Iron (II)-chelating activity of buffalo α_S -casein hydrolysed by corolase PP, alcalase and flavourzyme

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Abstract Iron is a vital substance for human health which participates in many biochemical reactions. It also act as initiator for many harmful oxidative process. Buffalo α_S -casein enriched fraction (80 %) was hydrolysed independently by corolase PP (H1), alcalase (H2), flavourzyme (H3) and sequentially by alcalase-flavourzyme (H4). After ultrafiltration (10 and 3 kDa) hydrolysates were analysed for their iron chelation activity using ferrozine. For H1 group of hydrolysates highest iron (II)-chelation activity ($265.58 \mu\text{M Fe}^{2+}/\text{mg protein}$) was found after 8 h of hydrolysis for H2 ($267.56 \mu\text{M Fe}^{2+}/\text{mg protein}$) and H3 group of hydrolysates ($380.68 \mu\text{M Fe}^{2+}/\text{mg protein}$) after 6 h of hydrolysis. Sequential hydrolysis was not effective for iron (II)-chelation activity. 3 kDa fractions show higher iron (II)-chelation activity than 10 kDa fraction. Flavourzyme was more effective for generation of iron (II)-chelating peptides from buffalo α_S -casein.

Keywords Iron (II)-chelating · Enzymatic hydrolysis · α_S -casein · Ferrozine · Ultrafiltration

Introduction

Iron is a valuable element for human health by involving in many biochemical reactions like binding and transport of oxygen in haemoglobin, many electron transport reactions, cell growth and differentiation. Iron is a vital substrate for haemoglobin production and sufficient iron stores are necessary to achieve and maintain adequate levels of haemoglobin (Tay et al. 2011). Deficiencies of dietary minerals can lead to numerous diseases affecting many bodily organs (Shenkin

2008; Guo et al. 2014). Deficiency of dietary iron lead to several diseases such as microcytic hypochromic anaemia, glossitis, koilonychia, angular stomatitis, cognitive impairment in children, impaired physical activity and endurance in adults (Torres-Fuentes et al. 2012). Many other physiological expressions including pregnancy complications, increased absorption of lead and cadmium, impaired mental and immune system (Beard 2001). A variety of factors can cause mineral deficiencies. For instance, many staple foods in the diet, such as cereals, corn, rice and legumes, often contain phytate (Miquel and Farré 2007).

Iron also act as pro-oxidant in lipid oxidation by generating reactive oxygen species (ROS) which includes superoxide anion radicals, hydroxyl radicals and non-free radical species such as hydrogen peroxide (Rival et al. 2001). ROS adversely affect cellular components in biological system and flavour, nutritive value and shelf life of food products (Chung et al. 2002). Iron (II)-chelating power of casein hydrolysates also measures its free radical scavenging activity (Li and Zhao 2011). Li et al. (2013) also found that stronger the metal chelating ability the stronger the antioxidant activity.

Metal salts and multi-mineral supplements adverse effects the physical and sensory properties of foods (Hurrell 2002). Reduced bioavailability of multi-mineral supplementation is also a barrier to overcome the issue of mineral deficiencies (Poitou Bernert et al. 2007). Therefore, inclusion of metal element-rich food in their daily diets will be a better choice (Keller et al. 2002). From the general health considerations and dietary preferences, food-derived nutritional supplements are more acceptable. Dietary components such as protein, peptides and amino acids can form soluble complexes with iron and keep away from ROS generation (Storcksdieck et al. 2007). Metal chelating peptides have been identified as potential functional ingredients to improve bivalent mineral bioavailability. Mineral chelating peptides derived from a variety of food protein sources such as sesame (Wang et al. 2012),

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chickpea (Megias et al. 2007; Torres-Fuentes et al. 2011, 2012), ovine milk caseinate (Corrêa et al. 2011), barley glutelin (Xia et al. 2012), soybean (Fidler et al. 2003), meat muscle tissue (Storcksdieck et al. 2007) and porcine blood plasma (Lee and Song 2009) proteins have been reported.

From milk proteins many peptides (caseinophosphopeptides-CPPs) with mineral (Ca^{2+} , Fe^{2+} , Zn^{2+}) chelation properties have been reported which also act as antioxidant due to metal chelation. Casein and its derived peptides are most studied and have most potent mineral binding properties in particular CPP's (Farooq et al. 2013). CPPs are suitable supplements for fortifying foods, since Fe bound to CPPs presents good bioavailability with increased solubility and the prevention of interactions with other minerals (Lopez and Martos (2004). Bouhallab and Bouglé (2004) reported that purified phosphopeptide β -CN (f 1–25) have a positive effect on iron bioavailability in vivo.

Both low (<0.5 kDa) and high molecular weight (10–30 kDa) peptides shows iron chelating activity (Lv et al. 2009; Torres-Fuentes et al. 2012; Guo et al. 2013). Cys, Met, His, Gln, Glu, Lys, Arg and Ser within the sequences of peptides are believed to contributing the metal chelating activity (Torres-Fuentes et al. 2011; Wu et al. 2012; Xia et al. 2012; De la Hoz et al. 2014). Sulph-hydryl (Cys) groups, carboxylate groups (Asp, Glu) and nitrogen-rich imidazole group of the His act as principal site for iron binding (Zachariou and Hearn 1996; Seth and Mahoney 2001; Huang et al. 2011).

Many mineral binding peptides have been reported from bovine α_S -casein (Kitts 2005; Cross et al. 2007). α_S -Casein fraction is composed of sub fraction α_{S1} - and α_{S2} -casein. There are amino acid variations in the primary structure of bovine and buffalo α_S -casein. Buffalo α_{S1} -casein fraction is a 199 residue single polypeptide chain having high homology to bovine milk α_{S1} -casein (Variant-B) (D'Ambrosio et al. 2008). Only 9 substitutions i.e., Gln⁴-His, Gly¹⁴-Glu, Thr⁴²-Lys, Ile⁷⁴-Asn, Leu¹¹⁵-Ser, Gln¹¹⁹-Arg, Gln¹⁴⁸-Glu, Pro¹⁷⁴-Thr and Gly¹⁹²-Glu have been reported between buffalo and bovine α_{S1} -casein, which corresponds to 97.20 % homology (El-Salam and El-Shibiny 2011; D'Ambrosio et al. 2008). Phosphorylation of buffalo α_{S1} -casein occurs at similar sites as in bovine α_{S1} -casein, except that at Ser¹¹⁵. The absence of phosphorylation at Ser¹¹⁵ strengthens the hydrophobic nature of buffalo α_{S1} -casein (Ferranti et al. 1998) and explains the higher sensitivity of buffalo α_S -casein towards Ca^{++} in comparison to that of bovine milk. Similarly buffalo α_{S2} -casein is also a single polypeptide chain of 207 amino acid residues and has very high homology (97.9 %) compared with bovine α_{S2} -casein (Sukla et al. 2007; D'Ambrosio et al. 2008). Only 10 substitutions at His²-Asn, His²⁹-Asn, Ile⁴⁴-Val, Ile¹⁴⁷-Phe, Asp¹⁵⁷-Glu, His¹⁷⁰-Arg, Thr¹⁷⁵-Ala, Try¹⁷⁶-Leu, Tyr¹⁸²-His

and Asn¹⁹⁹-Lys have been reported between buffalo and bovine α_{S2} -casein (El-Salam and El-Shibiny 2011; D'Ambrosio et al. 2008). But there is no difference in phosphorylation between buffalo and bovine α_{S2} -casein. These variations in the primary structure of bovine and buffalo α_S -casein, together with phosphorylation level (Leu¹¹⁵-Ser), might be responsible for difference in metal chelating (Fe^{2+}) behavior between buffalo and bovine α_S -casein. Sakamoto et al. (2012) reported higher bioavailability of iron in ferrous form (Fe^{2+}) than that in ferric form. Therefore, the present study focused on determining iron (II)-chelating properties of buffalo α_S -casein enriched fraction hydrolysates generated by different commercially available proteolytic enzymes.

Materials and methods

Chemicals

The commercial enzyme, Corolase PP (catalog number P1750) was gifted from AB Enzymes (ABF ingredients company, Germany), Alcalase 2.4 L FG and flavourzyme 1,000 L were gifted from Novozyme, Bangalore. O-phthaldialdehyde (OPA), β -mecaptoethanol, L-serine and ferrous chloride were purchased from HiMedia (Mumbai, India). Sodium dodecyl sulfate (L5750), Folin and Ciocalteu's phenol reagent (47641), bovine serum albumin (A-2153) and 3-(2-Pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-p, p'-disulfonic acid monosodium salt hydrate (Ferrozine) (160601) was provided by Sigma (St. Louis, MO, USA). Unless noted otherwise, other chemicals were of analytical grade and purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Highly purified water prepared with arium® pro VF (Sartorius Weighing Technology GmbH, Germany) was used to prepare all buffers and solutions.

Preparation of α_S -Casein

Fresh whole pooled buffalo milk collected from Cattle Yard, National Dairy Research Institute, Karnal, Haryana was defatted in a refrigerated centrifuge (Sorvall, Model- RC 6 Plus, Thermo scientific) at 5,000 $\times g$ for 30 min at 4 °C. After centrifugation defatted milk was diluted in 1:1 ratio with distilled water. Whole casein was obtained from this milk by acid precipitation method at pH 4.6 as described by Wangoh et al. (1998). The casein precipitates were washed several times with distilled water and re-precipitated to remove whey trapped in the curd mass. From this buffalo milk whole casein, α_S -casein was fractionated on the basis of differential solubility at 3.3 M urea concentration, pH 4.6 by the method of Zittle

and Custer (1963) as described by Burr (2001). Urea was removed from α_S -casein by adjusting pH 7.5 with 2 M NaOH and extensive dialysis against several change of distilled water. After dialysis sample was freeze dried. Protein in the dried sample was estimated by Kjeldahl method. The purity of the α_S -casein enriched preparation was 80 % as determined following UREA-PAGE electrophoresis method (Andrews 1983) and analysed by 1D-gel analysis software (Nonlinear Dynamics Ltd.).

Preparation of hydrolysates

To prepare hydrolysates, α_S -casein was dissolved at 0.5 % w/v in 0.01 M sodium phosphate buffer (pH 7.5) and hydrolysed by commercial protease of animal/microbial origin in alone and in sequentially at their optimum activity conditions. The enzyme was dissolved/diluted in 0.01 M sodium phosphate buffer (pH 7.5). Hydrolysis was performed with gentle agitation, constant temperature (55 °C) without pH adjustment for 2–12 h. Hydrolysates were collected at a regular interval of 2 h from each hydrolysis followed by inactivation of enzyme. For single use of enzymes corolase PP, alcalase were used at 1 % level and flavourzyme at 2.5 % level. Whereas for sequential hydrolysis with α_S -casein was initially hydrolysed with alcalase for 2 h at enzyme level of 7.25 units/g of casein followed by 4 h hydrolysis with flavourzyme at enzyme level of 0.97 units/g of casein after inactivation of alcalase. The enzyme was inactivated by keeping at 95 °C, for 10 min in water bath (Tauzin et al. 2003). After cooling hydrolysate was centrifuged at 10,000 $\times$ g at 4 °C for 20 min. The supernatants were passed through 0.45 μ filter, lyophilized (Model- Ecospin 3180C, Hanil Science International, Korea), and stored at –20 °C until used. The proteolytic activity was measured by using casein as substrate. For corolase PP, alcalase and flavourzyme at corresponding level proteolytic activity was 11.55, 7.25 and 4.57 units/g of substrate, respectively at 1 % enzyme level.

Determination of protein content

Protein content in each hydrolysate was determined using the Folin phenol reagent method of Lowry et al. (1951) with slight modifications using bovine serum albumin (BSA) as standard. Briefly, clear hydrolysate sample (protein concentration ≤ 500 μ g/mL) and complex forming reagent (98 part 2 % Sodium carbonate: 1 part 1 % CuSO_4 : 1 part 2 % Sodium potassium tartrate) was mixed in 1:10 ratio with continuous mixing. After 10 min incubation at 25 °C, 0.1 N Folin's reagent equal to sample volume was further added with continuous mixing and incubated for

next 30 min. The change in colour was measured spectrophotometrically at 750 nm (protein concentration ≤ 500 μ g) using a microplate reader (Model: Infinite F-200 Pro, Tecan, Austria). The results were expressed as mg protein or peptide mL^{-1} of hydrolysate.

Degree of hydrolysis (DH)

The DH of hydrolysates was determined by o-phthaldialdehyde (OPA) method reported by (Nielsen et al. 2001), with some modifications. The OPA reagent was prepared by combining the following reagents to a final volume of 100 mL with water: 50 mL 0.1 M sodium tetraborate buffer solution (pH 9.5), 5 mL 20 % SDS, 80 mg OPA (in 2 mL 95 % ethanol) and 200 μ L of 2- β -mercaptoethanol. The reagent was prepared daily, protected from light and kept for 1 h with continuous stirring before use. The assay was carried out by mixing 0.4 mL of a 0.45 μ filtered sample (or control) to 3.0 mL OPA reagent. After vortexing for 5 s and incubation at 25 °C for exactly 2 min, the absorbance of the mixture was measured in an UV spectrophotometer (Model: UV-2700, Shimadzu, Tokyo, Japan) at 340 nm. L-serine (0.9516 meq/L) was used as positive control. The DH in enzymatic hydrolysates was then calculated by the following equation:

$$\text{DH}(\%) = h/h_{\text{tot}} \times 100$$

Where, $h = (\text{serine-NH}_2 - b) / \text{ameqv/g/protein}$

$$\text{arraySerine-NH}_2 = (A_{340\text{sample}} - A_{340\text{Blank}}) / (A_{340\text{standard}} - A_{340\text{Blank}}) \times 0.915 \text{ meqv.l}^{-1} \times S \times D \times (P/V),$$

Where, Serine-NH₂ is meqv serine NH₂ g^{–1} protein; S is sample volume in liter; D is dilution volume; P is protein content in the volume of the sample; V is sample volume in assay; α , β and h_{tot} constants for casein are 1.039, 0.383 and 8.2, respectively.

Determination of iron (II)-chelating activity

Iron (II)-chelating activity of the samples was estimated by the ferrozine method measuring the formation of the Fe²⁺-ferrozine complex (Carter 1971), with modifications. Briefly the assay was carried by mixing 100 μ g peptide in 250 μ L distilled water and 30 μ L of FeCl₂ (0.01 %, w/v or 49.5 μ M). Ferrozine (25 μ L, 20 mM) was added after incubation for 30 s at room temperature. After adding 0.1 ml of 5 mM ferrozine,

vortexing, and incubation at room temperature (25 °C) for 10 min, the absorbance was measured at 562 nm using plate reader (Model: Infinite® M200 Pro, Tecan Austria GmbH, Austria). The control was determined similarly by replacing the protein sample with 250 µL distilled water, EDTA was used as positive control. The chelating activity was calculated as follows:

$$\text{Iron (II)-chelation} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times \text{Iron (II) conc. } (\mu\text{M}) \times \text{Iron (II)-chelation}$$

Where A_{sample} and A_{control} are the absorbance of test sample and control, respectively. Iron (II)-chelating activity was expressed as µM iron/mg.mL⁻¹ protein.

Statistical analysis

All data were expressed as mean ± standard deviation from at least three independent experiments. Differences between the mean values of multiple groups were analysed by two-way analysis of variance (anova) with duncan's new multiple range test at the 95 % confidence interval using SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA).

Results and discussion

Enzymatic hydrolysis of buffalo α_S-casein

To prepare casein hydrolysate with better iron (II)-chelation activity buffalo α_S-casein enriched fraction (80 %) was hydrolysed independently by corolase PP, alcalase, flavourzyme and sequentially with alcalase followed by flavourzyme. Four group of hydrolysates, H1 group by proteolytic action of corolase PP, H2 group by alcalase, H3 group by flavourzyme and H4 group by alcalase-flavourzyme were generated. The extent of protein breakdown in these hydrolysates were monitored by measuring the degree of hydrolysis (DH) by OPA assay (Church et al. 1985) with modification described by Nielsen et al. (2001). This assay is based on increase in absorbance at 340 nm due to reduction of α-amino groups of peptides in an alkaline environment. As shown in Fig. 1 DH for the hydrolysate (H1) generated by corolase PP action ranges from 16.57 to 19.43 %, by alcalase (H2) 12.37–7.25 % and by flavourzyme (H3) 14.91–18.58 % on independent use of enzyme. Relatively lower hydrolysis achieved by alcalase was due to endoproteinase activity which preferentially cleaves peptide bond when P1 is occupied by mainly hydrophobic amino acid (Doucet et al. 2003). Similarly the

DH achieved by corolase PP was higher due to very broad specificity as corolase PP is a technical preparation having mixture of trypsin, chymotrypsin, elastase, carboxypeptidase (van der Ven et al. 2002). For hydrolysates (H4) generated by sequential hydrolysis with alcalase followed by flavourzyme about 2.75 fold increase (33.31 %) in DH was found than by the single use of alcalase. For all hydrolysates there was sharp increase in DH during initial 2 h of hydrolysis. After 8 h of hydrolysis increase in DH was very slow but the increase was significant ($p < 0.05$). Among the three enzymes on independent use corolase PP was more effective than flavourzyme followed by alcalase to achieve higher DH. It was also noticed that sequential use of enzyme was more effective than individual use. Pedroche et al. (2002) also reported higher DH by sequential hydrolysis with alcalase and flavourzyme. During initial hydrolysis by alcalase, the number of terminal sites available increased, which assists the further hydrolysis with flavourzyme (Zhang et al. 2012).

Iron (II)-chelating activity

Stronger chelating ability of food proteins to some macro- and micro-elements, such as Ca²⁺ and Fe²⁺, might improve bio-availability of these elements to body, or have the opportunity to provide body with sufficient minerals, as the ligands that from soluble chelates with metals may enhance absorption from some foods (Kumagai et al. 2004). Along with nutritional role iron (II) also act as initiator for Lox (soybean lipoxygenase) and metal catalysed lipid oxidation (Rival et al. 2001). Metal chelating ability may be involved in

Fig. 1 Degree of hydrolysis achieved by independent use of corolase PP, alcalase and flavourzyme at 1 % enzyme level for 2–12 h of hydrolysis. Different superscripts on each group of hydrolysates (H1-corolase PP, H2-alcalase, and H3-flavourzyme) represents significant difference in DH ($p < 0.05$) over different time of hydrolysis. Error bars are standard deviation from three replicates

antioxidant activity and it affects other functions that contribute to the antioxidant activity. The stronger the metal chelating ability stronger, the stronger the antioxidant activity (Li et al. 2013). Hence iron (II)-chelation activity of unhydrolyzed buffalo whole casein, their individual fractions and ultrafiltrate fractions (10 and 3 kDa) of α_S -casein hydrolysates was evaluated. Iron (II)-chelating activity was determined by measuring the formation of the Fe^{2+} – ferrozine complex (Carter 1971) as adapted by Torres-Fuentes et al. (2012) with slight modifications. The reaction of ferrozine/ Fe^{2+} complex resulting in reduction of violet colour, was used as an indicator to evaluate the iron (II)-chelation activity.

Results as presented in Table 1 shows that for unhydrolyzed caseins maximum iron (II)-chelating activity was observed in α_S -sodium caseinate ($454.91 \mu\text{M Fe}^{2+}/\text{mg}$) followed by β - ($385.54 \mu\text{M Fe}^{2+}/\text{mg}$), whole sodium caseinate ($304.45 \mu\text{M Fe}^{2+}/\text{mg}$) and κ -sodium caseinate ($278.67 \mu\text{M Fe}^{2+}/\text{mg}$). The difference in iron (II)-chelation activity might be due different degree of phosphorylation of casein fractions, with maximum in α_S -casein fraction, composed of α_{S1} - and α_{S2} - casein fractions. High amount of polar amino acids (Gln, Gly, Thr and His) in buffalo α_S -casein may be reason for higher chelation activity. Berner and Miller (1985) and Van Campen (1973) also reported that these are polar amino acids that may also be implicated in the high iron (II)-binding capacity. Glahn and Van Campen (1997) recognised cysteine as an important amino acid in the generation of iron chelates.

Hydrolysates obtained from buffalo α_S -casein enriched fraction hydrolysis with different enzyme were ultra-filtered through 10 and 3 kDa MWCO membrane (Sartorius Stedim Biotech GmbH, Germany). These fractions were freeze dried and stored at -20°C . Prior to iron (II)-chelation activity analysis hydrolysate was reconstituted in minimum amount of HPLC grade water.

Effect of enzyme

Iron (II)-chelation activity for each group of hydrolysates is shown in Fig. 2. It was observed that there was decrease in iron (II)-chelation activity of α_S -casein due to enzymatic

Table 1 Iron (II)-chelating activity (μM chelation per mg/mL protein) of buffalo casein fractions

Buffalo casein fractions	Iron (II)-chelation
Whole casein	304.46 ± 0.71^a
α_S -casein	454.91 ± 1.07^b
β -casein	385.54 ± 1.05^c
κ -casein	278.67 ± 0.45^d

Mean with different superscripts in each column (a, b, c, d) were significantly different ($p < 0.05$) from each other. Data are presented as means $\pm$ SD ($n=3$)

Fig. 2 Iron (II)-chelation activity of buffalo Na- α_S -caseinate enriched fraction before and after hydrolysis in **a** 10 kDa permeate, **b** 3 kDa permeate by independent use of corolase PP, alcalase and flavourzyme at 1 % enzyme level for 2–12 h of hydrolysis. Different superscripts on each group of hydrolysates (H1-corolase PP, H2-alcalase, and H3-flavourzyme) represents significant difference in activity ($p < 0.05$) over different time of hydrolysis. Error bars are standard deviation from three replicates

hydrolysis. This reduction in chelating activity was speculated to be caused by decreased peptides length. For H1 group of hydrolysates (corolase PP) iron (II)-chelation activity ranges over $134\text{--}265 \mu\text{M iron}/\text{mg protein}$ with significant increase in activity upto 8 h of hydrolysis afterward activity decreased significantly with the increase in time of hydrolysis ($p < 0.05$). For H2 group of hydrolysates (alcalase) highest iron (II)-chelation activity was found in 6 h hydrolysate with the activity ranging between 99 and $267 \mu\text{M iron}/\text{mg protein}$. Increase in iron (II)-chelation activity was significant upto 6 h of hydrolysis later on decreased significantly ($p < 0.05$). Similar trend was also observed for H3 hydrolysates with higher iron (II)-chelation activity ranges over $268\text{--}380 \mu\text{M iron}/\text{mg protein}$. Maximum iron (II)-chelating activity observed in 6 h hydrolysates. There was significant difference

($p < 0.05$) in iron (II)-chelation activity over the time period of 2–12 h. While in H4 hydrolysates iron (II)-chelation activity was relatively lower (187.45 μM iron/ mg protein) than that of other groups of hydrolysates. This decrease in iron (II)-chelation activity in H4 group of hydrolysate was due excessive breakdown resulting in loss of phosphorylation sites. Similar trend of decrease in iron (II)-chelation activity at with increase in hydrolysis time was reported by Luo et al. (2014) for casein hydrolysates prepared by pancreatin and trypsin. Similarly Wang et al. (2011) reported that, if the hydrolysis time is too long, the ferrous-binding capacity of yak casein hydrolysate decrease with the increase of the DH. Zambrowicz et al. (2012) also reported increase in iron (II)-chelating capacity upto 4 h of hydrolysis chymotryptic and tryptic hydrolysates of egg yolk protein. By comparison of iron (II)-chelation activity between the hydrolysates group it was observed that hydrolysates obtained from flavourzyme have highest iron chelation activity (380.68 μM iron/ mg protein) followed by alcalase (267.56 μM iron/ mg protein) and corolase PP (265.87 μM iron/ mg protein). In some reports it is reported that hydrolysates have better iron (II)-chelation capacity than the unhydrolyzed protein. Kim et al. (2007a, b) reported better iron (II)-binding properties from alcalase hydrolysed whey protein concentrate than the unhydrolysed whey protein concentrate. They also evaluated the influence of various enzymes on the hydrolysis of whey protein concentrate to quantify the peptides having iron (II)-binding ability and found the highest iron solubility was noticed when the hydrolysates were derived with alcalase (up to 95 %). Li and Zhao (2011) also reported better iron (II)-chelating activity from casein hydrolysates than original casein.

Effect of membrane fractionation

Iron (II)-chelation activity of 10 and 3 kDa membrane passed permeate is shown in Fig. 2. It was observed that activity of 3 kDa permeate fractions were relatively higher than corresponding 10 kDa permeate fractions for all groups of hydrolysates. For H1 group of hydrolysates increase in iron (II)-chelation activity of 3 kDa fractions ranges between 1.1 and 1.9 times (average 1.4 fold) than 10 kDa fractions. In H2 group of hydrolysate 3 kDa fractions have 1.2–1.9 fold (average 1.6 fold) higher iron (II)-chelation activity than corresponding 10 kDa fractions. This increase in iron (II)-chelation activity of 3 kDa fractions ranges over 1.0–1.3 for H3 group of hydrolysates. Similarly for H4 group of hydrolysate iron (II)-chelation activity of 3 kDa permeate fractions were 1.2 fold higher than 10 kDa permeate fractions. From above effect it was noticed that increase in iron (II)-chelation activity in 3 kDa fractions were higher by proteolytic action of alcalase followed by corolase PP, alcalase-flavourzyme and flavourzyme. Torres-Fuentes et al. (2012) reported that in

general, smaller peptides, below 500 Da from chickpea hydrolysates show higher iron (II)-chelating activities than above 500 Da.

Conclusions

Iron (II)-chelation activity of unhydrolysed α_s -casein was significantly higher than all ultrafiltrate fractions obtained from different group of hydrolysates (H1, H2, H3 and H4) from α_s -casein. For all hydrolysates significant increase in iron (II)-chelation activity was noticed upto 6–8 h afterwards activity decreases. Hydrolysates obtained from proteolytic action of flavourzyme shows higher iron (II)-chelation activity than from alcalase and corolase PP. Iron (II)-chelation activity of 3 kDa peptides were higher than corresponding 10 kDa peptides with the highest increase by proteolytic action of alcalase. Although sequential use of alcalase with flavourzyme was effective for achieving higher DH in less time but not effective for production of iron (II)-chelating peptides. Flavourzyme could be a used for the generation of iron (II)-chelating peptides from buffalo α_s -casein fraction.

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References

- Andrews AT (1983) Proteinases in normal bovine milk and their action on caseins. *J Dairy Res* 50(01):45–55
- Beard JL (2001) Iron biology in immune function, muscle metabolism and neuronal functioning. *J Nutr* 131(2):568S–580S
- Berner L, Miller D (1985) Effects of dietary proteins on iron bioavailability-a review. *Food Chem* 18(1):47–69
- Bouhallab S, Bouglé D (2004) Biopeptides of milk: caseinophosphopeptides and mineral bioavailability. *Reprod Nutr Dev* 44(5):493–498
- Burr R (2001) Protein purification from milk. In: Roe S (ed) *Protein purification applications: a practical approach*, 2nd edn. Oxford University Press, UK, pp 87–115
- Carter P (1971) Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). *Anal Biochem* 40(2):450–458
- Chung YC, Chang CT, Chao WW, Lin CF, Chou ST (2002) Antioxidative activity and safety of the 50 ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. *J Agric Food Chem* 50(8):2454–2458
- Church FC, Porter DH, Catignani GL, Swaisgood HE (1985) An o-phthalaldehyde spectrophotometric assay for proteinases. *Anal Biochem* 146(2):343–348
- Corrêa APF et al (2011) Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. *J Sci Food Agric* 91(12):2247–2254

- Cross K, Huq N, Reynolds E (2007) Casein phosphopeptides in oral health-chemistry and clinical applications. *Curr Pharm Des* 13(8): 793–800
- D'Ambrosio C et al (2008) A proteomic characterization of water buffalo milk fractions describing PTM of major species and the identification of minor components involved in nutrient delivery and defense against pathogens. *Proteomics* 8(17):3657–3666
- De la Hoz L et al (2014) Iron-binding properties of sugar cane yeast peptides. *Food Chem* 142:166–169
- Doucet D, Otter DE, Gauthier SF, Foegeding EA (2003) Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. *J Agric Food Chem* 51(21):6300–6308
- El-Salam MHA, El-Shibiny S (2011) A comprehensive review on the composition and properties of buffalo milk. *Dairy Sci Technol* 91(6):663–699
- Farooq I, Moheet IA, Imran Z, Farooq U (2013) A review of novel dental caries preventive material: casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) complex. *King Saud Univ J Dental Sci* 4(2):47–51
- Ferranti P et al (1998) The primary structure of water buffalo α_{S1} - and β -casein: Identification of phosphorylation sites and characterization of a novel β -casein variant. *J Protein Chem* 17(8):835–844
- Fidler MC, Davidsson L, Walczyk T, Hurrell RF (2003) Iron absorption from fish sauce and soy sauce fortified with sodium iron EDTA. *Am J Clin Nutr* 78(2):274–278
- Glahn RP, Van Campen DR (1997) Iron uptake is enhanced in Caco-2 cell monolayers by cysteine and reduced cysteinyl glycine. *J Nutr* 127(4):642–647
- Guo L et al (2013) Preparation, isolation and identification of iron-chelating peptides derived from Alaska pollock skin. *Process Biochem* 48(5):988–993
- Guo L et al (2014) Food protein-derived chelating peptides: biofunctional ingredients for dietary mineral bioavailability enhancement. *Trends Food Sci Technol* 37(2):92–105
- Huang G, Ren Z, Jiang J (2011) Separation of iron-binding peptides from shrimp processing by-products hydrolysates. *Food Bioprocess Technol* 4(8):1527–1532
- Hurrell R (2002) How to ensure adequate iron absorption from iron-fortified food. *Nutr Rev* 60(s7):S7–S15
- Keller JL, Lanou AJ, Barnard ND (2002) The consumer cost of calcium from food and supplements. *J Am Diet Assoc* 102(11):1669–1671
- Kim SB, Seo IS, Khan MA et al (2007a) Enzymatic hydrolysis of heated whey: iron-binding ability of peptides and antigenic protein fractions. *J Dairy Sci* 90(9):4033–4042
- Kim SB, Seo IS, Khan MA, Ki KS, Nam MS, Kim HS (2007b) Separation of iron-binding protein from whey through enzymatic hydrolysis. *Int Dairy J* 17(6):625–631
- Kitts D (2005) Antioxidant properties of casein-phosphopeptides. *Trends Food Sci Technol* 16(12):549–554
- Kumagai H, Koizumi A, Suda A, Sato N, Sakurai H, Kumagai H (2004) Enhanced calcium absorption in the small intestine by a phytate-removed deamidated soybean globulin preparation. *Biosci Biotechnol Biochem* 68(7):1598–1600
- Lee SH, Song KB (2009) Purification of an iron-binding nona-peptide from hydrolysates of porcine blood plasma protein. *Process Biochem* 44(3):378–381
- Li D, Zhao XH (2011) Glutaminase-induced deamidation and hydrolysis of casein and metal-chelating or ACE-inhibitory activity of the hydrolysates in vitro. *Int J Food Sci Technol* 46(2): 324–332
- Li Z et al (2013) Purification and identification of five novel antioxidant peptides from goat milk casein hydrolysates. *J Dairy Sci* 96(7): 4242–4251
- Lopez MAA, Martos FC (2004) Iron availability: an updated review. *Int J Food Sci Nutr* 55(8):597–606
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193(1):265–275
- Luo Y, Pan K, Zhong Q (2014) Physical, chemical and biochemical properties of casein hydrolyzed by three proteases: partial characterizations. *Food Chem* 155:146–154
- Lv Y et al (2009) Identification and characteristics of iron-chelating peptides from soybean protein hydrolysates using IMAC-Fe³⁺. *J Agric Food Chem* 57(11):4593–4597
- Megías C et al (2007) Affinity purification of copper chelating peptides from chickpea protein hydrolysates. *J Agric Food Chem* 55(10): 3949–3954
- Miquel E, Farré R (2007) Effects and future trends of casein phosphopeptides on zinc bioavailability. *Trends Food Sci Technol* 18(3):139–143
- Nielsen P, Petersen D, Dambmann C (2001) Improved method for determining food protein degree of hydrolysis. *J Food Sci* 66(5):642–646
- Pedroche J et al (2002) Utilisation of chickpea protein isolates for production of peptides with angiotensin I-converting enzyme (ACE) - inhibitory activity. *J Sci Food Agric* 82(9):960–965
- Poitou Bernert C et al (2007) Nutritional deficiency after gastric bypass: diagnosis, prevention and treatment. *Diabetes Metab* 33(1):13–24
- Rival SG, Boeriu CG, Wichers HJ (2001) Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *J Agric Food Chem* 49(1):295–302
- Sakamoto LM et al (2012) Iron peptide complex does not increase blood iron concentration at the same extent as ferrous sulfate after oral ingestion in healthy adult males. *J Nutrol* 4(6):87–92
- Seth A, Mahoney RR (2001) Iron chelation by digests of insoluble chicken muscle protein: the role of histidine residues. *J Sci Food Agric* 81(2):183–187
- Shenkin A (2008) Basics in clinical nutrition: physiological function and deficiency states of trace elements. *E Spen Eur E J Clin Nutr Metab* 3(6):255–258
- Storcksdieck S, Bonsmann G, Hurrell R (2007) Iron-binding properties, amino acid composition, and structure of muscle tissue peptides from in vitro digestion of different meat sources. *J Food Sci* 72(1): S019–S029
- Sukla S et al (2007) Molecular cloning and characterization of buffalo α_{S1} -casein gene. *Mitochondrial DNA* 18(5):334–340
- Tauzin J, Miclo L, Roth S, Mollé D, Gaillard JL (2003) Tryptic hydrolysis of bovine α_{S2} -casein: identification and release kinetics of peptides. *Int Dairy J* 13(1):15–27
- Tay EL et al (2011) Replacement therapy for iron deficiency improves exercise capacity and quality of life in patients with cyanotic congenital heart disease and/or the Eisenmenger syndrome. *Int J Cardiol* 51(3):307–312
- Torres-Fuentes C, Alaiz M, Vioque J (2011) Affinity purification and characterisation of chelating peptides from chickpea protein hydrolysates. *Food Chem* 129(2):485–490
- Torres-Fuentes C, Alaiz M, Vioque J (2012) Iron-chelating activity of chickpea protein hydrolysate peptides. *Food Chem* 134(3):1585–1588
- Van Campen D (1973) Enhancement of iron absorption from ligated segments of rat intestine by histidine, cysteine, and lysine: effects of removing ionizing groups and of stereoisomerism. *J Nutr* 103:139–142
- van der Ven C, Gruppen H, de Bont D, Voragen AG (2002) Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. *Int Dairy J* 12(10):813–820
- Wang X et al (2011) Preparation and characteristics of yak casein hydrolysate-iron complex. *Int J Food Sci Technol* 46(8): 1705–1710
- Wang C, Li B, Ao J (2012) Separation and identification of zinc-chelating peptides from sesame protein hydrolysate using IMAC-Zn²⁺ and LC–MS/MS. *Food Chem* 134(10):1231–1238

- Wangoh J, Farah Z, Puan Z (1998) Iso-electric focusing of camel milk proteins. *Int Dairy J* 8(7):617–621
- Wu H, Liu Z, Zhao Y, Zeng M (2012) Enzymatic preparation and characterization of iron-chelating peptides from anchovy (*Engraulis japonicus*) muscle protein. *Food Res Int* 48(2):435–441
- Xia Y, Bamdad F, Gänzle M, Chen L (2012) Fractionation and characterization of antioxidant peptides derived from barley glutelin by enzymatic hydrolysis. *Food Chem* 134(3):1509–1518
- Zachariou M, Hearn MT (1996) Application of immobilized metal ion chelate complexes as pseudocation exchange adsorbents for protein separation. *Biochemistry* 35(1):202–211
- Zambrowicz A, Pokora M, Eckert E, Szołtysik M, Dąbrowska A, Chrzanowska J, Trziszka T (2012) Antioxidant and antimicrobial activity of lecithin free egg yolk protein preparation hydrolysates obtained with digestive enzymes. *Funct Foods Health Dis* 2(12):487–500
- Zhang M, Mu TH, Wang YB, Sun MJ (2012) Evaluation of free radical-scavenging activities of sweet potato protein and its hydrolysates as affected by single and combination of enzyme systems. *Int J Food Sci Technol* 47(4):696–702
- Zittle C, Custer J (1963) Purification and Some of the Properties of α -casein and κ -casein. *J Dairy Sci* 46(11):1183–1188