Assessing the iron chelation capacity of goat casein digest isolates

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ABSTRACT

We isolated goat phosphopeptides via calcium and ethanol precipitation from a caseinate digest and investigated their feasibility as an iron-fortification ingredient in nutritional foods. Goat tryptic-digested phosphopeptides could bind 54.37 ± 0.50 mg of Fe/g of protein compared with goat milk, which could bind 3.83 ± 0.01 mg of Fe/g of protein, indicating that isolation did increase iron binding. However, the >13-fold increase in iron binding was only partly explained by the increased concentration of phosphoserine-rich residues in the isolated fraction: we observed a 77% increase in serine residue content and a 5.9-fold increase in phosphorus in the goat peptide isolate compared with the starting caseinate material. We investigated the effect of potential industrial processing conditions (including heating, cooling, holding time, and processing order) on iron binding by the tryptic-digested phosphopeptides. In addition, we tested the effect of ionic strength and the addition of peptides to a milk system to understand how food formulations could affect iron binding.

Key words: iron, chelation, goat casein, peptides

INTRODUCTION

Anemia is a worldwide issue: an estimated 2 billion people have low iron, and this has many repercussions, including decreased energy levels, fatigue, and cold intolerance (Miller, 2013). In children, low iron can affect neurological development, immunity, and growth (Kibangou et al., 2005). Although iron is found in a variety of foods, cohorts such as the elderly (Busti et al., 2014), women of childbearing age (Patterson et al., 2001), and infants unable to breastfeed may find it difficult to achieve adequate iron intake. As well, iron fortification in food can negatively affect product quality. For example, one problem in applications containing oil is that soluble iron forms can act as pro-oxidants, leading fats to become rancid more quickly. This results

in a loss of nutrient value and consumer acceptance because of negative effects on organoleptic quality and gastrointestinal discomfort in people with compromised health or sensitive digestive systems, such as infants (McClements and Decker, 2000).

Phosphopeptides from α_{S1} -, α_{S2} -, and β -caseins contain highly polar acidic sequences of 3 phosphoserine groups followed by 2 glutamic acid residues (Sp-SpSpEE; Bouhallab and Bouglé, 2004; Miquel et al., 2006) that enable the peptides to bind cationic minerals. Phosphoserine groups bind iron via coordination and ionic linkages (Baumy and Brule, 1988; Pérès et al., 1999), and calcium binds via ionic bonding only (Baumy and Brule, 1988); both calcium and iron can also bind to the carboxylic groups on the caseins, but with less affinity (Baumy and Brule, 1988; Swaisgood, 2003). Phosphopeptides have been shown to be stable and soluble over a range of physicochemical conditions, such as changes in pH (Aït-Oukhatir et al., 2000).

In terms of nutritional significance, iron-fortified phosphopeptides increased iron absorption in rats compared with iron gluconate (Pérès et al., 1999). Similarly, Aït-Oukhatir et al. (1999) evaluated the ability of iron-deficient rats to restore hemoglobin levels using a low-molecular-weight phosphopeptide compared with FeSO_4 and intact β -CN. The β -CN(1–25) peptide fortified with iron improved iron tissue storage and hemoglobin levels compared with FeSO_4 . Whole casein bound to iron also showed an improvement, but not to the same extent on an equal-protein diet. These findings were supported by Bouhallab et al., (2002), who found that iron absorption was greater using the β -CN(1–25) peptide than with a whole digest of β -CN and a mixture of casein phosphopeptides.

Methods for isolating phosphopeptides that are highly saturated with calcium to remineralize enamel have been developed (Reynolds et al., 1994; Adamson and Reynolds, 1995; Adamson and Reynolds, 1996) and used by different authors (McDonagh and FitzGerald, 1998; Corsetti et al., 2003; Kim et al., 2007). Corsetti et al. (2003) studied the yield and calcium-binding properties of phosphopeptides derived from different species and found that goat caseinate produced the highest yield but had a lower capacity to bind calcium than

Received September 30, 2016.

Accepted December 21, 2016.

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casein phosphopeptides derived from bovine caseinate. Iron-bound phosphopeptides from cow caseinate have been prepared for absorption studies. Bouhallab et al. (2002), Chaud et al. (2002), and Kibangou et al. (2005) produced iron-bound phosphopeptides by digesting caseinate, adding iron, and then filtering the solution or isolating the complex by dialysis (Ait-Oukhatar et al., 1999). The purpose of our study was to assess how goat caseinate performed as a precursor for phosphopeptides that could bind iron under simulated industrial processing conditions. We carried out the same process using cow caseinate as a reference during the isolation stage, because of the greater depth of information in the literature.

MATERIALS AND METHODS

Materials

Goat and cow caseinate were prepared by isoelectric precipitation of reconstituted goat (predominantly Saanen) or cow milk powder obtained from Dairy Goat Co-operative (Hamilton, New Zealand), and Fonterra Co-operative (purchased from Milligans Food Group Ltd., Oamaru, New Zealand), respectively. Briefly, the skim milk powders were hydrated to 10% (wt/wt) and acidified to pH 4.6 for cow and 4.2 for goat milk at 15°C. The solutions were warmed to 30°C and the precipitate collected by centrifugation. The precipitates were caseinated by addition of sodium hydroxide to pH 6.7 and freeze-dried. We obtained ammonium bicarbonate, ammonium molybdate, disodium tetraborate, and sodium sulfite from BDH Chemicals (Poole, UK); hydrochloric acid, sodium hydroxide, 2-propanol, acetonitrile, trifluoroacetic acid, and ferrous sulfate from Fisher Scientific (Leicestershire, UK); hydrogen peroxide, disodium EDTA, and sodium chloride from LabServ (Biolab, Australia); *o*-phthaldialdehyde (OPA), dithiothreitol, serine, ferrozine, and neocuprione from Sigma (St. Louis, MO); Eriochromeback T indicator from Riedel-de Haën (Seelze, Germany); and trypsin (Pancreatic Trypsin Novo 6.0S) from Novozymes (Bagsvaerd, Denmark). All water was MilliQ grade, and all other chemicals were analytical grade. The spectrophotometer used throughout was Pharmacia UV/visible Ultrospec II (LKB, Biochem, Cambridge, UK).

Hydrolysis

The goat and cow caseinates were hydrolyzed using the methods of Reynolds et al. (1994), Adamson and

Reynolds (1996), and Strader et al. (2006) with modifications. Sodium caseinates (35 mg/mL) were dissolved in 50 mM ammonium bicarbonate solution, pH 8.0. Commercial grade trypsin hydrated in 1 mM HCl at 4 mg/mL was added to the solution at an enzyme to protein ratio of 1:200 with rapid stirring. Digestion was maintained at 37°C overnight, and the reaction stopped by heating the solutions to 75°C for 10 min. The pH was lowered to pH 4.6 ± 0.1 and the solutions centrifuged at $12,000 \times g$ for 10 min (Sorvall RC 6+, ThermoFisher Scientific, Waltham, MA) to remove undigested material. The serum pH was increased to pH 6.74 ± 0.03 and a 10% calcium chloride solution was added slowly at room temperature to a final concentration of 100 mM. The solution was incubated at room temperature for 1 h, 95% ethanol was added to a final 50% (vol/vol) concentration by slow addition. The solution was centrifuged at $12,000 \times g$ for 15 min (Thermo Sorvall RC 6+, rotor type: F21S-8x50, ThermoFisher Scientific) and the pellet was collected and freeze-dried. The hydrolysis was carried out in triplicate for each species.

Calcium Ion Exchange of Phosphopeptides

A weight of 50 mg of peptide solids was dissolved in 5 mL of deionized water. Amberlite IRC-50 resin (Rohm & Haas Company, Philadelphia, PA) was prepared by washing the resin in 1 M NaOH for 1 h to charge it to the sodium form and then rinsing it with water until the pH was close to pH 7.0 (Mittal et al., 2015). Then, 0.1 mL of wet resin was added to the peptide solution, mixed gently, and incubated overnight at 4°C; finally, the solutions were decanted from the resin.

Degree of Hydrolysis

We evaluated the degree of hydrolysis (DH) according to Nielsen et al. (2001). The OPA reagent was prepared by mixing 1.14 g of disodium-tetraborate decahydrate and 30 mg of SDS in 22.5 mL of water until fully dissolved. The OPA (24 mg) was dissolved in 0.6 mL of ethanol and followed by addition of 26.4 mg of dithiothreitol, and the solution was made up to 30 mL. Samples were diluted to 0.8 mg/mL in water; 0.75 mL of OPA reagent was added to 100 μ L of sample, mixed for 5 s, and measured at 340 nm exactly 2 min after addition. The standard used was 0.01% (wt/vol) serine solution with a milli-equivalence of 0.9516 mEq/L. The degree of hydrolysis was determined as a percentage of cleaved peptide bonds:

$$\text{Serine-NH}_2 = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \times 0.9516 \text{ mEq/L} \\ \times 0.1 \times \frac{100}{X} \times P,$$

where serine-NH₂ was the equivalents of serine-N per gram of protein, OD is optical density, X was the amount (g) of the sample, P was the protein (%) in the sample, and 0.1 was the sample volume in L.

$$H = \frac{\text{serine-NH}_2 - \beta}{(\alpha \text{ mEq/g of protein})},$$

$$\text{DH (\%)} = \frac{H}{h_{\text{tot}}} \times 100\%,$$

where α , β , and h_{tot} were defined by Nielsen et al. (2001) as 1.039, 0.383, and 8.2 for casein, respectively, and H was the number of hydrolyzed bonds. After 18 h of digestion, the DH was $27.1 \pm 0.6\%$ for cow and $26.3 \pm 0.8\%$ for goat caseinate ($P > 0.05$).

Phosphorus Content

The phosphorus content was determined using the method of Bartlett (1959). Samples were prepared by dissolving peptide powders in water to 0.7 mg/mL, and 2 mL of the solution was pipetted into boiling tubes. Then, 0.5 mL of 5 M sulfuric acid was added to the boiling tubes and heated at 155°C for 3 h. Hydrogen peroxide was added (0.1 mL or until the solution was close to clear) and the solution was further heated for 90 min. Ammonium molybdate (0.22%, 4.6 mL) was added, followed by addition of 0.2 mL of Fiske reducer [0.0625 g of 1-amino-2-naphthol-sulfonic acid and 0.125 g of sodium sulfite in 25 mL of 15% sodium bisulfite (3.75 g); filtered]. The solution was heated at 100°C for 7 min and then read at 830 nm.

Calcium Content

Calcium was determined using calcium-EDTA titration with Eriochromeblack T indicator. Borate buffer was prepared by dissolving 3.332 g of sodium tetraborate decahydrate in 80 mL of water. Sodium hydroxide (0.54 g) and sodium sulfide (0.4165 g) were dissolved and added to the borate solution and made up to 100 mL. The indicator solution was made by dissolving 0.1 g of Eriochromeblack T powder in 3 mL of water and 0.1 mL of 1 N sodium carbonate and made up to 10 mL

with 2-propanol. A 1 mM or 5 mM EDTA titrant was prepared by with disodium EDTA dehydrate. For a 25-mL sample, 0.5 mL of borate buffer was added followed by addition of 0.025 mL of indicator and titrating until a light blue endpoint was achieved.

Protein Content of Peptide Isolates

The protein concentration of the ion-exchanged solutions was determined using the Lowry method with the Ohnishi and Barr modification using the total protein kit by Sigma (TP0200) according to the manufacturer's directions (<http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/tp0200bul.pdf>). Samples were diluted in 0.85% sodium chloride solution in the range of 150 to 1,000 µg/mL, and 0.2 mL of sample was combined with 2.2 mL of Biuret reagent and incubated for 10 min followed by addition of 0.1 mL of Folin-Ciocalteu reagent and incubated for 30 min. The samples were measured against a standard curve prepared from a stock protein standard provided. The absorbance was measured at 550 nm.

Amino Acid Profile

The Massey University Nutrition Laboratory (Palmerston North, New Zealand) performed the amino acid analysis. The methodology used was standard AAA hydrochloric acid hydrolysis, followed by reversed-phase HPLC separation using AccQ Tag derivatization (AOAC International, 2012; method 994.12).

Reversed-Phase HPLC

The RP-HPLC was performed with a 1200 Series apparatus from Agilent Technologies (Santa Clara, CA) equipped with an G1311A Quaternary pump and 1200 Series sample injector and UV detector. The chromatographic software used was EZChrom (Agilent Technologies Inc., Santa Clara, CA). The apparatus was equipped with a RP column, Prevail C18 (150 × 4.6 mm i.d., 5-µm pore size; Chromservis, Prague, Czech Republic). The samples were prepared by dissolving in 90% H₂O and 10% acetonitrile to 2 mg/mL. The samples were centrifuged at 13,300 × g for 10 min. Two buffers: A containing 95% MilliQ water, 5% acetonitrile, and 0.1% trifluoroacetic acid (TFA) and B containing 95% acetonitrile, 5% MilliQ water, and 0.08% TFA were used for elution.

A linear gradient was performed with 50 mL/L buffer B in 5 min followed by 50 to 600 mL/L buffer B within 120 min, followed by 600 to 900 mL/L within 121 min.

At a flow rate of 1 mL/min, a 5- μ L sample was injected onto the column, maintained at 25°C, and the absorbance was measured at 214 and 280 nm.

Iron Chelation

Samples were diluted to 0.2 mg/mL in a modified simulated milk ultrafiltrate buffer (20 mM imidazole, 30 mM NaCl, pH 6.7) to maintain a constant pH. A 0.93-mL aliquot was mixed with 0.025 mL of 1 mM FeSO₄ in 1 mM HCl, and held for 3 min. Variations of these conditions were adjusted as required, significant changes to the method are detailed below.

Effect of pH, Temperature, and Ionic Strength.

The peptides were subjected to buffer conditions at various combinations of pH (pH 2.75, 4.6, and 6.7), ionic strength (50 mM, 400 mM) and temperature (4°C, 20°C, 37°C, 72°C) to which ferrous sulfate was added.

Addition of Peptides to a Milk Model. Milk powder was reconstituted in water to 10% (wt/wt) solids at 20°C and mixed for 2 h before being chilled overnight at 4°C. After overnight storage, the milk solutions were diluted to 0.2 mg/mL in imidazole buffer and peptides solubilized in buffer were added where the volumes were varied to produce different ratios of milk protein to peptide to achieve a final 0.2 mg/mL protein concentration. Iron binding was carried out at 20°C to limit the effects of temperature on oxidation; equal iron fortification of 28 μ M was used across all the samples. The order of addition of iron to the solution was varied to reflect the various process options that are possible and whether these process options affect iron binding by the peptides. Accordingly, ferrous sulfate was either added to the peptide solution and held for 3 min and then added to the milk solution or the peptide and milk solution were mixed then the iron was added to this.

Ferrozine (6.09 mM, 0.04 mL) was added to stop the binding reaction; the mixture was held for 10 min to allow color development and then read at 562 nm. There is potential for error as oxidized iron in the ferric form will not be detected and therefore could produce a false positive for bound iron. Due to the nature of the technique, it is difficult to assess how much iron is oxidized or bound other than doing a baseline calculation under identical conditions by using a blank.

We calculated the iron chelation and activity as follows (Abs = absorbance):

$$\text{Fe chelation} = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}},$$

$$\text{Fe chelation activity} = \frac{\text{Fe chelation} \times \text{Fe added } (\mu\text{M})}{\text{Protein (mg/mL)}}.$$

Statistical Analysis

Data were analyzed using a 2-sample *t*-test after checking for homogeneity of variances using F-test or ANOVA where appropriate. Analysis was carried out using R (Harrell et al., 2014; R Development Core Team, 2014).

RESULTS AND DISCUSSION

Yield

The yield of goat phosphopeptides from the original caseinate solution was $15.3 \pm 0.2\%$ (wt/wt). By changing the enzyme system and processing conditions, the yield of casein phosphopeptides can vary greatly. Reynolds et al. (1994) reported a yield of $10.50 \pm 0.4\%$ (wt/wt) from a tryptic digest of cow caseinate at pH 3.5. Adamson and Reynolds (1995), who also used trypsin, obtained a yield of $11.04 \pm 0.30\%$ (wt/wt) at pH 4.6, which increased to $13.85 \pm 0.48\%$ (wt/wt) when the pH was raised to 8.0. The composition of the precipitate differed in terms of the phosphopeptide clusters; at higher pH, the extraction loses specificity, whereby di- and monophosphopeptides can also be isolated (Reynolds et al., 1994). These authors also reported a slight increase in yield for a digest held at pH 4.6 by substituting trypsin for alcalase because of its lower specificity (Adamson and Reynolds, 1996). Zhu and FitzGerald (2010) found that casein phosphopeptides were more stable against heating when the peptides were isolated at pH 7.0. McDonagh and FitzGerald (1998) used a wide range of enzymes, along with calcium and ethanol precipitation, and found a yield range of 3.4 to 16.0%. Kim et al. (2007) found that the yield increased linearly from pH 3 (5%) to 7 (12%) and remained at 12% at pH 8.0. It is likely that the peptides produced in our study may have contained a greater amount of di- and monophosphopeptides because of the higher precipitation pH and yield, but would be more stable during heating, an important property in the production of nutritional foods. Corsetti et al. (2003) reported a yield of casein phosphopeptides from goat caseinate of 1.86% (wt/wt). They used *Lactobacillus helveticus* PR4 on different species of sodium caseinate and found a yield of 0.6 to 1.86% (wt/wt). Their overall low yield compared with our study and other studies may have been because they used different culture and digestion conditions.

Phosphorus Content

The phosphorus content of the goat caseinate was 7.84 ± 0.31 mg/g of solids, and of the cow caseinate was

7.03 $\pm$ 0.8 mg/g of solids. These figures were similar to those reported in the literature: McMeekin and Polis (1949) and Díaz et al. (2003) reported values of 8.6 and 7 mg/g of cow casein, respectively. After digestion and calcium and ethanol precipitation, the phosphorus content of both the goat- and cow-derived phosphopeptides increased by more than 500% to 45.39 $\pm$ 2.37 mg/g of solids and 40.08 $\pm$ 1.57 mg/g of solids, respectively ($P > 0.05$). On a percentage basis, the phosphorus content for both isolates, at 4.64% (goat) and 4.01% (cow), were similar to the content of approximately 5% for cow-casein-derived phosphopeptides obtained at pH 6.7 reported by Kim et al. (2007).

Amino Acid Profile

Our results showed the relative change in composition of the amino acid residue content of cow and goat phosphopeptides compared with the starting material of the respective caseinates (Figure 1). Of the 16 amino acids measured in the goat peptide isolate (and 10 in the cow peptide digest), 9 decreased in concentration, indicating exclusion of the non-binding peptides. The predominant amino acid that increased was the serine residues, which increased by 77.7 and 72.3% for goat- and cow-derived peptides, respectively. In addition to serine residues, glutamic acid, glycine, histidine, threonine, and isoleucine increased in goat-derived peptides and aspartic acid, glutamic acid, glycine, threonine, and isoleucine increased in cow-derived peptides. It is likely that the increase in these latter amino acids reflected their proximity to the serine residues on the primary amino acid sequence and thus the likelihood of coprecipitating as part of a serine-containing peptide. According to the sequence of caseins from UniProtKB (Apweiler et al., 2004), serine residues that make up the phosphoserine clusters are surrounded by acidic residues such as glutamic acid (Reynolds et al., 1994). Comparing the theoretical amino acid profile and the expected cutting pattern of trypsin, we found that relative increases and decreases of the residues of the phosphoserine-containing peptides were similar to those observed in our study.

Reversed-Phase HPLC

Figure 2 shows the reversed-phase HPLC chromatograms for goat caseinate digests and the isolated peptides. We observed approximately 70 peaks in the original digest, similar to the number of theoretical peptides produced in a tryptic digest according to UniProtKB (Apweiler et al., 2004). After calcium and ethanol precipitation, the number of peaks reduced, indicating an isolation of peptides. The peptide isolate produced 1 in-

tense peak, about 6 major peaks, and approximately 33 peaks in total. At 214 nm, the peptide bonds were the absorbing species and were significantly more sensitive than at 280 nm. Few peaks appeared at 280 nm (data not shown), as aromatic amino acids are detected, indicating that the peptides in the phosphopeptide fraction contained very few aromatic residues (Højrup, 2004; Mennella et al., 2010). This finding was supported by the amino acid analysis: phenylalanine and tyrosine decreased by 81% and 79%, and histidine increased by 52% (tryptophan was not measured). Phosphopeptides were all eluted after 45 min, whereas peptides from the full digest required up to 75 min before they were fully eluted. This observation indicates that the eluted phosphopeptides were small and more hydrophilic than those in the overall digest, as expected given the characteristics of phosphoserine.

In terms of the isolation mechanism, calcium binds to negatively charged phosphoserine residues or acidic residues via ionic bonding. At a low pH, only multiply phosphorylated peptides extracted as acidic residues are protonated, and monophosphoserines do not have a strong negative charge (Reynolds et al., 1994). At pH 6.7, the acidity at which the peptides were extracted in our study, selectivity would not be expected to be high, and monophosphopeptide groups would be extracted along with the phosphoserine clusters. For a

Figure 1. Percentage change in AA residues after calcium and ethanol precipitation compared with starting material for goat and cow caseinate.

Figure 2. Chromatograms of goat caseinate (top) and goat phosphopeptide (bottom) at 214 nm, bandwidth of 8 nm, reference at 360 nm with a bandwidth of 50 nm.

phosphorylated peptide to be precipitated by calcium and ethanol, at least 1 phosphoserine group must be present, followed by an acidic residue, and binding may be restricted if the acidic residue is not next in sequence to the phosphoserine residue (Adamson and Reynolds, 1996). On this basis, and in the absence of mass spectrometry analysis, it is reasonable to assume that the isolated peptides contained phosphoserine residues or clusters, and that the peptides that contained no phosphoserine groups were removed.

Iron Chelation

Typically, iron-binding measurements in milk systems use ultracentrifugation or filtration to partition species based on size to determine bound and non-bound phases (Hegenauer et al., 1979; Gaucheron et al., 1996, 1997; Raouche et al., 2009). However, because the peptides of interest are small, they may be incorrectly measured using these techniques. Therefore, we used a more direct method using ferrozine, which does not require separation of sample components. Ferrozine measures only unbound ferrous iron, so an estimation of protein chelation of iron can be obtained. Many authors, including Decker and Welch (1990), Farvin et al. (2010), and O'Loughlin et al. (2015), have used variations of this method in dairy-based systems to measure the iron-binding capacity of the protein of interest.

Effect of pH, Temperature, and Ionic Strength.

As shown in Figure 3, temperature, pH, and ionic strength were significant factors ($P < 0.001$) in the iron-binding capacity of the peptides. At pH 2.75, almost all of the iron remained free and in the reduced form over the temperature range tested. At 4°C, the iron remained unbound to the peptides, and at 20°C, under ionic-strength conditions of 50 mM, the amount of iron bound to the peptides was also below the level of detection (0.01 ± 0.08 mg of Fe/g of protein). These findings indicate that iron does not bind to protein under highly acidic conditions. This may be because of protein precipitation (Taborsky, 1980), which could result in steric hindrance to the binding site, or protonation of the serine phosphate groups, which would also prevent binding (Castellani et al., 2004). The isoelectric points of the isolates were 3.67 and 3.55 for cow and goat peptides, respectively (data not shown). The peptides would be positively charged at pH 2.75, so protonation of the side chains appears to inhibit iron binding. Interestingly, Ait-Oukhtar et al. (2000) found that lowering the pH to 2.5 after iron was added to β -CN(1–25) casein did not cause a release of iron because of covalent bonding; therefore, it is likely that binding the iron to the peptides before lowering the pH would not cause the same effect as observed in this experiment. This finding would have important implications for processing and nutrition.

At pH 4.6, iron binding by the peptides increased and was particularly sensitive to temperature: in a 50 mM buffer, binding at 20°C was 0.64 ± 0.06 mg of Fe/g of protein, increasing to 2.92 ± 0.43 mg of Fe/g of protein at 37°C and 6.17 ± 0.01 mg of Fe/g of protein at 72°C. Increasing the ionic strength to 400 mM inhibited iron binding (0.53 ± 0.06 mg of Fe/g of protein at 37°C), possibly due to salting out of the protein, preventing access to the binding sites for iron. At pH 4.6, above the isoelectric point, the peptides are slightly negatively charged; affinity between the phosphate and side-chain groups and iron cations would be greater.

At pH 6.7, the iron-binding activity was greater compared with the acidic buffers, because the proteins have more available binding sites, being further away from the isoelectric point (Castellani et al., 2004). At low ionic strength, iron binding increased in a curvilinear and likely sigmoidal fashion with temperature. We observed a significant difference in binding: for example, the binding increased from 8.87 ± 0.02 mg of Fe/g of protein at 20°C to 10.09 ± 0.11 mg of Fe/g of protein ($P < 0.01$) at 72°C. The higher ionic strength of 400 mM resulted in decreased iron binding: at 20°C, the binding was 4.05 ± 0.05 mg Fe/g protein, with a sharp increase in binding at 37°C of 7.44 ± 0.01 mg of Fe/g of protein and then a more gradual increase to 72°C. This finding may indicate that under pasteurization conditions,

the binding of iron will not be significantly affected by low ionic strength, because within the duration of the experiment (3 min) we observed no dissociation of iron at 72°C.

Over the range of parameters we studied (pH 2.75–6.7, ionic strength of the buffer 50 and 400 mM, temperature 4–72°C), we measured maximum iron binding at neutral pH in a buffer with low ionic strength. This observation was likely due to the lower competition of salt for binding sites in an unprotonated environment. Castellani et al. (2004) looked at similar conditions using phosvitin from egg yolks and found that the optimal conditions for iron binding were at pH 6.5 at a low ionic strength of 0.15 M. At this pH, Castellani et al. (2004) suggested that the phosphates are completely ionized and that little salt remains to compete with iron for binding sites. Wang et al. (2011) found optimal iron-binding conditions for yak casein digests of pH 6.0 at 40°C. Similarly, the temperature for binding appeared to be optimal at 37°C or above in our study. Iron will bind to peptides in solutions that are mildly acidic and ideally low in ionic strength at temperatures above 20°C.

Effect of Holding Time on Iron Binding. Holding stages regularly occur during manufacturing, so the point at which iron is added will determine what temperature/time profiles the iron-peptide mixture will be subjected to. It is therefore critical to understand the effect of process delays (holding time) on iron-peptide mixtures held at constant temperatures.

As shown in Figure 4a, the effect of temperature on the response of iron binding depends on the holding time ($P < 0.001$). At 20°C, the binding of iron to the protein increased by 48% as the holding time increased with an ionic strength of 50 mM; the binding after 3 min was 13.10 ± 0.09 mg of Fe/g of protein and increased to 19.46 ± 0.01 mg of Fe/g of protein ($P < 0.001$) after 25 min. Likewise, the binding at 37°C also increased significantly from 15.38 ± 0.32 mg of Fe/g of protein to 20.65 ± 1.33 mg of Fe/g of protein over the same time frame ($P < 0.05$). Within this period, ferrous iron in an imidazole buffer is relatively stable, and over 80% of the added iron remains in the ferrous form (Figure 4b). At 50°C and 72°C, the binding decreased with time, caused in part by the blank buffer; at these elevated temperatures, the rate of iron oxidation was greater. The confounding factor of the ferrous iron oxidizing must be taken into account. Based on the data we obtained, at 72°C the iron dissociated from the protein as time progressed; however, it is more likely that when free ferrous ions are in solution with no chelators, they will experience a faster rate of oxidation than when protein is present. Ruegg et al. (1977) found

Figure 3. Iron bound by goat phosphopeptides in buffers at pH 2.75 (□), 4.60 (○), and 6.70 (△) at ionic strength of 50 mM (open symbols) and 400 mM (solid symbols) after blank subtraction of iron in respective buffers. Error bars indicate SE.

Figure 4. (a) Effect of holding time of iron and goat phosphopeptides on iron chelation capacity, with blank subtraction with respect to time; (b) background curves of ferrous iron oxidation rate as a fraction of the initial ferrous iron concentration. Error bars indicate SE.

that iron dissociated from saturated lactoferrin upon fast-rate heating, using calorimetry with a monoferric species developing in simulated milk ultrafiltrate. However, lactoferrin was a structured protein, so it would likely behave differently than a peptide. Due to the uncertainty of the oxidation and binding kinetics of

iron, solutions should not be incubated at temperatures above 50°C because oxidation, or unbinding of ferric iron, could occur, or both.

Maximum Iron Binding of Phosphopeptides.

We investigated iron loading of the peptide to determine if a saturation point could be determined. This would allow us to quantify how much iron could be added to the peptide mixture before significant amounts remained unbound and susceptible to binding to other components. The interaction of temperature and iron loading was significant for iron binding ($P < 0.001$).

At 20°C, binding at the higher iron loadings of 100 to 265 μM was significantly lower ($P < 0.05$) than at 37°C and 50°C (Figure 5). At 20°C, iron binding increased from 10.36 ± 0.09 mg of Fe/g of protein at 25 μM to 42.24 ± 0.97 mg of Fe/g of protein at 212 μM iron. Similarly, at 37°C, the binding at the lowest iron loading was 10.36 ± 0.01 mg of Fe/g of protein, but in contrast to the findings at 20°C, binding at the maximum loading increased to 50.16 ± 0.63 mg of Fe/g of protein. At 50°C, iron binding was initially similar to that at both 20 and 37°C (9.94 ± 0.02 mg of Fe/g of protein), but at the high iron level of 212 μM , the binding increased further to 54.37 ± 0.50 mg of Fe/g of protein. Increasing the iron concentration to 265 μM resulted in a decrease in binding, indicating that the maximum iron binding was approximately 212 μM . Emery (1992)

Figure 5. Effect of higher iron loading on goat phosphopeptides at pH 6.70 and ionic strength of 50 mM. Error bars indicate SE.

performed similar work with casein and ferrous iron and found that the iron was rapidly oxidized to Fe^{3+} in the presence of casein using ferrozine; the reaction can serve as a thermodynamic trap where the oxidation of iron promotes further oxidation. The rate of oxidation was highly dependent on the concentration of casein, and in our study the different iron loadings may have affected iron-binding capacity.

Gaucheron et al. (1996) found that adding iron to caseins caused precipitation at 4 mM, and Raouche et al. (2009) found that milk could bind 20 mmol/kg of milk under chilled conditions. Hekmat and McMahon (1998) added 100 mg/L FeCl_3 to milk and found that 83% remained in the casein pellet within the pH range of 5.3 to 6.7 and Emery (1992) estimated that casein could bind 10 mg/g of casein. Mittal et al. (2015) found that more than 90% of added iron could be bound to a calcium-depleted milk solution at 11% (wt/wt), which roughly corresponds to 26 mg of Fe/g of protein at 5°C. These results show significant binding of iron using peptides compared with intact casein proteins.

The peptides in our study had significantly improved iron-binding capacity over milk and caseinate systems, but they did not match the findings of Bouhallab et al. (2002), who reported binding of 70 mg of iron/g of phosphopeptide. These authors used ferrous chloride with a molar ratio of 4 that was bound to $\beta\text{-CN}(1-25)$ at 37°C at pH 6.5. This finding indicates that using a single peptide enabled complex formations with higher binding capacities than an extracted peptide mix with no further purification. Chaud et al. (2002) bound ferric iron with a digest and found that the resultant precipitate contained 5.61% ferric chloride hexahydrate by weight, or 51.6 mg of Fe/g of protein, similar to the results obtained in our study.

Addition of Peptides to a Milk Model. We investigated the ability of the phosphopeptides to bind iron in a milk system to determine their effectiveness at delivering iron in a model commercial-style application. We postulated that adding the iron to a peptide solution first would, in the absence of competition from intact casein, increase the probability for iron-peptide interactions and result in more effective iron binding in the final milk system.

The fraction of peptide addition and the point at which the iron was added during manufacture significantly affected iron binding ($P < 0.001$) in the model iron-fortified milk formulation. The iron binding of goat milk without peptide addition was 3.83 ± 0.01 mg of Fe/g of protein. At all levels of peptide addition, we observed an improvement in the amount of iron bound when the iron was added to the peptide solution first, rather than adding it directly to a peptide and milk mixture (Figure 6). This finding supports the

Figure 6. Iron binding by milk and peptide mixtures. Squares indicate where iron was added to a 0.2 mg/mL solution of milk protein and peptides containing 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mass fraction of peptide; circles indicate where the iron and peptide solution were first mixed and added to a milk solution with a final peptide mass fraction as before. Error bars indicate SE.

hypothesis that iron binding can be improved by adding it to a peptide stream before adding it to milk to ensure binding between these 2 ingredients and to limit interactions with other components. The percentage improvement in iron binding in the final milk solution varied with peptide concentration: at 0.1 g of peptide/g of total protein, improvement was 6.9% when the iron and peptide were added first and reached a maximum at 0.2 g peptide/g total protein at 10.3% improvement; at 0.5 g peptide/g total protein, we observed a 1.5% difference ($P > 0.05$). Binding of iron added to the pre-mixed milk and peptide solution at a 50% peptide addition was 11.11 ± 0.04 mg of Fe/g of protein, slightly less than when the peptide and iron were mixed first (11.27 ± 0.02 mg of Fe/g of protein). This difference was statistically significant ($P < 0.05$), but we do not believe it is nutritionally significant.

It is likely that as the peptide concentration increases, the relative importance of creating an iron-peptide solution first decreases, because the chance of an iron-peptide interaction when iron is added directly to the milk peptide solution would be higher.

A significant amount of peptide must be added into the solution before the iron binding is significantly higher than in normal milk; the food would have to

contain about 10% as peptides of the total protein to bind 5 mg of iron/g of protein. Statistically, the order of addition appeared to affect iron binding, but realistically these conditions would produce little difference; if a 20% mass fraction of phosphopeptide was added, a 10% increase in binding may improve the stability of the product. However it is likely that this level of addition is not commercially economical. In the context of a nutritional drink, a 250-mL serving containing 3.5% protein and a peptide fraction of 0.1 could deliver 5.25 mg of peptide-bound iron, equating to 30% recommended daily intake for a female New Zealander aged 19 to 50 yr. However, the absolute amount of iron binding is only one of the factors that would influence the commercial success of a peptide-based iron-binding product. Also of importance is the reactivity of the bound iron: iron-fortified nutritional formulations typically contain fats that are prone to oxidizing, and the presence of free iron leads to increased oxidation rates. The effect of peptide addition on the iron-mediated oxidation of fat will be the subject of future investigation.

CONCLUSIONS

A fundamental understanding of how an iron-phosphopeptide compound would behave in an industrial setting under different processing conditions is important for determining where it could be added to the bulk mix. Isolation of the peptides improved iron binding compared with milk at the same concentration, to approximately 3.8 mg of Fe/g of protein. This study showed that the iron-phosphopeptide complex has potential for binding considerable amounts of iron, but it can be sensitive to temperature and holding times. We found that optimal maximum binding temperature was approximately 50°C; the highest iron loading tested at 125 mg of Fe/g of protein bound 54 mg of Fe/g of protein. Elevated temperatures may negatively affect the rate of iron oxidation, and a lower temperature may reduce the potential oxidation of unbound iron. At temperatures below 37°C, the iron and peptide compound can be held for up to 25 min without significant detrimental effects. The order of iron and peptide addition to a milk system had no significance in terms of industrial relevance, but even the addition of peptide at a fraction of 0.1 in a milk system improved iron binding by 27 to 33%.

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