Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics

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Minerals and trace elements in cow's milk occur as inorganic ions and salts or form complexes with proteins and peptides, carbohydrates, fats and small molecules. The main mineral binder or chelators of calcium are the caseins, α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein, but also whey proteins and lactoferrin bind specific minerals like calcium, magnesium, zinc, iron, sodium and potassium. Less documented is the binding of trace elements. Peptides obtained by *in vitro* or *in vivo* hydrolysis act as mineral trappers through specific and non-specific binding sites. They may then function as carriers, chelators, of various minerals and thus enhance or inhibit bioavailability. Peptides from milk proteins have found interesting new applications in the food industry as products with improved functionality or as ingredients of dietary products, or used in pharmaceutical industry. Fortification of foods with minerals in a low concentration has for a long time been used in some countries to overcome mineral deficiency, which is an increasing problem in humans. These types of foods are being used to create a new generation of super foods in the industry today.

Milk: Proteins and peptides: Mineral status

Introduction

Peptides are formed from milk proteins by various chemical or biological treatments. Enzymes (*in vitro*) are the most efficient and commonly used method to obtain controlled protein hydrolysis. Proteolytic enzyme extracts from microbial, vegetable or gastric gland juices vary in specificity and thus give peptides of different characteristics and properties. Peptides from both cow and goat milk proteins have been derived and characterised. The caseins are easily degradable proteins due to their random coil structure. The phosphoseryl-containing peptides from α_{s1}-casein, α_{s2}-casein and β-casein are of special interest. These peptides are reported to have different physiological functions in the human body: both positive and negative effects. However, because these peptides have a high content of negative charges they will efficiently bind divalent cations with the formation of soluble complexes. Complexes of peptides and minerals of Fe, Mg, Mn, Cu and Se are reported. The high content of negative charges makes these phosphopeptides resistant to further hydrolysis. However, peptides from the caseins have the disadvantage that they may give rise to a bitter taste. The whey proteins, β-lactoglobulin, α-lactalbumin and lactoferrin also give rise to peptides with mineral binding abilities. These proteins are regarded as more resistant to enzymatic attack and undergo hydrolysis much more slowly than the caseins. Since whey is a by-product from the cheese industry and may create an environmental waste problem, it is of fundamental importance to find new applications of these highly valuable nutritional proteins and peptides. However, very little information exists on the effect of processing on peptides generated from the milk proteins with regards to structure and conformation, functionality, digestibility and nutritional value.

Mineral status of milk

The minerals in bovine milk are present in a solution in which there exists an equilibrium between the free ions and complexes with various components such as proteins, carbohydrates and low-molecular-weight ligands like citrate and amino acids (Brulé & Fauquant, 1982; West, 1986; Baumy & Brulè, 1988; Loennerdal, 1989; Flynn, 1992). The mineral content of milk is not constant but varies according to several different factors such as stage of lactation, feed, genetic variance etc. The role of cow's milk in nutrition is often discussed. Based on the knowledge that nearly all of the twenty minerals that are considered as essential in the human diet, are present in higher amounts in cow's milk than in human milk (Flynn, 1992), a comparison between the status of cow's milk and human milk may give insight into the topic. The average mineral contents of human milk and cow's milk are given in Table 1.

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Table 1. Mean concentration of minerals and trace elements in human milk and cow's milk according to (Flynn, 1992)

Component	Mature human milk	Cow milk
Sodium (mg/l)	180	500
Potassium (mg/l)	525	1500
Chloride (mg/l)	420	950
Calcium (mg/l)	280	1200
Phosphorus (mg/l)	140	950
Magnesium (mg/l)	35	120
Iron (mg/l)	0·3	0·5
Zinc (mg/l)	1·2	3·5
Copper (mg/l)	0·25	0·09
Manganese (μg/l)	6	30
Iodine* (μg/l)	64–178	100–770
Fluoride (μg/l)	16	20
Selenium (μg/l)	16	10
Cobalt (μg/l)	0·1	0·5
Chromium (μg/l)	0·27	2
Molybdenum (μg/l)	2	50
Nickel (μg/l)	1·2	26
Arsenic (μg/l)	0·2–0·6	20–60
Silicon (μg/l)	700	3000
Boron (μg/l)	60–80	1000

* Minerals or elements whose concentration is strongly influenced by dietary intake.

Protein and peptide sources

A number of peptides from milk proteins with mineral binding abilities have been reported. The different bioactive peptides formed from *in vitro* proteolysis are hidden in an inactive state within the amino acid sequence of the proteins. The caseins, α_{s1}-, α_{s2}-, β- and κ-casein, and their genetic variants, are most often reported as precursors of peptides containing binding sites, phosphoseryl and carboxyl, for different minerals (Baumy & Brulè, 1988; Meisel & Schlimme, 1990; Bouhallab *et al.* 1991; Schlimme & Meisel, 1995; Peres *et al.* 1997). In addition, peptides from whey proteins, β-lactoglobulin, α-lactalbumin and lactoferrin, have been derived (Chung & Raymond, 1993; Kawakami *et al.* 1993; Nagasako *et al.* 1993; Feng *et al.* 1995; Svenning & Vegarud, 1998). Since they are not phosphorylated, the minerals seem to bind through other binding sites than in the caseins (Baumy & Brulè, 1988; Paulsson, 1990; Dufour *et al.* 1994; Feng *et al.* 1995; Cayot & Lorient, 1997). Some minerals cause a change in conformation, and thereby alter (inhibit/accelerate) the enzymatic attack on the protein and peptide. The physiological significance of the ion metal-binding phenomena is still not understood (Hirai *et al.* 1992; Chung & Raymond, 1993; Dufour *et al.* 1994). Among the whey proteins lactoferrin is regarded as the most important iron-binding protein (Kontoghiorghes, 1986; Nagasako *et al.* 1993; Feng *et al.* 1995). Conformational changes of lactoferrin have been shown to have a role in iron binding and release (Chung & Raymond, 1993). The role of peptides obtained from the other milk proteins, for example bovine serum albumin (BSA), immunoglobulins, lysozyme and lactoperoxidase are currently unknown. A summary of the milk proteins as precursors of peptides with mineral binding abilities is shown in Table 2.

Isolation of peptides

Different isolation methods have been described in the literature to obtain bioactive peptides (Fig. 1). *In vitro* proteolysis could use either non-specific or specific enzymes from different sources. A combination of commercial proteolytic enzymes and proteolytic enzymes from starter bacteria has also been used (Kahala *et al.* 1993; Korhonen *et al.* 1994). The use of immobilised enzymes or enzymes in batch processes has been reported. It appears that termination of the hydrolysis at a proper time is in all cases crucial for obtaining peptides with specific properties. The degree of hydrolysis (DH) can be calculated by different methods and may be used as a controlling index for protein hydrolysis (Adler-Nissen, 1986; Vegarud *et al.* 1991; Leclerc, 1997). Isolation of mineral binding peptides includes selective solubilisation and precipitation methods by the use of different solvents, chelating complexes (Ca/Ba), pH and ionic strengths (Juillerat *et al.* 1989; Hirai *et al.* 1992; Kawakami *et al.* 1993; Dufour *et al.* 1994; Reynolds *et al.* 1994; Schlimme & Meisel, 1995; Gaucheron *et al.* 1996). Peptides of different molecular sizes, ionic and hydrophobic characteristics are obtained by dialysis or filtration techniques and by the use of selective chromatography by ionic, affinity, hydrophobic interactions and chelating column techniques (Miller *et al.* 1981; Kontoghiorghes, 1986; Gutch, 1994; Zhang & Allan, 1995).

Physico-chemical characterisation

Peptides with mineral binding abilities from *in vitro* proteolysis by enzymes are summarised in Table 3. Several phosphopeptides with specific sequences from α_{s1}-casein, α_{s2}-casein and β-casein have been reported. Trypsin is the most common enzyme used for liberating multi-phosphoryl containing peptides (Meisel & Schlimme, 1993, 1996; Reynolds *et al.* 1994; Zhang & Allan, 1995; Schlimme & Meisel, 1995; Gaucheron *et al.* 1996). The peptides vary in size and in content of phosphoseryl groups and are reported to be resistant to further proteolysis. Since the charges of the peptides vary with the number of phosphoryl residues, the ability to bind minerals also varies. Sequence analysis of casein phosphopeptides (CPP) is reported to be associated with a number of problems (West, 1986; Reynolds *et al.* 1994). Characterisation of peptides from whey proteins reveals that they are lower in number and much less specific. Lactorphiner derived from β-lactoglobulin or α-lactalbumin are not reported to bind minerals, neither are peptides from the glycoprotein κ-casein (Antila *et al.* 1991; Pilhlanto-Leppala *et al.* 1994). However, it has been reported that glycylated amino acids, peptides and proteins possess metal-binding properties different from those of their non-glycylated analogues (Mossini & Feather, 1998). Seventeen different peptides have been identified by hydrolysis of β-lactoglobulin with thermolysin using two different concentrations of calcium (Dufour *et al.* 1994). Also peptides from α-lactalbumin using trypsin, chymotrypsin and pepsin have been reported (Hirai *et al.* 1992). Preliminary studies with peptides obtained by hydrolysis of β-lactoglobulin and α-lactalbumin have shown a higher affinity for Fe than the native proteins

Table 2. Milk proteins as sources of mineral binding and peptide precursors

Native protein	Concn. (g/l)		Active protein	Active peptide	Mol. wt.	Phospho residues	Charge/ residue	Charge at pH 6	Mineral binding	References
	Human	Cow								
Total protein	8·8	33	+	+					Mg, Mn, Zn, Ca	Flynn, 1992; Walstra & Jenness, 1984; Swaisgood, 1992; Brulé & Fauquant, 1982
Caseins	2·0–2·5	26·5		+						
α_{s1} -Casein		11		+	23 614	8	−0·1	−42·6	Fe, Zn, Ca	Schlimme & Meisel, 1995; Gaucheron *et al.* 1996; Swaisgood, 1992; Zhang & Allan, 1995
α_{s2}-Casein		2·5		+	25 230	11	−0·07	−31·2	Zn, Ca	Swaisgood, 1992; Zhang & Allan, 1995
β-Casein	1·5	9		+	23 983	5	−0·06	−30·5	Fe, Zn, Ca, Mg, Mn, Cu	Schlimme & Meisel, 1995; Gaucheron *et al.* 1996; Zhang & Allan, 1995; Baumy & Brulè, 1988; Bouhallab *et al.* 1991
κ-Casein	0·5	4		+	19 023	1	−0·02	−6·9	Ca	Swaisgood, 1992; Fox & McSweeney, 1998
Whey proteins	6·3	6·5	+	+					Fe	Svenning & Vegarud, 1998
α-Lactalbumin	1·9	1·2	+	+	14 176		−0·02	−2·6	Ca, Zn, Mg, Cu, Mn, Fe	Fox & McSweeney, 1998; Hirai *et al.* 1992; Baumy & Brulè, 1988; Svenning & Vegarud, 1998
β-Lactoglobulin		3·2	+	+	18 363		0·04	−19	Mg, Cu, Zn, Fe, Mn, Ca	Dufour *et al.* 1994, Baumy & Brulè, 1988; Svenning & Vegarud, 1998
BSA	0·4	0·4	+	+	66 276				Fe, Cu, Al	Nagasako *et al.* 1993; Mossini & Feather, 1997
Lactoferrin	1·7	0·1	+	+	77 000–93 000				Fe, Zn	Nagasako *et al.* 1993; Feng *et al.* 1995; Zhang & Allan, 1995
Immunoglobulins	1·3	0·9	+	?	75 000–900 000					
Lysozyme	0·4		+	?					Ca	Swaisgood, 1992
Lactoperoxidase			?	?						
Hormones			?	?						
Miscellaneous	0·6	0·7	?	?						

BSA, bovine serum albumin.

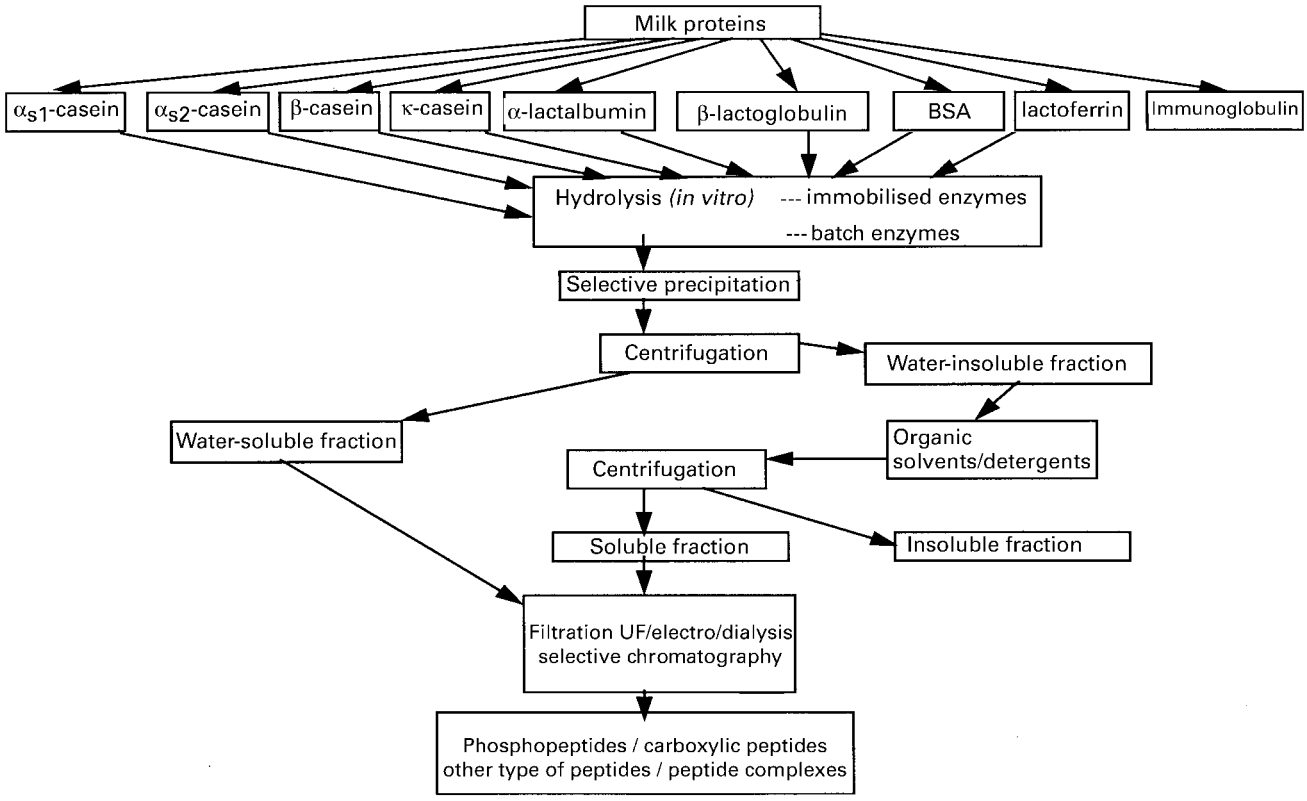

Fig. 1. Possible isolation methods of mineral binding peptides from milk proteins. BSA, bovine serum albumin; UF,

(Svenning & Vegarud, 1998). Peptides of the size 30–50 kDa have been reported from pepsin and trypsin treatment of lactoferrin (Kawakami *et al.* 1993; Feng *et al.* 1995; Zhang & Allan, 1995).

Measurements of the mineral content of the various peptides are complex and dependent on several factors. Among these the equilibrium status, red/ox form, solubility and binding affinity are regarded as some of the most important ones. Also sensitivity of the selective method is of importance. Analysis of the total calcium, iron, zinc, magnesium and some trace elements are most commonly detected by atomic absorption (AA) spectroscopy (Aziz-Alrahman, 1994). Radioactive isotopes (Amine & Hegsted, 1975; Sanyal *et al.* 1990) and other markers (Fairweather-Tait *et al.* 1997) are used to follow the binding mechanisms. Ion activities, such as Ca^{2+} and Mg^{2+}, are measured by ion-sensitive electrodes (Holt *et al.* 1981). However, this method has certain limitations. Capillary electrophoresis will probably become an even more important analysis in the future for detecting the ionic form of minerals. Chelating agents in chromatography are important selective techniques in use today (Miller *et al.* 1981; Kontoghiorghes, 1986; Gutch, 1994). Colorimetric methods have been used to measure the ferrous form of iron (Miller *et al.* 1981; Clydesdale & Nadeau, 1984). ESR (electron spin resonance) or affinity chromatography are also reported methods (Zhang & Allan, 1995). Differences in mineral affinity, competition at binding sites, pH and ionic strength of the solvent are reported to affect the mineral binding and release. CPP have shown significant differences in their affinity for calcium and iron binding (Kawakami *et al.* 1993; Schlimme & Meisel 1995; Zhang & Allan, 1995; Gaucheron *et al.* 1996; Meisel & Schlimme, 1996). Studies of CPP using immobilised enzymes have shown lower calcium- and higher iron-binding capacity than enzymes used in batch processes (Meisel & Schlimme, 1993). Also trace elements compete for the same absorption site, and these include iron–zinc, iron–manganese and copper–zinc (Loennerdal, 1991). The binding of bivalent cations to β-casein is reported to be affected by pH and ionic strength (Baumy & Brulé, 1988). Some amino acids like histidine appear to enhance zinc absorption by forming a complex that is readily absorbed (Zhang & Allan, 1995).

Technological properties and applications

Food fortification dates back to the 1920s and 1940s with the addition of vitamin D and A to low fat milk. Today the FDA has identified 21 nutrients as candidates for addition to foods among which are several minerals (Ternus, 1996). The minerals can be added as salts or in combination with metal-binding peptides and proteins.

The technology for production of mineral-binding peptides may induce certain changes, which may influence the functional behaviour of the final product. Common processes in production of peptides are enzyme hydrolysis, heat, acid and alkali treatment and drying. All these treatments may introduce specific changes in the functional properties of the product and this may change the

Table 3. Mineral binding peptides from milk proteins

	Enzyme	Peptide	Phosphoresidues	Net charge	Mineral binding	References
Casein-derived phosphopeptides					Fe, Mn, Cu, Se	Brulé & Fauquant, 1982
α_{s1} -Casein	Trypsin	43–59			Ca, Fe	Schlimme & Meisel, 1995; Zhang & Allan, 1995; Meisel & Schlimme, 1996
α_{s1} -Casein	Immob.trypsin	43–58			Ca, Fe	Meisel & Schlimme, 1993
α_{s1} -Casein	Trypsin	43–58	2	−7		Juillerat *et al.* 1989
α_{s1} -Casein	Trypsin	43–79	7			Reynolds *et al.* 1994
α_{s1} -Casein	Trypsin	48–58	2		Ca,	Schlimme & Meisel 1995; Gaucheron *et al.* 1996; Meisel & Schlimme, 1996
α_{s1} -Casein	Trypsin	59–64	4		Ca,	Schlimme & Meisel 1995; Gaucheron *et al.* 1996
α_{s1} -Casein	Trypsin	59–79			Ca, Fe	Kawakami *et al.* 1993; Schlimme & Meisel, 1995; Gaucheron *et al.* 1996; Meisel & Schlimme, 1996
α_{s1} -Casein	Trypsin	59–79	5	−9		Juillerat *et al.* 1989; Reynolds *et al.* 1994
α_{s2}-Casein	Trypsin	1–21	4			Reynolds *et al.* 1994
α_{s2}-Casein	Trypsin	46–70	4	−11		Juillerat *et al.* 1989
α_{s2}-Casein	Trypsin	46–70	4			Reynolds *et al.* 1994
α_{s2}-Casein	Trypsin	66–74	3		Ca	Schlimme & Meisel, 1995; Gaucheron *et al.* 1996
β-Casein	Trypsin	1–25	4	−9	Ca, Fe	Bouhallab *et al.* 1991; Reynolds *et al.* 1994; Schlimme & Meisel, 1995; Gaucheron *et al.* 1996; Meisel & Schlimme, 1996; Peres *et al.* 1997
β-Casein		1–28	4		Ca	Chung & Raymond, 1993
β-Casein		1–28	4	−8		Juillerat *et al.* 1989
β-Casein		33–48	2	−6		Juillerat *et al.* 1989; Meisel & Schlimme, 1996
α-Lactalbumin	Pepsin trypsin/ chymotrypsin				Cu, Ca, Zn, Fe	Hirai *et al.* 1992; Svenning & Vegarud, 1998
β-Lactoglobulin	Thermolysin	17 peptides fragments				Dufour *et al.* 1994
β-Lactoglobulin	Pepsin trypsin/ chymotrypsin				Fe	Svenning & Vegarud, 1998
Lactoferrin	Pepsin/trypsin	30 kDa			Fe	Kawakami *et al.* 1993; Feng *et al.* 1995; Zhang & Allan, 1995
	Trypsin	40 kDa			Fe	Kawakami *et al.* 1993
	Trypsin	50 kDa			Fe	Kawakami *et al.* 1993

nutritional properties. Proteolytic enzymes will produce peptides with different ion-binding capacity. However, the use of the same enzyme in immobilised form or in batch may produce different products as reported for trypsin. Caseinophosphopeptides obtained with immobilised trypsin showed lower calcium and a higher iron-binding capacity than fractions obtained from dissolved trypsin (Meisel & Schlimme, 1993).

Heating or alkaline treatment may generate indigestible peptides and proteins and will therefore impair the nutritional value (Tomè *et al.* 1987; Swaisgood & Catignani, 1991). It is well known that carbanion formed from cysteine and phosphoserine can undergo a β-elimination reaction to form the extreme reactive dehydroalanine which may form cross-links with lysine to form lysinoalanine (Damodaran, 1996).

Melanoidins formed from heat treatment of milk are reported to have metal-ion absorbing and antioxidant properties (Hidalgo *et al.* 1997; Homma *et al.* 1998; Srinivasan *et al.* 1998). Maillard reaction products derived from peptides are also reported to have antioxidant effects (Cämmerer & Kroh, 1996). Norwegian brown whey cheese is a high-heated iron-fortified whey concentrate. Different processing conditions of the milk have resulted in differences in the mineral composition of the cheese. This may be of importance for the bioavailability of minerals as reported for iron (Borch-Iohnsen *et al.* 1994; Svenning *et al.* 1999*b*). However, there is little information on the effect of processing.

The solubility of the protein- and peptide-binding mineral complexes are important for obtaining good functional properties like emulsification and foaming (Vegarud *et al.* 1991; Swaisgood, 1992). Solubility also seems to be of great importance for *in vivo* absorption and bioavailability of both proteins and minerals (Sanyal *et al.* 1992; Loennerdal *et al.* 1994; Peres *et al.* 1997). In addition, gastric digestion has been shown to increase the solubility of an iron-binding Maillard product, such as

brown whey cheese and thereby increase the mineral release from the product (Svenning *et al.* 1999*a*).

Natural mineral-rich components such as CPP have already found interesting applications in the food and pharmaceutical industry (Meisel & Schlimme, 1990, 1996). An increasing number of patents are seen in the literature on application of mineral-rich peptides (Reynolds, 1987). The use of CPP as ingredients or fortifiers in different low mineral-containing foods and drinks are increasing, for example: flour dry mixed CPP, breakfast cereals sprayed with a CPP solution and dried, and tooth paste with CPP (Reynolds, 1987). Reports on the mineral content and absorption of Ca, Fe, Mg, Mn and Zn have shown a lot of variation among different protein diets, fortified milk and infant formulas (Miller *et al.* 1981; Hurrell *et al.* 1989; Miller *et al.* 1990; Jackson & Lee, 1992; Pantako *et al.* 1992; Loennerdal *et al.* 1994; Zhang & Allan, 1995; Gotelli *et al.* 1996; Boccio *et al.* 1996; Schaafsma, 1997). The bioavailabilities of ions are also dependent on specific proteins, the oxidation state of the ion and other components, such as lactose, fat etc. For instance β-casein seems to enhance Mg absorption better than the other milk casein fractions and iron-absorption is reported to be dependent on the oxidation state (Amine & Hegsted,1975; Pantako *et al.* 1992; Sanyal *et al.* 1992; Arizono *et al.* 1996). Another important area for the use of mineral-binding peptides are the potential effect on micro-organisms; the phosphopeptides may bind ions such as Zn, Cu, Sn, Mn, Mg, Fe and thereby depress microbial growth (Reynolds, 1987; Vassilakos *et al.* 1993).

However, more information and documentation is needed in this area. Foods rich in minerals and trace elements are important for human health, even though their regulation and mechanisms *in vivo* are not well understood. Conflicting results have reported that protein-rich diets may either depress or enhance mineral absorption. It is therefore of great importance to get a better knowledge of the mineral-binding components in the diet, the effects of processing on the digestibility, nutritional value and bioavailability for optimal and safe uses.

Conclusion

A number of peptides from milk proteins with mineral-binding abilities have been reported. The whey proteins, β-lactoglobulin, α-lactalbumin and lactoferrin also give rise to peptides with mineral-binding abilities. Among the whey proteins lactoferrin is regarded as the most important iron-binding protein. Peptides are formed from milk proteins by various chemical or biological treatments. Isolation of mineral-binding peptides includes selective solubilisation and precipitation methods by the use of different solvents, chelating complexes (Ca/Ba), pH and ionic strengths. Lactorphiner derived from β-lactoglobulin or α-lactalbumin are not reported to bind minerals, neither are peptides from the glycoprotein κ-casein. Casein phosphopeptides have shown significant differences in their affinity for calcium and iron binding. Solubility also seems to be of great importance for *in vivo* absorption and bioavailability of both proteins and minerals

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