MILK PROTEIN MODIFICATION TO IMPROVE FUNCTIONAL AND BIOLOGICAL PROPERTIES

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I. INTRODUCTION

The actual and potential use of milk proteins as food ingredients has been a popular topic for research over the past 30 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

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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 (EA). Reviews on classical food protein modifications for improved functionality are available in the literature (Means and Feeney, 1971; Feeney and 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, which 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, enzymatic and genetic methods that modify dairy proteins with an emphasis on current developments.

II. 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.

A. PHOSPHORYLATION

1. Reaction conditions

Phosphorylation is an effective way to increase negative charges in a protein molecule and thereby to improve functionality, particularly solubility. Either O- or N-esterification reactions can transfer inorganic phosphate (P_i)

to proteins. In an O-esterification reaction, P_i reacts with the primary or secondary hydroxyl on seryl or threonyl residues, respectively; or with the weakly acidic hydroxyl on tyrosyl residue, forming a –C–O–Pi bond. In N-esterification, P_i combines with the ε-amino group of lysyl residue, the

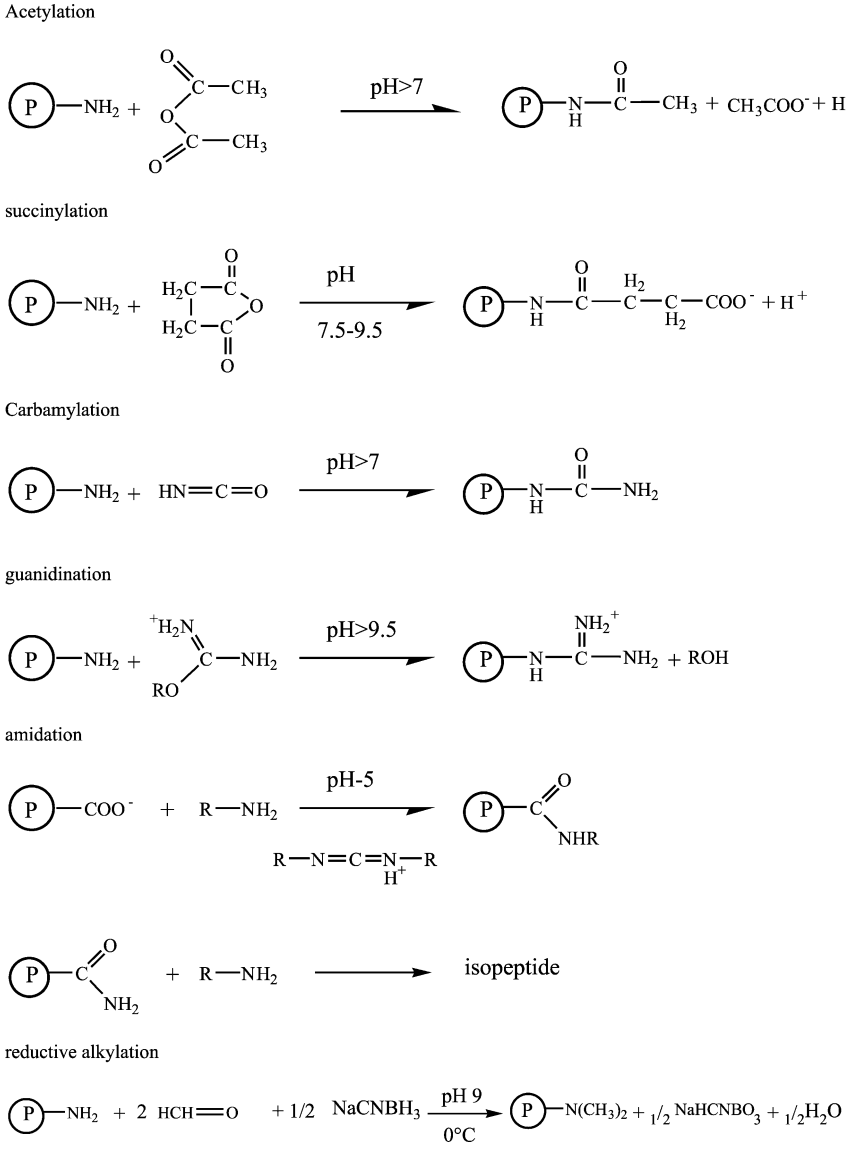
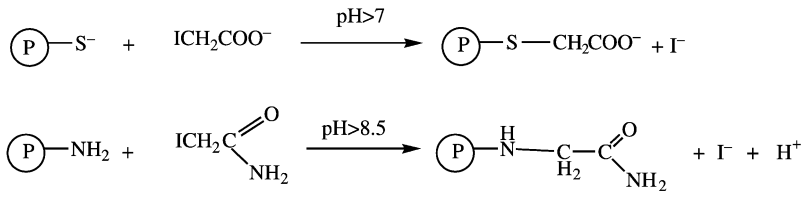
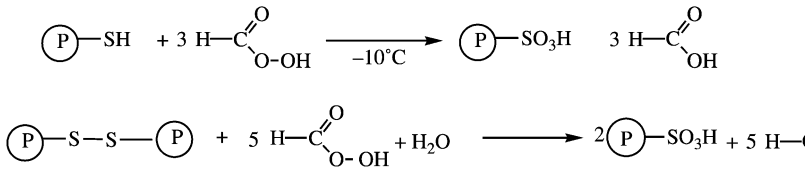


FIG. 1. Main chemical modifications of food proteins.

Carboxymethylation



Oxidation



Esterification

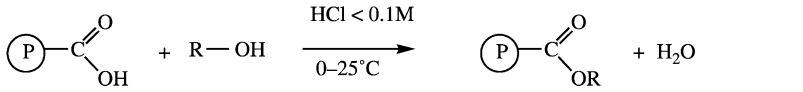


FIG 1. (continued)

imidazole group of histidyl residue, or the guanidino group or arginyl residue, forming a $-C-N-P_i$ bond. The nitrogen-bound phosphates are acid labile and are readily hydrolyzed at pH values at or below 7. Proteins containing oxygen-bound phosphate are acid stable and are the modification of choice for food proteins since the pH of most food systems is 3–7 (Shih, 1992).

Enzymatic phosphorylation by phosphorylases and phosphatases produces phosphoesters such as phosphoserine and phosphothreonine. Chemical phosphorylation of proteins changes their functional properties, improving them sometimes (Yoshikawa *et al.*, 1981; Hirotsuka *et al.*, 1984; Huang and Kinsella, 1986; Chobert *et al.*, 1989; Matheis, 1991). However, the properties of the phosphorylated proteins depend entirely on the degree of denaturation and substitution defined by the reaction conditions and the protein (Medina *et al.*, 1992; Sitohy *et al.*, 1994). Casein was phosphorylated by the commonly used methods, characterized by use of excessive amounts of phosphorus oxychloride and with important additions of concentrated inorganic bases (Matheis *et al.*, 1983; Medina *et al.*, 1992). Thus, obtained phosphorylated caseins were highly cross-linked and partially insoluble and difficult to characterize. Hence, there arose a need to produce monomeric over-phosphorylated caseins more suitable for use and for study of their

functional properties. The monomeric forms are more hydrophilic, and easier to study and used as additives. It was found that the outcome of phosphorylation can be directed by the reaction conditions either towards the formation of polymeric or predominately monomeric phosphoproteins (Sitohy *et al.*, 1994).

Whole casein, which originally contained 6 mol P/mol protein, bound an additional 4, 7 and 11 mol P/mol protein when prepared with 25, 50 and 100 mol $POCl_3$/mol protein in the presence of triethylamine (Sitohy *et al.*, 1995a). α_s-, β- and κ-casein fractions containing 7, 4 and 1 mol P/mol protein, respectively, bound an additional 21, 20 and 9 mol P/mol protein when reacted with 100 mol $POCl_3$/mol protein. The relatively lower extent of phosphorylation achieved for the whole casein, as compared to α_s- or β-casein fractions, might be due to the complexing effect between casein components making some fragments of the protein molecule inaccessible for the reacting reagents. Alternatively, the relatively poor extent of κ-casein phosphorylation might be due to its hydrophobic nature (Kato and Nakai, 1980). Caseins with such properties were obtained not only by use of low molar ratios of $POCl_3$ (25–100 mol $POCl_3$/mol protein) but also due to the presence of triethylamine allowing the reaction to proceed with such low $POCl_3$ molar ratios. The phosphorylation yields obtained in these conditions (low $POCl_3$/protein molar ratios; 25–100 in the presence of triethylamine) were higher than those obtained by Matheis *et al.* (1983) and even higher in some cases than those obtained by Medina *et al.* (1992) who used extremely high $POCl_3$/protein molar ratios (1000–2000) in the presence of an inorganic base.

The SDS-PAGE patterns of the same samples showed small intermolecular associations in the phosphorylated caseins especially when compared with the results obtained by Matheis *et al.* (1983) and Medina *et al.* (1992) whose phosphorylated samples were entirely unable to enter the SDS-PAGE gel due to high cross-linking.

The possibility of using basic amino acids in the form of free bases as the only base of the reaction was studied in order to eliminate the use of tertiary amines, which are nutritionally unacceptable (Sitohy *et al.*, 1995b,c; Haertlé and Chobert, 1999).

The extent of phosphorylation was proportional to the applied $POCl_3$/protein molar ratios. Phosphoamidation was proportional to the basic amino acid/$POCl_3$ molar ratio, which is in agreement with the previously observed importance of triethylamine acting as the proton scavenger (Sitohy *et al.*, 1994). The highest phosphorylation yield was observed for 80 mol $POCl_3$/mol protein and 6 mol lysine or arginine acid/mol $POCl_3$. However, the highest phosphorylation achieved varied according to the (basic) amino acid used. This difference is clearly due to the different nucleophilicity of the three

amino acids used. The p*K* of the lateral groups of arginine, lysine and histidine are 12.48, 10.53 and 6.0, respectively. Hence, the pH of the starting reaction media using L-arginine, L-lysine and L-histidine in the form of free bases were 10.8, 9.7 and 7.6, respectively. It is well known that protonated primary amines are unreactive. Thus, basic pH is essential for the rapid formation of the phosphoamide bond. The obtained phosphorylation yields might seem low but they may be acceptable for food purposes and they are close to the highest phosphorylation (6 mol P/mol casein) reported by Matheis *et al.* (1983) using 2000 mol $POCl_3$/mol protein. The secondary phosphoamide bond formation depends on the initial substitution of protein with $-POCl_2$ and $=POCl$ showing higher yields for arginine than lysine and confirming the role of activated phosphate groups in secondary grafting of the amino acids on β-lactoglobulin. The use of arginine or lysine may be advised as a reaction base for low phosphorylation yield. The secondary grafting of the basic amino acid used for the reaction improved this method of eliminating the unacceptable quaternary amines (triethylamine). When other amino acids such as leucine, methionine, threonine or tryptophan were added (at a molar ratio of 2 mol/mol $POCl_3$) in addition to the basic amino acid (molar ratio of 5 mol/mol $POCl_3$) to the reaction medium (80 mol $POCl_3$/mol protein), the phosphorylation yield was reduced to 2 mol P/mol protein. The arginine and lysine grafting yields were reduced to 2 mol amino acid/mol protein and the added amino acids did not form significant amounts of secondary phosphoamide bonds. This indicates the importance of the appropriate buffering by arginine and lysine conferred by the basicity of their lateral side-chains.

2. *Effect of phosphorylation of casein on its functional properties*

The pH–solubility curves presented in Figure 2 generally show that phosphorylated whole casein has greater solubility than native casein in neutral and basic pHs resulting from the displacement of the isoelectric point towards acidic values. The gradual displacement of the isoelectric points towards the acidic side demonstrates the gradual increase in the negative charges with increasing phosphorylation yield. However, highly phosphorylated proteins are not resolubilized below their isoelectric point indicating some cross-linking. The solubility curves of the phosphorylated casein fractions (α_s, β and κ) show the same dependence of solubility profiles pointing to the monomeric state of the modified proteins since the polymeric form is poorly soluble and shows diffuse changes in the isoelectric points as previously found by Medina *et al.* (1992).

The emulsifying activity index (EAI) curves for phosphorylated whole casein solution versus pH showed shift of their minima towards the acid

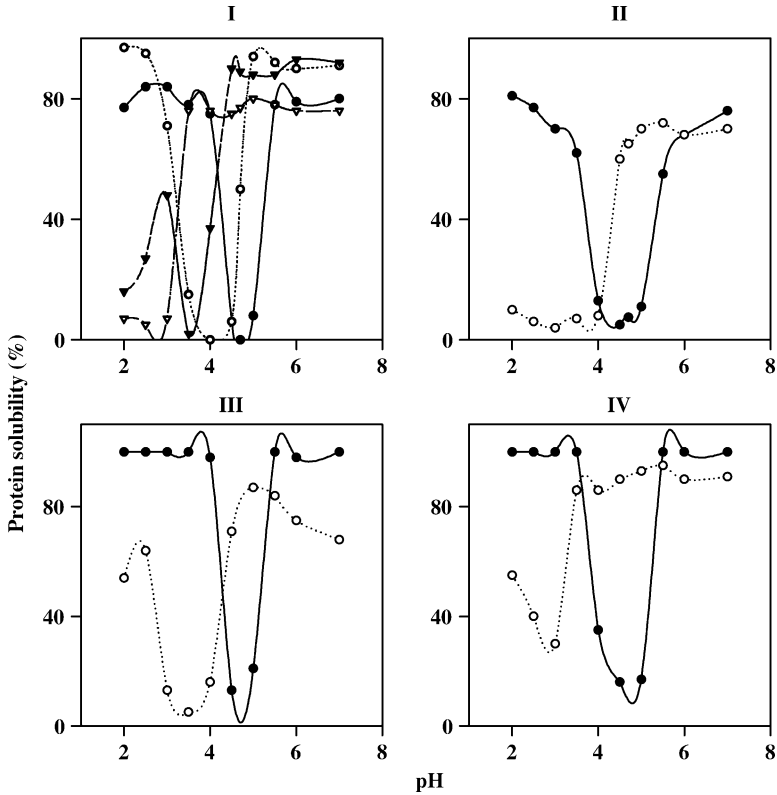


FIG. 2. pH–solubility curves of phosphorylated caseins. I, whole native (•) and phosphorylated caseins [4 (○), 7 (◆) and 11 (▽) mol P/mol protein]. II, III and IV are α_s-, β- and κ-casein in the native (•) and phosphorylated (○) forms. (*Source*: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.)

values (Figure 3). It was proportional to the extent of phosphorylation and to the shift of isoelectric point. EAI of the phosphorylated whole casein was lower in the pH range 6–7 than that of the native whole casein. This is quite visible in the case of the highly phosphorylated derivatives and was due to significant hydrophilicity increase. Near the isoelectric point of the native whole casein, EAI of the phosphorylated whole casein was higher than that of the native caseins. EAI decreased in the acidic pH range 2–4, which is close to the isoelectric points of the modified proteins. All the phosphorylated whole casein samples showed high stability at the pH range 3–6, and lower stability at the pH range 6–7, when compared to the native sample. Generally, all the phosphorylated caseins behave similarly, with phosphorylated κ-casein showing the biggest improvement of emulsifying properties.

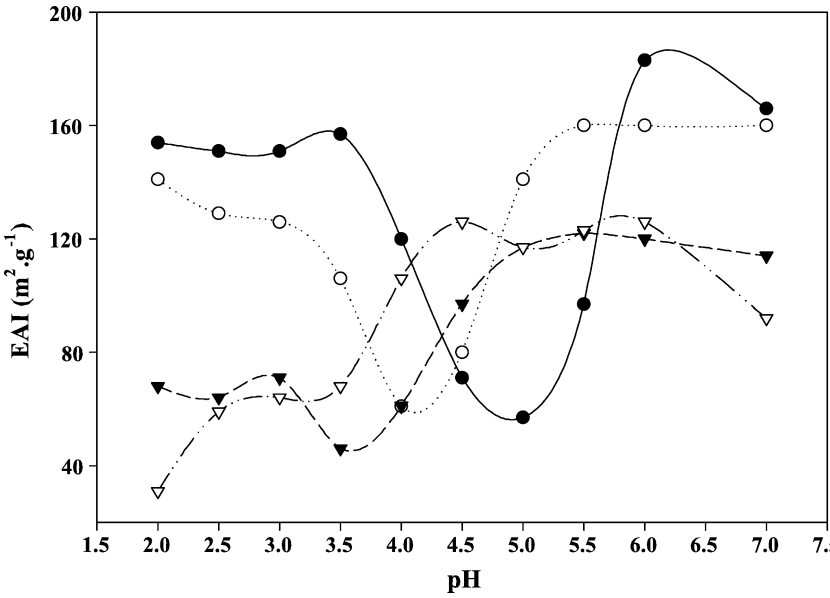


FIG. 3. Emulsifying activity index of native (●) and phosphorylated whole caseins [4 (○); 7 (◆) and 11 (▽) mol P/mol protein]. (*Source*: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.)

Since the emulsifying properties depend on the balance between hydrophobicity and hydrophilicity at the protein surface, a very high yield of phosphorylation may disrupt this balance. The higher improvement of emulsifying properties of κ-casein was due to its original high hydrophobicity, which diminished after phosphorylation.

Table I shows that the foaming properties of whole casein improved by slight phosphorylation. The lowest phosphorylated form of casein (4 mol P/mol protein) showed higher foam hydration and stability than the native whole casein. However, the highly phosphorylated whole casein (11 mol P/mol protein) showed poor foaming properties. The foam hydration of α_s-casein deteriorated while that of κ-casein improved by phosphorylation. This discrepancy seemed to be caused by a different initial hydrophobic/hydrophilic balance of the proteins in their native states. However, foam stabilities of all casein fractions were reduced by phosphorylation, with κ-casein being only slightly affected.

The highest phosphorylated samples of β-lactoglobulin in the presence of arginine and lysine showed EAI of 254 and 229 m^2/g, respectively, which represented an improvement as compared to EAI of native

TABLE I

FOAMING PROPERTIES OF NATIVE AND PHOSPHORYLATED CASEINS

Protein sample	1[a] TF(s)	2 FV max	3 LV max	4 FV 20 min	5 LV 20 min	6 HL (s)
Native whole casein	201	50	3.7 ± 0.05	30.6 ± 0.08	0.71 ± 0.05	460 ± 15
Phosphocaseins:						
(4 mol P/mol protein)	201	50	4.25 ± 0.04	36.5 ± 0.11	0.78 ± 0.07	471 ± 10
(7 mol P/mol protein)	204	50	4.11 ± 0.05	34.1 ± 0.15	0.71 ± 0.04	460 ± 11
(11 mol P/mol protein)	218	50	2.75 ± 0.02	15 ± 0.09	0.24 ± 0.02	453 ± 12
Native α-casein	206	50	6.75 ± 0.06	41.3 ± 0.09	1.63 ± 0.05	556 ± 14
Phospho α-casein	212	50	5.2 ± 0.05	21.2 ± 0.08	0.79 ± 0.07	421 ± 13
Native β-casein	203	50	6.14 ± 0.09	47 ± 0.21	1.72 ± 0.04	552 ± 11
Phospho β-casein	206	50	6.24 ± 0.16	34.7 ± 0.34	1.1 ± 0.05	476 ± 5
Native κ-casein	206	50	4.42 ± 0.04	48.7 ± 0.15	1.33 ± 0.11	475 ± 16
Phospho κ-casein	205	50	5.65 ± 0.09	45.1 ± 0.13	1.1 ± 0.12	445 ± 14

Source: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.

[a]1: Time of foaming (s); 2: Maximum foam volume (ml); 3: Maximum liquid volume in foam; 4: Foam volume after 20 min; 5: Liquid volume in foam after 20 min; 6: Half-life of foam (s).

β-lactoglobulin (160 m^2/g). The stability of emulsions prepared with phosphorylated β-lactoglobulin was also higher than the stability of emulsions prepared with native β-lactoglobulin. Emulsion prepared with phosphoarginyl β-lactoglobulin amide exhibited the highest stability. Such improvement in the emulsifying properties of the phosphorylated proteins agrees well with the results reported by Sung *et al.* (1983), Hirotsuka *et al.* (1984), and Huang and Kinsella (1987). According to these authors, the improvement in emulsifying properties could be explained in light of the increased negative charges on the phosphorylated proteins, which tends to cause electrostatic repulsion between the negatively charged emulsion faces, reducing the flocculation and coalescence of the emulsions. Consequently, a significant improvement of emulsifying properties can be achieved with moderate phosphorylation.

B. ESTERIFICATION

The esterification reaction is an important tool for modifying food proteins. Esterification with different alcohols leads to the blocking of free carboxyl groups thus raising the net positive charge, making the modified proteins more basic (Wilcox, 1967; Mattarella *et al.*, 1983; Halpin and 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 (Briand *et al.*, 1995). While β-lactoglobulin is resistant to peptic hydrolysis in aqueous and physiological conditions, an ethylated β-lactoglobulin derivative was highly susceptible to pepsin hydrolysis in aqueous conditions. The esterified β-lactoglobulin 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 β-lactoglobulin was performed in hydro-ethanolic conditions (Dalgalarrondo *et al.*, 1995). Hence, the reaction might be a good tool for increasing 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 physico-chemical and functional properties (Mattarella and Richardson, 1983; Halpin and Richardson, 1985; Chobert *et al.*, 1990).

Since the initial work of Fraenkel-Conrat and 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 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 and 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 deesterification (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 β-lactoglobulin 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 β-lactoglobulin (3% protein concentration) dispersed in 95% ethanol in the presence of 0.7N HCl. The extent of esterification increased gradually with the length of reaction as 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, 0.7N HCl final concentration) were carried out by esterifying β-lactoglobulin 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 may 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 presence of water. Water is required for the reaction at proportions depending on the type of alcohol (ethanol, methanol, and *n*-propanol) used. Water is required to dissociate hydrochloric acid supplying

the protons needed for the activation of the carboxyl groups during the esterification reaction. Water in the medium may also be used to form a hydration layer around the protein molecules, which helps us to organize the hydrophobic moieties in the globulin core (Tanford, 1980), thus making the carboxyl groups more accessible for esterification. The importance of water for the esterification reaction was indirectly considered by Halpin and Richardson (1985) who used 95% ethanol to esterify β-lactoglobulin while using anhydrous methanol for the same purpose.

d. Influence of the type of alcohols. The comparison of different alcohols used for esterification showed that methanol is the most reactive and can esterify most of the carboxyl groups of β-lactoglobulin, 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 also depends on its quantity contained in the added acid.

The difference between reactivity of alcohols agrees with the results of Fraenkel-Conrat and Olcott (1945) and Halpin and Richardson (1985) who found that methanol was most reactive as an esterifying agent followed by ethanol and then *n*-butanol. Mattarella *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 isopropanol.

e. Effect of the protein concentration. The effect of the protein concentration was investigated using different conditions. Esterification was performed for 24 h at 4°C in 95% ethanol and 0.7N HCl. β-Lactoglobulin was added at 2, 3, 4 and 5% (Table II). Increase of protein concentration from 2 to 3% slightly enhanced the extent of esterification. However, a further increase till 4 and 5% had an adresse effect on the reaction, resulting in poorer extents of esterification. During esterification, carboxyl groups of the protein should first be activated with protons before reacting with the alcohol. Consequently, the most important factor for the reaction is not the protein concentration *per se*, but the concentration of activated carboxyl groups. Taking into account that acid concentration was equal in all preparations with different protein concentrations, it can be concluded that the ratio H^+/COOH became lower when the protein concentration was increased up to 4 or 5%. Hence, with higher protein concentrations, the reactivity of carboxyl groups was lower and, consequently, the extent of esterification was reduced. In this series of preparations, the initial H^+/COOH ratio was equal to 24 when using 2% protein and 0.7N HCl,

TABLE II

EXTENT OF ESTERIFICATION OF β-LACTOGLOBULIN WITH 95% ETHANOL AS INFLUENCED BY PROTEIN AND HYDROCHLORIC ACID CONCENTRATIONS IN THE REACTION MEDIUM AT 4 °C

Acid normality	0.7N	1.4N		1.75N	
Time (h)	24	14	24	14	24
Protein concentration (%)		Extent of esterification (%)			
2	36 ± 0.71				
3	38 ± 0.87				
4	21 ± 1.04	44 ± 0.46	51 ± 1.10		
5	21 ± 0.75			45 ± 1.01	57 ± 0.80

Source: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.

decreasing to 12 when switching to 4% protein and 0.7N HCl. In the latter case, the activated carboxyl groups available for the reaction are expected to be less numerous and, consequently, the relatively lower extent of esterification is justified.

In another series of experiments, the H^+/COOH ratio was kept a constant (24 mol H^+/mol COOH group, as in the case of a 2% preparation) during the esterification of 4 and 5% β-lactoglobulin. Results in Table II show that protein concentration in such conditions is an essential factor for the reaction. The extent of β-lactoglobulin esterification in 95% ethanol was remarkably enhanced up to 51% after 24 h reaction when using 4% protein. Increasing the protein concentration up to 5% with the same H^+/COOH molar ratio equal to 24, further increased the extent of esterification up to 57%. Even by decreasing the reaction time to 14 h, protein was more esterified when present at 4 or 5%, compared to that obtained after 24 h with 2% protein. Consequently, protein concentration is an important driving force for the reaction if supplied in the form of activated carboxyl groups. This factor can not only maximize the esterification yield but also shorten the time required to attain this maximum.

2. *Application to α-lactalbumin*

α-Lactalbumin (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). α-Lactalbumin was more resistant to esterification than β-lactoglobulin 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 α-lactalbumin, which may be better protected than β-lactoglobulin against the nucleophilic attack by the alcohols. α-Lactalbumin may be subject to conformational changes in acidic 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 α-lactalbumin, either the reaction time should be prolonged or the molar ratio of HCl should be increased.

SDS-PAGE patterns of esterified α-lactalbumin 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*

Extent of esterification of β-casein increased gradually parallel to the increase in the molar ratio of HCl (Sitohy *et al.*, 2001a). As observed with β-lactoglobulin, methanol achieved the highest rates of esterification. However, propanol was more efficient than ethanol, which was the opposite of the results obtained with β-lactoglobulin. 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% β-casein using a 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 β-casein 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 β-lactoglobulin.

In order to examine the effectiveness of the third important factor playing a role in esterification yield, β-casein (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 β-lactoglobulin*

Esterification generally enhances the susceptibility of proteins to peptic action (Briand *et al.*, 1995; Chobert *et al.*, 1995). This was most evident with

β-lactoglobulin 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) independent of the substrate protein concentration. Hence, esterification can be a good method for improving the peptic digestibility of pepsin-resistant proteins, such as β-lactoglobulin 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 III.B.). Tricine-SDS PAGE of peptic hydrolysates of esterified β-lactoglobulin 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.*, 2001e). 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 β-lactoglobulin (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 β-lactoglobulin 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 β-lactoglobulins differed from those of native β-lactoglobulin. 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 β-lactoglobulin 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. Peptic hydrolysis of ester derivatives of β-casein and α-lactalbumin

In a recent work (Sitohy *et al.*, 2001b) 100% methyl-, 59% ethyl- and 56% propyl-esters of β-casein, and 52% methyl-, 36% ethyl- and 25% propyl-esters of α-lactalbumin were prepared. The degree of pepsinolysis (% DH) was enhanced considerably after esterification. Methyl esters of both proteins yielded the highest levels of DH. Compared to SDS-PAGE of peptic hydrolysates of native proteins, those of esterified β-caseins demonstrated the

disappearance of the bands corresponding to peptides of medium and high molecular masses. SDS-PAGE of peptic hydrolysates of esterified α-lactalbumin showed the disappearance of the bands corresponding to peptides of low molecular masses. Compared with native protein, RP-HPLC profiles of peptic hydrolysates of esterified β-casein showed more hydrophobic peptides. The major changes in RP-HPLC of peptic hydrolysates of esterified α-lactalbumin concerned peptides eluted lately (hydrophobic), while the distribution of peptides eluted early (hydrophilic) remained constant.

6. *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, β-casein 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 β-lactoglobulin to fat globules increases with increased pH, exhibiting good EA in the alkaline pH range (Yamauchi *et al.*, 1980; Nagasawa *et al.*, 1996; Hattori *et al.*, 1997). Adsorption of β-lactoglobulin 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 β-lactoglobulin molecules are more rigid at acid pH (Shimizu *et al.*, 1981, 1985; McCrae *et al.*, 1999). Recently, it was confirmed that pH influences the interfacial composition and stability of emulsions prepared from egg yolk proteins (Le Denmat *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. EA also depends 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 and Dalgleish, 1994). When β-lactoglobulin adsorbs at an oil–water interface, it denatures partially exposing its highly reactive free sulfhydryl groups (Corredig and Dalgleish, 1995), which can then interact with similar groups of neighboring molecules forming polymers. The second major whey protein, α-lactalbumin, was found to inhibit β-lactoglobulin polymerization (Dickinson and 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 and 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 needed. Esterification of proteins was proved to block the protein negative charges thus increasing 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 β-lactoglobulin 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 and Richardson, 1983; Halpin and Richardson, 1985). Similar features were observed during esterification of β-casein (Chobert *et al.*, 1990). The magnitude of 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 β-lactoglobulin 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 β-lactoglobulins were more efficient in lowering the interfacial tension at the oil–water interface as a result of increased surface hydrophobicity (Halpin and Richardson, 1985). It was also reported that EA of esterified β-lactoglobulins is worse than that of the native form (Mattarella and 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 EA of esterified proteins was followed by measurement of turbidity according to the method of Pearce and Kinsella (1978). Since the oil droplet size may better reflect EA, Sitohy *et al.* (2001c) measured the change of this parameter in order to elucidate the changes in EA 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 and Mine, 1999; Le Denmat *et al.*, 1999).

Three milk proteins (β-lactoglobulin, α-lactalbumin and β-casein) were esterified to different extents with methanol, ethanol and propanol; then their solubility was studied in the pH range 3–10. Solubility of β-lactoglobulin esters depends on the degree of esterification as well as on the nature of the grafted ester groups. Samples of highly esterified β-lactoglobulin (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 (Figure 4). Consequently, two opposite effects of esterification on the solubility of

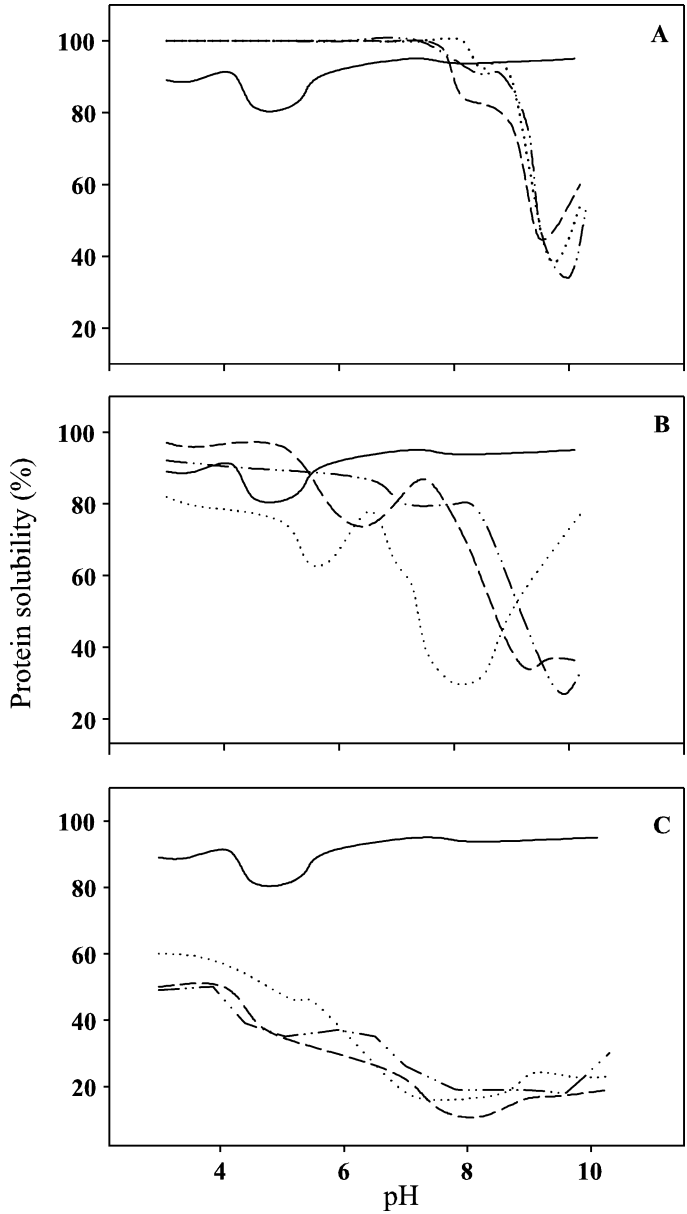


FIG. 4. Solubility profiles of β-lactoglobulin esterified to different extents with [A]: 0% (—), 85% (· · · · · ·), 94% (–··–) and 99% (– –) methanol; [B]: 0% (—), 44% (· · · · · ·), 49% (–··–) and 73% (– –) ethanol; [C]: 0% (—), 34% (· · · · · ·), 39% (–··–) and 49% (– –) propanol. (*Source*: From Sitohy *et al.* (2001c), by courtesy of Wiley-VCH Verlag, GmbH.)

β-lactoglobulin 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 and Richardson, 1983; Chobert *et al.*, 1990).

α-Lactalbumin 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 (Figure 5). 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 α-lactalbumin sample (36%) showed improved solubility in acid conditions and decreased solubility in the alkaline range of pH value. The solubility of the low ethylated derivative (11%) was improved whatever the pH. The solubility curves of α-lactalbumin propyl esters were similar to that of ethyl derivatives. However, the reduction of solubility of propyl esterified derivatives was more pronounced when compared to α-lactalbumin ethyl esters showing solubility levels of 47–50% and 67–70%, respectively. As observed with β-lactoglobulin ester derivatives, the nature of the grafted ester group is an important factor for the solubility of the modified α-lactalbumin. Comparison of solubility of α-lactalbumin propyl esters with that of β-lactoglobulin propyl esters shows a different behavior for these two families of esters. While grafted propyl ester groups decrease the solubility of β-lactoglobulin in the acidic pH range, they improve the solubility of α-lactalbumin. 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 β-lactoglobulin and α-lactalbumin, the isoelectric points of esterified β-casein shifted towards the alkaline pH range. The magnitude of this shift was proportional to the extent of esterification. β-Casein methyl esters (Figure 6A) 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 β-casein methyl esters, giving a minimum of solubility as low as 20% for the highly esterified samples (88 and 100% esterified). The minimum

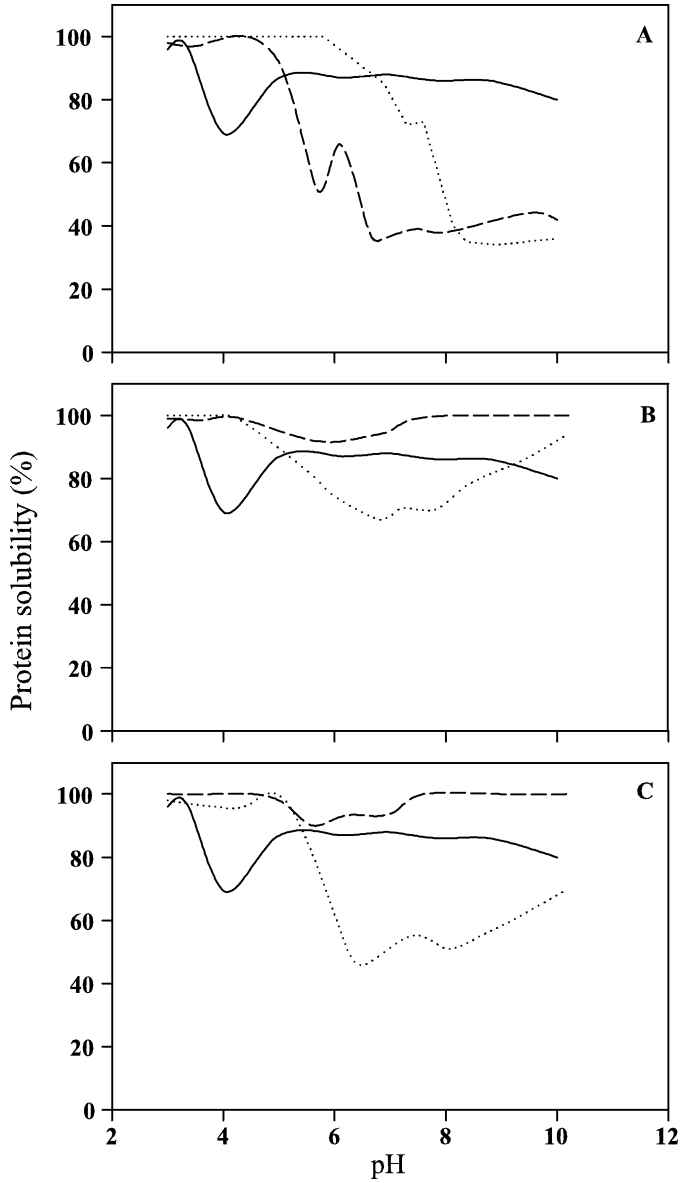


FIG. 5. Solubility profiles of α-lactalbumin esterified to different extents with [A]: 0% (—), 38% (– –) and 52% (· · · · · ·) methanol; [B]: 0% (—), 11% (– –) and 36% (· · · · · ·) ethanol; [C]: 0% (—), 8% (– –) and 24% (· · · · · ·) propanol. (*Source*: From Sitohy *et al.* (2001c), by courtesy of Wiley-VCH Verlag, GmbH.)

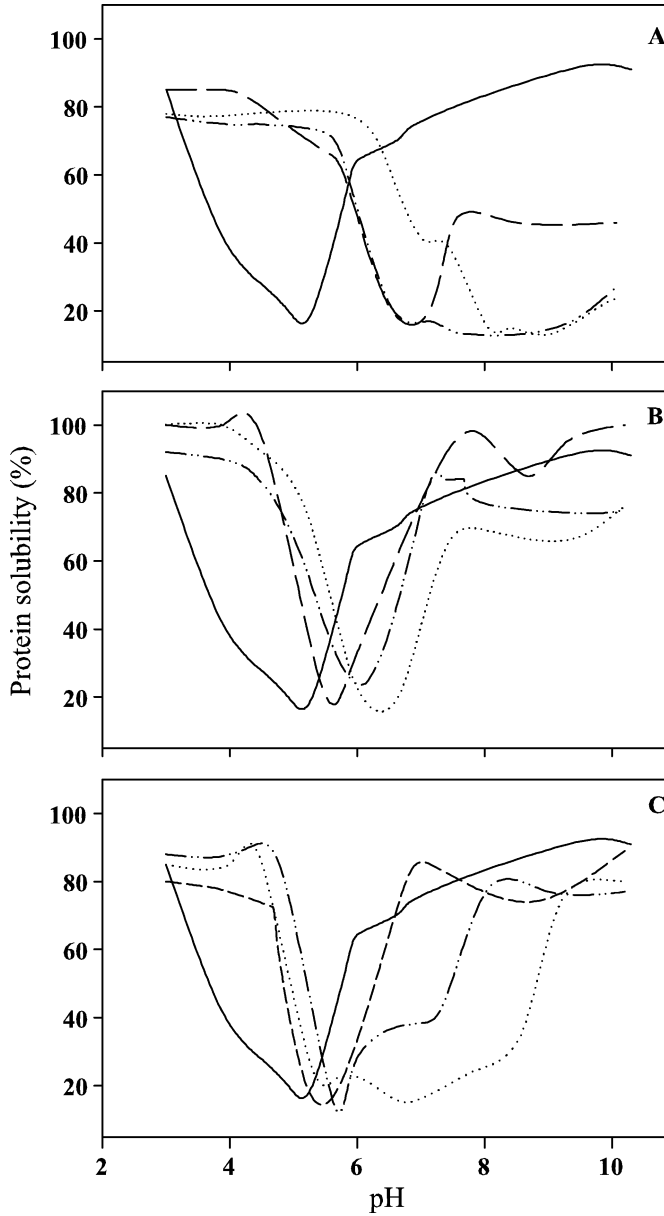


FIG. 6. Solubility profiles of β-casein esterified to different extents with [A]: 0% (—), 66% (– –), 88% (–··–) and 100% (·····) methanol; [B]: 0% (—), 17% (– –), 33% (–··–) and 59% (·····) ethanol; [C]: 0% (—), 26% (– –), 38% (–··–) and 56% (·····) propanol. (*Source*: From Sitohy *et al.* (2001c), by courtesy of Wiley-VCH Verlag, GmbH.)

of solubility of β-casein esters is mainly observed for one pH value or one pH range in contrast to two minima previously observed with β-lactoglobulin and α-lactalbumin. This may be because the flexible structure of β-casein makes the alcoholic groups more accessible to nucleophilic attack, giving rise to more homogeneous esterified populations. The hydrophobic core of β-lactoglobulin 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 β-casein shows a more important shift of isoelectric point for the methyl derivative due to the higher extent of modification. Moreover, the solubility of β-casein 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 β-casein displayed similar extents of substitution (~20 to ~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 (see Section III.C.).

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: β-lactoglobulin > α-lactalbumin > β-casein.

Emulsifying properties of esterified milk proteins were also studied in the pH range 3–7 by measuring the oil droplet size using laser light scattering (Table III). EA and stability of esterified milk proteins in the acidic pH range 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 EA improvement was observed for methyl ester derivatives at pH 5 where the native proteins have poor emulsifying properties (Sitohy *et al.*, 2001c).

7. *Change in biological properties*

a. Formation of complexes between DNA and esterified dairy proteins. As with many natural basic proteins such as histones and lysozymes, plasmid DNA can interact with methylated α-lactalbumin and methylated β-lactoglobulin forming complexes (Sitohy *et al.*, 2001d). The stability of the complex was tested at different pH, temperatures and salt concentration, and after enzyme digestion with DNase I and pepsin. Incubation at 37°C for long

TABLE III

EMULSIFYING ACTIVITY AND STABILITY OF NATIVE AND ESTERIFIED MILK PROTEINS MEASURED BY OIL DROPLET DIAMETER, DIRECTLY AFTER EMULSIFICATION OR AFTER A TWO-WEEK STORAGE AT 4 °C USING 4 MG/ML PROTEIN CONTENT AND 15% SUNFLOWER OIL

pH	Emulsifying activity (oil droplet diameter, μm)				Emulsifying stability (oil droplet diameter, μm)			
	Native	Methyl	Ethyl	Propyl	Native	Methyl	Ethyl	Propyl
β-Lactoglobulin								
4	1.37 ± 0.06	1.17 ± 0.04	1.13 ± 0.08	1.13 ± 0.04	1.40 ± 0.03	1.24 ± 0.03	1.40 ± 0.05	1.30 ± 0.03
5	1.64 ± 0.08	1.18 ± 0.06	1.15 ± 0.06	1.32 ± 0.03	1.93 ± 0.05	1.33 ± 0.03	1.34 ± 0.04	1.41 ± 0.04
7	0.97 ± 0.06	1.20 ± 0.04	1.25 ± 0.08	1.46 ± 0.06	1.02 ± 0.02	1.30 ± 0.05	1.48 ± 0.06	1.61 ± 0.05
α-Lactalbumin								
4	0.94 ± 0.02	1.11 ± 0.04	0.95 ± 0.07	1.21 ± 0.03	0.88 ± 0.02	1.07 ± 0.02	0.97 ± 0.02	1.22 ± 0.02
5	1.21 ± 0.03	0.92 ± 0.07	1.03 ± 0.06	1.24 ± 0.04	1.15 ± 0.03	0.93 ± 0.02	0.95 ± 0.02	1.21 ± 0.02
7	0.71 ± 0.02	1.06 ± 0.06	1.64 ± 0.05	1.67 ± 0.06	0.67 ± 0.01	1.07 ± 0.02	1.60 ± 0.04	1.67 ± 0.05
β-Casein								
4	U	1.13 ± 0.06	1.21 ± 0.01	1.35 ± 0.06	U	1.20 ± 0.02	1.29 ± 0.03	1.21 ± 0.02
5	U	1.25 ± 0.05	1.54 ± 0.05	1.85 ± 0.05	U	1.21 ± 0.02	1.89 ± 0.03	1.85 ± 0.11
7	0.96 ± 0.01	U	U	U	0.96 ± 0.01	U	U	U

U: Unmeasurable; *Source*: From Sitohy *et al.* (2001c), by courtesy of Wiley-VCH.

periods (up to 24 h) allowed the interaction of DNA with low concentrations of esterified proteins to take place. High temperature treatment (100°C) for short periods of time enhanced complex formation after 5 min of heating in case of both DNA-methylated α-lactalbumin and DNA-methylated β-lactoglobulin. The DNA-methylated β-lactoglobulin complex was more stable than the DNA-methylated α-lactalbumin complex when the thermal treatment at 100°C was extended to 10 min. Both complexes of DNA with methylated α-lactalbumin and DNA with methylated β-lactoglobulin were formed in large amounts and were more stable at acid pH (3–6). In contrast, their amounts were smaller and they were less stable at basic pH (8–9). Generally, larger amounts of stable DNA-methylated β-lactoglobulin complex were obtained at acid pH when compared with DNA-methylated α-lactalbumin complex. These complexes were still highly stable at very acidic pH (1–2) but not at very basic pH (10–11). Formation and stability of the complexes of DNA studied with esterified protein were generally salt-concentration-dependent. Magnesium chloride had the biggest inhibitory effect on the formation and stability of these complexes while potassium acetate had the smallest. The inhibitory effect of KCl on both the complex formation and stability was observed in the range 0.4–1.0 M. DNA complexed with esterified milk proteins and with lysozyme was more resistant to hydrolysis with DNase I than was unbound DNA. Additionally, DNA complexed with methylated α-lactalbumin was more resistant to DNase I digestion than its complex with methylated β-lactoglobulin or with lysozyme. All the studied proteins complexed with DNA were resistant to pepsin.

b. Inhibition of bacteriophage replication with esterified milk proteins. DNA-binding proteins can have antiviral and tumor suppressor activities. Milk proteins have already been reported to have antiviral effects, which were ascribed to lactoferrin (Hasegawa *et al.*, 1994; Harmsen *et al.*, 1995), a mammalian iron-binding protein of 80 kDa (Baker *et al.*, 1994). Human lactoferrins exhibit potent antiviral activities against cytomegalovirus (Hasegawa *et al.*, 1994; Harmsen *et al.*, 1995), HSV-1 infection (Fujihara and Hayashi, 1995; Marchetti *et al.*, 1996) and inhibit the replication of HIV-1 in a T-cell line if they are added prior to infection or during the virus adsorption step (Puddu *et al.*, 1998).

Lactoferrin-mediated protection of mice from cytomegalovirus infection was reported to be linked with T-cell-dependent increase in natural killer cell activity (Shimizu *et al.*, 1996). Lactoferrin potent antiviral inhibitory effect against HIV-1 in T-cell line was attributed to its action on HIV binding or on the entry of this virus into the cells no matter if it was in the apo or saturated form (Puddu *et al.*, 1998). Antiviral and antitumor activities are correlated to

each other. Factors that enhance protein–DNA cross-linking, e.g., the antibiotic Givocarcin V, show antiviral and antitumor activities (Morimoto *et al.*, 1981; Nakano *et al.*, 1981). RNA– and DNA–protein interactions are essential for some pathogenic viruses. Tat protein is essential for HIV viral replication and activates transcription by binding to the transactivation-responsive (TAR) site located at the 5′-end of the viral transcript. In hepatitis C virus (HCV), a ~350 nucleotide region in the 5′-end of the viral transcript is required for cap-independent translation and is vital for virus replication (Xavier *et al.*, 2000). Hence, the disruption of such interaction with external nucleic acid binding proteins can result in significant reduction of virus replication and infection.

DNA-binding properties of esterified milk proteins have been studied previously. These interactions are the result of increased net positive charge on the modified proteins caused by esterification (Sitohy *et al.*, 2001d). Hence, it was interesting to verify the possible use of esterified milk proteins in inhibiting viral replication. Since esterified milk proteins bind to DNA in a nonspecific way (Sitohy *et al.*, 2001d), this will probably endow them with broad specificity against viruses, whatever their mutated forms could be. This could be of interest since viruses adopt mutation strategies to escape immune control (Alcami and Koszinowski, 2000) and produce viral proteins that are no longer recognized by antibodies (Hill *et al.*, 1996; Zeidler *et al.*, 1997). Bacteriophage M13, which is specific for *Escherichia coli*, is a well-characterized filamentous 6407-nucleotide single-stranded bacteriophage (Van Wezenbeek *et al.*, 1980). Its structure and mechanism of replication are well known (Sambrook *et al.*, 1989; Makowski, 1994; Marvin, 1998). Hence, it can be used as a model phage for investigating the antiviral action of esterified milk proteins.

Esterified proteins showed an antiviral activity against bacteriophage M13 due to their DNA-binding properties (Sitohy, M., Chobert, J.-M., Karwowska, U., Gozdzicka-Jozefiak, A., and Haertlé, T., unpublished data). Methylated β-lactoglobulin, ethylated β-lactoglobulin and methylated α-lactalbumin formed complexes with the M13 DNA with an efficiency depending on their degree of esterification. The more the esterification extent of the protein, the higher the DNA complexing capacity and the higher its antiviral activity. The antiviral effect was associated with a simultaneous inhibitory effect on viral replication, which reinforces the previous conclusion that antiviral activity originates from interactions between esterified proteins and viral DNA. Methylated β-lactoglobulin showed even higher antiviral activity and a stronger inhibitory effect on the replication of bacteriophage M13 than native basic proteins such as calf thymus histone and chick egg lysozyme, probably as a result of the formation of a more stable DNA–protein complex in the case of esterified milk proteins. Complexes of esterified proteins with DNA

were previously found to be resistant to hydrolysis (Sitohy *et al.*, 2001d). Consequently, the formation of complexes with viral DNA will definitively exclude it from the replication system inhibiting the overall process. During replication, some regions of the viral DNA are exposed to interactions with proteins. Esterified proteins present in the viral medium can attack the viral DNA, forming complexes and stopping the replication machinery. The virus life cycle depends on the organization of its structures through the interactions with specific proteins (Johnson and Chiu, 2000). Consequently, the nonspecific interactions with esterified milk proteins may disrupt the virus life cycle through disrupting its structural organization.

C. GLYCATION OF β-LACTOGLOBULIN USING MILD CONDITIONS

The nonenzymatic 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 and 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 nonenzymatic reactions are responsible for numerous changes on food properties and may impair food safety. Although these reactions are of great importance in the 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 and Schleicher, 1990). Results of nonenzymatic 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 and Schleicher, 1990; Friedman, 1996). Through this reaction, the conjugation of 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 because they take place when a protein and a sugar are just heated together (Chuyen, 1998).

β-Lactoglobulin is a major whey protein. It is present in the milk of various ruminant species (Godovac-Zimmermann *et al.*, 1990a,b; Sawyer and Holt, 1993; Ochirkhuyag *et al.*, 1998). This protein constitutes a major waste product of the cheese industry. Only recently, its use increased as a food additive thanks to its good nutritional properties (Smithers *et al.*, 1996). Consequently, the improvement of β-lactoglobulin functional properties may be of considerable interest to industry.

To improve their functional and physico-chemical properties, dairy proteins have been modified by several methods (Haertlé and Chobert, 1999), such as phosphorylation (Sitohy *et al.*, 1995c), esterification (Chobert *et al.*, 1995; Sitohy *et al.*, 2001c), alkylation (Kitabatake *et al.*, 1985), and reductive amidation (Mattarella *et al.*, 1983); (see Section II.A., B.). However, most of these methods, using toxic chemicals, cannot be used in food processing.

β-Lactoglobulin 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 β-lactoglobulin 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 a 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 β-lactoglobulin (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 β-lactoglobulin 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 β-lactoglobulin. 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 β-lactoglobulin after heating. When β-lactoglobulin was heated in the presence of sugars, larger structural modifications were observed depending on the sugar used (Chevalier *et al.*, 2002). Conformational modification of β-lactoglobulin 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 microcalorimetry 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 β-lactoglobulin. According to previous work (Chevalier *et al.*, 2001a,c), glycation of β-lactoglobulin 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 β-lactoglobulin (Chevalier *et al.*, 2001c; see below), which demonstrated the importance of the sugar used for 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 and Lorient, 1982). According to Kinsella and 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 and Chobert, 1999). Many studies were carried out with β-lactoglobulin, 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 and Richardson, 1983; Sitohy *et al.*, 2001c), gluconic or melibionic acids (Kitabatake *et al.*, 1985), carbohydrates (Waniska and 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. Recently, some attempts were made to improve the functional properties of β-lactoglobulin by conjugation with glucose-6-phosphate (Aoki *et al.*, 1997).

In a recent work (Chevalier *et al.*, 2001d), the functional properties (solubility, heat stability, emulsifying and foaming properties) of β-lactoglobulin after glycation of the protein with several sugars (arabinose, galactose, glucose, lactose, rhamnose or ribose) were studied. 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 β-lactoglobulin was soluble over the whole pH range studied (Figure 7A). 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 (Figure 7A). After heating β-lactoglobulin in the presence of arabinose or ribose, the resulting glycated derivatives exhibited 35% solubility at pH 4 (Figure 7B), which is lower than the solubility obtained with heated β-lactoglobulin and β-lactoglobulin 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

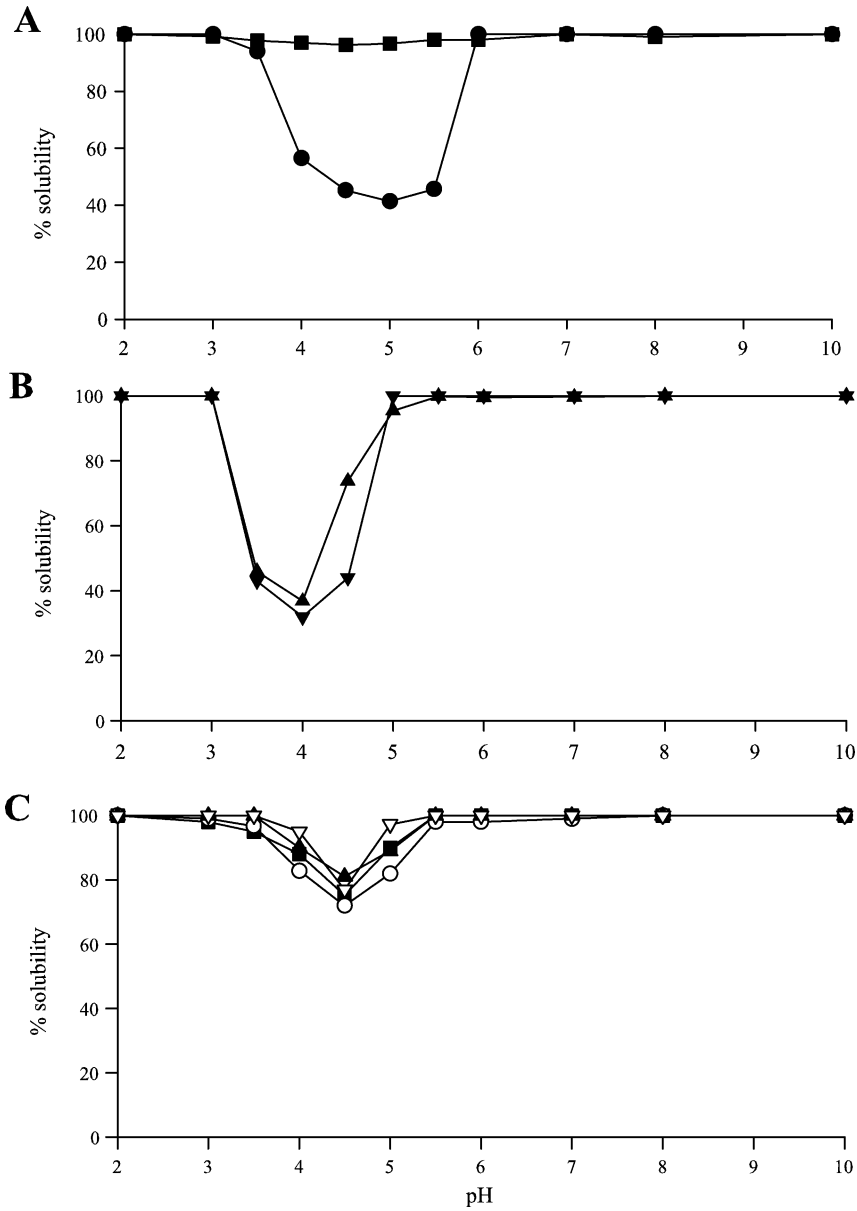


FIG. 7. Solubility of native, heated and glycated β-lactoglobulin (BLG) as a function of pH. The protein concentration was 2 mg/ml. **A**: native (■) and heated (•) BLG; **B**: BLG glycated with arabinose (▲) and ribose (▼); **C**: BLG glycated with galactose (■), glucose (○), lactose (▲) and rhamnose (▽). (Reprinted from Chevalier *et al.* (2001d), Copyright 2001, with permission from Elsevier Science.)

derivatives showed an increased solubility at pH 5 as compared with heated β-lactoglobulin. In the case of proteins modified with galactose, glucose, lactose or rhamnose, 75% solubility was observed at pH 4.5 (Figure 7C), 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 β-lactoglobulin 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 and Whitehead, 1989). Despite the fact that native β-lactoglobulin is highly soluble in the pH range 2–10, its EAI was low at acid pHs and increased with increasing pH (Shimizu *et al.*, 1985), suggesting that structural changes occur in β-lactoglobulin at acidic pH that influence its emulsifying properties. Such effects close to the pI of β-lactoglobulin could be at the origin of the decrease of emulsion stability (ES) (Klemaszewski *et al.*, 1992) observed in the $d_{3.2}$ values.

Recent studies have shown that lactosylation of β-lactoglobulin 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 β-lactoglobulin (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 β-lactoglobulin 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

The protein fraction of milk is known to contain many valuable components and biologically active substances, which confer special properties for the support of infant development and growth (Meisel and Schlimme, 1990). Many milk-born bioactivities are latent, requiring proteolytic release of bioactive peptides from inactive native proteins (Schanbacher *et al.*, 1998). Milk protein-derived bioactive peptides include a variety of substances that are potential modulators of various regulatory processes and reveal

multifunctional bioactivities (Meisel, 1998). Opioid agonist (Meisel, 1986), opioid antagonist (Tani *et al.*, 1990), inhibitor of angiotensin converting enzyme (ACE) (Yamamoto, 1997; Pihlanto-Leppälä *et al.*, 1998), immunomodulator (Brantl *et al.*, 1981), antimicrobial (Zucht *et al.*, 1995; Dionysius and Milne, 1997) and antithrombotic (Chabance *et al.*, 1995) activities have been largely described (Meisel, 1997). β-Lactoglobulin is known to contain an ACE-inhibitory sequence (Mullally *et al.*, 1997) but its biological function in milk is still not well known (Sawyer *et al.*, 1998). Some of the biological properties of milk proteins are discussed in Section III.E.

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 oxidoreductive modification in order to see whether new biological properties could be induced. The Maillard reaction is one of the major reactions modifying proteins in food and in nature. The study of how it influences the 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 on model systems, which consist of heating a single amino acid with a reducing sugar. The Maillard reaction induced: (1) antioxidative activity of glucose–glycine (Anese *et al.*, 1994), xylose–lysine (Yen and Hsieh, 1995) and xylose–arginine reacting systems (Beckel and Waller, 1983); (2) antimicrobial activity of xylose–arginine and glucose–histidine systems (Einarsson *et al.*, 1983; Einarsson, 1987a,b) and of glucose–glycine system (Stecchini *et al.*, 1993); (3) cytotoxic activity of glucose–lysine and fructose–lysine systems (Jing and Kitts, 2000); (4) 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 antimicrobial 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 and Augustin, 1991).

As shown in Section II.C.3., functional properties (such as thermal stability, emulsifying and foaming properties) of β-lactoglobulin modified by the Maillard reaction were improved, depending on the sugar used during modification. Glycated β-lactoglobulin used as a food ingredient for its functional properties may also change the oxidative conditions and stress and/or can influence cellular and microorganism growth. A recent study was carried out to determine the extent to which this reaction can convey antioxidant, antimicrobial, mutagenic or cytotoxic activities to β-lactoglobulin, and to its tryptic and peptic hydrolysates (Chevalier *et al.*, 2001b).

Antioxidant properties of β-lactoglobulin modified with six different sugars (see Section II.C.1.) were estimated using a radical scavenging activity test. Glycation induced a radical scavenging activity to β-lactoglobulin, whose intensity depended on the sugar used for modification (Figure 8). Proteins modified with ribose and arabinose showed higher radical scavenging activity, depicted by about 80 and 60% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) absorption decrease at 515 nm.

Einarsson *et al.* (1983) and Einarsson (1987a,b) observed an inhibition of bacterial growth by Maillard reaction products prepared with model systems of arginine–glucose, arginine–xylose and histidine–glucose. Antimicrobial activity has never been observed with glycated proteins except in the case of lysozyme modified with dextran by the Maillard reaction (Kitts *et al.*, 1993). Nonmodified lysozyme exhibits an inhibition of growth only for Gram-positive bacteria. Conjugation of dextran to lysozyme resulted in an extension of the antimicrobial spectrum to Gram-negative bacteria. In the study of Chevalier *et al.* (2001b), antimicrobial properties against different bacterial

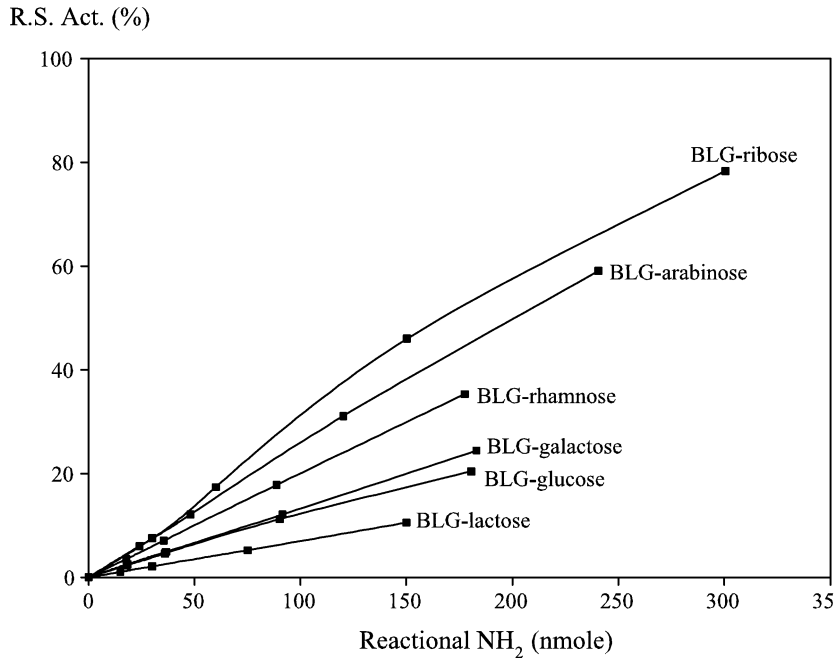


FIG. 8. Radical scavenging activity (R.S. Act.%) of β-lactoglobulin glycated with arabinose, galactose, glucose, lactose, rhamnose or ribose as a function of the number of reactional primary amino groups of β-lactoglobulin. (*Source*: From Chevalier *et al.* (2001b), by courtesy of American Chemical Society.)

strains were studied with a diffusion disk method. No antimicrobial effect of any glycated form of β-lactoglobulin against *E. coli*, *Bacillus subtilis*, *Listeria innocua* and *Streptococcus mutans* was observed. As native β-lactoglobulin did not show any antimicrobial activity, its modification by the Maillard reaction could not induce any extension of the antimicrobial spectrum. Only the appearance of new antimicrobial activity could be expected. Such an activity is certainly more difficult to induce by a nonspecific chemical modification because of the large spectrum of resistance mechanisms in bacteria.

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. Cytotoxicity test with MTT showed no enhancement of cytotoxicity by modified proteins and their peptic and tryptic peptides against COS-7 and HL-60 cells after glycation.

Maillard reactions are known to produce mutagenic, DNA-damaging and cytotoxic substances especially during food processing and cooking (O'Brien and Morrissey, 1989). This certainly concerns AMP. Heterocyclic amines are the most potent mutagens known to be formed during heat processing of food (Friedman, 1996). Such compounds should not be formed (at least in detectable quantity) in the conditions used in the study by Chevalier *et al.* (2001b) because of the mild heat treatment applied (72 h at 60°C). Consequently, in the case of products studied earlier, some innocuousness can be expected.

D. THERMAL MODIFICATIONS OF STRUCTURE AND CO-DENATURATION OF α-LACTALBUMIN AND β-LACTOGLOBULIN

Milk and dairy products are submitted to thermal treatments in order to remove undesirable microorganisms. For example, microbial agents rapidly spoil whey from the cheese industry unless it is treated or used rapidly. Currently, heating is the most commonly used pasteurization/sterilization treatment (heating at 135–150°C during 2 s). The development of new technologies should be able to solve this problem by more selective treatments such as filtration, high pressure, and magnetic or electric fields.

Although less frequently discussed, heat processes often influence the textures and chemistries of the intermediate and end products, and thermal treatments are not without consequences on milk proteins that are denatured. Denaturation of proteins occurs under precise conditions of pH, temperature and ionic strength leading to their unfolding. Denaturation is significantly slower when proteins are near their isoelectric point. Only β-lactoglobulin is irreversibly denatured at pH 7 and 70°C; α-lactalbumin is denatured at pH 6.7 and 65°C. Aggregation of these proteins, besides hydrophobic

interactions, is often stabilized by intra- and intermolecular bonds. This can be due to thiol–disulfide interchange catalyzed by free thiol groups (McKenzie, 1971) and hydrophobic or electrostatic interactions (McSwiney *et al.*, 1994). The capacity and rate by which whey proteins aggregate is controlled by factors such as protein concentration, pH, temperature and presence of other components. According to Aguilera (1995), aggregation can take place in three steps: denaturation, aggregation, and gelification. Aggregation is more rapid near the isoelectric point of proteins. For a review on the effect of thermal treatment on milk protein structure, see Cayot and Lorient (1998).

β-Lactoglobulin is the major whey protein. Hence, its molecular properties may control whey protein aggregation. β-Lactoglobulin resists denaturation at acidic pH but can be easily denatured at alkaline pH (McKenzie and Sawyer, 1967; Waissbluth and Grieger, 1974). It aggregates and is subjected to conformational changes at intermediate pHs. At pH 7.5, a reversible transition (Tanford transition; Tanford *et al.*, 1959) takes place increasing the reactivity of the free thiol group Cys 121. At alkaline pH, denaturation occurs in two steps: unfolding of α-helix and of exposed β-sheet portions followed by unfolding of other β-sheets. Thermal denaturation of β-lactoglobulin occurs in the temperature range 50–90°C with conformation changes similar to those observed during the first step of alkaline denaturation, such as unveiling of the thiol group. β-Lactoglobulin denaturation is reversible as long as its temperature is lower than 65–70°C. At neutral pH, three steps have been described by Roefs and de Kruif (1994): (1) Initiation: reversible reactions, which lead to monomeric β-lactoglobulin; then an irreversible modification of structure unveiling the free thiol group directed towards the exterior of the molecule; (2) Propagation: reactive free thiol group involved in chemical exchange with one of the two disulfide bridges of another nonreactive molecule of β-lactoglobulin. An intermolecular bridge is formed and a new free thiol group becomes reactive on the second molecule and so on; (3) End of reaction: reactive intermediates form a dimer or polymer without reactive free thiol group.

All whey proteins are not able to self-aggregate. α-Lactalbumin, that changes conformation at 64°C, is devoid of free cysteyl residues needed to initiate and propagate the exchange reaction. However, in the presence of proteins with a free thiol group (β-lactoglobulin, bovine serum albumin), α-lactalbumin may take part in aggregation (Gezimati *et al.*, 1997).

Study of heat denaturation of major whey proteins (β-lactoglobulin or α-lactalbumin) either in separated purified forms, or in forms present in fresh industrial whey or in recomposed mixture respecting whey proportions has been recently carried out (Bertrand-Harb *et al.*, 2002), indicating significant differences in their denaturation depending on pH,

temperature of heating, presence or absence of other co-denaturation partner, and existence of a previous thermal pre-treatment (industrial whey). α-Lactalbumin, usually resistant to tryptic hydrolysis, aggregated after heating at 85°C and over. After its denaturation, α-lactalbumin was susceptible to tryptic hydrolysis probably because of exposure of its previously hidden tryptic cleavage sites (Lys–X and Arg–X bonds). Heating over 85°C of β-lactoglobulin increased its aggregation and exposure of its peptic cleavage sites. The co-denaturation of α-lactalbumin with β-lactoglobulin increased their aggregation and resulted in complete exposure of β-lactoglobulin peptic cleavage sites and partial unveiling of α-lactalbumin tryptic cleavage sites. The exposure of α-lactalbumin tryptic cleavage sites was slightly enhanced when the α-lactalbumin/β-lactoglobulin mixture was heated at pH 7.5. Co-denaturation of fresh whey by heating at 95°C and pH 4.5 and above produced aggregates stabilized mostly by covalent disulfide bonds easily reduced by β-mercaptoethanol. The aggregates, stabilized by covalent bonds other than disulfide, arose from a similar thermal treatment but performed at pH 3.5. Thermal treatment of whey at pH 7.5 considerably enhanced the tryptic and peptic hydrolysis of both major proteins.

III. 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, nonspecific 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 nonenzymatic 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 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 and 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 describes 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 and Mozaffar, 1992; Agboola and Dalgleish, 1996; Slattery and FitzGerald, 1998; Smyth and FitzGerald, 1998), soybean proteins (Puski, 1975; Adler-Nissen and Olsen, 1979; Mohri and Matsushita, 1984; Adler-Nissen, 1986a; Kim *et al.*, 1990b), wheat gluten and gluten fractions (Popineau and Pineau, 1993), among others.

Hydrolysis of peptide bonds causes several changes in proteins: (1) the NH_3^+ and COO^- content of the protein increases, increasing its solubility; (2) the molecular mass of the protein decreases; and (3) 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 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, 1986a). Protamex™ (a *Bacillus* proteinase complex) hydrolysates of sodium caseinate (DH 9 and 15%) displayed 85–90% solubility between pH 4 and 5 (Slattery and 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 EA at all pH (Chobert *et al.*, 1987, 1988a), whereas the 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 decreasing hydrophobicity due to proteolysis (Mahmoud *et al.*, 1992). Since the *S. 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. While 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 and Mozaffar, 1992). Protamex hydrolysates (DH 0.5 and 1.0%) of casein showed higher EA at pH 2 as compared with unmodified casein (Slattery and 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 p*I* (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 β-lactoglobulin (Chen *et al.*, 1993, 1994; Huang *et al.*, 1994, 1996). The emulsifying properties of these peptides were two to three-fold larger than that of the native protein in the pH range 3–9. 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 β-lactoglobulin 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 β-lactoglobulin. 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 exhibit 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 and FitzGerald, 1998). Hydrophobic peptides resulting from

plasmin hydrolysis of β-casein improved foam formation and stabilization at pH 4 (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 and 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 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 cheese. During this process, chymosin hydrolyzes 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 and 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 55–77% reduction in the apparent viscosity (Hooker *et al.*, 1982).

B. ALLERGENICITY

One of the applications of proteases is to decrease the risk of allergenicity when cow's milk is used as a substitute for human milk. Human and cow's milk differ in their protein composition. β-Lactoglobulin, the major whey protein in cow's milk (9.8% of the total protein content) is absent from human milk. Hydrolyzing the protein can reduce allergenicity. Asselin *et al.* (1989) compared the allergenicity of whey protein hydrolysates produced by pepsin, chymotrypsin, trypsin, pancreatin and combinations of these proteases. DH was not the only important parameter influencing allergenicity. A hydrolysate with 20.5% DH produced with pancreatin had a higher residual content of α-lactalbumin and β-lactoglobulin as compared with a 11.2% DH chymotryptic hydrolysate. Ultrafiltration could be used to reduce the residual antigens in whey protein hydrolysates (Asselin *et al.*, 1989).

Nakamura *et al.* (1993) showed that using a combination of two nonspecific endoproteases and a DH of up to 25%, the antigenicity of whey proteins can be reduced by 1000 times. Antigenicity can be lowered further by including proteases specific for glutamyl, aspartyl, lysyl and arginyl residues, which play important roles in allergen peptides.

The potentiality of a protein to cause an allergic reaction is related to the size of the protein, its primary, secondary and tertiary structures. Antigenicity of casein hydrolysate prepared by using pancreatic proteases was studied by Mahmoud *et al.* (1992).

C. BITTER PEPTIDES

Depending on the nature of the protein and the protease used, progressive proteolysis can liberate bitter peptides from proteins, the bitterness of which is a function of amino acid composition and sequence as well as the peptide chain length (Adler-Nissen, 1986b). An excellent review of the chemistry of bitterness has been published (Roy, 1997) and the reader is directed to this for more details.

Bitterness has long been associated with the formation of hydrophobic peptides (Ney, 1971) and it has been reported (Ney and Rezzlaff, 1986) that peptides having a Q-value (calorific value of the amino acids in a peptide) greater than 1350 kcal/mol are bitter. Bitterness is also related to the DH; bitter peptides contain typically between 3 and 15 amino acids (Pawlett and Bruce, 1996). Treating casein with proteases with differing specificities results in increased bitterness with an increase in the DH. The level of bitterness is dependent on the hydrophobicity of the peptides formed, which in turn, is related to specificity. It is, therefore, possible to influence the level of bitterness by protease selection.

Although hydrolysis can result in improved functional properties and increased digestibility, the formation of bitterness usually has a negative impact on the product acceptability. A logical extension of the use of enzymes for the hydrolysis of food proteins is the use of enzymes for further hydrolysis of the bitter peptides formed. Peptidases are a class of enzymes that cleave single or a pair of amino acids from the ends of peptide chains. Exopeptidases are specific either for the N-terminal (aminopeptidases) or the C-terminal (carboxypeptidases) ends. Key to the use of peptidases in either debittering or bitter prevention restricts the DH. Peptidases have been applied to hydrolysis of α-lactalbumin and casein to produce a nonbitter and highly soluble ingredient for use in nutritional drinks and health-food products.

Among amino acids some L-amino acids are known to be bitter ones. Aromatic amino acids, basic amino acids, and branched-chain amino acids are bitter amino acids. Several bitter amino acids are essential for humans.

Therefore, when an amino acid mixture is administrated orally, the bitterness of these amino acids is crucial. According to Kirimura *et al.* (1969), γ-Glu-Phe is not bitter but sour and astringent. Improvement of the bitter taste of amino acids by γ-glutamylization and an enzymatic method for synthesizing γ-Glu-Phe from Gln and Phe involving bacterial glutamyltranspeptidase have been recently described (Suzuki *et al.*, 2002). Results indicate that γ-glutamylization of Phe can abolish its bitterness and improve the taste. According to several panel members γ-Glu-Phe has a lemon-like refreshing sourness.

Several patent applications pertaining to peptidases have highlighted the growing interest in this type of enzyme. A patent from Gist brocades (1996) demonstrated a process for producing an aminopeptidase preparation by *Aspergillus niger*. Röhm GmbH (1997) and Novo Nordisk (1998) used modern biotechnology to engineer strains of *Aspergillus*, which produce amino- and carboxy-peptidases, respectively. Both of these enzymes were shown to enhance food protein hydrolysis and help prevent the development of bitterness.

D. TRANSGLUTAMINASE AND DAIRY PRODUCTS

In nature, various enzymes are known to cross-link proteins. The physiological function of protein cross-linking is to enhance the strength of the molecular structure of proteins and protein networks. The substrate specificity of cross-linking enzymes is a key aspect in developing protein–protein cross-linking structures. Enzymes like transglutaminase and protein disulfide isomerase have a defined reaction mechanism and are therefore specific, whereas enzymes like peroxidase and lipoxygenase have a more random reaction mechanism resulting in a broad substrate specificity.

Transglutaminase (EC 2.3.2.13) catalyzes an acyl-transfer reaction in which the γ-carboxamide groups of peptide-bound glutaminyl residues are the acyl donors. The enzyme catalyzes *in vitro* cross-linking in whey proteins, soya proteins, wheat proteins, beef myosin, casein and crude actomyosin refined from mechanically deboned poultry meat (Zhu *et al.*, 1995). In recent years, based on the enzyme's reaction to gelatinize various food proteins through the formation of cross-links, this enzyme has been used in attempts to improve the functional properties of foods. Commercial transglutaminase is usually obtained from animal tissues. The complicated separation and purification procedure results in an extremely high price for the enzyme, which hampers a wide application in food processing. Recently, studies on the production of transglutaminase by microorganisms have started. The enzyme obtained from microbial fermentation has been applied in the treatment of food of different origins. Food treated with microbial transglutaminase appeared to have an improved flavor, appearance and texture. In addition,

this enzyme can increase shelf-life and reduce allergenicity of certain foods (Zhu *et al.*, 1995).

α_{s1}-Casein and soybean globulins were polymerized and gelatinized by Ca^{2+}-independent transglutaminase from a variant of *Streptoverticillium mobaraense*, which is totally independent of Ca^{2+}. In this aspect, microbial transglutaminase is quite unique from other mammalian enzymes. Such a property is very useful in the modification of functional properties of food proteins, because many food proteins, such as milk caseins, soybean globulins and myosins, are susceptible to Ca^{2+} (Nonaka *et al.*, 1989). This enzyme polymerized such albumins as bovine serum albumin, human serum albumin and conalbumin in the presence of dithiotreitol. Rabbit myosin was polymerized by the present enzyme but actin was not. An RP-HPLC analysis after enzymatic digestion of the polymerized α_{s1}-casein showed existence of the ϵ-(γ-Glu)Lys bond. Thus, it was confirmed that the polymerization was formed by a catalytic reaction of the transglutaminase.

Several suspensions and emulsions containing commercial sodium caseinate or skim milk were gelatinized by microbial transglutaminase treatment. The characteristics of the gels were largely affected by the enzyme concentrations employed (Nonaka *et al.*, 1992). For caseinate gels, generally the higher enzyme concentration gave steep decreases in breaking strength, strain and cohesiveness of the gels. The creep tests on emulsified gels prepared with two different enzyme concentrations showed that the gel made with a higher enzyme concentration was more viscoelastic. For skim-milk gels, the enzyme caused a substantial increase of the breaking and hardness, while the strain and cohesiveness showed little or no changes.

Microbial transglutaminase is capable of incorporating amino acids or peptides covalently into proteins (Nonaka *et al.*, 1996). This reaction can improve the nutritive values of food or feed proteins, because covalently incorporated amino acids or peptides behave like amino acid residues in a protein.

Many food protein substrates of microbial transglutaminases were gelled upon incubation with it. The characteristics of such a gelation procedure and the gels formed are as follows: proteins that are not gelled by heating can be gelled; gels that normally melt at elevated temperature no longer melt after the microbial transglutaminase gelation; protein in oil-in-water emulsions, even in the presence of sugars and/or sodium chloride, can be gelled; gel firmness increases after heating; the gels can no longer be solubilized by detergents or denaturants. Concerning dairy products, many researchers have shown that milk casein, which has no capability of gel formation even by heating, was a very good substrate for various transglutaminases. It was found that a heat-resistant firm gel was formed from casein in the transglutaminase reaction. An example is the use of microbial transglutaminase in

yoghurt production. Yoghurt is a milk gel formed by acidic fermentation with lactic starter, but it may suffer from problems of serum separation with a change in temperature or physical impacts. Adding microbial transglutaminase can solve this, because microbial transglutaminase improves the water-holding capacity of the gel. The microbial transglutaminase reaction also makes it possible to produce dairy products, such as ice cream and cheese with low-fat contents or a reduced content of nonfat-solids (for a review, see Motoki and Seguro, 1998).

Transglutaminases from different sources exhibit marked differences in substrate specificity. The relatively low substrate specificity of microbial transglutaminase means that this enzyme can cross-link a large variety of proteins and that its application potential is higher than that of transglutaminase from bovine blood plasma and pig erythrocytes. Previous cross-linking of β-casein with microbial transglutaminase increased the gel strength of low pH-gelled β-casein. This increase showed a maximum depending on the incubation time of cross-linking. In addition, cross-linking of β-casein resulted in protein with heat-induced gelling properties, whereas uncross-linked β-casein did not form a gel under these conditions (Wijngaards *et al.*, 2000).

Emulsifying properties of milk proteins were significantly altered after cross-linking with transglutaminase from *Streptoverticillium mobaraense*. With a low degree of cross-linking, the stability of β-lactoglobulin stabilized oil–water emulsions towards strong flocculation or coalescence was reduced after cross-linking (Færgemand *et al.*, 1997). With extensive cross-linking, the stability of milk protein stabilized oil–water emulsions was poorer than that of the native emulsions. The stability towards creaming and the stability in ethanol were generally improved after cross-linking.

Transglutaminase might be used in stabilizing products like yoghurt, fresh cheese, whipping cream and novel milk products. The preparation of cross-linked caseinates as functional ingredients in foods may also be worthwhile (Lorenzen *et al.*, 2000). However, extensive studies are necessary to get a better understanding of cross-linking in dairying. A possible mechanism by which transglutaminase affects heat stability of milk has been recently reported (O'Sullivan *et al.*, 2002). As potential nonfood uses of transglutaminase, the preparation of foils or films, coatings, medical polymers and carriers for immobilizing enzymes should be mentioned (Table IV).

Since application of microbial transglutaminase-catalyzed modification on food proteins is vast, intense attention must be focused on nutritional efficiency of such cross-linked proteins. It has been demonstrated that the glutamine–lysine dipeptide could be metabolized in rats, and that lysine was

TABLE IV
POTENTIAL USE OF TRANSGLUTAMINASE IN PROCESSING OF MILK PROTEINS AND DAIRY PRODUCTS

Caseinates	↗ Gelation, emulsifying properties, viscosity
α_{S1}-Casein	Films, coating, artificial skin, immobilizing enzymes
Whey proteins	Packaging film
Yoghurt, fresh cheese	↗ Gel strength, ↘ syneresis
Ice cream	↗ Water binding, gelation properties
Proteolysates	Covalent incorporation of lysyl peptides

integrated in rat tissues (Iwami and Yasumoto, 1986; Friedman and Finot, 1990). Moreover, rats fed with cross-linked caseins grew normally as compared to rats fed with native caseins (Seguro *et al.*, 1996), suggesting that the cross-linked proteins are cleaved and that the lysine in the moiety is metabolically utilized in the body.

In summary, enzymatic hydrolysis presents numerous possibilities to modify the properties of proteins. Several food-grade enzymes with different specificities are now available. The selection of an enzyme is mainly dictated by its cost, while the cost of an enzyme accounts for only a small percentage of the protein hydrolysate production cost.

E. LIBERATION OF BIOLOGICALLY FUNCTIONAL PEPTIDES

The isolation and characterization of encephalins in 1975 led to the detection in 1979 of the opioid activity of peptides issued from partial proteolysis of dairy proteins (Brantl *et al.*, 1979, 1981, 1982; Henschen *et al.*, 1979; Lottspeich *et al.*, 1980). Peptides that are inactive within the amino acid sequence of a protein may be released by digestive processes *in vivo* and may act as physiological modulators of metabolism. In analogy to the endogenous opioid peptides (endorphins), opioid peptides from food proteins have been called exorphins (Zioudrou *et al.*, 1979; Clare and Swaisgood, 2000). Peptides with antihypertensive, immunomodulating, antithrombotic, and opioid activities have been found in bovine and human milks, and in fermented products and cheese (Smacchi and Gobetti, 2000).

1. Peptides with opioid agonistic and antagonistic activities

Most of the known bioactive peptides derived from milk proteins are opioid peptides (Table V). Those derived from caseins are called casomorphins or casoxins, while those from whey proteins are lactorphins, lactoferroxins or serorphin. Major opioid peptides are fragments of β-casein.

TABLE V
MAIN BIOLOGICAL PROPERTIES OF PEPTIDES ISSUED FROM MILK PROTEINS

No.	Sequence	Fragment	Name	Biological activity			
				Opioid IC50[a]	ACE Inhibition IC50[b]	Immuno-modulatory (% control[c])	Ca^{2+} binding capacity
1	YPFPGPIPNSL	β-Cn (f60-70)	β-Casomorphin-11	10			
2	YPFPGPI	β-Cn (f60-66)	β-Casomorphin-7	14	500	−21/+26	
3	YPFPG	β-Cn (f60-64)	β-Casomorphin-5	1.1	0		
4	RYLGYLE	α_{S1}-Cn (f90-96)	α-Casein exorphin	1.2			
5	RYLGYL	α_{S1}-Cn (f90-95)	α-Casein exorphin	12			
6	YLGYLE	α_{S1}-Cn (f91-96)	α-Casein exorphin	45			
7	YLGF * NH2	α-La (f50-53)	α-Lactorphin	300	733		
8	YLLF * NH2	β-Lg (f102-105)	β-Lactorphin	160	172		
9	YL	β-Lg (f102-103) α_{S1}-Cn (f91-92)			122		
10	LF	β-Lg (f104-105)			349		
11	YGFQNA	SA (f399-404)	Serorphin	85			
12	SRYPSY * OCH3	κ-Cn (f33-38)	Casoxin 6	15			
13	YIPIQYVLSR	κ-Cn (f25-34)	Casoxin C	50			
14	AVPYPQR	β-Cn (f177-183)	β-Casokinin-7		15		
15	FFVAP	α_{S1}-Cn (f23-27)	α-Casokinin-5		6		
16	FPEVFGK	α_{S1}-Cn (f28-34)	α-Casokinin-7		140		
17	TTMPLW	α_{S1}-Cn (f194-199)	α-Casokinin-6		16	+121/+162	
18	YQQPVLGPVR	β-Cn (f193-202)	β-Casokinin-10		300	−28/+14	
19	PGPIPN	β-Cn (f63-68)	Immunopeptide			+122/+139	
20	LLY	β-Cn (f191-193)	Immunopeptide			+148	

TABLE V (*continued*)

MAIN BIOLOGICAL PROPERTIES OF PEPTIDES ISSUED FROM MILK PROTEINS

No.	Sequence	Fragment	Name	Biological activity			
				Opioid IC50[a]	ACE Inhibition IC50[b]	Immuno-modulatory (% control[c])	Ca^{2+} binding capacity
21	YG	α-La(f50-51) α-La(f18-19) κ-Cn (f38-39)		>1000		+101	
22	YGG	α-La (f18-20)				+35	
23	RELEELNVPGEIVES ∗ LS ∗ S ∗ S ∗ EESITR	β-Cn (f1-25)4P	Caseinophospho-peptide				629
24	DIGS ∗ ES ∗ TEDQAMEDIM	α_{S1}-Cn (f43-58)2P	Caseinophospho-peptide				328
25	QMEAES ∗ IS ∗ S ∗ S ∗ EEIVPNS ∗ VEQK	α_{S1}-Cn (f59-79)	Caseinophospho-Peptide				841
26	FKCRRWNRMKKL GAPSITCVRRAF	LF (f17-41)	Lactoferricin	Antimicrobial			
27	MAIPPKKNQDK	κ-Cn (f106-116)	Casoplatelin	Antithrombotic			

S ∗ phosphoseryl; *Source*: adapted from Meisel (1998).

[a]IC_{50} value (μmol/l) given for peptide concentration inhibiting [^{3}H]-naloxone binding, by 50%.

[b]IC_{50} value (μmol/l) given for peptide concentration inhibiting the activity of ACE, by 50%.

[c]Values indicate % stimulation (+) and inhibition (−) in relation to control (= 100).

These β-casomorphins were found in analogous positions in sheep, water buffalo and human β-casein. β-Casomorphins correspond to the fragment 60–70 of the β-casein sequence. These compounds are liberated from casein by enzymatic *in vitro* digestion (Henschen *et al.*, 1979; Teschemacher and Koch, 1990; Meisel and Schlimme, 1996; Meisel, 1998) as well as under *in vivo* conditions. Opioid antagonists have been found in bovine and human κ-casein (casoxins) and in α_{S1}-casein (Meisel and Schlimme, 1996). The opioid antagonist lactoferroxin has been found in human lactoferrin (Meisel, 1998). Various synthetic casoxins were isolated as C-terminally methoxylated peptides (fragments 33–38, 34–38 and 35–38 of κ-casein). They were more active than native fragments. Tryptic fragment 25–34 of bovine κ-casein (casoxin C) showed a relatively high opioid antagonistic activity in comparison to the esterified peptides (Meisel and Schlimme, 1996). Casoxins are opioid receptor ligands of the μ-type with relatively low antagonistic potency as compared with naloxone. Recently, a tryptic peptide with an anxiolytic activity has been isolated from bovine α_{S1}-casein (fragment 91–100, named α-casozepine; Miclo *et al.*, 2001).

2. *ACE-inhibitory activity*

These peptides exert their bioactivity through the inhibition of the ACE. ACE is a multifunctional enzyme (Meisel and Schlimme, 1996) located in different tissues (brain, plasma, lung, kidney, heart, skeletal muscle, pancreas, spleen, placenta, arteries, testes, uterus and brush border; Meisel and Schlimme, 1996), and plays a key physiological role in the regulation of local levels of endogenous bioactive peptides. Exogenous ACE inhibitors having a hypertensive effect *in vivo* were first discovered in snake venom (Ferreira *et al.*, 1970; Kato and Suzuki, 1971). Several food protein sources including fish, maize and milk protein contain sequences that inhibit ACE (Meisel and Schlimme, 1996). Peptides have been found in α_{S1}-, β- and κ-caseins, and in bovine serum albumin (Table V).

ACE-inhibitory peptides are produced during the manufacture of dairy products, e.g., secondary proteolysis during cheese ripening. The ACE-inhibitory activity in cheese was mainly associated with the low molecular mass peptide fraction.

3. *Peptides with immunomodulatory activities*

The bioactivity of immunopeptides has been characterized by different *in vitro* and *in vivo* test systems. Casein-derived immunopeptides including fragments of α_{S1}- and β-casein (Table V) have been shown to stimulate phagocytosis by murine macrophages, and to exert a protective effect against

Klebsiella pneumoniae infection in mice after intravenous treatment (Meisel and Schlimme, 1996). The mechanism by which milk protein derived peptides exert their immunomodulatory effects is not yet defined. However, opioid peptides may affect the immunoreactivity of lymphocytes via the opiate receptor. There is a remarkable relationship between the immune system and opioid peptides, because opioid μ receptors for endorphins are present on T lymphocytes and human phagocytic leukocytes (Meisel, 1998).

4. Peptides with antimicrobial activities

Antimicrobial peptides have been derived from lactoferrin (Meisel, 1998). An antimicrobial peptide fragment was produced *in vitro* upon enzymatic cleavage of lactoferrin with pepsin. The resulting peptide had better bactericidal properties than the undigested lactoferrin, suggesting that its smaller size may facilitate access to target sites on the microbial surface. The antimicrobial activity of lactoferrin seemed to be correlated with the net positive charge on the peptide. An α_{S2}-casein fragment (165–203), named casocidin-I, containing a high proportion of basic amino acids was also found to be an antibacterial agent which can inhibit the growth of *E. coli* and *Staphylococcus carnosus* (Meisel and Schlimme, 1996).

5. Peptides with antithrombotic activities

Casoplatelins are peptides obtained from the caseinomacropeptide (fragment 106–162) of bovine κ-casein (Meisel and Schlimme, 1996). These peptides are inhibitors of the aggregation of platelets as well as of the binding of human fibrinogen γ-chain to a specific receptor site on the platelet surface. The main antithrombotic peptides of κ-casein are the sequences 106–116, 106–112, 112–116 and 113–116. Three amino acid residues (Ile 108, Lys 112, Asp 115) of κ-casein fragment 106–116 are in homologous positions as compared with the γ-chain sequence of human fibrinogen (Meisel and Schlimme, 1996). These residues seem to be important for the inhibitory effect, which is due to the competition between antithrombotic peptides and the γ-chain for the platelet receptors.

6. Peptides with mineral-binding properties

Micelles in bovine milk contain physiologically significant amounts of calcium and phosphorous because of phosphorylated seryl residues in α_{S1}-, α_{S2}- and β-casein. These casein phosphopeptide (CPP) fragments help to create thermodynamically stable casein micelles super-saturated with calcium and phosphate, thus contributing to the stability of milk during

heat processing (Holt, 1994; Holt and Horne, 1996). *In vitro*, cleavage of casein with enzymes such as trypsin and alcalase leads to a number of CPP fragments (Adamson and Reynolds, 1995, 1996). If CPP exerts a function *in vivo*, one may expect that these casein-derived fragments are relatively resistant to proteolytic breakdown in the intestinal tract. Naito and Suzuki (1974) showed that this is indeed the case. In later rat studies, these results were confirmed; furthermore, it was shown that the formation of CPP in the intestine could increase the concentration of soluble calcium (Lee *et al.*, 1980, 1983; Sato *et al.*, 1991; Hirayama *et al.*, 1992). Meisel and Frister (1989) found that CPP fragments were also released in the intestine of mini pigs.

Cell culture and animal and human studies have subsequently addressed the question of whether CPP in the diet improves the absorption of calcium. The animal studies that tried to find positive effects of dietary CPP on intestinal Ca absorption, Ca balance and bone formation showed controversial results (see Steijns, 2001 for a review).

Complexes of Ca, CPP and phosphate have also been shown to reduce caries in a dose-dependent fashion, by increasing the level of calcium phosphate in the plaque, thus influencing the demineralization/remineralization process (Reynolds *et al.*, 1995), and to significantly reduce the adherence of *Streptococci* to tooth enamel (Schuepbach *et al.*, 1996).

Most published research on the anticariogenic properties of milk proteins concentrates on the whole casein, its hydrolysates, isolated fractions from the hydrolysates such as CPP, and some proteose peptone fractions (Scholz-Ahrens and Schrezenmeir, 2000; Grenby *et al.*, 2001; Warner *et al.*, 2001). Whey proteins have also been studied for their anticariogenic properties (Reynolds and Del Rio, 1984; Grobler, 1991; Loimaranta *et al.*, 1999). Therefore, it is possible that whey proteins, as a whole or after hydrolysis, could exhibit some cariostatic effects that might find applications in food and other formulations (Warner *et al.*, 2001). Although whey proteins gave a lower level of protection against caries than CPP, they are readily available and their preparation on a large scale is economically competitive.

IV. GENETIC ENGINEERING OF MILK PROTEINS AND PROTEASES

One of the most important factors that determine whether or not a protein is usable in the fabrication of a food product is its functionality. The functionality results from a combination of physico-chemical properties that define the behavior of the food protein in food systems. It is evident that a detailed understanding of food protein functionality requires an intimate knowledge of the protein structure. The bovine milk protein system has been studied in great detail. Although providing useful structure–function

information on proteins, chemical derivatization often results in polydisperse protein products because of more or less random reactions with protein functional groups of varying reactivity. Thus, specificity of modification is lost, which can hamper interpretation of structure–function information.

Recent developments in recombinant DNA technology can be used to systematically alter single amino acids in the primary sequence of proteins. It is known that single changes in amino acids can markedly alter the functional characteristics of proteins.

In the genetic engineering of enzymes, one probably requires the precision of modification inherent in recombinant DNA techniques to alter the K_m, V_{max}, specificity, etc. of the enzyme. It is now feasible to genetically engineer enzymes to change their thermal inactivation, kinetic characteristics, and specificity to make them more suitable for use in food processing and analysis. Manipulation of the functionality of food proteins for the most part will probably not require such precision and it becomes possible to alter their properties with more generalized changes. This section will discuss the use of some changes in β-lactoglobulin to improve its functional properties and the use of oligodeoxynucleotide directed mutagenesis for specific alterations in the primary structure of trypsin to change its characteristics.

A. THERMOSTABILITY OF β-LACTOGLOBULIN

β-Lactoglobulin serves as an excellent model system because the various requirements to carry out protein engineering have been established, including a high resolution structure and a system for expression of the recombinant protein.

Various preparations of whey proteins have found applications in food formulations for either their nutritional or functional properties. For example, the texture of yoghurt is highly dependent upon the incorporation of β-lactoglobulin in the curd. To achieve this, the milk is heated to 85°C to aggregate β-lactoglobulin onto the casein micelle. On the other hand, the use of whey proteins in beverage formulations is limited where thermal processing is required and a clear, nonturbid solution is desirable. Applications are limited due to its tendency to form aggregates that scatter light upon heating. The free thiol group (see Section II.D) initiates thermal-induced reactions leading to the formation of macromolecular complexes. β-Lactoglobulin cannot form effective gels when reducing conditions are used.

The events leading to the macromolecular associations observed with β-lactoglobulin appear to be mediated by disulfide interactions. The initial solution proposed to reduce thermoinduced aggregation was to remove the Cys 121 and replace it with alanine (Cho *et al.*, 1994). Unfortunately, recombinant protein could not be purified from inclusion bodies. The alternative

for enhancing thermostability was to engage the free thiol in a disulfide bridge, thus introducing a third disulfide linkage into the protein. Due to their close vicinity with Cys 121, Ala 132 and Leu 104 were chosen for replacement with a cysteine. A132C and L104C proteins were created. In contrast to C121A, both of these proteins refolded properly. The conformational stability of the mutant proteins against thermal denaturation was substantially increased (8–10°C) as compared with wild-type β-lactoglobulin. The midpoint of the denaturation profile of A132C β-lactoglobulin is at a guanidine hydrochloride concentration of 4.5 M as compared to 3.6 and 3.4 M for L104C and wild-type β-lactoglobulin, respectively. Neither the A132C nor the L104C polymerized upon heating at 65°C, in contrast to the wild-type protein. Thus, a valuable performance attribute was engineered into the protein by modification of its thermal stability (Cho *et al.*, 1994).

B. GELATION OF β-LACTOGLOBULIN

The curd formed during the manufacture of cheese and other fermented dairy products results in a partial denaturation and aggregation of proteins. Although the curd is primarily composed of caseins, some whey proteins may be entrapped depending on the process and particularly on the temperature to which the milk is heated prior to fermentation. Yoghurt has a significant amount of β-lactoglobulin in its curd due to the high temperature used in the process, which is sufficient to denature this protein. Incorporation of β-lactoglobulin into the curd is desirable for textural and flavor properties of the product.

The design of thermostable variants of β-lactoglobulin coupled with the fact that chemical thiolation could increase the gel strength, suggested a strategy for engineering proteins with enhanced gelation ability (Kim *et al.*, 1990a; Lee *et al.*, 1993). Increasing the free thiol group content might enhance gelation. Two mutations F82C and R40C were engineered and the resulting gel strength measured by a penetrometer that measured the weight necessary for a flat-tipped needle to puncture the gel inside a capillary tube. Wild-type β-lactoglobulin when heated at 90°C for 15 min formed gels with strengths of 14–19 g over a concentration range 9.4–10%, but it did not form a gel below 9%. In contrast, F82C β-lactoglobulin formed a gel at concentrations down to 8% with a gel strength of 23.7 g. Gels of R40C/F82C β-lactoglobulin were formed at concentrations as low as 6.8% and had a gel strength of 16.5 g (Batt, 1997).

The key issue with a number of modifications that have been made to β-lactoglobulin is their performance as food ingredients, especially when added to complex formulations (Lee *et al.*, 1994). As a model system, yoghurt is simple and could be improved by reducing whey syneresis. Since the double

mutant R40C/F28C formed stronger gels at lower temperatures as compared to the wild-type β-lactoglobulin, its functionality as a food additive for yoghurt has been explored. Very small amounts of R40C/F28C β-lactoglobulin reduced the amount of whey formed in yoghurt that was processed at 70°C, a temperature 15°C lower than the standard regimen.

C. DESIGN OF RECOMBINANT ENZYMES USED FOR PROTEIN MODIFICATION

The cost of enzyme purification is a major factor in the expense of most enzyme processes. If a bioreactor is incorporated into the process, then costs associated with bioreactor preparation and regeneration as well as the operational stability of the immobilized enzyme are major factors in the economics of the bioprocess (Swaisgood, 1991).

One of the approaches has been to genetically design fusion proteins with an affinity domain linked to the enzyme. This technology provides for one-step purification as opposed to the multistep processes required for purification of commercial enzymes. Furthermore, immobilization can be achieved at the same time, leading to a minimization of bioreactor preparation costs. Fusion proteins with streptavidin as an affinity domain have been designed (Sano and Cantor, 1991; Walsh and Swaisgood, 1994; Lee and Swaisgood, 1998).

Cross-linking of the polypeptide chains can dramatically alter the functionality of food proteins. This result may be achieved by sulfhydryl–disulfide interchange (see Section II.D.). More extensive cross-linking can be obtained by using transglutaminase to catalyze formation of isopeptide bonds between the γ-carboxamide group of glutaminyl residues and the ε-amino group of lysyl residues (see Section III.D.). An immobilized streptavidin-transglutaminase with a specific activity nearly 50% of that obtained for purified guinea pig liver enzyme has been designed and expressed (Oh *et al.*, 1993; Huang *et al.*, 1995).

The functional behavior of a food protein depends on a number of molecular properties including the chemical characteristics of the surface such as the distribution of the charge and polar and nonpolar residues, and the flexibility of the structure (Huang *et al.*, 1996). These characteristics are modified by limited hydrolysis (see Section III.A.). Size and structure of a polypeptide are important for good functionality. Consequently, DH must be carefully controlled (Huang *et al.*, 1996). Such a control is best achieved by using immobilized enzymes, which in turn avoids the necessity of a downstream enzyme inactivation step that may destroy the structure of the polypeptide, prevents enzyme autolysis, and avoids contamination of the product

with the proteinase (Swaisgood, 1991). An effort to develop enzyme purification and immobilization technology has been done, and the genetic construction and expression of a trypsin–streptavidin has been investigated (Higaki *et al.*, 1989).

One of the major goals in protease engineering is to understand how structure determines function. Site-directed mutagenesis can provide a powerful approach to design enzymes with novel specificities (Perona and Craik, 1995). The aspartyl 189 residue of trypsin (S_1) is crucial for substrate orientation and specific lysis of its Arg–X and Lys–X bonds (P_1). Sequence alignments of other seryl proteases reveal that the vicinal amino acid residue 188 (S_2) is highly conserved and can be either a lysine or an arginine (Perona and Craik, 1995). Consequently, the amino acid in position 188 plays an important role differing, however, from outright definition of protease specificity determination. K188 was replaced with aromatic amino acids (Chobert *et al.*, 1998a) or histidine (Briand *et al.*, 1997) in order to change the interactions of the substrate-binding site and, hence, modulate catalytic properties of this protease. In other series of experiment the highly conserved G187, K188 and D189 were replaced with aromatic amino acid residues (Chobert *et al.*, 1998b) in order to perturb the electrostatics and to amplify hydrophobic interactions of the substrate-binding site.

Caseins were and are widely used as industrial and food substrates because they are inexpensive and easily obtained in highly purified forms. β-Casein is an abundant and well-characterized milk protein with a pronounced amphiphilic character and containing a large number of prolyl residues (1 proline per about 6 amino acid residues). The peptides arising from fragmentation of β-casein have interesting physiological and biological activities (see Section III.E.). All these reasons mean that β-casein may constitute a model of choice for relatively sophisticated hydrolysis studies.

The trypsin mutants K188F, K188Y and K188W (Chobert *et al.*, 1998a) were characterized first by analyzing the kinetic parameters k_{cat}, K_m, and k_{cat}/K_m for comparable artificial tetrapeptide substrates containing arginine (Suc-Ala-Ala-Pro-Arg-*pNA*) and lysine (Suc-Ala-Ala-Pro-Lys-*pNA*) (Table VI). Compared with trypsin, mutants K188F, K188Y and K188W exhibited a 1.6- to 3-fold increase of K_m for arginine and lysine containing substrates, respectively. These mutants had k_{cat} similar to that observed with wild-type except for a 3- to 4-fold decrease for K188W mutant. While the second-order rate constant k_{cat}/K_m of K188W trypsin mutant decreased $\approx$7- to 12-fold for substrates containing arginine and lysine, respectively, 2-fold decrease was observed for K188F and K188Y trypsin mutants. Wild-type and K188Y trypsins showed 6-fold arginine/lysine preference whereas K188F and K188W trypsins preferred the arginine over the substrate containing lysine by a factor of 8 and 10, respectively.

TABLE VI
KINETIC PARAMETERS OF WILD-TYPE AND MUTANT TRYPSINS MEASURED AT pH 8

Enzyme	Substrate[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
Wild-type	Arg	32.8 ± 7.1	163 ± 31	4.97 ± 1.21
K188F		55.4 ± 1.3	144 ± 15	2.63 ± 0.31
K188Y		53.4 ± 2.2	114 ± 12	2.14 ± 0.33
K188W		98.0 ± 8.1	58 ± 3	0.59 ± 0.01
WFY[b]		47.0 ± 7.0	4.9 ± 0.4	0.11 ± 0.001
Wild-type	Lys	105 ± 15	74 ± 15	0.71 ± 0.21
K188F		188 ± 20	61 ± 12	0.32 ± 0.07
K188Y		107 ± 10	43 ± 4	0.41 ± 0.03
K188W		292 ± 25	17 ± 4	0.059 ± 0.013
WFY[b]		123 ± 0.3	1.7 ± 0.1	0.013 ± 0.0005

[a]Substrates: Suc-Ala-Ala-Pro-Arg-*pNA* and Suc-Ala-Ala-Pro-Lys-*pNA*.
[b]WFY: G187W/K188F/D189Y.

The same lysyl residue 188 was replaced with histidine in order to build a metal chelation site in the substrate-binding pocket of trypsin (Briand *et al.*, 1997). K188H mutation did not affect catalytic efficiency at all. In the presence of Cu^{2+}, trypsin K188H exhibited a 30- to 100-fold increase of K_m, while k_{cat} was only slightly decreased (Table VII). Hydrolytic activity of this mutant could be fully restored by addition of EDTA. Thus, in contrast to the chelation of the active site, a different mode of metal-dependent inhibition of the activity of trypsin by building a co-ordination site in the substrate-binding pocket of the protease was achieved.

In other series of experiment, the highly conserved G187, K188 and D189 were replaced with aromatic amino acid residues in order to perturb the electrostatic interactions and to amplify hydrophobic interactions of the substrate-binding site (Chobert *et al.*, 1998b). The mutant G187W/K188F/D189Y exhibited 1.3-fold increase in K_m values for tetrapeptide synthetic substrates. This mutant shows a 30- to 40-fold decrease of its k_{cat} and its second-order rate constant k_{cat}/K_m decreased ≈40- and 55-fold for substrates containing arginine and lysine, respectively (Table VI).

Synthetic substrates allow rapid determination of the catalytic constants of an enzyme. Nevertheless, it is known that the environment of the peptide bond depends largely on physico-chemical conditions of the applied media, and imposed steric hindrance. Since these parameters are important, the hydrolysis of purified β-casein was studied at different pHs. The kinetic analysis revealed that the mutant conserved the native trypsin capacity to hydrolyze peptide bonds containing arginyl and lysyl residues. The optimal pH of activity changed considerably according to the mutation.

TABLE VII
KINETIC PARAMETERS OF WILD-TYPE AND K188H TRYPSINS MEASURED AT pH 8, WITH 0, 5 OR 200 μM Cu^{2+}

$[Cu^{2+}]$ (μM)	Substrate[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
Wild-type	Arg			
0		41 ± 1	132 ± 5	3.22 ± 0.17
5		49 ± 2	109 ± 16	2.22 ± 0.43
200		42 ± 1	79 ± 5	1.88 ± 0.17
K188H				
0		30 ± 2	169 ± 12	5.63 ± 0.84
5		118 ± 3	150 ± 11	1.27 ± 0.32
200		~2200	~131 ± 10	0.06 ± 0.13
Wild-type	Lys			
0		75 ± 7	39 ± 2	0.52 ± 0.08
5		102 ± 4	34 ± 2	0.33 ± 0.03
200		104 ± 3	26 ± 5	0.25 ± 0.06
K188H				
0		74 ± 1	48 ± 5	0.65 ± 0.05
5		409 ± 42	54 ± 3	0.13 ± 0.02
200		~7500	~54 ± 5	0.007 ± 0.001

[a]Substrates: Suc-Ala-Ala-Pro-Arg-*pNA* and Suc-Ala-Ala-Pro-Lys-*pNA*.

Surprisingly, as demonstrated only by proteolysis of a natural substrate (β-casein), all aromatic mutants and the triple mutant G187W/K188F/D189Y acquired the capacity to hydrolyze β-casein at C-termini of amidated amino acids (Q and N) (Figure 9).

The use of β-casein as a test substrate presents, besides the importance of this protein in the food industry, advantages of releasing the hydrolysis from several structural limitations characteristic of many other potential native protein substrates. The use of this protein enabled a better understanding of the scope and validity of the results obtained with synthetic substrates. Additionally, the harnessing of mutated trypsins into the processing of β-casein diversified the peptide products obtained. Most of the observed new cleavage sites were located in the hydrophobic portion of the protein.

Modifications introduced by the mutations were central to the alteration of the specificities of the enzymes studied, which were capable of cleaving β-casein at many new sites, for example, hydrolyzing the fragment Arg1-Lys105, reported to be a trypsin inhibitor (Bouhallab *et al.*, 1997). Since many tryptic inhibitors contain amidated Glu and Asp, and form amyloid structures, the mutants of this type could be used for the hydrolysis of the lytically resistant protein structures.

FIG. 9 Cleavage sites obtained from 48 h of hydrolysis of β-casein with K188F (***F***), K188Y (***Y***), K188W (***W***) and G187W/K188F/D189Y (↘) at pH 8. β-Casein (1 mg/ml, initial concentration) was dissolved in 0.2 M Tris-HCl. Active enzyme was added to β-casein solution at an enzyme/substrate ratio of 0.01, 0.02, 0.02 and 1% for K188F, K188Y, K188W and G187W/K188F/D189Y (w/w), respectively. *Source*: From Haertlé and Chobert (1999), by courtesy of Food & Nutrition Press, Inc.

V. CONCLUSION

Proteins are fundamentally as important as nutrients but now they are considered 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. Relatively mild phosphorylation of dairy proteins with $POCl_3$, in the presence of natural nucleophiles such as basic amino acids, alters the net charge of substituted proteins but also grafts significant amounts of arginine and lysine through phosphoamide bonds. Simple glycations of β-lactoglobulin with reducing sugars under mild conditions (pH 6, 60°C) can substitute up to 70% of ε-amino groups and significantly improve the solubility of transformed proteins. As us known for over 90 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 manipulation of disulfide bridges of abundant whey globulins such as α-lactalbumin and β-lactoglobulin through astute use of baric treatment for conformational destabilization and of appropriate redox conditions scrambles disulfide bridges creating intermolecular arrays of disulfide connections. By varying applied pressure, temperature, pH, concentrations and initiating reducer sulfide, different viscosities or gel types of the protein solutions can be obtained. Alternatively, the blocking of free cysteine in β-lactoglobulin increases its stability and eliminates the capacity to promote the aggregation process described before. 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 more acceptable in the eye of the nonscientist 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 nonbioactive 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 pre-requisite 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.

We have seen how oligodeoxynucleotide site-directed mutagenesis can be used to engineer the primary sequences of proteins, and examples were given of how this powerful technique can be used to engineer enzymes and their protein substrates.

The modification of the contact surfaces between substrate protein and protease triggers important variations in the hydrolytic processing of dairy proteins. This is achieved either by modifying the conformation of dairy globulins by mild physico-chemical treatments or by well-conceived site-directed mutagenesis of the S2 proteinase substrate-binding site. Finally, microbial transglutaminase, as well as other transglutaminases, can catalyze formation of the ε-(γ-glutamyl) lysine bond in many food proteins; the resulting cross-link drastically alters protein functionalities. Applications are emerging in the development of novel foods and nonfood processing methods. There may be many applications in the incorporation of various amines, amino acids, lysine-containing peptides, glutamine-containing peptides and heterologous polypeptides. There is no doubt that microbial transglutaminase technology will be an essential tool for protein modification in both food processing and nonfood processing in the future.

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 before. They are finally a source of essential amino acids. This means milk proteins are highly functional substances.

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