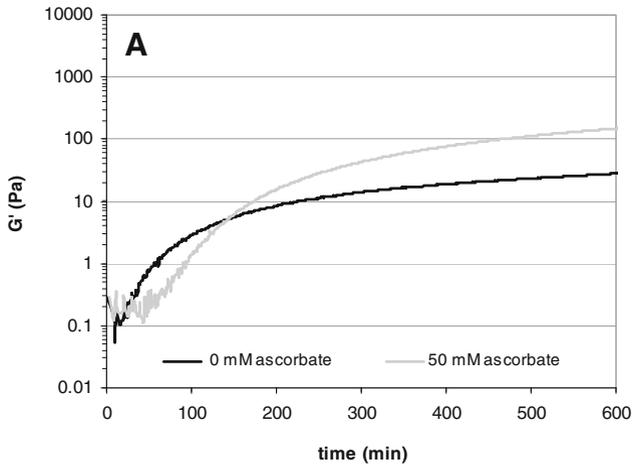


**Fig. 2** Maximum force at fracture from texture analysis of whey protein isolate (WPI) cold-set gels containing different ratios of  $\text{Fe}^{2+}$  (10–30 mM Fe) and ascorbate (0–50 mM) in final gel



**Fig. 3** Force–distance gradient (elasticity) from texture analysis of WPI cold-set gels containing different ratios of Fe<sup>2+</sup> (10–30 mM Fe) and ascorbate (0–50 mM) in final gel

the ascorbate concentration improves the gel strength ( $F_{\max}$ ) but not the  $F$ – $d$  gradient, i.e., at 50 mM ascorbate stronger gels were formed than at the lower concentrations and without ascorbate. Also for 20 mM Fe<sup>2+</sup>, ascorbate improves the gel strength ( $F_{\max}$ ), but above 20 mM ascorbate, this effect levels off. For the  $F$ – $d$  gradient, an opposite effect was observed, i.e., from 0 to 20 mM ascorbate no significant effect is observed, whereas at higher ascorbate concentration, a lower gradient was found. At higher ascorbate concentrations, the gels apparently fracture at higher deformation. At 30 mM Fe<sup>2+</sup>, there is no clear effect of ascorbate addition. In contrast to the two lower iron concentrations, the gel strength is not enhanced compared to the reference without ascorbate. From these results, three iron/ascorbate ratios were chosen to study the influence of ascorbate on the rate of gel formation. The ratios were based on the optimal ascorbate concentration for each iron concentration with regard to both the highest  $F_{\max}$  and  $F$ – $d$  gradient and were found to be: 10 mM Fe<sup>2+</sup>/50 mM ascorbate (10Fe50A), 20 mM Fe<sup>2+</sup>/20 mM ascorbate (20Fe20A), and 30 mM Fe<sup>2+</sup>/50 mM ascorbate (30Fe50A). Figure 4 shows the increase in storage modulus  $G'$  with time for the three ratios of iron/ascorbate with regard to their reference without ascorbate. In general,  $G'$  was the highest for 20 mM Fe<sup>2+</sup> in accordance to the results observed with the texture analyzer. For 10 mM Fe<sup>2+</sup>, the initial increase in  $G'$  was delayed when ascorbate was added, but after 150 min, a higher  $G'$  was obtained. At 20 mM Fe<sup>2+</sup>, only a small effect of ascorbate was observed on the gelation rate as ascorbate slightly enhanced  $G'$ . At 30 mM Fe<sup>2+</sup>, ascorbate initially induced an increase in the gelation rate, but at longer time intervals, the addition of ascorbate resulted in a lower  $G'$ . Table 1 summarizes the values for  $G'$  and  $G''$  at short and long time scales as derived from Fig. 4. It appears that  $G'$  increased in the order 10Fe50A < 30Fe50A < 20Fe20A.



**Fig. 4** Gel strength given by storage modulus  $G'$  (in Pascal) for 6% WPI cold gelled by **A** 10 mM Fe<sup>2+</sup> with and without the presence of 50 mM ascorbate; **B** 20 mM Fe<sup>2+</sup> with and without the presence of 20 mM ascorbate; **C** 30 mM Fe<sup>2+</sup> with and without the presence of 50 mM ascorbate

Summarizing, ascorbate affects the gel formation process and increases the gel strength ( $F_{\max}$ ) at 10 and 20 mM Fe<sup>2+</sup>. At 30 mM Fe<sup>2+</sup>, no effect of ascorbate was observed. The extent to which the gel strength is increased depends on the iron/ascorbate ratio.

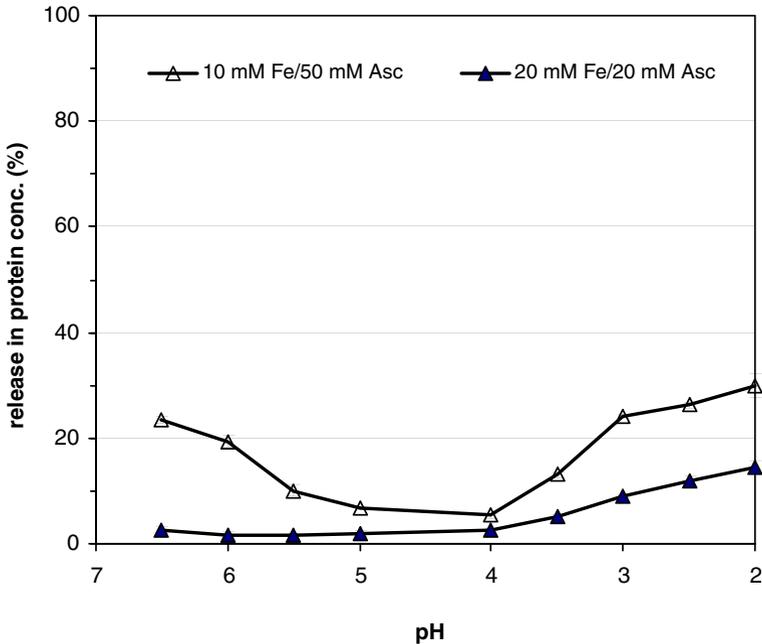
### 3.2 Protein stability of gel particles

Based on the characteristics of the cold set whey protein gels, two extreme cases were chosen to study the effect of gel strength on the stability of the gel particles and thus the encapsulation capability. The main question to be answered is whether the particles are stable enough to be able to keep iron entrapped at conditions in food products and unstable at conditions where iron should be released and delivered. For the stability test, protein particles from 10Fe50A and 20Fe20A gels were subjected to a pH range from 7 to 2. Figure 5 shows the stability of both gel particles by plotting the release of protein into the supernatant as a function of the pH. The amount of protein present in the supernatant after centrifugation is a measure for the stability of the protein particles. The more protein present in the supernatant, the less stable the protein particles as they fall apart. Figure 5 shows that the amount of protein in the supernatant was generally higher for 10Fe50A than for 20Fe20A. Moreover, going from neutral pH towards pH and lower, the stability of the 10Fe50A particles first increased and then decreased again. The 20Fe20A particles are stable throughout a broad pH range and only slightly decrease in stability at very low pH.

Screening for the optimal iron/ascorbate ratio, the 20Fe20A particles showed to give strongest gels and highest stability. For these particular particles, the next steps are (1) to determine the mol binding ratio of Fe/protein and (2) to determine the effect of ascorbate on the oxidation rate of Fe<sup>2+</sup>, in the presence of whey protein.

**Table 1** Storage modulus  $G'$  and loss modulus  $G''$  as a function of time (t) for whey protein isolate (WPI) cold gelled by three different combinations of Fe<sup>2+</sup> and ascorbate concentrations (derived from Fig. 4)

Protein (%)	Fe (mM)	Ascorbate (mM)	t=4 h		t=10 h	
			$G'$ (Pa)	$G''$ (Pa)	$G'$ (Pa)	$G''$ (Pa)
6	10	0	11	1	28	3
6	10	50	25	4	181	19
6	20	0	1,131	166	1,919	258
6	20	20	1,810	249	2,435	303
6	30	0	1,050	117	1,700	175
6	30	50	448	66	563	73



**Fig. 5** Stability of protein particles at different pH: the release of protein from the cold-set gelled protein particles into the supernatant is a measure for the stability (*Asc* = ascorbate)

### 3.3 Characteristics gel particles

For the optimal combination of iron and ascorbate, the protein particles were prepared according to a more extended procedure (see Section 2.4). Washing of the particles was performed to remove any unbound iron. It appeared that not all iron was effectively enclosed by the protein as after two washing steps, 30% of the iron was removed from the particles. From washing steps 3 to 5, this amount did not increase further assuming that all unbound iron was completely removed. At the same time, the protein content (as determined by Kjeldahl) in the washing fluids decreased from 0.05% (step 1) to 0.01% (step 5) showing that the protein gel particles stay intact during the washing. These protein gel particles were freeze dried and Kjeldahl analysis showed that the protein content of these particles was 82%. The mol binding ratio of Fe/protein can now exactly be determined as both iron concentration and protein concentration are known. Using 14 mM Fe and 4.9% protein, the mol binding ratio of Fe/protein was found to be  $\sim 5.7:1$ .

As for sensory properties and food fortification, the size of the gel particles is very important. The size of the particles was determined:  $d_{0.5} \sim 850 \mu\text{m}$ . For the application in fluid (dairy) products, this size is too large. Therefore, milling of the (freeze dried) particles was done in order to obtain a  $d_{0.5} \sim 75 \mu\text{m}$ .

### 3.4 Effect of ascorbate on $\text{Fe}^{2+}$ oxidation

To study the effect of ascorbate on the oxidation of  $\text{Fe}^{2+}$  in the presence of whey protein, analysis of Fe was performed on unheated whey protein and heated whey

protein samples. To the unheated whey protein, Fe<sup>2+</sup> was added as such without any gel formation. The addition of Fe<sup>2+</sup> to the pre-heated whey protein induced cold-set gelation. For comparison, also unheated iron and iron/ascorbate were analyzed. The total iron content and Fe<sup>2+</sup> content were determined, and from those results, the amount of Fe<sup>3+</sup> was derived. Table 2 summarizes the results showing the composition of the samples and the iron recovery. When Fe<sup>2+</sup> was added by itself to a non-heated protein solution, a large part of the Fe<sup>2+</sup> was oxidized. The addition of ascorbate to unheated protein prevented oxidation and the amount of Fe<sup>3+</sup> recovered was decreased to a large extent. When Fe<sup>2+</sup> was added to a pre-heated whey protein solution, a gel was formed. The total amount of iron that was recovered was much lower than that of the non-heated protein solutions. This is due to the fact that iron is trapped in a gel network and not available for analysis. The gels were ground to fine particles, and for Fe analysis, the supernatant of the centrifuged gel particle suspensions was taken. Only a part of the iron was released into the supernatant. Similar to the non-heated protein samples and the sample without protein, ascorbate clearly prevented oxidation of Fe<sup>2+</sup> and enhanced the recovery of Fe<sup>2+</sup>. The results show that it is possible to analyze different forms of Fe and to measure the effect of ascorbate on the oxidation state of iron.

### 3.5 Iron release from the gel particles

With the 20Fe20A gel particles being stable at a broad pH range with regard to protein (see Section 3.3) and iron essentially entrapped in the ferrous form (Section 3.4), the release of iron needs to be studied as a function of storage time. This will indicate the encapsulation efficiency and the capability of whey protein to keep iron bound at different conditions and time scales. Both wet and freeze-dried gel particles were subjected to pH 2 and to pH 6.5. To the latter also, 1 mg.mL<sup>-1</sup> CaCl<sub>2</sub> was added to mimic the situation in milk products. This was done to study the possible exchange of Ca<sup>2+</sup> and Fe<sup>2+</sup> from the particles as Ca<sup>2+</sup> can also be used for cold-set gelation and has affinity for the whey protein.

**Table 2** Analysis of Fe content in different combinations of protein, iron, and ascorbate; heated indicates the pretreatment of WPI to form cold-set gels; the Fe<sup>2+</sup> and Fe<sup>3+</sup> recovery are given as percentage of the total Fe recovery and add up to 100%

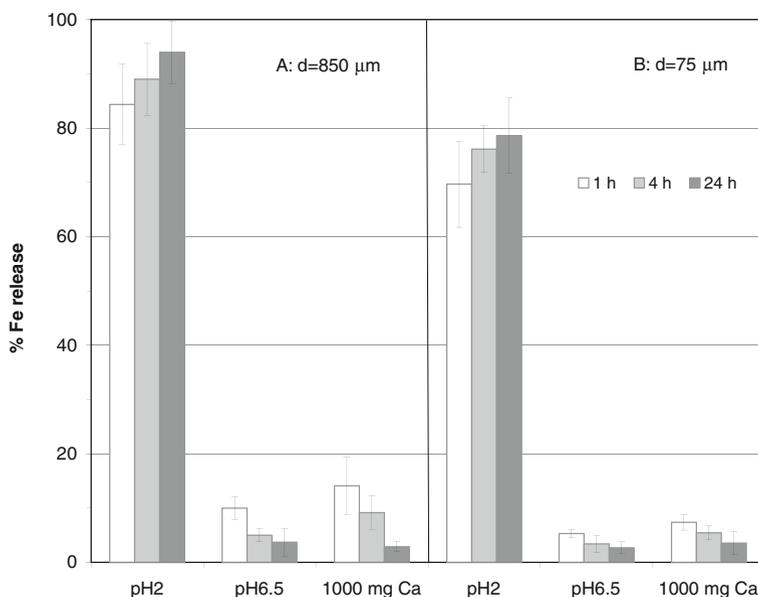
Heat treatment	Samples			Recovery		
	Protein (%)	Fe <sup>2+</sup> (mM)	Ascorbate (mM)	total Fe (%)	Fe <sup>2+</sup> (%)	Fe <sup>3+</sup> (%)
Not heated	0	20	0	23	100	0
	0	20	20	53	81	19
	6	0	0	0	0	0
	6	20	0	89	28	72
	6	20	20	88	83	17
	6	0	20	0	0	0
Heated	6	20	0	16	53	47
	6	20	20	28	90	10

No significant difference in iron release patterns was observed between the wet gel particles and the freeze-dried particles. Therefore, Fig. 6a shows the iron release patterns for the 20Fe20A freeze-dried gel particles ( $d_{0.5} \sim 850 \mu\text{m}$ ), only. The iron release is given in percentage of the actual amount of iron present (14 mM Fe) as determined before. As a function of time, the iron release at pH 2 reaches an equilibrium where more than 90% of the iron is released from the protein particles. At pH 6.5, less than 10% of iron is released, meaning that the particles are very stable over 24 h. The presence of 1,000 mg.L<sup>-1</sup> calcium (similar to concentration in milk) at neutral pH did not affect the release of iron, which is positive for the use in milk products. The size of the gel particles (see Fig. 6b) did not seem to have a large effect either on the iron release patterns, although the release at pH 2 appeared to be somewhat lower for the smaller particles ( $d_{0.5} \sim 75 \mu\text{m}$ ).

In general, at pH 2, an increase in iron release with time was observed, whereas in comparison to pH 6.5, the iron release decreased slightly with time. At the latter pH value also, a light brown coloring is observed, which indicates the oxidation of Fe<sup>2+</sup> into Fe<sup>3+</sup>. The decrease in iron release could be explained by the formation of insoluble Fe<sup>3+</sup> oxide complexes which are removed during the centrifugation step when preparing the samples for spectrophotometry.

### 3.6 Tiny-TIM studies

Protein gel particles have shown to be very stable at neutral pH and release iron at pH 2 at conditions where no enzymes are present. To study iron availability at gastrointestinal conditions in the presence of digestive enzymes and to validate the



**Fig. 6** Iron release from WPI gel particles (20 mM Fe/20 mM ascorbate) as a function of time when exposed to pH 2, pH 6.5, or pH 6.5 with 1,000 mg.L<sup>-1</sup> added calcium; 100% Fe release is 14 mM Fe; A = particle diameter ( $d$ ) of 850 μm; B = particle diameter of 75 μm

potential of protein particles for food fortification, the Tiny-TIM system was used. Four different test products were run through the Tiny-TIM system, of which two protein-free controls with Fe<sup>2+</sup> and Fe<sup>2+</sup>/ascorbate and two test products containing protein gel particles in which Fe was trapped with and without the presence of ascorbate. During the TIM runs, the dialysate from the upper part of the intestine was retrieved at different time intervals and the remaining residues (gastric and intestine) after finishing the TIM run. The dialysates represent the iron that could be transferred from the intestine through the intestine cells, which is the bio-accessible fraction. In Table 3, the values of the different Fe concentration as percentage relative to the start concentration are given for the four samples, respectively, (1) Fe, (2) Fe/ascorbate, (3) protein + Fe, and (4) protein + Fe/ascorbate. The values for recovery of Fe in Table 3 can be compared with those in Table 2, which are in fact the same samples but when undigested. Table 2 shows that the recovery of iron from the undigested samples is not very high: ~80% for unheated and ~20% for heated samples. Even test product 1 (Fe), which contains only an unheated iron solution has a recovery of around 23%. The addition of ascorbate to an unheated iron solution enhances the recovery to 53%, which shows that the recovery problems are related to the oxidation of Fe<sup>2+</sup>. A similar effect with the recovery is observed for the undigested samples containing protein. When ascorbate was added together with iron to form protein gel particles, the recovery of Fe increased from 4% to 14%.

For the test products that were digested with TIM, the amount of iron that was collected in the dialysates is generally also low. When TIM was fed with iron sulfate only, only ~4% of the iron is recovered, of which the largest part was Fe<sup>3+</sup>. It seems that most of the iron was oxidized and thereafter precipitated which made it impossible to penetrate the dialysis membrane. It would be logical that the missing iron would be still in the gastric and intestine residue.

**Table 3** Analysis of Fe concentration (in percent) relative to the start concentration in dialysate and residues after TIM runs

		Total Fe (%)	Fe <sup>2+</sup> (%)	Fe <sup>3+</sup> (%)
Fe	Total from dialysate	4	1	3
	Gastric residue	7	3	4
	Intestinal residue	23	2	21
Fe + Asc	Total from dialysate	39	30	8
	Gastric residue	6	5	1
	Intestinal residue	35	19	16
WPI + Fe	Total from dialysate	11	2	9
	Gastric residue	11	9	2
	Intestinal residue	9	4	6
WPI + Fe + Asc	Total from dialysate	78	45	33
	Gastric residue	0	0	0
	Intestinal residue	14	8	6

Start concentrations were 20 mM Fe<sup>2+</sup>, 20 mM ascorbate (Asc), and 6% WPI  
Asc ascorbate, whey protein isolate (WPI)

Because the iron analysis only detects the solubilized iron, the precipitated iron in the residues was not determined. It is now assumed that the largest part of the iron was precipitated in the residue fractions.

When a mixture of Fe/ascorbate was analyzed with the TIM system, almost 40% of the iron was recovered in the dialysis fluid of which the largest part was  $\text{Fe}^{2+}$ . This shows that ascorbate clearly favors iron to a  $\text{Fe}^{2+}$  state which facilitates the absorption in the dialysis fluid. The digested test product of combined  $\text{Fe}^{2+}$ /protein gives a low recovery of iron (~10%), of which most iron is in the oxidized  $\text{Fe}^{3+}$  state. When ascorbate is additionally incorporated into the gel particles, the recovery of iron is increased to 78%; moreover, the largest part of the recovered iron is in  $\text{Fe}^{2+}$  state. Similar to the samples without protein, ascorbate enhanced iron absorption; moreover, using protein as a carrier for  $\text{Fe}^{2+}$  increased the total recovery and hence the in vitro iron accessibility.

#### 4 Discussion

In previous work (submitted), we have shown that the type of protein structure from whey protein as starting point for the iron-induced cold-set gelation influences the gel strength and the stability of gel particles. The type of protein structure was determined by different pretreatment conditions, a combination of pH and heating time and temperature. Both fibril-like and random aggregates formed cold-set gels when iron was added, of which the latter showed the highest gel strength and stability of the gel particles. Remondetto et al. (2004) have shown that the microstructure of the gels formed by pretreated  $\beta$ -lactoglobulin and different iron concentrations affects iron release from these particles. Not only the microstructure plays a role but this study reveals that the gel strength is also of relevance for the final gel particle stability and iron release. Figure 5 shows that the stability of the gel particles is related to the gel strength of the cold-set gel: a lower  $G'$  decreases the stability of the gel particles. As the gel strength is determined by protein–Fe and protein–protein interactions, and these interactions are changed by shifts in the net charge of the protein polypeptide chain at acidic conditions, it seems that weaker gels are more susceptible to these changes and hence fall apart more easily. This agrees with Katzhendler et al. (2000) who showed a correlation between the elasticity and the gel strength and release kinetics from proteinaceous matrices, indicating that the matrices that are more elastic and with higher gel strength retain more strongly entrapped the molecules.

This study showed that ascorbate influenced the gel strength of the cold-set gels, and moreover, it favored the iron recovery and absorption. The ratio protein/iron/ascorbate can be used to tune the gel strength, and therefore, the iron release properties from the gel. It is recognized that ascorbate is added as a salt and therefore also  $\text{Na}^+$  is added. From literature, it is known that also  $\text{Na}^+$  can induce cold-set gelation (Ako et al. 2010; Bryant and McClements 2000). However, this only occurs at much higher concentrations (>300 mM NaCl) than used in this study. If the addition of  $\text{Na}^+$  would have contributed to the gelation process, then a consistent increase in gel strength with ascorbate concentration would have been expected for all samples as a function of total ionic strength. This was not the case though. Bivalent

ions such as Fe<sup>2+</sup> and Ca<sup>2+</sup> are far more effective in inducing cold-set gelation than mono-valent ions such as Na<sup>+</sup>, so the addition of small amounts of Na<sup>+</sup> does most likely not contribute to the gel formation.

The mechanism by which ascorbate enhances gel strength was thought to be attributed to its role in reducing the ferric iron to ferrous iron. In this way, more Fe<sup>2+</sup> would be available for inducing cold-set gelation. It is not known in what state iron is when present in the protein gel particles without ascorbate. The actual oxidation rate of iron depends on the type of ligand to which it is bound. Chelators that contain oxygen tend to stabilize the ferric form, whereas chelators that contain nitrogen or sulfur ligands tend to stabilize the ferrous form (Miller et al. 1990). The stability of the form in which iron is in when bound to protein, is unsure. As literature does not report on trivalent ions and their capability of inducing cold-set gelation, we assume that in the case of absence of ascorbate, a part of the iron is bound as Fe<sup>2+</sup> to the protein and the free Fe<sup>2+</sup> has a chance to move to the ferric state. This would also support the higher numbers found of ferric iron released from protein gel particles without ascorbate, as more free iron is present compared to the ones with ascorbate.

The stability of the iron form is also very important with regard to fortification of food products. When ferrous iron is present and reactive, it may react with other components (e.g. fat) present in the food product. For fortification in dairy products, this study focused on the presence of Ca<sup>2+</sup> and its effect on iron release. Calcium can also induce cold-set gelation of whey protein (Bryant and McClements 2000; Hongsprabhas and Barbut 1997a; Hongsprabhas and Barbut 1997b) and forms much stronger gels than those with iron. Apparently, the binding of iron to protein is very effective as hardly no exchange between calcium and iron took place when exposing the protein particles to a calcium concentration similar to that in milk. This is very promising for the fortification of dairy products. To increase the iron content of, e.g., milk to a concentration of ~5 mg.100 mL<sup>-1</sup> by using these gel particles, the mol binding ratio of Fe/protein is important. In the case of a mol binding ratio of ~5.7:1, it means that 0.32% WPI will be added in the form of gel particles. Concerning the binding ratio, literature does not report on the exact mol binding ratio when cold-set gelation is used. Although Remondetto et al. (2004) looked at the bio-accessibility of iron from iron–protein gels, they report the release in iron as percentage and do not give an absolute value. Sugiarto et al. (2009) did not use cold-set gelation but common binding and found a binding ratio for iron to WPI of 6:1, similar to the results presented here. They, however, state in their research that they faced precipitation of oxidized iron, which is also commonly observed by others (Gaucheron et al. 1996; Raouche et al. 2009). As they never determined the amount of precipitated iron, they assume all iron was bound to the protein when calculating the mol binding ratio. Although the binding ratio is high, the stability of the binding was never tested at extreme conditions, such as those at gastric conditions.

The combination of using ascorbate, cold-set gelation for entrapping iron, and the new procedure for preparing stable protein gel particles seems promising as is shown by the high in vitro iron bio-accessibility as shown by TIM. The mechanism by which ascorbate enhances the uptake of ferrous iron absorption could work in two ways: (1) reduction of ferric iron to ferrous iron and (2) formation of a ferric iron and ascorbate complex, which could be transported directly into the intestinal cells. In the latter case, ascorbate functions as a chelating agent for ferric iron (Thumser et al. 2010).

This mechanism is, however, thought not to contribute to a large extent as iron is bound to protein. The role of ascorbate is therefore to keep the iron in its ferrous state leading to optimal binding of iron to protein.

## 5 Conclusions

This research showed that cold-set gelation of whey protein as induced by  $\text{Fe}^{2+}$  and ascorbate proved to be successful in enhancing the recovery and in vitro bio-accessibility of iron. The ratio whey protein/iron/ascorbate can be tuned for optimal gel strength creating stable gel particles that release iron only at acidic conditions. The new procedure of washing and freeze drying renders protein particles, of which the particle size can be controlled without affecting the iron release. Using this preparation procedure, the mol binding ratio of  $\text{Fe}$ /protein in the particles was found to be 5.7:1. For food fortification purposes, this means an addition of 0.32% protein to obtain an iron concentration of  $50 \text{ mg.L}^{-1}$  in the food product.

As for in vitro iron bio-accessibility, the TIM results showed that  $\text{Fe}^{2+}$  uptake in the presence of protein particles (with ascorbate) is a factor 2 higher than without the protein particles: almost 80% of the iron was absorbed by the dialysis. Moreover, ascorbate plays an even more important role in the recovery of iron. The  $\text{Fe}^{2+}$  uptake in the presence of ascorbate in iron–protein particles increases from 10% to almost 80%. This suggests that the concept of using protein particles with iron and ascorbate can successfully and effectively be used to fortify food products with iron.

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