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Biological Roles and Production Technologies Associated with Bovine Glycomacropeptide

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1.1 Introduction

Glycomacropeptide (GMP) is a casein-derived whey peptide found in 'sweet' whey. The addition of chymosin to milk during cheese making enzymatically hydrolyses or cleaves the milk protein (kappa-casein) into two peptides, an insoluble peptide (para-kappa-casein) and a soluble hydrophilic glycopeptide (GMP), as shown in Figure 1.1. The larger peptide, para-kappa-casein, contains the amino acid residues 1–105 and becomes coagulated and incorporated into the cheese curd. The smaller peptide, which contains the amino acid residues 106–169 (GMP), becomes soluble and is incorporated into the whey (Walstra et al. 2006). GMP is the third most abundant whey protein, after beta-lactoglobulin and alpha-lactalbumin, accounting for approximately 15–25% (1.2–1.5 g L−1) of the total whey protein (Thomä-Worringer et al. 2006). GMP is highly polar and has unique characteristics due to the absence of phenylalanine, tryptophan, tyrosine, histidine, arginine or cysteine residues (Neelima et al. 2013). The peptide is rich, however, in branched chain amino acids, such as isoleucine and valine (Marshall 2004; Krissansen 2007).

At least 13 genetic variants of bovine kappa-casein have been identified which have different post-translational modifications (PTMs) and vary in their level of phosphorylation and glycosylation (Thomä-Worringer et al. 2006). The average molecular weight for GMP is 7500 Da, whereas the highest recorded molecular weight is 9631 Da (Mollé and Léonil 2005). It has been suggested that the peptide has the ability to associate and dissociate under certain pH conditions, possibly explaining why molecular weights of between 14 and 30 kDa are observed via SDS-PAGE (Galindo-Amaya 2006; Farias et al. 2010).

Given the heterogeneity of GMP, there is no single isoelectric point (pI) assigned to GMP but the pI of the peptide portion is approximately 4, varying with PTM. Approximately 60% of GMP consists of O-linked carbohydrate chains which are composed of mainly galactose (gal), N-acetyl galactosamine (GalNAc) and N-neuraminic acid (Neu5Ac) attached at threonine residues. Saito et al. (1991) determined via high-performance liquid chromatography (HPLC) the distribution of monosaccharide,
Figure 1.1 Bovine kappa-casein structure which varies depending on its post-translational modifications (phosphorylation and glycosylation). During cheese making, hydrolysis by chymosin releases the water-soluble fragment para-kappa-casein and the hydrophilic glycomacropeptide.

disaccharide, trisaccharide (straight and branched) and tetrasaccharide chains as 0.8%, 6.3%, 18.4%, 18.5% and 56.0%, respectively, while Mollé and Léonil (1995) identified five potential glycosylation sites using electrospray-ionisation mass spectrometry (ESI–MS) (Saito et al. 1991; Molle and Leonil 1995). Glycosylation influences the physical properties of GMP such as solubility (Taylor and Woonton 2009) and its emulsifying and foaming properties (Kreuß and Kulozik 2009). Moreover, variations in glycosylation can occur over the course of lactation (Recio et al. 2009; Neelima et al. 2013). For instance, colostrum GMP has an elevated glycan content (Guerin et al. 1974). Only GalNAc, Gal and Neu5Ac have been identified in GMP glycans from mature milk, but glycans from colostrum samples in addition contain N-acetylglucosamine (GlcNAc) and fucose (Fuc). Furthermore, a greater number of glycans and more complex structures have been identified in colostrum GMP (Fiat et al. 1988). A disialylated tetrasaccharide is the most abundant glycan present in mature GMP (Saito and Itoh 1992), and this high level of sialylation is vital for some of GMP’s biological activities, as will be discussed later. Commercially available forms of GMP contain approximately 8% sialic acid (Arla Food Ingredients and Agropur Ingredients).

The aim of this chapter is to provide an overview of the state of the art in research regarding the functional role of GMP in maintaining and improving human health which is summarised in Table 1.1 and providing better knowledge on the isolation and detection of GMP as an ingredient in functional or medical foods.

1.2 Biological Properties Associated with Glycomacropeptide

1.2.1 Management of Phenylketonuria

Phenylketonuria (PKU) (OMIM 261600) is an autosomal recessive disorder caused by mutations in the phenylalanine hydroxylase (PAH) gene that encodes the enzyme which
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catalyses the conversion of phenylalanine (Phe) to tyrosine (Tyr) in a reaction dependent on the essential PAH co-factor tetrahydrobiopterin (Blau et al. 2010). Tyr is an essential amino acid in PKU. Normal intake of dietary protein in untreated PKU causes Phe to accumulate in blood, leading to toxic concentrations of Phe in the brain and intellectual disability (Vockley et al. 2014). The main therapy for PKU is long-term adherence to a low-Phe diet that limits Phe intake from natural foods that contain protein, and supplements with special medical formulas that supply vitamins, minerals and all essential amino acids except Phe (MacLeod et al. 2009; Singh et al. 2014). The absence of Phe in GMP makes this peptide a valuable dietary ingredient for patients who are suffering from PKU. GMP can be made into a variety of palatable GMP medical foods that are low in Phe and high in protein content (Etzel 2004; Lim et al. 2007).

Table 1.1 Biofunctional roles of GMP in improving human health.

<table>
<thead>
<tr>
<th>Bioactivity</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Management of PKU</td>
<td>Etzel (2004); Ney et al. (2008); Ney et al. (2016)</td>
</tr>
<tr>
<td>Ability to bind cholera toxin and <em>E. coli</em> enterotoxins</td>
<td>Kawasaki et al. (1992) – cholera toxin, Isoda et al. (1999) – <em>E. coli</em> heat labile enterotoxins</td>
</tr>
<tr>
<td>Promotion of bifidobacterial growth</td>
<td>Brody (2000) – review has several examples, Thomä‐Worringer et al. (2006) – review has several examples, Recio et al. (2009) – review has several examples, O’Riordan et al. (2014) – review has several examples</td>
</tr>
<tr>
<td>Reduction in intestinal epithelial cell barrier dysfunction</td>
<td>Rong et al. (2015) – barrier function, Feeney et al. (2017) – barrier function</td>
</tr>
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</table>
A number of preclinical studies in a PKU mouse model demonstrated that GMP supplemented with limiting amino acids supports growth and reduces concentrations of Phe in plasma and brain, improves bone status and reduces metabolic stress compared with an amino acid diet (Ney et al. 2008; Solverson et al. 2012a,b). Clinical evaluation of GMP found that in 11 PKU subjects, safety, acceptability, improved satiety and greater protein retention were observed with GMP medical formulas compared with amino acid medical formulas (Ney et al. 2008; MacLeod et al. 2009; van Calcar et al. 2009). Based on the results of these studies, GMP medical formulas first became available in the United States in 2010, with Cambrooke Therapeutics, Agropur Ingredients and Nestlé supplying formulas containing GMP for the treatment of PKU.

1.2.2 Anti-Infective Properties

It is now accepted that mucosal surface adherence of bacteria is required for colonisation and subsequent development of disease. When in the adherent state, these bacteria are more likely to survive as their resistance to cleansing mechanisms, immune factors, bacteriolytic enzymes and antibiotics is higher (Ofek et al. 2003). Bacterial surface components that mediate adherence are collectively known as adhesins (Moran et al. 2009). Several bacterial species utilise specific adhesins, or proteinaceous lectins, that bind glycan structures on the surface of host tissues to facilitate attachment. Milk glycans, such as those associated with GMP, have also been shown to obstruct specific host–pathogen interactions, including bacterial adhesion to the host ligands (Cravioto et al. 1991; Simon et al. 1997; Coppa et al. 2006). The glycans can structurally mimic epithelial cell surface glycans and thus function as decoys that pathogens can bind to instead of the host and thereby prevent infection (Sharon and Ofek 2000). GMP-derived peptides have been found in the intestinal lumen and blood of human (Chabance et al. 1998; Ledoux et al. 1999) and animal subjects (Fosset et al. 2002) after ingestion of the peptide and milk products, suggesting that GMP survives digestion and can be produced in the gastrointestinal tract where it can be absorbed by intestinal cells. It is believed that because GMP is O-glycosylated, some protein fragments are non-digestible, and so they reach the distal segment of the gastrointestinal tract intact where they can exert their anti-infective properties (Boutrou et al. 2008).

In terms of inhibiting bacterial adhesion, GMP has been shown to reduce the adherence of pathogens such as Salmonella typhimurium, Shigella flexneri and E. coli to certain intestinal cell lines (Nakajima et al. 2005; Rhoades et al. 2005; Bruck et al. 2006a,b). Strömqvist et al. (1995) demonstrated that GMP inhibited adhesion of Helicobacter to sections of stomach tissue. GMP has also been shown to inhibit binding of cholera toxin to Chinese hamster ovary cells at concentrations as low as 20 ppm (Kawasaki et al. 1992). Nakajima et al. (2005) found that GMP inhibits the association of EHEC O157 with Caco-2 cells and the association of EPEC with Caco-2 cells based on pathogen binding to its sialic acid component. The glycopeptide was also found to inhibit the adhesion of certain strains of EPEC to HT-29 cells (Rhoades et al. 2005) and the ETEC strain K88 to porcine intestinal cells and porcine mucus (Gonzalez-Ortiz et al. 2013, 2014). Recently, Feeney et al. (2017) found that GMP reduced intestinal epithelial cell barrier dysfunction and adhesion of enterohemorrhagic and enteropathogenic E.coli in vitro.

Another important property associated with GMP is the ability to inhibit the adhesion of cariogenic bacteria such as Streptococcus mutans, S. sanguis and S. sobrinus to
oral surfaces, therefore modifying the composition of plaque bacteria to control acid production and, in turn, reducing the demineralization of enamel and promoting remineralization (Moynihan et al. 2000; Kashket and DePaola 2002; Janer et al. 2004). In this respect, GMP as an ingredient in dental hygiene products such as toothpaste and mouthwash to protect against tooth decay and plaque formation has received much attention in recent years.

Glycomacropeptide has also been shown to possess antibacterial properties and can inhibit the growth of both gram-positive, such as \textit{Streptococcus mutans}, and gram-negative bacterial species, such as \textit{Porphyromonas gingivalis} and \textit{E. coli} (Malkoski et al. 2001). In addition, GMP is effective in preventing haemagglutination by \textit{Actinomyces viscosus}, \textit{Streptococcus sanguis} and \textit{Streptococcus mutans} (Neeser et al. 1988, 1994, 1995). Furthermore, bioactive peptides released by the pepsin treatment of GMP have been shown to have an antibacterial effect on \textit{E. coli} in acidic media and also improve the resistance of \textit{Lactobacillus rhamnosus} to acid stress (Robitaille et al. 2012). GMP is also known to have antiviral activity against human rotavirus (HRV) infection \textit{in vitro} (Inagaki et al. 2014). Desialylated kappa-casein obtained by neuraminidase treatment exhibited anti-HRV activity, whereas deglycosylated kappa-casein obtained by \textit{O}-glycosidase treatment lacked antiviral activity, indicating that glycans other than sialic acid were responsible for the activity. Kawasaki et al. (1993a,b) demonstrated that GMP also inhibits haemagglutination by four strains of human influenza virus while Dosako et al. (1992) demonstrated that GMP prevents Epstein–Barr virus from inducing morphological transformations in peripheral lymphocytes.

1.2.3 Prebiotic

There are contradictory data on the effects of GMP on the growth promotion of \textit{Bifidobacterium} and \textit{Lactobacillus} strains (Azuma et al. 1984; Poch and Bezkorovainy 1991; Idota et al. 1994; Bruck et al. 2006a,b; Cicvárek et al. 2010; Hernandez-Hernandez et al. 2011). The differences observed between studies may be as a result of the quality and/or purity of the GMP used. Robitaille et al. (2012) demonstrated that highly purified GMP exhibits dose-dependent growth-promoting activity for lactic acid bacteria in a minimal culture medium. This study also concluded that the presence of glycans linked to caseinomacropeptide is not required for the growth-promoting activity. When added to diets given to infants (Bruck et al. 2006b) and piglets (Gustavo Hermes et al. 2013), caseinomacropeptide also increased lactobacilli populations in faeces and in ileal and proximal colonic digesta, respectively, suggesting that caseinomacropeptide could also be a growth promoter for lactic acid bacteria \textit{in vivo}.

To address the influence of hydrolysis on the growth-promoting activity of caseinomacropeptide, effects of peptic and tryptic digests of the peptide on probiotic lactic acid bacteria growth were investigated (Robitaille and Champagne 2014). Pepsin treatment was effective in promoting the growth in milk of all probiotic bacteria tested, with biomass levels being improved significantly, by 1.7 to 2.6 times (P < 0.05), depending on the strain. Another study by Tian et al. (2014) demonstrated that GMP significantly improved the growth of probiotic bacteria supplemented in yogurt. GMP increased growth of \textit{Streptococcus thermophilus} (P < 0.05), while it had little effect on the growth of \textit{Lactobacillus bulgaricus} (P > 0.05). An addition of 1.5% GMP increased
Bifidobacterium animalis subsp. lactis (Bb12) growth in the yogurt fourfold relative to the control (no GMP). The authors concluded that the growth-promoting effect of GMP was not linked to its sialic acid content but might be related to its high Glutamic acid, Leucine, and Alanine content.

Recently, Sawin et al. (2015) reported that GMP feeding resulted in a significant decrease in the abundance of Proteobacteria and the genus Desulfovibrio in the caecal and faecal microbiota of wild-type and phenylketonuric mice. Increased short chain fatty acids (SCFA) and lower indices of inflammation were also observed in comparison to the casein and amino acid-based control diets. Ntemiri et al. (2017) used an in vitro batch fermentation (artificial colon model) to simulate colonic fermentation processes of two GMP products, a commercially available GMP concentrate and a semi-purified GMP concentrate, and lactose. Faecal samples were collected from healthy and frail older people. Sequencing analysis revealed that the commercial GMP preparation had a positive effect on the growth of health-promoting taxa such as Coprococcus and Clostridium cluster XIVb. GMP also increased SCFA production and sustained the diversity of the microbiota from healthy elderly inocula and to a lesser extent in inocula from frail elderly subjects under in vitro conditions. Taken together, a unifying feature and potential mechanism for the reported health-promoting properties associated with GMP may be its role in the development of a more host-friendly flora that may increase our ability to resist acute infection.

1.2.4 Immunomodulatory Activities Associated with GMP

1.2.4.1 Inflammation and Allergy

Most studies which examine the effects of pure GMP on cells of the immune system are performed in vitro and focus on lymphocytes. Allergic disorders can be identified by screening for regulation of allergen-specific immunoglobulin antibodies and T helper type 2 cells. There have been several in vivo studies which demonstrate GMP’s ability to inhibit splenocyte propagation and how it can be used to suppress immune responses such as allergic reactions. GMP has been shown to inhibit lipopolysaccharide-induced splenocyte proliferation (Otani and Monnai 1993; Mikkelsen et al. 2005), to suppress interleukin (IL)-2 receptor expression in mouse CD4+ T-cells (Otani et al. 1996) and to block serum IgG antibody production by mouse lymphocytes (Monnai et al. 1998).

Investigations carried out on the effect of GMP on macrophages have shown that GMP increases the release of IL-1 receptor while leaving IL-1-beta unaffected, in a mouse monocytic cell line (Monnai and Otani 1997). GMP also increases the phagocytic activity and propagation of human macrophage cells (U937) at 10 μg mL⁻¹ (Li and Mine 2004a). Li and Mine (2004a) found that pepsin hydrolysis of GMP increased cell proliferation and phagocytic action while sialidase treatment decreased it; however, 70% of the activity was still retained suggesting that both the terminal sialic acids and polypeptide portions of GMP are required for optimal stimulatory effects.

Requena et al. (2009) demonstrated that GMP induced cytokine production in human monocytes via stimulation of the MAPK and the NF-kappa-B signal transduction pathways by upregulating the secretion of TNF, IL-1-beta, and IL-8 in a concentration-dependent fashion. The effect of GMP on cytokine secretion was confirmed using human primary blood monocytes (Requena et al. 2009). Jimenez et al. (2012) demonstrated that orally consumed GMP prevented allergic sensitization and reduced severity
of the early phase reaction induced by antigen in cutaneous hypersensitivity and in anaphylaxis in ovalbumin-sensitized rats. GMP also displays immunoregulatory activity in allergic asthma models, as it effectively suppresses blood and lung eosinophilia, goblet cell hyperplasia, and collagen deposition in airways. The beneficial effect of GMP in asthma is associated with downregulation of IL-5 and IL-13 and upregulation of IL-10 expression in asthmatic lung tissue (Roldan et al. 2016). A study in rats demonstrated that the prebiotic action of GMP leads to an allergy-protective microbiota, resulting in an increase in TGF-beta production and a reduction in mast cell response to allergens (Jimenez et al. 2016). Results from a separate rat trial have recently indicated that GMP has an inhibitory effect on atopic dermatitis through downregulating the Th2-dominant immune response (Munoz et al. 2017).

1.2.4.2 Colitis

In ulcerative colitis (UC), both innate and adaptive immunity dysfunctions may contribute to disease pathogenesis (Baumgart and Carding 2007; Abraham and Cho 2009). With the exception of 5-aminosalicylate (5ASA)-containing drugs, most medications used for UC have side effects and significant complication risks. Therefore, a novel strategy for UC is highly desired. GMP is a promising candidate for UC treatment because of its ability to modulate gut microbiota and regulate immune responses (Daddaoua et al. 2005). Hvas et al. (2016) used GMP as a nutritional therapy to treat patients with active distal UC and found that it was well tolerated and accepted by patients, and the disease-modifying effect of GMP was similar to that of 5ASA. Ortega-Gonzalez et al. (2014) found that GMP exhibited intestinal anti-inflammatory effects in a lymphocyte transfer mouse model of colitis by reducing the activity of colonic myeloperoxidase and the percentage of CD4+ interferon (IFN)-gamma+ cells in mesenteric lymph nodes. Cui et al. (2017), in a recent study of famoxadone (OXZ)-induced mouse experimental UC, found that it could significantly improve morphological injury to intestinal mucosa in OXZ-induced UC mice to the same extent as regular treatment of UC. The study found that GMP could significantly reduce the expression of human mucosal addressin cell adhesion molecule-1 (MAdCAM-1), Cluster of differentiation 4 (CD4) and Cluster of differentiation 8 (CD8) in the lamina propria of the intestinal mucosa and significantly stimulate the secretion of slgA to increase intestinal immunity. Furthermore, GMP was found to be directly involved in inhibiting the MAPK pathway and activating the TGF-beta-1/Smad signal transduction cascade, which could maintain immunological regulation of the intestinal mucosa and protect the function of the intestinal mucosal barrier.

1.2.5 Satiety

Glycomacropeptide is believed to stimulate the release of the hormone CCK in the gastrointestinal tract. CCK slows gastric emptying which may in turn promote satiety (Keogh et al. 2010). However, studies investigating the effect of GMP on food intake and satiety have resulted in mixed findings. Degen et al. (2001) demonstrated that oral GMP stimulates CCK hormone and increased satiety in human test subjects. Royle et al. (2008) found that GMP was associated with reduced fat mass in Wistar rats fed ad libitum for seven weeks with diets differing in protein type and amount. In addition, Veldhorst et al. (2009) showed a decrease in food energy intake at a subsequent meal 180 minutes after consumption of a test breakfast containing whey protein with GMP...
compared to whey protein without GMP. However, Burton-Freeman (2008) found that GMP had no effect on satiety or on food intake 75 minutes after consumption but did reduce daily food intake.

More recently, Keogh et al. (2010) compared the ability of GMP and a GMP-depleted whey protein concentrate to stimulate CCK, by making subjective measures of satiety and food intake for 20 overweight/obese male test subjects. Blood samples, CCK levels record, and subjective measures of satiety were collected before and 15, 30, 60, 90, 120, and 180 minutes after GMP consumption. A lunchtime meal of hot food was provided from which subjects ate ad libitum until satisfied. Energy and nutrient intakes from the food consumed were calculated. There was no significant difference in CCK levels, subjective measures of satiety, food intake, and energy intake between treatments across all GMP concentrations.

Chungchunlam et al. (2009), however, showed that the ingestion of preload drinks enriched with whey protein containing naturally present GMP resulted in subjects consuming a lower energy intake at a subsequent meal and reporting a greater feeling of fullness compared with a maltodextrin carbohydrate-enriched control beverage. However, the natural presence of GMP in whey hinders investigation of the satiating effect of whey protein, and the influence of GMP alone on satiety and food intake. The group (Chungchunlam et al. 2014) later found that whey GMP alone did not reduce subsequent food intake compared with a drink enriched with maltodextrin, but whey protein had a greater satiating effect than maltodextrin. Therefore, this study suggests that the presence of GMP in whey does not appear to be the cause of the observed effect of whey protein on satiety. Despite this controversy, several companies still claim that GMP promotes weight loss.

### 1.2.6 Anticarcinogenic

The development of colorectal cancer is a complex pathological process which involves multiple steps and stages, with changes from normal crypt foci to aberrant crypt foci (ACF), adenoma formation, expansion and eventual development to colorectal cancer (Belinsky et al. 1998; Takayama et al. 1998). Chen et al. (2014) utilised dimethylhydrazine (DMH)-induced colorectal cancer (CC) model rats to explore the effects of GMP on colorectal cancer. Rats with CC were orally given various concentrations of GMP or the same volume of phosphate-buffered saline for 15 weeks. The total numbers of ACF and crypts per focus in colon were scored, the methylation level of DNA extracted from colon was detected and the expression of p16 and mucin 2 (MUC2) proteins was measured. The results showed that although ACF were found in rats treated with GMP, their number was significantly decreased compared to that of the control rats. In addition, methylation and expression levels of p16 and MUC2 were also inhibited by GMP, which were more obvious in rats treated with higher concentrations of GMP. The study highlights the potential of GMP as nutritional therapy for preventing colorectal cancer.

### 1.3 Glycomacropeptide Production

Several methodologies have been employed to isolate and purify GMP from cheese whey, with the main challenge arising from separating GMP from the other whey proteins (Abd El-Salam (2006); Tullio et al. (2007); Neelima et al. (2013); Nakano and
Ozimek (2014). GMP is known to be soluble in 8% (w/v) trichloroacetic acid (TCA) solution, while all other whey proteins are precipitated. Therefore, GMP can be separated from sweet whey proteins by deproteinization with TCA (Nakano et al. 2002). GMP can also be separated from other whey proteins by cellulose acetate electrophoresis in pyridine/acetic acid because of pI differences between the proteins (Nakano et al. 2009). These methods are useful for preparation of GMP at laboratory scale, but are not suitable for large-scale production of GMP for commercial use. For this reason, isolation methods to generate highly pure GMP for use in humans have become an area of interest for many whey producers. Techniques such as thermal treatment, ultrafiltration (UF), ethanol precipitation, complexation, supercritical carbon dioxide processing, various chromatographic methods, and combinations thereof have been used to isolate GMP.

1.3.1 Thermal Treatment and Ethanol Precipitation

Thermal treatment and ethanol precipitation may be useful to eliminate significant amounts of proteins/peptides from GMP-containing streams (Saito et al. 1991; Berrocal and Neeser 1993; Martín-Diana et al. 2002; Li and Mine 2004b). Rojas and Torres (2013) evaluated the isolation and recovery on whey GMP by means of thermal treatment (90 °C). Eighteen samples (2 L each) of sweet whey, resuspended commercial whey (positive control), and acid whey (negative control) were processed. The indirect presence of GMP was verified using chemical tests and 15% SDS-PAGE. At 90 °C, bands of 14, 20, and 41 kDa bands were observed in sweet whey (Rojas and Torres 2013). These bands may correspond to oligomers of GMP. Peptide recovery showed an average of 1.5 g L⁻¹ (34.08%). The results indicate that industrial-scale GMP production is feasible. However, it should be noted that the yield and glycosylation characteristics of GMP are significantly influenced by the severity of milk heat treatment. The greater the severity of heating, the lower the quantity of GMP recovered, and the lower the quantity of sialic acid associated with the soluble GMP fraction (Taylor and Woonton 2009). Previously, Saito et al. (1991) reported complete loss of sialic acid from GMP by heating sweet whey at pH 3.0 and 98 °C for one hour. Since a considerable number of biological activities associated with GMP can be attributed to sialic acid (see above), determination of this glycan residue during isolation of GMP is important.

1.3.2 Complexation

Another approach for the isolation of GMP involves the use of an enzymatic cross-linking technique (Tolkach and Kulozik 2005). This method involves the pretreatment of WPC with the enzyme transglutaminase (Tgase) followed by microfiltration. GMP can be cross-linked to Tgase due to the presence of two glutamine and three lysine residues in its amino acid sequence. The native whey proteins show much less sensitivity to cross-linking by this enzyme due to their globular structure despite the presence of glutamine and lysine. The covalent linked GMP aggregates can be removed by means of microfiltration or diafiltration. However, the procedure only separates native whey proteins from GMP and may not be applicable to heated whey. As cross-linking of other milk proteins by Tgase changes their functional properties (Bönisch et al. 2007; Czernicka et al. 2009), it is possible that the same may occur for GMP isolated by Tgase.
Chitosan, a polysaccharide comprising co-polymers of glucosamine and N-acetylglucosamine, is a derivative of the naturally abundant biopolymer chitin (Singh 2015). The polycationic character of chitosan at acidic pH values allows the formation of complexes with negatively charged GMP molecules, inducing their flocculation (Nakano et al. 2004; Casal et al. 2005). Glycosylated GMP was found to have a higher affinity for chitosan when compared to non-glycosylated forms. The carboxylic groups in the carbohydrate moiety of the GMP increase the negative charge of the molecule and may play a role in the selective precipitation. The authors found that at pH 5.0, 0.08 mg mL$^{-1}$ of chitosan completely removed the GMP whereas 70% of non-glycosylated GMP remained in solution. As the pH increased, the amount of chitosan required to ensure complete removal of GMP also increased by up to 0.19 and 0.34 mg mL$^{-1}$ for pH 6.0 and 6.6, respectively (Casal et al. 2005). In another study by Nakano et al. (2006a), chitosan was mixed with sweet whey at pH 3.0 to form soluble chitosan-GMP complex. This complex was then separated from the other whey proteins and peptides by UF (molecular weight cut-off (MWCO) 100 000). The chitosan–GMP complex recovered in the retentate was then dialysed to give an insoluble complex, from which GMP was separated by elution with sodium chloride. The GMP fraction obtained accounted for an average 6.4% of dry non-dialyzable fraction of sweet whey, and contained 7.9% (w/w) sialic acid. The adsorption capacity and selectivity of these chitosan materials, however, are generally low.

A further study by Li et al. (2010) used beta-cyclodextrin which was immobilised to native chitosan beads by cross-linking with 1,6-hexamethylene di-isocyanate (HMDI). The resultant modified beads had a superior adsorption affinity for GMP. At pH 3.0, 90.23% of GMP was adsorbed, with a maximum adsorption capacity corresponding to 12.87 mg of sialic acid/g-adsorbent. Subsequent desorption experiments demonstrated that the modified beads could be regenerated and used for further cycles without significant decreases in capacity and selectivity.

### 1.3.3 Aqueous Two-Phase Systems

Aqueous two-phase extraction has been used widely as a mild separation method in many research fields (Oliveira et al. 2002). An aqueous two-phase system (ATPS) is formed when two water-soluble polymers, such as polyethylene glycol (PEG) and dextran, or a polymer and a salt are dissolved in water beyond a critical concentration at which two immiscible phases are formed (Gu and Glatz 2007). Da Silva et al. (2009) explored the possibility of partitioning of GMP using ATPS. In their study, the use of PEG and sodium citrate as ATPS for the partitioning of GMP was proposed. The results demonstrated that the partitioning of GMP depends on PEG molar mass, tie line length, pH, sodium chloride concentration, and temperature. The data indicated that GMP is preferentially partitioned into the PEG phase without addition of sodium chloride at pH 8.0. Larger tie line lengths and higher temperatures favour GMP partition to the PEG phase. Furthermore, it was verified that PEG molar mass and concentration have a slight effect on GMP partition. The increase in the molar mass of PEG induces a reduction of the protein solubility in the top PEG-rich phase, showing that the use of PEG1500 is beneficial for the extraction of GMP. A protein recovery higher than 85% was obtained in the top phase (PEG-rich phase) of these systems, demonstrating its suitability as a starting point for the separation of GMP.
A more recent study by Wu et al. (2012), using PEG/ammonium sulfate to separate GMP, found also that molecular weight of PEG and concentration of both the phases influence the partitioning of GMP. The study showed that 18% (w/w) PEG6000 and 15% (w/w) ammonium sulfate were the optimum conditions for protein recovery (69.2%) (Wu et al. 2012). However, the use of this system still requires another purification step, as do most of the methods described above.

1.3.4 Ultrafiltration

Ultrafiltration is a commonly used process for concentrating a dilute product in dairy streams, particularly in whey streams. UF is used to separate different sized whey proteins in a solution based on the membrane pore size or MWCO. The molecular weight of GMP is pH dependent and at neutral pH, self-association occurs, which forms oligomers through non-covalent bonds, whereas at low pH this is prevented through partial disassociation (Xu et al. 2000). Kawasaki et al. (1993b) used this molecular weight-pH dependency to influence the separation of GMP from other whey proteins including bovine serum albumin, immunoglobulin, beta-lactoglobulin, and alpha-lactalbumin in a UF system with a cut-off range of 20–50 kDa. This resulted in a GMP purity of between 18% and 63%. Once the permeate containing the GMP was adjusted to pH7, it was passed through the same membrane where the purity of the GMP recovered was reported to be between 81% and 86%.

Li and Mine (2004c) compared the efficiency of three techniques of GMP isolation from WPI: TCA, ethanol precipitation, and UF. The TCA pretreatment recovered only sialo-GMP (glycosylated) and eliminated all contaminated proteins; however, the recovery rate was the lowest (6.7% of the initial WPI). Ethanol precipitation recovered 20.4% of GMP from WPI and 75.7% was glycosylated, but the heating process may lead to degradation of the glycans. UF was found to be the most effective in recovering GMP. The recovery rate was 33.9% with 81.6% sialo-GMP. The authors concluded that the carbohydrate profile of GMP varied widely and depended on the isolation method. Based on the high recovery of sialo-GMP, a combination of UF and anionic chromatography was deemed to be a suitable approach on an industrial scale.

Javanmard et al. (2012) isolated GMP for use as a source of protein for PKU patients from a whey protein solution (10% protein w/v) using dual UF disc membranes with 50 and 10 kDa cut-offs. The whey was passed through the membranes at pH 3.5, 4, and 4.5 with an ambient temperature of 25°C. Diafiltration was used for purification of GMP in both UF phases. After UF, the Phe content of the whey at pH 4 was lowest, indicating a high purity and recovery rate of GMP. The disadvantage of employing some of these processes is that salts, solvents, buffers, and acids may be required, which introduce contaminants into the GMP product that must be removed through additional processing steps.

Supercritical carbon dioxide (SCO2) is an established method for the fractionation of alpha-lactalbumin and beta-lactoglobulin from WPC and WPI (Bonnaillie and Tomasula 2012). Bonnaillie et al. (2014) used SCO2 to further fractionate WPI via acid precipitation of alpha-lactalbumin, beta-lactoglobulin, and the minor whey proteins to obtain GMP-enriched solutions. The process was optimised and alpha-lactalbumin precipitation maximised at low pH and a temperature (T) ≥65°C, where beta-lactoglobulin with 84% purity and GMP with 58% purity were obtained, after UF and
diad filtration to separate beta-lactoglobulin from the GMP solution. At 70 °C, beta-lactoglobulin also precipitated with alpha-lactalbumin, leaving a GMP-rich solution with up to 94% purity after UF.

1.3.5 Chromatography

1.3.5.1 Gel Filtration

Gel filtration chromatography separates molecules based on their size by filtration through a gel. In 1998, Nakano and Ozimek performed a study to determine the molecular weight of GMP and whether it is in fact influenced by pH (Nakano and Ozimek 1998). The authors found that GMP could be purified from sweet whey using a Sephacryl S-200 gel. To purify GMP from a solution of hydrolysed caseinate, the same group (Nakano and Ozimek 2000) used a number of processes involving (i) exclusion chromatography with Sephacryl S-200 at pH 7, (ii) addition of acid solution, pH 3.5 to precipitate contaminating proteins and peptides, and (iii) chromatography in Sephacryl S-200 at pH 3.5. The resulting GMP preparation was considered highly pure based on subsequent sialic acid determination, amino acid analysis, and SDS-PAGE. A follow-up study by the group (Nakano and Ozimek 2002) demonstrated that GMP could also be isolated from sweet whey by taking advantage of the pH-dependent difference in elution patterns between GMP and other whey proteins using Sephacryl S-200 gel chromatography. The isolated fraction contained a high molecular weight GMP accounting for 0.02% (w/v) of sweet whey. The GMP fraction contained 106 μg sialic acid/mg dry weight. Again, a high level of purity was observed through the use of gel electrophoresis and amino acid analysis.

1.3.5.2 Affinity

Affinity chromatography (AC) is a selective purification, separation, and enrichment technique in which the compound of interest must have a specific property that can be exploited during the affinity procedure (Hernández-Ledesma et al. 2014). Morr and Seo (1988) were among the first to use affinity chromatography for the isolation of GMP. The method involved a combination of chromatography steps including Con A-Sepharose affinity chromatography (Morr and Seo 1988). Saito et al. (1991) fractionated GMP into sialo- and asialo-GMP using peanut (*Arachis hypogoea*) lectin-affinity chromatography. Recently, Baieli et al. (2017) developed a GMP purification process based on the affinity of sialic acid for wheat germ agglutinin (WGA). After formation of chitosan beads and adsorption of WGA, the agglutinin was covalently attached with glutaraldehyde. Two matrices with different WGA density were assayed for GMP adsorption. GMP was recovered as an aggregate with an overall yield of 64%.

1.3.5.3 Hydrophobic Interaction

Hydrophobic interaction chromatography (HIC) relies on hydrophobic interactions between immobilised hydrophobic ligands and non-polar regions on the surface of proteins (Hernández-Ledesma et al. 2013). Nakano and Ozimek (2000) investigated purification of GMP from the non-dialysable fraction of sweet whey by chromatography on phenyl-agarose and found that most of the whey proteins were adsorbed on the column, while the GMP was not. The estimated yield of the GMP fraction was approximately 1.6 g L⁻¹ of sweet whey. Another study by the group, (Silva-Hernandez et al. 2002) used
a combination of anion exchange and HIC to purify GMP from goat sweet whey. Approximately 0.06% (w/v) of sweet whey was recovered as GMP. Amino acid analysis of the GMP preparation demonstrated that the GMP was of high purity.

1.3.5.4 Ion Exchange

Ion exchange chromatography (IEX) separates proteins on the basis of their ionic charge. In anion exchange chromatography, negatively charged proteins are attracted to a positively charged group. Conversely, in cation exchange chromatography, positively charged proteins bind to a negatively charged medium. For preparation of GMP for use in food, ion exchange is one of the most common techniques used, considering, as mentioned, that GMP has a pI ~4, which is lower than the pI (>4.1) of the other major whey proteins (beta-lactoglobulin, alpha-lactalbumin, serum albumin, immunoglobulins, etc.) (Eigel et al. 1984) and therefore can be separated from these whey proteins based on the difference in pI.

Both cation and anion exchange chromatography have been used to purify GMP. Léonil and Mollé (1991) isolated GMP from sweet whey using cation exchange HPLC on a Mono-S column. More recently, LaClair et al. (2009), in their experiment on PKU diet preparation, reported that the Phe concentration (5 mg g⁻¹ of product) in a commercially available GMP was too high, and thus, refined the product by using cation exchange chromatography to reduce the Phe level to 2.7 mg g⁻¹ protein equivalent (43% reduction) (Léonil and Mollé 1991; LaClair et al. 2009).

Glycomacropeptide has more commonly been purified from sweet whey and its fractions by anion exchange chromatography (Kawasaki et al. 1992; Nakano and Ozimek 1999b; Ayers et al. 2003). In 1992, Tanimoto et al. used whey prepared from lactic casein for large-scale production of GMP (Tanimoto et al. 1992). The whey was filtered, desalted, and lyophilised, resulting in a GMP preparation with a Phe content of 2.4% (w/w). The GMP was then purified by ion exchange chromatography, using QSepharose®, presenting Phe levels of 0.9% (w/w). In 1995, Outinen et al. developed a simple method for isolating a peptide fraction, consisting largely of GMP from emmental cheese whey (Outinen et al. 1995). The whey was first clarified by microfiltration, the pH was then adjusted to 5.0 and it was then passed through a polystyrene basic anion exchange resin column. The GMP which was selectively adsorbed on the column was released with dilute sodium chloride solution, subsequently desalted, concentrated by UF and then dried. Other whey components remained intact. About 70% of GMP originally present in whey was recovered in the prepared GMP fraction with a yield of 253 mg from 200 mL of clarified whey. The authors claimed a GMP purity of 70–80%.

The GMP purification process for scaled-up production proposed by Ayers et al. (2003) also involves using anion exchange (QA GiboCel TM, Life Technologies Ltd) under conditions in which GMP is adsorbed, followed by elution, removal of impurities by numerous processes and recovery of purified GMP (Ayers et al. 2003). At pH 5.1, there was elution of 91% of the GMP. Anion exchange chromatography was also used by Nakano and Ozimek (1999a,b) to purify GMP from the non-dialysable fraction of whey using a DEAE-Sephacel column at two pH values (6.4 and 3.0). The column at pH 3.0 resulted in GMP of high purity and yielded 1 g L⁻¹ of whey (Nakano and Ozimek 1999b). The authors concluded that chromatography with DEAE Sephacel at pH 3.0 is an easy method that can be applied for large-scale production of GMP. The same authors (Nakano and Ozimek 1999b) also purified GMP from sweet whey dialysed in water by
anion exchange chromatography on DEAE Sephacel at pH 2.0–4.5. Dialysis of sweet whey was shown to be important to maximise the yield of GMP adsorbed to the anion exchanger. Only highly sialylated GMP, accounting for approximately 55% of total sialic acid content, was adsorbed on the anion exchanger from non-dialysed sweet whey.

Xu et al. (2000) reported that GMP was selectively adsorbed from Cheddar cheese whey at pH 4.7 on a polystyrene anion exchange resin IRA93. The adsorbed material was then released with dilute sodium chloride solution, desalted and concentrated by UF using an Amicon YM 100 membrane. Tek et al. (2005) investigated the effect of whey conductivity, pH, and salt concentration of the elution buffer on GMP recovery and its extent of contamination using anion exchange chromatography. The results demonstrated that GMP recovery increased substantially with decreasing conductivity and increasing pH of the whey feed stream. Increasing the pH, but not increasing the conductivity, increased contamination of the GMP by primarily beta-lactoglobulin. A salt concentration of at least 0.1 M was required for complete elution of bound GMP. This study helped define conditions required for GMP recovery by a process chromatography system that uses food-grade buffers, operates at industrially relevant flow rates, and achieves up to 98% recovery.

A direct-capture anion exchange membrane adsorption process for the separation of a pure glycosylated GMP fraction of caseinomacropeptide was successfully developed at pilot plant scale (Kreuß and Kulozik 2009). The process included a desalting and concentration step, which was performed by a 10 kDa UF/diafiltration. The efficiency of the UF was strongly influenced by the pH of the solutions and displayed optimum performance at pH 4.1 for the eluate. A recent study by Nakano and Ozimek (2016) was undertaken to determine whether commercially available GMP can be refined by anion exchange chromatography with high reproducibility to provide GMP with no contaminating amino acids. Anion exchange chromatography on DEAE Sephacel was employed to remove impurities. The results demonstrated that Phe-containing proteins or peptides do not bind to the column, while most GMP, accounting for 93% of total recovered sialic acid, can bind to the column.

Overall, it may be that a combination of approaches for the isolation of GMP from whey may provide the best results. Etzel (2001) developed a process for producing a substantially pure GMP from whey using two steps. First, the GMP was recovered from whey using a strong anion exchanger (quaternary amino ethyl cellulose ion exchanger). The column was washed with water to remove contaminants and the GMP was then eluted with 0.5 M sodium chloride. The elute was adjusted to pH 7.15 using 1 M sodium hydroxide. In the second step, the crude GMP solution was then chromatographed using metal affinity chromatography (iminodiacetic acid agarose beads containing immobilised Cu sup2+ metal ion). The beads were washed with 0.02 M sodium phosphate and 0.5 M sodium chloride pH 7.15. The effluent up to this point was the substantially purified GMP product. The invention also describes a method for large-scale production of GMP in a substantially pure form using fewer steps than methods of similar capability in purity.

Doultani et al. (2003) also used a two-step approach to purify GMP through the use of cation and anion exchange. A cation exchange resin was used to recover WPI from sweet whey and the effluent was fed to an anion exchange resin to recover GMP. The authors reported that nearly all of the major whey proteins (alpha-lactalbumin, beta-lactoglobulin, immunoglobulin G, and serum albumin) and about half of the total Kjeldahl nitrogen were recovered by the cation exchange resin. The anion exchange
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Resin recovered nearly all the GMP from the effluent of the cation exchanger. This was considered the first process to simultaneously manufacture WPI and GMP from a single stream of whey, increasing the value obtained from whey.

Combining ion exchange and UF, however, remains the most popular choice for large-scale production of GMP (Kawasaki et al. 1994; Etzel 2004). In view of the different processes available for the production of GMP and the importance of its structure (in particular glycosylation) in its various biological activities, it is necessary to know whether the methodology used to obtain GMP influences its structure and activity (Recio et al. 2009). Similarly, storage and technological processes, such as heating, may affect the glycosylation degree or the chemical stability of GMP (Lieske et al. 2004) and should be considered when selecting conditions for large-scale production of GMP.

1.4 Detection of Glycomacropeptide

Isolation of GMP requires assays or methods to track its presence at each step of purification. Monitoring the presence of GMP is also important in the detection of cheese whey in milk as a marker of adulteration (Neelima et al. 2013). The section below summarizes the various methods employed for measuring GMP. However, the reader is referred to Neelima et al. (2013) and Nakano and Ozimek (2014) for further detail.

The quantification of GMP in dairy protein fractions and products, including enzymatically treated casein, cheese whey, WPC or WPI, is generally performed through the identification and measurement of characteristic peaks with HPLC or capillary zone electrophoresis, coupled with mass spectrometry or gel electrophoresis to determine the molecular weight and identity of isolated peptides. Colorimetric, immunological, and biosensor-based methods are also available. The content of GMP can also be monitored by measuring ultraviolet (UV) absorbance at wavelength 205–230 nm to determine its peptide amide bond (Peterson 1983). UV absorbance at 280 nm can be used to estimate the amount of contaminating protein/peptide other than GMP (El-Salam et al. 1996; Nakano and Ozimek 1999b, 2000) as GMP has no aromatic amino acids and therefore displays no UV absorbance at 280 nm (Léonil and Mollé 1991; Abd El-Salam 2006).

1.4.1 Chromatography

Kawasaki et al. (1992) described a method for determination of glycosylated and non-glycosylated GMP by size exclusion chromatography using two coupled TSK gel G3000 PW columns and anion exchange chromatography on a Mono Q HR 5/5 column. The method was able to distinguish heterogeneous GMP by the variations in carbohydrate moieties and to determine GMP content without pretreatment of milk samples with thiobarbituric acid. In 2003, Ferreira and Oliveira used RP-HPLC with a polystyrene divinylbenzene column on adulterated samples (Ferreira and Oliveira 2003). The authors used pH 4.6 filtrate from the samples, and eluted using the gradient of two solvents. Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 95% acetonitrile–5% water–0.1% TFA. The eluate was monitored by a UV detector at 214 nm and enabled the separation of three peaks, two of which corresponded to the nonglycosylated forms of GMP and the third to the less well-resolved glycosylated GMP components.
1.4.2 Capillary Electrophoresis

In capillary electrophoresis (CE), small amounts of fluids are injected into a separation channel using plug injection. Substances are separated based on their electrophoretic mobility, which is proportional to their charge to size ratio in the interior of a small capillary filled with an electrolyte (Neelima et al. 2013).

In 1995, Otte described a CE method for the separation of the major whey proteins using acidic and basic conditions (Otte et al. 1995). Under acidic conditions (70 mM phosphate, pH 2.5), a GMP peak was separated from the other whey proteins. Also in 1995, van Riel and Olieman used the CE method for the detection of rennet whey solids in skim milk and buttermilk powder, based on the non-glycosylated variants of GMP (van Riel and Olieman 1995). Cherkaoui et al. (1997) then validated a method for the analysis and quantitative determination of a GMP mixture. Recio et al. (1996, 2000) demonstrated the suitability of the CE method to identify GMP in liquid milk samples and observed the presence of detectable GMP in stored UHT milk samples incubated with psychrotrophs.

1.4.3 SDS-PAGE

Galindo-Amaya (2006) developed a SDS-PAGE-based method for the detection of GMP in milk. The authors isolated GMP from milk and adulterated milk samples with sequential precipitation in TCA, treatment with ethanol-ether, and re-suspension in Tris–HCl buffer. Precipitates were analysed by SDS-PAGE and GMP was evidenced as a trimer of 20.8 kDa in samples of sweet whey, and mixtures of whey and milk (1%, 5%, 10%, and 50%), but absent in samples of acid whey and raw milk. The authors claim that the detection of GMP in milk using SDS-PAGE is a sensitive and specific method of detecting milk adulteration in whey, to levels as low as 1%.

1.4.4 Colorimetric

Other methods to measure GMP are based on determination of its glycan moieties, namely sialic acid, which are reviewed extensively by (Nakano and Ozimek 2014). The use of a sialic acid assay to track GMP isolation and purification is important considering the numerous biological activities of GMP which can be attributed to sialic acid (Fukuda et al. 2004). GMP sialic acid has been analysed using the acidic ninhydrin reaction (Yao et al. 1989), a fluorimetric method involving 14% (w/v) TCA and sodium metaperiodate (Rao et al. 2012), and also by HPLC (Fernando and Woonton 2010). Nakano and Ozimek (2014) describe the thiobarbituric acid reaction with 1-propanol as a chromophore extracting solvent as an inexpensive, practical and specific technique. Sephacryl S-200 gel filtration chromatography, cellulose acetate electrophoresis, and SDS-PAGE are the major techniques used to identify sialic acid specific to GMP (Nakano and Ozimek 1999a; Nakano et al. 2006b, 2007, 2009).

1.4.5 Immunological

A number of methods based on immunochemical assays have been developed to detect and quantify GMP, such as biosensor-based immunoassays (Haasnoot et al. 2004), immunoblot (Chávez et al. 2008), immunochromatographic testing (Oancea 2009), and
inhibition enzyme-linked immunosorbent assay (ELISA) (Bitri et al. 1993; Picard et al. 1994; Bremer et al. 2008). Immunoblot and immunochromatographic tests have low sensitivity and biosensor methods require expensive equipment. ELISA is the most frequently used immunoassay in routine test quantification because it is a simple, sensitive, and reliable assay system that allows the use of small sample volumes and high sample numbers. In 2012, Chávez et al. developed and validated a sandwich ELISA system using polyclonal antibodies toward pure bovine GMP that affords a more sensitive analysis of raw milk for the routine detection of GMP as an indicator of adulteration with cheese whey (Chávez et al. 2012).

1.5 Conclusion

In summary, GMP is a bioactive peptide from cheese whey with unique chemical properties in terms of glycosylation, absence of aromatic amino acids and self-association. It is well known that GMP exhibits several useful biological activities, including protection from toxins, bacteria, and viruses and regulation of the immune system. In addition to these biological properties, the peptide has a number of functional physical properties (emulsification and foaming properties, gel formation, wide pH range, solubility, wide heat stability, etc.) that make it a valuable ingredient. For these reasons, dairy companies have invested heavily in methods of selectively isolating intact GMP from whey in order to produce a premium functional ingredient in a commercially viable manner. The main aim is to market GMP for application in the diet of hepatic and PKU patients and also for inclusion in infant formula and sports supplements.

Currently, anion exchange is the technique of choice for large-scale production of GMP from whey. However, commercial production of Phe-free GMP still remains a challenge. Agropur Ingredients recently received the Breakthrough Award for Dairy Ingredient Innovation for its development of highly pure GMP. The product contains just 1–2 mg of Phe/g protein, making it highly functional for PKU products. PKU sphere™ is a powdered, low-phenylalanine protein substitute developed by Nestlé which also contains approximately 1 mg of Phe/g protein. Following on from this, further research should explore ways of developing efficient techniques to purify GMP with undetectable levels of Phe on a commercial scale.

The addition of GMP to infant formula has also raised concern due to its high threonine content (12–13 threonine residues) which increases the occurrence of hyperthreoninaemia in infants fed formula containing GMP versus breastfed infants (Rigo et al. 2001). However, more recent studies suggest this increased occurrence of hyperthreoninaemia is due to differences in threonine metabolism among the infants tested (Sandström et al. 2008). Taking this into consideration, future studies are required to increase our knowledge of the biological and structural functions of GMP while also focusing on the safety of its inclusion as a food ingredient.

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