Emulsifying Properties of Pure and Mixed α_{s1}- and β-Casein Fractions: Effects of Chemical Glycosylation

Philippe Cayot,* Jean-Luc Courthaudon, and Denis Lorient

Département de Biochimie et Toxicologie Alimentaires, Ecole Nationale Supérieure de Biologie Appliquée à la Nutrition et à l'Alimentation, Campus Universitaire de Montmuzard, 21000 Dijon, France

α_{s1}- and β-caseins were prepared from whole casein by batchwise ion-exchange chromatography before being chemically galactosylated. Solubility of these pure fractions was found to be minimal around the isoelectric point (pH 4–5). The glycosylation and the increase of the ionic strength improved the solubility in the range of isoelectric pH and had a small effect on both sides of this pH. Glycosylation had little effect on the emulsifying index. Emulsions made from α_{s1}-caseins do not have the same stability as emulsions from β-caseins. Nevertheless, glycosylation does not influence the behavior of each fraction as far as emulsion stability is concerned. Binary mixtures of α_{s1}- and β-caseins had a lower emulsifying activity index than the arithmetical calculated value. Adding galactosylated β-casein to those binary mixtures improved the emulsifying activity of the resulting ternary mixtures. Then, glycosylation would limit the intermolecular interactions rather than improve the properties of the monomeric casein fractions.

It is possible to explain the good emulsifying properties of caseinates since their primary structure has been determined: α_{s1}-casein (Mercier et al., 1971), β-casein (Ribadeau-Dumas et al., 1972), and κ-casein (Mercier et al., 1973). Due to their amphiphilic character and the absence of any ordered secondary structure, the principal α_{s1}- and β-casein fractions have an unfolded structure facilitating their adsorption at the water–oil interface. This relationship has been confirmed by Lee et al. (1987), who pointed out that β-casein surface properties were lost by enzymatic hydrolysis of either the very hydrophobic C-terminal fragment (193–209) or the very hydrophilic N-terminal fragment (1–25). The removal of the N-terminal (1–40) or the C-terminal (134–199) regions by a limited proteolysis has been found to alter α_{s1}-casein tensioactive properties (Shimizu et al., 1983).

The investigation of the competitive adsorption of individual caseins in oil-in-water emulsions showed that β-casein preferentially adsorbs at the oil–water interface when a mixture of α_{s1}- and β-caseins is used (Dickinson et al., 1988); the protein which adsorbs most rapidly remains predominant at the interface (Brock and Enser, 1987). Despite the importance of kinetic phenomena, β-casein added to an emulsion made with α_{s1}-casein is able to replace it at the interface, whereas the inverse phenomenon is not observed (Dickinson, 1989a,b). There is nevertheless no preferential adsorption at the oil–water interface during homogenization (Robson and Dalgleish, 1987).

Casein fractions are efficient at interfaces under monomeric state. However, by associating very easily to each other in aqueous solution as composite complexes, mixed casein fractions often have unforeseeable behaviors; so a mixture of α_{s1}-, β-, and κ-caseins in a 4/4/1 ratio has a surface viscosity close to that of entire caseinate, while a 1/1 α_{s1}- and β-casein mixture has a lower interfacial shear viscosity (Dickinson, 1989a,b). This can lead to the prediction of the preponderant role of κ-casein, whose excellent foaming properties are already known (Lorient et al., 1989).

This study aims at a better understanding of the role played by the major α_{s1}- and β-casein fractions in emulsions and at a simulation of the role of κ-casein. The latter can be mimed by using chemically galactosylated α_{s1}- and β-casein fractions since glycosylation was found to improve solubility and viscosity of whole casein (Courthaudon et al., 1989) or to enhance the emulsifying properties (between pH 1 and 6) of the β-lactoglobulin (Bertrand-Harb et al., 1990). In the same way, the emulsifying activities of ethylalkylated β-casein were higher than that of native β-casein, though the solubility was not modified (Chobert et al., 1990). The chemical modification of caseins by covalent attachment of hydrophobic groups did not increase conversely the emulsifying properties (Chobert et al., 1987). We looked for the improvement of the emulsifying properties by binding hydrophilic groups like aldoses.

MATERIALS AND METHODS

Preparation of Whole Caseinate. Bovine casein was obtained from fresh (nonrefrigerated) skimmed milk by isoelectric precipitation at pH 4.6. Then it was washed with deionized water and dissolved at pH 7 by addition of sodium hydroxide. This process of precipitation and resolubilization was repeated twice, and the caseinate solution was eventually freeze-dried.

Purification of α_{s1}- and β-Casein Fractions. Casein fractions were obtained by batchwise chromatography following a method inspired by that of Mercier et al. (1968) as modified by Davis and Law (1977). The separation was made by using strong ion-exchanger Q-Sepharose Fast Flow from Pharmacia in a dissociating buffer containing 4 M urea, 6.5×10^{-5} M dithiothreitol, and 20×10^{-3} M Bis-Tris-propane hydrochloride, pH 8, containing 0.01 wt % sodium azide. Each casein fraction was both washed and concentrated by ultrafiltration (Millipore Pellicon System equipped with a polysulfone Millipore PTGC membrane with a cutoff point of 10 000 Da) and then freeze-dried. The purity of each casein fraction was checked by polyacrylamide gel electrophoresis (7.5% w/v) at pH 8.3 in a 6 M urea and 2-mercaptoethanol dissociating medium according to the method of Maurer (1971).

Glycosylation of Casein Fractions. The covalent binding of galactose to the ϵ-lysyl residues of the α_{s1}- and β-caseins as well as the control of the glycosylation level was made following the methods described by Courthaudon et al. (1989).

The level of modification, τ, of the α_{s1}- and β-casein (i.e., the percentage of "hidden" ϵ-lysyl residues) was calculated according to the relationships

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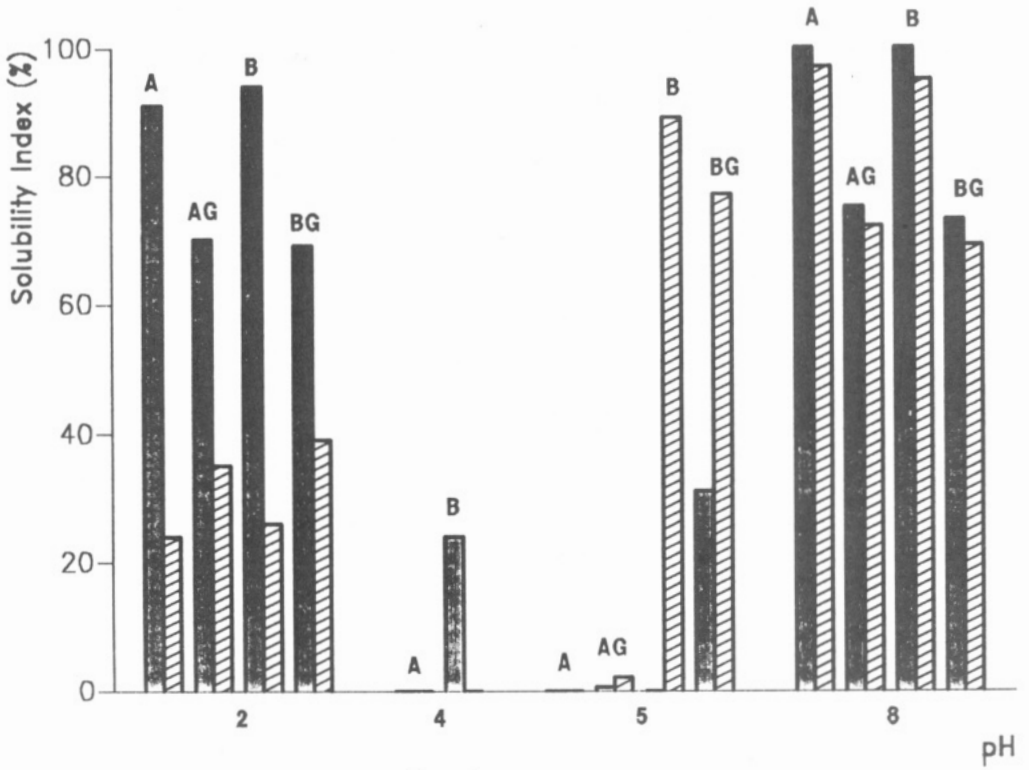

Figure 1. Influence of pH and ionic strength, $\mu = \sum_{i=1}^{n} {}^1/_2 c_i z_i^2$ (z_i = ionic valence; c_i = ionic concentration) on solubility of α_{s1}-casein (A), galactosylated α_{s1}-casein (AG), β-casein (B), and galactosylated β-casein (BG). Conditions: temperature, 20 °C; pH 2, 4, 5, and 8; μ = 0 (solid bars) and 0.1 M (hatched bars); protein concentration, 2.5 g L^{-1}. Values for solubility were set at 100%.

$$\tau_{\alpha_{s1}} = \frac{A_R - A_G}{A_R + 0.0978A_G}$$

$$\tau_{\beta} = \frac{A_R - A_G}{A_R + 0.0752A_G}$$

where A_R is the absorbance of reference TNP-casein (2,4,6-trinitrophenylcasein) at 420 nm and A_G is the absorbance of TNP-galactosylated casein at 420 nm.

Solubility Measurement. A 2.5 g L^{-1} protein solution was centrifuged (4000g, 1 h, 20 °C), and the solubility value was obtained from the soluble protein content in the supernatant determined according to the method of Lowry et al. (1951).

Emulsifying Properties. The emulsifying properties were assessed with 25% v/v oil-in-water emulsions. The 2.5 g L^{-1} protein solution was mixed with soya oil by using a Polytron PVC 2 homogenizer at 19 500 rpm during 30 s for emulsion activity and at 12 000 rpm during 2 min for emulsion stability.

The emulsifying activity was defined by the emulsifying activity index (EAI) of Pearce and Kinsella (1978).

To appraise the emulsion stability, we defined an emulsifying stability index (ESI) by

$$\mathrm{ESI} = 1/\int_{t=0}^{t=60\ \mathrm{min}} V(t)\,dt$$

where $V(t)$ is the curve representing the volume of coalesced oil as a function of time, after centrifugation (3800g) for 60 min (one measure per 10 min). The higher the value of ESI, the better the stability.

RESULTS AND DISCUSSION

Glycosylation Level of the Pure Casein Fractions. The levels of glycosylation were 69 ± 4% with α_{s1}-casein (i.e., 9.7 ± 0.6 mol of glucide/mol of protein; MM = 23 900) and 84 ± 2% with β-casein (i.e., 9.2 ± 0.2 mol of galactose/mol of protein; MM = 24 100).

Influence of pH and Glycosylation on the Solubility of α_{s1}- and β-Casein. The highest solubility values were obtained at pH 8 and pH 2, due to a very high charge density (electrostatic repulsions): at pH 8, β-casein has a net charge of −6 and α_{s1}-casein −15, whereas they have, respectively, +15 and +20 net charge at pH 2. At pH 5, close to the isoelectric point of α_{s1}- (4.96) and β-casein (5.19), the electrostatic attractions are maximum and cause a lowering of solubility (Figure 1). The solubilizing effect of sodium chloride is only observed at pH 5 and especially with β-casein. On the contrary, the solubility is decreased by NaCl at pH 2, while NaCl has very little effect at pH 8; as a matter of fact, the number of total charges is higher at pH 8 than at pH 2.

The solubility is increased by glycosylation and NaCl at pH 5 (especially with α_{s1}-casein) owing to a partial reduction of the electrostatic attractions. An opposite effect is observed at pH 2 and pH 8 since the charges are lowered by glycosylation. The results are slightly different with whole caseinate. Courthaudon et al. (1989) actually noticed a highly improved solubility at the isoelectric point (pH 4.6) without salt because of the high solubility of glycosylated β-casein.

Influence of pH and Glycosylation on Emulsifying Properties of Caseins. It is noteworthy by comparing Figures 1 and 2 that the curves of EAI and solubility against pH are superimposable (standard deviations of Figures 1 and 2 are very low and so are not indicated). The diffusion of proteins to the oil–water interface depends indeed on their solubility. A higher interfacial area can be covered by α_{s1}-casein at pH 8 than by β-casein (in the same quantities in solution) because of a superior charge density for α_{s1}-casein (electrostatic repulsions are more important) and because of a more pronounced amphipolarity for α_{s1}-casein. The better emulsifying activity of α_{s1}-casein would also be explained by the presence of two hydrophobic "anchorage" sites (residues 1–40 and 139–199) in its primary structure. The effect of glycosylation seems to be minimal at pH 2 and pH 8 (Figure 2), where the emulsifying activity is already high.

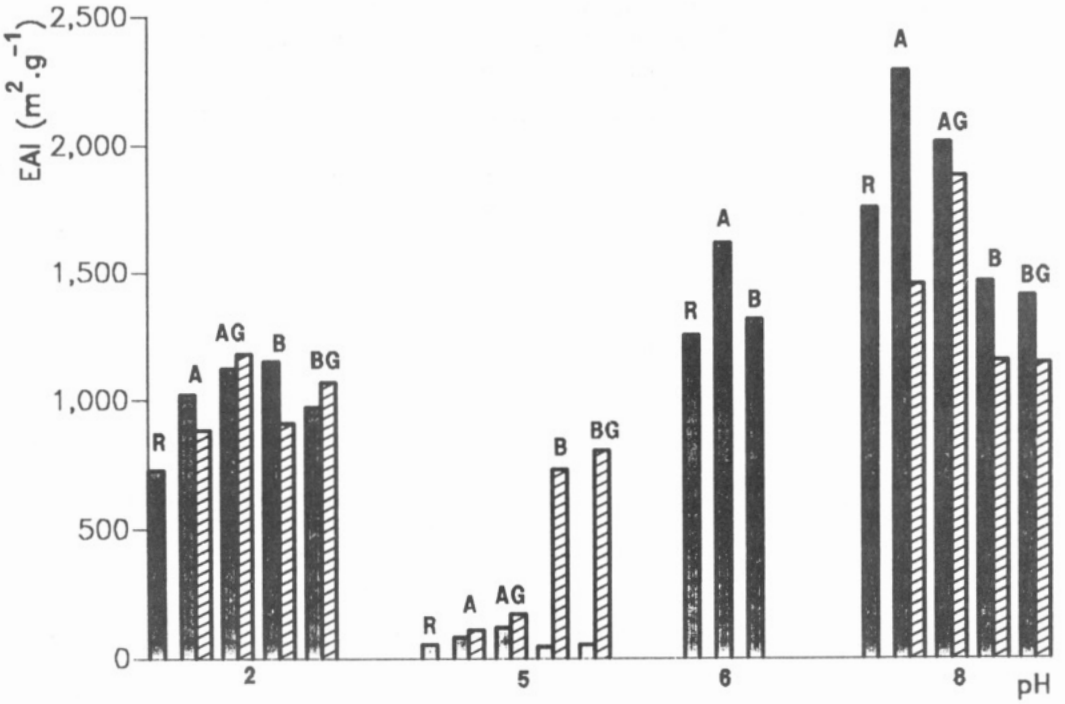

Figure 2. Influence of pH and ionic strength, μ, on the emulsifying activity index (EAI) of α_{s1}-casein (A), galactosylated α_{s1}-casein (AG), β-casein (B), galactosylated β-casein (BG), and whole casein (reference = R). Conditions: temperature, 20 °C; pH 2, 4, 5, and 8; μ = 0 (solid bars) and 0.1 M (hatched bars); protein concentration, 2.5 g L^{-1}.

Table I. Comparison of Emulsifying Stability between α_{s1}-Casein (α_{s1}), β-Casein (β), Galactosylated α_{s1}-Casein (α_{s1}g), and Galactosylated β-Casein (βg) as Functions of pH and μ[a]

ESI, 10^{-2} mL^{-1} min^{-1}		caseins			
pH	ionic strength (μ), M	α_{s1}	α_{s1}g	β	βg
2	0	2.1	4.0	40.0	15.4
	0.1	6.7	7.4	19.0	15.4
5	0	0.7	0.6	0.6	0.7
	0.1	0.9	2.1	6.9	6.3
8	0	+∞	66.7	13.8	5.0
	0.1	+∞	50.0	6.8	5.4

[a] Conditions: temperature, 20 °C; protein concentration, 2.5 g L^{-1}.

β-casein gives higher emulsion stabilities than α_{s1}-casein at pH 2 (Table I). Since the net positive charges of both β-casein and α_{s1}-casein are about the same at this pH, this effect could be attributed to enhanced hydrophobic interactions of β-casein (segment 193–209 in β-casein is more hydrophobic than segments 1–40 and 139–199 in α_{s1}-casein). At pH 8, the importance of electrostatic repulsions gives a opposite effect (Table I). Whatever the pH value, the emulsion stability is generally unchanged by the glycosylation.

Emulsifying Properties of Binary α_{s1}- and β-Casein Mixtures (Figure 3). For possible competitive or synergistic effects to be observed, the conditions of the experimental medium were chosen so that the α_{s1}- and β-casein emulsifying properties were very different.

Whatever the relative ratio of two fractions, the emulsifying activity of the mixture at pH 8 was less than the arithmetical average calculated from the emulsifying activities of α_{s1}- and β-caseins (dotted line; Figure 3I). This effect is even more enlarged at pH 6, where the electrostatic repulsions are lower (Figure 3II).

This effect could be ascribed to a competition between the casein fractions. Preferential adsorption of β-casein at the oil–water interface (Dickinson, 1989a,b) would attenuate the influence of α_{s1}-casein, although it has a better surface activity (Figure 2) and a better emulsifying stability (Table I) at high pH values. This result could be

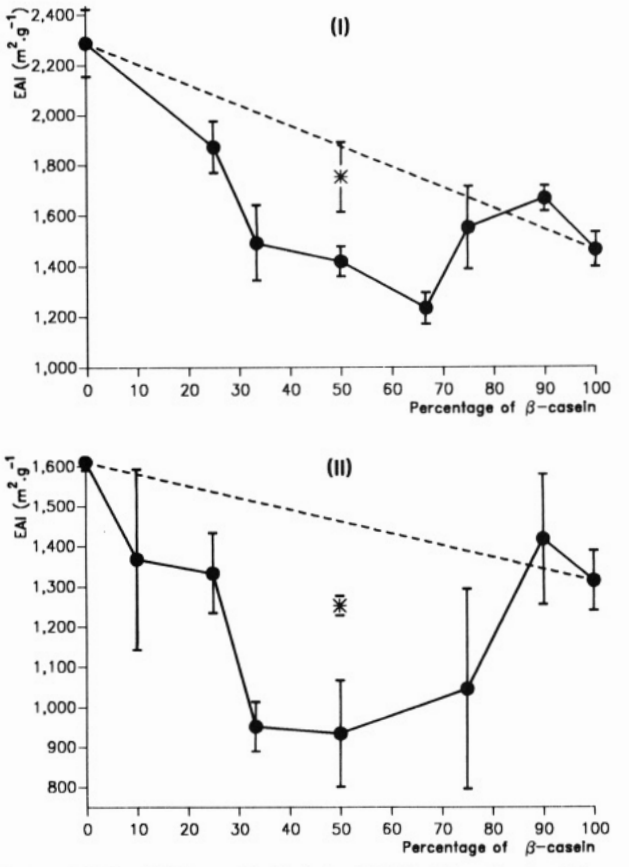

Figure 3. Emulsifying activity index (EAI) of standard mixtures of α_{s1}-casein and β-casein (●), whole casein (*), and the mixture's calculated arithmetical EAI (- - -). Conditions: temperature, 20 °C; μ = 0 M; protein concentration, 2.5 g L^{-1}. (I) Plots of EAI at pH 8; (II) plots of EAI at pH 6.

Table II. Comparison of Emulsifying Activity Index (EAI) between Pure Caseins and Binary and Ternary Mixtures of Caseins[a]

pure or mixed constituents	EAI, $m^2 g^{-1}$ measd	EAI, $m^2 g^{-1}$ theor
α_{s1}-casein	2288 ± 133	
β-casein	1463 ± 68	
galactosylated β-casein	1407 ● 32	
α_{s1} + β-casein (1/1)	1419 ● 59	1875
β + βg-casein (1/1)	1401 ± 121	1453
α_{s1} + β + βg-casein (1/1/1)	1741 ± 192	1731
whole casein	1752 ± 139	

[a] Conditions: temperature, 20 °C; pH 8; μ = 0 M; protein concentration, 2.5 g L^{-1}.

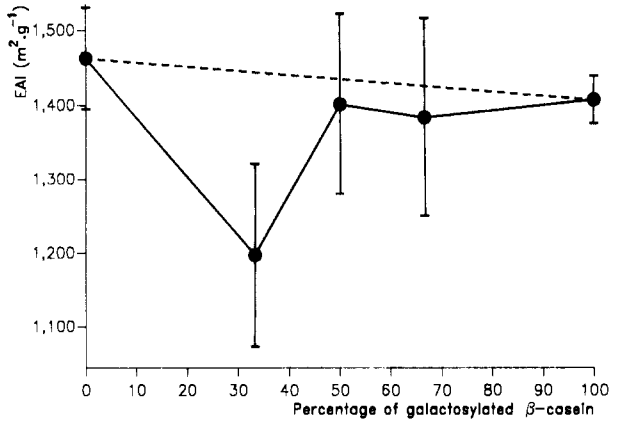

Figure 4. Emulsifying activity index (EAI) of standard mixtures of β-casein and galactosylated β-casein (●) and the mixture's calculated arithmetical EAI (- - -). Conditions: temperature, 20 °C; pH 8; μ = 0 M; protein concentration, 2.5 g L^{-1}.

put in relation with the interfacial shear viscosity of α_{s1}-casein 10 times higher than that of β-casein at pH 7 (Dickinson, 1989a,b).

However, it seems likely that association of casein fractions in solution would rather be responsible for the depressing effect of EAI in α_{s1}- and β-casein mixtures. According to Schmidt (1982), α_{s1}–β-casein heterogeneous associations (with α_{s1}- to β-casein molar ratios greater than 1) develop preferentially to α_{s1}-casein/α_{s1}-casein or β-casein/β-casein homogeneous associations. In this way, although α_{s1}-casein is a better surfactant than β-casein, α_{s1}-casein would be more associated in complexes (than β-casein) and therefore less available in monomeric state (the only surface active state) to participate at the emulsion.

Emulsifying Properties of Ternary Casein Mixtures. The EAI of a ternary equimolar mixture of galactosylated β-casein/α_{s1}-casein/β-casein (1/1/1) is close to the EAI arithmetical average of the components (1741 instead of 1731 $m^2 g^{-1}$) and also close to the EAI of whole caseinate (1752 $m^2 g^{-1}$) (Table II). This means that the depressing effect is canceled by equimolar proportional addition of galactosylated β-casein to α_{s1}-casein/β-casein mixture. Galactosylated β-casein would lead to a preferential β-casein/galactosylated β-casein association, which would be low if we refer to the small decrease in EAI (as compared to the arithmetical average) especially for the mixture of 50% β-casein/50% galactosylated β-casein (Figure 4).

Whole caseinate and ternary mixture of α_{s1}-casein/β-casein/galactosylated β-casein (1/1/1) do not behave as a α_{s1}-casein/β-casein (1/1) mixture does. These differences would be likely consequent upon the dissociating role played by galactosylated β-casein in the ternary mixture. In this mixture, the higher proportion of monomeric molecules could be explained by the destructuring effect of glycosylation—for a high level of modification. According to Colas et al. (1988), glycosylation is indeed known to increase the hydrodynamic volume (actually the voluminosity) of the casein fractions (lower Huggins coefficient). The hydrophobic bindings should be therefore reduced. The κ-casein undergoes nevertheless aggregation and then associates to about 30 monomers of β-casein (Fox and Mulvihill, 1983). The dissociating role of κ-casein would be due especially to a preferential association in the whole caseinate which set free the α_{s1}-casein to the interface.

CONCLUSION

The interfacial behavior in oil-in-water emulsions of whole caseinate or binary casein mixtures cannot be predicted from that of native or galactosylated α_{s1}- or β-casein. This makes quite obvious the effect of associations on the interfacial behavior of mixtures and especially if mixtures contain destructured components such as highly chemically glycosylated proteins. Therefore, chemical glycosylation would improve the emulsifying properties of whole caseinate by diminishing the α_{s1}-casein/β-casein association rather than improving the emulsifying properties of the casein fractions. Consequently, κ-casein plays an essential role, albeit in a minority. Being itself a naturally glycosylated protein, one may wonder whether this effect is due to its natural glycosylation or to its protein structure.

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