SPECIAL GUEST EDITOR SECTION

Bioactive Peptides Derived from Food

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As interest in the ability of functional foods to impact on human health has grown over the past decade, so has the volume of knowledge detailing the beneficial roles of food-derived bioactive peptides. Bioactive peptides from both plant and animal proteins have been discovered, with to date, by far the most being isolated from milk-based products. A wide range of activities has been described, including antimicrobial and antifungal properties, blood pressure-lowering effects, cholesterol-lowering ability, antithrombotic effects, enhancement of mineral absorption, immunomodulatory effects, and localized effects on the gut. Although there is still considerable research to be performed in the area of food-derived bioactive peptides, it is clear that the generation of bioactive peptides from dietary proteins during the normal digestive process is of importance. Therefore, it will become necessary when determining dietary protein quality to consider the potential effects of latent bioactive peptides that are released during digestion of the protein.

In the last decade, in particular, scientific interest in food peptides that have a bioactive role, such as reducing the risk of disease, has grown considerably. As the consumer becomes more knowledgeable and, with continued developments in food technology and nutritional science, the market for a range of products with health benefits beyond basic nutritional functions is growing, as is the manufacturer's ability to make a functional food product. The role of bioactive peptides introduces a completely new dimension to be considered in the description of dietary protein quality.

Bioactive peptides are generally 3–20 amino acid residues in length and, although both animal and plant proteins are known to contain potential bioactive sequences, to date, milk proteins have provided the greatest source of biologically active peptides. As well as resulting from the digestion of food proteins, there are a number of bioactive peptides that are present naturally in foods, such as milk growth cofactors (1).

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The methods for studying bioactive peptides are varied, although many rely on in vitro methods for demonstrating biological activity. Generation and identification of bioactive peptides have been performed in a number of ways. Peptides have been produced in vitro by hydrolysis methods using digestive enzymes and, in some cases, individual peptides have then been isolated and characterized. Often, peptides have been synthesized and used for in vitro studies. In some cases, the peptides have been identified based on comparison of sequences with those of known biological activity, e.g., opioid peptides. Other methods rely on food processing techniques, such as using heat, pH changes, or the ability of microbial enzymes to hydrolyze proteins, e.g., during fermentation. However, food processing can also cause structural and chemical changes with detrimental effects on potential bioactive peptides, often preventing their release due to the formation of hydrolysis-resistant covalent bonds, e.g., dephosphorylation and subsequent loss of mineral binding capacity (2). Fermentation of milk products is a natural way in which bioactive peptides can be generated, and different microorganisms are likely to generate different ranges of peptides, possibly with different health effects. Bioactive peptides may have more than one biological activity, such as β-casomorphin-7, which exhibits opioid, angiotensin converting enzyme (ACE), inhibitory and immunomodulatory effects (3). Often, these peptides are from overlapping peptide sequences in so-called strategic zones that are partially protected from proteolysis by virtue of their primary structure (2, 4).

Food-derived peptides have been shown to impact on a range of physiological functions, including influencing intestinal transit, modifying nutrient absorption and excretion, immunomodulatory effects, and antihypertensive activity. A number of products specifically designed to contain or generate bioactive peptides during digestion are currently being marketed, including hypoallergenic infant formulas and dental enamel enhancers. The present contribution provides an overview of the various reported physiological effects of food bioactive peptides.

Bioactive Peptides with Antimicrobial and Antifungal Properties

Lactoferrin, a naturally occurring iron-binding glycoprotein found in milk, is widely considered to be an important antimicrobial component for protecting the host

against microbial infections (5). The antimicrobial mechanism is more complex than simply iron-binding however, as peptides derived from lactoferrin (e.g., lactoferricin) that have a highly potent antimicrobial activity [100 to 1000 times higher than intact lactoferrin (6)] have also been isolated against a range of both Gram-negative and Gram-positive bacteria and certain yeast and fungi (7–11). Nuclear magnetic resonance (NMR) studies have shown that the pepsin-liberated peptides from lactoferrin, which carry a net positive charge, adopt a structure that allows them to disrupt the cell membrane and enter the cell (12). Therefore, peptides such as lactoferricin may kill bacteria by increasing membrane permeability. There is evidence to suggest that lactoferricin is generated by digestion of lactoferrin in the human stomach (13) and gastrointestinal contents of rats (14). The in vivo bactericidal properties of lactoferricin have been questioned, however, because both the addition of 5% cow's milk or increasing concentrations of mucin to the assay reduced the antimicrobial effects (7).

Casecidin, which is derived from a chymosin digest of casein, exhibits in vitro antimicrobial activity against a range of microorganisms, including *Staphylococcus* spp. and *Streptococcus pyogenes* (15). In vivo studies using isracidin (casein peptide) have been shown to protect mice against *S. aureus* and *Candida albicans*, and sheep and cows against mastitis (15).

Glycomacropeptide (GMP), which is formed from κ-casein during either cheese manufacture (action of chymosin) or digestion, bears a number of oligosaccharide structures that are thought to bind to pathogenic bacteria and, thereby, prevent them from binding to sites on the mucosal membrane. GMP has been shown to bind to enterotoxins from *Vibrio cholerae* and *Escherichia coli* (16, 17), with the carbohydrate structures mimicking the enterotoxin receptor sites. Via this mechanism, GMP has been shown to prevent gastrointestinal bacterial infections in several animal challenge trials. Isoda et al. (16) demonstrated that GMP was able to protect mice from *V. cholerae* and *E. coli* enterotoxin-associated diarrhea.

Researchers have also suggested that GMP may help to reduce dental caries, following in vitro studies demonstrating that GMP was able to prevent cariogenic bacterial adhesion (18). In this case, GMP is thought to promote the growth of *Actinomyces viscous* over the more cariogenic *Streptococcus mutans* and *S. sanguis,* thus effectively altering the bacterial populations in the dental plaque.

GMP has also been shown to inhibit hemagglutination of 4 human influenza virus strains (19), *Mycoplasma gallisepicum* (20), and *M. pneumoniae* (21). The fact that GMP lacks any aromatic amino acids is also noteworthy, as it may be useful for populations with certain dietary restrictions, such as phenylketonuria.

Bioactive Peptides Influencing Gut Integrity and Anti-Inflammatory Bowel Disease

A number of food-derived growth factors and peptides have been shown to be important for the maintenance of gut integrity in inflammatory diseases. These include epidermal growth factor (EGF) and transforming growth factor-β (TGF-β), both of which are present in bovine colostrum. EGF, among other activities, exerts a trophic effect on epithelia, accelerates cell maturation, and stimulates cellular proliferation (22). TGF-β has been implicated in epithelial cell growth and differentiation, and aids in the repair of injured tissue (23). Preliminary trials investigating the effects of diets containing TGF-β on various forms of inflammatory bowel disease have shown favorable results, i.e., inducing remission and promoting mucosal healing (23). Therefore, it appears there may be a possible role for bioactive peptides in enteral diets used as therapy for inflammatory bowel disease (IBD); however, a considerable amount of evidence is still required to support this. EGF and TGF-β are present in colostrum, and there is evidence to suggest that EGF can survive the gastrointestinal (GI) tract in neonatal animals, including human infants and suckling rats, lambs, and pigs (24–27).

Bioactive Peptides with Blood Pressure-Lowering Effects

ACE Inhibitors

There is considerable market potential for functional foods containing food-derived hypotensive peptides, particularly ACE-inhibitory peptides, and consequently, considerable resources have been dedicated to identifying potential ACE-inhibitory peptides. ACE converts angiotensin I to angiotensin II, which increases blood pressure and aldosterone and inactivates the depressing action of bradykinin.

A number of food-derived peptides have been shown to have ACE-inhibitory activity, including peptides from bovine and human casein, yeast, gelatin, bonito, tuna, ovalbumin, zein, corn, wheat, soybean, soy sauce, snake venom, chickpeas, and muscle myosin (28–34). However, many of these putative ACE inhibitors were identified using in vitro assays (35–37), and subsequent studies have shown that a number were merely competing with the synthetic substrate used in the assay (38) rather than actually inhibiting the ACE activity. Some in vivo studies, however, have demonstrated the ability of several peptides, particularly di- and tripeptides with Pro or Trp as the C-terminal amino acid, to inhibit ACE activity and lower blood pressure in spontaneously hypertensive rats (30, 38–40) when given intravenously or orally. Di- and tripeptides are readily absorbed in the intestine (41), and it has been shown that peptides containing Pro and, in particular, C-terminal Pro-Pro sequences, tend to be resistant to digestive proteases and proline-specific peptidases (42, 43). Therefore, ACE-inhibiting peptides present in fermented milk products, for example, are not

degraded but absorbed directly and inhibit ACE in the aorta (44), and they may also act by stimulating blood flow in the GI tract, thus enhancing gut function (45). However, work by Miyoshi et al. (29) indicated that the blood pressure-lowering effect was of short duration (5 min) and weaker than that achieved by the use of commercially available antihypertensive drugs.

A number of ACE-inhibitors derived from bovine α_{s1}-, β-, and κ-casein have been reported (46) and are referred to as casokinins. A number of lactokinins, which is the term given to ACE inhibitors derived from the whey proteins α-lactalbumin, β-lactoglobulin, and bovine serum albumin, have also been identified (46).

Because lactic acid bacteria have extracellular protease activity and are able to release peptides from casein, fermented milk products provide an obvious source of casokinins. Indeed, several trials using fermented milk products have demonstrated the ability of these products to lower blood pressure in rats and humans. In vivo studies with spontaneously hypertensive rats have shown that administration of Calpis™ (contains the peptides valine-proline-proline and isoleucine-proline-proline) milk fermented using *Lactobaccillus helveticus* and *Saccharomyces cerevisiae* to rats 22–26 weeks of age was able to significantly lower the systolic blood pressure of the rats between 4 and 8 h after administration (47). Furthermore, long-term feeding studies showed that this product had an antihypertensive prophylactic effect in spontaneously hypertensive rats (48). Several similar studies using a range of fermented milk products and associated peptides have also been carried out (39, 40, 49–51). From these studies, it is clear that not all strains of lactic acid bacteria are capable of fermenting milk to yield antihypertensive peptides (44).

A study in which normotensive and mildly hypertensive volunteers ingested 10 g trypsin-hydrolyzed casein/day (containing ACE-inhibitory peptides) for 4 weeks gave promising results, suggesting the casein peptides have an ability to prevent hypertension (2). A placebo-controlled study examining the effects of Calpis fermented milk on the blood pressure of 30 hypertensive patients over an 8-week period showed a significant blood pressure-lowering effect in the Calpis-treated patients (52). In a randomized placebo-controlled study, 39 hypertensive subjects consuming milk fermented with *L. helveticus* LBK-16H on a daily basis for 21 weeks were shown to have significantly lower systolic and diastolic blood pressure than the control subjects (53). However, more studies are needed with a larger sample size, as well as studies with proteolytic hydrolyzates and fractions that have been enriched for ACE-inhibitory peptides (46).

An ACE-inhibitory peptide isolated from a thermolysin digest of dried bonito exists in 2 forms: a 5 amino acid residue peptide that exhibits its maximum blood pressure-lowering effects in spontaneously hypertensive rats 4 h after oral administration, and a derivative of this peptide that is only 3 amino acid residues long and gives its maximum effect only 2 h after administration (32). The authors also reported that oral administration of a thermolysin digest of dried bonito to mildly hypertensive patients was able to prevent hypertension.

Vasorelaxation

Yoshikawa and coworkers (32) isolated 2 peptides from ovalbumin (ovokinin I and II) that caused vasorelaxation and lowered the blood pressure of spontaneously hypertensive rats following oral administration. Vasorelaxation was mediated via the bradykinin B1 receptor and prostacylin for ovokinin I and an unknown receptor and nitric oxide for ovokinin II (32).

Cholesterol-Lowering Bioactive Peptides

Consumption of a number of food proteins, particularly plant proteins, is known to contribute to a lowering of serum cholesterol levels (54). Soybean protein is a typical example of a hypocholesterolaemic protein. The high molecular weight core peptides that remain after digestion are able to prevent the reabsorption of bile acids and, therefore, are able to lower cholesterol (55). Lower molecular weight peptides, such as α-lactotensin (β-lactoglobulin fragment), and a peptide from soybean glycinin have both been shown to reduce serum cholesterol levels in mice (32), although neither increases excretion of fecal cholesterol or bile acids.

Antithrombotic Bioactive Peptides

Casoplatelin, which is a κ-casein fragment, is able to inhibit platelet aggregation. It does this by both inhibiting adenosine diphosphate (ADP)-activated platelets and inhibiting the binding of human fibrinogen γ-chain to a specific receptor on the surface of the platelets (56). A smaller peptide (casopiastrin) derived from a tryptic digest of κ-casein also exhibits antithrombotic activity by inhibiting fibrinogen binding. Interestingly, another peptide containing much of the same sequence as casopiastrin inhibits platelet aggregation but does not affect fibrinogen binding to the platelet receptor (57). Several κ-casein-derived glycopeptides from different species, including bovine, human, and sheep (58, 59), have also been shown to inhibit platelet aggregation, and at least 2 of these peptides have been identified in newborn human infants fed breast milk or cow's milk-based formula (60). A peptide derived from a pepsin digest of sheep and human lactoferrin was shown to inhibit thrombin-induced platelet aggregation (58).

Bioactive Peptides Affecting Mineral Absorption

Caseinophosphopeptides (CPPs) are formed during the digestion of casein in the GI tract, and they have been detected in the intestinal contents of animals fed both intact casein and purified β-casein (61–65). CPPs are able to chelate minerals, in particular Ca, and, because this complex remains soluble, there is the potential for enhanced Ca absorption across enterocytes in the distal intestine. This enhancement of Ca solubility, absorption, and, hence, bioavailability has been seen in animals

fed casein diets compared to those fed soy-based diets (66). A trial in humans in which CPPs were incorporated into a rice-based infant food also led to an increase in both Ca and Zn absorption, although incorporation into a whole-grain infant cereal did not achieve the same result (67).

The use of CPPs for the prevention of dental caries has also been proposed, because CPPs are able to inhibit the formation of caries through recalcification of the dental enamel (68). CCPs have already been included in dietary and pharmaceutical supplements and have been found in cheese (69, 70).

Due to the large number of acidic amino acid residues in CPPs, they bear a number of negative charges, which renders the peptides resistant to further proteolysis and makes them excellent candidates for inclusion as bioactive ingredients in functional foods. It has been suggested that CPPs could be used as a supplement in bread, flour, cakes, beverages, and chewing gum, as well as in toothpaste.

Immunomodulatory Bioactive Peptides

Bovine milk is known to contain a number of peptide fractions that can affect immune function. The majority of these are derived from the hydrolysis of the major milk proteins through proteolysis, although some are naturally occurring and require little or no modification to become active (71, 72).

Of the peptides found to be naturally occurring in milk, proline-rich polypeptide (PRP) enhances splenic antigen responses to sheep red blood cells (RBC) in mice (73), induces cytokine production by murine macrophages (74), and induces the growth and differentiation of resting B lymphocytes (75). Milk growth factor (MGF) has been shown to suppress human T-lymphocyte function in vitro (71).

A number of peptides derived from casein have been found to be immunostimulatory. Not only do each of the caseins produce different bioactive peptides, but those produced and, hence, their effects vary depending on the enzymatic process used to generate them (76). For example, pancreatin and trypsin digests of α_{s1}-casein and β-casein were shown to inhibit proliferation of murine spleen cells and rabbit Peyer's patch cells (77), whereas digests prepared using pepsin and chymotrypsin had no effect.

Specific peptides derived from α_{s1}-casein have been shown to have a wide range of immunomodulatory actions, including suppression in vitro of mitogen-stimulated human peripheral blood mononuclear cell proliferation (78); promotion of antibody formation; enhancement of phagocytosis in vitro; in vivo protection against *Klebsiella pneumoniae* infection in mice (3, 45, 79); and enhancement of lymphocyte proliferative responses, natural killer cell activity, and neutrophil locomotion (80, 81).

Likewise, other peptides derived from β-casein have been shown to suppress mitogen-induced proliferation of human lymphocytes (78), stimulate proliferation of rat lymphocytes in the absence of mitogens (3), promote antibody formation, enhance murine peritoneal cell phagocytic activity, and enhance antigen-dependent T-cell proliferation (80, 82, 83).

In vitro studies with κ-caseinoglycopeptide (generated via a chymosin digest) have shown it to be a potent inhibitor of lipopolysaccharide (LPS)- and phytohemagglutinen (PHA)-induced proliferation of murine splenic lymphocytes and rabbit Peyer's patch cells (84), as well as suppressing antibody production by murine spleen cell cultures (84). In contrast, Sutas et al. (85) found that peptides from a pepsin-trypsin digest of κ-casein were able to significantly enhance human lymphocyte proliferation in vitro. In vitro studies by other workers have also shown that specific peptides generated by digestion of κ-casein are able to enhance cellular proliferation (3, 78) or promote antibody formation and enhance phagocytic activity of murine and human macrophages (82, 83).

As previously mentioned, a number of the bioactive peptides from casein appear to be multifunctional. In vivo β-casomorphins have been shown to protect mice against *K. pneumoniae* infection, an effect that was suppressed by the administration of an opioid antagonist, hence suggesting the effect is mediated via an opioid receptor (81, 86). β-casein-derived ACE inhibitory peptides have also been shown to stimulate phagocytosis and protect mice against *K. pneumoniae* infection (45, 79).

Hydrolysis of the whey proteins α-lactalbumin and lactoferrin has led to preparations that are capable of modulating the immune system. A diet of hydrolyzed α-lactalbumin was able to modulate T and B lymphocyte function (87). In an in vitro assay, bovine lactoferricin (residues 17–41 of lactoferrin) was shown to suppress interleukin (IL)-6 production by a human monocyte cell line following stimulation with LPS (88), stimulate the release of IL-8 from human polymorphonuclear leucocytes (89), and promote the phagocytic activity of human neutrophils (90).

A preliminary trial in which pre-AIDS (acquired immune deficiency syndrome) patients were treated with 2 bioactive peptides (Tyr, Gly, Gly and Tyr, Gly) from a dialyzed leucocyte extract known as IMREG-1 gave promising results, as measured by a significant decrease in progression of the disease (91, 92). In another trial, as well as exhibiting fewer symptoms, the rate of reduction of $CD4^+$ cell numbers was also slowed in AIDS-related complex patients receiving these peptides (93). In a more recent trial, Gottlieb et al. (94) demonstrated the ability of IMREG-1 to restore the expression of the IL-2 receptor on $CD4^+$ cells towards their normal level in a patient with advanced human immunodeficiency virus (HIV).

The mechanism of action through which bioactive peptides elicit their effects on the immune system is unknown, but several hypotheses have been suggested, including stimulation of the maturation and proliferation of T cells and natural killer cells. It has also been suggested that milk-derived opioid peptides may act on lymphocytes via the opiate receptor, thereby affecting the immunoreactivity of the cells (3).

Because milk is an important nutritional source for the neonate, it is likely that the primary role of immunomodulatory milk peptides is to assist in the development of the GI tract and aid in the prevention of hypersensitivity to nutrients. Immunostimulating peptides from sources other than milk have also been shown to exist. Soymetide, a peptide derived from soybean protein, has been shown to stimulate the phagocytic activity of human polymorphonuclear leukocytes in vitro (32).

Opioid Peptides

Peptides with opioid activity have been identified from a number of digested food proteins, including cereal (95); bovine, human, ovine, and water buffalo milk (63, 96–98); hemoglobin from bovine blood (hemorphins; 99); gluten and gliaden from wheat; zein from maize; hordein from barley; and soy α-protein and cytochrome b (100). They have also been found occurring naturally in the brain, plasma, and cerebrospinal fluid (101, 102).

Peptides with opioid activity may affect appetite, behavior, and gastrointestinal motility. A number of bioactive peptides derived from milk proteins are opioid agonists (casomorphins, α-lactorphin, β-lactorphin, and serorphin from serum albumin), that is, they bind to opioid receptors and exhibit morphine-like effects. Others are opioid antagonists (lactoferroxins and casoxins); these are able to depress the agonist activity of enkephalin (103, 104). Generation of these opioid peptides is achieved by digesting the parent protein with digestive enzymes: pepsin, pepsin followed by trypsin, or chymotrypsin alone (105). Hamel et al. (106) demonstrated that casomorphins are produced during cheese ripening due to the proteolytic activity of certain bacteria. The β-casomorphins were the first opioid peptides identified from food proteins and, to date, they are the most studied of all the opioid peptides, with β-casomorphin-11 (63, 107) and β-casomorphin-7 (108, 109) being characterized as in vivo digestion products.

There are a number of opioid receptors responsible for specific physiological effects: the μ-receptor for emotional behavior and suppression of intestinal motility, the σ-receptor for emotional behavior, and the κ-receptor for sedation and the regulation of food intake. The physiological effects of α-lactorphin and β-lactorphin appear to be quite different, despite the fact that the amino acid sequences of these tetrapeptides differ by only 1 amino acid. α-Lactorphin appears to exert a weak inhibitory effect on contractions of guinea pig ileum in vitro, while β-lactorphin causes a nonopioid stimulatory effect (105). Their action appears to occur via binding to the μ-receptor because both are able to displace ^{3}H-naloxone from its binding site. It has been calculated (105) that, with a 100% efficiency in peptide generation, 1 L milk would give rise to concentrations of lactorphins sufficient to achieve the effects observed in vitro. However, studies of hydrolyzates have revealed that only 5–14% of the theoretical yield is achieved and, more importantly, the generation of α-lactorphin and β-lactorphin during gastrointestinal digestion is yet to be proven (105), whereas the release of casomorphins has been shown (107).

In adult humans, the effects of the casomorphins are limited to the GIT, as they are either not absorbed or are subject to enzymatic degradation in the intestinal wall (98). There is, however, evidence to suggest that β-casomorphins can be transported from the blood to the brain stem (110) and the cardiovascular compartment in infants (98). Passive transport across intestinal mucosa occurs in neonates and may provide an analgesic effect on the nervous system, resulting in calmness and sleep in infants (98). In pregnant/lactating women, β-casomorphins pass through the mammary gland and could potentially influence the release of prolactin and oxytocin.

Milk-derived opioid peptides have been shown to play a role in appetite regulation by modifying the endocrine activity of the pancreas to increase insulin output (111). Studies in rats indicate that β-casomorphins may have a role in modulating dietary fat intake, with β-casomorphin 1-7 stimulating intake of a high-fat diet and suppressing intake of a high-carbohydrate diet in satiated rats (112). Casomorphins have been shown to modulate social behavior and produce increased analgesia in experimental animals (113, 114) and infants (111). Bovine β-casomorphins have also been shown to have depressive effects on the central respiratory system, causing a slowing of respiratory frequency and tidal volume in rats and rabbits (115). However, despite the large volume of research, particularly on β-casomorphins, a functional role for the opioid peptides is yet to be demonstrated. Part of the role for casomorphins could be to slow the passage of digesta through the gut, thereby enabling maximum production of other bioactive peptides and increasing the time for these peptides to assert their action (4).

Several peptides with opioid activity have been isolated from enzymatic digests of wheat gluten (116, 117). These peptides have structures quite different from other peptides. A further investigation into the effects of 2 of these gluten exorphins, A5 and B5, has shown that oral and intravenous administrations to rats led to a stimulation of postprandial insulin release (118).

Hemorphins have been shown to bind and stimulate opioid receptors in a number of in vitro binding studies (99, 119, 120). As well as exhibiting agonistic effects, the hemorphins can also act as opioid receptor antagonists (121). Hemorphin-6 and LVV-hemorphin-6 have been shown to have a higher affinity for the σ-binding site than β-casomorphins and other opioid peptides (119). Hemorphins appear to have analgesic properties similar to classical opioid peptides (122) and may also have a role in the modulation of acute inflammatory responses (102). In addition, hemorphins have been shown to have ACE inhibitory activity (102, 123).

Bioactive Peptides and Gastrointestinal Function

A key physiological role for opioid peptides and other bioactive peptides is likely to be in the GI tract, where they

Table 1. Mean endogenous ileal amino acid flows (μg/g dry matter intake) in the growing rat and pig, determined using purified diets, each of which is devoid of a specific amino acid or based on a protein-free dietary control

	Rat[b]		Rat[c]		Pig[d]	
Amino acid[a]	Synthetic diet	Control	Synthetic diet	Control	Synthetic diet	Control
Lysine	212	228	—	—	284	252
Aspartic acid	704	585	—	—	—	—
Serine	282	290	254	220	—	—
Glutamic acid	597	615	593	524	—	—
Alanine	—	—	195	200	—	—

[a] None of the differences between the mean flows for each amino acid was significant ($P > 0.05$).
[b] Reference (146).
[c] Reference (147).
[d] Reference (148). The 15 kg body-weight pigs received lysine intravenously.

may have localized effects, effects mediated via gut hormones, or systemically mediated effects following their absorption. There have been several reports in the literature describing a role for bioactive peptides in regulating gastric emptying rate and gastrointestinal motility in animals (95, 124–131), gut secretory and absorptive activity (132–138), and gut tissue growth (139).

It has been shown (140) that fragments of bovine casein macropeptide (CMP) appear in both the intestinal lumen and blood of the rat following oral administration. CMP and smaller CMP-derived peptides influence modulation of gastrointestinal function (140). It is suspected (137) that CMP modulates the release of gastrointestinal hormones like cholecystokinin, gastrin, and somatostatin, thus affecting gastric secretion, pancreatic secretion, and gastrointestinal motility.

There is an interaction of casomorphins with opiate receptors located on the serosal side of the intestinal epithelium with a subsequent increase in electrolyte transport and a potential effect on antisecretory activity (133, 141). Casomorphins exert an antidiarrhea (antisecretory) action (126) by enhancing water and electrolyte absorption in both the small and large intestines. The casomorphins are also able to modulate amino acid transport and have been shown to stimulate secretion of insulin and somatostatin in dogs (142, 143).

A number of milk-derived peptides have also been shown to affect gastrointestinal function: atrial natiuretic factor (atriopeptin) is a strong diuretic and vasorelaxant (144). Peptides from both β-lactoglobulin (β-lactotensin) and serum albumin (albutensin-A) have been reported to induce contraction of smooth muscle (145). Our own group working at Massey University has described a central role for peptides in influencing gut protein dynamics. In our first set of studies, we sought to determine the effect of nitrogenous alimentation per se (i.e., excluding effects of proteins and peptides) on the net effect of gut protein secretion and reabsorption (i.e., endogenous protein loss measured at the end of the small bowel). A semisynthetic, nitrogen-free diet (control) was formulated to mimic the effect of the nonprotein component of a diet, along with a series of similar diets containing synthetic free amino acids as the sole source of nitrogen. By not including certain dietary nonessential amino acids in some of the diets, and by also omitting certain dietary essential amino acids in others (but with accompanying intravenous infusion), we could directly and unambiguously measure endogenous amino acid loss at the terminal ileum. The studies were undertaken with laboratory rats and pigs as generalized mammalian model animals. The animals consumed the amino acid-containing diets readily, grew normally, and were in a positive body nitrogen balance. A comparison of endogenous ileal amino acid loss for animals receiving the synthetic amino acid-based diets and the protein-free control is given in Table 1. Despite the treatment groups being in positive body nitrogen balance and receiving a gut luminal amino acid supply, the endogenous amino acid flows (the net result of overall gut secretion and reabsorption) were not higher than for the control (protein free) animals that were in negative body nitrogen balance and deprived of a direct dietary amino acid supply to the gut. The results of these studies indicated that gut protein dynamics do not appear to be influenced by nitrogenous alimentation per se.

The second series of experiments sought to determine whether feeding the animal protein rather than amino acids would have any effect. The complete transformation of lysine in dietary proteins to the analog homoarginine allowed a direct measure of the endogenous loss of lysine. Homoarginine is absorbed in a similar manner to lysine and is partially converted in the liver to lysine. In this case, animals would consume a protein-containing diet and would be in a positive body nitrogen balance. We were successful in completely guanidinating proteins (149) and, subsequently, several studies were conducted with the laboratory rat using this approach. There are also naturally occurring proteins that are completely devoid of certain amino acids. An example of such a protein is zein, which can be isolated from the maize grain

Table 2. Mean endogenous flows of lysine (μg/g dry matter intake)[a] at the terminal ileum of the growing rat fed guanidinated protein-based diets, a zein-based diet, or a protein-free control diet

Guanidination study[b]	Guanidinated gelatine	Guanidinated soy	Guanidinated casein	Protein-Free
Lysine flow	488[a]	442[a]	472[a]	239[b]
Zein study[c]	Zein	Protein-free		
Lysine flow	389[a]	252[b]		

[a] Means with different superscript letters were significantly different ($P < 0.01$).
[b] References (149) and (150). Lysine was supplied by liver conversion from homoarginine.
[c] Reference (148).

and is virtually devoid of lysine. Accordingly, we prepared semisynthetic zein-based diets and fed them to young pigs, which were simultaneously infused intravenously with lysine. Here again, the gut tissues were supplied with nitrogenous material, and the animals were in positive body nitrogen balance. The ingestion of protein led to an almost doubling of the endogenous ileal lysine flow (Table 2).

The question now arose as to whether the pronounced effect of dietary protein on gut protein dynamics may be caused by peptides. To test this hypothesis, we prepared and fed to the test animals an enzymic hydrolyzate of protein as the sole source of dietary nitrogen. The size of the peptides in the hydrolyzate was <5000 daltons (Da). Ileal digesta were subsequently collected from the animals, and the material was centrifuged and ultrafiltered (10 000 Da molecular weight cutoff). Any unabsorbed dietary peptides were discarded in the ultrafiltrate, with the precipitate plus retentate being an estimate of gut endogenous loss. This estimate is slightly low due to the loss of a small amount of endogenous free amino acids and peptides in the ultrafiltrate. This technique has been applied widely in a number of studies, with the consistent result of a significant ($P < 0.05$) effect of the peptides on gut endogenous amino acid loss (Table 3). Net endogenous ileal amino acid flows, determined after administering peptides, for

Table 3. Mean endogenous ileal amino acid flows (μg/g dry matter intake) for rats and pigs determined after administering dietary peptides versus a protein-free control[a,b]

	Rat		Pig	
Amino acid	Hydrolyzate	Protein-free	Hydrolyzate	Protein-free
Lysine	275	172**	461	312*
Methionine	127	53**	—	—
Cysteine	142	56**	—	—
Histidine	223	133**	319	231 ns
Phenylalanine	237	212**	278	238ns
Tyrosine	179	161ns	244	181ns
Threonine	525	311**	909	572*
Leucine	386	256**	528	400*
Isoleucine	313	159**	504	230***
Valine	341	234**	593	321**
Alanine	349	213**	485	436ns
Aspartic acid	748	636**	1531	754**
Arginine	274	217**	373	480ns
Serine	759	374**	1383	550***
Glutamic acid	1366	701**	3378	786***
Glycine	796	765ns	682	1660*
Proline	493	584*	1419	3558*

[a] References (151) and (152). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; NS = nonsignificant.
[b] Animals were fed a semisynthetic enzyme-hydrolyzed casein (molecular weight 5000 Da)-based diet. Digesta were centrifuged and ultrafiltered (molecular weight cutoff = 10 000 Da), and the precipitate + retentate was taken as the endogenous component.

Table 4. Endogenous amino acid loss (μg/g dry matter intake) at the end of the small bowel, determined using the enzyme-hydrolyzed protein (casein) method with the laboratory rat, pig, cat, chicken, and human[a]

Amino acid	Laboratory rat[b]	Pig[b]	Domestic cat	Chicken	Human[b]
Lysine	312	455	1101	303	614
Methionine	125	—	411	—	269
Cysteine	188	—	853	—	367
Histidine	216	339	897	288	561
Threonine	689	951	2127	606	857
Valine	538	640	1687	604	978
Phenylalanine	236	332	1015	278	442
Tyrosine	220	301	1046	293	439
Isoleucine	486	510	1205	530	564
Leucine	560	636	1823	514	808
Arginine	303	442	948	—	478

[a] References (148) and (151–155).

[b] Overall means representing 3 rat and 2 pig studies and a mean for 6 ileostomized human subjects. The chicken and human data are unpublished.

a range of simple-stomached animal species, including humans, are shown in Table 4.

Further studies have demonstrated that the dietary peptides have a profound effect on gut secretory and reabsorptive activity, and that the effect is dose-dependent (156). It appears that the breakdown products of dietary proteins themselves may assist in regulating the digestive processes. Current research is investigating which of the peptides present in the complex hydrolyzates are responsible for the effect, what are the mechanisms of action, and what components of the endogenous milieu are affected.

General Discussion

Bioactive peptides are inactive within the parent molecule until released by enzymatic hydrolysis, generally via the digestive process (either in vivo or in vitro) or by food processing; peptides produced following digestive proteolysis often are quite different compared to those released following bacterial fermentation (2). Once released, the bioactive peptides are theoretically capable of influencing a range of behavioral, gastrointestinal, hormonal, immunological, neurological, physiological, vasoregulatory, and nutritional responses. These peptides have, therefore, been put forward as candidates for a variety of food and pharmaceutical applications. However, the introduction of bioactive peptides or the parent protein into food products is not straightforward. First, in the case of an ingested parent protein, the bioactive peptide in question must be generated during normal digestive proteolytic action. For bioactive peptides added directly to foods, they must survive the digestive process and not be further degraded in the gut. It is notable, however, that many bioactive peptides appear to be somewhat resistant to enzymatic hydrolysis. There is also evidence to suggest that peptides generated from in vitro digests yield different peptides compared to those that are achieved in vivo. For example, β-casomorphins isolated from intestinal contents following ingestion of casein (107) and plasma following the ingestion of milk (157) have been shown to be larger fragments than those found in in vitro digests.

Peptides ingested orally or produced via proteolytic action may exert their effect locally on the GI tract, may exert systemic effects following absorption into the peripheral blood, or may bind directly to cellular receptor sites in the gut. Any of these cases requires that the peptide reaches its target site in its active form. Some studies indicate that small peptides can be absorbed from the GI tract intact following oral administration (158, 159). There is also evidence to suggest that some bioactive peptides are absorbed as longer-chain peptides, which are then hydrolyzed in the intestinal tissue to yield the bioactive molecule. Generally, however, it is difficult to measure the absorption of bioactive peptides, as they are difficult to detect and rapidly degraded once in the bloodstream (113, 160).

In order to release bioactive peptides, digestive enzymes can be secreted by the digestive system or produced by resident microflora in the gut. Experimental findings have clearly shown that intestinal microflora are able to modify the immunomodulatory effects of foodborne bioactive peptides (85, 161), and one way by which probiotic bacteria may be effective is by enhancing the formation of efficacious bioactive peptides.

In addition to the generation of bioactive peptides by the digestive process, these peptides can also be generated during the food manufacturing process itself (e.g., partially hydrolyzed milk products for hypoallergenic infant formulas). In such cases, the bioactive components are ingested as part of the food. Cheese ripening is another process that results in the

generation of various peptides, including ACE inhibitors. A number of bacterial species used in the cheese manufacturing process are also capable of generating bioactive peptides.

However, food processing can also damage proteins to such an extent as to render the bioactive peptides either inactive following digestion or prevent them from being released from the parent protein. Damaged proteins are frequently digested in a different manner, as resistant peptide bonds can be generated from heat and/or alkali treatments. Hence, peptides that would not normally occur naturally may be generated. The consequences of food processing on the generation of bioactive peptides, therefore, require careful attention.

There is considerable evidence, mainly from in vitro studies, for the existence and efficacy of bioactive peptides from milk, and some experimental evidence demonstrating that, during the normal digestive process, bioactive casein peptides are naturally generated. Hence, milk proteins are currently the main known source of biologically active peptides, although other plant and animal proteins contain bioactive sequences. Peptides generated from casein, lactoferrin, and β-lactoglobulin predominate. These peptides are able to affect a range of responses, such as ACE inhibition (casokinins); antimicrobial activity (casecidin, isracidin, lactoferricin); antithrombotic activity (casoplatelins); calcium absorption (CPPs); immunomodulatory activity (casein and lactoferrin hydrolyzates); and opioid activity (β-casomorphins). Many of the milk bioactive peptides, in particular, are multifunctional.

Some in vivo clinical trials have been performed with encouraging results, such as those with ACE-inhibitor peptides, which showed a significant decrease in blood pressure in hypertensive subjects, and immunomodulatory peptides which gave encouraging results in pre-AIDS and AIDS patients. However, there is in general a paucity of the in vivo studies that are required from a safety perspective as well as for generating the necessary evidence required to make a health claim. Further information is needed to demonstrate that the bioactive peptides are, indeed, generated in vivo and that they reach their target sites. Safety aspects must also be investigated, such as potential allergic or cytotoxic effects of the bioactive peptides, as well as the amount of product necessary to elicit a response.

The process of demonstrating the effect of bioactive peptides on gastrointestinal health is not an easy one. While in vitro tests may provide a valuable screening tool, they are not necessarily a true indicator of in vivo function. The use of animal models is also somewhat controversial, due to physiological differences between species. The process of investigating the anti-infection properties of bioactive peptides in humans is also difficult, because studies performed in areas with high rates of infections (such as developing countries) are confounded, in that malnutrition is also frequently encountered. Studies in normal healthy, well-nourished communities tend to require a large sample size and also a long trial period, which means high costs and poor compliance. One group in the United States (162) has performed anti-infection studies in infant rhesus monkeys, using a range of milk protein components as test products and infecting the monkeys with enteropathic *E. coli*. Although indicating trends, the results did not give statistically significant results, and the continuation of such trials in monkeys is likely to raise ethical issues.

Some manufacturers believe that, because the starting product (i.e., the food) is safe, any peptides generated from that product must also be safe. However, this is not necessarily the case. One of the main potential problems with milk-derived bioactive peptides is the potential for allergenicity in susceptible individuals. Many milk products, which are hydrolyzed during manufacture and consist almost entirely of peptides, will also contain bioactive peptides. Because these products are often destined for distinct markets, such as hypoallergenic infant formulas or enteral nutrition, care must be taken to ensure that undesirable side effects do not ensue in the consumer, who is often either sick or immunocompromised in some way.

Conclusions

The potential of bioactive peptides to impact on the prevention and possible treatment of infection and disease is clear. However, a significant amount of in vivo research is required first to demonstrate the safety and efficacy of the products before the benefits can be realized. There is no doubt that the generation of bioactive peptides during the normal digestion of food in the human alimentary canal is an important function of dietary proteins, and these peptides have numerous regulatory and stimulatory effects. Food proteins are far more than merely a supply of amino acids for body protein synthesis. The rapidly accumulating information on the bioactive peptides released from proteins during digestion is an important aspect to be considered when describing dietary protein quality.

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