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PRODUCTION AND PROPERTIES OF HEALTH-PROMOTING PROTEINS AND PEPTIDES FROM BOVINE COLOSTRUM AND MILK

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Abstract

The high nutritive value and diverse functional properties of milk proteins are well known. Beyond these qualities, milk proteins have attracted growing scientific and commercial interest as a source of biologically active molecules. Such proteins are found in abundance in colostrum which is the initial milk secreted by mammalian species during late pregnancy and the first few days after birth of the offspring. The best characterized colostrum-based bioactive proteins include alpha-lactalbumin, beta-lactoglobulin, immunoglobulins, lactoferrin, lactoperoxidase and growth factors. All of them can nowadays be enriched and purified on an industrial scale from bovine colostral whey or cheese whey. These native proteins exhibit a wide range of biological activities that are known to affect the digestive function, metabolic responses to absorbed nutrients, growth and development of organs and disease resistance. Also, some of these proteins may prove beneficial in reduction of the risks of chronic human diseases reflected by the metabolic syndrome. It is speculated that such potentially beneficial effects are partially attributed to bioactive peptides derived from intact proteins. These peptides can be liberated during gastrointestinal digestion or fermentation of milk by starter cultures. The efficacy of a few peptides has been established in animal and human studies and the number of commercial products supplemented with specific milk peptides is envisaged to increase on global markets. Bovine colostrum appears as a highly potential source of biologically active native proteins and peptide fractions for inclusion as health-promoting ingredients in various food applications.

Key words: Colostrum, bioactive proteins, peptides, isolation, functionality, health effects.

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INTRODUCTION

Colostrum is the milk formed in the mammary gland during late pregnancy and secreted after parturition for the first 5-7 days after delivery of the offspring. The biological function of this early milk is to provide the newborn at its most vulnerable phase of life with immune protection against pathogens and to boost its physiological performance, growth and development (88, 149). It is, therefore, well understood that the composition of colostrum is different from that of mature milk. Colostrum contains plenty of physiologically active components. These include immunoglobulins, lactoferrin, lysozyme, cytokines, growth factors, hormones, oligosaccharides and lipid components (56, 60, 109).

The advent of functional foods in the 1990's has created the demand for natural bioactive components as potential ingredients for health-promoting foodstuffs and biopharmaceuticals. In this context, bovine whey proteins derived either from colostrum or cheese whey have gained increasing interest owing to evidence accumulating from scientific research about their potential health benefits (29, 43, 52, 59, 99, 100, 155, 159). Animal model and human studies suggest that bovine whey protein-based formulations are beneficial in weight management and regulation of other events related to the metabolic syndrome, e.g. cardiovascular disease and type two diabetes which health disorders are of global concern (23, 68, 81, 83, 96, 113, 131, 132, 151). Animal model and epidemiological studies also suggest that milk proteins may play a role also in prevention of certain cancer types, e.g. colon cancer (8, 112). As a result, commercial applications of these proteins have rapidly increased and efforts have been made to develop suitable technologies for large-scale isolation of specific protein compounds. At present, industrial-scale technologies are in place for major protein components from cheese whey and some specific components from colostral whey (25, 54, 65, 69).

In addition to bioactivities exerted by native protein molecules, it is now well established that milk proteins exert further physiological functions resulting from the numerous bioactive peptides encrypted within the intact protein molecules (64). The discovery of natural bioactive peptides is now opening new opportunities for exploitation of such peptides in promotion of human health through a regular diet and food supplements or even in the form of biopharmaceuticals.

This review article attempts to summarize the current scientific knowledge about the isolation of bovine colostral and whey proteins and production of bioactive peptides from these proteins. Also, current and potential future applications of these proteins and peptides are highlighted.

MAJOR PROTEINS IN MAMMARY SECRETIONS DURING LACTATION

Whey proteins represent about 60-80 % of the total pro-

tein in bovine colostrum, whereas in normal, mature milk the caseins are the predominant protein fraction accounting for about 80 % (26-28 grams per litre) of total protein. Also, the concentrations of different whey proteins in colostrum differ greatly from those present in mature milk. The foremost protein fraction in colostrum are the immunoglobulins (IgG, IgM, IgA) followed by lactoferrin (LF), α-lactalbumin (α-la), β-lactoglobulin (β-lg) and enzymes lactoperoxidase (LP), lysozyme (LZM) and other minor components. Figure 1 depicts the average concentration of major antimicrobial components in bovine lacteal secretions over the lactation period (56). The Igs carry the biological function of antibodies and are mainly derived from blood serum while part of IgA antibodies are synthesized locally in the mammary gland (13). The transport of Igs from serum to milk is a selective process favouring homologous IgG in most species. In the first milking, the total Ig amount may vary between 20 and 200 g/L and the IgG concentration *post partum* ranges from 15 to 180 g/L, the mean being approximately 60 g/L. Thereafter, the IgG concentration falls sharply to the level below 1g/L in mature milk. Also, the concentrations of α-la and β-lg are highest (on average 3.0 and 8.0 g/L, respectively) in the first milkings of colostrum and decrease sharply thereafter to the average levels of 1.2 g/L for α-la and 3.3 g/L for β-lg, respectively. In parallel, the LF concentration in colostrum varies from 1 to 5 g/L, decreasing soon after parturition to the level of about 0.1 g/L in mature milk of healthy cows. LP is the major enzyme in bovine mammary secretions. Colostrum contains about 30 mg/L of LP, and about the same amount is found in mature milk. In contrast to a relatively high concentration (0.36 g/L) in human colostrum, the concentration of LZM enzyme is about a thousand times less in cow's colostrum, ranging from 0.3 to 0.8 mg/L, and declining to 0.1mg/L in mature milk (32, 89, 116).

Colostrum and milk contain many factors which influence cell growth and differentiation. At present, the following growth factors have been identified in bovine mammary secretions: BTC (beta cellulin), EGF (epidermal growth factor), FGF1 and FGF2 (fibroblast growth factor), IGF-I and IGF-II (insulin-like growth factor), TGF-β1 and TGF-β2 (transforming growth factor) and PDGF (platelet-derived growth factor). The concentrations of all known growth factors are highest in colostrum during the first hours after calving and decrease substantially thereafter (109, 121). In cow's colostrum, the EGF concentration varies from 4 to 320 µg/L and that of mature milk from 2 to 155 µg/L. BTC is present in colostrum (2.30 µg/L) and at the same level in cheese whey (2.59 µg/L). The IGF-I and IGF-II are single chain polypeptides structurally resembling insulin. Bovine colostrum (32-800 µg/L) contains much higher concentrations of IGF-I than does human colostrum (17-52 µg/L) but the concentration decreases in mature bovine milk (4-27 µg/L). IGF-II is present in bovine colostrum and milk at concentrations of 150-600 µg/L and 2-100 µg/L, respectively. TGF-β1 is detected in bovine colostrum and milk (12-43 µg/L and 0.8-4 µg/L, respectively) but TGF-β2 is the predominant isoform present at high concentrations in bovine colostrum and milk (150-1150 µg/L and 13-71µg/L, respectively (22, 36).

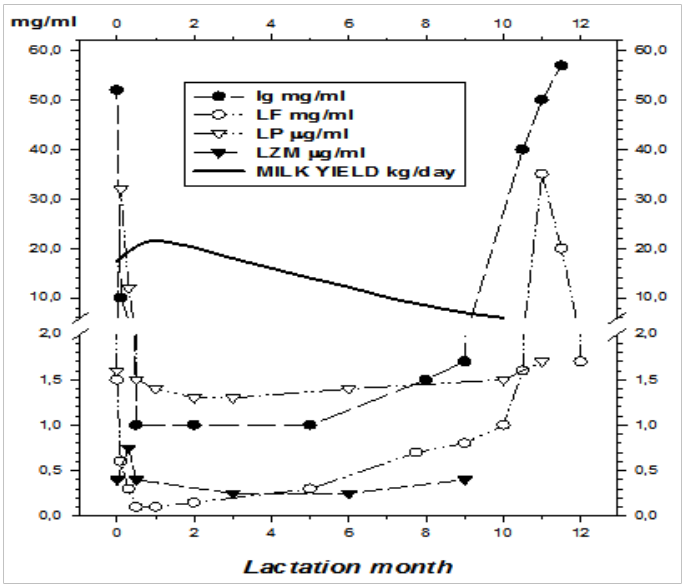

Figure 1. Antimicrobial factors in milk during lactation period.

Recently, modern molecular biology methods have revealed a great number of other chemically characterized molecules in bovine colostrum. Their concentration in colostrum and milk is marginal and their importance is scientifically not well established, as yet (31, 140).

Table 1 summarizes the major bioactive whey proteins found in bovine colostrum and milk and provides information about their concentration and molecular weights. Table 2 describes suggested or proven health benefits associated with these proteins.

FRACTIONATION AND ISOLATION OF WHEY PROTEINS

Fractionation and isolation of bioactive, health-enhancing milk proteins have in recent years emerged as a new lucrative sector for dairy industries and specialized bio-industries. To this end, modern chromatographic and membrane separation techniques have been applied successfully and many components, e.g. Igs, LF, LP and some growth factor fractions are now commercially available (17, 40, 57, 70, 142). Products based on total whey proteins isolated from cheese whey are widely applied in various industries. Technologies employed in the manufacture of these products include membrane separation processes, such as ultrafiltration, reverse osmosis, diafiltration and electrodialysis followed by a drying process which usually is spray-drying. These techniques are applicable for ordinary whey powder and whey protein concentrate (WPC) with a protein content of 30-80%. Gel filtration and ion-exchange chromatography techniques are employed in the manufacture of whey protein isolates (WPI) with a protein content of 90-95% (25, 144). The chemical composition and functionality of whey protein preparations are largely affected by the method used in the manufacturing process. The biological properties of such preparations may also be affected and are difficult to standardize due to the complex nature of bioactivities exerted by different proteins (30, 57). For example, heat treatment may alter the nutritional properties of α-la and β-lg by lactosylation of Lys residues. On the other hand, heating can reveal new properties such as the inhibition of the *E. coli* enterotoxin provoked diarrhea by lactosylated α-la and β-lg (136). Therefore, growing interest has been focused on development of specific techniques for isolation of pure whey protein components. Industrial or semi-industrial scale processing techniques

Table 1. Major whey proteins.

Protein	Concentration (mg/ml)		Molecular weight
	Colostrum	Milk	Daltons
Immunoglobulins (IgG, IgM, IgA)	20-200	0.5–1.0	150.000–900.000
β-lactoglobulin	8.0	3.3	18.400
α-lactalbumin	3.0	1.2	14.200
Glycomacropeptide	2.5	1.2	8.000
Lactoferrin	1.5	0.1	80.000
Lactoperoxidase	0.02	0.03	78.000
Lysozyme	0.0004	0.0004	14.000
Growth factors	50 µg -40 mg/L	<1 µg-2mg/L	6.400-30.000

Compiled from Marnila and Korhonen (88) and Pihlanto and Korhonen (116).

Table 2. Health benefits of whey proteins.

Effect	Proteins involved	Reference (*)
Prevention of muscular atrophy, improved physical performance, faster recovery after exercise	Total whey protein hydrolysates, Immunoglobulins, Growth factors	Buckley *et al.*, 2002, (11) Ha and Zemel, 2003, (43) Krissansen, 2007, (68) Shing *et al.,* 2006, (137)
Satiety and weight management	Total whey protein concentrates, Glycomacropeptide, Lactoferrin	Schaafsma, 2006, (131,132) Luhovyy *et al*., 2007, (81) Royle *et al.,* 2008, (126) Pilvi *et al.*, 2009, (118)
Reduction of blood pressure	α-lactalbumin and β-lactoglobulin hydrolysates and peptides	Murray and FitzGerald, 2007, (102) Korhonen, 2009, (58)
Stimulation of insulin production and reduction of postprandial glycemia	Total whey protein concentrates	Blouet *et al*., 2007, (5) Frid *et al.*, 2005, (33)
Prevention of oral and intestinal microbial infections	Immunoglobulins, Glycomacropeptide, Lactoferrin, Lactoperoxidase system	Korhonen and Marnila, 2009, (62) Ochoa and Cleary, 2009, (105) Seifu *et al.*, 2005, (134) Setarehnejad *et al*. 2010, (135)
Prevention of mucosal inflammation	Whey protein concentrates, α-lactalbumin, Lactoferrin	Chatterton *et al.*, 2006, (17) Mezzaroba *et al.*, 2006, (98)
Wound care and repair	Growth factors	Pouliot and Gauthier, 2006, (121) Smithers, 2008, (141)
Anti-cancer effects	Total whey proteins and hydrolysates	Bounous, 2000, (8) Parodi, 2007, (112) Zimecki and Kruzel, 2007, (162)
Improved cognitive functions, anti-stress effects	Lactoferrin α-lactalbumin	Markus *et al*., 2002, (87) Scrutton *et al*., 2007, (133)
Hypoallergenic effects	Whey protein hydrolysates	Ha and Zemel, 2003, (43) Krissansen, 2007, (68)
Psoriasis symptoms relief	Whey protein extract	Drouin *et al.*, 2007 (21)

(*) Literature reference number in parentheses.

are already available for purification of α-la, β-lg, Igs, LF, LP, some growth factor fractions and glycomacropeptide (GMP) from bovine colostral or cheese whey (65, 69). The progress of proteomics has facilitated the use of modern techniques, for example two-dimensional gel electrophoresis, in identification and characterization of novel whey protein molecules (76, 106)**.** Using this technique, Fong *et al*. (31) identified a large number of minor whey proteins after first fractionating bovine whey by semi-coupled anion and cation exchange chromatography. Among the identified minor proteins were several proteose peptone fractions, e.g. PP3 (lactophorin) and PP5. Also, complement C3, butyrophilin, fibrinogen, precursors of many major proteins and a cluster of novel osteopontin peptides were identified. Such proteomic display appears useful in the future design of strategies for purification of selected milk proteins or their fractions containing targeted bioactivities.

IMMUNOGLOBULINS

Immunoglobulins represent the biological function of antibodies and are present in colostrum of all lactating species to provide passive immunity against invading pathogens (13, 89). Colostral Ig preparations designed for farm animals are commercially available, and similar products have found growing worldwide market as dietary supplements for humans (130, 145, 149, 158). Some bovine colostral preparations have in clinical trials shown beneficial effects in recovery from long durance physical exercises (11, 137) and upper respiratory tract infections in adults (9), and in reduction of symptoms of endotoxemia in surgery patients (14, 15), and symptoms of rotavirus infections (6). More clinical studies are, however, required to confirm the health-promoting benefits of these preparations. The concentration of specific antibodies against pathogenic microorganisms can be raised in colostrum and milk by immunizing cows with vaccines made of pathogens or their antigens (67). These antibodies can be enriched and purified using membrane separation and chromatographic techniques and thus formulated to obtain so-called immune milk preparations (93). Traditionally, the globulin fraction was isolated from colostral whey by precipitation with either ammonium sulphate or ethanol. Chemical methods are, however, economically not suitable for large scale-production of Igs although they yield rather pure Ig fractions. Other challenges in isolation of active Igs from colostrum have been its complex composition and the sensitivity of Igs to heat treatments.

In high temperature / short time (HTST) pasteurization (72°C / 15 sec) about 25-40% of the Ig activity is lost, whereas ultra high temperature (UHT) treatment (138°C / 4 sec) and evaporation processing destroy most of the specific immune activity of milk due to Ig denaturation (75). In contrast, other studies (84, 85) have reported that bovine milk Igs could resist the HTST pasteurization treatment without affecting their structure. Only 1% of IgG, 2% of IgA, and 14% of IgM concentrations were denatured in laboratory experiments. Also, the HTST pasteurization had little effect on the activity of bovine colostral IgG as the original rotavirus neutralising activity was reduced by only 0.5%. McMartin *et al.* (92) demonstrated that a rapid heat inactivation of IgG started at temperatures higher than 65°C, and at 81°C, as much as 90% of the virus neutralization activity of Igs was lost in less than two minutes. On the other hand, heating moderate or high quality colostrum at 60°C for at least 120 min had no effect on mean IgG concentration or titer of neutralizing antibodies against bovine viral diarrhea virus type 1.

A great number of pilot- or industrial-scale methods have been developed and patented for fractionation and isolation of Igs from colostral or cheese whey (65). In these methods, the recovery rate of Igs has varied from 40% to 70% of the level present in the starting material (22). Specific chromatographic techniques, such as immobilised metal chelate affinity chromatography, immunoaffinity chromatography and cation-exchange chromatography have been applied to improve the yield and purity of immunoglobulin preparations further (3, 39). Microfiltration (MF) combined with ultrafiltration (UF) of bovine, equine and caprine colostrum has led to IgG/total solids purity of more than 90% (119). Korhonen and Marnila (62) used various MF methods, such as UF, MF and reverse osmosis, and a cation-exchange resin as a molecular sieve to concentrate Igs from colostral whey. The Ig level of the final freeze-dried concentrates varied from 45% to 75%. Expanded Bed Adsorption Chromatography (EBAC) is a new technology which has been applied to isolate Igs from cheese whey using adsorbent with tailored ligand chemistry. EBAC provides significant advantages over conventional packed bed column chromatography and an Ig-purity from 50% to 70% can be achieved with this upscalable method.

Most of the current commercial Ig products are prepared from colostrum of non-immunized cows by removing the fat followed by microfiltration or pasteurization under conditions that retain the biological activity of Igs. These products include whole colostrum powder, fat-free colostrum powders, fat-free colostral protein concentrate and colostral whey concentrates and are marketed as supplements for humans or colostrum-deprived calves. Some of these preparations have been tested clinically for certain physiological functions or prevention/treatment of microbial infections. Accordingly, a few products boast specific health or nutrition function claims, but clinical evidence related to these products is very limited or not proven, so far.

The specificity of natural antibodies found in milk and colostrum of different cows reflects the wide spectrum of antigens the animals have encountered in the past in their environment and ingested feedstuffs. In contrast, the immune milk products have fundamentally different antimicrobial properties and efficacies against pathogens than normal colostrum and these two concepts should, therefore, be differentiated from each others. Normal colostrum preparations are in most countries regarded as food supplements, whereas the products from immunized cows are often regarded as pharmaceuticals, e.g. in EU and USA, or their regulatory status is not defined (93).

Since 1980's an increasing number of clinical studies have demonstrated that immune milk preparations can be effective in prevention of human and animal diseases caused by different pathogenic microbes, e.g. rotavirus, *Escherichia coli, Candida albicans, Clostridium difficile, Shigella flexneri, Streptococcus mutans, Cryptosporidium parvum* and *Helicobacter pylori*. On the other hand, the therapeutic efficacy of these preparations has proven quite limited (44, 61, 62, 67, 143, 158). Table 3 provides a list of recent clinical studies on the efficacy of colostral prepara-

tions made from immunized or non-immunized cows. A few commercial immune milk products are on the market in some countries but the unclear regulatory status in many countries has emerged as a constraint for their global commercialization. The globally increasing problem of antibiotic-resistant strains, reflected by endemic hospital infections, may offer a promising approach to develop appropriate immune milk products to combat these serious infections. In a recent study (160) an immune milk preparation was found safe for oral use.

α-LACTALBUMIN

α-la is the major whey protein in human milk and accounts about 20 % of the proteins in bovine whey. α-la is fully synthesized in the mammary gland where it acts as coenzyme for biosynthesis of lactose. α-la can be enriched from cheese whey using membrane separation and pH/ heat-treatment process (51). Recently, Konrad and Kleinschmidt (55) described a novel method for isolation of native α-la from sweet whey using membrane filtration and treatment of permeate with trypsin. After a second UF and diafiltration of the hydrolysate, the calculated overall recoveries were up to 15 % of α-la with a purity of 90-95%. The biological functions of α-la have long been speculated but recent research suggests that this molecule can provide many beneficial effects in the form of a) intact whole protein, b) peptides of the partly hydrolysed protein and c) amino acids of the fully digested protein (17). α-la is a good source of the essential amino acids tryptophan and cysteine which are precursors of serotonin and glutathione, respectively. As these biomolecules are important for the cognitive functions, it has been suggested that the oral administration of α-la could improve the ability to cope with stress. This hypothesis has been supported by one study (87) whereas in another clinical study (133) daily administration of 40 g of α-la to healthy women increased plasma tryptophan levels and its ratio to neutral amino acids but no changes in emotional processing was observed. Furthermore, there is some evidence from animal model studies that α-la can provide protection against induced gastric mucosal injury caused by intake of ethanol or non-steroid anti-inflammatory drugs (91, 98). α-la is rich in cysteine and it has been shown that an intake of cysteine-rich whey protein diet tends to improve glycaemic control and development of insulin resistance in rats fed a high sucrose diet (5). Also, α-la may play a role in regulation of appetite and weight management. In a recent mouse model study, Pilvi *et al.* (118) investigated the effect of different whey protein-containing high-Ca diets on the weight loss and weight regain in a model of diet-induced obesity. Weight loss by energy restriction was performed on four different high-Ca diets (1.8 % $CaCO^3$) containing different whey proteins (18 % of energy): α-la, β-lg, LF and WPI. The results showed that the mice on the LF diet lost significantly more weight than mice on the WPI but only the α-la diet reduced fat accumulation during weight regain. These animal studies are encouraging in view of the potential use of α-la in dietary regimes targeting at reducing the risk of development of diabetes type two and obesity. To this end, intervention trials in humans are required. Bovine α-la has high degree of amino acid homology to human α-la. This characteristic makes bovine α-la and its hydrolysates a suitable source material for development of infant formulae, in particular.

β-LACTOGLOBULIN

β-lg is the major whey protein in bovine milk accounting for about 50 % of the proteins in whey but is not found in human milk. β-lg can be isolated in industrial scale from cheese whey using chromatographic and membrane separation techniques but the yield and purity have been relatively poor. In a recently published method by Lozano *et al*. (80) β-lg was isolated from bovine whey using differential precipitation with ammonium sulphate followed by cation-exchange chromatography. The overall yield of purified β-lg was 14.3% and purity higher than 95%. β-lg exhibits a variety of functional and nutritional characteristics that have made this protein a multi-functional ingredient material for many food and biochemical applications. β-lg has excellent heat-set gelation properties and has found a wide range of applications in products which require good water-binding and texturizing properties. Regarding biological functions, β-lg has been associated with antiviral, prevention of pathogen adhesion, anticarcinogenic and hypocholesterolemic effects (17). Also, β-lg has the ability to bind hydrophobic components, including retinol and long-chain fatty acids. It has been speculated that this whey protein may play a role in the absorption and subsequent metabolism of fatty acids. Furthermore, as described earlier, β-lg has proven an excellent source of peptides with a wide range of bioactivities, such as antihypertensive, antimicrobial, antioxidative, anticarcinogenic, immunomodulatory, opioid, hypocholesterolemic and other metabolic effects (47, 100).

LACTOFERRIN

LF is an iron-binding glycoprotein found in colostrum, milk and other body secretions and cells of most mammalian species. In comparison to bovine colostrum (1.5 g/L) human colostrum is a very rich source (up to 5 g/L) of this multifunctional compound (82). LF can be isolated from colostral and cheese whey by various chromatography and membrane separation techniques and is commercially available from many companies. LF is known to confer many biological activities, such as antimicrobial, antioxidative, antiinflammatory, anticarcinogenic and immunomodulatory properties (73, 111, 154, 157, 162). In addition, several antimicrobial peptides, such as lactoferricin B f(18-36) and lactoferrampin f(268-284) can be cleaved from LF by the action of digestive enzyme pepsin. The biological properties of LF have been subject of scientific research since the discovery of this "red protein" in the early 1960's (41). LF is considered to play an important role in the body's innate defence system against microbial infections and degenerative processes induced, e.g. by free oxygen radicals. Initially, the role was confined largely to antimicrobial activity alone but now the multifunctionality of LF has been well recognized. As reviewed by Pan *et al*. (111) the bactericidal effect of LF can be augmented by the action of lysozyme or antibodies. LF can also increase susceptibility of bacteria to certain antibiotics, such as vancomycin, penicillin and cephalosporins. The *in vitro* antimicrobial activity of LF and the derivatives has been demonstrated against a wide range of pathogenic microbes, including enteropathogenic *E.coli, Clostridium perfringens, Candida albicans, Haemophilus influenzae, Helicobacter*

Table 3. Human studies with preparations based on colostrum from non-immunized and immunized cows.

Bacteria used in immunization	Target disease	Treatment regime	Efficacy	Reference (*)
No immunization	Upper respiratory tract infections	60 g of colostral protein daily for eight weeks	Reduced significantly incidence of self-estimated symptoms of respiratory infections but no difference in duration	Brinkworth and Buckley (2003), (9)
No immunization	HIV-associated diarrhoea	Orally for 4 weeks	Reduced diarrhoea and increase in body weight, decrease in fatigue	Florén *et al*. (2006), (28)
Shigella dysenteriae	Shigellosis	100 ml orally 3 times/day for 3 days in combination with antibiotics	No significant difference in any clinical parameter in infected children	Ashraf *et al*. (2001), (4)
Five or one strains of *E. coli*	Diarrhea	Once per day orally 0,5g of IgG per kg of body weight, follow up period for 6 months	Lower incidence of diarrhea and shorter duration of diarrhea episodes	Tawfeek *et al*. (2003), (146)
Clostridium difficile toxin and *C. difficile* whole cells	*C. difficile* diarrhoea	Orally for two weeks as supportive treatment after antibiotic treatment	*C. difficile* toxins eradicated from 15 of 16 patients and no relapses in any patient during 11 month follow up period	Van Dissel *et al*. (2005), (150)
Clostridium difficile toxin and *C. difficile* whole cells	*C. difficile* diarrhoea	Orally 5g of whey powder - 3 times daily for two weeks for supportive treatment after antibiotics	Relapse in 10% of Ig treated patients whereas relapse in 20-25% of control group	Numan *et al*. (2007), (104)
Clostridium difficile toxin and *C. difficile* whole cells	*C. difficile* diarrhoea	Orally 5g of whey powder - for two weeks as supportive treatment after antibiotics	Clinical evidence for the safety for use in *C. difficile* patients	Young *et al*. (2007), (160)

(*) Literature reference number in parentheses.

pylori and *Listeria monocytogenes* and viruses, including hepatitis C, HIV-1, cytomegalovirus, poliovirus, rotavirus and herpes simplex virus (26, 48, 105). Furthermore, LF has been studied intensively for its anticarcinogenic activity and several mechanisms have been suggested, e.g. iron-chelation related antioxidative property, immunoregulatory and anti-inflammatory functions (74, 154).

Many animal and human studies performed during the last three decades have shown that oral administration of LF can exert several beneficial effects on the health of humans and animals (90, 157). Animal studies have shown that orally administered LF can suppress the overgrowth and translocation of certain intestinal bacteria, but does not affect intestinal bifidobacteria. Further, oral administration of LF and lactoferricin reduce the infection rate of *H. pylori, Toxoplasma gondii,* candidiasis and tinea pedis as well as prevent clinical symptoms of influenza virus infection. Animal studies have also demonstrated that orally ingested LF can improve nutritional status by reducing iron-deficient anemia and drug induced intestinal inflammation, colitis, arthritis, decrease mortality caused by endotoxin shock, stimulate weight gain in pre-weaning calves and regulate the bone cell activity as well as increase bone formation in mice (90). An earlier clinical study (125) in human infants demonstrated that oral administration of bovine LF increases the number of bifidobacteria in fecal flora and the serum ferritin level while the ratios of *Enterobacteriaceae, Streptococcus* and *Clostridium* tend to decrease. In a recent clinical study by King *et al*. (53) it was shown that LF supplementation to healthy infants for 12 months was associated with fewer lower respiratory tract illnesses and higher hematocrits as compared to the control group which received regular infant formula. In other human studies reviewed by Wakabayashi *et al.* (154) LF has been shown to increase eradication rate of *H. pylori* gastritis when administered in connection of triple therapy. Also, LF ingestion decreased the incidence of bacteremia and severity of infection in neutropenic patients. In further human studies LF has been shown to alleviate symptoms of hepatitis C virus infection, and to reduce small intestine permeability in drug induced intestinal injury. Also, LF ingestion and topical application have proven beneficial in the cure of tinea pedis. Another recent *in vitro* finding is that LF effectively inhibits biofilm formation and reduces the established biofilm of periodontopathic bacteria *Porphyromonas gingivalis* and *Prevotella intermedia* (153).

Owing to increasing experimental and clinical evidence about the potential health benefits of LF, commercial production of purified bovine LF has attracted considerable interest in recent years. As a result, many products containing added LF have been launched on the market in Asian countries, in particular. Current commercial applications of LF include e.g. yoghurt products marketed in Japan and Taiwan, baby foods and infant formulas marketed in South Korea, Japan and China. In addition, LF has been applied in different dietary supplements which combine LF and bovine colostrum and/or probiotic bacteria. Due to potential synergistic actions LF has been incorporated together with lysozyme and lactoperoxidase into human oral health care products, such as toothpastes, mouth-rinses, moisturising gels and chewing gums (154). U.S. Food and Drug Administration (FDA) have approved the use of bovine LF (at not more than 2% by weight) as a spray to reduce microbial contamination on the surface of raw beef carcasses. Accordingly, FDA has granted to bovine LF a "Generally Recognized As Safe" (GRAS, GRN 67) status for uses at defined levels in beef carcasses, sub-primals, and finished cuts (147). It can be envisaged that the usage of LF as an ingredient in functional foods and pharmaceutical preparations will increase significantly in the near future.

LACTOPEROXIDASE

Lactoperoxidase (EC 1.11.1.7) is a peroxidase with broad substrate specificity. LP represents the most abundant enzyme in bovine milk (approx. 30 mg/L) and can be recovered in substantial quantities from whey using chromatographic techniques. Bovine LP is relatively heat-resistant, retaining about 50% of its original activity after the HTST pasteurization (71). LP catalyzes an antimicrobial system consisting of the thiocyanate anion (SCN^-) and hydrogen peroxide to generate short-lived oxidation products, primarily hypothiocyanate ($OSCN^-$), which kill or inhibit the growth of a wide range of microorganisms, including bacteria, viruses, fungi, molds and protozoa (134). Nowadays, the LP system is considered to be an important part of the natural host defence system in mammals and the protective function seems to be mediated by several mechanisms (7). The LP system is known to provide a natural method for preserving raw milk as it naturally contains all necessary components to make the system functional. As the natural concentrations in milk of thiocyanate and hydrogen peroxide can be below the required level, the system usually requires activation by addition of a source of these components. The effectiveness of the activated LP system has been demonstrated in many field trials worldwide (3, 134). The LP system is approved by the Codex Alimentarius Committee for preservation of raw milk under conditions where facilities for milk cooling are insufficient. For this purpose, the method is being employed nowadays in a number of developing countries. The LP system has also found applications in dental health care products and animal feeds. Novel applications have been envisaged for preservation of different easily perishable products, for example meat, fish, vegetables, fruits and flowers (7).

GLYCOMACROPEPTIDE

GMP is a C-terminal glycopeptide f (106-169) released from the κ-casein molecule by the action of chymosin. GMP is hydrophilic and remains in the whey fraction in the cheese manufacturing process. GMP contains a significant (50-60 % of total GMP) carbohydrate fraction which is composed of galactose, *N*-acetyl-galactosamine and *N*-neuraminic acid. Pure GMP can be recovered in large quantities from cheese whey by chromatographic or ultrafiltration techniques (148). GMP is suggested to possess many biological properties but clinical evidence about potential health benefits is still rather limited. In *in vitro* studies GMP has been shown to inactivate microbial toxins of *E.coli* and *V. cholerae*, inhibit adhesion of cariogenic bacteria and influenza virus, modulate immune system responses, promote growth of bifidobacteria, suppress gastric hormone activities and regulate blood circulation through antihypertensive and antithrombotic activity (10, 86, 124). Owing to its glycoprotein nature GMP has interesting nutritional and physico-chemical properties. GMP is rich in branched –chain amino acids and low

in methionin, which makes it a useful ingredient in diets for patients suffering from hepatic diseases. GMP contains no phenylalanin making it suitable for patients suffering from phenylketonuria. Animal model studies have suggested that the high sialic acid content of GMP may deliver beneficial effects for brain development and improvement of learning ability (156). GMP is speculated to regulate appetite and this potential effect has been investigated in a number of studies, as reviewed by Recio *et al*. (124). Animal model and *in vitro* studies have shown that GMP inhibits gastric secretions and slows down stomach motility by means of stimulating the release of cholecystokinin (CCK)(12, 42, 161). This hormone is involved in controlling food intake and digestion in the duodenum of animals and humans. A recent study (135) using hydroxyapatite as a model system suggested that caseinomacropeptide and its fractions have a protective effect against dental erosion, for example when applied in acidic drinks.

In recent years, GMP containing products or supplements have been launched on the market in many countries for the purpose of appetite control and weight management. The efficacy of these products remains, however, to be established in clinical studies. An animal model study (126) demonstrated that both GMP and WPI decreased weight gain and altered body composition in male Wistar rats. GMP had a significant additional effect on fat accumulation when combined with WPI. The mechanism of this effect was not suggested by the authors. On the other hand, GMP may have a beneficial role in modulation of gut microflora, as this macropeptide has been shown to promote the growth of bifidobacteria (86). There is some indication from mouse model and human studies that bifidobacteria residing in the gut may affect the weight gain (124). To this end, further research in human subjects is needed to confirm these findings.

GROWTH FACTORS

It is now well documented that mammalian colostrum and milk contain many compounds with growth-promoting or growth-inhibitory activity for different cell types (121). At least some of these growth factors seem to withstand relatively well pasteurization and even UHT heat treatment of milk (36). Chemically the growth factors are polypeptides and their molecular masses range between 6000 and 30000 Daltons. In recent years, several pilot or semi-industrial methods have been developed for extraction of different growth factors from bovine colostrum and cheese whey (1, 119). The growth factors have been associated with many physiological functions affecting e.g. skin, intestinal tract and bone health (121, 149). Contradictory results are reported from studies concerning the stability of growth factors in the gastrointestinal tract. Many animal model studies have shown that EGF, IGF-I, and both TGF forms can provoke various local effects on the gastrointestinal tract and can be absorbed intact or partially from intestine into blood circulation. Increasing evidence supports the view that orally administered growth factors would retain their biological activity and exhibit in the body a variety of local and systemic functions. A number of health-related applications have been suggested for the growth factors derived from bovine colostral or cheese whey but just a few have been commercialized until now. The main health targets have been skin disorders, gut health and bone health. Playford *et al*. (120) suggested that colostrum based growth factors could be applied to prevent the side-effects of non-steroid anti-inflammatory drugs (NSAIDs) and symptoms of arthritis. An acid casein extract rich in TGF-β2 has been tested successfully in children suffering from Crohn's disease. Another growth factor extract from cheese whey has shown promising results in animal models and humans in treatment of oral mucositis and wound healing, for example leg ulcers (141). Other potential applications could be treatment of psoriasis, induction of oral tolerance in the newborn children against allergies and protection against intestinal damages caused by chemotherapy.

PRODUCTION AND FUNCTIONALITY OF BIOACTIVE PEPTIDES

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (54). The activity of peptides is based on their inherent amino acid composition and sequence. The size of active sequences may vary from two to twenty amino acid residues, and many peptides exhibit multi-functional properties. At present, milk proteins are considered the most important source of bioactive peptides and during the last two decades a great number of peptide sequences with different bioactivities have been identified in different milk proteins. The best characterized peptides have been shown to exert e.g. antihypertensive, antithrombotic, antimicrobial, antioxidative, immunomodulatory, mineral-binding and opioid activities. The properties of these peptides have been reviewed in many recent review articles (45, 58, 63, 66, 94, 103). Milk-derived bioactive peptides may deliver many physiological effects *in vivo* on the gastrointestinal, cardiovascular, endocrine, immune, central nervous and other body systems. Recent studies suggest that bioactive milk peptides may be beneficial also in reducing the risk of metabolic syndrome as well as targeting obesity and type two diabetes (24, 46, 100, 103). Casein hydrolysates are known as an abundant source of bioactive peptides. Their formation and properties have been reviewed in many recent articles (50, 77, 79, 102, 114, 129, 139) and will not be discussed here further. Also, the whey proteins, α-la and β-lg, in particular, have proven rich precursor proteins of bioactive peptides with a wide range of bioactivities, as referred to in recent study reports and review articles (47, 51, 128).

Bioactive peptides are inactive within the sequence of the parent protein molecule and can be released from precursor proteins using the following means: (a) hydrolysis by digestive enzymes (b) fermentation of milk with proteolytic starter cultures, and (c) proteolysis by enzymes derived from microorganisms or plants. In many studies, the above methods have been combined successfully. As for digestive enzymes, pepsin, trypsin and chymotrypsin and also other proteolytic enzymes, such as alcalase, thermolysin and subtilisin have been shown to liberate from whey proteins a great number of peptide sequences which exert different bioactivities under *in vitro* conditions (19, 37, 78, 108, 115).

The release of different bioactive peptides from milk proteins through fermentation by lactic acid bacteria (LAB) is now well documented (27, 39). Many studies have demonstrated that *Lactobacillus helveticus* strains are capable

of releasing antihypertensive peptides, the best known of which are ACE-inhibitory tripeptides Val-Pro-Pro and Ile-Pro-Pro. The antihypertensive capacity of these peptides has been demonstrated in many *in vitro* and rat model studies (50, 102, 129). Also yoghurt bacteria, cheese starter bacteria and commercial probiotic bacteria have been shown to produce different bioactive peptides in milk or cheese during fermentation (16, 18, 20, 34, 117, 129).

Bovine colostrum is a rich source of whey proteins and could be a preferred source of bioactive peptides. In order to obtain a suitable whey protein fraction from colostrum, it is advisable to remove first the casein fraction. For this purpose, the use of anionic polysaccharides, κ-carrageenan and pectin, have been tested successfully recently (72).

APPLICATIONS OF BIOACTIVE PEPTIDES

A great variety of naturally formed bioactive peptides have been found in fermented dairy products, such as yoghurt, sour milk, dahi, kefir, quark and different types of cheese (39, 110, 123, 138). The occurrence, specific activity and amount of bioactive peptides in fermented dairy products depend on many factors, such as type of starters used, type of product, time of fermentation and storage conditions (107, 138). It is noteworthy that in these products peptides with different bioactivities, e.g. calcium-binding, antihypertensive, antioxidative, immunomodulatory and antimicrobial, can be found at the same time. The formation of peptides can be regulated to some extent by starter cultures used but the stability of desired peptides during storage seems difficult to control (16, 127).

Over the last decade, large-scale technologies, based on membrane separation techniques, have been developed for the purpose of enrichment and isolation of peptides with a specific molecular weight range and bioactivity (122, 152). In particular, nanofiltration and ultrafiltration techniques are being employed to produce specific bioactive peptides derived from casein or whey protein hydrolysates. Such preparations are now commercially available and are being used as ingredients in different consumer products, such as dairy and fruit based drinks, confectionery, chewing gum, pastilles and capsules. Table 4 enlists examples of commercial whey protein-derived bioactive fractions and peptides which have shown to confer e.g. antihypertensive or anticariogenic activity or acne, psoriasis or stress-relieving properties (21, 58, 66, 95).

CONCLUSIONS

The current global interest in developing functional foods provides a timely opportunity to exploit the great arsenal of native bioactive milk components for promotion of human health and wellbeing. This approach has been facilitated by rapidly increasing knowledge about the fundamental biological properties and mechanism of action of major milk proteins, bioactive peptides and growth factors. It is evident that more minor molecules with specific bioactivities will be exploited in coming years and also new bioactive molecules are discovered with the help of modern analytical and biomolecular methods. Examples of such molecules are butyrophilin, colostrinin, cytokines, hormones, osteopontin, oligosaccharides, milk basic protein, mucin cytokines and lipids of the fat globule membrane (49, 97, 124). Commercial applications based on these components can be developed now more easily using advanced industrial or semi-industrial scale processing techniques available for fractionation and isolation of specific molecules from colostrum, milk and whey. Modern non-thermal, clean and green technology based methods are well suited for manufacturing heat-sensitive ingredients from these sources. Fractionation and marketing of bioactive milk ingredients is now emerging as a new lucrative business for the dairy industries and specialized bio-industries. Much as a result of this development the dairy industry has already achieved a leading role in the development of functional foods and has commercialized many milk protein and peptide-based products which can be consumed as part of a regular healthy diet. It can be envisaged that in the near future more similar products will be launched on worldwide markets. They could be targeted to infants, elderly and immune-compromised people as well as to maintain good health status and prevent diet-related chronic diseases. In view of the current increasing prevalence of obesity and related diseases, type two diabetes, in

Table 4. Examples of commercial milk protein products with specific functionality.

Brand name	Type of product	Functional bioactive proteins /peptides	Suggested or proven health/function claim	Reference /manufacturer (*)
BioZate	Hydrolysed whey protein isolate	β-lactoglobulin fragments	Reduction of blood pressure	**Davisco, USA**
BioPURE-GMP	Whey protein isolate	κ-casein f(106-169) (Glycomacropeptide) anticariogenic	Satiety regulation through CCK,	Burton-Freeman, 2008, (12)/ Davisco, USA
Vivinal Alpha	Ingredient	α-lactalbumin rich whey protein hydrolysateBorculo	Aids relaxation and sleep	Korhonen and Pihlanto, 2006, (65)/ Domo Ingredients (BDI), the Netherlands
Praventin	Food supplement/ capsule	Lactoferrin enriched Whey protein hydrolysate	Helps reduce acne	Korhonen and Pihlanto, 2006 (65)/ DMV International, the Netherlands
Dermylex	Food supplement/ tablet	Whey protein extract XP-828L	Helps reduce symptoms of mild to moderate psoriasis	Drouin et al., 2007 (21) / Advitech Inc., Canada

(*) lierature reference number in parentheses.

particular, more experimental research should be focused on bioactive milk peptides which can regulate appetite, reduce inflammation signals and manage blood glucose balance. Also, the potential benefits of bioactive milk proteins and peptides should be studied in relation to impairment of cognitive functions, memory-related diseases and mood control. In development of food supplements containing milk bioactives technological challenges are anticipated in formulation of products that can withstand the harsh environment of the gastrointestinal tract. To this end, potential solutions may be found in micro- or nanoencapsulation techniques which have developed rapidly in recent years.

An emerging research field is the effect of milk proteins on the expression of animal and human genome. Research in this field may in future open new opportunities for optimal exploitation of bovine milk proteins for human nutrition and health, when compared in analogy with human milk components (38, 99).

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