Jean-Marc Chobert

Milk protein tailoring to improve functional and biological properties

Author's address:
UR 1268, INRA Biopolymères Interactions Assemblages-équipe Fonctions et Interactions des Protéines, Nantes, France.

Correspondence:
Jean-Marc Chobert
UR 1268, INRA Biopolymères Interactions Assemblages-équipe Fonctions et Interactions des Protéines, rue de la Géraudière, B.P. 71627, 44316 Nantes Cedex 03, France.
Tel.: +33 2 4067 5230
e-mail: chobert@nantes.inra.fr

ABSTRACT

Proteins are involved in every aspects of life: structure, motion, catalysis, recognition and regulation. Today's highly sophisticated science of the modifications of proteins has ancient roots. The tailoring of proteins for food and medical uses precedes the beginning of what is called biochemistry. Chemical modification of proteins was pursued early in the twentieth century as an analytical procedure for side-chain amino acids. Later, methods were developed for specific inactivation of biologically active proteins and titration of their essential groups. Enzymatic modifications were mainly developed in the seventies when many more enzymes became economically available. Protein engineering has become a valuable tool for creating or improving proteins for practical use and has provided new insights into protein structure and function. The actual and potential use of milk proteins as food ingredients has been a popular topic for research over the past 40 years. With today's sophisticated analytical, biochemical and biological research tools, the presence of compounds with biological activity has been demonstrated. Improvements in separation techniques and enzyme technology have enabled efficient and economic isolation and modification of milk proteins, which has made possible their use as functional foods, dietary supplements, nutraceuticals and medical foods. In this review, some chemical and enzymatic modifications of milk proteins are described, with particular focus on their functional and biological properties.

Key words: milk protein, modification, functional properties, biological properties

INTRODUCTION

The actual and potential use of milk proteins as food ingredients has been a popular topic for research over the past 40 years. Milk and dairy products have numerous advantages over competitors when used as ingredients: they are colorless, have a bland taste, are rather stable to processing, are free of toxins and have constituents that can be easily fractionated. As ingredients, dairy products are used mainly for their physico-chemical properties.

The effective utilization of proteins in food systems is dependent on tailoring the protein's functional characteristics to meet the complex needs of the manufactured food products. Many food proteins require modification to improve such functional properties as solubility, foaming and emulsifying activity. Reviews on classical food protein modifications for improved functionality are available in the literature (Means & Feeney, 1971; Feeney & Whitaker, 1977, 1982, 1986).

Functional properties of proteins are closely related to their size, structural conformation, and level and distribution of ionic charges. Chemical treatments that could cause alteration of these properties include reactions that either introduce a new functional group to the protein or remove a component part from the protein. Consequently, reactions such as acylation, phosphorylation, esterification, glycation, limited hydrolysis, and deamidation have been used to impart improved functional properties to the dairy proteins.

This review concerns some chemical and enzymatic methods that modify dairy proteins with an emphasis on current developments.
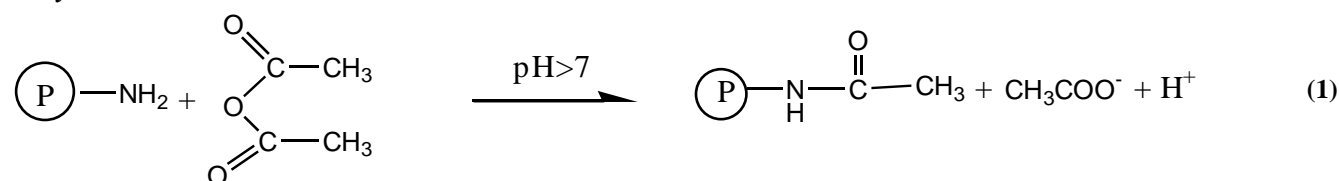
I. CHEMICAL MODIFICATION OF MILK PROTEINS

Primary structure of proteins can be chemically modified in order to improve their functional properties. This approach has been used with success to study the structure-function relationships (enzymatic function, biological function, physico-chemical and functional properties). Deliberate chemical modification of food proteins can result in alteration of the nutritive value, formation of potentially toxic amino acid derivatives, and contamination by toxic chemicals.

Alteration of amino acid residues can be obtained by heating at acid or alkaline pH. Main classes of reactions used to chemically modify the side chain of amino acids are acylation, alkylation, oxidation and reduction (Figure 1). Some of them are described in this chapter.
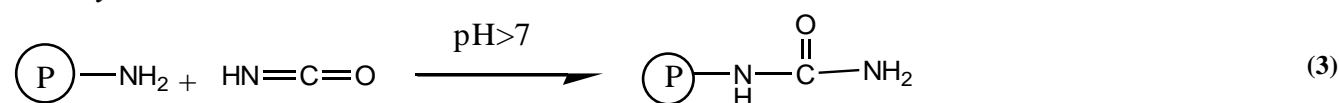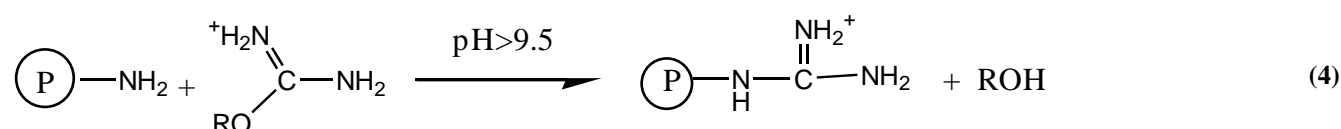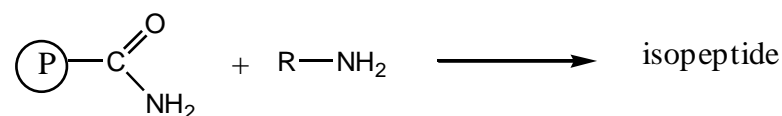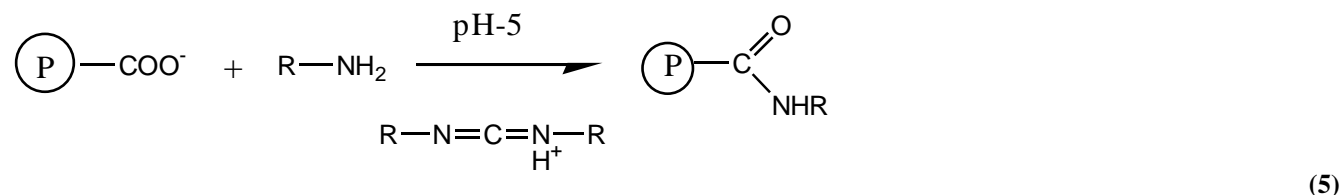
Acetylation
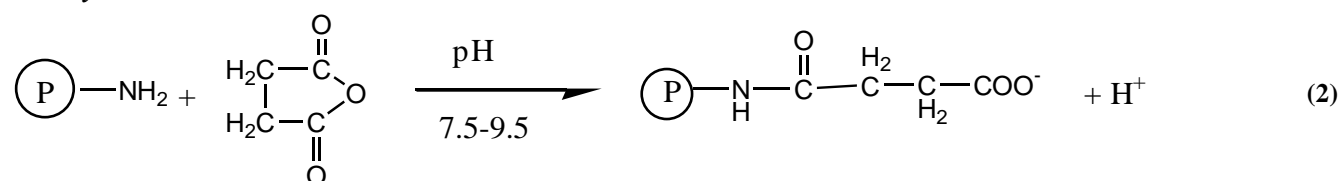
(1)

Succinylation

(2)

Carbamylation

(3)

Guanidination

(4)

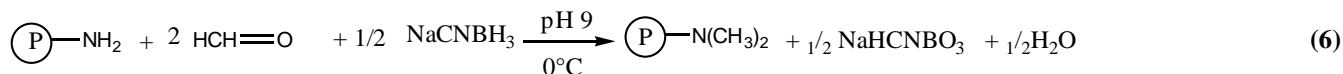*Amidation*

(5)

Figure 1. *Main chemical modifications of food proteins (1 – 5).*

Reductive alkylation

$$\boxed{P}-NH_2 \ + \ 2 \ HCH{=}O \ + 1/2 \ NaCNBH_3 \xrightarrow[0°C]{pH \ 9} \boxed{P}-N(CH_3)_2 \ + {}_1/_2 \ NaHCNBO_3 \ + {}_1/_2 H_2O \qquad (6)$$

Carboxymethylation

$$\boxed{P}-S^- \ + \ ICH_2COO^- \xrightarrow{pH>7} \boxed{P}-S-CH_2COO^- \ + \ I^-$$

$$ (7) $$

$$\boxed{P}-NH_2 \ + \ ICH_2C\!\!\begin{smallmatrix}O\\ \\NH_2\end{smallmatrix} \xrightarrow{pH>8.5} \boxed{P}-N\!\!\begin{smallmatrix}H\\ \\ \end{smallmatrix}-C\!\!\begin{smallmatrix} \\H_2\\ \end{smallmatrix}-C\!\!\begin{smallmatrix}O\\ \\NH_2\end{smallmatrix} \ + \ I^- \ + \ H^+$$

Oxidation

$$\boxed{P}-SH \ + \ 3 \ H-C\!\!\begin{smallmatrix}O\\ \\O-OH\end{smallmatrix} \xrightarrow{- \ 10°C} \boxed{P}-SO_3H \ + \ 3 \ H-C\!\!\begin{smallmatrix}O\\ \\OH\end{smallmatrix}$$

$$ (8) $$

$$\boxed{P}-S-S-\boxed{P} \ + \ 5 \ H-C\!\!\begin{smallmatrix}O\\ \\O-OH\end{smallmatrix} \ + H_2O \longrightarrow 2\boxed{P}-SO_3H \ + \ 5 \ H-C\!\!\begin{smallmatrix}O\\ \\OH\end{smallmatrix}$$

Esterification

$$\boxed{P}-C\!\!\begin{smallmatrix}O\\ \\OH\end{smallmatrix} \ + \ R-OH \xrightarrow[0-25°C]{HCl<0.1M} \boxed{P}-C\!\!\begin{smallmatrix}O\\ \\OR\end{smallmatrix} \ + \ H_2O \qquad (9)$$
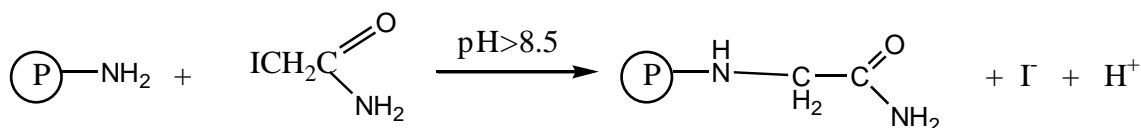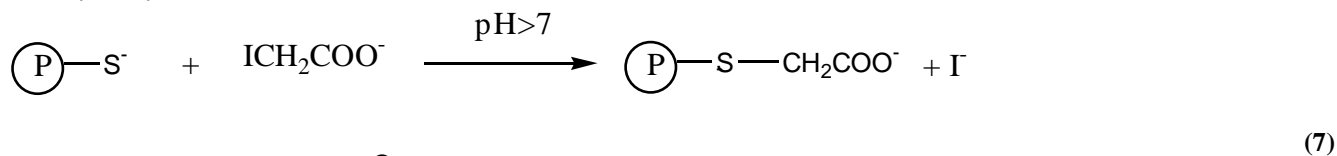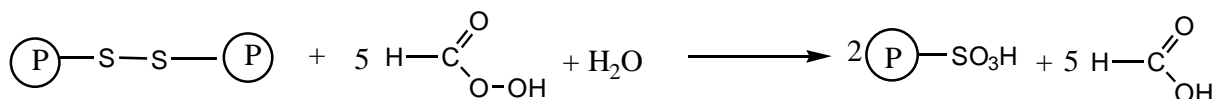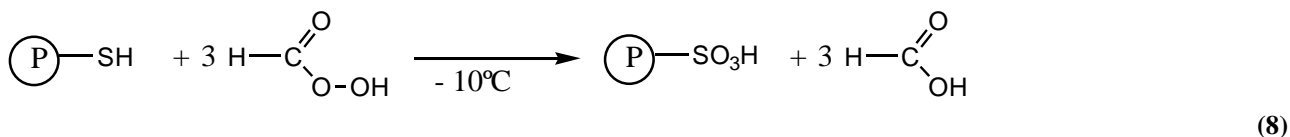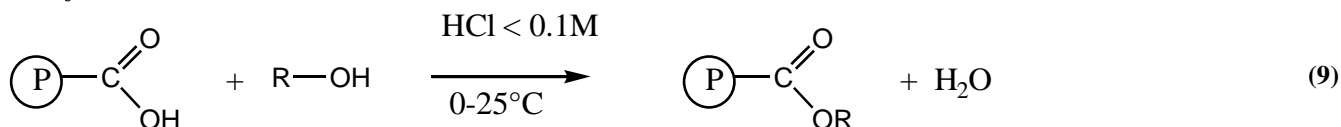
Figure 1. *Main chemical modifications of food proteins (6 – 9).*

A. ESTERIFICATION

The esterification reaction is an important tool for modifying food proteins. Esterification with different alcohols leads to the blocking of free carboxyl groups raising thus the net positive charge, making the modified proteins more basic (Wilcox, 1967; Mattarella et al., 1983; Halpin & Richardson, 1985). The basicity of the modified protein depends on the degree of esterification and on the original content of basic amino acid residues on the protein molecules. This modification has shown its impact on the folding and peptic hydrolysis of β-lactoglobulin (BLG) (Briand et al., 1995). While BLG is resistant to peptic hydrolysis in aqueous and physiological conditions, an ethylated BLG derivative was very susceptible to pepsin

hydrolysis in aqueous conditions. The esterified BLG exhibited 22 new sites of pepsin cleavage (Briand et al., 1995; Chobert et al., 1995) as compared to the peptic peptides obtained when hydrolysis of native BLG was performed in hydro-ethanolic conditions (Dalgalarrondo et al., 1995). Hence, the reaction might be a good tool for the increase of peptic digestibility of food proteins or producing specific new unconventional peptides exhibiting novel activities. Moreover, the high isoelectric points of the esterified proteins might endow them with new physicochemical and functional properties (Mattarella & Richardson, 1983; Halpin & Richardson, 1985; Chobert et al., 1990).

Since the initial work of Fraenkel-Conrat & Olcott (1945), protein esterification has been described in a number

of studies (Mattarella et al., 1983; Chobert et al., 1990, 1995; Bertrand-Harb et al., 1991; Briand et al., 1994, 1995). The conventional procedure involves three steps. The first step is the mixing of reactants (protein, alcohol and acid). The second step is the esterification reaction itself, which generally ranges in length from one to several days, at 4°C. The last step is reaction termination and product recovery. The length of the reaction, which may reach 10-12 days, could limit the reproducibility and the applicability of this reaction. Additionally, the method used to recover the product at the end of the reaction can lead to side-reactions. In some procedures (e.g. Halpin & Richardson, 1985; Bertrand-Harb et al., 1991), the reaction medium at the end of the reaction time was combined with an equal volume of water, then dialyzed against water for several days, which can lead to de-esterification (Bertrand-Harb et al., 1991). Another way to recover the reaction product was by drying under vacuum (Briand et al., 1995).

Protons are essential catalysts of the esterification process, which will not proceed to any extent if they are absent in the reaction medium. Consequently, determining the optimum amount of protein catalyst required for the reaction is extremely important (Sitohy et al., 2000).

1. Factors influencing esterification. Study of BLG as a model

***a*. Influence of time-course of reaction.** The reaction time-course (8, 16, 24, 48, 72 and 96 h) was followed in an esterification process using BLG (3% protein concentration) dispersed in 95% ethanol in the presence of 0.7 N HCl. Esterification extent increased gradually with the length of reaction to be 11, 14, 23, 28, 32 and 36%, respectively. It is obvious that under the conditions used, the esterification did not reach its maximum even after 96 h of reaction. Consequently, in order to increase the extent of modification after shorter reaction times, conditions should be modified drastically. For example, protein and acid concentrations can be increased. The length of reaction is alcohol-dependent since it was observed that esterification with methanol reached its maximum within 24 h by using less concentrated solutions.

***b*. Influence of temperature.** Comparative experiments (2% protein, 0.7 N HCl final concentration) were carried out esterifying BLG with 99.7% ethanol at three different temperatures (4, 10 and 20°C). The reaction was stopped after 8 h. The extent of esterification increased with the increase of temperature, being 2, 4 and 8% at 4, 10 and 20°C,

respectively. However, the products obtained in higher reaction temperatures developed violet coloration after drying showing that high temperatures may induce side-reactions. Wilcox (1967) stated that higher temperatures might give insoluble esterified products. Consequently, although higher temperatures may increase the reaction rates they should not be used in order to avoid undesired side-reactions.

***c*. Influence of the type of alcohols.** The comparison of the different alcohols used for esterification showed that methanol is the most reactive and can esterify most of the carboxyl groups of BLG, leading to a maximum esterification extent of 97% when traces or 1% water is present in the starting alcohol. The final amount of water in the reaction medium depends also on its quantity contained in the added acid.

The difference between reactivity of alcohols agrees with the results of Fraenkel-Conrat & Olcott (1945) and Halpin & Richardson (1985) who found that methanol was most reactive as an esterifying agent followed by ethanol then n-butanol. Matarella et al. (1983) found that in methanol the esterification reaction progressed quicker than in ethanol, while there was no evidence for esterification in propanol, butanol or pentanol even after one week reaction time. The reason for such a difference according to the alcohols used is due to their different polarity and structural hindrance, which is especially evident in iso-propanol.

2. Application to α-lactalbumin (ALA)

α-Lactalbumin (ALA) (5%, w/v) was esterified for 6 h at 4°C with >99.5% alcohols (methanol, ethanol, n-propanol) using 20, 40 and 60 molar ratio (MR, mole acid/mole carboxyl group) (Sitohy et al., 2001a). ALA was more resistant to esterification than BLG with the different alcohols used. Even in the presence of methanol, the extent of esterification did not exceed 52% with the highest concentration of HCl. This may be due to the more compact conformation of ALA, which may be better protected than BLG against the nucleophilic attack by the alcohols. ALA may be subject to conformational changes in acid conditions followed by a strong increase of surface hydrophobicity. Hence, the contact with hydrophilic alcohols should be limited. However, methanol was still more reactive as an esterifying agent than the other two alcohols used with both proteins. In order to achieve higher extents of esterification in the case of ALA, either the reaction time should be prolonged or the molar ratio of HCl should be increased.

SDS-PAGE patterns of esterified ALA did not differ from those observed for native protein, indicating no sign of hydrolysis or polymerization. Hence, the conditions used for esterification did not affect the molecular size of the modified protein.

3. Application to β-casein (BCN)

Extent of esterification of β-casein (BCN) increased gradually parallel to the increase in the molar ratio of HCl (Sitohy et al., 2001a). As observed with BLG, methanol achieved the highest rates of esterification. However, propanol was more efficient than ethanol, which was the opposite of the results obtained with BLG. This discrepancy might be due to conformational differences between these two proteins.

The relatively low extents of esterification obtained using ethanol and propanol could be due to the following factors: 1. Low polarity of the reaction medium; 2. Inadequate protein concentration; 3. Insufficient amount of catalyst HCl.

In order to increase the polarity of the medium, ethanol and propanol were used at a concentration of 95% during esterification of 5% BCN using molar ratio of 30, 40 and 50. The extent of esterification decreased considerably (about 50% decrease) when compared with the values obtained in the presence of >99.5% alcohol. Although water might be needed to enhance the polarity of the reaction medium, it should not exceed a certain limit.

The role of protein concentration was examined by esterifying BCN for 6 h with 99.7% ethanol and 99.5% propanol using a molar ratio of 70 and protein concentrations of 3, 4 and 5%. The respective extents of esterification were 26, 45 and 56% with ethanol and 36, 49 and 54% with propanol indicating that a protein concentration of 5% was the most efficient as was observed with BLG.

In order to examine the effectiveness of the third important factor playing a role in esterification yield, BCN (5%) was esterified using higher molar ratio (60, 70 and 80). The modified product was recovered either by centrifugation or by filtration under vacuum. The extent of esterification could be increased up to 59 and 56% in the presence of ethanol and propanol, respectively, when using a molar ratio of 80 and when the resulting product was recovered by centrifugation. These values were slightly lower (51 and 53%) when the modified product was recovered by filtration. Since centrifugation is more time consuming than filtration under vacuum, and leaves the reactants in contact for an additional period, this may be one of the causes of a higher esterification yield. However, the use of filtration under vacuum may have the advantage of reducing the time and energy costs associated with freeze-drying (3–4 days).

4. Factors influencing pepsinolysis of ester derivatives of BLG

Esterification generally enhances the susceptibility of proteins to peptic action (Briand et al., 1995; Chobert et al., 1995). This was most evident with BLG methyl ester, which underwent the highest degree of esterification. It was easily hydrolyzed by as low as 0.125% Enzyme/Substrate ratio (E/S) of pepsin, at a low temperature (4°C) and for short incubation times (15-30 min) reaching a high degree of hydrolysis (DH) independently of the substrate protein concentration. Hence, esterification can be a good method for improving the peptic digestibility of pepsin-resistant proteins, such as BLG and some legume proteins (Sakai et al., 1997; Vieths et al., 1999). Consequently, it can eliminate their allergenicity and enhance their biological value (see Section II. B.). Tricine-SDS PAGE of peptic hydrolysates of esterified BLG were characterized mainly by low molecular mass bands in the case of methyl and ethyl derivatives, while there were additional bands of medium molecular mass in the case of propyl ester derivatives. Hence, both the degree of esterification and the nature of the ester group are responsible for this difference (Sitohy et al., 2001c). A high extent of esterification (100%) increased the amount of peptic cleavage sites throughout the protein molecule, giving rise to low molecular mass peptides of about 3 kDa. In contrast, peptic hydrolysates of propyl ester derivatives of BLG (44% esterified) showed an additional band of a medium molecular mass of 5-6 kDa. Reversed phase High Performance Liquid Chromatography (RP-HPLC) profiles of peptic hydrolysates of esterified BLG showed a broad population of hydrophilic peptides independent of the nature of the ester group or of the extent of esterification. This was due mainly to the increased availability and to the spread of the peptic cleavage sites throughout the esterified molecules, leading to their lysis into small hydrophilic peptides. RP-HPLC profiles of esterified BLGs differed from those of native BLG. This means that esterification gives rise to new peptides. There were only slight differences between the profiles of methyl and ethyl ester hydrolysates. However, RP-HPLC profile of hydrolysate of BLG propyl ester was different from those obtained with methyl and ethyl esters. In order to obtain more hydrophobic peptides by peptic hydrolysis, the esterifying agent should have a relatively long aliphatic chain and the conditions of hydrolysis should be mild.

5. Improvement of functional properties of milk proteins by esterification

Milk proteins have good inherent functionalities as well as high nutritional values. Hence, they are used as ingredients in a wide range of food products (Huffman 1996; McCrae et al., 1999). Due to its amphiphilic properties, BCN is one of the most surface-active milk proteins (Mitchell et al., 1970; Benjamin et al., 1975). However, because of the acid isoionic points of milk proteins, their functional properties are worst in that range of pH, when compared to the alkaline range. Adsorption of BLG to fat globules increases with increased pH, exhibiting good emulsifying activity in the alkaline pH range (Yamauchi et al., 1980, Nagasawa et al., 1996, Hattori et al., 1997). Adsorption of BLG on the oil/water interfacial layer is strongly reduced with decreasing pH reaching 61.6 and 12.9% at pH 9 and 3, respectively. This can be attributed to pH-dependent structural changes since BLG molecules are more rigid at acid pH (Shimizu et al., 1981, 1985; McCrae et al., 1999). Generally, the most important factors determining the emulsifying properties of whey proteins are protein concentration, pH, ionic strength and history of processing and storage conditions. Emulsifying activity depends also on the flexibility of protein molecules since flexible molecules such as caseins can spread over the oil-water interface in contrast to more rigid proteins such as the whey globulins (Hunt & Dalgleish, 1994). When BLG adsorbs at an oil-water interface, it denatures partially exposing its highly reactive free sulfhydryl groups (Corredig & Dalgleish, 1995), which can then interact with similar groups of neighboring molecules forming polymers. The second major whey protein, ALA, was found to inhibit BLG polymerization (Dickinson & Matsumura, 1991, Monahan et al., 1993). Hence, the ability of a protein molecule to form and stabilize oil droplets is closely linked to the structure and conformation of the molecule (Das & Kinsella, 1990).

Since good solubility and emulsification properties at acidic pHs are required for some food applications such as acid soft drinks and acid foods, modification of proteins improving their functional properties in this pH range may be required. Esterification of proteins was proved to block the protein negative charges increasing thus the net positive charge and raising the protein isoionic points. This should render the modified proteins more soluble and likely much more tensio-active in the acidic range of pH. The isoionic point of BLG was raised from 5.2 in its native state to 6.2, 8.7 and 9.8 after its esterification with butanol, ethanol and methanol, respectively (Mattarella & Richardson, 1983;

Halpin & Richardson, 1985). Similar features were observed during esterification of BCN (Chobert et al., 1990). The magnitude of the change in the isoionic point values depends on the degree of esterification (Chobert et al., 1990; Bertrand-Harb et al., 1991; Sitohy et al., 2000). Alternatively, methyl and ethyl esterification of BLG resulted in randomized protein structures (Mattarella et al., 1983). Generally, partial denaturation of proteins results in increased surface activity compared to the native globular forms (Mitchell et al., 1970). Hence, esterified BLGs were more efficient in lowering the interfacial tension at the oil/water interface as a result of increased surface hydrophobicity (Halpin & Richardson, 1985). It was reported also that emulsifying activity of esterified BLGs is worse than that of the native form (Mattarella & Richardson, 1983; Chobert et al., 1990). This discrepancy might be due to differences in pH or any other limiting factors used in each study. In previous studies the change of emulsifying activity of esterified proteins was followed by measurement of turbidity according to the method of Pearce & Kinsella (1978). Since the oil droplet size may better reflect emulsifying activity, Sitohy et al. (2001b) measured the change of this parameter in order to elucidate the changes in emulsifying activity and stability of different esterified milk proteins in the acidic and neutral ranges of pH. This yielded information on the suitability of using esterified proteins as ingredients in acid foods or to fortify acid soft drinks. Moreover, it was reported that oil droplet size influences the organoleptic perception of food emulsions and that the interfacial layer surrounding the oil droplet may be a determinant factor controlling the stability of the emulsion towards flocculation and coalescence (Mine, 1998; Aluko & Mine, 1999; Le Denmat et al., 1999).

Three milk proteins (BLG, ALA and BCN) were esterified to different extents with methanol, ethanol and propanol then their solubility was studied in the pH-range of 3-10. Solubility of BLG esters depends on the degree of esterification as well as on the nature of the grafted ester groups. Samples of highly esterified BLG (99%) gave rise to a more homogenous protein population with a minimum of solubility near pH 10, while those with low degrees of esterification gave a heterogeneous population showing two solubility minima. Consequently, two opposite effects of esterification on the solubility of BLG in the acidic pH range could be perceived. The first factor improving solubility results from the isoelectric point shift towards the alkaline pH. The second factor lowering solubility results from replacing hydrophilic carboxylic groups by hydrophobic ester

groups. The overall protein solubility is a result of the interplay between these two effects. The first effect is more efficient in the case of the most esterified derivatives and when the grafted ester group is of a less hydrophobic nature (e.g. methyl). The second effect is more prominent in case of derivatives with a low degree of esterification and when the grafted ester groups are more hydrophobic (e.g. propyl). This may explain the reduced solubility reported in case of some esterified proteins at acid pHs (Mattarella & Richardson, 1983; Chobert et al., 1990).

ALA methyl ester was more soluble in the acidic range of pH and less soluble in the alkaline range of pH as compared to unmodified protein. The low methylated sample (38%) showed two regions with a minimum of solubility (pH 5.6 and 6.8) compared to pH 7.2 and 8.8 for the 52% methylated derivative. Similarly, the highly ethylated ALA sample (36%) showed improved solubility in acid conditions and decreased solubility in the alkaline range of pH. The solubility of the low ethylated derivative (11%) was improved whatever the pH. The solubility curves of ALA propyl esters were similar to that of ethyl derivatives. However, the reduction of solubility of propyl-esterified derivatives was more pronounced when compared to ALA ethyl esters showing solubility levels of 47-50% and 67-70%, respectively. As observed with BLG ester derivatives, the nature of the grafted ester group is an important factor for the solubility of the modified ALA. Comparison of solubility of ALA propyl esters with that of BLG propyl esters shows a different behavior for these two families of esters. While grafted propyl ester groups decrease solubility of BLG in the acidic pH range, they improve the solubility of ALA. Consequently, the conformation of the esterified protein might play an important role on the solubility of the protein. Hence, it is not only the nature of the grafted ester group that determines the solubility of the modified protein but also the nature of the protein itself.

As observed with BLG and ALA, the isoelectric points of esterified BCN shifted towards the alkaline pH range. The magnitude of this shift was proportional to the extent of esterification. BCN methyl esters being the more esterified samples (60, 88 and 100% esterification) showed higher magnitudes of isoelectric point shift compared with other types of esters. The decrease of solubility in the alkaline pH range (7-9) was more evident for BCN methyl esters, giving a minimum of solubility as low as 20% for the highly esterified samples (88 and 100% esterified). The minimum of solubility of BCN esters is mainly observed for one pH value or one pH

range in contrast to two minima previously observed with BLG and ALA. This may be because the flexible structure of BCN makes the alcoholic groups more accessible to nucleophilic attack, giving rise to more homogeneous esterified populations. The hydrophobic core of BLG is certainly more resistant to the nucleophilic attack of the alcoholic groups at the beginning of the reaction, becoming only accessible with the progress of esterification or at higher protein concentrations giving rise to different molecular populations.

Comparison of methyl and ethyl esters of BCN shows a more important shift of isoelectric point for the methyl derivative due to the higher extent of modification. Moreover, the solubility of BCN methyl esters is smaller in the alkaline range of pH (7-9) due also to the high extent of modification. However, although ethyl and propyl esters of BCN displayed similar extents of substitution (~20 - ~60%) and had a similar shift of isoelectric points, the propyl ester derivatives were less soluble in the alkaline pH range than the ethyl ester derivatives probably because of the more hydrophobic nature of grafted propyl groups. It can be concluded that the solubility was improved only between pH 4 and 5. This property might find some practical application when using esterified proteins as additives for beverages instead of protein hydrolysates that often have a bitter taste.

Comparison of the solubility of all three esterified proteins showed that the magnitude of the shift in the isoelectric points decreased in the following order:

BLG > ALA > BCN.

Emulsifying properties of esterified milk proteins were also studied in the pH-range of 3-7 by measuring the oil-droplet size using laser light scattering. Emulsifying activity and stability of esterified milk proteins in the acidic pH range of 3-5 were generally higher compared to the corresponding native proteins. This improvement was associated with the degree of solubility, the degree of esterification, the type of ester group grafted and the nature of the used protein. The highest emulsifying activity improvement was observed for methyl ester derivatives at pH 5 where the native proteins have poor emulsifying properties (Sitohy et al., 2001b).

6. Change in biological properties. Interactions between esterified milk proteins and nucleic acids. Inhibition of virus

DNA replication was studied *in vitro* in the presence of native and esterified milk proteins (ALA, BLG and BCN). Addition of unmodified proteins to the PCR medium did not

change the result of the reaction seen by electrophoresis, even at excessive ratios of basic amino acids in proteins:phosphate groups in DNA as high as 100:1. Addition of esterified proteins greatly reduced the intensity of the bands corresponding to the newly synthesized DNA, at ratios as low as 1:1 and 5:1 in case of methylated-BLG and methylated-ALA, respectively. The inhibitory effect of esterified proteins was directly proportional to their extent of esterification and strongly related to their DNA-binding capacity. Generally, inhibition of PCR with esterified proteins was similar to what can be observed with histones. However, stronger inhibition was observed with highly esterified proteins when using a higher ratio of basic:acid residues (1:1) when compared to 0.5:1 ratio in case of histones. Highly esterified BCN did not exert any inhibitory effect because of its relatively lower pI when compared to that of other esterified milk proteins and due to its lower positive net charge at the pH used for PCR. During a second PCR run, only the addition of new DNA template was able to reinitiate the reaction, giving rise to new synthesized DNA. Addition of Taq DNA polymerase did not enhance DNA synthesis, showing that inhibition was performed only by binding of DNA template and not by the inhibition of the polymerase.

Herpes simplex virus (HSV) causes a wide spectrum of clinical manifestations in the central nervous system of infants (encephalitis with or without disseminated visceral infection) and adults. *Herpes simplex* virus type 1 (HSV-1) is a large enveloped DNA virus replicating in the nuclei of mammalian cells. It is a significant human pathogen causing cutaneous lesions primarily in the oral mucosa but also in other sites. More serious manifestations of HSV-1 infection include encephalitis and blinding keratitis. Nucleoside analogues of guanine e.g. acyclovir (ACV) have been used for therapeutic use against HSV.

Antiviral activity of methylated ALA, methylated and ethylated BLGs was evaluated against sensitive and resistant strains of HSV-1 and compared to that of acyclovir and polylysine (4-15 kDa) using fixed or suspended Vero cell lines. Esterified proteins and their peptic hydrolysates displayed protective action against sensitive HSV-1, which was relatively lower than that induced by acyclovir or polylysine. Higher activity of polylysine was maintained against a resistant strain of HSV-1 while acyclovir lost much of its activity. The mean 50% inhibitory concentration (IC_{50}) was about 0.8-0.9 µg/ml for polylysine against two strains of HSV-1 when using two concentrations of virus (50 and 100% cytopathic effect, CPE). IC_{50} values of acyclovir against the

sensitive strain of HSV-1 were of 3 and 15 µg/ml when using the low and high concentrations of virus, respectively. When using the lower concentration of virus (50% CPE), IC_{50} values for esterified milk proteins ranged from 20 to 95 µg/ml, depending on the nature of the ester group, the degree of esterification, and the nature of the protein. Esterified milk proteins were active against both sensitive and resistant strains of HSV-1. By using the real-time PCR technique, it was shown that methylated ALA inhibited the HSV-1 replication (Sitohy et al., 2007).

The enteroviruses, RNA viruses of the *Picornaviridae* family, are ubiquitous pathogens, which include more than 70 different serotypes (3 of which are polio serotypes) infecting people of all ages. Enteroviruses can produce severe and sometimes fatal illnesses such as meningitis, encephalitis, myocarditis, neonatal sepsis and polio. Enteroviruses belong to the group of enteric viruses, which are excreted in feces of infected individuals and may directly contaminate drinking water supplies. Numerous studies described the presence of enteroviruses in raw and in inadequately treated drinking water, waste waters and sludges. Enteroviruses create a public health risk as they can be transmitted via the fecal-oral route through contaminated water, initiating severe infections in humans.

Poliovirus is a small positive strand RNA virus replicating in the human oropharynx and extending to the central nervous system in 1 to 4% of cases.

Adenoviruses present as great public health hazard as do enteroviruses. Both contaminate water and cause epidemics. Adenoviruses are an important cause of morbidity and mortality in the immuno-compromised hosts. There are no approved antiviral agents with proven efficiency for treatment of severe adenovirus diseases.

Coxsackie B viruses (CVB) have been associated with such pathologies as aseptic meningitis, non-specific febrile illness and myocarditis (Morens & Pallansch, 1995). Most CVB infections are asymptomatic, but different organs may be affected (e.g. myocardium, pancreas). Some acute and persistent CVB infections are particularly severe in infants (Woodruff, 1980).

The antiviral activity of esterified whey proteins (IC_{50} = 20-40 µg/ml) observed against poliovirus type-1 and Coxsackie virus B6 infecting Vero cells depended on five factors (in order of importance): concentration of esterified proteins, multiplicity of infection, duration of incubation, timing of esterified proteins addition after infection, and extent of esterification of proteins. Renewal of cell growth

medium after 24 h of incubation with new medium containing the same amount of esterified proteins enhanced the anti Coxsackie virus effect. Difference between start of infection of the reporter Vero cells and the time of addition of esterified proteins decreased significantly their antiviral efficacies. The poliovirus titer reduced gradually in response to the increase of the concentrations of esterified proteins. The relative amounts of viral RNA were less affected by the esterified proteins concentration than was the virus infectious titer, indicating that inhibitory proteins may slow down viral multiplication yielding reduced amounts of viral RNA (Sitohy et al., 2008).

The extent of inhibition of poliovirus and Coxsackie virus was related to the degree of cationisation of esterified whey proteins as well as to the size of the backbone protein. Hence, the results obtained in this study agree well with previous results obtained during studies of antiviral action of other cationic proteins.

In this preliminary study, the mechanism of antiviral activity of esterified whey proteins could be due to:

1- Saturating binding to viral RNA by purely coulombic interactions, inhibiting its replication and transcription;

2- Hydrophobic interactions with viral capsid proteins;

3- Perturbation of viral RNA–protein interactions, hence inhibition of the translation of viral proteins;

4- Interference with/saturation of viral entry sites on the cellular membranes.

However, whichever effect is prevalent esterified whey proteins may present several advantages over other cationic proteins described by other authors: they are available in large quantities and simplicity of their modification favors them as cheap and efficient antiviral agents that may be used with success for the disinfection of infected solid surfaces and liquids. Their susceptibility to proteases can be an additional asset allowing their rapid recycling in the environment (Chobert & Haertlé, 2003).

In the last 100 years there have been 3 major influenza pandemics: Spanish Flu in 1918, Asian Flu in 1957 and Hong Kong Flu in 1968. These claimed the lives of approximately 50 million, 2 million and 1 million people, respectively. Added to this is the annual death toll of 250,000 to 500,000 people worldwide with a further 3 to 4 million people suffering severe illness. In 1997 the alarming emergence of a new, highly pathogenic subtype, H5N1, which has a 50% mortality rate, provided a major impetus for renewed influenza research. Re-emergence of fatal human influenza A

subtype H5N1 disease was reviewed (Peiris et al., 2004). At the same time another subtype, H1N1, has emerged (Neumann et al., 2009). This latter subtype causes a relatively mild infection in humans, however it is highly transmittable between people and a new influenza pandemic was declared by the World Health Organization. Development of effective new drugs for the treatment and/or prevention of epidemics and pandemics of influenza is a major sanitary objective.

Addition of methylated BLG (Met-BLG) in the medium of MDCK cell lines infected with influenza virus subtype H1N1 reduced hemagglutination activity (HA) in a concentration dependent manner. Antiviral activity of Met-BLG depended on its concentration, viral load, and duration of infection. Using 17 µg/ml of Met-BLG inhibited 50% of HA of H1N1 grown in MDCK cells at 1 MOI after 24 h of incubation at 37°C and in 5% CO_2. Extension of incubation time enhanced antiviral action since the same concentration of Met-BLG inhibited about 61% of viral activity after 48 h. This viral inhibition was accompanied by a protection of MDCK cells as observed by using neutral red or by direct microscope examination. Reduction of viral RNA replication upon the addition of Met-BLG (50 µg/ml) was observed by real time-PCR showing a reduction of viral log value of about 0.9. When viral stock solution was mixed with 25 µg/ml Met-BLG in absence of cell lines, the morphology and viability of virus particles were significantly affected as observed by electron microscopy, and the number of intact virus particles was reduced by roughly 65% (Sitohy et al., 2010).

B. GLYCATION OF BLG USING MILD CONDITIONS

The non-enzymatic browning or Maillard reactions are of great importance in food manufacturing. This reaction was first described by the French biochemist Louis Camille Maillard at the beginning of the 20th century (Maillard, 1912). The browning reaction can be defined as the sequence of events that begins with the reaction of the amino group of amino acids, peptides, or proteins with a glycosidic hydroxyl group of sugars; the sequence terminates with the formation of brown nitrogenous polymers or melanoidins (Ellis, 1959; O'Brien & Morrissey, 1989; Ames, 1990; Friedman, 1996; Chuyen, 1998). This is also one of the principal pathways of final degradation of organic matter in nature.

These non-enzymatic reactions are responsible for numerous changes on food properties and may impair food safety. Although these reactions are of a great importance in production of aroma, taste and color, they are often

accompanied by a reduction of the nutritive value of different foods and by the formation of toxic compounds harmful for human health (Ledl & Schleicher 1990). Results of non-enzymatic browning can be either desirable or undesirable. The brown crust formation on bread is desirable; the brown discoloration of evaporated and sterilized milk is undesirable. For products in which the browning reaction is favorable, the resulting color and flavor characteristics are generally experienced as pleasant. In other products, color and flavor may become quite unpleasant.

The reaction velocity and pattern are influenced by the nature of the amino acid or protein and carbohydrate involved in the reaction. Generally, lysyl residue is the most reactive amino acid because of the free ε-amino group. Reaction of the ε-amino group of lysyl residues of proteins with reducing sugar results in the so-called "Amadori product" via the formation of a Schiff's base and the Amadori rearrangement (Ledl & Schleicher, 1990; Friedman, 1996). Through this reaction, the conjugation of the sugar to the protein does not require chemical catalysis, but just heating in order to accelerate the spontaneous reaction. A well-controlled Maillard reaction can thus be a good method for protein processing in the food industry. Since lysine is the limiting essential amino acid in many food proteins, its destruction can reduce the nutritional value of proteins. Foods that are rich in reducing sugars are very reactive and this explains why lysine in milk is destroyed more easily than in other foods (de Man, 1999).

Study and characterization of the Maillard reaction products could allow one to control the formation of Advanced Maillard Products (AMP), responsible in part for noxious effects in diabetes and in age-related cardiovascular diseases (Al Abed et al., 1999). This knowledge could lead to a better control of these reactions in order to modify food proteins and their functions. Maillard reactions are one of the simplest ways to modify food proteins since they take place when a protein and a sugar are just heated together (Chuyen, 1998).

BLG is the major whey protein. It is present in the milk of various ruminant species (Godovac-Zimmermann et al., 1990a, b; Sawyer & Holt, 1993; Ochirkhuyag et al., 1998). This protein constitutes a major waste product of the cheese industry. At the end of the 90's, its uses increased as a food additive thanks to its good nutritional properties (Smithers et al., 1996). Consequently, the improvement of BLG functional properties may be of considerable interest to industry.

To improve their functional and physicochemical

properties, dairy proteins have been modified by several methods (Haertlé & Chobert, 1999), such as phosphorylation (Sitohy et al., 1995c), esterification (Chobert et al., 1995, Sitohy et al., 2001b), alkylation (Kitabatake et al., 1985), and reductive amidation (Mattarella et al., 1983); (see Section I.). However, most of these methods, using toxic chemicals, cannot be used in food processing.

BLG showed better emulsifying properties after glycation with glucose-6-P (Aoki et al., 1997) and better heat stability and solubility after glycation with glucose, mannose or galactose (Nacka et al., 1988). Study of the Maillard reactions between BLG and lactose revealed the presence of α-lactulosyllysine (Jones et al., 1998), a unique lactosylation site in the early step of the reaction (Léonil et al., 1997; Fogliano et al., 1998) and heterogeneity of protein glycoforms (Morgan et al., 1997).

1. Reaction conditions. Glycation in the presence of different sugars

Protein polymerization and glycation site specificity were investigated according to the nature of sugar used for modification of BLG (Chevalier et al., 2001c). Among the six common sugars used, arabinose and ribose induced the highest degree of modification. Glucose, galactose and rhamnose were less reactive and lactose generated the lowest degree of modification. Proteins substituted with ribose or arabinose formed polymers stabilized by sugar-induced covalent bonds. When other sugars were used, part of the aggregated proteins were stabilized only by hydrophobic interaction and disulfide bonds. According to mass spectrometry analysis, leucine 1 (N-terminal amino acid), lysine 14 and lysine 47 were modified in the presence of galactose, glucose or lactose. Lysines 69, 75 and 135 were modified only in the case of protein glycated with glucose. Lysine 100 was modified only when protein was glycated with lactose. No glycation site could be detected for proteins glycated with ribose or arabinose due to the higher degree of modification, which inhibited the tryptic hydrolysis used before mass spectrometry analysis.

2. Change in protein structure

Heating of BLG for 3 days at 60 °C did not induce major conformational changes, as observed by peptic hydrolysis, since only an additional 5% of the protein was hydrolyzed when compared with native BLG. Only minor modifications could be observed in far- and near- UV Circular Dichroism spectra of these proteins. Nevertheless, some changes in aggregation behavior were observed by SDS-PAGE, showing

polymerization of BLG after heating. When BLG was heated in the presence of sugars, larger structural modifications were observed depending on the sugar used (Chevalier et al., 2002). Conformational modification of BLG was related to the degree of glycation. The more reactive the sugar was, the more denatured was the glycated protein. According to peptic hydrolysis data, near- and far- UV Circular Dichroism spectra and micro-calorimetry analysis, proteins modified with ribose or arabinose (the most reactive sugars) showed important conformational changes. In contrast, proteins modified with lactose or rhamnose (the less reactive sugars) had similar three-dimensional structures to native BLG. According to Chevalier et al. (2001a,c), glycation of BLG induced polymerization of protein monomers. Moreover, as observed by calorimetry analysis, Maillard glycation increased the temperature of denaturation of proteins glycated with galactose, glucose, lactose or rhamnose. These results correlated well with those obtained in a previous study of the functional properties of glycated BLG (Chevalier et al., 2001c; see below), which demonstrated the importance of the sugar used on the improvement of emulsifying and foaming properties of the derivatives.

3. Change in functional properties

The whey produced during cheese and casein manufacturing contains approximately 20% of all milk proteins. It represents a rich and varied mixture of secreted proteins with wide-ranging chemical, physical and functional properties (Smithers et al., 1996). Due to their beneficial functional properties, whey proteins are used as ingredients in many industrial food products (Cheftel & Lorient, 1982). According to Kinsella & Whitehead (1989), functional properties of foods can be explained by the relation of the intrinsic properties of the proteins (amino acid composition and disposition, flexibility, net charge, molecular size, conformation, hydrophobicity, etc.), and various extrinsic factors (method of preparation and storage, temperature, pH, modification process, etc.).

Numerous attempts were made to improve the functional properties of whey proteins through physical, chemical and/or enzymatic treatments (Haertlé & Chobert, 1999). Many studies were carried out with BLG, the major whey protein. Focusing on the improvement of solubility, heat stability, foaming properties and emulsifying properties, this protein has been conjugated with ester (Mattarella & Richardson, 1983; Sitohy et al., 2001b), gluconic or melibionic acids (Kitabatake et al., 1985), carbohydrates

(Waniska & Kinsella, 1988; Bertrand-Harb et al., 1990) and phosphoric acid (Sitohy et al., 1995a-c).

However, most of these methods utilize toxic chemicals and are not permitted for potential industrial applications. Some attempts were made to improve the functional properties of BLG by conjugation with glucose-6-phosphate (Aoki et al. 1997).

The functional properties (solubility, heat stability, emulsifying and foaming properties) of BLG after glycation of the protein with several sugars (arabinose, galactose, glucose, lactose, rhamnose or ribose) were studied (Chevalier et al., 2001d). Protein samples were heated in the presence or in the absence (heated control) of different sugars for three days at 60°C. Subsequent glycation induced a modification of the solubility profile, shifting the minimum solubility towards more acidic pH. Native BLG was soluble over the whole pH range studied. After heating, a 50% decrease of solubility was observed in the pH range 4.0 - 5.5 with a minimum observed at pH 5, which is near the pI of the protein. After heating BLG in the presence of arabinose or ribose, the resulting glycated derivatives exhibited 35% solubility at pH 4, which is lower than the solubility obtained with heated BLG and BLG glycated with the other sugars. Major conformational modification induced by the glycation could explain such a decrease. However, due to the shift of their isoelectric point, these derivatives showed an increased solubility at pH 5 as compared with heated BLG. In the case of proteins modified with galactose, glucose, lactose or rhamnose, 75% solubility was observed at pH 4.5, showing a protective effect of glycation by these sugars against the decrease of solubility due to heating.

Glycated proteins exhibited a better thermal stability when heated at pH 5 as compared to native or heated control.

Glycation of BLG with arabinose or ribose (the most reactive sugars) improved its emulsifying properties. Solubility is a determining factor for a protein's ability to form an emulsion (Kinsella & Whitehead, 1989). Despite the fact that native BLG is highly soluble in the pH range 2-10, its emulsifying activity index was low at acid pHs and increased with increasing pH (Shimizu et al., 1985), suggesting that structural changes occur in BLG at acidic pH that influence its emulsifying properties. Such effects close to the pI of BLG could be at the origin of the decrease of emulsion stability (Klemaszewski et al., 1992) observed in the $d_{3.2}$ values.

Other studies have shown that lactosylation of BLG in a dry system did not significantly alter the three-dimensional

structure of the protein. In contrast, in an aqueous system, an important structural change was observed at the protein-protein interface (Morgan et al., 1999a-c). This suggests that protein modified with galactose, glucose, lactose or rhamnose could present structural changes as compared with the native protein. Polymerization observed with glycated BLG (Bouhallab et al., 1999; Chevalier et al., 2001a) could be involved in these changes.

Proteins modified with galactose, glucose, lactose or rhamnose were less glycated. Consequently, they may have conserved a globular three-dimensional structure close to the conformation of the native protein, accounting for the increase in $d_{3.2}$ at pH 5.

Foaming properties were better when BLG was glycated with glucose or galactose (moderately reactive sugars). These results suggest that the nature of the sugar is an essential factor for improving the functional properties of glycated proteins by processes of Maillard reaction.

4. Change in biological properties

Since many attempts are made to control food storage and to preserve food from oxidation and microorganism contamination, it is interesting to subject protein to oxido-reductive modification in order to see whether new biological properties could be induced. The Maillard reaction is one of major reactions modifying proteins in food and in nature. The study of how it influences biological properties of derived proteins and peptides is of particular interest. The consequences of this reaction on the biological properties of the modified products have been largely studied mainly on model systems, which consist in heating a single amino acid with a reducing sugar. The Maillard reaction induced: (i) anti-oxidative activity of glucose-glycine (Anese et al., 1994), xylose-lysine (Yen & Hsieh, 1995) and xylose-arginine reacting systems (Beckel & Waller, 1983); (ii) anti-microbial activity of xylose-arginine and glucose-histidine systems (Einarsson et al., 1983; Einarsson, 1987a, b) and of glucose-glycine system (Stecchini et al., 1993); (iii) cytotoxic activity of glucose-lysine and fructose-lysine systems (Jing & Kitts, 2000); (iv) clastogenic activity of ribose-lysine (Vagnarelli et al., 1991) and of glucose-lysine systems (Kitts et al., 1993). Proteins modified by the Maillard reaction can also present some of these properties. For example, lysozyme modified with dextran by glycation revealed a significant anti-microbial activity against both Gram-negative and Gram-positive bacteria (Nakamura et al., 1991); glycation of casein with glucose or lactose resulted in enhancement of antioxidant activity when compared with native casein (McGookin & Augustin, 1991).

Maillard reaction occurs during many industrial and domestic thermal treatments of foods. It is widely used because of its role in creating colors, flavors, textures and other functional properties in foodstuffs. Proteins glycated without the use of conventional chemical reagents have demonstrated to improve techno-functional properties as heat stability, emulsifying and foaming properties. A study was carried out to determine the extent in which this reaction can convey antioxidant, anti-microbial or cytotoxic activities to BLG, and to its tryptic and peptic hydrolysates. BLG was modified with six different sugars in solution at 60°C. Antiradical properties were estimated using a radical scavenging activity test. Anti-microbial activities against different bacterial strains were studied with a diffusion disk method. Cytotoxic tests were performed using two cell lines and the MTT [3, (4,5-dimethylthiazoyl-2-yl) 2,5 (diphenyl-tetrazolium bromide)] rapid colorimetric assay. Glycation induced a radical scavenging activity to BLG which intensity depended on the sugar used for modification. Proteins modified with ribose and arabinose showed the highest radical scavenging activities depicted by about 80 and 60% of DPPH (2,2-diphenyl-1-picrylhydrazyl) absorption decrease at 515 nm. No anti-microbial effect of any glycated form of BLG against *Escherichia coli*, *Bacillus subtilis*, *Listeria innocua* and *Streptococcus mutans* was observed. MTT test showed no enhancement of cytotoxicity by modified proteins and peptides against COS-7 and HL-60 cells. Thus, glycated proteins could be used in formulated food as functional ingredients with a radical scavenging activity able to delay deterioration due to oxidation. This use could be even more advisable considering the lack of toxicity to eukaryotic and prokaryotic cell cultures demonstrated in this work (Chevalier et al., 2001b).

II. ENZYMATIC PROTEIN PROCESSING

Protein modification, traditionally carried out by direct chemical means, can also be accomplished using enzyme catalysis. Chemical modification is not very desirable for food applications because of the harsh reaction conditions, non-specific chemical reagents, and the difficulty to remove residual reagents from the final product. Enzymes provide several advantages including fast reaction rates, mild conditions and high specificity. Under optimum conditions, enzyme–catalyzed reactions proceed 10^8-10^{11} times more

rapidly than the corresponding non-enzymatic reactions, resulting in reduction of energy costs and increased processing efficiency. Enzymatic methods are consequently more attractive to the food processor. Moreover, most enzymes can be produced in large quantities, each having appropriate physical, chemical and catalytic properties. The cost of enzyme production is reasonable if one uses microbial fermentation or biotechnological processes.

Besides the proteases, which have been investigated extensively and which are the only modifying enzymes currently in use commercially, there are transglutaminase, protein kinase, and peptidoglutaminase. These enzymes have only been reported for use in food protein modification on a laboratory scale. Feeney & Whitaker (1977, 1982, 1986) addressed possible approaches applicable to the modification of proteins and discussed the impact of these potential modifications on the structure and properties of the proteins in great detail.

A. LIMITED PROTEOLYSIS AND FUNCTIONAL PROPERTIES

A large number of papers describe the effect of proteolytic breakdown of food proteins on their functional properties. They comprise animal proteins such as casein (Chobert et al., 1987, 1988a, b; Haque & Mozaffar, 1992; Agboola & Dalgleish, 1996; Slattery & FitzGerald, 1998; Smyth & FitzGerald, 1998), soybean proteins (Puski, 1975; Adler-Nissen & Olsen, 1979; Mohri & Matsushita, 1984; Adler-Nissen, 1986; Kim, et al., 1990), wheat gluten and gluten fractions (Popineau & Pineau, 1993), among others.

Hydrolysis of peptide bonds causes several changes in proteins: (i) the NH_3^+ and COO^- content of the protein increases, increasing its solubility; (ii) the molecular mass of the protein decreases; (iii) the globular structure of the protein is altered, exposing the previously hidden hydrophobic groups.

Proteolytic modification has special importance for the improvement of solubility of proteins. This effect becomes significant even after very limited proteolysis. Hydrolysis of casein to degree of hydrolysis (DH) of 2 and 6.7% with *Staphylococcus aureus* V8 protease increased the isoelectric solubility to 25 and 50%, respectively (Chobert et al., 1988a). However, it should be noted that the solubility profiles were not identical, due to a shift of the isoelectric point of the modified proteins. Solubility of a protein hydrolysate depends on the enzyme used (Adler-Nissen, 1986).

Protamex™ (a *Bacillus* proteinase complex) hydrolysates of sodium caseinate (DH 9 and 15%) displayed 85-90% solubility between pH 4.0 and 5.0 (Slattery & FitzGerald, 1998).

Emulsifying properties of proteins are sensitive to proteolytic modification (Mietsch et al., 1989). Limited hydrolysis (DH 2 and 6.7%) of casein decreased the emulsifying activity (EA) at all pH (Chobert et al., 1987, 1988a), whereas the emulsion stability (ES) at DH = 2% was higher than that of native casein (Chobert et al., 1987). The EA of casein was reported to decrease with increasing net charge and with the decreasing hydrophobicity due to proteolysis (Mahmoud et al., 1992). Since the *Staphylococcus aureus* V8 protease is highly specific for glutamate residues, and as these residues are uniformly distributed in the sequence of caseins, a poor EA was not expected at these DH values. This could be due to some properties of the obtained peptides, which had the glutamyl residue at the C-terminal end. In the case of using trypsin for hydrolysis of dairy proteins (Chobert et al., 1988b), it was reported that a limited hydrolysis of casein (DH of 4.3, 8.9, and 9.9%) improved its EA, showing the importance of the specificity of the enzyme used. However ES of such hydrolysates was lower than that of unmodified casein. This could be attributed to the fact that the obtained peptides were not amphiphilic enough to impart a high stability to the emulsion. Hydrolysis of casein with trypsin, chymotrypsin and Rhozyme-41 resulted in an improvement in EA (Haque & Mozaffar, 1992). Protamex hydrolysates (DH 0.5 and 1.0%) of casein showed higher EA at pH 2.0 as compared with unmodified casein (Slattery & FitzGerald, 1998). However, at higher DH of 9.0 and 15%, hydrolysates exhibited lower EA compared to caseinate.

Generally, the pH of protein solutions during emulsification affects their emulsifying properties via electrostatic effects. The emulsifying capacity of protein hydrolysates is usually low at the isoelectric point. Addition of salt improves the emulsifying properties at pI (Turgeon et al., 1992). Several studies have shown no correlation between emulsifying capacity and high solubility (Chobert et al., 1988a, b; Turgeon et al., 1992).

The effect of limited proteolysis of whey proteins with covalently immobilized trypsin has been investigated. A carefully controlled limited proteolysis yielded a fraction (residues 41-100 with a disulfide bond to residues 149-162) of the core β-barrel of BLG (Chen et al., 1993, 1994; Huang et al., 1994, 1996). The emulsifying properties of these peptides were 2-3 fold larger than that of the native protein in

the pH range of 3 to 9. Furthermore, the stabilities of emulsions formed with the domain peptide fraction were greater as indicated by the lack of separation of an oil phase after standing at ambient temperature for one week, whereas emulsions formed with the native protein separated (Huang et al., 1996).

Because of the lower structural stability and altered molecular characteristics of the domain peptides, their interactions and thus their gelling properties were significantly different from those of the native protein. Solutions of a limited proteolysate of BLG formed gels at 60°C, whereas the untreated protein required temperatures of 70-80°C for gelation (Chen et al., 1994). The domain peptides, even in the presence of other whey proteins, alter the gelation properties of BLG. Thus, limited proteolysis of whey protein isolate exhibited gelation characteristics that were different from those of untreated whey protein isolate. For example, 10% solutions of enzyme-treated whey protein isolate at pH 7.0 exhibited a gelation point at 77°C, while untreated whey protein isolate only gelled after holding at 80°C for 1.4 min. The enzyme-treated whey protein isolate solution formed an opaque particulate gel at 80°C whereas the whey protein isolate gel was clear and fine stranded. Values for hardness, cohesiveness, gumminess and chewiness were significantly greater for enzyme-treated whey protein isolate gels (Huang et al., 1999).

Enzymatic hydrolysis modifies the foaming properties of casein. Protamex hydrolysates of sodium caseinate (DH 0.5 and 1.0%) displayed increased foam expansion at pH 2, 8 and 10 as compared with unhydrolyzed caseinate (Slattery & FitzGerald, 1998). Hydrophobic peptides resulting from plasmin hydrolysis of BCN improved foam formation and stabilization at pH 4.0 (Caessens et al., 1999).

A commercial range of hydrolysates of whey proteins with DH ranging from 8 to 45% was used to make emulsions with soybean oil (Singh & Dalgleish, 1998). As estimated by the particle sizes, the maximum emulsifying capacity was obtained from hydrolysates with a 10 or 20% DH. Higher hydrolysis resulted in peptides that were too short to act as effective emulsifiers, and, at lower proteolysis, the somewhat reduced solubility of the hydrolysates slightly decreased their emulsifying power. All of the emulsions were unstable when they were subjected to heat treatment at high temperatures (122°C for 15 min), but emulsions prepared from the less hydrolyzed peptide mixtures were stable to heat treatment at 90°C for 30 min.

One of the applications of enzymes in the preparation of food gels is the production of cheeses. During this process, chymosin hydrolyses a specific bond of κ-casein, resulting in the destabilization of the micelle structure followed by aggregation and formation of an insoluble coagulum.

Enzymatic gelation of partially heat-denatured whey proteins by trypsin, papain, pronase, pepsin, and a preparation of *Streptomyces griseus* has been studied (Sato et al., 1995). Only peptic hydrolysate did not form a gel. The strength of the gel depended on the enzyme used and increased with increasing DH. Hydrolysis of whey protein concentrate with a glutamic acid specific protease from *Bacillus licheniformis* at pH 8 and 8% protein concentration has been shown to produce plastein aggregates (Budtz & Nielsen, 1992). The viscosity of the solution increased dramatically during hydrolysis and reached a maximum at 6% DH. Incubation of sodium caseinate with pepsin or papain resulted in a 55 to 77% reduction in apparent viscosity (Hooker et al., 1982).

B. ALLERGENICITY

It should be highlighted that the onset of food allergies is rising significantly amongst the world populations. It is concerning particularly infant group of the European Community population. In some cases, very severe allergic reactions against consumed foods and to dairy proteins in particular, lead to anaphylaxis. In this context well described and well-studied influence of the fermentative processing of foods, and in particular the dairy food systems, could help to circumvent or limit the severity of some adverse allergenic reactions. This could be achieved thanks to pre-prandial proteolysis occurring in fermented dairy (and other food) systems changing the allergen presentation or cleaving the allergenic protein epitopes. Hence, it could be expected that well managed fermentative transformations of dairy (and other food) products with appropriate lactic acid bacteria (LAB) strains, could produce hypoallergenic products at least for some classes of allergic patients.

Proteolysis is considered to be one of the most important biochemical processes involved in the manufacture of many fermented dairy products, irrespective of the contribution of the proteolytic/peptidolytic enzymes of LAB to organoleptic properties of the final milk products. The ability to produce extracellular proteinases is a very important feature of LAB. They catalyze milk proteins, providing the amino acids essential for growth of LAB (Fira et al., 2001). Proteolysis by microbial enzymes in yogurt is a desirable process improving

INVITED REVIEW

milk digestibility and enhancing nutritional quality of yogurt. It is accepted that proteolytic system of LAB degrades proteins and hence, changes the texture, the taste and the aromas of fermented products.

Allergy to cow milk (CMA) concerns ~2.5% of children below 3 years of age, hence the feeding of an infant allergic to cow milk casein may create serious trouble. Most studies revealed that caseins and BLG are the main allergens in cow milk (Cocco et al., 2003, Gaudin et al., 2008). Different attempts have been made to reduce the allergenicity of dairy proteins, and various technological processes have been applied. Attempts to modify the protein components of cow milk in an effort to reduce their allergenic potential have included the application of heat treatment, enzymatic treatment with a variety of enzymes and some combination of these processes, such as heating and glycation (Taheri Kafrani et al., 2009). Heating has only a small effect on the antigenic/allergenic potential of cow's milk proteins, even though some authors have shown a reduction in whey protein antigenicity. Furthermore, heating processes can only modify conformational epitopes, which lose their binding capacity to specific IgEs. However, linear epitopes remain unaffected by structural changes and maintain their allergenicities after heating. Milk proteins contain both types of epitopes and, even though a slight reduction of antigenicity can be observed in case of whey proteins, only very insignificant changes in binding properties were reported for caseins (Restani et al., 2009).

Another method for reduction of antigenic/allergenic properties of milk proteins is bacterial fermentation. During microbial fermentations proteolytic enzymes can be produced and they can degrade milk protein allergens. Proteolytic systems of lactobacilli are complex and are composed of proteinases and peptidases with different sub-cellular locations. Cellular proteases grown on milk showed similar hydrolytic activities towards α_S- and β-caseins. Proteolysis is often followed by a reduction of the number of epitopes and consequently by a decrease in allergenicity of hydrolyzed proteins or could contribute to preventing allergenic problems frequent in children under 3 years of age due to poor digestion of milk proteins. Protein hydrolysates are also included in specific formulations, as well as hypoallergenic infant formulas to reduce their antigenicity as compared with intact protein (Bertrand-Harb et al., 2003; Cocco et al., 2003; Peñas et al., 2006; Pescuma et al., 2009, 2010, 2011; El-Ghaish et al., 2010a,b, 2011).

It was already reported that some LAB reduce the antigenic response of milk proteins (Wróblewska et al., 1995). The reduction of milk protein antigenicity depends on the species of LAB and on conditions of fermentation (Kleber et al., 2006, Bu et al., 2010).

Lactic acid fermentation was applied to reduce the BLG antigenicity of sweet whey and skim milk, and furthermore, its products were reported to have beneficial effects on the consumer including the activation of the immune system (Shida et al., 1998). BLG is in about 80% of all cases the main cause of milk allergies in children and infants. BLG is the major whey protein in milk and in dairy products and it is of particular interest since it is the only whey protein of cow's milk absent in human milk. Kleber et al. (2006) studied the ability of some LAB strains to reduce the antigenicity of BLG in skim milk and in sweet whey by indirect competitive ELISA, using polyclonal antibodies. They observed the reduction of antigenicity of BLG exceeding 70% in sweet whey and higher than 90% in skim milk compared to the initial value. In addition, the synergic reduction of the antigenicity was observed when co-cultures of LAB were used. The proteases produced by different LAB are more or less specific and the using of co-cultures yields higher hydrolysis of native allergens as well as of their epitopes. Fermentation of sterilized cow's milk using a mixture of meso- and thermophilic LAB resulted in almost complete disappearance of antigenicity of ALA and BLG (ELISA, rabbit antibodies) (Jedrychowski & Wróblewska, 1999). Different studies revealed that the combination of *Lactobacilli* strains with *Streptococcus thermophilus* during fermentation of milk and whey resulted in higher immunoreactivity reduction of native allergenic proteins. It was found in a recent study (Bu et al., 2010) that combined strains of *Lactobacillus helveticus* and *Streptococcus thermophilus* were the most effective in the reduction of the antigenicity of ALA and BLG in skim milk as compared with *Lactobacillus helveticus* and *Streptococcus thermophilus* alone. ALA antigenicity was reduced by 87% with combined strains fermentation by *Lactobacillus helveticus* and *Streptococcus thermophilus.* However, ALA antigenicity was inhibited by 71% and 49% with *Lactobacillus helveticus* and *Streptococcus thermophilus* alone, respectively.

During fermentation, release of proteases and peptidases results in more effective hydrolysis of milk proteins with higher possibility for cleavage of epitopes. Nevertheless, it is also possible that by further cleavage of the peptides into smaller peptides and amino acids by peptidases, some hidden epitopes or linear epitopes could be exposed. Bu et al. (2010)

found that at the beginning of the fermentation, antigenicity of BLG decreased gradually but at longer fermentation time it slightly increased, what indicates that there was no linear correlation between the degree of hydrolysis of milk protein and the antigenicity of whey proteins during the fermentation (Bu et al., 2010). Ehn et al. (2005) used different strains of *Lactobacillus helveticus* to hydrolyze milk whey proteins and observed 80% hydrolysis of BLG. However, this did not affect the epitope recognition by IgE as judged by the unchanged inhibition pattern (ELISA, human serum) after this treatment. This suggests that the extracellular proteolytic activity in the fermentation process did not degrade enough the IgE epitopes. It is also possible that the degradation was only partial, leaving peptides long enough to bind the antibodies, or it is possible that better access to some buried internal epitopes compensated any hydrolytic reduction of external epitopes. It is also possible that the preheating temperature might have a significant impact on the changes of the antigenicity of whey proteins. Kleber et al. (2006) studied the effect of different LAB on the antigenicity of BLG in skim milk heated 40 min at 90°C. They found that the antigenicity of BLG decreased by 84–98% as compared with unfermented skim milk. Jedrychowski & Wróblewska (1999) claimed that after the fermentation with LAB the antigenicity of sterilized milk (110°C, 10 min) was reduced by over 99%. However, when prick test with whey samples from fermented whole milk were done on allergic to milk patients their allergic reactions were only slightly attenuated. Further *in vitro* and *in vivo* studies are necessary to understand better the mechanism of allergenicity of dairy proteins and the methods to decrease it. Additionally, it would be interesting to study the combination of various methods, such as fermentation and hydrolysis with animal and plant proteases, heat treatment, high pressure, and microwave combined with enzymatic proteolysis (El Mecherfi et al., 2011).

C. LIBERATION OF BIOLOGICALLY FUNCTIONAL PEPTIDES

Milk contains components that provide critical nutritional elements, immunological protection, and biologically active substances to both neonates and adults (Meisel & Schlimme, 1990). In general, the major protein fractions in bovine milk include ALA, BLG, caseins (α_{S1}, α_{S2}, β, and κ), immunoglobulins, lactoferrin, proteose-peptide fractions (heat-stable, acid soluble phosphoglycoproteins), and minor

whey proteins such as transferrin and serum albumin. From these, bioactive peptides may be generated *in vivo* through gastrointestinal processes. These peptides, encoded within the sequence of native protein precursors, may also be generated *in vitro* by enzymatic hydrolysis. The number of such peptides identified in caseins and whey proteins continues to grow (Gobetti et al., 2002). Lastly, physiologically active peptides have been chemically synthesized to confirm the biological properties associated with a specific amino acid sequence. Many bioactive peptides serve in multifunctional capacities and often share common structural features based on a defined, bio-specific role.

1. Milk defense peptides - antimicrobial peptides

Jones & Simms (1930) reported the first dairy protein derived antibacterial factor, named lactenin, which slowed the growth of streptococci. Other antimicrobial milk proteins, such as lactoferrin, were described early in the literature in the 70's. Discovery of basic glycopeptides with bactericidal activity against various strains of *Staphylococcus aureus* and *Streptococcus* have been also detailed at this time. Later, there has been a new interest in using bioactive peptides for application within the health care industry (Table 1 and references therein).

Casecidin, obtained by chymosin digestion of casein at neutral pH, was among the first defense peptides actually purified and exhibited activity *in vitro* against *Staphylococcus*, *Sarcina*, *Bacillus subtilis*, *Diplococcus pneumoniae*, and *Steptococcus pyogenes*. Casocidin I from bovine milk, a cationic α_{S2}-CN derived peptide, inhibited growth of *Escherichia coli* and *Staphylococcus carnosus*. Isracidin, an N-terminal segment of α_{S1}-CN-B, protected mice against *Staphylococcus aureus* and *Candida albicans*.

Hydrolysate of bovine lactoferrin was active against both Gram positive (*Bacillus*, *Listeria*, and *Streptococcus*) and Gram negative (*E. coli*, *Klebsiella*, *Salmonella*, *Proteus*, and *Pseudomonas*) microorganisms *in vitro*. A bactericidal peptide generated by peptic hydrolysis of lactoferrin (named lactoferricin-B) was active against Gram-positive and Gram-negative bacteria.

Milk also contains peptides that exhibit anti-fungal properties (Table 1). Anti-fungal activity of lactoferrin or its peptides (ex: lactoferricin B), in combination with azole anti-fungal agents, has been demonstrated with *Candida albicans*. Several filamentous fungi, including agents of skin disease (dermatophytes), were also found to be susceptible to this mixture.

INVITED REVIEW

Table 1. *Antimicrobial milk peptides (according to Clare and Swaisgood, 2000).*

Milk peptide fragment	Release protease	Gram (+) activity	Gram (–) activity	Yeast and fungi *
Casecidin[a] α_{S1}- and κ-CN (MW $\cong$ 4000-6000)	Chymosin and chymotrypsin	*Staphylococcus Sarcina* **Bacillus subtilis** *Diplococcus pneumoniae Streptococcus pyogenes*		
Casocidin-I[b] α_{S2}-CN (f 165-203)	Synthetic peptide	*Staphyloccocus carnosus*	*Escherichia coli*	
Isracidin[a] α_{S1}-CN (f 1-23)	Chymosin and chymotrypsin	*Staphylococcus aureus*		*Candida albicans*
Lactoferricin B[c] Lactoferrin (f 17-41)	Pepsin	*Bacillus Listeria Streptococci Staphylococci*	*E. coli 0111 E. coli 0157H :7 Klebsiella Proteus Pseudomonas Salmonella*	*Candida albicans Dermatophytes* * *Cryptococcus uniguttulatus* * *Penicillum pinophilum* * *Trichophyton mentagrophytes*

a Lahov & Regelson, 1996
b Zucht et al., 1995
c Bellamy et al., 1994; Shin et al., 1998; Tomita et al., 1991

The most recent data showed that lactoferrin-related compounds, combined with triazoles, inhibited growth of azole-resistant *Candida albicans*.

Several antimicrobial peptide databases have been created over the past several years. Some of these list peptides from various sources (Wang et al., 2009; Thomas et al., 2010; Piotto et al., 2012; Seshadri Sundararajan et al., 2012) while others are dedicated to a specific category of peptides (Whitmore and Wallace, 2004; Seebah et al., 2007; Hammami et al., 2010) as previously reviewed (Hammami & Fliss, 2010). A new database that contains valuable information on antimicrobial peptides of dairy origin, including microbiological and physico-chemical data is freely available at http://milkampdb.org/.

2. Physiologically active milk peptides

In addition to providing immuno-defense systems, milk also contains other major peptide fractions that elicit behavioral, neurological, physiological, and vaso-regulatory responses (Table 2 and references therein).

Anti-hypertensive peptides

Anti-hypertensive peptides inhibit the angiotensin-converting enzyme (ACE). ACE is a dipeptidyl-dipeptidase that cleaves dipeptides from the carboxy terminal end of the substrate. ACE converts angiotensin I to angiotensin II, increasing blood pressure and aldersterone, and inactivating the depressor action of bradykinin. ACE inhibitors derived from casein, or casokinins have been identified within the sequences of human β- and κ-CN. They are also generated by tryptic digestion of bovine α_{S1}- and β-CN. The C-terminal tripeptide sequence is the primary structural feature governing this inhibitory response. Reports indicated that the ACE-binding pocket exhibited a preference for hydrophobic amino acids at each of these sites.

A second characteristic of ACE inhibitory casokinins is the presence of a positively charged lysine or arginine at the carboxy terminal end. Removal of this amino acid residue from bradykinin, an endogenous ACE inhibitor, resulted in production of an analogue that was essentially inactive.

ACE inhibitory peptides are also derived from both α_{S1}- and β-CN that are generated by the hydrolysis of sour milk with the *Lactobacillus helveticus* CP790 cellular protease. These peptides exhibited anti-hypertensive activity in spontaneously hypertensive rats as monitored by systolic blood pressure. A synthetic seven amino acid peptide, equivalent to a segment found in the β-CN hydrolysate, exhibited potent anti-hypertensive activity in these rats over an 8-h interval after oral administration.

Table 2. *Examples of physiologically active milk peptides (according to Clare and Swaisgood, 2000).*

Sample number	Peptide sequence[1]	Name	AA[2] segment	Physiological classification	Release protease
1	FFVAP	α_{S1}-Casokinin-5	α_{S1}-CN (f 23-27)	ACE inhibitor	Proline endopeptidase
2	AVPYPQR	β-Casokinin-7	β-CN (f 177-183)	ACE inhibitor	Trypsin
3	YGLF	α-Lactorphin	α-LA (f 50- 53)	ACE inhibitor and opioid agonist	Synthetic peptide
4	ALPMHIR	β-Lactorphin	β-LG (f 142-148)	ACE inhibitor	Trypsin
5	KVLPVPQ	Antihypertensive peptide	β-CN (f 169-174)	Antihypertensive peptide	*Lactobacillus* CP790 protease & synthetic peptide
6	MAIPPKKNQDK	Casoplatelin	κ-CN (f 106-116)	Antithrombotic	Trypsin & synthetic peptide
7	KDQDK	Thrombin inhibitory peptide	κ-CN glyco-macropeptide (f 112-116)	Antithrombotic	Trypsin
8	KRDS	Thrombin inhibitory peptide	Lactotransferrin (f 39–42)	Antithrombotic	Pepsin
9	QMEAES*IS*S*S*EEIVPNS*VEQK	Caseinophospho-peptide	α_{S1}-CN (f 59-79)	Calcium binding and transport	Trypsin
10	LLY	Immunopeptide	β-CN (f 191-193)	Immunostimulatory (+)	Synthetic
11	FKCRRWQWRMK KLGAPSITCVRRAF	Lactoferricin B	Lactoferrin (f 17-41)	Immunomodulatory (+) and antimicrobial	Pepsin
12	YQQPVLGPVR	β-Casokinin-10	β-CN (f 193-202)	Immunomodulatory (+/-) & ACE Inhibitor	Synthetic
13	RYLGYLE	α-Casein exorphin	α_{S1}-CN (f 90-96)	Opioid agonist	Pepsin
14	YGFQNA	Serorphin	BSA (f 399-404)	Opioid agonist	Pepsin
15	YLLF.NH$_2$	β-Lactorphin (amide)	β-LG (f 102-105)	Opioid agonist = ACE Inhibitor	Synthetic or Trypsin
16	YIPIQYVLSR	Casoxin C	κ-CN (f 25-34)	Opioid antagonist	Trypsin
17	[YVPF PPF]	Casoxin D	α_{S1}-CN (f 158-164)	Opioid antagonist	Pepsin-chymotrypsin
18	YLGSGY-OCH$_3$	Lactoferroxin A	Lactoferrin (f 318-323)	Opioid antagonist	Pepsin

[1]The one-letter amino acid codes were used ; S* = phosphoserine
[2] Amino-acid
Sample number/references: 1-2/*Maruyama et al., 1985*; **3**/*Mullally et al., 1996*; **4**/*Mullally et al., 1997*; **5**/*Maeno et al., 1996*; **6**/*Jollès et al., 1986*; **7**/*Qian et al., 1995b*; **8**/*Qian et al., 1995a*; **9**/*Schlimme & Meisel, 1995*; **10**/*Migliore-Samour et al., 1989*; **11**/*Bellamy et al., 1992, Miyauchi et al., 1998*; **12**/*Meisel & Schlimme, 1994*; **13**/*Loukas et al., 1983*; **14**/*Tani et al., 1994*; **15**/*Mullally et al., 1996*; **16**/*Chiba et al., 1989*; **17**/*Yoshikawa et al., 1994*; **18**/*Yamamoto et al., 1994*.

A third subclass, β-lactorphins, are sequestered within the primary structure of bovine BLG and released by trypsin. Novel ACE inhibition was detected in synthetic peptides that corresponded to sequences within both BLG and ALA.

Anti-thrombotic peptides

Early on, it was learned that the mechanisms involved in milk clotting, defined by the interaction of κ-CN with chymosin and blood clotting processes, defined by the interaction of fibrinogen with thrombin, were comparable. In this regard, the C-terminal dodecapeptide of human fibrinogen γ-chain (residues 400 to 411) and the undecapeptide (residues 106 to 116) from bovine κ-CN are structurally and functionally quite similar. This casein-derived peptide sequence, termed casoplatelin, affected platelet function and inhibited both the aggregation of ADP-activated platelets and the binding of human fibrinogen λ-chain to its receptor region on the surface of platelets.

A smaller κ-CN fragment (residues 106 to 110), named casopiastrin, was obtained from tryptic hydrolysates and exhibited anti-thrombotic activity by inhibiting fibrinogen binding. A second segment of the trypsin κ-CN fragment, residues 103 to 111, inhibited platelet aggregation but did not

affect fibrinogen binding to the platelet receptor. Later, it was reported that biologically active peptides, isolated from both casein and lactotransferrin, inhibited platelet function.

Antithrombotic peptides have also been derived from κ-caseinoglycopeptides that were isolated from several animal species. Bovine κ-caseinoglycopeptide the C-terminal end of κ-CN (residues 106 to 169), inhibited platelet aggregation. Two anti-thrombotic peptides derived from human and bovine κ-caseinoglycopeptides have been identified in the plasma of 5-d-old newborns after breast-feeding and ingestion of cow's milk based formula, respectively. The C-terminal residues (106 to 171) of sheep κ-CN decreased thrombin- and collagen-induced platelet aggregation in a dose dependent manner. Thrombin-induced platelet aggregation was inhibited with pepsin digests of sheep and human lactoferrin.

Caseinophosphopeptides

Caseinophosphopeptides (CPP) have been identified after trypsin release from α_{S1}-, α_{S2}-, and β-CN. The phosphate residues, which are present as monoesters of serine, occur mainly in clusters. Most CPP contain three serine phosphate clusters followed by two glutamic acid residues, form soluble organophosphate salts, and probably function as carriers for different minerals, especially calcium. These fractions exhibit different degrees of phosphorylation, and a direct relationship between the degree of phosphorylation and mineral chelation ability has been described. In this event:

α_{S2}-CN>α_{S1}-CN>β-CN>κ-CN.

The specific amino acid composition associated with the phosphorylated binding site also influences the degree of calcium binding. CPP are mostly resistant to enzymatic hydrolysis in the gut and most often found in a complex with calcium phosphate. This complex formation results in an increased solubility, which, in turn, provides enhanced absorption of calcium across the distal small intestines of animals, fed casein diets in comparison to control animals fed soy-based diets. This passive transport system is the primary means of calcium absorption under physiological conditions and provides calcium required for bone calcification. Caseinophosphopeptides also inhibit caries lesions through re calcification of the dental enamel. Hence, their application in the treatment of dental diseases has been proposed.

Immunomodulatory peptides

Immunomodulatory milk peptides affect both the immune system and cell proliferation responses. β-Casokinins inhibit

ACE enzymes that are responsible for inactivating bradykinin, a hormone with immune enhancing effects. Thus, this chain of events indirectly produces an overall immuno-stimulatory response. Peptides derived from casein hydrolysate were shown to increase phagocytotic activity of human macrophages against aging red blood cells and augment phagocytosis of sheep red blood cells by murine peritoneal macrophages *in vitro*. Immuno-stimulatory activity against *Klebsiella pneumoniae* was demonstrated *in vivo* using rats treated intravenously with a hexapeptide obtained by hydrolysis of human β-CN. Lactoferricin B, obtained by hydrolysis of lactoferrin with pepsin, was found to promote phagocytic activity of human neutrophils via dual mechanisms that may involve direct binding to the neutrophil and opsonin-like activity.

Small peptides corresponding to the N-terminal end of bovine ALA (dipeptide) and κ-CN (tripeptide) significantly increased proliferation of human peripheral blood lymphocytes, while the C-terminal part of bovine β-CN (193 to 209), obtained by hydrolysis with pepsin-chymosin, induced a similar response in rats. Bioactive peptides in yoghurt preparations decreased cell proliferation with IEC-6 or Caco-2 cells. This may explain in part why consumption of yoghurt has been associated with a reduced incidence of colon cancer. In general, the mechanisms by which these milk-derived peptides exert their immuno-potentiating effects or influence proliferative responses are not currently known. However, one example suggests that the opioid milk peptide, β-casomorphin (Table 3), may exert an inhibitory effect on the proliferation of human lymphocytes *in vitro* via the opiate receptor. This anti-proliferative response was reversed by the opiate receptor agonist, naloxone.

Opioid milk peptides

The major opioid peptides are fragments of β-CN, called β-casomorphins, due to their exogenous origin and morphine-like properties; however, they have also been obtained from pepsin hydrolysis of bovine α_{S1}-CN. Similar peptides have been reported from human β-CN and the Y-P-F sequence, which is common to bovine β-casomorphin, was also found to be present in the primary structure of human β-CN. Various synthetic derivatives have been made and among these, Y-P-F-V-NH$_2$ (valmuceptin) and Y-P-F$_{(D)}$-V-NH$_2$ (D-valmuceptin) show high affinity for their receptor. Brantl and co-workers (1979, 1981, 1982) reported opioid activity from synthetic tetra-and penta-peptide fragments of human β-CN.

Table 3. *Opioid milk peptides (according to Clare and Swaisgood, 2000).*

Protein substrate	Peptide name	Amino acid segment
1.Bovine α_{S1}-CN	α_{S1}-Casein exorphins	f 90-95, f 90-96, and f 91-96
2. Human α_{S1}-CN	Casoxin D	f 158-164
3. Human β-CN	β-Casomorphin (4,5)	f 51-54, and f 51-55
4. Bovine and human κ-CN	Casoxin A, B, and C	f 25-34, f 35-41, and f 57-60
5. Bovine and human α-LA	α-Lactorphin	f 50-53
6. Bovine β-LG	β-Lactorphin	f 102-105
7. Lactotransferrin	Lactoferroxins A, B and C	f 318-323, f 536-540, f 673-679
8. BSA	Serophin	f 399-404

Protein substrate/references: 1/*Loukas et al., 1983*; **2**/*Yoshikawa et al., 1994*; **3**/*Brantl, 1984*; **4**/*Yoshikawa et al., 1986b, Yoshikawa et al., 1986a, Chiba et al., 1989*; **5**/*Chiba & Yoshikawa, 1986, Fiat & Jollès, 1989*; **6**/*Fiat et al., 1993; Yoshikawa et al., 1986b*; **7**/*Tani et al., 1990*; **8**/*Tani et al., 1994*.

Opioid peptides have been generated *in vitro* by enzymatic digestion of β-caseins from cow, water buffalo, and sheep. In general, the α- and β-CN fragments produce agonist responses, while those derived from κ-CN elicit antagonist effects. Opioid peptides may be further subdivided into classifications according to the specific milk from which they were derived, and these categories are summarized in Table 3 (and references therein). It is noteworthy that bioactive peptides are generated from most of the major proteins in both bovine and human milk.

Structure and function

"Typical" opioid peptides or endorphins exhibited a definite N-terminal sequence Y-G-G-F. Milk-derived peptides generated by hydrolysis of α_S- and β-CN, ALA, and BLG, are called "atypical", exomorphic, agonist peptides and exhibit morphine-like activity. Their primary structure (Y-X1-F or Y-X1-X2-F or Y) differs from the N-terminal sequence of the typical endogenous opioid peptides. With the exception of α_{S1}-CN, most share a common sequence feature, defined by a N-terminal tyrosyl residue, that is absolutely essential for activity. Typically, a second aromatic amino acid residue, such as phenylalanine or tyrosine, is also present in the third or fourth position. This structural motif fits well into the binding pocket of the opioid receptor.

β-Casomorphin-4-amide (morphiceptin) also contains a proline that is crucial for its function. This residue maintains the proper orientation of the tyrosyl and phenylalanyl side chains.

Exorphins have been isolated from peptic hydrolysates of α_S-CN fractions. In general, their structures differ considerably from those of β-caseinomorphins. Active fractions were shown to be a mixture of two separate peptides derived from α_S-CN fragments 90-95 and 90-96 [R-Y-L-G-Y-L-(E)]. The N-terminal arginyl residue was reported to be essential for activity.

Opioid agonists

β-CN peptides were among the first reported opioid peptides. β-Casomorphins are fragments corresponding to the 60 to 70th amino acid residues of bovine β-CN, the "strategic zone", and are classified as μ-type receptor ligands. Three exorphins, derived from bovine α_{S1}-CN were shown to be selective for δ-receptors. Certain proteolytic bacteria, such as *Pseudomonas aeruginosa* and *Bacillus cereus*, also produce high levels of β-casomorphins when inoculated and grown in milk. β-Casomorphins are resistant to enzymes of the gastrointestinal tract and have been detected *in vivo* in human small intestines. Since their absorption in the gut has not been observed in adults, it is concluded that the physiological influences are limited to the gastrointestinal tract with important effects on intestinal transit time, amino acid uptake, and water balance. Once they enter the bloodstream, they are rapidly degraded.

In contrast, passive transport of β-caseinomorphins across intestinal mucosal membranes does occur in neonates, which may experience physiological responses such as an analgesic effect on the nervous system resulting in calmness and sleep in infants. In pregnant or lactating woman, β-casomorphins originate in the milk, pass through the mammary tissue, and possibly influence the release of prolactin and oxytocin. Many peptides derived from α_{S1}-, β- or κ-CN, and κ-caseinoglycomacropeptide can be detected in the stomach of adults after consumption of milk or yoghurt. Casomorphins,

as opioid ligands, modulate social behavior, increase analgesic behavior, extend gastrointestinal transient time by inhibiting intestinal peristalsis and motility, exert anti-secretory (anti-diarrheal) action, modulate amino acid transport, and stimulate endocrine responses such as the secretion of insulin and somatostatin. Opioid-like milk peptides also play a regulatory role regarding appetite by modifying endocrine activity of the pancreas, resulting in an increase of insulin output.

Opioid antagonists

Opioid antagonists suppress the agonist activity of enkephalin. Casoxins A and B have been chemically synthesized and correspond to amino acid sequences within both bovine and human κ-CN. Casoxin C is an opioid antagonist obtained from tryptic digests of bovine κ-CN. Casoxin D, purified from human α_{S1}-CN fractions, elicits an opioid antagonist response. In general the modified casoxins are more active than their non-methylated derivatives. Lactoferroxins are antagonists generated from human lactoferrin. Peptides derived from peptide digestion, alone, were minimally effective, while those purified from a methyl-esterified fraction were significantly more potent. Lactoferroxin A, residues 318 to 323, showed a preference for μ-receptors. Lactoferroxins B and C, derived from residues 536 to 540, and 673 to 679, respectively, exhibited a higher propensity for κ-receptors.

CONCLUSION

Proteins are fundamentally important as nutrients but now they are seen to be much more. Through their unique functional properties, they can become key ingredients that determine many parameters of food quality. However, most proteins still have scope for further improvement in their physical and functional properties. The application of several simple physico-chemical and hydrolytic treatments to caseins and whey proteins can change significantly their functional properties. The simplest approach involves the modification of protein/peptide surface polarity. This may be achieved either by changing the ionization of polar amino acid side chains or by blocking ε-amino groups by glycation or substitution of carboxylates by esterification. Esterified dairy proteins or any abundant food proteins can become almost unlimited substrates for the production of diversified and amplified peptide libraries. Simple glycations of BLG with reducing sugars under mild conditions (pH 6.0, 60°C) can

substitute up to 70% of ε-amino groups and significantly improve the solubility of transformed proteins. As known for 100 years due to the pioneering work of L.C. Maillard, these processes also operate during food storage and aging. It is clear that they may be either beneficial to food protein functionality or harmful to their perception as food. The surfactant properties of proteins have been given a special attention since they determine the physical and functional properties of proteins in many food and biological systems. The sophisticated methodology available to improve the surface properties of proteins emphasizes the potential of chemical (and enzymatic) modifications of their functionality.

Enzymes have long been used for modifying food proteins and their use is many times more acceptable in the eye of the non-scientist than is the use of chemicals. The use of modified enzymes for processing foods is developing rapidly. In each case, the tailored proteins are used for processing purposes and not as part of the food, *per se*, such as immobilized enzyme. The use of enzymes for preparing peptides for research purposes has been employed for the formation of opioids from food proteins such as caseins. As stated by Steijns (2001), before commencing the development of nutraceuticals from milk or whey, a number of technological and marketing issues should be addressed. The question of the cost of the process required to manufacture the desired protein or peptide should be dealt with at a very early stage, together with how to productively utilize the non-bioactive residual raw material. Food safety is also an important feature. Cost effectiveness of the ingredient and ease of incorporation into a good tasting end product are vital. Claiming a message that can be understood by consumers or is allowed by legal authorities is a further prerequisite for successful market introduction. According to the present state of knowledge, caseinophosphopeptides and ACE-inhibitory peptides are the favorite bioactive peptides for diet supplementation and application to foodstuffs formulated to provide specific health benefits.

Much additional research is necessary before successful genetic manipulation of plants and animals to tailor-make more functional proteins becomes a reality.

In summary, intact milk proteins have their specific functions such as casein micelle formation and regulation of lactose synthesis. Milk proteins exhibit various biological activities when they are partially digested as described above. They are finally a source of essential amino acids. This means milk proteins are highly functional substances.

References

Adler-Nissen J. 1986. Enzymatic Hydrolysis of Food Proteins. Elsevier, London.

Adler-Nissen J, Olsen, HS. 1979. The influence of peptide chain length on taste and functional properties of enzymatically modified soy protein. - In Pour-El, A. (ed.), Functionality and Protein Structure. Advances in Chemical Series 92; American Chemical Society, Washington DC, pp. 125-146.

Agboola SO, Dalgleish, DG. 1996. Enzymatic hydrolysis of milk proteins used for emulsion formation. 1. Kinetics of protein breakdown and storage stability of the emulsions. J. Agric. Food Chem., 44: 3631-3636.

Al-Abed Y, Mitsuhashi T, Li H. 1999. Inhibition of advance glycation end product formation by acetaldehyde: role in the cardioprotective effect of ethanol. Proceed. Nat. Acad. Sci. USA, 96: 2385-2390.

Aluko RE, Mine Y. 1999. Characterization of oil-in-water emulsions stabilized by hen's egg yolk granule. Food Hydrocoll., 12: 203-210.

Ames JM. 1990. Control of the Maillard reaction in food systems. Trends Food Sci. Technol. Dec, 150-154.

Anese M, Nicoli MC, Lerici, CR. 1994. Influence of pH on the oxygen scavenging properties of heat-treated glucose-glycine systems. Ital. J. Food Sci., 3: 339-343.

Aoki T, Kitahata K, Fukumoto T, Sugimoto Y, Ibrahim H.R, Kimura T, Kato, Matsuda T. 1997. Improvement of functional properties of β-lactoglobulin by conjugation with glucose-6-phosphate through the Maillard reaction. Food Res. Intern., 30: 401-406.

Beckel RW, Waller GR. 1983. Antioxidative arginine-xylose Maillard reaction products: conditions of synthesis. J. Food Sci., 48: 996-997.

Bellamy WR, Takase M, Yamauchi K, Kawase K, Shimamura S, Tomita M. 1992. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. Biochim. Biophys. Acta, 1121: 130-136.

Bellamy WR, Yamauchi, K, Wakabayashi H, Takase M, Shimamura S, Tomita M. 1994. Antifungal properties of lactoferricin, a peptide derived from the N-terminal region of bovine lactoferrin. Lett. Appl. Microbiol., 18: 230-233.

Benjamin J, de Feijter JA, Evans MTA, Graham DE, Phillips MC. 1975. Dynamic and static properties of proteins adsorbed at the air-water interface. Faraday Discuss. Chem. Soc., 59: 218-229.

Bertrand-Harb C, Charrier B, Dalgalarrondo M, Chobert JM, Haertlé T. 1990. Condensation of glycosidic and aromatic structures on amino groups of β-lactoglobulin B via reductive alkylation. Solubility and emulsifying properties of the protein derivatives. Lait, 71: 205-215.

Bertrand-Harb C, Chobert JM, Dufour E, Haertlé T. 1991. Esterification of food proteins: Characterisation of the derivatives by a colorimetric method and by electrophoresis. Sci. Aliments, 11: 641-652.

Bertrand-Harb C, Ivanova IV, Dalgalarrondo M, Haertlé T. 2003. Evolution of β-lactoglobulin and α-lactalbumin content during yoghurt fermentation. Int. Dairy J., 13 : 39-45.

Bouhallab S, Morgan F, Henry G, Mollé D, Léonil J. 1999. Formation of stable covalent dimer explains the high solubility at pH 4.6 of lactose-β-lactoglobulin conjugates heated near neutral pH. J. Agric. Food Chem., 47, 1489-1494.

Brantl V. 1994. Novel opioid peptides derived from human beta-casein: human beta-casomorphins. Eur. J. Pharmacol., 106: 213-214.

Brantl V, Teschemacher H, Henschen A, Lottspeich F. 1979. Novel opioid peptides derived from casein (β-casomorphins): I. Isolation from bovine casein peptone. Hoppe-Seyler's Z. Physiolog. Chem., 360: 1211-1216.

Brantl V, Teschemacher H, Blasig J, Henschen A, Lottspeich F. 1981. Opioid activities of beta-casomorphins. Life Sci., 28: 1903-1909.

Brantl V, Pfeiffer A, Herz A, Henschen A, Lottspeich F. 1982. Antinociceptive potencies of β-casomorphin analogs as compared to their affinities towards μ and δ opiate receptor sites in brain and periphery. Peptides, 3: 793-797.

Briand L, Chobert JM, Haertlé T. 1994. Tryptic hydrolysis of esters of β-casein and β-lactoglobulin. Milchwissenschaft, 49: 367-371.

Briand L, Chobert JM, Haertlé T. 1995. Peptic hydrolysis of esterified β-casein and β-lactoglobulin. Int. J. Peptide Protein Res., 46: 30-36.

Bu G, Luo Y, Zhang Y, Chen F. 2010. Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins. J. Sci. Food Agric., 90: 2015–2020.

Budtz P, Nielsen PM. 1992. Protein preparations. Int. Patent Application W092/13964.

Caessens PWJR, Visser S, Gruppen H, van Aken GA, Voragen AGJ. 1999. Emulsion and foam properties of plasmin derived β-casein peptides. Int. Dairy J., 9: 347-351.

Cheftel JC, Lorient D. 1982. Aspects Technologiques. Les propriétés fonctionnelles des protéines laitières et leur amélioration. Lait, 62: 435-483.

Chen SX, Hardin CC, Swaisgood HE. 1993. Purification and characterization of β-structural domains of β-lactoglobulin liberated by limited proteolysis. J. Prot. Chem., 12: 613-625.

Chen SX, Swaisgood HE, Foegeding EA. 1994. Gelation of β-lactoglobulin treated with limited proteolysis by immobilized trypsin. J. Agric. Food Chem., 42: 234-239.

Chevalier F, Chobert JM, Dalgalarrondo M, Haertlé T. 2001a. Characterization of the Maillard reactions products of β-lactoglobulin glucosylated in mild conditions. J. Food Biochem., 25: 33-55.

Chevalier F, Chobert JM, Genot C, Haertlé T. 2001b. Scavenging of free radicals, antimicrobial, and cytotoxic activities of the Maillard reaction products of β-lactoglobulin glycated with several sugars. J. Agric. Food Chem., 49: 5031-5038.

Chevalier F, Chobert JM, Mollé D, Haertlé T. 2001c. Maillard glycation of β-lactoglobulin with several sugars: comparative study of the properties of the obtained polymers and of the substituted sites. Lait, 81: 651-666.

Chevalier F, Chobert JM, Popineau Y, Nicolas MG, Haertlé T. 2001d. Improvement of the functional properties of β-lactoglobulin glycated through the Maillard reaction is related to the sugar nature. Int. Dairy J., 11: 145-152.

Chevalier F, Chobert JM, Dalgalarrondo M, Choiset Y, Haertlé T. 2002. Maillard glycation of β-lactoglobulin induces conformation changes. Nahrung, 46: 58-63.

INVITED REVIEW

Chiba H, Yoshikawa M. 1986. Biologically functional peptides from food proteins. - in Protein Tailoring for Food and Medical Uses. Feeney, R.E. and Whitaker, J.R. (eds.). Marcel Dekker, New York. pp. 123-153.

Chiba H, Tani F, Yoshikawa M. 1989. Opioid antagonist peptides derived from κ-casein. J. Dairy Res., 56: 363-366.

Chobert JM, Haertlé T. 2003. Composition pour l'inactivation de particules virales et applications d'une telle composition. French patent # FR-03/06786.

Chobert JM, Sitohy MZ, Whitaker JR. 1987. Specific limited hydrolysis and phosphorylation of food proteins for improvement of functional and nutritional properties. J. Am. Oil Chem. Soc., 64: 1704-1711.

Chobert JM, Sitohy MZ, Whitaker JR. 1988a. Solubility and emulsifying properties of caseins modified enzymatically by *Staphylococcus aureus* V8 protease. J. Agric. Food Chem., 36: 220-224.

Chobert JM, Bertrand-Harb C, Nicolas MG. 1988b. Solubility and emulsifying properties of caseins and whey proteins modified enzymatically by trypsin. J. Agric. Food Chem., 36: 883-892.

Chobert JM, Touati A, Bertrand-Harb C, Dalgalarrondo M, Nicolas MG, Haertlé T. 1990. Ethyl substituted β-casein. Study of differences between esterified and reductively alkylated β-casein derivatives. J. Agric. Food Chem., 38: 1321-1326.

Chobert JM, Briand L, Grinberg V, Haertlé T. 1995. Impact of esterification on the folding and susceptibility to peptic proteolysis of β-lactoglobulin. Biochim. Biophys. Acta, 1248: 170-176.

Chuyen NV. 1998. Maillard reaction and food processing. Application aspects. Adv. Exp. Med. Biol., 434: 213-235.

Clare DA, Swaisgood HE. 2000. Bioactive milk peptides: a prospectus. J. Dairy Sci., 83: 1187-1195.

Cocco RR, Järvinen KM, Sampson HA, Beyer K. 2003. Mutational analysis of major, sequential IgE-binding epitopes in α_{S1}-casein, a major cow's milk allergen. J. Allergy Clin. Immunol., 112: 433-437.

Corredig M, Dalgleish DG. 1995. A differential microcalorimetric study of whey proteins and their behavior in oil water emulsions. Colloids Surface, 4: 411-422.

Dalgalarrondo M, Dufour E, Chobert JM, Bertrand-Harb C, Haertlé T. 1995. Proteolysis of β-lactoglobulin and β-casein by pepsin in ethanolic media. Int. Dairy J., 5: 1-14.

Das KP, Kinsella JE. 1990. Stability of food emulsions: physicochemical role of protein and non-protein emulsions. Adv. Food Nutrition Res., 34: 81-201.

De Man JM. (ed), 1999. Principles of Food Chemistry. Third edition, Aspen Publishers, Gaithersburgh, Maryland, USA.

Dickinson E, Matsumura Y. 1991. Time dependent polymerization of β-lactoglobulin through disulphide bonds at oil water interface in emulsions. J. Biol. Macromol., 13: 26-31.

Ehn BM, Allmere T, Telemo E, Bengtsson U, Ekstrand B. 2005. Modification of IgE binding to β-lactoglobulin by fermentation and proteolysis of cow's milk. J. Agric. Food Chem., 53: 3743-3748.

Einarsson H. 1987a. The effect of time, temperature, pH and reactants on the formation of antibacterial compound in the Maillard reaction. Lebensm.-Wiss. u.-Technol., 20: 51-55.

Einarsson H. 1987b. The effect of pH and temperature on the antibacterial effect of Maillard reaction products. Lebensm.-Wiss. u.-Technol., 20: 56-58.

Einarsson H, Snygg BG, Eriksson C. 1983. Inhibition of bacterial growth by Maillard reaction products. J. Agric. Food Chem., 31: 1043-1047.

El-Ghaish S, Dalgalarrondo M, Choiset Y, Sitohy M, Ivanova I, Haertlé T, Chobert JM. 2010a. Screening of strains of lactococci isolated from Egyptian dairy products for their proteolytic activity. Food Chem., 120: 758-764.

El-Ghaish S, Dalgalarrondo M, Choiset Y, Sitohy M, Ivanova I, Haertlé T, Chobert JM. 2010b. Characterization of a new isolate of *Lactobacillus fermentum* IFO 3956 from Egyptian Ras cheese with proteolytic activity. Eur. Food Res. Technol., 230: 635-643.

El-Ghaish S, Rabesona H, Choiset Y, Sitohy M, Haertlé T, Chobert JM. 2011. Proteolysis by *Lactobacillus fermentum* IFO3956 isolated from Egyptian milk products decreases α_{S1}-casein immuno-reactivity. J. Dairy Res., 78: 203-210.

Ellis GP. 1959. The Maillard Reaction. Adv. Carbohydr. Chem., 14: 63-134.

El Mecherfi KE, Saidi D, Kheroua O, Boudraa G, Touhami M, Rouaud O, Curet S, Choiset Y, Rabesona H, Chobert JM, Haertlé T. 2011. Combined microwave and enzymatic treatments of β-lactoglobulin and bovine whey proteins and their effect on the IgE immunoreactivity. Eur. Food Res. Technol., 233, 859-867.

Feeney RE, Whitaker JR. (eds) 1977. Food Proteins: Improvement through Chemical and Enzymatic Modification. Advances in Chemistry Series 160; American Chemical Society, Washington, DC.

Feeney RE, Whitaker JR. (eds) 1982. Modification of Proteins: Food, Nutritional, and Pharmacological Aspects. Advances in Chemistry Series 198; American Chemical Society, Washington, DC.

Feeney RE, Whitaker JR. (eds) 1986. Protein Tailoring for Food and Medical Uses. Marcel Dekker, New York.

Fiat AM, Jollès P. 1989. Caseins of various origins and biologically active casein peptides and oligosaccharides: structural and physiological aspects. Mol. Cell. Biochem. 87: 5-30.

Fiat AM, Migliore-Samour D, Jollès P, Drouet L, Sollier CB, Caen J. 1993. Biologically active peptides from milk with emphasis on two examples concerning antithrombotic and immunomodulating activities. J. Dairy Sci., 76: 301-310.

Fira D, Kojic M, Banina A, Spasojevic I, Strahinic I, Topisirovic L. 2001. Characterization of cell envelope-associated proteinases of thermophilic lactobacilli. J. Appl. Microbiol., 90: 123–130.

Fogliano V, Monti SM, Visconti A, Ritieni A. 1998. Identification of a β-lactoglobulin lactosylation site. Biochim. Biophys. Acta, 1388: 295-304.

Fraenkel-Conrat H, Olcott HS. 1945. Esterification of proteins with alcohols of low molecular weights. J. Biol. Chem., 161: 259-268.

Friedman M. 1996. Food browning and its prevention: an overview. J. Agric. Food Chem., 44: 631-653.

Gaudin JC, Rabesona H, Choiset Y, Yeretssian G, Chobert JM, Sakanyan V, Drouet M, Haertlé T. 2008. Assessment of the IgE-mediated immune response to milk-specific proteins in allergic patients using microarrays. Clin. Exp. Allergy, 38: 686-693.

Gobetti M, Stepaniak L, de Angelis M, Corsetti A, di Cagno R. 2002. Latent bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. Crit. Rev. Food Sci. Nutr., 42: 223-239.

INVITED REVIEW

Godovac-Zimmermann J, Conti A, Sheil M, Napolitano L. 1990a. Covalent structure of the minor monomeric β-lactoglobulin II component from donkey milk. Biol. Chem. Hoppe-Seyler, 371: 871-879.

Godovac-Zimmermann J, Krause I, Buchberger J, Weiss G, Klostermeyer H. 1990b. A novel wild-type β-lactoglobulin W and its primary sequence. Biol. Chem. Hoppe-Seyler, 371: 255-260.

Haertlé T, Chobert JM. 1999. Recent progress in processing of dairy proteins: a review. J. Food Biochem., 23: 367-407.

Halpin MI, Richardson T. 1985. Elected functionality changes of β-lactoglobulin upon esterification of side chain carboxyl groups. J. Dairy Sci., 68: 3189-3198.

Hammami R, Fliss I. 2010. Current trends in antimicrobial agent research: chemo- and bioinformatics approaches. Drug discovery today, 15: 540-546.

Hammami R, Zouhir A, Le Lay C, Ben Hamida J, Fliss I. 2010. BACTIBASE second release: a database and tool platform for bacteriocin characterization. BMC Microbiology, 10: 22.

Haque ZU, Mozaffar Z. 1992. Casein hydrolysate. II. Functional properties of peptides. Food Hydrocoll., 5: 559-571.

Hattori M, Ogino A, Nakai H, Takahashi K. 1997. Functional improvement of β-lactoglobulin by conjugating with alginate lyase-lysate. J. Agric. Food Chem., 45: 703-708.

Hooker PH, Munro PA, O'Meara GM. 1982. Reduction of the viscosity of sodium caseinate solutions by enzymatic hydrolysis. N. Z. J. Dairy Sci. Technol., 17: 35-40.

Huang XL, Catignani GL, Swaisgood HE. 1994. Comparison of the size and rate of formation of peptides released by limited proteolysis of β-lactoglobulins A and B with immobilized trypsin. J. Agric. Food Chem., 42, 1281-1284.

Huang XL, Catignani GL, Swaisgood HE. 1996. Improved emulsifying properties of the β-barrel domain peptides obtained by membrane-fractionation of a limited tryptic hydrolysate of β-lactoglobulin. J. Agric. Food Chem., 44: 3437-3443.

Huang XL, Catignani GL, Swaisgood HE. 1999. Modification of the rheological properties of whey protein isolates by limited proteolysis. Nahrung, 43: 79-85.

Huffman LM. 1996 Processing whey protein for use as a food ingredient. Food Technol., 50: 49-52.

Hunt JA, Dalgleish DG. 1994. Effect of pH on the stability and surface composition in emulsions made with whey protein isolate. J. Agric. Food Chem., 42: 2131-2135.

Jedrychowski L, Wróblewska B. 1999. Reduction of the antigenicity of whey proteins by lactic acid fermentation. Food Agric. Immunol., 11: 91-99.

Jing H, Kitts DD. 2000. Comparison of the antioxidative and cytotoxic properties of glucose-lysine and fructose-lysine Maillard reaction. Food Res. Int., 33: 509-516.

Jollès P, Levy-Toledano S, Fiat AM, Soria C, Gillesen D, Thomaidis A, Dunn FW, Caen, J. 1986. Analogy between fibrinogen and casein: effect of an undecapeptide isolated from κ-casein on platelet function. Eur. J. Biochem., 158: 379-384.

Jones AD, Tier CM, Wilkins JPG. 1998. Analysis of the Maillard reaction products of β-lactoglobulin and lactose in skimmed milk powder by capillary electrophoresis and electrospray mass spectrometry. J. Chromatogr., 822: 147-154.

Jones FS, Simms HS. 1930. The bacterial growth inhibitor (lactenin) of milk. J. Exp. Med., 51: 327-339.

Kim SY, Park PSW, Rhee KC. 1990. Functional properties of proteolytic enzyme modified soy protein isolate. J. Agric. Food Chem., 38: 651-656.

Kinsella JE, Whitehead DM. 1989. Protein in whey: chemical, physical and functional properties. Adv. Food Nutr. Res., 33: 343-438.

Kitabatake N, Cuq JL, Cheftel JC. 1985. Covalent binding of glycosyl residues to β-lactoglobulin: effects on solubility and heat stability. J. Agric. Food Chem., 33: 125-130.

Kitts DD, Wu CH, Stich HF, Powrie WD. 1993. Effect of glucose-lysine Maillard reaction product on bacterial and mammalian cell mutagenesis. J. Agric. Food Chem., 41: 2353-2358.

Kleber N, Weyrich U, Hinrichs J. 2006. Screening for lactic acid bacteria with potential to reduce antigenic response of β-lactoglobulin in bovine skim milk and sweet whey. Innov. Food Sci. Emerging Technol., 7: 233-238.

Klemaszewski JL, Das KP, Kinsella, JE. 1992. Formation and covalence stability of emulsions stabilized by different milk proteins. J. Food Sci., 57: 366-371.

Lahov E, Regelson W. 1996. Antibacterial and immunostimulating casein-derived substances from milk: casecidin, isracidin peptides. Food Chem. Toxicol., 34: 131-145.

Le Denmat M, Anton M, Beaumal V, Gandemer G. 1999. Characterization of the interface of oil-in-water emulsions prepared with hen egg yolk at different pH and NaCl concentrations. Proceedings VIII European Symposium on the Quality of Eggs and Eggs Products, pp. 325-332. Bologna, Italy.

Ledl F, Schleicher E. 1990. New aspects of the Maillard reaction in foods and in the human body. Angewandte Chemie International (Edition in English) 29: 565-594.

Léonil J, Mollé D, Fauquant J, Maubois JL, Pearce RJ, Bouhallab S. 1997. Characterization by ionization mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. J. Dairy Sci., 80: 2270-2281.

Loukas L, Varoucha D, Zioudrou C, Straty RA, Klee, WA. 1983. Opioid activities and structures of alpha-casein-derived exorphins. Biochemistry, 22: 4567-4573.

Maeno M, Yamamoto N, Takano T. 1996. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. J. Dairy Sci., 79: 1316-1321.

Mahmoud MI, Malone WT, Cordle CT. 1992. Enzymatic hydrolysis of casein: effect of degree of hydrolysis on antigenicity and physical properties. J. Food Sci., 57: 1223-1229.

Maillard LC. 1912. Action des amines sur les sucres: formation des mélanoïdines par voie méthodique. C. R. Acad. Sci., 154: 66-68.

Maruyama S, Nakagomi K, Tomizuka N, Suzuki H. 1985. Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. Agric. Biol. Chem., 49: 1405-1409.

Mattarella NL, Creamer KL, Richardson T. 1983. Amidation or esterification of bovine β-lactoglobulin to form positively charged proteins. J. Agric. Food Chem., 31: 968-972.

Mattarella NL, Richardson T. 1983. Physicochemical and functional properties of positively charged derivatives of bovine β-lactoglobulin. J. Agric. Food Chem., 31: 972-978.

McCrae CH, Law AJR, Leaver J. 1999. Emulsification properties of whey proteins in their natural environment: effect of whey protein concentration at 4 and 18% milk fat. Food Hydrocoll.,

INVITED REVIEW

13: 389-399.

McGookin BJ, Augustin MA. 1991. Antioxidant activity of casein and Maillard reaction product from casein-sugar mixtures. J. Dairy Res., 58: 313-320.

Means GE, Feeney RE. 1971. Chemical Modification of Proteins. Holden Day, San Francisco.

Meisel H, Schlimme E. 1990. Milk proteins: precursors of bioactive peptides. Trends Food Sci. Tech., 1: 41-43.

Meisel H, Schlimme E. 1994. Inhibitors of angiotensin I-converting enzyme derived from bovine casein (casokinins). - In β-Casomorphins and Related Peptides: Recent Developments. Brantl, V. and Teschemacher H. (eds.). VCH-Weinheim, Germany, pp. 27-33.

Mietsch F, Feher J, Halasz A. 1989. Investigation of functional properties of partially hydrolyzed proteins. Nahrung, 33: 9-15.

Migliore-Samour D, Floch F, Jollès P. 1989. Biologically active casein peptides implicated in immunomodulation. J. Dairy Res., 56: 357-362.

Mine Y. 1998. Emulsifying characterization of hen's egg yolk proteins in oil-in-water emulsions. Food Hydrocoll., 12: 409-415.

Mitchell J, Irons L, Palmer GJ. 1970. A study of the spread and adsorbed films of milk proteins. Biochim. Biophys. Acta, 200: 138-150.

Miyauchi H, Hashimoto S, Nakajima M, Shinoda I, Fukuwatari Y, Hayasawa H. 1998. Bovine lactoferrin stimulates the phagocytic activity of human neutrophiles: identification of its active domain. Cell. Immunol., 187: 34-37.

Mohri M, Matsushita S. 1984. Improvement of water absorption of soybean protein by treatment with bromelain. J. Agric. Food Chem., 32: 486-490.

Monahan FR, McClements DJ, Kinsella JE. 1993. Polymerisation of whey protein-stabilised emulsions. J. Agric. Food Chem., 41: 1826-1829.

Morens DM, Pallansch MA. 1995. Epidemiology of human enterovirus infections. –In: Rotbart HA. (ed.), Human Enterovirus Infections. Washington, DC: American Society for Microbiology, pp. 3-23.

Morgan F, Léonil J, Mollé D, Bouhallab S. 1997. Nonenzymatic lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. Biochem. Biophys. Res. Commun., 236: 413-417.

Morgan F, Léonil J, Mollé D, Bouhallab S. 1999a. Modification of bovine β-lactoglobulin by glycation in a powdered state or in an aqueous solution: effect on association behavior and protein conformation. J. Agric. Food Chem., 47: 83-91.

Morgan F, Mollé D, Henry G, Vénien A, Léonil J, Peltre G, Levieux D, Maubois JL, Bouhallab S. 1999b. Glycation of bovine β-lactoglobulin: effect on the protein structure. Int. J. Food Sci. Technol., 34: 429-435.

Morgan F, Vénien A, Bouhallab S, Mollé D, Léonil J, Peltre G, Levieux D. 1999c. Modification of bovine β-lactoglobulin by glycation in a powdered state or in aqueous solution: immunological characterization. J. Agric. Food Chem., 47: 4543-4548.

Mullally MM, Meisel H, FitzGerald RJ. 1996. Synthetic peptides corresponding to alpha-lactalbumin and beta-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity. Biol. Chem. Hoppe Seyler, 377: 259-260.

Mullally MM, Meisel H, FitzGerald RJ. 1997. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin. FEBS Lett., 402: 99-101.

Nacka F, Chobert JM, Burova T, Léonil J, Haertlé T. 1988. Induction of new physicochemical and functional properties by the glycosylation of whey proteins. J. Prot. Chem., 17: 495-503.

Nagasawa K, Takhashi K, Hattori M. 1996. Improved emulsifying properties of β-lactoglobulin by conjugating with carboxymethyl dextran. Food Hydrocoll., 10: 63-67.

Nakamura S, Kato A, Kobayashi K. 1991. New antimicrobial characteristics of lysozyme-dextran conjugate. J. Agric. Food Chem., 39: 647-650.

Neumann G, Noda T, Kawaoka Y. 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature, 459: 931-939.

O'Brien J, Morrissey PA. 1989. Nutritional and toxicological aspects of the Maillard browning reaction in foods. Crit. Rev. Food Sci. Nutr., 28: 211-248.

Ochirkhuyag B, Chobert JM, Dalgalarrondo M, Choiset Y, Haertlé T. 1998. Characterization of whey protein from Mongolian yak, khainak, and bactrian camel. J. Food Biochem., 22: 105-124.

Pearce KN, Kinsella JE. 1978. Emulsifying properties of proteins: evaluation of a turbidimetric technique. J. Agric. Food Chem., 26: 716-723.

Peiris JSM, Yu WC, Leung CW, Cheung, CY, Ng WF, Nicholls, JM, Ng TK, Chan KH, Lai ST, Lim WL, Yuen KY, Guan Y. 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. The Lancet, 363: 617-619.

Peñas E, Préstamo G, Baeza ML, Martínez-Molero MI, Gomez R. 2006. Effect of combined high pressure and enzymatic treatment on the hydrolysis and immuno-reactivity of dairy whey proteins. Int. Dairy J., 16: 831−839.

Pescuma M, Hébert EM, Dalgalarrondo M, Haertlé T, Mozzi F, Chobert JM, Font de Valdez G. 2009. Effect of exopolysaccharides on hydrolysis of β-lactoglobulin by *Lactobacillus acidophilus* CRL 636 in an *in vitro* gastric/pancreatic system. J. Agric. Food Chem., 57: 5571−5577.

Pescuma M, Hébert EM, Mozzi F, Font de Valdez G. 2010. Functional fermented whey-based beverage using lactic acid bacteria. Int. J. Food Microbiol., 141: 73−81.

Pescuma M, Hébert EM, Rabesona H, Drouet M, Choiset Y, Haertlé T, Mozzi F, Font de Valdez G, Chobert JM. 2011. Proteolytic action of *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 reduces antigenic response to bovine β-lactoglobulin. Food Chem., 127: 487-492.

Piotto SP, Sessa L, Concilio S, Iannelli P. 2012. YADAMP: yet another database of antimicrobial peptides. Int. J. Antimicrobial Agents, 39: 346-351.

Popineau Y, Pineau F. 1993. Emulsifying properties of wheat gliadins and gliadin peptides. –In: Schwenke KD. & Mothes R. (eds). Food Proteins. Structure and Functionality. VCH, Weinheim, pp. 290-296.

Puski G. 1975. Modification of functional properties of soy proteins by proteolytic enzyme treatment. Cereal Chem., 52: 655-664.

Qian ZY, Jollès P, Migliore-Samour D, Fiat AM. 1995a. Isolation and characterization of sheep lactoferrin, an inhibitor of platelet aggregation and comparison with human lactoferrin. Biochim. Biophys. Acta, 1243: 25-32.

INVITED REVIEW

Qian ZY, Jollès P, Migliore-Samour D, Schoentgen F, Fiat AM. 1995b. Sheep kappa-casein peptides inhibit platelet aggregation. Biochim. Biophys. Acta, 1244: 411-417.

Restani P, Ballabio C, di Lorenzo C, Tripodi S, Fiocchi A. 2009. Molecular aspects of milk allergens and their role in clinical events. Anal. Bioanal. Chem., 395: 47-56.

Sakai K, Ogawa M, Takashima H, Mizunuma T, Manabe S. 1997. Peptic digestibility of proteins in different heat-treated commercial cow's milks. J. Jap. Soc. Food Sci. Techn., 45: 330-333.

Sato K, Nakamura M, Nishiya T, Kawanari M, Nakajima I. 1995. Preparation of a gel of partially heat-denatured whey protein by proteolytic digestion. Milchwissenschaft, 50: 389-392.

Sawyer L, Holt C. 1993. The secondary structure of milk proteins and their biological functions. J. Dairy Sci., 76: 3062-3078.

Schlimme E, Meisel H. 1995. Bioactive peptides derived from milk proteins. Structural, physiological, and analytical aspects. Die Nahrung, 39: 1-20.

Seebah S, Suresh A, Zhuo SW, Choong YH, Chua H, Chuon D, Beuerman R, Verma C. 2007. Defensins knowledge base: a manually curated database and information source focused on the defensins family of antimicrobial peptides. Nucleic Acids Res., 35: D265-D268.

Seshadri Sundararajan V, Gabere MN, Pretorius A, Adam S, Christoffels A, Lehvaslaiho M, Archer JAC, Bajic VB. 2012. DAMPD: a manually curated antimicrobial peptide database. Nucleic Acids Res., 40: D1108-D1112.

Shida K, Makino K, Morishita A, Takamizawa K, Hachimura S, Ametani A, Sato T, Kumagai Y, Habu *S*, Kaminogawa *S*. 1998. *Lactobacillus casei* inhibits antigen-induced IgE secretion through regulation of cytokine production in murine splenocyte cultures. Int. Arch. Allergy Immunol., 115: 278-287.

Shimizu M, Kamiya T, Yamauchi K. 1981. The adsorption of whey proteins on the surface of emulsified fat. Agric. Biol. Chem., 45: 2491-2496.

Shimizu M, Saito M, Yamauchi K. 1985. Emulsifying and structural properties of β-lactoglobulins at different pHs. Agric. Biol. Chem., 49: 189-194.

Shin K, Yamauchi K, Teraguchi S, Hayasawa H, Tomita M, Otsuka Y, Yamazaki S. 1998. Antibacterial activity of bovine lactoferrin and its peptides against enterohaemorrhagic *Escherichia coli* 0157:H7. Lett. Appl. Microbiol., 6: 407-411.

Singh AM, Dalgleish DG. 1998. The emulsifying properties of hydrolysates of whey proteins. J. Dairy Sci., 81: 918-924.

Sitohy M, Chobert JM, Haertlé T. 1995a. Physico-chemical characterization of monomeric chemically phosphorylated caseins. J. Food Biochem., 19, 97-108.

Sitohy M, Chobert JM, Haertlé T. 1995b. Amino acid grafting on β-lactoglobulin mediated with phosphorus oxychloride. Int. J. Biol. Macromol., 5: 269-272.

Sitohy M, Chobert JM, Haertlé T. 1995c. Phosphorylation of β-lactoglobulin under mild condition. J. Agric. Food Chem., 43: 59-62.

Sitohy M, Chobert JM, Haertlé T. 2000. Study of factors influencing protein esterification using β-lactoglobulin as a model. J. Food Biochem., 24: 381-398.

Sitohy M, Chobert JM, Haertlé T. 2001a. Simplified short-time method for the esterification of milk proteins. Milchwissenschaft, 56: 127-131.

Sitohy M, Chobert JM, Haertlé T. 2001b. Improvement of solubility

and of emulsifying properties of milk proteins at acid pHs by esterification. Nahrung, 45: 87-93.

Sitohy M, Chobert JM, Dalgalarrondo M, Haertlé T. 2001c. Factors influencing pepsinolysis of methyl-, ethyl- and propyl-ester derivatives of β-lactoglobulin. J. Food Biochem., 25: 181-198.

Sitohy M, Billaudel S, Haertlé T, Chobert JM. 2007. Antiviral activity of esterified α-lactalbumin and β-lactoglobulin against *herpes simplex* virus type 1. Comparison with the effect of acyclovir and L-polylysines. J. Agric. Food Chem., 55: 10214-10220.

Sitohy M, Dalgalarrondo M, Nowoczin M, Besse B, Billaudel S, Haertlé T, Chobert JM. 2008. The effect of bovine whey proteins on the ability of poliovirus and Coxsackie virus to infect Vero cells cultures. Int. Dairy J., 18: 658-668.

Sitohy M, Scanu M, Besse B, Mollat C, Billaudel S, Haertlé T, Chobert JM. 2010. Influenza virus A subtype H1N1 is inhibited by esterified β-lactoglobulin. J. Dairy Res., 77: 411-418.

Slattery H, FitzGerald RJ. 1998. Functional properties and bitterness of sodium caseinate hydrolysates prepared with a *Bacillus* proteinase. J. Food Sci., 63: 418-422.

Smithers GW, Ballard FJ, Copeland AD, de Silva KJ. 1996. Symposium: Advances in dairy foods proceeding and engineering. New opportunities from the isolation and utilization of whey proteins. J. Dairy Sci., 79: 1454-1459.

Smyth M, FitzGerald RJ. 1998. Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. Int. Dairy J., 8: 819-827.

Stecchini ML, Giavedoni P, Sarais I, Lerici CR. 1993. Antimicrobial activity of Maillard reaction products against *Aeromonas hydrophilia*. Ital. J. Food Sci., 2: 147-150.

Steijns JM. 2001. Milk ingredients as neutraceuticals. Int. J. Dairy Technol., 54: 81-88.

Taheri-Kafrani A, Gaudin JC, Rabesona H, Nioi C, Agarwal D, Drouet M, Chobert JM, Bordbar AK, Haertlé T. 2009. Effects of heating and glycation of β-lactoglobulin on its recognition by IgE of sera from cow milk allergy patients. J. Agric. Food Chem., 57: 4974-4982.

Tani F, Iio K, Chiba H, Yoshikawa M. 1990. Isolation and characterization of opioid antagonist peptides derived from human lactoferrin. Agric. Biol. Chem., 54: 1803-1810.

Tani F, Shiota A, Chiba H, Yoshikawa M. 1994. Serophin, an opioid peptide derived from serum albumin. -In: Brantl V. & Teschemacher H. (eds). β-Casomorphins and Related Peptides: Recent Developments. VCH-Weinheim, Germany, pp. 49-53.

Thomas S, Karnik S, Barai RS, Jayaraman VK, Idicula-Thomas S. 2010. CAMP: a useful resource for research on antimicrobial peptides. Nucleic Acids Res., 38: D774-D780.

Tomita M, Bellamy WR, Takase M, Yamauchi K, Wakabayashi H, Kawase K. 1991. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. J. Dairy Sci., 74: 4137-4142.

Turgeon SL, Gautier SF, Paquin P. 1992. Emulsifying properties of whey peptide fractions as a function of pH and ionic strength. J. Food Sci., 57: 601-604, 634.

Vagnarelli P, de Sario A, Cuzzoni MT, Mazza P, de Carli L. 1991. Cytotoxic and clastogenic activity of ribose-lysine browning model system. J. Agric. Food Chem., 39: 2237-2239.

Vieths S, Reindl J, Mueller U, Hoffmann A, Haustein D. 1999. Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure. Eur. Food Res. Techn., 209: 379-388.

Wang GS, Li X, Wang Z. 2009. APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res., 37: D933-D937.

Waniska RD, Kinsella JE. 1988. Foaming and emulsifying properties of glycosylated β-lactoglobulin. Food Hydrocoll., 2: 439-449.

Whitmore L, Wallace BA. 2004. The Peptaiboldatabase: a database for sequences and structures of naturally occurring peptaibols. Nucleic Acids Res., 32: D593-D594.

Wilcox PE. 1967. Esterification. Methods Enzymol., 11: 605-617.

Woodruff JF. 1980. Viral myocarditis: a review. Am. J. Pathol., 101: 425-483.

Wróblewska B, Jedrychowski L, Bielecka M. 1995. The effect of selected microorganisms on the presence of immunoreactive fractions in cow and goat milks. Polish J. Food Nutr. Sci., 4/45(3): 21-28.

Yamamoto N, Akino A, Takano T. 1994. Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. J. Dairy Sci., 77: 917-922.

Yamauchi K, Schimizu M, Kamiya T. 1980. Emulsifying properties of whey proteins. J. Food Sci., 45: 1237-1242.

Yen GC, Hsieh PP. 1995. Antioxidative activity and scavenging effects on native oxygen of xylose-lysine Maillard reaction products. J. Sci. Food Agric., 67: 415-420.

Yoshikawa M, Tani F, Ashikaga T, Yoshimura T, Chiba H. 1986a. Purification and characterization of an opioid antagonist from a peptide digest of bovine κ-casein. Agric. Biol. Chem., 50: 2951-2954.

Yoshikawa M, Tani F, Yoshimura T, Chiba H. 1986b. Opioid peptides from milk proteins. Agric. Biol. Chem., 50: 2419-2421.

Yoshikawa M, Tani F, Shiota H, Suganuma H, Usui H, Kurahashi K, Chiba H. 1994. Casoxin D, an opioid antagonist ileum-contracting/vasorelaxing peptide derived from human α_{S1}-casein. − In: Brantl V. & Teschemacher H. (eds). β-Casomorphins and Related Peptides: Recent Developments. VCH-Weinheim, Germany, pp. 43-48.

Zucht HD, Raida M, Adermann K, Mägert HJ, Forssmann WG. 1995. Casocidin-I: a casein-α_{S2} derived peptide exhibits antibacterial activity. FEBS Lett., 372: 185-188.