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Functional and biological activities of casein glycomacropeptide as influenced by lipophilization with medium and long chain fatty acid

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Abstract

This study determined the functional and different biological activities of casein glycomacropeptide (GMP) after conjugation with fatty acids. Medium (i.e. caproic, lauric and myristic acid) and long (i.e palmitic and stearic acid) fatty acids were conjugated to GMP at the available amino group. Functionalities of lipophilized GMP conjugates included foaming and emulsifying activities, and biological activities for bacterial growth inhibition, cell damage and anti-invasion. Greater lipophilization of GMP was achieved with medium chain fatty acids ($p < 0.05$), which resulted in reduced GMP solubility regardless of fatty acid conjugate. Foaming activity of GMP was lost after lipophilization, but emulsification activity of GMP was enhanced ($p < 0.05$). A parallel increase in growth inhibition of *Salmonella* spp. coupled with anti-invasion of *Salmonella enteritidis* (*Inv A*) into mammalian cells was induced by lipophilization of GMP with long chain fatty acid. Our results show that GMP modified by lipophilization with specific fatty acids provides improved functionality as a surfactant with enhanced antibacterial activity towards gram negative bacteria.

1. Introduction

Casein glycomacropeptide (GMP) represents the 106–169 amino acid fragment of κ-casein, which can be produced by the enzymatic cleavage at the Phe_{105}-Met_{106} peptide. The liberated 64 amino acid residue is highly hydrophilic and various forms of GMP can be isolated based on the degree of glycosylation and phosphorylation in the κ-casein (Pedersen et al., 2000).

Numerous bioactive peptides derived from milk have been reviewed (Kitts & Weiler, 2003) and several in vivo biological and physiological activities of GMP have been summarized (El-Salam, El-Shibiny, & Buchheim, 1996). Earlier studies on biological activities of GMP reported the inhibition of both bacterial adhesions by oral *actinomyces* and *Streptococci* species responsible for dental plaque and dental caries (Neeser, 1987; Neeser, Chambez, Delvedova, Prigent, & Guggenheim, 1988) and binding of Cholera toxin from *Vibrio cholera* to Chinese hamster ovary-K1 cells (Kawasaki et al., 1992). Furthermore, a growth-promoting effect of GMP in both *Bifidobacteria* (Idota, Kawakami, & Nakajima, 1994) and *Lactococcus* species (Bouhallab, Favrot, & Maubois, 1993) was demonstrated and this prebiotic activity was attributed to glycosylated *N*-acetylneuraminic acid (Bouhallab et al., 1993). Physiological benefits of GMP have been reported to include reduced platelet aggregation through the binding of platelet membrane glycoprotein GPIbα (Chabance, Qian, Migliore-Samour, Jolles, & Fiat, 1997) and binding of

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^{125}I-fibrinogen to platelets (Sollier et al., 1996), stimulation of pancreatic secretion (Pedersen et al., 2000) and inhibition of splenocytes proliferation induced by lipoposaccharides (Otani, Monnai, & Hosono, 1992).

In light of the reported biological and physiological activities of bioactive peptides, there is little information on the functional activities of GMP in food systems. Functional activities of proteins in foods are often dependent on the amphipathic nature of the compound. The hydrophilic nature of GMP has been shown to reduce emulsifying activity, compared to native amphipathic κ-casein (Chobert, Touati, Bertrand-Harb, Dalgalarrondo, & Nicolas, 1989). Modification of protein involving glycosylation of amino groups at the N-terminus and lysine residues with a reducing sugar, by the Maillard reaction, reported an improved functionality of hydrophilic proteins (Nakamura, Kato, & Kobayashi, 1992). In particular, glycosylation of GMP with lactose can lead to an increased emulsifying activity without significantly reducing the macropeptide solubility (Moreno, Lopez-Fandino, & Olano, 2002).

Proteins that are modified at the amino groups of the N-terminus and lysine residues with the attachment of a fatty acid represent the lipophilization reaction. Unlike the Maillard reaction, which leads to loss of solubility associated with polymerization of glycosylated proteins (Jing & Kitts, 2002), the lipophilization reaction will result in no colour development, but a loss of peptide solubility. Novel functionalities in emulsification and foam formation of hydrophilic proteins was observed after the lipophilization of lysozyme with palmitic acid (Ibrahim, Kato, & Kobayashi, 1991) and stearic acid (Takahashi, Lou, Ishii, & Hattori, 2000), soybean glycinin with palmitic acid (Haque, Matoba, & Kito, 1982) and β-lactoglobulin with stearic acid (Akita & Nakai, 1990). The improved functionalities and biological activities of lipophilized proteins were attributed to the amphipathic nature of the modified proteins (Akita & Nakai, 1990; Haque et al., 1982), which was further supported by the improvement in bactericidal activity of lysozyme after the attachment of a hydrophobic pentapeptide to the C-terminus (Ibrahim, Yamada, Kobayashi, & Kato, 1992, 1994). Lipophilization of lysozyme was shown to enhance the bactericidal activity to include gram negative bacteria (Ibrahim et al., 1991, Ibrahim, Kobayashi, & Kato, 1993; Liu, Sugimoto, Azakami, & Kato, 2000). Furthermore, lipophilization of β-lactoglobulin and lysozyme with stearic acid exhibited a resistance to proteolysis (Akita & Nakai, 1990; Takahashi et al., 2000) and a decrease in allergencitiy of β-lactoglobulin in foods (Akita & Nakai, 1990).

The objectives of this study were to determine the functional and biological activities of native GMP and following lipophilization with fatty acids of different chain length. The effects of lipophilization with medium and long chain fatty acids on specific functionalities in foaming and emulsifying activities, as well as specific biological activities that included antibacterial and anti-invasion activities of GMP is described.

2. Materials and methods

2.1. Materials

All reagent grade chemicals and cell culture media were purchased from Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Toronto, ON). Bacterial culture media were purchased from Difco Laboratories (Detroit, MI). Bacterial culture of *Bacillus subtilis* (ATCC 10774) and *Salmonella typhimurium* (ATCC 13311) were obtained from American Type Tissue Culture (Manassas, VA). Bacterial culture of *Salmonella enteritidis* (*Inv A*) was provided courtesy of Shimane Perfectural Institute of Public Health and Environmental Science (Matsue, Japan). Kidney fibroblast cell CV-1 (ATCC CCL-70) from *Cercopithecus aethiops* was obtained from American Type Tissue Culture (Manassas, VA).

2.2. Preparation of lipophilized GMP

2.2.1. Preparation of GMP from κ-casein

Purified κ-casein was isolated from acid-precipitated whole casein according to the method of Zittle and Custer (1963). GMP was then prepared from purified κ-casein by an enzymatic digestion with chymosin and the liberated macropeptide was purified by dialysis and confirmed by electrophoresis (Coolbear, Elgar, Coolbear, & Ayers, 1996).

2.2.2. Esterification of fatty acids

Free fatty acids of caproic, lauric, myristic, palmitic and stearic (31.5 mmol) were individually esterified to *N*-hydroxysuccinimide fatty esters according to the method of Haque et al. (1982).

2.2.3. Lipophilization of GMP

N-hydroxysuccinimide fatty ester (0.05 mmol) of each fatty acid was covalently attached to the lysyl residue of GMP (0.01 mmol) by a base-catalyzed ester exchange according to the method of Liu et al. (2000). The lipophilized GMP was dialyzed against distilled deionized water and 20 mM phosphate buffer (pH 7), centrifuged, and the supernatant was freeze-dried. Freeze-dried lipophilized GMP was used for experimentation.

2.3. Characterization of lipophilized GMP

2.3.1. Macropeptide concentration

Macropeptide concentration of lipophilized GMP was determined by the Bradford protein assay. In brief, a 160 μl aliquot of diluted lipophilized GMP was mixed

with 40 μl of coomassie blue dye. After 15 min incubation at ambient temperature, the colour intensity was measured at 595 nm. Macropeptide concentration was determined from a standard curve developed from GMP. All lipophilized GMP used for experimentation was prepared on a macropeptide-equivalent basis.

2.3.2. Degree of lipophilization

The degree of lipophilization by *N*-hydroxysuccinimide fatty esters was estimated by quantifying the unbound lysyl residue in a 0.1% macropeptide-equivalent lipophilized GMP sample using trinitrobenzene sulfonic acid (TNBS) as described by Takahashi et al. (2000). Confirmation of attachment of individual fatty acids was made by gas–liquid chromatography analysis.

2.3.3. Total conjugate and protein solubility

Total conjugate and protein solubility of lipophilized GMP were determined in a 2.5% macropeptide-equivalent buffered solution at pH 7. Total conjugate solubility was first estimated by turbidity measurements at 500 nm. Protein solubility in the same sample was determined by the macropeptide absorbance, at 280 nm, in the supernatant after centrifugation at 12,000*g* for 15 min.

2.4. Functional activities of lipophilized GMP

2.4.1. Foaming capacity and stability

A 0.1% macropeptide-equivalent solution of lipophilized GMP was aerated at high speed in a Polytron tissue homogenizer (Brinkmann Instruments, NY) and the foaming capacity and stability was recorded according to the procedure of Haque et al. (1982).

2.4.2. Emulsification capacity and stability

A 0.1% macropeptide-equivalent solution of lipophilized GMP was homogenized with corn oil in a Polytron tissue homogenizer (Brinkmann Instruments, NY) and the emulsification capacity and stability was determined according to the turbidmetric method reported by Shu, Sahara, Nakamura, and Kato (1996).

2.5. Biological activities of lipophilized GMP

2.5.1. Bacterial growth inhibition

An aliquot of a 2.5% macropeptide-equivalent solution of lipophilized GMP was applied to growing cultures of *B. subtilis*, *S. typhimurium* and *S. enteritidis* (*Inv A*) in a Luria–Bertani medium. Growth inhibition were monitored by reading absorbance values at 660 nm over a 24 h period.

2.5.2. Detection of bacterial cell damage

Mid-exponential phase of *B. subtilis*, *S. typhimurium* and *S. enteritidis* (*Inv A*) cultured in Luria–Bertani medium were washed twice in 20 mM sodium phosphate buffer (pH 7) and re-suspended in the same buffer. An aliquot of a 2.5% macropeptide-equivalent solution of lipophilized GMP was added to the bacterial cell suspensions and bacterial cell damage was detected according to the procedure of Nakamura et al. (1992), after 30 min incubation at 50 °C to increase the sensitivity of the bacteria to the presence of macropeptide.

2.5.3. Anti-invasion activity

Confluent CV-1 cells, grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, were seeded in a 24-wells plate at a density of 10^5 cells/ml and allowed to grow to a monolayer. On the day of experimentation, all cells were thoroughly washed with PBS prior to exposure to *S. enteritidis* (*Inv A*) (10^6 CFU/ml) and an aliquot of a 2.5% macropeptide-equivalent solution of lipophilized GMP was made in antibiotic-free MEM supplemented with 10% FBS for 1 h. All cells were then washed twice with PBS and gentamicin (100 g/ml) in PBS was added to destroy surface attached, but non-invaded bacteria. After 2 h of incubation with antibiotics, all cells were again washed twice with PBS and 1% Trintion X-100 was applied. The resulting lysed kidney cell suspension was serially diluted in a Luria–Bertani medium and plated onto nutrient agar. Viable bacterial count on nutrient agar represented the population of *S. enteritidis* (*Inv A*) that invaded the CV-1 kidney cell.

2.6. Statistical analysis

All data are presented as means ± SEM ($n = 6$). One-way analysis of variance (ANOVA) was used to determine treatment effect of lipophilization and the analysis of means by Tukey's test was used to determine significant effects between individual fatty acids.

3. Results and discussion

3.1. Characterizations of modified macropeptides

Protein modification can occur at the N-terminal amino group and lysine residues via the Maillard reaction involving a reducing sugar, or lipophilization with a fatty acid. Theoretically, GMP contains 4 potential modification sites (i.e. 3 lysine residues and 1 N-terminus) that represent potential lipophilization sites if the stioichiometric ratio between the protein and fatty acid is optimized (Ibrahim et al., 1991, 1993). To maximize the degree of lipophilization, a stoichiometric ratio of 1:5 for GMP to fatty acid esters was chosen, based on the number of available modification sites. Our results indicated that the close proximity of two lysine residues in the macropeptide chain likely restricted lipophiliza-

tion to only 3 active sites (Table 1). Increasing chain length of the fatty acid beyond 14 carbons also reduced the degree of lipophilization of GMP, possibly due to stearic hindrance at the active site (Table 1). Other studies employing a different protein, lysozyme, have also shown that lipophilization reaction was greater with medium chain fatty acids (e.g., caproic>capric>myristic acid). Ibrahim et al. (1993) also reported that the rate of incorporation of fatty acids to lysozyme was greater for myristic acid than stearic acid.

GMP is a hydrophilic macropeptide derived from κ-casein and possesses biological activities (El-Salam et al., 1996). Lipophilization with medium chain (i.e., caproic, lauric and myrisitc acid) fatty acids and palmitic acid, in general, did not significantly ($p > 0.05$) reduce the total solubility of GMP in aqueous solutions (Table 2). However, the lipophilization of GMP with the stearic acid greatly decreased ($p < 0.05$) total solubility (Table 2). Minor losses in both total conjugate and more specifically, protein solubility, have been reported by Ibrahim et al. (1991) with the attachment of palmitic acid to lysozyme, and by Akita and Nakai (1990) with the attachment of stearic acid to β-lactoglobulin. The observed reduction in protein solubility is likely due to increased hydrophobic interactions between lipophilized macropeptide, which caused micelle formation in solution and precipitation of GMP.

3.2. Functional properties of modified macropeptides

The primary purpose of lipophilizing hydrophilic GMP was to enhance solubility in non-aqueous solution, thus enhancing the range of potential bioactivities of the original macropeptide to include a less polar

Table 1
Physicochemical characterization of GMP and lipophilized GMP

Sample	Binding ratio (GMP:fatty acid)	Turbidity of conjugate (A_{500})	Protein solubility (%)
GMP	N/A	0.23 ± 0.06^{c}	97.2 ± 2.9^{a}
GMP-caproic acid	1:3	0.26 ± 0.00^{c}	94.6 ± 0.8^{b}
GMP-lauric acid	1:3	0.37 ± 0.00^{b}	93.0 ± 1.8^{b}
GMP-myristic acid	1:3	0.25 ± 0.00^{c}	90.7 ± 1.1^{c}
GMP-palmitic acid	1:2	0.23 ± 0.00^{c}	93.5 ± 0.8^{b}
GMP-stearic acid	1:2	0.68 ± 0.02^{a}	88.2 ± 1.3^{c}

N/A, not applicable.
[a–d] Data (mean ± SEM) in a column with different superscripts are significantly ($p < 0.05$) different.

Table 2
Growth inhibitory effects of GMP and lipophilized GMP on gram-positive and gram-negative bacteria

Time (h)	Turbidity of bacterial suspension (A_{660})						
	Control	GMP	GMP-caproic acid	GMP-lauric acid	GMP-myristic acid	GMP-palmitic acid	GMP-stearic acid
Bacillus subtilis							
0	0.04 ± 0.01^{cE}	0.06 ± 0.01^{bE}	0.07 ± 0.01^{bD}	0.10 ± 0.01^{aD}	0.06 ± 0.01^{bE}	0.07 ± 0.01^{bE}	0.05 ± 0.01^{bE}
4	0.21 ± 0.01^{aD}	0.11 ± 0.05^{cD}	0.07 ± 0.03^{dD}	0.09 ± 0.04^{cD}	0.19 ± 0.09^{bD}	0.21 ± 0.07^{aD}	0.08 ± 0.05^{dD}
8	0.44 ± 0.02^{aC}	0.22 ± 0.09^{cC}	0.21 ± 0.10^{cC}	0.21 ± 0.10^{eC}	0.41 ± 0.10^{bC}	0.40 ± 0.05^{bC}	0.29 ± 0.09^{dC}
12	0.60 ± 0.05^{aB}	0.31 ± 0.10^{dB}	0.40 ± 0.09^{cB}	0.39 ± 0.09^{cB}	0.54 ± 0.11^{bB}	0.51 ± 0.13^{bB}	0.39 ± 0.09^{cB}
24	0.86 ± 0.10^{aA}	0.53 ± 0.11^{eA}	0.62 ± 0.09^{dA}	0.62 ± 0.09^{dA}	0.63 ± 0.08^{dA}	0.70 ± 0.01^{bA}	0.65 ± 0.01^{cA}
Salmonella typhimurium							
0	0.04 ± 0.02^{cE}	0.05 ± 0.02^{cE}	0.07 ± 0.02^{cE}	0.11 ± 0.05^{aD}	0.06 ± 0.03^{cE}	0.07 ± 0.03^{bE}	0.01 ± 0.01^{dE}
4	0.26 ± 0.10^{aD}	0.11 ± 0.09^{cD}	0.11 ± 0.08^{cD}	0.14 ± 0.08^{bD}	0.20 ± 0.10^{dD}	0.23 ± 0.07^{aD}	0.09 ± 0.042^{eD}
8	0.46 ± 0.10^{aC}	0.20 ± 0.07^{eC}	0.28 ± 0.07^{dC}	0.29 ± 0.06^{dC}	0.39 ± 0.08^{cC}	0.42 ± 0.10^{bC}	0.28 ± 0.09^{dC}
12	0.68 ± 0.14^{aB}	0.25 ± 0.11^{gB}	0.46 ± 0.01^{dB}	0.43 ± 0.01^{eB}	0.50 ± 0.01^{cB}	0.54 ± 0.07^{bB}	0.39 ± 0.01^{fB}
24	0.86 ± 0.08^{aA}	0.37 ± 0.06^{dA}	0.66 ± 0.09^{bA}	0.67 ± 0.09^{bA}	0.63 ± 0.06^{bA}	0.68 ± 0.08^{bA}	0.51 ± 0.06^{cA}
Salmonella enteritidis (*Inv A*)							
0	0.04 ± 0.01^{eE}	0.05 ± 0.01^{dE}	0.07 ± 0.00^{bE}	0.12 ± 0.01^{aD}	0.06 ± 0.01^{cE}	0.07 ± 0.01^{cE}	0.01 ± 0.01^{fE}
4	0.22 ± 0.07^{aD}	0.10 ± 0.04^{bD}	0.14 ± 0.04^{cD}	0.17 ± 0.04^{bD}	0.18 ± 0.03^{bD}	0.23 ± 0.03^{aD}	0.06 ± 0.02^{eD}
8	0.46 ± 0.01^{aC}	0.22 ± 0.08^{eC}	0.32 ± 0.07^{dC}	0.31 ± 0.06^{dC}	0.39 ± 0.01^{cC}	0.41 ± 0.03^{bC}	0.27 ± 0.04^{eC}
12	0.63 ± 0.11^{aB}	0.30 ± 0.07^{eB}	0.45 ± 0.06^{cB}	0.44 ± 0.10^{cB}	0.52 ± 0.09^{bB}	0.52 ± 0.10^{bB}	0.39 ± 0.07^{dB}
24	0.84 ± 0.10^{aA}	0.57 ± 0.09^{dA}	0.84 ± 0.10^{aA}	0.78 ± 0.10^{bA}	0.69 ± 0.07^{cA}	0.62 ± 0.09^{cA}	0.54 ± 0.11^{dA}

[a–f] Data (mean ± SEM, $n = 4$) in a row with different superscripts are significantly ($p < 0.05$) different.
[A–E] Data (mean ± SEM, $n = 4$) in a column with different superscripts are significantly ($p < 0.05$) different.

medium. Moreover, similar amphipathic macropeptides often function as a surfactant molecule, which aids in the foam and emulsion formation and stabilization, thus contributing to additional functionalites as well.

The formation of foams requires a surfactant molecule that lowers the surface tension of water, thereby facilitating the incorporation of air droplets into an aqueous system. Foam stabilization requires a strong adherence of surfactant molecules at the air/water interface to prevent the collapse of entrapped air droplets. Native GMP exhibited high foaming capacity and stability at neutral pH (Fig. 1). The lipophilization of GMP with medium chain fatty acids, however, reduced ($p < 0.05$) the foaming capacity, whereas lipophilization with long chain fatty acids completely abolished the foaming capacity of the native macropeptide. This finding is contrary to the enhanced foaming capacity observed with palmitoyl soybean glycinin, which was attributed to the amphipathic nature of the modified protein (Haque et al., 1982). In the present study, the amphipathic nature of the lauric and myristic acid lipophilized GMP was shown to actually decrease foam stability (Fig. 1). We attribute our present observation to the observed decreased in solubility of the long chain fatty acid lipophilized GMP complex, which may reduce the availability of modified macropeptide to interact with the air/water interface. The net result of this effect is the inhibition of GMP to promote both the incorporation and stabilization of entrapped air droplets in the foam.

Chobert et al. (1989) attributed the amphipathic nature of whole casein and κ-casein to a greater emulsification activity than that observed for hydrophilic GMP. Similarly, the increased amphipathic nature of lipophilized β-lactoglobulin, lysozyme and soybean glycinin was responsible for the reported improved emulsification (Akita & Nakai, 1990; Haque et al., 1982; Ibrahim et al., 1991). We found that the hydrophilic nature of GMP was a limiting factor for this macropeptide behaving like an effective emulsifier (Fig. 2). Lipophilization of GMP introduced an amphipathic group that enhanced the emulsion capacity and stability (Fig. 2). The greater overall emulsification activities (i.e. both capacity and stability) of lipophilized GMP in this study could not be attributed to the chain length of the fatty acid.

3.3. Biological properties of modified macropeptides

Past studies have shown that GMP exhibits antibacterial activities by inhibiting adherence of *Streptococci sobrinus* in the oral cavity (Schupach, Neeser, Golliard, Rouvet, & Guggenheim, 1996) and adherence of Cholera toxin to mammalian cell lines (Kawasaki et al., 1992). Recent studies involving enzymatic digestion products of GMP have further revealed novel bactericidal activity of less polar GMP fragments on both gram-positive and gram-negative bacteria (Malkoski et al., 2001; Matin & Otani, 2002). We propose, therefore, that

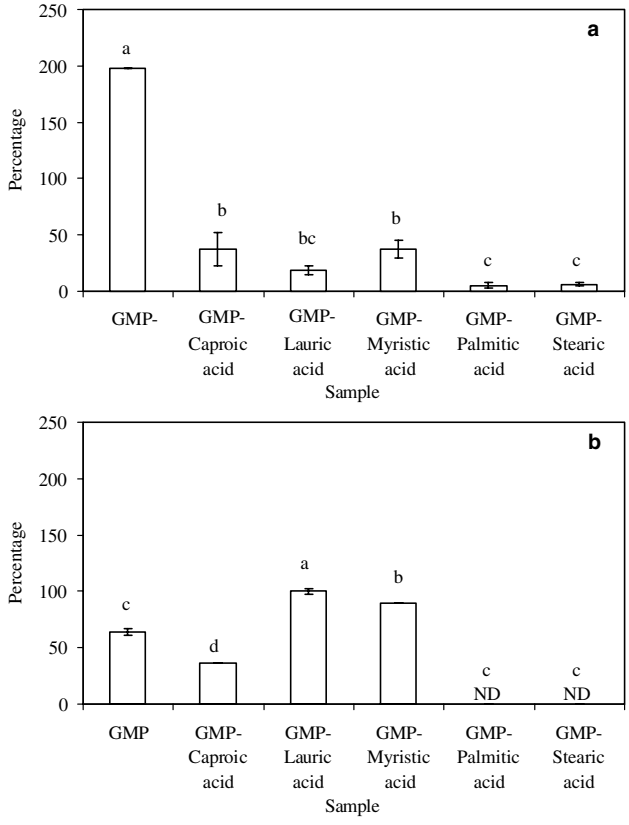

Fig. 1. Foaming capacities (a) and stabilities (b) of GMP and lipophilized GMP. [a–d]Columns with different letters are significantly different ($p < 0.05$). ND denotes none detected.

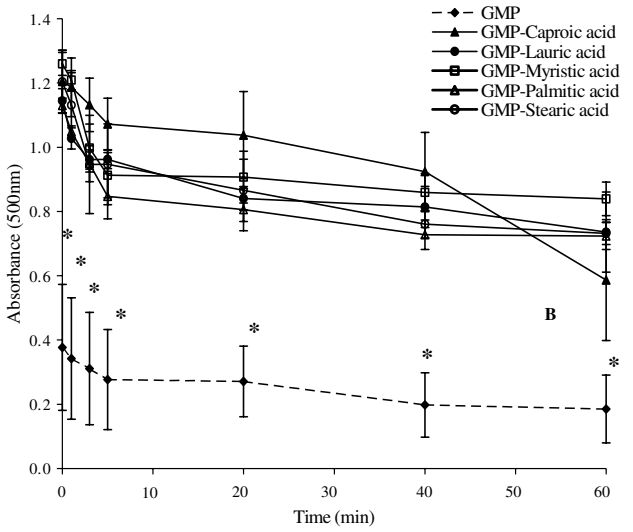

Fig. 2. Emulsification activities of GMP and lipophilized GMP. *Emulsification activity of GMP was significantly different ($p < 0.05$) from all lipophilized GMP. No significant difference ($p > 0.05$) in emulsification activity was found between all lipophilized GMP at each time interval measured.

any modification to the polarity of GMP could affect the antibacterial activity. Furthermore, the free fatty acid form of lauric, myristic and palmitic acid is known to have antibacterial activity (Kabara, 1981; Kabara & Vrable, 1977), which could result in an additive antibacterial activity.

GMP exhibited bacteriostatic effects for both gram-positive (i.e. *B. subtilis*) and gram-negative (*S. typhimurium* and *S. enteritidis Inv A*) organisms (Fig. 3), which are similar to the bacteriostatic activity reported for *Streptococcus mutans Porphyomonas* and*Escherichia coli* (Malkoski et al., 2001). Myristic, palmitic and stearic acids lipophilized GMP had a significantly greater ($p < 0.05$) bacteriostatic effect against both gram-positive and gram-negative bacteria, compared to caproic and lauric acids in this study (Table 2). A parallel increase in cellular damage, as measured by the leakage of intracellular materials, of gram negative bacteria was also observed with long chain fatty acid lipophilized GMP (Fig. 3). Taken together, these findings suggest that an increase in cellular damage caused by the longer chain fatty acids lipophilized GMP conjugates was the reason for the specific bacteriostatic effect noted for lipophilized GMP. Similar findings have been reported for lysozyme conjugated to both medium and long chain fatty acids (Ibrahim et al., 1991, 1993; Liu et al., 2000) and hydrophobic pentapeptide (Ibrahim et al., 1992, Ibrahim, Yamada, Matsushita, Kobayashi, & Kato, 1994). The insertion of modified lysozyme into lipopolysaccharide membrane of gram negative bacteria also facilitated the leakage of cellular materials, which led to the enhanced bactericidal activity of the native lysozyme (Ibrahim et al., 1991, 1993; Liu et al., 2000).

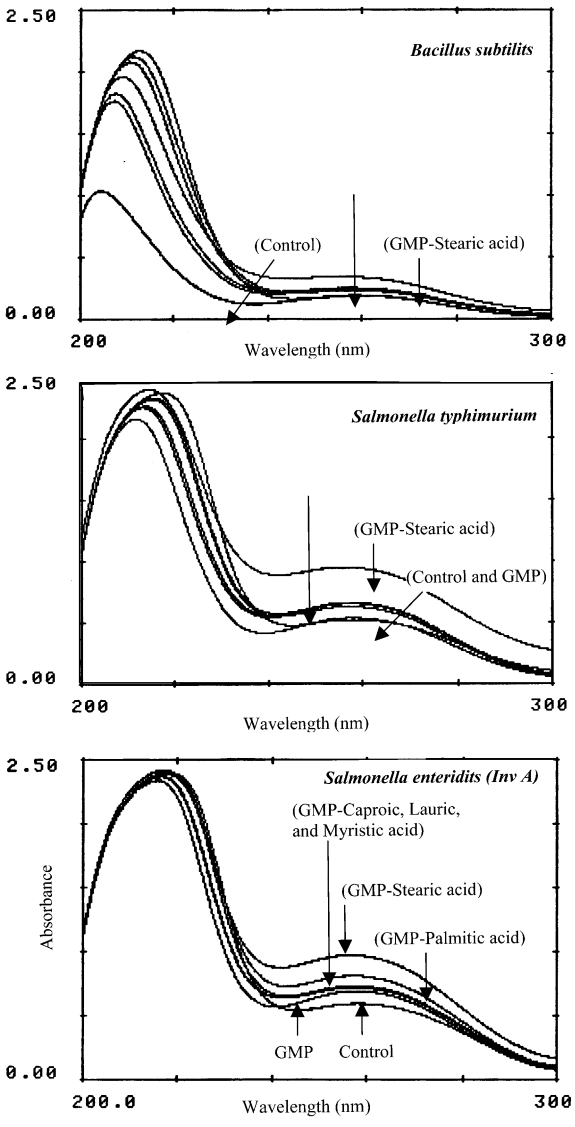

Fig. 3. Bacterial cell damage induced by GMP and lipophilized GMP.

The inhibition of both bacterial adhesion by oral *actinomyces* and *Streptococci* species responsible for dental plaque and dental caries (Neeser, 1987; Neeser et al., 1988) and binding of cholera toxin from *V. cholera* to Chinese hamster ovary-K1 cells (Kawasaki et al., 1992) are important properties of GMP to act as an antibacterial agent, or prevent food borne illness. Kawasaki et al. (1992) suggested that the hydrophilicity of GMP might affect the antibacterial activity by decreasing cholera toxin binding affinity. Decreasing the hydrophilicity of lipophilized GMP in this study slightly enhanced the anti-invasion property of native GMP in mammalian CV-1 cell lines (Fig. 4). An increased membrane permeability of *S. enteritidis* (*Inv A*) in the presence of lipophilized GMP may have been responsible for the decreased invasion into the mammalian cell observed herein. Earlier investigations have also suggested that GMP can be adsorbed onto the surface of oral pellicle, which masks the ligand receptors on salivary

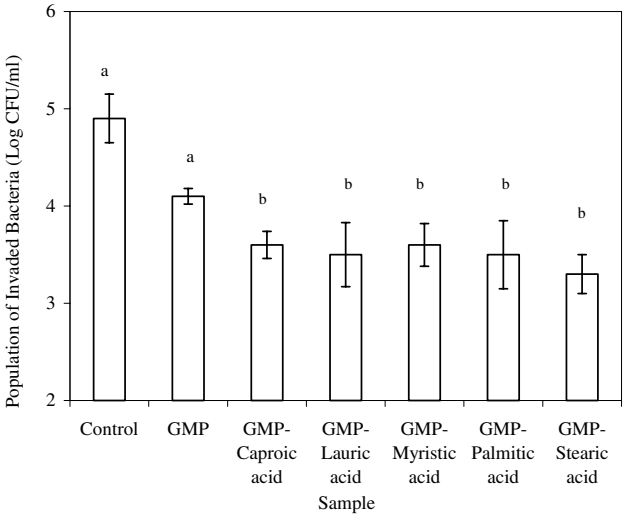

Fig. 4. Anti-invasion activity of GMP and lipophilized GMP against *Salmonella enteritidis* (*Inv A*) in CV-1 cells. [a–b]Columns with different letters are significantly different ($p < 0.05$).

molecules for bacteria recognition (Schupach et al., 1996; Neeser et al., 1988; Neeser, 1987). Thus, an interaction between lipophilized GMP and lipid membrane of CV-1 cell line could also act to decrease invasion by *S. enteritidis* (*Inv A*), reported herein. Taken together, both observations likely are a result of a similar non-specific inhibition of bacterial attachment by polymers with varying physicochemical characteristics (Neeser et al., 1988).

4. Conclusion

The specific chain length of fatty acids used for lipophilization of GMP influenced the degree of GMP lipophilization. Lipophilization decreased the total solubility of GMP in aqueous solution, regardless of chain length, due to the aggregation and precipitation resulting from the increased hydrophobicity induced by the attached fatty acid. Functionality in foam formation and stabilization was greatly reduced by lipophilization of GMP; especially with medium to long chain fatty acids. However, emulsification capacity was greatly enhanced by the amphipathic nature of the lipophilized GMP. A parallel increase in bacteriostatic effect and bacterial cell damage in gram-negative bacteria was induced specifically by long chain fatty acids lipophilized to GMP. This result suggests a disruption of bacterial lipid membrane by the inserted lipophilized macropeptide. Similarly, lipophilized macropeptides incubated with a mammalian cell membrane resulted in reduced invasions by *S. enteritidis* (*Inv A*). The lipophilization reaction of macropeptide with a fatty acid resulted in amphipathic GMP conjugates that provided improved functionality as a surfactant and an antibacterial agent against gram negative bacteria.

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References

Akita, E. M., & Nakai, S. (1990). Lipophilization of β-lactoglobulin: effect on hydrophobicity, conformation and surface functional properties. *Journal of Food Science, 55*, 711–717.

Bouhallab, S., Favrot, C., & Maubois, J. L. (1993). Growth-promoting activity of tryptic digest of caseinomacropeptide for *Lactococcus lactis* subsp *lactis*. *Lait, 73*, 73–77.

Chabance, B., Qian, Z. Y., Migliore-Samour, D., Jolles, P., & Fiat, A. M. (1997). Binding of the bovine caseinoglycopeptide to the platelet membrane glycoprotein GPIbα. *Biochemistry and Molecular Biology International, 42*, 77–84.

Chobert, J. M., Touati, A., Bertrand-Harb, C., Dalgalarrondo, M., & Nicolas, M. G. (1989). Solubility and emulsifying properties of kappa casein and its caseinomacropeptide. *Journal of Food Biochemistry, 13*, 457–473.

Coolbear, K. P., Elgar, D. F., Coolbear, T., & Ayers, J. S. (1996). Comparative study of methods for the isolation and purification of bovine κ-casein and its hydrolysis by chymosin. *Journal of Dairy Research, 63*, 61–71.

El-Salam, M. H. A., El-Shibiny, S., & Buchheim, W. (1996). Characteristics and potential uses of the casein macropeptide. *International Dairy Journal, 6*, 327–341.

Haque, Z., Matoba, T., & Kito, M. (1982). Incorporation of fatty acid into food protein: palmitoyl soybean glycinin. *Journal of Agricultural and Food Chemistry, 30*, 481–486.

Ibrahim, H. R., Kato, A., & Kobayashi, K. (1991). Antimicrobial effects of lysozyme against gram negative bacteria due to covalent binding of palmitic acid. *Journal of Agricultural and Food Chemistry, 39*, 2077–2082.

Ibrahim, H. R., Kobayashi, K., & Kato, A. (1993). Length of hydrocarbon chain and antimicrobial action to gram negative bacteria of fatty acylated lysozyme. *Journal of Agricultural and Food Chemistry, 41*, 1164–1168.

Ibrahim, H. R., Yamada, M., Kobayashi, K., & Kato, A. (1992). Bactericidal action of lysozyme against gram-negative bacteria due to insertion of a hydrophobic pentapeptide into its C-terminus. *Bioscience, Biotechnology and Biochemistry, 56*, 1361–1363.

Ibrahim, H. R., Yamada, M., Matsushita, K., Kobayashi, K., & Kato, A. (1994). Enhanced bactericidal action of lysozyme to *Escherichia coli* by inserting a hydrophobic pentapeptide into its C-terminus. *The Journal of Biological Chemistry, 269*, 5059–5063.

Idota, T., Kawakami, H., & Nakajima, I. (1994). Growth-promoting effects of *N*-acetylneuraminic acid-containing substances on Bifidobacteria. *Bioscience, Biotechnology and Biochemistry, 58*, 1720–1722.

Jing, H., & Kitts, D. D. (2002). Chemical and biochemical properties of casein-sugar maillard reaction products. *Food and Chemical Toxicology, 40*, 1007–1015.

Kabara, J. J. (1981). Food-grade chemicals for use in designing food preservative systems. *Journal of Food Protection, 44*, 633–647.

Kabara, J. J., & Vrable, R. (1977). Antimicrobial lipids: Natural and synthetic fatty acids and monoglycerides. *Lipids, 12*, 753–759.

Kawasaki, Y., Isoda, H., Tanimoto, M., Dosako, S., Idota, T., & Ahiko, K. (1992). Inhibition by lactoferrin and κ-casein glycomacropeptide of binding *Cholera* toxin to its receptor. *Bioscience, Biotechnology and Biochemistry, 56*, 195–198.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Application of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design, 9*, 1309–1323.

Liu, S. T., Sugimoto, T., Azakami, H., & Kato, A. (2000). Lipophilization of lysozyme by short and middle chain fatty acid. *Journal of Agricultural and Food Chemistry, 48*, 265–269.

Malkoski, M., Dashper, S. G., O'Brien-Simpson, N. M., Talbo, G. H., Macris, M., Cross, K. J., et al. (2001). Kappacin, a novel antibacterial peptide from bovine milk. *Antimicrobial Agents and Chemotherapy, 45*, 2309–2315.

Matin, M. D. A., & Otani, H. (2002). Cytotoxic and antibacterial activities of chemically synthesized κ-casecidin and its partial peptide fragements. *Journal of Dairy Research, 69*, 329–334.

Moreno, F. J., Lopez-Fandino, R., & Olano, A. (2002). Characterization and functional properties of lactosyl caseinomacropeptide conjugates. *Journal of Agricultural and Food Chemistry, 50*, 5179–5184.

Nakamura, S., Kato, A., & Kobayashi, K. (1992). Bifunctional lysozyme-galactomannan conjugate having excellent emulsifying properties and bactericidal effect. *Journal of Agricultural and Food Chemistry, 40*, 735–739.

Neeser, J. R. (1987). Caseinoglycopeptides as dental plaque and dental caries inhibiting agents. European patent application EP, 283, 675.

Neeser, J. R., Chambez, A., Delvedova, S., Prigent, M. J., & Guggenheim, B. (1988). Specific and non-specific inhibition of adhesion of oral actinomyces and streptococci to erythrocytes and polystyrene by caseinoglycopeptide derivatives. *Infection and Immunity, 56*, 3201–3208.

Otani, H., Monnai, M., & Hosono, A. (1992). Bovine κ-casein as inhibitor of the proliferation of mouse splenocytes induced by lipopolysaccharides stimulation. *Milchwissenschaft, 47*, 512–515.

Pedersen, N. L., Nagain-Domaine, C., Mahe, S., Chariot, J., Roze, C., & Tome, D. (2000). Caseinomacropeptide specifically stimulates exocrine pancreatic secretion in the anesthetized rat. *Peptides, 21*, 1527–1535.

Schupach, P., Neeser, J. R., Golliard, M., Rouvet, M., & Guggenheim, B. (1996). Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci. *Journal of Dental Research, 75*, 1779–1788.

Shu, Y. W., Sahara, S., Nakamura, S., & Kato, A. (1996). Effects of the length of polysaccharide chains on the functional properties of the maillard-type lysozyme-polysaccharide conjugate. *Journal of Agricultural and Food Chemistry, 44*, 2544–2548.

Sollier, C. B. D., Drouet, L., Pignaud, G., Chevalier, C., Caen, J., Fiat, A. M., et al. (1996). *Thrombosis Research*, 427–437.

Takahashi, K., Lou, X. F., Ishii, Y., & Hattori, M. (2000). Lysozyme-glucose stearic acid monoester conjugate formed through the maillard reaction as an antibacterial emulsifier. *Journal of Agricultural and Food Chemistry, 48*, 2044–2049.

Zittle, C. A., & Custer, J. H. (1963). Purification and some of the properties of α_s-casein and κ-casein. *Journal of Dairy Science, 46*, 1183–1188.