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Peptides From Milk Proteins and Their Properties

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INTRODUCTION

Food proteins are increasingly being used in the food industry for their functionality. Functionality of food proteins refers comprehensively to the physico-chemical properties of the proteins such as hydrophilicity and hydrophobicity (influencing water-protein interactions), interfacial (air-water and oil-water interfaces), intermolecular interactions (viscosity, fiber formation, film formation, gelation, cohesion, adhesion, etc.) and sensory (taste and smell). These physico-chemical properties are influenced by the intrinsic nature and type of protein and the processing according to the protein. The type of protein implies the amino acid composition, conformation and the bonds and forces involved therein. Whereas the processing factors encompass protein source and variety, extraction process (time, temperature, pH, ionic strength, etc.), impurities, conditions of storage and the method of use and other ingredients present in a food formulation.

It has been realized that commonly used food proteins have room for improvement in their functionalities. Physical, chemical and biological methods of altering the proteins to enhance their functionalities have been attempted. Of sustained interest and importance among these is the hydrolysis of proteins by enzymes. Enzymatic modification of proteins has been covered extensively (Adler-Nissen, 1985; Arai and Fujimaki, 1991; Reimerdes, 1990; Margot et al., 1994; Panyam and Kilara, 1996). Changes in functionality of proteins upon hydrolysis result from the reduced molecular weight of the peptides, loss or alteration of native structure and

enhanced interaction of peptides with themselves and with the environment. The functional properties of milk proteins have been well characterized, understood and utilized in food applications, but the properties of peptides generated by enzymatic modification are still relatively enigmatic.

Casein is used in a variety of foods and has been widely studied for its functional properties. Its amino acid composition and sequences are known (Mercier et al., 1971; Ribedeau-Dumas et al., 1972; Brignon et al., 1977). Nakai and Li Chan (1989) have comprehensively reviewed research on chemical and enzymatic modification of caseins to improve their functionality. Limited proteolysis improved such surface properties as emulsification and foaming (Shimizu et al., 1984, 1986, 1989; Chobert et al., 1988a,b, 1989b; Lee et al., 1987a; Haque and Mozaffar, 1992). The foaming property of caseins is important in whipped cream, ice cream, whipped toppings and mousses.

Proteolysis of purified samples of α -lactalbumin; β -lactoglobulin A and B by pepsin at pH 2.5; and papain at pH 8.0 were studied (Schmidt and van Markwijk, 1993). Papain did not hydrolyze α -lactalbumin and native β -lactoglobulin was not hydrolyzed by pepsin.

Mutilangi et al. (1995) reported on the hydrolysis of heat denatured whey proteins by trypsin, chymotrypsin, Alcalase and Neutrase to various degrees of hydrolysis. Molecular weights of the hydrolyzates were characterized using polyacrylamide gel electrophoresis. Predictably, as the degree of hydrolysis increased, the molecular weight of the products of hydrolysis decreased.

Rhozyme P-41, a broad-spectrum protease, did not hydrolyze α -lactalbumin or bovine serum albumin in whey protein isolate even at 5% degree of hydrolysis. Heat treatment to destroy enzyme activity resulted in the formation of high molecular weight aggregates. Heating of whey proteins at pH 6.0 resulted in much lower solubility, which was attributed to the large proportion of high molecular weight aggregates. Solubility of the hydrolyzate was higher at pH 4.5 when the pH during heating was 4.0, suggesting that the formation of large aggregates was mediated by disulfide bonds.

A great deal of attention has been placed on the extent of hydrolysis, the type of enzymes used and the conditions for the hydrolysis. Once the proteins were hydrolyzed, their functionality vis-à-vis unhydrolyzed controls were evaluated. Often, no efforts were made to separate the peptides from the rest of the reaction mixture. In many instances the key functionality that was targeted for improvement was solubility, and enzymatic hydrolysis did improve solubility of proteins as a rule (Panyam and Kilara, 1996). In some instances, hydrolysis was attempted to reduce the allergenicity and improve the digestibility of the proteins.

Turgeon, Gauthier and Paquin (1991) hydrolyzed whey protein concentrate and heat-treated whey protein concentrate with trypsin and chymotrypsin in an ultrafiltration reactor to fractionate the total hydrolyzates to a fraction that contained peptides larger than 5000 Daltons and another fraction consisting of small peptides and free amino acids. Heat treatment of the substrates modified the peptide profile by increasing the content of larger peptides (>5,000 MW).

Mannheim and Cheryan (1990) have also hydrolyzed casein with Alcalase in a continuous reactor. Using a statistical design, four variables (enzyme concentration, substrate concentration, permeate flux and reactor volume) were optimized. High conversion of casein could be achieved after one hour at 50°C and pH 8. The reactor activity was most affected by the enzyme substrate ratio. The decay of activity with time was a function of enzyme leakage through the membrane, thermal inactivation and enzyme-membrane interaction.

Caseinomacropptide is a peptide that results from the action of chymosin on κ -casein and is recovered as a by-product in cheese whey. When this macropptide is subjected to tryptic hydrolysis, bioactive peptides can be produced. Bouhallab et al. (1992) reported a method of continuous hydrolysis of this peptide and, as compared to a batch reactor, the continuous process was three times more pro-

ductive after 3.5 h. hydrolysis, but only 50% of the substrate was converted to smaller peptides.

In this article, the functional properties, biological activities, allergenicity and sensory properties will be examined in greater detail. Perhaps a better understanding of the physiochemical basis for these properties of peptides will allow a judicious choice of enzymes to hydrolyze food proteins. Such a review may also aid in the search of specific enzymes capable of producing desirable peptides. As biotechnology advances and proteins are modified through genetic engineering, specific protease cleavage sites may be engineered into transgenic proteins. A combination of these techniques may lead to a more efficient use of valuable protein resources.

PEPTIDE FUNCTIONALITY

Controlled enzyme modification of casein and whey proteins by proteolysis generates a total hydrolyzate, comprising certainly of unhydrolyzed substrates, hydrolyzed substrate and the low molecular weight materials. The total hydrolyzate can be further fractionated into low molecular weight peptides and high molecular weight materials. Because the proteolytic reactions are terminated with thermal destruction of the enzyme, sometimes in conjunction with an adjustment of pH, the state of the products in the reaction milieu is almost always different than the unreacted substrates. A majority of the published research pertains to the total hydrolyzate while more recent research has investigated the nature of the other two fractions from enzyme-modified proteins.

The nature of the enzyme modification is dependent upon reaction conditions employed, type of enzyme used, duration or extent of hydrolysis, conditions used to terminate the reaction and the post reaction processing accorded to the mixture. Because of these variables, an infinite number of processes can be devised to achieve similar results. Another important consideration is the type of products that will be made from the reaction product. For example, a hydrolyzed protein for use in an infant formula will have different considerations than one used as a whipping or foaming agent in nougat.

Solubility

Solubility is considered an essential prerequisite for the manifestation of many functional properties, and enzyme hydrolysis has been directed towards improving solubility of milk protein

substrates. Hydrolysis of micellar casein dispersion by alkaline milk proteinase (native to milk) increased the rennet coagulation time after extensive hydrolysis, but had no effect on its ethanol stability (Grufferty and Fox, 1988). Such hydrolysis resulted in a decrease in viscosity of the micellar casein suspension. Further studies showed that the observed increases in soluble nitrogen were due to the production of proteose peptones and not due to the breakdown of the components of the micellar casein. It was also speculated that micellar size might be affected by such hydrolysis. The hydrolyzed micellar casein lowered interfacial tension to a greater extent than the unhydrolyzed controls leading these researchers to conclude that the peptides generated by such hydrolysis had good surface activity.

Abert and Kneifel (1992) studied the physicochemical and functional properties of casein hydrolyzates as obtained by treatment of acid casein from pasteurized milk with different enzymes. Two of the proteases were Corolase PN (Rohm, Darmstadt, Germany) and Bromelain. Corolase is a neutral protease derived from *Aspergillus* species and Bromelain was derived from *Ananas comosus*. The enzyme substrate ratio was maintained at 2.2, pH was controlled at 6.7 and the temperature for the Corolase reaction was 55°C while for the Bromelain reaction it was 52°C. Enzyme inactivation conditions were 80°C for 20 min and the hydrolyzates were spray dried. As was expected, an increase in the degree of hydrolysis resulted in an increase in the nitrogen solubility index.

Beta casein A-1 was hydrolyzed by trypsin to various degrees of hydrolysis (Chobert et al., 1989a). Beta casein with a molecular weight of approximately 23,000 was easily hydrolyzed by trypsin and within 4 min the molecular weight was reduced to less than 8,000. Peptides ranging

from 15,000 to 2,500 were separated by molecular size exclusion chromatography. This difference in the molecular weights could be due to the characteristics of the two methods used to estimate the molecular weights. In both instances, the fundamental assumption is that the shape of molecules being separated is spherical to ovoid. This may not often be the case in the case of peptides. Solubility of all hydrolyzates was high and none of the hydrolyzates exhibited a pH of minimum solubility. Unhydrolyzed β -casein A1 has a pH of minimum solubility around 5.2.

Chobert et al. (1989b) also studied the solubility of κ -casein and its caseinomacropeptide. The minimum solubility for κ -casein was at pH 5.3 and the caseinomacropeptide was soluble over the range of pH tested. The caseinomacropeptide is generated by the hydrolysis of κ -casein by chymosin. Solubility of the para κ -casein was not reported.

Lactalbumin, a product prepared by heat denaturing whey proteins, has a similar name as the whey protein α -lactalbumin and is the cause of some confusion. The term heat denatured whey protein (HDWP) avoids the confusion created by homonym protein products. Heat denatured whey protein was hydrolyzed by a number of commercial proteases and the solubility of the hydrolyzed products were greatly improved (Mullally et al., 1994). They also demonstrated that depending on the number and ratio of activities within a protease preparation, the molecular characteristics such as molecular mass of the peptides, DH values and concentration of amino acids in hydrolyzates differ. Improvement in solubilities reported by Mullally et al. (1994) is shown in Table 1.

Heat denatured whey protein isolate was hydrolyzed with trypsin, chymotrypsin, Alcalase and Neutrase (Mutilangi et al., 1996). Hydrolyzates

TABLE 1
Soluble Nitrogen (N) and Degree of Hydrolysis (DH%) After a 240 min Incubation of Lactalbumin with Different Proteases

Protease	Supplier	N	DH%
PTN 3.0S	Novo Nordisk, Copenhagen	89.00	8.83
Trypsin (bovine)	Sigma Chemical Co., St. Louis	100.00	6.04
Chymotrypsin (bovine)	Sigma Chemical Co., St. Louis	93.00	3.93
Elastase (porcine)	Sigma Chemical Co., St. Louis	78.00	6.78
Corolase PP	Rohm, GmbH, Darmstadt	81.00	15.82
PEM 2500S	Novo Nordisk, Copenhagen	78.00	7.66
Debitterase DBP .20	Imperial Biotechnology, London	0.15	0.83
PEM 2700S	Novo Nordisk, Copenhagen	78.50	7.22
PEM 800S	Novo Nordisk, Copenhagen	78.70	8.08
Pancreatin	Chemicon Ltd., Dublin	100.00	13.22

were fractionated by ultrafiltration. This insoluble substrate was rendered soluble by hydrolysis with all proteases. Upon fractionation with a 10,000 molecular weight cutoff membrane it was reported that all permeates were 100% soluble whereas the solubility of the retentate increased with the degree of hydrolysis and was dependent on the type of enzyme used. Trypsin retentate was the most soluble followed by Neutrase, Alcalase, and chymotrypsin.

These serve as just a few examples of the increase in solubility of dairy proteins caused by proteolysis. There are many more studies that report similar results based on that can be summarized that solubility of almost all dairy proteins could be improved by treatment with proteases. The increased solubility of enzyme-modified proteins is advantageous in the manufacture of whey protein enriched nutritional supplements that are otherwise vulnerable to coagulation and fouling during thermal processing.

Emulsification

Stable coexistence of an oil phase and an aqueous phase in a food system necessitates good emulsification properties. Dispersion of one phase into another with which it is normally immiscible requires input of mechanical energy and results in the increase of interfacial area by several orders of magnitude. Thermodynamic instability would lead to the rapid separation of the two phases unless stabilized by surfactants capable of lowering the interfacial tension between the phases. Proteins, by virtue of their amphipathic nature, exhibit good emulsifying properties. Behavior of proteins at the oil-water interface in forming stable emulsions is generally studied under two aspects. Emulsifying capacity is a quantitative measure of how much oil can be emulsified by unit weight of the protein; the second is

emulsion stability, which measures the ability of the formed emulsion to remain unchanged over time.

Many proteins are surface active and are widely used to control or influence interfacial behavior. The primary and secondary structures of proteins have been believed to be important in the observed emulsifying functionality. However, parts of a protein molecule may consist of amphipathic α -helices and β -sheets with high surface activity so that a peptide designed to have a maximally stabilized structure should have a higher potential specific surface activity. Enser et al. (1990) tested this hypothesis by synthesizing various peptides of differing chain lengths and amino acid sequences. Eight amphipathic peptides were synthesized with 8, 11, 15, 18, 22, 26 and 29 amino acid residues (Table 2). The potential for the α -helix formation at the interface was maximized through the use of α -helix forming amino acids, a relatively large 200° arc of hydrophobic surface and ion pairs of basic and acidic amino acids on the hydrophilic surface.

Peptides 1 and 2 showed no emulsification activity, peptide 4 showed activity at pH 7.0, but not at pH 5, and peptides 5,6 and 7 showed superior emulsifying activity indices when compared with bovine serum albumin. The emulsions formed were stable at pH 7. Peptide 5 stabilized the emulsions for 34 days which was over eight times longer than that of bovine serum albumin (2 days). At pH 5 emulsions made with peptides 5, 6 and 7 were 16–19 times more stable than those formed by bovine serum albumin. At pH 5, peptide 8 showed behavior similar to peptide 4, but at pH 7, no emulsification activity was observed with peptide (Enser et al., 1990). Based on this work the authors conclude that emulsifying activity is present only in samples which show some helical confirmation in solution (peptides 3–7) and sufficient residues are present to support at least three turns of the helix. Conclusions about

TABLE 2
The Sequence and Structural Properties of Synthesized Peptides (Enser et al., 1990)

Peptide Number	Number of Residues	Sequence	Possible α -Helical Turns	% Helix
1	8	SWAEALSK	2	0.2
2	11	SWAEALSKLLS	3	0
3	15	SWAEALSKLLESLAK	4	18.3
4	18	SWAEALSKLLESLAKALS	5	27.3
5	22	SWAEALSKLLESLAKALSELAK	6	37.5
6	26	SWAEALSKLLESLAKALES	7	37.1
7	29	SWAEALSKLLESLAKALESLLK	8	47.4
8	18	SWAEAWSKWESAKAWS	5	NA*

*NA = not analyzed.

the relationship between peptide length and emulsifying activity or emulsion stability were not made.

When β -casein was modified by hydrolysis with trypsin, the emulsifying activity of the hydrolyzates was higher than that of β -casein in the range of pH 1.5 to 3.5 and 6.5 to 10 but all the emulsions obtained with trypsin-treated β -casein were less stable than those obtained with unhydrolyzed substrate (Chobert et al., 1989a). For whole casein and κ -casein at pH >5.5, the emulsifying activity increased as the pH increased and the maximum value was obtained for very alkaline solutions. For pH values below 4.5, the increase in emulsifying activity was much more pronounced at pH 2.5, and activity decreased below pH 2.5 (Chobert et al., 1989b). The emulsifying activity of the caseinomacropptide increased in the acidic range rather than the basic range. The caseinomacropptide is a hydrophilic fragment resulting from the hydrolysis of κ -casein by chymosin.

A peptide corresponding to the 23 N terminal residues of α_s1 -casein was purified from a peptic hydrolyzate of α_s1 -casein (Shimizu et al., 1984). The emulsifying activity was concentration dependent. At concentrations greater than 2%, this peptide fragment was as effective as the unhydrolyzed protein in emulsifying vegetable oil. The peptide maintained its emulsifying activity in the acidic pH range of 3.5–5.5, whereas the unhydrolyzed protein lost its ability to emulsify oil in this acidic range.

A hydrophobic peptide of 17 residues (f193–209) and a hydrophilic peptide of 25 residues (f1–25) were isolated from chymosin hydrolyzates of bovine β -casein (Lee et al., 1987c). Both peptides were not very efficacious in emulsifying oil at neutral pH. In the acidic or alkaline pH range, however, f193–209 showed high emulsifying activity. The hydrophilic fragment (f1–25) showed high emulsifying activity values in the acidic pH range. Both peptides were more surface active at pH 3 than at pH 7 (Table 3). It is interesting to note that the f1–25 segment is rich in serine phosphates.

The fragment f193–209 when combined with the hydrophilic glycomacropptide was observed to increase the emulsifying activity of the mixture greatly at neutral pH (Lee et al., 1987b). These authors hypothesized that an amphipathic structure was formed by the two peptides in mixture.

Chobert et al. (1988b) treated casein with a *Staphylococcus aureus* V8 protease to obtain 2 and 6.7% degrees of hydrolysis. This enzyme has specificity for peptide bonds in which the carbonyl group is contributed by glutamic acid. After 2% degrees of hydrolysis, a majority of the fragments had molecular weights <12,500, as determined by molecular size exclusion chromatography. At all pH values tested the hydrolyzed caseins had poor emulsifying activity when compared with the unhydrolyzed control. The reasons for this observation were not evident to the authors and they theorize that the peptides may not have been amphiphilic.

When casein was treated with trypsin to three levels of hydrolysis 4.3, 8.0 and 9.9% degree of hydrolysis, the hydrolyzates had molecular weights <15,000 (Chobert et al., 1988a). The emulsion capacity of the hydrolyzates was improved over the controls in the pH range of 4.0–5.0. At all other pH values tested, the hydrolyzates had inferior emulsion capacities than the unhydrolyzed control. As the degree of hydrolysis increased, the emulsion capacity in the pH range of 5.5–8.0 decreased. In the same study, whey protein concentrate was also hydrolyzed by trypsin to obtain 2.5, 3.9 and 5.3% degree of hydrolysis. In all instances, the hydrolyzed whey proteins had better emulsion capacities than the unhydrolyzed control. They also suggested that in order to maintain good emulsifying properties, the apparent molecular weights of the peptides should not be less than 5,000. In addition, the important role played by enzyme specificity in generating desirable peptides with amphiphilic properties was also reinforced.

Proteose-peptone is a heat-stable and acid-soluble fraction of milk that has important functional

TABLE 3
Emulsifying and Structural Properties of the Peptide Fragments Generated by Hydrolysis of β -Casein by Chymosin (Lee et al., 1987c)

Peptide	Emulsifying Activity Index (m ² /g)	Average Hydrophobicity	Molecular Weight	Surface Tension at pH (Dynes/cm)	
				7.0	3.0
f193–209	8.2	1,768	1880	54.4	50.1
f1–25	10.4	938	3115	56.1	51.8

properties (Innocente et al., 1998). These fragments are believed to arise from the hydrolysis of a milk fat globule membrane protein. Component 3 of proteose peptone was reported to be a good surfactant at both air-water and oil-water interfaces. No emulsions or foams were prepared from the fractions investigated in this study.

Sodium caseinate was hydrolyzed with Protamex (*Bacillus subtilis* protease) to obtain 0.5, 1, 3, 9 and 15% degree of hydrolysis (Slattery and Fitzgerald, 1998). Improved functional properties over the unhydrolyzed controls were noted at low degrees of hydrolysis. At higher degrees of hydrolysis, the emulsion activity decreased. At high pH values, the emulsion activity progressively decreased with increase in hydrolysis. Similar to many studies cited in this review the exact nature of the peptides generated was not described.

Bovine β -casein was hydrolyzed by plasmin (Caessens et al., 1997). The hydrolyzates were fractionated by reverse phase high performance liquid chromatography. Soluble peptides containing a variety of peptides from the "middle part" of the β -casein sequence in different proportions possessed improved emulsion capacity at pH 6.7 when compared to that of intact β -casein. Fractions containing hydrophilic N-terminal portion of the β -casein showed inferior emulsion properties at pH 6.7. The approximate sequences of the peptide fragments generated are also reported in this paper. The fractions for which functional properties were determined were mixtures of peptides generated. The middle part of the sequence included f29–105 and f29–107, which are proline and glutamine rich portions of the β -casein molecule.

Acid casein was hydrolyzed using immobilized trypsin, chymotrypsin and Rhozyme-41 (Haque and Mozaffar, 1992). Between 2 and 5%, degree of hydrolysis was attained by varying the retention time of the substrate in the immobilized enzyme columns. Modifications markedly increased the emulsion activity index, while the emulsion stability decreased slightly or remained the same. As the concentration of the modified proteins increased, a decrease in the surface per unit mass reflected the thickening of the interface. Similarly, the oil holding capacity was also markedly improved by limited proteolysis.

Mozaffar and Haque (1992) synthesized peptides from a casein hydrolyzate and measured the functional properties of these synthesized peptides. Two different kinds of plastein type peptides, one water dispersible and the other indispersible, were synthesized from casein hydrolyzates and papain. The oil holding capacity was considerably improved

for both preparations over native casein or its hydrolyzate. The dispersible peptide was highly soluble at neutral pH and was more thermostable than casein. The emulsion activity and stability of both peptides was decreased over that of casein. The sequence or the structural properties of the peptides were not reported in this study.

Casein was hydrolyzed by trypsin to 5% degree of hydrolysis and the hydrolyzate was fractionated by ultrafiltration (Turgeon et al., 1996). While screening for the best emulsifying properties, the retentate fraction was further separated by ammonium sulfate fractionation, ion exchange and reverse phase high performance liquid chromatography. Residue from the 30% ammonium sulfate saturation yielded three fractions on anion exchange chromatography. Preparative reverse phase high performance liquid chromatography was used to isolate peptides with good emulsifying activity. Using computational methods and amino acid sequencing, the identity of the peptides possessing good emulsifying activities were determined. These peptides originated from α_s1 – casein (f167–208), β -casein (f48–63 and f129–184).

Whey proteins are less surface-active than caseins because of their globular structure. Mutilangi et al. (1996) hydrolyzed heat denatured whey protein isolate with several proteases. The hydrolyzates were fractionated by ultrafiltration with a 10,000 molecular weight cutoff membrane to obtain a retentate and a permeate fraction. Retentates had higher emulsifying activity indices than permeates, while permeates did not form stable emulsions. Fractions generated by hydrolysis with trypsin generally had the best emulsifying properties.

Salad dressings are emulsions with a large dispersed phase volume and model salad dressings can be prepared by mixing oil, vinegar, spices and sweetener in a Waring blender. Turgeon et al. (1996) prepared tryptic and chymotryptic hydrolyzates of whey protein concentrate and heat-treated whey protein concentrate. Salad dressings made with 0.5% of unhydrolyzed whey protein concentrate or heat-treated whey protein concentrate showed low stability (<17 days). Emulsions made with whey protein concentrate were unstable (<4 days), while higher stability was obtained when the whey proteins were heat-treated. Tryptic hydrolyzates resulted in increasing the stability of the emulsions made. If low molecular weight materials (<1,000 MW) were removed, the performance of the hydrolyzate improved markedly. When chymotrypsin was used to hydrolyze substrates, the resultant products were not capable of producing

stable emulsions. This may indicate the role of enzyme specificity in generating peptides that are useful in salad dressing applications.

There is some indication from the work with total hydrolyzates and their fractions that low molecular weight materials may impede the emulsification performance of the peptides. This lends further credence to the possibility that an optimal peptide chain length range may exist for forming stable emulsions (Panyam, 1998). Further work on elucidating peptide sequences and the structure of such sequences will be of great interest in the study of structure-function relationships of food proteins (Table 4).

Commercial hydrolyzates of whey proteins varying between 8 and 45% degree of hydrolysis were used to make emulsions with soybean oil (Singh and Dalgeish, 1998). The concentration of hydrolyzates was varied between 0.02 and 5% (wt/wt). The stability of these emulsions was measured by determining the average size of the emulsion droplets and their size distribution both immediately after formation and after storage. The effects of heating on the stability of the emulsions were also determined. As estimated by the particle sizes, the maximum emulsion capacity was observed with 10 or 20% degree of hydrolysis. Higher degrees of hydrolysis resulted in peptides that were reported to be too small to act as emulsifiers. At lower than 10%

degree of hydrolysis, these researchers reported that a lowered solubility diminished the ability of hydrolyzates emulsify oil. All of the emulsions prepared were unstable to heat at temperatures of 122°C for 15 min, but emulsions prepared with less hydrolysis were stable to heat at 90°C for 30 min. To a limited extent, mixing different peptides post emulsification could alter emulsion stability. This report points out the possibility of making stable emulsion with hydrolyzates for most food uses. If emulsion, such as an infant formula, has to undergo a thermal process it may be unstable.

A whey protein hydrolyzate with 27% degree of hydrolysis was used at 4% concentration (wt/wt) to emulsify 4% soy oil using a two-stage homogenizer. Emulsions containing 0.1 or 0.25% of commercial soy lecithin or hydroxylated lecithin were also prepared with this protein hydrolyzate. The effect of retorting at 121°C for 16 min on creaming stability, droplet size distribution, microstructure, surface concentration and surface composition were then studied. The effect of retorting was an immediate destabilization of the lecithin-free emulsions. Addition of unmodified lecithin slightly improved the stability of the retorted emulsions, but did not prevent coalescence. Addition of hydroxylated lecithin markedly improved the creaming stability after retorting and retarded coalescence. These authors

TABLE 4
Summary of Physical Properties of Milk Protein Peptide Sequences Reported in Literature to be Effective Emulsifying Agents

Fragment Number	Non-Polar: Polar Residue	Hydrophobicity (kcal/mol)	Approx. Molecular Weight	Reference
α_s -casein				
1-23	0.77	12.2	2261	10
154-199	0.69	18.2	4843	13
167-208	0.82	18.1	4733	36
β -casein				
1-25	0.41	8.9	1511	29
29-105	1.0	31.6	7740	33
29-107	0.95	31.6	7897	33
48-63	1.29	9.8	1733	36
129-184	1.35	31.7	5447	36
183-209	1.69	7.0	1691	29
κ -casein				
106-169	0.75	13.1	6497	15
β -lactoglobulin				
41-60	0.82	10.7	1852	42
41-100	0.81	25.2	5752	46
149-162	0.50	5.8	1099	46

report that the most important factor affecting creaming stability was the particle diameter, in accordance with Stokes law. Incorporation of unmodified lecithin encouraged coalescence and increased average droplet diameter, but the mechanism by which creaming stability is preserved in systems containing hydroxylated lecithin was not evident.

It has generally been reported in literature that surface activity is positively correlated with peptide chain length. A minimum chain length of greater than 20 residues has been suggested as necessary for good surface activity. The amino acid composition and sequence of this peptide may also be an important consideration. Evidence is being provided with studies in which peptides with good surface activity have been isolated and characterized with respect to their chain length and sequence. Environmental conditions of the test play an important role as well in the functional properties of the peptides. Additionally, mixtures of peptides may have synergistic or antagonistic effects on surface activity.

Gauthier et al. (1993) hydrolyzed commercial 35% whey protein concentrate and heat-treated 35% whey protein concentrate with trypsin and chymotrypsin. The hydrolyzates were ultrafiltered with a 1,000 molecular weight cut off membrane. The interfacial properties of these peptides revealed that tryptic hydrolyzates had rates of adsorption at the oil water interface than intact proteins. Enzyme specificity was considered important because the tryptic hydrolyzates were more surface active than the chymotryptic hydrolyzates. Turgeon et al. (1991, 1992a) also reported earlier similar observations.

The predominant component in whey protein is β -lactoglobulin. Turgeon et al. (1992b) demonstrated that an oligopeptide from β -lactoglobulin (f41-60) with a size of 2300 Daltons possessed improved surface activity over the native β -lactoglobulin. The three dimensional structure of β -lactoglobulin reveals that the core of the molecule consists of eight strands of anti-parallel β -structure that form a calyx with a short length of α -helix on the surface. It has been suggested that the core of the β -barrel domain is the hydrophobic region that binds small hydrophobic molecules (e.g., retinol, fatty acids). A number of reports have shown that major fragments of the β -barrel domain can be produced by limited trypsinolysis of β -lactoglobulin (Chen et al., 1993; Huang et al., 1994, 1996).

Fragments of the β -barrel domain of β -lactoglobulin were obtained by membrane fractionation of a limited proteolysate prepared with an immobilized trypsin reactor (Huang et al., 1996). Analysis of the fraction by size exclusion chro-

matography under physiological conditions indicated that the fraction contained a predominant peptide with a size of 8400 Daltons and several other peptides with sizes ranging from 2,000 to 30,700 Daltons. When the fraction was analyzed by gel electrophoresis under reducing conditions, a major peptide with a molecular weight of 6,400 Daltons was observed. The authors suggest the presence of a smaller peptide linked to this 6,400 Dalton peptide via a disulfide linkage. The isolated peptide had a reduced surface hydrophobicity and a slightly higher surface and interfacial tension than native β -lactoglobulin. The emulsifying activity index for the domain peptide was twofold larger than that of the intact protein. Droplet size measurement revealed that domain peptides formed smaller emulsion droplets than the intact protein. Their results suggested that the emulsifying properties of the f41-100 + f149-162 were greater than those of β -lactoglobulin f41-60.

Foaming

Dispersion of air in a continuous liquid phase generates a large interfacial area. Stabilization of this air-water interface by surfactants like proteins or peptides results in the formation of foam. The role and behavior of proteins at the interface is governed by the primary and secondary structure of the peptide chain. A diverse array of products ranging from ice cream to cappuccino gain their identity and structure from the foaming properties of milk proteins. Enzymatic modification of proteins generates peptides with altered foaming properties.

Casein was hydrolyzed by trypsin, chymotrypsin, Alcalase, Neutrase, Pronase, pepsin, acid fungal protease or Prozyme to 5 or 10% degrees of hydrolysis (Patel, 1994). Permeates were 100% soluble and the hydrolysates were soluble at pH 4.2. Several enzymes produced hydrolysates that could foam as well as the controls. Generally, the foam capacities were lower at 10% DH compared to the 5% DH. Only hydrolyzates of acid-pH optimum enzymes formed stable foams. By using acid fungal protease peptides of <10,000 molecular weight that formed copious and stable foams were observed. Systematic purification of this fraction led to the identification of three peptides, all of which originated from β -casein: (f101-145), (f107-145) and (f107-135).

Kuehler and Stine (1974) treated casein, laboratory prepared whey protein concentrate and commercial whey protein concentrate with pepsin,

Pronase and Prolase. Pepsin and Pronase hydrolyzates produced foams that were inferior to the untreated control. Hydrolysis of whey protein by Prolase resulted in improving the foaming capacity and foam stability of the hydrolyzates over the control.

Whey protein isolate was modified to enhance foaming characteristics by controlled hydrolysis using Alcalase, acid fungal protease, chymotrypsin, pepsin and trypsin to achieve 2.5–3.0% degree of hydrolysis (Althouse et al., 1995). Hydrolyzates were separated by ultrafiltration (10,000 molecular weight cutoff) to obtain a permeate and a retentate fraction for each enzyme treatment. Foam capacity, foam stability and surface tension at the air water interface were measured. Permeate from Alcalase treatment exhibited the best foaming characteristics, comparable to that of egg white.

Innocente et al. (1998) report that proteose peptone 3, a component that results from the milk fat globule membrane protein, has good foaming properties. At concentrations of 0.05, 0.10 and 0.20 g/100 ml of proteose peptone 3, the equilibrium surface tension at the air-water interface were reported to be 44.75, 36.14 and 32.11 mN/m, respectively. The compression isotherms for the total proteose peptone films were different from those of the proteose peptone 3 films. The maximum surface elasticity of the proteose peptone 3 films was at surface values of 0.3 and 0.6 m²·mg⁻¹. This property was considered important for the foaming capacity and foam stability parameters.

When casein was hydrolyzed by a protease obtained from *Bacillus subtilis* (Slattery and Fitzgerald, 1998) various degrees of hydrolysis were observed. At low degrees of hydrolysis (0.5 or 1.0) and at pH 8 or 10, hydrolyzates displayed increased foam expansion and decreased foam drainage compare to unmodified substrate. In general, higher than 1% degree of hydrolysis resulted in poor foaming properties.

Caessens et al. (1997) hydrolyzed casein with plasmin and then fractionated the hydrolyzate by ultrafiltration. Plasmin preferentially hydrolyzes Lys-X and Arg-X bonds. The substrates most affected by plasmin are α_2 -casein and β -casein. They observed that the peptides from the C-terminal end of β -casein, which were hydrophobic and poorly soluble in water had improved foam forming and stabilizing properties. The fraction containing the hydrophilic N-terminal part of β -casein showed inferior foam and surface-active properties at pH 6.7. The C-terminal half of β -casein corresponds to

γ -casein and therefore the surface pressures generated by this peptide is higher.

In contrast to the emulsification properties of enzyme modified dairy proteins, relatively little work has been done in the area of foaming properties. It will be interesting to understand the structural and sequence properties necessary to form good foams from these substrates. With advances in genomics, it may be possible to screen for proteases that can specifically hydrolyze the bonds desired, leading to a target production of enzyme modified ingredients.

Gelation

A gel is easier to recognize than to define. Qualitative descriptions of gels can help with this recognition. Gels are solids, able to store the work expended in their deformation and to recover to their original shape. Often gels are soft solids that can be deformed and contain a large proportion of low molecular weight liquid. Thus, gels exhibit both elastic and viscous properties described by the term viscoelasticity. The gelation process can immobilize a large volume of liquid.

A classic example of gelation of milk occurs in the cheese making process wherein the enzyme chymosin causes the formation of a soft gel. This gelation occurs due to the hydrolysis one specific peptide bond (Phe105-Met106) in κ -casein by chymosin. The scission of the glycomacropeptide causes the casein micelle to become sensitive to ionic calcium in the environment leading to coagulation or gel formation (Fox et al., 2000).

Because of the vast amount of available literature, textbooks and monographs on cheese science, this article will not look at the gelation of milk. The focus is on modification of milk proteins (other than κ -casein) that can result in the formation of gels.

Kitabatake and Doi (1993) authored an excellent article on enzymatic methods of gel formation using egg proteins. In this paper they discussed various physical and enzymatic treatments that can result in improving the gelation of ovalbumin and egg whites. An intriguing sentence in this article states that a "limited proteolysis of milk whey protein changed the gel properties markedly." Unfortunately no reference was provided for this statement.

Any alteration in the native confirmation of a protein has the potential to induce gelation (Foegeding and Hamann, 1992). Enzymes have thus been used to modify the conformation of proteins

and the altered conformation increases the propensity of the protein to form gels under appropriate conditions. Sometimes other enzymes, e.g. transglutaminase, can be employed to alter the properties of the formed gels (Tanimoto and Kinsella 1988). Once more, such modifications as those affected by transglutaminase are not considered in this article.

Ju et al. (1997) investigated the enzyme-induced gelation of unheated and denatured whey proteins. Solutions of whey protein isolate (9% w/v) were denatured by heating at 80°C for 2–30 min and gelation was induced by incubation with a *Bacillus licheniformis* protease at 40°C and pH 7.0. The heat treatments denatured up to 98% of the whey proteins. *Bacillus licheniformis* protease (BLP) was able to induce gelation of both unheated and pre-heated whey proteins, but the gelation process was strongly dependent on the extent of denaturation. Higher percent of denaturation resulted in earlier gelation and a faster increase in gel firmness. The relationship between the extent of denaturation and the rate of gel firming was reported to be linear. The strength of the enzyme induced gels increased continuously during a 30 h incubation. After 9 h, the gel from 98% denatured whey protein was 3.2 times stronger than with 47% denaturation and 18 fold stronger than the gel from unheated whey protein isolate. The gel from the unheated whey protein isolate exhibited a particulate microstructure, whereas the gel from the 8% denatured whey protein isolate had a fine stranded structure.

Whey protein isolate (WPI) solutions were hydrolyzed at 55°C by Alcalase, Esperase, Multifect P-3000, or Protease N (Ma et al., 1997). During hydrolysis, turbidity increased, followed by formation of viscous solutions, and, finally, soft gels. Protease N exhibited the quickest gelation. Gels formed more rapidly at higher protein concentrations. Size-exclusion high performance liquid chromatography showed an aggregate peak in the void volume, whose area increased but elution time decreased during hydrolysis. Enzyme-induced aggregates were probably formed mainly via hydrophobic interactions and also some disulfide linkages. Differences in the properties of aggregates and gels induced by the four enzymes indicated that the specific peptides formed were important. Degree of hydrolysis at gel points for the four enzymes were in the range of 12.1–15.4%. For BLP induced whey protein gelation, a DH of 2% was reported (Otte et al., 1996). BLP is a protease that preferentially hydrolyses Asp-X and Glu-X bonds. This specificity probably caused aggregation and gelation at a low DH.

In these instances, the exact nature of the peptides that result in gelation are not easy to understand. The processing pre-treatments accorded to proteins pre- or post-modification confound the understanding of gelation process. In general, it seems that enzymatic modification results in a conformation of the protein that can form aggregates. The formed aggregates have a propensity to form gels under a variety of conditions. Altering the environment by changing pH, ionic strength, and type of ions, or by physical treatments such as heat, causes the aggregates to form gels. Structural information in this context is of limited use in delineating the mechanism of gelation.

BIOLOGICAL ACTIVITIES

There are many comprehensive reviews on the effects of fragments from milk proteins on various biologic functions (Clare and Swaisgood, 2000; Shah, 2000; Smacchi and Gobetti, 2000; Brody, 2000; Tome and Ledoux 1998; Meisel and Schlimme, 1990; Meisel and Frister, 1989). These reviews and the insights they provide are highly recommended to researchers interested in learning more about the potent effects of peptides derived from milk proteins. In general, these peptides can be opioids, mineral carriers, antihypertensives, immuno-stimulants, anti-thrombotics, and antigestrics. These bioactive peptides are generated in-vivo, in-vitro and during food processing. Bioactive peptides are largely found in milk, fermented milks and cheeses. Proteolytic enzymes naturally occurring in milk and enzymes from lactic acid bacteria or exogenously added enzymes contribute to the generation of bioactive peptides. Milk contains many hormones and some of these are peptides (Table 5). The physiological significance of the presence of these peptides is not fully understood (Koldovsky, 1998).

Dunbar et al. (1999) report on the presence of a major peptide growth factor in milk. Betacellulin (BTC), a member of the epidermal growth factor (EGF) family of peptide growth factors, was purified from a growth factor enriched whey fraction of bovine milk by a combination of ion exchange chromatography, gel-filtration chromatography, affinity chromatography and reverse-phase-HPLC. Bovine BTC (bBTC) had an apparent molecular mass of 21–22 kDa on SDS PAGE and existed in a glycosylated form. The cDNA encoding bBTC was obtained by a combination of 5' and 3' rapid amplification

TABLE 5
Peptide Hormones in Bovine Milk (Koldovsky, 1998)

Bovine Growth Hormone (BST)	Vasoactive Intestinal Peptide
Prolactin	Relaxin
Thyrotropin releasing hormone	Epidermal growth factor
Gastric inhibitory peptide	Epidermal growth factor (EGF-I)
Somatostatin	Epidermal growth factor (EGF-II)

of cDNA ends. The primary translation product consists of 178 residues containing a putative signal sequence, a transmembrane domain, the mature BTC domain and a cytoplasmic domain containing a highly hydrophilic Arg-Lys rich region similar to that of mouse BTC and human BTC. The amino acid sequence of bBTC precursor was 88% identical with human BTC and 79% identical with mouse BTC. The bBTC gene was found to be expressed in a wide range of tissues including the mammary gland. The identification of BTC in milk raises the possibility that it has a major role in the growth and development of the neonatal gastrointestinal tract.

Antimicrobial and Probiotic Peptides

Peptides produced *in vivo* from the ingestion of milk have been shown to exert a physiological effect. Lactoferricin, a peptide cleaved from lactoferrin by pepsin, interacts with microbial surfaces and provides an antimicrobial effect (Tomita et al., 1991). Lactoferricin markedly inhibited the growth of *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Staphylococcus haemolyticus*, *Streptococcus thermophilus*, *Corynebacterium ammoniagenes*, *Bacillus subtilis* and *Bifidobacterium infantis* (Tome and Ledoux, 1998; Tomita et al., 1994). Similarly, Cascocidin, a α_s2 -casein fragment (f165–203) is produced by the action of chymosin and inhibits the growth of *Escherichia coli* and *Staphylococcus carnosus* (Zucht et al., 1995). Isracidin derived from α_s1 casein B (f1–23) is effective against a lethal infection of *Staphylococcus aureus* and protected mice from *Candida albicans* by stimulation of phagocytosis and immune responses (Lehov and Regelson, 1996). Another study reported that caseinomacropptide from human milk (para κ -casein) generated from the action of chymosin on κ -casein (f106–169) enhances the growth of *Bifidobacterium* significantly (Azuma et al., 1984). *Bifidobacterium infantis* S₁₂ protects against the development of *Escherichia coli* in the gastroin-

testinal tract. Caseinomacropptide has been shown to exert antiviral effects by inhibiting haemagglutination of influenza virus or by blocking receptor to cholera toxin. Leucocytes may also play a role in host protection by secreting antibodies to cytokines (Lestrade, 1988).

In breast fed infants, about 90% of the microflora present in the intestinal tract is constituted by *Bifidobacterium bifidum* (Zbikowski and Ziajka, 1986). Casein hydrolyzed by papain and pepsin contained fractions that were capable of enhancing the growth of *Bifidobacterium bifidum*. Traditional promoters of the growth of bifidobacteria have included a variety of oligosaccharides and the inclusion of peptides generated by enzyme hydrolysis of casein adds a new dimension to probiotics.

Lactoferricin, a peptide derived from bovine lactoferrin, inhibited thiobarbituric acid reactive substances (TBARS) formation in an iron/ascorbate induced liposomal phospholipid peroxidation system (Wakabayashi et al., 1999). The inhibition of TBARS formation occurred with N-acetylated 9-mer peptides with a core sequence of lactoferricin. These peptides exerted their antioxidant effect at a concentration almost 100 times lower than lactoferricin. This study also revealed a novel role for acetylated peptides.

Siciliano et al. (1999) studied the interaction of bovine lactoferrin with Herpes Simplex virus type I. Bovine lactoferrin prevents the infection of some enveloped and naked viruses. To identify the sequence responsible for the antiviral activity, 31 HPLC fractions were tested from tryptic digestion of bovine lactoferrin towards herpes simplex virus type I. Only a few HPLC purified fragments were effective against this virus even at concentrations lower than undigested lactoferrin. Two large fragments, one corresponding to the C-lobe (f345–689) and the other corresponding to a large portion of the N-lobe (f1–280) inhibited herpes virus. The smaller part of the N-lobe (f86–258) was ineffective in suppressing the herpes virus. Among the low molecular weight fragments, only two small peptides, which coeluted in a single chromatographic peak, were effective in

inhibiting herpes virus. These peptides, both present in the N-lobe were identified as peptides 222–230 (ADRDQYELL) and 264–269 (EDLIWK). The same peptides, chemically synthesized were able to inhibit herpes simplex virus type 1.

Digestive and Metabolic Effects

Some milk peptides are thought to affect the gastric hormones. These hormones include gastrin, cholecystokinin, and secretin and gastrin inhibitory peptide. These hormones control gastric and intestinal motility and gastric and bilio-pancreatic secretions (Hara et al., 1992). Specific bioactive peptides resulting from the proteolysis of various food proteins (including caseins) are thought to exert these effects either in the lumen of the intestine or post absorption (Daniel et al., 1990). Ingestion of large amounts of casein enhances nitrogenous secretions when compared with ingestion of a similar amount of whey protein (Mahe et al., 1996). This was considered to be suggestive of the role of casein-derived peptides in enhancing the secretion of gastric hormones (Daniel et al., 1990; Mahe et al., 1995; Yvon et al., 1990). In the contents exiting the stomach after casein ingestion, caseinomacropeptide, glycomacropeptide, opioid peptides and peptide inhibitors have been identified (Yvon et al., 1990; Beucher et al., 1994).

Numerous studies have focused on the presence of opioid peptide sequences in the sequence of milk proteins including β -casein, α -casein, β -lactoglobulin, α -lactalbumin, lactoferrin, and serum albumin (Meisel, 1997). Opioid peptides derived from bovine casein are called casomorphins or casoxins while those derived from whey proteins are called lactorphins. Since they originate from exogenous food sources and exhibit naloxone inhibition, they are also termed exorphins in contrast to endogenous derived opioid peptides, which are called endorphins, enkephalins and dynorphins.

Meisel and Schlimme (1990) point out that there are opioid agonists (molecules that bind to naloxone receptors) and opioid antagonists (blocks the action of an agonist). All fragments from κ -casein acts as antagonists, whereas β -casomorphins, two α_s1 -casein exorphins and the lactophorins derived from α -lactalbumin and β -lactoglobulin act as opioid agonists. The physiologic role of casomorphins is speculated to be the exertion of anti-diarrhea action and in so doing may also affect nutrient adsorption. Casoxin C has been identified as an ileum contracting peptide

(Takahashi et al., 1997). Casomorphins react with opioid receptors on the serosal side of the intestinal epithelium causing an increase in electrolyte transport. This can in turn be related to the antisecretory activity that is observed (Tome and Ledoux, 1998; Tome et al., 1987; Ben Mansour et al., 1988; Mahe et al., 1989). Casomorphins also modulate postprandial insulin, somatostatin and pancreatic polypeptide release (Morley et al., 1983; Schusdziarra et al., 1983a, b). Casomorphins have also been thought to be involved in food intake regulation. It has been reported that reticular contraction frequency, duration and amplitude in abomasums of animals infused with casomorphins decreased, thereby reducing gastric mobility and emptying (Froetschel, 1996).

Meisel and Frister (1989) studied the *in vivo* formation of biologically active caseinopeptides. They proved that bioactive peptides were released in the small intestine of minipigs in the course of luminal digestion of diets containing bovine casein. An opioid peptide and a phosphopeptide were isolated from jejunal chyme and were chemically characterized. The opioid peptide was identified as f60–70 from β -casein. This peptide named β -casomorphin II, displayed substantial opioid activity in an opiate binding receptor assay. The casinophosphopeptide was f66–74 of α_s1 -casein. Casein derived phosphopeptides form soluble complexes with calcium and trace minerals, thus regulating micronutrient entry.

Pihlanto-Leppala et al. (1994) have investigated the formation of opioid peptides by *in vitro* proteolysis of bovine caseins. Bovine α_s1 -, β -, κ -caseins were predigested with pepsin and subsequently treated with either trypsin or trypsin+chymotrypsin to simulate digestive processes. Peptides generated were separated and analyzed by high performance liquid chromatography and the separated peptides were sequenced. Digestion of α_s1 -casein with pepsin alone yielded f90–95. Proteolysis of β -casein with pepsin and trypsin produced f59–68, which contains the opioid f60–66 segment. Digestion of κ -casein for 24 h with pepsin yielded f33–38.

Peptides derived from milk proteins also affect blood pressure in humans and animals. The observed lowering of blood pressure is attributed to angiotensin-converting enzyme inhibition (ACE inhibition). This group of peptides is also called casokinins and is believed to arise from β - and α_s1 -caseins (Maruyama and Suzuki, 1982; Maruyama et al., 1985; Kohmura et al., 1989; Ariyoshi, 1993; Yamamoto, 1997; Yamamoto et al., 1994; Nakamura et al., 1995; Masuda et al., 1996). Specifically two tripeptides (Valine-Proline-Proline and Isoleucine-Proline-Proline) have demonstrated

anti-hypertensive properties in animals and humans (Yamamoto, 1997; Yamamoto et al., 1994; Nakamura et al., 1995; Masuda et al., 1996). The tripeptide sequence is present in both κ - and β -casein. This concept has been included in a drink manufactured and marketed by Calpis. Calpis cultured milk AMEALS, which has been approved by Japan's Health and Welfare Ministry as a Food for Specified Health Use (FOSHU). When consumed, this beverage reduced blood pressure in humans, and the Institute of Food Technologists awarded the Industrial Achievement Award to Calpis at its annual meetings in New Orleans in 2001.

Peptides from whey with angiotensin I-converting enzyme (ACE) inhibition have also been reported by Pihlanto-Leppala et al. (2000). The bovine whey proteins α -lactalbumin and β -lactoglobulin were hydrolyzed with pepsin, trypsin, chymotrypsin, pancreatin, elastase or carboxypeptidase alone and in combination. The total hydrolyzate was fractionated in a two-step ultrafiltration process, first with a 30,000 MWCO membrane and then with a 1,000 MWCO membrane. Inhibition of ACE was monitored spectrophotometrically. The peptides were isolated by chromatography and analyzed by mass spectrometry and sequencing. Inhibition of ACE was observed after hydrolysis with trypsin alone and with an enzyme combination containing pepsin, trypsin and chymotrypsin. Whey protein digests gave a 50% inhibition of ACE activity at concentration ranges of 345–1733 $\mu\text{g/ml}$. Values for 1–30,000 fractions ranged from 485–1134 $\mu\text{g/ml}$ and for the <1,000 fraction from 109–837 $\mu\text{g/ml}$. Potencies of the purified peptide fractions indicated that concentrations required to achieve 50% inhibition of ACE ranged from 77–1062 μM . The ACE inhibitory peptides arising from α -lactalbumin hydrolysis were f50–52, f99–108, and f104–108, while those from β -lactoglobulin were f22–25, f32–40, f81–83, f94–100, f106–111 and f142–146 (Pihlanto-Leppala et al., 2000).

In a previous study, Abubakar et al. (1998) also digested whey proteins by different proteases. The digests were assayed for ACE inhibition and also for changes in systolic blood pressure in spontaneously hypertensive rats after gastric intubation. The strongest depressive effect on the systolic blood pressure was a decrease of 55 mm Hg and was observed 6 h after gastric intubation of the whey protein that was digested with Proteinase K. The amino acid sequences and their origins were reported as Val-Tyr-Pro-Phe-Pro-Gly (f59–64 from β -casein), Gly-Lys-Pro (f18–20 β_2 -microglobulin), Ile-Pro-Ala (f78–80 of β -lactoglobulin), Phe-Pro (f221–

222 from bovine serum albumin; f62–63, f157–158, f205–206 β -casein), Val-Tyr-Pro (f59–61 from β -casein) and Thr-Pro-Val-Val-Val-Pro-Phe-Leu-Gln-Pro (f80–90 from β -casein). The strongest antihypertensive activity was reported for a chemically synthesized Ile-Pro-Ala derived from β -lactoglobulin.

These studies show promising evidence of antihypertensive activities of numerous fragments derived from caseins and whey proteins. The assays used measure ACE inhibition, which may be one mechanism by which blood pressure is lowered. Fitzgerald and Meisel (2000) think that casokinins and lactokinins represent a group of bioactive peptides that have significant potential in naturally derived agents for the prevention/control of blood pressure and related diseases. The widespread use of these peptides in functional foods/nutraceuticals requires ongoing studies including extended clinical trials to demonstrate their long-term efficacy and safety.

Two peptides isolated from κ -casein and human lactoferrin affect platelet function and could have anti-thrombic properties (Jolles et al., 1986; Raha et al., 1988). Kappa-casein fragments 106–116 and its subsets (f106–112, f112–116, f113–116) and f39–42 of human lactotransferrin are implicated in this anti-thrombic activity. Presence of these antithrombic peptides arising from κ -casein has been shown in infants fed cow's milk or human milk (Tone and Ledoux, 1998).

Peptides from milk affecting coagulation have been reviewed recently (Rutherford and Gill, 2000). Based on amino acid sequence similarities between the fibrinogen γ -chain and κ -casein, and also functional similarities between milk and blood coagulation, considerable effort has been made to investigate the effects of milk proteins on platelet function and thrombosis. A number of peptides derived from the glycomacropeptide portion of κ -casein have been shown to inhibit platelet aggregation and thrombosis. A peptide from lactoferrin (KRDS) has also been shown to inhibit platelet aggregation but to a lesser extent than its fibrinogen analog (RGRDS). Despite their functional and structural similarities they do not act in the same way in platelet function and are thought to affect thrombus formation differently.

Fosset and Tome (1998) report that an undecapeptide corresponding to the fragment f106–116 of κ -casein inhibited the ADP-treated platelet aggregation and the fixation of fibrinogen to platelets. A tetrapeptide with a sequence corresponding to f39–42 of κ -casein also has antithrombotic activity.

Biological evaluation in humans has indicated that some peptides are poorly degraded in the gastrointestinal tract and have been found *in vivo* in blood. These findings suggest a potential application of milk protein-derived peptides.

Effectors of the Immune System

A regular ingestion of milk protein components could impact on the development of tissues and organs (Britton and Kastin, 1991). Components such as lactoferrin, prolactin and IGF-1 are involved in DNA synthesis, proliferation, and differentiation. Prolactin acts as an immunoregulatory agent on T and B cells (Gonella et al., 1989; Hartman et al., 1989; Grove et al., 1991; Gala and Shevach, 1993a,b; Gaufo and Diamond, 1996; Kooijman et al., 1996). Proliferation of endothelial cells, fibroblasts, muscle cells and lymphohemopoietic cells is stimulated by insulin like growth factor 1 (IGF-1), as is DNA synthesis (Kooijman et al., 1996; Burrin, 1997).

Lactoferrin has been recognized as a growth factor for a number of cell types (Nichols et al., 1987; Oguchi et al., 1995; Hagiwara et al., 1995; Kohno et al., 1993; Azuma et al., 1989a). Epithelial cells, hepatocytes, B-lymphocytes and macrophages/monocytes all have been shown to bind lactoferrin through a specific receptor.

Peptides arising from the hydrolysis of β -casein have demonstrated their ability to stimulate mitosis of different cell types (Azuma et al., 1989b; Nagaune et al., 1989; Coste et al., 1992). Takanaka et al. (1991) report the presence of another peptide that inhibits proteolysis and protein synthesis and enhances ureogenesis. Glycomacropeptide and the parent (k-casein) inhibited *in vitro* mouse mitogen-induced proliferation of mouse spleen B lymphocytes and rabbit Peyer's patch cells and antibody responses to sheep red blood cells in mouse splenocyte cultures (Otani et al., 1986, 1988). Other peptides have been shown to act on immunocompetent cells and have been suggested to play role in the stimulation of the immune system.

Hata et al. (1999) used a commercially available preparation of casein phosphopeptide, CPP-III, which mainly contained residues 1–32 of bovine α_s2 -casein and residue 1–28 of β -casein to study the proliferation of cells in culture. The proliferative response induced by lipopolysaccharide (LPS), phytohaemagglutinin (PHA) and Concanavalin-A (ConA), and the immunoglobulin production in mouse spleen cell cultures, was significantly en-

hanced in the presence of the casein phosphopeptides. In addition, CPP-III displayed a mitogenic activity in mouse thymocyte, nude mouse spleen cell and rabbit Peyer's patch cell cultures without LPS, PHA or ConA. However, CPP-III displayed little mitogenic activity in mouse plastic-adherent cell cultures. The mitogenic activity for mouse spleen cells was hardly influenced by added proteases but was significantly affected by the addition of acid phosphatase. Based on these observations, Hata et al. (1999) conclude that CPP-III possess an immunostimulating activity in cell cultures and the activity is attributable to the O-phospho-L-serine residue, suggesting that the immunostimulating activity of CCP-III is relatively stable to proteinase action in the intestinal tract.

The immunomodificatory effect of dietary bovine κ -caseinoglycopeptide (f106–169) was examined in mice (Monnai, 1998). Mice fed a mixture of this peptide and ovalbumin gained weight at a similar rate as those fed ovalbumin alone, and no significant difference in body weight was observed between the two groups. The proliferative response to Concanavalin A, however, was enhanced in mice fed the glycomacropeptide, but such a response was not observed when the mice were fed phytohaemagglutinin or lipopolysaccharide. Moreover, the ingestion of the glycomacropeptide suppressed the level of serum IgG antibodies specific to dietary ovalbumin and intraperitoneally injected b-lactoglobulin when compared with a diet of ovalbumin alone. In contrast, IgM, IgA and IgE antibody responses were not influenced by the ingestion of the caseinoglycopeptide. Based on these observations, Monnai et al. (1998) conclude that dietary caseinoglycopeptide has an immuno-suppressing activity in mice, suggesting that caseinoglycopeptide from whey protein concentrate may be a useful ingredient in preparing immunosuppressive foods.

A recent review summarizes the relevant research pertaining to the effect of bovine milk derived peptides on the immune system (Gill et al., 2000). Bovine milk is known to contain a number of peptide fractions that can affect immune function. The vast majority of immunoregulatory peptides that have been characterized are hydrolyzate derivatives of major milk proteins. The metabolic activity of probiotic lactic acid bacteria can generate *de novo* immunoregulatory peptides from milk via enzymatic degradation of parent milk proteins. In contrast, relatively little is known of endogenous preformed immunoregulatory peptides in milk that may be relevant to modulating human health. The natural *in vivo* role of preformed and enzymatically

derived peptides is likely to be one of regulation of bovine neonatal gastrointestinal tract immune system, in order to modulate immune function with respect to the development of immunocompetence and avoidance of undesirable immunological responses. The authors conclude that a fertile field of research exists in dealing with the characterization of both the origin and function of milk derived peptides so that effects on human health can be fully studied.

ALLERGENICITY ISSUES

Concerns about food allergies have been heightened recently. The term "food allergy" is often used to denote any adverse reaction to food. A true food allergy, however, is a food intolerance that has immunological consequences. This should be clearly distinguished from "food intolerance" which is a broader term applicable to any reproducible adverse reaction to a food that is not psychologically based (Walker, 1992). Early concern for milk protein allergies was expressed due to the use of these proteins in infant foods. Digestive intolerance to food proteins may occur in childhood as a result of a wide range of etiologic and pathophysiologic mechanisms. Cow milk protein intolerance is the most common form of food intolerance in children. Food allergy and food intolerance may be confused because both produce similar symptoms, especially in young children with clinical manifestations of food allergies localized to the gastrointestinal tract (Polanco, 1992). On the other hand, food-sensitive enteropathy may be defined as the clinical food-related syndromes associated with an abnormal small intestinal mucosa. Although several foods have been reported to damage the intestinal mucosa in infancy (soy, rice, fish, chicken, meat, egg), cow milk-sensitive enteropathy is the most common cause. Whatever the mechanism, digestive intolerance to food proteins with or without enteropathy is primarily a temporary condition of infancy, in contrast to most forms of food allergy. In children with these disorders, symptoms usually resolve by 1–3 year age (Polanco, 1992). The variation in the prevalence rates of this disorder in different countries can be explained by different diagnostic criteria. The classic food-sensitive enteropathy syndromes with chronic diarrhea and failure to thrive in infancy have become rare in some European countries. Some risk factors for the development of these conditions appear to be early exposure to cow milk feedings, acute infectious diarrhea and malnutrition.

Sera from patients with cow milk protein allergy ($n = 6$) and cow dander allergy ($n = 5$) were analyzed for reactivity of IgE antibodies specific for allergens derived from milk and cow dander (Szpfalusi et al., 1993). The cow milk and cow dander allergic patients exhibited elevated specific IgE levels to milk and cow dander, respectively, as determined by radioallergosorbent test (RAST). Immunoglobulin E (IgE) immunoblot analysis revealed that cow-milk allergic patients exhibited IgE binding to milk allergens casein, β -lactoglobulin, α -lactalbumin and bovine serum albumin. Four of six cow milk reactive patients also exhibited IgE binding to cow dander proteins with molecular weights of 20, 22, 36, 50 and >200 kD. IgE from the sera of cow dander allergic patients reacted with the major allergens of cow dander (20 and 22 kD) and with other proteins of molecular weights of 24, 36, 42, 50 and 70 kD. Only one out of five sera displayed reactivity with cow milk proteins of molecular weight of 69, 92 and >200 kD. Inhibition studies revealed the cross-reactive nature of the IgE antibodies. Pre-incubation of cow milk sera with cow milk and subsequent immunoblotting led to complete blocking of IgE binding to cow dander proteins and to cow milk proteins. Pre-incubation of cow milk reactive sera with cow dander proteins led to blocking of IgE binding only to defined cow milk proteins: these could be identified in two cases as casein and one case to β -lactoglobulin. Enzyme linked immunosorbent assay (ELISA) inhibition assays confirmed the results and provided quantitation. The results demonstrate the existence of cross-reactive IgE antibodies reacting with similar or identical cow milk and cow dander allergens, which may cause clinical manifestations.

Oldaeus et al. (1992) screened several hypoallergenic milk-based formulas for adverse reactions in sensitized children. These formulas were as follows: Beba HA (an enzyme hydrolyzed whey based formula sold by Carnation under the trade name Good Start HA), partially hydrolyzed milk protein formula (consisting of 60% whey and 40% casein with a degree of hydrolysis (DH) of 8.2 from Mead Johnson), partially hydrolyzed milk protein formula B (60% whey and 40% casein enzymatically modified by pancreatin and a DH of 18.1), Alfare D (Nestle formula containing 100% whey protein enzymatically hydrolyzed), Nutramigen (extensively hydrolyzed casein based formula with a DH > 50 from Mead Johnson), Enfamil (a cow milk formula from Mead Johnson) and Similac (a cow milk based formula from Ross Laboratories). A skin prick test with wheel diameters of greater than 3 mm was taken

as a positive reaction in 15 sensitive children tested. Nutramigen and Alfare did not produce any reactions while Formula B, Beba HA and Formula A (products with partially hydrolyzed proteins) produced reactions in seven out of the fifteen subjects tested. As anticipated, Similac and Enfamil produced extensive reactions in virtually all the subjects tested. From this study it appears that a DH > 50 is required to avoid allergic responses in sensitive individuals. Up to a DH of 15 the hydrolyzed proteins can still elicit a reactive response from sensitive individuals.

To evaluate the role of specific antibody response to milk proteins in patients with milk-protein intolerance, allergen specific IgE, IgG and IgG4 to these proteins was measured by ELISA (Burks et al., 1990). Bovine casein, gamma globulin (GG), β -lactoglobulin and α -lactalbumin were the milk proteins used. Antibody production to these proteins were analyzed in 18 patients who underwent milk-protein challenges (8 positive and 10 negative) and in five normal children used in the analysis. ELISA results for specific IgE, IgG and IgG4 to these specific proteins demonstrated no statistically different response to the four milk proteins among the three patient groups by multivariate analysis. When the antibody results from the positive challenge group, the negative challenge group and the normal group were combined, the IgE and IgG4 to GG and the IgG to casein were significantly higher than the corresponding specific antibody to the other proteins tested. The IgG or IgG4 to GG would differentiate the positive from the negative challenge group, but were not significantly different from the normal control group. Contrary to previous published reports, these challenges indicate IgG specific for the milk proteins are not increased in patients with milk-protein intolerance. The data also support the concept that IgE and IgG4 are not elevated in these patients. Therefore, there appears to be no pathogenic role for these specific immunoglobulins in milk-protein intolerance.

The importance of peptide chain length in hypoallergenic infant formulae has been reviewed (Siemensma et al., 1993). Dietary proteins are broken down in three phases; in the gut lumen, at the brush border and in the cytoplasm of the enterocyte. The digestion and or absorption of di- and tri-peptides is more rapid than that of an equivalent amount of free amino acids. Therefore, the control and knowledge of the lengths of the peptides in a hydrolyzate is important for the development of physiologically optimal hydrolyzates for use in peptide based hypoallergenic infant formulae.

A new method to determine peptide lengths has been used to develop "third generation" hypoallergenic infant formulae-short chain hydrolyzates that imitate the intestinal lumen digest very closely and that have an improved taste profile. The three generations of hydrolyzates described in this review are as follows: first generation infant formulae are casein based characterized by more than 70 mol% free amino acids and detectable peptides up to 5–8 amino acids long. The second-generation formulae contain mostly whey protein hydrolyzates with 40–60 mol% free amino acids and detectable peptides up to 10–12 amino acids long. The third generation hypoallergenic formulae have 20 mol% free amino acids and detectable peptides up to 10–15 amino acid residue long. The third generation hypoallergenic formulae are the result of a better understanding of the enzymatic processes involved in hydrolysis and advances in ultrafiltration to yield hydrolyzates with a low level of free amino acids and a main fraction of short peptides. This also allows the control of taste and prevention of formation of bitter peptides. The third generation formulae resemble the intestinal luminal digests closely, especially in their low content of free amino acids.

Otani et al. (1986; Otani and Hosono, 1989) studied the antigenicity of α_s 1-casein and some peptides derived from the C-terminal region of this protein. By using a combination of chemical agents and enzymes f136–151, f152–193, f165–199, f180–199 and f194–199 were isolated from α_s 1-casein. The antigenicity of these fragments was tested with antiserum for intact bovine α_s 1-casein. On quantitative precipitation between α_s 1-casein and its antiserum, peptides f152–193 and 165–199 inhibited the formation of immunoprecipitates by 12.1% and 11.4%, respectively, whereas little inhibitory activities could be detected for f136–151, f180–199, and f194–199. Further peptides f152–193 and f165–199 had essentially equal antibody binding abilities in an enzyme-linked immunoabsorbent assay, but little antibody binding activities were observed for peptides f136–151, f180–199 and f194–199. From these results the authors concluded that the most likely location of the antigenic site is in the region 136–199 and probably in the 165–179 region of α_s 1-casein. An extrapolation of this study would be that the functional peptides responsible for emulsifying activities (Chobert et al., 1988b; Turgeon et al., 1996) might also contain the antigenic site.

Bovine β -casein has at least 6 antigenic sites in its molecule (Otani and Hosono, 1989). At least one antigenic site is associated with each of the regions 1–25, 26–60, 61–93, 94–102, 103–109, 110–144

and 157–185. Antigenic sites of native protein antigens are usually composed of 6–7 amino acid residues. Hence, these regions, especially 1–25, 26–60, 61–93, 110–144, and 157–185 may carry non-reactive fragments. The immunoassays involved in this study utilized rabbit IgG antibodies specific to peptide f110–144. Peptide f114–144 has a strong binding ability with individual IgG antibodies specific to peptide f114–144 showed strong binding ability, whereas, peptides f110–121, f122–133, and f132–144 exhibited no inhibitory activity. These results indicate that the antigenic sites in the region 110–144 are associated with glutamyl peptide bonds at positions 121 and/or 131. Once again this study also points to the possibility that the functional peptides identified in the earlier section may also be antigenic.

The effects of in vitro proteolysis on the allergenicity of major whey proteins by simulating the human gastrointestinal conditions were evaluated (Asselin et al., 1989). The proteolysis of demineralized whey with pepsin (pH 2.0 for 30 min) and/or various pancreatic enzymes (pH 7.5 for 60 and 240 min) was performed by the pH stat technique at 37°C. The enzyme inactivation was performed by heating at 80°C for 20 min. Allergenicity of the hydrolyzates was evaluated by RAST inhibition using sera obtained from children allergic to whey proteins. Selective proteolysis of whey by pepsin and α -chymotrypsin was the most efficient combination of enzymes to reduce the allergenicity of both α -lactalbumin and β -lactoglobulin. The authors suggest that this hydrolyzate could be used as an ingredient for infant milk formula with a lower allergenicity. The degree of hydrolysis ranged from 1–20% depending on the enzyme combinations and the time of incubation.

Otani and Hosono (1989) studied the immunological properties of enzyme digests of α_s 1-casein. Peptides were separated from α_s 1-casein digested with pepsin, trypsin and/or chymotrypsin by ultrafiltration with a 5,000 MWCO membrane. Molecular weights of most of the peptides in the trypsin or chymotrypsin digests ranged from 3,460 to 243 Daltons while those of most peptides from pepsin digests ranged from 1,400 to 181 Daltons, and those in the digests of the combination of the three proteases were smaller than 1,400 Daltons. The binding between intact α_s 1-casein and its IgG antibody was not inhibited by the digest of the combination of the three proteases and inhibited slightly by the pepsin, trypsin and chymotrypsin digests; each digest required 1,000 times the concentration of intact α_s 1-casein to give a 25% inhibition. The IgG

antibody titer in antisera obtained via the 10th injection of pepsin, trypsin or chymotrypsin digest was only one or two orders of magnitude lower than that in antiserum obtained by the injection of intact α_s 1-casein, while the antibody titer induced by the digest obtained from the combination of the three proteases was four orders of magnitude lower. On the other hand, the anaphylactic sensitizing capacity of the antisera with regard to digests was more than three orders of magnitude lower than that of the antiserum with regard to intact α_s 1-casein.

The effect of the degree of hydrolysis on the antigenicity of casein was investigated by Mahmoud et al. (1992). Casein was enzymatically hydrolyzed with porcine pancreatin and samples of the digests at different hydrolysis times (HT) were tested for antigenicity, hydrophobicity, net charge (zeta potential) and emulsifying activity. Casein antigenicity loss could be approximated by three-phased first order kinetics. Extensive antigenicity loss occurred during the first 10% of HT and relatively small changes occurred during the remaining 90% of the HT. Hydrophobicity and emulsifying activity decreased while the magnitude of the net charge increased with increasing degree of hydrolysis. Strong correlations were reported between hydrophobicity, emulsifying activity and zeta potential. While the antigenic reactivity of the protein hydrolyzate is a crucial factor in the formulation of hypoallergenic infant formula, the functional properties, particularly emulsification, are also very important to preparing a stable formulation. Extensive enzymatic hydrolysis of casein, which is essential to render it nonantigenic, resulted in a considerable reduction in emulsifying property. The emulsifying properties of the hydrolyzates were closely related to the hydrophobicity and zeta potential. These findings suggest that the emulsifying properties of extensively hydrolyzed food proteins can be predicted by simple measurements of certain physicochemical properties.

The reduction of whey protein antigenicity has been attempted by hydrolyzing these proteins with different proteases. Whey protein components were hydrolyzed with Corolase 7092 (peptidase from *Aspergillus* strains), pepsin and Corolase PP (a mixture of pancreatic enzymes), either individually or in combination, in trials to eliminate protein allergenicity. The hydrolyzates were characterized by physico-chemical and by immunological techniques using sera from patients allergic to milk proteins. Enzyme specificity rather than degree of hydrolysis or molecular mass distribution of hydrolyzates determined the residual antigenicity of whey proteins. Ultrafiltration was a prerequisite for obtaining

hypoallergenic whey protein hydrolyzates. Enzymatic proteolysis may also increase protein antigenicity by exposing more antigenic sites during hydrolysis, as was observed in this study for bovine serum albumin and IgG. Antigenic sites for individual whey proteins and bovine serum albumin and IgG were completely eliminated by combination of pepsin and Corolase PP, but were hardly affected by Corolase 7092. Intact epitopes of α -lactalbumin and β -lactoglobulin remained present in both hydrolyzates. These enzymes did not result in completely hypoallergenic hydrolyzates.

The above survey clearly indicates that depending upon enzyme specificity, and the extent of hydrolysis, allergenicity of milk proteins can be decreased, if not eliminated. Almost all the work has been performed in context of the hypoallergenic infant formulae. While these learnings can be applied to functional peptide ingredients derived from milk proteins, further work is required to specifically assess the allergenicity potential of these ingredients prior to their use in foods.

TASTE OF PEPTIDES

Murray and Baker (1952) were the first to identify the bitterness associated with enzyme hydrolyzates. They observed that gelatin and egg albumin hydrolyzates had a relatively bland taste while the taste of casein and lactalbumin had a bitter taste. Subsequent treatment of the casein hydrolyzate with activated carbon reduced the bitterness. Elution of the adsorbed material from the activated carbon yielded a peptide rich fraction with extreme bitterness. When these bitter peptides were hydrolyzed with acid, a "brothy" taste was detected with no bitterness.

According to Solms (1969), the common amino acids have the following taste properties near neutral pH: no taste or barely perceptible taste (D-Ala, D and L-Arg, D and L-Asp, D-Glu, L-His, D and L-Ile, D and L-Lys, D and L-Pro, D and L-Ser, D and L-Thr, D and L-Val), sweet taste (in order of decreasing sweetness: D-Tyr, D-His, D-Phe, D-Tyr, D-Leu,

L-Ala, Gly), bitter taste (in order of decreasing bitterness: L-Tyr, L-Phe, L-Tyr, L-Leu) and sulfurous taste (D and L-CySH, D and L-Met). L- Glutamic acid has a unique taste potentiating property; a similar effect has been reported for trichloric acid and ibotenic acid.

The sweet peptide aspartame is L-Asp-L-Phe-oMe, or aspartyl phenylalanine methyl ester, and tastes 180 times sweeter than sugar. Thermal degradation of this intense sweetener leads to the formation of either aspartyl phenylalanine or diketopiperazine, neither of which is sweet. Thaumatin and monellin are sweet proteins isolated from plants native to Western Africa. Thaumatin and monellin are 1600 and 3000 times sweeter than sucrose, respectively. Other than these, there are no reports of naturally occurring sweet tasting peptides in foodstuffs.

A number of protein hydrolyzates have been reported to be bitter. Ney (1972) formulated the Q rule, which assigns a hydrophobic value arrived at by totaling the free energy values of constituent amino acids in the peptides and dividing it by the number of residues. This number expresses the average hydrophobicity of a residue in the bitter peptide sequence. If the Q value is greater than 1400, the peptide will elicit a bitter taste. The bitter peptides isolated from casein hydrolyzates are shown in Table 6.

Dipeptides containing glutamic acid and/or aspartic acid have been reported to have a sour taste. Sometimes this sour taste is accompanied by bitterness or by bitterness and astringency. The acidic sensation is thought to be associated with the binding of the hydronium ion when an acid amino acid binds to the taste receptor (Kato et al., 1989).

It has been reported that peptides contain salty stimuli (Kato et al., 1989). Synthesized peptides L-ornithyl- β -alanyl hydrochloride, L-ornithyl-Tauryl hydrochloride, Lysyl-tauryl hydrochloride, L-ornithyl-glycyl hydrochloride and lysyl-tauryl hydrochloride had the same taste intensity as sodium chloride. The degree of disassociation of the carboxyl or sulfonyl group in peptides was theorized to contribute to the intensity of salty taste. These peptides may be beneficial as seasonings for

TABLE 6
Bitter Peptide Sequence from Casein Hydrolyzates (Kato et al., 1989)

Enzyme Used for Hydrolysis	Bitter Peptide Sequence
Papain	AQTQSLVYFPFGPIPNSLPQNIPPLTQ
Trypsin	GPFPVIPPVAPPEVPGK and PALPEYLK
Bacterial proteinase	RGPPFIV, VYFPFPPGINH and cyclo (LWLW)

diabetics and hypertensives because they contain no sodium ions. Some researchers claim that these peptides are not salty and the method of synthesis can inadvertently result in the presence of sodium chloride and, hence, the associated salty taste.

A number of reports exist in literature for the umami taste peptides. These are the savory peptides that have caused a lot of controversy in literature. Many researchers in Japan have conducted studies on the taste peptides, but the Western researchers are unable to discern the taste properties in the same peptides.

Proline containing peptides have been reported to be bitter (Ishibashi et al., 1988b). In order to investigate the role of proline residue in bitter taste of peptides, some oligopeptides containing proline were synthesized and their taste evaluated. Proline peptides exhibited bitterness, this aspect being different from other hydrophobic amino acids. The most significant role of proline residue in peptide bitterness was dependent on the conformational alteration of the peptide molecule by folding the peptide skeleton due to the imino ring of the proline molecule. These workers demonstrated that two bitter taste determinant sites were essential for peptide bitterness. Further, these sites had to be adjacent in the steric conformation of the peptides.

To estimate the steric distance between the bitter taste determinant sites in peptides, some cyclic Dipeptides, amino acid anilides, amino acid cyclohexylamides and benzoyl amino acids were synthesized and their taste evaluated (Ishibashi et al., 1988a). The diketopiperazine ring of the cyclic dipeptides acted as a bitter taste determinant site due to its hydrophobicity. The steric hindrance between the two sites was estimated to be 4.1 Å from molecular models of cyclic dipeptides composed of typical amino acids in bitter peptides. Due to the hypothesis of two bitter taste determinant sites, which bind with bitter taste receptor via a binding unit and a stimulating unit, a mechanism for bitterness in peptides was postulated.

In order to investigate the role of the side chain of amino acids in the bitterness of peptides, some oligopeptides containing Glycine, alanine, aminobutyric acid, valine, isoleucine, norvaline and norleucine were synthesized and their tastes evaluated (Ishibashi et al., 1988c). For the bitter taste to be exhibited, the side chain skeleton of the amino acid should contain at least three carbon atoms. The peptides consisting of amino acids having a side chain of less than three carbon atoms did not exhibit bitterness. Valine exhibited an intermediate feature; the taste of valine-containing peptides varied de-

pending upon the structure of the peptide. Peptide bitterness was found to be less affected by the degree of branching of the side chains.

In order to investigate the effect of leucine residues on the taste of peptides, some oligopeptides containing leucine residues were synthesized and their taste evaluated (Ishibashi et al., 1987a). The hydrophobicity of leucine residues markedly caused the bitterness of peptides, and the intensity of the bitterness was always greater when the residue was located at the C-terminus of the peptide.

Ishibashi et al. (1987b) also synthesized peptides containing phenylalanine and tyrosine to evaluate the effect of these residues on bitterness. They concluded that the hydrophobicity of these residues markedly cause bitterness in peptides containing them. The bitterness was more intense when the phenylalanine occurred at the C-terminus and when the content of phenylalanine and tyrosine were increased in the peptides. The hydrophobic residue in the peptides studied functioned as the bitter taste determinant site. The experimental results suggested the existence of an additional site for bitterness in these peptides.

Bitterness has been observed in protein hydrolyzates and in ripened cheeses. In the instance with cheeses, microbial proteases from the starter cultures, as well other organisms that may grow in cheese, act on the substrate casein. Bovine β -casein is very hydrophobic and hydrolysis of this substrate has the potential of generating bitter peptides. Bitter tastes of two peptides (β -Casein f196–209 and f61–67) were demonstrated by Kato et al. (1985) and Shinoda et al. (1985).

Bitter taste of individual peptides may be related to the hydrophobic amino acid content and chain length of the peptides. However, in protein hydrolyzates, there is a mixture of peptides. Casein is not bitter. Upon hydrolysis with proteases in the initial stages, the peptides liberated may be long enough to assume structures that result in buried hydrophobic groups. This assumption of structure driven by hydrophobic forces may result in less bitterness than when hydrolysis progresses to a stage where these structures cannot form as readily. When hydrolysis is extreme to the extent of generating free amino acids, the bitterness decreases further (Adler-Nissen, 1986). In peptides with five to seven amino acid residues, bitterness masking is extremely difficult. Ney (1979) reported that hydrolyzates from common food proteins containing a preponderance of peptides below an apparent molecular weight of 6000 would certainly be bitter. The question of the balance between degree of hydrolysis and bitterness

is not easy to resolve and is multifaceted. It is dependent on the substrate, type of enzyme, hydrolysis conditions and post hydrolysis separations, among other variables.

Tamura et al. (1990) suggested several approaches to debittering peptides. In these experiments model bitter peptides were used and the masking of bitterness was attempted with a variety of substances such as cyclodextrins, starches, other proteins and peptides, fatty substances and acidic amino acids. About 1.5 equivalents of α -cyclodextrins were necessary to mask bitterness of amino acids, while with starch, heating at 100°C for extensive periods (>16 h) decreased the intensity of bitterness. Skim milk, casein and casein hydrolyzate, when added to model peptides, were also effective in mitigating bitterness. Fatty substances such as creaming powder, vegetable oil and margarine were hypothesized to decrease bitterness by binding the hydrophobic amino acid residues, but in practice this hypothesis was proven to not be correct.

The conclusions that can be drawn from the studies on taste of peptides are that there is always a possibility of generating bitter taste compounds during the manufacture of peptides. It is also more than likely that the peptides that are described in the literature as being functional and possessing biological activities may also be bitter. Inherently, caseins are rich sources of hydrophobic amino acids and therefore alteration of casein with proteases will likely result in intense bitterness. Whey proteins may be less hydrophobic than caseins but can still generate peptides with bitter taste properties. Methods to debitter hydrolyzates have yielded encouraging results but have failed to remove all traces of bitterness. Bitterness of enzyme modified proteins continue to be an impediment in wider commercial applications of hydrolyzates. In hypoallergenic infant formulas, hydrolysis becomes a clinical necessity and the resulting product, though bitter, may not be too objectionable since infants do not perceive bitter taste. Most of the commercially available whey protein hydrolyzates impart a bitter, brothy or salty taste from the peptides which has to be masked in the product.

SUMMARY AND CONCLUSIONS

This review has attempted to study the literature pertaining to peptides derived from milk proteins. Hydrolysis of milk proteins to generate peptides has been practiced for a long time and it was recognized early on in this process that the taste of hydrolyzates might hinder use of these products in

food formulations. Modification of protein is necessary to form a more acceptable or utilizable product, to form a product that is less susceptible to deteriorative reactions and to form a product that is of higher nutritional quality. Modifications may be achieved by a number of chemical and enzymatic means. This review has considered only enzymatic modification of dairy proteins.

Modified proteins contain peptides and some of these peptides have been purified and their functionalities have been compared with unmodified proteins. This paper has examined the literature pertaining to improvement in functionality of enzyme-modified proteins. Improvements in solubility, emulsification, foaming and gelation were examined. There is limited information available on the sequence of the peptides necessary to improve the functional characteristics of proteins. Knowing the sequences of desirable functional peptides can lead to genetic alteration of proteins to improve functionality. Addition of synthetic peptides to intact proteins may be another way in which the functionality of proteins can be augmented.

Some of the peptides in milk proteins are capable of affecting biological functions of an organism. These effects can be antimicrobial and probiotic, i.e., prevent the growth and proliferation of undesirable and pathogenic organisms, or they may promote the growth of desirable bacteria in the digestive tract of humans and animals. Peptides derived from milk protein have been shown to exert digestive and metabolic effects as well. They may also influence the immune system. These biological effects may play an important role in the development of medical foods that treat or mitigate the effects of diseases.

Proteins are allergens and therefore it is possible that products derived from modification of proteins may also be allergens. The known literature about the allergenicity of peptides derived from milk proteins has been examined in this article.

Last, but not the least, the taste attributes of peptides is also considered. Bitterness of hydrolyzates is a common occurrence and the origins of these bitter peptides and possible ways of mitigating this sensory defect has been discussed. Many of the peptides that enhance functionality and exert biological activity are likely to be bitter. Therefore, the bitter taste of hydrolysis products has to be dealt with in boosting the functional or nutraceutical aspects of foods containing these peptides.

Analytical techniques for sequencing peptides have become more accessible and purification of peptides is commercially feasible. Computer based

modeling techniques have aided the prediction of structures in these peptides. These advances, coupled with the advances in biotechnology, promise to revolutionize the future of nutraceutical and functional foods.

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