Acylation and Alkylation of Bovine β-Lactoglobulin in Organic Solvents

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Bovine β-lactoglobulin (BLG) was solubilized in solvent systems of varying polarity and hydrophobicity. An amount of 2 mg/mL initially added could be solubilized up to 70% (1.4 mg/mL) in $CHCl_3/CH_3OH$ (7/3, v/v) acidified with HCl and up to 40% (0.8 mg/mL) in $CHCl_3/CH_3OH$ (3/7, v/v) in the presence of triethylamine. After solubilization in organic solvents, the yield of reductive alkylation is much higher (98%) than that in aqueous conditions (75%). Acylation of BLG with stearic acid anhydride was also performed in binary mixtures of methanol and chloroform. The efficiency of substitutions of ε-amino groups in studied organic systems amounted to 80% compared to none in water. Besides, the emulsifying activity of 40% stearylated BLG was 3–6-fold superior (depending on the pH) than that of unmodified BLG, even after a 30-min heating at 80 °C.

INTRODUCTION

β-Lactoglobulin (BLG) is a compact globular protein found in the milk of several mammal species. It represents as much as 50% of the total whey proteins in bovine milk. Depending on conditions, BLG (162 amino acids) exists in an oligomeric form or as a monomer (MW 18 200). Two orthogonal β-sheets, one of which is flanked by an α-helix, shape a hydrophobic pocket (Papiz et al., 1986; Monaco et al., 1987) which is probably involved in the binding of various ligands (Futterman and Heller, 1972; Brown, 1984; O'Neill and Kinsella, 1987; Dufour and Haertlé, 1990a; Dufour et al., 1990).

A significant amount of already accumulated structural information and the availability of BLG make it a model of choice to study chemical modifications of globular proteins and their influence on the protein functional properties. Since the pioneering works of Fraenkel-Conrat and Olcott (1945) and Fraenkel-Conrat and Feeney (1950), many studies have been done in this field, namely lipophilization of glycinin (Haque et al., 1982) or of BLG (Akita and Nakai, 1990a,b), amidation and esterification of BLG (Mattarella and Richardson, 1983; Mattarella et al., 1983), alkylation of β-casein and β-casein tryptic peptides (Chobert et al., 1990; Touati et al., 1990), and glycosylation of BLG (Bertrand-Harb et al., 1990; Waniska and Kinsella, 1988). Most of these protein modifications were performed to improve their foaming and/or emulsifying properties. The outcome of these studies supports the idea that the hydrophilic/lipophilic balance and hydrophobic/hydrophilic interactions are the most important factors of the surface activity of proteins (Akita and Nakai, 1990a; Kinsella, 1979; Kato et al., 1981; Chobert et al., 1987).

Numerous studies on the conformation of proteins solubilized in hydroorganic media have demonstrated that proteins undergo changes in their secondary structure, the extent of which depends on the solvent system used. Initially, the structural changes of BLG under the influence of weakly protic solvents in acid pHs were studied by Tanford et al. (1960) and Townend et al. (1967). Then, Inoue and Timasheff (1967) demonstrated that BLG acquired a highly α-helical conformation in 2-chloroethanol/water (1/4 v/v) or in methanol/water (2/3 v/v). More recently, Dufour and Haertlé (1990b) showed that in hydroalcoholic mixtures BLG undergoes a reversible structural transition depending on the polarity and the dielectric constant (ε) of the solvent. In aqueous media, native BLG contains 52% β-sheets and 8% α-helices. It refolds in a hydroalcoholic mixture with $\epsilon = 50$, reaching 50% α-helices. According to Nozaki and Tanford (1971), the hydrophilicity of the peptide backbone is one of the factors that may explain why proteins acquire a highly α-helical structure when dissolved in organic solvents.

It has been demonstrated that alkylation could increase the strength of interactions between BLG and some of its ligands (Dufour and Haertlé, 1990a,b) but that it was incomplete (75%) when performed in aqueous solutions, unless a large excess of reagents is applied during a long reaction time. Consequently, it was tempting to submit the globulin structure to strong solvent perturbations to disorganize its folding, with the hope that randomized side chains were going to be less protected against full and fast substitution.

The present work establishes conditions of reasonable solubility of BLG in binary organic mixtures (methanol and chloroform) of varying hydrophobicity and polarity. Then it describes alkylation and acylation yields obtained in such solvent systems, as well as interface properties of some BLG derivatives.

MATERIALS AND METHODS

Materials. Bovine β-lactoglobulin was a kind gift of Dr. J. Faucant and J. C. Maubois, INRA (Rennes, France). It has been isolated from lactoserum by ultrafiltration, as a mixture of two variants: A (38.5%) and B (35.7%). It also contained α-lactalbumin (2.5%) as the main protein contaminant, as judged from reversed-phase HPLC (RP-HPLC) chromatograms (Figure 2A) and electrophoresis data (Figure 3). Except for the α-lactalbumin band, the electrophorogram (Figure 3) only shows a faint band (MW ≈ 37 000), which is probably a BLG dimer. Hence, the purity of the used BLG preparation appears, on the gels, to exceed 75% calculated on the basis of HPLC data.

Stearic anhydride was from Fluka (Mulhouse, France). Succinic and glutaric anhydrides as well as acetaldehyde and amines were from Aldrich (Strasbourg, France). The solvents, methanol and chloroform, were from Carlo Erba (Milan, Italy).

Solubility Assays. Solubility studies were performed in binary mixtures of chloroform and methanol containing 0, 10, ..., 100% (v/v) methanol, with the addition of an acid (5 N HCl, 10 μL/mL of solvent) or a tertiary amine (triethyl-, tripropyl-, or tributylamine (TEA, TPA, TBA), 25 μmol/mg of BLG). During the solubility tests, 10 mg of BLG ($m_i = 10$ mg) was dissolved in 5 mL of solvent mixtures ($V_i = 5$ mL). After 4 h of stirring at room temperature, a known volume (V_f) of solution was filtered

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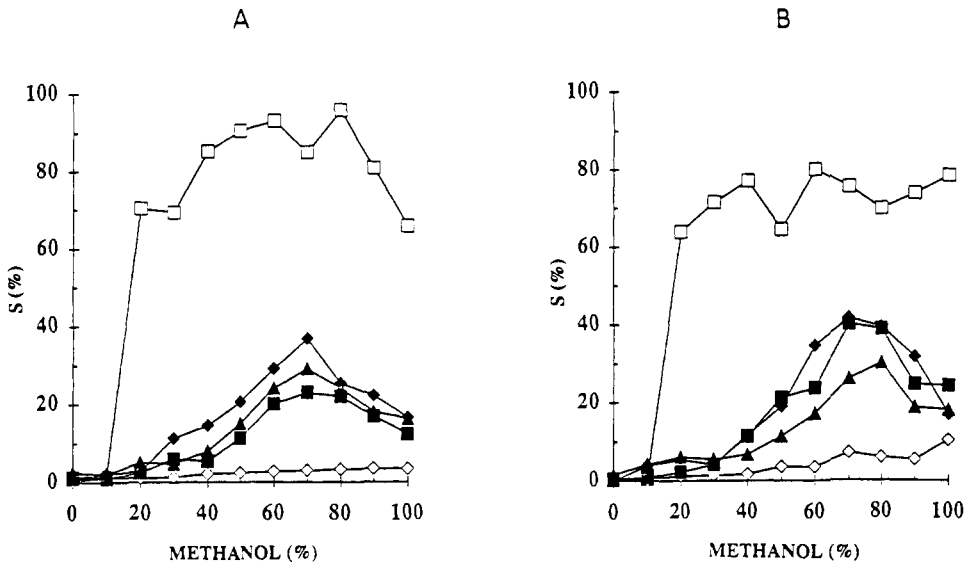

Figure 1. Solubility of BLG in methanol/chloroform mixtures with addition of HCl (□), TEA (■), TPA (◆), or TBA (▲) or no addition at all (◇). (A) Native BLG (pH 6.7); (B) BLG that has been adjusted to its isoionic point (pH 4.9) in water and lyophilized before use for the solubility tests.

through a glass fiber filter until a clear solution was obtained. Then, the solution was dried under vacuum and the residue was dissolved in 3 mL (V_d = 3 mL) of water. The determination of the absorbance at 280 nm (A) enabled us to calculate the amount of protein and its solubility (S) as

$$S(\%) = 100A(\mathrm{MW})V_dV_i/(\epsilon m_i V_f)$$

where MW = 18 200 and ϵ = 17 600 mol^{-1} L cm^{-1}; m_i is expressed in milligrams, and V_i, V_f, and V_d are expressed in milliliters. Some tests were performed on native BLG (pH 6.7 in water), others after the pH of BLG solution in water was adjusted to different values (for example pH_i = 4.9). BLG solutions were then lyophilized before the tests.

Fluorescence Spectra of the Solubilized BLG. The changes in fluorescence spectra of BLG dissolved in $CHCl_3/CH_3OH$ were recorded at 20 °C on an Aminco SLM 4800C spectrofluorometer in the ratio mode. Both excitation and emission slits were set at 4 nm. Tryptophan fluorescence emission spectra were measured at an excitation wavelength of 287 nm.

Chemical Modifications. Reductive alkylation (Means and Feeney, 1968) of BLG was performed with acetaldehyde in the presence of a reductive agent (1 M sodium cyanoborohydride in tetrahydrofuran). The reagent molar ratios were 1/2/4 (NH_2/CHO/$NaBH_3CN$). During alkylation in aqueous medium, BLG was solubilized at 50 mg/mL in 0.4 M sodium borate buffer, pH 8.0. After 3 h at room temperature, the reaction was stopped by acidification and the solution was dialyzed for 48 h at 4 °C and then lyophilized. During the alkylation of BLG in organic media, the reaction was carried out in conditions of highest solubility of the protein: $CHCl_3/CH_3OH$ 7/3 (v/v) plus HCl or 3/7 (v/v) plus TEA. The reaction was stopped by quick evaporation of the solvents under vacuum. Acylations with anhydrides were performed in $CHCl_3/CH_3OH$ (3/7 v/v) with added TEA. Stearic, succinic, and glutaric anhydrides were used at molar ratios ranging from 3/1 to 1/15 (anhydride/NH_2). The anhydrides were solubilized in chloroform just before use and added to the vigorously stirred solution. After 2 h at room temperature, the solvents were evaporated under vacuum. The modified protein was solubilized in water and lyophilized.

The degree of protein alkylation was assessed by measuring the decrease in free amino groups with trinitrobenzenesulfonic acid (TNBS), according to the method of Adler-Nissen (1979). It was also determined by measuring the loss of lysine by amino acid analysis: after an acid hydrolysis under vacuum in the presence of 6 N HCl for 24 h, at 110 °C, in a Pico-Tag station (Waters), the amino acids were derivatized with phenyl isothiocyanate (PITC) (Bidlingmeyer et al., 1984) and quantified by RP-HPLC on a Pico-Tag C_{18} column (3.9 mm i.d. × 15 cm, Waters).

A qualitative analysis of alkylation was made by trypsinolysis and subsequent analysis by RP-HPLC (Dalgalarrondo et al., 1990), since apparently the alkylation of the lysyl residues prevents the action of trypsin. The BLG samples were dissolved at 1 mg/mL in 0.1 M Tris buffer, pH 8.0, and were hydrolyzed with trypsin for 10 h at 37 °C. The enzyme/substrate ratio was 0.01 (w/w). The peptides were then separated by RP-HPLC.

The yield of acylations was determined by measuring the decrease in free amino groups with o-phthaldialdehyde (OPA) (Church et al., 1983), after solubilization of the studied proteins in 50 mM sodium phosphate buffer, pH 7.8, with 1% SDS. The presence of 1% SDS did not interfere with the OPA procedure.

Electrophoresis. SDS-PAGE was performed according to the procedure of Laemmli (1971).

Emulsifying Activity. The emulsifying activity was determined for native BLG and for BLG acylated with stearic anhydride (40% substitution). The determination of the emulsifying activity was performed according to the method of Pearce and Kinsella (1978), with a mixture of soybean and rapeseed oil, in pH ranging from 2.0 to 12.0. It was studied with suspensions of BLG in water (1 mg/mL) or with the soluble fraction of pelleted suspensions (solutions), after centrifugation (4 °C, 5000 rpm, 15 min). Aliquots of the soluble fraction were used to measure the protein concentration according to the bicinchoninic acid method, using the Pierce BCA protein assay reagent (Smith et al., 1985). The emulsifying activity was expressed as an index: EAI = $2T/\phi C$, where T = 2.3 A/l, A is the absorbance at 500 nm, l = 10^{-2} m (light path), ϕ = 0.25 (oil phase volume fraction), and C is the concentration of soluble protein (g/m^3). EAI is expressed in m^2/g. The emulsifying activity was measured just after the formation of the emulsion (EAI_{0s}) and after a 30-min heating at 80 °C following a 24-h storage at room temperature ($EAI_{80°C}$).

The stability was calculated as follows: $\Delta EAI = 100(EAI_{80°C} - EAI_{0s})/EAI_{0s}$.

ΔEAI is the variation of the EAI relative to the initial EAI.

RESULTS AND DISCUSSION

Solubility of BLG in Mixtures of Organic Solvents. The determination of the optimal proportions of methanol and chloroform leading to the highest solubility of BLG was the main goal of solubility measurements. Figure 1 shows that BLG's solubility is weak in binary mixtures of $CHCl_3/CH_3OH$, unless an acid (HCl) or a tertiary amine is added to the solvent. Several advantages can rise from the insolubility of some enzymes in pure organic solvents. Klibanov (1986), Russel and Klibanov (1988), and Zaks and Klibanov (1988) have indeed shown that the native

conformation of some enzymes (lipases, esterases) can be conserved in highly hydrophobic and highly anhydrous organic solvents in which these enzymes are insoluble. When the substrates are solubilized in organic solvents, the insoluble but highly dispersed enzymes are fully active and can be recovered easily by filtration.

In this study, the solubilization of BLG was a preliminary step to its chemical modifications in an organic phase. It was observed that the solubility of BLG in the studied binary system could be enhanced by addition either of HCl or of a tertiary amine. Figure 1 shows that HCl had a favorable effect on BLG solubility. The solubility reached 70% (1.4 mg/mL) as soon as the solvent contained 30% methanol, whatever the initial pH of the protein. The effect of tertiary amines on solubility was weaker compared to that of HCl. But, as seen in the case of TEA, the solubility depends on the initial pH of the protein. The closer this pH is to the isoionic point of BLG (pH_i = 4.9), the stronger is the effect of TEA on BLG solubility. Hence, the addition of TEA could improve solubility up to 40% (0.8 mg/mL) in $CH_3OH/CHCl_3$ (7/3 v/v), providing the used protein has been lyophilized previously at its isoionic point.

The obtained results seem to depend also on the initial amount of protein used since solubility tests made with an initial amount of 20 mg of BLG/mL of solvents, with 110 μmol of TEA/mg of protein in suspension, led to still higher BLG content per milliliter of solvent (3.5 mg/mL, data not shown). As it amounts to 17.5% solubility only, we chose an initial quantity of 2 mg/mL to minimize the excessive use of protein.

The action of HCl on solubility can be explained by the reversal of dissociation of carboxylic groups and by the formation of salts with the protonated amino groups. Both effects nullify a substantial part of the protein charges. This seems also to be the main reason why the enhancement of BLG solubility by HCl does not depend on the initial ionic status of the protein.

In the case of tertiary amines, the situation looks more complicated. The used tertiary amines, due to their free doublet of electrons on the nitrogen atom, induce the dissociation (deprotonation) of charged BLG amino groups. Once they trap the protons of the BLG amino groups, they are in a quaternary form and can act as counterions of the ionized carboxylic groups of the protein, forming mixed salts of a type $COO^-NH^+(R)_3$. This also results in a decrease of the whole charge of the protein, but not in its zeroing. As it has been observed, a previous decrease in charge improves the solubilization of BLG in the studied solvent system. Thus, the effect of the amines on BLG solubility depends on the initial ionic status of the protein.

Fluorescence spectra of BLG solubilized in organic solvents were recorded at an excitation wavelength of 287 nm, corresponding to the preferential excitation of the tryptophanyl residues of BLG. When BLG is dissolved in water, the maximum of its tryptophan emission can be observed at 332 nm. After its solubilization in 100% $CHCl_3$, the maximum of emission is blue-shifted to 330 nm. When BLG is solubilized in 100% CH_3OH, the maximum is red-shifted to 335 nm (data not shown). These shifts indicate polarity changes of the close neighborhood of the tryptophanyl residues. Blue shifts of the tryptophan emission maximum indicate change toward the more unpolar environment of the indole moiety and red shifts, toward the more polar one (Lakowicz, 1986). The obtained results imply that the two residues of tryptophan are getting in closer contact with the solvent; otherwise, such shifts would not have been observed.

Table I. BLG Alkylation Yields in Aqueous Solution or in Mixtures of Methanol and Chloroform[a]

solvent	TNBS	amino acid composition
sodium borate buffer 0.4 M, pH 8	74	75
$CHCl_3/CH_3OH$ (3/7 v/v) + TEA	81	98
$CHCl_3/CH_3OH$ (7/3 v/v) + HCl	27	12

[a] Quantification by two procedures: TNBS method (Adler-Nissen, 1979) and amino acid composition. The results are expressed as a percentage of the amino groups susceptible to alkylation.

Previous studies involving circular dichroism measurements have shown that, as a function of the polarity of the solvent in which BLG is solubilized, BLG undergoes structural transitions, leading to a highly α-helical conformation (Tanford et al., 1960; Townend et al., 1967; Dufour and Haertlé, 1990b). For example, the α-helical content of BLG increases to 50% in 50% ethanol, whereas it is only 8% in an aqueous medium (Dufour and Haertlé, 1990b). This can be correlated with fluorescence spectra of BLG when the maximum of emission is red-shifted (336 nm) upon addition of methanol or ethanol (data not shown). Therefore, the shifts in the maximum of emission of the tryptophanyl residues can be considered indicators of the solvent-induced protein conformation changes.

In addition to a blue shift, the emission spectra of a BLG solution in $CH_3OH/CHCl_3$ (3/2 v/v) displays a shoulder at 317 nm (data not shown). A possible interference of tyrosyl residues was excluded since such a shoulder at 317 nm was not observed in the tyrosinamide spectra. Consequently, it was concluded that this shoulder was due to a variation of polarity in the environment of at least one of the tryptophans.

All of these results indicate that significant BLG conformational changes occur under the influence of organic solutions of varying polarity. Complementary structural studies (CD, IR) might be necessary to confirm this hypothesis.

Chemical Modifications in Organic Solvents. Reductive alkylation involves first the formation of a Schiff base after the reaction of an aldehyde or a ketone with an amino group of a protein. Reactivity of the amino group is dependent on the existence of a free doublet of electrons on the nitrogen (deprotonation of the amino groups). Subsequent reduction of the Schiff base by $NaBH_3CN$ leads to a stable covalent bond of secondary amine type (Means and Feeney, 1968). Only the N_α atom of the N-terminal amino acid and the N_ϵ atoms of the lysyl residues are modified, what amounts to 16 susceptible residues in BLG. Acylation proceeds through a nucleophile addition of a carbonyl group to the nitrogen atom. This reaction involves the free doublet on the amino group too and concerns the same amino residues modified also by reductive alkylation.

Table I shows the alkylation yields measured by the TNBS assay and by analysis of the amino acid composition. It shows that the alkylation can take place in an organic mixture, in the presence of TEA, and that the yield obtained is significantly higher than that observed in aqueous conditions. It reaches 98% in $CH_3OH/CHCl_3$ (7/3) with TEA. The favorable effect of TEA on alkylation is due to the deprotonation of the amino groups. Protonation of the amino groups by addition of HCl makes reductive alkylation impossible in a similar way as it was postulated for aqueous media (Means and Feeney, 1968).

Higher alkylation yield in the organic medium containing TEA suggests that the accessibility of the amino groups to the used reagents and their reactivity are higher than in aqueous solutions. Tryptic hydrolysis and sub-

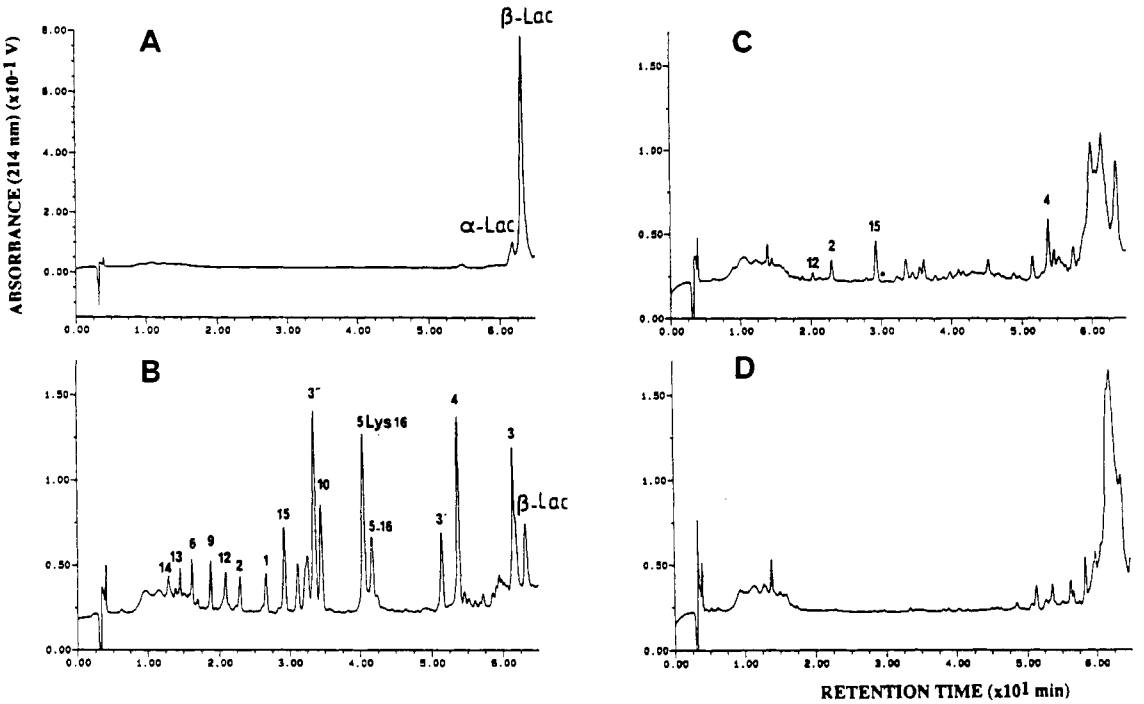

Figure 2. C_{18} HPLC chromatograms of BLG after 10 h of proteolysis with trypsin according to the procedure of Dalgalarrondo et al. (1990). (A) Reference, no trypsin; (B) trypsinolysis of native BLG; (C) trypsinolysis of BLG alkylated in 0.4 M borate buffer, pH 8; (D) trypsinolysis of BLG alkylated in $CHCl_3/CH_3OH$ (3/7 v/v) plus TEA. The peptide assignments are those described by Dalgalarrondo et al. (1990).

sequent analysis of resulting peptides by C_{18} HPLC could demonstrate that some lysyl residues unmodified in aqueous solutions are alkylated in organic medium (Figure 2). The action of trypsin, usually hydrolyzing the peptide bond on the C-terminal side of lysyl and arginyl residues, is prevented by alkylation of the lysyl ε-amino groups. Hence, the alkylation of a particular lysine will provoke the disappearance of both the peptide that contains this residue at its C-terminal side and the next peptide in the sequence, even if it has a C-terminal arginine. The simplification of HPLC profiles observed after a more intense alkylation in organic medium can be explained by the decrease in trypsin cleavage sites in more intensely substituted BLG (Figure 2). For example, already assigned peptide peaks (Dalgalarrondo et al., 1990) which conserve their retention times after a moderate alkylation are no more present after a more complete substitution: the disappearance of peaks 2, 4, 12, and 15 demonstrates that the lysyl residue numbers 14, 60, 135, and 141 are now substituted in the organic reaction system (Figure 2). This correlates well with the 23% increase in the alkylation yield (Table I).

The results of acylation of BLG in studied organic solvents are presented in Table II. The reaction yields are weak when succinic and glutaric anhydrides are used. Glutaric anhydride leads to 58% substitution provided that the anhydride is present in large excess. Stearic anhydride, otherwise insoluble in aqueous media, reveals to be a good acylating reagent in the studied solvents. The degree of substitution at equimolar ratio reaches 65% and can be enhanced to nearly 80% when anhydride is used in excess. Figure 3 presents the increase in molecular weight of BLG derivatized with growing amounts of stearic anhydride. The presence of a single band for each sample suggests that the substitution is rather homogeneous. Lipophilization of BLG had already been reported by Akita and Nakai (1990a). Their approach was different from this one since they used dicyclohexylcarbodiimide (DCC) activated stearic acid instead of stearic anhydride. Both

Table II. BLG Acylation Yields in $CHCl_3/CH_3OH$ (3/7 v/v)[a]

	anhydride		
anhydride/NH_2	stearic	glutaric	succinic
3/1	77	58	20
2/1	80	30	ND[b]
1/1	65	17	18
1/2	40	9	12
1/5	21	5	6
1/10	5	7	7
1/15	2	4	4

[a] Quantification by the OPA procedure (Church et al., 1983) after solubilization of the samples in 50 mM sodium phosphate buffer, pH 7.8, 1% SDS. The results are expressed as a percentage of the amino groups susceptible to acylation. [b] ND, not determined.

methods give comparable results; however, the reaction in organic solvents is simpler since it involves a single reaction step after solubilization of BLG in the organic mixture and gives practically no byproducts. The use of stearic acid implies the previous preparation of an *N*-hydroxysuccinimide ester of the acid and its activation with DCC in tetrahydrofuran (THF), and it requires a long reaction time (10 h) in a 0.05 M Tris-HCl (pH 8.0)/THF solution (1/1 v/v). The use of DCC leads to the formation of dicyclohexylurea, which is difficult to eliminate. In contrast, in the solvent system studied, the solvents can be removed easily by evaporation and a pure reaction product is obtained, only with free fatty acid present.

The fully acylated protein is weakly soluble in water unless SDS is added (Akita and Nakai, 1990a). That is why the OPA assay used to quantify the substitution yield was performed after the solubilization of the acylated protein in sodium phosphate buffer supplemented with 1% SDS. SDS disrupts hydrophobic interactions and hydrogen bonds, preventing the oligomerization and precipitation of proteins. Hydrophobic interactions are strengthened by the introduction of important hydrophobic moieties on lysyl side chains. As they play a great

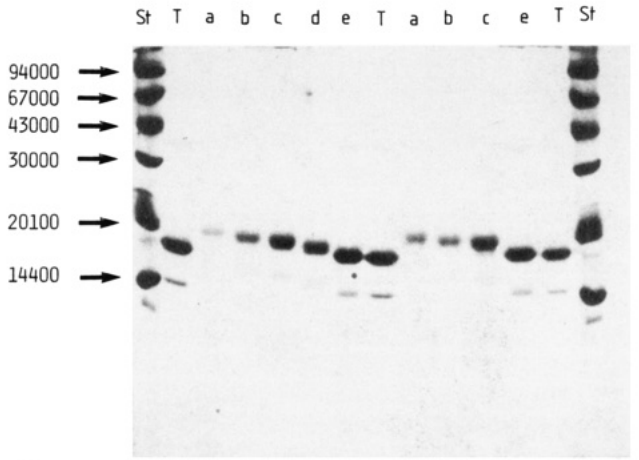

Figure 3. SDS–PAGE (Laemmli, 1971) of stearylated BLG. The acylation was performed in $CHCl_3/CH_3OH$ (3/7 v/v) with varying anhydride/NH_2 ratios: (a) 3/1; (b) 2/1; (c) 1/1; (d) 1/2; (e) 1/15. T, native BLG; St, standard (phosphorylase *b*, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 100; α-lactalbumin, 14 400).

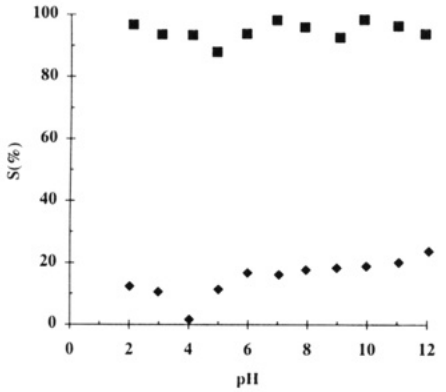

Figure 4. Solubility in water of native (■) or stearylated (◆) BLG, expressed as a percentage of the total weight of protein in suspension (1 mg/mL).

role in the determination of the functional properties of the proteins (Kinsella, 1979; Kato et al., 1981; Chobert et al., 1987), the emulsifying properties of acylated BLG were studied in comparison with those of native BLG.

Emulsifying Activity of the Acylated BLG. The emulsifying activity was measured for native and acylated BLG (40% substitution with stearic anhydride), according to the method of Pearce and Kinsella (1978). The results were expressed as an emulsifying activity index (EAI) in the following way: $EAI = 2T/\phi C$. The meaning of the symbols is explained under Materials and Methods. It has to be pointed out that C is the concentration of the soluble protein and *not* the total concentration of BLG in the suspension. As solubility of the acylated protein varies from 10 to 20% (Figure 4), approaching the neighborhood of zero at the isoionic point, the results and their interpretation are dependent on the right choice being made for C (soluble fraction or total amount in the suspension).

Our choice to take only the soluble fraction into account was supported by the observation that the results were the same when the EAIs were measured on the soluble fraction only or on the total unpelleted suspension (Figure 5). It was concluded, therefore, that the insoluble fraction does not play any significant role in the formation and stabilization of the emulsion. Hence, it does not need to be taken into account in the calculations of the EAIs.

Figure 5 presents the results of EAI measurements obtained for acylated and native BLG, just after formation

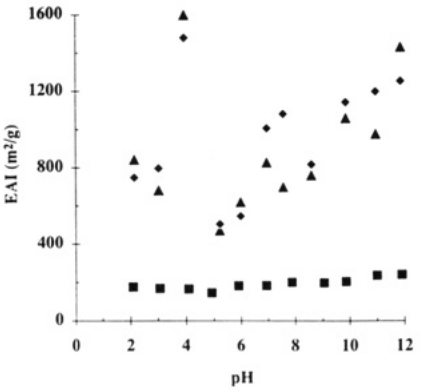

Figure 5. Emulsifying activity index of the native BLG (■) or of the soluble fraction of stearylated BLG (◆) or of its total suspension (▲). Measurements at time 0 s. The results are expressed in m^2/g.

of the emulsion. The high EAI value obtained at pH 4 for acylated BLG can be judged as insignificant since the solubility is approaching zero at this pH. At other pHs, the EAI values of the acylated BLG range from 470 to 1433 m^2/g, with slight increase from acid to alkaline pH. These values are 3–6-fold greater than those obtained in the case of the native BLG (150–250 m^2/g). It may be concluded that at equal concentrations of soluble protein the emulsifying activity of acylated BLG is much higher than that of native BLG. It is probably the hydrophobic chains linked to the amino groups that decrease the polarity of the protein and enhance its capacity to participate in hydrophobic interactions at the oil-water interface. It should be pointed out that moderately stearylated BLG (40%) was used for this study. A higher level of substitution diminishes the protein solubility in the aqueous phase and may not lead to much higher EAIs. It seems that an optimum hydrophobicity/hydrophilicity ratio, correlated with sufficient amphiphilicity, is needed to improve the surface properties of a protein (Nakai, 1983). Above this threshold, the poor solubility of the protein in water may have a negative effect on its emulsifying activity. If one considers the total amount of protein in the suspension, the high EAI of the soluble fraction of acylated BLG does not compensate for the inactivity of the insoluble protein fraction. The use of this approach for EAI calculations is leading, therefore, to an underestimation of the true EAI values, which should be rather reported to soluble protein. Consequently, much smaller EAI values than those observed for more water-soluble native BLG are obtained.

Figure 6 presents the results of emulsion stability studies. Apparently, the emulsion stability is pH-dependent, and the best stability is obtained for native BLG at alkaline pH. However, the absolute EAI values are always higher for acylated BLG than for native BLG, even at alkaline pH (data not shown). It may be concluded that a moderate derivatization of BLG with fatty acid aliphatic chains has a positive effect on its emulsifying properties.

As shown in the present study, bovine BLG could be solubilized to a reasonable extent in mixtures of methanol and chloroform and chemically modified straight in this solvent system. First, alkylation, which has been shown to reinforce BLG interactive properties toward some of its ligands (Dufour and Haertlé, 1990a), could be performed in organic media with higher yields than in aqueous conditions. It should then be interesting to examine whether these interactions are further strengthened after a more extensive alkylation in the organic system. Second, the solvent system used is valuable since it allows for the

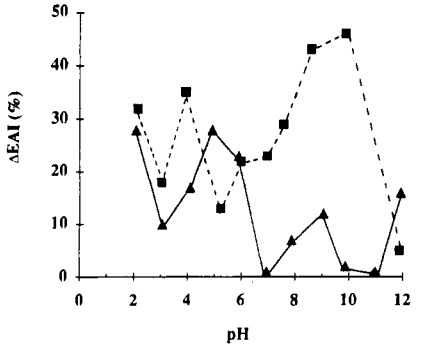

Figure 6. Stability of the emulsions after 24 h at room temperature and 30 min of heating at 80 °C. The stability index (ΔEAI) is calculated as the variation in the EAI relative to the initial EAI. ΔEAI is expressed in percent. (■) Acylated BLG; (▲) native BLG.

chemical modification of BLG with water-insoluble reagents, such as stearic anhydride. Even after the binding of hydrophobic groups such as $CH_3(CH_2)_{16}CO$, BLG still conserves enough of its hydrophilic and amphiphilic properties. Consequently, acylated BLG has better emulsifying properties than native BLG.

The data reported in this paper demonstrate that the functional properties of proteins can be improved by simple chemical means in relatively unsophisticated organic systems. This may have significant importance in the production of novel or more intensely substituted protein derivatives. These derivatizations may open additional possibilities for fuller understanding of factors controlling the induction of new functional properties in important alimentary proteins.

ABBREVIATIONS USED

BLG, β-lactoglobulin; DCC, dicyclohexylcarbodiimide; EAI, emulsifying activity index; HPLC, high-performance liquid chromatography; OPA, o-phthaldialdehyde; PITC, phenyl isothiocyanate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TEA, triethylamine; TBA, tributylamine; THF, tetrahydrofuran; TNBS, trinitrobenzenesulfonic acid; TPA, tripropylamine.

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