β-lactoglobulin is not known, although it behaves as a globular protein with strong self-association properties near its isoelectric point (McKenzie, 1971). Phosphorylation of ε-amines in β-lactoglobulin (e.g., Lys-60) also decreases ellipticity, but because dephosphorylation does not restore the original ellipticity, it is more likely that secondary and tertiary protein structures are being modified (Woo et al., 1982). Thus, the near-UV CD spectrum of proteins is an indicator of the extent of protein denaturation. The CD spectra of the modified β-lactoglobulins compared to those of the native protein and the protein completely randomized with Gdn·HCl indicated a more random structure as a result of amidation or esterification.

Since these modified proteins are more positively charged and are partially denatured, some of their functional properties in foods may also be altered. Systematic studies of alterations in protein functionality that result from structural changes may reveal structure–function relationships useful for increasing the utilization of whey proteins in foods.

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Physicochemical and Functional Properties of Positively Charged Derivatives of Bovine β-Lactoglobulin

Nina L. Mattarella[1] and Thomas Richardson*

Physicochemical and functional properties of positively charged amidated, ethyl-esterified, and methyl-esterified derivatives of bovine β-lactoglobulin were markedly different from those of β-lactoglobulin. Mean isoionic points for the proteins were 9.8, 8.7, and 9.5, respectively, for the derivatives compared to 5.2 for β-lactoglobulin. Blocking exposed carboxyl groups on β-lactoglobulin decreased the absorbance between 260 and 310 nm, increased the rate of tryptic hydrolysis, and decreased the rate of peptic hydrolysis. Methyl or ethyl ester groups were hydrolyzed only 7–14% when solutions of proteins were held at pH 10 for 24 h at 26 °C. Porcine liver esterase did not cleave ester bonds under conditions tested. Emulsion activities of modified proteins were less than that of β-lactoglobulin, but the stability of emulsions prepared with ethyl-esterified protein was greater, and this protein was 3.7-fold more concentrated at the oil/water interface than the other proteins. Binding of heptane by the proteins revealed that amidated β-lactoglobulin was most hydrophobic followed closely by ethyl-esterified β-lactoglobulin.

Bovine β-lactoglobulin, the major protein in cheese whey, is a well-characterized globular protein although its three-dimensional structure has not been defined with X-ray crystallography. Available knowledge on the structure of β-lactoglobulin coupled with its ready availability make it a useful protein to study structure–activity relationships of protein functionality in foods. Secondly, the utilization of surplus whey proteins in general, and β-lactoglobulin in particular, may be increased through a fundamental understanding of structural factors contributing to their functionality. Futhermore, useful nontoxic

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derivatives of whey proteins may find applications as food additives. In the long term, functionality studies of food protein derivatives may yield valuable structural information for designing and producing food proteins by using recombinant DNA technology.

Numerous studies on functionality of chemically modified proteins have established the usefulness of this approach (Kinsella, 1976, 1979; Sen et al., 1981; Watanabe et al., 1981a,b). Most previous research has involved the acylation or alkylation of lysine residues in various proteins. Although this may not necessarily have an adverse effect on the nutritional quality of the modified protein (Matoba et al., 1980), derivatization of nonessential amino acid residues such as glutamate and aspartate may represent a more desirable approach to novel yet nutritious proteins.

Early research on availability of exposed carboxyl groups in proteins involved simple, acid-catalyzed esterification of the protein suspended in the alcohol (Fraenkel-Conrat and Olcott, 1945; Sri Ram and Mauer, 1957). More recently, caseins and other proteins have been esterified and amidated to yield positively charged food proteins which interact strongly with negatively charged food proteins (Di Gregorio and Sisto, 1981; Mattarella et al., 1983). Indeed, positively charged proteins have been proposed as electrostatic coagulants for milk proteins to make cheese (Di Gregorio and Sisto, 1981).

The research reported in this paper is an extension of studies on the functional properties of positively charged, esterified, or amidated derivatives of β-lactoglobulin (Mattarella et al., 1983). The solubilities, UV difference spectra, stabilities of ester groups to pH and enzymic treatments, proteolysis by trypsin and pepsin, emulsifying characteristics, and hydrophobicities of the modified proteins were examined.

MATERIALS AND METHODS

Bovine β-lactoglobulin (3× crystallized, Sigma Chemical Co., St. Louis, MO) was modified by amidation or esterification (Mattarella et al., 1983). Isoionic points of the amidated, methyl-esterified, and ethyl-esterified β-lactoglobulsin used in this study were 9.8, 9.5, and 8.7, respectively.

Protein Solubility. Solubilities of proteins were measured by a modification of the method of Sen et al. (1981). Protein samples (10% w/v) in the various buffers of $\mu = 0.55$ (Sen et al., 1981) were incubated 1 h at 37 °C and then for 48 h at 4 °C. Samples were centrifuged 27000*g* for 1 h at 4 °C, each supernatant fluid was diluted to 10 mL with deionized, distilled water, and the protein content of the supernatant fluid was determined by using the method of Lowry et al. (1951).

Solubilities of proteins were also measured by adjusting the pH between 3 and 10, with 1 M HCL and 1 M NaOH, of protein solutions (1.0% w/v) at low ionic strength. These samples were incubated at 26 °C for 24 h and then centrifuged at 10000*g* for 15 min. The supernatant liquid was diluted to 10 mL and protein concentration determined in the supernatant solution as before.

Ultraviolet Difference Spectra. Difference spectra between modified and native β-lactoglobulins (0.1% protein in 0.01 M phosphate buffer, pH 7.0) were measured at 26 °C by using a Beckman Model 25 double-beam spectrophotometer.

Chemical Stability of Ester Groups. The stability of the ester groups at various pH values was determined by adding 2 mL of esterified protein solution (5 mg/mL) to 2 mL of buffer (0.1 M sodium phosphate, pH 7.0 and 8.0; 0.1 M sodium citrate, pH 3.0, 4.0, 5.0, and 6.0; 0.1 M sodium bicarbonate, pH 9.0 and 10.0) and incubating 24 h at 26 °C. Mixtures were dialyzed against 0.001 M HCl, and the isoionic points of the treated proteins were determined by using the mixed-bed ion-exchange resin (Mattarella et al., 1983) to follow any increase in negative charge.

Enzymic Stability of Ester Groups. To 3-mL solutions of each esterified protein (10 mg/mL) in 0.1 M sodium phosphate buffers, pH 7.0 and 8.0, or 0.1 M sodium citrate, pH 6.0, was added 3 mg of porcine liver esterase (Type I or Type II, Sigma Chemical Co., St. Louis, MO). After 24 h at 26 °C the protein solutions were dialyzed against 0.001 M HCl and the isoionic points of the mixtures were measured as before to detect any decrease in isoionic points. Methyl *n*-butyrate (0.1 mL) suspended in the various buffers was readily hydrolyzed by the esterases.

Proteolysis in Vitro. The modified proteins and native β-lactoglobulin were treated with trypsin or pepsin according to the method of Sri Ram and Maurer (1957). Digestion was at 26 °C, and at specific intervals 4 mL of each reaction mixture was added to 3 mL of 10% trichloroacetic acid (TCA) to stop the reaction. Each acidified mixture was centrifuged in a clinical centrifuge (Model CL, International Equipment Co., Boston, MA), and the absorbance of each clear supernatant fluid was measured at 280 nm by using a Beckman Model 25 double-beam spectrophotometer.

Emulsifying Activity. Ten milliliters of protein solution (0.50% protein) in 0.10 M sodium chloride, pH 7.0, and 20 mL of Mazola corn oil were homogenized for 30 s with a Brinkman Polytron homogenizer at room temperature. Ten-microliter samples of each emulsion were diluted with 10 mL of 0.10 M NaCl containing 0.05% sodium dodecyl sulfate ($NaDodSO_4$), pH 7.0, and the apparent absorbance was measured at 600 nm (Pearce and Kinsella, 1978). Protein adsorbed to the fat globules was determined by centrifuging emulsions 25000*g* for 15 min, measuring the protein concentration in samples of the aqueous layer (Lowry et al., 1951), and subtracting this from the total protein concentration in the system. The oil contents of emulsions were measured by drying 1-mL samples of the emulsions and protein solutions to constant weight at 120 °C and calculating the oil volume fraction (Pearce and Kinsella, 1978).

Emulsion Stability. Emulsions were prepared as before at pH 7.0 and also at the isoionic point of each protein. The emulsions were heated at 80 °C for 1 h and centrifuged at 1300*g* for 10 min, and the volume of the oil separated was recorded.

Protein Hydrophobicity Measured by Use of Fluorescent Probes. Protein hydrophobicity was measured with *cis*-parinaric acid (*cis,trans,trans,cis*-9,11,13,15-octadecatetratraenoic acid) and 8-anilino-1-naphthalenesulfonate (ANS) (Daban and Guasch, 1980) as hydrophobic probes. *cis*-Parinaric acid (Molecular Probe, Plano, TX) was dissolved in ethanol (3.6×10^{-3} M) with equimolar butylated hydroxytoluene to retard oxidation, purged with nitrogen, and stored at 0 °C in 1-mL aliquots until used. The ANS (0.01 M) (Sigma Chemical Co., St. Louis, MO) in deionized water was prepared freshly before each use.

Three sodium phosphate buffers (0.01 M, pH 7.0) of increasing ionic strength were prepared by adding NaCl to final concentrations of 0, 0.55, and 2 M, respectively. Each buffer contained 0.002% $NaDodSO_4$. The concentration of *cis*-parinaric acid or ANS in each fluorometer cell containing 3 mL of the appropriate buffer was 1.2×10^{-5} and 1.5×10^{-5} M, respectively. Small additions (1–20

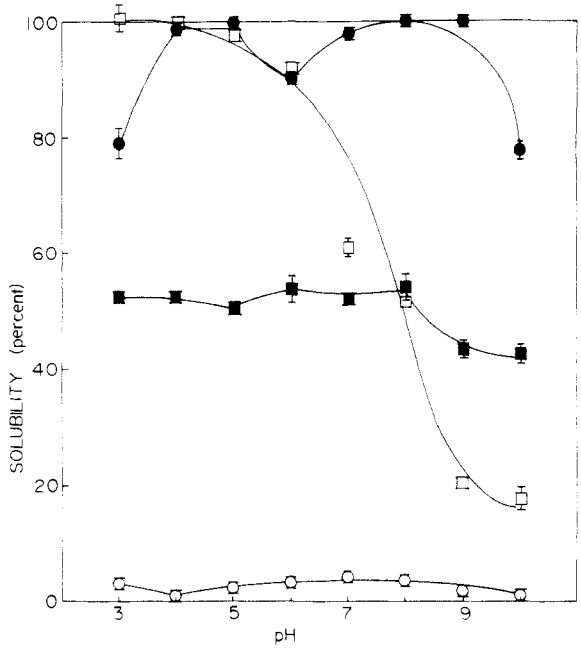

Figure 1. Solubilities of 10% w/v suspensions of native (●), methyl-esterified (○), amidated (□), and ethyl-esterified (■) β-lactoglobulin at various pH values and 0.55 μ. $n = 2$; reported as mean and range.

μL) of native, amidated, ethyl-esterified, or methyl-esterified β-lactoglobulin solutions (2.5 mg/mL) were made sequentially, and the fluorescent emission from each cell was again measured. This procedure was repeated until 350 μL of protein solution had been added or until addition of protein solution yielded only a small enhancement of fluorescence.

Increases in fluorescence were recorded at 26 °C with a Varian SF-330 spectrofluorometer. Three-nanometer excitation and 20-nm emission spectral bandwidths were used at 325 and 410 nm, respectively, for *cis*-parinaric acid and at 370 and 470 nm, respectively, for ANS. The initial slope (S_0) was calculated for each protein from a plot of the fluorescence intensity of *cis*-parinaric acid vs. protein concentration (Kato and Nakai, 1980).

Hydrophobic Binding of Heptane by Proteins. A total of 1.2 mL of protein solutions (2.0% w/v) in 0.1 M sodium phosphate, pH 6.8, was equilibrated with 0.9 mL of heptane (Aldrich Chemical Co., Milwaukee, WI) for 10 or 24 h (Mohammadzadeh-K. et al., 1969a). After equilibration, the supernatant layer was removed and discarded. The lower phase was recovered and 1 μL analyzed directly by using gas–liquid chromatography. Controls containing buffer, but no protein, were also equilibrated and analyzed.

The heptane was quantified with a Varian (Model 2740) gas–liquid chromatograph equipped with a flame ionization detector and a 3 m × 2 mm (inner diameter) glass column packed with Chromosorb W (100–120 mesh) coated with 3% OV-101. After each analysis at 60 °C, with a helium flow rate of 15 mL/min, the column temperature was increased to 200 °C and held for 4 min to facilitate elution of water vapor.

RESULTS AND DISCUSSION

Solubility. The solubility patterns of the modified β-lactoglobulins suspended at 10% w/v protein in various buffers at $\mu = 0.55$ are shown in Figure 1. The amidated and esterified derivatives decreased in solubility close to their isoionic points—pH 8–10. The methyl-esterified protein was much less soluble at all indicated pH values than the ethyl-esterified β-lactoglobulin in these experiments, suggesting a more random and more hydrophobic structure for the methyl-esterified protein. Under the same conditions, the ethyl-esterified protein was only about 50% as soluble as the native and amidated β-lactoglobulin between pH values of 3 and 6. Amidated β-lactoglobulin gradually decreased in solubility with increasing pH compared to a rather constant solubility for each of the other proteins between pH 5 and pH 8.

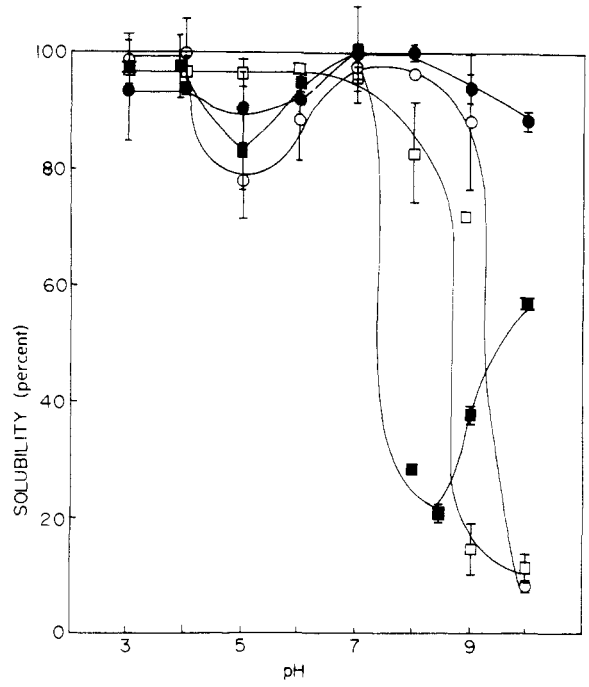

Figure 2. Solubilities of 1% w/v suspensions of native (●), methyl-esterified (○), amidated (□), and ethyl-esterified (■) β-lactoglobulin at various pH values and low ionic strength. $n = 2$; reported as mean and range.

The solubilities of proteins as 1% w/v suspensions at low ionic strength and adjusted to various pH values with acid or base are shown in Figure 2. At this concentration and at low ionic strength, a greater percentage of each modified protein was soluble at pH values below 8; however, their solubilities decreased as their isoionic points were approached. The ethyl-esterified β-lactoglobulin with an isoionic point of 8.7 increased in solubility above its isoionic point. The differences in solubilities of the same proteins under different conditions (Figures 1 and 2) underline the importance of environment in determining their solubilities. Alterations in solubilities of the proteins, but in different ways, also stresses how slight modifications of reagents and reaction conditions can differentially affect the physical properties of protein derivatives.

UV Difference Spectra. The modified proteins have lower absorbance than the native β-lactoglobulin in the 260–310-nm region (Figure 3). The extent of deviation correlated directly with the change in isoionic points. However, the amidated β-lactoglobulin had a higher absorbance in the 240–250-nm region. The aromatic residues of the modified proteins are apparently in a different environment as evidenced by the change in absorbance, suggesting a change in conformation of the derivatized proteins. This is consistent with circular dichroism spectra of similar proteins in previous studies (Mattarella et al., 1983). Enhancement of net positive charges on the surface of β-lactoglobulin could alter its globular structure by changes in the distribution of electrostatic forces.

Chemical Stability of Ester Groups. The extent of hydrolysis of ester groups, as measured by a decrease in the isoionic points of the treated, esterified proteins, in-

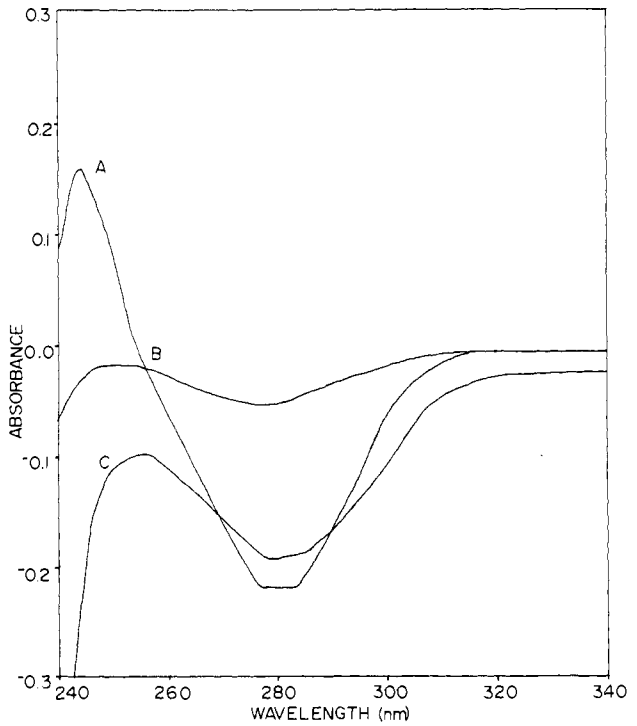

Figure 3. Ultraviolet difference spectra of amidated (A), ethyl-esterified (B), and methyl-esterified (C) β-lactoglobulin with native β-lactoglobulin as the reference.

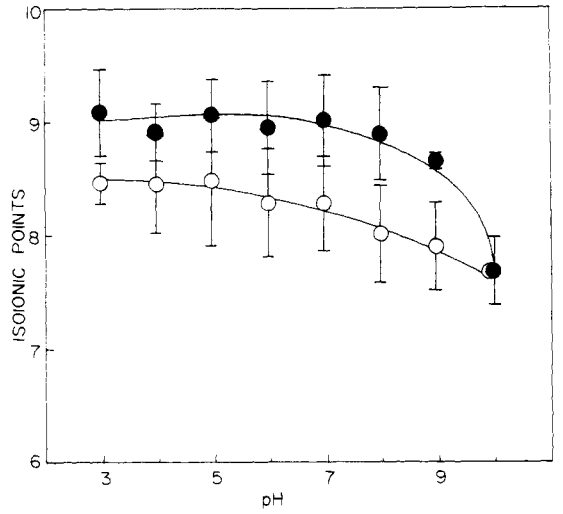

Figure 4. Stability of methyl (●) or ethyl (○) residues in esterified β-lactoglobulin to various pH values for 24 h as measured from isoionic points of treated proteins. $n = 3$; reported as mean ± SD.

creased at pH values above 7.0 (Figure 4). After 24 h at pH 10.0 the isoionic points of the ethyl-esterified and methyl-esterified β-lactoglobulin had decreased 7% and 14%, respectively. This confirms the slow rate of hydrolysis of ester groups of proteins in aqueous solutions at pH values greater than 7 as reported by Sri Ram and Maurer (1959). However, the esters appear relatively stable at pH values between 5 and 7.

Stability of Ester Groups to Treatment with an Esterase. Under the conditions used, no hydrolysis of protein ester groups by porcine liver esterase was detectable. Methyl *n*-butyrate, however, was readily hydrolyzed to *n*-butyric acid as measured by a rapid decrease in pH of an aqueous suspension of the ester. Because the enzyme was active on simple substrates under these experimental conditions, enzymic specificity or steric hindrance may

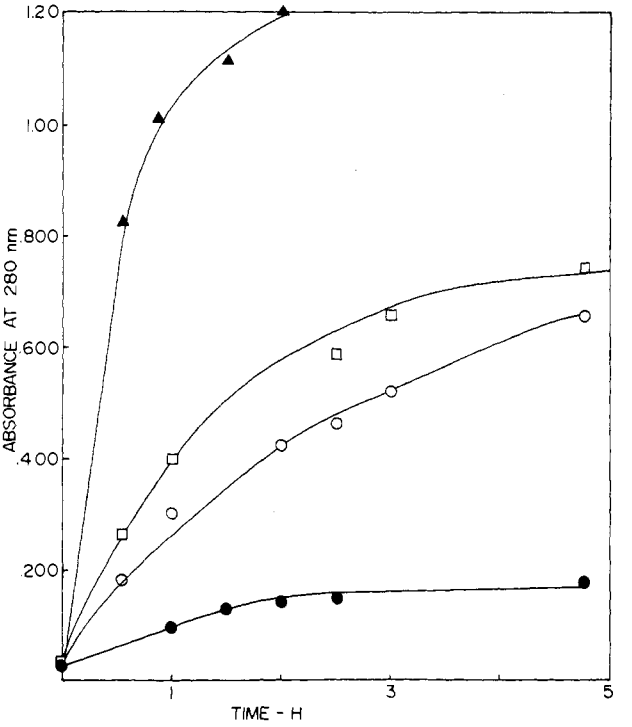

Figure 5. Rates of hydrolysis of native (●), methyl-esterified (○), ethyl-esterified (□), and amidated (▲) β-lactoglobulin by trypsin.

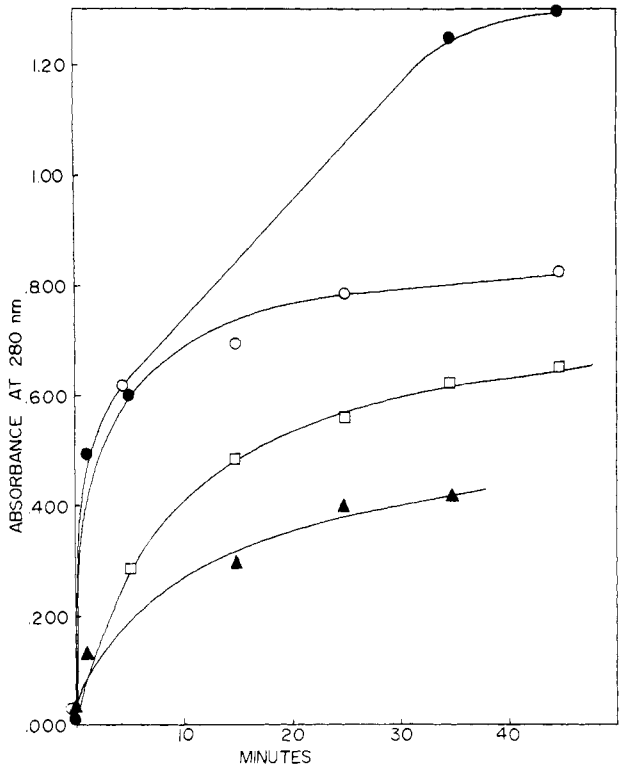

Figure 6. Rate of hydrolysis of native (●), methyl-esterified (○), ethyl-esterified (□), and amidated (▲) β-lactoglobulin by pepsin.

have prevented hydrolysis of ester groups in the proteins.

Proteolysis in Vitro. The rate of tryptic hydrolysis of native β-lactoglobulin was much slower than for amidated, ethyl-esterified, or methyl-esterified β-lactoglobulin (Figure 5). This increase in rate of hydrolysis may be due, partially, to denaturation or randomization of the modified proteins (Mattarella et al., 1983). Sri Ram and Maurer (1959) also observed increased rate of tryptic hydrolysis for methyl-esterified bovine serum albumin. On the other

Table I. Properties of Emulsions[a] Formed with Native and Modified β-Lactoglobulin

protein sample	relative emulsifying activity[b]	% protein adsorbed to emulsion	mL of oil after heating
β-lactoglobulin	1.00	11	1.7
amidated β-lactoglobulin	0.84	2	1.7
methyl-esterified β-lactoglobulin	0.92	11	3.0
ethyl-esterified β-lactoglobulin	0.86	41	0.5

[a] 0.5% protein in 0.1 M sodium chloride, pH 7.0. [b] Calculated from the emulsifying activity index (Pearce and Kinsella, 1978). β-Lactoglobulin thus serves as the standard for comparison.

hand, the rate of peptic hydrolysis was decreased by modification of the β-lactoglobulin (Figure 6). This may be due to steric hindrance or a modified affinity of pepsin for the positively charged proteins. The isoelectric point of porcine pepsin is 2.2 compared to 8 for trypsin (Malamud and Drysdale, 1978). A very high affinity of the negatively charged pepsin for the positively charged substrates may retard the rate of dissociation of the enzyme–substrate complex.

Emulsifying Activity and Emulsion Stability. Native β-lactoglobulin had slightly better emulsifying activity than any of the modified proteins under the conditions used (Table I). Although the differences in emulsifying activities among the proteins were not large, the percentage of protein adsorbed by the oil droplets varied greatly. Both native and methyl-esterified β-lactoglobulins were equally adsorbed whereas amidated β-lactoglobulin was adsorbed to a lesser extent. Over 40% of the estyl-esterified β-lactoglobulin was adsorbed to the oil phase. These differences were observed repeatedly; possibly, the larger percentage of ethyl-esterified β-lactoglobulin adsorbed could be due to smaller oil droplets in this emulsion. However, the lower emulsion activity observed for this protein would not be consistent with such an interpretation. Since the isoionic point of ethyl-esterified β-lactoglobulin was the lowest at 8.7, the average net charge of this protein was less than the other positively charged modified proteins. Perhaps reduced charge repulsion among molecules of the ethyl esterified may favor protein association with the oil phase. Additional modified protein may continue to bind to protein associated directly with oil at the interface to yield a thick proteinaceous coat enrobing the oil droplet. This observation will clearly require more study.

The amount of free oil released from each emulsion after heating was used as an index of emulsion stability. On this basis (Table I) ethyl-esterified β-lactoglobulin forms the most stable emulsion which might result from its greater association with the oil phase. Emulsions stabilized by ethyl-esterified β-lactoglobulin formed a thick gel upon being heated at pH 7.0 in contrast to the other emulsions. On the basis of this observation, emulsions from native and modified β-lactoglobulins prepared as before and adjusted to the respective isoionic points of the proteins were heated to induce gel formation. Native and ethyl-esterified β-lactoglobulin formed gellike emulsions on heating. However, the gel from heating the ethyl-esterified β-lactoglobulin emulsion was more viscous. Emulsions prepared by using the methyl-esterified or amidated β-lactoglobulin did not visibly change in viscosity. The greater adsorption of the more hydrophobic, ethyl-esterified β-lactoglobulin in the oil phase may result in enhanced gelation of the emulsion upon heating.

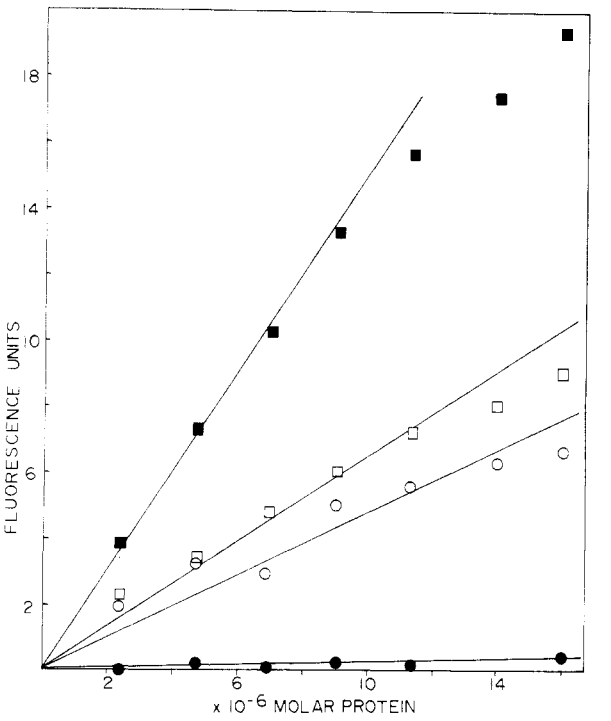

Figure 7. Increase in fluorescence of 8-anilino-1-naphthalenesulfonate with addition of native β-lactoglobulin (●), amidated β-lactoglobulin (○), ethyl-esterified β-lactoglobulin (□), and methyl-esterified β-lactoglobulin (■).

Table II. Hydrophobic Parameters[a] of Native and Modified β-Lactoglobulin Using *cis*-Parinaric Acid[b]

protein	0.01 M sodium phosphate (pH 7)–0.002% $NaDodSO_4$		
	no NaCl	0.55 M NaCl	2 M NaCl
β-lactoglobulin	57	144	584
ethyl-esterified β-lactoglobulin	132	168	260
methyl-esterified β-lactoglobulin	420	407	225
amidated β-lactoglobulin	384	360	158

[a] Initial slope (S_0). [b] Kato and Nakai (1980).

Protein Hydrophobicity. When ANS was used as the hydrophobic probe (Figure 7), the methyl-esterified β-lactoglobulin was the most hydrophobic protein studied followed by ethyl-esterified and amidated β-lactoglobulin which had similar increases in fluorescence. Native β-lactoglobulin increased the least in fluorescence with additional protein and, therefore, was considered to be the least hydrophobic. Penzer (1972) argued, however, that molecular rigidity rather than polarity is the dominant factor influencing the energy and quantum yield of the fluorescence of ANS. Since the modified proteins possess a high net positive charge, some of the increased fluorescence may be due to electrostatic binding of the probe and not to true hydrophobicity.

cis-Parinaric acid has been used as a fluorescent membrane probe (Sklar et al., 1976) and to determine effective hydrophobicity of proteins (Kato and Nakai, 1980). Kato and Nakai reported a good correlation for initial slope of fluorescence enhancement vs. increased protein concentration and effective hydrophobicity as determined by

Table III. Binding of Heptane by Various Proteins[a]

protein	molecular weight	amount bound: mmol/10 000 g of protein	amount bound: mol/mol of protein	relative hydrophobicity scale[b]
bovine serum albumin	69 000	354	2.45	0.84
β-lactoglobulin (bovine)	36 000	282	1.01	0.35
amidated β-lactoglobulin	36 000	810	2.92	1.00
ethyl-esterified β-lactoglobulin	36 000	630	2.26	0.77
methyl-esterified β-lactoglobulin	36 000	378	1.36	0.46

[a] Data reported are for the 10-h equilibration period; however, no differences were evident in binding after 24-h equilibration. [b] Based on moles of heptane bound per mole of protein.

hydrophobic partition experiments using poly(ethylene glycol)palmitate (Keshavarz and Nakai, 1979). High effective hydrophobicity, in turn, correlated with superior emulsifying activity of proteins. In hydrophobic chromatography high concentrations of sodium chloride are used to favor binding of proteins to the support since at high salt concentrations hydrophobic interactions are favored and electrostatic ones are suppressed (Hjertin, 1981). Since the carboxylate anion of *cis*-parinaric acid might interact electrostatically with the positively charged proteins, increasing salt concentrations were used in the determination of effective hydrophobicity of the various proteins (Table II). Addition of sodium chloride, indeed, lowered the apparent hydrophobicity of both methyl-esterified and amidated β-lactoglobulin which had isoionic points of 9.5 and 9.8, respectively. Thus, the interaction of these proteins with *cis*-parinaric acid at low salt concentrations may be partially electrostatic in nature. On the other hand, fluorescence increased in solutions of native β-lactoglobulin and ethyl-esterified β-lactoglobulin as the sodium chloride concentrations increased, which suggests hydrophobic interactions between probe and protein. However, all positively charged derivatives had lower effective hydrophobicities in 2 M NaCl compared to that of native β-lactoglobulin. This is somewhat unusual since the positively charged proteins were more random in structure as measured by circular dichroism spectra (Mattarella et al., 1983) and increased hydrophobicity due to exposure of hydrophobic residues might be expected. Although these observations may be confounded by the effects of varying ionic strength on protein conformation, measurements of effective hydrophobicity of proteins in low salt concentrations must be interpreted cautiously because of possible electrostatic interactions between the protein and *cis*-parinaric acid.

Because of potential complications inherent in the use of charged fluorescent probes, hydrophobic chromatography (Keshavarz and Nakai, 1979), hydrophobic partition of poly(ethylene glycol)palmitate (Keshavarz and Nakai, 1979), or hydrocarbon solubility in proteins (Mohammadzadeh-K. et al., 1969b) may provide more accurate information about effective hydrophobicity than anionic fluorescent probes. The hydrophobic binding of heptane by native and modified β-lactoglobulins (Mohammadzadeh-K. et al., 1969b) provided another comparison of the relative hydrophobicities of these proteins (Table III). Binding of heptane is reported for 10 h; however, there was no observed difference after 24-h equilibration. Although the millimoles of heptane bound per 10 000 g of protein for β-lactoglobulin and bovine serum albumin are lower than those reported by Mohammadzadeh-K. et al. (1969b), the relative differences between proteins should be valid. This method revealed that amidated β-lactoglobulin was the most hydrophobic followed closely by ethyl-esterified β-lactoglobulin. Methyl-esterified β-lactoglobulin was slightly more hydrophobic than native β-lactoglobulin. The greater binding of heptane by amidated β-lctoglobulin may be due to greater randomization of this protein compared to the others as indicated by circular dichroism spectra (Mattarella et al., 1983) and UV difference spectra. On the other hand, ethyl-esterified β-lactoglobulin bound more heptane than methyl-esterified β-lactoglobulin. At the same time, UV difference spectra and circular dichroism spectra (Mattarella et al., 1983) indicated that the ethyl-esterified β-lactoglobulin was less randomized than either the amidated or methyl-esterified β-lactoglobulin. Although less derivatized as indicated by a smaller increase in isoionic point, the ethyl-esterified β-lactoglobulin may possess a higher hydrophobicity, in part, from contributions of the more hydrophobic ethyl residues. Indeed, greater surface hydrophobicity of the ethyl-esterified derivative might favor a more compact, less random structure in an aqueous environment.

Modification of β-lactoglobulin to form positively charged proteins by amidation or esterification changed the hydrophobicity by randomizing the protein and, in the case of ethyl esterified, by introduction of a more hydrophobic residue. Although modifications were of nonessential amino acids, the inability of porcine liver esterase to cleave the esters and the decrease in peptic hydrolysis point out the necessity of toxicological as well as nutritional evaluations of these novel, modified proteins. Proteins of greater hydrophobicity can probably be prepared by transesterification reactions involving longer chain alcohols to help elucidate the mechanism of the unusual adsorption to the oil and the greatly increased emulsion stability observed for ethyl-esterified β-lactoglobulin.

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Determination of Acetaldehyde in *Streptococcus lactis* Cultures as 2,4-Dinitrophenylhydrazone by High-Performance Liquid Chromatography

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A method is described for acetaldehyde analysis in *Streptococcus lactis* cultures that involves distillation and formation of acetaldehyde 2,4-dinitrophenylhydrazone with separation by high-performance liquid chromatography (HPLC). Relative standard deviations for data obtained from HPLC analysis were slightly lower than for data obtained by a conventional spectrophotometric method for *S. lactis* broth cultures. Acetaldehyde recovery data were comparable for both techniques. Acetaldehyde data for two strains of *S. lactis* incubated at 32 °C are compared.

The importance of acetaldehyde as a flavor contributor in cultured dairy products has been well documented (Lindsay et al., 1965; Keenan and Bills, 1968; Lees and Jago, 1978). However, a wide variation in levels of acetaldehyde production and metabolism has been reported for different strains of lactic acid bacteria. The detectable acetaldehyde levels in microbial cultures may also vary with culturing conditions. Much of the variability in reported acetaldehyde data may relate to variation in method of analysis used since acetaldehyde is a highly volatile and reactive compound (Lees and Jago 1969). The most commonly employed methods involve distillation and subsequent reaction of acetaldehyde with reagents such as 3-methyl-2-benzothiozolone hydrazone (MBTH; Lindsay and Day, 1965) or 2,4-dinitrophenylhydrazine (DNPH; Harvey, 1960) followed by spectrophotometric analysis. These techniques are nonspecific and, therefore, are subject to background interference. Direct gas chromatographic (GC) analysis requires specialized sample handling because of the high volatility of acetaldehyde. While this problem can be overcome by hydrazone derivatization, difficulties have been reported for GC analysis of carbonyl 2,4-dinitrophenylhydrazone (DNP) derivatives because of formation of isomers with heating (Papa and Turner, 1972a,b). High-performance liquid chromatography (HPLC) has been reported as an acceptable method for carbonyl DNP separation and analysis (Fung and Grosjean, 1981). HPLC analysis techniques involving DNPH derivatization have been reported for formaldehyde in shrimp (Radford and Dalsis, 1982) and for methyl ketones in dry food model systems (Reinedccius et al., 1978). The objective of this investigation was to evaulate the use of DNPH derivatization and HPLC separation for estimation of acetaldehyde levels in cultures of lactic acid bacteria.

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MATERIALS AND METHODS

Microorganisms and Growth Conditions. The organisms, *Streptococcus lactis* 60 (Hansen's Laboratories, Milwaukee, WI) and *S. lactis* C2 (University of Minnesota, St. Paul, MN) were subcultured (12 h at 32° C) 3 successive times in Elliker Broth (Difco). In experimental trials, 3 L of Elliker broth was inoculated with *S. lactis* at 1.0%. Incubation was in a New Brunswick Model 19 fermentor (New Brunswick Scientific Co., Edison, NJ). with constant agitation (200 rpm) at 32 °C. Duplicate samples were removed from the fermentor at incubation times of 0, 3, 6, and 9 h.

Acetaldehyde Analysis. *Reagents and Standards.* Carbonyl-free hexane was prepared by elution of nanograde hexane through a 2,4-DNPH–Celite column followed by reaction with 1.0% activated charcoal and filtering. The DNPH reagent mixture was prepared as a mixture of 2,4-DNPH (Aldrich Chemical Co., Milwaukee, WI), H_3PO_4, and Celite (Schwartz and Parks, 1961). Distilled water, used as a diluent or in chromatography, and the chromatographic solvent (HPLC-grade CH_3CN; Fisher Scientific Co., Fairlawn, NJ) were further purified through Porapak Q (Supelco, Inc., Bellefonte, PA). Acetaldehyde stock standard solution was prepared by weighing cold acetaldehyde (Aldrich Chemical Co., Milwaukee, WI) into cold water. The stock solution was calibrated by iodometric titration (McCloskey and Mahaney, 1981) and appropriately diluted for standard curve preparation.

Distillation. Two distillation procedures were compared. A low-temperature purge system was assembled as described by Pack et al. (1964) and used by Lindsay and Day (1965). The system involved nitrogen purging of tubes containing culture in a 65 °C water bath into a collection trap containing derivatizing reagents. For steam distillation, a micro-Kjeldahl apparatus fitted with a cold water condenser and graduated collection trap containing derivatizing reagents was used.

3-Methyl-2-benzothiozolone Hydrazone (MBTH) Spectrophotometric Procedure. The MBTH procedure

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