Bioactive Properties of Milk Proteins with Particular Focus on Anticariogenesis

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ABSTRACT Beyond nutrition, there is an increasing amount of data and information to demonstrate a bioactive role for dairy components in adults including a role in prevention of dental caries. Specifically, the casein fraction and hydrolysates thereof have been the focus of researchers investigating cariogenicity prevention. Tooth enamel is a polymeric substance consisting of crystalline calcium phosphate embedded in a protein matrix. Dental caries develop by acidic demineralization (calcium and phosphorous solubilization) of tooth enamel. Demineralization occurs directly (acidic food consumption) or indirectly (by fermentation products of dental plaque odontopathogenic bacteria growing on residual food particles between teeth or adhering to the plaque). Research efforts with milk derived bioactive peptides have focused on inhibition of cariogenic, plaque-forming bacteria, inhibition of tooth enamel demineralization, and subsequent enamel remineralization. Caseinophosphopeptides (CPP) and glycomacropeptide (GMP) have been patented for use in common personal hygiene products to prevent dental caries. Research has shown CPP and GMP to be growth inhibitory to the cariogenic bacteria Streptococcus mutans and other species. Additionally, CPP forms nanoclusters with amorphous calcium phosphate (AMP) at the tooth surface to provide a reservoir of calcium and phosphate ions to maintain a state of super saturation with respect to tooth enamel. This would buffer plaque pH, and also provide ions for tooth enamel remineralization. Glycosidic structures attached to GMP are important to numerous bioactive properties of the peptide including anticariogenicity. Like CPP, GMP has shown inhibitory activity to enamel demineralization and promotes tooth enamel remineralization. J. Nutr. 134: 989S–995S, 2004.

KEY WORDS: • milk • bioactives • anticariogenicity • caseinophosphopeptide • glycomacropeptide

Dental caries (tooth decay) are a major public health problem that plagues all countries in the world. Industrialized nations have controlled the problem with fluoride enriched water and personal hygiene products since early in the 1960s, but cariogenicity remains a crisis that economically burdens the health care system to an extent greater than many publicized diseases such as heart disease, cancer, and hypertension. Dental disease remains a “silent epidemic” in the United States that threatens children and adults (1). As developing countries begin consuming more developed foods, tooth decay also is becoming an issue (2). High risk of dental caries is accentuated by a number of sociodemographic variables including ethnicity and low socioeconomic status. Certain individuals are also at risk as a complication of other disease states including diabetes (3,4), obesity (5), and osteoporosis (6). Researchers and product developers continue to search for products to reduce overall severity and prevalence of dental caries. A major emphasis has been placed on developing products that are convenient to the consumer such as chewing gums and sugar-free confections that offer a degree of protection from the causative agents of tooth decay.

Milk is an excellent protein food that provides essential amino acids and organic nitrogen for humans and animals of all ages. Milk also contains factors that have anticariogenic properties: calcium, phosphate, casein, and lipids. Dairy products were recognized in the late 1950s as a food group that is effective in preventing dental caries. Shaw et al. (7) observed that milk, ice cream, and cheese lowered incidence of dental caries in rats. Epidemiological studies in recent years indicate children (8) and adolescents (9) with low incidence of dental caries drank more milk. Several reviews describe the role of milk and dairy products in dental caries prevention (11–13). The purpose of this paper is to review the role of minor milk proteins and bioactive peptides embedded in the major milk proteins that inhibit cariogenicity.

Dental caries pathogenesis

Children’s teeth become infected with potential odontopathogenic bacteria between middle of y 2 and end of y 3 of life—the “window of infectivity” (14). Primary source of in-
fection for infants is maternal, but certain environmental conditions, such as infants born into a high caries-prone population, can also favor nonfamilial infection (15). Children that are not infected by a high maternal dose by 3 y of age remain minimally colonized by odontopathogenic bacteria until eruption of their secondary teeth.

Caries lesions, or tooth decay, are the clinical manifestation of a pathogenic process that may have been occurring as a series of interactions on the tooth surface for months or years. Plaque is a biofilm over tooth enamel composed of viable and nonviable bacteria, mucopoly saccharides, and other cellular debris and metabolites. The first step in cariogenicity is that indigenous oral bacteria begin decay by interacting with dietary constituents (e.g., sucrose) at the tooth enamel’s surface. Dental plaque appearance on tooth enamel is the first overt clinical evidence of this interaction. Plaque bacteria metabolize dietary sugars to produce organic acids that solubilize tooth enamel composed of hydroxyapatite crystals of calcium phosphate. When enamel is exposed to organic acids, solid calcium phosphate is solubilized to free calcium that is removed from the mouth by saliva movement. This process is termed demineralization, but can be reversed by presence of salivary sodium bicarbonate that aids in remineralization.

The role of bacteria in causing dental caries is a source of continual controversy. At issue has been whether a specific bacterial species or a nonspecific mixed bacterial flora is the agent responsible. Also debated is if dental caries is an infectious bacterial disease in the classical sense or an ecological overgrowth (16). Frequent presence of Lactobacillus acidophilus and Streptococcus mutans with caries activity gave credibility to their being specific cariogens. However, many other indigenous oral bacteria are capable of producing substantial amounts of organic acid from fermentable carbohydrates providing arguments for nonspecificity. Numerous studies have shown some indigenous bacteria are capable of remineralizing tooth enamel to prevent dental caries. To date, more research in understanding mixed-bacterial ecology and metabolism is needed to develop therapeutic strategies to counter excess acid accumulation and tooth demineralization.

Dental caries is still the predominant cause of tooth loss in all populations worldwide. Numerous approaches have been used to protect children and adults from cavities. Milk and dairy products have been identified as having cariostatic factors. However, milk-derived cariostatic factors have limited effectiveness in their natural source because they would require large consumption of dairy products. Researchers have focused on isolating protective factors from milk to use as food additives or in personal hygiene products to reduce cariogenicity.

Milk proteins

Milk is synthesized in mammary secretory epithelial cells and contains 2 major protein groups distinguished by their solubility in unheated milk at pH 4.6 and 20°C: caseins (insoluble) and whey proteins (soluble). Both groups have unique physiochemical and biological properties. Caseins account for ~80% of the total protein in bovine milk, and exist primarily as calcium phosphate stabilized micellar complexes. Caseins are a heterogeneous family of proteins predominated by $\alpha_s^1$, $\alpha_s^2$, $\beta$, and $\kappa$-caseins. Individual casein proteins are small molecules with a molecular mass of 20 to 25 kDa, and primary amino acid sequences that are high in proline content. Proline prevents casein molecules from having much secondary structure ($\alpha$-helices, $\beta$-sheets, and $\beta$-turns). All caseins show genetic polymorphism and have post-translational modification with either phosphorus and/or carbohydrate moieties. Caseins are relatively hydrophobic, but have primary sequence clusters that have high surface hydrophobicity that contributes to functional properties such as emulsification and foaming. Fox (18) and Wong et al. (19) review the relationship of individual casein’s structure and function.

Whey proteins (20% of total milk protein) are also a heterogeneous, polymorphic group of proteins composed of $\alpha$-lactalbumin ($\alpha$-LA, $\beta$), $\beta$-lactoglobulin ($\beta$-Lg, 50%), serum albumin (BSA, 10%), immunoglobulins (10%), and protease peptides (<10%). Unlike caseins, whey proteins have high levels of secondary, tertiary, and quaternary structures, and are typically heat-labile globular structures. All whey proteins contain intermolecular disulfide bonds that stabilize their structure. Whey proteins are not extensively glycosylated, and none are phosphorylated. The dominant proteins ($\alpha$-LA and $\beta$-Lg) are responsible for functional properties, predominantly foaming and gelation, that have been commercialized in whey protein concentrate and isolate products.

Milk contains numerous minor proteins found mainly in the whey and milk fat globule membrane fractions. These minor proteins do not have significant functional properties like casein and whey fractions, but many have been identified as having physiological effects. The minor proteins include enzymes, metal-binding proteins, enzyme inhibitors, vitamin-binding proteins, and numerous growth factors (18). Several minor dairy proteins have been included as bioactive ingredients in nutraceutical products.

Dairy protein bioactivities

Bioactive proteins and peptides are embedded in casein and whey primary sequences. A variety of regulatory activities are exerted by milk-derived bioactive sequences. Biological activities identified include modulators of digestive and gastrointestinal functions, hemodynamics (hypertension and gastric blood flow), anticariogenicity, analgesic properties, growth factors, immunoregulation, and nonimmune disease defense. Most bioactivities are only expressed by peptides derived from the amino acid sequence of native milk proteins. Digestive proteases or in vitro proteolysis liberates bioactive peptides to the host’s benefit. Milk-derived peptides are now commercially produced and these peptides are being used as dietary supplementation in functional foods and personal products. However, very few clinical trials have been done to prove safety of milk-derived peptides. Most commercial companies have presumed safety on basis of a “safe” starting raw material. Acute toxicity, allergenicity, and nutritional studies have not been adequately conducted (20). Extensive reviews on milk-derived bioactivities are written (21,22).

Lactoferrin is an iron-binding protein found in milk of many species including bovine and human, and has been observed to possess numerous bioactive properties. The bioactive role of lactoferrin appears to be dependent on exceptional iron-binding activity by the molecule. Iron availability from an infant formula supplemented with bovine lactoferrin has been evaluated by iron balance studies in human infants (23). Iron retention was 36% in the supplemented group versus 28% in the nonsupplemented group. Lactoferrin has also been

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3 Abbreviations used: ACP, amorphous calcium phosphate; $\alpha$-LA, alpha-lactalbumin; $\beta$-Lg, beta-lactoglobulin; BSA, bovine serum albumin; CPP, caseinophosphopeptides; GMP, glycomacropeptide; SCN-, thiocyanate; S-HA, saliva-coated hydroxyapatite beads.
catalyzes the oxidation of thiocyanate (SCN–) produced by the mammary gland. This enzyme is part of a complex. Lactoperoxidase is a porphrin-containing peroxidase secreted by the mammary gland or digestive tract, or by in vitro digestion with commercial proteases. The resulting peptides influence particular physiological or metabolic processes. Immunological derived bioactive components result from vaccinating cows to induce production of antibodies to the vaccine antigen.

Other minor proteins in bovine milk have antibacterial activity. Lysozyme is an effective antibacterial enzyme isolated from milk, tears, and saliva. Human milk is a better source of lysozyme (2.5 mg/100 mL) than bovine milk (0.025 mg/100 mL). The mechanism of action for lysozyme is to hydrolyze β(1→4)-glucosidic linkages in bacterial cell wall peptidoglycan. Lactoperoxidase is a porphrin-containing peroxidase secreted by the mammary gland. This enzyme is part of a complex that ultimately has antibacterial effects. Lactoperoxidase catalyzes the oxidation of thiocyanate (SCN–) to hypohiacyanate by using hydrogen peroxide produced by endogenous bacteria (Fig. 1).

Induced bioactive components are defined as activities derived by proteolytic or immunological means (30). Protein modification by proteases can occur in situ in the mammary gland or digestive tract, or by in vitro digestion with commercial proteases. The resulting peptides influence particular physiological or metabolic processes. Immunological derived bioactive components result from vaccinating cows to induce production of antibodies to the vaccine antigen.

Bovine κ-casein treated with chymosin is cleaved into 2 peptides during the cheese making process. Para-κ-casein (residues 1–105) remains with the curd, while a unique peptide, glycomacropeptide (residues 106–169), elutes with the whey (17). Glycomacropeptide (GMP) is a major component of cheese whey as it is 15–20% of the total protein. Glycomacropeptide is a glycosylphosphate with no aromatic amino acids. This could have positive implications for certain dietary restricted populations.

Glycosidic structures (Table 1) attached to the peptide are important in GMP bioactivity. The ability to bind enterotoxins from Vibrio cholerae and Escherichia coli has been reported with GMP (31). Carbohydrates attached to GMP mimic receptor sites for enterotoxins. Therefore, in animal challenge studies GMP binds enterotoxin and the complex is washed from the intestinal tract. GMP treated with neuraminidase to remove sialic acid carbohydrate moiety from the terminal end of the glycosidic side chain on GMP lost its bioactivity. Feeding mice GMP (1 mg/d) during in vivo trials protected the animals from E. coli and V. cholerae enterotoxin associated diarrhea (32).

Kawasaki et al. (33) demonstrated that GMP inhibits hemagglutination of 4 human influenza virus strains. The effect was noted with low GMP concentrations (80 mg/L). Hemagglutination of Mycoplasma gallisepticum (34) and M. pneumoniae (35) is not inhibited by GMP.

The role of GMP as a prebiotic is elusive. Prebiotics are compounds that promote growth of desirable gastrointestinal bacteria (probiotics). Kehagias et al. (36) reported bifidobacteria growth was promoted by a casein fraction that was similar to GMP. Poch and Bezkorovainy (37) demonstrated that bovine casein and whey digest promoted bifidobacteria growth. Petchow and Talbott (38) found that growth promoting activity for some bifidobacteria species is present in bovine milk ultrafiltration permeate and retentate. GMP would be present in the retentate. Therefore, it appears permeate may contain amino acids and other compounds also necessary for bifidobacterial growth.

Milk micelles contain physiologically significant amounts of calcium and phosphorus. Phosphorus in milk is bound via monoester linkages to casein serum residues. The presence of phosphorus and calcium bound to casein helps to maintain thermodynamically stable casein micelles in fluid milk. Caseinophosphopeptides (CPPs) are phosphorylated casein-derived peptides produced by proteolytic digestion of αs1, αs2, and β-casein in vitro or in the digestive tract (Table 2). CPP complexes and solubilizes minerals, especially calcium. CPP improves bioavailability in intestinal absorption by keeping minerals soluble and preventing precipitation. Most CPPs contain a serine phosphate cluster and glutamyl residues in the sequence of 3 phosphoserine groups followed by 2 glutamic acid residues. These amino acids serve as mineral-binding sites because of negatively charged side chains. However, there is a difference in calcium binding activity depending on the casein phosphopeptide source (39). The order of mineral chelating ability is αs2-casein > αs1-casein > β-casein > κ-casein.

Casein phosphopeptides released from casein molecules are resistant to further proteolytic breakdown in the intestinal tract.
Anticariogenic activity of milk bioactive proteins

Early studies recognized that dairy products (milk, casein, caseinates, and cheeses) exhibited anti-caries activity (50, 51). Acid casein (insoluble) as an active ingredient in toothpaste was effective at reducing dental caries, but was required at very high levels for activity (52, 53). Sodium caseinate solubilized in water and fed to rats in a caries model was shown to be anticariogenic (54). Sodium caseinate as an ingredient in a chocolate confectionary reduced cariogenicity, but high levels of caseinate (17%) were required to elicit an effect and the product was unpalatable (55, 56). Therefore, early studies demonstrated that casein was an effective anticariogenic substance, but casein’s adverse organoleptic properties and the large amount required for efficacy precluded its use as a food or toothpaste.

Tryptic digestion of caseinate did not destroy the proteins’ ability to prevent enamel demineralization in a human oral caries model (57). Analysis of human dental plaque samples found elevated concentrations of casein peptides, calcium, and phosphorus. It was concluded that these peptides were caseinophosphopeptides derived from specific tryptic activity on αs1-, αs2-, and β-caseins. This prompted investigators to focus on casein peptides in subsequent research.

Many of the reported physiologically active or bioactive components account for a very minor fraction of total milk constituency. This group includes lactoferrin, lysozyme, lactoperoxidase, folate-binding protein, growth factors, and other proteins (Table 3). Immunoglobulin proteins also have a protective role in vivo. Bioactive components that have a role in prevention of dental caries would need to mechanistically inhibit odontopathogenic bacteria and/or bind minerals.

Prevention of dental caries by milk-derived bioactive peptides is a complex physical and chemical sequence of cascading events. In general, bioactive peptides with anticariogenic activity have multiple functions to prevent dental lesions including bacterial inhibition, competitive exclusion to enamel binding sites, improved buffering capacity in the pellicle surrounding teeth, reduced enamel demineralization, and enamel remineralization. Antiangiogenicity studies with dairy bioactive peptides have been accomplished with a number of in vitro, in situ, and in vivo model systems. Much of the work has been done with caseinophosphopeptides-colloidal amorphous calcium phosphate (CPP-ACP). Caseinophosphopeptides inhibit dental caries lesions by influencing the demineralization/remineralization process of dental enamel. A 1% (wt/v) CPP solution can stabilize 60 mmol/L CaCl2 and 36 mmol/L sodium phosphate at pH 7.0 to form CPP-ACP complexes (58).

Specifick pathogen-free rats orally infected with Streptococcus sobrinus had a reduced incidence of smooth surface caries after CPP-ACP solutions were applied to the animal’s teeth twice daily. A dose dependent response was observed with a 0.1% (wt/v) CPP-ACP producing a 14% reduction, and 1.0% CPP-ACP a 55% reduction relative to a distilled water control. These results were similar to a 50 ppm fluoride control. A synergistic effect was reported when CPP-ACP (0.5%) and fluoride (300 ppm) were applied together to rat’s teeth. Nonphosphorylated casein peptides had no anticariogenic effects. A synthetic octapeptide, Ac-Glu-Ser(P)Ile-Ser(P)Ser(P)-Ser(P)-Glu-Glu-NHMe, significantly reduced caries activity in the rat model. This confirmed that the xnSer(P)Ser(P)-Ser(P)-Glu-Glu-xn portions of CPP are associated with anticariogenicity. However, the synthetic octapeptide was not as effective in binding ACP or in anticariogenicity as CPP-ACP. This indicates other residues and/or conformational specificity such as in the longer αs1- and β-peptides are required for full activity (58).

A human in situ caries model has been used to study the ability of 1% CPP-ACP to prevent enamel demineralization (59). Humans are fitted with a removable dental appliance that contains a left and a right pair of enamel slabs placed in the mouth to produce a plaque retention site. Frequent exposure to sucrose solutions elevated levels of oral mutans streptococci and lactobacilli. Subsurface enamel demineralization resulted in an incipient “caries-like” lesion. Two daily exposures of CPP-ACP solution to 1 side of the enamel slab reduced (51 ± 19%) enamel mineral loss compared to the control side. Plaque exposed to CPP-ACP had 2.5 times more Ca and phosphorus than control plaque. Caseinophosphopeptides stabilize ACP; in turn this is used to localize ACP in dental plaque, provides a large calcium reservoir within plaque, and slows diffusion of free calcium (60). Restricted mineral loss during a cariogenic episode is likely to occur, and conversely provide a source of calcium for remineralization. Therefore, the mechanism of anticariogenicity for CPP-ACP is that this bioactive peptide substantially increases the level of amorphous calcium phosphate in plaque depressing enamel demineralization and enhancing remineralization. As the concentration of CPP-ACP in contact with tooth enamel increases so does remineralization (Fig. 2).
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Salivary pellicle, and reduced adherence of S. sobrinus enamel (63).
This could also control acid formation (buffering) in dental metabolism of less cariogenic species such as oral actinomyces (62).

Commercialization of milk bioactives

There is commercial interest in production of dairy-derived bioactive peptides with the purpose of using them in commercial products such as toothpastes, gels, or mouth rinses. Casein GMP was patented as an antimicrobial agent effective against S. mutans (73,74). Product developers incorporated GMP into commercial products that were protected by patents (75). The uniqueness of this patent was the use of hydrolyzed gelatin as a stabilizer in the compositions. A patent was issued (76) for a multicomponent anticaries dentifrice composition and methods to use that contained GMP and fluoride in combination. Another patent (77) was issued when it was disclosed that GMP in combination with xylitol would act synergistically to yield enamel remineralization results greater than those observed with xylitol and fluoride in combination (Fig. 3). These patent’s claims were supported by in vitro enamel remineralization data. A patent has been filed (Warner-Lambert Company) to use CPP in chewing gum compositions to promote anticariogenicity. Lactoperoxidase and lactoferrin have been formulated into toothpaste to be bactericidal to cariogenic bacteria. Glucose oxidase in this toothpaste activates the lactoperoxidase system to produce hypothiocyanous acid and hypoiodite ions in the oral environment (Fig. 4), resulting in inhibition of odontopathogenic bacteria.

Future research

Tooth decay is a series of interactions that occur on a tooth surface, and remains a critical health problem despite advances made in fluoridation of water and toothpastes. Research data outlined in this paper presents a strong case that dairy-derived bioactive peptides reduce dental caries in humans using in situ...
**FIGURE 4** The effectiveness of tooth brushing for 1 min with a commercial toothpaste that contains glucose oxidase (10,000 U), lactoperoxidase (15,000 U), and lysozyme (16 mg) as active ingredients on the generation and decomposition of hypohydrocyanic acid (HOSCN) and hypohydrocyanic ions (OSC(N)H) (69).

methods. However, future work should be focused on in vivo and epidemiological effects of bioactive consumption in reducing or eliminating dental caries wherein artificial plaque-developing environments are not depended upon to study caries prevention. Minimally, an effect should be observed in reducing caries severity. Products developed with CPF to date have enabled the peptides to remain in prolonged contact (such as chewing gum and toothpastes) with the plaque environment to prevent dental caries. The primary mechanisms of action are a reduction in enamel demineralization and improved enamel remineralization.

**LITERATURE CITED**

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